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Development of an Integrative DNA Transformation System for the Yeast *Candida tropicalis*

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We developed the alkane and fatty-acid utilizing yeast *Candida tropicalis* as a host for DNA transformations. The system is based on an auxotrophic mutant host of *C. tropicalis* which is defective in orotidine monophosphate decarboxylase (*ura3*). The *ura3* host was isolated by mutagenesis and a double-selection procedure that combined nystatin enrichment selection and 5-fluoro-orotic acid resistance selection. As a selectable marker, we isolated and characterized the *C. tropicalis URA3* gene. Plasmid vectors that contained the *C. tropicalis URA3* gene transformed the *C. tropicalis* mutant host at a frequency of 10^3 to 10^4 transformants per μg of plasmid DNA. Vectors that contained the *Saccharomyces cerevisiae URA3* gene could not transform *C. tropicalis*. DNA transfer was accomplished by modified versions of either spheroplast generation ($CaCl_2$-polyethylene glycol)-fusion or cation (LiCl) procedures developed for *S. cerevisiae*. Plasmid vectors that had been cut within the *C. tropicalis URA3* fragment integrated by homologous recombination at the *URA3* locus.

Candida tropicalis, an asporogenous dimorphic yeast, is of medical, academic, and industrial interest. The *C. tropicalis* strain used in this study was isolated from the soil and is not known to be pathogenic; however, certain strains of *C. tropicalis* are well-known opportunistic pathogens of humans and are recognized as second only to *C. albicans* among virulent yeasts (16, 18, 20, 26, 32–35). An unusual and interesting feature of *C. tropicalis* metabolism is the ability to utilize alkanes and fatty acids as sole carbon and energy source. Many of the β-oxidation pathway enzymes required to degrade these substrates are highly regulated and compartmentalized in a subcellular organelle called the peroxisome. Growth on alkanes or fatty acids leads to induction of pathway enzymes and proliferation of peroxisomes inside the cells, a situation that is being exploited to study biogenesis of and protein import into peroxisomes of *C. tropicalis* (21, 27, 28, 31). Of industrial interest, *C. tropicalis* might have advantages over other organisms as a host for production of heterologous proteins. Furthermore, the yeast efficiently uses alkanes or fatty acids as sole carbon and energy source and has a rapid growth rate.

Studies of *C. tropicalis* at the molecular level would be enhanced significantly by the ability to transfer and maintain DNA sequences in the yeast. In this report, we describe an efficient transformation system for *C. tropicalis*. The system is based on an orotidine monophosphate decarboxylase (OMD)-defective (*ura3*) host of *C. tropicalis* and modified versions of DNA transfer procedures developed for *Saccharomyces cerevisiae* (13, 14). As a selectable marker, we isolated and characterized the *C. tropicalis URA3* gene. Plasmids that contain the *URA3* gene transform the mutant *C. tropicalis* host at high frequencies and can be directed to integrate by homologous recombination into the *URA3* locus.

MATERIALS AND METHODS

Strains, plasmids, and media. The wild-type *C. tropicalis* strain used was ATCC 20336. *C. tropicalis* SU-2 (*ura3*) was generated by mutagenesis of ATCC 20336 with 1-methyl-3-nitro-1-nitrosoguanidine (NTG). The isolation of the mutant is described below. *S. cerevisiae* SHY-3 (*his3 ura3 leu2 ade1 trp1*) was obtained from the Yeast Genetic Stock Center at the University of California, Berkeley. *Escherichia coli* CSH28 [Δ(*lac pro*) *supF trp pyrF strA thi*], which has a defect in OMD (19), was a gift from D. Kwoh (Salk Institute Biotechnology/Industrial Associates, Inc.). *E. coli* MC1061 [F⁻ *araD139* Δ(*araABDIC-leu*)*7679* Δ*lacX74 galU galK rpsL hsdR*] was provided by M. Casadaban and was used for all recombinant DNA experiments that required a bacterial host.

YEp24, which consists of the *S. cerevisiae URA3* gene on a 1,200-base-pair fragment inserted in the *Eco*RI and *Hin*dIII sites of pBR322 (5, 6), was the source of the *S. cerevisiae* gene and was obtained from G. Thill (Salk Institute Biotechnology/Industrial Associates, Inc.). YEp13 (7) and pUC18 (36) have been described previously. The complete medium (YPD) and minimal medium (SD) used to grow *S. cerevisiae* and *C. tropicalis* have been described by Rose et al. (23). *E. coli* was cultured in either LB medium (19) or 2B medium (8).

