REGULATION IN AROMATIC AMINO ACID BIOSYNTHESIS

IN *Saccharomyces cerevisiae*

Judy Forsyth Brown

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1987

ACKNOWLEDGEMENTS

I would like to express my grateful thanks to Dr Ian W Dawes for his helpful advice, guidance and encouragement throughout this project and the preparation of this thesis.

I would also like to thank Prof J F Wilkinson in whose department the work was carried out, and I acknowledge the receipt of a studentship grant fron the Science and Engineering Research Council and G D Searle & Co.

Further thanks are extended to all members of the Microbiology Department, past and present, for making it such an enjoyable place to work. A special thankyou to everyone in the EPGL for their appreciation of fashion and music.

Finally, I am grateful to my friends and family for their support, both moral and financial, throughout my education.

DECLARATION

I hereby declare that this thesis has been composed by myself, and that the work presented herein is my own. Any collaborative work has been duly acknowledged.

JUDY F. BROWN

1987

LIST OF CONTENTS

ABSTRACT

In *Saccharomyces cerevisiae* the enzymatic pathway for biosynthesis of aromatic amino acids is branched, culminating in the formation of phenylalanine, tyrosine and tryptophan. The last biosynthetic reaction common to phenylalanine and tyrosine, the formation of prephenate from chorismate, is catalsyed by the enzyme chorismate mutase, encoded by the *ARO7* gene..

An *aro7* mutant of *Saccharomyces cerevisiae* was used to screen a wild-type gene bank, based on the vector YEp13, for complementation of the *aro7* phenotype. One such transformant was isolated; *aro7* complementation being correlated with the presence of the plasmid (pJFB1). Yeast strains transformed with pJFB1 displayed increased levels of chorismate mutase activity compared to wild-type.

A restriction map of pJFB1 has been obtained and the cloned insert estimated to 5.3 kb. Deletion analysis localised the the complementing activity to a region spanning a *Hin*dIII site at 12.7 on the map. Transcriptional analysis identified a polyadenylated transcript of approximately 1 kb.

In vitro mutagenesis of pJFB1 resulted in several transformants resistant to levels of the phenylalanine analogue β-thienylalanine inhibitory to the wild-type transformant. Chorismate mutase assays indicated that two of the mutants (J14-26IV6 & J14-26IV9) were resistant to the feedback-inhibition by tyrosine displayed by wildtype strains. Analysis of the effect of other aromatic amino acids on chorismate mutase activity showed that tryptophan counteracted this inhibition.

Analysis of the effect of tyrosine in the growth media on enzyme activity indicated that the wild-type *ARO7* gene was repressed by tyrosine, a phenomenon not previously reported. Two of the β-thienylalanine resistant mutants (J14-26IV3 & J14-26IV9) appeared to be resistant to this repression.

This was confirmed by transcriptional analysis which showed that the level of *ARO7* transcript decreased with increasing tyrosine concentration. In strain J14-26IV9 the *ARO7* transcript level was not affected. J14-26IV9, therefore, appears to be a double mutant, resistant to both feedback-inhibition and repression by tyrosine.

1. INTRODUCTION

The systematic investigation of how amino acids are biosynthesised was well under way long before the field of biotechnology emerged. From its earliest beginnings, however,the commercial production of amino acids has been an important area of biotechnology. The first report of the production of an amino acid describes the preparation from sea tangles of the flavouring agent sodium glutamate (Ikeda, 1908). This work led to the commercial production of monosodium glutamate as a seasoning agent from acid hydrolysates of wheat gluten or soyabean protein. Today glutamate, cysteine, glycine, alanine, lysine, methionine, threonine and tryptophan are routinely used throughout the world as flavour enhancers and additives in both human and animal feedstuffs.

The amino acid phenylalanine was, however, of relatively minor importance until the dynamic rise in popularity of the new artificial sweetener, Aspartame, caused interest in the commercial production of phenylalanine to increase dramatically. Aspartame, an aspartyl phenylalanine methyl ester produced by G.D. Searle & Co., now dominates the market for low calorie sweeteners, being some two hundred times sweeter than sugar. The demand for phenylalanine has, therefore, risen accordingly. Several different methods of producing phenylalanine have been developed by the main suppliers of the amino acid (Klausner, 1985) but their cost still remains the limiting factor in the production of Aspartame.

In an effort to increase production and reduce expenditure, research has turned to genetics with the aim of isolating bacterial

strains that overproduce phnylalanine. This has already been achieved in *E. coli* (B. Carter, personal communication). In such a highly competitive market, however, new ways are being sought to maximise production. These must remain financially feasible when applied to industrial manufacture.

Recently interest has been shown in the yeast *Saccharomyces cerevisiae* as a means of producing phenylalanine. The potential advantages of using yeast as opposed to bacterial systems include :

1) The biosynthetic pathway for amino acid production in yeast is fairly well understood and characterised by genetic mutations (Jones & Fink, 1982).

2) Its eukaryotic life cycle means that cells can grow as stable haploids or diploids. This allows the application of many classical genetic techniques. In the isolation of phenylalanine overproducers, the stable diploid phase offers a distinct advantage over haploid bacterial strains by allowing the preferential selection of dominant mutations.

3) Many of the recombinant DNA techniques commonly used with bacteria can now be applied successfully to yeast systems. Combined with classical methods, these result in a wide range of procedures available for the genetic manipulation of the cell.

4) Large scale fermentation technology in yeast is well established and there are no toxicity or phage problems associated with its use.

The original aim of this project was, therefore, to genetically manipulate the yeast *Saccharomyces cerevisiae* in a directed manner to maximise overproduction and secretion of phenylalanine with the long

term view that this may result in a very efficient method of producing the amino acid at commercially useful levels.

1.1 THE AROMATIC AMINO ACID BIOSYNTHETIC PATHWAY.

The enzymatic pathway for the biosynthesis of phenylalanine in yeast is branched, culminating in the formation of the three aromatic amino acids; phenylalanine, tyrosine and tryptophan. It is similar but not identical to the pathway in *E. coli* in which it has ben studied in more detail. The pathway has been well characterised by the isolation of mutant strains which are defective in various key biosynthetic steps throughout the system. The complete aromatic amino acid biosynthetic pathway for *Saccharomyces cerevisiae* is illustrated in Fig. 1.1 which gives gene designations and enzymes involved. Table 1.1 lists the enzymes produced by the specific genes.

Synthesis of phenylalanine, tyrosine and tryptophan proceeds via a common route to chorismate, at which point the pathway branches. One branch leads to tryptophan, the other to phenylalanine and tyrosine.

The common pathway begins with the condensation of erythrose-4-phosphate, an intermediate of the pentose phosphate pathway, and phospho*enol*pyruvate, an intermediate in glycolysis, to yield deoxy-D-*arabino*-D-heptulosonate-phosphate (DAHP) (Jones & Fink, 1982). This reaction is catalysed by either of two isoenzymes of DAHP synthase (Lingens *et al.*, 1967) encoded by the *ARO3* and *ARO4* genes (Meuris *et al.*, 1967). Meuris and his coworkers isolated mutant strains bearing deficiencies in both synthases and showed that a double mutant strain was auxotrophic for all three aromatic amino

Figure 1.1 : Biosynthetic pathway of aromatic amino acids in *Saccharomyces cerevisiae*. Genetic blocks are indicated. (Adapted from Jones & Fink, 1982)

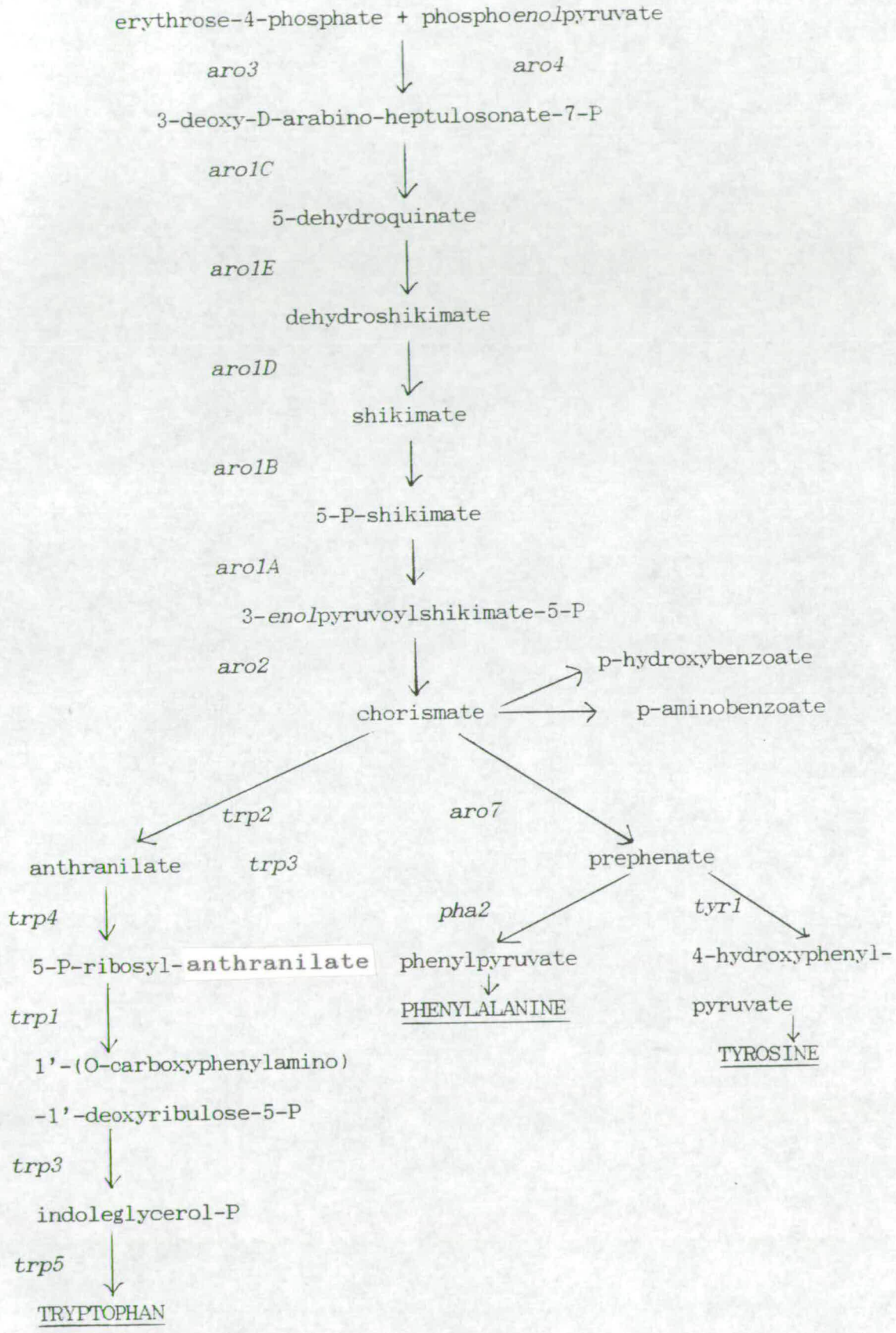

erythrose-4-phosphate + phospho*enol*pyruvate
aro3
aro4
3-deoxy-D-arabino-heptulosonate-7-P
aro1C
5-dehydroquinate
aro1E
dehydroshikimate
aro1D
shikimate
aro1B
5-P-shikimate
aro1A
3-*enol*pyruvoylshikimate-5-P
aro2
chorismate
p-hydroxybenzoate
p-aminobenzoate
trp2
aro7
anthranilate
trp3
prephenate
trp4
pha2
tyr1
5-P-ribosyl-anthranilate
phenylpyruvate
4-hydroxyphenyl-
trp1
PHENYLALANINE
pyruvate
1'-(O-carboxyphenylamino)
TYROSINE
-1'-deoxyribulose-5-P
trp3
indoleglycerol-P
trp5
TRYPTOPHAN

TABLE 1.1 Genes and enzymes in the aromatic amino acid biosynthetic pathway

GENE	ENZYME
ARO3/ARO4	deoxy-D-heptulosonatephosphate synthase
ARO1C	dehydroquinate synthase
ARO1E	dehydroquinate dehydratase
ARO1D	shikimate dehydrogenase
ARO1B	shikimate kinase
ARO1A	3-*enol*pyruvoylshikimate-5-P synthase
ARO2	chorismate synthase
ARO7	chorismate mutase
PHA2	prephenate dehydratase
TYR1	prephenate dehydrogenase
TRP2/TRP3	anthranilate synthase
TRP4	anthranilate transferase
TRP1	anthranilate isomerase
TRP3	indole-3-glycerol-P synthase
TRP5	tryptophan synthase

acids plus p-aminobenzoate and p-hydroxybenzoate.

The *ARO1* cluster of *Saccharomyces cerevisiae* is thought to be a single transcription unit encoding one multifunctional polypeptide of molecular weight 15,000**Da**(De Leeuw, 1967). The five sequential enzymatic activities convert DAHP to 3-*enol*pyruvoyl shikimate-5-phosphate (Gaertner & Cole, 1977). Genetic and biochemical evidence indicates that this organisation is analogous to that found in *Neurospora crassa* (Lumsden & Coggins, 1969) and probably other fungi (Ahmed & Giles, 1969). In contrast to this, the bacterial arrangment is one of scattered genes and separate products (Pittard & Wallace, 1966). The next step is the generation of chorismate. This is catalysed by chorismate synthase, the product of the *ARO2* gene (de Leeuw, 1967).

From chorismate, the last common precursor, the pathway branches. One branch leading to tryptophan production, the other to the production of phenylalanine and tyrosine.

The products of the *TRP2* and *TRP3* genes form a protein complex, anthranilate synthase, even though they are transcribed from two separate genes (Doy & Cooper, 1966). Together they catalyse the formation of anthranilate from chorismate, the first step in the tryptophan branch.

The *TRP3* gene product is also active irrespective of its aggregation with anthranilate synthase. After the two enzymatic steps catalysed by *TRP4* and *TRP1*, the *TRP3* protein functions again to catalyse the formation of indole-3-glycerol-phosphate. Accordingly there exists mutants in *TRP3* influencing either the indole-3-glycerol-phosphate synthase activity, or the anthranilate synthase activity or both activities together (Schurch, 1982).

Although apparently a single polypeptide, tryptophan synthase catalyses a two step reaction resulting in the production of tryptophan (Zalkin & Yanofsky, 1982).

The branch in the pathway leading to phenylalanine and tyrosine proceeds by rearrangment of chorismate to yield prephenate. The enzyme responsible for this step is chorismate mutase, specified by the *ARO7* gene (Kradolfer *et al.*, 1977). Mutants in the gene are auxotrophic for both phenylalanine and tyrosine.

Again the pathway branches. The route leading to the synthesis of tyrosine begins with the conversion of prephenate to 4-hydroxyphenyl pyruvate, the immediate precursor of tyrosine, by the enzyme prephenate dehydrogenase, the *TYR1* gene product (Lingens *et al.*, 1966).

Alternatively, prephenate dehydratase, encoded by the *PHA2* gene (Lingens *et al.*, 1966), catalyses the formation of phenylpyruvate which is transaminated to phenylalanine. There is genetic and biochemical evidence for the existence, in yeast, of multiple transaminase activities which carry out this final step (Jones & Fink, 1982).

1.2 REGULATION OF THE PATHWAY.

The regulation of metabolic pathways in eukaryotic cells is still poorly understood compared with that of prokaryotes. A problem in studying the regulation of a biosynthetic pathway in yeast is that the structural genes are generally scattered over the genome and each may be seperately regulated by a different set of signals, as well as by different receptors for the same signals. In bacteria, the genes for a given pathway are often linked in an operon, and all respond to the same metabolic signals mediated through the same receptors (operators and promoters). For this reason the analysis of one gene's regulation in *E. coli* may provide sufficient information on the regulation of an entire pathway. In *Saccharomyces cerevisiae* no operons exist and no polycistronic mRNA seems to be synthesised (Peterson & McLaughlin, 1973). This makes a study of regulation of a pathway much more difficult since each gene may be regulated differently.

In yeast and bacteria the biosynthesis of amino acids is regulated by both feedback inhibition and repression. Both methods respond to the amino acid levels in the cell. In feedback inhibition the amino acid interacts with its target enzyme allosterically so that the enzyme loses its affinity for its biosynthetic substrate. The loss of activity is therefore immediate. The most common situation is that the first enzyme of a pathway is inhibited by the end product of the pathway as a whole. In repression the endproduct of the biosynthetic pathway inhibits transcription of certain enzymes thereby reducing their levels of expression.

In yeast, feedback inhibition is reported to be the principle

regulatory control in the formation of the aromatic amino acids. Although the aromatic pathways are subject to general amino acid control there is no evidence for specific transcriptional regulation (Deneu & Demain, 1981). Repression is a less important control since the amplitude of the effect is low. Unlike bacteria in which repression can cause more than a hundredfold decrease in enzyme levels, repression in yeast may be only a twofold effect (Miozarri *et al.*, 1978).

The feedback inhibition of both DAHP synthase enzymes provides the prime control and limits entry into the system (see Fig.1.2). Both the *ARO3* and the *ARO4* genes code for DAHP synthase but the product of the former is inhibited by phenylalanine (Meuris, 1967) and uncharged tRNA-phe (Meuris, 1973) while the latter product is inhibited by tyrosine (Lingens *et al.*, 1966) and by tyrosyl-tRNA (Meuris, 1973). Mutants defective in either gene are prototrophs because of the compensating isoenzyme. However, *aro3* mutants are unable to grow in the presence of tyrosine since it inhibits the functional *ARO4* gene. Reciprocally *aro4* mutants are phenylalanine sensitive. So far as is known, tryptophan plays no part in this regulation but rather exerts control in an indirect fashion (see later). The subsequent *ARO1* and *ARO2* encoded steps of the common pathway seem to be insensitive to allosteric effects.

At chorismate the pathway branches. In the normal yeast cell tryptophan is preferentially synthesised (Lingens *et al.*, 1967), due to the kinetic parameters of the pathway-specific enzymes in their reactions with the common chorismate intermediate. Anthranilate synthase (*TRP2*, *TRP3*) shows normal Michaelis-Menten kinetics, that is,

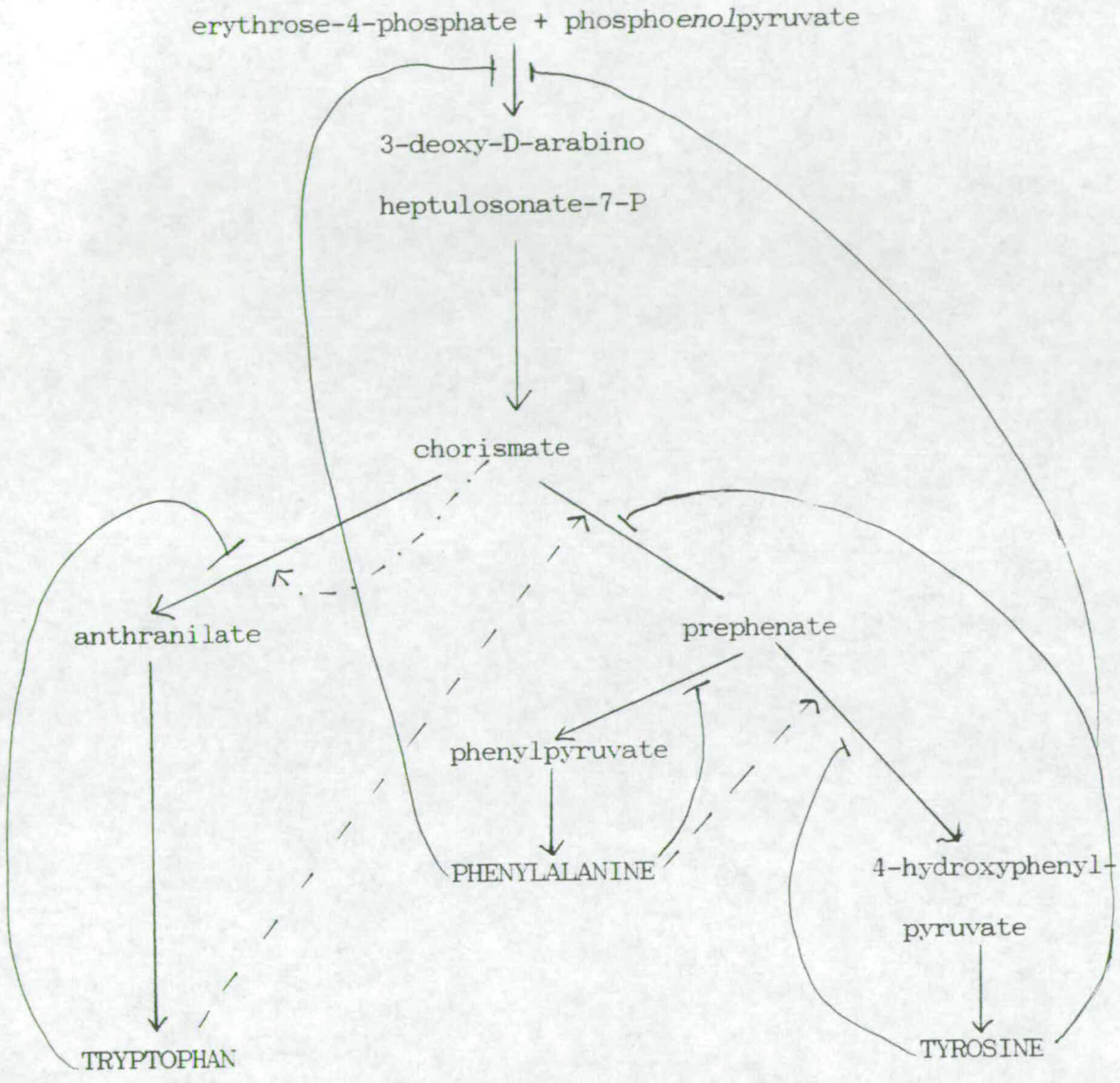


Figure 1.2 : Regulation of the aromatic amino acid pathway. (Adapted from Jones & Fink, 1982). ——⊣ Feedback inhibition, — · — · → Counteracts feedback inhibition, — — → overcomes feedback inhibition and activates.

it can act on the substrate at low concentrations. Chorismate mutase (*ARO7*) displays sigmoidal kinetics and, consequently, is active only when chorismate concentrations increase. Anthranilate synthase, therefore, has a higher affinity than chorismate mutase for chorismate, but once the cell's requirment for tryptophan is satisfied, excess tryptophan feedback inhibits the *TRP2* product resulting in increased concentrations of chorismate for chorismate mutase activity. In addition this activity may be enhanced by tryptophan-induced conformational changes (Kradolfer *et al.*, 1977). As well as being tryptophan activated, chorismate mutase is inhibited by tyrosine (Lingens *et al.*,1966) so that the balance between these end products controls the direction of chorismate metabolism. Phenylalanine does not seem to have any regulatory role up to this point (Lingens *et al.*, 1966).

Prephenate, the product of the chorismate mutase reaction, is the branchpoint between phenylalanine and tyrosine. Once again the kinetic properties of the enzymes determine the preferential synthesis of phenylalanine over tyrosine. Prephenate dehydratase (*PHA2*) displays normal Michaelis-Menten kinetics whereas prephenate dehyrogenase shows positive co-operativity (Lingens *et al.*,1967). Production of phenylalanine is determined by the end product inhibition of prephenate dehydratase while tyrosine synthesis is controlled by the allosteric inhibition, by tyrosine, of prephenate dehydrogenase. Phenylalanine eliminates this inhibition and normalises the sigmoidal kinetics of prephenate dehydrogenase in the absence of tyrosine (Lingens *et al.*,1967). Thus when phenylalanine is in excess prephenate is channelled into the tyrosine arm, due to the fact that the dehydratase is inhibited. The activity of the dehyrogenase is

enhanced in a manner analogous to the tryptophan effect on chorismate mutase. When phenylalanine is limiting, however, prephenate will pass into the phenylalanine arm, whether tyrosine is present or not, because of the low affinity of the dehydrogenase for prephenate when phenylalanine is absent. As a consequence of these reactions the aromatic amino acid pools normally fill in the order : tryptophan, phenylalanine, tyrosine (Jones & Fink, 1982).

Knowledge of the regulatory mechanisms controlling the level of transcription in yeast is not extensive and the more complex arrangement of the genetic material makes extrapolation of data obtained from prokaryotes difficult.

Preliminary work by Lingens *et al.*(1967) found that transcription of the *ARO3* and *ARO4* genes is not affected by any of the aromatic amino acids, either singly or in combination. Chorismate mutase seems to be induced by tryptophan and prephenate dehydrogenase by phenylalanine, while prephenate dehydratase is repressed by phenylalanine. The significance of these effects *in vivo* is still unclear.

More information, however, is available on the control of the tryptophan pathway. Doy & Cooper (1966) reported that anthranilate synthase production is repressed by tryptophan. In contrast, Fantes *et al.* (1976) found no repression of the enzymes of the pathway by tryptophan. In an effort to resolve these contradictory reports, Miozzari and his co-workers (1978) showed that the activity of all the tryptophan enzymes is proportional to their gene dosage, indicating that they are transcribed and translated constitutively. An increase in enzyme levels was elicited by growth under tryptophan limiting

conditions but it was shown that this derepression is not a specific response of the cell to tryptophan limitation, since derepression of the enzymes also occurred upon limitation of unrelated amino acids such as leucine, histidine and arginine.

1.3 GENERAL CONTROL OF AMINO ACID BIOSYNTHESIS

Co-regulation of enzymes of different amino acid biosynthetic pathways in fungi, called metabolic interlock, was first described in *Neurospora crassa* (Carsiotes & Jones, 1974). A similar regulatory system was described in *Saccharomyces cerevisiae* by Schurch *et al.* (1974) and has been termed "the general control of amino acid biosynthesis" by Delforge *et al.* (1975).

In yeast, a number of enzymes in different amino acid biosynthetic pathways are under this common control and derepress when any single amino acid becomes limiting. For example, starvation for histidine leads not only to elevated levels of histidine biosynthetic enzymes but also causes elevated levels of the tryptophan, arginine, lysine and isoleucine-valine pathways (Jones & Fink, 1982). This cross-pathway regulation is known to affect at least twenty-four different enzymes in the basic, the aromatic and other branched chain amino acid pathways (Jones & Fink, 1982).

Regulation appears to occur at the level of transcription because increased levels of several of the enzymes subject to the general control have been correlated with corresponding increases in mRNA levels (Messenguy & Dubois, 1983), and has been shown to be elicited by the under-charging of single tRNA molecules (Messenguy &

Delforge,1976).

Five trans-acting regulatory genes that act as positive effectors for derepression have been identified; *GCN1-5* (Schurch *et al.*, 1974; Wolfner *et al.*, 1975; Penn *et al.*, 1983). The wildtype products of *GCN1, 3* and *4* are necessary for initiation of biosynthetic gene mRNA derepression, whereas *GCN2*, itself under general control, is required for maintenance of elevated mRNA levels (Penn *et al.*, 1984). *GCN2* has been shown to be transcriptionally regulated, whereas *GCN4* is translationally regulated (Hinnebusch, 1984). There are also trans-acting control genes which are negative regulators, acting to limit the expression of genes under general control. Three such genes, *GCD1, 2* and *3* have been identified (Wolfner *et al.*, 1975; Myers *et al.*,1986).

Analysis of the interactions between different mutations by several research groups (Wolfner, *et al.*, 1975, Niederberger *et al.*, 1981, Hinnebusch & Fink, 1983; Myers *et al.*, 1986) has shown that, in the cases studied, constitutive mutations are epistatic to the mutations causing non-derepressible synthesis. This has allowed the construction of a model concerning the roles of the different regulatory molecules in general amino acid control (see Fig. 1.3).

The coincident control of a large number of genes involved in amino acid biosynthesis suggests a signal common to all of the genes. Cis-acting regulatory mutations have been identified in the 5' non-coding regions of the *HIS3*(Struhl, 1982) and *HIS4* (Donahue *et al.*, 1983) genes which prevent normal derepression of these genes. Comparative analysis of the upstream regions has defined a recognition site for a positive regulatory factor that mediates general amino acid

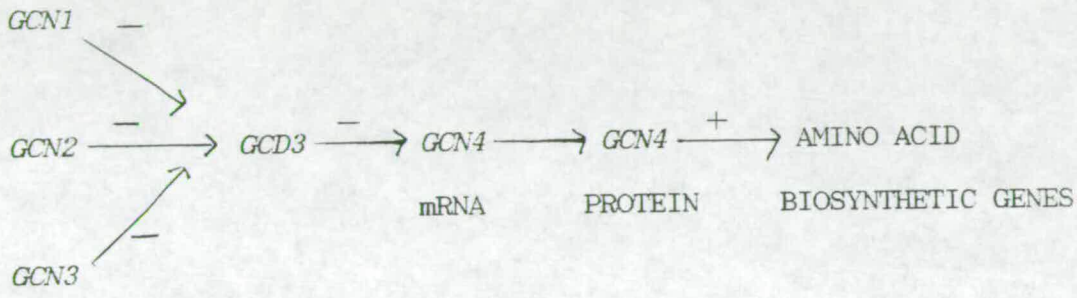


Figure 1.3 : Model of general amino acid control. (Adapted from Myers *et al.*, 1986).
- negative control, + positive control.

control. This site has a consensus sequence 5'TGACTC 3' and has been found in multiple copies upstream from sixteen different genes, all subject to general control (Hill *et al.*, 1986). The *GCN4* protein binds to this sequence, activating transcription (Hope & Struhl, 1985).

