**Rhabdothermus arcticus** gen. nov., sp. nov., a member of the family **Thermaceae** isolated from a hydrothermal vent chimney in the Soria Moria vent field on the Arctic Mid-Ocean Ridge

Bjørn O. Steinsbu,1,2 Brian J. Tindall,3 Vigdis L. Torsvik,1,4 Ingunn H. Thorseth,1,2 Frida L. Daae1,4 and Rolf B. Pedersen1,2

Correspondence
Bjørn O. Steinsbu
bjorn.steinsbu@geo.uib.no

1Centre for Geobiology, University of Bergen, Allégaten 41, N-5007 Bergen, Norway
2Department of Earth Science, University of Bergen, Allégaten 41, N-5007 Bergen, Norway
3DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstrasse 7b, D-38124 Braunschweig, Germany
4Department of Biology, University of Bergen, Thormøhlensgate 53 A/B, N-5020 Bergen, Norway

A novel thermophilic member of the family **Thermaceae**, designated strain 2M70-1\(^T\), was isolated from the wall of an active white smoker chimney collected in the Soria Moria vent field at 71°N in the Norwegian–Greenland Sea. Cells of the strain were Gram-negative, non-motile rods. Growth was observed at 37–75 °C (optimum 65 °C), at pH 6–8 (optimum pH 7.3) and in 1–5 % (w/v) NaCl (optimum 2.5–3.5 %). The isolate was aerobic but could also grow anaerobically using nitrate or elemental sulfur as electron acceptors. The strain was obligately heterotrophic, growing on complex organic substrates like yeast extract, Casamino acids, tryptone and peptone. Pyruvate, acetate, butyrate, succrose, rhamnose and maltodextrin were used as complementary substrates. The G+C content of the genomic DNA was 68 mol%. Cells possessed characteristic phospholipids and glycolipids. Major fatty acids constituted saturated and unsaturated iso-branched and saturated anteiso-branched forms. Menaquinone 8 was the sole respiratory lipoquinone. Phylogenetic analysis of 16S rRNA gene sequences placed the strain in the family **Thermaceae** in the phylum ‘**Deinococcus–Thermus**’, which is consistent with the chemotaxonomic data. On the basis of phenotypic and phylogenetic data, strain 2M70-1\(^T\) (JCM 15963\(^T\) = DSM 22268\(^T\)) represents the type strain of a novel species of a novel genus, for which the name **Rhabdothermus arcticus** gen. nov., sp. nov. is proposed.

The family **Thermaceae** currently comprises five genera: **Thermus** (Brock & Freeze, 1969), **Meiothermus** (Nobre et al., 1996), **Marinithermus** (Sako et al., 2003), **Oceanithermus** (Miroshnichenko et al., 2003a) and **Vulcanithermus** (Miroshnichenko et al., 2003b). The first species described as a member of this family was **Thermus aquaticus**, isolated from a hot spring in Yellowstone National Park, USA (Brock & Freeze, 1969). Species of the genera **Thermus** and **Meiothermus** are typically found in various heated terrestrial habitats (Williams & Da Costa, 1992), whereas members of the other three genera have only been cultured from marine hydrothermal environments. All members of the family **Thermaceae** are heterotrophic, thermophilic, non-sporo-forming, rod-shaped bacteria that grow by aerobic respiration, although members of the genera **Vulcanithermus** and **Oceanithermus** are microaerophilic. Many strains of these genera can also grow anaerobically using nitrate or nitrite as electron acceptors (Da Costa & Rainey, 2001; Miroshnichenko et al., 2003a, b; Mori et al., 2004). Modest growth by sulfur reduction has been reported for **Oceanithermus desulfurans** and **Oceanithermus profundus** (Mori et al., 2004). **O. profundus** can also grow lithoautotrophically using H\(_2\) as an energy source. Moreover, reduction of sulfur and metals, including iron and manganese, has been reported for certain strains of the genus **Thermus** (Kieft et al., 1999). In the present study, a novel member of the family **Thermaceae**, isolated from a chimney structure in the Soria Moria hydrothermal vent field located ~700 m below sea-level on the Arctic Mid-Ocean Ridge (Pedersen et al., 2010), is described.

