

First description of giant *Archaea* (*Thaumarchaeota*) associated with putative bacterial ectosymbionts in a sulfidic marine habitat

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Summary

Archaea may be involved in global energy cycles, and are known for their ability to interact with eukaryotic species (sponges, corals and ascidians) or as archaeal–bacterial consortia. The recently proposed phylum *Thaumarchaeota* may represent the deepest branching lineage in the archaeal phylogeny emerging before the divergence between *Euryarchaeota* and *Crenarchaeota*. Here we report the first characterization of two marine thaumarchaeal species from shallow waters that consist of multiple giant cells. One species is coated with sulfur-oxidizing γ -*Proteobacteria*. These new uncultured thaumarchaeal species are able to live in the sulfide-rich environments of a tropical mangrove swamp, either on living tissues such as roots or on various kinds of materials such as stones, sunken woods, etc. These archaea and archaea/bacteria associations have been studied using light microscopy, transmission electron microscopy and scanning electron microscopy. Species identification of archaeons and the putative bacterial symbiont have been assessed by 16S small subunit ribosomal RNA analysis. The sulfur-oxidizing

ability of the bacteria has been assessed by genetic investigation on alpha-subunit of the adenosine-5'-phosphosulfate reductase/oxidase's (AprA). Species identifications have been confirmed by fluorescence *in situ* hybridization using specific probes designed in this study. In this article, we describe two new giant archaeal species that form the biggest archaeal filaments ever observed. One of these species is covered by a specific biofilm of sulfur-oxidizing γ -*Proteobacteria*. This study highlights an unexpected morphological and genetic diversity of the phylum *Thaumarchaeota*.

Introduction

Since Woese's work in the mid 1970s the biological world is divided in three major domains: *Archaea*, *Bacteria* and *Eukarya*. The domain *Archaea* includes the most abundant and well-distributed types of organisms on Earth, and may be involved in global energy cycles (Robertson *et al.*, 2005). The relative abundance of archaea is dependent on the environment and the season and can represent approximately 20% of the total microbial planktonic population (Karner *et al.*, 2001; Robertson *et al.*, 2005). It can dominate the total microbial community in some environments, such as those characterized by high temperatures or low pH (Robertson *et al.*, 2005). In view of their broad distribution and abundance in soils and oceans, archaea have been proposed to contribute substantially to global energy cycles (Schleper *et al.*, 2005), such as those of nitrogen and carbon (Martínez-García *et al.*, 2008).

The domain *Archaea* is currently composed of three major distinct phyla, the *Euryarchaeota*, the *Crenarchaeota* and the phylum of *Thaumarchaeota* that was recently described by Brochier-Armanet and colleagues (2008a). Emergence of the mesophilic *Thaumarchaeota* lineage has been proposed to precede the speciation of *Crenarchaeota* and *Euryarchaeota*, and may represent the deepest branching lineage in the archaeal phylogeny (Brochier-Armanet *et al.*, 2008b).

Although some archaea can form cell aggregates, e.g. the sphere forming aggregates by *Methanosarcina mazei* (Robinson *et al.*, 1985), the cell-fusing hyperthermophilic archaeon *Thermococcus coalescens* (Kuwabara *et al.*,

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2005), or multiple cells filaments as *Methanothermobacter thermautotrophicus* (Lundgren and Bernander, 2005; Majernik *et al.*, 2005), the domain *Archaea* is known to be entirely composed of single-celled microorganisms. Archaeal cell dimensions range from 0.1 µm up to 15 µm wide and can reach 100 µm length, as observed in Candidatus *Korarchaeum cryptofilum* (Elkins *et al.*, 2008).

Archaea are known to interact with other species. Some interactions between eukaryotes and archaea have been observed in scleractinian coral-associated microbial communities (Kellogg, 2004) and associated with marine sponges in waters of the coastal Pacific (Preston *et al.*, 1996), Mediterranean and Great Barrier Reef (Kellogg, 2004). Some interactions between bacteria and archaea have been observed, such as endosymbioses between ciliates and euryarchaea (Embley and Finlay, 1994), the euryarchaea/bacteria filamentous aggregate of String-of-Pearls (Rudolph *et al.*, 2001) or the consortia between methanotrophic archaea and specific syntrophic sulfate-reducing bacterial partners (Boetius *et al.*, 2000). In this latter case, there is no host and no symbiont as both partners (archaea and bacteria) have similar size and are present in almost equal numbers: an average archaea/bacteria consortium consists of an inner sphere containing about 100 coccoid archaeal cells surrounded by about 200 bacterial cells (Boetius *et al.*, 2000). To date, at least one case of symbiotic association involving archaea as host has been proposed for the relationship between *Nanoarchaeum equitans* and the host crenarchaeon *Ignicoccus hospitalis* (Huber *et al.*, 2002). However, whereas *N. equitans* is in obligate dependence on the presence of the host for its proliferation, it appears that this association does not provide benefits for the host (Jahn *et al.*, 2008).

The only two well-characterized *Thaumarchaeota* are *Cenarchaeum symbiosum* hosted as a symbiont by the marine sponge *Axinella mexicana* (Preston *et al.*, 1996; Martínez-García *et al.*, 2008) and *Nitrosopumilus maritimus* (Konneke *et al.*, 2005). The *A. mexicana*/*C. symbio-*

sum symbiotic partnership may be involved in ammonia oxidation and nitrification. Aside of a few marine clones, the only other well-characterized thaumarchaeon is Candidatus *Nitrosopumilus maritimus* that was the first observed nitrifier within the domain *Archaea* (Konneke *et al.*, 2005).

Results

Our investigations in the mangrove swamp of Guadeloupe (French West Indies) showed the presence of a mat of long and thin white filaments (Fig. 1A) attached to sunken wood substrates or to *Rhizophora mangle* roots. High concentrations of sulfide in various habitats of the mangrove swamp have been reported, from sediments to mangrove peat (Vopel *et al.*, 2005) or even on massive pieces of sunken woods (Laurent *et al.*, 2009).

Archaeal characterization.

Genetic investigations based on the archaeal 16S small subunit ribosomal RNA (ss-rRNA) (Fig. 2) indicated that these long white filaments are composed of two archaeal species that form a monophyletic branch within the recently described mesophilic phylum of *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008a). Only two different sequences of *Thaumarchaeota* have been found in our clone library.

