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Distribution and Physiological Characteristics of Hyperthermophiles in the Kubiki Oil Reservoir in Niigata, Japan

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The distribution of culturable hyperthermophiles was studied in relation to environmental conditions in the Kubiki oil reservoir in Japan, where the temperature was between 50 and 58°C. Dominant hyperthermophilic cocci and rods were isolated and shown to belong to the genera *Thermococcus* and *Thermotoga*, respectively, by 16S rDNA analyses. Using the most-probable-number method, we found that hyperthermophilic cocci were widely distributed in several unconnected fault blocks in the Kubiki oil reservoir. In 1996 to 1997, their populations in the production waters from oil wells were 9.2×10^3 to 4.6×10^4 cells/ml, or 10 to 42% of total cocci. On the other hand, hyperthermophilic rods were found in only one fault block of the reservoir with populations less than 10 cells/ml. Dominant *Thermococcus* and *Thermotoga* spp. grew at reservoir temperatures and utilized amino acids and sugars, respectively, as sole carbon sources. While organic carbon was plentiful in the environment, these hyperthermophiles were unable to grow in the formation water due to lack of essential nutrients. Concentrations of some organic and inorganic substances differed among fault blocks, indicating that the movement of formation water between fault blocks was restricted. This finding suggests that the supply of nutrients via fluid current is limited in this subterranean environment and that the organisms are starved in the oil reservoir. Under starved conditions at 50°C, culturable cells of *Thermococcus* sp. remained around the initial cell density for about 200 days, while those of *Thermotoga* sp. decreased exponentially to 0.01% of the initial cell density after incubation for the same period. The difference in survivability between these two hyperthermophiles seems to reflect their populations in the fault blocks. These results indicate that hyperthermophilic cocci and rods adapt to the subterranean environment of the Kubiki oil reservoir by developing an ability to survive under starved conditions.

Many thermophilic bacteria with optimum growth temperatures from 45 to 80°C have been isolated from oil fields (4, 8, 9, 11, 12, 27, 40, 41, 43, 44). In 1993, hyperthermophiles which grew optimally over 80°C were found in the production fluids of North Sea and Alaskan oil reservoirs (48). However, it was pointed out that these organisms might have been introduced into the oil reservoir during drilling or enhanced oil recovery with water injection, because their characteristics are similar to those of organisms found in hydrothermal fields (42). The hyperthermophilic archaea *Thermococcus litoralis* and *Archaeoglobus fulgidus* were isolated from oil wells in the East Paris Basin where no water had been injected (33). While heterotrophic microorganisms have been studied in 37 crude oil reservoirs at temperatures between 37 and 148°C, hyperthermophiles have been detected only in the North Sea oil reservoir (16).

In the North Sea oil reservoir, sulfate reducers in different fault blocks were different, though no correlation was found between the seawater injection and their population densities in the production water (39). Flora of hyperthermophiles at several wells of Thistle offshore were similar (48). However, their distribution in relation to the environmental conditions has not been studied.

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The ecological system of these hyperthermophiles under the ground is still unknown. We studied the distribution of hyperthermophiles and the environmental conditions in an oil reservoir in Japan. Their physiological growth characteristics, including nutritional requirements and survivability in the formation water, were also studied. Their strategies for living in the environment of the oil reservoir are discussed.

MATERIALS AND METHODS

Kubiki oil reservoir. The Kubiki oil reservoir is located near the coast of the Sea of Japan in Niigata prefecture, Japan. This reservoir has many faults and is divided into many unconnected fault blocks (49) (Fig. 1). The depths of oil wells were between 1,000 and 1,200 m, with in situ temperatures between 50 and 58°C. The average in situ pressure was 6 MPa. This oil reservoir consists of three major fault blocks, the KP, OZ, and CC blocks, which are subdivided into smaller blocks (Fig. 1). In the OZ block, 650,000 kl of seawater had been injected into some wells to enhance oil recovery from 1977 to 1994 (Fig. 1). Wells in the KP and CC blocks have not been injected with seawater.

Samples of formation waters. To study the microbial community in formation water, production fluids were collected from the no. 3 storage tank (Fig. 1), where production fluids from all oil wells are stored for approximately 24 h. To determine the microbial distribution, we collected six production fluids from the drains of four separation tanks and two wellheads (Fig. 1). Formation water was collected in sterile glass bottles, which were then sealed with sterile silicon stoppers and plastic screw caps. Samples were chilled in a cooler box and transferred to the laboratory.

