Resolution of the Varietal Relationship within the Species *Pichia opuntiae* and Establishment of a New Species, *Pichia thermotolerans* comb. nov.

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The varietal relationship within the species *Pichia opuntiae* was determined by a study of deoxyribonucleic acid relatedness and by a comparison of the electrophoretic mobilities of 12 metabolic enzymes. The nuclear genomes of *P. opuntiae* var. *opuntiae* and *P. opuntiae* var. *thermotolerans* showed about 28% relative binding to hydroxylapatite in intervarietal deoxyribonucleic acid reassociation experiments, and the allozymes encoded by eight loci were different in the two taxa. Based on these molecular criteria, as well as allopatry, different host plants, and weak sexual interaction, *P. opuntiae* var. *thermotolerans* is raised to the rank of species as *Pichia thermotolerans* (Starmer et al.) Holzschu et al. comb. nov.

Starmer et al. (20) described a new heterothallic cactus-specific yeast which they named *Pichia opuntiae*. Two varieties were designated based on differences in physiological properties, habitat, and geographic distribution. *P. opuntiae* var. *opuntiae* strains have been found only in necrotic tissue of *Opuntia inermis* (tribe Opuntieae, subtribe Opuntiinae) in Australia. This variety has a maximum temperature for growth of 30 to 33°C, and assimilates citric acid strongly, and cellobiose utilization is latent, weak, or negative. *P. opuntiae* var. *thermotolerans* is associated with rotting tissue of several species of columnar cacti (tribe Pachycereeae, subtribe Pachycereinae) in a number of locations in the North American Sonoran Desert. It grows well at 37°C (but not at 39°C) and assimilates cellobiose strongly but not citric acid. All other assimilation reactions of carbon compounds are the same. The reasons for considering these two taxa as varieties of a single species were the virtually identical guanine-plus-cytosine contents of their nuclear deoxyribonucleic acids (DNAs) and the observation that intervarietal matings of haploid heterothallic cells of opposite mating types produced a moderate number of zygotes, although extremely few asci were produced. In addition, the morphological properties of these varieties are similar, and the difference in physiological properties is small and may be based on a difference in only a few genes (15). However, there is a significant difference in sexual reproduction; haploid *P. opuntiae* var. *opuntiae* strains of opposite mating types conjugate well, and numerous viable spores are produced, whereas mating types of *P. opuntiae* var. *thermotolerans* conjugate infrequently and asci are therefore extremely rare. Presumably because of the strong mating ability of *P. opuntiae* var. *opuntiae*, intervarietal matings produce more zygotes than intravarietal matings of *P. opuntiae* var. *thermotolerans*. Starmer et al. (18) suggested that the two varieties of *P. opuntiae* could represent the final stages in the speciation process. In this study we conducted DNA-DNA hybridization experiments and compared the electrophoretic mobilities of a number of key metabolic

enzymes. Our results suggest that *P. opuntiae* var. *thermotolerans* should be raised to the rank of species.

MATERIALS AND METHODS

Strains. Yeast strains were obtained from the yeast culture collection of the Department of Food Science and Technology, University of California, Davis. The strains studied included all of those listed in the original taxonomic description of *P. opuntiae* (20), as well as a number of more recently isolated strains (Table 1).

Purification of DNA, base composition determination, and procedure for reassociation. DNA extraction and purification were carried out by using the methods described by Price et al. (15), except the 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. (10). The guanine-plus-cytosine contents of the nuclear DNAs were calculated from buoyant density values in cesium chloride gradients established in a Spinco model E analytical ultracentrifuge equipped with photographic optics (16, 22) and were based on three separate

TABLE 1. Localities and sources of isolates of *P. opuntiae* var. *thermotolerans* which were not included in the original taxonomic description

Strain(s) ^a	Geographic area of isolation	Source
79-251, 79-253	Tucson, Ariz.	<i>Carnegiea gigantea</i> (saguaro) ^b
80-203	El Arenoso, Baja California Norte, Mexico	<i>Pachycereus pringlei</i> (cardon)
80-204, 80-205	El Arenoso, Baja California Norte, Mexico	<i>Drosophila</i> larvae from <i>Pachycereus pringlei</i> (cardon)
80-206, 80-207	Punta Prieta, Baja California Sur, Mexico	<i>Pachycereus pringlei</i> (cardon)

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^a Culture Collection, Department of Food Science and Technology, University of California, Davis.

