APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2005, p. 1084–1088 Vol. 71, No. 2
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.2.1084–1088.2005

Phylogenetic Characterization of 16S rRNA Gene Clones from Deep-Groundwater Microorganisms That Pass through 0.2-Micrometer-Pore-Size Filters

Tatsuo Miyoshi,[1] Teruki Iwatsuki,[2] and Takeshi Naganuma[1,2]*

Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima,[1] and Tono Geoscience Center, Japan Nuclear Cycle Development Institute, Izumicho, Toki,[2] Japan

Received 8 June 2004/Accepted 16 September 2004

A total of 247 clones of 16S rRNA genes from microorganisms captured by 0.2- and 0.1-μm-pore-size filters from sedimentary and granite rock aquifers were amplified and yielded 37 operational taxonomic units (OTUs). Fifteen OTUs captured by 0.1-μm-pore-size filters were affiliated with the candidate divisions OD1 and OP11, representing novel lineages. On the other hand, OTUs captured by 0.2-μm-pore-size filters were largely affiliated with *Betaproteobacteria*.

The existence of microorganisms passing through 0.2-μm-pore-size filters has been questioned based on the minimum requisite size for a complete set of genome, ribosomes, proteins, and other intracellular components (21). Geologically, 0.05- to 0.2-μm-diameter spheres on mineral surfaces are presumably involved in biomineralization (6, 7). Similarly, <0.2-μm-diameter spheres mineralized with apatite have been observed in medical samples and affiliated with *Alphaproteobacteria* (1, 4, 17). Certain aquatic bacteria are filterable by 0.2-μm-pore-size filters (5, 9, 10, 18, 24, 30, 31), and the archaeon *Nanoarchaeum equitans* is also smaller than 0.2 μm (13).

Molecular techniques phylogenetically characterize the microbial communities filterable by 0.2-μm-pore-size filters, and a Mediterranean community was thus affiliated with *Alphaproteobacteria*, *Gammaproteobacteria*, and *Cytophaga-Flavobacterium-Bacterioides* (11). On the other hand, few or no studies have focused on the microorganisms in deep groundwater that are filterable by 0.2-μm-pore-size filters. We have characterized the microorganisms captured by 0.2- and 0.1-μm-pore-size filters in deep groundwaters of the Tono uranium mine, Japan (35.4°N, 137.2°E), where total counts of cells captured by 0.2-μm-pore-size filters are in the range of 10^5 to 10^6 ml^{-1} (22, 23).

Groundwaters were collected from aquifers in sedimentary rock (161 to 163 m below ground level) and granite rock (177 to 227 m below ground level) through separate Teflon tubes after >1,000 passages of the aquifer waters. The temperature and pH of the anaerobic groundwaters are 19.0 to 20.1°C and 7.8 to 9.6, respectively (23). Sterivex filters (0.2-μm pore size; Millipore Corp., Bedford, Mass.) were connected to Teflon tubes to capture >0.2-μm-diameter cells in 10 liters (each) of groundwater. In parallel, 0.1-μm-pore-size Sterivex filters captured microorganisms filterable by 0.2-μm-pore-size filters in 100 liters (each) of the 0.2-μm filtrate.

Bulk DNA was extracted from the 0.2- and 0.1-μm-pore-size Sterivex filters washed with 1 ml of SET buffer (20% sucrose, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.6) after cell lysis according to the method of Somerville et al. (28). The lysates were used for DNA preparation by phenol-chloroform extraction and isopropanol precipitation. The 0.2-μm-pore-size filter-captured cells in 10 liters of sedimentary and granite rock groundwater yielded 0.93 and 1.17 μg of DNA, respectively, while 0.1-μm-pore-size filter-captured cells in 100 liters of the 0.2-μm filtrate yielded 0.70 and 0.62 μg of DNA, respectively.

