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GENETIC AND BIOCHEMICAL STUDIES
OF MITOCHONDRIA IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Summary

Previous studies have shown that mitochondrial petites of the yeast Saccharomyces cerevisiae appear to have some advantage over their wild type counterparts in terms of fermentative performance.

In this thesis evidence is presented that inhibition of the respiratory chain by chemical or genetic means leads to an increase in ethanol formed per unit sugar and a decrease in biomass per unit sugar. The genetic studies involve a mutation in formation of subunit III of the cytochrome c oxidase complex (pet122) and a mutation in putative mitochondrial pyruvate transport (tpyl). The chemical study involves inhibition of mitochondrial pyruvate transport by a specific inhibitor alpha-cyano-4-hydroxycinnamic acid.

To try and discover more of the nature of the tpyl mutation a gene was cloned from a yeast centromeric genomic library which complemented the mutation. The insert containing the complementing gene was reduced in size and sequenced. An open reading frame containing 1698 nucleotides coding for 566 amino acids was discovered. After comparison to databases the gene appeared to be undiscovered to date. No similarity was found between the translated protein and others on databases with the exception of the FAS2 gene of Penicillium patulum.

Studies of isolated mitochondria and cell growth pattern, of the tpyl mutant, revealed data typical of a classical petite mutation rather than a mutation in a regulatory gene as described by the original paper describing the mutant.

Work presented in this thesis has been included, or is to be included in the following publications;

Hill J, Donald K.A.G. and Griffiths D.E., DMSO-enhanced whole cell yeast transformation, (1991). Nucl. Acid Res., 19, 5791

Donald K.A.G., Hill J and Griffiths D.E., Cloning and Sequencing of a gene complementing the petite like mutation tpy1 in Saccharomyces cerevisiae, in preparation

The DNA sequence of the TPY1 gene has been submitted to the EMBL DNA database. The accession number is X62430.

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LIST OF ABBREVIATIONS.

A	adenine
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CCCP	m-chloro carbonyl cyanide phenylhydrazone
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide triphosphates
DSMP	2-(dimethylaminostyryl)-1-methylpyridinium
DTI	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide (oxidised)
FADH ₂	flavin adenine dinucleotide (reduced)
G	guanine
GIP	general insertion protein
GTP	guanosine triphosphate
IPTG	isopropyl-Beta-D-thio-galactoside
kb	kilo bases
kDa	kilo Dalton
mA	milliampere
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NAD ⁺	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
OD	optical density
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
SDS	sodium dodecylsulphate
SDW	sterile distilled water
T	thymine
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2'-amino-2(hydroxymethyl)-1,3-propane diol
Triton	polyoxyethylene p-t-octyl phenol
tRNA	transfer ribonucleic acid
U	unit

UTP	uridine triphosphate
U.V.	ultraviolet
V	volts
v/v	volume/volume
w/v	weight/volume
X-GAL	5-bromo-4-chloro-3-indolyl-Beta-galactoside

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Work reported in this thesis is my own except where I have acknowledged other peoples work.

INTRODUCTION

Many yeasts are facultative anaerobes, that is as well as having the ability to respire in the presence of oxygen they are also able to survive in its absence. In the presence of oxygen yeasts can oxidise sugars to carbon dioxide and water and in its absence some yeasts can ferment sugars to carbon dioxide and ethanol.

Saccharomyces cerevisiae is a yeast which has been used for centuries in the processes of baking (aerobic respiration) and alcoholic fermentation (anaerobic respiration) with little knowledge of biochemical principles involved. However over the last century studies have revealed much of this yeast's complex machinery. During the last twenty years or so S.cerevisiae has become an important tool in genetic studies and is perhaps second only to Escherichia coli in this field.

1.1 The Biochemistry of Yeast Fermentation

In order to utilize the energy contained in sugars external to S.cerevisiae the sugars must be either digested externally and then be imported or they may be imported directly. Either way once inside the cell the energy the sugars contain is released slowly in a stepwise process. The metabolic pathway from the entry of the sugar to the formation of pyruvate is known as glycolysis. In

S.cerevisiae glucose, fructose and galactose are taken up by facilitated diffusion (Heredia et al 1968) whereas maltose is imported by proton co-transport (Serrano 1977) and sucrose is digested extracellularly to glucose and fructose. The principle sugars occurring in brewers wort are maltose (approximately 6%) and glucose (approximately 1%) (Lancashire personal communication). Once inside the cell the sugar is broken down to a monosaccharide, if it is not already one, and phosphorylated by the appropriate kinase at the expense of ATP.

Glucose 6-phosphate is converted to fructose 6-phosphate by the action of phosphoglucose isomerase. Another phosphate group is then added by one of the key enzymes of glycolysis phosphofructokinase (PFK) and fructose 1,6-biphosphate is produced. PFK is a potential candidate for the rate limiting enzyme of glycolysis being inhibited by high levels of ATP and activated by phosphate ions and AMP. In other words when the cell is producing a lot of energy the glycolytic flux is slowed down but when it needs energy the glycolytic flux is increased. However it has been demonstrated that an increase in the level of PFK does not lead to an increase in the level of ethanol produced (Hainisch et al 1986). Although this may rule out PFK as the main site of regulation of glycolysis in yeast, studies have shown concentration dependent kinetic behaviour with respect to ATP (Aragon and Sanchez, 1985)

Following the production of fructose 1,6-biphosphate there

are several further reactions which end with the conversion of phosphoenolpyruvate to pyruvate using the enzyme pyruvate kinase (See figure 1.1.1). This step is another controlling one which is activated by fructose 1,6-biphosphate thus allowing the enzyme to keep up with the pace of the glycolytic flux.

The result of glycolysis is that two molecules of pyruvate are formed from one molecule of glucose with a net gain of two molecules of ATP.

In yeast there are two main routes for the utilization of pyruvate. The first route for pyruvate is for it to enter the mitochondria and join the TCA cycle (described later) and the second is for it to be converted to ethanol. The path that is taken depends on the prevailing conditions and the yeast species.

Ethanol production from pyruvate depends on the presence of two enzymes pyruvate decarboxylase and alcohol dehydrogenase. The first step is the removal of carbon dioxide from pyruvate to form acetaldehyde. Secondly, the acetaldehyde is acted on by alcohol dehydrogenase to form ethanol.

Of the two pathways available the one that produces more energy is the mitochondrial pathway which gives rise to 36 mol ATP/mol glucose against 2 mol ATP/mol glucose produced during fermentation. So, as would be expected, some yeasts will preferentially convert glucose all the way to carbon dioxide and water in the presence of oxygen, and if the

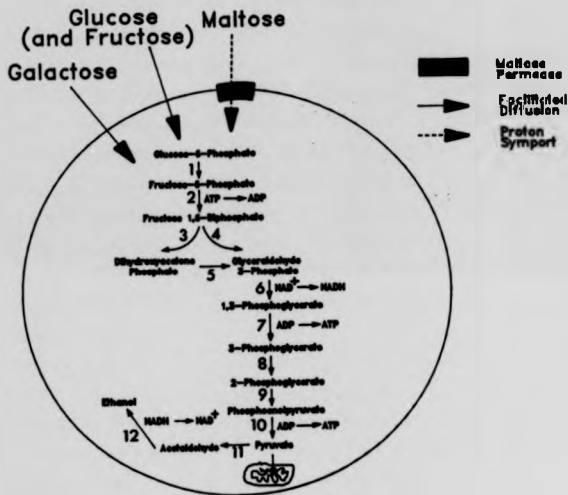
Figure 1.1.1

Transport of sugars and the glycolytic path in yeast. The transport of the major sugars utilized by *S.cerevisiae* is shown and their possible fates once inside the cell. The enzymes catalysing the various steps are as follows;

- 1) Phosphoglucose isomerase (R),
- 2) Phosphofructokinase,
- 3 and 4) Aldose,
- 5) Triose phosphate isomerase (R),
- 6) Glyceraldehyde 3-phosphate Dehydrogenase(R),
- 7) Phosphoglycerate kinase (R),
- 8) Phosphoglyceromutase (R),
- 9) Enolase (R),
- 10) Pyruvate kinase,
- 11) Pyruvate decarboxylase,
- 12) Alcohol dehydrogenase (R)

Enzymes that catalyse reversible reactions are marked R

Fate of Principal Sugars in Brewing Yeast Cell



yeast had been previously fermenting then the introduction of air into the system will cause the yeast to oxidise the glucose. This is known as the 'Pasteur effect'. This describes the control of the rate of glycolysis by oxygen and states that the rate of glycolysis in yeast is 7 times faster in the absence of oxygen than in its presence. However this is not the case in S.cerevisiae. The main catabolic route, in the presence of glucose, is fermentation even during aerobic growth. This is known as the "glucose effect". The mechanism of the effect is still unclear. It does not appear to be the same as the situation in bacteria where glucose phosphorylation causes a drop in the level of cyclic AMP, which is required for transcription of the genes affected (Gottschalk et al 1979). However the effect seems to be restricted to glucose and does not occur to the same extent in the brewing process as the main constituent is maltose.

1.2 Principles of Brewing

The basics of beer brewing have changed little over the last few centuries. Barley is allowed to undergo limited germination or "malting" during which time the starch contained in the kernels is hydrolysed by beta-amylase to maltose. Further degradation of starch comes about when the malt is dried, cooked and placed in water at 60-70°C. This process of "mashing" produces mainly maltose along with small oligosaccharides and dextrins. The soluble extract

obtained from mashing, called wort, is cooled to a temperature suitable for yeast fermentation and inoculated with brewers yeast. Fermentation is allowed to continue for 5-7 days during which time the ale type yeast S.cerevisiae tends to rise to the surface.

During this type of so called batch fermentation 5% of the available sugar may be used for cell growth and maintenance. This is a considerable part of the energy available to yeast cells which is ultimately lost or used for animal feed.

Over the last century attempts have been made to improve the performance of industrial yeasts by a variety of means. The more traditional methods employed are hybridisation and non-specific mutagenesis while modern methods are protoplast fusion and genetic transformation.

1.3 Methods of Improving Industrial Yeasts

1.3.1 Hybridisation

During the period 1934-1935 Satava working in Czechoslovakia and Winge in Denmark discovered the nature of the sexual cycle of Saccharomyces (Phaff et al 1978). Their studies led to the idea that an alteration of generations occurs in Saccharomyces ie the vegetative cells are diploid where as the ascospores are haploid. Winge and his co-workers developed a micro-manipulator which could be used to dissect apart the four ascii and then showed that these could be crossed to form a diploid cell. Further work by Lindgren in the USA showed that heterothallism exists in Saccharomyces

ie ascospores are one of two types referred to as a and alpha. The consequences of this work for brewers was that they could now take two haploid yeasts, each with a desirable quality, hybridise them and use the resultant diploid for brewing.

An example of the use of hybridisation in brewing technology was reported by Tubb et al in 1981. The authors introduced the ability to utilize dextrans into a brewing strain of S.cerevisiae by hybridisation of this strain with another that produced the enzyme dextrinase. However their work also showed the disadvantage of this type of improvement in that the hybridisation also introduced an undesirable change which in this case was a phenolic-off flavour.

Hybridisation in brewing yeasts is made difficult by the tendency of these strains to be polyploid or even aneuploid. As a consequence of this these strains do not have a mating type, have a low degree of sporulation and poor spore viability. These factors make genetic analysis difficult. However this level of ploidy protects brewing strains from potentially harmful mutations and thus offers the brewer a consistent strain of yeast to work with.

1.3.2 Mutagenesis

Non-specific mutagenesis can be used to produce novel strains of yeast albeit in rather a random manner. Mutagens employed are either radiation (ultra-violet light or x-rays) or chemicals (usually ethylmethane sulphonate,

nitrosoguanidine or diethyl sulphate). An example of the use of mutagenesis is the work of Jones and co-workers (1987). This group reported the isolation of a glucose derepression mutant in a brewing strain of yeast. The mutant was insensitive to the non-metabolizable but repressing analogue of glucose, 2-deoxyglucose. By avoiding glucose repression of other sugar transporters the mutant strain was able to utilize a range of sugars in the presence of glucose. The disadvantage of this type of mutagenesis is its non-specificity is more than one mutation may have been induced. This can be avoided by directing the mutagenesis, which will be discussed later.

1.3.3 Spheroplast Fusion

Spheroplast fusion of yeasts was first demonstrated by Van Solingen and Van Der Platt in 1977. Since then many workers have used this technique to fuse yeast cells together. It has been found that it is not only possible to fuse together yeast of the same genus (intergeneric fusion) but it is also possible to fuse yeast of different genera (intragenic fusion).

Spheroplast fusion is basically a type of hybridisation except in this case there is no need for the yeasts to be of opposite mating types or to be haploid. The procedure for fusion is to remove the cell wall enzymatically and then fuse the spheroplasts together in the presence of polyethylene glycol and calcium chloride.

An example of intergeneric fusion is demonstrated by the work of Russell and Stewart in 1979. The authors fused together a polyploid brewing strain of yeast, that was non-flocculant, with a flocculant haploid yeast. The resultant fusant was able to ferment maltose and was flocculant. Another important consequence of the fusion was that the yeast sporulated well unlike its polyploid parent thus making genetic analysis possible. Unfortunately the fusion yeast produced an unpalatable drink with a heavy phenolic off flavour. This demonstrates the limitations of spheroplast fusion namely that undesirable characteristics can be introduced during fusion.

An example of intragenetic fusion is demonstrated by the work of Farahnak et al in 1986. The authors were trying to combine the characteristics of two different genera of yeast. They wanted the fermentative power of S.cerevisiae combined with the ability of Kluveromyces fragilis to ferment lactose. Although K.fragilis is able to ferment lactose only a fraction of the available lactose is used. A hybrid produced from the two genera may be able to utilize a greater fraction of the lactose available than K.fragilis alone. This would have important consequences for industry since whey, the source of lactose, is at best wasted and can be a serious pollutant. The authors found that their fusion yeast produced 30% more ethanol than the K.fragilis parent during growth on lactose. This appeared to be due to an increase in ethanol tolerance rather than the rate of ethanol

production. Although the fusant appeared stable the long term stability was in doubt.

1.3.4 Transformation

Genetic transformation is now probably the most widely used method for improving industrial yeasts. S.cerevisiae was first shown to be transformable by Hinnen and co-workers (1978) who showed that leucine dependent cells could become independent of leucine when treated with wild type DNA in the presence of calcium chloride. Soon after this discovery, Beggs (1978) demonstrated that it was possible to swap vectors between E.coli and S.cerevisiae by creating a chimeric plasmid called a shuttle vector. The vector was able to exist in both types of cell because it contained both bacterial and yeast origins of replication.

With the ability to introduce DNA into yeast came the opportunity to change the genetic make-up of industrial organisms in a way that had never previously been possible. There are two basic ways in which the genetic make-up of a yeast cell can be changed using a plasmid. Firstly it is possible to introduce a plasmid borne gene into a cell which will autonomously replicate. This requires the presence of a chromosomal autonomously replicating sequence (ARS) or part of the native 2 μ m plasmid containing the origin of replication. Secondly it is possible to integrate DNA into the genome of yeast using an integrating plasmid or just linear DNA.

1.3.4.1 Autonomously Replicating Plasmids

ARS plasmids transform at a high efficiency but are not very stable. If the selection pressure that was used to isolate them in the first place is withdrawn they are easily lost. However stability can be introduced by the use of a plasmid containing a gene such as the CEN4 chromosomal centromeric gene from chromosome IV of S.cerevisiae. Without the centromeric sequences ARS plasmids exist at a high copy number per cell however with the sequence they generally exist at one or two copies per cell.

A practical application of these genetics techniques is demonstrated by the work of Hollenberg and Strasser (1990). In both brewing and baking there are polysaccharides that S.cerevisiae cannot utilize directly by itself. Instead expensive enzymes must be added to produce glucose. To avoid this situation the authors isolated two genes from Schwanniomyces occidentalis which together completely degrade starch to glucose. The genes were attached to galactose promoters and transformed into a laboratory strain of S.cerevisiae using a centromeric shuttle vector. The expression of the genes was found to be inducible by the presence of galactose and the extracellular activity of the enzymes was comparable with that of wild type Sch. occidentalis.

1.3.4.2 Integrating Plasmids

Lactose fermentation, as mentioned earlier, is an area in

which there is a great deal of commercial interest. The protoplast fusion method of combining lactose fermentation with ethanol tolerance and a high rate of ethanol production could be improved upon by taking just the characteristics wanted from a Kluveromyces yeast and adding them to S.cerevisiae. This was accomplished by Jeong and co-workers (1991). Two types of plasmid were constructed both containing lactose utilization genes from K.lactis. The first type of plasmid was an integrating one containing no yeast origin of replication. This means transformants can only occur by integration into the genome. The second type was a normal ARS plasmid. The integrating plasmid is far more stable than an ARS plasmid but transforms at a much lower efficiency. Both plasmids successfully transformed a haploid strain of S.cerevisiae into a lactose utilizing strain. The integrated plasmid produced a strain able to ferment lactose at a faster rate than the strain transformed with an ARS plasmid, probably due to the stability of the integrated strain.

