Rhodothermus profundus sp. nov., a thermophilic bacterium isolated from a deep-sea hydrothermal vent in the Pacific Ocean

Viggó Thór Marteinsson,¹ Snaedis H. Bjornsdottir,^{1,2} Nadège Bienvenu,³ Jakob K. Kristjansson⁴ and Jean-Louis Birrien³

Correspondence

Viggó Thór Marteinsson
viggo.th.marteinsson@matis.is

¹Matis Vinlandsleid 12, IS-113, Reykjavik, Iceland

²Institute of Biology, University of Iceland, Sturlugata 7, IS-101, Reykjavik, Iceland

³UMR 6197, Centre National de la Recherche Scientifique, Université de Bretagne Occidentale, Ifremer, IUEM, BP70, 29282 Plouzané, France

⁴Arkea Technology Park, Reykir, PO Box 200, IS-810 Hveragerdi, Iceland

Nine thermophilic strains of aerobic, non-sporulating, heterotrophic bacteria were isolated after enrichment of chimney material sampled from a deep-sea hydrothermal field at a depth of 2634 m on the East-Pacific Rise (13°N). The bacteria stained Gram-negative. They were rod-shaped and measured approximately 0.5 µm in width and 1.5–3.5 µm in length. They grew at 55–80 °C, pH 6–8 and 1–6 % NaCl. Optimal growth was observed at 70–75 °C, pH 7.0 and 1–3 % NaCl. The organisms were identified as members of the genus *Rhodothermus*, having a 16S rRNA gene similarity of 98.1 % with *Rhodothermus marinus* DSM 4252^T. The novel isolates differed morphologically, physiologically and chemotaxonomically from *R. marinus*, e.g. in lack of pigmentation, response to hydrostatic pressure, maximum growth temperature and DNA G+C content. DNA–DNA hybridization revealed a reassociation value of 37.2 % between strain PRI 2902^T and *R. marinus* DSM 4252^T, which strongly suggested that they represent different species. Furthermore, AFLP fingerprinting separated the novel strains from *R. marinus* reference strains. It is therefore concluded that the strains described here should be classified as representatives of a novel species for which the name *Rhodothermus profundus* sp. nov. is proposed; the type strain is PRI 2902^T (=DSM 22212^T =JCM 15944^T).

At the time of writing, the assignment of the family ‘*Rhodothermaceae*’, phylum ‘*Bacteroidetes*’, is uncertain (Ludwig *et al.* 2008). The family contains two genera, *Rhodothermus* and *Salinibacter*. The former comprises a single species, *Rhodothermus marinus*. Since *R. marinus* DSM 4252^T was first isolated from a shallow submarine hot spring in Iceland, many additional strains have been reported from marine geothermal sites around the world (Alfredsson *et al.*, 1988; Bjornsdottir *et al.*, 2006). Although originating from distantly located sites, *R. marinus* strains have been shown to be closely related on the basis of highly similar 16S rRNA gene sequences and DNA–DNA reassociation values of 68–96 % (Moreira *et al.*, 1996; Petursdottir *et al.*, 2000; Silva *et al.*, 2000). *R. marinus* strains are also very similar in their phenotypic characters.

Most are orange-reddish-pigmented, although colourless strains have been isolated (Petursdottir *et al.*, 2000). *R. marinus* is non-spore-forming, heterotrophic, slightly halophilic, thermophilic and obligately aerobic (Alfredsson *et al.*, 1988). Therefore, its growth in marine hot springs is confined to aerobic zones of suitable temperatures. It has been shown that aerobic heterotrophic bacteria, for example of the genera *Thermus* and *Bacillus*, are present in deep-sea hydrothermal vent environments (Marteinsson *et al.*, 1995, 1996, 1999). Thus, we investigated whether the distribution of *Rhodothermus* encompassed such environments. This paper reports the isolation and characterization of thermophilic bacteria isolated from a deep-sea hydrothermal vent at the East-Pacific Rise at 13°N (12.449844° N 103.56812° W) and assigns them to a novel species within the genus *Rhodothermus*.