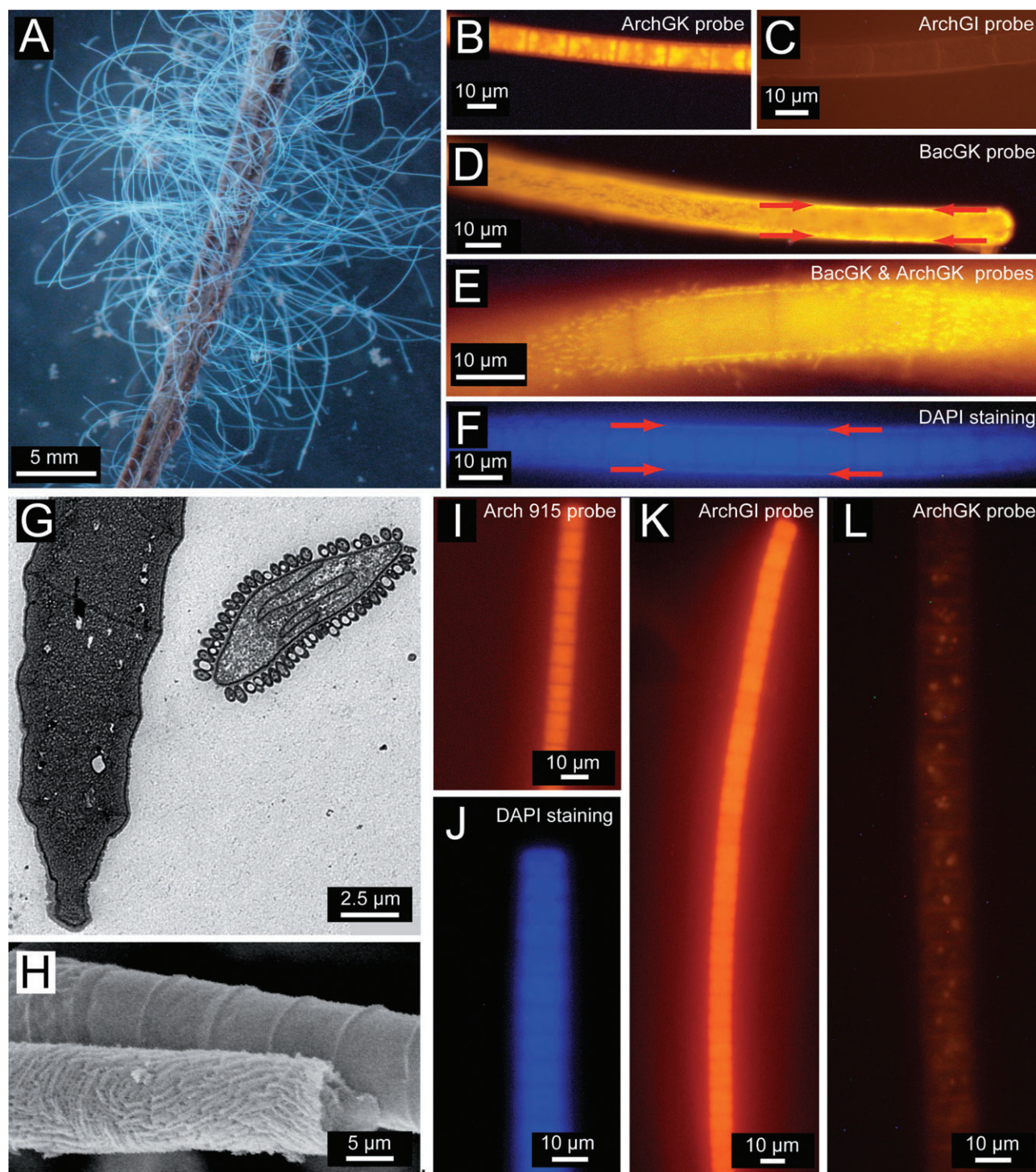
The phylogenetic relationship of these two species has been verified by *in situ* hybridization using either the universal probe for archaea (Arch 915) or specific probes designed from the archaeal sequences obtained in this study (ArchGK probe in Fig. 1B and ArchGI in Fig. 1K). Probe specificity has been confirmed by the lack of cross-hybridization with the second archaeal species of the community (ArchGI probe in Fig. 1C and ArchGK probe in Fig. 1L). Moreover, no hybridization of the two *Thaumarchaeota* could be obtained using universal probes designed for either eukaryotic (Euk 1379) or bacterial

Fig. 1. Ultrastructural analysis, optical and Fluorescence *in situ* hybridization of Candidatus *Giganthauma karukerense* and Candidatus *Giganthauma insulaporcus*.

- A. Light micrograph of Candidatus *G. karukerense*/*G. insulaporcus* filaments on sunken wood from mangrove swamp.
- B. Positive hybridization with Candidatus *G. karukerense*. (Probe ArchGK cyanine 3 dye labelled only). Scale bar = 10 µm.
- C. Negative hybridization showing Candidatus *G. karukerense*. (Probe ArchGI cyanine 3 dye labelled only). Scale bar = 10 µm.
- D. Hybridization with the putative Candidatus *G. karukerense* ectosymbionts. (Probe bacGK cyanine 3 dye labelled only). Red arrows indicate bacterial coat. Scale bar = 10 µm.
- E. Dual hybridization showing Candidatus *G. karukerense* (Probe archGK cyanine 3 dye labelled) filament covered by putative Candidatus *G. karukerense* ectosymbionts (Probe bacGK cyanine 3 dye labelled). Scale bar = 10 µm.
- F. Whole filament of Candidatus *G. karukerense* cells after DAPI stain demonstrating the lack of nuclei inside. Red arrows indicate bacterial coat. Scale bar = 10 µm.
- G. TEM micrograph of a transverse section of Candidatus *G. insulaporcus* and Candidatus *G. karukerense*.
- H. SEM micrograph of Candidatus *G. karukerense* and Candidatus *G. insulaporcus*.
- I. Positive hybridization with Candidatus *G. insulaporcus*. (Probe Arch 915 cyanine 3 dye labelled only). Scale bar = 10 µm.
- J. Whole filament of Candidatus *G. insulaporcus* cells after DAPI stain demonstrating the lack of nuclei inside. Scale bar = 10 µm.
- K. Positive hybridization with Candidatus *G. insulaporcus*. (Probe ArchGI cyanine 3 dye labelled only). Scale bar = 10 µm.
- L. Negative hybridization showing the Candidatus *G. insulaporcus*. (Probe ArchGK cyanine 3 dye labelled only). Scale bar = 10 µm.

cells (Eub 338) (data not shown). Sequence identity matrix performed on both 700 bp archaeal clone sequences obtained in this study showed 98.4% of sequence homology. A sequence identity matrix performed between the two clone sequences obtained in this study and the single cell Candidatus *N. maritimus* showed 97.7% sequence homology.

