A genetic strategy to reduce sulfite reductase activity in *Saccharomyces cerevisiae.*

by

CATHERINE M. SUTHERLAND

BSc.(Hons) (Adel.)

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resources Sciences

Department of Plant Science

The University of Adelaide

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THESIS SUMMARY

A genetic strategy to reduce sulfite reductase activity in *Saccharomyces cerevisiae.*

The production of hydrogen sulfide by *Saccharomyces cerevisiae* during wine fermentation has long been a problem for wine makers as H_2S has a low odour threshold. The problem occurs when yeast attempt to make the sulfur containing amino acids methionine and cysteine via the sulfate assimilation pathway, in a low nitrogen grape juice. In the presence of nitrogen, sulfide produced by the enzyme sulfite reductase combines with nitrogenous precursors to form methionine. If the nitrogenous precursors are not available the sulfide diffuses from the cell as hydrogen sulfide.

This study was undertaken to derive a strategy to reduce the potential of *S. cerevisiae* to produce hydrogen sulfide under oenological conditions by altering the levels of active sulfite reductase in the cell. To achieve this, the study also looked at the structure/function relationships of sulfite reductase. A reduction in the levels of active sulfite reductase rather than the elimination of the enzyme was necessary, as methionine and cysteine are essential amino acids and yeast must be able to synthesise these if they are not available in the growth medium. The dominant negative strategy was chosen to accomplish this.

Sulfite reductase is a member of the flavoenzyme family of proteins and catalyses the six electron reduction of sulfite to sulfide. *S. cerevisiae* sulfite reductase is a heterotetramer with an $\alpha_2\beta_2$ structure. The α subunit, encoded by *MET10*, binds the cofactors NADPH and FAD, whilst the β subunit binds FMN and siroheme to form the active enzyme. The interaction between the sulfite reductase subunits was examined using the yeast two-hybrid system.

Previous experiments had indicated that the β subunit of sulfite reductase was encoded by *MET5*, and results from this study provide further evidence for this

as an interaction between Met5p and Met10p was seen in the two-hybrid system. However the interaction could only be detected in the absence of methionine. Models that may explain why Met5p and Met10p were only seen to interact when cells were grown under conditions in which the methionine biosynthetic pathway is on are proposed.

The homology of sulfite reductase to other members of the flavoenzyme family enabled the three dimensional structure of the cofactor binding region to be modelled. Amino acids in the predicted NADPH and FAD binding sites were substituted to prevent cofactor binding, whilst still allowing the α and β subunits to bind to each other, and the altered Met10p over-expressed from a plasmid in a haploid *S. cerevisiae* strain containing the wild-type Met10 protein.

The dominant negative effect in these cells of the amino acid substitutions within Met10p on the activity of sulfite reductase was determined with a sulfite reductase enzyme assay and a sulfite accumulation assay. No difference could be detected between strains of *S. cerevisiae* encoding altered Met10p and a wild-type *S. cerevisiae*, therefore further experiments were undertaken to find the reason for this. To ensure the amino acid substitutions had not altered the folding properties of Met10p such that it could no longer bind to the β subunit, subunit binding was tested. Yeast two-hybrid experiments showed that the altered α subunits could bind and therefore sequester the sulfite reductase β subunit.

A strain of *S. cerevisiae* which had *MET10* deleted and appropriate auxotrophic markers was constructed and the effect of the amino acid substitutions on sulfite reductase activity estimated in this strain by sulfite accumulation assays. It was found that all of the mutants retained some activity, although three of the amino acid substitutions affected the activity of sulfite reductase to the extent that when these altered α subunits were expressed in the *Δmet10* strain, growth in the absence of methionine was not supported.

The amino acid substitutions introduced into Met10p were all shown to reduce activity of sulfite reductase and would therefore be expected to produce a dominant negative phenotype when overexpressed in *S. cerevisiae*. The most likely explanation that a phenotype was not observed is that the level of expression of the *met10* genes was not high enough when driven by the *MET3* promoter.

Based on the results presented it would appear that the dominant negative strategy is feasible to reduce the production of H_2S in yeast, however increased expression of the altered subunit is required. The subsitution of selected amino acids within predicted co-factor binding sites in Met10p, and the determination of their effect on sulfite reductase activity has provided some insights into the structure/function relationships within this enzyme.

The observation of an interaction between Met10p and Met5p in the yeast two-hybrid system provides further evidence to support the function of Met5p as the sulfite reductase β subunit. Models are proposed to explain why the interaction could only be observed in the absence of methionine.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Catherine Sutherland.

April 2000.

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CHAPTER ONE Introduction

1.0 INRODUCTION

The production of hydrogen sulfide (H_2S) by *Saccharomyces cerevisiae* during grape juice fermentation has long been a problem for wine makers. Only a very low concentration of H_2S is required before its odour threshold is reached (50-80μg), and an undesirable sulfurous off-flavour, similar to rotten eggs is imparted to the wine (Rauhut, 1993). Whilst improvements in the vineyard and in winemaking practice have been important in the reduction of the occurrence of H_2S, it is still a frequently encountered problem in the wine industry.

A relationship between a lack of assimilable nitrogen and H_2S production was first established by Vos and Gray (1979). The production of sulfide is a necessary intermediate in the assimilation of sulfur. The sulfide produced is combined with a nitrogen precursor to form the essential amino acids cysteine and methionine. However, if a nitrogen precursor is not available the H_2S formed will diffuse from the cell into the wine. Consequently many wine makers now routinely add a source of nitrogen in the form of diammonium phosphate (DAP) to grape juice. Whilst there are currently no guidelines on the quantity of DAP which may be added to grape juice in Australia, the total phosphate content of wine is limited to 0.4g/L as phosphorous, and European law restricts the addition of DAP to 0.3g/L (Jiranek and Henschke, 1991). In addition, there is currently a market trend towards wine that is 'organically' produced and additive free.

There are problems associated with the supplementation of grape juice with DAP such as an increase in the concentration of higher alcohols and esters in the wine (Monk, 1986). Addition of DAP late in fermentation can also result in higher residual ammonia concentration which may contribute to microbial instability. In addition to the supplementation of juice with DAP, copper sulfate, a

potentially toxic compound, may be used to remove excess H_2S from wine, however flavour compounds are thought to be removed by this process.

Studies in the 1960's led to the isolation of four H_2S-negative *S. cerevisiae* strains and demonstrated that the quantity of H_2S produced by yeast is genetically determined (in Zambonelli, *et. al.*, 1975). Later studies showed that a deficiency of nitrogen in combination with an active yeast sulfite reductase enzyme, resulted in the production of excess H_2S (Jiranek, *et. al.*, 1995; Jiranek, *et. al.*, 1996).

The use of recombinant DNA technology may enable a wine yeast strain that produces minimal H_2S to be engineered. The most feasible way to accomplish this is to target the key enzyme in the production of H_2S such that the activity of the enzyme is reduced.

2.0 SULFUR ASSIMILATION

The sulfur containing amino acids methionine and cysteine are essential for yeast growth. Methionine initiates the synthesis of nearly all proteins in all organsims and is also the precursor of S-adenosylmethionine, a chief donor of methyl groups in numerous biochemical pathways. Cysteine plays a critical role in the structure, stability and catalytic function of many proteins. If these amino acids are not present in the growth medium then sulfur must be assimilated and methionine and cysteine synthesised (Figure 1.01) however the production of sulfide is a necessary intermediate in this pathway. In *S. cerevisiae* extracellular sulfate, the most common sulfur source, is transported into the cell by sulfate permease. Once inside the cell, sulfate is activated to adenosine-5'-phosphosulfate (APS) by ATP sulfurylase. The next step in the pathway is the phosphorylation of APS to 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and is catalyzed by APS-kinase. PAPS reductase uses NADPH to reduce PAPS to sulfite and 3'-5' diphosphoadenosine (PAP). Under oenological conditions, sulfite in the grape juice can freely diffuse into the yeast cell and enter the pathway at this point, thus by-passing all regulatory mechanisms up to this stage

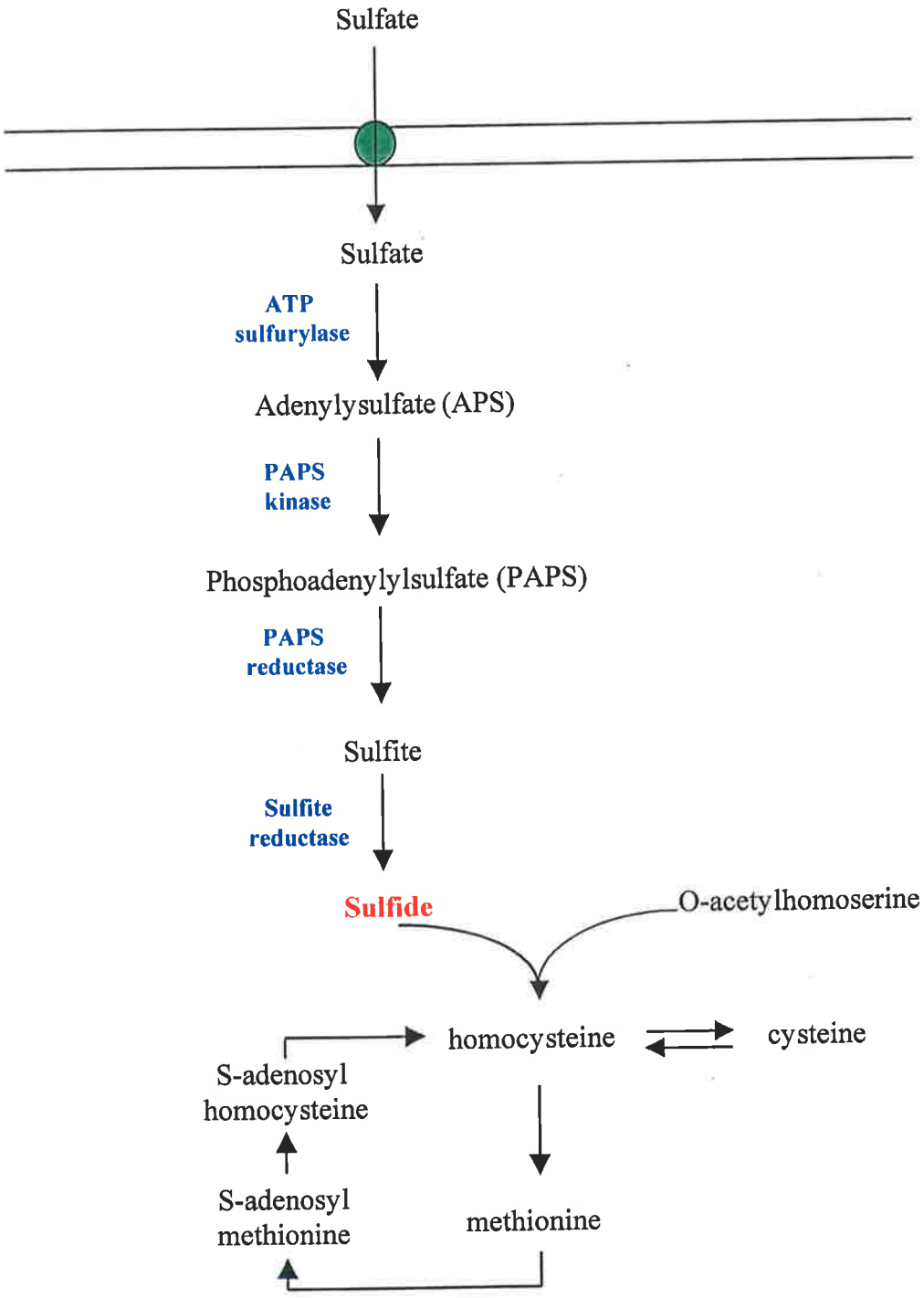


Figure 1.01 The sulfate assimilation pathway in *S. cerevisiae* and the biosynthesis of the sulfur containing amino acids, cysteine and methionine. The production of sulfide (highlighted in red text) is a necessary intermediate in this pathway.

(Figure 1.02) (Jiranek and Henschke, 1991). Sulfite is then reduced to sulfide by sulfite reductase.

Sulfur that has been assimilated and reduced is then incorporated into carbon compounds by reaction of sulfide with nitrogenous precursors to synthesise the amino acids cysteine and methionine. However, if the nitrogenous precursors are not available, the sulfide formed is able to freely diffuse from the cell as hydrogen sulfide (Figure 1.02).

3.0 BIOSYNTHESIS OF CYSTEINE AND METHIONINE

Whilst the sulfate assimilation pathway appears to be identical in all microorganisms, the way in which the sulfur atom is incorporated into the amino acid backbone varies. Incorporation into a three carbon derivative yields cysteine and incorporation into a four carbon derivative will produce homocysteine. One or both of these mechanisms may exist in a single organism (Cherest and Surdin-Kerjan, 1992). The transsulfuration pathway is able to convert cysteine to homocysteine and the reverse reaction, homocysteine to cysteine, with the intermediary formation of cystathionine.

In both plants and bacteria, *O*-acetylserine reacts with sulfide to form cysteine, in a reaction catalysed by cysteine synthase. Homocysteine, which is the immediate precursor of methionine, is then synthesised by transsulfuration. In plants, cysteine is combined with *O*-phosphohomoserine through the action of cystathionine γ-synthase to form cystathionine. Cystathionine-β-lyase then catalyses the synthesis of homocysteine (Giovanelli, 1987) which can then be methylated to methionine. The same series of reactions occurs in bacteria, however the donor of the four-carbon moiety is either *O*-succinylhomoserine (bacteria) or *O*-acetylhomoserine (bacteria and fungi) (Soda, 1987). In both plants and bacteria the transsulfuration proceeds only in the direction of cysteine to homocysteine. However the reverse transsulfuration, the formation of cysteine from homocysteine, catalysed by cystathionine β-synthase and cystathionine γ-lyase, occurs in fungi and animals. Mammals and humans cannot synthesise methionine and it is an essential amino acid in the diet. Cysteine however, can

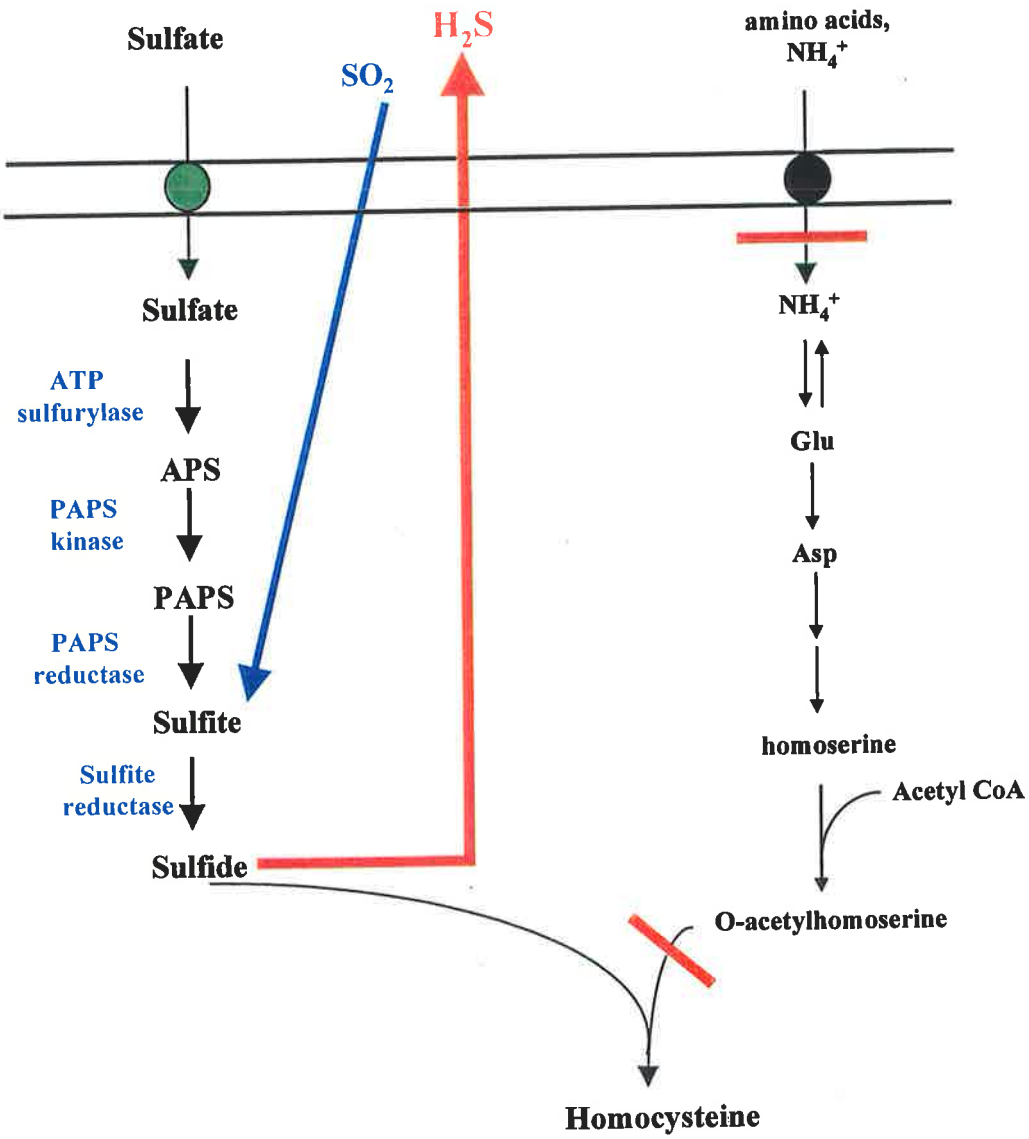


Figure 1.02 The production of H_2S by the sulfate assimilation pathway under oenological conditions in which nitrogen is limiting If there is a limiting amount of *O*-acetylhomoserine for the sulfide to combine with, it will diffuse from the cell as H_2S (represented by a red arrow). Sulfite, used to inhibit the growth of non-*Saccharomyces* yeasts in grape juice, can freely diffuse into the yeast cell where it will be reduced to sulfide (represented by a blue arrow), further exacerbating the H_2S problem.

be synthesised but the cysteine sulfur is derived from methionine (Griffith, *et. al.*, 1987).

While the enzymatic steps involved in the assimilation of sulfur are fairly well understood, the metabolism of sulfur in *S. cerevisiae* is complex and has been a matter of controversy (Ono, *et. al.*, 1996). Yamagata and colleagues (Yamagata *et. al.*, 1974) isolated a single protein with both *O*-acetylhomoserine sulfhydrylase and *O*-acetylserine sulfhydrylase activity, later shown to be encoded by the *MET25* gene (Kerjan, *et. al.*, 1986). This implied that sulfur is incorporated in both *O*-acetylserine and in *O*-acetylhomoserine by the action of *O*-acetylserine-*O*-acetylhomoserine sulfhydrylase to form cysteine and homocysteine (Figure 1.03).

A study of *met25 S. cerevisiae* mutants also impaired in the transsulfuration pathway showed that cysteine is synthesised from homocysteine only by the C4 to C3 transsulfuration pathway (Cherest *et. al.*, 1992). This implied that the sulfur atom is incorporated only in the four carbon backbone, O-acetylhomoserine, and not in *O*-acetylserine. Further support for these results was the inability to detect the enzyme *O*-acetylserine sulfydrylase *in vitro*, even in wild type strains. According to these results, the pathway for the metabolism of sulfur should be represented as shown in figure 1.04.

Ono *et. al.* (1996) reported the detection of serine *O*-acetyl transferase activity in a number of *S. cerevisiae* strains, and suggested that the enzyme is indirectly involved in the regulation of the sulfate assimilation pathway. In *Escherichia coli* and *Salmonella typhimurium*, cysteine inhibits serine *O*-acetyltransferase to lower the level of *O*-acetylserine, responsible for the induction of all enzymes involved in cysteine biosynthesis. Ono *et. al.* (1996) have proposed that a similar mechanism exists in *S. cerevisiae*. According to their studies, the pathway for the metabolism of sulfur should remain as it has previously been accepted, as shown in figure 1.03.

In a recent study of strains of *S. cerevisiae* disrupted in *CYS3* (encoding cystathionine γ-lyase) or *CYS4* (encoding cystathionine β-synthase), the authors

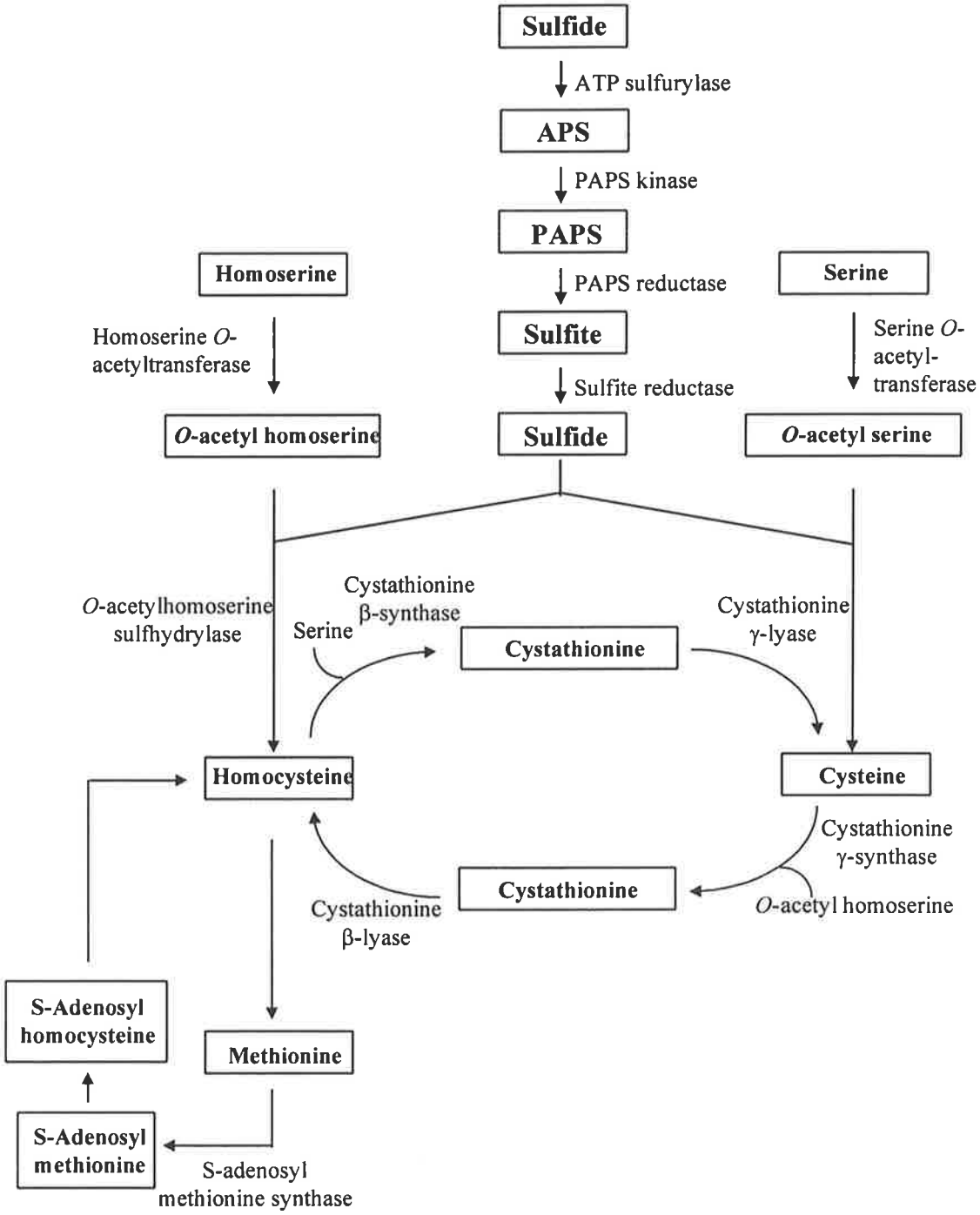


Figure 1.03 Pathway for the biosynthesis of sulfur-containing amino acids in *Saccharomyces cerevisiae* (adapted from Cherest and Surdin-Kerjan, 1992).

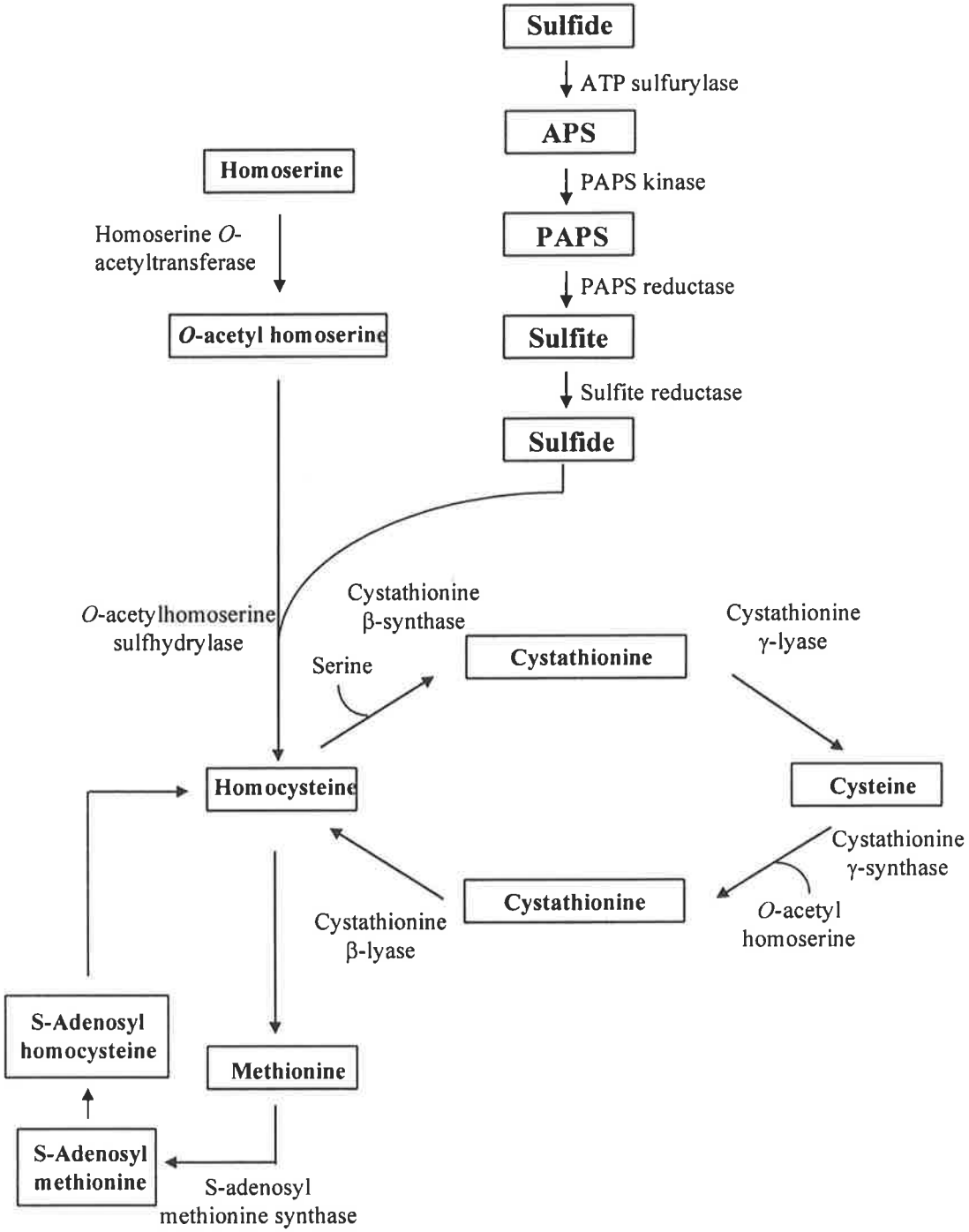


Figure 1.04 Modified pathway for the biosynthesis of sulfur-containing amino acids in *Saccharomyces cerevisiae* (adapted from Cherest and Surdin-Kerjan, 1992).

concluded that cysteine is exclusively synthesised through the transsulfuration pathway in *S. cerevisiae* (Ono, *et. al.*, 1999) (Figure 1.04). The enzyme encoded by *MET25* appears to have *O*-acetylserine sulfhydrylase and *O*-acetylhomoserine sulfhydrylase activity *in vitro*, but only *O*-acetylhomoserine sulfhydrylase *in vivo,* which may be due to the cellular localisation of the enzyme or its substrates (Ono, *et. al.*, 1999).

4.0 GENETICS OF SULFATE ASSIMILATION

The genetics of sulfate assimilation appear to be quite complex. Earlier studies with methionine auxotrophs identified 21 complementation groups (Masselot and de Robichon-Szulmajester, 1975). This was thought to indicate that some enzymes require more than one protein to be fully active since the number of complementation groups is greater than the number of enzymatic steps in the pathway (Masselot and Surdin-Kerjan, 1977). However, the regulation of the methionine biosynthetic pathway is controlled by a number of transcriptional regulators and repressors (Thomas, *et. al.*, 1992; Thomas, *et. al.*, 1995; Kuras, *et. al.*, 1996; Blaiseau, *et. al*, 1997). The genes required for sulfate assimilation and metabolism are shown in Table 1.01.

MET16 is the structural gene required for active PAPS reductase, encoding the enzyme itself. *MET4* encodes the transcriptional activator of the sulfate assimilation pathway, therefore no PAPS reductase is detected in a strain mutated in this gene. In a *met1* or *met8* strain of *S. cerevisiae* the *MET16* transcript increases compared to a wild type strain, however active PAPS reductase could not be detected (Thomas, *et. al.,* 1990). The products of *MET1* and *MET8* have been shown to be required for siroheme synthesis (Hansen, *et. al.*, 1997).

The requirement of the *MET22* (*HAL2*) gene for an active PAPS reductase was first reported by Masselot and Surdin-Kerjan (1977). However Thomas *et. al.*, (1990) found that in the presence of the *met22* mutation, the enzyme activity was comparable to that of the wild type, even though the *MET16* transcript could not be detected in this mutant on a Northern. The *MET22* (*HAL2*) gene encodes 3'(2')5'-biphosphate nucleotidase, which is required for the turnover of

Gene	Protein	Reference
MET1 (MET20)	Siroheme synthase	Hansen, *et. al.*, 1997
SFP2	Putative sulfate permease	Jin, *et. al.*, 1995
MET2	Homoserine acetyltransferase	Hansen and Kielland-Brandt, 1994
MET3	ATP Sulfurylase	Cherest, *et. al.*, 1987
MET4	Member of the leucine zipper family of transcriptional activators	Thomas, *et. al.*, 1992
MET5 (ECM17)	Putative sulfite reductase β-subunit	Mountain, *et. al.*, 1991
MET6	Homocysteine methyltransferase	Csaikl, and Csaikl, 1986
MET7	Dihydrofolate reductase	Barclay, *et. al.*, 1988
MET8	Siroheme synthase	Hansen, *et. al.*, 1997
MET10	Sulfite reductase α-subunit	Hansen, *et. al.*, 1994
MET14	APS kinase	Korch, *et. al.*, 1991
MET16	PAPS reductase	Thomas, *et. al.*, 1990
MET18	Regulator of TFIIH	Lauder, *et. al.*, 1996
MET19 (ZWF1)	Glucose-6-phosphate dehydrogenase	Thomas, *et. al.*, 1991
MET22 (HAL2)	3'(2')5'-bisphosphate nucleotidase	Murguia, *et. al.*, 1995
MET25 (MET17)	O-acetylhomoserine-O-acetyl serine sulfhydrylase	Kerjan, *et. al.*, 1986
MET27 (VPS33)	Vacuolar sorting protein	Banta, *et. al.*, 1990
MET28	Transcriptional activator	Kuras, *et. al.*, 1996
MET30	Trascriptional inhibitor	Thomas, *et. al.*, 1995
MET31	Zinc finger DNA binding factor	Blaiseau, *et. al.*, 1997
MET32	Zinc finger DNA binding factor	Blaiseau, *et. al.*, 1997

Table 1. List of cloned genes which are required in the sulfate assimilation pathway, and the enzymes they encode.

adenosine 3'5' biphosphate (pAp) (Dichtl, *et. al.*, 1997). Deletions in this gene are lethal unless the cell is supplied with exogenous methionine, thus turning off the sulfate assimilation pathway. pAp is a toxic metabolite produced in the reduction of PAPS to sulfite. Accumulation of this metabolite results in the inhibition of the RNA processing enzymes, Xrn1p and Rat1p (Dichtl, *et. al.*, 1997).

MET25 encodes O-acetyl homoserine sulfhydrylase which catalyses the incorporation of sulfide into O-acetyl homoserine, a step farther along the pathway than the reduction of PAPS. The requirement for the *MET25* gene for PAPS reductase activity is unclear, however it has been postulated that a large protein complex exists which includes all the sulfate reducing enzymes and serves to channel intermediates, some of which are toxic to the cell (sulfite) (Masselot and Surdin-Kerjan, 1977). A similar complex may also be present in *E. coli*, which would explain why *E. coli* PAPS reductase is purified as a high molecular weight product (Tsang and Schiff, 1976). Thomas, *et. al.*, (1990) proposed that this complex may also prevent the accessibility of a sulfur source to certain enzymes, leading to an apparent absence of activity.

Sulfite reductase also requires several loci to be intact for an active enzyme. These loci are *MET1, MET4, MET5, MET8, MET10, MET18* and *MET20* (Thomas, *et. al.*, 1992a). The gene *MET10* encodes the α subunit of sulfite reductase and *MET5* is thought to encode the β subunit (Hansen, *et. al.*, 1994), however it has also been implicated in cell wall biosynthesis (Lussier, *et. al.*, 1997). The *MET18* gene is involved in the nucleotide excision repair of UV-damaged DNA and is also a regulator of TFIIH (Lauder, *et. al.*, 1996). The role of this gene in the methionine biosynthetic pathway is unclear. However, a *met18* mutant is a methionine auxotroph, accumulates sulfite *in vivo* and no sulfite reductase activity can be detected *in vitro* (Thomas, *et. al.*, 1992a). The enzyme seems to be partially active *in vivo* as a *met18* mutant strain gives a pale brown colour when grown on bismuth medium. As discussed above *MET1* and *MET8* are both required for siroheme biosynthesis. Sulfite reductase uses a siroheme coupled to an iron-sulfur cluster, Fe_4S_4, to perform the six electron

reduction of sulfite to sulfide. The *MET20* gene has been shown to be identical to *MET1* (Hansen, *et. al.*, 1997).

5.0 REGULATION OF THE SULFATE ASSIMILATION PATHWAY

5.1 The role of S-adenosylmethionine

In the presence of methionine, the synthesis of the enzymes of the sulfate assimilation pathway is repressed. How methionine exerts this effect is still not certain. The enzymes of the sulfate assimilation pathway can be divided into two groups. The enzymes which are specifically involved in methionine biosynthesis are termed the sulfate group I enzymes, and respond more extensively to methionine than enzymes common to threonine and methionine biosynthesis, which are called group II enzymes (Cherest, *et. al.*, 1971).

Four group I enzymes, homoserine-O-transacetylase, homocysteine synthetase, ATP sulfurylase and sulfite reductase were examined and it was shown that the synthesis of these enzymes are controlled by the same regulatory system (Cherest, *et. al.*, 1971). Methionyl-tRNA appears to have a role in this regulation, since the synthesis of the four group I enzymes are not repressed in a mutant with a defective methionyl tRNA synthetase under conditions which lead to full repression in wild type strains (Cherest, *et. al.*, 1971).

S-adenosylmethionine (SAM) was proposed as a corepressor in the regulation of sulfate assimilation since studies with *E. coli* had implicated SAM as a participant in the regulation of methionine biosynthesis (Greene, *et. al.*, 1970). The involvement of SAM in the repression of enzymes of the sulfate assimilation pathway in yeast was confirmed in a later study (Cherest, *et. al.*, 1973). The addition of 0.1mM SAM to minimal medium was shown to repress the enzymes of the sulfate assimilation pathway in a wild type strain of *S. cerevisiae*. Under such conditions the intracellular methionine pool remains constant, as SAM is transformed into free methionine. However, the free methionine pool remains at the same level as in unrepressed cells, which indicates that the observed repression is due to SAM and not the free methionine. Further evidence

supporting the role of SAM in the regulation of the sulfate assimilation pathway was provided by the work of Thomas, *et. al.* (1988). A yeast strain was constructed in which two genes which encode the two forms of S-adenosylmethionine synthetase, *SAM1* and *SAM2*, were disrupted. In such strains, SAM cannot be synthesized and O-acetylhomoserine sulfhydrylase is no longer repressed in the presence of methionine.

Ono and colleagues (1996) claim that SAM is unlikely to play a direct role in the regulation of *S. cerevisiae* sulfur metabolism. O-acetylserine has been observed to cause coordinate induction of the sulfate assimilation enzymes (Ono, *et. al.*, 1996), and hence is a positive effector for regulation of the sulfate assimilation enzymes. The addition of 10mM SAM has been shown to result in a slight activation of cystathionine β-synthase *in vitro* (Ono, *et. al.*, 1994). Therefore, the addition of SAM to the growth medium may stimulate cysteine synthesis, which would result in the inhibition of O-acetylserine sulfhydrylase. The sulfate assimilation enzymes would no longer be induced in strains having the active reverse transsulfuration pathway (Ono, *et. al.*, 1996). However, the control mechanisms proposed are dependent on the pathway for sulfur metabolism in *S. cerevisiae* proposed by Cherest and Surdin-Kerjan (1992) (Figure 1.01) being incorrect.

5.2 Transcriptional control of *MET* genes

Investigations into the transcriptional responses of some of the genes encoding enzymes in the sulfate assimilation pathway found that methionine is the only amino acid which markedly affects the transcript level of the genes involved in methionine biosynthesis (Mountain, *et. al.*, 1991). The transcription of these genes is strongly reduced upon the addition of 2mM methionine to the growth medium. Since it has been demonstrated that SAM and not methionine regulates the sulfate assimilation pathway, it appears that SAM acts mainly by decreasing the level of transcription of these co-regulated genes.

Deletion analysis of the *MET25* gene led to the identification of a *cis*-acting sequence, 5'-TCACGTGA-3', which is crucial for transcriptional control (Thomas, *et. al.*, 1989). This same sequence can also be found upstream of the genes *MET2, MET3, MET8, MET14, MET16,* and *SAM2* (Thomas, *et. al.*, 1992b), which suggests that it has a role in the regulation of many of the *MET* genes. This sequence is known as CDE1 (centromere DNA element 1), is completely conserved in all known yeast centromeres, and binds the helix-loop-helix protein, Cbf1 (centromere binding factor 1). A *cbf1* null mutant is defective in chromosomal segregation and is auxotrophic for methionine (Baker, and Masison, 1990). Cbf1p is a member of the general regulatory factor family. Members of this family are associated with loci involved in chromosome maintenance, and are also implicated as positive and negative regulators of transcription (O'Connell, *et. al.*, 1995). Thomas and colleagues (1989) have shown that whilst the inactivation of the *CBF1* gene does not affect the activity of the ATP sulfurylase or sulfite reductase, there is a threefold decrease in homocysteine synthase (*MET25*) activity and a considerable decrease in the activity of PAPS reductase (*MET16*) activity. Mutational analysis of Cbf1p indicated that a DNA-bound form of Cbf1p is required for transcriptional activation of the methionine biosynthetic genes, (Masison, *et. al.,* 1993) however Cbf1p is not solely responsible for the activation of the sulfate assimilation pathway. This protein plays a 'recruitment' role, as it lacks a transcription activation domain (Baker and Masison, 1990).

