Microbiology, Vol. 73, No. 5, 2004, pp. 616–620. Translated from Mikrobiologiya, Vol. 73, No. 5, 2004, pp. 716–720.

EXPERIMENTAL ARTICLES

Detection of a Culturable Hyperthermophilic Archaeon of the Genus *Sulfophobococcus* in an Anaerobic Digestor Operated in a Thermophilic Regime

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Received November 3, 2003

Abstract—A stable association of hyperthermophilic microorganisms (82°C), which contained mostly cocci and a minor amount of non-spore-forming rods, was obtained from the digested sludge of an anaerobic digestor used to process municipal wastewater under thermophilic conditions (50°C). PCR amplification of 16S rRNA genes using total DNA isolated from this association and archaea-specific primers, followed by sequencing of the product obtained, showed that the archaeal component was represented by a single nucleotide sequence, which was 99.9% homologous to the 16S rRNA gene of *Sulfophobococcus zilligii.* Thus, a hyperthermophilic archaeon was for the first time detected in a system of anaerobic biological treatment of wastewater. In addition, this is the first report on the detection of a culturable member of *Crenarchaeota* in anthropogenic habitats with neutral pH.

Key words: hyperthermophilic archaea, *Sulfophobococcus*, anaerobic digestor.

In nature, hyperthermophilic microorganisms (with a temperature growth optimum of at least 80°C) inhabit various freshwater and marine hydrothermal vents and geothermal subsurface waters [1, 2]. There have been several reports about the isolation of hyperthermophilic archaea from cold seawater [3, 4], where they most probably survive after efflux from submarine hydro- or geothermal systems. Thermophilic microorganisms capable of growing at 60–70°C are mostly spore-forming and are routinely isolated from various nonthermal biotopes, such as soils, sediments, and intestinal tracts of animals [5]; however, hyperthermophilic prokaryotes have never been found in these habitats.

The high-temperature microbial habitats of anthropogenic origin include heaps of self-heated organic materials (compost, grain, peat, and coal), hot water supply systems, and biological waste treatment plants operated in a thermophilic regime. Currently, bacteria of the genera *Thermus* and *Hydrogenobacter* (maximum growth temperature of up to 80°C), identified in compost, and the acidophilic crenarchaeota *Metallosphaera prunae* (optimal growth at 75°C), isolated from a uranium mine dump, represent the most high-temperature microorganisms developing in anthropogenic biotopes [6–8]. Although the microflora of biological waste treatment systems, including anaerobic digestors, has been studied by both cultural [9] and molecular ecological methods [10], no culturable hyperthermophilic microorganisms or nucleotide sequences of their 16S rRNA genes have been found so far in these systems.

In this work, we report the detection of a culturable representative of the hyperthermophilic archaeal genus *Sulfophobococcus* in the digested sludge of an anaerobic digestor used to process municipal wastewater under thermophilic conditions. Some physiological characteristics of this microorganism are presented.

MATERIALS AND METHODS

Sampling. Samples of digested sludge were taken from an anaerobic digestor operated in a thermophilic regime (52.6°C) to process activated sludge formed during the treatment of municipal wastewater at Kur'yanovskaya plant (Moscow). The samples were kept in tightly closed flasks at –4°C for 18 h and then used to initiate enrichment cultures.

Medium composition and cultivation. Hyperthermophilic cultures were grown on medium of the following composition (per liter of distilled water): NH_4Cl, 0.33 g; KCl, 0.33 g; $MgCl_2 \cdot 6H_2O$, 0.33 g; KH_2PO_4, 0.33 g; $NaHCO_3$, 2 g; trace element solution [11], 1 ml; vitamin solution [12], 1 ml; yeast extract (Sigma), 0.2 g; peptone, 10 g. The medium was prepared under anaerobic conditions, dispensed into Hungate tubes under a flow of 100% CO_2, and sterilized in an autoclave at 2 atm for 1 h. The medium contained no reducing agents. The pH of the autoclaved medium was 6.7–6.9 at 20°C. Initial enrichment cultures were grown on the above medium supplemented with 90 mM amorphous Fe(III) oxide prepared as described in [13].

