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Detection of 16S rDNA Sequences Representing the Novel Phylum "Nanoarchaeota": Indication for a Wide Distribution in High Temperature Biotopes

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Summary

We screened samples from high temperature biotopes for 16S rRNA genes of the novel archaeal phylum "Nanoarchaeota". Positive PCR amplifications were obtained from Yellowstone National Park, Uzon Caldera, and an abyssal vent system. These sequences form a cluster with the sequence of *"Nanoarchaeum equitans"*, indicating a wide distribution of this phylum.

Key words: "Nanoarchaeota" – PCR – 16S rRNA – Archaea – hyperthermophilic – ecology

Recently a novel phylum of Archaea was discovered, the "Nanoarchaeota" [7], which is currently represented by a single species, *"Nanoarchaeum equitans"*. It was obtained from hot rocks taken at the Kolbeinsey Ridge, north of Iceland. This hydrothermal system is located at the shallow sub polar Mid-Atlantic Ridge at a depth of 106 m [5]. *"N. equitans"* is a nano-sized hyperthermophilic symbiont that grows attached to the surface of a new *Ignicoccus* species [7]. It has a cell diameter of only 400 nm and grows under strictly anaerobic conditions at temperatures between 75 and 98 °C. Molecular investigations revealed that *"N. equitans"* harbours a highly divergent 16S rDNA sequence which exhibits several base exchanges even in previously "universal" sequence signatures (primer sequences). As a consequence, representatives of the "Nanoarchaeota" previously remained undetectable by commonly used PCR-based ecological studies [1] and nothing is known on their phylogenetic diversity or their distribution in nature.

Here we report the first ecological studies using "Nanoarchaeota"-specific primers to amplify 16S rDNA from environmental DNAs from a wide variety of high temperature biotopes.

Occurrence of "Nanoarchaeota" 16S rDNA sequences in environmental samples

We isolated DNA from more than 30 marine and continental samples from neutral pH high temperature environments: sandy submarine sediments, venting water, and material from black smokers from the East Pacific Rise (N 9°, W 104°; depth 2500 m), Guaymas Basin (between N 20°49′, W 109°06′ and N 27°01′, W 111°24′; depth 2000 m), and Vulcano Island, Italy (depth up to 15 m) and muds, sediments, and spring waters from continental solfataric areas at Yellowstone National Park, USA (Obsidian Pool), Kamchatka Peninsula, Russia (Uzon Caldera, Makinski, Puchino), Atacama Desert, Chile (Thermas de Jurasi, Thermas de Pollequere, Puchuldiza Geyser area, Tatio volcanic area), and Lihir Island, Papua New Guinea (original temperatures 70 to 98 °C). From 5 g of original material, 50 to 100 ng of DNA were extracted as described previously [1], with modifications [11]. 10 ng of purified DNA was used as a template for PCR using a standard protocol [3] and primers 7mcF, 518mcF, 1116mcR, and 1511mcR (Table 1). In addition, the archaeal specific primer combination 8aF-1512uR was used as a positive control. By applying our "Nanoarchaeota"-specific primers for amplification, we obtained PCR products from one marine sample from the East Pacific Rise, designated LPC33 (black smoker fragment), and two continental samples: Obsidian Pool, Yellowstone National Park, USA, designated OP9 (temperature 80 °C; pH 6.0) and the Uzon Caldera, Kamchatka, Russia, designated CU1 (temperature 85 °C; pH 5.5). The amplified 16S rDNA fragments were purified (QIAquick PCR Purification Kit), cloned into pDrive (QIAGEN, Hilden, Germany) and transformed into competent *E. coli* cells. 30 clones were randomly chosen for plasmid preparation

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Table 1. Primer sequences used in PCR amplifications and sequencing.

