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DNA Transformations of *Candida tropicalis* with Replicating and Integrative Vectors

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The alkane-assimilating yeast *Candida tropicalis* was used as a host for DNA transformations. A stable *ade2* mutant (Ha900) obtained by UV-mutagenesis was used as a recipient for different vectors carrying selectable markers. A first vector, pMK16, that was developed for the transformation of *C. albicans* and carries an *ADE2* gene marker and a *Candida* autonomously replicating sequence (CARS) element promoting autonomous replication, was compatible for transforming Ha900. Two transformant types were observed: (i) pink transformants which easily lose pMK16 under non-selective growth conditions; (ii) white transformants, in which the same plasmid exhibited a higher mitotic stability. In both cases pMK16 could be rescued from these cells in *Escherichia coli*. A second vector, pADE2, containing the isolated *C. tropicalis ADE2*, gene, was used to transform Ha900. This vector integrated in the yeast genome at homologous sites of the *ade2* locus. Different integration types were observed at one or both *ade2* alleles in single or in tandem repeats.

KEY WORDS — *Candida tropicalis*; auxotrophic mutants; DNA transformation; replicative and integrative plasmid.

INTRODUCTION

Candida tropicalis is an asporogenous yeast with the potential to use a very large variety of carbon sources. They include mono- and disaccharides such as xylose, maltose, cellobiose and polysaccharides such as starch (Gancedo and Serrano, 1989) or aromatics such as phenols (Neujahr, 1990). Growth of *C. tropicalis* on these compounds and on other sugars results in high biomass yields since the metabolism of this yeast is mainly oxidative when oxygen availability is non-limiting (Fiechter *et al.*, 1987). *C. tropicalis* is also able to utilize alkanes and alkane derivates as sole carbon and energy source. Many studies have been carried out characterizing the metabolism of this yeast, especially on these non-conventional carbon sources. They include the study of the alkane-inducible cytochrome P450 monooxygenases (Sanglard *et al.*, 1986) and of the peroxisomal proteins, of which several have been identified as enzymes associated with the β-oxidation pathway (Kamyro and Okazaki, 1984). The genes encoding for the cytochrome P450 monooxygenases, which participate in the oxidation of alkanes or fatty acids, have been cloned recently and include the cytochrome *P450alk* genes (Sanglard and Loper, 1989; Seghezzi *et al.*, 1991) and the NADPH cytochrome P450 reductase gene (Sutter *et al.*, 1989). The cytochrome *P450alk* genes belong to the *CYP52* gene family and some of its members, now seven in number, have unknown functions (Sanglard and Fiechter, 1989).

Besides these properties, *C. tropicalis* could be attractive for the expression of intra- or extracellular heterologous proteins, as well as for the overexpression of endogenous proteins, particularly when other hosts do not possess the metabolic versatility of this yeast. The isolation of the *ACP* gene for the secreted acid protease and the use of its secretion signal is aimed partly in this direction (Togni *et al.*, 1991). Moreover, *C. tropicalis* is an opportunistic pathogen of medical importance (Odds, 1987) and the study of its virulence by genetic means has been totally ignored. However, the development of this yeast for these purposes is dependent on the establishment of efficient DNA transformation systems.

We are interested in developing a DNA transformation system for this yeast, so that the tools of molecular genetics may be used in this organism. These methods will allow gene disruption experiments to be performed for investigation of the biological significance of the recently cloned alkane-

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inducible cytochrome P450 genes, as well as of the gene for the secreted acid protease considered as a potent virulence factor (Borg and Rüchel, 1990). Furthermore, the limits of application of this yeast for heterologous gene expression could be tested. Here we report the development of a transformation system for this yeast based on the complementation of an auxotrophic mutant with both replicative and integrative vectors.

MATERIALS AND METHODS

Strains, media and plasmids

Candida tropicalis ATCC750 and strains 1405 (*ade1,ino,pro*) and 1407 (*ade2,ino,pro*), kindly provided by R. Poulter (University of Otago, Dunedin, New Zealand), were used in this study. *Saccharomyces cerevisiae* IH2 (**a**, *ade2-100,leu2-3,leu2-112,trp1,ura3-251,ura3-328,ura3-373,his4-519*), kindly provided by A. Hinnen (Ciba-Geigy, Basel), was utilized for complementation experiments. *Escherichia coli* DH5α (Hanahan, 1985) was used for DNA subcloning and plasmid propagations. Minimal medium for the growth of yeasts was yeast nitrogen base (Difco) supplemented with glucose and the required amino acids. Regeneration agar contained 3% noble agar (Difco) and 1 M-sorbitol. Plasmid pMK16 contained a 0·6 kb *Rsa*I fragment with a *Candida* autonomously replicating sequence (CARS) element and the *C. albicans ADE2* gene as a 2·4 kb *Eco*RV fragment in the *Pvu*II and *Eco*RV sites of pBR322, respectively. pMK16 was constructed by Kurtz *et al.* (1987) and was obtained from the Squibb Institute for Medical Research (Princeton). Plasmid ♯1056 containing the *C. albicans ADE1* gene and a CARS element from the same yeast was kindly provided by S. Scherer (University of Minnesota, Minneapolis).