^b The common names of the cacti are given in parentheses.

TABLE 2. DNA base compositions and sources of yeast strains used in DNA-DNA hybridization experiments

Organism	Isolation locality	Source	Strain ^a	Guanine-plus-cytosine content (mol%) ^b
<i>P. opuntiae</i> var. <i>opuntiae</i>	Yarrowonga, Australia	<i>Opuntia inermis</i>	77-40 ^T	33.4 ± 0.5
	Yarrowonga, Australia	<i>Opuntia inermis</i>	77-42	33.3 ± 0.4
	Martindale, Australia	<i>Opuntia inermis</i>	77-159	33.0 ± 0.1
	Martindale, Australia	<i>Opuntia inermis</i>	77-162	33.1 ± 0.1
	Yarrowonga, Australia	<i>Opuntia inermis</i>	77-177	33.1 ± 0.2
	Yarrowonga, Australia	<i>Opuntia inermis</i>	78-304	32.9 ± 0.6
<i>P. opuntiae</i> var. <i>thermotolerans</i>	Baja California Sur, Mexico	<i>Lophocereus schottii</i>	76-211 ^T	33.0 ± 0.3
	Baja California Sur, Mexico	<i>Lophocereus schottii</i>	76-288	32.8 ± 0.2
	Sonora, Mexico	<i>Pachycereus pringlei</i>	76-411	32.9 ± 0.2
	Sonora, Mexico	<i>Pachycereus pringlei</i>	76-385-B	32.9 ± 0.3
	Tucson, Ariz.	<i>Carnegiea gigantea</i>	79-251	32.9 ± 0.2
	Tucson, Ariz.	<i>Carnegiea gigantea</i>	79-253	32.8 ± 0.2
<i>P. heedii</i>	Baja California Sur, Mexico	<i>Lophocereus schottii</i>	76-356 ^T	32.1 ± 0.3
<i>P. cactophila</i>	Baja California Sur, Mexico	<i>Stenocereus thurberi</i>	76-243A ^T	36.0 ± 0.3
<i>P. amethionina</i> var. <i>amethionina</i>	Sonora, Mexico	<i>Stenocereus alamosensis</i>	76-401B ^T	33.2 ± 0.1
<i>P. amethionina</i> var. <i>pachycereana</i>	Baja California Sur, Mexico	<i>Pachycereus pringlei</i>	76-384A ^T	32.4 ± 0.5

^a Culture collection, Department of Food Science and Technology, University of California, Davis.

^b Mean ± standard deviation, based on three independent determinations.

determinations. DNA from *Micrococcus luteus* strain 2039 (International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis), with a buoyant density of 1.7310 g/ml, was used as a reference. The buoyant density of the *M. luteus* DNA was derived from a 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, and analysis of the renaturation kinetics, reannealing reactions, and quantitation of single- and double-stranded DNAs were carried out according to the protocol of Price et al. (15). Samples were counted with a gamma ray counter (90% efficiency).

