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Heterogeneity within the alkane-inducible cytochrome P450 gene family of the yeast *Candida tropicalis*

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The reexamination of a genomic λgt11 *Candida tropicalis* expression library for the presence of genes related to the previously reported alkane-inducible cytochrome P450alk gene (*P450alk*), which is the first member of the P450LII gene family, was undertaken. A positive clone with a DNA fragment having 69% similarity with a portion of *P450alk* was isolated. As in the case of *P450alk*, this new putative P450 gene was also induced by tetradecane when *C. tropicalis* was grown on this carbon source and was therefore named *P450alk2*, *P450alk1* corresponding to the first isolated P450 gene. In addition to *P450alk2*, the existence of other P450alk-related genes is suggested by the hybridization pattern of *P450alk1* and *P450alk2* probes with the *C. tropicalis* genomic DNA. The P450LII gene family in *C. tropicalis* appears therefore to include several different members. This heterogeneity is presently a unique feature within yeast P450 gene families and resembles the situation existing in P450 gene families of higher eukaryotes.

Alkane; Cytochrome P450 gene family; Yeast; (*Candida tropicalis*)

1. INTRODUCTION

The assimilation of alkane by yeast requires the presence of cytochrome P450 monooxygenases responsible for the first oxidation of the substrate [1]. *Candida tropicalis* has been shown to contain such a system consisting of an alkane-inducible cytochrome P450 (P450alk) and an NADPH cytochrome P450 oxidoreductase (NCPR) that provides electrons in the catalytic cycle of the hemoprotein [2]. To allow the study of this system at the molecular level, the cloning of an alkane-inducible P450 gene (*P450alk*) from *C. tropicalis* was undertaken and reported recently [3]. This gene was shown to be the first member A1 of a new P450 gene family, namely the P450LII gene family [3]. Upon expression of this gene in *Saccharomyces cerevisiae*, P450alk was functional for the terminal hydroxylation of lauric acid but was exhibiting a higher molecular mass than was expected from a major P450 protein isolated from *C. tropicalis* grown on alkane [3]. With the assumption that P450alk produced in *S. cerevisiae* was not altered by post-translational modifications, this discrepancy could be hypothetically explained by the existence of other P450alk isoenzymes in *C. tropicalis* being the products of distinct putative members of the P450LII gene family. The possible existence of different P450 isoenzymes from alkane-grown *C. tropicalis* presented earlier [4] and the possible occurrence of multiple members within P450 gene families as well described in higher eukaryotes led us to formulate this hypothesis. Here we show that, by reexamination of a genomic λgt11 *C. tropicalis* expression library that was used previously for the isolation of

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Abbreviations: aa, amino acid; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-thiogalactopyranoside; kb, kilobase; nt, nucleotide; Mops, 3-morpholinopropanesulfonic acid; ORF, open reading frame; P450, cytochrome P450; P450alk, alkane-inducible P450, also named P450LIIA1 according to the nomenclature proposed by Nebert et al. [16]; *P450alk1*, gene coding for P450alk1; *P450alk2*, gene coding for P450alk2; P450cam, camphor-inducible P450 from *Pseudomonas putida*

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P450alk [5], another P450alk-related gene could be identified. Moreover, the presence of additional P450alk-related genes in *C. tropicalis* could be observed, supporting therefore our hypothesis.

2. MATERIALS AND METHODS

2.1. *Strains and media*

The wild-type yeast *Candida tropicalis* ATCC 750 was used in this study. *E. coli* XL1-Blue (*rec*A1, *lac*$^-$, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, [F′proAB, *lac*I^Q, *lac*ZΔM15, Tn10]) was used as a recipient for plasmid subcloning and was grown on LB-medium supplemented when required with ampicillin. Synthetic medium [6] was used for the growth of *C. tropicalis* on glucose (3%) or tetradecane (1%).

