GENETIC MANIPULATION AND BIOCHEMICAL STUDIES OF *SACCHAROMYCES CEREVISIAE*

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This work is dedicated to the memory of my father James I. Hill (6th October 1942-18th December 1991) and to my mother, Jessie.

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LIST OF ABBREVIATIONS

AAC	ADP/ATP carrier
ADP	Adenosine-5'-diphosphate
ALDC	α-acetolactate decarboxylase
Amp	Ampicillin
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine-5'-triphosphatase
BSA	Bovine serum albumine
CIP	Calf intestinal alkaline phosphatase
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
ETC	Electron transport chain
$FAD/FADH_2$	Flavin adenine dinucleotide oxidised/reduced forms
GC	Gas Chromatography
HEPES	N-2-Hydroxyethylpiperazine-N'-2 ethane sulphonic acid
LMP	Low melting point
oli	Oligomycin
PEG	Polyetheylene glycol
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SX-1	Di-(2,3,5,6-tetrafluorophenyl) amine
Tris	Tris (hydroxymethyl)methylamine
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

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DECLARATION

All the work described in this thesis is original and was carried out by the author.

James Hill

(September 1991)

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James Hill, K., Allen G. Donald, David, E. Griffiths (1991) "DMSO-Enhanced Whole Cell Yeast Transformation" *Nucleic Acids Research* 19 (20), 5791.

SUMMARY

The brewing properties of an industrial strain of *Saccharomyces cerevisiae* were investigated by laboratory scale brewing trials in the presence or absence of an uncoupler of oxidative phosphorylation (SX-1). When SX-1 was added the change in specific gravity of brewing wort with respect to time was less, yeast produced less biomass and more ethanol per unit drop in specific gravity than the control. Similar fermentation properties were observed for C2, a haploid laboratory yeast strain with the ability to ferment maltose. Recombinant DNA technology was used to generate a C2 *pet* mutant, specifically in the *ATP12* gene, which encodes a protein essential for mitochondrial ATP synthesis. In brewing trials a comparison of C2 and C2:Δ*ATP12* shows similar results to C2 fermentations in the presence or absence of SX-1 although the effects of SX-1 are more dramatic than with C2:Δ*ATP12*. Together, the results of chemical studies and gene disruption mutagenesis suggest that mitochondrial ATP synthesis affects nuclear functions, although the possibility that results obtained are a consequence of changes to the yeast mitochondrial DNA cannot be eliminated.

Problems experienced with yeast DNA transformation protocols lead to the development of a new transformation method that is quicker and more efficient than the standard protocol. Initial studies revealed that DMSO could enhance yeast transformation efficiency, and that the optimal concentration of DMSO used is strain specific. The point at which DMSO was added was found to be important, with maximal transformation efficiency achieved when DMSO was added just before heat shocking. The optimised protocol for *S. cerevisiae* JRY188 routinely enhanced transformation 15- to 25-fold compared with a control transformation protocol. The osmotic condition was found to be important for DNA uptake as transformation was inhibited if yeast were washed in 1M sorbitol and selected on plates containing the same. Significantly, DMSO enhanced transformation even in the absence of cations, therefore this method may prove useful for yeasts which transform poorly by existing cationic-based yeast transfomation methods.

CHAPTER 1
INTRODUCTION

The brewing process, one of the oldest and largest biotechnologies, is continually being modified to incorporate information from research and development. Improvements in brewery design, and better control of fermentation are two areas that have improved the quality and efficiency of beer production. A current area of active research is the production of new yeast strains with improved brewing properties. This chapter is written to explain the brewing process and highlight recent innovations that afford the brewer oppurtunities to improve the brewing process, with particular emphasis placed on genetic engineering of brewing yeast. The initial aim of this project was to investigate the importance of yeast mitochondrial function during fermentation. Biochemical and genetic studies were made to analyse the effect of yeast deficient in oxidative phosphorylation, as described in chapters 2, 3 and 4. During the course of these studies an improved method for introducing heterologous DNA into yeast was discovered, as discussed in chapter 5.

1.1 HISTORY OF BREWING

Ethanol production by fermentation is widely believed to have been discovered by the early Neolithic people as they spread through Western Europe, bringing with them the skill to cultivate wheat and barley (Whitbread & Co., 1951). Beer and wine production have been dated at around 8000 B.C, preceding baking by about 5000 years (Enari, 1987). Yeast is therefore one of Mankind's oldest domestic

"pets", although its role, or even its presence during fermentation were not discovered until relatively recent times.

Large scale brewing of beer was first introduced into Britain by the Normans, who built abbeys and monasteries, each with a brewery to produce beer for "the refreshment of both monk and wayfarer" (Whitbread & Co., 1951). Surprisingly little has changed with regard to the fundamental principles of brewing since Norman times, although present-day brewing is on a far greater scale. Fermentation has remained a batch process in which an aqueous extract of barley is converted into ethanol, carbon dioxide and essential flavour components.

The first major changes in the process resulted as a consequence of the work of Louis Pasteur, who in the 1860's proved that "alcoholic fermentation is an act correlated with the life and organisation of the yeast cells" (Anderson, 1989). Prior to this, the generally accepted theory was that fermentation was caused by decomposition of the sugar solution upon exposure to air, with subsequent release of carbon dioxide and ethanol; sedimented yeast was regarded as a non-crystalline solid and not as a living organism (Anderson, 1989). The work of Pasteur, and others, such as Horace Brown at Burton upon Trent, contributed towards better standards of hygiene within breweries with subsequent reductions in the waste of beer due to microbial spoilage. This era is regarded as the Golden Age of Brewing, during which the industry was at the forefront of biochemical research.

The most significant application of Pasteur's findings was the

introduction of pure culture fermentation (using a single, defined strain), primarily at the Carlsberg brewery in Denmark in 1883. This practise spread to other Scandinavian breweries, completely transforming the lager brewing industry. Pure culture brewing was greeted with enthusiasm in Britain, but attempts to reproduce the distinctive taste of nineteenth century beer by pure culture methods failed. This type of beer was brewed at a high sugar concentration, typically 1.060 specific gravity (c.f. 1.040 today), and had a distinctive flavour as a consequence of secondary fermentation by opportunistic wild yeast (Anderson, 1989). Attempts to reproduce the same quality of beer by pure culture methods would have been difficult, as the effect of wild yeast was not fully understood at that time.

Today, much more is known about the role of yeast in the brewing process. This knowledge has been combined with advances in engineering technology to produce beer of more consistent quality at a higher level of efficiency. This is highlighted by the current trend in modern brewing for fermentations to be performed in large cylindrical vessels, which typically hold between 100,000 and 200,000 litres of beer (Hammond, 1988). Increasing the scale of fermentation has many advantages, such as cheaper running costs and reductions in beer loss. There is, however, the risk of increased losses if anything goes wrong. A number of examples of advances in engineering technology being applied to the brewing process are discussed below. Brewers are also looking to yeast as a means of improving fermentation. Current recombinant DNA technology allows brewers to stably intoduce foreign DNA into brewers yeast or modify existing yeast genes. A number of potential applications of genetic

engineering are given, but first we outline the various stages used to produce beer from essentially four ingredients: barley, water, hops and yeast.

1.2 THE BREWING PROCESS

The brewing process consists of a series of distinct batch processes that culminate in the production of a beer of characteristic alcohol content and taste. **Figure 1.2.1** details the various stages and the raw materials that go into making beer.

Germinating barley grain is used as a source of sugar in the brewing process. The barley grains are soaked in water and allowed to germinate. A large number of hydrolytic enzymes are produced during germination, which begin to degrade the starch stores of the seed. Germinated barley is dried in a kiln by gentle heating to yield malted barley, which can be stored for long periods. The skill of malting is to produce enough starch-degrading enzymes without too much digestion of the barley starch reserves.

Malted barley is mixed with warm water (typically at 65°C) during mashing. The barley enzymes produced during malting start to degrade the barley starch and proteins to produce a sugary liquid known as wort. Mashing conditions are carefully selected by the brewer to produce a distinctive sugar profile for each beer that consists of mixtures of fermentable and non-fermentable sugars. The majority of non-fermentable sugars present in the wort after the mashing is a polymer of glucose known as dextrin. Brewers who produce low carbohydrate beers add commercially available

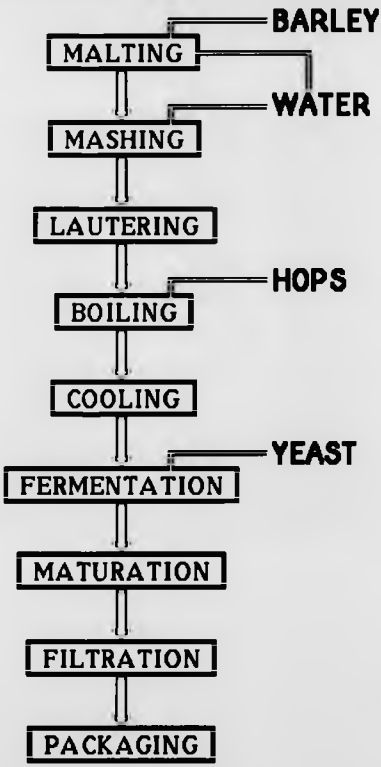

Figure 1.2.1: Schematic representation of the brewing process. Details of each step are given in the text.

amyloglucosidases that break down dextrin into fermentable sugars, thus reducing the carbohydrate content of the final product. After washing, the wort is separated from the spent grain by a filtration process known as lautering.

Hops are added to the filtered wort which is then boiled. The boiling step releases bitter flavour and antimicrobial compounds from the hops and sterilizes the wort. It also serves to fix the level of fermentable sugar produced during mashing by inactivating the barley amyloglucosidases, and is used to fix the specific gravity of the wort by boiling off excess water used to extract maximum sugar levels during lautering. The sterile wort is then cooled, and yeast are added to start fermentation.

During the course of fermentation the fermentable sugar present in the wort is converted into ethanol, carbon dioxide, biomass and trace amounts of essential flavour components. The state of fermentation can be followed by measuring the specific gravity of the wort, as wort gravity is directly proportional to the sugar concentration, or by following ethanol and carbon dioxide production.

At the end of fermentation the "green" beer is separated from the spent yeast and matured for a period of up to six weeks at 0-1°C. This allows removal of various undesirable flavour components produced during fermentation, and precipitation of wort solids by sedimentation.

After maturation many beers are finely filtered and pasteurized, then packaged into cans, bottles or kegs. This type of beer is very

stable and typically has a long shelf-life. A different approach is taken when brewing so-called "real-ale". The "green" beer is matured in casks (cask conditioned ale). More sugar is added to casks to allow secondary fermentation that gives the beer a distinctive taste, and naturally carbonates the beer.

1.3 PROBLEMS ASSOCIATED WITH BEER PRODUCTION

The simplified picture of brewing does not dwell on any of the problems faced in brewing. Due to the scale involved in brewing, the potential losses are great, especially when one considers that the tax paid by the brewer is based on the initial gravity of the wort. Recently, the Chancellor of the Exchequor indicated that taxation may soon be based on the alcohol content of the final beer (Lamont, 1991), the only change to beer taxation since that made by Gladstone (Whitbread & Co., 1951). The major problems faced by brewers are:

1. *Production of off-flavours.* Fermentation produces a number of compounds that give the beer a distintive flavour. Other compounds, such as diacetyl, acetaldehyde and sulphur-containing compounds are undesirable and have to be removed during a maturation period typically at 0-1°C for up to six weeks. This step adds considerable time and cost to the brewing process.

2. *Formation of hazes.* A number of components of wort are implicated in the formation of undesirable hazes. Sugars, proteins, and large ring-structured chemicals are all known to influence the susceptibility of beer to produce haze formation,

especially during chill storage.

3. *Microbial spoilage*. Several species of bacteria can be found in breweries and some, notably acetic acid bacteria, can contaminate beer, altering the taste and ethanol yield. Similar changes are induced by opportunistically-infecting wild yeast strains. These often contain a "killer-factor" which facilitates their genetic takeover by inhibiting growth of the brewing strain.

4. *Inconsistent fermentation*. Variation in the time taken to complete fermentation, and the quality of the beer produced, can be a consequence of a number of factors such as the physiological state of the yeast and the composition of the wort. More consistent fermentation times can reduce the cost of brewing considerably.

Many of the above problems are being tackled by the brewing industry. A number of examples of applied technology (engineering and genetic) being used as a solution to existing problems are now discussed.

1.4 TECHNICAL IMPROVEMENT OF BREWING

Technology is available that can be applied to solve problems incurred during the brewing process, or make it more efficient. A number of these are now discussed. Initially non-genetic methods are dealt with; the use of genetically modified barley and yeast will be covered later.

Brewing Low Alcohol Beers

Low Alcohol beers are traditionally produced by premature termination of fermentation or by removing alcohol from the final beer (Muller, 1990). Premature termination of fermentation often produces a beer that is microbiologically unstable, due to the presence of fermentable sugars in the final product. Alcohol removal is an expensive process and not always specific for ethanol, which can result in the loss of desirable flavour compounds. An alternative approach based on the difference in thermostability of barley α- and β-amylases solves these problems.

It has been shown that at 75-80°C α- and β-amylase enzymes behave quite differently; β-amylase is inactivated rapidly whereas α-amylase remains active (Muller, 1991). These amylolytic enzymes are produced by the germinating barley seed to hydrolyse the starch reserves in the endosperm. Barley α-amylase activity produces a mixture of fermentable and non-fermentable sugars, whereas β-amylase only produces fermentable sugars. Careful control of mashing conditions can therefore be used to control the level of fermentable sugar in the wort and hence the ethanol content of the final beer. This process can be used to produce beer that is low in alcohol (<1.2%) and high in unfermentable dextrins, which give the beer more body, compensating for the reduction in alcohol content.

Preventing Beer Spoilage by Bacterial and Wild Yeast Infection

During fermentation yeast produce a hostile environment that is low in oxygen and generally below pH4. High levels of ethanol and hop-derived microbially active compounds contribute to make beer an

inherently microbiologically stable liquid. Despite these conditions, problems of microbial contamination do exist in brewing, mainly in the form of gram-negative acetic acid bacteria of the genera *Lactobacillus* and *Pediococcus*, which can cause fermentations to have excess turbidity, acidity, and off-flavours (Ogden *et al*, 1988).

Nisin, an internationally accepted food preservative, has been shown to be active against a wide range of gram-positive and some gram-negative bacteria encountered in breweries (Ogden *et al*, 1988). Nisin is a bacteriocin produced by strains of *Streptococcus lactis* and is widely used in the dairy industry. It is a 34 amino acid polypeptide which contains five internal disulfide bridges, which confer thermal stability under acid conditions (Gross and Morrel, 1971). Heat stability and low pH optimal activity make this bacteriocin a good candidate for controlling infection in the brewing process. Commercially available nisin could be used at various stages of the brewing process, or added to the final product to enhance the shelf-life of beer (Atkinson, 1988). A yeast washing step prior to pitching recycled yeast would probably be the best point for application of nicin as this would prevent recycling of any acetic acid bacteria trapped in the yeast slurry at the end of the previous fermentation (Hammond, 1988; Atkinson, 1988). Brewing trials have shown that nisin has no detrimental effect on fermentation or flavour, but inhibits growth of acetic acid bacteria (Ogden, 1986 and 1987).

Some yeast contain a killer factor, which is a cytoplasmically coded polypeptide that kills sensitive yeast (Bostian *et al*, 1980). Strains with killer factor can infect a brew, and kill the indigenous yeast population; beer produced has properties very different to

uninfected fermentations (Taylor and Kirsop, 1979). Killer factor has been introduced into brewing yeast by rare mating techniques (Young, 1981; Hammond and Eckersley, 1984) to produce yeast that prevent beer infection by a number of wild yeast, and have resistance to the action of other killer strains. Beer produced by modified yeast is very similar to that produced by parental strains.

Currently, demands are placed on breweries to produce a wide range of beers, which increases the risk of cross-contamination of different brewing strains. Methods are being developped to identify the presence of different yeast in a fermentation by differential uptake of dyes on agar plates by different yeast strains (Anderson, 1990) and by genetic fingerprinting (Meaden, 1990).

Improving the Consistency of Fermentation

One of the factors affecting reproducibility of fermentation is the physiological state of the yeast used. Physiology is correlated with the ability of a yeast population to acidify a glucose solution, as measured by the acidification power test (Kara *et al*, 1988). Care is taken to select yeast that can produce a sufficiently low pH during this test, thus eliminating yeast of "poor health" prior to fermentation. A number of factors have been shown to influence the acidification power of yeast, such as the temperature and length of yeast storage periods (Fernandez *et al*, 1991).

The course of fermentation can be followed by various means, including on-line measurement of CO_2 evolution (Stassi *et al*, 1991) and ethanol production (Criddle *et al*, 1987). Work from laboratory scale up to experimental brewing scale (10,000 litres) has

demonstrated a correlation between evolution of CO_2 and changes in the specific gravity (Daoud and Searle, 1990). From the onset of CO_2 evolution to the end of fermentation the evolved CO_2 yield is ca. 1.0 g CO_2/litre/degree gravity drop. Knowing this relationship is possible to maintain fermentation on a pre-determined course using a feedback temperature control system. Such control has been achieved at pilot scale brewing level using on-line measurement of specific gravity to correct deviations from a preset fermentation profile (Daoud, 1987). Implementation of on-line sensing will result in better control of fermentation, with subsequent improvement in product consistency and reduction in brewing costs.

More Efficient Use of Raw Materials

Research has provided ways of improving the use of raw materials more efficiently, an example being the extraction of carbohydrate from spent grain. Even after mashing and lautering, the spent grain consists of 55% carbohydrate by dry weight, of which 17% is cellulose and 38% β-glucans, starch and pentosans (Atkinson, 1988). Treatment with less than 1% sulphuric acid at 160°C for ten minutes effectively converts the β-glucans, starch, and pentosans into soluble carbohydrates, of which 50% are glucose and 50% pentose These can then be used for future fermentations either alone or as an adjunct to normal wort.

Extrusion cooking of hops is another example of improvement of an existing method. When hops are boiled in wort the non-bitter α-acids present in the hop cone are converted into bitter iso-α-acids (see **Figure 1.4.1**), although only 30-40% of the bittering potential is realised (Atkinson, 1991). During extrusion cooking the hops are

cooked to produce a hop powder, in which all the α-acids are converted into iso-α-acids. In evacuated packs hop extrudate is stable and when added to wort prior to boiling, 50-60% of the bittering potential is realized. More efficient use of raw material, time and energy have been, and always will be important to brewers.

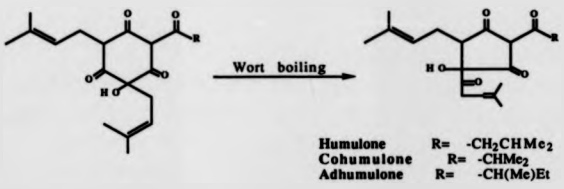

Figure 1.4.1: Conversion of hop α-acids to iso-α-acids by boiling. The R groups of three components of wort are shown (from Atkinson, 1981).

1.5 IMPROVING BARLEY STRAINS

The concept of improving the brewing process by creating improved barley varieties is relatively old. Plant breeders have been experimenting with barley since the end of the nineteenth century. Early success was achieved using hybrid strains that yielded 5% more fermentable sugars than pre-existing strains (Whitbread & Co, 1951). Twenty years ago further success was achieved using γ-radiation to produce the agronomically useful "dwarf" factor of the variety Golden Promise (Atkinson, 1991). Increasingly, barley varieties are being "tailor" made to the needs of the brewers.

Currently mutagenesis is being used to create new strains that are

then empirically selected for improved qualities. A good example of this is the production of varieties containing low levels of anthocyanogens. **Figure 1.5.1** shows the complex structure of two antocyanogens found in barley. These large compounds can survive through the various stages of beer processing to combine with proteins and carbohydrates to form hazes in the beer. Plant breeders have managed to introduce specific mutations in the anthocyanogen synthesis pathway, leading to the production of barley varieties with very low levels of anthocyanogens (von Wettstein, D. *et al*, 1985).

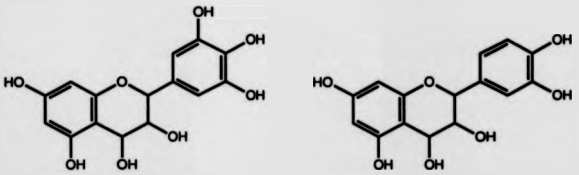

Figure 1.5.1: Structure of two anthocyanogens found in barley.

An active area of research is the development of transformation systems for cereal crops (reviewed by Potrykis, 1990). The development of such a system for barley would be of immense value to brewers, as it would provide an ideal oppotunity to design barley to their needs. A possible application would be the introduction of amylolytic enzymes with improved qualities.

Proteins present in beer after fermentation are also known to cause haze. Addition of crude proteases to fermentation have been shown to reduce chill haze formation in bottled beer (Bilinski *et al*, 1987).

Caution must be taken if a genetically engineered yeast was introduced that secreted a protease into wort, as a number of polypeptides play important roles in beer production (Dale, 1990). Such a protease would be required to be inactive against any other genetically engineered product that enhances the brewing process, some of which are now discussed.

1.6 GENETIC ENGINEERING

With the exception of pure culture selection, yeast strains used in brewing have undergone very little systematic breeding. Attempts at classical genetic manipulation have been frustrated by the genetic polyploidy or aneuploidy, and virtual homothallicity of most brewing yeast (Hinchliffe, 1988). These properties serve to make brewing yeasts genetically stable, which is important for industrial applications. Recombinant DNA technology has provided the oppurtunity to modify brewers yeast in a controlled and specific manner. Genetic engineering can be applied to solve problems encountered by brewers, and provide new commercial opportunities.

It was shown, as early as 1978, that recombinant DNA molecules could be introduced into yeast as an autonomously replicating plasmid (Beggs, 1978), or integrated into the yeast genome in a site-specific manner (Hinnen *et al*, 1978). These approaches were made possible by the availability of the *LEU2* auxotrophic marker (Ratzkin and Carbon, 1977), which was used to transform leucine auxotrophs to prototrophy. Auxotrophic mutations are extremely rare in brewing yeast, therefore a different approach was needed to successfully transform these strains. The first transformation of a brewing yeast

was achieved using the *CUP1* gene, a dominant marker that confers resistance to high levels of copper (Henderson *et al*, 1985). Common dominant markers currently used are given in **Table 1.6.1.**

Marker	Function	Reference
CUP1	copper resistance	Henderson et al (1985)
APT3	resistance to antibiotic G418	Jiminez and Davies (1980)
CAT	resistance to chloramphenicol	Hadfield et al (1986)
DEX1	confers ability to utilise starch or dextrin as a carbon source	Meaden *et al* (1985)
IL2	methotrexate resistance	Zhu *et al* (1985)

Table 1.6.1: Dominant markes for selection of yeast transformation.

The goal of genetic engineering is that the new yeast has improved brewing properties in some way, such as enhanced beer quality, reduced process costs, enhanced efficiency of fermentation, longer shelf-life of the product, or an increase in the commercial value of spent yeast.