Culture conditions of the hyperthermophiles. All culture media for hyperthermophiles (Table 1) were based on artificial seawater (ASW) (26) and supplemented, with 10 ml per liter of a Wolfe trace mineral solution (51), 10 ml of a

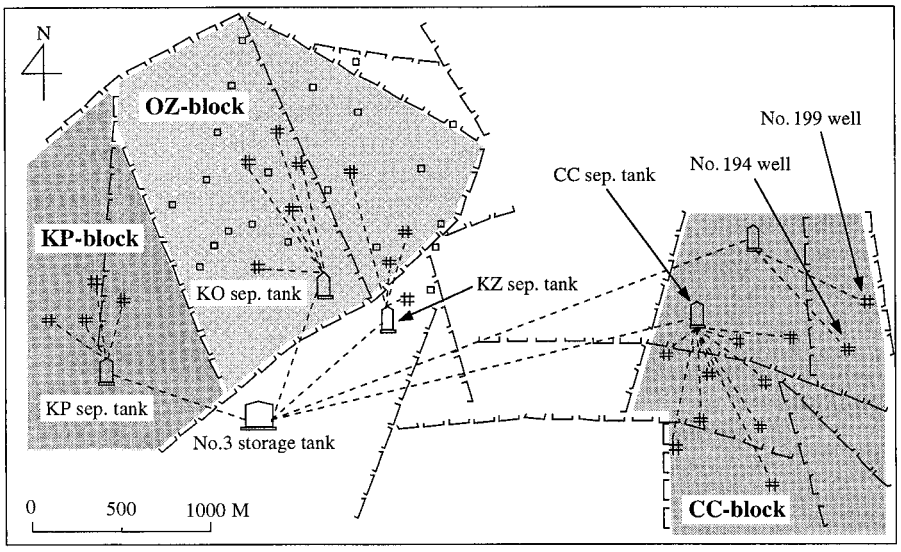

FIG. 1. Map of studied area and sampling points in Kubiki oil reservoir. ⌊⌋⌊⌋⌊⌋, fault line; #, production well; □, well injected with seawater; ----, production tubing; sep., separation.

vitamin mix solution (2), and 60 mg of resazurin. HC and HA media were flushed with an oxygen-free mixture of hydrogen and carbon dioxide (80%:20%) instead of oxygen-free nitrogen. Gas phase of the AY medium was air.

Isolation and cultivation of the hyperthermophiles. One milliliter of the formation water was inoculated into 9 ml of culture medium in a Hungate tube and incubated at 85°C for 1 to 7 days. When the hyperthermophiles grew, the cell suspension was transferred to fresh medium.

The hyperthermophiles were isolated on Gelrite plates. Gelrite (Kelco Division of Merck & Co., Rahway, N.J.) was dissolved at 0.8% (wt/vol) in ASW medium containing 0.6% (wt/vol) yeast extract. After autoclaving at 121°C for 20 min, the solution was dispensed into glass petri dishes in an anaerobic glove box (ANX-1; Hirasawa, Tokyo, Japan) and solidified at room temperature. In the glove box, 10 to 100 μl of the enrichment culture for hyperthermophiles was placed on the plates. Plates were then placed in a Gas Pack jar (AJ9023; MART Jar Systems, Lichtenvoorde, The Netherlands) and incubated at 85°C for 1 to 7 days. This procedure was repeated at least three times.

Electron microscopy. The isolates were fixed with 2.5% (vol/vol) glutaraldehyde for 20 min at room temperature and stained with 4% (wt/vol) uranyl acetate as described by Kurr et al. (32). They were observed in a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan) operated at 75 kV.

Growth characteristics of the isolates. Effects of temperature, pH, and NaCl on microbial growth were examined on YE-based medium. The temperature was controlled with a mineral oil bath (OH-16; Taitec, Saitama, Japan). Buffers for the pH experiments are described elsewhere (19). The cultures were incubated at the optimum growth temperatures for each isolate.

TABLE 1. Media used for detection of hyperthermophiles from the Kubiki oil reservoir

Medium[a]	Gas phase	Carbon source
HC	H_2:CO_2 (4:1,200 kPa)	Free
HA	H_2:CO_2 (4:1,200 kPa)	Acetate (0.1%) + lactate (0.1%)
AY	Air (100 kPa)	Yeast extract (0.2%)
YE	N_2 (100 kPa)	Yeast extract (0.2%)
AA	N_2 (100 kPa)	20 amino acids[b] (total, 0.2%)
GS	N_2 (100 kPa)	Glucose (0.1%) + starch (0.1%)
AL	N_2 (100 kPa)	Acetate (0.1%) + lactate (0.1%)
CO	N_2 (100 kPa)	Crude oil[c] (10% [vol/vol])

[a] Media based on artificial seawater were incubated for 1 to 7 days at 85°C in a dry oven.

[b] The amino acids Gly, Ala, Ser, Thr, Cys, Asn, Gln, Leu, Ile, Val, Met, Phe, Tyr, Trp, Pro, Asp, Glu, His, Lys, and Arg were added at a final concentration of 0.01% (wt/vol) each.

