Interspecific Complementation Analysis by Protoplast Fusion of Candida tropicalis and Candida albicans Adenine Auxotrophs

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A protocol employing inositol starvation was used to isolate proline and adenine auxotrophs of Candida tropicalis. Interspecific hybrids between red adenine auxotrophs of C. tropicalis and Candida albicans were formed by protoplast fusion. These C. tropicalis red adenine auxotrophs were shown to fall into two complementation groups by crossing them with a known C. albicans ade1 tester strain. It is suggested that these two groups correspond to the ade1 and ade2 mutants of Saccharomyces cerevisiae and C. albicans and that these defined mutants may be useful in attempts to develop transformation systems for C. tropicalis.

The asexual yeast Candida tropicalis is an opportunistic human pathogen that causes both superficial and deep-seated systemic infections. It is considered the second most virulent Candida species, after the more commonly isolated Candida albicans (9). C. tropicalis is also of interest as a model organism for studies on peroxisome biogenesis. This organelle contains enzymes involved in the breakdown of alkanes and fatty acids. The synthesis of peroxisomes in C. tropicalis is induced manyfold on growth on these substrates as sole carbon and energy sources (11). Recently, several genes encoding C. tropicalis peroxisomal proteins have been cloned into Escherichia coli (5, 10, 14, 19). The development of genetic systems appropriate to C. tropicalis may be of value in elucidating mechanisms of pathogenesis, the basis of drug resistance, and the induction and targeting of peroxisomal proteins. More generally, further genetic analysis of C. tropicalis may contribute to our understanding of the genetic organisation of imperfect species. Previous parasexual genetic studies of C. tropicalis (2, 20) were limited to the formation of fusion products by protoplast fusion and the recovery of parental markers in recombinants that either occurred spontaneously or were induced by γ radiation or p-fluorophenylalanine. Transformation systems for C. tropicalis have not yet been described.

A barrier to transforming C. tropicalis is the absence of suitable genetically marked recipients for selection of rare transformants. Commonly, recipient strains in yeast transformation systems have been strict nonreverting monogenic recessive auxotrophs, with the transforming DNA including sequences that complement the genetic defect. For example, the first transformation of C. albicans was recently accomplished (7) with a red adenine auxotroph mutant at the ade2 locus as recipient for a plasmid carrying a C. albicans ADE2 gene. Transformation was by integration at the chromosomal ADE2 locus and occurred at a low frequency. The successful transformation of C. albicans red adenine auxotrophs suggested that ade1 and ade2 mutants of C. tropicalis may be similarly useful, as well as having uses in further parasexual genetic analyses. The value of red adenine auxotrophs lies in the ease with which they may be visually selected: they develop a red color on media containing limiting amounts of adenine. They have been studied extensively in Saccharomyces cerevisiae, where the basis of the phenotype has been found to be a defect in either of two sequential enzymes of purine biosynthesis, phosphoribosylaminomimidazole carboxylase (corresponding to the ADE1 locus) and phosphoribosylaminomimidazole succinocarboxamide synthetase (ADE1 locus) (1). Genetically, these two types of red adenine auxotroph can be distinguished on the basis of their complementation behavior. Mutant alleles of the ade1 locus of S. cerevisiae do not exhibit intragenic complementation, whereas alleles of the ade2 locus frequently do (21). This complementation pattern was also noted in several other species of fungi and was the way in which red adenine auxotrophs of C. albicans were characterized (13). Protoplast fusion between different C. albicans red adenine auxotrophs was used to determine complementation patterns, while linkage analysis confirmed the existence of two loci (13). The validity of these assignments to ade1 or ade2 classes was confirmed by transformation of one of the putative ade2 C. albicans strains (7).

Although characterization of C. tropicalis red adenine auxotrophs could presumably be done by means analogous to those used for C. albicans, the construction of full complementation grids in order to unambiguously distinguish intragenic from intergeneric complementation is time-consuming. Furthermore, no linkage information is available yet for C. tropicalis. An alternative approach was suggested by the recent report of interspecific hybrid formation by protoplast fusion of C. albicans and C. tropicalis (6). This makes it possible to use a C. albicans ade1 strain as a defined tester strain and cross all the C. tropicalis strains with this strain. This simple complementation analysis effectively eliminates the complications of intragenic complementation that can occur with two ade2 alleles.

