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Isolation of *Candida tropicalis* Auxotrophic Mutants

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An enrichment scheme using nystatin was designed for the isolation of auxotrophic mutants from the diploid-alkane-utilizing yeast *Candida tropicalis*. A collection of 194 auxotrophs representing 7 phenotypes was isolated. One class of mutants was identified as having a defect in histidinol dehydrogenase activity and a second class of mutants was identified as having a defect in orotidine monophosphate decarboxylase activity. These strains are good candidates to be carrying mutations corresponding to the *HIS4* and *URA3* genes of *Saccharomyces cerevisiae*.

Candida tropicalis is an asexual diploid yeast of significant relevance to the fields of medicine, cell biology, and industry. As an opportunistic human pathogen, it is a major hazard to immunocompromised patients and is recognized as second only to *C. albicans* with respect to pathogenicity and virulence (9, 11, 21, 22). Also, *C. tropicalis* has been used as a model organism for the study of peroxisomes. These microbodies undergo enormous proliferation upon transfer of cells to alkane- or fatty acid-containing media. Many of the β-oxidation pathway enzymes required to degrade these substrates are highly regulated and compartmentalized in the peroxisome (16, 17, 20). Several of these genes have been cloned (6, 7, 12–14, 19), and additional proteins thought to be involved in the biogenesis of peroxisomes have been isolated. Finally, the fatty acid or alkane utilization pathway of *C. tropicalis* is of interest to the chemical industry as a potential source of oligochemicals which are intermediates in the pathway. The development of strains which overproduce specific pathway intermediates via a biotransformation process might offer an attractive alternative to a chemical route to their production.

The development of techniques for the molecular genetic manipulation of the *C. tropicalis* genome will be of significant value to all aspects of *C. tropicalis* biology. Genetic systems may aid in elucidating the mechanisms of pathogenesis and drug resistance, biogenesis, and differentiation of peroxisomes and the manipulation of metabolic pathways for industrial use. Parasexual studies of *C. tropicalis* have been conducted on a limited number of strains (4, 5). However, the absence of suitable genetically marked strains has hindered the development of a transformation system. As a preliminary step towards developing a transformation system, we describe here techniques for the efficient isolation of auxotrophic mutants of *C. tropicalis* and a preliminary characterization of two classes of mutants which may be suitable hosts for transformation.

The wild-type *C. tropicalis* strain ATCC 20336 was mutagenized by harvesting exponentially growing cells in 1% yeast extract–2% peptone–2% glucose (YPD) and by washing in 10 ml of sterile H_2O and twice with an equal volume of citrate buffer (0.067 M sodium citrate, 0.03 M citric acid, pH 5.5). The cells were mutagenized with 100 μg of nitrosoguanidine ml^{-1} in citrate buffer for 40 min. After nitrosoguanidine treatment, the culture was washed twice with sterile H_2O, suspended in YPD medium, and incubated for 2 h at 30°C. Cells were then plated onto YPD plates (2% agar) at a dilution which gave ca. 200 colonies per plate after growth at 30°C. Colonies were screened for auxotrophs by replicating to minimal medium (0.67% yeast nitrogen base, 2% glucose, 2% agar) and scoring for lack of growth after 2 days of incubation. A series of 14 independent mutagenesis treatments resulted in auxotrophic mutants being isolated at a frequency of 0.01%. We attempted to increase the yield of auxotrophs by using a nystatin suicide enrichment procedure. This drug is known to kill growing yeast cells selectively under conditions in which nongrowing mutant cells survive (15, 18).

To obtain an efficient level of enrichment, the procedure was optimized for application to *C. tropicalis*. By using a population of prototrophic cells dosed with a known concentration of an adenine-requiring strain, Ade2, isolated in the screening procedure described above, it was possible to determine the level of enrichment for each of a variety of regimens tested. The parameters optimized were concentration of nystatin used; time of incubation in nystatin; the period of growth in minimal medium prior to nystatin treatment; and the use of nitrogen depletion as opposed to carbon depletion prior to selective growth and treatment of cells with nystatin.

From these studies it was found that depletion for nitrogen gave a ninefold-higher enrichment than depletion for carbon. The dose of nystatin was optimal around 20 U ml^{-1} and at 30 U or above the level of enrichment was reduced (Fig. 1). A key factor for enrichment was found to be the time of incubation in minimal medium prior to nystatin treatments (Fig. 2). No significant enrichment was observed until after at least 5 h of incubation. At 7 h, the level of enrichment was high; however, this decreased with further incubation. These results suggest that there is an optimal time of incubation in minimal medium, which generates the most significant contrast between auxotrophic and prototrophic cells. This is reflected in the relative kill of wild-type to auxotrophic cells and a corresponding high level of enrichment. Combining these optimal parameters, we were able to obtain significant (500-fold) enrichment for auxotrophic strains of *C. tropicalis* in a mutagenized culture.