5.3 The role of the Met4 protein

A second factor required for the activation of the *MET* genes is the Met4 protein. A *met4* mutant strain lacks all enzymatic functions required to assimilate sulfate. *MET4* encodes a *trans*-acting factor responsible for the transcriptional activation of inorganic sulfur metabolism. The Met4 protein consists of 666 amino acids, has a molecular mass of 73400 Da, and is related to the basic region leucine-zipper protein family (bZIP) (Thomas, *et. al.*, 1992b). The leucine zipper allows the formation of a stable dimer, while the basic region is responsible for specific DNA binding. The construction of LexA-Met4 fusion proteins have shown that the bZIP domain of Met4 is responsible for its DNA recognition, either by

binding directly to the DNA or through its interaction with another DNA-bound protein factor (Thomas, *et. al.*, 1992b). Activation of transcription of the *MET16* gene can occur through two different mechanisms. The pathway specific control requires functional Met4p and is triggered by methionine starvation, whereas general control responds to starvation for many different amino acids and requires Gcn4p. Both of these pathways were shown to be dependent on Cbf1p for optimum activity. (Baker and Masison, 1990) Transcription of the *MET25* gene is absolutely dependent on Met4p. The interaction of the Cbf1 protein with Met4, and how the intracellular level of SAM affects Met4 remains unclear.

Another group also cloned and sequenced the *MET4* gene and mapped the 5' end of the *MET4* transcript (Mountain, *et. al.*, 1993). The Met4 protein was found to be transcriptionally regulated by the general amino acid control (GC). Thus, a leucine zipper-containing *trans*-activator protein that specifically regulates the *MET* genes is itself transcriptionally regulated by GCN4, a bZIP protein controlling the general amino acid response.

Functional analysis of the Met4 protein by successive internal deletions of LexA-Met4 fusion proteins revealed three separate domains of functional importance (Kuras and Thomas, 1995). The activation domain is in the N-terminal region between residues 95 and 144. The Met4 activation function is inhibited fourfold when intracellular SAM increases. The inhibtion of the Met4 activation function requires a distinct region, located between residues 188 and 235. Deletions in this region eliminate most of the SAM responsiveness of LexA-Met4 derivatives. An auxiliary domain lies between residues 312 and 375. In the absence of this domain, the inhibitory region prevents the operation of the Met4 activation domain, even when the intracellular SAM level is low.

Kuras and Thomas (1995) have proposed a model to explain the function of Met4. When the intracellular concentration of SAM is high, the inhibitory region of Met4 would control an interaction with a regulatory protein that would prevent the activation domain from having access to components of the basal transcription machinery. When intracellular SAM concentrations are low, the

auxiliary domain would facilitate the reversal of the inhibition of the activation domain.

5.3.1 Recruitment of Met4p to the promoter region

Met4p and Met28p are found in a high molecular weight complex that binds DNA and also contains Cbf1p. The yeast two-hybrid system has shown that Met4p interacts with Met28p, and also with Cbf1p via their leucine zippers (Kuras, *et. al.*, 1996), however Met28p and Cbf1p do not interact directly. The function of Met28p is to stimulate the DNA binding activity of Cbf1p by decreasing the dissociation rate of the Cbf1-DNA complex (Kuras, *et. al.*, 1997). Foot-printing experiments showed that the Cbf1p-Met4p-Met28p complex is assembled on the TCACGTG 5' upstream sequence (Kuras, *et. al.*, 1997).

Mutational analysis of the upstream region of the *MET25* gene revealed another *cis*-acting element which could be found upstream of most methionine biosynthetic genes (Thomas, *et.al.*, 1989). This upstream region contains the core sequence 5' AAACTGTGG 3'. Two zinc finger proteins, Met31p and Met32p, have been shown to bind to this DNA sequence (Blaiseau, *et. al.*, 1997). Neither of these proteins have intrinsic transcriptional activation function, and their role appears to vary from one gene to another. Both proteins function as negative *trans*-regulatory factors at the *MET25* promoter, and as positive factors at the *MET3* and *MET14* promoters (Blaiseau, *et. al.*, 1997). Mobility shift assays demonstrated that Met31p and Met32p recruit Met4p to the promoter regions of *MET3* and *MET28* by forming Met4p-Met28p-Met31/32 complexes (Blaiseau, *et. al.*, 1998). Although the promoter regions of these genes contain the Cbf1p DNA binding site, Cbf1p-Met4p-Met28p complexes do not assemble upstream of these genes (Kuras and Thomas, 1995). Cbf1p bound upstream of *MET3* and *MET28* appears to stabilise the binding of Met4p-Met28p-Met31/32p complexes to the DNA (Blaiseau, *et. al.*, 1998).

The apparently simple pathway of methionine biosynthesis appears to have complex regulatory mechanisms, where not all genes in the one pathway are regulated in the same manner. Whilst all of the genes of the methionine

biosynthetic pathway are activated by the Met4 protein, the cell has devised different mechanisms of recruiting this activator to the DNA.

Microarray hybridisation experiments have revealed that the methionine biosynthetic genes appear to be cell cycle regulated, even in the presence of methionine (Spellman, *et. al.*, 1998). An increase in the transcription of these genes was detected at the G1/S phase boundary. It was postulated that activation of methionine biosynthesis may ensure sufficient methionine for protein synthesis in the next cell cycle, or depletion of S-adenosylmethionine as cells enter S phase, may activate the *MET* genes (Spellman, *et. al.*, 1998). Another possibility proposed is that the Met30 protein, an F-box protein involved in the negative regulation of the *MET* genes and in the targeting of Swe1 for degradation, may become limiting, enabling transcription of the methionine biosynthetic genes. Transcription of *SWE1* peaks just before S phase (Spellman, *et. al.*, 1998). It is interesting to note that the RNA used in the microarray experiments was isolated from cells which had been grown in a nutrient rich medium (YPD), yet previous experiments have demonstrated that the methionine biosynthetic pathway is turned off by low levels of methionine (Cherest, *et. al.*, 1971; Cherest, *et. al.*, 1973; Cherest, *et. al.*, 1985; Mountain, *et. al.*, 1991).

5.4 The Role of the Met30 polypeptide

The Met30 polypeptide has been shown to participate in the negative regulation of sulfur assimilation as a transcriptional inhibitor (Thomas, *et. al.*, 1995). *MET30* is predicted to encode a protein of 640 amino acids with a molecular mass of 72800 Da. Sequence analysis of *MET30* revealed that the encoded product belongs to a family of proteins that comprise several repeats of a 40 amino acid segment, called the WD40 motif (Thomas, *et. al.*, 1995). All WD40 proteins to date have a regulatory function. A *MET30* mutation prevents the SAM-mediated inhibition of Met4 activation function. The use of the two-hybrid system has demonstrated that Met30 and the inhibitory region of Met4 interact *in vivo* to repress the activation domain when the intracellular SAM level is high (Thomas, *et. al.*, 1995).

Met30 is an F-box protein which can be found within an E3 ubiquitin ligase complex composed of Skp1, Cdc53, and an F-box protein (SCF complex) (Patton, *et. al.*, 1998). There are three F-box proteins identified that provide specificity to this ubiquitin ligase, Cdc4, Met30 or Grr1. The SCF^{Met30} complex is required for repression of the methionine biosynthetic genes and has been demonstrated to target *MET4* for ubiquitin dependent proteolysis when intracellular S-adenosylmethionine increases (Rouillon, *et. al.*, 2000). Met30 has also been shown to interact with the Cdk-inhibitory kinase, Swe1, and target it for degradation by the Cdc34 dependent ubiquitination pathway (Kaiser, *et. al.*, 1998).

The function of Met30 is not restricted to the sulfate assimilation pathway, but is also involved in cell cycle regulation, the methyl cycle and the SAM regulation of *MET19*, encoding the first enzyme of the pentose phosphate pathway. This implies that Met30 has the ability to interact with other transcriptional factors, most likely targeting them for ubiquitin dependent proteolysis as described above. The lethality associated with a *MET30* gene disruption is consistent with the involvement of Met30 in the regulation of numerous sets of genes. This lethality may originate from the metabolic importance of SAM which is second only to ATP in the number of reactions in which it participates (Cantoni, 1977).

5.5 Post-transcriptional control

It appears that, in addition to tightly regulated transcriptional control, a strong post-transcriptional control may be operative for at least some of the *MET* genes. Overexpression of the *MET2* gene on a galactose inducible plasmid, increased *MET2* mRNA at least 100-fold compared to wild-type strains, yet the specific activity of the enzyme, homoserine O-acetyltransferase, increased only 2-fold (Forlani, *et. al.*, 1991). Post-transcriptional control has also been reported for *MET25* (Thomas, *et. al.*, 1989), and *MET10* (Hosseini-Mazinani, *et. al.*, 1995).

6.0 SULFITE REDUCTASE

Zambonelli (in Zambonelli, *et. al.,* 1975) was the first to report the existance of yeast strains which could not produce hydrogen sulfide from sulfates. Naiki (1965) also isolated several mutants of *S. cerevisiae* incapable of reducing sulfite. The enzyme sulfite reductase, which catalyzes the six electron reduction of sulfite to sulfide was first purified from *S. cerevisiae* by Yoshimoto and Sato (1968a) and extensively characterized (Yoshimoto and Sato, 1968a, 1968b, 1970). The yeast sulfite reductase (SiR) is a complex protein. It contains two molecules each of FAD (flavin adenosine dinucleotide), FMN (flavin mononucleotide) and haem, per enzyme molecule. The enzyme was also found to possess multiple catalytic activities, such as the reductions of sulfite, nitrite, hydroxylamine, ferricyanide, cytochrome c and quinones by NADPH; and the reduction of sulfite by reduced methylviologen or reduced benzyl viologen. Initial findings suggested the operation of a complicated intramolecular electron-transfer pathway consisting of several carriers or reactive sites (Yoshimoto and Sato, 1968a).

The study of partially purified yeast SiR from *S. cerevisiae* mutants previously isolated by Naiki (1965) resulted in the conclusion that intact NADPH-SiR has at least three components: a FAD containing component, a FMN containing component and a component containing the chromophore (Yoshimoto and Sato, 1968b). Some of the mutant yeast SiR's lacked the FAD component and therefore were incapable of reducing sulfite by NADPH but were able to reduce sulfite by reduced methyl viologen, an alternative electron donor. Other mutant yeast SiR's lacked both the FAD and FMN component and were unable to reduce sulfite with either NADPH or reduced methyl viologen as the electron donor.

The yeast SiR enzyme activity is inhibited by chelating agents and the production of H2S by yeast cells is reduced by iron inhibitors and by iron deficiency. Studies by Prabhakararao and Nicholas (1969) confirmed that yeast SiR is a haemoflavoprotein. SDS-polyacrylamide gel electrophoresis of native or cross-

linked enzyme indicated that yeast SiR has a subunit stucture of $\alpha 2\beta 2$ (Kobayashi and Yoshimoto, 1982a).

Although yeast SiR has many properties similar to those of other NADPH dependent sulfite reductases (*E. coli, Aspergillus nidulans* and *S. typhimurium*), it shows an unusual instability to low ionic strength (Kobayashi and Yoshimoto, 1982b). In the native enzyme, FAD and FMN are close enough, or bridged to exchange electrons. However, when exposed to a solvent of low ionic strength, the enzyme undergoes dissociation into two components, resulting in the interception of the electron flow between FAD and FMN, and therefore ceases NADPH-linked reductase activities. The process is rapid and can be reversed by raising the ionic strength (Kobayashi and Yoshimoto, 1982c). The prosthetic groups of the yeast SiR make up an electron transport chain in the enzyme, from NADPH to FAD, and then to FMN, siroheme and finally to the sulfite molecule.

6.1 *S. cerevisiae* sulfite reductase

A number of genes are required for an active sulfite reductase (Table 1). One of these genes, *MET10*, has been cloned by complementation of a *S. cerevisiae met10* mutant (Hansen, *et. al.*, 1994). Deletion within the gene resulted in a yeast strain that accumulated 180 times more sulfite than a wild-type strain. Growth in the presence of methionine at concentrations greater than or equal to 2mM almost completely abolishes transcription of *MET10*, therefore the regulation of the transcription of *MET10* appears to be similar to that of other *MET* genes.

The *MET10* gene was shown to encode a polypeptide of 114.8kDa (Hansen, *et. al.*, 1994), which is very close to the molecular weight that Kobayashi and Yoshimoto (1982a) determined for the SiR α-subunit (116kDa). The polypeptide was also shown to have flavoprotein homology and an NADPH binding site. Thus the Met10 polypeptide may interact with NADPH and FAD, which supports the hypothesis that *MET10* encodes the α subunit of yeast SiR.

FMN is another prosthetic group of the yeast SiR holoenzyme, and is needed for the integrity of the electron transport chain and for sulfite reduction to take place. However, no FMN binding motifs could be found in the Met10 polypeptide. Therefore, either a novel mechanism of FMN binding exists in the Met10 polypeptide or, more likely, FMN binding is present in the yeast SiR on another subunit (Hansen, *et. al.*, 1994). Since disintegration at low ionic strength results in two identical αβ pairs and the electron flow between FAD and FMN is interrupted, the electron flow may be from FAD on the α subunit (Met10) of one αβ pair to the FMN on the β subunit of the other αβ pair (Hansen, *et. al.*, 1994).

Another research group also cloned the *MET10* gene (Hosseini-Mazinani, *et. al.*, 1995). The α-subunit of SiR was purified from *S. cerevisiae* by SDS-PAGE, and its partial amino acid sequence determined. This sequence was used to PCR amplify a segment of the *MET10* gene, which was then used to screen a *S. cerevisiae* library. Positive clones were sequenced and found to be identical to the *MET10* sequence reported by Hansen *et. al.* (1994), except at five positions. The differences detected are most likely to be due to strain divergence (Hosseini-Mazinani, *et. al.*, 1995).

6.2 *Escherichia coli* sulfite reductase

The *E. coli* sulfite reductase has the subunit structure $\alpha_8\beta_4$, in which the 66kDa α -subunit is a flavoprotein containing FAD and FMN and the 64kDa β-subunit is a haemoprotein containing sirohaem and a Fe_4S_4 cluster. The holoenzyme contains four FAD molecules, four FMN molecules, four Fe_4S_4 clusters and four sirohemes (Ostrowski, *et. al.*, 1989).

The *E. coli* SiR is different from the yeast SiR in that two α subunits cooperate to form a single active FAD and FMN pair. The FAD serves as an entry for electrons from the NADPH, while FMN serves as a mediator for rapid transfer of these electrons to the haemoprotein component where sulfite is reduced. The amino acid sequence of the α-subunit, encoded by *cysJ*, contains regions homologous to the FAD and FMN binding domains of NADPH-cytochrome P-

450 oxidoreductase. Therefore it appears that the binding of one flavin to an α-subunit precludes the binding of a second to the same polypeptide chain (Ostrowski, *et. al.*, 1989). The *E. coli* SiR is also capable of catalysing a number of other NADPH-dependent reduction reactions including the activation of ribonucleotide reductase and the reduction of ferrisiderophores (Eschenbrenner, *et. al.*, 1995). These activities of SiR are dependent on its general flavin reductase activity.

Studies by Eschenbrenner, *et. al.* (1995) have shown that the flavin substrates mainly receive the electrons directly from reduced FAD. Cytochrome c, ferricyanide, and the β-subunit of SiR, belong to a second class of substrates that receive their electrons from FMN. Another model was proposed for the SiR flavoprotein structure, in which two flavins (1 FAD and 1 FMN) are bound per α-subunit (Eschenbrenner, 1995). However, the SiR flavoprotein octamer only binds four FAD and four FMN cofactors (Ostrowski, *et. al.*, 1989).

Study of the structure of the *E. coli* SiR haemoprotein (SiRHP) at 1.6Å revealed that the protein uses two-fold symmetry to associate co-factors and enhance their reactivity for catalysis (Crane, *et. al.*, 1995). Conserved residues between the symmetry-related halves of SiRHP, and also between sulfite reductases and nitrite reductases highlight regions of sequence required for stability and function, called sulfite or nitrite reductase repeats (SNiRR). Crane *et. al.* (1995) suggested this repeat is present in molecules as divergent as nitrate reductases and mammalian sulfite oxidases, and that SiRHP may be representative of an ancient ancestral enzyme involved in early multi-electron reductions of inorganic substrates.

Whilst the *E. coli* sulfite reductase is significantly different from the yeast enzyme, there are also some similarities which may provide clues about the structure and function of yeast sulfite reductase.

7.0 GENETIC ENGINEERING OF YEAST TO REDUCE H_2S PRODUCTION

A greater understanding of the sulfate assimilation pathway increases the possibility of genetically engineering a wine yeast strain with reduced H_2S production. Since sulfite reductase is the key enzyme in the production of sulfide, this enzyme is a likely target in the genetic manipulation. The *MET10* gene has been inactivated in *S. carlsbergensis* brewer's yeast with the aim of increasing sulfite levels in beer (Hansen and Kielland-Brandt, 1996). Allotetraploid strains of *S. carlsbergensis* with one to four copies of *MET10* inactivated by internal deletions were constructed. Strains with no functional *MET10* gene showed no sign of H_2S production and produced more than six times the amount of sulfite than the control strain under brewing conditions (Hansen and Kielland-Brandt, 1996).

The inactivation of all copies of *MET10* renders the yeast strain a methionine auxotroph. Whilst this doesn't appear to be a problem in wort, a wine yeast strain which is a methionine auxotroph may not perform well in grape must. The average concentration of methionine in wort (59mg/L, Garza-Ulloa, *et. al.*, 1986) is considerably greater than that found in Australian grape juice (1-5mg/L, Henschke, and Jiranek, 1993). Therefore a reduction in the activity of sulfite reductase, rather than its elimination, may be a more feasible approach.

The reduction of H_2S production by approximately two-fold in a brewing yeast has been achieved by constitutive expression of the *MET25* gene (Omura, *et. al.*, 1995). The *MET25* gene product (OAH/OAS sulfhydrylase) directly utilises H_2S as its substrate therefore overexpression of the enzyme should reduce the amount of sulfide able to be released. However, the function of the sulfate transporter was found to be impaired in strains which overexpress *MET25*, (Omura, *et. al.*, 1995), probably due to a feedback inhibition mechanism, which would also contribute to a decrease in H_2S production. Another gene, *NHS5* (*cys4*), which encodes cystathionine β-synthase has also been cloned. Overexpression of this

gene enhances cystathionine synthesis and therefore removes excess H_2S (Tezuka, *et. al.*, 1992).

Overexpressing the above genes in wine yeast may not overcome the problem of excess H_2S production during fermentation. H_2S is often produced when there is a lack of assimilable nitrogen (Vos, and Gray, 1979) however overexpression of *MET25* would require the availability of an abundance of assimilable nitrogen precursors to be combined with the sulfide. Likewise, overexpression of *NHS5* also requires the presence of sufficient nitrogenous precursors. Reducing the production of the sulfide is a better approach than trying to consume it in a later metabolic step. The six electron reduction of sulfite to sulfide is a high energy consuming step, therefore targeting the sufite reduction step, would save considerable energy expenditure within the cell and may reduce the production of H_2S.

8.0 CONCLUSIONS

The production of hydrogen sulfide has long been a problem for wine makers, since even low levels impart off-flavours to the wine. Whilst improvements in vineyard management and wine making techniques have reduced the occurrence of the problem, the routine addition of DAP to grape must is an indication that the problem is still widespread. The possibility that DAP addition could be restricted in the future, as well as the restrictions that already exist in Europe means that alternative solutions need to be found.

The aim of this work is to reduce the activity of sulfite reductase in *S. cerevisiae* using a genetic approach that will not eliminate the enzyme. The dominant negative strategy (Herskowitz, 1987), discussed in further detail in chapter three, will not eliminate sulfite reductase activity. Manipulation of *MET10* encoding the α subunit of sulfite reductase such that it can no longer bind the cofactors NADPH or FAD, yet still binds to the β subunit, may reduce the activity of the enzyme in the cell and provide information on the predicted active sites of the enzyme.

CHAPTER TWO Materials and methods

2.1 *ESCHERICHIA COLI* RECOMBINANT TECHNIQUES

2.01.1 Ligation of DNA into plasmid

Ligation of restriction digested DNA into the appropriate sites of vectors was performed as described in Maniatis *et.al.* (1982).

2.01.2 Dephosphorylation of vector DNA

Vector DNA digested with the appropriate restriction enzymes and gel purified, was transferred to a 1.5ml eppendorf tube (routinely 10µl). Calf Intestinal Phosphatase (CIP) (1µl), and 10 x CIP buffer (Appendix 1) (2µl) were added to the vector DNA, and the total volume of the reaction made up to 20µl by the addition of water. The reaction was incubated at 37°C for 30 minutes. The enzyme was heat inactivated by incubation at 65°C for 10 minutes. An additional 0.5µl of CIP was added and the reaction incubated at 37°C for 15 minutes. The enzyme was heat inactivated as above, and the volume of the reaction made up to 100µl with water, and phenol:choroform:isoamyl alcohol (24:24:1) extracted twice. The DNA was precipitated by the addition of one tenth volume of sodium acetate (pH 7.0), then 2.5 volumes of ethanol. The DNA was pelleted by centrifugation at 12 000 rpm for 10 minutes at 4°C, and washed with 1ml of 70% ethanol. The pellet was briefly dried under vacuum and resuspended in 10µl of nanopure water.

2.01.3 Transformation of *E. coli*

2.01.3.1 Preparation of chemically competent cells

Chemically competent *E. coli* DH5α cells were prepared as described by Inoue *et.al.* (1990). Briefly, ten to twelve large (2-3mm in diameter) colonies were isolated from a fresh plate of DH5α and inocluated into 250ml of SOB medium (Appendix 1) in a 2 liter flask. The cells were grown to an OD_{600} of 0.6 at 23°C, with vigorous shaking (200-250 rpm). The flask was then placed on ice for 10 min. The culture was transferred to a pre-cooled 500ml centrifuge bottle and spun at 3000 rpm in a JA-14 rotor for 10 min at 4°C. The pellet was resuspended in 80 ml of ice cold transformation buffer (Appendix 1), incubated in an ice bath for 10 min, and spun down as above. The cell pellet was gently resuspended in 20 ml of transformation buffer, and DMSO was added with gentle swirling to a final concentration of 7%. After incubating in an ice bath for 10 min, the cell suspension was dispensed in 500-1000µl aliquots in pre-cooled tubes and immediately chilled by immersion in liquid nitrogen. The frozen competent cells were stored at –80°C.

2.01.3.2 Transformation of chemically competent *E. coli*

A tube of competent cells was thawed at room temperature. Once cells had thawed they were gently mixed. 200µl of cells were added to ligation mix in 1.5ml reaction tubes and incubated on ice for 30 min. Cells were heat shocked at 42°C for 30 seconds and returned to the ice bath for 5 min. SOB (800µl) was added to the tube and incubated at 37°C for 60 min. Transformed cells were spread onto LB plates (Appendix 1) containing 50µl/ml ampicillin. Plates were incubated overnight at 37°C. Potential recombinant colonies were identified by a rapid size screen protocol (section 2.01.6).

2.01.3.3 Preparation of electro-competent cells for transformation

When transformation efficiency was expected to be low, especially after a ligation reaction, *E. coli* cells were electroporated. The protocol for the preparation of electro-competent was essentially the same as that given in the Bio-Rad pulse controller operating instructions and applications guide.

A single colony of DH5α was inoculated into 10ml of LB (Appendix 1) and incubated overnight at 37°C with shaking. The 10ml overnight culture was used to inoculate 1L of LB and the culture grown at 37°C with vigorous shaking until an OD_{600} of 0.5 to 1.0 was reached. The flask was chilled on ice for 15 to 30 minutes and cells were transferred to pre-cooled 500ml centrifuge bottles and spun at 3000 rpm in a JA-14 rotor for 10 min at 4°C. As much of the supernatant was removed as possible and the pellets resuspended in 1L of sterile cold water, and centrifuged again as described. The cell pellets were resuspended in 500ml of ice cold water and centrifuged as above. The pellets were resuspended in 20ml of cold 10% glycerol and centrifuged as above. Cells were then resuspended to a final volume of 2 ml in cold 10% glycerol. The cell suspension was dispensed in 45µl aliquots in pre-cooled tubes and immediately chilled by immersion in liquid nitrogen. The frozen competent cells were stored at –80°C.

2.01.4 Purification of DNA before electroporation

The volume of the reaction mix was made up to 500µl with water and an equal volume of phenol:chloroform:iso-amyl alcohol (24:24:1) was added and mixed by vortexing. After centrifugation the upper phase was transferred to a fresh tube and the DNA precipitated with 7.5M ammonium acetate (pH 4.8) and ethanol. The pellet was washed in 70% ethanol, dried under vacuum and resuspended in nanopure water (10µl).

2.01.5 Transformation of electro-competent *E. coli*

The protocol for the preparation of electro-competent was essentially the same as that given in the Bio-Rad pulse controller operating instructions and applications guide.

The cells were gently thawed at room temperature and immediately placed on ice. The DNA was then added to the tube of cells and mixed well, before transferring the mix to a chilled cuvette (Bio-Rad, 0.2cm gap). The electro-transformation was done according to the manufacturer's instructions. After electro-transformation, 1ml of SOC (Appendix 1) was added to the cells and the suspension was transferred to a 1.5ml reaction tube. Transformed cells were incubated at 37°C with gentle shaking for 1 hour, before being spread onto LB plates containing 50μg/ml ampicillin.

2.01.6 Rapid size screen for the detection of recombinant plasmids

Single colonies were picked with a yellow pipette tip, and patched onto an agar plate containing 50μg/ml ampicillin. The remainder of the colony was resuspended in 15μl of cracking solution (Appendix 1). The tip was left in the solution and the tube incubated at 65°C for 15 minutes. Samples were loaded onto a 1% agarose gel, with buffer coming up the sides of the gel only, not covering it. The samples were first run into the gel at 40-50V, the gel was covered with 1 x TAE buffer and the voltage increased to 90-100V. Recombinant plasmids were identified as running higher on the gel than vector alone. Colonies containing recombinant plasmids were picked and grown overnight in LB containing 50μg/ml ampicillin.

2.02 TRANSFORMATION OF *SACCHAROMYCES CEREVISIAE*

2.02.1 Preparation of cells for transformation

The protocol used to transform *Saccharomyces cerevisiae* was essentially the same as the method described in the Clontech MATCHMAKER Two-hybrid system 2 instruction manual.

YPD (50ml) was inoculated with several yeast colonies and the culture grown overnight at 30°C with shaking. Enough overnight culture was transferred into 300ml of YPD to give an OD_{600} 0.2-0.3. The culture was incubated at 30°C with shaking for 3 hours.

The cells were collected by centrifugation in a Beckman JA-14 rotor at 3000rpm for 5 minutes at room temperature. The supernatant was discarded and the cells resuspended in 50ml of sterile nanopure water. The cells were again pelleted by centrifugation as described above. The cells were resuspended in 1.5ml of 1xTE/LiAc (Appendix 1).

2.02.2 Transformation of cells

The vector(s) (0.1μg) to be transformed was transferred to a 1.5ml eppendorf tube. Denatured herring testes carrier DNA (0.1mg) was added to the plasmid DNA and mixed. The yeast competent cells (0.1ml) were also added to the DNA and mixed well. Sterile PEG/LiAc (Appendix 1) (0.6ml) was added to the tube, vortexed and the competent cells/DNA mix incubated at 30°C with shaking for 30 minutes. DMSO was added to a final concentration of 10%, mixed gently by inversion, and the cells were heat shocked at 42°C for 15 minutes. After the heat shock, the cells were chilled on ice, then pelleted by centrifugation for 5 seconds at 14 000rpm. The supernatant was removed and the cells

resuspended in 0.5ml of 1 x TE. The cells were plated onto selective media and incubated at 30°C for 5-7 days.

2.02.3 Colony cracking for PCR analysis

One half of a transformed colony which had been growing on selective medium for 5 – 7 days, was resuspended in 20µl of sterile nanopure water, and the other half of the colony kept at 4°C for further work. The resuspended colony was snap frozen in liquid nitrogen for approximately 10 seconds, boiled for 10 minutes and cooled on ice. The solution (1µl) was then used as template DNA in a PCR.

2.03 ISOLATION OF PLASMID DNA FROM *E. COLI*

2.03.1 Small scale isolation of plasmids

The protocol for small scale isolation of plasmid DNA was the alkaline lysis method described by Maniatis *et.al.* (1982).

A single colony of *E.coli* strain DH5α containing the plasmid of interest was inoculated into LB (5ml) (Appendix 1) containing ampicillin (50µg/ml). The culture was grown overnight at 37°C with rapid shaking. 1.5ml of the culture was transferred to a 1.5ml eppendorf tube and the cells pelleted. The supernatant was removed and cells resuspended in ice cold plasmid solution 1 (100µl) (Appendix 1), by vortexing. Freshly prepared plasmid solution 2 (200µl) was added, mixed gently by inversion, and incubated on ice for 5 minutes. Ice cold plasmid solution 3 (150µl) (Appendix 1) was added and immediately mixed by inversion. The tubes were centrifuged at 14 000 rpm for 5 minutes, and the supernatant transferred to a clean tube. An equal volume of phenol:chloroform:iso-amyl alcohol (24:24:1) was added and mixed by vortexing, and the tubes centrifuged as above. The upper layer was transferred to a fresh tube. The DNA was precipitated by the addition of

2.5 volumes of ice cold ethanol, and allowed to stand at room temperature for 5 minutes. The DNA was pelleted by centrifugation at 14 000 rpm for 10 minutes and washed with 1ml of ice cold 70% ethanol. The pellet was dried briefly under vacuum and resuspended in 20µl of TE (pH 8.0) containing RNase A (20µg/ml).

2.04 ISOLATION OF NUCLEIC ACIDS FROM YEAST

2.04.1 Preparation of chromosomal DNA

A single colony of *S. cerevisiae* or *Kluveromyces lactis* was inoculated into 10ml of YPD (Appendix 1), and grown at 30°C with shaking to stationary phase. The cells were harvested by centrifugation at 3000rpm for 5 minutes, and resuspended in 500µl of water. The resuspended cells were then transferred to a 1.5 ml eppendorf tube and pelleted by centrifuging for 5 seconds. The pellet was disrupted by vortexing briefly. The cells were resuspended in 200µl of yeast lysis solution (Appendix 1) and 0.3g of acid washed glass beads (400-625µm) added. Phenol:chloroform:iso-amyl alcohol (200µl) was added and the tube vortexed at high speed for 3 minutes. TE buffer (200µl) was added and the tube vortexed briefly. Cell debris and glass beads were removed by centrifugation for 5 minutes at 14 000 rpm. The aqueous phase was transferred to a clean eppendorf tube. The DNA was precipitated by the addition of 1ml of ethanol, and pelleted by centrifugation for 5 minutes at 14 000 rpm. The pellet was resuspended in 400µl of TE buffer. Contaminating RNA was degraded by the addition of 3µl of 10mg/ml RNaseA and incubation at 37°C for 5 minutes. DNA was recovered by the addition of ammonium acetate to a final concentration of 2.5M. Ethanol (1ml) was added and the tubes were mixed by inversion. Centrifugation pelleted the DNA, which was then washed in 70% ethanol and dried under a vacuum. The DNA was resuspended in 100µl of TE buffer and kept at 4°C overnight or incubated at 65°C for 1 hour and put on ice.

2.04.2 Preparation of plasmid DNA from *S. cerevisiae*

The method used to isolate plasmid DNA from *S. cerevisiae* was essentially the same as the protocol provided in the Clontech yeast two-hybrid manual, which is based on the method of Hoffman and Winston (1987) and Kaiser and Auser (1993).

Synthetic drop-out media (Appendix 1) (5ml), excluding selective amino acids to maintain the plasmid, was inoculated with a single, well isolated yeast transformant colony and the culture incubated at 30°C with shaking, overnight. The overnight culture was spun transferred to a 10ml plastic tube and the cells pelleted by centrifugation at 5000rpm for 5 minutes. The supernatant was poured off and the pellet resuspended in the residual liquid by vortexing. The cell suspension was transferred to a 1.5ml eppendorf tube, and 200µl of yeast lysis solution (Appendix 1) was added. Phenol:chloroform:isoamyl alcohol (24:24:1) (200µl) and 0.3g of acid washed glass beads was added and the tube vortexed for 2 minutes. Cell debris and glass beads were removed by centrifugation at 14 000 rpm for 5 minutes. The supernatant was transferred to a clean 1.5ml eppendorf tube and the DNA precipitated by the addition of one tenth of the volume of 3M NaOAc (pH 5.2) and 2.5 volumes of ethanol. The DNA was pelleted by centrifugation as above. The pellet was washed with 70% ethanol and dried under a vacuum. The DNA was resuspended in 20µl of nanopure water. Plasmid DNA from yeast is often contaminated by chromosomal DNA and only small quantities can be recovered, therefore the isolated plasmid DNA was electroporated into *E. coli* as described in section 2.01.7 and isolated from *E. coli* as described in section 2.03.1.

2.04.3 Preparation of RNA from *Saccharomyces cerevisiae*

The method used to isolate RNA from yeast was based on the method of Schmitt *et.al.* (1990).

A single colony of yeast 15d*a* was used to inoculate 20ml of synthetic drop-out medium without methionine, and incubated at 30°C with shaking, until the culture had reached mid-exponential phase. The cells were harvested by centrifugation at 5000*g* for 10 minutes. The supernatant was discarded and the pellet resuspended in 400µl of 50mM sodium acetate/10mM EDTA, pH4.8. All subsequent steps were done on ice. SDS (10%) (40µl) was added to the cell suspension and the tube vortexed. Phenol:chloroform:isoamyl alcohol (24:24:1) (440µl) was added and the tube vortexed again. The tube was then incubated at 65°C for exactly 4 minutes. The mixture was snap frozen in liquid nitrogen, then allowed to thaw on ice. The tube was centrifuged at 4°C for 2 minutes and the upper layer transferred to a fresh 2ml eppendorf. An equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) was added and the tube vortexed. The mixture was incubated at room temperature for 5 minutes, centrifuged as above and the upper layer transferred to a fresh 2ml eppendorf tube. The nucleic acid was precipitated by the addition of one tenth volume of 3M sodium acetate and 2.5 volume of ice cold ethanol, and stored at –20°C overnight. The next morning, the nucleic acid was pelleted by centrifugation at 12 000rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet dried briefly under vacuum. The nucleic acid was resuspended in 30µl of water. The DNA was then digested by the addition of 4 units of RNase-free DNase (1U/µl), 5.0µl Promega buffer B (Appendix 1), and 52 units of Promega RNAsin (26U/µl). The total volume of the reaction was made up to 50µl by the addition of water, and the reaction incubated at 37°C for 5 hours. The volume of the mix was made up to 500µl by the addition of water and an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1)

added. The phases were separated by centrifugation at 12 000rpm for 10 minutes at 4°C. The aqueous phase was removed to a clean 1.5ml eppendorf tube and extracted with an equal volume of chloroform. The RNA was precipitated by the addition of one tenth the volume of 3M sodium acetate and 2.5 volumes of ice cold ethanol, and stored at –20°C overnight. The RNA was pelleted by centrifugation at 12 000 rpm for 15 minutes at 4°C, and washed with 1ml of ice cold 70% ethanol. The supernatant was removed and the pellet dried briefly under a vacuum. The RNA was resuspended in nanopure water (50µl) and then stored at –80°C.

2.04.4 Isolation of polyA$^+$ RNA from total RNA

PolyA$^+$ RNA was purified from total RNA using the Promega PolyATract mRNA Isolation System. PolyA$^+$ RNA was isolated according to the manufacturers recommendations.

2.05 PREPARATION OF *S. CEREVISIAE* CELL EXTRACT

2.05.1 Enzymatic lysis of *S. cerevisiae*

The method used was essentially the same as that described by Maniatis *et.al.* (1982).

Cells from a 1ml culture in exponential phase were collected by centrifugation at 14 000 rpm for 1 minute, and resuspended in 1ml of ice cold phosphate buffered saline. The cells were recovered by centrifugation as above and the supernatant discarded. The pellet was resuspended in a volume of stabilising buffer A (Appendix 1), equal to the volume of the original culture, and incubated at 30°C for 10 minutes. The cells were recovered by centrifugation as above and resuspended in a volume of stabilising buffer B (Appendix 1), equal to the volume of the

original culture, and the suspension incubated in a 30°C water bath for 2 minutes.

Cell wall was digested by the addition of Zymolyase 100T (Seikagaku Kogyo Co.) (10mg/ml) (Appendix 1) (0.25 volume) and the suspension incubated at 30°C. After 15 minutes, the extent of cell lysis was examined by removing two small aliquots of the suspension. Nonidet P-40 added to one, at a final concentration of 1%. The two samples were examined by phase contrast microscopy, with protoplasts being visible in the Nonidet P-40 untreated sample. The zymolyase incubation was continued until the majority of the cells had been converted to protoplasts. The protoplasts were collected by centrifugation at 500*g* for 10 minutes at 4°C. The pellet was resuspended in a volume of high salt lysis buffer (Appendix 1) equivalent to 0.01 volume of the original culture. The suspension was incubated at 0°C for 30 minutes. Cell debris was removed by centrifugation at 12 000 rpm for 10 minutes at 4°C and the yeast lysate transferred to a fresh tube.

2.05.2 Mechanical lysis of *Saccharomyces cerevisiae*

The method used was essentially the same as that described in Harlow and Lane (1988).

Cells from a 10ml culture in exponential phase were collected by centrifugation at 14 000 rpm for 1 minute, and resuspended in 10ml of ice cold phosphate buffered saline (Appendix 1). The cells were recovered by centrifugation as above and the supernatant discarded. The yeast cell pellet was resuspended in 200µl of ice-cold RIPA buffer (Appendix 1), and an equal volume of acid washed glass beads were added to the tube. The tube was vortexed vigorously for 3 minutes, incubating on ice for at least one minute after vortexing for one minute. Cell debris and the glass beads were removed by centrifugation at 12 000 rpm for 5 minutes at 4°C, and the yeast lysate transferred to a fresh tube.

2.06 YEAST MATING

2.06.1 Mating

The yeast strains ΔM10-1 and 90844 were streaked onto a YPD plate, such that both strains were mixed in the middle of the plate, and separately on each side of the plate. The plate was incubated at 30°C for two days. The yeast strains were replica plated to selective media, synthetic complete medium, and incubated for a further two days at 30°C.

2.06.2 Sporulation

Cells were replica plated onto sporulation medium (Appendix 1), and incubated for 3 days at 30°C. Ascus formation was monitored microscopically.

2.06.3 Dissolution of the ascus wall

A small toothpick-full of tetrads was gently resuspended in 50μl of 0.5mg/ml Zymolyase-100T solution (Appendix 1). Cells were examined under a phase contrast microscope to detect intact asci, then incubated at 30°C for 10 minutes. Sterile water (800μl) was gently added by slowly running in down the side of the tube, and the tube stored on ice.

2.06.4 Microdissection

A loopful of the spore suspension was streaked along the top of a YPD plate, which had been thinly poured and free of imperfections. Well isolated spores were dissected with the aid of a micromanipulator (Micro Video Instruments) using an inverted phase contract microscope (Olympus, CHT213E).