Mutant isolation. Log growth phase *C. tropicalis* cells in YPD were prepared for NTG mutagenesis by being washed once with 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 NTG per ml in citrate buffer for 40 min (11). After NTG treatment, the culture was washed twice with sterile H_2O, suspended in YPD medium, and incubated for 2 h at 30°C. For nystatin enrichment of auxotrophs, mutagenized cells were transferred to yeast carbon base medium (Difco Laboratories, Detroit, Mich.) and grown overnight. The culture was then transferred by centrifugation to SD medium to allow growth of prototrophs. After 7 h of incubation in SD, 20 to 40 U of nystatin per ml was added and the culture was incubated with shaking for 20 to 30 min to enrich for auxotrophs. The culture was then washed twice and directly plated on SD agar plates containing 0.75 mg of 5-fluoro-orotic acid (5-FOA) per ml, 0.5 mg of uracil per ml, and 0.5 mg of uridine per ml. Colonies which grew on these 5-FOA-containing plates were then tested for uracil auxotrophy.

Uracil auxotrophs to be tested for loss of OMD activity were grown in YPD medium to an A_{600} of 2.0 and washed in

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potassium phosphate buffer (0.1 M K_2HPO_4, pH 7.5, 5 mM β-mercaptoethanol). Cells were extracted by adding 1 g of 0.5-mm-diameter glass beads and 0.5 ml of potassium phosphate buffer to 1 g of cells and vortexing them for 5 min. One milliliter of potassium phosphate buffer was added, and the mixture was vortexed again for 5 min. One milliliter of cell extract was centrifuged in an SS34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 20,000 rpm for 40 min at 4°C in an RC-5B refrigerated superspeed centrifuge. The supernatant was removed and passed through a Sephadex G25 column. The resulting cell extract was then assayed for OMD activity by the method of Yoshimoto et al. (37).

DNA isolation. Large-scale DNA from *C. tropicalis* was prepared by the method of Cregg et al. (8). Small- and large-scale *E. coli* cultures were grown as described previously (17). Plasmids from all preparations were isolated by the alkaline lysis method of Birnboim and Doly (2).

DNA methods. Restriction enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and digestions were performed as recommended by the suppliers. Restriction fragments were purified by electroelution from agarose gels onto NA45 membrane strips as recommended by the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.). The conditions used for DNA-DNA (Southern blot) hybridizations were described by Cregg et al. (8).

The method used to construct the *C. tropicalis Sau*3AI DNA-YEp13 library has been described previously (8). The library consisted of 5- to 20-kilobase (kb) *Sau*3AI partial-digestion fragments of wild-type *C. tropicalis* DNA inserted into the *Bam*HI site of *S. cerevisiae-E. coli* shuttle vector YEp13. Approximately 98% of the colonies which were pooled for the library were tetracycline sensitive. YEp24-based recombinant plasmids containing *C. tropicalis* genomic DNA fragments were constructed by digesting *C. tropicalis* genomic DNA to completion with *Bam*HI and ligating the DNA into the *Bam*HI site of YEp24.

Yeast transformations. *S. cerevisiae* transformations were performed by the spheroplast generation method of Hinnen et al. (13). To transform *C. tropicalis*, a modified form of this procedure was used as follows.

A colony of *C. tropicalis* was inoculated into 10 ml of YPD and grown at 30°C with shaking overnight. On the next day, cells were diluted in YPD to an optical density at 600 nm of 0.01 to 0.1 and maintained in the logarithmic growth phase throughout the day. In the afternoon, this seed culture was inoculated into 100 ml of YPD to a final optical density at 600 nm of 0.00003 and grown overnight at 30°C with shaking. On the next morning, the culture at an optical density at 600 nm of 0.2 to 0.3 was harvested by centrifugation at 1,500 × *g* for 5 min and washed with 10 ml of H_2O. The cells were then washed once in 10 ml of freshly prepared filter-sterilized SED (1 M sorbitol, 25 mM EDTA, and 50 mM dithiothreitol), washed once in 10 ml of sterile 1 M sorbitol, and suspended in 5 ml of SCE buffer (1 M sorbitol, 100 mM sodium citrate, 10 mM EDTA, pH 5.8). To this we added 3 μl of Zymolyase 6000 at 4 mg/ml, and the cells were incubated at 30°C. This is a critical step in the transformation procedure, and the formation of spheroplasts needs to be monitored by adding 100-μl aliquots of cells to 900 μl of 0.2% sodium dodecyl sulfate and 900 μl of 1 M sorbitol before addition of Zymolyase and at times during the incubation period. Incubation should be stopped when cells lyse in sodium dodecyl sulfate (showing a drop in the optical density at 600 nm) but not in sorbitol (usually 15 to 20 min of incubation). The spheroplasts were washed once in 10 ml of sterile 1 M sorbitol by centrifugation at 1,000 × *g* for 10 min and then washed in 10 ml of sterile CaS (1 M sorbitol, 10 mM $CaCl_2$) and suspended in a total of 0.6 ml of CaS. DNA samples of up to 20 μl are added to 100-μl volumes of the spheroplasts and incubated at room temperature for 20 min.

