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A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont

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According to small subunit ribosomal RNA (ss rRNA) sequence comparisons all known Archaea belong to the phyla Crenarchaeota, Euryarchaeota, and—indicated only by environmental DNA sequences—to the 'Korarchaeota'[1,2]. Here we report the cultivation of a new nanosized hyperthermophilic archaeon from a submarine hot vent. This archaeon cannot be attached to one of these groups and therefore must represent an unknown phylum which we name 'Nanoarchaeota' and species, which we name '*Nanoarchaeum equitans*'. Cells of '*N. equitans*' are spherical, and only about 400 nm in diameter. They grow attached to the surface of a specific archaeal host, a new member of the genus *Ignicoccus*[3]. The distribution of the 'Nanoarchaeota' is so far unknown. Owing to their unusual ss rRNA sequence, members

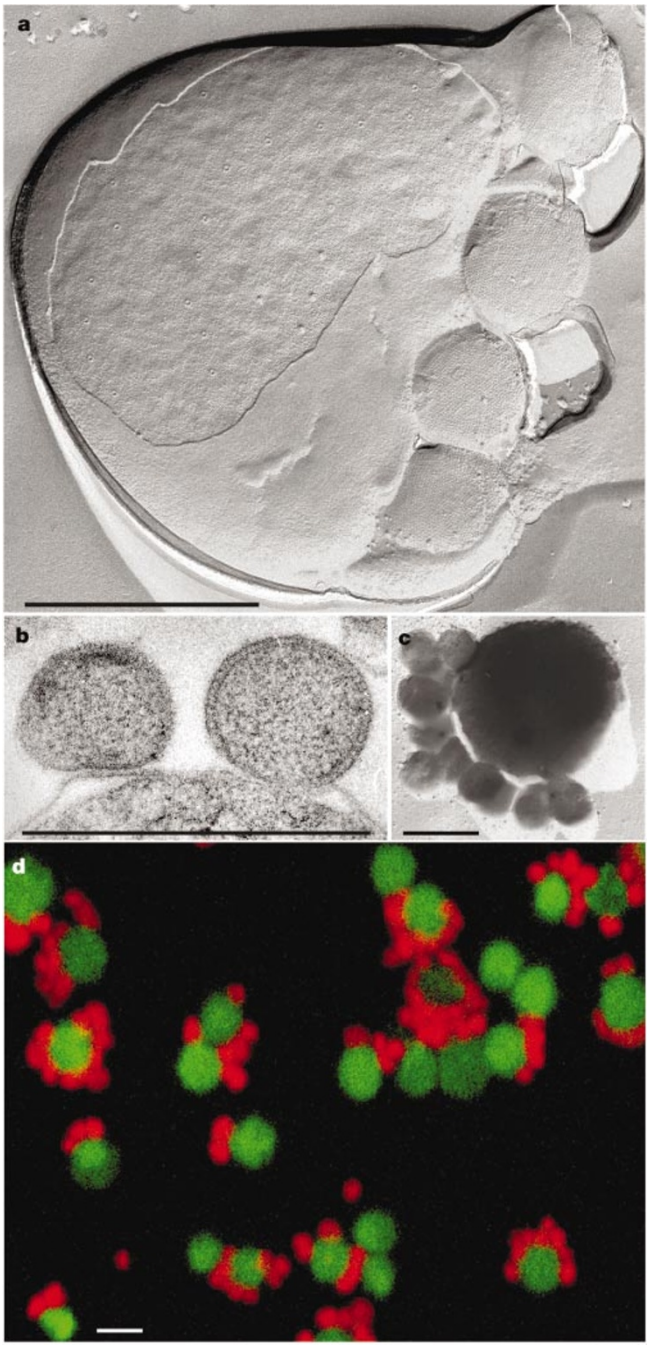


Figure 1 Electron microscopy and fluorescence light microscopy of the '*Nanoarchaeum equitans*'–*Ignicoccus* sp. coculture. **a**, Freeze-etched cell of *Ignicoccus* and four attached cells of '*Nanoarchaeum*', showing their crystalline S-layer with sixfold symmetry. **b**, Ultrathin section of two cells of '*Nanoarchaeum*' attached to the outer membrane of *Ignicoccus*. **c**, Cell of *Ignicoccus*, with several cells of '*Nanoarchaeum*' attached on the left side; platinum-shadowed. **d**, Confocal laser scanning micrograph after hybridization with the CY3-labelled probe 515mcR ('*Nanoarchaeum*') and rhodamine-green-labelled probe CREN499R (*Ignicoccus*). **a**–**d**, Scale bar, 1.0 μm.

