Polyphasic evidence for the reclassification of *Rhodothermus obamensis* Sako *et al.* 1996 as a member of the species *Rhodothermus marinus* Alfredsson *et al.* 1988

Zélia Silva,¹ Célia Horta,¹ Milton S. da Costa,¹ Ana Paula Chung¹ and Fred A. Rainey²

Author for correspondence: Milton S. da Costa. Tel: +351 2398 24024. Fax: +351 2398 26798.
e-mail: milton@cygnus.ci.uc.pt

¹ Centro de Neurociências de Coimbra, Departamento de Zoologia, and Departamento de Bioquímica, Universidade de Coimbra, 3000 Coimbra, Portugal

² Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

DNA–DNA reassociation studies, 16S rRNA gene sequence comparisons and fatty acid analysis were used to reassess the taxonomic status of the type strain of *Rhodothermus obamensis* and several strains of the genus *Rhodothermus* isolated from widely distributed shallow marine hot springs. The results show that the type strain of *R. obamensis*, JCM 9785^T, has a DNA–DNA reassociation value of 78% with the type strain of *R. marinus*, DSM 4252^T. The other strains examined had DNA–DNA reassociation values that varied between about 68 and 94% with *R. marinus*. The 16S rRNA gene sequence was determined for the type strain of *R. obamensis* and found to share 99.5% similarity with the type strain of *R. marinus*. The fatty acid composition of *R. obamensis* was slightly different from that of the other strains examined, but indicated that this strain is very closely related to the other strains examined in this study. On the basis of DNA–DNA reassociation values, 16S rRNA gene sequence comparison and fatty acid profiles, it was concluded that *R. obamensis* and *R. marinus* represent the same species and that the name *Rhodothermus obamensis* should be regarded as a junior synonym of *Rhodothermus marinus*.

Keywords: *Rhodothermus marinus*, *Rhodothermus obamensis*, 16S rRNA gene sequence, DNA–DNA reassociation

INTRODUCTION

The first strains of the thermophilic and slightly halophilic species *Rhodothermus marinus* were isolated from shallow marine hot springs off the coast of Iceland by Alfredsson *et al.* (1988). This organism is a strictly aerobic chemo-organotroph, requiring about 0.5–2.0% NaCl for optimum growth. The optimum growth temperature is about 65–70 °C and the maximum growth temperature is around 77 °C. On the basis of 16S rRNA gene sequence analysis, *R. marinus* is considered to represent a deep lineage within the *Cytophaga–Flavobacterium–Bacteroides* phylum, (Andrésson & Fridjónsson, 1994).

Organisms similar to *Rhodothermus marinus* were later isolated from marine hot springs at a beach on the island of São Miguel in the Azores and from a saline hot spring about 100 m from the Bay of Naples at Campi Flegri, Italy (Nunes *et al.*, 1992a, b; Moreira *et al.*, 1996). DNA–DNA reassociation values and phenotypic characteristics indicated that these organisms belonged to the species *R. marinus*, originally isolated from Iceland, and implied that *R. marinus* had a wide distribution in saline hot springs with neutral pH.

A new species, isolated from shallow marine hydrothermal vents in Japan and designated *Rhodothermus obamensis*, was described by Sako *et al.* (1996). The description of this species was essentially based on 16S rRNA gene sequence analysis and on the higher cardinal growth temperatures of strain OKD7^T. *Rhodothermus obamensis* was reported to have an

The EMBL accession numbers for the 16S rDNA sequences of strains JCM 9785^T, DSM 4252^T, DSM 4253, PRQ-34, PRQ-55, NR-29 and NR-32 are AF17493–AF17499, respectively.

optimum growth temperature of 80 °C and a maximum growth temperature of about 85 °C (Sako *et al.*, 1996). The optimum growth temperature was later confirmed to be about 76 °C (Takai *et al.*, 1998). The extremely high growth temperature range of *R. obamensis* made it one of the most thermophilic bacteria described, which was particularly remarkable because this organism belongs to a phylum which, apart from *R. marinus*, contains only two other thermophilic species, both of the genus *Thermonema*, with optimum growth temperatures in the vicinity of 60 °C (Patel *et al.*, 1994; Tenreiro *et al.*, 1997).

During a study of the effect of the salt concentration of the medium and the growth temperature on the accumulation of compatible solutes by the type strains of *R. marinus* and *R. obamensis*, we noticed that we could not grow the latter strain above about 77 °C, irrespective of the medium used (Silva *et al.*, 1999). This observation lead us to perform a study using representative strains of *Rhodothermus* originating from Iceland, the island of São Miguel, Naples, the island of Monserrat and the type strains of *R. marinus* and *R. obamensis* to assess the species status of the latter. The results of DNA–DNA reassociation and 16S rRNA gene sequence comparison indicate that all the strains, including the type strain of the species *R. obamensis* OKD7^T (= JCM 9785^T), belong to the species *R. marinus*.