The first type of archaeal filament is associated with bacteria (Fig. 1B–F), is characterized by a total length of up to 30 mm and is an assemblage of cells with a diameter up to 10 μm (Fig. 1E and Fig. 3D). Each of the numerous archaeal cells forming a single long filament measures 20–24 μm long and 8–10 μm wide (Fig. 3B–D). As no obvious overlapping could be observed between



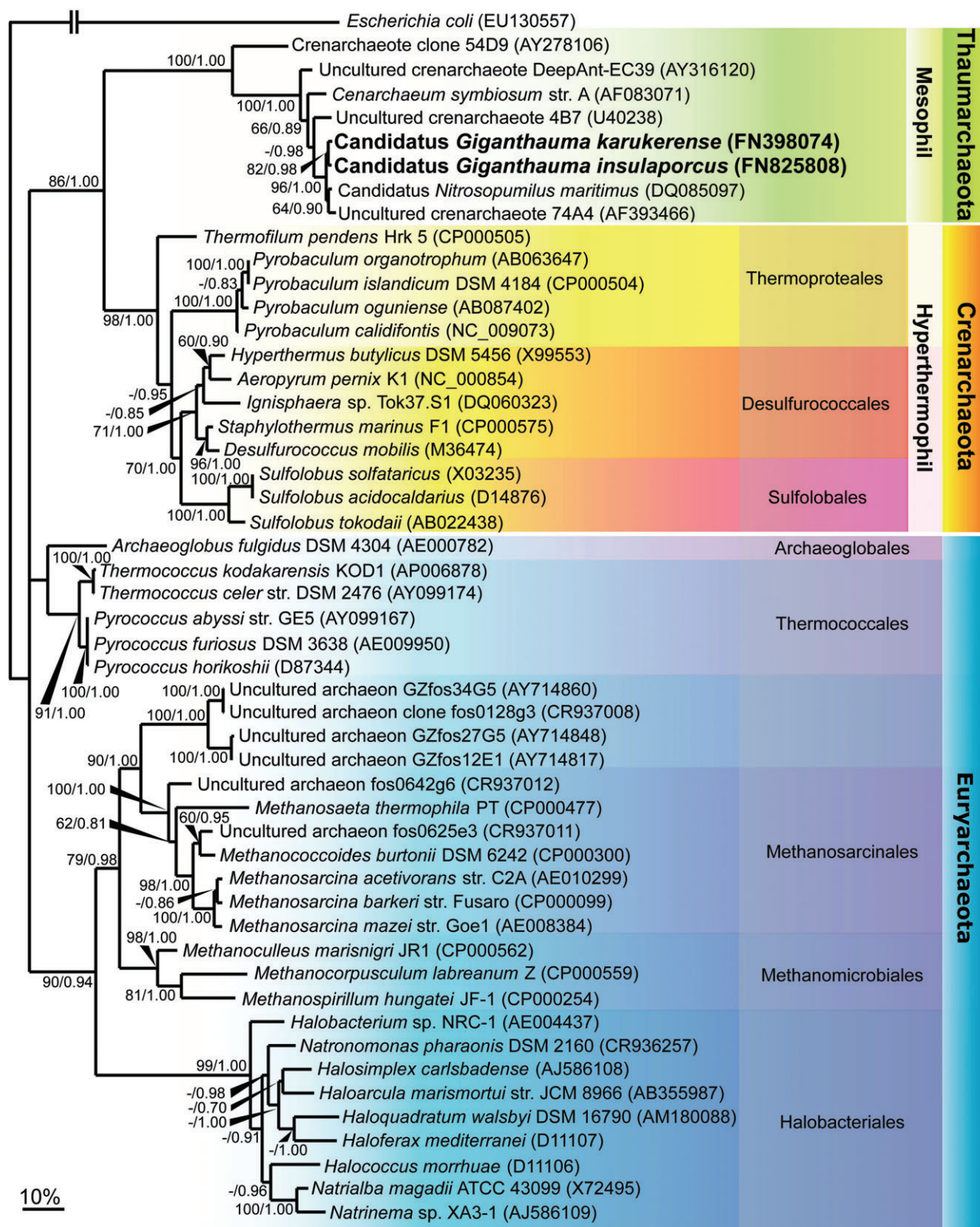


Fig. 2. Tree displaying the phylogenetic relationship between Candidatus *G. karukerense* (in bold) and Candidatus *G. insulaporcus* (in bold) with other Thaumarchaeota, Crenarchaeota and Euryarchaeota based on the analysis of 16S ss-rRNA gene sequences of 700 nucleotides. *Escherichia coli* is used as outgroup. Values at nodes indicate bootstrap values and posterior probabilities (ML)/(Bayesian). Only bootstrap values and posterior probabilities of more than 60% are shown.

two adjacent cells by using SEM, each filament is probably composed of ~1500 cells.

The second type of archaeal filament also has a total length of up to 30 mm but has no external bacterial biofilm. It is compact assemblage of single cells with a diameter up to 13 µm and 6–8 µm length (Fig. 1G–L). This bacteria-free phenotype occurs much less frequently than the bacteria associated one (F. Muller and O. Gros, personal observations).

The cell walls of both archaeal cell genotypes are considerably different from bacterial cell walls described for Gram-negative or Gram-positive marine bacteria (Fig. 1G–H and Fig. 3B–C). The thick outer layer covering the cytoplasmic membrane appears different from the peptidoglycan-rich cell wall of Gram-positive bacteria. Further biochemical analyses will be necessary to clarify the exact nature of the polymers present in these *Thaumarchaeota* species. Most of the archaeal cells overlap each other with infolding-like structures between two adjacent cells (Fig. 3A). Cytoplasmic membranes are separated by electron dense extracellular material (Fig. 3C) corresponding to cell–cell adherens junctions. No nuclei or organelles, except numerous ribosomes, were detectable inside of the cytoplasmic volume of the archaeal cells whatever section orientation was used.

The absence of nuclei in these large cells was confirmed by DAPI staining of whole filaments (Fig. 1F and J). DNA occupies the entire cytoplasm, which is a characteristic of prokaryotic cells. Clear affiliation with the clade *Thaumarchaeota* by phylogenetic analyses as well as positive *in situ* hybridization with a 16S rRNA-specific probe supports the archaeal status of these filaments. A non-eukaryotic status is also suggested by the absence of 18S rDNA polymerase chain reaction (PCR) amplifications. The presence of a gene indicative for ammonia oxidation (AmoA) has been tested using rDNA PCR amplifications with universal primers, but all our results were negative. So, at this time, we have no evidence to state if the two giant *Thaumarchaeota* presented in this study can perform ammonia oxidation as observed in other *Thaumarchaeota* species (Martínez-García *et al.*, 2008; You *et al.*, 2009).

