Examination of "Hydrocarbon-Utilizing Mutants of *Saccharomyces cerevisiae*"

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Taxonomic and genetic studies were carried out on hydrocarbon-utilizing yeasts received from the Department of Genetics and Selection, A. A. Zhdanov Leningrad State University, Leningrad, U.S.S.R. These strains have been referred to by Inge-Vechtomov and colleagues as "hydrocarbon-utilizing mutants of *Saccharomyces cerevisiae*." However, we were unable to confirm this identification. According to the taxonomic criteria in general use, including deoxyribonucleic acid base composition, these strains closely resemble those of *Candida maltosa*.

Yeasts able to grow on hydrocarbons are now being used as animal fodder and are being investigated as possible sources of protein in human nutrition (5, 9). The hydrocarbon-utilizing yeasts most used for "single-cell protein" are species of *Candida* and include *C. lipolytica*, *C. tropicalis*, and *C. maltosa* (5, 11). In some countries, authorities have been reluctant to approve these *Candida* yeasts as new sources of protein because some of the species used were thought to be closely related to potentially pathogenic yeast species. Another reason was the fear of possible carry-over of carcinogens from the petroleum substrate. Investigators at the Leningrad State University have reported on the isolation of mutants of bakers' yeast (*Saccharomyces cerevisiae*) that were capable of utilizing hydrocarbons as a growth substrate (8, 15, 16, 18-20). These could prove to be important strains since *S. cerevisiae* is already heavily employed as a food yeast, and its nutritional value is thoroughly understood.

This report covers genetic and taxonomic studies of hydrocarbon-utilizing yeasts received from the Leningrad State University. We were unable to confirm the identification of these strains as members of *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains. The yeasts received from the Department of Genetics and Selection, A. A. Zhdanov Leningrad State University, are listed in Table 1.

The following strains of *S. cerevisiae* were obtained from the Yeast Genetics Stock Center, Donner Laboratory, University of California, Berkeley, Calif.:

X2928-3D(1A)	α <i>adel1 gal1 leu1 his2 ura3 trp1 met14</i>
X2928-3D(1C)	α <i>adel1 gal1 leu1 his2 ura3 trp1 met14</i>
S1795A	α <i>ade6 his4 trp5 ura1 gal2</i>
S1799D	α <i>ade6 his4 trp5 gal2</i>

The mating types of the strains are designated by "a" and " α ." The other genetic symbols refer to auxotrophic and fermentation-negative genetic markers.

Media. The capacity of the strains to utilize hydrocarbons was tested on CAAH₁₆ medium (20) consisting of 0.1% yeast autolysate (Difco), 0.67% yeast nitrogen base without amino acids (Difco), and 2.0% agar. As an additional supplement for certain nutritional requirements present in the strains, we added the following: adenine, 30 μ g/ml; methionine, 20 μ g/ml; arginine, 30 μ g/ml; and histidine, 20 μ g/ml. Hydrocarbon substrate was present in the vapor phase of the culture system. Hexadecane (1 ml) was added to a 9-cm-diameter filter paper disk placed in the lid of petri dishes containing CAAH₁₆ medium, and the plates were then sealed.

Sporulation was tested by replica plating to McClary sporulation medium containing 1.0% potassium acetate, 0.1% glucose, 0.25% yeast extract (Difco), and 2.0% agar, and to raffinose-acetate medium containing 0.3% potassium acetate, 0.022% raffinose, and 2.0% agar. Yeast cultures were grown and stored on YEPD medium which contained 2.0% glucose, 2.0% peptone (Difco), 1.0% yeast extract (Difco), and 2.0% agar. The synthetic medium utilized for scoring auxotrophic genetic markers consisted of 1.0% glucose, 0.66% yeast nitrogen base without amino acids, and 2.0% agar. Amino acids and purines were added so that auxotrophic markers could be individually scored.