METHODS

Strains and growth conditions. The type strain of *Rhodothermus obamensis*, OKD7^T (= JCM 9785^T) was obtained on two separate occasions from the Japanese Collection of Microorganisms, Riken, Japan. The first culture was obtained in December 1996 for the study on osmoadaptation and the second culture was obtained in May 1998 to confirm the identity of the first culture. The lyophilized cultures were revived using Degryse medium 162 containing 1.0% (w/v) NaCl (Nunes *et al.*, 1992b) and cultured on the same medium solidified with 2.0% (w/v) agar at 65 °C. The strains were subsequently stored at –70 °C in the medium described above containing 15% glycerol. *Rhodothermus marinus* strains DSM 4252^T and DSM 4253 were obtained from the DSMZ, Braunschweig, Germany. Strain RM-25 was isolated from a marine hot spring on the island of Monserrat and kindly provided by Neil Raven (CAMR, Salisbury, UK). Strains PRQ-34 and PRQ-55 were isolated from the marine hot springs at the Praia da Ribeira Quente on the island of São Miguel in the Azores (Nunes *et al.*, 1992b); strains NR-29 and NR-32 were isolated from the Stufe di Nerone, on the Bay of Naples, Italy (Moreira *et al.*, 1996), while strain FRR-15 was recently isolated from the marine hot spring at Ferrara on the island of São Miguel in the Azores.

16S rRNA gene sequence determination and phylogenetic analysis. The extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rDNA and sequencing of the purified PCR products were carried out as described previously by Rainey *et al.* (1996). Purified sequence reaction products were electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems).

The 16S rRNA gene sequences obtained in this study were aligned against previously determined sequences available from the public databases and pairwise sequence similarity values were calculated using the ae2 editor component of the Ribosomal Database Project (RDP-II) (Maidak *et al.*, 1999). The accession numbers and strain designations of the 16S rRNA gene sequences used as reference sequences in the comparisons are: *R. marinus* strain DSM 4252^T, X80994; and *R. obamensis* strain JCM 9785^T, X95071.

DNA–DNA reassociation studies. DNA was extracted and purified by the procedure of Marmur (1961). The degree of DNA reassociation was determined spectrophotometrically from the initial renaturation rates, according to De Ley *et al.* (1970). The renaturation rates were measured in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) using a Uvikon 940 spectrophotometer (Kontron) equipped with a thermostat-controlled cuvette chamber. The optimal renaturation temperature used in each case was calculated from the G+C content (De Ley *et al.*, 1970). Each hybridization experiment was executed at least twice. The published G+C content values of Alfredsson *et al.* (1988) and Sako *et al.* (1996) were used for DNA–DNA reassociation studies.

Fatty acid analysis. Cultures for fatty acid analysis were grown on Degryse 162 medium plates, in sealed plastic bags, submerged in a water bath at 65 °C for 24 h. Fatty acid methyl esters (FAMES) were obtained from fresh wet biomass by saponification, methylation and extraction as described by Kuykendall *et al.* (1988). The fatty acid methyl esters were separated using a Hewlett Packard model 5890 gas chromatograph equipped with a flame-ionization detector fitted with a 5% phenylmethyl silicone capillary column (0.2 mm × 25 m; Hewlett Packard). The carrier gas was high-purity H₂, the column head pressure was 60 kPa, the septum purge was 5 ml min^{–1}, the column split ratio was 55:1 and the injection port temperature was 300 °C. The temperature of the oven was programmed from 170 to 270 °C at a rate of 5 °C min^{–1}. Identification and quantification of the FAMES, as well as the numerical analysis of the fatty acid profiles, were performed by using standard MIS Library Generation Software (Microbial ID).

RESULTS AND DISCUSSION

16S rRNA gene sequence comparison

Almost complete 16S rRNA gene sequences of 1471 or 1472 nt were determined for six *Rhodothermus* strains. These included the type strains of the two *Rhodothermus* species *R. marinus* and *R. obamensis* and four *Rhodothermus* strains, PRQ-34, PRQ-55, NR-29 and NR-32. Partial 16S rRNA gene sequences of 1150 nt were determined for two additional strains, FRR-15 and RM-25. The 16S rRNA gene sequence of *R. obamensis* was determined twice. The sequences determined in this study for strain JCM 9785^T were found to be identical for this strain when obtained on two separate occasions from the Japanese Collection of Microorganisms, Riken, Japan.

