Rhodotorula arctica sp. nov., a basidiomycetous yeast from Arctic soil

H. S. Vishniac¹ and M. Takashima²

Correspondence

H. S. Vishniac

helen.s.vishniac@okstate.edu

¹Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK 74075, USA

²Japan Collection of Microorganisms, RIKEN BioResource Center, Hirosawa 2-1, Wako, Saitama 351-0198, Japan

Three isolates, typified by Pro94 Y29^T (=JCM 13290^T =CBS 9278^T =DBVPG 7841^T), that represent a novel species, *Rhodotorula arctica* sp. nov., were studied. *R. arctica* differed from its only close relative, *Bensingtonia yamatoana*, by requiring thiamine and by failing to assimilate maltose and quinate, but strain Pro94 Y29^T could be most readily identified using the rDNA sequence of the LSU D1/D2 region, which differed from that of *B. yamatoana* CBS 7423^T at four positions, and the ITS sequence, which differed at nine positions. One *R. arctica* isolate, Pro94 Y56 (=JCM 13292 =CBS 9280 =DBVPG Y7843), was unique in requiring either L-arginine or L-citrulline as a source of nitrogen.

A previously published latitudinal survey of soil yeasts included undescribed yeasts from sites in Beringia, the area from which the earliest Americans infiltrated the North American continent; D1/D2 sequences of a cluster of three isolates, represented by Pro94 Y29^T (GenBank accession no. AY204483) and referred to as *Bensingtonia* cf. *yamatoana*, were similar. This cluster, comprising Pro94 Y29^T, the representative and designated type strain, Pro94 Y49 and Pro94 Y56, has been examined further and the strains are here described as representatives of a novel species most closely related to *Bensingtonia yamatoana*. The strain designation 'Pro' refers to the origin of all three strains from soil in the vicinity of Providenya, Russian Far East. Canonical correspondence analysis located this site slightly to the side of Principal Component Axis 1 and slightly below the median of Axis 2, reflecting a mean annual temperature of -5.16°C , rainfall of $71.23\text{ cm year}^{-1}$, net primary production of approximately $230\text{ g C m}^{-2}\text{ year}^{-1}$ and electrical conductivity of 1272 mmho cm^{-1} (1.27 S cm^{-1}) (Vishniac, 2006).

A general description of methods used to isolate Pro94 Y29^T, Pro94 Y49 and Pro94 Y56 is given by Vishniac (2006). More specifically, after the soil sample had been returned under refrigeration to Oklahoma, USA, isolates were obtained by direct plating on M3C agar spread with an antibiotic mixture (on which this group of phenotypically similar isolates constituted over 21 % of soil sample contents) or by enation in liquid M3C plus antibiotics (on which such isolates were nearly 31 % of the soil sample

contents). They constituted one of the two dominant species in this soil sample. The choice of exemplary isolates was based on differences in biotype.

Morphology was examined after growth in YM broth and by using conventional methods for growth on cornmeal agar and YM agar (Kurtzman & Fell, 1998; Barnett *et al.*, 2000). Sexual reproduction was assessed after mixing strains manually on YM agar at 17°C .

Physiological profiles were produced using conventional methods (Kurtzman & Fell, 1998; Barnett *et al.*, 2000) and by the methods of Vishniac (1985), modified by the addition of $10\text{ }\mu\text{M NiSO}_4$ to Y-2 mineral base and the use of $2\text{ mM L-arginine hydrochloride}$ as a nitrogen source where necessary. Strain Pro94 Y56 was unable to grow in conventional Wickerham's medium.

Grading growth in both liquid and solid media can be complicated by the presence in laboratory air of both carbon sources (ethanol, propane-1,2-diol) and nitrogen sources (ammonia) utilized by most of the yeasts studied. The use of commercial agar as a solid substrate introduces the additional complication of utilizable substances staining brown with I_2/KI ; these strains cleared the agar around their colonies for about 5 mm. Because of these factors, we have considered responses, whether in the liquid media of conventional tests or on multipoint-inoculated agar media, to be positive only if growth is not only significantly greater than in the negative controls but also continues, albeit slowly, over time. Liquefaction of 12 % gelatin (Knox) and digestion of casein (5 % non-fat dry milk) were examined by comparing growth using Vishniac's base without other nitrogen sources and with $2\text{ mM L-arginine hydrochloride}$ added (positive control).

