***Cryptococcus statzelliae* sp. nov. and three novel strains of *Cryptococcus victoriae*, yeasts isolated from Antarctic soils**

Skye Thomas-Hall,¹ Kenneth Watson¹ and Gloria Scorzetti²

¹ School of Biological Sciences, University of New England, Armidale, NSW 2351, Australia

² Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Key Biscayne, FL, USA

Author for correspondence: Skye Thomas-Hall. Tel: +61 2 6773 2729. Fax: +61 2 6773 3267. e-mail: sthomas2@metz.une.edu.au

A morphological and physiological characterization of yeast strains CBS 8908, CBS 8915, CBS 8920, CBS 8925^T and CBS 8926, isolated from Antarctic soils, was performed. Phylogenetic analyses of the sequences of the D1/D2 regions and the adjacent internal transcribed spacer (ITS) regions of the large-subunit rDNA of these strains placed them into the Tremellales clade of the Hymenomycetes. The sequence data identified strains CBS 8908, CBS 8915 and CBS 8920 as belonging to the species *Cryptococcus victoriae*. Strains CBS 8925^T and CBS 8926 were found to represent an unique clade within the Hymenomycetes, with *Dioszegia crocea* CBS 6714^T being their closest phylogenetic relative. Fatty acid composition and proteome fingerprint data for these novel strains were also obtained. No sexual state was observed. A novel basidiomycetous species, *Cryptococcus statzelliae*, is proposed for strains CBS 8925^T and CBS 8926.

Keywords: *Cryptococcus statzelliae* sp. nov., rDNA sequencing, fatty acids, PAGE, scanning electron microscopy

INTRODUCTION

A survey of yeasts in soil and snow samples from Antarctica resulted in the collection of over 500 isolates. Two morphologically similar isolates with red colonies, CBS 8925^T and CBS 8926, were shown to be closely related by PAGE analysis of whole-cell proteins. Phylogenetic analyses of the D1/D2 and internal transcribed spacer (ITS) regions of the large-subunit rDNA of these strains confirmed their relatedness and showed them to represent a novel species. Based on carbon assimilation data, the absence of fermentation, positive Diazonium blue B and urease reactions, production of starch-like compounds and the lack of an inducible sexual state or the formation of ballistoconidia, these isolates were classified as be-

longing to the genus *Cryptococcus* (Kurtzman & Fell, 1998). The two strains, CBS 8925^T and CBS 8926, are described in this communication as *Cryptococcus statzelliae* sp. nov., in honour of Adele Statzell-Tallman, a yeast biologist at the University of Miami, FL, USA.

Three yeast isolates, CBS 8908, CBS 8915 and CBS 8920, were shown by phylogenetic analyses of the D1/D2 and ITS regions of their large-subunit rDNA to be closely related to the type strain of *Cryptococcus victoriae*, CBS 8685^T. PAGE proteome fingerprinting of these strains showed them to be very similar, thus supporting the relationship between them. On the basis of these data and the results of carbon-assimilation tests, CBS 8908, CBS 8915 and CBS 8920 are presented as variant strains of *C. victoriae*.

METHODS

Isolation techniques. The yeast strains used in this study can be found in Table 1. Soil and snow samples were collected from Vestvold Hills, Davis Base, Antarctica, in December 1997. Samples were stored at -10 °C in quarantine and screened for yeasts in April 1998. Soil samples (0.1 g) were added to YEP (yeast extract/peptone/2% glucose) broth containing 250 µg streptomycin ml⁻¹ and 500 µg ampi-

Published online ahead of print on 21 June 2002 as DOI 10.1099/ijs.0.02293-0.

Abbreviation: ITS, internal transcribed spacer.

The GenBank accession numbers for the sequences of the D1/D2 domains of *C. statzelliae* CBS 8925^T, *C. statzelliae* CBS 8926, *C. victoriae* CBS 8920, *C. victoriae* CBS 8915 and *C. victoriae* CBS 8908 are AY029341, AY029344, AY040650, AY040652 and AY040653, respectively. The GenBank accession numbers for the ITS sequences of strains CBS 8925^T, CBS 8926, CBS 8908, CBS 8915 and CBS 8920 are AY029342, AY029343, AY040654, AY040655 and AY040656, respectively.