Characterization of associated bacteria

The first type of archaeal filaments are covered by numerous small bacteria located outside of their cell walls (Fig. 3A–B). These are clearly visible using transmission electron microscopy (TEM). Scanning electron microscopy (SEM) observations show that these bacteria are regularly distributed along the archaeal cells (Fig. 3D–H) up to the terminal 'apex' of the filament (Fig. 3G). They form a monolayer of extracellular prokaryotic cells (Fig. 3E–H) that is in close contact with the cell wall of the

host (Fig. 3B). The putative ectosymbionts are rod-shaped (Fig. 3E–H) with a double membrane characteristic of Gram-negative bacteria (Fig. 3A–B). They are 1.5–4.5 µm long and 0.3–0.5 µm wide (Fig. 3B and E–F). The ovoid shapes observed in thin sections (Fig. 3A–B) are caused by the orientation of the respective section since no cocci-shaped bacterial cells were observed with SEM (Fig. 3E–H). The cytoplasm of the bacterial symbionts shows large electron lucent granules indicative of elemental sulfur granules as described in thioautotrophic free-living bacteria (Pasteris *et al.*, 2001), in gill-endosymbionts of various bivalves (Vetter, 1985) or in the cytoplasm of eukaryotic host cells (Lechaire *et al.*, 2008). The density of this bacterial population varies from scattered (Fig. 3H) to dense (Fig. 3E) hiding the underlying archaeal cells (Fig. 3G).

The small cells surrounding these archaeal cells only hybridized with the *γ-Proteobacteria* probe (Gam 42) and with the probe specific for sulfur-oxidation designed here (Fig. 1D–E). The PCR amplification and direct sequencing show the presence of one single bacterial 16S ss-rRNA sequence in our sample. The 16S ss-rRNA characterization of the small bacteria verifies that they belong to the *γ-Proteobacteria* (Fig. 4A). They are in a monophyletic group formed by other endo- and ecto-symbiotic thioautotrophic and free-living sulfur-oxidizing bacteria retrieved from sulfide-rich habitats. Sulfur-oxidizing ability is indicated by the presence of the gene for adenosine-5'-phosphosulfate reductase/oxidase's alpha-subunit (AprA), which groups within the Apr lineage I (Fig. 4B) found in other endo- and ecto-symbiotic thioautotrophic and free-living sulfur-oxidizing bacteria (Meyer and Kuever, 2008; Rinke *et al.*, 2009). It has been proposed to contain the 'authentic' Apr gene loci not affected by lateral gene transfer (Meyer and Kuever, 2008; Rinke *et al.*, 2009). Unfortunately, no fluorescent *in situ* hybridization (FISH) experiment using probes directed against mRNA AprA were done in order to localize this AprA gene in the bacteria associated with the *Thaumarchaeota*. However, PCR amplification and direct sequencing show the presence of one single AprA sequence in the archaea/bacteria DNA sample. Both AprA and the 16S ss-rRNA sequences of the *Thaumarchaeota*-associated bacteria are closely related to their homologues in the Candidatus *Thiobios zoothamnii* ectosymbiont described by Rinke and colleagues (2009).

The PCR amplifications of a unique bacterial 16S rDNA sequence, and one single AprA gene and their corresponding phylogenetic analyses, positive *in situ* hybridizations using both universal *γ-Proteobacteria* and 16S rRNA specifically designed probes confirm the status of the *Thaumarchaeota*-associated bacteria within the clade of *γ-Proteobacteria*. The presence of a specific *γ-proteobacterium* closely related to sulfur-oxidizing bac-