Allozyme analysis. For allozyme analyses, yeast cells were transferred from a stock slant to 5 ml of yeast autolysate (0.5%) supplemented with 5% glucose and incubated for 20 to 24 h at 30°C on a Rollor drum. Each sample was then used as an inoculum for 100 ml of the same medium in a 250-ml flask, which was incubated on a rotary shaker at 180 rpm for 20 to 24 h at 30°C. The cells were harvested by centrifugation, suspended in approximately 15 ml of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4), and disrupted in a Bronwill cell homogenizer containing glass beads (diameter, 0.5 mm) for 3 min with CO₂ cooling. After cell breakage, a 1- to 2-ml portion of each suspension was removed with a Pasteur pipette, placed in a single well of a plastic tray (24 samples per tray), covered, and frozen immediately. The samples were stored at -20°C for not more than 3 days before transfer to -80°C.

The protocols used for starch gel preparation, buffer preparation, electrophoresis, gel cutting, and staining have been described by Ayala et al. (4), Tracey et al. (23), Harris and Hopkinson (8), and Shaw and Prasad (17). The enzymes studied were resolved by using the following four buffer systems (see references 4, 8, 17, and 23 for buffer strengths): discontinuous tris(hydroxymethyl)aminomethane-citrate electrode buffer (pH 8.65) and borate (NaOH) gel buffer (pH 8.1) (buffer system A); continuous tris(hydroxymethyl)aminomethane-borate-ethylenediaminetetraacetate (pH 9.1) electrode and gel buffer (buffer system B); continuous tris(hydroxymethyl)aminomethane-citrate-ethylenediaminetetraacetate electrode buffer (pH 7.0) and,

for gel buffer, a 15-fold dilution of the electrode buffer (buffer system C); and continuous phosphate-citrate (pH 7.0) electrode and gel buffer (buffer system D). The banding patterns of the following enzymes were resolved by specific staining after horizontal electrophoresis in the buffer system indicated in parentheses: alcohol dehydrogenase (buffer system B); fumarase (buffer system C); glucose-6-phosphate dehydrogenase (buffer system A); hexokinase (buffer system B); leucine aminopeptidase (buffer system A); malate dehydrogenase (buffer system B); glucose phosphate isomerase (buffer system D); phosphoglucosmutase (buffer system C); tetrazolium reductase (buffer system B); and triose phosphate isomerase (buffer system B). Activity stains (4) were prepared 30 to 60 min before use, and horizontal slices of the starch gels were incubated in the staining solutions in the dark until the indicator dyes appeared. Gels were fixed and stored as described by Ayala et al. (4). A total of 26 samples were run in each starch gel (19.5 by 17.5 by 1 cm) by placing wicks soaked in cell extracts approximately 0.3 cm apart. The genetic basis of zymograms was inferred from their similarity to zymograms of other species whose Mendelian inheritance had been analyzed. In this study, however, we referred to the allozyme bands as electromorphs rather than as alleles because the genetic basis of the bands is incompletely known in *P. opuntiae* and *Pichia thermotolerans*. The nomenclature used for the gene-enzyme systems follows that of Ayala et al. (4), in which the loci encoding the enzymes are designated by italicized enzyme abbreviations.

The type strain of *Pichia heedii* Phaff et al. (strain 76-356) was used as the reference strain for determining the relative mobilities of the corresponding enzymes from other yeasts. The mobility for each gene-enzyme system detected in strain 76-356^T (T = type strain), was arbitrarily assigned a value of 100; designations for allozymes from other strains were obtained by adding or subtracting the number of millimeters separating the corresponding band from the band at a value of 100 (23). Controls to determine the identities of electromorphs were included in every gel by including the standard strain, 76-356^T, and by cross-controlling each set of gels. In addition, representatives of each species were run side by side on a series of gels so that direct comparisons of enzyme mobilities could be made.