Strain 2M70-1\(^T\) was enriched from an active white smoker chimney (270 °C) collected in the Soria Moria vent field (71° 15.53’ N 5° 48.73’ W) in the Norwegian–Greenland
Sea during the BioDeep2006 cruise in June 2006. The chimney was collected with the remotely-operated vehicle (ROV) 'Bathysaurus' and brought to the surface in a partially closed basket. Parts from the outer 1 cm of the chimney wall were crushed with sterile equipment and placed in a 50 ml serum bottle whilst being vigorously flushed with N2. The bottle was closed with a butyl rubber stopper and sealed with an aluminium crimp cap. A slurry was then prepared by adding enrichment medium ('M-medium') to the bottle by use of a flushed syringe. 'M-medium' was composed of (l−1); PIPES (Sigma) (3.03 g), NaCl (25 g), MgSO4, 7H2O (2.7 g), MgCl2, 6H2O (4.3 g), NH4Cl (0.25 g), KCl (0.5 g), CaCl2, 2H2O (0.14 g), K2HPO4, 3H2O (0.14 g), yeast extract (1.0 g), trace element solution (Steinsbu et al., 2010) (1 ml), Fe(NH4)2(SO4)2.6H2O (0.002 g) and resazurin (0.001 g).

Several reagents were then added from sterile anaerobic stocks: sucrose, 0.1 %; sodium pyruvate, 10 mM; vitamin solution (Balch et al., 1979), 10 ml l−1; NaHCO3, 12 mM. The pH was readjusted to 7.0 and the medium was dispensed into 27 ml Balch tubes (Bellco) in 10 ml portions whilst flushing with N2 gas. The tubes were sealed with butyl rubber stoppers and aluminium crimp caps. Samples (1 ml) of the prepared slurry were used as inocula and filter-sterilized air was added to the headspace to give a final O2 concentration of ~4 % (v/v). Tubes were incubated horizontally at 70 °C without shaking. After 2 days, the culture medium was turbid and non-motile rods could be seen by phase-contrast microscopy. All attempts to grow the enriched cells on Gelrite gellan gum (Sigma) or agar, on plates under aerobic or microaerobic conditions, or anaerobically in shake tubes with nitrate provided as electron acceptor, failed. Seven subsequent 'dilution-to-extinction' series (Baross, 1995) were performed in 'M-medium' to get a pure culture. The dilution series was made in 10-fold steps, except for series 4, 5 and 6, for which 1:2 (v/v) dilution steps were used for tubes in the series with a predicted total cell number in the range 10^2–10^7. Cell numbers in the undiluted cultures were determined by counting in a phase-contrast microscope (Leitz Laborlux K) using a Thoma counting chamber (0.02 mm depth). Culture purity was checked by phase-contrast microscopy and by PCR amplification and sequencing of the 16S rRNA gene with several different primers. The purified strain, designated 2M70-1T, was characterized in detail.

Cells of strain 2PM70-1T were non-motile rods or filaments of various lengths (~0.5–0.7 × 1.5–15.0 μm). Turbid cultures and pellets prepared from them had a creamy colour. Circular structures, resembling those referred to as 'rotund bodies' often formed by strains of Thermus (Brock & Edwards, 1970) and Oceanithermus (Miroshnichenko et al., 2003a; Mori et al., 2004), could sometimes be observed in cultures of strain 2M70-1T; however, the structures were small and generally contained no more than two or three cells. On the other hand, a bubble could often be seen protruding from single cells or between pairs of cells. Such cells often turned into spherical structures. Huge and tightly packed aggregates of cells could be observed during fast growth or when cultures were stored at room temperature after reaching late exponential growth phase. Transmission electron microscopy analyses of ultrathin-section preparations and cells placed on grids were performed as described previously (Steinsbu et al., 2010). These analyses revealed that the cells possessed a Gram-negative cell wall (not shown). Flagella were not observed.