Primer	Target site	Sequence 5′ → 3′	Applied for	Reference
7mcF	7–23	CTC CCG TTG ATC CTG CG	All "Nanoarchaeota"	This study
8aF	8–23	TCY GGT TGA TCC TGC C	Archaea (as control)	[3]
344aF	344–363	CGG GGY GCA SCA GGC GCG AA	Clone OP9, clone CU1 (two mismatches)	[3]
518mcF	518–536	GCA GCC GCC GCG GGA ACA C	*"N. equitans"*, clone LPC33	This study
1116mcR	1100–1116	GCG GGT CTC GCC TGT TT	*"N. equitans"*, clone LPC33	This study
1119aR	1101–1119	GGY RSG GGT CTC GCT CGT T	Clone OP9, clone CU1 (one mismatch)	[2]
1511mcR	1490–1511	CGG CTA CCT TGT GTC GAC TTA G	All "Nanoarchaeota"	This study
1512uR	1493–1513	ACG GHT ACC TTG TTA CGA CTT	Archaea (as control)	[3]

Table 2. Sequence similarities between the "Nanoarchaeota" sequences and representatives of major phylogenetic groups.

	"Nanoarchaeota"			Cren-archaeota	Eury-archaeota	"Kor-archaeota"	Bacteria
"Nanoarchaeota"	*"N. equitans"*/ Clone LPC33	Clone OP9	Clone CU1				
	"N. equitans"/ Clone LPC33	–	82.7	83.2	80–71	80–67	75–72	70–60
	Clone OP9	82.7	–	92.7				
	Clone CU1	83.2	92.7	–				
Crenarchaeota		80–71			–	84–69	82–75	73–62
Euryarchaeota		80–67			84–69	–	80–70	74–60
"Korarchaeota"		75–72			82–75	80–70	–	72–64
Bacteria		70–60			73–62	74–60	72–64	–

Table 3. Comparison of standard 16S rDNA primers with the corresponding sequences of the "Nanoarchaeota".

Primer (Reference)	**8aF** [3]	**344 aF** [3]
Sequence	TCY GGT TGA TCC TGC C	CGG GGY GCA SCA GGC GCG AA
"N. equitans" / LPC33	TCC [C]GT TGA TCC TGC [G]	CGG GGC GCA CCA GGG GCG AA
Clone OP9 / CU1	n.d.	CGG G**A**T GCA CCA GGG GCG AA
Primer (Reference)	**519uF** [3]	**934aR** [13]
Sequence	CAG CMG CCG CGG TAA TAC	GTG CYC CCC CGC CAA TTC CT
"N. equitans" / LPC33	CAG CCG CCG CGG GAA [C]AC	GTG CTC CCC CGC CTA TTC CT
Clone OP9 / CU1	CAG **T**CG CC**A** CGG GAA TAC	GTG CCC CCC CGC CTA TTC CT
Primer (Reference)	**1044 aF** [2]	**1119aR** [2]
Sequence	GAG AGG WGG TGC ATG GCC G	GGY RSG GGT CTC GCT CGT T
"N. equitans" / LPC33	GAG AGG AGG TGC ATG GCC G	GGC GCG GGT CTC GC[C] [T]GT T
Clone OP9 / CU1	GAG AGG AGG TGC ATG GC**T** G	GGT GCG GGT CGC **G**CT CGT T
Primer (Reference)	**1406uR** [3]	**1512uR** [3]
Sequence	ACG GGC GGT GTG TRC AA	ACG GHT ACC TTG TTA CGA CTT
"N. equitans" / LPC33	ACG GGC GGT GAG TGC AA	ACG GCT ACC TTG T[GT] CGA CTT
Clone OP9 / CU1	ACG GGC GGT GAG **A**GC AA	n.d.

Base exchanges in *"N. equitans"* are boxed; in the sequences of clones OP9 and CU1 they are written in bold. Base exchanges shared among all "Nanoarchaota" are highlighted.
n. d. = not determined.