Studies with mutant strains defective in general control showed clearly that all the tryptophan biosynthetic genes except *TRP1* are subject to general control (Miozzari *et al.*, 1978), although in a non-coordinated manner (Niederberger *et al.*, 1981); each enzyme being derepressed to a different extent under various limiting conditions. This situation is similar for several pathways including enzymes in the isoleucine-valine pathway (Magee & Hereford, 1969). This demonstrates that under the influence of external amino acid imbalance the general control may derepress specifically appropriate target enzymes rather than all enzymes potentially under its control (Niederberger *et al.*, 1981). Possibly this is because, under the more physiological conditions of external aminoacid imbalance, the threshholds for derepression of most enzymes under the general control are not reached.

Recent work with *Saccharomyces cerevisiae ARO3* and *ARO4* genes (Teshiba *et al.*, 1986) has shown that both the DAHP synthase isoenzymes also respond equally well to the general control. This explains earlier results (Niederberger, 1981) which showed that addition of tyrosine and phenylalanine led to severe growth inhibition in *ndr* (*gcn*) mutant strains, in which there is no derepression, but not in the wild-type strain. Since normal growth was restored by tryptophan it suggests that growth inhibition was caused by tryptophan limitation due to feedback inhibition of the DAHP synthases by

phenylalanine and tyrosine. This does not happen in the wild-type presumably because tryptophan limitation will trigger the general control and the resulting derepression of the *ARO3* and *ARO4* genes will counteract the feedback inhibition allowing tryptophan to be synthesised.

Tyrosine alone also inhibits growth in *ndr* strains possibly due to phenylalanine limitation. This suggests that chorismate mutase is also under general control although it has not been tested directly. To date, there is no direct evidence showing that any of the other aromatic amino acid biosynthetic enzymes are under general control.

1.4 METHODS OF OBTAINING PHENYLALANINE OVERPRODUCTION

There are essentially three ways that over production of phenylalanine can be achieved in yeast:

1. The isolation of mutants resistant to phenylalanine analogues.
2. Isolation of regulatory mutants in defined genes in the pathway.
3. Using recombinant techniques, wild-type alleles or feedback resistant alleles of key genes in the pathway may be cloned.

I.4.1 **Analogue Resistant Mutants**

Traditionally, overproducers of amino acids have been isolated by selecting for resistance to amino acid analogues even when there has been no prior knowledge of the regulation of the biosynthetic pathway. On the assumption that certain phenylalanine analogues inhibit growth by being incorporated into the protein instead of phenylalanine and that the extent of this incorporation is dependent on the relative intracellular levels of phenylalanine and the analogue, it would be expected that one class of analogue-resistant mutants would include ones with a greatly increased rate of phenylalanine synthesis. Overproducing strains of *E. coli*, *B. subtilis* and some Corynebacteria have been successfully isolated from mutants resistant to phenylalanine analogues (B. Carter, personal

communication). It seems reasonable, therefore, that such an approach should be feasible in yeast.

The major drawback in this approach, however, is that the genetic basis of over-production is unknown. Consequently it would be difficult to integrate this kind of programme with the more reasoned approaches of classical genetics or genetic engineering.

A programme of this nature would require the isolation and investigation of large numbers of mutants resistant to one or other of multiple analogues, so it is important that a simple screening procedure should be designed (see section 2.11).

There is a possibility that some of the resistant colonies might be analogue-uptake mutants instead of overproducers. These could be weeded out by replica-plating onto a range of different phenylalanine analogues. Overproducers should be cross-resistant whereas the majority of uptake mutants should not.

An alternative approach would be to mutagenise and screen diploid cells. Uptake mutants are by their nature recessive and consequently in a diploid cell both copies of the appropriate gene would require the introduction of a mutation. If the frequency of a mutation in one gene copy is 10^{-6} survivor^{-1} then a mutation in both copies will occur in 10^{-12} survivor^{-1} i.e. very rarely. It is likely that phenylalanine overproduction will result from regulatory mutants particularly feedback-resistant mutations. These by their nature may be dominant and therefore only one copy of the gene would need to be mutagenized to obtain a phenylalanine overproducer and thus an analogue-resistant mutant. It therefore seems reasonable to expect that mutagenesis of a diploid strain would preferentially result in the selection of

analogue-resistant mutants due to phenylalanine overproduction.

The above is a good example of the way one can use the life-cycle of yeast to advantage. Under appropriate conditions diploids will undergo meiosis and sporulation, resulting in the production of four haploid spores. If analogue-resistance is due to a single nuclear gene, two of the haploid spores will give rise to resistant colonies and two will give rise to sensitive colonies according to Mendelian segregation. It would, therefore, be easy to recover the mutant allele in haploid cells.

The ease of genetic analysis in yeast along with the fact that the genetic regulation of the phenylalanine biosynthetic pathway is well understood, should permit the more reasoned approach to phenylalanine overproduction through the isolation of particular gene mutations.

1.4.2 Regulatory Mutants

Flux through the phenylalanine pathway is achieved mainly through feedback inhibition and activation of key enzymes in the pathway. Thus by introducing primarily dominant mutations in the genes coding for these enzymes, it should be possible to manipulate yeast in a directed manner and so control the flow through to the phenylalanine branch, bypassing the controls that exist in wild-type cells.

The first point of regulation in *Saccharomyces cerevisiae* is the reaction catalysed by DAHP synthase. Separate, unlinked, genes code for this, *ARO3* and *ARO4*, their products being feedback inhibited by

NOTE - The following two pages are out of sequence.

should be feasible to isolate them using the same rationale as *aro7* mutants; as the flow is directed away from tyrosine and towards phenylalanine such mutants will have enhanced sensitivity to tyrosine analogues. Feedback insensitive mutants of *PHA2* should also be resistant to analogues of phenylalanine.

Such mutants have not been isolated in yeast although similar work has been carried out using *E. coli* (Im & Pittard, 1971). In *E. coli* chorismate is converted through prephenate to phenylpyruvate by the multifunctional enzyme chorismate mutase prephenate dehydratase. This enzyme is feedback inhibited by phenylalanine. Mutants were isolated which grew on concentrations of *p*-fluorophenylalanine inhibitory to wild-type, and found to excrete phenylalanine. After further analysis, however, these mutants turned out to be regulatory mutants; being insensitive to repression rather than inhibition by phenylalanine.

It should be possible, using these regulatory mutants, to divert the flow through the pathway to phenylalanine overproduction. This could be assisted by blocking the flow into the other branches with the use of defective alleles in the tryptophan and tyrosine pathways. This would involve, however, the further complication of adding tyrosine and tryptophan to the medium. It may, therefore, be advantageous to use "leaky" mutants. Such bradyotrophic strains would permit the synthesis of adequate levels of tryptophan and tyrosine without excessive utilization of chorismate and prephenate.

phenylalanine and tyrosine respectively. Cells which have a defective allele at *ARO4* will require *ARO3* encoded enzyme activity to provide the flux through the pathway to the three aromatic amino acids. The presence of high levels of phenylalanine in the medium will inhibit the activity of this enzyme and so cells will not grow in the absence of tyrosine. The required mutation is one that will make the *ARO3* enzyme resistant to inhibition by phenylalanine. Auxotrophic strains were isolated by Meuris *et al.* (1967) and feedback-insensitive strains obtained by selecting for growth of *aro4* strains in the presence of excess phenylalanine and absence of tyrosine (Meuris, 1973). Similar insensitive *aro4* mutants were selected by growth of *aro3* auxotrophs in an excess tyrosine but not of phenylalanine.

Chorismate mutase is subject to activation by tryptophan and inhibition by tyrosine (Lingens *et al.*, 1966). In order to facilitate the flux preferentially through this branch it should be feasible to introduce regulatory mutations in the structural gene *ARO7*. The type of mutation required is one that enhances the catalytic activity to such an extent that even in the presence of tyrosine and the absence of tryptophan, the flux is diverted towards the production of phenylalanine. This will result in the production of very little tryptophan and so such mutants can be selected by looking for enhanced tryptophan-analogue sensitivity. Such a protocol was used by Kradolfer and his collegues (1977) and analysis of the resulting mutants showed them to be insensitive to feedback inhibition by tyrosine.

The next regulatory point which could be abolished is the reaction catalysed by *PHA2*. Desirable mutations are those which make prephenate dehydratase resistant to inhibition by phenylalanine. It

1.4.3 Recombinant DNA Methods

The approach outlined in the previous section can be usefully allied to a recombinant DNA approach. Since defective alleles of each gene have been isolated (Jones & Fink, 1982) it should be possible to clone any gene in the pathway by complementation , using a gene bank from wild-type cells to transform the appropriate yeast mutant.

This has already been achieved with *ARO3* (Teshiba *et al.*, 1986), *TRP2* and *TRP3* (Aebi *et al.*, 1982). The *ARO1*, *TRP1* and *TRP4* genes have also been isolated by functional complementation of *E. coli* mutants (Larimer *et al.*, 1983, Struhl *et al.*, 1979, Walz *et al.*, 1978), an alternative method for isolating genes from a wild-type yeast DNA clone bank.

While it is possible to isolate a wild-type gene and introduce this on a multicopy plasmid into yeast, the enzyme produced may still be susceptible to feedback inhibition and multiple copies may not increase the flux of metabolites significantly. What is required is the introduction, by transformation, of the feedback-resistant allele. This is now quite possible using *in vitro* mutagenesis.

Traditional mutagenesis procedures involve chemical treatment of a whole cell and a means of isolating a mutant with the desired phenotype from a population of randomly mutagenized and wild-type cells. This isolation is aided by the ability to predict the growth phenotype of the mutant on certain nutrients or in the presence of drugs.

In vitro mutagenesis is extremely powerful in that only the DNA base sequence of a single gene need be altered. The mutagenised gene, on its plasmid vector, is reintroduced into the organism and mutants

of the required phenotype are selected or screened. Methods exist for introducing either random mutations throughout the gene, or directed to specific regions in the promoter, effector or active sites in the coding sequence (Shortle *et al.*, 1981). The mutations can be deletions or alterations in a single base (point mutations). Such methods could be used to generate feedback-resistant mutations or otherwise alter the regulation of specific enzymes.

Although the original aim of the project was to engineer overproduction of phenylalanine during the course of this work several new and interesting aspects of the regulation of the *ARO7* gene were discovered. *ARO7* encodes the enzyme chorismate mutase which catalyses the conversion of chorismate to prephenate (Fig. 1.4) and is known to be inhibited by tyrosine (Lingens *et al.*, 1966). Very little, however, is known about the regulation of expression of th *ARO7* gene in *Saccharomyces cerevisiae*.

In *E. coli* there are two enzymes with chorismate mutase activity; one, involved in the biosynthesis of tyrosine, also incorporates prephenate dehydrogenase activity (T-protein). The other is also a bifunctional protein, in the phenylalanine biosynthetic pathway, and contains prephenate dehydratase activity (P-protein) (Gibson & Pittard, 1968). The gene for the T-protein, *tyrA*, together with the structural gene for tyrosine-sensitive DAHP synthetase, *aroF*, and transaminase A form a single operon, the expression of which is controlled by the product of a regulator gene, *tyrR* (Im *et al.*, 1971). The *pheA* gene encoding the P-protein does not form an operon with any

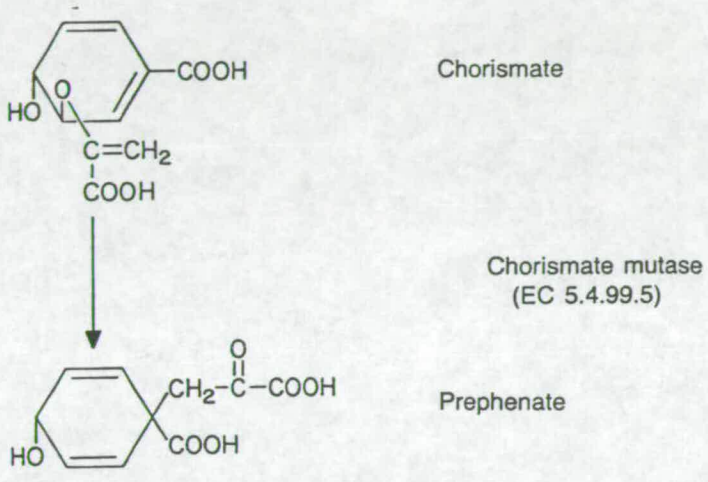


Figure 1.4 : Reaction catalysed by the *ARO7* encoded enzyme, chorismate mutase.

other genes but is controlled by the *pheR* gene product (Brown & Somerville, 1971). The *tyrR* and *pheR* genes are thought to encode protein aporepressors which, when combined with either of three aromatic amino acids, repress the appropriate biosynthetic genes (Brown & Somerville, 1971). In the T-protein, the chorismate mutase activity is not inhibited by tyrosine (Christopherson, 1985) while the P-protein can only be reduced to 70% activity by phenylalanine (Im & Pittard, 1971).

This project was begun in collaboration with the pharmaceutical company G.D. Searle & Co. who commercially produce Aspartame. After a year, however, they were taken over by Monsanto and subsequently withdrew from the project. The most advantageous consequence of this was that the pressure to concentrate research exclusively on phenylalanine overproduction was removed. This allowed a more thorough investigation of the control of *ARO7* expression in *Saccharomyces cerevisiae* and its implications for the regulation of aromatic amino acid biosynthesis.

2. MATERIALS AND METHODS

2.1 STRAINS, PLASMIDS AND DNA

2.1.1 Yeast Strains

The strains of *Saccharomyces cerevisiae* used are listed below :

Table 2.1: *Saccharomyces cerevisiae* strains used in this work

STRAIN	GENOTYPE	SOURCE
312	*MATα ade1 trp1 his4*	I.W.Dawes
314	*MATα ade5*	"
315	*MATa ade1 trp1 his4 leu2*	"
322	*MATa cdc28-15 tyr1 lys2 cyh1*	"
DC5	*MATa leu2-3 leu2-13 his3 can1*	"
SF747-19D	*MATa leu2-3 leu11-2 his4 ura3-52*	
	gal2 MEL4 suc0	"
A236-57B	*MATa leu2-3 aro7 trp1 met4*	"
	lys11 his8 suc2 MAL3	B.L.A.Carter
J14-26	*MATa leu2-3 leu11-2 aro7 trp1*	From SF747
	ura3-52 met4 lys11	X A237-57B

2.1.2 Bacterial Strains

The strains of *Escherichia coli* used are listed below :

Table 2.2: *E.coli* strains used in this work.

STRAIN	GENOTYPE	SOURCE
JA226	*recBC leuB6 trpE5 hsdR⁻ hsdM⁺ lacY600*	I.W.Dawes
HB101	F⁻ *recA13 hsdS20 ara1 proA2 lacY1 galK2 rpsh20 xyl5 mtl-1 supE44*	"

2.1.3 Cloning Vectors

The cloning vector used in the isolation of the *ARO7* gene was YEp13. This is a yeast episomal plasmid derived by Broach *et al.*, (1979) from the endogenous yeast 2 μm plasmid. The plasmid is 10.7 kb in size and has three distinct regions as shown in Figure 2.1 :

1. The yeast origin of replication contained in the 2 μm region.

2. Sequences from the bacterial plasmid pBR322 containing the ColE1 origin of replication and also the genes encoding ampicillin and tetracycline resistance. Single *Bam*HI and *Sal*I sites are located within the Tet^R gene providing a direct screening procedure for inserted DNA via insertional inactivation of the gene.

3. The yeast *LEU2* gene which allows selection in *leu2* mutants of *Saccharomyces cerevisiae*

YEp13 is a shuttle vector. The presence of both yeast and bacterial origins of replication allows autonomous replication in both hosts and the *leu2* gene and those coding for antibiotic resistance provide selectable markers in yeast and *E.coli* respectively. This "shuttle" property is very important, allowing rare recombinant molecules to be amplified in *E.coli* after initial transformation and isolation from yeast.

2.1.4 *S. cerevisiae* Gene Library

The library used to screen for the *ARO7* gene was constucted by Nasmyth & Tatchell,(1980) by the partial digestion of total yeast DNA with the restriction enzyme *Sau*3A. The fragments generated were cloned into the unique *Bam* H1 site in YEp13, producing a pool of plasmids containing sequences representing, theoretically, the complete genome of *S.cerevisiae*.

2.1.5 Probe for rRNA

A shuttle vector comprising of a 1 kb *Xba*I/*Sst*I pYIrG12 fragment, containing the 18s rRNA gene (Peteset *al.*, 1978), cloned into pSP65 (Santiago *et al.*, 1986) was obtained from A.J.P. Brown, Dept of Genetics, University of Glasgow.

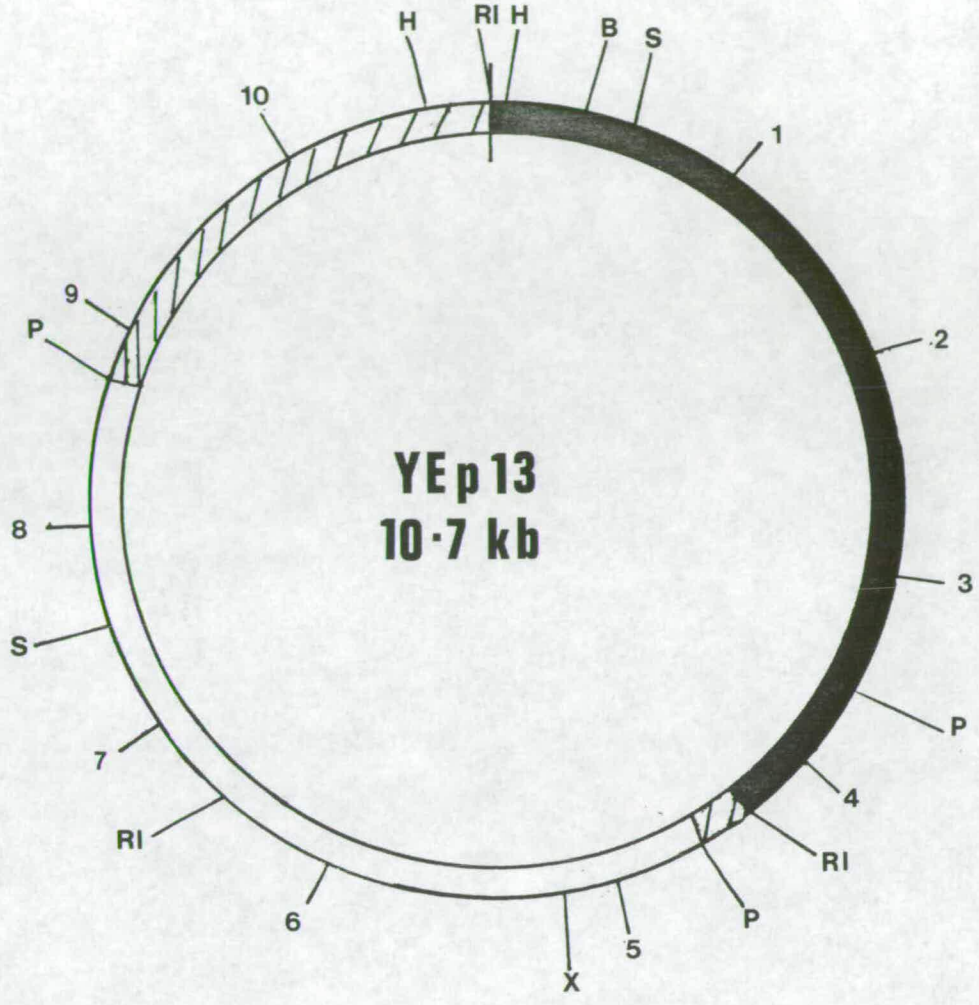


Figure 2.1 : Restriction map of YEp13.

pBR322

2 um

Leu2

RI - *Eco*RI B - *Bam*HI

S - *Sal*I P - *Pst*I

X - *Xho*I H - *Hin*dIII

2.2 GROWTH MEDIA AND CULTIVATION

2.2.1 Cultivation of *Saccharomyces cerevisiae*

The various growth media used are listed below. Amounts given are per litre :

MINIMAL : 20 g D-glucose, 1.7 g yeast nitrogen base (w/o amino acids & $(NH_4)_2SO_4$), 5 g ammonium sulphate, 15 g agar (for solid media), amino acid supplements added where necessary at 50 mg each.

COMPLETE (YEPD) : 20 g D-glucose, 20 g peptone, 10 g yeast extract, 15 g agar (for solid media).

REGENERATION AGAR : 20 g D-glucose, 182 g D-sorbitol, 1.7 g yeast nitrogen base, 5 g ammonium sulphate, 20 g agar.

SPORULATION MEDIUM : 0.5 g D-glucose, 2.2 g yeast extract, 20 g potassium acetate, 20 g agar.

Unless otherwise stated, all strains were grown at 30°C with vigourous shaking of liquid cultures.

2.2.2 Cultivation of *E.coli*

The various growth media used are listed below :

LURIA BERTANI (LB) MEDIUM : Per litre, 10 g tryptone, 10 g sodium chloride, 5 g yeast extract, 15 g agar (for solid media).

For selection of antibiotic resistance the appropriate antibiotic was added to the above media at the following concentrations : ampicillin 50 μgml^{-1} ; chloramphenicol 50 μgml^{-1}; kanamycin 30 μgml^{-1}.

2.3 GENETIC ANALYSIS OF *Saccharomyces cerevisiae*

Standard techniques were used for the mating, sporulation and tetrad dissection of yeast strains (Sherman & Lawrence, 1974; Mortimer & Hawthorne, 1975). Tetrad dissection was carried out using a Leitz micromanipulator (Sherman, 1975) and resulting recombinant phenotypes analysed by replica plating onto drop-out media - minimal media supplemented with all the amino acids except the one selecting for the auxotrophic marker.

2.4 TRANSFORMATION OF *Saccharomyces cerevisiae*

Yeast strains were transformed by the method of Beggs (1978).

2.5 TRANSFORMATION OF *E. coli*

E. coli strains were made competent according to the method of Messing (1983), and stored at -70 °C. They were transformed as described by Dagert & Ehrlich (1979).

2.6 DNA ISOLATION AND PURIFICATION

Total DNA from yeast was prepared by the method of Winston *et al.* (1983)

Plasmid DNA was isolated according to the method of Nasmyth & Reed (1980).

Small-scale preparations of plasmid DNA from *E. coli* were obtained using the alkaline-SDS method of Birnboim & Doly, (1975). Large-scale preparations were obtained in the same manner followed by centrifugation in CsCl-ethidium bromide gradients (Maniatis *et al.*,

1982).

DNA concentations were estimated from the absorbance of the solution at 256 nm. Calculations were based on an OD_{256} = 1 being equivalent to 50 $\mu g ml^{-1}$.

2.7 GENERAL RECOMBINANT DNA TECHNIQUES

2.7.1 Restriction Digests of DNA

Restriction enzymes were used as recommended by the supplier (Boehringer, N.B.L.)

2.7.2 Ligation of DNA

Ligation was carried out using T4 DNA ligase following the protocol of Maniatis *et al.* (1982).

2.7.3 Agarose Gel Electrophoresis

DNA fragments were separated by horizontal, gel electrophoresis using a Tris/borate buffer system as detailed in Maniatis *et al.* (1983). Generally 0.7% (w/v) agarose gels were used, however 1% and 1.5% gels were used to give improved resolution of smaller (less than 2 kb) fragments. Gels contained 0.5 $\mu g ml^{-1}$ ethidium bromide allowing DNA to be visualised under UV illumination and fragment sizes were calculated by comparison with standards of *Hin*dIII digested lambda DNA.

2.7.4 Electro-elution of DNA

Digested fragments of DNA were recovered from agarose gels by

electroelution onto dialysis membrane (Smith, 1980), and subsequently purified and concentrated by running through an Elutip-d column according to manufacturers instructions (Schleicher & Schuell).

2.7.5 Radio-active Labelling of DNA

Plasmids and fragments of DNA were labelled with (α-^{32}P) dATP (Amersham) by nick-translation using the BRL kit ingredients and protocol. Incorporation of the radio-active isotope was estimated by measuring Cerenkov radiation using a Packard 3330 scintillation spectrometer. Measurments were made on a sample of DNA fixed to a glass microfibre filter (Whatman), before and after 2 X 5% (w/v) TCA washes; the ratio giving approximate percentage incorporation.

2.8 METHODS FOR ANALYSIS OF RNA

2.8.1 Isolation of RNA From *Saccharomyces cerevisiae*

Total yeast cellular RNA was prepared following the method of Weir-Thompson & Dawes, (1984).

PolyA$^+$-RNA was isolated by passing total RNA samples through an oligo-dT cellulose column equilibrated with binding buffer (0.4 M NaCl, 10 mM Tris/HCl, 1 mM EDTA, 0.2% SDS, pH 7.4). The bound polyA$^+$-RNA was eluted by washing the column with elution buffer (10 mM Tris/HCl, 1 mM EDTA, 0.2% SDS, pH 7.4),the RNA species were identified by their absorbance at 260 nm.

2.8.2 Gel Electrophoresis of RNA

RNA fractions were denatured then separated by electophoresis through 1% agarose gels containing 2.2 M formaldehyde as described by

Maniatis *et al.* (1982).

2.8.3 Northern Hybridisation

Following electrophoresis, the RNA gel was treated with alkali and then neutralised according to Maniatis *et al.* (1983). The RNA fractions were transferred to nitrocellulose and hybridised with labelled DNA probes following the protocol of Thomas (1980) except that dextran sulphate was omitted from the hybridisation solution. The hybridised filters were exposed to Agfa-Gevaert, Curix RP1 X-ray film at -70°C. Films were developed using Kodak LX-24 and fixed with Kodak FX-40.

2.8.4 Slot Blots

15 µl 20xSSC and 10 µl 37% (w/v) formaldehyde were added to 40 µg RNA and made up to a final volume of 50 ml. Samples were denatured by incubating them at 60°C for 15 min. 25 µl aliquots were transferred to nitrocellulose using BRL's "Hybri-slot" apparatus and fixed by baking at 80°C for 3 h under vacuum. Filters were probed with labelled DNA using the procedures described above (2.8.3). Densitometry was performed by scanning autoradiographs using Loebl Chromoscan 3 apparatus.

2.9 CHORISMATE MUTASE ASSAY

2.9.1 Preparation of Crude Enzyme Extracts

Strains were grown to late log phase in minimal media with appropriate supplements, washed and resuspended in 2 ml enzyme buffer per gram wet weight of cells (125 mM Tris-HCl, 2.5 mM EDTA, 60 mM β-mercaptoethanol). 3.3 ml were added to Braun breakage tubes and filled to within 1cm from the top with glass beads. Cells were broken by shaking for 35 sec in a Braun homogeniser. The extracts were decanted and spun through a stainless steel grid to remove the glass beads. Extracts were spun at 12 000 rev min^{-1} for 10 min and the soluble protein, to be assayed, was separated from the pellet and lipid layer.

2.9.2 Chorismate Mutase Assay

Crude cellular extracts from *Saccharomyces cerevisiae* strains were assayed for chorismate mutase activity using a method adapted by M. Edwards (personal communication.) from the assay procedure developed for *E. coli* (Cotton & Gibson, 1964).

In *E. coli*, chorismate mutase activity is associated with the bifunctional polypeptide encoded by the *phe*A gene which also has prephenate dehydratase activity (Im & Pittard, 1971). The *phe*A gene product, therefore, catalyses the overall conversion of chorismate to phenylpyruvate. Chorismate mutase activity is measured as the extract-dependent formation of prephenate and phenylpyruvate in the presence of chorismate. At the end of the reaction any prephenate is chemically converted to phenylpyruvate by incubation in acid solution. The concentration of the phenylpyruvate is then measured

spectrophotometrically at 320 nm in alkaline solution.