Brewing Low Carbohydrate Beers

β-Glucan, a β-(1,3-1,4)-linked glucose polymer, is a major component of the barley endosperm wall. During the mashing process β-glucan is hydrolysed by barley β-(1,3-1,4)-glucanase. A high proportion of β-glucanase activity is lost as a consequence of high temperatures used in mashing. As a result, unhydrolysed β-glucans can survive into the final product, causing hazes and reducing beer filtration rates. Currently β-glucan hydrolysis is achieved in industry by adding crude β-glucanase enzyme preparations from strains of *Bacillus*

(Meadon, 1991). β-Glucanase genes have been cloned from a number of sources, including *Trichoderma reesi* (Penttilä *et al*, 1986) and *Bacillus subtillis* (Cantwell and McConnell, 1983). The major β-glucanase from *T.reesi* hydrolyses β-1,4-glycosidic linkages and has a pH optimum between 4 and 5, which makes it a good candidate for expression in a brewing yeast (Arsdell *et al*, 1987). The gene was successfully expressed in a brewer's yeast under the control of yeast regulatory signals to enhance gene expression (Penttilä *et al*, 1987). Recombinant yeast were shown to hydrolyse 70-76% of the β-glucan present in the wort, to produce a beer with improved filtration rates. A similar approach has been adopted using the endo-(1,3-1,4)-β-glucanase gene from *B.subtillus* to create a genetically engineered brewer's yeast that secretes a functional β-glucanase into the wort (Lancashire and Wilde, 1987). Transformants produce a beer with reduced β-glucan content, with an associated improvement in filterability.

Current practise in the brewing industry is to add crude enzyme preps of β-glucanases to reduce β-glucan content. β-glucanase is currently isolated from *B.amyloliquifaciens* and *B.subtillis* for use in the brewing industry. An alternative approach to reduce the level of β-glucan in wort, using a hybrid β-glucanase has recently been suggested (Meadon, 1991). The β-glucanase from *B.macerans* has greater thermostability than the enzyme of *B.amyloliquifaciens* and *B.subtillis* at neutral pH. At pH4, however, the *B.macerans* enzyme is unstable, and loses activity even quicker than the *B.amyloliquifaciens* and *B.subtillis* β-glucanase. Attempts at creating a novel β-glucanase by "shuffling" existing genes (as shown in **Figure 1.6.1**) were extremely successful, as clearly demonstrated

by the result shown in **Table 1.6.2.** The most simple application of this approach would be to over-express Hybrid H1 in a commercial strain of *Bacillus* and use the isolated enzyme to degrade wort.

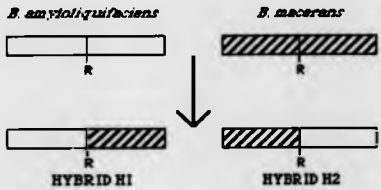

Figure 1.6.1: Creating new genes from old. The cloned β-(1,3-1,4)-glucanase genes from the *Bacillus* strains indicated were digested by a restriction enzyme (indicated R) then rejoined to produce the hybrid genes shown. The properties of parental and hybrid gene products are shown in **Table 1.6.1.**

Source of β-glucanase	Activity (%)[a] remaining after 30 minutes	pH optimum
B.amyloliquifaciens	25	6.0 to 7.0
B.macerans	5	6.0 to 7.5
Hybrid H1	100	5.6 to 6.6
Hybrid H2	not detected	7.0 to 8.0

Table 1.6.2: β-glucanase activity of parental and hybrid genes (based on Meadon, 1991).

[a] activity measured at 65°C, pH4.

Expression of the gene by a brewing yeast would be of less value than degradation of the β-glucan during mashing. The ideal situation would be to produce a recombinant barley strain that expresses this β-(1,3-1,4)-glucanase. The recombinant barley enzyme would be more active in the mash than existing barley enzymes, with potential reductions in mashing times. Such applications will follow the development of a DNA transformation protocol for barley.

Off-Flavour Problems

As previously mentioned, the products of fermentation are ethanol, carbon dioxide and various flavour components. Not all of these flavour components are desirable, and have to be removed by the maturation process. A period of approximately ten days is required to remove volatile off-flavours such as acetaldehyde and sulfur compounds. A six week maturation is required for removal of diacetyl, a diketone which gives beer a distinct butterscotch flavour when present above a taste threshold of approximately 0.1 mg/litre (Sone *et al*, 1988). Attempts to reduce the diacetyl level in beer have focussed on two separate aspects of diacetyl metabolism. The biochemistry of diacetyl production is summarised in **Figure 1.6.2.** Two approaches to overcome the diacetyl problem using recombinant DNA technology are discussed.

An intermediate in the valine biosynthetic pathway (α-acetolactate) can leak out of the cell into the wort during the course of fermentation (step 2, **Figure 1.6.2**), where it is converted into diacetyl by a non-enzymatic decarboxylation (step 3). During maturation, diacetyl is taken up by the yeast cell (step 4) and is converted into acetoin by diacetyl reductase (step 5).

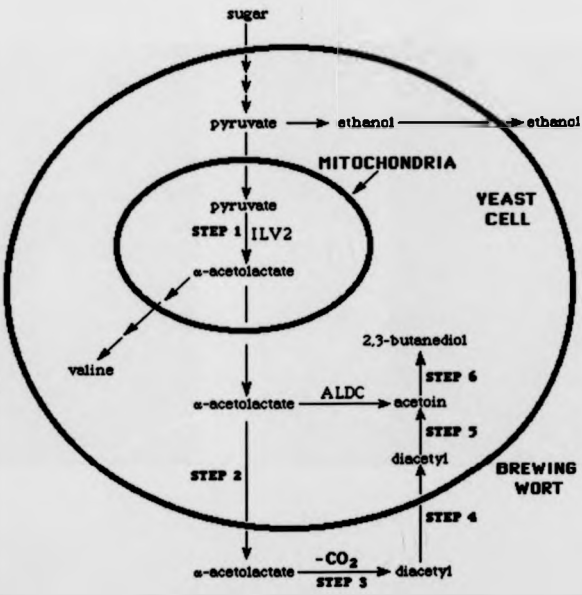

Figure 1.6.2: Biochemistry of diacetyl formation and removal. The natural process for diacetyl removal by yeast is indicated in **STEPS 1 to 6** (see text for more information). The diacetyl problem is being tackled by geneticists on two fronts. Firstly, the *ILV2* gene has been mutated to prevent α-acetolactate formation. Secondly, the bacterial α-acetolactate decarboxylase gene, which converts α-acetolactate directly to acetoin, as shown, has been expressed in yeast.

Acetoin is finally converted into 2,3-butanediol by acetoin reductase (step 6). **Figure 1.6.2** shows that α-acetolactate can be converted directly into acetoin by α-acetolactate decarboxylase (ALDC). This enzyme is found in several species of *Enterobacter*, *Lactobacillus* and *Bacillus* (Godtfredsen *et al*, 1983a). Studies have shown that addition of purified ALDC directly to freshly fermenting beer reduces the concentration of α-acetolactate, with subsequent levels of diacetyl below the taste threshold (Godtfredsen *et al*, 1983b). The ALDC gene from *Enterobacter aerogens* has been cloned (Sone *et al*, 1987), and expressed in a yeast brewing strain, under the control of yeast regulatory signals. In small scale trials, the mutant produced beer with significantly reduced levels of diacetyl, without altering fermentation (Sone *et al*, 1988). The ALDC activity was also shown to be localised in the cytoplasm, therefore allaying initial fears that ALDC activity might interfere with valine biosynthesis (Shimizu *et al*, 1989).

Step 1 shows that pyruvate is converted into α-acetolactate by the action of acetoxyhydroxy acid synthase which is encoded by the *ILV2* gene. Inactivation of the *ILV2* in laboratory strains by gene disruption techniques has shown that such strains do not produce diacetyl. Attempts to disrupt all the copies of *ILV2* in a brewing strain have been successful (Gjermansen *et al*, 1991), although the brewing properties of the mutant have not been reported.

Improving the Value of Spent Yeast.

The yeast biomass produced is of little commercial value to the brewer, and is used mainly as a livestock feed. A potential use of yeast is in the production of pharmaceutical products. This has been

demonstrated by the expression of a component of blood, which is expressed under the control of a galactose-inducible promoter (Coghlan, 1991). Brewers wort contains no galactose, therefore spent yeast grown on galactose-containing medium will express the gene of interest; no expression is observed during fermentation.

1.7 THE FUTURE OF BREWING

Brewing has continually changed through the ages as understanding of the processes involved has increased. The most significant changes in recent years have come about because of improvements in engineering technology (reviewed by Atkinson, 1988). Changes in the design of brewing equipment have reduced costs by minimizing beer losses, and reduced the time of specific steps, such as lautering. Improvements in technology will be a continual goal of the brewing industry.

A number of examples where recombinant yeast might be used to improve the brewing process have been discussed. The likehood that any of these yeast may be used routinely in the future cannot be determined at present, as decisions taken by breweries are influenced by factors beyond the brewing process itself. Decisions are taken with the ultimate aim of being profitable, and to do this brewers must continue to make products that satisfy customer demand. Government legislation has a major influence in long term strategy, in particular the taxation placed on beer. Recombinant yeast are potential "gold mines" for brewers as the new yeasts will improve the process without a significant investment to modify existing plant equipment.

The use of genetically engineered yeast is a good example of external influences on decisions taken by brewers. The release of genetically engineered organisms into the environment is a very topical issue, with many arguments for and against. The release of recombinant yeast would be more acceptable if foreign DNA inserted into the new yeast is kept to a minimum. A potentially greater problem, however, would perhaps come when selling a "genetically engineered" beer to the public. In recent years the media has tended to highlight the more sensational potentials of genetic engineering rather than emphasing its potential uses. The public attitude is perhaps indicated by a recent survey in The Netherlands, which showed that people are very suspicious of genetically engineered food products (Coghlan, 1991). On a scale from 1 (totally unacceptable) to 38 (totally acceptable), people rated genetically engineered food at 5.6. Brewers may experience difficulties when trying to sell a genetically engineered beer. The current increase in demand for traditionally brewed beers in Britain can be seen by the focus on "traditional" brewing, typified by Banks' Bitter, which, the manufacturers claim, is "unspoilt by progress". With better education and imaginative advertising the public may come to accept recombinant beer.

A positive aspect of genetic engineering may be realized if brewers develop a method that allows them to produce low or non-alcoholic beers relatively cheaply. These could then be sold at reduced prices, encouraging people to consume less alcohol, in keeping with increasing public awareness about diet and health. Provided such beers were of a high quality, this could significantly reduce the alcohol intake, with all the associated advantages.

CHAPTER 2

EFFECT OF CHEMICAL INHIBITION OF RESPIRATION ON FERMENTATION PROPERTIES OF YEAST

2.1 INTRODUCTION

Objectives of Current Research

Yeast derives free energy from the oxidation of fuel molecules such as carbohydrates and lipids, and store liberated energy in the form of ATP. Depending on the growth environment, yeast can generate ATP by oxidative phosphorylation or by fermentation, as outlined in **Figure 2.1.1**. For ATP generation by oxidative phosphorylation, it is crucial that the yeast cells have functional mitochondria. Mutations in genes involved in mitochondrial function abolish the ability of the yeast cell to produce ATP by oxidative phosphorylation; it relies on fermentation to generate its ATP requirements. It is also known that a group of chemicals, collectively known as uncouplers, can abolish oxidative phosphorylation. Uncouplers have also been shown to reduce the growth yield of *S.cerevisiae* (Lancashire and Griffiths, 1975), and further studies have indicated that fermentation performed in the presence of uncouplers induce small but commercially significant increases in fermentation capacity (Dr W.E. Lancashire, personal communication). These, and other observations, suggest that inhibiting respiration affects non-mitochondrial cell functions. The objective of current research is to further investigate the action of uncouplers and use recombinant DNA technology to produce yeast deficient in oxidative phosphorylation so as to evaluate the potential use of such yeast in the brewing process.

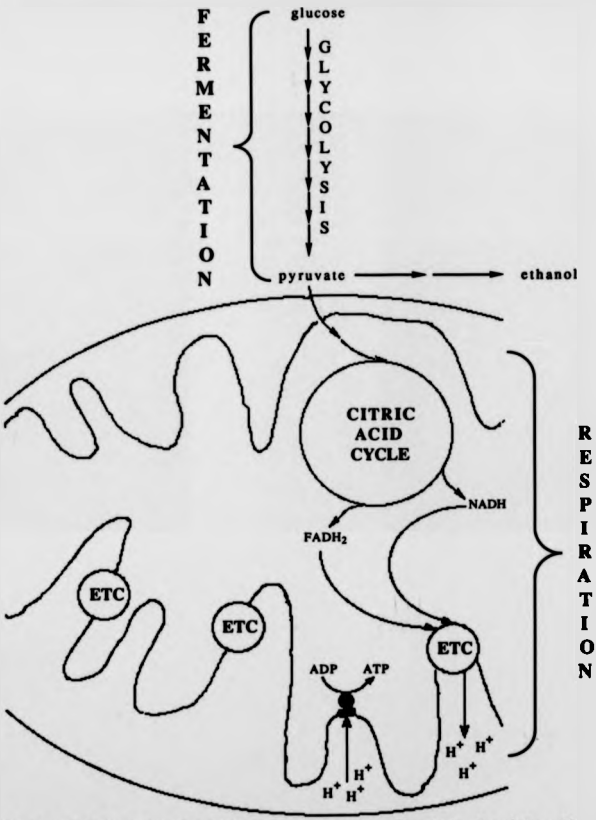

Figure 2.1.1: Pathways for ATP generation in yeast. Glycolysis, the citric acid cycle, and the electron transport chain (ETC) are detailed by Stryer (1981); aspects of fermentation and respiration are discussed later.

Nuclear-Mitochondrial Interactions

Mitochondria are double membrane eukaryotic organelles that house the cells apparatus for ATP production *via* oxidative phosphorylation (or respiration). They are unique amongst aerobic non-photosynthesising organelles in having their own genetic material, which consists of a histone-free double stranded circular DNA molecule which varies in size from 73kb to 88kb in a strain dependent manner (reviewed by Tzagoloff and Myers, 1986). The genome codes for approximately 10% of the mitochondrial protein and components of the mitochondrial protein translation apparatus. The remainder of mitochondrial requirements are transcribed from nuclear genes and imported into the mitochondria by an elaborate and highly efficient targetting system which directs imports specifically to the various compartments or membranes of the mitochondria. (reviewed by Reid, 1985; Schatz, 1987). The need for nuclear genes products puts mitochondrial biogenesis and function under nuclear regulation. But to what extent does mitochondrial structure and function control the regulation of nuclear processes?

A major argument against a mitochondrial involvment in nuclear regulation is that nuclear genes involved in mitochondrial ETC biogenesis (*PET* genes) are expressed in cells with non-functional mitochondria (Tzagoloff and Myers, 1986). However arguments favouring mitochondrial-nuclear interactions have been presented. For example, cytochrome c reductase, a nuclear coded component of the mitochondrial ETC, is expressed at much lower levels in a yeast strain with deletions in the mitochondrial genome (Van Loon *et al*, 1982). Lesions in the mitochondrial genome have also been

associated with changes in drug resistance and inability to import and utilize certain sugars (Evans and Wilkie, 1976; Mahler and Wilkie, 1978). The tendency of certain yeast strains to flocculate (i.e. stick together to form clumps) is controlled by the products of nuclear genes (Lewis *et al*, 1976) ; deficiencies in the mitochondrial genome have been shown to alter the flocculation properties of yeast (Evans *et al*, 1980). The idea that mitochondria may inhibit or alter nuclear function by virtue of a mitochondrially encoded protein that is transported to the nucleus (Barath and Kuntzel, 1972) has not been supported by experimental evidence. Another suggestion is that the mitochondrial DNA or RNA regulates nuclear gene expression (Evans *et al*, 1980; Parikh *et al*, 1987).

In the brewing industry it is observed that the mitochondrial genome of lager yeasts is highly conserved between strains, and are quite different to those of ale yeasts which vary considerably in sequence. Indeed polymorphism of ale yeast mitochondria has been used to distinguish two closely related ale strains using a variation of DNA finger printing methodology (Lee and Knudsen, 1985). Furthermore, it is known from work within the brewing industry that lager mitochondria confer certain characteristics which give advantages in brewing performances as compared to ale yeasts (Gancedo and Serrano, 1984). Under conditions of high ethanol concentrations and temperature, yeast viability depends on the state of the mitochondrial genome; mutations lead to decreased growth and viability in high ethanol concentrations (Jimenez and Benitez, 1988). The toxic effects of ethanol are believed to be felt on the inner mitochondrial membrane (Cabeca-Silva *et al*, 1982).High ethanol concentrations have been shown to induce the yeast plasma

membrane ATPase, with an increase in ethanol yield and a decrease in biomass production.

The mitochondrial genome of *S.cerevisiae* is not essential for viability, in contrast to other eukaryotic organisms. Respiratory-deficient mutants arise at a high frequency, although such mutants can survive and grow almost as well as wild type yeast under optimum growth conditions (Evans *et al*, 1985). The examples of yeast mitochondria interfering with cellular functions indicate that the mitochondria is not an isolated organelle, and that it may exert control over nuclear gene expression, by an unknown mechanism. It is possible that mitochondrial DNA or RNA directly affect nuclear gene expression, or that observed changes in phenotype are caused by metabolic products generated by the mitochondria. The main metabolic products of yeast mitochondria are ATP and NAD^+. The two pathways by which yeast generate ATP are now considered.

Generation of ATP *via* Fermentation

S.cerevisiae is a facultative anaerobe, which means it is able to utilize glucose under aerobic or anaerobic conditions. During aerobic growth by fermentative yeasts such as those used in brewing, respiration accounts for less than 10% of glucose catabolism (Gancedo and Serrano, 1984), therefore the major route of ATP production during brewing is fermentation. Glucose is converted to pyruvate by the process of glycolysis, which theoretically generates 2 moles of ATP per mole of glucose. NADH is also produced, which has to be oxidised to NAD^+ for glycolysis to continue. In yeast, the recycling of NADH is achieved as shown in **Figure 2.1.2**. The process of fermentation is not very efficient compared with respiration, but is ideally suited to

the life style of brewing yeast.

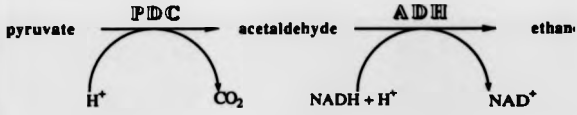

Figure 2.1.2: Regeneration of NAD^+ during fermentation. Pyruvate is converted to acetaldehyde by the enzyme pyruvate decarboxylase (PDC). Acetaldehyde is then converted to ethanol by alcohol dehydrogenase (ADH).

Generation of ATP *via* Oxidative Phosphorylation

An alternative mechanism to fermentation for ATP generation exists in yeast. Under certain environmental conditions, pyruvate can be taken up into the mitochondrion, where it is incorporated into the citric acid cycle, and fully oxidised (Stryer, 1981). The energy released by oxidation is coupled to the reduction of NAD^+ and FAD^+ to NADH (and H^+) and $FADH_2$. These molecules then act as intermediates between the citric acid and the electron transfer chain (*ETC*). The electrons from NADH and $FADH_2$ feed into the *ETC* and are passed along the chain in a series of reactions that slowly release energy. This energy is somehow used to drive the synthesis of ATP.

The coupling of energy release to ATP production has stimulated much interest, and a great deal of research. It was clear that ATP is not generated directly by an enzyme catalysed step as in glycolysis as electron transport occurs in the absence of inorganic phosphate. There must therefore be an intermediate factor. A number of theories exist as to the nature of this intermediate (reviewed by

Slater, 1987) some are now discussed.

The *chemical hypothesis* (Slater, 1953) proposed that electron transfer generated an intermediate with a high energy bond which then converted ADP to ATP as shown below:

$$AH_2 + B + C \rightarrow A\sim C + BH_2$$
$$A\sim C + ADP + P_i \rightarrow A + C + ATP$$

Lack of experimental evidence however, argued against the presence of such a high energy intermediate.

The above theory was modified to give the *conformational theory* (Boyer, 1965), which suggested the high energy intermediate was a specific conformation of a member of the *ETC*, induced as electrons flowed down the *ETC* to oxygen, the terminal electron acceptor. No such intermediate has been found to date.

An earlier idea was the *chemiosmotic hypothesis* proposed by Mitchell (1961), which states that as electrons are transferred from NADH along the *ETC* to oxygen there is a loss in free energy which is used to expel protons (H^+) from the matrix, thus creating a H^+ gradient across the inner mitochondrial membrane, which is highly specialised and impervious to H^+. Protons can only pass back into the matrix through the multienzyme ATP synthase complex, which utilises the energy to drive the synthesis of ATP. There are disagreements about the exact way protons are expelled, and the mechanism of coupling proton import to ATP synthesis (reviewed by Senior, 1988). With minor modifications, the chemiosmotic

hypothesis is the widely accepted mechanism of ATP synthesis by respiration.

Respiratory-Deficient Yeast

The inability of yeast to respire arises spontaneously in a population, and when such yeast are grown on agar plates they produce colonies that are smaller than wild type cells grown under the same conditions. Such mutants are said to have a *petite* phenotype compared with the *grande* phenotype of the wild type yeast. *Petites* can arise as a consequence of mutations in nuclear or mitochondrial genes essential for respiration. Mitochondrial DNA mutations affecting respiration are inherited in a non-Mendelian fashion and can be divided into three categories :

1. Deletions in the wild type mitochondrial genome can result in *petite* mutants that retain part of the mitochondrial genome (ρ^-) or lose it completely (ρ^o). Such mutants have a non-revertable respiratory deficient phenotype.

2. Point mutations in essential genes can produce *mit*$^-$ strains which, in contrast to the above mutants, can perform mitochondrial protein synthesis. The nature of *mit*$^-$ mutations means they can revert to wild type.

3. Mutation in the mitochondria rRNA or tRNA genes produce mutants that are specifically deficient in mitochondria protein synthesis (*syn*$^-$).

Respiration is dependent on the products of a number of nuclear

genes which have been termed *PET* genes (Sherman, 1963). *PET* mutations from a number of studies have been placed into complementation groups (Tzagoloff and Dieckmann, 1990). Each member of a complementation group is deficient for the same nuclear activity, and this activity is essential for respiration. The function of *PET* genes can be generated into three groups:
(1) structural, (2) regulatory, and (3) component of protein synthesis.

1. *PET* gene products can be structural components of the respiratory apparatus, such a component of the ETC or a subunit of the ATP synthase. For example, complementation group G1 is known to code the β-subunit of the mitochondrial F_1-ATPase (Saltzgaber-Muller *et al*, 1983).

2. Certain *PET* genes are necessary for the production of functional mitochondrial DNA products. Mutants studied to date indicate that these proteins exert their effect post-transcriptionally by mRNA processing, or enhancing translation (reviewed by Fox, 1986) or by post-translational modification (Pratje *et al*, 1983).

3. Groups of genes are involved in mitochondrial protein synthesis and affect expression of a number of mitochondrial proteins.

4. PET genes that are non-structural, but required for assembly of multisubunit mitochondrial complexes exist

An example of the number of enzymes involved in mitochondrial biogenesis is provided by studies of cytochrome c oxidase, which has nine structural subunits, three of which are encoded by

mitochondrial DNA, and six are nuclear-coded. McEwen *et al* (1986) have identified thirty four complementation groups essential for the production of a functional cytochrome c oxidase holoenzyme. Therefore at least twenty six non-structural gene products are required for cytochrome c oxidase activity. Mitochondrial ATP synthase mutants have recently been identified as essential for assembly of the subunits of the ATP synthase complex (Ackermann and Tzagoloff, 1990; Bowman *et al*, 1991).