2.07 ESTIMATION OF HYDROGEN SULFIDE PRODUCTION

2.07.1 Indicator media

2.07.1.1 BiGGY

Hydrogen sulfide producing colonies become discoloured when grown on bismuth sulfite, ranging from off-white to near black, depending on the concentration of sulfide produced (Jiranek, *et. al.*, 1995). Bacto BIGGY agar (Difco) was prepared according to the manufacturer's instructions. Yeast were grown on the BIGGY medium at 30°C for 2-3 days.

The lack of selection on the BIGGY medium was a problem due to plasmid loss. To overcome this, bismuth sulfite (8g/L) was added to synthetic dropout medium when the agar had cooled to 55°C, however this was not successful as the phosphates and sulfates in Yeast Nitrogen Base (YNB) (Difco) precipitate out.

2.07.1.2 Pb^{2+} medium

The accumulation of hydrogen sulfide ions can also form a PbS precipitate in the presence of divalent lead ions, giving the colony a dark brown to black pigmentation (Cost and Boeke, 1996). Yeast were grown on Pb^{2+} medium (Appendix 1) at 30°C for 2-3 days. Plasmid loss on this medium was a problem due to lack of selection. Phosphates and sulfates in YNB also precipitate in the presence of lead ions.

2.08 ESTIMATION OF SULFITE REDUCTASE ACTIVITY

Two methods were used to estimate sulfite reductase activity. The first method was essentially the same as that described by Jiranek and Henschke (1995).

Cultures were grown to mid log phase and 1 x 10^{10} cells collected by centrifugation. The cells were washed twice in 0.25M phosphate buffer (pH 7.3) (Appendix 1), and the pellet resuspended in 5ml of resuspension buffer (Appendix 1). The cell suspension was then transferred to homogeniser shaking flask made of duran glass and 10g of glass beads (425-600μm) added. The glass bead/cell slurry was then shaken in a cell homogeniser (MSK, B.Braun) with CO_2 cooling at 2000 rpm until >99% disruption was achieved (as determined by microscopic examination) after 10 minutes. The glass beads were removed by filtration through a glass sinter. The cellular debris was removed by centrifugation in a Beckman JM-21 centrifuge, at 15 000 rpm for 30 minutes at 4°C. The supernatant was dialysed against 1000 volumes of fresh resuspension buffer for two periods of 75 minutes, and then stored on ice before being assayed.

Cell-free extract was added to 3ml of reaction mixture (Appendix 1), and the total volume adjusted to 4ml with resuspension buffer. The tubes were immediately stoppered with septa (Suba-seal, number 29) and gently mixed by inversion before being incubated at 30°C for one hour. Working strength amine reagent (300μl) (Appendix 1) was injected into the tubes, the contents mixed and incubated at room temperature for at least one hour. Samples were centrifuged at 5000 rpm for 5 minutes to remove any precipitate that may have formed, and the OD_{672} measured. The spectrophotometer (Beckman, DU-64) was calibrated with a blank that was identical to the reaction, except that the extract had been boiled for 3 minutes to inactivate sulfite reductase. Standards were also identical, except extract had been substituted with known amounts of hydrogen sulfide. Hydrogen sulfide formation was calculated from a standard curve.

The second method used to estimate sulfite reductase activity was an adaptation of the method described by DeVito and Dreyfus (1964), and Cherest *et.al.* (1992).

Cells were lysed mechanically as described in section 2.05.2, with a few modifications to the lysis buffer (Appendix 1). After removal of the cell debris and glass beads by centrifugation, the lysate was transferred to a clean 1.5ml tube, and the volume made up to 400µl by the addition of PBS (Appendix 1) containing protease inhibitors [PMSF (100µg/ml), aprotinin (2µg/ml) and leupeptin (2µg/ml)].

The sulfite reductase activity was determined by the addition of 1-4mg of total yeast protein (routinely 250µl of lysate) to sulfite reductase reaction mixture (Appendix 1). The final volume of the reaction was made up to 2.0ml by the addition of water, and the reaction incubated at 37°C for 30 minutes. A reaction without yeast lysate was a negative control.

The amount of sulfite reduced was analysed by the method of Grant (1947), described in section 2.09. The amount of sulfite in the lysate before the sulfite reductase reaction was also measured. The difference between the two results gave the amount of sulfite which had been converted to sulfide by sulfite reductase.

2.09 QUANTITATION OF SULFITE ACCUMULATION

The method used to measure sulfite accumulation is essentially the same as that described in Hansen *et.al.* (1994) which is an adaptation of the method described by Grant (1947).

Fresh, single colonies of yeast were used to inoculate 10 cultures of synthetic B medium (Appendix 1) supplemented with the appropriate amino acids for the yeast strain and to maintain plasmid selection. The inclusion of DL-homocysteine thiolactone in the medium enables cells

with a disruption in the methionine biosynthetic pathway to grow in the absence of methionine, whilst the pathway remains active (Cherest, *et. al.*, 1985; Hansen, *et. al.*, 1994). The cultures were grown in 50ml flasks at 30°C with shaking for 4 days. A sample (200µl) was taken from each flask for cell counting, and the flasks were cooled at 4°C. The cells were pelleted and the supernatant removed to a fresh tube. Mercuric Chloride reagent (Appendix 1) (100µl) was added to a plastic 10ml tube. To this, 100µl of 0.1M H_2SO_4 was added and the solutions mixed gently. The sample was then added (200µl) and the tubes mixed. NaOH was then added (0.1M, 300µl), the tubes mixed. After standing for 1 minute, 0.1M H_2SO_4 (200µl) was added, the tubes mixed gently and 1100µl of autoclaved nanopure water added. Colour reagent (400µl) (Appendix 1) was added, the tubes mixed and then 400µl of freshly prepared 0.2% formaldehyde added. An additional 1200µl of autoclaved nanopure water was added and the tubes incubated at room temperature for 30 minutes. The absorbance at 570nm was then measured.

A standard curve was constructed by the addition of known amounts of sulfite to the reaction. Sodium bisulfite (9g) was dissolved in a 500ml volumetric flask of volumetric flask. The sulfite solution (5ml) was transferred to a 100ml conical flask and potassium iodide (1g) was added. Potassium iodate (0.1M) (20ml) and 500µl of 1% soluble starch were also added to the flask, and the solution titrated with 0.1M sodium thiosulfate to endpoint. The concentration of sulfite in the solution was then determined using the equation shown in Appendix 1. Mercuric chloride reagent (250ml) was added to a 500ml volumetric flask and 1ml of sulfite solution added. The total volume was made up to 500ml with autoclaved nanopure water. Standards were prepared by further dilutions (1 – 15%) of the dilute sulfite solution. From each standard solution, 2ml was transferred to a 10ml plastic tube, and the sulfite assay was continued from the addition of the colour reagent.

2.10 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein gels were prepared as described in the Bio-Rad Protean II gel system manual. The gels routinely consisted of an 8% resolving gel (0.375M Tris-HCl, pH 8.8) and a 4% stacking gel (0.125M Tris-HCl, pH 6.8) (Appendix 1). Gels which were run overnight (20mV) were poured in plates 20cm x 20cm with 1mm spacers. Mini-gels were poured in plates 5cm x 10cm with 1mm spacers. Molecular weight markers used were the Promega high molecular weight markers, or the Novex SeeBlue pre-stained standards. Proteins were visualised by staining with Coomassie blue (Appendix 1), or BioRad Biosafe Coomassie.

2.11 OVEREXPRESSION OF MET10p IN *E. COLI*

2.11.1 Cloning and Transformation

E. coli strains BL21(pLysS) and B834 were transformed by electroporation (section 2.01.5) with the pET-14b vector (Novagen) containing the *MET10* gene. A *Xho* I site was introduced into the 5'region of the *MET10* gene by PCR and cloned into the *Xho* I and *BamH* I sites of the vector.

2.11.2 Rapid screening of small scale expression cultures

Five colonies from a plate of freshly transformed cells were picked and used to inoculate 5ml cultures of LB containing 100μg/ml ampicillin [BL21(pLysS) and B834] and 34μg/ml chloramphenicol [BL21(pLysS) only]. Cultures were incubated at 37°C overnight with shaking. The next day the overnight cultures were subcultured into 20ml of LB containing the appropriate antibiotics, and incubated with shaking until the OD_{600} reached 0.4. The expression of Met10p was induced by the addition of IPTG to a final concentration of 1mM. A 1ml sample was taken from each culture just prior to addition of IPTG and this served as a t_0 control.

The cultures were induced for 3 hours, with 1ml samples taken at hourly intervals. The cells from each 1ml sample were pelleted and stored at –20°C until the end of the time course.

The pelleted cells were defrosted and briefly vortexed to resuspend the cells in the residual liquid. *E. coli* cracking buffer (100µl) (Appendix 1) was added to the resuspended cells and then boiled for 3 minutes. Samples (20µl) were loaded onto a Laemmli SDS-PAGE gel (Stacking gel – 4%, Resolving gel – 8%).

2.11.3 Screening of small cultures by purification of 6xHis-tagged proteins

Colonies which had been shown to be expressing Met10p were picked and grown overnight at 37°C in 5ml of LB containing the appropriate antibiotics. The next morning, 15ml of LB plus antibiotics was inoculated with 1.5ml of the overnight culture. When the OD_{600} was between 0.4 and 0.6 expression of Met10p was induced by the addition of IPTG to a final concentration of 1mM. Samples (1ml) were taken just before the addition of IPTG (t_0), and 1, 2, and 3 hours after induction (t_1, t_2, t_3). Cells were pelleted by centrifugation at 14 000rpm for 30 seconds, the supernatant discarded and the cells stored at –20°C until all samples were ready for processing.

The cells were resusupended in 200µl of Buffer B (Appendix 1), and lysed by gently vortexing. The lysate was centrifuged at 14 000rpm for 10 minutes to pellet the cellular debris. The supernatant was transferred to a fresh 1.5ml eppendorf tube and 50µl of a 50% Ni-NTA resin added to each tube. The tubes were mixed gently on a rotary mixer for 10 minutes at room temperature, and 20 minutes at 4°C. The resin was pelleted by centrifugation for 10 seconds at 12 000rpm, and 20µl of the supernatant transferred to a fresh tube and stored on ice. The remainder of the supernatant was discarded, and the resin was washed 3x with 1ml

of Buffer C (pH6.3) (Appendix 1). Buffer C containing 100mM EDTA (20µl) was added to each tube. The tubes were incubated at room temperature on a rotary mixer for 2 minutes at room temperature. The resin was pelleted by centrifugation at 12 000rpm for 10 seconds and 20µl of the supernatant transferred to a fresh tube. Loading buffer (20µl) was added to all samples, boiled for 5 minutes, and loaded onto an SDS-PAGE.

2.11.4 Determination of target protein solubility

LB medium (10ml) containing 100µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with BL21(pLysS)[pET*MET10*] and incubated overnight at 37°C with shaking. The overnight culture was used to inoculate 100ml of LB plus ampicillin and chloramphenicol. The culture was grown until the OD_{600} reached 0.4-0.6 and expression of the protein induced as described above. Induction was for 3 hours. The cells were harvested by centrifugation at 4000*g* for 20 minutes, and resuspended in 5ml of lysis buffer for native purification (Appendix 1). The sample was then frozen in liquid nitrogen and thawed in cold water. The cell suspension was then sonicated on ice for 6 x 20 seconds, with 20 second pauses at 50W. The lysate was centrifuged at 10 000*g* for 30 minutes at 4°C. The supernatant, which contains the soluble protein, was decanted and stored on ice. The pellet, which contains insoluble protein was resuspended in 5ml of lysis buffer. Loading buffer (20µl) was added to samples of soluble and insoluble protein (20µl), the protein boiled for 5 minutes, and loaded onto an SDS-PAGE.

2.11.5 Preparation of cleared lysates under denaturing conditions

Cultures (500ml) were grown and protein expression induced as described above for the smaller scale cultures. The cells were harvested by centrifugation, the supernatant discarded and the pellet stored at –20°C overnight.

The pellet was thawed on ice for 15 minutes and resuspended in buffer B at 5ml per gram of wet weight. The cells were mixed on a rotary mixer for 60 minutes at 4°C. The cellular debris was pelleted by centrifugation at 10 000*g* for 30 minutes at 4°C. The lysate was decanted into 10ml sterile plastic tubes.

2.11.6 Batch purification under denaturing conditions

The 50% Ni-NTA slurry was added to the cleared lysate (1ml per 4ml of cleared lysate), and gently mixed on the rotary mixer at 4°C for 1 hour. The Ni-NTA slurry was pelleted by centrifugation for 10 seconds. The supernatant was poured off, and 20µl was transferred to a fresh tube for SDS-PAGE analysis. The Ni-NTA pellet was washed six times in Buffer C (Appendix 1) containing 0.3M NaCl. The Ni-NTA resin/buffer C slurry was mixed on a rotary mixer for 2 minutes at room temperature each wash. Washes were kept for SDS-PAGE analysis. Elution of the protein from the Ni-NTA resin was achieved by washing the pellet in Buffer E (Appendix 1) (100µl) twice, mixing on a rotary mixer for 5 minutes at room temperature each time. The supernatant was transferred to a fresh tube, and an aliquot (20µl) used in SDS-PAGE analysis. The remainder was stored at –20°C.

2.11.7 Preparation of cleared lysates under native conditions

A –20°C glycerol stock of BL21(pLysS)[pET*MET10*] was used to inoculate LB (50ml) containing ampicillin (100µg/ml) and chloramphenicol (34µg/ml) and the culture grown overnight at 37°C with shaking. This culture was used to inoculate 500ml of LB containing ampicillin and chloramphenicol, and grown at 30°C until the OD_{600} reached 0.6. The expression of the protein was induced by the addition of IPTG to a final concentration of 1mM and grown for a further 3 hours.

Cells were harvested by centrifugation at 3500rpm for 5 minutes at room temperature, the supernatant discarded and the pellet stored at –20°C overnight.

The pellet was thawed on ice and lysis buffer (Appendix 1) (5ml/gram of wet weight) added. Lysozyme was added to a final concentration of 1mg/ml, and the cell suspension incubated on ice for 30 minutes. Cells were sonicated at 50W for 3 x 20 seconds, with 1 minute cooling time between each burst. Cellular debris was pelleted by centrifugation at 9500 rpm in a Beckman JA-20 rotor for 30 minutes at 4°C. The lysate was decanted into 10ml sterile plastic tubes.

2.11.8 Batch purification under native conditions

2.11.8.1 Preparation of Zinc resin

Ni-NTA resin slurry (50%) (1ml) was incubated with 1ml of 1M $ZnCl_2$ on a rotary shaker for 1 hour at room temperature. The resin was pelleted and washed 3 times in 1ml of lysis buffer (Appendix 1) and the Zn-NTA resin resuspended as a 50% slurry in lysis buffer.

2.11.8.2 Purification

The 50% Zn-NTA slurry was added to the cleared lysate (1ml per 4ml of cleared lysate), and gently mixed on the rotary mixer at 4°C for 1 hour. The Zn-NTA slurry was pelleted by centrifugation for 10 seconds. The supernatant was poured off, and 20µl was transferred to a fresh tube for SDS-PAGE analysis. The Zn-NTA pellet was washed 4 times in Wash buffer (1ml) (Appendix 1) containing 0.3M NaCl. The Zn-NTA resin/wash buffer slurry was mixed on a rotary mixer for 2 minutes at room temperature each wash. Washes were kept for SDS-PAGE analysis. Elution of the protein from the Zn-NTA resin was achieved by washing the pellet in Elution buffer (Appendix 1) (200µl) twice, mixing

on a rotary mixer for 5 minutes at room temperature each time. The supernatant was transferred to a fresh tube, and an aliquot (20µl) used in SDS-PAGE analysis. The remainder was stored at –20°C.

2.12 TRANSFER OF PROTEINS TO NITROCELLULOSE

2.12.1.1 Wet transfer

The Bio-Rad mini Protean II gel system was used to transfer protein from mini SDS-PAGE gels to nitrocellulose (Nitrobind, Micron Separations Inc.). The SDS-PAGE gel was carefully removed from the glass plates and the stacking gel cut off using a scalpel. The gel was equilibrated by soaking in cold western transfer buffer (Appendix 1) for 30 minutes. The Whatman paper, nitrocellulose , and the sponges were also soaked in cold western transfer buffer for 30 minutes. The transfer 'sandwich' was set up as described by the manufacturer. Transfer was at 100 V for 90 minutes in western transfer buffer.

2.12.1.2 Semi-dry transfer

The Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell was used to transfer protein from large and mini SDS-PAGE gels to nitrocellulose. The gel, nitrocellulose and whatman paper, were soaked in cold western transfer buffer as described above. The transfer 'sandwich' was set up as described in the instruction manual provided by Bio-Rad. Transfer was at 15V for 45 minutes for mini-gels and 20V for 90 minutes for large gels.

2.12.1.3 Ponceau S staining

The membrane was incubated in enough Ponceau S solution (Appendix 1) to cover the membrane for 5-30 minutes. The Ponceau S solution was poured off and the membrane destained in water until the background become white and the protein bands could be distinguished.

2.13 IMMUNISING RABBITS WITH MET10p

2.13.1 Native Met10p

The initial injection with native purified Met10p (approximately 500μg) was injected into rabbits with Freunds complete adjuvant. Boosts were every three weeks (between 300 - 500μg of protein) with Freunds incomplete adjuvant. Test bleeds monitored antibody production. All injections and test bleeds were done at the University of Adelaide Central Animal House, Waite campus.

2.13.2 Denatured Met10p

Purified protein was run on an 8% SDS-PAGE, transferred to nitrocellulose and stained with Ponceau S. The band corresponding to Met10p was cut from the membrane and destained in sterile water. When completely destained, the nitrocellulose was placed in a baked mortar and pestle and frozen by the addition of a small amount of liquid nitrogen. The frozen nitrocellulose containing Met10p was then ground to a fine powder. The powder was then combined with approximately 250μl of sterile saline and Freunds complete adjuvant, ready for immunising the rabbit. Boosts were every three weeks, however the adjuvant was Freunds incomplete adjuvant. Test bleeds monitored antibody production.

2.14 WESTERN BLOTTING

Western blots were done as described in Harlow and Lane (1988).

The nitrocellulose membrane was rinsed 2 x 5 minutes in 1 x PBS (Appendix 1). Blocking buffer, Blotto (Appendix 1), which had been heated to approximately 55°C and cooled on ice, was added such that the membrane was covered, and incubated at room temperature overnight with agitation.

The blot was removed from the blocking solution and washed 2 x 5 minutes in 1 x PBS. The blot was placed into a plastic bag and the primary antibody solution (Appendix 1) added. The bag was sealed and the blot incubated with the antibody for 2 hours at 37°C with gentle agitation. The blot was then washed 4 x 5 minutes in 1 x PBS. The blot was again placed into a plastic bag, and incubated with the secondary antibody solution (Appendix 1) for 1 hour at 37°C with gentle agitation. The blot was then washed 1 x 5 minutes in 1 x PBS containing 0.1% Tween-20, 3 x 5 minutes in 1 x PBS, and 2 x 5 minutes in 150mM NaCl, 50mM Tris (pH 7.5).

The blot was developed by the addition of alkaline phosphatase buffer (Appendix 1) containing bromochloroindolyl phosphate (165μg/ml) and nitro blue tetrazolium (330μg/ml). The blot was developed at room temperature with agitation until the bands were suitably dark (routinely 10 – 15 minutes). The reaction was stopped by the addition of 1ml of 0.5M EDTA, and the blot washed in nanopure water.

2.15 IMMUNOPRECIPITATION

2.15.1 Metabolic labelling of yeast cells

Synthetic dropout medium without leucine (Appendix 1) (10ml) was inoculated with a single yeast colony, and incubated overnight at 30°C with shaking. The next morning, the cells were diluted in the same medium and grown to a density of 10^7 cells/ml. The cells were pelleted by centrifugation at 5000rpm for 5 minutes, resuspended in 10ml of 1 x PBS and centrifuged again. The cells were then resuspended at 10^7 cells/ml in synthetic dropout medium without leucine or methionine, and incubated at 32°C for 20 minutes. An aliquot of the cell suspension (1ml) was transferred to a 2ml eppendorf tube and 50µCi of [^{3}H-Leu] added to the cells. The cells were incubated at 32°C for 2-3 hours with shaking, then collected by centrifugation and washed once in 1 x PBS.

2.15.2 Lysis of yeast cells

The cells were lysed mechanically, as described in section 2.05.2.

2.15.3 Collection of immune complexes

Serum (5µl) was added to the yeast lysate and incubated on ice for 60 minutes. Protein A beads (100µl) (10% vol/vol in lysis buffer) were added to the antibody-antigen reaction, and incubated at 4°C for 60 minutes with rocking. The beads were collected by centrifugation at 12 000 rpm for 15 seconds at 4°C, and washed three times with ice cold lysis buffer. As much of the lysate and wash buffers as possible were carefully removed. Laemmli sample buffer (Appendix 1) (50µl) was added to the beads, and the solution heated to 85°C for 10 minutes. The beads were

pelleted by centrifugation and the supernatant containing the immune complexes run on an SDS-PAGE gel overnight.

2.15.4 Fluorography

The gel was washed by soaking in water 2 x 5 minutes, and then soaked in 10 gel volumes of the scintillant, 1M sodium salicylate, for 30 minutes, or the commercially available scintillant, Entensify, solutions A and B (Dupont). The gel was then dried and exposed to X-ray film.

2.16 THE YEAST TWO-HYBRID SYSTEM

2.16.1 Construction of the cDNA library

Total RNA was extracted from *S. cerevisiae* grown in the absence of methionine, as described in section 2.04.3. The mRNA was isolated from total RNA as described in section 2.04.4, and was used to construct a cDNA library following the Clontech two-hybrid cDNA library construction kit. The synthesis of cDNA was monitored with [α-^{32}P]dCTP, and routinely gave counts per minute of 100 000 or higher, which is indicative of successful cDNA synthesis. However, the number of colonies appearing after transformation of the library into *E. coli* was routinely low (50 000 colonies).

2.16.2 Cloning *MET5* and *MET10* into yeast two-hybrid system vectors

The cloning steps used to insert *MET5* and *MET10* into the yeast two-hybrid system vectors are described in sections 2.18 and 2.19.

2.16.3 Co-transformation of *S. cerevisiae*

S. cerevisiae was co-transformed with the cDNA library and pAS*MET10* (library screen), or the pAS*MET5* and pACT*MET10* vectors (testing interaction between Met5p and Met10p), as described in section 2.02.

2.16.4 Interaction controls

Yeast two-hybrid system interaction controls were transformed into the same host strain of *S. cerevisiae* as the activation and binding domain vectors containing *MET5* and *MET10*. These controls confirm that the two-hybrid system is working properly. Positive controls were transformation with pCL1, which encodes full length, wild-type Gal4 protein, and co-transformation with pVA3-1 and pTD1-1 which are known to interact (MATCHMAKER GAL4 two-hybrid user manual, Clontech). The negative controls were co-transformation with pAS2-1 and pACT-2, pVA3-1 and pACT-2 and pTD1-1, and pLam5'-1 and pTD1-1.

2.16.5 Selection media for *S. cerevisiae* strains

Synthetic dropout media, with appropriate amino acids omitted, were used to select for transformants carrying the plasmid(s) of interest, or activation of the *HIS3* reporter gene when using *S. cerevisiae* strain Y190. Expression of the *HIS3* reporter gene is leaky in Y190, therefore the His3p inhibitor, 3-amino-1,2,4-triazole (3-AT) was included in the medium (25mM).

2.16.6 Identification of positives

The binding domain vector was 'cured' from the positive transformants by growth in the presence of leucine, and the vector containing the cDNA encoding the unknown interacting protein isolated from Y190.

2.16.7 β-galactosidase assay

Colonies which had been growing on selective medium for 7 days were used in the assay. A clean, dry Whatman #5 filter was laid over the surface of the agar plate containing the colonies to be assayed. Holes were poked through the filter paper and into the agar to enable the filter paper and the agar plate to be oriented. The filter paper was carefully lifted from the surface of the agar with tweezers and submerged in liquid nitrogen with the colonies facing up, for approximately 10 seconds. The filter paper was removed from the liquid nitrogen and placed into the lid of a petri dish where it was allowed to thaw at room temperature.

While the filter papers were thawing, Whatman #5 filters were presoaked in Z buffer/X-gal solution (Appendix 1). The Z buffer/X-gal solution was added to the base of a petri dish (5ml for 150mm plates, 2.5ml for 100mm plates), and a filter paper carefully laid over the top of the solution to soak it up, avoiding air bubbles. The thawed filter paper with the colonies was then carefully placed over the top of the pre-soaked filter paper, avoiding air bubbles, colony side up. The plates were sealed with parafilm and incubated at 30°C.

2.17 AMPLIFICATION OF *MET5* AND *MET10* FROM THE GENOME

The *MET5* gene was amplified from the genome with the primers MET5F and CMS3 (Table 2.01). These primers introduced a *Bgl* II site at both ends of the *MET5* gene. A PCR reaction mix (50µl) containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 4.0mM $MgCl_2$, 250mM of each dNTP, 10pmole of each primer and *Taq* DNA polymerase (0.5 units) (Advanced Biotech) was added to *S. cerevisiae* 90844 chromosomal DNA (0.5µg), and the reactions performed in a MJ-Research thermal cycler

Oligonucleotide	Sequence (5' – 3')
MET5F	AAC AAA AGA TCT GGC GAA TGA CTG C
CMS3	CCA ATA GAT CTG TCC TAC TAT GTC
MET10FB	CAA CGC CAT GGC AGT TGA GTT TGC
MET10RB	AAT GTT CAA GAC AGG ATC CAT AAA TAG
MET10XhoI	AGA TGC CAC TCG AGT TTG CT
CMS1*	CCT TTT GGC GAG GCC AAA AAT GCA ACT TC**G TGA TTC TGG GTA GAA GAT CGG**
CMS2*	CTA TAT TAA TTT AGT AGA CTT CTA AAA TG**T TGC TTC TTT TCT TAT CCG GGG**
G891893A	TTA TTA TGA GTG GTT TAG CTA CTG CTT TGG CAC CAT TCA AGG
G891893A complementary	CCT TGA ATG GTG CCA AAG CAG TAG CTA AAC CAC TCA TAA TAA
S820V	AAG AGA AGA GAA TAC GTG ATT GCC TCC TCT CAG AAA
S820V complementary	TTT CTG AGA GGA GGC AAT CAC GTA TTC TCT TCT CTT
S953K	ATC ACA CAC ATC GGC GCT GCT TTC AAA AGA GAC CAA CCT CAA AAA ATT
S953K complementary	AAT TTT TTG AGG TTG GTC TCT TTT GAA AGC AGC GCC GAT GTG TGT GAT
K959S	TCA AGA GAC CAA CCT CAA TCT ATT TAC ATT CAA GAT CGT ATC
K959S complementary	GAT ACG ATC TTG AAT GTA AAT AGA TTG AGG TTG GTC TCT TGA
C987A	AAT AAA GGT TCA TTT TAC TTG GCT GGC CCT ACT TGG CCA GTT
C987A complementary	AAC TGG CCA AGT AGG GGC AGC CAA GTA AAA TGA ACC TTT ATT
MET10 sequence	CCA ATC TCA TAA CCT TGC TGC

Table 2.01 Oligonucleotides used in PCR.

* Bold type indicates the region of the primer with homology to the *K. lactis* *URA3* gene.

programmed as follows: denaturation of DNA at 94°C for 4 minutes, followed by 30 cycles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 60°C and 3 minutes DNA extension at 72°C. The final chain extension was allowed to proceed for 10 minutes and then the tubes were cooled to 4°C.

The *MET10* gene was amplified from the genome, introducing a *Nco* I site at the 5' end of the gene, using the primers MET10FB and MET10RB (Table 2.01). A *Xho* I site was introduced at the 5' end of *MET10* using the primers MET10XhoI (Table 2.01) and MET10RB. The PCR reaction mix was as described above except the $MgCl_2$ concentration was 2.0mM. The thermal cycler was programmed as follows: denaturation of DNA at 94°C for 4 minutes, followed by 36 cycles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 55°C and 2 minutes DNA extension at 72°C. The final chain extension was allowed to proceed for 10 minutes and then the tubes were cooled to 4°C.

2.18 CLONING *MET5* INTO VECTORS

The amplified *MET5* gene was run on a 1% agarose gel, then gel purified using the Qiagen QIAquick gel extraction kit. The DNA was eluted from the column with 30µl of Tris-HCl. The purified PCR fragment was cloned into the pGEM-T vector (Promega), as described in the instruction manual provided by the manufacturer. The plasmid was called pGEM-*MET5* (Appendix 2).

The *MET5* gene was cut out of pGEM-*MET5* by digestion with *Bgl* II. The *Bgl* II fragment was gel purified with the Qiagen QIAquick gel extraction kit and cloned into the *BamH* I site of pAS2-1 (Clontech), resulting in pAS-*MET5* (Appendix 2).

2.19 CLONING *MET10* INTO VECTORS

The amplified *MET10* gene with the *Nco* I site introduced, was cloned into the pGEM-T vector (Promega) as described above. The plasmid was called pGEM-*MET10* (Appendix 2). Digestion with *Nco* I and *BamH* I excised *MET10* from this plasmid, and the fragment was cloned into p463 (Appendix 2) (kindly provided by Steve Dalton, Biochemistry Department, The University of Adelaide), which had also been digested with *Nco* I and *BamH* I. This plasmid was called pCS*MET10* (Appendix 2).

The *MET3* promoter and *MET10* gene was cut from the pCS*MET10* vector by digestion with *Sma* I followed by *Sac* I. This fragment was cloned into the pRS425 phagemid vector (ATCC 77106) which had also been digested with *Sma* I and *Sac* I, and the resulting plasmid called pRSP3M10 (Appendix 2).

The *met10* mutants were excised from pGEM-T by digestion with *Nco* I and *BamH* I, and gel purified using the Qiagen QIAquick gel purification kit. They were then cloned into pRSP3M10 which had been similarly digested to remove the wild-type copy of the gene already in this plasmid. The resulting plasmids were called pRS*met10*G891893A, pRS*met10*S820V, pRS*met10*S953K, pRS*met10*K959S and pRS*met10*C987A (Appendix 2).

The *Nco* I-*BamH* I *MET10* fragment was also cloned into pAS2-1 (Clontech) and pACT-2 (Clontech), which had both been digested with *Nco* I and *BamH* I. The resulting plasmids were called pAS*MET10* and pACT*MET10*, respectively (Appendix 2).

The amplified *MET10* gene with the *Xho* I site introduced, was cloned into the pGEM-T vector (Promega) as described above. The plasmid was called pGEM-*MET10X* (Appendix 2). The *MET10* gene was cut from

this plasmid by digestion with *Xho* I and *BamH* I, and cloned into pET-14b (Novagen) which had been similarly digested. The resulting plasmid was called pET*MET10* (Appendix 2).

2.20 DELETION OF *MET10* FROM *S. CEREVISIAE*

The method used for deletion of the *MET10* gene was targeted deletion by micro-homology mediated PCR (Manivaskam, *et.al.*, 1995; Langle-Rouault and Jacobs, 1995).

Oligonucleotides (52mers) were designed with 29bp homologous to the *MET10* gene of *S. cerevisiae* (Nucleotides 25 – 53, and 3090 – 3115) and 22bp homologous to the *URA3* gene of *Kluveromyces lactis* (CMS1 and CMS2, Table 2.01).

Chromosomal DNA from *Kluveromyces lactis* was isolated as described in section 2.04.1. The *K. lactis URA3* gene was amplified from the genome using the above primers. The PCR reaction mix was as described above except the $MgCl_2$ concentration was 2.0mM. The thermal cycler was programmed as follows: denaturation of DNA at 94°C for 4 minutes, followed by 30 cycles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 60°C and 3 minutes DNA extension at 72°C. The final chain extension was allowed to proceed for 10 minutes and then the tubes were cooled to 4°C.

The PCR product was run on a 1% agarose gel, then gel purified using the Qiagen QIAquick gel extraction kit. The DNA was eluted from the column with 30µl of Tris-HCl.

S. cerevisiae ATCC 90844 was then transformed with the amplified *K. lactis URA3* gene containing the *MET10* targeting 'tags' (30µl), as described in section 2.02. A control transformation of YCplac33

(*URA3*$^+$) was also included. The transformed cells were plated onto SD-Ura plates (Appendix 1), and incubated at 30°C for 5 days.

One half of the colony of a potential transformant was cracked as described in section 2.02.3, and analysed by PCR. The *URA3* primers were used to identify colonies containing the *K. lactis URA3* gene, and the *MET10* primers (Table 2.01) were used to determine that the *URA3* gene had disrupted the *S. cerevisiae MET10* gene.

2.21 SITE-DIRECTED MUTAGENESIS OF *MET10*

2.21.1 Megaprimer method

To introduce amino acid substitutions into *MET10*, the one-step polymerase chain reaction site-directed mutagensis method described by Ling and Robinson (1995) was attempted. The template used was pGEM*MET10* (100ng). The PCR profile was as described by Ling and Robinson (1995). Several different conditions were employed to amplify the full length product. Initially, the DNA polymerase, *Pwo* I was tried with 8, 24, 80, or 500ng of mutagenic primer. Other DNA polymerases used were *Vent* (New England Biolabs), *Tli* (Promega), and *Pfu* (Stratagene).

2.21.2 QuikChange site directed mutagenesis

Selected amino acids of Met10p were mutated using the Stratagene QuikChange Site-Directed Mutagenesis kit.

The template DNA used in the PCR was pGEM-*MET10* (10ng) which had been purified using the Qiagen QIAprep columns, as described in the miniprep handbook supplied by the manufacturer. The primers used to introduce the mutations were: G891893A, G891893A complementary,

S820V, S820V complementary, S953K, S953K complementary, K959S, K959S complementary, C987A, and C987A complementary (Table 2.01).

The mutagenic PCR was done as described in the QuikChange site-directed mutagenesis kit instruction manual, except that the extension time was changed to 3 minutes/kb of DNA. An aliquot of the PCR product was run on a 1% agarose gel to check that the PCR had worked. The remainder of the PCR product was digested with the *Dpn* I restriction enzyme, as instructed in the kit manual, and transformed into the Epicurian Coli XL1-Blue supercompetent cells provided with the kit.

Transformants were picked and grown in 10ml of LB containing 50μg/ml of ampicillin overnight. The plasmid DNA was purified using either the Qiagen QIAprep miniprep kit, or the Promega Wizard plus minipreps DNA purification kit. The DNA was sequenced with the *MET10* sequencing primer (Table 2.01) by the Nucleic Acid and Protein Chemistry Unit, The University of Adelaide, Waite campus.

CHAPTER THREE Mutagenesis and expression of *MET10* in *Saccharomyces cerevisiae*

3.01 INTRODUCTION

The enzyme sulfite reductase catalyses the six electron reduction of sulfite to sulfide, which is subsequently used in sulfur amino acid biosynthesis. The properties of the *Saccharomyces cerevisiae* enzyme have been well characterised, however little information on the structure/function relationships of the enzyme is available.

The *S. cerevisiae* NADPH dependent sulfite reductase is a heterotetramer, consisting of two α and two β subunits ($\alpha_2\beta_2$), with a holoenzyme molecular weight of 604 kDa. The α-subunit is encoded by the *MET10* gene and has a molecular weight of 116 kDa. The molecular mass of the β-subunit is 167 kDa (Kobayashi and Yoshimoto, 1982c) and is predicted to be encoded by *MET5* (Mountain, *et. al.*, 1991; Hansen, *et. al.*, 1994). Sulfite reductase has been demonstrated to be a hemoflavoprotein, binding two molecules of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Yoshimoto and Sato, 1968a), and two molecules of siroheme (Prabhakararao and Nicholas, 1969) per molecule of enzyme. The enzymes necessary for the synthesis of siroheme from uroporphyrinogen III have been shown to be encoded by *MET1* (uroporphyrinogen III transmethylase) and *MET8* (cobaltochelatase) (Hansen, *et. al.*, 1997; Raux, *et. al.*, 1999), which are essential for an active sulfite reductase (Thomas, *et. al.*, 1992). An iron sulfur cluster (Fe_4S_4), is another prosthetic group required for an active sulfite reductase.

The flow of electrons within sulfite reductase is from NADPH, which binds to the α-subunit, to the prosthetic group FAD, also bound on the α-subunit. Electrons are then transferred to FMN bound on the β-subunit. FMN mediates the transfer of electrons to the siroheme, where sulfite is reduced at the metal centre (Kobayashi and Yoshimoto, 1982b). The *S. cerevisiae* enzyme shows unusual instability in that it reversibly dissociates into αβ pairs at low ionic

strength, inactivating the enzyme due to a disruption of electron flow (Yoshimoto and Sato, 1970; Kobayashi and Yoshimoto, 1982a).

Whilst little information is available on *S. cerevisiae* sulfite reductase, the enzyme from *Escherichia coli* has been extensively characterised. Much structural information about the bacterial sulfite reductase has been gained through X-ray crystallography studies (McRee, *et. al.*, 1986; Crane, *et. al.*, 1995). The *E. coli* sulfite reductase is also an NADPH enzyme, and binds the prosthetic groups FAD, FMN, siroheme and Fe_4S_4. It too is made up of two different subunits, α and β, however the structure of the bacterial enzyme is $\alpha_8\beta_4$. *E. coli* sulfite reductase holoenzyme binds four molecules of FMN, FAD, siroheme and Fe_4S_4 per molecule of enzyme. The α-subunit (*cysJ*) is a 66 kDa flavoprotein, and binds NADPH, FAD and FMN. The β-subunit (*cysI*) is a 64 kDa hemoprotein, which binds the siroheme and the Fe_4S_4 cluster (Siegel, *et. al.*, 1982). The bacterial enzyme also differs from that of *S. cerevisiae,* in that it is possible to separate the enzyme into a functional flavoprotein component, and a functional hemoprotein component (Siegel, *et. al.*, 1974).

The mechanism of electron transfer within *E. coli* sulfite reductase is the same as that found in the yeast enzyme, from NADPH, to FAD and then FMN, followed by transfer to the siroheme and substrate. The bacterial enzyme differs from that found in *S. cerevisiae* in that it can be dissociated into functional flavoprotein and hemoprotein components (Siegel and Davis, 1974). Crystallographic structures of the *E.coli* sulfite reductase hemoprotein showed that a cysteine thiolate supplied by the protein covalently links the siroheme to the Fe_4S_4, facilitating their ability to transfer electrons to the substrate (Crane, *et.al.*, 1997).

The *S. cerevisiae* sulfite reductase is a member of the ferredoxin reductase family (Karplus, *et. al.*, 1991; Porter, 1991; Eschenbrenner, *et. al.*, 1995). Members of this family include ferredoxin reductase, cytochrome P450 reductase, *E. coli* sulfite reductase, cytochrome b_5 reductase and nitrate reductase. Although the overall homology between these enzymes is quite low, there are highly

conserved regions, which incorporate the cofactor binding sites. Therefore, a consensus sequence for cofactor binding can be generated (Figure 3.01).