The assay mixture comprised of 625 µl of crude extract, 25 µl 5% (w/v) BSA and 350 µl enzyme buffer. Samples were prewarmed at 30°C for 5 min and the reaction started by adding 250 µl 5 mM potassium chorismate (prepared from a solution of the barium salt in 0.01 M potassium sulphate). At time 0 a 0.25 ml sample was withdrawn and added to 0.25 ml prewarmed 3 M HCl, mixed and incubated for a further 10 min at 30°C. Further samples were removed at 2 and 5 min and treated similarly. 0.25 ml of acid treated solution was mixed with 0.75 ml 1 M sodium hydroxide and the A_{320} measured immediately. The rate of increase in A_{320} was then calculated and corrected for the control rate in the absence of added extract. This is necessary due to the spontaneous (non-enzymatic) conversion of chorismate to prephenate.

The chorismate mutase activity is calculated using an extinction coefficient for phenylpyruvate of 17,500 $M^{-1}cm^{-1}$

$$\text{Activity (Units mg}^{-1}) = \frac{A_{320} \times 0.343}{VP} \left(\frac{A_{320} \times 6}{17.5} \times \frac{1}{V} \times \frac{1}{P} \right)$$

where A_{320} = net increase in absorbance

V = volume of extract (ml)

P = protein concentration ($mgml^{-1}$)

2.9.3 Estimation of Protein Concentration

The protein concentration of each of the samples was measured using the method of Lowry *et al.* (1951).

2.10 MUTAGENESIS

2.10.1 *In Vitro* Mutagenesis

Plasmid DNA was mutated by treatment with o-methylhydroxylamine according to the method of J. Mandelstam (personal communication). O-methylhydroxylamine induces C-T transitions in DNA.

63 mg o-methylhydroxylamine.HCl was treated with 2 M sodium hydroxide until pH5, the volume added being noted. DNA was added and the final volume adjusted to 1 ml with water. The sample was heated at 50 °C for 2 h then precipitated with 2 ml ethanol and cooled at -20°C for a further 2 h. The DNA was pelleted by centrifugation and redissolved in a small volume of water. The sample was then reprecipitated and finally redissolved in a suitable volume of TE buffer (10 mM Tris, 1 mM EDTA). This DNA was then used to transform suitable yeast strains, using conditions selective for the desired mutant phenotype.

2.10.2 Mutagen Ring Test

Various strains of *Saccharomyces cerevisiae* were exposed to different mutagens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethylmethanesulphonate) as follows :

1 µl of mutagen was placed in the centre of a YEPD plate spread with the yeast strain to be mutated and the plates incubated at 30 °C for 48 h. This resulted in a lawn of colonies except for an area of no growth around the mutagen. Colonies around the perimeter of the no growth zone which were not killed but mutagenised were then isolated by replica plating onto media selective for the desired mutation. After incubation at 30°C for a further 48 h, colonies of the required phenotype formed a ring of growth on the plate and separate colonies

could be easily isolated.

2.11 METHODS FOR ASSESSING PHENYLALANINE OVER-PRODUCTION

2.11.1 NINHYDRIN TEST

On reaction with amino acids, ninhydrin solution produces a characteristic blue colour. 5 ml samples of extracellular supernatants were boiled for 2 min in a 1% ninhydrin (indanetrione hydrate) solution and their absorbance read at 440 nm.

2.11.2 Bioassay of Extra cellular Supernatants

Wild type yeast cells cannot grow on synthetic complete media lacking phenylalanine if it contains 100 $\mu g ml^{-1}$ β-thienylalanine, an analogue of phenylalanine. Filter paper discs (3 mm diameter) impregnated with a solution of phenylalanine placed on a lawn of yeast spread on such media will allow growth in the surrounding area; the diameter of this growth "halo" being related to phenylalanine concentration.

The ability of mutant yeast strains to secrete phenylalanine was tested by impregnating the filter paper discs with their extracellular supernatantand noting if they supported a "halo" of wild-type growth around them. The amount of phenylalanine secreted could be roughly estimated by comparison with phenylalanine standards.

2.11.3 Bioassay Using Phenylalanine Auxotrophs

Mutant yeast strains overproducing phenylalanine will be surrounded by a high local concentration of this amino acid in agar medium. This assay is based on the fact that this secreted

acetone. The paper was left to dry at room temperature then placed at 100°C for 20 sec. The resulting amino acid spots could be identified by comparison with the known standards.

2.11.4 Thin Layer Chromatography

Amino acids secreted by mutant yeast strains were also analysed using a thin layer chromatography system.

10 µl (5x2 µl) aliquots were spotted on TLC aluminium sheets precoated with silica gel F_{254} (Merck), 3 cm from the base. This was placed in a chromatography tank 1 cm deep in the solvent (butanol: acetic acid: water of 4:1:1 by volume) and left until the solvent front had reached half-way up the sheet (approx. 2 h). The sheet was then dried and sprayed with a solution of 0.2% ninhydrin in acetone. On drying the resulting spots could be identified by comparison with amino acid standards. Intracellular supernatants obtained by the method of Dickinson *et al.* (1985) were also analysed this way.

3. ISOLATION AND ANALYSIS OF ANALOGUE RESISTANT MUTANTS

3.1 INTRODUCTION

Preliminary work carried out at G.D. Searle & Co. indicated that it was possible to isolate *Saccharomyces cerevisiae* strains which overproduced phenylalanine by mutating yeast cells and selecting for resistance to the phenylalanine analogue β-thienylalanine. In this feasibility trial, one analogue resistant mutant, out of only eight studied, overproduced pheneylalanine to such an extent that it secreted 1 g l^{-1} into the medium (B. Carter, personal communication). The aim of this section was, therefore, to continue with these experiments; isolating new analogue-resistant strains, improving their phenylalanine output and developing simple assay procedures to detect and measure this overproduction.

3.2 ISOLATION OF MUTANTS

Mutant strains resistant to the phenylalanine analogue β-thienylalanine were obtained by exposing *Saccharomyces cerevisiae* strain 312 to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (mutagen ring test - see 2.10) and selecting for growth on media containing 200 μg ml^{-1} β-thienylalanine. Prior to mutagenesis 312 failed to grow at β-thienylalanine concentrations above 60 μg ml^{-1}. Ten mutant colonies were isolated.

Since high levels of phenylalanine are often deleterious to yeast cells, strain 312 was previously tested to establish that it

could tolerate any excess phenylalanine that the resulting mutants may have produced.

3.3 ASSAY FOR SECRETION OF PHENYLALANINE

The mutant strains and wild-type 312 were grown in minimal media (plus appropriate supplements) for 36 h, their extracellular supernatants treated with a 1% ninhydrin solution (see 2.11.1) and their absorbance read at 440 nm (Table 3.1). On reaction with amino acids ninhydrin solution produces a charateristic blue colour. This experiment, therefore, should give an indication of whether phenylalanine was excreted by the β-thienylalanine resistant mutants.

TABLE 3.1 : Absorbance at 440 nm of extracellular supernatants on reaction with ninhydrin solution.

STRAIN	ABSORBANCE	STRAIN	ABSORBANCE
312NG1	0.61	312NG7	0.21
312NG2	0.18	312NG8	0.46
312NG3	0.66	312NG9	0.21
312NG4	0.20	312NG10	0.35
312NG5	0.32		
312NG6	o.35	312	0.17

Although a range of absorbances was observed, three strains (312NG1, 312NG3 & 312NG8) produced a much more intense blue colour than the others. The unmutated 312 gave a very low absorbance, producing no discernable blue colour at all. This was not a direct test for phenylalanine but it did show that the mutants may be

excreting an amino acid of some sort.

3.4 IDENTIFICATION OF SECRETED AMINO ACIDS

High voltage paper electrophoresis (HVPE) was used to separate and identify the amino acids present in the extracellular supernatants. The resulting chromatograph, when stained, showed that although there was a slight amount of phenylalanine present, there were several other amino acids being produced in much larger quantities (Fig. 3.1), including alanine, valine, proline, glutamate and tyrosine. The mutants with the higher absorbance at 440 nm did not produce more phenylalanine than the others, indicating that it was probably the presence of the other amino acids which was the main factor in the intensity of the blue colour produced.

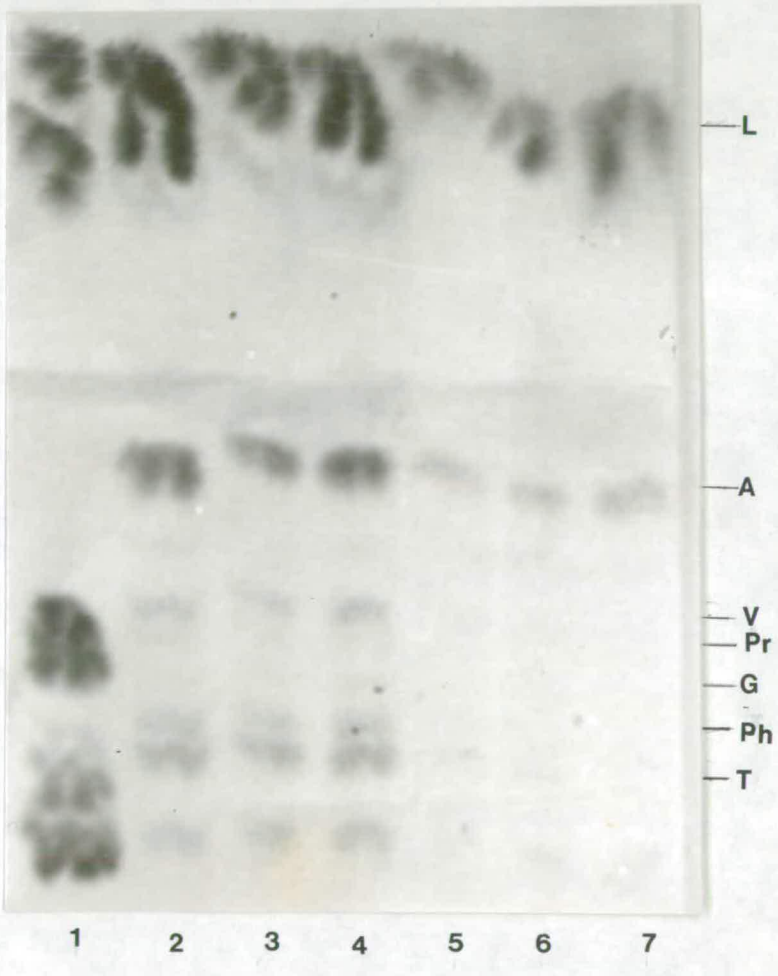
3.5 GENETIC ANALYSIS

Strains 312NG1, 3 and 8 were crossed with 315, a strain of the opposite mating type. The resulting tetrads were dissected into separate groups of four spores and after incubation on YEPD media the four haploid colonies were replica-plated onto minimal media containing 200 μg ml^{-1} β-thienylalanine. The growth patterns produced showed that resistance to β-thienylalanine segregated in a 2:2 manner indicating that resistance was due to a single mutation.

Single diploid colonies were plated on minimal media containing 200 μg ml^{-1} β-thienylalanine. All the diploid colonies grew indicating that the β-thienylalanine resistant mutation (Th^r) was dominant over the sensitive allele (Th^s).

Figure 3.1 : Separation, by high voltage paper chromatography, of amino acids secreted by β-thienylalanine resistant strains. 1 - standards, 2 - 312NG1, 3 - 312NG6, 4 - 312NG10, 5,6 & 7 - 312.

L - lysine, A - alanine, V - valine, Pr - proline, G -glutamate, Ph - phenylalanine, T - tyrosine.


L
A
V
Pr
G
Ph
T
1
2
3
4
5
6
7

3.6 SENSITIVITY TO OTHER PHENYLALANINE ANALOGUES

The above three Th^r strains were replica-plated onto media containing concentrations of *m*-fluorophenylalanine (300 μg ml^{-1}) and *p*-fluorophenylalanine (500 μg ml^{-1}) which inhibited wild-type growth. The mutant strains grew on both types showing they were resistant to both analogues. This indicated that β-thienylalanine resistance was possibly due to intracellular phenylalanine overproduction rather than a permease mutation affecting the uptake of the analogue. Multiple resistance, in the latter case, would involve several separate mutations occurring independently, the frequency of which would be very low. Fluorophenylalanine may be taken up by the same permease as β-thienylalanine but such a mutation is unlikely to be dominant in diploid heterozygotes since there would always be a wild-type gene present to produce a functional permease.

3.7 ISOLATION OF *Tyr1*/Th^r DOUBLE MUATANTS

The Th^r strains appeared to produce more tyrosine than phenylalanine, hence, by blocking the tyrosine pathway the flux might be diverted through the phenylalanine branch. This would possibly increase phenylalanine production especially when coupled with the muation causing the Th^r phenotype.

The previous method of selecting analogue-resistant mutants was repeated, this time using the *Tyr1* strain 322 which is deficient in the enzyme prephenate dehydrogenase. 30 colonies resistant to 200 μg ml^{-1} β-thienylalanine were obtained and tested to confirm they had retained their tyrosine auxotrophic phenotype.

3.8 AMINO ACID ANALYSIS OF DOUBLE MUTANTS

The extracellular supernantants from liquid cultures of the double mutants were analysised using HVPE as before. Most of the mutants showed the same pattern of amino acids present as before but 11 of the 30 showed a slight increase in phenylalanine as well as a decrease in the amount of tyrosine produced.

3.9 TIME COURSE OF PHENYLALANINE SECRETION

A 500 ml culture of the double mutant strain 322NG11 was incubated at 30°C, samples being removed at regular time intervals up to 140 h. The sample supernatants were analysed by HVPE.

The resulting chromatograph (Fig. 3.2) showed that phenylalanine did not appear until 27-31 h (i.e. late log phase) then steadily increased up to 56 h. By 80 h the amount had decreased substantially, very little being present at 140 h. Tyrosine was present at high levels initially but decreased with time. This was expected since tyrosine is added to the media and will be gradually consumed. The other amino acids did not appear until approximately 31 h except alanine which was present from the start. Repeating this experiment with the other ten double mutants produced the same results.

3.10 EFFECT OF TYROSINE ON PHENYLALANINE SECRETION

The fact that phenylalanine was not excreted until late log phase may be a function of cell growth itself or it may be due to the decrease in external tyrosine initially inhibiting the pathway. Strains 322NG11 and 322NG14 were grown over a range of tyrosine

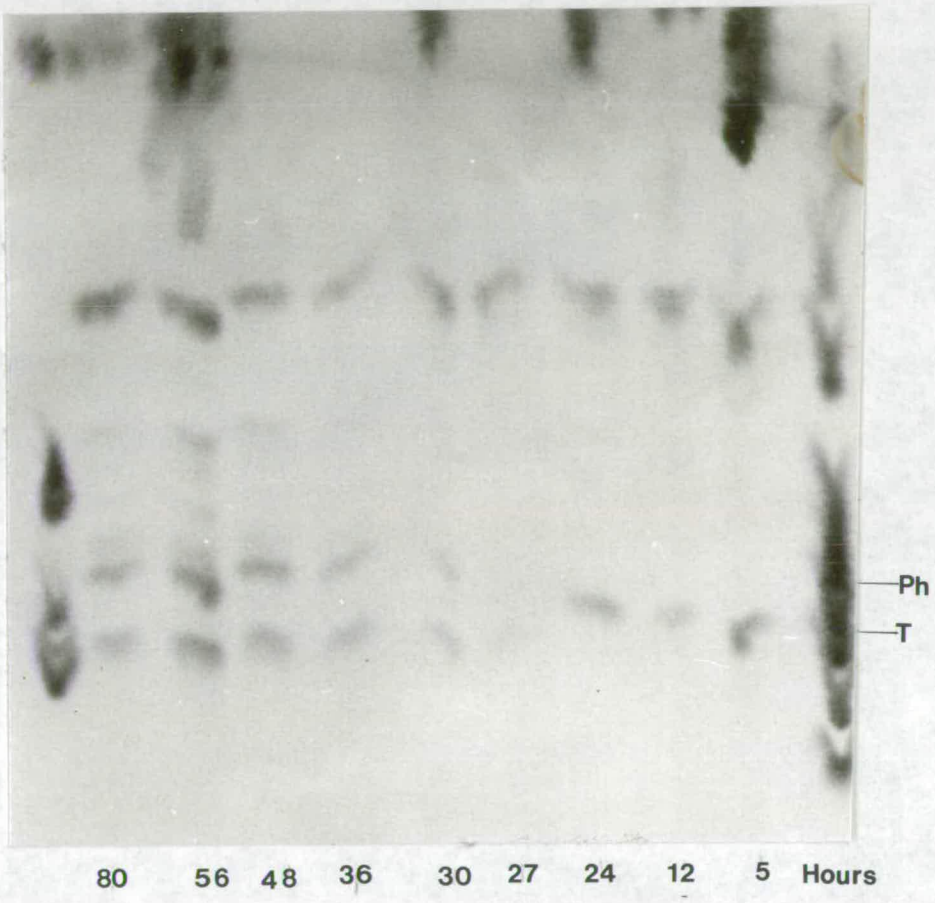


Figure 3.2 : HVPC showing time course of phenylalanine secretion from strain 322NG11.

concentrations (0 to 40 μg ml^{-1}) and samples were taken at various time intervals. the supernatants were then subjected to HVPE.

The initial concentration of tyrosine did not seem to affect the production of phenylalanine. At all concentrations of tyrosine, phenylalanine was present by 38 h. If tyrosine was inhibiting the pathway, then phenylalanine would have been expected to appear earlier at the lower tyrosine concentrations but this was not so. Tyrosine concentration did not seem to affect the appearance of the other amino acids either.

3.11 IMPROVEMENT OF PHENYLALANINE PRODUCTION

With the aim of increasing the amount of phenylalanine produced, strain 322NG11 was subjected to a second round of mutagenesis. Cultures were exposed to EMS (see 2.10) then aliquots plated on Phe^- media which had been previously spread with a phenylalanine auxotrophic tester strain A236-57B. The plates were incubated at 30°C for 48 h.

Mutant colonies could be identified by a "halo" of tester strain growth around them due to cross-feeding (see 2.11). Although there seemed to be an overall background growth, 12 colonies had a much more concentrated area of growth around them. This was much more evident when viewed with a binocular microscope (x5 magnification). The 12 colonies were picked and streaked out on phe^- media to separate them from cross-feeding contaminants.

3.12 ASSESSMENT OF PHENYLALANINE OVERPRODUCTION

Master plates were made by spotting single colonies of the "overproducing" strains (OP) onto YEPD media at regular intervals

around the circumference of the plate. Controls of 322NG11 and DC5 were also on each plate. These were allowed to grow then replica-plated onto phe⁻ plates containing different concentrations of β-thienylalanine and spread with the phenylalanine auxotrophic tester strain A236-57B. The amount of tester growth around each colony was taken as a measure of the phenylalanine excreted.

The strains which secreted the most phenylalanine were OP1, 2a, 2c, 5 and 6 (Fig. 3.3). These five strains were able to support the growth of the tester strain up to β-thienylalanine concentrations of 100 μg ml^{-1}. Strains OP2c and OP9 failed to cross-feed the phenylalanine auxotrophic strain above 50 μg ml^{-1}. No "halos" were observed around strain 322NG11 at 50 μg ml^{-1} β-thienylalanine. Since these mutants were derived from strain 322NG11, it appears that this second round of mutagenesis has, in the strains mentioned, improved the yield of phenylalanine.

It is interesting to note that some mutants were resistant to 250 μg ml^{-1} β-thienylalanine (322NG11, OP7) even though they failed to support the growth of the tester strain at 100 ug ml^{-1}, while other strains (OP2c) had large "halos" around them but did not grow above 100 μg ml^{-1} β-thienylalanine. This may be due to the strains supporting the tester strain being able to secrete phenylalanine more readily than the other strains.

3.13 QUANTITATIVE ANALYSIS OF PHENYLALANINE PRODUCTION

The extracellular supernatants of the OP strains were analysed by thin layer chromatography (TLC) with the aim of quantifying the amount of phenylalanine produced. A range of phenylalanine concentrations

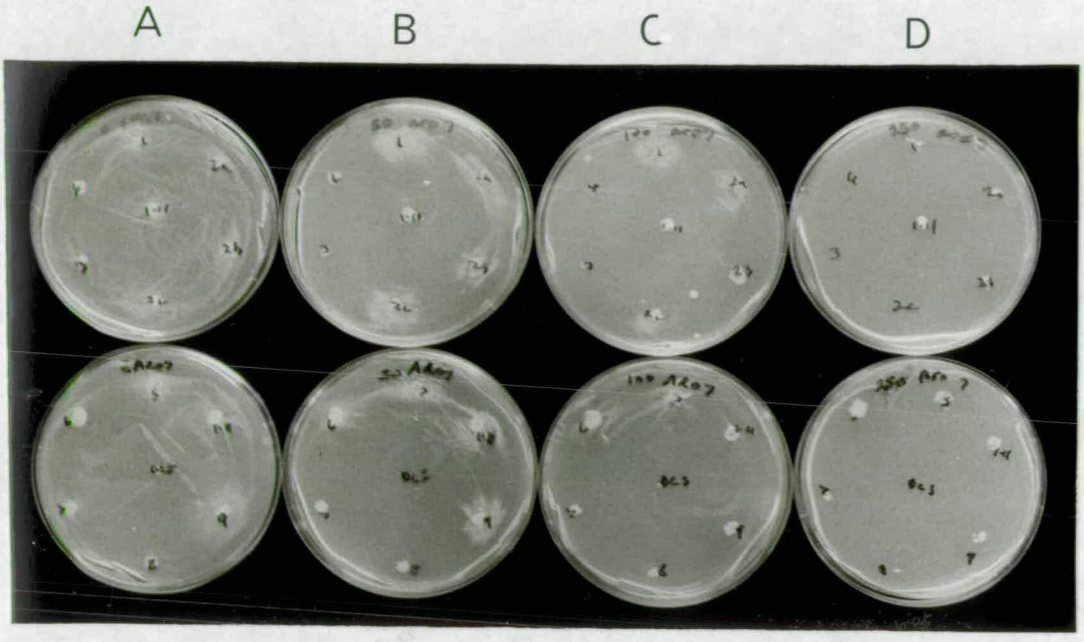


Figure 3.3 : Cross-feeding of phenylalanine auxotrophic strain, A236-57B, by OP strains. Top row, in clockwise direction; OP1, 2a, 2b, 2c, 3, 4 and 322NG11 (centre). Bottom row; OP5, 14, 9, 8, 7, 6 and DC5 (centre). A-O β-thienylalanine, B-50 μg ml^{-1}, C-100 μg ml^{-1} and D-250 μg ml^{-1}.

were also run, to act as standards against which the samples could be measured.

Strain OP5 appeared to produce the most extracellular phenylalanine, the concentration was estimated as 0.25 mg ml^{-1} (Table 3.2). This strain plus OP1, 2a, 2c, 6 and 9 all produced more phenyalanine than the original strain 322NG11 showing that the second round of mutagenesis had improved phenylalanine output. All the mutant strains produced much more phenylalanine than the unmutated strain 322.

The results of 3.12 indicated that there may be differences in the amount of phenylalanine produced by the cell compared to the amount that is secreted. The above experiment was, therefore, repeated this time analysing the intracellular levels of phenylalanine. No significant difference was observed in the amount of phenylalanine measured for each strain. This indicated that most of the available phenylalanine was secreted by the cells.

3.14 **DISCUSSION**

In summary, several mutant strains of *Saccharomyces cerevisiae* have been created which show a significantly increased level of phenylalanine production compared to wild-type yeast strains. Although the phenylalanine levels obtained (approx. 0.25 mg ml^{-1}) were not as high as those previously achieved by researchers at G.D. Searle (1mg ml^{-1}), these results werevery encouraging and indicate that an attempt to produce large quantities of phenylalanine using yeast may be rewarding.

The bioassay proved an efficient method for the detection of colonies overproducing and secreting phenylalanine and analysis of the supernatant by TLC allowed the amount of phenylalanine produced to be

Table 3.2 : Concentration of phenylalanine (mg ml^{-1}) secreted by Th^r/*Tyr1*second round mutants.

STRAIN	PHENYLALANINE CONC.(mg ml^{-1})	STRAIN	PHENYLALANINE CONC. (mg ml^{-1})
322NG11	0.1	OP5	0.25
OP1	0.2	OP6	0.2
OP2a	0.2	OP7	<0.1
OP2b	0.1	OP8	<0.1
OP2c	0.2	OP9	0.1
OP3	<0.1	OP14	0.1
OP4	<0.1	322	undetectable

quantified. Further measurments using an amino acid analyser would, however, give a more accurate profile of the amino acids produced and their relative concentrations.

The phenotype of the mutant strains indicate that the overproduction of phenylalanine is probably caused by a mutation in one of the control genes (*ARO3, ARO4, ARO7, PHA2 or TYR1*) rendering its protein insensitive to feedback-inhibition by phenylalanine. Further analysis involving the appropriate enzyme assays should confirm this and indicate which gene has been mutated.

The *tyr1*/Th^r double mutant strains appeared to produce more phenylalanine than the original Th^r strains, therefore, it may be possible to improve phenylalanine production further by crossing in mutations in other relevant pathways. By introducing the *trp2* or *trp3*

mutations, for example, the tryptophan biosynthetic pathway would be blocked, possibly directing the flux towards phenylalanine production.

Unfortunately, the mutant strains' ability to mate was seriously affected during mutagenesis. The strains failed to form diploids when crossed to strains of the opposite mating type. Mutations in the *ARO1* and *ARO2* genes of *Saccharomyces cerevisiae* have been reported to affect mating (Lucchini *et al.*, 1978) but this seems to be a failure to sporulate rather than formation of diploids. It is more probable that one of the genes involved in mating is responsible.

Theoretically, it should also be possible to improve the yield of phenylalanine by continuing rounds of mutagenesis, selecting for strains which crossfeed phenylalanine auxotrophs at increasing concentrations of β-thienylalanine. The overproducing strain finally obtained, however, would be a conglomeration of many different mutations, the genetical basis of which is unknown. In addition, it would contain numerous other mutations, which have not been selected for, affecting its general physiology in an undetermined manner. The above evidence also indicates that repeated rounds of mutagenesis are likely to lead eventually to sterile strains. A more genetically controlled approach may, therefore, be advantageous in the long term.

The regulation of aromatic amino acid biosynthesis is fairly well understood and so it should be possible to engineer phenylalanine overproduction in a more directed manner by constructing regulatory altered yeast strains with mutations in the relevant genes. This approach is covered in the following chapters.

4. CLONING AND CHARACTERISATION OF THE *Saccharomyces cerevisiae* *ARO7* GENE

4.1 INTRODUCTION

Mutant alleles exist for most of the main enzymatic steps in the aromatic amino acid pathway (Jones & Fink, 1981), and thus it is possible to isolate the genes encoding the enzymes responsible for each reaction using current molecular techniques. The expression of specific genes can then be studied in a much more direct manner than the classical methods used in the previous chapter.

The aim of this section was to isolate one of the genes in the pathway and study its regulation in more detail, with the purpose of then altering its control in a manner directed towards the eventual overproduction of phenylalanine.

Since other research groups were working extensively on the earlier sections of the pathway, especially the *ARO3* and *ARO4* genes, research was concentrated on one of the lower branches of the pathway. The *ARO7* gene codes for chorismate mutase (Kradolfer, 1977) which catalyses the formation of prephenate from chorismate. Since this enzymatic step occurs at the branchpoint where the tryptophan biosynthetic pathway separates from the phenylalanine and tyrosine pathway, it is likely that it plays a key role in the control of aromatic amino acid production. It is, therefore, an ideal candidate for the research purposes outlined above.

4.2 ISOLATION OF A SEQUENCE COMPLEMENTING THE *aro7* MUTATION IN *Saccharomyces cerevisiae*

4.2.1 Transformation of *Saccharomyces cerevisiae* with a Yeast Gene Bank

The *aro7 leu2* auxotrophic strain J14-26 was transformed with DNA from a wild-type gene-bank (Nasmyth & Tatchell, 1980), contained within the shuttle vector YEp13 (see section 2.1.4). Transformants were selected by their ability to grow in the absence of leucine. The conditions used and the resulting number of transformants are displayed in Table 4.1 .

Several controls were included: Sample 1 showed that the host strain could not grow without leucine and sample 2 showed that strain J14-26 was indeed transformable. Sample 6 was a positive control to show that the host strain could grow in the presence of leucine. By using these controls, the sixty four colonies which grew on the leu- plates (samples 3 and 4) were identified as transformants containing a plasmid vector from the gene bank.

4.2.2 Analysis of the Leu$^+$ Transformants

The sixty four Leu$^+$ transformants were screened for simultaneous complementation of the *aro7* mutation by testing their ability to grow on medium lacking leucine, phenylalanine and tyrosine. Two such colonies were isolated.