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PCR primers used in this study and the results of amplification (approximate size of the amplification product, bp)

Primer	Nucleotide sequence (5' ⟶ 3')	Specificity, reference	Template DNA from			
			sample	enrichment culture		
				after three transfers on medium with Fe(III)	after five transfers	KP4
U 515F	GTGBCAGCMGCCGCGGTAA	SSU rRNA gene of prokaryotes and eukaryotes [16]	1000	1000	1000	1000
U 1492R	GGTTACCTTGTTACGACTT					
Arch 2F	TTCCGGTTGATCCYGCCGGA	16S rRNA gene of archaea [16]	–	950	950	950
Arch 958R	YCCGGCGTTGAMTCCAATT					
Cren 7F	TTCCGGTTGATCCYGCCGGACC	16S rRNA gene of *Crenarchaeota* [14]	–	500	500	500
Cren 518R	GCTGGTWTTACCGCGGCGGCTGA					
TcPc 173F	TCCCCCATAGGYCTGRGGTACTGGAAGGTC	16S rRNA gene of the family *Thermococcaceae* [15]	–	–	–	–
TcPc 589R	GCCGTGRGATTTCGCCAGGGACTTACGGGC					

Note: "–", no amplification products.

To obtain colonies, 20-ml flasks containing agarized (2.5% Bacto Agar) anaerobic medium of the same composition under 100% CO_2 and closed with rubber stoppers and aluminum caps were inoculated with tenfold serial dilutions of enrichment cultures. After solidification of the agar, the flasks were incubated at 82°C. Grown individual colonies were selected with a syringe and transferred to liquid medium. If not otherwise specified, cultivation was performed at 82°C.

The pure culture of *Sulfophobococcus zilligii* DSM 11193^T was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown on an appropriate medium (770) according to DSMZ recommendations (www.dsmz.de).

Light and electron microscopy. Microscopic examinations and cell counts were performed using a LOMO AU-12 phase-contrast microscope (Russia). Transmission electron microscopy was performed with a JEM-100C electron microscope.

DNA isolation and amplification and sequencing of 16S rRNA genes. Isolation and purification of genomic DNA and selective PCR amplification of the 16S rRNA gene and its sequencing were performed as described previously [13, 17]. Oligonucleotide primers used in PCR analysis of the enrichment culture are shown in the table. Amplification conditions are described in [14–16].

RESULTS

Obtaining of a culture of hyperthermophilic microorganisms. Samples (1 ml) of digested sludge from an anaerobic digestor were introduced into Hungate tubes containing 10 ml of sterile anaerobic medium supplemented with amorphous Fe(III) oxide. After incubation at 82°C for seven days, transformation of the brown nonmagnetic amorphous Fe(III) oxide to a black magnetic precipitate of a smaller volume occurred in inoculated variants, which was a result of the reduction of iron from ferric to ferrous state. After three successive 5% transfers on medium of the same composition, stable reduction of Fe(III) to Fe(II) was observed in the enrichment culture, which was a mixture of cocci and non-spore-forming rods of two or three morphotypes. Subsequent experiments were aimed at obtaining a culture of the cocci, which were likely to be archaea. Domination of cocci was achieved after cultivation on medium containing no amorphous Fe(III) oxide. After tenfold serial dilutions, growth in liquid medium occurred only in the 10^{-1} dilution. Individual colorless colonies 0.4–0.7 mm in diameter were obtained on agarized medium from the 10^{-3} and 10^{-4} dilutions. Nine colonies were selected and incubated in the liquid medium at 82°C for eight days. Examinations under light and electron microscopes revealed domination of regular-shaped cocci 2–5 μm in diameter, which occurred individually or in pairs, and a minor amount (no more than 2%) of non-spore-forming rods (Fig. 1). Repeated obtaining of individual colonies

Fig. 1. Electron micrograph of culture KP4. Negative staining with phosphotungstic acid. Bar, 1 μm.

