Rhodothermus bifroesti sp. nov., a thermophilic bacterium isolated from the basaltic subsurface of the island Surtsey.

Pauline Bergsten[1,2,] Pauline Vannier[1], Alan Mougeolle[1], Louise Rigaud[1], Viggó Thór Marteinsson[1,3*]

[1] Matís, Exploration & Utilization of Genetic Resources, Reykjavík, Iceland

[2] Faculty of Life and Environmental Sciences, University of Iceland, Reykjavík, Iceland

[3] Faculty of Food Science and Nutrition, University of Iceland, Reykjavík, Iceland

*Corresponding author: viggo@matis.is

Running title: *Rhodothermus bifroesti* sp. nov.

Subject category: New taxa-*Rhodothermaceae*

Supplementary Text.

Extended fatty acid analysis, quinones and polar lipids composition were carried out by the Identification service of Leibniz-institute DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Analysis of Cellular Fatty Acids by MIDI and GC-MS Cellular fatty acids

Cellular fatty acid were analyzed after conversion into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction using minor modifications of the method of Miller (1982) and Kuykendall et al., (1988) (1,2). The fatty acid methyl esters mixtures are separated by gas chromatography and detected by a flame ionization detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.). Peaks are automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID).

For identity confirmation and to resolve summed features of the MIDI analysis, the analysis is supplemented by a GC-MS run on a Agilent GC-MS 7000D using an Agilent HP-5ms UI 30 m x 250 µm x 0,25 µm column with a helium flow of 1.2 ml with an injection of 1 µl with split ratio of 7.5:1. The oven program was as follows: initial temperature 170°C, ramp 3°C/min to 200°C, ramp 5°C/min to 270°C, ramp 120°C/min to 300°C and hold for 2 min. The inlet temperature was set to 170 °C and then linearly increased with 200 °C/min up to 350 °C and hold for 5min. The mass spectrometry parameters were set to aux temperature 230°C, source temperature 230°C, and electron impact ionization at 70 eV with mass range of m/z 40-600 or 40-800, respectively. Peaks were identified based on retention time and mass spectra. The position of single double bounds was confirmed by a derivatization to the corresponding dimethyl disulfide adduct (3). Branched-chain fatty acid positions, cyclopositions and multiple double bounds were determined by derivatization to their 3-pyridylcarbinol ("picolinyl") and/or 4,4-dimethyloxazoline (DMOX) derivatives (4–6).

Analysis of Respiratory Quinones

Respiratory quinones are extracted from freeze dried cell material using hexane and are further purified by a silica-based solid phase extraction. Purified samples are further analysed by HPLC

using a reverse phase column recording absorption spectra. 270 nm for ubiquinones and 326 nm for menaquinones are used for a relative quantification. For complex mixtures, samples are further analysed on an UHPLC-ESI-qTOF system.

Analysis of polar lipids

Polar lipids are extracted from freeze dried cell material using a choroform:methanol:0.3% aqueous NaCl mixture, polar lipids are recovered into the chloroform phase (modified after Bligh and Dyer, 1959 (7)).

Polar lipids are separated by two dimensional silica gel thin layer chromatography. The first direction is developed in chloroform:methanol:water, and the second in chloroform:methanol:acetic acid:water. Total lipid material is detected using molybdatophosphoric acid and specific functional groups detected using spray reagents specific for defined functional groups (8).

Supplementary Figures.