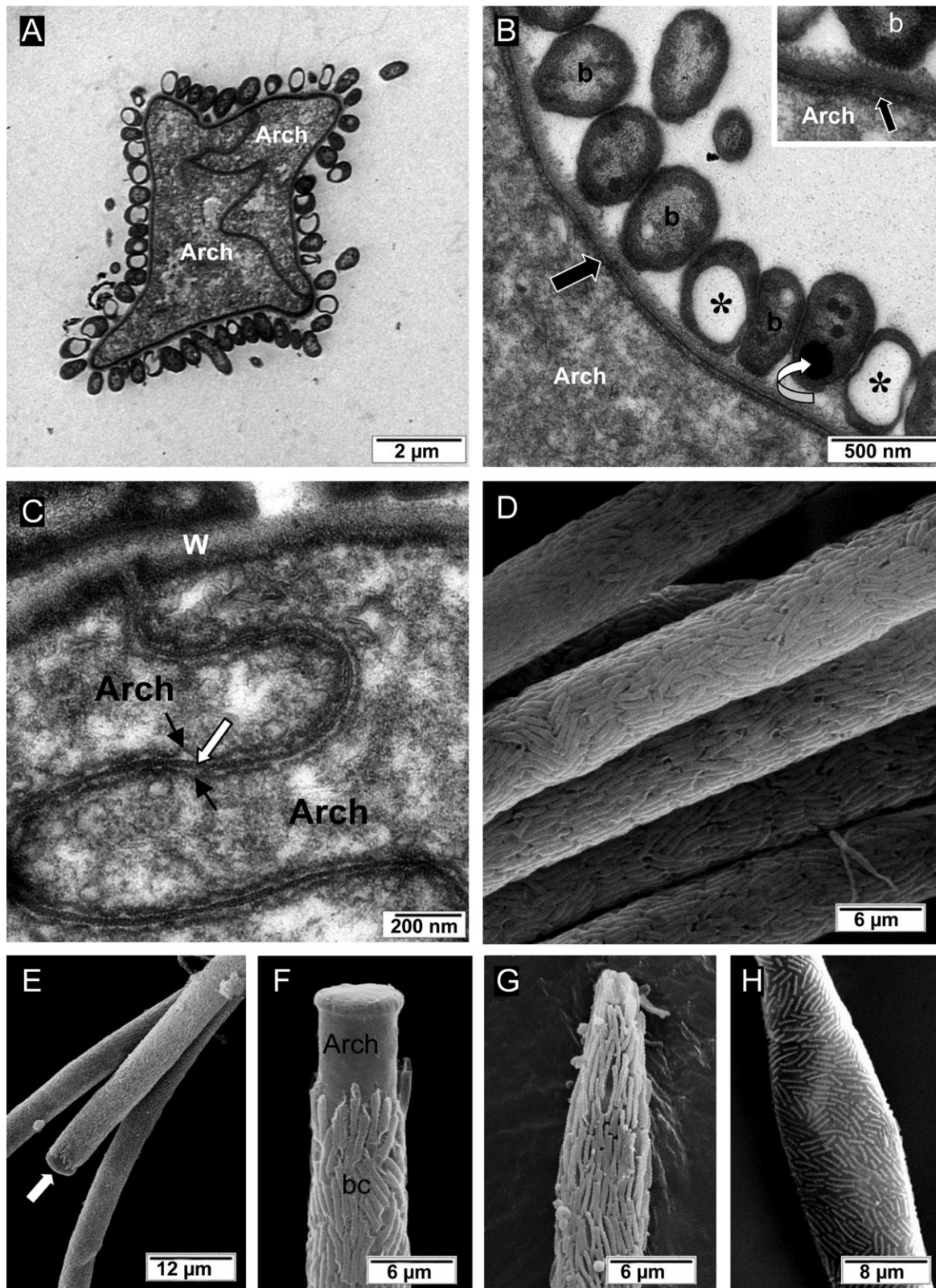


Fig. 3. Ultrastructural analysis of *Candidatus Giganthauma karukerense*.

A. TEM micrograph of a transverse section of *Candidatus G. karukerense* showing two archaeal cells (arch) covered by a monolayer of bacteria. The extracellular bacterial coat surrounding the archaeal cells is composed of transverse sections of the same bacterial species. B. TEM micrograph focusing on the putative bacterial ectosymbionts. Some of the bacteria (b) located outside the archaeal cell (Arch) show sulfur granules (asterisks) that appear empty in ultrathin sections because elemental sulfur storage granules (white curved arrows) dissolve in solvents commonly used in electron microscopy. The archaeal cell wall is indicated by black straight arrows. Insert shows a higher magnification focusing on the thick polymer layer covering the cytoplasmic membrane (black arrows). C. TEM micrograph of two adjacent archaeal cells showing the two cytoplasmic membranes (black arrows) separated by electron dense extracellular material (white arrow) resembling cell–cell *adherens* junction. W, archaeal cell wall. D. SEM micrograph of *Candidatus G. karukerense* filaments entirely covered by their putative bacterial ectosymbionts. All symbionts have a similar size and rod shape. No cocci-shaped bacterial cells could be observed with SEM. E and F. SEM micrographs of broken *Candidatus G. karukerense* filaments with an archaeal cell (white arrow). The bacterial coat (bc) covers the underlying archaeal cell (Arch) except in areas of break. G and H. SEM micrographs showing the variations in density and numbers of the putative thioautotrophic bacterial ectosymbionts from a dense population at the 'apex' of the archaeal filaments to a more scattered one.

teria has been confirmed from more than 30 samples collected during one year of monthly observations from three different sampling sites.

Chemical characterization of the habitat

Short-term measurements indicate that the microenvironment of the *Thaumarchaeota* described is substantially enriched in sulfide. Free sulfide concentration reach several hundred micromolar to millimolar values (Fig. 5A). This enrichment is observed at the sediment surface among filaments as well as in the upper millimeters of the sediment layer just underneath the filaments, suggesting that sulfide is diffusing from the sediment up to the filamentous mat covering it. Sulfide remained undetectable ($< 1 \mu\text{M}$) in the seawater above the archaeal mats.

The continuous record reveals a significant variation of sulfide exposure levels in the mat over a 6 h period (Fig. 5B). Compared with sulfide concentrations, the pH is more stable and remains close to neutral (6.6 to 6.8), about 1 unit below local sea water pH (8.0 to 8.3). This stable neutral pH suggests quite stable microscale hydrodynamic conditions at the scale of the mat, in comparison to those described in other mangrove sulfidic microhabitats hosting sulfide-dependent symbiotic ciliates (Vopel *et al.*, 2005; Laurent *et al.*, 2009). The observed fluctuation of sulfide concentration may therefore reflect the modulation of sulfide diffusion intensity from the sediment by some physical or biological processes.

Discussion

The two new thaumarchaeal species described here form filaments that are up to 30 mm long, more resembling filamentous bacteria, such as those described within the cyanobacteria (except of the presence of heterocysts) and within giant filamentous sulfur oxidizers bacteria (*Beggiatoa*; *Thioploca*). To date, each of the numerous cells of the archaeal species described here (20–24 μm long for 8–10 μm wide and 6–8 μm long for more than 10 μm wide) are the biggest archaeal cells ever described

in a natural environment. Before this study, the largest cells were *Staphylothermus marinus* sp. with a diameter up of 15 μm under laboratory growing conditions while measuring only 1 μm in natural environment (Fiala *et al.*, 1986).

Following the International Code of Nomenclature of Prokaryotes, we propose for the shallow water mesophilic archaeal species with associated bacteria, the name of *Candidatus Giganthauma karukerense* gen. nov., sp. nov., in reference to the large size of these cells and the resulting huge size of the archaeal filament, to the phylum they belong to, and to the Pre-Columbian name of Guadeloupe (Karukera) for the species name.

For the shallow water mesophilic archaeal species that is not associated with bacteria, we propose the name of *Candidatus Giganthauma insulaporcus* gen. nov., sp. nov., in reference to the large size of these cells, the phylum they belong to and the first sampling site in Guadeloupe (îlet cochon) for the species name.

The consistent presence of a biofilm on *Candidatus G. karukerense* that is made of one specific γ -proteobacterium closely related to sulfur-oxidizing bacteria could suggest a symbiotic relationship between the large archaeon (host) and the associated bacteria (ectosymbionts). It is as yet unclear how the putative symbiotic partners interact with each other. The associated bacteria could allow this new species of the phylum *Thaumarchaeota* to colonize an environment with relatively high and fluctuating sulfide concentrations thus minimizing sulfide toxicity for the host, as has been proposed for some sulfide-oxidizing symbioses (Stewart *et al.*, 2005; Dubilier *et al.*, 2008).

To date, *Candidatus G. insulaporcus* have only been observed in limited proportion mixed in with 'large' *Candidatus G. karukerense* dominated populations. This observation could possibly support a local decontamination of sulfide performed by *Candidatus G. karukerense* bacteria that could allow growth of *Candidatus G. insulaporcus*.