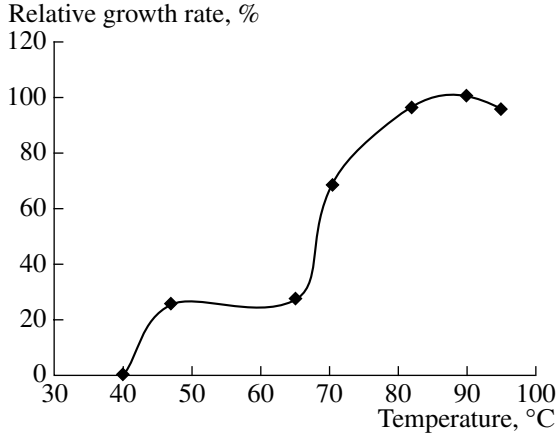

Fig. 2. Effect of temperature on the growth of *Sulfophobococcus* sp. in culture KP4. The growth rate corresponding to a doubling time of 3.4 h was taken as 100%.

and their transfer to liquid medium yielded the same results. We suggested that the culture under study was a stable association of two microorganisms, likely based on symbiotic relationships, and that the isolation of a pure culture of the coccoid component is impossible. This mixed culture was designated as KP4.

PCR analysis of the enrichment cultures. At different stages of the enrichment culture purification, total genomic DNA was isolated and used in PCR analyses with 16S rDNA–targeted oligonucleotide primers: universal and specific to archaea, *Crenarchaeota*, and *Thermococcaceae* (table). For the original sample of digested sludge taken from the anaerobic digestor, a positive result of PCR was recorded only with the universal primers. For the enrichment cultures that experienced three successive transfers on medium containing amorphous Fe(III) oxide or five transfers on iron-free medium, as well as for culture KP4, PCR products were obtained with all primers except the primer pair specific to *Thermococcaceae.* In all positive PCR reactions, a single product of the expected size was obtained.

Amplification and sequencing of the 16S rRNA genes. Selective PCR amplification of the 16S rRNA gene was performed using total genomic DNA isolated from enrichment culture KP4 and *Archaea*-specific primers [17]. Sequencing of the obtained product (884 nucleotides) showed that the archaeal component was represented by a single nucleotide sequence that was 99.9% homologous to the 16S rRNA gene of *Sulfophobococcus zilligii.*

Physiological characteristics of the growth of culture KP4. Culture KP4 grew within a temperature range of 47–98°C with an optimum at 82°C (Fig. 2). No growth was observed at 40°C or above 98°C. In the temperature range 87–95°C, good growth of only coccoid cells (2×10^8 cells/ml) was observed after the first culture transfer; however, after the next transfer, the culture failed to grow. The pH growth optimum was 7.4–7.6 (measured at 80°C); at pH below 6.5 and above 8.5, neither cocci nor rods grew. Culture KP4 grew only under anaerobic conditions; the growth was neither stimulated nor inhibited by the reducing agents $Na_2S \cdot 9H_2O$ (0.5 g/l), titanium(III) citrate (2 mM), or dithiothreitol (1 mM). Elemental sulfur (10 g/l), sodium sulfate (14 mM), sodium thiosulfate (20 mM), sodium fumarate (20 mM), and amorphous Fe(III) oxide (90 mM) neither stimulated nor inhibited growth with peptone as an electron donor. In the absence of peptone, the culture could grow on beef extract (1%) but not on yeast extract (1%). After the first culture transfer, the growth of the coccoid component of culture KP4 was not inhibited by kanamycin (200 μg/ml), neomycin (200 μg/ml), streptomycin (200 μg/ml), or chloramphenicol (100 μg/ml); however, after the next transfer, the culture failed to grow even in the absence of antibiotics.

DISCUSSION

The stable association of hyperthermophilic microorganisms obtained by us (KP4) consists of predominating cocci and a minor amount of non-spore-forming rods, which are most probably bacteria. In this associa-

