

Pichia barkeri, a New Yeast Species Occurring in Necrotic Tissue of Opuntia stricta

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We describe Pichia barkeri, a new cactophilic yeast species occurring in necrotic tissue of Opuntia stricta in New South Wales, Australia, and on several islands in the Caribbean Sea. The new species is homothallic and appears to occur in nature in the haploid state. After conjugation between a mother cell and a bud, four hat-shaped spores are produced that are rapidly released from the ascus. The range of guanine-plus-cytosine contents for the nuclear deoxyribonucleic acids of 22 strains is 35.7 to 36.6 mol% (average, 36.1 mol%; standard deviation, 0.2 mol%). The type strain of P. barkeri is strain UCD-FST 83-994.3 (ATCC 64111, CBS 7256) isolated at Discovery Bay, Jamaica.

The rotting stems and cladodes of Opuntia species are specific habitats for a diversity of cactus-specific yeast species (1, 2, 7, 11). During surveys of yeasts associated with rots of Opuntia species in Australia (1, 2), we isolated a number of strains that were initially identified by the system of Lodder (5) as Pichia kluyveri. Because P. kluyveri had been recovered only rarely in earlier surveys of columnar cacti (12), we initially concluded that these strains might have converged on the P. kluyveri phenotype or might be fermentative forms of Pichia cactophila. Subsequent characterization by molecular criteria (6) showed that the isolates in question differed from P. kluyveri in having a nuclear deoxyribonucleic acid (DNA) guanine-plus-cytosine (G+C) content approximately 6 mol% higher than that of P. kluyveri (~36 versus ~30 mol%), virtually precluding conspecificity (6). Ensuing experiments, using the technique of DNA-DNA hybridization, provided additional evidence that the Australian isolates represented a different species, although retaining a distant relationship with P. kluyveri. DNA-DNA hybridization experiments also showed that the Australian isolates are distinct from P. cactophila, which has a similar base composition (~36 mol% G+C). During subsequent explorations of the yeast floras associated with cacti occurring on various islands in the Caribbean Sea, we isolated several additional strains of the new species, Pichia barkeri, also associated with necrotic tissue of Opuntia stricta.

MATERIALS AND METHODS

Samples of necrotic Opuntia tissue yielding Pichia barkeri were collected by J. S. F. Barker and colleagues in eastern Australia and airmailed to the Davis laboratory. They normally arrived within 4 or 5 days. Strains 84-670.2 and 84-663.4 of P. kluyveri were collected by W. T. Starmer in New South Wales, Australia. The samples yielding P. barkeri on Caribbean islands were collected by W. T. Starmer, H. J. Phaff, and M. A. Lachance in November 1983 during cruise CF-8314 of the research vessel Cape Florida. The techniques used for yeast isolation and purification have been described previously (1, 2, 11, 12). The locations and isolation sources of P. barkeri, P. kluyveri, and others are given in Table 1. The strains used in DNA-DNA hybridization studies and those analyzed for nuclear G+C content are listed in Table 2.

The usual phenotypic characterization of the isolates was carried out by methods currently used in yeast taxonomy (14, 15), but additional carbon compounds were used in assimilation tests. These included d-glucosamine hydrochloride, N-acetyl-d-glucosamine, methanol, 2-propanol, acetone, and ethyl acetate. The last three compounds could be tested only by incorporating them at concentrations of approximately 1.0% in yeast nitrogen base agar (Difco Laboratories, Detroit, Mich.), because in liquid nitrogen base medium they appeared to inhibit growth even in those instances where good growth occurred on solid medium.