Genetic techniques. The procedures for tetrad dissection and for scoring genetic markers were described previously (12). Mitotic recombinants were induced by irradiating a distilled-water suspension of stationary-phase cells with ultraviolet light (560 ergs/mm², ca. 70% survival). Irradiated cells were spread on YEPD plates and incubated at 30°C. Finally, mature colonies were replica plated to minimal synthetic media to detect auxotrophic sectors and clones.

Yeast identification and DNA base composition. Identification of the strains at the species level was performed by standard methods currently used in yeast taxonomy (22, 23). Deoxyribonucleic acid (DNA) extraction and purification were done by a combination of the procedures of Marmur (10) and Bernardi

TABLE 1. Genetic and physiological properties as determined by the authors, of yeast strains received from Leningrad State University

Strains	Genotypes (a)	Growth (+ or -)		Hydrocarbon	Hydrocarbon (hyc) Phenotype	Marker recovery-- dissected spores	Marker recovery-- UV-treated cells	Total clones	Autotrophic clones
		Minimal	Methionine	CAH ₁₆					
D131	$\frac{a}{a} \frac{ade2}{+} \frac{his7}{+} \frac{metA1}{+} \frac{Hth^{+}}{+}$	+	+	-	hyc-negative (f)	<i>ade his met</i> (b)		1,450	40 (d)
D131-3-4	$\frac{a}{a} \frac{ade2}{+} \frac{his7}{+} \frac{metA1}{+} \frac{Hth^{+}}{+}$	-	+	+	hyc-positive <i>met</i>	- (c)		3,200	0
(IV-2)-4-6	$\frac{a}{a} \frac{ade2}{+} \frac{met}{+} \frac{ura}{+} \frac{his}{+} \frac{Hth^{+}}{+}$	+	+	+	hyc-positive	- (c)		2,780	0
(IV-2)-4-9	$\frac{a}{a} \frac{ade2}{+} \frac{met}{+} \frac{ura}{+} \frac{his}{+} \frac{Hth^{+}}{+}$	+	+	+	hyc-positive	- (c)		-- (e)	-- (c)

^a Genotypes listed in the letters that accompanied strains received from the Department of Genetics, Leningrad State University, Leningrad, U.S.S.R. *Hth*⁺/*Hth*⁻ refers to heteroalleles at a locus determining heterothallic (*Hth*⁺) or homothallic (*Hth*⁻) mating.

^b *his*, *ade*, and *met* markers segregated in dissected ascospores. The spore clones from D131 mated with strains of *S. cerevisiae* listed in Materials and Methods. Mating was detected by the formation of prototrophic diploids in mixtures of haploid auxotrophs with complementing nutritional requirements.

^c Strains did not sporulate on raffinose-acetate or McClary sporulation media.

^d *his*, *ade*, and *met* phenotypes, and no other nutritional requirements, were detected among the 40 auxotrophic clones.

^e Not studied.

^f hyc-negative, Non-hydrocarbon utilizing; hyc-positive, hydrocarbon utilizing.

et al. (3). The guanine plus cytosine (G+C) content of the DNA was calculated from buoyant-density values in CsCl (17, 21) and was based on three 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.

RESULTS

Genetic experiments. Hydrocarbon-utilizing mutants in homothallic diploid strains of *S. cerevisiae* were first reported in 1973 (20). The mutation to hydrocarbon utilization involved an intermediate stage which was hydrocarbon utilizing and required methionine. Subsequently, hydrocarbon-utilizing prototrophic clones were recovered from the methionine-requiring strains (18). The hydrocarbon-utilizing mutants were of spontaneous origin, and their frequency was reported as 10⁻⁹ per cell per generation (18, 20).

The hydrocarbon-utilizing mutants differed from their parental strains in a considerable number of phenotypic properties in addition to the ability to utilize hydrocarbons: the mutants were found to be altered in their ability to assimilate several carbon sources; they had become resistant to various inhibitors and to "killer" strains of yeast; they had acquired the ability to form pseudomycelia; and they had lost the capacity to sporulate, copulate, and show mitotic recombination (15, 16, 20). With the exception of a methionine requirement, the hydrocarbon-utilizing strains requiring this amino acid were phenotypically indistinguishable from the prototrophic hydrocarbon-utilizing strains (15, 16). The absence of sexuality as well as of mitotic recombination made it impossible to detect the presence of parental heterozygous recessive markers in the hydrocarbon-utilizing mutants (16).

