陸上温泉から分離された新規好熱細菌Rhodothermus clarus sp. nov.

誌名	三重大学大学院生物資源学研究科紀要 = The bulletin of the Graduate School of Bioresources Mie University
ISSN	
著者名	田中,礼士 左子,芳彦 前田,広人
発行元	三重大学大学院生物資源学研究科
巻/号	35号
掲載ページ	p. 39-45
発行年月	2008年12月

Bull. Graduate School of Bioresources, Mie Univ.
No.35 : 39 − 45
December, 2008

Rhodothermus clarus sp. nov., a strictly aerobic, thermophilic bacterium isolated from a terrestrial hot spring

Reiji TANAKA[1]*, Yoshihiko SAKO[2] and Hiroto MAEDA[1]

[1] Graduate School of Bio resources, Mie University, 1577 Kurima-matchiya, Tsu, Mie, 514-8507, Japan
[2] Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwake, Sakyo, Kyoto, 606-8502, Japan

Abstract

A thermophilic, strictly aerobic, heterotrophic bacterium (AR3^T) was isolated from a terrestrial hot spring at Arima, Hyogo prefecture, Japan. The cells were Gram-negative, non-spore-forming, and rod-shaped, about 0.6-1.2 μm in diameter and 3.0-5.0 μm long. Flagella were not observed, and also motility was not observed. The isolate grew at temperatures between 43 and 80℃. The new isolate grew over the salinity range of 0 to 8.0% (w/v), and the pH range of pH 5.5 to 9.2. The G + C content of the genomic DNA of the new isolate was 66 mol%. Physiological properties and sequence analysis of the 16S rRNA gene, as well as DNA-DNA hybridization experiments, indicated that the isolate represents a new species of the genus *Rhodothermus* for which the name *Rhodothermus clarus* is proposed. The type strain is AR3^T (=JCM 13927^T=DSM 18403^T).

Key Words: *Rhodothermus*; thermophile; aerobic; heterotroph.

Introduction

The genus *Rhodothermus* belongs to the class Sphingobacteria and family Crenotrichacea. It is an aerobic, chemoorganotrophic, gram-negative eubacterium and has been isolated from marine habitats. The type species *Rhodothermus marinus* was isolated from submarine alkaline hot springs at 2-4 m depth in Isafjardardjup, in Iceland[1)]. *R. marinus* grew at 54-77℃ and from 0.5% NaCl to over 6 % NaCl. *R. marinus* strains were isolated from marine hot springs at Praia de Ribeira Quente[2)] and Ferraria[3)] on the island of São Miguel in the Azores, Portugal, from Stufe di Nerone, Italy[4)] and from the island of Monserrat in the Caribbean Sea[3)]. *Rhodothermus* sp. has recently isolated from a deep-sea hydrothermal vent chimney[5)], and junior synonym '*R obamensis*' was isolated from Tachibana Bay in Japan[6)]. The habitat of the genus *Rhodothermus* is highly restricted to shallow hydrothermal vents, and they have been isolated from similar habitats in distant locations around the world[7)].

We isolated the first *Rhodothermus* strain from a terrestrial hot spring, and describe that it is a novel species of *Rhodothermus*.

2008年2月28日受理
* For correspondence (e-mail: tanakar@bio.mie-u.ac.jp)

Materials and Methods

Hot fluid samples were collected from the hot springs at Arima, Hyogo, Japan. The temperature of the fluid water was 74℃. Salinity of the fluid water was 6 %. Samples were stored aerobically at room temperature for 4 h prior to incubation. One ml of water sample was inoculated in 9 ml of MJYPV medium[5] prepared in 20 ml test tube, and the culture was incubated at 70℃ in a dry oven. Growth of aerobic thermophiles was observed after 1 day of incubation. To obtain a pure culture of the cells, the enriched cells were streaked onto MJYPV plates hardened with 0.5% (w/v) Gellan Gum (Wako-chemical, Osaka, Japan). The plates were incubated at 70℃ in a tightly sealed polycarbonate jar to prevent evaporation. After 3 to 5 days of incubation, small, spherical (1-2 mm in diameter) and colorless colonies were formed on the surface of the plates. Well-isolated colonies were picked, and the cells were incubated in fresh liquid MJYPV medium at 70℃. In order to ensure purity, the streaking and isolation step was repeated at least three times. The first pure culture was designated strain AR3^T (=JCM 13927^T=DSM 18403^T) and was investigated in detail. Cells were observed using a differential interference microscope (UFX; Nikon).