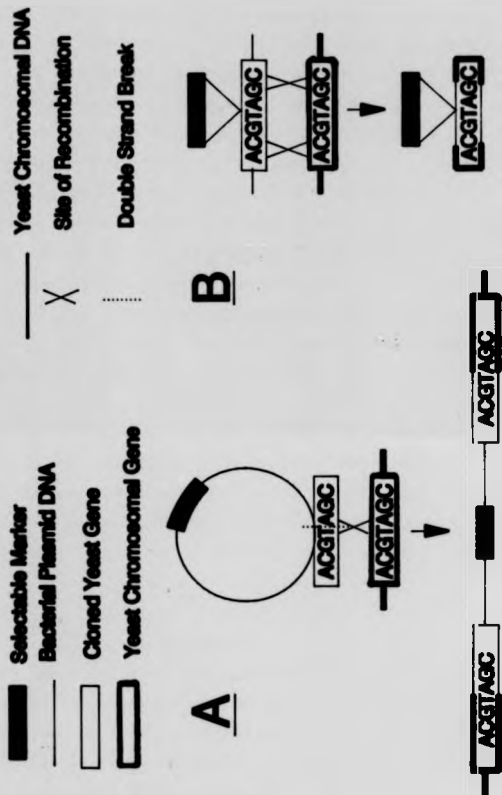
1.3.4.3 Gene Disruption

Apart from introducing foreign genes into Saccharomyces yeasts, transformation can also be used to delete genes from the genome by gene disruption (for a summary of this and another integration event see figure 1.3.1). This technique was used by Gjermansen et al (1991) to produce a diacetyl-less lager. Diacetyl is formed from acetolactate by a non-

Figure 1.3.1

Examples of two types of integration into the yeast genome. A) In this case an integrating vector cut in the gene of interest recombines with an homologous area in the yeast genome. The result is the integration of the whole plasmid. B) In this case the selectable marker is contained within the gene of interest. A fragment containing the selectable marker and ideally about 250 bp of the gene of interest is used to transform yeast (Based on Ausubel et al 1987).

Integrative Transformation in Yeast



enzymatic oxidative decarboxylation. Acetolactate is formed by the action of the enzyme acetohydroxy acid synthase encoded by the ILV2 gene. Diacetyl produces an off-flavour in lager and normally an expensive and time consuming lagering period is required to get rid of it. However if the enzyme responsible for production of acetolactate could be genetically removed then no diacetyl would be formed. The authors used a dominant marker G418 resistance present on an integrating plasmid, along with a mutated form of the gene, to produce mutations in three of the four wild type genes. However deletions of the fourth gene proved impossible as this condition was lethal. Subsequent examination showed that Saccharomyces cerevisiae has a low uptake rate for isoleucine and valine. Acetohydroxy acid synthetase is part of the biosynthetic pathway for these amino acids. Therefore deleting the gene coding for this enzyme leaves the cell short of essential amino acids. However the authors were able to isolate ilv auxotrophic strains by sporulation and plating on media with large amounts of isoleucine and valine.

1.4

Mitochondria

Mitochondria are made up of two membrane components, inner and outer, and two aqueous components, the intermembrane space and the matrix. These organelles contain the apparatus needed to oxidise sugars completely to water and carbon dioxide. They also provide building blocks for amino acid

synthesis and for gluconeogenesis.

There is strong evidence to suggest that mitochondria were derived from free living organisms as they contain their own DNA and protein synthesizing apparatus. However the mitochondrial genome contains few of its original genes and most proteins are now nuclear encoded and are imported into the mitochondria. S.cerevisiae mitochondrial DNA (mtDNA) codes for 2 rRNAs, 25 tRNAs, 6 known inner membrane proteins and 10 more unassigned reading frames or URFs. (Bowman 1989) The mitochondria of yeasts perform many tasks and probably contribute to others as yet unknown, but the two major functions they perform are the generation of ATP via the TCA cycle and oxidative phosphorylation and the oxidation of fatty acids.

The generation of ATP can be divided into two distinct sectors the tri-carboxylic (TCA) cycle or Krebs cycle and oxidative phosphorylation

1.4.1 TCA Cycle

The route by which pyruvate enters the mitochondria was thought to be via a specific monocarboxylate carrier (Papa et al 1971). This was supported by work done using an inhibitor that specifically blocked mitochondrial pyruvate transport (Halestrap and Denton 1974).

The link between glycolysis and the TCA cycle is the oxidative decarboxylation of pyruvate to form acetyl Co-A which occurs in the mitochondrial matrix. This is

irreversibly catalysed by the enzyme complex pyruvate dehydrogenase. The fact that this reaction takes place via an enzyme complex suggests a complex route, and this is indeed the case. There are four steps in the conversion of pyruvate to acetyl Co-A.

Once formed the acetyl Co-A condenses with an important intermediate of the TCA cycle, oxaloacetate. This is a key intermediate because as well as being the 'starting point' of the TCA cycle it is also required for gluconeogenesis, the formation of glucose which is carried out at times of excess energy levels. Oxaloacetate condenses with acetyl Co-A to form citrate in a reaction catalysed by the enzyme citrate synthetase. There are several further steps described in figure 1.4.1 which finally lead to the regeneration of oxaloacetate. In the course of the cycle the two carbons gained from acetyl Co-A are lost as carbon dioxide and five hydrogens are lost as three NADH and one FADH₂.

The TCA cycle is constantly being depleted of intermediates for biosynthetic purposes and these must be replaced by anaplerotic reactions. Two main anaplerotic pathways exist in yeast. The first is catalysed by pyruvate carboxylase which at times of high levels of acetyl Co-A produces oxaloacetate from pyruvate. The second pathway is the glyoxylate cycle which provides succinate and malate from acetyl Co-A.

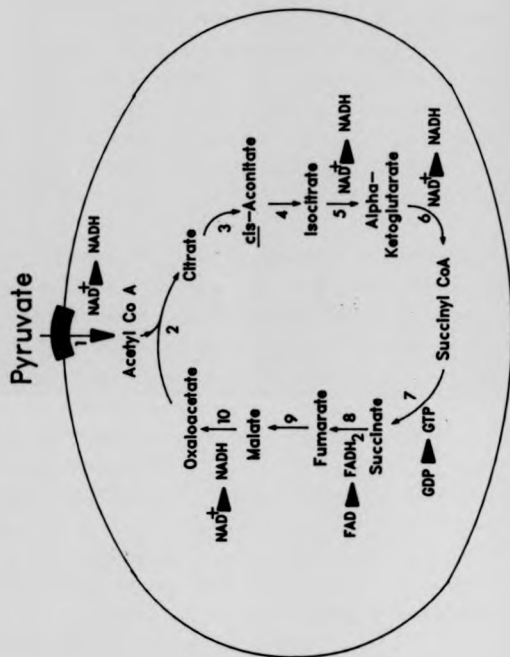
Figure 1.4.1

TCA cycle in mitochondria showing generation of NADH and FADH. The Dark band at the edge of the mitochondria represents the monocarboxylate (pyruvate) transporter. The enzymes and enzyme complexes catalysing the various reactions of the cycle are as follows;

- 1) Pyruvate dehydrogenase complex,
- 2) Citrate synthase,
- 3+4) Aconitase (R),
- 5) Isocitrate dehydrogenase (R),
- 6) Alpha-Ketoglutarate dehydrogenase complex (R),
- 7) Succinyl CoA synthetase (R),
- 8) Succinate dehydrogenase (R),
- 9) Fumarase (R),
- 10) Malate Dehydrogenase (R).

Enzymes catalysing reversible reactions are marked R

TCA (Krebs) Cycle in Mitochondria



1.4.2 Oxidative Phosphorylation

The NADH and FADH_2 formed during the oxidation of glucose are energy rich molecules each containing a pair of electrons with a high transfer potential. When these are donated to molecular oxygen a large amount of free energy is liberated which is used to generate ATP. This is the major source of ATP in the yeast cell providing 32 of the 36 molecules of ATP formed during the oxidation of a molecule of glucose.

The outer membrane is freely permeable to most small molecules as it is transversed by many porin molecules. These are proteins with a large pore that allows the passage of most small molecules. However the inner membrane is not so permeable and specific carriers such as the monocarboxylate and dicarboxylate carriers are required. The inner membrane also contains the apparatus needed for oxidative phosphorylation.

The molecules NADH and FADH_2 donate their energy by passing electrons down a chain of proteins and protein complexes known as the respiratory chain. The respiratory chain consists of three enzyme complexes linked by two mobile electron carrier (Figure 1.4.2). The pathway is as follows;

- 1) NADH reduces NADH-Q reductase (complex I)
- 2) NADH-Q reductase reduces ubiquinone, a mobile hydrophobic carrier
- 3) Reduced ubiquinone reduces cytochrome reductase (Complex III)

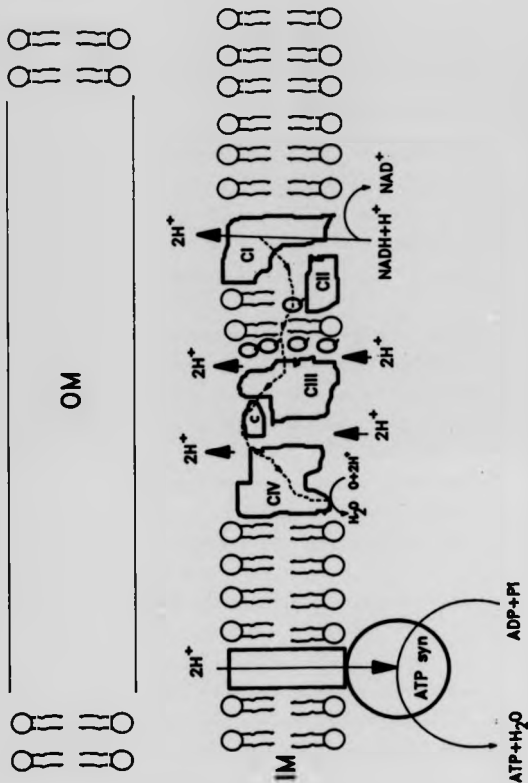
Figure 1.4.2

Diagrammatic representation of the respiratory chain showing the generation of a proton gradient by the passage of electrons and subsequent generation of ATP. The components of the respiratory chain are represented as follows;

- CI) complex 1, NADH-Q reductase,
- Q) ubiquinone,
- CII) complex 2, succinate-Q reductase,
- CIII) complex 3, cytochrome reductase,
- c) cytochrome c,
- CIV) complex 4, cytochrome c oxidase.

ATP synthetase is represented as ATP syn.

The Respiratory Chain of Mitochondria



- 4) Cytochrome reductase reduces cytochrome c
- 5) Cytochrome c reduces cytochrome oxidase (Complex IV).
- 6) Electrons from FADH_2 are passed into the respiratory chain via succinate-Q reductase complex (Complex II).

This complex does not act as a proton pump and hence only two molecules of ATP are formed per molecule of FADH_2 .

In yeast NADH external to the mitochondria is utilized with the help of an externally located NADH-ubiquinone oxidoreductase, thus eliminating the need for complex shuttle systems.

The passage of electrons is coupled to ATP synthesis by an intermediate factor. This factor has been the subject of long and heated debate, however most workers agree that as a base the Chemiosmotic Theory is the most likely explanation of events. This theory was proposed by Peter Mitchell in 1961 (Mitchell 1961). He suggested that electron transport and ATP synthesis were coupled by a proton gradient across the inner membrane of the mitochondria. Evidence for this theory has built up over the years:

- 1) ATP is synthesised when a pH gradient is imposed on the mitochondria in the absence of electron transport.
- 2) Synthetic vesicles containing bacteriorhodopsin (which pumps protons when illuminated) and mitochondrial ATPase was able to synthesise ATP (Racker and Stoekenius 1974).
- 3) Solubilized mitochondrial preparations do not synthesise ATP.
- 4) Substances that are known to carry protons from high

concentrations to low, stop the synthesis of ATP, presumably by dissipating the membrane potential.

There are discrepancies in the theory such as accounting for ATP synthesis in some halophilic bacteria (Halgeron 1983) which have a very small membrane potential. Thus there is no absolute theory for the coupling of electron transport to ATP synthesis and it may be some time yet before the full story is known.

1.4.3 Oxidation of Fatty Acids

Kennedy and Lehninger showed in 1949 (Stryer 1988) that fatty acids are oxidised in the mitochondria. The fatty acid is linked to coenzyme A by the enzyme acyl Co A synthetase at the expense of ATP. If the acyl chain of the fatty acid is long then the inner membrane is not easily transversed. In order for these molecules to get across the membrane a specific transporter, carnitine, is needed. This molecule releases the coenzyme A and allows the passage of the newly formed acyl carnitine through the inner membrane via a translocase. Once on the matrix side the acyl group is transferred to coenzyme A again and the oxidation of the acyl chain can begin.

The chain is shortened by two carbon units each time it goes round the so-called beta-oxidation chain. This oxidation releases FADH_2 and NADH which can then transfer their high energy electrons to the electron transport chain and thus generate ATP.

1.4.4 Petites

Some yeasts including all strains of Saccharomyces are able to survive without the presence of viable mitochondria. In other words they derive enough energy from fermentation to be able to survive on a substrate such as glucose. Such yeasts are termed petites because their growth on glucose agar is distinctive in that they produce smaller colonies than the wild type grandes. The non-functioning mitochondria can arise from a mutation in the mitochondrial genome (rho⁰, rho⁻, or mit⁻) or a deletion in a nuclear gene that is targeted to the mitochondria (pat mutant).

Petites occur naturally in a population of yeast at 1-2% but can arise from treatment with mutagenizing reagents such as ethidium bromide, acriflavine and ethyl methane sulphonate. Examination of mtDNA from some spontaneous petite mutants (Bernadi et al 1968) revealed a gross alteration in the GC content from its former level of 18% to 4%. The reason for this change became clear when it was revealed that the mechanism by which the mutation had occurred was excision of an AT spacer. These areas rich in AT bases multiplied and eventually became the petite genome so that there are no, or few, coding regions left in the genome.

Prolonged treatment with ethidium bromide can remove all of the mtDNA, these are the rho⁰ yeasts mentioned earlier with no functional mtDNA. If there is some coding region left the yeast is termed a rho⁻. If there is a mutation in a single gene then the yeast is termed mit⁻.

The pet mutants are mutations in nuclear genes which affect, directly or indirectly, the functioning of the mitochondria. A recent review by Tzagoloff and Dieckman (1991) claimed to represent all PET genes present in the yeast genome. Some pet mutations are straightforward, in that the genes they are mutated in, code for a protein directly involved in respiration. For instance the PET9 or OP1 gene codes for the ADP/ATP translocator in yeast (O'Malley et al 1982). A mutation in this gene leads to an absence of ADP essential for ATP synthesis and hence mutants are unable to grow on a non-fermentable media. Other mutations are rather more convoluted. Such is the case of the PET122 gene (McKevan et al 1988) which codes for a protein necessary for the translation of a single mitochondrial mRNA that encodes subunit III of the cytochrome c oxidase complex. Although this gene does not code for a protein used in respiratory function it does code for a protein necessary for translation of a respiratory chain subunit mRNA.

1.5 Genetic Control of the Nucleus Over the Mitochondria

The vast majority of mitochondrial proteins are nuclear encoded. How is it then that these proteins reach their destination? Workers in the early eighties discovered that amino terminal sequences of nuclear encoded mitochondrial proteins determine their ultimate destination. Recently an exception has been discovered in the shape of the ATP/ADP translocator. This protein possesses targeting sequences

within the mature protein (Pfanner et al 1987). Most mitochondrial import studies over the last decade have been carried out on Neurospora crassa and yeast.

Many imported mitochondrial proteins are synthesised with amino terminal presequences of 20-80 amino acid residues. These presequences carry targeting information to direct proteins into mitochondria and are made of mainly positively charged basic residues with few acidic residues.

Specific receptors exist on the outer membrane of the mitochondria which recognise the target sequence. Two receptors have been identified to date. MOM19 (Sollner et al 1989) is the receptor for proteins with an amino terminal presequence and MOM72 (Sollner et al 1990) is the receptor for the ATP/ADP translocator. The equivalent proteins in yeast are probably MOM17 (Kiebler et al 1990) and MAS70 (Hase et al 1984).

After being received at the outer membrane, precursor proteins are inserted into the membrane. Nearly all proteins appear to use the same insertion site. The site was given the name "general insertion protein" or GIP (Pfanner et al 1988). Recent work has suggested the protein is MOM38 (Kiebler et al 1990). It is conceivable that the import site protein 42, or ISP42, of yeast (Ohba and Schatz 1987) is the analogue of the GIP in N. crassa.

Apocytochrome c, the precursor of cytochrome c, is imported in a unique way. It is able to insert into membranes without the help of any other proteins such as a protease sensitive

receptor or a GIP (One of several). The mature protein is found in the intermembrane space.

Proteins are translocated into the matrix by crossing both membranes at a contact point (Schleyer and Neupert 1985) is the point where the outer and inner membranes are extremely close.

Presequences are cleaved off by a mitochondrial processing peptidase, MPP (Yang et al 1988) which has a low protease activity unless accompanied by a second enzyme protease enhancing peptidase, PEP (Yang et al 1988). The equivalents in yeast are MAS2 and MAS1 (Pollock et al 1988). Some presequences are cleaved in two steps. The first removes the matrix targeting sequence and the second removes the intermembrane targeting sequence. Most intermembrane proteins have this two part presequence (Hartl et al 1989). The second presequence differs from its partner in that it is relatively hydrophobic.

Once the mature protein is present in the mitochondrial matrix it is folded or refolded to their mature configuration and are sometimes built into multimeric complexes. In yeast this appears to require the presence of ATP and a protein called hsp60 (Osterman et al 1989). Intermembrane proteins do not become folded by this protein but hsp60 may induce a conformation change that allows passage across the inner membrane.

1.6 The Influence of the Mitochondrial Genome on Nuclear Genes

As early as the mid seventies it was known that the mitochondrial genome exerted some affect on nuclear genes. Colson and co-workers (Colson 1974) produced a yeast mutant that was resistant to oligomycin and venturicidin. In the diploid state the venturicidin resistance was abolished by the introduction of mitochondrial oligomycin resistance. Another area of mitochondrial control was discovered later in the same decade. Yeast cells that were rho⁻ were found to be unable to utilize certain sugars such as maltose although they could still utilize glucose (Khan and Greener 1977). Treatment of cells with the solvent dimethylsulphoxide (DMSO) allowed cells to grow suggesting that the apparatus for utilization was present but the inducible permease was not. Later work revealed the involvement of nuclear genes in this system. Khan (1982) discovered a nuclear gene which was necessary for maltose utilization in rho⁻ cells, he called this gene PMU1 (petite maltose utilization).

