

**The application of the Universal Method to
tryptophan biosynthesis in yeast**

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Declaration

This thesis has been composed wholly by the candidate. All the work reported has been performed by the candidate alone.

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Abbreviations

5-MT	5-methyltryptophan
A, Ad	adenine
<i>aat, AAT</i>	(mutations in) genes encoding aromatic aminotransferases
amp	ampicillin
<i>aro, ARO</i>	(mutations in) genes encoding enzymes of the shikimate pathway
AROM	the pentafunctional enzyme comprising five activities of the shikimate pathway
<i>ARS</i>	autonomous replicating sequence
AMP, ATP	adenosine monophosphate, (triphosphate)
C	(usually) control coefficient
<i>CEN</i>	sequences sufficient for segregation at mitosis in yeast
dNTP	deoxy-nucleotide triphosphate
DAHHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
DAHPS	DAHHP synthase
DHQ	3-dehydroquininate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	(usually) enzyme activity
E4P	erythrose-4-phosphate
EDTA	ethylenediaminetetraacetic acid(disodium salt)
EPSP	5-enolpyruvylshikimate phosphate
F	phenylalanine
FMN	flavin mononucleotide
FOA	5-fluoro-orotic acid
<i>gcn, GCN</i>	(mutations in) genes encoding mediators of general control
GCRE	general control responsive elements
IPTG	isopropyl β -D-thiogalactopyranoside
J	flux
kan	kanamycin
KGB	potassium glutamate buffer (+ supplements used for restriction digest)
L	leucine
LB	Luria-Bertani medium (+ supplements as defined)
MCA	Metabolic Control Analysis
MCS	multiple cloning site
MV	yeast minimal medium
MW	molecular weight
NAD(P)(H)	β nicotinamide adenine dinucleotide (phosphate) (reduced)
OD ₅₄₆	optical density (absorbance) at 546 nm
PEG	polyethyleneglycol
PEP	phosphoenolpyruvate
PTS	phosphotransferase system
RNA	ribonucleic acid

RNAse	ribonuclease
SC	synthetic complete (MV medium + complete set of nutritional supplements - amino acids + adenine and uracil)
SDH	Shikimate dehydrogenase
SDW	sterile distilled water
TBE	Tris/boric acid/EDTA buffer for agarose electrophoresis
TCA	trichloroacetic acid
TE	TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0)
<i>trp</i> , <i>TRP</i>	(mutations in) genes encoding enzymes of the tryptophan pathway
U	uracil
UV	ultra-violet
V	valine
W	tryptophan
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
Y	tyrosine
YAC	yeast artificial chromosome
YEPD	yeast extract peptone glucose medium

Abstract

Recent developments of Metabolic Control Analysis include the derivation of a general method (the 'Universal Method') which aims to define the conditions under which a pre-determined change in the flux to a specific end-point metabolite may be achieved without changing other fluxes or metabolite concentrations. This thesis describes the application of this method in the yeast, *Saccharomyces cerevisiae*, by overexpressing genes of the shikimate and tryptophan pathways and, by analysing the outcomes, attempts an evaluation of the method's validity.

A single-copy plasmid, pPH28, bearing the 4 genes encoding the enzymes of the shikimate pathway was constructed and introduced into a strain along with a multi-copy plasmid, pME554, bearing the 5 genes of the tryptophan pathway. In this way the activity of the shikimate enzymes and tryptophan enzymes were overexpressed by factors of about 2-5 and 20-60, respectively. The stability of the plasmids, both separately and together, under selective and non-selective conditions in different media was studied, along with enzyme activities. Results showed higher losses of plasmids for growth without selection, greater loss of the 2-micron plasmid than for the centromeric plasmid, and, in general, corresponding rates of loss of enzyme activities.

The effects of the plasmids upon the production of tryptophan, phenylalanine and tyrosine were studied. pME554 and, subsequently, pPH28 had no observable effect upon the total flux to tryptophan, phenylalanine and tyrosine. pME554 did not significantly change the intracellular concentrations of these amino acids. However, when pPH28 was also introduced, intracellular tryptophan, phenylalanine and tyrosine concentrations increased by factors of 2-3. There were also corresponding changes in the amounts excreted into the medium. The failure to achieve increases in flux arises because flux to protein contributes most of the total flux, namely 97 - 99%. The dynamics of the production, excretion and re-uptake of aromatic amino acids are considered by estimating intracellular, extracellular and total culture concentration during culture. The role of anthranilate synthase in regulating intracellular

concentrations of tryptophan, and its effects upon tyrosine and phenylalanine concentrations are also investigated by feeding cells on anthranilic acid.

The results show that, for tryptophan biosynthesis at least, the Universal Method is not an appropriate method of making large specific changes in flux. The method's failure is likely to arise from the lack of increased excretion of tryptophan at wild-type intracellular concentrations. The study suggests that the flux control coefficients of all the shikimate and tryptophan enzymes may be very low, and that the intracellular concentrations of tryptophan, tyrosine and phenylalanine are tightly regulated.

Chapter 1

Introduction

The application of microbiological fermentation by humans has a long history. Both yeast and bacteria, for instance, have long been used in the production of bread, milk products and alcoholic drink. Recently, there has been a trend toward a systematic study of micro-organisms and, more recently, plants to address the possibilities of other applications such as the production of food and nutrients, fuel, polymers, chemicals, drugs and antibiotics. This is now taking place in a context where a number of important dynamics are at play. The economics of industrial production are increasingly constrained by the costs and sustainability of raw materials, disposal of waste products and measures designed to improve health and safety. There is an awareness that the range and complexity of useful products such as enzymes and therapeutic agents produced by organisms outstrips conventional technologies. For instance, our growing understanding of enzymology may allow the design and production of new or modified catalysts. Finally, our exploitation of this potential by generating useful strains of bacteria, fungi, plants and animals with classical genetics is giving way to a more directed approach which uses our ability to identify, characterise and manipulate relevant genes. There is therefore considerable interest in the possibility of large and specific increases in the production of metabolites, using recombinant DNA technology to overexpress particular gene products, which may allow biotechnological exploitation.

Initial attempts to generate increased production of metabolites by increasing the copy number of 'key' genes have generally been disappointing (Westerhoff, 1995). There is a growing appreciation that successful outcomes may be limited by the structure and organisation of metabolism which may regulate the operation of specific pathways and/or constrain the functioning of metabolism as a whole. These factors may limit the effects of rather simplistic strategies which seek to 'knock out' genes or to generate large increases in expression of key genes. However, it may be possible

to increase yields by large amounts if changes are made within a context which seeks to minimise the effects on other pathways.

The project described here attempts to address experimentally the question 'how may we increase the production of a metabolite in a general, predictable and specific way'? Generality requires that a strategy should be applicable to many different situations and problems. Predictability demands that we should be able to define the magnitude of the increase to be achieved. Specificity means that only the production of the defined metabolite should change. Such a strategy is in contrast to the traditional genetic methods by which strain manipulation, or mutation followed by selection, may produce a required phenotype, but which may give unexpected yields and produce other ill-defined changes. It is also in contrast with the simple application of recombinant DNA technology which may manipulate the activities of one or more 'key' enzymes. Such methods do not usually give predicted increases in production, nor are wild-type characteristics such as the growth rate or other pathway fluxes necessarily maintained.

The context of the project is shaped by two fields. The first is the study of how metabolic systems respond *in vivo* to changes in enzyme activities. Metabolic Control Analysis (MCA) is one such approach. Although MCA deals with the effects of small changes in enzyme activity or metabolite concentrations, more recent developments have attempted the analysis of large changes. Furthermore, attempts have been made to predict how specific and predictable increases in metabolic flux or metabolite concentrations may be made. The model employed in this work, the 'Universal Method', attempts to define how increases in flux to any end-point metabolite may be achieved without concomitant change in other pathways. Secondly, the system chosen for the evaluation of this method is tryptophan biosynthesis in *Saccharomyces cerevisiae*. Studies of the enzymes, genes and regulation of this pathway have produced a large body of knowledge. It includes some progress in the understanding of the ways in which the pathway is regulated, attempts to overproduce tryptophan and more recently, analysis of the data, using the concepts, definitions and theorems of MCA.

The present work described in this thesis therefore attempts to apply a theory, called the 'Universal Method', whose roots lie in MCA, to tryptophan biosynthesis as an experimental model, building upon the successes of previous work.

The project has the following goals

- to apply the 'Universal Method' to tryptophan biosynthesis in yeast
- to test the validity of the 'Universal Method'
- to achieve increases in tryptophan production
- to minimise changes in other closely-related pathways
- to understand what factors may limit the effectiveness and applicability of the method

The remainder of this introduction is organised as follows. The theoretical basis of Metabolic Control Analysis, its experimental confirmation and its application to the treatment of large changes are presented in section 1.1. This concludes with a description of the 'Universal Method'. Section 1.2 describes our present understanding of the structure of aromatic amino acid biosynthesis, including a brief introduction to transport and catabolism. Descriptions of the possible regulatory mechanisms are given. Section 1.3 attempts to analyse the control and regulation of flux and metabolite pools in the tryptophan pathway using the work of Niederberger and others who considered various mutations and their effects upon flux and metabolite pools within the pathway and the effects of overexpressing the enzymes. Finally, the objectives of the project are developed and experimental strategies outlined in section 1.4. Section 1.5 explains the structure of the thesis and the contents of each chapter.

1.1 Theoretical Aspects of Metabolic Control

Traditionally, many biochemical texts and courses treated the question of regulation and control as 'add-on' features to the central concerns which were the identification of metabolic pathways and gene-enzyme relationships which culminated in the 'metabolic map'. It is sometimes difficult to perceive much difference today, even

with the present concern for the control of gene expression, cell signalling and structure-function studies of biomolecules. Once the modulation of enzyme activity by effectors was understood, along with the phenomena of allostery, co-operativity, cascades, co-valent modification of protein and some principles of the regulation of gene expression, it proved all too easy to convince ourselves that we understood how metabolic pathways are controlled and regulated. According to this view, all that remained was to identify the feedback and regulatory circuits.

This problem was (and to some extent still is) compounded by a number of factors. The dominant paradigm for the control of metabolic flux was the 'rate-limiting' step. For example, Lehninger (1975) wrote

"There is now good evidence that the phosphofructokinase reaction is the major rate limiting reaction in glycolysis."

Thus, if control mechanisms could alter the activity of an enzyme catalysing a 'rate-limiting' step in a pathway, then this would change the flux in that pathway. Disturbing questions may be asked, such as; 'how is a rate-limiting step defined?' and 'how much increase in flux is observed when an enzyme activity is increased by a given amount?'. It is probable that the discomfort wrought by these questions has contributed to the 'toning-down' of the terminology. Thus, Stryer (1988) wrote

"Phosphofructokinase is the most important control element in glycolysis".

Confusion is often increased when concepts such as first committed step, irreversible reactions and regulatory properties of an enzyme are introduced and applied uncritically. For instance, there often appeared to be an implicit (if not explicit) assumption that the regulatory properties of an enzyme was assurance that these enzymes control flux. For instance, Stryer (1988) explained that

"the activity [of phosphofructokinase] increases when the ATP/AMP ratio is lowered. In other words, glycolysis is stimulated when the energy charge of the cell is low."

Without a rigorous definition of the question or terminology along with an attempt to quantify the variables involved, the discussion of how metabolism is controlled or regulated becomes fruitless. For instance, a relevant question might be ‘what effect does the manipulation of a specific enzymatic step have upon metabolism’? This question is important to those wishing to understand metabolism and its regulation. It should also concern biotechnologists who wish to achieve specific goals such as increased metabolite concentrations or flux to end product.

A number of systematic approaches have been developed. These provide a theoretical framework for the asking of questions, design of experiments and analysis and interpretation of results. The one most commonly used by both theoretical and experimental biochemists is Metabolic Control Analysis (MCA) (Kacser and Burns, 1973; Heinrich and Rapoport, 1974) which is a form of sensitivity analysis. However other approaches such as Biochemical Systems Theory (Savageau, 1976) or the ‘flux oriented’ approach of Crabtree and Newsholme (1987) are available. MCA combines ease of use, mathematical simplicity and comprehensive theoretical and experimental development, and can be ‘mapped’ quite easily onto an intuitive concept of metabolism. For these reasons the other approaches are not discussed further.

1.1.1 Metabolic Control Analysis

MCA provides a comprehensive framework for the understanding and quantitation of metabolic control. It tackles the basic question ‘what is the effect on pathway flux or metabolite concentration if the activity of a specific enzyme is changed’?

In addition to the original formulation of MCA (Kacser and Burns, 1973; also Kacser *et al.*, 1995), there are a number of excellent reviews which discuss the central tenets of MCA, its development and its implications (Fell, 1992, 1997; Kacser, 1995; Westerhoff *et al.*, 1984)

MCA depends upon (Fell, 1992)

- a set of explicit assumptions regarding the metabolic system

- a set of definitions which quantify the response of the system and its elements and hence,
- a set of derived theorems

Assumptions

Metabolism consists of a set of enzymatic reactions which are interconnected by metabolites, the concentrations of which influence the rates of individual reactions. MCA studies the system at steady state, the characteristics of which include constant concentrations of metabolites, fixed source (substrates or nutrients) and sink (products or excretants) pools and constant fluxes within the pathway of the system. The system may be perturbed by changing a parameter (such as enzyme activity) by a small (strictly, limiting) amount, after which the system attains a new steady state, with new fluxes and metabolite concentrations. MCA deals with the comparison between these two states although subsequent developments have characterised the dynamic behaviour as the system relaxes (Melendez-Hevia *et al.*, 1990; Easterby, 1990). Parameters may be altered by the experimentalist; variables are dependent and will vary from one steady state to another when parameter values are changed. Enzyme activities are treated as parameters. They can be altered by site-directed mutagenesis, manipulation of copy number, inducible promoters, antisense RNA and specific inhibitors etc. Metabolites must be homogenous within compartments and enzyme activities should be proportional to concentration. Enzyme-enzyme interactions therefore need special treatment. Further developments in MCA have allowed some of these assumptions to be relaxed and have widened the scope of the analysis (Hofmeyr *et al.*, 1986; Fell and Sauro, 1990; Kacser *et al.*, 1990; Sauro and Kacser, 1990).

Definitions of MCA

A central task of MCA is to quantitate how a system variable such as metabolic flux or metabolite concentration responds to the variation in a parameter (usually the enzyme activity). If a simple linear pathway consisting of enzymes with Michaelis-Menten type kinetics is modelled then, for any of the enzymes in the pathway, there exists a hyperbolic relationship between flux through the pathway and enzyme

activity, as illustrated in Figure 1-1A. A number of important points may be understood by considering this relationship. The sensitivity of flux to small changes in the activity of an enzyme is related to the gradient of this graph at a particular point. When the flux and the enzyme activity are high, a change in the activity of an enzyme gives a small change in the flux. When the flux and enzyme activity are smaller, then the changes in flux brought about by small changes in enzyme activity are somewhat greater. The actual values will, of course, depend upon the precise kinetic parameters of the enzymes within the pathway, the activities of all the other enzymes in the pathway and the concentrations of the initial substrate and final product.

The changes which occur when the system is perturbed by altering the value of one enzyme's activity may be considered by defining a 'flux control coefficient', $C_{E_i}^J$ as

$$C_{E_i}^J = \lim_{\delta E \rightarrow 0} \frac{\delta J / J^0}{\delta E / E_i^0} \quad (1)$$

where E_i is the activity of a defined enzyme, E_i^0 is its initial value, δE is a small change in E_i , J is a defined flux, J^0 is its initial value and δJ is the small change arising.

Therefore

$$C_{E_i}^J = \frac{dJ}{dE} \cdot \frac{E_i^0}{J^0} \quad (2)$$

Thus the flux control coefficient is equal to the gradient of the function of pathway flux with respect to enzyme activity (Figure 1-1A), multiplied by a scaling factor (E_i/J^0) so that the values obtained are independent of absolute fluxes, enzyme activities and the units used. The values of the control coefficients for the hypothetical pathway are given in Figure 1-1B. As the enzyme activity increases, the control coefficient decreases from unity when the flux is zero towards a value of zero at high enzyme activities and maximum flux. Three out of the four examples show coefficients of less than 0.1 at an enzyme activity of 1 (wild-type activity). This is reflected in the rather small changes in flux which result when enzyme activity is increased or decreased from wild-type activity.

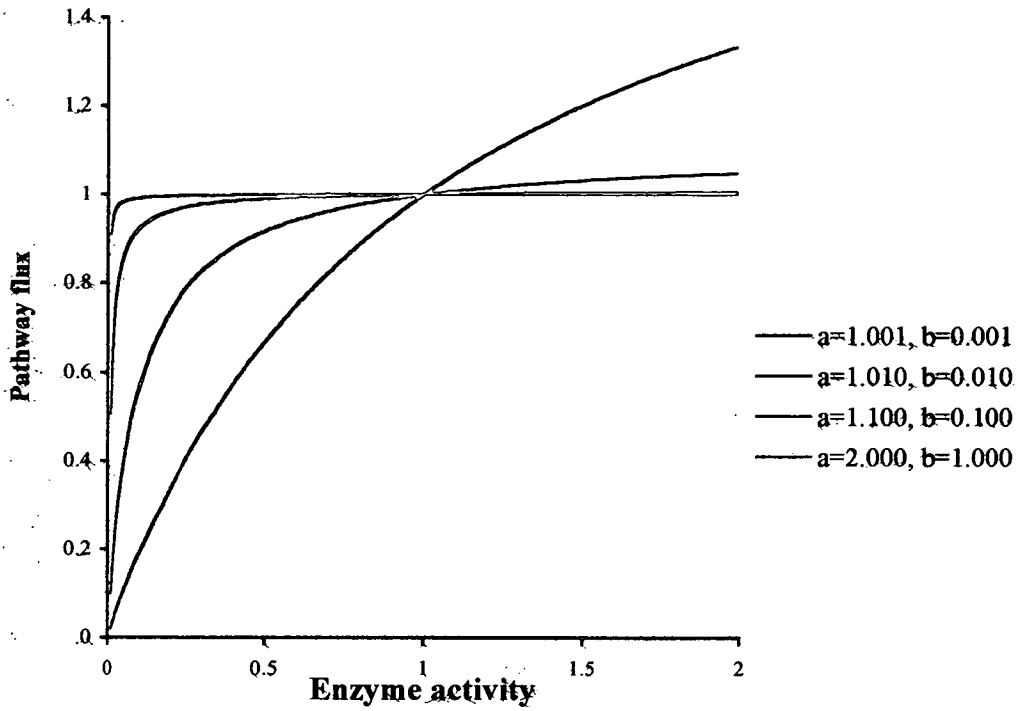
Figure 1-1 · The relationships between pathway flux, flux control coefficient and enzyme activity

These graphs show the relationship between flux (A) or flux control coefficient (B) and the activity of a single enzyme within a hypothetical pathway consisting of a linear sequence of enzymes whose kinetics behave according to Michaelis - Menten equations. An enzyme activity of 1 represents wild-type activity.

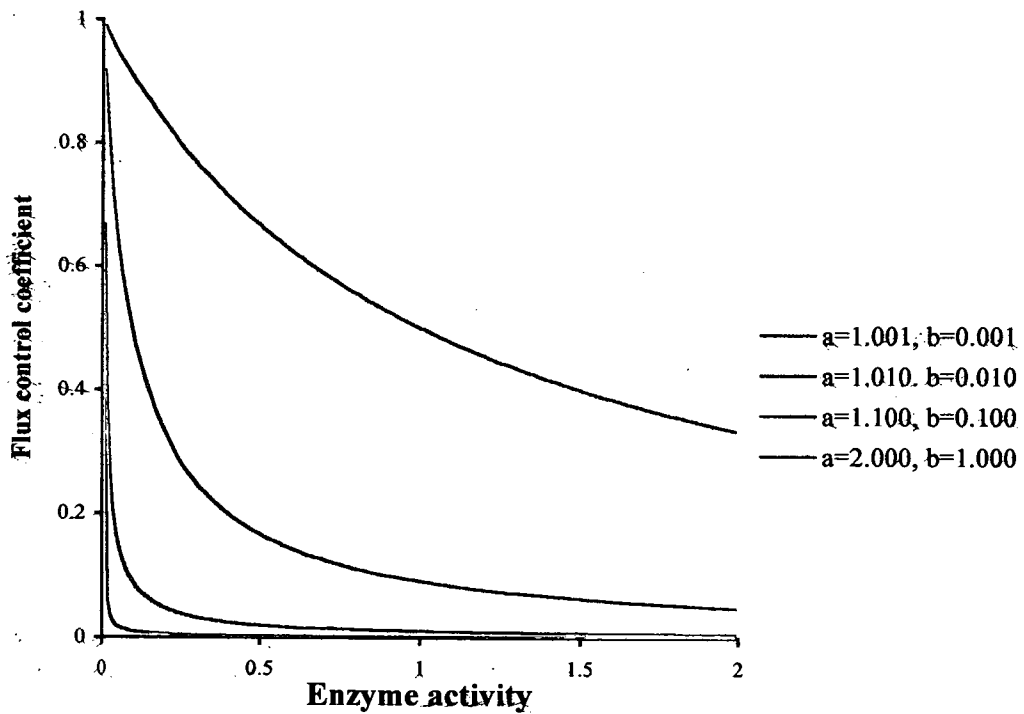
A. The flux through the pathway is calculated according to the hyperbolic function $J = aE/(b+E)$ where J = the pathway flux, E = enzyme activity, a = the maximum flux through the pathway and b = the enzyme activity giving half of the maximum flux. Thus the graphs are normalised so that a wild-type enzyme activity gives an arbitrary flux of 1.

B. The control coefficients are calculated by multiplying the gradients of the graphs in A by a scaling factor = J/E

A. The relationship between flux and enzyme activity



B. Change in flux control coefficient with enzyme activity



There will be a coefficient for every flux-enzyme pair. Thus for example, C_{as}^W is a measure of the sensitivity of the flux (W) through the tryptophan pathway to an infinitesimally small change in the activity of anthranilate synthase (as). Flux control coefficients will often be between zero and one. However, in some branched pathways a positive change in one enzyme activity may produce a reduction in flux in the other branch. This would be represented by a negative coefficient. Similarly, it is conceivable that a coefficient will be greater than one. This would occur if the fractional flux change in a pathway were greater than the fractional change in an enzyme activity.

In general, there will be a control coefficient C_P^V for every variable/parameter pair, where V may be a flux or metabolite concentration and P may be any parameter but, typically, is an enzyme activity. Thus there is also a metabolite concentration control coefficient, defined as follows.

$$C_{E_i}^S = \frac{dS}{dE} \cdot \frac{E_i^0}{S^0} \quad (3)$$

This defines the extent to which the concentration of a specific metabolite (S^0) depends upon the activity of a given enzyme. This metabolite may or may not be a substrate, product or effector. Since an increase in enzyme activity will tend to decrease metabolite concentrations upstream of a particular step (*e.g.* the substrates) and increase metabolite concentrations downstream (*e.g.* products), these values may be positive or negative.