tion, PCR analysis revealed the presence of *Crenarchaeota* and sequencing of the amplified 16S rRNA gene fragment showed that *Sulfophobococcus zilligii* was the only archaeal component of the culture. The genus *Sulfophobococcus*, belonging to the family *Desulfurococcaceae*, is represented by a single species *Sulfophobococcus zilligii* isolated from a hot spring in Iceland [18]. No other isolates of this genus have been reported. Although the coccoid component (*Sulfophobococcus* sp.) of culture KP4 is similar in morphology to the type strain of *Sulfophobococcus zilligii* (and their 16S rRNA genes are almost completely homologous), it exhibits certain physiological distinctions: (1) its growth is not inhibited by sulfur; (2) it grows on peptone and beef extract and does not grow on yeast extract; and (3) it does not require reduced medium for growth. *Sulfophobococcus* sp. from culture KP4 can grow at temperatures above 87°C only after the first culture transfer; its resistance to antibiotics also is lost after the second culture transfer. Evidently, a symbiotic relationship exists between the bacterial and archaeal components of culture KP4 and this accounts for the inability of the components to grow individually. Under conditions inadequate for the bacterial component (high temperature, presence of antibiotics in the medium), the crenarchaeotal component also perishes after the next culture transfer.

All known cultivated *Crenarchaeota* are thermophilic or hyperthermophilic organisms. They were isolated from continental or submarine hydrothermal systems or from hot anthropogenic biotopes with acidic pH [1]. With the development of methods of molecular biology, reports appeared on the detection of crenarchaeotal 16S rRNA genes in various ecosystems, such as marine picoplankton, continental shelf sediments, estuaries, freshwater lake sediments, and forest soils (see [19] for references). The 16S rRNA genes of *Crenarchaeota* were also detected in a laboratory anaerobic bioreactor where winery wastes were treated at 35°C [20]. However, no culturable members of the kingdom *Crenarchaeota* have been identified so far in anthropogenic habitats with neutral pH.

Our results extend the list of habitats populated by the phylogenetic group *Crenarchaeota*, as well as by the physiological group of hyperthermophilic microorganisms. Although the PCR method failed to reveal *Crenarchaeota* directly in the digested sludge of the anaerobic digestor, it may be assumed that the culturable *Sulfophobococcus* sp. identified by us, which has a lower growth temperature limit of 47°C, is capable of developing in this ecosystem both in the bulk of sludge and in local superheated zones. Thus, hyperthermophilic *Crenarchaeota* are part of the microbial community that degrades organic municipal wastes.

ACKNOWLEDGMENTS

This work was supported by the Program on Physicochemical Biology of the Russian Academy of Sciences and by a grant awarded as a result of the 1999 competition of basic and applied research projects submitted by young scientists of the Russian Academy of Sciences (project no. 196).