To reduce the activity of sulfite reductase in *S. cerevisiae*, the dominant negative approach was chosen. To create a dominant negative mutation, the cloned gene must be altered such that the protein it encodes will inhibit the wild type gene product in a cell when over-expressed (Herskowitz, 1987). The cloned gene is most frequently altered in the substrate or cofactor binding sites (Appling, 1999). This approach has been used by many researchers to reduce the levels of an enzyme within a cell, and to study the function of genes (Fotedar, *et. al.*, 1996; Studamire, *et. al.*, 1998; Van Hoof, *et. al.*, 1998; Mao, *et. al.*, 1999; Scheibel, *et. al.*, 1999).

The dominant negative approach also enables sulfite reductase activity to be reduced in different wine yeast strains with fewer genetic manipulations, and without completely abolishing sulfite reductase activity. The approach used by Hansen and Kielland-Brandt (1996) to inactivate *MET10* by gene replacement in brewing yeast could not be applied to wine yeast as the methionine concentration in grape juice (1-5mg/L [Henschke and Jiranek, 1993]) is considerably lower than that found in wort (59mg/L [Garza-Ulloa, 1996]). To maintain yeast viability once the methionine in the medium has been used, the yeast must be able to synthesise methionine from organic sulfate. Deletion of the *MET10* gene disrupts this pathway, therefore the dominant negative approach was more appropriate to this situation. Amino acid substitution also enables protein structure/function relationships to be established.

By altering the predicted FAD or NADPH binding sites on the Met10 protein, the α subunit should still be able to bind to the β subunit, however the resulting enzyme will be inactive as one of the cofactors cannot bind to the α subunit. If this mutated Met10 protein is over-expressed in the cell, the altered α subunit will have the effect of 'mopping up' the functional, chromosomally encoded β subunit, and the majority of sulfite reductase formed will be inactive. A small proportion of the sulfite reductase will be active, as a functional, chromosomally

Figure 3.01 The FAD and NADPH consensus binding sequence. The absolutely conserved residues are highlighted in red, and the highly conserved amino acids are in bold type.

encoded α subunit is also produced (Figure 3.02). This active sulfite reductase will enable the cells to grow once methionine in the medium has been exhausted, without producing excess sulfide.

3.02 RESULTS

3.02.1 Modelling the 3-dimensional structure of Met10

The availability of X-ray crystallography data for some enzymes within the ferredoxin reductase family (Karplus, *et. al.*, 1991; Aliverti, *et. al.*, 1995; Crane, *et. al.*, 1995) enabled a model of the 3-dimensional structure of Met10p to be generated (Figure 3.03). Swiss-Model was the program used to generate the model, and RasMol and Swiss PDB viewer were used to view and manipulate the model. Only the last 400 amino acids (of 1035) of Met10p were modelled as the remainder of the protein did not show homology to any known structures in the Protein Database (Bernstein, *et. al.*, 1977; Berman, *et. al.*, 2000). However, the NADPH and FAD binding sites were within the modelled region.

3.02.2 Selection of amino acids to alter

Using the model generated (Figure 3.03) and the consensus FAD and NADPH binding sequence, amino acids were selected which were predicted to inhibit cofactor binding (Figure 3.04).

Serine820: The substitution of this amino acid could not be modelled as it was outside the region of homology enabling Met10p to be modelled. The equivalent serine in ferredoxin reductase (Ser96) has been shown by X-ray crystallography to interact through its amide nitrogen and the side chain, with the isoalloxazine ring of FAD (Karplus, *et. al.*, 1991). This contact stabilises the interaction between the isoalloxazine ring of FAD and the nicotinamide ring of NADPH as well as influencing the reduction properties of FAD (Aliverti. *et. al.*, 1995). The replacement of this amino acid with valine in ferredoxin reductase resulted in a mutant enzyme with only 0.05% of the wild type activity (Aliverti, *et. al.*, 1995). How valine exerted its effect remains unclear.

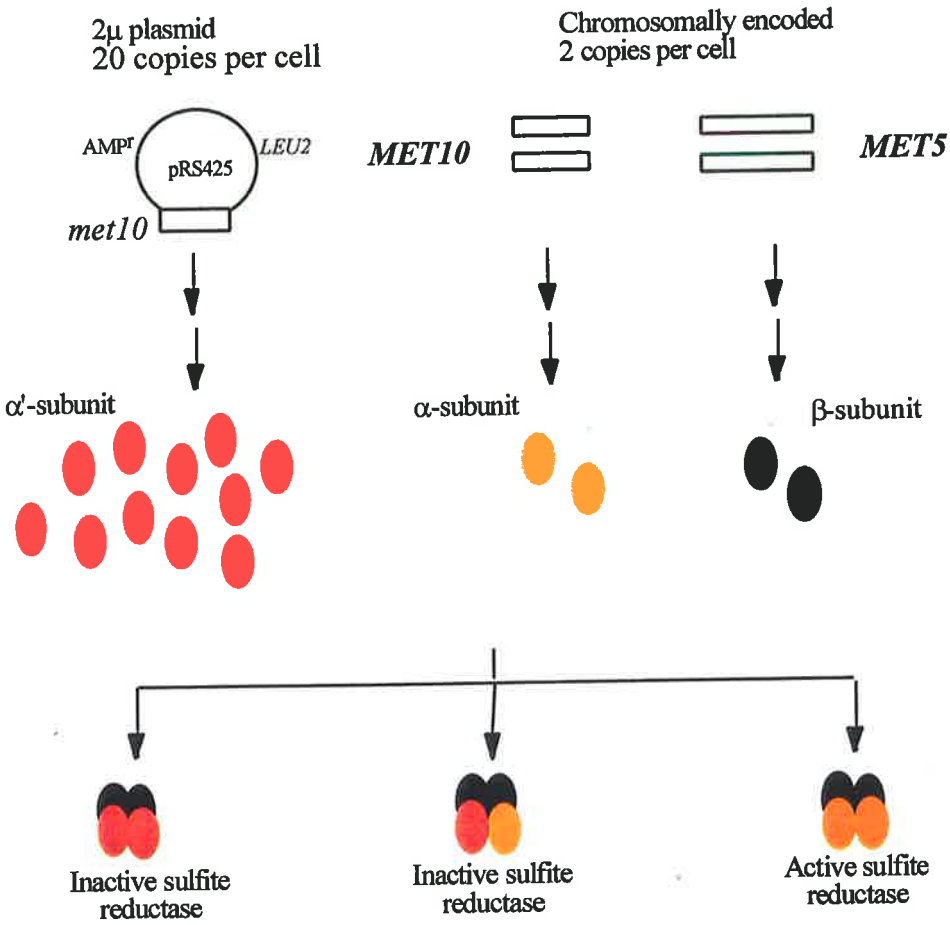


Figure 3.02 An outline of the dominant negative strategy. A multicopy vector containing a *met10* gene encoding a protein with altered cofactor binding sites is transformed into a strain of *Saccharomyces cerevisiae* with a *MET10* background. When over-expressed, the majority of α subunit which binds to chromosomally encoded β subunit will not bind one or more of the cofactors, making the enzyme inactive. Some of the enzyme formed will be active due to the functional, chromosomally encoded Met10p available, which will ensure that the cells can grow in the absence of methionine.

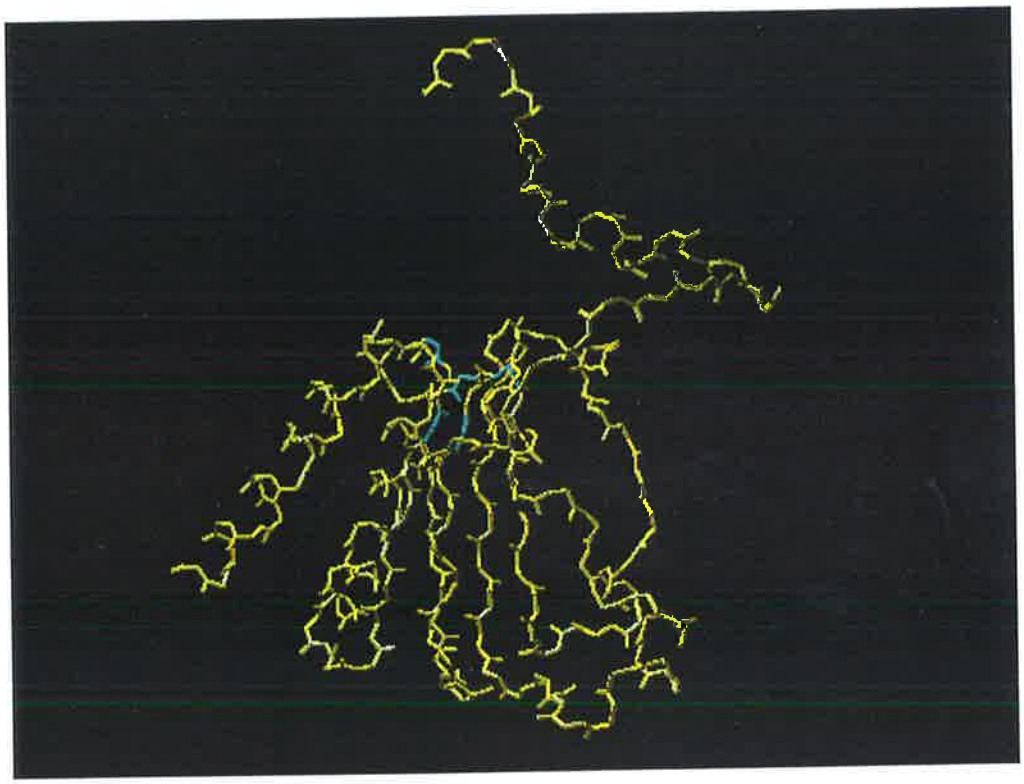


Figure 3.03 Truncated model of the three-dimensional structure of Met10p, generated using SwissModel. Highlighted in blue are the amino acids in the predicted NADPH binding region which were selected to be altered by site directed mutagenesis.

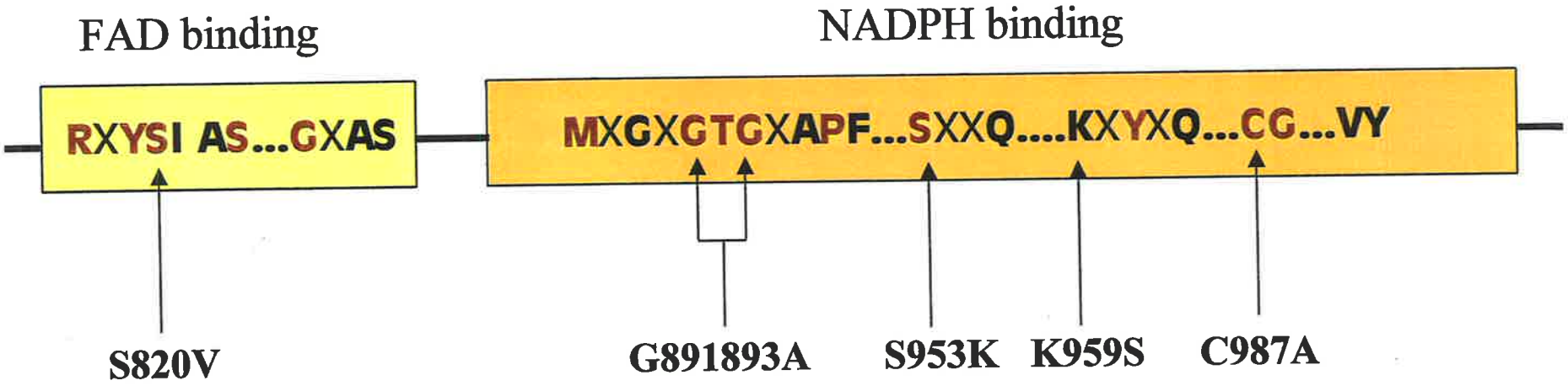
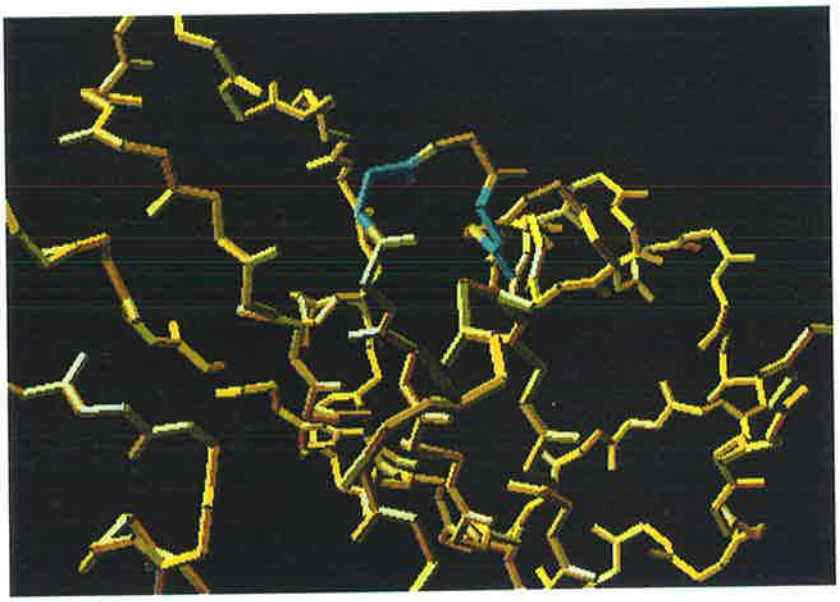


Figure 3.04 The FAD and NADPH consensus binding sequence. The absolutely conserved residues are highlighted in red, and the highly conserved amino acids are in bold type. Arrows indicate the amino acids selected to be substituted. The substitution of these residues is described in the text.

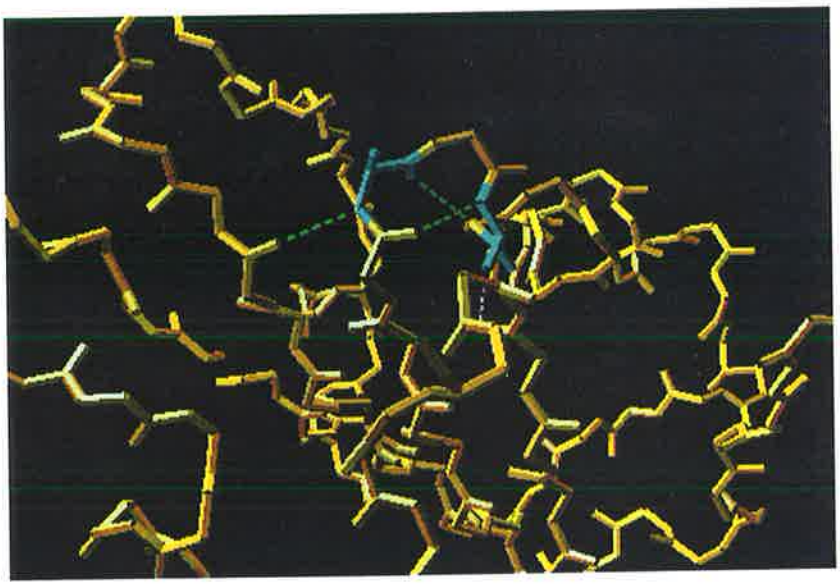
Glycine891 and Glycine893: Most enzymes which utilise the dinucleotides NAD or NADP comprise a glycine-rich loop segment, the GXGXXG motif of a Rossmann fold, which binds the cofactors diphosphate moiety (Chu and Hwang, 1998). The Rossmann fold (βαβ) is a highly similar structure between enzymes, however the sequences may be very dissimilar (Rossmann, *et. al.*, 1974; Wierenga, *et. al.*, 1985). This structure, also called a dinucleotide binding motif, has a symmetrical α/β structure, made up of a six-stranded parallel-pleated sheet and four helices (Creighton, 1984), which are on both sides of the sheet (Branden and Tooze, 1991). The first glycine of the motif is absolutely conserved in all dinucleotide binding enzymes, allowing for a tight turn of the main chain with special (ϕ,ψ) angles. The second glycine of the motif is conserved since steric interference with a bound dinucleotide would occur with a side chain in this position (Wierenga, *et. al.*, 1985). The third glycine may be replaced with an alanine (GXGXXA) in enzymes in which hydrogen bonding to the adenine ribose occurs by a direct mechanism (Baker, *et. al.*, 1992). Recognition of adenine ribose by enzymes containing the GXGXXG motif occurs indirectly via an acidic residue.

The GXGXXG motif can vary in several families of enzymes. The aldehyde dehydrogenases contain the GXXXXG motif (Vedadi, *et. al.*, 1997), and glutamate dehydrogenase from *Psychrobacter* sp. has serine in the last position of the motif (GXGXXS) (Di Fraia, *et. al.*, 2000). *E. coli* quinone oxidoreductase has the nucleotide binding motif, AXXGXXG (Thorn, *et. al.*, 1995), and this motif is GXGXXV in pyridine nucleotide transhydrogenase from *E. coli* (Bragg, *et. al.*, 1997). In the ferredoxin reductase family, the dinucleotide binding motif is GXGXXP (Figure 3.05).

The glycines in the GXXXXG motif of *Vibrio harveyi* aldehyde dehydrogenase were mutated to alanine, and the effect on enzyme activity measured (Vedadi, *et. al.*, 1997). Replacement of the first glycine with alanine prevented NADPH binding, and the replacement of the second glycine in the motif resulted in a three-fold reduction in enzyme activity. Based on these results and the



Met10



Met10 G891893A

Figure 3.05 The SwissModel program was used to model the glycine to alanine amino acid substitutions in the predicted NADPH binding pocket. The amino acid substitutions are highlighted in blue. Green dashed lines represent hydrogen bonds, and purple dashed lines indicate steric interference.

conservation of the motif within dinucleotide binding enzymes, the glycines in the GXGXXP motif of the ferredoxin reductase family were substituted with alanine (Figure 3.06).

Serine953: This serine is highly conserved in this family of enzymes and functions in the binding of NADPH by hydrogen bonding. The side chain of the equivalent serine (Ser234) in ferredoxin reductase forms a strong hydrogen bond with the 2'-phosphate of NADPH (Karplus, *et. al.*, 1991). The conservation of this residue within the ferredoxin reductase family suggests that Ser953 has an identical function in sulfite reductase. The side chain of serine is small with a hydroxyl group able to function as both a hydrogen donor and an acceptor in hydrogen bonding (Creighton, 1983). Replacement of serine with lysine is expected to disrupt hydrogen bonding due to its positive charge (Creighton. 1983; Branden and Tooze, 1991) (Figure 3.07). Steric interference caused by the replacement of a small amino acid with a residue containing a long side chain may further disrupt the interaction, since optimal hydrogen bonds are stronger than bent hydrogen bonds (Creighton, 1983), resulting in instability of NADPH binding to the α subunit.

Lysine959: The lysine residue 244 in spinach ferredoxin reductase is equivalent to lysine959 and has been proposed to interact with NADPH, binding the 5'- and 2'-phosphates (Karplus, *et. al.*, 1991). The interaction is thought to be a polar interaction between the positively charged side chain of lysine and the oxygen atoms of phosphate in NADPH (Karplus, *et. al.*, 1991). The separation distance is 4 Å, therefore the interaction may be weak, however the side chains move towards the ligand during binding (Karplus, *et. al.*, 1991). If this interaction occurs as predicted by X-ray crystallography, substitution of Lys959 with the small polar amino acid, serine should disrupt this interaction as the electrostatic forces will no longer operate and the smaller side chain increases the distance between the side chain and the phosphate (Figure 3.08). Since the altered α subunit must still be able to bind to the β subunit of sulfite reductase to function as a dominant negative, the overall conformation of the enzyme cannot be altered by the amino acid substitution. The substitution of lysine for serine in several

Ferredoxin-NADP$^+$ reductase

	167	
Spinach	..MLGTGTGIAPF..	
Spirulina maxima	..MMATGTGIAPF..	

NADPH-cytochrome P450 reductase

	529	
Human	..MVGPGTGVAPF..	
Yeast	..MIGPGTGVAPF..	
Bacillus megaterium	..MVGPGTGVAPF..	

NADPH-sulfite reductase

	457	
E.coli	..MIGPGTGIAPF..	

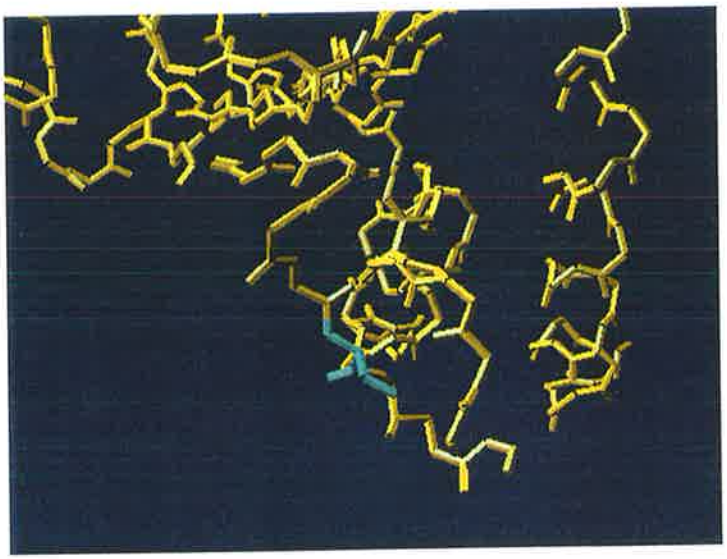
NADH-cytochrome b_5 reductase

	176	
Human	..MIAGGTGITPM..	

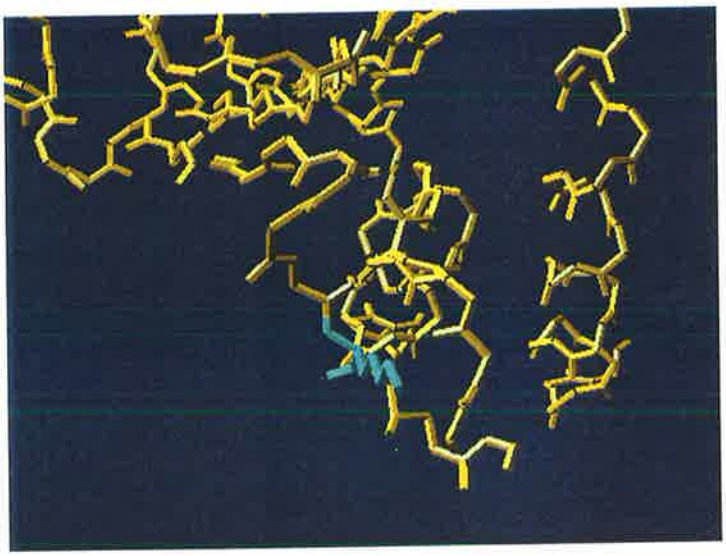
NADH-nitrate reductase

	781	
Arabidopsis thaliana	..MLAGGTGITPV..	
Met10	887	
S.cerevisiae	..MSGLGTGLAPF..	

Figure 3.06 The dinucleotide binding motif found in the ferredoxin reductase family. The motif is highlighted in red.

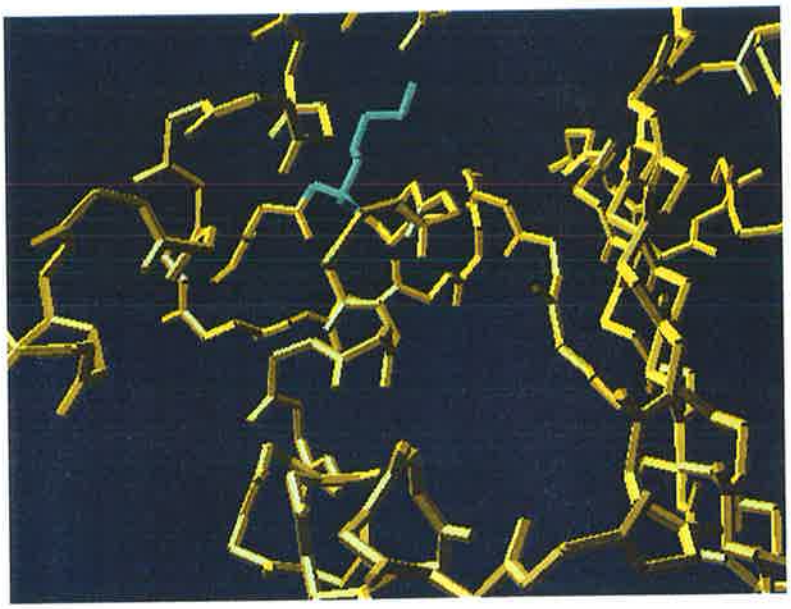


Met10

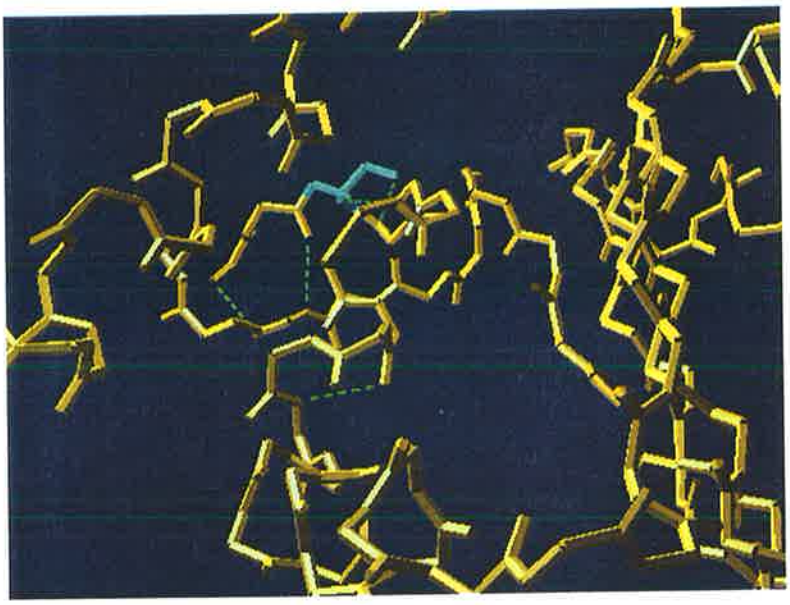


Met10 S953K

Figure 3.07 The SwissModel program was used to model the serine to lysine amino acid substitution in the predicted NADPH binding site. The amino acid substituted, is highlighted in blue.



Met10



Met10 K959S

Figure 3.08 The SwissModel program was used to model the lysine to serine amino acid substitution in the predicted NADPH binding site. The amino acid substituted, is highlighted in blue. Dashed green lines represent hydrogen bonds.

studies has not caused gross conformational changes to the enzymes (Hartman, *et. al.*, 1987; Soper, *et. al.*, 1988).

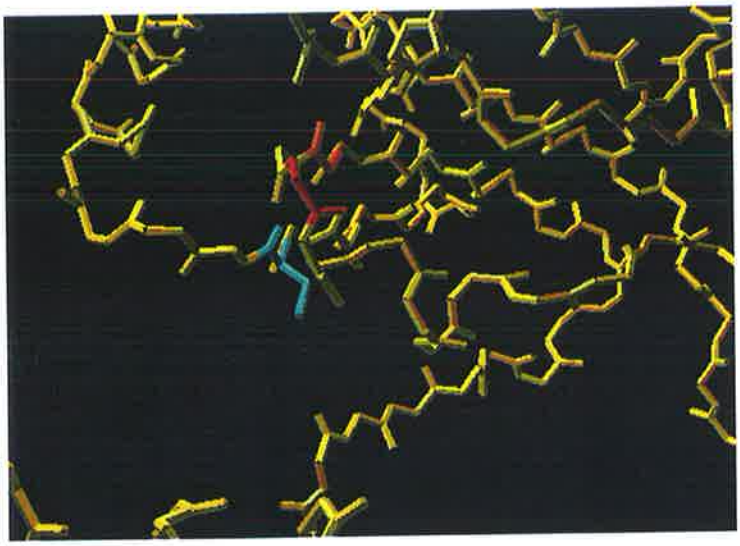
Cysteine987: This cysteine residue is conserved in all members of the ferredoxin reductase family, and is one of only two cysteine residues in Met10p. It has been shown to function in the binding of NADPH such that it can effectively interact with FAD (Aliverti, *et. al.*, 1993, Pollegioni, *et. al.*, 1997), positioning the two rings of NADPH and FAD to facilitate hydride transfer between C_4 of the nicotinamide and N_5 of the isoalloxazine. This amino acid was substituted with alanine (Figure 3.09) which should maintain the quaternary structure of the enzyme while impeding hydride transfer (Tai, *et. al.*, 1998; Iwaoka, *et. al.*, 1999; Wei and Swiedler, 1999).

3.02.3 Site directed mutagenesis – the 'megaprimer' method

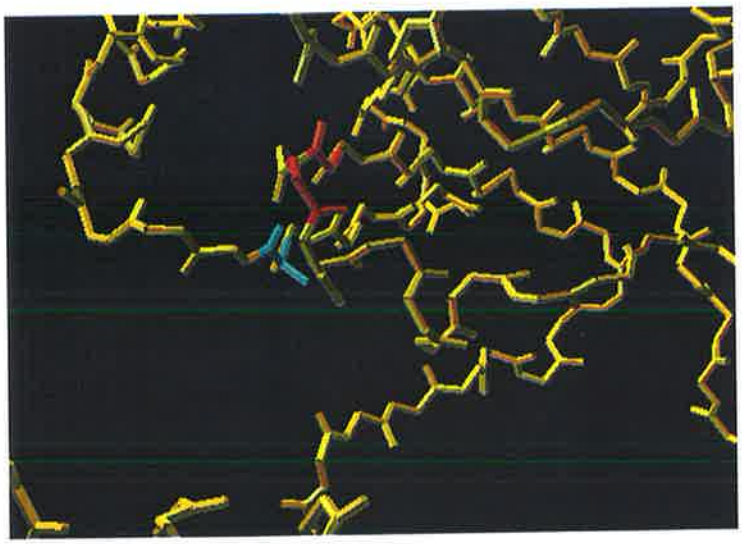
The method selected to introduce the amino acid substitutions was an adaptation of the 'megaprimer' method (Sarkar and Sommer, 1990) described by Ling and Robinson (1995) which is a one step, three stage PCR method for site directed mutagenesis, with an intermediate megaprimer product.

To establish the method and adjust PCR conditions, a single amino acid substitution was selected to work with initially (K959R). The primer was designed such that the mismatches were in the centre of the oligonucleotide, and the codon usage of *S. cerevisiae* was preserved (Table 2.01, chapter two). The one step, three stage PCR conditions were as described in materials and methods. Thermostable DNA polymerases with 3'-5' exonuclease activity ('proof-reading') were used to minimise the incorporation of additional, undesired mutations in the PCR product.

Several different conditions were employed to amplify the full length product, however only the megaprimer was able to be amplified (Figure 3.10).



Met10



Met10 C987A

Figure 3.09 The SwissModel program was used to model the cysteine to alanine amino acid substitution in Met10p. The amino acid substituted, is highlighted in blue. The proximity to the tight glycine turn required for the 2'-phosphate of NADPH to bind (residues highlighted in red) is shown.

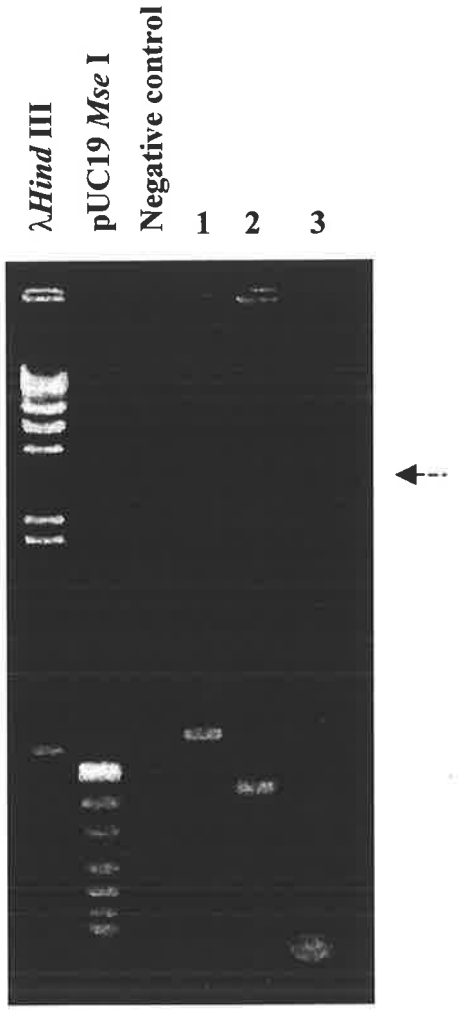


Figure 3.10 Megaprimer PCR using the K959R mutagenic primer. Only non-specific low molecular weight products were seen when 80ng or 100ng of the K959R primer was included in the reaction (Lanes 1 and 2). When 500ng of K959R was used, only the megaprimer was formed in the reaction (Lane 3). An arrow indicates where the full length product would be expected to be a seen on a gel.

3.02.4 Site directed mutagenesis – oligonucleotide extension technique

An alternative method to the 'megaprimer' method for site directed mutagenesis is an oligonucleotide extension technique (Stratagene). Purified plasmid DNA (pGEM-*MET10*) (5 – 50 ng) was used as the template with the site directed mutagenesis kit. The primers were designed such that both mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid. The oligonucleotides were 36 - 48 bp in length (Table 2.01, Chapter 2). Efficiency of mutagenesis for all of the substitutions was between 75 – 100% (Figure 3.11).

3.02.5 Effect of expression of *met10* genes in *Saccharomyces cerevisiae*

To study the effects of the amino acid substitutions in Met10p, and to test if the altered α subunits could function as a dominant negative in *Saccharomyces cerevisiae*, the mutated *met10* genes were expressed from a multicopy plasmid under the control of an inducible promoter. The plasmid selected was pRS425 (ATCC 77106) (Christianson, *et. al.*, 1992), a 2μm, high copy number (approximately 20 per cell) yeast shuttle vector. The *met10* genes were cloned into this vector in front of the inducible *MET3* promoter. This promoter was selected as it is regulated in the same manner as the other genes of the methionine biosynthetic pathway, with induction in the absence of methionine (Cherest, *et. al.*, 1985; Black, *et. al.*, 1995). Since the dominant negative effect in wine yeast would only be required under winemaking conditions where methionine was no longer available, the *MET3* promoter is an ideal choice.

3.02.5.1 Viability of dominant negative mutants in the absence of methionine

To function as a dominant negative, *S. cerevisiae* transformed with the mutated *met10* constructs must be viable in the absence of methionine. To test this and to determine if any phenotype due to the mutations could be detected, such as slow growth in the absence of methionine, *S. cerevisiae* 90844 transformed with the

G891893A

CCCCAATGGC CTTGAATGGT GCCAA**AGC**AG T**AGC**TAAACC ACTCATAATA
ACTGGTTGCT TTGGAGATGG TGGTAATTTC ATAACAGATG GTTTAACGCT
AACGACCAAT TCTGAACCGA CAGCAAGGTC TGAGATATAC TTAGAAGCTT
GACCGTACCT TTTTCTTCCT TTATTATCCA CCCAATCAAC AACAACGATC
AATAAATGAA CTTCATTTGG ATGAACTTTC TGAGA

S820V

TCATAACAGA TGGTTTAACG CTAACGACCA ATTCTGAACC GACAGCAAGG
TCTGAGATAT ACTTAGAAGC TTGACCGTAC CTTTTTCTTC CTTTATTATC
CACCCAATCA ACAACAACGA TCAATAAATG AACTTCATTT GGATGAACTT
TCTGAGAGGA GGCAAT**CAC**G TATTCTCTTC TCTTCAATGG TTCAATGATA
GTAACAAGTT CCTCAAGAGA TGGGCGAACA GATGGGAACA ATTCAAAAAT
GTCAGCATAT GTTATAATAC TCCACATCTT GAAATCTCTT CCAAATCTAC
TGCACCGGCA GGAATTAACC

S953K

TAGGGCCACA CAGGTAAAAT GAACCTTTAT TATCAATCAT TGCAGTTTTT
AATTCATCCA AATTCTCTTT GATACNATCT TGAATGTAAA TTTTTTGAGG
TTGGTCTCT**T** **TT**GAAAGCAG CGCCGATGTG TGTGATAATA CCTGCATCTT
TGTAAGCCTC CCATAACTCA CCACATAAAT ATNCTTCTCT TTTGTGTCTT
GAACCTAAAT ATAGGAA

K595S

TGGAACTGGC CAANTAGGGC CACACAGGTA AAATGAACCT TTATTATCAA
TCATTGCAGT TTTTAATTCA TCCAAATTCT CTTTGATACG ATCTTGAATG
TAAAT**AGA**TT GAGGTTGGTC TCTTGAAAAA GCANCGCCGA TGTGTGTGAT
AATACCTGCA TCTTTGTAAG CCTCCCATAA CTCACCACAT AAATATCTTC
TCTTTGTGTT CTTGACCTA

C987A

GCTTCCTTTA ATTCTTCAAT TGCGGCATCC AANTCNACTT TGATGCCTCT
TTCCTCGGCG TCTTTAGCCA NAATGTCTTG CAAACCTTGA GTAATATCTG
GAACTGGCCA AGTAGGGCC**A** **GC**CAAGTAAA ATGAACCTTT ATTATCAATC
ACTGCAGTTT TTAATTCATC CAAATTCTCT TTGATACGAT CTTGAATGTA
AATTTTTTGA GGTTGGTCTC TTGAAAAAGC AGCGCC

Figure 3.11 DNA sequence of the *met10* genes with the mutations introducing the amino acid substitutions (highlighted in red). The reverse complement sequence is shown.

met10 constructs were replica plated onto synthetic medium with and without methionine.

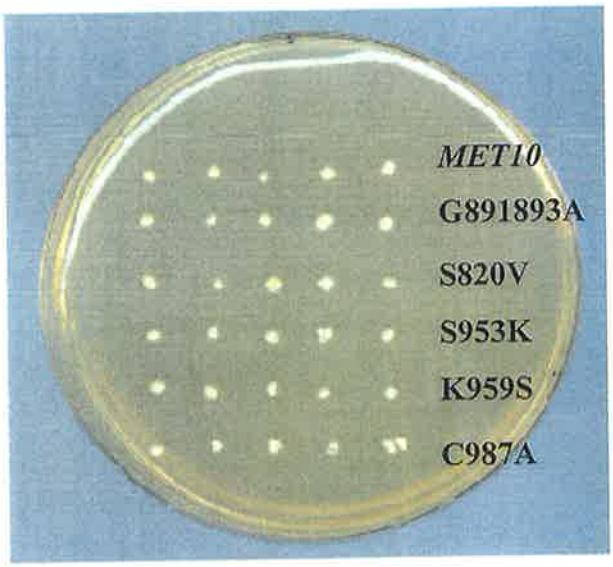
The growth of the mutants was the same in the presence or absence of methionine (Figure 3.12), and no difference in growth characteristics from the wild-type parent strain could be detected.