The 16S rRNA gene of strain 2M70-1T was amplified by whole-cell PCR with primers B27mF (5'-AGAGTTTGACTCMTGGCTCAG-3'; Suzuki & Giovannoni, 1996) and Hr (5'-AAGAGGTTGATCCACGCGCA-3'; Edwards et al., 1989). The resulting amplicon was sequenced by cycle sequencing using a BigDye Terminator sequencing kit 3.1 (Applied Biosystems) and analysed on an ABI PRISM 377 DNA sequencer. A 1472 bp fragment was assembled using standard methods and then compared with sequences in GenBank (Benson et al., 2008) using BLAST (Altschul et al., 1997). The 16S rRNA gene sequences of strain 2M70-1T and type strains of other members of the phylum 'Deinococcus–Thermus' were aligned against the SILVA 100 alignment using the SINA web-based aligner (http://www.arb-silva.de) (Pruesse et al., 2007). The alignment was exported to ARB 5.0 (Ludwig et al., 2004) where it was corrected manually. A 50 % positional conservation filter was made from the selected dataset (30 sequences) and used to exclude highly variable positions and columns containing gaps. A maximum-likelihood tree was reconstructed from the resulting 1258 nt alignment by use of PhyML 3.0 aLRT (Guindon & Gascuel, 2003; Anisimova & Gascuel, 2006), available at http://www.phylogeny.fr (web server version 2) (Dereeper et al., 2008). Calculations were done using the ‘HKY85’ substitution model assuming an estimated proportion of invariant sites and four gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. A distance tree and a maximum-parsimony consensus tree were reconstructed using MEGA4 (Tamura et al., 2007); the Jukes–Cantor distance matrix (Jukes & Cantor, 1969) and the neighbour-joining algorithm (Saitou & Nei, 1987) were calculated by global pairwise alignments in MatGat 2.02 (Campanella et al., 2003). Only parts of the sequences corresponding to Escherichia coli positions 40–1405 were considered. 16S rRNA gene sequence similarities between strain 2M70-1T and the type strains of reference species were as follows: ‘Thermophilic bacterium IH55’, 95.6 %; Marinithermus hydrothermalis, 90.4%; Vukanithermus mediatlanticus, 89.9%; O. profundus,
For growth tests and maintenance of strain 2M70-1 T, a medium termed 'Cam-medium' was used, unless otherwise noted in the text. 'Cam-medium' contained (1 l⁻¹): HEPES (2.38 g), NaCl (30 g), MgSO₄·7H₂O (2.7 g), MgCl₂·6H₂O (4.3 g), NH₄Cl (0.25 g), KCl (0.5 g), CaCl₂·2H₂O (0.14 g), K₂HPO₄·3H₂O (0.14 g), yeast extract (0.1 g), peptone (Bacto) (2.0 g), trace element solution (Steinsbu et al., 2010) (1 ml) and Fe(NH₄)₂(SO₄)₂·6H₂O (0.002 g). The pH was adjusted to 7.3 at room temperature and the medium was dispensed aerobically in 10 ml portions in Balch tubes which were then sealed and autoclaved. Vitamins (Balch et al., 1979) were added to each tube before use.

Growth of strain 2M70-1 T was determined by measuring changes in OD₆₀₀ by placing the culture tubes directly into a Spectronic 21 spectrophotometer (Milton Roy). The impact of temperature on growth was determined in the range 20–80 °C. The strain grew at 37–75 °C and optimal growth was observed around 65 °C. No growth was detected at 30 or 80 °C. Salt tolerance was tested at 65 °C using 'Cam-medium' prepared with various concentrations of NaCl in the range 0–6.0 %. The strain grew optimally in the presence of 2.5–3.5 % (w/v) NaCl; the lower and upper limits for growth were 1.0 and 5.0 % (w/v) NaCl, respectively. Growth of strain 2M70-1 T was determined at pH 4–9 at 65 °C in 'Cam-medium' prepared with various buffers (trisodium citrate for pH 4.0–5.0, MES for pH 5.5–6.3, PIPES for pH 6.3–7.3, HEPES for pH 7.3–8.0, AMPSO for pH 8.0–9.0) at a concentration of 10 mM. Optimal growth was observed at pH 7.3 and the limits for growth were pH 6.0–8.0. All tests were performed in triplicate.