[c] Crude oil was collected from the no. 3 storage tank and autoclaved at 121°C for 30 min.

Nutritional growth requirements of the isolates. To determine nutritional requirements, the cultures were incubated at 80°C unless otherwise stated. Growth on various carbon sources was examined in 10 ml of ASW medium (pH 7.0) containing 0.1% (wt/vol) yeast extract, peptone, casein, 20 amino acids, glucose, sucrose, maltose, starch, acetate, lactate, formate, propionate, or pyruvate without shaking. Growth on hydrocarbons was also examined in the presence of 10% (vol/vol) kerosene, light oil, or A-heavy oil (Nisseki Oil Co., Tokyo, Japan) in 30 ml of ASW medium (pH 7.0) with shaking.

The amino acid requirements of the isolates were determined as described previously (19, 21, 26).

Metabolic products of the isolates. After growth of the isolates in YE medium, low-molecular-weight (C_2 to C_5) organic acids in the culture were analyzed on a high-pressure liquid chromatograph (HPLC; Hitachi L-6000) equipped with a UV detector at 210 nm. Production of H_2 and CO_2 was assessed by gas chromatography (Hitachi 263-30 chromatograph with a thermal conductivity detector). Hydrogen gas was analyzed with a molecular sieve 13X-S column (3-mm inside diameter by 2 m) with N_2 as the carrier gas, at a flow rate of 30 ml/min (injection temperature, 82°C; column temperature, 42°C). Carbon dioxide was analyzed by using a WG-100 column (0.25 in. [outside diameter] by 1.8 m) with He as the carrier gas, at a flow rate of 40 ml/min (injection temperature, 50°C; column temperature, 50°C).

Sulfur requirements of the isolates. One gram of steam-autoclaved elemental sulfur was added to 100 ml of YE medium in 125-ml bottles. Cultures of the isolates, with or without elemental sulfur, were incubated with shaking at the optimum growth temperature. Hydrogen sulfide production was analyzed with a gas analysis kit (Gastec, Kanagawa, Japan).

Sensitivities of the isolates to antibiotics. Growth of the isolates was examined after incubation at 70°C for 7 days in the presence of 100 μg of rifampin, streptomycin, vancomycin, or chloramphenicol per milliliter.

Isolation of DNA. Genomic DNA was extracted from the isolate by using a procedure described elsewhere (1), with slight modification. RNA was digested with DNase-free RNase (20 μg/ml) at 37°C for 1 h after extraction with chloroform-isoamyl alcohol.

DNA base composition (GC content). Nucleotide composition was determined with an HPLC equipped with an UV detector (UV 8010; Tosoh, Tokyo, Japan) at 270 nm with a Develosil ODS-HG-5 column (4.6 by 250 mm) after digestion of DNA with nuclease P1 (53).

16S rDNA analysis. PCR was used to amplify 16S rDNA from the genomic DNA of the isolates by using a pair of primers (A3R [*Escherichia coli* positions 518 to 536, *Thermococcus celer* positions 461 to 479; 5′CAGCCGCCGCGGTA ATACC3′] and A8 [*E. coli* positions 1387 to 1406, *T. celer* positions 1337 to 1356; 5′GACGGGCGGTGTGTGCAAGG3′]) for archaea and another pair of primers (B0R [*E. coli* positions 8 to 27; 5′AGAGTTTGATCCTGGCTCAG3′] and B9 [*E. coli* positions 1491 to 1512; 5′TACGGCTACCTTGTTACGACTT3′]) for bacteria. Amplification consisted of 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. After purification of the PCR products with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), the 16S rDNA sequences were det ermined with an ABI Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer Biosystems, Norwalk, Conn.) and an ABI 377 DNA sequencer (Perkin-Elmer). The determined sequences were aligned with the reported 16S