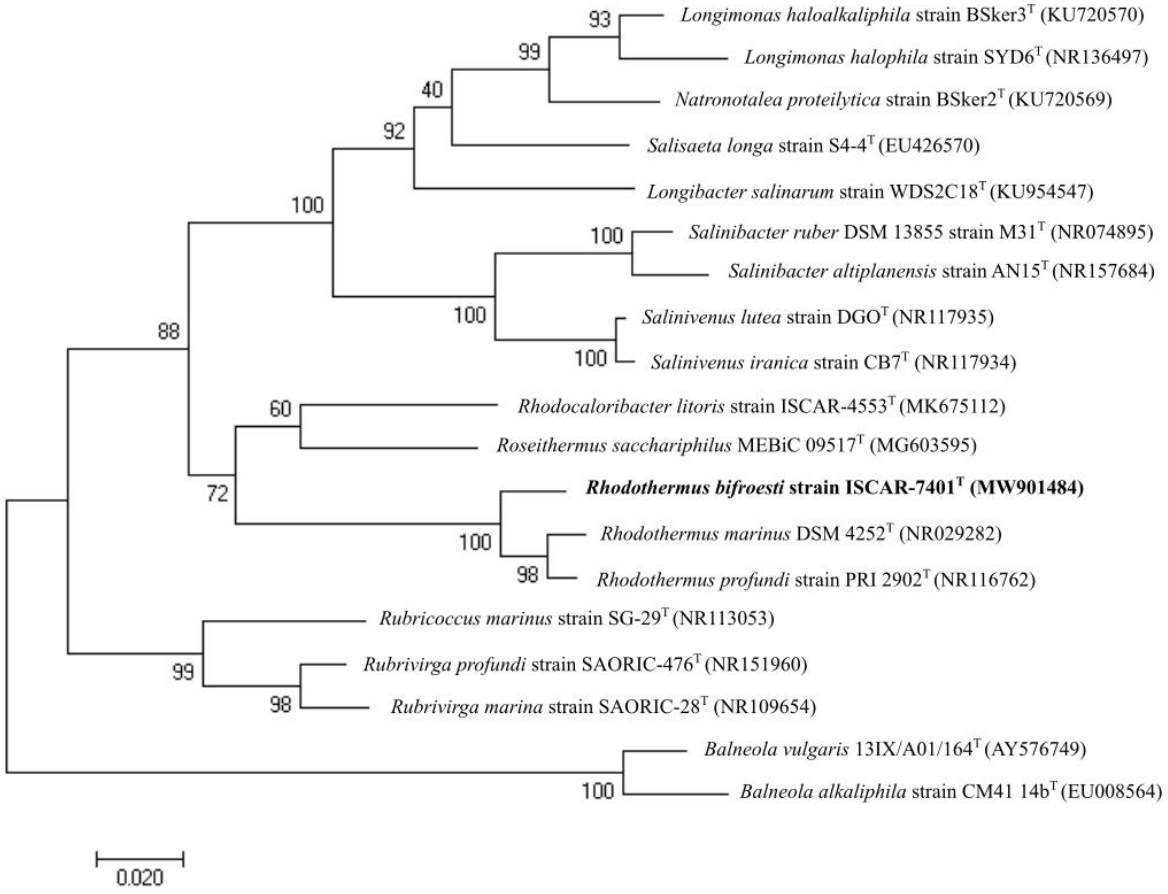

Fig. S1. Maximum Likelihood phylogenetic tree constructed based on the 16S rRNA gene sequences indicating the relationship of strain ISCAR-7401^T with the type strains within *Rhodothermaceae* and other species within the *Rhodothermales*. GenBank accession numbers are given in parentheses. Bootstrap was carried out 1000 replicates using a total of 1354 positions in the final dataset. The sequences of *Balneola* species were used as an outgroup. Bar, 0.02 represented the nucleotide substitution per position.

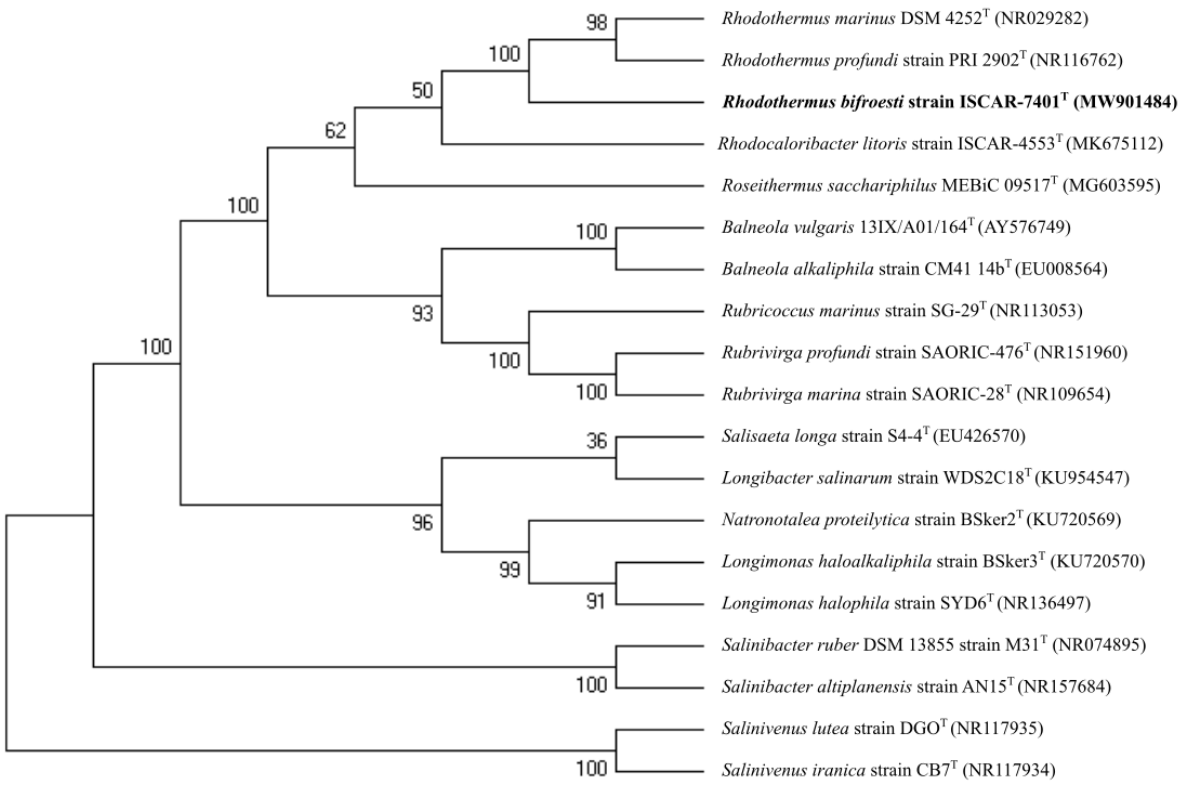

Fig. S2. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences of strain ISCAR-7401^T and closely related species. GenBank accession numbers are given in parentheses. Bootstrap was carried out 1000 replicates using a total of 1354 positions in the final dataset.