DNA preparations were used for PCR amplification of 16S rRNA genes using the *Archaea*-specific primers Arch21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and Arch958R (5′-YCCGGCGTTGAMTCCAATT-3′) and the *Bacteria* universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (3). Bacterial (archaeal in parentheses) 16S rRNA gene sequences were amplified with ExTaq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) as follows: 1 cycle of 96°C for 3 min, 56°C for 25 s (53 to 56°C for 40 s), and 72°C for 15 s (40 s) and 27 cycles of 96°C for 30 s (40 s), 56°C for 25 s (53°C for 30 s), and 72°C for 15 s (40 s), followed by 72°C for 10 min. The PCR products were purified by agarose gel electrophoresis and cloned into pCR2.1-TOPO in the *Escherichia coli* TOP10 transformants (Invitrogen Corp., San Diego, Calif.).

The cloned inserts were grouped by restriction fragment length polymorphism (RFLP) to form operational taxonomic units (OTUs) using the type II restriction enzymes (subtype P) HaeIII and BglII simultaneously. After restriction digestion at 37°C for 4 h, the banding patterns were compared by electrophoresis with a resolution of 1 bp using an Agilent Technologies (Palo Alto, Calif.) 2100 Bioanalyzer, and clones showing the same RFLP pattern were grouped into a single OTU. Cloned inserts were sequenced using a multicapillary DNA sequencer (RISA-384; Shimadzu Corp., Kyoto, Japan) with a Dynamic ET Terminator Cycle Sequencing kit (Amersham Bioscience Corp., Piscataway, N.J.), applying the following primers: 27F, 357F (5′-TACGGGAGGCAGCAG-3′), 926F

* Corresponding author. Mailing address: Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan. Phone: 81-82-424-7986. Fax: 81-82-422-7916. E-mail: takn@hiroshima-u.ac.jp.

TABLE 1. Clone number distributions and phylogenetic affiliations of OTUs among the 0.2- and 0.1-μm-pore-size filter-captured microorganisms from sedimentary and granite rock aquifers in the Tono uranium mine