We added 1 ml of PEG solution (20% polyethylene glycol 3350, 10 mM $CaCl_2$, 10 mM Tris hydrochloride, pH 7.4) to each sample and incubated it at room temperature for 15 min. Samples were then centrifuged at 1,000 × *g* for 10 min, the PEG solution was decanted, and the samples were suspended in 150 μl of SOS (1 M sorbitol, 30% YPD medium, 10 mM $CaCl_2$) and incubated at room temperature for 30 min, after which 850 μl of 1 M sorbitol was added. The samples were then added to 10 ml of melted regeneration agar (3% Bacto-Agar, 2% glucose, 0.67% yeast nitrogen base [YNB], 0.6 M KCl), kept at 48°C, poured onto a base plate containing a bottom layer of 10 ml of regeneration agar, and incubated for 2 to 4 days at 30°C.

Transformation of *C. tropicalis* by a modification of the whole-cell LiCl treatment procedures of Ito et al. (14) was as follows.

Mid-exponential growth phase *C. tropicalis* cells in YPD prepared as described above were harvested by centrifugation at 1,500 × *g* for 10 min, washed once in 5 ml of sterile TE (10 ml Tris hydrochloride [pH 7.4], 1 mM EDTA), suspended in 2.5 ml of 100 mM LiCl, and incubated for 1 h at 30°C with shaking.

DNA samples were mixed with 100-μl samples of the cells and incubated at 30°C for 30 min. We added 900 μl of 40% PEG (40% polyethylene glycol 3350, 100 mM LiCl) to each sample and incubated it for 1 h at 30°C with shaking. The samples were then subjected to a heat shock step, 42°C for 5 min, after which the cells were centrifuged and washed in 1 ml of sterile H_2O before being plated onto SD plates. Colonies appeared after 2 to 3 days of growth at 30°C.

RESULTS

Isolation of a *ura3* mutant strain. As a first step in the development of a transformation system for *C. tropicalis*, a uracil auxotrophic host with a defect in OMD activity (*ura3*) was isolated. To isolate this strain, double selection with nystatin and 5-FOA was developed. Nystatin, a drug which selectively kills growing yeast cells under conditions in which nongrowing cells survive (25, 29), was used to enrich for auxotrophic mutants in a population of cells which had been mutagenized with NTG. We estimate that the nystatin enrichment factor was approximately 800-fold, a value obtained in control experiments in which a population of wild-type cells was dosed with a known concentration of auxotrophic cells and then subjected to the enrichment regimen. After nystatin enrichment, a portion of the resulting culture (approximately 4×10^8 cells) was then spread on agar plates containing 5-FOA (3, 4), and after 5 days at 30°C, the colonies that resulted were analyzed for the ability to grow on medium without uracil. Of 100 yeast colonies that resulted from the double-enrichment procedure, 11 were uracil auxotrophs (11%). To identify *ura3* mutants, cell-free extracts of these 11 strains were assayed for OMD activity and 4 were observed to be defective in this enzyme activity (33%). Overall, we estimate that the frequency of *ura3* mutants in the mutagenized cell population was approximately 5×10^{-7}. Of the four *ura3* strains, one, named SU-2, appeared to be best suited as a host for transformation. In addition to having low OMD activity, it exhibited an absolute requirement for uracil for growth and reverted to uracil prototrophy at a frequency of less than 10^{-9}.

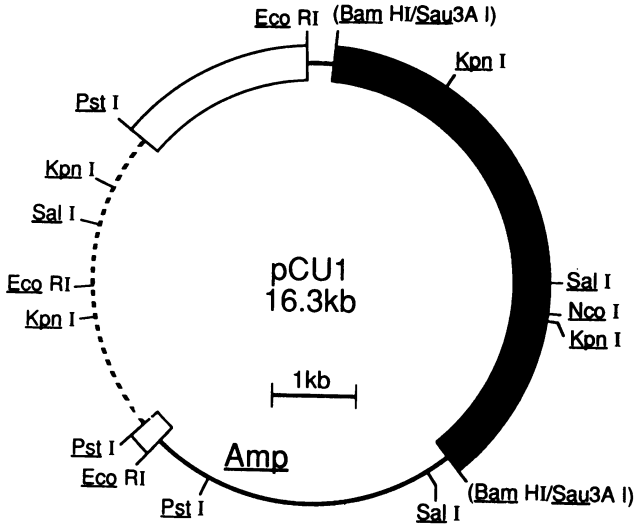

FIG. 1. Restriction enzyme map of pCU1. The plasmid contains sequences from the following sources: pBR322, thin line; *S. cerevisiae LEU2* gene, broken line; *S. cerevisiae* 2μm plasmid, open boxes; *C. tropicalis URA3* gene, thick line. Amp, Ampicillin.