Negatively stained cells were examined by transmission electron microscopy, as described by Zillig *et al*[8]; cells were stained with 1 % (w/v) uranyl acetate and examined using an H-700 electron microscope (Hitachi, Japan) at an accelerating voltage of 100 kv. Growth of the new isolate under various conditions was determined by direct cell counting, after staining with 4', 6-diamidino-2-phenylindole[9], using an epifluorescence microscope (Eclipse E800 system; Nikon, Japan) equipped with color chilled 3CCD camera system (C5810; Hamamatsu Hotonikusu, Japan).

The effects of pH and salinity on the growth of the isolate were determined at 70℃. The pH of MJYPV medium containing 20 mM of MES (pH 5.0-6.0), PIPES (pH 6.3-7.0), HEPES (pH 7.3-8.0) or Tris (pH 8.5 and above) was adjusted to the designed values with H_2SO_4 or NaOH at room temperature. To determine the effect of salinity on growth, the isolate was incubated in MJYPV medium containing various dilutions of 3×MJ synthetic seawater[10].

In an attempt to find organic substrates that could support the growth of the isolate, various organic substrates were tested instead of both yeast extract and tryptone in MJYPV medium. Each of the following substrates was added at concentrations of 0.1% (w/v): L-alanine, L-arginine, L-asparagine, L-asparaginate, L-asparate, L-glutamin, L-glutamate, L-phenylalanine, L-proline, L-serine, L-valine, Casamino acid, gelatin, D-(-)-fructose, D-(+)-glucose, galactose, inositol, D-sorbitol, D-(+)-xylose, D-(+)-cellobiose, lactose, maltose, D-(+)-trehalose, sucrose, chitin, starch, sodium acetate, citrate, malate, sodium pyruvate, casamino acid, casein, yeast extract (Difco), and tryptone (Difco). These tests were performed at temperatures of 70℃, and run in duplicate.

In an attempt to examine whether or not the new isolate was able to grow under anaerobic condition, 200 kPa of N_2 was tested as a gas phase instead of air with MJYPV medium in the presence or absence of possible alternative electron acceptors such as 0.1% (w/v) of $NaNO_3$, Na_2SO_3, $NaNO_2$ or $Na_2S_2O_3$.

Genomic DNA of strain AR3^T, *R. marinus* DSM 1452^T and *R. marinus* JCM 9785 was isolated as described by Lauerer *et al.*[11] and the G + C content (mol%) was determined by the method of Tamaoka and Komagata[12] using a DNA-GC kit (Yamasa shouyu, Japan). The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using 27F and 1492R primers[13] and the nucleotide sequence of the PCR product was directly determined in both strands using dideoxynucleotide chain termination method with an ABI 373A DNA sequencer (Applied Biosystems, CA, USA). Neighbor-joining analysis[14] of 1441 bases of sequence from each organism was accomplished using Bio NJ[15]. Purified DNA from the isolate,

strain AR3^T, was compared with that of *R. marinus* DSM 1452^T and *R. marinus* JCM 9785 by fluorometric DNA-DNA hybridization using photobiotin-labelled DNA, as described by Ezaki *et al.*[16]. Relatedness values were measured under optimal condition (Tm-25℃).

Results

Cells stained gram-negative, which were 3.0-5.0 μm in length and 0.6-1.2 μm in width. Flagella were not observed (Fig. 1), and motility was not observed under the differential interference microscopic observation. These morphological properties of the new isolate were generally similar to those of *R. marinus* (1). Strain AR3^T grew at 43-80℃ with optimum at 70℃. The strain did not grow at temperatures below 42℃ or above 81℃. The isolate grew at pH values between pH 5.5 and 9.2, with an optimum at pH 7.0. No growth was detected below pH 5.0 or above pH 9.5. The isolate grew at 0-8.0% (w/v) salinity, with an optimum at 3.5%. Growth was not observed at salinities above 9.0%. The isolate was found to be unable to grow under any of the anaerobic conditions tested in this study. The isolate was found to be able to utilize D-(+)-glucose, galactose, D-(+)-xylose, maltose, L-alanine, L-asparaginate, L-glutamin, L-glutamate, sodium acetate, citrate, malate, sodium pyrvate, casamino acid, yeast extract, and tryptone.