2.2. *DNA subcloning and sequencing*

The plasmid Bluescript M13 + /KS (Stratagene) was used for subcloning of DNA fragments obtained by digestion of λgt11 recombinant clones with *Eco*RI. These clones were obtained by immunoscreening the λgt11 gene library with a P450alk antibody as described previously [5]. The sequencing of a recombinant Bluescript plasmid was performed with the Sequenase™ sequencing kit from United States Biochemical Corporation (USB, Cleveland, USA). For sequencing, plasmids were isolated with mini-preparations and purified with NACS columns (BRL) and 1 μg of each alkali-denatured with 0.2 M NaOH and 2 mM EDTA. After neutralization and precipitation, plasmids were annealed with the reverse primer (Pharmacia) and SK primer (USB) and sequenced with [^{35}S]dATP (Amersham) according to the recommendations of the supplier.

2.3. *Preparation of yeast DNA and RNA*

Genomic DNA from *C. tropicalis* was prepared according to Rothstein [7]. Harvest of the cells from glucose- and alkane-grown cultures for RNA isolation was performed as described previously [5]. RNA was extracted in 50 ml sterile Falcon tubes by vortexing approx. 1 g cell wet wt with 2.5 ml LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.2% SDS), 3 ml phenol and 11 g glass beads and precipitated with LiCl as described by Sherman et al. [8].

2.4. *Southern and Northern hybridizations*

1% agarose gel electrophoresis of restricted genomic DNA was performed according to standard protocols [9]. RNA was electrophoresed after formaldehyde denaturation in 1% agarose containing 20 mM Mops buffer (pH 7.0) and 0.60 M formaldehyde. Ethidium bromide was added at a concentration of 10 μg/ml to allow direct UV-visualization of ribosomal RNA bands [10]. Southern transfer of DNA and Northern transfer of RNA were performed on Genescreen Plus™ membranes according to the recommendations of the supplier (New England Nuclear). DNA probes were labelled with ^{32}P-dCTP by random primer labelling as described by Feinberg and Vogelstein [11]. The hybridization buffer for Northern blot contained 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, 100 μg denatured salmon sperm DNA and 10^6 cpm probe per ml. The buffer composition for the low stringency hybridization experiments of Southern blots was similar except that it contained 20% formamide and 5 × SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, pH 7.0, 50 mM EDTA). Hybridization temperatures for both hybridization types were kept overnight at 42°C.

3. RESULTS AND DISCUSSION

3.1. *Isolation of an additional P450alk-related gene*

After reexamination of the clones obtained from a screening of a λgt11 *C. tropicalis* expression library [5], a recombinant clone, from which an *Eco*RI insert of about 0.5 kb could be isolated, was characterized. This clone, upon infection of a lysogenic *E. coli* strain Y1089 and induction with IPTG, was producing a 135 kDa β-galactosidase fusion protein immunoreacting with a P450alk antibody (data not shown). To examine the relatedness of the insert DNA from this clone with the DNA sequence of *P450alk*, it was subcloned in a Bluescript vector. Surprisingly, two types of recombinant plasmids were recovered, namely pDS10 and pDS11 (fig.1). Each was carrying a fragment of 0.4 to 0.5 kb, the fragment of pDS11 having however an additional *Hin*dIII site (fig.1). When the nucleotide sequences of inserted DNA fragments from both pDS10 and pDS11 were compared with the *P450alk* nucleotide sequence, they exhibited a 78.5 and 61.5% homology with its C-terminal coding and 3′-flanking regions, respectively. Both fragments could be joined in fact by a common *Eco*RI site (figs 1 and 3A). These fragments were practically equal in size, thus explaining why they were recovered as a single apparent *Eco*RI fragment from the recombinant λgt11 clone.

3.2. *Inducibility of the new isolated P450 gene by alkane*

To show that these isolated fragments were part of a gene coding for a P450alk-like protein, a probe from the pDS11 *Eco*RI fragment was hybridized with total RNA from glucose- and tetradecane-grown cells. As shown in fig.2, transcripts were detected with this probe in total RNA from tetradecane-grown cells and not from glucose-grown cells, a characteristic shared with *P450alk*, which was first isolated from *C. tropicalis* [3]. Therefore, this second alkane-inducible P450 gene can be tentatively named

