Protoplast Fusion between a Petite Strain of *Candida utilis* and *Saccharomyces cerevisiae* Respiratory-competent Cells

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**Abstract.** Prototrophic RD mutant cells of *Candida utilis* NRRL-Y-1084 and auxotrophic mutant respiratory-competent cells of *Saccharomyces cerevisiae* 4003-5B *his4* *leu2* *can5* *meth2* *trps* *ade1* *ura3* *gal* were turned into protoplasts to be further fused with the aid of polycylene glycol (PEG) and Ca$^{2+}$ ions. Minimal medium containing glycerol as the carbon source was employed for fusion product selection. The respiratory-competent fusion products, mainly oval cells, resembled *Candida utilis* and had the fermentative abilities of this strain (dextrose, sucrose, raffinose). Five fusion products were analyzed as to their ability to metabolize dextrose, xylose, cellobiose, trehalose, glycerol, succinic acid, citric acid, salycin, and maltose. Fusion products partially restored the respiratory-competent *Candida utilis* capacity to grow by use of these carbon compounds, and none of the *Saccharomyces cerevisiae* fermenting abilities were found. Our results would suggest either a partial recombination between parental mitochondria or some occurring phenomenon affecting the cell membrane function after somatic fusion without concomitant nuclear fusion.

Intraspecific and interspecific somatic fusions of yeast protoplasts have been widely used in studying the restoration of respiratory capacity of petite strains (rho−) of the petite-negative yeast *Kluyveromyces lactis* and of the petite-positive yeasts *Saccharomyces cerevisiae* and *Saccharomyces diastaticus* [1, 11, 17, 18]. Other workers have observed mitochondrial transfer (transfusion of mitochondria) during formation of hybrids by protoplast fusion [2, 7]. Gunge in 1979 [4] and Yoshida in 1979 [25] showed the transfer of isolated mitochondria obtained from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Hansenula wingii* to petite strains of *Saccharomyces cerevisiae* by PEG-mediated transformation. Also, Fukuda and Kimura in 1980 [3] and Maráz and Ferenczy in 1980 [7] reported transfusion of mitochondria from miniprotoplasts of *Saccharomyces cerevisiae* to petite strains of the same species. In 1981, Yamashita et al. [24] observed intergeneric transfusion of mitochondria of *Hansenula wingii* to petite strains of *Saccharomyces cerevisiae*, and in 1979 Lancashire and Matoon [5] employed the cytoduction phenomenon for introducing certain mitochondrial genes to isogenic strains of *Saccharomyces cerevisiae*. Matsumoka et al. in 1982 [10] reported on the cytoplasmic transfer of oligomycin resistance by PEG-mediated fusion of protoplasts of *Saccharomycopsis lipolytica* in the absence of karyogamy. Other authors [9] observed transmission and recombination of mitochondrial genes in *Saccharomyces cerevisiae* after protoplast fusion. Successful intergeneric fusion of auxotrophic complementing mutant strains of *Saccharomycopsis lipolytica* (formerly *Candida lipolytica*) and *Saccharomyces cerevisiae* has been reported [23]. Recently we also reported having obtained a petite-like strain of *Candida utilis* as a prior step to using it as a partner for somatic fusion [13]. Here we describe an intergeneric fusion that led to the isolation of hybrids between a petite-like strain of *Candida utilis* with respiratory-competent cells of *Saccharomyces cerevisiae*. This is of great interest because it offers a method to increase the knowledge about the aerobic metabolism of this imperfect yeast and allows its hybridization by means other than mating.

**Materials and Methods**

**Strains.** *Candida utilis* var Major NRRL-Y-1084, prototrophic, "petite-like" mutant obtained at this laboratory [13]. *Saccharo-
myces cerevisiae 4003-5B a his4 leu2 can^2 ade2 methy1 ura3 trp5 gal was provided by the Yeast Genetic Stock Center, Donner Laboratory, CA, USA.

Protoplast formation. The strains were maintained on 1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar (YEPD). For production of cells for protoplast formation, the cells were grown in YEPD broth, 10 ml of medium in a 25-ml Erlenmeyer flask, until the middle log phase was reached (12 h at 30°C) on an Incubator Rotary Shaker, New Brunswick (120 rpm). A number of 1.5 x 10^9 cells were harvested by centrifugation and washed twice with 1.5 ml of sterile distilled water. A pretreatment solution containing 0.1 M Tris-HCl, 5 mM EDTA, 50 mM DTT (dithiothreitol), pH 8, was used to resuspend the cells, and the suspension was incubated 10 min at 30°C with gentle agitation. The cells were again centrifuged, washed once, and resuspended with 1.5 ml of 0.6 M KCl, 0.1 M sodium citrate, pH not adjusted, protoplasting buffer solution, and 0.15 ml of enzyme preparation (Helicase; Suc Digestif d' Helix pomatia) was added. The suspension was incubated with gentle agitation at 30°C, usually for 30-40 min, until protoplast formation was complete. The course of protoplast formation was followed microscopically and washed twice with 1.5 ml of sterile distilled water. A pretreatment of 1.5 x 10^9 cells were harvested by centrifugation and washed twice with 1.5 ml of sterile distilled water. A pretreatment solution containing 0.1 M Tris-HCl, 5 mM EDTA, 50 mM DTT (dithiothreitol), pH 8, was used to resuspend the cells, and the suspension was incubated 10 min at 30°C with gentle agitation.