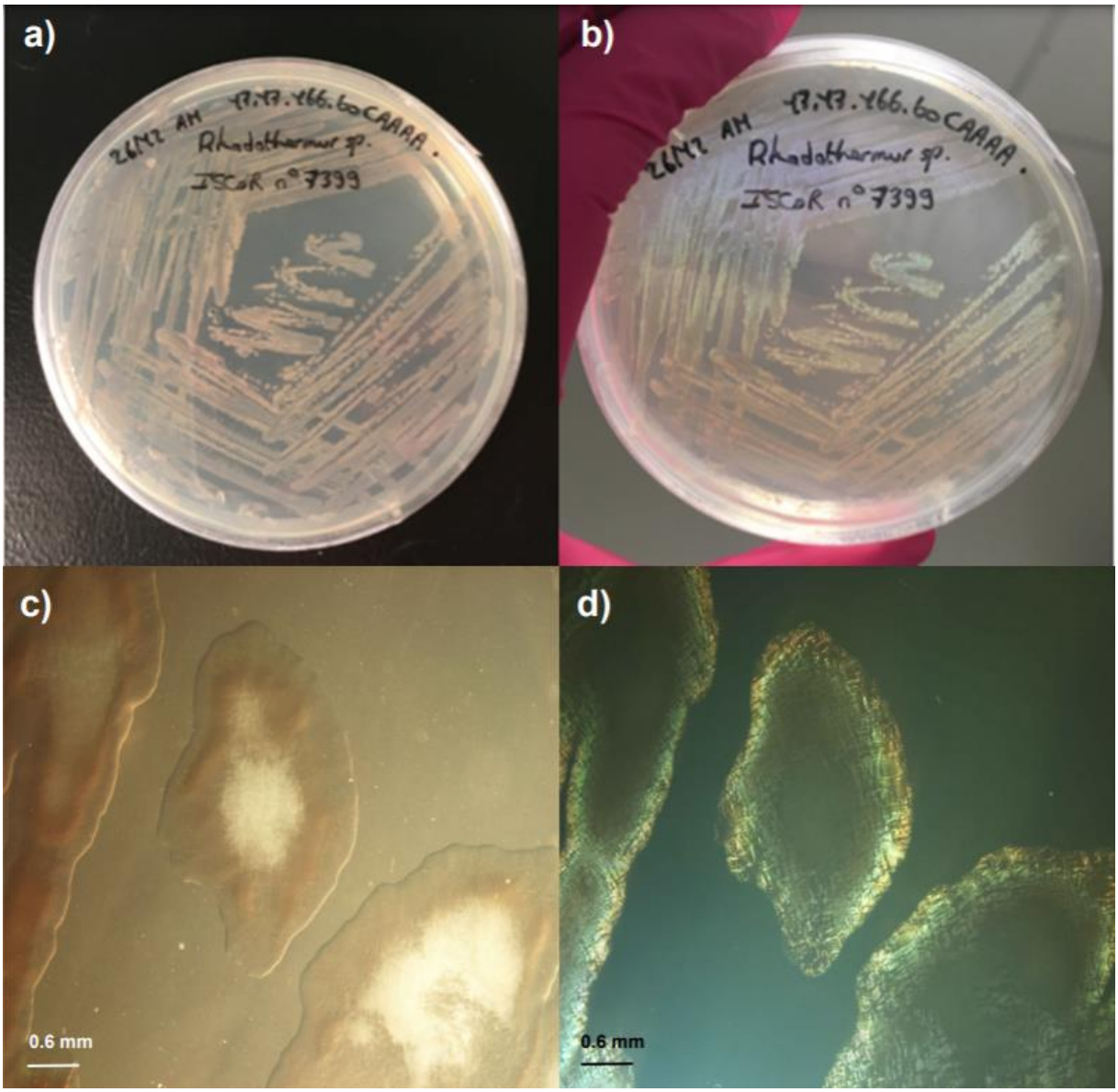

Fig. S3. Colonies of *Rhodothermus bifroesti* strain ISCAR-7399 at different scales and under different conditions of illumination. Colonies on a black background (a), under artificial light (b), observed with binocular microscope under condition of tran-illumination (c) and with a 60°angle-illumination (d), showing the iridescent characteristic of the species.