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remained undetectable by commonly used ecological studies based on the polymerase chain reaction[4]. '*N. equitans*' harbours the smallest archaeal genome; it is only 0.5 megabases in size. This organism will provide insight into the evolution of thermophily, of tiny genomes and of interspecies communication.

Hyperthermophilic microorganisms with optimal growth temperatures above 80 °C occur in both prokaryotic domains, the Bacteria and Archaea. In phylogenetic trees based on ss rRNA they represent all the short deep lineages, forming a cluster around the root[5]. This feature and their ancient biotopes indicate that hyperthermophiles are still primitive[5]. Within the Archaea, hyperthermophilic members of the Crenarchaeota and the Euryarchaeota have been cultivated, which exhibit unusual cell morphologies such as disks, networks, 'golf clubs', irregular spheres, and branched and irregular rods[6]. Variations in cell volume by up to four orders of magnitude even within a pure culture were observed (for example, *Thermoproteales* and *Desulfurococcales*)[6].

To investigate hot submarine vent microbial communities, we carried out experiments to cultivate hyperthermophiles from samples of originally hot rocks and gravel taken at the Kolbeinsey ridge, north of Iceland[7]. By anaerobic incubation at 90 °C in the presence of S, H_2 and CO_2, a new autotrophic sulphur-reducing species of the archaeal genus *Ignicoccus*[3] could be enriched. In contrast to known species, several large spherical *Ignicoccus* cells were covered by very tiny cocci which could be stained by DNA-specific fluorescence microscopy (DAPI)[8]. Few such tiny cocci existed in the free state.

These tiny cocci could be physically isolated either by using 'optical tweezers'[9,10] or by ultrafiltration (pore width, 0.45 μm). All attempts to grow them in pure culture employing various inorganic and organic energy sources failed. However, isolation of a combination of a tiny coccus attached to an *Ignicoccus* sphere after incubation under enrichment conditions, resulted in a defined coculture in which about half of the *Ignicoccus* cells appeared to be colonized by the tiny cocci. The final density of both the tiny cocci and the *Ignicoccus* cells was about 3×10^7 cells ml^{-1} resulting in about two tiny cocci per *Ignicoccus* cell on average. The purified coculture was used in all further investigations. Cloning of single *Ignicoccus* cells gave rise to cultures which never contained tiny cocci.

By electron microscopy, a close attachment of the tiny cocci to the surface of the *Ignicoccus* cells became evident (Fig. 1a, b, c). The tiny cocci consistently exhibited a cell diameter of about 400 nm. In contrast to *Ignicoccus*, they were covered by a regular surface layer (S-layer) with sixfold symmetry and a lattice constant of 15 nm (Fig. 1a). Ultrathin sections showed the presence of cytoplasmic membranes inside the tiny cocci and the *Ignicoccus* cells (Fig. 1b). No specific attachment structures could be detected thus far at the sites of contact which suggested a loose connection of the tiny cocci with the *Ignicoccus* cells. In line with this assumption, the tiny cocci could be removed by mild sonification (30 W).

The phylogenetic relationships of the tiny cocci were investigated by ss rRNA sequence comparisons[1,11]. Total DNA of the coculture was extracted and ss rRNA genes were PCR-amplified using primers addressed to highly conserved gene sections. Surprisingly, however, only the *Ignicoccus* ss rRNA gene sequence was amplified even when primers considered general for all Archaea as well as for all organisms ('universal') had been employed (for example, 8aF[12] and 1513uR[12]). More directly, we examined the coculture-derived DNA for ss rRNA genes by Southern blot hybridization[13], taking advantage of the generally high sequence homology of all ss rRNA genes. Two different hybridization signals became visible, indicating two non-identical ss rRNA genes (Fig. 2; lanes 2 and 4). As a control, DNA isolated from the separate *Ignicoccus* sp. by the same procedure yielded only one hybridization signal (Fig. 2; lanes 1 and 3) which was identical with one from the mixed culture. Therefore, the tiny cocci must have given rise to the second hybridization signal, indicating that they hold a ss rRNA gene different from *Ignicoccus*.