TABLE 3. Reassociation of ¹²⁵I-labeled nuclear DNA isolated from *P. opuntiae* var. *opuntiae* 77-40^T with DNAs of other cactophilic yeast strains^a

Organism or tissue	Strain	Level of Relative binding (%) ^b
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-40 ^T	100
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-42	97.4 ± 1.4
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-159	100.3 ± 0.3
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-162	95.3 ± 0.6
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-177	94.1 ± 1.0
<i>P. opuntiae</i> var. <i>opuntiae</i>	78-304	93.6 ± 0.7
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-211 ^T	28.1 ± 0.3 (26.4 ± 2.1) ^c
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-385B	28.8 ± 0.7 (26.7 ± 1.0)
<i>P. amethionina</i> var. <i>amethionina</i>	76-401B ^T	6.1 ± 0.1
<i>P. amethionina</i> var. <i>pachycereana</i>	76-384A ^T	6.9 ± 0.2
<i>P. heedii</i>	76-356 ^T	2.2 ± 0.3
<i>P. cactophila</i>	76-243A ^T	2.2 ± 0.3
Calf thymus DNA		1.0 ± 1.0

^a ¹²⁵I-labeled DNA (0.2 µg) and unlabeled DNA (200 µg) were incubated at 61.2°C for 25 h in 0.5 ml of 280 mM phosphate buffer.

^b Mean ± standard deviation. Average of triplicate tests, corrected for zero-time binding (0.5%) and for self reassociation of labeled DNA (2.0 ± 0.8%) (15).

^c The data in parentheses are the results from an independent experiment done under comparable conditions (zero-time binding, 0.6%; self-reassociation, 3.8 ± 0.5%).

RESULTS

DNA hybridization experiments. The guanine-plus-cytosine contents of the nuclear DNAs of the yeast strains included in this study are shown in Table 2. A number of representative strains were examined by DNA-DNA reassociation for intravarietal homogeneity, intervarietal affinity, and interspecific similarity.

The results of experiments in which we compared the base sequence similarity of *P. opuntiae* var. *opuntiae* and *P. opuntiae* var. *thermotolerans* are shown in Tables 3 and 4. The cactus-specific species *P. heedii*, *Pichia amethionina*, and *Pichia cactophila* are not closely related to *P. opuntiae* var. *opuntiae* or *P. opuntiae* var. *thermotolerans*. The two varieties of *P. opuntiae* are well-defined taxa, as indicated by

TABLE 4. Reassociation of ¹²⁵I-labeled nuclear DNA isolated from *P. opuntiae* var. *thermotolerans* 76-211^T with DNAs of other cactophilic yeast strains^a

Organism or tissue	Strain	Level of relative binding (%) ^b
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-211 ^T	100
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-411	99.0 ± 2.5
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-288	99.7 ± 0.7
<i>P. opuntiae</i> var. <i>thermotolerans</i>	79-251	95.5 ± 1.7
<i>P. opuntiae</i> var. <i>thermotolerans</i>	79-253	95.3 ± 2.6
<i>P. opuntiae</i> var. <i>thermotolerans</i>	76-385B	90.0 ± 0.7
<i>P. opuntiae</i> var. <i>opuntiae</i>	77-40 ^T	34.0 ± 0.9
<i>P. amethionina</i> var. <i>amethionina</i>	76-401B ^T	9.0 ± 0.2
<i>P. heedii</i>	76-356 ^T	9.7 ± 1.0
<i>P. cactophila</i>	76-243A ^T	9.1 ± 1.5
Calf thymus DNA		1.6 ± 0.9

^a ¹²⁵I-labeled DNA (0.2 µg) and unlabeled DNA (200 µg) were incubated at 61.2°C for 25 h in 0.5 ml of 280 mM phosphate buffer.

^b Mean ± standard deviation. Average of triplicate tests, corrected for zero-time binding (1.0%) and for self-reassociation of labeled DNA (5.0 ± 0.3%) (15).