Table 1. Yeast strains examined in this study

All strains were isolated from Antarctica, except for *D. crocea*. Lichen Valley, 68° 29' S 78° 25' E; Echo Lake, 68° 31' S 78° 16' E.

Details	<i>D. crocea</i> CBS 6714 ^T	<i>C. statzelliae</i> CBS 8925 ^T	<i>C. statzelliae</i> CBS 8926	<i>C. victoricae</i>			
				CBS 8685 ^T	CBS 8908	CBS 8915	CBS 8920
Substrate	Strawberry	Soil	Soil	Soil	Soil	Soil	Soil, lichen
Source/isolated from	UK	Lichen Valley	Echo Lake	Victoria Land	Lichen Valley	Lichen Valley	Lichen Valley
ITS accession no.	–	AY029342	AY029343	–	AY040654	AY040655	AY040656

cillin ml⁻¹; the samples were incubated at 10 °C. Serial dilutions of the broth cultures were inoculated onto YEP/streptomycin/ampicillin agar plates at 0, 5 and 10 days post-inoculation, and the plates were incubated at 10 °C until colony growth was visible. Yeast isolates were then streaked onto YEP agar plates.

Morphology. Cell size and morphology were determined by scanning electron microscopy (JEOL model JSM 5800LV, accelerating voltage 15 kV) using an improved fixation procedure for yeasts (Hanschke & Schauer, 1996).

Physiological tests. Physiological tests were performed in duplicate according to the protocols described by Yarrow (1998).

Protein extraction. Cells in the exponential growth phase (YEP broth at 15 °C for 3–5 days) were pelleted by centrifugation at 2000 g for 5 min, washed once with distilled water (dH₂O) and then centrifuged. To the pellet was added 150 µl ice-cold lysis buffer (0.1% Triton X-100, 100 mM KCl, 8 mM MgCl₂, 150 mM NaCl, 20 mM Tris/HCl, pH 7.4, 1 mM PMSF) and an equal volume of 0.5 mm glass beads. Samples were vigorously vortexed for 30 s, followed by 30 s on ice; this procedure was repeated eight times. The lysed cells were centrifuged (1500 g for 5 min) and the supernatant was taken for protein analysis. Protein concentrations were determined by a modified Bradford Coomassie method (Pierce). Equal volumes of 3 × gel loading buffer (150 mM Tris/HCl, pH 6.8, 300 mM DTT, 6% SDS, 3% bromophenol blue, 30%, v/v, glycerol) were added to the samples and they were boiled for 3 min to denature the proteins.

PAGE. PAGE was performed according to Laemmli (1970). Proteins (10 µg) were loaded into each well on an SDS resolving acrylamide gel with a 4% stacking gel. Gels were run at 30 mA, 400 V at 10 °C for about 4 h, until the dye had migrated to the bottom of the gel. Gels were silver-stained (Bio-Rad silver stain kit) and dried; the images were scanned for later analysis.

PCR products. A loopful of yeast cells from a pure culture was washed with dH₂O and then resuspended in 1 ml dH₂O. Amplicons were obtained by using 5 µl of this cell suspension and the Finnzyme system (Geneworks) [5 µl 10 × buffer 511, 1 µl dNTP mix, 1 µl Dynazyme II (polymerase), 25 pmol forward primer NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and 25 pmol reverse primer LR6 (5'-CGCC-AGTTCTGCTTACC-3') in a final volume of 50 µl made up with dH₂O]. The PCR reaction steps were as follows: initial denaturation at 94 °C for 1 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final step at 70 °C

for 8 min. The amplification of products of approximately 2.2 kb in size was confirmed by agarose gel electrophoresis; the PCR products were purified using the QIAquick purification kit (Qiagen).