The corresponding DNA fragment was cloned and sequenced. Its sequence turned out to be (so far) unique, harbouring many base exchanges even in the so-called 'highly conserved regions' that are usually employed as primer targets for ss rDNA PCR (Table 1). This explains our initial failure to amplify this gene by PCR. Comparison of the new ss rRNA sequence with those of other microorganisms established the tiny cocci as previously unknown members of the archaeal domain indicated by sequence identities of 0.69 to 0.81 with the Archaea in contrast to sequence identities of only 0.59 to 0.70 with bacterial species. The secondary structure of the ss rRNA sequence conformed to the standard two-dimensional structural model[11]. It exhibited features characteristic of Archaea, especially helices numbers 17, 18, 36 and 47 (ref. 11; Fig. 3). The sequence identities to the Crenarchaeota (0.73–0.81), the Euryarchaeota (0.69–0.81), and the 'Korarchaeota' (0.73–0.75) were in the same range as those between these three known phyla of Archaea (0.69–0.83). Therefore, the tiny cocci represent a new archaeal phylum. On the basis of its extremely small cell size, we name it 'Nanoarchaeota' (the dwarf archaea) and the corresponding species '*Nanoarchaeum equitans*' (riding the fire sphere).

In ss rRNA phylogenetic trees that are founded on the maximum

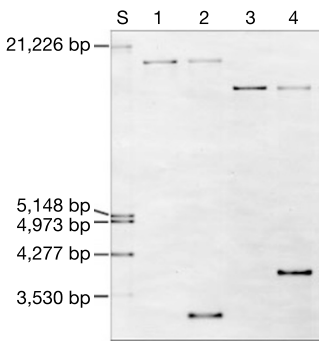


Figure 2 Southern blot analysis of DNA from *Ignicoccus* sp. and the '*N. equitans*'–*Ignicoccus* sp. coculture, treated with restriction enzymes. S, length standard (DNA relative molecular mass marker III, Roche). Lane 1 and 3, *Ignicoccus* DNA. Lane 2 and 4, DNA of the coculture. Lane 1 and 2, digested with *Eco*RI. Lane 3 and 4, digested with *Hin*dIII.

Table 1 Sequence specificity of ss rDNA primers

Designation	Sequence (5′ → 3′)	Specific against				
		C	E	K	N	B
8aF[12]	TCY GGT TGA TCC TGC C	+	+	+	−	−
8mcF	TCC CGT TGA TCC TGC G	−	−	−	+	−
9bF[12]	GRG TTT GAT CCT GGC TCA G	−	−	−	−	+
	CCC GTT GAT CCT GCG GGA G	−	−	−	+	−
217kF[29]	GAG GCC CCA GGR TGG GAC CG	−	−	+	−	−
	CCC GCC CGA GGA TGG GCC GG	−	−	−	+	−
519uF[12]	CAG CMG CCG CGG TAA TAC	+	+	−	−	+
519mcF	CAG CCG CCG CGG GAA CAC	−	−	−	+	−
1114aR	GGG TCT CGC TCG TTR CC	+	+	+	−	−
1114mcR	GGG TCT CGC CTG TTT CC	−	−	−	+	−
1406uR[12]	ACG GGC GGT GTG TRC AA	+	+	+	−	+
1406mcR	ACG GGC GGT GAG TGC AA	−	−	−	+	−
1513uR[12]	ACG GHT ACC TTG TTA CGA CTT	+	+	+	−	+
1513mcR	ACG GCT ACC TTG TGT CGA CTT	−	−	−	+	−
EURY498R[15]	CTT GCC CRG CCC TT	−	+	−	−	−
511mcR	CTT GCC CAC CGC TT	−	−	−	+	−
CREN499R[15]	CCA GWC TTG CCC CCC GCT	+	−	−	−	−
515mcR	CCC CTC TTG CCC ACC GCT	−	−	−	+	−
ARCH915R[16]	GTG CTC CCC CGC CAA TTC CT	+	+	−	−	−
934mcR	GTG CTC CCC CGC CTA TTC CT	−	−	−	+	−

C, Crenarchaeota; E, Euryarchaeota; K, 'Korarchaeota' sequences; N, 'Nanoarchaeota'; B, Bacteria. Base exchanges in '*N. equitans*' are underlined.