The optimized method, as indicated by the above results, is as follows. After growing mutagenized culture on complete medium for 2 h, the cells were depleted for nitrogen by growth in 1% yeast carbon base for 17 h. Transfer to minimal medium for 7 h allowed prototrophic cells to grow; the cells were then treated with 25 U of nystatin for 20 min. Mutants

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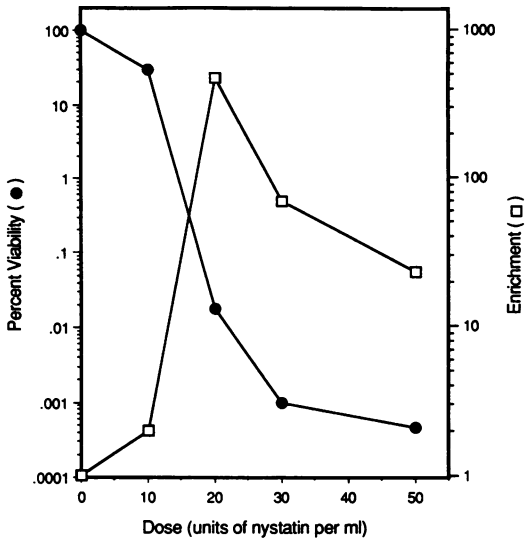

FIG. 1. Effect of various nystatin concentrations on viable count of *C. tropicalis* (●) and enrichment of the adenine-requiring strain, Ade2, added to the population of wild-type cells (□). Cultures were preincubated overnight in yeast carbon base and transferred to minimal medium for 7 h prior to treatment with differing doses of nystatin for 45 min.

were isolated from this treatment at a frequency of 3.9 to 5.3%. From two independent aliquots of mutagenized cells, a total of 183 mutants with a single auxotrophic requirement were isolated. The distribution of mutant types is indicated in Fig. 3. The majority of mutants (55%) were adenine requiring. This bias has been noted in a number of organisms undergoing mutagenesis (*Saccharomyces cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha*; 5, 7; J. M. Cregg, unpublished results) and, taking into account that the procedure was optimized for selection of a preexisting Ade^- subgroup, was not unexpected. Histidine, methionine, arginine, lysine, and proline mutants were also found. In contrast, no leucine, tryptophan, or uracil mutants were detected. This may reflect a susceptibility of these mutants to the nystatin treatment, a poor initial phenotype which interferes in the first plate screening, an inability to take up the

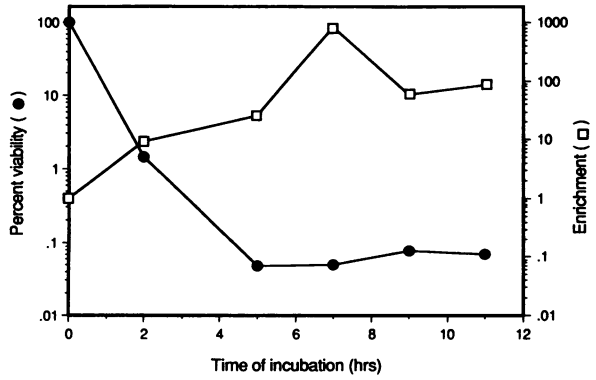

FIG. 2. Effect of incubation period in minimal medium prior to nystatin treatment. The viable count of *C. tropicalis* (●) and the enrichment of the adenine-requiring strain, Ade2, added to the population of wild-type cells (□) are shown after treatment with nystatin, 50 U ml^{-1}, for 40 min.

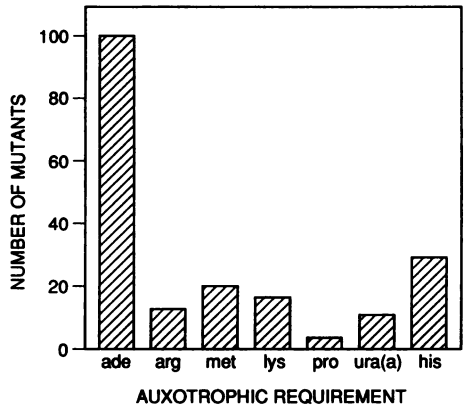

FIG. 3. Distribution of auxotrophic mutants isolated in *C. tropicalis*. The mutant phenotypes are indicated below each bar, and the number of mutants allocated to each phenotype is indicated along the vertical axis. The suffix (a) denotes that the uracil-requiring auxotrophs were isolated independently by using a double-selection regimen (see text).

supplement at a sufficient rate to support growth, or a lower frequency at which such mutants arise from this type of mutagenesis procedure.