DNA transformations

E. coli was transformed by electroporation with a Bio-Rad Gene Pulser. *S. cerevisiae* was transformed by the LiAc-method as described by Rothstein (1985). *C. tropicalis* was transformed by protoplasting as follows: from a growing culture at OD_{600nm} 2 in complex medium (YEPD), 10 ml of the cell culture was sedimented and washed with TE. The protoplasts were obtained by digestion of cell walls with 0·75 mg 100T-Zymolyase in PRO buffer (1 M-sorbitol, 25 mM-ETDA, 100 mM-Na-citrate buffer, pH 6·5) supplemented with 15 mM-β-mercaptoethanol for 15–30 min. The formation of protoplasts was followed under the microscope. The protoplasts were washed three times with PRO buffer and resuspended in 0·2 ml CAS buffer (PRO buffer supplemented with 10 mM-$CaCl_2$). DNA was added, the mixture incubated for 30 min at 30°C and 0·5 ml of 40% polyethylene glycol (PEG) 4000 and CAS buffer were added. After a 30-min incubation, the cells were pelleted and protoplasts regenerated in 1 M-sorbitol in YPE for 30 min. The cells were pelleted again, resuspended in CAS buffer, mixed in melted regeneration agar kept at 45°C and plated on minimal agar. Transformants appeared after 3–4 days of incubation at 30°C.

Protoplast fusions

Protoplasts from yeast cells were obtained as outlined above and each fusion partner was incubated in 20% PEG at 30°C for 1 h. Thereafter, the proplasts were regenerated and fusionants selected in appropriate selective and osmotic medium as described by Gil *et al.*, (1988).

Mutagenesis and nystatin enrichment of auxotrophic mutants

UV-mutagenesis was performed with a Philips TUV-lamp (15 W). Each mutagenesis was performed with a UV-dose killing approximately 90% of the cells. After UV-treatment the cells were incubated overnight in YEPD complex medium at 30°C in the dark and transferred to minimal YNB medium for 8 h. 40 U nystatin (Sigma) was then added for 30 min and the cells were washed with YNB medium. These cells were incubated for 3 days on YEPD agar and replica plated on YNB plates lacking defined amino acids.

Other methods

Isolation of DNA from yeast was performed using 5 ml cultures according to Rothstein (1985), with the exception that phenol extraction was avoided and DNA was precipitated after RNAse treatment by the addition of one volume of isopropanol. Electrophoresis of DNA was performed in 1% agarose (Biorad). Southern blots were performed on GeneScreen Plus membranes (New England Nuclear). Membranes were hybridized at 42°C in 50% formamide and washed according to the supplier's recommendations. DNA probes were labelled by random primer extension using α-^{32}P]dCTP (Feinberg and Vogelstein, 1984).

Table 1. Protoplast fusion between *ade*⁻ *C. tropicalis*.

Fusion partners	Ade⁺ colonies*
Ha900	—†
1407	—‡
1405	—‡
Ha900 × 1407	—‡
Ha900 × 1405	100
1407 × 1405	170‡

*Fusionants were recorded by their ability to grow without the addition of adenine. About 10^7 cells were plated in each case.
†No growth was detected after 4 days of incubation.
‡Inositol and proline were added to minimal medium to allow the growth of strains 1407 and 1405 (Corner and Poulter, 1989).

Mapping of λDashII clones was performed by partial digestion and hybridization with T3 and T7 phosphatase-linked oligonucleotides according to the supplier's recommendations (Stratagene). Restriction enzymes and DNA-modifying enzymes were from Boehringer Mannheim. All other chemicals were of analytical grade.

RESULTS

Mutant characterization

A first screening of mutants in different biosynthetic pathways generated by UV-treatment and nystatin enrichment gave several auxotrophs mainly for uracil, adenine and histidine. These mutants were rather unstable, with a reversion rate higher than 10^{-6}. We chose to stabilize these mutants by a second cycle of UV-mutagenesis. After this treatment, the *ade* mutant strain Ha900 particularly exhibited a higher stability, with a reversion rate less than 10^{-8} and this strain was used for further investigations. To define this strain as an *ade1* or *ade2* mutant, two different approaches were chosen, namely a protoplast fusion with known *C. tropicalis ade1* or *ade2* strains described by Corner and Poulter (1989) and complementation experiments with both the *ADE1* and *ADE2* genes of *C. albicans*. Since complementation of the *ade* mutation in Ha900 could be obtained only by fusion of the *ade1* *C. tropicalis* strain (Table 1), this result suggested strongly that Ha900 was *ade2*. This phenotype was later confirmed by the growth restoration of Ha900 in minimal medium by pMK16 and not by ♯1056, which are plasmids carrying the *C. albicans ADE2* and *ADE1* genes and a CARS element, respectively.

Table 2. Stability of pMK16 in the *C. tropicalis* transformants.

Type of transformant	Mitotic stability in non-selective medium*
White	50 ± 10%
Pink	10 ± 5%

*The mitotic stability of pMK16 was measured by cultivating single colonies in non-selective medium (YEPD) to saturation and plating the same aliquot of these cells in minimal and complex agar. The number of colonies appearing after 4 days in both media was estimated and the differences between selective and non-selective media were expressed as a percentage. The higher the percentage, the higher the plasmid mitotic stability. The values given represent means of three separate experiments.