It is vital to understand that control coefficients (flux or metabolite concentration) are system properties in that they may depend upon the species or strain, developmental stage, metabolic state, growth condition, media composition and genetic background etc. There is no simple way of predicting the control coefficients even if all the parameters of the system are identified and known.

However, the system properties do emerge from the behaviour of enzymes in response to changes in the concentrations of effectors, as substrates, products,

activators and inhibitors. These effects may be considered using 'elasticity coefficients', which describe the effect of a change, δS , in a metabolite S_j , upon the rate of a reaction v_i , catalysed by the enzyme E_i , at constant and prevailing concentrations of other effectors, and are defined as

$$\mathcal{E}_{S_j}^{v_i} = \frac{dv_i}{dS} \cdot \frac{S_j^0}{v_i^0} \quad (4)$$

An elasticity coefficient therefore defines the responsiveness of the enzyme activity to changes in the concentration of an effector. Values will be positive for substrates (except when substrate inhibition occurs) and activators and negative for products and inhibitors. Although the elasticity coefficient is a 'local' property in that it does not depend upon the behaviour or properties of other elements in the system (such as other enzymes), it is essential to understand that it is dependent upon the enzyme's cellular milieu. Therefore, it is not possible to assume that the elasticity coefficient may be derived from kinetic parameters obtained from enzyme assays *in vitro*. Firstly, the enzyme's elasticity with respect to a given effector may be a complex function of the concentrations of any number of the cytosolic components. Secondly, the task of measuring these cellular concentrations is far from straightforward. Thirdly, even if these were definable, the effect of cellular location, supra-molecular organisation, and, even cytosolic structure may modify an enzyme's kinetic parameters. The conclusion is that, in most cases, elasticity coefficients must be measured not derived.

In conclusion, two control coefficients have been defined. They measure how much the flux or concentration of a metabolite changes when an enzyme activity is modified by a small amount. Elasticity coefficients describe the response of an enzyme activity to small changes in the concentration of any effector within the system.

Theorems of MCA

The theorems of MCA were originally derived for simple linear pathways, and were gradually extended to more complex systems. They include the summation theorems which relate the flux (or metabolite concentration) control coefficient of all the enzymes in the system. Thus, the flux control coefficient summation theorem,

$$\sum_{i=1}^n C_{E_i}^J = 1 \quad (5)$$

formalises the statement that the sum of every enzyme's control coefficient, with respect to one specified flux, is equal to 1. Also, the metabolite control coefficient summation theorem,

$$\sum_{i=1}^n C_{E_i}^S = 0 \quad (6)$$

summarises the finding that the sum of every enzyme's metabolite control coefficient, with respect to a single specified metabolite concentration, is equal to zero. These theorems constrain the allowable values of control coefficients for a particular flux or metabolite concentration. For instance, if an enzyme is truly rate-limiting (control coefficient = 1) for a particular flux, then all other control coefficients must be equal to zero, unless another enzyme has a negative control coefficient. They also allow the possibility that many or all enzymes contribute to the control of flux or metabolite concentration.

Other theorems have been derived, including the important connectivity theorems. These relate elasticities and control coefficients. They suggest how values of control coefficients depend in a complex manner upon the elasticities (and hence the kinetic properties) of the enzymes and place limits upon the values of control coefficients and emphasise how they are systemic properties, not properties of isolated enzymes. They also allow the determination of control coefficients provided that the elasticities of the enzymes are known. In some cases, it may be easier to determine these, rather than to make direct and accurate estimates of the control coefficients. Essentially they can form a set of simultaneous equations which may be represented and solved in matrix form (Fell and Sauro, 1985, Sauro *et al.*, 1987).

Implications of the theorems of MCA

The flux summation theorem (Equation 5) formalises the following observations.

- All enzymatic steps have the potential for some control

- There are constraints on the values which the control coefficients may have
- Most enzymes will have very low flux control coefficients
- The number of enzymes with significantly high flux control coefficients will be limited
- If there is a change in one control coefficient, there will be complementary changes in one or more of the others

These implications have now been supported by experimental determination of control coefficients in defined pathways.

Experimental applications of MCA

The theoretical results of MCA guide the development of relevant experiments designed to probe how control is distributed in biological systems. Conversely, experimental work has confirmed the central theorems of MCA and suggested new directions for development. A full review of the experimental justification of MCA is outside the scope of this review. Therefore, only some data are presented to illustrate the implications of the theorems of MCA, in contrast to Fell (1992) whose excellent and comprehensive review is organised on the basis of the various methodologies employed.

The summation theorems suggest that control may be shared amongst all the enzymes of the system. This has been confirmed for the control of oxidative phosphorylation in intact mitochondria, studied by Groen *et al.* (1982), using specific inhibitors for the adenine nucleotide translocator, dicarboxylate carrier, cytochrome c oxidase and the bc_1 complex. In respiratory state 3, values of the control coefficients were 0.29, 0.33 and 0.17 respectively. The control coefficients for the bc_1 complex, proton leakage and hexokinase were 0.03, 0.04 and 0.0, respectively. These values sum to 0.86. Values for succinate dehydrogenase and ATP synthase were left undetermined. Thus there is no 'rate-limiting' step, in the sense that one particular step, and no other, has control of flux. Alternatively, most of the steps are 'rate-limiting' in the sense that they show some control of flux.

Another implication of MCA is that many control coefficients will have low values. Since most experiments deal with limited pathways and measure only enzymes closely associated with the relevant flux, the flux control coefficients for only small numbers of enzymes are available. Nevertheless, the occasions when these have values greater than 0.5 are rare. Flint *et al.* (1981) used heterokaryons in *Neurospora crassa*, containing various doses of genes encoding four enzymes of arginine synthesis. The control coefficients of all of these enzymes were less than 0.1. This, of course, also suggests that significant control is contributed by enzymes more distal to the arginine pathway.

Of particular relevance to this study is the work of Miozzari *et al.* (1978a) who considered the effect of gene dosage in the tryptophan pathway in *S. cerevisiae* by constructing a tetraploid gene dosage series and measuring growth rate. The data fit rectangular hyperbolae (as in Figure 1-1A) and allow the determination of control coefficients with respect to growth rate. Of the five enzymes of the pathway, four have coefficients less than 0.04, while the other, phosphoribosyltransferase, has a coefficient of only 0.174. The sum of the five coefficients is 0.258, indicating that most of the control lies outwith those steps specific to tryptophan biosynthesis (Niederberger *et al.* 1992).

In view of the status of phosphofructokinase as the classic example of a 'rate-limiting' enzyme, the result that a five-fold increase in the enzyme leads to no change in glucose consumption or ethanol production in yeast in anaerobic culture and only a 30% increase under aerobic conditions, is important (Davies and Brindle, 1992). It suggests that its control coefficient is probably no more than 0.2 even under aerobic conditions.

The distribution of control coefficients may change as the environmental conditions, genetic composition or metabolic state of the system changes. For example, Stitt *et al.* (1991) considered the control coefficient of ribulose biphosphate carboxylase (rubisco) upon carbon assimilation under different conditions of light intensity, partial pressure of carbon dioxide and relative humidity. At wild-type rubisco levels, control

coefficients changed from 0.7 to 0.1 on reduction of light intensity, 0.8 to 0.1 when the partial pressure of carbon dioxide was reduced and 0.75 to 0.5 when humidity was reduced. The contributions of other steps were not considered.

Finally, MCA suggests, as a corollary, that large changes in activities may have rather small effects on flux. Thus in glycolysis, separate large increases in the expression of most of the enzymes of glycolysis, including hexokinase, phosphoglucose isomerase, phosphofructokinase, pyruvate kinase etc., gave small changes in flux (Schaaff *et al.*, 1989). Similar results have been obtained when the enzymes of tryptophan biosynthesis are overexpressed (Niederberger *et al.*, 1992). This effect can, of course, work in the opposite direction. An example which cautions against the uncritical use of enzyme inhibitors for therapeutic applications shows that even a small reduction in purine biosynthesis (10% reduction in flux) requires a very large reduction (86%) in the activity of the enzyme phosphoribosylglycinamide transformylase by the use of a specific inhibitor (Smith *et al.*, 1990). However, the two situations are not symmetrical. In overexpression of enzyme activity, a control coefficient may well decrease as the enzyme activity rises, leading to a situation of 'diminishing returns'. However, when an enzyme activity is decreased, the flux control coefficient tends to increase. Thus the effect on flux becomes greater as increased inhibition is obtained. These effects may be simply visualised with reference to Figure 1-1.

Results such as those described above illustrate why most deleterious mutants in genes for enzymes are recessive in diploid organisms. For many enzymes and fluxes, a halving of activity will result in an insignificant change in flux or metabolite concentration. The phenotype is thus not apparent. Indeed, the explanation for the dominance of wild-type genes in terms of the central concepts and theorems of MCA has been comprehensively developed (Kacser and Burns, 1981). The phenomenon can be viewed as a simple consequence of the summation theorem which formally acknowledges that control may be spread over many enzymes, often in pathways remote to the local pathway in question; hence, most enzymes have low flux control coefficients.

Taken as a whole, the data from experimental work described here show why the analysis of control in pathways according to the concept of rate-limiting enzymes is often misleading, ambiguous or simplistic.

1.1.2 Large changes

Classical MCA deals with small (strictly speaking, infinitesimal) changes in parameters. However, the changes occurring in organismal development and cell differentiation or demanded by industrial concerns may be very large. Is there any way of extending MCA into a treatment of such changes?

Small and Kacser (1993) defined a deviation index, D , as follows

$$D_{E_i}^J = \frac{\Delta J / J^r}{\Delta E_i / E_i^r} \quad (7)$$

where ΔJ and ΔE represent the difference between final values of flux, J^f , and enzyme activity, E^f , and initial values (J^0 and E^0). Note that this is analogous to the definition of a flux control coefficient. Large defined differences have replaced small changes and the scaling factor (J^f/E^f) refers to the flux and enzyme activity at the values after the large change, while the control coefficient uses a scaling factor which refers to these parameters before the small change. Given important simplifying assumptions about the kinetics of enzymes in the pathway (with respect to linearity etc.), they show that the deviation coefficient is equal to the control coefficient.

$$D_{E_i}^J = C_{E_i}^J \quad (8)$$

The theorems of control analysis are then derived for the deviation indices. These results were tested and supported by consideration of data from tryptophan catabolism and glucose metabolism in rat liver and the growth rate responses to β -galactosidase activity in *Escherichia coli*. This gives some support to the idea that large changes may be considered in control analysis, in certain limited situations where the qualifying assumptions may be justified.

Subsequently, Kacser and colleagues have applied these concepts to the possibility of generating large changes in flux (Kacser and Acerenza, 1993) or metabolite concentration (Small and Kacser, 1984) by using DNA technology in a biotechnological context.

1.1.3 'The 'Universal Method''

The 'Universal Method' (Kacser and Acerenza, 1993) defines the changes in enzyme activities required to achieve a predetermined and large increase in a specific output whilst maintaining all metabolite pools and (thereby) all other fluxes at unchanged levels. It is illustrated in Figure 1-2 and its features are described below.

- Sections of pathways between branch point metabolites are the units of manipulation. Increases in enzyme activities (enzyme multipliers, r_i) are applied to all enzymes in a given section.
- Sections upstream of the output flux may be considered in turn. In order to maintain constant fluxes in side-branches, the absolute increase in flux (namely ΔJ) will be the same in all sections of the **main** pathway.
- Since fluxes of upstream sections are larger (by the magnitude of flux in branch pathway), *i.e.* $J_3 = J_2 + J_1$, the ratio of the absolute flux increase to the original flux ($\Delta J/J_i$) decreases as upstream sections are considered in turn.
- The enzyme multipliers to be applied within each section are a function of this ratio, namely

$$r_1 = 1 + \frac{\Delta J}{J_1^o} \quad (9)$$

$$r_2 = 1 \quad (10)$$

$$r_3 = 1 + \frac{\Delta J}{J_3^o} \quad (11)$$

Figure 1-2 The universal method

A model branched pathway is represented schematically. Substrates (S) are converted to the branchpoint metabolite (B) in a series of enzymatic reactions (section 3). The branchpoint metabolite forms product (P) through section 1. A side-branch (section 2) produces other metabolites (M). The original fluxes in the three sections are shown (in green) as J^o (with subscripts 1,2 or 3). The original enzyme activities are represented (in yellow) as E^o (with subscripts). At steady state

$$J_3^o = J_2^o + J_1^o$$

Changes are made to all enzyme activities within sections of the pathways in order to generate new fluxes. These are represented as J^r (with subscripts 1,2 or 3). The absolute change in flux in section 1 is ΔJ , such that

$$J_1^r = \Delta J + J_1^o$$

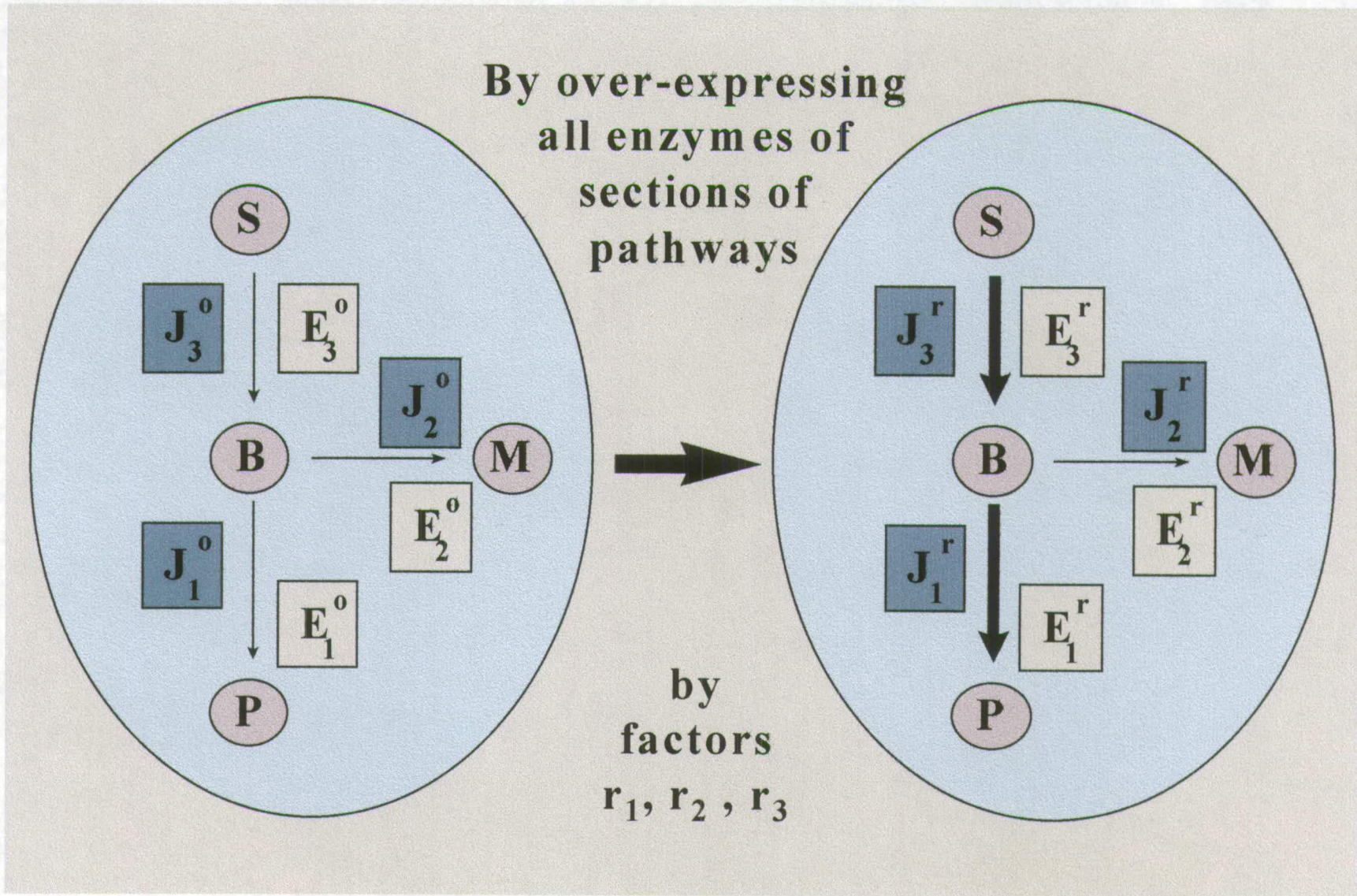
The new enzyme activities within the sections of the pathway are E^r (with subscripts 1, 2 or 3). Original and final enzyme activities are related to the 'enzyme multipliers' r_1 , r_2 and r_3 , thus

$$r_i = \frac{E_i^r}{E_i^o}$$

Note however, that the flux in the side-branch remains unchanged and therefore

$$J_2^r = J_2^o$$

Also the concentrations of the metabolites, S, B, M and P remain unchanged as well as those of all intermediate metabolites in all three sections.



- As sections further upstream are considered, J_i^0 becomes progressively larger and, therefore, r_i becomes smaller (as ΔJ becomes smaller in relation to J_i^0). Smaller increases in enzyme activities are therefore required.
- A given increase in output flux may be achieved by overproducing enzymes of the final section by a specified amount, r_l where

$$r_l = 1 + \frac{\Delta J}{J_1^0} \quad (12)$$

- r_l is therefore equal to the 'flux multiplier', f_l , given by

$$f = \frac{J_1^r}{J_1^0} \quad (13)$$

- Enzymes in side branches are left unchanged since $\Delta J = 0$. Metabolite pools concentrations within sections of pathways and at branch points will remain unchanged. Therefore, fluxes in branch pathways will also remain unchanged.

To summarise, the overall output flux in the pathway may be pre-set. The fluxes in side-branches and all metabolite concentrations are required to remain unchanged. Most importantly therefore, a knowledge of the regulatory network is unnecessary. The maintenance of metabolite pools ensures that factors such as enzyme inhibition or activation, modulation of enzyme expression by induction, de-repression and transcriptional activation etc. should remain unchanged. The only data required are the relative fluxes of the various branches with respect to the fluxes in sections of the main pathway. The methodologies required to implement the defined increases in enzyme activity are not defined but may include changing the copy number of the corresponding genes and the use of heterologous, variable and inducible promoters.

The organisation and regulation of aromatic amino acid biosynthesis is discussed in the following sections so that the experimental application of the 'Universal Method' to this system may be introduced in section 1.4.

1.2 Aromatic Amino Acid Biosynthesis

The considerable interest shown in aromatic amino acid biosynthesis probably derives from a number of factors. It has a central importance in the biosynthesis of aromatic (and related) compounds which are essential to the production of a range of 'secondary metabolites' among which are included many candidate compounds with antibiotic or therapeutic properties. It also has industrial relevance for the production of economically important chemicals such as indigo, tryptophan, *p*-aminobenzoic acid, adipic acid etc. Thus, aromatic biosynthesis serves as a paradigm for how biocatalysts can be manipulated to achieve the yield, rate and purity criteria central to chemical manufacture (Frost and Draths, 1995). Also, because the biosynthesis of tryptophan, phenylalanine and tyrosine is limited to archaeobacteria, eubacteria, plants and fungi (Braus, 1991), chemical species which specifically inhibit the enzymes of these pathways may be promising candidates for herbicides, and anti-bacterial or -fungal agents. Finally, the low concentrations of tryptophan within the cell, the low percentage of all three aromatic amino acids in protein, the high energetic costs of making tryptophan, phenylalanine and tyrosine, and the branched-nature of the pathway make it an excellent system for the study of biosynthetic regulation.

An outline of the tryptophan biosynthetic pathway is illustrated in Figure 1-3. The genes, enzymes and effectors of enzyme activities in *S. cerevisiae* are shown in Figure 1-4. The synthesis involves a common pathway, called the shikimate pathway which was first identified and characterised by Davis and co-workers during the early 1950's (Davis *et al.*, 1955). The shikimate pathway uses intermediates of glycolysis (phosphoenolpyruvate (PEP)) and the pentose phosphate pathway (erythrose-4-phosphate (E4P)) to produce a branch-point metabolite, chorismic acid. From here, tryptophan is synthesised via the tryptophan pathway and tyrosine and phenylalanine are synthesised via the prephenate pathway. Several important metabolites, such as *p*-aminobenzoate and ubiquinone, are also synthesised from chorismic acid. A number of reviews focus on specific parts of the pathway. For example Bentley (1990) gives a detailed review of the shikimate pathway including details of the multiple branches,

Figure 1-3 The tryptophan biosynthetic pathways

Above, from the precursors, phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), the shikimate pathway produces the branch-point metabolite chorismic acid (CHA). Intermediates shown are 3-deoxy-*D-arabino*-heptulosonate 7-phosphate (DAHP), 3-dehydroquinate (DHQ), 3-dehydroshikimate (DHS), shikimate (SHK), shikimate 3-phosphate (S3P) and 5-*enol*pyruvyl shikimate 3-phosphate (EPSP).

Below, tryptophan is synthesised from chorismic acid through the tryptophan pathway. Intermediates shown are 2-amino-2-deoxy-isochorismic acid (A), anthranilate (B), phosphoribosylanthranilate (C), 1-(2-carboxyphenylamino)-1-deoxyribulose 5-phosphate (D) and indoleglycerol phosphate (E).

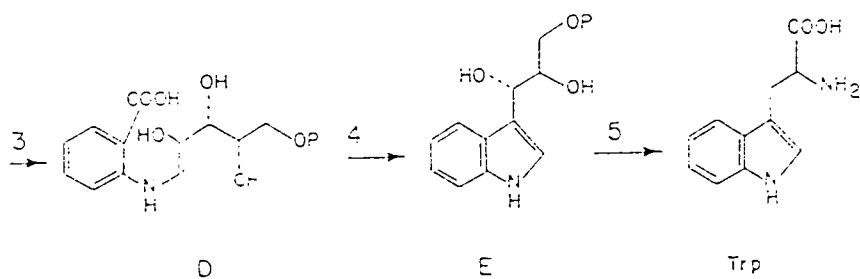
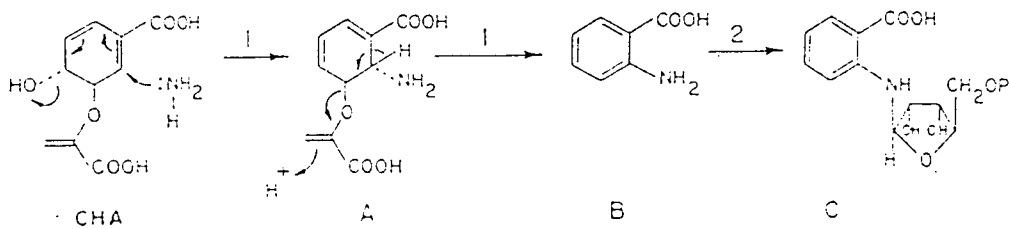
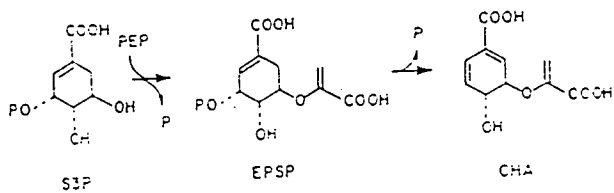
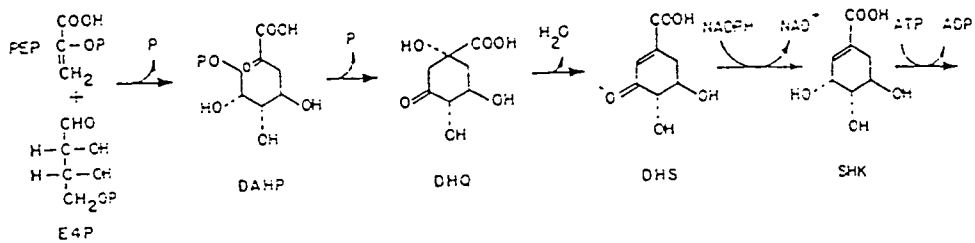


Figure 1-4 Enzymes and genes of aromatic amino acid biosynthesis

The genes of the shikimate pathway (*ARO*) and the tryptophan pathway (*TRP*) are shown in blue, while the activities encoded are shown in green. For the prephenate pathway, only the gene (*ARO7*) encoding the first enzyme, chorismate mutase, is shown.