3.02.5.2 Sulfite reductase measurements

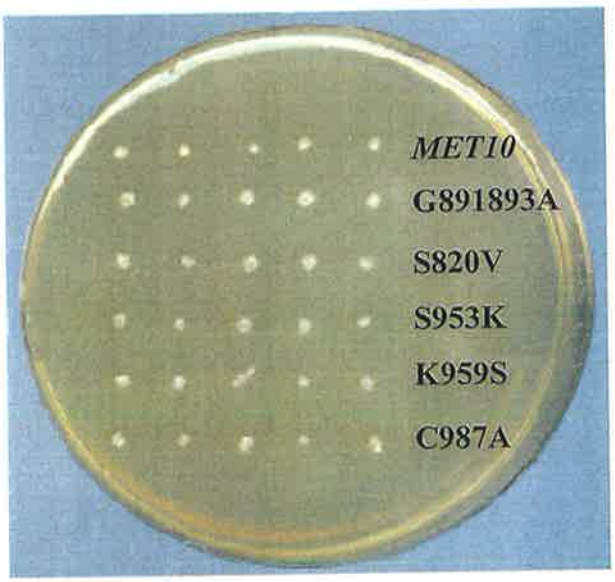
To determine if the mutants were functioning as dominant negatives, sulfite reductase activity in the absence of methionine was measured (Jiranek, 1992; Jiranek, *et. al.*, 1996). An enzymatic method was used to lyse cells, since using the homogenisation method (Jiranek, *et. al.*, 1996) resulted in no enzyme activity being detected. Homogenisation may have inactivated the enzyme, possibly due to heat generated using this method. Protease inhibitors were included in extraction buffers, however this did not enable any active enzyme to be detected, indicating that protease degradation was not the cause of the problem. Variations in the recovery of cell lysates using the homogenisation method was also a concern. Attempts to optimise the assay with the control strains (ΔM10-1 and 90844) and two mutants [90844(pRS*met10*G891893A) and 90844(pRS*met10*S820V], proved unsuccessful with considerable variation between replica samples measured. Therefore alternative methods of estimating sulfite reductase activity were investigated.

3.02.5.3 Determination of sulfide accumulation by growth on indicator medium

Yeast strains overproducing the mutant *met10* genes, and the control strains, 90844 and ΔM10-1, were plated onto two types of indicator media, BiGGY and Pb^{2+} medium. Neither of these methods were found to be conclusive as nutrient rich medium had to be used, which may have resulted in plasmid loss. Dropout lead medium is not feasible since phosphates and sulfates in YNB precipitate when the lead is added (Gregory, 1999). A precipitate was also seen when bismuth sulfite was added to dropout medium. The negative control strain,



+Methionine



-Methionine

Figure 3.12 Comparison of *S. cerevisiae* 90844 transformed with pRS*MET10*, pRS*met10*G891893A, pRS*met10*S820V, pRS*met10*S953K, pRS*met10*S953K, pRS*met10*K959S, and pRS*met10*C987A on media with and without methionine. No obvious growth phenotype could be detected.

ΔM10-1, remained white on BiGGY and Pb^{2+} medium, indicating no sulfide production as expected. However, the 90844 positive control strain was not a high sulfide producing strain, making comparisons between this strain and those with the *met10* genes difficult.

3.02.5.4 Determination of sulfite accumulation

An alternative method to estimate the activity of sulfite reductase is to measure the production of sulfite (Hansen, *et. al.*, 1994; Hansen and Kielland-Brandt, 1996). Strains with an inactive sulfite reductase cannot reduce sulfite to sulfide, therefore sulfite accumulates and is able to diffuse from the cell at high concentrations, as well as being exported from the cell as part of a stress response (Avram and Bakalinsky, 1996).

The pararosanilin method (Grant, 1947) was adapted (Hansen, *et. al.*, 1994; J. Hansen, personal communication) for determination of sulfite produced by the control strains, 90844 and ΔM10-1, and 90844 over-expressing the *MET10* and *met10* genes. ΔM10-1 was found to accumulate 5mg of $SO_2/10^{10}$ cells, however no sulfite was detected for the other strains. The possibility that sulfite was accumulating in these strains, but could not be detected by this method was further investigated.

3.02.5.5 Determination of SO_2 accumulation by gas chromatography

A more sensitive method to determine sulfite concentration is available which utilises a gas chromatograph, a headspace sampler and a Sievers chemiluminescence detector (Lowe and Dreyer, 1997). Some of this equipment was not available therefore the method was adapted to a GC (Hewlett-Packard 6890 GC) and a solid phase micro-extraction (SPME) portable field sampler (Supelco, Bellefonte, USA) fitted with a 100 μm polydimethylsiloxane (PMDS) fibre assembly (Supelco, Bellefonte, USA). The method was not very precise, due to the high volatility of SO_2 and the differences in affinity between SO_2 and DMDS (the internal standard) for the SPME fibre. Perhaps a better method for

future investigations would be to use the above extraction method, except using static headspace with a gas-tight syringe (eg. 25 μL), rather than SPME. Despite these difficulties the results indicated (Table 3.01) that 90844 over-expressing the *met10* genes were not accumulating any sulfite.

These results were confirmed externally by the established and more sensitive method of SO_2 analysis by headspace gas chromatography and a chemiluminescence sulfur detector. Only the ΔM10-1 control strain could be shown to accumulate any sulfite by this method (8ppm). The 90844 control strain and 90844 over-expressing each *met10* mutant gene did not accumulate any sulfite.

3.03 DISCUSSION

The introduction of mutations into proteins is important to understand the relationship between structure and function. Selected amino acids have been modified within the α subunit of sulfite reductase using a PCR oligonucleotide extension technique, with the aim of preventing the cofactors, NADPH or FAD binding to the α subunit whilst maintaining both the three-dimensional structure of Met10p and its ability to bind to the β subunit. This dominant negative approach may reduce the levels of active sulfite reductase in *S. cerevisiae*.

3.03.1 Site-directed mutagenesis

The first attempt to alter amino acids in Met10p using the megaprimer method of site-directed mutagenesis did not prove to be successful. Why the megaprimer only and not the full length product could be synthesised using this method is unclear, however other researchers have also had trouble with extension of the megaprimer (Upender, *et. al.*, 1995). It has been reported that when the megaprimer is very large (> 300 bases), inefficient priming of template DNA by the megaprimer occurs due to self annealing primer strands (Smith and Klugman, 1997).

Yeast strain	SO_2 (ppm)
ΔM10-1	5.03 ± 2.50
90844	0.32 ± 0.04
90844 (pRS*met10*G891893A)	0.37 ± 0.01
90844 (pRS*met10*S820V)	0.45 ± 0.08

Table 3.01 Accumulation of sulfite by strains of *S. cerevisiae* as determined by GC (Hewlett-Packard 6890 GC) and a solid phase micro-extraction (SPME) portable field sampler (Supelco, Bellefonte, USA) fitted with a 100 μm polydimethylsiloxane (PMDS) fibre assembly (Supelco, Bellefonte, USA. The results indicated that the strains expressing the altered *met10* genes were not functioning as dominant negatives.

The concentration of the megaprimer has been shown to be a critical factor in the success of the PCR, with a high ratio of megaprimer to template DNA producing the best results. Smith and Klugman (1997 did not see full length product until 2μg of megaprimer was used in the reaction, and the best result was achieved when 6μg of megaprimer was included. It is likely that insufficient megaprimer was formed in the initial stage of the mutagenic PCR, making the amplification of the *MET10* genes encoding the amino acid substitutions improbable. The use of an alternative PCR-based method did result in efficient mutagenesis of the *MET10* gene.

3.03.2 The effect of amino acid substitution in Met10p on sulfite reductase activity in *S. cerevisiae* 90844

Several different methods were used to analyse the effect the amino acid substitutions in Met10p may have on the activity of sulfite reductase. The aim of this work was to produce a strain with a dominant negative mutation such that sulfite reductase activity was reduced in a *MET10* wild-type background, yet growth rates in the absence of methionine remain at wild type levels. A decrease in the rate of growth of *S. cerevisiae* 90844 expressing an altered Met10p would have been interesting in terms of structure-function relationships of sulfite reductase, however no growth phenotype could be detected in any of the 90844 transformants expressing altered Met10p in the absence of methionine. Whilst wild-type growth rates were the long-term goal, the results gave no indication whether the dominant negative approach may be working, or if the sulfite reductase activity supporting growth in the absence of methionine was from chromosomally encoded sulfite reductase only.

To further study the effects of the amino acid substitutions in Met10p, the yeast expressing *met10* genes were grown on indicator media that select colonies producing hydrogen sulfide, and therefore have an active sulfite reductase. Sulfite reductase activity and sulfite accumulation were also examined.

The use of a bismuth sulfite containing media is effective for the selection of hydrogen sulfide producing yeast, with sulfide producing strains appearing as

brown to near-black colonies on solid medium. The growth of yeast strains on bismuth sulfite containing media has been reported to give a visual measure of the genetically determined maximal activity of sulfite reductase, and therefore its potential to produce hydrogen sulfide, of any given strain (Jiranek, *et. al.*, 1995).

Lead ions can also be used as a marker for hydrogen sulfide production, which is the basis behind the use of the *MET15* gene (also called *MET25*) as a marker gene in molecular biology and genetic applications (Smith and Boeke, 1997). Yeast which are *met15* overproduce hydrosulfide ions which leak from the cell, and are able to combine with lead ions included in the culture medium, forming a dark brown/black precipitate (Ono, *et. al.*, 1991; Cost and Boeke, 1996). Yeast with an active sulfite reductase may accumulate sulfide also, therefore growth on a medium containing lead ions can identify sulfide accumulating colonies by their colour. This method has been used to identify sulfide producing wine yeast by oenologists (Rupela and Tauro, 1984; Zambonelli, *et. al.*, 1984). Neither of these methods were conclusive, possibly due to these media being non-selective for the plasmids containing the *met10* genes, potentially resulting in plasmid loss.

Attempts to measure the activity of sulfite reductase using the assay described were not successful. The variations in sulfite reductase activity measured in replica samples may be due to cold and heat sensitivity of the enzyme (Jiranek, *et. al.*, 1996), low ionic strength sensitivity (Yoshimoto and Sato, 1970; Kobayashi and Yoshimoto, 1982b and c), or oxidation of the enzyme (Bauer, *et. al.*, 1999).

Since the activity of sulfite reductase could not be reliably measured, an indirect approach was attempted. Sulfite reductase is responsible for the reduction of sulfite to sulfide, therefore an impairment in the enzyme will result in the accumulation of sulfite if the cells are grown under conditions in which the sulfate assimilation pathway is on. Several different methods were attempted to measure sulfite accumulation, since the pararosanilin method initially used could only detect sulfite in *S. cerevisiae* ΔM10-1. This may indicate that either the mutations in the *met10* gene were not having an effect and sulfite reductase was

still functional, the dominant negative approach was not working, or the assay was not sensitive enough to detect the quantity of sulfite accumulated by 90844 and 90844 expressing mutated *met10*. The indicator media had shown that 90844 did not accumulate high levels of sulfide, therefore the amount of sulfite produced may not be detected using the pararosanilin method.

Gas chromatography is a sensitive method to detect and quantitate compounds. The established method to detect sulfur dioxide required the use of equipment which was not available, therefore an adaptation of this method was tried using a solid phase micro-extraction (SPME) portable field sampler (Supelco, Bellefonte, USA) fitted with a 100 μm polydimethylsiloxane (PMDS) fibre assembly. However SO_2 is volatile and did not bind to the fibre well, producing variable results. Finally, the samples were able to be analysed externally using the established method, the results confirming that no sulfite was accumulated in 90844 or 90844 expressing the *met10* genes.

3.03.3 Conclusions

A lack of sulfite accumulation by yeast over-expressing *met10* genes implies that sulfite reductase in these strains is fully functional. This may be due to a number of reasons, the first being the amino acid residues selected in Met10p are not essential and their substitution for another amino acid had no effect on enzyme function. Another possible reason is that the mutations have altered the conformation of the α subunit such that it can no longer bind to the β subunit, and the sulfite reductase activity is therefore identical to the wild-type, 90844 level. Alternatively, the mutated α subunits still bind to the β subunit, but retain some level of activity. Finally, the altered *met10* genes may not be over expressed at a sufficient level to sequester enough of the β subunit to produce the dominant negative phenotype. These possibilities were investigated in further experiments.

CHAPTER FOUR Identification of proteins that interact with Met10p

4.01 INTRODUCTION

Sulfite reductase from *Saccharomyces cerevisiae* is a heterotetramer with an $\alpha_2\beta_2$ structure (Kobayashi and Yoshimoto, 1982a). The genes *MET1, MET5, MET8, MET10, MET18* and *MET20*, have all been shown to be required for functional sulfite reductase (Thomas, *et. al.*, 1992), yet only the product of the *MET10* gene (α subunit) had been assigned a function at the time of starting this project (Hansen, *et. al.*, 1994; Hosseini-Mazinani, *et. al.*, 1995).

The β subunit binds FMN and siroheme (Yoshimoto and Sato, 1968b), and was predicted to be encoded by the *MET5* gene (YJR137c) (Mountain, *et. al.*, 1991; Hansen, *et. al.*, 1994), yet no conclusive evidence that *MET5* is the β subunit has been reported. A *met5* mutant has been shown to be devoid of sulfite reductase activity and accumulate sulfite *in vivo* (Thomas, *et. al.*, 1992), demostrating that functional sulfite reductase is dependent on an intact *MET5* gene.

The *MET5* transcript is 5.5kb in length (Mountain, *et. al.*, 1991), which could encode a 167kDa protein, agreeing with the predicted molecular weight of the sulfite reductase β subunit (Kobayashi and Yoshimoto, 1982). A protein similarity search revealed significant homology to the *E. coli* sulfite reductase hemoprotein, and NADPH cytochrome P450 reductase (Figure 4.01), and a search for functional motifs in Met5p revealed iron-sulfur/siroheme binding motifs within YJR137c (Figure 4.02). The motif found in residues 1343 – 1359 of YJR137c can also be found in the *E. coli* sulfite reductase hemoprotein, where it has been shown to wrap tightly around the Fe_4S_4 cubane. Therefore, Cys1349 in YJR137c may be the counterpart of the bridging cysteine (Cys483) between Fe_4S_4 and siroheme found in the *E. coli* protein.

```
 59  LSSLPEIDKSLVVFCMATYGEGDPTDNAQDFYDWLQ-ETDVDLTGVKFAVFGLGNKTY- 115
     L  LP  +   VVF  +T G+G+   + + F++ L+ +TD+DL  +  AVFGLG+  Y
721  LEELPGEEN-VVFITSTAGQGEFPQDGKSFWEALKNDTDLDLASLNVAVFGLGDSEYWP 778

116  ----EH-FNAMGKYVDQRLEQLGAQRIFELGLGDD---DGNLEEDFITWREQFWPAV  164
        +H FN   + + +RLE L A+ +  LGLGDD   DG  +  +  W  + W A+
779  RKEDKHYFNKPSQDLFKRLELLSAKALIPLGLGDDQDADG-FQTAYSEWEPKLWEAL  834

869  KDTSSGGVTHANEQLMKFHGI  889
      ++T  G +   N Q  +FHGI
 32  ENTIYGSIRLTNRQTFQFHGI 52

443   RALVPMFVRKSQFRLPFKSTTP 464
      + L P ++  ++ RLP   TTP
904   QGLEPYYMFMARARLPGGKTTPQQWLALDHLS-DTSGNGTLKLTTRATFQIHGVLKKNLKHT 964
        LEP +  + R RLPGG  T +QW A+D  + + +  G+++LT R TFQ HG+LKKN+K
  1     LEPRHAMLLRCRLPGGVITTKQWQAIDKFAGENTIYGSIRLTNRQTFQFHGILKKNVKPV 60

965   LRGMNAVLMDTLAAAGDVNRNVMVSALPTNAKVHQQIADMGKLISDHFLPKTTAYHEVWL 1024
       + +++V +D LA A D+NRNV+ ++ P  +++H +  +  K IS+H LP+T AY E+WL
 61   HQMLHSVGLDALATANDMNRNVLCTSNPYESQLHAEAYEWAKKISEHLLPRTRAYAEIWL 120

1025  EGPEEQDDDPSWPSIFENRKDGPRKKKTLV 1054
      +  +    D   P + +     PRK KT V
 121  DQEKVATTDEE-PILGQTYL--PRKFKTTV 147

1058  ALVDIEPIYGPTYLPRKFKFNIAVPPYNDVDVLSIDVGLVAIVNPETQIVEGYNVFVGGG 1117
      A  D EPI G TYLPRKFK  + +PP ND+D+ + D+  VAI   E   + G+N+ VGGG
 126  ATTDEEPILGQTYLPRKFKTTVVIPPQNDIDLHANDMNFVAIA--ENGKLVGFNLLVGGG 183

1118  MGTTHNNKKTYPRLGSCLGFVKTEDIIPPLEGIVIVQRDHGDRKDRKHARLKYTVDDMGV 1177
      +   H NKKTY R  S  G++  E  +   E +V  QRD G+R DRK+A+ KYT++ +GV
 184  LSIEHGNKKTYARTASEFGYLPLEHTLAVAEAVVTTQRDWGNRTDRKNAKTKYTLERVGV 243

1178  EGFKQKVEEYWGKKFEPERPFEFKSNIDYFGWIKDETGLNHFTAFIENGRVEDTPDLPQK 1237
      E FK +VE   G KFEP RP+EF    D  GW+K       H T FIENGR+ D P  P K
 244  ETFKAEVERRAGIKFEPIRPYEFTGRGDRIGWVKGIDDNWHLTLFIENGRILDYPARPLK 303

1238  TGIRKVAEYMLKTNSGHFRLTGNQHLVISNITDEHVAGIKSILKTYKLDNTDFSGLRLSS 1297
      TG+ ++A    K + G FR+T NQ+L+I+ + +   A I+ I K   L N   + R +S
 304  TGLLEIA----KIHKGDFRITANQNLIIAGVPESEKAKIEKIAKESGLMNA-VTPQRENS 358

1298  SSCVGLPTCGLAFAESERFLPDIITQLEDCLEEYGLRHDSIIMRMTGCPNGCSRPWLGEL 1357
       +CV  PTC LA AE+ERFLP  I  +++ + ++G+  + I+MR+TGCPNGC R  L E+
 359  MACVSFPTCPLAMAEAERFLPSFIDNIDNLMAKHGVSDEHIVMRVTGCPNGCGRAMLAEV 418

1358  ALVGKAPHTYNLMLGGGYLGQRLNKLYKANVKDEEIVDYIKPLFKRYALEREEGEHFGDF 1417
       LVGKAP  YNL LGG  +G R+ ++YK N+ + EI+  +  L  R+A ERE GE FGDF
 419  GLVGKAPGRYNLHLGGNRIGTRIPRMYKENITEPEILASLDELIGRWAKEREAGEGFGDF 478

1418  CIRVGIIKPTTE 1429
       +R GII+P  +
 479  TVRAGIIRPVLD 490
```

Figure 4.01 Alignment of Met5p (shown in red) with sulfite reductase hemoprotein (shown in bold black type) and NADPH-cytochrome P450 reductase (shown in blue) from a BLASTPed against clustered PDB protein sequences, performed by the Saccharomyces Genome Database. Conservative amino acid substitutions are indicated by a +, identical amino acids are also shown. Underlined are the nitrite and sulfite reductases iron-sulfur/siroheme binding motifs.

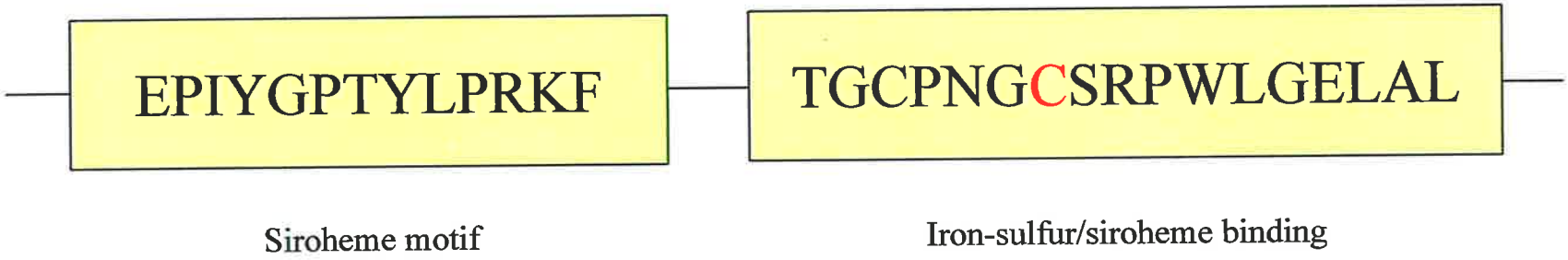


Figure 4.02 Nitrite and sulfite reductases iron-sulfur/siroheme binding motifs within YJR137c, found by an EMOTIF search (Biochemistry, Stanford University). Highlighted in red is the cysteine residue predicted to bridge the siroheme and Fe_4S_4 cluster.

The above evidence suggests that *MET5* encodes the β subunit of sulfite reductase in *S. cerevisiae*. However, the YJR137c ORF has also been identified as a gene involved in cell wall biosynthesis (*ECM17*) in a screen for altered sensitivity to calcofluor white, a cell surface perturbing agent (Lussier, *et. al.*, 1997), making the biological role of Met5p uncertain.

The identification of proteins and the protein-protein interactions mediating a biological process is important in understanding how the process functions. Immunoprecipitation is a powerful technique that can be used to obtain information about a protein to which an antibody has been raised. Protein-protein interactions within the cell can be determined by antibody induced coprecipitation of two or more proteins (Williams, 1999). By using immunoprecipitation in combination with SDS-polyacrylamide gel electrophoresis, the presence and quantity of an antigen can be determined, the molecular weight of the protein estimated and interactions with other proteins can be detected (Harlow and Lane, 1988).

The yeast two-hybrid system (Fields and Song, 1989) has been used extensively to identify protein-protein interactions, providing insights into various molecular mechanisms (Baksh, *et. al.*, 1995; De Vries, *et. al.*, 1995; Tu and Carlson, 1995; Das, *et. al.*, 1997; Fu, *et. al.*, 1997; Datta, *et. al.*, 1998; Tu and Wu, 1999). The use of this approach in a cDNA library screen enables proteins interacting with the target to be identified. An advantage of the two-hybrid system is that it is able to detect transient interactions that may not be recognised by other methods (Aelst, *et. al.*, 1993; Fields and Sternglanz, 1994).

The aim of this work was to identify other proteins able to interact with the α subunit of sulfite reductase, and to confirm the identity of the β subunit of sulfite reductase. Immunoprecipitation and the yeast two-hybrid system were used in an attempt to achieve this. As there was considerable evidence to support the identity of *MET5* as the β subunit of sulfite reductase, a direct interaction between Met10p and Met5p was also tested in the two-hybrid system.

4.02 RESULTS

4.02.1 Expression of Met10p in *E. coli*

The Met10 protein was expressed in *E. coli*, purified and prepared for injection into rabbits to raise a polyclonal antibody. To increase the likelihood of the antibody recognising the protein in yeast, antibodies were raised against the native protein and the denatured protein.

The *MET10* gene was cloned into the expression vector, pET-14b (Novagen), and the pET*MET10* vector transformed into *E. coli* strains BL21 (pLysS) and B834. The expression of Met10p was induced in these strains, and the level of expressed protein compared (Figure 4.03A and B). The over-expressed protein appeared slightly smaller in the B834 strain and could not be recovered by binding cell lysates to a nickle resin. Protein from B834 transformants had most likely been truncated, possibly by losing the histidine tag. Work with the *E. coli* B834 strain was not continued.

4.02.2 Purification of denatured and native Met10p

Before doing a large scale purification of Met10p, the solubility of the protein in *E. coli* cells induced at 37°C was tested. The majority of Met10p which bound to the nickle resin was found to be insoluble (Figure 4.04), with a small fraction of the nickle-bound protein soluble. To increase the proportion of Met10p in the soluble fraction, BL21 [(pLysS) pET*MET10*] was induced at 30°C. This resulted in an increase in the quantity of Met10p found in the soluble fraction to approximately 50%.

To optimise the purification of denatured Met10p, variations of the Ni-NTA wash buffer (Buffer C) used to remove proteins bound to the Ni-NTA resin without the histidine tag were tried, including 20mM imidazole, 0.1% Triton X-100, 10% glycerol and 0.3M NaCl. The best result was achieved when 0.3M NaCl was included in Buffer C. On a small scale (50ml culture), very few contaminating proteins could be seen on the SDS-PAGE gel. However, a large

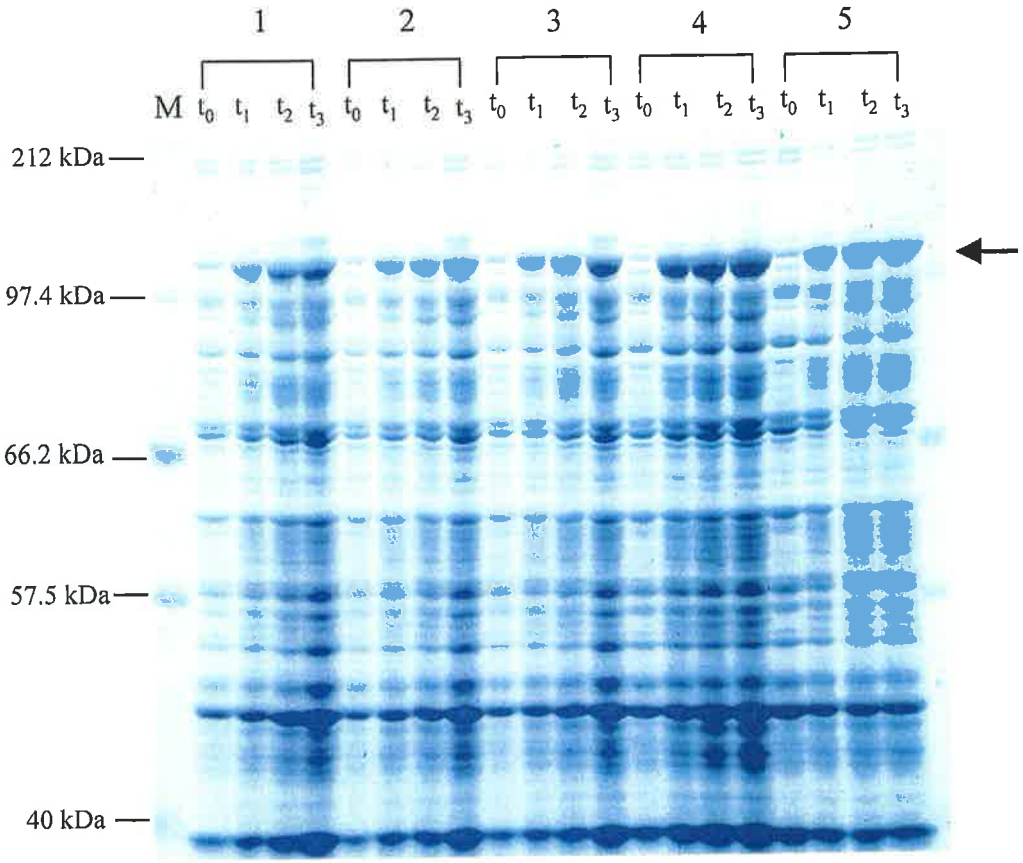


Figure 4.03A Expression of *S. cerevisiae* Met10p in different transformants of *E. coli* B834. After the addition of IPTG to induce expression of Met10p, culture samples were taken at t_0#, t_1, t_2, and t_3*. All of the colonies selected were shown to be expressing Met10p. The band which corresponds to Met10p is indicated by an arrow.

M - Promega high molecular weight markers.
Sample taken just prior to induction of protein expression by IPTG.
*Time 1, 2 and 3 hours after induction of protein expression.

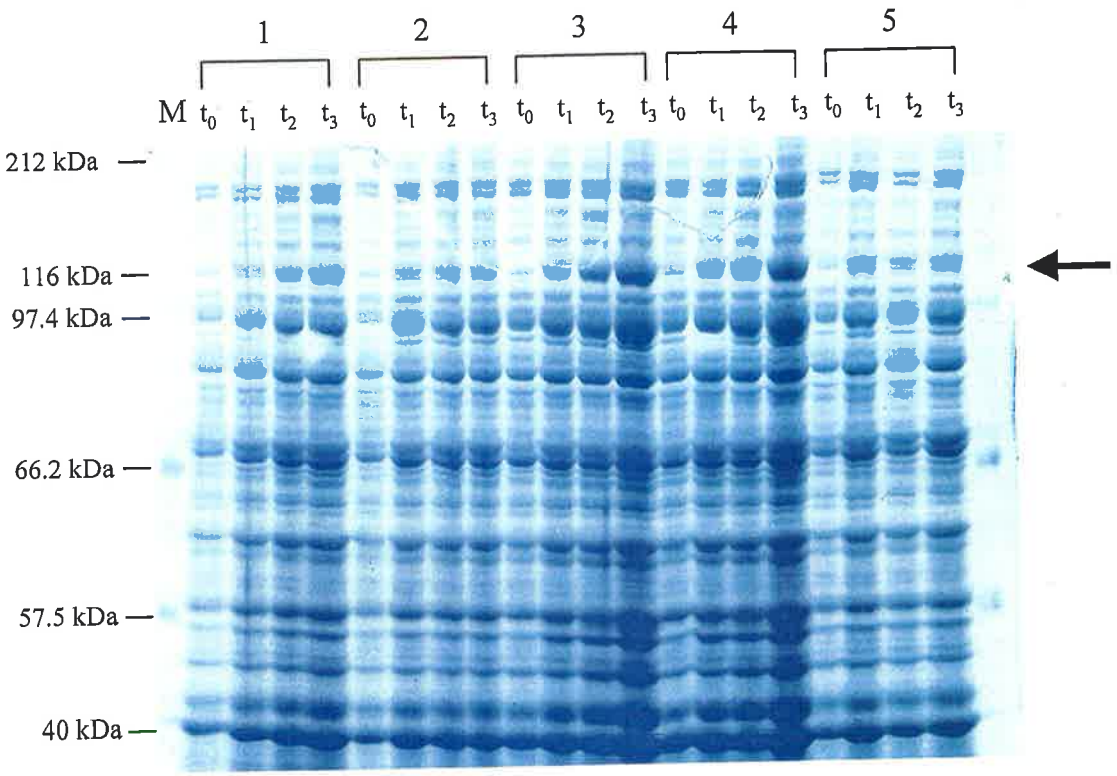


Figure 4.03B Expression of *S. cerevisiae* Met10p in *E. coli* BL21(pLysS). After the addition of IPTG to induce expression of Met10p, culture samples were taken at t_0#, t_1, t_2, and t_3*. Three of the colonies selected were shown to be expressing Met10p. The band which corresponds to Met10p is indicated by an arrow.

M - Promega high molecular weight markers.
Sample taken just prior to induction of protein expression by IPTG.
*Time 1, 2 and 3 hours after induction of protein expression.

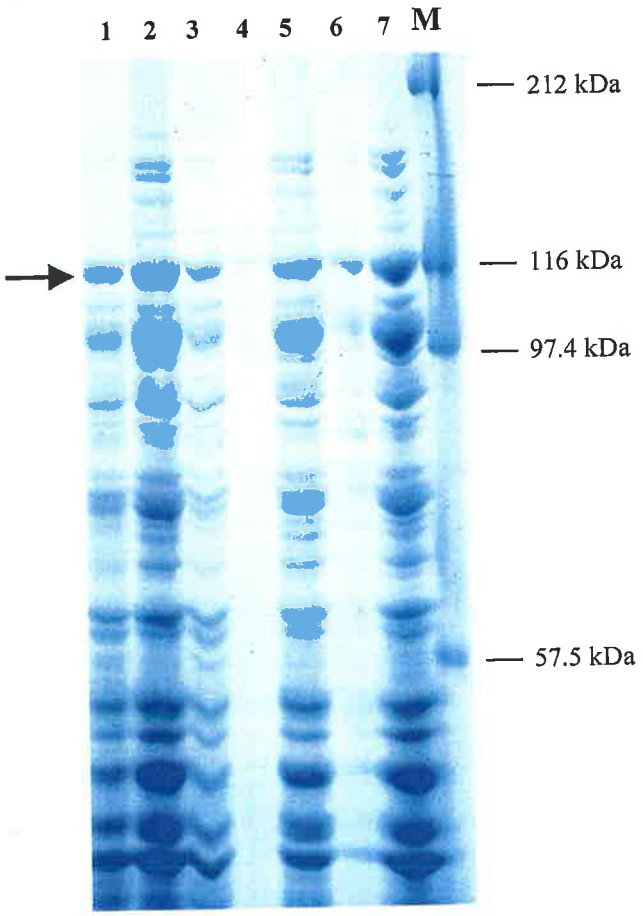


Figure 4.04 Solubility of Met10p expressed in BL21(pLysS) cells grown at 37°C. Lane 1, soluble fraction of cell protein. Lane 2, insoluble fraction of cell protein. Lane 3, soluble fraction which did not bind to the Ni-NTA resin. Lane 4, Ni-NTA bound soluble proteins. Lane 5, insoluble proteins which did not bind to the Ni-NTA resin. Lane 6, insoluble protein bound protein. Lane 7, total cell protein in induced cells three hours after induction of protein expression. The band corresponding to Met10p is indicated by an arrow.

M - Promega high molecular weight markers

scale purification (500ml), increased the number of contaminating bands (Figure 4.05).

Native purification of Met10p from BL21 (pLysS) did not give a pure preparation and the number of contaminating proteins was very high (Figure 4.06). To overcome this, higher stringency wash buffers were used which contained increased concentrations of imidazole and 0.3M NaCl, however still no protein was recovered. Met10p seemed to bind poorly to the Ni-NTA resin and was displaced by low concentrations of imidazole (20mM imidazole displaced any bound Met10p).

A different resin in which the Ni^{2+} had been displaced by Zn^{2+} was used to purify native Met10p. The recovery of Met10p increased when the Zn-NTA resin was used in the purification, and the intensity of contaminating bands was slightly reduced (Figure 4.07).

4.02.3 Detection of Met10p by the anti-Met10p antibody

Recognition of Met10p by the anti-Met10p antibody was first tested on Met10p expressed from *E. coli* by western blot (Figure 4.08). The antibody against native Met10p showed a very low specificity to the protein and work with this antibody was not continued. Met10p expressed from BL21(pLysS) could be detected in approximately 100ng of total cell protein by the anti-denatured Met10p antibody (α-dMet10p) (Figure 4.09). This antibody was not highly specific and recognised several other bands on a western blot, some of which were present in the pre-immune serum (Figure 4.10). The ability of α-dMet10p to recognise Met10p in *S. cerevisiae* was then tested. The detection level was much lower (Figure 4.11), most likely due to a lower level of the protein being expressed in yeast. Glycosylation of the *S. cerevisiae* protein, predicted to have 12 glycosylation sites, may also function to reduce the ability of α-dMet10p to recognise Met10p in *S. cerevisiae*. Surprisingly, the antibody also detected Met10p in the ΔM10-1 strain. This is a deletion strain, although the region of DNA deleted is 218 nucleotides in length and is in frame, therefore the protein may be translated, but folded incorrectly. Since the yeast lysates were run on a

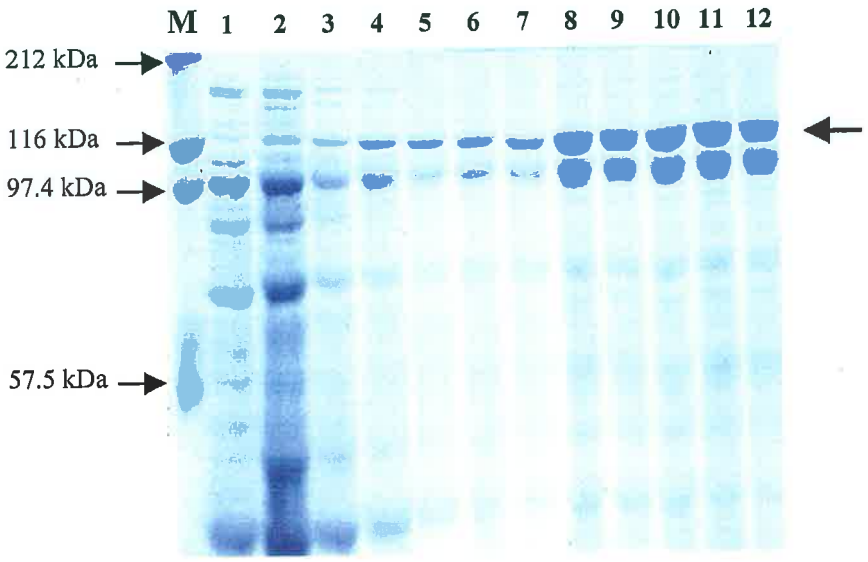


Figure 4.05 Purification of denatured Met10p from *E. coli* BL21(pLysS) from a 500ml culture, divided into 100ml aliquots. Lane 1 shows total cell protein before induction. Lanes 2 - 7 are the washes with Buffer C containing 0.3M NaCl. Lanes 8 - 12 show protein elution off the Ni-NTA resin. The band corresponding to Met10p is indicated by an arrow.

M - Promega high molecular weight markers

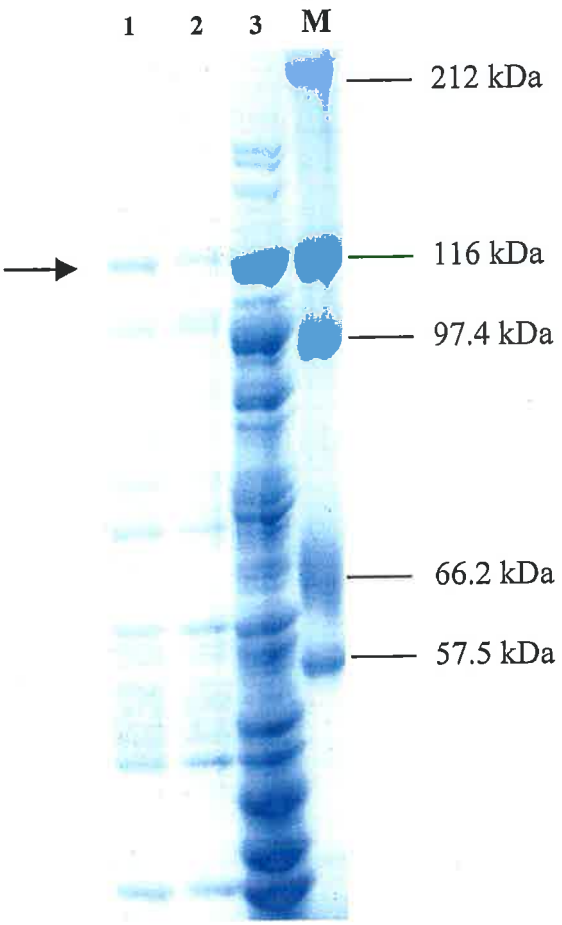


Figure 4.06 Purification of native Met10p from a 500ml culture of *E. coli*(pLysS), grown at 30°C. Lanes 1 and 2 show the purified Met10p eluted off the Ni-NTA resin. Lane 3 is the total cell protein three hours after induction. The band corresponding to Met10p is indicated by an arrow.