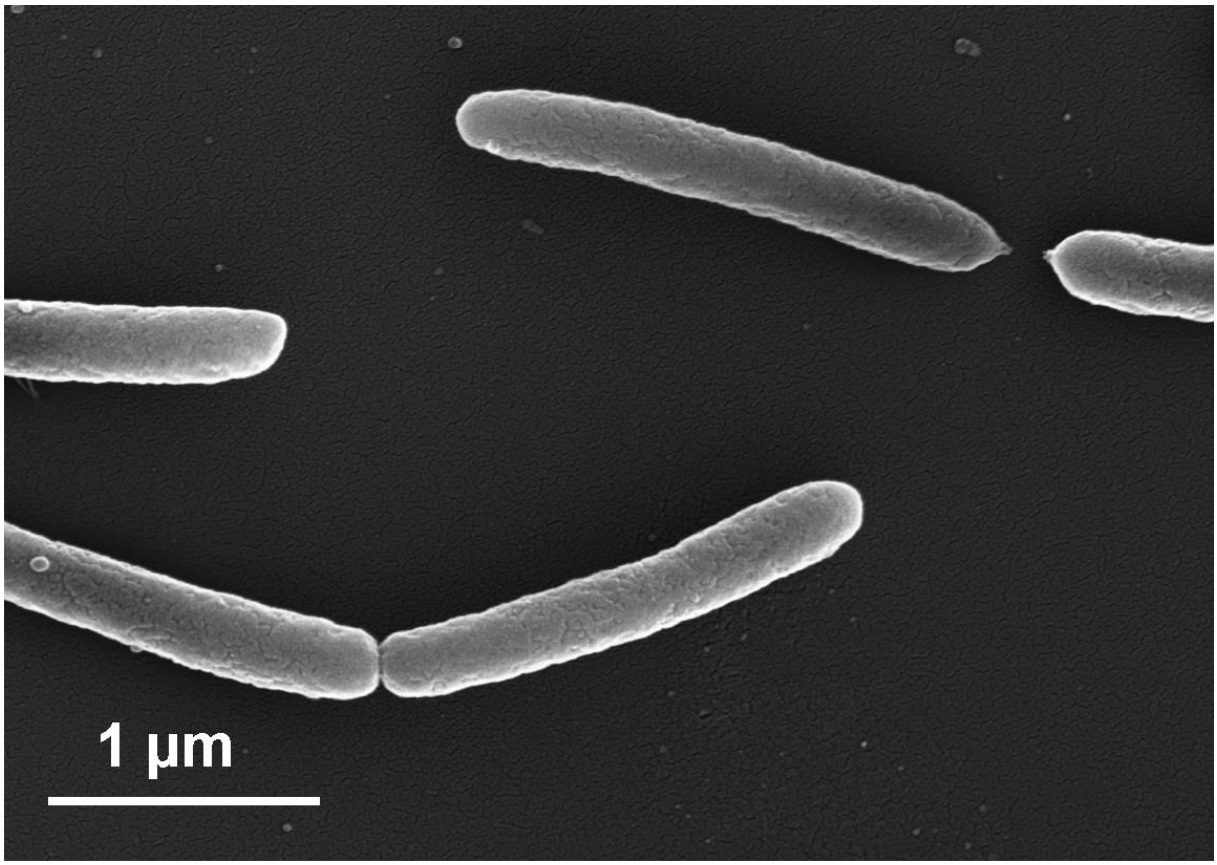

Fig. S4. Cell morphology of strain ISCAR-7401^{T} observed using scanning electron microscope Supra25 Gemini FE-SEM (ZEISS) at electron high tension voltage of 20 kV.

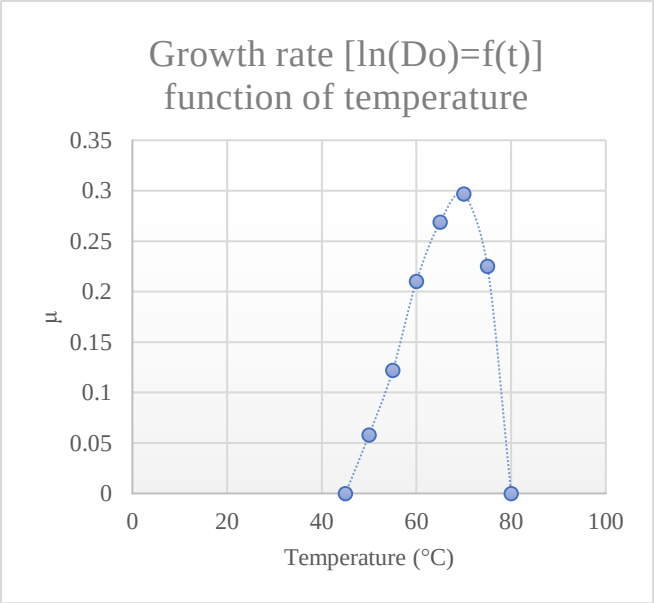

Fig. S5. Growth rates of the strain ISCAR-7401^{T} function of the temperature.

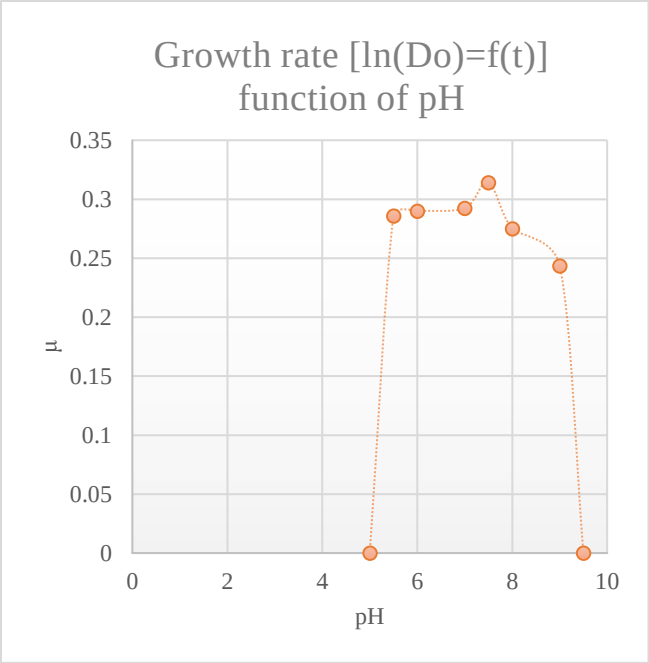

Fig. S6. Growth rates of strain ISCAR-7401^{T} function of pH.

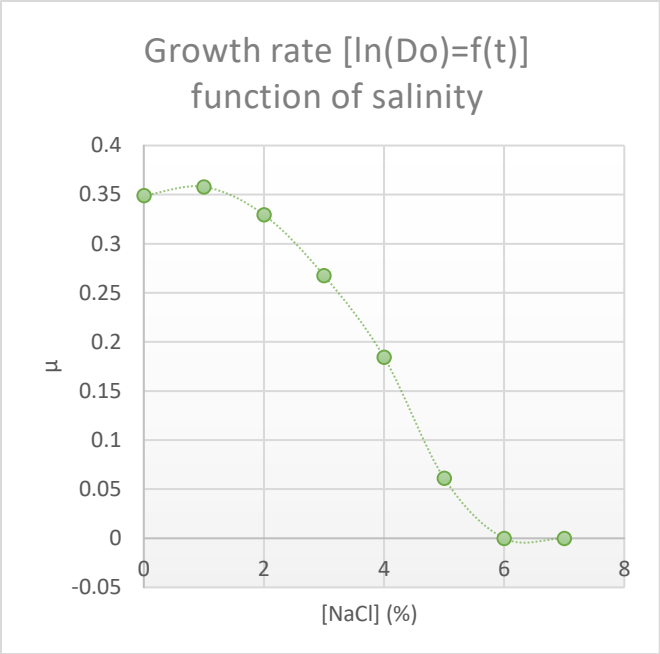

Fig. S7. Growth rates of strain ISCAR-7401^{T} function of salinity.