[12]. Inserts were reamplified from the plasmids, digested separately with *Sau*3AI and *Rsa*I and compared on agarose gels by amplified rDNA restriction analysis (ARDRA) [8]. ARDRA analyses of the clones representing each environmental sample indicated that only one sequence type was found in each PCR product. The 16S rDNA clones were sequenced with primers 7mcF, 344aF, 518mcF, 1116mcR, 1119aR, and 1511mcR (Table 1). Complete sequences were submitted to the CHECK_CHIMERA program at the Ribosomal Database Project [10] to detect possible chimeras and then aligned with about 11,000 sequences (ARB project; [9]). The new "Nanoarchaeota" 16S rDNA sequences were deposited in the EMBL database, accession numbers AJ458437 (clone CU1) and AJ458436 (clone OP9).

Analysis of "Nanoarchaeota" sequences and phylogenetic position of the "Nanoarchaeota" phylum

The sequence from the East Pacific Rise was identical to the "*N. equitans*" sequence, while the others represented previously unknown primary structures. Sequence similarities among the new sequences, calculated by using distance matrix analysis without correction factors and filters, ranged from 93% (OP9 to CU1) to 83% (OP9 and CU1 to "*N. equitans*" and LPC33) (Table 2). However, in spite of their sequence diversity, the three sequences were more closely related to one another than to sequences representing other archaeal phyla or Bacteria (Table 2). Distance matrix (neighbour joining, Fitch-Margoliash algorithm [4], using Jukes-Cantor correction), maximum parsimony, and maximum-likelihood (fastDNAml) methods were carried out for tree reconstruction as implemented in the ARB package and PAUP 4.0b9 [14] with and without 50% domain-specific filters. Furthermore, bootstrap analyses (100 repeats) were carried out to check the robustness of the branching patterns. In all phylogenetic analyses the three sequences grouped together with high bootstrap support (98–100%) and the whole "Nanoarchaeota" branch was placed very deep within the archaeal domain. However, placement of the branch within the Archaea is problematic. Its position varied significantly depending on the analytical method used and the domain-specific filters applied. Also, insignificant bootstrap values were obtained for the branching point of the "Nanoarchaeota" with all calculations, making it impossible to define the branching position for the "Nanoarchaeota". Further organisms and/or sequences of this novel lineage may stabilize the 16S rRNA-based trees. However, it is also possible that the phylogenetic position of the "Nanoarchaeota" may never be resolved using 16S rDNA sequences alone. Analysis of the already sequenced genome of "*N. equitans*" (490 kb) may give insights into this problem (http://www.Diversa.com; 03.05.2002).

Like "*N. equitans*", the new environmental sequences exhibited base exchanges in nearly all primer sequences previously considered "Archaea-specific" or "universal" (Table 3). However, in most of these primer regions OP9 and CU1 differed from "*N. equitans*" (Table 3). Nevertheless, primers 7mcF and 1511mcR yielded specific PCR products from the environmental DNAs and seem to be specific for "Nanoarchaeota", although no final statements on this can be made at present. The occurrence of sequence heterogeneity in conserved regions permits the possibility that there is a much wider "nanoarchaeotal" diversity still to be detected and might explain why we never obtained more than one sequence from each environmental sample.

The presence of "Nanoarchaeota" 16S rDNA sequences in hydrothermal biotopes in the deep sea (LPC33), in shallow marine areas *("N. equitans")* and in solfataric fields (OP9, CU1) located on different continents indicates a wide distribution of members of the "Nanoarchaeota". Since FISH experiments were unsuccessful, it is unclear whether the new "Nanoarchaeota" sequences represent small organisms similar to "*N. equitans*". Also, a symbiotic lifestyle cannot be deduced from the current information, particularly for the clones OP9 and CU1, which came from continental hydrothermal habitats that are not known biotopes for *Ignicoccus* [6]. Therefore, a better understanding of the morphological, physiological, and molecular diversity of this group awaits the cultivation and study of the corresponding organisms.

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