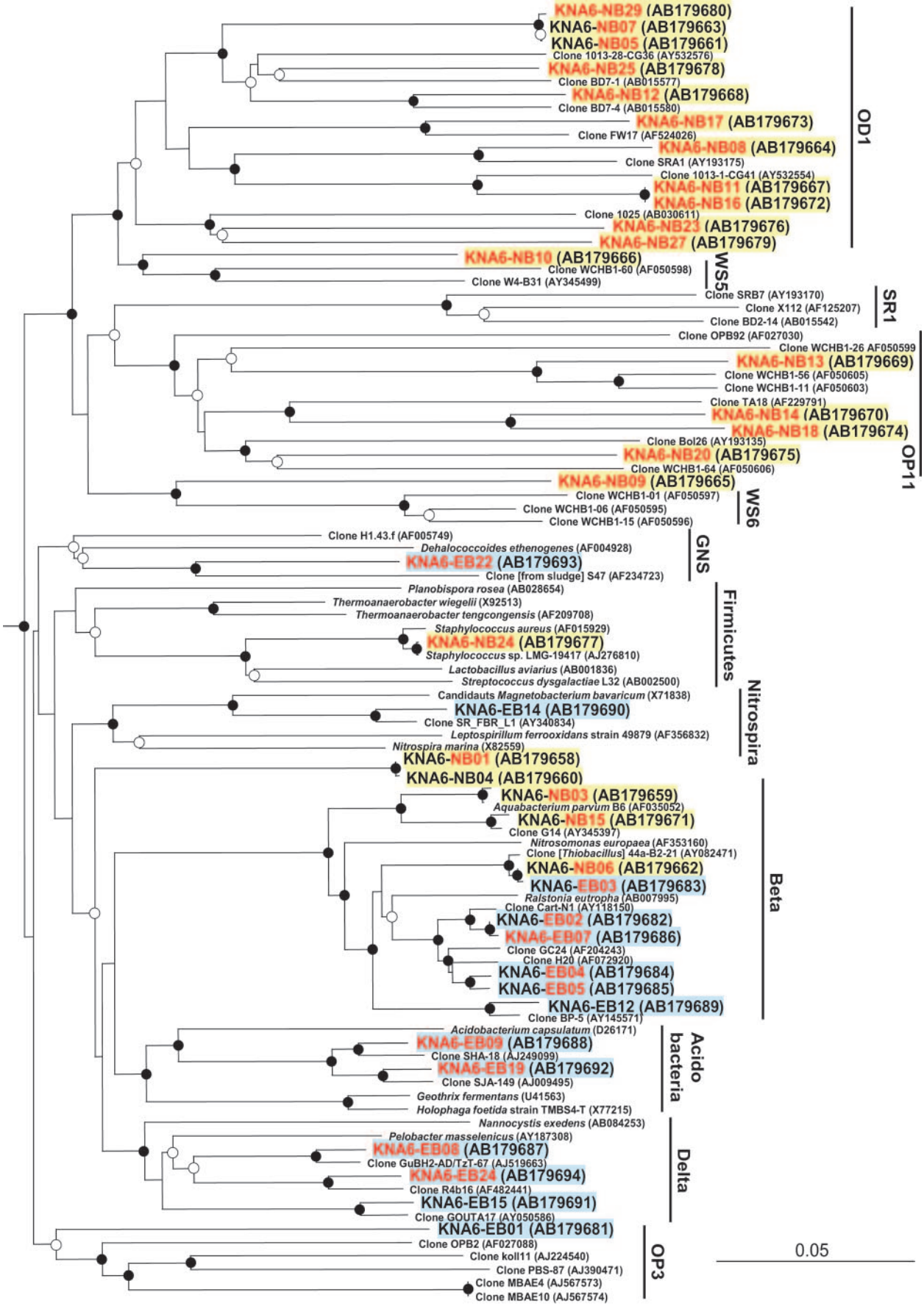
Filter size (μm)	OTU KNA6-	No. of sediment clones	No. of granite clones	DDBJ accession no.	Putative affiliation[a]	Closest sequence (>95% similarity)	Similarity (%)	Closest organism (>95% similarity)	Similarity (%)
0.1	NB05	1	1	AB179661	Cand. div. OD1				
	NB07	1	2	AB179663	Cand. div. OD1				
	NB08		3	AB179664	Cand. div. OD1				
	NB11		7	AB179667	Cand. div. OD1				
	NB12		2	AB179668	Cand. div. OD1				
	NB16		2	AB179672	Cand. div. OD1				
	NB17		2	AB179673	Cand. div. OD1				
	NB23		1	AB179676	Cand. div. OD1				
	NB25		1	AB179678	Cand. div. OD1				
	NB27		1	AB179679	Cand. div. OD1				
	NB29		1	AB179680	Cand. div. OD1				
	NB13		3	AB179669	Cand. div. OP11				
	NB14		10	AB179670	Cand. div. OP11				
	NB18		2	AB179674	Cand. div. OP11				
	NB20		2	AB179675	Cand. div. OP11				
	NB03	5	24	AB179659	β-*Proteobacteria*	Clone B35 (AY375072)	98.3	*Aquabacterium parvum* B6 (AF035052)	99.1
	NB06	2	2	AB179662	β-*Proteobacteria*	Clone [*Thiobacillus*] 44a-B2-21 (AY082471)	98.7	*Thiobacillus denitrificans* NCIMB	97.5
	NB15	1	4	AB179671	β-*Proteobacteria*	Clone G14 (AY345397)	99.0	Acidovorax sp. "smarlab 133815" (AY093698)	98.6