16S rRNA gene sequence comparisons based on a data set comprising 1502 aligned and unambiguous nucleotides between positions 38 and 1510 (*Escherichia coli* numbering; Brosius *et al.*, 1978) showed all

Table 1. 16S rRNA gene sequence similarity values for *Rhodothermus* strains

Strain	1	2	3	4	5	6	7
1. <i>R. marinus</i> DSM 4252 ^T	–						
2. <i>R. marinus</i> DSM 4253	99.5	–					
3. <i>R. marinus</i> PRQ-34	99.2	98.9	–				
4. <i>R. marinus</i> PRQ-55	99.3	99.5	98.9	–			
5. <i>R. marinus</i> NR-29	99.5	99.6	99.0	99.9	–		
6. <i>R. marinus</i> NR-32	99.3	99.3	99.2	99.6	99.7	–	
7. <i>R. obamensis</i> JCM 9785 ^T	99.5	99.5	99.1	99.5	99.7	99.7	–

Table 2. DNA–DNA reassociation values between *Rhodothermus* strains

Values are the means of at least two determinations. ND, Not determined.

Strain	Degree of DNA–DNA reassociation (%)								
	1	2	3	4	5	6	7	8	9
1. <i>R. marinus</i> DSM 4252 ^T	–								
2. <i>R. marinus</i> DSM 4253	94	–							
3. <i>R. marinus</i> PRQ-34	69	68	–						
4. <i>R. marinus</i> PRQ-55	70	ND	86	–					
5. <i>R. marinus</i> NR-29	83	ND	82	72	–				
6. <i>R. marinus</i> NR-32	81	ND	77	96	79	–			
7. <i>R. marinus</i> FRR-15	78	ND	89	92	89	89	–		
8. <i>R. marinus</i> RM-25	81	78	91	90	96	86	78	–	
9. <i>R. obamensis</i> JCM 9785 ^T	78	ND	71	78	84	70	84	81	–

sequences determined in this study to have 98.9–99.9% similarity (Table 1). 16S rRNA gene sequence similarities between strains PRQ-34, PRQ-55, NR-29 and NR-32 were 98.9–99.9%; these strains shared sequence similarities to the type strain of *R. marinus* DSM 4252^T of 99.2–99.5%. The two partial 16S rRNA gene sequences (~1150 nt) determined for strains FRR-15 and RM-25 have 98.8–100% similarity to the other sequences determined in this study. The 16S rRNA gene sequence of the previously described *R. marinus* strain DSM 4253 was shown to have 99.5% similarity to DSM 4252^T and 98.9–99.6% similarity to the other strains. Sequence similarity of 99.9% was found between the 16S rRNA gene sequence for the *R. marinus* type strain determined in this study and the sequence available from the public databases under the accession number X80994. The 16S rRNA gene sequence of *R. obamensis* strain JCM 9785^T determined in this study shared only 97.2% similarity with the previously determined sequence of the same strain available from the public databases under the accession number X95071. This newly determined sequence of strain JCM 9785^T had 99.5% similarity to the 16S rRNA gene sequence of the type strain of *R. marinus* and similarities in the range 99.1–99.7% to the other *Rhodothermus* strains used in this study. These data demonstrate a high level of 16S rRNA sequence

similarity (>98.9%) between all strains having similar characteristics to the two species of the genus *Rhodothermus*. There is a clear difference (2.8%) between the previously determined 16S rRNA gene sequence of *R. obamensis* (X95071) and that determined in this study. Close examination of the sequence deposited under accession number X95071 by alignment with other 16S rRNA gene sequences shows that it includes additional nucleotides (which may be attributed to automated base calling errors) not found within the other members of the domain *Bacteria* and a number of clearly erroneous nucleotides.

DNA–DNA reassociation data

These data demonstrate a high level of relatedness (68–96%) between the *Rhodothermus* strains for which DNA–DNA reassociation values were determined (Table 2). The results show that strain JCM 9785^T had a DNA–DNA reassociation value of 78% with the type strain of *Rhodothermus marinus* and values in the range 70–84% with the other strains studied, indicating that these strains are closely related (Table 2). Moreover, all other strains examined had high DNA–DNA reassociation values with each other and with the type strain of *R. marinus*. The lowest DNA–DNA reassociation values of 68 and 69% were observed

Table 3. Fatty acid composition of *Rhodothermus* strains grown at 65 °C

Values are the means of two determinations. Values below 0.5 % were not considered. Abbreviations: i, iso; a, anteiso; n, straight chain.