TABLE 4.1 : Results obtained from the transformation of the *Saccharomyces cerevisiae* strain J14-26 with a wild-type yeast gene bank. Transformations were performed as described in section 2.4 using 5 µg DNA in each sample.

SAMPLE	DNA SOURCE	REGENERATION MEDIUM	NO. OF COLONIES
1	0	Leu$^-$	0
2	YEp13	Leu$^-$	40
3	Gene Bank	Leu$^-$	28
4	Gene Bank	Leu$^-$	36
5	Gene Bank	Leu$^-$ Phe$^-$ Tyr$^-$	0
6	Gene Bank	Leu$^+$	Complete Lawn

Growth on complex medium relieves selection for episomal plasmids and these are lost from 5 to 10% of the population per generation. When transferred to selective media, only those colonies which have retained the plasmid will grow. The two colonies were streaked out onto YEPD plates, to obtain single colonies, and replica-plated onto leu$^-$ phe$^-$ tyr$^-$ plates to check that the *aro7* complementing phenotype was conferred by the plasmid and not due to a chromosomal mutation.

Of the two transformants studied, one isolate did show a correlation between plasmid loss and loss of *aro7* complementation, proving that the DNA complementing the *aro7* mutation was carried on the plasmid. The other isolate, however, showed no correlation; all the colonies transferred from YEPD were able to grow in the absence of phenylalanine, tyrosine and leucine. This was probably due to integration of the plasmid into the yeast genome since the *LEU2* marker failed to segregate with the plasmid.

4.2.3 Retransformation of yeast with pJFB1

To confirm the wild-type *ARO7* phenotype was due to the presence of complementing DNA cloned in the plasmid, designated pJFB1, plasmid DNA was extracted from a culture of the transformed strain, J14-26TR1, and used to retransform the *aro7* auxotrophic strain J14-26. All transformants obtained were able to grow without phenylalanine and tyrosine supplementation proving that pJFB1 did indeed carry a region of DNA which complemented the *aro7* mutation in *Saccharomyces cerevisiae*.

The *E. coli* strain HB101 was transformed with pJFB1 and sufficient plasmid DNA was obtained from a two litre culture for subsequent analysis. This DNA was purified by CsCl gradient centrifugation.

4.3 CONSTRUCTION OF A RESTRICTION MAP

To localise the putative *aro7* gene within the insert, it was first necessary to obtain a restriction map of the cloned insert.

Twelve restriction enzymes were used; nine were found to have recognition sites within the DNA insert (see Fig. 4.2). The sizes of the digested fragments were calculated by comparison with standards from phage lambda DNA digested with *Hin*dIII, following gel electrophoresis. By using the known restriction sites present in YEp13, an accurate map of pJFB1 was constructed (see Fig.4.1). Any ambiguities were resolved by digesting with two or more enzymes simultaneously.

Fig. 4.2 shows that a *Hin*dIII digest gives four fragments of approximately 10.3, 3.1, 2.3 and 0.2 kb (the 0.2 kb fragment has not incorporated sufficient ethidium bromide to be seen), and a *Stu*I digest gave two fragments of approximately 8.5 and 7.5 kb. By carrying out a double digest with both enzymes, the *Stu I* site in the insert was located within the 2.3 kb *Hin*dIII fragment and the orientation of the *Hin*dIII sites resolved.

The *Bam*HI digest did not produce a fragment the size of the complete insert, showing that at least one of the original YEp13 *Bam*HI sites had not been maintained. By performing a *Bam*HI/*HindIII double digest it became clear that neither site had been retained; both Bam*HI sites being within the insert-derived 3.1 kb *Hin*dIII fragment.

TABLE 4.2 Number and location of restriction enzyme sites in pJFB1.

	NUMBER OF RESTRICTION SITES		
ENZYME	YEp13	INSERT	TOTAL pJFB1
*Bam*HI	1*	2	2
*Bgl*II	2	1	3
*Cla*I	3	3	6
*Eco*RI	3	2	5
*Eco*RV	3	1	4
*Hind*III	2	2	4
*Pst*I	3	0	3
*Pvu*II	1	0	1
*Sal*I	2	0	2
*Stu*I	1	1	2
*Xba*I	1	1	2
*Xho*I	1	1	2

* Although YEp13 has a single *Bam HI* site, this was lost during the construction of the gene bank (see text).

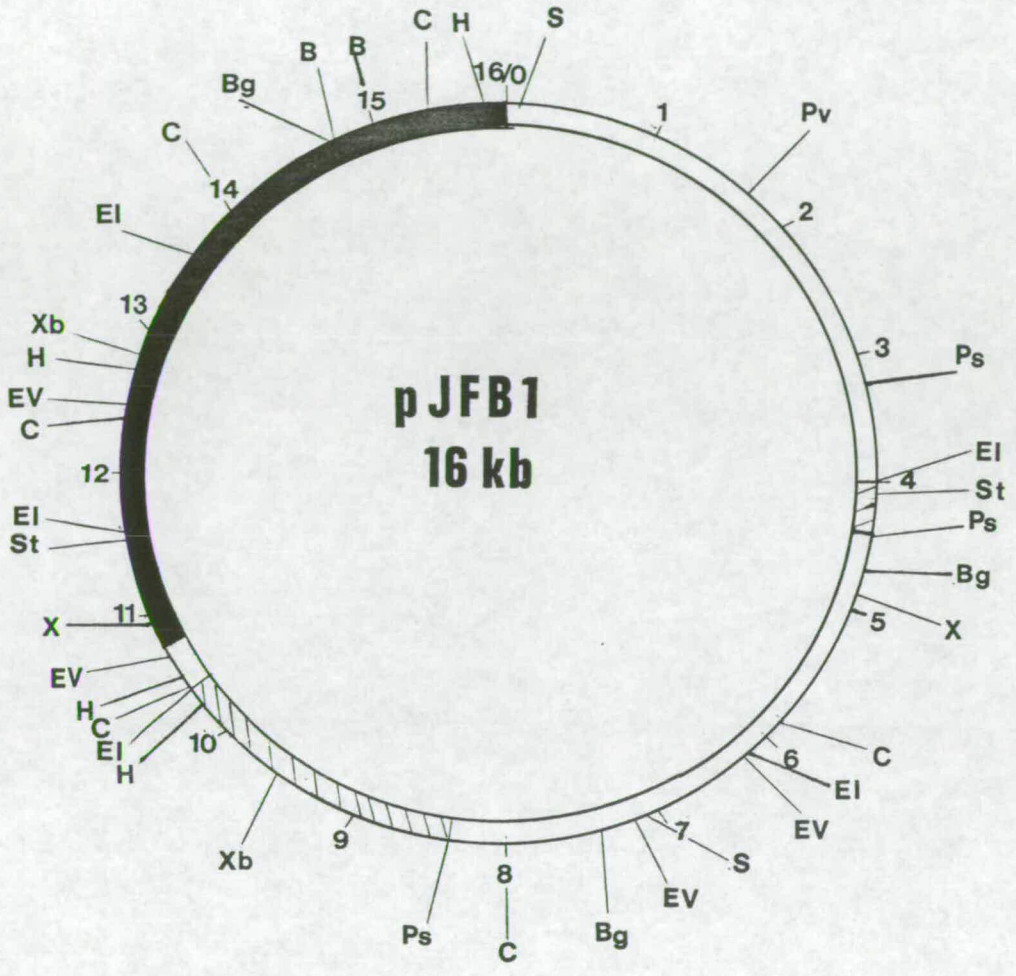


Figure 4.1 : Restriction map of pJFB1.

Cloned Insert

EI - *Eco*RI	B - *Bam*HI	Pv - *Pvu*II
S - *Sal*I	Ps - *Pst*I	St - *Stu*I
X - *Xho*I	H - *Hin*dIII	Bg - *Bgl*II
C - *Cla*I	EV - *Eco*RV	Xb - *Xba*I

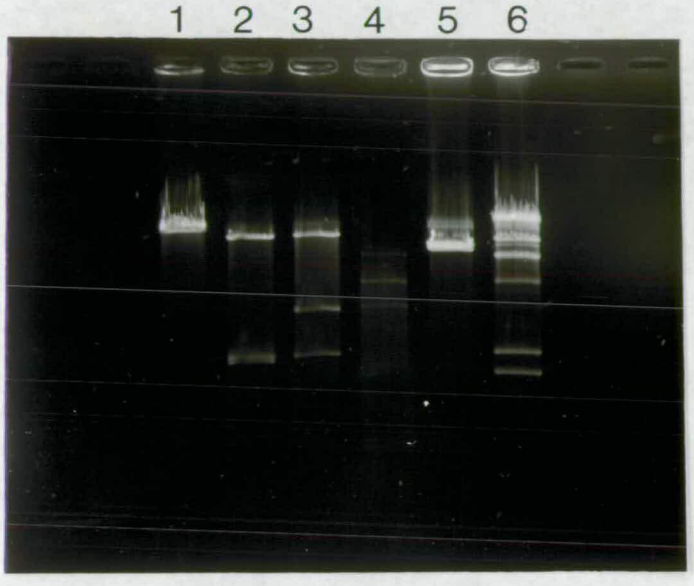


Figure 4.2 : Gel electrophoresis of restriction digests of pJFB1. 1-*Bam*HI, 2-*Bam*HI/*HindIII*, 3-*Hin*dIII, 4-*Hin*dIII/*Stu*I, 5-*Stu*I and 6-*Hin*dIII digested lambda DNA.

4.4 DELETION ANALYSIS OF pJFB1

To locate the gene responsible for complementation within the cloned 5.3 kb fragment, deletions were engineered in the insert and the resulting deletion plasmids transformed back into J14-26 and tested for complementation of the *aro7* mutation.

The insert is conveniently divided by *Hin*dIII sites into two fragments of 2.3 and 3.1 kb. Deletion plasmids lacking either of these two fragments would be helpful in locating the desired sequence. These were obtained by partial digestion of pJFB1 with *Hin*dIII (5 µg DNA, 5 units enzyme), followed by ligation. Gel electrophoresis of samples digested for 8, 16 and 30 min showed progressive appearance of partial digestion products

The 30 min sample was ligated and transformed into *E. coli* strain HB101. Plasmid DNA was prepared from twenty four isolates and screened by gel electrophoresis for plasmids smaller than the original pJFB1. Eight of the isolates yielded smaller plasmids which were subsequently analysed for the desired deletions by *Hin*dIII digestion.

As can be seen from Fig. 4.4, most of the plasmids showed a complete deletion of both fragments but isolate 1 had the 3.1 kb fragment missing while isolate 3 lacked the 2.3 kb fragment (see Fig. 4.5).

Plasmid DNA from isolates 1 and 3 (denoted pKH1 and pKH3 respectively) was used to transform the *Saccharomyces cerevisiae* strain A236-57B and tested for its ability to complement the *aro7* mutation by testing for growth of the transformant in the absence of phenylalanine and tyrosine.

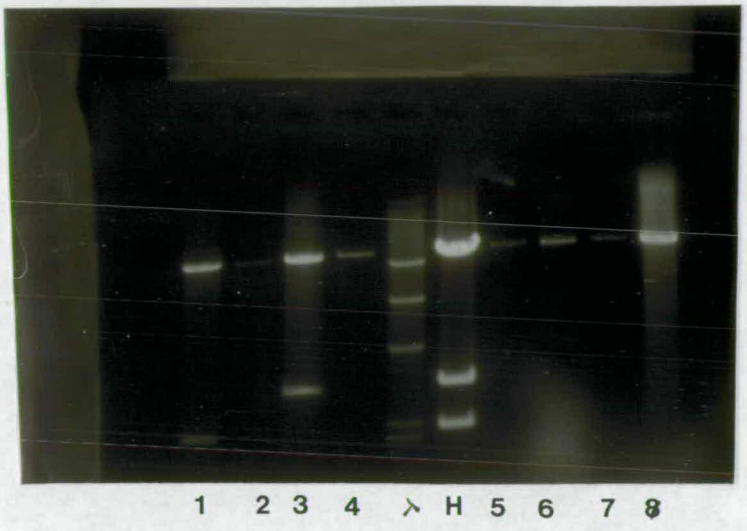


Figure 4.4 : Gel electrophoresis of a *Hin*dIII digest of pJFB1 deletion plasmids.

1. pKH1 2. pKH2 3. pKH3 4. pKH4

λ. Lambda DNA H. pJFB1

5. pKH5 6. pKH 7. pKH7 8. pKH8

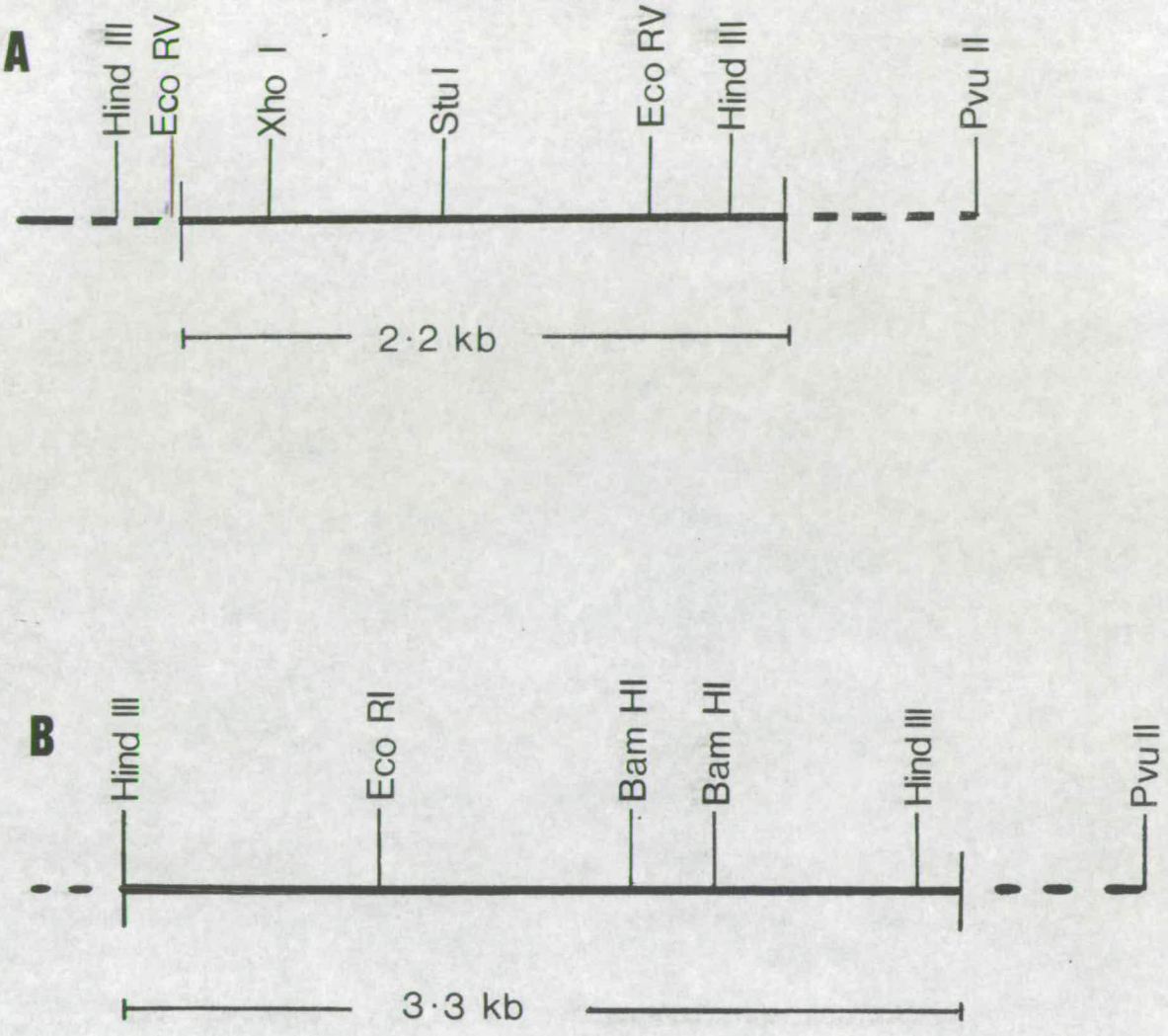


Figure 4.5 : Restriction maps of the pJFB1 inserts in deletion plasmids (A) PKH1 and (B) pKH3.

TABLE 4.3 : Deletion analysis results of *aro7* complementation test. + = growth, - = no growth, -* = some growth observed (see text). *() represents strain A236-57B transformed with the relevent plasmid.

	Saccharomyces cerevisiae STRAINS				
MEDIUM	A236-57B	*(pKH1)	*(pKH3)	*(pJFB1)	314
Complete	+	+	+	+	+
Leu⁻	-	+	+	+	+
Phe⁻ Tyr⁻	-	-*	-	+	+
Leu⁻ Phe⁻ Tyr⁻	-	-*	-	+	+

From Table 4.3 it can be seen that neither construct complemented the *aro7* mutation; none of the yeast transformants were able to grow without phenylalanine and tyrosine supplementation. This indicated that the complementing sequence spans the region of the *Hin*dIII site at 12.7 on the pJFB1 map.

Strain A236-57B(pKH1) showed some growth in the absence of leucine, phenylalanine and tyrosine but this was not correlated with the presence of the plasmid. The growth was in the form of papillae which indicates that mitotic recombination had probably taken place (Fogel *et al.*, 1983). That recombination between pKH1 and the *ARO7* locus produces functional chorismate mutase whereas recombination with pKH3 was not detected, localises the original *aro7* mutation to the 2.3 kb fragment

4.5 TRANSCRIPTIONAL ANALYSIS

4.5.1 Preparation of RNA Filters

Total RNA was isolated from 500 ml cultures of *Saccharomyces cerevisiae* strains 314 and J14-26TR1 and poly-adenylated mRNA fractions of each were separated on an oligo-dT-cellulose column. 20 µg samples of total RNA and 2 µg of poly(A)$^+$ RNA from each strain were run on a denaturing formaldehyde-agarose gel then transferred to nitrocellulose filters.

4.5.2 Northern Hybridisation Using A 2.3 kb pJFB1 Probe

To obtain the necessary radioactively labelled probe needed to hybridise with the pJFB1 transript, a *Hin*dIII digest was carried out on pJFB1 DNA. After gel electrophoresis, the 2.3 kb fragment was electroeluted, purified and nick-translated using (α-^{32}P)dATP. The filter was hybridised for 24 h then exposed to X-ray film for 20 h.

As can be seen from Fig. 4.6, the probe hybridised to several bands, all of which were represented in track 4.

Band A was present only in tracks 2 and 4 and was, therefore, not polyadenylated RNA. Its very high molecular weight indicated that it may have been DNA contamination.

Of the remaining bands, only C, E and F were represented in the poly-A tracks 1 and 3, and so may possibly have been transcripts from the complementing gene.

Bands C and E clearly increased in intensity in tracks 3 and 4, compared to tracks 1 and 2, suggesting that the transformed strain was producing more of this transcript than the wild-type. Since the

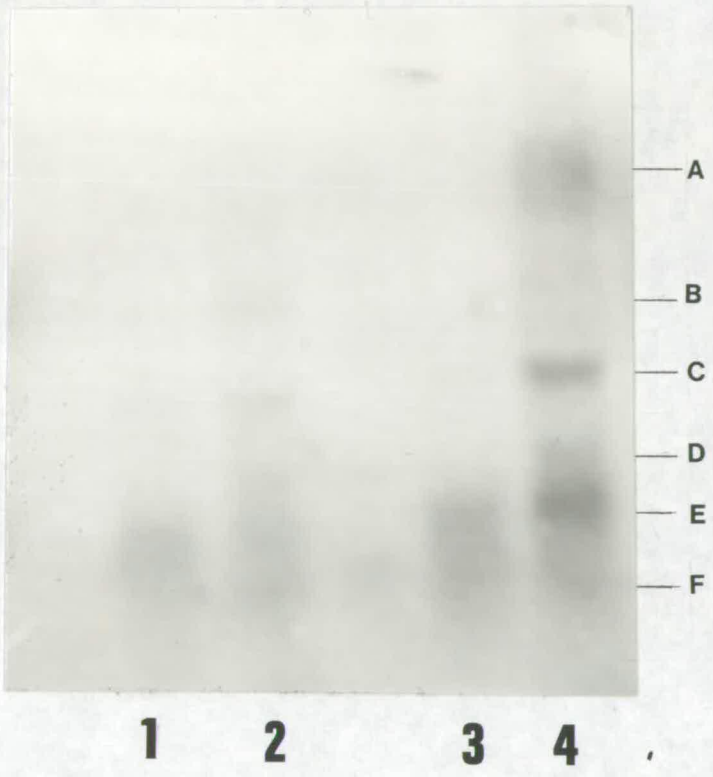


Figure 4.6 : Northern hybridisation with 2.3 kb pJFB1 probe.

TRACK 1 - 314 poly-A$^+$ RNA

2 - 314 total RNA

3 - J14-26TR1 poly-A$^+$ RNA

4 - J14-26TR1 total RNA

plasmid was present in multiple copies in the cell, this increase in plasmid-derived mRNA would be expected. Transcript E, however, was present in the poly-A RNA at much higher levels than transcript C which made it a much more likely candidate for the putative *ARO7* transcript.

Band F also followed the pattern expected of the pJFB1 transcript. Since this band was rather diffuse, however, some doubt was expressed as to whether it was not an artifact due to degradation of the RNA.

By comparison with DNA markers, the size of transcript E was estimated to be between 0.5 and 2 kb.

4.5.3 Northern Hybridisation Using A 3.1 kb pJFB1 Probe

To clarify the previous results, the Northern hybridisation procedure was repeated using the 3.1 kb *Hin*dIII pJFB1 fragment. If this fragment contained a larger ratio of *ARO7* sequence to any extraneous gene sequences it would, possibly, only hybridise to one single transcript.

Examination of the resulting autoradiograph (see Fig. 4.7) showed, however, that this probe also hybidised to several RNA bands on the filter. As with the previous probe though, only two of these transcripts were polyadenylated. Also in accordance with the previous result was the observation that the transcripts from the transformed strain was present in much higher quantities than those from the untransformed strain. This not only showed that those mRNAs were transcribed from plasmid DNA but indicated that the chromosomally encoded message was present at very low levels within the cell.

The fact that a large proportion of band E was present as

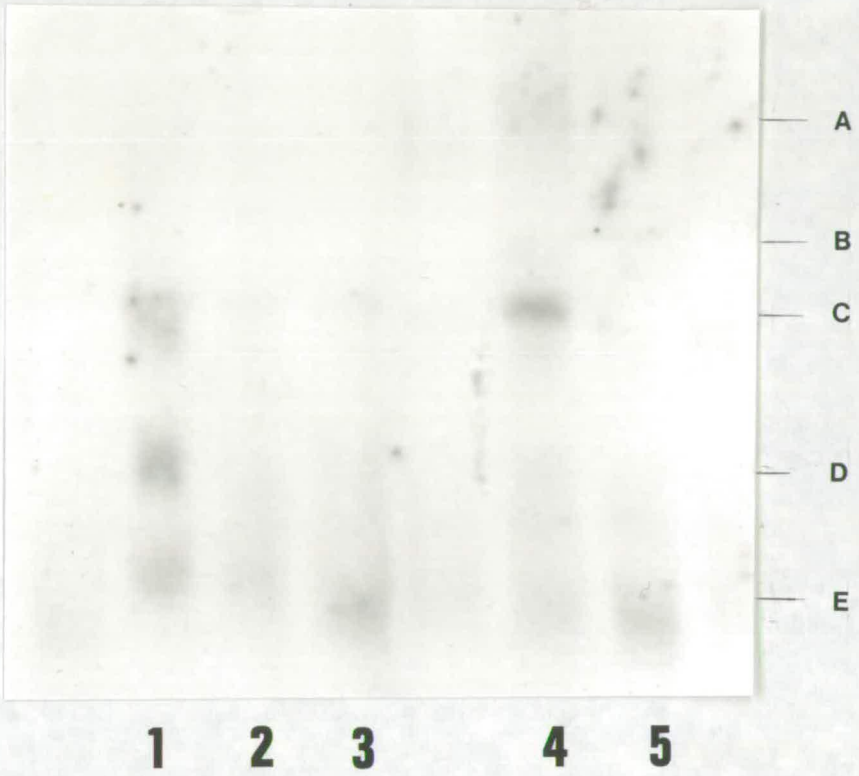


Figure 4.7 : Northern hybridisation using 3.1 kb pJFB1 probe.

TRACK 1 - J14-26TR1 total RNA

2 - J14-26TR1 poly-A^+ RNA

3 - J14-26TR1 poly-A^+ RNA

4 - 314 total RNA

5 - 314 poly-A^+

Extracts of soluble proteins were prepared from the yeast strains 314, J14-26 and J14-26TR1 grown, until late log phase, in minimal media containing the appropriate supplements. These were then assayed for chorismate mutase activity by measuring the conversion of chorismate to phenylpyruvate (Table 4.4).

TABLE 4.4: Chorismate mutase activity in *Saccharomyces cerevisiae*. Data presented are the average of a minimum of four separate assays.

STRAIN	SPECIFIC ACTIVITY OF CHORISMATE MUTASE
	nmol product min^{-1} $(mg\ protein)^{-1}$
314	8.57
J14-26	0
J14-26TR1	24.2

Strain J14-26TR1 showed a three fold increase in chorismate mutase activity compared to wild-type (Table 4.4). This result would have been expected if the strain was carrying multiple copies of the structural gene for chorismate mutase and hence it provided further evidence that pJFB1 carried the *ARO7* gene. As expected J14-26 had showed no detectable chorismate mutase activity.

4.7 DISCUSSION

In summary, a fragment of DNA has been isolated from a wild-type yeast gene bank which complements an *aro7* auxotrophic mutant of *Saccharomyces cerevisiae*. A restriction map of the plasmid pJFB1 and the cloned insert has been obtained and the size of the insert estimated to be 5.3 kb. Deletion analysis of pJFB1 has shown that the complementing region is located around a *Hind III* site 2.0 and 3.3 kb, respectively, from each end of the insert. Transcriptional analysis revealed that pJFB1 produces a 1 kb transcript which is polyadenylated and enzyme assays have shown an increase in chorismate mutase activity in the *aro7* auxotrophic strain transformed with pJFB1.

As pointed out by Nasmyth and Reed (1980), complementation of a mutation in a specific gene by a cloned sequence is not sufficient proof that this sequence corresponds to the gene being sought. For genes cloned by complementation it is necessary to demonstrate that the fragment codes for the wild-type gene and not a phenotypic suppressor.

The defect in the *ARO7* gene of strain J14-26 is a nonsense mutation, which results in the premature termination of translation (B. Carter, personal communication) and, therefore, could be suppressed by an altered tRNA which would read through this stop codon. There are, however, several lines of evidence to suggest that this has not happened.

First, pJFB1 produces a transcript which is polyadenylated and approximately the correct size to code for the chorismate mutase protein. This suggests that it is mRNA and not tRNA since the latter is not polyadenylated.

Secondly, in the strain J14-26TR1, the increase in chorismate

mutase activity above the level displayed by wild-type cells cannot be explained by the presence of a suppressor tRNA. A suppressor would be unlikely to increase the activity of a mutant strain above its original wild-type level. The possibility of having cloned a positive regulator of chorismate mutase can also be ruled out since this would not complement an *aro7* mutation of this nature.

Thirdly, transformation of strain J14-26 with pJFB1 confers upon the cells an increased resistance to the phenylalanine analogue β-thienylalanine, compared to non-transformed strains (K.Linton, personal communication). This implies that multiple copies of the complementing gene within the cell result in a greater production of phenylalanine than found in wild-type cells, a property not readily associated with a tRNA suppressor.

Fourthly, the *met4* mutation of strain J14-26 was also an amber mutation but it was not complemented by pJFB1 (K.Linton, personal communication).

Finally, the deletion plasmid pKH1 recombined with the original *aro7* mutation to produce a functional enzyme. Such a recombination event would only take place at a region of homology between the insert and the chromosomal *ARO7* locus and so implies that pKH1 and hence pJFB1 carries the wild-type *ARO7* gene.

All of these points indicated that pJFB1 contains the stuctural gene for chorismate mutase but they are not conclusive. Proof that a fragment actually codes for the complemented gene is generally demonstrated by showing that a plasmid containing the fragment integrates at the corresponding chromosomal locus and shows genetic linkage to the gene. Construction of an integrating vector and

subsequent integration experiments would, therefore, have been the next logical step in proving that the *ARO7* gene had been cloned.