Killer activity was assayed by the methods described in various earlier papers on the killer phenomenon (8, 16). YM agar buffered to pH 4.2 with 0.05 M citrate buffer and containing 0.003% (wt/vol) methylene blue was used as the assay medium. Yeasts to be tested for susceptibility to potential killer yeasts were spread as a lawn (approximately 105 cells per plate). Potential killer yeasts were inoculated onto the lawn at high density. Both lawn strains and strains tested for killer activity were taken from exponentially growing cultures (1 to 3 days after transfer). Plates were incubated at 22°C for 48 to 72 h. If the tested strain was surrounded by a region of blue cells or by a clear zone of inhibition bounded by blue cells, the tested strain was designated a killer and the lawn was designated as susceptible. Candida glabrata (Y55 [NCYC 388], obtained from T. W. Young) was used as a susceptible strain for a general screening of isolates for killer activity because of its broad susceptibility to known toxins (16). This test was included in our study because it was found useful in separating similar yeast species such as P. barkeri and P. kluyveri.

Lipase activity was assayed on solid medium consisting of 1% peptone, 0.5% NaCl, 0.2% CaCl2, 0.45% Tween 80, and 2% agar. A zone of precipitate surrounding the test colony was used as an indicator of lipase activity.

DNA extraction and purification were carried out by the methods described by Price et al. (9), except that purified DNA was concentrated by electrophoresis in a concentrator (ISCO, Lincoln, Nebr.), and the reference DNA was labeled with 32P as described by Holzscheu et al. (4). The G+C contents of the nuclear DNAs were calculated from buoyant density values in cesium chloride gradients established in a Spinco model E analytical ultracentrifuge with photographic...
optics (10, 13), and were based on at least three separate determinations. DNA from Micrococcus luteus 2039 (International Collection of Phytopathogenic Bacteria, University of California, Davis) with a buoyant density of 1.7309 g/ml was used as a reference. The buoyant density of the M. luteus DNA was derived from comparison with plasmid-free Escherichia coli K-12 DNA, the buoyant density of which was taken to be 1.7100 g/ml. Denaturation of DNA, incubation for reassociation, analysis of the renaturation kinetics and renameling reactions, and quantitation of single- and double-stranded DNAs were carried out by using the methods described by Price et al. (9). Samples were counted with a TM Analytic model 1290 gamma ray counter (84% efficiency).

RESULTS

Phenotypic identification of some of the Australian isolates indicated that they represented strains of P. kluveri, although most of these isolates exhibited conjugation between a mother cell and a bud with a variable-length neck, whereas P. kluveri is normally isolated as a diploid. The G+C contents of the nuclear DNAs of strains of P. kluveri were approximately 30 to 31 mol%, while those of P. Barkeri were about 36 mol% (Table 2). A comparison of representative isolates of this group by DNA-DNA hybridization is also shown in Table 2. These reassociation experiments clearly showed that the isolates under consideration consist of two distinct species. Those designated P. kluveri showed greater than 90% DNA relatedness with the type strain of that species (C-7), whereas the other group, described below as P. Barkeri, showed approximately 20% DNA relatedness with P. kluveri. Pichia antillensis and Clavispora lusitaniae DNAs were used as negative controls and showed very low relatedness to the reference DNAs. A second DNA hybridization experiment (Table 2) was designed in which P. Barkeri DNA was labeled and annealed not only with P. kluveri DNA (30 mol% G+C) but also with DNAs of several cactus-specific yeasts (P. cactophila and Pichia pseudocactophila) and P. norvegensis (also found in cactus necroses) that have G+C contents similar to that of P. Barkeri. The strains of P. Barkeri demonstrated a high degree of DNA relatedness (greater than 90%) to each other, whereas the relatedness between P. Barkeri and P. kluveri again was found to be approximately 20%. Although their phenotypic properties initially were found to be nearly identical, we discovered that the two species can be distinguished on the basis of lipolytic activity (positive for P. Barkeri and negative for P. kluveri) and by killer activity on the susceptible strain C. glabrata Y55 (negative for P. Barkeri and usually positive, but sometimes negative, for P. kluveri). On the basis of the differences described above, we now describe P. Barkeri as a new species.


