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UNSTABLE PETITE AND GRANDE VARIANTS OF *Candida shehatae*

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SUMMARY

Two strains of *Candida shehatae* (ATCC 22984 and CSIR Y492) exhibit marked variability in colony size (petite, grande) and respiratory activity (tetrazolium reaction) when grown on glucose, xylose, and--especially--xylitol agar. The transitions occur in both directions at high frequency. Strains showing a negative or weak tetrazolium reaction on xylitol ferment xylose better than those showing a strong tetrazolium reaction. The type strain (ATCC 34887) shows stable colonial morphology with moderate respiratory and fermentative activities. The objective of this report is to demonstrate these variations.

INTRODUCTION

The recent description of *Candida shehatae* as a rapid fermenter of D-xylose (du Preez and van der Walt, 1983; du Preez et al., 1984) was a major step forward in the emergence of a practical process for the utilization of this abundant sugar. The specific rate of ethanol production by *C. shehatae* is about 2.5 to 3.5 times greater than the best values reported for *Pachysolen tannophilus*. *C. shehatae* attains higher ethanol concentrations than *P. tannophilus* when growing on xylose, but the ethanol yields are similar. Like *P. tannophilus*, *C. shehatae* uses xylose aerobically or anaerobically. Under aerobic conditions, *P. tannophilus* concomitantly forms and respires ethanol, (Maleszka and Schneider, 1982) resulting in lowered ethanol yields. Although it has not yet been demonstrated, *C. shehatae* could also exhibit concomitant ethanol formation and respiration.

One way to improve ethanol yields is to obtain a petite mutant. This is a yeast strain with deficient respiratory capacity and small colonial morphology. Selection of petite mutants was useful in the development of improved strains of *Candida pseudotropicalis* (Moulin et al., 1981), but it was less important in the case of *Saccharomyces cerevisiae* (Alexander and Detroy, 1983). Petite mutants have not been previously described in xylose-fermenting yeasts. *Pachysolen tannophilus*--the organism most studied in this regard--is petite-negative, *i.e.*, petite mutants die after a few generations (Neirinck et al., 1984).

This report describes the selection and characteristics of petite and grande strains of *C. shehatae* and a preliminary evaluation of their fermentation characteristics. It is concluded that the colonial and respiratory variations occur in both directions with high frequencies, that colony size and respiratory activity are separate but related traits, and that selection for a stable petite may be a useful technique for further strain development.

MATERIALS AND METHODS

Strains. Three parental strains of *C. shehatae* were employed: ATCC 22984, ATCC 33487 and CSIR Y492. The first two were obtained from the American Type Culture Collection, Rockville, Maryland, USA; the last was obtained from J.P. van der Walt, Council for Scientific and Industrial Research, Pretoria, South

Africa. Stock cultures were maintained on Difco Yeast Malt Agar (YMA). Slants were grown out at 32°C then stored at 5°C for up to 3 months. Isolates from these parental strains were obtained by streaking on Difco Yeast Nitrogen Base w/o ammonium sulfate or amino acids (YB) supplemented with 2.27 g/l of urea (U), 2% xylitol (Xi), xylose (Xo) or glucose (G) and 1.8 g/l agar.

Culture conditions. YMA plates were incubated at 32° C for 7 d; YBUXi, YBUXo and YBUG plates were incubated for 5–18 d in order to distinguish grande and petite colonies. Ethanol fermentation rates were tested in triplicate cultures of YBUXo broth as previously described (Jeffries, 1982, 1984). Inocula were adjusted to obtain the same initial OD for all organisms in each test (1 OD @525 nm=0.19 mg/ml cells). Triplicate flasks of each organism were used and the results reported as average values.

Isolation of variants. Petite and grande isolates were obtained from YMA-grown cells as small or large colony variants on YBUXi agar. Petite II isolates were obtained from petite I isolates. ATCC 22984-1, 2 and 3 were successive grande variants selected from a petite variant in the preceeding passage on YBUXi; ATCC 22984-SU was a petite isolate showing unstable morphology after four serial passages on YBUXi. CSIR Y492-1 and CSIR Y492-2 were isolated from CSIR Y492 by streaking on YBUXi. Other strains were obtained as described in the text.

Variant frequencies. The frequencies of conversions from petite to grande and grande to petite colonial morphologies were estimated for 18-day old cells grown on YBUXi agar. One petite and one grand colony from YBUXi agar were each suspended in sterile water and plated onto triplicate plates of YBUXi, YBUXo and YBUG agar. After five days, plates were scored for the numbers of small and large colonies. After scoring, diameters were measured under a dissecting microscope, and two plates from each set were overlaid with tetrazolium agar to determine the frequency of respiration positive- and respiration-negative colonies. After 18 days, the remaining plates were overlaid with tetrazolium agar and the frequency of petites and respiratory competent cells were again determined for each of the three carbon sources.