While attempting to do this, however, Wickner and his collegues reported that they had also cloned the *ARO7* gene (Ball *et al.*, 1986). Comparison of the restriction map of their plasmid (YpAR7-1) with pJFB1 revealed a large region of homology within the insert, proving that the cloned region complementing the *aro7* mutation was the same, although the actual orientation was reversed.

Wickner's group had shown that their cloned gene integrated at the chromosomal *ARO7* locus. Since the pJFB1 insert shared a homologous DNA region with YpAR7-1 it was not necessary to perform any integration experiments to conclude that pJFB1 definitely contained the structural *ARO7* gene.

More information on the localisation of the *ARO7* gene was obtained from Hutter's group who were also working with YpAR7-1. They obtained full chorismate mutase activity from a plasmid containing the 2.7 kb *Bgl*II - *Cla*I fragment while the 1.8 kb *Cla*I fragment gave only partial activity (T. Schmidheini, personal communication). This suggested that the promoter was located around the *Cla*I site at 12.4 on the map. The direction of transcription would, therefore, be from left to right (see Fig. 4.8).

Alternatively, transcription might be in the opposite direction; the deletion may have removed part of the stuctural gene and thus produced a slightly less active form of the protein. This is unlikely, however, due to the location of the original nonsense mutation within the 2.3 kb *Hin*dIII fragment. Unless the mutation is situated between the *Cla*I site at 12.4 and the *Hin*dIII site at 12.7, transcription in

this direction would result in the improbable situation that although the 1.8 kb *Cla*I fragment displays partial chorismate mutase activity, the longer polypeptide synthesised by original mutant strain displays no enzyme activity at all.

Wickner found full chorismate mutase activity with a 2.4 kb *Sau*3A partial digestion fragment (Ball *et al.*, 1986) but a detailed location of this fragment was not given, although it did span the 0.9 kb *Hin*dIII - *Eco* RI region. More information was needed, therefore, before precise localisation of the gene and direction of transcription could be conclusively resolved.

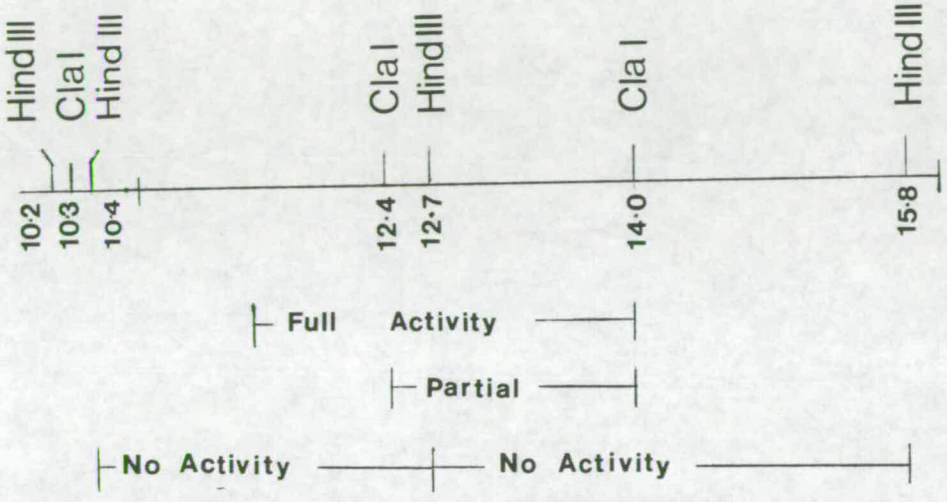


Figure 4.8 : Map of pJFB1 insert containing the *ARO7* gene. The levels of chorismate mutase activity, determined by complementation analysis, shown by different subclones are indicated.

5. ISOLATION AND ANALYSIS OF *aro7* REGULATORY MUTANTS

5.1 INTRODUCTION

Having cloned the *ARO7* gene from *Saccharomyces cerevisiae*, further molecular genetic analysis such as DNA sequencing could have been applied. It was considered important, however, not to lose sight of the original purpose of isolating the gene, which was to alter its regulation in a manner condusive to phenylalanine overproduction. Although multiple copies of the *ARO7* gene in the cell resulted in enhanced chorismate mutase activity, the enzyme should still be regulated by feedback inhibition by tyrosine. Any tendency towards increased phenylalanine production would be counteracted by the excess tyrosine also produced inhibiting the activity of the enzyme. Therefore, a yeast strain resistant to this feedback inhibition by tyrosine was required.

The aim of this section was to isolate regulatory mutants of *ARO7* by *in vitro* mutagenesis of pJFB1. During *in vitro* mutagenesis the only gene subject to mutation is the desired target gene and this procedure, therefore, offered a more specific and direct way to enrich for feedback-resistant mutations.

A mutation in the *ARO7* gene resulting in feedback insensitive chorismate mutase would be advantageous in several ways. Not only would it facilitate the overproduction of phenyalanine but the information obtained by analysing the phenotypes of the various regulatory mutants may result in a fuller understanding of the

regulation of aromatic amino acid biosynthesis in *Saccharomyces cerevisiae*.

5.2 ISOLATION OF aro7 REGULATORY MUTANTS

5.2.1 *In Vitro* Mutagenesis of PJFB1

400 µg pJFB1 were treated with the mutagen *O*-methylhydroxylamine, which induces C-T transitions in DNA. The *ARO7* auxotrophic yeast strain J14-26 was then transformed with the mutated plasmid and transformants were selected by their ability to grow in the absence of leucine.

The frequency of transformation was very low (Table 5.1). The 35 Leu$^+$ transformants were replica plated onto media lacking phenylalanine and tyrosine to check if they still complemented the *ARO7* gene. 24 transformants were able to grow without phenylalanine and tyrosine supplementation, the remaining eleven had probably lost chorismate mutase activity completely.

TABLE 5.1 : Number of transformants obtained in the transformation of yeast strain J14-26 with *in vitro* mutated (m) pJFB1.

DNA SOURCE	TRANSFORMANTS
0	0
5 µg pJFB1	240
2 µg pJFB1(m)	2
4 µg "	4
10 µg "	9
20 µg "	18
40 µg "	2

5.2.2 Selection of Regulatory Mutants

Mutants with feedback resistant chorismate mutase should contain elevated phenyalanine and tyrosine pools and, therefore, show increased resistance to analogues of these amino acids. This rationale formed the basis of the selection method used to isolate regulatory mutants of *ARO7*.

The 24 *Aro*$^+$ colonies transformed with the mutated pJFB1 were transferred to media containing a range of concentrations of β-thienylalanine and 5-methyltyrosine and checked for an increase in analogue resistance compared to the wild-type transformant J14-26TR1. Nine of the transformants showed an increased resistance to the phenylalanine analogue β-thienylalanine and were able to grow at a concentration of 50 mg ml^{-1} which is inhibitory to the mutant transformed with the wild-type plasmid (Fig. 5.1). No significant increase in resistance to 5-methyltyrosine was detected.

This phenotype was correlated with the presence of the plasmid by plasmid-loss experiments and retransformation. Strain 2, however, retained its resistant phenotype in all colonies, after growth on complex medium, and failed to retransform. Further analysis of its genotype revealed that it did not contain any of the auxotrophic markers carried by the original J14-26 strain. It was, therefore, assumed to be a contaminant.

Figure 5.1 : Growth of J14-26 transformed with *in vitro* mutated pJFB1 on different concentrations of β-thienylalanine.

A - 0 μg ml^{-1}

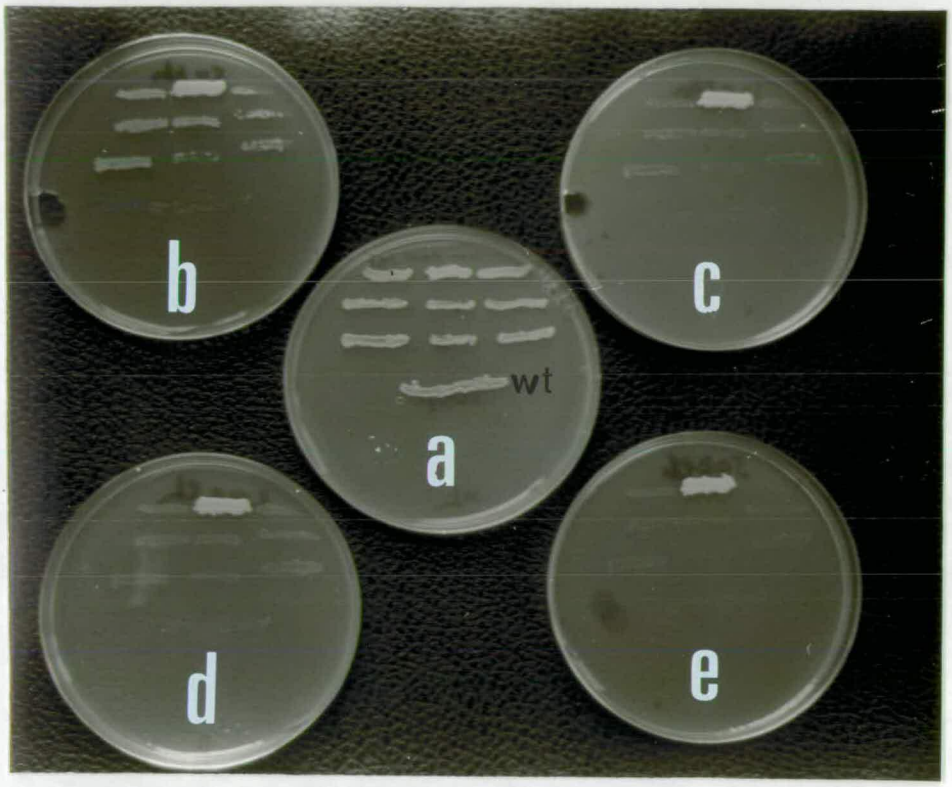
B - 50 μg ml^{-1}

C - 100 μg ml^{-1}

D - 250 μg ml^{-1}

E - 500 μg ml^{-1}

wt - wild-type


b
c
wt
a
d
e

5.3 REGULATION OF CHORISMATE MUTASE ACTIVITY IN *Saccharomyces cerevisiae*

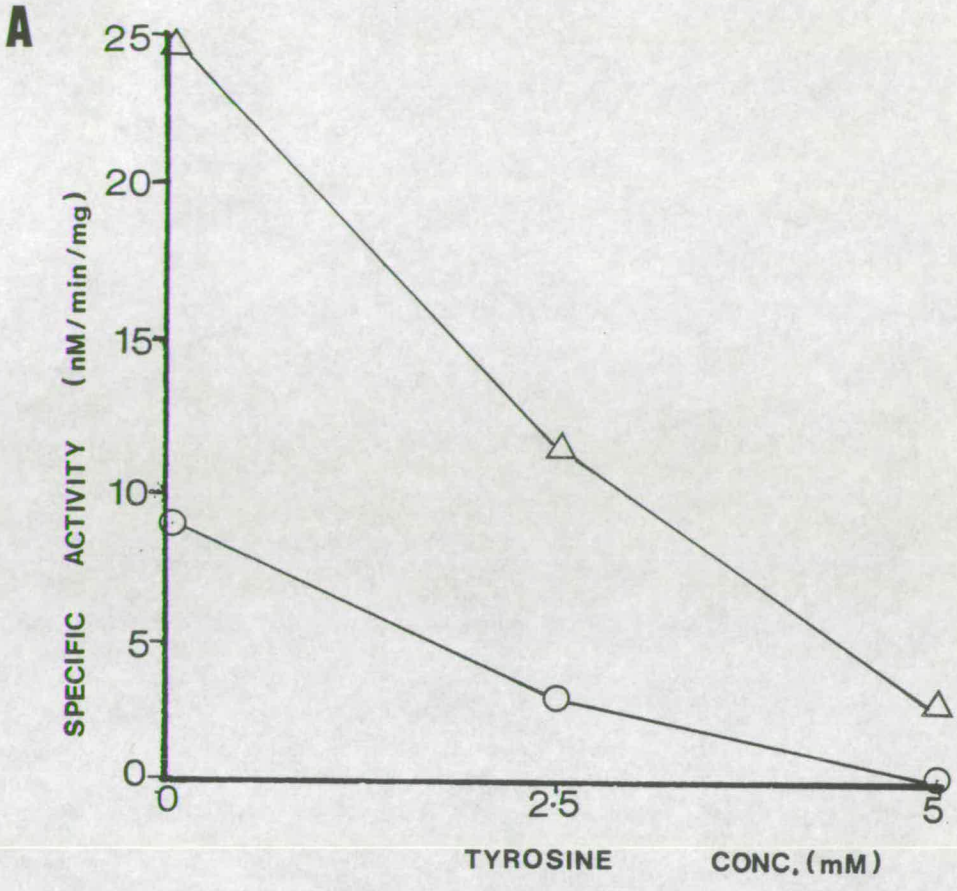
The four regulatory mutants which displayed the greatest increase in resistance to β-thienylalanine (J14-26IV3, J14-26IV5, J14-26IV6 and J14-26IV9) were used, along with J14-26TR1, containing the wild-type pJFB1, and the wild-type strain 314 to assay chorismate mutase activity under a variety of conditions. Unless otherwise stated, each result was the average of at least four repetitions of the assay concerned.

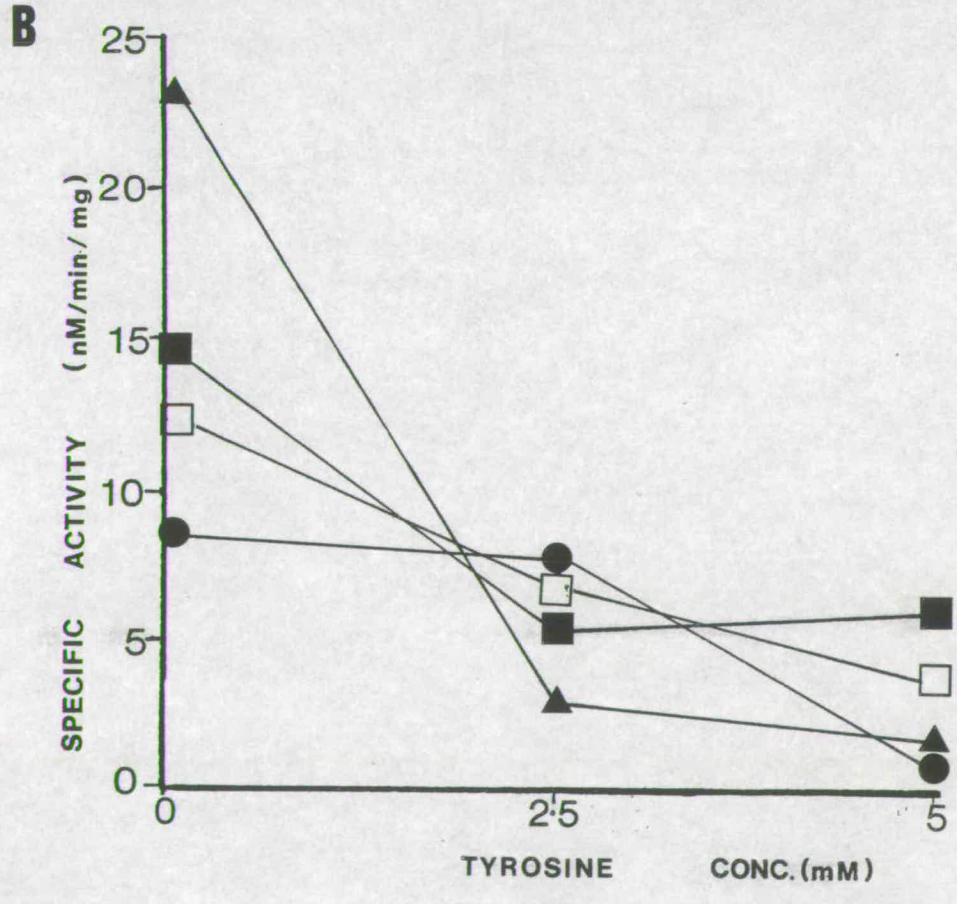
5.3.1 Effect of Tyrosine

Extracts of soluble proteins from the above strains were assayed for chorismate mutase acivity in the presence of a range of concentrations of tyrosine. The results are presented graphically in Figs. 5.2A and 5.2B.

Graph A shows that both strains displayed feedback inhibition of chorismate mutase by tyrosine. Strain J14-26TR1 had a higher initial activity and was not inhibited to the same degree as 314, still showing some activity at 5 mM tyrosine. The transformed strain carries multiple copies of the *ARO7* gene and thus produces more chorismate mutase, feedback inhibition, therefore, would not be so effective as with an nontransformed strain, at a given concentration of tyrosine.

Graph B shows the effect of tyrosine on the four strains representing possible regulatory mutants of *ARO7*. All four strains showed inhibition by tyrosine but to different extents. As the concentration of tyrosine increased, both J14-26IV5 and J14-26IV6 displayed a decrease in chorismate mutase activity similar to that of the unmutated strain. Strains J14-26IV3 and J14-26IV9, however,


A
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)


B
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)

appeared to have an increased resistance to inhibition by tyrosine, showing half their initial level of activity at 5 mM tyrosine compared to a decrease by a factor of approximately 10 in the other strains. These results indicate that these two strains contain mutations in the *ARO7* gene which render it resistant to feedback inhibition by tyrosine.

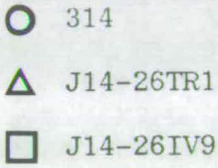
5.3.2 Effect of Tryptophan

The above experiment was repeated, this time assaying chorismate mutase activity in the presence of a range of tryptophan concentrations.

Fig.5.3 shows that tryptophan had vitually no effect on chorismate mutas activity, over the range of concentrations used. This result was surprising since tryptophan had previously reported to activate the enzyme (Jones & Fink, 1982), so an increase in activity was expected. Other groups, however, had stated that rather than stimulating chorismate mutase activity, tryptophan counteracts the inhibition by tyrosine (Miozzari *et al.*,1978). To resolve these contradictory results, the effect of both tyrosine and tryptophan on chorismate mutase activity was studied in all six strains.

Graph A in Fig.5.4 clearly shows that, in the wild-type strain, tryptophan did overcome the inhibitory effect of tyrosine; the extent to which it counteracted inhibition increasing with tryptophan concentration. Strain J14-26TR1, represented in graph B, also showed this reduction in tyrosine inhibition although to a lesser extent, with 5 mM tyrosine still causing inhibition even at levels of 5 mM tryptophan. In the wild-type strain 314, 5 mM tryptophan practically abolished tyrosine inhibition, chorismate mutase activity was restored

Figure 5.3 : Levels of chorismate mutase activity displayed by various yeast strains assayed in different concentrations of tryptophan.



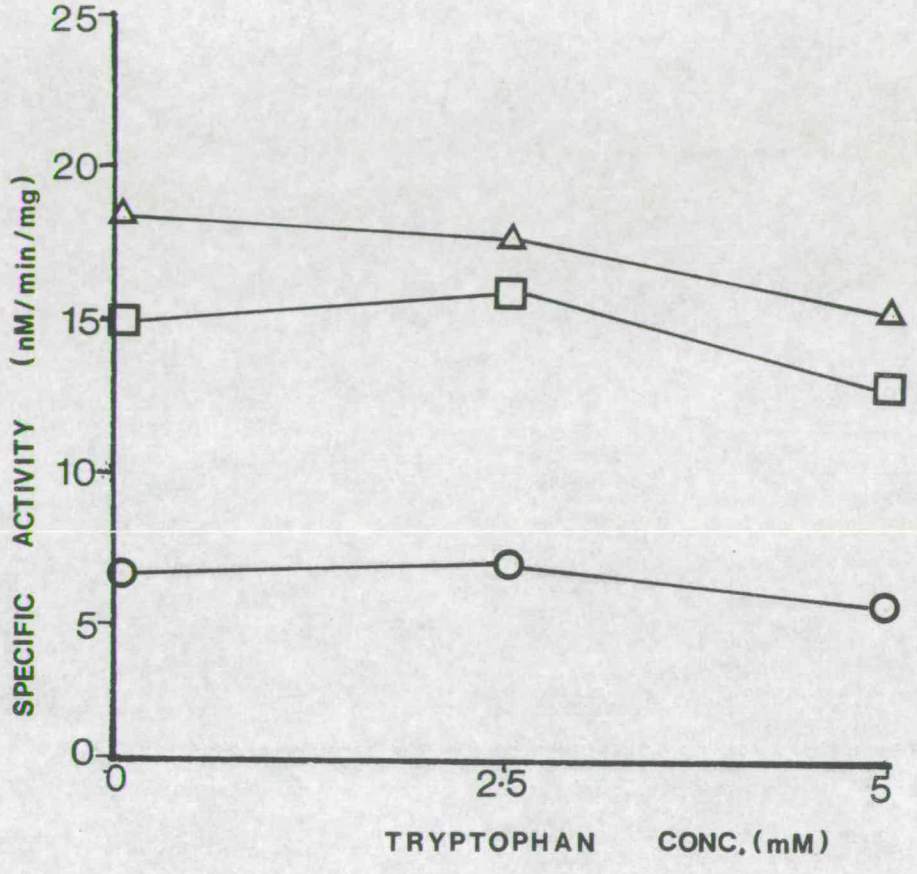

25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TRYPTOPHAN CONC.(mM)

Figure 5.4 : Levels of chorismate mutase activity displayed by various yeast strains assayed in different concentrations of tyrosine and tryptophan.

Graph A - 314

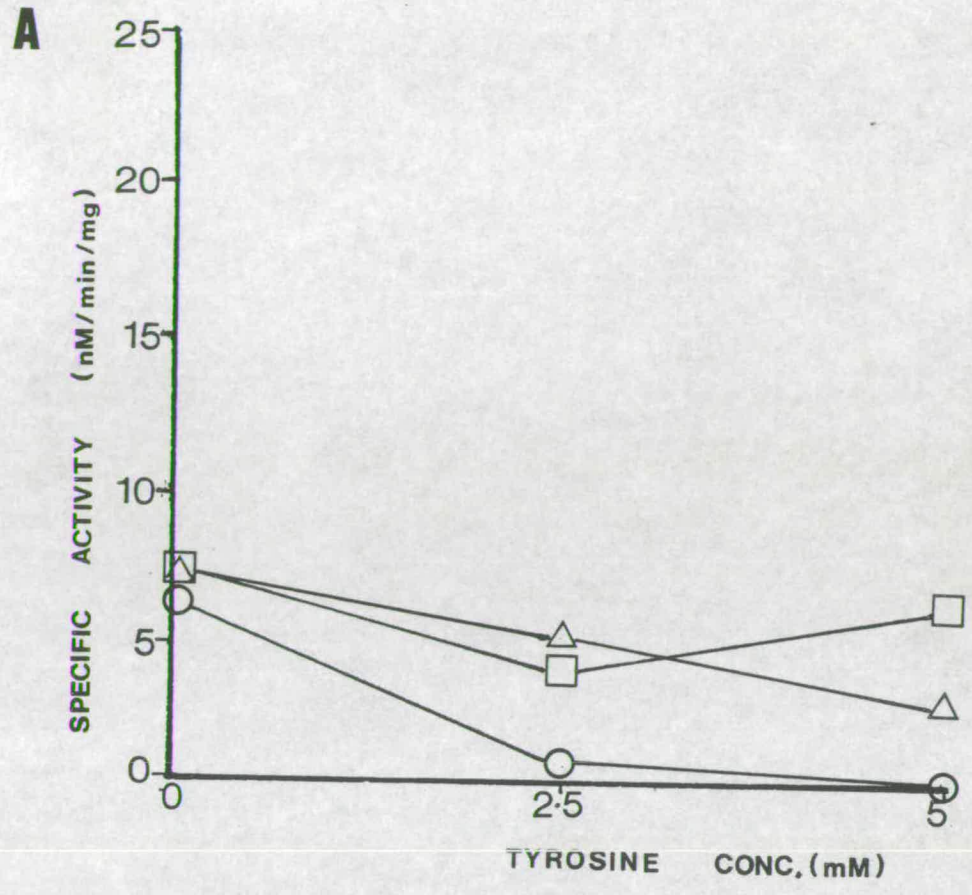
Graph B - J14-26TR1

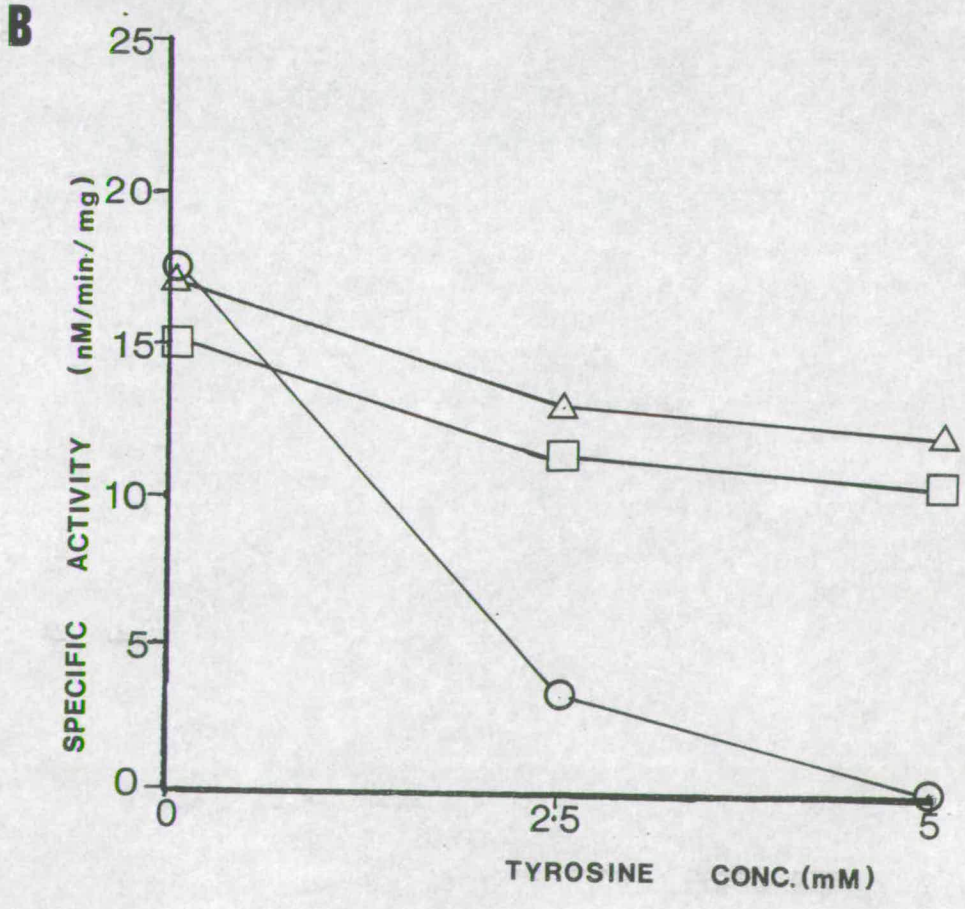
Graph C - J14-26IV9

○ 0 mM Tryptophan

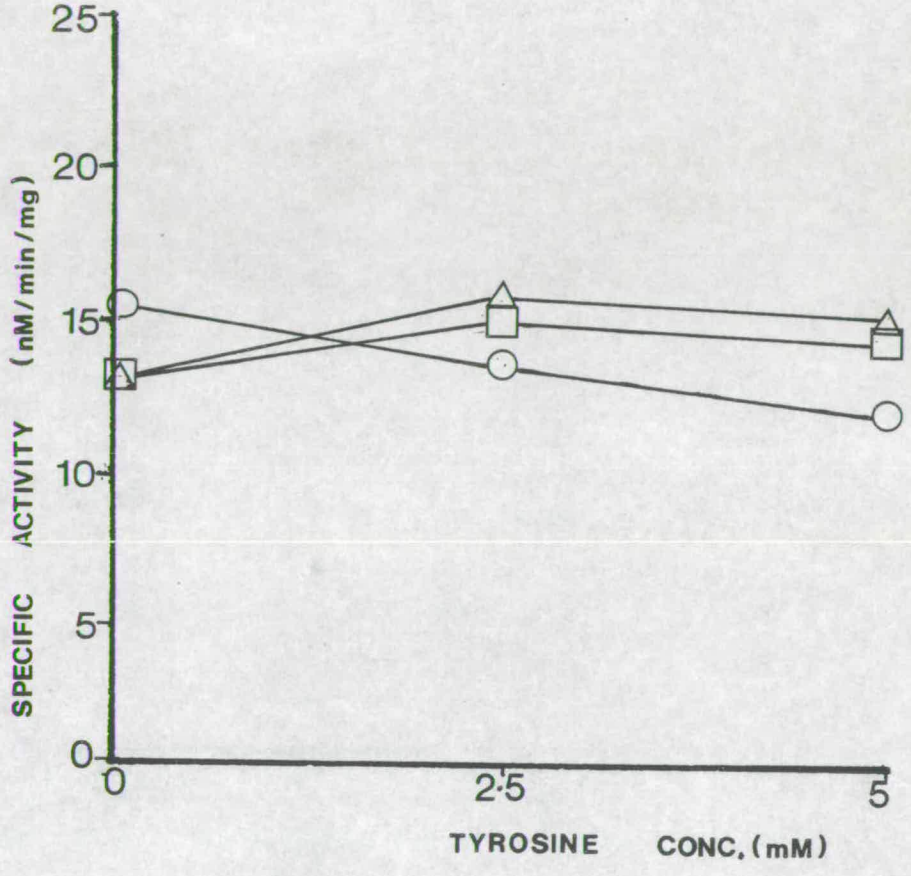
△ 2.5 mM Tryptophan

□ 5.0 mM Tryptophan


A
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)


B
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)

C


25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)

to its original level seen at 0 and 5 mM tyrosine.