We were interested in identifying specific biosynthetic pathway gene defects corresponding to known transformation gene markers in *S. cerevisiae*. Of 30 histidine auxotrophs isolated, 10 had a "tight" (no residual growth on minimal medium) phenotype. These strains were assayed for histidinol dehydrogenase activity (8) which corresponds to the *HIS4* gene activity of *S. cerevisiae*. As positive controls, two *HIS4* strains of *S. cerevisiae* were used; one was wild type and another had a defect in *his1* (FW29, a gift from F. Winston) and so was a histidine auxotroph. In addition, the wild-type *C. tropicalis* strain was used. A *his4* strain of *S. cerevisiae* (6657-9B, a gift from G. Fink) served as the negative control. Figure 4 shows the results of this assay. *C. tropicalis* wild type has a histidinol dehydrogenase activity approximately half that of the *S. cerevisiae* wild-type strain and equivalent to that of the *S. cerevisiae his1* strain. Mutants h1-5, h2-3, and h3-1 had significantly reduced levels of activity with respect to the wild type, while the other strains tested had levels of activity which were greater than the levels obtained in the wild-type strain. These strains represent potential hosts for transformation.

We considered that a mutant defective in orotidine monophosphate decarboxylase (equivalent to the *URA3* gene of *S. cerevisiae*) would be a particularly useful marker for transformation. This mutation confers resistance to the toxic metabolic pathway analog 5-fluoro-orotic acid, and so both a negative and positive selection can be applied to cells (1, 2). No uracil-requiring mutants were isolated by mutagenesis and nystatin enrichment or direct selection with 5-fluoro-orotic acid alone. To isolate uracil-requiring strains of *C. tropicalis*, a double selection involving, first, nystatin enrichment and then transfer to selective agar plates containing uracil and 5-fluoro-orotic acid (750 mg $liter^{-1}$) was used. One hundred colonies arising on these plates were screened for uracil auxotrophy, and those unable to grow without uracil supplement (11%) were assayed for orotidine monophosphate decarboxylase activity (23). Four strains were found to be defective for this activity.

We conclude from this work that the isolation of auxotrophic mutants from *C. tropicalis* ATCC 20336 can be

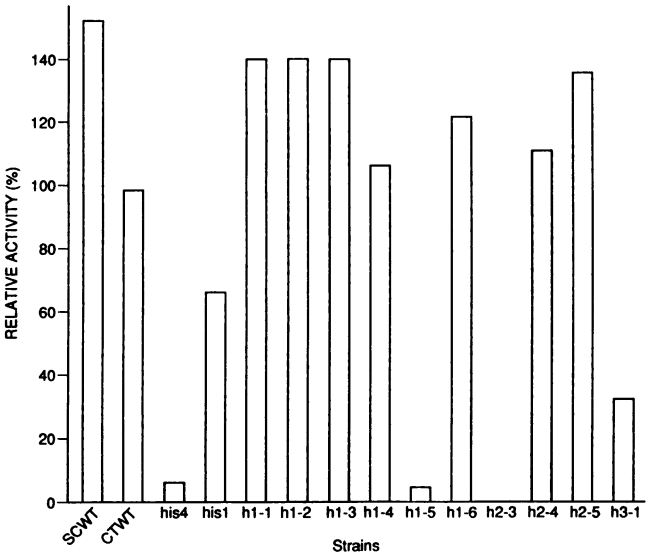

FIG. 4. Histidinol dehydrogenase activity in histidine-requiring auxotrophs of *C. tropicalis* relative to the wild-type strain. The relative histidinol dehydrogenase activity expressed as a percentage of wild-type activity is indicated along the vertical axis. The strain type is indicated below each bar. The first four bars represent the activity of control strains: SCWT, *S. cerevisiae* wild type; CTWT, *C. tropicalis* wild type; *his4*, *S. cerevisiae his4* mutant; *his1*, *S. cerevisiae his1* mutant. All other strains are histidine auxotrophs of *C. tropicalis*.

readily achieved by using the techniques described here. The generation of such mutants represents the first step towards development of genetic systems in *C. tropicalis*. Moreover, strains which appear analogous to the *S. cerevisiae his4* and *ura3* mutants represent candidates for the development of a DNA transformation system in *C. tropicalis*.

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