Red dashed lines indicate feedback inhibition of enzyme activity. Green dotted line indicates tryptophan relieving inhibition of chorismate mutase by tyrosine. The black bar indicates relief from an inhibitory interaction

Erythrose-4-phosphate

+

Phosphoenolpyruvate

ARO3, ARO4

DAHP synthases (phe, tyr inhibitable)

feedback inhibition

*ARO1
C,E,D,B,A*

AROM complex

(inc. DHQ synthase, DHQ-ase, shikimate dehydrogenase, shikimate kinase and EPSP synthase)

ARO2

Chorismate synthase

Chorismic acid

ARO7 Chorismate mutase

Phenylalanine and Tyrosine

TRP 2/3C

Anthranilate synthase

TRP 4

Phosphoribosyl-anthranilate transferase

TRP 1

Phosphoribosyl-anthranilate isomerase

TRP 3B

Indole-glycerolphosphate synthase

TRP 5

Tryptophan synthase

feedback inhibition

Tryptophan

while Hutter *et al.* (1986) reviews the tryptophan pathway. Specific aspects of the genomic organisation of the genes, association of enzymes and activities and the regulatory mechanisms (at the level of transcription, translation and the kinetic behaviour of enzymes) differ considerably in different species (Henner and Yanofsky, 1993). For instance, genes of the tryptophan pathway in *Escherichia coli* are located in the *trp* operon which allows co-ordinate regulation of gene expression, while *S. cerevisiae* has tryptophan genes distributed over the genome but whose expression may be co-ordinated by the action of a common transcriptional activator, GCN4 (Hinnebusch, 1988). Interestingly, *S. cerevisiae* and other fungi, such as *Aspergillus nidulans* and *Neurospora crassa* also have a multi-functional enzyme complex (the *AROM* complex) in the shikimate pathway, which has also been considered as a means of co-ordinate regulation (Duncan *et al.*, 1988). Therefore, other reviews limit themselves to a discussion of the organisation and regulation of the pathways in one species or within a group of closely related organisms. For example, Pittard (1987) and Henner and Yanofsky (1993) discuss biosynthesis of aromatic amino acids in enteric bacteria and gram-positive bacteria, respectively. The most relevant review in the context of this study is that of Braus (1991) who reviews aromatic amino acid biosynthesis in *S. cerevisiae* and focuses upon gene-enzyme relationships, the regulation of the pathway at the level of transcription, mRNA stability and translation and the modulation of enzyme activity by metabolite inhibition and activation. The following sections review knowledge of the genes and enzymes of the shikimate and tryptophan pathways relevant to this study. A brief review of tryptophan catabolism and the transport of aromatic amino acids into the cell is also included. Finally, the possible mechanisms of regulation and control are summarised.

1.2.1 The shikimate pathway

Seven activities are encoded by four genes. The first activity, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase is executed by two isoenzymes, encoded by the genes *ARO3* and *ARO4*, which have feedback sensitivity to phenylalanine and tyrosine, respectively. The next five activities, 3-dehydroquininate (DHQ) synthase, 3-dehydroquininate dehydratase (3-dehydroquinase), shikimate

dehydrogenase (SDH), shikimate kinase and 5-enolpyruvylshikimate phosphate (EPSP) synthase constitute the pentafunctional AROM complex, encoded by the gene *ARO1*. Lastly, chorismate synthase is encoded by *ARO2*. Details of the genes and enzymes are shown in Table 1-1.

DAHP synthase (*ARO3* and *ARO4*)

The condensation of erythrose-4-phosphate and phospho-enol-pyruvate in an aldol-type condensation is carried out by two differentially regulated isoenzymes encoded by the genes *ARO3* and *ARO4*. They share 61% amino acid identity. The cognate genes have both been cloned and sequenced (Paravicini *et al.*, 1988; Kunzler *et al.*, 1992).

The AROM complex (*ARO1*)

This multi-functional protein, encoded by *ARO1* located on chromosome IV, catalyses 5 activities; DHQ synthase (which achieves ring closure), 3-dehydroquinase (which introduces the first ring double bond), SDH, shikimate kinase and EPSP synthase. The gene has been cloned (Larimer *et al.*, 1983) and sequenced (Duncan *et al.*, 1987). The five genes corresponding to these activities are termed *ARO1C*, *ARO1E*, *ARO1D*, *ARO1B*, *ARO1A*, as judged by sequence similarity with the homologous genes in *E. coli*. These are not co-linear in the *ARO1* gene. Instead the order of genes is *ARO1C*, *ARO1A*, *ARO1B*, *ARO1D*, *ARO1E* (Duncan *et al.*, 1987).

The AROM complex has attracted speculation that it may show channelling characteristics (Welch and Gaertner, 1975). Channelling crudely means that an intermediate metabolite does not fully equilibrate with the bulk cytosol. It is a complex phenomenon. For example, definitions of channelling make a distinction between static and dynamic channelling. Also, the criteria used to determine and distinguish channelling phenomena experimentally are not obvious. It should, therefore, come as no surprise that some controversy has occurred over the question of whether channelling occurs in the AROM complex (for review, see Lamb *et al.*, 1997).

Gene	Gene product	Activity (inc. regulation)	E.C. number	Amino acids	homologous <i>E. coli</i> gene	Reference
<i>ARO3</i>	DAHP synthase	inhibited by phenylalanine	4.1.2.15	370	aroF	Paravicini <i>et al.</i> (1988)
<i>ARO4</i>		inhibited by tyrosine		370	aroG	Kunzler <i>et al.</i> (1992)
<i>ARO1</i>	AROM complex			1,588		Duncan <i>et al.</i> (1987, 1988)
<i>ARO1C</i>		DHQ synthase	4.6.1.3	1-392	aroB	
<i>ARO1E</i>		3-dehydroquinase	4.2.1.10	1059-1293	aroD	
<i>ARO1D</i>		Shikimate dehydrogenase	1.1.1.25	1306-1588	aroE	
<i>ARO1B</i>		Shikimate kinase	2.7.1.71	886-1059	aroL	
<i>ARO1A</i>		EPSP synthase	2.5.1.19	404-886	aroA	
<i>ARO2</i>	Chorismate synthase		4.6.1.4	376	aroC	Jones <i>et al.</i> (1991)

Table 1-1 Enzymes and genes of the shikimate pathway

The chromosomal locus of the genes are given in the text. The amino acids coding for activities on the multi-functional *AROM* complex are derived by similarity with the homologous *E. coli* genes.

Chorismate synthase (*ARO2*)

Chorismate is formed from EPSP by the loss of phosphate and the introduction of another double bond in the ring structure. Chorismate synthase is encoded by *ARO2* located on chromosome VII. It has been cloned and sequenced (Jones *et al.*, 1991). Although the enzymes from all of the species studied so far have a requirement for a reduced flavin cofactor, they may be classified into two groups. One, including the enzyme from bacteria, algae and plants, does not have an intrinsic NADPH-dependent flavin reductase (diaphorase) activity (Horsburgh *et al.*, 1996) while those from the fungi *Neurospora crassa* (Henstrand *et al.*, 1995) and *S. cerevisiae* (Jones *et al.*, 1991) both have this activity.

1.2.2 The tryptophan pathway

Five enzymatic steps convert chorismic acid into tryptophan. The five enzymes are anthranilate synthase, phosphoribosyl - anthranilate transferase, phosphoribosyl - anthranilate isomerase, indole glycerophosphate synthase and tryptophan synthase. Table 1-2 summarises data relating to the pathway's genes and enzymes. A review specific to the tryptophan pathway is given by Crawford (1989).

Anthranilate synthase (*TRP2/3C*)

Anthranilate synthase activity resides in a bifunctional complex consisting of two subunits encoded by *TRP2* and *TRP3*. This complex also catalyses the fourth reaction of the pathway (indole glycerophosphate synthase, see below). The genetically defined *TRP3C* contributes a glutamine amidotransferase activity to the complex. Both genes have been cloned and sequenced (Aebi *et al.*, 1984; Zalkin *et al.*, 1984).

Phosphoribosyl - anthranilate transferase (*TRP4*)

This enzyme catalyses the transfer of a 5-phosphoribosyl moiety from 5-phosphoribosylpyrophosphate to the amino group of anthranilic acid to give phosphoribosylanthranilate. The *TRP4* gene has been cloned and sequenced (Furter *et al.*, 1986).

Gene	Enzyme name	Activity	E.C. number	Amino acids (no.)	Homologous to E. coli gene	Reference
<i>TRP2</i>	Anthranilate synthase	anthranilate synthase	4.1.3.27	528	<i>trpE</i>	Aebi <i>et al.</i> (1984)
<i>TRP3C</i>		glutamine-amido transferase		1-206	<i>trpG(-D)</i>	Zalkin <i>et al.</i> (1984) Prantl <i>et al.</i> (1985)
<i>TRP4</i>	Anthranilate-phosphoribosyl transferase		2.4.2.18	380	<i>trp(G-D)</i>	Furter <i>et al.</i> (1986)
<i>TRP1</i>	Phosphoribosyl-anthranilate isomerase		4.2.1.10	224	<i>trp(C-F)</i>	Tschumper and Carbon (1980)
<i>TRP3B</i>	Indole-3-glycerol phosphate synthase		4.1.1.48	(218-484)	<i>trpC(-F)</i>	Zalkin <i>et al.</i> (1984)
<i>TRP5</i>	Tryptophan synthase		4.2.1.20	707	1-239 <i>trpB</i> 319-707 <i>trpA</i>	Zalkin and Yanofsky (1982)

Table 1-2 Enzymes and genes of the tryptophan pathway

The relationship between anthranilate synthase and indole glycerol phosphate synthase activities and the genes *TRP2* and *TRP3* are explained in the text.

Phosphoribosyl - anthranilate isomerase (*TRP1*)

This enzyme catalyses an essentially irreversible rearrangement, involving an internal redox reaction which yields carboxyphenylamino-1-deoxyribulose-5-phosphate. The gene was cloned by complementation of an *E. coli trp* mutant and was used in some first generation yeast vectors. The sequence of *TRP1* has been determined (Tschumper and Carbon, 1980).

Indole-3-glycerol phosphate synthase (*TRP3B*)

This enzyme catalyses the decarboxylation of carboxyphenylamino-1-deoxyribulose-5-phosphate and closure of the second ring to give indole-3-glycerol phosphate. It is encoded by the 3' half of the *TRP3* gene.

Tryptophan synthase (*TRP5*)

In the final reaction of the tryptophan pathway, indole-3-glycerol phosphate is cleaved and indole condensed with serine to yield tryptophan. In most organisms, these two reactions are catalysed by two separate proteins but in *S. cerevisiae* and *Neurospora crassa*, the two activities are present on one polypeptide. In all cases, however, there are two distinct active sites on opposite sides of the protein or complex. Indeed there is now overwhelming evidence that, in enteric bacteria the intermediate, indole, diffuses directly from one active site to the other (Yanofsky, 1989) through a 'tunnel' which passes through both subunits (Hyde *et al.*, 1988) of a complex. The function of this arrangement may be the avoidance of unwanted side reactions by the intermediate indole or the generation of favourable thermodynamic conditions (Pan *et al.*, 1997; West, 1997). The gene from *S. cerevisiae* has been cloned and sequenced (Zalkin and Yanofsky, 1982).

1.2.3 The prephenate pathway

Chorismic acid is converted into phenylalanine and tyrosine by five reactions. Firstly, chorismate mutase, encoded by the gene *ARO7* catalyses the intramolecular rearrangement of chorismate to form prephenate. This product is converted to phenylpyruvate and 4-hydroxyphenylpyruvate by prephenate dehydratase (encoded by

PHA2) and prephenate dehydrogenase (*TYR1*), respectively. Two aminotransferases then yield phenylalanine and tyrosine. There may be an important role for chorismate mutase in the regulation of the pathway. The regulatory properties of this enzyme are discussed below in section 1.2.6.

1.2.4 Tryptophan transport

A number of genes involved in the transport of amino acids across the cell wall and membrane have been identified by biochemical and genetic methods while others have been suggested on the basis of sequence data now available (André, 1995). Both non-specific and specific transport systems co-exist. There is a general amino acid permease, encoded by *GAP1* (Jauniaux and Grenson, 1990) which is repressed completely in the presence of ammonia (Grenson *et al.*, 1970). Among the specific permeases identified are *TAT1* and *TAT2* (also called *TAP2* or *SCM1*) which show specificity to the aromatic amino acids (Heitman *et al.*, 1993; Chen *et al.*, 1994; Schmidt *et al.*, 1994). *TAT2* shows particular specificity for tryptophan, while *TAT1* has specificity for tyrosine (Schmidt *et al.*, 1994).

It is generally assumed that these transporters (proton antiporters) function unidirectionally in favour of uptake and that excretion must therefore occur through non-specific diffusion mechanisms. However this question appears not to be fully resolved.

1.2.5 Catabolism of aromatic amino acids

Kradolfer *et al.* (1982) identified the products of degradation of tryptophan and partially characterised the enzymes involved. Tryptophol is the main product, the first step being catalysis by a trans- or de-aminase to indole-pyruvate, presumably followed by decarboxylation and reduction. Two aminotransferases are involved, encoded by the genes, *AAT1* and *AAT2*. The former is a low affinity enzyme with a $K_m = 6$ mM with respect to tryptophan with 2-oxoglutarate as amino acceptor. *AAT2* has a higher affinity with a $K_m = 0.2 - 0.4$ mM and phenylpyruvate as amino acceptor. They both show a greater affinity for phenylalanine and tyrosine. In contrast with the affinities to

tryptophan, *AAT1* shows a much greater affinity for phenylalanine and tyrosine than *AAT2*. *AAT2* is induced by media containing tryptophan or phenylalanine as nitrogen source. *AAT1* is therefore proposed to catalyse the final reactions in the biosynthesis of phenylalanine and tyrosine while *AAT2* is predominantly responsible for catabolic degradation. Kradolfer *et al.* (1982) also isolated an *aat2* mutant strain, RH805. This showed an increase in the excretion of tryptophan, due to increases in the intracellular concentration of tryptophan.

1.2.6 Regulatory mechanisms in the pathway

The biosynthesis of aromatic amino acids incur high energetic costs. It would therefore be surprising if the intracellular concentrations of these amino acids were not highly regulated. Indeed the intracellular concentrations of tryptophan, phenylalanine and tyrosine, reported to be 20, 500 and 600 μM , respectively (Fantes *et al.*, 1976; Messenguy *et al.*, 1980), are amongst the lowest of the amino acids. The possible regulatory mechanisms functioning in aromatic amino acid biosynthesis are now reviewed.

Regulation of enzyme activity

The network of effectors acting upon enzymes of the shikimate, prephenate and tryptophan pathway are shown as broken lines in Figure 1-4. *In vitro* analysis of purified or partly purified enzymes show that the first enzymes of the shikimate pathway, DAHP synthase, encoded by the genes *ARO3* and *ARO4* are inhibited by phenylalanine and tyrosine, respectively (Takahashi and Chan, 1971; Paravicini *et al.*, 1989b). Tryptophan has no detectable effect upon them. Anthranilate synthase activity is inhibited by tryptophan. This is competitive with respect to chorismate ($K_i = 56 \mu\text{M}$) (Prantl *et al.*, 1985). Mutants with feedback resistant anthranilate synthase activity have been isolated (Fantes, *et al.*, 1976). There is a *TRP2* allele which encodes a feedback-insensitive enzyme with a single amino acid substitution (Graf *et al.*, 1993). Chorismate mutase, which catalyses the first step of the prephenate pathway to phenylalanine and tyrosine, shows co-operative kinetics with respect to chorismic acid. The enzyme is inhibited by tyrosine. Tryptophan relieves this

inhibition and activates the enzyme by negating the positive co-operativity (Schmidheini *et al.*, 1990).

This network therefore conforms to some of the classical patterns of enzyme regulation in metabolism, whereby initial (first committed) steps are inhibited by end products and where cross regulation exists between two branches of a common pathway.

Regulation of gene expression and 'general control'

Most of the genes of the tryptophan pathway and the shikimate pathway are subject to de-repression of gene expression. This phenomenon is an example of 'general control of amino acid biosynthesis' (Schurch *et al.*, 1974; Delforge *et al.*, 1975; Hinnebusch, 1988) whereby a general derepression of enzymes in amino acid biosynthetic pathways such as histidine, arginine, lysine, tryptophan, isoleucine, valine and leucine occurs in response to starvation by one, or more, amino acids. Such limitations of an amino acid can occur in bradytrophic (slow growing in absence of exogenously supplied nutrient) mutants (Niederberger *et al.*, 1981), with the use of analogues such as 5-methyl tryptophan (Schurch, *et al.*, 1974) or in media lacking amino acids. A study of the genetics of this system has yielded two classes of mutants, *gcd* (general control derepressed) and *gcn* (general control non-derepressible, formerly *aas*, or amino acid analogue sensitive). The gene product GCN4 is a transcriptional activator, required for general control. It has similarities with the human *c-jun* oncoprotein (Struhl, 1987). It has a 'leucine zipper' structure characteristic of DNA binding proteins which require dimerisation (Agre *et al.*, 1989). It activates transcription in the general control aromatic amino acid biosynthesis (Hinnebusch and Fink, 1983) which leads to an increase in the activity of enzymes of the tryptophan and shikimate pathway (Miozzari *et al.*, 1978a), amongst others. The GCN4 binding site, the GCN responsive element (GCRE) is well characterised, having a consensus sequence ATGA(C/G)TCAT, usually varying from this at one or two positions (Arndt and Fink, 1986; Oliphant *et al.*, 1989) and has been found upstream of every structural gene known to be subject to general control (Hinnebusch, 1988). These include all the genes of the tryptophan and shikimate pathways, except for *TRP1*. The

locations of GCN4 binding sites upstream of the *ARO* genes are given in Chapter 3, Table 3-1.

In contrast to the regulation of amino acid biosynthesis in bacteria, *S. cerevisiae* exhibits a significant (basal) level of expression of the biosynthetic enzymes even when amino acids are present in excess concentrations. In *ARO3*, this basal level of transcription is also, unusually, GCN4 dependent (Paravicini *et al.*, 1989a). Transcription from *TRP1* is not regulated by GCN4. Instead, a transcriptional terminator element located in the 5' untranslated region is required for *TRP1* expression. Deletion of this element results in large, poorly translated transcripts (Braus *et al.*, 1988)

The biological significance of general control has been interpreted in terms of the co-ordinate regulation of most enzymes in a pathway. Thus, a functional relationship may exist between organisation of such genes into operons in bacteria (*e.g.* the *trp* operon in *E. coli*) and general control in fungi. The extension of MCA to the treatment of large changes in enzyme activity (section 1.1.2), and specifically, the relationship between group control coefficient, enzyme multipliers and flux amplification constitutes a theoretical treatment of the functional implications of general control and operons as forms of co-ordinate regulation (Kacser and Acerenza, 1993).

1.3 Control and regulation of tryptophan pools and flux to tryptophan

This section attempts to draw together a disparate collection of data and interpretations concerning the control and regulation within the tryptophan pathway. In section 1.3.1 some data on tryptophan concentrations and flux estimations are given, along with the changes which occur in various mutant strains. In section 1.3.2 results of experiments where enzymes of the pathway are overexpressed are given. Finally, section 1.3.3 attempts to evaluate the interpretations of the data which have been offered and gives a perspective through which the present work as well as past work may be interpreted.

1.3.1 Intracellular concentrations and flux

Intracellular concentration of tryptophan

The concentration of tryptophan in wild-type cells grown on minimal media (MV) is about 20 μM (Fantes *et al.*, 1976). However, this can be modulated by a number of factors. The presence of anthranilic acid, indole or tryptophan in the medium increases this by a factor of 30, 50 and 10, respectively. Mutants with a feedback resistant anthranilate synthase (*fbr*) gave 50 - 60-fold increases in tryptophan, whilst mutants with feedback supersensitive enzyme give tryptophan pools half that of the wild-type (Fantes *et al.*, 1976). These results suggest that feedback inhibition of anthranilate synthase regulates the tryptophan pool. Mutants with defects in general control (*ndr* mutants, now known as *gcn* mutants, were selected by increased sensitivity to 5-methyl tryptophan, 5-MT) showed no significant changes in the tryptophan pool, on MV alone, but showed no de-repression of enzymes subject to general control, and reduced pool size, compared to wild-type strains, under de-repressed conditions (5-MT). Finally constitutively de-repressed mutants (*cdr*, now known as *gcd*) gave 20 - 30-fold increases in tryptophan pools (Fantes *et al.*, 1976). Miozzari *et al.* (1978a) obtained similar results for mutants with a feedback resistant anthranilate synthase (*trp2_{fbr}*) but only 2-fold increases in tryptophan pools with *gcd* mutants. Other mutations which have given increased intracellular pools include *aro7* (chorismate mutase) and *aat2* (Kradolfer *et al.*, 1982). Unfortunately, the effect on the tryptophan pool of a single point mutation which abolishes the regulatory behaviour of chorismate mutase (Schmidheini *et al.*, 1989) has not been investigated.

Flux to tryptophan

Flux to tryptophan consists of a number of components. Theoretically, the total flux to tryptophan is the sum of the flux to protein, internal pool, excretion, degradation and fluxes into other pathways, such as nicotinamide synthesis. In practice, the fluxes into other pathways are assumed to be small and, therefore, ignored. The contribution of tryptophan degradation may be highly significant, but in many investigations, including the present one, the use of *aat2* mutants is assumed to minimise this factor. Therefore, flux is operationally defined as the flux to tryptophan

in protein plus flux to intracellular and extracellular pools. The sum of the free tryptophan pools can be used to calculate the 'accumulation rate'. Therefore the total flux can be considered to equal the flux to protein plus the accumulation rate. Unless excreted tryptophan forms a significant fraction of total tryptophan, the accumulation rate is largely composed of flux to intracellular pool.

