

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

Dominique Sanglard and Armin Fiechter

Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Received 30 June 1989; revised version received 11 August 1989

The reexamination of a genomic  $\lambda$ gt11 *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  $\lambda$ gt11 *C. tropicalis* expression library that was used previously for the isolation of

*Correspondence address:* D. Sanglard, Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

*Abbreviations:* aa, amino acid; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl  $\beta$ -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*

*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 (*recA1*, *lac*<sup>-</sup>, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, [F'<sup>+</sup>proAB, *lacI*<sup>Q</sup>, *lacZ*ΔM15, 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 *EcoRI*. 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<sup>TM</sup> 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 [<sup>35</sup>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<sup>TM</sup> membranes according to the recommendations of the supplier (New England Nuclear). DNA probes were labelled with <sup>32</sup>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<sup>6</sup> 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<sub>2</sub>PO<sub>4</sub>, 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 *EcoRI* 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 *HindIII* 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 *EcoRI* site (figs 1 and 3A). These fragments were practically equal in size, thus explaining why they were recovered as a single apparent *EcoRI* 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 *EcoRI* 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

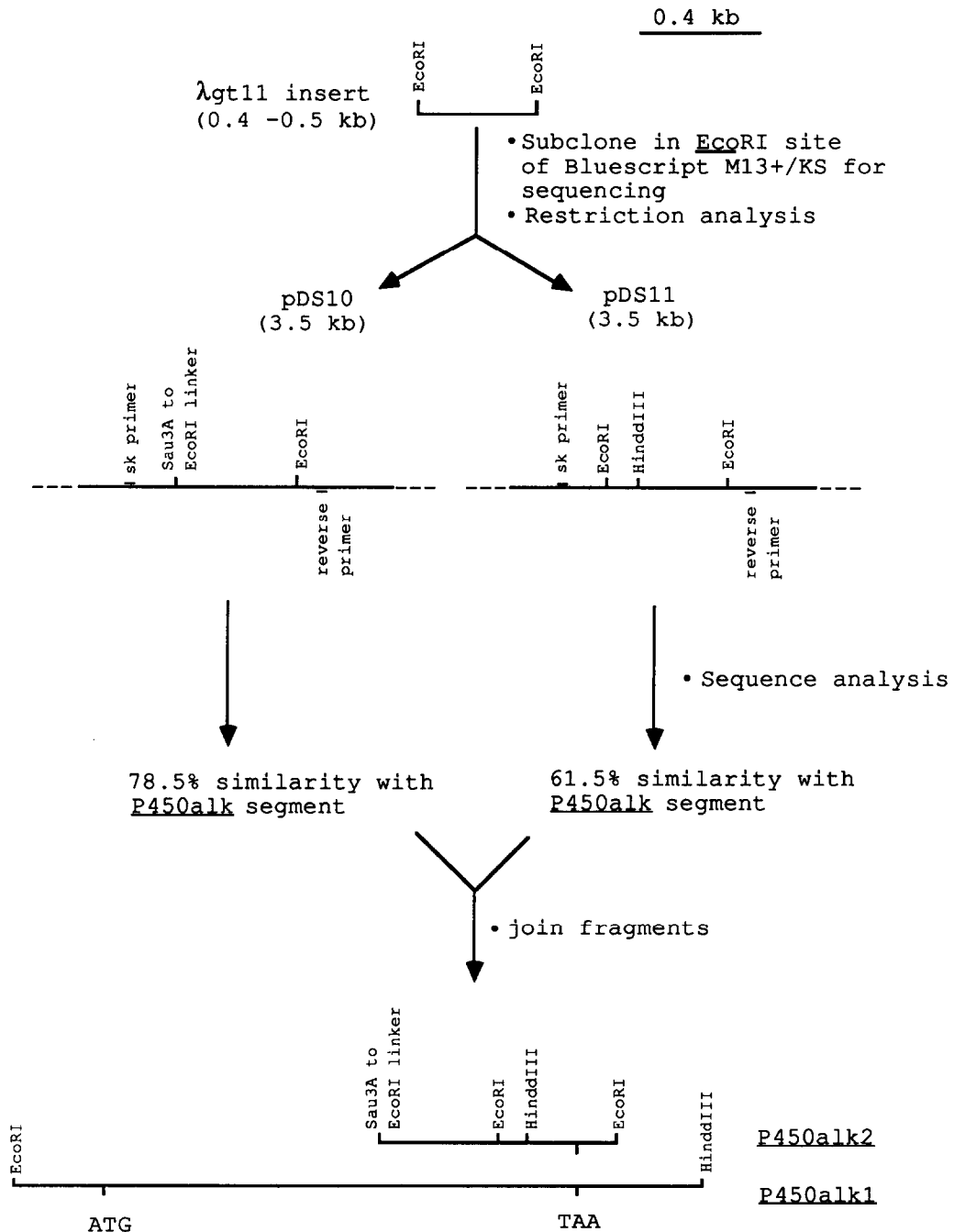
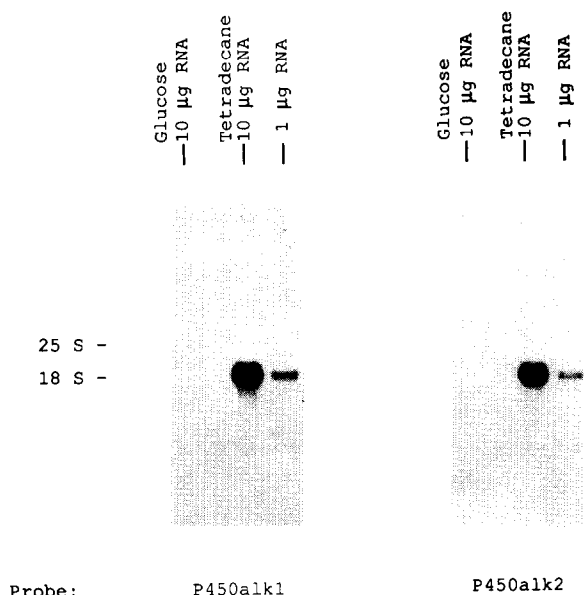