Table 1 summarizes our experiments on the genetic properties of the strains received from the Leningrad State University. The strain which did not utilize hydrocarbons sporulated and gave rise to spore clones that hybridized with strains of *S. cerevisiae*. This strain also showed mitotic recombination of three heterozygous, recessive markers. The hydrocarbon-utilizing strains, however, neither sporulated nor showed mitotic recombination.

In addition to selecting hydrocarbon-utilizing mutants, Rabinovich et al. reported on the recovery of non-hydrocarbon-utilizing revertants that had regained the ability to sporulate and showed segregation of the markers present in the original non-hydrocarbon-utilizing parental strain (16). Table 2 summarizes data from our

TABLE 2. Selection of hydrocarbon-utilizing mutants and non-hydrocarbon-utilizing, sporulating revertants

Strain ^a	Direction of mutation ^b	No. of mutants/no. of cells plated
D131 #26 ^c	hyc-neg → hyc-pos	0/2 × 10 ¹⁰
D131 #26 <i>his7 ade2</i> ^c	hyc-neg → hyc-pos	0/1.02 × 10 ¹¹
D131-3-4 #25 ^d	hyc-pos, spo-neg → hyc-neg, spo-pos	0/4.1 × 10 ⁴

^a Numbers after strain designations refer to single clones isolated from the cultures received from the Leningrad State University. Strain D131 #26 *his7 ade2* is a doubly auxotrophic, red mitotic recombinant selected after exposure of D131 #26 to ultraviolet light.

^b hyc-neg, Non-hydrocarbon utilizing; hyc-pos, hydrocarbon utilizing; spo-neg, no sporulation; spo-pos, sporulates.

^c Cultures were grown on YEPD plates for 3 days, suspended, counted, and plated (5×10^8 cells per plate) to CAAH₁₆ medium. The plates were incubated at 30°C for at least 20 days. Reconstruction experiments using mixtures of D131-3-4 #25 *met* and D131 #26 were carried out to ascertain that hydrocarbon-utilizing mutants could be detected against a background of 5×10^8 non-hydrocarbon-utilizing cells.

^d D131-3-4 #25 was grown on YEPD plates for 3 days, suspended, counted, exposed to ultraviolet light, and plated to YEPD. Survival was 67% at a dose of 560 ergs/mm². After incubation for 2 days, the YEPD plates were printed on CAAH₁₆ medium. Twenty-six non-hydrocarbon-utilizing revertants were found, but none sporulated. Four of the revertants were tested for the presence of ultraviolet-induced mitotic recombination. At least 1,000 clones of each were examined, and no auxotrophic clones or sectors were found.

experiments designed to repeat both the mutation to hydrocarbon utilization and the reversion to cells that were non-hydrocarbon-utilizing and could sporulate.

Two parental strains, D131 #26 and D131 #26 *his7 ade2*, were used during attempts to select hydrocarbon-utilizing mutants. The latter strain is a doubly auxotrophic mitotic recombinant recovered by us following ultraviolet irradiation of D131 #26. D131 #26 *his7 ade2* is red because the *ade2* mutation results in the accumulation of a pigmented compound (6). The advantage of this strain is that marker expression is not dependent on sporulation or mitotic recombination, and any contaminant yeast can be clearly identified. In the experiments summarized in Table 2, neither hydrocarbon-utilizing mutants nor non-hydrocarbon-utilizing, sporulation-positive revertants were found.

In Table 3 are summarized the data on the physiological properties of the strains received from the Leningrad State University. The hydrocarbon-utilizing strains showed differences in 20 phenotypic characteristics when compared to the non-hydrocarbon-utilizing strain. Sixteen of these physiological characteristics are useful for taxonomic purposes. In all of these cases the phenotypic characteristics of the non-hydrocarbon-utilizing strain agreed with those reported for *S. cerevisiae* whereas the hydrocarbon-utilizing strains differed.

