Ignicoccus hospitalis sp. nov., the host of 'Nanoarchaeum equitans'

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A novel chemolithoautotrophic and hyperthermophilic member of the genus *Ignicoccus* was isolated from a submarine hydrothermal system at the Kolbeinsey Ridge, to the north of Iceland. The new isolate showed high similarity to the two species described to date, *Ignicoccus islandicus* and *Ignicoccus pacificus*, in its physiological properties as well as in its unique cell architecture. However, phylogenetic analysis and investigations on the protein composition of the outer membrane demonstrated that the new isolate was clearly distinct from *I. islandicus* and *I. pacificus*. Furthermore, it is the only organism known so far which is able to serve as a host for 'Nanoarchaeum equitans', the only cultivated member of the 'Nanoarchaeota'. Therefore, the new isolate represents a novel species of the genus *Ignicoccus*, which we name *Ignicoccus hospitalis* sp. nov. (type strain KIN4/I^T = DSM 18386^T = JCM 14125^T).

INTRODUCTION

Both prokaryotic domains, the *Bacteria* as well as the *Archaea*, contain hyperthermophilic micro-organisms, with an optimal growth temperature above 80 °C (Stetter, 1989). The cultivated hyperthermophiles cluster around the root in 16S rRNA-based phylogenetic trees (Stetter, 1992). Within the *Bacteria*, only members of the phyla *Thermotogae* and *Aquificae* can be characterized as hyperthermophiles, whereas hyperthermophilic organisms are found within all archaeal phyla, the *Crenarchaeota*, *Euryarchaeota* and 'Nanoarchaeota'. Until recently, all cultivated crenarchaeotes were exclusively extremely thermophilic or hyperthermophilic (Huber, 2005). Interestingly, a mesophilic, autotrophic, ammonium-oxidizing, marine crenarchaeote was described recently (Könneke *et al.*, 2005). Within the *Crenarchaeota*, three orders have been described so far: the *Sulfolobales*, the *Thermoproteales* and the *Desulfurococcales*. The *Desulfurococcales* can be subdivided into two families: the *Pyrodictiaceae* and the *Desulfurococcaceae* (Huber & Stetter, 2001). Within the *Desulfurococcaceae*, only members of the genus *Ignicoccus* harbour obligately chemolithotrophic sulfur reducers (Huber *et al.*, 2000). All strains of this genus exhibit a cell envelope unique among the *Archaea*: it consists of a cytoplasmic membrane, a periplasmic space with a variable width of between 20 and 400 nm and, as the outermost sheath, an outer membrane (Rachel *et al.*, 2002).

Here we report on the isolation and properties of a novel species of the genus *Ignicoccus*, which represents the host for 'Nanoarchaeum equitans', the only cultivated representative of the recently described phylum 'Nanoarchaeota' (Huber *et al.*, 2002).

METHODS

Source of sample. On a diving excursion to the hydrothermal system at Kolbeinsey Ridge, to the north of Iceland (67° 03' 46" N 18° 42' 64" E), samples of hot rocks and gravel were taken in a depth of about 106 m (Fricke *et al.*, 1989).

Strains and culture conditions. The new isolate and *Ignicoccus* type strains (obtained from our culture collection) were cultivated in strictly anaerobic ½ SME medium (Stetter *et al.*, 1983; Pley *et al.*, 1991; modified by Huber *et al.*, 2006), prepared according to Balch & Wolfe (1976). The medium contains the following components (l⁻¹): NaCl, 13.85 g; MgSO₄·7H₂O, 3.5 g; MgCl₂·6H₂O, 2.75 g; KH₂PO₄, 0.5 g; CaCl₂·2H₂O, 0.38 g; KCl, 0.33 g; (NH₄)₂SO₄, 0.25 g; NaBr, 0.05 g; H₃BO₃, 0.015 g; NaHCO₃, 0.16 g; SrCl₂·6H₂O, 7.5 mg; KI, 25 µg; elemental sulfur, 5.0 g. Reduction of the medium was carried out by addition of 0.5 g Na₂S; afterwards, the pH was adjusted at room temperature to pH 5.5–6.0 with sulfuric acid. Growth conditions were similar to those described for the other two described *Ignicoccus* species (Huber *et al.*, 2000): routinely, the organisms were grown in 120 ml serum bottles containing 20 ml ½ SME medium pressurized with H₂/CO₂ (250 kPa; 80:20, v/v). Incubation was carried out at 90 °C under shaking (100 r.p.m.). Heterotrophic growth was tested under a gas phase of N₂/CO₂ (200 kPa, 80:20, v/v), using the following substrates: yeast extract, Casamino acids (Difco), gelatin (Sigma Aldrich), formate, acetate, meat extract, starch, peptone and glucose (VWR). Unless otherwise noted, substrates were added