Since J14-26IV9 appeared to be resistant to feedback inhibition by tyrosine, there was the possibility that tryptophan may, in counteracting inhibition, have led to a further increase in chorismate mutase activity. The results displayed in Fig.5.3 showed that tryptophan had very little effect on chorismate mutase but when tyrosine was present (Fig.5.4C) there was a very slight increase in enzyme activity. Since the data was the result of only two assays, however, it was doubtful whether this increase was significant.

5.3.3 Effect of Tyrosine on a Tryptophan Prototrophic Strain

The yeast strain J14-26 carries a mutation in the *TRP1* gene, therefore supplementation with tryptophan was necessary. Tryptophan was shown to affect tyrosine inhibition, so the additional tryptophan in the media may have been affecting the results obtained in these studies, making comparisons between strains less easy to interpret. The plasmid pJFB1 was, therefore, transformed into the tryptophan prototrophic strain SF747-19D and the effect of tyrosine on chorismate mutase activity analysed (Fig.5.5).

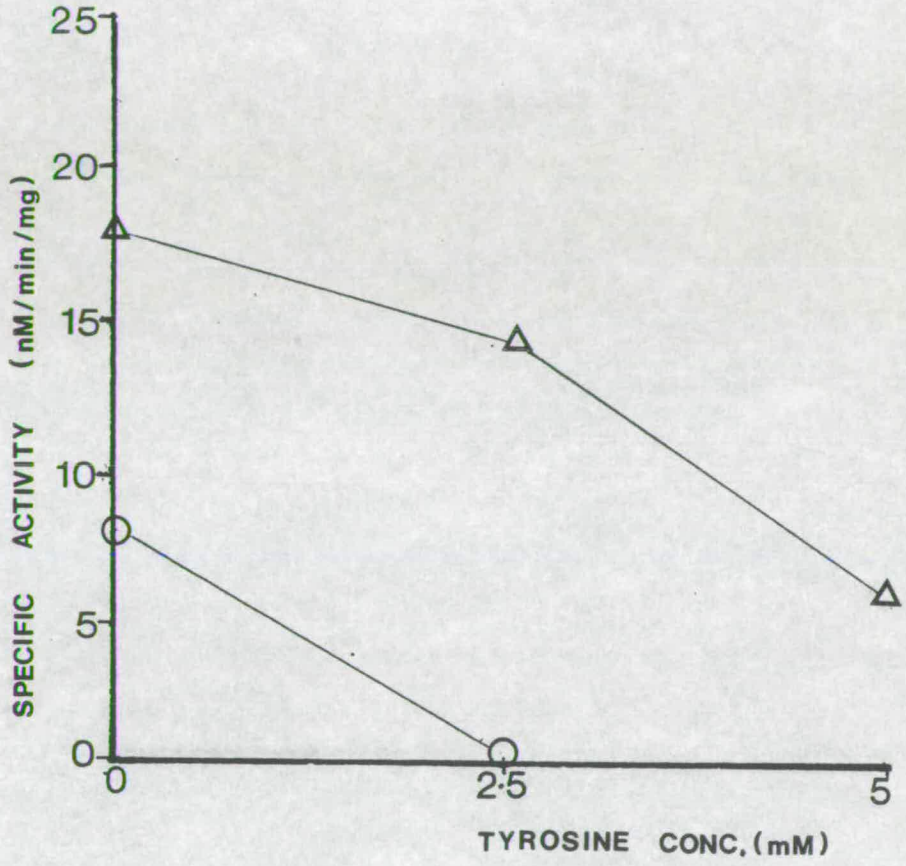
No significant difference in tyrosine inhibition was found between tryptophan auxotrophic and prototrophic backgrounds. As expected the untransformed SF747-19D showed a reduction in chorismate mutase activity with increasing tyrosine concentration similar to that displayed by 314.

Chorismate mutase activity in SF747-19D carrying pJFB1 was also inhibited in a manner similar to J14-26TR1. The presence of tryptophan in the growth media, therefore, did not affect tyrosine inhibition. Presumably this was because it was present in quantities just

Figure 5.5 : Levels of chorismate mutase activity displayed by tryptophan prototrophic yeast strains assayed in different concentrations of tyrosine.

○ SF747-19D

△ SF747-19DTR1


25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)

sufficient to allow growth. Also, if there was any exogenous tryptophan this would be removed during the initial washes.

5.3.4 Effect of Phenylalanine

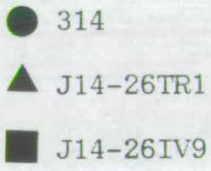
The mutant pJFB1 plasmids were selected on the basis of conferring increased β-thienylalanine resistance to their host yeast strain. This was, presumably, due to the transformed strains overproducing phenylalanine. Therefore, the possibility that chorismate mutase was regulated in some way by phenylalanine was investigated.

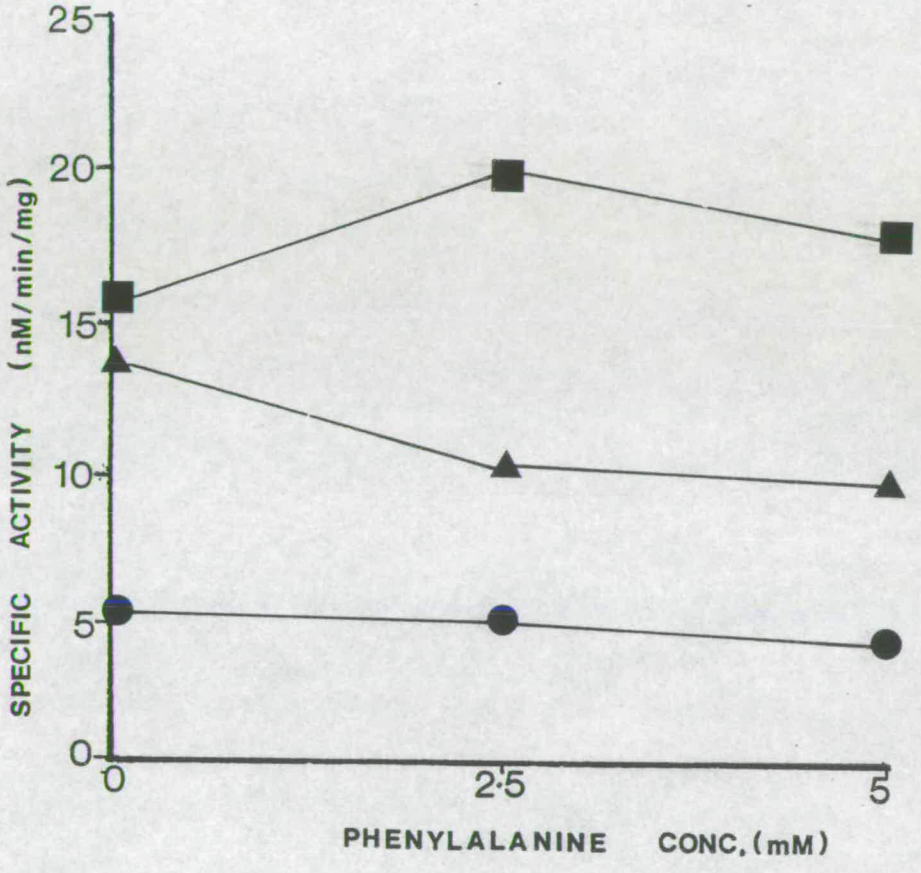
Fig.5.6 shows the results from chorismate mutase assays carried out over a range of phenylalanine concentrations. Over the range used, phenylalanine had very little effect on chorismate mutase activity; there was a slight decrease in activity in strain J14-26TR1 which was not shown in strain J14-26IV9 but since 314 was not affected it was unlikely that this decrease represented inhibition by phenylalanine.

5.4 REGULATION OF *ARO7* EXPRESSION IN *Saccharomyces cerevisiae*

In addition to the inhibition or activatoin of enzyme activity, a metabolic pathway may be regulated by controlling the transcription rate of specific genes. Expression of the *ARO7* gene was studied by assaying chorismate mutase activity in various yeast strains grown in the presence of the appropriate amino acids. These added amino acids were removed before assaying, therefore any alteration in chorismate mutase activity was due to a change in the amount of enzyme produced.

Figure 5.6 : Levels of chorismate mutase activity displayed by various yeast strains assayed in different concentrations of phenylalanine.




25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
PHENYLALANINE CONC.,(mM)

5.4.1 Effect of Tyrosine

Strains 314, J14-26TR1, J14-26IV3, 5, 6 and 9 were grown in minimal media containing different cocncentrations of tyrosine, plus appropriate amino acid supplements. The cells were washed several times to remove exogenous amino acids before assaying for chorismate mutase. The results are represented graphically in Fig. 5.7.

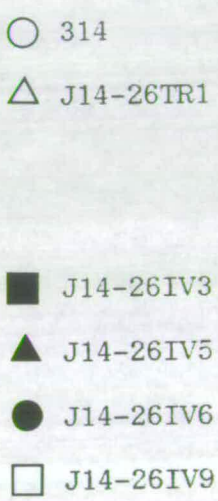
As the concentration of tyrosine in the growth medium was increased, there was a corresponding decrease in chorismate activity (Graph A). The tyrosine was removed before the activity was measured so the reduction in activity was unlikely to be caused by inhibition but due to a decrease in the actual amount of enzyme present. Therefore, tyrosine was probably repressing transcription of the *ARO7* gene. Overexpression of *ARO7*, in J14-26TR1, led to the production of higher levels of enzyme at all tyrosine concentrations but repression was still seen.

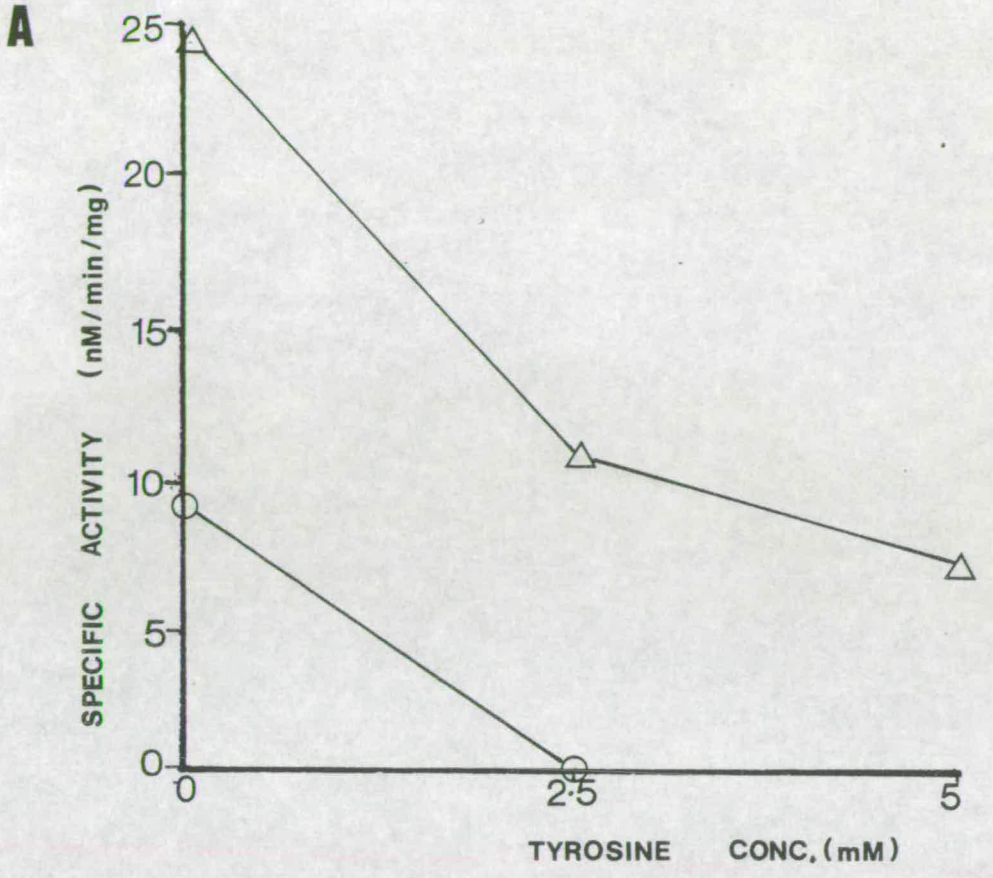
Fig. 5.7B shows the effect of incresing the concentration of tyrosine in the medium on the chorismate mutase activity of the mutated strains. Both J14-26IV3 and J14-26IV5 seem to be repressed by tyrosine in a similar manner to the unmutated strain. J14-26IV6 and J14-26IV9, however, appeared to be resistant to this effect, showing no appreciable decrease in activity with increasing tyrosine concentration.

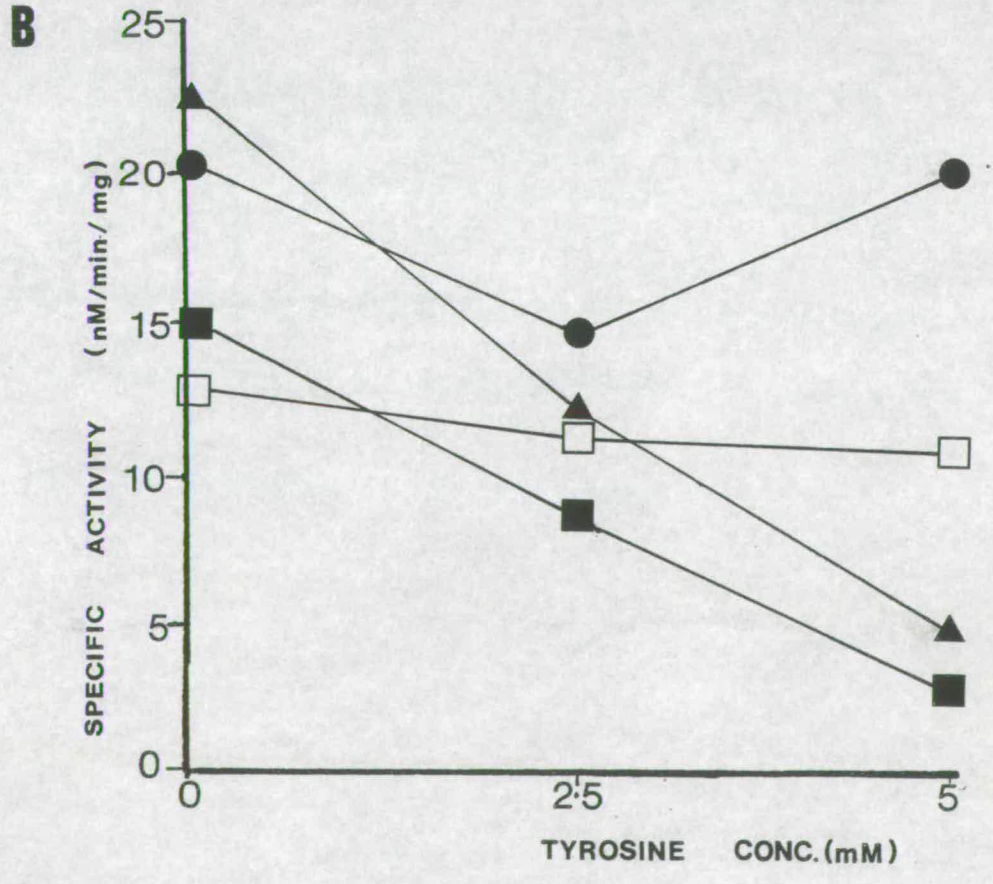
5.4.2 Effect of Tryptophan

Chorismate mutase activity was studied in the three strains, 314, J14-26TR1 and J14-26IV9, this time grown in various concentrations of tryptophan. (The concentrations of tryptophan cited do not include the low level of tryptophan supplement (10 mgl^{-1}), required by strains

Figure 5.7 : Levels of chorismate mutase activity displayed by various yeast strains grown in different concentrations of tyrosine.




A
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC.(mM)


B
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC.(mM)

J14-26TR1 and IV9)

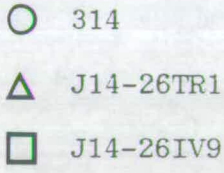
Fig.5.8 shows that tryptophan caused a very slight increase in chorismate mutase activity in all three strains. There was, however, considerable variation in the values for addition of 5 mM tryptophan hence it was doubtful whether this increase represented a valid increase in *ARO7* expression. Lingens *et al.* (1967) described an induction of *ARO7* expression but this could not be confirmed by later work (Kradolfer, 1977).

Since tryptophan had been shown to counteract inhibition by tyrosine, the same three strains were grown in the presence of both tyrosine and tryptophan to find out whether tryptophan also reduced tyrosine repression (Fig.5.9A). With strain 314, tryptophan had little effect on the rate of tyrosine repression, although higher enzyme activity was noticed with increasing tryptophan concentration, at all levels of tyrosine. Strain J14-26TR1 showed similar results (Graph B) while J14-26IV9 still appeared to be resistant to the repression effect. It also showed no increase in activity with increased levels of tryptophan.

5.4.3 Effect of Phenylalanine

Chorismate mutase was assayed over a range of phenylalanine concentrations (0 to 5 mM) in the growth media but no significant change in *ARO7* expression was observed (Fig.5.10) since all strains displayed an approximately constant level of activity over the range of phenyalanine concentrations used.

Figure 5.8 : Levels of chorismate mutase activity displayed by various yeast strains grown in different concentrations of tryptophan.



Tryptophan concentrations do not include the additional 0.1 mM tryptophan supplement required by strains J14-26TR1 and J14-26IV9.

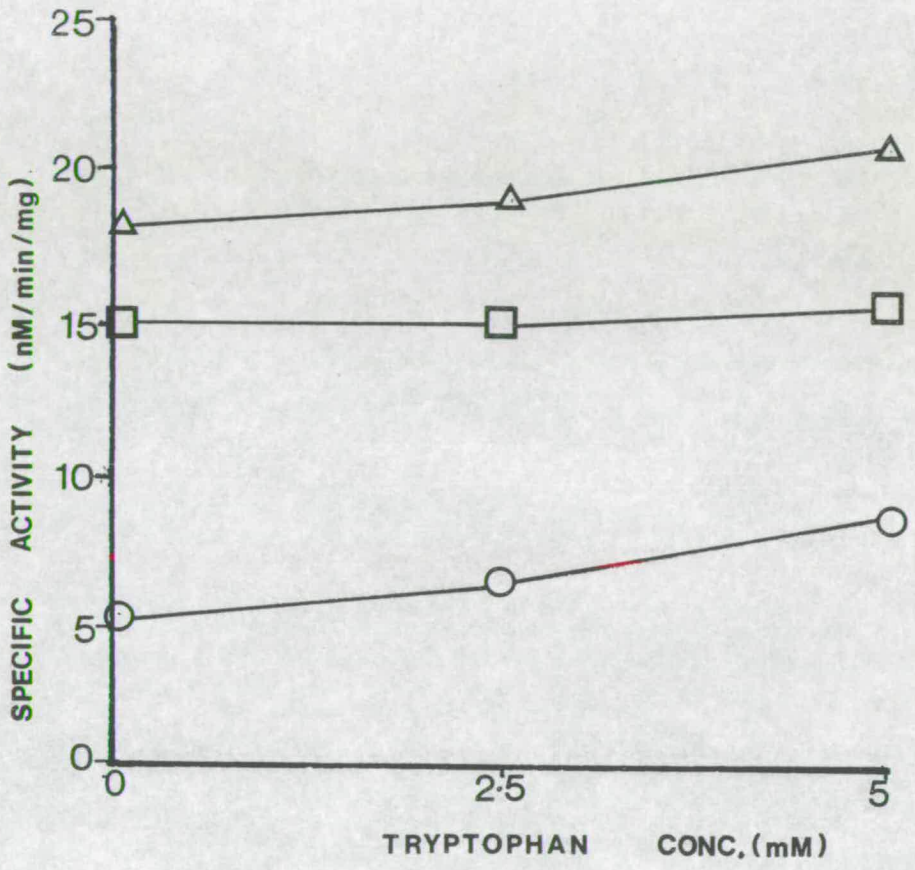

25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TRYPTOPHAN CONC. (mM)

Figure 5.9 : Levels of chorismate mutase activity displayed by various yeast strains grown in different concentrations of tyrosine and tryptophan.

Graph A - 314

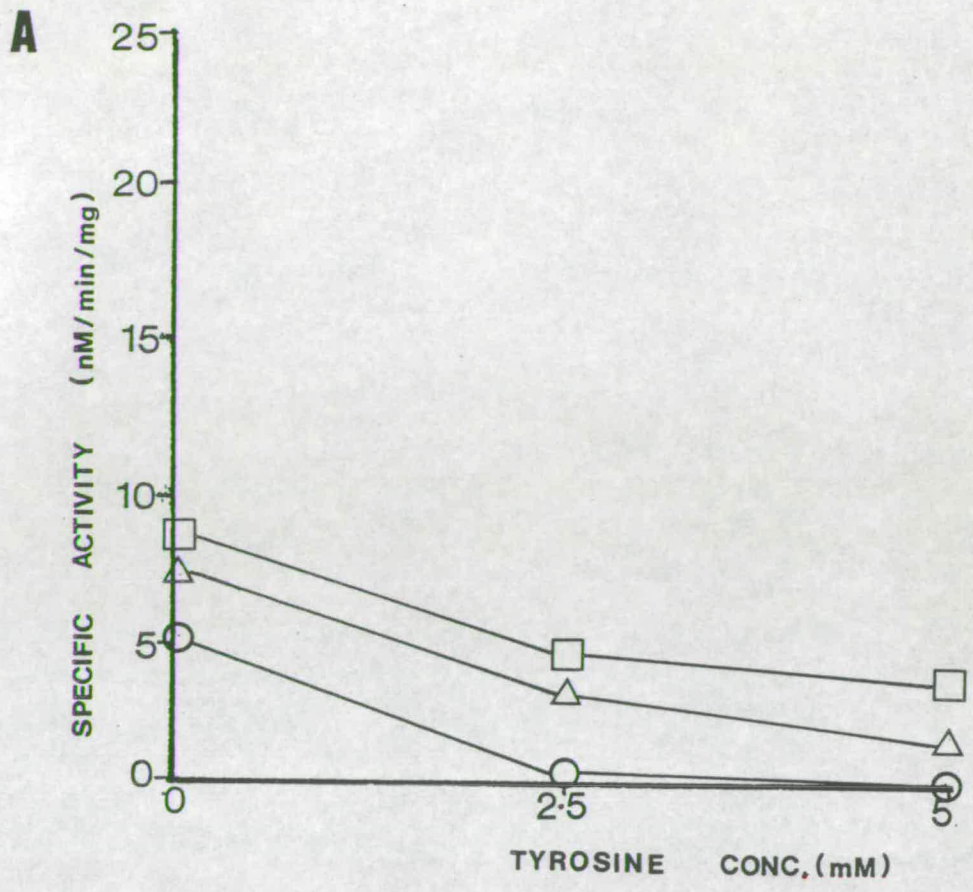
Graph B - J14-26TR1

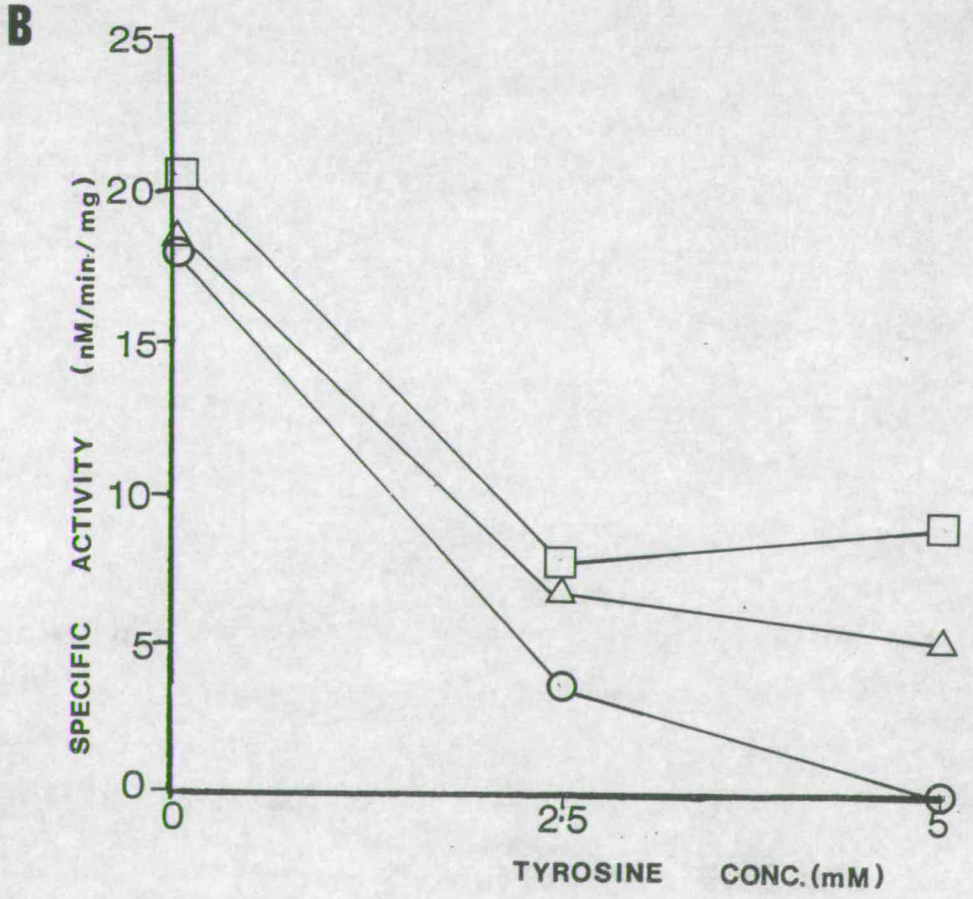
Graph C - J14-26IV9

○ 0 mM Tryptophan

△ 2.5 mM Tryptophan

□ 5.0 mM Tryptophan


A
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC.(mM)


B
25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC.(mM)

C

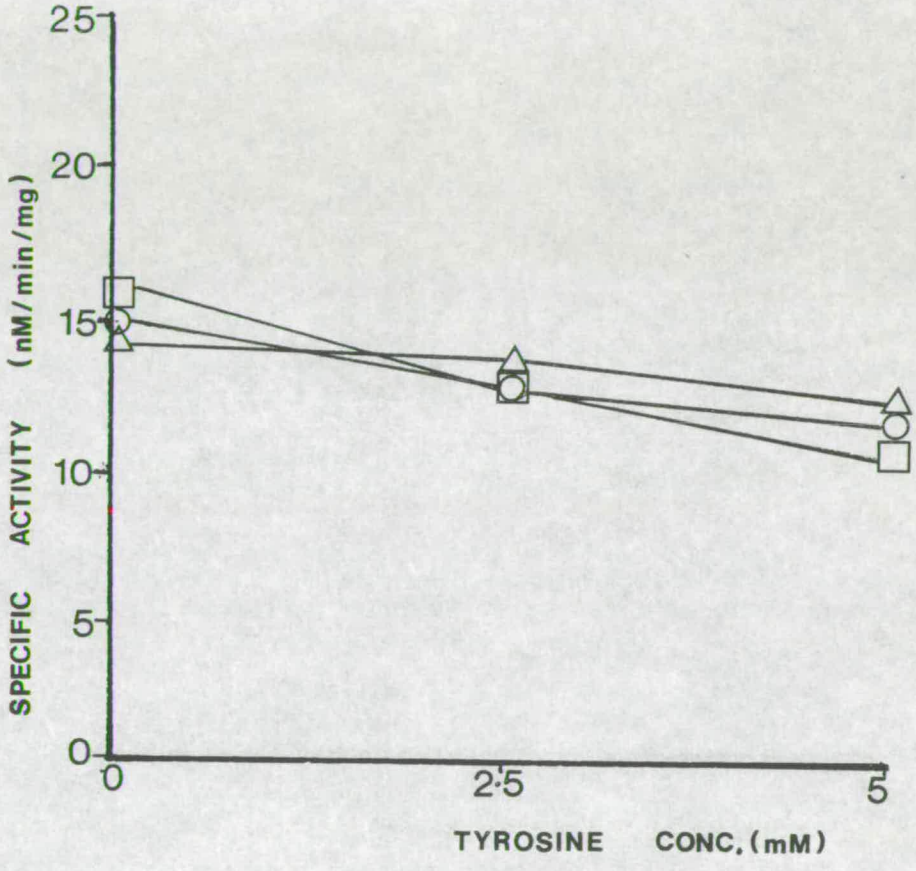
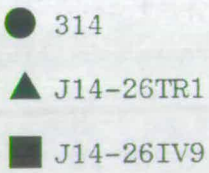
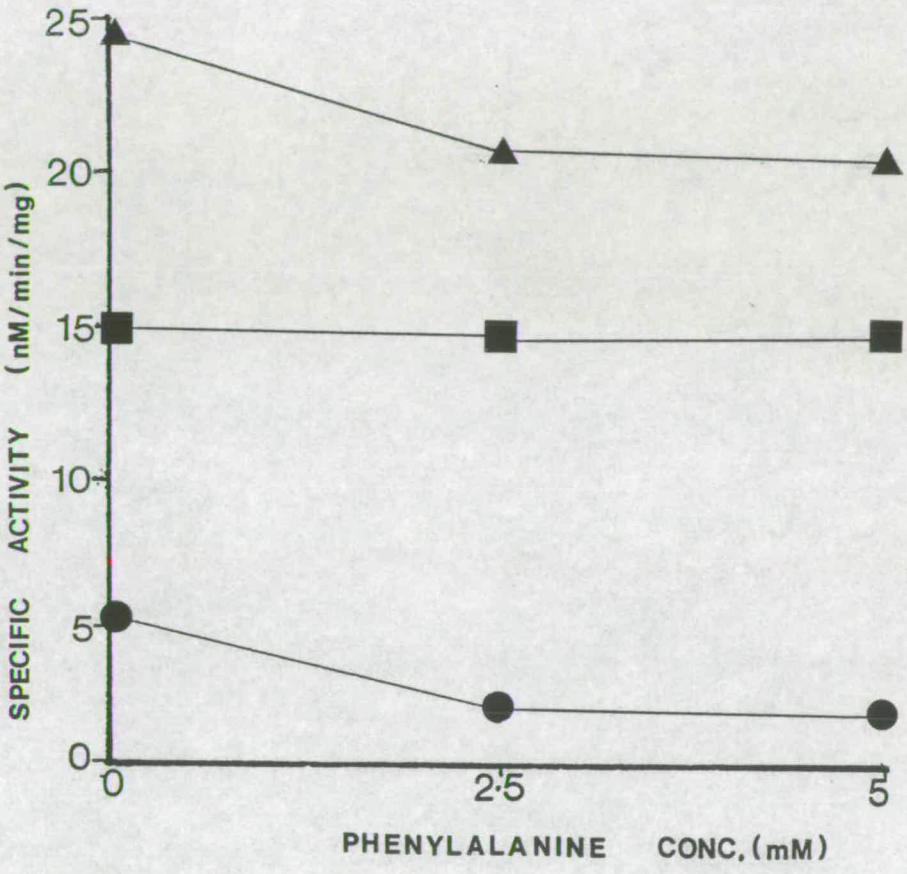

25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
TYROSINE CONC. (mM)

Figure 5.10 : Levels of chorismate mutase activity displayed by various yeast strains grown in different concentrations of phenylalanine.