Tetrazolium agar overlay. This method was performed as described by Ogur et al. (1957) using 5- to 18-day-old cultures streaked on YBU agar with 2% Xi, Xo or G as the carbon source. Color reactions were scored on an arbitrary scale of 1+ to 4+. Respiration-deficient colonies remain white (1+, Tet-) while respiration-sufficient colonies turn red (4+,Tet+).

Assays. Cell density, ethanol, xylose, and xylitol determinations were performed as previously described (Jeffries, 1982, 1984; Verhaar and Kuster, 1980).

RESULTS

Colonial morphologies. ATCC 22984 and CSIR Y492 formed convex, smooth, entire, cream-colored colonies on YMA. ATCC 34887 formed convex, rugose, cream- to light yellow-colored colonies with an undulate edge. Within each strain, isolated colonies on YMA were essentially similar. When streaked onto YBUXi, however, ATCC 22984 exhibited distinct heterogeneity in colony size and morphology (Fig. 1). Small colonies on older plates tended to be lobate and irregular, indicating colonial sectoring and reversion to more extensive growth. The colonial transformation occurred in both directions, i.e. large colony strains could be derived from small and vice versa. ATCC 22984-SU continued to give rise to large and small colonies as long as it was subcultured (eight passages). Large (1) and small (2) variants of CSIR Y492 were also obtained on

YBUXi. ATCC 34887 formed colonies with irregular shapes, but no consistant distinction could be made between large and small colonies of this strain on YBUXi agar.

Xylose fermentation rates. One petite and three grande strains derived from ATCC 22984, one grande and one petite strain derived from CSIR Y492 and the stable ATCC 34887 strain were compared for their aerobic and anaerobic xylose fermentation rates. ATCC 22984-SU and ATCC 22984-3 followed by CSIR Y492-1 showed the highest fermentation rates under aerobic conditions. CSIR Y492-2 was the best under anaerobic conditions followed by ATCC 22984-3 and CSIR Y492-1 (Fig. 2). Differences among the strains were substantial in some instances. In three separate experiments CSIR Y492-1 showed initial aerobic fermentation rates 30-50% greater than CSIR Y492-2. Anaerobically, the relationship was reversed with CSIR Y492-2 producing ethanol 15-40% faster than CSIR Y492-1.

Tetrazolium reaction. The Tet reaction varied with the strain, the carbon source, the age of the plate and the density of the colonies on the plate. However, if separate strains were handled in a similar manner, distinct and reproducible differences could be observed (Table 1). In general, petite colonies, or grande variants derived from successive petite isolates, gave weaker Tet reactions; however, occasional petite colonies with Tet+ color reactions were observed and infrequent Tet+ colonies could be obtained from Tet- isolates.

Table 1. Tetrazolium color reactions (10 d) for *C. shehatae* strains.

Strain	Designation	Glucose	Xylitol	Strain	Designation	Glucose	Xylitol
22984-1	grande	3+	4+	34887	stable	3+	2-4+
22984-2	grande	2+	1+	Y492-1	grande	3+	4+
22984-3	grande	1+	1+	Y492-2	petite	1+	2+
22984-SU	petite	ND	ND				

Petite/grande variant frequency. The apparent frequency of petite and grande colonies depended on the origin of the cells, the carbon source on which they were plated and the age of the plates. A petite strain from ATCC 22984 (petite I) gave rise to larger colonies (grande I) and even smaller colonies (petite II) when plated on YBUXi. The grande I strain gave rise to petite I variants, but no petite II variants (Table 2).

Table 2. Variant frequencies and colony diameters (5 d) for ATCC 22984

Source of cells (colonies/plate)	YBU-Xylitol agar freq.	dia. (mm)	YBU-Xylose agar freq.	dia. (mm)	YBU-Glucose agar freq.	dia. (mm)
YBUXi--Grande I	.86	1.44 ±.20	.84	1.23 ±.16	.92	1.38 ±.20
(119±12)	.14	1.12 ±.22	.16	0.71 ±.13	.08	0.64 ±.23
YBUXi--Petite I	.92	0.97 ±.11	.94	1.46 ±.26	.93	1.76 ±.41
(333±27)	.03	0.41 ±.12	.06	0.67 ±.28	.07	0.44 ±.31
	.05	1.44 ±.10				

Petite and grande colonies could also be distinguished on YBUXo and YBUG in about the same frequency as observed on YBUXi. As would be expected from their origin, cells derived from the grande I colony gave larger colonies than cells derived from the petite I colony when both were plated on YBUXi; however, petite I cells gave rise to larger colonies than grande I cells when both were plated on

YBUG or YBUXo agar. Comparisons of colony sizes derived from the two cell sources were complicated by different plating densities obtained. Grande I cells, however, showed smaller colonies on YBUXo and YBUG than did petite I-derived cells, even though the grande I plating density was lower.