Heterologous transformation of C. tropicalis *by the* C. albicans *replicative vector pMK16*

As mentioned above, *C. tropicalis* Ha900 could be transformed by pMK16 to an Ade⁺ phenotype. The transformation efficiencies obtained varied from 10^2 to 3×10^2 per μg plasmid DNA. These values are comparable to those obtained when transforming *C. albicans* with the same plasmid (Kurtz *et al.*, 1987). The transformants appeared as pink colonies from which white mycelium could grow after prolonged incubation time. White colonies also appeared after repeated plating of pink transformants on selective minimal plates. The mitotic stability of pMK16 was investigated by incubation of the transformants in non-selective YEPD complex medium. As summarized in Table 2, the pink transformants lose pMK16 very rapidly. In the white transformants the mitotic stability of pMK16 was much higher, since only 40–60% of the white cells returned to an *ade2* phenotype with a characteristic red colour when plated on complex agar. This result indicates that the white transformants are probably not the result of the integration of pMK16 in the yeast genome, but rather suggests that this plasmid exists in an episomal state. This contrasts with the observations made in *C. albicans* where most of the white colonies originating from the pink transformants were shown to be the result of pMK16 integration events (Kurtz *et al.*, 1987, 1990).

To examine further the fate of pMK16 in the *C. tropicalis* transformants, DNA was extracted and probed with labelled pBR322 DNA (Figure 1). In every transformant the presence of plasmid could be observed. Genomic DNA digested with *Hin*dIII

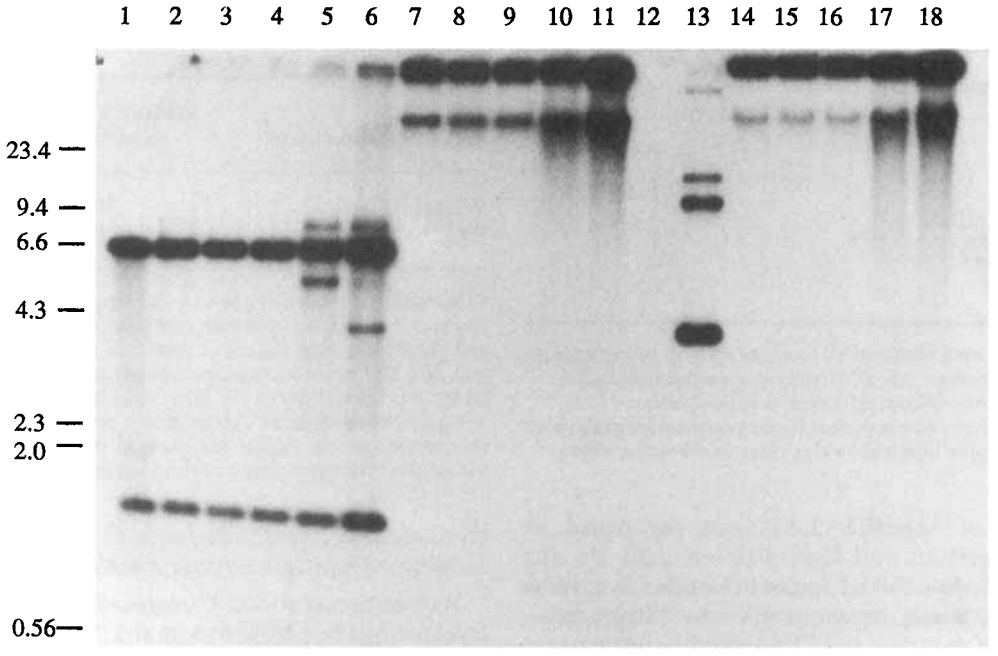

Figure 1. Analysis of *C. tropicalis* colonies obtained after transformation with pMK16. DNA was isolated from growing pink colonies, 2 µg of it was cut with the corresponding restriction enzymes, run on a 1% agarose gel and transferred to a Genescreen Plus membrane. The membrane was hybridized with labelled pBR322, washed and exposed to an X-ray film for 5 h. Lanes 1 and 13: pMK16 cut with *Hin*dIII and undigested. Lanes 2–6, 7–11 and 14–18: *Hin*dIII, *Pvu*II and undigested DNA from independent transformants, respectively. Lane 12: DNA from *C. tropicalis* wild type. Molecular weight standards (λ DNA cut with *Hin*dIII) are shown at the left side.

gave a pattern essentially similar to that exhibited by pMK16 (lanes 1–6). For two transformants, however, additional bands could be observed (lanes 5 and 6). The appearance of these bands can be attributed to different mechanisms such as recombinations between the plasmid and chromosomal DNA or between plasmid molecules themselves. The possible integration of this plasmid in the yeast genome would be revealed by eventual adjacent genomic *Pvu*II sites, since *Pvu*II does not cut within pMK16. Genomic digests performed with this restriction enzyme failed to detect such fragments and have a pattern similar to undigested DNA (lanes 7–11 and 14–18). Therefore it is unlikely that pMK16 integrated at a location in the yeast chromosomes. Undigested DNA exhibited signals of high molecular weights migrating very slowly in agarose gel electrophoresis (lanes 14–18). Such a picture was observed for the *C. albicans* transformed with pMK16, where it was shown to be present as large molecules made of plasmid tandem repeats. It is possible that a similar conformation exists for pMK16 in the *C. tropicalis* transformants.