The ability of strain 2M70-1 T to use alternative substrates was tested in 'Cam medium' prepared without peptone and with only 0.05 g yeast extract l⁻¹. This was the minimum amount of yeast extract necessary for growth. Substrates from concentrated stock solutions were added to each tube prior to inoculation. The strain grew very well on yeast extract (0.1 %), Casamino acids (0.1 %), tryptone (0.1 %), bactopeptone (0.1 %) and beef extract (0.1 %). Pyruvate (10 mM) and acetate (20 mM) increased final growth yields when compared with controls containing only the required 0.005 % yeast extract. The same was observed for butyrate (10 mM), succrose (0.2 %), maltodextrin (0.2 %) and rhamnose (0.2 %) but the stimulatory effect of these substrates was less pronounced. The following substrates did not stimulate growth: H₂/CO₂ (80:20, v/v; 200 kPa), formate (20 mM), propionate (10 mM), lactate (10 mM), fumarate (10 mM), methanol (0.1 %), ethanol (0.1 %), mannitol (0.1 %), glucose (0.2 %), galactose (0.2 %), maltose (0.2 %), arabinose (0.2 %), cellobiose (0.2 %), lactose (0.2 %) and mannose (0.2 %). Succinate (10 mM), malate (10 mM), fructose (0.2 %) and xylose (0.2 %) seemed to have an inhibitory effect on growth.

To test the ability of strain 2M70-1 T to use alternative electron acceptors, a medium with the same composition as the one used for the substrate tests was prepared anaerobically as described for 'M-medium'. Electron acceptors were added from anaerobically and separately sterilized stock solutions. Elemental sulfur (~0.2 g) was prepared in pre-sterilized Balch tubes as described previously (Steinsbu et al., 2010). H₂/CO₂ (80:20, v/v; 200 kPa), yeast extract (0.1 %), bactopeptone (0.1 %) and pyruvate (10 mM) were tested as substrates. No growth was observed on thiosulfate (10 mM), sulfite (4 mM), sulfate (20 mM) or iron(III) citrate (20 mM). Nitrate reduction was assessed by measuring the decrease in nitrate and production of nitrite by ion-exchange chromatography (IC25 chromatograph/AS11-HC4 × 250 column; Dionex) using UV detection (Spectra-physics UV150; Thermo Separation Products). A 1 ml culture sample was collected with a syringe and stored at −20 °C until analysis. The strain grew rather poorly on nitrate (10 mM) and the measurements showed that equivalent amounts of nitrite were formed from the consumed nitrate. Negligible amounts of nitrite were detected in controls without cells. No growth was observed in the presence of the reducing agents Na₂S (2 mM), FeCl₂ (0.2 mM) or cysteine hydrochloride (0.05 %). When elemental sulfur was provided as electron acceptor, growth was strictly dependent on the presence of CO₂, provided as 1:4 (v/v) mixture of N₂/CO₂. Sulfide was produced in growing cultures but not in control tubes without cells. Replacing the N₂/CO₂ mixture with a 1:4 (v/v) mixture of H₂/CO₂ did not have any noticeable effect on growth. No growth was observed in the presence of 100 % N₂ or N₂. When the above-mentioned gas mixtures were tested using nitrate instead of elemental sulfur in the medium, the presence of CO₂ had no stimulatory effect on growth. The strain was not able to grow by sulfur oxidation in the presence of elemental sulfur and 4 % O₂ (provided as air).

Strain 2M70-1 T undoubtedly preferred aerobic respiration with complex organic substrates for growth. Nitrate and elemental sulfur were less efficient than O₂ as electron acceptors, which could be explained by a possible sensitivity of the strain to the toxic compounds produced during growth with these electron acceptors. However, sulfur and nitrate could potentially play a beneficial role in the hydrothermal vent chimney from which the strain was isolated. It is assumed that, inside the chimney wall, produced sulfide is continuously immobilized by precipitation as metal sulfides and both sulfide and nitrite are diluted by seawater circulation. Moreover, nitrite and sulfide produced by the strain during growth could be removed by other micro-organisms by either ammonification/denitrification or sulfur oxidation, respectively. Such interactions could make sulfur and nitrate reduction excellent alternatives for energy conservation by strain 2M70-1 T in situations where access to oxygen is limited. The strain's need for CO₂ during growth on elemental sulfur is, however, more difficult to explain. It could be, for
instance, that CO₂ or HCO₃⁻ is needed for enzymic reactions or an ion transport mechanism essential for growth on this electron acceptor. It could also be that the CO₂ lowers the pH slightly and thereby counteracts to some extent the pH increase caused by the sulfide produced; however, further investigation is necessary to support these assumptions.