Fig.1. Schematic representation for the isolation and identification of the second *P450alk* gene from *C. tropicalis*. Relevant steps are indicated. Reverse primer and SK primer were used to sequence pDS10 and pDS11 inserts in both directions. The pDS10 insert was carrying an *EcoRI* linker at its 5'-end joined to a *Sau3A* 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*.



Probe: P450alk1 P450alk2

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 tetradeceane [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 *EcoRI* 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 14 $\alpha$ -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 *EcoRI* 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

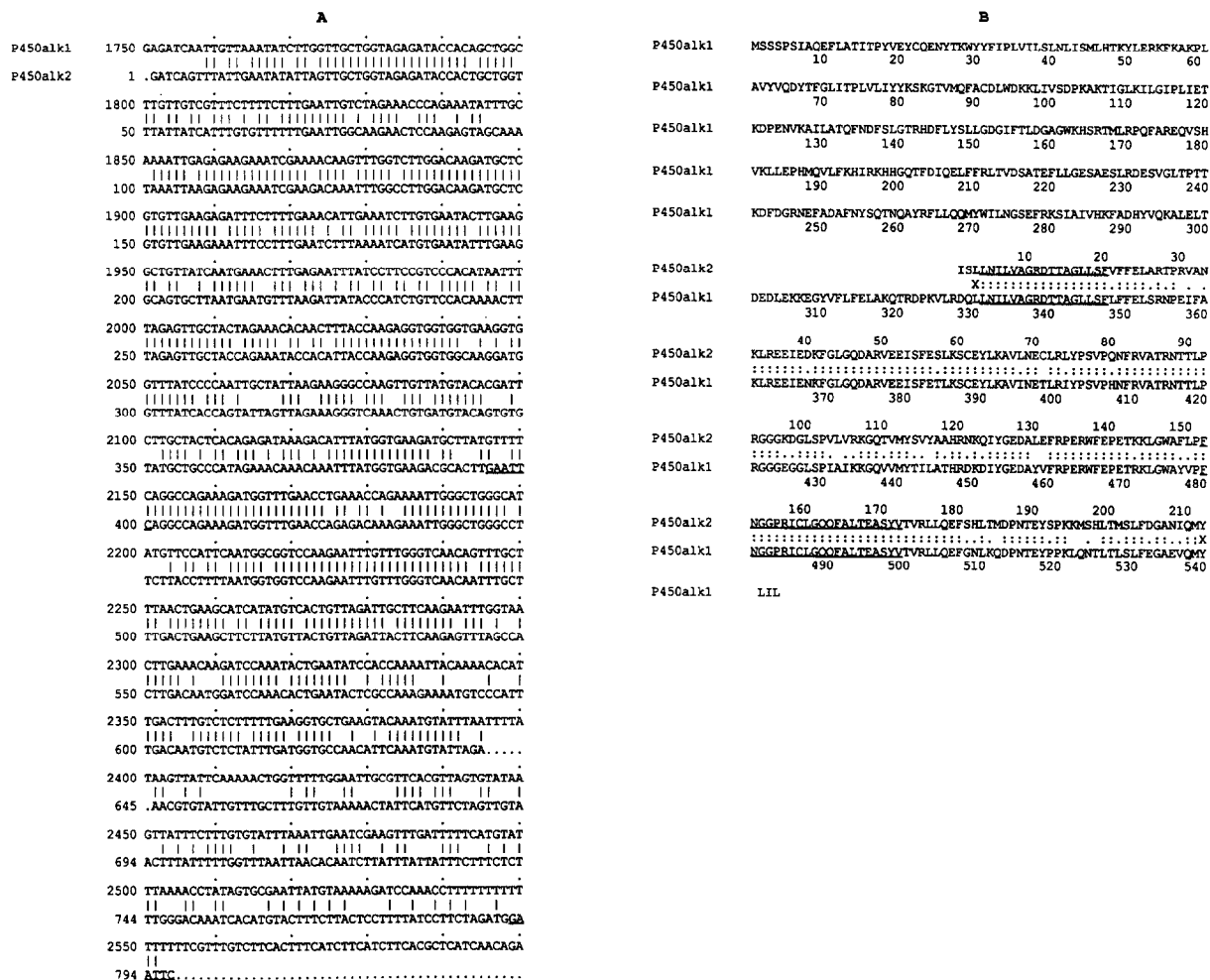
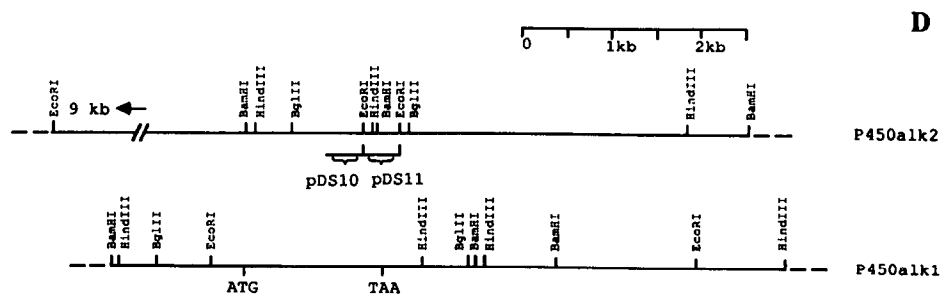
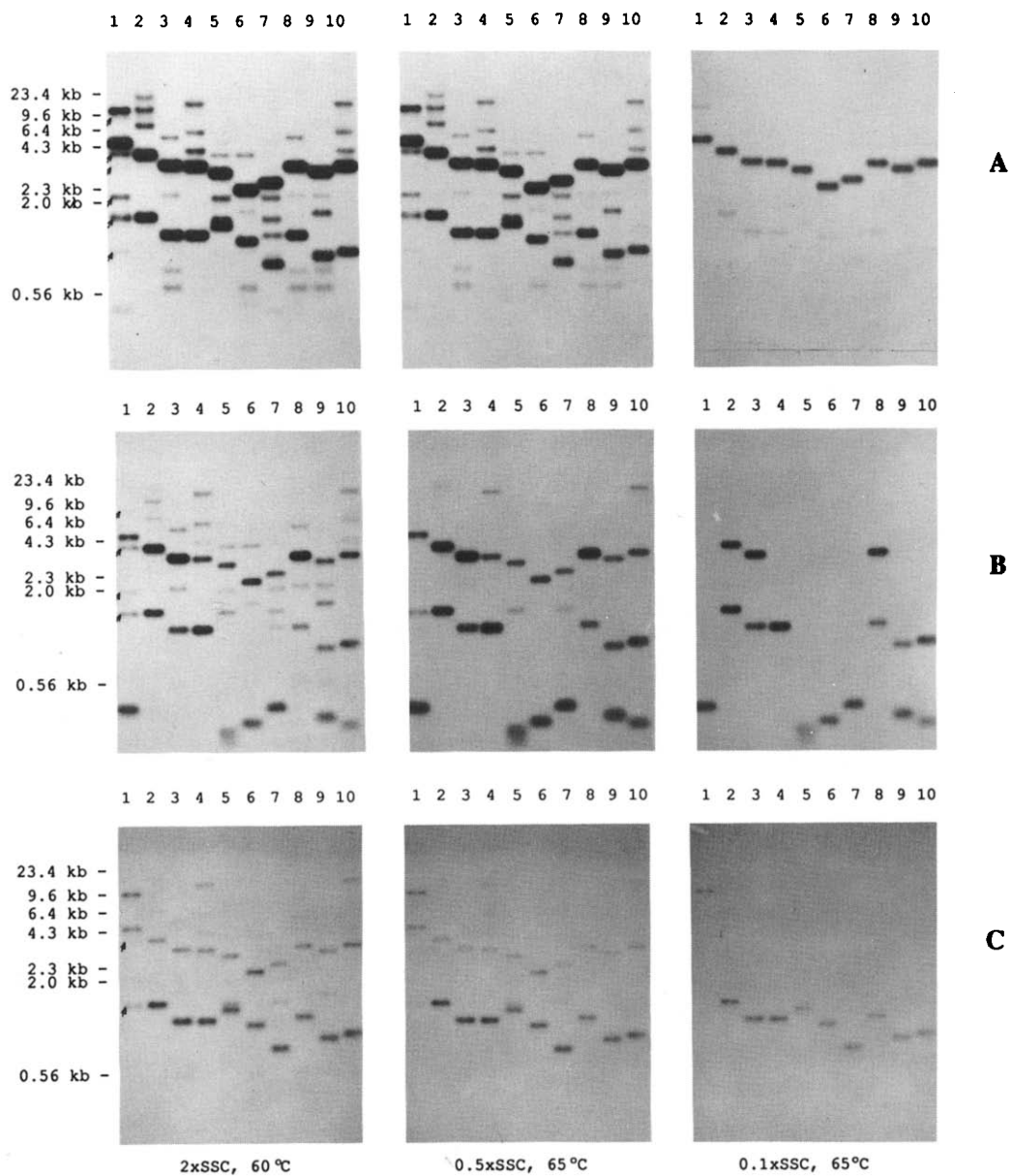