In general, the contribution of the accumulation rate to total flux to tryptophan is small (< 1%) (Miozzari *et al.*, 1978a). Flux to protein in strains which grow at wild-type rates is about $0.3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ while the accumulation rate is about $0.001 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. This means that large changes in the tryptophan pool may have quite small effects upon flux. Even a *trp2_{fbr}* mutant gave only a 50% increase in flux despite a 40-fold increase in intracellular pool. A *gcd* mutant with a *trp2_{fbr}* allele showed a 2-fold increase in flux with no further increase in intracellular pool (Miozzari *et al.*, 1978a).

1.3.2 Overexpression of enzymes of the tryptophan pathway

The results of the 'down-modulation' experiment obtained by constructing a tetraploid gene dosage series for enzymes of the tryptophan pathway (Miozzari *et al.*, 1978a; Niederberger *et al.*, 1992) were discussed in the context of an experimental justification of MCA in section 1.1.1. The control coefficients for all of the enzymes were low. This result is not exceptional and is equivalent to the finding that functional alleles are usually genetically dominant (Kacser and Burns, 1981) with respect to null alleles.

Niederberger and colleagues also conducted a series of experiments with strains overexpressing enzymes of the tryptophan pathway from multi-copy plasmids. A summary of these experiments is given in Table 1-3 (Niederberger *et al.*, 1992). Strains were grown in minimal media with added anthranilic acid. Therefore, in all cases, the feedback of anthranilic acid was by-passed. Overexpression of genes raised enzyme activities by factors of 10-60 by using high copy number plasmids. Overexpression of each gene alone does not increase tryptophan flux significantly above wild-type levels. The overexpression of some combinations of genes results in

Upmodulated genes					Relative enzyme levels				Flux to tryptophan				
					Anthranilate synthase <i>TRP2/3B</i>	PRAT <i>TRP4</i>	PRAI <i>TRP1</i>	IndGPS <i>TRP3C</i>	Tryptophan synthase <i>TRP5</i>	Trp accumula- tion	Flux to Trp in protein	Total flux to Trp	Relative flux
Wild type					1.0	1.0	1.0	1.0	1.0	0.10	0.33	0.43	1.0
<i>TRP4</i>					1.2	19.9	1.2	1.6	1.2	0.27	0.30	0.57	1.3
<i>TRP1</i>					1.5	1.2	10.6	1.6	0.7	0.20	0.30	0.50	1.3
<i>TRP3</i>					1.5	0.8	1.8	51.5	1.1	0.13	0.31	0.44	1.0
<i>TRP5</i>					1.3	0.8	1.7	1.6	37.1	0.23	0.29	0.52	1.2
<i>TRP1 TRP3</i>					1.5	0.6	58.6	56.0	0.5	0.58	0.29	0.87	2.0
<i>TRP2</i>	<i>TRP4</i>	<i>TRP1d</i>	<i>TRP3</i>		25.0	34.0	1.7	47.0	1.0	0.72	0.31	1.03	2.4
<i>TRP2f</i>		<i>TRP1</i>	<i>TRP3</i>		29.4	1.0	30.6	40.9	1.0	0.22	0.31	0.53	1.2
<i>TRP2f</i>	<i>TRP4</i>	<i>TRP1</i>	<i>TRP3</i>		26.5	26.4	30.3	37.4	0.8	0.59	0.32	0.91	2.1
<i>TRP2</i>	<i>TRP4</i>	<i>TRP1d</i>	<i>TRP3</i>	<i>TRP5</i>	20.0	15.2	1.6	20.7	18.1	3.25	0.29	3.54	8.2
<i>TRP2f</i>	<i>TRP4</i>	<i>TRP1</i>	<i>TRP3</i>	<i>TRP5</i>	24.0	19.6	22.1	26.9	23.9	3.51	0.28	3.79	8.8

Table 1-3 Changes in flux upon over-expression of genes of the tryptophan pathway (from Niederberger *et al.*, 1992)

PRAT is phosphoribosyl- anthranilate transferase, PRAI is phosphoribosyl-anthranilate isomerase, IndGP synthase is indole glycerol phosphate synthase. *TRP2fbr* refers to a mutant gene which encodes an anthranilate synthase with resistance to feedback inhibition by tryptophan. *TRP1d* refers to cloned *TRP1* gene without a fully functional promoter. It gives greatly reduced expression as shown. The experimental details, strains and plasmids are given in Niederberger *et al.* (1992) and in references contained therein.

a significant but not a dramatic increase in tryptophan flux (up to 2.4-fold). Large increases in flux (8-fold) were only achieved by overexpression of four (not *TRP 1*) or all five genes of the pathway. In all cases, the growth rate remained unchanged and, therefore, increases in tryptophan concentration accounted for the flux increases.

These results show how control is still distributed amongst four of the enzymes of the pathway, and how, despite the limited extent of the defined pathway (anthranilic acid - tryptophan), significant control lies elsewhere (*i.e.* the flux multipliers obtained, about 8, fall far short of the probable enzyme multipliers, about 25-50). The data do not tell us how control will be distributed when strains are growing on normal media. In this situation, other enzymes of metabolism are likely to share some control and the effect of inhibition of anthranilate synthase will come into play. Nevertheless, some general comments may be made for the purpose of defining the context of the present project.

They are

- control is distributed over the pathway
- up-modulation of most, if not all, enzymes is required for significant flux amplification
- although control of flux, with respect to enzymes outwith the limited pathway defined by the experimental situation, may be limited before the manipulation, it clearly becomes significant over the range of increases achieved here.

Prasad *et al* (1987) report an experiment where a strain RH1095 (*aro7, trp5, leu2, aat2*) was transformed with a multi-copy plasmid, pME554, containing a copy of all five *TRP* genes, both in the presence and absence of anthranilic acid. At least during exponential phase, flux to free tryptophan was less than $0.05 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). In the presence of anthranilic acid, flux to free tryptophan increased to between 0.5 and $3.5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. When the multi-copy plasmid, pME559, bearing a *TRP2_{br}* allele was used (in a different strain), the fluxes to free tryptophan were similar and were not greatly increased by the presence of anthranilic acid in the medium.

1.3.3 Interpreting the regulatory mechanisms

The elements which may contribute to the regulation of tryptophan biosynthesis were identified in section 1.2.6. These include feedback inhibition; two DAHP synthase isozymes are inhibited by phenylalanine and tyrosine, anthranilate synthase is inhibited by tryptophan and chorismate mutase by tyrosine. Secondly, all of the enzymes of the shikimate and tryptophan pathway, except for phosphoribosyl anthranilate isomerase (encoded by *TRP1*), are subject to the general control of amino acid biosynthesis. They are de-repressed under conditions of amino acid starvation. Finally, there are the roles of tryptophan transport and catabolism to be considered.

How do these factors contribute to the control and regulation of both intracellular concentration of tryptophan and pathway flux?

Interpretations of the regulatory behaviour of the pathway in terms of this network of feedback interactions have been advanced. For instance Jones and Fink (1982) give a qualitative account of the regulation of amino acid biosynthesis, a summary of which follows. If the intracellular concentration of tryptophan falls, then anthranilate synthase is released from feedback inhibition which redirects flux into the tryptophan pathway. At the same time, chorismate mutase will become relatively inhibited (by tyrosine in the absence of tryptophan). As tryptophan concentration increases, flux becomes redirected back to the prephenate pathway by inhibition of anthranilate and activation of chorismate mutase. Furthermore, they describe other mechanisms of regulation within the prephenate pathway. Firstly, prephenate dehydratase (leading to phenylalanine) has a higher affinity for prephenate than prephenate dehydrogenase. Secondly, prephenate dehydrogenase is modulated by tyrosine and phenylalanine in an analogous way to the regulation of chorismate mutase by tyrosine and tryptophan, respectively, and prephenate dehydratase is inhibited by phenylalanine alone. Therefore, they suggest that if phenylalanine concentrations drop, then this pool will be regulated by inhibition of prephenate dehydrogenase (by tyrosine) until phenylalanine concentrations increase sufficiently to release this inhibition.

The work of Fantès, Miozzari, Niederberger and Hutter, discussed above, leads to the following conclusions (Miozzari *et al.*, 1978a).

Flux through the tryptophan pathway is adjusted to the rate of protein synthesis by means of feedback inhibition of the first enzyme [anthranilate synthase] by [tryptophan].

Increasing or lowering the concentration of individual enzymes had no noticeable influence on the overall flux to tryptophan.

Our results [.....] suggest that de-repression does not serve as an instrument for the specific regulation of the flux through the tryptophan pathway.

In summary, the following perspectives are offered. One is that feedback inhibition of anthranilate synthase (and perhaps chorismate mutase and other enzymes) is an important regulatory mechanism whereby tryptophan concentrations (and those of phenylalanine and tyrosine) are maintained at the levels normally found within the cell. Another, borne out by the over- (and under-) expression of enzymes of the tryptophan pathway is that no single enzyme contributes significant control of tryptophan flux. Thirdly, de-repression mediated by GCN4 does not result in increases in flux, except under very low flux conditions obtained in bradytrophic mutants or by the action of tryptophan analogues. In other words, precise regulation of flux and metabolite concentrations occurs, despite low control coefficients for all enzymes of the pathway.

1.4 Aims of the project

The central task of this project is to apply the 'Universal Method' to the shikimate and tryptophan pathway and to evaluate its success.

Briefly, the plasmids pME554 or pME559 (Niederberger *et al.*, 1984; Prasad *et al.*, 1987) may be used to overexpress all of the enzymes of the tryptophan pathway.

Given values for the fluxes in the shikimate, tryptophan and prephenate sections of the pathway, it is possible to use the 'Universal Method' to calculate a factor by which the activities of enzymes of the shikimate pathway should be increased to achieve the

conditions set by the constraints of the model. These constraints are, a predicted large increase in flux to tryptophan, minimised changes in flux to phenylalanine and tyrosine and maintenance of metabolite concentrations within and outwith the pathway.

The plasmids (pME554, pME559) may be used to apply an enzyme multiplier of between 20 and 50 to the tryptophan pathway (Niederberger *et al.*, 1984; Prasad *et al.*, 1987). This factor sets the flux multiplier for flux to tryptophan and, given the wild-type flux to tryptophan, the absolute increase in flux, ΔJ . This value, ΔJ , and estimations of the flux in the prephenate pathway allows the determination of an enzyme multiplier for the shikimate pathway using Equation 9.

As will be described in Chapter 2, fluxes to tryptophan, phenylalanine and tyrosine during exponential phase may be calculated from an estimation of the intracellular concentrations within the cell, the concentration of the amino acid within protein, the growth rate and the relationship between cell volume and protein concentration, given certain simplifying assumptions. These predicted fluxes are shown in Table 1-4.

These predicted data allow the calculation of an enzyme multiplier for the shikimate pathway in order to give a flux multiplier of 25 in the tryptophan pathway, given the overexpression of tryptophan pathway enzymes by a similar factor. This enzyme multiplier is predicted to be 3.5.

The central strategy of the project is, therefore, to construct a single copy plasmid containing one copy of all four shikimate pathway genes, which can be introduced along with the multicopy plasmids pME554 or pME559. This has the theoretical consequence of introducing an enzyme multiplier of 2, close enough to the estimated required enzyme multiplier to allow a reasonable evaluation of the method. Attempts to overexpress the shikimate pathways enzymes by a factor of 3.5 precisely would have involved the introduction of slightly stronger constitutive or inducible promoters which, even if available, may have distorted the metabolic state of the strains in unpredictable ways.

Amino acid	Intracellular concentration (μM)	Accumulation rate	Protein flux	Total flux	Accumulation rate as % of total flux
		$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$			
Tryptophan	20	0.0004	0.31	0.31	0.13%
Phenylalanine	500	0.0097	1.50	1.51	0.64%
Tyrosine	600	0.0116	1.13	1.14	1.01%
Prephenate pathway (phenylalanine + tyrosine)				2.65	
Shikimate pathway (tryptophan + phenylalanine + tyrosine)				2.96	

Table 1-4 Proposed fluxes in the shikimate pathway

The data in this table are calculated from the intracellular pools previously reported (Fantes *et al.* 1976; Jones and Fink, 1982), an estimate for the intracellular volume at a given OD_{546} (de Koning and van Dam; 1992), an estimate of protein concentration at a given OD_{546} (Fantes *et al.*, 1976) and a growth rate (μ) in minimal media of 0.31 hr^{-1} , according to the methods set out in Chapter 2. It assumes no excretion of amino acids into the medium, no degradation of these amino acids, zero fluxes to other metabolites and maintenance of steady state with respect to intracellular concentrations of these amino acids and of protein composition.



The transformation of the appropriate strains with the multi-copy plasmid carrying the *TRP1-5* genes and/or single-copy plasmids carrying the *ARO1-4* genes will be followed by culture and measurement of enzyme activities and concentrations of tyrosine, phenylalanine and tryptophan. These experiments will be carried out without the use of anthranilic acid as an external source for phosphoribosyl-anthranilate transferase. It was planned to evaluate the effects of these changes in 'batch' culture initially.

Finally, the strict application of the 'Universal Method' demands that intracellular concentrations of tryptophan remain at wild-type levels. In these circumstances, an increase in flux to tryptophan can be achieved by excretion of tryptophan. That work was planned independently of this project.

1.5 Plan of the thesis

The remainder of the thesis is organised as follows. Chapter 2 describes the materials and experimental methods used in the project. Chapter 3 describes the design and construction of a yeast plasmid used to overexpress enzymes of the shikimate pathway. Chapter 4 describes the construction and validation of yeast strains used in the project, the validation of a plasmid used to overexpress enzymes of the tryptophan pathway and the measurement of enzyme activities in all transformed strains. Chapter 5 analyses the stability of both *TRP* and *ARO* plasmids in different media. Chapter 6 discusses the data relating to flux and metabolite pools obtained in transformed and untransformed strains during exponential phase of batch culture. Chapter 7 considers the dynamic changes in these variables during other phases of batch culture. Chapter 8 offers an overall evaluation of the 'Universal Method' in the context of this study and considers other approaches to the problem of over-producing aromatic amino acids in micro-organisms.

Chapter 2

Materials and methods

2.1 Bacterial strains

Various strains of *E. coli* were acquired and employed in the construction of plasmids. They are listed in Table 2-1.

Strains were grown in Luria-Bertani (LB) medium (1% tryptone, 1% NaCl, 0.5% yeast extract and, for solid media, 1.5% agar). Media were sterilised by autoclaving. Ampicillin (stock 150 mg ml⁻¹) or kanamycin (50 mg ml⁻¹) were filter sterilised and added after partial cooling, as required, to give LB-amp or LB-kan with final antibiotic concentrations of 150 µg ml⁻¹ or 50 µg ml⁻¹, respectively.

Glycerol stocks (triplicate) of host strains and transformants were made by adding 750 µl of an overnight culture to 250 µl of sterile 80% glycerol. After mixing, cells were frozen on dry ice and stored at -70°C. When required, cells were retrieved with a sterile toothpick and streaked onto solid selective media.

2.2 Yeast strains and standard media

Yeast strains used as hosts for flux analysis and for testing the functionality of cloned genes are shown in Table 2-2.

Untransformed yeast strains were grown in YEPD media (1% yeast extract, 2% bacteriological peptone, 2% glucose plus 2% agar for solid media) or in minimal medium plus supplements required for growth. Minimal medium (MV) is Yeast Nitrogen Base (without ammonium sulphate) (1.45 g l⁻¹), ammonium sulphate (5.25 g l⁻¹), glucose (20 g l⁻¹), succinic acid (10 g l⁻¹), pH to 4.0 (liquid) or 5.5 (solid media) with potassium hydroxide. Nutrients required for growth were filter sterilised and added, as required. These included adenine (20 µg ml⁻¹), uracil (20 µg ml⁻¹), tryptophan (20 µg ml⁻¹), tyrosine (30 µg ml⁻¹), phenylalanine (50 µg ml⁻¹), histidine (20 µg ml⁻¹), arginine (20 µg ml⁻¹), methionine (20 µg ml⁻¹), leucine (30 µg ml⁻¹),

Strain	Genotype	Source
CB-51	<i>dam</i> ⁻ strain	Sheila Carmichael
JM109	<i>endA1, relA1, recA1, supE44, hsdR17 (r_k⁻ m_k⁻), thi, gyrA96, Δ(lac-proAB), [F', traD36, lacI^f ZΔM15, proA⁺ B⁺]</i>	Stratagene
XL-1	<i>endA1, relA1, recA1, supE44, hsdR17 (r_k⁻ m_k⁻), thi, gyrA96, lac, [F', traD36, lacI^f ZΔM15, proA⁺ B⁺, ::Tn10(Tet^r)]</i>	Stratagene

Table 2-1 Bacterial strains used in this study

Strain	Genotype	Source or Reference
RH805	<i>aat2</i> (mating type not defined)	Kradolfer <i>et al.</i> (1982)
RH1207	<i>trp1, trp2, trp3, trp4, trp5, leu2, aat2</i> (mating type not defined)	Prasad <i>et al.</i> (1987)
HK1	<i>trp1, trp2, trp3, trp4, trp5, leu2, aat2, ura3</i> (mating type not defined)	This study
RH1220	<i>a, aro3, aro4, leu2-2</i>	G. Braus
RH1380	<i>α, aro4, ura3-52, gcn4-101</i>	G. Braus
RH1129	<i>a, aro1C, trp5, leu1, hom2, ade6, lys1, ura3, arg4-1, thr1, his6, met1, gal2</i>	G. Braus
RH1181	<i>α, aro1, trp1, ura3</i> and/or <i>ura4, his4-15, can1, ade2, kar1-1</i>	G. Braus
RH1182	<i>a, aro1, trp1, ura3</i> and/or <i>ura4, leu2, lys1^a, can1, ade2, kar1-1</i>	G. Braus
RH1610	<i>a, aro2</i>	G. Braus

Table 2-2 Yeast strains used in this study

^a Nutrient requirement for growth as determined in this work showed that this strain was not auxotrophic for lysine, but was auxotrophic for methionine (see Chapter 3).

lysine ($30 \mu\text{g ml}^{-1}$), valine ($150 \mu\text{g ml}^{-1}$) and threonine ($200 \mu\text{g ml}^{-1}$). Investigations of plasmid stability and flux analysis sometimes used media which contained different concentrations of leucine, valine, tyrosine and phenylalanine (all $100 \mu\text{g ml}^{-1}$) and tryptophan and uracil ($40 \mu\text{g ml}^{-1}$).

In this thesis, media are abbreviated as follows. For minimal media plus additives, the abbreviation is MV + XZ (concentration in $\mu\text{g ml}^{-1}$). Thus, for example, MV + LV(100) is MV medium plus leucine and valine at $100 \mu\text{g ml}^{-1}$.

Growth of strains was monitored by measuring the optical density at 546 nm (OD_{546}) using a Pye Unicam spectrophotometer (SP8-100). Where cultures had reached an OD_{546} greater than 0.8, optical densities were determined on appropriately diluted culture, with medium as diluent.

Glycerol stocks of yeast strains and transformants were prepared and maintained as for bacterial stocks.

2.3 Plasmids

A list of the plasmids employed within this study are shown in Table 2-3. Relevant data are also included on the reference card held in the end flap pocket, for convenience.

2.4 Chemicals and media

Table 2-4 gives details of the sources or suppliers of chemicals, reagents and materials.

2.5 DNA techniques

The DNA techniques used during this study were drawn from standard protocols (Sambrook *et al.*, 1989). The descriptions given here, therefore, only outline the (Sambrook *et al.*, 1989). The descriptions given here, therefore, only outline the particular method employed and emphasise important points or variations from

Plasmid		<i>ARO/TRP</i> genes	Reference
pK19	general cloning vector		Pridmore (1987)
pRS416	centromeric yeast/bacterial shuttle vector		Sikorski and Hieter (1989)
pZH2	source plasmid for <i>ARO2</i>	<i>ARO2</i>	Jones <i>et al.</i> (1991)
pME631	source plasmid for <i>ARO3</i>	<i>ARO3</i>	Teshiba <i>et al.</i> (1986)
pME1199	source plasmid for <i>ARO4</i>	<i>ARO4</i>	Kunzler <i>et al.</i> (1992)
pME173	source plasmid for <i>ARO1</i>	<i>ARO1</i>	Duncan <i>et al.</i> (1987)
The following plasmids are derived from the <i>ARS/CEN</i> single copy plasmid, pRS416			
pPH16	derived from pRS416		This study
pPH17	derived from pPH16		This study
pPH18	derived from pK19 and pZH2	<i>ARO2</i>	This study
pPH19	derived from pPH18	<i>ARO2</i>	This study
pPH20	derived from pPH29	<i>ARO2</i>	This study
pPH22	derived from pPH20 and pME631	<i>ARO2,3</i>	This study
pPH23	derived from pPH22 and pME1199	<i>ARO2,3,4</i>	This study
pPH24	derived from pK19 and pME173	<i>ARO1</i>	This study
pPH27	derived from pPH24	<i>ARO1</i>	This study
pPH28	<i>ARO</i> plasmid derived from pPH23 and pPH27	<i>ARO1,2,3,4</i>	This study
pPH29	derived from pPH17 and pPH19	<i>ARO2</i>	This study
pPH30	<i>ARO</i> plasmid derived from pPH23 and pPH27	<i>ARO1,2,3,4</i>	This study
pPH31	derived from pRS416 and pPH27	<i>ARO1</i>	This study
The following plasmids are derived from the 2μ multi-copy plasmid			
pME554	<i>TRP</i> plasmid 2 μ m multicopy	<i>TRP2,4,1d,3,5</i>	Niederberger <i>et al.</i> (1984)
pME559	<i>TRP</i> plasmid 2 μ m multicopy	<i>TRP2fbr,4,1,3,5</i>	Prasad <i>et al.</i> (1987)

Table 2-3 Details of plasmids used and constructed

Because pPH28 was used to investigate the effects of over-expression upon the production of tryptophan, phenylalanine and tyrosine in investigations described in Chapter 6 and 7, it is shown in bold. For easy reference, these data are also given on a card in the inside back cover of volume 2, along with data on the yeast strains used.

Chemical or reagent	Use	Grade	Supplier
Chemicals including amino acids (except for quantitative analysis)	General laboratory		BDH, Fluka, Fisons, Sigma, Biometra
Restriction enzymes	Molecular biology		NBI, New England Biolabs, Boehringer Mannheim
Klenow, Alkaline Phosphatase, DNA ligase, T4 polymerase	Molecular biology		NBI
Tryptone, yeast extract, bacteriological peptone, agar	Bacterial media		Oxoid
Yeast nitrogen base			Difco
Agarose			Amresco, Biometra
Glucose, ammonium sulphate, succinic acid	Yeast media		Biometra
o-phthaldialdehyde	reagent for amino acid analysis	99% purity	Sigma
boric acid, sodium hydroxide and citric acid	amino acid analysis	highest quality	BDH, Fisons
Standard amino acids (inc. chlorotyrosine)	amino acid analysis	highest quality	Sigma
NADPH, chorismic acid, shikimic acid, erythrose-4-phosphate, phosphoenolpyruvate	enzyme analysis	highest quality	Sigma
EPSP	chorismate synthase assay		gift of John Coggins
DAHP	DAHP synthase assay		gift of John Coggins

Table 2-4 Chemicals and reagents

2.5.1 DNA preparation

In order to propagate enough DNA of suitable quality for molecular cloning (mid-preps), bacterial strains containing the required plasmid were grown from a single colony overnight in 100 ml cultures of LB-amp or LB-kan. Plasmids were purified from these cultures using 'Qiagen-tips 100' (Qiagen). The manufacturer's protocols were followed. Typically, over 100 µg of DNA were recovered and dissolved at a concentration of 1 µg ml⁻¹ in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Samples were analysed on agarose gels after digestion with restriction enzymes used to distinguish between relevant plasmids.