Samples were collected during the French oceanographic cruise AMISTAD in June 1999. Chimney rocks, hot fluids and invertebrates were collected by the man-operated submersible *Nautilie* and brought to the surface in an insulated box. Samples were immediately processed for

Abbreviation: AFLP, amplified-fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PRI 2902^T is FJ624399.

A scanning electron micrograph of strain PRI 2902^T is available with the online version of this paper.

enrichment aboard the ship and for transportation to the laboratory. Various media were used for aerobic enrichments at 65 °C and some growth was observed after 1–30 days of incubation. The novel isolates were obtained from an active chimney rock sample collected at a depth of 2634 m. Rock pieces and sterile seawater were mixed together and kept at 4 °C until processing 2 weeks later in the laboratory. The mixture was inoculated into medium 160 (Degryse *et al.*, 1978) containing 1% NaCl and 2.5 g l⁻¹ each of tryptone and yeast extract (designated 160-1). The sample was serially diluted in 25 ml culture tubes and incubated aerobically at 65 °C for 2 weeks. As resistance of *R. marinus* to aminoglycoside antibiotics had been reported previously (Alfredsson *et al.*, 1988), streptomycin (1.56 µg ml⁻¹) was added to the enrichment medium to facilitate growth of *Rhodothermus*-like species and to prevent overgrowth of fast-growing opportunist-like *Bacillus* species. Previous experiments showed that *Bacillus* sp. and *R. marinus* exhibited different levels of resistance to streptomycin of up to 0.78 µg ml⁻¹ and 12.5 µg ml⁻¹, respectively (data not shown). Growth was not observed in the dilutions, but was observed after concentrating the cultures and subsequent spreading on agar plates containing marine agar (DSMZ medium 514; Bacto marine broth, Difco 2216) diluted to half strength with water (designated MB1/2) (Marteinsson *et al.* 1995). Homogeneous colonies appeared on the plates and nine colourless colonies were randomly picked and purified by restreaking on the same medium at least six times. Isolates PRI 2894, PRI 2895, PRI 2896, PRI 2897, PRI 2898, PRI 2901, PRI 2902^T, PRI 2904 and PRI 2908 were further examined. *R. marinus* DSM 4252^T was used as a reference strain throughout this work.

For phenotypic analysis, the novel isolates were grown on agar medium MB1/2. They showed very similar phenotypic characteristics, which were consistent with their assignment to the genus *Rhodothermus*. They stained Gram-negative and were positive for catalase and negative for oxidase. Colonies were circular, smooth, viscous and 2–3 mm in diameter after incubation at 70 °C for 4 days. Colonies were colourless, whereas those of *R. marinus* 4252^T are orange-red (Alfredsson *et al.*, 1988). Sensitivity of the strains to antibiotics was examined at 65 °C on plates using standard antibiotic discs (6 mm) from Oxoid. The inhibition zone was measured after 24 h and strains scored sensitive to an antibiotic if the zone was larger than 20 mm in diameter (Kristjansson *et al.*, 1994). The strains were sensitive to ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), novobiocin (30 µg), penicillin G (10 IU) and rifampicin (2 µg). They were resistant to gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), polymyxin B (300 IU) and streptomycin (10 µg). Phase-contrast microscopy revealed that cells of strain PRI 2902^T occurred singly or in short chains during the exponential phase. Motility and endospores were never observed. However, motility has been observed for *R. marinus* DSM 4252^T. Cell morphology of strain PRI 2902^T

was examined using a Leo Supra 25 scanning electron microscope. Cells appeared as irregular rods and measured approximately 0.5 µm in width and 1.5–3.5 µm in length (Supplementary Fig. S1, available in IJSEM Online). The DNA G+C content of strain PRI 2902^T was 60.9 mol%, determined at the DSMZ according to Mesbah *et al.* (1989). Phenotypically, the novel isolates differed from *R. marinus* 4252^T by characteristics represented in Table 1.