Chemical Uncouplers and Inhibitors of Oxidative Phosphorylation

Chemicals can eliminate ATP synthesis by respiration at three stages:

1. Transfer of electrons down the *ETC* can be blocked.

2. H^+ can be prevented from re-entering into the mitochondria by compounds that bind to the F_0 ion channel of the ATP synthase, blocking the transmembrane pore.

3. The H^+ gradient across the inner mitochondrial membrane can be dissipated.

In present studies respiration is uncoupled by the action of a chemical belonging to the latter group of respiration inhibitors. Di-(2,3,5,6-tetrafluorophenyl) amine (SX-1) is a very potent inhibitor of oxidative phosphorylation. The structure of SX-1 and its mechanism of uncoupling the H^+ gradient are shown below:

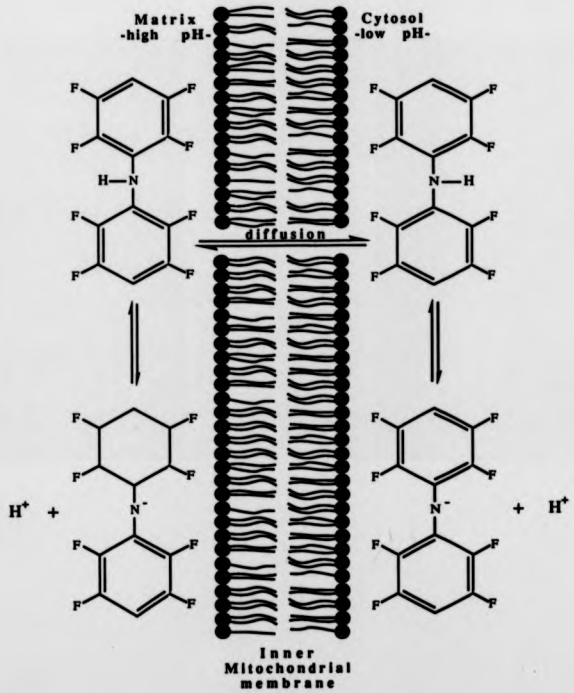

Figure 2.1.3: Structure of SX-1 and its mechanism of uncoupling the H^+ gradient across the inner mitochondrial membrane.

Evidence of a shuttle type mechanism of uncoupling has been

presented for a number of uncouplers including weakly acidic uncouplers (Skulachev, 1971), substituted phenols (Miyoshi *et al*, 1987), and long chain fatty acids (Schonfeld *et al*, 1989). The results of brewing trials uncoupled by the action of SX1 are presented below.

2.2 MATERIALS AND METHODS.

Yeast Strains

Y9 is an industrial ale strain of brewers' yeast and C2 (MATa, *leu2*, *lys1 MAL*) is a haploid laboratory strain of *S.cerevisiae*. Both were gifts from Dr. W.E. Lancashire, Whitbread Research Centre, Luton.

Chemicals

All chemicals used were high purity AnalarR grade, and used without further purification unless otherwise stated. SX-1 was obtained as a gift from Shell, U.K.

Yeast Growth Conditions

Yeast were grown on either complete (YEP) or synthetic (mm) media:

YEP	*mm* (minimal medium)
1% Bacto Yeast Extract	0.67% Difco Yeast nitrogen
2% Bactopeptone	base without amino acids
	+/- amino acids

Lab-*M* agar was used at 1.5% when solid media was required. YEP was supplemented with 2% Dextrose, 3% Glycerol (vol/vol), or 2% Galactose to produce YEPD, YEPG and YEPGal respectively. *mm* was

supplemented with a carbon source (2% glucose or 2% maltose), and amino acids, as recommended by Sherman *et al* (1986). Yeast grown on agar plates were incubated at 30°C; yeast were shaken in an orbital shaker at 30°C and 250rpm, unless otherwise stated.

Storage of Yeast Cultures

Yeast strains were maintained at 4°C for 3 months on YEPD slopes supplemented with adenine, transferred to fresh slopes and grown for 1 day at 30°C before re-storing. In addition, glycerol stocks were made by suspending yeast cells in 15% glycerol and storing at -70°C (Sherman *et al*, 1986).

Sterilisation

All media, solutions, Eppendorf tubes etc., were sterilised as required by autoclaving at 120°C, 150 psi for 15 minutes (Rodwell Scientific Instruments, Series 2001 autoclave). G-tubes used in fermentation were washed with boiling water, then 100% ethanol, inverted and allowed to dry completely.

Brewing Trials of Yeasts

Brewing trials were performed in G-tubes under conditions designed to mimic industrial brewing conditions. The side arm of the G-tube allows samples to be removed for analysis at any time without disturbing fermentation. **Figure 2.2.1** shows the structure of a G-tube and, the advantages of this reaction vessel. The three main stages of fermentation trials are as follows :

I. Starting a Fermentation

The first step of a trial involves preparing the wort and pitching yeast. Munton-Fison Cederex B ale wort concentrate was obtained as a thick syrup from Whitbread Research and Development Centre, Luton. The concentrate wort is added to approximately 1 litre of boiled water, stirred to obtain a homogeneous solution, then added with an equal volume of water to a "home brew" fermentation bin. The wort is then allowed to cool and the specific gravity measured (see below). The value is adjusted to approximately 1.040g/cm^3 at 20°C by addition of water. At this gravity reading 1 litre of wort is made from approximately 100ml of concentrate. The wort is then placed in 2 litre conical flasks (1.6 litres per flask) and steamed for 1 hour at 80°C in an autoclave. The wort is allowed to cool, and is aerated by pouring from beaker to beaker just before yeast is added. Yeast is prepared by growing in YEPD or YEPG medium (depending on the strain) and harvested at stationary phase in pre-weighed centrifugation pots. The weight of yeast harvested is calculated, then sterile water is added to give a 0.5mg/ml yeast suspension. To start a fermentation 2.4ml of yeast suspension (i.e. 1.2g) is added to exactly 500ml of wort, which has been supplemented with 1ml of a 440g/litre $ZnSO_4$ solution, as zinc is important for brewing (Raspor, 1990). The sterile G-tubes are sealed with parafilm and inverted to mix the contents.

II. In-line Analysis

The trial can be followed visually and biochemically to determine the stage of fermentation. The rate of CO_2 production is proportional to the amount of ethanol produced. Fermentation is judged as finished when release of CO_2 bubbles is negligible. Measuring the sugar

content of the wort gives a more quantitative determination of the state of fermentation. Sugar content is directly correlated to the specific gravity of the wort. At various times wort is removed *via* the side arm of the G-tube (see **Figure 2.2.1**), degassed, and gravity determined using a densitometer. Gravity readings were made at the times indicated for each experiment. After 24 hours at 20°C, yeast are resuspended and aerated by pouring the contents of the G-tube from beaker to beaker, then returned to the tubes and sealed with parafilm.

III. End of Fermentation

When fermentation is judged to have stopped, the specific gravity, ethanol content, and biomass production are determined.

Measuring Specific Gravity

A hand-held densitometer (Paar Scientific Ltd., Density Meter DMA 35) was kindly provided by Whitbread plc. This machine is used to measure the specific gravity of a solution. Beer is degassed prior to injection into the machine and care taken to avoid air bubbles entering the cell, as this affects accuracy.

Biomass Determination

Biomass is determined using pre-weighed centrifuge pots. The contents of each G-tube are spun down at 5000 rpm for 10 minutes in a Sorval GSA rotor and the wet weight of yeast produced determined. The dry weight is measured by drying the yeast pellet at 90°C until the weight remains constant.

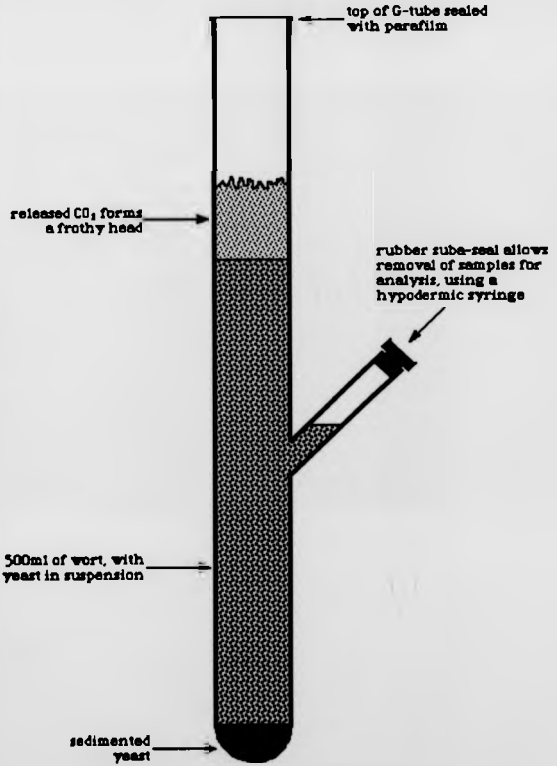

Figure 2.2.1: G-tube fermentation. The important features of a G-tube are shown. The side arm allows on-line analysis of wort as a means of following fermentation by sugar uptake, as described in the text.

Ethanol Determination

Gas Chromatography (GC) was used to accurately measure the ethanol content of beer produced during brewing trials. The concept of Gas Chromatography can be described by considering the diagram below (**Figure 2.2.2**):

Ethanol Determination by Gas Chromatography

The technique of Gas Chromatography is no different from other forms of chromatography in that it is a process for separating the individual components of complex mixture. The sample is injected through a rubber septum into the stream of a carrier gas using a microlitre syringe. The sample is carried into a column which separates the various components of the sample. Separation is achieved by correct selection of column package. The column is packed with a solid support that has varied affinity for different compounds. Components of the mixture with higher affinity are retained in the column longer than those with less affinity. The speed and efficiency of separation are temperature dependent. The column is therefore mounted in an oven, which allows precise temperature control and reproducibility of separation. The column effluent passes into a detector used to detect the presence of compounds coming off the column. A 5 feet, 4 mm internal diameter column packed with POROPAK Q (80-100 mesh size) was bought from Jones Chromatography. POROPAK Q allows separation of the alcohols tested in these studies. The column is compatible with the PYE series 204 gas chromatographer used in these studies. The type of detector used in these studies was Flame Ionisation Detection (F.I.D.), which is based on the observation that when organic compounds are burned in a hydrogen and air flame, ions are produced.

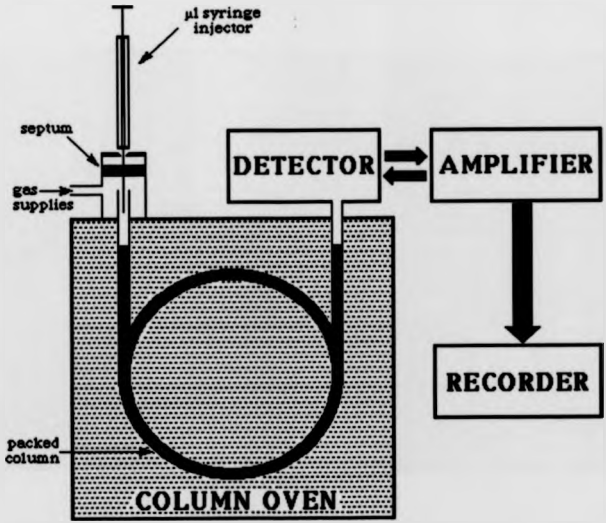

Figure 2.2.2. Diagramatic representation of Gas Chromatography. Sample is injected into the stream of a carrier gas that carries the sample through the packed column, where resolution is achieved by virtue of good selection of package material and control of oven temperature. The quantity of a component in a solution and time it takes to come off the column (retention time) are determined by a detector. The signal output is amplified under the control of an amplifier which passes the amplified signal to a chart recorder and an integrator for accurate calculation of retention time and quantity. (Above diagram and description of GC in the text are based on Ashby, 1977)

GC conditions: column temperature 150°C, injector temperature 200°C, detector temperature 200°C, N_2 gas at 10 psi, H_2 gas at 12 psi, air at 6 psi. Under these conditions, retention times of ethanol and propan-1-ol were 170 and 320 seconds respectively.

A simplified equation of flame ionisation is given:

$$\text{Organic sample} + H_2 + O_2 \rightarrow CO_2 + H_2O + (\text{ions})^+ + (\text{ions})^- + e^-$$

$$\Sigma\ (\text{ions}^- + e^-) \rightarrow \text{current flow}$$

The ions produce a current flow that is amplified before passing to a chart recorder. The amplifier output also passes to an electronic integrator which automatically calculates the area under each peak. Using an internal standard, this peak area value can be used to determine the ethanol content of a sample.

Calibration of Ethanol:Propanol Ratios

As previously mentioned an internal standard was used in ethanol determination. Propan-1-ol was chosen as a standard. When run together under experimental condition there is good separation of both samples.

Standard Ratio

A 0.5% ethanol, 0.5% propanol solution was accurately made and analysed by GC. The area of each peak was determined by an integrator and from these a standard ratio (S.R) calculated:

$$\text{standard ratio (S.R.)} = \frac{\text{area of ethanol peak}}{\text{area of propanol peak}}$$

The average S.R. value of a 0.5% ethanol, 0.5% propanol solution was calculated from ten separate injections. Other 0.5% ethanol, 0.5% propanol solutions were made and compared. The standard deviation between the S.R. values of different solutions was small. The value of

S.R. for a 0.5% ethanol:0.5% propanol solution was calculated as 0.800 ± 0.016. Before determining the ethanol content of beer a 0.5% ethanol, 0.5% propanol solution was analysed to ensure the same S.R. value was attained.

The ethanol content of beer produced was calculated using the S.R. value and 0.5% propan-1-ol as an internal standard to produce a sample ratio (area of ethanol peak/area of propanol peak). Beer was initially assumed to be approximately 3.5% ethanol. From this assumption a "0.5% ethanol" : 0.5% propanol solution was made. The actual ethanol content was calculated using the following equation:

$$\% \text{ ethanol} = \frac{\text{sample ratio}}{\text{standard ratio}} \times 0.5 \times \text{dilution factor}$$

The amount of beer added was varied to achieve a sample ratio approximately equal to the S.R. value, whilst maintaining the propanol content at 0.5%. The new dilution factor was used, with the S.R. and sample ratio values to accurately determine the ethanol content of the beer.

2.3 RESULTS

Uncoupling Whole Yeast Cells using SX-1

SX-1 has been shown to be a potent uncoupler of isolated rat liver mitochondria at a concentration between 2 to 4 x 10^{-9}M (Dr D.E. Griffiths, unpublished results). Preliminary experiments indicated that a concentration of 10^{-9}M SX-1 was not sufficient to uncouple the mitochondria of whole cell brewing yeast during fermentation. The

effective uncoupling concentration was determined for yeasts Y9 and C2 by streaking them onto YEPG plates containing various concentrations of SX-1. Testing yeasts over an SX-1 concentration range from 10^{-9} to 10^{-3}M, it was found that Y9 and C2 were unable to grow at or above SX-1 concentrations of 10^{-5}M. This concentration was used in subsequent brewing trials where uncoupling of oxidative phosphorylation was required.

Effect of SX-1 on Y9 Fermentation

The initial aim of this work was to investigate the effect of an uncoupler on the fermentation properties of an industrial ale yeast. These studies were based on previous findings, indicating that fermentation performed in the presence of uncouplers of oxidative phosphorylation produce less biomass and small, but commercially significant improvements in fermentation performance, compared with the control (Dr. W.E. Lancashire, personal communication).

Laboratory scale brewing trials were performed to determine the effect of the uncoupler SX-1 on Y9 fermentation using laboratory scale brewing trials. The description of brewing trials given in **SECTION 2.2** was followed in this and subsequent brewing trials, with minor variations as indicated in the text.

To assay for uncoupling effect in brewing trials 50µl of 0.1M SX-1 (in 100% ethanol) was added per 500ml. Wort of control experiments was supplemented with 50µl of 100% ethanol per 500ml. The results summarised in **Figure 2.3.1** and **Table 2.3.1** indicate that SX-1 has a dramatic effect on the fermentation profile of Y9.

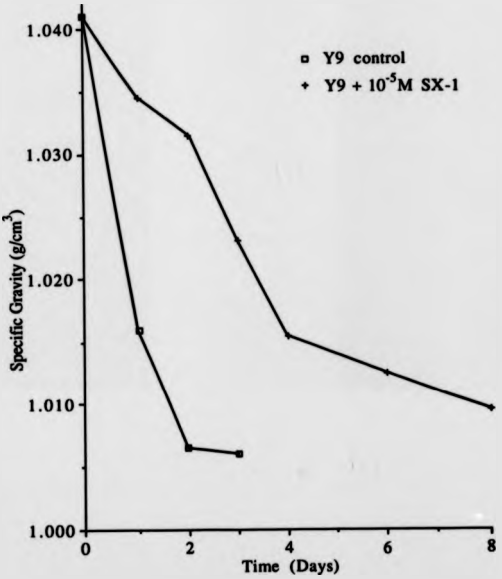

Figure 2.3.1: Change in specific gravity with time: Y9 fermentation in the presence or absense of SX-1.

	Y9					Y9 + 10^{-5}M SX-1						
G-tube	1	2	3	4	Mean ±σ	1(a)	2(a)	1(b)	2(b)	Mean ±σ (a)	Mean ±σ (b)	P[1]
Sugar Used (units)[2]	35	35	35	35	35	15	16	31	31	15.5 ±0.5	31	-
Dry Weight (g)	3.521	3.597	3.770	3.797	3.671 ±0.116	1.243	1.338	1.343	1.608	1.291 ±0.048	1.476 ±0.133	-
Ethanol Yield (%)	4.7	4.7	4.9	4.9	4.8 ±0.1	2.2	2.3	4.7	4.8	2.25 ±0.05	4.75 ±0.05	0.7 - 0.6
Ethanol (%) per Sugar Used	0.134	0.134	0.140	0.140	0.137 ±0.003	0.147	0.143	0.151	0.154	0.145 ±0.002	0.153 ±0.002	0.01 - 0.001
Dry Weight (g)per Sugar used ($x10^2$)	10.1	10.3	10.8	10.8	10.5 ±0.3	8.3	7.0	4.3	5.0	7.7 ±0.7	4.7 ±0.4	< 0.001

Table 2.3.1: Brewing trials of Y9 in the presence or absence of SX-1. SX-1 was added (at 10^{-5}M) to four G-tubes, as described in SECTION 2.2; four control tubes were also set up. All fermentations were allowed to run to completion, except tubes 1a and 2a, which were analysed at the same time as Y9 (see Figure 2.3.1). Mean values are arithmetic means with standard deviation. The four Y9 tube results were compared with tubes1(b) and 2(b) for calculation of stastistical significance

[1] ***P*** **is a calculation of probability using the Student *t* test. Using this method of analysis, values of *P* less than 0.05 are regarded as statistically significant *i.e.* there is less than a 5% chance that the values are from the same population. Values of *P* greater than 0.05 do not necessarily indicate that observed differences are not significant.**

[2] **one unit of sugar unit is equivalent to a change in specific gravity of 0.001g/cm3.**

In the Y9 trial, the time taken to complete fermentation increases greatly, and the final gravity of the wort is higher, indicative of differential sugar uptake between uncoupled and control yeast. Results also indicate that these changes are associated with very little difference in ethanol concentration of the final beer produced. Comparisons of fermentation ratios indicates that uncoupling results in more efficient use of sugar with regards to ethanol yield. In the presence of SX-1, Y9 produces 11.7% more ethanol per sugar used. This observation is of interest to the brewing industry as it provides an opportunity for potential savings on raw materials.

The next step in our work was the creation of a yeast that mimics the effects of uncoupling. Recombinant DNA technology facilitates the creation of such yeasts. For initial studies it was proposed to disrupt a haploid laboratory strain of yeast due to the difficulties associated with such studies in polyploid or aneuploid industrial strains (Hinchliffe, 1986). The strain chosen for those studies was *S.cerevisiae* C2.

Effect of SX-1 on C2 Fermentation

Prior to genetic engineering of C2, it was necessary to determine the effect of uncouplers on C2 fermentation. Brewing trials were performed essentially the same as the Y9 ± SX-1 brewing trial. A notable variation was that 1.5g of C2 (cf. 1.2g of Y9) was pitched as starting innoculum, as C2 has a slower fermentation rate than Y9. The results are shown in **Figure 2.3.2** and **Table 2.3.2.**

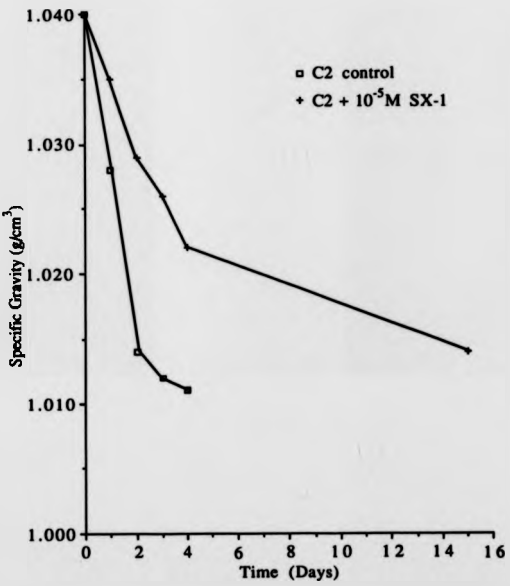

Figure 2.3.2: Change in specific gravity: C2 fermentation in the presence or absense of SX-1.

	C2				C2 + 0.5M SX-1				
G Tube	1	2	3	Mean ± σ	1	2	3	Mean ± σ	*P*
Sugar Used (units)	28	29	29	28.7 ±0.5	25	25	25	25	-
Dry Weight (g)	1.938	2.105	2.128	2.057 ±0.084	1.356	1.232	1.288	1.292 ±0.051	-
Ethanol Yield (%)	3.57	3.74	3.63	3.65 ±0.07	3.48	3.56	3.63	3.56 ±0.06	0.30 - 0.20
Ethanol (%) per Sugar Used	0.127	0.129	0.125	0.127 ±0.002	0.139	0.142	0.145	0.142 ±0.002	0.01 - 0.001
Dry Weight (g) per Sugar used ($x10^2$)	6.92	7.26	7.34	7.17 ±0.18	4.93	5.42	5.15	5.17 ±0.20	< 0.001

Table 2.3.2: Brewing trials of C2 in the presence or absence of SX-1. Three G-tubes were used as replicas. Brewing conditions and analysis of data were performed as previously described (see **Table2.3.1**).

The time of C2 fermentation increases considerably in the presence of SX-1, indicative of reduced growth rate. A decrease in biomass is observed, although there is little change in the ethanol concentration of the beer. As with Y9, the laboratory strain used less sugar, as measured by the final gravity of the wort. In the presence of SX-1, Y9 produces 11.8% more ethanol per sugar used, similar to the result observed for Y9 Reasons for improved ethanol yields, and the observed differential sugar use of uncoupled Y9 and C2 are considered in chapter3.

2.4. DISCUSSION

The results of the Y9 and C2 brewing trials indicate that SX-1 has a profound effect on fermentation. For present studies these results are important as they indicate uncouplers produce a similar effect on fermentations using C2 and Y9. This makes C2 a suitable yeast for disruptions of specific *PET* genes. Work of this nature is described in the following two chapters.