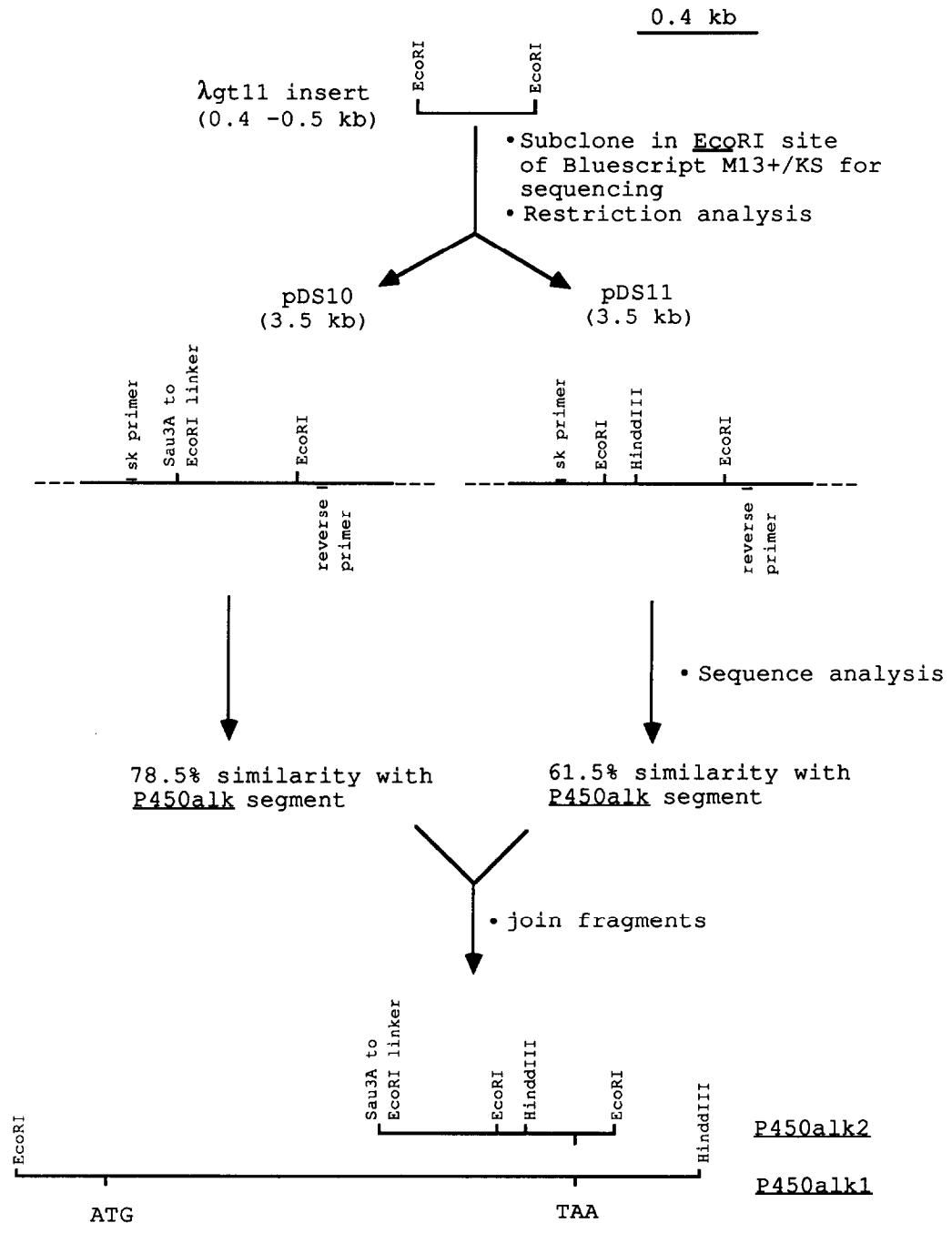


Fig.1. Schematic representation for the isolation and identification of the second *P450alk* gene from *C. tropicalis*. Relevant steps are indicated. Reverse primer and SK primer were used to sequence pDS10 and pDS11 inserts in both directions. The pDS10 insert was carrying an *Eco*RI linker at its 5'-end joined to a *Sau*3A site originating from the construction of the λgt11 library [5]. Partial restriction maps of *P450alk1* and *P450alk2* are presented at the bottom of the figure. ATG and TAA indicate the position of the respective start and stop codons of *P450alk1*.