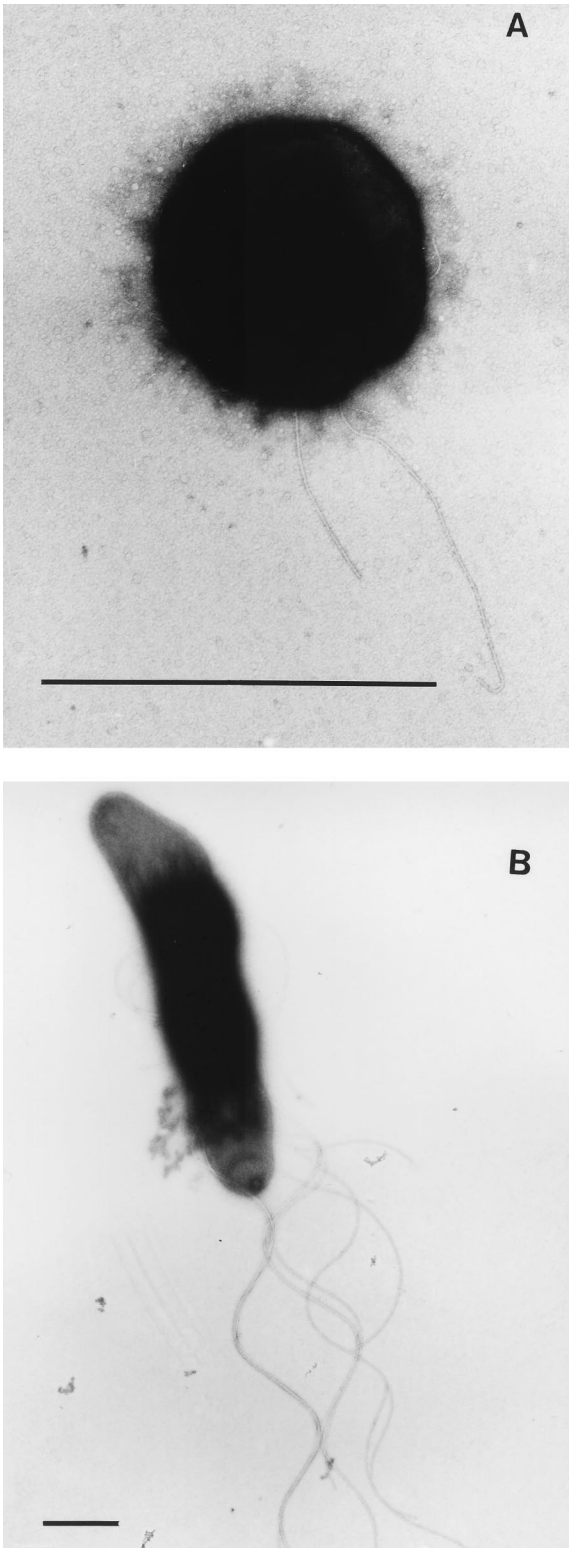

FIG. 2. Transmission electron micrographs of strains CKU-1 (A) and RKU-1 (B); negative staining with 4% uranyl acetate. Bars, 1 μm.

rRNA gene sequences collected from DDBJ (DNA data bank of Japan), and the sequence similarities were determined with GENETYX-MAC 8.0.

Chemical analysis of formation water. Prior to chemical analysis, the samples were passed through a sterile filter (Disk Capsule, 0.2-μm pore size; Fuji, Tokyo, Japan). Concentrations of Cl^- and SO_4^{2-} were analyzed with an HPLC equipped with a TSKgel IC-Anion-PW (4.6-mm [inside diameter] by 5 cm) column, using 4 mM potassium phosphate buffer (pH 9.1) as an elution buffer, at a flow rate of 1.0 ml/min at 40°C. They were detected with a conductivity detector (Tosoh CM 8010). Concentrations of Na^+ and Mg^{2+} in the samples were measured with an inductively coupled sequential plasma spectrometer (model ICPS-4000; Shimadzu, Kyoto, Japan). Dissolved organic carbon (DOC) was analyzed with a total organic carbon analyzer (Shimadzu TOC 5000). Amino acids were analyzed with a reverse-phase HPLC equipped with an AccQ-Tag column (Nihon Millipore Kogyo, Tokyo, Japan) and a fluorescence detector (Tosoh FS8010). The reducing sugars were analyzed by a modified fluorescence HPLC method based on the postcolumn reaction with benzamidine (28).

Estimation of total cell density. Total cell density of microorganisms was determined by the acridine orange direct count method (22). Cells were fixed with 1% (vol/vol) glutaraldehyde, stained with 0.05% acridine orange (vol/vol), and then filtrated through polycarbonate filters (Nuclepore Track-Etch membrane; 0.2-μm pore size; Corning Costar, Boston, Mass.). The filter was observed under an epifluorescence microscope (BX60; Olympus, Tokyo, Japan).

Estimation of culturable cell density. Culturable cell density was determined by the three-tube most-probable-number method (10). Hyperthermophilic cocci were detected by using YE medium containing vancomycin (100 μg/ml) at 85°C; for hyperthermophilic rods, we used GC medium at 80°C. Growth was monitored by turbidity of the cultures. In this study, cells that grew in YE medium at 45 and 20°C were defined as anaerobic heterotrophic thermophiles and mesophiles, respectively.

