Pichia amethionina, a New Heterothallic Yeast Associated with the Decaying Stems of Cereoid Cacti

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A new species of the genus *Pichia* has been recovered 38 times in the Sonoran Desert from "rot pockets" of cereoid cacti and from *Drosophila* species which utilize the cacti. We have named the species *Pichia amethionina* due to its absolute requirement for methionine or cysteine. *P. amethionina* is heterothallic and demonstrates an agglutination reaction when opposite mating types are mixed. Two varieties are designated based on the combination of mannitol assimilation and ecological habitat. *P. amethionina* var. *amethionina*, the type variety, was recovered from cacti in the subtribe *Stenocereinae* and cannot assimilate mannitol, whereas *P. amethionina* var. *pachycereana* was found in cacti of the subtribe *Pachycereinae* and can assimilate mannitol. Results are given which demonstrate that the assimilation of mannitol is controlled by a single genetic locus. An evaluation of the interfertility and postmating viability among the two varieties and possibly identical organisms was made. The base composition of the nuclear deoxyribonucleic acid (average of 10 strains) is 33.05 ± 0.19 mol% guanine plus cytosine. The type strain of *P. amethionina* and of the type variety, *P. amethionina* var. *amethionina*, is UCD-FST 76-401B (=ATCC 36080 = CBS 6940). The type strain of *P. amethionina* var. *pachycereana* is UCD-FST 76-384A (=ATCC 36079 = CBS 6943).

Most yeasts we have recovered from cactus necroses are cactus specific (6, 7). The host specificity ranges from species which occur predominantly in one major host plant (e.g., *Pichia heedii* [8]) to species which inhabit many cactus substrates but which are limited to cacti in general (e.g., *P. cactophila* [12]). The ultimate causes for specific associations have yet to be determined and probably involve interaction at various levels of biological organization. Nevertheless, the first important step for uncovering causal components is a thorough taxonomic treatment of the yeast floras found in the cactus "soft-rot" habitat. Some of the factors which are necessary for species determination are: (i) physiological profiles as revealed by tests of assimilation, fermentation, and physiological tolerance of various compounds; (ii) sexuality and interfertility among isolates; (iii) characterization of the nuclear deoxyribonucleic acids (DNAs) of representative strains; (iv) ecological constraints and flexibilities exhibited by the natural habitat and physical environment, and (v) genetic cohesiveness indicated by coordinated observations on the physiology, ecology, and sexual fertility of geographically widespread representative strains.

An earlier survey of cactus soft-rots (3, 11)

revealed a yeast similar to *P. delftensis* Beech occurring in the necroses of *Carnegie gigantea* (Engelm.) Britt. and Rose and the associated fly *Drosophila nigrospiracula* Patterson and Wheeler. The cactus yeast strains were found to have nuclear DNA base compositions almost identical with that of *P. delftensis* and only minor differences in physiological profile. However, the type strain of *P. delftensis* had been isolated from apple juice in England, and it has a lower maximum temperature for growth (4). The dissimilarity of habitats for the cactus yeast and *P. delftensis* was suggestive of different species; yet taken alone, the evidence for different species was not sufficient. Additional surveys of cactus soft-rots and subsequent laboratory studies revealed that the yeast isolates from cactus were unique in several respects. Probably the most striking characteristic is an absolute requirement for methionine or cysteine, the sulfur-containing amino acids.

In addition, single-spore isolates of representative strains showed the species to be heterothallic and to possess a strong agglutination reaction when opposite mating types are mixed. This provided the necessary material for demonstrating fertility or infertility among strains and with possibly identical organisms. Matings between single-spore clones derived from the cactus isolates with *P. delftensis* asporogenous single-spore clones were unsuccessful.

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When the habitat, methionine requirement, and sexual fertility are considered together, the cactus isolates constitute a distinct species different from *P. delftensis*. We have named this new species *P. amethionina* due to the inability of the organism to synthesize methionine from ammonium sulfate. Furthermore, we have recognized two varieties based on ability to assimilate D-mannitol and on ecological associations with two different subtribes of cacti. *P. amethionina* var. *amethionina*, the type variety, is found mainly in some cacti belonging to the subtribe *Stenocereinae* and cannot assimilate D-mannitol. *P. amethionina* var. *pachycereana* can assimilate D-mannitol and is found predominantly in soft-rots of some cactus species belonging to the subtribe *Pachycereinae*.

