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A Genetic approach to Improvements of *Candida tropicalis* as a Biocatalyst

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This thesis is dedicated to my husband David.

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ABSTRACT

It is well documented in the literature that, in addition to carbon sources such as glucose, *Candida* species are able to utilise a wide variety of n-alkanes which are degraded via β-oxidation. Prior to β-oxidation a primary oxidation system acts on the end terminal methyl groups to generate carboxyl groups. The main industrial aim of this work was to optimise the bioconversion of pelargonate [$CH_3(CH_2)_7COOH$] to azeleate [$HOOC(CH_2)_7COOH$], ie. the primary oxidation steps, by blocking the β-oxidation pathway in *C. tropicalis*. To this end a library of *Sau* 3AI partially digested *C. tropicalis* NCYC997 genomic DNA in pBR322 was constructed from which it was hoped to isolate and disrupt the long-chain fatty acid acyl-CoA oxidase genes, *POX4* and *POX5*, which catalyse the first step of β-oxidation. The library was probed with oligonucleotides specific to *POX4* and *POX5*, and a putative *POX4* clone was isolated.

Several marker systems for identifying *C. tropicalis* transformants were investigated for use in gene transplacement and on general purpose cloning vectors for this yeast with a view to developing its molecular biology. The sensitivity of *C. tropicalis* to copper, chloramphenicol, kanamycin, neomycin, and G418 was assessed with a view to developing these as dominant selectable markers in this yeast. It was also attempted to isolate the *HEM1* gene from *C. tropicalis* genomic DNA libraries constructed to be enriched for these sequences in order to produce a non-revertible *C. tropicalis hem1* strain by gene disruption which would require 5-amino levulinic acid for growth. The cloned gene would then act as a selectable marker in this strain by alleviating this requirement. *C. tropicalis ura3* mutant strains were isolated after exposure to UV-irradiation and 5-fluoro orotic acid selection. However, attempts to transform these strains with vectors carrying the *S. cerevisiae URA3* gene were unsuccessful as were attempts to isolate the *C. tropicalis URA3* gene from the genomic library constructed in pBR322 by complementation of *E. coli* B15 which carries the *pyrF* mutation.

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ABBREVIATIONS

BIOCHEMICALS

AA:	α-amino adipic acid
ALS:	α-acetolactate synthase
ATP:	adenosine 5'-triphosphate
B:	brij 58 (polyoxyethylene 20 cetyl)
Can:	L-canavanine sulphate
CoA, CoA-SH, acyl-CoA:	coenzyme A and its acyl derivatives
D:	D-Glucose
DAB:	3,3'-diaminobenzidine
DEAE:	diethylaminoethyl
DMSO:	dimethyl sulphoxide
DNA:	deoxyribonucleic acid
dsDNA:	double stranded DNA
E:	ethanol
EDTA:	diaminoethanetetra acetic acid disodium salt
EMS:	ethyl methanesulphonate
ER:	endoplasmic reticulum
FAD, $FADH_2$:	flavin adenine dinucleotide and its reduced form
FOA:	5-fluoro orotic acid
G:	glycerol
dGTP:	2'-deoxyguanosine 5'-triphosphate
IPTG:	isopropyl b-D-thiogalactopyranoside
dITP:	2'-deoxyinosine 5'-triphosphate
NAD^+, NADH:	nicotinamide adenine dinucleotide and its reduced form
$NADP^+$, NADPH:	nicotinamide adenine dinucleotide phosphate and its reduced form
O:	oleic acid
PEG:	polyethylene glycol
PMSF:	phenylmethylsulphonyl fluoride
R:	D-raffinose
RNA:	ribonucleic acid
dsRNA:	double stranded RNA
mRNA:	messenger RNA
rRNA:	ribosomal RNA

SDS:	sodium dodecylsulphate
SM:	sulphometuron methyl, N-[(4,6-dimethylpyrimidin-2-yl)-amino-carbonyl]-2-methoxycarbonylbenzenesulphonamide
TCA:	tricarboxylic acid
Tris:	tris(hydroxymethyl)amino-methane
U:	uracil
X-gal:	5-Bromo-4-chloro-3-indoyl-b-d-galactoside

MEDIA

BBL:	Baltimore Biological Laboratories medium
CM:	complete medium
L:	Luria Bertani medium
M9:	minimal glucose medium
NEP:	nutrient extract-peptone
S:	semi-defined minimal medium
YNB:	yeast nitrogen base
YEP:	yeast extract peptone

ANTIBIOTICS

Ap:	ampicillin
Cm:	chloramphenicol
G418:	geneticin G418-sulphate
Km:	kanamycin sulphate
Nm:	neomycin sulphate
Tc:	tetracycline hydrochloride

MISCELLANEOUS

ARS:	autonomously replicating sequence
bp:	base pairs
IMS:	industrial methylated spirit
PAGE:	polyacrylamide gel electrophoresis
PFGE:	pulsed field gel electrophoresis
PXP:	peroxisomal polypeptide

SCP:	single-cell protein
UV:	ultraviolet
YCp:	yeast centromeric plasmid
YEp:	yeast episomal plasmid
YIp:	yeast integrating plasmid
YLp:	yeast linear plasmid
YRp:	yeast replicating plasmid

PREFIXES USED IN INTERNATIONAL SYSTEM OF UNITS

k:	kilo (10^3)
c:	centi (10^{-2})
m:	milli (10^{-3})
μ:	micro (10^{-6})
n:	nano (10^{-9})
p:	pico (10^{-12})
f:	femto (10^{-15})

UNIT ABBREVIATIONS

A:	ampere	min:	minute
°C:	degrees Celsius	mol:	mole
Ci:	Curie	pH:	-log H^+ concentration
Da:	dalton	psi:	pounds per square inch
g:	gram	rpm:	revolutions per minute
h:	hour	RT:	room temperature
J:	joule	s:	second
l:	litre	U:	unit
ln:	logarithm to the base *e*	V:	volt
log:	logarithm to the base 10	v/v:	volume for volume
m:	meter	w/v:	weight for volume
M:	molar concentration		

AIMS OF THIS WORK

The primary objective of this work was to produce a non-revertible β-oxidation mutant of the n-alkane assimilating yeast *Candida tropicalis* by genetic manipulation of the long-chain fatty acid acyl-CoA oxidase gene, whose product catalyses the first step of peroxisomal β-oxidation. This mutant was to be used in the bioconversion of pelargonate [$CH_3(CH_2)_7COOH$] to azeleate [$HOOC(CH_2)_7COOH$] by ω-oxidation of the terminal methyl group.

A secondary objective, which was in part a means of achieving the primary objective, was to develop the molecular biology of this yeast by finding suitable dominant selectable markers; producing auxotrophic mutants and cloning the complementary genes to act as genetic markers on a plasmid vector system; and ultimately isolating autonomously replicating sequences (*ARS*) and appropriate signal sequences to optimise vector expression and stability in this yeast.

CHAPTER 1. INTRODUCTION

1.1 *CANDIDA TROPICALIS*

1.1.1 Classification and Homology to other *Candida* Yeasts

Candida yeasts are characterised as white imperfect (asporogenous) yeasts capable of forming pseudohyphae. The genus is artificial in the sense that assignment of the generic name does not imply descent from a common ancestor. Its members are considered to be imperfect forms of ascomycete species from a variety of other genera, some of which have been identified by DNA reassociation studies, for example, the perfect forms of *C. pseudotropicalis* (*Kluyveromyces fragilis*), *C. guilliermondii (Pichia guilliermondii)* and *C. krusei* (*Issatchenkia orientalis*). The apparent absence of any closely related ascosporogenous yeast species argues that *C. albicans* has been imperfect for a prolonged time (Riggsby, 1985; Shepherd *et al.*, 1985).

Taxonomic classification of *Candida* yeasts is difficult. The absence of a sexual cycle makes classification schemes based on the use of teleomorphic characteristics impossible, and therefore, within the genus *Candida*, species are characterised primarily on colonial morphology, carbohydrate utilisation, and fermentation. Nutritional characteristics such as fermentation and assimilation of various substrates are useful, but studies of perfect genera show that nutritional variation can occur among members of a clearly defined species. Cell morphology is probably less sensitive to mutation than nutritional markers.

The imperfect nature of *Candida* yeast not only excludes the possibility of using morphological criteria associated with ascospore formation but also prevents experimental taxonomy based on crossing of isolates, and has retarded genetic analysis. *Candida* species have lost the ability to undergo meiosis and form the haplophase. Although sexually active strains have been reported in both *C. tropicalis* and *C. albicans*, there is no obvious evidence of a sexual life cycle (Kucsera and Ferenczy, 1986). No evidence of natural crossing-over has been reported by any laboratory using auxotrophically characterised strains, and this absence suggests that no natural sexual cycle exists as passage through the haplophase would eliminate recessive auxotrophic alleles (Shepherd *et al.*, 1985).

Restriction fragment length polymorphisms in the ribosomal DNA are a useful criterion for distinguishing various isolates of *C. albicans*, and similar digestion of other *Candida* species, *C. tropicalis*, *C. stellatoidea*, and *C. guilliermondii*, show that each can be identified on the basis of its restriction patterns. Since these are highly reiterated genes, the differences are apparent on ethidium bromide-stained gels and Southern transfers are unnecessary (Magee *et al.*, 1987). Similarly, the restriction patterns of *C. albicans* and *C. stellatoidea* mitochondrial DNA are almost identical, but have few restriction fragments in common with *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, or *C. glabrata* (Riggsby, 1985).

The base composition of *C. tropicalis* and of *C. albicans* is approximately 35 mol% (G + C). The nuclear genome of *C. albicans* contains mostly single-copy DNA, with only 1-2% of the sequence complexity organised in the form of repetitive sequences (Barnett *et al.*, 1983; Riggsby, 1985). Most of the medically important *Candida* species show very little homology with one another, with the exception of *C. albicans* and *C. stellatoidea* (Riggsby, 1985). The relative DNA-DNA homology is only 8% between *C. tropicalis* and *C. albicans*. However, on the basis of morphological criteria and physiological tests such as serology, and PMR-spectra of the cell wall mannans, *C. tropicalis* and *C. albicans* are closely related species (Kucsera and Ferenczy, 1986).

Crossed-line immunoelectrophoresis has been used to compare antigenic determinants of *C. tropicalis* and *C. albicans* with antigens from other *Candida* species. *C. tropicalis*, *C. albicans*, *C. clausenii*, and *C. stellatoidea* form one of three groups sharing 85-100% antigenic similarity. Using a monoclonal antibody to a surface antigen of *C. albicans*, it was shown that this antigen is also produced by *C. tropicalis* and *C. glabrata*, but not by *C. parapsilosis* or *C. krusei*. It seems strange that *C. albicans* and *C. tropicalis*, which have low DNA homology, should be so physiologically and immunologically similar (Riggsby, 1985). The acyl-CoA oxidases of *C. tropicalis* and *C. lipolytica* are antigenically highly dissimilar (Fujiki *et al.*, 1986).

C. albicans DNA has been analysed by pulsed field gel electrophoresis (PFGE). Unlike *S. cerevisiae* DNA which is very amenable to PFGE analysis, several problems were encountered with *C. albicans*, the chief of which was the much larger size of some of the chromosomes. The result represented at least six and possibly eight distinct chromosomes in *C. albicans* (Snell and Wilkins, 1986).

1.1.2 General Characteristics

Candida tropicalis forms white or cream colonies, shows multilateral budding, elaborate pseudohyphae and septate hyphae, and no sexual reproduction. The cells can ferment D-glucose (D) but cannot ferment or grow aerobically on D-raffinose. *C. tropicalis* has been isolated from sauerkraut, molasses, miso, water, fruit, bakers' yeast, man and other mammals, unlike *C. albicans* which is believed to be an obligate associate of warm-blooded animals (Barnett *et al.*, 1983).

Pseudohyphae are elongated yeast cells formed by polar budding, constricted at the cell junctions and joined in chains and clusters. Germ-tube formation, the initial stage in the yeast to hyphal transition, is characterised by the absence of constriction at the mother cell junction, random rather than polar evagination from the mother cell, and the positioning of the first septum with an accompanying filament ring approximately 2 μm from the mother cell junction. Septal pores are present in mature unseparated yeast cells, pseudohyphae and in the true hyphal form (Shepherd *et al.*, 1985).

C. albicans is usually present as a harmless asymptomatic commensal but can manifest as a pathogen. Such infections (candidoses) take two forms: superficial (oral and vaginal thrush, and chronic mucocutaneous candidoses) and invasive (*Candida* myocarditis and acute disseminated *Candida* septicaemia among others). *C. albicans* can infect virtually every tissue in the human body, but the most common candidoses are superficial lesions of the mucous surfaces. An increased incidence of candidoses has been attributed to the widespread use of antibiotics and immunosuppressive agents. Candidoses are caused primarily by *C. albicans*, and less frequently by *C. tropicalis* and *C. parapsilosis*. Accumulating clinical and experimental evidence suggests that species such as *C. tropicalis* are increasingly causes of candidoses in the compromised host (Shepherd *et al.*, 1985).

1.1.3 Ploidy

It was assumed for a long time that *Candida* yeasts were haploid, but several lines of evidence suggest that they are in fact stable diploids.

Candida auxotrophs are largely unavailable. Several authors have reported unsuccessful efforts to derive the customary diverse spectrum of such mutations by conventional techniques. One explanation for this lack of auxotrophic strains is that *Candida* yeasts are of a higher ploidy than haploid. Diploid strains yield auxotrophic mutants at a far lower rate, because mutations either have to be introduced in both alleles present for the given locus, or a dominant mutation is required for the mutation to be expressed. Both of these conditions occur at a low rate when compared with the isolation of recessive auxotrophic mutations from comparable haploid cell lines (Olaiya and Sogin, 1979).

Candida auxotrophs have been isolated by a variety of mutagenesis protocols involving ultraviolet- (UV) irradiation, ethyl methanesulphonate (EMS), nitrous acid, and N-methyl-N'-nitro-N-nitrosoguanidine, some involving enrichment procedures. Following exposure to UV-irradiation, some but not all strains give rise to an unusually large number of auxotrophs, almost all of which are of the same type (a biased auxotroph spectrum). Different strains have distinctive biased auxotroph spectra. Strains of *C. albicans* showing biased auxotroph spectra are heterozygous for fully recessive defective biosynthetic alleles and are therefore thought to be diploid. Exposure to UV-irradiation induces mitotic crossing-over, and as a consequence the heterozygous state is brought to homozygosity (Whelan and Magee, 1981).

A number of naturally occurring multiply auxotrophic isolates of *C. albicans* are unstable after exposure to UV light, giving rise to prototrophic revertants. These revertants are mitotically unstable, and following UV-irradiation give rise to the parental auxotroph with a high frequency. They frequently give rise to sectored colonies after UV-irradiation, one component of the colony being the parental auxotroph and the other component being a stable prototroph. Some prototrophic revertants of a double auxotroph show coincident appearance of the two parental auxotrophies, and after UV-induced mitotic instability they give rise to doubly auxotrophic derivatives. Prototrophic revertants of a double auxotroph show sectored colonies after UV-irradiation, each sector corresponding to one of the parental auxotrophies.

These observations can be interpreted by assuming that *C. albicans* is naturally diploid and that the prototrophic revertants are heterozygotes. This heterozygosity explains their instability after UV induced mitotic crossing over, as this generates reciprocal products, a stable prototrophic sector homozygous for the functional allele and a sector homozygous for the defective allele. Prototrophic revertants of a strain auxotrophic for two linked loci will be of two types, either *cis* linked or *trans* linked for the defective alleles, which are expected to occur with approximately equal frequency. Revertant strains carrying the mutant allele in *cis* linkage give rise to homozygous double auxotrophs after mitotic crossing over between the centromere and the proximal gene and single auxotrophs for the distal gene if the crossing over occurs between the loci. Strains carrying the mutant alleles in *trans* linkage would give rise to sectored colonies, each component displaying one of the parental auxotrophies (Poulter *et al.*, 1982; Poulter and Rikkerink, 1983).

Analysis of UV-induced mitotic crossing-over in *C. albicans* prototrophic fusion products suggest that there are at least two types of heterozygote, simplex and duplex. The simplex (single functional allele) prototrophic heterozygotes give rise to a high frequency of auxotrophs, and the duplex (two functional alleles) prototrophic heterozygotes give rise to a low frequency of auxotrophs following UV-irradiation. This supports the hypothesis that *C. albicans* is naturally diploid and that prototrophic fusion products are of higher ploidy (Shepherd et al., 1985).

Total cellular DNA content, determined by a colourimetric method, has been used as an index of ploidy in *C. albicans*. Five mononucleate hybrids formed by sphaeroplast fusion of diploid parent strains were taken to be tetraploid, and one was taken to be hexaploid or near-hexaploid on the basis of estimated DNA content. Selection for increased resistance to 5-fluorocytosine in the hybrids, which were heterozygous for resistance, resulted in the isolation of variants which were of lower ploidy than the hybrids from which they originated (Whelan *et al.*, 1985). The total amount of DNA in the *C. albicans* cell has been measured by chemical analysis and compared to the kinetic complexity of the DNA, as measured by DNA reassociation kinetics. The complexity of single-copy *C. albicans* DNA is half the *C. albicans* single-copy DNA content per cell which suggests diploidy (Riggsby, 1985). The complexity of *C. albicans* DNA gives a $C_0t_{1/2}$ value of 14, compared with a $C_0t_{1/2}$ value of 13 for *S. cerevisiae*, suggesting that the single copy genome size is approximately the same. The DNA content of *C. albicans* measured by flow microfluorometry, a technique capable of analysing single cells, was compared with flow microfluorometric DNA determinations on haploid, diploid, triploid and tetraploid *S. cerevisiae*. By this criterion, *C. albicans* was found to contain a diploid amount of DNA (Olaiya and Sogin, 1979).

If *C. albicans* were haploid with a genome size twice that of *S. cerevisiae*, *C. albicans* would be far more susceptible to mutagens. Survival of UV-irradiation and exposure to the chemical mutagens nitrosoguanidine and EMS is proportional to ploidy for strains of *S. cerevisiae*. The greater the ploidy number, the more resistant these strains are. *C. albicans* has inactivation kinetics nearly identical to those of diploid *S. cerevisiae* (Olaiya and Sogin, 1979). Integration occurs at only one chromosomal homologue providing evidence at the molecular level for diploidy in the *Candida* genome (Kurtz *et al.*, 1986). Although some of these results can also be interpreted on the basis that *Candida* yeasts are haploid, taken together they provide convincing evidence that they are in fact stable diploids.

1.1.4 Peroxisomes and n-Alkane Metabolism

1.1.4.1 Biogenesis and Structure of Peroxisomes

Candida tropicalis assimilates fatty acids or n-alkanes as the sole source of carbon and energy, and is a valuable microorganism for studying fatty acid metabolism in eukaryotic cells. Peroxisomes are organelles whose development can be controlled under various conditions, providing a model for the investigation of transportation of precursor proteins encoded by nuclear genes (Kamiryo and Okazaki, 1984; Ueda *et al.*, 1985).

Microbodies, subcellular respiratory organelles for catabolic pathways, are present in most eukaryotic cells, and have many properties in common. They are 0.5-1.5 μm in diameter having a single, boundary, lipid bilayer membrane surrounding a granular matrix of soluble proteins, with no inner membranes but sometimes a dense core or crystalloid structure of protein. They are permeable to most small substrates (Tolbert, 1981).

Yeasts grown on a precursor substrate for glyoxylate (e.g. malate) develop glyoxysomes, on methanol they develop gigantic microbodies that fill the cell and contain an alcohol oxidase, and on fatty acids or alkanes they develop numerous microbodies containing the long-chain fatty acid β-oxidation pathway. The latter are termed peroxisomes and are characterised by the presence of flavin oxidases that form hydrogen peroxide, and excess catalase to remove it (Tolbert, 1981). The localisation of catalase activity in peroxisomes can be visualised by the intense deposition of the 3,3'-diaminobenzidine (DAB)-reaction product in the peroxisomes (Osumi *et al.*, 1974).

The appearance and activity of microbodies is substrate dependent. The development of peroxisomes in *C. tropicalis* and other *Candida* yeasts has been studied by several groups (Osumi *et al.*, 1974; Teranishi *et al.*, 1974a and b; Tanaka *et al.*, 1982; Ueda *et al.*, 1985). Cells grown on malt extract, D, ethanol (E) or lauryl alcohol contain only a few peroxisomes and have low catalase activity, peroxisomes are repressed and respiration is mainly mitochondrial. In cells transferred to a medium containing alkanes (C_{10}-C_{13}) or fatty acids and incubated aerobically, peroxisome number and catalase activity increase, especially during exponential growth, although there is no significant change in peroxisome size. The hydrocarbon-induced morphological change of *C. tropicalis* is also observed on a hydrocarbon medium supplemented with D.

These groups also showed that the development of peroxisomes and the enhancement of catalase activity in alkane-utilising cells is completely repressed by 0.2-0.5% D or 1 μgml^{-1} cycloheximide, an inhibitor of cytoplasmic protein synthesis, but not by 5% galactose or 1 $mgml^{-1}$ chloramphenicol. Cycloheximide does not inhibit the catalase activity *in vitro*. The catalase activity of *C. tropicalis* is inhibited completely by cyanide and azide.

E-grown and acetate-grown cells of *C. tropicalis* pK233 are elongated, contain more peroxisomes and have catalase activities which are higher than D-grown cells, but lower than hydrocarbon-grown cells. In contrast, the catalase activities of E- and acetate-grown cells of *C. lipolytica* NRRL Y-6795 are the same as in D-grown cells. (Osumi *et al.*, 1974).

The endoplasmic reticulum (ER) membrane and the outer membranes of the nucleus, mitochondria, and microbody all have a common origin. The single membrane of microbodies is similar in composition to the ER containing phosphatidylcholine, phosphatidylethanolamine, phosphatidyl-inositol, and antimycin A-insensitive NADH:cytochrome c reductase. This is evidence that microbodies are formed by budding from a dilated region of the smooth ER, to which they may remain attached and, in consequence, the enzymatic composition of new and old microbodies may interchange via the ER channels. Peroxisomes of *C. tropicalis* are often located along the ER (Osumi, *et al.*, 1974; Tolbert, 1981; Tanaka *et al.*, 1982).

Electronmicroscopical observations made by Tanaka *et al.* (1982) suggest that yeast peroxisomes may also arise by division of pre-existing peroxisomes during growth of *C. tropicalis* on n-alkanes. Fujiki *et al.* (1984) reported that the 21.7 kDa peroxisomal membrane polypeptide from rat liver, like other peroxisomal proteins, is inserted post-translationally into the organelle. This is further evidence that peroxisomes, like mitochondria and chloroplasts, can form by fission from pre-existing organelles.

Initial studies of the peroxisome membrane were limited by a significant amount of ER contamination relative to the small amount of available peroxisome membrane. Some enzymes associate with microbody ghosts by ionic forces after osmotic shock, but are not thought to be intrinsic membrane components (Tolbert, 1981). Van Veldhoven *et al.* (1987) showed that the high permeability of the rat liver peroxisomal membrane is based on the presence of a 22 kDa integral membrane, pore-forming protein, unique to the peroxisomal membrane which allows the diffusion of hydrophilic molecules up to a molecular weight of 800. The same group detected large conductance channels with electric properties distinct from those of the mitochondrial porin, but with a similar pore diameter, in planar lipid bilayers after fusion with purified peroxisomal membranes and in liposomes, reconstituted with peroxisomal membranes or peroxisomal integral membrane protein fractions. These are probably responsible for the permeability of intact peroxisomes to solutes. Mannaerts *et al.* (1982) have shown that acyl-CoA synthetase of proliferated rat liver peroxisomes is located in the peroxisomal membrane with its active site towards the cytoplasm.

Osumi, *et al.* (1974) did not observe crystalline inclusions in any yeast microbody, although electron-dense areas which appear in the peroxisome matrix after treatment with DAB may be related to crystalline inclusions. Kawamoto *et al.* (1977b) reported that peroxisomes isolated from n-alkane-grown *C. tropicalis* have an homogeneous matrix, whereas, those isolated from methanol-grown yeasts contain crystalloids with a lattice structure.

The development of peroxisomes is accompanied by a markedly high expression of peroxisomal proteins. When *C. tropicalis* is grown on fatty acids or n-alkanes 9-18 abundant mRNAs are strikingly induced (Kamiryo and Okazaki, 1984; Fujiki *et al.*, 1986; Okazaki *et al.*, 1986; Rachubinski *et al.*, 1987; Small and Lazarow, 1987).

Yeast peroxisomes purified to near homogeneity (less than 1% mitochondrial contamination) from oleic acid-grown *C. tropicalis* contain small amounts of DNA, which yield restriction fragments indistinguishable from those of mitochondrial DNA indicating they are not peroxisomal in origin. Results from caesium chloride density gradient centrifugation of the organelles lysed with a detergent, staining of the organelles with DNA specific fluorescent dyes (ethidium bromide and 4',6-diamidino-2-phenyl-indole), and labelling of the DNA with [^{3}H]adenine also indicate that peroxisomes contain no DNA (Kamiryo *et al.*, 1982). Peroxisome biogenesis must therefore be controlled entirely by nuclear genes.

Some peroxisomal genes are clustered. Genes within a cluster show a high degree of functional and structural homology. In *C. tropicalis* pK233 the gene for peroxisomal peptide- (PXP-) 4 is adjacent to that for PXP-2 on the genome and hybridises to PXP-5 mRNA, suggesting that gene clusters arose by duplication of a few ancestral genes (Kamiryo and Okazaki, 1984).

Some peroxisomal and mitochondrial enzymes are products of the same nuclear gene. For example, peroxisomal and mitochondrial carnitine acetyltransferases, purified from alkane-grown *C. tropicalis*, have similar amino acid compositions and isoelectric points, the same molecular weight, amino-terminal residue and heat stability, and are indistinguishable immunochemically (Ueda *et al.*, 1984). There are no differences in the enzymological and immunological properties of the peroxisomal and cytosolic forms of catalase from *C. tropicalis* (Mauersberger *et al.*, 1987). Mice have five catalase isoenzymes, the products of a single structural gene, which differ in sialic acid content, peroxisomal catalase being most highly sialated (Tolbert, 1981). Peroxisomal acyl-CoA synthetase purified from rat liver was reported to be identical with the enzyme purified from microsomes and mitochondria, the same enzyme being distributed to microsomes, mitochondria, and peroxisomes (Hashimoto, 1982).

Peroxisomal proteins, such as acyl-CoA oxidase (76 kDa subunit), trifunctional hydratase-dehydrogenase-epimerase (102 kDa subunit), and catalase (57 kDa subunit) from purified peroxisomes of *C. tropicalis* (Fujiki *et al.*, 1986) and the 22 kDa membrane polypeptide from rat liver (Fujiki *et al.*, 1984), are synthesised in the cell cytosol on free polyribosomes and, except thiolase, at their mature sizes, rather than as larger precursors. All are transported into the organelle post-translationally without proteolytic processing.

All peroxisomal enzymes (except acyl-CoA synthetase) are located in the matrix, at the matrical side of the membrane or in the core (Fujiki *et al.*, 1984; Fujiki *et al.*, 1986; Okada *et al.*, 1986). Peroxisomal CoA is tightly bound to a peroxisomal matrix protein (Van Veldhoven *et al.*, 1987).

Secretory proteins and certain integral membrane proteins have been shown to a have short-lived amino-terminal signal sequence which targets them within the cell. However, ovalbumin is synthesised with an internal rather than an amino-terminal signal sequence (Lingappa *et al.*, 1979). There is sufficient information in the carboxy-terminal portion of acyl-CoA oxidase from *C. tropicalis* to target it to peroxisomes (Small and Lazarow, 1987). The inferred amino acid sequence of catalase from *C. tropicalis* is homologous to the carboxy-termini of mammalian and *S. cerevisiae* catalases (Rachubinski *et al.*, 1987). The information for the targeting of proteins to peroxisomes is therefore presumed to reside in the mature amino acid sequence as in the case of ovalbumin, but the details are unknown.

1.1.4.2 The Metabolic Roles of Peroxisomes and Microbodies and their Interaction with Mitochondria

To demonstrate the metabolic functions of peroxisomes and microbodies it is necessary to prove the presence of specific enzymes. Microbody associated metabolic pathways are variable compared with the relative constancy of mitochondrial metabolism, and the activities of microbody enzymes are often duplicated in other cellular compartments. In order to associate them unequivocally with microbodies, enzymes must therefore be isolated from the purified organelle fraction, and shown to be either isoenzymic with or different from enzymes showing the same activity elsewhere in the cell.

The enzymes catalysing the initial steps of alkane oxidation in alkane-utilising yeasts and mammalian liver cells are enriched in the microsomal fraction. Alkane-induced cytochrome P-450 and alkane hydroxylase are involved in the oxidation of alkanes to the corresponding primary fatty alcohols and alcohols are further oxidised to fatty acids via aldehyde by NAD^+-linked long chain alcohol dehydrogenase and aldehyde dehydrogenase (Tolbert, 1981; Tanaka *et al.*, 1982; Mauersberger *et al.* 1987). Kawamoto *et al.*, (1977b) could not detect either cytochrome P-450 or NADPH-cytochrome c reductase, the components involved in the n-alkane hydroxylation system of *C. tropicalis* in the microbody fraction. Mauersberger *et al.*, (1987) have suggested that the most likely site of localisation of cytochrome P-450 in *C. maltosa* is the ER, but report that the enzymes catalysing the next steps of terminal alkane oxidation show a distinct subcellular distribution. Microsomes of anaerobically grown *S. cerevisiae*, a yeast which does not oxidise alkanes, contain cytochrome P-450 the function of which, with regard to alkane oxidation, is unknown (Rehm and Reiff, 1981). Yamada *et al.* (1980) reported that the initial oxidation system for alkanes, alkane hydroxylation composed of cytochrome P-450 and NADPH-cytochrome c reductase, is localised mainly in the microsomal fraction, and is absent in the peroxisomes of alkane-grown *C. tropicalis* pK233.

The results above suggest that alkane hydroxylation takes place in the ER, the resulting alcohols being oxidised to fatty acids in microsomes. These fatty acids are activated *in situ* to the corresponding CoA esters by acyl-CoA synthetase located in the peroxisomal membrane (Kawamoto *et al.*, 1978; Mannaerts *et al.*, 1982; Tanaka *et al.*, 1982).

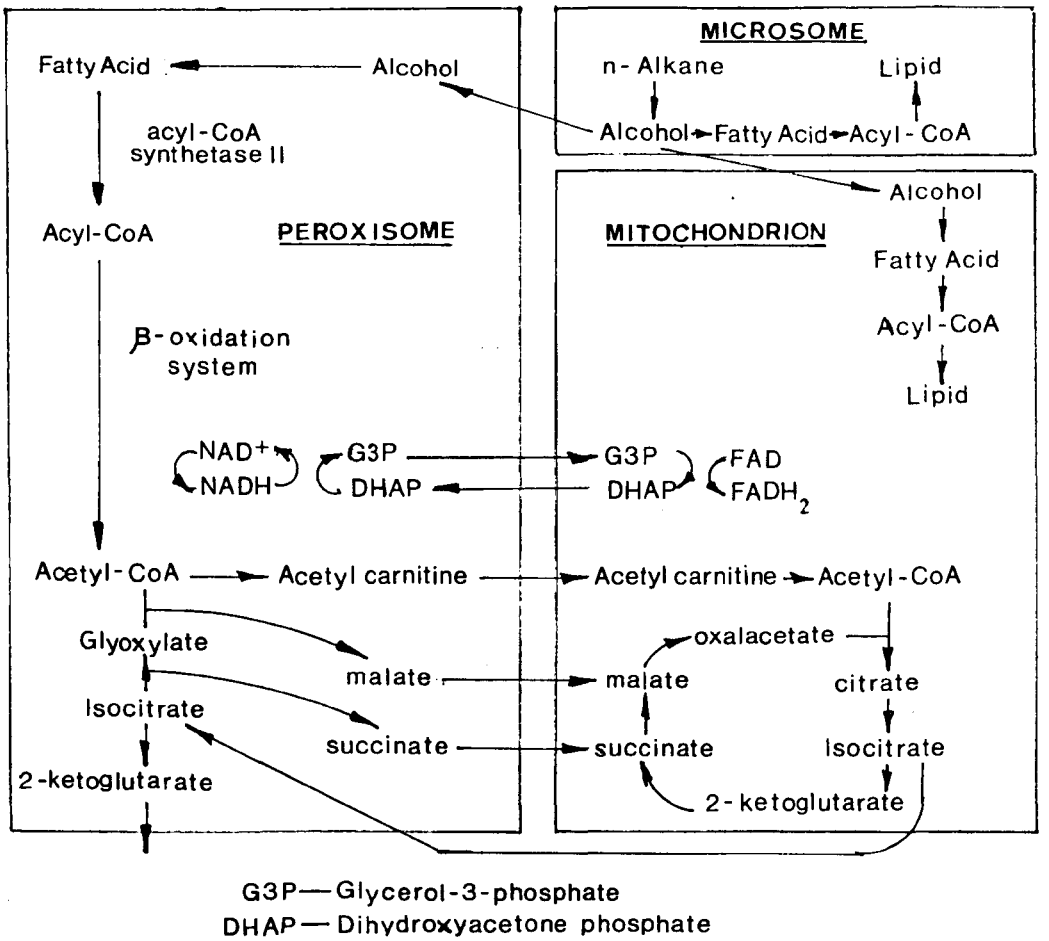


***Figure* 1.1 The interactions between peroxisomes, microbodies and mitochondria (after: Kawamoto *et al.*, 1978; Fukui and Tanaka, 1981; Tanaka *et al.*, 1982; Ueda *et al.*, 1984)**

The glyoxylate cycle plays an important role in the biosynthesis of cellular components by the production of one C_4-compound such as malate or succinate. Two molecules of acetyl-CoA, formed by β-oxidation, are condensed by the action of malate synthase with glyoxylate, which is derived from isocitrate by isocitrate lyase reaction. Malate and succinate are transported to mitochondria to undergo further conversion (Kawamoto *et al.*, 1978; Bühler and Schindler, 1984). Acetyl-CoA can also be processed by the TCA cycle which produces carbon dioxide and reducing power linked to a respiratory system to yield energy. Results suggest that in alkane utilising yeasts the glyoxylate cycle is more active than the TCA cycle (Bühler and Schindler, 1984).

Various workers have shown that the peroxisomes, but not other microbody fractions or mitochondria, of *C. tropicalis* contain various fatty acid β-oxidation enzymes including catalase (Tolbert, 1981; Fujiki *et al.*, 1986; Okada *et al.*, 1986), palmitoyl-CoA oxidase (Tolbert, 1981), carnitine acetyltransferase (Kawamoto *et al.*, 1977b; Tolbert, 1981; Okada *et al.*, 1986), D-amino acid oxidase (Kawamoto *et al.*, 1977b), $NADP^+$-linked isocitrate dehydrogenase (Kawamoto *et al.*, 1977b), trifunctional hydratase-dehydrogenase-epimerase (De La Garza *et al.*, 1985; Ueda *et al.*, 1985; Fujiki *et al.*, 1986), acyl-CoA oxidase (Ueda *et al.*, 1985; Fujiki *et al.*, 1986), 3-ketoacyl-CoA thiolase (Ueda *et al.*, 1985), and acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase (Kurihara, *et al.*, 1989). Fukui and Tanaka, 1981 discuss enzymes essential for fatty acid metabolism in peroxisomes of both *C. tropicalis* and *C. lipolytica* . Okazaki *et al.* (1987) detected more than 18 different kinds of PXPs from purified peroxisomes of *C. tropicalis*. 2,3-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase are also present in the peroxisomes of *C. tropicalis* enabling this yeast to degrade saturated as well as unsaturated fatty acids (Dommes *et al.*, 1983).

Other workers have demonstrated that specific enzyme activities of fatty acid β-oxidation are located solely in the peroxisomes of *C. tropicalis* for example, catalase activity (Osumi *et al.*, 1974), the reduction of NAD^+, the formation of acetyl-CoA and hydrogen peroxide, and the consumption of oxygen depending on fatty acyl-CoA in the presence or absence of sodium azide, an inhibitor of catalase (Ueda *et al.*, 1985), and palmitate-dependent activities of NAD^+ reduction, acetyl-CoA formation and oxygen consumption (Kawamoto *et al.*, 1978).

The results above suggest that in *C. tropicalis* cells fatty acids derived from alkanes are degraded to acetyl-CoA by β-oxidation exclusively in the peroxisomes.

In contrast the mammalian peroxisomal system plays a distinct role in degrading long-chain fatty acids to middle-chain fatty acids, and the mitochondrial system in degrading middle-chain fatty acids, transported from peroxisomes, to acetyl-CoA (Ueda *et al.*, 1985), although the β-oxidation pathways of mitochondria and peroxisomes both form acetyl-CoA (Tolbert, 1981). The same intermediates are formed during β-oxidation in both the peroxisomal and mitochondrial pathway, but the enzymes are different, and some, for example, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, have been demonstrated to be immunochemically distinguishable (Ueda *et al.*, 1985). Microbody enzymes have pH optima around 8.5, higher than in other subcellular compartments. Mitochondrial β-oxidation *in vitro* is increased by added albumin, whereas the converse is true for peroxisomal β-oxidation (Tolbert, 1981). The initial step of β-oxidation in peroxisomes is mediated by acyl-CoA oxidase and that in mitochondria by acyl-CoA dehydrogenase. The latter enzyme has not been detected in any subcellular fraction from *C. tropicalis* (Ueda *et al.*, 1985), which is further evidence that there is no mitochondrial β-oxidation pathway in this yeast.

Catalase and urate oxidase, together with low activities of D-amino acid oxidase and L-α-hydroxyacid oxidase (glycollate oxidase), and increased acyl-CoA oxidase and catalase A activity were associated with peroxisomes isolated from derepressed *S. cerevisiae* cells. Peroxisomes were destroyed as the cells became fermentative during sphaeroplast preparation. Fatty acids are not utilised by *S. cerevisiae*, so the peroxisomal β-oxidation of fatty acids may be a detoxification pathway in this yeast (Parish, 1975; Veenhuis *et al.*, 1987; Skoneczny *et al.*, 1988).

Isocitrate lyase (Kawamoto *et al.*, 1977b) and malate synthase (Kawamoto *et al.*, 1977b; Kawamoto *et al.*, 1978; Okada *et al.*, 1986), the key enzymes of the glyoxylate cycle, are also present in the peroxisomes but not the mitochondria of *C. tropicalis*. The mitochondria possess the tricarboxylic acid cycle enzymes and carnitine acetyltransferase (Okada *et al.*, 1986). The glyoxylate cycle, which supplies C_4-compounds for gluconeogenesis, is thought to function between the peroxisomes and mitochondria, the transport of acetyl-CoA formed in yeast peroxisomes being via an "acetylcarnitine shuttle" composed of peroxisomal and mitochondrial carnitine acetyltransferases (Kawamoto *et al.*, 1978; Ueda *et al.*, 1985; Okada *et al.*, 1986). The interactions between the microbodies, peroxisomes and mitochondria in *C. tropicalis* are shown schematically in *figure* 1.1.

During peroxisomal β-oxidation energy released from fatty acyl-CoA oxidase, which would be conserved in ATP synthesis if the oxidation occurred during mitochondrial respiration, is lost as heat (Tolbert, 1981). Peroxisomes of *C. tropicalis* and other n-alkane-utilising yeasts provide a pathway not limited by electron transport for production of acetyl-CoA, and reducing equivalents therefore play indispensable roles in the degradation of the carbon skeleton of alkanes and in supplying TCA cycle intermediates for the syntheses of cellular constituents, such as carbohydrates and amino acids.

1.2 INDUSTRIAL APPLICATIONS OF *CANDIDA TROPICALIS*

1.2.1 Production of Single-Cell Protein and other Products from Industrial Waste

Bacteria, yeasts and fungi have been employed in foods and drinks for millenia, yielding secondary metabolites such as flavouring agents (the aromas of butter and cheese), desirable metabolites (E and glutamic acid), preservatives (acetic, lactic and other acids in pickles and sauerkraut) or carbon dioxide produced by a microbial leavening agent. *Candida* yeasts have been used extensively for the production of single-cell protein (SCP) and also various useful cellular components or secondary metabolites (Teranishi *et al.*, 1974a).

There has been a lot of research into the production of SCP from yeasts and fungal hyphae. SCP is the biomass comprising the microorganism itself, and may be employed as a protein source in animal feedstuffs or in human foods. SCP products can replace meat, milk, casein, egg white and wheat gluten (Anderson and Cuthbertson, 1987).

C. tropicalis, *C. utilis*, *Chaetomium cellulolyticum*, and *Paecilomyces varioti* have been used to produce SCP from wood-pulping sulphite waste liquors. This substrate has been extensively used because of consistent availability of large quantities, low investment and production costs, and availability of organisms capable of high growth rates on the substrate (Crueger and Crueger, 1984; Vasey and Powell, 1984; Sturley and Young, 1986; Anderson and Cuthbertson, 1987).

C. utilis and *S. cerevisiae* have been used for SCP production from molasses. Four types of molasses are available: cane, beet, corn, and citrus. However, because of its commodity status molasses may often reach a value in excess of that suitable for an SCP substrate, and it may require pre-treatment to reduce mineral content and remove suspended solids before use (Vasey and Powell, 1984).

C. tropicalis, *C. oleophila*, and *Saccharomycopsis lipolytica* have been used in the production of SCP from n-alkanes in diesel oil. This is a fraction derived from crude oil containing 10-25% C_{15}-C_{30} alkanes. A disadvantage of n-alkanes is that they are not easily soluble (Crueger and Crueger, 1984; Vasey and Powell, 1984; Anderson and Cuthbertson, 1987).

The use in feeding stuffs of protein products obtained from *Candida* yeasts cultivated on n-alkanes has been prohibited in the European Community for several reasons: Some *Candida* yeasts are opportunist pathogens, and because it is not possible to completely eliminate contamination of the environment or the product these organisms are banned; high numbers of odd-number carbon atom fatty acid residues which have been reported to affect neurobehavioural parameters in rat offspring, are found in tissues of animals given diets based on *Candida* yeasts from n-alkane fermentation; and an adverse effect when poultry were fed *Candida* yeasts grown on n-alkanes resulting from a methionine deficiency in the yeast protein has also been reported (Vasey and Powell, 1984; Anderson and Cuthbertson, 1987). *C. lipolytica* has been used to produce biomass with a low odd-chain fatty acid content (Fukui and Tanaka, 1980).

C. tropicalis, *Aspergillus niger*, and an unidentified filamentous fungus grown in continuous culture have been used to breakdown *Pinus radiata* bark, a major waste product of the New Zealand forestry industry, for use as a horticultural compost or animal feed. Conifer bark is rich in polyphenolic materials which inhibit microbial growth by binding to proteins or polysaccharides. The ability of *C. tropicalis* to degrade tannin molecules and to significantly reduce the dark colouration of the extract may be of value in the treatment of similar tannin based industrial effluents (Broderick and Sinclair, 1984).

Many *Candida* species can utilise D-xylose for growth, and as xylose is one of the major constituents of renewable biomass, they can play a significant role in its efficient utilisation. *C. tropicalis* which ferments D-xylose to E, produces this alcohol from crude hardwood hemicellulose hydrolysate in batch culture, with variable amounts of xylitol as a by-product. *C. tropicalis* also has the ability to metabolise phenolic compounds (Fein *et al.*, 1984).

C. tropicalis has been used to ferment cane juice and molasses to E both at 30°C and 40°C. Industrial E fermentations by yeasts are usually carried out at 25-35°C since temperatures close to 40°C have adverse effects. In tropical and sub-tropical regions the cost of cooling systems may be restrictive and the use of thermotolerant strains in these situations could be advantageous (Kar and Viswanathan, 1985).

C. tropicalis pK233 has been used to produce extracellular glutathione, a medicine for hepatic diseases and a potent and natural reductant in biological research, during growth in filamentous form caused by adding E. The industrial production of glutathione is currently either by chemical synthesis which is inefficient, or by extraction from yeast cells. *C. tropicalis* pK233 also produces extracellular polysaccharides and proteins under the same cultural conditions (Yamada *et al.*, 1984).

1.2.2 n-Alkanes and Peroxisomal Enzymes

Petroleum is a highly important and non-renewable feedstock for chemistry. Most research into the biotransformation of this substrate has been done with n-alkanes with a chain length of 9-18 carbon atoms because of their better degradability compared to lower n-alkanes. This behaviour has been ascribed to the higher toxicity of the short chain n-alkanes due to their better solubility in aqueous solutions.

n-Alkanes have been utilised as carbon sources for the production of a variety of useful products which can be classified into three groups: products of conventional microbial processes using carbohydrate substrates, cell mass, carbohydrates, amino acids, nucleic acids, antibiotics, several kinds of enzymes, organic acids and vitamins; products related to physiological and metabolic features of alkane assimilation, acetyl-CoA, isoprenoids such as carotenoids, xanthophylls, steroids and coenzyme Q; and products specific to alkane substrates, dicarboxylic acids and peroxisomal enzymes specifically induced by alkanes.

Intracellular enzymes are used for industrial, analytical and medical purposes. The peroxisomes of alkane-grown yeasts contain various enzymes, including β-oxidation enzymes, which have practical uses. *C. tropicalis* has been used to produce acyl-CoA synthetase and acyl-CoA oxidase, useful enzymes for the diagnostic analysis of nonesterified fatty acids in serum (Tanaka *et al.*, 1982). *C. tropicalis* and *C. lipolytica* have been used to produce catalase which is used to remove hydrogen peroxide employed as a food processing agent (Fukui and Tanaka, 1980; Tanaka *et al.*, 1982); D-amino acid oxidase which is used for the determination and degradation of D-amino acids, testing the optical purity of L-amino acids, and the resolution of racemic amino acids (Kawamoto *et al.*, 1977a; Fukui and Tanaka, 1980; Tanaka *et al.*, 1982); and uricase (urate oxidase), which is used for the analytical determination of serum or urinary uric acid (Tanaka *et al.*, 1977; Fukui and Tanaka, 1980; Tanaka *et al.*, 1982).

n-Alkane-grown yeast have also been used for the production of a variety of other products such as L-tryptophan, coenzyme Q, and vitamin B_2 from *C. tropicalis*; ergosterol from *C. tropicalis* and *C. petrophilum*; α-ketoglutarate, citrate and isocitrate from *C. lipolytica*, *C. zeylanoides*, *C. hitachinica*, and *C. citrica*; fumarate from *C. hydrocarbofumarica* and *C. utilis*; malate from *C. hydrocarbofumarica* and *C. brumptii*; succinate from *C. brumptii*; anglyceric acid from *C. tenuis*; polyols from *C. lipolytica* and *C. zeylanoides*; vitamin B_6 from *C. albicans* and *C. guilliermondii*; cytochrome c from *C. albicans* and *C. lipolytica*; and an emulsifying factor from *C. petrophilum* (Fukui and Tanaka, 1980).

1.2.3 Industrial Importance of Dioic acids

Long-chain dioic acids can be produced by microbial transformation of n-alkanes and fatty acids. For example, tetradecane 1,14-dicarboxylic acid has been produced from n-tetradecane by *C. cloacae* using acetic acid as carbon source for growth (Uchio and Shiio, 1972); and other long-chain dicarboxylic acids have been produced by *C. tropicalis* OH23, *C. rugosa*, *Torulopsis candida*, and *Pichia* species (Fukui and Tanaka, 1980).

Dicarboxylic acids with a carbon chain length of $n>10$ are of interest as chemical intermediates. For example, Dodecanedioic acid ($n = 12$) and brassylic acid ($n = 13$) are manufactured on an industrial scale (Hill *et al.*, 1986). Dioic acids are basic substrates for the synthesis of perfumes, plastisisers, lubricants, polyurethanes, and polyamides (Bühler and Schindler, 1984).

1.3 RECOMBINANT DNA TECHNOLOGY AS A TOOL FOR THE GENETIC MANIPULATION OF INDUSTRIAL YEAST STRAINS

1.3.1 Genetic manipulation of Industrial Yeast strains

The use of *S. cerevisiae* as a basic research tool in genetics has led to deliberate selection of heterothallic haploid cultures which mate and produce diploids, sporulate readily and give high spore survival. Industrial *Saccharomyces* yeast strains are often polyploid or aneuploid and, as a consequence, like *Candida* strains do not possess a mating type and have a low degree of sporulation and poor spore viability, rendering normal genetic analysis and standard breeding procedures, which are widely used in other species extremely difficult.

Industrial strains of *Saccharomyces* yeast have been selected for consistently good performance, and strain improvement has been primarily achieved through direct selection for desirable characteristics. Empirical selection has not employed deliberate or systematic breeding programs, and it is this process of selection within the asexual stage which has resulted in sterile strains. Mutagenesis has not been widely applied because of the difficulties encountered with mutagenic treatment of polyploid yeasts. However, some laboratories have had success with mutagenic treatment of brewing strains (Stewart, 1981; Rank *et al.*, 1988).

The types of genetic improvement possible in industrial yeasts vary with the product and the production facility. Some general objectives include increasing process efficiency or productivity, lowering the cost of or increasing the diversity of raw materials, developing new products, proprietary marking of strains, improving product quality and/or quality control and adding value to surplus yeast (Stewart, 1981; Rank *et al.*, 1988).

The technique of rare mating can overcome natural barriers to the crossing of strains. Two cell lines, one auxotrophic and respiratory sufficient and the other prototrophic and respiratory deficient are mixed, and rare mating events selected by plating the mixture onto minimal medium (the auxotrophic parent cannot grow) containing E or glycerol as sole source of carbon for growth (the respiratory deficient parent cannot grow). The hybrids are both prototrophic and respiratory sufficient (Young, 1986).

The techniques of sphaeroplast fusion and transformation have a total disregard for ploidy and mating type and in consequence have great applicability to industrial yeast strains because of their polyploid nature and absence of mating type characteristic.

Sphaeroplast fusion provides a means of producing hybrids from imperfect strains. The partial digestion of the cell wall with "helicase" or zymolyase in the presence of an osmotic stabiliser such as sorbitol or mannitol produces sphaeroplasts. In the presence of polyethylene glycol sphaeroplasts fuse and fusion products can be induced to regenerate cell walls if embedded in agar. Haploid *S. cerevisiae* strains have been fused with *S. cerevisiae* (ale) and *S. carlsbergensis* (lager) strains. An auxotrophic haploid strain of *K. lactis* was fused with a laboratory strain of *Saccharomyces* but these fusion products were unstable and reverted to their constituent fusion partners (Stewart, 1981; Young, 1986).

The products of sphaeroplast fusion and rare mating combine both desirable and undesirable characters of the parents. Problems may also be encountered in terms of progeny stability since there is no control over the number of cells involved in the fusion process. Fusion is not specific enough to genetically modify yeast strains in a controllable fashion, and it would be difficult to introduce a single trait into a yeast strain by this method (Stewart, 1981; Young, 1986).

Genetic engineering has allowed the development of laboratory strains of the yeast *S. cerevisiae* as host organisms for the production of high value proteins such as growth hormones, immune modulators and vaccines, and has become a practical proposition. Most of the recombinant DNA technology developed with haploid laboratory strains of *S. cerevisiae* can be used in commercial *Saccharomyces* strains (Stewart, 1981; Yocum, 1986; Rank *et al.*, 1988).

The genetic analysis of non-mating, non-sporulating polyploid yeast by "conventional" genetic techniques such as rare-mating, selection, and exposure to mutagens has only limited potential to create new strains with a markedly different genetic character because they can only modify or abolish existing genetic traits or rearrange a limited pool of genetic variation by intraspecific hybridization. Industrial yeast are more amenable to recombinant DNA technology than "classical" genetic techniques.

1.3.2 Yeasts as Host Organisms

The most widely developed systems for expression of genetically engineered proteins have used prokaryotes as host organisms. *E. coli* systems pose several problems including degradation of the protein, contamination with endotoxin and the need to disrupt the cells to recover the protein. Intracellular, "over-produced" proteins may accumulate in insoluble aggregates called inclusion bodies which must be solubilised with denaturants and the protein fractionated and renatured to regain biological activity. *Bacillus subtilis* secretes proteins and has fewer toxin problems. However, instability of transformed strains, and degradation of secreted foreign proteins has caused problems (Davidow *et al.*, 1985).

As alternative hosts, yeasts are eukaryotic cells amenable to biochemical and genetic analyses, both of the traditional sort and of the kind involving recombinant DNA methodology. Most are non-toxic, can be grown to very high densities and some species can secrete proteins (Barnes *et al.*, 1982; Davidow *et al.*, 1985). Compared with other major groups of microorganisms (algae, bacteria and protozoa) the yeasts are represented by comparatively few genera and species. The two main yeast genera of commercial interest are *Saccharomyces* and *Candida*. *Candida* yeasts are asexual, stable diploids and are in many ways analogous to industrial *Saccharomyces* yeast strains which are polyploid or aneuploid, lack a mating type, and sporulate poorly. All of these factors confer phenotypic stability on industrial strains, making them less susceptible to mutation and recombination, and enabling them to be used routinely with a high degree of confidence (Stewart, 1981).

1.4 MOLECULAR BIOLOGY OF YEASTS

1.4.1 Molecular Biology of *Candida tropicalis*

At present, the molecular biology of *Candida* species has not been developed to the same extent as the molecular biology of *S. cerevisiae* or of *E. coli*, and, because *C. tropicalis* is asexual, diploid and does not have a system for DNA-mediated transformation, genetic analysis is difficult.

Limited genetic analysis of *Candida* species, including *C. tropicalis*, has been performed using complex protocols with sphaeroplast fusions as described in section 1.3.1. For example, the technique has been used to demonstrate complementation among isoleucine-valine, methionine, and arginine mutants from two different *C. albicans* isolates (Kakar and Magee, 1982), killer plasmids pGKL1 and pGKL2 of double-stranded linear DNA were able to propagate in *C. pseudotropicalis* after transfer from *K. lactis* via sphaeroplast fusion (Sugisaki, *et al.*, 1985), and sphaeroplasts from auxotrophic mutants of *C. tropicalis* and *C. albicans* have been fused and, nutritionally complemented interspecific hybrids obtained by selective regeneration on minimal media which contained one nucleus, and had a DNA content per cell that was higher than in the parents. The hybrids were partial alloploids containing the total chromosomal set of either of the parental species and one or a few chromosomes of the other (Kucsera and Ferenczy, 1986).

Genetic analysis requires three capabilities: mutagenesis, complementation, and recombination. Mutagenesis is difficult to achieve effectively in *C. tropicalis* because of its stable diploid nature, and sphaeroplast fusion can be used to investigate complementation between independently isolated mutants as discussed above. Recombination analysis can be performed in the tetraploid or aneuploid products of sphaeroplast fusion by exposing the yeasts to UV-irradiation which induces mitotic crossing-over allowing linkage analysis of genes located on the same chromosome arm and their positioning relative to each other and relative to the centromere. Briefly heat shocking tetraploid yeasts in logarithmic growth induces chromosome loss, which results in the expression of auxotrophic alleles that were carried heterozygously in the original tetraploid. This system permits linkage analysis of whole chromosomes and reveals *trans* centromeric linkage (Shepherd et al., 1985).

1.4.2 Yeast Transformation

1.4.2.1 Transformation of *Saccharomyces cerevisiae*

Transformation is the process by which naked DNA from one organism is introduced into a recipient cell and incorporated as part of the inheritable genetic material. Hinnen *et al.*, (1978) first transformed *S. cerevisiae* with plasmid pYE*leu10*, which was maintained in the cell by integration into the chromosome, and Beggs, (1978) transformed *S. cerevisiae* with a 2-μm based chimeric plasmid which was capable of self-maintenance. These transformation procedures rely upon enzymic removal of the cell wall, as in sphaeroplast fusion, and treatment of the sphaeroplasts with 10 mM $CaCl_2$ and PEG 4000 to induce the uptake of DNA.

More recently it has been shown that intact *S. cerevisiae* cells can be induced to take up plasmid DNA. Iimura *et al.* (1983) transformed a *trp1* strain by incubating intact cells with plasmid DNA carrying the *TRP1* gene in chilled 200 mM $CaCl_2$ solution and then raising the incubation temperature to 37°C. PEG and the age of the cells showed little effect on the transformation. Ito *et al.* transformed intact cells with plasmid YRp7 by treating them with alkali cations Li^+, Cs^+, Rb^+, K^+, and Na^+ (1983a), or 2-mercaptoethanol (1983b). In both cases PEG was absolutely required. Klebe *et al.* (1983) transformed intact cells of *S. cerevisiae* with plasmid YEp13 by a 1 hour PEG treatment. No obvious divalent cation requirement was observed during yeast transformation. This method is also applicable to *E. coli*.

The transformation efficiencies of the methods described above are, on the whole, comparable to those of conventional sphaeroplast methods. The effect of PEG can probably be attributed to changes in membrane charges caused by interactions among negatively charged PEG, monovalent cations, and the yeast cell surfaces. Such changes may induce both conformational changes and aggregation of cells (Ito *et al.*, 1983a). All these methods have been found to be applicable to both laboratory and industrial strains of *S. cerevisiae*.

1.4.2.2 Advantages of Transforming Intact cells over Sphaeroplasts

Transformation of intact cells has several advantages over transformation of sphaeroplasts: it is simple and easy; it is applicable to cells resistant to lytic enzymes; drug-resistant transformants can be isolated (because sphaeroplasts are fragile and particularly sensitive to drugs, it is difficult to recover transformants by selecting for drug-resistance); as transformants can be spread on the surface of agar plates, it is very easy to isolate or replica-plate them; colonies grow rapidly (the preparation of sphaeroplasts and subsequent regeneration of cell walls in a solid medium is tedious and time consuming); transformants can sometimes be selected on the basis of the size of the colonies formed on agar surfaces (this is not applicable to sphaeroplasts, as regenerated colonies are variable in size and shape, depending on how deep they are embedded in the agar); the method is applicable to any strain regardless of its genetic markers (Sakai and Yamamoto (1986) reported that the sphaeroplast method of transformation is not suitable for two strains of *S. carlsbergensis*); the ploidy of the transformants does not change during the transformation (the fusion of sphaeroplasts occurs very easily, so that transformants are sometimes polyploid, which is not desirable); sphaeroplasts often show a low regeneration efficiency; competent cells treated with lithium compounds can be preserved longer than sphaeroplasts (Kimura, 1986; Sakai and Yamamoto, 1986).

Transformation, particularly with the aid of recombinant DNA techniques, is a more subtle method for introducing single genetic traits into industrial yeast strains. However, before such manipulations can be realistically undertaken, detailed biochemical knowledge of the system to be incorporated or modified must be available. It is undesirable to meddle with established strains embodying numerous desirable characteristics. Therefore, for the introduction of completely novel characteristics or the modification of the function of a small number of genetic elements, recombinant DNA technology offers industry a very powerful tool. Genetic change is brought about using specific cloned sequences of DNA. Therefore, in principle, all the desirable attributes of the parental strain are retained and the alteration of proven strains is minimal (Knowles and Tubb, 1986; Rank *et al.*, 1988).

1.4.2.3 Transformation of Novel Yeasts and filamentous Fungi

The ability to transform wild-type yeasts provides a means of introducing discrete, predetermined genetic material without significant disruption of the yeast genome. A wide range of organisms can be considered as sources of additional genetic information, providing a large genetic 'pool' of great diversity for strain development. New characteristics can be added and undesirable ones specifically modified or eliminated.

Based on the transformation systems developed for *S. cerevisiae*, several workers have achieved the transformation of other species of yeast and filamentous fungi. Sphaeroplasts of the following species have been successfully transformed *Neurospora crassa* (Case *et al.*, 1979), *Schizosaccharomyces pombe* (Beach and Nurse, 1981; Fournier *et al.*, 1982), *Kluyveromyces lactis* (Das and Hollenberg, 1982), *Ustilago maydis* (Banks, 1983), *Aspergillus nidulans* (Tilburn *et al.*, 1983), *Yarrowia lipolytica* (Gaillardin *et al.*, 1985), and *Acremonium chrysogenum* (Isogai *et al.*, 1987). The alkali cation method has been used to successfully transform *Kluyveromyces fragilis* (Das *et al.*, 1984), and *Yarrowia lipolytica* (Davidow *et al.*, 1985; Gaillardin *et al.*, 1985).

There have been several reports of the successful transformation of various *Candida* species, the earliest of which pre-date the first reported transformations of *S. cerevisiae* by Hinnen *et al.* and Beggs (1978). A *C. albicans* transformant, was obtained in the presence of a preparation containing nucleic acids and proteins of *Mycobacterium tuberculosis* (Kwapinski, 1968), and more recently *C. albicans* sphaeroplasts were transformed by the method of Beggs (Kurtz *et al.*, 1986). *C. pseudotropicalis* lactose-negative cells were transformed to lactose-positive cells by mixing DNA from prototrophic cells in media with auxotrophic cells (Frye *et al.*, 1973). *C. utilis* has been transformed successfully to G418-resistance by both sphaeroplast (Ho *et al.*, 1984), and alkaline cation methods (Zhang and Reddy, 1986). *C. maltosa* has been transformed successfully by both sphaeroplast (Takagi *et al.*, 1986a) and alkaline cation methods (Takagi *et al.*, 1986a; Kawai *et al.*, 1987). To date there have been no reports of the successful transformation of *C. tropicalis*.

1.4.3 Yeast Vectors

1.4.3.1 Vectors for the Transformation of *S. cerevisiae*

A desirable vector should have as many as possible of the following attributes: good transformation efficiency; stable inheritance of transferred genes; suitable selectable markers; good expression of plasmid encoded genes; and usefulness in a wide range of industrial strains. Five classes of vectors have been constructed for gene transfer in *S. cerevisiae* strains.

YIp (yeast integrating plasmid) vectors do not contain an origin of replication and integrate into the genomic DNA of the host strain by reciprocal recombination within an area of genetic homology. Although the frequency of transformation is low (1-10 transformants μg^{-1} DNA), integration is targeted and enhanced by linearising the vector at a restriction endonuclease site within the plasmid integrating gene prior to transformation. The location of the double-stranded cut generated determines where the plasmid integrates, because of the recombinogenic nature of free DNA termini. In all cases, the integrated structure has a direct duplication of the integrated gene sequence which flanks other plasmid borne markers thus including any non-yeast sequences on the original vector. Transformants obtained by integration are very stable. The segregation rate is usually much less than 1% per generation when grown in non-selective media. Using this type of plasmid, only one or two copies of the gene of interest integrate into the chromosome, although multiple integration events sometimes occur, particularly within the repeated DNA sequences (Struhl *et al.*, 1979; Fleming, 1988; Rank *et al.*, 1988).

YEp (yeast episomal plasmid) vectors contain all or part of the yeast 2-μm plasmid DNA. YEp plasmids transform yeast very efficiently (10^3-10^4 transformants μg^{-1} DNA). However, under non-selective growth conditions these plasmids are segregated from the cell at high frequency, showing approximately 1% loss per generation. The copy number of these plasmids after transformation is high, ranging from 25-200 copies per cell. YEp plasmids can be made to integrate in [*cir*^{0}] strains which have been cured of native 2-μm DNA (Fleming, 1988; Rank *et al.*, 1988).

YRp (yeast replicating plasmid) vectors contain *S. cerevisiae* chromosomal autonomously replicating sequences (ARS) and transform yeast very efficiently (10^3-10^4 transformants μg^{-1} DNA). However, YRp transformants are unstable, with a segregation rate in non-selective media of 1-10% per generation and of up to 1% under selective conditions. The copy number of these plasmids after transformation is low and variable, ranging from 3-100 copies per cell. YRp plasmids also integrate at low frequency (Fleming, 1988; Rank *et al.*, 1988).

The instability of YRp vectors can be rectified by combining ARS elements with yeast centromeric sequences (*CEN*) to form YCp (yeast centromeric plasmid) vectors. YCp plasmids transform yeast with similar efficiency to YRp plasmids, and are stably maintained during mitosis, segregating at less than 1% per generation under non-selective conditions. They are present as a single-copy per cell (Fleming, 1988; Rank *et al.*, 1988).

YLp (yeast linear plasmid) vectors have been constructed using telomeres from *Tetrahymena*. YLp plasmids transform yeast at high frequency, and those containing *CEN* sequences are very stable with a segregation rate of 0.006-0.015% per generation under non-selective conditions. These plasmids are maintained as linear extra-chromosomal elements with a copy number of 20-60 copies per cell (Fleming, 1988; Rank *et al.*, 1988).

1.4.3.2 ARS and Signal Sequences on Yeast Vectors

ARS are necessary to support autonomous replication of recombinant plasmids, and there are many examples of isolated sequences which act as ARS in heterologous hosts, for example, a *C. maltosa* DNA segment in *S. cerevisiae* (Kawamura *et al.*, 1983), DNA sequences of a mitochondrial origin of *C. utilis* in *S. cerevisiae* (Tikhomirova *et al.*, 1983), an *Aspergillus nidulans* mitochondrial fragment in *S. cerevisiae* (Tilburn *et al.*, 1983), *KARS2* of *K. lactis* in *K. fragilis*, *K. lactis* and *S. cerevisiae* (Das *et al.*, 1984), ARS-like sequences of *Yarrowia lipolytica* in *S. cerevisiae* (Gaillardin *et al.*, 1985), *C. albicans* replicator regions in *S. cerevisiae* (Gorman and Koltin, 1986), and the TRA region of *C. maltosa* in *S. cerevisiae* (Takagi *et al.*, 1986b). However, with the exceptions of the TRA region of *C. maltosa*, and *KARS2* of *K. lactis* none of these sequences promote the autonomous replication of plasmids in their species of origin, and this is probably not a good strategy for isolating ARS for use in *C. tropicalis* vectors.

Gene expression in yeast requires that in front of the gene there is a regulatory DNA sequence, "the promoter" which is recognised by yeast RNA polymerase as a "start" signal for messenger RNA (mRNA) synthesis under the appropriate conditions, and a terminator sequence to end mRNA synthesis. With homologous genes the natural promoter is recognised by the transcription machinery as has been demonstrated for the *SMR1.410* allele which confers resistance to the herbicide sulphometuron methyl (Casey *et al.*, 1988), and as it is not necessary to modify these genetic markers for expression they can be used directly for transformant selection.

It has not been established that the same promoter sequences function in different hosts. For example, an isolated *C. maltosa* DNA fragment promotes expression of a *LEU* gene in *S. cerevisiae* but efficient expression in *E. coli* depends on the Tc promoter (Kawamura *et al.*, 1983); the *S. cerevisiae LYS2* gene present on YIp333 is probably not expressed from its own promoter in *Yarrowia lipolytica*, and although the *S. cerevisiae URA3* promoter drives efficient expression of the *E. coli XylE* gene in *S. cerevisiae* no expression is observed in *Yarrowia lipolytica* (Gaillardin *et al.*, 1985); and expression of the *S. cerevisiae URA3* gene in *E. coli* is dependent on a fortuitous promoter sequence which is not present in all strains (Kurtz *et al.*, 1986).