REFERENCES

1. Stetter, K.O., Hyperthermophilic Prokaryotes, *FEMS Microbiol. Rev.*, 1996, vol. 18, pp. 149–158.
2. Slobodkin, A.I., Zavarzina, D.G., Sokolova, T.G., and Bonch-Osmolovskaya, E.A., Dissimilatory Reduction of Inorganic Electron Acceptors by Thermophilic Anaerobic Prokaryotes, *Mikrobiologiya*, 1999, vol. 68, pp. 600–622.
3. Huber, R., Stoffers, P., Cheminee, J.L., Richnow, H.H., and Stetter, K.O., Hyperthermophilic Archaebacteria within the Crater and Open-Sea Plume of Erupting Macdonald Seamount, *Nature*, 1990, vol. 345, pp. 179–182.
4. Stetter, K.O., Huber, R., Blochl, E., Kurr, M., Eden, R.D., Fiedler, M., Cash, H., and Vance, I., Hyperthermophilic Archaea Are Thriving in Deep North Sea and Alaskan Oil Reservoirs, *Nature*, 1993, vol. 300, pp. 743–754.
5. Mishustin, E.N., *Termofil'nye mikroorganizmy v prirode i praktike* (Thermophilic Microorganisms in Nature and in Agricultural and Industrial Practice), Moscow: Akad. Nauk SSSR, 1950.
6. Feffa, T., Blanc, M., Lyon, P.-F., Vogt, G., Marchiani, M., Fisher, J.L., and Aragno, M., Isolation of *Thermus* Strains from Hot Composts (60 to 80°C), *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 1723–1727.
7. Feffa, T., Blanc, M., and Aragno, M., Obligately and Facultatively Autotrophic, Sulfur- and Hydrogen-Oxidizing Thermophilic Bacteria Isolated from Hot Composts, *Arch. Microbiol.*, 1996, vol. 165, pp. 34–40.
8. Fuchs, T., Huber, H., Teiner, K., Burggraf, S., and Stetter, K.O., *Metallosphaera prunae* sp. nov., a Novel Metal-Mobilizing, Thermoacidophilic Archaeum, Isolated from a Uranium-Mine in Germany, *Syst. Appl. Microbiol.*, 1995, vol. 18, pp. 550–556.
9. Kalyuzhnyi, S.V., Danilovich, D.A., and Nozhevnikova, A.N., Anaerobic Biological Treatment of Wastewaters, *Itogi Nauki Tekh., Ser. Biotekhnol.*, 1991, vol. 29.
10. Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H., and Nakamura, K., Phylogenetic Diversity of Mesophilic and Thermophilic Granular Sludges Determined by 16S rRNA Gene Analysis, *Microbiology* (UK), 1998, vol. 144, pp. 2655–2665.
11. Slobodkin, A., Reysenbach, A.-L., Strutz, N., Dreier, M., and Wiegel, J., *Thermoterrabacterium ferrireducens* gen. nov., sp. nov., a Thermophilic Anaerobic Dissimilatory Fe(III)-Reducing Bacterium from a Continental Hot Spring, *Int. J. Syst. Bacteriol.*, 1997, vol. 47, pp. 541–547.
12. Wolin, E.A., Wolin, M.J., and Wolfe, R.S., Formation of Methane by Bacterial Extracts, *J. Biol. Chem.*, 1963, vol. 238, pp. 2882–2886.
13. Slobodkin, A.I., Tourova, T.P., Kuznetsov, B.B., Kostrikina, N.A., Chernyh, N.A., and Bonch-Osmolovskaya, E.A., *Thermoanaerobacter siderophilus* sp. nov., a Novel Dissimilatory Fe(III)-Reducing, Anaerobic, Thermophilic Bacterium, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 1471–1478.

14. Perevalova, A.A., Lebedinsky, A.V., Bonch-Osmolovskaya, E.A., and Chernyh, N.A., Detection of Hyperthermophilic Archaea of the Genus *Desulfurococcus* by Hybridization with Oligonucleotide Probes, *Mikrobiologiya*, 2003, vol. 72, pp. 383–389.
15. Slobodkina, G.B., Subbotina, I.V., Lebedinskii, A.V., Slobodkin, A.I., Bonch-Osmolovskaya, E.A., and Chernych, N.A., PCR-Based Detection and Identification of Hyperthermophilic Archaea of the family *Thermococcaceae*, *Abstr. 4th Int. Congress on Extremophiles, September 22–26, 2002, Naples, Italy*, p. 181.
16. Reysenbach, A.-L. and Pace, N.R., Reliable Amplification of Hyperthermophilic Archaeal 16S rRNA Genes by the Polymerase Chain Reaction, *Archaea: A Laboratory Manual*, Robb, F.T. *et al.*, Eds., Cold Spring Harbor: Cold Spring Harbor Lab., 1995, pp. 101–105.
17. Kolganova, T.V., Kuznetsov, B.B., and Tourova, T.P., Designing and Testing Oligonucleotide Primers for Amplification and Sequencing of Archaeal 16S rRNA genes, *Mikrobiologiya*, 2002, vol. 71, pp. 283–286.
18. Hensel, R., Matussek, K., Michalke, K., Tacke, L., Tindall, B.J., Kohlhoff, M., Siebers, B., and Dielenschneider, J., *Sulfophobococcus zilligii* gen. nov., spec. nov., a Novel Hyperthermophilic Archaeum Isolated from Hot Alkaline Springs of Iceland, *Syst. Appl. Microbiol.*, 1997, vol. 20, pp. 102–110.
19. Abreu, C., Jurgens, G., De Marco, P., Saano, A., and Bordalo, A.A., Crenarchaeota and Euryarchaeota in Temperate Estuarine Sediments, *J. Appl. Microbiol.*, 2001, vol. 90, pp. 713–718.
20. Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R., Molecular Microbial Diversity of an Anaerobic Digestor as Determined by Small-Subunit rRNA Sequence Analysis, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 2802–2813.