	NB24		1	AB179677	*Firmicutes*	Clone 2e (AJ223451)	99.4	*Staphylococcus* sp. strain LMG-19417 (AJ276810)	99.7
	NB01	20	2	AB179658	Unidentified				
	NB04	2		AB179660	Unidentified				
	NB09		13	AB179665	Unidentified				
	NB10		4	AB179666	Unidentified				
Total		32	90						
0.2	EB01	3		AB179681	Cand. div. OP3				
	EB02	32	47	AB179682	β-*Proteobacteria*	Clone Cart-N1 (AY118150)	97.3		
	EB03	9	2	AB179683	β-*Proteobacteria*	Clone [*Thiobacillus*] 44a-B2-21 (AY082471)	98.4	*T. denitrificans* NCIMB 9548 (AJ243144)	97.3
	EB04	14	1	AB179684	β-*Proteobacteria*	Clone GC24 (AF204243)	95.5		
	EB05	3	1	AB179685	β-*Proteobacteria*	Clone H20 (AF072920)	95.5		
	EB07		2	AB179686	β-*Proteobacteria*	Clone Cart-N1 (AY118150)	97.1		
	EB12	1		AB179689	β-*Proteobacteria*	Clone 244ds10 (AY212692)	96.3		
	EB08		3	AB179687	δ-*Proteobacteria*	Clone GuBH2-AD/TzT-67 (AJ519663)	97.0		
	EB15	1		AB179691	δ-*Proteobacteria*				
	EB24		1	AB179694	δ-*Proteobacteria*				
	EB09		2	AB179688	*Acidobacteria*	Clone SHA-18 (AJ249099)	96.0		
	EB19		1	AB179692	*Acidobacteria*	Clone SJA-149 (AJ009495)	97.2		
	EB22		1	AB179693	Green nonsulfur				
	EB14	1		AB179690	*Nitrospira*				
Total		64	61						