In all cases, these preparations proved to be satisfactory for all subsequent manipulations such as restriction digestion and modification with DNA polymerase (Klenow fragment), T4 polymerase, alkaline phosphatase, ligation and transformation.

For the selection of transformants containing the required recombinant plasmid, mini-preparations (minipreps) of DNA were made from transformants for restriction analysis. DNA was prepared from 10 ml overnight cultures by the alkaline lysis procedure (Ish-Horowicz and Burke, 1981). After removing the supernatant after potassium acetate treatment, DNA was precipitated with 0.7 volumes of propan-2-ol, and washed with 70% (v/v) ethanol. The pellets were dissolved in 100 µl sterile distilled water (SDW) and RNA was digested with 1 µl of RNase A (10 mg/ml) at 37 °C for 30 minutes. DNA was recovered by phenol treatment, as follows. DNA solutions were made to 250 µl with SDW and 250 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) added. After shaking, the aqueous phase was recovered after centrifugation and traces of phenol removed with 500 µl of chloroform/isoamyl alcohol (24:1). 225 µl of aqueous phase was made to 0.3 M sodium acetate and DNA was precipitated with 2 volumes of ethanol. The pellet was disturbed and washed with 500 µl of 70% (v/v) ethanol. After centrifugation, the last traces of 70% (v/v) ethanol were carefully removed and pellets were air dried for 5 minutes. DNA was dissolved in 20 µl sterile TE buffer prior to restriction analysis, usually of 1 µl

aliquots. In all cases, DNA prepared in this way was a suitable substrate for restriction enzymes.

2.5.2 Restriction analysis and agarose gel electrophoresis

In most cases, DNA (usually 0.5 - 1.5 μg) was digested in 20 μl reactions containing appropriate buffer, and approximately 2-10 units of enzyme for 1 hour at the appropriate temperature (usually 37 °C). The buffer system was chosen from those supplied along with the enzyme, the 'universal buffer' supplied by Strategene, Promega, or 'KGB' buffer (McClelland *et al.*, 1988). These buffers were supplied or made as 10x concentrates and were appropriately diluted to 0.5x - 2x, depending on the enzyme used. Where two or more enzymes were included in a reaction, the most appropriate compromise buffer was used, or occasionally, after digestion with one enzyme, DNA was retrieved by ethanol precipitation and resuspended in different buffer before digestion with the other enzyme. Also, a non-standard incubation temperature was occasionally required (for example, *Sma*I), in which case, two separate incubations were necessary.

After digestion, reactions were quenched by the addition of 5 μl of gel loading buffer (25% Ficoll, 50 mM EDTA (pH8.0), and 0.05% bromophenol blue and/or xylene cyanol). The reactions were heated to 75 °C for 5 minutes and quenched on ice prior to loading on agarose gels.

Agarose gels were prepared from solutions of agarose in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 2 mM EDTA (pH 8.0)). The concentration of agarose depended upon the size of the distinguishing fragments. 0.7% agarose was most commonly used. Particular care was taken to avoid small particles of undissolved agarose and to ensure a homogenous gel. Ethidium bromide was added to a concentration of 0.5 $\mu\text{g ml}^{-1}$. Mini-gels containing 4 - 14 samples were run at 80 - 120 V for a period of about 20 -60 minutes, alongside DNA size markers such as lambda phage DNA digested with *Hind*III, or '1 kb ladder'. Gels were removed and bands viewed under UV light. Photographs were taken for analysis and record-keeping.

2.5.3 DNA modifications and cloning

For the construction of recombinant plasmids, at least 2 μg of plasmid DNA was restricted with appropriate enzymes. As well as the enzymes generating the required fragment, those cutting within unwanted fragments but not within the required fragment were sometimes included in order to reduce the number of unwanted ligation products and/or to avoid the purification of fragments from gels. Where necessary, and particularly where a background of non-recombinant plasmids was expected, samples were analysed by gel electrophoresis prior to subsequent steps.

The filling-in of cohesive ends was required in a number of steps. 5' - protruding ends were 'blunt-ended' with Klenow fragment in the presence of dATP, dCTP, dGTP and dTTP (dNTPs). Restriction enzymes were heat inactivated or the DNA was extracted with phenol/chloroform and precipitated prior to resuspension in reaction buffer (50 mM Tris-Cl (pH 7.5), 10 mM MgCl_2 , 1 mM DTT, 50 $\mu\text{g ml}^{-1}$ BSA). 10 units of enzyme and 33 μM dNTPs were added per μg DNA and incubated for 15 minutes at room temperature. 3' - protruding ends were 'blunt-ended' with T4 polymerase (2 units per μg DNA in 10 μl KGB buffer plus 100 μM of each dNTPs, 5 minutes at 37 C) In both cases, DNA was recovered by phenol extraction and ethanol precipitation.

When a single enzyme site was used to generate a cloning site for a donor fragment, a background of non-recombinant transformants could be avoided by dephosphorylation of 5' ends of the host plasmid after restriction digestion, using calf intestinal alkaline phosphatase (CIP). This was performed in CIP buffer (10 mM Tris-Cl (pH8.3), 1 mM ZnCl_2 , 1 mM MgCl_2) with 1 unit of enzyme/100 picomoles of 5'-protruding ends, or 1 unit/2 picomoles of 3'-protruding ends or blunt ends. The amount of phosphatase used in the reactions was sometimes increased in order to reduce background intramolecular ligation to an acceptable level. Control mock dephosphorylation reactions were always included. Reactions were incubated for 30 min at 37 °C. The reactions were stopped by the addition of 0.1 volume of 50 mM

EDTA (pH8.0). DNA was recovered prior to ligation by phenol extraction and ethanol precipitation.

DNA ligations were performed according to standard protocols. The concentration and amounts of plasmid and fragment DNAs were adjusted according to the size of the fragments, the numbers of molecular species competing for cohesive ends and the possibility of intramolecular ligation etc. As a general guide, 250 ng of DNA were used in a 10 μ l volume with vector and insert in molar ratios of 1:3. Ligation reactions were usually set up by co-precipitating DNA components by ethanol precipitation followed by resuspension in ligase buffer (supplied with DNA ligase). Where high concentrations of reacting ends were thought to be desirable, polyethyleneglycol (PEG) was added to a final concentration of 5%. Control ligation reactions always included vector alone without ligase to monitor undigested DNA, vector alone with ligase to monitor intramolecular ligations, and the required reaction containing vector, insert and ligase. When the insert was derived from another plasmid which could produce transformants too, a control including the insert alone plus ligase was used. Where dephosphorylation reactions were performed, another control was employed, where mock dephosphorylated vector was ligated. This reaction could be compared with dephosphorylated vector, allowing an evaluation of the dephosphorylation efficiency.

2.5.4 Bacterial transformation

The propagation of recombinant DNA requires its introduction into an appropriate bacterial host which has been made competent. Competent cells were prepared by the calcium chloride method of Cohen *et al.* (1972). Approximately 1 ml of a 10 ml overnight culture was transferred to 500 ml of medium and grown to an OD₅₅₀ of 0.3 - 0.5. Cultures were cooled on ice and cells pelleted (Beckmann JA-14, 2,500 rpm, 5 min, pre-cooled 0 °C). They were resuspended in 400 ml of 100 mM calcium chloride (ice-cold) and left on ice for 15 minutes. Cells were pelleted and resuspended in 9 ml calcium chloride. This was mixed with 1 ml sterile glycerol on ice and stored at -70°C in 100 μ l aliquots for use in transformation reactions.

Small aliquots (typically, 2 μ l) of ligation reactions (including controls) were incubated with an aliquot of competent cells for 45 min on ice. After 90 seconds of heat-shock at 42 °C and a further 10 minutes on ice, 1 ml LB (37 °C) was added (no antibiotic) and samples were incubated at 37 °C for 1 hour. Samples were spun briefly and supernatant replaced with 100 μ l fresh pre-warmed LB. The suspension was plated onto selective plates. Three control plates were processed. A mock transformation with no DNA was plated on both selective and non-selective plates. Furthermore an aliquot of a standard plasmid dilution (with the appropriate antibiotic resistance) was used to monitor the competence of the cells and the effectiveness of the transformation procedure.

On occasions, selection of recombinant plasmids was possible using 'blue/white' selection on media containing IPTG and X-gal. In such cases LB-amp or LB-kan plates were spread with 40 μ l of IPTG (24 mg ml⁻¹) and 40 μ l of X-Gal (20 mg ml⁻¹)

Transformants derived from the selective plate were taken and grown in 10 ml cultures of selective media, for analysis of minipreps and identification of the required recombinant plasmid. Single colonies were isolated on selective solid media for establishing glycerol stocks and propagation of midi-prep DNA for future cloning steps.

2.5.5 Yeast transformation

Some plasmids contained suitable genes for transformation of auxotrophic yeast strains to prototrophy and selection on media lacking specific nutrients. A lithium acetate procedure was used throughout. Host strains were grown with aeration in 100 ml YEPD to an OD₅₄₆ of 1-2. For generating transformants containing two plasmids ('double' transformant), it was necessary to grow the host strain, containing one plasmid ('single' transformant), on selective media in order to maintain the original plasmid. Cells were harvested and resuspended in 40 ml lithium acetate (100 mM in TE buffer) and incubated for 90 minutes at 30 °C to establish competence. After harvesting, cells were resuspended in 2 ml of 100 mM lithium acetate in TE. 100 μ l

aliquots were transformed with 5 µg of plasmid DNA and incubated at 30 °C for 10 minutes. 0.5 ml of 50% PEG (MW 4000) was added and incubated for a further 30 mins at 30 °C. The cells were subjected to heat shock (42 °C) for 5 minutes and were pelleted gently and resuspended in 200 µl of 0.8M sorbitol prior to the plating onto selective media, which was often, but not always, synthetic complete (SC) medium (Sherman, 1991). Transformants appeared within 3-5 days. These were picked onto plates for evaluation of their growth requirements and individual colonies were isolated from appropriate transformants and glycerol stocks established. Selected transformants were grown in 50 ml cultures for assay of enzyme activities. In some cases DNA was isolated for transformation of bacteria in order to rescue plasmid for analysis.

2.5.6 Plasmid rescue

When appropriate strains were transformed with plasmids containing all of the *TRP* genes and/or those containing the *ARO* genes, it was appropriate to analyse the structure of the plasmid after yeast transformation in order to validate the strain obtained. Cultures were grown in selective media and plasmid DNA extracted by the method of Strathern and Higgins (1991). Yeast cells were resuspended in TENS buffer (10 mM Tris-Cl (pH 8.0), 100mM NaCl, 1 mM EDTA, 0.1% SDS) and disrupted by vortexing with glass beads and again in the presence of phenol. After extraction with more phenol and then chloroform, total DNA was purified by extraction using 'glassmilk' (Geneclean), according to the manufacturer's instructions. DNA was precipitated with ethanol, dissolved in TE and small amounts transformed into a bacterial host for propagation, in parallel with a preparation obtained from the untransformed strain, as an appropriate control. Miniprep DNA from bacterial transformants was analysed by restriction digests to confirm the identity of plasmid(s) contained within the yeast strains. In the case of transformants containing both the multi-copy *TRP* plasmid and the single-copy *ARO* plasmid, an aliquot of DNA was additionally digested in *Bam*HI prior to the transformation of *E. coli*, in order to reduce the background of the multi-copy *TRP* plasmid and, thereby, to allow the

detection of the less abundant single-copy *ARO* plasmid, in which there is no *Bam*HI site.

2.6 Plasmid stability

To assess the loss of plasmid during long-term culture, two experiments were performed. In the first, the basic growth medium was MV + LV(100). In the second, the base medium was MV + LVFY(100). In both experiments, there were eight strain/media combinations, as follows. HK1/pME554 was grown in both selective and non-selective media (-/+ W(40)), HK1/pPH28 was similarly grown in selective and non-selective media (-/+ U(40)) and the double transformant, HK1/pME554/pPH28, was grown under four conditions, namely, non-selective conditions (+ U(40), + W(40)), selection for both plasmids (- U(40), - W(40)), and selection for one plasmid only (- U(40), + W(40)), and (+ U(40), - W(40)).

Before inoculation of flasks containing the above media, yeast strains containing one or two plasmids were grown from a fresh colony in 10 ml MV medium with leucine, valine, phenylalanine and tyrosine (all at 100 $\mu\text{g ml}^{-1}$) (LVFY(100)). Uracil and/or tryptophan (both at 40 $\mu\text{g ml}^{-1}$) were added if required for growth of the strain.

These were subcultured at least once (when $\text{OD}_{546} < 1.5$) into similar media. Prior to inoculation of 50 ml flasks containing selective and non-selective media, samples were taken for estimation of enzyme activities (see section 2.10) and dilutions were made in LVFY(100) for spreading onto selective and non-selective plates. For inoculation of main cultures, cells were pelleted, washed and resuspended in MV + LV(100) or LVFY(100). Pre-warmed and aerated flasks, containing 50 ml of medium were inoculated with a suitable numbers of cells. Amounts of inoculum were calculated to keep growth in exponential phase ($\text{OD}_{546} < 1.5$). After 24 hours, appropriate dilutions were made for plating and counting of cells, samples were processed to measure enzyme activities and a suitable inoculum was prepared and delivered to another aerated and pre-warmed flask containing similar medium. This process was repeated daily for a total of 5 days culture.

To determine the loss of plasmid and the appearance of auxotrophy, cells were plated daily on selective and non-selective media. Dilutions were calculated to produce between 200 and 500 colonies on the non-selective plates. Plates used were LVFY(100), LVFY(100) + U (40), LVFY(100) + W (40) and LVFY(100) + U,W (40). Plates were spread in triplicate using 100 μ l of dilutions and incubated for 3 days at 30°C. After counting the numbers of colonies, the means of triplicate plates were expressed as a percentage of the means of the counts obtained from the LVFY + U,W plates.

The mitotic instability (η , fractional loss of prototrophy per generation) may be calculated as follows.

$$\eta = \frac{10^{m \mu / \ln 2}}{100}$$

% prototrophy is plotted logarithmically (base10) against time. The gradient of this line (m) may be divided by the doubling time ($\ln 2 / \mu$).

2.7 Enzyme activity and protein determination

Enzyme assays and protein determinations were carried out on permeabilised cell preparations as described by Miozzari *et al.* (1978b). These were prepared from cultures as follows. Appropriate volumes of culture (< 50 ml) were cooled on ice for 20 minutes and cells pelleted in a bench top centrifuge (2,500 rpm, 4 min). The supernatant was discarded (or sampled for amino acid analysis) and cells washed in 15 - 50 ml ice-cold SDW. After centrifugation, the cells were washed again in 15 ml ice-cold SDW and pelleted once more. These pellets were drained thoroughly. The following steps were carried out on ice using ice-cold reagents. Thorough mixing and agitation were carried out in order to ensure a homogenous cell suspension at all times. Appropriate volumes of cell suspension buffer (100 mM potassium phosphate, pH 7.6, 2.1 mM β -mercaptoethanol and 1.2 mM phenylmethylsulphonyl fluoride) were added. The cells were resuspended and the volume measured. Further volumes of cell suspension buffer were mixed with the appropriate volumes of 1% Triton X-

100 (in cell suspension buffer) in order to standardise the concentration of protein and to maintain Triton X-100 at a concentration of 0.05%. The formulae used to calculate the final volumes of suspension and 1% Triton to be added were

$$\begin{aligned}\text{Vol of cell suspension buffer} &= \text{Vol of culture taken} \times \text{OD}_{546} \times 0.019 \\ \text{Vol of 1\% Triton X-100 added} &= \text{Vol of culture taken} \times \text{OD}_{546} \times 0.001\end{aligned}$$

This procedure was adopted to ensure that an accurate and reliable concentration of Triton X-100 was achieved. The cell suspension and extra buffer were then thoroughly mixed, dispensed into aliquots of appropriate volumes for protein analysis and enzyme assays, placed on dry ice and stored at -70 °C until required. Before analysis, the samples were thawed on ice for at least 2 hours prior to assay, in order to ensure proper permeabilisation which occurs during the thawing process.

2.7.1 DAHP synthase assay

DAHP synthase catalyses the reaction between erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to yield DAHP, which is measured according to the method of Gollub *et al.* (1970). The assay mixture (total volume 200 μ l) contained 20 μ l of 25 mM E4P, 40 μ l of 10 mM PEP, 100 μ l 100 mM bis-tris propane (pH7.4) and the reaction initiated by the addition of 10 μ l of permeabilised cell suspension. The reaction was allowed to proceed for 5 minutes at 37 °C. 40 μ l of 20% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. The stopped assay was incubated on ice for 5 mins and spun in a microfuge for 2 mins. 75 μ l of supernatant was removed and 75 μ l of sodium periodate (200 mM in 9 M phosphoric acid) was added. This was incubated at 37 °C for 10 minutes. 150 μ l of 800 mM sodium arsenite (in 0.5 M Na₂SO₄, 50 mM H₂SO₄) were added, mixed and incubated for 5 minutes at room temperature. 600 μ l of 80 mM thiobarbituric acid (in 500 mM Na₂SO₄) were added, mixed and incubated in a boiling water bath for 10 mins. The mixture was cooled to 55 °C and 1 ml of cyclohexanol was added, mixed and allowed to stand for 2 minutes at 55 °C. The optical density of the organic layer was measured in a spectrophotometer at 549 nm within a cuvette holder at 55 °C. A calibration curve using known concentrations of DAHP (gift from John Coggins and

Chris Abel, University of Glasgow) was obtained which allowed the determinations of DAHP concentrations in samples. Control incubations included the stopping of reactions at t=0 and the use of null mutants of *ARO3* and/or *ARO4*.

The specific estimation of phenylalanine inhibitable DAHP synthase and of tyrosine inhibitable DAHP synthase was achieved by adding tyrosine or phenylalanine to the reaction mixtures at concentrations of 1mM or 5 mM, respectively (Teshiba *et al.*, 1986). Concentrations of phenylalanine and tyrosine which gave complete but specific inhibition of one activity were sought unsuccessfully. Generally these two activities did not summate to the total (uninhibited) enzyme activity and the conditions given above represent a reasonable compromise.

Specific activities were calculated according to the formula

$$\text{Specific activity}_{\text{DAHPs}} = \frac{\text{OD}_{549}}{\text{grad}} \times \frac{1000}{v} \times \frac{1}{10} \times \frac{1}{\text{prot}}$$

where 'grad' represents the gradient of the standard curve for OD₅₄₉ against amount of DAHP (nmoles) in the assay mixture, 'v' is the volume (μl) of cell suspension used in the assay and 'prot' represents the protein concentration in the undiluted cell suspension in mg ml⁻¹. The average of triplicate determinations was used.

Experimental reproducibility was poor, with variability between similar samples of up to 30%.

2.7.2 Shikimate dehydrogenase activity

Dehydroshikimate is reduced to shikimate by the action of SDH. The activity is assayed in the reverse direction to that occurring in the cell, using NADP as the electron acceptor. SDH activity was simply determined in a 1 ml reaction volume in a cuvette containing 200 μl of 500 mM sodium carbonate (pH 10.6), 100 μl of 40 mM shikimic acid, 100 μl of 20 mM NADP and SDW to the final volume. The reaction was initiated with the addition of permeabilised cell suspension (usually 10 or 20 μl). Because temperature control proved to be important, the buffer and SDW were prewarmed to 32 °C and the reaction cuvette was placed in a temperature controlled holder round which water was supplied at 32 °C, within the spectrophotometer. The

appearance of NADPH was followed by measuring the increase in absorbance at 340 nm using a chart recorder. A straight line was drawn tangentially through at the starting point to represent the initial velocity and the specific activity was calculated according to the following formula.

$$\text{Specific activity}_{\text{sdh}} = \frac{a_2 - a_1}{t} \times \frac{\text{aufs}}{v} \times \frac{10^6}{6.2} \times \frac{1}{\text{prot}}$$

where a_1 and a_2 represent the absorbance units indicated on the straight line at an interval of t minutes, aufs indicates the absorbance units at full scale deflection, v defined the volume (μl) of enzyme suspension added to the reaction and prot indicates the protein concentration of the suspension in mg ml^{-1} . The formula was derived using the value given for the molar extinction coefficient for NADPH. Triplicate samples gave enzyme activities which varied by up to 10%

2.7.3 Chorismate synthase activity

Chorismate synthase stop assays were performed based upon the methods of Jones *et al.* (1991) where the appearance of chorismate is measured spectrophotometrically. A reaction mixture (final volume 200 μl) containing 100 mM potassium phosphate (pH7.6), 500 μM NADPH, 10 μM FMN, 100 μM EPSP (added to initiate the reaction) and between 10 and 50 μl of permeabilised cell suspension (depending on the specific activity). The reaction was carried out at 30°C, stopped by the addition of 40 μl of 1 M HCl. The mixture was centrifuged for 2 minutes and the supernatant removed from the cell debris, made up to 1 ml with water and the amount of chorismate was monitored spectrophotometrically at 275 nm (extinction coefficient of 263 $\text{M}^{-1} \text{cm}^{-1}$) after subtraction of negative control assay using the *aro2* mutant, RH1610.

$$\text{Specific activity}_{\text{cs}} = \frac{A}{283} \times \frac{10^3}{v} \times \frac{10^6}{10} \times \frac{1}{\text{prot}}$$

The specific activity is expressed in units of $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ where A represents the absorbance measured, ' v ' is the volume (μl) of cells used in the assay and 'prot' is the concentration of protein in the cells (mg ml^{-1})

2.7.4 Anthranilate synthase activity

Anthranilate synthase activity was assayed according to the method of Egan and Gibson (1970). Chorismic acid is converted by anthranilate synthase to anthranilic acid in the presence of glutamine and magnesium ions. Anthranilic acid may be sensitively quantitated by fluorescence spectroscopy. A 3 ml reaction volume in a fluorescence cuvette contains 1.5 ml PK buffer (200 mM potassium phosphate, pH7.6), 0.58 ml PG buffer (200 mM potassium phosphate, pH7.6, 15% glycerol), 0.24 ml 250 mM glutamine, 0.06 ml 250 mM magnesium sulphate and 0.6 ml 50 mM chorismic acid. The PK and PG buffers, glutamine and magnesium sulphate were pre-warmed to 33 °C while the chorismic acid was maintained on ice. The reaction was initiated by the addition of 20 µl of permeabilised cell suspension and the cuvette was placed in a heated holder. The fluorescent emission at 408 nm (excitation 340 nm) was monitored by chart recorder. A straight line tangential to the initial increase in fluorescence was used to estimate rate of fluorescence change. The fluorescence of standard concentrations of anthranilic acid under assay conditions was used to obtain a calibration curve relating anthranilic acid concentration to fluorescence change.