Strain*	Fatty acid composition (% of the total)										
	iC14	nC14	iC15	aC15	iC16	nC16	iC17	aC17	iC18	nC18	aC19
<i>R. marinus</i> DSM 4252 ^T	1.4	—	8.1	20.0	11.4	6.1	25.3	19.9	5.1	—	—
<i>R. marinus</i> DSM 4253	0.9	—	6.6	21.6	7.6	6.3	24.3	26.1	3.5	1.1	0.6
<i>R. marinus</i> PRQ-34	0.9	—	9.1	15.2	9.7	5.8	30.2	20.5	4.8	1.3	0.9
<i>R. marinus</i> PRQ-55	0.5	—	9.1	23.6	9.0	6.1	30.0	23.2	1.1	0.7	—
<i>R. marinus</i> NR-29	—	—	9.1	21.9	2.9	1.9	30.3	20.1	1.0	1.4	—
<i>R. marinus</i> NR-32	—	—	8.7	23.5	2.2	9.0	30.6	22.1	0.8	1.3	—
<i>R. marinus</i> FRR-15	—	0.6	12.2	22.7	4.0	10.8	29.2	17.3	0.8	0.8	—
<i>R. marinus</i> RM-25	0.6	—	8.8	23.0	6.5	6.0	29.3	22.7	1.5	—	—
<i>R. obamensis</i> JCM 9785 ^T	—	—	12.3	19.4	3.6	3.4	41.0	17.3	1.2	—	—

between strains PRQ-34, strain DSM 4253 and the type strain of *R. marinus*. However, these strains lack distinctive phenotypic characteristics (Nunes *et al.*, 1992a) and the DNA–DNA reassociation values obtained are within the range proposed for the definition of a bacterial species (Wayne *et al.*, 1987). On the basis of the DNA–DNA reassociation values obtained, all of the strains examined in this study, including *R. obamensis* strain JCM 9785^T, can be considered strains of a single species, namely *R. marinus*.

Fatty acid composition

The fatty acid composition of strains of *R. marinus* has been difficult to reproduce in replicate determinations because of the erratic growth of some of the organisms on solid media (Nunes *et al.*, 1992b; Moreira *et al.*, 1996; our unpublished results). To minimize this difficulty, all strains were grown in duplicate on the same batch of medium. The results show that the fatty acid composition of *R. obamensis* is slightly different from the other strains examined. In this organism the odd-chain/even-chain branched fatty acid ratio is higher than in the other organisms (Table 3). However, highly variable fatty acid compositions are known to occur in strains belonging to the same species. A very large variation in the fatty acid composition is found, for example, among the strains of the species of *Thermus thermophilus* that share very high DNA–DNA reassociation values (Manaia *et al.*, 1994; Nobre *et al.*, 1996). In the case of *R. obamensis* the fatty acid composition, although slightly different from the other strains examined in this study, is in congruence with the results that show all the strains examined to be genetically closely related.

Optimum growth temperature

The cardinal growth temperatures of all micro-organisms are influenced by the composition of the medium. In the study on the accumulation of com-

patible solutes by the type strains of the two *Rhodothermus* species, we utilized medium MJ, used in the description of *R. obamensis*, in parallel with modified medium 162 to grow *R. marinus* (Silva *et al.*, 1999). The results show that strain JCM 9785^T was not more thermophilic than *R. marinus* and, if anything, had a slightly lower maximum growth temperature in both media. However, strain JCM 9785^T had a slightly different NaCl requirement than the type strain of *R. marinus*, which had already been observed by Sako *et al.* (1996). The differences in the optimum growth temperature obtained by Sako *et al.* (1996) and by Silva *et al.* (1999) may be due to slightly different growth conditions and we may not have been able to completely reproduce the original growth conditions. Nevertheless, strains JCM 9785^T and the type strain of *R. marinus* were always grown in parallel on the same batch of medium.

It is sometimes not possible to compare all the type strains of genera with numerous species when a new species of that genus is characterized. We recommend that type strains of as many species as possible, especially those known to be closely related to the new isolates be used to confirm and to compare the characteristics of new isolates, with those that have been previously reported, to reveal possible differences in characteristics due to modifications of methodologies used in different laboratories. In the case of the genus *Rhodothermus*, only one species had been described and it would have been useful to use it as a control. The failure to use the type strain, and perhaps the reference strain of *R. marinus*, may have resulted in the assertion that strain JCM 9785^T had higher cardinal growth temperatures. Strain JCM 9785^T may have higher growth temperatures than those reported by Silva *et al.* (1999), because medium MJ could not be exactly reproduced or because of differences in other growth parameters, but it is almost certain that the cardinal growth temperatures of strain JCM 9785^T will not be very different from those of *R. marinus*.

Taxonomic conclusions

On the basis of the DNA–DNA reassociation values, which are well within the limits proposed for the definition of a bacterial species (Wayne *et al.*, 1987), the high level of 16S rDNA sequence similarity and the highly similar fatty acid profiles, we propose that strain JCM 9785^T (= OKD7^T) belongs to the species *R. marinus* and that the name *R. obamensis* should be regarded as a junior synonym of the name *R. marinus*.

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