The GenBank/EMBL/DDBJ accession numbers for the ITS and D1/D2 region sequences of strain Pro94 Y29^T are AB478857 and AB478858, respectively.

For sequencing and phylogenetic analysis, nuclear DNA was extracted using PrepMan Ultra Reagent (Applied Biosystems). The ITS regions, including the 5.8S rDNA, were amplified by PCR according to Sugita & Nakase (1999). The D1/D2 domain of LSU rDNA was amplified following Kurtzman & Robnett (1997). PCR products were sequenced directly using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and analysed with an Applied Biosystems sequencer model 3100 according to the manufacturer's instructions. Reference sequences used for the phylogenetic study were obtained from GenBank/EMBL/DDBJ (Fig. 1). Sequences were aligned with those of related strains using the program CLUSTAL W version 1.83 (Thompson *et al.*, 1994) and the alignment was adjusted manually. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). Evolutionary distances were calculated according to Kimura (1980). Sites where gaps existed in any sequence were excluded. Bootstrap analyses (Felsenstein, 1985) for the neighbour-joining method were performed from 100 random resamplings.

The ITS and D1/D2 regions of LSU rDNA sequences were identical for all three strains. These sequences were similar to those of the *B. yamatoana* CBS 7243^T D1/D2 (accession no. AF189896; shown in Fig. 1a) and ITS (accession no. AF444634; shown in Fig. 1b) regions, but differed in four positions (including two gaps) and nine positions (including six gaps), respectively. This degree of difference is

generally considered to indicate lack of conspecificity; accordingly, we consider these strains as representatives of a novel species, described below.

Cells and rudimentary pseudohyphae of Pro94 Y29^T are illustrated in Fig. 2. Differences in physiological profiles between the novel isolates and *B. yamatoana* are shown in Table 1.

In ascribing this novel species to the genus *Rhodotorula*, it has been relegated to a very heterogeneous taxon for want of a better solution. Since it lacks ballistoconidia, it should not be described as a member of the genus *Bensingtonia*, which is also not monophyletic; *B. yamatoana* is, in any case, not closely related to the type species of *Bensingtonia* (Sampaio *et al.*, 2003; Wang *et al.*, 2006), but is closer to certain *Rhodotorula* and *Sporobolomyces* species. This clade (of the *Agaricostilbum* lineage) has been most recently included in Eukaryota, Fungi, Dikarya, Basidiomycota, Pucciniomycotina, Agaricostilbomycetes, Agaricostilbomycetes *incertae sedis*, mitosporic Agaricostilbomycetidae (Scorzetti *et al.*, 2002; Hibbett *et al.*, 2007). *R. arctica* sp. nov. can be most readily identified by its characteristic sequences, but does exhibit physiological differences both within the species and in comparison with *B. yamatoana* (Table 1).

The most unusual, indeed unique, feature of strains of *R. arctica* sp. nov. is the requirement of Pro94 Y56 for specific organic nitrogen sources. Its inability to use any of

Fig. 1. Neighbour-joining trees of *Rhodotorula arctica* sp. nov. Pro94 Y29^T (=JCM 13290^T) and related species based on the D1/D2 domain of LSU rDNA sequences (a) and the ITS region, including 5.8S rDNA sequences (b). Bars, evolutionary distance of 0.02 (a) and 0.01 (b) (calculated according to Kimura, 1980). Numbers at branch-points are from 100 replicate bootstrap samplings (frequencies less than 50% are not shown). Sequence accession numbers are given for each strain.