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KIN4/I^T is AJ318042.

at final concentrations of 0.1%. The same concentration was used for the alternative electron acceptors thiosulfate, sulfite, sulfate, nitrate and nitrite. Resistance to ampicillin, rifampicin and vancomycin was tested at final concentrations of 50 µg ml⁻¹ each. For investigations of the pH dependence of growth, the pH was adjusted with diluted sulfuric acid as indicated; additional buffers were not used. Batch cultures were grown in a 50 l enamel-protected fermenter (HTE Bioengineering) at 90 °C under stirring (150 r.p.m.) and gassing with H₂/CO₂ (80:20, v/v; 2 l min⁻¹).

Light and electron microscopy. Cells were routinely observed with an Olympus BX 60 phase-contrast microscope with a UPlanFl 100/1.3 oil-immersion objective. Bacterial growth was followed by direct cell counting using a Thoma chamber (depth 0.02 mm). Samples were prepared for transmission electron microscopy as described previously (Rieger *et al.*, 1997; Huber *et al.*, 2000; Rachel *et al.*, 2002). Electron micrographs were digitally recorded using a slow-scan CCD camera (Tietz) attached to a CM12 transmission electron microscope (FEI Co.) with an acceleration voltage of 120 kV.

H₂S determination. Formation of H₂S was monitored qualitatively by dripping the sample onto lead acetate tape, yielding a dark brown colouring.

Nitrogen fixation. The capacity of isolate KIN4/I^T for nitrogen fixation was tested in ½ SME medium without (NH₄)₂SO₄. This medium was pressurized with N₂/H₂/CO₂ (300 kPa; 65:15:20, by vol.). Experiments were carried out with and without Na₂MoO₄ and VOSO₄ (1 µM each).

DNA isolation and DNA base composition. DNAs were prepared as described previously (Wildgruber *et al.*, 1982). The G+C content of genomic DNA was determined by melting-point analysis (Marmur & Doty, 1962) and by direct analysis of the nucleotides after digestion of the DNA with nuclease P1 and separation by HPLC (Völkl *et al.*, 1993). Calf thymus DNA (G+C content of 42 mol%) was used as a reference.

16S rRNA gene sequence analysis. The nearly complete 16S rRNA gene sequence of the new isolate was amplified by PCR. The primers used in the amplification corresponded to positions 8–23 (5'-TCYGGTTGATCCTGCC; Eder *et al.*, 1999) and 1406–1390 (5'-ACGGGCGGTGTGTRCAA; Lane, 1991) in the 16S rRNA sequence of *Escherichia coli* (Brosius *et al.*, 1981). Both strands of the PCR product were sequenced directly as described by Burggraf *et al.* (1997). The sequence was aligned with a set of representative archaeal 16S rRNA gene sequences using the ARB program (Ludwig *et al.*, 2004). Dendrograms were computed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods included in the ARB package.

Outer-membrane protein composition. After homogenizing the cells using a glass homogenizer, outer membranes were purified by gradient centrifugation on a continuous sucrose gradient, as described previously (Näther & Rachel, 2004). Protein gel electrophoresis under denaturing conditions was performed according to Schägger & von Jagow (1987).