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<th>UCD-FST strain no.*</th>
<th>Locality</th>
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<td>C. lusitaniae 80-84°</td>
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* UCD-FST, Department of Food Science and Technology, University of California, Davis.
Ad crescendiam vitaminea additae necessariae sunt. Crescere potest in 36°C at non in 39°C. Crescere potest in 10% NaCl, at non in 12.5% NaCl. G+C acidi deoxyribonucleati 36.1 ± 0.2 mol% (22 stirpes).

Habitatio: species opuntiae. Typus: stirps UCD-FST 83-994.3 ex tabidosus sacculis cacti Opuntia stricta isolata est. In collectione zymotica Centraalbureau voor Schimmeleculures, Delphi, butavorum sub no. 7256 deposita est. Description of Pichia barkeri. In YM liquid medium after 3 days at 25°C, a thin, dull, smooth, creeping pellicle and a sediment are formed.

Cells are oval, elongate, and cylindrical, 2.5 to 6.5 by 5.0 to 13 μm, and occur singly, in pairs, and in small and large clusters, sometimes with numerous cells in pseudomycelical formations.

On malt agar after 3 weeks at 25°C, streak cultures are flat to raised, with very little spreading; the periphery is entire, with irregular spotty tufts of pseudomycelia. Surface varies from nearly smooth with some transverse striations to slightly warty; semidull to semiglossy, greyish to light tan. Texture is pasty to butyrous.

In Dalmau plate cultures on corn meal agar after 18 days, strongly branched pseudomycelium is abundant under the cover slip, and virtually lacking or very compact and dense in the aerobic portion. True mycelium is lacking.

The vegetative cells are haploid. Sporulation appears to occur best on acetate agar at about 25°C after approximately 4 to 10 days. Cells begin the sporulation process by producing a tubelike protuberance of variable length which undergoes a terminal swelling until a dumbbell-shaped zygote is formed (Fig. 1). Four hat-shaped spores are formed in one of the two cells constituting the dumbbell-shaped ascus, usually the original mother cell. The asc rupture rapidly, releasing groups of four spores (Fig. 1D). There is a tendency for loss of sporulating ability during storage of strains under sterile mineral oil. The life cycle just described suggests that P. barkeri is homothallic.

Glucose is rapidly fermented; no other sugars are fermented. The carbon compounds D-glucose, ethanol, glyceral (latent), lactic acid, succinic acid, citric acid (slow), D-glucosamine (weak to moderate; dull, chalky growth) and ethyl acetate are assimilated.

The following carbon compounds are not assimilated: D-galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xyllose, L-arabinose, D-arabinose, D-ribose, D-xylose, D-erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl-α-D-glucoside, salicin, glucono-δ-lactone, potassium gluconate, 2- and 5-ketogluconate, meso-inositol, N-acetylglucosamine, acetone, 2-propanol, and hexadecane.

Assimilation of nitrogen compounds: potassium nitrate and sodium nitrite negative; ethylamine, cadaverine, lysine, and ammonium sulfate positive.

Growth in a vitamin-free medium is negative. Growth in amino acid-free medium supplemented with (NH₄)₂SO₄ is positive. Growth on 50% (wt/wt) glucose-yeast extract agar strongly branched pseudomycelium is abundant under the cover slip, and virtually lacking or very compact and dense in the aerobic portion. True mycelium is lacking.

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is negative. Growth on glucose-yeast extract agar containing 10% NaCl is positive; growth on the same medium containing 12.5% NaCl is negative.

Maximum temperature for growth: positive at 36 to 37°C, negative at 39°C.


Growth in the presence of 1 μg of cycloheximide per ml is weak; with 10 μg/ml growth is negative.

G+C content of the nuclear DNA is 35.7 to 36.6 mol% (22 strains) (Table 2); average ± standard deviation for 22 strains, 36.1 ± 0.2 mol%.

Killer activity for C. glabrata Y55: negative.

A total of 22 strains was recovered from necrotic tissues of Opuntia stricta in eastern Australia and on several islands in the Caribbean Sea (Table 1).