Quinones and polar lipids were extracted using the two-stage method described by Tindall (1990a, b). Respiratory lipoquinones were separated into their functional classes using TLC, eluted from the appropriate bands and then analysed by reversed-phase HPLC. Polar lipids were analysed by two-dimensional TLC using the solvents chloroform/methanol/water (65 : 25 : 4, v/v/v) in the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v/v/v) in the second dimension. All lipids were stained using 5 % methanol/acetic acid/water (80 : 12 : 15 : 4, v/v/v/v) in the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v/v/v) in the second dimension. All lipids were stained using 5 % molybdophosphoric acid in ethanol and heated at 150 °C for 15 min. Specific spray reagents were used to visualize functional groups, such as phosphates and sugars (Tindall et al., 2007). Fatty acids were extracted and analysed using the standard MIDI Sherlock Microbial Identification system (version 4.5) and peaks were identified on an Agilent 6890N GC using the peak naming table TSBA50. The sole respiratory lipoquinone detected was menaquinone 8 (MK-8), a compound found in all members of the phylum ‘Deinococcus–Thermus’ examined to date. Strain 2M70-1T possessed eight unidentified glycolipids and two unidentified phospholipids (Fig. 1). The fatty acids comprised mainly saturated and unsaturated iso-branched forms and saturated anteiso-branched forms (Table 1). Strain 2M70-1T and V. mediatlanticus possessed a similar lipid content comprising both glycolipids and phospholipids. Only phospholipids have been detected in O. profundus (Miroshnichenko et al., 2003a) and no information on lipid content is available for O. desulfurans and M. hydrothermalis. The major phospholipids of members of the genera Thermus and Meiothermus has an Rₖ value that corresponds with phospholipid PL1 in strain 2M70-1T (Fig. 1), as well as with a major phospholipid in V. mediatlanticus, labelled PL2 by Miroshnichenko et al. (2003b), and O. profundus, labelled PL3 by Miroshnichenko et al. (2003a). This compound has recently been identified as 2’-O-(1,2-diacyl-sn-glycero-3-phospho)-3’-O-(α-N-acetylglicosaminyl)-N-glycerol alkylamine, a compound that appears to be characteristic of members of the family Thermaceae examined to date (Yang et al., 2006). Leone et al. (2006) proposed a slightly different structure in a strain of the genus Thermus and a related structure is also found in Deinococcus radiodurans (Anderson & Hansen, 1985). The lipid labelled PL2 in Fig. 1 has the same Rₖ value as that labelled PL3 in V. mediatlanticus (Miroshnichenko et al., 2003b). Characteristic glycolipids have been detected in species of the genera Thermus and Meiothermus (Carreto et al., 1990, Ferreira et al., 1999) that have the structure dihexosyl-N-acyl-hexosaminyl-hexosyl-diacylglycerol, with important variations in the hexose sugars as well as in the nature of the amide-linked fatty acid. The Rₖ values are similar to those of glycolipids GL5, GL6 (although both run slightly faster than the glycolipids in species of the genera Thermus and Meiothermus where hydroxy fatty acids are not amide linked) and GL7 (running similarly to the glycolipid that contains amide-linked 2-ΟH fatty acids in strains of the genus Meiothermus, not present in this organism) present in strain 2M70-1T. This implies that further modifications of the basic structures are present. The presence of significant amounts of unsaturated fatty acids is characteristic of genera currently placed within the family Thermaceae that grow under reduced oxygen concentrations, such as the genera Oceanithermus and Vukaniathermus, as well as for strain 2M70-1T (Table 2). However, the fatty acid patterns of the species in these genera have some clear differences: V. mediatlanticus can be discriminated from other species by the presence of significant amounts of branched C₁₇:0 cyclo fatty acids (Miroshnichenko et al., 2003b), whereas strain 2M70-1T can be distinguished from the other species by the presence of small amounts of iso-C₁₇:0-3-ΟH (Table 1).