This report describes the isolation of red adenine auxotrophs of C. tropicalis by UV mutagenesis coupled with auxotroph enrichment by inositol starvation. Ten of these red adenine auxotrophs were subsequently classified as ade1 or ade2 mutants on the basis of complementation by interspecific hybridization with C. albicans. The terms ade1 and ade2 are used to denote genetic loci in C. tropicalis that correspond to the ade1 and ade2 mutations of C. albicans and S. cerevisiae.

UV mutagenesis and inositol enrichment for C. tropicalis strains with proline and adenine auxotrophic requirements.
The origins and genotypes of strains used in this work are described in Table 1. UV mutagenesis was used to isolate through serendipity the inositol auxotroph T1400. Cells from a stationary-phase culture of C. tropicalis CDC B397 grown in YEP medium (a rich medium comprising 20 g of glucose, 10 g of peptone, 2.5 g of yeast extract, 1 g of NaCl, 0.5 g of MgSO₄, and 1 g of KH₂PO₄ per liter of water) were suspended in sterile distilled water and plated at approximately 1,000 per YEP plate. Plates were immediately exposed to 60 s of short-wavelength UV light at 13 ergs/mm² per s and incubated at 28°C. The survival rate after this treatment was approximately 10%. After 5 days, colonies were replica plated onto a minimal medium GSB [15 g of glucose, 1 g of (NH₄)₂SO₄, 2 g of KH₂PO₄, 0.05 g of MgSO₄, 0.05 g of CaCl₂, and 25 μg of biotin per liter of water]. Potential auxotrophs were purified and retested on GSB supplemented as required with amino acids and adenine at 3 \times 10^{-3} \text{ M} and with inositol at 3 \times 10^{-4} \text{ M}.

Inositol starvation enrichment (3, 4, 8, 16) was used to isolate proline auxotrophic derivatives of T1400. Proline auxotrophy was chosen because these mutants in S. cerevisiae can be partially grouped by growth supplementation testing into three classes; two of them (pro1 and pro2) respond to arginine as well as proline, while pro3 mutants respond only to proline (17). To enrich for proline auxotrophs by inositol starvation, approximately 10⁶ stationary-phase YEP-grown T1400 cells were spread on each of 10 GSB plates, UV irradiated for 60 s, and incubated at 28°C for 4 days. At this time, inositol (approximately 0.5 mg) plus proline (approximately 5 mg) were added to the center of each plate. After an additional 3 days of incubation, a total of 15,000 colonies grew and were replica plated onto GSB plus inositol and onto GSB plus inositol plus proline. Four proline auxotrophs were isolated; one of these, T1402 (an arginine or proline auxotroph), was used in a similar inositol starvation enrichment to isolate a series of red adenine auxotrophs. In this way, 13 red adenine auxotrophs were detected by replica plating onto YEP medium the 40,000 survivors from an initial 3 \times 10⁶ mutagenized cells. Ten of these red adenine auxotrophs, strains T1404 through T1413, were used in the complementation analyses. Long-term maintenance of strains was by lyophilization or by 6-monthly subculturing on YEP slopes.

In both of these examples, use of inositol enrichment resulted in an auxotroph yield of about 0.03%. By comparison, only one nonleaky red adenine auxotroph of T1402 was isolated from 33,000 survivors after UV mutagenesis in the absence of inositol enrichment. Efficiency of enrichment is in part limited by the reversion frequency of the inositol allele. This is the main advantage of doing the starvation on agar plates rather than in liquid media as has been described previously for S. cerevisiae (3). Revertant colonies are easily disregarded during replica plating, whereas in liquid media descendants of a single revertant can disperse and multiply in the culture. It is likely that other specific mutants of C. tropicalis may be isolated by inositol starvation enrichment.

**Complementation of C. tropicalis red adenine auxotrophs by interspecific hybridization with a C. albicans mutant.** Protocols essentially similar to those used for intraspecific C. albicans crosses (12) were used for intraspecific C. tropicalis crosses and interspecific crosses of C. albicans with C. tropicalis. Exponential-phase 5-ml YEP cultures (containing about 5 \times 10^5 cells) were harvested, washed by centrifugation, and suspended in 5 ml of M sorbitol−10 M Tris hydrochloride (pH 8.0) buffer. This suspension was treated with 0.01% β-mercaptoethanol for 15 min, and then zymolyase 100T was added to a final concentration of 5 μg/ml. After incubation for 1 to 2 h at 37°C, approximately 70 to 80% of cells were converted to spheroplasts as assessed by phase-contrast microscopy and osmotic lability. The spheroplasts were harvested by gentle centrifugation and suspended in 0.5 M CaCl₂−0.1 M Tris hydrochloride (pH 7.2) buffer (CT buffer). Halves of each spheroplast suspension were mixed pairwise as required. The remainder of each preparation was kept as a control to detect reversion. The spheroplasts were again harvested by gentle centrifugation, and the resultant soft pellets were gently suspended in 0.2 ml of CT buffer with 1 ml of 30% polyethylene glycol (6000). After 20 min of incubation at room temperature, this suspension was combined with 6 ml of molten 1% agar in CT buffer and quickly poured onto GSB-plus-adenine plates for regeneration of spheroplasts. Regeneration plates were incubated for 5 to 6 days at 28°C. Typically, 20 to 100 colonies grew on the fusion plates.