MATERIALS AND METHODS

Samples of necrotic cactus tissue were collected in various areas of the North American Sonoran Desert (Fig. 1). Most of the isolations were accomplished by streaking a loopful of necrotic tissue directly on acidified yeast extract-malt extract agar (Difco YM agar + 0.7% [vol/vol] 1 N HCl = AYM, pH 3.7 to 3.8) in the field. In some cases, dilutions of the tissue were made in sterile water as described earlier (11). The plates were stored at ambient temperatures (24 to 30°C) until colonies appeared. Counts of morphologically distinct colony types were then made, and a representative of each type was brought into pure culture by two successive platings on YM agar for identification. Based on the subsequent identification and recognition of *P. amethionina* as a separate species, the colonies of this species on the isolation medium were recognized as convex, highly glossy on

FIG. 1. Collection localities for isolates of *Pichia amethionina* listed in Table 1.

glossy, and smooth but sometimes with a textured surface.

Some isolates were obtained from adult drosophilae captured near cacti with necrotic tissue. The flies were surface sterilized in 70% ethanol (for about 1 min), rinsed in 10 ml of sterile water, and then squashed and streaked directly on AYM agar plates in the field.

Identification was done by standard methods currently used in yeast taxonomy (14). DNA extraction and purification were done by a combination of the procedures of Marmur (5) and Bernardi et al. (1). The guanine-plus-cytosine (G+C) content of the DNA was calculated from buoyant-density values in cesium chloride (10, 13) and was based on two to six separate determinations. *Micrococcus lysodeikticus* DNA, with a buoyant density of 1.7311 g/ml, was used as a reference. The buoyant density of the *M. lysodeikticus* DNA was derived from comparison with plasmid-free *Escherichia coli* K-12 DNA, the buoyant density of which was taken to be 1.7100.

Single ascospores were isolated from mature asci with a micromanipulator (2) and sometimes after treatment of the sporulating suspension of cells and asci with the cell wall-digesting enzyme Zymolyase.

RESULTS

Latin diagnosis of *Pichia amethionina* sp. nov.

Pichia amethionina var. *amethionina* var. nov. In extracto malti cellulae cylindricae aut ovoideae, 1.6–4.3 × 4.0–9.3 μm, interdum longitudo 12.2 μm, singulae, binae, in catenis brevis, aut in fasciculae; pellicula nulla aut tenuis, non-nitida.

Cultura in agar malti post unum mensem (25°C) crocea (in centro) et cana (in margine), butyrosa vel mollis, umbonata, glabra cum lineis radiatis, patula, nitida vel semi-nitida, margine glabro vel undulato.

In agar farinae Zea mais post dies 10, pseudomycelium nullum; interdum paucae cellulae oblongae formantur. Species heterothallica, diploidea aut haploidea. Asci ex cellulae diploidae inconjugati fiunt, habentes 4 sporos pileiformae; asci rumpunter post 5–7 dies.

Fermentatio glucosi nullum.

Glucosum, ethanolum, glycerolum, glucono-δ-lactonum, acidum lacticum, et acidum succinicum assimilantur at non galactosum, L-sorbosum, maltosum, saccharum, cellobiosum, trehalosum, lactosum, melibiosum, raffinose, melezitosum, inulinum, amyllum solubile, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum, methanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, α-methyl-D-glucosidum, salicinum, 2- et 5-ketogluconatum, D-glucosaminum, acidum citricum nec inositolum.

Kalium nitricum et natrium nitrosum non assimilantur.

Ethyl aminum assimilatur.

Ad crescentiam pyridoxinum et thiaminum necessariae sunt.

Crescere potest in 39°C, interdum in 42°C.

G+C acidi deoxyribonucleati = 33.0–33.1 mol% (6 stirpes, vide tabulum 3).

Habitatio in cacti subtribus *Stenocereinae*.

Typus: Stirps UCD (FST) 76-401B ex tabidosus sacculis cacti *Rathbunia alamosensis* isolata est.

In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum sub. no 6940 deposita est.

Pichia amethionina var. *pachycereana* var. nov. Varietas a *P. amethionina* var. *amethionina* differt: D-mannitolum assimilatur. G+C acidi deoxyribonucleati = 32.8–33.6 mol% (4 stirpes, vide tabulum 3). Habitatio in cacti subtribus *Pachycereinae*.

Typus: Stirps UCD (FST) 76-384A ex tabidus sacculis cacti *Pachycereus pringlei* isolata est.

In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum sub no. 6943 deposita est.

Standard description of *Pichia amethionina* sp. nov.