Some foreign (heterologous) genes can be expressed in *S. cerevisiae* without modification, but the efficiency of expression is usually low. It is desirable to obtain efficient gene expression of selectable markers. Heterologous genes may therefore require modification to replace the natural promoters and termination sequences with ones derived from the recipient species in order to produce enough of the appropriate genetic determinant. Heterologous genes have been expressed in *S. cerevisiae* by placing them under the control of various yeast gene promoters.

1.4.3.3 Auxotrophic vs. Dominant Selectable Markers in Diploid and Polyploid Strains

The manipulation of industrial yeast strains by recombination technology can be successfully applied to improve cells for use in a bioreactor for the production of large amounts of metabolites. This requires a reliable and efficient method for transformation of yeast cells, and genetic markers which can be used to identify a successful vector-mediated transformation of the host cells.

The most commonly used markers are prototrophic genes such as the *LEU2* (Hinnen *et al.*, 1978), *HIS3* (Struhl, 1982), *ARG4* (Hsiao and Carbon, 1979), *HIS4* (Hinnen *et al.* 1979) and *URA3* (Struhl *et al.*, 1979) genes from *S. cerevisiae* . These require an auxotrophic mutant as host, the introduction of the hybrid plasmid being detected by complementation of the auxotrophic lesions by the wild-type allele carried on the vector. However, industrial yeast strains are prototrophic and complementation of auxotrophic mutations, by plasmid encoded genes cannot be used to select transformants.

Treatment with mutagens such as N-methyl-N-nitro-N-nitrosoguanidine, EMS, nitrous acid and UV-irradiation increases the proportion of mutants in a population. These mutations are frequently recessive and only expressed in diploid or polyploid strains when the effects of the dominant alleles are lost via mutation, gene conversion or mitotic recombination. Mutation can often cause further rearrangements in the chromosomes, resulting in undesirable changes to the strain and therefore it is difficult to obtain stable recessive mutations without adversely influencing the physiological and fermentation characteristics of the yeast. Therefore it is impractical to introduce auxotrophic mutations into industrial yeast for the purpose of transformation, and in order to select transformants it is preferable to have a dominant gene which confers on them the ability to grow in otherwise inimical conditions.

Transformation of an industrial (brewing) yeast was not achieved until 1985 (Henderson *et al.*, 1985). The lack of suitable genetic markers was the main reason for this delay. The transformation efficiency of yeast is variable and it is difficult to introduce plasmid DNA into some strains, so success often depends on having a good selection system. Several dominant selectable markers have been investigated including genes conferring drug resistance to antibiotics or other toxic substrates, and genes conferring the ability to ferment novel substrates.

Selection of antibiotic-resistant transformants is commonplace in bacterial transformation systems as prokaryotes are sensitive to many antibiotics such as ampicillin, tetracycline, and kanamycin, and plasmid-bourne antibiotic-resistance genes have been widely used as genetic markers for bacterial transformation. Eukaryotes, including yeast and *Candida*, are naturally resistant to the antibiotics commonly used for the selection of bacterial transformants and these selections have been unavailable for yeast due to the lack of a toxic antibiotic and a resistance gene to complement it.

Industrial strains vary considerably in their sensitivity to different agents and it is usually necessary to test several different resistance markers against any particular strain. The medium in which transformation and selection is carried out also has an effect on the level of resistance/sensitivity exhibited by both the transformed and untransformed strain. Ideally a selective agent should block growth of the untransformed cells at relatively low concentrations and the system should be effective over a wide range of concentrations.

Screening for the effects of toxic substances and their corresponding resistance determinants on yeast cells and exploiting the sensitivity of eukaryotic cells to some heavy metal ions has led to the development of several dominant selectable markers for industrial strains of *S. cerevisiae*.

Ernst and Chan (1985) isolated *S. cerevisiae* mutants with supersensitivity to the antibiotic G418, and Danilenko and Barshtein (1986) isolated mutants of *C. tropicalis* D-2 with supersensitivity to several antibiotics. These have some potential as host cells for dominant selection systems.

Transplacement of normal chromosomal genes with *in vitro* generated mutant alleles as discussed below requires that the selectable marker is efficiently expressed in single-copy, and is aided by using an heterologous vector-borne marker that does not contain sequences homologous to the host chromosome as this helps to direct integration to the target genes, in this case the peroxisomal acyl-CoA oxidases of *C. tropicalis*.

Some resistance determinants must be present in multiple copies for maximum efficiency of transformation which limits the use of these markers to situations where they are introduced on multi-copy plasmids, and makes them unsuitable for single-copy integrative transformation unless expression can be improved by placing the resistance gene under the control of a high efficiency promoter.

Hollenberg (1982) demonstrated that bacterial antibiotic resistance genes can be functionally expressed in *S. cerevisiae* permitting the use of these genes as markers on vectors for *S. cerevisiae* transformation, and indeed many of the dominant selectable markers developed for use in industrial yeast strains are encoded by non-yeast (heterologous) DNA, unlike auxotrophic markers which are usually homologous to chromosomal sequences in the recipient strain. These give good phenotypic expression when present in single copy thus embodying the two requirements outlined above. In the case of *C. tropicalis*, dominant markers encoded by *S. cerevisiae* genes are also heterologous.

Optimising transformation by adjusting the media used to select transformants and allowing for gene expression prior to the application of selection pressure can also improve the expression of some unmodified heterologous genes, for example *E. coli* aminoglycoside phosphotransferase genes (Rank *et al.*, 1988).

The expression of heterologous genes in yeast can cause complications as the accumulation of the gene product within the yeast cell can adversely affect growth rate and this can exert a selective pressure against cells with high-copy number plasmids.

1.4.4 Introducing Genes on Plasmids in an Industrial Process

Most recombinant DNA vectors have been developed by taking advantage of auxotrophic mutations which are unavailable and undesirable in industrial strains. There is also a reluctance to introduce non-yeast genes into industrial strains of yeast used for the production of food and beverage products (Rank *et al.*, 1988).

Genes of interest carried on plasmids in an industrial process need to be maintained stably in the host strain. However, unintegrated plasmids segregate at high frequency under non-selective conditions. Selection methods requiring addition of drugs or metals to growth media are efficient for the introduction of desired plasmids, but it is expensive to maintain vectors carrying resistance markers by addition of cellular inhibitors and such selections would seldom be feasible. Stability of these vectors can be improved in some strains if they contain only yeast sequences.

An alternative strategy to maintain plasmids in complex medium makes use of a double mutant host, which selects for cells that maintain the plasmid. For example, a *ura3 fur1* cell is lethal in the presence of uracil (U) since U can not be obtained by the normal *de novo* pathway (*ura3*) or by the salvage pathway (*fur1*). The killer/immunity phenotype also provides an autoselection for cells containing the killer factor. However, these systems do not prevent cells from losing plasmids but select against them, and this continuous segregation of lethal cells may become a serious limiting factor in production efficiency and/or yield (Bussey and Meaden, 1985; Rank *et al.*, 1988).

Integration of plasmids into the genome of the host organism is far more stable under non-selective conditions, and these are the vectors of choice for industrial systems. However, yeast has the ability to copy from one genetic locus to a second homologous locus at the expense of information residing at the second locus. The frequency of this type of gene conversion event is roughly 0.001% per cell per generation. This segregation rate may still be inefficient for large scale industrial applications, especially where cells from one large scale fermentation are reused to inoculate the next fermentation, as there is probably a slight growth advantage for cells that shed the integrated plasmid (Bussey and Meaden, 1985; Yocum, 1986; Rank *et al.*, 1988).

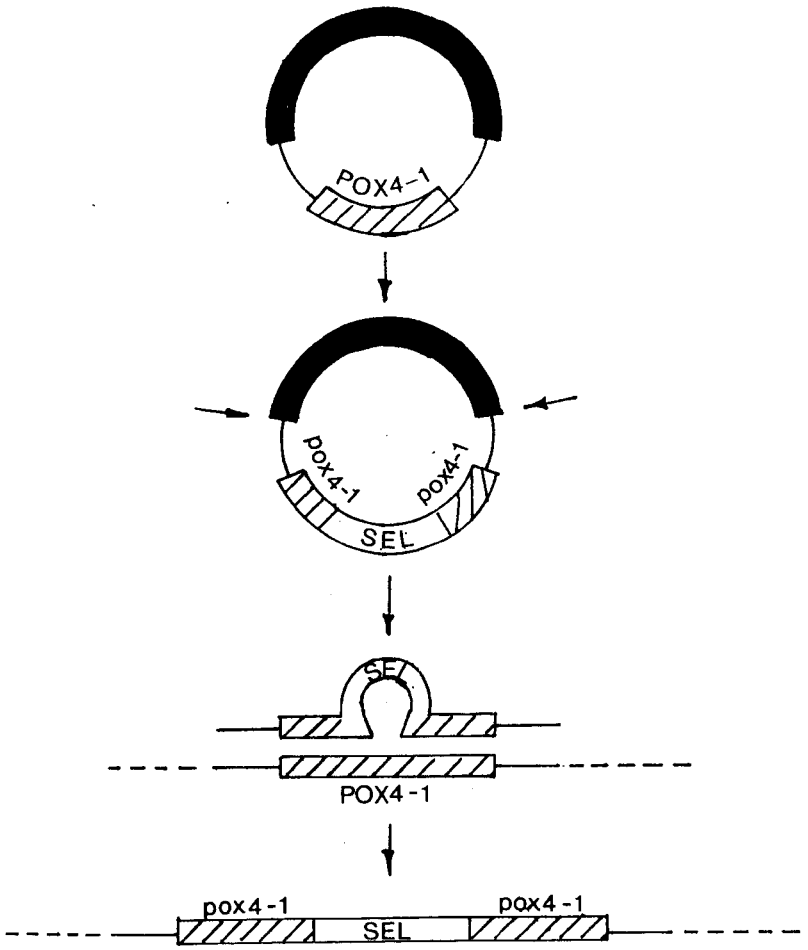


***Figure* 1.2 One step gene disruption (after: Rothstein, 1983)**

A selectable marker (SEL) is cloned into the gene of interest (*POX4-1*), and this disrupted gene is liberated from the plasmid sequences by restriction endonuclease digestion such that homology to the *POX4-1* region remains on both sides of the insert. This linear fragment is transformed into a suitable yeast strain where it undergoes homologous recombination resulting in the substitution of the linear disrupted sequence for the resident chromosomal *POX4-1* sequence.

1.4.5 Gene Disruption and Transplacement

1.4.5.1 Advantages of Introducing Specific, Non-Revertible Genetic Mutations

Some strains of yeast form α,ω-alkanedioic acids as by-products which are excreted into the culture medium when grown on n-alkanes. This can be substantially enhanced by the use of suitable mutants. *C. tropicalis* strains can be subjected to mutation and selection procedures to produce these mutants. For example, *C. tropicalis*, 7/34 is a double negative mutant unable to grow with dodecanedioic acid or n-alkanes (C_{12}-C_{16}). However, the exact genetic blocks of the strain are not known, and the strain uses a part of the substrate to form biomass and products other than long-chain dioic acids. The metabolic block of this mutant is incomplete and it has to be designated as a leaky mutant (Hill *et al.*, 1986). This approach does not produce specific, well-defined changes in these strains.

Methods have been developed which can be used to determine whether a cloned fragment contains a specific gene; to determine whether a cloned gene is essential; and to alter or completely delete a specific region. By *in vitro* manipulation of cloned genes a wide variety of well-defined changes in a DNA sequence can be introduced with precision. Foreign sequences or deletions, constructed *in vitro* can be stably introduced into the chromosomes at the exact location from which the parent sequence was derived. The chromosomal sequences are replaced with the altered sequences unambiguously and at high efficiency. Any isolated gene can be deleted or altered and used to replace the wild-type chromosomal copy.

1.4.5.2 One Step Gene Disruption

In one step gene disruption (Rothstein, 1983; *figure* 1.2) the gene of interest is cloned and digested within its DNA sequence with a restriction enzyme. A second DNA fragment carrying a selectable gene is ligated into the cleaved gene disrupting it.

The *in vitro* disrupted gene is liberated from the bacterial plasmid sequences by restriction enzyme digestion. This linear fragment must contain sequences homologous to the chromosomal gene of interest on both sides of the inserted fragment to direct integration. It is desirable to purify the fragment carrying the disrupted gene if after restriction homologous chromosomal sequences are left on both ends of the vector DNA. The digested DNA is transformed into a suitable yeast strain and transformants are selected, among which are cells that simultaneously lose the function of the gene of interest which has been replaced with the disrupted version.

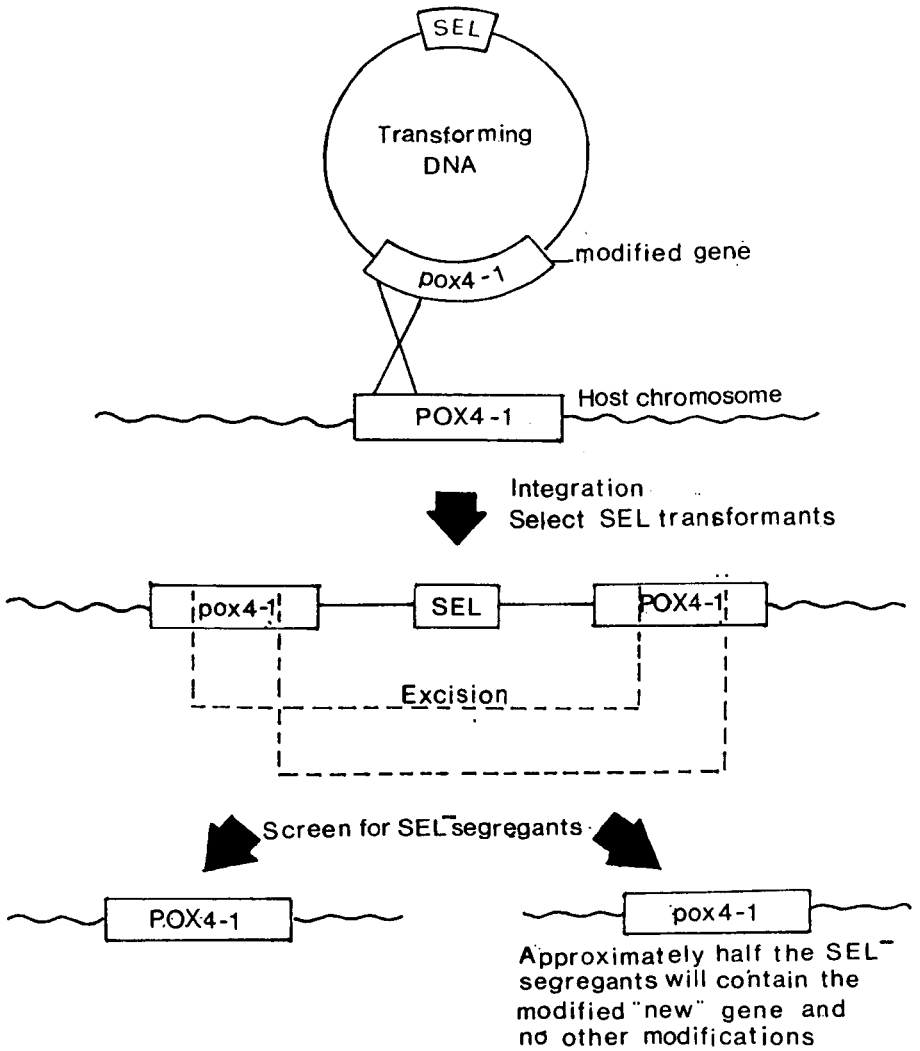


***Figure* 1.3 Gene Transplacement (after: Scherer and Davis, 1979; Knowles and Tubb, 1986)**

In this case the plasmid-borne selectable marker (SEL) is not cloned into the gene of interest. After transformation into a suitable yeast strain, the modified gene sequences undergo homologous recombination resulting in a direct, non-tandem duplication separated by the vector and marker DNAs. A second recombinational event, on the other side of the modified gene relative to the first, generates a chromosome with the altered sequence replacing those in the untransformed cell. The vector and marker DNAs are lost

This method is versatile as a disruption can be made simply by the appropriate cloning experiment. The resultant chromosomal insertion is non-reverting and contains a genetically linked marker. Detailed knowledge of the restriction map of a fragment is not necessary. As the selectable marker is not lost, sequential disruptions are not possible unless a range of selectable markers are available.

1.4.5.3 Gene Transplacement

In gene transplacement (Scherer and Davis, 1979; *figure* 1.3) an *in vitro* generated mutation on a plasmid is substituted for a chromosomal gene by transforming and integrating the plasmid into the corresponding chromosomal locus. Colonies are screened for those excisions that leave the mutated gene copy in the chromosome, replacing completely the wild-type copy.

Transformants are selected by the expression of plasmid-encoded genes. The integrated structure arises by a single crossover event involving the cloned yeast segment and a homologous sequence in the genome. This results in a direct, non-tandem duplication of the cloned segment, with copies bracketing integrated plasmid sequences which is genetically unstable.

A single recombination event between the repeated regions resolves the duplication and excises the integrated plasmid, which is then lost during cell division. If the selectable marker is outside the homologous sequences it segregates along with the vector sequences such that segregants losing the selectable marker also lack vector sequences. If the recombination event falls in the appropriate genetic interval, a segment of the yeast genome will have been altered to contain sequence information formerly present in the plasmid.

Transplacement requires several manipulations or detailed information about the cloned fragment. Detailed maps of restriction endonuclease cleavage sites and transcribed regions facilitate the analysis of transformants and make it possible to predict the phenotypes of the segregants. The major disadvantage of the method is that several recombinants must be examined in order to detect the correct replacement. A positive selection for loss of a marker on the plasmid enriches for, but does not ensure, successful replacement of the mutant gene. The efficiency of introduction, however, is sufficiently high that examination of a few segregants which have lost the selectable marker easily identifies the desired strains.

To screen for spontaneous loss of a prototrophic marker transformants are grown permissively for several generations and then screened for the auxotrophic phenotype. This screening can be facilitated by procedures which enrich for auxotrophy, or positively select for a specific auxotrophy as described in Chapter 7 (Scherer and Davis, 1979; Winston *et al.*, 1983). Sequential transplacements may be made because the selective marker is always lost with all foreign sequences in the final product. Therefore, a single selective marker is sufficient for repeated reactions.

These methods should be applicable to diploid as well as haploid organisms. In diploids, however, mitotic recombination between the marker of interest and the centromere of that chromosome may generate segregants identical to the undesired class of cells which have a DNA structure indistinguishable from that of the untransformed strain (Scherer and Davis, 1979).

1.4.5.4 Requirements for Gene Transplacement

Gene transplacements require: a molecular clone of the gene under investigation; a recipient strain for integrative transformation; yeast transformation or another process to introduce DNA into the recipient strain; integration mediated by homologous recombination at a specified site in the yeast genome; and suitable selectable markers available as cloned DNA fragments.

An advantage of using a dominant selectable marker of bacterial origin rather than a yeast gene is that if the selectable marker is also a yeast gene, integration can occur at a site homologous to the marker as well as at the site homologous to the gene of interest (the desired location of integration). However, it is possible to direct the integration to the desired location by various methods.

Integration at *ura3* is low compared to integration at other sites probably because *ura3* sequences are only 1.2 kb long. By using *URA3* as the selectable marker with a recipient strain one carrying a non-reverting *ura3* mutation such as *ura3-52* or *ura3-50* in *S. cerevisiae*, most integrants are directed to the location of choice unless the other yeast homology is less than 1000 bp (Scherer and Davis, 1979; Stiles, 1983; Winston *et al.*, 1983).

Integration can be directed to one specific site in the genome by making a double-strand break within the sequence homologous to the target site on the plasmid. The free ends generated have increased recombinogenic potential. This approach is most easily achieved by using a plasmid which has a unique restriction site in the cloned yeast segment (Orr-Weaver *et al.*, 1983; Stiles, 1983; Winston *et al.*, 1983). The region on the plasmid that is homologous to the desired chromosomal integration site must be large enough to permit a recombination event and to avoid degradation past the end of the homologous sequences. If the double-strand break is close to the edge of homology, degradation may occur, and substitutions rather than integrations will be obtained (Orr-Weaver *et al.*, 1983). Linearised plasmids sometimes give rise to transformants with multiple tandem insertions of the plasmid making it necessary to screen the transformants to find those that have only a single insertion of the plasmid (Winston *et al.*, 1983). The frequency of integration at a particular site is also proportional to the length of the homologous sequence and therefore manipulation of sequence lengths can give some direction to the integration (Stiles, 1983).

The ability of different yeast strains to undergo transformation is very variable, and it is advisable to check the transformation efficiency of the recipient strain (Orr-Weaver *et al.*, 1983; Winston *et al.*, 1983).

CHAPTER 2. MATERIALS

Water is sterile, deionised, distilled throughout this thesis.

2.1 STRAINS AND VECTORS

2.1.1 Yeast Strains

2.1.1.1 *Candida tropicalis*

Strain NCYC547: Prototrophic, killer strain. (Isolated as *Trichosporon lodderi* by Phaff *et al.*, 1952).
Strain NCYC997: Prototrophic (Teranishi *et al.*, 1974a. and b.; Tanaka *et al.*, 1977.). Also known as strain pK233, and ATCC strain 20336.

Both prototrophic strains of *Candida tropicalis* were obtained from the National Collection of Yeast Cultures. *C. tropicalis* strains PMPU1 and PMPU2 were prepared during this study.

2.1.1.2 *Saccharomyces cerevisiae*

Strain S150-2B: *MATa, ura 3-52, trp 1-289, his3-Δ1, leu 2-3, leu 2-122,* 2 μm$^+$ (McLeod *et al.*, 1984).
Strain X-2180-1A: *MATa, SUC2, mal, mel, gal 2, CUP1* (Yeast Genetic Stock Centre, Berkeley, California).
Strain YNN27: *MATα, trp 1-289, ura 3-52, gal2,* 2-μm$^+$ (Stinchcomb *et al.*, 1980).

Strains S150-2B and X-2180-1A were obtained from A. Boyd, Department of Biochemistry, University of Edinburgh. Strain YNN27 was obtained from P. A. Meacock, Leicester Biocenter, University of Leicester.

2.1.2 *Escherichia coli* Bacterial Strains

Strain NM522: *hsd Δ5, Δ(lac-proAB), F', lac ZΔM15, lac IQ, thi, SupE* (Gough and Murray, 1983).
K12 strain 5K: *hsdR, SupE, thr, leu, thi, tonA.*
Strain B15 : *trp, lac, hsdR, hsdM, pyrF::μ* also known as PNN36 and BNN46 (Struhl *et al.*, 1979).

EPICURIAN COLI™ AG1: competent cells, *endA1, hsdR17* (r_k- m_k+), *supE44, thi-1, recA-1, gyrA96, relA-1.*

EPICURIAN COLI™ AG1 competent cells were supplied by Stratagene cloning systems (formerly Vector cloning systems). Strains 5K and NM522 were obtained from A. Boyd, strain B15 was obtained from P. A. Meacock.

2.1.3 Plasmid and Viral Vectors

All plasmids used are listed below, except for those isolated during the course of this work which are described in the text where appropriate.

2.1.3.1 Plasmid Vectors for use in *Escherichia coli*:

pBR322 (Bolivar *et al.*, 1977a. and b.) supplied by New England Biolabs, *Bam* H1 cleaved and dephosphorylated, and pUC12 (Messing, 1983) obtained from T. Pillar, Leicester Biocenter, University of Leicester.

2.1.3.2 *E. coli-Saccharomyces cerevisiae* Plasmid Shuttle Vectors:

pCH100 (Hadfield *et al.*, 1986) conferring chloramphenicol resistance, and pCH192 and pCH200 conferring G418 resistance obtained from C. Hadfield, Leicester Biocenter, University of Leicester.

2.1.3.3 Plasmid Vectors carrying the *S. cerevisiae URA3* gene:

pEMBLYe23 (Baldari and Cesareni, 1985), and YEp24 (Botstein *et al.*, 1979) obtained from T. Pillar. YCp50, and YIp5 (Struhl *et al.*, 1979) obtained from P. A. Meacock.

2.1.3.4 Sequencing Vector:

M13mp18 (Yanish-Perron *et al.*, 1985). Supplied by Pharmacia LKB.

2.2 MEDIA

Where appropriate media were solidified by the addition of 1.5% w/v Bacto agar. Unless otherwise indicated ingredients were dissolved in deionised distilled water, and were sterilised by autoclaving at 15 psi for 15 minutes. Filter sterilisation was through a 2 μm mesh. If not used immediately after autoclaving, solid media were subsequently melted by microwaving. Bacto yeast extract, bacto peptone, yeast nitrogen base, bacto agar, and agar noble, were purchased from Difco.

2.2.1 Yeast Media

Semi Defined minimal medium (S):
0.67% w/v Yeast Nitrogen Base (YNB) with ammonium sulphate, without amino acids.

Yeast Extract Peptone (YEP), complete medium for the maintenance of strains:
1% w/v Bacto yeast extract.
2% w/v Bacto peptone.

Complete Medium (CM):
0.67% w/v YNB with ammonium sulphate, without amino acids.
10 ml^{-1} 100x amino acid mix (section 2.2.3.2).
10 ml^{-1} L-tryptophan stock solution (section 2.2.3.3).
10 ml^{-1} uracil stock solution (section 2.2.3.2).
The uracil was omitted (CM-U) when the medium was required as a selection for *URA3* transformants.

α-amino adipic acid medium (AA), for selection of *lys2* mutants (Chattoo *et al.*, 1979):
0.5 g YNB without ammonium sulphate and amino acids.
6.0 g D-glucose.
9.0 mg lysine.
6.0 g Bacto agar.
290 ml water.
20 ml α-amino adipic acid stock solution (section 2.2.3.3) added after autoclaving.

5-fluoro orotic acid medium (FOA), for selection of *ura3* mutants (Boeke *et al.*, 1984):

7.0 g YNB with ammonium sulphate, without amino acids.

0.5 g 5-fluoro orotic acid monohydrate, purchased from PCR incorporated.

50 mg uracil.

20 g D-glucose.

500 ml water.

The above was filter sterilised, and added to 500 ml 4% w/v sterile Bacto-agar.

Nutrient extract-peptone medium (NEP) for the selection of copper resistant yeast (Naiki and Yamagata, 1976):

2.00 gl^{-1} magnesium sulphate, $MgSO_4.7H_2O$.

2.00 gl^{-1} ammonium sulphate, $(NH_4)_2SO_4$.

3.00 gl^{-1} potassium dihydrogen orthophosphate, KH_2PO_4.

0.25 gl^{-1} calcium chloride, $CaCl_2.2H_2O$.

2.00 gl^{-1} Yeast extract.

3.00 gl^{-1} Bacto peptone

20.0 gl^{-1} agar-noble.

After autoclaving, D-glucose stock solution, was added to give a final concentration of 4% w/v.

2x Yeast preservation mix for the storage of yeast strains and transformants:

12.6 gl^{-1} dipotassium hydrogen orthophosphate, K_2HPO_4.

3.6 gl^{-1} KH_2PO_4.

0.9 gl^{-1} sodium citrate, $Na_3C_6H_5O_7.2H_2O$.

0.18 gl^{-1} $(NH_4)_2SO_4$.

200 ml glycerol, $CH_2OH.CHOH.CH_2OH$.

Sterilised by autoclaving at 10 psi for 10 minutes.

2.2.2 *Escherichia coli* Media

Luria Bertani Medium (L) for the maintenance of strains (except NM522):

1.0% w/v Bacto tryptone.

0.5% w/v Bacto yeast extract.

1.0% w/v sodium chloride, NaCl.

pH adjusted to 7.2 with sodium hydroxide, NaOH (5 M).

Minimal Glucose (M9) medium, for the maintenance of the F' plasmid in *E. coli* NM522:
180 ml water or water agar (1.5% Bacto agar in water).
25 ml 10x M9 salts (section 2.2.3.4).
2.5 ml 100x M9 additive (section 2.2.3.4).
2.5 ml D-glucose stock (section 2.2.3.1).
0.225 ml 2 $mgml^{-1}$ thiamin (B1) in water (stored at 20°C, filter sterilised).
Thiamin was purchased from Sigma.

Media for the transformation and selection of Epicurian coli^{TM} AG1 competent cells were made up as directed by the suppliers.

2x YT Medium for growth of M13 transfected *E. coli* for template preparation:
16 gl^{-1} tryptone.
10 gl^{-1} yeast extract.
5 gl^{-1} NaCl.
pH adjusted to 7.2 with NaOH (5 M).

Baltimore Biological Laboratories (BBL) medium for M13 transfected *E. coli*:
10 gl^{-1} BBL trypticase.
5 gl^{-1} NaCl.
10 gl^{-1} Bacto agar.

BBL-soft medium for M13 overlays was prepared as above using 6.5 gl^{-1} Bacto agar.

2.2.3 Media Supplements

2.2.3.1 Carbon Sources

Unless otherwise indicated, the stock solutions were made up in deionised, distilled water, and sterilised by autoclaving at 10 psi for 10 minutes. D-glucose, glycerol and ethanol were purchased from Fisons. Oleic acid, brij 58 and D-raffinose were purchased from Sigma. Stock solutions were as follows:

D-Glucose (D): 40% w/v stored at room temperature (RT), and used at a final concentration of 2% w/v unless otherwise stated.

Glycerol (G): 50% v/v stored at RT, and used at a final concentration of 1% v/v.

D-Raffinose (R): 40% w/v stored at RT, and used at a final concentration of 2% w/v.

Ethanol (E): Stored neat at RT after filter sterilisation, and used at 2% v/v.

Oleic acid (O): Stored neat at -20°C, and used at 1% v/v. It was found to be unnecessary to sterilise the oleic acid prior to use.

Brij 58, (polyoxyethylene 20 cetyl; B) added with the oleic acid as an emulsifier: 10% w/v stored at RT, and used at a final concentration of 0.1% w/v, after filter sterilisation.

2.2.3.2 Amino Acids and Bases

These stock solutions were made up in deionised distilled water, and filter sterilised. All amino acids and bases were purchased from Sigma.

100x amino acid mix (without uracil and tryptophan):
2 gl^{-1} adenine sulphate.
2 gl^{-1} L-histidine.
3 gl^{-1} L-arginine-HCl.
3 gl^{-1} L-methionine.
3 gl^{-1} L-tyrosine.
3 gl^{-1} L-isoleucine.
3 gl^{-1} L-lysine.
3 gl^{-1} L-leucine.
5 gl^{-1} L-Phenylalanine.
10 gl^{-1} L-aspartic acid.
10 gl^{-1} L-glutamic acid.
15 gl^{-1} L-valine.
20 gl^{-1} L-threonine.
40 gl^{-1} L-serine
Stored in aliquots at -20°C.

Uracil (U): The stock solution (2 $mgml^{-1}$) was stored at RT, and used at a final concentration of 0.02 $mgml^{-1}$.

L-Tryptophan: The stock solution (2 $mgml^{-1}$) was stored at 4°C in a light proof bottle, and used at a final concentration of 0.02 $mgml^{-1}$.

Orotidine-5'-monophosphate: The stock solution (4 $mgml^{-1}$ in 0.1 M Tris-HCl pH 8.6, 1.7 M magnesium chloride, $MgCl_2$), was stored at -20°C, and used as specified in the text.

2.2.3.3 Amino Acid Analogues

These were purchased from Sigma. The stock solutions were as follows:

α-Amino adipic acid: 1.2 g in 20 ml water, adjusted to pH 6.0 with KOH (10 M) was used in AA medium (section 2.2.1).

L-Canavanine sulphate (Can): 100 $mgml^{-1}$ stored at -20°C, and used as specified in the text.

2.2.3.4 Salts

These stock solutions were made up in water, sterilised by autoclaving at 15 psi for 25 minutes, and stored at RT.

10x M9 salts:
70 gl^{-1} disodium hydrogen orthophosphate, Na_2HPO_4.
30 gl^{-1} KH_2PO_4.
5 gl^{-1} NaCl.
10 gl^{-1} ammonium chloride, NH_4Cl.

100x M9 additive: 0.1 M $MgSO_4.7H_2O$
0.01 M $CaCl_2$.

Copper sulphate, $CuSO_4.5H_2O$: The stock solution (1 M) was used as specified in the text, for the selection of copper resistance.

2.2.3.5 Antibiotics

Unless otherwise indicated stock solutions were made up in deionised, distilled water, filter sterilised, stored at -20°C and used as specified in the text.

Chloramphenicol, neomycin sulphate, kanamycin sulphate, ampicillin, tetracycline hydrochloride, and IPTG were purchased from Sigma. X-gal, and G418-sulphate were purchased from BRL/Gibco. Stock solutions were as follows:

Chloramphenicol (Cm): 50 $mgml^{-1}$ in absolute ethanol (keeps 1 year).

Geneticin G418-sulphate (G418): 100 $mgml^{-1}$.

Neomycin sulphate (Nm): 50 $mgml^{-1}$.

Kanamycin sulphate (Km): 50 $mgml^{-1}$.

Ampicillin (Ap): 10 $mgml^{-1}$ of the sodium salt.

Tetracycline hydrochloride (Tc): 10 $mgml^{-1}$ in 50% v/v ethanol, stored in a light proof bottle (keeps one week).

For the selection of pUC and pEMBL E. coli transformants, the following were added to Luria agar containing ampicillin (100 μgml^{-1}, LAp): Isopropyl β-D-thiogalactopyranoside (IPTG) stored as a stock solution (0.1 M, -20°C) and used at a final concentration of 300 nM after filter sterilisation; and 5-Bromo-4-chloro-3-indoyl-β-d-galactoside (X-gal) stored as a stock solution in dimethyl formamide (2% w/v, -20°C) and used at a final concentration of 0.015% w/v.

2.3 CHEMICALS

Chemicals and solvents were analar grade, purchased from Fisons, BDH, or Sigma. Buffers and chemical solutions were sterilised by autoclaving at 15 psi for 15 minutes.

2.3.1 Buffers:

These were made up with deionised, distilled water unless stated otherwise in the text.

1.0 M Tris-HCl:
48.44 g trizma base (tris[hydroxymethyl]amino methane).
200 ml water.
Stir whilst monitoring the pH. Add hydrochloric acid, HCl (1 M) until the correct pH (variable depending on use) is reached. Make up to 400 ml with water.

0.5 M Diaminoethanetetra acetic acid disodium salt (EDTA):

148.896 g EDTA.

200 ml water.

Prepare as for Tris-HCl adjusting the pH (variable) with NaOH (1 M or 20 g per 500 ml). Make up to 400ml with water.

TE: 10.0 mM Tris-HCl pH 7.6.
0.1 mM EDTA pH 7.6.

Nb. this is not standard TE which contains 1 mM EDTA.

Caesium Saturated Isopropanol:

Dissolve caesium chloride, CsCl, in 200 ml TE buffer to saturation. Add an equal volume of isopropanol and mix. Use the upper layer.

20x SSC: 175.3 gl^{-1} NaCl.
88.2 gl^{-1} sodium citrate.

2.3.2 10x Buffers for Restriction Endonuclease Digestion

React buffers were supplied by Gibco BRL.

React 1: 50 mM Tris-HCl pH 8.0.
10 mM $MgCl_2$.

React 2: 50 mM Tris-HCl pH 8.0.
10 mM $MgCl_2$.
50 mM NaCl.

React 3: 50 mM Tris-HCl pH 8.0.
10 mM $MgCl_2$.
100 mM NaCl.

React 4: 20 mM Tris-HCl pH 7.4.
5 mM $MgCl_2$.
50 mM potassium chloride, KCl.

Low salt buffer: 10 mM Tris-HCl pH 7.5.
10 mM $MgCl_2$.
1 mM dithiothreitol (DTT).

2.3.3 Solutions for Electrophoresis of DNA

10x TBE: 108 gl^{-1} trizma base.
55 gl^{-1} boric acid, H_3BO_3.
9.3 gl^{-1} EDTA.

10x Loading Buffer: 0.25% w/v bromophenol blue.
0.25% w/v xylene cyanol.
25% w/v Ficoll (type 400).

30% Acrylamide: 29 g acrylamide.
1 g N,N'-methylene bisacrylamide.
Water to 100 ml.

Electran grade acrylamide was purchased from BDH.

2.3.4 Buffers for Hexanucleotide Priming of dsDNA

Solution O: 1.25 M Tris-HCl pH 8.0.
0.125 M $MgCl_2$.

Stored at 4°C.

Deoxynucleotide Triphosphates (dNTPs): 100 mM in water.
Stored at -20°C.

Solution A: 1 ml solution O.
18 μl 2-mercaptoethanol.
5 μl each dNTP except dCTP.

Stored at -20°C.

Solution B: 2 M HEPES-NaOH pH 6.6.
Stored at 4°C.

Solution C: Hexanucleotide mixture (Pharmacia #2166; A_{260} = 90 ml^{-1} in TE buffer).

Stored at -20°C.

OLB Buffer: A 10:25:15 mixture of solution A:B:C.

Stored at -20°C.

Stop Buffer: 20 mM Tris-HCl pH 7.5.
2 mM EDTA pH 7.5.
0.25% w/v sodium dodecylsulphate (SDS).
1 μM dCTP.

Stored at -20°C.

2.3.5 Solutions for DNA Hybridization

Denaturing Solution: 1.5 M NaCl.
0.5 M NaOH.

Neutralising Solution: 0.5 M Tris-HCl pH 7.4.
1.5 M NaCl.

50x Denhardt's Solution:
10 gl^{-1} ficoll.
10 gl^{-1} polyvinylpyrrolidone.
10 gl^{-1} bovine serum albumen (pentax fraction V).

Prehybridization/hybridization Solution for dsDNA Probes:
5x Denhardt's solution.
6x SSC.
0.5% w/v SDS.
100-500 μgml^{-1} salmon sperm DNA sheared by passing through an 18 guage needle several times and denatured by incubating in a polyethylene glycol-bath (105°C, 10 minutes).

Prehybridization/Hybridization Solution for Oligonucleotide Probes:

1x Denhardt's solution.

6x SSC.

0.5% w/v SDS.

100 μgml^{-1} denatured salmon sperm DNA.

0.05% w/v sodium pyrophosphate, $Na_4P_2O_7.10H_2O$.

2.3.6 Solutions and Buffers for Protein Methodology.

Lysis Buffer:	0.1 M Tris-HCl pH 7.0.
	2 mM EDTA pH 7.0.
	5 μlml^{-1} 2-mercaptoethanol added
	immediately before use.

5x Protease Inhibitors:

20 mM EDTA.

20 mM ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra acetic acid (EGTA).

20 mM phenylmethylsulfonyl fluoride (PMSF).

10 μgml^{-1} pepstatin.

10 μgml^{-1} leupeptin.

10 μgml^{-1} chymostatin.

10 μgml^{-1} antipain.

Stored in aliquots at -20°C.

Separating Gel Buffer:	0.75 M Tris-HCl pH 8.8.
	0.2% w/v SDS.
Stacking Gel Buffer:	0.25 M Tris-HCl pH 6.8.
	0.2% w/v SDS.
Acrylamide Stock Solution:	44% w/v acrylamide.
	0.8% w/v bisacrylamide.

When dissolved 15 ml amberlite mixed bed resin is mixed in to deionise, and the solution clarified by filtration through Whatman No.1 filter paper. Stored at 4°C in opaque containers.

Electrophoresis Buffer:	0.025 M Tris-HCl.
	0.129 M glycine.
	0.1% w/v SDS.
2x Sample Buffer:	12.5 ml stacking gel buffer.
	12.5 ml water.
	4.0 g SDS.
	5.0 ml 2-mercaptoethanol.
	20.0 ml glycerol.
	0.1% w/v bromophenol blue.

2.4 ENZYMES

Zymolyase 100T was purchased from ICN biochemicals.
Proteinase K was purchased from BCL.
Lysozyme was purchased from Sigma.

Ribonuclease A (RNase) was purchased from Sigma. The 10 $mgml^{-1}$ stock solution in deionised, distilled water was boiled for 10 minutes to destroy any DNase activity, and stored at -20°C. It was used at a final concentration of 1 $mgml^{-1}$.

Restriction endonucleases were purchased from BRL/Gibco.

Buffer (section 2.3.2)	Restriction endonuclease
React 1	*Rsa* I
React 2	*Ava* I, *Hha* I, *Hin* dIII, *Hin* fI, *Pst* I
React 3	*Bam* HI, *Bgl* II, *Eco* RI, *Sal* I
React 4	*Kpn* I, *Sau* 3AI
10x low salts	*Bal* I, *Xmn* I

Calf intestinal alkaline phosphatase was purchased from BCL.
T4 DNA ligase was purchased from BRL/Gibco.
T4 DNA polynucleotide kinase was purchased from BRL/Gibco.
Large fragment DNA polymerase (Klenow fragment) was purchased from Amersham International.

2.5 SUPPLIERS

Amersham International, Buckinghamshire, UK.

Boehringer Mannheim, BCL, Lewes, East Sussex, UK.

BDH Chemicals Ltd, Atherstone, Warwickshire, UK.

Bethesda Research Laboratories, BRL/Gibco life technologies, Paisley, Scotland, UK.

Difco Laboratories, Detroit, Michigan, USA.

Fisons Scientific, Loughborough, Leicestershire, UK.

FMC Bioproducts, 5, Maple Street, Rockland, USA.

National Collection of Yeast Cultures (NCYC), Agricultural and Food Research Council, Food Research Institute, Colney Lane, Norwich, NR4 7UA.

New England Biolabs, Beverly, Massachusetts, USA.

P. C. R. incorporated, P. O. box 1466, Gainesville, Florida 32602, USA.

Pharmacia LKB, Uppsala, Sweden.

Sigma Chemical Company, Poole, Dorset, UK.

CHAPTER 3. METHODS

3.1 CULTURE CONDITIONS AND STORAGE OF STRAINS

3.1.1 Culture Conditions

All yeast strains were grown at 28°C, and *Escherichia coli* strains at 37°C. Broth cultures were incubated in a controlled environment rotary shaking incubator unless otherwise indicated in the text.

3.1.2 Storage and Recovery of Yeast Strains

For short term storage, the strains were streaked to single colonies over suitable agar media, incubated until growth was visible, the plates were sealed with parafilm, and stored (4°C) for up to four weeks.

For long term storage, the strains were grown to mid-log in a suitable liquid medium. Aliquots of culture (1 ml) were added to aliquots of 2x yeast preservation mix (1 ml; section 2.2.1) in small screw-capped glass vials, mixed, fast frozen in dry ice/industrial methylated spirit (IMS), sealed with parafilm, and stored (-70°C).

To revive the cells a small piece of frozen culture was chipped off, thawed rapidly (37°C), streaked over a suitable agar plate, and incubated.

3.1.3 Storage and Recovery of *Escherichia coli* Strains and Transformants

Short term storage of strains was as described for yeast (section 3.1.2).

Luria broth (L-broth; section 2.2.2; 10 ml) was inoculated with a single colony of the strain or transformant, and incubated overnight. Transformants were grown up under appropriate selection. The culture was centrifuged (4000 rpm, 10 min), the cell pellet washed in M9 medium (10 ml; section 2.2.2), and resuspended in M9 medium (1.5 ml). Aliquots (0.4 ml) of this cell suspension were mixed into aliquots (2.5 ml) of 50% v/v glycerol (G; section 2.2.3.1) in screw-capped glass vials, mixed, sealed, fast frozen and stored as described for yeast (section 3.1.2).

To recover a strain, cells were scraped from the surface of the frozen culture, inoculated into a suitable liquid medium, and incubated.

3.1.4 Growth of *Candida tropicalis* on D-Glucose, and Oleic Acid

Both strains of *C. tropicalis* were aseptically inoculated from yeast extract peptone glucose (YEPD) agar into YEPD, semi-defined medium with glucose (SD), or S medium with oleic acid and Brij 58 (SBO) broths (10 ml; sections 2.2.1 and 2.2.3.1), and incubated overnight.

Cell density of each starter culture was calculated with a haemocytometer, and appropriate volumes were added from each starter culture to the same broth (25 ml) to give initial concentrations of about 4.0×10^6 cells ml^{-1} in YEPD, and SD, and about 2.0×10^6 cells ml^{-1} in SBO.

Growth was monitored by measuring cell density with a haemocytometer, and absorbance at 600 nm (A_{600}; read against a blank of the appropriate broth), over an 11 h period.

O is immiscible with water, forming a suspension of droplets which interferes with A_{600} readings, and for this reason, growth in SBO was monitored by haemocytometer readings only. Samples were diluted into YEPD broth when necessary for ease of counting with the haemocytometer, but were never diluted for A_{600} readings. They were evenly suspended through the broth before removal of samples, dilution, and A_{600} readings by shaking or inverting.

To determine viable cells ml^{-1} samples were serially diluted into YEPD broth and aliquots (500 μl) of the final dilution spread over YEPD agar in duplicate. YEPD broth (500 μl) was plated out as a control. After incubation (72 h) the colonies on each plate were counted and these counts multiplied by the dilution factor to give viable cells ml^{-1}.

3.1.5 Quick Test for *Candida tropicalis*

To check that a strain was *C. tropicalis* and not a contaminating yeast, cells were streaked over semi-defined medium, supplemented with growth requirements as necessary, with 2% w/v D-raffinose (R; section 2.2.3.1). *C. tropicalis* is unable to utilise this carbon source therefore only contaminents grow (Barnett *et al.*, 1983).

3.2 ISOLATION OF NUCLEIC ACIDS

Ethanol (E) precipitation and phenol-chloroform extraction of DNA was performed essentially as described by Maniatis *et al.* (1982).

3.2.1 Isolation of Total DNA from *Candida tropicalis* NCYC997

Chromosomal DNA was extracted using a modified method of Cryer *et al.* (1975) followed by isopycnic equilibrium density gradient centrifugation.

YEPD broth (800 ml) was inoculated with strain NCYC997, and incubated overnight. An aliquot (5 ml) of the overnight culture was harvested by centrifugation (6000 rpm, 3 min) in a pre-weighed tube to determine wet weight of the cells. An appropriate volume of culture was harvested as above to yield 10 g wet weight of cells, which were resuspended in 0.05 M EDTA pH 7.5 (25 ml), and repelleted. The washed cell pellet was incubated in 0.35 M 2-mercaptoethanol, 0.05 M EDTA pH 9.0 (22.5 ml, 20 min, RT), pelleted (6000 rpm, 1 min), resuspended in 1.0 M sorbitol, 0.1 M EDTA pH 7.5 (SE buffer, 20 ml), and freshly prepared Zymolyase in the same buffer (1 ml of a 5 $mgml^{-1}$ solution) was added, mixed, and the tube incubated (40 min, 37°C). The resulting sphaeroplasts were harvested (3000 rpm, 5 min), resuspended in SE buffer (20 ml), and repelleted (3000 rpm, 5 min).

The spheroplasts were resuspended in 0.15 M NaCl, 0.1 M EDTA pH 7.5 (10 ml), and freshly prepared proteinase K (2 ml of a 2 $mgml^{-1}$ solution in water) added and mixed. Sodium dodecylsulphate (SDS) in water (0.5 ml; 20% w/v) was added dropwise down the side of the tube with gentle agitation to release the DNA from the sphaeroplasts. This lysate was incubated (2 h, 37°C; 30 min, 65°C).

An equal volume of a 24:1 mixture of chloroform : isoamyl alcohol was added to the lysate, and shaken gently to form a white emulsion which was centrifuged (12 000 rpm, 20 min) in a corex tube to separate the phases. The upper (aqueous) phase was carefully removed into clean corex tubes using a 1 ml Gilson pipette with the end cut off the tip. Care was taken to avoid shearing the high molecular weight DNA.

Two volumes of cold 95% E were mixed into the aqueous phase and the tube incubated overnight (-20°C) to precipitate the DNA which was retrieved by centrifugation (2000 rpm, 1 min), and freeze dried.

The DNA pellet was resuspended in 0.1 M Tris, 0.01 M EDTA pH 8.0 (17 ml), by incubating (overnight, 4°C, 5 min, 65°C), and dispersing the DNA by gentle agitation. Caesium chloride, CsCl (20 g) was dissolved into the DNA solution, and Bis Benzamide added (0.5 ml of a 1 $mgml^{-1}$ solution in water). This was transferred to a 35 ml Sorval ultracentrifuge tube, placed in a TV850 rotor, and spun in a Sorval ultracentrifuge (50 000 rpm, 18 h, 20°C).

DNA bands were visualised under long wave ultraviolet (UV) light. The upper (mitochondrial), and middle (chromosomal) DNA bands were removed separately by piercing the side of the tube with a large bore needle, and allowing the DNA to flow out into sterile corex tubes. The lower band of RNA, and high molecular weight polysaccharides was discarded.

Each band was diluted with 2 volumes of water. 3 volumes of 95% E were added, mixed well, and the tube incubated (RT, 10 min). The DNA was pelleted (12 000 rpm, 10 min, RT), washed with 70% E (5 ml, 12 000 rpm, 5 min, RT), air dried (15 min, RT), and resuspended overnight in TE buffer (1 ml, section 2.3.1, 4°C).

DNA concentration was calculated by measuring the A_{260} of the solution, and stored (-20°C).

3.2.2 Isolation of Plasmid DNA from *Escherichia coli*

3.2.2.1 Large Scale Triton Lysis

Large scale preparations of plasmid DNA were made using the Triton lysis method of Kahn *et al.* (1979), scaled down for a 400 ml broth culture, with minor modifications, followed by isopycnic equilibrium density gradient centrifugation.

The *Escherichia coli* strain carrying the plasmid was grown up overnight in L-broth under appropriate selection. The cells were harvested by centrifugation (6000 rpm, 10 min, 4°C), and washed once with cold TE buffer (10 ml). The cell pellet was resuspended in cold 25% w/v sucrose, 50 mM Tris-HCl pH 8.0, lysozyme (2 ml of a 20 $mgml^{-1}$ solution in water) was added, mixed, and the tube incubated on ice (10 min), followed by 0.5 M EDTA pH 8.5 (2 ml) which was added, mixed, and the tube was again incubated on ice (5 min). Triton lysis cold detergent (16 ml; 1 ml 10x triton, 12.5 ml 0.5 M EDTA pH 8.5, 5 ml 1.0 M Tris-HCl pH 8.0, 81.5 ml water) was added whilst mixing vigourously, and the preparation incubated on ice (10 min). This lysate was centrifuged (17000 rpm, 30 min, 4°C), and the supernatant was decanted into a fresh tube.

A half volume of cold 30% w/v polyethylene glycol (PEG) 6000, 1.5 M NaCl was mixed into the supernatant which was centrifuged (6000 rpm, 15 min, 4°C). The pellet was drained, and resuspended in 0.1 M Tris-HCl, 0.01 M EDTA pH 8.0 (18 ml). Caesium chloride (20 g) was dissolved completely in the solution, and ethidium bromide added (1 ml of a 10 $mgml^{-1}$ solution). The preparation was decanted into Sorval 35 ml ultracentrifuge tubes, placed in a TV850 rotor, and spun in a Sorval ultracentrifuge (49 000 rpm, 18 h, 20°C). The upper band of chromosomal DNA was removed through a large bore needle as described in section 3.2.1, and discarded. The plasmid band was then removed by piercing the side of the tube with a large bore needle and withdrawing the DNA into a 5 ml syringe. This was transferred into a 5 ml Sorval ultracentrifuge tube, placed in a TV865 rotor, and spun in a Sorval ultracentrifuge (49 000 rpm, 6 h, 20°C). The plasmid band was then removed through a large bore needle into a tube in as small a volume as possible.

Ethidium bromide was removed by extracting several times with an equal volume of caesium saturated isopropanol (section 2.3.1). The DNA was then diluted with 2 volumes of water, E precipitated, its concentration measured, and stored as described in section 3.2.1.

3.2.2.2 Mini-Preparation by Alkaline Lysis

Rapid small scale extraction of plasmid DNA (miniprep) was performed using a modified method of Birnboim and Doly (1979).

E. coli carrying the required plasmid was grown up in L-broth under appropriate selection (3-5 ml, overnight), and two successive aliquots (1.5 ml) were pelleted in a 1.5 ml Eppendorf tube (6000 rpm, 5 min).

The pellet was resuspended in lysis buffer (100 μl; 2 $mgml^{-1}$ lysozyme, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM D), and the tube incubated on ice (30 min). 0.2 M NaOH, 1% w/v SDS (200 μl) was mixed in thoroughly, and the tube was incubated on ice (5 min). Sodium acetate (150 μl, 3.0 M) was mixed in well by repeatedly inverting the tube, which was then incubated on ice (60 min).

The lysate was centrifuged (12 000 rpm, 10 min), and the supernatant decanted into a fresh tube. E (1 ml, 95%) was added, and the contents of the tube mixed and frozen solid in a dry ice / IMS bath to precipitate the DNA. The DNA was pelleted by centrifugation (12 000 rpm, 10 min).

The pellet was drained and resuspended in TE (100 μl). RNase A (10 μl, section 2.4) was added and the tube was incubated (30 min, 37°C).

The DNA solution was extracted with an equal volume of phenol, followed by an equal volume of 25:24:1, phenol : chloroform : isoamyl alcohol, and finally an equal volume of 24:1, chloroform : isoamyl alcohol. Ammonium acetate (250 μl 0.3 M in 95% E) was added and the DNA was precipitated and pelleted as described above.

The pellet was washed with 70% E (50 μl, 12 000 rpm, 5 min), air dried (15 min, RT), resuspended in TE (50 μl) and stored (-20°C).

3.3 DNA MANIPULATIONS

3.3.1 Restriction Endonuclease Digestion

Restriction endonucleases, were used according to manufacturers instructions. Buffers (10x React buffers) supplied by the manufacturers, were used except where indicated in section 2.4. Composition of the buffers is detailed in section 2.3.2.

3.3.1.1 Digestion to Completion

Reactions were performed in 1.5 ml Eppendorf tubes. DNA was diluted to give a final concentration of 100 ngμl^{-1}. One tenth final volume (typically 10-30 μl) 10x buffer was added, followed by the enzyme (5-10 unitsμg^{-1} DNA). The tube was briefly vortexed to mix and centrifuged for a few seconds to bring the contents down.

The reaction was incubated (37°C) for times ranging from 1 h to overnight depending on the quantity and quality of the DNA in the digestion.

The reactions were stopped by the addition of a tenth volume of loading dye (section 2.3.3) if the sample was to be run on an electrophoresis gel, or by extraction with an equal volume of 25:24:1, phenol : chloroform : isoamyl alcohol, followed by precipitation with ammonium acetate (0.3 M in 95% E) as described for the recovery of plasmid DNA in section 3.2.2.2 to remove the restriction endonuclease.

After washing and air drying, the pellet was resuspended in a suitable volume of TE buffer and stored (-20°C), or used as specified.

3.3.1.2 Partial Digestion of Genomic DNA

A *Sau* 3A partial digest of *C. tropicalis* NCYC997 DNA was set up and fractionated in a sucrose density gradient (section 3.3.4.1) as described by Maniatis *et al.* (1982).

To establish the correct conditions for the partial digestion of *C tropicalis* NCYC997 DNA (titration of the *Sau* 3AI enzyme), a reaction mixture was prepared containing genomic DNA (10 μg), and React 4 buffer in a final volume of 150 μl. This was mixed well by inverting the tube several times. The reaction mix was then dispensed into 9 tubes (30 μl into tube 1, 15 μl into tubes 2-9). All the tubes were chilled on ice. *Sau* 3AI (4 units) was added to tube 1, mixed, and 15 μl transferred to tube 2. This two-fold serial dilution was continued through to tube 8, nothing being added to tube 9. Tubes 1-8 were incubated in a waterbath (60 min, 37°C). The tubes were chilled on ice, and EDTA was added to a final concentration of 20 mM to stop the reactions. A tenth volume of loading dye (section 2.3.3) was added to each sample which were analysed by electrophoresis (section 3.3.5) on an 0.4% agarose gel against λ *Eco* RI/*Hin* dIII restriction marker fragments. Electrophoresis was carried out slowly (1-2 Vcm^{-1}) until the bromophenol blue had just migrated off the gel. The gel was photographed, and the amount of enzyme needed to produce the maximum intensity of fluorescence in that part of the gel containing the fragment sizes of interest was ascertained.

To obtain the maximum number of molecules in this size range half the concentration of enzyme that produced the maximum amount of fluorescence was used with *C. tropicalis* NCYC997 genomic DNA (500 μg), incubation time, temperature, and DNA concentration being identical to those used in the pilot reactions. An aliquot (0.5 μg DNA) was analysed by electrophoresis through 0.4% agarose to check that the size distribution of the restriction fragments was correct. The partial digest was then extracted twice with 25:24:1, phenol : chloroform : isoamyl alcohol, precipitated and air dried as described for plasmid DNA in section 3.2.2.2. The dried DNA pellet was resuspended in TE buffer (0.5 ml).

3.3.2 Treatment with Calf Intestinal Alkaline Phosphatase

Calf intestinal alkaline phosphatase was used exactly according to manufacturers instructions.

3.3.3 Ligation

This was performed as described by King and Blakesley (1986).

Vector DNA (60 fmol) and insert DNA (20 fmol) were placed in a 1.5 ml Eppendorf tube (12 μl total volume). reaction buffer (4 μl, 5x; 250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 25% w/v PEG 8000, filter sterilised and stored at RT), ATP (2 μl of a 10 mM solution, stored at -70°C), and dithiothreitol (2 μl of a 10 mM solution, stored at -20°C) were added, followed by T4 DNA ligase (0.1 units).

The tube was briefly vortexed to mix the components, and centrifuged for a few seconds to bring the reaction mixture to the bottom of the tube, and incubated (4 h, RT; overnight, 15°C).

0.5 M EDTA pH 8.0 (1 μl) was added to stop the reaction.

3.3.4 Size Fractionation of Restriction Fragments by Sucrose Gradient Centrifugation

This was performed as described by Maniatis *et al.* (1982).

Solutions of sucrose (40% w/v, and 10% w/v in 1 M NaCl, 20 mM tris-HCl pH 8.0, 5 mM EDTA) were prepared and sterilised by autoclaving (10 psi for 10 min). The 40% solution (38.5 ml) was placed in the mixing chamber of a cylinder gradient mixer, and the 10% solution (34.5 ml) in the other chamber. Two gradients were poured simultaneously by slow mixing of the solutions in 36 ml Sorval ultracentrifuge tubes.

The DNA preparation to be fractionated was added to the top of one of the gradients, the tubes balanced with the 10% solution, placed in an AH627 rotor, and spun (26 000 rpm, 24 h, 20°C).

The fractions (0.5 ml) were collected from the bottom of the gradient into Eppendorf tubes. Aliquots (10 μl) from every third fraction were analysed by electrophoresis through 0.4% agarose. All fractions containing the fragments of interest were pooled, diluted with two volumes of water, and precipitated with 0.3 M ammonium acetate in 95% E, washed, air dried, resuspended in a suitable volume of TE, and stored as described above.

3.3.5 Electrophoresis of DNA in Agarose Gels

This was performed essentially as described by Maniatis *et al.* (1982).

Agarose gel electrophoresis was carried out in 1x TBE (section 2.3.3) running buffer. This was diluted from the 10x stock with water. Ethidium bromide was added to the buffer (100 $ngml^{-1}$) to allow staining and visualisation of the DNA. Agarose was purchased from FMC Bioproducts. Sea Kem ME agarose was used throughout unless otherwise specified.

Agarose was dissolved in 1x TBE at the desired concentration (0.4-2.0% w/v) by heating in a microwave oven, poured into a perspex gel former or over a microscope slide (slide gel), and allowed to set at RT.

10x loading buffer (section 2.3.3) was added to the DNA to be analysed, and the sample loaded into a well in the agarose slab. Suitably sized marker fragments were also loaded onto the gel as molecular weight standards.

Slide gels were run at 50-100 mA until the bromophenol blue was near the end of the gel. Large format gels were run at 20-30 mA overnight.

DNA bands were visualised with long wave UV light, and photographed using either a polaroid MP-4 land camera with polaroid type 55 pos/neg film, or a polaroid CU-5 hand held camera with polaroid 667 professional film.

3.3.6 DNA Restriction Fragment Isolation from Agarose Gels

Specific DNA fragments were isolated from 0.8% agarose gels prepared and run as described above (section 3.3.5.1).

The DNA was visualised with long wave UV light, and the band of interest cut out using a sharp razor blade, and placed into a bag of dialysis tubing with a minimal volume of 1x TBE.

The bag was placed in an electrophoresis tank such that its length was parallel to the electrodes, just covered with 1x TBE, and run (100 V, 30 min). The polarity was reversed for 30 s to bring the DNA off the side of the tubing.

The sample was extracted with an equal volume of phenol, an equal volume of 25:24:1, phenol : chloroform : isoamyl alcohol, and an equal volume of 24:1, chloroform : isoamyl alcohol, E precipitated, washed, air-dried, and resuspended in a suitable volume of TE buffer.

3.4 PREPARATION, STORAGE, AND TRANSFORMATION OF COMPETENT CELLS

3.4.1 *Escherichia coli*

E. coli was made competent and transformed using the method of Mandel and Higa (1970) as described by Maniatis *et al.* (1982).

Sterile, plastic tubes and very clean glassware were used throughout.

The recipient strain was inoculated into L-broth (10 ml) and incubated overnight. This starter culture was used to inoculate L-broth (150 ml) which was incubated until the culture reached 0.265 (A_{600} against a blank of L-broth).

The cells were harvested by centrifugation (4000 rpm, 10 min, 0°C), washed in cold $MgCl_2$ (60 ml, 0.1 M), resuspended in cold $CaCl_2$ (60 ml, 0.1 M), and incubated on ice (30 min).

The cells were pelleted as above, resuspended in cold $CaCl_2$ (0.1 M in 12.5% v/v G), and incubated on ice overnight.

Those cells not required for use straight away were fast frozen in aliquots (0.5 ml) in dry ice / IMS, and stored (-70°C).

To transform, DNA (1-10 μl) was added to an aliquot of the competent cells (100 μl), mixed gently by inversion, and incubated on ice (30 min). The cells were heat-shocked (2 min, 42°C), incubated on ice (5 min), L-broth was added (900 μl) and the tube incubated (45 min, 37°C) for phenotypic recovery. The cells were spread over L-agar containing a suitable selective agent and incubated overnight to allow the transformed cells to grow up.

Epicuran coli™ AG1 competent cells were transformed in accordance with the suppliers instructions.

3.4.2 Yeast Strains

3.4.2.1 Lithium Acetate Method

Yeast strains were made competent and transformed using a modified method of Ito *et al.* (1983a).

Sterile plastic tubes were used throughout.

The recipient strain was incubated overnight in YEPD broth (10 ml), subcultured into fresh YEPD (50 ml) to give a cell density of 10^6 ml^{-1}, and incubated until cell density reached 10^7 ml^{-1}.

The cells were harvested by centrifugation (5000 rpm, 5 min, RT), washed with TE buffer (10 ml), resuspended in 0.1 M lithium acetate in TE (5 ml), and incubated in a rotary shaking air incubator (1 h, 28°C).

The cells were harvested as above, resuspended in 0.1 M lithium acetate, 15% v/v G in TE buffer (5 ml), dispensed into aliquots (0.3 ml), fast frozen in dry ice / IMS, and stored (-70°C).

To transform, 50% w/v PEG 4000 (700 μl) was added to each aliquot of competent cells with the DNA (10-20 μg). The tube was mixed by inversion, and incubated (1 h, 30°C). The cells were heat-shocked (5 min, 42°C), and aliquots (0.2 ml) spread over dry selective plates. When using a dominant selectable marker 2 volumes of YEP broth containing 4% w/v D was added to each transformation and incubated (2 h, 28°C) prior to plating out.

3.4.2.2 Polyethylene Glycol Induced Transformation

Yeast strains were made competent and transformed using a modified method of Klebe *et al.* (1983).

The recipient strain was incubated overnight in YEPD broth (10 ml), subcultured into fresh YEPD (20 ml per transformation), and incubated until the culture reached 0.600 (A_{600} against a blank of YEPD).

The cells were harvested by centrifugation (1500 rpm, 10 min, RT), resuspended in solution A (5 ml; 1.25 M KCl, 30 mM $CaCl_2$, 10 mM bicine pH 8.35, 3% v/v ethylene glycol, 5% v/v dimethyl sulphoxide), incubated (10 min, RT), pelleted as above, resuspended in solution A (0.2 ml) and frozen in dry ice / IMS. The cells were kept frozen for a minimum of 10 min. The competent cells can be stored for 1 month (-70°C), or used immediately.

DNA (10-20 μg) was added to the cells which were thawed by rapid agitation in a shaking waterbath (5 min, 37°C). 40% v/v PEG 1000, 0.2 M bicine (1.5 ml) was mixed in gently, and incubated (1 h, 28°C) mixing gently every so often to ensure the cells did not settle out.

The cells were harvested as above, resuspended in YEP-broth with 4% w/v D and incubated (2 h, 28°C) prior to plating out over selective plates.

3.5 DNA HYBRIDISATION TECHNIQUES

3.5.1 Preparation of Radioactive Probes

3.5.1.1 5'-Labelling of Oligonucleotide Probes Using T4 Polynucleotide Kinase

The oligonucleotide (0.5 μl of a 1 $mgml^{-1}$ solution), $MgCl_2$ (10 μl, 50 mM), tris-HCl pH 7.6 (5 μl, 1 M), 2-mercaptoethanol (5 μl, 10.2 M), and γ-^{32}P ATP (5 μl, 50 μCi, high specific activity) were placed into a 1.5 ml Eppendorf tube with water (to a final volume of 50 μl), and T4 polynucleotide kinase (10 units), and incubated (1 h, 37°C).

Incorporation of the label was checked by spotting the probe (1 μl) on to a strip of DE81 paper about 2 cm from the end, then eluting the chromatogram using ammonium formate (0.3 M). The solvent front was run 8 cm past the sample spot. The paper was wrapped in Saran Wrap, and exposed to X-ray film (20 min). Percentage incorporation was estimated from the intensity of the spots (incorporated material stays at the origin, and unincorporated nucleotides move up the paper). If incorporation was >50% then the probe was ready for use without further modification.

3.5.1.2 Hexanucleotide Priming of dsDNA Probes Using the Klenow Fragment of DNA Polymerase I

Probes were labelled as described by Feinberg and Vogelstein (1983).

The DNA (25 ng in 16 μl) was boiled (1 min) in a 1.5 ml Eppendorf tube, centrifuged briefly to pool the solution in the bottom of the tube, and placed on ice. OLB buffer (5 μl, section 2.3.4), and bovine serum albumin (1 μl of a 10 mgml^{-1} solution) were dispensed into a fresh 1.5 ml Eppendorf tube, and the boiled DNA was added, followed by α-^{32}P dCTP (2.5 μl, 10 μCiμl^{-1}). The contents of the tube were mixed by vortexing briefly, and brought back to the bottom of the tube by centrifuging briefly. Klenow fragment (0.5 μl, 2 units) was added and mixed in very gently by pipetting up and down. The tube was incubated (20 h, RT), and the reaction stopped by the addition of stop buffer (100 μl, section 2.3.4).