25
20
15
10
5
0
SPECIFIC ACTIVITY (nM/min/mg)
0
2·5
5
PHENYLALANINE CONC.(mM)

5.5 OVERPRODUCTION OF PHENYLALANINE

The mutant colonies were isolated on the basis of their increased resistance to the phenylalanine analogue β-thienylalanine. This is, probably, due to the various strains producing increased levels of phenylalanine compared to the wild-type strain.

The amino acid content of extracellular supernatants from the various strains, grown with and without additional tyrosine (50 μg ml^{-1}) in the media, was analysed by thin layer chromatography, in an attempt to identify and quantitate the phenylalanine.

All the mutated strains showed detectable levels of phenylalanine excreted into the medium (between 0.01 and 0.1 mg ml^{-1}). Phenylalanine was not detected in the supernatants of 314 and J14-26TR1 indicating that the regulatory mutants are overproducing phenylalanine. The supernatants of the mutant strains grown in the presence of tyrosine showed a small increase in phenylalanine excretion although the amount was still less than 0.1 mg ml^{-1}. This increase in phenylalanine production is, presumably, due to the excess tyrosine inhibiting prephenate dehydrogenase (see Fig. 1.2), making more prephenate available for phenylalanine biosynthesis.

5.6 DISCUSSION

Several regulatory mutants of *ARO7* have been isolated. The phenotypes of the four strains analysed can be summarised as follows: J14-26IV3 showed a partial resistance to feedback inhibition by tyrosine while J14-26IV6 appeared to have an increased resistance to tyrosine repression. J14-26IV5 displayed characteristics similar to the unmutated J14-26TR1, being subject to both inhibition by tyrosine and repression. J14-26IV9, however, showed resistance to tyrosine repression and

possibly resistance to inhibition as well.

It is not yet known whether tyrosine acts as a competetive or non-competetive inhibitor of chorismate mutase. Research in *E. coli* (Christopherson, 1985) suggests that it interacts with the substrate binding site, however, extrapolation to yeast is complicated by the fact that, in *E. coli*, chorismate mutase is part of a bifunctional protein which also incorporates prephenate dehydrogenase activity (Camakaris & Pittard, 1983).

If tyrosine does bind to the active site then any mutation reducing its binding may also affect the binding of the substrate and hence reduce tha activity of the enzyme. This was observed in the mutant strains J14-26IV3 and IV9. These strains both showed resistance to inhibition by tyrosine but their initial level of activity was much lower than that displayed by the unmutated strain. Alternatively, tyrosine may bind to an allosteric site which has been altered in the mutants, reducing their capacity to bind tyrosine. Depending on the nature of the conformational change, this could also affect the enzyme's affinity for chorismate and reduce its activity.

The most likely mechanism for loss of inhibition is by introducing a mutation into the stuctural gene for the enzyme concerned. The feedback-resistant mutants were obtained by *in vitro* mutagenesis of pJFB1 and so provide strong evidence that the plasmid carries the structural gene for chorismate mutase in *Saccharomyces cerevisiae*.

A surprising result was the observation that addition of tryptophan to the assay mixture did not actvate chorismate mutase to any significant extent. Other groups had reported a 20-27 fold

increase in enzyme activity in the presence of 1 mM tryptophan (Kradolfer *et al.*, 1977; Ball *et al.*, 1986). In strain J14-26TR1 this lack of activation could be due to the tryptophan present in the medium causing maximum enzyme activity throughout. Strain 314, however, was not supplemented with tryptophan so an increase in activity would be expected on the addition of tryptophan.

In contrast to the above reports it was noticed, however, that tryptophan counteracted inhibition by tyrosine. In strains 314 and J14-26TR1, the enzyme activity in cells grown in 2.5 mM tryptophan, 5 mM tyrosine was reduced by 50 and 25% respectively compared to a complete loss of activity when no tryptophan was present. Tryptophan restored the slight loss of activity in J14-26IV9 but did not increase it, to any significant extent, beyond its original (no additional tyrosine or tryptophan) level.

Phenylalanine had been reported to play a role in the regulation of chorismate mutase in several species (Gething & Davidson, 1977; Singh *et al.*, 1985) but showed no detectable effect on enzyme activty in the *Saccharomyces cerevisiae* strains used.

Strain J14-26IV9 displayed resistance to feedback inhibition by tyrosine and was selected on the basis of its increased resistance to β-thienylalanine, an analogue of phenylalanine. This strain showed, at the concentrations used, no increase in resistance to 5-methyltyrosine, compared to the unmutaed strain, and so indicates that it is overproducing more phenylalanine than tyrosine. The conversion of chorismate into prephenate, catalysed by chorismate mutase, is the last step in the common phenylalanine/tyrosine biosynthetic pathway. Prephenate is subsequently converted into

phenylpyruvate and 4-hydroxyphenylpyruvate by prephenate dehydratase and prephenate dehydrogenase respectively (Jones & Fink, 1982). Prephenate dehydratase, encoded by the *PHA2* gene, has a higher affinity for prephenate than the dehydrogenase (Jones & Fink, 1982)) and so phenylalanine is preferentially synthesised. This alone may explain the increase in phenylalanine production although excess phenylalanine is reported to inhibit prephenate dehydratase. The overproduction, therefore, may be due to the flux through this pathway being more sensitive to the increase in prephenate than the flux through the tyrosine pathway.

Although the aim of these experiments was to isolate mutants resistant to feedback inhibition by tyrosine, the analysis of the mutants obtained has revealed much information not only on the regulation of chorismate mutase activity but also on the control of *ARO7* expression. The most interesting of these being the fact that, as well as chorismate mutase being inhibited by tyrosine, its production appears to be repressed by tyrosine. Tyrosine mediated repression of chorismate mutase has been well documented in *E. coli* (Camakaris & Pittard, 1983; Hudson & Davidson, 1984) but not considered to operate in yeast, inhibition being cited as the major control process (Lingens *et al.*, 1967).

Two of the mutants studied, J14-26IV6 and IV9 showed resistance to this repression. Strain J14-26IV9 also showed resistance to inhibition by tyrosine. This indicates that the *ARO7* gene on this plasmid probably contains at least two separate mutations; one in the structural gene affecting its conformation and another in its upstream regulatory sequences. This is not unlikely since treatment

with O-methylhydroxylamine *in vitro* probably causes several random mutations throughout the plasmid.

These results do not prove conclusively that the decrease in chorismate mutase activity is caused by a reduction in *ARO7* expression, many other factors including post-translational modif cation must be taken into consideration. The existence of these "repression" resistant mutants, therefore, will be advantageous in analysing this effect further and facilitate a more thorough understanding of the regulation of *ARO7* expression.

6. TRANSCRIPTIONAL ANALYSIS OF THE *ARO7* GENE IN *Saccharomyces cerevisiae* STRAINS J14-26TRI AND J14-26IV9

6.1 INTRODUCTION

Although the regulatory mutants were isolated with the aim of overproducing phenylalanine, analysis of their phenotypes revealed many new aspects to the regulation of the *ARO7* gene. With the withdrawal of G.D. Searle from the project, however, the pressure to concentrate research on phenylalanine overproduction was removed and hence it was considered important to study the tyrosine repression phenomenom in more detail. The main point to ascertain was whether the decrease in chorismate mutase activity was due to tyrosine illiciting a decrease in the actual transcription of the *ARO7* gene and, if so, was transcription of the repression-resistant gene unaffected ? This was answered by directly investigating the effect of tyrosine on *ARO7* transcription in both the wild-type strain J14-26TR1 and the mutated strain J14-26IV9, which displayed resistance to both repression and inhibition by tyrosine.

6.2 NORTHERN ANALYSIS AND HYBRIDISATION

Total RNA was isolated from 250 ml cultures of *Saccharomyces cerevisiae* strains J14-26TR1 and J14-26IV9 grown in a range of concentrations of tyrosine (0 to 5 mM). 20 µg RNA samples from each strain were run on a denaturing formaldehyde/agarose gel and then tranferred to nitrocellulose filters.

The additional information on the localisation of the *ARO7* gene

within pJFB1 (T. Schmidheini, personal communication) allowed the construction of a more specific DNA probe. The probe was obtained by a *Stu*I/*Bam*HI double digest of pJFB1 and subsequent isolation of the 3.1 kb insert-derived fragment which was nick-translated using (α-^{32}P)-dATP. This fragment spans the *Hin*dIII site known to be in the structural gene and possibly has less extraneous DNA at either end . It therefore has an increased likelihood of hybridising specifically to *ARO7* mRNA, compared with previous probes.

The filter was hybridised with this probe for 36 h and then exposed to X-ray film for 20 h.

As can be seen from Fig.6.1, the probe only hybridised to one band, assumed to be the *ARO7* transcript. In lanes 1 to 4 there is a decrease in the intensity of the bands indicating that there is less mRNA present. It appears, therefore, that tyrosine is repressing the transcription of the wild-type *ARO7* gene in strain J14-26TR1.

The band in lane 5, representing the transcript produced from strain J14-26IV9, had an increased intensity indicating that there was a larger amount of mRNA present compared to the other lanes. It was deduced, therefore, that the mutant *ARO7* gene in J14-26IV9 has an increased level of transcription.

Preliminary analysis revealed that the levels of the *ARO7* transcript in this mutant strain did not decrease significantly as the concentration of tyrosine in the growth media increased but more substantial evidence was required.

6.3 ANALYSIS OF TRANSCRIPTION USING THE SLOT-BLOT METHOD

One of the advantages of isolating a specific *ARO7* probe which hybridised to a single transcript was that further transcriptional

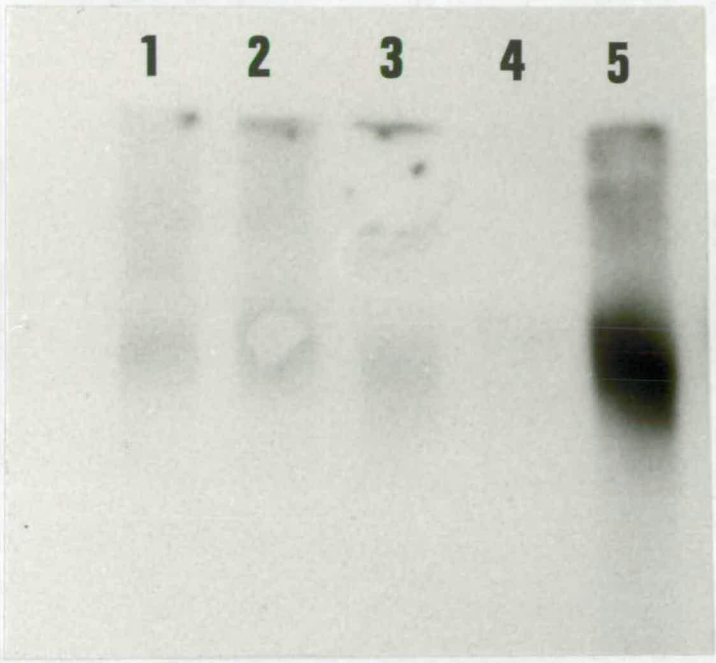


Figure 6.1 : Northern hybridisation of 3.1 kb *Stu*I/*Bam*HI pJFB1 fragment to total RNA from J14-26TR1 grown in different tyrosine concentrations.

TRACK 1 - 0 mM tyrosine

2 - 1.0 mM

3 - 2.5 mM

4 - 5.0 mM

5 - J14-26IV9 total RNA

0 mM tyrosine

analysis could be performed using the Amersham "Hybri-slot" apparatus. In this "slot-blot" procedure discrete amounts of RNA are applied directly to the nitrocellulose filter without the requirement for gel electrophoresis. Since the probe is specific for the *ARO7* transcript, any changes in the intensity of the hybridised band can be assumed to be due to changes in the amounts of *ARO7* mRNA present.

A filter containing 20 µg aliquots of denatured RNA, isolated from strains J14-26TR1 and J14-26IV9 grown under different concentrations of tyrosine (see 6.2), were probed with the radioactively labelled 3.1 kb *Bam*HI/*Stu*I pJFB1 fragment. A second similar filter was probed with the radioactively labelled plasmid pSP65 which carries the gene encoding 18 *S* rRNA (see 2.1.5). Transcription of the 18*S* rRNA gene should not be affected by changing the concentration of tyrosine present in the media and hence remain at a constant level throughout. The levels of rRNA are, therefore, indicative of the total amounts of RNA present. Comparison between the two filters would, therefore, indicate whether any decrease in *ARO7* mRNA levels is due to a decrease in the transcription of the gene or caused by discrepancies in the total amount of RNA in the sample.

The filters were hybridised for 48 h and then exposed to X-ray film for 16 h.

The resulting autoradiograph (Fig. 6.2) showed that the amount of *ARO7* mRNA produced by J14-26TR1 decreased with increasing tyrosine concentration while the *ARO7* transcript from the mutated strain J14-26IV9 did not show this decrease. The levels of rRNA did not show any corresponding decrease in intensity, remaining at an approximate constant level in both strains.

The intensities of the various bands were measured using a

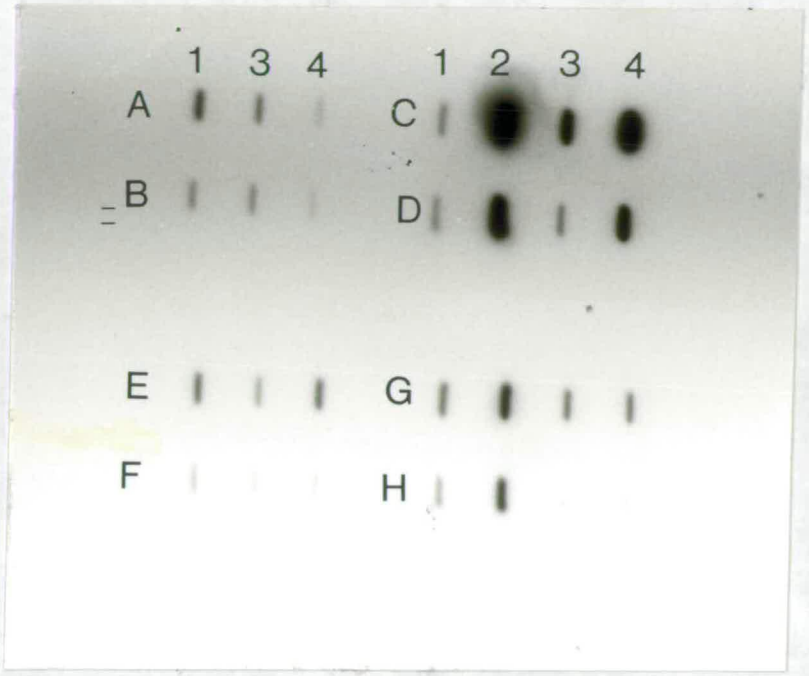


Figure 6.2 : Transcriptional analysis using the slot-blot hybridisation method.

A - 20 µg total RNA from J14-26TR1 grown in (1) 0 mM, (3) 2.5 mM and (4) 5 mM tyrosine, probed with 3.1 kb pJFB1 fragment.

B - As above with 0.2 µg RNA

C - 20 µg total RNA from J14-26IV9 grown in (1) 0 mM, (2) 1 mM, (3) 2.5 mM and (4) 5.0 mM tyrosine, probed with 3.1 kb pJFB1 fragment.

D - As above with 0.2 µg RNA

E - As A probed with 18*S* rRNA probe.

F - As B "

G - As C "

H - As D "

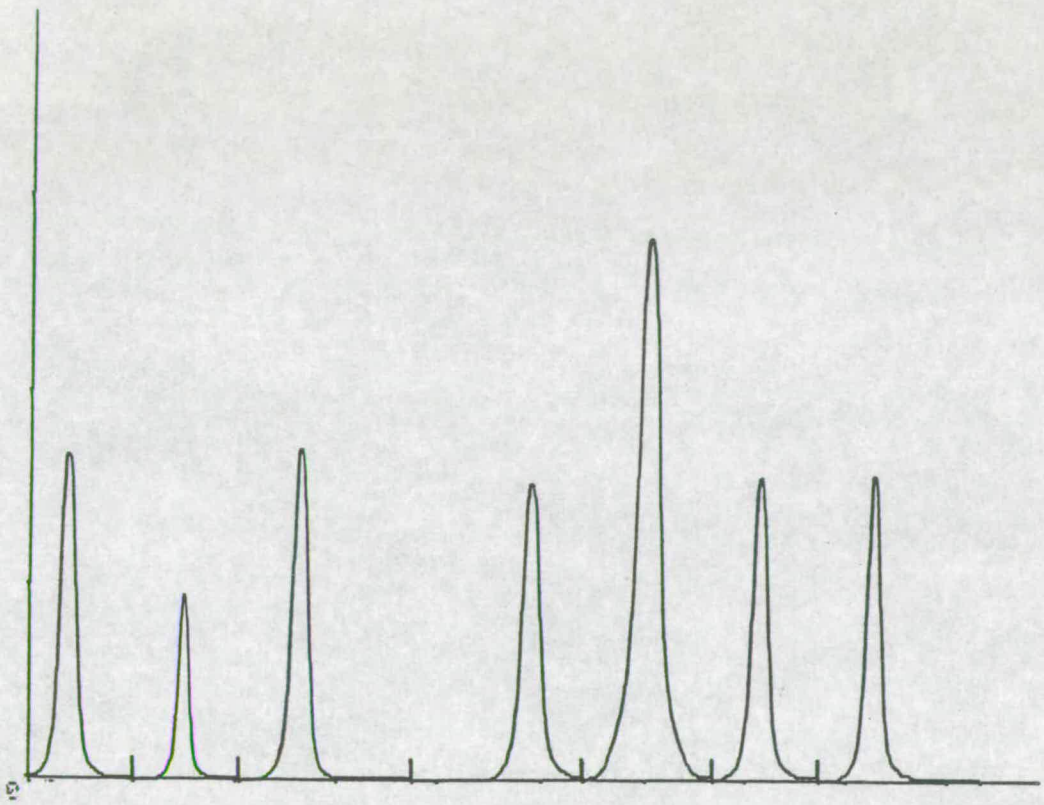


Figure 6.3 : Densitometer scan corresponding to rRNA probe hybridisation in Fig. 6.2.

densitometer (Loebl Chromoscan 3). Specific peaks were cut from the resulting graphs (see Fig. 6.3) and weighed, their weight being proportional to the area under the curve and, therefore, representative of the intensity of the bands. By assigning numerical values to specific bands, a more accurate estimate of the relative levels of *ARO7* mRNA and rRNA could be calculated (Table 6.1).

TABLE 6.1: Levels of *ARO7* mRNA relative to rRNA. Data is standardised relative to the value obtained for J14-26TR1 under conditions of no additional tyrosine.

TYROSINE CONC. (mM)	J14-26TR1	J14-26IV9
0	1.00	2.12
2.5	0.62	1.93
5.0	0.07	3.60

In strain J14-26TR1 the level of *ARO7* mRNA decreased considerably as the concentration of tyrosine present in the medium increased. The amount of transcript present at 5 mM tyrosine being approximately ten fold lower than the level produced under conditions of no additional tyrosine. This indicates that tyrosine was repressing the expression of the *ARO7* gene.

The strain J14-26IV9, containing the mutated plasmid, did not show this decrease but a slight increase in the transcript at 5 mM tyrosine was noted. This implies that the strain is resistant to repression by tyrosine.

When the levels of *ARO7* mRNA produced by each strain under conditions of no additional tyrosine were compared it was observed

that strain J14-26IV9 was producing approximately twice as much transcript as J14-26TR1, indicating that there was an increase in the expression of the gene in the mutated strain. This is in agreement with observations made with the Northern analysis.

6.4 ANALYSIS OF PLASMID COPY NUMBER

Although the previous section showed that *ARO7* mRNA decreased with increasing tyrosine concentration, this was not conclusive proof that tyrosine was repressing transcription of the *ARO7* gene. The *ARO7* gene is present on an episomal plasmid and hence the level of *ARO7* mRNA within the cell is not just a function of the transcription rate of the gene but also of the number of copies of the *ARO7* gene within the cell. Any factor causing a change in the copy number of the plasmid would, therefore, cause a corresponding change in the levels of *ARO7* mRNA. This "repression" by tyrosine could, therefore, be due to the additional tyrosine causing a decrease in the muber of copies of pJFB1 within the cell.

Total DNA was prepared from 100 ml cultures of the strain J14-26TR1 grown in minimal media (plus appropriate supplements) containing 0, 2.5 and 5 mM tyrosine respectively. 1 µg aliquots of each sample were electrophoresed on a 0.7% agarose gel. The ratio of plasmid to chromosomal DNA was calculated by densitometry, outlined in the previous section (Table 6.2).

TABLE 6.2 : Amount of pJFB1 within the cell relative to total DNA. Results are standardised relative to the value obtained with J14-26TR1 under conditions of no additional tyrosine. The data presented are the average of three separate aliquots of each sample.

TYROSINE CONC. (mM)	J14-26TR1	J14-26IV9
0	1.00	0.92
2.5	1.25	0.75
5.0	1.35	0.60

The number of copies of pJFB1 did not decrease with increasing tyrosine concentration (Table 6.2). There was a slight increase in the amount of plasmid DNA relative to chromosomal DNA but this was probably due to discrepancies during the preparation of the DNA rather than a significant increase in the plasmid copy number.

Fluctuations in plasmid copy number could also be responsible for the phenotype of strain J14-26IV9. If mutagenesis of the plasmid had resulted in a mutation which caused tha amount of plasmid to increase with increasing tyrosine concentration, then this would result in the cell being less sensitive to tyrosine repression and so account for the apparent constant level of *ARO7* mRNA. The above experiment was repeated, therefore, using DNA from strain J14-26IV9 grown in the presence of different concentration of tyrosine (0 to 5 mM). No increase in plasmid DNA relative to total DNA was noted

(Table 6.2).

When the relative amounts of pJFB1 present in each strain, under conditions of no additional tyrosine were compared, the two values were approximately equal, indicating that the initial copy number was the same in each case.

6.5 DISCUSSION

In summary, direct analysis of mRNA levels in the *Saccharomyces cerevisiae* strains J14-26TR1 and J14-26IV9 has revealed that increasing the concentration of tyrosine present in the medium resulted in a decrease in the level of transcript produced. A decrease in the copy number of pJFB1 was not responsible for this decrease in transcript level since this remained approximately constant throughout the increase in tyrosine concentration. This indicates, therefore, that tyrosine is repressing the transcription of the *ARO7* gene.

In strain J14-26IV9, however, this repression was not observed; the level of the *ARO7* transcript did not decrease with increasing tyrosine concentration. The level of plasmid within the cell did not increase and hence was not responsible for the maintenance of *ARO7* mRNA levels. This implies that the *in vitro* mutagenesis of pJFB1 with *O*-methylhydroxylamine resulted in a mutation in the *ARO7* gene rendering it resistant to repression by tyrosine. By the nature of its phenotype, this mutation probably lies within the regulatory DNA sequences situated upstream from the structural gene. Assuming that repression is effected by a protein molecule binding to the DNA and so preventing transcription of the gene, the mutation is likely to be an

alteration in the DNA sequence of the binding site, rendering it unable to bind the repressor effectively.

It was also noted, in both the Northern and slot-blot analysis, that the mutated strain J14-26IV9 produced higher levels of *ARO7* mRNA than J14-26TR1 when no extra tyrosine was added. Comparison of plasmid copy number showed no difference between the two strains and hence it was concluded that mutagenesis of the plasmid pJFB1 had resulted in increased expression of the *ARO7* gene.

This increased rate of transcription may be the result of the original mutation which conferred resistance to repression, or it may be caused by a second mutation in an independent control region of the *ARO7* gene. The latter is more probable since the former assumes a basal level of repression, even in the absence of additional tyrosine.

The enhanced expression of the *ARO7* gene in strain J14-26TR1 may also possibly form the basis of the strain's partial resistance to inhibition by tyrosine. If this increase in *ARO7* mRNA is all translated into protein, then a specific concentration of tyrosine would not inhibit the enzyme as effectively as it would the unmutated strain expressing lower levels of chorismate mutase.

This point, however, raises the question of why, if there is increased transcription of the *ARO7* gene in J14-26IV9, is there not a corresponding increase in chorismate mutase activity ? The activity displayed by this strain, under conditions of no additional tyrosine, was almost half that displayed by strain J14-26TR1. There are several reasons for this :

First, perhaps not all the mRNA produced is available for translation into protein.

Secondly, if resistance to inhibition is caused by a mutation in the structural gene, the resulting conformational change may also affect the enzyme's activity. Thirdly, there may be other independent mutations in the structural gene which reduce the activity of the enzyme.

Whichever reason is correct, these experiments provide strong evidence that the enzyme chorismate mutase is regulated at both the level of gene expression and post-translationally.