Double auxotrophic requirements in both parents (in addition to the unselected adenine characters) were used to avoid problems of reversion when the interspecific fusions were done. Spontaneous reversion frequencies of the mutant alleles used here were each less than 10⁻⁴⁻⁻. The series of C. tropicalis Ade⁻ Ino⁻ Pro⁻ strains (T1404 through T1413) were crossed by protoplast fusion with the previously described (12) C. albicans hOG6 ade1 ura lys. This strain is derived from hOG1 and is therefore an ade1 mutant on the basis of complementation studies (13). Since fusion products were selected for growth on minimal medium supplemented with adenine, they were expected to be in two categories, Ade⁺ or Ade⁻, reflecting the presence or absence of adenine complementation. Results from this analysis are presented in Table 2. While some strains gave only Ade⁺ fusion products (suggesting that they were ade2 mutants) and others gave only Ade⁻ fusion products (suggesting that they were ade1 mutants), several strains gave both Ade⁺ and Ade⁻ fusion products. In the latter cases, the majority of fusion products were Ade⁺, suggesting that they are ade2 strains and that the Ade⁺ fusion products are a result of incomplete transfer of genetic material between strains. Interestingly, these incomplete hybrids generally formed larger, faster-growing colonies than the presumably more complete Ade⁺ hybrids, suggesting that there is some form of incompatibility between the two species. Previous work (15) on C. albicans interspecific hybrids has shown that

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<th>TABLE 1. Origins and genotypes of strains</th>
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<td>Strain</td>
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<td>C. albicans</td>
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<td>hOG6</td>
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<td>C. tropicalis</td>
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<td>CDC B397</td>
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<td>T1405I</td>
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*The C. tropicalis genotypes were deduced from the present analysis.
*Originally from the Centers for Disease Control, Atlanta, Ga.
such partial hybrids are frequently detected when one selective element is absent, as in these experiments. UV irradiation of several interspecific Ade+ fusion products led to the appearance of the red adenine phenotype at a high frequency (data not shown), confirming that the fusion products were hybrids rather than contaminants.

**Intraspecific hybrids of *C. tropicalis* adenine auxotrophs.** As confirmation that the complementation grouping done by interspecific fusion was correct, one probable ade2 C. tropicalis strain, T1419, was used in a series of intraspecific adenine complementations. T1419 (Adε- Pro+ His+) was derived by UV mutagenesis of T14051, a spontaneous Ino+ revertant of strain T1405. This strain was crossed with Pro+ revertants of the entire series of *C. tropicalis* red adenine auxotrophs, and fusion products were selected on GSBL or adenine. These results are presented in Table 3. The observations that both other putative ade1 strains did not complement T1419, while all the ade2 strains did, are strong support that the initial assignments were in fact correct. These fusions were also necessary to establish that suppression by hOG6 was not giving spurious complementation results with any of the *C. tropicalis* strains. Natural tRNA suppressor activity in two strains of *C. albicans hOG6* previously been noted (18). The use of isogenic *C. tropicalis* strains makes it unlikely that suppression is occurring in these intraspecific fusions.

**Conclusions.** This report provides results of methods for specific mutant enrichment in the asexual yeast *C. tropicalis*}

and describes the use of complementation by protoplast fusion with *C. albicans* to genetically define these mutants. Both ade1 and ade2 mutants of *C. tropicalis* have been isolated and defined by these means. An important use of the ade1 and ade2 mutants of *C. tropicalis* described here may be in developing transformation systems. The low reversion frequencies of T1405 (ade1) and T1409 (ade2) suggest that they may be particularly suitable for this purpose. Exploratory work with other members of the genus *Candida* suggests that several other interspecific hybrids can be formed (unpublished results), and it may therefore be relatively simple to genetically define mutants in most of these species by analogous means.

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**LITERATURE CITED**