When hybridizing the pBR322 with DNA from white and pink transformants, no difference could be observed in their hybridization patterns (Figure 2). This indicates that the higher mitotic stability of pMK16 in the white transformants is not the result of observable insertions or deletions in this plasmid but of other factors not yet characterized. At least the pBR322-hybridizing bands from the genomic digests of a pink transformant exhibited a more pronounced intensity than in the case of the white transformant, thus suggesting a difference in the plasmid copy number. The scanning densitometric values obtained for these bands indicated a six-fold reduction of intensity (data not shown). Comparing these values with a DNA digest of a yeast transformed with an integrative plasmid to which the labelled pBR322 probe could hybridize (see next section), the pMK16 copy number was estimated to be 15–20 and 90–120 for white and pink transformants, respectively.

pMK16 could be rescued from transformants in *E. coli* by electroporation at relatively high frequencies (10^2–10^3 ampicillin-resistant colonies per µg

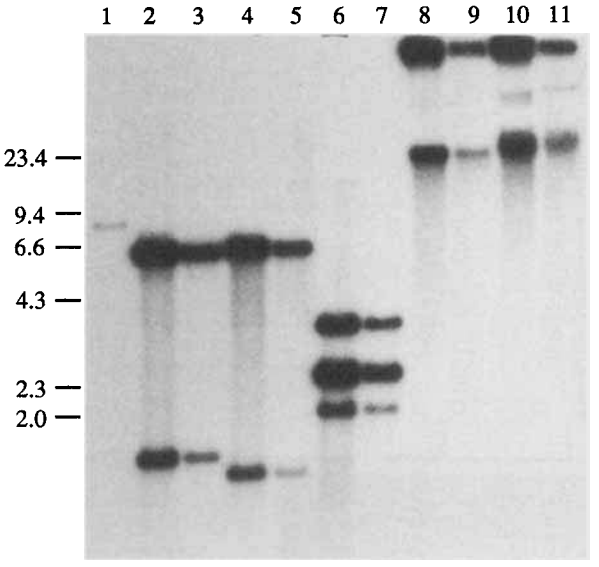

Figure 2. Southern blot analysis of *C. tropicalis* white and pink transformants. Lanes 2, 4, 6, 8, 10 and 3, 5, 7, 9, 11: *Hin*dIII, *Bam*HI, *Eco*RI, *Pvu*II digested and undigested DNA from a pink and a white transformant, respectively. The probe was ^{32}P-labelled pBR322. The banding pattern of the digested DNA corresponds to the size expected from pMK16. In lane 1 a *Bam*HI digest of DNA from a transformant obtained by integrative transformation containing a pBR322-derived copy has been included for estimating the copy number of pMK16. The transformant is identical to the one analysed in lane 6 of Figures 5 and 6. Molecular weight standards (λ DNA cut with *Hin*dIII) are shown at the left side.

genomic DNA). A striking difference was obtained from plasmids rescued from white and pink cells. Whereas the plasmids from pink cells were rescued in high molecular weight multimeric structures, plasmids from white cells were obtained with low molecular weight structures, most probably in the monomeric form (Figure 3). After digestion with *Hin*dIII, however, the pMK16 restriction pattern could be observed (Figure 3, right side). Since undigested genomic DNA from white cells was exhibiting high molecular weight plasmid DNA (Figure 2, lane 11), it is surprising to find that the plasmids rescued from these cells are in an apparent monomeric form. It is possible, however, that a minor fraction of the plasmids are replicating effectively in a monomeric form which is barely detectable in these cells and that this fraction is much more efficiently rescued in *E. coli*.

This last observation led us to hypothesize that the appearance of white transformants could have been the result of single base alterations in pMK16, improving its replication and maintenance. To test this hypothesis, a plasmid rescued from a white transformant was propagated in *E. coli* and *C. tropicalis* Ha900 transformed again with this plasmid preparation. Obtaining directly white transformants with this plasmid would imply that some changes affecting the mode of its propagation had occurred. However, this plasmid gave transformants with similar properties to those observed with the original pMK16. Consequently, the appearance of white transformants is probably due to a transient mode of propagation of pMK16 into a monomeric form.

Cloning of the C. tropicalis ADE2 *gene for homologous integrative transformation*

To clone the *ADE2* gene from *C. tropicalis*, the *ADE2* gene from *C. albicans* was used as a probe. A λDashII gene library was screened at low stringency and positive signals were purified. Two similar clones were picked, from which the restriction map is shown in Figure 4A. The entire 12 kb insert could be recovered by digestion with the flanking λDashII *Sal*I sites and was cloned into the same site of the yeast replicating vector pEMBLYe31, thus creating pADE2 (Figure 4B). This vector could transform the *ade2 S. cerevisiae* mutant strain IH2 to adenine-independent growth, thus indicating that in fact this fragment contained a functional *ADE2* gene. As summarized in Figure 4A, the subcloning of a 8 kb *Hin*dIII fragment failed to complement the *S. cerevisiae ade2* mutant but not a 6·9 kb *Sal*I-*Pvu*II fragment. The minimal functional size of the *C. tropicalis ADE2* gene could be further delimited to a 3·5 kb *Eco*RV fragment.