TABLE 5. Occurrence of electromorphs with frequencies of at least 0.05 in populations of *P. opuntiae* var. *opuntiae* and *P. opuntiae* var. *thermotolerans*

Locus ^a	No. of haploid strains examined	Electromorph(s) ^b	Frequency in:	
			<i>P. opuntiae</i> var. <i>opuntiae</i>	<i>P. opuntiae</i> var. <i>thermotolerans</i>
<i>Adh</i>	12	100	— ^c	1.00
<i>Fum</i>	29	92		1.00
		100	1.00	
<i>Tr</i>	29	104	1.00	1.00
<i>G-6-pdh</i>	17	110	1.00	—
<i>Hk-1</i>	29	99		1.00
		100	1.00	
<i>Hk-2</i>	29	85	1.00	
		96		1.00
<i>Hk-3</i>	29	85	0.50	
		87	0.50	
		96		1.00
<i>Lap</i>	29	96		1.00
		97	0.44	
		100	0.56	
<i>Mdh</i>	29	101		1.00
		106	1.00	
<i>Pgm</i>	29	128	0.94	1.00
		135	0.06	
<i>Gpi</i>	29	108		0.08
		110		0.92
		113	1.00	
<i>Tpi</i>	29	102		0.75
		104		0.25
		113	1.00	

^a The locus designations are for the following enzymes: *Adh*, alcohol dehydrogenase; *Fum*, fumarase; *Tr*, tetrazolium reductase; *G-6-pdh*, glucose-6-phosphate dehydrogenase; *Hk-1*, hexokinase 1; *Hk-2*, hexokinase 2; *Hk-3*, hexokinase 3; *Lap*, leucine aminopeptidase; *Mdh*, malate dehydrogenase; *Pgm*, phosphoglucosyltransferase; *Gpi*, glucose phosphate isomerase; *Tpi*, triose phosphate isomerase.

^b Electromorphs were designated in reference to the standard electromorphs from *P. heedii* 76-356^T, which were arbitrarily assigned values of 100 (23) (see text).

^c —, No data (band not resolved in the gel-buffer system used).

the high degree of DNA complementarity among the strains tested within the two varieties (more than 90% relative binding). The mean relative binding level when the two varieties were compared was 28.0 ± 3.1%, based on five pair-wise comparisons (including reverse experiments). The low level of base sequence similarity supports our proposal that the two varieties of *P. opuntiae* should be divided into two separate but closely related species.

Allozyme analysis. *P. opuntiae* var. *opuntiae* and *P. opuntiae* var. *thermotolerans* were compared by allozyme analyses at 12 loci (Table 5). The allozyme pattern of *P. opuntiae* var. *thermotolerans* was unique and was easily distinguished from that of *P. opuntiae* var. *opuntiae*. The allozymes encoded by the loci *Fum*, *HK-1*, *HK-2*, *HK-3*, *Lap*, *Mdh*, *Gpi*, and *Tpi* were different in the two taxa and thus allowed unequivocal identification of the proposed species. The experiments described here were undertaken to obtain additional measures of genetic diversity between the two varieties of *P. opuntiae*. The lack of interfertility between *P. opuntiae* var. *opuntiae* and *P. opuntiae* var. *thermotolerans* observed in our laboratory (a moderate number of zygotes are produced, but very few asci are observed) indicates that these two varieties have become genetically isolated, perhaps due to allopatry and different host plants. *P. opuntiae* var. *opuntiae* occurs in rots of *O.*

inermis in Australia, and strains of this variety have not been isolated thus far from *Opuntia* species in Arizona and Texas (19) or on various Caribbean islands (H. J. Phaff and W. T. Starmer, unpublished data). On the other hand, *P. opuntiae* var. *thermotolerans* has been found only in columnar cacti of the North American Sonoran Desert. The molecular data presented here and the reproductive isolation (7, 14) of the two varieties lead us to propose that *P. opuntiae* var. *thermotolerans* should be elevated to the rank of species as *Pichia thermotolerans* (Starmer et al.) Holzschu et al. comb. nov. (basonym, *Pichia opuntiae* var. *thermotolerans* Starmer et al. Int. J. Syst. Bacteriol. 29:161, 1979).