Pichia amethionina var. *amethionina* var. nov. Growth in malt extract: After 3 days at 25°C the cells are thin, cylindrical, or ovoidal, 1.6 to 4.3 by 4.0 to 9.3 μm. Occasionally cells occur which are up to 12.2 μm long. The thallus consists of single cells, pairs, branched chains, and starlike clusters (Fig. 2a, b, and c). A pellicle is usually not formed, but extremely thin, smooth, and dull surface growth may occur. Little sediment is formed.

Growth on malt extract agar: After 1 month at 25°C, the streak culture is yellowish (central portion) to grayish (periphery), butyrous to soft, umbonate, smooth with slight radial striations, spreading, and glossy to semiglossy; the border is entire to lobulate.

Dalman plate culture on cornmeal agar: Most strains form no pseudomycelium either aerobically or anaerobically. In a few strains a very rudimentary pseudomycelium is present consisting of a few elongated cells.

Formation of ascospores: The vegetative cells are heterothallic and they are isolated as diploid as well as haploid cells. Ascospores are formed best on YM agar after 5 to 7 days at about 25°C. The spores are hat-shaped, and there are usually four per ascus. Two of the spores are of mating type a and two are of mating type α. The asci rupture at maturity, releasing the spores soon after formation.

Other characteristics: Ability to ferment is

lacking. Assimilation of carbon compounds: D-glucose, ethanol, glycerol, glucono- δ -lactone, D-lactic acid, and succinic acid are assimilated. The following compounds are not assimilated: D-galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, methanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α -methyl-D-glucoside, salicin, 2- and 5-ketogluconate, D-glucosamine, citric acid, and *meso*-inositol.

Assimilation of nitrogen compounds: Potassium nitrate, —; sodium nitrite, —; ethylamine, +; ammonium sulfate, +.

Growth in vitamin-free medium: Absent. Pyridoxine and thiamine are required for growth.

Other growth requirements: Sulfate does not serve as a source of sulfur; synthetic media must be supplemented with 10 mg of L-methionine or L-cysteine per liter.

Growth on 50% (wt/wt) glucose-yeast extract agar: Negative. Growth on 10% NaCl plus 5% glucose in yeast nitrogen base agar: Negative.

Maximum temperature for growth: 39 to 42°C, depending on the strain.

Acid formation on chalk agar: Negative, occasionally weak.

Hydrolysis of urea: Negative.

Gelatin liquefaction: Negative.

Casein hydrolysis: Negative.

Lipolytic activity: Negative.

Growth in the presence of 0.1 mg of cycloheximide per ml: Negative.

G+C content of the nuclear DNA: 33.0 to 33.1 mol% (six strains, Table 3).

Habitat: Twenty-eight strains were recovered in Baja California, and the State of Sonora, Mexico (Tables 1 and 2, Fig. 1) from necrotic tissue of various species of cacti, predominantly members of the subtribe *Stenocereinae*.

Type: The type strain of *P. amethionina*, and thus also of *P. amethionina* var. *amethionina*, the type variety, is strain UCD (FST) 76-401B; it was isolated from necrotic tissue of *Rathbunia alamosensis* near Guaymas (Fig. 1, Table 1). It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures in Delft, The Netherlands, as CBS 6940, and in the American Type Culture Collection,

Rockville, Md., as ATCC 36080.

Etymology: *amethionina*. L. prep. *a* without: M.L. n. *methioninum* methionine; M.L. adj. *amethionina* without methionine, i.e., pertaining to the organism's lack of biosynthesis of methionine from sulfate.

Pichia amethionina var. *pachycereana* var. nov.

Assimilation of D-mannitol: Positive.

G+C content of the nuclear DNA: 32.8 to 33.5 mol% (four strains, Table 3).

Habitat: Ten strains were recovered in various locations of the North American Sonoran Desert (Tables 1 and 2, Fig. 1) from necrotic tissue of various species of cacti, predominantly members of the subtribe *Pachycereinae*.

Type: The type strain, UCD (FST) 76-384A, was isolated from *Pachycereus pringlei* near Mulegé, Baja California (Table 1, Fig. 1). It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures in Delft, The Netherlands, as CBS 6943 and in the American Type Culture Collection, Rockville, Md., as ATCC 36079.

Etymology: *pachycereana*. M.L. *Pachycereinae* subtribe of cactus: L. adj. *ana* association with; M.L. *pachycereana* referring to its habitat in cactus species of the subtribe *Pachycereinae*.