The specific activity within the enzyme sample is calculated from the following expression

$$\text{specific activity}_{AS} = \frac{f_2 - f_1}{\text{grad}} \times \frac{Vfs}{t} \times \frac{1000}{v} \times \frac{3}{\text{prot}}$$

where f_1, f_2 represent the units of fluorescence from the chart recorder, Vfs is the fluorimeter output voltage giving full scale deflection, ' v ' the volume of permeabilised cells used in the assay, ' prot ' the protein concentration of the permeabilised cells in mg ml^{-1} and ' grad ' the gradient of the calibration curve ($\text{output volts nm}^{-1}$). Triplicate determinations rarely gave a variation greater than 10%.

2.7.5 Tryptophan synthase activity

The tryptophan synthase activity was estimated using a stop assay (Yanofsky, 1955). The full reaction catalysed by the enzyme is the conversion of indolyl

glycerophosphate into tryptophan with indole produced as an intermediate. Here, only the second reaction was assayed and the disappearance of indole over a fixed time period was measured. The volume of cells used in the assay was calculated in order to produce a fairly small change in indole concentration. In this way, any inaccuracies incurred as a result of a non-linear decrease in indole over time, were minimised.

The reaction mixtures were composed of 250 μ l of 4 mM indole (all reagents dissolved in PK buffer (200 mM potassium phosphate, pH7.6), 1.25 ml of 120 mM serine, 250 μ l of 15 mM pyridoxal-5-phosphate, 250 μ l of 100 mM EDTA. The reaction was initiated by the addition of 500 μ l of appropriately diluted cells. At two time points (0 and 20 minutes), 500 μ l of reaction mixture was aliquoted into 100 μ l of 5% sodium hydroxide and indole was extracted by the addition of 2 ml of toluene. After vortexing for 30 seconds, 500 μ l of upper (toluene) layer was removed and placed on ice. Indole concentrations were measured by the addition of 1 ml ethanol and 2 ml of Ehrlich's reagent (prepared by dissolving 36 g of *p*-dimethylamino-benzaldehyde in 500 ml of ethanol, followed by the addition of 180 ml of concentrated HCl and adjustment to 1 litre with ethanol). Colour development occurred over 60 minutes and was estimated spectrophotometrically at 570 nm. A series of standard indole solutions were used to obtain a calibration curve under assay conditions. Triplicated assays gave a variation of less than 20%.

2.7.6 Protein determination

Protein determinations were carried out according to the method of Herbert *et al.* (1971). 100 μ l of a permeabilised cell preparation were diluted to 500 μ l with 100 mM potassium phosphate, 0.05% Triton X-100, pH 7.6. 250 μ l of 3M NaOH was added, mixed and placed in boiling water bath for 5 minutes. The tubes were removed and cooled for 5 minutes. 250 μ l of 2.5% copper (II) sulphate was added, mixed and incubated at room temperature for 5 minutes. The tubes were centrifuged for 2 minutes and the OD₅₅₅ of the supernatant determined against a sample containing no protein as 'blank'. A calibration graph was generated using a set of

samples containing known concentrations of bovine serum albumin as standard. The gradient of the linear portion of this graph was determined and was used to determine protein concentrations from the OD₅₅₅. Samples were performed in duplicate and averages taken. Duplicates were usually within 5%.

2.8 Analysis of flux to tryptophan, phenylalanine and tyrosine

2.8.1 Culture methods and sample collection

Cultures were generated from a freshly grown single colony in a 10 ml starter culture. The medium used was similar to that used in the final large-scale culture, except for those strains harbouring the multi-copy plasmid, pME554, where phenylalanine and tyrosine were added at 100 µg ml⁻¹. These starter cultures were sub-cultured at least once prior to the inoculation of pre-warmed and pre-aerated 100 - 200 ml pre-cultures containing identical medium to that of the final cultures. These were grown (usually overnight) until the OD₅₄₆ reached about 1. Cells from these cultures were then pelleted by centrifugation and used to inoculate pre-warmed and pre-aerated main (200-400 ml) cultures to an OD₅₄₆ of between 0.1 and 0.5. In this way two conditions were satisfied. Firstly, the cells were adapted to their final medium. Secondly, the starter cultures used medium (MV+LVFY (100)) which was thought most likely to reduce the loss of plasmid (Prasad *et al.*, 1987).

Cultures were monitored for OD₅₄₆ throughout the growth phase and samples taken at appropriate times for generation of permeabilised cells for enzyme assays, for protein determination and for analysis of phenylalanine, tyrosine and tryptophan concentrations, as follows.

For the analysis of the amounts of intracellular amino acids, a measured volume of culture (10 - 50 ml) was vacuum filtered through 4 cm Millipore HA (4 µm pore size) filters and cells washed in 2 x 15 ml aliquots of ice-cold water. The filters were rapidly removed and placed into 1.35 ml of ice-cold TCA (6.67%) for rapid disruption of cells and cessation of metabolism. After thorough agitation and a 30 min

incubation, the liquid was removed to a labelled sample tube and stored at -20°C . For analysis of the amount of amino acids in the culture (supernatant + intracellular), $900\ \mu\text{l}$ of culture was dispensed into pre-cooled tubes containing $300\ \mu\text{l}$ of TCA (20%) and mixed. They were also incubated on ice for 30 mins before storage at -20°C . Supernatant samples (extracellular pools) were treated in a similar way to those of the whole culture, except that supernatants were removed from centrifuged culture and further centrifuged (2 min, $\times 16,000\text{g}$) prior to TCA treatment.

Before estimation of amino acid concentrations, samples were centrifuged at 4°C for 5 minutes and supernatants removed into new tubes. This was repeated twice.

2.8.2 Analysis of amino acid content

Samples were analysed by chromatography (involving ion-exchange and reverse phase components) using a sulphonated polystyrene solid phase ($8\ \mu\text{m}$ bead size; The Locarte company, London, UK) and citrate buffer in the mobile phase. The flow rate was $1.4\ \text{ml}\ \text{min}^{-1}$. Amino acids were derivatised by post-column reaction with *o*-phthaldialdehyde (OPA) reagent in borate buffer and detected by on-line fluorescence at $440\ \text{nm}$.

For analysis of all samples, the running buffer was citric acid ($20\ \text{g}\ \text{l}^{-1}$), sodium hydroxide ($9.4\ \text{g}\ \text{l}^{-1}$) and sodium chloride ($2.92\ \text{g}\ \text{l}^{-1}$), pH 5.67. Buffer was filtered ($0.4\ \mu\text{m}$) under partial vacuum. The reagent buffer was $0.4\ \text{M}$ sodium borate, pH 10.5. After thorough saturation with nitrogen, OPA ($12.5\ \text{mg}\ \text{ml}^{-1}$ in absolute ethanol) was added to a final concentration of $125\ \text{mg}\ \text{l}^{-1}$. The solution was sparged with more nitrogen before the addition of $250\ \mu\text{l}\ \text{l}^{-1}$ of β -mercaptoethanol.

For all determinations, the sample was prepared in a final volume of $300\ \mu\text{l}$, including a suitably diluted sample in the running buffer plus a standard addition of chloro-tyrosine which acted as an internal standard. The actual sample volume passed onto the column (loop volume) was $150\ \mu\text{l}$. After elution of amino acids, the column was washed with $0.4\ \text{M}$ sodium hydroxide (22 min) and equilibrated with buffer (29 min) prior to the loading of another sample. Occasionally during analysis, and particularly

for calibration purposes, known concentrations of tryptophan, phenylalanine or tyrosine were included.

The elution times for tyrosine, phenylalanine, chloro-tyrosine and tryptophan were 23, 27, 37 and 67 mins, respectively.

Amino acid concentrations were estimated from peaks obtained as follows. A baseline was drawn across the bottom of the peak and the peak height measured in mm. The width of the peak at half peak height was estimated. In most cases, repeated measurements of the same small peak was +/- 0.3 mm. The height and width were multiplied to give an area indicator (loosely called 'area'). The area was divided by the area of the chloro-tyrosine peak to give a relative area. In order to generate a concentration for each amino acid within the sample, the relative area was divided by factors specific for each amino acid, derived empirically, by calibration. The concentration within the culture could then be calculated from the volume of culture used to generate the sample and the dilution used to form the injected sample. This means that internal pools are expressed as if they were diluted throughout the culture volume. They may be subtracted from the 'total' culture concentrations to give an estimate of the concentration within the supernatant. These values generally gave reasonable agreement with the empirical determination of supernatant concentrations.

The estimation of the actual intracellular concentration is based upon the relationship between cellular volume and protein mass ($3.75 \mu\text{l} \cdot \text{mg protein}^{-1}$) (de Koning and van Dam, 1992) and the relationship between protein concentration and OD_{546} (160 mg l^{-1} at $\text{OD}_{546} = 1$) obtained in this study, thus -

$$\text{Intracellular conc.} = \frac{\text{Culture conc.}}{\text{OD}} \times 1667$$

These values are also used in this thesis, where defined, in order to monitor intracellular concentrations during culture of different strains.

Terminology of metabolite pools

In discussion of the various metabolite pools, the following terminology is used throughout the rest of the thesis. Intracellular concentrations with respect to culture

volume is referred to as an 'internal pool' and is expressed in μM ($\mu\text{moles of amino acid/litre of culture}$) while the corresponding value with respect to cell volume is termed 'intracellular concentration' (μM , or $\text{pmoles of amino acid}/\mu\text{litre of cell volume}$). Extracellular and total concentrations are also termed extracellular and total pools (extracellular pool + internal pool), respectively. They are expressed with reference to culture volume. The flux to total pool is termed 'flux to free metabolite' or, 'accumulation rate'.

2.8.3 Flux calculations

Flux to tryptophan, phenylalanine and tyrosine was calculated by adding the accumulation rate and the flux to protein. This assumes that no degradation of tryptophan occurs, and ignores all other fluxes from tryptophan.

Flux to protein may be calculated from the composition of yeast protein and the growth rate, assuming steady state. The values used for the tryptophan, phenylalanine and tyrosine content of yeast protein were 1.22, 4.74 and 3.96% by weight (Cortassa *et al.*, 1995). These yield values of 59.75, 288.7 and 218.5 $\text{nmol} \cdot \text{mg protein}^{-1}$, respectively. These may be converted into flux by multiplying by the growth rate (μ) and dividing by 60 to give the flux per minute.

Accumulation rates are calculated from the metabolite pools (c) at a given OD_{546} as follows.

$$\text{Accumulation rate} = \frac{c \times \mu}{60} \times \frac{1000}{\text{prot}}$$

where c represents the concentration of metabolite pool (intracellular + extracellular) in μM (with respect to culture), μ represents the growth rate (hr^{-1}) and 'prot' represents the protein concentration in mg l^{-1} . The protein concentration was assumed to equal $160 \times \text{OD}_{546}$. The accumulation rate is expressed in $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

Chapter 3

Plasmid and strain construction

The requirements of the 'universal method' predict that in order to generate a given change in flux to tryptophan, the enzymes of the shikimate pathway should be overexpressed by a factor which depends upon the relative fluxes to tryptophan and prephenate (tyrosine + phenylalanine) as well as the factor by which enzymes of the tryptophan pathway should be overexpressed. Estimates made in Chapter 1 (section 1.4) suggest that with enzymes of the tryptophan pathway overexpressed from a multi-copy plasmid by a factor of 25, shikimate pathway enzymes might have to be overexpressed by a factor of about 3 - 4.

Given these requirements, this chapter describes the cloning strategy used to construct a plasmid bearing a copy of each of the genes (*ARO1-4*) encoding enzymes of the shikimate pathway, and reports upon its implementation. It describes the strains of yeast acquired for functional testing of cloned genes, the verification of their genotype and the results of their transformation with the plasmids constructed.

3.1 Plasmid construction

The options available for the introduction of *ARO* genes into the parent strain include integration into the host genome, the use of single or multi-copy plasmids or employing yeast artificial chromosomes (YACs). Because the 'universal method' demands that enzymes of the shikimate pathway should be overexpressed by a small factor (relative to the enzyme multiplier of the tryptophan pathway), centromeric plasmids or yeast artificial chromosomes (YACs) were judged to be most appropriate. YACs require special techniques and need very large inserts (> 100kb) for stability while stable centromeric plasmids may be smaller, are easily transferable from *E. coli* to yeast and back and allow simple manipulation and analysis. For these reasons, the pRS413-6 series of centromeric plasmids (Sikorski and Hieter, 1989) was chosen as suitable vectors. They contain autonomous replicating sequences (*ARS*) and centromeric (*CEN*) sequences, which confer the properties of autonomous replication

and stable segregation at mitosis in yeast, and feature a comprehensive multiple cloning site (MCS) for ease of cloning fragments, blue/white colour selection for identification of recombinant transformants and a high copy number in *E. coli*. Four different vectors are available with different selectable markers in yeast; *HIS3*, *TRP1*, *LEU2* and *URA3* (pRS413-6, respectively). Finally, because they each have a corresponding vector which lacks the *ARS/CEN* sequences, integration of cloned sequences into the chromosome is possible, if desired.

Some of the general features of the four genes, *ARO1-4* were discussed in Chapter 1. The sequences of the genes were mapped and analysed using the University of Wisconsin GCG package (UWGCG). This information was used both to design possible cloning strategies and to identify restriction digests which could be used to validate the source plasmids containing fragments of choice.

3.1.1 Planning the strategy

The final cloning strategy adopted was developed to fulfil the following criteria.

- Fragments containing genes were chosen from the source plasmids so that all likely upstream promoter sequences (*i.e.* GCRC and TATA sequences) were included.
- Similarly, downstream sequences thought to play a role in transcriptional termination and mRNA 3'-end formation (Zaret and Sherman, 1982; Henikoff and Cohen, 1984; Guo and Sherman, 1996) were included.
- The final plasmid should contain a 'cassette' containing all 4 genes which could, in principle, be transferred to other vectors with ease, using restriction enzymes which only cut at one site.
- It was anticipated that control of flux by each of the shikimate pathway enzymes could be investigated if it were possible to 'disrupt' each of the additional plasmid-borne genes easily and selectively. This is possible if the coding regions may be cut at a unique site in the final plasmid, followed by 'blunt-ending' and

ligation. This may require the destruction of some restriction sites in plasmid or cloned genes during construction.

- The order in which genes were introduced into the plasmid added an additional constraint. Monitoring the functionality of genes by transforming specific mutants requires that *ARO3* should be cloned before *ARO4*. The former relies upon GCN4 for basal transcription. It should therefore be possible to evaluate expression of the *ARO4* gene in an *aro4*, *gcn4* host in the presence of an *ARO3* gene, whilst the reverse is not possible.
- The cloning steps were designed to be as rapid, simple and cheap as possible by avoiding partial digestions, filling in of cohesive ends, intermolecular blunt-ended ligations and isolation of bands on agarose gels and by using directional cloning where possible.
- The sizes of fragments containing genomic *ARO* gene sequences were kept to a minimum given the fulfilment of the first two criteria above.

The source and extent of the genomic sequences used in the construction of the final plasmids are shown in Table 3-1, while the final strategy adopted is illustrated in Figure 3-1. Maps of source plasmids and the cloning vectors used are given in Figures 3-2 - 3.7. They show relevant features such as restriction sites and coding regions of genes. Some selective disruptions to restriction sites within the yeast vector, pRS416, and upstream of the *ARO2* gene were necessary. Also, some preliminary manipulations involving the fragment containing the *ARO1* gene were necessary for its incorporation into the final plasmids, pPH28 and pPH30. These two plasmids contain all four *ARO* genes on fragments which should have all sequence features required for 'wild-type' expression. A map of pPH28 is given in Figure 3-8. The plasmids should be stable when maintained in media without uracil. The plasmids will allow transfer of a 12.5 kb *XhoI/NotI* '*ARO* gene cassette' from the *URA3* plasmid pRS416 to the homologous centromeric plasmids pRS413-5 if a different selectable marker is required, or to pRS403-6 for integration of sequences into the chromosome, if necessary. Moreover the final plasmid can be used to generate any

Gene	Gene product	DNA sequence accession number	Coding sequence length (bp) ^a	Source plasmid ^b	Amount of DNA cloned (bp)	5'-UT sequence used (bp) ^c	3'-UT sequence used (bp) ^c	Promoter sites data ^d	Reference
<i>ARO3</i>	DAHP synthase inhibited by phenylalanine	X13514	1113	pME631	1720	232	375	GCREs: -180 - -188 (-ve orientation) TATA sites: -210 - -207, -156 - -151, -138 - -134, -88 - -85, -51 - -48, -45 - -42, -9 - -6	Paravicini et al. (1988)
<i>ARO4</i>	DAHP synthase tyrosine inhibition	X61107	1113	pME1199	1903	546	244	GCREs: -312 - -304	Kunzler et al (1992)
<i>ARO1C, A, B, E, D</i>	AROM complex	X13802 X13803 X06077 Y00313	4767	pME173	6489	912	810	GCREs: -531 - -523, -410 - -402, -224 - -216, -196 - -188 TATA sites: -138 - -132	Duncan et al. (1987, 1988)
<i>ARO1C</i>	DHQ synthase								
<i>ARO1E</i>	3-dehydroquinase								
<i>ARO1D</i>	SDH								
<i>ARO1B</i>	Shikimate kinase								
<i>ARO1A</i>	EPSP synthase								
<i>ARO2</i>	Chorismate synthase	X60190	1131	pZH2	1742	389	226	GCREs: -326 - -318, -218 - -210, -143 - -135 TATA sites: -105 - -100, -87 - -82	Jones et al. (1991)

Table 3-1 *ARO* genes encoding enzymes of the shikimate pathway

The table shows some details of the *ARO* genes which are cloned into the same plasmid in this study. ^a The coding sequence length includes the termination codon. ^b The source plasmid indicates the plasmid used to obtain the cloned fragment. ^c The number of bases from the 5'- and 3'-UT regions cloned into the final plasmid, pPH28, are shown. ^d Those sites suspected or, in some cases, known to participate in the transcription of genes at basal levels (TATA) and during activation by the transcriptional activator *GCN4* (GCREs), are shown.

Figure 3-1 Cloning strategy

This diagram summarises the cloning steps planned to create pPH28 and pPH30, each bearing a copy of the four *ARO* genes. Reference may also be made to the Plasmid/Strain Card contained in the back pocket.

Briefly, pZH2 contains *ARO2* which is incorporated (*via* pK19) into pRS416 (modified) to give pPH29. *ARO3* and *ARO4*, derived from pME631 and pME1199 respectively, are then introduced sequentially to give pPH22 and pPH23. *ARO1*, derived from pME173 is incorporated (*via* pK19) to give pPH28 and pPH30 (different orientations of *ARO1*).

Figure 3-2 pME173, the *ARO1* source plasmid

BamHI sites destroyed in previous cloning steps are shown in brackets. The cloning vector is pAT153. The *ARO1* fragment is indicated (white box). Both the ampicillin resistance gene (*amp^r*) and the *ARO1* coding region are shown as black arrows.

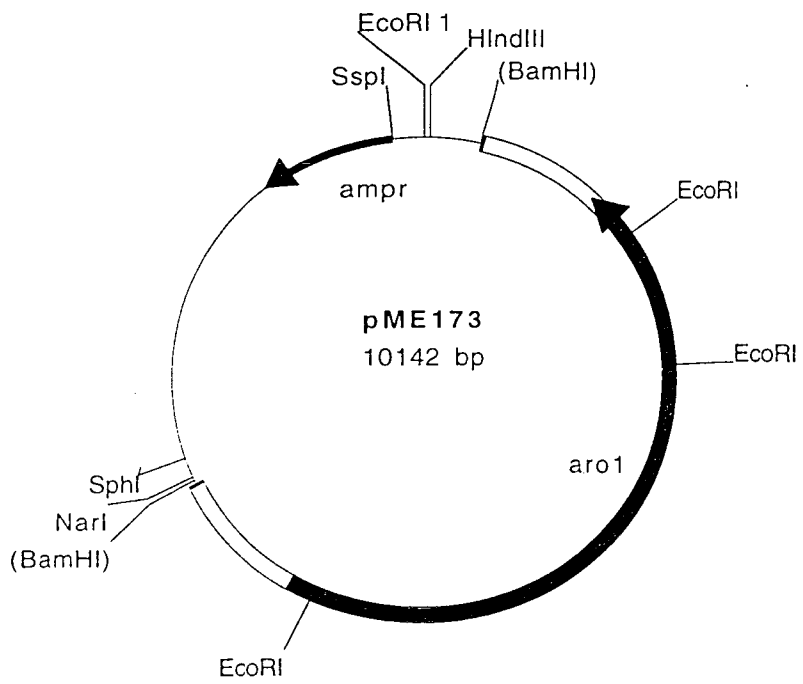


Figure 3-3 pZH2, the *ARO2* source plasmid

BamHI sites destroyed in previous cloning steps are shown in brackets. The cloning vector is YEp13. The *ARO2* fragment is indicated (white box). Both the ampicillin resistance gene (*amp^r*), the yeast selectable marker, *LEU2*, and the *ARO2* coding region are shown as black arrows.

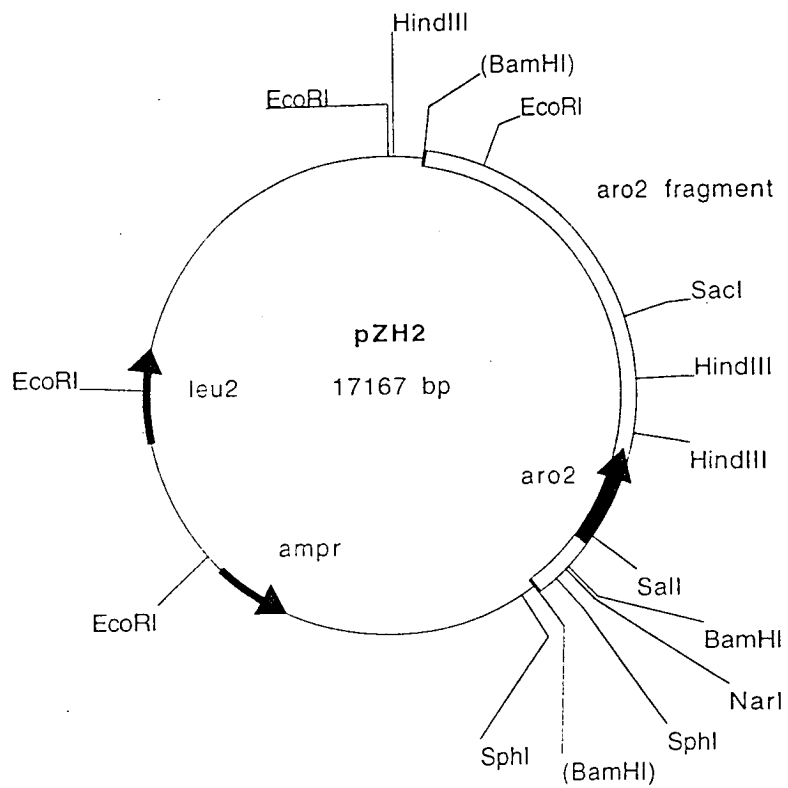


Figure 3-4 pME631, the *ARO3* source plasmid

The cloning vector is pUC18. The *ARO3* fragment is indicated (white box). Both the ampicillin resistance gene (*amp^r*) and the *ARO3* coding region are shown as black arrows.