The G + C content of strain AR3^T was 66 mol%, which was similar to that of *R. marinus* strain DSM 1452^T (Table 1). The phylogenetic tree demonstrated that the new isolate was a close relative of *R. marinus* strain DSM 1452^T (Fig. 2). The similarity of the sequence for 16S rRNA gene between the strain AR3^T and *R. marinus* strain DSM 1452^T was 99.0%. The DNA of strain AR3^T yielded relatively low hybridization signals with DNA from *R. marinus* DSM 1452^T and *R. marinus* JCM 9785 (57 and 60% relatedness, respectively). These findings indicated that the new isolate could be differentiated from other *Rhodothermus* species.

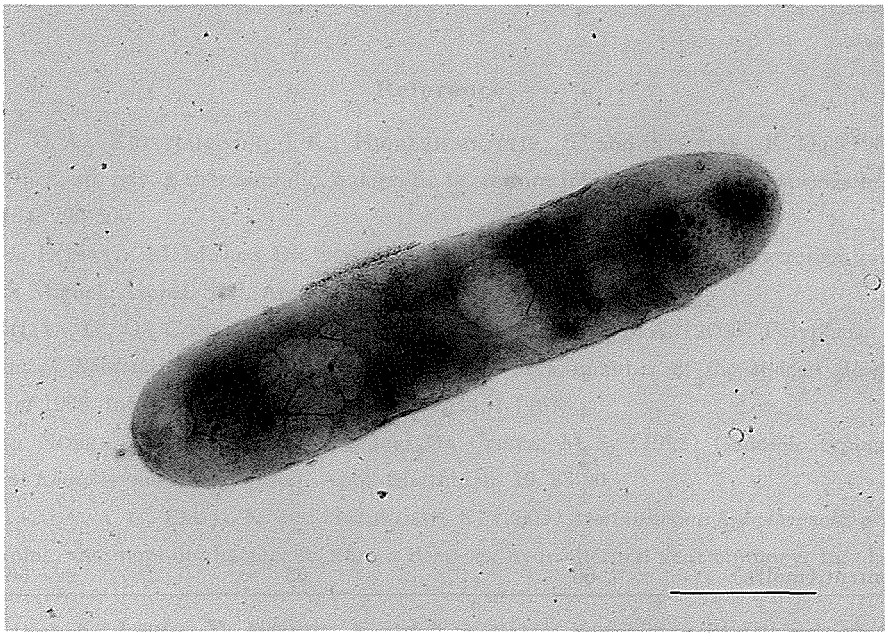

Fig. 1. Transmission electron micrograph of a negative-stained cell of strain AR3^T. Bar, 1 μm.

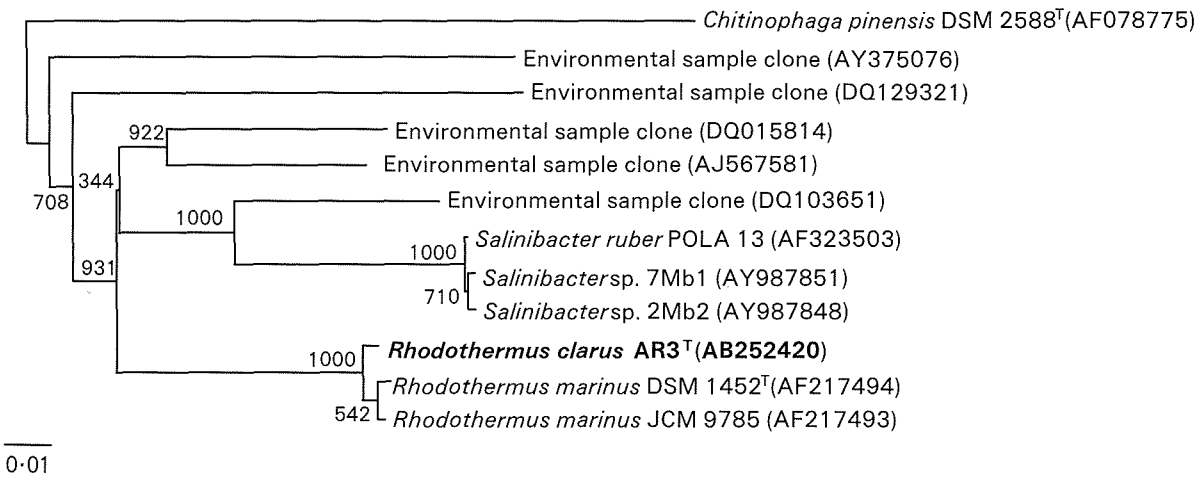

Fig. 2. Neighbor-joining tree showing the phylogenetic position of strain AR3^T and related taxa based on 16 S rRNA gene sequences. Numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 1. Characteristics of *Rhodothermus clarus* AR3^T and *R. marinus*. Data were taken from this study, Alfredsson *et al.* (1988) and Sako *et al.* (1996 a)