DISCUSSION

P. amethionina has an absolute requirement for methionine or cysteine, sulfur-containing amino acids (Table 4). This is due to the inability of the species to convert hydrogen sulfite to hydrogen sulfide, a complex step in the metabolism of sulfate (9). It is noteworthy that some variability exists for the conversion of cystathionine into the required amino acid. The variability is present in both varieties of *P. amethionina* and is exhibited by latent growth (mutation followed by selective adaptation) when cystathionine is used as the precursor. The methionine requirement was found in all strains of *P. amethionina* and is thus widespread (Fig. 1) throughout the Sonoran Desert. This property may be an adaptive character which has allowed the species to assume its present niche in the cactus soft-rot microcosm. The reasons for this

FIG. 2. Vegetative reproduction (3 days at room temperature in malt extract) and ascospore germination of *P. amethionina*. The vegetative cells occur singly, in pairs, or in branched chains (a) and frequently in asteroid 110 formations (b). The extent of asteroid formation is strain dependent. (c) Illustrates the arrangement of the formations (b). The extent of asteroid formation is strain dependent. (c) Illustrates the arrangement of the asteroid clusters as a tendency of buds to form at polar shoulders and remaining undissociated. Tightly packed hat- to helmet-shaped spores are formed in diploid cells (d). As the asci dehisce (e) the spores remain associated. Upon mixing of haploid clones derived from single spores, rapid conjugation occurs (f), reestablishing the diploid condition. The bars represent 5 μ m in all illustrations.

TABLE 1. *Localities and sources for isolates of Pichia amethionina*

Variety	Serial no.	UCD-FST no.	Locality ^a	Habitat/substrate
<i>P. amethionina</i> <i>amethionina</i>	var. 1 (type)	76-401B	Guaymas, Sonora, Mexico (a)	<i>Rathbunia alamosensis</i>
	2	71-152	Bahia Kino, Sonora, Mexico (b)	<i>Machrocereus gummosus</i>
	3	76-217C	Rancho Cuñaño, Baja California Sur, Mexico (e)	<i>M. gummosus</i>
	4	76-219C	Las Planos, Baja California Sur, Mexico (g)	<i>M. gummosus</i>
	5	76-223A	Punta Pescadero, Baja California Sur, Mexico (h)	<i>M. gummosus</i>
	6, 7	76-233A, 76-240B	Todos Santos, Baja California Sur, Mexico (i)	<i>M. gummosus</i>
	8, 9	76-266A, 76-267A	Mission San Luis Gonzaga, Baja California Sur, Mexico (k)	<i>M. gummosus</i>
	10	76-279A	La Paz, Baja California Sur, Mexico (l)	<i>M. gummosus</i>
	11	76-284A	El Centenario, Baja California Sur, Mexico (m)	<i>M. gummosus</i>
	12	76-296A	North of La Paz, Baja California Sur, Mexico (n)	<i>Lemaireocereus thurberi</i>
	13, 14, 15	76-297B, 76-299A, 76-300A	Santiago, Baja California Sur, Mexico (o)	<i>M. gummosus</i>
	16	76-309C	Palmilla, Baja California Sur, Mexico (p)	<i>M. gummosus</i>
	17	76-315B	East of Insurgentes, Baja California Sur, Mexico (q)	<i>M. gummosus</i>
	18	76-320A	Loreto, Baja California Sur, Mexico (r)	<i>M. gummosus</i>
	19	76-344	San Borja, Baja California Norte, Mexico (t)	<i>Lophocereus schottii</i>
	20	76-353B	Las Flores, Bahia de Los Angeles, Baja California Norte, Mexico (u)	<i>M. gummosus</i>
	21	76-354A	Mulegé, Baja California Sur, Mexico (s)	<i>M. gummosus</i>
	22, 23, 24, 25, 26, 27, 28	77-352A, 77-354B, 77-360E, 77-367A, 77-390A, 77-399A, 77-401B	El Socorro, Baja California Norte, Mexico (r)	<i>Drosophila mojavensis</i>
<i>P. amethionina</i> <i>pachycereana</i>	var. 29	72-109	Caborca, Sonora, Mexico (c)	<i>L. schottii</i>
	30, 31, 32, 33, 34	72-318, 72-319, 72-321, 72-322, 73-63	Tucson, Ariz. (d)	<i>Carnegie gigantea</i>
	35	76-216	Rancho Cuñaño, Baja California Sur, Mexico (e)	<i>L. schottii</i>
	36	76-227A	San Pedro, Baja California Sur, Mexico (f)	<i>Pachycereus pringlei</i>
	37	76-247B	Isla San Jose, Baja California Sur, Mexico (j)	<i>M. gummosus</i>
	38 (type)	76-384A	Mulegé, Baja California Sur, Mexico (s)	<i>P. pringlei</i>

^a The letters in parentheses correspond to the geographic locations shown in Fig. 1.