The type strain of P. barkeri is UCD-FST 83-994.3, isolated from rotting cladodes of O. stricta at Discovery Bay, Jamaica; this strain has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, as CBS 7256 and in the American Type Culture Collection, Rockville, Md. as strain ATTC 64111.

The specific epithet of Pichia barkeri (bar.ker’ i. L. gen. m. n. of Barker) refers to the important contributions made by J. S. F. Barker, University of New England, Armidale, Australia, to the cactus-yeast-Drosophila model system.

**DISCUSSION**

The habitat of P. barkeri appears to be restricted to rotting cladodes of O. stricta. Thus far, it has been isolated in a restricted region of New South Wales, Australia, and on several islands in the Caribbean Sea (Table 1). Although large areas in New South Wales and Queensland were surveyed for yeasts associated with necroses in various Opuntia species (1, 2), P. barkeri was found only in the Yarrawonga area of New South Wales (Table 1). Cacti were not native to Australia until they became introduced about 1840. The original introduction of O. stricta into Australia is not certain but it is presumed to have come from the vicinity of trade ports in Texas, Florida, and Cuba (3). The first record of O. stricta appears to be that of a plant brought from Sydney to Scone, New South Wales, in 1839. Scone is in the vicinity of Yarrawonga, where the Australian P. barkeri strains were found. The rapid spread of the Opuntia species led to the prickly pear eradication program (ca. 1926 to 1930), when infested plants containing the destructive Cactoblastis moth and other insects parasitic on cacti were shipped to Australia from Argentina and various areas in the southern United States (3). Since the destruction of plants was widespread and the rotting process was effectively accelerated by the introduction of the infested material from the Americas, most of the microflora of cactus necroses identified in recent years (1, 2) must have originated from areas that have not yet been surveyed (i.e., regions such as Florida and Louisiana, or South America) or possibly from the Caribbean region at the time of the introduction of O. stricta in the mid 1800s.

On the basis of the molecular data (Table 2), P. kluyveri and P. barkeri clearly represent different species. The DNA relatedness of approximately 20% relative binding between P. barkeri and P. kluyveri is interesting (Table 2). P. kluyveri, a diploid heterothallic species (Starmer, unpublished data) appears to be very stable because strain 77-165 (from a cladode rot in Australia), strain 77-348D (from Arroyo Seco, Baja California, Mexico), and strains 84-663.4 and 84-670.2 (from Opuntia fruit collected at O’hara near Denman, New South Wales, Australia) show greater than 90% DNA complementarity with that of the type strain C-7, which had been isolated from fermenting olives in California some 40 years earlier. The finding that P. barkeri shares about 20% of its DNA base sequences with P. kluyveri indicates a significant, although distant, relationship because numerous experiments in our laboratory have shown that relatively unrelated yeasts show DNA relatedness values below 10% (Table 2). This is the more surprising as the G+C contents of the two species are 6 mol% apart, and in our experience, such a large difference virtually precludes a significant relatedness between yeast species (6). The habitat of P. kluyveri in the cactus ecosystem is mainly in rotting cladodes and occasionally in Opuntia, whereas that of P. barkeri is limited to rotting cladodes. P. barkeri is more commonly found in various necrotic fruit tissues outside the cactus habitat. It is possible that P. barkeri is derived from P. kluyveri, perhaps from the progeny of a haploid spore that may have mutated to homothallism and evolved independently in a new niche, namely, the necrotic tissue of O. stricta with a distinctly different chemistry from its fruits. Alternatively, P. kluyveri and P. barkeri could both be derived from a common ancestor we have not yet detected.

**ACKNOWLEDGMENTS**

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We appreciate the collaboration and helpful discussions with M. A. Lachance. We thank Alice Moffitt and Jim Haudenshield for their competent technical assistance in one of the DNA hybridization experiments.

**LITERATURE CITED**