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parsimony, distance matrix, and maximum likelihood methods[14], '*N. equitans*' represents an isolated, very deeply branching lineage. However, because of its unique ss rRNA, a large variation of its branching point, challenged by insignificant bootstrap values below 50%, was observed (not shown). Therefore, the determination of the accurate branching position of the 'Nanoarchaeota' must await the discovery of further members of this group.

Owing to their great divergences in ss rRNA—in contrast to the *Ignicoccus* host—cells of the 'Nanoarchaeota' did not stain by fluorescence *in situ* hybridization using ss rRNA-targeted oligonucleotide probes directed against Crenarchaeota and Euryarchaeota (for example, EURY498R[15], CREN499R[15] and ARCH915R[16];

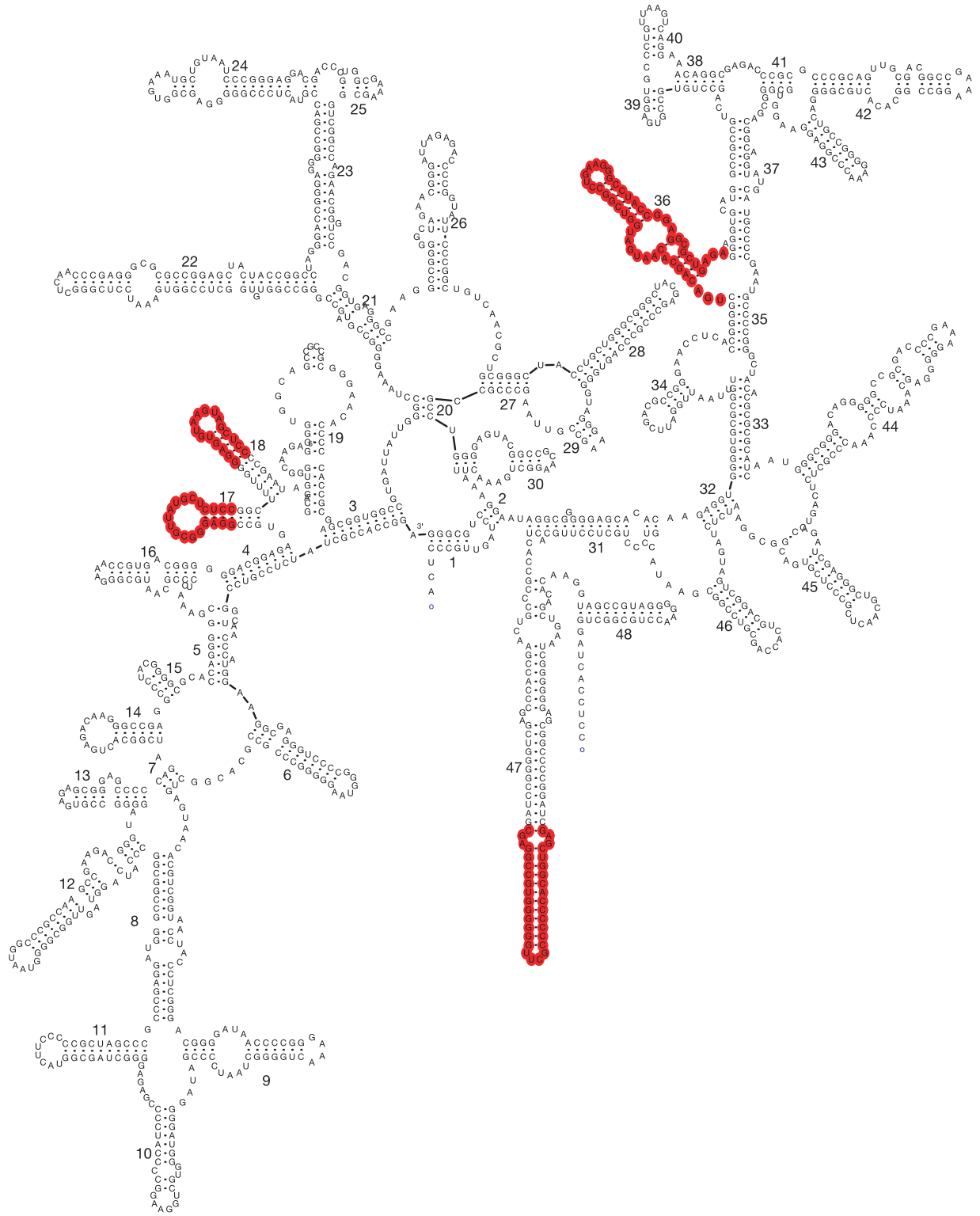


Figure 3 Secondary structure model for the ss rRNA of '*N. equitans*'. Highlighted positions indicate characteristic archaeal secondary structures[11]. Numbers correspond to the helix numbers. The model structure was determined using RnaViz[28].

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Table 1). However, after redesigning these oligonucleotide probes on the basis of the 'Nanoarchaeota' sequence (for example, 511mcR, 515mcR and 934mcR; Table 1), the tiny cocci exhibited a bright fluorescence (Fig. 1d), indicating that they possessed ribosomal RNA harbouring the target sequence.

Few physiological properties of the '*Nanoarchaeum*' strain are known thus far. Isolated cells did not grow on cell homogenates of *Ignicoccus* sp., so they require an actively growing *Ignicoccus* culture. Within pure cultures of *Ignicoccus*, '*Nanoarchaeum*' cells locally separated by dialysis bags were unable to propagate (not shown). Therefore, a direct cell–cell contact with the host appears to be a prerequisite for growth. No negative influence on *Ignicoccus*, such as slower growth, lower final cell density, or increased cell lysis, could be observed in the coculture compared to the pure culture, rendering a parasitic mode of living of '*Nanoarchaeum*' unlikely. Therefore, the organism is most probably symbiotic. So far, archaeal symbionts are only known at