[a] Cand. div., candidate division.

(5′-ACTCAAAGGAATTGACGG-3′), 1100R (5′-AGGGTTGCGCTCGTTG-3′), and 1492R.

The OTUs with chimeric 16S rRNA gene sequences were removed by the program Chimera Check at the Ribosomal Database Project II and Bellerophon server (14). The remaining sequences were further chimera checked by trisecting (nucleotide positions 28 to 500, 501 to 1000, and 1001 to 1491), with the sequences showing different trisect trees removed (15). The surviving OTUs were searched for homology using the program FASTA at the DNA Database Bank of Japan (DDBJ), aligned with ClustalW (29), and used to construct a phylogenetic tree by the neighbor-joining algorithm (27) with TreeView (25). The 16S rRNA gene sequences of the chimera-checked OTUs were registered in DDBJ as listed in Table 1.

This study yielded 247 chimera-checked clones of bacterial 16S rRNA genes. No archaeal clones were obtained despite repeated attempts. Of the 247 clones, 122 were from the microorganisms captured by 0.1-μm-pore-size filters (32 and 90 from sedimentary and granite aquifers, respectively), while 125 were from the microorganisms captured by 0.2-μm-pore-size


KNA6-NB29 (AB179680)
KNA6-NB07 (AB179663)
KNA6-NB05 (AB179661)
Clone 1013-28-CG36 (AY532576)
KNA6-NB25 (AB179678)
Clone BD7-1 (AB015577)
KNA6-NB12 (AB179668)
Clone BD7-4 (AB015580)
KNA6-NB17 (AB179673)
Clone FW17 (AF524026)
KNA6-NB08 (AB179664)
Clone SRA1 (AY193175)
Clone 1013-1-CG41 (AY532554)
KNA6-NB11 (AB179667)
KNA6-NB16 (AB179672)
Clone 1025 (AB030611)
KNA6-NB23 (AB179676)
KNA6-NB27 (AB179679)
KNA6-NB10 (AB179666)
Clone WCHB1-60 (AF050598)
Clone W4-B31 (AY345499)
Clone SRB7 (AY193170)
Clone X112 (AF125207)
Clone BD2-14 (AB015542)
Clone OPB92 (AF027030)
Clone WCHB1-26 AF050599
KNA6-NB13 (AB179669)
Clone WCHB1-56 (AF050605)
Clone WCHB1-11 (AF050603)
Clone TA18 (AF229791)
KNA6-NB14 (AB179670)
KNA6-NB18 (AB179674)
Clone Bol26 (AY193135)
KNA6-NB20 (AB179675)
Clone WCHB1-64 (AF050606)
KNA6-NB09 (AB179665)
Clone WCHB1-01 (AF050597)
Clone WCHB1-06 (AF050595)
Clone WCHB1-15 (AF050596)
Clone H1.43.f (AF005749)
Dehalococcoides ethenogenes (AF004928)
KNA6-EB22 (AB179693)
Clone [from sludge] S47 (AF234723)
Planobispora rosea (AB028654)
Thermoanaerobacter wiegelii (X92513)
Thermoanaerobacter tengcongensis (AF209708)
Staphylococcus aureus (AF015929)
KNA6-NB24 (AB179677)
Staphylococcus sp. LMG-19417 (AJ276810)
Lactobacillus aviarius (AB001836)
Streptococcus dysgalactiae L32 (AB002500)
Candidauts Magnetobacterium bavaricum (X71838)
KNA6-EB14 (AB179690)
Clone SR_FBR_L1 (AY340834)
Leptospirillum ferrooxidans strain 49879 (AF356832)
Nitrospira marina (X82559)
KNA6-NB01 (AB179658)
KNA6-NB04 (AB179660)
KNA6-NB03 (AB179659)
Aquabacterium parvum B6 (AF035052)
KNA6-NB15 (AB179671)
Clone G14 (AY345397)
Nitrosomonas europaea (AF353160)
Clone [Thiobacillus] 44a-B2-21 (AY082471)
KNA6-NB06 (AB179662)
KNA6-EB03 (AB179683)
Ralstonia eutropha (AB007995)
Clone Cart-N1 (AY118150)
KNA6-EB02 (AB179682)
KNA6-EB07 (AB179686)
Clone GC24 (AF204243)
Clone H20 (AF072920)
KNA6-EB04 (AB179684)
KNA6-EB05 (AB179685)
KNA6-EB12 (AB179689)
Clone BP-5 (AY145571)
Acidobacterium capsulatum (D26171)
KNA6-EB09 (AB179688)
Clone SHA-18 (AJ249099)
KNA6-EB19 (AB179692)
Clone SJA-149 (AJ009495)
Geothrix fermentans (U41563)
Holophaga foetida strain TMBS4-T (X77215)
Nannocystis exedens (AB084253)
Pelobacter masselenicus (AY187308)
KNA6-EB08 (AB179687)
Clone GuBH2-AD/TzT-67 (AJ519663)
KNA6-EB24 (AB179694)
Clone R4b16 (AF482441)
KNA6-EB15 (AB179691)
Clone GOUTA17 (AY050586)
KNA6-EB01 (AB179681)
Clone OPB2 (AF027088)
Clone koll11 (AJ224540)
Clone PBS-87 (AJ390471)
Clone MBAE4 (AJ567573)
Clone MBAE10 (AJ567574)
OD1
WS5
SR1
OP11
WS6
GNS
Firmicutes
Nitrospira
Beta
Acido bacteria
Delta
OP3
0.05

filters (64 and 61 from sedimentary and granite aquifers, respectively). RFLP of the 122 and 125 clones yielded 23 (0.1-μm-pore-size filter-captured) and 14 (0.2-μm-pore-size filter-captured) OTUs, respectively. No overlaps in the 0.1- and 0.2-μm-pore-size filter-captured OTUs were found, and thus a total of 37 OTUs were obtained (Table 1).

Among the 0.1-μm-pore-size filter-captured OTUs, 15 of 23 OTUs (with 13 of 15 only from the granite aquifer) were related to the candidate divisions OD1 and OP11 of putative bacterial phyla (Fig. 1). The candidate division OP11 phylum consisted of five subdivisions (16), and subdivisions 4 and 5 have recently been considered independent candidate divisions SR1 and OD1, respectively (12). SR1, OD1, and OP11 contain clones from various anaerobic habitats (2, 15, 19, 20, 26, 32, 33), as from the Tono subsurface.

The OTUs KNA6-NB09 (the common prefix KNA6- is omitted hereafter) and NB10 in particular branched deeply from the candidate divisions WS6 and WS5, respectively, probably forming novel candidate divisions (Fig. 1). Novel lineages among the 0.1-μm-pore-size filter-captured microorganisms were also suggested by four other OTUs. The OTUs NB09 and NB10 were only weakly related to the candidate divisions WS6 and WS5, with similarities of 79.7 and 81.0%, respectively (Table 1). The OTUs NB01 and NB04 were closely related to each other at a similarity of 99.9% and are considered a single group, although NB04 was found only in the sedimentary aquifer while NB01 was recovered from both aquifers (Table 1).