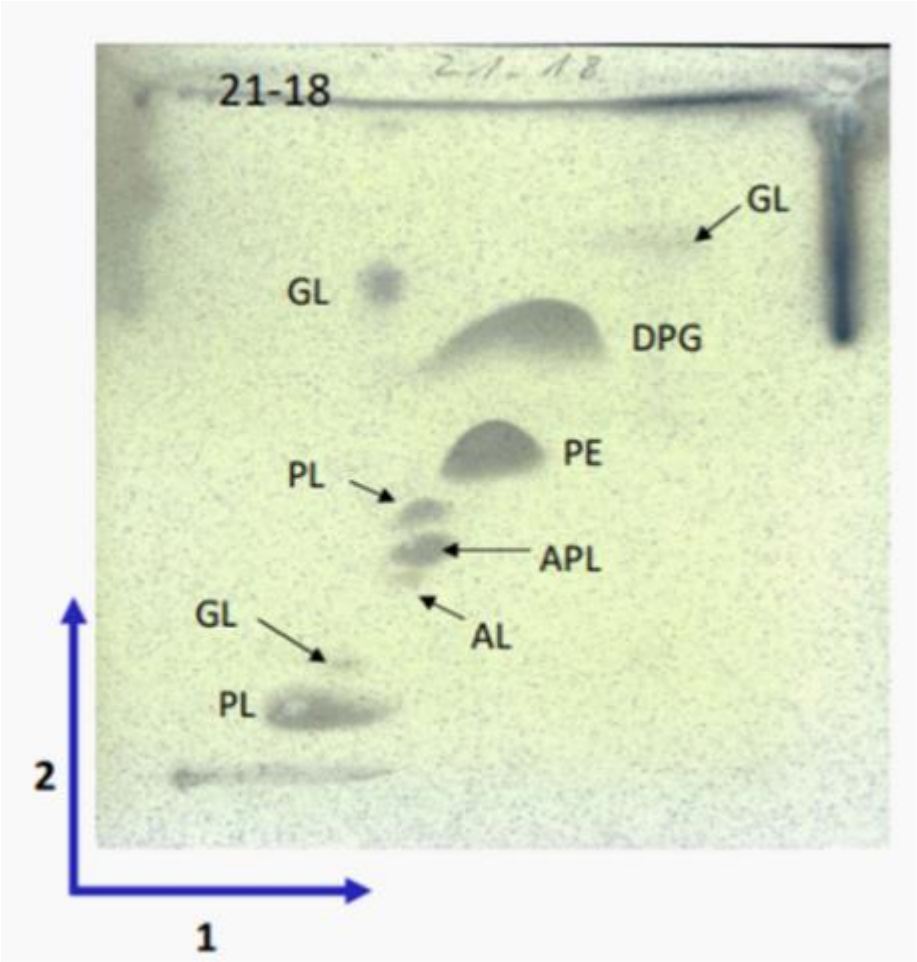

Fig. S8. Polar lipid profiles of strain ISCAR-7401^{T}. Lipid identification legends: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; AL, unidentified aminolipid; APL, unidentified aminophospholipid; GL, unidentified glycolipid; PL, unidentified phospholipid. 1, first dimension of TLC; 2, second dimension of TLC.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.965E+8	0.027	----	7.007	SOLVENT PEAK	----	< min rt		
2.206	852	0.023	----	7.870		----	< min rt		
2.689	866	0.026	----	8.813		----	< min rt		
2.953	270	0.024	----	9.327		----			
3.210	297	0.026	----	9.826		----			
4.910	387	0.035	----	11.973		----			
6.953	636	0.031	0.982	13.619	14:0 iso	0.55	ECL deviates 0.000	Reference -0.005	OK
7.106	1062	0.036	----	13.730		----			
8.446	13211	0.040	0.964	14.623	15:0 iso	11.20	ECL deviates 0.000	Reference -0.004	OK
8.587	19256	0.040	0.963	14.713	15:0 anteiso	16.31	ECL deviates 0.000	Reference -0.004	OK
10.081	9166	0.043	0.952	15.627	16:0 iso	7.68	ECL deviates 0.000	Reference -0.004	OK
10.703	5317	0.045	0.949	15.999	16:0	4.44	ECL deviates -0.001	Reference -0.005	OK
11.800	48141	0.046	0.946	16.631	17:0 iso	40.07	ECL deviates 0.001	Reference -0.003	OK
11.961	19382	0.045	0.946	16.723	17:0 anteiso	16.13	ECL deviates 0.000	Reference -0.004	OK
12.444	692	0.044	0.945	17.001	17:0	0.58	ECL deviates 0.001	Reference -0.003	OK
13.557	2683	0.046	0.943	17.632	18:0 iso	2.23	ECL deviates 0.000	Reference -0.005	OK
14.204	987	0.041	0.943	17.999	18:0	0.82	ECL deviates -0.001	Reference -0.006	OK