TABLE 2. Sources and frequencies of isolation of strains of the two varieties of *Pichia amethionina*

Source	No. of strains of:	
	<i>P. amethionina</i> var. <i>amethionina</i>	<i>P. amethionina</i> var. <i>pachycereana</i>
<i>Macherocereus gummosus</i> (Engelm.) Britt. & Rose [agria] ^a	18	1
<i>Drosophila mojavensis</i> Patterson & Crow	7	0
<i>Lemaireocereus thurberi</i> (Engelm.) Britt. & Rose [organpipe]	1	0
<i>Rathbunia alamosensis</i> (Coulter) Britt. & Rose [cina]	1	0
<i>Carnegia gigantea</i> (Engelm.) Britt. & Rose [saguaro]	0	5
<i>Lophocereus schottii</i> (Engelm.) Britt. & Rose [senita]	1	2
<i>Pachycereus pringlei</i> (S. Wats.) Britt. & Rose [cardon]	0	2
Total	28	10

^a Common names of the cacti are in brackets following the scientific names.

are unclear, but additional studies into this unique character should provide insight.

Both diploid and haploid strains of *P. amethionina* were recovered from the natural habitats. Haploid strains of both varieties were shown to be interfertile among one another. However, varying degrees of postmating breakdown were indicated by low viability of ascospores. For example, a number of four-spored asci were dissected from a hybrid of the two varieties. The parents (strain 27 $\alpha \times$ strain 35 α) of the hybrid were isolated over 800 km apart on the peninsula of Baja California, Mexico. Fifty dissected asci, containing four spores each, yielded 103 viable spores (viability = $103/200 = 51.5\%$). Parental strains showed about 80% spore viability. All spore viability classes were present (Table 5), which demonstrates that the inviability is not due to a simple genetic phenomenon such as a reciprocal translocation, where only the zero, two, and four spore viability classes are expected. In fact, the distribution of viability classes fits a binomial distribution ($\chi^2 = 9.98$; $df = 4$; $\alpha = 0.05$), indicating the possibility of multiple genetic factors. One cross between the two varieties (strain 2 $\alpha \times$ strain 38 α) produced four-spored asci but 0% viability (0/40 spores viable). Other intervarietal crosses demonstrated higher

TABLE 3. Nuclear DNA base compositions of representative strains of *P. amethionina*^a

Strain	Locality	Substrate	Mol% G+C $\pm$ SD ^b	Assimilation of mannitol
1	Guaymas, Sonora, Mexico	<i>Rathbunia alamosensis</i>	33.1 $\pm$ 0.14	—
2	Bahia Kino, Sonora, Mexico	<i>Macherocereus gummosus</i>	33.0 $\pm$ 0.33	—
17	East of Insurgentes, Baja California Sur, Mexico	<i>M. gummosus</i>	33.1 $\pm$ 0.13	—
20	Las Flores, Bahia de Los Angeles, Baja California Norte, Mexico	<i>M. gummosus</i>	33.1 $\pm$ 0.08	—
27	El Socorro, Baja California Norte, Mexico	<i>Drosophila mojavensis</i>	33.0 $\pm$ 0.13	—
28	El Socorro, Baja California Norte, Mexico	<i>D. mojavensis</i>	33.0 $\pm$ 0.13	—
31	Tucson, Ariz.	<i>Carnegia gigantea</i>	33.54 $\pm$ 0.09 ^c	+
34	Tucson, Ariz.	<i>C. gigantea</i>	32.83 $\pm$ 0.04	+
35	Rancho Cuñaño, Baja California Sur, Mexico	<i>Lophocereus schottii</i>	32.9 $\pm$ 0.16	+
38	Mulegé, Baja California Sur, Mexico	<i>Pachycereus pringlei</i>	33.0 $\pm$ 0.19	+

^a Strains 31, 34, 35, and 38 represent *P. amethionina* var. *pachycereana*; the remaining strains represent *P. amethionina* var. *amethionina*.

^b The value for strain 1 is based on six separate determinations; the value for strain 31 is based on four determinations; the values for strains 17, 20, 35, and 38 are based on three determinations; and the values for strains 2, 27, and 28 are based on two determinations.