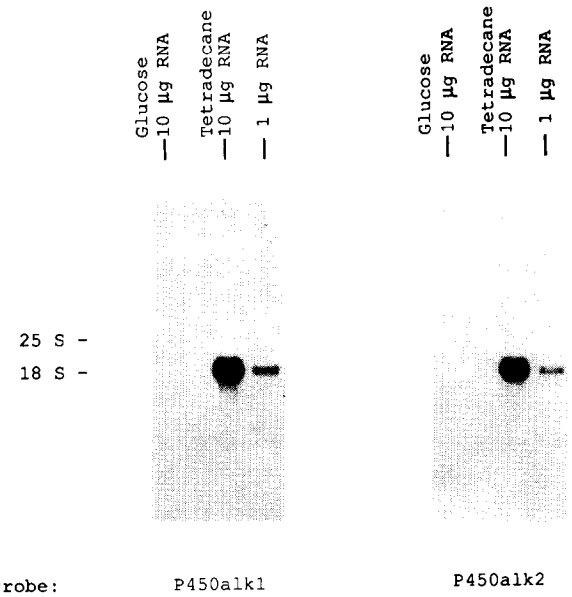


Fig.2. Induction of *P450alk2* by alkane. The origin of total RNA is indicated with the corresponding loaded quantity above each slot of the Northern blot. The position of the yeast 18 S (1.7 kb) and 25 S (3.4 kb) ribosomal RNA is indicated on the left. A *P450alk1* probe was used first as a control (left panel) for its inducibility by tetradecane [5]. Washing of unbound probes was performed at 65°C with 0.1 × SSC and 1% SDS during 1 h to allow hybridization only to homologous *P450alk* transcripts. The same membrane was used for hybridization of the *P450alk2* probe after removal of the first probe (right panel). In both cases, the Northern blot was exposed to a Fuji HR-L X-ray film for approx. 5 h.

P450alk2 and the previously reported *P450alk* [3], *P450alk1*.

3.3. *Sequence analysis and restriction map of the* P450alk2 *gene*

The nucleotide sequence of the joined fragments from pDS10 and pDS11, that now constitutes a part of *P450alk2*, is shown in fig.3A aligned with the corresponding partial *P450alk1* nucleotide sequence. The most conserved segment is situated in the coding regions of both P450s genes. An ORF from the *P450alk2* segment was highly similar to the *P450alk1* amino acid sequence and had an identity of 79.1% in 211 overlapping amino acids (fig.3B). The region corresponding to the proximal heme-binding domain, which is also called HR2, with a cysteine residue as a fifth ligand of the iron molecule [3] and the region mostly related to the distal heme-spanning region of the bacterial P450cam [12], remained practically unchanged.

The genomic restriction map of *P450alk2* was constructed using both *Eco*RI fragments from pDS10 and pDS11 as probes (fig.4D). The comparison with the *P450alk1* restriction map revealed that both genes did not share the same restriction sites. Therefore, *P450alk2* is not likely to be a *P450alk1* allele, as often observed for genes of *Candida* species generally considered as di- or polyploid organisms [13]. Kirsch et al. [14] reported for example that the gene coding for P450 lanosterol 14α-demethylase (*P45014DM*) from *C. albicans* existed in two different allelic forms that were differentiated only by a single restriction site polymorphism. On the other hand, *C. tropicalis* ATTC 750, from which *P450alk1* and *P450alk2* have been isolated, did not show a restriction site polymorphism for a corresponding *P45014DM*, showing that this yeast may be homozygous at the *P45014DM* locus [15]. This observation is also valid for *P450alk1*.