Sequencing. The D1/D2 region of the 26S rDNA was amplified by cycle sequencing using forward primer F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3'); the ITS regions were analysed using forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Fell *et al.*, 2000). The sequences were obtained with a Li-Cor automated sequencer using a standard protocol. The sequences were analysed and corrected using Li-Cor ALIGNR, and aligned to the sequences of their closest phylogenetic relatives using MEGALIGN (DNASTAR). Phylogenetic analyses were computed with PAUP 4.0 using parsimony analysis (heuristic search, random stepwise additions).

Lipid extraction and fatty acid analysis. Cells for fatty acid analysis were grown in YEP broth and then washed twice with dH₂O. Fatty acid extractions were performed according to Rule (1996). Samples were analysed as described by Swan & Watson (1997).

RESULTS AND DISCUSSION

PAGE analysis of whole-cell proteins

Proteome fingerprinting by PAGE was used as a rapid, initial screening step for the preliminary characterization of the Antarctic yeasts isolated as part of our ongoing studies (S. Thomas-Hall & K. Watson, unpublished results). Protein profiles of strains CBS 8925^T and CBS 8926 and of *Dioszegia crocea* CBS 6714^T were obtained by PAGE for comparative proteome fingerprinting (Fig. 1). Strains CBS 8925^T and CBS 8926 were shown to have essentially identical proteome fingerprints, thus supporting their relatedness. By contrast, the proteome fingerprint of *D. crocea* CBS 6714^T, although sharing some similar banding patterns with strains CBS 8925^T and CBS 8926, was different from those of the novel strains, and the majority of its protein bands did not align with those of the novel strains. This phenotypic difference supports the classification of strains CBS 8925^T and CBS 8926 as a novel species that is taxonomically distinct from *D. crocea*.

Fig. 1. PAGE (12.5% acrylamide) analysis of whole-cell-protein extracts. Lanes: 1, protein extract of strain CBS 8925^T; 2, protein extract of strain CBS 8926; 3, protein extract of *D. crocea* CBS 6714^T; 4, low-molecular-mass markers.

Fig. 2. Phylogenetic analysis of the D1/D2 region of the large-subunit rDNA. Bootstrap values are expressed as a percentage of 100 full heuristic bootstrap replications. The sequences of *Mrakia gelida* and *Cryptococcus aquaticus* were used as outgroups.

classification of strains CBS 8925^T and CBS 8926 as a taxonomically distinct species within the *Dioszegia* clade, as shown in Fig. 2. Recently, it has been reported that the defining characteristic of members of the *Dioszegia* clade is the secondary structure of the 18S rDNA (Takashima, Deak & Nakase, 2001); thus, further analyses on the 18S rDNA of the strains used in this study would be informative.

Fatty acid analysis

Cells were grown at 15 °C to stationary phase and their fatty acid profiles determined by GC. The total fatty acids of the novel strains comprised ~ 50% oleic acid (C_{18:1}), ~ 30% linoleic acid (C_{18:2}), ~ 7% linolenic acid (C_{18:3}) and ~ 10% palmitic acid (C_{16:0}). These results were consistent with previous findings for the fatty acid compositions of Antarctic yeasts (Watson, 1987). In contrast, palmitoleic acid (C_{16:1}), a major fatty acid in *Saccharomyces cerevisiae*, was found only in trace quantities (< 0.5%), a characteristic of members of the genus *Cryptococcus* (Ratledge & Evans, 1989).

Sequence analysis

Phylogenetic analyses based on the D1/D2 region of the large-subunit rDNA sequences of the novel strains indicated that strains CBS 8925^T and CBS 8926 belonged to the Tremellales clade of the hymenomycetous yeasts (Fell *et al.*, 2000). The closest phylogenetic relative of these two strains was *D. crocea*, which differed by 10 nt from strain CBS 8925^T and by 12 nt from strain CBS 8926 (Fig. 2). The nucleotide sequences from the ITS1–5.8S–ITS2 regions of strains CBS 8925^T and CBS 8926 were identical. Utilizing the same strains as shown in Fig. 2, phylogenetic analysis of the ITS1–5.8S–ITS2 sequences (data not shown) confirmed the relationships observed in D1/D2 phylogenetic tree (Fig. 2). The sequence data supported the

Sexual state

Numerous experimental conditions were tested in an attempt to induce a sexual state in strains CBS 8925^T and CBS 8926. These tests included the plating of each strain and a mixture of both strains onto YEP agar, corn meal agar, malt agar, carbon base agar and nitrogen base agar, and their inoculation into YEP broth and distilled water. The plates and broth were incubated at 6 and 15 °C. Plates and broths were examined at 1, 3, 6 and 9 months post-inoculation; although pseudohyphae were noted infrequently, no sexual structures were observed for the two strains.