Isolates PRI 2894, PRI 2895, PRI 2898, PRI 2901, PRI 2902^T, PRI 2904 and PRI 2908 were grown in liquid medium MB1/2 at several temperatures, pH and NaCl concentrations. Growth was examined as described previously (Marteinsson *et al.*, 1995). Growth was observed at 55–80 °C, with optimum growth at 70–75 °C. All isolates grew in the presence of 1% and up to 6% NaCl. However, a slight difference was observed for the optimal NaCl concentration which ranged from 1 to 3% NaCl. Growth occurred between pH 6.0 and 8.0; optimum growth occurred around pH 7.0. Growth on single carbon sources was tested as described previously (Alfredsson *et al.*, 1985; Marteinsson *et al.*, 1995) on plates containing MB1/2 without peptone and yeast extract. The strains utilized asparagine, aspartate, casein, Casamino acids, gelatin, glutamate, glutamine, pyruvate and starch. Anaerobic growth was examined as described previously (Marteinsson *et al.*, 1995). The isolates could not grow anaerobically and oxygen could not be replaced by nitrate.

Growth of isolates PRI 2895, PRI 2901 and PRI 2902^T and *R. marinus* DSM 4252^T was examined under *in situ* hydrostatic pressure (25 MPa), as well as under atmospheric hydrostatic pressure (0.1 MPa). Experiments were performed in duplicate as described previously (Marteinsson *et al.*, 1999). Growth was monitored by microscopic observation and by measuring the turbidity at 600 nm. All strains exhibited growth at both 0.1 and 25 MPa. *R. marinus* DSM 4252^T produced about 30% fewer cells under hydrostatic pressure of 25 MPa. Furthermore, cell deformations were observed, which were never observed at atmospheric pressure. However, the *in situ* hydrostatic pressure of 25 MPa did not affect the growth rates of isolates PRI 2901 and PRI 2902^T and strain PRI 2895 produced about 35% more cells than at atmospheric hydrostatic pressure. No cell deformations were observed for isolates PRI 2895, PRI 2901 and PRI 2902^T when grown under *in situ* hydrostatic pressure, although the cells appeared shorter.

Fatty acids were extracted from strain PRI 2902^T and *R. marinus* DSM 4252^T according to the protocol of the MIDI system. Analysis by GC was controlled by the MIS software and the peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990). The general fatty acid profiles were similar to those presented by Silva *et al.* (2000) for *R. marinus* strains and indicated that PRI 2902^T belonged to the genus *Rhodothermus*. However, the comparison was inadequate as PRI 2902^T could not grow in the medium used by Silva *et al.* (2000). The fatty acid profiles of PRI 2902^T and *R.*

Table 1. Characteristics that distinguish the novel strains from *Rhodothermus marinus*

Strains: 1, *Rhodothermus profundus* sp. nov. PRI 2902^T; 2, *R. profundus* sp. nov. PRI 2901; 3, *R. profundus* sp. nov. PRI 2898; 4, *R. profundus* sp. nov. PRI 2895; 5, *R. marinus* DSM 4542^T. Data from this study. ND, Not determined.

Characteristic	1	2	3	4	5
Pigmentation	None	None	None	None	Orange-red
Optimum growth temperature (°C)	70	70	70	75	65
Growth at 50 °C	—	—	—	—	+
Maximum growth temperature (°C)	80	80	80	80	77
Growth in 6% NaCl	—	—	—	+	+
Resistance to:					
Rifampicin	—	—	—	—	+
Erythromycin	—	—	—	—	+
Motility	—	—	—	—	+
DNA G + C content (mol%)	60.9	ND	ND	ND	64.4
Utilization of:					
Acetate	—	—	—	—	+
D-Galactose	—	—	—	—	+
Sucrose	—	—	—	—	+
Maltose	—	—	—	—	+
Lactose	—	—	—	—	+
Effect of hydrostatic pressure	None	None	None	Positive	Negative

marinus DSM 4252^T grown in parallel in liquid medium MB1/2 at 70 °C are given in Table 2. Both strains contained anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0} as predominant fatty acids, although in different ratios. The most abundant fatty acid in PRI 2902^T was iso-C_{17:0}, whereas it was iso-C_{16:0} in *R. marinus* DSM 4252^T. Furthermore, the proportion of straight-chain fatty acids was lower in PRI 2902^T. Quinone and polar lipid analyses were carried out by the Identification service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Strain PRI 2902^T contained menaquinone MK-7 (100%), which is the predominant respiratory quinone in *R. marinus* DSM

4252^T (97%) (Tindall, 1991). The polar lipid pattern of PRI 2902^T was similar to that of *R. marinus* DSM 4252^T (Tindall, 1991) with diphosphatidylglycerol and phosphatidylethanolamine as major phospholipids. Phosphatidylglycerol was also detected, as well as phospholipids PL1–PL2 and glycolipids GL1–GL3. The chemotaxonomic properties of strain PRI 2902^T support its affiliation to the genus *Rhodothermus*.