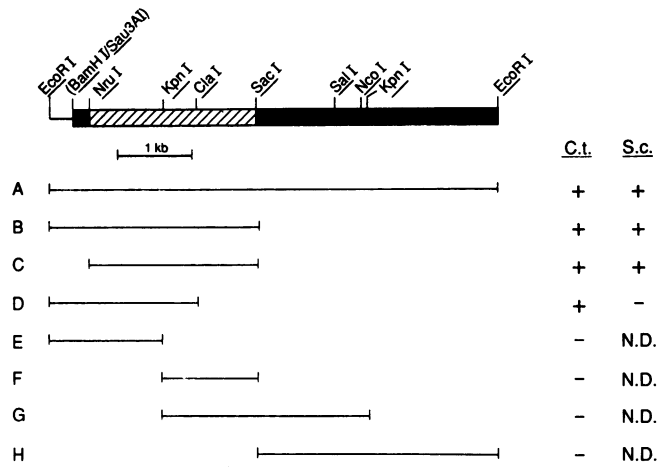

FIG. 2. Transformation with *C. tropicalis* DNA fragment. Abilities of DNA fragments A to H to complement the *ura3* defect in a *C. tropicalis* (C.t.) or *S. cerevisiae* (S.c.) host are indicated by plus and minus signs. For transformations of *C. tropicalis*, the indicated DNA fragments were directly mixed with spheroplasts. These plasmids were linearized within the *C. tropicalis* DNA insert and also transformed into SU-2. For transformations of *S. cerevisiae*, fragments A to D were first subcloned into plasmids containing a portion of the 2μm circle. N.D., Not determined; pBR322, thin line; *C. tropicalis* DNA, thick line; approximate position of *C. tropicalis URA3* gene, hashed line.

Isolation of the *C. tropicalis URA3* gene. As a marker for transformation of the *C. tropicalis ura3* host, the *C. tropicalis URA3* gene was isolated and characterized. DNA fragments which contained the *C. tropicalis URA3* gene were isolated from a *C. tropicalis* DNA YEp13 library by the ability to complement *ura3*-carrying *S. cerevisiae* SHY-3. Spheroplasts of SHY-3 were mixed with the *C. tropicalis* DNA library and allowed to regenerate in uracil-deficient medium. The transformation resulted in approximately 10^3 Ura^+ yeast colonies from a population of 5×10^7 total regenerable spheroplasts. Total yeast DNA was extracted from individual Ura^+ colonies and transformed into *E. coli*. Ampicillin-resistant colonies from four of the yeast DNA preparations were examined for plasmids, and each contained a plasmid which comprised YEp13 plus insert DNA. Two plasmids appeared to be identical by restriction enzyme analysis and had an insert size of 5.8 kb. The remaining two had insert sizes of approximately 10 and 20 kb. All four plasmids retransformed SHY-3 to uracil prototrophy at a frequency indistinguishable from that of controls in which leucine prototrophy was selected.

As additional evidence that the *C. tropicalis URA3* gene and not a DNA fragment with suppressor activity had been isolated, each of the four plasmids was transformed into a *pyrF* mutant strain of *E. coli* which is defective in OMD activity. Transformants that contained any one of the putative *C. tropicalis URA3* plasmids grew on uracil-deficient plates, whereas control strains that did not harbor a plasmid did not grow. As described below, these plasmids transformed our *C. tropicalis ura3*-carrying strain.

Characterization of the *C. tropicalis URA3* gene. One of the *C. tropicalis URA3* gene-YEp13 recombinants that contained the gene on a 5.8-kb *Sau*3AI partial fragment was named pCU1 and subjected to further analysis (Fig. 1). To facilitate this analysis, a 6.1-kb *Eco*RI fragment from pCU1 (Fig. 2) that contained most of the *C. tropicalis* DNA was subcloned into the polylinker of pUC18. This *Eco*RI fragment was composed of 377 base pairs of YEp13 at one end and all of the *C. tropicalis* DNA except for approximately 50 base pairs between the *Eco*RI site and the *Bam*HI-*Sau*3AI junction at the other end. This plasmid was called pCU2. The approximate position of the *URA3* gene was mapped by transforming selected subfragments isolated from pCU2 into SU-2 and determining which subfragments could complement the mutant defect (Fig. 2). Fragments derived from the middle or right-hand end of the *C. tropicalis* DNA insert did not transform SU-2 to uracil prototrophy. However, fragments from the left-hand end of the insert did, suggesting that the *URA3* gene existed near the left-hand end of the *C. tropicalis* fragment. Interestingly, neither the *Eco*RI-*Kpn*I fragment nor the *Kpn*I fragment derived from the left side of the *C. tropicalis* fragment transformed SU-2 (Fig. 2). This result might be explained if the *URA3* gene mutation in SU-2 exists very close to or spans the *Kpn*I site, so that the wild-type sequence on the transforming DNA fragment is usually removed by exonuclease digestion before the fragment can recombine into the genome.