The constructed vector pADE2 was used to complement the *ade2* strain Ha900. The transformation of this strain with pADE2 gave rise to well-growing white colonies but with a low transformation efficiency (10–20 transformants per μg DNA). The high stability of the Ade$^+$ phenotype of these transformants after growth in non-selective complex medium was an indication that pADE2 integrated in the yeast genome. Southern blot analysis of genomic DNA from these strains showed that they contained plasmid DNA (Figure 5A). Genomic digests with *Bam*HI indicated that, when integrating in the yeast genome according to the model of Figure 5C, pADE2 leaves an endogenous 21 kb *Bam*HI fragment and a 12 kb plasmid-derived *ADE2* gene copy, or multicopies, as observed by the intensity of the plasmid-specific bands (Figure 5B, lane 4). Since *C. tropicalis* is probably a diploid yeast, the *Bam*HI digests are not conclusive on whether

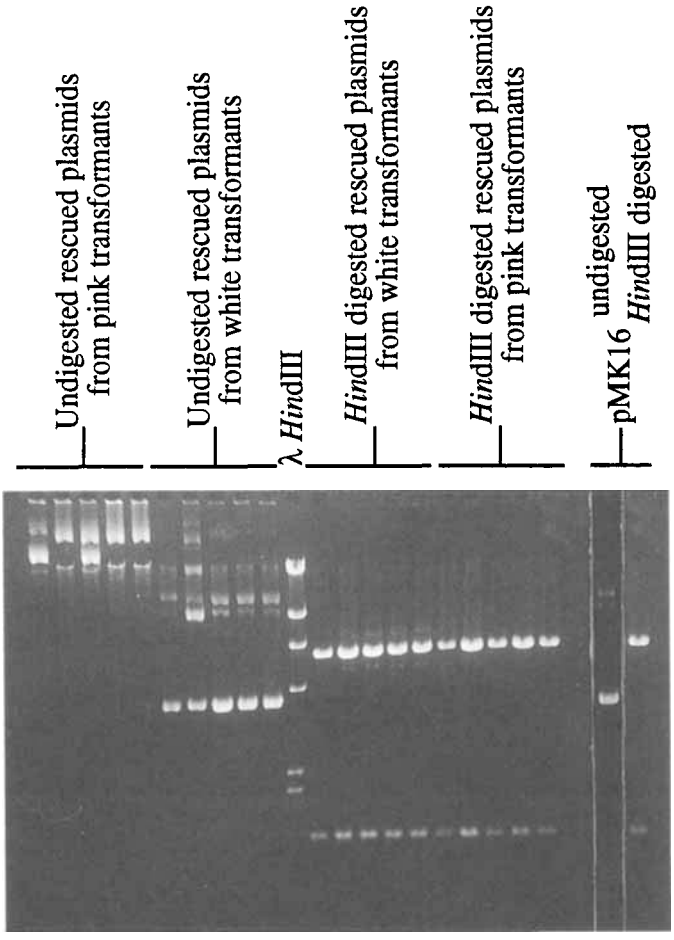

Figure 3. Plasmid rescue from *C. tropicalis* transformants. The origin of plasmid DNA, i.e. from white and pink transformants, is indicated. Plasmid DNA was obtained by minipreparation after electroporation in *E. coli* DH5α of total DNA obtained from the yeast transformants. The digestion of the minipreparations by *Hin*dIII gave a restriction pattern identical to authentic pMK16, whereas undigested pMK16 migrated at the same position as undigested plasmid DNA rescued from white transformants. *Hin*dIII-cut λ DNA was loaded as a molecular weight standard in the middle of the gel.

pADE2 integrated in one or both alleles. Therefore genomic DNA from the same transformants was digested with *Nco*I, which does not cut within pADE2 and probed with an *ADE2*-labelled fragment (Figure 6). Thus any migration shift of the *ADE2 Nco*I fragment can be considered as an integration event at this locus. The results of Figure 6 indicated that, besides the direct evidence of the diploid nature of this yeast (lanes 4 and 5), different integration types occurred: (i) cases where the plasmid integrated in both alleles (lanes 2, 3 and 6); (ii) where pADE2 integrated at only one allele (lanes 4 and 5); and (iii) where the plasmid integrated in tandem repeats in one allele only, as judged by the slow migration of one of the *ADE2*-hybridizing *Nco*I fragments of lane 4. In the analysis of these transformants, no banding patterns other than the ones expected appeared, thus suggesting that pADE2 integrated at homologous sites only.

It is surprising that the integration of pADE2 is observed for three out of five transformants at the two *ade2* alleles. In most of the integrative transformations performed in *Candida* species, the integration events occurred at one allele only. Possible