Property	*R. clarus* AR3^T	*R. marinus* DSM 4252^T	*R. marinus* JCM 9785
Habitat	Terrestrial hot springs	Shallow hydrothermal vent	Shallow hydrothermal vent
Cell size (μm)	3.0-5.0×0.6-1.2	2.0-7.0×0.3-0.6	1.0-4.0×0.2-0.5
Colony color	Colorless	Reddish	Reddish
Growth in the presence of:			
0 % NaCl	+	−	−
3 % NaCl	+	+	+
6 % NaCl	+	+	−
8 % NaCl	+	−	−
Growth at 80℃	+	−	+
Growth on:			
Xylose	+	+	−
Sorbitol	−	+	−
Casein	+	+	−
Casamino Acids	+	−	−
Glutamine	+	−	−
Acetate	+	−	−
Pyruvate	+	−	−
Malate	+	−	−
DNA G+C content (mol%)	66	65	66.6

Discussion

In spite of the high similarity of 16S rRNA gene sequence between strain AR3^T and *R. marinus*, there were many differences in phenotype between these organisms (Table 1). These organisms differed in the utilization of carbon source and the effect of NaCl concentration and temperature on growth. The growth of strain AR3^T was supported by several carboxylic acids (acetate, citrate and malate). By contrast, the growth of *R. marinus* was not supported by these substrates. These physiological properties strongly suggest that strain AR3^T can be classified into different species from *R. marinus*. Finally, DNA-DNA hybridization analysis clearly indicated that the new isolate could be genotypically differentiated from *R. marinus*.

On the basis of these physiological and genetic properties, we propose a new species of the genus *Rhodothermus*, to be designed *R. clarus*; the type strain is strain AR3^T (= JCM 13927^T=DSM 18403^T).

Description of *Rhodothermus clarus* sp. nov. *Rhodothermus clarus* (cl. a'. rus. a. um. adj. clarus color-ress, relating to its colony color.). Cells are Gram-negative, non-motile rods 3.0-5.0 μm in length and 0.6-1.2 μm in width. Growth occurs between 43℃ and 80℃, at pH 5.5 to 9.2 and in the presence of 0-8.0% salinity. Growth is heterotrophic in the presence of D-(+)-glucose, galactose, D-(+)-xylose, maltose, L-alanine, L-asparaginate, L-glutamin, L-glutamate, sodium acetate, citrate, malate, sodium pyrvate, casamino acid, yeast extract, and tryptone. The G + C content of the genomic DNA of the type strain is 66 mol% (HPLC). The type strain is AR3^T (=JCM 13927^T=DSM 18403^T), was isolated from a terrestrial hot spring at Arima, Hyogo prefecture, Japan.

Acknowledgements

We are grateful to Dr. Tomoo Sawabe, Graduate School of Fisheries, Hokkaido University, Japan, for the electron micrographs. This work was supported by a research fellowship (No. 15-4791) for young scientists and a Grant-in-Aid for scientific research (No. 16208020) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Reference

1) ALFREDSSON, G. A., KRISTJANSSON, J. K., HJÖRLEIFSDOTTIR, S. and STETTER, K. O. (1988) *Rhodothermus marinus*, gen. nov., sp. nov, a thermophilic, halophilic bacterium from submarine hot springs in Iceland. J. Gen. Microbiol. **134**: 299-306.

2) NUNES, O. C., DONATO, M. M. and DA COSTA, M. M. (1992) Isolation and Characterization of *Rhodothermus* strains from S-Miguel, Azores. Syst. Appl. Microbiol. **15**: 92-97.