Growth ability of isolates in the Kubiki oil reservoir. Growth ability of the isolates in the oil reservoir was estimated by using KFW medium containing the formation water and crude oil from the Kubiki oil reservoir. Filtrate obtained after passage through a membrane filter (Fuji Disk Capsule; 0.2 μm pore size) (90 ml) was added, along with sterile resazurin (60 mg/liter) and 10 ml of autoclaved crude oil, into sterile 125-ml vials. These vials were flushed with oxygen-free nitrogen, and sterile Na_2S solution was added at a final concentration of 1 mM. Limitation of the nutrients in the formation water was examined with KFW medium containing 20 amino acids (0.1% each amino acid) or 2% sucrose. The isolates were inoculated into the vials with the initial cell density at approximately 10^5 cells/ml. They were incubated at their optimum growth temperatures for 10 days, and growth was monitored by the acridine orange direct count method (22).

Survivability of isolates in the Kubiki oil reservoir. To estimate survivability in the Kubiki oil reservoir, the isolates were inoculated in KFW medium at an initial cell density of approximately 10^5 cells/ml. After incubation at 50°C for about 200 days, cell densities were monitored by the three-tube most-probable-number method.

Nucleotide sequence accession numbers. The 16S rDNA sequences of strains CKU-1 and RKU-1 were registered at the DDBJ under accession no. AB027014 and AB027016, respectively. The sequence of strain CKU-199 (isolated from the no. 199 well) was registered at the DDBJ under accession no. AB027015.

RESULTS

Isolation of hyperthermophiles. Two types of hyperthermophiles were isolated from the Kubiki oil reservoir after cultivation at 85°C. Cocci grew in both YE and AA media. Rods with togas at both ends grew in YE and GS media. No hyperthermophiles grew in HC, HA, AY, AL, or CO media.

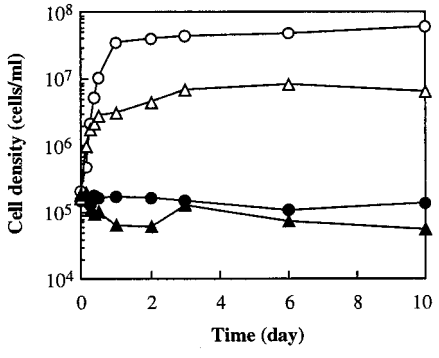

FIG. 3. Growth curves of strains CKU-1 and RKU-1 in filtered sterile formation water with 10% (vol/vol) crude oil under anaerobic conditions (KFW medium) at their optimum temperatures. ▲, CKU-1 in KFW medium; △, CKU-1 in KFW medium containing 20 amino acids (each at 0.1 g/liter); ●, RKU-1 in KFW medium; ○, RKU-1 in KFW medium containing sucrose (1 g/liter).

TABLE 2. Characteristics of strains CKU-1 and RKU-1

Strain	Diam (μm)	Flagella	Temp (°C)		pH		NaCl concn (%)		Doubling time (h) at:	
			Range	Optimum	Range	Optimum	Range	Optimum	Optimum temp	In situ temp (52°C)
CKU-1	0.5–3.0	+	46–95	85	5.2–9.0	6.0	0.1–6.0	2.0	0.6	11.4
RKU-1	2.0–7.0 × 0.7–1.0	+	47–88	80	5.2–9.0	7.0	0.1–5.5	1.0	0.9	22.1

[a] R, rifampin; S, streptomycin; V, vancomycin; C, chloramphenicol.
[b] Peptone or casein.
[c] Glucose, sucrose, maltose, or starch.

Eleven cocci and six rods were isolated from the enrichment cultures which had been inoculated with the sample from the no. 3 storage tank. The partial 16S rDNA sequences were determined for these isolates. Homologies between the 16S rDNA sequences (455 bp) of the isolated 11 cocci and that of *T. celer* were 98.5 to 99.1%, which indicated that they belonged to the genus *Thermococcus*. Homologies between the 16S rDNA sequences (440 bp) of the isolated six rods and that of *Thermotoga maritima* were 98.6 to 99.3%, which indicated that they belonged to the genus *Thermotoga*.

Characteristics of isolated coccus and rod. Strain CKU-1 was one of the 11 cocci isolated from the formation water of the Kubiki oil reservoir, and strain RKU-1 was one of the six rods (Fig. 2; Table 2). Identity of the 16S rDNA sequence (857 bp) between strain CKU-1 and *T. celer* was 98.7%. Identity of the 16S rDNA sequence (1482 bp) between strain RKU-1 and *Thermotoga maritima* was 99.3%.

Formation waters in the fault blocks. To estimate the movement of formation water in the reservoir, concentrations of organic and inorganic components in the formation waters from several fault blocks were analyzed (Table 3). All samples were collected from the same oil layer, but the oil wells in the KP block were a little deeper than those in the other blocks (Table 3). The in situ temperature was between 50 and 58°C and increased with depth. The pHs in all of the formation waters were about 8. Concentrations of DOC were between 36 and 755 mg/liter and differed among the samples even in the same fault block. Concentrations of Cl^-, Na^+, Mg^{2+}, and SO_4^{2-} in the OZ block were higher than those in the KP block. The higher concentrations of these ions in the OZ block were possibly caused by seawater injection. Concentrations of these ions in KP block were similar to those in the CC block.