Unincorporated nucleotides were removed by passing the probe through G50 sephadex in TE buffer, and collecting 0.5 ml aliquots. These samples were Cerenkov counted in a Scintillation counter which gives two peaks of high radioactivity. Four samples with high counts were selected from the first peak, pooled, and boiled (5 min) prior to use.

3.5.2 Transfer and fixing of DNA to Nitrocellulose and Nylon Filters

3.5.2.1 Colony Hybridisation

This was performed essentially as described by Grunstein and Hogness (1975).

Nitrocellulose filters were placed onto a blank agar plate to pre-wet them prior to being placed on an L-agar plate containing ampicillin (100 μgml^{-1}; LAp).

If only a small number of *E. coli* transformants were to be screened (method 1), they were picked over two filters in a regular array, and also on to another plate from which the "positives" could be recovered. Positive and negative control colonies were also included.

For screening large numbers of transformants the method of Woods, D. (1984; method 2) was used. Four 9 cm Whatman No. 1 filters were placed in a Buchner funnel, wetted with L-broth, and excess liquid removed under vacuum. A filter (pre-wetted if nitrocellulose) was placed on top of the filter papers. The transformants to be screened were titered and suitably diluted in L-broth. An aliquot of this dilution (5 ml) containing 2000-5000 cells was pipetted evenly over the filter, and the liquid filtered through under vacuum. The filter was placed on an L-agar plate containing the selective antibiotic, and incubated until the colonies were visible. The filter was pierced with a sterile needle for identification and orientation, and two replicas were made. The original filter was replaced on its plate, and stored (4°C).

All filters (methods 1 and 2) were placed onto LAp plates, and incubated (5-6 h, 37°C) until the colonies were easily visible. The filters were transferred to L-agar containing chloramphenicol (150 μgml^{-1}) and incubated (overnight, 37°C).

The filters were placed colony side up on filter paper soaked in denaturing solution (section 2.3.5, 7 min), transferred to filter paper soaked in neutralising solution (section 2.3.5, 3 min), transferred to fresh filter paper soaked in neutralising solution (3 min), rinsed in 2x SSC (section 2.3.1), and finally blotted between two sheets of filter paper.

The DNA was fixed to nitrocellulose filters by baking (2 h, 80°C, under vacuum), and to Hybond-N nylon filters by air drying, wrapping the filter in Saran Wrap, placing colony side down on a transilluminator, and exposing to long wave UV light (30 s).

Prior to hybridisation the filters were pre-washed in a large volume of 3x SSC, 0.1% w/v SDS (2 h, 65°C), the wash being changed several times during this period.

3.5.2.2 Dot-blotting

For a small number of samples the DNA was boiled (5 min), and chilled on ice (2 min). The Hybond-N filter was placed on to a filter paper, and the samples spotted on in aliquots (2 μl). Each spot was allowed to dry between the application of each aliquot.

Larger numbers of samples were denatured in 0.5 M NaOH (200 μl, 10 min, 37°C) in a microtitre plate, and transferred to Hybond-N with a Bio Rad Bio-Dot apparatus used exactly according to the manufacturers instructions.

Filters were wetted in denaturing solution (1 min), neutralising solution (1 min), air dried, and fixed as described above (section 3.5.2.1).

3.5.2.3 Southern Blotting

This was performed essentially as described by Southern, E. M. (1975).

The DNA to be blotted was run on a 0.7% agarose gel and photographed (section 3.3.5). The gel was shaken gently in denaturing solution (30 min), rinsed with water and shaken gently in depurinating solution, (0.25 M HCl; 10 min), rinsed and shaken gently in neutralising solution (30 min), and finally rinsed and shaken gently in 20x SSC (20 min).

Several sheets of blotting paper were soaked in 20x SSC and supported on a solid surface with their ends resting in a reservoir of 20x SSC. The gel was placed on top of this ensuring that no air bubbles were trapped between the gel and the blotting paper. Strips of parafilm were placed around the edges of the gel to prevent the buffer passing directly from the blotting paper to the wick. Nitrocellulose or Hybond-N was cut to fit exactly over the gel. Nitrocellulose was wetted in 2x SSC before being placed on top of the gel. Hybond-N was wetted in 20x SSC by being floated on the surface (1 h) as advised by Khandjian (1987). Again ensuring that no air bubbles were trapped between the gel and the filter. Three pieces of blotting paper cut to size and soaked in 20x SSC were placed on top of the filter, and a stack of paper towels was placed on top of this. A weight was placed on top and blotting allowed to proceed (overnight, 4°C).

The filters were removed, rinsed in 2x SSC, and the DNA fixed as described above (section 3.5.2.1).

3.5.2.4 Vacu-Blotting

The DNA to be blotted was run on an 0.5% w/v Sea Kem HGT, 1.5% w/v NuSieve agarose gel, and photographed as described above (section 3.3.5).

The DNA was transferred to a Hybond-N filter using a Pharmacia LKB Bromma 2016 vacugene vacuum blotting apparatus used exactly according to the manufacturers instructions.

3.5.3 Hybridisation Conditions

Prehybridisation and hybridisation were performed as described by Maniatis *et al.* (1982).

Filters to be hybridised were placed in a sandwich box with a minimal volume of prehybridisation solution (section 2.3.5), and incubated with gentle shaking (at least 2 h, 65°C for dsDNA probes, 42°C for oligonucleotide probes).

dsDNA probes were boiled (2 min) before being added directly to the prehybridisation solution and incubated with gentle shaking (16 h, 65°C). Oligonucleotide probes were added directly to the prehybridisation solution without boiling and incubated with gentle shaking (16 h, 42°C).

Filters hybridised to dsDNA probes were washed twice with 2x SSC (500 ml, 15 min, 65°C), once with 2x SSC, 0.1% w/v SDS (500 ml, 30 min, 65°C), and once with 0.1x SSC (50 ml, 10 min, 65°C). Filters hybridised to oligonucleotide probes were washed twice with a large volume of 6x SSC, 0.05% w/v sodium pyrophosphate (1 h each), the first wash at the hybridisation temperature (42°C), and the second wash at the hybridisation temperature but with the buffer prewarmed to 5°C below the oligonucleotide T_m (50°C).

Washed filters were blotted dry, wrapped in Saran Wrap and autoradiographed.

3.5.4 Autoradiography

Autoradiography was performed at -70°C in a Kodak X-ray cassette with intensifying screens. X-ray film (Kodak XAR-5, or Fuji RX) was exposed to the filter (30 min to several days). Films were developed using an Agfa-Gevert Gevamatic 60 automatic film developer.

3.5.5 Removal of Probes from Hybond-N filters

Radioactive probes were removed from hybond-N filters by one or other of the two protocols described by the manufacturers (Membrane transfer and detection methods, Amersham International). Removal was checked by autoradiography, and the filters stored at 4°C.

3.6 DNA SEQUENCING IN M13

3.6.1 Template Preparation

M13mp18 was digested to completion with *Eco* RI and *Sal* I (section 3.3.1.1), dephosphorylated with calf intestinal alkaline phosphatase (section 3.3.2), and ligated to donor DNA with compatible ends (section 3.3.3).

Competent *E. coli* NM522 (300 μl, section 3.4.1) were added to each ligation, incubated on ice (30 min), heat shocked (2 min, 42°C), and incubated on ice (5 min). The transfection was transferred to a pre-warmed test tube and a log-phase culture of *E. coli* NM522 in 2x YT broth (200 μl, section 2.2.2), 0.1 M IPTG (20 μl, section 2.2.3.5), 2% w/v X-gal (80 μl, section 2.2.3.5), and BBL-soft agar (6 ml, section 2.2.2) were added, the tube was rotated to mix the contents without forming bubbles, the contents poured as a top layer over BBL-agar plates (section 2.2.2), and the plates incubated (overnight, 37°C).

A culture of NM522 in mid-log phase was diluted 100 fold with 2x YT, and aliquots (5 ml) dispensed into NUNC universal tubes. A white plaque from the transfection plates was picked into each aliquot by piercing the centre of the plaque with a sterile toothpick then dropping the toothpick into one of the prepared tubes. A blue plaque was also picked as a control. The tubes were incubated with vigourous shaking (6 h, 37°C).

Cells from an aliquot of each culture (1.5 ml) were pelleted by centrifugation (12 000 rpm, 10 min), and the supernatant (1.2 ml) drawn off into a fresh 1.5 ml Eppendorf tube without disturbing the cell pellet. 20% w/v PEG 6000, 2.5 M NaCl (300 μl) was added, mixed, and incubated (overnight, 4°C). The remainder of each culture was stored (4°C) as a stock of phage.

Precipitated phage were pelleted by centrifugation (12 000 rpm, 10 min), and the supernatant drawn off with a Pasteur pipette which had been drawn out to a fine point. This procedure was repeated to ensure all the PEG had been removed. TE buffer (100 μl) was added to each pellet, the tubes incubated (RT, 15 min), vortexed, incubated (RT, 5 min), and revortexed to resuspend the pellet, which was extracted twice with a half volume of phenol, and once with a half volume of 24:1, chloroform : isoamyl alcohol. The phage DNA was precipitated with three volumes of 0.3 M ammonium acetate in 95% E, pelleted, and air-dried as described above (section 3.2.2.1), then resuspended in TE buffer (25 μl).

10x loading buffer was added to an aliquot (1 μl) of each template preparation which were analysed by electrophoresis on an 0.8% agarose gel, including an aliquot from the template preparation from a blue phage (no insert DNA) to determine which preparations had insert DNA.

3.6.2 Sequencing.

Sequencing of templates was performed using a sequenase™ kit purchased from Amersham International, exactly according to the manufacturers instructions, using dGTP rather than dITP with reaction conditions adjusted for reading beyond 300 nucleotides.

6% w/v acrylamide, 8 M urea in 1x TBE (50 ml), 10% ammonium persulphate (250 μl), and TEMED (25 μl), were mixed and poured between scrupulously clean, sealed glass plates, and allowed to set at RT to form a 0.4 mm sequencing gel. The gel was pre-run in 1x TBE (1 h, 1200 V), and the wells washed out with 1x TBE prior to loading the samples. Half of each sample was loaded and run (4 h, 1200 V), then the second half of each sample were loaded into the remaining wells and the gel run (4 h, 1200 V). The gel was dried under vacuum (1 h, 80°C), and autoradiographed at RT overnight using Kodak XAR-5 X-ray film.

3.7 MUTAGENESIS OF *CANDIDA TROPICALIS*

3.7.1 Exposure to Nitrous Acid

Cells were exposed to nitrous acid as described by Kakar *et al.* (1983) with modifications.

The cell density of overnight cultures of *C. tropicalis* NCYC547 and NCYC997 in YEPD broth (10 ml) were calculated using a haemocytometer. An aliquot of culture containing 2.5 x 10^8 cells was harvested by centrifugation (4000 rpm, 10 min), and the pellet washed with sodium acetate buffer pH 5.0 (1 ml, 0.1 M). The washed cell pellets were resuspended in sodium acetate buffer pH 5.0 (0.2 M), or sodium acetate buffer pH 5.0 (0.2 M); dimethyl sulphoxide (DMSO; 5% v/v). Sodium nitrite (0.6 M) was added (0.06 M final concentration), the reaction mixed, and incubated at RT. To stop the reaction the mixture was diluted 1:10 with potassium phosphate buffer pH 7.5 (0.1 M), washed with sodium chloride solution (0.9% w/v), resuspended in YEPG broth (1 ml, section 2.2.3.1), added to YEPG (50 ml) and incubated (40 h) to allow recovery and segregation (Kamiryo *et al.* 1977). Cells were not UV-irradiated following exposure to nitrous acid.

Kill curves were produced as described by Gibbons and Howard (1986) using the above method with and without 5% v/v DMSO, with no recovery time. Aliquots (0.1 ml) of the reaction mixture were removed into potassium phosphate buffer (0.1 M) at 5 min intervals, including one removed immediately prior to adding the sodium nitrite. These samples were serially diluted, plated over YEPD-agar plates, and incubated (72 h).

3.7.2 Exposure to Ultra-Violet Irradiation

The cell densities of overnight cultures of *C. tropicalis* NCYC547 and NCYC997 in YEPD broth (10 ml) were calculated using a haemocytometer. The cells were harvested by centrifugation (4000 rpm, 10 min), and the pellet washed with water (5 ml). The washed cell pellets were resuspended in 0.9% w/v sodium chloride to give a concentration of 10^8 cells ml^{-1}, and an aliquot (1 ml) was exposed to UV-irradiation (0.5 $Jm^{-2}s^{-1}$) in a small plastic petri dish with the lid removed. Exposed cells were allowed to recover in YEP-broth with 4% w/v D (10 ml, 2 h, 28°C).

Kill curves were produced by exposing aliquots of cells as described above to UV-irradiation (0.5 $Jm^{-2}s^{-1}$) for varying amounts of time, allowing each aliquot to recover in YEP with 4% D as described above, serially diluting, plating over YEPD-agar plates, and incubating (72 h).

3.7.3 Screening for Auxotrophic Mutants without Enrichment or Positive Selection

To screen for mutants 10^8 cells were exposed to nitrous acid allowing 20% survival (25 min), or to UV-irradiation (5 min). Cells were spread over YEPG agar and incubated (72 h, 28°C). The colonies were screened for red adenine mutants and then picked onto SD and YEPD agar over grids and incubated to screen for other auxotrophs. G was provided as the carbon source to eliminate petite mutants.

3.7.4 Positive Selections for the Isolation of Specific Mutations

3.7.4.1 α-Amino Adipic Acid

α-amino adipic acid medium (AA, section 2.2.1) was used to select for *lys2* mutants (Chattoo *et al.*, 1983). Cultures growing exponentially in YEPD broth were serially diluted, spread over SD, and AA-agar plates, and incubated (72 h) to ascertain whether or not *C. tropicalis* was sensitive to α-amino adipic acid. *S. cerevisiae* X-2180-1A was used as a control.

3.7.4.2 L-Canavanine Sulphate

L-canavanine sulphate (Can, section 2.2.3.3) was used to select for Can resistant (Can^R) mutants. Cultures growing exponentially in YEPD broth were serially diluted, spread over SD, and SDCan-agar plates containing various concentrations of Can (Ahmad and Bussey, 1986), and incubated (72 h) to ascertain whether *C. tropicalis* was sensitive to Can. *S. cerevisiae* X-2180-1A was used as a control.

C. tropicalis NCYC547 exposed to nitrous acid (15 min) was serially diluted and spread over SDCan and YEPG agar. A culture of unexposed cells was also serially diluted and plated out. Plates were incubated (72 h) and then checked for the appearance of Can^R mutants. Exposure to nitrous acid for 15 min allows 50% cell survival, which Gradova and Robysheva (1980) found gave a higher proportion of *Candida* mutants.

3.7.4.3 5-Fluoro Orotic Acid

5-fluoro orotic acid (FOA, section 2.2.1) was used to select for *ura3* mutants (Boeke *et al.*, 1984; Boeke *et al.*, 1987). Cultures of *C. tropicalis* NCYC547 and NCYC997 growing exponentially in YEPD broth were serially diluted, spread over YEPD, and FOA-agar plates, and incubated (72 h) to ascertain whether or not *C. tropicalis* was sensitive to FOA. *S. cerevisiae* strains YNN27 and X-2180-1A were used as controls.

C. tropicalis NCYC997 was exposed to nitrous acid with 5% v/v DMSO (25 min), or UV-irradiated (5 min), and aliquots containing 1.5×10^5 viable cells (assessed by serially diluting the exposed culture and plating known numbers on YEPD) were spread over SD and FOA-agar. Plates were incubated (72 h) and checked for the appearance of FOA^R mutants.

3.7.5 Stability of *Candida tropicalis* Auxotrophic Mutants

YEPD-broth (100 ml) was inoculated with the mutant strain and incubated (22.5 h). The spontaneous reversion rate was established by plating 10^9 cells from this culture over four SD plates and counting the number of colonies that grew. This procedure was repeated four times using cells from the YEPD culture to inoculate fresh YEPD each time, cells plated out from the final culture had therefore been growing without selection for a total of 90 h. Each revertant was retested and confirmed by streaking over SD, FOA, and semi-defined medium supplemented with uracil (U; 2 $mgml^{-1}$, section 2.2.3.2), and 2% w/v R (section 2.2.3.1).

3.8 MINIMUM INHIBITORY CONCENTRATIONS OF POTENTIAL DOMINANT SELECTABLE MARKERS

Cultures growing exponentially in YEPD-broth were serially diluted, and aliquots containing about 1000 cells spread over agar-plates containing various amounts of the substance being tested. Cell density was calculated from haemocytometer readings. The plates were incubated (28°C, 72 h). The number of colonies on each plate was counted to ascertain the minimum inhibitory concentration.

3.9 PROTEIN METHODOLOGY

3.9.1 Extraction of Proteins from Whole Cells

Protein was extracted from 50 ml cultures, inoculated from overnight YEPD-broth cultures (10 ml) and grown up until cell density was 10^8 cells ml^{-1}, using a modified small scale method for preparation of yeast cell crude extracts (Herrero, Leicester Biocentre Laboratory Manual).

Two successive aliquots (20 ml) were harvested by centrifugation (4000 rpm, 10 min) in NUNC universal tubes. The cell pellet was transferred to, and re-harvested in a pre-weighed 1.5 ml Eppendorf tube for wet weight determination. The cells were resuspended in 0.1 M Tris-HCl pH 8.5, 10 mM EDTA pH 8.5, 10 mM 2-mercaptoethanol (100 mg wet weight ml^{-1}). An aliquot (1 ml) of this suspension was pelleted in a fresh 1.5 ml Eppendorf tube (8000 rpm, 2 min), and the cell pellet resuspended in lysis buffer (100 μl, section 2.3.6). 10mM phenylmethylsulphonyl fluoride (PMSF, 12.5 μl in isopropanol), 5x protease inhibitors (25 μl, section 2.3.6), and 0.45 mm diameter glass beads (700 mg) were added to the tube. The tube was cooled on ice (10 min) then vortexed (2 min). This was repeated for three cycles of cooling and vortexing. The tube was punctured with a fine needle, placed in a small falcon tube, and the lysate was collected by centrifugation (1500 rpm, 10 min). After the addition of an equal volume of 2x sample buffer (section 2.3.6), the lysate was boiled (5 min), centrifuged (12000g, 5 min), the supernatant transferred to a fresh Eppendorf tube, and stored (-20°C) to minimise proteolysis. This procedure denatures the proteins, and removes cell debris and high molecular weight carbohydrate (Hames and Rickwood, 1981).

The amount of protein present in a lysate was determined by a Bio-Rad Protein assay, which was performed exactly according to the manufacturers instructions.

3.9.2 Sodium Dodecylsulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A "Mighty Small" miniature slab gel electrophoresis unit SE200 (manufactured by Hoeffer Scientific Instruments) was used as directed in the instruction manual.

A 15% polyacrylamide separating gel was prepared by mixing separating gel buffer (2.5 ml, section 2.3.6), acrylamide stock solution (1.7 ml, section 2.3.6), water (0.7 ml), 10% w/v ammonium persulphate (0.125 ml), and TEMED (2.5 μl), poured, covered with a layer of isopropanol, and allowed to set at RT. The isopropanol was washed off with water and the top of the gel blotted dry. A stacking gel was prepared by mixing stacking gel buffer (2.5 ml, section 2.3.6), acrylamide stock solution (0.75 ml), water (1.7 ml), 10% w/v ammonium persulphate (50 μl), and TEMED (2.5 μl), poured on top of the 15% polyacrylamide (SDS/PAGE) separating gel, and allowed to set at RT.

Samples were boiled (5 min), and the sample wells were cleaned out extensively with electrophoresis buffer (section 2.3.6) before loading. Samples were loaded as the smallest possible volume (typically 1-4 μl), and empty wells were loaded with 2X sample buffer (2 μl) to prevent 'smiling' (Hames and Rickwood, 1981). The best volume of each extract to load was determined by running and silver staining a trial gel.

3.9.3 Silver Staining

Silver staining was performed as described by Wray *et al.* (1981).

The gel was soaked in 2-3 volumes (50 ml) of 50% methanol (RT, 1 h) to fix the bands and remove glycine from the gel.

Silver nitrate, $AgNO_3$ (0.8 g) was dissolved in water (4 ml). Concentrated aqueous ammonia (1.4 ml, 880 ammonia) was added to 0.36% w/v sodium hydroxide (21 ml) in a widemouth Erlenmeyer. The silver nitrate solution was added dropwise whilst gently swirling the contents of the flask, and the stain made up to its final volume with water (74 ml). This stain was used within 5 min of preparation.

The gel was soaked in the stain with gentle agitation (15 min), rinsed with water, and soaked in water (5 min). Developer was prepared whilst the gel was soaking by mixing water (500 ml), 1% w/v citric acid (2.5 ml), and formaldehyde (0.25 ml). The gel was again rinsed with water, and agitated gently in the developer to develop the stained bands. Just before the optimum band intensity was reached the developer was replaced with 50% methanol to stop the reaction.

The stained gel was placed on a light box and photographed as previously described (section 3.3.5.1).

3.9.4 Assay for Orotidine-5'-Phosphatase Activity

This assay was based on the procedures described by Beckwith *et al.* (1962), and Lacroute (1968).

Proteins were extracted from 50 ml cultures of the test strains using the method described in section 3.9.1, except that the harvested cells were washed, resuspended and lysed in Tris-HCl pH 8.6 (0.1 M), $MgCl_2$ (1.7 M). PMSF (5 μl, 10 mM in isopropanol) was added along with the glass beads but not the 5x protease inhibitors. After collection by centrifugation into falcon tubes, the lysates were transferred into 1.5 ml Eppendorf tubes and centrifuged (13 000 rpm, 10 min, 4°C) to pellet any debris that might interfere with A_{285} readings.

The cleared lysates were made up to 2.4 ml with Tris-HCl pH 8.6 (0.1M), $MgCl_2$ (1.7 M), and divided between two quartz cuvettes. Changes in A_{285} at RT over time, after the addition of orotidine-5' monophosphate (10 μl, section 2.2.3.2) to one cuvette, the other half of each sample being used as a blank, were monitored on a Shimadzu UV-visible recording spectrophotometer UV-240. Buffer alone was monitored as a control.

The amount of protein in each lysate was ascertained by a Bio-Rad assay.

CHAPTER 4. CLONING LONG CHAIN FATTY ACID ACYL-CoA OXIDASE II FROM *CANDIDA TROPICALIS* NCYC997

4.1 INTRODUCTION

4.1.1 Aims

The purpose of the work described in this chapter was to clone the long-chain fatty acid acyl-CoA oxidase (*POX*) genes of *C. tropicalis* NCYC997 from a genomic DNA library as the first step towards creating a non-revertible β-oxidation mutant by gene disruption or transplacement to optimise the biotransformation of pelargonate [$CH_3(CH_2)_7COOH$] to azeleate [$HOOC(CH_2)_7COOH$].

4.1.2 Alkane Metabolism

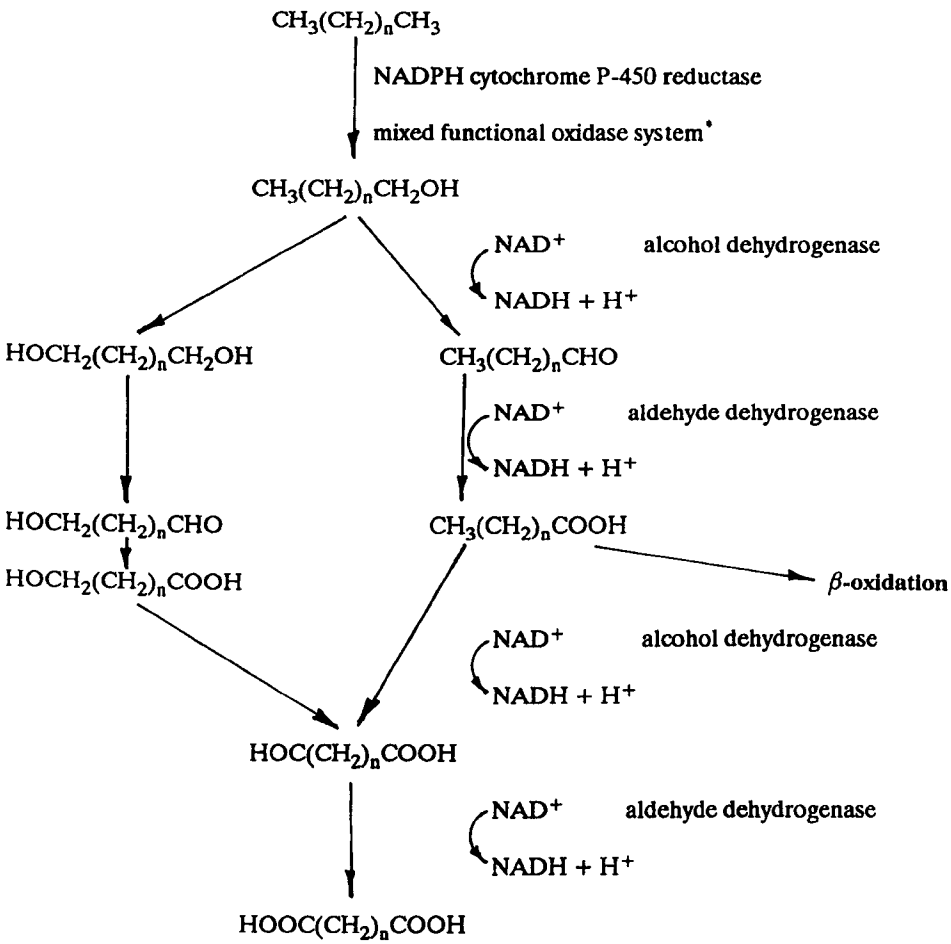
4.1.2.1 Uptake Mechanisms

Alkanes are taken up through direct contact between alkane droplets and microbial cells, via accommodated alkane phase and via pseudo-solubilised alkanes. Alkanes are insoluble, and cells produce emulsifying substances to convert them into droplets of 0.01-0.5 μm to facilitate their passage through the cell wall. An emulsifying factor has been detected in the culture medium of *Candida petrophilum*. *C. tropicalis* builds up microemulsions of n-hexadecane by synthesis of surface active substances (Bühler and Schindler, 1984; Crueger and Crueger, 1984).

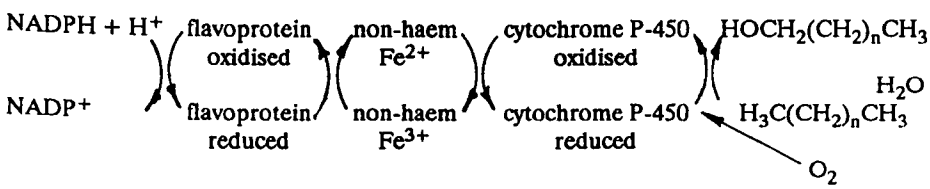
The alkane microemulsion adheres by a non-enzymatic mechanism to the cell wall of *C. tropicalis*. Adhesion is aided by an alkane-induced lipopolysaccharide-fatty acid complex located at the cell surface. The lipopolysaccharide is a mannan containing approximately 4% covalently linked fatty acids which may provide a more hydrophobic region through which the lipophillic substances permeate more easily. n-Alkane-grown cells of *C. tropicalis* contain twice as many lipids as glucose-(D) grown cells which exhibit a 25% lower adsorption capacity (Fukui and Tanaka, 1981; Bühler and Schindler, 1984).

Figure 4.1 **Oxidation of n-alkanes to fatty acids in *C. tropicalis***

Alkanes are degraded to primary alcohols via a mixed functional oxidase system. Primary alcohols are further degraded via aldehydes to monocarboxylated fatty acids catalysed by alcohol and aldehyde dehydrogenases as shown on the right (α-oxidation). These monocarboxylated fatty acids are either degraded via β-oxidation (*figure* 4.2), or undergo ω-oxidation to form dicarboxylic acids (Fukui and Tanaka, 1981; Rehm and Reiff, 1981; Bühler and Schindler, 1984). Dicarboxylic acids are also produced in far lower amounts via α,ω-diols as shown on the left (Yi and Rehm, 1982c). Dicarboxylic acids are excreted into the growth medium (some undergo β-oxidation).



* Mixed functional oxidase system for the primary oxidation of alkanes (Rehm and Reiff, 1981).



The formation of canals, pores, protrusions, membrane vesicles, and intricate membrane complexes has been observed in the cell walls of various hydrocarbon-grown microorganisms. Electronmicroscope observation has revealed special channels in the cell wall of alkane-grown cells of *C. tropicalis* and, at an early phase of growth, similar channels accompanied by slime-like outgrowths have also been detected together with protrusions on the cell surface. The slime-like outgrowths reach the cell membrane through electron dense channels which have ER arranged regularly beneath them. It has been suggested that alkanes attached to the protrusions (or slime-like outgrowths) migrate through the channels to the ER, the site of alkane hydroxylation. The protrusions are not alkanes or lipids attached to the cell wall, as washing with organic solvents or detergents fails to remove them (Fukui and Tanaka, 1981; Bühler and Schindler, 1984). Hydrocarbons accumulate at the interfaces between hydrophobic and hydrophillic regions after penetrating the cell wall (Bühler and Schindler, 1984).

4.1.2.2 Mono-Terminal Oxidation to Fatty Acids

Alkane molecules are susceptible to oxidation at either or both termini (mono-terminal or di-terminal oxidation), sub-terminal or internal positions of the carbon chain (Fukui and Tanaka, 1981; Crueger and Crueger, 1984). Mono-terminal oxidation of n-alkanes, fatty alcohols and fatty acids is predominant and diterminal oxidation proceeds as a minor reaction in *C. maltosa* and other n-alkane-utilising yeasts (Fukui and Tanaka, 1981; Rehm and Reiff, 1981; Crueger and Crueger, 1984; Blasig *et al.*, 1988). *Candida*, *Saccharomycopsis*, *Endomycopsis*, *Pichia* and *Torulopsis* yeasts infrequently form intermediates in the 2-position. Sub-terminal oxidation other than at the 2-position has not been detected (Rehm and Reiff, 1981). Most yeasts oxidase alkanes very quickly.

Three mechanisms have been reported for n-alkane oxidation: hydroxylation by a mono-oxygenase system (mixed function oxidase); dehydrogenation to the corresponding alkene followed by hydration; and hydroperoxidation via a free-radical mechanism and subsequent reduction to alcohols (Fukui and Tanaka, 1981; Bühler and Schindler, 1984).

In mono-terminal oxidation, the corresponding mono-carboxylic acid is produced by hydroxylation to the primary alcohol followed by two oxidative steps via the aldehyde, mediated by NAD^+-linked long-chain-alcohol and aldehyde dehydrogenases (*figure* 4.1). The fatty acids formed from the alkanes can be oxidised to acetyl-CoA units via β-oxidation (Fukui and Tanaka, 1981; Rehm and Reiff, 1981; Bühler and Schindler, 1984; Crueger and Crueger, 1984).

The formation of the primary alcohol, the first step of n-alkane degradation in bacteria, moulds and alkane-utilising yeasts is catalysed by a complex mixed-functional mono-oxygenase (hydroxylase) system (Bühler and Schindler, 1984; Blasig *et al.*, 1988). In *C. tropicalis*, *C. guilliermondii* and *Cunninghamella bainieri* this hydroxylase system consists of cytochrome P-450, $NADPH_2$-cytochrome c (cytochrome P-450) reductase, and a heat stable phospholipid and is presumed to be analogous to the rat liver or rabbit liver microsomal systems (Fukui and Tanaka, 1981; Rehm and Reiff, 1981; Bühler and Schindler, 1984).

The involvement of cytochrome P-450 rather than rubredoxin can be demonstrated by the characteristic spectra of its oxidised and reduced state, its sensitivity to carbon monoxide, and its insensitivity to cyanide. The reaction requires NADPH and molecular oxygen. The latter is incorporated into n-alkanes to give the corresponding primary alcohol of the same chain length as the substrate, (Bühler and Schindler, 1984; Blasig *et al.*, 1988).

Long-chain alkanes, alkenes, secondary alcohols, and ketones induce the formation of P-450 and NADPH-cytochrome c-reductase in *C. tropicalis*. The cytochrome P-450 concentration increases linearly with increasing specific hexadecane uptake rate, especially under conditions of oxygen limitation. In consequence, cytochrome P-450 may be the rate-limiting step of alkane uptake and oxidation (Rehm and Reiff, 1981).

Alcohol and aldehyde dehydrogenases are inducible by alkanes, long-chain alcohols or aldehydes. Alcohol dehydrogenase from *C. tropicalis* is NAD^+-dependent. The long-chain alcohol and aldehyde dehydrogenases of *C. tropicalis* have no or only weak activities on substrates with a chain length of C_{15} or more, although the yeast can assimilate C_{15}-C_{19} alkanes. Alcohol dehydrogenase has a preference for C_{11}-C_{16} alcohols as substrate (Fukui and Tanaka, 1981; Rehm and Reiff, 1981; Bühler and Schindler, 1984).

The oxidation of long chain primary alcohols in *C. maltosa* occurs without addition of $NADP^+$ and is accompanied by stoichiometric oxygen consumption and hydrogen peroxide production, which suggests that an alcohol oxidase and not an alcohol dehydrogenase may catalyse these reactions in this yeast (Blasig *et al.*, 1988).

The fatty acid patterns show a relation to the number of carbon atoms in the n-alkanes, but this relationship may be blurred by additional diterminal pathways and oxidation products arising from the secondary alcohol (Rehm and Reiff, 1981).

4.1.2.3 Dioic Acid Formation

Diterminal oxidation (*figure* 4.1) can be observed in some bacteria and yeasts, leading to the production of the corresponding dicarboxylic acid, which is further broken down into acetate units and succinate by means of β-oxidation (Rehm and Reiff, 1981; Yi and Rehm, 1982a; Crueger and Crueger, 1984).

Dicarboxylic acids with a carbon chain length of C_{10} or greater are of interest as chemical intermediates, and there are many industrial patents for the production of dioic acids by microbial transformation of n-alkanes. Dodecanedioic acid (C_{12}) and brassylic acid (C_{13}) are manufactured on an industrial scale. Little information is available on intermediates and on the metabolism which leads to the formation of dioic acid from n-alkanes, and cell lipids give no indications regarding this pathway (Rehm and Reiff, 1981; Yi and Rehm, 1982a; Hill *et al.*, 1986).

Several groups have isolated yeast strains with a reduced ability to assimilate dioic acids: *C. tropicalis* 1230 and *C. tropicalis* S76 derived from *C. tropicalis* 1230 (Yi and Rehm, 1982a; Hill *et al.*, 1986); *C. cloacae*, and *Torulopsis candida* (Bühler and Schindler, 1984); *Torulopsis candida* (Ogata *et al.*, 1973); *Pichia* species Y-3 (Ogino *et al.*, 1965). These strains excrete large quantities of α,ω-dioic acids into the medium along with the 3-hydroxy derivatives of long-chain α,ω-dioic acids and dioic acids with a shortened carbon chain.

n-Dodecanol and n-dodecanoic acid were detected in the n-dodecane medium of *C. tropicalis* S76 (Yi and Rehm, 1982a). *C. tropicalis* S76 also produces α,ω-dodecanediol from n-dodecane and α,ω-tridecanediol from either n-tridecanol or n-tridecanoic acid along with other intermediates, monoic acid, ω-hydroxy acid and α,ω-dioic acid (Yi and Rehm, 1982b and c). This indicates that formation of α,ω-dioic acids in *C. tropicalis* can occur via the monoic acids where one methyl group is oxidised to the corresponding fatty acid followed by oxidation of the ω-methyl group (ω-oxidation, Yi and Rehm, 1982a; Buhler and Schindler, 1984), and that an alternative pathway exists via α,ω-diols which holds true for alkanes with even and odd carbon chain lengths (Yi and Rehm, 1982b and c).

C. tropicalis mutant S76 can convert oleic acid (O) to Δ^9-1,18-octadecenedioic acid by ω-oxidation of the terminal methyl group. This shows that the strain does not possess a marked substrate specificity to ω-oxidation of fatty acids, the terminal methyl group of both saturated acids and unsaturated acids can be attacked. The strain can also produce long-chain dioic acid from alkane derivatives such as alcohol, monoic acid, α,ω-diol, and α,ω-hydroxy acid (Yi and Rehm, 1988).

4.1.3 Fatty Acid β-Oxidation

4.1.3.1 Fatty Acid Activation

The key step for β-oxidation and other metabolic transformations of fatty acids is the synthesis of the acyl-CoA thioester at the expense of ATP catalysed by acyl-CoA synthetase (Bühler and Schindler, 1984). As acyl-CoA lies before the metabolic branch between esterification and oxidation, regulation of acyl-CoA synthetase does not provide independent control of either process. In rat liver, the conversion of acyl-CoA to acylcarnitine represents the rate-limiting step of fatty acid oxidation (Numa and Yamashita, 1974).

Peroxisomes isolated from alkane-grown *C. tropicalis* are able to degrade palmitate to acetyl-CoA in the presence of CoA and ATP, indicating the presence of acyl-CoA synthetase in these organelles (Fukui and Tanaka, 1981). Mannaerts *et al.* (1982) demonstrated the following: proliferated rat liver peroxisomes contain CoA available for the thiolase reaction in the peroxisomal matrix, but not for acyl-CoA synthetase; mechanical disruption of purified peroxisomes results in the release of catalase but not of acyl-CoA synthetase; acyl-CoA synthetase is not latent although at least one of its substrates has a limited membrane permeability; and pronase, a proteinase that does not penetrate the peroxisomal membrane, almost completely inactivates the acyl-CoA synthetase of intact peroxisomes. This evidence suggests that acyl-CoA synthetase is located in the peroxisomal membrane with its catalytic site facing the cytoplasm, and that the CoA ester is the species that has to cross the membrane. Acyl-CoA synthetase requires a chain length of C_8 or longer, and has only minimal activity with shorter-chain substrates (Tolbert, 1981).

Mishina et al. (1978) demonstrated that *C. lipolytica* acyl-CoA synthetase I is distributed among different subcellular fractions, including microsomes and mitochondria (active sites of phospholipid synthesis) where glycerolphosphate acyltransferase is located whereas, acyl-CoA synthetase II is localised in peroxisomes along with the acyl-CoA oxidising system. Mutants of *C. lipolytica* lacking either acyl-CoA synthetase I (Kamiryo *et al.*, 1977) or acyl-CoA synthetase II (Kamiryo *et al.*, 1979) have been isolated.

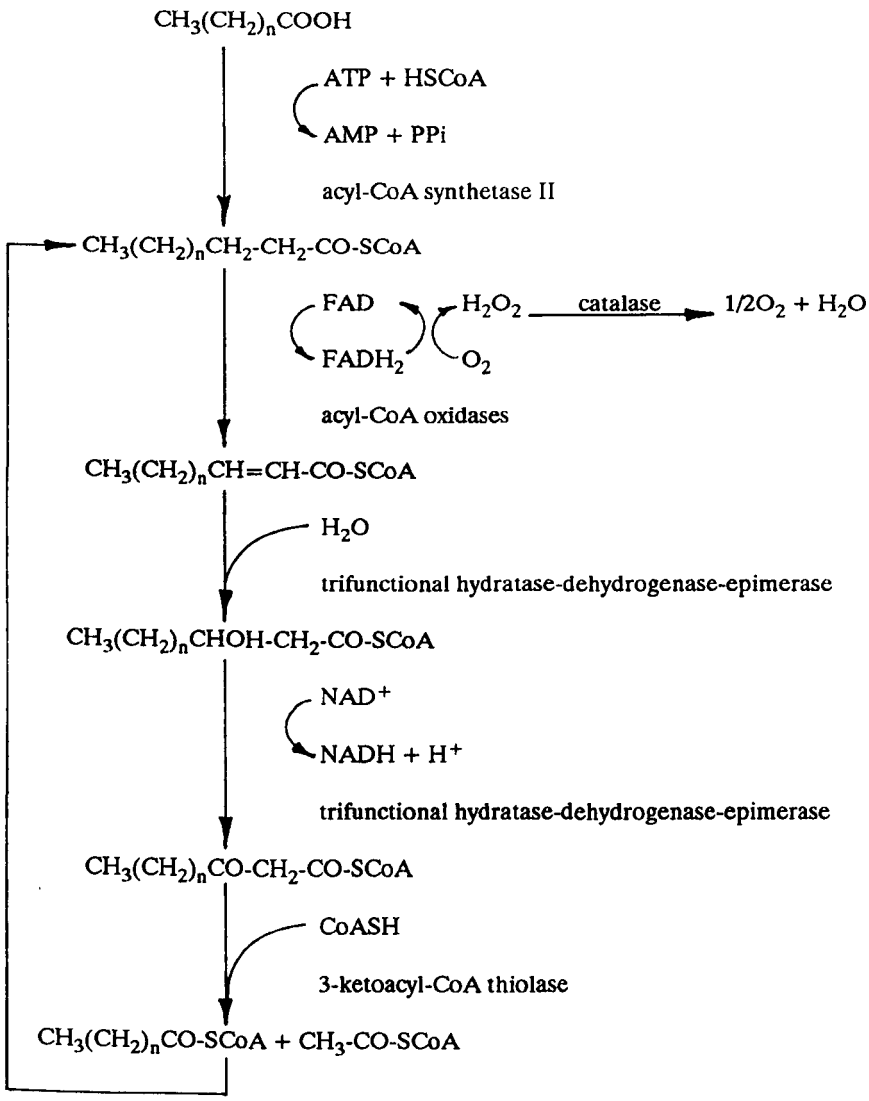


***Figure* 4.2 β-Oxidation sequence of long-chain fatty acids in peroxisomes of *C. tropicalis* (Fukui and Tanaka, 1981; Hashimoto, 1982)**

The mutants lacking acyl-CoA synthetase I are capable of growing on fatty acid or n-alkane as a sole carbon source but cannot incorporate exogenous fatty acid or the fatty acid derived from n-alkane as a whole into cellular lipids. They utilise fatty acid synthesised *de novo* from acetyl-CoA produced by degradation of exogenous fatty acid (Kamiryo *et al.*, 1977). The mutants lacking acyl-CoA synthetase II fail to grow on fatty acid as a sole carbon source but are capable of incorporating exogenous fatty acid into cellular lipids (Kamiryo *et al.*, 1979).

In contrast to acyl-CoA synthetase I which is constitutive and phosphatidylcholine-independent, acyl-CoA synthetase II is induced by fatty acid, exhibits a broad substrate specificity with respect to fatty acid, and is dependent on phosphatidylcholine (Fukui and Tanaka, 1981; Tanaka *et al.*, 1982).

C. tropicalis also has two long-chain acyl-CoA synthetases which differ in subcellular localisation and metabolic function, but neither enzyme is phosphatidylcholine-dependent (Yamada *et al.*, 1980). Yu and Hao (1986) have reported an inducible diacyl-CoA synthetase with activity towards dicarboxylic acids with chain lengths C_4-C_{16} from *C. tropicalis* 1230. Human cells have also been shown to possess two long-chain acyl-CoA synthetases differing in subcellular localisation and metabolic function (Wanders et al., 1988).

Peroxisomal acyl-CoA synthetase purified from rat liver was reported to be identical with the enzyme purified from microsomes and mitochondria, the same enzyme being distributed to microsomes, mitochondria, and peroxisomes (Hashimoto, 1982). Differences in the CoA requirement in the activation of different long chain fatty acids suggest that there may be two different activating enzymes in rat liver peroxisomes, as the properties of the enzyme that activated palmitate, stearate, and erucate are similar but distinct from that/those which activated oleate, linoleate, linolenate, and arachidonate (Pande and Mead, 1968).

Acyl-CoAs produced by acyl-CoA synthetase I in mitochondria are incorporated into lipids by the action of glycerol-3-phosphate acyl transferase whereas, acyl-CoAs formed in peroxisomes by acyl-CoA synthetase II are exclusively metabolised to yield acetyl-CoA (and propionyl-CoA from odd-chain alkanes or fatty acids) via the fatty acid β-oxidation pathway (Kamiryo *et al.*, 1977; Kamiryo *et al.*, 1979; Tanaka *et al.*, 1982).

4.1.3.2 β-Oxidation

A fraction of the fatty acids derived from alkanes is incorporated into cellular lipids without degradation, but most are degraded by β-oxidation to acetyl-CoA (from even- and odd-chain alkanes) and propionyl-CoA (from odd-chain alkanes) (Fukui and Tanaka, 1981; *figure* 4.2).

Peroxisomal catalase activity is induced in *C. tropicalis* by C_{10}-C_{13} alkanes (Rehm and Reiff, 1981). Peroxisomal β-oxidation is most active with long-chain (C_{10}-C_{22}) fatty acyl-CoA substrates, the C_{14} acyl-CoA producing maximum activity (Tolbert, 1981). The respiration of *C. tropicalis* cells grown on C_{10}-C_{13} alkanes is more sensitive to various respiratory inhibitors than D-grown cells (Rehm and Reiff, 1981).

Peroxisomes of n-alkane-grown *C. tropicalis* reduce NAD^+ in the presence of palmitate, CoA, ATP, $MnCl_2$ and molecular oxygen under aerobic conditions. Formation of acetyl-CoA is also dependent on the presence of palmitate. The amount of acetyl-CoA formed is comparable to that of NAD^+ reduced (Kawamoto *et al.*, 1978).

The initial step of β-oxidation in liver and yeast peroxisomes, and seed glyoxysomes is catalysed by an FAD-linked acyl-CoA oxidase that transfers electrons to molecular oxygen (O_2) forming hydrogen peroxide. The oxidase has low activity and may be rate limiting. Acyl-CoA produced by acyl-CoA synthetase II is oxidised with consumption of O_2 to *trans*-2-enoyl-CoA yielding hydrogen peroxide (H_2O_2) which is degraded by peroxisomal catalase (Kawamoto *et al.*, 1978; Fukui and Tanaka, 1981; Tolbert, 1981; Hashimoto, 1982; Tanaka *et al.*, 1982; Ueda *et al.*, 1985). Catalase may protect thiolase and its substrate, 3-ketoacyl-CoA, by removing the H_2O_2 abundantly produced during peroxisomal enzyme reactions (Hashimoto and Hayashi, 1987).

The acyl-CoA oxidases of *C. tropicalis* oxidise C_4-C_{20} acyl-CoAs, and those of *C. lipolytica* oxidise C_4-C_{18} acyl-CoAs. In both cases, lauroyl-CoA is the most effective substrate. The enzyme is inactive toward acetyl- and succinyl-CoAs (Shimizu *et al.*, 1979; Fukui and Tanaka, 1981). Peroxisomal fatty acyl-CoA oxidase, and thiolase have only minimal activity with substrates with a chain length shorter than C_8, and have a high affinity for O_2 (Tolbert, 1981).

The next two reactions are catalysed by a multifunctional protein displaying the activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in rat liver (Hashimoto, 1982). In *C. tropicalis* this enzyme is trifunctional having enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase activities (De La Garza *et al.*, 1985).

In the final reaction, 3-ketoacyl-CoA was cleaved by 3-ketoacyl-CoA thiolase into acetyl-CoA and a saturated acyl-CoA having two carbons less than the original molecules. This newly formed acyl-CoA re-enters the pathway (Hashimoto, 1982).

O-grown *C. tropicalis* can degrade unsaturated as well as saturated fatty acids as 2,3-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase, an obligatory enzyme for the degradation of unsaturated fatty acids possessing double bonds at even numbered carbon atoms, are induced simultaneously with the other β-oxidation enzymes. 2,4-dienoyl-CoA reductase which has a specific requirement for NADPH, catalyses the conversion of 2-*trans*, 4-*cis*-decadienoyl-CoA to 3-*trans*-dienoyl-CoA esters. Erucic acid (C_{22}:1) in particular is metabolised (Tolbert, 1981; Dommes *et al.*, 1983). β-oxidation of long-chain unsaturated dioic acid is similar to that of saturated dioic acids and can run from either of the terminal carboxylic groups. Adipic acid can be produced either gradually via β-oxidation of sebacic acid, or through enzymic isomerisation, hydration and thiolysis, from 1,10-decenedioic acid and 1,8-octenedioic acid (Yi and Rehm, 1988).

4.1.4 Production of a Non-Revertible β-Oxidation Mutant of *C. tropicalis*

4.1.4.1 Acyl-CoA Oxidase: The Best Target for Gene Transplacement

The methods of one-step gene disruption and gene transplacement were described in Chapter 1 of this thesis. It can be seen in *figure* 4.2 that there are two potential targets for gene transplacement, long-chain fatty acid acyl-CoA oxidase and long-chain fatty acid acyl-CoA synthetase II. The oxidase, the first enzyme of the β-oxidation pathway, was chosen as the best target for gene disruption to produce a non-revertible β-oxidation mutant rather than acyl-CoA synthetase II for several reasons.

The nature of acyl-CoA oxidase from *C. tropicalis* is well documented. It can be crystalised from a 30% saturated ammonium sulphate solution at 5°C, with no increase in specific activity, and is homogeneous on ultracentrifuge and disc electrophoresis. It is an octameric flavoprotein containing 8.5 mol FAD per mol enzyme, which is sulphydryl dependent and inactivated by sulfhydryl reagents such as silver and mercury compounds, and by the acetylenic substrate analog, 3-octynoyl-CoA, which acts as an active site-directed inhibitor. The addition of glutathione protects the enzyme from inhibition. The enzyme has a molecular weight of 552 kDa with an isoelectric point of 5.5, and has absorption maxima of 277, 365, and 445 nm typical of a flavoprotein. Maximum activity occurs at 50°C and pH 8.0. On purification it forms yellow rod-shaped crystals. The addition of palmitoyl-CoA to the enzyme under anaerobic conditions bleached the visible colour. Photochemical induction of the oxidase flavin yields a red semiquinone. (Shimizu *et al.*, 1979; Coudron *et al.*, 1983; Jiang and Thorpe, 1983).

Several groups have cloned and sequenced genes encoding acyl-CoA oxidases from *C. tropicalis* pK233: *POX4-1* (acyl-CoA oxidase II) and *POX5* (acyl-CoA oxidase I; Kamiryo and Okazaki, 1984; Okazaki *et al.*, 1986); *POX4-2* (Rachubinski *et al.*, 1985; Murray and Rachubinski, 1987; Small and Lazarow, 1987); *POX2* (Okazaki *et al.*, 1987), providing sequence data to aid disruption and facilitate the cloning of the gene.

The number of target genes would necessitate using gene transplacement sequentially, however, this might be simplified because the acyl-CoA oxidase genes exist in clusters. *POX4* is linked to *POX2* in a convergent arrangement, and *POX5* forms a similarly arranged doublet with another oleate-inducible gene *OLE3*. The two gene pairs are so similar in appearance that they are thought to result from a gene duplication (Kamiryo and Okazaki, 1984; Okazaki *et al.*, 1986; Okazaki *et al.*, 1987). The genes have a significant homology to each other. *POX4-2* is 97.2% homologous to *POX4-1* and 55.6% homologous to *POX5* (Murray and Rachubinski, 1987; Small and Lazarow, 1987). *POX2* has significant homology to both *POX4* and *POX5* (Okazaki *et al.*, 1987). The sequence of *POX4* hybrid-selects mRNAs for *POX2*, the sequence of *POX5* selects mRNAs for *POX4*, *POX2* and *POX5*, and mRNA transcribed from *POX2* cross-hybridises with *POX4* and *POX5*, but the *POX2* transcript does not hybridise to *OLE3* and *vice versa* (Kamiryo and Okazaki, 1984; Okazaki *et al.*, 1986; Okazaki *et al.*, 1987). It has been suggested that the regions of greatest homology might represent internal signal sequences which direct these enzymes to the peroxisomes (Murray and Rachubinski, 1987; Small and Lazarow, 1987). It might, therefore, be possible to alter a cloned gene *in vitro* such that it is targeted to more than one member of the multigene family, thus avoiding the necessity of cloning and altering all the acyl-CoA oxidase genes.

O induces more peroxisomal polypeptide- (PXP) 4 relative to PXP-5 than n-alkanes with shorter chain lengths (C_{10}-C_{12}) (Okazaki *et al.*, 1986). Therefore, there is a possibility that by cloning the appropriate oxidase gene, ie. that most active with pelargonate (chain length C_9), and disrupting it by gene transplacement, one could enrich for the formation of azeleate.

Very little information is available about the acyl-CoA synthetase enzymes or their genes, the other possible targets for gene transplacement. In *C. lipolytica* acyl-CoA synthetase II, but not I, is phosphatidylcholine-dependent whereas, neither *C. tropicalis* enzyme is phosphatidylcholine-dependent (Yamada *et al.*, 1980) which makes distinguishing between acyl-CoA synthetase I (mitochondrial) and II (peroxisomal) more difficult, and acyl-CoA synthetase II is therefore a less attractive target for gene transplacement.

Table 4.1 Growth of *Candida tropicalis* in YEPD

Growth was measured as described in Chapter 3. Cells from cultures growing in YEPD were found to be 100% viable up to densities of 5×10^8 cells ml^{-1}.

Time/h	NCYC547		NCYC997	
	Cells ml^{-1}	A_{600}	Cells ml^{-1}	A_{600}
0	4.8×10^6	0.30	3.5×10^6	0.20
1	3.8×10^6	0.36	1.6×10^6	0.11
2	7.5×10^6	0.47	4.0×10^6	0.23
3	1.0×10^7	0.61	8.0×10^6	0.54
4	1.5×10^7	0.87	2.1×10^7	0.96
5	2.6×10^7	1.19	3.8×10^7	1.40
6	4.8×10^7	1.50	8.0×10^7	1.70
7	8.2×10^7	1.79	1.3×10^8	1.95
8	1.0×10^8	2.06	2.3×10^8	2.17
9	2.0×10^8	2.27	2.8×10^8	2.31
10	2.4×10^8	2.40	3.7×10^8	2.39
11	3.3×10^8	2.50	5.0×10^8	2.44

Table 4.2 Growth of *Candida tropicalis* in SD

Growth was measured as described in Chapter 3. * denotes that the culture was hyphal rather than single cells.

Time/h	NCYC547		NCYC997	
	Cells ml^{-1}	A_{600}	Cells ml^{-1}	A_{600}
0	4.1×10^6	0.44	4.2×10^6	0.35
1	5.5×10^6	0.44	1.3×10^6	0.14
2	6.0×10^6	0.47	1.7×10^6	0.15
3	7.0×10^6	0.50	2.7×10^6	0.26
4	9.0×10^6	0.69	6.5 x 106	0.45
5	*1.1×10^7	0.90	8.5×10^6	0.65
6	*9.4×10^6	1.14	1.5×10^7	0.92
7	*2.3×10^7	1.34	2.4×10^7	1.18
8	*2.1×10^7	1.54	3.8×10^7	1.43
9	*2.1×10^7	1.71	7.0×10^7	1.61
10	*2.8×10^7	1.86	7.5×10^7	1.75
11	*2.6×10^7	1.99	1.0×10^8	1.87

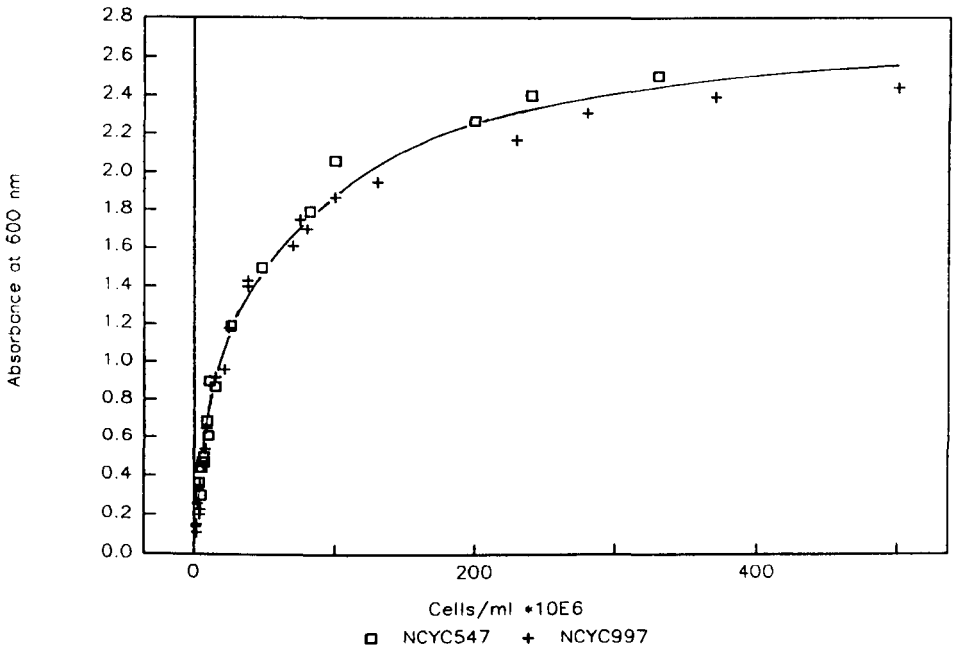


***Figure* 4.3 Graph of A_{600} vs. cells ml^{-1} *C. tropicalis* NCYC547 and NCYC997**

Plotted from the data presented in tables 4.1 and 4.2 to facilitate the conversion of A_{600} readings of cultures of *C. tropicalis* to cells ml^{-1}.

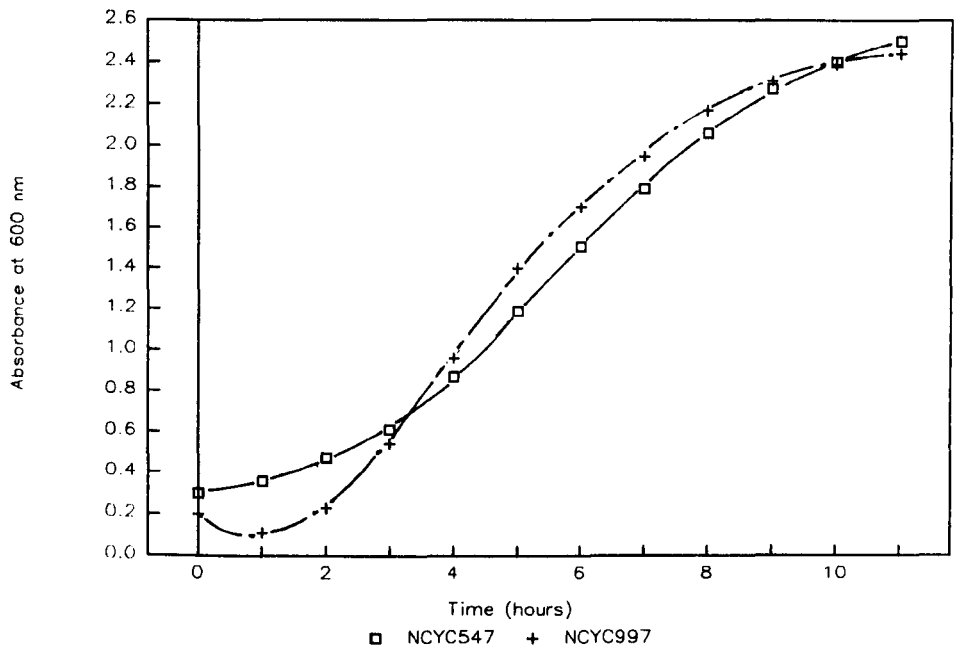


***Figure* 4.4 Growth of *C. tropicalis* NCYC547 and NCYC997 in YEPD**

Plotted from the data presented in table 4.1. The initial drop in A_{600} for NCYC997 is probably due to lysis of dead cells in the inoculum from the overnight starter culture. Doubling times for each strain were calculated from this graph and are presented in table 4.4.

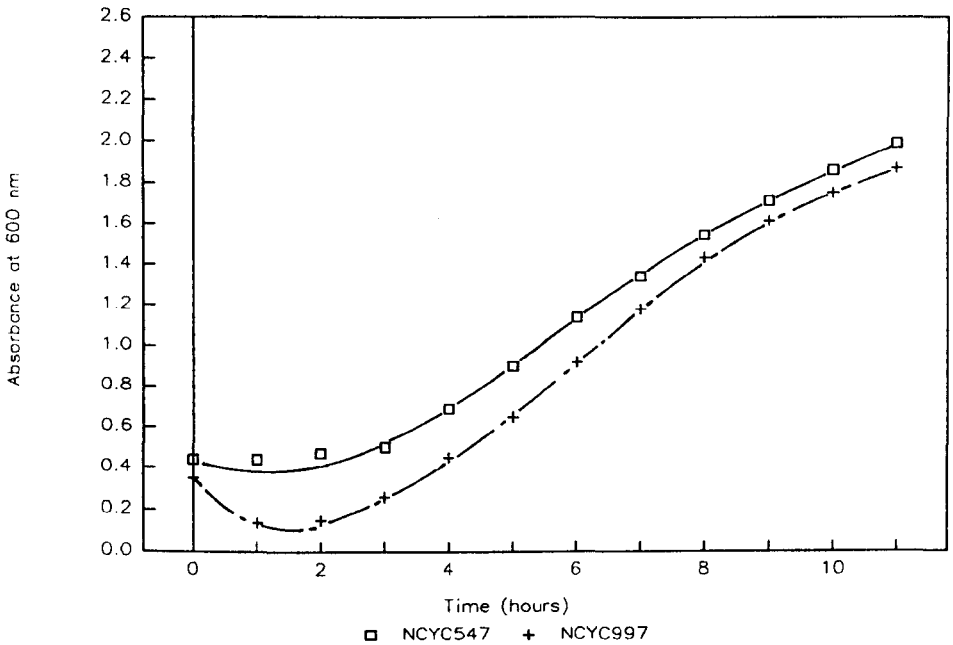


***Figure* 4.5 Growth of *C. tropicalis* NCYC547 and NCYC997 in SD**

Plotted from the data presented in table 4.2. The initial drop in A_{600} for NCYC997 is probably due to lysis of dead cells in the inoculum from the overnight starter culture. Doubling times for each strain were calculated from this graph and are presented in table 4.4.

Table 4.3 Growth of *Candida tropicalis* in SBO

Growth was measured as described in Chapter 3

Time/h	Cells ml^{-1}	
	NCYC547	NCYC997
0	2.7×10^6	1.4×10^6
1	3.2×10^6	1.9×10^6
2	3.4×10^6	1.9×10^6
3	4.8×10^6	2.7×10^6
4	5.0×10^6	2.6×10^6
5	7.8×10^6	3.1×10^6
6	1.1×10^7	4.5×10^6
7	1.3×10^7	5.8×10^6
8	1.5×10^7	8.0×10^6
9	1.8×10^7	8.0×10^6
10	2.0×10^7	9.0×10^6
11	2.2×10^7	1.0×10^7

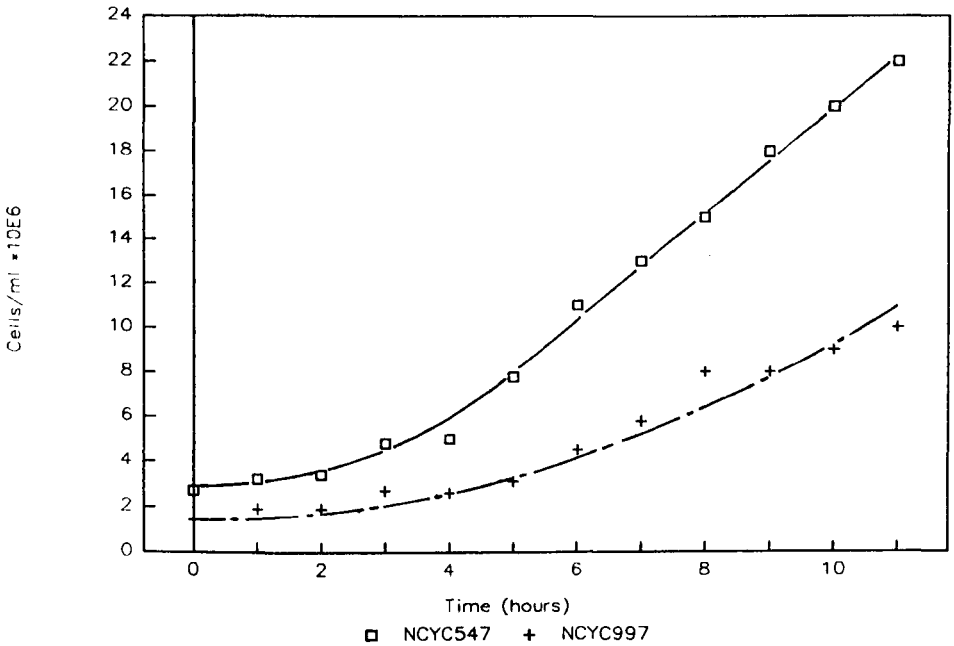


***Figure* 4.6 Growth of *C. tropicalis* NCYC547 and NCYC997 in SBO**

Plotted from the data presented in table 4.3. Doubling times for each strain were calculated from this graph and are presented in table 4.4.

Table 4.4 Doubling times of NCYC547 and NCYC997 in various media

These were calculated from *figures* 4.4, 4.5, and 4.6. A_{600} readings were converted to cells ml^{-1} by using the graph of A_{600} vs. cells ml^{-1} (*figure* 4.3). For both strains the doubling times in SBO were much slower than those in YEPD and SD, and there was a substantially longer lag-phase. This is probably a result of the cells having to adapt to the new carbon source by producing emulsifiers, and synthesising peroxisomes and peroxisomal enzymes. Kamiryo and Numa (1973) found that addition of Brij 58 to YEP with 2% w/v sucrose caused a slight delay of growth which may in part account for the longer doubling times observed in SBO.

Strain	Medium	Doubling time (min)
NCYC547	YEPD	96
	SD	104
	SBO	192
NCYC997	YEPD	104
	SD	112
	SBO	224

Table 4.5 Molecular weights of protein standards

Bovine Serum Albumin is negatively stained by silver.