CHAPTER 3
FERMENTATION PROPERTIES OF A *PET* MUTANT

3.1 INTRODUCTION

Previous results indicate that chemical uncoupling of oxidative phosphorylation of brewer's yeast alters its fermentation properties, such that less biomass is produced in conjunction with an increase in ethanol yield. This chapter reports the creation of a genetically engineered yeast strain deficient in oxidative phosphorylation, and the biochemical properties of this mutant, particularly it's fermentation profile. The results are compared with those presented in the previous chapter.

The advances in yeast recombinant DNA technology have given scientists the power to genetically engineer changes in any cloned sequence of DNA *in vivo*; deletions, insertions and substitutions of single nucleotides are possible (Watson *et al*, 1983). Transfomation of yeast with *in vitro*-modified DNA, followed by recombination at the target sequence, can be used to alter chromosomal DNA (reviewed by Rothstein, 1991). A gene disruption strategy was used to produce a mutant yeast defective for the previously cloned *ATP*12 *PET* gene (Bowman, 1989).

PET Genes of *Saccharomyces cerevisiae*

Ethyl methane sulphonate and nitrosoguanidine, chemical mutagens that specifically create mutations in nuclear DNA, were used to generate a series of mutants in *S.cerevisiae* (Tzagoloff *et al*, 1975a). Mutants positive for mitochondrial protein synthesis but unable to

grow on YEPG were shown to have a mutation in either nuclear DNA or mitochondrial DNA by crossing each strain with a ρ^o tester strain. Both nuclear (Tzagoloff *et al*, 1975b) and cytoplasmic mutations (Tzagoloff *et al*, 1975c) were assigned to deficiencies in cytochrome c oxidase, coenzyme QH_2-cytochrome c reductase, oligomycin-sensitive ATPase, or a combination of all three. Mutants were crossed with one another and diploid yeast unable to complement their respiration mutations were placed in the same complementation group. Of 215 complementation groups identified, at least 77 have been completely or partially sequenced, and a specific function assigned (reviewed by Tzagoloff and Dieckmann, 1990). Respiratory lesions have been assigned to particular proteins. For example members of complementation group G1 lack the β-subunit of the mitochondrial F_1-ATPase (Saltzgaber-Muller *et al*, 1983).

The *ATP12* gene was cloned using a yeast genomic DNA library to complement a member of group G57 of Tzagoloff *et al* (1977b) and was shown to encode an activity essential for production of a functional mitochondrial ATPase. Immunological studies show that *ATP12* encodes a protein that is not a structural component of the ATPase, but is essential for assembly of the cytoplasmic F_1 domain (Bowman *et al*, 1991). The availability of the *ATP12* clone and the fact that members of complementation group G57 have virtually no mitochondrial ATPase activity made this gene suitable for gene disruption studies.

3.2 MATERIALS AND METHODS

E.coli Growth Medium

E.coli was grown in LB broth supplemented with the antibiotics ampicillin (50μg/ml) and Kanamycin (25μg/ml), as recommended by Maniatis *et al* (1982), when required. *E.coli* NM522 was used for all transformations.

LB broth
0.5% Yeast extract (Difco)
1.0% Bacto Tryptone (Difco)
1.0% Sodium Chloride
1.5% Agar (Lab-M), for solid media.

Molecular Biology Enzymes and Reagents

T4 DNA ligase, *E.coli* DNA polymerase Klenow fragment, Calf intestinal alkali phosphatase (CIP) and T4 DNA polymerase were purchased from BCL. Restriction enzymes and buffers were bought from BRL. Lysozyme and CTAB were obtained from Sigma, as were Bovine Serum Albumin (BSA) fractions IV and V. Zymolyase was obtained from the Kirin Brewery, Japan. [α^{32}P]-dCTP (ca. 3000 mCi/ml) was bought from Amersham. Care was taken to follow the COSHH guidelines for safety when handling all chemicals and radiochemicals.

Agarose Electrophoresis (Maniatis *et al.*,1982)

Agarose (normal and LMP) was purchased as Electrophoresis grade from BRL. Agarose was used at 0.7-1.0% in 1x TBE buffer, DNA was loaded onto gels in 1x Loading Buffer (Type III). Gels were submerged in a running buffer of 1x TBE containing 0.5μg/ml EtBr.

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***E.coli* Transformation** (based on Maniatis *et al*,1982)

1ml of an overnight *E.coli* culture was used to innoculate 100ml of LB. Cells were shaken at 200rpm in a 37°C rotary shaker until the A_{600} value was between 0.2-0.25 (ca. 2 hours). Cells were placed in 4 sterile 30ml plastic tubes then placed on ice for 20 minutes. Cells were pelleted at 4°C (10 minutes at 4000rpm), washed with 20ml (total) of 0.1M $MgCl_2$, pooled in a single tube, pelleted as before, then resuspended in 2ml of ice-cold 0.1M $CaCl_2$. Competent cells were then stored at 4°C. *E.coli* remains competent for several days but optimum transformation efficiency was obtained with cells used after 24 hours at 4°C. Transformations were performed in sterile pre-cooled 1.5ml Eppendorfs. 1-10µl of DNA was made up to a volume of 100µl with sterile dH_2O and 200µl of competent cells added. The contents were mixed gently then placed on ice for 60 minutes, followed by a 2 minute heat shock (42°C), then returned to ice for 60 minutes. The time on ice is not critical and was frequently varied to suit the work programme. 300µl of LB was added and the tubes incubated at 37°C for 60 minutes. This allows expression of plasmid-coded antibiotic resistance before the cells are finally spread (200µl per plate) on LB plates containing antibiotic selection.

Mini-prep of Plasmid DNA from *E.coli* (modified from Maniatis *et al*, 1982)

E.coli was transformed with vector DNA and plated on LB plus antibiotic. 10ml of antibiotic-supplemented LB was innoculated with a single transformant and grown up overnight at 37°C with shaking (250rpm). A total of 3ml of cells were harvested in a single 1.5ml Eppendorf tube by low speed centrifugation in a Microcentaur centrifuge for 1 minute (2 spins). The cells were then resuspended in

200µl of Lysozyme Solution by vortexing. After 5 minutes at room temperature 400µl of fresh alkaline solution was added and the contents gently mixed by inverting the tube 3-6 times.

Lysozyme Solution	Alkaline Solution
50mM D-Glucose	0.2N NaOH
5mM Tris-HCl (pH 8.0)	1% SDS
10mM EDTA	
4mg/ml Lysozyme	

After 5 minutes on ice 300µl of 7.5M ammonium acetate was added and contents of the tube mixed by inversion. Incubation on ice for 10 minutes precipitates most of the protein, high molecular weight RNA and chromosomal DNA, which were then pelleted by spinning at 10,000rpm for 3 minutes in an Eppendorf (5415C) variable speed centrifuge. The supernatant was removed to a fresh Eppendorf tube and 0.6 volumes of isopropanol added. The tubes were maintained at room temperature for 10 minutes then centrifuged at 15,000rpm for 10 minutes; the supernatant was removed by aspiration and the pellet washed with 70%(v/v) ethanol solution. The ethanol solution was removed by aspiration and pellets dried under vacuum for 5 minutes then resuspended in 50µl TE buffer. This technique produces plasmid DNA that cuts with restriction enzymes for identification of plasmid constructs. Twelve clones can be screened in an hour, which is useful because of the low efficiency of blunt-end ligations. An advantage of this technique compared with the alkali lysis method (Birnboim and Doly, 1979) is that no phenol is used, eliminating the risk of burns.

Large Scale Plasmid Preparation

A scaled up version of the mini-prep was used to produce large amounts of plasmid DNA. 200ml of a stationary culture of transformed *E.coli* were harvested by spinning at 4000rpm for 10 minutes in a Sorval GSA rotor. The pellet was resuspended in 10ml lysozyme solution and left for 5 minutes at room temperature. 20ml of freshly prepared alkaline solution was added and the tube mixed by inversion. After 5 minutes on ice, 15ml of 7.5M ammonium acetate was added, mixed by inversion then incubated at room temperature for 10 minutes. After spinning at 6000rpm at 4°C the supernatant was removed and aliquoted into two Sorval SS34 tubes. RNAaseA was added (50µl of a 5mg/ml solution), the tubes incubated at 37°C for 60 minutes, after which a 0.6 volume of isopropanol was added. The tubes were maintained at room temperature for 10 minutes and spun at 12000rpm for 10 minutes. The DNA pellet was washed in 70% ethanol, dried under vacuum, then resuspended in 2ml of TE buffer.

DNA Cloning Techniques

The following techniques were performed, as recommended by Ausubel *et al* (1989):

1. Blunt-ended DNA molecules were produced by treating molecules with 5' overhangs with *E. coli* DNA polymerase Klenow fragment, and 3' overhangs with T4 DNA polymerase.

2. CIP treatment was used to remove the 5' phosphate groups of DNA used as a vector in ligations. This was done to reduce the frequency of self-ligation.

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2. CIP treatment was used to remove the 5' phosphate groups of DNA used as a vector in ligations. This was done to reduce the frequency of self-ligation.

3. DNA ligations were created using T4 DNA ligase, chosen for its ability to ligate blunt-ended DNA.

Recovery of DNA from LMP Agarose (Dr. A.T. Carter, personal communication: modified from Langridge *et al*, 1979)
Stock solutions were made by mixing 150ml of butan-1-ol and 150ml of dH_2O by shaking, the phases were allowed to settle out and then separated. 1g of CTAB was added per 100ml of butanol phase, and 50µl of antifoam A (SIGMA) per 100ml of aqueous phase. The solutions were then re-mixed and separated as before. The butanol and water phases were bottled separately and stored at 37°C.

DNA was digested with the indicated restriction enzyme and separated on a horizontal 0.7% LMP agarose gel as previously described. The band of interest was visualised under UV illumination and trimmed from the gel. Excess agarose was removed and the agarose slice placed in an Eppendorf tube. A quick spin at high speed collapses the LMP agarose and allows an estimation of volume. An equal volume of equilibrated dH_2O was added and the tube was incubated at 65°C for 5-15 minutes. For volumes between 0.5-1.0ml, 300µl of CTAB/butanol (pre-warmed at 65°C) was added. The tube was shaken vigorously by hand for a few seconds, spun in a Microcentaur for 45 seconds, the top phase removed to a fresh Eppendorf tube, and maintained at 37°C to keep the agarose molten. This extraction was repeated with a further 300µl of CTAB/butanol which results in isolation of 95% of the DNA. A further extraction with 200µl of CTAB/butanol is optional. Next an equal volume of equilibrated dH_2O solution was used to extract the pooled CTAB/butanol phases, as before. The butanol phase containing the

DNA fragment can now be stored at room temperature. This was extracted with a quarter volume of 200mM NaCl, mixed by hand as before, and spun for 45 seconds in a Microcentaur. The bottom phase was recovered and placed in a fresh Eppendorf tube. The top phase was re-extracted with 200mM NaCl as before. The aqueous fractions were pooled and CTAB removed with an equal volume of chloroform at 0°C by occasional shaking of tubes that were put on ice for 10-30 minutes. The tube was centrifuged for 30 seconds, the aqueous phase was removed and precipitated by adding 2 volumes of ethanol. The DNA was pelleted, washed in 70% ethanol, dried under vacuum, and resuspended in a suitable volume of TE buffer.

Yeast Mitochondrial Isolation (Bowman, 1989)

1% (v/v) of stationary yeast were added to 500ml YEPG (YEPGal for *petite* mutants) and grown up overnight by shaking in a 30°C orbital shaker at 200rpm. Cells were harvested by spinning at 2000rpm for 10 minutes in a Sorval GSA rotor. The cell pellet was washed once in cold Breaking Buffer, then resuspended in 0.5ml of Breaking Buffer per gramme of wet weight yeast. The preparation was then continued in a cold room (4°C). Yeast cells were lysed by the Langs Handshake method. Resuspended yeast were transferred to a 250ml screw-capped bottle containing a half-volume of Ballotoni beads (diameter 0.45-0.50mm) that had previously been washed in cold breaking buffer. The yeast cells were broken by shaking the bottles

Breaking Buffer
0.5M Sorbitol
10mM Tris.HCl (pH 7.5)
1mM EDTA
0.1% BSA (Sigma, grade IV, fatty acid free)

by hand with three cycles of shaking (2 minutes shaking with a one minute interval on ice). The yeast suspension was decanted in Sorval SS34 polycarbonate tubes. The beads were washed with breaking buffer and the pooled suspension spun at 4°C for 15 minutes at 2000rpm. The supernatant was transferred to a fresh tube and spun as before. This was repeated until no pellet appeared. The isolated mitochondria were pelleted by spinning the final supernatant at 4°C, 12000rpm for 15 minutes. The pellet was washed in mitochondrial storage buffer (Breaking Buffer without BSA), and resuspended in a minimal volume of the same. Mitochondria were used immediately or stored at -70°C.

Protein Concentration Determination

The protein concentration of solutions was determined using the Biuret method (Lowrey, 1969). BSA was used to generate a standard curve, from which concentrations of mitochondrial protein were determined.

Mitochondrial ATPase Assay

This method measures the ATP hydrolytic activity of mitochondrial ATPase by determining the amount of inorganic phosphate liberated per mg of mitochondrial protein per minute. The P_i concentration was determined by the method of Heinonnen and Lahti (1981). Approximately 0.1mg of mitochondrial protein was incubated with 0.5ml 2X ATPase buffer, plus or minus inhibitor, and the volume was made up to 0.980ml with dH_2O. The reaction was started by adding 20µl of a 0.5M ATP solution.

2X ATPase Buffer
100mM Tris.H_2SO_4 (pH 7.5)
8mM $MgSO_4$

AAM Solution
1vol 10mM NH_4 Molybdate
1vol 2.5M H_2SO_4
2vol Acetone

After 15 minutes, 0.2ml of the assay was removed and added to 50µl 1M citric acid to stop the reaction. 3ml of AAM solution was added and the colour change was measured at A_{355} after 15 minutes. ATPase activity was determined in Kletts Units (K.U.); 1 K.U. is equivalent to a change in the A_{355} value of 0.005 absorbance units per mg of protein per minute. The oligomycin-sensitive ATPase activity was determined by including oligomycin (5µg/ml) in the assay mixture.

Making ρ^o Yeasts (Goldring *et al*, 1970)

A stationary phase yeast culture was used to innoculate 10ml of *mm* plus 2% glucose and 25µg/ml ethidium bromide, which was then grown at 30°C shaking at 250rpm until a stationary yeast culture was produced. This was then repeated and the second mutagenised culture was diluted and plated on YEPD to produce single colonies.

Mating Haploid Yeast Strains

Yeast of opposite mating type were picked as single colonies into 0.5ml of YEPD in an Eppendorf tube and grown for 3 hours at 30°C. Cells were then pelleted by spinning in a microcentrifuge at high speed for 5 seconds, washed in sterile dH_2O and resuspended in 50µl dH_2O. 5µl aliquots of one cell suspension were placed on plates selective for successful mating of haploids. 5µl of the other yeast was spotted onto the same area of the plates, allowed to dry, then incubated at 30°C. Productive crosses were observed after 2-3 days.

3.3 RESULTS

The plasmid YEp351/ST4 shown in Figure 3.3.1, was obtained as a gift from Dr. S. Bowman. It contains the *ATP12* gene of *S.cerevisiae* cloned into the polylinker of the *E.coli-S.cerevisiae* shuttle vector YEp351 (Hill *et al*, 1986) YEp351 is a suitable vector for *ATP12* disruption because it contains no *Nco*I restriction sites. The 70bp *Nco*I fragment within the coding region of *ATP12* can therefore be removed, and foreign DNA inserted to create a disrupted form of the *ATP12* gene. The convenient *Pvu*II sites allow any hybrid *ATP12* construct to be removed from other plasmid DNA making it suitable for one-step gene disruption transformations.

The transposable element Tn601(903) encodes aminoglycosidase phosphotransferase-3'(I) (*APT-3*) that confers resistance to the antibiotic G418 in *S.cerevisiae* (Jiminez and Davies, 1980). When driven under the control of strong yeast promoter and terminator sequences, the *APT-3* construct confers G418 resistance in single copy (Hadfield *et al*, 1990), thus making it suitable as a dominant marker for gene disruption studies. A construct containing the *APT-3'* gene under the control of the constitutive phosphoglycerate kinase PGK promoter and terminator sequences was engineered on a 2.0kb cassette (*APT2*) that was cloned into the *E.coli* vector pIC-19R. The resulting plasmid (pCH217; Figure 3.3.1) was obtained from Dr. Chris Hadfield.

This section describes the disruption of *ATP12* with the 2.0kb expression cassette of pCH217 and creation of a Δ*ATP12* mutant.

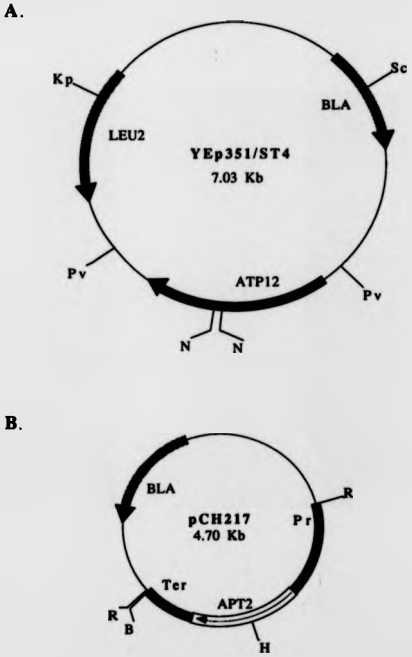


Figure 3.3.1: Vectors used in the construction of pJH2. **A**: Yeast-*E.coli* shuttle vector **YEp351/ST4** contains the *ATP12* gene on a 1.4kb fragment cloned into the polylinker of YEp351. The *LEU2* and *bla* genes allow plasmid selection in yeast and *E.coli* respectively. **B** : **pCH217** contains the *APT-3* gene under the control of the PGK1 promoter and terminator signals. This *APT2* cassette can be separated from the pIC-19R parental DNA on a 2.0kb *EcoR*I fragment. The *BLA* gene allows selection for ampicillin resistance in *E.coli*. The *APT2* construct confers resistance to G418 in yeast, and kanamycin in *E.coli*. Restriction enzyme abbreviations used are: **B** (*Bam*HI), **H** (*Hin*DIII), **Kp** (*Kpn*I), **N** (*Nco*I), **Pv** (*Pvu*II), **R** (*Eco*RI) and **Sc** (*Sca*I).

Construction of Disrupted *ATP12* DNA

The strategy used to disrupt *ATP12* with the G418^{res} marker is outlined in **Figure 3.3.2:**

1. YEp351/ST4 was digested with restriction enzyme *Nco*I in T4 DNA polymerase buffer, then blunt ended molecules produced by treatment with T4 DNA polymerase. The DNA was run on a 0.7% LMP agarose gel and vector DNA visualised under UV and isolated using the CTAB/butanol method. Vector DNA was then treated with CIP to remove 5'-phosphate groups.

2. pCH217 was digested with *Eco*RI then run on a 0.7% LMP gel. The 2.0 kb band containing the *APT3* gene cassette (*APT2*) was purified by the CTAB/butanol method as before. The fragment was then treated with T4 DNA polymerase klenow fragment to produce blunt-ended insert DNA.

3. YEp351/ST4 and pCH217 isolated fragments were joined by blunt-end ligation using T4 DNA ligase to produce a plasmid containing the *ATP12* gene disrupted by *APT2*. Ligation mixes were used to transform competent *E.coli* NM522. Bacteria were first selected for ampicillin resistance on LB+ampicillin plates, then replica-plated onto LB+kanamycin to select for expression of *APT2*. It was observed that direct selection on kanamycin immediately after transformation yielded no transformants.

Plasmid DNA was isolated from Kanamycin resistant NM522 colonies by the small scale plasmid isolation technique, and verified by restriction digest analysis. The new construct was named pJH2.

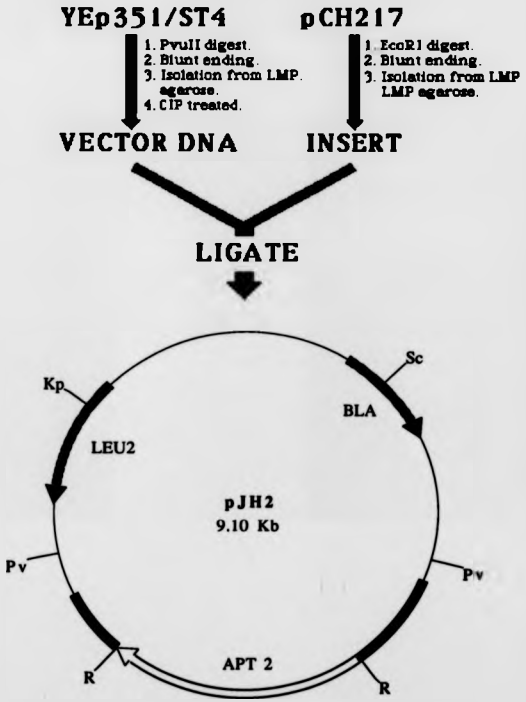


Figure 3.3.2. Strategy for disruption of *ATP12*. Details of blunt ending etc. are given in the text. The *Pvu*II restriction sites are used to liberate the *APT2*-interrupted *ATP12* fragment for one-step gene disruption studies. The *Bgl*II (B) anb *Sca*I (Sc) sites were used to determine the orientation of the *APT2* gene.

A *Pvu*II digest of pJH2 liberates a fragment containing the disrupted form of *ATP12*; this digest was used in transformations to create a new yeast strain that is deficient in *ATP12*.

One-step Gene Disruption of *ATP12*

S.cerevisiae strain C2 was transformed using the lithium acetate method (Ito *et al*, 1983) with *Pvu*II-digested pJH2; G418 resistance was used as a marker for gene disruption. It was determined that 0.3mg/ml G418 in YEPD plates prevented background growth of spontaneous G418 resistant C2. Uncut pJH2 and TE buffer were used as controls during transformation to ensure the efficiency of the technique was high enough, and that no naturally-occuring $G418^{res}$ yeast were produced. This one-step gene disruption procedure provided $G418^{res}$ C2 with the desired phenotype, as shown in (**Table 3.3.1**).

Transforming DNA	Efficiency (colonies/µg DNA)	Selection	
		YEPD	YEPG
pJH2 *Pvu*II digest	10	25	1
pJH2	120	10	10
None	0	-	-

Table 3.3.1: One-step disruption of the *ATP12* gene of C2. Efficiency of transformation was determined as the number of $G418^{res}$ transformants/µg DNA. *Petite* phenotype of transformants was shown by differential growth on YEPD and YEPG plates.

Twenty-five $G418^{res}$ transformants obtained using *Pvu*II-digested pJH2 as transforming DNA were grown on YEPD and YEPG to identify

petite mutants. Of these, twenty four failed to grow on YEPG plates after 5 days. The one colony observed to grow might be G418 resistant because of incomplete digestion of pJH2 with *Pvu*II or the *Pvu*II fragment coding for G418 resistance may have integrated non-specifically into the C2 genome at a site outside the *ATP12* gene locus. The twenty-four possible *ATP12* mutants were named C2:Δ*ATP12:1* to C2:Δ*ATP12:24* and maintained for further investigation.

Mating C2:Δ*ATP12* with a ρ° Yeast.