RESULTS

Enrichment and isolation

Enrichment attempts were carried out with samples of hot rocks and gravel taken at the hydrothermal system at Kolbeinsey Ridge. About 1 g of the material was added to 20 ml anoxic, organic compound-free culture medium. Enrichment attempts were incubated under shaking at

90 °C. After 2 days, cocci about 1–2 µm in diameter were visible in the enrichment attempt of sample KIN4 and large amounts of H₂S could be detected qualitatively in the culture medium. Surprisingly, several cells were covered by very tiny cocci. These tiny cocci had meanwhile been described as '*Nanoarchaeum equitans*', the first representative of the novel archaeal phylum '*Nanoarchaeota*' (Huber *et al.*, 2002). A pure culture of the new isolate could be obtained by the use of optical tweezers (Huber *et al.*, 1995). Separations were carried out with and without attached '*Nanoarchaeum*' cells. As expected, cells without '*Nanoarchaeum*' cells always led to '*Nanoarchaeum*'-free subcultures (designated KIN4/I^T). Surprisingly, the same result was obtained for cells with attached '*Nanoarchaeum*' cells (only one co-culture was obtained from about 50 separations). In these subcultures, '*N. equitans*' was also no longer detectable by specific PCR amplifications of the 16S rRNA gene. These cultures were initially designated KIN4/AH, but later turned out to be identical to strain KIN4/I^T.

Morphology

Cells of isolate KIN4/I^T were regular to irregular cocci, usually occurring singly or in pairs. They exhibited cell diameters of 1–4 µm, rarely up to 6 µm. The organisms stained Gram-negative. Like *I. islandicus*, up to nine flagella-like appendages, each with a diameter of 14 nm, were observed to be anchored at one pole into the cell. The cell architecture of isolate KIN4/I^T was quite similar to that of the type strains of *I. islandicus* and *I. pacificus* (Huber *et al.*, 2000; Rachel *et al.*, 2002). The densely packed cytoplasm was enclosed by a membrane (Fig. 1a). The width of the periplasm varied between 20 and 500 nm and contained round or elongated membrane-coated vesicles, about 50 nm in diameter and up to 300 nm long. They were seen to be released from the cytoplasmic membrane and to come into close proximity with the outer membrane, the outermost sheath of the cells. It had a weak double-layer appearance in sections; the two leaflets were seen to become separated in freeze-etched samples (Fig. 1b). As in *I. islandicus*, this membrane contained numerous tightly packed proteins (Näther & Rachel, 2004) and lipids which, according to LC-MS analyses, are derivatives of archaeol (2,3-di-*O*-phytanyl glycerol diether) (Jahn *et al.*, 2004). In addition, caldarchaeol (glyceroldibiphytanyl glycerol tetraether) was found in the cytoplasmic membrane in small but significant amounts. The predominant polar headgroups of the core lipids consisted of one or more sugar residues attached either directly to the core lipid or via a phosphate group (Jahn *et al.*, 2004). The quantitatively dominant protein of the outer membrane is Imp1227 (*Ignicoccus* outer-membrane protein; molecular mass 6.23 kDa), which occurs in several thermostable oligomeric complexes, with a total mass of more than 50 kDa (Burghardt *et al.*, 2007).

Metabolism

Isolate KIN4/I^T was an obligately anaerobic chemolithoautotroph, growing exclusively by sulfur reduction with

Fig. 1. Transmission electron micrographs of strain KIN4/I^T. (a) Ultrathin section; (b) freeze-etching. Cy, Cytoplasm; P, periplasm; V, vesicles; OM, outer membrane; *, fracture face through the outer membrane. Bars, 1 µm.

molecular hydrogen as the sole electron donor (final cell concentrations up to 1 to 2×10^7 cells ml⁻¹). Thiosulfate, tetrathionate, sulfite, sulfate, nitrate or oxygen (0.5–5 % v/v) could not serve as electron acceptors. In the presence of H₂ and S⁰, growth was stimulated by the addition of meat extract (0.1 %) or yeast extract (0.02 %). No heterotrophic growth was observed on organic substrates such as meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate and glucose when cultures were pressurized with hydrogen-free gas (N₂/CO₂, 80:20; 200 kPa). Ampicillin, rifampicin and vancomycin [final concentration 50 µg (ml medium)⁻¹] were not inhibitory to growth. The organism showed no ability to fix molecular nitrogen.