Petite and grande colonies were more readily distinguished on older (18 d) plates. Figure 3 shows one set of plates used in the experiment summarized in Table 2. At 18 d, positive Tet reactions were only observed with grande colonies on YBUXi. At 5 d, positive tet reactions were observed with petite colonies on glucose. With streak plates at 5 d, grande I colonies gave a stronger Tet reaction on xylitol than on glucose. Petite I and petite II colonies gave weak reactions on xylitol and strong reactions on glucose. A positive Tet reaction was not observed with cells grown on xylose.

Colony types and fermentation characteristics. From the 18 d YBUXi plates with cells from a petite I colony, five populations could be distinguished, based on their colony size and their Tet reactions (Table 3).

Table 3. Growth and fermentation characteristics of strains derived from ATCC 22984 petite I cells grown on xylitol (18 d).

Designation	Freq.	Dia.(mm)	Tet. rxn.	Ethanol g/l-h◇	Ethanol g/g	Xylitol g/g	Glycerol g/g
Grande I Tet+	.08	2.44 ±.30	3+	.43	.23	.26	.11
Grande II Tet+	.03	1.61 ±.14	4+	.18	.20	.40	.16
Petite I Tet-	.84	1.15 ±.01	1+	.38	.18	.14	.04
Petite I Tet+	.01	0.92 ±.05	4+	.02	.11	0*	0*
Petite II Tet-	.04	0.65 ±.19	1+	.37	.17	.10	.04

◇determined from 24-48 h *other products formed

The most frequent colonies resembled the petite I, Tet- parent. When three isolates of this type were tested, they were not the best fermenters. The grande I and II, Tet+ strains were somewhat better. Petite I, Tet+ strains occurred in the lowest frequency. These strains were relatively stable on subsequent subculture, but exhibited very low rates of xylose fermentation. Petite II, Tet- strains were very unstable and showed moderate to poor xylose fermentation

DISCUSSION

These studies show that the grande and petite variants of ATCC 22984 are unstable. To a certain degree, this appeared to be a function of the isolate. Colony age or the carbon source on which it was grown may also be important. One can conclude from the experiment summarized in Tables 2 and 3, that the change occurs in both directions, that at least five combinations of colony size and respiratory types are formed, and that variants are observed on glucose and xylose in addition to xylitol, albeit in lower frequency. Because the grande I variant actually showed relatively smaller colonies and diminished Tet reaction on glucose with larger colonies and a stronger Tet reaction on xylitol, it appears that this variation facilitates xylitol utilization at the expense of glucose.

The change from large to small and small to large colonial morphologies is similar to the conventional petite variation observed with *S. cerevisiae* and other yeasts because colonies exhibiting diminished colony diameter on a given

carbon source also exhibit diminished Tet color reactions on it. A metabolic effect on respiration is therefore indicated. Unlike conventional petites, (Wilkie, 1983) the change described here occurs in both directions. It should be noted, however, that unstable or partial petites have been previously described in *S. cerevisiae* (Johnson et al., 1971). Interpretation of the metabolic basis for these changes is complicated by grande I cells showing relatively larger colonies on xylitol and smaller on glucose. Under a conventional designation, these might be termed petites, based on their colony size on glucose. This interpretation is also complicated by the petite I Tet+ strains which exhibit small colonies on xylitol but a strong Tet reaction with this carbon source.

It is concluded that the Tet reaction and the petite/grande variation are separate, but not metabolically independent, traits. Generally petite I variants were Tet- on xylitol, but occasional petite, Tet+ variants were observed. Classically, petite colonies would be Tet- (Ogur et al, 1957). Of these two traits, the Tet reaction showed less stability, but this may have been due in part to changes in the color reaction with time and carbon source.

The relationships among colony size, the Tet reaction, and the ability of the strain to ferment a given carbon source are complex. It is clear, however, that fermentative capacities are strongly related to these other factors because strains showing strong Tet reactions on xylitol were poor fermenters. Additional research is required to determine the biochemical bases for differing colony sizes and Tet teactions and the genetic basis for the strain variability. Selection on the basis of colony size or Tet reaction may be useful for further strain development.