DNA fragments that transformed SU-2 to Ura^+ may have contained only that portion of the *URA3* gene that was defective in SU-2. To locate the boundaries of *URA3*, selected DNA fragments were tested for *URA3* function in *S. cerevisiae* on autonomous plasmids. Since these plasmids do not integrate, it is probable that the entire *URA3* gene must exist within the *C. tropicalis* DNA fragment for Ura^+ complementation to occur. *C. tropicalis* DNA fragments were subcloned into pBR322 or pUC18, along with a DNA fragment containing a portion of the 2μm circle capable of giving plasmids the ability to replicate autonomously in *S. cerevisiae*. Transformation of these plasmids into *S. cerevisiae* SHY-3 revealed that a 2.3-kb *Nru*I-*Sac*I fragment was the smallest fragment that could complement the *ura3* mutation. A pUC18-derived plasmid containing this fragment was constructed, mapped, and named pCU3 (Fig. 3).

C. tropicalis genomic DNA was examined for homology to the *S. cerevisiae URA3* gene on plasmid YIp5 (30). The *S. cerevisiae URA3* gene did not hybridize significantly with *C.*

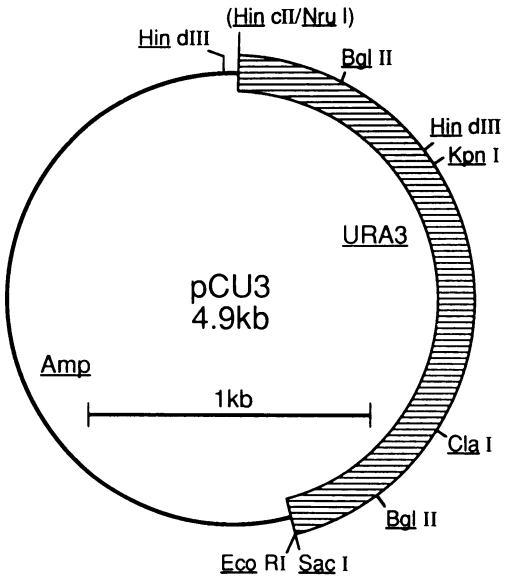

FIG. 3. Restriction enzyme map of pCU3. The plasmid consists of pUC18 (thin line) and a 2.4-kb fragment which contains the *C. tropicalis URA3* gene (hashed line). Amp, Ampicillin.

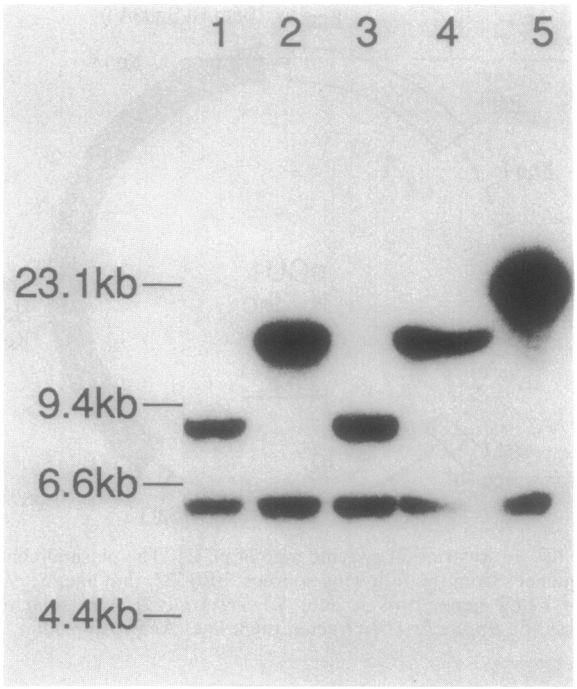

FIG. 4. Southern blot hybridization of yeast cellular DNAs with ^{32}P-labeled pCU2Δ*Sac*I. Each lane contained 5 μg of *C. tropicalis* DNA restricted with *Nco*I. Lane 1 contained *C. tropicalis* SU-2 DNA. Lanes 2 to 5 contained DNAs from four SU-2 transformants, as described in the text.

tropicalis DNA, even at the lowest stringency examined (37°C, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 30% formamide). Thus, the *URA3* genes from these yeast species do not appear to be closely homologous at the DNA sequence level.