The location of the *pet* mutation in each C2:Δ*ATP12* mutant isolated was determined by crossing each mutant with a ρ° strain of *S.cerevisiae*. Crossing these strains with a ρ° mutant will produce a diploid yeast which either can or cannot grow on YEPG. If the diploid yeast can grow on YEPG this implies the *pet* mutation is located in the chromosomal DNA and the integrity of the mitochondrial DNA is unaltered. If, however, the diploid cannot grow on YEPG this indicates the *pet* mutant lacks a complete mitochondrial genome but does not exclude the possibility of additional nuclear *pet* lesions.

S.cerevisiae X2180-1A was treated with ethidium bromide to produce a ρ° yeast (Goldring *et al*, 1970), which was then crossed with each of the C2:Δ*ATP12* mutants isolated. Successful mating was selected for by isolating diploid cells that complemented deficiencies of the other. C2 requires leucine and lysine for growth on *mm* plates and X2180-1A cannot utilize maltose as a carbon source, so diploids were selected for growth on *mm* plates containing 2% maltose as the sole carbon source. Crosses grew after 2 days at 30°C but haploid C2 and X2180-1A failed to grow after 1 week on the same plates.

Diploids were then streaked onto YEPG plates. Crosses that grew on YEPG consist of C2:Δ*ATP12* with intact mitochondrial DNA and the ρ^{o} X1280-1A with no *PET* mutation. Of the twenty-four C2:Δ*ATP12* diploids tested, twenty were found to be respiration-competent. These were further examined to determine the location of the nuclear *pet* lesion, as described below.

Phenotypic Rescue of C2:Δ*ATP12*

To determine whether the *pet* mutation is in the *ATP12* gene, C2:Δ*ATP12.1* was transformed with YEp351/ST4 and pJH2. The only difference between these plasmids is that the *ATP12* gene of pJH2 is disrupted. Thus, YEp351/ST4 contains the *ATP12* gene in a form that will rescue the *pet* phenotype of a Δ*ATP12* mutant, whereas with pJH2 the gene is disrupted with subsequent loss of function. The *LEU* marker of both plasmids was used to assess successful transformations on *mm* plates supplemented with lysine. Both plasmids transformed C2:Δ*ATP12.1* to leucine prototrophy, but only YEp351/ST4 rescued the *pet* phenotype of C2:Δ*ATP12.1*, determined by replica plating *LEU* transformants onto YEPG plates. This indicates that the *pet* lesion of C2:Δ*ATP12.1* is in the *ATP12* gene.

A YEp351/ST4 transformant was picked into 5ml of YEPD and grown under non-selective conditions for 24 hours. Cells were harvested, washed, and plated out at various dilutions on YEPD plates. A mixed population of *grande* and *petite* colonies was observed at a frequency of approximately 70% to 30% respectively. The phenotype of one hundred of these colonies, chosen at random, was determined by streaking on the various selective plates indicated in **Table 3.3.2.** The plates were grid-referenced to allow identification of individual

transformants. It was observed that colonies that did not grow on *mm* supplemented with lysine also failed to grow on YEPG plates. These results demonstrate co-retention or co-deletion of the *LEU* and *ATP12* phenotypes under condition of non-selective growth. Also, rescue of the *pet* phenotype is plasmid coded, and confirms that C2:Δ*ATP12.1* is deficient in a functional *ATP12* gene product. All colonies screened were G418 resistant, therefore the *APT2* expression cassette has stably integrated into the chromosomal DNA, and is not lost under the non-selective growth conditions used. Stability of integration is a major advantage of a one-step gene disruption strategy, and essential for industrial application.

Selection	No. of cells	Phenotype
mm+Lys	68	*grande* : 68%
YEPG	68	*petite* : 32%
YEPD+G418	100	$G418^{res}$: 100%

Table 3.3.2 : Non-selective growth of C2:Δ*ATP12.1*:YEp351/ST4. A single colony of YEp351/ST4-transformed C2:Δ*ATP12.1* (LEU, *grande*), was grown non-selectively in YEPD for 24 hours, then plated on YEPD. 100 colonies, chosen at random, were screened for retention of the LEU2 marker (on *mm*+lysine), *ATP12* (on YEPG), and *APT2* (on YEPD+0.3mg/ml G418). Each colony was picked onto plates underlayed with a reference grid that allowed identification of individual colonies. Plates were incubated at 30°C for 5 days before scoring for growth.

ATPase Assay of C2 and C2:Δ*ATP12.1*

Mitochondria were isolated from wild type C2 and C2:Δ*ATP12.1* by a mechanical disruption technique (Bowman, 1989). The concentration of mitochondrial protein was determined and approximately 0.5mg of protein used per assay. ATPase activity was measured in Kletts Units (K.U.). Oligomycin-sensitive activity was calculated as the difference between total ATPase activity in the presence of 20μg/ml

oligomycin. **Table 3.3.3** shows that mutant C2:Δ*ATP12.1* has approximately 2.8% oligomycin-sensitive activity of the wild type. This agrees with the ATPase activity previously found for members of the complementation group G57 (Bowman, 1989).

Yeast strain	S. A. ± oligomycin		oli[s] S.A.
	-	+	
C2	29.060	8.547	20.513
C2:Δ*ATP12.1*	1.524	0.952	0.572

Table 3.3.3: ATPase activities of C2 and C2:Δ*ATP12.1*. Specific activity (S.A.) was calculated in Klett Units (K.U.) (ΔA335/mg protein/min). 20μg/ml oligomycin (oli) was added to the assay to determine the oligomycin-sensitive ATPase activity (oli[s] S.A.).

Brewing Trial *1*: C2 versus C2:Δ*ATP12.1*

One reason for choosing C2 for gene disruption studies is that this haploid laboratory strain of *S.cerevisiae* has the ability to utilize maltose as a carbon source (*MAL*). Using a *MAL* strain allows the wild type and mutated yeast to be studied in brewing trials to determine the effect of mutagenesis on brewing performance, which is assessed by trials designed to mimic industrial fermentation conditions.

Brewing trials were carried out as previously described (see **SECTION 2.2**) with the C2:Δ*ATP1* mutant and wild type C2 to assess the effect disrupting *ATP12* has on the fermentation properties of C2. The trial was started by pitching 1.5g of yeast into 500ml of wort. Three separate G-tubes were set up for both C2 and C2:Δ*ATP12*. Fermentation was followed qualitatively by the evolution of carbon

dioxide gas, and quantitatively by accurate determination of the specific gravity of the wort. As sugar is taken up by the yeast there is a drop in the specific gravity, which was measured using a densitometer throughout the course of fermentation. The rate of fermentation of the wild type and mutant, followed by sugar uptake, is shown in **Figure 3.3.3**. The time taken for the mutant to ferment the sugar is shown to be far greater than the time taken by C2.

After completion of fermentation, yeast were harvested in pre-weighed centrifuge pots. Wet and dry weights, ethanol production, and final specific gravity values were determined (**Table 3.3.4**). The following ratios were calculated from these values to assess the efficiency of fermentation production:

(a) ethanol produced : sugar used
(b) ethanol produced : biomass produced

Conclusions drawn from this data were as follows:

1. The mutant does not ferment wort to the same gravity as the control, and it takes far longer to complete fermentation.

2. Less biomass is produced by the mutant with approximately the same ethanol yield.

3. The efficiency of ethanol production per unit of sugar used is greater when fermenting with the mutant.

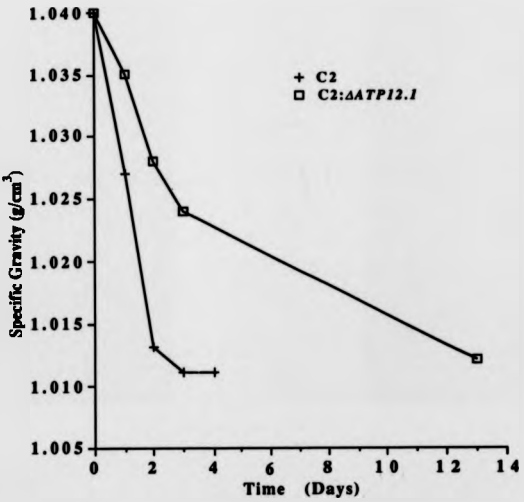


Figure 3.3.3: Sugar uptake by yeasts during Brewing Trial *1*. The specific gravity of each G-Tube was determined at the times indicated. C2 and C2:Δ*ATP12.1* fermented the wort to a specific gravity of 1.010 and 1.012g/cm^3 respectively (difference of 6.7%). Each of the three replica G-tubes had identical values at all time points indicated.

	C2				C2:Δ*ATP12.1*				
G Tube	1	2	3	Mean ± σ	1	2	3	Mean ± σ	*P*
Sugar Used (units)	29	29	29	29	27	27	27	27	-
Dry Weight (g)	2.184	2.161	2.174	2.177 ±0.009	1.984	2.002	1.994	1.993 ±0.007	-
Ethanol Yield (%)	3.47	3.40	3.68	3.52 ±0.12	3.56	349	347	3.507 ±0.039	1.00 - 0.90
Ethanol (%) per Sugar Used	0.120	0.117	0.127	0.121 ±0.004	0.132	0.129	0.129	0.130 ±0.001	0.10 - 0.05
Dry Weight (g) per Sugar used ($x10^2$)	7.53	7.45	7.50	7.49 ±0.04	7.53	7.42	7.39	7.47 ±0.06	0.40 - 0.30

Table 3.3.4: Brewing Trial *I*: C2 versus C2:Δ*ATP12.1*. The fermentations were ran to completion, and the values given calculated as before (see **Table 2.3.1**).

A major disadvantage when brewing with C2:*ΔATP12.1* was the length of fermentation, therefore attempts were made to reduce the fermentation period by pitching excess of mutant yeast at the start of fermentation.

Brewing Trial *II*: Increasing the Amount of Mutant Yeast
A scaled down version (100ml) of the 500ml brewing trial was performed using varying amounts of mutant yeast and a fixed amount of C2. A 5-fold increase in C2:*ΔATP12.1* was found to ferment wort at approximately the same rate as C2 pitched at the normal concentration. Trial *II* was performed as normal, except 1.5g of C2 and 7.5g of C2:*ΔATP12.1* was pitched into each G-tube. The conditions of fermentation and analysis of data were the same as described for Trial *I*. **Figure 3.3.4** indicates that pitching a five fold excess of mutant accelerates fermentation, as expected, but the fermentation profile of mutant and wild type yeasts are different. A difference in the change in the gravity of the wort was again observed, with the mutant again having a higher final gravity. The values of sugar used, and biomass and ethanol yields produced, were analysed as before to give an indication of brewing performance **(Table 3.3.5)**

As shown in **Figure 3.3.4**, the original gravity of the wort containing mutant yeast is 1 unit lower than the control, as a consequence of adding a five-fold excess of yeast made in sterile distilled water. This altered the density of the wort, although the sugar present in both sets of G-tubes was equivalent. The mutants fermented the wort to 1 unit higher than the wild type yeast, which accounts for the two unit difference (7.7%) in sugar use observed.

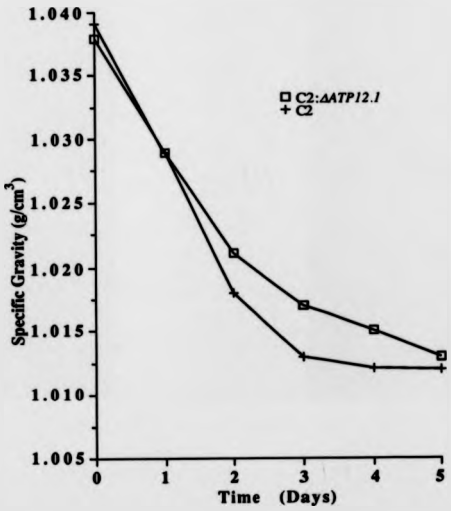


Figure 3.3.4: Change in specific gravity during Brewing Trial *II*. A five-fold excess of mutant was pitched. Both yeast ferment the wort at approximately the same rate although the mutant uses two units of sugar less than C2, as explained in the text.

	C2				C2Δ*ATP12.1*				
G Tube	1	2	3	Mean ± σ	1	2	3	Mean ± σ	*P*
Sugar Used (units)	27	27	27	27	25	25	25	25	-
Dry Weight (g)	1.805	1.861	1.767	1.811 ±0.039	0.682	0.701	0.687	0.690 ±0.008	-
Ethanol Yield (%)	3.78	3.76	3.77	3.77 ±0.01	3.65	3.74	4.09	3.83 ±0.23	0.70 - 0.60
Ethanol (%) per Sugar Used	0.140	0.139	0.140	0.140 ±0.001	0.146	0.150	0.164	0.153 ±0.007	0.10 - 0.05
Dry Weight (g) per Sugar used ($x10^2$)	6.69	6.89	6.54	6.71 ±0.14	2.73	2.80	2.75	2.76 ±0.03	< 0.001

Table 3.3.5: Brewing Trial *II*: pitching a five-fold excess of C2:Δ*ATP12.1*. Each C2 G-tube fermentation was started with 1.5g of yeast as usual; 7.5g of C2:Δ*ATP12.1* was pitched per tube. The values of biomass shown for C2:Δ*ATP12.1* are one fifth the actual values determined; this allows comparisons to be made between both sets of data. Other values shown were determined as before (see Table 2.3.1).

Analysis of the fermentation data (Table 3.3.5) shows that as for Trial *1*, the [ethanol produced : sugar used] ratio is greater when brewing with C2:Δ*ATP12.1*. The significance of these results are discussed below, with reference to the results of SX-1 brewing trials.

3.4 DISCUSSION.

Summary of *ATP12* Studies

Gene cloning techniques have been used to disrupt the *APT12* gene (Bowman *et al*, 1991) with the *APT2* cassette (Hadfield *et al*, 1990) that confers resistance to the antibiotic G418. The *APT2:APT12* construct was used to transform *S.cerevisiae* C2, with transformants selected for G418 resistance. Respiratory-deficient *ATP12 pet* mutants were identified by biochemical and genetic analysis. Brewing trials of a C2:Δ*ATP12* mutant against the wild type revealed that the mutant has advantageous properties for brewing. The rate of fermentation, however, is slow and the mutant is unable to ferment wort to the same gravity as the wild type. Brewing with a five-fold excess of mutant, reduces the fermentatation period to a time comparable to fermentation with the wild type, thus overcoming the problem of slow fermentation by the mutant. A noticable difference between the parental C2 and the mutant is that the rate of change in specific gravity is more constant throughout the course of fermentation when brewing with the mutant yeast strain. In addition mutant yeast consistently fermented wort to a higher final gravity value than the wild type control yeast. Brewing trials did indicate that the [ethanol produced : sugar used] ratio significantly increases when brewing with the mutant.

The conclusions drawn from uncoupler studies and *ATP12* mutagenesis indicate that fermentation is altered in a similar manner in each case. The rate of fermentation is reduced, as is sugar uptake (measured by specific gravity). This is balanced with favourable fermentation characteristics, as indicated by the calculated ratios of [ethanol produced : sugar used] and [biomass produced : sugar used]. There is, therefore, more efficient use of available sugar *i.e.* reduction in biomass and increase in ethanol production.

Mitochondrial Structure or Mitochondrial Function?

It has been previously reported that a ρ^- mitochondrial genome is associated with differential uptake of sugar (Evans and Wilkie, 1976; Mahler and Wilkie, 1978) and that this phenotype is affected by the quality of the ρ^- genome. Similarily, different classes of respiratory-deficient yeast have been found to produce different levels of nuclear-coded mRNA transcripts (Parikh *et al*, 1987). This evidence infers a role for mitochondrial DNA or RNA in intergenomic regulation. The possibility that changes to the mitochondrial genome affects expression of a mitochondrial product that regulates nuclear function are unlikely. Most of the yeast mitochondrial genome has been sequenced, and functions assigned to gene products (reviewed by Grivell, 1989), and no such protein has been detected.

The evidence presented in **Chapters 2 and 3** does not directly support the theory that nuclear DNA is regulated by the mitochondrial genome. Changes in nuclear processes (*i.e* sugar uptake and fermentation rate) are induced by chemical uncoupling of oxidative phosphorylation, or when a *pet* mutant is used for brewing. These effects are different in their mode of action but both

effectively stop ATP production *via* respiration during brewing trials, suggesting that mitochondrial function, and not structure, is affecting fermentation performance. The possibility, however, that ρ^- mitochondria are formed during fermentation cannot be eliminated, as the proportion of cytoplasmic petites present at the end of fermentations was not determined.

Work has shown that industrial yeast strains with ρ^- mitochondria have improved efficiency of ethanol production compared to parental ρ^+ yeast (Bacila and Horii, 1979). It has, however, been suggested that these results are a consequence of the Pasteur effect but this does not appear to be the case in the conditions used in our laboratory scale brewing trials. A colleague has used an inhibitor of pyruvate transport (α-cyan-4-hydroxy cinnamic acid) in brewing trials similar to those described in **SECTION 2.2**. Results obtained resemble those reported for SX-1 brewing trials. The control and inhibited yeast were observed to continually produce ethanol throughout fermentation (Allen Donald, Ph.D thesis, Warwick University, in preparation), arguing against the Pasteur effect giving apparent increases in ethanol yields.

Viegas and Sa-Correia (1991) have recently demonstrated that treatment of growing yeast cells with octanoic acid reduces the growth rate and improves ethanol yields (16.7% increase per unit of glucose used), results similar to those presented in these studies. The toxicity of octanoic acid is thought, in part, to be due to dissipation of the H^+ gradient across the plasma membrane. Octanoic acid increases the level of the plasma membrane ATPase, whose function is to maintain the H^+ gradient across the plasma membrane at the

expense of ATP. The resulting plasma membrane H^+ gradient drives secondary membrane transport and regulates the activity of many intracellular enzymes (Serrano, 1988). Interestingly, uncouplers inhibit uptake of maltose, the major carbohydrate in wort, by affecting the H^+ gradient (Serrano, 1977). One possibility is that abolishing mitochondrial respiration, genetically or chemically, impedes the mantainence of the plasma membrane proton gradient, as less ATP per unit of sugar is generated.

Future Studies

The eventual aim of any study to create a novel yeast for brewing is a full-scale brewing trial using the genetically engineered yeast to compare its properties with those of the parental yeast. For these studies, mutation of an industrial yeast strain would be essential, to evaluate the multiple factors important in brewing, such as taste and appearance. Attempts were made to create a Y9 strain disrupted for *ATP12* using *Pvu*II digests of pJH2 (see **Figure 3.3.2**). Colonies containing stably integrated copies of the *APT2* cassette were obtained but none had a *pet* phenotype. The exact copy number of *ATP12* in Y9 was not determined, but is probably greater than two. Creating an *ATP12* null phenotype would require much work, and more than one round of gene disruption. One approach would be to use different antibiotic resistance genes in each stage. A similar approach would be to sequentially disrupt the *ATP12* genes with the same antibiotic marker and remove the antibiotic gene after each round of disruption. A different approach would be to use an antisense RNA approach. Disruption of *ILV2* function in yeast has been demonstrated (Xiao and Rank, 1988). This may be a good approach for *ATP12* disruption as there is a good selection system

for loss of *ATP12* activity and the gene is present in single copy per haploid genome. A disadvantage of an anti-sense approach is that the suppression is often partial (Kim and Wold, 1985).

Brewing with a *pet* mutant may be beneficial to the brewing industry if such a mutation does not alter the characteristics of the beer. The differences in ethanol yields are very significant on an industrial scale, although the exact nature of the difference in sugar use needs to be determined. It is possible that uptake of a specific sugar (or sugars) is affected, or that mutated yeast take up all sugar to a lesser extent. Elucidation of this apparent inhibition could possibly lead to a modification of the brewing process, such as addition of a carbohydrate adjunct that is taken up by a fermenting Δ*ATP12* mutant, with a subsequent increase in ethanol yield.

CHAPTER 4
GENE DISRUPTION OF THE ADP/ATP TRANSLOCASE

4.1 INTRODUCTION

The ADP/ATP carrier (AAC) protein[1] transports ADP and ATP across the inner mitochondrial membrane, and is the most abundant mitochondrial protein (Klingenberg, 1985). At the beginning of these studies a gene encoding an AAC protein of *S.cerevisiae* had been cloned (O'Malley *et al*, 1982) by complementation of an *op1* (Kovac *et al*, 1967) or *pet9* (Beck *et al*, 1968) mutation. The gene was sequenced and found to be homologous to a number of AAC proteins from other eukaryotes (Adrian *et al*, 1986). The AAC protein was therefore considered a good target for gene disruption studies as it's gene was shown to be nuclear, mutants have a distinguishing phenotype, and the complete sequence had been published.

Subsequent studies have revealed the presence of a second *AAC* gene which has been isolated and sequenced (Lawson and Douglas, 1988). The new gene was named *AAC2*, and the original gene (*PET9*) was renamed *AAC1*. The *AAC1* and *AAC2* genes are very homologous, but *AAC2* will complement the *op1* or *PET9* mutation when expressed from a single copy number plasmid whereas *AAC1* only does so when expressed on a multicopy vector (Lawson and Douglas, 1988). Furthermore, disruption of *AAC1* has no effect on yeast respiration (Lawson and Douglas, 1988), whereas Δ*AAC2* mutants cannot grow on a non-fermentable carbon source (Lawson *et*

[1]synonomous with ADP/ATP translocator protein and ADP/ATP translocase

al, 1990). Further studies have shown that *ΔAAC1*:*ΔAAC2* mutants have essentially the same phenotype as *ΔAAC2* strains (Lawson *et al*, 1990). This chapter details attempts to create a C2:*ΔAAC2* strain to evaluate the fermentation properties of yeast deficient for the AAC protein.

4.2 MATERIALS AND METHODS

Xgal Selection For Ligation

The *LacZα* gene of pUC7 (**Figure 4.3.1**) contains a restriction enzyme polylinker near the 5' end of the gene. The unique sites in the polylinker can be used for insertion of any DNA fragment. Disruption of the *LacZα* gene, which codes for β-galactosidase activity, can be detected by transforming *E.coli* with ligation mixtures of pUC7 (digested with a restriction enzyme that cuts in the polylinker) plus insert DNA, followed by selection on plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). Functional β-galactosidase cleaves Xgal to release a blue indolyl derivative that can be distinguished from white colonies with no β-galactosidase activity (Old and Primrose, 1985). Inactivation can be caused by insertion of a DNA fragment within the *LacZα* gene, or by religation of pUC7 DNA that has been blunt ended. Xgal was obtained from Sigma, and used as recommended.

Preparation of High Specific Activity Probes by Random Hexanucleotide Priming

The protocol followed for preparation of DNA probes was based on that of the Leicester University Biocentre handbook for Standard

Molecular Biology (Dr. W.E. Lancashire, personal communication), with some variations:

Solutions used to make Probe DNA

Solution O

1.25M Tris.HCl (pH8.0)

0.125M $MgCl_2$

dNTPs

100mM solutions

(bought from BCL)

Solution A

1ml Solution O

18µl β-mercaptoethanol

5µl each dNTPs (except dCTP)

Solution B

2M HEPES-NaOH (pH6.6)

Solution C: Hexanucleotides (from Pharmacia) at 90 A_{260} units/ml in TE buffer

OLB Buffer (ratios)

Solution A : 10

Solution B : 25

Solution C : 15

Stop Buffer

20mM Tris.HCl (pH7.5)

20mM NaCl

2mM EDTA

0.25% SDS

1µM dCTP

1. Probe DNA was isolated from plasmids by digesting with the appropriate restriction enzyme(s), then digests were run on a 0.7% LMP agarose gel stained with ethidium bromide dye. The band of interest was visualised by UV illumination and the amount of DNA estimated. The band was then removed in a minimum volume of agarose.