Standard	Molecular Weight (kDa)
Phosphorylase B	97.4
Bovine Serum Albumin	68.0
Ovalbumin	43.0
Chymotrypsinogen	25.7
Cytochrome c	11.7

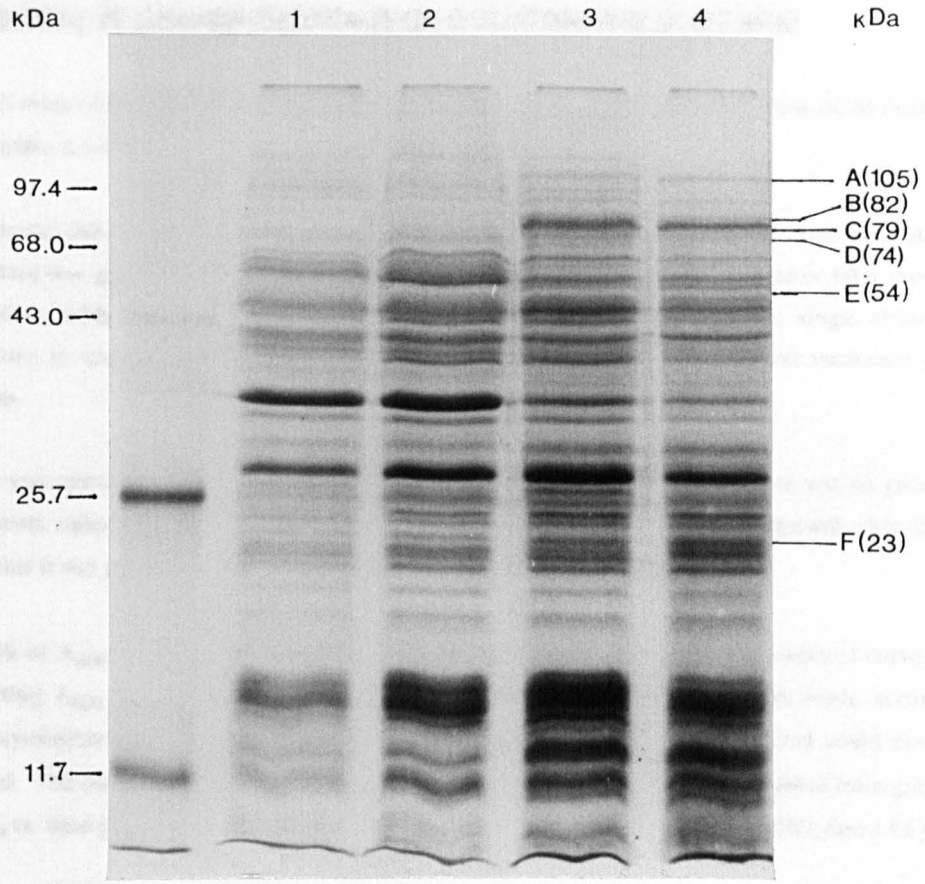


***Figure* 4.7 Silver-stained 15% SDS-PAGE of *C. tropicalis* NCYC547 and NCYC997 whole cell protein extracts**

Lanes: 1, SD-grown NCYC547 (4 μl); 2, SD-grown NCYC997 (3 μl); 3, SBO-grown NCYC547 (2 μl); 4, SBO-grown NCYC997 (1 μl). Table 4.5 lists the proteins used as size markers. Some proteins unique to the SBO-grown cells, identified from their molecular weights, are indicated: A, multifunctional protein - enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase, 102 kDa (De La Garza *et al.*, 1985; Fujiki *et al.*, 1986); B, acyl-CoA oxidase (*POX2*), 82 kDa (Okazaki *et al.*, 1987); C, acyl-CoA oxidase II (*POX4-1*), 78.544 kDa, 77 kDa, (Kamiryo and Okazaki, 1986), acyl-CoA oxidase II (*POX4-2*), 79.155 kDa (Murray and Rachubinski, 1987); D, acyl-CoA oxidase I(*POX5*), 74.048 kDa (Kamiryo and Okazaki, 1986); E, catalase, 57 kDa (Fujiki *et al.*, 1986; Rachubinski *et al.*, 1987); F, integral membrane pore-forming protein, 22 kDa (Van Veldhoven *et al.*, 1987).

4.2 GROWTH OF *CANDIDA TROPICALIS* ON D-GLUCOSE AND OLEIC ACID

Growth rates of both strains of *Candida tropicalis* on D and O were studied and compared (section 3.1.4; tables 4.1-4.3).

Both strains were streaked on YEP and S plates containing no carbon source and incubated. After 65 h there was good growth on the YEP plates compared to the S plates which after 90 h showed only some hyphal growth where the cells had been thickly streaked, and no single colonies. Therefore, to ensure that no other carbon source was available, growth on O was measured in S medium.

The O was streaked over two YEP plates and incubated (28°C). After 90 h there was no growth. YEP broth inoculated with O and similarly incubated also showed no signs of growth after 40 h. From this it was concluded that there was no need to sterilise the O prior to use.

A graph of A_{600} v. cells ml^{-1} (*figure* 4.3) was constructed for both strains as a standard curve for converting A_{600} readings into cells ml^{-1}. Pseudohyphae and multilateral buds made accurate haemocytometer readings difficult as the exact number of individual nuclei present could not be counted. The doubling time for each culture during exponential growth was calculated from graphs of A_{600} vs. time (SD and YEPD; *figures* 4.4 and 4.5), and $\log_{10}$ cells ml^{-1} vs. time (SBO; *figure* 4.6).

4.3 COMPARISON OF THE WHOLE CELL PROTEIN PROFILES OF *CANDIDA TROPICALIS* GROWN ON D-GLUCOSE AND OLEIC ACID

Whole cell protein was extracted (section 3.9.1) from both strains of *C. tropicalis* growing in SD or SBO broth, separated by electrophoresis (section 3.9.2), silver stained (section 3.9.3) and photographed (*figure* 4.7).

Several proteins unique to the alkane grown cells can be identified (*figure* 4.7), and have been sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility (table 4.5). This is linear between 12-45 kDa in a 15% polyacrylamide gel, and may therefore be inaccurate for sizing proteins outside this range (Hames and Rickwood, 1981). There are very few differences between the protein profiles of strains NCYC547 and NCYC997 under both sets of conditions.

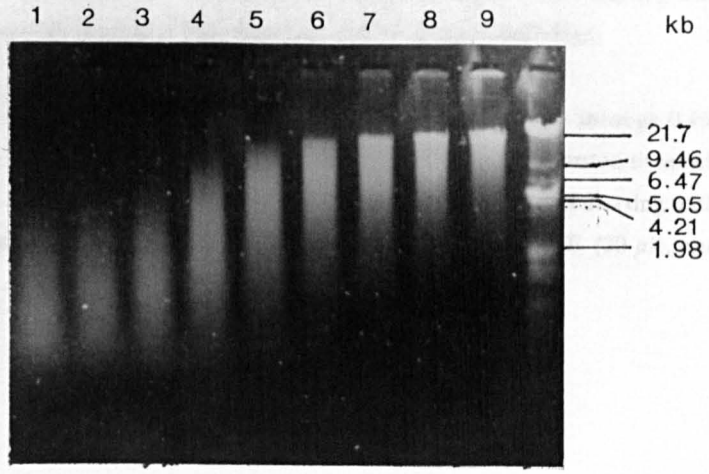


***Figure* 4.8 To define conditions for a *Sau* 3AI partial digestion**

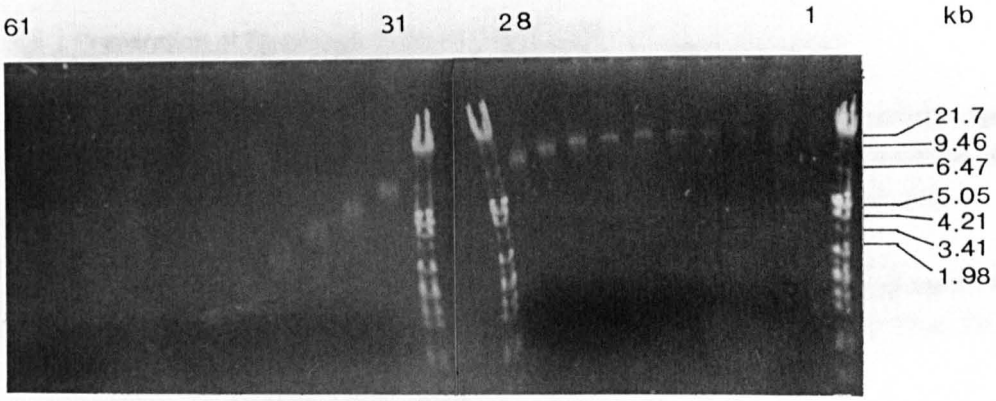
C. tropicalis NCYC997 genomic DNA digested with various amounts of *Sau* 3AI (section 3.3.1.2), subjected to electrophoresis through 0.4% agarose against λ *Eco* RI/*Hin* dIII markers. Lanes: 1, 1.000 U μg^{-1} DNA; 2, 0.500 U μg^{-1} DNA; 3, 0.250 U μg^{-1} DNA; 4, 0.125 U μg^{-1} DNA; 5, 0.063 U μg^{-1} DNA; 6, 0.031 U μg^{-1} DNA; 7, 0.016 U μg^{-1} DNA; 8, 0.008 U μg^{-1} DNA; 9, 0.000 U μg^{-1} DNA. Lane 5 has most fluorescence between the 3.41 and 9.46 kb markers, therefore to generate the greatest number of fragments of this size 0.031 U *Sau* 3AI μg^{-1} DNA were used (1 h, 37°C).

***Figure* 4.9 *C. tropicalis* NCYC997 genomic DNA (500 μg) partially digested with *Sau* 3AI, subjected to sucrose gradient density centrifugation (section 3.3.4), and collected as 0.5 ml fractions**

4.9a. Aliquots (5 μl) of every third fraction were subjected to electrophoresis through 0.4% agarose against λ *Eco* RI/*Hin* dIII markers, the salt content of which was adjusted to match that of the fractions. Fractions were loaded sequentially from right to left starting with Fraction 1, the bottom of the gradient. Fractions 1-14 and 33-62 were discarded.

4.9b. Aliquots (5 μl) from fractions 15-32 were subjected to electrophoresis through 0.4% agarose against λ *Eco* RI/*Hin* dIII markers, the salt content of which was adjusted to match that of the fractions. Fractions were loaded sequentially from right to left starting with Fraction 15. Fractions 29-31 were pooled, precipitated, resuspended in TE (20 μl), and stored.

4.9a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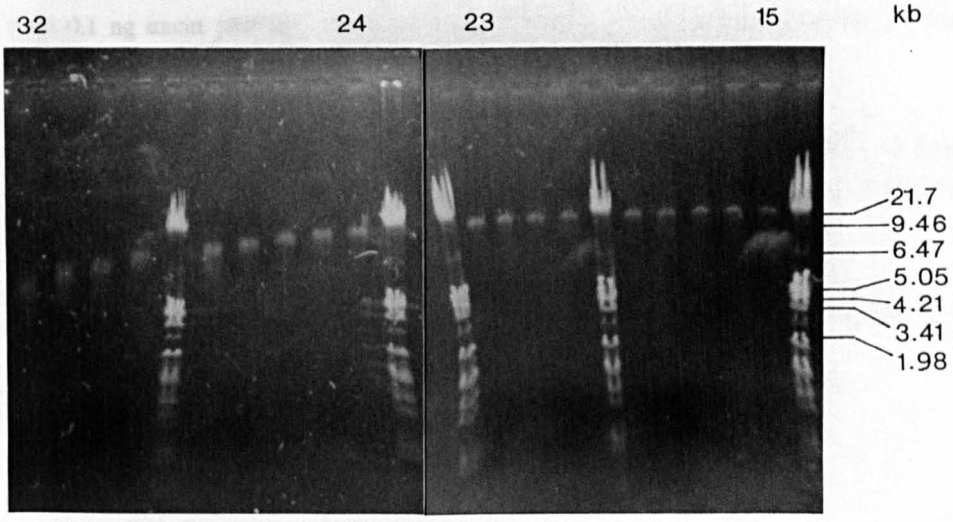
3.41
1.98

4.9b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4.4 CONSTRUCTION OF A *CANDIDA TROPICALIS* NCYC997 GENOMIC DNA LIBRARY IN pBR322

4.4.1 Preparation of Fragments of *C. tropicalis* DNA

Chromosomal DNA extracted from *C. tropicalis* NCYC997 (section 3.2.1) was partially digested (section 3.3.1.2) with 15.5 U *Sau* 3AI (0.031 Uμg^{-1} DNA) to generate fragments of 3.5-9.5 kb (*figure* 4.8), and size fractionated over a 10-40% sucrose density gradient (section 3.3.4, *figure* 4.9).

5 μl from fractions 32-15 were subjected to electrophoresis in an 0.4% w/v agarose gel against λ *Eco* RI/*Hin* dIII markers. Fractions 29-31 were pooled, precipitated, resuspended in 20 μl TE, and stored (section 3.3.4).

4.4.2 Ligation and Generation of Transformants

150 ng *Sau* 3AI partially digested DNA was ligated (section 3.3.3) to 350 ng pBR322 cleaved with *Bam* HI and dephosphorylated.

10 μl aliquots of this ligation were used to transform (section 3.4.1) Epicurian coli^{TM} AG1, and competent *E. coli* 5K. The efficiency of the competent cells was assessed by transforming an aliquot with 0.1 ng uncut pBR322. Transformants were selected on L-agar containing 100 μgml^{-1} Ap (LAp).

The efficiency of the Epicurian coli^{TM} was 2.08 x 10^8 transformants per μg DNA. (The suppliers stated the efficiency to be 3.40 x 10^8), and the efficiency of the competent 5K was 2.13 x 10^7 transformants per μg DNA.

$\simeq$6444 Ap^R transformants were generated from the Epicurian coli^{TM} AG1 competent cells, and $\simeq$11836 Ap^R transformants from the 5K competent cells.

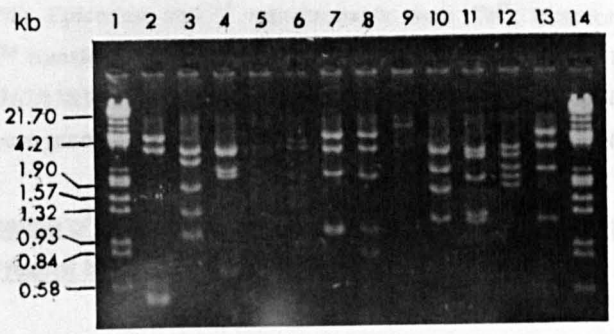


***Figure* 4.10 *Eco* RI/*Sal* I digested plasmid DNA from 12 Ap^R, Tc^S transformants subjected to electrophoresis through 0.8% agarose**

Lanes: 1 and 14, λ *Eco* RI/*Hin* dIII markers; 2-13, plasmid DNA. Insert sizes were calculated to be: lane 2, 4.64 kb; lane 3, 6.11 kb; lane 4, 3.64 kb; lane 7, 8.22 kb; lane 8, 8.90 kb; lane 10, 4.22 kb; lane 11, 6.50 kb; lane 12, 8.98 kb; lane 13, 8.28 kb. Lanes 5, 6, and 9 were not assessed to determine insert size because they are too faint.

According to the suppliers specifications the cleaved, dephosphorylated pBR322 can account for 0.35% of the Ap resistant (Ap^R) transformants generated. An aliquot of Epicurian coli™ transformed with 100 ng unligated vector generated 2219 transformants which is within this limit taking the efficiency of transformation of the cells to be 2.08×10^8. Cloning into the *Bam* HI site of pBR322 disrupts the Tc resistance gene, therefore Ap^R, Tc^R transformants carry plasmid DNA that has no insert. 1000 Ap^R transformants from each strain were picked in a regular pattern onto LAp and L-agar plates containing 12.5 μgml^{-1} Tc, and incubated (37°C, overnight), and plasmid preparations were carried out to determine the percentage of transformants carrying insert DNA. 514/1000 (48.5%) Epicurian coli™ transformants were Tc^R, therefore ≃3132/6444 (51.5%) Epicurian coli™ transformants have insert DNA. 141/1000 (14.1%) 5K transformants were Tc^R, therefore ≃10167/11836 (85.9%) 5K transformants have insert DNA. In total ≃13299 transformants were generated which were carrying *C. tropicalis* genomic DNA.

4.4.3 Determination of the Average Insert Size of the Library and Probability that Any Gene will be Present in the Library

Plasmid DNA was extracted from 12 Tc sensitive (Tc^S) transformants using the miniprep method (section 3.2.2.2), digested with *Eco* RI and *Sal* I, and subjected to electrophoresis in a 0.8% agarose slide gel (section 3.3.5) alongside λ *Eco* RI/*Hin* dIII restriction fragments (*figure* 4.10). The size of each insert was determined from a standard curve of mobility v. reciprocal fragment size of the λ markers and the average insert size was determined to be 6.61 kb.

According to Clarke and Carbon (1976), "given a preparation of cell DNA that has been fragmented to a size such that each fragment represents a fraction (f) of the total genome, then the probability (P) that a given unique DNA sequence is present in a collection of N transformant colonies is given by the expression:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

Assuming the haploid genome size of *C. tropicalis* is 15 000 kb (Kamiryo and Okazaki, 1984) 10448 Ap^R, Tc^S transformants are required to represent 99% of the genome, and therefore, using the above equation, the library should represent 99.7% of the genome.

4.4.4 Storage of the Library

Frozen cultures of the *E. coli* strains carrying the library (section 3.1.3) were prepared and stored.

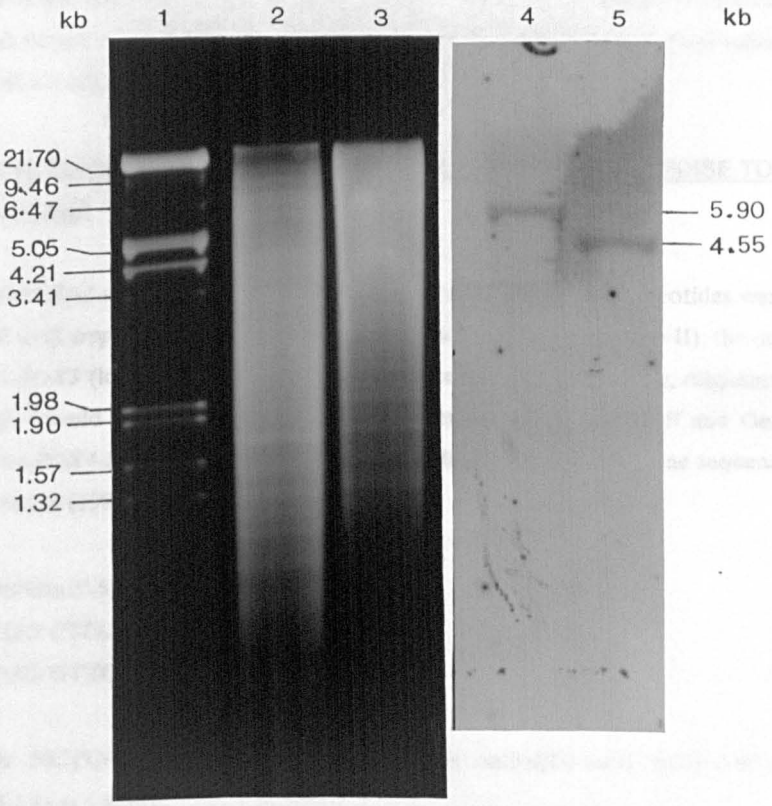
***Figure* 4.11 *C. tropicalis* NCYC997 genomic DNA digests subjected to electrophoresis through 0.8% agarose and Southern blots showing hybridisation to the oligonucleotide probes**

4.11a. *C. tropicalis* NCYC997 genomic DNA hybridised to the oligonucleotide specific to *POX4*. Lanes: 1, λ *Eco* RI/*Hin* dIII markers; 2 and 4, *Bam* HI digest; 3 and 5, *Bam* HI/*Hin* dIII digest.

4.11b. *C. tropicalis* NCYC997 genomic DNA hybridised to the oligonucleotide specific to *POX5*. Lanes: 1, λ *Eco* RI/*Hin* dIII markers; 2 and 3, *Bgl* II digest. The larger bands lighting up on the autoradiograph probably represent partially digested DNA.

Zeff and Geliebter (1987) recommend loading at least 30 μg genomic DNA per lane when probing genomic DNA blots with oligonucleotides. However, good hybridisation was seen with the amounts of DNA on these filters.

4.11a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4
5
kb
21.70
9.46
6.47
5.05
4.21
3.41
1.98
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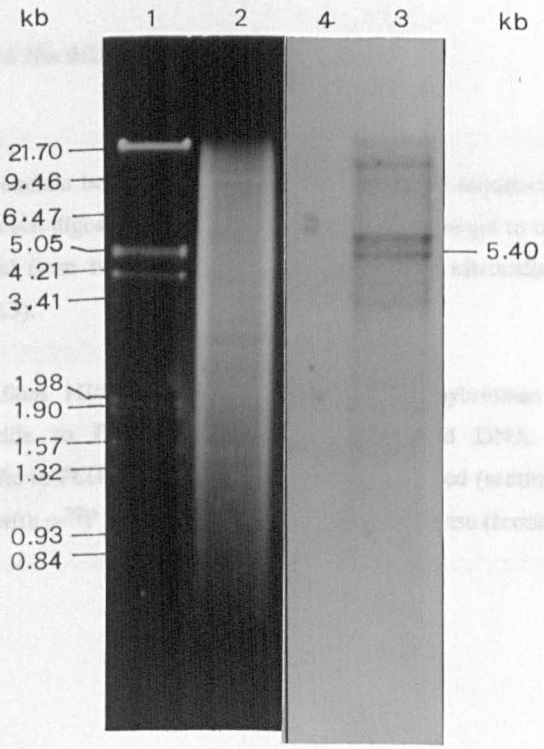
1.57
1.32
5.90
4.55

4.11b


kb
1
2
4
3
kb
21.70
9.46
6.47
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.93
0.84
5.40

Plasmid DNA was extracted by large scale triton lysis (section 3.2.2.1) from two 400 ml LAp cultures (one of each strain) of the library. The DNA was pooled, resuspended in a final volume of 1 ml TE, and stored at a concentration of 1.25 $\mu g\,\mu l^{-1}$.

4.5 DEMONSTRATION THAT SPECIFIC OLIGONUCLEOTIDES HYBRIDISE TO *CANDIDA TROPICALIS* DNA

Based on published sequences (Okazaki *et al.*, 1986) two 20 bp oligonucleotides were synthesised, one specific to *C. tropicalis POX4-1* (long chain fatty acid acyl Co-A oxidase II), the other specific to *C. tropicalis POX5* (long chain fatty acid acyl Co-A oxidase I). Statistically, oligonucleotides 19-21 bp in length should be specific for only a single genomic sequence (Zeff and Geliebter, 1987). However, the *POX4-1* oligonucleotide will also hybridise to the *POX4-2* gene sequenced by Murray and Rachubinski (1987).

Oligonucleotides 3'-5':
POX4 TTTGG CTCCA ACTGT GGTTA
POX5 TTTGG GTTCA ACTGT GGCTA

C. tropicalis NCYC997 genomic DNA was digested overnight with restriction endonucleases (section 3.3.1.1) as follows:
10 μg with *Bam* HI.
10 μg with *Bam* HI and *Hin* dIII.
10 μg with *Bgl* II.

These enzymes were chosen because according to the published sequences they do not cut within the genes. 100 ng of each digest was run on an 0.8% agarose slide gel to confirm digestion (section 3.3.5), and transferred from two large format agarose gels to nitrocellulose filters by Southern blotting (section 3.5.2.3).

The *Bam* HI and *Bam* HI/*Hin* dIII digested DNA was hybridised (section 3.5.3) to the oligonucleotide specific to *POX4*, and the *Bgl* II digested DNA was hybridised to the oligonucleotide specific to *POX5*, and the filters autoradiographed (section 3.5.4, *figure* 4.11). The probes were labelled with γ-^{32}P ATP using T4-polynucleotide kinase (section 3.5.1.1).

4.6 SCREENING THE *C. TROPICALIS* NCYC997 GENOMIC LIBRARY FOR *POX4* AND *POX5*

4.6.1 Colony Hybridisations of the Library to Mixed Oligonucleotide Probes

Aliquots of the frozen library containing 2000-5000 cells, were inoculated onto 8 nitrocellulose filters for colony hybridisation (section 3.5.2.1, method 1, 16 000-40 000 colonies). Only one replica of each master was made, and there were no positive or negative controls. The method does not lend itself to including negative colonies on the filters, and it was assumed that most of the colonies on the filters would be negative.

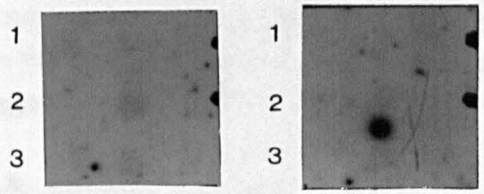
Both oligonucleotides were labelled with γ-^{32}P ATP (section 3.5.1.1), the filters were hybridised (section 3.5.3) to both probes, marked with radioactive ink for orientation, and autoradiographed (section 3.5.4, Kodak XAR-5, 72 h).

Where a positive reaction was seen to have occurred the cells were scraped off the master filters into LAp-broth. The resulting culture was serially diluted, plated over LAp-agar plates, and incubated overnight to assess the cell density. 10 nitrocellulose filters were inoculated with approximately 200 cells each, and the filters processed as above. Two replicas of each master were made. All the filters were colony hybridised to mixed oligonucleotide probe and autoradiographed as above. As above, no controls were included on the filters.

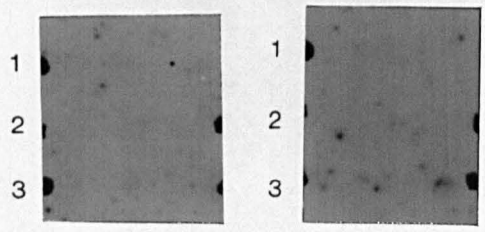
Colonies (200) which appeared to be lighting up more strongly than background on both filters were picked over nitrocellulose filters in duplicate (50 colonies per filter, section 3.5.2.1, method 2). A negative control (Tc^R colony carrying pBR322 but no insert DNA) was included on each filter. The filters were hybridised to mixed oligonucleotide probe and autoradiographed as above.

4.6.2 Dot-blots of Positive Colonies

Plasmid DNA was extracted from 34 colonies which appeared to be stronger than the negative control on both colony hybridisation filters, using the miniprep method (section 3.2.2.2), and dot-blotted (section 3.5.2.2) onto nitrocellulose in duplicate. pBR322 was included as a negative control. The filters were hybridised to mixed oligonucleotide probe and autoradiographed (Kodak XAR-5, 24 h).



Filters probed with the oligonucleotide specific to *POX4*



Filters probed with the oligonucleotide specific to *POX5*

***Figure* 4.12 Dot blots of DNA from colonies which hybridised to the oligonucleotides specific to *POX*4 and *POX*5**

Rows: 1, pBR322; pBR322; pBR322 (negative controls); 2, miniprep 7; miniprep 13; miniprep 16; 3, miniprep 18; miniprep 23; miniprep 30.
DNA from minipreps 13 and 23 which hybridised to the oligonucleotide specific to *POX4* on both filters. These plasmids were designated pMP13 and pMP23. None of the miniprep DNAs hybridised to the oligonucleotide specific to *POX5*.

Figure 4.13 Restriction map of pMP13

4.13a. Restriction endonuclease digests of pMP13 subjected to electrophoresis through 0.7% agarose. Lanes: 1, *Eco* RI digested pBR322; 2, *Eco* RI digested pMP13; 3, *Eco* RI/*Sal* I digested pMP13; 4, *Sal* I digested pMP13; 5, *Eco* RI/*Sal* I digested pBR322; 6, λ *Eco* RI/*Hin* dIII markers. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility.

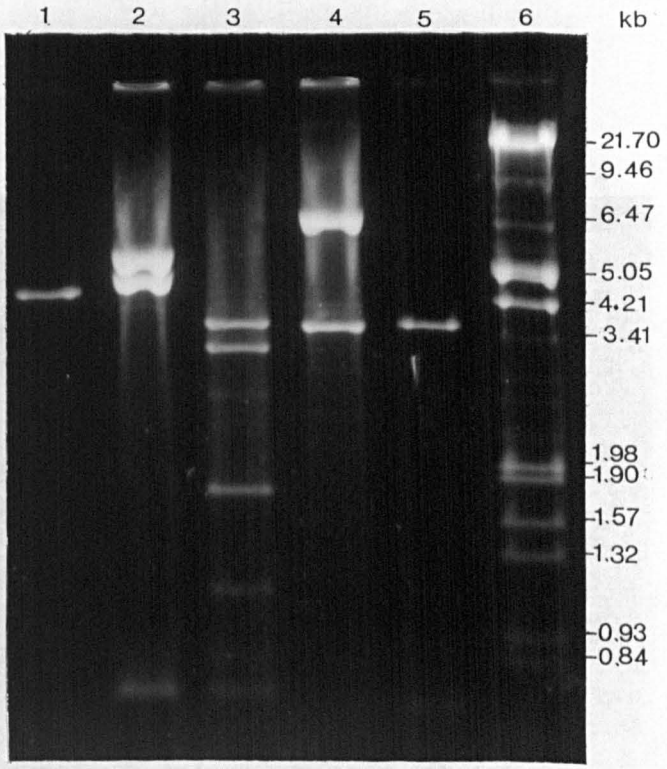
4.13b. Restriction map of pMP13 deduced from the information in 4.13a. The heavy line represents pBR322 sequences, the remainder of the plasmid is the *C. tropicalis* NCYC997 genomic DNA insert. The plasmid is drawn to scale.

Restriction sites:

E = *Eco* RI

S = *Sal* I

4.13a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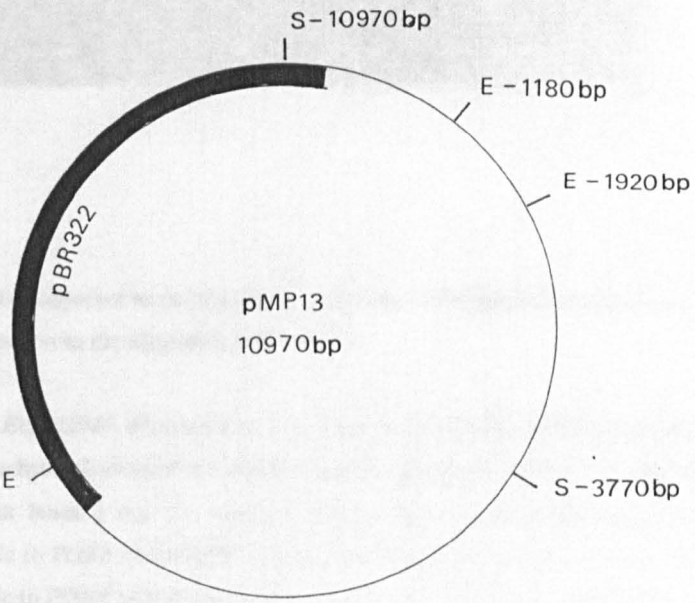
4.13b


S – 10970 bp
E – 1180 bp
E – 1920 bp
pBR322
pMP13
10970 bp
7260 bp – E
S – 3770 bp

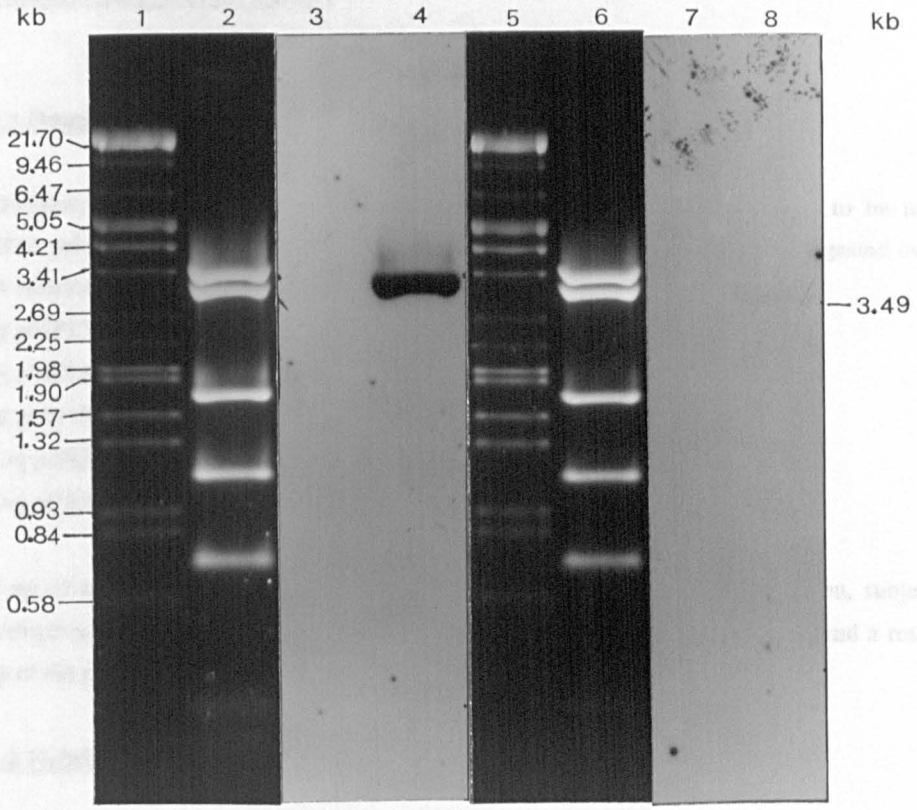


***Figure* 4.14 pMP13 DNA subjected to electrophoresis through 0.8% agarose and Southern blots showing hybridisation to the oligonucleotide probes**

Lanes: 1, 3, 5 and 7, λ *Eco* RI/*Hin* dIII markers; 2, 4, 6 and 8, *Eco* RI/*Sal* I digested pMP13. Lanes 3 and 4 show the hybridisation of the oligonucleotide specific to *POX4* to the pMP13 DNA transferred from lanes 1 and 2. Lanes 7 and 8 show the hybridisation of the oligonucleotide specific to *POX5* to the pMP13 DNA transferred from lanes 5 and 6. The oligonucleotide specific to *POX4* hybridised strongly to the 3.49 kb fragment of pMP13.

The six best clones and three negative controls were dot-blotted onto nitrocellulose in quadruplicate. Two of the filters were hybridised to the oligonucleotide specific to *POX4*, and the other two to the oligonucleotide specific to *POX5* (*figure* 4.12).

4.7 IDENTIFICATION OF PMP13

4.7.1 Restriction Map of pMP13

Preliminary digests with restriction endonucleases showed pMP13 and pMP23 to be identical. pMP13 prepared by large scale triton lysis (section 3.2.2.1), and pBR322 were digested overnight with restriction endonucleases (section 3.3.1.1) as follows:

1 μg pMP13 with *Eco* RI.

1 μg pMP13 with *Sal* I.

1 μg pMP13 with *Eco* RI and *Sal* I.

0.5 μg pBR322 with *Eco* RI.

0.5 μg pBR322 with *Eco* RI and *Sal* I.

100 ng of each digest was run on an 0.8% agarose slide gel to confirm digestion, subjected to electrophoresis in a large format 0.7% agarose gel (section 3.3.5), photographed, and a restriction map of the plasmid was drawn up using the information on the gel (*figure* 4.13).

4.7.2 Hybridisation of pMP13 to the Oligonucleotide Specific to *POX4*

5 μg pMP13 prepared by large scale triton lysis was digested overnight with restriction endonucleases *Eco* RI and *Sal* I.

100 ng of the digest was run on an 0.8% agarose slide gel to confirm digestion, and transferred from a large format agarose gel to a Hybond-N filter by Southern blotting (section 3.5.2.3).

The filter was divided in half and each half was hybridised (section 3.5.3) to one of the oligonucleotides labelled with T4 polynucleotide kinase (section 3.5.1.1), and autoradiographed (section 3.5.4, *figure* 4.14). The λ *Eco* RI/*Hin* dIII markers were also blotted to act as a negative control.

pMP13 was stored as DNA prepared by large scale triton lysis, and as a frozen preparation of the transformant carrying the plasmid (section 3.1.3).

***Figure* 4.15 *C. tropicalis* NCYC997 genomic DNA digests subjected to electrophoresis through 0.7% agarose and Southern blot showing hybridisation to the 3.49 kb fragment of pMP13**

Lanes: 1 and 7, λ *Eco* RI/*Hin* dIII markers; 2 and 8, *Bam* HI digest; 3 and 9, *Bgl* II digest; 4 and 10, *Eco* RI digest; 5 and 11, *Hin* dIII digest; 6 and 12, *Sal* I digest. The 3.49 kb fragment of pMP13 hybridised strongly to the genomic DNA confirming that the origin of the cloned DNA was *C. tropicalis* NCYC997.

4.15

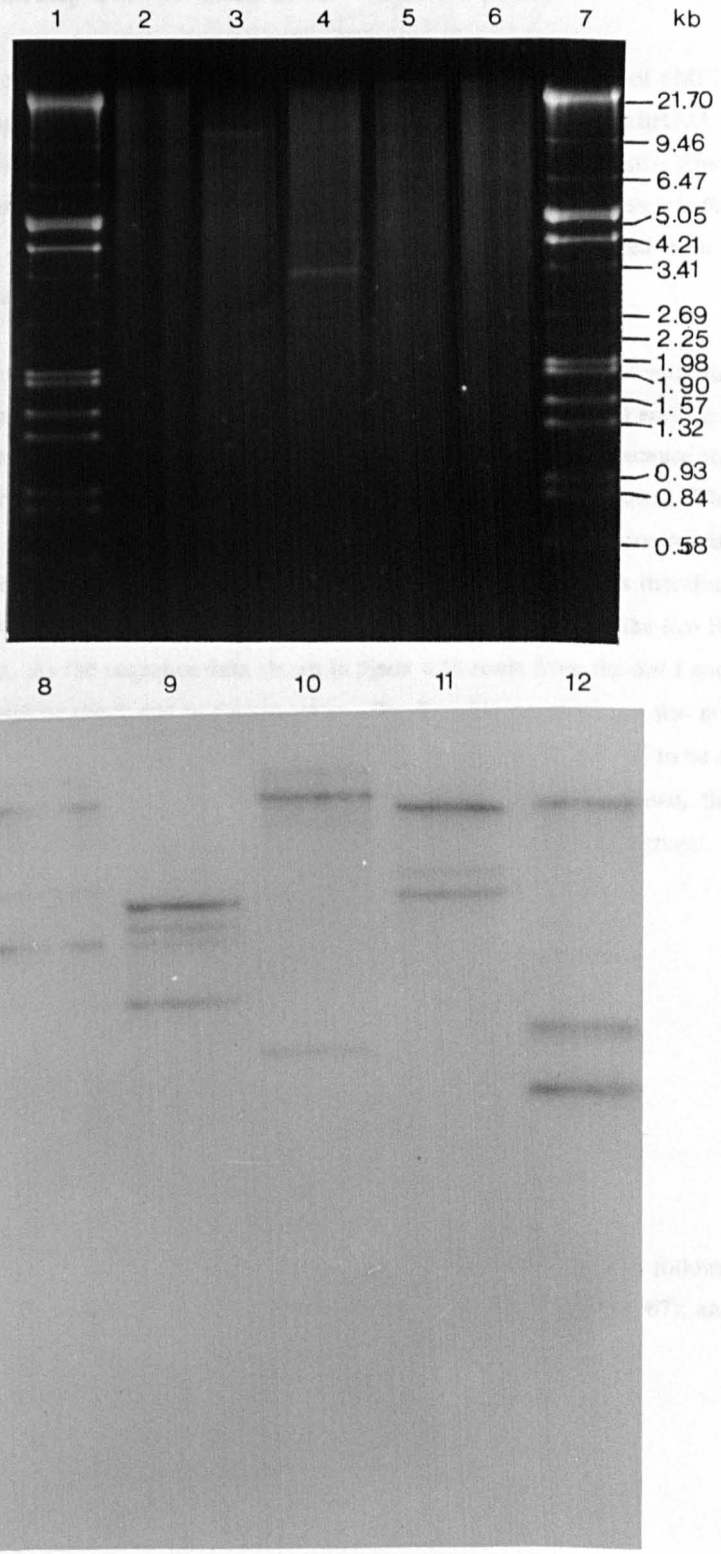

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***Figure* 4.16 Restriction map of the 3.49 kb *Eco* RI/*Sal* I fragment of pMP13**

4.16a. Restriction endonuclease digests of the 3.49 kb *Eco* RI/*Sal* I fragment of pMP13 subjected to electrophoresis through 2.0% agarose. Lanes: 1, *Hin* fI digested pBR322; 2 and 13, *λ Eco* RI/*Hin* dIII markers; 3, *Bal* I digest; 4, *Bam* HI digest; 5, *Hha* I digest; 6, *Kpn* I digest; 7, *Rsa* I digest; 8, *Sau* 3AI digest; 9, *Xmn* I digest; 10, *Bal* I/*Bam* HI digest; 11, *Bal* I/*Xmn* I digest; 12, *Bam* HI/*Xmn* I digest. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility.

4.16b. Restriction map of the 3.49 kb *Eco* RI/*Sal* I fragment of pMP13 deduced from the information in 4.16a compared with the restriction maps of the other fatty acid acyl-CoA oxidase genes. The maps are drawn to scale. *Kpn* I failed to cut the DNA because the enzyme was inactive, and the fragment is not cleaved by *Hin* dIII (results not shown). The *Eco* RI end of the 3.49 (3.25) kb fragment is within the pBR322 DNA and is approximately 375 bp from the *Bam* HI site that the *C. tropicalis* DNA was cloned into. It is therefore likely that as there is a *Bam* HI site 380 bp from one end of the map that this is the *Eco* RI end of the fragment. As the sequence data shown in *figure* 4.18 reads from the *Sal* I end, the *Eco* RI site should be the 5'-end of the sequence. The *Bam* HI site at 380 bp was not seen in the sequence data obtained which also suggests that the *Sal* I site is unlikely to be at this end of the map . The map most resembles that of *POX4-1*. As drawn, the oligonucleotide specific to *POX4* should hybridise to the 530 bp *Xmn* I/*Bam* HI fragment.

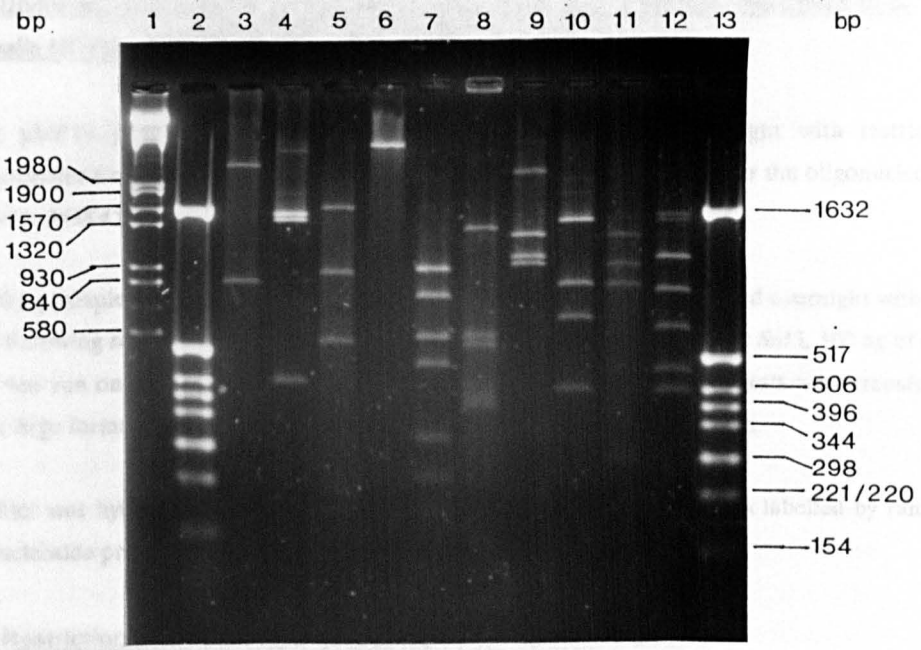
Restriction sites:

A = *Bal* I
B = *Bam* HI
E = *Eco* RI
H = *Hin* dIII
S = *Sal* I
X = *Xmn* I

O = region of DNA homologous to the oligonucleotide specific to *POX4*

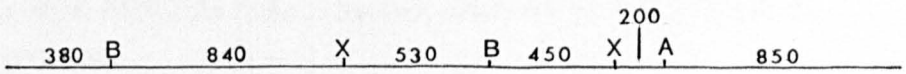
Restriction maps of acyl-CoA oxidases were deduced from published sequences as follows: *POX4-1* and *POX5* (Okazaki *et al.*, 1986); *POX4-2* (Murray and Rachubinski 1987); and *POX2* (Okazaki *et al.*, 1987).

4.16a

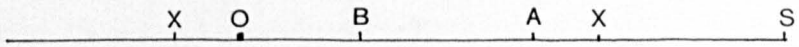


4.16b Scale: 200 bp 5′→3′

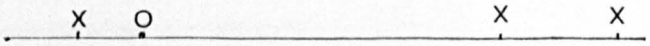
3250 (3490) bp fragment of pMP13



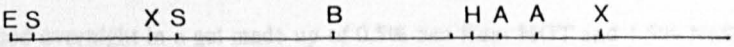
POX4-1, 2845 bp



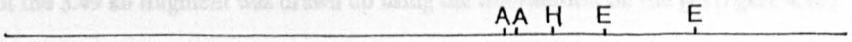
POX4-2, 2288 bp



POX5, 2624 bp



POX2, 3076 bp



4.7.3 Demonstration that the 3.49 kb *Eco* RI/*Sal* I Fragment of pMP13 Hybridises to *C. tropicalis* NCYC997 Genomic DNA

40 μg pMP13 prepared by large scale triton lysis was digested overnight with restriction endonucleases *Eco* RI and *Sal* I and the 3.49 kb fragment which hybridised to the oligonucleotide specific to *POX4* was isolated by electroelution (section 3.3.6).

Five 10 μg samples of *C. tropicalis* NCYC997 genomic DNA were each digested overnight with one of the following restriction endonucleases: *Hin* dIII, *Bgl* II, *Bam* HI, *Eco* RI, or *Sal* I, 100 ng of each digest was run on an 0.8% agarose slide gel to confirm digestion, and the digests were transferred from a large format 0.7% agarose gel to a Hybond-N filter by Southern blotting.

The filter was hybridised to the 3.49 kb fragment of pMP13 which had been labelled by random hexanucleotide priming (section 3.5.1.2), and autoradiographed (*figure* 4.15).

4.7.4 Restriction Map of the 3.49 kb *Eco* RI/*Sal* I Fragment of pMP13

220 μg pMP13 prepared by large scale triton lysis was digested overnight with restriction endonucleases *Eco* RI and *Sal* I. The 3.49 kb band which hybridised to the oligonucleotide specific to *POX4* was isolated by electroelution and the DNA resuspended in a final volume of 0.1 ml TE.

10 μl aliquots of the DNA were digested overnight with the following restriction endonucleases:
Bal I.
Bam HI.
Hha I.
Kpn I.
Rsa I.
Sau 3AI.
Xmn I.
Bal I and *Bam* HI.
Bal I and *Xmn* I.
Bam HI and *Xmn* I.

The digests were separated overnight in a gel made up of 0.5% Sea Kem HGT and 1.5% NuSieve agarose alongside pBR322 *Hin* fI and λ *Eco* RI/*Sal* I marker fragments, photographed, and a restriction map of the 3.49 kb fragment was drawn up using the information on the gel (*figure* 4.16).

Figure* 4.17 Restriction fragments of the 3.49 kb fragment of pMP13 homologous to the oligonucleotide specific to *POX4

4.17a. Southern blot of restriction endonuclease digests of the 3.49 kb *Eco* RI/*Sal* I fragment of pMP13 subjected to electrophoresis through 2.0% agarose (*figure* 4.16a) and hybridised to the oligonucleotide specific to *POX4*. Lanes: 1, *Bal* I digest; 2, *Bam* HI digest; 3, *Hha* I digest; 4, *Kpn* I digest; 5, *Rsa* I digest; 6, *Sau* 3AI digest; 7, *Xmn* I digest; 8, *Bal* I/*Bam* HI digest; 9, *Bal* I/*Xmn* I digest; 10, *Bam* HI/*Xmn* I digest. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility. The bands hybridising to the oligonucleotide are as follows: *Bal* I, 0.85 kb; *Bam* HI, 1.48 kb; *Hha* I, 0.91 kb; *Rsa* I, 0.78 kb; *Sau* 3AI, 0.60 kb; *Xmn* I, 1.05 kb. These results are not in agreement with those predicted from the published sequences of *POX4-1* (Okazaki *et al.*, 1986): *Bal* I, >1.92 kb; *Bam* HI, >1.30 kb; *Hha* I, >2.17 kb; *Rsa* I, 54 bp; *Sau* 3AI, 0.53 kb; *Xmn* I, 1.54 kb; and *POX4-2* (Murray and Rachubinski 1987): *Bal* I, no site; *Bam* HI, no site; *Hha* I, no site; *Rsa* I, 54 bp; *Sau* 3AI, 0.75 kb; *Xmn* I, no site. The oligonucleotide specific to *POX4* did not hybridise to the 530 bp *Xmn* I/*Bam* HI as was suggested by *figure* 4.16b.

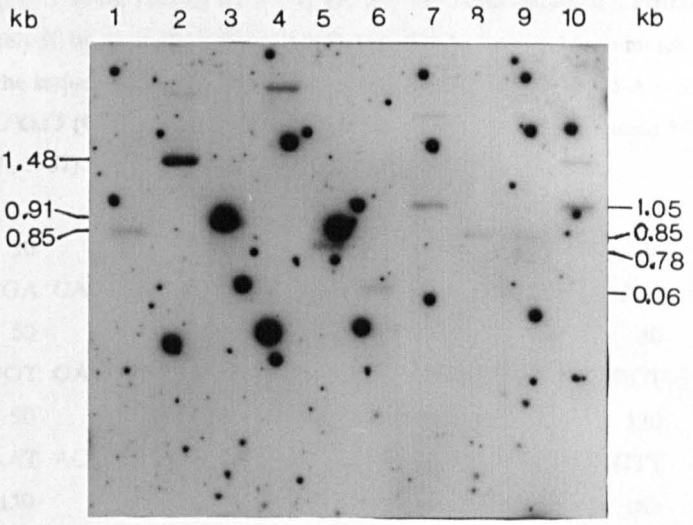
4.17b. Restriction map of the 3.49 kb *Eco* RI/*Sal* I fragment of pMP13 compared with the restriction maps of *POX4-1* (Okazaki *et al.*, 1986) and *POX4-2* (Murray and Rachubinski 1987), taking into account the region of DNA homologous to the oligonucleotide specific to *POX4* as determined from 4.17a. The maps are drawn to scale lining up the *Xmn* I sites such that the 850 bp *Bal* I-end fragment coincides with the sequences homologous to the oligonucleotide in *POX4-1* and *POX4-2*. However, neither *POX4-1* or *POX4-2* has a *Bal* I site in this position.

Restriction sites:

A = *Bal* I
B = *Bam* HI
S = *Sal* I
X = *Xmn* I

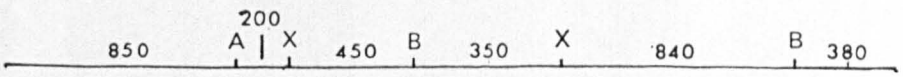
O = region of DNA homologous to the oligonucleotide specific to *POX4*

4.17a

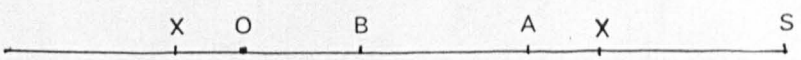


4.17b Scale: ⌴ 100 bp 5′→3′

3250 (3490) bp fragment of pMP13



POX4-1, 2845 bp



POX4-2, 2288 bp

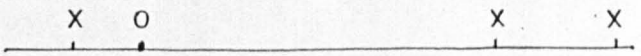
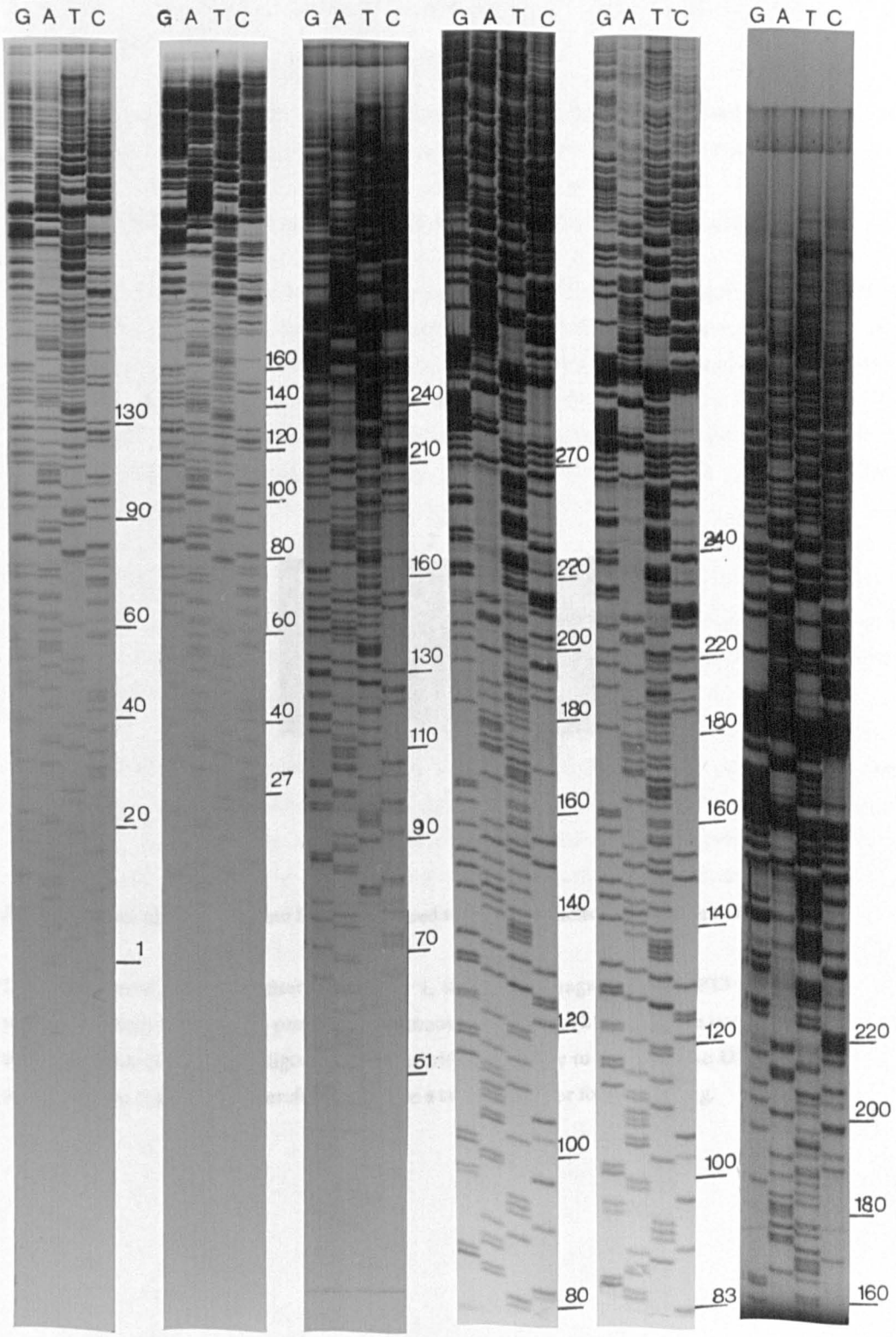


Figure 4.18 **Sequence of part of the 3.49 kb fragment of pMP13**

The sequence is from the *Sal* I end of the fragment which is furthest away from the pBR322 DNA. From the sequence of the control template, the sequence data obtained is estimated to begin approximately 50 bp from the primer. It was found to be impossible to match this sequence to any of the sequences published for the long chain fatty acid acyl Co-A oxidase genes: *POX4-1* and *POX5* (Okazaki *et al.*, 1986); *POX4-2* (Murray and Rachubinski 1987); *POX2* (Okazaki *et al.*, 1987).

```
          5        10        15        20        25        30        35        40
3'-  AATTC ACCGA CAAGT ACGGT GAAGT CTGCC CAGCT AACTG
          45        50        55        60        65        70        75        80
     GCACC CAGGT GATGA AACCA TCAAG CCAAG CCCAG ACGCT
          85        90        95       100       105       110       115       120
     TCCAA GGAAT ACTTT GGCAA GGTCA ACAAA TAAGT AGGTT
         125       130       135       140       145       150       155       160
     ACGGC CTAGG CTAGT TTTAT AGATA GTAGC TATGC ATTGT
         165       170       175       180       185       190       195       200
     TGGTA TTTCT AATAT AAATA TGTAT TCATC TTGTC CATGT
         205       210       215       220       225       230       235       240
     CTGCA AATTG CAACC CCTTG TTGGT TCCTT TTTGG GCCCG
         245       250       255       260       265       270       275       280
     GTTTC TTTTT GGGTT TGTTC TTTTT TCCGA TTCGT TCGTT
         285
     CGAAG-5'
```


4.18
G A T C
130
90
60
40
20
1
G A T C
160
140
120
100
80
60
40
27
G A T C
240
210
160
130
110
90
70
51
G A T C
270
220
200
180
160
140
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160
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120
100
83
G A T C
220
200
180
160

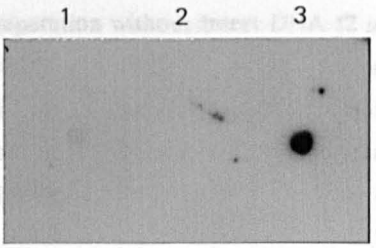


Figure* 4.19 Dot blots of template DNA hybridised to the oligonucleotide specific to *POX4

DNA was dotted onto each filter as follows: 1, the 3.49 kb fragment of pMP13 (400 ng; positive control); 2, template preparation without insert DNA (2 μl; negative control); 3, template DNA (2 μl). The oligonucleotide hybridises strongly to the template DNA, and to the positive control, and therefore should be a suitable primer for sequencing.

4.7.5 Hybridisation of the Oligonucleotide Specific to *POX4* the 3.49 kb *Eco* RI/*Sal* I Fragment of pMP13

The DNA from the gel of restriction digests run in section 4.7.4 was transferred to Hybond-N by vacu-blotting (section 3.5.2.4), and hybridised to the oligonucleotide specific to *POX4* (*figure* 4.17).

4.7.6 Nucleotide Sequence of Part of the 3.49 kb *Eco* RI/*Sal* I Fragment of pMP13

10 μg pMP13 prepared by large scale triton lysis was digested overnight with restriction endonucleases *Eco* RI and *Sal* I. The 3.49 kb band which lit up when probed with the oligonucleotide specific to *POX4* was isolated by electroelution, and 4 ng was used to make template DNA (section 3.6.1) to obtain some sequence data to clarify the identity of the *C. tropicalis* DNA cloned in pMP13. The template was sequenced (section 3.6.2) using universal primer alongside control DNA supplied with the kit (*figure* 4.18).

Template (2 μl), template preparation without insert DNA (2 μl), and the 3.49 kb fragment of pMP13 (400 ng) were transferred to Hybond-N by dot-blotting (section 3.5.2.2), hybridised to the oligonucleotide specific to *POX4*, and autoradiographed (*figure* 4.19) to determine whether or not it could be used as a primer to obtain sequence data from the region of the cloned DNA that hybridises to the oligonucleotide.

Despite the fact that the oligonucleotide lit up the template DNA strongly, no sequence data could be obtained using the oligonucleotide as primer, although sequence was obtained from template primed with universal primer set up at the same time. The oligonucleotide DNA may have deteriorated with age which would have prevented it from acting as an efficient primer for the sequencing reaction.

4.8 DISCUSSION

4.8.1 The Identity of the Cloned *C. tropicalis* NCYC997 Genomic DNA Fragment

Statistically, the *C. tropicalis* NCYC997 genomic DNA library constructed in section 4.4 should have included the *POX4* and *POX5* genes along with other members of this multigene family. The results from section 4.7.2 clearly show that the cloned *C. tropicalis* DNA in pMP13 , specifically a 3.25-3.49 kb *Eco* RI/*Sal* I fragment, hybridises strongly to the oligonucleotide specific for *POX4-1* (Okazaki *et al.*, 1986), and the results from section 4.7.3 show that this 3.49 kb fragment hybridises specifically to *C. tropicalis* NCYC997 DNA confirming that this was the origin of this DNA.

The oligonucleotide probes were constructed as 20mers to statistically ensure hybridisation to only one member of the multigene family under conditions that only allow annealing to perfect complementary sequences (Zeff and Geliebter, 1987). The oligonucleotide specific to *POX4-1* is also complementary to *POX4-2* (Murray and Rachubinski, 1987). It was therefore assumed that the cloned DNA in pMP13 would contain all or part of one of the above alleles.

The restriction map generated for the 3.49 kb fragment of pMP13 in section 4.7.4 is similar but not identical to that for the *POX4-1* gene based on the published sequences. However, the pMP13 restriction fragments which hybridised to the oligonucleotide (section 4.7.5) were not consistent with the restriction maps of *POX4-1*, and *POX4-2* (*figure* 4.16), and the small amount of sequence data obtained in section 4.7.6 could not be matched with any of the published sequences of the multigene family.

The results obtained in this chapter leave the question of the identity of the cloned fragment unanswered. The fatty acid acyl-CoA oxidase gene clusters probably arose by gene duplication (Kamiryo and Okazaki, 1984), and it is possible that the cloned fragment has arisen as a result of this, and is another, as yet unidentified, member of the gene family or a redundant sequence which has arisen as a result of gene duplication and has some of the original sequence deleted and/or inverted. The restriction maps of the *OLE3* gene which is clustered with *POX5* (Okazaki, *et al.*, 1986) and those of other *POX* and *OLE* genes (Kamiryo and Okazaki, 1984) are very dissimilar to those of pMP13, which is therefore very unlikely to be carrying any of these genes.

Another possibility is that the oligonucleotide encodes a repeated sequence. However, this is unlikely as the autoradiographs in section 4.5 show that the oligonucleotide hybridises to the *C. tropicalis* NCYC997 genomic DNA forming a limited number of bands, and not several bands of high intensity as might be expected for a repeated sequence.

4.8.2 Peroxisomal Fatty Acid Acyl-CoA Synthetase: a better target for gene transplacement?

For several reasons it was decided to abandon the attempts to clone the *POX* genes.

C. tropicalis was a good choice of organism for this project as this yeast has more peroxisomal enzymes than methylotropic yeast (Kamiryo and Okazaki, 1984), contains the most abundant microbodies and the highest catalase activity when compared with other n-alkane-utilising yeasts (Osumi *et al.*, 1974), and has enzyme activities induced by hydrocarbons or ethanol which are much higher than those of other *Candida* yeasts grown on these substrates (Teranishi *et al.*, 1974a). However, these attributes also present problems.

It has been shown that integration occurs at only one chromosomal homologue in *C. albicans* (Kurtz *et al.*, 1986), and as *Candida* yeasts are stable diploids this means that the number of targets for gene disruption is effectively doubled. Even though it is possible to disrupt genes sequentially by transplacement the number of targets involved in this case (at least three and possibly four genes, i.e. six or more targets), and the lack of a good selectable marker for the identification of transformants, made this option appear less feasible (when the project was conceived it was thought that only two target genes existed).

If the *POX* genes are to be the targets for gene transplacement then it might be better to choose a different yeast as the host organism such as *C. maltosa*. Although this yeast does not utilise n-alkanes as efficiently as *C. tropicalis*, it only has one *POX* gene which would greatly facilitate gene transplacement (Hill *et al.*, 1988).

The presence of two functionally distinct fatty acid acyl-CoA synthetase activating enzymes has been demonstrated in rat liver cells (Pande and Mead, 1968); patients with X-linked adrenoleukodystrophy which is characterised by the accumulation of very long chain fatty acids in tissues and body fluids caused by deficient peroxisomal (but not microsomal) activation of very long chain fatty acids (Wanders et al., 1988); *C. lipolytica* (Kamiryo *et al.*, 1977; Kamiryo *et al.*, 1979) and *C. tropicalis* (Yamada *et al*, 1980).

The acyl-CoA synthetases of *C. lipolytica* and *C. tropicalis* are functionally distinct producing two long-chain fatty acid acyl-CoA pools in the cell which do not take the place of each other. Acyl-CoA formed by acyl-CoA synthetase II in peroxisomes is degraded exclusively via β-oxidation to yield acetyl-CoA, whereas that produced by acyl-CoA synthetase I is utilised for the synthesis of cellular lipids, and does not undergo β-oxidation. If β-oxidation is blocked, then the long-chain acyl-CoA produced by acyl-CoA synthetase II accumulates when the cells are grown in the presence of exogenous fatty acid (Kamiryo *et al.*, 1977; Kamiryo *et al.*, 1979; Yamada *et al*, 1980). These results suggest that a β-oxidation mutant would not produce dioic acids more efficiently, but would accumulate long-chain acyl-CoA instead.

The gene defects in the *C. tropicalis* mutant strains which secrete large amounts of dioic acids (Yi and Rehm, 1982a; Hill *et al.*, 1986) are unknown, and provide no information as to the best target for gene transplacement. A better target for gene transplacement to produce a mutant which would efficiently transform pelargonate to azeleate may therefore be the gene encoding fatty acid acyl-CoA synthetase II. Disrupting this gene would render the cells unable to activate fatty acids so there would be no substrate molecules for β-oxidation, and these cells would effectively be β-oxidation mutants. There is no evidence to suggest that the synthetase genes are a multigene family in the same way as the acyl-CoA oxidase genes, and although it might be necessary to disrupt the inducible diacyl-CoA synthetase reported by Yu and Hao (1986) to prevent the β-oxidation of the dioic acids formed the evidence discussed above shows that it would be unnecessary to disrupt the acyl-CoA synthetase I gene as it does not cross-react with acyl-CoA synthetase II.

The situation regarding the production of a mutant strain that will efficiently convert pelargonate to azeleate is complicated by the presence of different degradation pathways (Yi and Rehm, 1982a, b, and c: Hill *et al.*, 1986). The latter group reports that with mutant 7/34 of *C. tropicalis* grown on substrates purified by rectification of the raw material to >99.8% purity of the individual n-alkanes, the main product fraction of the transformation of a single n-alkane contains about 20 different dioic acids, including dioic acids with a shortened carbon chain and small amounts of acids with longer carbon chains.

This group also found that the moiety of the dioic acids produced increases with higher n-alkanes used as substrate. 91.13% of the acid precipitable products from dodecane was dodecanedioic acid, but only 12.2% of hexadecanedioic acid was formed from hexadecane. These results suggest that the biotransformation of pelargonate (C_9) would result in >91.13% of the acid precipitable products being azeleate. However, the presence of 3-hydroxydioic acids in the microbial transformation would limit the utilisation of the product because preparation of polymers such as polyamides and polyesters requires very pure monomers.

Although it maybe possible to produce a strain of *C. tropicalis* which is optimised for the production of azeleate from pelargonate this will not necessarily be cost effective as azeleate might form a relatively low percentage of the products of the biotransformation, and the cost of purifying the azeleate from the other dioic acids maybe as expensive as converting the pelargonate by a chemical method.