3.4. *Multiplicity of P450alk-related genes*

Since a second *P450alk* gene was present in *C. tropicalis*, we tested the presence of possible additional P450alk-related genes in this yeast by performing low stringency hybridization of restricted genomic DNA with *P450alk1* and *P450alk2* specific probes. As shown in fig.4A, B and C, additional hybridization bands (marked with arrows only in the *Eco*RI DNA digests of the left panel) can be detected that are neither corresponding to those expected from the *P450alk1* or *P450alk2* restriction maps. The increasing disappearance of these bands was related to the degree of hybridization stringency, as mostly apparent in fig.4B. Thus, the existence of at least a third and possibly a fourth P450alk-related gene could be suggested. These additional bands were not observed in previous hybridization experiments [5], since hybridization stringency was much higher and the type of probe different. It seems therefore that the P450LII gene family in *C. tropicalis*, from which *P450alk* (now *P450alk1*) is the first reported member [3], shows a heterogeneity not observed in the other yeast P450 gene family [15] but characteristic of P450 gene families of higher eukaryotes [16]. In addition, the complexity of the P450LII gene family is enlarged by two *C. maltosa* P450alk-related genes that were isolated recently by Takagi et al. [17] and Schunck et al. [18]. They

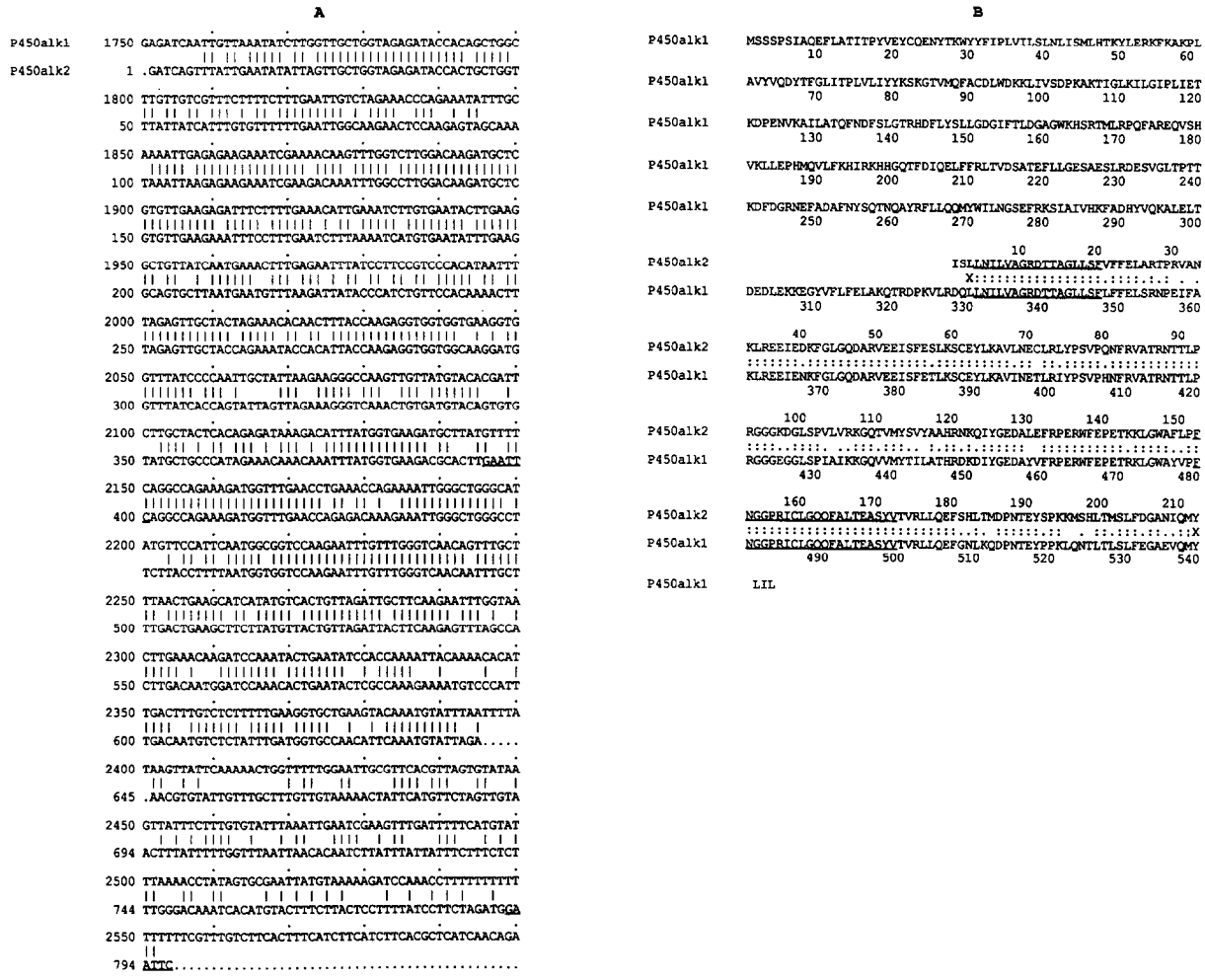
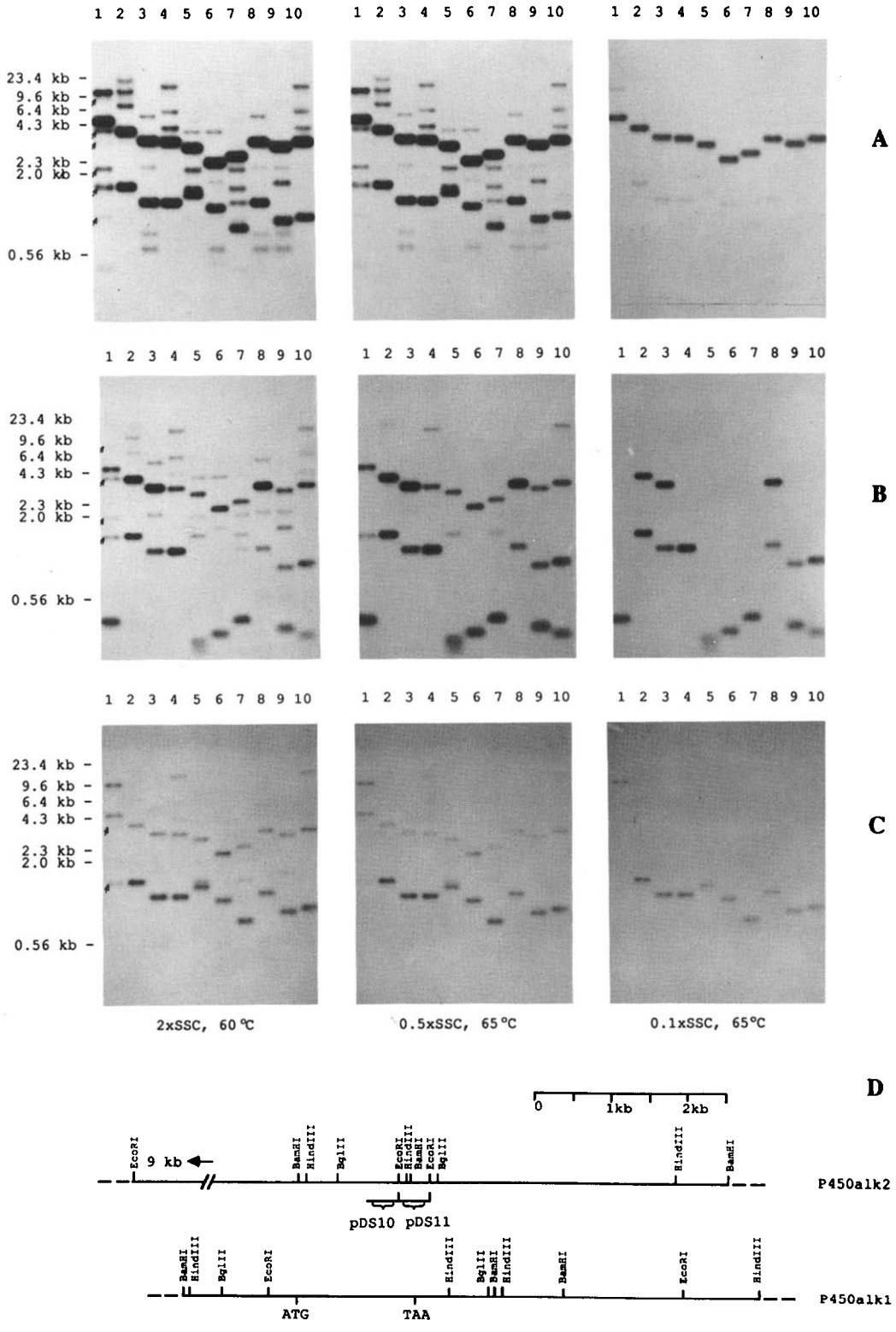