2. The agarose slice was placed in a pre-weighed Eppendorf tube then 1.5ml of dH_2O was added per gram of gel. The Eppendorf was heated at 100°C for 7 minutes then held at 37°C for 10-60 minutes. Excess probe DNA can be stored at -20°C but needs re-boiling before further use.

3. The probe was usually labelled overnight at room temperature in an Eppendorf tube placed inside a lead container. The following ingredients were added in the order given:

   Xµl dH2O (to give 25µl final volume)
   5µl OLB
   1µl BSA
   Yµl probe DNA solution
   2.5µl [α-^{32}P] dCTP (10µCi/µl, ~3000Ci/mmol) (Amersham)

4. The reaction was stopped by adding 100µl of Stop Buffer, and incorporation of [α-^{32}P] dCTP checked. A small sample of probe solution (<1µl) was spotted onto a pencil mark on a strip of DE81 paper which was then placed in a solution of 0.3M Ammonium Formate, with the pencil mark above the level of the solvent. After approximately 20 minutes the solvent front moves about 5cm. Radioactivity incorporated into DNA remains at the bottom of the DE81 paper, whereas unincorporated material moves with the solvent front.

5. Level of dNTP incorporation can be determined using X-ray film. DE81 paper is overlaid with X-ray film in an autorad cassette, which was encased with lead, as the probe DNA is very 'hot'.

6. Unincorporated dNTPs were removed by two ethanol precipitation steps, then resuspended in a suitable volume of TE. This method was prefered over that used at Leicester (Sephadex G50 column purification).

Probe DNA prepared by the above method is suitable for Southern Blotting and Colony Hybridization studies.

Preparation of Yeast Genomic DNA (Ausubel *et al*, 1989)

A single yeast colony was used to innoculate 5ml of YEPD in a 30ml plastic universal. The yeast were grown for 16-24 hours in a 30°C orbital shaker (250rpm) then harvested by spinning at 4000rpm for 5 minutes at room temperature in a Griffin and George VJ1 bench centrifuge. The pellet was resuspended in a 0.5ml sorbitol solution and 100µl of yeast spheroplast solution added.

Sorbitol Solution	Spheroplast Solution
0.9M Sorbitol	3ml Sorbitol Solution
0.1M Tris.HCl, pH.8.0	10µl β–mercaptoethanol
0.1M EDTA	6mg Zymolyase

The tubes were shaken at 37°C for 60 minutes at 200rpm. After checking for spheroplast formation, cells were harvested as before. 0.5ml of TE buffer was added and the pellet gently resuspended by repeatedly pipetting up and down. The suspension was then transferred to an Eppendorf tube; 50µl of 10% SDS added and the contents were mixed by gentle inversion. After incubation at 65°C for 20 minutes, 200µl of 7.5M ammonium acetate was added. The contents of the tube were mixed by inversion, then placed on ice for 30 minutes. The Eppendorf was then spun for 3 minutes and the

supernatant removed to a fresh tube. Two volumes of ethanol were added (ca. 1ml) and the tubes gently shaken by hand. The DNA appears as threads of fibre at this stage. The Eppendorf was then spun for 10 seconds, the supernatant removed by aspiration, and the pellet partially dried under vacuum. The DNA was then redissolved in 300µl of TE. DNA can be left overnight to dissolve or the tubes can be placed at 65°C and the DNA dissolved by finger-flicking the tube periodically. Time to resuspend the pellet by this method varied between 1-2 hours. Next 5µl of a 10mg/ml solution of RNAaseA was added and the tube incubated for 1 hour at 37°C. 0.5ml isopropanol was added and the contents gently mixed until the DNA appeared as a single clump. The DNA was then pelleted by a 10 second spin and the supernatant removed by aspiration. The pellet was washed twice with 70% ethanol, dried under vacuum for 5 minutes, and resuspended in 100µl of TE. DNA prepared by this method is of relatively high molecular weight and is suitable for restriction enzyme digestion, and Southern Blotting.

Southern Blotting (Southern, 1975)

Yeast genomic DNA was digested with the restriction enzymes indicated and resolved by gel electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and photographed with a ruler placed adjacent to the DNA markers. The size of hybridizing bands can be determined by comparing the photograph and the final autoradiograph. Detection of hybridrizing bands is accomplished by transfer to a Hybond[1] nylon membrane (HybondN), followed by hybridization to radiolabelled probe, washing off of unbound

[1]Hybond is a trade name of Amersham

radioactivity, and finally detection of hybridizing bands with X-ray film. The method below is essentially the same as that described in the Amersham hybridisation handbook:

I. Transfer of DNA to Nylon Support

The gel was marked for orientation purposes then immersed in 0.25M HCL for 15 minutes; this improves the efficiency of transfer of DNA fragments greater than 10kD. The HCL solution was then replaced by denaturing solution for 15 minutes followed by further 15 minutes incubation in fresh denaturing solution. Next the gel was equilibrated in transfer solution for one hour.

Denaturing Solution	Neutralizing Solution
1.5M NaCl	1.5M NaCl
0.5M NaOH	0.5M Tris.HCl (pH7.2)
	10mM EDTA

Transfer Buffer (0.025M Sodium Phosphate, pH6.5)
16ml 0.5M Na_2HPO_4
34ml 0.5M NaH_2PO_4
made to 1 litre with dH_2O

HybondN was cut to the size of the gel and layered on top of the gel ensuring that no air bubbles were trapped between the gel and the membrane. Two sheets of 3MM filter paper were placed on either side and the sandwich was completed by placing thick nylon pads on each side. The sandwich was then inserted into a vertical electrophoresis tank. Care was taken to ensure that the nylon mumbrane was between the gel and the anode. DNA transfer was

performed at 4°C for 2 hours at 1 Amp. After transfer the membrane was washed with Transfer Buffer to remove residual agarose then air dried DNA side up and placed DNA side down on a Fotodyne 3-3002 UV transilluminator for 1-2 minutes. Calibrations indicated that longer exposure to UV dramatically reduces the hybridization signal obtained.

II. Hybridization and washing

Prior to addition of probe DNA the hybondN membrane was prehybridized in 100ml of "Blotto" solution (Dr D. Whitcombe, personal communication) for at least one hour in a sealed sandwich box placed in a 65°C shaking water bath.

"Blotto" Solution
0.5% (w/v) Cadburys Marvel
1.0% (w/v) SDS
6.0% (w/v) PEG4000
0.27M NaCl
1mM EDTA
1.5mM Sodium Phosphate, pH.7.7
100µg/ml heat denatured Herring Sperm DNA (Sigma)

Radioactive probe DNA, synthesised by random hexanucleotide priming, was heat denatured by boiling for 3 minutes, chilled rapidly on ice, then added to the prehybridization mix. Hybridization was performed for 12-24 hours, after which the hybridization mixture was removed and the membrane washed; twice with 100ml 2xSSC at 65°C for 15 minutes, followed by 100ml of 0.1xSSC at 65°C for 30 minutes.

III. Dection of Hybridizing DNA

The washed nylon membrane was dried and placed in Saran Wrap then immobilized within an autoradiography cassette case. Fugi X-ray film was overlayed noting the exact position by marking the borders of the film with respect to the nylon membrane. An intesifying screen was put on top of the X-ray film, and the cassette was placed at -70°C for varying exposure times, that depended on the intensity of the radioactive signal. X-ray film was developed by immersing it in Kodak X-ray film developer for 3 minutes, followed by a one minute wash in dH_2O. Film was then in placed in Kodak Fixer solution for 5 minutes, then finally under running water for 10 minutes, as recommended by Maniatis *et al* (1982).

Colony Hybridization

Ligation mixtures were used to transform *E.coli* NM522. Vector DNA containing the correct insert were detected by screening transformants by colony hybridization. Insert DNA, labelled by the random hexanucleotide method, was used as a probe to detect functional ligations. Amp^{res} transformants were isolated and streaked onto two identical sets of LB plates overlayed with gridded circular HybondN membranes. Both sets of plates were grown overnight at 37°C then one set was stored at 4°C as a master copy. The other set were subjected to colony hybridization as according to the Amersham hybridisation handbook.

Membranes were overlayed colony side up onto 3MM filter paper soaked in Denaturating Solution for 7 minutes. The membranes were then transferred colony side up to filter paper soaked in neutralizing solution for 3 minutes. This step was then repeated with a fresh pad

soaked in the same solution. Filters were washed in 2xSSC, transferred to dry filter paper, and allowed to dry colony side up. DNA cross-linking to HybondN and detection of hybridizing DNA was achieved by the methods used in Southern Blotting.

4.3 RESULTS

As mentioned in the introduction, the *AAC1* gene has been cloned and sequenced (O'Malley *et al*, 1982; Adrian *et al*, 1986). We wrote away for a copy of the cloned gene but received no response. A *pet9* mutant was obtained from Dr. J. Haslam, and attempts made to obtain the *AAC1* gene by complementing with a yeast genomic DNA library (Nasmyth and Reed, 1980). Problems experienced with yeast transformation (as discussed in **CHAPTER 5**) hindered all attempts to clone the *AAC1* gene. We decided to write again to Dr. Douglas, and eventually received a copy of the *AAC2* clone prior to publication of the sequence (Lawson and Douglas, 1988). We decided to carry out work to create a C2:Δ*AAC2* mutant. At this stage the phenotype of Δ*AAC1*:Δ*AAC2* was unknown. The initial aim of this study, therefore, was to disrupt *AAC2* as this appeared to provide yeast with most, if not all, mitochondrial ADP/ATP translocase activity.

Disruption of Cloned *AAC2*

The *AAC2* gene was kindly provided by Dr. Janet Lawson, cloned into the polylinker of the yeast-*E.coli* shuttle vector pSEY8. The coding region of the gene is contained on a 3.5kb *Eco*RI fragment (Lawson *et al*, 1988). A restriction map of pSEY8/AAC2 (**Figure 4.3.1**) shows that the restriction sites of pSEY8/AAC2 are not suitable for gene disruption, therefore it is necessary to clone the *AAC2* gene into another vector.

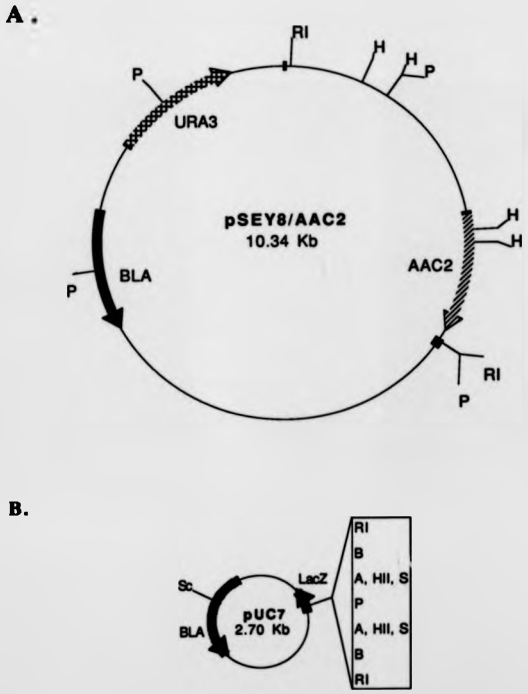


Figure 4.3.1: Plasmids used in *AAC2* studies. A. pSEY8/AAC2 contains the *AAC2* gene cloned into the polylinker of pSEY8 on a 3.5kb *Eco*RI fragment. B. pUC7 contains a polylinker and the *BLA* gene that confers ampicillin resistance. All plasmids drawn in this chapter are scaled 1kb of DNA is equivalent to 3.142cm. Restriction site abbreviations: A=*Aac*I, B=*Bam*HI, H=*Hin*DIII, HII=*Hin*DII, P=*Pst*I, and S=*Sal*I.

pUC7 (Figure 4.3.1) was chosen, as it has no *Hin*dIII sites. Cloning the 2.6kb *Pst*I/*Eco*RI fragment of pSEY8/AAC2 into pUC7 allows disruption of *AAC2*, by inserting the 2.0kb *Eco*RI fragment of pCH217 (Figure 3.3.1) between the two internal *Hin*dIII sites shown. Figure 4.3.2 summarises the two-step procedure used to disrupt *AAC2* with *APT2*:

1. An *Eco*RI/*Pst*I double digest of pSEY8/AAC2 was performed in T4 DNA polymerase buffer. Digested DNA was then treated with T4 DNA polymerase to produce blunt ended molecules. Similarly, pUC7 was digested with *Pst*I and blunt ended.

2. Both digests were run on a LMP gel. The 2.6kb *Eco*RI/*Pst*I fragment and *Pst*I-linearised pUC7 were isolated from a 0.7% LMP agarose gel by CTAB butanol extraction.

3. Digested pUC7 DNA was treated with CIP to remove 5' phosphate groups, then mixed with the *AAC2* fragment and ligated overnight using T4 DNA ligase.

4. Ligation mixtures were used to transform *E.coli* NM522 with selection on LB-ampicillin plates containing X-gal. White colonies were chosen for colony hybridization studies using the 2.6kb *Eco*RI-*Pst*I fragment as a probe (Figure 4.3.3). Positive colonies were isolated and verified as having the *AAC2* gene by restriction digests analysis. A clone was named pJH3 (Figure 4.3.4) and chosen for further studies.

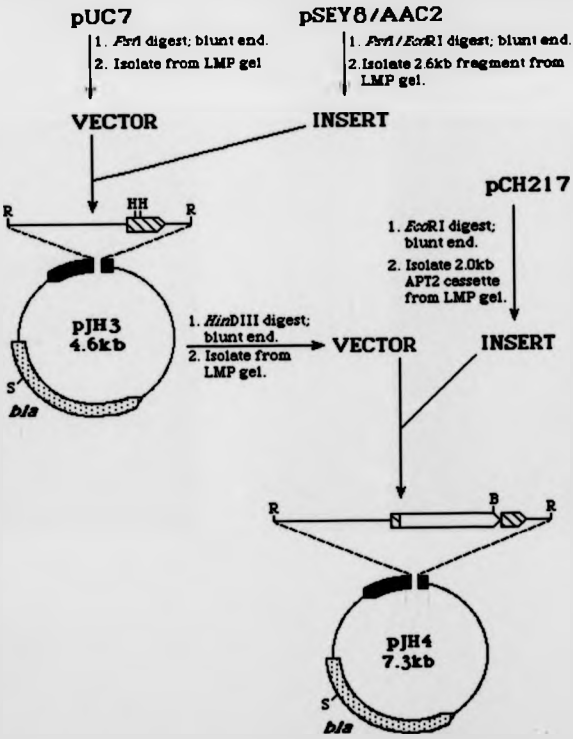


Figure 4.3.2: Summary of *AAC2* gene disruption. Restriction maps of constructs are shown in **Figure 4.3.4.** (Note that plasmids shown are not to scale).

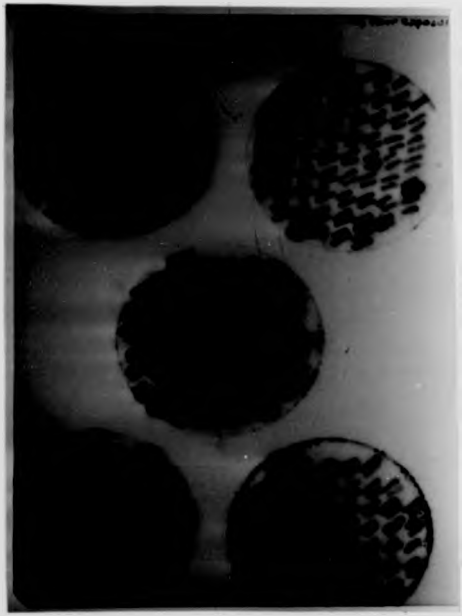
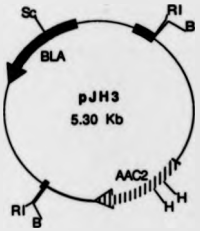


Figure 4.3.3: Colony hybridization used to screen DNA ligations. *E.coli* NM522 transformed with pUC7-*AAC2* ligations was selected on LB plus ampicillin and Xgal. White colonies were subjected to colony hybridization using the 2.6kb PstI-*Eco*RI fragment of pSEY8/*AAC2* (Figure 4.3.1). *E.coli* transformed with pSEY8/AAC2 was streaked in an X pattern at the top righthand of each membrane as a positive control for detection of the *AAC2* fragment, and for easy identification of the streaks.

A.



B.

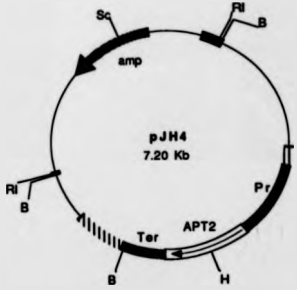


Figure 4.3.4: Plasmids constructed during *AAC2* gene disruption. A: pJH3 contains the *AAC2* gene on a 2.6kb *Eco*RI fragment. B: pJH4 contains the *AAC2* gene disrupted by the *APT2* gene. Digesting with *Eco*RI releases a 4.5kb hybrid molecule suitable for disruption of genomic *AAC2*.

5. A *Hin*dIII digest of pJH3 was performed in T4 DNA polymerase buffer and the digested plasmid blunt-ended using T4 DNA polymerase, as before. The 2.0kb *Eco*RI fragment of pCH217 containing the *APT2* cassette was isolated and blunt ended as previously described.

6. pJH3 and *APT2* DNA were ligated overnight, then mixtures used to transform *E.coli* NM522.

7. Transformants were selected for Amp^{res} initially, then replica plated onto LB supplemented with Kanamycin to select bacteria containing a functional *APT2* cassette.

8. Restriction digest analysis was used to verify the disruption of *AAC2*. The plasmid chosen for further study was named pJH4 (**Figure 4.3.4**).

Disruption of the *AAC2* Gene of C2.

Previous results indicated that *AAC2* is the major ADP/ATP translocator protein in *S.cerevisiae*, and that yeast with a disrupted *AAC1* gene were able to survive on a non-fermentable carbon source (Lawson *et al*, 1988) but the effect of disrupting the *AAC2* gene was initially unknown. *S.cerevisiae* C2 was used as an experimental system for *AAC2* gene disruption studies. *Eco*RI digests of pJH4 were used to transform C2 using a variation of the LiOAc method described elsewhere (Method *1a*, SECTION 5.2); yeast were washed and resuspended in 0.5ml YEPD before plating out on YEPD agar plates that contained 0.3mg/ml G418. Yeast expressing the *APT2* cassette appeared as colonies after 2 days growth at 30°C.

Genetic Properties of Transformants

A number of G418^{res} colonies were obtained when C2 was transformed using *Eco*RI digests of pJH4 as transforming DNA. Controls using pUC7 as transforming DNA gave no G418^{res} colonies, making it unlikely that resistance to G418 was due to anything other than expression of the *APT2* cassette. G418^{res} colonies were tested for growth on YEPG plates. A number of G418^{res} cells were identified that could not grow on YEPG, indicating that they cannot generate ATP by oxidative phosphorylation.

The location of the lesion was determined by crossing mutants with X2180-1A (ρ^o). Successful crosses were selected on *mm* plates containing 2% maltose as a carbon source. Of these none were found that grew on YEPG plates. This indicates that all the mutant C2 isolated had either ρ^o or ρ^- mitochondria, as mutants were unable to complement the ρ^o phenotype of X2180-1A. Three G418^{res} colonies (C2:*ΔAAC2.1-.3*) were isolated for further study. They were each picked into a universal bottle containing 5ml YEPD and shaken at 300rpm for 24 hours at 30°C. Dilutions were spread onto YEPD plates such that single colonies could be isolated after 2 days growth. Thirty colonies were then tested for G418 resistance by picking onto YEPD plates suplemented with 0.3mg/ml G418. All colonies retained their G418^{res} phenotype, indicating stable integration of the *APT2* cassette in mutants C2:*ΔAAC2.1*, C2:*ΔAAC2.2* and C2:*ΔAAC2.3*.

Southern Blot Analysis of Mutants

Genomic DNA preparations of C2:*ΔAAC2.1-.3* and C2 were subjected to Southern Blotting (**Figure 4.3.5**) using the 2.6kb *Pst*I-*Eco*R I fragment of pSEY8/AAC2 as probe DNA.

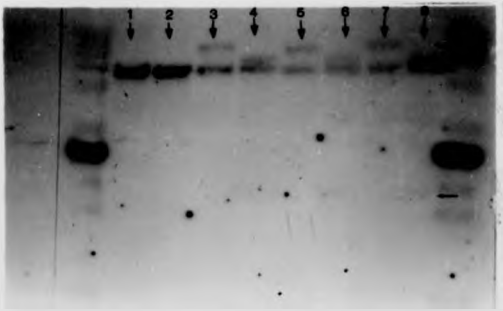


Figure 4.3.5. Southern blot analysis of G418-resistant transformants. The outer lanes are BRL 1kb DNA ladder used to determine the size of DNA on agarose gels. Non-specific hybridization was observed. Lanes 1 and 2 consist of C2 genomic digests (*Eco*RI and *Eco*RI/*Bam*HI respectively. Lanes 3 to 8 contain alternate *Eco*RI and *Eco*RI/*Bam*HI digests of C2::Δ*AAC2.1*, C2::Δ*AAC2.2*, and C2::Δ*AAC2.3* (in that order). Digests were probed with radioactively labelled *AAC2* DNA.

Restriction Enzymes	Yeast Strain	
	C2	C2::Δ*AAC2.1-3*
*Eco*RI	3.5	3.5 , 8
*Eco*RI/*Bam*HI	3.5	3.5 , 4.5 , 1.0

Table 4.3.1: Hybridization bands to *AAC2* probe DNA. The size of Hybridizing bands (kb) is shown for mutant and wild type yeast.

Hybridization bands are visible for all four yeasts, although the patterns observed for C2 and G418^{res} yeast are different. For *Eco*RI and *Eco*RI/*Bam*HI digests of C2, a hybridizing band at 3.5kb was observed. This band was also detected at lower intensity in digests of G418^{res} yeast. The three G418^{res} yeast have identicle profiles for both restriction enzymes examined. The estimated size of all hybridizing DNA is shown in **Table 4.3.1.**

The fragmentation of the genome by *Eco*RI and *Eco*RI/*Bam*HI digests indicate the following :

1. C2 has the *AAC2* gene on a 3.5kb *Eco*RI fragment with no internal *Bam*HI sites.

2. G418^{res} mutants contain two *Eco*RI fragments that hybridize to *AAC2* DNA, at 3.5kb and approximately 8kb.

3. The 8kb hybridizing band contains a *Bam*HI restriction site located 1kb from one of the *Eco*RI sites.

The restriction enzyme *Bam*HI was chosen in these studies because there is a site 1kb from the 3' end of the *APT2* cassette, as shown in **Figure 3.3.1.** The above observations are therefore consistent with integration of pJH4 into a copy of the *AAC2* gene. The presence of a stronger intensity band in C2 genomic DNA was believed to indicate the presence of a second *AAC2* gene, as equal quantities of DNA were loaded onto each lane of the gel. A number of possibilities were considered:

1. An extremely *AAC2*-homologous gene is present on a 3.5kb EcoRI fragment. The *APT2* gene has integrated into this sequence in G418^{res} strains examined.