The DNA G+C content of strain 2M70-1T was determined from the melting point (Tₘ) by thermal denaturation (Mandel et al., 1970) using a Cary 300 UV-Vis spectrophotometer (Varian). DNA from E. coli (Sigma D-2001) was used as a reference for the measurements. DNA was isolated from a frozen cell pellet, which was thawed and suspended in TE buffer (pH 8) containing 1 g lysozyme l⁻¹ and incubated at 37 °C for 15 min. Further treatment was performed as described by Marmur (1963), except that an additional deproteinization step with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) was performed prior to the chloroform/isoamyl alcohol (24 : 1, v/v) treatment. The DNA G+C content of strain 2M70-1T was 68 mol%.

Phylogenetic analyses (Fig. 2) placed strain 2M70-1T on a distinct branch within the family Thermaceae, clearly separated from all other described species. The rather low 16S rRNA gene sequence similarity values (84.2–90.4 %) between 2M70-1T and the other type strains of members of the family clearly indicate that the strain represents a new genus (Yarza et al., 2008). The cell shape, thermophilic

![Fig. 1. Two-dimensional TLC of the polar lipids of strain 2M70-1T.](image-url)
nature, as well as the pH and salinity range and optima for growth of strain 2M70-1T support its affiliation with this family (Table 2). Strain 2M70-1T and strains belonging to the genera *Vulcanithermus* and *Marinithermus* differ from members of the genus *Oceanithermus* by having higher optimal temperatures for growth and a higher tolerance to oxygen. Even though *M. hydrothermalis* has been described as being incapable of anaerobic growth, it should be noted that, according to Mori *et al.* (2004), this was tested only in the presence of sulfide. It is therefore possible that *M. hydrothermalis* is capable of anaerobic growth under unreduced conditions, as has been observed for strain 2M70-1T and all the other type strains in Table 2. Strain 2M70-1T and all described species belonging to the genera *Vulcanithermus*, *Oceanithermus* and *Marinithermus* have an absolute requirement for NaCl for growth. This distinguishes them from members of the genera *Thermus* and *Meiothermus*, which do not require NaCl. The presence of MK-8 as the sole respiratory lipoquinone and iso- and anteiso-branched fatty acids as the dominant fatty acid types further support the strain’s close relationship to members of the family *Thermaceae*.

Based on its phenotypic and genotypic characteristics and its distinct phylogenetic position, strain 2M70-1T represents a novel species of a new genus, for which the name *Rhabdothermus arcticus* gen. nov., sp. nov. is proposed.

**Description of *Rhabdothermus* gen. nov.**

*Rhabdothermus* (Rhab.do.ther9mus. Gr. fem. n. rhabdos rod; Gr. adj. thermos hot; N.L. masc. n. Rhabdothermus a hot rod).

Cells are non-motile, Gram-negative rods, 0.5–0.7 μm wide and of various lengths. They are thermophilic, neutrophilic,
The cell membrane contains both phospholipids and glycolipids; the major phospholipid has an iso-branched, anteiso-branched and unsaturated iso-branched respiratory lipoquinone present. Major fatty acids used as complementary substrates. The major fatty acids present are iso-C<sub>17</sub>:0, iso-C<sub>15</sub>:0 and iso-C<sub>17</sub>:1<sup>v</sup>9c. Polar lipids comprise two unidentified phospholipids and eight unidentified glycolipids.

The type strain, 2M70-1<sup>T</sup> (=JCM 15963<sup>T</sup> =DSM 22268<sup>T</sup>), was isolated from the wall of an active white smoker chimney (270 °C) located ~700 m below sea-level in the Soria Moria vent field at 71 °N in the Norwegian–Greenland Sea. The DNA G+C content of the type strain is 68 mol% (T<sub>m</sub>).

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References