3) SILVA, Z., HORTA, C., DA COSTA, M. S., CHUNG, A. P. and RAINEY, F. A. (2000) Polyphasic evidence for the reclassification of *Rhodothermus obamensis* Sako *et al.* 1996 as a member of the species *Rhodothermus marinus* Alfredsson *et al* 1988. Int. J. Syst. Evol. Microbiol. **50**: 1457-1461.

4) MOREIRA, L., NOBRE, M. F., SACORREIA, I. and DA COSTA, M. S. (1996) Genomic typing and fatty acid composition of *Rhodothermus marinus*. Syst. Appl. Microbiol. **19**: 83-90.

5) SAKO, Y., NAKAGAWA, S., TAKAI, K. and HORIKOSHI, K. (2003) *Marinithermus hydrothermalis* gen. nov., sp. nov., a strictly aerobic, thermophilic bacterium from a deep-sea hydrothermal vent chimney. Int. J. syst. Bacteriol. **53**: 59-65.

6) SAKO, Y., TAKAI, K., ISHIDA, Y., UCHIDA, A. and KATAYAMA, Y. (1996 a) *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. Int. J. Syst. Bacteriol. **46**: 1099-1104.

7) PETURSDOTTIR, S. K., HREGGVIDSSON, G. O., DA COSTA, M. S. and KRISTJANSSON, J. K. (2000) Genetic diversity analysis of *Rhodothermus* reflects geographical origin of the isolates. Extremophiles **4**: 267-274.

8) ZILLIG, W., YEATS, S., HOLZ, I., BOCK, A., RETTENBERGER, M., GROPP, F. and SIMON, G. (1986) *Desulfurolobus ambivalens*, gen. nov., sp. nov., an autotrophic archaebacterium facultatively oxidizing or reducing sulfur. Syst. Appl. Microbiol. **8**: 197-203.

9) PORTER, K. G. and FEIG, Y. S. (1980) The use of DAPI for identifying and counting microflora. Limnol. Oceanogr. **25**: 943-948.

10) SAKO, Y., NOMURA, N., UCHIDA, A., ISHIDA, Y., MORII, H., KOGA, Y., HOAKI, T. and MARUYAMA, T. (1996 b) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperature up to 100℃. Int. J. Syst. Bacteriol. **46**: 1070-1077.

11) LAUERER, G., KRISTJANSSON, J. K., LANGWORTHY, T. A., KÖNIG, H. and STETTER, K. O. (1986) *Methanothermus sociabilis* sp. nov., a second species within the *Methanothermaceae* growing at 97℃. Syst. Appl. Microbiol. **8**: 100-105.

12) TAMAOKA, J. and KOMAGATA, K. (1984) Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. **25**: 125-128.

13) NAKAGAWA, S., NAKAMURA, S., INAGAKI, F., TAKAI, K., SHIRAI, N. and SAKO, Y. (2004) *Hydrogenivirga caldilitoris* gen. nov., sp. nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field. Int. J. Syst. Evol. Microbiol. **54**: 2079-84.

14) SAITOU, N. and NEI, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406-425.

15) GASCUEL, O. (1997) "BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data" Mol. Biol. Evol. **14**: 685-695.

16) EZAKI, T., HASHIMOTO, Y. and YABUUCHI, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. **39**: 224-229.

陸上温泉から分離された新規好熱細菌 *Rhodothermus clarus* sp. nov.

田中　礼士[1*]・左子　芳彦[2]・前田　広人[1]

[1] 三重大学大学院生物資源学研究科,
[2] 京都大学大学院農学研究科

要　旨

好熱性，好気性の従属栄養細菌（AR3^T）が兵庫県の有馬温泉から新規分離された。細胞はグラム陰性，胞子形成のない幹菌で，細胞の直径は0.6～1.2 μm，細胞の長さは3.0～5.0 μm であった。鞭毛は形成せず，運動性も見られなかった。分離株は43～80℃で増殖した。0～8.0%（w/v）の塩分濃度で増殖が可能であった。増殖可能なpHは5.5～9.2であった。ゲノムDNA中のGC含量は66 mol%であった。生化学的性状，リボゾーム小サブユニットRNA遺伝子塩基配列による系統解析ならびに，DNA-DNA相同性試験より本分離株は*Rhodothermus*属の新種と考えられた。そこで本分離株を*Rhodothermus clarus*と命名することを提案する。標準株はAR3^T（＝JCM 13927^T＝DSM 18403^T）とする。

* For correspondence (e-mail: tanakar@bio.mie-u.ac.jp)