How is it that the mitochondrial genome can influence nuclear genes? There is no direct evidence of a protein or other molecular signal as of yet. However there is evidence of a path of communication between the mitochondria and the nucleus which might influence gene regulation. Work carried out by Butow and co-workers (Parikh et al 1987) showed that abundance of some transcripts of the nuclear genome varied

up to five time according to the condition of the mtDNA. The authors work suggests that the nucleus reacts in response to the quality and quantity of mtDNA. The greatest abundance of transcript was reported to be in a rho⁻ yeast and the smallest amount was shown to be in the parental grande yeast. The mechanism by which this control is exerted has been suggested to involve export of mitochondrial RNA that functions as a regulator (Parikh et al 1987).

1.7

Aims and Approaches

To study the affect petite and petite type mutations would have on a brewing performance, it will be necessary to introduce specific mutations into S.cerevisiae strains capable of fermenting brewers wort. This would be carried out by a one step gene disruption (Rothstein 1983). Initially this would be carried out in a haploid yeast, if this were successful then mutations would be introduced into a polyploid brewing yeast.

The two particular mutations studied in this thesis are the pet122, which prevents expression of subunit III of the cytochrome c oxidase complex (Ohmen et al 1988), and tpy1, a putative mutation in mitochondrial pyruvate transport (Wills et al 1986).

These mutations were chosen because of their different areas of action. The pet122 mutation prevents the passage of electrons past cytochrome c in the electron transport chain. The tpy1 mutation prevents entry of pyruvate into the

mitochondria. However reducing equivalents are able to enter the mitochondria, thus not all respiratory function is absent.

As well as trying to introduce the tpy1 mutation into a brewing yeast it would be interesting to try and establish its true nature. The paper describing the mutation was by no means certain of its origin. An attempt will be made to try and clone the TPY1 gene and to study isolated mitochondria.

To examine the affect a mutation in mitochondrial pyruvate transport would have on fermentation performance, a specific inhibitor of this function, alpha-cyano-4-hydroxycinnamic acid, will be used in brewing trials.

CHAPTER 2

Disruption of PET122 Gene and Subsequent Brewing Trials

2.1

Introduction

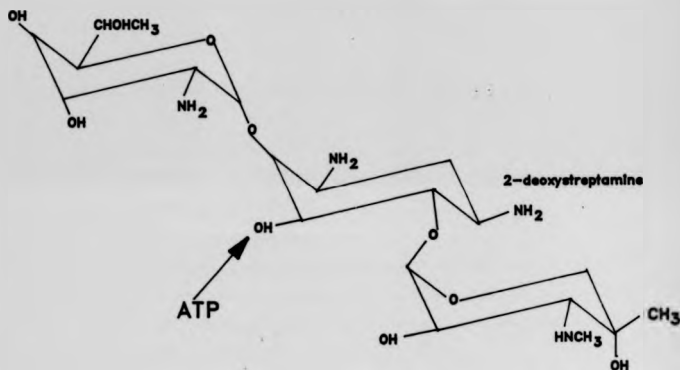
The respiratory chain of yeast mitochondria responsible for the passage of electrons from NADH to oxygen is made up of three major enzyme complexes which are located in the inner mitochondrial membrane.

The final complex, which is responsible for passing electrons to molecular oxygen, is cytochrome c oxidase. The complex is made up of nine subunits in S.cerevisiae three of which are coded for by the mitochondrial genome.

Genetic disruption of the complex in S.cerevisiae leads to formation of petite cells unable to produce a proton gradient and hence unable to produce mitochondrial ATP. If the level of intracellular ATP is lower than normal then phosphofructokinase (PFK) will not be inhibited and, consequently, neither will glycolytic flux. As well as this the mitochondria will be non-functional and so pyruvate would be mostly fermented.

If a nuclear gene encoding a protein necessary for function of the cytochrome c oxidase complex were to be mutated in a brewing strain of yeast, then this mutation's effect on fermentation performance could be observed. The gene selected was cloned by Ohmen and co-workers (1987) and is called PET122. The gene is required for the expression of the mitochondrial COX3 gene in S.cerevisiae. In order to disrupt the PET122 gene a marker is required. The marker

used in the disruption described in this chapter is resistance to the antibiotic G418. The resistance is associated with the APT2 gene. This gene codes for an enzyme which provides resistance by catalysing the transfer of a phosphate group from cellular ATP to the 2-deoxystreptamine moiety at the 3' hydroxyl side group of the antibiotic thus rendering it inactive (See figure 2.1.1).



**Figure 2.1.1 inactivation of the Antibiotic G418
by Cellular ATP**

2.2

Materials and Methods

Bacteriological peptone, yeast extract, bacto-agar, and bacto-tryptone were purchased from Difco labs, Detroit Michigan, USA. Ampicillin sulphate, kanamycin sulphate, and G418 sulphate were purchased from Sigma chemicals, Poole, Dorset, UK. Polyethylene glycol was purchased from Fisons, Loughborough, UK. All other reagents were of 'AnalaR' grade. All restriction enzymes and buffers were supplied by Gibco-BRL.

2.2.1 Maintenance of Escherichia coli StrainsEscherichia coli Strains.

TG1 (Sambrock et al 1989)

TG2 (Sambrock et al 1989)

II.1.b. Short-Term Storage:

Stock strains of Escherichia coli were maintained by plating out individual colonies onto glucose/minimal medium plates followed by incubation at 37°C overnight and storage at 4°C. This process was repeated monthly.

Glucose/Minimal Medium.10xM9 Salts.

15g Minimal Agar in 900ml SDW

6% (w/v) Na_2HPO_4

100ml 10xM9 Salts

3% (w/v) KH_2PO_4

1ml 1M MgSO_4

1% (w/v) NH_4Cl

1ml 0.1M CaCl_2

0.5% (w/v) NaCl

1ml 1M Thiamine HCl

10ml 20% (w/v) Glucose

All components of glucose/minimal medium plates were made up with SDW, autoclaved separately and cooled before mixing aseptically.

Long-Term Storage:

A single bacterial colony was grown overnight at 37°C in 10ml L-broth in an orbital shaker at 200rpm. 850ul of overnight culture were transferred to a vial containing 150ul of sterile glycerol and mixed. Glycerinated cultures were stored at -80°C.

L-Broth.

1% (w/v) NaCl

1% (w/v) Bacto-Tryptone

0.5% (w/v) Yeast Extract

(To make L-agar, 2% agar was added to the above components before autoclaving).

2.2.2 Preparation of Competent Cells. (Cohen et al 1972)

Escherichia coli cells of the appropriate strain were picked from a single colony and used to inoculate 10ml of L-broth which was grown overnight, to stationary phase, at 37°C in an orbital shaker at 200rpm. 40ml of L-broth were inoculated with 2ml of this overnight culture and grown at 37°C in an orbital shaker at 200rpm for 1½-2 hours, to mid-log phase (until the OD₅₅₀ was approximately 0.3). Cells were pelleted by centrifugation at 2000xg for 5 minutes at 4°C, then resuspended in 20ml sterile 100mM CaCl₂ which had been pre-

chilled on ice. The resuspended cells were left on ice for 20 minutes before repeating the centrifugation step and resuspending in 4ml cold 100mM CaCl_2 . Cells were left on ice for at least an hour before use, or stored at 4°C and used within 48 hours. The efficiency of transformation obtained, using cells prepared in this way, increases with time of storage then declines after 24 hours.

2.2.3 Transformation of Escherichia coli with Plasmid DNA.

(Cohen et al 1972)

Approximately 1ng of the DNA was gently mixed with 100ul competent cells in pre-chilled Eppendorfs and incubated on ice for 45 minutes with intermittent gentle mixing. Cells were heat-shocked by incubation at 42°C for 2 minutes then diluted with 1ml L-broth and incubated at 37°C for 40 minutes. The transformed cells were then pelleted in an Eppendorf centrifuge by spinning for 1 minute at low speed, and then resuspended in 100ul L-broth prior to spreading on L-agar plates (see L-broth), containing ampicillin if required.

When insertional inactivation of the lacZ gene in the plasmid was to be used for selection of recombinant DNA molecules, isopropyl-1-thio-B-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-GAL) were added to the plates.

Ampicillin and Kanamycin.

Ampicillin was prepared as a 50mg/ml stock solution in SDW and stored at -20°C. This stock solution was added to liquid media or cooled agar-containing media just before pouring, to give a final concentration of 50µg/ml. Kanamycin was prepared as a 30mg/ml stock solution in SDW and stored at -20°C. This stock solution was added to liquid media or cooled agar-containing media to give a final concentration of 30µg/ml.

IPTG.

100mM stock (23.8mg/ml in SDW)

X-GAL.

100mM stock (20mg/ml in
dimethylformamide)

(Both stock solutions were prepared just before use and added to cooled L-agar just before the plates were poured). The following control transformations were always carried out:

-A transformation with no added DNA to check for contamination.

-A transformation containing log ds DNA to monitor cell competence. (expect 10^4 colonies per ng). If IPTG and XGAL were used, the control DNA used also conferred beta-galactosidase activity on the cell so that the effectiveness of the blue/white selection could also be monitored .

2.2.4. Small Scale Isolation of Plasmid DNA from E.coli.

(Based on Holmes and Quigley 1981)

10ml aliquots of L-broth containing ampicillin at 50µg/ml were inoculated with E.coli cells from individual colonies

selected from L-agar plates containing 50µg/ml ampicillin. Cells were grown overnight at 37°C in an orbital shaker at 200rpm and then harvested by centrifugation at 2000g for 10 minutes at room temperature. Each pellet was resuspended in 100µl of 25% (w/v) sucrose/50mM Tris-Cl pH8.0.(filter-sterilised) and transferred to an Eppendorf. 600µl of MSTET and 7µl of freshly prepared lysozyme 50mg/ml in Tris-Cl pH8.0 were added to each sample just before boiling for 60 seconds. The lysed cells were placed on ice to cool and then pelleted by centrifugation for 1 hour at room temperature in an Eppendorf centrifuge. The supernatant from each sample was removed and incubated at 37°C for 30 minutes after the addition of 2µl ribonucleaseA. Each sample was phenol/chloroform extracted. DNA was precipitated by the addition of 1/10 volume 3M sodium acetate pH6.0 (autoclaved),and an equal volume of isopropanol followed by freezing on dry-ice for 30 minutes. Samples were pelleted by centrifugation as above for 15 minutes and dried under vacuum before resuspending in 100µl SDW. The samples were ethanol precipitated then resuspended in 100µl SDW or TE.

MSTET.

5% (w/v) Sucrose
50mM Tris-HCl pH8.0
50mM EDTA
5% (v/v) Triton X-100
(All components were autoclaved. The sucrose solution was filter-sterilised.

TE.

10mM Tris-Cl pH8.0
10mM EDTA pH8.0
(autoclaved)

RibonucleaseA.

Made up in SDW to 10mg/ml then boiled for 10 minutes and cooled before use.

2.2.5 Preparation and use of Phenol.

100g of chromatography grade phenol was dissolved in 100ml of 1.5M Tris-Cl pH8.0 and 0.1% (w/v) 8-Hydroxyquinoline was added to aid identification of the organic phase and to act as an antioxidant. The top (aqueous) phase was replaced with fresh 1.5M Tris-Cl pH8.0 and mixed with the organic phase; this process was repeated until the aqueous phase was between pH7.5 and pH8.0. The aqueous layer was then replaced with 10mM Tris-Cl pH8.0. After mixing, the phenol was stored in aliquots at -20°C. When required, an aliquot was thawed, used at room temperature and stored for the short-term at 4°C. (Tris-Cl solutions had been autoclaved).

Phenol/Chloroform Extaction of DNA.

All the following manipulations were carried out at room temperature, unless otherwise stated. The sample was mixed with an equal volume of phenol in an Eppendorf tube until an emulsion formed, then spun at high speed in an Eppendorf

centrifuge for 3 minutes. The upper, aqueous phase was transferred to a clean Eppendorf, care being taken to avoid the interface, and an equal volume of a 1:1 mixture of phenol and chloroform added. The sample was mixed, spun, and then the upper, aqueous phase transferred, as above. This step was repeated if there was a large amount of protein at the interface. An equal volume of chloroform was added to the aqueous phase and the sample which was then mixed, spun and transferred as above. The DNA was recovered by ethanol precipitation of the aqueous phase.

Ethanol Precipitation of DNA.

2 volumes of ice-cold ethanol and 1/10 volume 3M sodium acetate pH 6.0 (autoclaved) were added to the sample which was then placed on dry-ice for 20 minutes or at -20°C for several hours. The sample was then spun at high speed in an Eppendorf centrifuge at 4°C for 15 minutes and the supernatant carefully removed. The pellet was then washed with 200µl 80% (v/v) ethanol, which had been pre-chilled to -20°C. The supernatant was removed and the pellet dried under vacuum before resuspension in SDW or TE.

2.2.6 Large Scale Plasmid DNA Isolation from E.coli.

(Based on Promega Biotec Technical Bulletin N°9).

Cells containing plasmid were grown overnight in 10ml of L-broth and 50µg/ml ampicillin at 37°C in an orbital shaker at 200rpm. 250ml of L-broth containing 50µg/ml ampicillin was then inoculated with 2.5ml of this overnight culture and

grown overnight at 37°C as above. Cells were pelleted by centrifugation at 2000g for 10 minutes at 4°C, then resuspended in 4ml of lysis buffer and placed on ice for 15 minutes. 8ml of alkali detergent solution were then added and the suspension which was gently mixed until transparent. After 10 minutes on ice, 5ml of 3M sodium acetate pH4.6 (autoclaved) was added and the suspension mixed until it was converted into a dense white precipitate. After 15 minutes on ice, this precipitate was pelleted by centrifugation at 17000g for 15 minutes at 4°C. The clear supernatant was transferred to a sterile universal and 5µl of a 10mg/ml solution of ribonucleaseA added to it before incubation at 37°C for 30 minutes. 0.5 volumes of phenol/chloroform were then mixed with the supernatant which was then left at room temperature for 15 minutes. The mixture was centrifuged at 3000g for 10 minutes at room temperature, the upper phase transferred to a clean universal and the phenol/chloroform treatment repeated until the top phase was transparent. DNA was precipitated from the top phase by the addition of 0.6 volumes of isopropanol and incubated for 15 minutes on ice. The sample was then spun at 17000g for 10 minutes at 4°C and the resulting pellet resuspended in 1.6ml of SDW, to which 0.4ml of 4M NaCl and 2ml of 13% polyethylene glycol (autoclaved) were then added. The sample was left on ice for an hour and then spun at room temperature for 10 minutes at high speed in an Eppendorf centrifuge. The supernatant was removed with a drawn-out Pasteur pipette and the pellet

washed twice with 80% (v/v) ethanol, dried under vacuum and resuspended in 50 μ l of SDW or TE. DNA concentration was estimated by running an aliquot of the sample along-side samples of known concentration on a agarose gel. This gel was then visualised under U.V. light and the DNA concentration estimated by eye.

Lysis Buffer.

25 mM Tris-Cl pH 8.0

10 mM EDTA

15% Sucrose

2mg/ml Lysozyme

Alkali Detergent Solution.

0.2 M NaOH

1% SDS

(Tris-Cl, EDTA, sucrose and SDS solutions were autolaved).

2.2.7 Digestion of DNA with Restriction Endonucleases.

The following were pipetted into a clean Eppendorf tube:

Xul DNA (0.1 μ g to 4 μ g in SDW or TE)

2 μ l 10x Restriction Buffer

(18-X) μ l SDW (so that the total volume was 20 μ l)

Restriction endonuclease was added at 1 to 5 units per μ g DNA and the reaction incubated at the temperature recommended by the enzyme manufacturers (usually 37°C but 30°C for SmaI) for 1 to 4 hours.

The volume of restriction enzyme(s) added was less than 1/10 the reaction volume. Appropriate restriction buffers were supplied with the enzyme by the manufacturers.

In principle, 1 unit of restriction endonuclease completely digests 1 μ g of purified DNA in 60 minutes, using the

recommended assay conditions. However, crude DNA preparations, such as the small-scale preparations outlined in section 2.2.4, often required more enzyme and/or more time for complete digestion.

If digestion by two restriction endonucleases was required, a restriction buffer was chosen in which both enzymes are relatively active. If the buffers of the enzymes were too dissimilar, the DNA was first digested by the enzyme active at the lower salt concentration, then sufficient 1M NaCl (1 to 3 μ l for a 20 μ l reaction) was added so that the final concentration was appropriate for digestion by the second enzyme which was then added to the incubation mixture.

2.2.8 Preparation and Running of Agarose Gels.

A 1% (w/v) agarose gel was prepared by adding 0.5g agarose to 5ml 10xTBE and 45ml SDW and heating in a microwave oven with intermittent mixing until the agarose was completely dissolved. The solution was allowed to cool to approximately 50°C and then ethidium bromide added (from a stock solution of 10mg/ml in SDW) to give a final concentration of 0.5 μ g/ml. Gels were cast in a BRL "Horizon 58" horizontal slab gel apparatus with the required well-former inserted. When the gel was completely set, the well-former and end-sealers were removed and the gel immersed in 1xTBE containing ethidium bromide at 0.5 μ g/ml, to cover the gel to a depth of 1mm. DNA samples and appropriate DNA molecular weight markers, were mixed with at least 1/6 volume of 6x

DNA sample buffer, loaded into the wells and the gel run at a constant voltage of 1-6 V/cm. Since DNA is negatively charged, it migrates towards the anode. DNA in the gel was visualised with an ultra-violet transilluminator. since ethidium bromide bound to DNA displays an increased fluorescent yield compared to that free in solution.

10xTBE.

0.89M Tris Base

0.89M Boric Acid

0.02M EDTA (pH8.0)

(autoclaved)

6x DNA Sample Buffer.