Optimal growth conditions

Minimal doubling times for isolate KIN4/I^T of around 1 h were observed at 90 °C, 1.4 % NaCl and pH 5.5 (Fig. 2). It grew at temperatures of 73–98 °C, salt concentrations of 0.5–5 % NaCl (w/v) and pH 4.5–7.0 in the medium. No growth was obtained at or below 70 °C or at or above 100 °C or at or below pH 4 or at or above pH 7.5. Furthermore, strain KIN4/I^T was not able to propagate when NaCl was omitted from the medium or when its concentration exceeded 6.0 %.

Storage

Stock cultures contained 5 % (v/v) DMSO and were stored at –140 °C over liquid nitrogen. They served as viable inocula for at least 3 years.

DNA base composition

The G + C content of genomic DNA of isolate KIN4/I^T was 56 mol%, calculated by melting-point analysis and by direct analysis of the mononucleotides.

Phylogenetic analysis

Comparison of the 16S rRNA gene sequences of isolate KIN4/I^T and the two other representatives of the genus *Ignicoccus* (*I. islandicus* and *I. pacificus*; Huber *et al.*, 2000) clearly showed that the genus *Ignicoccus* represents a separate branch within the *Desulfurococcales* (Fig. 3) (phylogenetic distances to all other members of the *Desulfurococcales* at least 6.1 %). Isolate KIN4/I^T exhibits phylogenetic distances of 4.0 % from *I. islandicus* Kol8^T and 2.8 % from *I. pacificus* LPC33^T. Investigations were also carried out on the phylogenetic distances between isolate KIN4/I^T, isolate KIN4/AH and the host organism of the co-culture with '*N. equitans*'. Identical 16S rRNA gene sequences were obtained for all three strains, demonstrating that strain KIN4/I^T is the host organism for '*N. equitans*'.

Outer-membrane protein composition

The outer membrane of cells of *I. islandicus* Kol8^T, *I. pacificus* LPC33^T and isolate KIN4/I^T showed clearly distinctive protein patterns after separation under denaturing conditions. While the most prominent bands in all strains were in the mass range of 50–80 kDa, there were obvious differences in the masses of the observed bands and also in the quantities of these bands (Fig. 4).

DISCUSSION

Based on 16S rRNA gene sequence comparisons, the new hyperthermophilic isolate KIN4/I^T is closely related to the known *Ignicoccus* strains Kol8^T (*I. islandicus*) and LPC33^T and LPC37 (*I. pacificus*), with phylogenetic distances of 4.0 and 2.8 %, respectively. It also resembles members of the genus *Ignicoccus* in its coccoid shape, its ultrastructure (e.g. the unique cell envelope with an outer membrane), the

Fig. 2. Optimal growth conditions of strain KIN4/I^T during growth on H₂ and sulfur. Doubling times were calculated from the slopes of the growth curves. (a) Effect of temperature at pH 5.5 and 1.4 % NaCl. (b) Effect of NaCl concentrations at 90 °C and pH 5.5. (c) Effect of pH at 90 °C and 1.4 % NaCl.

negative Gram reaction and its physiological properties. Like the other members of the genus *Ignicoccus*, strain KIN4/I^T is a hyperthermophilic, moderately halophilic and moderately acidophilic organism, and it grows optimally at 90 °C, 1.4 % NaCl and pH 5.5. The short doubling time of about 1 h is also identical to results for the other *Ignicoccus* species (Huber *et al.*, 2000). Furthermore, isolate KIN4/I^T is an obligate chemolithoautotroph and grows under strictly anoxic conditions. However, isolate KIN4/I^T can clearly be distinguished from the type strains of *I. islandicus* and *I. pacificus* by the significantly higher G + C content of its DNA and the different composition of the outer membrane (Fig. 4; Näther, 2003; Burghardt *et al.*, 2007). Therefore, it represents a novel species of the genus *Ignicoccus*. Due to the fact that it is the only host organism for '*N. equitans*', which is always attached directly to its surface, we name this novel species *Ignicoccus hospitalis* sp. nov.