Fig.3. Comparative analysis of *P450alk1* and *P450alk2*. (A) Nucleotide sequence alignment of a *P450alk1* segment with the *P450alk2* partial nucleotide sequence. Numbering of *P450alk1* is as previously reported [3]. The position of the *Eco*RI sites in *P450alk2* as a junction between the pDS10 and 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, *Hind*III; 4, *Bgl*II; 5, *Eco*RI/*Bam*HI; 6, *Eco*RI/*Hind*III; 7, *Eco*RI/*Bgl*II; 8, *Bam*HI/*Hind*III; 9, *Bam*HI/*Bgl*II; 10, *Hind*III/*Bgl*II. The washing temperatures of the hybridized membranes are indicated at the bottom of each panel. Molecular weight standards (phage  $\lambda$ , *Hind*III cut) are indicated on the left of each Southern blot. 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.



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 lipolytica* (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.

**Acknowledgements:** Our thanks to Drs M. Takagi and W.-H. Schunck for providing their data prior to publication. This research is supported by the Swiss National Foundation grant 2000.5.532 to D.S.

## REFERENCES

- [1] Rehm, H.J. and Reif, I. (1981) Adv. Biochem. Eng. 19, 175–215.
- [2] Sanglard, D., Käppeli, O. and Fiechter, A. (1986) Arch. Biochem. Biophys. 251, 276–286.
- [3] Sanglard, D. and Loper, J.C. (1989) Gene 76, 121–136.
- [4] Loper, J.C., Chen, C. and Dey, C.R. (1985) Hazardous Wastes and Hazardous Materials 2, 131–141.
- [5] Sanglard, D., Chen, C. and Loper, J.C. (1987) Biochem. Biophys. Res. Commun. 144, 251–257.
- [6] Hug, H., Blanck, H.W. and Fiechter, A. (1974) Biotechnol. Bioeng. 16, 965.
- [7] Rothstein, R. (1985) in: DNA Cloning, Cloning in Yeast, vol.II (Glover, D.M. ed.) pp.45–66, IRL Press, Oxford.
- [8] Sherman, F., Fink, G. and Hicks, J.B. (1986) in: Laboratory Course Manual for Methods in Yeast Genetics, pp.143–144, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- [9] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning, A Laboratory Manual, 10th edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- [10] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) in: Basic Methods in Molecular Biology, pp.129–152, Elsevier, Amsterdam.
- [11] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
- [12] Poulos, T.L., Finzel, B.C. and Howard, A.J. (1987) J. Mol. Biol. 195, 687–700.
- [13] Magee, P.T., Rikkerink, E.H.A. and Magee, B.B. (1988) Anal. Biochem. 175, 361–372.
- [14] Kirsch, D.R., Lai, M.H. and O'Sullivan, J. (1988) Gene 68, 229–237.
- [15] Chen, C., Turi, T.G., Sanglard, D. and Loper, J.C. (1987) Biochem. Biophys. Res. Commun. 146, 1311–1317.
- [16] Nebert, D.W., Nelson, D.R., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R. and Waterman, M.R. (1989) DNA 8, 1–13.
- [17] Takagi, M., Ohkuma, M., Kobayashi, N., Wanatabe, M. and Yano, K. (1989) Agric. Biol. Chem., in press.
- [18] Schunck, W.-H., Kärger, E., Gross, B., Wiedmann, B., Mauersberger, S., Köpke, K., Kiessling, U., Strauss, M., Gaestel, M. and Müller, H.-G. (1989) Biochem. Biophys. Res. Commun., in press.
- [19] Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol. 48, 443–453.
- [20] Lipman, D.J. and Pearson, W.R. (1985) Science 227, 1435–1441.