mesophilic temperatures in association with eukaryotes (see ref. 17 for example) and bacteria[18]. Cocultures of '*Nanoarchaeum*' grew within the same temperature range (70–98 °C) as its host. During mass-culturing in a 300-litre enamel-protected fermentor[3], final cell concentrations of '*Nanoarchaeum*' could be improved about tenfold by an increased gassing rate (20 l min^{-1}; $H_2 : CO_2 = 80 : 20$), while that of *Ignicoccus* remained unchanged. This procedure improved hydrogen supply and efficiently removed H_2S, the main metabolic end product of *Ignicoccus*[3]. During the late exponential growth phase, about 80% of the '*Nanoarchaeum*' cells detached from their host cells and occurred freely in suspension. They could be collected (for example, 1.5 g wet weight) by high-speed centrifugation, after removal of the *Ignicoccus* cells at low speed. By this method, cell masses of '*Nanoarchaeum*' became available for biochemical and molecular investigations. A first analysis of its genome size by adding up the sizes of restriction fragments determined after pulse field gel electrophoresis resulted in only 500 kilobases (kb), one of the smallest genomes of all prokaryotes known so far (not shown)[19].

With its tiny cell and genome size, '*Nanoarchaeum*' resembles an intermediate between the smallest living organisms like *Mycoplasma genitalium*[19] and big viruses like the pox virus and *Chlorella* virus CVK2 (refs 20 and 21), and is close to the theoretical minimum genome size calculated for a living being[22]. Small cell size and reduced genomes are common features observed in many symbiotic or parasitic Bacteria[23], but have so far not been reported for Archaea or hyperthermophiles. No final conclusions can be drawn about a primitive or derived state of evolution of '*Nanoarchaeum*'. However, its high growth temperature and anaerobic mode of life correlates with probable early environmental conditions which suggest that the 'Nanoarchaeota' are possibly still a primitive form of microbial life. Analysing its genome and its symbiotic relationship should help. Other members of the 'Nanoarchaeota' may still exist on Earth and may be discovered by using our knowledge of their unique ss rRNA. □