M - Promega high molecular weight markers

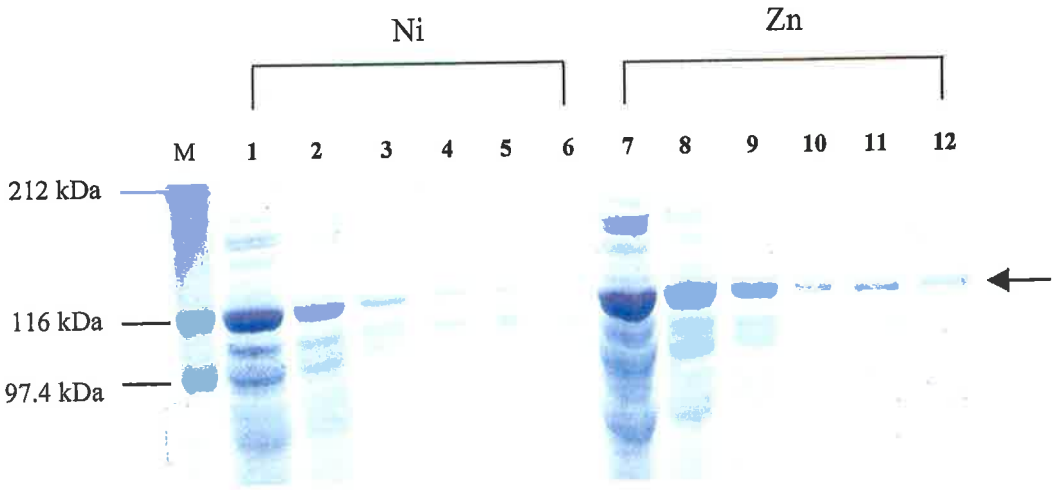


Figure 4.07 Comparison of the yield and purity of purified native Met10p, using NTA resin with Ni^{2+}(Lanes 1 - 6) or Zn^{2+} (Lanes 7 - 12). Lanes 5 and 6 show the purified Met10p eluted off the Ni-NTA resin, and Lanes 7 - 8 show Met10p eluted off the Zn-NTA resin. The yield and purification were better when the Zn-NTA resin was used, compared to the Ni-NTA resin. The band corresponding to Met10p is indicated by an arrow.

M - Promega high molecular weight markers

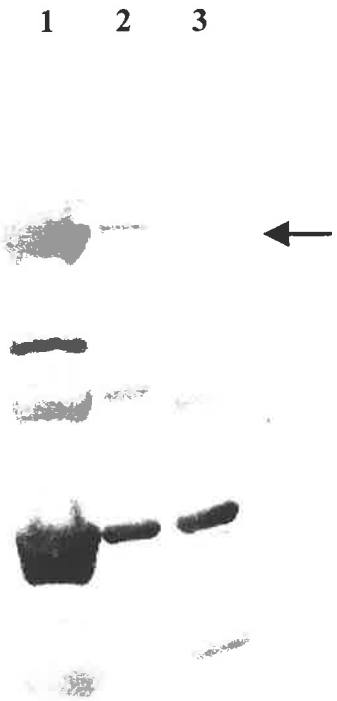


Figure 4.08 The specificity of the anti-Met10p antibody to Met10p expressed in *E. coli* was tested by western blot. Met10p expression was induced in BL21(pLysS) and cell samples were taken before induction (Lane 2) and 3 hours after induction (Lane 1). Total cell protein was run on an 8% SDS-PAGE, and after transfer to nitrocellulose, the detection of the protein by anti-Met10p was tested. The band corresponding to Met10p is indicated by an arrow (Lanes 1 and 2). This band was not detected in the BL21(pLysS) with no Met10p expression vector cell sample (Lane 3).

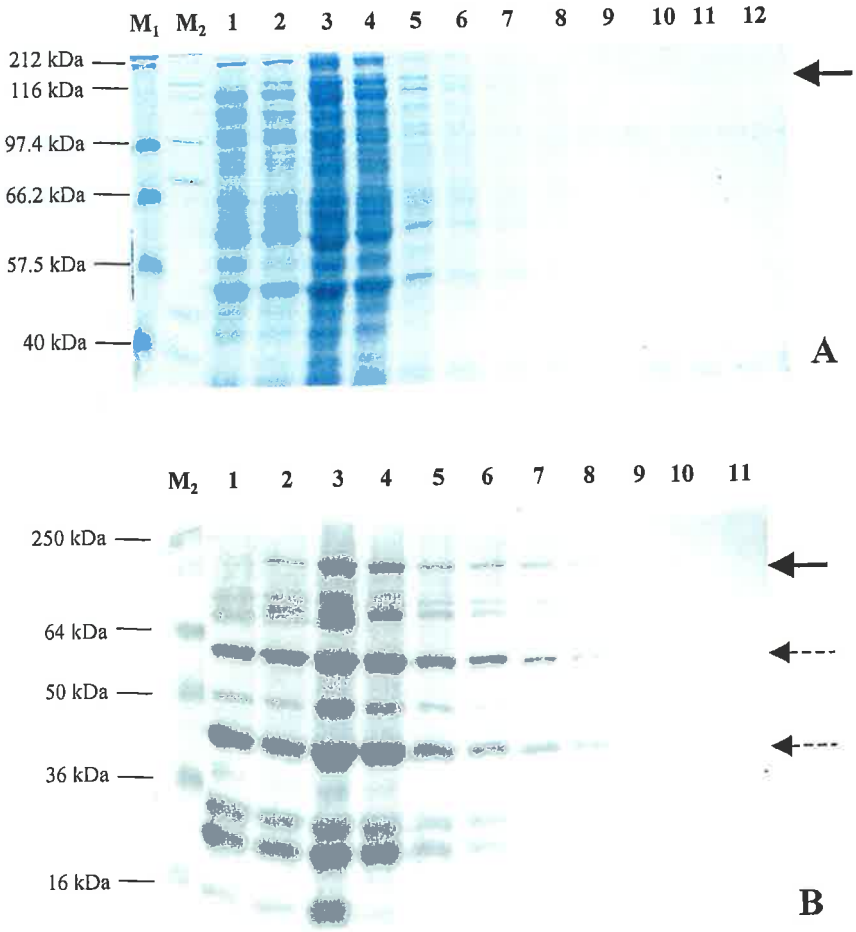


Figure 4.09 Detection of Met10p expressed in *E. coli* BL21(pLysS) by anti-Met10p antibody (B). The band corresponding to Met10p is indicated by an unbroken arrow. This band was not detected in BL21(pLysS) without the pET*MET10* expression vector (Lane 1), but a band was seen in BL21(pLysS) with pET*MET10* before induction (lane 2), indicating the expression was slightly leaky. The band was considerably more intense at t_3 (lane 3). Lanes 4 - 11 show the dilutions of the total cell protein; 0.5, 10^{-1}, 0.05, 0.02, 10^{-2}, 5 x 10^{-3}, 2 x 10^{-3}, 10^{-3}, 2 x 10^{-4}, 10^{-4} dilution respectively. A - the protein on an 8% SDS-PAGE. The load order was the same as the western blot (B).

M_1 - high molecular weight markers (Promega)
M_2 - SeeBlue pre-stained markers (Novex).
Dashed line - bands detected in pre-immune serum (see also, Fig 6.10)

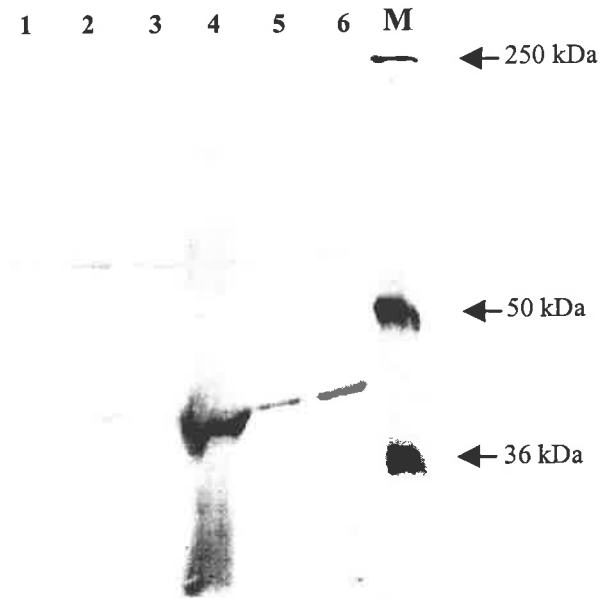


Figure 4.10 Western blot with anti-Met10p pre-immune serum. Whole cell protein from both *E. coli* and *S. cerevisisae* lysates were tested. Lanes 1- 3 show the yeast lysate extracted from CS222, 90844 and ΔM10-1, respectively. Total cell protein from BL21(pLysS) expressing Met10p, three hours after induction is shown in Lane 4, and t_0 is shown in Lane 5. Lane 6 is BL21(pLysS) with no Met10p expression vector before induction.

M - SeeBlue pre-stained standards (Novex).

Figure 4.11 Comparison of the specificity of the anti-Met10p antibody for the Met10p protein from *S. cerevisiae.* In Lane 1, the detection of Met10p in total cell protein from BL21(pLysS) expressing Met10p (t_3) by the antibody is shown. The detection of Met10p is also shown in *S. cerevisiae* 90844 (Lane 2) and ΔM10-1 (Lane 3). The band of interest is indicated by an arrow. Surprisingly, a Met10p signal was also detected in ΔM10-1.

denaturing gel before transfer to nitrocellulose for the western, correct folding of the protein may not be a factor in the recognition of Met10p by α-dMet10p.

4.02.4 Immunoprecipitation

Immunoprecipitation with the α-dMet10p antibody was used to determine the presence of proteins interacting with Met10p. The yeast cells were labelled with ^{3}H-leucine rather than ^{35}S-methionine, as the presence of the methionine in the medium would result in the methionine biosynthetic pathway not being expressed.

No purified immune complex signal could be detected. Western blots had shown that the affinity of the antibody for Met10p was low, which may prevent this antibody coprecipitating Met10p and proteins interacting with it.

4.02.5 Screening a yeast cDNA library with Met10p as the 'bait'

Interaction between proteins can also be detected by the yeast two-hybrid system, and some interactions, especially transient ones, were previously detected by this method and not by immunoprecipitation (Aelst, *et. al.*, 1993). A cDNA library was constructed from mRNA isolated from *S. cerevisiae* grown in the absence of methionine in order to increase the proportion of methionine biosynthesis pathway clones. After amplification of the library in *E. coli*, it was co-transformed into *S. cerevisiae* strain Y190 with the yeast two-hybrid binding domain vector containing the *MET10* gene (pAS*MET10*).

Screening the library revealed six colonies (from 100 000 transformants) which gave a positive signal in the β-galactosidase assay. The size of the cDNA insert in the activation domain vector from each positive transformant was estimated by restriction digest and gel electrophoresis (Figure 4.12). Some clones appeared to lack an insert (Figure 4.12, Lane 2), which may indicate that the positive yeast colony may have arisen from more than one yeast transformant, some containing an insert and some without. Clones with an insert were sequenced and a DNA

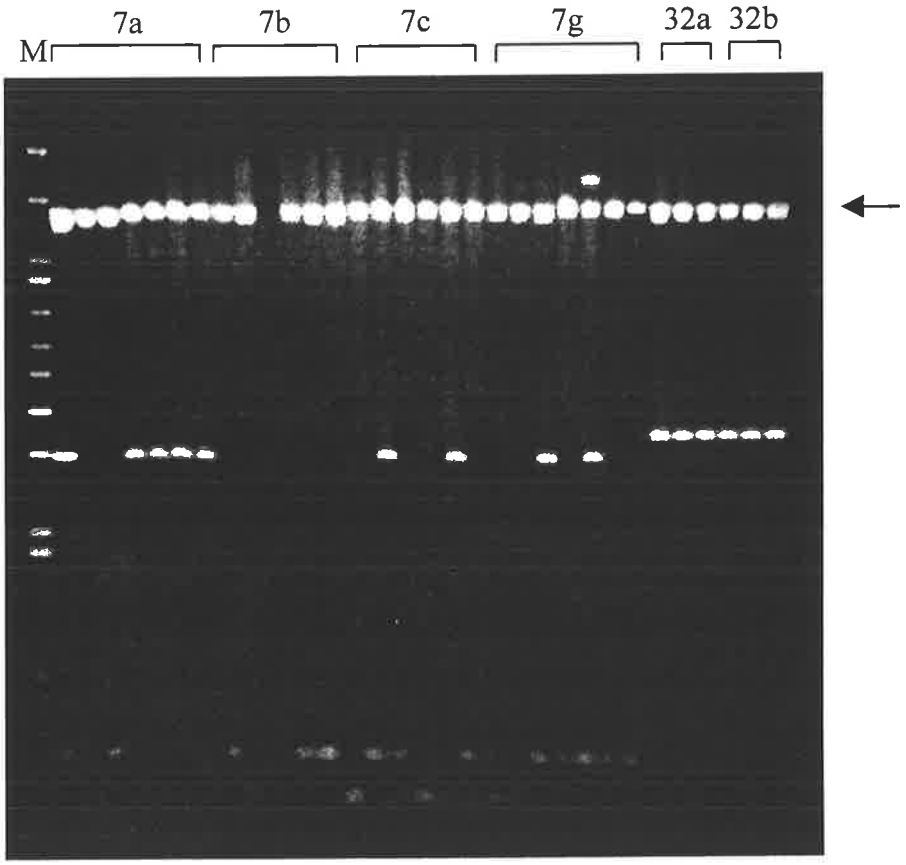


Figure 4.12 Activation domain vector from *S. cerevisiae* transformants which gave a positive signal in the yeast two-hybrid system, digested with *Bgl* II to release the cDNA insert. The plasmid DNA was first isolated from yeast then transformed into *E. coli*. DNA was isolated from several *E. coli* transformants. The band corresponding to the plasmid DNA is indicated by an arrow.

similarity search done within the *Saccharomyces* Genome Database identified the inserts, shown in Table 4.01.

The insert within the vector of one transformant giving a positive signal in the yeast two-hybrid screen was shown to encode *MET10* (Table 4.01), indicating that the system was working. Sulfite reductase has a $\alpha_2\beta_2$ structure therefore it was reasonable to assume that Met10p would interact with itself. However further sequencing revealed that the *MET10* gene contained the *Nco* I site introduced at the 5' end of the gene to facilitate cloning *MET10* into the pAS2-1 vector. It is unlikely that the pAS*MET10* vector was retained in the cell and sequenced instead of the activation domain vector, since digestion with *Bgl* II did not produce the fragments expected on a gel if this was the case.

The inserts within the activation domain vector of the remaining colonies producing a positive signal in the yeast two-hybrid screen encoded *GAL4* and ribosomal proteins that did not appear to be biologically relevant and were therefore false-positives. These proteins have been identified as frequently encountered false positives in yeast two-hybrid screens (Hengen, 1997).

4.02.6 A direct interaction between Met10p and Met5p is only detected in the absence of methionine

To ascertain whether the *MET5* gene did encode the β subunit, a direct interaction between Met10p and Met5p was tested in the two-hybrid system. During attempts to detect an interaction between Met10p and Met5p, different host *S. cerevisiae* strains were used. The *S. cerevisiae* strain Y187 was co-transformed with pACT*MET10* and pAS*MET5* and an interaction was detected between Met10p and Met5p, however this was inconclusive since an interaction was consistently seen in the negative controls (Figure 4.13).

Y190 contains two reporter genes, *HIS3* and *lacZ*, however expression of the *HIS3* allele is leaky and the anti-metabolite 3-amino-1,2,4-triazole (3-AT) must be included in the selection media (Harper, *et. al.*, 1993). An interaction could not be detected between Met10p and Met5p in this strain indicating that these

Positive clone number	Identity
1 (7a)	*GAL4* (YPL248c)
2 (7b)	*GAL4* (YPL248c)
3 (7c)	*MET10*
4 (7g)	*GAL4* (YPL248c)
5 (32a)	*RPS28A* (YOR167c) Ribosomal protein S28A
6 (32b)	Ribosomal binding protein (YBR084c) RPL19B

Table 4.01 The identity of the cDNA inserts within the two-hybrid activation domain vector which gave a positive signal in a two-hybrid library screen. Sequence results were used in a BLAST search within the Saccharomyces Genome Database.

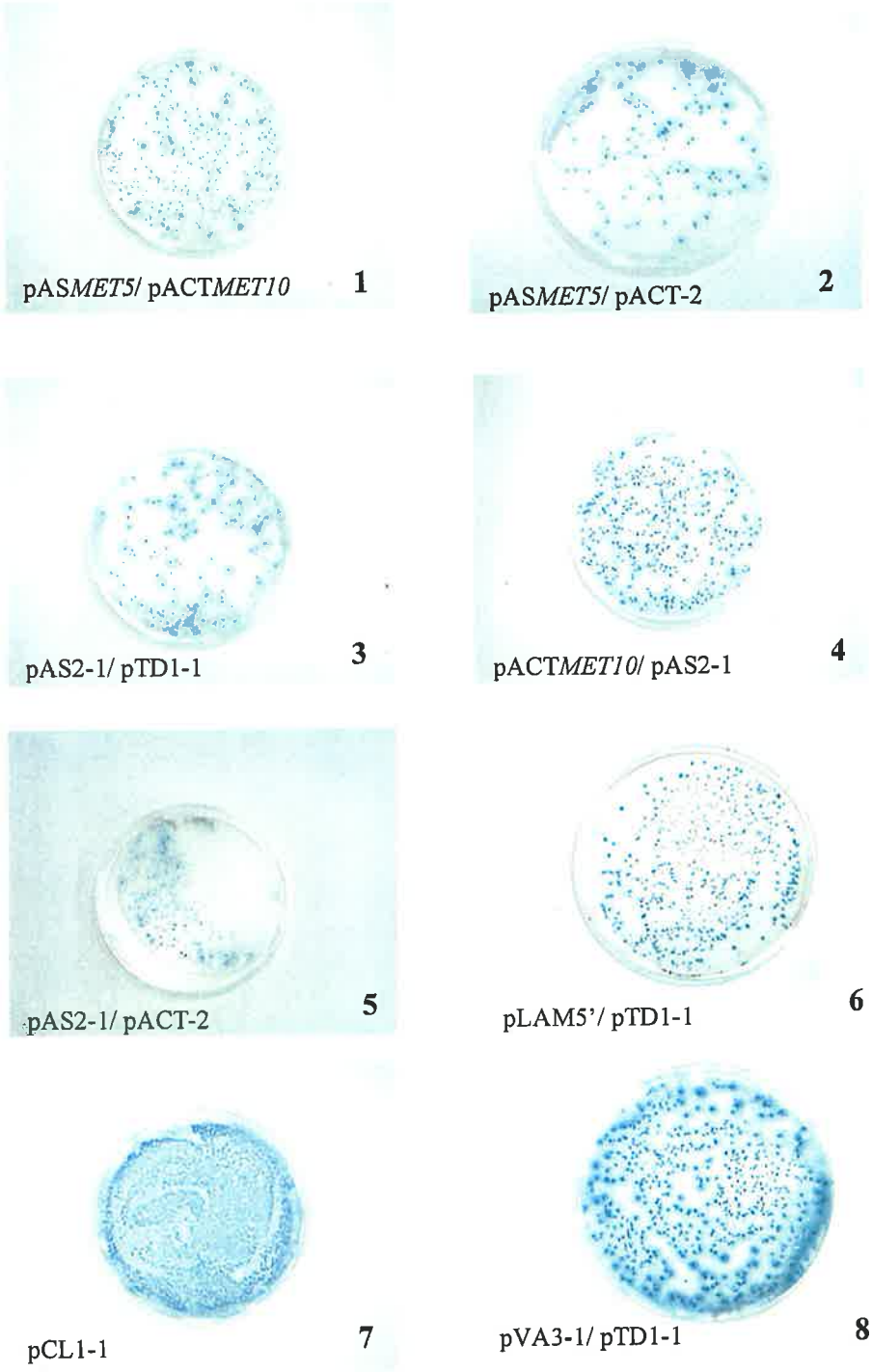


Figure 4.13 Testing an interaction between Met10p and Met5p using the yeast two-hybrid system. The host strain was *S. cerevisiae* Y187. Using this strain, an interaction between Met10p and Met5p was detected (Plate 1), however this was inconclusive as all of the negative controls were also positive (Plates 2-6). Positive controls were as expected (Plates 7 and 8).

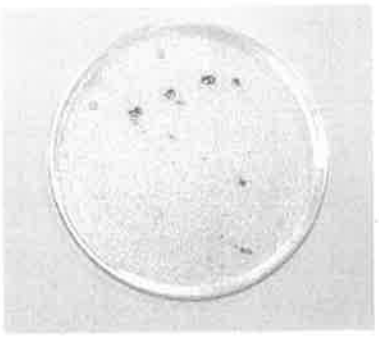
proteins did not interact under the conditions tested. Since Y190 requires the use of 3-AT in the medium and Y187 is a methionine auxotroph, the *S. cerevisiae* strain CG-1945 was chosen as the reporter strain. CG-1945 contains two reporter genes, *HIS3* and *lacZ*, although the *lacZ* reporter gene is weaker in this strain. The expression of *HIS3* in CG-1945 is not leaky, thus overcoming the 3-AT requirement.

An interaction between Met10p and Met5p was detected in the CG-1945 strain in the absence of methionine, which was not seen when the transformants were grown on a medium with methionine (Figure 4.14). This finding indicates that the interaction of Met10p and Met5p is regulated by the availability of methionine.

4.03 DISCUSSION

Several genes have been shown to be required for an active sulfite reductase in *S. cerevisiae* (Thomas, *et. al.*, 1992), however at the time of starting this work, only the gene product of *MET10* had been assigned a function. *MET5* had been predicted to encode the β subunit (Mountain, *et. al.*, 1991; Hansen, *et. al.*, 1994), but had also been shown to have a role in cell wall biosynthesis (Lussier, *et. al.*, 1997). In this work different techniques were used in an attempt to detect proteins which interact with the α subunit of sulfite reductase.

Attempts to co-precipitate proteins bound to Met10p using an antibody that recognises the α subunit were not successful. The α subunit antibody may not recognise native Met10p since the protein to which it was raised was denatured. Another antibody was raised against the native protein, however it was highly non-specific and could not be used in further experiments. Western blots showed that Met10p expressed in *E. coli* appeared to bind the anti-Met10p antibody more effectively than Met10p in *S. cerevisiae*. A possible explanation would be post-translational modifications of Met10p in yeast that do not occur in bacteria, or perhaps the protein expressed in *E. coli* was folded incorrectly. Met10p contains 12 predicted N-glycosylation motifs (*Saccharomyces* Genome Database), suggesting that this protein is glycosylated.



A

B

C

Figure 4.14 Interaction of Met10p and Met5p in *S. cerevisiae* strain CG-1945. Plate A is the two-hybrid positive control, Plate B is the interaction in the absence of methionine. Plate C shows that no interaction was detected between Met5p and Met10p in the presence of methionine.

The low affinity of the antibody, demonstrated in western blot experiments, may prevent Met10p from being precipitated as an immune complex. Low affinity antibodies bound to the antigen produce a less stable complex and dissociate more rapidly than high affinity antibodies (Harlow and Lane, 1988). An important factor determining the avidity of an antibody is its multimeric interactions, which can stabilise an antibody-antigen complex (Harlow and Lane, 1988). Immobilisation of the antigen on solid supports at high concentrations, as in a Western blot, is more likely to allow multivalent interactions. This enables low affinity antibodies to work reasonably well, whereas they may not work for other techniques. A low avidity anti-Met10p antibody may explain why the immunoprecipitation experiments did not precipitate Met10p.

The yeast two-hybrid screen was also used to identify proteins that interact with Met10p, however the library screen did not reveal interacting proteins of any biological significance. One positive clone contained the *MET10* gene, which may be expected, given that sulfite reductase has a $\alpha_2\beta_2$ structure, and the detection of this clone was initially thought to be an indication that the two-hybrid system was functioning as expected. However the insert was found to contain the *Nco* I site introduced at the 5' end of *MET10* by PCR to facilitate cloning. It seems possible that an unexpected recombination event has occurred between the two vectors in the yeast two-hybrid system, a problem identified in other laboratories (T. Lithgow, personal communication).

The identification of the cDNA from the other positive transformants suggested that these were false positives, a frequently encountered problem in the two-hybrid system (Vidal and Legrain, 1999; Hengen, 1997). Ribosomal proteins and heat shock proteins appear to be the most common false positives identified in the two hybrid system, although the reason for this is uncertain (Hengen, 1997). The failure to detect *MET5* and other interacting proteins may be due to the small size of the cDNA library. The quality of the cDNA constructed was thought to be good, based on radioactivity counts, however the efficiency of the

transformation of the cDNA into *E. coli* was consistently low, producing a cDNA library that was smaller than expected.

As the *MET5* gene had been reported in the literature to be the putative sulfite reductase β subunit (Mountain, *et. al.*, 1991; Hansen, *et. al.*, 1994), a direct interaction between Met5p and Met10p was tested in the yeast two-hybrid system. In the course of identifying an interaction between Met10p and Met5p, different *S. cerevisiae* host strains were used. Y187 was selected as the host strain initially as it contained a stronger *lacZ* promoter construct, and therefore would exhibit a higher level of β-galactosidase activity than other reporter strains. A positive signal was detected when this strain was co-transformed with the two-hybrid vectors containing *MET5* and *MET10*, however the negative controls also gave a positive signal and the results could not be interpreted. The strong *lacZ* promoter in this strain may contribute to the constant positive signal detected in the negative controls. Another contributing factor could be the use of only one reporter gene in this strain.

It was therefore decided to test the interaction in a more stringent strain, Y190 that has both the *lacZ* and *HIS* reporter genes. Results obtained when an interaction between Met10p and Met5p was tested indicated that these proteins do not interact, and that *MET5* did not encode the β subunit of sulfite reductase. An interaction between Met5p and Met10p could be demonstrated only in the absence of methionine. Since *MET5* and *MET10* are usually only expressed in the absence of methionine, and genetic studies have shown there are several genes required for a functional sulfite reductase (Thomas, *et. al.*, 1992), Met10p and Met5p may interact via one or more of these factors rather than directly.

The interaction may only be seen in the absence of methionine as the additional factor required for the interaction could only be present within the cell, or in a conformation which can mediate the interaction, in the absence of methionine.

4.03.1 Interpretation of the Met10p and Met5p interaction

It is not known why Met10p and Met5p could only be shown to interact in the yeast two-hybrid system in the absence of methionine, however possible reasons are outlined below.

4.03.1.1 Protein modification

The subunits of sulfite reductase may be modified in the absence of methionine, enabling them to combine to form an active sulfite reductase. The mechanisms behind this can only be hypothesised, however phosphorylation of one or both subunits in the absence of methionine may enable them to assemble into active sulfite reductase. Phosphorylation of protein subunits has been demonstrated to be necessary for protein interaction in several biological systems (Leusen, *et. al.*, 1996; Post, *et. al.*, 1999; Virkamäki, *et. al.*, 1999). Acetylation of proteins has also been shown to regulate protein-protein interaction (Kouzarides, 2000) and may be a mechanism which regulates the interaction of sulfite reductase α and β subunits. Alternatively, sulfite reductase may only be active if it is membrane associated, which is seen in some NADPH dependent redox enzymes, for example the P450s (Peterson, *et. al.*, 1997). Members of this family of enzymes are also found in the flavoenzyme family with sulfite reductase (Karplus, *et. al.*, 1991). How the enzyme would be targeted to the membrane only in the absence of methionine is uncertain.

The end product of the sulfate assimilation pathway, S-adenosylmethionine could potentially have a role in the suppression of sulfite reductase. In the presence of methionine, S-adenosylmethionine can be made directly from methionine by the action of S-adenosylmethionine synthetase (EC 2.5.1.6). S-adenosylmethionine may bind to one or both of the sulfite reductase subunits, preventing the assembly of the enzyme, perhaps by binding at the site(s) where α and β bind each other. This possible post-transcriptional control mechanism would enable sulfite reductase to be rapidly inactivated in the presence of methionine when this high energy consuming enzyme is not required.

4.03.1.2 Interaction of the α and β subunits via siroheme

In a study of four interacting proteins using the two-hybrid system (Pause, *et. al.*, 1999) it was noted that the two-hybrid system fails to detect protein-protein interactions that require the presence of additional components of a multi-subunit complex. This problem was overcome by the integration of the other components expected to interact into the genome of the yeast reporter strain. Similarly, growing the host strain under conditions where other interacting components may be present in the cell would enable proteins of a multi-subunit complex to be detected. Therefore CG-1945 transformed with the two-hybrid vectors containing *MET5* and *MET10* were grown in the absence of methionine, turning on the methionine biosynthetic pathway.

The requirement of an additional factor to mediate an interaction between Met10p and Met5p is the favoured model to understand the observation that the interaction occurs only in the absence of methionine. Two genes shown to be required for an active sulfite reductase, *MET1* and *MET8*, are essential for siroheme biosynthesis in *S. cerevisiae*, (Hansen, *et. al.*, 1997; Raux, *et. al.*, 1999), and are only transcribed in the absence of methionine. Therefore siroheme may be a good candidate for the intermediate factor required for the interaction between the α and β subunits. Furthermore, over-expression of sulfite reductase in *E. coli* requires the inclusion of the *Salmonella typhimurium cysG* gene in the plasmid to achieve maximum catalytic activity of the enzyme (Wu, *et. al.*, 1991). The *S. typhimurium cysG* gene encodes an enzyme necessary for siroheme biosynthesis, indicating that the availability of siroheme is the limiting factor in the activity of sulfite reductase.

The sulfite and nitrite reductases are unique in that these enzymes catalyse a four or six electron transfer without the release of inorganic compounds with an intermediate oxidation state between substrate and product (Murphy, *et. al.*, 1974). They are able to accomplish this due to the prosthetic group, siroheme, an iron-tetra-hydroporphyrin of the isobacteriochlorin type (Murphy, *et. al.*, 1973) (Figure 4.15). Studies with the *E. coli* sulfite reductase hemoprotein revealed

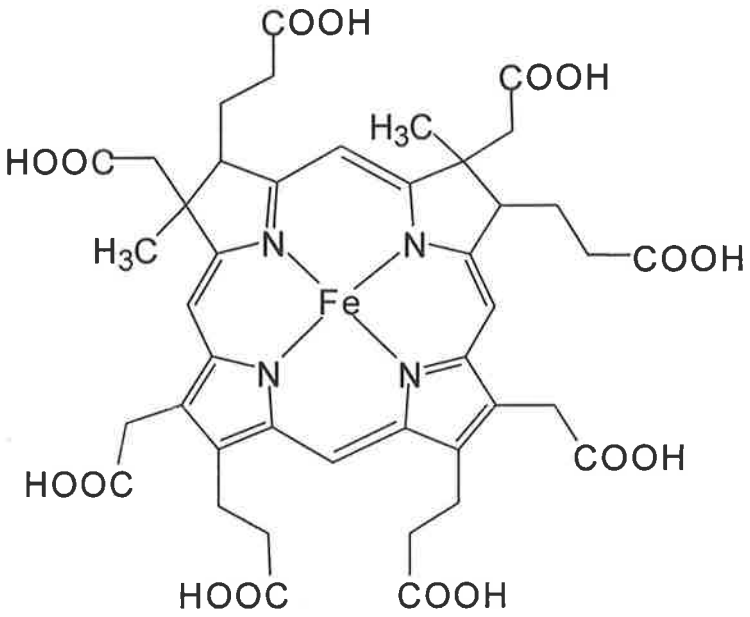


Figure 4.15 Sulfite reductase prosthetic group, siroheme.

that the siroheme is covalently linked to an iron-sulfur cluster (Fe-S) via a conserved cysteine residue. Whilst a detailed structural analysis of yeast sulfite reductase has not been reported, the yeast enzyme has been shown to contain two Fe-S clusters (Yoshimoto and Sato, 1968a; Thomas and Surdin-Kerjan, 1997), and the amino acid sequence of Met5p contains the conserved cysteine in the siroheme/Fe-S cluster binding motif (Figure 4.02).

A variety of different clusters exist, however almost all clusters consist of a common building block, which is a single iron atom tetrahedrally ligated by four sulfur atoms (Mouesca and Lamotte, 1998). The Fe-S clusters bind cysteine ligands, which may be from different subunits of the proteins. If this is the case, the cofactor binding can mediate dimer formation (Beinert, *et. al.*, 1997). The sulfite and nitrite reductases are the only enzymes to create a catalytically active redox centre by the coupling of an Fe-S cluster to siroheme (Figure 4.16). Crystallography studies of *E.coli* sulfite reductase hemoprotein showed that the cofactors are covalently linked by a bridging cysteine thiolate supplied by the protein (McRee, *et. al.*, 1986; Crane, *et. al.*, 1997).

As stated above, siroheme can also have a bridging role between protein subunits if both subunits have binding sites for the cofactor (Beinert, *et. al.*, 1997). One subunit may bind the siroheme, and the other may bind the iron-sulfur cluster, with the link between the two being the bridging cysteine. However Met10p does not contain a siroheme binding motif, which suggests that binding of this cofactor does not mediate dimer formation between the sulfite reductase subunits. It is possible that the binding of siroheme to Met5p induces a conformational change in the protein which then enables it bind to the sulfite reductase α subunit. If Met5p is in fact involved in cell wall biosynthesis (Lussier, *et. al.*, 1997), a siroheme induced conformational change may be a mechanism allowing dual functions of this protein.

4.03.2 Met5p and cell wall biosynthesis

The identification of *MET5* (*ECM17, ExtraCellular Mutant*) as a gene involved in cell wall biosynthesis was unexpected (Lussier, *et. al.*, 1997). The *met5*

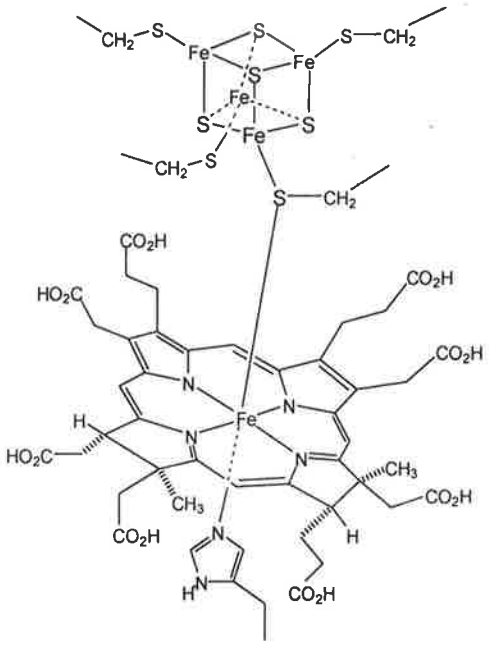


Figure 4.16 The catalytically active redox centre of sulfite reductase, showing the iron-sulfur cluster coupled to the siroheme.

mutation was detected in a screen for altered sensitivity to calcofluor white, and the mutant was also shown to be hypersensitive to zymolyase and hygromycin B. No alterations in the relative proportions of the three main cell wall hexoses, glucose, mannose and N-acetylglucosamine were detected. However, the altered sensitivity to cell wall perturbing agents indicated that a *met5* mutant is defective in the incorporation of cell wall proteins, has N-glycosylation defects and reduced levels of branched β1,3-glucan polymers (Lussier, *et. al.*, 1997) which have not been tested in other *met5* mutants. Methionine auxotrophy of the *met5* mutant detected in this screen was not tested. How a mutation in *MET5*, predicted to be involved in sulfite reduction, also impairs cell wall biosynthesis is unknown. However the accumulation of the toxic metabolite, sulfite in a strain defective in sulfite reductase, may offer a possible explanation.

4.03.3 Sulfite toxicity

Sulfite is a reducing agent often used in the food industry as an anti-oxidant and anti-microbial agent, however the molecular mechanisms behind the action of sulfite are not well understood (Taylor, *et. al.*, 1986; Thomas and Surdin-Kerjan, 1997). The addition of sulfite to yeast cells has been demonstrated to result in a drastic drop in intracellular ATP, and the inhibition of the enzymes glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase and NAD^+ glutamate dehydrogenase. Respiration and aerobic glucose consumption were also retarded (Maier, *et. al.*, 1986). The large decrease in the ATP level of yeast cells has been shown to be a consequence of the inhibition of ATP synthesis linked to the glycolytic degradation of glucose (Hinze and Holzer, 1986).

A study of several mutant strains of *S. cerevisiae* sensitive to sulfite identified the gene *FZF1*(*Five Zinc Fingers*), which when over-expressed, suppressed the sulfite sensitivity of several sensitive mutants, but not that of *ssu1* or *met20* (Avram and Bakalinsky, 1996). The authors suggested that Fzf1p may therefore have a role as a positive regulator of these genes. *SSU1* (*Sensitive to SUlfite*) has been shown to encode a plasma membrane protein which functions as a transporter involved in sulfite efflux (Avram and Bakalinsky, 1997). The *MET20* gene has been shown to be allelic to *MET1* (Hansen, *et. al.*, 1997),

encoding S-adenosylmethionine uroporphyrinogen III transmethylase, essential for the biosynthesis of siroheme (Raux, *et. al.*, 1999).

The *FZF1* gene product has since been demonstrated to be a positive regulator of *SSU1* transcription and shown to bind to the promoter of *SSU1* (Avram, *et. al.*, 1999). In a *met5* strain of *S. cerevisiae*, sulfite accumulation due to an inactive sulfite reductase may result in the activation of *SSU1* by *FZF1* in an attempt to detoxify the cell. It is tempting to speculate that an increase in Ssu1p in the cell membrane may also result in an increase in sensitivity to cell wall perturbing agents such as calcofluor white.

Whilst the presence of methionine in the medium would be expected to repress the genes in the methionine biosynthetic pathway, these genes have also been identified as cell cycle regulated genes in the presence of methionine (Spellman, *et. al.*, 1998). It appears that the regulation of the methionine biosynthetic genes is considerably more complex than initially thought.

4.04 CONCLUSIONS

An interaction between Met10p, the α subunit of sulfite reductase, and Met5p, was detected using the yeast two-hybrid system. Met5p has been predicted to encode the β subunit of sulfite reductase (Hansen, *et. al.*, 1994; Thomas and Surdin-Kerjan, 1997), and its interaction with Met10p shown here, adds further evidence that it is in fact the sulfite reductase β subunit. As the interaction was only seen in the absence of methionine it is possible that another protein, transcriptionally regulated in a similar manner to the methionine biosynthetic genes, may mediate the interaction. *MET1* and *MET8* encode enzymes required to synthesise siroheme, and are also necessary for an active sulfite reductase. The expression of these genes in a two-hybrid reporter strain of *S. cerevisiae*, from a promoter not under methionine control may allow an interaction between the two sulfite reductase subunits in the presence of methionine to be detected.

The role of *MET5* in cell wall biosynthesis remains unclear, however the sensitivity to cell wall perturbing agents may be an indirect effect of the sulfite

detoxifying mechanism, switched on due to the accumulation of sulfite in cells with an inactive sulfite reductase.

CHAPTER FIVE Expression of *met10* constructs in Δ*met10* strain of *S. cerevisiae*

5.01 INTRODUCTION

Amino acids in the predicted NADPH and FAD binding sites of *Saccharomyces cerevisiae* sulfite reductase α subunit, encoded by *MET10,* were altered so the cofactors may no longer bind (Chapter Three). Over-expression of the *met10* genes in the cell may produce a dominant negative phenotype, reducing the activity of sulfite reductase so that excess sulfide is not produced by the methionine biosynthetic pathway yet cell growth can be maintained in the absence of methionine. However no reduction in the activity of sulfite reductase was observed when the altered *met10* genes were expressed in an *S. cerevisiae* strain with a chromosomally encoded wild-type *MET10* gene.

The dominant negative approach may have failed to give a phenotype for several reasons. Expression of the mutated gene from the vector may be a problem, or the altered gene product may be targeted for degradation soon after translation. Alternatively, the introduced amino acid substitutions in Met10p may alter the overall conformation of the α subunit to such an extent that it is no longer able to bind to the β subunit. If this were occurring only wild-type levels of sulfite reductase normally found in the host strain would be present. Another possibility is that the amino acids selected for substitution may not play an esssential role in the function of sulfite reductase from *S. cerevisiae*. Finally, if the mutated α subunits are able to combine with the wild-type β subunit within the cell and still produce an enzyme that retains some activity, the dominant negative effect phenotype will not be seen.