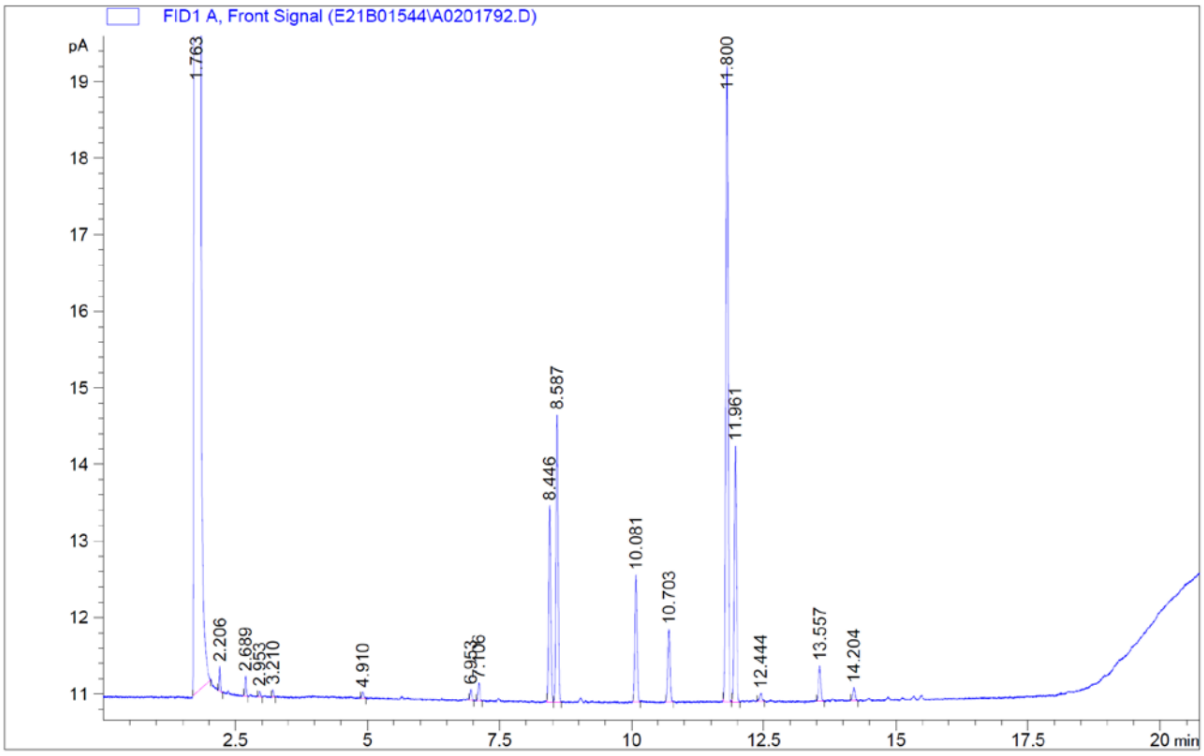

Fig. S9. Fatty acids analyses of *Rhodothermus bifroesti* strain ISCAR-7401^{T}.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.924E+8	0.028	----	7.005	SOLVENT PEAK	----	< min rt		
2.205	952	0.022	----	7.868		----	< min rt		
2.690	2366	0.025	----	8.811		----	< min rt		
2.957	210	0.025	----	9.333		----			
3.215	336	0.029	----	9.834		----			
4.911	488	0.030	----	11.973		----			
6.954	3900	0.038	0.982	13.619	14:0 iso	1.70	ECL deviates 0.000	Reference -0.004	OK
7.107	1900	0.036	----	13.730		----			
8.447	15603	0.041	0.964	14.623	15:0 iso	6.68	ECL deviates 0.000	Reference -0.004	OK
8.587	31513	0.040	0.963	14.714	15:0 anteiso	13.48	ECL deviates 0.001	Reference -0.003	OK
10.081	35466	0.043	0.952	15.627	16:0 iso	15.00	ECL deviates 0.000	Reference -0.004	OK
10.702	7267	0.044	0.949	15.998	16:0	3.06	ECL deviates -0.002	Reference -0.006	OK
11.801	77994	0.046	0.946	16.631	17:0 iso	32.78	ECL deviates 0.001	Reference -0.003	OK
11.961	44723	0.045	0.946	16.723	17:0 anteiso	18.79	ECL deviates 0.000	Reference -0.004	OK
12.442	975	0.055	0.945	17.000	17:0	0.41	ECL deviates 0.000	Reference -0.004	OK
13.555	15777	0.047	0.943	17.631	18:0 iso	6.61	ECL deviates -0.001	Reference -0.006	OK
14.206	1668	0.049	0.943	18.000	18:0	0.70	ECL deviates 0.000	Reference -0.005	OK
15.321	565	0.041	0.942	18.636	19:0 iso	0.24	ECL deviates 0.002	Reference -0.004	OK
15.485	1325	0.043	0.942	18.729	19:0 anteiso	0.55	ECL deviates -0.002	Reference -0.007	OK