Ballistoconidia formation

The inverted-plate method of Phaff & do Carmo-Sousa (1962) was employed to determine whether ballistoconidia were formed by strains CBS 8925^T and CBS 8926; corn meal agar plates inverted over YEP plates at 6 and 15 °C were used. *D. crocea* was used as the positive control; this strain formed a mirror image of the streaks (indicating the presence of ballistoconidia) within 4 weeks incubation at both temperatures tested. Neither of the novel strains nor a mixed culture of the two produced ballistospores after up to 9 months incubation.

Based on the phenotypic and molecular data presented above, it is proposed that strains CBS 8925^T and CBS 8926 be classified a novel species within the genus *Cryptococcus*, *Cryptococcus statzelliae*.

Fig. 3. Scanning electron micrographs of strains CBS 8925^T and CBS 8926 and of *C. victoriae* CBS 8685^T, CBS 8908, CBS 8915 and CBS 8920 showing budding cells and bud scars. Bars, 1 µm.

Latin diagnosis of *Cryptococcus statzelliae* Thomas-Hall, Watson et Scorzetti sp. nov.

Haec species e Lichen Valley (68° 29' S 78° 25' E) *et* Echo Lake (68° 31' S 78° 16' E), Vestvold Hills, Davis Base, Antarctica, *separata est*. In YEP agar post dies 3 (15 °C), *cellulae globosae vel ovoidae capsulatae, singulae vel binae sunt* (2–3 × 3–4 µm); *breves pseudohyphae* (10–20 µm) *post dies formatae sunt*. *Flocculi (plerumque polares) quos iungit breve tuber, cicatrices linquunt*. In YEP agar *species fert convexas rotundas, rubentes colonias, quae 1–2 mm diametron plerumque habent*. *Status sexualis non apparet*. *Assimilat: glucosum, galactosum, sucrosum, cellobiosum, trehalosum, raffinsum, D-xylosum, L-arabiosum, N-acetyl-D-glucosaminum, galacticum, D-mannitolum, D-glucitol, D-gluconatum, DL-lactatum, D-glucuronatum, 10 % NaCl 5 % glucosum*. *Exigue respondent: maltosum, melibiosum, melezitum, D-arabiosum, D-ribosum, D-glucosaminum, ribitolum, salicinum, succinatum*. *Non assimilantur: L-sorbosum, lactosum, inulinum, amyllum, solubile, methyl α-D-glucosidum, ethanolum, glycerolum, erythritolum, methyl α-D-glucosidum, citratum, inositolum, hexadecanum, nitratum*. *Ad crescentiam vitaminarum externarum necessariae sunt*. *Amyli instar composita creantur*. *Fermentatio nulla*. *Incrementum in 25 °C non respondet, maxima incrementi temperatio est 22 °C, optime crescit in 15–18 °C*. In collectione zymotica Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Trajectum ad Rhenum, Neerlandia, CBS

8925^T *est Cryptococcus statzelliae*, *et* CBS 8926 *est Cryptococcus statzelliae*.

Description of *Cryptococcus statzelliae* Thomas-Hall, Watson & Scorzetti sp. nov.

Cryptococcus statzelliae (stat.zell'i.ae. N.L. gen. fem. n. *statzelliae* in honour of Adele Statzell-Tallman).

