

Rhodotorula pacifica sp. nov., a novel yeast species from sediment collected on the deep-sea floor of the north-west Pacific Ocean

Takahiko Nagahama,¹ Makiko Hamamoto² and Koki Horikoshi¹

Correspondence
Takahiko Nagahama
nagahama@jamstec.go.jp

¹Extremobiosphere Research Center, Japan Agency for Marine–Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

²Department of Life Sciences, School of Agriculture, Meiji University, 1-1 Higashi Mita, Tama-ku, Kawasaki 214-8571, Japan

A novel species of the genus *Rhodotorula* was isolated from sediments collected on the deep-sea floor in the north-west Pacific Ocean. Strains SY-96^T, isolated from the Yap Trench, and SY-246, isolated from the Iheya Ridge, had almost identical nucleotide sequences for their internal transcribed spacers and their 5·8S rDNA. Their physiological characteristics were also almost identical. The strains were assumed to be related to *Rhodotorula mucilaginosa* and *Rhodotorula dairenensis* based on sequence similarities in the D1/D2 region of the 26S rDNA. The low DNA–DNA relatedness and sequence similarity between strain SY-96^T and related species revealed that strains SY-96^T and SY-246 represent a hitherto unknown species. As ballistoconidia and sexual reproduction were not observed in strains SY-96^T and SY-246, these strains are described as *Rhodotorula pacifica* sp. nov. The type strain is SY-96^T (=JCM 10908^T = CBS 10070^T).

We have previously investigated yeast distribution in deep-sea environments in the north-west Pacific Ocean (Nagahama *et al.*, 2001a) and found red yeast strains belonging to the *Erythrobasidium* clade or the Sporidiobolales in sediments and benthic animals such as tubeworms or giant white clams. So far, four novel species have been described for isolates of the *Erythrobasidium* clade (Nagahama *et al.*, 2001b, 2003). Most deep-sea Sporidiobolales strains were identified as anamorphic strains of *Rhodospiridium diobovatum* and members of a cluster with *Rhodospiridium sphaerocarpum* and *Rhodotorula mucilaginosa*, based on the internal transcribed spacer (ITS) and 5·8S rDNA sequences. Of the *Rhodotorula mucilaginosa*-related strains, it appeared possible that strains SY-96^T, SY-100 and SY-101, found in sediment from the northern Yap Trench, represented undescribed species (Nagahama *et al.*, 2001a). More recently, strain SY-246, similar to strain SY-96^T, was collected at a depth of 991 m from the Iheya Ridge, off Japan.

Recently, *Rhodotorula glutinis* var. *dairiensis* was elevated to the *Rhodotorula* species *Rhodotorula dairenensis* (Gadanhó & Sampaio, 2002) which is the species most closely related to *Rhodotorula mucilaginosa*. We examined deep-sea isolates of this species to determine the phylogeny of the 26S rDNA and ITS sequences, DNA–DNA relatedness and physiological characteristics and found that strains SY-96^T and SY-246 represent a novel species of the genus *Rhodotorula*, named *Rhodotorula pacifica* sp. nov.

Sample collection and yeast isolation

SY-96^T, SY-100 and SY-101 were isolated from sediment collected at a depth of 3702 m on the northern Yap Trench (11° 46·303' N 139° 07·300' E; 1·7 °C) by means of a sampling system which prevents contamination by open water, as previously described (Nagahama *et al.*, 2001a). At this sampling site, the deep-sea benthos was scarce, as in the majority of deep-sea floors in the Pacific Ocean. *Rhodotorula mucilaginosa* and the anamorph of *Rhodospiridium diobovatum* also appeared in the same sediment sample. Following the same procedure, strain SY-246 was isolated from sediment collected at a depth of 991 m on the Iheya Ridge (27° 27·240' N 126° 53·892' E; 4·7 °C). This sample was collected near a fertile spot teeming with macrobenthos, such as clams, crabs and tubeworms, integrated in a deep-sea hydrothermal ecosystem in contrast to the largely deserted ocean floor at the northern Yap Trench. In this sample,

Published online ahead of print on 9 September 2005 as DOI 10.1099/ijs.0.63584-0.