^c The G+C value of this strain differs from all other values ($\alpha = 0.05$). An earlier determination for this strain (11) was reported as 33.57 ± 0.10 .

TABLE 4. Precursors used in determining the metabolic block in the pathway leading to the biosynthesis of methionine for all strains of *Pichia amethionina*

Precursor added to basal medium ^a	Ability to grow
None	—
Homoserine	—
SO ₄ ⁻²	—
SO ₃ ⁻²	—
S ₂ O ₃ ⁻²	+
Cysteine	+
Homocysteine thiolactone	+
Cystathionine	+ ^b
Methionine	+

^a Yeast nitrogen base without amino acids (Difco) plus 0.5% glucose and precursor (10 mg/liter).

^b Strains 11, 17, 29, 32, and 38 exhibited latent growth, whereas strain 8 did not grow at all.

spore viabilities (e.g., strain 24 a × strain 36 a yielded 20/32 viable spores = 63%). Since only four-spored asci were dissected, the spore survivals reported above represent high estimates. Nevertheless, the potential for gene flow between the two varieties is demonstrated, as is some degree of postmating breakdown, which results in partial isolation of the two gene pools. We do not know if hybrids occur in nature, but this could be easily ascertained by scoring segregants on a selective isolation medium in the field.

The two varieties of *P. amethionina* differ from each other physiologically in their ability to assimilate the sugar alcohol D-mannitol. Mannitol utilization was tested for in clones derived from spores dissected from the hybrid of the mannitol-positive strain 35 and the mannitol-negative strain 27. A single locus was implicated since five tetrads, with four viable spores each, segregated into two mannitol-positive and two mannitol-negative ascospore types. In addition, random spore analysis of 91 spores (39 mannitol negative; 52 mannitol positive) indicated that a single gene was responsible for the observed difference (corrected $\chi^2 = 1.58$; df = 1; $\alpha = 0.05$).

Another interesting attribute of *P. amethionina* is the phenomenon of agglutination or clumping when opposite mating types are mixed together. The reaction is similar to the agglutination Wickerham (15) reported in *Hansenula wingei*. We found that the reaction is stronger for older cultures than for freshly streaked ones. Zygotes become apparent in a few (2 to 4) hours after mixing, and ascospores could sometimes be observed within 24 h (Fig. 2).

Comparative observations of the two varieties show that *P. amethionina* var. *amethionina* takes from 5 to 7 days to sporulate on YM agar

TABLE 5. Viability classes for spores dissected from four-spored asci of a cross (strain 27 a × strain 35 a) between the two varieties of *P. amethionina*

Determination	0 ^a	1	2	3	4
Observed frequency	1	18	15	9	7
Expected frequency ^b	2.8	11.8	18.7	13.2	3.5

^a Numbers of viable spores per ascus.

^b Expectation is based on the assumption that spore viability had a binomial distribution $B(\chi; n, p)$ with $n = 50$, $\hat{p} = 0.515$.

after mixing opposite mating types, whereas mixes of *P. amethionina* var. *pachycereana* only require 24 h to 3 days to sporulate on the same medium. The intervarietal mixes show an intermediate time to complete the sporulation cycle. In general, we have found it more difficult to induce sporulation in *P. amethionina* var. *amethionina*, and we presume that its optimum sporulation conditions are somewhat different from those of the mannitol-positive variety, *P. amethionina* var. *pachycereana*.

Table 2 lists the frequency of isolation of the *P. amethionina* varieties from various cacti, but it does not give the number of cacti sampled. When one considers that 100 agria and 16 saguaro soft-rots were examined, it is apparent that the two varieties occupy two different niches in approximately the same frequency. In addition, saguaro, senita, and cardon belong to the subtribe *Pachycereinae* whereas the other cactus species are in the subtribe *Stenocereinae*. The subtribes are based on phylogenetic considerations, and the time of divergence of the two cactus tribes may also indicate the time of divergence for the two varieties of *P. amethionina*.

We attempted matings of haploid clones of *P. amethionina* with haploid clones of *P. delftensis* and found no conjugation. We also attempted matings with *P. heedii* (8), which has a G+C value similar to that of *P. amethionina*, but without success. This clearly demonstrates the distinctness of these species and confirms our suspicions based on ecological considerations.

P. amethionina can be distinguished from other *Pichia* species by its unique requirement for methionine and by its cactus habitat. In addition, it differs from *P. delftensis* by its inability to assimilate ribitol and by its much higher maximum growth temperature.

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REPRINT REQUESTS

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