Pichia caribaea, a New Species of Yeast Occurring in Necrotic Tissue of Cacti in the Caribbean Area

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We describe *Pichia caribaea*, a new species of yeast which is closely related to *P. amethionina*. *P. caribaea*, of which 92 strains were isolated, is heterothallic and occurs in nature in both the haploid state and the diploid state. It produces asci with four hat-shaped spores, which are gradually released upon maturity. *P. caribaea* occurs in rotting tissue of cereoid and opuntia cacti on various islands in the greater Caribbean area and on coastal land masses surrounding it. It resembles *P. amethionina* var. *pachycereana* in its assimilation pattern of carbon compounds but differs in its ability to ferment glucose strongly. The DNAs of *P. caribaea* and the two described varieties of *P. amethionina* show about 40% complementarity. The type strain of *P. caribaea* is strain UCD-FST 81-62 (= ATTC 76713 = CBS 7692).

In 1978, Starmer et al. (15) described *Pichia amethionina*, a naturally occurring auxotroph that has an absolute requirement for organic forms of sulfur, such as the sulfur-containing amino acids L-methionine and L-cysteine. This nonfermentative species is represented by two varieties, *P. amethionina* var. *amethionina* and *P. amethionina* var. *pachycereana*. The former is commonly recovered from rot pockets in cacti of the subtribe *Stenocereinae*, whereas the latter has its principal habitat in members of the subtribe *Pachycereinae* (2, 15). All isolates of these two varieties came from decaying columnar and *Opuntia* cacti in the North American Sonoran desert, including the Baja California peninsula. The DNA relatedness between the two varieties was determined by Holzschu and Phaff (3), who reported a mean DNA-DNA complementarity value of 64.9% $\pm$ 3.2% on the basis of 16 pairwise comparisons, including reverse DNA reassociations. Nearly all strains of *P. amethionina* var. *amethionina* lack the ability to utilize D-mannitol for growth and are resistant to triterpene glycosides or digitonin at 25°C, while *P. amethionina* var. *pachycereana* is D-mannitol positive and is inhibited by these compounds (12).

During two expeditions in 1982 and 1983 to collect yeasts from cactus necroses on various islands in the Caribbean Sea and the Bahamas, we discovered another yeast with an absolute requirement for sulfur-containing amino acids that was similar to *P. amethionina* var. *pachycereana*, except that glucose was vigorously fermented. Additional strains with these properties had been isolated by J. W. Fell and later by M. A. Lachance in the Exuma chain in the Bahamas, by Starmer and Phaff (14) on the Gulf of Mexico coast of Texas, and more recently by Starmer et al. (10) in Florida. In the publications cited, these isolates were referred to as *P. amethionina* var. "f" or "fermentans." As a formal description with a Latin diagnosis was not provided, this name constitutes a nomen nudum.

To substantiate the basis for a valid description of this group of isolates, we carried out mating tests and DNA-DNA hybridizations between typical strains of the fermenting isolates and the two varieties of *P. amethionina*. Mating

between the fermenting isolates and either of the two varieties of *P. amethionina* was limited. The genome comparisons revealed that both varieties exhibited approximately 40% DNA complementarity with several isolates of the glucose-fermenting strains. On this basis, we conclude that these strains comprise a separate species in the genus *Pichia*, for which we propose the name *P. caribaea* sp. nov., after the geographic area where most of the strains were found.

MATERIALS AND METHODS

Samples of necrotic cactus tissue yielding *P. caribaea* came from the host plants and locations recorded in Table 1. The samples from Caribbean islands and the Bahamas were collected in May 1982 during cruise CF-8205, in November 1983 during cruise CF-8314 of the research vessel Cape Florida, and in June 1990 during cruise CA-9008 of the research vessel Calanus in that region. The techniques used for yeast isolation and purification have been described previously (14). The strains analyzed for nuclear DNA base composition and those used in the DNA-DNA hybridization studies are listed in Table 2.