7. DISCUSSION

The original aim of this project was to engineer overproduction of phenylalanine in *Saccharomyces cerevisiae*. The results of chapter three indicated that it was possible to isolate mutant yeast strains secreting increased levels of phenylalanine by the relatively simple procedure of mutagenesis and selection for mutants resistant to the phenylalanine analogue, β-thienylalanine. Mutant yeast strains secreting approximately 0.25 mg^{-1} phenylalanine were obtained, phenylalanine secretion in wild-type strains was undetected, but the basic bioassay developed to detect such mutants could be adapted to select for ever-increasing levels of phenylalanine secretion. Repeated rounds of mutagenesis may result in a yeast strain secreting a relatively large amount of the amino acid but the genetic basis of the phenotype would be difficult to determine and the acquirement of additional, unselected and possibly deleterious, random mutations could not be prevented. These problems led to the decision to attempt to achieve phenylalanine overproduction by manipulating yeast in a much more controlled manner.

By isolating and mutating specific genes coding for regulatory enzmes in the pathway, it was considered possible to direct the metabolic flux through to the phenylalanine branch, bypassing the controls that exist in wild-type cells. The results are summarised as follows :

The *Saccharomyces cerevisiae ARO7* gene coding for chorismate mutase, a key regulatory enzyme, was cloned by screening a wild-type

gene-bank for complementation of an *aro7* auxotrophic mutant. A restriction map of the plasmid and cloned insert (pJFB1) was obtained and the size of the insert estimated to be 5.3 kb. Deletion analysis of pJFB1 showed the complementing activity was located around a *Hin*dIII site at 12.7 on the plasmid map with full activity present in a subclone containing the 2.7 kb *Bgl*II - *Cla*I fragment. The location of the promoter was estimated to be around the *Cla*I site at 12.4 on the map. Transcriptional analysis revealed that pJFB1 produced a 1 kb transcript which is poly-adenylalated and enzyme assays showed an increase in chorismate mutase activity in the *aro7* auxotrophic strain transformed with pJFB1.

The plasmid pJFB1 was mutated *in vitro* with *O*-methylhydroxylamine to obtain a yeast strain producing chorismate mutase that was resistant to the feedback-inhibition by tyrosine displayed by wild-type cells. The chorismate mutase activity from four mutant strains were analysed, two of which, J14-26IV3 and J14-26IV9, showed increased resistance to inhibition by tyrosine compared to the yeast strain transformed with the wild-type pJFB1 (J14-26TR1).

No activation of chorismate mutase by tryptophan was observed but the presence of tryptophan did counteract inhibition by tyrosine. Phenylalanine did not appear to have any significant effect on chorismate mutase activity.

During the course of this enzyme analysis it was discovered that addiing tyrosine to the growth media also resulted in a decrease in chorismate mutase activity. This excess tyrosine was removed before assaying and therefore indicated that repression rather than inhibition was the cause of the decrease in enzyme activity.

The effect of tyrosine on transcription of the *ARO7* gene was subsequently analysed in more to detail to ascertain whether this "repression" effect was the result of an actual decrease in the amount of *ARO7* transcript produced. A combination of Northern and slot-blot analysis revealed that the level of transcript decreased approximately ten-fold from 0 to 5 mM tyrosine, compared to total RNA levels. The plasmid copy number did not change throughout indicating that this decrease was due to an actual decrease in the transcription rate of the *ARO7* gene rather than a decrease in the number of genes transcribed.

Two of the regulatory mutants isolated appeared to be resistant to this repression by tyrosine, increasing tyrosine in the growth medium having much less effect on their chorismate mutase activity than in the wild-type transformant. A more thorough transcriptional analysis of one of these strains, J14-26IV9, showed that the level of *ARO7* transcript did not decrease significantly as the concentration of tyrosine increased. Again the plasmid copy number was shown to be approximately constant over the range of tyrosine concentrations. It was, therefore, concluded that transcription of the *ARO7* gene in this strain was resistant to tyrosine repression.

Although tyrosine-mediated repression of chorismate mutase has been well documented in *E. coli* (Camakaris & Pittard, 1983; Hudson & Davidson, 1984) it has not been reported in *Saccharomyces cerevisiae*. Another research group working with the *ARO7* gene reported that the gene was constitutively expressed, showing no difference in *ARO7* mRNA levels between strains grown with and without 1 mM tyrosine and phenylalanine (Ball *et al.*,1986). This failure to detect tyrosine

repression may have been due to several reasons. First, an external concentration of 1 mM tyrosine may not repress transcription sufficiently enough to be detected. Secondly, they were analysing samples of total RNA extracted from wild-type cells. The *ARO7* transcript appears to be expressed at very low levels so a slight change in the transcript level may have been difficult to detect.

Repression by tyrosine indicates the existence of a repressor molecule which, in the presence of tyrosine, binds to the DNA preventing transcription. Positive control, however, is the more common form of regulation in eukaryotes (Lewin, 1983) indicating that tyrosine may possibly inhibit the binding of regulatory protein necessary for *aro7* transcription. This situation, however, leads to the conclusion that *in vitro* mutagenesis of the *ARO7* gene has resulted in a DNA alteration enabling it to bind the protein in the presence of tyrosine. This requires a more complex alteration than a mutation preventing binding of a repressor molecule but the possibility cannot be disregarded. The isolation of other *ARO7* regulatory mutants, especially mutations causing constitutive expression, would help to determine which method of control is operating.

There are very few reports of regulation of gene expression in any of the other genes for aromatic amino acid biosynthesis. The DAHP synthase isoenzymes are not repressed by any of the amino acids, either singly or in combination, nor is dehydroquinate dehydratase (Doy & Cooper, 1966). Lingens *et al.* described an induction of chorismate mutase by tryptophan but this could not be confirmed by this work or others (Kradolfer *et al.*, 1977; Ball *et al.*, 1986). Prephenate dehydratase is, however, reported to be repressed by

phenylalanine while prephenate dehydrogenase is induced by phenylalanine but not repressed by tyrosine (Lingens *et al.*, 1967). These effects are of the order of two to three-fold whereas feedback inhibition can be a hundred-fold effect. The latter has, therefore, always been cited as the main mode of control. The results of this work, however, indicate that repression may be more important in the regulation of aromatic amino acid biosynthesis than previously thought.

Transcriptional control has been implicated in the derepression of tryptophan biosynthetic enzymes in response to tryptophan starvation (Miozzari *et al.*, 1977). Derepression of these enzymes also occurred, however, in response to starvation for unrelated metabolites such as arginine and histidine and, therefore, it was concluded to be not a tryptophan-specific response but elicited by the general amino acid control system. This raises questions of whether the *ARO7* gene is under general amino acid control and if the observed repression by excess tyrosine is associated with it ?

This could be ascertained by comparing the chorismate mutase activity of wild-type yeast strains grown on complete medium to the same strain grown under phenylalanine/tyrosine limiting conditions. Analysis of the tryptophan biosynthetic pathway (Miozzari *et al.*, 1977) revealed that the enzymes were present at levels in excess of that required for normal growth. Changes in the tryptophan pool size were, therefore, not reflected in enzyme levels until tryptophan became growth limiting. If this is also the case in phenylalanine biosynthesis then creating phenylalanine/tyrosine limiting conditions may involve using bradyotrophic strains or phenylalanine/tyrosine

analogues which inhibit specific steps in the pathway.

It would also be useful to check if there was a response in *ARO7* expression to starvation for other amino acids known to be under general control. The same analysis could also be carried out using *gcn* mutant strains. The enzymes normally under general control are not derepressed in these mutant strains so any difference in chorismate activity between these strains and wild-type may be attributed to the effect of this cross-pathway control.

Any indication of derepression mediated by the general control system could be investigated further by analysing directly the levels of the *ARO7* transcript produced by cells grown under the appropriate conditions.

Even if the *ARO7* gene is under general amino acid control, the observed repression by tyrosine may not be directly associated with this control. The yeast cell's internal tyrosine pool concentration is apoximately 0.7 mM (Jones & Fink, 1982). The tyrosine concentrations which evoked this repression response may, therefore, be well above the normal physiological levels encountered by the cell. It may be that this repression is part of an emergency responseto extreme conditions.

The mutant strain J14-26IV9 appears to be resistant to both repression and feedback-inhibition by tyrosine, indicating that there are at least two separate mutations within the plasmid pJFB1, one in the upstream regulatory sequences and the other in the structural *ARO7* gene. More information, however, is required before these two mutations can be separated and analysed independently. Once the transcription initiation site has been elucidated it may be possible

to isolate the mutated upstream controlling regions, link them to the wild-type *ARO7* structural gene sequences and observe if the resulting construct still showed resistance to repression but produced a protein sensitive to feedback-inhibition. Alternatively, the existance of two separate mutations could also be proved by linking the mutated structural gene to wild-type upstream sequences and showing that there was inhibition without repression.

Given that repression exists, it is not surprising that a double mutant has been isolated since mutant strains resistant only to feedback-inhibition by tyrosine would still be repressed and, therefore, difficult to select. Similarly, feedback-inhibition may be suppressing the phenotype of repression resistant mutants. This is assuming, however, that loss of feedback-inhibition results in an overproduction of tyrosine sufficient to repress transcription of the *ARO7* gene and vice versa.

The transcriptional analysis was carried out using the mutant yeast strain J14-26IV9. The other mutant strains must, therefore, be further analysed to check that a) the enzyme activity results displayed by J14-26IV6, indicating resistance to repression, are reflected in the *ARO7* transcript levels, and b) the apparent resistance to inhibition by tyrosine shown by J14-26IV3 is not due to increased expression of the gene or an increase in the plasmid copy number.

A relatively simple method of determining whether the feedback-inhibition resistant strains are producing an enzyme with an altered tyrosine binding site involves the use of tyrosine-linked agarose beads. By reacting cellular extracts of soluble protein with

the agarose beads and comparing bound and unbound fractions by polyacrylamide gel electrophoresis, the binding status of each enzyme may be determined. The chorismate mutase protein produced by a wild-type strain will be able to bind tyrosine and, therefore, bind to the tyrosine-linked agarose. If the mutant enzymes produced by strain J14-26IV3 and J14-26IV9 are unable to bind tyrosine they will not bind to the beads and, therefore, the protein will be present in the unbound fraction.

Tryptophan-linked agarose could be used in a similar manner to detect if the tryptophan binding has been altered. Gel electrophoresis of soluble protein extracts would also reveal any possible differences in the electrophoretic mobility of the mutant enzymes due to conformational changes.

Tryptophan may counteract inhibition by tyrosine in several ways: If tyrosine effects inhibition by binding to an allosteric site then tryptophan may prevent this by competitively binding to the same site. Alternatively tryptophan may bind to a separate allosteric site, changing the conformation of the enzyme in such a way that tyrosine can no longer bind. If tyrosine is a competitive inhibitor, tryptophan may counteract this by either of the two routes mentioned above. This is unlikely, however, since it is difficult to visualise how the binding of tryptophan may prevent tyrosine binding but preserve a fully functional active site. In the absence of specific enzyme kinetic parameters and structural information, however, this remains just speculation.

Ball *et al.* (1986) report that *OSM2*, a gene required for growth

in hypertonic medium, maps to the *ARO7* locus indicating that they are the same gene. A possible explanation for the osmotic sensitivity of *aro7* mutants may be that depletion of an intermediate in the tyrosine/phenylalanine pathway leads to the absence or malfunction of a membrane component. Mutant yeast strains hypersensitive to naladixic acid map to the same complementation group as *aro7* mutants (J.A. Prendergast, personal communication) and Meade & Manney (1983) showed that at least a quarter of phenethylalcohol hypersensitive mutants are amino acid auxotrophs. Growth inhibition by the latter compound is generally believed to be mediated by its effect on the cell membrane, thus the aromatic amino acid pathway might be of particular importance for its normal function. This may explain why the phenylalanine overproducing mutants, discussed in chapter three, secreted many other amino acids. It would be interesting to test these and other known mutants in the aromatic amino acid pathway for similar osmotic and hypersensitivity effects.

Although strain J14-26IV9 is insensitive to feedback-inhibition and repression by tyrosine, it only secreted 0.1 mg ml^{-1} phenylalanine, indicating that deregulation of chorismate mutase alone may not alter the flux of the pathway sufficiently to achieve significant overproduction of phenylalanine. To obtain overproducing strains adequate for commercial purposes, which require 20-30 g l^{-1}, it may , therefore, be necessary to deregulate other key enzymes in the pathway. These would include *ARO3*, *ARO4* and *PHA2*. The *ARO3* gene has been cloned by complementation of an *aro3* mutation with a wild-type gene bank (Teshiba *et al.*, 1986) so it should be possible

to isolate the other genes in a similar manner and obtain the required mutant by *in vitro* mutagenesis. To maximise phenylalanine production, it may also be necessary to restrict the flow of metabolites into the tryptophan and tyrosine pathway by using bradytrophic strains. The deregulation of the phenylalanine pathway through multiple feedback-resistant mutations may, however, result in the flux of intermediates to phenylalanine becoming limiting.

Miozarri *et al.* (1978) demonstrated, by both biochemical and genetic analysis, that most enzymes of the tryptophan pathway are present in excess of the levels necessary for wild-type cell growth. In a feedback-resistant mutant, however, on activation, anthranilate phospho-ribosyl transferase (*TRP4*), did limit the maximum capacity of the pathway. Kradolfer *et al.* (1977) reported that mutants containing high chorismate mutase activity have a reduced chorismate pool, thus a similar situation may arise in a multiple feedback-resistant, phenylalanine overproducing cell.

If the limitation is due to a low concentration of an enzyme, this may be increased by two methods. The gene dosage may be increased by introducing the specific gene on a high copy number plasmid, or, if a detailed restriction map of the gene is available, the natural promoter may be removed and replaced with a stronger or constitutive one such as the phosphoglycerate kinase (*PGK*) promoter. Thus, gene expression is increased either by increasing the gene copy or elevating the transcription of mRNA.

The results of this project also suggest that overproduction of phenyalanine could affect the production of the pathway at the level of transcription, thus over-riding the former method of increasing

gene expression. This may necessitate the use of either specific amino acid repression resistant mutants or general control mutants (*gcd1*, *gcd2*) which are no longer subject to this repression control and have high, constitutive levels of amino acid biosynthetic enzymes (Wolfner *et al.*, 1975; Miozarri *et al.*, 1978; Myers *et al.*, 1986).

No effort to achieve high yields of an amino acid from *Saccharomyces cerevisiae* can neglect the effect of carbon metabolism on biosynthesis. The carbon precursors of the aromatic amino acids are erythrose-4-phosphate and phospho*enol*pyruvate, intermediates of the pentose phosphate pathway and glycolysis respectively.

A pyruvate kinase deficient mutant may also be used to divert PEP from the glycolytic pathway into the phenylalanine pathway. Mutants in pyruvate kinase (*pyk*) cannot grow on glucose because glycolysis is blocked and their growth on non-fermentable carbon sources is inhibited by glucose due to the build up of toxic levels of glycolytic intermediates (Maitri & Lobo, 1977). Temperature-sensitive alleles of pyruvate kinase (*cdc19*) could be used to increase cellular PEP concentrations so that pyruvate kinase activity can be adjusted to allow sufficient glycolysis for energy generation, and to prevent the accumulation of toxic levels of PEP. An investigation of the effect of the above mutation on phenylalanine biosynthesis would, therefore, be extemely useful in maximising phenylalanine production.

The simplest method of amalgamating these necessary mutations would be to combine the genes for phenylalanine biosynthesis, using feedback-insensitive and other deregulated mutants where necessary, on

the one high copy plasmid. This could then be transformed into a host yeast strain with the appropriate genetic background. This has already been achieved with the tryptophan biosynthetic genes (Prasad *et al.*, 1987). This may result, however, in problems with plasmid stability, an important point if the strain is to be used in industrial-scale fermentation procedures. Prasad *et al.* (1987) reported that presence of tryptophan in the media, either added externally or excreted from the cells, resulted in the loss of the plasmid from 90% of the cells in 24 h (approx. ten doublings). This indicates that the phenylalanine produced by the transformed cells would result in the loss of the plasmid responsible from the cell.

Integrating genetically engineered constructs into one site in the yest genome is the common solution to this problem, but this generally results in the loss of advantages gained by placing them on a high copy number vector. It should be possible, however, to introduce multiple copies of a given construct into a strain by using integrating vectors. Tandem copies of a gene can be lost through intrachromosomal recombination and may, therefore, prove to be just as unstable as plasmids. Integrating vectors often carry several segments of yeast DNA and are, therefore, capable of integrating at several sites in the yeast genome. A given transformant will probably contain only one copy of the construction, but the integration events from several different transformations can be combined into a single strain through successive rounds of mating and sporulation. This should eventually lead to a stable multicopy integrant. Ultimately, it may be feasible to construct an artificial chromosome (Murray & Szostak, 1983) incorporating all of the biosynthetic genes, in single or

multiple copies as required.

The results of this project and the above discussion indicate that the genetic manipulation of yeast to obtain overproduction of phenylalanine at commercially useful levels is a complex procedure. Many different aspects of gene expression, enzyme regulation and cell physiology have to be considered. The information obtained from studying these different areas, however, should lead not only to commercial success but also to a fuller understanding of the regulation of aromatic amino acid biosynthesis in *Saccharomyces cerevisiae*.

REFERENCES

Aebi, M., Niederberger, P. & Hutter, R. (1982). Current Genetics 5, 39-46.

Ahmed, S.I. & Giles, N.H. (1969). Journal of Bacteriology 99, 231-237.

Aibi, S., Imanaka, T. & Tsunekawa, H. (1980). Biotechnology Letters 2, 525-550.

Aibi, S., Tsunekawa, H. & Imanaka, T. (1982). Applied Environmental Microbiology 43, 289-297.

Ambler, R.P. (1972).Methods in Enzymology 25, 143-154.

Baldwin, G.S. & Davidson, B.E. (1983). Biochimica et Biophysica Acta 742, 374-383.

Ball, S.G., Wickner, R.B., Cottarel, G., Schau, M. & Titiaux, C. (1986). Molecular and General Genetics 205, 326-330.

Barker, C. & Lewis, D. (1974). Journal of General Microbiology 82, 337-343.

Beggs, J.D. (1978). Nature 275, 104-108.

Berry, A., Byng, G.S. & Jensen, R.A. (1985). Archives of Biophysics and Biochemistry 246, 470-479.

Birnboim, H.C. & Doly, J. (1970). Nucleic Acid Research 7, 1513-1523.

Bolivar, F &, Backman, K. (1978). Methods in Enzymology 68, 245-258.

Broach, J.R., Strathearn, J.N. & Hicks, J.B. (1979). Gene 8, 121-133.

Brown, K.D. & Somerville, R.L. (1971). Journal of Bacteriology 108, 386-399.

Bulot, E, & Cooney, C.L. (1985). Biotechnology Letters 7, 93-98.

Calvin, K.H. & Davidson, B.E. (1985). Biochimica et Biophysica Acta 827, 1-7.

Camakars, H, & Pittard, J. (1983). in Amino Acids+ Biosynthesis and Genetic Regulation. Herrman, K.M., Somerville, R.L., eds. pp339--350. Addison-Wesley, Reading, MA.

Carsiotis, M. & Jones, R.F. (1974). Journal of Bacteriology 119, 889-892.

Chai, Y.J. & Tribe, D.E. (1982). Biotechnology Letters 4, 223-228.

Christopherson, R.I. (1985). Archives of Biochemistry and Biophysics 240, 654-646.

Cotton, R.G.H. & Gibson. F. (1964). Biochimica et Biophysica Acta 100, 76-88.

Dagert, M. & Ehrlich, S.D. (1979). Gene 6, 23-28.

Davidson, B.E., Blackburn, E.H. & Dopheide, T.A. (1972). Journal of Biological Chemistry 247, 4441-4446.

De Leeuw, A. (1967). Genetics 56, 554-567.

Delforge, J., Messenguy, F. & Wiame, J.M. (1975). European Journal of Biochemistry 57, 231-239.

Dickinson, J.R., Ambler, R.P. & Dawes, I.W. (1985). European Journal of Biochemistry 148, 405-406.

Donahue, T.F., Daves, R.S., Lucchini, G. & Fink, G.R. (1983). Cell 32, 89-98.

Dopheide, T.A.A., Crewther, P. & Davidson, B.E. (1972). Journal of Biological Chemistry 247, 4447-4452.

Fantes, P.A., Roberts, L.M. & Hutter, R. (1976). Archives of

Microbiology 107, 207-214.

Fishe, M.J. & Kane, J.F. (1984). Journal of Bacteriology 160, 676-681.

Fogel, S. (1983). In Yeast Genetics. Spencer, J.F.T., Spencer. D.M., Smith, A.R.W. eds. Springer-Verlag, New York.

Folliette, M.T. & Sinskey, A.J. (1986). Journal of Bacteriology 167, 695-702.

Gaertner, F.H. & Cole, K.W. (1977). Biochemical and Biophysical Research Communications 75, 259-264.

Gething, M.H. & Davidson, B.E. (1977). European Journal of Biochemistry 78, 111-117.

Gibson, F. & Pittard, J. (1968). Bacteriology Reviews 32, 465-492.

Grove, C.L. & Gunsalus, R.P. (1987). Journal of Bacteriology 169, 2154-2164.

Heyde, E. (1979). Biochemistry 18, 2766-2775.

Hill, D.E., Hope, I.A., Macke, J.P. & Struhl, K. (1986). Science 234, 451-457.

Hinnebusch, A.G. (1984). Proceedings of the National Academy of Sciences USA 81, 6442-6446.

Hinnebusch, A.G. & Fink, G.R. (1983a). Proceedings of the National Academy of sciences USA 80, 5374-5378.

Hinnebusch, A.G. & Fink, G.R. (1983b). Journal of Biological Chemistry 258, 5238-5247.

Hoch, S.O. (1974). Journal of Bacteriology 117, 315-317.

Hope, I.A. & Struhl, K. (1985). Cell 43, 177-189.

Hudson, G.S. & Davidson, B.E. (1984). Journal of Molecular Biology 180, 1023-1051.

Ikeda, K. (1908). Journal of the Tokyo Chemical Society 30, 820-836.

Im, S.W.K., Davidson, H. & Pittard, J. (1971). Journal of Bacteriology 108, 44-409.

Im, S.W.K. & Pittard, J. (1971). Journal of Bacteriology 106, 784-790.

Jones, E.W. & Fink, G.R. (1982). In The Molecular Biology of the Yeast *Saccharomyces cerevisiae*. (Strathearn, J.N., Jones, E.W., Broach, J.R., eds.) Cold Spring Harbour, New York.

Kasian, P.A., Davidsin, B.E. & Pittard.J. (1986). Journal of Bacteriology 167, 556-561.

Kasian, P.A. & Pittard, J. (1984). Journal of Bacteriology 160, 175-183.

Klausner, A. (1985). Biotechnology 3, 301-307.

Kradolfer, P., Zeyer, J., Miozzari, R. & Hutter, R. (1977). FEMS Microbiolgy Letters 2, 211-216.

Larimer, F.W., Morse, C.C., Beck, A.K., Cole, K.W. & Gaertner, F.H. (1983). Mollecular and Cellular Biology 3, 1609-1614.

Lewin,B. (1983). In Genes, John Wiley & Sons Ltd., New York.

Lingens, F., Goebel, W. & Uesseler, H. (1966). Biochem. Z. 346, 356-367.

Lingens, F., Goebel, W. & Uesseler, H. (1967). European Journal of Biochemistry 1, 363-374.

Lowry, O.H., Rosebrough, N.J., Farr, A.C. & Randall, R.J. (1951). Journal of Biological Chemistry 193, 265-275.

Lucchini, G., Biraghi, A., Carbone, M.L., De Scrille, A. & Magni, G.E. (1978). Journal of Bacteriology 136, 55-62.

Lucchini, G., Hinnebusch, A.G., Chen, C. & Fink, G.R. (1984). Molecular and Cellular Biology 4, 1326-1338.

Lumsden, J. & Coggins, J.R. (1977). Biochemical Journal 161, 599-607.

Magee, P.T. & Hereford, L.M. (1969). Journal of Bacteriology 98, 857-862.

Maiti, P.K. & Lobo, Z. (1977). Molecular and General Genetics 152, 193-200.

Maniatis, T., Fritsch, T.F. & Sambrook, J. (1982). Molecular Cloning; A Laboratory Manual. Cold Spring Harbour, New York.

Meade, J.H. & Manney, T.R. (1983). Genetics 104, 235-240.

Messenguy, F., Colin, D. & Ten Have, J. (1980). European Journal of Biochemistry 108, 439-451.

Messenguy, F. & Delforge, J. (1976). European Journal of Biochemistry 67, 335-339.

Messenguy, F. & Dubois, E. (1983). Molecular and General Genetics 189,

148-156.

Messing, J. (1983). Methods in Enzymology 101, 20-78.

Meuris, P. (1973). Molecular and General Genetics 121, 207-218.

Meuris, P. (1974). Genetics 76, 735-44.

Millar, G., Lewendon, A., Hunter, M.G. & Coggins, J.R. (1986). Biochemical Journal 237, 427-437.

Miozzari, G., Wiederberger, P. & Hutter, R. (1978). Journal of Bacteriology 134, 48-59.

Mortimer, R.K. & Hawthorne, D.C. (1975). Methods in Cell Biology 11, 221-233.

Murray, A.W. & Szostk, J.W. (1983a). Nature 305, 189-193.

Murray, A.W. & Szostak, J.W. (1983b). Cell 34, 961-970.

Nasmyth, K.A. & Reed, S.I. (1980). Proceedings of the National Academy of Sciences USA 77, 2119-2123.

Nasmyth, K.A. & Tatchell, K. (1980). Cell 19, 753-764.

Niederberger, P., Miozzari, G. & Hutter, R. (1981). Molecular and

Cellular Biology 1, 584-593.

Penn, M., Galgoci, B. & Green, H. (1983). Proceedings of the National Academy of Sciences USA 80, 2704-2708.

Penn, M., Thireos, G. & Green, H. (1984). Molecular and Cellular biology 4, 520-580.

Peterson, N.S. & McLaughlin, C.S. (1973). Journal of Molecular Biology 8, 33-45.

Pittard, J., Camakaris, J. & Wallace, B.J. (1969). Journal of Bacteriology 97, 1242-1247.

Pittard, J. & Wallace, B.J. (1966). Journal of Bacteriology 91, 494-508.

Prasad, R., Niederberger, P. & Hutter, R. (1987). Yeast 3, 95-105.

Schurch, Y. (1972). Archiv. fur Genetik. 45, 129-159.

Schurch, A., Miozzari, J. & Hutter, R. (1974). Journal of Bacteriology 117, 1131-1140.

Sherman, F. (1975). Methods of Cell Biology 11, 189-200.

Sherman, F. & Lawrence, C.W. (1974) Handbook of Genetics vol. 1

pp359-393 (King, R.C., ed.) Plenum Press, New York,

Shortle, D., Di Mario, D. & Nathans, D, (1981). Annual Reviews of Genetics 15, 265-278.

Singh, B.K., Connely, J.A. & Conn, E.E, (1985). Archives of Biochemistry and Biophysics 243, 374-384.

Smith, H.O. (1980). Methods in Enzymology 65, 371-380.

Southern, E. (1975). Journal of Molecular Biology 98, 503-515.

Struhl, K. (1982). Nature 300, 284-286.

Struhl, K., Stinchcomb, D.T., Scherrer, S. & Davis, R.W. (1979). Proceedings of the National Academy of Sciences USA 76, 1035-1039.

Teshiba, S., Furter, R., Niederberger, P., Braus, G., Paravicini, G. & Hutter, R. (1986). Molecular and General Genetics 205, 353-357.

Thomas, P.S. (1980). Proceedings of the National Academy of Sciences USA 77, 5201-5205.

Tribe, E. & Pittard, J. (1979). Applied Environmental Micribiology 38,

181-190.

Walz, A., Ratzkin, B. & Carbon, J. (1978). Proceedings of the National Academy of Sciences USA 75, 6172-6176.

Weir-Thompson, E.M. & Dawes, I.W. (1984). Molecular and Cellular Biology 4, 695-702.

Welch, A.R. & Gaertner, F.H. (1976). Archives of Biochemistry and Biophysics 172, 476-489.

Whipp, M.J., Halsall, D.M. & Pittard, A.J. (1980). Journal of Bacteriology 143, 1-7

Wiemken, A. (1980). In Cell Compartmentation and Metabolic Channeling. (Nayer, L. ed.) Elsevier Biomedical Press, Amsterdam.

Winston, F., Chumley, F. & Fink, J.R. (1983). Methods in Enzymology 101, 211-228.

Wolfner, M., Yep, D., Messenguy, F. & Fink, G. (1975). Journal of Molecular Biology 96, 273-290.

Wookey, P.J., Pittard, J., Forrest, S.M. & Davidson, B.E. (1984). Journal of Bacteriology 160, 169-174.

Zalkin, H. & Yanofsky, C. (1982). Journal of Biologiacl Chemistry 257, 1491-1500.