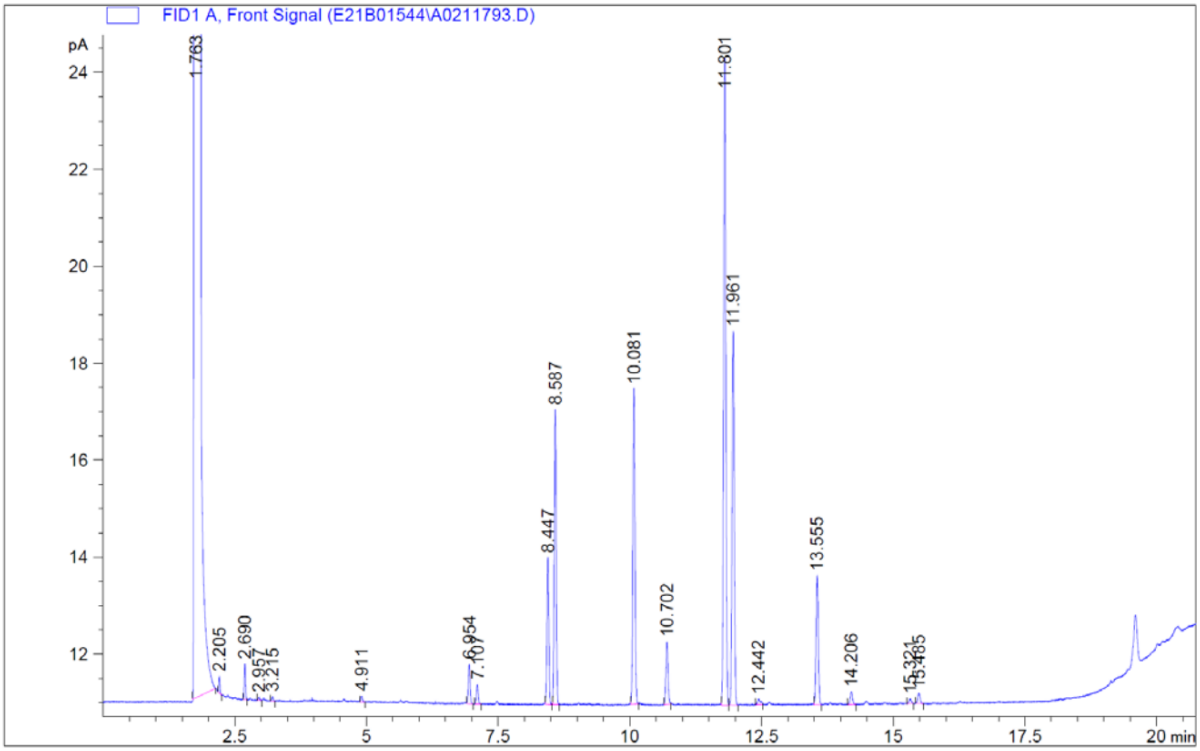

Fig. S10. Fatty acids analyses of *Rhodothermus marinus* DSM 4252^{T}.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.763	4.938E+8	0.028	----	7.006	SOLVENT PEAK	----	< min rt		
2.206	583	0.023	----	7.869		----	< min rt		
2.690	1243	0.026	----	8.813		----	< min rt		
2.958	283	0.032	----	9.334		----			
3.214	320	0.026	----	9.834		----			
4.909	450	0.032	----	11.971		----			
6.954	2907	0.036	0.982	13.619	14:0 iso	1.07	ECL deviates 0.000	Reference -0.004	OK
8.447	20639	0.040	0.964	14.623	15:0 iso	7.46	ECL deviates 0.000	Reference -0.004	OK
8.588	28456	0.041	0.963	14.714	15:0 anteiso	10.28	ECL deviates 0.001	Reference -0.003	OK
10.081	55186	0.043	0.952	15.627	16:0 iso	19.72	ECL deviates 0.000	Reference -0.004	OK
10.702	6567	0.043	0.949	15.998	16:0	2.34	ECL deviates -0.002	Reference -0.006	OK
11.801	92022	0.046	0.946	16.631	17:0 iso	32.66	ECL deviates 0.001	Reference -0.003	OK
11.961	45055	0.045	0.946	16.723	17:0 anteiso	15.98	ECL deviates 0.000	Reference -0.004	OK
13.558	25916	0.047	0.943	17.632	18:0 iso	9.17	ECL deviates 0.000	Reference -0.004	OK
14.204	1402	0.045	0.943	17.998	18:0	0.50	ECL deviates -0.002	Reference -0.007	OK
14.850	607	0.037	----	18.367		----			
15.326	1155	0.047	0.942	18.638	19:0 iso	0.41	ECL deviates 0.004	Reference -0.001	OK
15.485	1170	0.043	0.942	18.729	19:0 anteiso	0.41	ECL deviates -0.002	Reference -0.007	OK

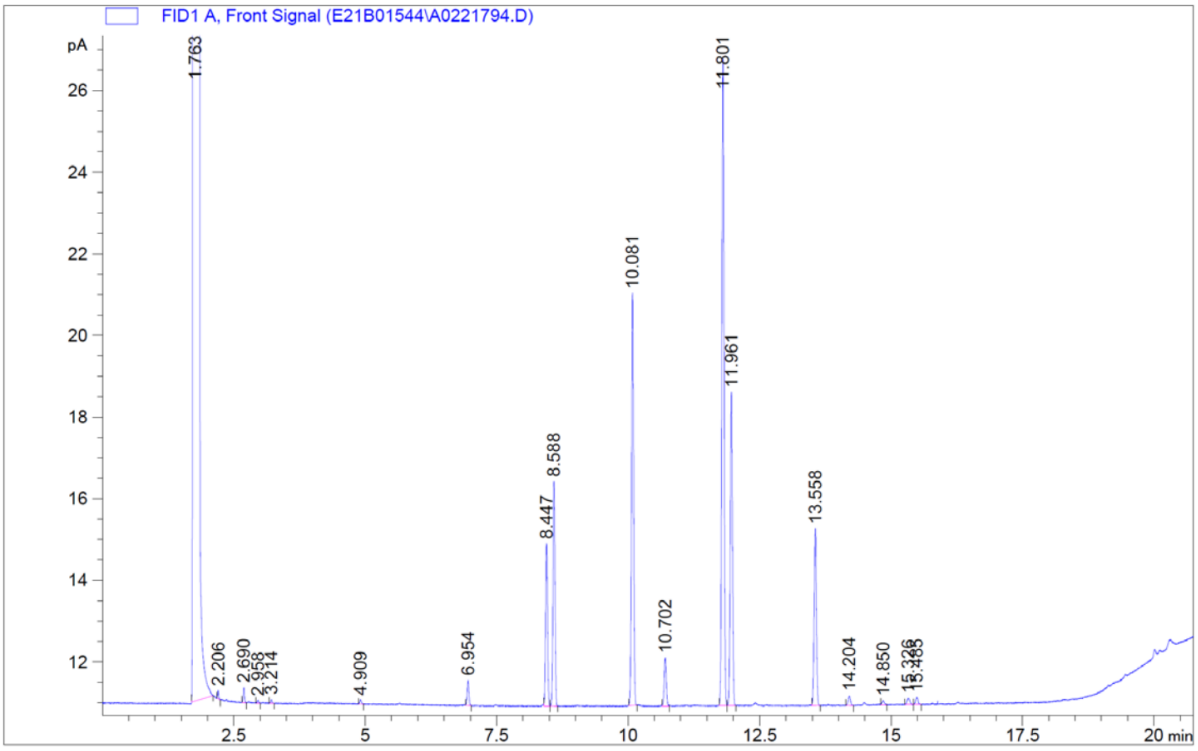

Fig. S11. Fatty acids analyses of *Rhodothermus profundi* strain PRI 2902^{T}.