30% (v/v) Glycerol

0.25% (w/v) Bromophenol Blue

0.25% (w/v) Xylene Cyanol

2.2.9 Isolation of DNA from Agarose Gels.

(Based on Grivitz et al 1980)

The required DNA band was visualised using a U.V. transilluminator. A slot was cut just in front of the band (on the anode side) and a strip of Whatman number 1 paper which had been pre-soaked in sterile 1xTBE placed in the slot. A strip of dialysis tubing which had been prepared by boiling in 1mM EDTA and rinsed thoroughly in SDW, was placed in the slot in front of the paper. The gel was returned to the tank but 1xTBE added to the tank so that it was level with the top of the gel on each side but not immersing it. The gel was run at 50V for 5-10 minutes then the current reversed for 10 seconds. The gel was then viewed using a U.V. transilluminator. If the band had not run into the paper, the gel was run for a further period. The paper and

dialysis tubing were transferred to a 1.5ml Eppendorf with a hole in the bottom made with a pin. The 1.5ml Eppendorf was placed inside a 2ml Eppendorf. DNA was removed from the paper by adding 100ul TE to the sample and spinning the Eppendorfs at high speed in an Eppendorf centrifuge for 1 minute. This step was repeated twice then the upper Eppendorf containing the paper discarded. The sample was spun at high speed for 2 minutes, the liquid transferred to a clean Eppendorf, phenol/chloroform extracted and ethanol precipitated.

2.2.10 Dephosphorylation of Plasmid DNA.

(Sambrook et al 1989)

When a ligation was carried out using a plasmid which had been cut yielding compatible ends, calf intestinal phosphatase (CIP) was used to remove 5' phosphate groups from DNA to prevent recircularisation of the plasmid without insertion of the required fragment during the ligation reaction.

Plasmid DNA (approximately 1µg) was digested to completion with the appropriate enzyme(s), phenol/chloroform extracted and ethanol precipitated. The DNA was dissolved in a minimum volume of 10mM Tris-Cl pH8.0, then 5µl 10xCIP buffer and SDW added to give a final volume of 48µl.

DNA with 5' overhangs was dephosphorylated by the addition of 0.1 units of CIP and incubation at 37°C for 30 minutes followed by addition of another 0.1 units and the incubation

continued for a further 30 minutes.

DNA with 5' recessed termini or blunt ends was dephosphorylated by the addition of 0.1 units of CIP and incubation at 37°C for 15 minutes followed by incubation at 56°C for 15 minutes. After addition of another 0.1 units of CIP, incubation at both temperatures was repeated.

40µl of SDW, 10µl of 10x STE and 5µl of 10% (w/v) SDS were added to the sample before heating to 68°C for 15 minutes. The sample was then phenol/chloroform extracted, ethanol precipitated and resuspended in 10µl of SDW.

10xCIP Buffer.

0.5M Tris-Cl pH9.0

10mM MgCl₂

1mM ZnCl₂

10mM spermidine

10xSTE.

100mM Tris-Cl pH8.0

1M NaCl

10mM EDTA

(filter-sterilised)

2.2.11 Blunt-Ending of 5' or 3' Overhangs.

(Sambrook et al 1989)

2µg of DNA was digested to completion with the appropriate restriction enzyme(s) in a 20µl reaction volume. 1µl of a solution containing dATP, dCTP, dGTP and dTTP each at 2mM in SDW was then added.

To blunt-end 5' overhangs, the recessed 3' termini were filled using the Klenow fragment of E.coli DNA polymerase 1. 1 unit of Klenow fragment was added for each microgram of DNA in the reaction and the sample incubated at room temperature for 15 minutes.

To blunt-end 3' overhangs, the protruding 3' termini were removed by utilising the 3' to 5' exonuclease activity of bacteriophage T4 DNA polymerase. 2 units of this enzyme were added for each microgram of DNA in the reaction and the sample incubated at 12°C for 15 minutes.

After the blunt-ending reaction, enzymes in the sample were inactivated by heating to 75°C for 10 minutes.

2.2.12 Ligation of DNA Blunt or Cohesive Ends.

(Ausubel et al 1987).

The enzyme T4 DNA ligase was used for both blunt-end ligations and ligation of cohesive ends. Between 30ng and 100ng of cut plasmid and fragment were used in a ligation reaction. Cut plasmid and fragment were generally used at ratios of 1:1 and 1:2 (plasmid:fragment) in terms of approximate numbers of molecules. The required amounts of cut plasmid and fragment were made up to a volume of 9µl using SDW, then 10µl 2x Ligase buffer, and 1 unit of T4 ligase for cohesive-end ligations and 100 units T4 ligase for blunt-end ligations were added. Blunt-end ligations were incubated at room temperature for 3 hours while cohesive-end ligations were incubated at 15°C overnight. Incubation at the lower temperature favours the annealing of cohesive ends but diminishes enzyme activity. Blunt-end ligations require 10 to 100 times more enzyme to achieve the same efficiency as cohesive-end ligations since the ends are not brought together by annealing.

After ligation, E.coli TG2 were transformed with 1 μ l and 10 μ l aliquots of the sample .

2x Ligase Buffer.

100mM Tris-Cl pH 7.5

20mM MgCl₂

20mM DTT

0.5mM ATP

2.2.13 Growth Media for Yeasts

Two types of media were routinely used for growing yeasts. YPD was used when cells were respiratory deficient or when the presence of these type of cells did not create a problem. YPG was used only when cells were respiratory competent.

YPD

1% (w/v) Yeast Extract

2% (w/v) Bacto-Peptone

2% (w/v) Glucose

2% (w/v) Agar

YPG

1% (w/v) Yeast Extract

2% (w/v) Bacto-Peptone

3% (w/v) Glycerol

2% (w/v) Agar

When liquid media was required the agar was omitted.

Addition of Antibiotics

G418 sulphate was added directly to hand hot media as a solid to obtain a final concentration of 0.5 mg/ml

Yeast Strains Employed

C2 - Mat alpha, Mal⁺, leu 2, lys 2

X2180-1A - Mat a, mal, gal, SUC 2, CUP 4 (Both Supplied by Whitbread PLC)

Preservation of strains

Yeast strains were streaked onto YPD plates every two months and placed on YPD slopes in Bijou bottles every six months.

2.2.14 PET122 Gene and G418 Cassette

The PET122 gene was obtained from Joan McEwen (Department of Microbiology and the Molecular Biology Institute, University of California). The gene was contained in the yeast shuttle vector Ycp50 and was named pJDO8 (Figure 2.3.1).

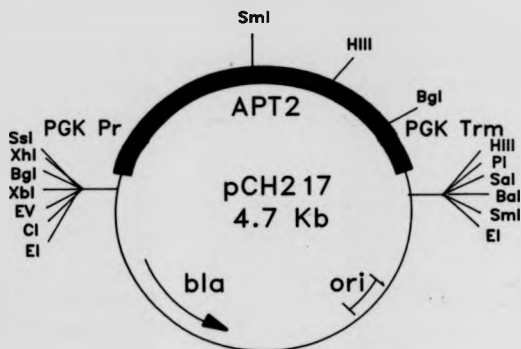
The G418 cassette containing the APT2 gene was obtained from Chris Hadfield (Biocentre, University of Leicester). The cassette was contained in the vector pUC7 and was named pCH217 (Figure 2.2.1).

2.2.15 Transformation of Yeast (Ito et al 1983)

Cells were grown overnight to stationary phase in 10ml of YPD. The volume of overnight culture used to inoculate a fresh one depended on the strain of yeast used but generally 50ml of fresh YPD was inoculated with 5µl of overnight culture and was grown until there were $1-2 \times 10^7$ cells present (approximately 15 hours).

Cells were washed once in TE and once in LA before being resuspended in 0.5ml of LA. The cells were made competent by shaking for an hour at 30°C then 0.1ml was added to 1-10µg of DNA. The cells and DNA were incubated at 30°C for 10 minutes after which 0.7ml of PEG solution was added. This suspension was incubated for a further 30 minutes at the

Plasmid Map of pCH217



EI=EcoR I CI=Cla I EV=EcoR V Xbi=Xba I Bgl=Bgl I
 Xhi=Xho I Sal=Sst I Sml=Sma I HIII=Hind III PI=Pst I
 Sal=Sal I Bal=BamH I

Figure 2.2.1

Map of pCH217 containing the APT2 gene sandwiched between the PGK promoter and terminator. The APT2 gene confers resistance to the antibiotic G418.

same temperature and was then heat shocked at 42°C for 5 minutes. The cells were washed with YPD and plated out onto selective media. If expression of antibiotic resistance was required the cells were allowed to recover for one hour in YPD prior to plating.

TE

10 mM Tris-HCl pH 8.0

1 mM EDTA pH 8.0

LA

0.1 M Lithium Acetate

Dissolved in TE

PEG Solution

40% Polyethylene Glycol 4000

Dissolved in LA

2.2.16 DMSO Enhanced Transformation of Yeast (Hill et al 1991)

Cells were grown in the same way as in the lithium acetate transformation procedure. Cells were harvested by transferring cultures into two sterile 30ml universal plastic tubes (Sterilin) and centrifuged at 4000rpm in a bench centrifuge for 5 minutes. The pellets were washed in 5 ml of lithium acetate solution and pooled into one tube. The suspension was centrifuged as above and then resuspended in 0.5ml of LA solution. 100µl of the yeast suspension is added to 10µl of a DNA solution contained in an 1.5ml Eppendorf tube. The contents were mixed and then left at room temperature for 5 minutes. 280µl of PEG4000 solution (50%

PEG4000 in LA solution) were added, the contents were mixed 4-6 times, then the tube was placed at 30°C for 45 minutes. DMSO was added to the tube to give a final volume of 10%, the contents mixed and then heat shocked at 42°C for 5 minutes. If antibiotic resistance was to be selected for the cells were allowed to 'recover' by resuspending in 1ml of YPD, otherwise cells were plated directly onto selective media.

2.2.17 Crossing of Haploid Strains of Yeast

Yeast strains that were to be crossed were grown up for a few hours in YPD. A drop of one strain was then placed on media that was selective for the cross. A drop from the second strain was then placed on top of the first. When the plate was dry it was incubated for two days at 30°C.

2.2.18 Formation of rho⁰ Yeast by Ethidium Bromide Treatment (Slominski et al 1968)

A stationary yeast culture was used to inoculate 10 ml of YPD containing 25µg/ml ethidium bromide. This was shaken overnight at 30°C overnight. This culture was then used to inoculate another 10 ml of YPD containing 25µg/ml ethidium bromide. After a further 24 hours cells were spread on solid YPD. After 48 hours small colonies were transferred to YPD and YPG solid media. Colonies able to grow on YPD but not on YPG were assumed to be rho⁰.

2.3

Results2.3.1 Disruption of PET122 Gene in Plasmid pJD08

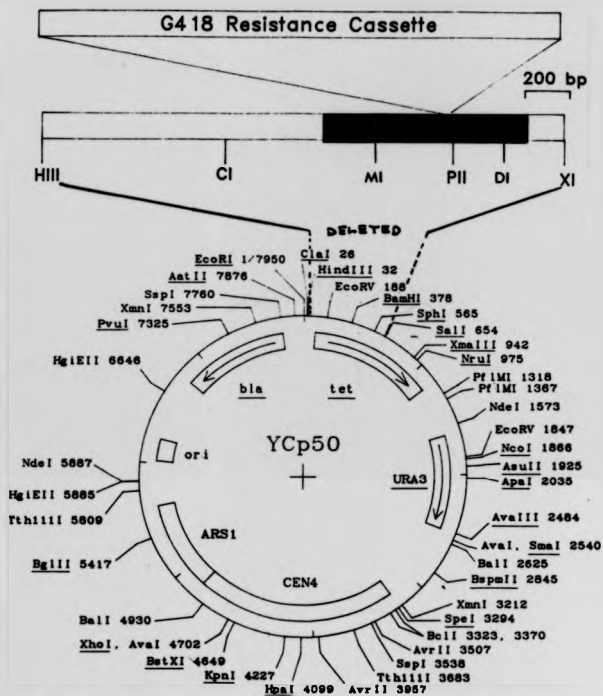
The plasmid pJD08 was disrupted with the G418 resistance gene as shown in figure 2.3.1. The plasmid was cut at the PvuII site contained in the gene generating blunt ends. This was treated with CIP to prevent self ligation. The G418 cassette was cut out of the plasmid pCH217 using EcoRI and blunt ended with Klenow fragment in the presence of the four dNTP's. The fragment was gel isolated and ligated into the PvuII site of pJD08 using T4 DNA ligase.

Transformation of E.coli was carried out by the calcium chloride method and cells containing a plasmid were selected by their ability to grow on LB plates with 50µg/ml ampicillin. Colonies from these plates were placed on LB plates containing 30µg/ml kanamycin. Colonies growing on this media were presumed to be carrying the G418 resistance gene which produces resistance to kanamycin in bacteria. Large scale plasmid preparations were carried out on a colony that showed both ampicillin and kanamycin resistance. Restriction digests of these preparation and those of plasmid pJD08 are shown in figure 2.3.2, the new plasmid was named pAD1.

2.3.2 Disruption of Genomic PET122 Gene of a Haploid Maltose Positive Strain of Yeast

The plasmid AD1 was digested with the restriction enzymes

Ligation of the G418 Resistance Cassette into the PET122 Gene



MI=MluI XI=Xho I HIII=Hind III CI=Cle I PII=Pvu II DI=DraI

Figure 2.3.1

Ligation of the G418 resistance cassette into the PET122 gene. The cassette was ligated into the unique PvuII site in the PET122 gene. The plasmid pJD08 was constructed by ligation of the PET122 into the HindIII and Sall sites of the plasmid Ycp50 (Ohmen et al 1988). The shaded area represents the PET122 coding region. The plasmid construct shown here was named pAD1 (plasmid map taken from Rose et al 1987).

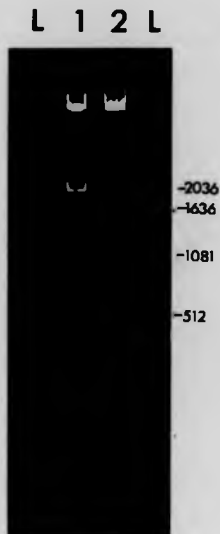


Figure 2.3.2

Restriction digests of the plasmids pJD08 (1) and pAD1 (2) using HindIII. Lanes marked L contain the 1kb ladder.

MluI and DraI producing a fragment of approximately 2.7 Kb, containing the G418 resistance cassette and flanked by regions of the disrupted PET122 gene. The fragment was isolated from an agarose gel and 20µg were used to transform the maltose utilizing strain C2. Transformants were selected by their ability to grow on YPD agar plates containing 0.5mg/ml G418 sulphate. Transformants were transferred to YPG agar plates to test for respiratory competence. All fifteen transformants were of a petite nature ie they were not able to grow on YPG plates.

2.3.3 Crossing of Mutant Yeast with a rho⁰ Yeast

In order to establish whether the petite formed by transformation was of nuclear origin (pet mutant) or of cytoplasmic origin (rho⁰, rho⁻, or mit⁻) it was necessary to cross the petite with a rho⁰ strain of yeast. To this end the petite was crossed with X2180-1A rho⁰ on agar plates that selected for growth of the cross ie minimal maltose. The diploid was streaked onto YPG agar plates and it grew.

2.3.4 Transformation of Petite with PET122 Gene

To establish if the respiratory incompetence of the petite was due to disruption of the genomic copy of the PET122 gene the petite was transformed with the plasmid containing the original PET122 gene, pJD08. The transformants produced were respiratory competent ie they were able to grow on YPG.

2.3.5 Breving Trial with Mutated Yeast See Appendix 2.

2.4

Discussion

From the results presented in chapter 2 it can be seen that the maltose positive yeast strain C2 has been converted to a pet122 mutant.

The restriction digest pattern, of plasmid DNA isolated from G418 resistant colonies, is consistent with insertion of the 2kb G418 resistance cassette in the unique PvuII site of the PET122 gene.

A cross between the C2 pet122 strain and a rho⁰ produces a diploid with a phenotype that is a combination of its parental strains. Only if the C2 pet122 has no deletions in its mitochondrial genome, can the diploid grow on non-fermentable media.

The final proof of the disruption is the phenotypic rescue using the pJD08 plasmid, containing the PET122 gene. When the yeast C2 pet122 was transformed with this plasmid it restored respiratory competence. The only gene on the plasmid that is related to respiratory function is the PET122 gene. Therefore it may be concluded that the PET122 gene has been disrupted in the yeast strain C2.

For a discussion of the brewing trials see Appendix 2.

CHAPTER 3

Studies of the tpv1 Mutant and an Inhibitor of Mitochondria
Pyruvate Transport

3.1

Introduction

It has been known for some time that petite type yeast cells have some advantage over their wild type counterparts in terms of fermentative performance (Bacilli and Horri 79, Lancashire Personal Communication). In order to try and exploit this a putative mutant in mitochondrial pyruvate transport was chosen. The mutant was created by Christopher Wills at the University of California by ethyl methane sulphonate mutagenesis and named tpv1 for transport of pyruvate.

The mutant produced by Wills had the right characteristics for a mitochondrial pyruvate transport mutant. It was unable to grow on pyruvate or glycerol but was able to grow on ethanol. Some mitochondrial functions remained intact as was demonstrated by its ability to grow on glucose in the presence of antimycin A even when the mutant lacked ADH activity. Antimycin A blocks electron transport at the level of cytochrome b, so if the yeast can grow under these conditions then reducing equivalents must be getting into the mitochondria. Studies of levels of pyruvate dehydrogenase and NADH dehydrogenase revealed these enzymes to be at levels comparable with that of the wild type yeast. Absence of one of these two enzymes could have explained the

missing link between pyruvate and the mitochondria. Therefore some other factor must be responsible for this phenotype.

From a brewing point of view it would seem logical that if there is a blockage in the pathway to the mitochondria then there will be more pyruvate available for fermentation. However not all mitochondrial functions would be absent since reducing equivalents could still be passed to the mitochondria.

The brewing trials performed were designed to simulate the affect such a mutation would have in a brewing yeast. The inhibitor, alpha-cyano-4-hydroxycinnamic acid, acts by binding to an essential thiol group of the pyruvate transporter and thus inactivates it (Halestrap 1978). A different brewing trial involved the use of the mutant royi and its wild type counterpart.