2. C2 has two copies of *AAC2*, both of which are present on a 3.5 kb *Eco*RI fragment, possibly as a consequence of gene duplication.

3. Transformed C2 strains are diploid due to self mating (Phaff *et al*, 1978) and G418^{res} transformants are therefore haploid for *AAC2*.

The presense of a second band is unlikely to be due to the *AAC1* gene as previous studies have shown that large *AAC1* and *AAC2* probes only show very slight hybridization to other genes under the conditions used (Adrian *et al*, 1986; Lawson and Douglas, 1988). The possibility that C2 had self-mated was investigated by attempting to sporulate transformants. C2:Δ*AAC2.1-3* failed to sporulate, indicating that these yeast are either haploid or are MATa/a or MATα/α diploids. It is also possible that the strong band in C2 is the result of an artifact of Southern Blotting.

The presence of two *AAC2* genes would explain our difficulties in obtaining a *pet mutant* after several attempts. The problems encountered, though, may be a consequence of the disruption strategy used. *Eco*RI digests of pJH4 were used as transforming DNA; a similar approach using pJH2 successfully produced C2:Δ*APT12* mutants. Southern Blott results are consistent with the integration of uncut pJH4 at the *AAC2* locus. The mutants investigated were isolated from the same transformation, and may actually be the same clone, as a 90 minute incubation was incorporated into the yeast

transformation protocol to allow expression of the *APT2* gene prior to selection on G418.

Further Analysis of C2:*ΔAAC2.1*

Yeast grew equally well on YEPD, irrespective of whether the *AAC2* gene was disrupted. The properties of C2:Δ*AAC2.1* strains was tested against C2 in a labolatory scale brewing trial. The yeasts were found to have no differences in the observed rate of fermentation, sugar uptake or ethanol yield (result not shown). A fermentation profile similar to that of C2 in previous chapters was observed for both yeast.

4.4 DISCUSSION

Genetic engineering was used to create a disrupted form of the *AAC2* gene of Lawson and Douglas (1988). Attempts to create a *PET* mutant defective for the *AAC* protein were unsuccessful. The biochemical properties of an *AAC2* mutant have been characterised (Gawaz, et al, 1990). This deletion is not lethal to cells but does inhibit growth. It was also noted that Δ*AAC1*:Δ*AAC2* mutants are viable. This indicates that mitochondria have some other means of transporting ATP across the inner mitochondrial membrane.

For present studies it would have been interesting to compare the phenotye of two different *pet* mutants. Another approach to studying genetic disruption of mitochondrial function was considered: expression of the rat brown fat uncoupler protein in *S.cerevisiae*. We requested a copy of the uncoupler protein gene from a group that had engineered it for targeting to yeast mitochondria. Unfortunately

they were unable to provide us with a copy. The brewing properties of such a mutant would indicate the precise effect of uncoupling oxidative phosphorylation, as SX-1 and other uncouplers also uncouple the plasma membrane H^+ gradient.

CHAPTER 5
DMSO-ENHANCED YEAST TRANSFORMATION

5.1 INTRODUCTION

The industrial nature of this project has focussed discussions upon the role of yeast in the brewing process, with little emphasis placed on features that make yeast valuable as a system for experimental biology. In particular, *S.cerevisiae* has been extensively used by biologists interested in many aspects of eukaryotic cell biology as this unicellular organism displays many features common to multicelluar eukaryotes, such as compartmentalisation. An attractive feature of yeast is the availability of DNA transformation techniques that allow heterologous DNA molecules to be introduced and expressed in yeast. As discussed in Chapter 1, DNA transformation is currently being exploited by the brewing industry to improve yeast brewing strains. This chapter describes experiments that culminated in the development of a modified yeast transformation protocol that is very efficient and quick. In addition, yeast transformation techniques and factors affecting yeast transformation are considered.

Versatility of Yeast as an Experimental System

Much of yeast's popularity is based on the ease of handling and propagation on well defined simple growth media (Sherman *et al*, 1986). It exists as two distinct haploid forms (a or α), that can be converted to a diploid state (a/α) by mating. Diploids can be induced to sporulate by nitrogen starvation, where they undergo meiosis to produce four spores. The understanding of this system has allowed the simple construction of yeast strains suitable for various studies,

including gene isolation by complementation. Chemical- or UV-induced *in vivo* mutagenesis (Lawrence, 1991) can be used to create mutants with a desired phenotype. Mutant phenoypes can then be complemented by transforming with a yeast genomic library. Library DNA is commercially available but can also be synthesised *de novo* as random yeast genomic DNA fragments, or enriched for genes of interest (Rose and Broach, 1991).

Vectors exist for overproduction and underproduction of cloned genes that can be expressed under the control of a range of constitutive or inducible promoters (reviewed by Schnieder and Guarante, 1991; Schena *et al*, 1991). Other types of vectors can be used to direct secretion of gene products making protein purification much simpler (Moir and Davidow, 1991). These expression cassettes contain a strong yeast promoter 5' of a DNA Leader Sequence that codes for the 5' end of naturally secreted yeast protein. Restriction enzyme sites 3' of the leader sequence facilitate the cloning of foreign DNA, such that in-frame ligations will produce heterologous proteins that are secreted by virtue of the 5' portion of the secreted yeast protein. A carbohydrate binding domain engineered between the Leader Sequence and the protein sequence has been used to further simplify recombinant protein purification (Taylor and Drickamer, 1991). Integration of such secretion cassettes into genomic DNA, coupled with rounds of chemical mutagenesis has been used to achieve high level secretion of foreign protein in yeast (Smith *et al*, 1985; Melnick *et. al.*, 1990). Ease of handling and growth allows screening of many colonies for identification of super-secreting strains of yeast, especially if the secreted protein has an enzyme activity or antibodies are available.

Vector systems also exist for the identification of DNA elements involved in gene expression. These vectors use a reporter gene such as *Escherichia coli lacZ*, whose activity can be readily measured by standard techniques. This approach has been used to identify yeast upstream activation sites, termination signals, and elements associated with protein secretion.

The most popular type of yeast vector are those based on the natural 2μm yeast plasmid, as it is fairly stable, and can be propagated at high copy number (reviewed by Rose and Broach, 1990). An alternative to 2-μm circle plasmids is provided by *Ty* transposition vectors, which can be used to insert expression cassettes at high copy number throughout the genome (Boeke *et. al*, 1988). This approach facilitates more stable propagation of introduced gene copies than the more commonly used multicopy plasmid approach.

The plethora of vectors available and the ease of transformation have attracted many workers from diverse fields towards yeast as a system of choice. An additional attraction of yeast is that many of the techniques previously designed for bacterial genetic studies can be adapted for use.

Yeast Transformation Techniques

The development of yeast transformation techniques have considerably improve the usefulness of *S.cerevisiae*. There are three main techniques commonly used to introduce recombinant DNA into yeasts; each with advantages and disadvantages:

I. Spheroplasting

Spheroplast transformation involves forming yeast spheroplasts by enzymatic digestion of the cell wall under osmotically stabilized conditions (Beggs, 1978; Hinnen *et al*, 1978). Cells are exposed to DNA then selected for transformants by resuspending in osmotically stable regeneration agar containing a selection system. This technique yields high efficiency of DNA transformation ($1\text{-}5x10^4$ transformants per μg of DNA) but is very tedious, and analysis of large numbers of transformants is time consuming as transformants are embedded in agar.

II. Electroporation

Transformation of yeasts by electroporation has been reported to yield $>3x10^5$ transformants per μg plasmid DNA (Becker and Guarante, 1991). This improvment over previously obtained efficiencies (50-100 transformants per μg ; Hashimoto *et al*, 1985) is achieved by stabilizing the cell membrane during and after administration of the electric pulse using sorbitol. Efficiency is further improved when selection plates contain sorbitol. This technique is ideal because it is quick, relatively simple compared to spheroplasting, and since transformants are present on the surface of agar plates, replica plating and colony hybridization can be used to study large numbers of transformants. The major disadvantage of this method is that specialized equipment is required and that transformation efficiencies decrease with increases of input DNA above 100ng.

III. Whole Cell Methods

Whole cell yeast transformation, first demonstrated by Ito *et al*,

(1983) is preferred to spheroplasting because transformants grow on the surface of agar plates, and are therefore easier to analyse in large numbers. This procedure is analogous to calcium chloride-mediated bacterial transformation (Mandel and Higa, 1970), in that cells are treated with cations to promote DNA uptake. In contrast to previous results (Ito *et al*, 1983) it was demonstrated that *S.cerevisiae* can also be transformed with DNA when treated with Ca^{++} provided the divalent cations are removed before DNA is added (Bruschi *et al*, 1987). This work also demonstrated that incubation in PEG is essential for stable transfection of yeast. It was proposed that PEG promotes binding of DNA to the cell wall but plays no role in DNA uptake. Transformation with Ca^{++} is strain specific, as is Li^{+} transfection, and large variations in efficiency are found with different strains. Also, strains that transform poorly by the Li^{+} method transform well with the Ca^{++} protocol and *vice versa*; some strains transform poorly by both methods. Additionally, it has also been shown that yeast can be transformed with PEG in the absence of cations (Klebe *et al*, 1983). The major disadvantage of these techniques is the relatively poor transformation efficiency (10^2-10^3colonies/μg). As will be dicussed later, modifications of Li^{+} transformation enhance efficiency considerably. The protocol for Li^{+}-mediated transformation is straightforward and quick, making it ideal for routine transformations.

DMSO-Induced Transformation in Eukaryotes and Bacteria

DMSO has been shown to enhance transfection of DNA into prokaryotic and eukaryotic cells. In bacteria, DMSO enhances transformation in conjunction with divalent cations, possibly as a consequence of the good metal ion solvating properties of DMSO,

which lead to more stabilized ionic interactions between the phosphate backbone of transforming DNA and phospholipids of the lipid bilayer (Hanahan, 1983). Similar studies have used DMSO in a protocol designed for rapid transformation and storage of competent *E.coli* (Chung and Miller, 1988 ; Chung *et al*, 1989).

DMSO has also been used to improve transformation of eukaryotic cells. Myeloid cell lines have been successfully transformed (Chisholm and Symonds, 1988) as have mouse L cells (Lopata *et al*, 1984). Both methods incorporate a DMSO or glycerol heat shock step. These observation sent us along a path that ended with the development of a rapid and efficient DMSO-Li^+ whole cell transformation technique.

5.2 MATERIALS AND METHODS

Ingredients Used in Transformation Assays

I.Solutions

TE Buffer	Lithium Acetate Solution
10 mM Tris.Cl, pH8.0	0.1M LiOAc
0.1 mM EDTA	made in TE buffer

PEG Solution
50% PEG4000
made in Lithium Acetate Solution

All solutions were sterilized by autoclaving.

II. Chemicals.

PEG4000 was purchased from Fisons plc, as was DMSO solution. Lithium acetate was bought from BDH Ltd.

III. Yeast Strain

JRY188 (MATα: *his3*Δ1, *leu2-3,112*, *trp*1-289, *ura*3-52).

IV. Transforming DNA

pJH2 (**Figure 3.3.1**) containing the *LEU2* marker, was used to transform JRY188 to leucine prototrophy.

V. Selection of Transformation.

JRY188 transformed with pJH2 were selected for on *mm* agar supplemented with histidine, tryptophan and leucine (*mm*+HUT). After spreading yeast cells the plates were incubated at 30°C. Small colonies started to appear after 48 hours. Efficiency was determined as the number of colonies per µg of transforming DNA counted after 3 days growth.

Whole Cell Yeast Transformation Methods

DMSO was incorporated into a Li^+ transformation protocol to evaluate the effect the solvent has on transformation efficiency. The techniques used in these studies are now described:

I. Method *Ia* (Ausubel *et. al*, 1989)

A modified version of the Li^+ method of Ito *et al* (1982) was used as a base line for comparison of transformation results:

1. A single colony of the recipient strain was used to inoculate 5ml of YEPD which was grown up overnight at 30°C with shaking (200rpm).

2. The cell density was determined and knowing the doubling time

of the recipient yeast it was possible to inoculate a flask of YEPD medium with enough cells so that transformations could be started at a desired time the following day. The volume of YEPD was varied, depending on the number of transformations required. The cells were shaken in a flask at 200rpm until the cell density was 1-4x10^7 cells/ml.

3. The cells were transferred to sterile plastic tubes (30ml capacity) and centrifuged by spinning at 3500rpm for 2 minutes in a Griffin and George bench centrifuge (UJ1). All centrifuge steps were performed at room temperature.

4. The supernatant was removed, cells were washed in TE solution, and then resuspended in a final volume of 10ml TE per 100ml original volume.

5. The cells were pelleted as before and resuspended in 10ml LiOAc solution.

6. Cells were centrifuged as before then resuspended by adding 1ml of lithium acetate solution per 100ml of YEPD.

7. The cell suspension was then shaken gently (50rpm) for 1 hour at 30°C. Competent yeast cell suspensions were used for transformation.

8. Transformations were performed in Eppendorf tubes; 100µl competent yeast cell suspension and 10µl of DNA solution were mixed by a brief vortexing step.

9. Eppendorfs were placed at 30°C for 30 minutes without shaking, 290µl of PEG solution was added, and the contents mixed by inverting the tubes 4-6 times.

10. After incubating for 45 minutes at 30°C (no shaking), tubes were placed at 42°C for 5 minutes without shaking.

11. Cells were then washed in sterile dH_2O by first spinning the yeast/PEG solution for 5 seconds (no longer) in a Microcentaur (ca. 12000rpm). 1ml of dH_2O was added, tubes inverted twice, then spun again for no longer than 3 seconds. Longer spins make resuspension of yeast very difficult.

12. Yeast were resuspended in a final volume of 1 ml sterile dH_2O by gentle finger flicking and 100µl of cell suspension spread onto selective plates. When high efficiencies were expected serial dilutions were performed in dH_2O .

Method *1a* was changed by adding DMSO at various stages. The altered protocols were named *1b*, *1c*, and *1d*.

II. Method *1b*

Method *1a* is varied by addition of DMSO after DNA and cells are mixed (see step 8, Method *1a*). An equal volume of dH_2O was added to assess non-specific effects in changes of volume. The method previously described is continued with an increase in the volume of PEG to give a 35% solution as before.

III. Method *Ic*

Method *Ia* is varied by adding DMSO just after PEG (step 10), which is adjusted to give a 35% final solution. It is important to add PEG before DMSO, as transient exposure to high levels of DMSO reduces transformation efficiency. This is also the case with Method *IIa* transformation.

IV. Method *Id*

This method is virtually the same as Method *Ia* except DMSO is added just before the 5 minute heat shock (step 11).

V. Method *IIa*

Method *IIa* differs significantly from Method *Ia*; cells are not exposed for 1 hour to 0.1M LiOAc, and the DNA-competent cell incubation step is reduced to 5 minutes. The protocol described below for 100ml of yeast transformationcan be performed in approximately one hour:

1. YEPD was inoculated with yeast that were grown up and harvested as previously described (Method *Ia*, steps 1-3).

2. Cells were washed in 10ml of LiOAc solution, pelleted, and resuspended gently after the addition of 1ml lithium acetate solution.

3. 10µl of DNA was mixed with 100µl of cells by brief vortexing in a 1.5ml Eppendorf tube, then maintained at room temperature for 5 minutes.

4. PEG solution was added and the contents mixed by inverting the tube 4-6 times. The amount of DMSO added in step 5 was taken into account when determining the volume of PEG solution added to give 35% final concentration.

5. DMSO was added immediately at the final concentration indicated, and the contents again mixed by inversion.

6. After 45 minutes at 30°C the tubes were heat shocked at 42°C for 5 minutes (both incubations without shaking).

7. The cells were pelleted by a 5 second spin in a Microcentaur and most of the PEG/DMSO solution decanted.

8. The pellet was washed with 1ml dH_2O and pelleted with a 3 second spin (as in Method *Ia*).

9. Cells were resuspended in a final volume of 1ml dH_2O by gentle finger flicking. 100µl of cells were plated on selective plates. Dilutions were made when high efficiencies were anticipated.

VI. Method *IIb*

This method is a variation of Method *IIa* in which DMSO is added just before the 42°C heat shock (step 6).

When assessing factors affecting transformation efficiency, each data point given in **SECTION 5.3** is the average of more than one Eppendorf tube (2-5). The mean and standard deviation values are represented in graph form as points with error bars, or tabulated.

Efficiency values are shown per μg of transforming DNA.

5.3 RESULTS

Initial attempts at yeast transformation using the lithium acetate method (Ito *et al*, 1983) yielded very low transformation efficiencies. This was initially attributed to inexperience in yeast genetics. However, after training at the Leicester Biocentre under the guidance of Dr. W.E. Lancashire and Dr. A.T. Carter, the high transformation efficiencies obtained could not be reproduced in our laboratory. Various changes to the transformation procedure were carried out including the type of water used, the treatment of glassware (detergent free) and make of lithium acetate. The problem was eventually traced to the type of PEG used.

Other techniques were incorporated into the transformation protocol such as sonicating cells to break up clumping, which improved transformation of flocculating yeast slightly (results not shown). It was also observed that adding DMSO to the transformation mixture increased the transformation efficiency slightly. The increase observed was small and the values were insignificant compared to efficiencies obtained at Leicester. When transformations were obtained at "normal" frequencies using new PEG, further investigations showed that adding DMSO significantly increased the efficiency of transformation. This chapter describes the optimization of a transformation protocol using JRY188 as a recipient strain.

Effect of DMSO on Yeast Transformation

JRY188 transformed with pJH2 using Method *Ia* yielded

approximately 800-2000 transformant/μg of pJH2 when transformants were selected on *mm*+HUT. Observed differences could be due to a number of factors, such as variations in the exact stage of growth that yeast were harvested. When JRY188 was transformed by different methods, yeast were grown in the same flask, split into batches, then transformed by the appropriate method. **Figure5.3.1** compares yeast cells transformed by Methods *1a* and *1b*.

A fixed volume of dH_2O and 100% DMSO was added to give the percentage DMSO indicated. This ensured that the same volume of DMSO solution was added per tube. It was observed that yeast were transformed at improved efficiency only when 20% DMSO was present. Adding an equal volume of dH_2O (0% DMSO), thus altering the cell density, was shown to reduce transformation efficiency. This explains DMSO's apparent inhibition at less than 10% DMSO.

It was considered that the final % DMSO after PEG addition might be more important than that present initially. This was tested by adding more DMSO to a tube containing 10% initially to give a concentration of 10% DMSO during PEG incubation. The results shown in **Figure 5.3.1(B)** compare the percentage DMSO present after addition of PEG, and indicate that there is significant improvement in transformation efficiency at 10% DMSO.

JRY188 was transformed by Methods *1b* and *1c* to determine the importance of DMSO presence during the DNA-yeast incubation step (**Figure 5.3.2**). The results confirm our previous observation that changes to the transformation volume reduce efficiency. Even so, when DMSO is added after the preincubation step, an approximate

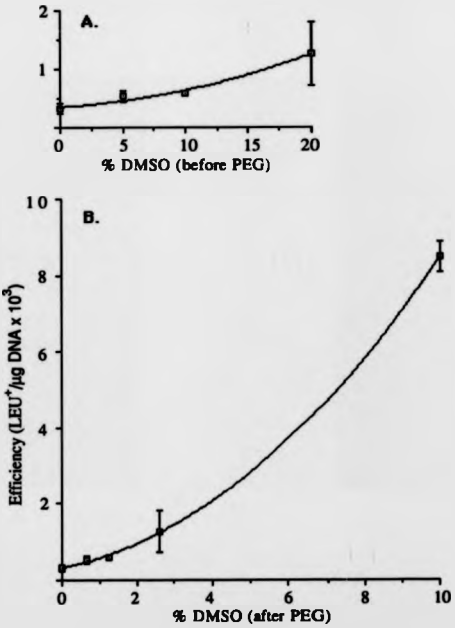


Figure 3.3.1: DMSO Effect on Yeast Transformation. Cells transformed by Method *1a* (efficiency 855±121/µg DNA) were compared with cells transformed by Method *1b* A. Efficiency of transformation is shown against % DMSO added to cells and DNA. B. The efficiency of transformation against % DMSO present after adding PEG. The value at 10% DMSO (after PEG) had 10% DMSO present initially, then after PEG addition more DMSO was added. Points are the average of 4 separate Eppendorf tubes. The error margins in all transformation experiments are mean deviations.

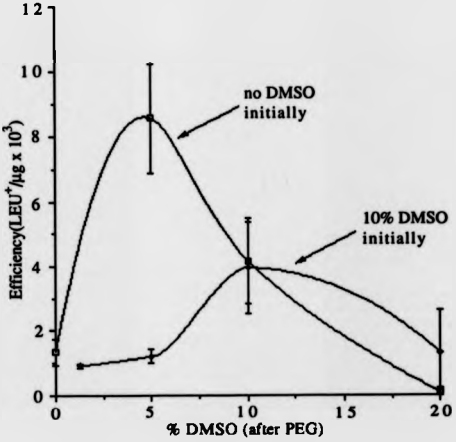


Figure 5.3.2: Comparing transformation by Methods *1b* and *1c*. Cells plus DNA were incubated with 0% or 10% DMSO (equivalent volume of dH_2O was added to the 0% tubes). After PEG addition 100% DMSO solution was added giving the percentage DMSO during the PEG incubation step. The number of LEU^+ transformants per µg of pJH2 is shown. JRY188 transformed at 1348±403 transformants per µg with Method *1a*. All points are the average of four tubes.

6-fold increase in transformation efficiency was observed (at 5% DMSO). This is double the maximum value obtained when cells are exposed to 10% DMSO before and after PEG addition. Interestingly, both *Ib* and *Ic* transform JRY188 at the same efficiency at 10% DMSO. These results are interpreted better if considered with the findings of a later experiment (see **Table 5.3.1**).

Importance of One Hour Li^+ Incubation Step

The washing steps and one hour LiOAc incubation steps add considerable time to the Li^+ transformation procedure. Preliminary results suggested that these steps were not necessary for DMSO-mediated high efficiency transformation. This modification cuts almost 2 hours off the time taken to perform Method *Ia*. These observations were followed up by a series of experiments to determine the optimal conditions for Method *IIa* transformation.

JRY188 was transformed by Method *IIa* with or without a 5 minute heat shock over a range of DMSO concentrations (**Figure 5.3.3**). This experiment examined whether a heat shock step was necessary, in addition to determining the optimal percentage DMSO for Method *IIa*. The efficiency of transformation was greatest at 10% DMSO with a heat shock. At 10% DMSO in the absence of heat shock, JRY188 transformation was approximately one third the equivalent heat shock value. The results also indicated that increasing the percentage DMSO in the absence of heat shock improves efficiency considerably, with optimum results obtained at 15% DMSO. Efficiency is almost as high as those obtained at 10% DMSO with a heat shock. **Figure 5.3.4** indicates that optimum efficiency is achieved with a 5 minute heat shock step and a 45 minute PEG/DMSO incubation.