Isolated from soil from Lichen Valley (68° 29' S 78° 25' E) and Echo Lake (68° 31' S 78° 16' E), Vestvold Hills, Davis Base, Antarctica. After 3 days growth at 15 °C in YEP broth, the cells are sphaeroidal to ovoid, occur singly or in parent-bud pairs and measure 2–3 × 3–4 µm; short pseudohyphae (10–20 µm) are formed infrequently after 4 weeks incubation at 6 °C. Budding is polar, a short protuberance connects budding cells, leaving raised bud scars (Fig. 3). Grows on YEP agar giving rise to convex, circular, shiny, orange/red colonies that are generally 1–2 mm in diameter. No sexual state is observed. Carbon compounds assimilated: glucose, galactose, sucrose, cellobiose, trehalose, raffinose, D-xylose, L-arabinose, L-rhamnose, N-acetyl-D-glucosamine, galactitol, D-mannitol, D-glucitol, D-gluconate, DL-lactate, D-glucuronate and 10 % NaCl 5 % glucose. Growth response is weak for maltose, melibiose, melezitose, D-arabinose, D-ribose, D-glucosamine, ribitol, salicin and succinate. Compounds not assimilated: L-sorbose lactose, inulin, soluble starch, methanol, ethanol, glycerol, erythritol, methyl α-D-glucoside, citrate, inositol, hexadecane, and vitamin-free, biotin-free and thiamin-free media. Nitrate assimilation is negative. Starch-like compounds are formed. Diazonium blue B- and urease-positive. Growth on 50 % (w/w) glucose/yeast extract agar is negative. Gelatin liquefaction is negative. Fermentation is absent. Growth at 25 °C is negative, maximum growth temperature is 22 °C, optimal growth temperature is between 15 and 18 °C. Strains CBS 8925^T and CBS 8926 are available from the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

Three novel strains of *C. victoriae* Montes et al. (1999), CBS 8908, CBS 8915 and CBS 8920

Morphology. *C. victoriae* CBS 8685^T gives rise to convex, smooth, round, light-pink, butyrous colonies; cell size is 3–5 × 2–3 µm; budding is polar (Fig. 3). Strain CBS 8908 gives rise to convex, smooth, round, peach (yellow/orange), butyrous colonies; cell size is 3–5 × 2–3 µm; budding is polar (Fig. 3). Strain CBS 8915 gives rise to umbonate, irregular, pink, pasty colonies; cell size is 3–5 × 2–3 µm; budding is bipolar (Fig. 3); cells aggregate into clusters and form a granular sediment in YEP broth after 1 week incubation at 15 °C. Strain CBS 8920 gives rise to convex, smooth, round, apricot (pink/orange), butyrous colonies; cell size is generally 3–5 × 2–3 µm,

Table 2. Assimilation tests that varied among the strains of *C. victoriae* studied here

Strains: 1, *C. victoriae* 8685^T; 2, *C. victoriae* 8908; 3, *C. victoriae* 8915; 4, *C. victoriae* 8920. +, Positive reaction; –, negative reaction; w, weak reaction; s, slow reaction.

Growth test	1	2	3	4
L-Sorbose	–	w	–	–
Cellobiose	+	+	+	w
Lactose	+	+	+	–
Soluble starch	w	s/w	–	–
L-Arabinose	+	w	+	+
L-Rhamnose	+	w	+	+
D-Glucosamine	w	w	+	w
Galactitol	+	+	w	w
Methyl α -D-glucoside	+	+	+	w
Succinate	+	w	w	+
Citrate	+	w	w	+
Inositol	+	w	w	–
Vitamin-free medium	+	w	+	w
D-Glucuronate	+	w	+	w
10 % NaCl 5 % glucose	w	w	+	+
50 % (w/w) glucose/yeast extract agar	–	–	w/–	w

with large cells ($10 \times 4 \mu\text{m}$) formed occasionally; budding is polar (Fig. 3).

Phenotypic characterization. Table 2 displays the carbon assimilation results that varied between the four *C. victoriae* strains tested. These tests were performed in three separate experiments, to confirm the results. Carbon compounds assimilated by the four *C. victoriae* strains: glucose, galactose, sucrose, trehalose, melibiose, raffinose, melezitose, inulin, D-xylose, D-arabinose, D-ribose, *N*-acetyl-D-glucosamine, glycerol, erythritol, ribitol, D-mannitol, D-glucitol and D-gluconate. Carbon compounds not assimilated: methanol, ethanol, soluble starch, DL-lactate and hexadecane. Nitrate assimilation, growth in biotin-free media and the urease test are all positive. Starch compounds are not formed, gelatin is not liquefied and growth at 25 °C is negative.