Abbreviations: ITS, internal transcribed spacer; ML, maximum-likelihood; TBR, tree bisection-reconnection.

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA D1/D2 domain, the internal transcribed spacer and the 5·8S rDNA gene sequences of *Rhodotorula pacifica* sp. nov. strains SY-96^T and SY-246 are AB026006 and AB193175, respectively.

anamorphs of *Rhodosporidium diobovatum*, *Rhodosporidium sphaerocarpum* and *Rhodosporidium toruloides* were found with strain SY-246.

Physiological and biochemical characteristics

The strains were characterized morphologically and physiologically using standard methods with some modifications (Yarrow, 1998). Assimilation of nitrogen compounds was examined on solid media using a starved inoculum (Nakase & Suzuki, 1986). Vitamin requirements were investigated according to the method of Komagata & Nakase (1967). Ubiquinones were extracted according to Yamada & Kondo (1973), with slight modifications, and determined by HPLC as described previously (Hamamoto & Nakase, 1995).

Nucleic acid analyses

DNA extraction and purification for the analysis of DNA G+C content and DNA–DNA relatedness were performed following the procedure described by Hamamoto & Nakase (1995), with slight modifications. The DNA G+C content was determined using the HPLC method of Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were performed using fixed DNA microplates (Ezaki *et al.*, 1989). As a representative of *Rhodotorula dairenensis*, CBS 4406^T was replaced by strain SY-100 due to a procedural problem in these experiments. Strains CBS 4406^T and SY-100 were identical, according to the nucleotide sequences of the ITS, 5·8S rDNA and the D1/D2 region. Strains *Rhodotorula mucilaginosa* JCM 8115^T, *Rhodosporidium sphaerocarpum* JCM 8202^T and *Rhodotorula* sp. JCM 8164 (=IGC 5597) were used for the analyses.

Phylogenetic analysis

DNA extraction for PCR was performed using a QIAamp DNeasy tissue kit (Qiagen), with some modifications (Nagahama *et al.*, 2001b). The primers used for amplification and sequencing of the 5·8S rDNA and ITS regions were those described by White *et al.* (1990); the primers for the D1/D2 region of the 26S rDNA were those described by Fell *et al.* (2000).

All sequences were aligned using CLUSTAL W 1.81 (Thompson *et al.*, 1994) and optimized manually on a sequence alignment editor, SE-AL version 1.0α1 (Rambaut, 1996). Positions where one or more species contained a length mutation or ambiguously aligned regions were not included in the subsequent phylogenetic analysis.

Nucleotide sequence phylogenies were derived using PAUP* 4.0b10 (Swofford, 2003). Maximum-likelihood (ML) analyses (Felsenstein, 1981) were performed using heuristic searches with random stepwise addition of 100 replicates and tree bisection-reconnection (TBR) rearrangements. The optimal model of nucleotide evolution for the ML analyses was determined using hierarchical likelihood ratio tests as implemented in MODELTEST 3.06 (Posada & Crandall, 1998). The model selected as the best fit for the 26S rDNA dataset was TrN + G. For the bootstrap analyses (Felsenstein, 1985), 250 replicates were generated with five random additions and TBR.

Phylogenetic positions of deep-sea yeast strains related to *Rhodotorula mucilaginosa* and proposal of *Rhodotorula pacifica* sp. nov.