The cells were again centrifuged, washed once, and resuspended with 1.5 ml of 0.6 M KCl, 0.1 M sodium citrate, pH not adjusted, protoplasting buffer solution, and 0.15 ml of enzyme preparation (Helicase; Suc Digestif d' Helix pomatia) was added. The suspension was incubated with gentle agitation at 30°C, usually for 30-40 min, until protoplast formation was complete. The course of protoplast formation was followed microscopically and macroscopically, the latter by comparison of water and protoplasting buffer 1/10 dilutions of samples. The protoplasts were washed once and resuspended in protoplasting buffer (2 x 10^8 prot/ml). Cellular density was estimated by counting the cells in a hemocytometer.

Protoplast fusion and selection of fusion products (FP). Equal numbers of protoplasts were mixed to a total of 4 x 10^8 protoplasts, spun down in an Eppendorf Microcentrifuge for 20 s at 9980 g, and the pellet was gently resuspended in 4 ml of 30% PEG 4000 containing 10 mM CaCl_2 in Tris-HCl, pH 8.1, added after being filter-sterilized. The protoplasts were incubated for 25 min at 30°C. Aliquots of 1 ml were diluted in 100 ml of melted minimal medium of regeneration (MMR) containing 0.67% yeast nitrogen base (YNB), 3% glycerol, 0.1% dextrose, 0.6 M KCl, and 3% agar and were immediately poured over already solidified MMR plates [16]. After the plates had cooled, they were incubated at 30°C until colonies of regenerants developed. Colonies were picked up and transferred from the MMR plates to MMR, master agar plates (without KCl and with 1.8% agar) and were replica-plated five times on the same medium. This step was called stabilization. After the fusion products were stabilized, they were reisolated on YEPD and MMR, and retested for respiratory deficiency with the tetrazolium assay.

Five isolated fusion products were further tested for growth on nonfermentable substrates and submitted to morphology and metabolic tests.

Tetrazolium assay (TTC). It was performed according to Ogor et al. [12].

Growth on nonfermentable substrates. Fusion products were tested for growth by replica-plating on 0.67% yeast nitrogen base (YNB) containing 3% glycerol, 3% ethanol, or 3% lactate and 1.8% agar (YNB-Gly; YNB-Eth; YNB-Lac). The plates were incubated at 30°C, and after 24-48 h the absence of growth was recorded.

Assimilation of carbon compounds and fermentation tests. The assays were recorded during a month, taking weekly readings, and during a week, taking daily readings, respectively [6].

Reversion controls. The genetic stability of Candida utilis “petite-like” was controlled on YNB-Gly, and the Saccharomyces cerevisiae nutritional characteristics on minimal agar medium (0.67% YNB; 2% dextrose, 1.8% agar) (MM).

Fusion control. Parental protoplasts were submitted to the same treatment as described for protoplast fusion, but in the absence of Ca^2+ ions.

Results and Discussion

Saccharomyces cerevisiae 4003-5B is a heterothallic, petite-positive yeast and therefore has a known perfect stage, whereas Candida utilis is an imperfect and petite-negative one, since it cannot go through a meiotic stage or render spontaneous petite strains like the former. Fusion frequency between respiratory-competent Saccharomyces cerevisiae 4003-5B and respiratory-deficient (RD) Candida utilis was 1.5 x 10^-5, in view of the number of protoplasts submitted to the PEG and Ca^2+ ions action. From 4 x 10^8 protoplasts used in fusion experiments, 160 fusion products were recovered after 4-5 days' incubation on MMR. After stabilization, only 37% (60) remained viable, probably owing to heterokaryon segregation in MMR. Heterokaryons carrying more than one nucleus could arise after cell wall regeneration from somatic fusion between yeasts belonging to different genera [22]. These heterokaryons could be forced towards nuclear fusion if placed under the selective pressure of a medium like MMR or MMR1. If, on the other hand, heterokaryons were placed on complete medium (YEPD), parental nuclei should segregate; thus, a different number of colony-forming units ought to appear after simultaneous fusion product colony dissemination on YEPD and MMR1, but this was not the case. Equal numbers and similar colony morphology and cell shape pointed out the absence of heterokaryons among the fusion products. This result was confirmed by nuclear staining, which revealed uninuclear cells. Isolated fusion product colonies in the presence of TTC proved to be respiratory-competent like the parental strain Saccharomyces, and the cell morphology resembled that of the original Candida utilis strain. The capacity to assimilate trehalose, maltose, and glyceral was recovered by the five fusion products analyzed (Table I); assimilation of xylose, cellobiose, succinic acid, citric acid, and salycilic acid was partially recovered. Whether there is some special reason for trehalose, maltose, and glyceral to be assimilated...