PAGE of whole-cell proteins. The protein profiles of *C. victoriae* CBS 8658^T, CBS 8908, CBS 8915 and CBS 8920 (Fig. 4) showed an overall similarity, with only a few unique bands for each of the strains. The similar protein fingerprints obtained indicated that these strains were closely related.

Sequence analysis. Phylogenetic analysis of the D1/D2 sequences (Fig. 2) of the four strains showed that *C. victoriae* CBS 8658^T, CBS 8915 and CBS 8920 have identical sequences, while CBS 8908 differed from these strains by 2 nt. Analysis of the ITS1–5.8S–ITS2 sequences showed that *C. victoriae* CBS 8658^T, CBS 8908, CBS 8915 and CBS 8920 differed from each other by 1 nt. These phylogenetic and morphological data supported the classification of CBS strains 8908, 8915 and 8920 as strains of the species *C. victoriae*.

Fig. 4. PAGE (10% acrylamide) analysis of whole-cell-protein extracts. Lanes: 1, low-molecular-mass markers; 2 and 6, protein extract of *C. victoriae* CBS 8685^T; 3, protein extract of *C. victoriae* CBS 8908; 4, protein extract of *C. victoriae* CBS 8920; 5, protein extract of *C. victoriae* CBS 8915.

ACKNOWLEDGEMENTS

This work was supported in part by the Australian Research Council (K.W.), an UNE postgraduate scholarship (S.T.-H.) and the National Science Foundation, Division of Ocean

Sciences (J.W. Fell). We thank John Bowman and Tom McMeekin of the Antarctic CRC, University of Tasmania, Hobart, Australia, for supplying soil samples and helpful discussions. Special thanks to Jack W. Fell for the use of his laboratory and database. Allan Treloar is acknowledged for the Latin translation.

REFERENCES

- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Hanschke, R. & Schauer, F. (1996). Improved ultrastructural preservation of yeast cells for scanning electron microscopy. *J Microsc* **184**, 81–87.
- Kurtzman, C. P. & Fell, J. W. (1998). Summary of species characteristics. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 924–927. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Montes, M. J., Belloch, C., Galiana, M., Garcia, M. D., Andrés, C., Ferrer, S., Torres-Rodriguez, J. M. & Guinea, J. (1999). Polyphasic taxonomy of a novel yeast isolated from Antarctic environment; description of *Cryptococcus victoriae* sp. nov. *Syst Appl Microbiol* **22**, 97–105.
- Phaff, H. J. & do Carmo-Sousa, L. (1962). Four new species of yeast isolated from insect frass in bark of *Tsuga heterophylla* (Raf.) Sargent. *Antonie van Leeuwenhoek* **28**, 193–207.
- Ratledge, C. & Evans, C. T. (1989). Lipids and their metabolism. In *The Yeasts*, vol. 3, 2nd edn, pp. 367–455. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Rule, D. C. (1996). Direct transesterification of total fatty acids of adipose tissue, and freeze-dried muscle and liver with boron-trifluoride in methanol. *Meat Sci* **46**, 23–32.
- Swan, T. M. & Watson, K. (1997). Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast. *Can J Microbiol* **43**, 70–77.
- Takashima, M., Deak, T. & Nakase, T. (2001). Emendation of *Dioszegia* with redescription of *Dioszegia hungarica* and two new combinations, *Dioszegia aurantiaca* and *Dioszegia crocea*. *J Gen Appl Microbiol* **47**, 73–84.
- Watson, K. (1987). Temperature relations. In *The Yeasts*, vol. 2, pp. 42–71. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Yarrow, D. (1998). Methods for the isolation, maintenance, classification and identification of yeasts. In *The Yeasts: a Taxonomic Study*, 4th edn, pp. 77–101. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.