To investigate which of these possibilities may have been preventing the dominant negative phenotype from being observed, experiments were undertaken to establish whether the mutated α subunits were still able to bind to the β subunit, using the yeast two-hybrid system, and if the altered α subunits were

being expressed. Finally, the role of the mutated Met10p on the activity of sulfite reductase was further investigated in a strain of *S. cerevisiae* which had the *MET10* gene deleted, removing any 'interference' from the chromosomally encoded Met10p.

5.02 RESULTS

5.02.1 Micro-homology PCR-mediated targeting of *S. cerevisiae MET10*

Several methods exist to create a gene disruption in *S. cerevisiae*, all of which are dependent on homologous recombination. Integrative disruption generates a deletion at the 5' or 3' end of the gene by the integration of a plasmid with an internal fragment of the gene to be replaced and a selectable marker (Shortle, *et. al.*, 1982). This results in two incomplete copies of the gene and the marker left in the chromosome. Recombination between a cloned gene disrupted by a marker, and the chromosomal gene is called one-step gene disruption, and results in stable, non-reverting mutants (Rothstein, 1983). The similar method, PCR-mediated one-step gene disruption, uses a PCR generated gene disruption cassette containing a marker flanked by 5' and 3' ends of the target gene. This method is widely used (Huang, *et. al.*, 1997; McNeil, *et. al.*, 1997; Girke, *et. al.*, 1998; Broco, *et. al.*, 1999; Vandenbool and Portetelle, 1999; Wilson, *et. al.*, 1999), and was chosen to disrupt the *MET10* gene in *S. cerevisiae* strain 90844.

The micro-homology mediated, PCR targeting technique was used in which the amount of homology required to obtain a good frequency of recombination had been determined to be 30bp (Manivasakam, *et. al.*, 1995). The *URA3* selectable marker was chosen. However to avoid the necessity of using a host strain in which the *URA3* gene had been completely deleted, preventing the possibility of gene conversion, the *Kluveromyces lactis URA3* gene, which is 68% identical at the nucleotide level, was used (Längle-Rouault and Jacobs, 1995).

PCR oligonucleotides with 30bp homologous to the 5' or 3' ends of the *S. cerevisiae MET10* gene, were designed to amplify the *K. lactis URA3* gene.

Amplification and purification of the *URA3* fragment followed by transformation into 90844 routinely resulted in very few, slow growing transformants. Colonies that grew on selective media were further analysed by PCR.

To determine if the *URA3* gene had integrated into the genome, the primers used to generate the *MET10-URA3* deletion cassette were used to amplify the *URA3* fragment from the transformants. To ensure that the marker gene had integrated into the *S. cerevisiae MET10* gene, the *MET10* forward and reverse oligonucleotides were used in a PCR. If *URA3* has integrated into *MET10* the primers used to amplify this gene should still bind to the ends of the gene. However extension will be across the *URA3* gene, and a smaller band (1500bp rather than 3170bp) will be observed on a gel.

S. cerevisiae 90844 was transformed with the purified *K. lactis URA3* fragment with 30bp of homology to the 5' and 3' ends of the *S. cerevisiae MET10* gene. The transformation was repeated many times since only a very low number of transformants were obtained each time (2 - 5 transformants/μg DNA). DNA was extracted from fifty transformants and analysed by PCR. Manivasakam *et. al.*, (1995) reported an integration efficiency of 54% when 30bp of homology to the gene to be disrupted was used in the disruption cassette. Whilst the *K. lactis URA3* gene could be detected by PCR in all of the transformants, the full length *S. cerevisiae MET10* gene was amplified each time also (Figure 5.01), indicating that either the disruption cassette had not integrated, or had integrated elsewhere in the genome. The use of longer regions of homology to the gene to be disrupted increases the frequency of integration (Manivasakam, *et. al.*, 1995) and may have been a better approach to use to generate a *met10::URA3* disruption cassette.

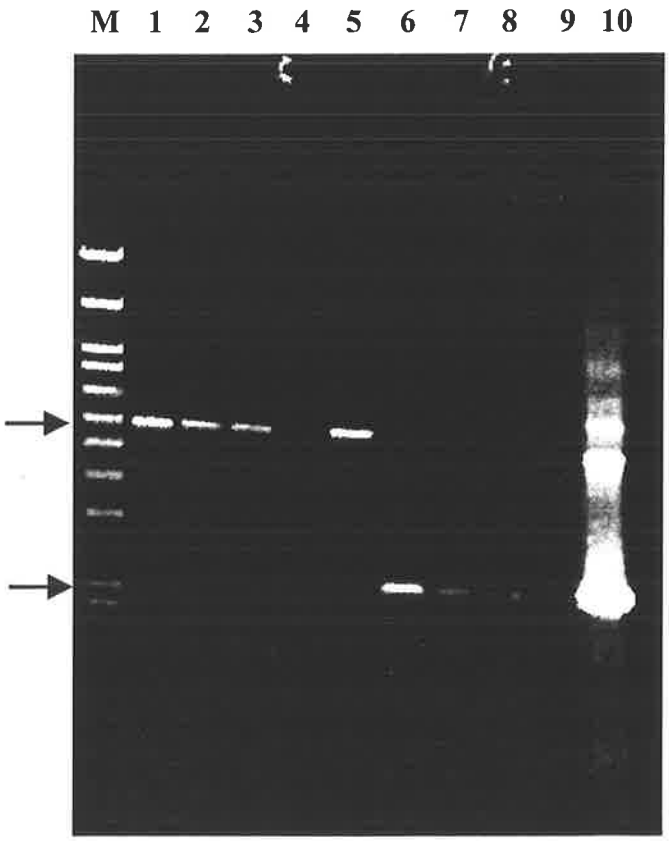


Figure 5.01 Amplification of DNA isolated from *S. cerevisiae* transformed with the *K. lactis URA3* fragment. Lanes 1 - 3, amplification of DNA from transformants with primers to amplify the *MET10* gene. Lane 4, negative control, *MET10* primers. Lane 5, positive control, amplification of the *MET10* gene from *S. cerevisiae* 90844. Lanes 6 - 8, amplification of the *K. lactis URA3* gene from *S. cerevisiae* transformants. Lane 9, negative control, *URA3* primers. Lane 10, amplification of the *URA3* gene from *K. lactis* 2359. The *K. lactis URA3* gene could be amplified from each of the transformants, however the full length *MET10* gene could also be amplified, indicating that the *MET10* gene had not been disrupted.

M - λ *Bste* II/*Sal* I markers

5.02.2 Mating *S. cerevisiae* strains ΔM10-1 and 90844

A strain of *S. cerevisiae* with the *MET10* gene disrupted is available (Hansen, *et. al.*, 1994), however this strain has no other auxotrophic markers which are necessary for the selection of plasmids. The ΔM10-1 strain was kindly provided by Jørgen Hansen (Carlsberg Research Laboratory), and was subsequently mated with 90844. The spores were dissected and analysed for auxotrophy for the different markers (Lys, Met, Leu, His, Trp and Ura). Thirty seven strains were analysed with various combinations of the auxotrophic markers. One of these (CS120) was auxotrophic for all six markers, however this strain was very slow growing and was not considered for future work. The strain CS222 had a -Met, -Leu, -Lys, -Ura phenotype and was selected to examine the effect of the amino acid substitutions in Met10p.

5.02.3 Expression of *met10* genes in *S. cerevisiae* CS222

The CS222 strain was transformed with pRSMET10, pRS*met10*G891893A, pRS*met10*S820V, pRS*met10*S953K, pRS*met10*K959S, pRS*met10*C987A and the growth of the transformants examined in the presence and absence of methionine. If the amino acid substitutions introduced into Met10p have reduced or eliminated sulfite reductase activity, growth of the transformants in the absence of methionine, when the methionine biosynthetic pathway is functional, may be affected.

When the transformants were patched from a medium with methionine to one without, three of the mutants, G891893A, S820V and S953K did not grow (Figure 5.02). No phenotype was observed for the remaining mutants, K959S and C987A, when grown in the absence of methionine. These results indicated that sulfite reductase activity had been disrupted in three of the mutants.

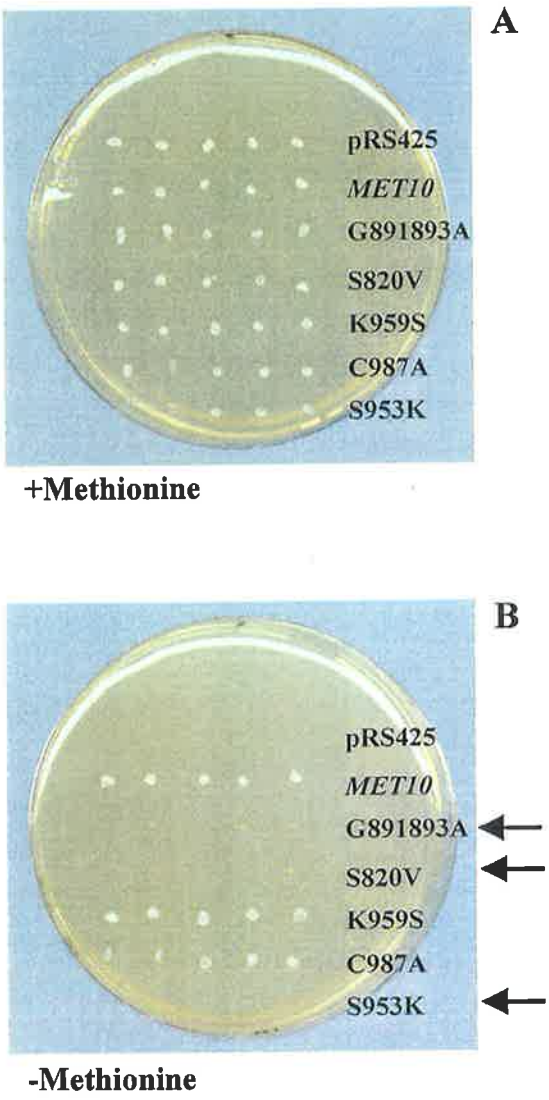


Figure 5.02 Expression of *met10* genes in CS222. *S. cerevisiae* CS222 was transformed with the pRS425 vector alone, and with pRS425 containing *MET10*, *met10*G891893A, *met10*S820V, *met10*S953K, *met10*K959S or *met10*C987A. Growth of the transformants in the presence (A) and absence (B) of methionine was compared. Three of the mutants, indicated by arrows, failed to grow in the absence of methionine. The pRS425 vector alone did not grow in the absence of methionine as expected.

5.02.4 The altered α subunits still bind to the β subunit of sulfite reductase

The yeast two-hybrid system was used to determine if the altered Met10 proteins could still bind to the β subunit of sulfite reductase. The *met10* genes were cloned into the two-hybrid activation domain vector, pACT-2, and subsequently co-transformed with pAS*MET5* into *S. cerevisiae* CG-1945. The transformed cells were grown in the presence and absence of methionine. Previous results had shown that an interaction between the α and β subunits is only observed when cells are grown under conditions in which the methionine biosynthetic pathway is on (Chapter Four).

All of the altered α subunits were shown to bind to the β subunit of sulfite reductase in the yeast two-hybrid system in the absence of methionine (Figure 5.03). This result indicates the amino acid substitutions have not produced conformational folding aberrations in Met10p to prevent the sequestering of the functional β subunit required for the dominant negative approach.

5.02.5 Determination of expression of *met10* genes in *S. cerevisiae* by Western blot

To determine if the dominant negative phenotype was not observed due to *met10* expression problems, an antibody against Met10p was used to detect the protein. The α-dMet10p antibody was shown in previous experiments (Chapter Four) to detect only low levels of Met10p expressed in *S. cerevisiae* compared to the detection of Met10p expressed in *E. coli*. This is most likely due to the lower level of expression of the sulfite reductase α subunit in *S. cerevisiae*, however any post-translational modifications in yeast which do not exist in bacteria, such as glycosylation, may contribute to the lower level of recognition.

Although the level of recognition of Met10p by α-dMet10p was lower, it was used in experiments to determine if the altered Met10p was over-expressed. Whilst Met10p from *S. cerevisiae* could be detected with the α-dMet10p antibody, the level of over-expression of the mutant Met10 proteins was difficult

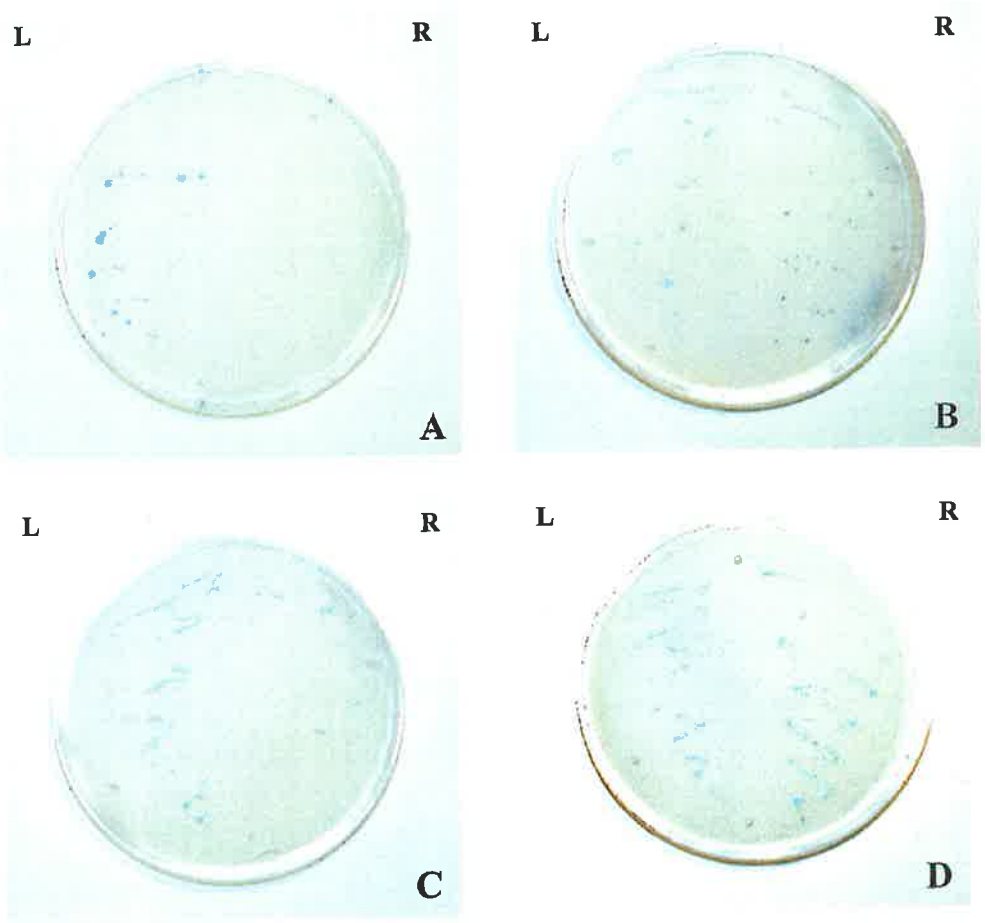


Figure 5.03 Interaction of altered Met10p with Met5p in *S. cerevisiae* strain CG-1945 in the absence of methionine. Frame A, positive control (L), negative control (R). Frame B, pAS*MET5*/ pACT*MET10* (L), pAS*MET5*/ pACT*met10*G891893A (R). Frame C, pAS*MET5*/ pACT*met10*S820V (L), pAS*MET5*/ pACT*met10*S953K (R). Frame D, pAS*MET5*/ pACT*met10*K959S (L), pAS*MET5*/ pACT*met10*C987A (R).

to ascertain. The band corresponding to Met10p on a western blot was quite smeared making the interpretation of the results difficult. However it appeared that the expression of altered Met10p was similar to that seen in the wild-type strain of *S. cerevisiae.*

5.02.6 Sulfite reductase activity of the *met10* mutants in a Δ*met10* background

As described earlier (Chapter Three), several methods were used to measure sulfite reductase activity. The most reliable method was found to be an indirect method in which the accumulation of sulfite is measured. A reduction in the activity of sulfite reductase would be expected to result in an increase in the quantity of sulfite accumulated within the cell. The pararosanilin method (Grant, 1947) was adapted (Hansen, *et. al.*, 1994; Hansen, pers. communication) and used to determine sulfite accumulation in the Δ*met10* CS222 strain transformed with pRSMET10, pRS*met10*G891893A, pRS*met10*S820V, pRS*met10*S953K, pRS*met10*K959S, and pRS*met10*C987A, in synthetic B medium with or without methionine.

The amount of sulfite accumulated by these strains is shown in Table 5.01. In the presence of methionine, only the ΔM10-1 deletion control strain accumulated a low level of sulfite. This strain does grow in the absence of methionine, although growth is very slow and the colonies formed are small, suggesting that the *met10* deletion is partially active. High levels of sulfite were accumulated in the absence of methionine by the control *met10* deletion strains, ΔM10-1 and CS222, and also by three of the CS222 strains expressing an altered *met10* gene. These mutants, *met10*G891893A, *met10*S820V, and *met10*S953K had also failed to grow in the absence of methionine (Figure 5.02). The level of sulfite accumulated by these mutants was not as high as that accumulated by ΔM10-1 and CS222, indicating that some residual activity is retained in these mutated proteins. It was interesting that the *met10*K959S and *met10*C987A mutants accumulated low levels of sulfite since no obvious growth phenotype was noticed when these cells were grown in the absence of methionine (Figure 5.02).

	SO_2 (μg/OD_{600} 2.0)	
***S. cerevisiae* strain**	+Methionine	-Methionine
ΔM10-1	13.925	241.957
CS222	0.0	235.830
90844	0.0	0.0
CS222 (pRS*MET10*)	0.0	29.377
CS222 (pRS*met10* G891893A)	0.0	187.344
CS222 (pRS*met10*S820V)	0.0	171.542
CS222 (pRS*met10*S953K)	0.0	202.817
CS222 (pRS*met10*K959S)	0.0	49.774
CS222 (pRS*met10*C987A)	0.755	37.276

Table 5.01 Sulfite accumulation in CS222 expressing the *MET10* gene and the mutated *met10* genes. CS222, ΔM10-1, and 90844 are control strains. The data was analysed by a one way analysis of variance for the effect of mutation, followed by a means comparison test using the Tukey Honestly Significant difference test ($p = 0.05$). The least significant difference was determined to be 31.2μg in the absence of methionine by the Tukey HSD test.

The CS222 strain over-expressing the *MET10* gene was found to accumulate a low level of sulfite in the absence of methionine, which was unexpected. Further experiments revealed that this is most likely due to plasmid loss, found to be between 10-20%.

5.02.7 Sulfite reductase assay

Measurements of the sulfite accumulated in a Δ*met10* host strain of *S. cerevisiae* expressing the altered *met10* genes showed that the activity of sulfite reductase had been reduced in all of the mutants. To quantify the amount the altered α subunit had reduced the activity of the enzyme, an alternative method to measure sulfite reductase (DeVito and Dreyfuss, 1964; Thomas, *et. al.*, 1989) was used. This method is also colourimetric but follows the disappearance of sulfite from the reaction mix.

Repeated attempts to duplicate this method were unsuccessful. Precipitation of SDS in the yeast lysate extraction buffer led to problems with optical density readings. To overcome this, SDS was replaced with the non-ionic detergent, Tween-20. However no reproducible measurements of sulfite reductase activity could be obtained.

5.03 DISCUSSION

The aim of this work was to find reasons why the dominant negative phenotype was not observed, and to determine if this strategy may still be useful if the problems could be identified. The dominant negative phenotype may not have been seen when the *met10* genes were over-expressed in *S. cerevisiae* 90844 for several reasons, which are discussed below.

The first potential problem to be considered was whether changes in amino acid residues in Met10p have disrupted the folding of the polypeptide. If Met10p had been altered such that it could no longer bind to and sequester the α subunit, the

dominant negative approach would not work. However the two-hybrid system demonstrated that the altered α subunits were still able to bind to the β subunit.

Another potential problem may have been poor expression of the mutated proteins from the *MET3* promoter. It is known that the *met10* genes were being expressed since the CS222 Δ*met10* strain expressing the wild–type Met10p and two of the mutants were able to grow in the absence of methionine, whereas CS222 could not. The level of expression however, is uncertain as the western blots were inconclusive.

Finally, the amino acid residues selected for substitution may not have had an affect on the activity of sulfite reductase. However estimation of the activity of sulfite reductase by sulfite accumulation assays showed that the altered α subunits still retained some activity.

When the methionine biosynthetic pathway was on, in the absence of methionine, both of the Δ*met10* strains CS222 and ΔM10-1 accumulated high levels of sulfite. Under the same growth conditions, expression of *met10* genes encoding amino acid substitutions G891893A, S820V and S953K in CS222 also resulted in high levels of sulfite accumulation, however not to the level observed in the deletion strains, CS222 and ΔM10-1. CS222 expressing these *met10* genes were unable to grow in synthetic dropout medium without methionine.

Expression of Met10p K959S and Met10p C987A in CS222 did support growth of this strain in the absence of methionine which indicated that sulfite reductase was functional, yet sulfite accumulation was seen in these strains. The quantity of sulfite accumulated was four-fold less than the three mutants that were auxotrophic for methionine, although not as high as the CS222 Δ*met10*. This indicates that the activity of sulfite reductase has been reduced in these mutants, although sufficient enzyme activity remains to support growth in the absence of methionine. Sulfite accumulation was also seen in the CS222 strain expressing *MET10* in the absence of methionine, which is unexpected for functional sulfite reductase. This result may be an aberration of the sulfite assay, or may be

accounted for by plasmid loss, which was determined to be between 10 and 20 percent.

In the presence of methionine a low level of sulfite was accumulated by ΔM10-1. This was not expected as the sulfate assimilation pathway is expected to be off in the presence of methionine, and the *MET10* gene in this strain has a 218 bp deletion. However this strain does form small colonies on solid media in the absence of methionine, indicating that the mutation is 'leaky'. It is not yet understood why this occurs (Hansen, personal communication). A secondary mutation may produce the 'leaky' phenotype in ΔM10-1 or mask it in CS222, as the Δ*met10* strain CS222, derived from ΔM10-1, did not accumulate sulfite in the presence of methionine, and did not grow in the absence of methionine. The very low level of sulfite accumulated by CS222 expressing the *met10*C987A mutant is most likely an artefact of the sulfite assay.

The amino acid substitutions introduced into Met10p have resulted in significant reductions to sulfite reductase activity, and identified these amino acids as important to the activity of the enzyme. However none of these changes knocked out enzyme activity completely. Since the *met10* mutants do retain some activity, cofactor binding must still be possible, even if not optimal, in the mutant polypeptides.

To achieve a dominant negative phenotype, the mutated *met10* gene must encode the majority of the α subunit available within the cell, and this requires high expression of the altered *met10* genes. The level of the expression of altered Met10p could not be measured precisely by western blot due to the low level of detection by the α-dMet10p antibody. The band corresponding to Met10p on western blots was consistently smeared which may be due to modifications of the protein in yeast such as glycosylation or phosphorylation that do not occur in bacteria. The results indicate that the over-expression was not very much greater than wild-type gene expression.

The altered *met10* genes may need to be under the control of a promoter stronger than the *MET3* promoter to achieve a dominant negative phenotype. This

promoter was chosen as it was regulated in the same manner as the *MET10* gene, however the strength of the promoters of the two genes may be too similar to enable the majority of the α subunit encoded within the cell to be under the control of the *MET3* promoter on the 2μ plasmid. The copy number of the plasmid encoded *met10* genes may not be sufficient to ensure this, and as both promoters utilise the same transcriptional machinery, competition for these factors may also reduce the proportion of altered Met10p in the cell. The use of an unrelated and stronger promoter such as *GAL1*, *CHA1* or *GPH1* (Farfán, *et. al.*, 1999) may overcome these limiting factors.

Whilst the dominant negative approach did not produce the expected decrease in sulfite reductase, and subsequently reduced potential to produce hydrogen sulfide, the substitution of conserved amino acids in predicted cofactor binding sites confirmed the identity of amino acids important for sulfite reductase function.

5.03.1 Glycine to alanine substitution within the predicted NADPH binding motif

From the results of other researchers it was expected that altering both the first and second glycines within the GXGXXP motif of sulfite reductase would inactivate the enzyme. Whilst cells expressing *met10*G891893A did not grow in the absence of methionine, surprisingly the G891893A Met10p mutant retained some activity which indicates that NADPH is still capable of binding to the α subunit.

The GXGXXP glycine-rich loop segment involved in dinucleotide binding in sulfite reductase is absolutely conserved in the flavoenzyme family (Karplus, *et. al.*, 1991; Hansen, *et. al.*, 1994). The motif, and its variations (Chapter Three), which denotes the Rossmann fold is also conserved in dinucleotide binding proteins (Wierenga, *et. al.*, 1985). The involvement of the conserved glycine residues in enzyme activity and nucleotide binding has been investigated in other enzymes.

Previous experiments designed to modify the glycine-rich loop of NADPH binding sites have highlighted the importance of the glycines in the motif. The first glycine and the last glycine in the GXGXXG dinucleotide binding motif within aldehyde dehydrogenase from *Vibrio harveyi* were mutated to alanines (Vedadi, *et. al.*, 1997), resulting in decreases in the activity of the enzyme. No enzyme activity could be detected when the first glycine was changed to alanine, and the mutant enzyme did not bind the dinucleotide. Altering the last glycine to alanine produced a mutant enzyme capable of binding the dinucleotide, however activity of the enzyme was three-fold lower than the wild-type enzyme.

The first glycine in the dinucleotide binding motif has also been substituted with alanine in pyridine nucleotide transhydrogenase from *E. coli*, resulting in a greater than 95% loss of enzyme activity (Bragg, *et. al.*, 1997). A similar reduction in enzyme activity was found when the first glycine in the GXGXXG motif in flavin-containing monooxygenase 1 (FMO1) was changed to alanine (Kubo, *et. al.*, 1997). Substitution of the second glycine in this motif from FMO1 with alanine knocked out enzyme activity (Kubo, *et. al.*, 1997).

5.03.2 Serine to valine substitution in the predicted FAD binding site

Study of the crystal structure of ferredoxin-$NADP^+$ reductase from spinach identified this enzyme as a prototype structure for the flavoenzyme family (Karplus, *et. al.*, 1991), which includes sulfite reductase from *S. cerevisiae* and *E. coli*. The Ser96 residue was predicted to be an esssential amino acid in the function of this enzyme as it could be shown to interact with the flavin of FAD (Karplus, *et. al.*, 1991). Changing the serine to valine, reduced the activity of ferredoxin-$NADP^+$ reductase to 0.05% of the wild-type enzyme (Aliverti, *et. al.*, 1995), confirming predictions from the crystallography studies. Alignment of the amino acid sequence of Met10p with other proteins within the flavoenzyme family identified Ser820 in Met10p as the equivalent residue to Ser96 in ferredoxin reductase, and it was mutated to valine.

Cells expressing *met10*S820V did not grow in the absence of methionine. However, levels of sulfite accumulation by these cells were less than the ΔM10-1 control strain, indicating that sulfite reductase still retained some activity. Aliverti *et. al.* (1995) reported that the S96V ferredoxin reductase mutant had 0.05% activity. Perhaps the S820V sulfite reductase mutant retains a similar level of activity, yet this is enough to convert some sulfite to sulfide, but not enough activity to support growth in the absence of methionine.

5.03.3 Serine to lysine substitution in the predicted NADPH binding site

Ser234 in spinach ferredoxin reductase has been shown by x-ray diffraction to form a strong hydrogen bond with the 2'-phosphate of NADPH (Karplus, *et. al.*, 1991). Sequence alignment shows that Ser953 in *S. cerevisiae* sulfite reductase is equivalent to spinach ferredoxin reductase Ser234, and it was predicted that substitution of this amino acid with lysine would disrupt the hydrogen bonding and prevent NADPH binding (Chapter Three). Sulfite accumulation levels indicated that S953K sulfite reductase retained some activity, although cells expressing *met10*S953K did not grow in the absence of methionine.

X-ray diffraction studies on spinach ferredoxin reductase also revealed three other amino acids that formed hydrogen bonds with NADPH (Karplus, *et. al.*, 1991). If this also occurs in *S. cerevisiae* sulfite reductase the remaining hydrogen bonds may be sufficient for NADPH to bind, albeit inefficiently. The reduction in activity of the enzyme is most likely due to inefficient transfer of electrons from NADPH to FAD.

5.03.4 Lysine to serine substitution in the predicted NADPH binding site

The equivalent residue to Lys959 in sulfite reductase in spinach ferredoxin reductase is Lys244 by homologous sequence alignement, which has been identified as possibly having a role in the binding of NADPH through electrostatic forces (Karplus, *et. al.*, 1991). However the distance between this residue and bound NADPH was found to be 4Å by x-ray diffraction, which may be too far away for the interaction to take place, even though the side chain of the amino acid moves toward the ligand during binding. This residue is conserved

within the flavoenzyme family (Karplus, *et. al.*, 1991; Hansen, *et. al.*, 1994), therefore its role was investigated by substituting Ser959 with lysine.

The mutant sulfite reductase was found to retain significant activity by sulfite accumulation measurements, and cells expressing *met10*K959S grew normally in the absence of methionine. The results found in the site-directed mutagenesis studies indicate that the role of this lysine is not essential to the function of sulfite reductase, even though it has been conserved across the family of enzymes. As more sulfite was found to accumulate in yeast cells expressing *met10*K959S than the wild type strain, activity of this mutant enzyme was slightly reduced. Given the distance of the side chain from the bound ligand in the crystal structure of ferredoxin reductase, the contribution of Lys959 to NADPH binding may be minimal.

5.03.5 Substitution of cysteine with alanine in the predicted NADPH binding site

Cysteine residues equivalent to C987 in Met10p are highly conserved across the flavoenzyme family, and C987 is one of only two cysteine residues in Met10p. This cysteine has been shown in spinach ferredoxin reductase to function in the binding of NADPH such that it can effectively interact with FAD (Aliverti, *et. al.*, 1993, Pollegioni, *et. al.*, 1997). It positions the two rings of NADPH and FAD to facilitate hydride transfer between C_4 of the nicotinamide and N_5 of the isoalloxazine. This amino acid does not appear to be essential in Met10p since substitution of C987 with alanine still supported growth in the absence of methionine when over-expressed in *S. cerevisiae*, and the quantity of sulfite accumulated by yeast expressing *met10*C987A was relatively low. In a study of nitrite reductase from *Neurospora crassa* (Colandene and Garrett, 1996), a highly conserved cysteine within the FAD-/NAD-binding domain was substituted with alanine, however activity of the enzyme was not abolished. The authors concluded that the mutation influenced electron flow within the FAD-/NAD-binding domain, but was not essential for function.

5.04 CONCLUSIONS

A dominant negative phenotype was not observed when altered α subunits were over-expressed in a strain of *S. cerevisiae* with a wild-type *MET10* background. The two-hybrid system results presented demonstrate that the altered α subunits are able to bind to the β subunit, which is a prerequisite for the dominant negative approach. Furthermore, all of the altered proteins were shown to accumulate sulfite, although none of the mutants accumulated as much sulfite as the control deletion mutant. This result indicates that all have retained some sulfite reductase activity, and further supports that the altered α subunits and β do bind each other.

The level of expression of the *met10* genes may offer the most likely explanation for the lack of a dominant negative phenotype. The sulfite accumulation results indicate that three amino acid substitutions substantially reduced the activity of sulfite reductase, and would therefore be expected to produce a dominant negative phenotype. Although the level of expression of the *met10* genes could not be conclusively determined, the results indicate that it may not have been high enough to produce predominantly inactive sulfite reductase within the cell. A stronger promoter than the *MET3* promoter may produce the desired result.

CHAPTER SIX GENERAL DISCUSSION

The aim of this work was to develop a strategy that would reduce the production of hydrogen sulfide by *Saccharomyces cerevisiae*. Sulfide is produced by *S. cerevisiae* as an intermediate in the sulfate assimilation pathway, which is required to synthesise the essential amino acids methionine and cysteine. The enzyme sulfite reductase catalyses the reduction of sulfite to sulfide in this pathway, and has been implicated in the production of excess H_2S. To reduce the potential for *S. cerevisiae* to produce H_2S, the activity of sulfite reductase was targeted. A reduction rather than elimination in the activity of this enzyme was sought for application in the wine industry. The dominant negative approach was selected to achieve this and in efforts to achieve such a phenotype, some conclusions could be reached regarding sulfite reductase.

Met5p appears to be the β subunit of sulfite reductase

The results of the yeast two-hybrid experiments demonstrate that Met5p and Met10p do interact. This finding and other researcher's results (Masselot and Surdin-Kerjan, 1977; Mountain, *et. al.*, 1991; Thomas, *et. al.*, 1992) provide considerable evidence that *MET5* encodes the β subunit of *S. cerevisiae* sulfite reductase. Interestingly, this interaction could only be detected in the absence of methionine, when the sulfate assimilation pathway in the two-hybrid host strain would be expected to be on. Whilst further investigation will be required to understand this finding, some models have been proposed which may offer an explanation.

The preferred model proposed is that an additional factor is required to mediate the interaction between the α and β subunits which is only present in the absence of methionine. Since the absence of methionine turns the sulfate assimilation pathway on, the proposed additional factor may be encoded by a gene(s) within this pathway to ensure its availability when required. The genes *MET1* and

MET8 are required for a functional sulfite reductase and encode the enzymes necessary to synthesise siroheme. The binding of siroheme to Met5p may induce a conformational change in the protein that enables the β subunit to bind to Met10p. The fact that over-expression of sulfite reductase in *E. coli* also requires over-expression of the genes encoding siroheme to achieve maximal catalytic activity of the enzyme (Wu, *et. al.*, 1991), supports the proposed role of siroheme in *S. cerevisiae* sulfite reductase.

Potential reduction of H_2S production with the dominant negative approach

To achieve a dominant negative phenotype, amino acids in the predicted NADPH and FAD binding sites were altered by site-directed mutagenesis, and the *met10* genes over-expressed in *S. cerevisiae*. These amino acid substitutions within the predicted cofactor binding sites of Met10p were shown to increase sulfite accumulation within a Δ*met10* strain of *S. cerevisiae* over-expressing the altered *met10* genes. The increase in sulfite accumulation indicates a reduction in the activity of sulfite reductase. However when the *met10* genes were over-expressed in a wild-type *MET10* background, no sulfite accumulation could be detected, indicating that sulfite reductase was fully functional.

One possibility that would prevent the dominant negative approach working is the failure of the mutated α subunits to bind to the β subunit. As the altered α subunits were shown to bind to Met5p in the two-hybrid system, it is not likely that conformational change preventing the subunits binding had occurred. The amino acid substitutions could be shown to affect sulfite reductase activity, therefore it is probable that the level of expression of the altered α subunits was not high enough to give a dominant negative phenotype.

The *MET3* promoter is a well characterised, relatively strong promoter and was selected to drive expression of the altered *met10* genes. This promoter is thought to be controlled in the same manner as the majority of genes encoding enzymes of the sulfate assimilation pathway (Cherest, *et. al.*, 1985). With the long-term view of transforming wine yeast with the *met10* genes encoding the

altered α subunits, the *MET3* promoter seemed an appropriate choice. The promoter is tightly regulated by methionine (Mumberg, *et. al.*, 1994; Black, *et. al.*, 1995). Therefore when methionine in the grape juice becomes limiting, the endogenous, chromosomally encoded *MET10* and *MET5* genes should be switched on at the same time as the altered *met10* gene. The *MET3* and *MET10* promoters are very similar (Thomas and Surdin-Kerjan, 1997). They both contain two copies of the upstream *cis*-acting regulatory sequence TCACGTG required for derepression of gene expression, however the *MET3* promoter also contains a copy of the AAACTGTGG motif required for full repression of gene expression (Thomas, *et. al.*, 1989; Thomas, *et. al.*, 1990; Thomas and Surdin-Kerjan, 1997). Whilst the strength of the two promoters are similar, the presence of multiple copies of the *met10* genes was thought to provide sufficient altered α subunit to produce a dominant negative phenotype.

The dominant negative approach required over-expression of altered *met10* in a *MET10* strain of *S. cerevisiae.* This will result in the presence of altered α subunit and wild-type α subunit within the cell and therefore three types of sulfite reductase may be formed. These are functional $\alpha_2\beta_2$ sulfite reductase, and mutated sulfite reductase containing a wild-type copy of the α subunit and one ($\alpha^m\alpha\beta_2$) or two copies of the mutant α subunit ($\alpha^m{}_2\beta_2$).

The $\alpha^m{}_2\beta_2$ form of sulfite reductase in this study has been shown to retain some activity, although three of the mutants did not retain enough activity to support growth in the absence of methionine. It is unknown if the $\alpha^m\alpha\beta_2$ form of sulfite reductase is fully functional, although one functional subunit of the human C1 zymogen dimer has been shown to be sufficient for full activity (Dobó, *et. al.*, 1999). Cofactor binding to the wild-type α subunit may occur, but may not enable enough electrons to be transferred within the enzyme to perform the six electron reduction of sulfite to sulfide. Nevertheless, it must be kept in mind that $\alpha^m{}_2\beta_2$ did retain some activity, even though cofactor binding sites had been mutated.

Driving expression of the *met10* genes from a stronger promoter may shift the subunit 'balance' within the cell to produce predominantly the $\alpha^{m}_{2}\beta_{2}$ form of sulfite reductase, incapable of binding cofactors. Whilst a sulfite reductase dominant negative phenotype was not achieved in this study, it is likely that this approach will work using the selected amino acid substitutions within Met10p if the altered genes are expressed from a stronger promoter.

Although the activity of the enzyme could not be measured, the accumulation of sulfite in strains of *S. cerevisiae* expressing the altered *met10* genes indicated that sulfite reductase activity had been reduced. To confirm the identity of the predicted cofactor binding sites, NADPH and FAD binding assays may be required. However, this study has provided useful information on several amino acid residues within the predicted NADPH and FAD binding sites of sulfite reductase. Three of the amino acids altered reduced the activity of sulfite reductase to the extent that growth in the absence of methionine was not supported, indicating these residues may play a role in binding the cofactors required for a functional enzyme. The remaining two residues altered appeared to have decreased the activity of sulfite reductase, however cells expressing these altered *met10* genes were not auxotrophic for methionine. These amino acids may have a role in the function of sulfite reductase, however they are not essential. The combination of computer modelling tools and site directed mutagenesis has provided an insight into the structure/function relationships that exist within *S. cerevisiae* sulfite reductase.

APPENDIX ONE

10 x CIP buffer

10 mM $ZnCl_2$

10 mM $MgCl_2$

100 mM Tris.Cl (pH 8.3)

SOB medium

To 950ml of deionized water, add:

tryptone, 20g

yeast extract, 5g

NaCl, 0.5g

Adjust pH to 7.0, adjust volume to 1000ml

Before use, add 5ml of sterile 2M $MgCl_2$.

SOC medium

To 1L of SOB medium, add:

20ml of sterile 1M glucose

LB medium

To 950ml deionized water, add:

tryptone, 10g

yeast extract, 5g

NaCl, 10g

Adjust pH to 7.0, adjust volume to 1000ml.