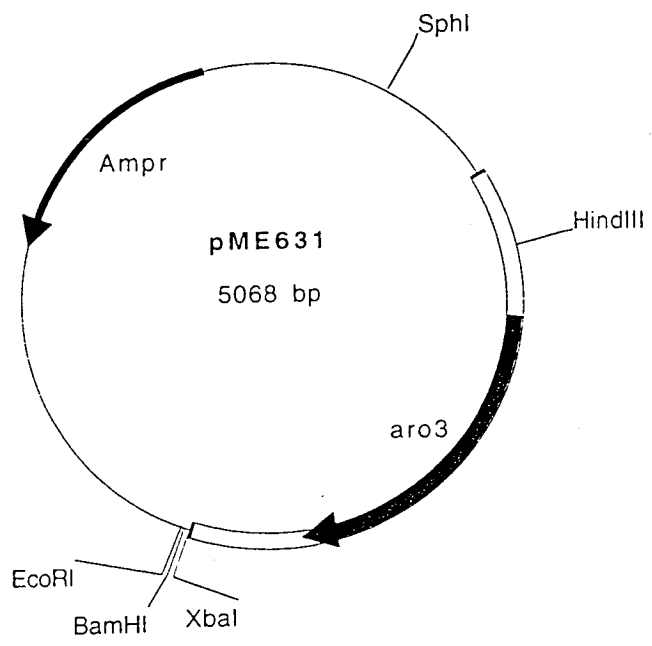


Figure 3-5 **pME1199, the *ARO4* source plasmid**

The cloning vector is pGEM-7Zf(+). The *ARO4* fragment is indicated (grey box). Both the ampicillin resistance gene (*amp^r*) and the *ARO4* coding region are shown as black arrows.

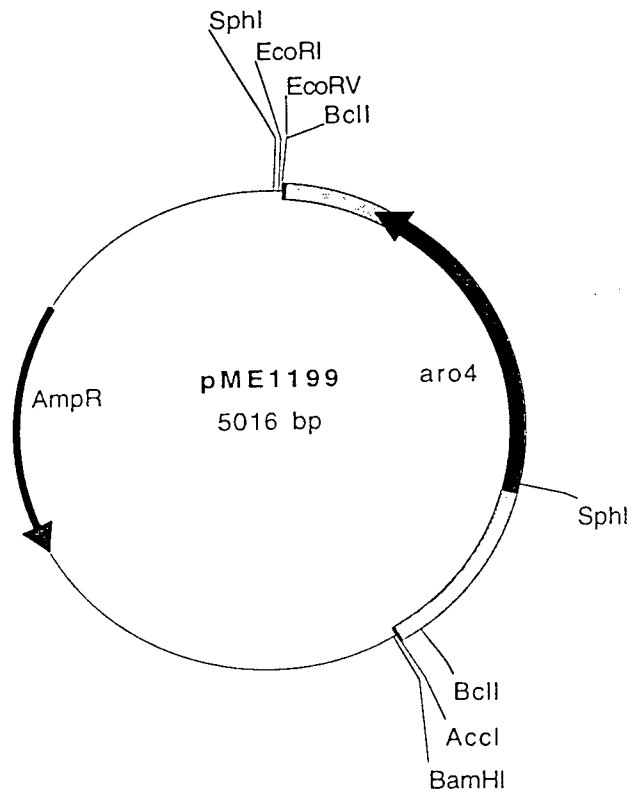


Figure 3-6 pK19 - general bacterial cloning vector

The kanamycin resistance gene is shown as a black arrow.

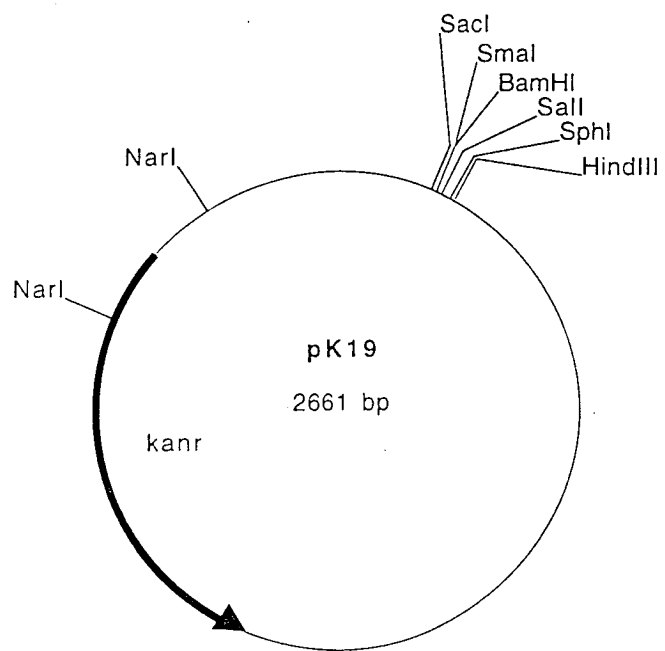


Figure 3-7 pRS416, a single-copy yeast plasmid

This plasmid contains autonomously replicating and yeast centromeric and autonomously replicating sequences (hashed box). Both yeast (*URA3*) and bacterial (ampicillin resistance) selectable markers are shown as black arrows.

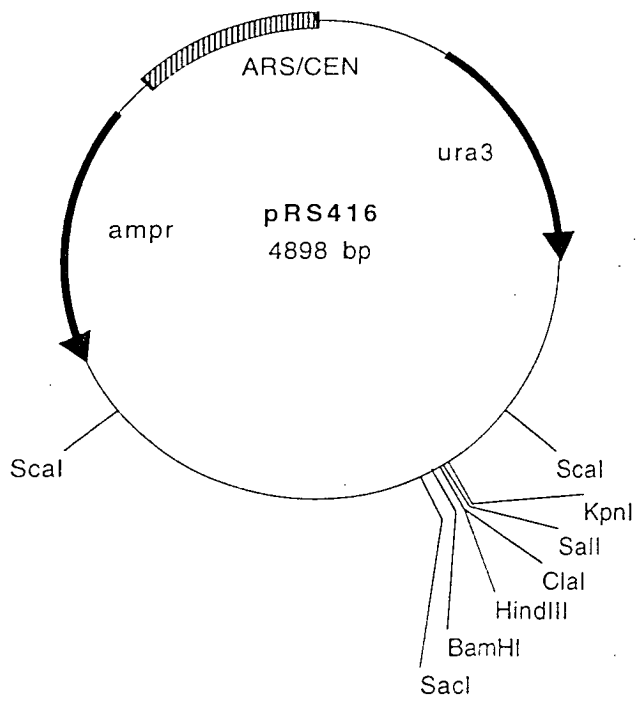
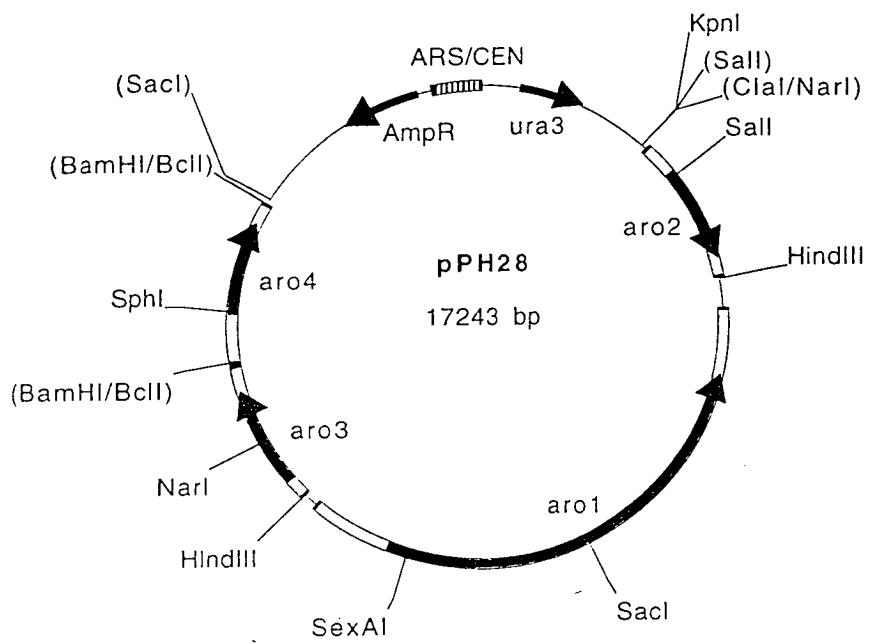


Figure 3-8 pPH28, the ‘*ARO*’ plasmid

This plasmid contains yeast centromeric and autonomously replicating sequences (hashed box). Both yeast (*URA3*) and bacterial (ampicillin resistance) selectable markers are shown as black arrows. The plasmid, pPH30 is identical except for reverse orientation of the *ARO1* gene.



permutation of individual or multiple gene disruptions using the restriction enzymes as defined in Table 3-2. These manipulations are predicted to cause premature chain termination well toward the 5' end of the genes, as shown. Thus the 'enzyme multiplier' may be independently varied for each of the four enzymes.

3.1.2 Plasmids constructed

During construction and validation of each plasmid, their predicted sequences were generated on computer using SEQED (UWGCG). This allowed the prediction of restriction maps which guided the restriction digests used to screen transformants. Miniprep plasmid preparations were validated by restriction digests, designed to discriminate between the required recombinant plasmid, the donor plasmids and/or vector, and other likely but unwanted recombinants. The cloned fragments were validated by running the same fragment from the parent plasmid in adjacent lanes. The orientations of fragments (where necessary) were determined by restriction analysis using a known asymmetric restriction site within the fragment. Restriction sites destroyed by filling in (*e.g. Bam*HI) of cohesive ends often generate new restriction sites (*e.g. Cla*I in *dam*⁻ host strains). This allowed further confirmation of the resulting construct in some cases. Finally the retention of sites to be used in future cloning steps was confirmed. After final selection of transformants, single colonies were used to generate glycerol stocks for storage at -70⁰C and to produce purified plasmid DNA from 100 ml cultures, prior to the next cloning step.

Construction of pPH16 and pPH17 from pRS416

Destruction of the *Sa*II and *Sac*I sites in pRS416 allows the use of these enzymes to disrupt the *ARO2* and *ARO1* genes respectively in the final plasmid, if required.

The *Sa*II site in pRS416 was cut, filled in by incubation with Klenow fragment and the four deoxyribonucleotide triphosphates (dNTPs), and the resulting blunt ends ligated. In order to reduce the number of transformants retaining an unmodified *Sa*II site, the re-ligated mixture was digested with *Sa*II prior to transformation into *E. coli* CB-51. Transformants tested gave the desired fragment sizes following digestion with *Sa*II

Gene	Enzyme	Normal protein length (aa)	Unique restriction site	Predicted length of truncated protein (aa)	Comment
<i>ARO3</i>	DAHP synthase (inhibited by phenylalanine)	370	<i>NarI</i>	183	
<i>ARO4</i>	DAHP synthase (inhibited by tyrosine)	370	<i>SphI</i>	20	
<i>ARO1</i>	<i>AROM</i> complex	1588	<i>SexAI</i>	90	<i>SexAI</i> is predicted to give loss of all activities. Although <i>SacI</i> disrupts only the final three activities, the entire complex may be non-functional
			<i>SacI</i>	818	
<i>ARO2</i>	Chorismate synthase	376	<i>SalI</i>	38	

Table 3-2 Selective gene disruption by restriction digest

The table above shows the restriction sites (unique in the plasmids pPH28 and pPH30) which are predicted to produce early chain termination of any combinations of the four *ARO* genes. This may be achieved by cutting with the appropriate restriction enzyme, blunt-ending with either Klenow fragment or T4 polymerase in the presence of 4 dNTPs, re-ligation and transformation. Transformants can be selected by cutting with the same enzyme prior to transformation.

and *ScaI*. One of these transformants was used to generate a glycerol stock and plasmid DNA, called pPH16.

The *SacI* site of pPH16 was destroyed in a similar manner. After digestion with *SacI*, T4 polymerase was used to digest 3' overhangs in the presence of the four dNTPs. DNA from transformants was analysed by digestion with *SacI* and *ScaI*. A transformant showing the required fragment sizes was used to generate a glycerol stock and plasmid DNA, called pPH17.

Construction of pPH18 and pPH19

Prior to the cloning of the *ARO2* fragment into the yeast shuttle vector, pRS416, it was necessary to destroy the *BamHI* site which lies about 350 bp upstream of the start of the coding sequence, so that a *BamHI* site within the multiple cloning site of the plasmid, pPH17, could be used at a later stage for the insertion of a *HindIII/BamHI* fragment containing the *ARO3* gene and for the insertion of a *BclI* fragment containing the *ARO4* gene. Since the unwanted *BamHI* site lies upstream of all three putative GCRE as well as two possible TATA elements, its modification was judged to be unlikely to affect normal transcription.

However, the *ARO2* donor plasmid, pZH2, contains other *BamHI* sites. Therefore, a 3.22 kbp *SphI/SacI* fragment containing *ARO2* was transferred from pZH2 to the general cloning vector, pK19 (Pridmore, 1987). pZH2 was digested with *SacI* and *SphI*. This reaction was ligated with *SacI/SphI* digested pK19. *E. coli* CB-51 cells were transformed with kanamycin selection. Transformants gave DNA which showed the required fragment sizes when digested with *SphI/SacI* or *HindIII*. One of these was used to give pPH18.

The single *BamHI* site in pK18 was destroyed by *BamHI* digestion, filling in with Klenow fragment in the presence of 4 dNTPs, ligation, re-digestion with *BamHI* and transformation in *E. coli* CB-51 under selection on LB-kan. A transformant giving the desired pattern of fragment size after digestion with *BamHI/EcoRI*, *SalI/HindIII* and *SacI/SphI*, using pZH2 and pPH18 as controls was selected to give pPH19. This

also showed the appearance of a predicted *ClaI* site, confirmed by digestion with *ClaI* and *EcoRI*.

Construction of pPH29 and pPH20

It was originally intended to transfer a 1.74 kb *NarI/HindIII* fragment containing the *ARO2* gene from pPH19 into the *ClaI/HindIII* site of pPH17 to give pPH20. This strategy involved the disruption of the *NarI* site during cloning and allows the use of another *NarI* site to disrupt the *ARO1* gene in the final plasmid. However, the close proximity of the *ClaI* site and *HindIII* sites within pPH17 inhibited efficient digestion, even with sequential digestion, and an alternative strategy was devised whereby a 3.49 kbp *NarI* fragment was first transferred to the *ClaI* site of pPH17, to give pPH29. Removal of 2 adjacent *HindIII* fragments (1.14 kbp and 0.61 kbp) by subsequent digestion with *HindIII* and intra-molecular ligation would yield pPH20.

pPH19 was digested with *NarI* and ligated with phosphatase-treated *ClaI*-digested pPH17. Ligation mixtures were transformed into *E. coli* JM109 cells and selected on LB-amp. A transformant giving the required insert and orientation of fragment as determined by digestion with *HindIII*, *XhoI/BamHI* and *XhoI/BamHI/HindIII* was designated pPH29.

This was digested with *HindIII* and religated at a reduced concentration. After further digestion with *SacI* to reduce unmodified transformants, *E. coli* JM109 cells were transformed with selection on LB-amp. Transformants were tested by *HindIII/XhoI* digestion. All gave the required fragment which is 20 bp longer than the *HindIII/NarI* fragment from pPH19. One of these transformants was used as pPH20.

These are the first plasmids constructed in this series to contain an *ARO* gene (*ARO2*) cloned into a pRS416 derivative.

Construction of pPH22 (contains *ARO2,3*)

Here, a 1.73 kbp *HindIII/BamHI* fragment containing the *ARO3* gene from pME631 was cloned into the *HindIII/BamHI* sites of pPH20 to give pPH22.

pME631 was simultaneously digested with *Hind*III and *Bam*HI (and *Eco*RI to cut the vector fragment) and ligated with *Hind*III/*Bam*HI-digested pPH20. The reactions were transformed into *E. coli* JM109 cells and transformants selected on LB + ampicillin. One transformant giving the required restriction fragments when digested with *Hind*III/*Bam*HI was called pPH22. This plasmid is predicted to contain the *ARO2* and *ARO3* genes.

Construction of pPH23 (contains *ARO2*, *ARO3*, *ARO4*)

A 1.90 kbp *Bcl*I fragment from pME1199 was cloned into the *Bam*HI site of pPH22, as follows.

pME1199 was propagated in the *dam*⁻ host *E. coli* CB-51 prior to restriction with *Bcl*I. This was required because *Bcl*I is sensitive to *dam* methylation (where adenine bases are methylated within GATC sequences). pPH22 was digested with *Bam*HI and then phosphatase treated. This was ligated with pME1199 digested with *Bcl*I, *Eco*RI and *Sac*I. *E. coli* JM109 were transformed with selection on LB + ampicillin. A transformant which showed the required restriction pattern with *Hind*III/*Xba*I and *Hind*III/*Sph*I digestion was used as pPH23. Only one orientation of the *Bcl*I fragment within pPH22 was obtained. This plasmid contains the *ARO2*, *ARO3* and *ARO4* genes.

Construction of pPH24 (contains *ARO1*)

It was necessary to manipulate an *ARO1* fragment into a vector in such a way as to remove it as a *Hind*III fragment for cloning into pPH23. pME173 was therefore digested with *Ssp*I/*Sph*I and this reaction was ligated with *Sma*I/*Sph*I digested pK19. White recombinant transformants were selected on LB + kanamycin + X-Gal/IPTG. A transformant giving the predicted fragment sizes with digestion with *Hind*III/*Sph*I, was designated pPH24.

It was necessary to destroy an *Sph*I site within a 7.02 kbp *Hind*III fragment so that the *Sph*I site in *ARO4* could be used to selectively disable the *ARO4* gene. pPH24 was therefore digested with *Sph*I and subsequently treated with T4 polymerase in the presence of dNTPs to digest 3' overhangs. Religation and transformation into *E. coli*

XL1 cells gave transformants which gave the required fragments after digestion with *SphI/EcoRI*. One was used to generate pPH27.

Construction of pPH31 (contains *ARO1*)

A 7.5 kbp *NarI* fragment from pPH27 bearing the *ARO1* gene was cloned into the *ClaI* site of pRS416 in order to produce a yeast plasmid containing only the *ARO1* gene. This was achieved by digesting pPH27 with *NarI* and pRS416 with *ClaI*. The vector was dephosphorylated with alkaline phosphatase. After ligation of the products and transformation of *E. coli* XL1 cells with selection on LB + ampicillin, transformants were obtained which showed the required pattern of restriction fragments after digestion of plasmid DNA with *SalI/BamHI*, *EcoRI* and *HindIII*. A transformant was used to generate a glycerol stock and plasmid DNA, called pPH31.

Construction of pPH 28 and 30 (contains *ARO1*, *ARO2*, *ARO3*, *ARO4*)

It was now possible to construct a plasmid containing all four *ARO* genes by transferring a 7.02 kbp *HindIII* fragment from pPH27 into the *HindIII* site of pPH23.

pPH23 was digested with *HindIII* and treated with alkaline phosphatase. It was ligated with pPH27 digested with *HindIII*. After ligation and transformation into *E. coli* XL1 cells, using selection on LB + ampicillin, transformants were analysed with *EcoRI/BamHI/HindIII*. All samples showed the required restriction pattern. Further analysis with *PstI* defined the orientation of the *HindIII* fragment containing the *ARO1* gene. Two transformants showing opposite orientations of this fragment were used to generate glycerol stocks and plasmid DNA, and were called pPH28 and 30. The map of pPH28 was previously shown in Figure 3-8.

3.1.3 Summary

Both of the plasmids pPH28 and pPH30 are designed to show the features defined in the cloning strategy. They should contain the coding regions, promoter sequences and downstream sequences required for normal expression from the chromosome of all the genes encoding enzymes of the shikimate pathway. They are expected to maintain a low copy number (possibly of 1 copy) within yeast cells in a stable manner

under the control of the *ARS/CEN* sequences. Each of the *ARO1-4* genes may be disabled selectively by disruption (blunt-ending) of the *SacI*, *SalI*, *NarI* or *SphI* restriction sites respectively. They can be propagated within *E. coli* using ampicillin resistance and can be transformed and selected in a yeast strain carrying a *ura3* mutation by growth on media lacking uracil.

Table 3-3 shows the salient features of the plasmids constructed.

The following section reports how plasmids (pPH20, 22, 23, 28 30 and 31) containing various combinations of the *ARO* genes were used to complement *aro* mutations in various strains of yeast.

3.2 Complementation of *aro* null mutations

Strains containing null mutations within genes encoding enzymes of the shikimate pathway may be used to test the identity and partial functionality of genes borne upon the plasmids pPH20, 22, 23, 28 and 30, by transformation and determination of growth requirements. Table 3-4 shows the strains used and which mutations they bear. It was first necessary to confirm their identity.

3.2.1 Strain verification

All strains used were verified for their nutritional requirements by growing strains in YEPD followed by transfer into 'drop-out' media, where one nutrient was omitted. Growth was noted. The results for RH1129, RH1181, RH1182, RH1610, RH1220 and RH1380 are shown in Table 3-5. RH1129, RH1181, RH1610, RH1220 and RH1380 showed growth requirements which are consistent with their designated genotypes. In contrast, RH1182 gave no growth on media lacking any of the following nutrients - phenylalanine, tryptophan, tyrosine, adenine, uracil or leucine, and poor growth when lacking methionine. This was not consistent with its given genotype. Auxotrophy for lysine was expected but not observed whilst auxotrophy for methionine was not expected but was observed, although this mutation appeared to be 'leaky'.