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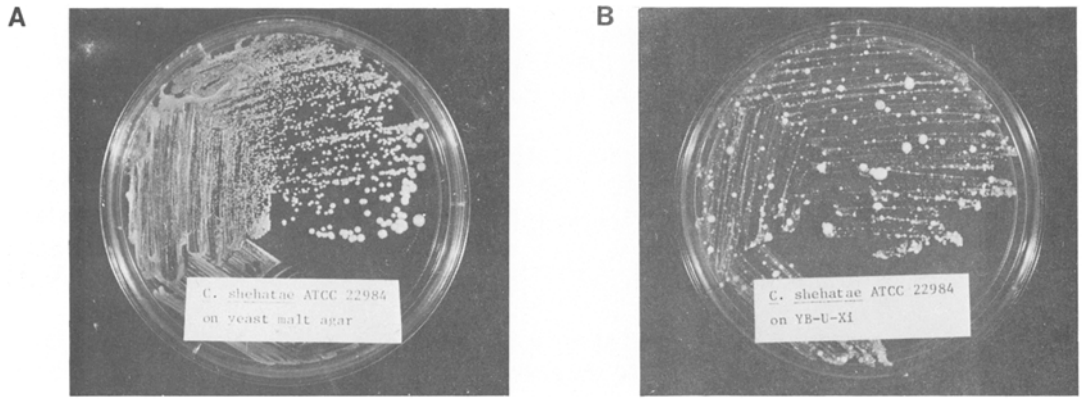

Figure 1. Comparison of colonial morphologies for ATCC 22984 grown on YMA (A) and YBUXi (B) agars.

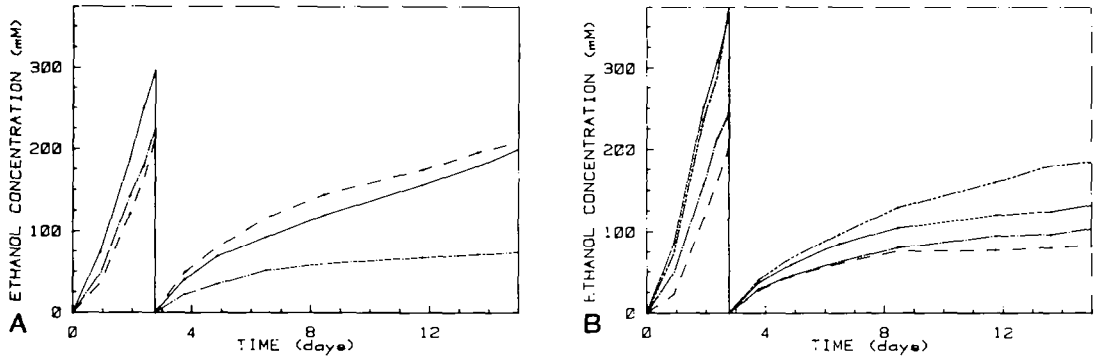

Figure 2. Comparison of fermentation rates of *C. shehatae* strains. Cultures were grown under low aeration on YBUXo-4.5% for 3 days then transferred to anaerobic conditions on the same medium. A) CSIR Y492-1 (——); CSIR Y492-2 (– – –); ATCC 34887 (— — —). B) ATCC 22984-SU (——); ATCC 22984-1 (– – –); ATCC22984-2 (— — —); ATCC 22984-3 (— · — ·). Average standard deviation was ± 6% of reported value.

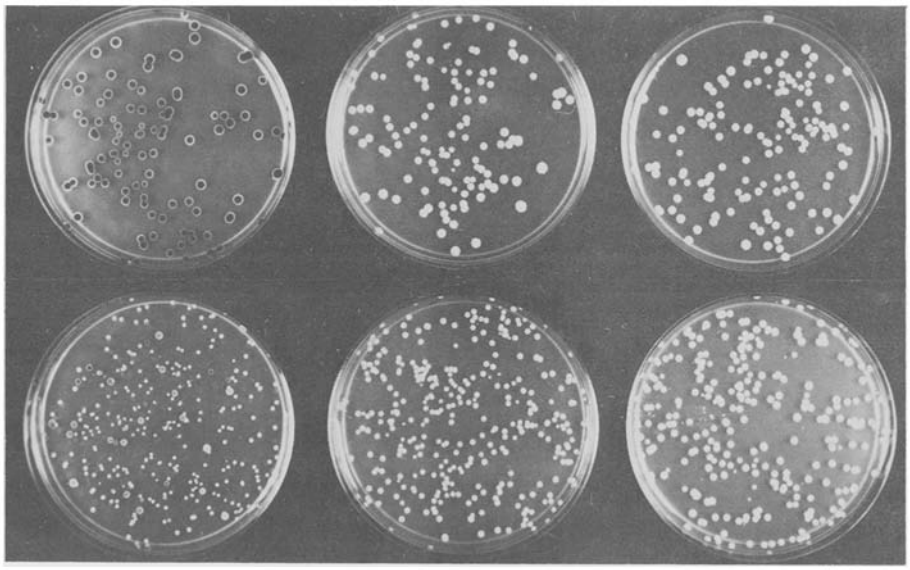

Figure 3. Colony sizes and Tet reactions of grande I and petite I strains. Top row, grande I; bottom row, petite I; left to right, Xi, Xo, G.