Comparison of the *C. tropicalis URA3* gene with other *URA3* loci. The *C. tropicalis URA3* locus was examined in a series of DNA-DNA (Southern) hybridizations. In these experiments, the labeled probe was pCU2Δ*Sac*I, a *URA3*-containing plasmid that was derived from pCU2 by deleting DNA sequences to the right of the *Sac*I site. Samples of *C. tropicalis* genomic DNA were digested with each of nine different restriction enzymes known to cut outside the cloned *URA3* gene fragment in pCU2Δ*Sac*I. We anticipated seeing a single band on a Southern blot of wild-type *C. tropicalis* DNA restricted with these endonucleases. However, we observed that most enzyme digests produced at least two hybridizing DNA fragments. Figure 4 is a photograph of such a Southern blot, in which *C. tropicalis* DNAs were digested with *Nco*I and hybridized with labeled pCU2Δ*Sac*I. Lane 1 contained *C. tropicalis* SU-2 DNA and showed three bands of 8.6, 6.1, and 2.4 kb (the smallest band is not shown). SU-2 DNA produced a banding pattern identical to that of wild-type *C. tropicalis* DNA on Southern blots (data not shown). Thus, it appeared that *C. tropicalis* has at least two *URA3*-hybridizing loci.

An alternative explanation for the multiple bands was that the *C. tropicalis* DNA in pCU2Δ*Sac*I contained an ectopic DNA fragment. That is, in the construction of the *C. tropicalis* genomic library, two *Sau*3AI genomic DNA fragments may have ligated together into the single *Bam*HI site of YEp13. If so, the probe would always hybridize to at least two fragments. To examine this possibility, another Southern blot experiment was performed in which two DNA probes composed of subfragments from the left and right sides of the *C. tropicalis* fragment in pCU2Δ*Sac*I were hybridized separately to the *Sal*I-digested *C. tropicalis* genomic DNA. These *Sal*I digests produced two bands when hybridized with labeled pCU2Δ*Sac*I. If an ectopic DNA fragment were present in the original *URA3* probe, it was expected that the subfragmented probes would hybridize preferentially to one or the other of the two genomic fragments. For this Southern blot experiment, radiolabeled probes were made from plasmids which contained either the 1.6-kb *Eco*RI-*Kpn*I or the 1.3-kb *Kpn*I-*Sac*I fragment. It was observed that both probes hybridized equally to both genomic *Sal*I fragments. Therefore, it was unlikely that the *C. tropicalis* DNA fragment in pCU2Δ*Sac*I contained an ectopic fragment. From this, we concluded that the *C. tropicalis* genome contains two loci which hybridize to the *URA3* fragment. By further analysis, we established that the smaller *Sal*I fragment contained the cloned *URA3* locus and that the larger band was from another locus of unknown identity.

Transformation of *C. tropicalis*. *C. tropicalis URA3*-containing DNA vectors transformed SU-2 at frequencies of 10^3 to 10^4 colonies per μg of DNA (Table 1). Either the spheroplast or LiCl procedure was satisfactory, although the spheroplast procedure produced approximately 10 times more colonies per microgram of DNA. Transformed colonies were of two distinct classes, large and small. There were far more small colonies than large ones, but the ratios varied (Table 1). In *S. cerevisiae*, the slower growth of some transformants is the result of the presence of the plasmids as autonomous elements and poor partitioning of these plasmids into daughter cells. Faster-growing cells are the consequence of integration of plasmids into the genome and the resulting mitotic stability. The large and small colonies seen with *C. tropicalis* appeared to be the consequence of the same phenomenon. As evidence of the autonomous nature of slow-growing transformants, we observed rapid loss of the Ura$^+$ phenotype when transformants were grown in nonselective medium (data not shown). Furthermore, a

TABLE 1. Transformation of *C. tropicalis* with selected vectors[a]

Vector(s)	Linearization within *C. tropicalis* DNA	No. of colonies/μg of DNA (10^4)	Large- to small-colony ratio
pCU1	−	4.0	0
pCU1	+	1.4	1:26
pCU2Δ*Sac*I	+	1.7	1:53
pCU3	+	1.8	1:59
YEp24	−	0	0
YEp24 + *C. tropicalis* DNA	+	0	0

[a] Cells were transformed by using the spheroplast generation procedure.

plasmid pattern was observed in Southern blots in which unrestricted total DNAs from strains transformed with uncut pCU1 were hybridized with labeled YEp13 (data not shown). When *C. tropicalis* was transformed with DNA vectors linearized in the *URA3* fragment, the proportion of large colonies was higher than with uncut plasmids. This result suggested that, as in *S. cerevisiae* (22), linearization facilitates integration of the vector into the host genome. It was surprising that, even after plasmid linearization, the ratio of large- to small-colony transformants remained low. The small colonies may have been due to (i) transformation with linear molecules that recircularized during the process, (ii) transformation with uncut plasmids that remained after restriction and transformed at a higher frequency than linear molecules, or (iii) a combination of both.