4.8.3 Further Work

In order to produce a *C. tropicalis* mutant optimised for the production of dioic acids the cloning and disruption of the gene encoding fatty acid acyl-CoA synthetase II should be undertaken. The main obstacle to this is the lack of information concerning this gene.

One approach would be to clone the gene by differential hybridisation in the same way as the *POX* genes were cloned (Kamiryo and Okazaki, 1984; Rachubinski *et al.*, 1985; Okazaki *et al.*, 1987), but this is hampered by the lack of a suitable probe. It might be possible to clone peroxisomal genes, in-vitro translate them, and try to identify the clones encoding the correct protein product by an enzyme assay.

Cohn *et al.* (1983) used a mixed-sequence synthetic oligonucleotide probe to isolate a clone containing the gene encoding the α subunit of bacterial luciferase from *Vibrio harveyi* and part of the gene coding for the β subunit from a library of total genomic DNA showing that mixed probes can be successfully used to isolate genes of interest if part of their protein sequences are known. If the appropriate data were available this approach would be feasible with *C. tropicalis* as codon usage in this organism is not random, with 87.4% of the amino acids specified by 25 principle codons which are similar to those used in highly expressed genes of *S. cerevisiae* (Murray and Rachubinski, 1987). It might be possible to isolate and sequence acyl-CoA synthetase II from peroxisomal membranes in order to produce such a probe.

CHAPTER 5. DOMINANT SELECTABLE MARKERS FOR *CANDIDA TROPICALIS*

5.1 INTRODUCTION

5.1.1 Aims

The purpose of the work described in this chapter was to find a suitable dominant selectable marker for *C. tropicalis*, initially for use in gene transplacement to produce a non-revertible β-oxidation mutant, but which could also be incorporated into a plasmid vector as the basis for the development of the molecular biology of this yeast species.

5.1.2 Types of Dominant Selectable Markers

5.1.2.1 Resistance Mechanisms

Dominant selectable markers effective in yeast transformation have been divided into two groups based upon their species of origin. In the first group the markers are encoded by genes from *Saccharomyces* species including resistance to killer toxin, cinnamic acid-resistance and growth on dextrin. In the second group the markers are encoded by genes from species other than *Saccharomyces* including chloramphenicol- (Cm) resistance, G418-resistance and methotrexate-resistance.

Resistance genes of bacterial origin are located on plasmids called R-factors (resistance transfer factors). These genes can be transferred by conjugation from one bacterium to another, irrespective of whether they belong to the same species. Other resistance genes are located on transposons (Esser and Dohmen, 1987).

There are several naturally-occurring resistance mechanisms as follows: The resistance gene encodes an enzyme which inactivates the toxic substance for example, resistance against β-lactam antibiotics such as penicillin, ampicillin and the cephalosporins, aminoglycoside antibiotics such as kanamycin (Km), neomycin (Nm) and G418, Cm and mercuric salts; binding of the toxic substance is prevented by modification of the target site for example, erythromycin and oligomycin; the resistance gene encodes specific transport proteins which actively excrete the toxic substance from the cell for example tetracycline, cadmium and arsenate, or the toxic substance is not taken up by the cell; the resistance gene encodes an enzyme which by-passes a blocked step in a biosynthetic pathway for example, trimethoprim, methotrexate, sulphonamides and sulphometuron methyl (SM); gene amplification, where multiple copies of a gene encoding the inhibited enzyme are introduced to the host cell resulting in over-production for example, resistance to tunicamycin and compactin; the resistance gene encodes a protein which complexes with the toxic agent thereby neutralising its toxicity for example, resistance against heavy metals (Davies, 1986; Esser and Dohmen, 1987).

Most antibiotic-producing organisms are resistant to the toxic products that they produce. Inactivation of the toxic substance and alteration of the target site are the most common mechanisms (Davies, 1986).

5.1.2.2 Resistance Determinants from *Saccharomyces*

Metallothioneins are low molecular weight proteins that have a high cysteine content (about 30%) and a great affinity for various metals such as Zn^{2+}, Cd^{2+}, Hg^{2+}, Ag^{2+} and Cu^{2+}. They play an important role in the protection of cells against heavy metal and zinc toxicity, and are present in all vertebrates, invertebrates, plants and lower eukaryotes such as yeast.

Naiki and Yamagata (1976) extracted copper-binding proteins from a copper-resistant strain of *S. cerevisiae*. In addition to the copper complexed with the copper-binding protein, they noted that copper-resistant strains of yeast accumulate considerable amounts of copper in the cells in the form of copper sulphide granules deposited under the cell walls.

The semi-dominant *CUP1* gene, located on chromosome VIII, which encodes the copper-binding protein in *S. cerevisiae* has been cloned and characterised, and used to successfully transform both laboratory and industrial strains of *S. cerevisiae* by several groups (Fogel and Welch, 1982; Welch *et al.*, 1983; Butt *et al.*, 1984a and b; Karin *et al.*, 1984; Henderson *et al.*, 1985; Meaden and Tubb, 1985; Knowles and Tubb, 1986; Fleming, 1988), as have the *S. cerevisiae* genes for resistance to cadmium and zinc (Kimura, 1986).

The synthesis of these metal-resistance genes is inducible by the ions for which they are specific at the transcriptional level. The regulation of *CUP1* is mediated by *cis*-acting control sequences located upstream of the coding sequence. The copper-chelating protein effects repression of the *CUP1* promoter in *trans*, acting as a negative regulator of *CUP1* expression (Butt *et al.*, 1984b).

In copper-resistant yeast the *CUP1* gene is amplified some 10 to 15 times as multiple, tandemly repeated copies of a 1.95 kb fragment of DNA which contains single sites for *Kpn* I and *Xba* I and two *Sau* 3AI sites (Welch *et al.*, 1983; Karin *et al.*, 1984). This results in the production of high levels of the copper chelating protein enabling growth in the presence of high concentrations of copper. Sensitive strains possess only one or two copies of the gene. *CUP1* is found in multiple copies in naturally occurring copper-resistant strains of *S. cerevisiae*, which suggests that multiple copies of *CUP1* have no detrimental effect on the yeast cell. Karin *et al.* (1984) reported a second distinct transcription unit within the amplified *CUP1* gene with a reading frame for a protein 246 amino acids in length whose function has not been determined.

The *CUP1* marker transforms laboratory and industrial strains of *S. cerevisiae* as efficiently as auxotrophic markers, but has to be present in multiple copies for maximum efficiency, and therefore is not a suitable marker for gene transplacement. Despite this the sensitivity of the *C. tropicalis* strains to copper was tested for several reasons: Copper-resistant transformants of industrial strains of *S. cerevisiae* maintain multiple copies of *CUP1* even in the absence of selective pressure demonstrating that the copper resistant phenotype is very stable, and the marker has no effect on fermentation characteristics (Karin *et al.*, 1984; Meaden and Tubb, 1985; Knowles and Tubb, 1986); industrial *S. cerevisiae* strains are reported to be extremely sensitive to copper compared with *cup1* laboratory strains (Henderson *et al.*, 1985; Fleming, 1988); and copper is readily available and relatively cheap to use. Copper resistance therefore suggested itself as a potential marker for the development of a general purpose cloning vector for *C. tropicalis*.

A second heavy-metal-resistance mechanism has also been reported in yeast-like cells, mycelium and chlamydospores of *Aureobasidium pullulans* which tolerates levels of cadmium toxic to fungi and yeasts via exclusion of cadmium in chlamydospores and restricted cellular uptake of cadmium by yeast-like cells and mycelium (Mowll and Gadd, 1984). Gadd *et al.* (1984) reported a stable copper-resistant (*cur1*) mutant of *S. cerevisiae* which took up less copper than the wild-type. The mechanism of tolerance involved changed membrane transport properties rather than alterations in the cell wall. These mutants have not been exploited in the development of dominant selection systems.

Some yeast strains produce toxins which are excreted into the environment where they are active against other yeasts. This killer phenotype is associated with cytoplasmic double-stranded RNAs (dsRNA) in *Saccharomyces* and *Ustilago* species and with DNA in *Kluyveromyces* species. The genetic determinant of killer character in other killer strains has not been established (Vondrejs, 1987). In *Saccharomyces* species killer character is determined by two species of cytoplasmically located dsRNA designated L- and M-dsRNA, which are found in encapsulated form as virus-like particles (VLPs). The dsRNA species of highest molecular weight (L) encodes the VLP capsid proteins and the smaller species (M) encodes a killer toxin protein and an immunity protein conferring toxin resistance to the host.

The cytoplasmically inherited killer character of a laboratory strain of *S. cerevisiae* has been transferred directly to a brewing yeast strain by rare mating between a respiratory sufficient, auxotrophic, haploid, killer strain carrying the *kar* mutation and a respiratory deficient brewing strain (Young, 1981). However, this approach is inappropriate for *Candida* strains which do not carry the necessary mutations.

cDNA of M-dsRNA has been cloned (Lolle *et al.*, 1984), and used to select transformants of sensitive yeast strains by exposure to added killer toxin (Bussey and Meaden, 1985). Transformants produce active toxin to which they are immune. The procedure requires 7-9 h of pre-incubation to allow the expression of immunity prior to selection, and a screening step to remove non-transformed survivors.

An advantage of this killer-immunity system, is that it permits selection for transformants containing multi-copy plasmids, as cDNA encoding yeast zymocin (killer toxin) and its immunity factor introduced into industrial strains on a multi-copy plasmid is 100% stable. Transformants which lose the plasmid are no longer immune to the zymocin and are killed by zymocin secreted by the plasmid-containing cells present in the extracellular medium. This results in self-selection of transformants (Bussey and Meaden, 1985; Knowles and Tubb, 1986; Tubb, 1987). Therefore, this marker provides a means of producing industrial strains resistant to contaminating killer-strains and of engineering strains capable of purging contaminant yeasts (Rank *et al.*, 1988).

Another advantage is that the killer phenotype can be used as a temporary marker for the purpose of selection and can be eliminated when no longer desired by curing via elevated temperature, cycloheximide or 5-fluorouracil treatment in *Saccharomyces* species, and by UV-irradiation in *Kluyveromyces* species. However, this marker is not suitable for gene transplacement as it must be present on a multi-copy vector for maximum efficiency.

The marker has other limitations to its application: Transformation efficiency is only 5-10% of that observed for selection of a prototrophic marker, and as cDNA transformants are not as fully immune as dsRNA containing killers some are killed by their own toxin, and there is some selection for increased resistance in the population. As the immunity gene must be expressed prior to selection with toxin, a compromise arises between obtaining a reasonable level of expression before other processes such as overgrowth of non-transformed cells leads to a loss of transformants. As killing is never complete there is more background survivor noise than is normally acceptable, and potential transformants need to be screened for the appropriate phenotype. Killer strains are still sensitive to zymocins of other immune classes, and the success in transfer of killer character between strains cannot be accurately predicted because the present knowledge of interactions among the components is poor (Bussey and Meaden, 1985; Vondrejs, 1987). For these reasons this system was not tested in *C. tropicalis*.

Glucoamylase (amyloglucosidase) is an extracellular enzyme that releases glucose (D) by sequential hydrolysis of α-1,4-linked sugars from the nonreducing end, whose activity can be monitored by a clear halo in starch-bromocresol medium (Rank *et al.*, 1988). Genes have been cloned from *Saccharomyces diastaticus* encoding extracellular glucoamylases which are capable of hydrolyzing starch. These are *STA1*, *STA2* and *STA3* which are allelic to *DEX2*, *DEX1* and *DEX3* respectively (Meaden *et al.*, 1985; Rank *et al.*, 1988).

DEX1 confers the ability to hydrolyse starch or dextrins and has been used to transform Dex^- haploid laboratory strains of *S. cerevisiae* directly by allowing plasmid treated sphaeroplasts to regenerate on G-sorbitol medium prior to selection on dextrin medium (Meaden *et al.*, 1985). However, several groups (Meaden and Tubb, 1985; Tubb, 1987; Rank *et al.*, 1988) reported failure to transform brewers' yeast using the same protocol. To overcome this problem, *CUP1* has been used as a selectable marker for introducing a *DEX* gene into brewers' yeast. Plasmids carrying both *CUP1* and *DEX1* give rise to copper-resistant transformants of *S. cerevisiae* which produce extracellular amyloglucosidase and are capable of hydrolysing wort dextrins (Meaden and Tubb, 1985; Tubb, 1987).

Bortol *et al.* (1988) carried out two successive sphaeroplast fusions in order to obtain an industrial strain able to utilise starch and produce killer toxin, the first between a wild-type killer strain and a laboratory strain (*S. cerevisiae STA2*), and the second between a Dex^+ hybrid carrying the killer factor and a bakers' yeast. The inefficiency of this system, especially when applied to industrial strains of *Saccharomyces* yeast, does not make it attractive as a selection system for *C. tropicalis*, and as *C. tropicalis* strains can grow on starch, and some ferment this substrate (Barnett *et al.*, 1983), this marker was not tested.

Melibiose produced by invertase cleavage of raffinose is further metabolised by the *MEL1* gene product, α-galactosidase (melibiase), to release D and galactose. α-galactosidase is absent in most industrial yeast strains except *Saccharomyces carlsbergensis* (Rank *et al.*, 1988). *MEL1*, which has been independently cloned and sequenced, is under the control of the *GAL* regulatory system and is both selectable and easily scorable (Knowles and Tubb, 1986).

Casey *et al.* (1988) used *Pvu* II cleavage of the *SMR1-410* gene to direct integration of *MEL1* to the *ILV2* locus of a haploid laboratory strain and two industrial strains of *S. cerevisiae*, demonstrating that single-copy expression of *MEL1* was sufficient to produce the *MEL1* phenotype making it a suitable marker for gene transplacement. However, Rank *et al.* (1988) were not able to use *MEL1* as a selectable marker in industrial strains due to a high background of untransformed cells. This was because the enzyme encoded by *MEL1* releases one D and galactose molecule per melibiose molecule hydrolysed and these two monosaccharides support the growth of non-transformed cells on melibiose selection media which impairs the use of *MEL1* as a selectable marker (Casey *et al.*, 1988; Rank *et al.*, 1988), therefore despite the fact that *C. tropicalis* is unable to ferment or grow on melibiose (Barnett *et al.*, 1983), and the *MEL1* phenotype is selectable in single-copy, it was decided not to test this marker.

Cinnamic acids are inhibitory to yeast growth. Non-brewing strains of *Saccharomyces*, yeasts of other genera and enterobacteria are able to decarboxylate ferulate to 4-vinylguaiacol, or cinnamate to styrene using the gene product encoded by *POF1*. Styrene can be easily detected in culture media by its pungent aroma. The yeast enzyme acts on cinnamate itself and has potential as a dominant selectable marker for industrial yeast strains, although this has yet to be proven (Goodey and Tubb, 1982; Tubb, 1987).

Methylglyoxal, a regulator of cell division, is a toxic α-ketoaldehyde found at extremely low concentrations in all organisms. It interacts with NH_2- or SH- groups, which may react with polyamines and which function in cell division.

A recombinant plasmid which confers resistance to methylglyoxal was isolated from an *S. cerevisiae* gene library. Transformed cells of sensitive strains, including industrial polyploids, grow in the presence of 5.0 mM methylglyoxal, and are also resistant to tetramethylthiuram disulphide, iodoacetamide, Zn^{2+}, Cd^{2+}, Co^{2+}, phenylglyoxal, N-ethylmaleimide and 8-hydroxyquinoline (Kimura, 1986). However, this marker is only efficiently expressed when present in multiple copies and is not suitable for gene transplacement, and therefore the sensitivity of *C. tropicalis* to methylglyoxal was not investigated.

The antibiotic polymyxin B kills sensitive cells by disrupting their membranes. The *S. cerevisiae* gene, *PBS2*, located on chromosome X, confers polymyxin B-resistance when propagated on a high-copy number plasmid probably via titration of the inhibitor by the excess of *PBS2* gene product. *PBS1* is not allelic to *PBS2*, and the inactivation of the *PBS2* gene in a *pbs1* background leads to a complete loss of polymyxin B-resistance, demonstrating that *PBS2* is necessary for *pbs1* activity (Boguslawski and Polazzi, 1987). The *PBS2* gene is only efficiently expressed when present in more than three copies and is not suitable for gene transplacement, and therefore the sensitivity of *C. tropicalis* to polymyxin B was not investigated.

The *ILV2* gene of *S. cerevisiae* encodes the enzyme α-acetolactate synthase (ALS) which catalyses the first common step in the biosynthesis of isoleucine and valine, and is unique in the yeast genome. Yeast ALS is localised in the mitochondria with the other isoleucine and valine biosynthetic enzymes and feedback inhibited by valine (Falco *et al.*, 1985).

ALS activity in bacteria, yeast and plants is the molecular target of sulphonylurea herbicides including SM, N-[(4,6-dimethylpyrimidin-2-yl) amino-carbonyl]-2-methoxycarbonyl-benzenesulphonamide, and also of a structurally unrelated class of herbicides, the imidazolinones. The sulphonylurea herbicides show a very high herbicidal activity combined with low mammalian toxicity, but the mechanism through which SM inhibits ALS is not known (Falco and Dumas, 1985).

3 μgml^{-1} SM is sufficient to inhibit the growth of most wild-type *S. cerevisiae* strains which have a normal level of ALS (Falco, 1986; Fleming, 1988). Sensitive cells are inhibited only on minimal media and this inhibition is reversed by addition of the branched chain amino acids (Falco and Dumas, 1985).

Mutations in several genes have been cloned whose phenotypic effects result in SM-resistance in *S. cerevisiae*. One locus, *pdr1.1* is allelic to a multiple drug resistant mutant with a plasma membrane permeability barrier (Rank *et al.*, 1988), and the physiological basis of the second mutation, *smr3*, is not known (Rank, 1986). The third group consists of at least twelve, phenotypically distinct mutant alleles of *ILV2*, which encode SM-resistant variants of ALS cloned from *E. coli* and *S. cerevisiae* (Falco and Dumas, 1985; Yadav *et al.*, 1986). These mutations result in normal levels of ALS activity in yeast and slightly reduced sensitivity to valine. *S. cerevisiae* strains carrying these mutant *ILV2* alleles are able to grow in the presence of 30 μgml^{-1} SM.

Both the mutant (*SMR1.410*) and the normal *ILV2* alleles have been sequenced, and this has shown that they are identical except for a C to T transition mutation at nucleotide 574 of the open reading frame of *ILV2*, resulting in a proline to serine change in the amino acid sequence of the protein which confers resistance to SM (Falco *et al.*, 1985; Yadav *et al.*, 1986).

The *SMR1.410* allele has been used as a dominant selection marker to successfully transform both laboratory and industrial strains of *Saccharomyces* yeast in both replicating and yeast integrating vectors (Falco, 1986; Casey *et al.*, 1988; Fleming, 1988).

SM-resistant transformants are easily detected because of the substantial difference between the resistance of untransformed and transformed cells, especially during the initial screening, and are also resistant to certain structurally related sulphonamide compounds (Falco, 1986). Integration can be directed to the *ILV2* locus of chromosome XIII in *S. cerevisiae* by restriction at the single *Pvu* II site within *SMR1.410* (Casey *et al.*, 1988). Depending on the homology between *SMR1.410* and *C. tropicalis* DNA this might be a problem for gene transplacement of the acyl-CoA oxidase genes.

5.1.2.3 Resistance Determinants from Sources Other than *Saccharomyces* Yeasts

A number of genes are not directly selectable, but can be used to verify transformants because their expression can be detected by a colour change for example, a *Pseudomonas* gene for catechol 2,3-dioxygenase has been used to transform industrial strains of *S. cerevisiae* (Knowles and Tubb, 1986), and the *E. coli lacZ* gene encoding β-galactosidase has been functionally expressed in *S. cerevisiae* (Hollenberg, 1982).

The *E. coli* β-lactamase enzyme, encoded by the *bla* gene, hydrolyses the β-lactam bond in penicillin antibiotics. Although *S. cerevisiae* is not sensitive to ampicillin, the expression of this gene in *S. cerevisiae* has been shown by applying nitrocefin to outgrown cell streaks. β-lactamase converts the yellow colour of the intact molecule into a red cleavage product within 15-60 min depending on the age of the cells. A second test detects the cleavage products of penicillin antibiotics via their uptake of iodine from an iodine starch complex (Hollenberg, 1979; Hollenberg *et al.*, 1981; Hollenberg, 1982).

Placing *bla* under the control of the yeast promoter for the structural gene encoding *ADH1* increases the amount of β-lactamase produced in *S. cerevisiae* 100-fold (Reipen *et al.*, 1982).

S. cerevisiae is susceptible to growth inhibition by aminopterin and methotrexate in rich media when the *de novo* synthesis of dihydrofolate is blocked by sulphanilamide. On the basis of this, mouse dihydrofolate reductase (dhfr), which confers resistance to the antimetabolite methotrexate has been developed for use as a dominant selectable marker by modification of dhfr cDNA for expression in yeast (Zhu *et al.*, 1985; Zhu *et al.*, 1986).

The endogenous dihydrofolate reductase is inhibited with 60 μgml^{-1} methotrexate and 0.2 $mgml^{-1}$ sulphanilamide on defined media plates supplemented with 20 μgml^{-1} of uridine, resulting in cessation of growth. Growth is then restored in transformants by over-expression of the mouse dhfr gene.

Transformation of *S. cerevisiae* cells with mouse dhfr on multi-copy transforming plasmids and with 2- to 3-copy integration of the mouse dhfr gene gives clear methotrexate resistant colonies against background cells. However, as resistance of the integrative transformants to methotrexate is dependent on the number of copies of the mouse dhfr gene, it is not a suitable marker for gene transplacement.

Phleomycin and bleomycin cause scission of DNA both *in vivo* and *in vitro*. The *ble* gene of transposon Tn*5* confers resistance to phleomycin and bleomycin, although the biochemical mechanism of resistance has not yet been elucidated (Davies, 1986; Gatignol *et al.*, 1987).

The *ble* gene has been expressed in *S. cerevisiae* using the *S. cerevisiae CYC1* promoter and terminator, giving rise to phleomycin-resistant transformants on complex media at the same efficiency as an auxotrophic marker. Transformation is more efficient in the presence of glycerol (G), a non-fermentable carbon source (Gatignol *et al.*, 1987).

On media supplemented with 1 μgml^{-1} phleomycin high background levels of untransformed cells were reported. Selection on 3 μgml^{-1} prevented the growth of these untransformed cells but greatly reduced the number of phleomycin-resistant transformants recovered. It has not been established that a single-copy of the *ble* gene is sufficient to confer phleomycin-resistance on transformants.

Several antibiotics inhibit protein synthesis by inhibiting ribosomal functions. These may be active against prokaryotes but not against eukaryotes or *vice versa*. The basis for this selective toxicity is that ribosomes from bacteria (and blue-green algae) are different and can be readily distinguished from those of higher organisms on the basis of their sedimentation coefficients ("S values"). Prokaryotes contain 70S ribosomes and eukaryotes contain 80S ribosomes. Chloroplasts and mitochondria have been shown to contain 70S ribosomes which resemble bacterial ones in their response to selectively toxic antibiotics (Gale *et al.*, 1981). This selective toxicity is not due to different membrane-permeabilities of different cell-types because protein synthesis in bacterial extracts has been shown to be sensitive to Cm, but not to cycloheximide, and the opposite has been demonstrated in systems derived from eukaryotic cells (Gale *et al.*, 1981).

Cm inhibits growth of a wide range of Gram-positive and Gram-negative bacteria and was the first broad-spectrum antibiotic to be used clinically. Experiments with [^{14}C] Cm demonstrated that 70S ribosomes bind Cm whereas 80S ribosomes from a variety of sources do not. The 70S ribosomes of chloroplasts and mitochondria are also sensitive to Cm which binds specifically to the 50S subunit of 70S ribosomes (Gale *et al.*, 1981).

Cm inhibits the peptide bond-forming reaction and acts directly upon the peptidyl transferase reaction *in vivo*, allowing binding of aminoacyl-tRNA into the ribosomal A site but preventing recognition by the peptidyl transferase of its acceptor substrate. Cm thus inhibits peptide chain elongation and the movement of ribosomes along mRNA (Gale *et al.*, 1981). Cm is normally inactive against eukaryotic cells, however under respiratory growth conditions *S. cerevisiae* has been shown to be sensitive to Cm. This is because cellular metabolism and growth on non-fermentable energy sources such as G and ethanol (E) requires functional mitochondria which, in yeast, is the cellular target of Cm (Cohen *et al.*, 1980; Hollenberg, 1982). Cm resistance is conferred by the bacterial Cm acetyltransferase (CAT) gene from transposon Tn*9* which inactivates Cm by acetylation (Hollenberg *et al.*, 1981; Hollenberg, 1982). CAT expression appears to be controlled via a post-transcriptional interaction between homologous sequences in the messenger RNA for the transacetylase and the 23S ribosomal RNA (Davies, 1986).

Rosanilin dyes such as crystal violet and basic fuchsin have been used as indicator dyes in solid growth medium for Cm-resistant enterobacterial colonies containing CAT. The colour difference is probably due to the binding of these dyes to CAT. However the contrast between *S. cerevisiae* colonies with and without CAT is insufficient for unambiguous distinction (Proctor and Rownd, 1982). A simple spectrophotometer assay for CAT exists which is applicable to yeast transformants. CAT has been functionally expressed in *S. cerevisiae* transformants (Hollenberg, 1979; Cohen *et al.*, 1980). The marker required time to express before being challenged with Cm, there was a problem with background growth of untransformed colonies, and the marker was not very efficient compared with auxotrophic markers.

The efficiency of transformation was greatly improved by the use of yeast promoter and terminator sequences to increase levels of CAT expression. This strategy allowed the transformation of both haploid and polyploid strains of *S. cerevisiae* and, when included in an integrating vector, single-copy transformants were formed as efficiently as with *LEU2* and *HIS3*. The Cm-resistance marker also efficiently transformed *E. coli* (Hadfield *et al.*, 1986). This group also reported that inhibition was slightly more effective on YEPGE than on SGE. Increasing Cm concentration in the medium does not result in an increase in the number of plasmids or the amount of CAT protein produced, showing that plasmid copy number and marker expression are regulated independently of the selection pressure (Hadfield *et al.*, 1987).

Cycloheximide inhibits protein synthesis in a wide variety of eukaryotic organisms by binding to the 60S subunits of 80S ribosomes inhibiting initiation as well as elongation of polypeptides (Gale *et al.*, 1981; Adoutte-Panvier and Davies, 1984). *Saccharomyces* species have a minimum inhibitory concentration (MIC) of 0.1-2 μgml^{-1} while *Kluyveromyces* species are resistant to concentrations of $>500\ \mu gml^{-1}$ (Adoutte-Panvier and Davies, 1984).

The recessive alleles *tcm1*, *cyh2* and *cry1* in *S. cerevisiae* result in resistance to trichodermin, cycloheximide and cryptopleurine (Rank *et al.*, 1988). The *RIM.C* (ribosome modification by cycloheximide) of *C. maltosa* which modifies ribosomes such that protein synthesis in the cells is no longer inhibited by cycloheximide has been cloned by using a host-vector system of *S. cerevisiae* (Takagi *et al.*, 1986b). *S. cerevisiae* cycloheximide-resistant transformants could be obtained by allowing sphaeroplasts to recover overnight prior to adding top agar containing 5 μgml^{-1} cycloheximide. However, cycloheximide is unsuitable as a dominant selection system for *C. tropicalis*, as strains are able to grow at concentrations of 0.1-1.0 $mgml^{-1}$ (Barnett *et al.*, 1983).

Various aminoglycosides such as Nm, Km and gentamicin inhibit translocation on bacterial ribosomes. Nms and gentamicins but not the Kms also inhibit translocation in *S. cerevisiae* extracts. Aminoglycosides bind to both 30S and 50S subunits of 80S ribosomes causing misreading of mRNA (Gale *et al.*, 1981). Aminoglycosides show multiphasic effects both upon protein synthesis and upon misreading of mRNA when examined over a broad range of drug concentrations. For example, gentamicin at low concentrations inhibits synthesis but gives little misreading; misreading increases with increasing gentamicin concentration accompanied by reversal of inhibition and at high gentamicin concentrations, inhibition of synthesis increases. These results and the failure to isolate single step mutants with high level resistance to Nm, Km, or gentamicin suggests that these drugs interact with multiple sites on 70S and 80S ribosomes (Gale *et al.*, 1981).

The aminoglycosides do not share a common mode of action in eukaryotic systems. For example, G418 is a 2-deoxystreptamine antibiotic that is structurally related to gentamicin but has inhibitory activity against a much wider variety of pro- and eukaryotic organisms (Jimenez and Davies, 1980).

Enzymes which modify aminoglycoside antibiotics have been detected in a wide range of resistant bacteria. The three known aminoglycoside-modification mechanisms are N-acetylation of amino groups, and O-phosphorylation and adenylation of hydroxyl groups. There is a very sensitive, rapid and specific assay for these enzymes which are poorly characterised from a physical and chemical point of view (Haas and Dowding, 1975; Davies, 1986). In some organisms, the ribosomes may become resistant as a result of modification of the ribosomal RNA (Davies, 1986). Two types of 3'-0-aminoglycoside phosphotransferases designated I and II, are encoded by the transposable elements Tn*601(903)* and Tn*5* respectively. 3'-0-aminoglycoside phosphotransferase I phosphorylates and inactivates a number of aminoglycoside antibiotics containing the 2-deoxystreptamine moiety, at the 3'-hydroxyl position and in yeast this phosphotransferase is most effective (Jimenez and Davies, 1980).

The 3'-0-aminoglycoside phosphotransferase I (Km-resistance, Km^R) gene of Tn*601*, used in combination with aminoglycosides such as Km, Nm, G418, etc., has been incorporated into vectors for use in bacteria, yeasts, fungi, plants, and mammalian cells (Davies, 1986). Some specific examples of its expression in yeast and industrially important fungal species are laboratory and industrial strains of *S. cerevisiae* (Hollenberg, 1979; Jimenez and Davies, 1980; Hollenberg, 1982; Webster and Dickson, 1983; Ernst and Chan, 1985; Yocum, 1986; Fleming, 1988), *Kluyveromyces lactis* (Sreekrishna *et al.*, 1984), *Candida utilis* (Ho *et al.*, 1984), *K. fragilis* (Das *et al.*, 1984), *Schizosaccharomyces pombe* and *S. carlsbergensis* (Sakai and Yamamoto, 1986) and *Acremonium chrysogenum* (Isogai *et al.*, 1987). The sensitivity of *S. cerevisiae* to Km or Nm is insufficient for the direct selection of transformants (Hollenberg, 1979; Hollenberg, 1982).

Media which contain the levels of salts found in yeast nitrogen base (YNB) confers partial or complete G418-resistance on *S. cerevisiae* cells and cannot be used for selecting G418-resistant transformants (Webster and Dickson, 1983). In the procedure of Hollenberg (1982) high background was caused in part by the salt component of YNB imparting drug resistance. This salt mediated G418-resistance has also been reported for *K. fragilis* (Das *et al.*, 1984) and *K. lactis* (Sreekrishna *et al.*, 1984). Hollenberg (1982) reported that the number of spontaneously resistant colonies could be greatly reduced on plates containing G as the sole carbon source.

Even on rich media G418-resistant mutants which appear spontaneously have been reported in laboratory and industrial strains of *S. cerevisiae* (Jimenez and Davies, 1980; Fleming, 1988), *C. utilis* (Ho *et al.*, 1984) and *S. carlsbergensis* (Sakai and Yamamoto, 1986). The number of background colonies can be decreased by selecting for higher resistance levels of G418 (Jimenez and Davies, 1980), and true transformants can be distinguished by replication on YEPD plates containing G418, or assaying for Km^R activity (Haas and Dowding, 1975).

A lag-phase is required prior to administration of G418 for expression of the Km^R gene in *S. cerevisiae* (Webster and Dickson, 1983) and *S. carlsbergensis* (Sakai and Yamamoto, 1986). G418-resistant transformants can be selected directly, but the number of transformants recovered is critically dependent upon the time of selective pressure administration (Webster and Dickson, 1983).

The salt constituents of YNB increase the viability of regenerating sphaeroplasts but, due to the inhibition of drug sensitivity, selection of G418 transformants is limited to YEPD or to YEPD supplemented with KPO_4, and in consequence, plating efficiencies are 5-60 fold lower for the drug selection than for auxotroph selection made on YNB. The results above indicate that transformation of sphaeroplasts is not suitable when G418 is to be used a dominant selectable marker. Even under optimum conditions where transformation does not involve sphaeroplasting, the frequency of G418-resistant transformants is lower than that for auxotrophic markers (Webster and Dickson, 1983).

Signals controlling expression of *E. coli* genes are different from those controlling expression in yeast cells, and in consequence the unmodified Km^R gene is poorly expressed. By expressing the Km^R gene on high-copy number plasmids in *S. cerevisiae*, high gene dosage compensates for poor gene expression (Hollenberg, 1979; Hollenberg, 1982). However, the Km^R resistance determinant has an adverse effect on growth rate (Fleming, 1988).

Under optimal conditions the Km^R gene confers G418-resistance on *S. cerevisiae* at low-copy numbers (Webster and Dickson, 1983; Ernst and Chan, 1985). Substituting the bacterial expression signals with yeast expression signals from the cytochrome c gene (*CYC1*) allows a single integrated copy of the Km^R gene to protect yeast cells from lethal doses of G418 (Yocum, 1986). This single-copy expression also improves the cell viability of transformants.

Since the bacterial Km^R gene is functionally expressed in several yeast species without modification provided conditions are optimal, it is reasonable to assume that this gene might also be expressed in *C. tropicalis*, and as the gene is heterologous it should be suitable for gene transplacement.

The sensitivity of yeast to G418 varies between strains. Haploid laboratory strains of *S. cerevisiae* are resistant to 100-500 μgml^{-1} G418 (Jimenez and Davies, 1980; Ernst and Chan, 1985; Fleming, 1988), whereas, the growth of brewing yeast strains is inhibited by 25 μgml^{-1} G418 (Sakai and Yamamoto, 1986; Fleming, 1988). *S. cerevisiae* strains carrying the mutations *AGS1, AGS2* and *AGS3* (aminoglycoside antibiotic sensitivity) have been isolated which are sensitive to 20 μgml^{-1} G418 (Ernst and Chan, 1985). Wild-type *K. fragilis* is sensitive to 50 μgml^{-1} G418 (Das *et al.*, 1984). Of the *Schwanniomyces* species strains investigated by Panchal *et al.* (1984), two (*Schwanniomyces castellii* 1402 and *Schwanniomyces castellii* 1436 a 2-deoxy-glucose-resistant mutant of strain 1402) were found to be resistant to 1 $mgml^{-1}$ G418.

Hygromycin B, an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus*, is a potent inhibitor of protein synthesis on endogenous, viral, or synthetic mRNA, inhibiting translocation and causing mistranslation of mRNA and phenotypic suppression in prokaryotic and eukaryotic cells. Hygromycin B and paromomycin promote misreading *in vitro* in various *Saccharomyces* and *Kluyveromyces* species. Hygromycin B appears to act at a single site on the 30S subunit of bacterial ribosomes (Gale *et al.*, 1981; Gritz and Davies, 1983; Adoutte-Panvier and Davies, 1984).

Hygromycin B is used as an anthelminthic agent in animal husbandry and is commercially available. 150 μgml^{-1} inhibits growth of *E. coli* and 60-100 μgml^{-1} inhibits growth of *Saccharomyces* yeast. Mammalian cells are also quite sensitive to hygromycin B. The permeability of different cell lines to hygromycin B determines in part their sensitivity (Gritz and Davies, 1983).

The plasmid-borne hygromycin B phosphotransferase gene, *hph*, of *E. coli* has been identified and its nucleotide sequence determined (Gritz and Davies, 1983). Hygromycin B phosphotransferase is linked in its R-plasmid of origin to a Km^R gene (Davies, 1986). Hygromycin resistance has been developed for use in a number of inter-species gene transfers and promises to be a useful selection method.

Table 5.1 Minimum inhibitory concentration of copper in NEP

Sensitivity to copper was measured as described in Chapter 3.

mM $CuSO_4$	Percentage survival			
	S. cerevisiae		*C. tropicalis*	
	S150-2B	X-2180-1A	NCYC547	NCYC997
0.0	100	100	100	100
0.5	85	100	84	98
1.0	84	78	96	3
1.5	100	72	-	0
2.0	92	81	32	1
2.5	0	51	2	0
3.0	0	50	0	0
3.5	-	6	1	-
4.0	0	5	12	0
5.0	-	0	0	-

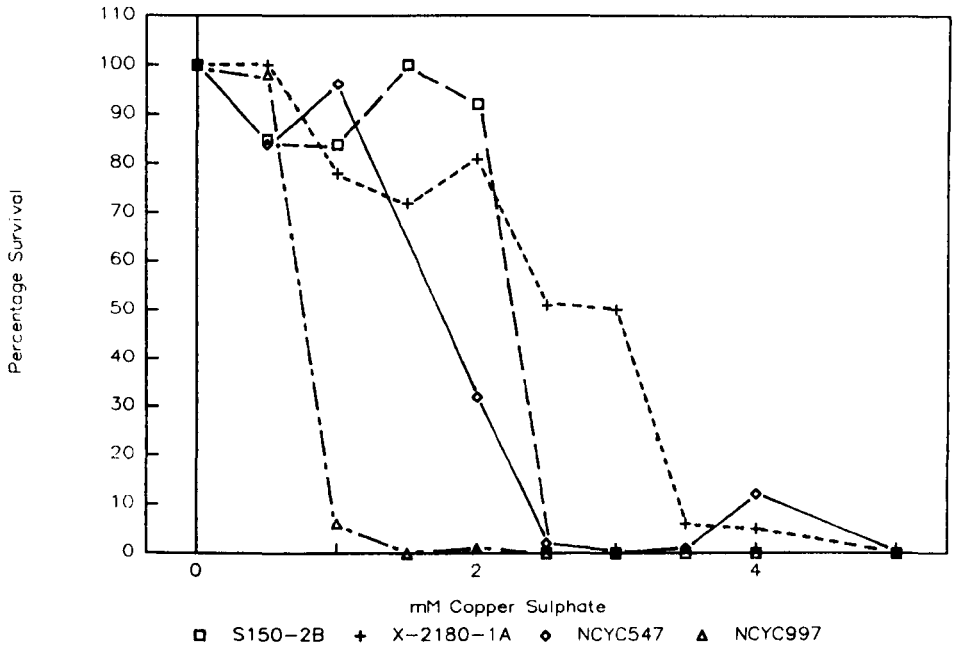


***Figure* 5.1 Minimum inhibitory concentration of copper in NEP for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B and X-2180-1A**

Plotted from the data presented in table 5.1. The MICs are: NCYC547, 3.0 mM $CuSO_4$; NCYC997, 1.5 mM $CuSO_4$; S150-2B, 2.5 mM $CuSO_4$; X-2180-2B, 5.0 mM $CuSO_4$.

Although the *hph* gene confers hygromycin B resistance in *E. coli*, the intact bacterial gene was not effective in conferring drug resistance to yeast cells. This was overcome by linking the gene up with *S. cerevisiae* promoter and terminator sequences (Gritz and Davies, 1983; Kaster *et al.*, 1984). Yeast cells receiving these plasmids can be directly selected after transformation on 1.5 $mgml^{-1}$ hygromycin B, and the *hph* gene can be used for insertional inactivation . Efficiency is improved by adding the hygromycin B 4-20 h after transformation.

Hygromycin B suppresses nonsense mutations in yeast and may induce its own expression in yeast by increasing the level of misreading in strains containing AUG codons in the region between the *CYC1* promoter and the *hph* coding sequence (Gritz and Davies, 1983). The relatively high concentrations of G418 and hygromycin B needed in selective growth media have hampered the use of phosphotransferase genes as dominant selectable markers in yeast (Ernst and Chan, 1985).

5.2 SENSITIVITY TO COPPER

The minimum inhibitory concentration of copper was determined as described in section 3.8 on NEP-agar (section 2.2.1) containing various concentrations of copper sulphate (section 2.2.3.4). It is necessary to use agar-noble since the copper ions are less toxic in the presence of Bacto-agar.

S. cerevisiae X-2180-1A (*MATα, SUC2, mal, mel, gal2, CUP1*; section 2.1.1.2) was included as a positive control.

In each case the value given for percentage survival is an average from several (2-8) plates. A graph of percentage survival vs. concentration of copper is shown (*figure* 5.1). *C. tropicalis* NCYC547 was as sensitive to copper as *S. cerevisiae* S150-2B (*MATα, ura3-52, trp1-289, his3-D1, leu2-3, leu2-122, cup1,* $2\mu m^+$; section 2.1.1.2), and *C. tropicalis* NCYC997 was more sensitive. However, the inhibition of the *C. tropicalis* strains is not as complete as it is in *S. cerevisiae*.

Table 5.2 Minimum inhibitory concentration of chloramphenicol in YEPG

Sensitivity to Cm in YEPG was measured as described in Chapter 3.

$mgml^{-1}$ Cm	Percentage Survival		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
0.0	100	100	100
1.0	0	100	-
2.5	0	100	100
3.0	0	81	100
3.5	0	95	100
4.0	0	40	37
4.5	0	35	0
5.0	0	29	0
6.0	-	5	0

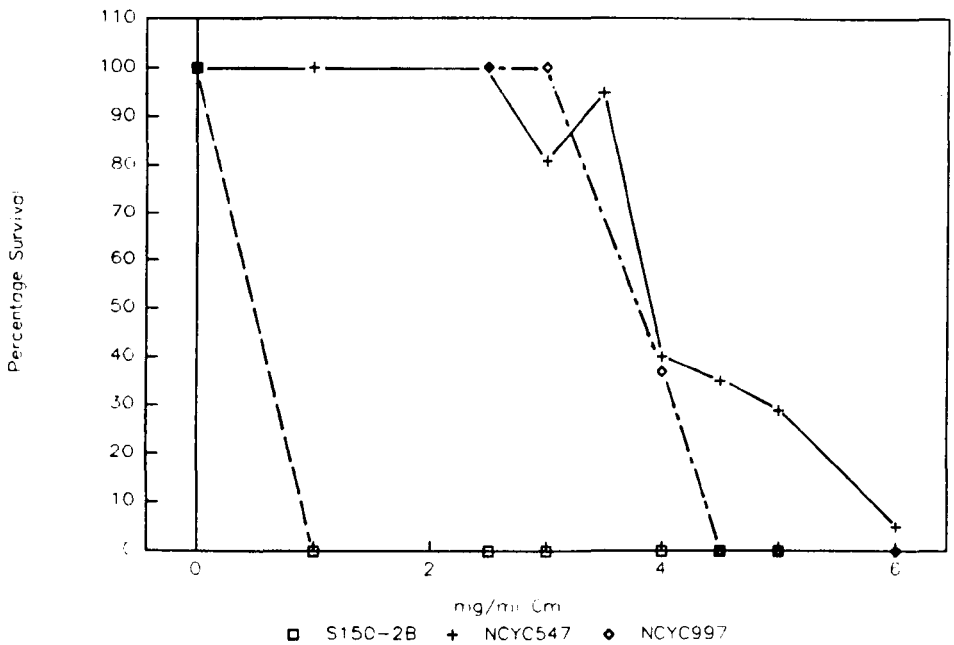


***Figure* 5.2 Minimum inhibitory concentration of Cm in YEPG for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B**

Plotted from the data presented in table 5.2. The MICs are: NCYC547, >6.0 $mgml^{-1}$ Cm; NCYC997, 4.5 $mgml^{-1}$ Cm; S150-2B, <1.0 $mgml^{-1}$ Cm.

5.3 SENSITIVITY TO SOME RIBOSOME INHIBITING ANTIBIOTICS

5.3.1 Chloramphenicol

5.3.1.1 Minimum Inhibitory concentration of Chloramphenicol in YEPG

The minimum inhibitory concentration (MIC) of Cm was initially determined in YEP-agar with G, section 2.2.3.1) as the sole carbon source to ensure that the cells were growing aerobically. Cellular metabolism and growth on the nonfermentable energy sources G and E requires functional mitochondria which, in yeast, is the cellular target of Cm; only cells that are resistant to the antibiotic will grow on GE plus Cm (Cohen *et al.*, 1980).

The Cm was added to give required concentrations from a stock solution of 50 $mgml^{-1}$ (section 2.2.3.5). All the test strains were streaked to single colonies over YEPG-agar and incubated overnight. All showed good growth. In YEPD, SD, and SG-agar all the test strains were found to be much more resistant to Cm (results not shown). This is consistent with the findings of other workers. In each case the value given for percentage survival is an average from several (2-8) plates. A graph of percentage survival vs. concentration of Cm is shown (*figure* 5.2).

C. tropicalis NCYC997 was approximately 4.5-fold, and NCYC547 was >6-fold more resistant to Cm in YEPG than the *S. cerevisiae* Cm^S control.

5.3.1.2 Transformations of the Test Strains Selecting Cm Resistance

The test strains were transformed using the lithium acetate method (section 3.4.2.1) as follows:
S. cerevisiae S150-2B with 2 μg pCH100 (section 2.1.3.2)
S. cerevisiae S150-2B with 10 μl TE (section 2.3.1)
C. tropicalis NCYC547 with 10 μl TE.
C. tropicalis NCYC997 with 10 μl TE.
S150-2B was selected on YEPG with 2.5 $mgml^{-1}$ Cm.
NCYC547 and NCYC997 were selected on YEPG with 5 $mgml^{-1}$ Cm. This should inhibit all untransformed NCYC997 cells, but is likely to allow the growth of some untransformed NCYC547 cells.
Each transformation was spread over five plates.

Table 5.3 Inhibition of growth by ethanol in YEP for *C. tropicalis* strains NCYC547 and NCYC997, and *S. cerevisiae* S150-2B

Sensitivity to ethanol was measured as described in Chapter 3. The *C. tropicalis* strains were much more E-tolerant than the *S. cerevisiae* strain tested.

% v/v E	Percentage cell Survival		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
1	100	100	100
2	65	94	100
4	0	97	100
5	-	100	100
7	-	100	100
8	0	50	47
10	0	0	0

Table 5.4 Minimum inhibitory concentration of chloramphenicol in YEPE

Sensitivity to Cm in YEPE was measured as described in Chapter 3.

$mgml^{-1}$ Cm	Percentage Cell Survival		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
0.0	100	100	100
2.0	0	100	88
4.0	0	100	100
6.0	0	100	100

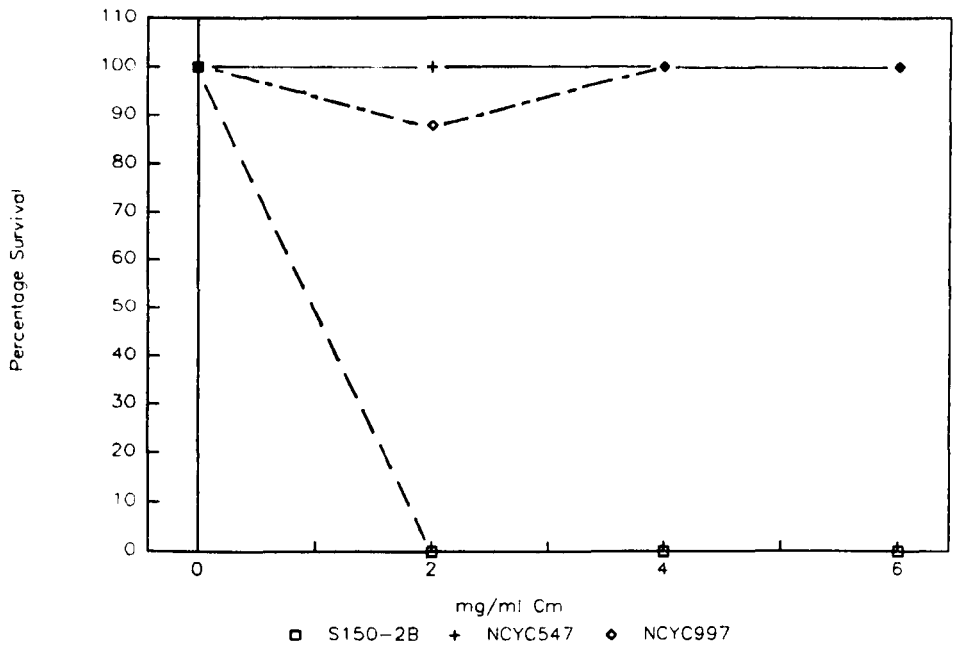


***Figure* 5.3 Minimum inhibitory concentration of Cm in YEPE for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B**

Plotted from the data presented in table 5.4. The MIC of Cm for *S. cerevisiae* S150-2B was <2.0 $mgml^{-1}$. The MIC of Cm was not reached for either *C. tropicalis* strain. Due to the insolubility of Cm in water it is difficult to raise the Cm concentration much higher and still be sure that all the antibiotic has dissolved.

pCH100 transformed S150-2B with an efficiency of 7.65 x 10^2 transformants per μg DNA. No colonies were isolated from the competent S150-2B control transformations to which no DNA was added. The *C. tropicalis* control transformations to which no DNA was added showed confluent growth over all plates. 10 S150-2B Cm^R transformants, and cells from 10 patches of each of the *C. tropicalis* transformations were streaked to single colonies over fresh selective media and incubated (72 h). All the restreaked cells grew well on the fresh plates.

5.3.1.3 Minimum inhibitory concentration of chloramphenicol in YEPE

As the concentration of Cm in the YEPG-agar used in the above section becomes higher, the v/v percentage of E present also increases. It was realised that cell inhibition could be due to high E concentrations rather than the Cm. This could explain the findings of the test transformations in the above section. To test the tolerance of the test strains to E, cell survival was ascertained on YEP-agar with 1-10% v/v E as the sole carbon source (table 5.2.1.2). All the test strains were streaked to single colonies on YEPE-agar and incubated overnight. All showed good growth. The *C. tropicalis* strains were much more E-tolerant than the *S. cerevisiae* strain, but were not 100% tolerant above 7% v/v E. 4% v/v E was considered to be the maximum concentration of E in the selective medium which would still ensure that any inhibition of growth would be entirely due to the effects of the Cm.

The MIC of Cm was ascertained in YEP-agar, the sole carbon source being the E added with the Cm. To ensure that the E concentration did not become toxic I did not exceed 4% v/v, and the concentration of the stock solution of Cm was increased to 150 $mgml^{-1}$: 2 $mgml^{-1}$ Cm = 1.335% v/v E; 4 $mgml^{-1}$ Cm = 2.67% v/v E; 6 $mgml^{-1}$ Cm = 4% v/v E; The medium without Cm contained 2% v/v E as sole carbon source.

These results demonstrate that both strains of *C. tropicalis* are too resistant to Cm for this antibiotic to be useful as a selective agent in this yeast.

Table 5.5 Minimum inhibitory concentration of kanamycin in YEPD

Sensitivity to Km was measured as described in Chapter 3.

$mgml^{-1}$ Km	Percentage cell survival.		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
0.0	100	100	100
0.1	94	89	97
0.5	99	100	100
1.0	77	95	100

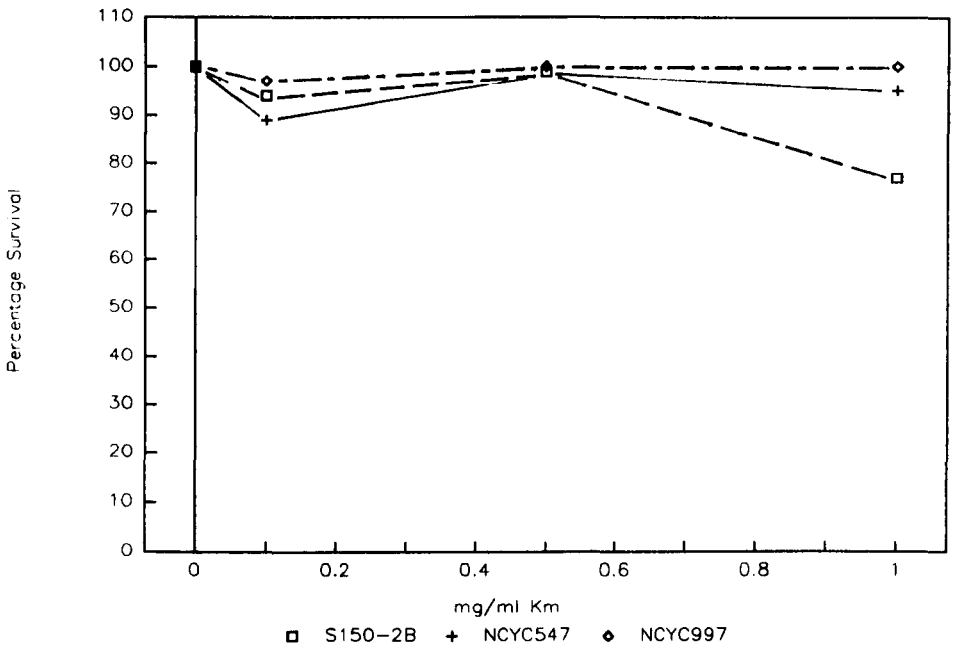


***Figure* 5.4 Minimum inhibitory concentration of Km in YEPD for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B**

Plotted from data presented in table 5.5. The MIC of Km was not reached for any of the yeast strains tested.

Table 5.6 Minimum inhibitory concentration of neomycin in YEPD

Sensitivity to Nm was measured as described in Chapter 3.

$mgml^{-1}$ Nm	Percentage cell survival		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
0.0	100	100	100
0.1	97	92	100
0.5	89	96	100
1.0	84	79	89
2.0	66	92	59
3.0	65	15	58
3.5	-	-	1
4.0	87	4	0

Table 5.7 Minimum inhibitory concentration of G418 in YEPD

Sensitivity to G418 in YEPD was measured as described in Chapter 3.

$mgml^{-1}$ G418	Percentage Survival		
	S. cerevisiae S150-2B	*C. tropicalis* NCYC547	*C. tropicalis* NCYC997
0.00	100	100	100
0.10	11	91	76
0.50	0	61	8
1.00	0	9	3
1.25	-	0	0
1.50	-	1	0
2.00	-	0	0

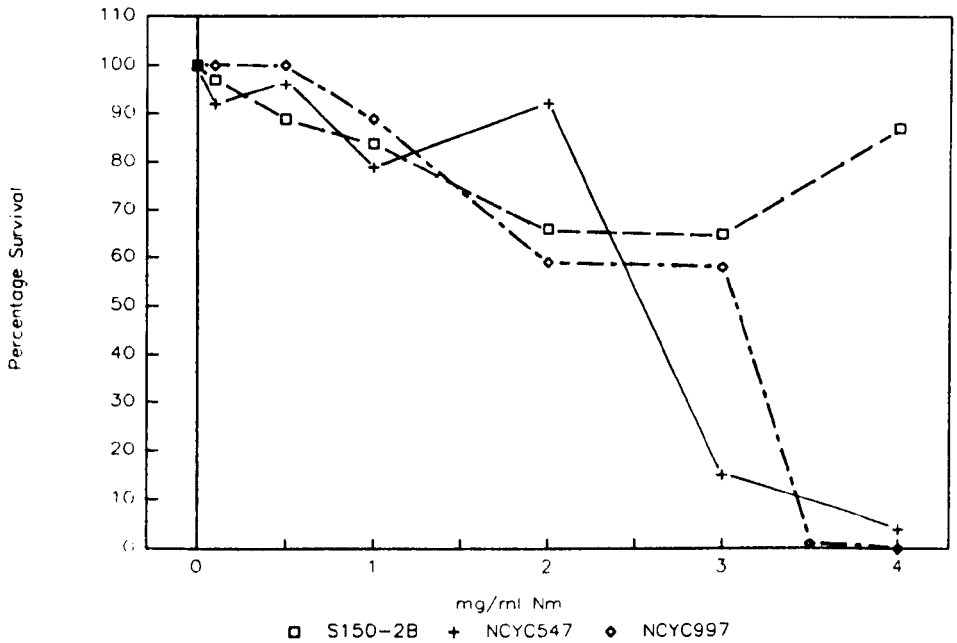


***Figure* 5.5 Minimum inhibitory concentration of Nm in YEPD for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B**

Plotted from the data presented in table 5.6. The MICs are: NCYC547, not reached; NCYC997, 4.0 $mgml^{-1}$ Nm; S150-2B, not reached.

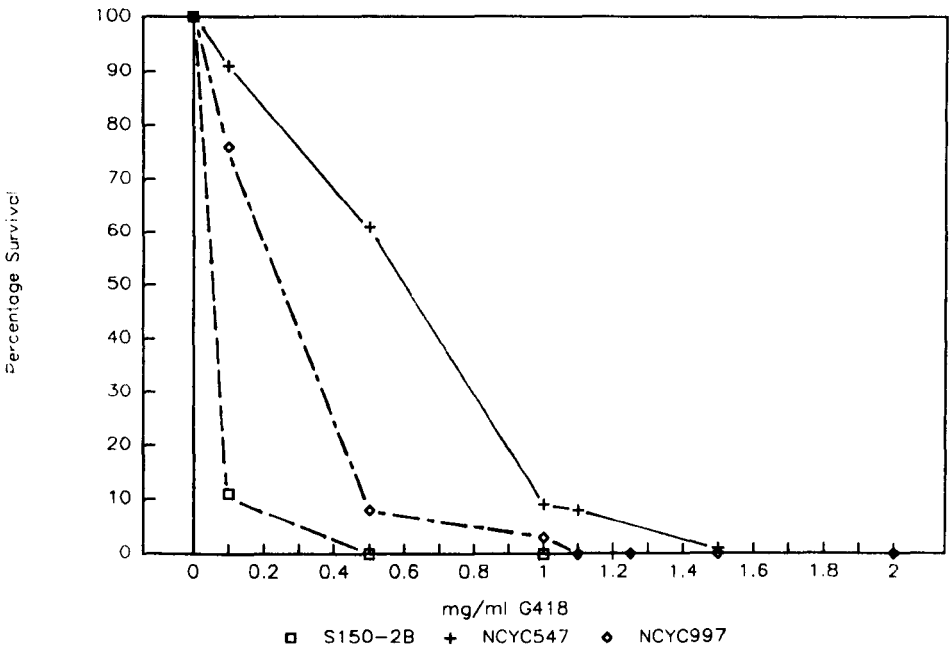


***Figure* 5.6 Minimum inhibitory concentration of G418 in YEPD for *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* S150-2B**

Plotted from the data presented in table 5.7. The MICs are: NCYC547, 1.25 $mgml^{-1}$ G418; NCYC997, 1.25 $mgml^{-1}$ G418; S150-2B, 0.5 $mgml^{-1}$ G418.

5.3.2 Neomycin, Kanamycin, and G418

5.3.2.1 Minimum Inhibitory Concentrations in YEPD

The antibiotics were added from stock solutions (section 2.2.3.5) to YEPD-agar to give the required concentrations. YEP was used rather than S medium as media which contain the levels of salts found in YNB have been found to render cells partially to completely G418-resistant. The salts $CaCl_2$, $MgSO_4$ and $(NH_4)_2SO_4$ in YNB are responsible for imparting drug resistance (Webster and Dickson, 1983). This resistance was observed for both *C. tropicalis* strains (results not shown).

In each case the value given for percentage survival is an average from several (2-8) plates. Graphs of percentage survival vs. concentration of Km, Nm and G418 are shown (*figures* 5.4-5.6).

All the yeast strains tested were resistant to Km, the *C. tropicalis* strains being slightly more resistant than *S. cerevisiae* S150-2B, and this is therefore not a suitable selective agent for these strains. S150-2B was much more resistant to Nm than either *C. tropicalis* strain although a point was not reached when NCYC547 was completely inhibited. G418 was the most effective antibiotic against all the strains tested, but the *C. tropicalis* strains were much more resistant than S150-2B. From these results it appeared that G418 might be a suitable selective agent for *C. tropicalis* transformants.

5.3.2.2 Transformations of the Test Strains Selecting on G418 in YEPD

The test strains were transformed using the lithium acetate method (section 3.4.2.1) as follows:
S. cerevisiae S150-2B with 2 μg pCH192 (section 2.1.3.2)
S. cerevisiae S150-2B with 10 μl TE (section 2.3.1)
C. tropicalis NCYC547 with 10 μl TE.
C. tropicalis NCYC997 with 10 μl TE.
S150-2B was selected on YEPD with 0.5 $mgml^{-1}$ G418. NCYC547 and NCYC997 were selected on YEPD with 2 $mgml^{-1}$ G418. Each transformation was spread over five plates (200 μl per plate).

pCH192 transformed S150-2B with an efficiency of 350 transformants per μg DNA. No colonies were isolated from the competent S150-2B control transformations to which no DNA was added. The *C. tropicalis* control transformations to which no DNA was added showed confluent growth over all plates. 10 S150-2B transformants were streaked to single colonies over fresh selective media and incubated (72 h). All the S150-2B transformants grew well on the fresh plates.

Table 5.8 Effect of DMSO and carbon source on the sensitivity of *C. tropicalis* NCYC547 and NCYC997 to G418

Sensitivity to G418 was measured as described in Chapter 3. The results for *S. cerevisiae* S150-2B confirm that the G418 is active. At 5% v/v the DMSO appeared to be slightly toxic to the *C. tropicalis* strains. 1% v/v DMSO decreases G418-sensitivity slightly. G418-sensitivity is most increased when the carbon-source is GE which is non-fermentable.

Media	%v/v DMSO	mgml-1 G418	Percentage cell survival		
			S150-2B	NCYC547	NCYC997
YEPD	0	0	100	100	100
YEPD	0	1	0	30	13
YEPD	0	2	0	0	3
YEPD	1	0	-	100	90
YEPD	1	1	-	33	19
YEPD	1	2	-	1	5
YEPD	5	0	-	82	100
YEPD	5	1	-	11	38
YEPD	5	2	-	2	6
YEPGE	0	0	100	100	100
YEPGE	0	1	0	0	0
YEPGE	0	2	-	0	0
YEPGE	1	0	-	100	100
YEPGE	1	1	-	0	0
YEPGE	1	2	-	0	0
YEPGE	5	0	-	99	77
YEPGE	5	1	-	0	0
YEPGE	5	2	-	0	0

Table 5.9 Minimum Inhibitory Concentration of G418 in YEPGE for *C. tropicalis* NCYC547 and NCYC997

Sensitivity to G414 in YEPGE was measured as described in Chapter 3.

$mgml^{-1}$ G418	Percentage Survival *C. tropicalis*	
	NCYC547	NCYC997
0.0	100	100
0.1	100	36
0.2	59	17
0.5	3	0
0.8	0	0
1.0	0	0
2.0	0	0

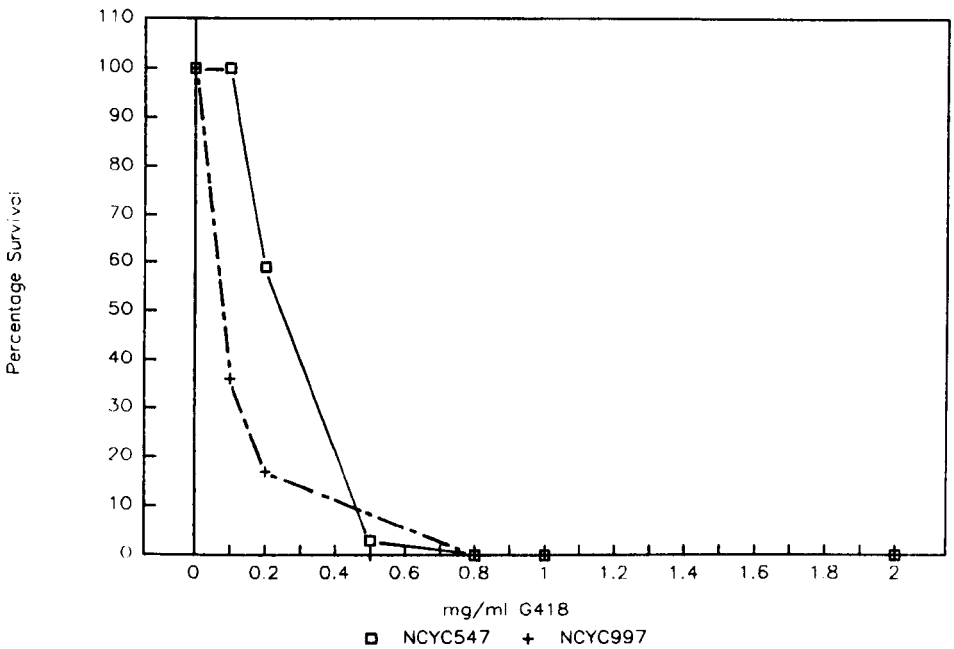


***Figure* 5.7 Minimum inhibitory concentration of G418 in YEPGE for *C. tropicalis* NCYC547 and NCYC997**

Plotted from the data presented in table 5.9. The MICs are: NCYC547, 0.8 $mgml^{-1}$ G418; NCYC997, 0.5 $mgml^{-1}$ G418.

5.3.2.3 Minimum Inhibitory Concentration of G418 with Different Carbon Sources, and in the Presence or Absence of DMSO

In order to increase the sensitivity of the *C. tropicalis* strains to G418 it was decided to determine the MIC using G and E as a combined carbon source to put greater pressure on the ribosomes, and also to determine the MIC with DMSO present in the media. In some fungal cell types, impermeability can be correlated with resistant behaviour for example, *Aureobasidium pullulans* (Mowll and Gadd, 1984), *Schwanniomyces castellii* (Panchal *et al.*, 1984), and *S. cerevisiae* (Gadd *et al.*, 1984), so the latter strategy should test this by increasing the uptake of the G418 into the cells by increasing the permeability of the cell membrane.

The test plates without G418 show that the presence of the DMSO has a slightly detrimental effect on cell growth at 5% v/v, but on the whole is not affecting cell viability. Rather than increasing the sensitivity of the cells to G418, the DMSO appears to be having the opposite effect, as was also noticed for 5-fluoro-orotic acid (section 7.2.4.3).

When growing on YEPGE the sensitivity of the cells appears to be increased compared with cells growing on YEPD. The MIC of G418 in YEPGE for the two *C. tropicalis* strains was investigated in more detail (table 5.9). In each case the value given for percentage survival is an average from several (2-8) plates. A graph of percentage survival vs. concentration of G418 in YEPGE is shown (*figure* 5.7).

These results show that both strains are much more sensitive to G418 on YEPGE than on YEPD with MICs comparable to that of *S. cerevisiae* S150-2B on YEPD.

5.3.2.4 Transformation of the Test Strains Selecting on G418 in YEPGE

The test strains were transformed using the polyethylene glycol (PEG) induced method (section 3.4.2.2) which allows some manipulation of cell density on the selective medium as follows:
Each of three aliquots *S. cerevisiae* S150-2B with 10 μg pCH192 (section 2.1.3.2)
S. cerevisiae S150-2B with 10 μl TE (section 2.3.1)
Each of six aliquots *C. tropicalis* NCYC547 with 10 μl TE, and six aliquots *C. tropicalis* NCYC997 with 10 μl TE, each aliquot of competent cells being harvested at a different A_{600}.