Methods

Cultivation conditions

Cultures were grown anaerobically in serum bottles as described[3]. Mass culturing was carried out in the same medium in a 300-litre enamel-protected fermentor (gassing rate routinely 2 l min^{-1} $H_2 : CO_2 = 80 : 20$). Cell mixtures were collected by centrifugation (9,000 r.p.m.; 30 min using rotor GS3 (Sorvall)) and resuspended in sulphur-free culture medium. The *Ignicoccus* cells were removed by centrifugation (2,000 r.p.m. for 30 min using rotor SS34 (Sorvall)). Then the '*Nanoarchaeum*' cells were precipitated (15,000 r.p.m. for 15 min using rotor SS34). Axenic cultivation of '*Nanoarchaeum equitans*' was tested autotrophically in the presence of $H_2 : CO_2$ (80 : 20) and elemental sulphur, nitrate, nitrite, and sulphate (each 0.1% w/v) as possible electron acceptors, and heterotrophically on single and complex organic nutrients such as sugars, amino acids, yeast extract, peptone, bacterial and archaeal (for example, from *Ignicoccus*) cell extracts (each 0.1% w/v; gas phase: $H_2 : CO_2 = 80 : 20$ and $N_2 : CO_2 = 80 : 20$).

Light and electron microscopy

Phase-contrast and electron microscopy were carried out as described[3]. Confocal image series were recorded on a LEICA TCS SP2 laser scanning microscope using the laser wavelengths 488 nm (argon) and 543 nm (HeNe) excitation. Fluorescence *in situ* hybridization (FISH) was performed as described[15]. CY3- and rhodamine-green-labelled oligonucleotides were obtained from Metabion (Germany).

Southern blot experiments

DNAs were digested with restriction endonucleases *Eco*RI and *Hin*dIII. The fragments were separated by electrophoresis (1% Seakem ME agarose) and transferred to a positively charged nylon membrane by Southern blot[13]. The membrane was hybridized with a digoxigenine-labelled single-stranded DNA fragment of an archaeal ss rRNA gene (*Metallosphaera sedula*; length 600 nucleotides, position 519 to 1119, *Escherichia coli* numbering[24]).

Estimation of the genome size

For pulse field gel electrophoresis '*Nanoarchaeum*' cells were embedded in 100-µl agarose plugs (0.8% Incert agarose, FMC; final concentration: 3×10^8 cells per plug). After cell lysis according to lysis method 'three'[25] genomic DNA was digested using the restriction enzymes *Asc*I, *Bss*HII, *Not*I, or *Sac*II (each 100 units, 18 h). The restriction fragments were separated as described[25].

Small subunit rDNA sequence analysis

The nearly complete ss rRNA gene of the new *Ignicoccus* species was PCR-amplified and sequenced as described[3]. To obtain the ss rRNA gene of '*N. equitans*', genomic DNA of the coculture was digested with *Eco*RI and separated as described (see 'Southern blot'). DNA fragments of the correct size, identified by Southern blot, were extracted, cloned into a plasmid (pBluescript SK(−)) and amplified in *Escherichia coli* (strain Dh5α). These inserts were sequenced by primer walking using the primers 1406uR[12], 934mcR, 1060aF[26], 515mcR, 519mcF and 8mcF (Table 1). The new sequences were aligned with approximately 10,000 homologous sequences available in public databases using the automatic alignment tools of the ARB package[27]. Distance matrix (Jukes–Cantor correction), maximum parsimony, and maximum likelihood (fastDNAml) methods were used for tree reconstruction as implemented in the ARB package. The secondary structure of the ss rRNA of '*N. equitans*' was displayed using the program RnaViz[28].

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for material should be addressed to K.O.S. (e-mail: karl.stetter@biologie.uni-regensburg.de). The 16S rRNA (ss rRNA) sequences for '*Nanoarchaeum equitans*' and *Ignicoccus* sp. were deposited at GenBank under accession numbers AJ318041 and AJ318042, respectively.

Feedback with soil biota contributes to plant rarity and invasiveness in communities

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Understanding the relative abundance of species in plant communities is an unsolved problem[1–10]. Mechanisms such as competition, resource partitioning[5], dispersal ability[10] and predation tolerance[6–9] do not adequately explain relative abundance under field conditions[11,12]. Recent work suggests that interactions between plants and soil microbes is important[13–21]. Here I show that such interaction explains a significant proportion of the variance in the relative abundance of species in plant communities. Rare plants exhibited a relative decrease in growth on 'home' soil in which pathogens had had a chance to accumulate, whereas invasive plants benefited from interactions with mycorrhizal fungi. Some plant species accumulate pathogens quickly and maintain low densities as a result of the accumulation of species-specific pathogens, whereas others accumulate species-specific pathogens more slowly and do not experience negative feedback until plant densities reach high levels[13,15,21]. These results indicate that plants have different abilities to influence their abundance by changing the structure of their soil communities, and that this is an important regulator of plant community structure.