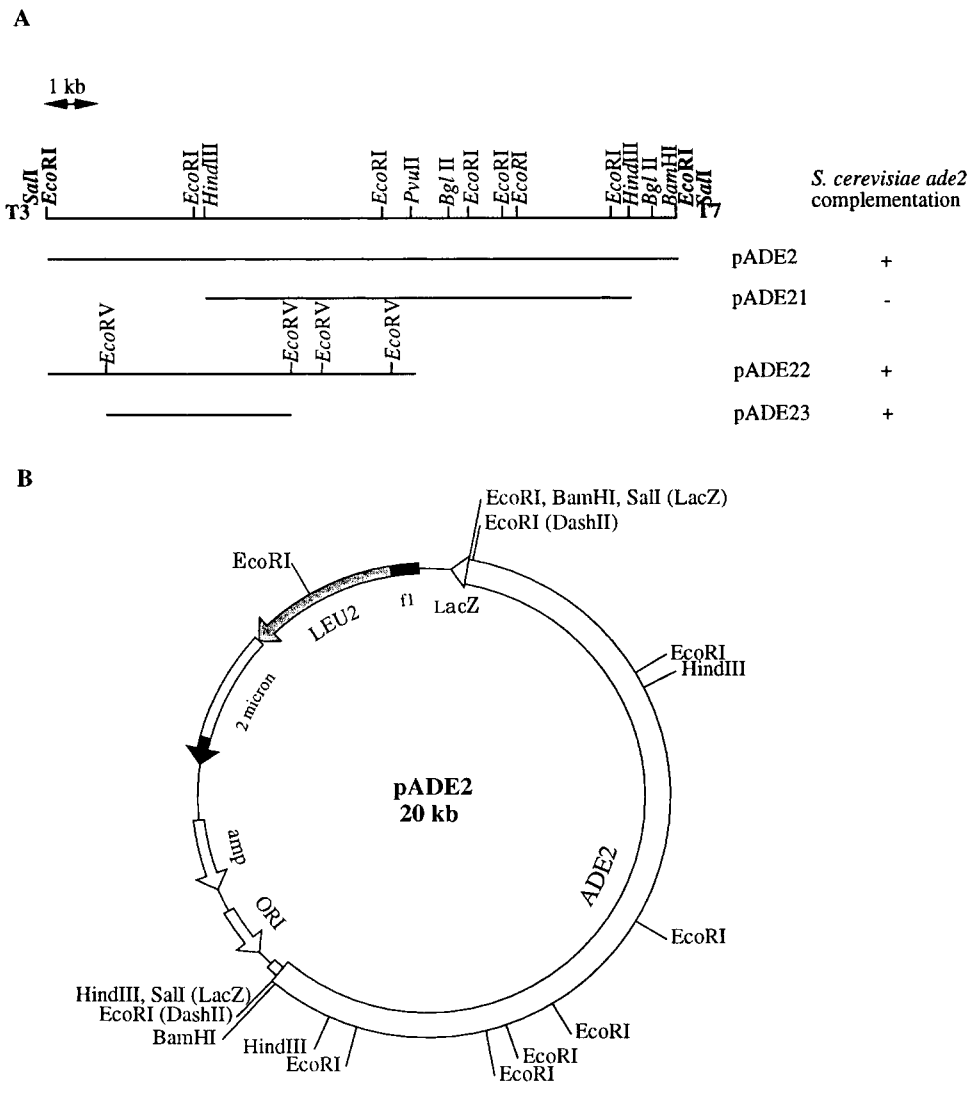

Figure 4. (A) Restriction map of an *ADE2*-containing λDashII clone. The position of the T3 and T7 elements flanked by λDashII *Eco*RI and *Sal*I sites (bold type) are shown. *Eco*RI sites were determined by partial digest mapping as described in Materials and Methods, whereas other sites were assigned by restriction analysis. The restriction map of the λDashII clone corresponds to maps established with genomic DNA (data not shown). The fragments that were used for complementation tests in *S. cerevisiae* were subcloned in pEMBLYe31 and are aligned to the λDashII clone. The construction of pADE2 is described in the text. pADE21, pADE22 and pADE23 were obtained by subcloning 8 kb *Hin*dIII, 6·9 kb *Sal*I-*Pvu*II and 3·5 kb *Eco*RV fragments in the *Hin*dIII, *Sal*I-*Sma*I and *Sma*I sites of pEMBLYe31, respectively. (B) Map of the yeast shuttle vector pADE2, where the *C. tropicalis* 12 kb *Sal*I *ADE2*-containing fragment has been subcloned; the origin of some restriction sites is shown in parentheses. The parent vector pEMBLYe31 has been described by Baldari and Cesarini (1985).

explanations for our results are a simultaneous integration of pADE2 in both alleles or successive events leading to the plasmid integration in one allele first followed by a mitotic recombination altering the second *ade2* allele. Given the fact that spontaneous mitotic recombination in *Candida*