Synthetic drop-out medium

To 850ml of deionized water, add:

6.7g Difco yeast nitrogen base without amino acids

0.67g appropriate amino acid dropout powder

20g agar (plates only)

Synthetic complete drop-out amino acid mix

Adenine hemisulfate, 2.0g

Arginine HCl, 2.0g

Histidine HCl, 2.0g

Isoleucine, 2.0g

Leucine, 4.0g

Lysine HCl, 2.0g

Methionine, 2.0g

Phenylalanine, 3.0g

Homoserine, 6.0g (or Serine, 2.0g and Threonine, 2.0g)

Tryptophan, 3.0g

Tyrosine, 2.0g

Uracil, 1.2g

Valine, 9.0g

Omit the appropriate amino acid(s) for selection.

Synthetic B medium

100ml:

10 x YNB made according to manufacturer's instructions, 10ml

200mM $(NH_4)_2SO_4$, 1ml

200mM DL-homocysteine thiolactone, 100µl

100 x amino acid mix supplement if required, 1ml

Adjust volume to 100ml

Transformation buffer

10 mM Pipes

55 mM $MnCl_2$

15 mM $CaCl_2$

250 mM KCl

1 x TE/LiAc

Diluted from a 10X stock of TE and LiAc:

10X TE:

0.1 M Tris-HCl

10 mM EDTA

Adjust to pH 7.5 and autoclave.

10X LiAc:

1M Lithium acetate

Adjust to pH 7.5 with dilute acetic acid and autoclave.

PEG/LiAc

40% PEG 4000

1 x TE buffer

1 x LiAc

***E.coli* cracking buffer** (for detection of recombinant plasmids)

0.05 M NaOH

0.5% SDS

0.005 M EDTA

10% glycerol

0.25% bromophenol blue

Plasmid solution 1

50 mM glucose

25 mM Tris.Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Plasmid solution 2

0.2 M NaOH

1% SDS

Plasmid solution 3

5M potassium acetate, 60ml

glacial acetic acid, 11.5ml

water, 28.5ml

Yeast lysis solution

2% Triton X-100

1% SDS

100 mM NaCl

10 mM Tris-HCl pH 8.0

1 mM EDTA

Promega buffer B

6 mM $MgCl_2$

50 mM NaCl

1 mM DTT

Stabilising buffer A

1 M sorbitol

10 mM $MgCl_2$

2 mM dithiothreitol

50 mM potassium phosphate (pH 7.8)

100 µg/ml PMSF

2µg/ml leupeptin

2µg/ml aprotinin

Stabilising buffer B

1 M sorbitol

10 mM $MgCl_2$

2 mM dithiothreitol

25 mM potassium phosphate (pH 7.8)

25 mM sodium succinate (pH 5.5)

100 µg/ml PMSF

2µg/ml leupeptin

2µg/ml aprotinin

Zymolyase 100T

10 mg/ml zymolyase 100T in 1M sorbitol

High salt lysis buffer

50 mM HEPES (pH 7.0)

1% NP-40

2μg/ml aprotinin

100 μg/ml PMSF

2μg/ml leupeptin

10 X Phosphate buffered saline

For 1L:

NaCl – 80g

KCl – 2g

KH_2PO_4 – 2g

Na_2HPO_4 - 14.4g

RIPA buffer

150 mM NaCl

1% NP-40

0.5% Deoxycholate

0.1% SDS

50 mM Tris-HCl (pH 8.0)

2μg/ml leupeptin

2μg/ml aprotinin

100 μg/ml PMSF

Sporulation medium

1 % potassium acetate

0.25% yeast extract

0.1% glucose

0.25M Potassium phosphate buffer

$NaH_2PO_4.2H_2O$ – 39 g/L

EGTA – 0.038 g/L

Resuspension buffer

$NaH_2PO_4.2H_2O$ – 39 g/L

EGTA – 0.038 g/L

20% Glycerol

Sulfite reductase reaction mix A

1.7 mM glucose-6-phosphate

1 mM $MgCl_2$

0.1 mM Na_2SO_3

0.1 mM $NADP^+$

166 units/L glucose-6-phosphate dehydrogenase

Prepared in resuspension buffer

Stock amine reagent

12g of N,N-diethyl-p-phenylene diamine-HCl dissolved in 80ml of 11.3 M H_2SO_4. Store in the dark at 4°C.

Working strength amine reagent

Dilute stock reagent 1:20 in 9 M H_2SO_4. Add $FeCl_3$ solution (60% w/v) at a rate of 25µl per 300µl immediately before use.

Yeast lysis buffer (for samples to be analysed for sulfite)

200 mM NaCl

1% NP-40

0.5% Deoxycholate

0.1% Tween-20

50 mM Tris-HCl (pH 8.0)

0.5% glycerol

2µg/ml leupeptin

2µg/ml aprotinin

100 µg/ml PMSF

Sulfite reductase reaction mix B

120 µmol sodium phosphate buffer (pH 7.4)

9.3 µmol glucose-6-phosphate

0.2 units glucose-6-phosphate dehydrogenase

0.5 µmol NADP

0.3 µmol $Na_2S_2O_5$

Mercuric chloride reagent

To make 1L:

Mercuric chloride – 10.8g

Sodium chloride – 4.7g

Colour reagent

To make 1L

0.4g of pararosanilin-HCl is dissolved in 800ml of 40°C water, and 80ml of concentrated HCl is added. Add water to a total of 1L.

SDS electrophoresis buffer (10X)

To make 1L:

30.2g Tris base

144g glycine

10g SDS

pH should be 8.3 (do not adjust)

Coomassie blue

50% methanol

0.05% Coomassie brilliant blue R-250

10% acetic acid

40% water

Destaining solution

5% methanol

7% acetic acid

88% water

Cell cracking buffer(SDS-PAGE)

60 mM Tris-HCl (pH 6.8)

1% β-mercaptoethanol

4% SDS

20% glycerol

0.01% bromophenol blue

Buffer B (purification of denatured protein)

8 M urea

0.1 M NaH_2PO_4

0.01 M Tris-HCl

pH to 8.0

Buffer C (purification of denatured protein)

8 M urea

0.1 M NaH_2PO_4

0.01 M Tris-HCl

pH to 6.3

Buffer E (purification of denatured protein)

8 M urea

0.1 M NaH_2PO_4

0.01 M Tris-HCl

pH to 4.3

Lysis buffer (native protein purification)

50 mM NaH_2PO_4 (pH 8.0)

300 mM NaCl

Wash buffer (native protein purification)

50 mM NaH_2PO_4 (pH 8.0)

300 mM NaCl

5 mM imidazole

Elution buffer (native protein purification)

50 mM NaH_2PO_4 (pH 8.0)

300 mM NaCl

20 mM imidazole

Western transfer buffer

25 mM Tris

192 mM glycine

20% methanol

Ponceau S solution

0.1% (w/v) Ponceau S

5% acetic acid

Blocking buffer (Blotto)

5% (w/v) non-fat dry milk in 1 x PBS

Alkaline phosphatase buffer

100 mM NaCl

5 mM $MgCl_2$

100 mM Tris (pH 9.5)

Z-buffer

To make 1L:

$Na_2HPO_4.7H_2O$ – 16.1g

$NaH_2PO_4.H_2O$ – 5.5g

KCl – 0.75g

$MgSO_4$ – 0.246g

Z-buffer/X-gal solution

100ml Z buffer

0.27ml β-mercaptoethanol

1.67ml X-gal (20mg/ml)

Pb^{2+} medium

3g peptone

5g yeast extract

200mg ammonium sulfate

20g agar

Make the volume up to 900ml with water and autoclave.

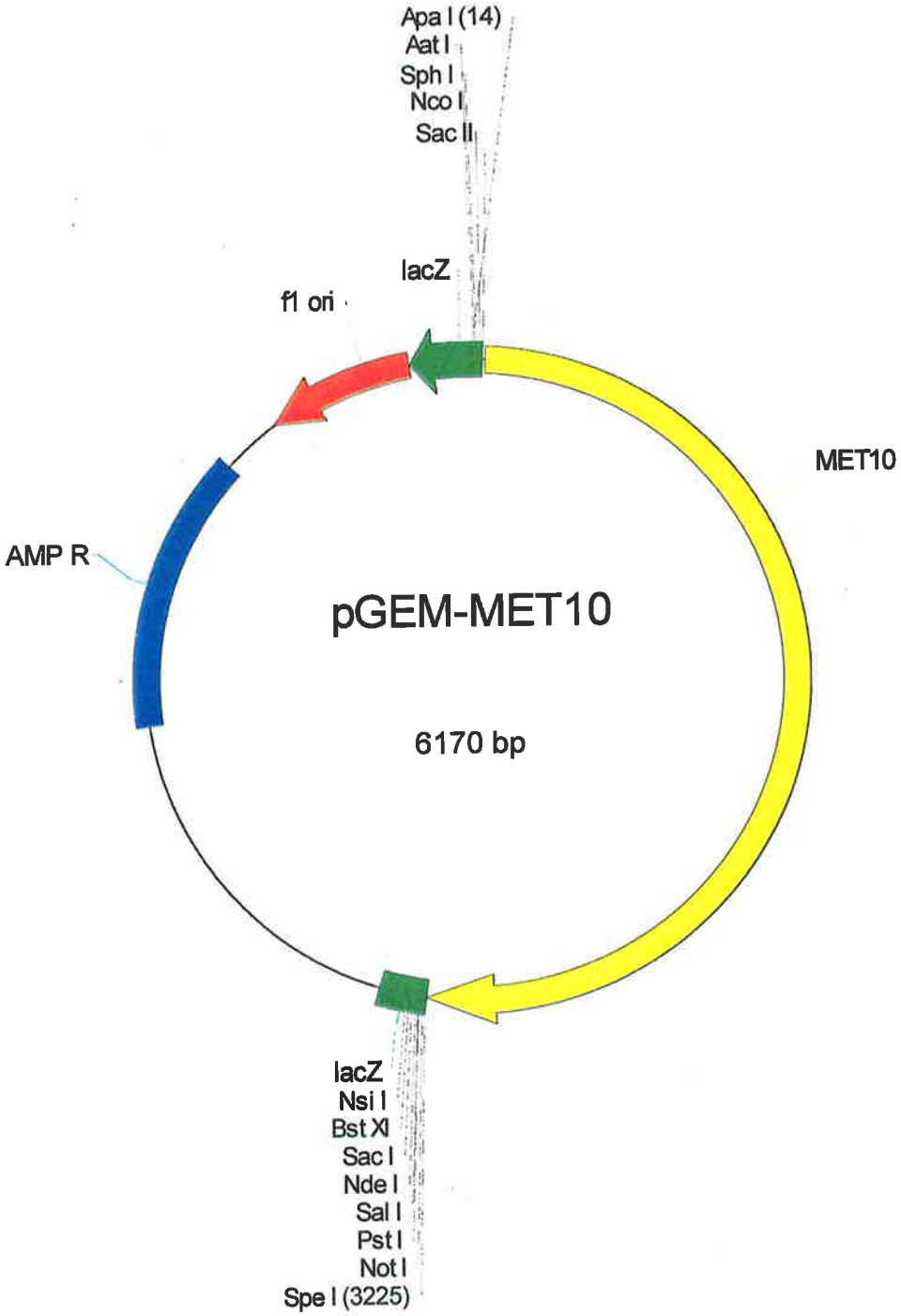
Add 40g glucose (100ml of a 40% solution)

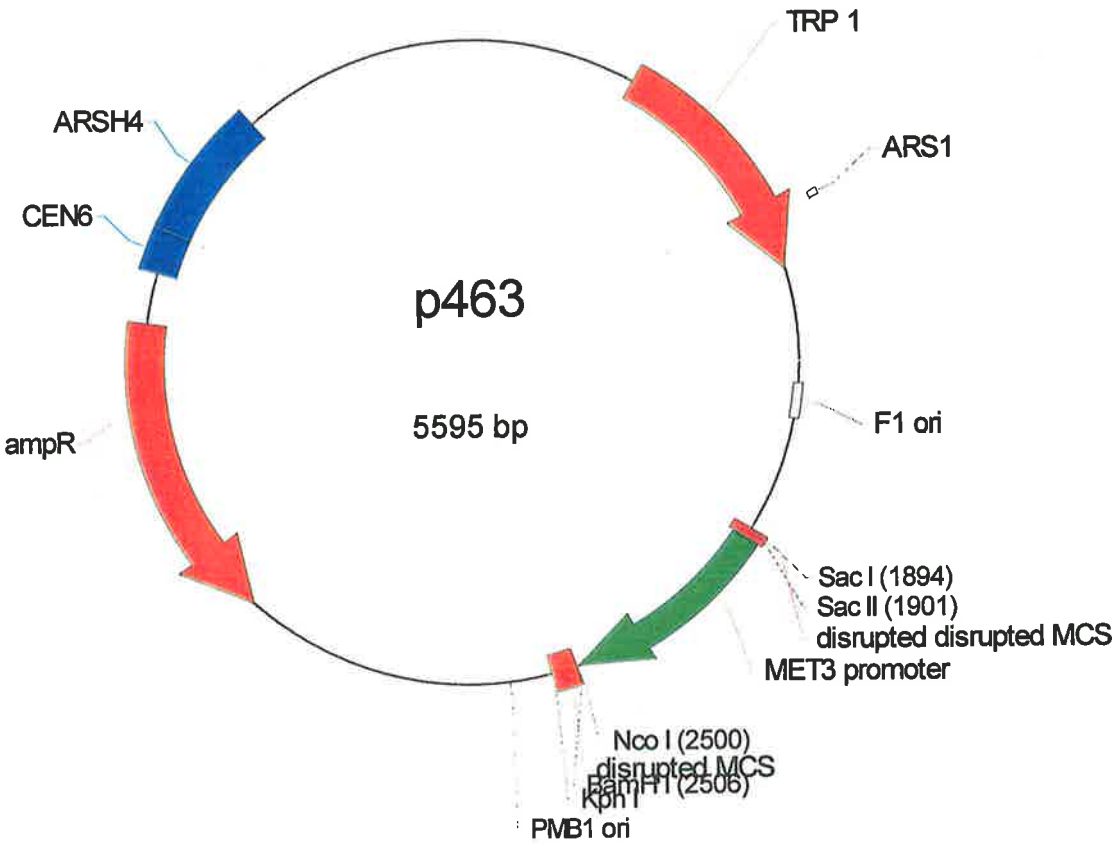
Add lead nitrate (2ml of 0.5g/ml solution) after agar has cooled to 55°C or less.

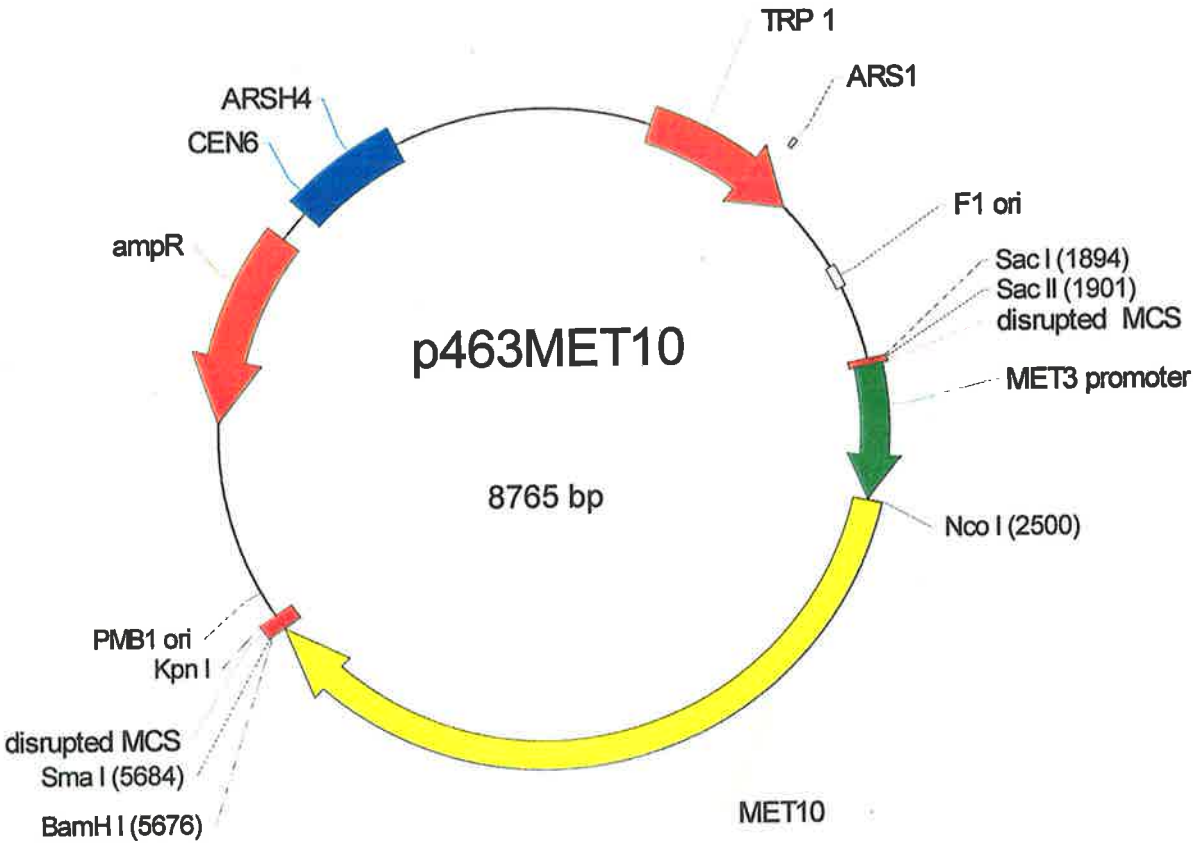
Mix well.

APPENDIX TWO

Plasmids used in this study:




TRP 1
ARS1
ARSH4
CEN6
p463
5595 bp
F1 ori
ampR
Sac I (1894)
Sac II (1901)
disrupted disrupted MCS
MET3 promoter
Nco I (2500)
disrupted MCS
BamH I (2506)
Kpn I
PMB1 ori


TRP 1
ARS1
F1 ori
Sac I (1894)
Sac II (1901)
disrupted MCS
MET3 promoter
Nco I (2500)
MET10
BamH I (5676)
Sma I (5684)
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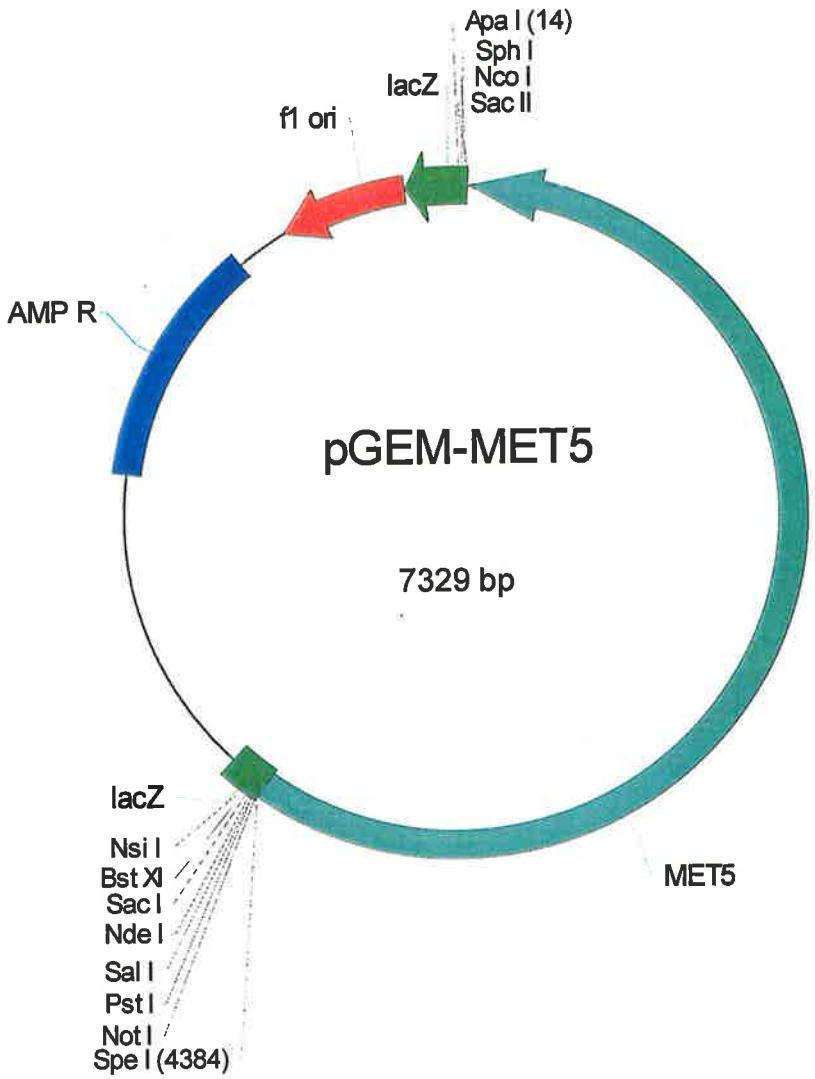
Kpn I
PMB1 ori
ampR
CEN6
ARSH4
p463MET10
8765 bp


Apa I (14)
Sph I
Nco I
Sac II
lacZ
f1 ori
AMP R
pGEM-MET5
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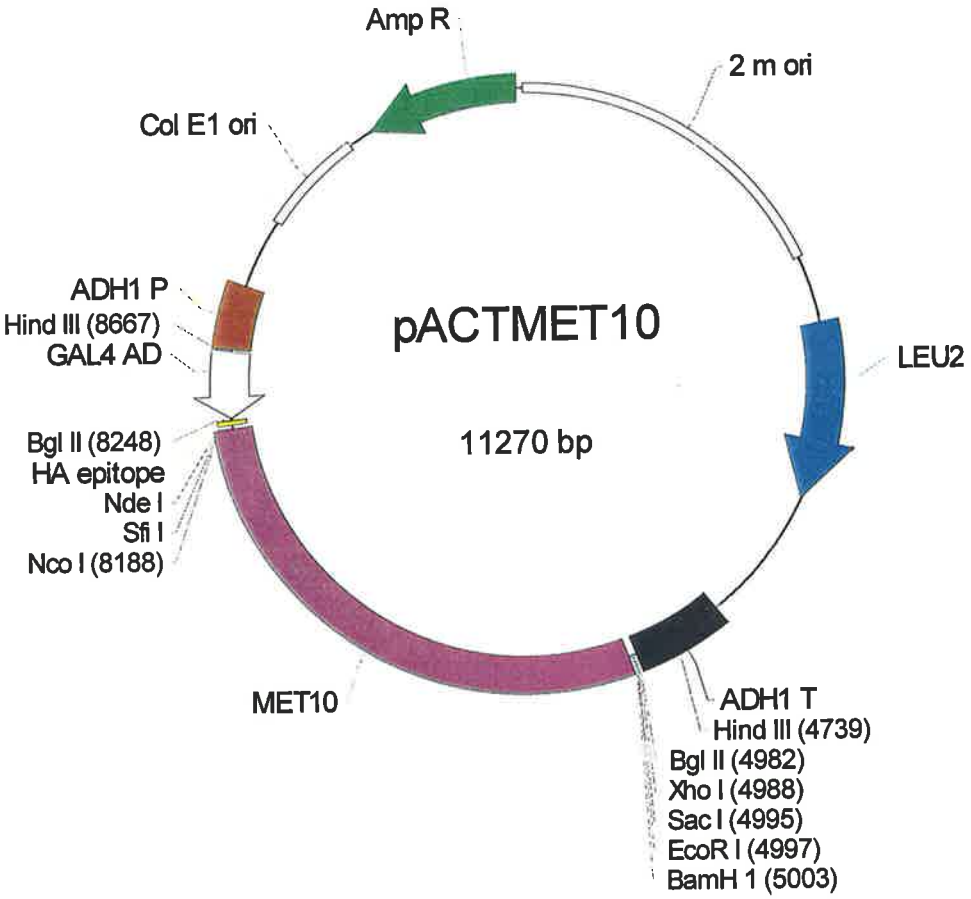
lacZ
Nsi I
Bst XI
Sac I
Nde I
Sal I
Pst I
Not I
Spe I (4384)
MET5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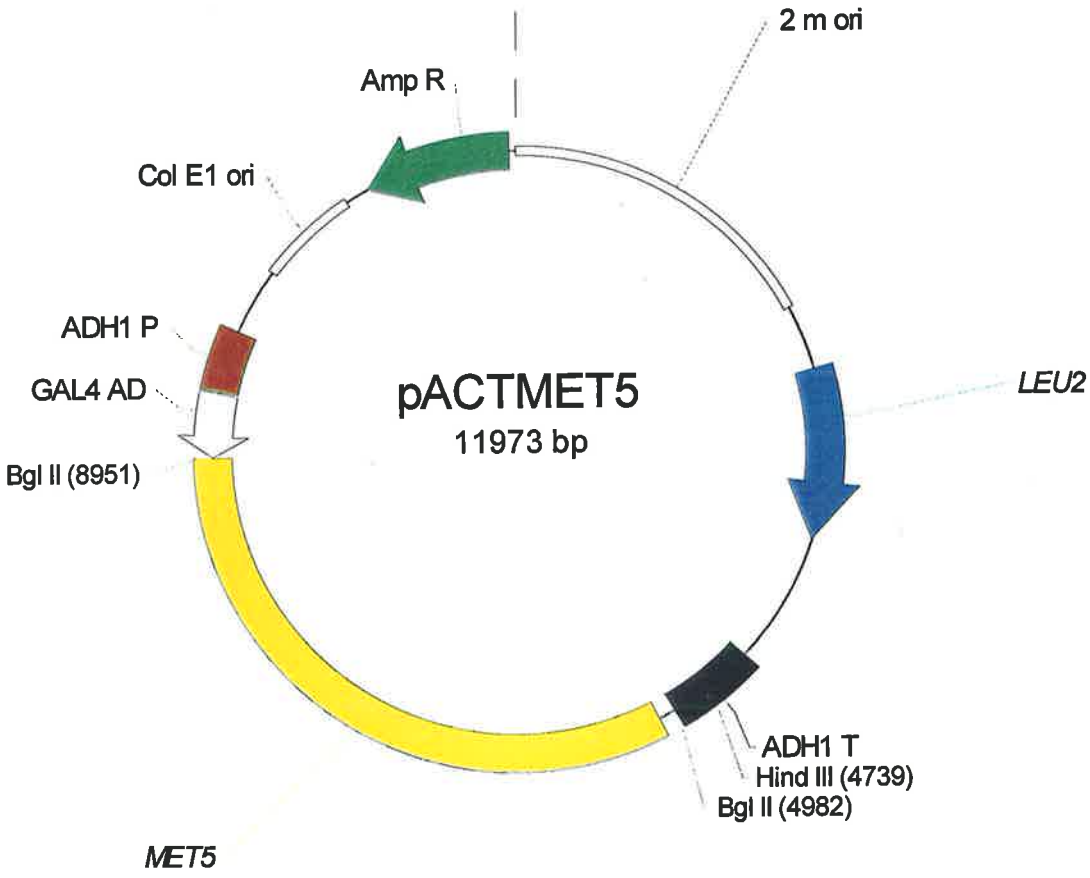
Col E1 ori
ADH1 P
Hind III (8667)
GAL4 AD
pACTMET10
11270 bp
LEU2
Bgl II (8248)
HA epitope
Nde I
Sfi I
Nco I (8188)
MET10
ADH1 T
Hind III (4739)
Bgl II (4982)
Xho I (4988)
Sac I (4995)
EcoR I (4997)
BamH 1 (5003)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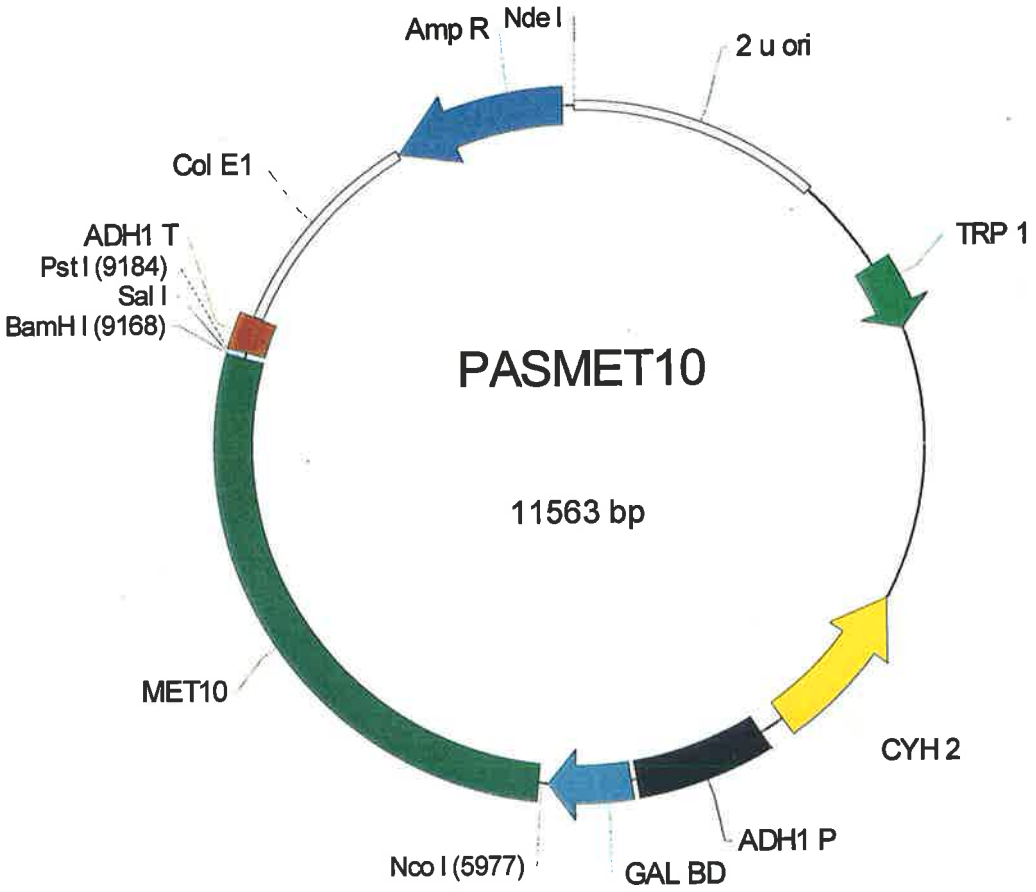
Amp R
Col E1 ori
ADH1 P
GAL4 AD
pACTMET5
11973 bp
LEU2
Bgl II (8951)
ADH1 T
Hind III (4739)
Bgl II (4982)
MET5


Amp R
Nde I
2 u ori
Col E1
ADH1 T
Pst I (9184)
Sal I
BamH I (9168)
TRP 1
PASMET10
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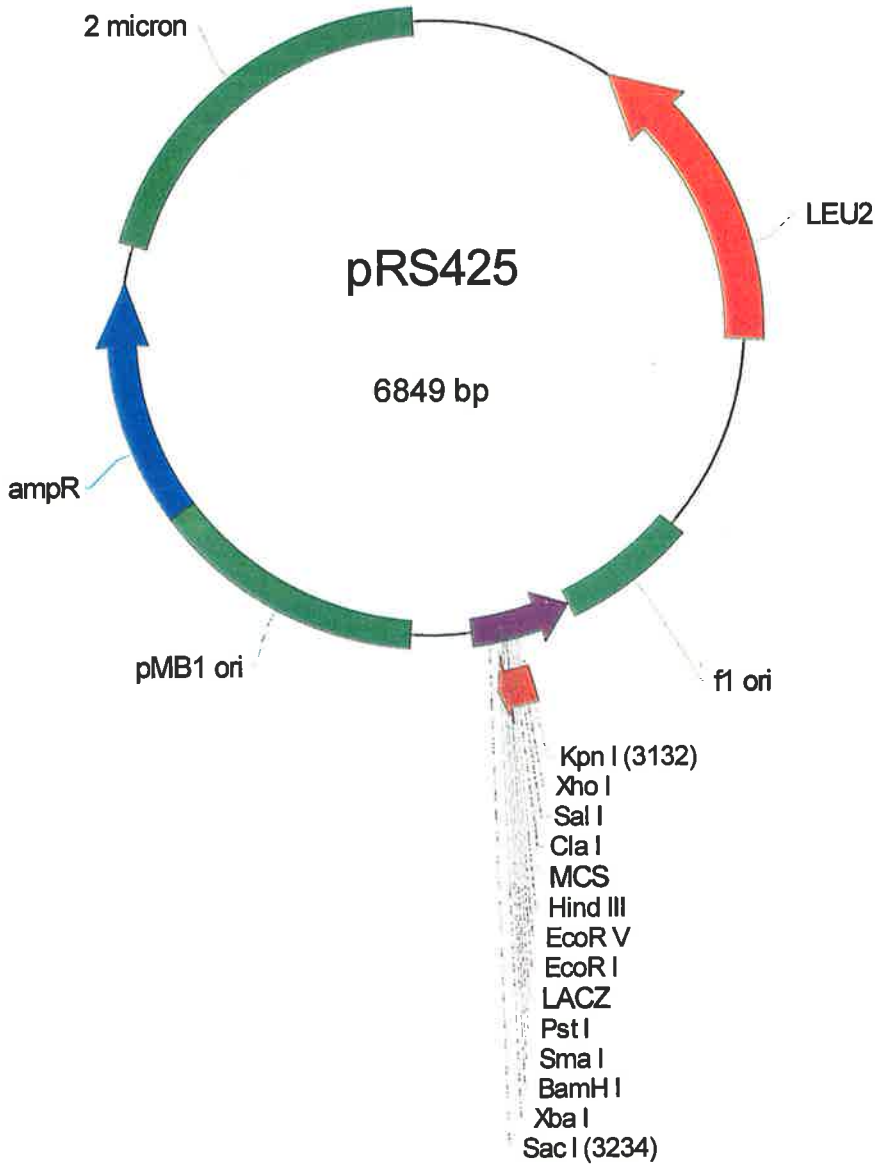
MET10
CYH 2
ADH1 P
Nco I (5977)
GAL BD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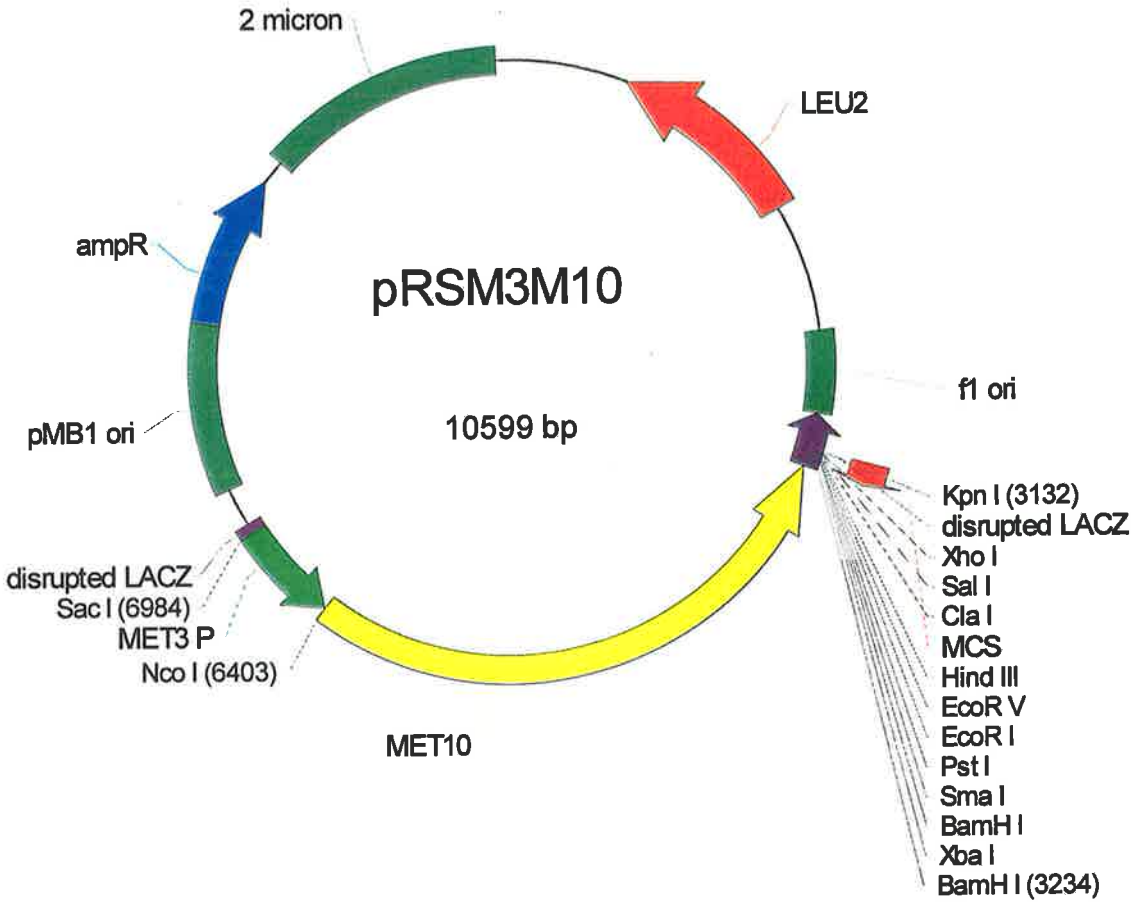
LEU2
pRS425
6849 bp
ampR
pMB1 ori
f1 ori
Kpn I (3132)
Xho I
Sal I
Cla I
MCS
Hind III
EcoR V
EcoR I
LACZ
Pst I
Sma I
BamH I
Xba I
Sac I (3234)


2 micron
LEU2
ampR
pRSM3M10
10599 bp
f1 ori
pMB1 ori
Kpn I (3132)
disrupted LACZ
Xho I
Sal I
Cla I
MCS
Hind III
EcoR V
EcoR I
Pst I
Sma I
BamH I
Xba I
BamH I (3234)
disrupted LACZ
Sac I (6984)
MET3 P
Nco I (6403)
MET10

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ERRATUM

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Table of genotypes of *E.coli* and *S.cerevisiae* strains used in this study.

Strain	Genotype
Escherichia coli	
DH5αF'	F' *endA1 hsdR17* ($r_K^- m_k^+$) *supE44 thi-1 recA gyrA* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR* (ϕ80*dlac*Δ(*lacZ*)*M15*)
B834	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm met*
BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm met* (DE3) pLysS (Cm^R)
Saccharomyces cerevisiae	
CG-1945	*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 cyh^{r}2 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 URA3::GAL4*$_{17\text{-}mers(x3)}$*CYC1*$_{TATA}$*-lacZ*
Y190	*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4Δ gal80Δ cyh^{r}2 LYS::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3 URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*
Y187	*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3 112 gal4Δ met$^-$ gal80Δ URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*
90844	*his3Δ leu2Δ1 lys2Δ202 trp1Δ63 ura3-52 GAL2*
ΔM10-1	*MATα met10-Δ1*
CS222	*Met10-Δ1 leu2Δ1 lys2 ura3-52*

Equation omitted from Appendix One

Equation to determine concentration of sulfite by titration in colorimetric sulfite assay:

Conc. of sulfite (g/l)= 0.64 x (20.00 - used sodium thiosulfate in ml)