The abundance of 0.1-μm-pore-size filter-captured microorganisms should be only minor compared with that of 0.2-μm-pore-size filter-captured microorganisms in water samples, as shown by the finding of 10^0 to 10^1 microorganisms filterable by 0.2-μm-pore-size filters liter^{-1}, as determined by the most-probable-number method, versus 10^8 to 10^9 0.2 μm-pore-size filter-captured cells l^{-1} (acridine orange direct counts) in coastal waters (8). Yet despite possibly being of quantitatively minor importance, the 0.1-μm-pore-size filter-captured microorganisms are a source of novel microbial diversity.

Of 14 OTUs among 0.2-μm-pore-size filter-captured microorganisms, 11 were affiliated with known *Betaproteobacteria*, *Deltaproteobacteria*, and *Acidobacteria* at high similarities of 93.2 to 98.4%. Three OTUs, EB01, EB14, and EB22, branched deeply from the candidate division OP3, *Nirospira*, and green nonsulfur bacteria, respectively. Although these deep-branching OTUs are likely minor components in the 0.2-μm-pore-size filter-captured populations (Table 1), their presence demonstrates the importance of deep groundwater as a source of unique and novel microorganisms even in the >0.2-μm-diameter regime.

We are obliged to Y. Murakami, Japan Nuclear Fuel Development Institute, for technical assistance.

This work was supported by the Special Coordination Fund Archaean Park Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

REFERENCES

1. **Åkerman, K. K., I. Kuronen, and E. O. Kajander.** 1993. Scanning electron microscopy of nanobacteria—novel biofilm producing organisms in blood. Scanning **15**(Suppl.)**:**90–91.
2. **Borneman, J., and E. W. Triplett.** 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. **63:**2647–2653.
3. **DeLong, E. F.** 1992. Archaea in costal marine environments. Proc. Natl. Acad. Sci. USA **89:**5685–5689.
4. **Drancourt, M., V. Jacomo, H. Lépidi, E. Lechevallier, V. Grisoni, C. Coulange, E. Ragni, C. Alasia, B. Dussol, Y. Berland, and D. Raoult.** 2003. Attempted isolation of *Nanobacterium* sp. microorganisms from upper urinary tract stones. J. Clin. Microbiol. **41:**368–372.
5. **Elsaied, H. E., M. Sato, and T. Naganuma.** 2001. Viable *Cytophaga*-like bacterium in the 0.2 μm-filtrate seawater. Syst. Appl. Microbiol. **24:**0–5.
6. **Folk, R. L.** 1992. Bacteria and nanobacteria revealed in hardgrounds, calcite cements, native sulfur, sulfide minerals, and travertines. Proc. Geol. Soc. Amer. Annu. Meet., p. 104.
7. **Folk, R. L.** 1993. SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks. J. Sedim. Petrol. **63:**990–999.
8. **Fukuba, T., M. Sato, H. E. Elsaied, and T. Naganuma.** 2002. Overlooked microbial agents in aquaculture: nanobacteria, p. 99–107. *In* C.-S. Lee and P. O'Bryen (ed.), Microbial approaches to aquatic nutrition within environmentally sound aquaculture production systems. World Aquaculture Society, Baton Rouge, La.
9. **Hahn, M. W.** 2003. Isolation of strains belonging to the cosmopolitan *Polynucleobacter necessarius* cluster from freshwater habitats located in three climatic zones. Appl. Environ. Microbiol. **69:**5248–5254.
10. **Hahn, M. W., H. Lünsdorf, Q. L. Wu, M. Schauer, M. G. Höfle, J. Boenigk, and P. Stadler.** 2003. Isolation of novel ultramicrobacteria classified as *Actinobacteria* from five freshwater habitats in Europe and Asia. Appl. Environ. Microbiol. **69:**1442–1451.
11. **Haller, C. M., S. Rölleke, D. Vybiral, A. Witte, and B. Velimirov.** 1999. Investigation of 0.2 μm filterable bacteria from the Western Mediterranean Sea using a molecular approach: dominance of potential starvation forms. FEMS Microbiol. Ecol. **31:**153–161.
12. **Harris, J. K., S. T. Kelley, and N. R. Pace.** 2004. New perspective on uncultured bacterial phylogenetic division OP11. Appl. Environ. Microbiol. **70:**845–849.
13. **Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter.** 2002. A new phylum of archaea represented by a nanosized hyperthermophilic symbiont. Nature **417:**63–67.
14. **Hugenholtz, P., and T. Huber.** 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int. J. Syst. Evol. Microbiol. **53:**289–293.
15. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:**366–376.
16. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:**4765–4774.
17. **Kajander, E. O., and N. Ciftçioglu.** 1998. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc. Natl. Acad. Sci. USA **95:**8274–8279.
18. **Kolter, R., D. A. Siegele, and A. Tormo.** 1993. The stationary phase of the bacterial life cycle. Annu. Rev. Microbiol. **47:**855–874.
19. **Kuske, C. R., S. M. Barns, and J. D. Busch.** 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Appl. Environ. Microbiol. **63:**3614–3621.
20. **Li, L., C. Kato, and K. Horikoshi.** 1999. Bacterial diversity in deep-sea sediments from different depths. Biodivers. Conserv. **8:**659–677.
21. **Maniloff, J.** 1997. Nanobacteria: size limits and evidence. Science **276:**1776.
22. **Murakami, Y., Y. Fujita, T. Naganuma, and T. Iwatsuki.** 2002. Abundance