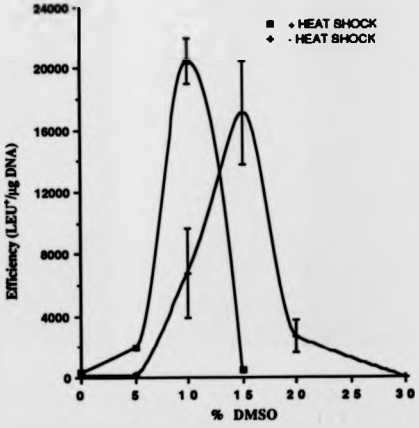


Figure 5.3.3: Effect of Heat Shock over a range of DMSO concentrations. Cells were transformed by Method *IIa* over a range of DMSO concentrations, with or without a 5 minute heat shock at 42°C. The efficiency and standard deviation values were obtained from two tubes. Cells transformed by Method *Ia* yielded 1935±276 LEU^+ transformants/µg of pJH2.

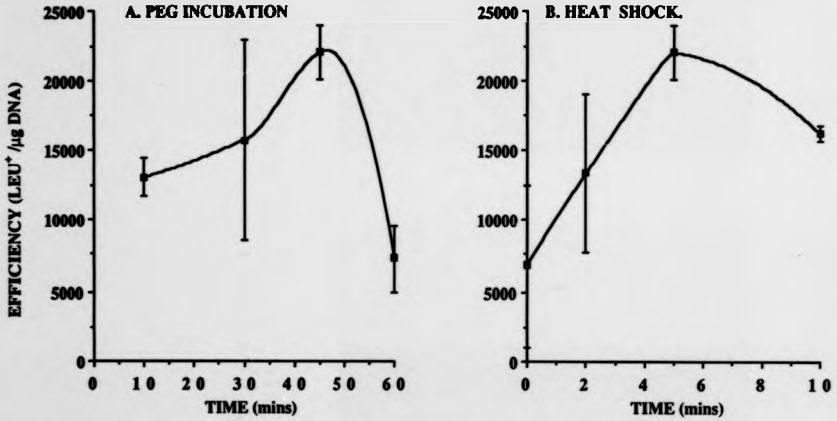


Figure 5.3.4. Optimising conditions of transformation for Method*IIa*. JRY188 was transformed using 10% DMSO in all cases. **A**: The time of the PEG incubation period was varied as shown. **B**: The duration of heat shock step was varied from 0-10 minutes as indicated. Both data sets were obtained from two points. Efficiency is given as the number of LEU^+ transformants per µg of pJH2.

Method *IIa* versus Method *IIb*

It was also considered that the presence of DMSO might only be important during the heat shock step. This was investigated by comparing JRY188 transformed by Method *IIa* and *IIb* (**Table 5.3.1**). The amount of DMSO used in Method *IIb* was varied to determine the optimal DMSO concentration.

Transformation Method	DMSO (%)	Efficiency (LEU$^+$/μg pJH2)	Enhancement Factor (c.f. *Ia*)
Ia	0	848 ± 64	-
IIa	10	5420± 640	6.5
IIb	5	17500 ± 1250	20.5
IIb	10	18350 ± 640	21.5
IIb	15	15800 ± 4940	18.5

Table 5.3.1: Comparison of transformation of JRY188 by Methods *Ia*, *IIa* and *IIb*. The enhancement of transformation by Method *II* protocols was calculated by dividing the efficiency value by 848 (Method *Ia*). All values are the average of three Eppendorf tubes.

The results for Method *IIb* transformation indicate an approximate 3-fold improvement compared to Method *IIa*, and a 20-fold improvement over the control. Optimal efficiency by this method was achieved using a 10% DMSO heat shock, although the enhancement

factor (*c.f.* Method *Ia*) with 5, 10, and 15% DMSO is almost identical. This is in contrast with results observed previously for yeast transformed by Method *IIa* (**Figure 5.3.3**), where there was a pronounced difference at various DMSO concentrations. These observations considered with previous results (**Figure 5.3.3**) can explain the role of DMSO to a certain extent.

DMSO Inhibits The Function of PEG

The addition of DMSO before heat shocking results in enhanced transformation efficiency, but effectively inhibits optimal achievable transformation. **Figure 5.3.2** indicates that the initial DNA-yeast incubation is not inhibited by DMSO, as transformation efficiency at 10% DMSO is the same whether DMSO is present or omitted from this step. The point of inhibition of DMSO is therefore during the PEG incubation step. Efficiencies produced by Method *Ic* transformation suggest that 10% DMSO inhibits transformation at least twice as much as 5% DMSO, and 20% DMSO virtually eliminates transformation. As there is little difference in the enhancement factor from 5-15% (**Table 5.3.1**), greater efficiency is therefore obtained at 5% DMSO.

Yeast Viability When Treated With DMSO.

The previous experiments have determined that DMSO is detrimental to transformation when added during the PEG incubation step. Possible explainations for this phenomenon are given below:

1. Exposure to DMSO in the presence of PEG may be harmful to yeast, increasing fatality levels, and therefore proportionally less transformants are obtainable.

2. The structure of the plasmid DNA is altered in the presence of DMSO in such a way that uptake or adhesion to the cell wall is impaired.

3. Components or general structure of the yeast cell wall is affected by long exposure to DMSO, and so the ability of PEG to mediate DNA-cell wall interactions is impaired.

To investiagte one these possibilities JRY188 was transformed with pJH2 using almost all the techniques previously described and the efficiency values obtained were compared with the viability of post-transformed yeast. Viability was calculated as:

$$\frac{\text{Observed number of colonies}}{\text{Expected number of colonies}} \times 100\%$$

The expected number of colonies was derived by measuring the cell density of the competent cell suspension (i.e. cells/ml in LiOAc solution). Cell density was approximately 1.2×10^9 cells/ml. Therefore 100µl of these cells finally redissolved in 1ml dH_2O has a final density of 1.2×10^8 cells/ml. Two serial dilutions (1:200) were made and 100µl of yeast suspension spread on YEPD plates. Colonies were counted after 2 days growth at 30°C. From these calculations approximately 300 colonies per 100µl should be observed. The results in **Figure 5.3.5** confirm that DMSO enhances transformation significantly, and that the level of enhancement varies according to when DMSO is added. The efficiency of Methods *1a*, *1c* and *1d* indicate a large difference in LEU+ yeast produced (10- to 15-fold) with relatively small differences in viability (2- to 3-fold).

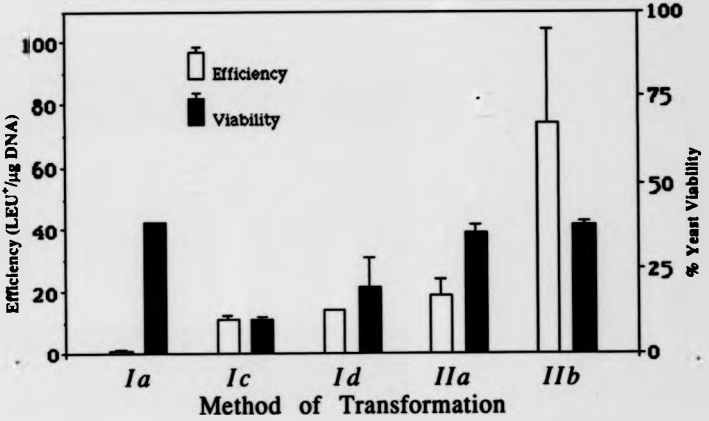


Figure 5.3.5. JRY188 transformed by a varity of methods. 1µg of pJH2 was used for each Method indicated . The efficeacy of transformation was compared with viability of post-transformed yeast. Viability calculations are explained in the text. Results are the average of two tubes

Similar comparisons of Methods *IIa* and *IIb* show that adding DMSO just before heat shock yields significantly improved transformation results but viability of yeast is virtually unaffected by a longer DMSO incubation. These results therefore argue against reduced efficiency of transformation as a consequence of DMSO toxicity.

Length of PEG Incubation Step is Important

Cells transformed by Method *IIb* in the previous experiment were also used to investigate the time required in PEG to achieve high efficiency transformation. The results shown demonstrate that even with a short PEG incubation (5 minutes) JRY188 transforms at greater efficiency than the control (**Figure 5.3.6**). Maximum transformation efficiency was however observed around 45 minutes, consistent with previous results for Method *IIa* (**Figure 5.3.4**). It appears therefore that a long PEG incubation step is essential for high efficiency transformation.

DMSO Transformation Without Li^+

Central to whole cell transformation protocols is the treatment of yeast with either monovalent (Li^+) or divalent (Ca^{++}) cations. The difference between *I*-type and *II*-type transformation methods is that exposure of cells to Li^+ is greatly reduced with the latter techniques. **Figure 5.3.5** indicates that short exposure techniques are more efficient than the equivalent *I*-type method. JRY188 was transformed in the presence or absence of Li^+ to determine the importance of Li^+ (cells were washed, resuspended in TE buffer and exposed to PEG dissolved in TE buffer).

The results presented in **Table 5.3.2** indicate that Li^+ do

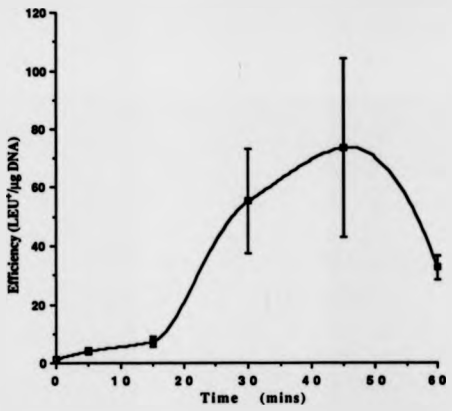


Figure 5.3.6: Method *IIb* optimum PEG incubation period. Two tubes were used to calculate each point.

Transformation Method	+/- Li^+	Efficiency ($LEU^+/\mu g$)	Enhancement factor
Ia	+	1589±338	-
IIb	-	18970±1350	x 11.9
IIb	+	41070±6700	x 25.8

Table 5.3.2: Method *IIb* transformation in the presence or absence of Li^+. JRY188 was transformed with 1µg of pJH2 by each Method indicated. Method *IIb* transformation was performed with Li^+ ions present(+) or absent(-). In the latter case yeast were washed and resuspended in TE buffer and incubated with PEG dissolved in TE. The efficiency values (transformants/µg) are the average for 2 tubes.

transformation efficiency. When Li^+ was present with DMSO the efficiency is improved by a factor of x25 compared with x12 if Li^+ was omitted. These results imply a cooperative effect between DMSO and Li^+.

Effect of Washing Cells In Sorbitol

Becker and Guarante (1991) increased the efficiency of yeast transformation by electroporation over 100-fold by osmotically stabilizing yeast during and after the electric pulse. The yeast and DNA mix was suspended in a 1M sorbitol solution before and after the "electro-shock". Transformants were selected on agar plates containing 1M sorbitol. The possibility that DMSO might destabilize the yeast plasma membrane was considered and that resuspending yeast in 1M sorbitol could lead to even higher transformation efficiencies. A sorbitol wash step was used to test this idea.

Yeast were transformed by Method *IIb* then washed in either dH_2O as usual, or 1M sorbitol. The results are shown below (**Table 5.3.3**) The transformation efficiency was assayed on selective plates with 1M sorbitol included (*mm*+HUTS) or omitted (*mm*+HUT). 100µl of sorbitol-washed cells were plated on *mm*+HUTS or *mm*+HUT; the remaining cells were washed in dH_2O, resuspended in an equal volume of dH_2O and plated on *mm*+HUT. Cells washed as normal in dH_2O were plated out for comparison.

When cells are washed and resuspended in 1M sorbitol, then plated on *mm*+HUTS, a 3-fold decrease in transformation efficiency is observed; plating on *mm*+HUT improves efficiency greatly. If these cells are then washed, resuspended in dH_2O and plated on *mm*+HUT,

the normal efficiency is restored. A possible explanation of these observations is that in the presence of sorbitol, cells remain in an osmotically stable state. When PEG is removed and replaced with dH_2O this state is destroyed. The results obtained are consistent with those of Bruschi *et al* (1987).

Wash Step	Selective Plates	Efficiency (LEU+/μg)
dH_2O	*mm*+HUT	36000 ± 4000
Sorbitol	*mm*+HUT	30800 ± 2800
Sorbitol	*mm*+HUTS	9420 ± 870
(+ dH_2O)[a]	*mm*+HUT	36000 ± 4000

Table 5.3.3: Differences between sorbitol and dH_2O washing. Yeast were transformed by Method *11b* as indicated in the text. At the washing step cells were either washed and resuspended in 1ml 1M sorbitol or 1ml dH_2O, then 100μl plated onto selective plates shown. Each value is the average of 4 tubes.

[a]cells were washed in sorbitol, repelleted and resuspended in dH_2O, then plated.

5.4 DISCUSSION

Main Conclusions From Results

From the experimental evidence presented four main conclusions can be drawn:

1. JRY188 transformation by the DMSO-modified protocol (Method

11b) is enhanced at least 20-fold compared with efficiencies obtained using the standard protocol (Ausubel *et al*, 1989).

2. DMSO enhances transformation when added at various stages of transformation, but optimal efficiency is attained when DMSO is added just before heat shocking.

3. There is a cooperative effect between Li^+ ions and DMSO.

4. The final wash step and the osmotic properties of selection plates are very important. Efficiency of DMSO-mediated transformation diminishes almost four-fold if cells are washed in 1M sorbitol after transformation and plated on sorbitol-containing plates. This inhibition is reversed by plating on agar plates without sorbitol or washing with water.

Osmotic Shock May Stimulate DNA Uptake

The observation that sorbitol washing inhibits transformation was the opposite of the results expected, and hoped for, based on previous observations (Becker and Guarante, 1991). The results however are consistent with other reports (Schiestl and Gietz, 1989) and could possibly explain the findings of Bruschi *et al* (1987), who demonstrated that *S.cerevisiae* transformation can be induced with Ca^{++}, provided the cations are removed before the addition of DNA. They propose a role for PEG in transformation based on experimental evidence using pancreatic DNAase, which digests unincorporated DNA. The following observations were made:

1. Adding DNAase at any time (from 0 to 80 minutes) during the

PEG incubation step virtually eliminates transformation, even when DNAase is added at the end of this step (80 minutes).

2. If the reaction was stopped at various times, PEG removed (by washing cells in TE) and the cells then treated with DNA_{ase} there was virtually no difference between the transformation efficiency of DNAase-treated and untreated control cells. Optimal efficiency was achieved at approximately 45 minutes, consistent with the result presented here.

3. Virtually no transformants were observed in the absence of PEG.

They concluded therefore that PEG was necessary for transformation but is not essential for DNA uptake. It appears to promote DNA adsorption to the cell surface, and, since washing does not prevent transformation, DNA must become firmly bound to the cells. It was suggested that after PEG removal DNA enters the cells or is bound to the surface in a form resistant to DNAase activity. In conclusion they proposed a transient status of DNA adsorption to the cell surface during transformation that is promoted by PEG.

Another explaination of these results is that washing cells in TE actually promotes DNA uptake. Yeast are present in 35% PEG and therefore approximately 65% water. When removing PEG and replacing with TE the aqueous content is almost 100%. This osmotic change in the environment surrounding yeast cells may induce DNA uptake. The molecular weight of sorbitol is 182.17 so a 1M solution contains 182.17 g/litre i.e. 18.2% sorbitol. Washing and resuspending in 1M sorbitol changes the aqueous content of the transformation

mix from 65% to approximately 82%. The relatively slight change in the aqueous content may explain the lower efficiency obtained. To test this further yeast would be transformed as before then washed with a 35% sorbitol solution (1.92M) solution, and plated on selective agar containing the same.

Factors Influencing Whole Cell Yeast Transformation

A number of treatments are found to enhance transformation, each to a different extent. Some of these are summarised in **Table 5.4.1.** Other factors are known to inhibit transformation to some extent are now discussed:

1. The size of transforming plasmid used is important. There is generally an inverse relationship between plasmid size and transformation efficiency.

2. Problems are often experienced with the type, and batch of PEG used. We were unlucky to receive a bad batch, as this severely handicapped initial attempts at yeast transformation. This problem did however lead to the findings presented here. No attempts were made to identify the reason why some batches are poor. Such studies may shed some more light on the mechanism of PEG-mediated transformation.

3. Components of the yeast cell wall are implicated in blocking DNA uptake since spheroplasts are transformed far more efficiently than whole cells (without modifications given in **Table 5.4.1**). It is interesting to note that treatment with β-mercaptoethanol and proteases have been shown to increase yeast cell wall porisity

Enhancing Factor	Description
Metal Cations	Li^+[1] and Ca^{++}[2] enhance transformation in a strain specific manner, as previously discussed.
β-mercaptoethanol	Has been shown to enhance transformation: (i) alone [3], or (ii) only in conjunction with 1M sorbitol [4].
Proteolytic Enzymes	Shown to increase efficiency [5]
Carrier DNA	Recommended in most protocols (eg. [6]). Single stranded, heat denatured carrier has been shown to be very good [7].
Ethanol	Similar results to those presented obtained using 5-10% ethanol [8].

References

[1] Ito *et al* (1983).

[2] Brushi *et al* (1987).

[3] Ito *et al* (1984).

[4] Brzobohaty and Kovac (1986a).

[5] Zlotnik *et al* (1984)

[6] Ausubel *et al* (1989).

[7] Schiestl and Gietz (1989)

[8] Lauermann (1991)

Table 5.4.1: Factors that enhance whole cell transformation.

and alter a mannoprotein of the yeast cell wall (Zlotnik *et al*, 1984).

4. Previously it has been shown that protoplast transformation efficiency is enhanced if naked plasmid DNA is first encapsulated in positively charged liposomes (Brzobohaty and Kovac, 1986b). This implies that the interactions of negative charges on DNA and phosphpolipids of the yeast plasma membrane discourage exchange of nucleic acid across the plasma membrane.

5. A prerequisite for optimal transformation by various yeast transformation techniques is that cells are harvested at mid to late log growth phase. Transformation by Methods *Ia* and *IIb* is greatly reduced if stationary cells are used (results not shown). The susceptibility of yeast to transformation may to be dependent on properties associated with active cell growth.

6. In these studies, carrier DNA (non-denatured sheared calf thymus DNA) decreased transformations efficiency slightly (results not shown). Previously it has been shown that the size and type of carrier DNA used is crucial, which may explained our results. Schiestl and Gietz (1989) showed that single stranded, heat denatured carrier DNA increases transformation efficiency routinely to more than 10^5 transformants per μg.

7. High concentrations of DMSO (**Figure 5.3.3**) and ethanol (Lauermann, 1991) have been shown to reduce efficiency of transformation. Reasons why DMSO inhibits transformation are discussed later.

The Role of Components of DMSO-Enhanced transformation

Very little is known about the mechanism of DNA uptake into whole cells. The possible roles played by the various components of the Method *IIb* transformation mixture are now considered.

I. PEG

Bruschi *et al*, (1987) showed that PEG is essential for transformation and proposed that its role was to bind DNA to the cell wall. A recent report has indicated that PEG causes precipitation or binding of carrier vector DNA onto yeast cells (Gietz and Schiestl, 1991). Longer than 45 minutes in PEG reduces transformation efficiency (**Figure 5.3.6**). This may be due to covalent binding of DNA to components of the yeast cell wall. The mechanism of DNA uptake may be associated with changes in the osmotic state upon removal of PEG.

II. Li^+

Li^+ in conjunction with DMSO, or on its own, enhances DNA uptake possibly by shielding the charges of DNA and phospholipids similar to the model proposed for *E.coli* transformation (Hanahan, 1983).

III. DMSO

DMSO enhancement of Li^+ transformation may partly be due to DMSO's metal solvating properties (Hanahan, 1983). This does not fully explain the role of DMSO as it improves transformation efficiency 12-fold even in the absence of Li^+ (**Table 5.3.2**). Transformation efficiency is lower if DMSO is added earlier than the heat shocking step. This cannot be attributed to increased levels of cell mortality by DMSO toxicity (**Figure 5.3.5**). Similar results established that high levels of ethanol inhibit transformation

(Lauermann, 1991). DMSO may act by altering cell wall components involved in binding (or uptake) of DNA. Inhibition is directly proportional to DMSO concentration, whereas the enhancing property of DMSO works almost equally well over a range of concentrations (**Table 5.3.1**). DMSO may act by stimulating the transfer of DNA molecules bound to the cell wall or the plasma membrane by disrupting the plasma membrane in some way. DNA bound to the outside of the membrane may enter the cell entrapped in lipid-coated vesicles by a form of endocytosis

IV. Reducing Transformation Time

The one hour Li^+ wash step (Method *1a*) was eliminated primarily to reduce transformation time to a minimum. Results however suggest that longer exposure to Li^+ reduces efficiency (**Figure 5.3.5**). It may be that Li^+ is detrimental to transformation, or that the transformation-enhancing properties associated with actively growing cells diminish with time.

Proposed Mechanism of DMSO-Enhanced Transformation

The results from this study, and factors enhancing or inhibiting whole cell transformation, are now used to propose a mechanism of whole cell yeast transformation:

> Cells are harvested when actively growing (i.e. mid to late log growth phase) then treated with Li^+ prior to the addition of transforming DNA. Reducing the time between harvesting and adding DNA to cells improves transformation. PEG acts to bring DNA and cells together. There may be a limited number of sites at which DNA can cross the cell wall. Single stranded carrier

DNA may enhance transformation by filling in crinkles on the cell wall that are non-functional for DNA translocation. Long PEG incubations inhibit transformation, possibly as a consequence of DNA becoming entangled or cross-linked with components of the cell wall. A number of factors contribute to the transfer of DNA across the plasma membrane, such as shielding of negative charges by Li^+, changes in the osmotic environment, heat shocking, and the presence of DMSO, all of which induce DNA uptake possibly by a form of endocytosis.

Uses of DMSO-Enhanced Transformation

A highly efficient whole cell transformation protocol is particularly useful, particularly for cloning genes by complimentation. One advantage is that there is a greater chance of isolating gene clones which are poorly represented in a genomic library. Also, less library DNA is required to isolate a given gene.

Recently Gietz and Schiestl (1991) demonstrated high efficiency transformation (using single stranded carrier DNA) could solve some problems of yeast molecular biology. For example, genomic DNA ligation mixtures could be directly transformed into yeast without first cloning in *E.coli*. This allowed complementation of a number of clones whose gene product is toxic to *E.coli*, such as the *RAD4* gene.

Using Method *IIb*, 5- to 10-fold improvements in *S.cerevisiae* C2 transformation efficiency was achieved (cf. Method *Ia*; Allen Donald, personal communication). A derivative of YCp50, containing the *APT2* cassette (Hadfield *et al*, 1990), was used as transforming DNA, with transformants selected on YEPD-G418 agar plates. A 90 minute

YEPD incubation step prior to plating is necessary for expression of the *APT2* cassette. Transformation occured better at 5% DMSO with C2, but systematic studies were not made to optimize the protocol. This result indicates that Method *IIb* works in yeast other than JRY188, and suggests that the optimal percentage DMSO may be strain specific.

DMSO mediated transformation may prove useful for transforming yeast strains that transform poorly by the Li^+ or Ca^{++} methods as improved efficiencies are obtained even in the absence of cations. In addition, it should be possible to adapt a DMSO approach to improve the efficiency of other Li^+ transformation techniques, such as transformation of colonies directly from agar plates (Baker, 1991).

The efficiency of DMSO-enhanced transformation approaches that achieved by spheroplast methods but is cheaper to perform and much less laborious. With further modification such as efficient use of carrier DNA, higher levels of transformation may be achieved.

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