Population change of microorganisms in the Kubiki oil reservoir. Total cell densities and culturable cell densities of hyperthermophilic, thermophilic, and mesophilic anaerobic heterotrophs in the formation water of the no. 3 storage tank were investigated from June 1996 to September 1997 (Table 4). In the Kubiki oil reservoir, only cocci were observed after incubation at 85°C in YE medium containing 100 μg of vancomycin per ml. Only rods with togas were observed after incubation in GS medium at 80°C. No coccus was observed after incubation in YE medium at 45 or 20°C. Total cell densities of cocci and rods were 8.0×10^4 to 2.0×10^5 cells/ml and 6.5×10^5 to 2.5×10^6 cells/ml, respectively in this period. Culturable cell densities of hyperthermophilic cocci, hyperthermophilic rods, thermophiles, and mesophiles did not change much and were 9.2×10^3 to 4.6×10^4, $<0.4 \times 10^0$ to 9.3×10^0, 2.4×10^4 to 1.1×10^5, and 1.1×10^4 to 4.0×10^4 cells/ml, respectively in this study period. Culturable cell densities of hyperthermophilic cocci were 10 to 42% of total cocci. Culturable cell densities of rods, including hyperthermophilic rods, thermophiles, and mesophiles, were 1.8 to 9.9% of total rods.

Distribution of hyperthermophiles and other anaerobic heterotrophs. Hyperthermophilic cocci were found in all of the blocks examined (Table 5). Their culturable cell densities were 1.5×10^0 to 1.1×10^5 cells/ml. Hyperthermophilic rods were detected only in the KP block, with culturable cell density of 1.5×10^0 cells/ml (Table 5). Anaerobic heterotrophic thermophiles and mesophiles were found in all samples (Table 5). Average culturable cell densities (9.1×10^4 cells/ml) of thermophiles in all samples were about the same as those in the no. 3 storage tank (5.4×10^4 cells/ml [Table 4]). Culturable cell densities of mesophiles in the production fluid collected at wellheads were much lower than those in separation tanks.

Six dominant hyperthermophilic cocci were isolated from four separation tanks and two wellheads, respectively (Fig. 1). The 16S rDNA sequence (455 bp) of the strain isolated from the KO separation tank was identical to that of strain CKU-1. Those of other strains isolated from the KP, KZ, and CC

TABLE 3. Properties of formation waters in fault blocks

Source	Sampling site	Depth (m)	Temp (°C)	pH	DOC (mg/liter)	Cl^- (g/liter)	Na^+ (g/liter)	Mg^{2+} (mg/liter)	SO_4^{2-} (mg/liter)
Formation water from:									
KP block	KP separation tank	1,210–1,245	57–58	7.9	43	7.8	3.1	9	ND[b]
OZ block	KO separation tank	1,022–1,126	51–54	7.9	112	12.0	4.6	137	161
	KZ separation tank	1,004–1,094	50–53	7.9	527	11.4	4.2	28	ND
CC block	CC separation tank	1,048–1,118	51–54	8.1	96	7.5	3.0	7	ND
	No. 194 well	1,080	53	7.9	755	7.0	2.9	15	ND
	No. 199 well	1,051	52	7.9	36	5.4	2.1	7	ND
Seawater[a]				8.0		19.4	10.8	1,300	2,700

[a] Data from reference 30.
[b] ND, not detected (<1 mg/liter).

TABLE 2—*Continued*

Substrates used	Metabolites	Growth response to elemental sulfur	Antibiotic sensitivity[a]	G+C content (%)
Yeast extract, proteinaceous substrates,[b] amino acids	Acetate, lactate, H_2, CO_2	No effect	R	37.8
Yeast extract, peptone, sugars,[c] cellulose	Acetate, lactate, H_2, CO_2	Inhibit	R, S, V, C	46.8

separation tanks and no. 194 and 199 wells were identical to each other, and identity to that of strain CKU-1 was 99.3%.

One hyperthermophilic rod was also isolated from the KP separation tank. Its 16S rDNA sequence (440 bp) was identical to that of strain RKU-1.

Growth ability of the isolates in the Kubiki oil reservoir. Strains CKU-1 and RKU-1 grew in YE medium at the in situ temperatures of the Kubiki oil reservoir (50 to 58°C). Their doubling times at 52°C were 11.4 and 22.1 h, respectively (Tables 2 and 3). Strain CKU-1 required yeast extract, proteinaceous substrates such as peptone or casein, or amino acids as sole carbon and energy sources (Table 2). Six amino acids (Leu, Met, Phe, Thr, Trp, and Tyr) were essential for the growth of strain CKU-1, which did not grow on sugars, organic acids, or hydrocarbons. On the other hand, strain RKU-1 required a sugar (glucose, sucrose, maltose, or starch) as sole carbon and energy source (Table 2). It also grew on yeast extract, peptone, or cellulose but did not grow on casein, amino acids, organic acids, or hydrocarbons.