The usual phenotypic characterization of the isolates was carried out by methods currently used in yeast taxonomy (18), but additional carbon compounds were used in assimilation tests. These included glucosamine, N-acetylglucosamine, methanol, 2-propanol, acetone, and ethyl acetate; we obtained the best differential results by adding the last three compounds tested to 1% (vol/vol) (based on medium volume) to the inverted cover of a glass petri dish after inoculation with the test cultures by multipoint inoculation, sealing the dishes with Parafilm M (American Can Co., Greenwich, Conn.), and incubating the dish upside down.

Sensitivity to triterpene glycoside or digitonin was tested at 25°C in YM (Difco) agar plates as described by Starmer et al. (12). Triterpene glycosides were tested in a concentration of 1% dried, powdered *Stenocereus gummosus* (agria) tissue (a columnar cactus species with a high content of these glycosides) or in a concentration of 8 mg of digitonin per liter (12).

Killer activity was tested against *Candida glabrata* NRRL Y-55 (11), a strain known for its broad sensitivity to known killer toxins.

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TABLE 1. Geographic and host plant distributions of *P. caribaea*

Locality	Host tissue ^a	No. of <i>P. caribaea</i> strains isolated
Quintana Beach, Tex.	<i>Opuntia lindheimeri</i>	3
Galveston Beach, Tex.	<i>Opuntia lindheimeri</i>	1
Canaveral National Seashores, Fla.	<i>Opuntia stricta</i>	6
Canaveral National Seashores, Fla.	<i>Opuntia stricta</i> fruit	1
Kennedy Space Center, Fla.	<i>Opuntia stricta</i>	5
Kennedy Space Center, Fla.	<i>Opuntia stricta</i> fruit	2
Flamingo, Fla.	<i>Opuntia stricta</i>	4
Tecucacán Pueblo, Mexico	<i>Escontria chiotilla</i>	1
Tuxtla Gutierrez, Chiapas, Mexico	<i>Nopalea</i> sp.	1
Airport, Montserrat	<i>Cephalocereus royenii</i>	5
Spanish Point, Montserrat	<i>Opuntia stricta</i>	2
Prickly Pear Island, B.V.I. ^b	<i>Cephalocereus royenii</i>	5
Prickly Pear Island, B.V.I.	<i>Melocactus intortus</i>	1
Beef Island, Virgin Gorda, B.V.I.	<i>Cephalocereus royenii</i>	1
Exuma chain, Bahamas	<i>Opuntia stricta</i>	19
Conception Island, Bahamas	<i>Opuntia stricta</i>	1
Conception Island, Bahamas	<i>Opuntia stricta</i> fruit	1
Matthew Town, Great Inagua, Bahamas	<i>Cephalocereus royenii</i>	1
Matthew Town, Great Inagua, Bahamas	<i>Opuntia stricta</i>	1
Matthew Town, Great Inagua, Bahamas	<i>Opuntia stricta</i> fruit	3
Navassa Island, Caribbean, United States	<i>Opuntia stricta</i>	8
Navassa Island, Caribbean, United States	<i>Drosophila straubae</i>	2
Guantanamo Bay, Cuba	<i>Cephalocereus robinii</i>	1
Discovery Bay, Jamaica	<i>Opuntia stricta</i>	1
Sandy Bay, Jamaica	<i>Opuntia stricta</i>	1
Sandy Bay, Jamaica	<i>Opuntia stricta</i> fruit	1
Cayman Brac, Cayman Islands	<i>Cephalocereus royenii</i>	4
Cayman Brac, Cayman Islands	<i>Opuntia stricta</i>	2
Little Cayman, Cayman Islands	<i>Cephalocereus royenii</i>	2
Little Cayman, Cayman Islands	<i>Opuntia stricta</i> fruit	1
El Anis, Venezuela	<i>Cereus repandus</i>	1
Oricao, Venezuela	<i>Cephalocereus langinosus</i>	1
Piritu, Venezuela	<i>Cereus repandus</i>	2
Prudencio, Venezuela	<i>Stenocereus griseus</i>	1

^a Tissue is the stem of columnar cacti or cladode of *Opuntia* spp. unless fruit is indicated.^b B.V.I., British Virgin Islands.TABLE 2. Reannealing between labeled DNA from *P. caribaea* UCD-FST 81-62^T and DNAs from seven yeast strains and calf thymus control DNA^a