3.2

Materials and Methods

DSMP⁺ was purchased from Gallard-Schlessinger Inc. and CCCP from Sigma. Both were made up in ethanol.

3.2.1 Media and Yeast strains

Growth media used was as described previously, with the addition that the non-fermentable media YPE was used in tests for growth of the tpyl mutant.

YPE

2% Ethanol

2% Bacto-peptone

1% Yeast Extract

Made up with distilled water. Where solid media was required 2% Bacto-Agar was added.

Strains

XW153-3C, MAT a, tpyl, SUC2, mal, gal2, CUP1, adel, trp1,
ure3

(Supplied by C. Wills University of California)

X2180-1A, MAT a, SUC2, mal, gal2, CUP1,

Y9, a polyploid ale type brewing yeast.

(Both supplied by Whitbread PLC)

3.2.2 Brewing Trials (Based on Lancashire and Wilde 1987)

Brewing trials to simulate industrial fermentation conditions were carried out in 1000ml tall tubes. Wort was diluted until the gravity was approximately 1040. Each tube

was filled with 500ml of aerated wort, 1ml of 1mM zinc sulphate, and 2.4 ml of yeast cell suspension containing 0.5g of cells per ml. When brewing trials were carried out with the mutant trvi an artificial wort was used containing 4% glucose, 2% bacto-peptone and 1% yeast extract. This was necessary because the wild type and mutant are unable to utilize maltose the main constituent of industrial wort. The tubes were maintained at between 18 and 22°C throughout the trial. After 24 hours the beer was roused to resuspend the yeast. Gravity and ethanol content were estimated after 24, 48 and 72 hours. After 72 hours only the biomass formed was measured.

3.2.3 Gravity Estimation (Lancashire Personal Communication)

Wort or beer was collected in a universal tube (Sterilin) and pumped up and down with a syringe to remove any bubbles. The sample was then injected into a density meter (Paar Scientific Ltd., Density Meter DMA 35, supplied by Whitbread PLC) and the reading was noted.

3.2.4 Alcohol Assay (Based on Bonnichsen 1965)

Beer samples were diluted 10^3 times using serial dilutions, 100 μ l into 1000 μ l total volume each time. From the final dilution 100 μ l were added to 2.9ml of ADH buffer in a cuvette. Next 10 μ l of 50mM NAD⁺ was added and then the cuvette (sample) was compared with another containing just buffer and sample (reference) in an SP1800

spectrophotometer. The reading taken here at 340_{nm} was called A1. When A1 was established 5ul of ADH were added to the sample cuvette. After one hour the reading of the sample against reference was noted (A2). The final reading (A2-A1) was compared against a standard curve of ethanol concentrations between 0.5% and 5% by volume.

ADH Buffer

20g Sodium Pyrophosphate

5g Semicarbaside Hydrochloride

1g Glycine

Made up in 500ml of distilled water and prior to making up to the final volume of 600ml the pH was adjusted to 9 with sodium hydroxide.

3.2.5 Preparation of Coupled Mitochondria (Based on Wills et al 1986)

Cells were grown to stationary phase in 500ml of YPD with good aeration, then spun down in a Sorvall GSA rotor at 4000rpm for 10 minutes. The pellet was washed once in 0.7M sorbitol and then resuspended in an equivalent volume of extraction buffer. The cells were placed on ice for 10 minutes and a weight of 0.3mm glass beads equal to that of the cells, was added. To disrupt the cells the handshake method was employed. This involved shaking the tubes containing the cells and glass beads for two minutes at 4°C by hand. This was followed by a period of one minute on ice.

The cycle was repeated three times before the beads and cell debris were spun down by two consecutive spins at 4°C in a Sorvall SS34 rotor at 4000rpm. The supernatant was kept on ice then spun at 10000rpm for 20 minutes. The resultant pellet was resuspended in a minimal amount of extraction buffer. Protein concentration was determined by use of the Biuret assay (Gornall et al 1949).

Extraction Buffer

0.1M Tris-HCl pH 8.0

0.7M Sorbitol

1mM EDTA pH 8.0

2% BSA

3.2.6 Measurement of Membrane Potential by Fluorescence Assay (Based on Neves and Rafael 1981)

The proton gradient across the inner membrane of mitochondria was measured by the change in fluorescence between coupled mitochondria and uncoupled mitochondria. Mitochondria prepared as described above were placed in a cuvette containing 2.8ml of extraction buffer and 2µl of 1mM DSMP⁺. Any fluorescence measured at this stage was due to simple diffusion of DSMP⁺. The addition of a substrate such as NADH causes DSMP⁺ to pass into the matrix of the mitochondria where it fluoresces. To neutralize the membrane potential, and hence measure the difference between coupled and uncoupled mitochondria, an uncoupler CCCP was added (1µl of a 1mM solution). Fluorescence was measured at 589 nm

after excitation at 479 nm. Fluorescence was measured using a Perkin Elmer LP5 fluorimeter.

3.3

Results3.3.1 Brewing Trials with alpha-cyano-4-hydroxycinnamic acid and the Mutant *tpyl*

In order to establish what concentration of inhibitor to use it was first necessary to titrate cell growth against different concentrations of the inhibitor. To do this cells were incubated with gentle shaking in YPD media containing concentrations of inhibitor between 1 and 50 mM. The cell concentration was estimated using a haemocytometer. The results are shown in table 3.3.1 and figure 3.3.1

From these results the concentration of 15 mM was taken as being the optimum level and was used in subsequent brewing trials

A number of brewing trials were carried out using the inhibitor and a control. Trials were carried out using either 100 ml or 500 ml of wort. The results obtained are shown in table 3.3.2 and in figures 3.3.2 and 3.3.3. An extended brewing trial involving the inhibitor and a control was carried out over a period of 120 rather than 72 hours. the results are shown in table 3.3.4 and figure 3.3.5.

Brewing trials were carried out using the mutant *tpyl* and the wild type X2180-1A. Trials were carried out using 100ml of artificial wort. The results are shown in table 3.3.3 and in figures 3.3.3 and 3.3.4

Table 3.3.1

This shows the results of a brewing trial carried out with the inhibitor alpha-cyano-4-hydroxycinnamic acid against a control. The inhibitor was dissolved in DMSO and used in the brew at a final concentration of 15mM. The equivalent amount of DMSO was added to the control. The yeast employed was an industrial ale yeast. These were large scale (500 ml) fermentations carried out over a period of 72 hours. Gravity of beer is a measure of the liquid density compared to water which is 1000. The ethanol content was estimated from a calibration curve of various concentrations from 1 to 5 %. Units of sugar are the difference between the original gravity and the gravity at the various times. The values of cells/unit sugar are given in terms of 100 ml of beer to allow comparison with the 100 ml scale fermentations shown later (See section 3.2.2 for further details).

Brewing Trials with alpha-cyano-4-hydroxycinnamic acid

		0	Time (hours)		72
			24	48	
Gravity of Beer	Inhibitor	1038	1021	1009	1008
	Control	1038	1008	1003	1002

		24	Time (hours)		72
			48		
% Ethanol in Beer	Inhibitor	0.65 +/-0.07	2.69 +/-0.12	3.17 +/-0.05	
	Control	1.24 +/-0.07	3.45 +/-0.02	3.65 +/-0.05	

		Time (hours)	
		72	
Wet Weight of Yeast (g)	Inhibitor	11.45	
	Control	16.5	

		24	Time (hours)		72
			48		
% Ethanol /unit sugar	Inhibitor	0.038 +/-0.004	0.093 +/-0.004	0.106 +/-0.002	
	Control	0.041 +/-0.002	0.099 +/-0.001	0.101 +/-0.002	

		Time (hours)	
		72	
Cells (g) /unit sugar	Inhibitor	0.076	
	Control	0.092	

Table 3.3.2

This shows the results of a brewing trial carried out with the mutant TPY1 against the wild type X2180-1A. These fermentations were of the small scale type (100 ml) and the wort used was composed of 4% glucose, 2% bacto peptone and 1% yeast extract. Ethanol concentration and units of suagr were estimated as previously described (See section 3.2.2 for further details).

Brewing Trials with TPY1 against the Wild Type

		0	Time (hours)		72
			24	48	
Gravity of Beer	TPY1	1022	1018	1009	1007
	WT	1022	1014	1006	1006

		24	Time (hours)		72
			48		
% Ethanol in Beer	TPY1	0.12 +/-0.062	1.81 +/-0.075	2.16 +/-0.075	
	WT	0.58 +/-0.030	2.10 +/-0.075	2.10 +/-0.075	

		Time (hours)	
		72	
Wet Weight of Yeast (g)	TPY1	1.05	
	WT	1.20	

		24	Time (hours)		72
			48		
% Ethanol /unit	TPY1	0.030 +/-0.015	0.14 +/-0.005	0.14 +/-0.005	
sugar	WT	0.072 +/-0.004	0.13 +/-0.005	0.13 +/-0.005	

		Time (hours)	
		72	
Cells (g) /unit	TPY1	0.070	
sugar	WT	0.075	

Figure 3.3.1

Graph representing the titration of cell growth against levels of α -cyano-4-hydroxycinnamic acid. The control contains an equivalent amount of the solvent DMSO used to dissolve the inhibitor in. The growth media was YPD. Cell density was measured after 24 hours using a haemocytometer.

Titration of Cell Growth Against Varying Levels of Inhibitor

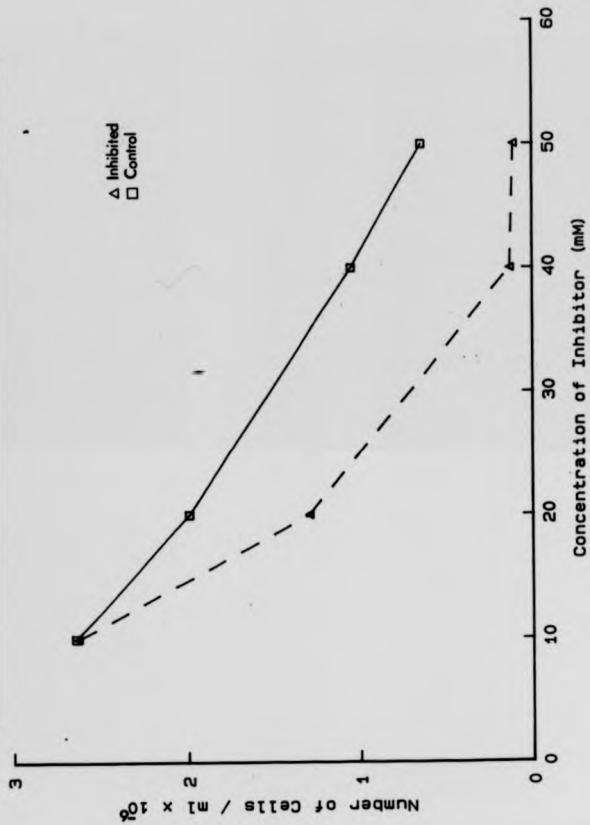


Figure 3.3.2

Graph representing total ethanol produced per total units of sugar used over 72 hours. Brewing trial involved alpha-cyano-4-hydroxycinnamic acid against a control. A unit of sugar is defined as a drop of one degree in gravity (See table 3.3.1 for further details).

Brewing Trial of an Inhibitor Against a Control

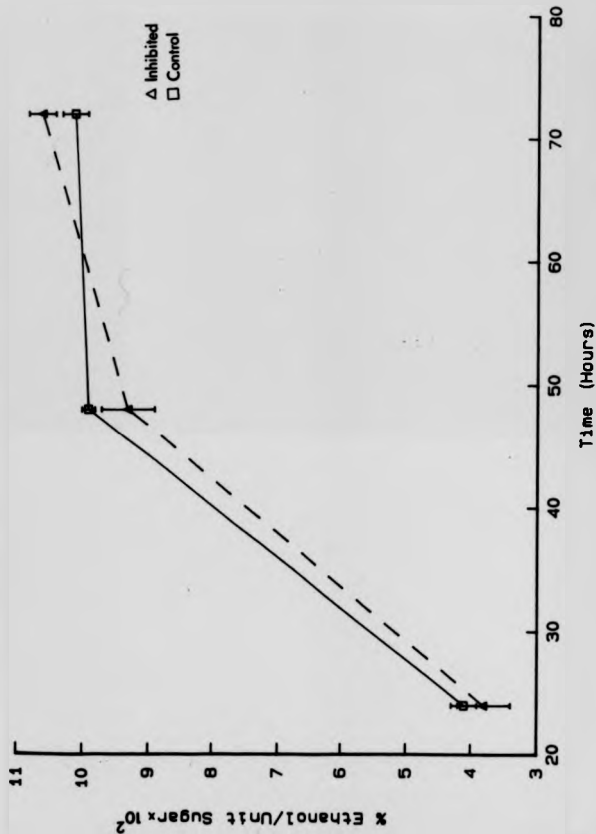
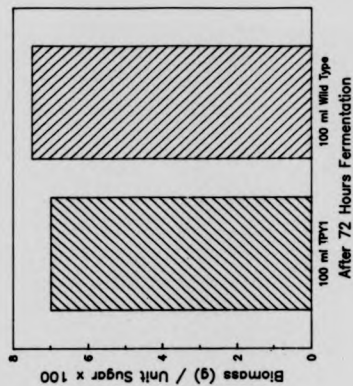


Figure 3.3.3

Graphs representing biomass formed per unit sugar used at 72 hours. Both brewing trials involving alpha-cyano-4-hydroxycinnamic acid and the *tpyl* mutant are shown (See tables 3.3.1 and 3.3.2 for further details).

Brewing Trial with the Mutant TPY1 and the
Wild Type



Brewing Trial with Alpha-cyano-4-hydroxycinnamic
acid and a Control

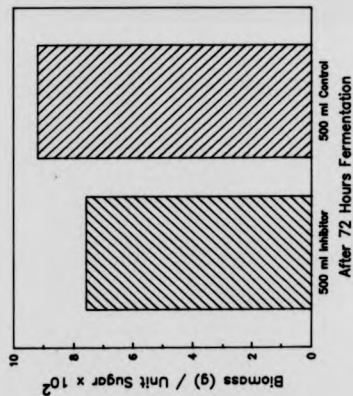


Figure 3.3.4

Graph representing total ethanol produced per total units of sugar used over 72 hours. Brewing trial involved the toy1 mutant and the wild type. A unit of sugar is defined as a drop of one degree in gravity (See table 3.3.2 for further details).

Brewing Trial of the *tpy1* Mutant Against the Wild Type

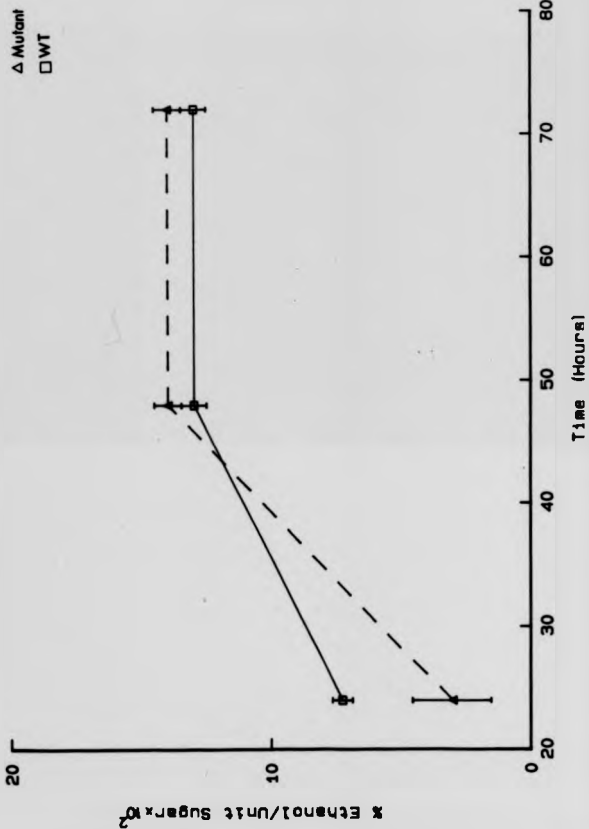
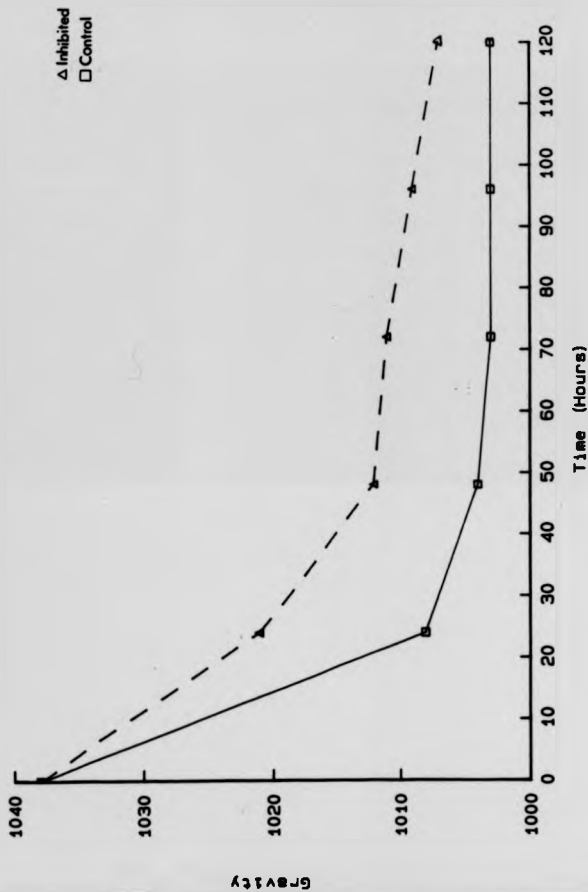


Figure 3.3.5

Graph of an extended brewing trial using alpha-cyano-4-hydroxycinnamic acid against a control. The inhibitor was dissolved in DMSO and added to wort to give a final concentration of 15mM. An equivalent amount of DMSO was added to the control. Brewing was carried out at temperatures between 18-22°C.