Table 4 presents data on the nuclear DNA base compositions of the strains. The three hydrocarbon-utilizing strains were very similar in their base compositions, and all three differed significantly from the non-hydrocarbon-utilizing strain. The non-hydrocarbon-utilizing strain

yielded DNA with a G+C value typical of that of *S. cerevisiae*.

DISCUSSION

Genetic and taxonomic studies were carried out on one non-hydrocarbon-utilizing and three hydrocarbon-utilizing yeasts received from the Leningrad State University. The non-hydrocarbon-utilizing strain appears to be a typical member of *S. cerevisiae*. The strain sporulated, and isolated spore clones hybridized with strains of *S. cerevisiae*. It also possessed a nuclear DNA base composition typical of that of *S. cerevisiae*. The three hydrocarbon-utilizing strains were all quite similar to each other in the phenotypic properties used in yeast taxonomy and in their base compositions. However, these strains differed from *S. cerevisiae* in numerous phenotypic characteristics and by about 4% in their nuclear DNA base compositions.

Simarov et al. suggested an explanation for the many phenotypic differences in the hydrocarbon-utilizing mutants compared to the non-hydrocarbon-utilizing parental strains. They argued that the low frequency of hydrocarbon-utilizing mutants (ca. 10^{-9}) indicated that more than one mutation was involved, and therefore the phenotypic complexity may be attributable to multiple independent mutations (19). Rabinovich et al. suggested that one mutation might represent an alteration in the α/α mating type locus (15). This locus is known to control a pheromone which regulates copulation and sporulation (4, 7) and which, in certain circumstances, can effect a substantial reduction in mitotic recombination (7). Rabinovich et al. further speculated that another mutation might

TABLE 3. *Physiological properties of tested strains*

Properties	Strains			
	D131 #26	D131-3-4 #25	(IV-2)-4-6 #21	(IV-2)-4- #23
Fermentation of:				
Glucose	+	+	+	+
Galactose	+	weak	weak	weak
Maltose	+	weak	weak	weak
Sucrose	+	+	+	+
Lactose	-	-	-	-
Melibiose	-	-	-	-
Raffinose	+	-a,b	-a,b	-a,b
Assimilation of:				
Glucose	+	+	+	+
Galactose	+	+	+	+
L-Sorbose	-	+a,b	+a,b	+a,b
Maltose	+	+	+	+
Sucrose	+	+	+	+
Cellobiose	-	latent	latent	latent
Trehalose	weak	+a,b	+a,b	+a,b weak
Lactose	-	-	-	-
Melibiose	-	-a,b	-a,b	-a,b
Raffinose	+	-a,b	-a,b	-a,b
Melezitose	-	weak	+a,b	+a,b
Inulin	-	-	-	-
Soluble Starch	-	-	-	-
D-xylose	-	+a,b	+a,b	+a,b
L-arabinose	weak	+	+	+
D-arabinose	-	-	-	-
D-ribose	-	-	-	-
L-rhamnose	-	-	-	-
Ethanol	+	+	+	+
Glycerol	-	+b	weak	weak
Erythritol	-	-a,b	-a,b	-a,b
Ribitol	-	+a,b	+a,b	+a,b
Galactitol	-	-a,b	-a,b	-a,b
D-mannitol	-	+a,b	+a,b	+a,b
D-glucitol	-	+a,b	+a,b	+a,b
α -methyl-D-glucoside	-	+a,b	+a,b	+a,b
Salicin	-	moderate	weak	weak
Glucono- α -lactone	-	latent	latent	latent
K-5-keto-gluconate	-	-	-	-
DL-lactic acid	latent	latent	latent	latent
Succinic acid	moderate	+	+	+
Citric acid	-	+a,b	+a,b	+a,b
Meso-Inositol	-	-a,b	-a,b	-a,b
Hexadecane	-	+a,b	+a,b	+a,b
Glucosamine	-	+a,b	+a,b	+a,b
Other properties:				
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	weak	weak
Growth at 39°C	+	-a,b	-a,b	-a,b
Acid formation	weak	weak	weak	weak
Gelatin liquefaction	-	-	-	-
Starch synthesis	-	-	-	-
Cycloheximide resistance	-	+a,b	+a,b	+a,b
Canavanine resistance ^(c)	-	+b	+b	+b
Casein hydrolysis	-	-a,b	-a,b	-a,b
Fat splitting	-	+a,b	+a,b	+a,b
Growth on:				
Glucose 50%	-	-	-	-
Glucose 60%	-	-	-	-
NaCl 10%	latent	-	-	-
Urease	-	-	-	-
Diazonium Blue B	-	-	-	-
Sporulation	+	-b	-b	-b
Mitotic recombination	+	-b	-b	-b
Pseudomycelium formation	-	+a,b	+a,b	-