Organism or tissue	Mol% G+C	Relative % binding with <i>P. caribaea</i> 81-62 ^{Tb}
<i>P. caribaea</i> strains		
UCD-FST 81-62 ^T	34.4	100.0
UCD-FST 81-38	34.4	94.1
UCD-FST 81-35	34.0	94.4
UCD-FST 81-27	34.0	93.0
<i>P. amethionina</i> var. <i>amethionina</i>	33.1	40.2
UCD-FST 76-401B ^T		
<i>P. amethionina</i> var. <i>pachycereana</i> UCD-FST 76-384A ^T	33.0	37.4
<i>P. cactophila</i> UCD-FST 76-243A ^T	36.3	2.3
Calf thymus DNA		0.0

^a All four *P. caribaea* strains used came from islands in the Exuma chain of the Bahamas.^b ¹²⁵I-labeled DNA (0.2 µg) and unlabeled DNA (200 µg) were incubated at 61.7°C for 25 h in 0.5 ml of 280 mM phosphate buffer, pH 6.8. The values are averages of three determinations and are corrected for zero-time binding and for self-renaturation (8).

DNA extraction and purification were carried out by the methods described by Price et al. (8), except that purified DNA was concentrated by electrophoresis in a concentrator (ISCO, Lincoln, Nebr.), and the reference DNA was labeled with ¹²⁵I as described by Holzschu et al. (5). The G+C contents of the nuclear DNAs were calculated from buoyant density values in cesium chloride gradients established in a Spinco E analytical ultracentrifuge equipped with photographic optics (9, 17) and were based on three separate determinations. DNA from *Micrococcus luteus* 2039 (International Collection of Phytopathogenic Bacteria, Department of Bacteriology, 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 1.700 g/ml. Denaturation of DNA, incubation for renaturation, analysis of the renaturation kinetics and reannealing reactions, and quantitation of single- and double-stranded DNAs were carried out by using the methods of Price et al. (8). Samples were counted with a TM Analytic 1290 gamma ray counter (84% efficiency).

Single ascospores were isolated from mature asci with a micromanipulator (1).

RESULTS

Single-spore clones of *P. caribaea* showed limited ability to mate with haploid clones of the opposite mating type of *P. amethionina* var. *amethionina* or *P. amethionina* var. *pachycereana*. In all combinations (best with *P. amethionina* var. *amethionina*), zygotes were produced after 2 to 5 days on YM agar, and in some combinations, aberrant asci with three or four malformed spores were observed. This is evidence for reproductive isolation of closely related species. Except for glucose fermentation, the physiological and morphological properties of the isolates listed in Table 1 were very similar to those of *P. amethionina* var. *pachycereana* (15) and the nuclear DNA base composition of selected strains (Table 2) was slightly higher by approximately 1 mol% G+C. These similarities induced us to compare their nuclear genomes by DNA-DNA hybridization. The results (Table 2) of this experiment indicate that *P. caribaea* shows about 40% DNA relatedness to both varieties of *P. amethionina*. *P. cactophila* was used as a negative control. This level of DNA complementarity shows significant evolutionary relatedness between the species and substantiates the other observations that indicate that the taxa represent distinct species. We therefore propose to describe *P. caribaea* as a new species of the genus *Pichia*.

Latin diagnosis of *Pichia caribaea* sp. nov. In YM (Difco) liquido post dies 3 ad 30°C, cellulae vegetativae ovoideae, pyriformes, clavatae vel elongatae, 2–4 × 4–9 µm, interdum longitudo 12–14 µm, singulae, binae, in catenis brevis, aut in fasciculae ad instar stellae; sedimentum; pellicula nulla aut tenuis. Cultura in agaro multi post dies 21 ad 25°C cana-cremea, butyrosa vel mollis, umbonata, glabra cum lineis radiatis, semi-nitida, margine glabro vel undulato.

In agaro farinae *Zea mays* post dies 10, pseudomycelium nullum. Species heterothallica, diploidea aut haploidea. Asci habentes 4 spores pileiformae; asci rumpunter post dies 5–10.