Fig. 2. Yeast cells (a) and segmented filaments (b) of *R. arctica* Pro94 Y29^T from sediment after 3 days in YM medium at 25 °C. Bars, 10 µm.

the conventional inorganic nitrogen sources as a sole nitrogen source results in failure to grow in Wickerham's medium. Pro94 Y56 responded to as little as 0.1 mM L-arginine hydrochloride, growing maximally at 2.0 mM; 2.0 mM L-citrulline was equally effective, but L-asparagine, L-glutamine, L-histidine, L-lysine, L-methionine and L-ornithine were not. Such a requirement might be satisfied by hydrolysis of proteins such as gelatin or casein (which do contain enough arginine to satisfy such a requirement), but none of the novel isolates liquefied gelatin (Knox) or cleared casein (non-fat dry milk). Pro94 Y56 was unable to grow using these proteins without the addition of

Table 1. Distinguishing characteristics of strains of *R. arctica* sp. nov. and *B. yamatoana*

Strains: 1, Pro94 Y29^T; 2, Pro94 Y49; 3, Pro94 Y56; 4, *B. yamatoana* CBS 7243^T (data from the CBS database). The degree of growth is indicated (in increasing magnitude) as clearly negative (0), failing to continue yet not clearly negative (NS; not significant compared with negative control), still poor after 3 weeks compared with the positive control (DW; delayed and weak), delayed beyond a week (D), weak (w) or clearly positive within 1 week (+). ND, No data available.

Characteristic	1	2	3	4
Growth on nitrogen sources				
NH ₄ Cl	+	+	0	+
Ethylamine	+	+	0	w/+
L-Asparagine	+	+	0	ND
L-Glutamine	+	+	0	ND
L-Lysine	+	+	0	w
Growth on carbon/energy sources				
Cellobiose	0	+	+	0
Glucitol	D	DW	DW	0/+
Maltose	NS/DW	NS/DW	0	+
Quinate	0	0	0	+
Growth in the absence of thiamine	0	0	0	+

L-arginine, although the commercial sources supplied sufficient utilizable nitrogen for growth of the other strains.

Latin diagnosis of *Rhodotorula arctica* Vishniac & Takashima sp. nov.

In liquido YM, post dies 3 ad 25 °C cellulae subglobosae, elongatae aut cylindricae (2.0–9.5 × 6.3–15.0 µm), singulae, binae, catenatae, vel fasciculatae; nonnumquam mycelia septata in sedimentis sed mycelium et pseudomycelium non aliter formantur. Post unum mensem ad 17 °C annullus fragilis, insulae et sedimentum formantur. *In agar* YM, post unum mensem ad 17 °C cultura xanthoalba, glabra aut rugoscula, subnitida, mollis et margo glabra. Non generant sed per modo blastico asexuali. Generant cum calore maxime maiore quam ca. 23–25 °C sed minore quam 30 °C. Amylosum non formatur est. Ureum finditur. Non fermentant. L-Arginino et L-citrullino utuntur (et nonnumquam chlorido ammoniae, L-asparagino, ethylamino, L-glutamino, L-lysino) sed non-cadaverino, creatinino, acido L-glutamico, D-lysino, kalio nitrati. Cellobiosum (nonnumquam), ethanolum, D-glucitolum (lente), glucosum, glucono-D-lactonum, acidum D-gluconicum, acidum D-glucuronicum, glycerolum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, D-mannitolum, melezitolum, acidum mucicum (nonnumquam lente), propane-1,2-diolum (nonnumquam lente), acidum protocatechuicum, acidum succinum (lente), sucrosum, trehalosum (lente), xylitolum (nonnumquam lente) assimilantur; non assimilantur (aut lentissime, sine significatione) N-acetyl-D-glucosaminum, amyllum solubile, L-arabinitolum, L-arabinosum, D-arabinosum, arbutinum, butane-2,3-diolum, acidum

citricum, *erythritolum*, *galactitolum*, *galactosum*, *acidum* D-*galacturonicum*, D-*glucosaminum*, *hexadecanum*, *inositolum*, *inulinum*, *methanolum*, *methyl* α -D-*glucosidum*, *acidum* DL-*lacticum*, *lactosum*, *maltosum*, *melibiosum*, *acidum* *quinicum*, *raffinoseum*, L-*rhamnosum*, *ribitolum*, D-*ribosum*, *acidum* *saccharicum*, *salicinum*, L-*sorbosum*, D-*xylosum*. *Non crescit sine thiamineo necque cum cyclohexamino* 0.1 aut 0.01 %. *Teleomorphosis ignota*.