^a Differs from *S. cerevisiae* (23).^b Differs from parental strain (D131 #26).^c -, Canavanine sensitive; +, canavanine resistant. Medium: Synthetic complete minus arginine (12). L-Canavanine, 60 μ g/ml.

TABLE 4. Nuclear DNA base compositions of yeast strains received from the Leningrad State University

Strain	Hydrocarbon-utilization phenotype ^a	mol% G+C ^b
D131 #26	hyc-neg	39.54 ± 0.12
D131-3-4 #25	hyc-pos <i>met</i>	35.90 ± 0.18
(IV-2)-4-6 #21	hyc-pos	35.15 ± 0.24
(IV-2)-4-9 #23	hyc-pos	35.25 ± 0.19

^a See footnote b of Table 2.^b The values are based on three centrifuge runs of the same sample of purified DNA.

affect cellular membranes (15). Membrane mutants of *S. cerevisiae* showing complex phenotypes have been studied. Examples are the *ole2* and *ole3* mutants, which require an unsaturated fatty acid or porphyrin intermediates. These mutants require methionine on a defined medium, are respiratory deficient, and, because of an altered membrane sterol composition, become resistant to the polyene antibiotic nystatin (1). The physiological basis for the pleiotropic effect of *ole2* and *ole3* appears to be a defect in porphyrin synthesis leading to a deficiency of hematin enzymes (2). Hematin enzymes are involved in fatty acid and sterol metabolism as well as in electron transport (14, 24).

However, the physiological relationships of mating-type-locus mutants or membrane mutations to the capacity to utilize hydrocarbons have not been established. Sporulation ability in yeasts is not necessarily related to the capacity to metabolize hydrocarbons. Some strains of the hydrocarbon-utilizing yeast *C. lipolytica* have been found to sporulate whereas others do not (25). It is not clear why the hydrocarbon-utilizing mutants of *S. cerevisiae* also exhibit multiple phenotypic changes which include such diverse properties as pseudomycelium formation, antibiotic resistance, sporulation, mitotic recombination, and a different spectrum of carbon-compound utilization.

The DNA base composition of a yeast or of another organism has great exclusionary value in taxonomic decisions because it reflects the overall composition of the genome to a much greater extent than do individual physiological or morphological characteristics. In many cases base composition data have been helpful in clarifying the taxonomic assignments of yeasts (13). The 4% difference in G+C content between the hydrocarbon-utilizing strains and *S. cerevisiae* is highly significant. On the basis of the taxonomic characteristics of the strains we received and because neither hydrocarbon-utilizing nor non-hydrocarbon-utilizing sporulating mutants were recovered, their identification as hydrocarbon-utilizing strains of *S. cerevisiae* could not be confirmed.

The physiological characteristics and the DNA base compositions of the hydrocarbon-utilizing yeasts received from the Leningrad State University closely match those reported for *C. maltosa* (11). *C. maltosa* utilizes hydrocarbons strongly and has been isolated from air, soil, and water. Given no other description of their origin, these hydrocarbon-utilizing strains could be identified as members of *C. maltosa*.

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REPRINT REQUESTS

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