Fate of transformed sequences. We were interested primarily in establishing an integrative transformation system in which transforming vectors could be targeted to a specific locus in the *C. tropicalis* genome. To determine whether or not vectors integrated by homologous recombination, we transformed SU-2 with pCU2Δ*Sac*I that had been cut once within the *URA3* locus by digestion with *Nru*I (Fig. 2). DNA was isolated from 14 large-colony transformants for analysis by Southern blot hybridization. The DNAs were cut with either *Nco*I or *Hpa*I, neither of which cuts pCU2Δ*Sac*I, and a Southern blot filter prepared with these digested DNAs was probed with labeled pCU2Δ*Sac*I. The results obtained with *Nco*I-digested DNAs are shown in Fig. 4. The blot indicated that all large-colony transformants were the result of integration events. Further examination of these blots revealed three types of transformation events. Type I transformants (lane 3) had a banding pattern that was identical to that of wild-type or SU-2 DNA (lane 1). This was most likely due to gene conversion. In type II transformation events, one *URA3* locus had been altered (lanes 2 and 4). The 8.6-kb band representing the locus was shifted to a position representing a larger DNA fragment of approximately 16 kb. The size of the new band suggested that type II events were the result of integration of a single copy of pCU2Δ*Sac*I at one *URA3* locus. In type III transformation events, the same *URA3* locus had also been altered (lane 5). However, the new DNA bands were shifted to positions representing larger DNA fragments consistent with multiple tandem integrations of pCU2Δ*Sac*I at *URA3*. Of the 14 transformations, 8 were single-integration events, 4 were multiple-integration events, and 2 were gene conversion events. All type II and III integration events occurred at the one *URA3* locus that corresponded to the cloned *URA3* gene and not at the second *URA3*-hybridizing locus. Most importantly, all integrative recombination events appeared to involve homologous recombination of linear plasmids.

Failure of the *S. cerevisiae URA3* gene to transform *C. tropicalis*. Transformation of SU-2 with vectors that contained the *S. cerevisiae URA3* gene were attempted. No URA^+ colonies were observed after transformation with YEp24, a pBR322-based plasmid containing the *S. cerevisiae URA3* gene and a portion of the 2μm circle (6). It was possible that YEp24 could not easily integrate into the *C. tropicalis* genome, because it shared no DNA homology. Furthermore, for YEp24 to replicate autonomously in *C. tropicalis*, an autonomous replication sequence element recognized by *C. tropicalis* must be present. To allow YEp24 to integrate into the *C. tropicalis* genome, *C. tropicalis* DNA was inserted into YEp24. Four YEp24-based recombinant plasmids were constructed that each contained a different *C. tropicalis* genomic DNA fragment with a unique restriction enzyme site within the *C. tropicalis* DNA. Transformation of SU-2 with each of these *C. tropicalis*-YEp24 DNA vectors linearized in the *C. tropicalis* DNA fragment also resulted in no transformants. Thus, it appeared that the *S. cerevisiae URA3* gene did not function in *C. tropicalis*.

DISCUSSION

A DNA transformation system for *C. tropicalis* was developed. The system is based on an auxotrophic mutant strain of *C. tropicalis* that is defective in OMD activity (*ura3*) and plasmid vectors that contain the *C. tropicalis URA3* gene as a selectable marker. To isolate *ura3* mutants, combined selection involving both nystatin enrichment and 5-FOA resistance selection was used. By using either selection method alone, we were unable to isolate *ura3* mutants from populations of mutagenized cells. We estimate that *ura3* mutants arose at a frequency of 5×10^{-7} in the mutagenized cell population before enrichment. The *ura3*-carrying *C. tropicalis* strain that we selected has ideal transformation host characteristics: a tight phenotype; a low reversion frequency; and other than its Ura^- phenotype, indistinguishability from the wild type.