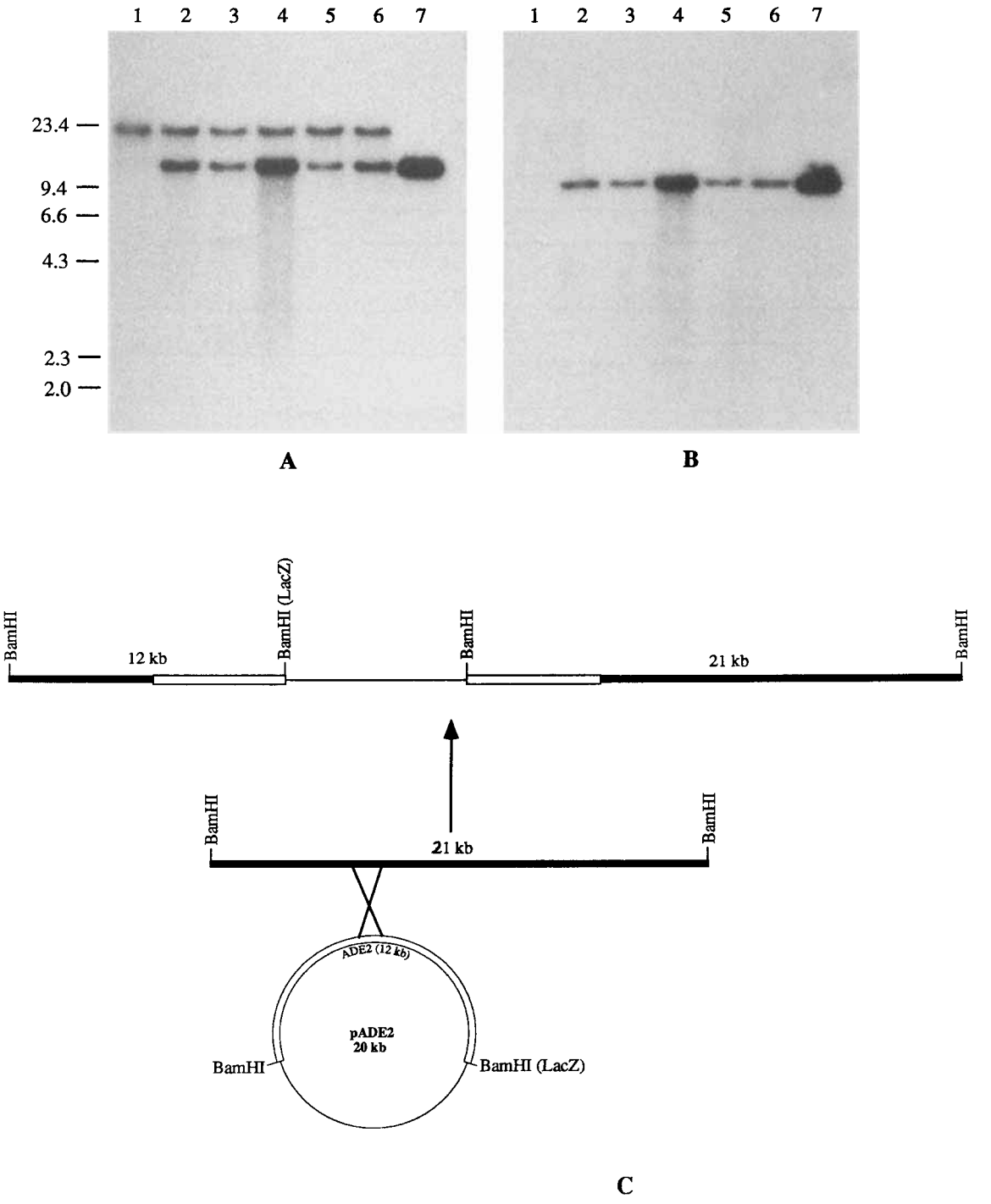

Figure 5. Integration of pADE2 in the *C. tropicalis* genome. DNA from isolated yeast transformants was subjected to electrophoresis in 1% agarose after digestion with *Bam*HI. The DNA was transferred as outlined in Figure 1 and probed with a labelled 12 kB *Sal*I *ADE2* fragment (A) and a labelled pBR322 probe (B). Lane 1: wild-type *C. tropicalis*; lanes 2–6: individual transformants; lane 7: *Bam*HI-digested pADE2. Molecular weight standards (λ DNA cut with *Hin*dIII) are shown at the left side. (C) Model for the integration of pADE2 in the yeast genome that corresponds to the pattern of (A).

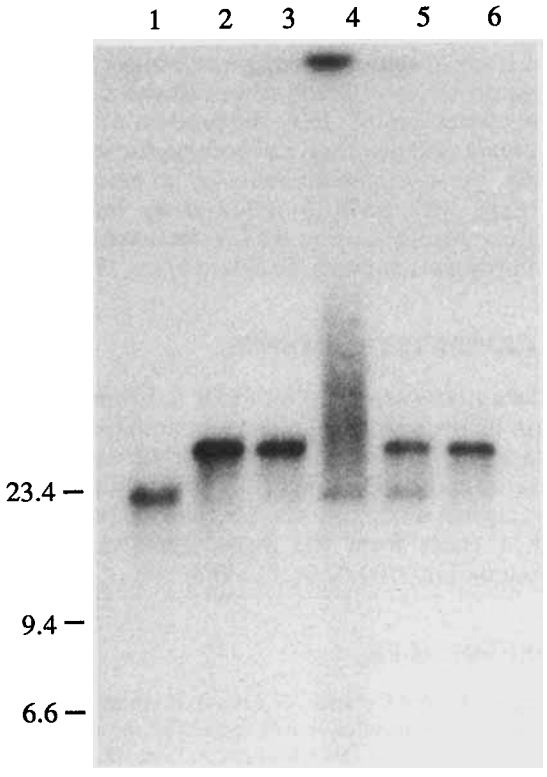

Figure 6. Integration types of pADE2 at the *C. tropicalis ade2* loci. DNA of transformants was cut with *Nco*I, which does not cut within pADE2, and separated by CHEF to resolve the large DNA fragments. The electrophoresis was run in 0·8% agarose at 200 V with a pulse time of 1–10 s increased over 8 h and terminated with a pulse time of 10 s for 1 h. The loading pattern is identical to Figure 5 (lanes 1–6; A and B). The probe was a labelled 12 kb *Sal*I fragment containing *ADE2* (Figure 4A). The two-banding patterns of lanes 4 and 5 indicate that one of the *ade2* alleles is unchanged as compared to the pattern of lane 1, and that the other allele has been altered by the integration of pADE2. Molecular weight standards (λ DNA cut with *Hin*dIII) are shown at the left side.

species takes place at frequencies ranging from 10^{-5} to 10^{-6} (Gorman *et al.*, 1991; Kurtz *et al.*, 1990), it makes this last possibility very unlikely to happen.