Genomic DNA was isolated with the Master Pure DNA Purification kit (Epicentre). The 16S rRNA gene was amplified with primers F9 and R1544 as described previously (Skirnisdottir *et al.*, 2000). Nucleotide sequences were determined using an Applied Biosystems 3730 DNA analyser and the BigDye terminator cycle sequencing kit. Primers F9 and R1544 were used for sequencing, as well as primers F338, R357, R805 and R1195 (Skirnisdottir *et al.*, 2000). The almost complete 16S rRNA gene sequences (1509 bp), which were determined for all nine isolates, were identical. Comparison of this sequence with sequences available from public databases demonstrated that the isolates belonged to the genus *Rhodothermus*. The sequence showed 98.1% similarity to the sequence of *R. marinus* DSM 4252^T (X80994), which was the most closely related species. This is below the threshold value of 98.7% that was recently suggested for species definition (Stackebrandt & Ebers, 2006). The two sequences were aligned with those of related bacteria using CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distance matrices were calculated using the Kimura two-parameter model (Kimura, 1980). The 16S rRNA gene sequence of strain PRI 2902^T exhibited 85% similarity to the sequence of the type strain of *Salinibacter ruber* of the family 'Rhodothermaceae' and 80% similarity to the type strain of *Thermonema*

Table 2. Cellular fatty acid composition (%) of strain PRI 2902^T and *R. marinus* DSM 4252^T

Data from this study. Cells were cultured in medium MB1/2 at 70 °C. Fatty acids represented by less than 0.5% were omitted. ND, Not detected.

Fatty acid	PRI 2902 ^T	DSM 4252 ^T
Straight-chain		
C _{16:0}	1.8	4.1
C _{18:0}	ND	0.6
Branched-chain		
iso-C _{14:0}	0.8	3.2
iso-C _{15:0}	7.4	6.0
anteiso-C _{15:0}	10.0	12.3
iso-C _{16:0}	15.8	27.8
iso-C _{17:0}	36.9	20.7
anteiso-C _{17:0}	18.3	13.9
iso-C _{18:0}	7.2	8.8

lapsum (Hudson *et al.*, 1989; Antón *et al.*, 2002; Ludwig *et al.*, 2008). DNA–DNA reassociation between strain PRI 2902^T and *R. marinus* DSM 4252^T was performed by the DSMZ, as described by De Ley *et al.* (1970) with the modifications of Huß *et al.* (1983); a value of 37.2 % was obtained, which clearly indicated that the isolates described here do not belong to *R. marinus* when a threshold value of 70 % DNA–DNA reassociation is adopted for defining species (Wayne *et al.*, 1987).

Five of the novel isolates (PRI 2894, PRI 2895, PRI 2898, PRI 2902^T and PRI 2908) were analysed by amplified-fragment length polymorphism (AFLP) fingerprinting. Three *R. marinus* reference strains were included: the type strain DSM 4252^T from north-west Iceland (Alfredsson *et al.*, 1988) and two colourless isolates, PRI 831 and PRI 1298, from south and north-east Iceland, respectively (Petursdottir *et al.*, 2000). AFLP analysis was performed essentially as described previously (Vos *et al.*, 1995). Genomic DNA was digested with *ApaI* and *TaqI* and ligated to double-stranded adapters. Primers A00 and T00 were used for pre-selective amplification and A01-6-FAM and T00 for selective amplification (Huys & Swings, 1999). The selective amplification products were separated on a 6 % acrylamide gel with 8 M urea. The AFLP fingerprints were visualized using the Typhoon 9400 Variable Mode Imager (Amersham). The presence of individual bands was scored manually. Clustering of the patterns was performed with NTSYSpc (Rohlf, 2008) using the Dice correlation coefficient and the UPGMA clustering algorithm (Fig. 1). Each fingerprint consisted of a mean of 80 ± 8 bands where only eight bands were common for all the strains. An identical banding pattern among the novel isolates was observed. However, a considerable genetic heterogeneity was seen between the novel isolates and the three Icelandic *R. marinus* reference strains (Fig. 1). The former were 26 % similar to *R. marinus* DSM 4252^T and 22 % and 26 % similar to the colourless isolates PRI 831 and PRI 1298, respectively. For some taxonomic groups, AFLP pattern similarity above 60 % indicates different strains of the same