Table 5.10 Results of control transformations of the *C. tropicalis* strains to which no DNA was added

Transformants were selected on YEPGE-agar containing G418.

c = confluent growth.

A_{600} is optical density of the culture when the cells were harvested prior to being made competent.

A_{600} culture	$mgml^{-1}$ G418	N°. resistant cells (one plate)	
		NCYC547	NCYC997
0.1	0.8	c	c
	1.0	512	432
	1.5	301	283
0.2	0.8	c	c
	1.0	c	c
	1.5	683	644
0.3	0.8	c	c
	1.0	c	c
	1.5	823	800
0.4	0.8	c	c
	1.0	c	c
	1.5	848	810
0.5	0.8	c	c
	1.0	c	c
	1.5	1296	1272
0.6	0.8	c	c
	1.0	c	c
	1.5	1692	1688

S150-2B was selected on YEPGE with 0.5, 1.0, or 2.0 $mgml^{-1}$ G418. NCYC547 and NCYC997 were selected on YEPGE with 0.8, 1.0, or 1.5 $mgml^{-1}$ G418. Each S150-2B transformation was spread over a single plate, but one fifth of each *C. tropicalis* transformation was spread over each concentration of G418.

pCH192 transformed S150-2B with an efficiency of 150 transformants per μg DNA on each G418 concentration. This demonstrates that even at high concentrations G418 has no effect on the number of resistant cells able to grow. No colonies were isolated from the competent S150-2B control transformations to which no DNA was added. 10 S150-2B transformants were streaked to single colonies over fresh selective media and incubated (72 h). All the S150-2B transformants grew well on the fresh plates. Results of the *C. tropicalis* control transformations to which no DNA was added are shown in table 5.10.

Even at high concentrations of G418 compared with the apparent MIC, and low plating densities compared with those at which it is possible to select S150-2B cells transformed to $G418^R$, a point was never reached where all or most of the *C. tropicalis* cells were inhibited by the G418.

5.4 DISCUSSION

5.4.1 Sensitivity of *Candida tropicalis* to Copper

The MICs of copper for the *C. tropicalis* strains tested (NCYC547 3.0 mM; NCYC997 1.5 mM) are similar to that for *S. cerevisiae* S150-2B (2.5 mM), a copper-sensitive laboratory strain (section 5.2), demonstrating that copper-resistance has potential as a dominant selectable marker for *C. tropicalis*. The inhibition of the *C. tropicalis* cells was not as complete as that of the *S. cerevisiae* laboratory strains tested however, which might indicate that some untransformed colonies might appear as background contamination following selection on copper.

It has been reported by several groups (Karin *et al.*, 1984; Meaden and Tubb, 1985; Knowles and Tubb, 1986) that the copper-resistance determinant is maintained very stably in industrial strains of *S. cerevisiae* without selective pressure being applied, a fact that makes copper an attractive marker for transforming industrial strains of yeast such as *C. tropicalis*. Most industrial strains of *S. cerevisiae* are inhibited by <0.08 mM $CuSO_4.5H_2O$ in NEP, a level of 0.3 mM on synthetic complete plates is sufficient to select transformants, and *CUP1* strains grow confluently on agar plates in the presence of up to 1.75 mM CuSO4 (Butt *et al.*, 1984a; Karin *et al.*, 1984; Henderson *et al.*, 1985; Fleming, 1988). These strains are therefore much more sensitive to copper than either laboratory strains of *S. cerevisiae* or the *C. tropicalis* strains tested in this thesis.

5.4.2 Sensitivity of *Candida tropicalis* to Ribosome Inhibiting Antibiotics

5.4.2.1 Sensitivity of *Candida tropicalis* to Chloramphenicol

Initially in section 5.3.1.1 it appeared that the *C. tropicalis* strains were being inhibited by Cm albeit at much higher concentrations than those required to inhibit *S. cerevisiae* S150-2B (4.5-6.0 $mgml^{-1}$ Cm cf. <1.0 $mgml^{-1}$ Cm in YEPG). *S. cerevisiae* Cm-resistant transformants are typically selected on YEPGE-agar containing 0.5-1.8 $mgml^{-1}$ Cm (Hollenberg, 1979; Cohen *et al.*, 1980), although some background problems have been reported (Hollenberg, 1979). However, control transformations of the *C. tropicalis* strains to which was added no DNA was added gave confluent growth over all the selective plates, although the results of transforming *S. cerevisiae* showed that both the transformation procedure and the selection were working.

The Cm is dissolved in E, and the results of testing the tolerance of the test strains to E in YEP-agar (section 5.3.1.3) suggested that at high Cm concentrations the E in the selective medium might be causing the inhibition of growth in the *C. tropicalis* strains rather than the action of the Cm. The toxicity of the E may have been diminished by the much greater number of cells plated out from the transformations compared with the relatively small numbers used to ascertain the MIC. The results from repeating the experiment in YEP-agar with the E concentration kept at 4% v/v or less confirmed that both *C. tropicalis* strains are resistant to Cm which is therefore unsuitable as a dominant selectable marker for these strains.

5.4.2.2 Sensitivity of *Candida tropicalis* to some Aminoglycoside Antibiotics

In YEPD the MIC of Km was not reached for any of the test strains demonstrating that this antibiotic is completely unsuitable for use as a dominant selectable marker for *C. tropicalis*. *S. cerevisiae* S150-2B was more resistant to Nm than either *C. tropicalis* strain, however a MIC was not reached for NCYC547, and at 4.0 $mgml^{-1}$ for NCYC997 was rather high. The sensitivity of *S. cerevisiae* to both Km and Nm is considered to be insufficient to select transformants (Hollenberg, 1979; Hollenberg, 1982), and this is also the case for the *C. tropicalis* strains tested.

The MICs of G418 in YEPD for the *C. tropicalis* strains tested (NCYC547 1.35 $mgml^{-1}$; NCYC997 1.25 $mgml^{-1}$) were higher than that for *S. cerevisiae* S150-2B (0.5 $mgml^{-1}$), but were low enough to consider using G418 as a dominant selectable marker in these strains. However, transformations of the *C. tropicalis* strains to which no DNA was added gave confluent growth over all the selective plates, although the results of transforming *S. cerevisiae* showed that both the transformation procedure and the selection were working.

Increasing the membrane permeability of the cells by adding DMSO to the selective medium did not lower the MIC of G418 for either *C. tropicalis* strain, but putting pressure on the mitochondrial ribosomes by selecting on YEPGE lowered the apparent MIC of G418 for both *C. tropicalis* strains to 0.5-0.8 $mgml^{-1}$, comparable to that of *S. cerevisiae* S150-2B on YEPD.

Transformation of the *C. tropicalis* strains was performed using the PEG-induced method of Klebe *et al.* (1983) which allowed some manipulation of the cell density on the YEPGE selective plates. Despite this, confluent or near confluent growth was still obtained, although the results of transforming *S. cerevisiae* showed that both the transformation procedure and the selection were working. The unacceptable numbers of untransformed *C. tropicalis* cells growing on the selective medium after these control transformations to which no DNA was added make G418 unsuitable as a dominant selectable marker for these strains.

Other groups have reported the appearance of spontaneous $G418^R$ mutants on rich media after transformation of laboratory and industrial strains of *S. cerevisiae* (Jimenez and Davies, 1980; Fleming, 1988), *C. utilis* (Ho *et al.*, 1984), and *S. carlsbergensis* (Sakai and Yamamoto, 1986), but this was not a problem to the extent seen in the results for *C. tropicalis* in this work.

The sensitivity to G418 of different yeast species has been reported by various groups and is very varied. Haploid *S. cerevisiae* strains are inhibited by 0.1-0.5 $mgml^{-1}$ G418 (Jimenez and Davies, 1980; Ernst and Chan, 1985; Fleming, 1988), industrial *S. cerevisiae* strains are inhibited by 25 μgml^{1} G418 (Sakai and Yamamoto, 1986; Fleming, 1988), *Kluyveromyces fragilis* is inhibited by 50 μgml^{-1} G418 (Das *et al.*, 1984), and two *Schwanniomyces castellii* strains were inhibited by 1.0 $mgml^{-1}$. *C. albicans* has also been reported to be naturally resistant to high levels of G418 (Kurtz *et al.*, 1986).

As reported for copper-sensitivity, polyploid industrial strains of *S. cerevisiae* are much more sensitive to G418 than haploid laboratory strains (Sakai and Yamamoto, 1986; Fleming, 1988). As *C. tropicalis* is diploid it was possible that the results obtained for this yeast might resemble those for the industrial *S. cerevisiae* strains however, the opposite would appear to be the case for most of the inhibitory substances tested.

5.4.2.3 Possible Resistance Mechanisms

Resistance mechanisms include inactivation, modification of the target site, active excretion of the inhibitor, impermeability to the inhibitor, by-passing a blocked step in a biosynthetic pathway, over-production of a resistance-determinant by gene amplification, and neutralisation of the inhibitor by complexing (Davies, 1986; Esser and Dohmen, 1987). Some naturally occurring resistant yeast strains may be mutants that arose during propagation in a micro-environment containing antibiotic-producing strains, and it has also been speculated that some yeast strains once produced antibiotics and the resistance mechanisms represent the natural means of self-protection commonly found in antibiotic-producing organisms. At the moment there is no evidence that yeasts produce such antibiotics (Adoutte-Panvier and Davies, 1984). Resistance has been correlated to impermeability in *Aureobasidium pullulans* challenged with cadmium (Mowll and Gadd, 1984), *Schwanniomyces castellii* challenged with G418 (Panchal *et al.*, 1984), *S. cerevisiae* challenged with hygromycin B (Gritz and Davies, 1983), and *S. cerevisiae curl* mutants challenged with copper (Gadd *et al.*, 1984). However, adding DMSO to selective media containing G418 did not increase the sensitivity of the *C. tropicalis* cells to this antibiotic although DMSO renders cells more permeable. In fact, the cells appeared to become slightly more resistant to the antibiotic. A similar finding was reported for sphaeroplasts of *S. cerevisiae curl* mutants which took up less copper and were more tolerant to copper than whole cells due to changed membrane transport properties (Gadd *et al.*, 1984). The resistance of the *C. tropicalis* strains to the aminoglycoside antibiotics and Cm is therefore unlikely to be due to the cells being impermeable to these inhibitors although it is possible that the cells are actively excreting the antibiotics.

Resistance can also arise through modification of ribosomal RNA (Davies, 1986) which might also explain why the *C. tropicalis* strains tested were resistant to the ribosome inhibiting antibiotics. For example, the *RIM.C* gene of *C. maltosa* modifies ribosomes such that they are resistant to cycloheximide (Rank *et al.*, 1988). This is one of the most common natural resistance mechanisms (Davies, 1986). The finding that DMSO does not increase G418-sensitivity allows for the possibility that the *C. tropicalis* strains tested have resistant ribosomes. The differences in the resistance to Cm, Km, Nm, and G418 can be explained by the fact that inhibitors of ribosome function act at different sites (Gale *et al.*, 1981) which might be modified to a greater or lesser extent. This phenomenon has also been reported for *Ustilago maydis* (Banks, 1983) which is sensitive to Nm and paromomycin whilst being resistance to 10-fold greater amounts of Km and gentamicin.

5.4.2.4 Results of the Transformations

Despite the apparent MIC of G418 obtained for the *C. tropicalis* strains, transformations, to which no DNA was added, selecting on G418 in YEPD and YEPGE-agar gave rise to large numbers of apparently G418-resistant untransformed cells. In a typical lithium acetate transformation 3×10^6 cells are spread upon each selection plate, of which only one tenth at most will be viable (Hadfield *et al.*, 1986). This means that up to 3×10^5 viable cells are spread over each plate following transformation, and this number will have increased during the time allowed for the recovery of the transformed cells before they are challenged with an inhibitor. This is considerably more than the 1000 cells spread over each plate to ascertain the MIC of an inhibitory substance. It could be that the large numbers of cells on the plate effectively dilute the inhibitor allowing the growth of untransformed cells. In the case of *C. tropicalis* strains the formation of pseudohyphae might also protect untransformed cells in some way for example, by cross-feeding.

A similar phenomenon was reported by Henderson *et al.* (1985) who found that copper sensitivity does not guarantee that copper resistance can be used to select *S. cerevisiae* transformants, and that the cut-off concentration above which no transformants were recovered is lower than the highest concentration tolerated by the transformants once sub-cultured.

5.4.3 Further Work

5.4.3.1 Development of the *CUP1* Allele as a General Dominant Selectable Marker for the Transformation of *Candida tropicalis*

The *CUP1* allele should be tested in *C. tropicalis* to establish whether it is a suitable marker for this yeast. As the work in this thesis was primarily concerned with producing a non-revertible β-oxidation mutant by gene transplacement and the *CUP1* allele is not suitable for this purpose because it is not sufficiently expressed in single-copy no trial transformations were carried out. It is also possible that the allele will not function efficiently in this yeast while it is under the control of *S. cerevisiae* promoters and terminators.

The *CUP1* gene is semi-dominant and the *CUP1/cup1* heterozygote displays a resistance level intermediate between that of the two homozygotes. Selection for resistance to progressively higher copper concentrations yields a family of isolates with graded resistance levels up to 12 mM $CuSO_4$ (Fogel and Welch, 1982). It should therefore be possible to develop strains of *C. tropicalis* which are either very resistant to, or very sensitive to Cu for use with this marker.

5.4.3.2 Other Dominant Selectable Markers

The sensitivity of *C. tropicalis* to some of the dominant selectable markers discussed in section 5.1 should be tested. *MEL1*, cinnamic acids, methylglyoxal, and polymyxin B might be of use as selectable markers on multicopy plasmids for this yeast although they are unsuitable for gene transplacement.

SM should be tested as it has been found to be a good marker in laboratory and industrial strains of *S. cerevisiae* (Falco, 1986; Casey *et al.*, 1988; Fleming, 1988), and has low mammalian toxicity. Only small amounts (3 μg) are required to inhibit sensitive cells whereas transformants are resistant to 30 μg making their identification very easy. SM-resistance has been used in both replicative and integrative transformation and is therefore suitable for gene transplacement. However, since the *IVL2* allele is of *S. cerevisiae* origin depending on the amount of homology, integrative transformation might be directed to the equivalent allele in *C. tropicalis* rather than the target gene.

Hygromycin B-resistance is also suitable for gene transplacement in *S. cerevisiae* although a relatively high concentration is required for yeast selection (Ernst and Chan, 1985). However, hygromycin B acts on the 30S subunit of 70S ribosomes, and given the resistance of *C. tropicalis* to the ribosome inhibiting antibiotics tested in the course of this work it is possible that this yeast will also be resistant to this antibiotic.

Phaff *et al.* (1952) describe *C. tropicalis* NCYC547 as a killer strain, and therefore it might be possible to develop this as a dominant selection system for *C. tropicalis* in the same way as the *S. cerevisiae* and *K. lactis* killer phenotypes have been developed.

5.4.3.3 Cloning *Candida tropicalis* Promoter and Terminator Sequences

Some resistance-determinants have been shown to be expressed in heterologous host cells without modification for example, the *bla* gene of *E. coli* encoding β-lactamase in *S. cerevisiae* (Hollenberg, 1979; Hollenberg *et al.*, 1981; Hollenberg, 1982), the CAT gene of Tn*9* in *S. cerevisiae* (Hollenberg, 1979; Cohen *et al.*, 1980), and the Km^R gene of Tn*601* in *S. cerevisiae* (Hollenberg, 1979; Jimenez and Davies, 1980; Hollenberg, 1982; Webster and Dickson, 1983; Ernst and Chan, 1985), and *Ustilago maydis* (Banks, 1983). However, in all the above cases expression was insufficient for selection of transformants from a single-copy of the gene. In order to improve expression in *S. cerevisiae* the genes had to be placed under the control of *S. cerevisiae* promoter and terminator sequences: *bla* (Reipen *et al.*, 1982), CAT (Hadfield *et al.*, 1986), and Km^R (Yocum, 1986). In the latter case as the Km^R gene has an adverse affect on growth rate (Fleming, 1988) cell viability was also improved by the low-copy number. The hygromycin B phosphotransferase gene of *E. coli* is not effective in *S. cerevisiae* unless it is linked to *S. cerevisiae* promoter and terminator sequences (Gritz and Davies, 1983; Kaster *et al.*, 1984).

From the evidence presented above it is clear that unless fortuitously *S. cerevisiae* and *E. coli* promoter and terminator sequences allow the efficient expression of heterologous resistance determinants in *C. tropicalis*, it will be necessary to clone these sequences from *C. tropicalis* strains and incorporate them into cloning vectors for this yeast. *S. cerevisiae* sequences are likely to be more compatible with *C. tropicalis* than those from *E. coli*.

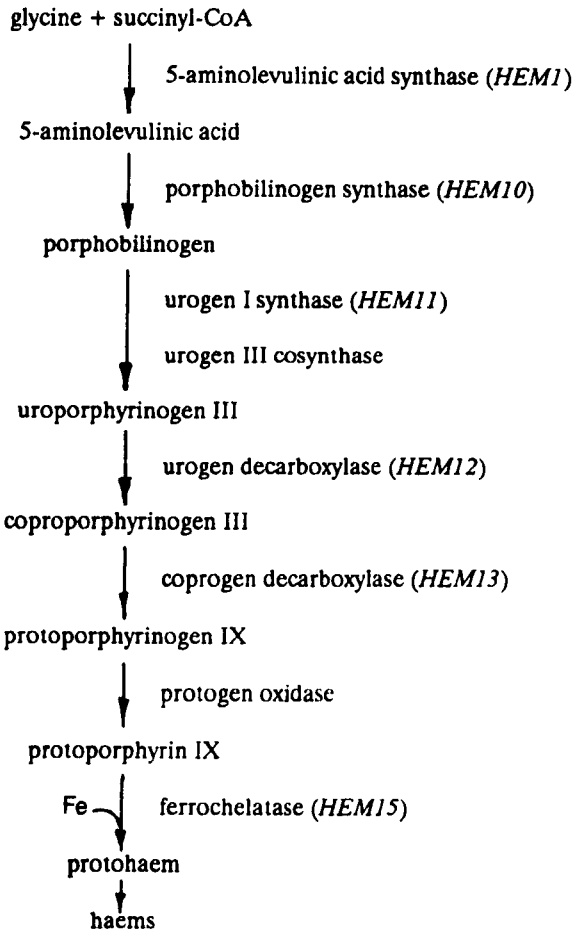


Figure* 6.1 Porphyrin biosynthesis in *S. cerevisiae

Haem biosynthetic intermediates are listed on the left, in the order of the haem biosynthesis reaction sequence (Lewis *et al.*, 1985).

CHAPTER 6. THE *HEM1* GENE AS A DOMINANT SELECTABLE MARKER

6.1 INTRODUCTION

6.1.1 Aims

The purpose of the work described in this chapter was to clone the *C. tropicalis HEM1* gene from a genomic library enriched for *HEM1* sequences and disrupt it via gene transplacement as described in chapter one of this thesis to produce a *C. tropicalis hem1* strain as a host for the complete *HEM1* allele which would then act as a dominant selectable marker. It was hoped that in addition, *C. tropicalis* promoter and terminator sequences would be cloned with the *HEM1* gene, which could be utilised in cloning vectors carrying other, heterologous, selectable markers for selection of *C. tropicalis* transformants.

6.1.2 Porphyrin Biosynthesis

The porphyrin biosynthetic pathway is shown in *figure* 6.1. The enzyme 5-aminolevulinic acid synthase, encoded by the *HEM1* gene, is important in the control of this metabolic pathway which leads to all haem compounds in yeast. This enzyme is located within the inner mitochondrial membrane, and catalyses the first committed step in haem synthesis (Astin *et al.*, 1977). The metabolic appearance of 5-aminolevulinic acid is unique to the haem biosynthetic pathway (Lewis *et al.*, 1985). Haem is involved in the interaction between cAMP and mitochondrial protein synthesis (Gopalan and Rajamanickam, 1985).

6.1.3 Effects of Lesions in *HEM1*

Mutations in haem biosynthesis comprise three complementation groups each of which represents a genetic locus. Within these groups are five distinct classes of mutation, each of which represents a structural gene defect designated *HEM1* to *HEM5* (Gollub *et al.*, 1977).

HEM1 represents the structural gene for 5-aminolevulinic acid synthase. Group I mutations including *hem1*, *cyd1*, and *ole3*, recover cytochromes when grown in the presence of exogenous 5-aminolevulinic acid and are therefore affected in 5-aminolevulinic acid synthesis (Woods *et al.*, 1975; Astin *et al.*, 1977; Gollub *et al.*, 1977; Urban-Grimal and Labbe-Bois, 1981). These *HEM1* mutations result in decreased 5-aminolevulinic acid synthetase activity *in vitro*. Two unlinked nuclear mutant genes, *cyd1* and *cyc4*, cause *S. cerevisiae* cells to be partially dependent upon exogenous 5-aminolevulinic acid for maximum cytochrome biosynthesis. In contrast, the *hem1* and *ole3* mutations cause an almost complete 5-aminolevulinic acid dependence (Woods *et al.*, 1975). 5-aminolevulinic acid synthase activity is barely detectable in cells carrying *hem1* or *ole3* (Gollub *et al.*, 1977; Urban-Grimal and Labbe-Bois, 1981).

The *hem1* mutation in *S. cerevisiae* results in the loss of all haem-containing enzymes, including the mitochondrial cytochromes, preventing the synthesis of components whose formation requires haem-containing enzymes, including unsaturated fatty acids, ergosterol and methionine (Astin *et al.*, 1977). *hem1* mutant cells grown in the absence of haem or haem precursor lack the characteristic absorption spectra for cytochromes a, a3, b, c and c1, do not produce any mature cytochromes and do not respire (Hörtner *et al.*, 1982; Guarente and Mason, 1983; Gudenus *et al.*, 1984). These cells grow on glucose (D) with ergosterol and Tween 80, but require 5 times as much 5-aminolevulinic acid compared with haem-sufficient cells for growth on ethanol (E) or glycerol, reflecting the greater requirement for precursors of the respiratory cytochromes in order to grow on a non-fermentable substrate (Astin *et al.*, 1977; Gollub *et al.*, 1977).

hem1, *hem2*, and *hem3* mutants have an additional requirement for methionine when grown on synthetic medium supplemented with either haem or ergosterol and Tween 80. This is the result of defective sulphur metabolism. Methionine and cysteine biosyntheses are dependent upon the metabolic pathway that reduces sulphur to sulphide. One of the enzymes of this pathway, sulphite reductase, contains sirohaem, a modified uroporphyrin III, and in consequence, defective porphyrin biosynthesis results in a lack of sirohaem for sulphite reductase and in the methionine requirement observed (Woods *et al.*, 1975; Gollub *et al.*, 1977).

The haem-dependent enzyme, catalase T, is also absent in haem-deficient cells (Gollub *et al.*, 1977; Hörtner *et al.*, 1982), and sterol uptake is not inhibited by 5-aminolevulinic acid in these cells as this is dependent on the ability to synthesize haem from 5-aminolevulinic acid (Lewis *et al.*, 1985).

6.1.4 Reasons for Cloning *HEM1*

6.1.4.1 Control Mechanisms

The synthesis of hemoprotein genes such as those coding for iso-1-cytochrome c, catalase T and catalase A, is regulated by D, oxygen and haem (Hörtner *et al.*, 1982). Haem is necessary for the proper processing of the precursor form of iso-1-cytochrome c and the correct assembly of cytochrome c oxidase (Lin *et al.*, 1982). The catalase T gene is positively controlled by haem (Spevak *et al.*, 1986).

Expression of the *S. cerevisiae* iso-1-cytochrome c *(CYC1)* gene is tightly regulated by intracellular haem at the level of initiation. Control of *CYC1* transcription by haem is mediated by an upstream activation site (UAS) which contains two homologous subsites, UAS1 and UAS2 (Guarente and Mason, 1983; Guarente *et al.*, 1984). UAS1, which is located about -270 bp in front of the transcriptional start, is responsible for haem induction, and UAS2, located at about -230 bp, is necessary for D derepression (Entian, 1986).

ANB1 (anaerobic gene coding for a mitochondrial component) expression in *S. cerevisiae* is repressed by haem, which is produced only in the presence of oxygen and which has been shown to be an inducer of aerobic genes. In combination with the *ROX1* gene product (a common *trans* acting factor) haem has an opposite effect on the expression of anaerobic and aerobic genes (Lowry and Lieber, 1986).

Haem has a modest repression-derepression effect on 5-aminolevulinic acid synthase (*HEM1*) expression which is typical of many regulatory systems in *S. cerevisiae* (Labbe-Bois *et al.*, 1986).

Genes of the porphyrin biosynthetic pathway are therefore of interest in investigating these control mechanisms in other yeasts such as *C. tropicalis*, and the regulatory sequences of this gene maybe useful for the development of expression vectors in this organism.

6.1.4.2 Catabolite Repression

Catabolite repression designates the inhibition of the synthesis of certain enzymes by D or other rapidly metabolised carbon sources. D-repression is a long-term regulatory mechanism during which carbohydrate metabolism of yeast cells is adapted to the utilisation of hexoses exclusively degraded by the glycolytic (Embden-Meyerhof) pathway yielding mainly E (Entian, 1986; Gancedo and Gancedo, 1986).

The mechanism of catabolite repression is a complex one where the expression of a given gene or set of genes is controlled by several regulatory genes, although it is uncertain whether these genes act in cooperation or sequentially. During D-repression, the biogenesis of mitochondria is prevented and pre-existing ones are broken down (Gopalan and Rajamanickam, 1985; Gancedo and Gancedo, 1986).

The mechanism of catabolite repression in yeast may be dissimilar to that found in *E. coli*. The addition of D to a yeast culture causes the synthesis of certain proteins to be repressed. When this D is exhausted the synthesis of some of these proteins may start and this process is known as derepression (Gancedo and Gancedo, 1986).

For the synthesis of certain D-repressed enzymes to occur, depletion of D is not enough, and the presence of an inducer is required. These enzymes are both repressible and inducible. Different mechanisms control repression by D and inducibility (Gancedo and Gancedo, 1986).

Gene expression is controlled by two types of *cis*-acting sequences, enhancers and silencers, the activity of which is determined by different genes acting in *trans*. These genes may act pleiotropically, affecting the expression of several genes, or only one. The expression of the genes acting in *trans* and the interaction of their products with the genes acting in *cis* may in turn be controlled by metabolites whose levels vary between repressed and derepressed conditions (Gancedo and Gancedo, 1986).

Genes of the porphyrin biosynthetic pathway are therefore of interest in investigating catabolite repression in *C. tropicalis*.

6.1.4.3 *HEM1* as a Dominant Selectable Marker

S. cerevisiae strains carrying the *hem1* or *ole3* mutations supplemented with 0.5-500 mgl^{-1} 5-aminolevulinic acid show a progressive increase in the cellular content of unsaturated fatty acids and accumulation of the respiratory cytochromes, cause the replacement of lanosterol and squalene by ergosterol, and an increase in total sterol content, and no longer require methionine when grown on synthetic medium (Woods *et al.*, 1975; Astin *et al.*, 1977; Gollub *et al.*, 1977; Gudenus *et al.*, 1984; Lewis *et al.*, 1985). *hem1* mutants transformed with a functional copy of the *HEM1* gene would no longer have a requirement for 5-aminolevulinic acid, and would be able to grow on unsupplemented minimal media. Thus the *HEM1* gene would act as a dominant selectable marker.

The only drawback in using this marker for gene transplacement is that it is homologous to the host genome, and therefore a plasmid carrying an altered fatty acid acyl-CoA oxidase gene and *HEM1* as the selectable marker might integrate at the *HEM1* locus. Integration could be directed to the fatty acid acyl-CoA oxidase gene by cutting the plasmid within this gene prior to transformation.

6.2 THE *S. CEREVISIAE HEM1* GENE AS A PROBE FOR THE *HEM1* GENE OF *C. TROPICALIS*

6.2.1 Preparation of *HEM1* Probe

A recombinant plasmisd comprising pUC12 and the *S. cerevisiae HEM1* gene on a 1.6 kb fragment cloned into the *Hin* dIII site (50 μg; kindly provided by T. Pillar) was cut to completion (section 3.3.1.1) with *Hin* dIII overnight. 100 ng of each digest was run on an 0.8% agarose slide gel (section 3.3.5) to confirm digestion, and the DNA fragment encoding *HEM1* was isolated by electroelution (section 3.3.6). The electroeluted DNA was of the expected size as determined by electrophoresis in a 0.8% agarose slide gel alongside λ *Eco* RI/*Hin* dIII marker fragments, and the original plasmid cleaved with *Hin* dIII. Concentration of the DNA was estimated by measuring A_{260}, and adjusted to 20 $ng\mu l^{-1}$ with TE (section 2.3.1). Aliquots of the electroeluted *S. cerevisiae HEM1* DNA were radioactively labelled by random hexanucleotide priming (section 3.5.1.2) as required.

6.2.2 Demonstration that the *S. cerevisiae HEM1* gene can Hybridise to *C. tropicalis* Genomic DNA

Chromosomal DNA extracted from *C. tropicalis* NCYC997 (section 3.2.1) was digested overnight with restriction endonucleases as follows:

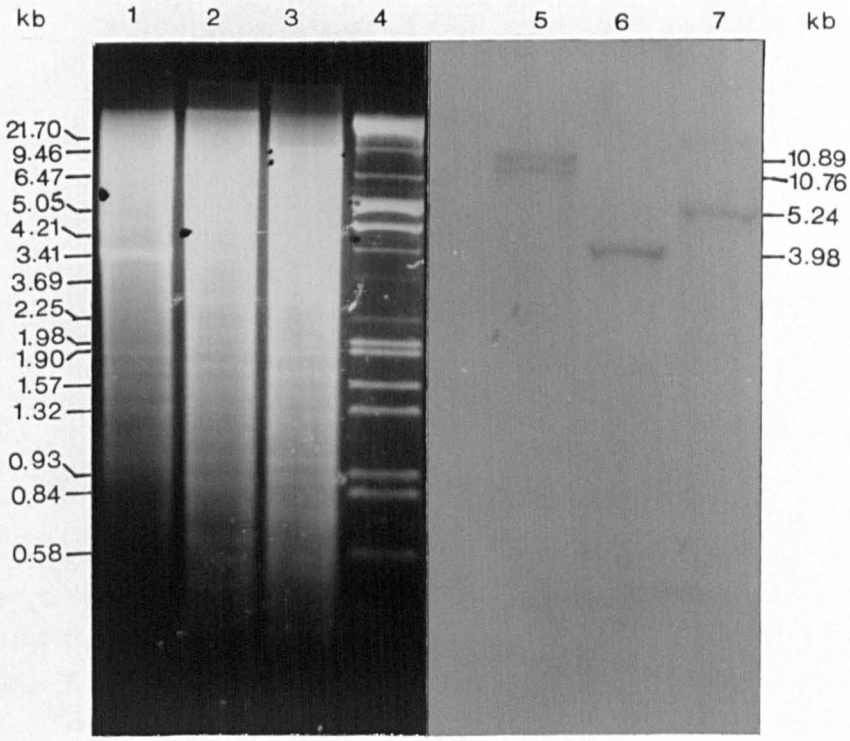


***Figure* 6.2 *C. tropicalis* NCYC997 genomic DNA digests subjected to electrophoresis through 0.8% agarose and Southern blot showing hybridisation to *S. cerevisiae HEM1* DNA**

Lanes: 1 and 7, *Eco* RI digest; 2 and 6, *Hin* dIII digest; 3 and 5, *Pst* I/*Sal* I digest; 4, λ *Eco* RI/*Hin* dIII markers. The blot demonstrates that the *S. cerevisiae HEM1* DNA hybridises to *C. tropicalis* DNA. The sizes of the *C. tropicalis* DNA fragments which hybridise to the *S. cerevisiae HEM1* gene were determined from a standard curve of mobility of the marker fragments vs. $\log_{10}$ molecular weight to be: *Hin* dIII, 3.98 kb; *Eco* RI, 5.24 kb; *Pst* I/*Sal* I, 10.89 and 10.76 kb.

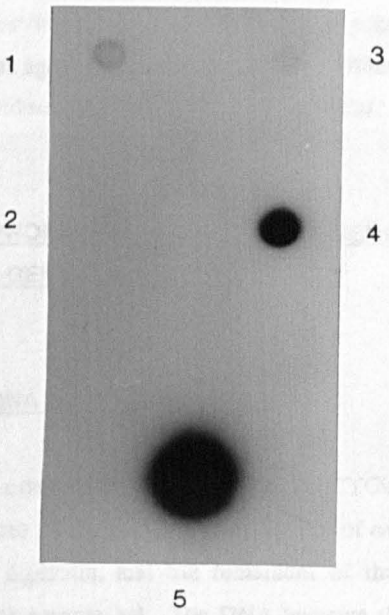


***Figure* 6.3 Dot-blot of electroeluted *C. tropicalis Hin* dIII digested genomic DNA hybridised to the *S. cerevisiae HEM1* gene**

Samples: 1, DNA electroeluted from immediately above the 5.05 kb marker; 2, pUC12 (negative control); 3, DNA electroeluted from immediately below the 3.41 kb marker; 4, DNA electroeluted from between the 3.41 and 5.05 kb markers; 5, pUC12 carrying the *S. cerevisiae HEM1* gene (positive control). The blot demonstrates that the *S. cerevisiae HEM1* gene hybridises strongly to the *C. tropicalis* genomic DNA electroeluted from that part of the 0.8% agarose gel containing fragments of between 3.41 and 5.05 kb which agrees with the size of the fragment which hybridised to the *S. cerevisiae HEM1* gene in *figure* 6.3.

10 μg with *Eco* RI.
10 μg with *Hin* dIII.
10 μg with *Pst* I and *Sal* I.

Aliquots (100 ng) of each digest were run on an 0.8% agarose slide gel to confirm digestion, and transferred from a large format agarose gel to a nitrocellulose filter by Southern blotting (section 3.5.2.3). The filter was hybridised (section 3.5.3) to the *HEM1* probe, and autoradiographed (section 3.5.4; *figure* 6.2).

6.3 CONSTRUCTION AND PROBING OF *C. TROPICALIS* GENOMIC DNA LIBRARIES ENRICHED FOR THE *HEM1* GENE

6.3.1 Preparation of Insert DNA by Electroelution

Chromosomal DNA (40 μg) extracted from *C. tropicalis* NCYC997 (section 3.2.1) was cut to completion (section 3.3.1.1) with *Hin* dIII. Aliquots (100 ng) of each digest was run on an 0.8% agarose slide gel to confirm digestion, and the remainder of the digested DNA subjected to electroelution in a large format agarose gel. The DNA between the 3.41 and the 5.05 kb λ *Eco* RI/*Hin* dIII marker fragments, and from strips taken from immediately above and below was isolated by electroelution (section 3.3.6). The electroeluted DNA was of the expected size range as determined by electrophoresis of aliquots (4 μl) of each sample in a 0.8% agarose slide gel alongside λ *Eco* RI/*Hin* dIII marker fragments, and the concentration of the DNA in each sample was estimated by measuring A_{260}, and adjusted to 25 ngμl^{-1} with TE (section 2.3.1).

Aliquots (50 ng) of each DNA sample obtained above was dot-blotted (section 3.5.2.2) onto a nitrocellulose filter with 50 ng of pUC12 carrying the *S. cerevisiae HEM1* gene (positive control) and 50 ng pUC12 (negative control), hybridised (section 3.5.3) to the *S. cerevisiae HEM1* gene isolated in section 6.2.1, and autoradiographed (section 3.5.4; *figure* 6.3).

DNA from the electroeluted fraction which hybridised to the *HEM1* probe was used as the insert DNA to construct a library of *C. tropicalis* genomic DNA enriched for the *HEM1* gene.

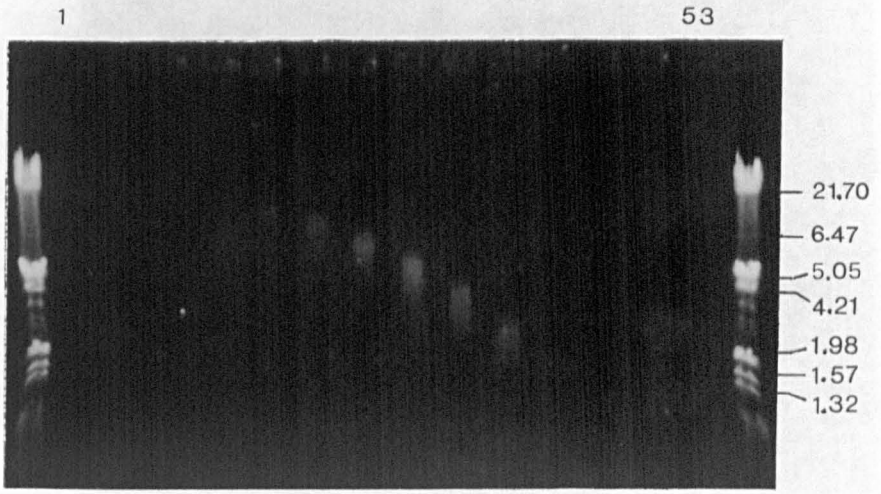


***Figure* 6.4 *C. tropicalis* NCYC997 genomic DNA (525 μg) digested to completion with *Hin* dIII, subjected to sucrose density gradient centrifugation and fractionated**

Fractions 1-9 and 50-56 were 1 ml, and the remainder were 0.5 ml. Aliquots (10 μl) of every fourth fraction were analysed by electrophoresis through 0.5% agarose against λ *Eco* RI/*Hin* dIII markers, the salt content of which was adjusted to match that of the fractions. Fractions were loaded sequentially from left to right starting with fraction 1, the bottom of the gradient. Fractions 1-13 and 45-56 were discarded.

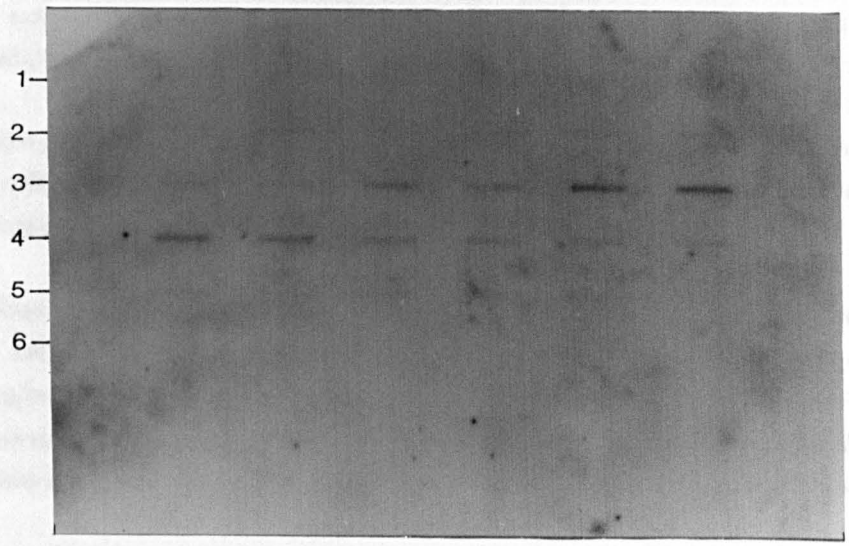


***Figure* 6.5 Slot-blot of *C. tropicalis Hin* dIII digested genomic DNA sucrose gradient fractions 14-44 hybridised to the *S. cerevisiae HEM1* gene**

Fractions were loaded sequentially from left to right starting with fraction 14. Rows: 1, fractions 14-19; 2, fractions 20-25; 3, fractions 26-31; 4, fractions 32-37; 5, fractions 38-43; 6, fraction 44. Fractions 30, 31, 32 and 33 hybridised to the *S. cerevisiae HEM1* gene. The size range of the DNA in each fraction correlates well with the size of the *Hin* dIII fragment which hybridised to the *HEM1* probe in *figure* 6.2 (3.98 kb), as judged from the information in *figure* 6.4.

6.3.2 Preparation of Insert DNA by Fractionation in a Sucrose Density Gradient

C. tropicalis NCYC997 genomic DNA (525 μg) was cut to completion with *Hin* dIII. Aliquots (100 ng) of each digest were run on an 0.8% agarose slide gel to confirm digestion, and the DNA was size fractionated over a sucrose density gradient (section 3.3.4). Aliquots (10 μl) of every fourth fraction were analysed by electrophoresis in 0.5% agarose (*figure* 6.4). Fractions 1-13, and 45-56 were discarded.

Aliquots (50 μl) of each of the remaining fractions were transferred and fixed to a Hybond-N filter using a Bio Rad Bio-Dot apparatus (section 3.5.2.2), hybridised to the *S. cerevisiae HEM1* gene, and autoradiographed (*figure* 6.5).

Fractions 30, 31, 32, and 33 were each diluted with two volumes of water, E precipitated, washed, dried and resuspended in 0.1 ml TE. Aliquots (5 μl) of each fraction were checked by electrophoresis in 1.0% agarose with λ *Eco* RI/*Hin* dIII markers. The sizes of the DNA in each fraction correlated well with the size of the *Hin* dIII fragment which hybridised to the *HEM1* probe in section 6.2.2 above (3.98 kb). The remainder of the fractions were stored in the sucrose solution.

6.3.3 Ligation, and Generation of Transformants

5 μg pEMBLYe23 (section 2.1.3.3), and 5 μg pUC12 (section 2.1.3.1) were digested to completion with *Hin* dIII, and 100 ng of each digest was run on an 0.8% agarose slide gel to confirm digestion. The pEMBLYe23 digest was treated with calf intestinal alkaline phosphatase (section 3.3.2).

The following ligations (section 3.3.3) were carried out:
440 ng insert DNA (section 6.3.1) to 800 ng *Hin* dIII cleaved pUC12.
400 ng insert DNA (section 6.3.1) to 300 ng *Hin* dIII cleaved and dephosphorylated pEMBLYe23.
12.5 μl of each of fractions 31 and 32 (section 6.3.2) to 300 ng *Hin* dIII cleaved and dephosphorylated pEMBLYe23
300 ng *Hin* dIII cleaved and dephosphorylated pEMBLYe23 to itself.

Aliquots (10 μl) of the above ligations were used to transform (section 3.4.1) competent *E. coli* NM522 (section 2.1.2). The efficiency of the competent cells was assessed by transforming an aliquot with 0.1 ng uncut pBR322. Transformants were selected on L-agar containing 100 μgml^{-1} ampicillin (Ap) with X-gal and IPTG (LAXI; section 2.2.3.5). Cloning DNA into the *Hin* dIII site of either pEMBLYe23 or pUC12 disrupts a segment of DNA derived from the *lac* operon of *E. coli* that codes for the amino-terminal fragment of β-galactosidase which is capable of α-complementation with a defective form of β-galactosidase in strain NM522, therefore transformants carrying plasmids with insert DNA cannot metabolise the X-gal in the medium and form white colonies which are easily distinguishable from transformants carrying plasmids with no insert DNA which convert the X-gal to a blue derivative, and hence form blue colonies. The IPTG is added to the medium as an inducer of the genes involved in this biochemical pathway.

The efficiency of the competent NM522 was found to be 1.59×10^6 transformants per μg DNA.

Altogether, 494 white pUC12 transformants, 288 white pEMBLYe23 transformants with insert DNA prepared by electroelution, and 1062 white pEMBLYe23 transformants with insert DNA prepared by sucrose gradient fractionation were generated. The transformation of self ligated pEMBLYe23 generated 2 blue, and 0 white transformants. Only 36 blue pEMBLYe23 transformants were generated on the other transformation plates, compared with >5000 blue pUC12 transformants. The latter results give a good indication of the success of dephosphorylating the cleaved pEMBLYe23 to prevent recircularisation.

6.3.4 Colony Hybridisations

All the white transformants generated above were picked onto LAp-agar, and Hybond-N gridded filters in duplicate (50 per filter). A blue transformant (negative control) was included on every filter, and a transformant carrying pUC12 with the *S. cerevisiae HEM1* gene (positive control) was included on five of the filters. The filters were placed on LAp-agar and incubated with the master plates. DNA from the colonies was transferred and fixed to the filters by colony hybridisation (section 3.5.2.1). The filters were marked with radioactive ink for orientation, hybridised to the *HEM1* probe, and autoradiographed (Kodak XAR-5, 33 h).

The positive controls hybridised strongly to the *HEM1* probe. None of the pUC12 transformants, or the pEMBLYe23 transformants with sucrose gradient insert DNA appeared to be hybridising more strongly than the negative controls.

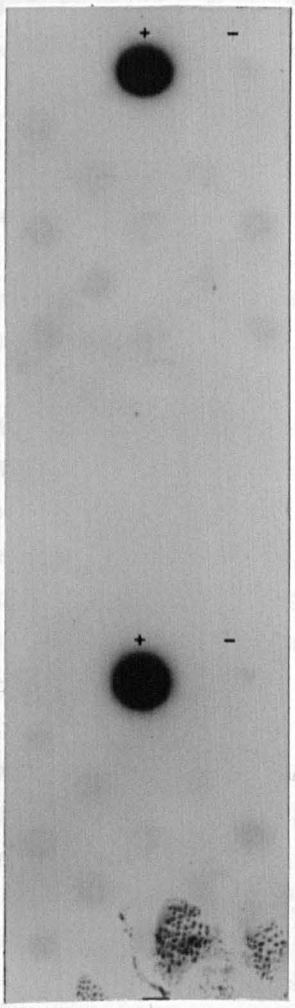


***Figure* 6.6 Dot-blot of miniprep DNA from 11 pUC12 clones possibly carrying the *C. tropicalis HEM1* gene hybridised to the *S. cerevisiae HEM1* gene**

The positive (pUC12 carrying the *S. cerevisiae HEM1* gene), and negative (pUC12) controls are indicated. Only the positive control hybridised to the *S. cerevisiae HEM1* gene.

Plasmid DNA was extracted by the miniprep method (section 3.2.2.2) from 11 pEMBLYe23 transformants with insert DNA prepared by electroelution, which appeared to be hybridising more strongly to the *HEM1* probe than the negative controls on both of a pair of filters. The DNA was dot-blotted onto Hybond-N filters in duplicate along with 50 ng of pUC12 carrying the *S. cerevisiae HEM1* gene (positive control), and 50 ng pUC12 (section 2.1.3.1; negative control). Both filters were hybridised to the *HEM1* probe, and autoradiographed (*figure* 6.6).

6.4 DISCUSSION

Figure 6.2 clearly demonstrated that the *S. cerevisiae HEM1* gene hybridises to a 3.98 kb fragment of *C. tropicalis* NCYC997 genomic DNA. Using this information the creation of *C. tropicalis* NCYC997 genomic DNA libraries enriched for sequences encoding the equivalent gene in both pUC12 and pEMBLYe23 was attempted.

The dot-blot of electroeluted DNA, and the slot-blot of DNA separated by sucrose density gradient centrifugation and fractionated, hybridised to the *S. cerevisiae HEM1* gene (*figures* 6.3 and 6.5) show that sequences in the correct size range, which hybridised to the *S. cerevisiae HEM1* gene were present in the DNA which was subsequently used to create the *HEM1* enriched libraries. However, despite this enrichment, no transformants were generated which carried DNA able to hybridise to the *S. cerevisiae HEM1* gene, although the positive controls included on the colony hybridisations and dot-blots all hybridised strongly to this DNA.

It may be the case that sequences included on the DNA fragment carrying the *C. tropicalis HEM1* gene were lethal to the *E. coli* cells such that any cells transformed with this DNA died and therefore were not picked up on the colony hybridisations. The *S. cerevisiae HEM1* gene is obviously not affecting the *E. coli* cells, and as this gene is probably quite homologous to the *C. tropicalis HEM1* gene as it hybridises strongly to this DNA it is possible that the *C. tropicalis* signal sequences such as promoter and terminator regions are deleterious to *E. coli*. If this should prove to be the case it could be a serious handicap to the development of vectors in this yeast, as a *C. tropicalis-E. coli* shuttle vector could not be developed which contained sequences deleterious to the growth of one of the host organisms.

Although not successful in this instance, the methodology used in this chapter to create small libraries enriched for genomic DNA carrying the *C. tropicalis HEM1* gene should be applicable to any gene of interest providing a suitable probe is available.

CHAPTER 7. DEVELOPMENT OF AN AUXOTROPHIC STRAIN OF *CANDIDA TROPICALIS* AS A RECIPIENT FOR VECTOR DNA CARRYING THE APPROPRIATE PROTOTROPHIC GENE

7.1 INTRODUCTION

7.1.1 Aims

The purpose of the work described in this chapter was to isolate *C. tropicalis* auxotrophic mutants, especially *ura3* mutants, to serve as host strains for transformation, and to clone the complementary genes to act as selectable markers on plasmid vectors for these auxotrophic strains.

7.1.2 Generation of Auxotrophic Mutants in *Candida tropicalis*

7.1.2.1 Auxotrophic Genes as Markers for Transformation

As discussed in Chapter One, the availability of a variety of tight mutations in *S. cerevisiae* auxotrophic genes has been utilised in the development of a transformation system in laboratory strains of this yeast. An auxotrophic strain transformed to prototrophy by the complementary gene can be easily picked out by its ability to grow on minimal media. These markers are recessive, and in a diploid or polyploid genetic background require that all the alleles present in the cell are auxotrophic. However, for some yeasts such as *Yarrowia lipolytica*, selection schemes based on antibiotic resistance cannot be used due to high resistance to antibiotics like G418 (Gaillardin *et al.*, 1985), and in these cases, auxotrophic markers are the only alternative.

Although useful for the selection of cells transformed with plasmid vectors, auxotrophic markers are not ideal for gene transplacement experiments as they are usually homologous to the host DNA which can be a problem for the accurate targeting of altered sequences to other sites in the genome. An exception to this in *S. cerevisiae* is *URA3*. No recombinational events between the *URA3* sequences in YIp5 and the *ura3* genomic sequences in the *ura3-52* allele have been detected, probably because the gene has been isolated as a very small DNA fragment which may act to suppress recombination during transformation at that locus (Scherer and Davis, 1979). This allele was therefore of particular interest for achieving the main industrial aim of this project, the creation of a non-revertible β-oxidation mutant of *C. tropicalis* by disrupting the genes encoding the fatty acid acyl-CoA oxidases *in vitro* and using these altered genes to replace the wild-type chromosomal genes via gene transplacement.

Selection for loss of plasmid marker is facilitated by protocols for positive selection of various auxotrophic mutants as described in section 7.1.2.4, since cells in a population which have lost the plasmid (and the prototrophic gene) by homologous recombination between the repeated segment become resistant to the selective agent.

7.1.2.2 Difficulties of Producing *Candida* Mutants

S. cerevisiae brewing strains are polyploid or aneuploid, and because mutations are usually recessive, these yeast are considerably genetically more stable and less susceptible to mutational forces than haploid strains (Fleming, 1988). *Candida* yeasts are stable diploids, and several authors have reported difficulty in mutating these yeasts by conventional methods. For example, Olaiya and Sogin (1979) were unsuccessful at isolating *C. albicans* auxotrophs, Gradova and Robysheva (1980) found that various strains of the yeast species *C. guilliermondii* and *C. tropicalis* isolated from soil had a high degree of resistance to nitrosomethylurea and ethylenimine, and Yano *et al.* (1981) only achieved poor results by treating *C. tropicalis* IFO 0589 and *C. maltosa* IAM 12247 with ethyl methanesulphonic acid (EMS).

Despite the problems involved, several groups have succeeded in producing a varied range of auxotrophic mutants from several *Candida* species. Fournier *et al.* (1977) isolated *C. tropicalis* methionine, lysine, histidine, adenine and cytosine auxotrophs using nystatin enrichment after two successive mutagenic treatments with γ rays (75 Krad), Okanishi and Gregory (1970) isolated mutants of *C. tropicalis* ATCC 1369, with up to 41% higher contents of methionine than the parent strain using nitrous acid, Vallin and Ferenczy (1977) isolated a series of adenine and cysteine auxotrophic mutants from *C. tropicalis* CBS 644 by ultraviolet (UV) treatment (Philips TUV 15 W lamp, 5% survival), and two groups (Kakar *et al.*, 1983; Gibbons and Howard, 1986) have induced auxotrophic mutants of *C. albicans* by a combination of treatments with nitrous acid and UV-irradiation. The latter groups reported that nitrous acid is a good mutagen in yeast, inducing mitotic crossing over, and that nitrous acid alone yields a broader spectrum of auxotrophs than UV alone and is therefore the mutagen of choice when only one is to be used.

7.1.2.3 Enrichments for Auxotrophic Mutants

Enrichment methods greatly facilitate the process of obtaining auxotrophic mutants. The various methods in use depend upon selectively killing growing cells and the survival of non-growing mutant cells. Mutants which die under non-permissive conditions will not be enriched by any of these procedures. Mutagenised cells are allowed a period of growth prior to selection since the efficiency of retrieval of the mutants depends on rapidly growing cells.

Some of the procedures for enrichment of yeast mutants include nystatin treatments where mutagenised cells are grown in YEP for about 18 h, then subjected to nitrogen starvation and nystatin selection (Snow, 1966), netropsin treatments (Young *et al.*, 1976), fatty acid-starvation of fatty acid-requiring yeast mutants where enrichment is the result of the protection conferred on fatty acid-starved cells by mutations affecting macromolecular synthesis (Henry and Horowitz, 1974), and inositol-less death of growing prototrophic cells and the preservation of non-growing mutant cells on inositol-less minimal medium (Megnet, 1964; Henry *et al.*, 1975).

The inositol-starvation procedure can be used with both mutagenised and unmutagenised cells. However, a drawback of both inositol-starvation and fatty acid-starvation is that the recipient yeast strain used in the transformation must already contain a stable mutation, namely a requirement for inositol or fatty acid, and therefore, these techniques cannot be applied to *C. tropicalis*.

Nystatin-treatment has been used to isolate *C. tropicalis* auxotrophs (Fournier *et al.*, 1977) and mutants of *Candida* yeast deficient in assimilation of n-alkane (Yano *et al.*, 1981). The latter group reported that the yeast strains were not efficiently killed by nystatin and that survival rate fluctuated. They found that pyrrolnitrin, an antifungal antibiotic like nystatin, was not effective at all, but using nystatin and pyrrolnitrin simultaneously had a synergistic effect which allowed the efficient isolation of mutants.

Netropsin binds to AT rich regions of DNA, although its exact mode of action as a killing agent is not known (Young *et al.*, 1976). As *C. tropicalis* has AT rich DNA (Barnett *et al.*, 1983) netropsin may have been effective in enriching for auxotrophic mutants in this yeast. However, it was not possible to examine netropsin in this study since a suitable supplier could not be found.

7.1.2.4 Positive Selection Systems for Specific Auxotrophic Mutations

Special systems have been devised for selecting auxotrophic mutants of specific genes in the presence of large numbers of wild-type cells. These techniques are extremely useful, and normally depend on the conversion of a non-toxic compound to one that is toxic to wild-type cells. Mutant cells which lack the ability to form the toxic compound can grow in the presence of the inert precursor. For example, *met2, met15*, and to a lesser extent *met6* mutants of *S. cerevisiae* are resistant to methyl mercury (Singh and Sherman, 1974).

At low adenine concentrations *S. cerevisiae ade1* and *ade2* mutants have distinct red or pink colony colours and can therefore be readily isolated from mutagenised cultures. Pink *C. albicans ade* mutants have also been reported (Poulter and Rikkerink, 1983), as have pink *C. tropicalis ade* mutants (Vallin and Ferenczy, 1977).

AA-medium which lacks a normal nitrogen source but contains lysine and α-aminoadipic acid allows the growth of *lys2* mutants but restricts the growth of normal strains. The *lys2* mutation permits yeast to use α-aminoadipic acid as a nitrogen source (Chattoo *et al.*, 1979).

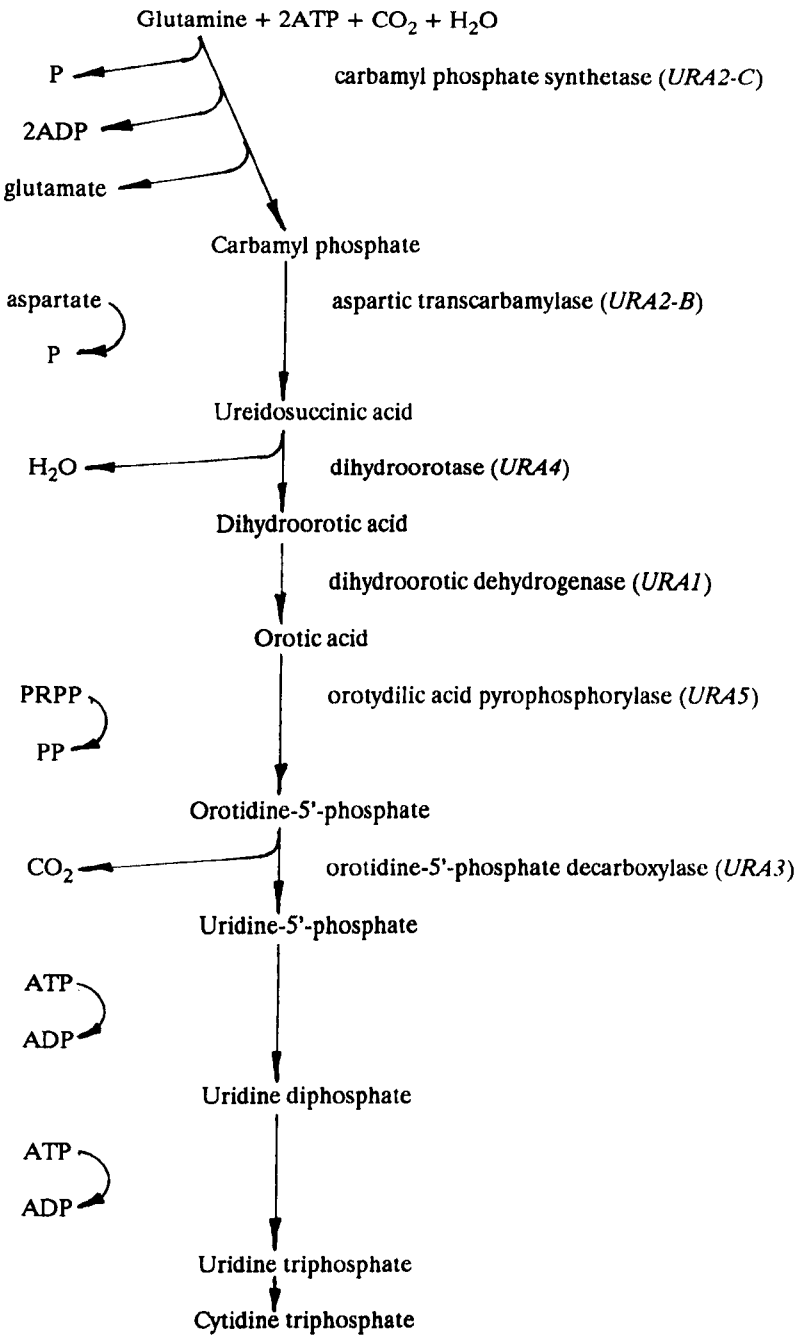


***Figure* 7.1 Pyrimidine Biosynthesis**

Pyrimidine biosynthetic pathway of *S. cerevisiae* (Lacroute, 1968; Greer and Fink, 1975; Jones and Fink, 1982).

The *CAN1* gene of *S. cerevisiae* codes for an arginine permease which transports arginine with high affinity, and during growth on SD, is the sole transport system for arginine into the cell. Only basic amino acids and the arginine analogue L-canavanine sulphate (Can) inhibit arginine uptake in a competitive manner. *CAN1* strains of yeast are sensitive to Can, since once it is transported into the cell it is incorporated into protein with deleterious results. *can1* strains of yeast are resistant to Can, since it is excluded from the cell by the absence of a functional arginine permease. Similarly, a *can1 arg1* double mutant cannot grow on SD, since it cannot transport arginine into the cell to supply its auxotrophic requirement. However, such a strain will grow on synthetic medium lacking ammonium sulphate, using the general amino acid permease to transport arginine which is relieved from repression by the absence of ammonium. A *can1 arg1* strain will also grow on YEPD either for the reason cited above or through use of peptides to supply its arginine requirement. A *CAN1 arg1* strain grows perfectly well on SD. Thus positive selections for both forward and reverse mutation of the *CAN1* locus exist (Broach *et al.*, 1979; Ahmad and Bussey, 1986).

Two positive selections for *ura* auxotrophs exist. Bach and Lacroute (1972) isolated *S. cerevisiae ura1*, *ura3* and *ura5* mutants by utilising their resistance to ureidosuccinic acid in a particular medium. However, this procedure is very sensitive to physiological conditions and does not work for strains that carry an *ade* mutation (Winston *et al.*, 1983). Boeke *et al.* (1984, 1987) reported that *ura3* and *ura5* mutants are resistant to the pyrimidine analogue 5-fluoro orotic acid (FOA; *ura5* mutants are only partially resistant). FOA is useful for isolating *ura3* mutants in industrial or brewing yeasts where no auxotrophic markers for transformation exist as it has a broad spectrum, inhibiting the growth of *Saccharomyces, Schizosaccharomyces*, *Candida* and *E. coli*.

7.1.3 Pyrimidine Biosynthesis

The biochemical steps of pyrimidine biosynthesis (*figure* 7.1) have been found to be the same in yeast as in bacteria (Lacroute, 1968).

In *S. cerevisiae*, the conversion of orotic acid to uridine-5'-phosphate in the pathway of pyrimidine nucleotide synthesis is believed to be catalysed by two enzymes, the first, orotidine-5'-phosphate:pyrophosphate phosphoribosyl-transferase (EC 2.4.2.10) catalyses the formation of orotidine-5'-phosphate from orotic acid and 5-phosphoribosyl-1-pyrophosphate by condensation. Orotidine-5'-phosphate is then decarboxylated to uridine-5'-phosphate and CO_2 by orotidine-5'-phosphate carboxylase (EC 4.1.1.23) (Lieberman *et al.*, 1955; Wolcott and Ross, 1966).

Table 7.1 Survival of *C. tropicalis* NCYC547 and NCYC997 cells exposed to nitrous acid

Sensitivity to nitrous acid was measured as described in Chapter 3.

Exposure	Percentage Survival		
	without DMSO		5% v/v DMSO
(min)	NCYC547	NCYC997	NCYC997
0	100	100	100
5	69	100	100
10	52	69	49
15	48	74	35
20	51	49	22
25	26	45	30
30	6	45	26
35	1	13	21

Table 7.2 Survival of *C. tropicalis* NCYC547 and NCYC997 cells exposed to UV-irradiation

Sensitivity to UV-irradiation was measured as described in Chapter 3.

Exposure	Percentage Survival	
(min)	NCYC547	NCYC997
0.0	100	100
0.5	50	58
1.0	63	54
2.5	55	27
5.0	29	10
10.0	11	1
15.0	-	0

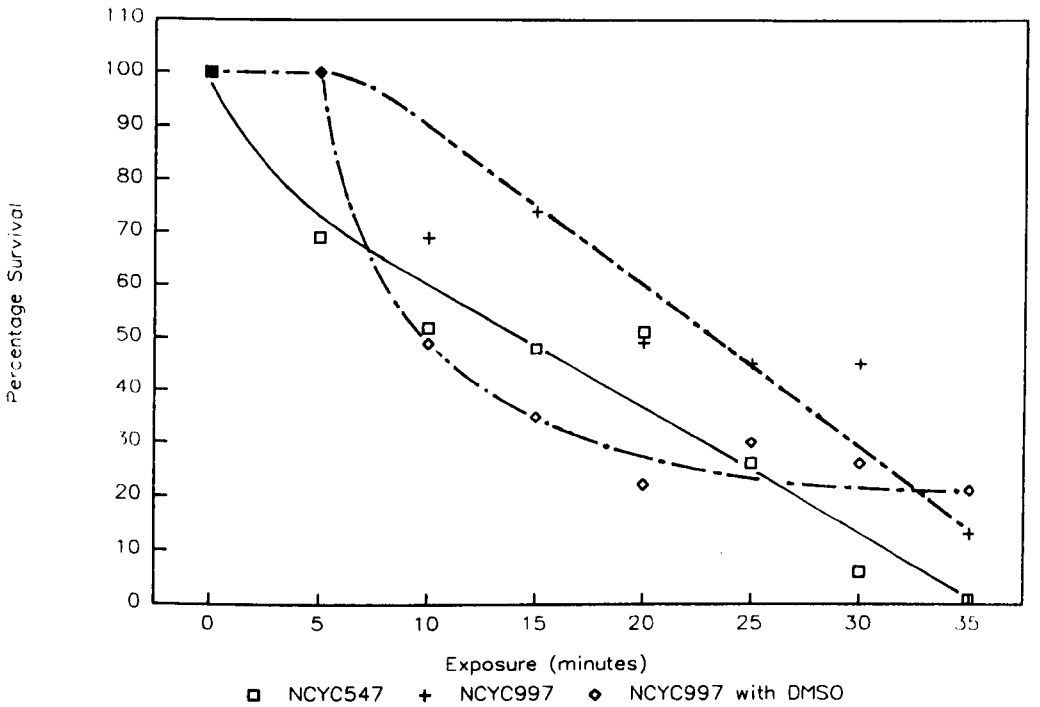


***Figure* 7.2 Survival of *C. tropicalis* cells exposed to nitrous acid**

Plotted from the data presented in table 7.1. Approximately 20% of the cells survive after 25 mins exposure.

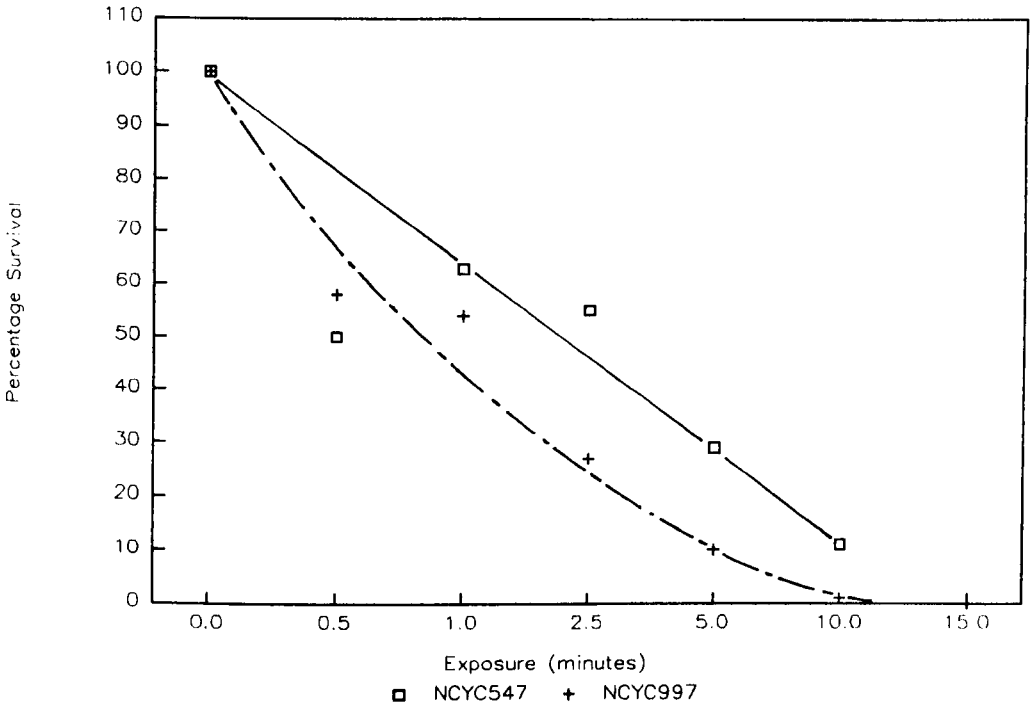


***Figure* 7.3 Survival of *C. tropicalis* cells exposed to UV-irradiation**

Plotted from the data presented in table 7.2. Approximately 20% of the cells survive after 5 mins exposure.