The DOC in formation water from the no. 3 storage tank was 480 mg of C/liter. Concentrations of low-molecular-weight organic acids, total free amino acids, and reducing sugars as carbon were 3.5, 0.14, and less than 0.01 mg of C/liter, respectively. Some amino acids (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val) were detected between 0.001 and 0.020 mg of C/liter. However, Arg, His, and Trp were not detected (<0.001 mg of C/liter). Most of other organic components in the formation water could not be identified.

Strains CKU-1 and RKU-1 did not grow in KFW medium but did grow in the same medium supplemented with their essential nutrients, amino acids and sugars, respectively (Fig. 3). This indicated that they were unable to grow in in situ formation water due to lack of essential nutrients.

Survivability of the isolates in the Kubiki oil reservoir. In the formation water at 50°C, the culturable cell density of strain CKU-1 fluctuated between 9.3×10^4 and 2.4×10^6 cells/ml within 50 days, but it did not change significantly for about 200 days (Fig. 4). On the other hand, the culturable cell density of strain RKU-1 decreased exponentially to 0.01% of the initial cell density after incubation for about 200 days (Fig. 4).

DISCUSSION

Hyperthermophilic cocci and rods which utilized amino acids and sugars as sole carbon and energy sources, respectively, were isolated from the Kubiki oil reservoir, where the temperature was between 50 and 58°C. An unidentified hyperthermophile (48) and a thermophilic sulfate reducer (47) have been reported to degrade hydrocarbons in the oil reservoirs. *Archaeoglobus* spp. and many thermophilic sulfate reducers utilize organic acids in oil reservoirs (3, 4, 41, 44). However, the hyperthermophiles in the Kubiki oil reservoir did not utilize acetate, lactate, or crude oil as a sole carbon and energy source.

16S rDNA analyses of 17 hyperthermophilic cocci and rods isolated from the storage tank showed that they belonged to the genera *Thermococcus* and *Thermotoga*, respectively. The GC content of strain CKU-1 was similar to that of *T. litoralis* (5, 38). *T. litoralis* has been isolated not only from shallow submarine solfatara but also from the oil reservoirs (33, 48). However, strain CKU-1 was different from *T. litoralis* in some characteristics, such as possession of flagella, pyruvate utilization, and effect of elemental sulfur on the growth (Table 2). While the optimum growth temperature and GC content of strain RKU-1 were similar to those of *Thermotoga maritima* (23), the organism differed from *T. maritima* in some characteristics such as cellulose utilization, effect of elemental sulfur on growth, and sensitivity to rifampin (Table 3).

Because culturable cell densities of mesophiles in tanks were much higher than those at wellheads (Table 5), they seemed to grow in the tanks. On the other hand, hyperthermophiles were unable to grow at the temperature in the tanks. Culturable cell densities of hyperthermophilic cocci in the storage tank were much higher than those of hyperthermophilic rods. Moreover, culturable hyperthermophilic cocci in the storage tank in March 1997 comprised 42% of total cocci. This indicates that they were dominant in this reservoir.

Microbial distribution in the reservoir must be affected by the geological structure (39). In the Kubiki oil reservoir, geological investigation has shown that the oil layers between the OZ and CC blocks are completely separated by the faults (49). The difference of concentrations of organic and inorganic substances between the OZ block and the adjacent KP block

TABLE 4. Total and culturable cell densities in formation waters of no. 3 storage tank

Time of collection	Total cell density (cells/ml)		Culturable cell density (cells/ml)			
	Cocci	Rods	Hyperthermophilic cocci	Hyperthermophilic rods	Thermophiles	Mesophiles
June 1996	8.0×10^4	2.5×10^6	9.2×10^3	ND[a]	3.5×10^4	1.1×10^4
November 1996	2.0×10^5	1.5×10^6	2.4×10^4	9.3×10^0	1.1×10^5	2.1×10^4
March 1997	1.1×10^5	6.5×10^5	4.6×10^4	ND	2.4×10^4	4.0×10^4
September 1997	9.0×10^4	1.7×10^6	9.2×10^3	0.4×10^0	4.6×10^4	2.4×10^4
Avg	1.2×10^5	3.1×10^6	2.2×10^4	2.4×10^0	5.4×10^4	2.4×10^4

[a] ND, not detected (<0.4×10^0 cells/ml).