We have previously sequenced a region comprising the ITS 1, 5·8S rDNA and ITS 2 of red yeast strains isolated from deep-sea environments (Nagahama *et al.*, 2001a). Of strains SY-96^T, SY-100 and SY-101, which are related to *R. mucilaginosa* but are rather distant from its type strain described in the previous study, the latter two had identical ITS sequences. Therefore, we sequenced the D1/D2 regions of the 26S rDNA of SY-96^T and SY-100 and a region comprising the ITS, 5·8S rDNA and D1/D2 of SY-246. An ML tree of the strains of *Rhodotorula mucilaginosa* and related species was generated (Fig. 1). All strains, except the two strains treated as an outgroup, were distributed into five lineages (in bold in Fig. 1), which were considered to represent separate species. The bootstrap values for the lineages in this tree were somewhat low, but were improved by using a combined dataset involving the ITS and 5·8S rDNA sequences (data not shown).

The D1/D2 and ITS sequences of strain SY-100, which was isolated from the Yap Trench, were almost identical to the sequences of *Rhodotorula dairenensis* CBS 4406^T and

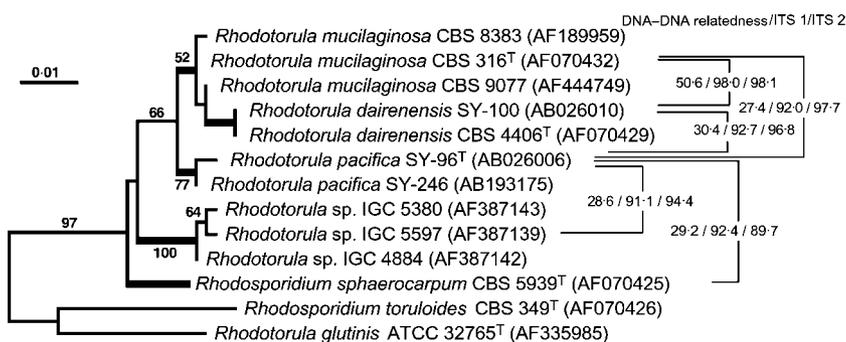


Fig. 1. Phylogenetic relationships between the strains of *Rhodotorula pacifica* and related species, based on the nucleotide sequences of the D1/D2 region of the 26S rDNA. The ML tree was constructed as described in the text. Numbers are the bootstrap values for the nodes supported by >50% (250 replicates). Bar, 0·01 substitutions per site. The DNA–DNA relatedness and nucleotide similarities in ITS 1 and ITS 2 between strain SY-96^T and some other strains are shown to the right of the strain names in the tree.

therefore this strain represents a new isolate of this species. As seen in Fig. 1, *Rhodotorula dairenensis* is closely related to *Rhodotorula mucilaginosa*; the difference between their D1/D2 sequences amounted to about 0.7% (four mismatches) and the difference between the ITS sequences was about 2%. Among the other *Rhodotorula mucilaginosa* strains, CBS 9077 was the closest to *Rhodotorula dairenensis* (Fig. 1), with a 0.5% difference (three mismatches) in the D1/D2 sequences. The DNA–DNA relatedness value between *Rhodotorula mucilaginosa* JCM 8115^T and *Rhodotorula dairenensis* SY-100 was 50.6%, whereas previously published studies have reported values of 66% (Hamamoto *et al.*, 1987) and 10–20% (Gadanhó & Sampaio, 2002) for these species. This may be due to procedural differences in the experiments or to differences in the strains used in the three studies. Of the three species used in this study, *Rhodotorula dairenensis* was most closely related to *Rhodotorula mucilaginosa*, although *Rhodotorula mucilaginosa* was distinguished from the other two species by an inability to use nitrate, unlike most of the species in the order Sporidiobolales.