Extended Brewing Trial of an Inhibitor and a Control



3.3.2 Estimation of Mitochondrial Membrane Potential and Growth Pattern of the *tpyl* Mutant

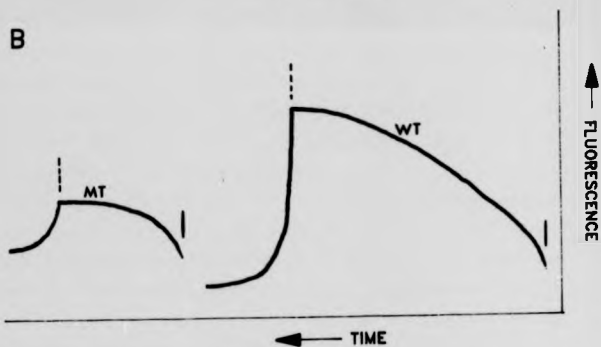
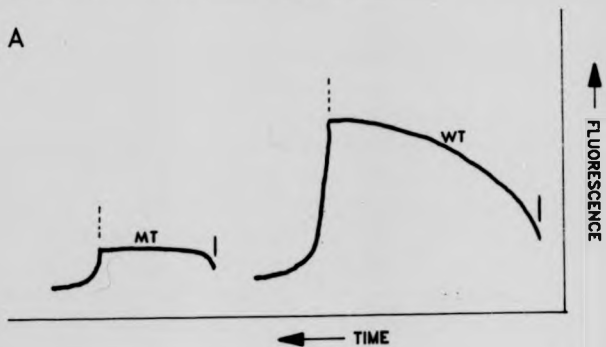
The membrane potential of isolated mitochondria from both *tpyl* and the wild type was estimated using the substrates pyruvate/malate and NADH. Protein content of the mitochondrial preparations was estimated by the biuret method. The results obtained are shown in figure 3.3.6.

The *tpyl* mutant was unable to grow on YPE solid media. It was able to grow on YPE after being crossed with a *rho*⁰.

Figure 3.3.6

Measurement of membrane potential using the substrates A) pyruvate/malate and B) NADH. Mitochondria were isolated from the wild type (WT) X2180-1A and the mutant (MT) *tpyl* strains of *S.cerevisiae*. Protein content was 80mg/ml for WT and 30mg/ml for MT. 20ul of mitochondrial suspension was used for each assay. The uncoupler used was CCCP. Addition of a second 20ul aliquot of mitochondrial suspension to the cuvette containing the mutant mitochondria, did not alter the decrease in fluorescence upon addition of the uncoupler. The solid line represents the addition of mitochondria, DSMP⁺ and substrate. The dashed line represents the addition of uncoupler (CCCP).

Fluorescence Assay for Mitochondrial Membrane Potential



3.4

Discussion3.4.1 Estimation of Mitochondrial Membrane Potential and Growth Pattern of the *tpyl* Mutant

Testing of *tpyl* mitochondria against X2180-1A revealed results differing from those reported by Wills and co-workers (1986) Although mitochondria isolated from *tpyl* were unable to produce a membrane potential using pyruvate/malate, as reported by the authors, the mitochondria were also unable to generate a membrane potential with NADH, contrary to the authors report. The wild type mitochondria were able to generate a membrane potential with both substrates.

It is possible that mitochondria isolated from the *tpyl* mutant were not coupled. A number of attempts were made to isolate coupled mitochondria from the mutant and the wild type and only once was this possible. In correspondence with Professor Wills he stated that mitochondria isolated from this mutant were often "unhealthy".

Growth was not observed on 2% ethanol although the authors of the above mentioned paper did observe this. Growth of the *tpyl* mutant, after crossing with a ρ^0 , on YPE was possible. This indicates that the *tpyl* mutation causing the phenotype is a nuclear mutation and not a deletion in the mitochondrial genome.

The type of mutation described by these results is more typical of a classical petite mutation rather than a

mutation in a regulatory gene as described by Wills et al (1986).

More investigation is required if the true nature of the mutant is to be discovered.

3.4.2 Brewing Trials with alpha-cyano-4-hydroxycinnamic acid and a control

From brewing trials using an ale type yeast in the presence and absence of the inhibitor, it can be seen that the amount of ethanol formed, in terms of sugar utilized, is greater in the presence of the inhibitor. This is consistent with the theory that blocking the route to the mitochondria may cause pyruvate to be diverted into the fermentation route. In the control experiment the yeast appear to utilize the vast majority of the available sugar in the first 48 hours and the rate of ethanol production is linear up to this point. The difference in the ethanol production between inhibited and control yeast is counterbalanced by the biomass of cells produced per unit sugar. At 72 hours the amount of cells produced per unit sugar by the inhibited yeast is less than that of the control yeast.

If the 72 hour results were taken on their own then there is a possibility that the difference in levels of ethanol could be due to ethanol utilization by the control yeast as observed by Alexander and Detroy (1983). However the method of estimating ethanol concentration every 24 hours eliminates this possibility.

From table 3.3.1 it can be seen that the inhibited yeast used considerably less sugar than the control. The reason for this difference could be a combination of slow growth and an inability to uptake sugars. Firstly there is the possibility that because the cells are growing slowly (due to changes in mitochondrial function) they are simply taking sugars up at a slower rate. However this seems an unlikely explanation as even with extended fermentations (figure 3.3.5) the inhibited yeast is unable to use all the available sugar. Secondly, in the inhibited yeast there will be lower levels of intracellular ATP because production via oxidative phosphorylation will be virtually zero. This may lead to a decrease in the proton gradient across the plasma membrane. Since the principle sugar in wort, maltose, is taken up by proton symport a drop in the plasma membrane proton gradient could reduce the amount of this sugar taken up. The third possibility is that the mitochondria are directly affecting nuclear genes such as those coding for permeases. This type of phenomena has already been observed by Khan and Greener (1977). They found that deletions in the mitochondrial DNA affected the yeast cells ability to grow on maltose even though the grande parental strain was able to utilize maltose. Other petites, unable to utilize alpha methylglucoside, were able to after treatment with dimethylsulphoxide (DMSO), which makes the plasma membrane 'leaky' to large molecules (Mahler and Wilkie 1978). This suggests the problem with petites is that permease genes are

repressed. It is not yet clear how the mitochondria affects the nuclear genes.

3.4.3 Brewing Trials with the Mutant TPY1 and the Wild Type

From the brewing trials of tpyl against the wild type it can be seen that the mutant produces the same or possibly slightly more ethanol compared with the wild type after 72 hours fermentation.

Since glucose is the only sugar present in the synthetic wort the difference in sugar utilization cannot be due to problems with uptake. Indeed the mutant is eventually able to utilize almost as much sugar as the wild type.

This experiment demonstrates a problem that may arise if a petite yeast were used in commercial brewing. There appears to be a critical density of cells required before fermentation accelerates to its peak. Thus the slower growth rate of petites means they take longer to reach this density. The solution may simply lie in pitching (introducing cells to wort) at a higher density if the yeast is of a petite nature.

CHAPTER 4

Cloning and Sequencing of a Gene Complementing the tpy1
Mutation

4.1

Introduction

From the results in chapter 3 it would appear that the mutation tpy1 may be advantageous in a brewing yeast. In order to introduce this mutation it is first necessary to clone the gene that is mutated in the TPY1 yeast. This can be carried out by complementation of the mutation with a gene contained in a genomic library of S.cerevisiae.

Once cloned the gene can be sequenced to try and establish its true nature. During the sequencing it will be possible to establish a unique site within the gene that may be used as a site for insertion of the G418 resistance gene.

The nature of the gene itself is of interest. In the original paper describing the mutation the authors suggested the mutation may be in the RAS2 gene (Wills et al 1986). Yeast mutants in this gene have been shown to have a reduced ability to grow on pyruvate .

4.2

Materials and Methods4.2.1 DNA vectors

The multicopy plasmid Yep13 containing S.cerevisiae genomic library (Nasmyth and Reed 1980) was supplied by the Leicester Biocentre. The low copy plasmid Ycp50 containing S.cerevisiae genomic library was supplied by Princeton University. The plasmids M13mp18 and M13mp19 were supplied by Boehringer-Mannheim.

4.2.2 Transformation of Yeast (Based on Beggs 1978)

Because of the high number of transformants required for the cloning of a gene from a library, the lithium acetate method of yeast transformation was inadequate and the spheroplast method was used instead.

Cells were grown to $1-2 \times 10^7/\text{ml}$, as previously described, and were then harvested in a bench centrifuge. After washing with 1M sorbitol the cells were placed in 1M sorbitol containing 10mg/ml zymolyase 6000 and 1 $\mu\text{l}/\text{ml}$ 2-mercaptoethanol and incubated at 30°C. Cells were observed under a microscope every 15 minutes. When the cells began to lyse in water they were presumed to have formed spheroplasts and were washed gently three times with 1M sorbitol. The cells were finally resuspended in 1ml of 1M sorbitol and 100 μl of this was added to plasmid DNA. After 20 minutes at room temperature 1ml of 40% PEG 4000 was added to each

transformation mix. After a further 20 minutes at room temperature the cells were heat shocked for 5 minutes at 42°C. Following two washes in 1M sorbitol cells were placed in molten regeneration agar which was then placed on plates containing set regeneration agar and incubated at 30°C for 4-6 days. When colonies had grown they could be removed with a toothpick and transferred to other plates for further testing.

Regeneration Agar

2% Glucose

0.7% Yeast Nitrogen Base Without Amino Acids

1M Sorbitol

20mg/l Uracil, Adenine, and Tryptophan

2% Agar

4.2.3 Small Scale Plasmid Isolation from S.cerevisiae

(Based on Hoffman et al 1986)

Yeast cells containing a plasmid were grown up overnight in selective media. From this culture 3ml were harvested by two high speed spins in a microfuge. The pellet was resuspended in the residual liquid and then 200µl of lysis solution, 200µl of phenol/chloroform and 0.3g of sterile glass beads were added. The mixture was vortexed for 2 minutes and then spun for 5 minutes. The aqueous phase was removed and phenol/chloroform treated. After ethanol precipitation and vacuum drying the pellet was resuspended in 20µl of TE.

Lysis Buffer

2% Triton X-100

1% SDS

100mM NaCl

10mM Tris pH 8.0

1mM EDTA pH 8.0

4.2.4 Transformation of Escherichia coli with M13 DNA.

Transformations using plasmids derived from M13 bacteriophage DNA were carried out essentially as described in chapter 2, with double-stranded or single-stranded plasmid, except that:

-300µl of competent cells were used.

-After heat-shock, Eppendorfs were placed on ice for 5 minutes then 2.8 mls of lawn mix were added. Samples were then transferred into 3mls of H-top maintained at 45°C, mixed quickly and poured onto pre-warmed H-agar plates. When the H-top had set, the plates were inverted and incubated overnight at 37°C. Plaques were stored on the plates at 4°C and used within 48 hours for preparation of DNA.

H-Top.

1% (w/v) Bacto-Tryptone

0.8% (w/v) NaCl

0.7% (w/v) Agar

H-Agar.

1% (w/v) Bacto-Tryptone

0.8% (w/v) NaCl

1.5% (w/v) Agar

2xTY

2% Bacto-Tryptone

1% Yeast Extract

0.5% NaCl

Lawn Mix.2ml E.coli culture

grown overnight in 2xTY

400µl 100mM IPTG

400µl 100mM XGAL

4.2.5 Single-Stranded DNA Preparation.

All steps were carried out at room temperature unless otherwise stated. Plaques were picked from H-top agar using sterile Pasteur pipettes and transferred to 3ml of 2xTY containing 30ul of an overnight E.coli culture. After incubation at 37°C in an orbital shaker at 300rpm for 5 hours, the cells were spun down at 2000g in a bench-top centrifuge leaving phage particles in the supernatant. 1.5ml of supernatant was transferred to an Eppendorf and spun at high speed in a Eppendorf centrifuge. If a cell pellet was visible, the supernatant was transferred to a clean Eppendorf and respun. 1ml of supernatant was transferred to a clean Eppendorf and 200µl of PEG/NaCl solution (20% w/v polyethylene glycol 6000/2.5M NaCl, autoclaved) were added. The tube was shaken and then left to stand for 15 minutes. Phage was precipitated by spinning in an Eppendorf centrifuge at high speed for 5 minutes. The supernatant was removed and the pellets respun for 3 minutes. Care was taken to remove any remaining supernatant, then the viral pellet was resuspended in 100ul TE. The

sample was phenol/chloroform extracted, ethanol precipitated and then resuspended in 30 μ l of TE or SDW.

4.2.6 DNA Sequencing.

Dideoxy Sequencing with Modified T7 DNA Polymerase.

Sequencing was carried out by the dideoxy chain termination method (Sanger et al 1977) using a SequenaseTM DNA sequencing kit from United States Biochemical Corporation.

The chain termination method for sequencing involves synthesis of a DNA strand by DNA polymerase in vitro using a single-stranded DNA template. A synthetic oligonucleotide, complementary to a portion of the DNA template, is annealed to the template and the DNA polymerase initiates synthesis from this of a complementary strand. The primer is extended using dCTP, dGTP, dTTP and radioactively labeled [α -³⁵S] dATP. The synthesis reaction is terminated by random incorporation of dideoxynucleotides triphosphates (ddNTPs) which are nucleotide analogues which lack the 3'-OH required for DNA chain elongation .

SequenaseTM is a modified form of bacteriophage T7 DNA polymerase (Tabor and Richardson 1987) which has low 3' to 5' exonuclease activity and, unlike Klenow fragment, has high processivity therefore DNA strands elongating from the template terminate only when a dideoxynucleotide is incorporated.

Sequencing M13 Single-Stranded DNA Templates.

Single-stranded M13 DNA was prepared as in 4.2.5.

Annealing of the primer to the template was carried out by adding 1 μ l (0.5 pmoles/ μ l) of M13 universal primer and 2 μ l of 5xSequenaseTM buffer (200mM Tris-Cl pH7.5/100mM MgCl₂/250mM NaCl) to 2 μ g of DNA template in 7 μ l SDW, heating to 65°C for 2 minutes then allowing the sample to cool to room temperature. The labelling reaction was carried out according to the manufacturer's instructions by adding 1 μ l 0.1M DTT, 2 μ l diluted labelling mix, 0.5 μ l [α ³⁵S]dATP (10uCi/ μ l Amersham International) and 2 μ l diluted SequenaseTM, followed by incubation at room temperature for 5 minutes. For each sequencing reaction, a set of four Eppendorfs, labelled A,C,G and T, were pre-warmed to 37°C each containing 2.5 μ l of ddATP, ddCTP ddGTP, ddTTP termination mixes respectively. When the labelling reaction was complete, 2.5 μ l of the sample was transferred to each termination mix and the incubation continued at 37°C for a further 5 minutes. 4 μ l of stop solution (95% (v/v) formamide/20mM EDTA/0.05% (w/v) Bromophenol Blue/0.05% (w/v) Xylene Cyanol) were then added to each Eppendorf and the samples stored at -20°C. Half of each sample was run on a sequencing gel (Section 4.2.7). Just before loading onto the sequencing gel, samples were heated to 80°C for 2 minutes.

Sequencing Double-Stranded Plasmid DNA Templates.

Dideoxy sequencing can be readily performed on a double-stranded, closed circular DNA template that has been denatured with alkali (Chen and Seeburg 1985).

To 2 μ g of DNA in a total volume of 9 μ l (made up with SDW),

1 μ l of 2M NaOH/0.02M EDTA was added and the sample incubated at room temperature for 5 minutes. The sample was then neutralised by the addition of 5 μ l 1M sodium acetate pH4.5 and ethanol precipitated by the addition of 40 μ l cold 100% (v/v) ethanol and placing on dry ice for 15 minutes. The sample was then spun at high speed in an Eppendorf centrifuge for 10 minutes at 4°C, the supernatant removed and the pellet dried under vacuum. The pellet was then resuspended in 7 μ l SDW; 1 μ l primer (0.5 μ moles/ μ l) and 2 μ l 5x SequenaseTM buffer were added and sequencing carried out using the SequenaseTM kit as above.

4.2.7 Preparation and Running of Sequencing Gels.

Sequencing gels are high-resolution polyacrylamide gels which are used to fractionate radiolabelled single-stranded oligonucleotides on the basis of size. To reduce the effects of DNA secondary structure on electrophoretic mobility, gels contain 7M urea and are run at sufficient voltage to heat them to approximately 65°C.

Sequencing gel plates (49.8cmx19.8cmx0.5cm) were cleaned thoroughly and wiped with ethanol. The cuspid plate had RepelcoteTM applied according to the manufacturer's instructions, then plates were assembled using 0.4mm spacers. A 6% sequencing gel was prepared by mixing 42ml of 6% acrylamide mix with 252 μ l 10% (w/v) ammonium persulphate and 11 μ l TEMED. The gel was poured immediately by sucking the mixture into a 60ml syringe then slowly squirting it

between the plates, avoiding trapping air bubbles. A shark-tooth comb 0.4mm thick, was inserted upside down 1cm into the top of the gel to create a well, and the gel left to polymerise at room temperature for 1 hour. Once polymerised, the comb and bottom spacer were removed and the exposed surfaces rinsed with distilled water. The gel was secured into the sequencing gel tank (BRL). The bottom reservoir was filled with 1xTBE and the top reservoir with 0.5xTBE. The buffer was squirted between the plates at the bottom of the gel to remove air bubbles. The gel was pre-warmed by running at 45V/cm until hot to the touch. Just before loading the gel, the comb-well was rinsed by squirting buffer into it then the shark-tooth comb was inserted the correct way up with the points just sticking into the gel. After the samples were loaded, the gel was run at 45V/cm for 1.5 hours (until the bromophenol blue dye in the sequencing stop solution was at the bottom of the gel). When a sequence of over 200 bases needed to be read, half the sample was loaded and the gel run for approximately 90 minutes then the other half loaded and the gel run for a further 90 minutes.