DISCUSSION

We described here the transformation of a *C. tropicalis ade2* mutant strain by two plasmid types. pMK16, a plasmid developed for the transformation of *C. albicans*, was able to complement a *C. tropicalis ade2* strain with a comparable efficiency and seems to exist in an episomal state as observed in *C. albicans* (Kurtz *et al.*, 1987). The integration of this plasmid was also reported in *C. albicans*, probably due to homologous sequences residing in both the plasmid and the yeast genome. The integration of this plasmid in the *C. tropicalis* strain investigated here was not detected. No *C. tropicalis* homologous sequence is present in pMK16, so that it is unlikely that this plasmid could integrate in the genome of this yeast. An unexpected banding pattern for pBR322-hybridizing signals was observed in two independent transformants, however (Figure 1). If integration is excluded as an explanation for these additional bands, recombination between plasmid molecules could have contributed to this result. This hypothesis must remain speculative, since no plasmids with an altered restriction pattern were rescued in *E. coli*. However, if such recombination had occurred, the ampicillin-resistance marker in such possible plasmid types might have been rendered inactive.

In *C. albicans*, pMK16 was replicating in the form of high molecular weight multimeric molecules (Kurtz *et al.*, 1987). This picture is similar to the replication of the same plasmid in *C. tropicalis*, as concluded from the pattern of pMK16 in undigested genomic DNA of the obtained transformants (Figures 1 and 2) or from the rescue of plasmids in *E. coli* (Figure 3). The formation of multimeric episomal plasmids has been reported in a number of yeast transformed with heterologous plasmids. *Hansenula polymorpha* has been transformed with plasmids that formed multimers after growth under selective conditions (Tikhomirtova *et al.*, 1986). Glumoff *et al.* (1989) and Ochsner *et al.* (1991) showed that, when plasmids carrying bacterial resistance gene markers or the *Aspergillus nidulans argB* gene marker were transferred into *Trichosporon cutaneum*, they were rescued in *E. coli* in different forms, including monomers and multimers, some of them showing an altered restriction pattern. Head-to-tail ARS-containing plasmid repeats have been demonstrated also in *Schizosaccharomyces pombe* (Sakaguchi and Yamamoto, 1982). These examples and our own observations suggest that the formation of plasmid multimers is a widely used mode of propagation in these organisms.

The property of extrachromosomal replication of pMK16 in *C. albicans* is due to the presence of a CARS element (Kurtz *et al.*, 1987). It seems that, by analogy, this element promotes a similar function in *C. tropicalis*, as shown in this study. Cannon *et al.* (1990) recently reported another form of replicative plasmid for *C. albicans* which contains an ARS different from the CARS element of pMK16. This ARS does not lead to the formation of multimers

but leaves the replicating plasmid in an apparent monomeric supercoiled form. No ARS element has yet been characterized in *C. tropicalis*. Now that the genetic tools have been made available, it is expected to be isolated in the near future.

Before undertaking this study and even during our investigations, several auxotrophic mutants of other *C. tropicalis* strains were reported (Corner and Poulter, 1989; Haas *et al.*, 1990). The reason for isolating mutants from the specific *C. tropicalis* strain used in this study is that several genes that are destined to be disrupted in the future have been isolated from this particular strain and that, for some of them, they are not identical between *C. tropicalis* strains. This factor is of importance, since homologous recombination is required when gene disruptions are performed by gene replacement. Different restriction patterns exist in the alkane-inducible P450 genes and members of the acyl-CoA oxidase genes (S. Piccatagio, personal communication) or between the *URA3* genes from our *C. tropicalis* strain (unpublished data) and from the one utilized by Haas *et al.* (1990). Along with the description of auxotrophic mutants, the same authors reported a transformation system for their *C. tropicalis* strain complementing an *ura3* mutant by the yeast homologous *URA3* gene. In contrast to the development of both replicative and integrative transformations undertaken here, their system was restricted to the integration of a marker-containing plasmid only, which limits the range of its applications (see below). As in our case plasmid integration was observed at homologous sites in single or tandem repeats but probably on one allele only. A major difference with the analogous integrative system reported here is the 100-fold increase of transformation efficiency obtained by these authors. Many factors such as the strain type, cell regeneration efficiency or plasmid size could have contributed to this difference.

The replicative plasmid for heterologous transformation investigated here is well suited for the increase of the copy number of an expression cassette or a defined gene. The integrative system for homologous transformation is of interest for stable integration of other properties and for gene disruptions. The integrative vector pADE23, which contains the minimal *ADE2* functional size (Figure 4A) and available cloning sites and which complements Ha900 by homologous recombination as demonstrated for pADE2 (data not shown), should be more adequate for this purpose than the larger pADE2. The usefulness of both transformation types including these replicative and integrative vectors will be tested in the future. We have utilized the transformation system reported here for the disruption of genes involved in metabolic functions. The disruption of *ACP*, the product of which is a secreted acid protease, has been performed successfully by co-transformation of a mutated *ACP* variant with pMK16 followed by induction of mitotic recombination by UV-treatment to obtain homozygous mutants (Sanglard *et al.*, 1992).

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