Fermentatio glucosi. Glucosum, ethanolum, glycerolum, D-mannitolum, acidum lacticum, acidum succinicum, acidum gluconatum, ethylacetat assimulantur at non galactosum, L-sorbosum, maltosum, saccharosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinose, melezitose, inulinum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, *i*-erythritolum, ribitolum, galactitolum, D-glucitolum, methyl- α -D-glucosidum, salicinum, 2-ketogluconatum, 5-ketogluconatum, acidum citricum, *meso*-inositolum, D-glucosaminum, *N*-acetylglucosaminum, methanolum, 2-propanolum, acetoneum, nec hexadecanum. Kalium nitricum, natrium nitrosum non assimilantur. Ethylaminum, L-lysinum, et cadaverinum assimilantur tarde. Ad crescentiam L-methioninum et vitaminae additae necessariae sunt. Crescere potest in 39°C, interdum in 41°C, exigue.

G+C acidi deoxyribonucleati 34.0–34.4 mol% (4 stirpes).

Habitatio in cacti tribus *Opuntiae* et subtribus *Pachycereinae*.

Typus: stirpes UCD-FST 81-62 ex tabidosus sacculis cacti *Opuntia stricta* isolata est. In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum sub no. 7692 deposita est.

Description of *Pichia caribaea* sp. nov. *Pichia caribaea* (ca.rib'ae.a. L. fem. adj., of the Caribbean, referring to the area of its common occurrence). In YM liquid medium after 3 days at 30°C, the cells are ovoid, pear-shaped, clavate, or short-cylindrical, 2 to 4 by 4 to 9 µm, sometimes elongate at 12 to 14 µm long, single, in pairs, short chains, or asteroid-like configurations, as illustrated in reference 15 for *P.*

amethionina; a sediment and a thin ring develop, a pellicle is lacking or very thin, smooth, and creeping. Streak culture on malt agar after 3 weeks at 25°C is cream colored to greyish, butyrous to soft, umbonate, smooth with slight lateral striations, and semiglossy, with an entire-to-lobulate periphery.

In Dalmat plate cultures on cornmeal agar after 10 days, pseudomycelium is lacking; occasionally, a few tufts of rudimentary chains of cells may be present.

The cells are haploid or diploid in natural populations. The type strain is diploid and heterothallic, producing three to four small hat-shaped spores per ascus; at maturity, asci gradually release the spores. Two spores are h^+ , and two are h^- . After mixing of the haploid clones, strong agglutination is noted, zygotes are formed, and the diplophase is reestablished. Sporulation is best on McClary's acetate agar after 3 to 5 days. Freshly isolated diploid strains also sporulate on YM agar or on dilute malt extract.

Glucose is fermented; a full tube of gas is produced after 3 to 5 days at 30°C. Assimilation of carbon compounds: positive for glucose, ethanol, glycerol, D-mannitol, lactic acid, succinic acid, gluconic acid, and ethyl acetate. Negative for galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, *i*-erythritol, ribitol, galactitol, D-glucitol, α -methyl-D-glycoside, salicin, 2-ketogluconate, 5-ketogluconate, citric acid, *meso*-inositol, D-glucosamine, *N*-acetylglucosamine, methanol, 2-propanol, acetone, and hexadecane.

KNO_3 and $NaNO_2$ are not utilized as sole sources of nitrogen; ethyl amine, L-lysine, and cadaverine are slowly utilized.

L-Methionine or L-cysteine and B vitamins are required in synthetic media. Growth occurs at 39°C and in some strains weakly at 41°C. G+C content of the nuclear DNA is 34.0 to 34.4 mol% (four strains, buoyant density).

Killer activity is lacking or extremely weak in all strains on *Candida glabrata* NRRL Y-55 as the sensitive strain.

Sensitivity to triterpene glycosides or digitonin is variable. The type strain is sensitive. Growth in the presence of 1 µg of cycloheximide per ml: negative.

Growth on YM agar–5% NaCl is weak to good, that on 7.5% NaCl is weak or negative, and that on 10% NaCl is negative.

Growth on 50% glucose is negative.