TABLE 5. Total and culturable cell densities in formation waters in fault blocks

Fault block	Sampling site	Total cell density (cells/ml)		Culturable cell density (cells/ml)			
		Cocci	Rods	Hyperthermophilic cocci	Hyperthermophilic rods	Thermophiles	Mesophiles
KP	KP separation tank	4.1×10^4	1.1×10^5	1.5×10^0	1.5×10^0	4.8×10^3	1.3×10^3
OZ	KO separation tank	1.7×10^5	1.7×10^6	1.1×10^5	ND[a]	4.6×10^5	4.6×10^4
	KZ separation tank	1.7×10^5	5.0×10^5	4.6×10^3	ND	4.6×10^4	3.3×10^2
CC	CC separation tank	3.8×10^5	9.5×10^5	2.4×10^4	ND	2.4×10^4	4.6×10^3
	no. 194 well	1.3×10^5	2.1×10^6	1.5×10^1	ND	1.1×10^4	1.5×10^1
	no. 199 well	3.4×10^4	1.6×10^5	1.5×10^3	ND	2.4×10^3	9.3×10^0

[a] ND, not detected ($<0.4 \times 10^0$ cells/ml).

indicated that the injected seawater did not move easily from one block to another through the faults. This indicates that even if microorganisms were introduced by injected seawater and grew in one block, it would be difficult for them to spread over the other blocks. Hyperthermophilic rods were found only in the KP block (Table 5). Their distribution might be restricted by unknown factors, or their densities in other fault blocks might be lower than the detection limit ($<0.4 \times 10^0$ culturable cells/ml). Hyperthermophilic cocci were found in all fault blocks of the Kubiki oil reservoir, but their culturable cell densities varied from 10^0 to 10^5 cells/ml, even in the same blocks. Partial 16S rDNA sequences of the dominant hyperthermophilic cocci isolated from the three different fault blocks were almost identical. These results suggested that they were widely distributed throughout the separated fault blocks in the Kubiki oil reservoir.

It is not clear how these hyperthermophiles dwell in the oil reservoir at temperatures lower than their optima. The minimum growth temperatures of strains CKU-1 and RKU-1 were 46 and 47°C, respectively (Tables 2 and 3). None of the known *Thermococcus* strains (5, 7, 13, 14, 15, 19, 24, 29, 35, 36, 45, 52) except *T. profundus*, *T. siculi*, and *T. barophilus* (17, 31, 34) grow below 55°C. Hyperthermophilic *Thermotoga* species (*T. maritima* and *T. neapolitana*) did not grow below 55°C (6, 23, 25). The growth ability below 55°C may be important in the Kubiki oil reservoir.

Growth of strains CKU-1 and RKU-1 was restricted due to lack of essential nutrients in the formation water of the Kubiki oil reservoir (Fig. 3). While the cell densities of the S^0-dependent heterotrophic hyperthermophiles in a shallow hydrothermal field in Kodakara-Jima, Japan, were high, concentrations of their essential nutrients, amino acids, were low (20). In these hydrothermal fields, water current may continuously supply nutrients to the microbial communities (18, 37). On the other hand, the flow rates of natural groundwater in a deep sedimentary basin were calculated to be 0.001 to 0.01 m per year (50). This indicates that the replenishment of organic carbons via fluid currents in the oil reservoir is likely to be much slower than that in the hydrothermal environment.

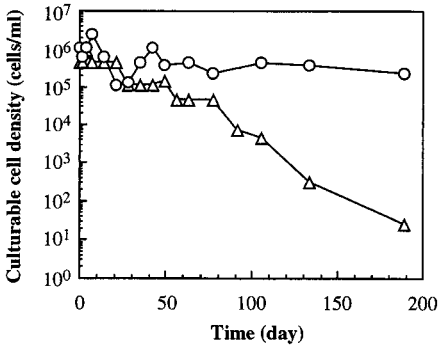

FIG. 4. Survival of strains CKU-1 (○) and RKU-1 (△) in KFW medium at 50°C.

Under starved conditions, the culturable cell density of heterotrophic microorganisms generally decreases in a logarithmic fashion (46). While the culturable cell density of strain RKU-1 slowly decreased under starved conditions in the formation water of the Kubiki oil reservoir, that of strain CKU-1 was almost constant for about 200 days (Fig. 4). Survivability under starved conditions may be important for their existence in the oil reservoir because supplies of the essential nutrients are likely to be limited. The difference of survivability between these two isolates seems to reflect their cell densities in the oil reservoir.

Distribution and physiological characteristics of hyperthermophilic archaea and bacteria in the Kubiki oil reservoir indicate that these organisms adapt to the subterranean environment where temperatures are lower than the optimum. The existence of hyperthermophiles in the oil reservoir at low temperature and under starved conditions suggests that they are distributed widely in the ground.

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