After running, the plate treated with RepelcoteTM was removed and the plate with the gel attached, submerged in gel fix for 15-20 minutes. The gel, still on the plate, was carefully removed from the gel fix. A piece of Whatman 3MM paper, slightly bigger than the gel, was laid on top of the gel then gently peeled off with the gel attached. The gel was dried onto the paper, under vacuum for 45 minutes at

80°C. When dry, the gel was exposed to X-ray film (Fuji 35.6cmx43.2cm Medical) overnight. The film was developed in a dark-room using LX24 developer (Kodak) and fixed with UnifixTM (Kodak) according to the manufacturer's instructions.

6X Acrylamide Mix

43g Urea

5ml 10xTBE

15ml 40X Acrylamide mix

Made up to 100ml with SDW

40X Acrylamide Mix.

38% (w/v) Acrylamide

2% (w/v) Bis-acrylamide

(Made up with SDW)

Gel Fix.

10% (v/v) Acetic acid

12% (v/v) Methanol

4.2.8 Reading Sequencing Gels

Reading of gels was carried out using the Beckman Micro-Genie computer programme. The photograph of the gel was read via a digitiser onto floppy disc. Analysis such as restriction enzyme sites and discovery of open reading frames could then be carried out using the package.

4.2.9 Analysis of Sequences

The sequence was further analyzed by use of the UWGCCG package of sequencing programs available on the Daresbury network (Devereux et al 1984). As well as doing analysis such as hydrophobicity and translation the package enables a protein or DNA sequence to be compared against the latest

databases such as Swissprot and NBRF (protein sequences) and EMBL and Genbank (DNA sequences).

4.3

Results4.3.1 Cloning of a Gene Complementing the *tpy1* mutation

Despite numerous attempts to clone the TPY1 gene using the multicopy library no colonies were obtained that were able to grow on glycerol using this genomic library. However one colony was obtained using the low copy library that was able to grow on glycerol. Plasmid DNA was extracted from the yeast cells able to grow on glycerol and was used to transform E.coli. A large scale preparation of plasmid DNA was carried out using E.coli able to grow on ampicillin plates. The plasmid DNA was used to transform the mutant strain of yeast TPY1 and colonies produced were all able to grow on glycerol. The plasmid was named pTPY1A

4.3.2 Restriction Mapping of TPY1 Gene

In order that the gene could be sequenced it was necessary to form a restriction map of the insert. Restriction enzymes used are shown in figure 4.3.1. The map of the gene formed from these digests is shown in figure 4.3.2.

4.3.3 Reducing the Size of the Complementing Fragment

In order that the gene could be sequenced it was also necessary to reduce the size of the fragment that contained the gene using restriction enzymes. This was accomplished as demonstrated in figure 4.3.3. The first change was to remove a 4kb fragment between the two EcoRI sites and ligate the

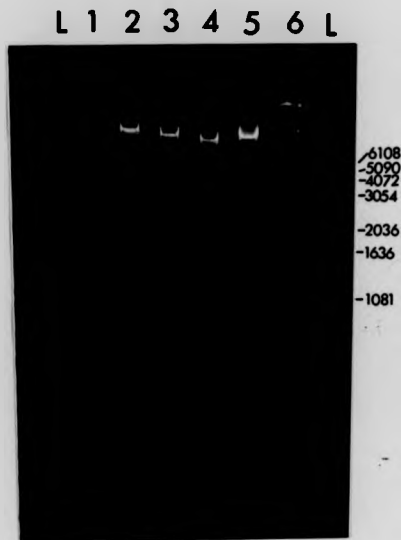


Figure 4.3.1

Restriction digests of pTPY1 a derivative of YCp50. The plasmid contains the insert that complements the tpy1 mutation. The digests are as follows; 1) HindIII, 2) BamHI, 3) ClaI, 4) EcoRI, 5) XbaI, 6) Uncut. Lanes marked L contain the 1kb ladder.

S.cerevisiae Genomic Library In Plasmid Ycp50

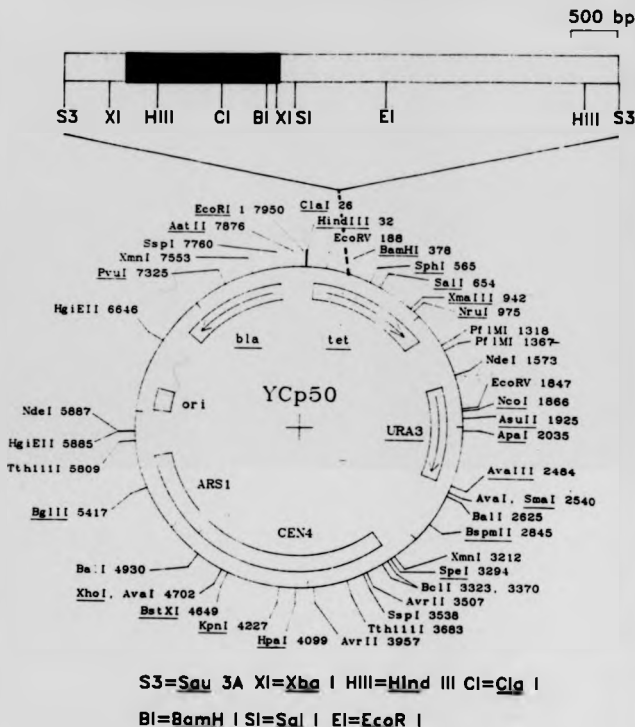


Figure 4.3.2

Plasmid Ycp50 containing fragments of *S.cerevisiae* genomic DNA. The library was constructed by ligation of fragments of a partial *Sau*3A digest of genomic DNA into the unique *Bam*HI site in Ycp50. The insert shown in this figure is the one that complements the *tpv*I mutation. The shaded area represents the open reading frame discovered by the sequencing described in figure (plasmid map taken from Rose et al 1987).

Figure 4.3.3

Complementation of the tpyl mutation by different fragments of the original insert contained in the YCp50 genomic library. Only the major restriction sites contained in the area of interest are shown. The shaded area within the insert represents the open reading frame that complements the tpyl mutation.

Cloning and Subcloning of DNA that Complements the *tpy* 1 Mutation

COMPLEMENTS

S3	XI	HIII	CI	BI	XI	SI	EI	S3	YES
									NO
									NO
									YES
									NO
									NO

S3 = Sau 3A XI = Xba I HIII = Hind III CI = Cla I BI = BamH I SI = Sal I EI = EcoR I

rest of the plasmid back together. This did not complement the mutation. The second change tried was to remove a 2kb fragment between the two ClaI sites and ligating the rest of the plasmid back together. Again this did not complement the mutation. Next the 4kb EcoRI fragment was removed and ligated into the EcoRI site of Ycp50. This did complement the mutation. The fourth change was to remove the 2kb ClaI fragment and ligate this into the ClaI site of Ycp50. This did not complement the mutation. Finally a 2.7kb fragment between the HindIII site and the EcoRI site was removed and cloned into Ycp50. This did not complement the mutation (See figure 4.3.2 for the restriction enzyme sites based in the plasmid).

These patterns suggested the gene was contained between the HindIII site and the ClaI site, with more of the gene in the areas immediately adjacent to this fragment.

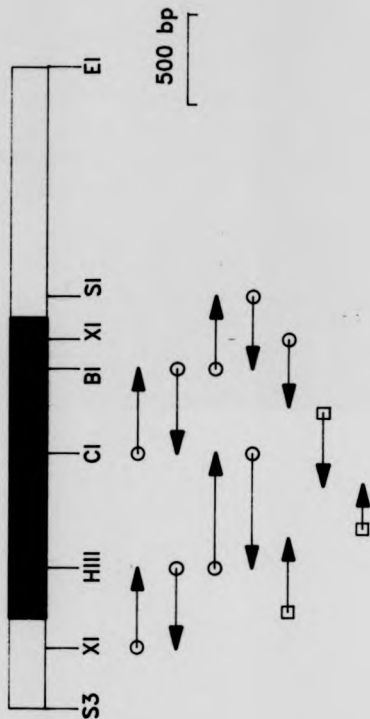
4.3.4 Sequencing Strategy

The sequencing strategy adopted was as shown figure 4.3.4. Four fragments were sequenced. The first was a 500bp fragment between the XbaI site and the HindIII site. The second was a 700bp fragment between the same HindIII site and the ClaI site. Thirdly a 500bp fragment between the same ClaI site and the BamHI site was sequenced. Finally a 300bp fragment between the BamHI and a SalI site was sequenced. The fragments were sequenced in both directions and overlaps were sequenced at the HindIII, ClaI and the BamHI sites.

Figure 4.3.4

The strategy for sequencing the TPY1 gene. Sequencing was carried out with the 'Universal' primer where circular symbols are shown and 'custom made' primers where the square symbols are shown. The shaded area within the insert represents the open reading frame that compliments the tpy1 mutation.

Sequencing Strategy for Gene Complementing *tpy1* Mutation



S3=Sau 3A XI=Xba I HIII=Hind III CI=Cla I BI=BamH SI=Sal I EI=EcoR I

4.3.5 Discovery of an Open Reading Frame

Computer analysis of the sequence showed an open reading frame beginning in the SalI-BamHI fragment and ending in the HindIII-XbaI fragment. This satisfies the conditions laid out by the complementation experiments. No other open reading frames satisfied these conditions. The 2kb of DNA sequenced along with the protein translation of the open reading frame are shown in figure 4.3.5

4.4

Discussion

The gene complementing the tpv1 mutation has been cloned and sequenced. It is 1698 nucleotides long which code for a protein of 566 amino acids (approximately 62 kDa).

The open reading frame does not appear to be an exon of a larger sequence as the consensus splicing signals, GTATGT and TACTAAC, are not present (Teem et al 1984).

The predicted protein obtained from the nucleotide sequence would appear to be soluble rather than membrane bound. There are 72 acidic residues and 66 basic ones. There are no large domains of hydrophobicity (figure 4.4.1). The amino terminus of the protein was inspected for possible targeting activity as this is the area from which most mitochondrial nuclear encoded proteins are directed. The characteristic pattern normally associated with mitochondrial targeting is a large number of positively charged residues (lysine, arginine, serine and threonine) with few if any negatively charged residues. The TPY1 sequence contains 7 threonine and serine residues and 6 lysine and arginine residues in the the first 25 amino acids. The sequence also contains an acidic residue aspartic acid at residue number 23. However this has been observed in previous targeting sequences (Ohmen et al 1988). Comparison with other DNA sequences revealed no similar sequence in the EMBL or Genbank DNA libraries. Comparison of the translated open reading frame with the Swissprot protein database revealed similarity with the FAS2 gene from

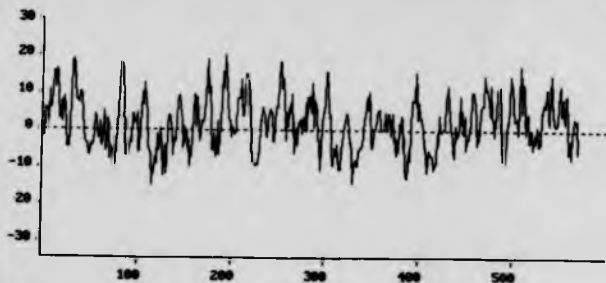


Figure 4.4.1

Hydrophobicity plot of the translated protein from the TPV1 gene. Hydrophobicity is represented as the negative values.

Cispecies: *PENICILLIUM PATULUM* (*PENICILLIUM* *GRISEOFULVUM*).
 CInccession: P15368
 R:WIESNER P., BECK J., BECI K.-F., RPKA S., MUELLER G., LUECKE S.,
 SCHMEIZER E.
 EUR. J. BIOCHEM. 177:69-79 (1988). . . .

SCOPES Init1: 48 Init2: 72 Out: 68
 10.5% identity in 252 aa overlap

	80	90	100	110	120
tpvdna	FELYSFQQLVDSEIRLDHFIHLNRLNHLRMVQWERS-KLSOEFLYPLNFFNVKFLRIF				
Fas2p	HNIVNGDSGLYKKVIAEPRANIKFEFPNLPDMEKEVKPLNENLKGMVNLKVVVVITGF				
	940	950	960	970	980 990
	170	140	150	160	170 180
tpvdna	ISN---VSENPQMGHDTNNEADLMALENATNTHRIEGRLLDNVQAND-PAREKFSFIEB				
Fas2p	SEVGFNGNERTAMEHESKGFLEBCVEMANIMBLIKHNGPLKSGAYSGNAKYTBEPV				
	1000	1010	1020	1030	1040 1050
	190	200	210	220	230 240
tpvdna	IUVDFKN--ENDNVPTKFN-AAPEENATEGSPDKLNLNLPLQFSLP--NGDNSTTTNT				
Fas2p	DDKQVKPKYKMHLEHTGIRLIEPLFKGYDPKKQLLQEIIVQEDLEPFEASKETAEF				
	1060	1070	1080	1090	1100 1110
	250	260	270	280	290 300
tpvdna	DQNNATMGEETAKDKHSTTPKLESVKMGVDPMFVDFDLGDIKRVGSENVCTISILRK				
Fas2p	KREHGDKVEIFEIPESBEYTVRLCKBATHLIP-KALQFDRLVAGQVPTGNDABRYBIPDD				
	1120	1130	1140	1150	1160 1170
	310	320	330	340	350
tpvdna	SSPE-EPFMSYSPQLTAIIGLKSGTSHDAIFSIYKYIHLNELLTNDESAFENLGNFNNH				
Fas2p	IISQDPVTLFLVLTAEAMLGGVDTF--VEFYKYVHLSEVGNICGGGIBGTHRLRMY				
	1180	1190	1200	1210	1220 1230
	360	370	380	390	400 410
tpvdna	NSHTISAKMLDAASSQSVIVKLTQLITLLPSLKESSPDTHKLTOLLINSTHLLPLQ				
Fas2p	IDFLDKPLQKDILOESFINTHSAMVNMLLSSTGPIKTPVGCCATAVESVDIGYETIVE				
	1240	1250	1260	1270	1280 1290

Figure 4.4.2

A comparison between the translated protein from the TPV1A gene and the FAS2 gene from Penicillium patulum.

Penicillium patulum (Figure 4.4.2). This gene codes for the enzyme complex fatty acid synthase, however the similarity would seem to be coincidental since there is less similarity between the FAS2 of *S.cerevisiae* and the TPY1 gene, than there is between the FAS2 gene of *P.patulum* and the TPY1 gene. There was no similarity to the RAS2 gene.

CHAPTER 5

General Conclusions and Discussion5.1 Petite Yeasts and Fermentation

It appears from the experiments discussed in chapter 3 that petite and petita type mutations can be used to improve fermentation performance. Small, but commercially significant, increases in ethanol production per unit sugar have been shown with brewing trials using a chemical inhibitor and petite mutations in haploid yeast strains.

Although there is little oxygen present in the brewing process the mitochondria obviously have some role to play. This can be seen from two facts. Firstly, as mentioned above, the petites produce more ethanol per unit sugar than their wild type counterparts. Secondly the petites produce less of their own biomass than the wild type. Thus although the mitochondria may not be respiratory functional during the brewing process, something about their condition affects the rest of the cell.

This type of regulation has been demonstrated before by Farikh and co-workers (1987, see introduction). The authors showed that abundance of some nuclear transcripts was affected by the condition of the mitochondrial genome. Although the petites reported in this thesis are nuclear they do affect the condition of the mitochondria. It may be that this is detected somehow by the cell and expression of enzymes involved in the fermentative pathway is enhanced. It

has been found previously that levels of the key fermentative enzymes PDC and ADH were important in determining the rate of fermentation (Sharma and Tauro 1986).

5.2 The Nature of the *tpv1* Mutation

If the work reported in this thesis were taken on its own, the *tpv1* mutation would probably be classified as a nuclear petite mutation. The inability of the mutant to grow on non-fermentable media, and the fact that isolated mitochondria are unable to produce a proton gradient in the presence of NADH, suggests this to be the case. However in the original paper describing the mutant (Wills et al 1986) and in correspondence with Professor Wills it is clear that the authors were able to isolate mitochondria that were capable of state 3 respiration.

The gene that complements the *tpv1* mutation is probably not a membrane bound transporter but a soluble protein targeted to the mitochondria. It has little similarity to the *RAS2* gene discussed as a possible site for the mutation in the original paper (Wills et al 1986). The only protein with which a significant amount of similarity was found was the *FAS2* gene from *Penicillium patulum* (Weisner et al 1988). There was 18.3% identity or 58.3% similarity over 252 amino acids. The area of this gene that was similar to *TPV1* has no specific function, although there was some overlap with the ketoacyl synthase domain of the *FAS2* gene.

5.3 Future Work

It is still not clear why petites and simulated petites (Chemically inhibited) use less sugar than their wild type counterparts, over the same period of time. They are pitched at the same cell density and, although there is some oxygen present in the wort at first, the respiratory competence of the wild type would not be enough to create the large differences in sugar utilization. One of the possible explanations, mentioned in chapter 3, was that uptake of a particular sugar could be responsible for the difference in sugar levels. This could be tested by examining the sugar profile at the end of a brewing trial. That is examine the proportions of sugars remaining after 72 hours of brewing. This could then be compared to proportions of sugars present at the start. Any sugar present in greater proportions at the end compared to the start could then be tested as a carbon source for the petites.

As mentioned earlier in this chapter it has been found that the quality and quantity of the mitochondrial genome can affect certain nuclear transcripts (Parikh et al 1987). Although the petites dealt with here are nuclear, their presence may cause damage to some obsolete mitochondrially coded genes. It would seem logical that if the mitochondria were damaged then the cell would detect this and try to increase the glycolytic flux. This could be achieved by increasing the levels of certain key enzymes. To try and detect such an increase of transcripts in petite cells,

probes could be made to various key fermentation enzymes and levels compared with the wild type.

The nature of the tpv1 mutant is still unknown. It appears to be targeted to the mitochondria but to be sure of this targeting experiments would have to be carried out.

To be certain that the cloned gene is the wild type form of the mutant, and not a suppressor of the mutation, the gene must be disrupted in another yeast (as described in chapter 2). This is being carried out at present.