DISCUSSION

The experiments described above were undertaken to obtain additional measures of the genetic diversity between *P. opuntiae* and *P. thermotolerans*. DNA-DNA complementarity values and allozyme analyses demonstrated that the two species are very different at the genetic level. The mean level of interspecific relative binding to hydroxylapatite of the reassociated DNAs of the two species was $28.0 \pm 3.1\%$ based on five pair-wise comparisons. Actual DNA-DNA complementarity values are probably somewhat higher because in experiments with distantly related species self-renaturation of the unlabeled DNA (driver DNA) occurs at a faster rate than the renaturation of the driver DNA with the heterologous labeled probe DNA. As a result, the driver DNA concentration is rapidly reduced in the reaction mixture, thereby preventing it from reannealing with the probe DNA. The renaturation kinetics of DNAs isolated from *P. opuntiae* 77-40^T and *P. thermotolerans* 76-211^T were followed by a C_0t analysis in one experiment (C_0t is the initial nucleotide concentration in moles per liter $\times$ time in seconds [6]) and were compared with the homologous *P. opuntiae* 77-40^T renaturation reaction (data not shown). The renaturation curve (6) of the heterologous reaction was shifted to the right of the homologous curve. The kinetics of renaturation are second order and depend on the concentration of driver DNA. The shift to the right by the heterologous reaction in comparison with the homologous reaction illustrates the point that the driver DNA was being depleted from the reaction, and therefore our complementarity values are lower than those that could theoretically be obtained. Nevertheless, the relatively low DNA complementarity value supports the species status of *P. thermotolerans*.

Another recently described heterothallic species, *Pichia antillensis* (21), appears to be more closely related to *P. opuntiae* than *P. thermotolerans* is. In reassociation experiments the DNA of *P. antillensis* showed about 55% relative binding with *P. opuntiae* reference DNA, compared with the value of approximately 28% observed between *P. opuntiae* and *P. thermotolerans*. Despite the higher level of DNA complementarity, the mating types of *P. antillensis* showed no sexual interaction with those of *P. opuntiae* (21).

Similar to our results with *P. opuntiae* and *P. thermotolerans*, Kurtzman et al. (13) observed interspecific mating between *Pichia amylophila* and *Pichia mississippiensis*, but ascospores from the crosses were infertile. These two species exhibited about 25% DNA relatedness. Similarly, *Hansenula bimundalis* and *Hansenula americana* (with about 21% DNA relatedness) showed poor conjugation, and ascospores were not produced (11). In contrast, *Issatchenkia scutulata* var. *scutulata* and *I. scutulata* var. *exigua* (with about 25% DNA relatedness) showed good conjugation between appropriate mating types, and the zygotes produced a low percentage of viable spores (12). On

the basis of spore viability, Kurtzman et al. retained these yeasts as varieties of *I. scutulata* (12). In matings between *P. thermotolerans* and *P. opuntiae* asci were so rare that spore viability could not be tested.

A study of the electrophoretic mobilities of 12 metabolic enzymes revealed that *P. opuntiae* and *P. thermotolerans* can be differentiated unequivocally by differences in the electrophoretic mobilities of eight enzymes. The allozyme patterns of fumarase, hexokinases 1, 2, and 3, leucine aminopeptidase, malate dehydrogenase, glucosephosphate isomerase, and triose phosphate isomerase can be used in the formal descriptions of *P. thermotolerans* and *P. opuntiae* as additional criteria to differentiate the two sibling species, as was done previously by Holzschu et al. (9) in the differentiation of *Pichia pseudocactophila* from *P. cactophila* and by Baptist and Kurtzman (5) in separating *Cryptococcus magnus* and *Cryptococcus heveanensis*. A previous use of this technique to differentiate subspecies of *Drosophila* has been described by Ayala and co-workers (1-3).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM-16370 from the National Institute of General Medical Sciences to H.J.P. and by grant 4803 from the Institute of Ecology, University of California, Davis, to D.H., H.J.P. and D.L.H.

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