FIG. 1. Neighbor-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences from OTUs in the 0.2- and 0.1-μm-pore-size filter-captured groundwater fractions from sedimentary and granite rock aquifers in the Tono uranium mine. The 16S rRNA gene sequences of the *Aquificales Aquifex aeolicus* (AJ309733) and *Hydrogenobacter hydrogenophilus* (Z30242) were used as outgroups but were pruned from the tree. Yellow and light-blue backgrounds indicate the OTUs captured by 0.1- and 0.2-μm-pore-size filters, respectively. Black and red letters indicate the OTUs from sedimentary and granite rock aquifers, respectively. The OTUs written in both black and red are from both sedimentary and granite rock aquifers. Bootstrap values at branching nodes after 1,000 resamplings are represented by filled circles (>75%), open circles (75%>○>50%), and no circles (<50%). GNS, green nonsulfur bacteria.

and viability of the groundwater microbial communities from borehole in Tono uranium deposit area, central Japan. Microb. Environ. **17:**63–74.
23. **Murakami, Y., Y. Fujita, T. Iwatsuki, and T. Naganuma.** 2003. Abundance and viability of subsurface microbial communities in sedimentary and igneous rock aquifers, p. 115–140. *In* M. Taniguchi, K. Wang, and T. Gamo (ed.), Land and marine hydrogeology. Elsevier B.V., Amsterdam, The Netherlands.
24. **Nyström, T., N. H. Albertson, K. Flärdh, and S. Kjelleberg.** 1990. Physiological and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp. S14. FEMS Microbiol. Ecol. **74:**129–140.
25. **Page, R. D. M.** 1996. Tree View: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
26. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. **183:**3770–3783.
27. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for constructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
28. **Somerville, C. C., I. T. Knight, W. L. Straube, and R. R. Colwell.** 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. Environ. Microbiol. **55:**548–554.
29. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1989. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
30. **Torrella, F., and R. Y. Morita.** 1981. Microcultural study of bacteria size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl. Environ. Microbiol. **41:**518–527.
31. **Velimirov, B.** 2001. Nanobacteria, ultramicrobacteria and starvation forms: a search for the smallest metabolizing bacterium. Microbes Environ. **16:**67–77.
32. **Watanabe, K., Y. Kodama, and S. Harayama.** 2001. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J. Microbiol. Methods **44:**253–262.
33. **Wise, M. G., J. V. McArthur, and L. J. Shimkets.** 1997. Bacterial diversity of a Carolina bay as determined by 16S rDNA gene analysis: confirmation of novel taxa. Appl. Environ. Microbiol. **63:**1505–1514.