APPENDIX 1

An Attempt to Transfer Mitochondria Between Industrial Yeast Strains

A1.1 Introduction

It has been known for some time that certain strains of S.cerevisiae have advantages over others in terms of fermentative performance. In one case an increase in the rate of ethanol production was found to be linked with the intracellular levels of the key fermentation enzymes pyruvate decarboxylase and alcohol dehydrogenase (Sharma and Tauro, 1986). In another case the ethanol tolerance of wine yeasts appeared to be linked to the mitochondrial genome (Jimenez and Benitez, 1988).

In the lager type yeasts, formerly known as Saccharomyces calshbergensis, the respiratory rate is particularly low compared with other brewing strains of S.cerevisiae. This suggests that fermentative rates may be higher in lager type yeasts than in other brewing yeasts. As respiration takes place in the mitochondria it may be possible to transfer this low respiratory capacity to another strain by transfer of the mitochondria. This may then lead to the strain with the lager type mitochondria possessing greater fermentative powers than before the transfer.

To transfer the mitochondria between two yeast strains requires the use of either hybridisation (Jimenez and Benitez, 1988) or spheroplast fusion (Ferenczy and Maraz 1977), both of which are described in the main introduction.

In the case of industrial yeasts, which are usually polyploid, spheroplast fusion must be used. To avoid nuclear fusion during transfer of mitochondria an intermediate strain of yeast is used as a carrier. The strain used must possess the mutation karl. Yeasts possessing this mutation are able to conjugate with other yeasts but there is no nuclear fusion (Conde and Fink, 1976). The technique of mitochondrial transfer involves fusion of the donor strain with a karl rho⁰ yeast (is a yeast strain with no functional mitochondrial genome). The receipt of the mitochondria is detected by the ability of the karl mutant to grow on a non-fermentable source such as glycerol. The karl rho⁺ strain is then fused with the receiving yeast strain which is a rho⁰ strain. The final strain is then selected by its ability to grow on glycerol. The process is described in figure A.1.1.

Transfer of Mitochondria Between Industrial Yeasts

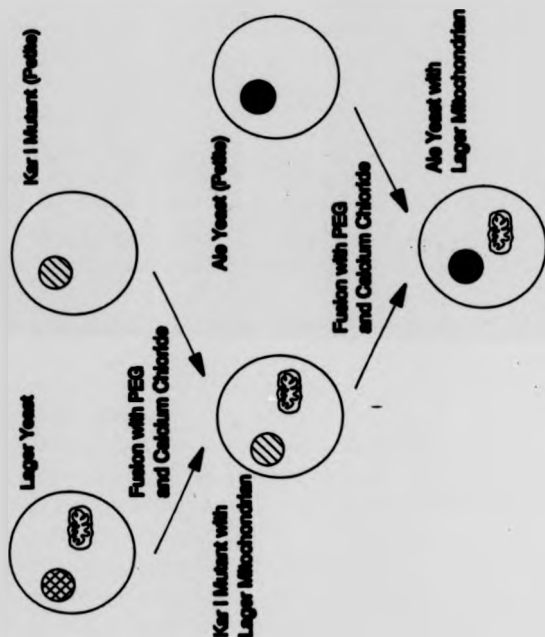


Figure A1.1

Method of transfer of mitochondria between two industrial strains of yeast (for details see text).

A1.2

Materials and Methods

Polyethylene glycol 4000 was supplied by BDH, Beta-glucuronidase was supplied by Boehringer-Mannheim, all other reagents were of 'AnalR' grade.

A1.2.1 Yeast Strains

Ale Yeast Y9- Industrial strain supplied by Whitbread PLC.

Lager Yeast Y7- Industrial strain supplied by Whitbread PLC.

K5-5A rho⁰- Mat alpha, his4-15, ade2-1, cav-1, kar1-1 (Conde and Fink 1976), supplied by Whitbread PLC.

A1.2.2 Spheroplast Preparation (Based on Farahnak et al 1986)

The treatment described below was carried out on the two strains to be fused.

Yeasts were grown in 10 ml of YPD media to stationary level. From these cultures 0.1 ml (strain dependent) were used to inoculate 10 ml of fresh YPD and these were incubated with aeration at 30°C overnight. Half of these cultures were pelleted by centrifugation in a bench centrifuge at 5000 rpm. The pellets were washed in 2 ml of a 0.1M sodium acetate, pH 5.3, then resuspended in 2 ml of the same solution containing 10mM Beta-Mercaptoethanol. After 10 minutes at room temperature the cells were washed twice in sodium acetate and finally resuspended in 0.8 ml of spheroplasting solution. After 40 minutes at 37°C the cells

were checked for spheroplasting by placing the cells in water and microscopically observing the number of intact cells. If more than 90% of the cells burst they were assumed to be ready for fusion.

Spheroplasting Solution

0.1M Sodium Acetate pH 5.3

0.7M Sorbitol

20 μ l Beta-Glucuronidase (10000 units/ml)

A1.2.3 Fusion of Spheroplasts (Based on Farahnak et al 1986)

After spheroplasting treatment the cells were washed three times in washing solution (3000 rpm in a bench centrifuge). After the final wash the cells were pelleted together at 3000 rpm. The cells were resuspended in residual fluid and then 1 ml of fusion solution was added.

After 20 minutes at 37°C the cells were diluted to 10^{-4} and 10^{-5} in washing solution. Aliquots of 0.2 ml were added to 10 ml of regeneration agar (maintained at 45°C) and the mixture was poured onto plates containing set regeneration agar.

Washing Solution

0.1M Sodium Acetate pH 5.3

10mM Calcium Chloride

0.7M Sorbitol

Fusion Solution

0.1M Sodium Acetate pH 5.3

50% PEG 4000

10mM Calcium Chloride

A1.2.4 Testing of Regeneration Products

After regeneration (4-6 days) colonies were removed from agar with a toothpick and placed on selective media.

A1.3**Results**

An attempt was made to introduce the mitochondria from a lager strain of yeast into an ale strain of yeast (as described in the introduction). However no transfer of mitochondria was observed at the first stage of transfer ie transfer of lager strain mitochondria into a kar1 mutant. Protoplasting and regeneration were demonstrated by controls in the above mentioned experiments. Spheroplast fusion was demonstrated by fusing two Mat alpha strains C2 and JRY188.

A1.4

Discussion

The frequency with which mitochondria are supposed to transfer between two strains of yeast has been worked out to be, on average, 10^{-4} by one laboratory (Iserantart, personal communication). It may be that the fusion event did not take place at a high enough frequency to allow transfer of mitochondria.

Another possibility for the lack of success could be poor cytoplasmic mixing. During experiments carried out by Sena (Sena 1982), the author found that transfer of mitochondria from a karl mutant to a petite was proving difficult. The problem proved to be poor cytoplasmic mixing in the zygote. This could be the situation in the case of fusion between the lager strain and the karl petite.

APPENDIX 2

Results and Discussion of Chapter 2

Preliminary brewing trials with the pet122 mutant, suggest there is little difference in fermentation performance between it and the wild type yeast C2 (Table A2.1).

Why should it be that there are differences in fermentation performance between inhibited yeast (inhibited with alpha-cyano-4-hydroxycinnamic acid) and uninhibited yeast, and the tpy1 mutant and the wild type, but not the pet122 mutant and its wild type?

The first possibility is that the change in performance goes unnoticed in this strain of yeast. The inhibitor trials were carried out with a polyploid brewing strain of yeast whereas the pet122 brewing trials were carried out with a haploid laboratory strain which was able to utilize maltose. This strain has recently been shown to be unable to utilize maltotriose (Whitbread PLC, personal communication). If the difference in previous brewing trials were due to an inability of the mutant/inhibited yeast to utilize maltotriose then this would not be apparent in a C2 brewing trial.

The second possibility is that the pet122 mutation does not cause the same changes as those observed previously. The mutation is effective at the cytochrome oxidase complex, the final complex in the chain of electron carriers. The inhibitor and tpy1 are both thought to work at the level of pyruvate transport into the mitochondria, although this

makes no real difference in terms of mitochondrial respiratory activity it may be that any signal telling the cell that the mitochondria are not active only occurs when certain parts of the respiratory chain are inactivated.

Table A2.1.

This shows the results of a brewing trial carried out with the mutant pet122 and the wild type C2. These were large scale (500 ml) fermentations carried out over a period of 144 hours at the Whitbread laboratories in Luton by Mr John Hossack. Gravity of beer is a measure of the liquid density compared to water which is 1000. The levels of ethanol were determined by Gas Chromatography.

Brewing Trials with the pet122 mutant and the Wild Type C2.

		0	Time (hours)		144
			24	48	
Gravity of Beer	pet122	1051	1035	1029	1016
	Wild Type	1051	1037	1028	1016
		Time (hours)			
		144			
% Ethanol in Beer	pet122	4.71			
	Wild Type	4.71			
		Time (hours)			
		144			
Wet Weight of Yeast (g)	pet122	7.0			
	Wild Type	6.9			
		Time (hours)			
		144			
% Ethanol /unit sugar	pet122	0.135			
	Wild Type	0.135			
		Time (hours)			
		144			
Cells (g) /unit sugar	pet122	0.2			
	Wild Type	0.2			

References

- Alexander, N. J. and Detroy, R. W. (1983) *Biotech. Letts.* 5: 165-168
- Aragon, J.J. and Sanchez, V. (1985) *Biochem. Biophys. Res. Comm.* 131: 849-853
- Ausebel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Editors) (1989) 'Current Protocols in Molecular Biology' John Wiley and Sons, New York.
- Baker, K.P. and Schatz, G. (1991) *Nature* 349: 205-208
- Beggs, J.D. (1978) *Nature* 275: 104-109
- Bernadi, G., Carnevali, F., Nicolaiff, A., Piperno, G. and Tecce, G. (1968) *J. Mol. Biol.* 37: 493-505
- Bonnichsen, R. (1965) 'Methods of Enzymatic Analysis' Ed. Bergmeyer, H-U.
- Bowman, S. (1989) PhD Thesis, University of Warwick, Coventry, England.
- Chen, E.Y. and Seeberg, P.H. (1985) *DNA* 4: 165-170
- Cohen, S.N., Chang, C.Y. and Hsu, L. (1972) *Proc. Natl. Acad. Sci.* 69: 2110-2114
- Colson, A.M., Goffeau, A., Briquet, M., Weigel, P. and Mattoon, J.R. (1974) *Mol. Gen. Genet.* 135: 309-326

Conde, J. and Fink, G.R. (1976) *Proc. Natl. Acad. Sci.* 73: 3651-3655

Devereux, J., Haeberli, P. and Smithes, O. (1984) *Nucl. Acid Res.* 12: 387-395

Farahnak, F., Seki, T., Ryu, D. D. Y. and Ogrydziak, D. (1986) *Appl. Enviro. Microbiol.* 51: 362-367

Ferenczy, L. and Maraz, A. (1977) *Nature* 268: 525-526

Gjermansen, C., Kielland-Brandt, M.C., Holmberg, S. and Nilsson-Tillgren, T. (1991) Yeast Genetics and Molecular Biology Meeting, Poster Presentation, San Francisco, California

Gottschalk, G. (1979) 'Bacterial Metabolism' Springer Verlag, Berlin.

Grivitz, S.C., Bacchetti, S., Rainbow, A.J. and Graham, F.L. (1980) *Anal. Biochem.* 106: 492

Hadfield, C., Jordan, B. E., Mount, R. C., Pretourius, G. H. J. and Burak, E. (1990) *Curr Genet.* 18: 303-313

Halestrap, A.P. (1978) *Biochem. J.* 172: 377-387

Halestrap, A.P. and Denton, R.M. (1974) *Biochem. J.* 138: 313-316

Hartl, F-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) *Biochem. Biophys. Acta.* 988: 1-45

Hase, T., Reizman, H., Suda, K. and Shatz, G. (1983) *EMBO J.* 2: 2169-2172

- Helgersson, S.L., Requadt, C. and Stoekenius, W. (1983) *Biochemistry* 22: 5746-5753
- Heniaisch, J. (1986) *Mol. Gen. Genet.* 202: 75-80
- Hill, J., Donald, K.A.G. and Griffiths, D.E. (1991) *Nucl. Acid Res.* 19: 5791
- Heredia, E.F., Sols, A. and De la Fuente, G. (1968) *Euro. J. Biochem.* 5: 321-326
- Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) *Proc. Natl. Acad. Sci. (U.S.A.)* 75: 1929-1933
- Hoffman, C. S. and Winston, F. (1987) *Gene* 57: 267-272
- Hollenberg, C. P. and Strasser A.W.M. (1990) *Food Biotech.* 4: 527-534
- Holmes, D.S and Quigley, M. (1981) *Anal. Biochem.* 114: 193-197
- Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bact.* 153: 163-168
- Jones, R.M., Russell, I. and Stewart, G.G. (1987) 'Yeast Biotechnology' (Ed. Berry, D.R., Russell, I and Stewart, G.G.) Allen and Unwin, London
- Jimenez, J. and Benitez, T. (1988) *Curr. Genet.* 13: 461-469
- Khan, N.A. (1982) *Mol. Gen. Genet.* 186: 40-43
- Khan, N.A. and Greener, A. (1977) *Mol. Gen. Genet.* 150: 107-108

- Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N. and Neupert, W. (1990) *Nature* 348: 610-616
- Mahler, H.R. and Wilkie, D. (1978) *Plasmid* 1: 125-133
- Mewes, H-W. and Rafael, J. (1981) *FEBS Letts.* 131: 7-10
- Mitchell, P. (1961) *Nature* 191: 144-148
- Nasmyth, K. and Reed, S.I. (1980) *Proc. Natl. Acad. Sci. (U.S.A.)* 77: 2119-2123
- Ohba, M. and Shatz, G. (1987) *EMBO J.* 6: 2109-2115
- O'Malley, K. Pratt, P., Robertson, J., Lilly, M. and Douglas, M.G. (1982) *J. Biol. Chem.* 257: 2097-2103
- Ohmen, J. D., Kloeckener-Gruissem, B. and McEwan, J.E. (1988) *Nucl. Acid Res.* 16: 10783-10802
- Ostermann, J., Horwick, A.L., Neupert, W. and Hartl, F-U. (1989) *Nature* 341: 125-130
- Papa, S., Francaville, A., Paradisi, G. and Meduri, B. (1971) *FEBS Letts.* 12: 285-287
- Parikh, V.S., Morgan, M.M., Scott, R., Clements, L.S. and Butow, R.A. (1987) *Science* 235: 576-580
- Pfanner, N., Hoeben, P., Tropschug, M. and Neupert, N. (1987) *J. Biol. Chem.* 262: 14851-14854
- Pfanner, N., Hartl, F-U. and Neupert, W. (1988) *Eur. J. Biochem.* 175: 205-212

- Pfanner, N. and Neupert, W. (1990) *Annu. Rev. Biochem.* 59: 331-353
- Phaff, H.J., Miller, M.W. and Mrak, E.M. (1978) 'The Life of Yeasts' Harvard University Press, Cambridge Massachusetts
- Pollock, R.A., Hartl, F-U., Cheng, M.Y., Ostermann, J., Horwich, A. and Neupert, W. (1988) *EMBO J.* 7: 3493-3500
- Racker, E. and Stoekenius, W. (1974) *J. Biol. Chem.* 249: 662-663
- Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. and Fink, G. R. (1987) *Gene* 60: 237-243
- Rothstein, R.J (1983) *Methods in Enzymology* 101: 202-209
- Russell, I. and Stewart, G.G. (1979) *J. Inst. Brev.* 85: 95-98
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) 'Molecular Cloning a Laboratory Manual' Second edition, Cold Spring Harbour Press.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci.* 74: 5463-5467
- Schleyer, M. and Neupert, W. (1985) *Cell* 43: 339-350
- Sena, E.P. (1982) *Curr. Genet.* 5: 47-52
- Serrano, R. (1977) *Euro. J. Biochem.* 80: 97-102
- Serrano, R., Gancedo, J.M. and Gancedo, C. (1973) *Euro J. Biochem.* 34: 479-482

- Sharma, S. and Tauro, P. (1987) *Biotech. Letts.* 9: 585-586
- Slominski, P.P., Perodin, G. and Croft, J.H. (1968) *Biochem. Biophys. Res. Comm.* 30:232-239
- Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N. and Neupert, W. (1989) *Cell* 59: 1061-1070
- Sollner, T., Pfaller, R., Griffiths, G., Pfanner, N. and Neupert, W. (1990) *Cell* 62: 107-115
- Stryer, L. (1988) 'Biochemistry' Third Edition, W.H. Freeman, New York
- Tabor, S. and Richardson, C.L. (1987) *Proc. Natl. Acad. Sci.* 84: 4767-4771
- Teem, J.L., Abrovich, N., Kaufer, N.F., Schwindinger, W.F., Warner, J.R., Levy, A., Woolford, J., Lear, R.J. van Raamsdonk-Duin, M.M.C., Mayer, W.H., Planta, R.J. and Schultz, L. (1984) *Nucl. Acid Res.* 12: 8295-8312
- Tubb, R.S., Brown, A.J.P., Searle, B.A. and Goodey, A.R. (1981) 'Current Developments in Yeast Research' (Ed. Stewart, G.G. and Russell, I) Pergamon Press, New York
- Tzagoloff, A. and Dieckman, C.L. (1990) *Microbiol. Rev.* 54: 211-225
- Van Soligen, P. and Van der Platt, J.B. (1977) *J. Bact.* 130: 946-947
- Wiesner, P., Beck, J., Beck, K-F., Ripka, S., Muller, G., Lucke, S., and Schweizer, E. (1988), *Eur. J. Biochem.* 177: 69-79.

Wills, C., Martin, T. and Melham, T. (1986) Arch. Biochem. Biophys. 246: 306-320

Yang, M., Jensen, R.E., Yaffe, M.P., Oppliger, W. and Shatz, G. (1988) EMBO J. 7: 3857-3862

Young, S. J., Vieth, W. R. and Matsuura, T. (1991) Biotech. Bioeng. 37: 587-590