The description of two large thaumarchaeal species and the possibility of a novel type of symbiotic relationship

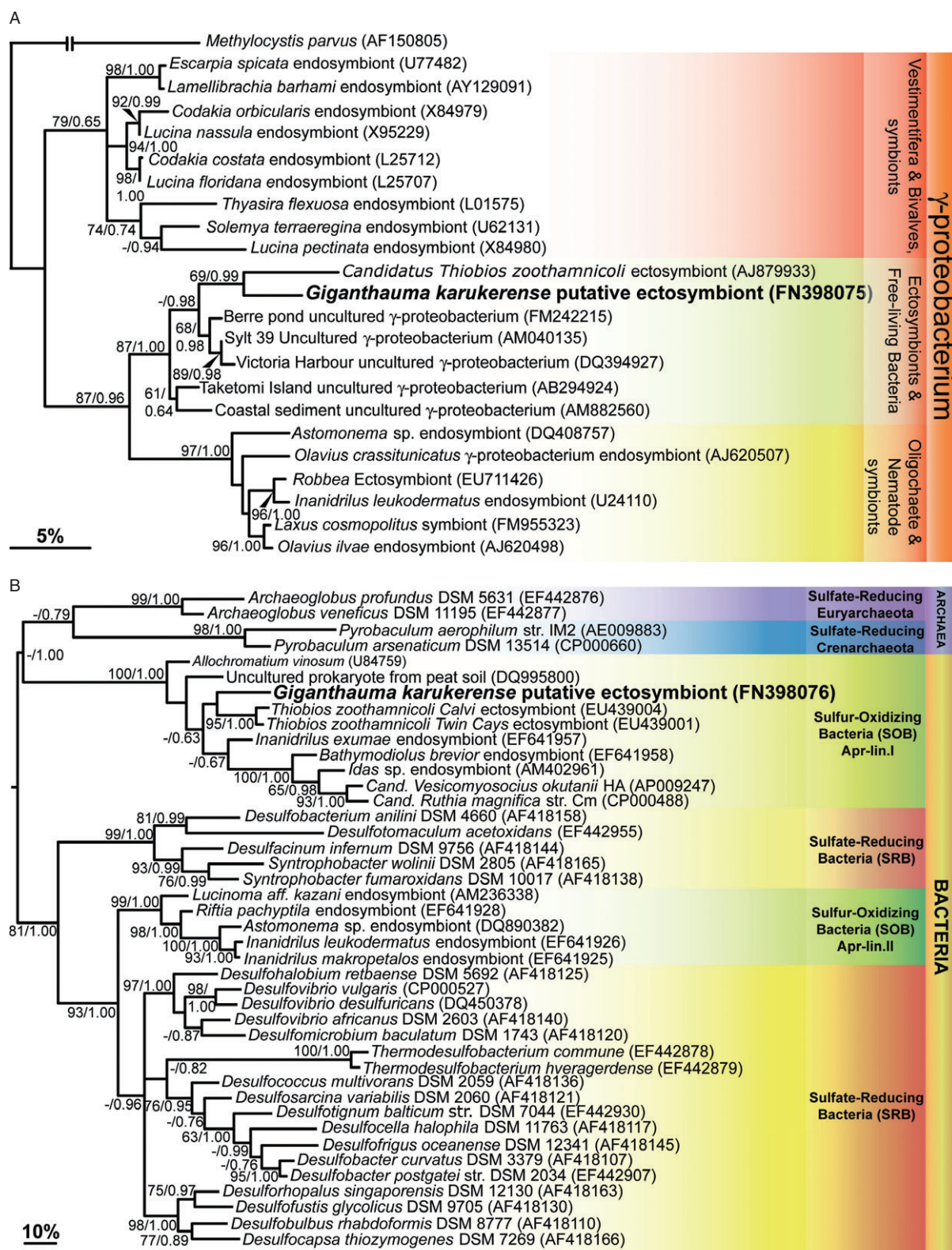


Fig. 4. A. Tree displaying the phylogenetic relationship between the putative *Candidatus G. karukerense* ectosymbiont (in bold), other γ -*Proteobacteria* symbionts and free living bacteria, based on the analysis on 16S ss-rRNA gene sequences of 800 nucleotides. *Methylocystis parvus* is used as an outgroup. Values at nodes indicate bootstrap values and posterior probabilities (ML)/(Bayesian). Only bootstrap values and posterior probabilities of more than 60% are shown. B. Midpoint rooted Bayesian tree displaying the phylogenetic relationship between putative *Candidatus G. karukerense* ectosymbiont (in bold) and other bacterial symbionts, free living bacteria, and archaea based on the analysis of adenosine-5'-phosphosulfate reductase/oxidase alpha subunit (AprA) gene sequences of 320 nucleotides. Values at nodes indicate bootstrap values and posterior probabilities (ML)/(Bayesian). Only bootstrap values and posterior probabilities of more than 60% are shown.

between archaea and bacteria highlight the unexpected diversity of the *Thaumarchaeota*. This result supports the proposal of a phylum status for this recently discovered archaeal group (Brochier-Armanet *et al.*, 2008a).

The multiple cell assembly of *Candidatus G. karukerense* and of *Candidatus G. insulaporcus* could be interpreted as a first evolutionary step to a vast increase in cellular complexity and origin of highly complex multicellular organisms as proposed by Cavalier-Smith (2002). A possible exchange of chemical compounds or a horizontal gene transfer between the putative symbiotic partners should be considered in further experiments.

Experimental procedures

Gene amplification, cloning and sequencing

Thaumarchaeal filaments were sampled in the mangrove swamps (sea water temperature ~28°C and salinity ~37‰) of Guadeloupe (French West Indies) and brought back to the lab in sea water. They were removed from their hard substrates by cutting their base under a dissecting microscope. Most sea water was removed by pipetting to obtain more

concentrated thaumarchaeal filaments. Approximately 20 mg of these filaments was used for DNA extraction using DNeasy Blood & Tissue (Qiagen).

The PCR amplifications used the following Touchdown PCR cycling conditions: initial denaturation at 94°C for 5 min, followed by 10 cycles at 94°C for 1 min, 65°C to 52°C for 1 min and 72°C for 1 min and by 25 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, then a final elongation step at 72°C for 7 min.

Paired primers 8F and 907R, and paired primers Arch21F and Arch806R (Table 1) were used for PCR amplification of bacterial and archaeal small ss-rRNA respectively. Paired primers 18S1F and 18SBIR (Table 1) were used for PCR amplification of eukaryote 18S small ss-rRNA. Paired primers Aps1F and Aps4R, and paired primers Arch-amoAF and Arch-amoAR were used for PCR amplification of bacterial AprA and AmoA sequence respectively (Table 1).

Bacterial 16S and bacterial AprA PCR products were purified with the QIAquick PCR purification kit (Qiagen) and directly sequenced by GATC Biotech (<http://www.gatc-biotech.com>).