Holotypus: isolatio Pro94 Y29^T ex *Providenio* in *collectionibus* *culturarum* ut JCM 13290^T quas 'Japan Collection of Microorganisms, RIKEN', Wako, Saitama, *Japonia conservata*, in *collectionibus* *culturarum* ut CBS 9278^T quas 'Centraalbureau voor Schimmelcultures', *Trajectum ad Rhenum*, *Hollandia conservata*, item in *collectionibus* *culturarum* ut DBVPG 7841^T quas 'Dipartimento di Biologia Vegetale e Biotecnologia Agroambientale', *Perugia, Italia conservata*.

Description of *Rhodotorula arctica* Vishniac & Takashima sp. nov.

Rhodotorula arctica (arc'ti.ca. L. fem. adj. *arctica* northern, Arctic, referring to the geographical area from which the first strains were isolated).

After 3 days of growth in liquid YM at 25 °C, cells are subglobose to elongate or cylindrical (2.0–9.5 × 6.3–15.0 µm), occurring singly or in pairs, chains or fascicles. After a month at 17 °C, a scanty annulus, some clumps and sediment are produced; a few septate hyphae are occasionally noted in the sediment, but neither hyphae nor pseudohyphae are otherwise produced. After 1 month on YM agar at 17 °C, colonies are cream-coloured, smooth or slightly rugulose, soft and not quite matt, with entire margins. Budding is monopolar; sexual reproduction has not been observed. The maximum temperature allowing growth is 23–25 °C (depending on strain) but less than 30 °C. Amylose is not produced. Urea is split. Fermentation does not occur. L-Arginine hydrochloride and L-citrulline are utilized as sole sources of nitrogen, but only Pro94 Y29^T and Pro94 Y49 can utilize ammonium chloride, L-asparagine, ethylamine, L-glutamine and L-lysine; cadaverine, creatinine, L-glutamate, D-lysine and potassium nitrate are not utilized. Cellobiose (variably), ethanol, D-glucitol (slowly and variably), D-glucose, gluconolactone, gluconic acid, glucuronate, glycerol, 2-ketogluconic acid, 5-ketogluconic acid, D-mannitol, melezitose, mucic acid (slowly and variably), propane-1,2-diol (sometimes slowly), protocatechuate, succinate (slowly and variably), sucrose, trehalose and xylitol (slowly and variably) are available as sole sources of carbon and energy. N-Acetyl-D-glucosamine, L-arabinitol, L-arabinose, D-arabinose, arbutin, butane-2,3-diol, citrate, erythritol, galactitol, galactose, D-galacturonate, D-glucosamine, hexadecane, *myo*-inositol, inulin, methanol, methyl α -D-glucoside, DL-lactate, lactose, maltose, melibiose, quinate, raffinose, L-rhamnose, ribitol, D-ribose, saccharate, salicin, soluble starch, L-sorbose and D-xylose are not, or not

significantly, available as sole sources of carbon and energy. Thiamine is required for growth. Cyclohexamine is inhibitory at both 0.1 and 0.01 %. No teleomorph is known.

The type strain is Pro94 Y29^T (=JCM 13290^T =CBS 9278^T =DBVPG 7841^T), isolated from Arctic soil. Strains Pro94 Y49 (=JCM 13291 =CBS 9279 =DBVPG 7842) and Pro94 Y56 (=JCM 13292 =CBS 9280 =DBVPG 7843), from the same source, are additional strains of the species.

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