The most important property of *I. hospitalis* in comparison with the other *Ignicoccus* species is its capacity to serve as a host for '*N. equitans*'. So far, not only no other *Ignicoccus* strain but no other hyperthermophilic archaeon can serve as a host organism. Since growth of '*N. equitans*' is strictly dependent on propagating *I. hospitalis* cells, these two organisms form the only known host–parasite or host–symbiont system of two archaea. The reason for this exclusivity is still unknown, although the obvious differences in the outer-membrane proteins of the different *Ignicoccus* species may provide an explanation (Näther, 2003). The fact that '*N. equitans*' has a highly reduced genome, lacking nearly all metabolic and biosynthetic pathways (Waters *et al.*, 2003), makes it reasonable that its growth depends on a host organism. Indeed, it has already been demonstrated that its lipids are most likely to be synthesized in *I. hospitalis* cells (Jahn *et al.*, 2004). In

Fig. 3. Phylogenetic position of *Ignicoccus* type strains Kol8^T, LPC33^T and KIN4/I^T calculated by the neighbour-joining method using the Jukes and Cantor correction, based on 16S rRNA gene sequences. Bar, 10 estimated substitutions per 100 nucleotides.

Fig. 4. SDS-PAGE of sucrose-gradient-purified outer membranes of strain KIN4/I^T (lane 1), *I. islandicus* Kol8^T (2) and *I. pacificus* LPC33^T (3). Lane M, molecular mass markers (sizes in kDa).

contrast, *I. hospitalis* can grow in pure culture as well as in co-culture with '*N. equitans*', which raises the question as to whether '*N. equitans*' is a symbiont of or a parasite on *I. hospitalis*. At present, this question cannot be answered satisfactorily. In principle, *I. hospitalis* shows identical growth parameters, such as doubling time or maximal cell density, whether it is cultivated with or without '*N. equitans*'. So far, no benefits for *I. hospitalis* could be detected in the co-culture, a characteristic for a symbiosis. On the other hand, '*N. equitans*' does not kill its host; the two organisms live in a stable co-culture. Therefore, '*N. equitans*' is neither a predator nor, in a strict sense, a parasite on *I. hospitalis*. It seems that the correct description of their relationship is between symbiosis and parasitism, which we hope to elucidate by our ongoing studies of this unique archaeal system.

Description of *Ignicoccus hospitalis* sp. nov.

Ignicoccus hospitalis (hos.pi.ta'lis. L. masc. adj. *hospitalis* relating to a guest, hospitable, referring to its ability to serve as a host for '*Nanoarchaeum equitans*').

Slightly irregular cocci, about 1–6 µm in diameter, with extracellular flagella-like appendages which insert at one cell pole. Gram-negative. Occurring singly and in pairs. No evidence for a regular arrayed surface protein. Grows at 73–98 °C, pH 4.5–7.0 and 0.5–5.5 % NaCl (optima: 90 °C, pH 5.5, 1.4 % NaCl). Strictly anaerobic.

Chemolithoautotrophic growth in the presence of H₂ and CO₂ with sulfur as electron acceptor. No chemo-organotrophic growth on meat extract, yeast extract, peptone, Casamino acids, gelatin, starch, formate, acetate or glucose. Sulfate, sulfite, sulfide, thiosulfate, tetrathionate, nitrate and oxygen are not used as electron acceptors. H₂S is formed during growth. Growth is stimulated by addition of 0.1 % (w/v) meat extract or 0.02 % (w/v) yeast extract. The G + C content of genomic DNA of the type strain is 56 mol%. The cell envelope consists of a cytoplasmic membrane, a periplasm of variable width and an outer membrane. The main core lipids are archaeol and caldarchaeol. Caldarchaeol does not appear in the purified outer membrane. The predominant polar headgroups consist of one or more sugar residues (mainly mannose and small amounts of glucose) attached either directly to the core lipid or via a phosphate group. Imp1227 (*Ignicoccus* outer-membrane protein; molecular mass 6.23 kDa) is the quantitatively dominant protein within the outer membrane, which occurs in several, stable oligomers. Host organism for '*N. equitans*'.

The type strain is isolate KIN4/I^T (= DSM 18386^T = JCM 14125^T), isolated from the Kolbeinsey Ridge, to the north of Iceland.

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