Supplementary Tables.

Table S1. Media 166, modified from Hjorleifsdottir et al., 2001 (9) (without proline). Grunnur base from medium 162 from Degryse et al., 1978 (10).

For 1L of liquid media:	
NaCl	10 g
K_2HPO_4	0.3 g
Yeast extract	1 g
Peptone	1 g
Tryptone	1 g
Glucose	0.5 g
Amidon (starch)	0.5 g
Na-pyruvate	0.6 g
Na_2CO_3	0.18 g
Base "Grunnur"	100 mL
Hot tap water	900 mL
pH adjusted to 7-7.5	

Grunnur (for 1L):	
Titriplex I (nitrilotriacetic acid)	1.32 g
$CaSO_4$ x $2H_2O$	0.4 g
$MgCl_2$ x $6H_2O$	2.0 g
Trace elements (Wolfe´s mineral solution)	5 ml
Ironcitrate	5 ml
H_2O	1000 ml
pH adjusted to 7.2	

Ironcitrate (for 1L):	
Na_3citrate x $2H_2O$	2.94 g
$FeCl_3$ x $6H_2O$	2.7 g

Table S2. Annotated genes from genome of strain ISCAR-7401^T (see Excel file).

Table S3. Characteristics that differentiate the characterized strains of *Rhodothermus bifroesti*. 1, *Rhodothermus bifroesti* sp. nov. strain ISCAR-7401^{T}; 2, Strains ISCAR-7397; 3, Strains ISCAR-7399; 4, Strains ISCAR-7403. Data were obtained in the present study. All strains are aerobic, stain Gram-negative, produce pinkish, translucent colonies, grew optimally at 70°C, and are oxidase- and catalase-negative. All strains could grow without NaCl (w/v) and showed growth on D-galactose, glucose, D-lactose, D-mannose, D-raffinose, D-rhamnose, D-sucrose, pyruvate, L-asparagine, L-aspartate, and L-glutamine. None exhibited growth on D-fructose, acetate, citrate, L-malic acid, L-alanine, L-arginine, L-proline, L-serine, L-threonine, and L-valine. All the strains were resistant to rifampicin, kanamycin, and streptomycin and were sensitive to gentamicin. W, weak growth.

	1	**2**	**3**	**4**
Growth at 6% NaCl (w/v)	-	-	-	w
Growth at pH 9.5	w	+	+	+
Utilization of D-maltose, L-glutamate	+	+	+	-
Utilization of L-asparagine, L-glutamine	+	+	w	w
16S rRNA gene similarity (%) with strain ISCAR-7401^{T}	100	100	99.76	99.68

Supplementary references.

1. Miller LT. Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. Journal of Clinical Microbiology. 1982;16(3):584–6. doi: 10.1128/jcm.16.3.584-586.1982

2. Kuykendall LD, Roy MA, O'Neill JJ, Devine TE. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. International Journal of Systematic Bacteriology. 1988;38(4):358–61. doi: 10.1099/00207713-38-4-358

3. Moss CW, Lambert-Fair MA. Location of double bonds in monounsaturated fatty acids of *Campylobacter* cryaerophila with dimethyl disulfide derivatives and combined gas chromatography-mass spectrometry. Journal of Clinical Microbiology. 1989;27(7):1467–70. doi: 10.1128/jcm.27.7.1467-1470.1989

4. Harvey DJ. Picolinyl esters as derivatives for the structural determination of long chain branched and unsaturated fatty acids. Biological Mass Spectrometry. 1982;9(1):33–8. doi: 10.1002/bms.1200090107

5. Spitzer V. Structure analysis of fatty acids by gas chromatography - Low resolution electron impact mass spectrometry of their 4,4-dimethyloxazoline derivatives - A review. Progress in Lipid Research. 1996;35(4):387–408. doi: 10.1016/S0163-7827(96)00011-2

6. Yu QT, Liu BN, Zhang JY, Huang ZH. Location of methyl branchings in fatty acids: Fatty acids in uropygial secretion of Shanghai Duck by GC-MS of 4,4-dimethyloxazoline derivatives. Lipids. 1988;23:804–810. doi: 10.1007/BF02536225

7. Bligh, E.G. and Dyer WJ. A rapıd method of total lıpıd extractıon and purıfıcatıon. Canadian Journal of Biochemistry and Physiology. 1959;37(8):911–7. doi: 10.1139/o59-099

8. Tindall BJ, Sikorski J, Smibert RA, Krieg NR. Phenotypic Characterization and the Principles of Comparative Systematics. In: Methods for General and Molecular Microbiology. 2007. doi: 10.1128/9781555817497.ch15

9. Hjorleifsdottir S, Skirnisdottir S, Hreggvidsson GO, Holst O, Kristjansson JK. Species composition of cultivated and noncultivated bacteria from short filaments in an icelandic hot spring at 88°C. Microbial Ecology. 2001;42(2):117–25. doi: 10.1007/s002480000110

10. Degryse E, Glansdorff N, Piérard A. A comparative analysis of extreme thermophilic bacteria belonging to the genus *Thermus*. Archives of Microbiology. 1978;117:189–196. doi: 10.1007/BF00402307