To test for the possibility that feedback with soil organisms will determine plant abundance, I conducted four separate and complementary experiments. In the first experiment I compared the direction and magnitude of soil feedback in five 'highly invasive' plant species versus five 'rare and endangered' plants in Canadian old-fields (meadows) and grasslands. I predicted that, in low-density monocultures and under previously uninhabited soil, rare plants will exhibit negative feedback limiting their growth and spread over time, whereas invasive plants will accumulate pathogens more slowly and display neutral or positive feedback. Each plant species was grown for ten weeks from seed in a pot with soil that either had a history of the same plant or a history of a different species of plant (see Methods for more details). Feedback was determined by comparing plant growth responses in their own versus foreign soils.

The results supported the stated prediction. The five rare plant species experienced strong negative feedback when grown in mono-

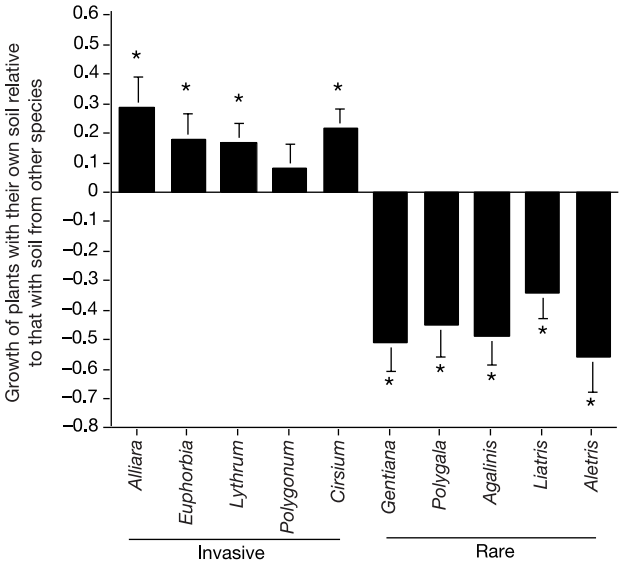


Figure 1 The soil feedback responses of five invasive and five rare plant species. $N = 10$ per treatment. Bars represent the mean with ± 1 s.e. Asterisks represent significant differences in plant growth when grown in home versus foreign soil after *t*-test analysis.

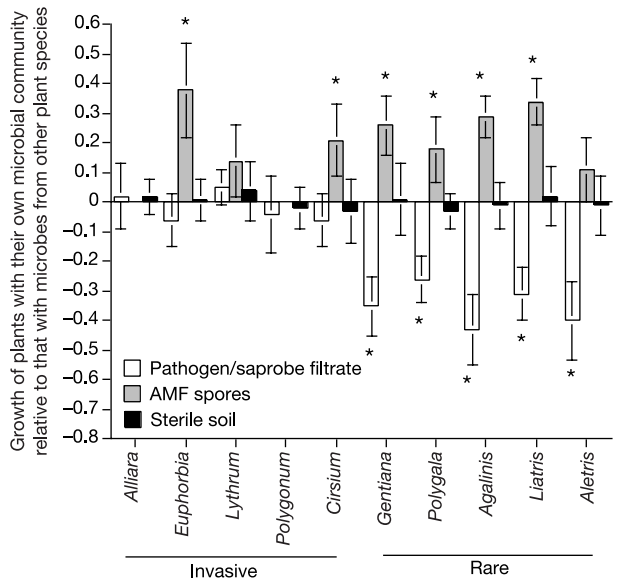


Figure 2 The feedback responses of five invasive and five rare plant species to pathogen/saprobes, mycorrhizal fungi, and sterile soil fractions. Bars represent the mean with ± 1 s.e. Asterisks represent significant differences in plant growth when grown with home versus foreign fractions after *t*-test analysis.

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