Casein hydrolysis is negative.

Gelatin hydrolysis is negative or latent and weak. Type strain is negative.

Lipolytic activity is negative.

Urease activity is negative.

Habitat is in necrotic tissue of cacti in the tribe *Opuntieae* and in columnar cacti of the subtribe *Pachycereinae*.

The type strain is UCD-FST 81-62, isolated from *Opuntia stricta* growing on one of the cays of the Exuma chain in the Bahamas. It has been deposited in the American Type Culture Collection under no. 76713 and in the Centraalbureau voor Schimmelcultures collection, Delft, The Netherlands, under no. 7692.

DISCUSSION

P. caribaea and *P. amethionina* are distinct but closely related species, as evidenced by their limited ability to mate and occasionally to form ascospores. Mating between haploid forms of closely related species is not uncommon, as reported by Kurtzman and co-workers (6, 7). When tested, spores in such asci were normally found to be nonviable (6).

DNA hybridization (Table 2) shows that *P. caribaea* exhibits approximately 40% base sequence complementarity with both varieties of *P. amethionina*. Although significant, this level of DNA relatedness is well below that usually found in conspecifics. Similar cases of significant degrees of DNA relatedness among cactophilic yeast species have been reported for *P. cactophila* and *P. pseudocactophila* (ca. 35%) (4) and between *P. antillensis* and *P. opuntiae* (ca. 50%) (16).

Phenotypically, *P. caribaea* is most similar to *P. amethionina* var. *pachycereana* in its assimilation pattern of carbon compounds, but it differs in its ability to ferment glucose vigorously. This ability by itself, however, does not guarantee that one is dealing with separate species. For example, Price et al. (8) showed that *Debaryomyces forficatus* and *D. vanrijae* exhibit 96% DNA complementarity, but the former ferments glucose (albeit slowly), while the latter does not.

Geographically, *P. caribaea* appears to be restricted to decaying tissue of *Opuntia* spp. and columnar cacti of the subtribe *Pachycereinae* on islands in the broad area composed of the Bahamas, the Caribbean Sea, the Gulf of Mexico, and coastal landmasses that border these areas (Table 1). Although widespread in this area, it has only rarely been isolated from members of the subtribe *Stenocereinae*. On certain Caribbean islands, where it was found in 9.5% of the samples tested, it accounted for 5.3% of the regional yeast community (13). In contrast, *P. amethionina* var. *amethionina* and *P. amethionina* var. *pachycereana* have not been found in the Caribbean area or in northern Venezuela but have overlapping distributions in the Sonoran desert and the southwestern United States, including the Baja California peninsula (15).

The principal host plants of *P. caribaea* are members of the subtribe *Pachycereinae* and *Opuntia* species which lack triterpene glycosides in their tissue (2, 12). On this basis, one would expect *P. caribaea* strains to be sensitive to these compounds (12), which are highly toxic to yeast strains not adapted to them, as are most strains of *P. amethionina* var. *pachycereana* (3). This was not the case, however, as we determined that approximately 40% of the strains listed in Table 1 were resistant. Even among the sensitive strains, of which most were totally inhibited from growing, some produced spotty resistant colonies that developed latently on plates containing triterpene glycosides, possibly owing to mutation. Resistance to triterpene glycosides is controlled by a single temperature-sensitive nuclear gene (12). To account for the occurrence of fully resistant strains from cacti that do not contain these inhibitory compounds, one could postulate that resistance to triterpene glycosides is the result of gene flow between strains of *P. amethionina* var. *amethionina* and *P. caribaea* by way of aneuploidy and nondisjunctive recombination, even without the sporulation process. However, our finding that these two species are not sympatric argues against this hypothesis. The few isolates of *P. caribaea* that did originate from cacti of the subtribe *Stenocereinae* were from the periphery of the geographic distribution, i.e., Venezuela and Mexico. Gene flow from resistant strains in these areas could also account for resistance in the centrally located strains. Because resistance to triterpene glycosides is most likely due to conformational changes in the cell membrane, it is also possible that the resistance is due to other membrane-associated adaptations of *P. caribaea*.

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