Table 7.3 Viability of *C. tropicalis* NCYC547 and *S. cerevisiae* X-2180-1A on AA

Sensitivity to AA was measured as described in Chapter 3. Growth of *C. tropicalis* NCYC547 was not inhibited by AA.

Test agar	Percentage Survival	
	C. tropicalis NCYC547	*S. cerevisiae* X-2180-1A
SD	100	100
AA	98	0

7.2 MUTAGENESIS OF *CANDIDA TROPICALIS*

Nitrous acid and UV-irradiation were chosen as suitable mutagens because they induce mitotic crossing over. This allows heterozygous loci to segregate producing a wider range of auxotrophic mutants in stable diploids such as *Candida tropicalis*.

7.2.1 Killing of *Candida tropicalis* Cells Exposed to Nitrous Acid

Kill curves (section 3.7.1) of percentage survival vs. exposure to nitrous acid with and without 5% v/v DMSO for both strains of *C. tropicalis* (*figure* 7.2) were produced from the results presented in table 7.1.

7.2.2 Killing of *Candida tropicalis* Cells Exposed to Ultraviolet Irradiation

Kill curves (section 3.7.2) of percentage survival vs. exposure to (UV) irradiation for both strains of *C. tropicalis* (*figure* 7.3) were produced from the results presented in table 7.2.

7.2.3 Screening of *Candida tropicalis* Cells for Auxotrophic Mutants without Enrichment or Positive Selection

No auxotrophs were recovered from either strain of *C. tropicalis* after several rounds of exposure to nitrous acid (with and without DMSO), or UV-irradiation (section 3.7.3). In one case 10^8 NCYC547 nitrous acid exposed cells were re-exposed. This produced a surge of growth during recovery in YEPG confirming the findings of Gradova and Robysheva (1980). No auxotrophs were recovered.

7.2.4 Positive Selections for the Isolation of Specific *Candida tropicalis* Mutants

7.2.4.1 α-Amino Adipic Acid

α-amino adipic acid (AA) medium (section 2.2.1) lacks a normal nitrogen source which restricts the growth of *LYS2* strains of *S. cerevisiae*. However, the AA and lysine allow the growth of *lys2* mutants which are able to utilise AA as an alternative nitrogen source (Chattoo *et al*, 1983).

Sensitivity of *C. tropicalis* NCYC547 to AA was ascertained (section 3.7.4.1), and the results are shown in table 7.3.

Table 7.4 Sensitivity of *C. tropicalis* NCYC547 and *S. cerevisiae* X-2180-1A to L-canavanine sulphate

Sensitivity to Can was measured as described in Chapter 3. *C. tropicalis* was not completely inhibited by Can at any concentration.

Concentration of Can (μgml^{-1}) in SD	Percentage survival	
	S. cerevisiae X-2180-1A	*C. tropicalis* NCYC547
0	100	100
50	0	51
100	0	63
200	0	13
250	0	17
300	0	17
350	0	16
400	0	14
450	0	12
500	0	8
550	0	3
600	0	1
650	0	1
700	0	1
1000	0	1

Table 7.5 Effect of nitrous acid on sensitivity of *C. tropicalis* NCYC547 to L-canavanine sulphate

Cells were exposed to nitrous acid (25 mins) as described in Chapter 3. The percentage of Can-resistant cells decreased slightly following exposure to nitrous acid.

Concentration of Can in SD ($mgml^{-1}$)	N°. of colonies per plate (average)	
	Nitrous acid exposed	Unexposed
0	812	783
1	9	15

Table 7.6 Sensitivity of *C. tropicalis* NCYC547 and NCYC997, and *S. cerevisiae* X-2180-1A and YNN27 to 5-Fluoro Orotic Acid

Sensitivity to FOA was measured as described in Chapter 3. The FOA inhibited the growth of all the strains except *S. cerevisiae* YNN27 which is $Ura3^-$. * indicates that the colonies on these plates were very small compared to YNN27.

FOA ($mgml^{-1}$)	Percentage survival			
	S. cerevisiae		*C. tropicalis*	
	YNN27	X-2180-1A	NCYC547	NCYC997
0.0	100	100	100	100
0.5	100	100*	87*	0.5
1.0	52	0	0	0

As expected *S. cerevisiae* X-2180-1A could not grow on AA, demonstrating that the medium was effective. All three colonies from the X-2180-1A AA plates were streaked to single cells over YEPD and SD agar and incubated. All grew well on YEPD, but not on SD confirming that these cells were either spontaneous mutants, or contaminants. *C. tropicalis* NCYC547 grew well on AA although colony size was slightly larger on SD. From these results it was concluded that it was not possible to positively select for *C. tropicalis lys2* mutants using AA.

7.2.4.2 L-Canavanine Sulphate

Can (section 2.2.3.3), an arginine analog, inhibits arginine uptake in a competitive manner in *S. cerevisiae*, and is highly toxic in the absence of arginine. Can resistant (Can^R) mutants have a defect in the arginine permease gene *CAN1* (Ahmad and Bussey, 1986). It is easy to select for these mutants by plating onto minimal media containing an appropriate concentration of Can.

Sensitivity of *Candida tropicalis* NCYC547 to Can was determined as described in section 3.7.4.2, (table 7.4) Colony size became progressively smaller as Can concentration increased. 50 μgml^{-1} Can completely inhibited *S. cerevisiae* X-2180-1A. *C. tropicalis* NCYC547 was never completely inhibited, although at the highest concentrations the colonies were extremely tiny.

The generation of Can^R *C. tropicalis* NCYC547 mutants after exposure to nitrous acid was investigated (section 3.7.4.2), and the results presented below (table 7.5)

The colonies growing on Can were very small, but no true Can^R mutants were picked up before or after exposure to nitrous acid. Exposure to nitrous acid did not increase the number of Can^R colonies. All the Can^R colonies were streaked to single colonies over SD-agar containing 1 $mgml^{-1}$ Can and incubated. Although growth was slow, all formed single colonies.

7.2.4.3 5-Fluoro Orotic Acid

Sensitivity of *Candida tropicalis* NCYC547 and NCYC997 to FOA was determined as described in section 3.7.4.3, (table 7.6)

The *S. cerevisiae* controls demonstrated the effectiveness of the selection. FOA was able to prevent the growth of X-2180-1A (*URA3*), but had little effect on YNN27 (*ura3*).

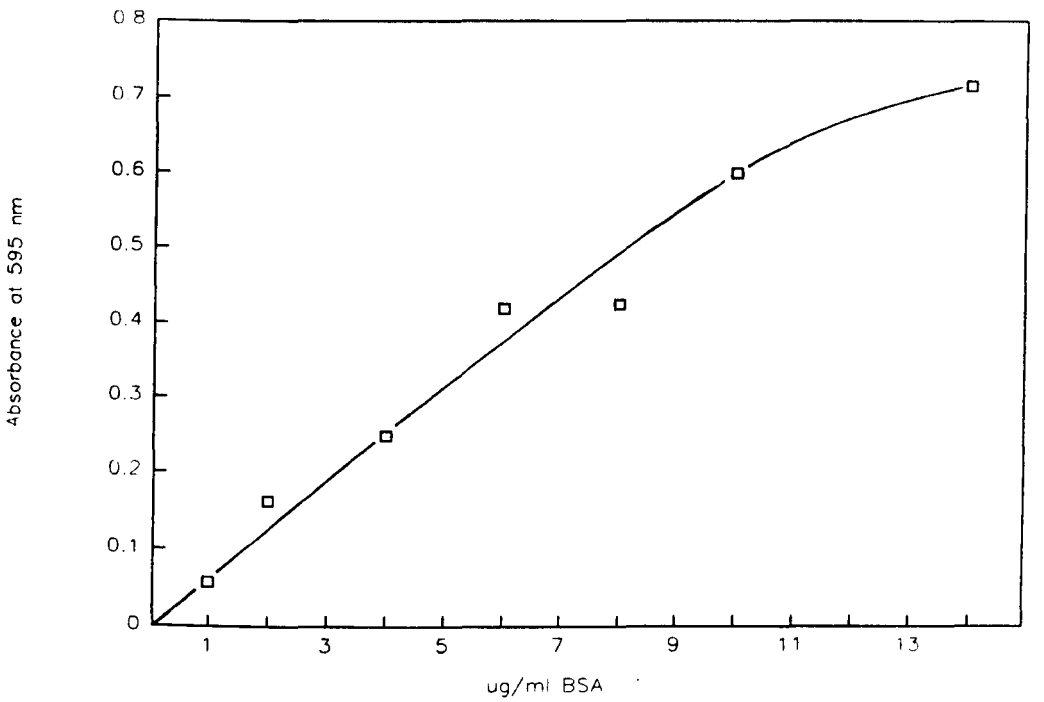


***Figure* 7.4 Bio-Rad assay standard curve of bovine serum albumin (BSA) protein standards**

The amount of protein (μgml^{-1}) present in cell-free extracts was calculated by performing a Bio-Rad assay exactly according to the manufacturers instructions, and comparing the results to the standard curve.

As FOA is costly it was decided to select for *C. tropicalis* NCYC997 *ura3* mutants on 0.5 $mgml^{-1}$ FOA, although 1 $mgml^{-1}$ gave clearer inhibition. Adding 5% v/v DMSO to FOA-agar containing 0.5 $mgml^{-1}$ did not improve inhibition of the *C. tropicalis* strains (results not shown), but appeared to slightly increase resistance to FOA, as was also noticed for G418 (section 5.2.2.3)

Approximately 5.5 x 10^7 nitrous acid exposed, and 10^7 UV-irradiated *C. tropicalis* NCYC997 cells were spread over FOA-agar (section 3.7.4.3). 500 Nitrous acid exposed colonies, and 500 UV-irradiated colonies growing better than background were picked in a regular array onto FOA-agar, and FOA-agar without uracil (U) and incubated. Two UV-irradiated colonies unable to grow without U were streaked to single colonies over FOA, and tested to check they were not contaminants (section 3.1.5). The FOA resistant mutants could not grow on R, and their cell and colony morphology appeared identical to that of *C. tropicalis* NCYC997. It seems reasonable to assume that these mutants derived from *C. tropicalis* NCYC997. The mutants were designated PMPU1, and PMPU2.

7.3 STABILITY OF THE *CANDIDA TROPICALIS* MUTANTS AND DEMONSTRATION THAT THE MUTATION IS IN THE *URA3* GENE

7.3.1 Stability of the *Candida tropicalis ura3* Mutants

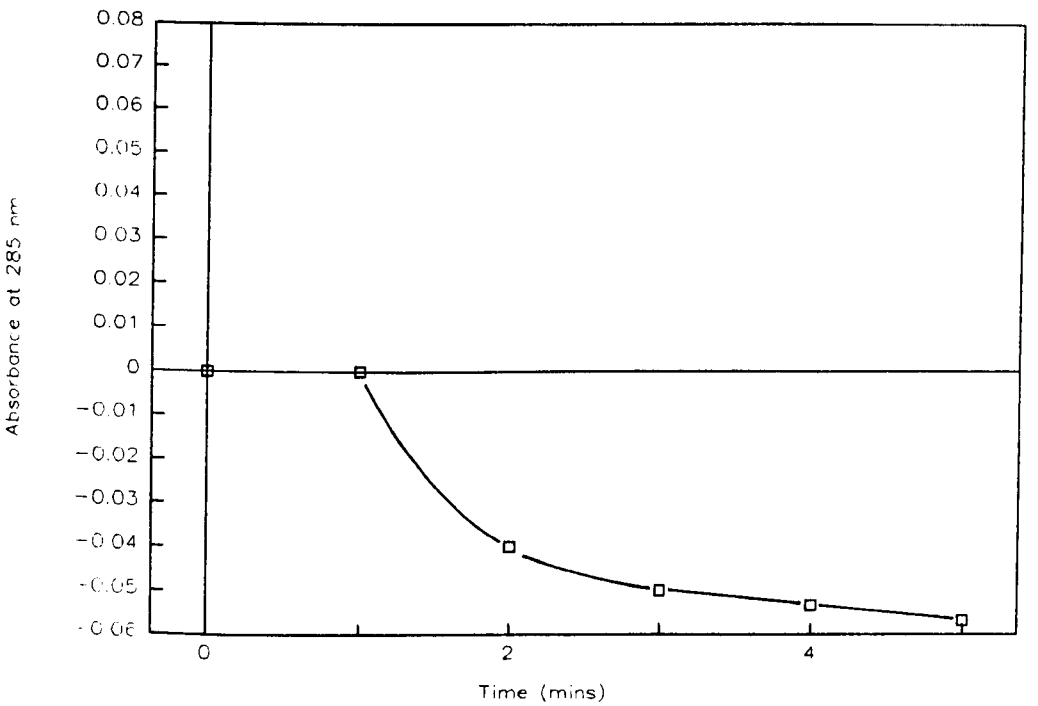
Stability was assessed as described in section 3.7.5. The spontaneous backward rate of mutation for PMPU1 was 5 per 10^9 cells after 22.5 h, and 5 per 10^9 cells after 45 h growth in non-selective medium. No revertants were isolated from cells growing under non-selective conditions for 67.5, or 90 h. The spontaneous backward rate of mutation for PMPU2 was 2 per 10^9 cells after 90 h growth in non-selective medium. From these results the mutations appear to be very stable, and the mutants should be suitable subjects for studies on transformation of *C. tropicalis*. It was observed that PMPU1 grew at a faster rate than PMPU2 as judged by colony size after overnight incubation.

7.3.2 Assay for Orotidine-5'-Phosphatase Activity (*URA3*)

A Bio-Rad assay standard curve (*figure* 7.4) was drawn up, and used to calculate the amount of protein present in each of the cell-free extracts listed below which were assayed for orotidine-5'-phosphatase activity (section 3.9.4), the enzyme encoded by *URA3*:

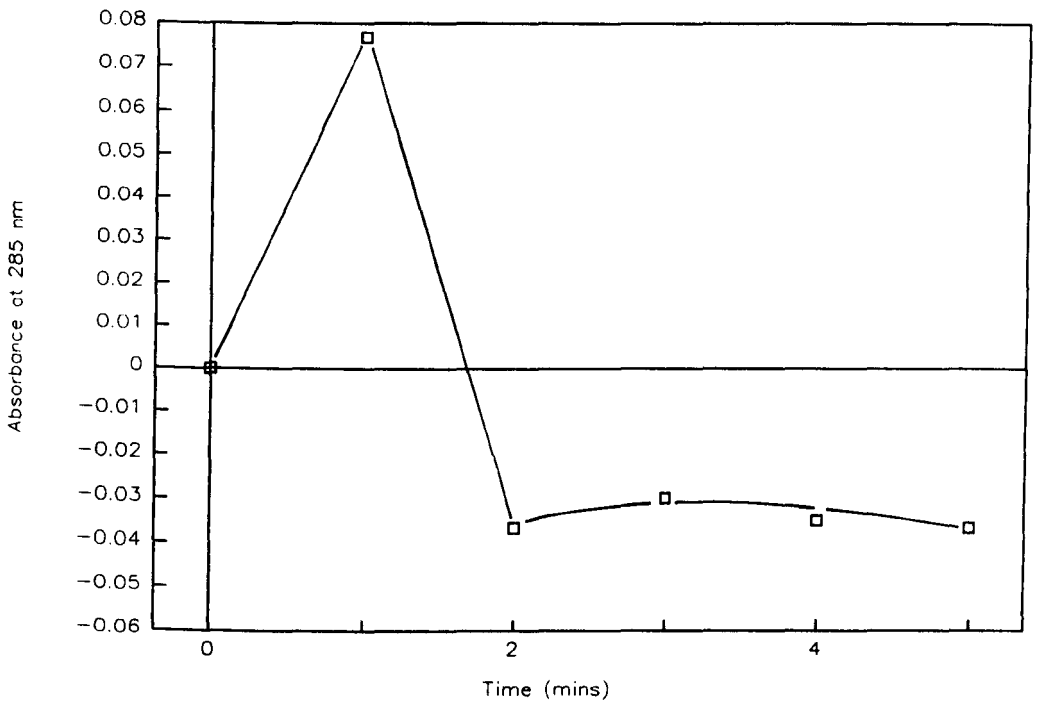
***Figure* 7.5 Change in absorbance at 285 nm over time for cell free extracts**

Changes in A_{285} were measured against a blank in a spectrophotometer as described in Chapter 3. Orotidine-5'-monophosphate, the substrate molecule of orotidine-5'-phosphate decarboxylase the enzyme encoded by the *URA3* gene, was added at time = 0. A drop in A_{285} represents the breakdown of the substrate by the enzyme, a change of -1.38 optical density units corresponding to 1 μmole of substrate decarboxylated (Beckwith *et al.*, 1962).

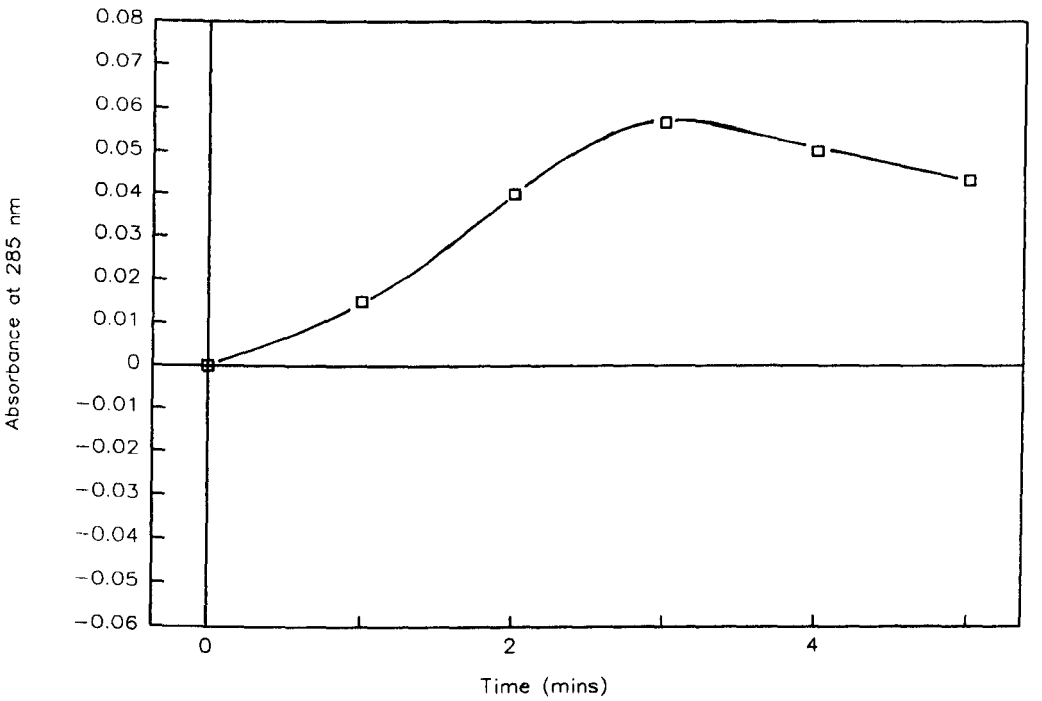


7.5a *S. cerevisiae* X-2180-1A

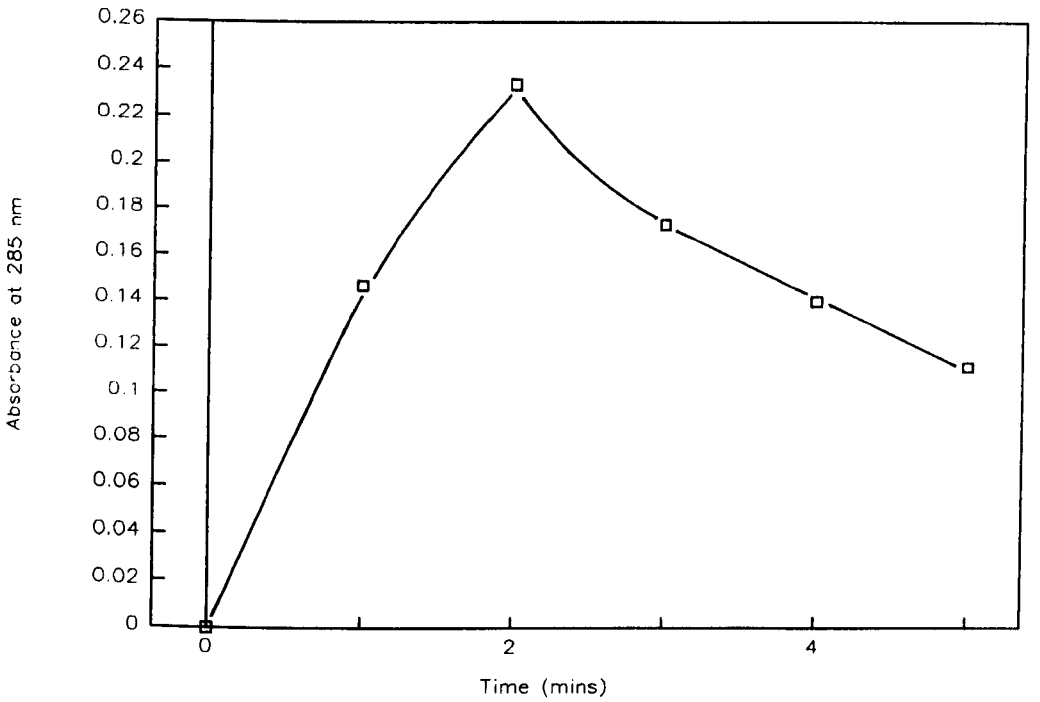
N.B. These results should be treated with caution
because the changes in optical density are not significant
enough for a firm conclusion to be drawn



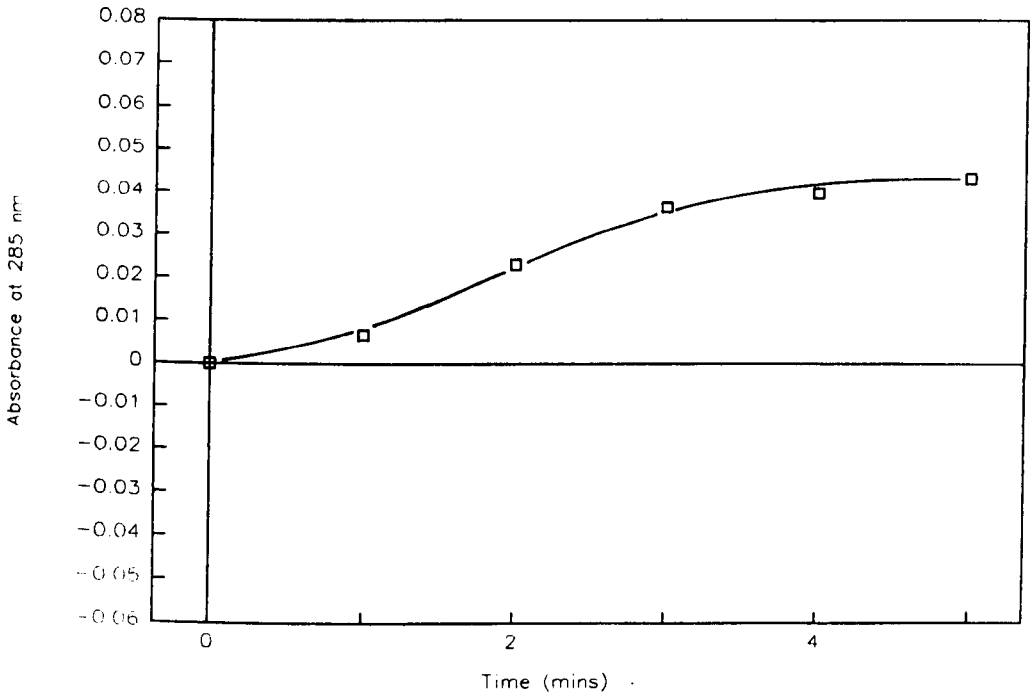
7.5b *C. tropicalis* NCYC997



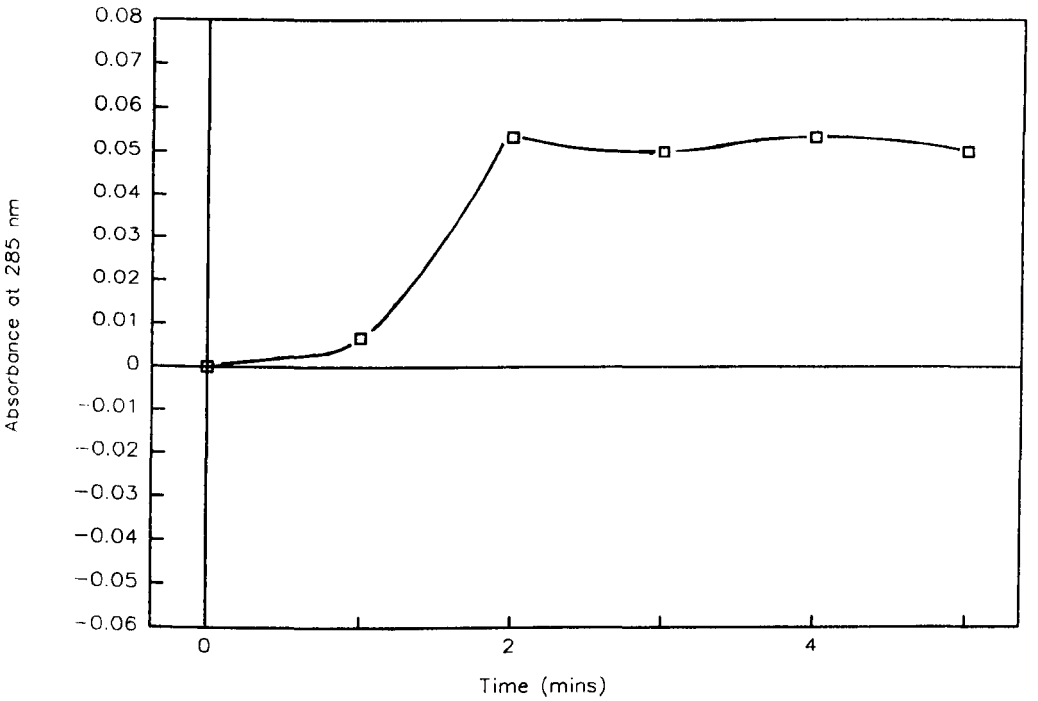
7.5c *S. cerevisiae* S150-2B


Absorbance at 285 nm
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
2
4
Time (mins)

7.5d Buffer



7.5e *C. tropicalis* PMPU1



7.5f *C. tropicalis* PMPU2

N.B. These results should be treated with caution because the changes in optical density are not significant enough for a firm conclusion to be drawn

Table 7.7 Change in absorbance at 285 nm of cell free extracts after the addition of orotidine-5'-monophosphate

The results presented in this table were calculated from the graphs in *figure* 7.5a-f, and show the change in A_{285} over time after the addition of orotidine-5'-monophosphate, the substrate molecule of orotidine-5'-phosphate decarboxylase, the enzyme encoded by the *URA3* gene. A change of -1.38 optical density units corresponds to 1 μmole of substrate decarboxylated (Beckwith *et al.*, 1962). Specific activities were calculated in nmoles of substrate transformed per min (Lacroute, 1968) calculated from *figure* 7.5a-f, per mg of protein calculated from a Bio-Rad assay (*figure* 7.4), and are presented in table 7.8.

Time	Absorbance at 285 nm					
	X-2180-1A	NCYC997	S150-2B	Buffer	PMPU1	PMPU2
(min)	*URA3*	*URA3*	*ura3*	*ura3*	?	?
0	0	0	0	0	0	0
1	0	0.077	0.015	0.147	0.007	0.007
2	-0.040	-0.037	0.040	0.233	0.023	0.053
3	-0.050	-0.030	0.057	0.173	0.037	0.050
4	-0.053	-0.035	0.050	0.140	0.040	0.053
5	-0.057	-0.037	0.043	0.112	0.043	0.050

Table 7.8 Specific Activity of Orotidine-5'-phosphatase

Cell Free Extract	Specific Activity
X-2180-1A	9.72
NCYC997	5.34
S150-2B	0
PMPU1	0
PMPU2	0
buffer	0

S. cerevisiae X-2180-1A (*URA3*).
C. tropicalis NCYC997 (*URA3*).
S. cerevisiae S150-2B (*ura3*).
C. tropicalis PMPU1.
C. tropicalis PMPU2.
The buffer was also assayed.

N.B. These results should be treated with caution because the changes in optical density are not significant enough for a firm conclusion to be drawn

Results of the enzyme assays are shown graphically (*figure* 7.5a-f), and in tables 7.7 and 7.8. According to Lacroute (1968) the presence of U does not significantly change the enzymatic levels of orotidine-5'-phosphatase.

7.4 TRANSFORMATIONS OF THE *CANDIDA TROPICALIS ura3* MUTANTS WITH VECTORS CARRYING THE *SACCHAROMYCES CEREVISIAE URA3* GENE

S. cerevisiae S150-2B, *C. tropicalis* PMPU1, and *C. tropicalis* PMPU2 were transformed using the lithium acetate method (section 3.4.2.1) and the polyethylene glycol (PEG) induced method (section 3.4.2.2). Before being made competent the *Candida* strains were grown to an optical density of 0.8 instead of 0.6 as the results of the growth curves (section 4.2) indicated that at this optical density the *Candida* cells should be in the optimum growth-phase. Each strain was transformed with the following:
10 μl TE (section 2.3.1);
10 μg YIp5 (section 2.1.3.3) cleaved with *Ava* I;
10 μg YEp24 (section 2.1.3.3);
10 μg YCp50 (section 2.1.3.3).
Transformants were selected on CM-U (section 2.2.1). Each transformation was spread over a single plate.

No transformants of any of the strains were isolated from the control transformations to which no DNA was added, or from the *C. tropicalis* strains transformed using the lithium acetate method. The vectors transformed S150-2B with the following efficiencies:
YIp5 (lithium acetate) 1 transformant per μg DNA.
YIp5 (PEG induced) 0 transformants per μg DNA.
YEp24 (lithium acetate) 424 transformants per μg DNA.
YEp24 (PEG induced) 5 transformants per μg DNA.
YCp50 (lithium acetate) 2.44×10^3 transformants per μg DNA.
YCp50 (PEG induced) 44 transformants per μg DNA.

A single transformant colony was isolated from each of the following PEG induced transformations: PMPU1 transformed with YIp5, YEp24, and YCp50 and PMPU2 transformed with YIp5.

10 S150-2B transformants, and all the *C. tropicalis* transformants were streaked to single colonies over fresh CM-U, and SR-agar plates and incubated (72 h). All the restreaked cells grew well on the fresh plates. That the *C. tropicalis* transformants could utilise R showed them to be contaminants and not true transformants (section 3.1.5).

The results demonstrate that vectors carrying the *S. cerevisiae URA3* gene were unable to transform *C. tropicalis ura3* mutants to *URA3* under the conditions employed.

7.5 SCREENING A *CANDIDA TROPICALIS* NCYC997 GENOMIC LIBRARY FOR THE *URA3* GENE

7.5.1 The *Saccharomyces cerevisiae URA3* Gene as a Probe for the *Candida tropicalis URA3* Gene

7.5.1.1 Preparation of *URA3* Probe

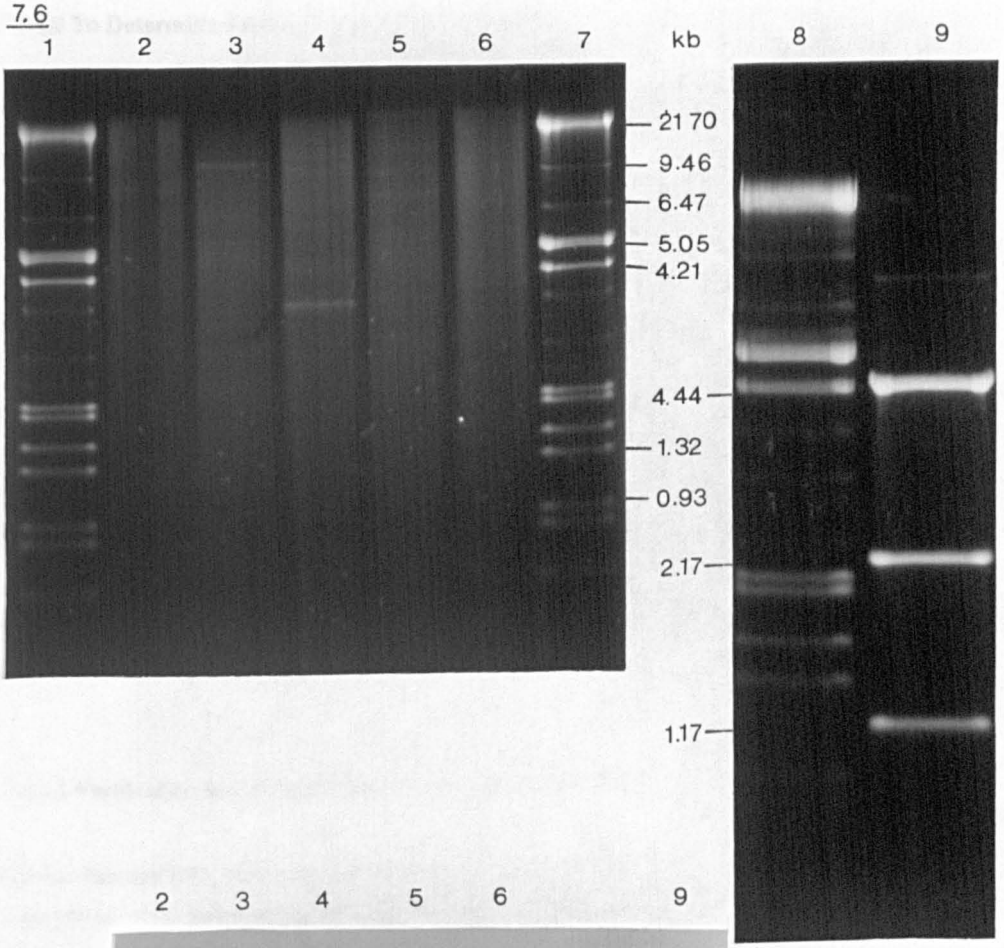
5 μg YEp24, which carries the *S. cerevisiae URA3* gene, was cut to completion (section 3.3.1.1) with *Hin* dIII overnight. 100 ng of the digest was run on an 0.8% agarose slide gel (section 3.3.5) to confirm digestion, and the *URA3* gene was isolated on a 1.1 kb fragment by electroelution (section 3.3.6). The electroeluted DNA was run on an 0.8% agarose slide gel alongside λ *Eco* RI/*Hin* dIII marker fragments, and the original plasmid cleaved with *Hin* dIII to confirm that the correct fragment had been eluted. The concentration of the electroeluted DNA was estimated by measuring A_{260}, and adjusted to 20 ngμl^{-1} with TE (section 2.3.1).

Aliquots of the electroeluted *S. cerevisiae URA3* DNA were radioactively labelled by random hexanucleotide priming (section 3.5.1.2) as required.

***Figure* 7.6 *C. tropicalis* NCYC997 genomic DNA and YEp24 digests subjected to electrophoresis through 0.8% agarose and Southern blots showing hybridisation to the *S. cerevisiae URA3* gene**

Lanes: 1,7 and 8, λ *Eco* RI/*Hin* dIII markers; 2-6 *C. tropicalis* NCYC997 genomic DNA; 2, *Bam* HI digest; 3, *Bgl* II digest; 4, *Eco* RI digest; 5, *Hin* dIII digest; 6, *Sal* I digest; 9, YEp24 *Hin* dIII digest. The probe hybridised strongly to itself but not to the genomic DNA.

7.6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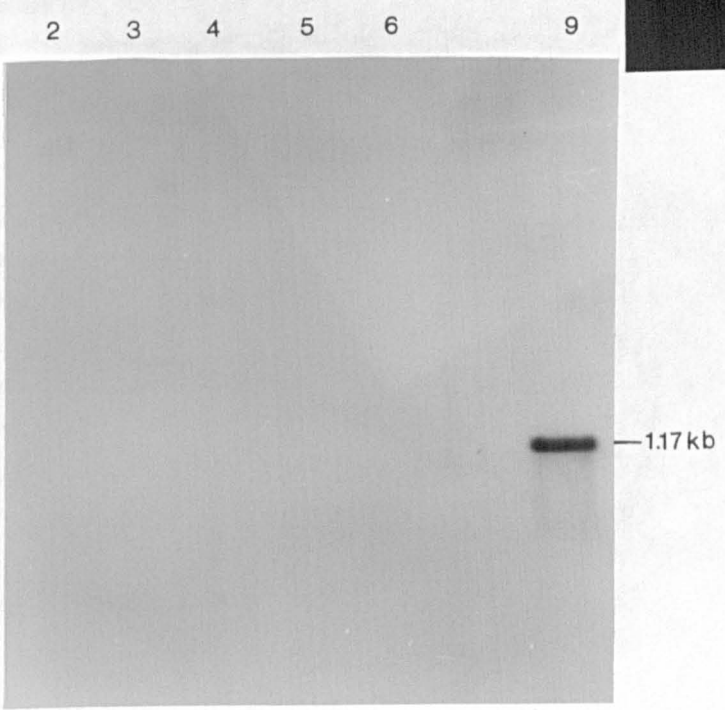
5
6
7
kb
8
9
21.70
9.46
6.47
5.05
4.21
4.44
1.32
0.93
2.17
1.17


2
3
4
5
6
9
1.17 kb

7.5.1.2 To Determine whether the *S. cerevisiae URA3* gene can Hybridize to *C. tropicalis* DNA

1 μg YEp24 was digested overnight with *Hin* dIII. 100 ng of the digest was run on an 0.8% agarose slide gel (section 3.3.5) to confirm digestion, and the remainder was transferred from a large format agarose gel to a nitrocellulose filter by Southern blotting (section 3.5.2.3). This filter and the Southern blot of completely digested *C. tropicalis* genomic DNA prepared in section 4.7.3 were hybridised (section 3.5.3) to the *URA3* probe, and autoradiographed (section 3.5.4; *figure* 7.6). The genomic blot had been stripped of all radioactivity and stored (section 3.5.5).

The results demonstrated that the *S. cerevisiae* DNA was unable to hybridise to *C. tropicalis* DNA under the conditions employed in this experiment, although it hybridised to itself demonstrating that the hybridisation was working. The *S. cerevisiae URA3* gene appears to be unsuitable as a probe for isolating the *C. tropicalis URA3* gene from a library of *C. tropicalis* genomic DNA.

7.5.2 Screening for the *Candida tropicalis URA3* Gene by Complementation of *Escherichia coli* B15

7.5.2.1 Verification and Preparation of the Recipient Strain

Escherichia coli B15, which carries the *pyrF* mutation (section 2.1.2), was streaked to single colonies over M9 medium (section 2.2.2) supplemented with tryptophan and U (M9TU, section 2.2.3.2), and M9T (without U), to verify that the strain does have a requirement for U. The cells grew well on M9TU, but not at all on M9T.

Strain B15 was made competent and stored (section 3.4.1). One aliquot of competent cells was transformed with 100 ng YEp24 which carries the *S. cerevisiae URA3* gene (section 2.1.3.3), and one aliquot with 10 μl TE. The cells transformed with TE were spread over a single Luria-agar (L, section 2.2.2) plate supplemented with 100 μgml^{-1} ampicillin (Ap, section 2.2.3.5), T and U. Half the YEp24 transformations was spread over five LApTU-agar plates, and the other half over five LApT plates. No transformants were isolated from the control cells transformed with TE. 4060 transformant colonies (8.12 x 10^4 transformants per μg DNA) were isolated from the LApT plates compared with 5590 (1.12 x 10^5 transformants per μg DNA) from the LApTU plates. On the basis of these results it was decided to select for Ap^R transformants on LApTU, and look for plasmids which complemented the *pyrF* mutation by replica plating onto M9T.

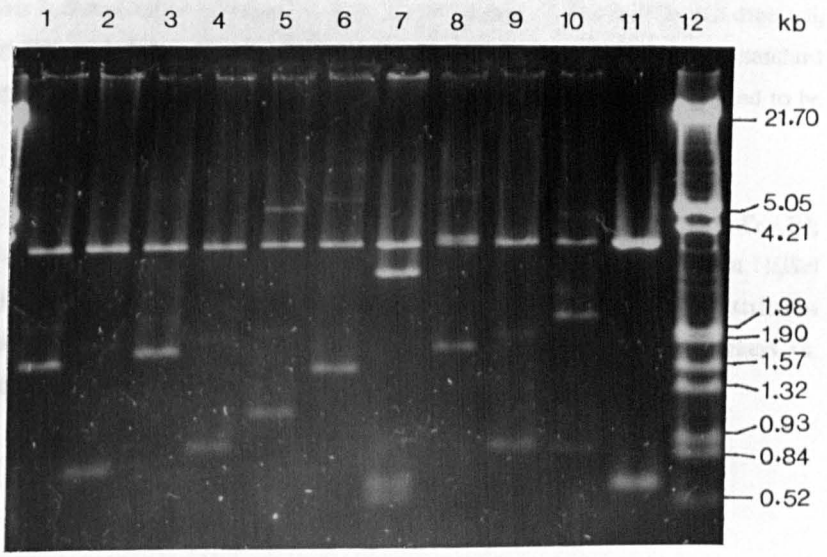


***Figure* 7.7 *Eco* RI/*Sal* I digested plasmid DNA from 10 Ap^R, Tc^S transformants subjected to electrophoresis through 0.8% agarose**

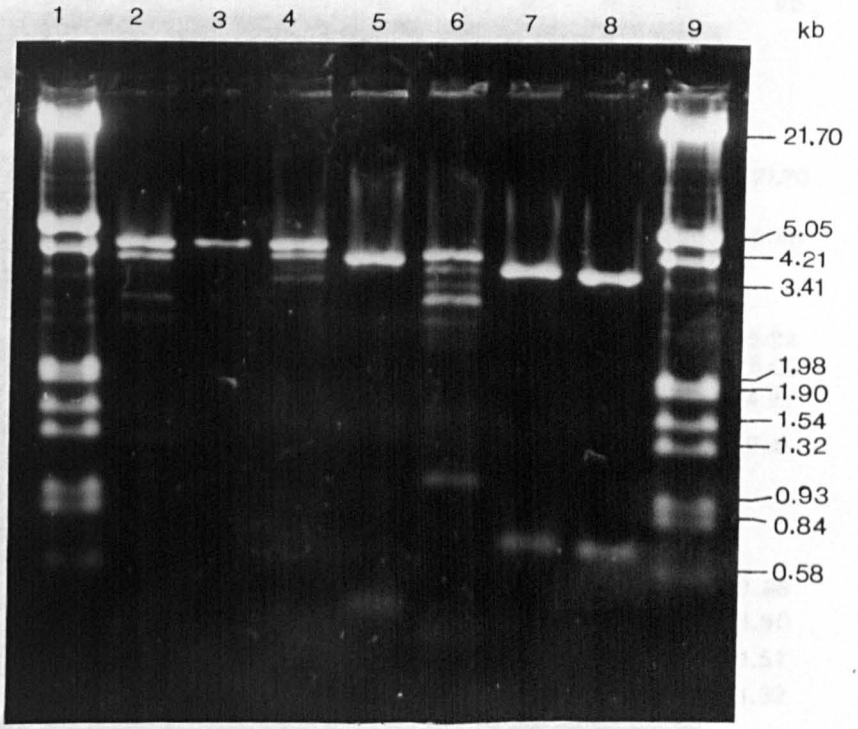
Lanes: 1-10, plasmid DNA; 11, *Eco* RI/*Sal* I digested pBR322; 12, *λ Eco* RI/*Hin* dIII markers. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility and insert sizes were calculated to be: lane 1, 0.86 kb; lane 2, 0.07 kb; lane 3, 1.11 kb; lane 4, 0.21 kb; lane 5, 5.49 kb; lane 6, 6.36 kb; lane 7, 3.62 kb; lane 8, 5.16 kb; lane 9, 2.22 kb; 10, 2.46 kb.

***Figure* 7.8 Restriction endonuclease digested plasmid DNA from two *E. coli* B15 transformants able to grow without uracil subjected to electrophoresis through 0.8% agarose**

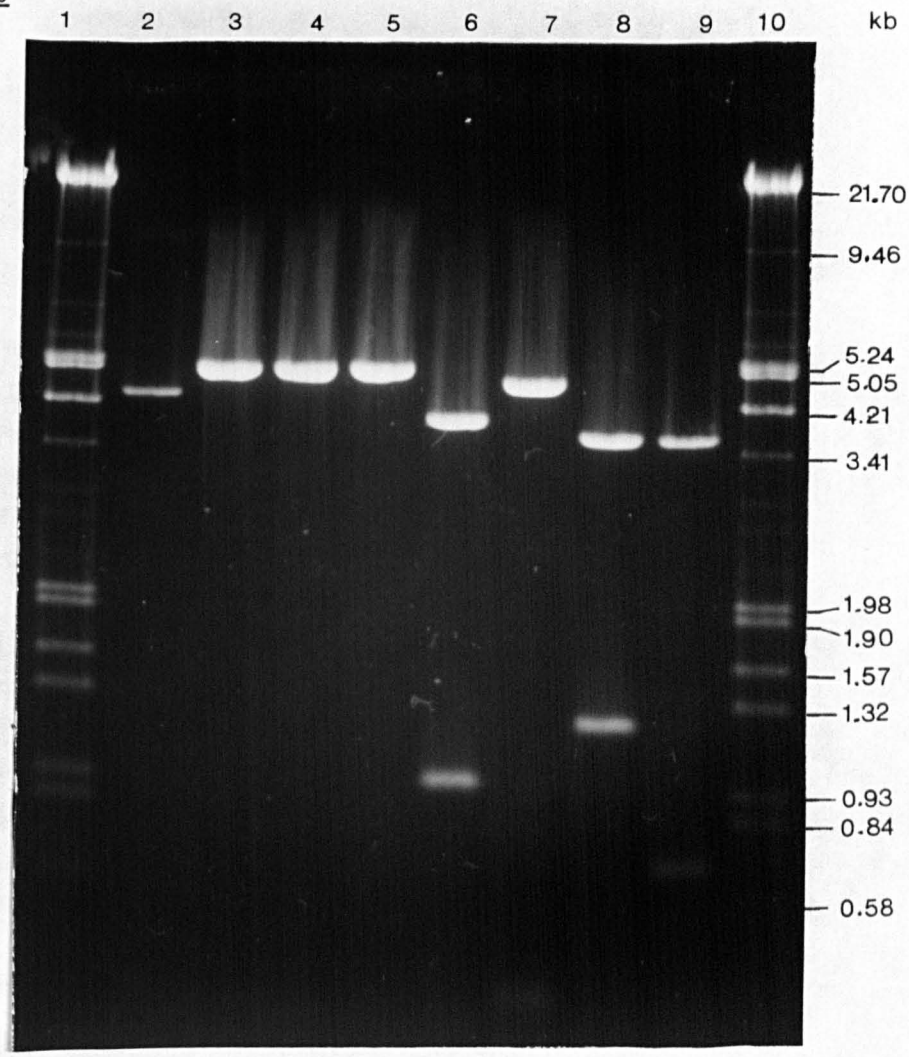
7.8a pMP1. Lanes: 1 and 9, λ *Eco* RI/*Hin* dIII markers; 2, *Bam* HI digest; 3, *Eco* RI digest; 4, *Sal* I digest; 5, *Bam* HI/*Eco* RI digest; 6, *Bam* HI/*Sal* I digest; 7, *Eco* RI/*Hin* dIII digest; 8, pBR322 digested with *Eco* RI/*Sal* I. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility and insert size was calculated to be 137 bp.

7.8b pMP2. Lanes: 1 and 10, λ *Eco* RI/*Hin* dIII markers; 2, pBR322 digested with *Eco* RI; 3, *Bam* HI; digest; 4, *Eco* RI digest; 5, *Sal* I digest; 6, *Bam* HI/*Eco* RI digest; 7, *Bam* HI/*Sal* I digest; 8, *Eco* RI/*Hin* dIII digest; 9, pBR322 digested with *Eco* RI/*Sal* I. The restriction fragments were sized from a standard curve of $\log_{10}$ molecular weight of markers vs. mobility and insert size was calculated to be 547 bp.

7.8a



7.8b


1
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8
9
10
kb
21.70
9.46
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.93
0.84
0.58

7.5.2.2 Isolation of Plasmids Able to Complement the *pyrF* Mutation

The remaining aliquots of competent B15 were each transformed with 100 ng DNA from the *C. tropicalis* genomic library the preparation of which is described in section 4.4. Ap^R transformants were selected by spreading each transformation over twenty LApTU-agar plates (approximately 500 colonies per plate). Altogether approximately 30 000 transformants were generated. Of these, two Tc^S colonies were isolated which were able to grow without added U after replica plating onto M9T.

Cloning into the *Bam* HI site of pBR322 disrupts the Tc resistance gene, therefore Ap^R,Tc^R transformants carry plasmid DNA that has no insert. 3833 Ap^R, Tc^S transformants were replica plated on L-agar plates containing TU, and 12.5 μgml^{-1} Tc (section 2.2.3.5), and incubated (37°C, overnight) to determine the percentage of transformants carrying insert DNA. The percentage of transformants with no insert was 27.0% (1033/3833 Tc^R), therefore, the remaining 35% (≈21900 transformants) have insert DNA.

Plasmid DNA was extracted from 10 Tc^S transformants using the miniprep method (section 3.2.2.2), digested with *Eco* RI and *Sal* I, and subjected to electrophoresis in a 0.8% agarose slide gel (section 3.3.5) alongside λ *Eco* RI/*Hin* dIII restriction fragments, and pBR322 digested with *Eco* RI and *Sal* I (*figure* 7.7). The size of each insert was determined from a standard curve of mobility vs. $\log_{10}$ molecular weight of the λ markers and the average insert size was determined to be 2.8 kb. Using the equation of Clarke and Carbon (1976) as in section 4.4.3, it was calculated that the B15 transformants generated above represent approximately 92% of the *C. tropicalis* genome.

7.5.2.3 Investigation of the B15 Transformants able to Grow without added Uracil

Plasmid DNA was prepared from both transformants by large scale triton lysis (section 3.2.2.1). 0.5 μg aliquots of the plasmids, designated pMP1 and pMP2, were digested overnight (section 3.3.1.1) with the following restriction endonucleases: *Bam* HI, *Eco* RI, *Sal* I, *Bam* HI and *Eco* RI, *Bam* HI and *Sal* I, *Eco* RI and *Sal* I.

100 ng of each digest was run on an 0.8% agarose slide gel (section 3.3.5) to confirm digestion, subjected to electrophoresis in a large format 0.8% agarose gel (section 3.3.5) alongside pBR322 with *Eco* RI, and *Eco* RI and *Sal* I, and λ *Eco* RI/*Hin* dIII restriction fragments, and photographed (*figure* 7.8).

pMP1 and pMP2 are both carrying a small insert.

100 ng of each plasmid was used to transform an aliquot of competent B15. Transformants were selected on LApTU and replica plated onto M9T. None of the transformants were able to grow in the absence of U. These results indicate that the apparent complementation of the *pyrF* mutation was probably due to natural reversion of the mutation in the original transformants, and not by complementation by the equivalent *C. tropicalis* gene carried on the transforming plasmid.

7.6 DISCUSSION

7.6.1 Mutagenesis of *Candida tropicalis*

No *C. tropicalis* auxotrophs were isolated after exposure to nitrous acid or to UV-irradiation without enrichment or positive selection. These results are in agreement with other workers (Olaiya and Sogin, 1979; Gradova and Robysheva, 1980; Yano *et al.*, 1981) who have also reported difficulties in isolating *Candida* mutants by conventional methods. This is probably due to *C. tropicalis* being a stable diploid so that the chance of both alleles at a locus being made recessive at the same time is very small. Whelan and Magee (1981) have reported that *C. albicans* strains show biased auxotroph spectra because genetic loci which are already heterozygous are more likely to become doubly recessive. The fact that no auxotrophs at all were isolated from the *C. tropicalis* strains used in this project by conventional mutagenesis without enrichment or positive selection suggests that they have very few heterozygous genes.

The *lys2* mutation permits *S. cerevisiae* to use the α-aminoadipic acid in AA-medium as a nitrogen source (Chattoo *et al.*, 1979). Prototrophic *C. tropicalis* was able to grow on AA-medium which suggests that a *lys2* mutation is not required for this yeast to utilise α-aminoadipic acid, or that something else was present in the medium which was a suitable nitrogen source for *C. tropicalis*. This selection was not suitable for *C. tropicalis*.

CAN1 strains of *S. cerevisiae* are sensitive to Can, since once it is transported into the cell it is incorporated into protein with deleterious results whereas, *can1* strains are resistant to Can, since it is excluded from the cell by the absence of a functional arginine permease (Broach *et al.*, 1979; Ahmad and Bussey, 1986). The results in section 7.2.4.2 suggest that *C. tropicalis* NCYC547 has the *CAN1* gene although this strain was not completely inhibited by Can. This incomplete inhibition made it difficult to select for *can1* mutants, none of which were isolated before or after exposure of the cells to nitrous acid.

S. cerevisiae ura3 and *ura5* mutants are resistant to the pyrimidine analogue FOA (*ura5* mutants are only partially resistant; Boeke *et al.*, 1984, 1987). *C. tropicalis* NCYC547 and NCYC997 were both sensitive to FOA, and two Ura$^-$ colonies were successfully isolated using this selection system after exposure of NCYC997 to UV-irradiation.

Kakar *et al.* (1983) and Gibbons and Howard (1986) report that nitrous acid is a good mutagen in *C. albicans*, inducing mitotic crossing over, and that nitrous acid alone yields a broader spectrum of auxotrophs than UV alone and is therefore the mutagen of choice when only one is to be used. The results obtained with *C. tropicalis* during the course of this work appear to contradict this. Exposing NCYC547 cells to nitrous acid failed to increase the number of Can^R colonies isolated in section 7.5.4.2, and Ura$^-$ colonies were only obtained from the UV-irradiated cells (section 7.5.4.3). No mutants of any description were ever recovered from nitrous acid exposed cells.

7.6.2 Identity of PMPU1 and PMPU2

As the Ura$^-$ mutants, designated PMPU1 and PMPU2, could not grow on raffinose, had cell and colony morphology which appeared to be identical to that of *C. tropicalis* NCYC997, and smelt similar to *C. tropicalis* NCYC997, it seemed reasonable to assume that they were derived from this strain. The results from section 7.3.1 showed that the mutations were very stable under non-selective conditions, and that therefore PMPU1 and PMPU2 should be suitable host strains for the development of a plasmid mediated transformation system for *C. tropicalis*, and for gene transplacement.

It was observed that PMPU2 grew slowly compared with PMPU1. Occasionally petites have been found among *ura3* colonies selected on FOA medium (Boeke *et al.*, 1984, 1987), so it is probable that as well as a requirement for U, strain PMPU2 is also respiratory deficient which would account for its slow growth rate.

It was possible that the mutation in PMPU1 and PMPU2 was in the *URA5* gene and not the *URA3* gene although *ura5* mutants are only partially resistant to FOA (Boeke *et al.*, 1984, 1987). The results of the orotidine-5'-phosphatase assays (section 7.3.2) showed that PMPU1 and PMPU2 did not have orotidine-5'-phosphatase activity and showed changes in optical density at 285 nm identical to *S. cerevisiae* S150-2B which carries a *ura3* auxotrophic mutation, and the buffer. Both the positive controls, *S. cerevisiae* X-2180-1A which is Ura$^+$, and *C. tropicalis* NCYC997 had orotidine-5'-phosphatase activity. These results confirm that PMPU1 and PMPU2 are mutated at the *URA3* and not the *URA5* locus.

7.6.3 Transformation of PMPU1 and PMPU2 with Vectors carrying *S. cerevisiae URA3*

Transformation of PMPU1 and PMPU2 with various vectors carrying the *S. cerevisiae URA3* gene (section 7.4) failed to produce any *C. tropicalis* Ura$^+$ transformants, although both the lithium acetate and the PEG-induced methods appeared to work well for the *S. cerevisiae* S150-2B control. The PEG-induced transformation method (Klebe *et al.*, 1983) was not found to be as efficient as the lithium acetate method (Ito *et al.*, 1983a).

YIp5 transforms *S. cerevisiae* at low frequency (1-10 transformants μg^{-1}) integrating into the genome by homologous recombination (Struhl *et al.*, 1979). However, YIp5 does not transform the *ura3* deletion mutant of *S. cerevisiae* YNN27 to Ura$^+$ (Hsu *et al.*, 1983). This is consistent with the results obtained for *S. cerevisiae* S150-2B. The results obtained in section 7.5.1.2 suggested that the *S. cerevisiae URA3* gene had no homology to the equivalent *C. tropicalis* gene, and therefore, no recombination could take place between the *S. cerevisiae URA3* gene on the plasmid and the *C. tropicalis* genome which may explain why no *C. tropicalis* Ura$^+$ transformants were obtained using this vector.

YEp24 contains the *S. cerevisiae* 2-μm DNA origin of replication which has been shown to promote autonomous plasmid replication in *Schizosaccharomyces pombe* (Beach and Nurse, 1981), *K. lactis* (Das and Hollenberg, 1982), and *Ustilago maydis* (Banks, 1983), resulting in low transformation frequencies and plasmid copy numbers. It was therefore possible that this vector might also support autonomous replication in *C. tropicalis*. However, no Ura$^+$ transformants were obtained from transformations with YEp24 or with YCp50 which carries *S. cerevisiae* centromeric DNA which acts as an origin of replication.

These results are consistent with the findings of most other groups who have attempted to transform other species with vectors that replicate autonomously in *S. cerevisiae*. For example, the vector YRp7 which contains an *S. cerevisiae* autonomously replicating sequence (ARS) only led to integrative transformation of *K. lactis* (Das and Hollenberg, 1982); *S. cerevisiae* 2-μm, *CEN4* and ARS1 replicons do not function in *K. lactis* (Sreekrishna *et al.*, 1984); attempts at using vectors developed for *S. cerevisiae* in *Yarrowia* (*Saccharomycopsis*) *lipolytica* (a dimorphic yeast) were unsuccessful because they failed to replicate (Gaillardin *et al.*, 1985); the 2-μm replicon does not replicate autonomously in *C. albicans* (Kurtz *et al.*, 1986); and the 2-μm replicon and the ARS1 site of *S. cerevisiae* are not active in *C. maltosa* (Takagi *et al.*, 1986a; Kawai *et al.*, 1987). These observations suggest that differences exist among various species of yeast in the handling of the 2-μm replicon.

Even if the plasmids were replicating autonomously in the *C. tropicalis* cells, the *S. cerevisiae* promoter and terminator sequences may not have been powerful enough in *C. tropicalis* to allow for the efficient expression of the *URA3* gene. For example, expression of a *C. albicans URA3* gene in *S. cerevisiae* and *E. coli* relied on promotion originating within the *C. albicans* DNA (Gillum *et al.*, 1984).

7.6.4 Cloning the *Candida tropicalis URA3* Gene

7.6.4.1 The *S. cerevisiae URA3* Gene as a Probe

The results obtained in Chapter six showed that the *S. cerevisiae HEM1* gene was able to hybridise to *C. tropicalis* genomic DNA. Hybridisation between genes from two yeast species has also been reported by other workers for example, the *Y. lipolytica LEU2* gene hybridizes weakly to the *S. cerevisiae LEU2* gene (Davidow *et al.*, 1985). However, other workers have found the converse, for example, a *C. utilis* ARS was found to have no detectable homology to total DNA from *C. albicans*, *Pachysolen tannophilus*, or *S. cerevisiae* (Hsu *et al.*, 1983), and the *LEU2* genes of *C. utilis* and *C. maltosa* have no detectable homology to the *LEU2* gene of *S. cerevisiae* (Zhang and Reddy, 1986). Given these results, it seemed reasonable to assume that the *S. cerevisiae URA3* gene might be able to hybridise to the equivalent gene in *C. tropicalis*, and would therefore be a suitable probe for the detection of the *C. tropicalis URA3* gene in a genomic library. However, the results from section 7.5.1 showed that the *S. cerevisiae URA3* gene was not homologous to *C. tropicalis* genomic DNA, although it hybridised strongly to itself.

Certain types of genes, such as those encoding mitochondrial enzymes like *HEM1*, may be more highly conserved between species than those involved in other pathways such as *URA3*.

7.6.4.2 Sequences that Complement Genes of Other Organisms

There are many reports of genes from one organism being functionally expressed in another, for example, the *Y. lipolytica LEU2* gene in *S. cerevisiae* (Davidow *et al.*, 1985), the *C. pelliculosa* β-glucosidase gene in *S. cerevisiae* (Kohchi and Toh-e, 1986), and the bacterial genes, chloramphenicol acetyltransferase, and aminoglycoside phosphotransferase in laboratory and industrial *Saccharomyces* species (Hollenberg, 1979; Rank *et al.*, 1988).

Genes from one organism that can complement auxotrophic mutations in others have also been reported, for example, *S. cerevisiae* genes can complement *E. coli* and *Salmonella typhimurium leuB* mutations, and *E. coli hisB* mutations (Ratzkin and Carbon, 1977), *S. cerevisiae LEU2* complements *Schizosaccharomyces pombe leu1* (Beach and Nurse, 1981), *S. cerevisiae URA3* complements *Schizosaccharomyces pombe ura4* (Fournier *et al.*, 1982), a *C. maltosa LEU* gene complements Leu⁻ mutations in *E. coli* and *S. cerevisiae* (Kawamura *et al.*, 1983), *S. cerevisiae LYS2* complements *Y. lipolytica lys2* (Gaillardin *et al.*, 1985), *S. cerevisiae ARG4* is expressed in *C. maltosa*, and *Pichia guilliermondii* (Kunze *et al.*, 1985), and *S. cerevisiae LEU2* complements Leu⁻ *C. maltosa* (Takagi *et al.*, 1986a).

The functional expression of heterologous genes as described above, has been utilised by several groups to isolate genes of interest from one organism by complementing gene defects in cells of a different strain, for example, *S. cerevisiae LEU2* in a *E. coli leuB* strain (Ratzkin and Carbon, 1977), *C. albicans URA3* in *S. cerevisiae ura3* and *E. coli pyrF* strains (Gillum *et al.*, 1984), *Y. lipolytica* (formerly *Saccharomycopsis lipolytica* or *Candida lipolytica*) *LEU2* in *E. coli leuB* and *S. cerevisiae leu2* strains (Davidow *et al.*, 1985), *C. albicans* genes for galactokinase, *HIS3*, benomyl resistance, sorbitol utilisation, glucoamylase, and isomaltase in suitable *S. cerevisiae* strains (Gorman and Koltin, 1986), *Candida albicans ADE2-2* and *ADE2-5* in *S. cerevisiae ade2-2* and *ade2-5* strains (Kurtz *et al.*, 1986), and *C. utilis LEU2* in *S. cerevisiae leu2* and *E. coli leuB* strains (Zhang and Reddy, 1986).

This method suggested itself as a simple way of isolating a prototrophic gene from a *C. tropicalis* NCYC997 genomic DNA library, as it is particularly applicable to strains for which a variety of auxotrophic mutants are not available.

The results obtained in section 7.5.2.1 confirmed that *E. coli* B15 has a U requirement, and was therefore a suitable host strain for cloning the *C. tropicalis URA3* gene by complementation. Statistically the number of transformants generated in section 7.5.2.2 was large enough, and contained inserts of a size to give a 92% probability of at least one cell possessing a copy of the *C. tropicalis URA3* gene.

Investigation of the two transformants able to grow without U (section 7.5.2.3) suggested that the apparent complementation of the *pyrF* mutation was probably due to its natural reversion in the original transformants, since the complemented strains obtained carried plasmids with either a very small or no insert. This was confirmed by transforming fresh aliquots of *E. coli* B15 with the plasmids isolated from these transformants. The fresh transformants were unable to grow without U.

The lack of homology between the *C. tropicalis URA3* gene and the *E. coli PYRF* gene should not have resulted in a lack of complementation. For example, Zhang and Reddy (1986) reported that the *LEU2* gene from either *C. utilis* or *C. maltosa* was able to complement the corresponding mutation in *S. cerevisiae* despite the fact that this gene from either *Candida* species had no detectable homology to the *LEU2* gene of *S. cerevisiae*. problems with gene expression, for example, have previously led to difficulties with complementation strategies in attempts at gene cloning. For example, the *S. cerevisiae URA3* gene does not complement the corresponding *Y. lipolytica* mutation (Davidow *et al.*, 1985), *SUC2* and *PHO5* of *S. cerevisiae* are not expressed in *Y. lipolytica* (Gaillardin *et al.*, 1985), and the *C. pelliculosa* β-glucosidase gene is not active in *E. coli* (Kohchi and Toh-e, 1986). Expression of the *S. cerevisiae URA3* gene in *E. coli* is dependent on a fortuitous promoter sequence which is not present in all strains (Kurtz *et al.*, 1986). That the *C. tropicalis URA3* gene could not be expressed in *E. coli* B15 might explain the failure to isolate this gene by complementation in this strain.

7.6.5 Further Work

Although the attempts described in this study to clone the *C. tropicalis URA3* gene were unsuccessful, whilst writing this thesis an abstract from the 1989 Cold Spring harbour Yeast Meeting, (Gleeson, M., Haas, L., and Cregg, J. Development of an efficient transformation system for *Candida tropicalis*), was published. This group produced *C. tropicalis ura3* mutants using a combination of nystatin enrichment and selection for FOA resistance, isolated clones of *C. tropicalis* DNA capable of complementing both the *S. cerevisiae ura3* and the *E. coli pyrF* mutations, and successfully transformed *C. tropicalis* with both integrating and autonomously replicating vectors. Therefore, by cloning this gene able to complement the mutations in PMPU1 and PMPU2 it will be possible to use these mutants for the development of a transformation system for *C. tropicalis*. In order to utilise the *URA3* gene from another organism as a selectable marker in *C. tropicalis*, it will be necessary to isolate *C. tropicalis* promoter and terminator sequences to ensure adequate expression of the heterologous sequences. Ideally, a clone of the homologous gene would be carrying these sequences intact for optimum expression on a plasmid vector in *C. tropicalis*. Ultimately, the *C. tropicalis ura3* strains and the complementary *URA3* gene could be used to produce a non-revertible β-oxidation mutant via gene transplacement.