A thaumarchaeal 16S ss-rRNA fragment of interest of about 700 bp was eluted using Nucleospin Extract II (Macherey-Nagel) and cloned with a pGEM-T TA cloning kit

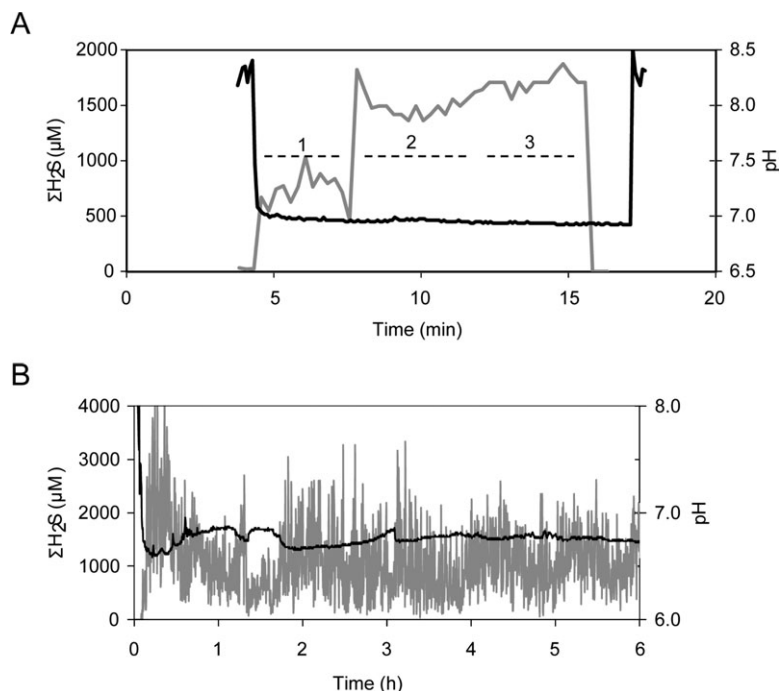


Fig. 5. Analysis of sulfide concentrations in the microenvironment of *Candidatus Giganthauma karukerense*/*Candidatus Giganthauma insulaporcus*. A. Short-term measurements of pH (black curve) and free sulfide concentration (grey curve) among filaments in two different locations inside a mat (1,2) and immediately below in the sediment (3). B. Six-hour record of pH and free sulfide concentrations in a *Candidatus G. karukerense*/*Candidatus G. insulaporcus* mat located on the surface of a wooden board.

Table 1. 16S, 18S ss-rRNA AprA and AmoA primer.

Primer names	Sequence (5' to 3')	References
8F	GGA TCC AGA CTT TGA TYM TGG CTC AG	Felske <i>et al.</i> (1997)
907R	CCG TCA ATT CMT TTR AGT TT	Lane <i>et al.</i> (1985)
A2F	TTC CGG TTG ATC CYG CCG GA	DeLong (1992)
UA1204R	TTM GGG GCA TRC IKA CC	Takai and Horikoshi (2000)
AprA-1-FW	TGG CAG ATC ATG ATY MAY GG	Meyer and Kuever (2007)
AprA-5-RV	GCG CCA ACY GGR CCR TA	Meyer and Kuever (2007)
18S1F	TAC CTG GTT GAT CCT GCC AGT AG	Giribet and Ribera (2000)
18SBiR	GAG TCT CGT TCG TTA TCG GA	Giribet and Ribera (2000)
Arch-amoAF	STA ATG GTC TGG CTT AGA CG	Francis <i>et al.</i> (2005)
Arch-amoAR	GCG GCC ATC CAT CTG TAT GT	Francis <i>et al.</i> (2005)

(Promega). A vector (pGEM-T; Promega) was used to transform Dh5 α competent *Escherichia coli* (Promega), and plasmids from 20 positive clones were extracted using Nucleospin plasmid (Macherey-Nagel). Inserts were then sequenced directly by Cogenics (<http://www.cogenics.com/>).

Sequences were compared with the Ribosomal Database Project (RDP) (Cole *et al.*, 2009) and with the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) databases using BLAST (Altschul *et al.*, 1990). Best hits were included in phylogenetic analyses. Sequences were aligned using CLUSTALX 2.0.8 (Larkin *et al.*, 2007) and alignments were checked manually.

A general time reversible (GTR) model with Γ -distributed rates of evolution, including invariable sites was used in maximum likelihood (ML) and Bayesian analyses of the archaeal 16S rRNA dataset (700 bp), bacterial 16S rRNA dataset (800 bp) and adenosine-5'-phosphosulfate reductase/oxidase's alpha-subunit (AprA) dataset (320 bp). Maximum likelihood analyses have been performed using phyML-aLRT software Version 2.4.5 (Guindon and Gascuel, 2003). Robustness of the nodes was assessed by performing 1000 bootstrap replicates.

Bayesian tree estimation and posterior probabilities were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), using the same evolutionary model parameters as used for the ML searches. Bayesian analysis comprised four Markov chains and each chain were run over 1 000 000 generations.

Fluorescent in situ hybridization

Thaumarchaeal samples were fixed for FISH in 2% paraformaldehyde in sterile sea water at 4°C for 2 h. After two washes in sterile sea water, samples were dehydrated in an increasing ethanol series (30%, 50, 70%, 95% and 3 changes of 100% ethanol, 10 min per bath). Before a hybridization experiment, samples were rehydrated in a reverse ethanol series, and incubated for 12 min in 0.2 M HCl, rinsed for 10 min in prehybridization buffer (20 mM Tris [pH 8.0]), incubated for 5 min at 37°C in 0.5 μ g ml⁻¹ of proteinase K (Sigma) in prehybridization buffer and rinsed in prehybridization buffer. Oligonucleotidic hybridization was done directly in 0.2 ml Eppendorf microtubes without any steps of embedding or sectioning of the samples analysed. Hybridizations were carried out for 4 h at 46°C in hybridization buffer [0.9 M NaCl, 0.02 M Tris-HCl buffer (pH 7.8), 0.01% SDS, and 20% or 35%

formamide in MilliQ water]. Probes at 50 ng μ l⁻¹ were diluted in hybridization buffer (1:15 v/v). At the end of the hybridization, samples were initially washed two times at 48°C for 15 min each in washing buffer [0.22 M NaCl, 0.02 M Tris-HCl buffer (pH 7.8), 0.005 M EDTA, and 0.01% SDS in MilliQ water]. Samples were observed in MilliQ water using an epifluorescence Eclipse 80i microscope.