Strain SY-96^T from the Yap Trench and strain SY-246 from the Iheya Ridge were very closely related and were placed near the cluster with *Rhodotorula mucilaginosa* and *Rhodotorula dairenensis*. Although strains SY-96^T and SY-246 showed a 0.4% difference (two mismatches) in the D1/D2 region nucleotide sequence, we estimated them to be identical because they showed only 0.3% difference (only one mismatch in ITS 2) in the ITS regions and no significant morphological, physiological or biochemical differences. We examined the DNA–DNA relatedness and nucleotide sequence similarities in both ITS 1 and ITS 2 between strain SY-96^T and other species. The low values, e.g. a DNA–DNA relatedness value of <30% and nucleotide sequence similarities of ITS 1 and ITS 2 of <95%, as shown in Fig. 1, confirmed that strains SY-96^T and SY-246 represent a novel species. Differences of <1% in the D1/D2 region or of 1–2% in the ITS regions are generally recognized to correspond to the borderline between conspecificity and species separation (Fell *et al.*, 2000; Fonseca *et al.*, 2000; Bai *et al.*, 2001a, b; Hamamoto *et al.*, 2002; Nagahama *et al.*, 2003). As ballistoconidia and sexual reproduction were not observed in strains SY-96^T and SY-246, a novel species, *Rhodotorula pacifica*, is described in the genus *Rhodotorula*.

Rhodotorula sp. strains IGC 4884, IGC 5380 and IGC 5597 (=JCM 8164) were also sufficiently separated from recognized species by considerable evolutionary distances, as previously reported in analyses using D1/D2 sequences (Gadanhó & Sampaio, 2002) and ITS sequences (Nagahama *et al.*, 2001a).

Comparison of physiological and biochemical characteristics between strains of *Rhodotorula pacifica* and related species

Some strains of *Rhodotorula dairenensis* were formerly classified as *Rhodotorula glutinis* on the basis of their ability to assimilate nitrate and nitrite. This ability is an important

criterion in distinguishing *Rhodotorula pacifica* and *Rhodotorula dairenensis* from *Rhodotorula mucilaginosa* (Gadanhó & Sampaio, 2002). It is not easy to discriminate *Rhodotorula pacifica* from *Rhodotorula dairenensis* in terms of phenotypic differences. We can only point out that L-rhamnose and galactitol are weakly assimilated as sole carbon sources by *Rhodotorula pacifica* but not by *R. dairenensis* and that 2-ketogluconate is slowly or weakly assimilated only by *Rhodotorula dairenensis*. No significant differences between these two species were observed in vegetative cells using optical microscopy (Fig. 2). Strains SY-96^T and SY-246 of *Rhodotorula pacifica* were almost identical in terms of their physiological characteristics but had different maximum growth temperatures: SY-246 grew at 37 but not at 40 °C, whereas SY-96^T grew at 35 but not at 37 °C. The habitat of *Rhodotorula pacifica* cannot be established with certainty as only two strains are known. The strains were found in geographically distant deep-sea-floor sites in the Pacific Ocean.

Latin diagnosis of *Rhodotorula pacifica* Nagahama et Hamamoto sp. nov.

In medio liquido YM post 3 dies ad 25 °C, cellulae ovoideae vel ellipsoideae (2–4 × 3–6 µm), singulae aut binae. Post unum mensem annulus tenuis et sedimentum formantur. Cultura in agaro YM ad 25 °C, subrosea vel subaurantiaca, nitida, mollis vel mucosa et margine glabra. Hyphae et pseudohyphae non formantur. Fermentatio nulla. Glucosum, galactosum, L-sorbosum (exiguum), sucrosus, maltosum, cellobiosum, trehalosum, raffinosum, melezitosum, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum (vel exiguum), ethanolum, glycerolum, ribitolium, galactitolium, D-mannitolium, D-glucitolium (vel exiguum), methyl α-D-glucosidum, salicinum, glucono-δ-lactonum, acidum DL-lacticum (exiguum), acidum succinicum, acidum citricum et acidum D-galacturonicum assimilantur, at non melibiosum, lactosum, inulinum, amyllum solubile, erythritolum, inositolium, acidum