To further develop the molecular biology of *C. tropicalis*, it would be useful to have available a wider range of strains carrying auxotrophic mutations. To achieve this, other positive selection systems such as methyl mercury (Singh and Sherman, 1974), or enrichments could be tried in conjunction with UV-irradiation.

CHAPTER 8. DISCUSSION

The primary aim of this project was to produce a non-revertible β-oxidation mutant of *C. tropicalis* to be used for the biotransformation of pelargonate [$CH_3(CH_2)_7COOH$] to azeleate [$HOOC(CH_2)_7COOH$] by *in vitro* disruption of the genes for long-chain fatty acid acyl-CoA oxidase the enzyme catalysing the first step of β-oxidation. These disrupted genes would then be used to replace the functional genes in the chromosomes by gene transplacement (Scherer and Davies, 1979). The fact that two of the long-chain fatty acid acyl-CoA oxidase genes, *POX4-1* and *POX5*, were sequenced just after the commencement of this work (Okazaki, *et al.* 1986) facilitated the first step towards achieving this aim by permitting cloning of these genes from a library of *C. tropicalis* genomic DNA. They provided the data for the construction of oligonucleotides specifically able to hybridise with these gene sequences. However, cloning the two acyl-CoA oxidase genes proved to be more difficult than at first anticipated, partly because when this project was conceived it was not realised that they comprise a multigene family in *C. tropicalis*, which is also a stable diploid, thus making it difficult to obtain a phenotypic mutant.

Statistically, the *C. tropicalis* NCYC997 genomic DNA library, the construction of which is described in Chapter 4, should have included all the fatty acid acyl-CoA oxidase genes, and the oligonucleotide probes were constructed as 20mers to statistically ensure hybridisation to only one member of this multigene family (Zeff and Geliebter, 1987). Therefore, as a clone (pMP13) was isolated which hybridised strongly to the oligonucleotide specific for *POX4-1* (Okazaki *et al.*, 1986), which is also complementary to *POX4-2* (Murray and Rachubinski, 1987), it was assumed that it would contain all or part of one of these alleles. However, the restriction maps and the small amount of sequence data obtained in Chapter 4 for pMP13 left the question of the identity of the cloned fragment unanswered. It is possible that it is another, as yet unidentified, member of the gene family or a redundant sequence which has arisen as a result of gene duplication and has some of the original sequence deleted and/or inverted.

The attempts to clone the *POX* genes were abandoned for several reasons. Even though it is possible to disrupt genes sequentially by transplacement the number of targets involved in this case (six or more alleles) and the lack of a good selectable marker for the identification of transformants made this option appear less feasible. The presence of two functionally distinct fatty acid acyl-CoA synthetase activating enzymes which produce two long-chain fatty acid acyl-CoA pools in the cell which do not take the place of each other has been demonstrated in *C. tropicalis* (Yamada *et al*, 1980). If β-oxidation is blocked, then the long-chain acyl-CoA produced by acyl-CoA synthetase II accumulates when the cells are grown in the presence of exogenous fatty acid (Kamiryo *et al.*, 1977; Kamiryo *et al.*, 1979; Yamada *et al*, 1980) which suggests that a β-oxidation mutant would not produce dioic acids more efficiently, but would accumulate long-chain acyl-CoA instead.

The gene encoding fatty acid acyl-CoA synthetase II may be a better target for gene transplacement as disrupting this gene would render the cells unable to activate fatty acids and these cells would effectively be β-oxidation mutants; furthermore there is no evidence to suggest that the synthetase genes are a multigene family.

It should be noted that the presence of different degradation pathways (Yi and Rehm, 1982a, b, and c: Hill *et al.*, 1986) suggests that the biotransformation of pelargonate (C_9) would result in >91.13% of the acid precipitable products being azeleate. However, the presence of impurities limits the utilisation of the product because the preparation of polymers requires very pure monomers and therefore, a strain of *C. tropicalis* optimised for the production of azeleate from pelargonate will not necessarily be cost effective as purifying the azeleate may be as expensive as converting the pelargonate by a chemical method.

The work presented in Chapters 5, 6 and 7 was concerned with the secondary aim of this project, finding a suitable selectable marker or markers for use in *C. tropicalis* primarily to be used in the gene transplacement experiment described above, but also with a view to the development of general purpose cloning vectors for this yeast. With gene transplacement in mind, emphasis was placed on markers which would be effective in single copy and, with the exception of copper-resistance, only these markers were tested.

In Chapter 5 the sensitivity of *C. tropicalis* to various dominant selectable markers was tested. The sensitivity of the *C. tropicalis* strains to copper indicated that copper-resistance has potential as a dominant selectable marker for *C. tropicalis*. It has been reported by several groups (Karin *et al.*, 1984; Meaden and Tubb, 1985; Knowles and Tubb, 1986) that the copper-resistance determinant is maintained very stably in industrial strains of *S. cerevisiae* without selective pressure being applied, a fact that makes copper an attractive marker for transforming industrial strains of yeast such as *C. tropicalis*.

The natural resistance of the *C. tropicalis* and *S. cerevisiae* strains tested to high concentrations of kanamycin (Km) and Neomycin (Nm) was found to be too great for the selection of transformants and the initial inhibition of the *C. tropicalis* strains by chloramphenicol (Cm) was shown by subsequent experiments in which the ethanol (E) concentration was kept at 4% v/v or less to be a result of the E in the selective medium causing inhibition of growth. Both *C. tropicalis* strains are resistant to Cm which is therefore unsuitable as a dominant selectable marker for these strains.

The MICs of G418 in YEPD for the *C. tropicalis* strains tested were low enough to consider using G418 as a dominant selectable marker, and although increasing the membrane permeability of the cells by adding DMSO to the selective media did not lower the MIC of G418 for either *C. tropicalis* strain, putting pressure on the mitochondrial ribosomes by selecting on YEPGE lowered the apparent MIC of G418 for both *C. tropicalis* strains to a level comparable to that for *S. cerevisiae* laboratory strains on YEPD. However, control transformations of the *C. tropicalis* strains to which no DNA was added using either the lithium acetate method of Ito *et al.* (1983) or the PEG-induced method of Klebe *et al.* (1983), which allowed some manipulation of cell density on the selective plates, gave confluent growth, although the results of transforming *S. cerevisiae* showed that both the transformation procedures and the selection were working. The large numbers of untransformed *C. tropicalis* cells growing on the selective media make G418 unsuitable as a dominant selectable marker for these strains.

Despite the MICs obtained for the *C. tropicalis* strains, control transformations to which no DNA was added gave rise to large numbers of untransformed resistant cells selecting in the presence of G418. The number of cells plated out from these transformations is considerably more than the 1000 cells spread over each plate to ascertain the MIC of an inhibitory substance. The toxicity of the inhibitors may have been diminished by the large numbers of cells on the plate effectively diluting the inhibitor and allowing the growth of untransformed cells. The formation of pseudohyphae by *C. tropicalis* may also protect untransformed cells in some way for example, by cross-feeding.

Resistance to antibiotics has been correlated to impermeability, but adding DMSO, which renders cells more permeable, to selective media containing G418 did not increase the sensitivity of the *C. tropicalis* cells. In fact, the cells appeared to become slightly more resistant to the antibiotic possibly due to changed membrane transport properties. The resistance of the *C. tropicalis* strains to the aminoglycoside antibiotics and Cm is therefore unlikely to be due to impermeability to these inhibitors, but it is possible that the cells are actively excreting the antibiotics. The latter hypothesis could be tested by pulse-chase experiments using radioactively labelled antibiotics. Resistance can also arise through modification of ribosomal RNA, one of the most common natural resistance mechanisms (Davies, 1986). For example, the *RIM.C* gene of *C. maltosa* modifies ribosomes such that they are resistant to cycloheximide (Rank *et al.*, 1988). The differences in the resistance to Cm, Km, Nm, and G418 can be explained by the fact that inhibitors of ribosome function act at different sites (Gale *et al.*, 1981) which might be modified to a greater or lesser extent.

Polyploid industrial strains of *S. cerevisiae* are much more sensitive to G418 and copper than haploid laboratory strains (Sakai and Yamamoto, 1986; Fleming, 1988). As a diploid, *C. tropicalis* might have been expected to have MICs intermediate between those for polyploid industrial and haploid laboratory *S. cerevisiae* strains. However, this was not case for most of the inhibitory substances tested.

The results presented in Chapter 6 involved the construction *C. tropicalis* NCYC997 genomic DNA libraries enriched for sequences encoding the *HEM1* gene, created to facilitate the cloning of this gene which could be used to create a *C. tropicalis hem1* mutant with a requirement for 5-aminolevulinic acid by gene transplacement. The cloned copy of the *HEM1* gene would then act as a dominant selectable marker in this strain by alleviating the nutritional requirement.

The results suggested that the *S. cerevisiae HEM1* gene would be a suitable probe for the equivalent *C. tropicalis* gene. However, no transformants were generated which carried DNA able to hybridise to the *S. cerevisiae HEM1* gene. Sequences included on the DNA fragment carrying the *C. tropicalis HEM1* gene may have been lethal to the *E. coli* cells although the *S. cerevisiae HEM1* gene does not affect them. Since this gene is probably quite homologous to the *C. tropicalis HEM1* because it hybridises strongly to it, it is possible that the *C. tropicalis* signal sequences such as promoter and terminator regions are deleterious to *E. coli*. Although not effective in this case, the methodology used in Chapter 6 to create libraries enriched for genomic DNA carrying the *C. tropicalis HEM1* gene should be applicable to any gene of interest providing a suitable probe is available.

The results presented in Chapter 7 describe the creation of *C. tropicalis* strains with a nutritional requirement for uracil (U) by mutation of a prototrophic strain by exposure to ultraviolet- (UV) irradiation and positive selection with 5-fluoro orotic acid (Boeke *et al.*, 1984, 1987). *C. tropicalis* is a stable diploid so that the chance of both alleles at a locus being made recessive at the same time is very small and as a consequence no auxotrophs were isolated after exposure to nitrous acid or to UV-irradiation alone. Positive selections for the *lys2* mutation using α-aminoadipic acid (Chattoo *et al.*, 1979), and *can1* using L-canavanine sulphate (Broach *et al.*, 1979; Ahmad and Bussey, 1986) were unsuccessful because of the resistance of *C. tropicalis* to these analogues.

Carbohydrate utilisation and cell and colony morphology strongly suggested that the Ura$^-$ mutants obtained, designated PMPU1 and PMPU2, were derived from *C. tropicalis* NCYC997, and the results showed that the mutations were very stable under non-selective conditions, making these strains suitable hosts for the development of a plasmid mediated transformation system for *C. tropicalis*, and for gene transplacement. The mutation in PMPU1 and PMPU2 was demonstrated to be in the *URA3* gene and not the *URA5* gene by the results of assays for orotidine-5'-phosphatase activity

Kakar *et al.* (1983) and Gibbons and Howard (1986) report that nitrous acid is a good mutagen in *C. albicans*, inducing mitotic crossing over, and that nitrous acid alone yields a broader spectrum of auxotrophs than UV alone and is therefore the mutagen of choice when only one is to be used. The results obtained with *C. tropicalis* in Chapter 7 appear to contradict this as no mutants of any description were ever recovered from nitrous acid exposed cells.

No *C. tropicalis* Ura$^+$ transformants were isolated with vectors carrying the *S. cerevisiae URA3* gene, although both the lithium acetate and the PEG-induced methods appeared to work well for the *S. cerevisiae* S150-2B control. One reason for this failure could be that neither the *S. cerevisiae* 2-μm DNA origin of replication or *S. cerevisiae* centromeric DNA, which acts as an origin of replication, can replicate in *C. tropicalis*. Even if the plasmids were replicating autonomously in the *C. tropicalis* cells, the *S. cerevisiae* promoter and terminator sequences may not have been powerful enough in *C. tropicalis* to allow for the efficient expression of the *URA3* gene. Unlike the *S. cerevisiae HEM1* gene (Chapter 6), the results obtained in Chapter 7 suggested that the *S. cerevisiae URA3* gene had no homology to the equivalent *C. tropicalis* gene, and therefore, no recombination could take place between the *S. cerevisiae URA3* gene on the plasmid and the *C. tropicalis* genome which may explain why no *C. tropicalis* Ura$^+$ transformants were obtained by integration of the *S. cerevisiae URA3* gene carried on the vectors. Certain types of genes, such as those encoding mitochondrial enzymes like *HEM1*, may be more highly conserved between species than those involved in other pathways such as *URA3*.

Having isolated *C. tropicalis ura3* mutants, and shown that vectors carrying the *S. cerevisiae URA3* gene were unable to transform these strains, an attempt was made to isolate the *C. tropicalis URA3* gene from a library of total genomic DNA. As the *S. cerevisiae URA3* gene was unable to hybridise to *C. tropicalis* DNA it was not a suitable probe for the detection of the *C. tropicalis URA3* gene in this library. Therefore, the method of choice involved the use of *E. coli* B15 carrying the *pyrF* mutation which is equivalent to *ura3*. This strain was transformed with DNA from the library of *C. tropicalis* NCYC997 genomic DNA. Statistically the number of transformants generated was large enough to give a 92% probability of at least one cell picking up a copy of the *C. tropicalis URA3* gene, which could then be isolated by complementation. Two transformants able to grow without U were isolated, but further investigation showed that the complementation of the *pyrF* mutation was due to reversion in the original transformants.

Genes from one species have been used to complement a corresponding mutation in another species despite having no detectable homology, so lack of homology between the *C. tropicalis URA3* gene and the *E. coli PYRF* gene should not have been a problem. Not all workers have managed to successfully express genes from one species in another. Expression of the *S. cerevisiae URA3* gene in *E. coli* is dependent on a fortuitous promoter sequence which is not present in all strains (Kurtz *et al.*, 1986). That the *C. tropicalis URA3* gene could not be expressed in *E. coli* B15 might explain the failure to isolate this gene by complementation in this strain. As discussed for the *HEM1* gene, *C. tropicalis* DNA sequences may be lethal to the *E. coli* cells, or promoter and terminator sequences may not have been present on the plasmid or powerful enough in *E. coli* to allow for the efficient expression of the *URA3* gene. Creating a library of *C. tropicalis* genomic DNA in an *E. coli* or *S. cerevisiae* expression vector which already carries signal sequences appropriate to the host cell would ensure the efficient expression of the *C. tropicalis* DNA, and improve the chances of isolating the *C. tropicalis URA3* gene by complementation.

In order to produce a *C. tropicalis* mutant optimised for the production of dioic acids the cloning and disruption of the gene encoding fatty acid acyl-CoA synthetase II should be undertaken by differential hybridisation or cloning peroxisomal genes, translating them *in-vitro*, and identifying the clones encoding the correct protein product by an enzyme assay or by using antibodies. It might be possible to isolate and partially sequence acyl-CoA synthetase II from peroxisomal membranes in order to produce a mixed-sequence synthetic oligonucleotide probe.

The *CUP1* allele should be further investigated as a possible marker for use on a general purpose cloning vector for *C. tropicalis*, and the sensitivity of *C. tropicalis* to some of the dominant selectable markers discussed in Chapter 5 including *MEL1*, cinnamic acids, methylglyoxal, and polymyxin B should also be tested for the same purpose. It is possible that the genes encoding these selectable markers will not function efficiently in this yeast while under the control of heterologous signal sequences.

In order to improve expression in *C. tropicalis*, plasmid-borne genes may have to be placed under the control of *C. tropicalis* promoter and terminator sequences. This can also improve cell viability by allowing plasmids to be maintained at low-copy number and preventing the over-expression of heterologous genes. It will be necessary to clone these sequences from *C. tropicalis* strains and incorporate them into cloning vectors for this yeast.

Sulphometuron methyl- and hygromycin B-resistance should be tested as suitable markers for gene transplacement. However, hygromycin B acts on the 30S subunit of 70S ribosomes, and as *C. tropicalis* is resistant to the ribosome inhibiting antibiotics tested in the course of this work it is probable that this yeast will also be resistant to this antibiotic. Phaff *et al.* (1952) describe *C. tropicalis* NCYC547 as a killer strain, and therefore it might be possible to develop this as a dominant selection system for *C. tropicalis* in the same way as the *S. cerevisiae* and *K. lactis* killer phenotypes have been developed.

Although the attempts described in this thesis to clone the *C. tropicalis URA3* gene were unsuccessful, it should eventually be accomplished. Using a gene able to complement the *ura3* mutations in PMPU1 and PMPU2 it will be possible to use these strains for the development of a *C. tropicalis* transformation system. To further develop the molecular biology of *C. tropicalis*, it would be useful to have a wider range of auxotrophic strains. To achieve this, other positive selection systems or enrichments could be tried in conjunction with UV-irradiation. Ultimately, auxotrophic *C. tropicalis* strains in conjunction with the complementary genes could be used to produce a non-revertible β-oxidation mutant via gene transplacement.

Whilst writing this thesis an abstract from the 1989 Cold Spring harbour Yeast Meeting, (Gleeson, M., Haas, L., and Cregg, J. Development of an efficient transformation system for *Candida tropicalis*), was published. This group also produced *ura3* mutants using a combination of nystatin enrichment and selection for 5-fluoro orotic acid resistance mutants. They isolated clones of *C. tropicalis* DNA capable of complementing both the *S. cerevisiae ura3* and the *E. coli pyrF* mutations, and successfully transformed *C. tropicalis* with both integrating and autonomously replicating vectors. The use of both enrichment and positive selection to obtain these mutants would suggest that this group also encountered problems in mutating this organism, and it is interesting that they too ended up with a selection system based on an auxotrophic requirement rather than a dominant selectable marker. That they successfully cloned the corresponding gene by complementation throws some doubt on the usefulness of the library of *C. tropicalis* NCYC997 genomic DNA in pBR322 (Chapter 1), from which genes of interest consistently could not be isolated.

REFERENCES

Adoutte-Panvier, A., and Davies, J. E. 1984. Studies of ribosomes of yeast species: Susceptibility to inhibitors of protein synthesis in vivo and in vitro. Mol Gen. Genet. **194**: 310-317.

Ahmad, M., and Bussey, H. 1986. Yeast arginine permease: nucleotide sequence of the *CAN1* gene. Current Genetics **10**: 587-592.

Anderson, D., and Cuthbertson, W. F. J. 1987. Safety testing of novel food products generated by biotechnology and genetic manipulation, in Biotechnology and Genetic Engineering Reviews (Russell, G. E., ed.), Vol. 5: 369-395, Intercept Ltd.

Astin, A. M., Haslam, J. M., and Woods, R. A. 1977. The manipulation of cellular cytochrome and lipid composition in a haem mutant of *Saccharomyces cerevisiae*. Biochem. J. **166**: 275-285.

Bach, M-L., and Lacroute, F. 1972. Direct selective techniques for the isolation of pyrimidine auxotrophs in yeast. Mol. Gen. Genet. **115**: 126-130.

Baldari, C., and Cesareni, G. 1985. Plasmids pEMBLY: New single-stranded shuttle vectors for the recovery and analysis of yeast DNA sequences. Gene **35**: 27-32.

Banks, G. R. 1983. Transformation of *Ustilago maydis* by a plasmid containing yeast 2-micron DNA. Current Genetics **7**: 73-77.

Barnes, D., Blair, L., Brake, A., Church, M., Julius, D., Kunisawa, R., Lotko, J., Stetler, G., and Thorner, J. 1982. Biosynthesis and mode of action of yeast α-factor mating pheromone: A model eukaryotic hormone. Rec. Adv. Yeast Mol. Biol. 1: 295-305.

Barnett, J. A., Payne, R. W., and Yarrow, D. 1983. Section 162 *Candida tropicalis*, in Yeasts: Characteristics and Identification, Chapter 7: 224-225, Cambridge University Press.

Beach, D., and Nurse, P. 1981. High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature **290**: 140-142.

Beckwith, J. R., Pardee, A. B., Austrian, R., and Jacob, F. 1962. Coordination of the synthesis of the enzymes in the pyrimidine pathway of *E. coli*. J. Mol. Biol. **5**: 618-634.

Beggs, D. J. 1978. Transformation of yeast. Nature **275**: 104-109.

Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening plasmid DNA. Nucleic Acids Research 7(6): 1513-1523.

Blasig, R., Mauersberger, S., Riege, P., Schunck, W-H., Jockisch, W., Franke, P., and Muller, H-G. 1988. Degradation of long-chain n-alkanes by the yeast *Candida maltosa*. Appl. Microbiol. Biotechnol. **28**(6): 589-597.

Boeke, J. D., La Croute, F., and Fink, G. R. 1984. A positive selection for mutants lacking orotidine-5'- phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**: 345-346.

Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. 1987. 5-Fluoro orotic acid as a selective agent in yeast molecular genetics. Methods in Enzymology **154**: 164-175.

Boguslawski, G., and Polazzi, J. O. 1987. Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. Proc. Nat. Acad. Sci. USA. **84**: 5848-5852.

Bolivar, F., Rodriguez, R. L., Betlach, M. C., and Boyer, H. W. 1977a. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene **2**: 75-93.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. V., Heynecker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. 1977b. Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. Gene **2**: 95-113.

Bortol, A., Nudel, C., Giulietti, A. M., Spencer, J. F. T., and Spencer, D. M. 1988. Industrial yeast strain improvement: construction of strains having the killer character and capable of utilizing starch. Appl. Microbiol. Biotechnol. **28**: 577-579.

Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K., and Davis, R. W. 1979. Sterile host yeasts (SHY): A eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**: 17-24.

Broach, J. R., Strathern, J. N., and Hicks, J. B. 1979. Transformation in yeast: Development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene **8**: 121-133.

Broderick, A. J., and Sinclair, E. B. 1984. Microbial biomass production by continuous fermentation of bark hydrolysate. Appl. Microbiol. Biotechnol. **20**(6): 384-388.

Buhler, M., and Schindler, J. 1984. Aliphatic hydrocarbons. In: Rehm, H-J., and Reed, G. (eds.), Biotechnology vol. 6a, Verlag Chemie, Weinheim, pp. 329-385.

Bussey, H., and Meaden, P. A. 1985. Selection and stability of yeast transformants expressing cDNA of an M1 killer toxin immunity gene. Current Genetics **9**: 285-921.

Butt, T. R., Sternberg, E., Herd, J., and Crooke, S. T. 1984a. Cloning and expression of a yeast copper metallothionein gene. Gene **27**: 23-33.

Butt, T. R., Sternberg, E. J., Gorman, J. A., Clark, P., Hamer, D., Rosenberg, M., and Crooke, S. T. 1984b. Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc. Nat. Acad. Sci. USA **81**: 3332-3336.

Case, M. E., Schweizer, M., Kushner, S. R., and Giles, N. H. 1979. Efficient transformation of *Neurospora crassa* by utilising hybrid plasmid DNA. Proc. Nat. Acad. Sci. USA. **76**(10): 5259-5263.

Casey, G. P., Xiao, W., and Rank, G. H. 1988. A convenient dominant selection marker for gene transfer in industrial strains of *Saccharomyces* yeast, *SMR1* encoded resistance to the herbicide sulfometuron methyl. J. Inst. Brewing **94**: 93-97.

Chattoo, B. B., Sherman, F., Azubalis, D. A., Fjellstedt, T. A., Mehvert, D., and Ogur, M. 1979. Selection of *lys2* mutants of the yeast *Saccharomyces cerevisiae* by the utilization of α-amino adipate. Genetics **93**: 51-65.

Clarke, L., and Carbon, J. 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9**: 91-99.

Cohen, J. D., Eccleshall, T. R., Needleman, R. B., Federhoff, H., Buchferer, B., and Marmur, J. 1980. Functional expression of the *Escherichia coli* plasmid gene coding for chloramphenicol acetyl transferase. Proc. Nat. Acad. Sci. USA. **77**: 1078-1082.

Cohn, D. H., Ogden, R. C., Abelson, J. N., Baldwin, T. O., Nealson, K. H., Simon, M. I., and Mileham, A. J. 1983. Cloning of the *Vibrio harveyi* luciferase genes: Use of a synthetic oligonucleotide probe. Proc. Nat. Acad. Sci.USA. **80**: 120-123.

Coudron, P. E., Frerman, F. E., and Schowalter, D. B. 1983. Chemical and catalytic properties of the peroxisomal acyl-coenzyme A oxidase from *Candida tropicalis*. Arch. Biochem. Biophys. **226**: 324-336.

Crueger, W., and Crueger, A. 1984. Single-cell protein, in Biotechnology: A Textbook of Industrial Microbiology (Brock, T. D. ed. of English edition), Chapter 16: 267-276, Science Tech. Inc.

Cryer, D. R., Eccleshall, R., and Marmur, J. 1975. Isolation of yeast DNA, in Methods in Cell Biology XII (Prescott, D. M., ed.), Chapter 3: 39-44, Academic Press, inc. (London) Ltd.

Danilenko, I. I., and Barshtein, Yu. A. 1986. Peculiarities of mutants of *Candida tropicalis* D-2 strain which are resistant to nystatin, levorin, amphotericin B. Genetika **22**(2): 336-338.

Das, S., and Hollenberg, C. P. 1982. A high frequency transformation system for the yeast *Kluyveromyces lactis*. Current Genetics **6**: 123-128.

Das, S., Kellermann, E., and Hollenberg, C. P. 1984. Transformation of *Kluyveromyces fragilis*. J. Bact. **158**: 1165-1167.

Davidow, L. S., Apostolakos, D., O'Donnell, M. M., Proctor, A. R., Ogrydziak, D. M., Wing, R. A., Stasko, I., and DeZeeuw, J. R. 1985. Integrative transformation of the yeast *Yarrowia lipolytica*. Current Genetics **10**: 39-48.

Davies, J. 1986. A new look at antibiotic resistance. F. E. M. S. Microbiology Reviews **39**: 363-371.

Dommes, P., Dommes, V., and Kunau, W-H. 1983. Partial Purification and biological function of an inducible 2,4-dienoyl coenzyme A reductase. J. Biol. Chem. **258**(18): 10846-10852.

Entian, K-D. 1986. Glucose repression: a complex regulatory system in yeast. Microbiol. Sci. **3**: 366-371.

Ernst, J. F., and Chan, R. K. 1985. Characterisation of *Saccharomyces cerevisiae* mutants supersensitive to aminoglycoside antibiotics. J. Bact. **163**(1): 4-14.

Esser, K., and Dohmen, G. 1987. Drug resistance genes and their use in molecular cloning. Process Biochemistry October: 144-148.

Falco, S. C. 1986. Selectable markers for yeast transformation. U. S. Patent No. 4, 626, 505.

Falco, S. C., and Dumas, K. S. 1985. Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics **109**: 21-35.

Falco, S. C., Dumas, K. S., and Livak, K. J. 1985. Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. Nucleic Acids Research **13**(11): 4011-4027.

Fein, J. E., Tallim, S. R., and Lawford, G. R. 1984. Evaluation of D-xylose fermenting yeasts for utilization of a wood-derived hemicellulose hydrolysate. Can. J. Microbiol. **30**(5): 682-690.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry **132**: 6-13.

Fleming, C. J. The Genetic Manipulation of Brewing Yeasts: The inheritance of 2μ plasmids. PhD thesis 1988.

Fogel, S., and Welch, J. W. 1982. Tandem gene amplification mediates copper resistance in yeast. Proc. Nat. Acad. Sci. USA. **79**: 5342-5346.

Fournier, P., Gaillardin, C., de Louvencourt, L., Heslot, H., Lang, B. F., and Kaudewitz. 1982. r-DNA plasmid from Schizosaccharomyces pombe: cloning and use in yeast transformation. Current Genetics **6**: 31-38.

Fournier, P., Provost, A., Bourguignon, C., and Heslot, H. 1977. Recombination after protoplast fusion in the yeast *Candida tropicalis*. Arch. Microbiol. **115**: 143-149.

Frye, B. L., Lancaster, J. H., and Larsh, H. W. 1973. Transformation in a nutritionally deficient mutant of *Candida pseudotropicalis*. J. Gen. Microbiol. **77**: 509-512.

Fujiki, Y., Rachubinski, R. A., and Lazarow, P. B. 1984. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. Proc. Natl. Acad. Sci. USA **81**: 7127-7131.

Fujiki, Y., Rachubinski, R. A., Zentella-Dehesa, A., and Lazarow, P. B. 1986. Induction, identification, and cell-free translation of mRNAs coding for peroxisomal proteins in *Candida tropicalis*. J. Biol. Chem. **261**(33): 15787-15793.

Fukui, S., and Tanaka, A. 1980. Production of useful compounds from alkane media in Japan. Adv. Biochem. Eng. **17**: 1-35.

Fukui, S., and Tanaka, A. 1981. Metabolism of alkanes by yeast. Adv. Biochem. Eng. **19**: 217-237.

Gadd, G. M., Stewart, A., White, C., and Mowll, J. L. 1984. Copper uptake by whole cells and protoplasts of a wild-type and copper-resistant strain of *Saccharomyces cerevisiae*. F. E. M. S. Microbiology Letters **24**: 231-234.

Gaillardin,C., Ribet, A. M., and Heslot, H. 1985. Integrative transformation of the yeast *Yarrowia lipolytica*. Current Genetics **10**: 49-58.

Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., and Waring, M. J. 1981. Antibiotic inhibitors of ribosome function, in The Molecular Basis of Antibiotic Action, 2nd edition, Chapter 6: 403-547, Wiley and sons Ltd.

Gancedo, J. M., and Gancedo, C. 1986. Catabolite repression mutants of yeast. F. E. M. S. Microbiology Reviews **32**: 179-187.

De la Garza, M. M., Schultz-Borchard, U., Crabb, J. W., and Kunau, W-H. 1985. Purification of a multifunctional protein possessing enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities. Eur. J. Biochem. **148**(2): 285-291.

Gatignol, A., Baron, M., and Tiraby, G. 1987. Phleomycin resistance encoded by the *ble* gene from transposon *Tn5* as a dominant selectable marker in *Saccharomyces cerevisiae*. Mol.Gen. Genet. **207**: 342-348.

Gibbons, G. F., and Howard, D. H. 1986. Arginine auxotrophs of *Candida albicans* deficient in argininosuccinate lyase. J. Gen. Mic. **132**: 263-268.

Gillum, A. M., Tsay, E. Y. H., and Kirsch, D. R. 1984. Isolation of the *Candida albicans* Gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyr F* mutations. Mol. Gen. Genet. **198**: 179-182.

Gollub, E. G., Liu, K-P., Dayan, J., Adlersberg, M., and Sprinson, D. B. 1977. Yeast mutants deficient in heme biosynthesis, and a heme mutant additionally blocked in cyclization of 2,3-oxidosqualene. J. Biol. Chem. **252**(9): 2846-2854.

Goodey, A. R., and Tubb, R. S. 1982. Genetic and Biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acid. J. Gen. Microbiol. **128**: 2615-2620.

Gopalan, G., and Rajamanickam, C. 1985. Heme mediated effect of cAMP on mitochondriogenesis during glucose repression-derepression in *Saccharomyces cerevisiae*. Indian J. Biochem. Biophys. **22**(4): 214-217.

Gorman, J. A., and Koltin, Y. 1986. Recombinant *Saccharomyces*: A method of cloning and expressing DNA sequences derived from *Candida* in *Saccharomyces*, a method for characterising the function of the DNA sequences, a *Saccharomyces* host microorganism transformed by the DNA sequence and a recombinant plasmid useful for performing such transformation. European Patent Application No. 0173668.

Gough, J. A., and Murray, N. E. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. **166**: 1-19.

Gradova, N. B., and Robysheva, Z. N. 1980. Study of mutagenesis in yeasts of the *Candida* Genus, in Current Developments in Yeast Research. Advances in Biotechnology. Proceedings of the Fifth International Yeast Symposium (Stewart, G. S., and Russell, I., eds,), pp. 205-211, Pergamon Press, Canada.

Greer, H., and Fink, G. R. 1975. Isolation of regulatory mutants in *Saccharomyces cerevisiae*. Section V: Pyrimidine Biosynthesis, in Methods in Cell Biology XI, Yeast Cells (Prescott, D. M., ed.), Chapter 14: 260-261, Academic Press Inc. (London) Ltd.

Gritz, L., and Davies, J. 1983. Plasmid encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene **25**: 179-188.

Grunstein, M., and Hogness, D. 1975. Colony hybridization: a method for the isolation of cloned DNAs that confirm a specific gene. Proc. Nat. Acad. Sci. USA. 72: 3961-3965.

Guarente, L., and Mason, T. 1983. Heme regulates transcription of the *CYC1* gene of *Saccharomyces cerevisiae* via an upstream activation site. Cell **32**: 1279-1286.

Guarente, L., Lalonde, B., Gifford, P., and Alani, E. 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene of *Saccharomyces cerevisiae*. Cell **36**: 503-511.

Gudenus, R., Spence, A., Hartig, A., Smith, M., and Ruis, H. 1984. Regulation of transcription of the *Saccharomyces cerevisiae CYC1* gene: identification of a DNA region involved in heme control. Current Genetics **8**(1): 45-48.

Haas, M. J., and Dowding, J. E. 1975. Aminoglycoside-modifying enzymes. Methods in Enzymology **43**: 611-628.

Hadfield, C., Cashmore, A. M., and Meacock, P. A. 1986. An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. Gene **45**: 149-158.

Hadfield, C., Cashmore, A. M., and Meacock, P. A. 1987. Sequence and expression of a shuttle chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. Gene **52**: 59-70.

Hames, B. D., and Rickwood, D., eds. 1981. Gel Electrophoresis of Proteins: A practical approach. IRL Press, Oxford.

Hashimoto, T. 1982. Individual peroxisomal β-oxidation enzymes. Ann. N. Y. Acad. Sci. **386**: 5-12.

Hashimoto, F., and Hayashi H. 1987. Significance of catalase in peroxisomal fatty-acyl-CoA β-oxidation. Biochim. Biophys. Acta **921**: 142-150.

Henderson, R. C. A., Cox, B. S., and Tubb, R. 1985. The transformation of brewing yeasts with a plasmid containing the gene for copper resistance. Current Genetics **9**: 133-138.

Henry, S. A., and Horowitz, B. 1975. A new method for mutant selection in *Saccharomyces cerevisiae*. Genetics **79**: 175-186.

Henry, S. A., Donahue, T. F., and Culbertson, M. R. 1975. Selection of spontaneous mutants by inositol starvation in yeast. Mol. Gen. Genet. **143**: 5-11.

Hill, D. E., Boulay, R., and Rogers, D. 1988. Complete nucleotide sequence of the peroxisomal acyl-CoA oxidase from the alkane-utilising yeast *Candida maltosa*. Nucleic Acids Research **16**(1): 365-366.

Hill, F. F., Venn, I., and Lukas, K. L. 1986. Studies on the formation of long-chain dicarboxylic acids from pure n-alkanes by a mutant of *Candida tropicalis*. Appl. Microbiol. Biotechnol. **24**: 168-174.

Hinnen, A., Hicks, J. B., and Fink, G. R. 1978. Transformation of yeast. Proc. Nat. Acad. Sci. USA. **75**(4): 1929-1933.

Ho, N. W. Y., Gao, H. C., Huang, J. J., Stevis, P. E., Chang, S. F., and Tsao, G. T. 1984. Development of a cloning system for *Candida* Species. Biotechnol. Bioeng. Symp. **14**: 295-301.

Hollenberg, C. P. 1979. The expression in *Saccharomyces cerevisiae* of bacterial β-lactamase and other antibiotic resistance genes integrated in a 2μm DNA vector. ICN-UCLA Symposium of Molecular and Cellular Biology **15**: 325-338.

Hollenberg, C. P. 1982. Cloning with 2lm DNA vectors and the expression of foreign genes in *Saccharomyces cerevisiae*. Cur. Top. Microbiol. Immunol. **96**: 119-144.

Hollenberg, C. P., Kustermann-Kuhn, B., Makedonski, V., and Erhart, E. 1981. The expression of bacterial antibiotic resistance genes in the yeast *Saccharomyces cerevisiae*, in Molecular Genetics in Yeast (von Wettstein, D., Friis, J., Kielland-Brandt, M., and Stenderup, A., eds.), Alfred Benzon Symposium **16**: 341-345, Munksgaard. Copenhagen.

Hortner, H., Ammerer, G., Hartter, E., Hamilton, B., Rytka, J., Bilinski, T., and Ruis, H. 1982. Regulation of synthesis of catalases and iso-1-cytochrome c in *Saccharomyces cerevisiae* by glucose, oxygen, and heme. Eur. J. Biochem. **128**: 179-184.

Hsiao, C. L., and Carbon, J. 1979. High-frequency transformation of yeast by plasmids containing the cloned yeast *ARG4* gene. Proc. Nat. Acad. Sci. USA. **76**(8): 3829-3833.

Hsu, W. H., Magee, P. T., Magee, B. B., and Reddy, C. A. 1983. Construction of a new yeast cloning vector containing autonomous replication sequences from *Candida utilis*. J. Bact. **154**(3): 1033-1039.

Iimura, Y., Gotoh, K., Ouchi, K., and Nishiya, T. 1983. Yeast transformation without the spheroplasting process. Agric. Biol. Chem. **47**(4): 897-901.

Isogai, T., Yoshida, M., Tanaka, M., and Aoki, H. 1987. Transformation of *Acremonium chrysogenum* and *Saccharomyces cerevisiae* using an antibiotic resistance marker. Agri. Biol. Chem. **51**: 2321-2329.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bact. **153**(1): 163-168.

Ito, H., Murata, K., and Kimura, A. 1983. Transformation of yeast cells treated with 2-mercaptoethanol. Agri. Biol. Chem. **47**: 1691-1692.

Jiang, Z., and Thorpe, C. 1983. Acyl-CoA oxidase from *Candida tropicalis*. Biochemistry **22**: 3752-3758.

Jimenez, A., and Davies, J. 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. Nature **287**: 869-871.

Jones, E. W., and Fink, G. R. 1982. Nucleotide Biosynthesis: Biosynthesis of pyrimidine nucleotides, in The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds.), pp. 264-271, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E., and Helinski, D. R. 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2, in Methods in Enzymology 68. Recombinant DNA (Wu, R., ed.), Chapter 17: 268-280, Academic Press inc. (London) Ltd.

Kakar, S. N., and Magee, P. T. 1982. Genetic analysis of *Candida albicans*: identification of different isoleucine-valine, methionine, and arginine alleles by complementation. J. Bact. **151**(3): 1247-1252.

Kakar, S. N., Partridge, R. M., and Magee, P. T. 1983. A genetic analysis of *Candida albicans*: Isolation of a wide variety of auxotrophs and demonstration of linkage and complementation. Genetics **104**: 241-255.

Kamiryo, T., Abe, M., Okazaki, K., Kato, S., and Shimamoto, N. 1982. Absence of DNA in peroxisomes of *Candida tropicalis*. J. Bact. **152**(1): 269-274.

Kamiryo, T., Mishina, M., Tashiro, S-I., and Numa, S. 1977. *Candida lipolytica* mutants defective in an acyl-coenzyme A synthetase: isolation and fatty acid metabolism. Proc. Nat. Acad. Sci. USA. **74**(11): 4947-4950.

Kamiryo, T., Nishikawa, Y., Mishina, M., Terao, M., and Numa, S. 1979. Involvement of long-chain acyl coenzyme A for lipid synthesis in repression of acetyl-coenzyme A carboxylase in *Candida lipolytica*. Proc. Nat. Acad. Sci. USA. **76**(9): 4390-4394.

Kamiryo, T., and Numa, S. 1973. Reduction of the acetyl coenzyme A carboxylase content of *Saccharomyces cerevisiae* by Exogenous Fatty Acids. F. E. B. S. Letters **38**(1): 29-32.

Kamiryo, T., and Okazaki, K. 1984. High-Level expression and molecular cloning of genes encoding *Candida tropicalis* peroxisomal proteins. Mol. Cell. Biol. **4**(10): 2136-2141.

Kar, R., and Viswanathan, L. 1985. Ethanolic fermentation by thermotolerant yeasts. J. Chem. Technol. Biotechnol., Biotechnol. **35B**(4): 235-238.

Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., and Fogel, S. 1984. Primary structure and transcription of an amplified genetic locus: The *CUP1* locus of yeast. Proc. Nat. Acad. Sci. USA. **81**: 337-341.

Kaster, K. R., Burgett, S. G., and Ingolia, T. D. 1984. Hygromycin B resistance as dominant selectable markers in yeast. Current Genetics **8**: 353-358.

Kawai, S., Hwang, C. W., Sugimoto, M., Takagi, M., and Yano, K. 1987. Subcloning and nucleotide sequencing of an ARS site of *Candida maltosa* which also functions in *Saccharomyces cerevisiae*. Agric. Biol. Chem. **51**(6): 1587-1591.

Kawamoto, S., Kobayashi, M., Tanaka, A., and Fukui, S. 1977a. Production of D-amino acid oxidase by *Candida tropicalis*. J. Ferment. Technol. **55**: 13-18.

Kawamoto, S., Nozaki, C., Tanaka, A., and Fukui, S. 1978. Fatty acid β-oxidation system in microbodies of n-alkane-grown *Candida tropicalis*. Eur. J. Biochem. **83**: 609-613.

Kawamoto, S., Tanaka, A., Yamamura, M., Teranishi, Y., Fukui, S., and Osumi, M. 1977b. Microbody of n-alkane-grown yeast. Enzyme localization in the isolated microbody. Arch. Microbiol. **122**: 1-8.

Kawamura, M., Takagi, M., and Yano, K. 1983. Cloning of a *LEU* gene and an ARS site of *Candida maltosa*. Gene **24**: 157-162.

Khandjian, E. W. 1987. Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Biotechnology **5**: 165-167.

Kimura, A. 1986. Molecular breeding of yeasts for production of useful compounds: novel methods of transformation and new vector systems, in Biotechnology and Engineering Reviews (Russell, G. E., ed.), Vol. **4**: 39-57, Intercept, Newcastle upon Tyne.

King, P. V., and Blakesley, R. W. 1986. Optimizing DNA ligations for transformation. Focus **8**(1): 1-3.

Klebe, R. J., Harriss, J. V., Sharp, Z. D., and Douglas, M. G. 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene **25**: 333-341.

Knowles, J. K. C., and Tubb, R. S. 1986. Recombinant DNA: gene transfer and expression techniques with industrial yeast strains. European Brewing Convention, Monograph XII, Symposium on Brewers Yeast, Helsinki, Finland. pp. 169-185.

Kohchi, C., and Toh-e, A. 1986. Cloning of *Candida pelliculosa* β-glucosidase gene and its expression in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **203**: 89-94.

Kucsera, J., and Ferenczy, L. 1986. Interspecific hybridization between *Candida albicans* and *Candida tropicalis*. F. E. M. S. Microbiology Letters **36**: 315-318.

Kunze, G., Petzoldt, C., Bode, R., Samsonova, T., Hecker, M., and Birnbaum, D. 1985. Transformation of *Candida maltosa* and *Pichia guilliermondii* by a plasmid containing *Saccharomyces cerevisiae ARG4* DNA. Current Genetics **9**: 205-209.

Kurihara, T., Ueda, M., and Tanaka, A. 1989. Peroxisomal acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase from an n-alkane-utilizing yeast, *Candida tropicalis*: purification and characterisation. J. Biochem. **106**: 474-478.

Kurtz, M. B., Cortelyan, M. W., and Kirsch, D. R. 1986. Integrative transformation of *Candida albicans*, using a cloned *Candida ADE2* gene. Mol. Cell. Biol. **6**(1): 142-149.

Kwapinski, J. B. 1968. A *Candida* transformant produced by mycobacterial nucleic acids. Antonie van Leeuwenhoek **34**: 57-65.

Labbe-Bois, R., Brouillet, N., Camadro, J. M., Chambon, H., Felix, F., Labbe, P., Rytka, J., Simon-Casteras, M., Urban-Grimal, D., Volland, C., and Zagorec, M. 1986. Molecular approaches to heme biosynthesis in the yeast *Saccharomyces cerevisiae*. Colloq. INSERM (Inst. Natl. Sante. Rech. Med.) **134**: 15-24.

Lacroute, F. 1968. Regulation of pyrimidine biosynthesis in *Saccharomyces cerevisiae*. J. Bact. **95**: 824-832.

Lewis, T. A., Taylor, F. R., and Parks, L. W. 1985. Involvement of heme biosynthesis in control of sterol uptake by *Saccharomyces cerevisiae*. J. Bact. **163**(1): 199-207.

Lieberman, I., Kornberg, A., and Simms, E. S. 1955. Enzymatic synthesis of pyrimidine nucleotides orotidine-5'-phosphate and uridine-5'-phosphate. J. Biol. Chem. **25**: 403-415.

Lin, K. I. P., Gollub, E. G., and Beattie, D. S. 1982. Synthesis of proteins of complex III of the mitochondrial respiratory chain in heme deficient cells. Eur. J. Biochem. **128**: 309-313.

Lingappa, V. R., Lingappa, J. R., and Blobel, G. 1979. Chicken ovalbumin contains an internal signal sequence. Nature **281**: 117-119.

Lolle, S., Skipper, N., Bussey, H., and Thomas, D. Y. 1984. EMBO J. **3**: 1383-1387.

Lowry, C. V., and Lieber, R. H. 1986. Negative regulation of the *Saccharomyces cerevisiae ANB1* gene by heme, as mediated by the *ROX1* gene product. Mol. Cell. Biol. **6**(12): 4145-4148.

Magee, B. B., D'Souza, T. M., and Magee, P. T. 1987. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species. J. Bact. **169**(4): 1639-1643.

Mandel, M., and Higa, A. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**: 159-162.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning - a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Mannaerts, G. P., Van Veldhoven, P., Van Broekhoven, A., Vandebroek, G., and Debeer, L. J. 1982. Evidence that peroxisomal acyl-CoA-synthetase is located at the cytoplasmic side of the peroxisomal membrane. Biochemical J. **204**: 17-23.

Mauersberger, S., Kargel, E., Matyashova, R. N., and Muller, H-G. 1987. Subcellular organisation of alkane oxidation in the yeast *Candida maltosa*. J. Basic Microbiol. **27**(10): 565-582.

McLeod, M., Volkert, F., and Broach, J. 1984. Components of the site specific recombination system encoded by the yeast plasmid 2-micron circle. Cold Spring Harbour Symposia on Quantitative Biology **49**: 779-787.

Meaden, P., Ogden, K., Bussey, H., and Tubb, R. S. 1985. A *DEX* gene conferring production of an extracellular amyloglucosidase on yeast. Gene **34**: 325-334.

Meaden, P. G., and Tubb, R. S. 1985. A plasmid vector system for the genetic manipulation of brewing strains. yeast genetics, in Proceedings of the European Brewing Convention, 20th Congress, Helsinki. pp. 219-226.

Megnet, R. 1964. A Method for the Selection of auxotrophic mutants of the yeast *Schizosaccharomyces pombe*. Experimentia **20**: 320-321.

Messing, J. 1983. New M13 vectors for cloning. Methods in Enzymology **101**: 20-78.

Mishina, M., Kamiryo, T., Tashiro, S., Hagihara, T., Tanaka, A., Fukui, S., Osumi, M., and Numa, S. 1978. Subcellular localization of two long-chain acyl-coenzyme A synthetases in *Candida lipolytica*. Eur. J. Biochem. **89**: 321-328.

Mowll, J. L., and Gadd, G. M. 1984. Cadmium uptake by *Aureobasidium pullulans*. J. Gen. Mic. **130**: 279-284.

Murray, W. W., and Rachubinski, R. A. 1987. The primary structure of a peroxisomal fatty acyl-CoA oxidase from the yeast *Candida tropicalis* pK233. Gene **51**: 119-128.

Naiki, N., and Yamagata, S. 1976. Isolation and some properties of copper-binding proteins found in a copper-resistant strain of yeast. Plant Cell Physiol. **17**: 1281-1295.

Numa, S., and Yamashita, S. 1974. Regulation of lipogenesis in animal tissues. Curr. Top. Cell. Regul. **8**: 197-246.

Ogata, K., Kaneyuki, H., Kato, N., Tani, Y., and Yamada, H. 1973. Accumulation of decanedioic acid from n-decane by *Torulopsis candida* N°.99. J. Ferment. Technol. **51**: 227-235.

Ogino, S., Yano, K., Tamura, G., and Arima, K. 1965. Studies on utilization of hydrocarbons by yeasts. Part II Diterminal oxidation of alkanes by yeasts. Agric. Biol. Chem. **29**: 1009-1015.

Okada, H., Ueda, M., and Tanaka, A. 1986. Purification of peroxisomal malate synthase from alkane grown *Candida tropicalis* and some properties of the purified enzyme. Arch. Microbiol. **144**: 137-141.

Okanishi, M., and Gregory, K. F. 1970. Isolation of mutants of *Candida tropicalis* with increased methionine content. Can. J. Microbiol. **16**(12): 1139-1143.

Okazaki, K., Takechi, T., Kambara, N., Fukui, S., Kubota, I., and Kamiryo, T. 1986. Two acyl-coenzyme A oxidases in peroxisomes of the yeast *Candida tropicalis*: Primary structures deduced from genomic DNA sequence. Proc. Nat. Acad. Sci. USA. **83**: 1232-1236.

Okazaki, K., Tan, H., Fukui, S., Kukota, I., and Kamiryo, T. 1987. Peroxisomal acyl-coenzyme A oxidase multigene family of the yeast *Candida tropicalis*; nucleotide sequence of a third gene and its protein product. Gene **58**(1): 37-44.

Olaiya, A., and Sogin, S. J. 1979. Ploidy determination of *Candida albicans*. J. Bact. **140**(3): 1043-1049.

Orr-Weaver, T. L., Szostak, J. W., and Rothstein, R. J. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods in Enzymology **101**:228-245.

Osumi, M., Miwa, N., Teranishi, Y., Tanaka, A., and Fukui, S. 1974. Ultrastructure of *Candida* yeasts grown on n-alkanes. Arch. Microbiol. **99**: 181-201.

Panchal, C. J., Whitney, G. K., and Stewart, G. G. 1984. Susceptibility of *Saccharomyces* species and *Schwanniomyces* species to the aminoglycoside antibiotic G418. Appl. and Env. Microbiol. **47**: 1164-1166.

Pande, S. V., and Mead, J. F. 1968. Long chain fatty acid activation in subcellular preparations from rat liver. J. Biol. Chem. **243**(2): 352-361.

Parish, R. W. 1975. The isolation and characterisation of peroxisomes (microbodies) from bakers' yeast *Saccharomyces cerevisiae*. Arch. Microbiol. **105**: 187-192.

Phaff, H. J., Mrak, E. M., and Williams, O. B. 1952. Yeasts isolated from shrimp. Mycologia **44**: 431-451.

Poulter, R., Hanrahan, V., Jeffery, K., Markie, D., Shepherd, M. G., and Sullivan, P. A. 1982. Recombination analysis of naturally diploid *Candida albicans*. J. Bact. **152**(3): 969-975.

Poulter, R. T. M., and Rikkerink, E. H. A. 1983. Genetic analysis of red, adenine-requiring mutants of *Candida albicans*. J. Bact. **156**(3): 1066-1077.

Proctor, G. N., and Rownd, R. H. 1982. Rosanilins: Indicator dyes for chloramphenicol-resistant enterobacteria containing chloramphenicol acetyltransferase. J. Bact. **150**(3): 1375-1382.

Rachubinski, R. A., Fujiki, Y., and Lazarow, P. B. 1985. Cloning of cDNA coding for peroxisomal acyl-CoA oxidase from the yeast *Candida tropicalis* pK233. Proc. Nat. Acad. Sci. USA. **82**: 3973-3977.

Rachubinski, R. A. , Fujiki, Y., and Lazarow, P. B. 1987. Isolation of cDNA clones coding for peroxisomal proteins of *Candida tropicalis*: identification and sequence of a clone for catalase. Biochim. Biophys. Acta **909**: 35-43.

Rank, G. H. 1986. Gene interactions in *Saccharomyces cerevisiae* resulting in high level resistance to the herbicide sulphometuron methyl. Can J. Genet. Cytol. **28**: 852-855.

Rank, G. H., Casey, G., and Xiao, W. 1988. Gene transfer in industrial *Saccharomyces* yeasts. Food Biotechnology **2**(1): 1-41.

Ratzkin, B., and Carbon, J. 1977. Functional expression of cloned yeast DNA in *Escherischia coli*. Proc. Nat. Acad. Sci. USA. **74**: 503-517.

Rehm, H. J., and Reiff, I. 1981. Mechanisms and occurrence of microbial oxidation of long-chain alkanes. Adv. Biochem. Eng. **19**: 175-215.

Reipen, G., Erhart, E., Breunig, K. D., and Hollenberg, C. P. 1982. Non-selective transformation of *Saccharomyces cerevisiae*. Curr. Genet. **6**: 189-193.

Riggsby, W. S. 1985. Some recent developments in the molecular biology of medically important *Candida*. Microbiological Sciences **2**(9): 257-263.

Rothstein, R. J. 1983. One-step gene disruption in yeast. Methods in Enzymology **101**: 202-211.

Sakai, K., and Yamamoto, M. 1986. Transformation of the yeast *Saccharomyces carlsbergensis* using an antibiotic resistance marker. Agri. Biol. Chem. **50**: 1177-1182.

Scherer, S., and Davis, R. W. 1979. Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Nat. Acad. Sci. USA. **76**: 4951-4955.

Shepherd, M. G., Poulter, R. T. M., and Sullivan, P. A. 1985. *Candida albicans*: biology, genetics, and pathogenicity. Ann. Rev. Microbiol. **39**: 579-614.

Shimizu, S., Yasui, K., Tani, Y., and Yamada, H. 1979. Acyl-CoA oxidase from *Candida tropicalis*. Biochem. Biophys. Res. Commun. **91**(1): 108-113.

Singh, A., and Sherman, F. 1974. Characteristics and relationships of mercury-resistant mutants and methionine auxotrophs of yeast. J. Bact. **118**(3): 911-918.

Skoneczny, M., Chelstowska, A., and Rytka, J. 1988. Study of the coinduction by fatty acids of catalase A and acyl-CoA oxidase in standard and mutant *S. cerevisiae* strains. Eur. J. Biochem. **174**: 297-302.

Small, G. M., and Lazarow, P. B. 1987. Import of the carboxy-terminal portion of acyl-CoA oxidase into peroxisomes of *Candida tropicalis*. J. Cell. Biol. **105**: 247-250.

Snell, R. G., and Wilkins, R. J. 1986. Separation of chromosomal DNA molecules from *C. albicans* by pulsed field electrophoresis. Nucleic Acids Research **14**(11): 4401-4406.

Snow, R. 1966. An enrichment method for auxotrophic yeast mutants using the antibiotic 'nystatin.' Nature **211**: 206-207.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503-517.

Spevak, W., Hartig, A., Meindl, P., and Ruis, H. 1986. Heme control of the catalase T gene of the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **203**: 73-78.

Sreekrishna, K., Webster, T. D., and Dickson, R. C. 1984. Transformation of *Kluyveromyces lactis* with the kanamycin (G418) Resistance Gene of *Tn 903*. Gene **28**: 73-81.

Stewart, G. G. 1981. The genetic manipulation of industrial yeast strains. Can. J. Microbiol. **27**: 973-990.

Stiles, J. I. 1983. Use of integrative transformation of yeast in the cloning of mutant genes and large segments of contiguous chromosomal sequences. Methods in Enzymology **101**: 290-300.

Stinchcomb, D. T., Thomas, M., Kelly, J., Selker, E., and Davis, R. W. 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Nat. Acad. Sci. USA. **77**(8): 4559-4563.

Struhl, K., Stinchcomb, D. T., Scherer, S., and Davis, R. W. 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Nat. Acad. Sci. USA. **76**: 1035-1039.

Sturley, S. L., and Young, T. W. 1986. Genetic Manipulation of commercial yeast strains, in Biotechnology and Genetic Engineering Reviews (Russell, G. E., ed.), Vol. **4**.: 1-38, Intercept Ltd.

Sugisaki, Y., Gunge, N., Sakaguchi, K., Yamasaki, M., and Tamura, G. 1985. Transfer of DNA killer plasmids from *Kluyveromyces lactis* to *Kluyveromyces fragilis* and *Candida pseudotropicalis*. J. Bact. **164**(3): 1373-1375.

Takagi, M., Kawai, S., Chang, M. C., Shibuya, I., and Yano, K. 1986a. Construction of a host-vector system in *Candida maltosa* by using an ARS site isolated from its genome. J. Bact. **167**(2): 551-555.

Takagi, M., Kawai, S., Shibuya, I., Miyazaki, M., and Yano, K. 1986b. Cloning in *Saccharomyces cerevisiae* of a cycloheximide resistance gene from the *Candida maltosa* genome which modifies ribosomes. J. Bact. **168**(1): 417-419.

Tanaka, A., Osumi, M., and Fukui, S. 1982. Peroxisomes of alkane-grown yeast: fundamental and practical aspects. Annals N. Y. Acad. Sci. **386**: 183-199.

Tanaka, A., Yamamura, M., Kawamoto, S., and Fukui, S. 1977. Production of uricase by *Candida tropicalis* using n-alkanes as a substrate. Appl. Environ. Microbiol. **34**: 342-346.

Teranishi, Y., Tanaka, A. Osumi, M., and Fukui, S. 1974a. Catalase activities of hydrocarbon-utilizing *Candida* yeasts. Agri. Biol. Chem. **38**(6): 1213-1220.

Teranishi, Y., Kawamoto, S., Tanaka, A. Osumi, M., and Fukui, S. 1974b. Induction of catalase activity by hydrocarbons in *Candida tropicalis* pK233. Agri. Biol. Chem. **38**(6): 1221-1225.

Tikhomirova, L. P., Kryukov, V. M., Strizhov, N. I., and Bayev, A. A. 1983. mtDNA Sequences of *Candida utilis* capable of supporting autonomous replication of plasmids in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **189**: 479-484.

Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A., and Davies, R. W. 1983. Transformation by integration in *Aspergillus nidulans*. Gene **26**: 205-221.

Tolbert, N. E. 1981. Metabolic pathways in peroxisomes and glyoxysomes. Ann. Rev. Biochem. **50**: 133-157.

Tubb, R. S. 1987. Gene technology for industrial yeasts. J. Inst. Brewing **93**: 91-96.

Uchio, R., and Shiio, I. 1972. Tetradecane-1,14-dicarboxylic acid production from hexadecane by *Candida cloacae*. Agric. Biol. Chem. **36**: 1389-1397.

Ueda, M., Tanaka, A., and Fukui, S. 1984. Characterisation of peroxisomal and mitochondrial carnitine acetyltransferases purified from alkane-grown *Candida tropicalis*. Eur. J. Biochem. **138**: 445-449.

Ueda, M., Yamanoi, T., Morikawa, T., Okada, H., and Tanaka, A. 1985. Peroxisomal localization of enzymes related to fatty acid β-oxidation in an n-alkane-grown yeast, *Candida tropicalis*. Agric. Biol. Chem. **49**(6): 1821-1828.

Urban-Grimal, D., and labbe-Bois, R. 1981. Genetic and biochemical characterisation of mutants of *Saccharomyces cerevisiae* blocked in six different steps of heme biosynthesis. Mol. Gen. Genet. **183**: 85-92.

Vallin, C., and Ferenczy, L. 1977. Diploid formation of *Candida tropicalis* via protoplast fusion. Acta Microbiol. Acad. Sci. Hung. **25**: 209-212.

Van Veldhoven, P. P., Just, W. W., and Mannaerts, G. P. 1987. Permeability of the peroxisomal membrane to cofactors of β-oxidation. J. Biol. Chem. **262**(9): 4310-4318.

Vasey, R. B., and Powell, K. A. 1984. Single-cell protein, in Biotechnology and Genetic Engineering Reviews (Russell, G. E., ed.), Vol 2: 285-312, Intercept Ltd.

Veenhuis, M., Mateblowski, M., Kunau, W. H., and Harder, W. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae*. Yeast **3**: 77-84.

Vondrejs, V. 1987. A killer system in yeasts: applications to genetics and industry. Microbiological Sciences **4**: 313-316.

Wanders, R. J. A., van Roermund, C. W. T., van Wijland, M. J. A., Schutgers, R. B. H., van der Bosch, H., Schram, A. W., and Tager, J. M. 1988. Direct demonstration that the deficient oxidation of very long chain fatty acids in X-linked adrenoleukodystrophy is due to an impaired ability of peroxisomes to activate very long chain fatty acids. Biochem. Biophys. Res. Comm. **153**(2): 618-624.

Webster, T. D., and Dickson, R. C. 1983. Direct Selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with a DNA vector carrying the kanamycin-resistance gene of *Tn903*. Gene **26**: 243-252.

Welch, J. W., Fogel, S., Cathala, G., and Karin, M. 1983. Industrial yeasts display tandem gene iteration at the *CUP1* region. Mol. Cell. Biol. **3**(8): 1353-1361.

Whelan, W. L., and Magee, P. T. 1981. Natural heterozygosity in *Candida albicans*. J. Bact. **145**: 896-903.

Whelan, W. L., Markie, D. M., Simpkin, K. G., and Poulter, R. M. 1985. Instability of *Candida albicans* hybrids. J. Bact. **161**(3): 1131-1136.

Winston, F., Chumley, F., and Fink, G. R. 1983. Eviction and transplacement of mutant genes in yeast. Methods in Enzymology **101**: 211-227.

Wolcott, J. H., and Ross, C. 1966. Orotidine-5'-phosphate decarboxylase from higher plants. Biochim. Biophys. Acta **122**: 532-534

Woods, D. 1984. Oligonucleotide screening of cDNA libraries. Focus **6**(3): 1-3.

Woods, R. A., Sanders, H. K., Briquet, M., Foury, F., Drysdale, B-E., and Mattoon, R. 1975. Regulation of mitochondrial biogenesis: enzymatic changes in cytochrome-deficient yeast mutants requiring δ-aminolevulinic acid. J. Biol. Chem. **250**(23): 9090-9098.

Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Analytical Biochemistry **118**: 197-203.

Yadav, N., McDevitt, R. E., Benard, S., and Falco, S. C. 1986. Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc. Nat. Acad. Sci. USA. **83**: 4418-4422.

Yamada, T., Nowa, H., Kawamoto, S., Tanaka, A., and Fukui, S. 1980. Subcellular localization of long-chain alcohol dehydrogenase and aldehyde dehydrogenase in n-alkane-grown *Candida tropicalis*. Arch. Microbiol. **128**: 145-151.

Yamada, Y., Tani, Y., and Kamihara, T. 1984. Production of extracellular glutathione by *Candida tropicalis* pK233. J. Gen. Microbiol. **130**(12): 3275-3278.

Yanish-Perron, C., Vieira, C., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**: 103-119.

Yano, K., Kawamura, M., and Takagi, M. 1981. Enrichment of n-alkane assimilation deficient mutants of *Candida* yeast by synergistic effect of nystatin and pyrrolnitrin. Agric. Biol. Chem. **45**(4): 1017-1018.

Yi, Z-H., and Rehm, H-J. 1982a. Metabolic formation of dodecanedioic acid from n-dodecane by a mutant of *Candida tropicalis*. Eur. J. Appl. Microbiol. Biotechnol. **14**: 254-258.

Yi, Z-H., and Rehm, H-J. 1982b. Degradation pathways from n-tridecane to α,ω-tridecanedioic acid in a mutant of *Candida tropicalis*. Eur. J. Appl. Microbiol. Biotechnol. **15**: 144-146.

Yi, Z-H., and Rehm, H-J. 1982c. A new metabolic pathway from n-dodecane to α,ω-dodecanedioic acid in a mutant of *Candida tropicalis*. Eur. J. Appl. Microbiol. Biotechnol. **15**: 175-179.

Yi, Z-H., and Rehm, H-J. 1988. Formation and degradation of Δ^9-1, 18-octadecenedioic acid from oleic acid by *Candida tropicalis*. Appl. Microbiol. Biotechnol. **28**(6): 520-526.

Yocum, R. R. 1986. Genetic engineering of industrial yeasts in Proceedings Bio. Expo. '86, Butterworth, Stoneham MA, pp. 171-180.

Young, J. D., Gorman, J. W., Gorman, J. A., and Bock, R. M. 1976. Indirect selection for auxotrophic mutants of *Saccharomyces cerevisiae* using the antibiotic netropsin. Mutation Research **35**: 423-428.

Young, T. W. 1981. The genetic manipulation of killer character into brewing yeast. J. of the Inst. Brewing **87**: 292-295.

Young, T. W. 1986. Genetical improvement of brewers yeast - fact or fantasy? In Brewers Yeast Genetics. Proceedings of the Second Aviemore Conference on Malting, Brewing, and Distiling (Priest, F. G., and Campbell, I., eds.), pp. 107-122, Institute of Brewing, London.

Yu, Z. H., and Hao, X. Z. 1986. Formation and characterization of chain diacyl-CoA synthetase from *Candida tropicalis*. Acta Microbiol. Sin. **26**(4): 333-340.

Zeff, R. A., and Geliebter, J. 1987. Oligonucleotide probes for genomic DNA blots. Focus **9**(2): 1-2.

Zhang, Y. Z., and Reddy, C. A. 1986. Cloning of a Candida utilis gene which complements *leu2* mutation in *Saccharomyces cerevisiae*. Current Genetics **10**: 573-578.

Zhu, J., Contreras, R., and Fiers, W. 1986. Construction of stable laboratory and industrial yeast strains expressing a foreign gene by integrative transformation using a dominant selection system. Gene **50**: 225-237.

Zhu, J. Contreras, R., Gheysen, D., Ernst, J., and Fiers, W. 1985. A system for dominant transformation and plasmid amplification in *Saccharomyces cerevisiae*. Biotechnology **3**: 451-456.

APPENDIX

SIZES OF RESTRICTION DIGEST FRAGMENTS USED AS MARKERS

λ Eco RI/*Hin* dIII digest	pBR322 *Hin* fI digest
21.70 kb	1632 bp
9.46 kb	517 bp
6.47 kb	506 bp
5.24 kb	396 bp
5.05 kb	344 bp
4.21 kb	298 bp
3.41 kb	221 bp
2.69 kb	220 bp
2.25 kb	154 bp
1.98 kb	75 bp
1.90 kb	
1.57 kb	
1.32 kb	
0.93 kb	
0.84 kb	
0.58 kb	
0.14 kb	