Seven oligonucleotide probes were used: Eub 338, targeting most members of the *Bacteria*; Gam 42, targeting most members of the γ -*Proteobacteria*; Euk 1379 designed for eukaryotes; Arch 915, targeting most members of archaea; and bacGK, ArchGK and ArchGI that were specifically designed from the 16S sequence of the γ -*Proteobacteria* associated with Candidatus *G. karukerense*'s and from the 16S sequence of the two thaumarchaeal species described in this study (Table 2).

For detailed information about Eub 338, Gam 42 and Arch 915 see Amann and Fuchs (2008).

Probe design was performed manually. Probe 16S ss-rRNA localization has been optimized according to Fuchs and colleagues (1998). The newly designed probe sequences obtained were checked against all 16S ss-rRNA sequences in RDP by the implemented tool Probe Match (Cole *et al.*, 2009).

The RDP Probe Match of the Candidatus *G. insulaporcus* probe (probe ArchGI) showed no hybridization with archaeal clones with no mismatches, one mismatch, or two mismatches. Candidatus *G. karukerense* probe (probe ArchGK) has been designed for the same locus of the ArchGI-specific probe, including two mismatches with the ArchGI sequence (Table 2) in order to discriminate the two thaumarchaeal sequences found in this community.

The results of the RDP Probe Match of the Candidatus *G. karukerense* probe (probe ArchGK) show the ability to hybridize with 121 uncultured archaeal clones and the isolated Candidatus *N. maritimus* with no mismatch. With one mismatch, probe archGK is able to hybridize 321 uncultured archaeal clones and the isolated Candidatus *N. maritimus*. With two mismatches, the probe archGK is able to hybridize with 459 uncultured archaeal clones and two isolated thaumarchaeal species, Candidatus *N. maritimus* and *C. symbiosum*. RDP Probe Match of the Candidatus *G. insulaporcus* probe (probe ArchGI) showed the inability to hybridize archaeal clones with no mismatches, one mismatch, or two mismatches. RDP Probe Match results of Candidatus *G. karukerense* associated bacterial probe (probe BacGK) hybridize *in silico* with three uncultured γ -*Proteobacteria*

Table 2. Probe sequences.

Probe names	Sequence (5' to 3')	Target	'RDP analysis'	References
Arch 915	GTG CTC CCC CGC CAA TTC CT	<i>Archaea</i>	–	Stahl and Amann (1991)
Eub 338	GCT GCC TCC CGT AGG AGT	<i>Eubacteria</i>	–	Amann <i>et al.</i> (1990)
Gam 42	GCC TTC CCA CAT CGT TT	<i>γ-Proteobacteria</i>	–	Manz <i>et al.</i> (1992)
Euk 1379	TAC AAA GGG CAG GGA C	Eukaryote	–	Hicks <i>et al.</i> (1992)
ArchGK	CAT TTG GAA CAC AAA CCA	Candidatus <i>G. karukerense</i>	0 mismatches (121) 1 mismatch (321)	This study
		16S	2 mismatches (459)	
ArchGI	CAT TTG GGG CAC AAA CCA	Candidatus <i>G. insulaporcus</i>	0 mismatches (0) 1 mismatch (9)	This study
		16S	2 mismatches (1030)	
BacGK	CTT GGG CTC ATC CAA TAG CGA TAA ATC	Candidatus <i>G. karukerense</i>	0 mismatches (3) 1 mismatch (34)	This study
		Associated bacteria	2 mismatches (43)	

clones with no mismatches. With one mismatch, the BacGK probe is able to hybridize with 34 uncultured *γ-Proteobacteria* clones while, with two mismatches, the BacGK probe is able to hybridize with 36 uncultured *γ-Proteobacteria* clones and 7 *α-Proteobacteria* clones (Table 2).

All FISH probes used in this study were labelled with the water-soluble fluorescent cyanine 3 dye.

Ultrastructural analysis

Archaeal filaments were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer, which was made isosmotic to sea water by addition of sodium chloride and calcium chloride. Following a brief rinse, samples were rinsed twice in the same buffer and then dehydrated through a graded acetone series before drying with CO₂ at critical point in a critical point drier (Polaron, Bio-Rad). The samples were then sputter-coated with gold (Sputter Coater SC500, Bio-Rad) before observation in an SEM Hitachi S-2500 at a 20 kV accelerating tension.

A few filaments of *Candidatus G. karukerense* and *Candidatus G. insulaporcus* were prefixed for a minimum of one hour at 4°C following the same procedure as for SEM, then fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer before being rinsed in distilled water, and post-fixed with 2% aqueous uranyl acetate for one more hour. After a rinse in distilled water, each sample was dehydrated through a graded ethanol series and embedded in Epon-Araldite according to Glauert (1975). Thin sections (60 nm thick) were contrasted 30 min in 2% aqueous uranyl acetate and 10 min in 0.1% lead citrate before examination in a TEM LEO 912 and a SEM Hitachi S-2500.

Sulfide and pH measurements

Autonomous electrochemical probes have been specially designed to monitor H₂S and pH in fluctuating environments (Le Bris *et al.*, 2008). Each of these probes is composed of a submersible potentiometric data logger (NKE, France) and a custom-made electrode. The pH electrode was adapted from a medical (stomachal) electrode (SOLAL, France) as described in Le Bris and colleagues (2001). The S²⁻-specific electrode is a conventional Ag/Ag₂S electrode. It was pre-

pared from a silver wire (Goodfellow, UK) preconditioned by immersion in a 200 mM Na₂S solution. Reference electrodes were also made of silver wires electrochemically coated with AgCl, with the ambient sea water being used as the reference electrolyte. These probes have been deployed for various durations in several shallow and deep environments, providing information about the temporal variability of sulfide and pH within a given microhabitat (Le Bris *et al.*, 2008). The sensing tips of the Ag/Ag₂S electrode and of the glass pH electrode are 0.8 and 1.5 mm in diameter, respectively, and about 2 mm in length. They were tightly attached and carefully placed among filaments. Both short-term (3 to 5 min) and long-term (overnight) deployments were performed within *Candidatus G. karukerense*/*G. insulaporcus* mats. Short-term measurements were made on a patch associated with organic debris covering the sediment surface, and immediately below in the sediment. The long-term measurement sequence was obtained on a patch colonizing the surface of a wooden board. During deployment, electrode potentials were recorded every 15 s. The Ag/Ag₂S electrode was calibrated before use in the laboratory from standard additions of a stock solution of Na₂S in natural sea water. This calibration allowed the *in situ* concentration of HS⁻ (the predominant form of free sulfide at sea water pH) to be quantified from both the electrode potential and pH. Free sulfide (i.e. H₂S + HS⁻) was then calculated from these values and the acidity constant for H₂S defined in Rickard and Luther (2007) for 28°C and a salinity of 35‰.

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