species (Savelkoul *et al.*, 1999; Thompson *et al.* 2004). Thus, the AFLP results presented here further support the species status of the isolates described.

On the basis of 16S rRNA gene phylogeny and chemotaxonomy, it is clear that the isolates described here belong to the genus *Rhodothermus*. DNA–DNA hybridization supports their status as a novel species. The strains can be differentiated genotypically from *R. marinus* by AFLP as well as phenotypically by their colony colour, fatty acid composition, their optimum and maximum temperatures for growth, response to hydrostatic pressure and sensitivity to rifampicin and erythromycin, as well as their inability to assimilate acetate, galactose, sucrose, maltose and lactose. Therefore, it is concluded that the strains examined in this study represent a novel species within the genus *Rhodothermus*. Because of the deep origin of the isolates, the name *Rhodothermus profundus* sp. nov. is proposed with PRI 2902^T (=DSM 22212^T =JCM 15944^T) as the type strain.

Description of *Rhodothermus profundus* sp. nov.

Rhodothermus profundus (pro.fun'di. L. n. *profundum* the depths of the sea; L. gen. n. *profundi* of/from the depths of the sea).

Cells are non-spore-forming, non-motile rods measuring 0.5 µm in width and 1.5–3.5 µm in length. Aerobic, oxidase-negative, catalase-positive. Cells stain Gram-negative. Colonies are cream, circular, smooth, viscous and 2–3 mm in diameter after incubation at 70 °C for 4 days on half-strength marine agar (diluted with water). Thermophilic, grows between 55 and 80 °C, with optimum growth at 70 °C. Neutrophilic, grows between pH 6.0 and 8.0, with optimum growth at pH 7.0. Minimum and maximum NaCl concentrations for growth are 1 % and 5 %, respectively; optimum growth is at 2–3 % NaCl. Grows in full-strength marine broth and half-strength marine broth (MB1/2), with or without agar. L-Asparagine, L-aspartate, casein, Casamino acids, gelatin, L-glutamate, L-glutamine,

Fig. 1. AFLP profiles (partial) and a corresponding dendrogram for isolates PRI 2894, PRI 2895, PRI 2898, PRI 2902^T and PRI 2908, and *R. marinus* DSM 4252^T. Two colourless *R. marinus* isolates, PRI 831 and PRI 1298, are also included. Cluster analysis was performed using the Dice correlation coefficient and the UPGMA clustering algorithm. The correlation coefficient is expressed as a percentage value.

pyruvate and starch are used as sole sources of carbon for energy and growth. Does not utilize acetate, L-alanine, adonitol, arabinose, L-arginine, butyrate, citrate, dulcitol, formate, fructose, galactose, gluconate, glucose, lactose, glycerol, L-histidine, inositol, inulin, L-isoleucine, L-leucine, L-lysine, malate, maltose, mannitol, L-phenylalanine, 2-oxoglutarate, L-proline, raffinose, rhamnose, L-serine, sorbitol, sucrose, tartrate, trehalose, L-threonine, L-valine or xylose. Sensitive to ampicillin, chloramphenicol, erythromycin, novobiocin and penicillin G. Resistant to gentamicin, kanamycin, nalidixic acid, polymyxin B and streptomycin. Contains MK-7. The major fatty acids are iso-C_{17:0}, anteiso-C_{17:0}, iso-C_{16:0} and anteiso-C_{15:0}.

The type strain is PRI 2902^T (=DSM 22212^T =JCM 15944^T), isolated from the hydrothermal vent fields of Genesis (12.449844° N 103.56812° W) in the Pacific Ocean. The DNA G + C content of the type strain is 60.9 mol%.

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