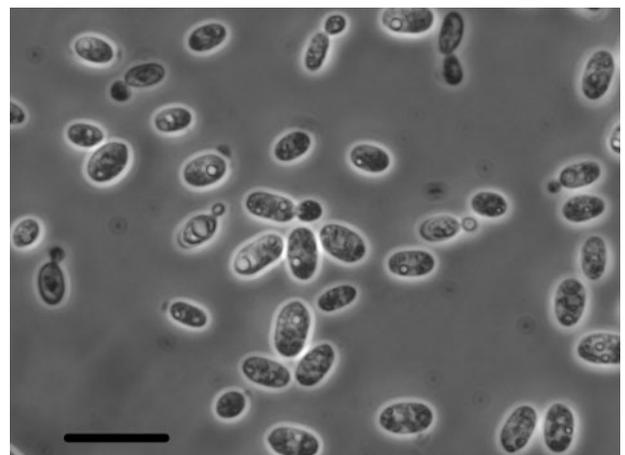


Fig. 2. Cells of *Rhodotorula pacifica* sp. nov. SY-96^T on YM agar after 3 days of culture. Bar, 10 µm.

2-ketogluconicum, *acidum* 5-ketogluconicum nec acidum D-glucuronicum. *Kalium nitricum*, *natrium nitrosum*, *ethylaminum*, *lysinum* et *cadaverinum* assimilantur. *Thiamin necessaria ad crescentiam*. G+C *acidi deoxyribonucleati* 57.5–57.8 mol% (per HPLC). *Ubiquinonum majus* Q-10.

Typus stirps SY-96^T ex *sedimentum*, *fossa* Yap, *Oceanus Pacificus*, *isolata est*. In *collectionibus culturarum* quas Japan Collection of Microorganisms, Wako, Saitama *sustentant*, JCM 10908^T (= CBS 10070^T) *deposita est*.

Description of *Rhodotorula pacifica* Nagahama & Hamamoto sp. nov.

Rhodotorula pacifica (pa.ci'fi.ca. L. fem. adj. *pacifica* peace-making, *pacific*, and by extension, referring to the Pacific Ocean).

In YM broth (Difco) after 3 days culture at 25 °C, cells are ovoidal to ellipsoidal (2–4 × 3–6 µm) and occur singly or in parent–bud pairs (Fig. 2). A sediment and thin ring are formed after 1 month. After 1 month on YM agar at 25 °C, streak culture is light pink to light orange, glistening, soft to slimy and has a complete margin. In Dalmau plate cultures on cornmeal agar (Difco), no branching hyphae or pseudo-hyphae are formed. Fermentation ability is negative. The following carbon compounds are assimilated: D-glucose, galactose, L-sorbose (weak), sucrose, maltose, cellobiose, trehalose, raffinose, melezitose, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose (or weak), ethanol, glycerol, ribitol, galactitol, D-mannitol, D-glucitol (or weak), methyl α-D-glucoside, salicin, glucono-δ-lactone, DL-lactic acid (weak), succinic acid, citric acid and D-galacturonic acid (weak); no growth occurs on melibiose, lactose, inulin, soluble starch, erythritol, inositol, 2-ketogluconic acid, 5-ketogluconic acid or D-glucuronic acid. The nitrogen compounds potassium nitrate, sodium nitrite, ethylamine, lysine and cadaverine are assimilated. Growth occurs at 35 but not at 40 °C. Thiamin is required for growth. No growth occurs on 50 % glucose/yeast extract agar or in the presence of 100 p.p.m. cycloheximide. Growth occurs in the presence of 10 % sodium chloride. No starch-like substances are produced. Diazonium blue B reaction is positive. Urease activity is positive. The nuclear DNA G + C content is 57.5–57.8 mol% (by HPLC). The major ubiquinone is Q-10.

The type strain, SY-96^T (= JCM 10908^T = CBS 10070^T), was isolated from sediments collected from the deep-sea floor in the Yap Trench, Pacific Ocean.

Acknowledgements

We thank Dr Y. Nogi for his useful suggestions regarding technical problems.

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