Fig.3. Comparative analysis of *P450alk1* and *P450alk2*. (A) Nucleotide sequence alignment of a *P450alk1* segment with the *P450alk2* partial nucleotide sequence. Numbering of *P450alk1* is as previously reported [3]. The position of the *Eco*RI sites in *P450alk2* as a junction between the pDS10 and pDS11 inserts and as a second site of the pDS11 insert are underlined. The stop codon of *P450alk1* is a nucleotide position 2402. Alignment was performed by the Needleman and Wunsch algorithm [19] implemented on the GCG DNA Analysis Software Package of the University of Wisconsin. The percentage of similarity between both sequences is 69.7%. (B) Amino acid alignment deduced from ORFs of *P450alk1* and *P450alk2*. The HR2 region (proximal heme-binding region) is underlined at the C-terminal end of both P450s, whereas the possible distal heme-binding site at the N-terminal end. Alignment was performed with the XFASTP algorithm of Lipman and Pearson [20]. There is 79.1% identity in 211 overlapping amino acids between both P450 segments.

Fig.4. Multiplicity of P450alk-related genes in *C. tropicalis*. Southern blots of restricted genomic DNA were hybridized with a *P450alk1* probe, i.e. a 1.5 kb *Eco*RI fragment from pDS535 described in Sanglard et al. [3] (A) and *P450alk2* probes, i.e. *Eco*RI fragments from pDS11 (B) and pDS10 (C). Genomic DNA was digested with (lanes): 1, *Eco*RI; 2, *Bam*HI; 3, *Hin*dIII; 4, *Bgl*II; 5, *Eco*RI/*Bam*HI; 6, *Eco*RI/*Hin*dIII; 7, *Eco*RI/*Bgl*II; 8, *Bam*HI/*Hin*dIII; 9, *Bam*HI/*Bgl*II; 10, *Hin*dIII/*Bgl*II. The washing temperatures of the hybridized membranes are indicated at the bottom of each panel. Molecular weight standards (phage λ, *Hin*dIII cut) are indicated on the left of each Southern blots subset. Arrows shown only in *Eco*RI DNA digests (lane 1) indicate the presence of additional fragments not corresponding to those expected from the *P450alk1* and *P450alk2* restriction maps. Southern blots were exposed to Fuji XR-L X-ray films. (D) Restriction map of *P450alk2* deduced from the restriction pattern exhibited in part (B) and (C). Below the *P450alk2* map, the *P450alk1* restriction map, as reported in Sanglard et al. [5], has been aligned to the corresponding segments of both genes.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23.4 kb
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EcoRI
BamHI
HindIII
BglII
pDS10 pDS11
P450alk2
P450alk1
ATG
TAA

were 57.6% and 60% similar to the *P450alk1* primary structure, respectively. Furthermore, we have observed that, using low stringency hybridization techniques, P450alk-related genes are also well conserved in other alkane assimilating yeasts such as *C. albicans, Lodderomyces elongisporus* and to a lesser extent in *Yarrowia lypolytica* (data not shown). Thus, P450alk-related genes are surprisingly well conserved even in unrelated yeast species.

When the isolation of P450alk-related genes from *C. tropicalis* is completed, it will be interesting to characterize the function of each of these gene products by their expression in *S. cerevisiae*. The main function of alkane-inducible P450s remains to hydroxylate aliphatic carbon chains at their terminal position, where the type of substrate can vary from alkanes or alkanols to fatty acids with different chain lengths [1]. It is still not certain whether a unique P450 type can utilize these different substrates and it is possible that each defined alkane-inducible P450 preferentially utilizes a specific substrate. Therefore, the characterization of these gene products will enable the detailed study of their heterogeneity and answer questions related to their substrate specificity.

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