Transformation of *C. tropicalis* with vectors containing the *C. tropicalis URA3* gene was efficient, resulting in up to 10^3 to 10^4 transformants per μg of DNA. Interestingly, the *S. cerevisiae URA3* gene could not transform our *C. tropicalis ura3* host, a result that was unexpected, since *S. cerevisiae* genes often function in other yeasts. For DNA transfer, two methods are available. In method 1, spheroplasts are mixed with DNA in the presence of $CaCl_2$ and polyethylene glycol and transformants are selected for uracil prototrophy (13). *C. tropicalis* spheroplasts regenerate and grow rapidly in top agar, and transformants are usually visible within 24 h. Transformants appear as filamentous colonies under top agar, a result that is not unexpected, since *Candida* yeasts are known to form pseudomycelia under stress (20). When these filamentous colonies are picked and either streaked on agar media or inoculated into liquid media, they regain their budding yeast morphology. Method 2 of DNA transfer is based on inducing competency when cells are treated with LiCl (14). Although transformation efficiency with LiCl is approximately 10-fold lower than with spheroplasts, transformants do not need to be recovered from agar embedding and are not filamentous.

Transformation of *C. tropicalis* results in colonies of two distinct sizes. The small colonies appear to contain the plasmid as an autonomously replicating element. Evidence for their autonomous state includes (i) slow growth of transformants on selective medium relative to integrated transformants, (ii) rapid loss of the Ura^+ phenotype by

transformants grown on nonselective medium, and (iii) a plasmid pattern in Southern blots of unrestricted total DNAs extracted from transformants. It may be possible to select for autonomous replication sequence elements for *C. tropicalis* and eventually to obtain vectors capable of autonomous replication in *C. tropicalis*, as achieved in other yeasts (8, 30). Large transformant colonies are the result of integration of vectors into the chromosome. Extensive Southern analysis of genomic DNAs from transformed strains has shown that vectors integrate by homologous recombination. Thus, it should be possible to perform a variety of precise manipulations of the *C. tropicalis* genome, such as gene replacements of the type that are routinely performed with *S. cerevisiae* (24).

A major consideration in developing the *C. tropicalis* transformation system around the *URA3* gene as a selectable marker was the potential that this marker system has for both positive and negative selections (3, 4). DNA can be introduced into the organism on vectors that contain *URA3* by selection for Ura^+, and with proper arrangement of sequences surrounding *URA3*, DNA can be excised from the *C. tropicalis* genome by selection for Ura^- with 5-FOA. An example of the power of this positive-negative selection capability of *S. cerevisiae* has been described by Alani et al. (1). In essence, their scheme allows the *URA3* selectable marker system to be used repeatedly for numerous independent genomic modifications. This capability is particularly important in *C. tropicalis*, since only one marker system is available to carry out all genomic manipulations.

Among alkane- or fatty acid-utilizing yeasts, transformation of *C. tropicalis* appears to be most similar to that reported for *Yarrowia lipolytica* (9, 10). Both yeasts are transformed at a high frequency by DNA vectors containing auxotrophic marker genes, and vectors integrate mostly by homologous recombination between sequences shared by vector and host genomes. In *C. albicans*, most transformed sequences integrate by homologous recombination, but reported transformation frequencies are lower than those of *C. tropicalis* and *Y. lipolytica* (14). *Candida maltosa*, *Pichia quillerimondii*, and *Hansenula polymorpha* were reported to be transformed by an autonomously replicating DNA vector (12, 15).

On the basis of Southern analysis, *C. tropicalis* appears to have two loci with homology to *URA3*. Restriction pattern analysis indicates that one is from the same cloned *URA3* gene. The other locus is distinct, and its identity is unknown. It could be a second functional *URA3* gene, a pseudogene, or an unrelated sequence that coincidentally shares homology with *URA3*. This second region of *URA3* homology is currently being isolated and tested for *URA3* function. The existence of two functional *URA3* genes in *C. tropicalis* might explain why *ura3* mutants are so rare, since their generation would require mutations in both genes.

In summary, a DNA transformation system is available for the first time for *C. tropicalis*. Genetic analysis of *C. tropicalis* has been hindered by the lack of a sexual cycle for this yeast. By allowing recombinant DNA-based manipulation of the genome, a transformation system could assist in providing some genetic capability. At least the following four research areas are of immediate interest: (i) understanding pathogenicity in yeasts, which should be aided by an ability to combine biochemical studies and genetic manipulations; (ii) regulation of gene expression by alkanes in *C. tropicalis*; (iii) development of *C. tropicalis* as a host for expression of heterologous proteins; and (iv) peroxisome studies in *C. tropicalis*, particularly the targeting and import of peroxisomal proteins. Small et al. (27) developed a peroxisomal in vitro import system in which cell-free translation products appear to associate specifically with *C. tropicalis* peroxisomes. By using the *C. tropicalis* transformation system, it would be possible to determine definitively which sequences are involved in peroxisomal import and targeting by constructing gene fusion vectors and examining their products in *C. tropicalis*. It is of particular importance that expression and testing of mutant constructs in vivo should produce sufficient protein to visualize targeting of gene fusion products to peroxisomes by immunocytochemical methods.

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