Plasmid	Antibiotic resistance marker	Yeast selection	ARO genes 1-4	Description and details of construction	Size (bp)
pPH16	ampicillin	<i>URA3</i>		pRS416 with <i>SalI</i> site destroyed	4902
pPH17	ampicillin	<i>URA3</i>		pRS416 with <i>SalI</i> and <i>SacI</i> sites destroyed	4898
pPH18	kanamycin		2	<i>SacI/SphI</i> fragment from pZH2 cloned in <i>SacI/SphI</i> site of pK19	5840
pPH19	kanamycin		2	pPH18 with <i>BamHI</i> site destroyed upstream of promoter in <i>ARO2</i>	5844
pPH20	ampicillin	<i>URA3</i>	2	pPH29 after removal of <i>HindIII</i> fragments	6634
pPH22	ampicillin	<i>URA3</i>	2,3	<i>HindIII/BamHI</i> fragment (<i>ARO3</i>) from pME631 cloned into <i>HindIII/BamHI</i> site of pPH20	8324
pPH23	ampicillin	<i>URA3</i>	2,3,4	<i>BclI</i> fragment (<i>ARO3</i>) of pME1199 cloned into <i>BamHI</i> site of pPH22	10223
pPH24	kanamycin		1	<i>SspI/SphI</i> fragment (<i>ARO1</i>) from pME173 cloned into <i>SmaI/SphI</i> site of pK19	9872
pPH27	kanamycin		1	pPH24 with <i>SphI</i> site destroyed	9868
pPH28	ampicillin	<i>URA3</i>	1,2,3,4	<i>HindIII</i> fragment (<i>ARO1</i>) of pPH27 cloned into <i>HindIII</i> site of pPH23	17243
pPH29	ampicillin	<i>URA3</i>	2	<i>NarI</i> fragment (<i>ARO2</i>) from pPH19 cloned into <i>Clal</i> site of pPH17	8385
pPH30	ampicillin	<i>URA3</i>	1,2,3,4	<i>HindIII</i> fragment (<i>ARO1</i>) of pPH27 cloned into <i>HindIII</i> site of pPH23 - frag reversed cf. pPH28	17243
pPH31	ampicillin	<i>URA3</i>	1	<i>NarI</i> fragment (<i>ARO1</i>) from pPH27 cloned into <i>Clal</i> site of pRS416	12400

Table 3-3 Details of plasmids constructed during this study

The plasmid pPH28, shown in boldface was used in future experiments in order to evaluate the universal method.

For easy reference, some of these data are also contained on a card in the inside back cover of volume 2, along with data on the yeast strains used.

Strain	Mating type	<i>aro</i>	<i>trp</i>	other mutations
RH1220	a	<i>aro3, aro4</i>		<i>leu2-2</i>
RH1380	α	<i>aro4-1</i>		<i>ura3-52, gcn4-101</i>
RH1129	a	<i>aro1C</i>	<i>trp5</i>	<i>leu1, hom2, ade6, lys1, ura3, arg4-1, thr1, his6, met1, gal2</i>
RH1181	α	<i>aro1</i>	<i>trp1</i>	<i>ura3</i> and/or <i>ura4, his4-15, can1, ade2, kar1-1</i>
RH1182	a	<i>aro1</i>	<i>trp1</i>	<i>ura3</i> and/or <i>ura4, leu2, lys1^a, can1, ade2, kar1-1</i>
RH1610	a	<i>aro2</i>		

Table 3-4 Yeast strains used to monitor functionality of cloned *ARO* genes

^a Nutrient requirement for growth as determined in this work showed that this strain was not auxotrophic for lysine, but was auxotrophic for methionine (see text and Table 3-5)

For easy reference, these data are also contained on a card in the inside back cover of volume 2, along with data on the plasmids used.

Strain	Relevant genotypic markers	Media lacking particular nutrients											
		F	W	Y	Ad	U	L	T	H	M	K	R	-
RH1220	<i>aro3, aro4, leu2</i>	-	-	-	+	+	-	+	+	+	+	+	+
RH1380	<i>aro4, ura3, gcn4</i>	+	-	-	nd	-	nd	nd	nd	nd	nd	+	+ ^a
RH1129	<i>aro1, trp5, leu1, hom2, ade6, lys1, ura3, arg4, thr1, his6, met1,</i>	-	-	-	-	-	-	-	-	-	-	-	+
RH1181	<i>aro1, trp1, ura3 and/or ura4, his4-15,, ade2,</i>	-	-	-	-	-	+	+	-/+	+	+	+	+
RH1182	<i>aro1, trp1, ura3 and/or ura4 leu2, lys1^b, ade2,</i>	-	-	-	-	-	-	+	+	-/+ ^b	+	+	+
RH1610	<i>aro2</i>	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	+ ^c

Table 3-5 Testing auxotrophy of *aro* strains

Strains were grown to about $OD_{546} = 1$ in 10 ml of permissive media. 20 μ l were then transferred into 10 ml of media lacking one of each of the nutrients specified above. These were then cultured until the permissive media (-) reached 1 unit OD_{546} . Cultures were then scored for growth. Visible growth approximating growth in permissive media +, invisible growth -, poor growth visible -/+ . The concentrations of the nutrients used are given in Chapter 2.

^a permissive media included F,W,Y,U and R only; ^b Growth properties suggest that this strain may be *LYS1, met* (see text), ^c permissive media included F,W and Y only.

RH1380 showed a complex pattern of growth requirements, which are illustrated in Table 3-6. Its absolute requirement for uracil is consistent with its *ura3* designation. Only slow growth is observed in the absence of phenylalanine, tryptophan and tyrosine. This is attributed to a combination of the *aro4* and *gcn4* mutations. Because basal expression (non-starvation conditions) of the *ARO3* gene is dependent upon GCN4 (Paravicini, *et al.*, 1989a), the DAHP synthase activity in RH1380 is very low and, in the absence of all three aromatic amino acids, there is only sufficient flux through the shikimate pathway to support low growth rates.

When phenylalanine is included in the media, however, growth became undetectable. Thus, the reduced activity of the *ARO3* gene product (phenylalanine inhibitable DAHP synthase) is sufficiently inhibited by phenylalanine to render the strain auxotrophic for both tyrosine and tryptophan. Thus the *aro3*, *gcn4* background only appears to provide sufficient flux for (slow) growth when phenylalanine is not present in the medium. These growth characteristics were useful for the purposes of monitoring the activity of *ARO3* and *ARO4* cloned into the plasmids constructed, as detailed in section 3.2.2.

In summary, the growth characteristics of the strains described above are consistent with their designated genotypes as given in Table 3-4 with the exception of RH1182 where weak methionine auxotrophy replaces lysine auxotrophy.

3.2.2 Transforming strains with *ARO* plasmids

Yeast strains having mutations in genes encoding enzymes of the shikimate pathway were transformed with plasmids bearing combinations of these genes, in order to confirm the identity of the cloned genes and to test their functionality. The results are summarised in Table 3-7.

RH1129 (*aro1* etc.)

Because RH1129 had been designated *ura3*, selection of transformants using media lacking uracil was used. However no transformants were obtained with pRS416, pPH20, pPH22, pPH28 or pPH31. Since RH1380 carries a *ura3* mutation which is

Media containing the following nutrients				Growth indication
F	W	Y	U	+
F	W	Y		-
	W	Y	U	+
F		Y	U	-
F	W		U	-
		Y	U	+
	W		U	+
F			U	-
			U	+/-

Table 3.6 Testing growth requirements of RH1380

The growth of RH1380 (*aro4*, *ura3*, *gcn4*) is tested in the media given above where each nutrient in turn is omitted or where two or three of the aromatic amino acids are omitted. The concentrations of the nutrients are as described in Chapter 2. + indicates normal growth, +/- indicates slow growth, - indicates no detectable growth compared to control

Strains		Plasmids transformed (with selectable markers)							
		pZH2	pPH20	pPH22	pPH23	pPH28	pPH30	pPH31	pRS416
relevant markers	Selection on media lacking	<i>ARO2</i> <i>LEU2</i>	<i>ARO2</i> <i>URA3</i>	<i>ARO2,3</i> <i>URA3</i>	<i>ARO2,3,4</i> <i>URA3</i>	<i>ARO1,2,3,4</i> <i>URA3</i>	<i>ARO1,2,3,4</i> <i>URA3</i>	<i>ARO1</i> <i>URA3</i>	<i>URA3</i>
RH1129	<i>aro1</i> <i>ura4?</i>		-	-	-	-			-
				-	-	+	+	+	
RH1181	<i>aro1</i> <i>ura4?</i>			-	-				
				-	-	+	+	+	
RH1182	<i>aro1</i> <i>ura4?</i>			-	-				
				-	-	+	+	+	
RH1610	<i>aro2</i>	F, Y, W	+	+	+	+	+	-	
RH1220	<i>aro3aro4</i>	F, Y, W		-	+	+	+	-	
RH1380	<i>aro4, gcn4, ura3</i>	uracil			+	+	+	+	+

Table 3-7 Transformation of *aro* strains with *ARO* plasmids constructed

Various strains (with their relevant auxotrophic markers) are transformed with various plasmids (with specific *ARO* or *URA* genes). Blanks indicate that a particular transformation was not attempted. All transformations included appropriate positive (growth of host strain on non-selective media) and negative (host strain plated onto selective media without plasmid in transformation) controls. Note that RH1129, RH1181 and RH1182 are given a *ura4?* designation to account for lack of complementation by *URA3* plasmid (see text).

complemented by pRS416, pPH22, 23, 28, 30 and 31, it was concluded that uracil auxotrophy in RH1129 arises through the mutation at another locus. Since RH1181 and RH1182 were supplied from the same source with a *ura3* and/or *ura4* designation, it seems reasonable to suppose that this mutation may be *ura4*.

Subsequent transformations of this strain with selection on media lacking phenylalanine and tyrosine show that plasmids pPH28, 30 and 31 convert phenylalanine and tyrosine auxotrophy to prototrophy, whilst pPH22 and 23 do not. This is entirely consistent with the prediction that only the former bear *ARO1*. All transformants required uracil in the medium when their nutritional requirements were tested by replica plating, further confirming that this strain's uracil auxotrophy arises through mutation of a gene other than *URA3*.

RH1181 (*aro1* etc.) and RH1182 (*aro1* etc.)

Neither of these strains were converted to uracil prototrophy by transformation with pPH22 and pPH23. Similar conclusions to those for RH1129 can be drawn, regarding the nature of the mutation giving rise to uracil auxotrophy. When transformants were selected on media lacking tyrosine and phenylalanine, pPH28, 30 and 31 gave many colonies (which still showed uracil auxotrophy) whilst pPH22 and 23 did not give transformants. These results support those above and confirm the presence of a functional *ARO1* gene in plasmids pPH28,30 and 31, as predicted.

RH1610 (*aro2*)

In an initial experiment conducted during plasmid construction, the functionality of the first *ARO* gene (*ARO2*) cloned into the yeast plasmid pRS416, was tested by transformation of the *aro2* mutant RH1610 with pPH20 and pZH2. Both plasmids gave complementation of the mutation on media lacking the three aromatic amino acids. This confirmed the presence of a functional *ARO2* gene and suggested that the system used was likely to be appropriate for carrying and expressing functional *ARO* genes.

Competent RH1610 were later incubated with pPH20, 22, 23, 28, 30 and 31. All these plasmids, except for pPH31, gave transformants on media lacking the three aromatic amino acids. This was entirely consistent with predictions. pPH31 bears

only the *ARO1* gene while the others all contain *ARO2* in various combinations with the other *ARO* genes. The results suggest, therefore, that *ARO2* is functional in all plasmids of this series.

RH1220 (*aro3, aro4*)

RH1220 was transformed with plasmids pPH20, 22, 23, 28, and 31. Only those plasmids expected to bear *ARO3* and/or *ARO4* (namely pPH22, 23 and 28) gave transformants on media which lacked the three aromatic amino acids. Since pPH22 (*ARO2, ARO3*) gives conversion from auxotrophy, this confirms that the *ARO3* gene is functional in this plasmid and that one of the two genes, *ARO3* or *ARO4*, is functional in plasmids pPH23 and 28.

RH1380 (*aro4, gcn4, ura3*)

RH1380 give transformants with pPH22, 23, 28, 30 and 31 when selected on Ura⁻ media, as expected. Selection on trp⁻ media was poor because of slow background growth of transformed cells. However, the functioning of *ARO4* may be independently established by monitoring the growth of these transformants in defined media (see below).

Distinguishing *ARO3* and *ARO4* activities on the basis of growth characteristics

In section 3.2.1 it was observed that growth of RH1380 (*aro4, gcn4, ura3*) was inhibited in media lacking tyrosine and/or tryptophan but containing phenylalanine. This was interpreted in terms of the inhibition by phenylalanine of the enzyme (phen-inhibitable DAHP synthase) encoded by *ARO3*, whose basal expression is almost entirely dependent upon GCN4. Those plasmids containing *ARO4* should rescue growth on media containing phenylalanine but lacking tryptophan +/- tyrosine, in contrast to plasmids containing only *ARO3*.

Transformants were plated onto solid media lacking uracil and replica-plated onto various media. Growth was noted relative to the growth of positive and negative controls. The results are shown in Table 3-8. They show clearly that all transformants grow without difficulty in all media with the exception of pPH22 transformants in MV+F, MV+FY and MV+FW. Thus, pPH23, 28 and 30 are

Strain	transformed with plasmid	Media								Comment ^a	
		MV + L +									
		-	F	W	Y	WY	FY...	FW	FWY		
RH1220 <i>aro3, aro4</i>	no plasmid	-	-	-	-	-	-	-	-	+	
	pPH22	+	+	+	+	+	+	+	+	+	
	pPH23	+	+	+	+	+	+	+	+	+	
	pPH28	+	+	+	+	+	+	+	+	+	
		MV (+U) ^a +									
		-	F	W	Y	WY	FY	FW	FWY		
RH1380 <i>aro4, gcn4, ura3</i>	no plasmid	+/-	-	+	+	+	-	-	+	Uracil present	
	pPH22	+/-	-	+	+	+	-	-	+	Uracil absent	
	pPH23	+	+	+	+	+	+	+	+	Uracil absent	
	pPH28	+	+	+	+	+	+	+	+	Uracil absent	
	pPH30	+	+	+	+	+	+	+	+	Uracil absent	

Table 3-8 Growth of *aro4* +/- *aro3* mutants and *ARO3,4* transformants

Transformants were replica-plated onto various media as shown. Normal, slow or insignificant growth (with respect to controls) are denoted by +, +/- or -, respectively. 12 independent transformants for each strain/plasmid pairing were used. The results shown were obtained by all transformants tested. The results for transformants of RH1220 are shown so that the effect of the *gcn4* mutation can be seen by comparing RH1220/pPH22 with RH1380 (both strains being *ARO3, aro4*).

^a Untransformed RH1380 requires uracil in all media. It is therefore included in all drop-out media used for this (untransformed strain). Both strains when transformed have no requirement for uracil. Therefore, this nutrient is omitted in order to maintain selection.

confirmed as carrying a functional *ARO4* gene. The pPH22 transformant matches the growth characteristics of the untransformed strain, except for uracil prototrophy. It was therefore concluded that *ARO3* on pPH22 was functional (it rescues the *aro3*, *aro4* mutations in RH1220) and that *ARO4* is functional in pPH23, 28 and 30 because they rescue 'wild-type' phenotype on specific media from the phenotype given by the background *ARO3*, *aro4*, *gcn4* genotype.

3.3 Summary

Strains carrying mutations in the *ARO* genes have been examined for their nutritional requirements. These are, in general, consistent with their designated genotype. Only RH1182 gave a conflicting result where a requirement for methionine was observed rather than for lysine, but this did not reflect upon the significance of the results obtained.

It was possible to detect the *aro4* mutation in RH1380 in the presence of *ARO3* in a *gcn4* background by culturing in MV+F, MV+FY or MV+FW. In these cases, growth was very slow or undetectable.

These strains were used in transformations with the plasmids pPH20, 22, 23, 28, 30 and 31 to test whether their genes could rescue their corresponding mutations. Since these plasmids used the *URA3* selective marker, it was also possible to test the presence of *ura3* mutations in RH1129, RH1181, RH1182 and RH1380. Only RH1380's auxotrophy for uracil could be converted by transformation with these plasmids.

It was possible to rescue all *aro1* mutants (RH1129, 1181 and 1182) with all plasmids containing *ARO1* (pPH28, 30 and 31) and the *aro2* mutant (RH1610) with plasmids bearing the *ARO2* gene (pPH20, 22, 23, 28 and 30), as predicted. *ARO3* and *ARO4*, the genes encoding the two isoenzymes of DAHP synthase, are more difficult to monitor. However, *ARO3* on pPH22 and *ARO4* on pPH23, 28 and 30 are positively confirmed. The presence of *ARO3* on pPH23, 28 and 30 may be confirmed by measuring phenylalanine-inhibitable DAHP synthase activity in transformants or by

disruption of the *ARO4* genes in pPH23, 28 and 30 followed by transformation into RH1220. However, there is no reason to suspect that the cloning of *ARO4* and *ARO1* into pPH22 should have disturbed expression of *ARO3*.

Since enzyme activities at a small fraction of wild-type levels may lead to phenotypic responses indistinguishable from those of a wild-type strain, it should be stressed that the rescue of mutations here does not yield any information regarding the level of expression from the plasmid encoded genes. Since the rationale for the method employed within this project depends upon large changes in enzyme activities, the suitability of the plasmid constructs described here can only be evaluated by measuring enzyme activities under similar conditions to those that will be used in future physiological experiments. These data are presented in Chapter 4, whilst the stability of the plasmid in strains appropriate for flux measurement is explored in Chapter 5.

Chapter 4

Construction and characterisation of transformed strains

Chapter 3 described the construction of a plasmid, pPH28, which bears the four genes (*ARO1-4*) encoding the enzymes of the shikimate pathway, and the subsequent demonstration of their function in strains carrying mutations in these genes.

The experimental strategy of this thesis requires the overexpression of the *ARO* genes, encoding enzymes of the shikimate pathway, by specific factors. In order to assess the suitability of the constructed plasmid, pPH28, appropriate strains were transformed with pPH28 (and other plasmids of this series) and activities of DAHP synthase, SDH and chorismate synthase measured, compared to wild-type levels, and expressed as enzyme multipliers. Furthermore, it was necessary to overexpress these enzymes in strains bearing the multi-copy plasmid pME554 (Niederberger *et al.*, 1984) used to overexpress all five enzymes of the tryptophan pathway. This required the isolation of a *ura3* derivative of the *trp1-5* quintuple null mutant, RH1207 (Niederberger *et al.*, 1992; Prasad *et al.*, 1987) in order to select pPH28 transformants.

It was also necessary to undertake a partial characterisation of RH1207, the reference strain RH805 and the multi-copy plasmid, pME554. This includes the determination of the activities of the enzymes anthranilate synthase and tryptophan synthase in these and other control strains before and after transformation with pME554 and/or pPH28 (and its precursors).

4.1 *trp* strains and *TRP* plasmids

Table 4-1 gives details of the strains discussed in this chapter. The same data are included on the reference card held in the end flap pocket.

Strain	Mating type	<i>aro</i>	<i>trp</i>	other mutations	source or reference
RH805	not defined			<i>aat2</i>	Kradolfer et al., (1982)
RH1207	not defined		<i>trp1, trp2, trp3, trp4, trp5</i>	<i>leu2, aat2</i>	Prasad et al., (1987)
RH1220	a	<i>aro3, aro4</i>		<i>leu2-2</i>	G. Braus
RH1380	α	<i>aro4</i>		<i>ura3-52, gcn4-101</i>	G. Braus
RH1129	a	<i>aro1C</i>	<i>trp5</i>	<i>leu1, hom2, ade6, lys1, ura3, arg4-1, thr1, his6, met1, gal2</i>	G. Braus
RH1181	α	<i>aro1</i>	<i>trp1</i>	<i>ura3</i> and/or <i>ura4, his4-15, can1, ade2, kar1-1</i>	G. Braus
RH1182	a	<i>aro1</i>	<i>trp1</i>	<i>ura3</i> and/or <i>ura4, leu2, lys1^a, can1, ade2, kar1-1</i>	G. Braus
RH1610	a	<i>aro2</i>			G. Braus

Table 4-1 Yeast strains used to assess over-expression of enzymes from *TRP* and *ARO* plasmids

^a Nutrient requirement for growth as determined in this work showed that this strain was not auxotrophic for lysine, but was auxotrophic for methionine (see text). For easy reference, these data are also given on a card in the inside back cover of this volume, along with data on the plasmids used.

The strain RH805 contains an *aat2* null mutation. The *AAT2* gene encodes the high affinity aromatic amino acid transferase which catalyses the catabolic conversion of tryptophan to tryptophol (Kradolfer *et al.*, 1982). This mutation was introduced in order to allow increased intracellular accumulation of tryptophan and to allow a more valid determination of flux to tryptophan, when the rate of tryptophan breakdown becomes smaller compared to the measurable rates of accumulation into free tryptophan pool and tryptophan in protein.

RH1207 is a derivative of RH805 which bears quintuple null mutations in the genes encoding enzymes of the tryptophan pathway. It also bears a *leu2* mutation. This strain has previously been used as a host for the *TRP* plasmids, pME554 and pME559 (Prasad *et al.*, 1987; Niederberger *et al.*, 1992).

The multi-copy plasmid, pME554 (Niederberger *et al.*, 1984), derived from the 2-micron yeast episome, contains a copy of the five *TRP* genes and gives overexpression of the five enzymes by factors of about 25-50 (Niederberger *et al.*, 1992).

4.1.1 Verification of RH805 and RH1207

Both of these strains showed nutrient requirements consistent with their genotypic designation. Thus, RH805 shows no auxotrophy. It grows well on MV medium. RH1207, on the other hand, requires leucine and tryptophan for growth on MV medium. Furthermore, RH1207 could be transformed with pME554 using selection on media lacking tryptophan. These transformants still required leucine for rapid growth. This result was entirely consistent with the findings of Prasad *et al.* (1987), who declared that pME554 and pME559 both contained a *LEU2d* allele with a defective promoter.

The activities of anthranilate synthase and tryptophan synthase, encoded by *TRP2/3C* and *TRP5* respectively, were determined in RH805 and RH1207. The results are shown in Table 4-2. There are detectable levels of both anthranilate synthase and tryptophan synthase activities (0.07 and 1.1 nmol • min⁻¹ • mg protein⁻¹, respectively)

Strain	Plasmid	Media	Anthranilate synthase	Tryptophan synthase
			activity	activity
$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$				
RH805		MV + LVFY(100)	0.07	1.1
RH1207		MV + LVFY(100) + W(40)	0.00	0.0
RH1207	pME554	MV + LVFY(100)	4.15	29.4

Table 4.2 Enzyme activities in RH805 ('wild type') and RH1207 (*trp1-5*) strains

in RH805. RH1207 shows no detectable activities of these two enzymes, and was subsequently used as a negative control for these assays.

4.1.2 Verification of plasmid pME554

The identity of pME554 was confirmed by restriction digestion with *Hind*III, *Eco*RI and *Bam*HI (and combinations of these enzymes) and comparison of the fragment sizes obtained, with the plasmid map as published (Niederberger *et al*, 1984). When RH1207 was transformed with pME554, the plasmid complemented the five null mutations of genes of the tryptophan pathway, allowing growth on media lacking tryptophan. Transformants gave large anthranilate synthase and tryptophan synthase activities compared to the wild-type activities in RH805 (see Table 4-2). The specific activity of anthranilate synthase was $4.15 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, representing an enzyme multiplier of 59 with respect to the wild-type, whilst the specific activity of tryptophan synthase was $29.4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, representing an enzyme multiplier of 27. This confirmed that pME554 bears the genes *TRP2*, *TRP3* and *TRP5* and suggests that this plasmid is maintained at high copy number. The activities of the other enzymes of the pathway, encoded by the genes *TRP1*, *TRP4* and *TRP3C* were not determined. For the purposes of this thesis, it was assumed that similar levels of overexpression were obtained for these enzymes relative to the factors obtained for anthranilate synthase and tryptophan synthase and comparable to the data of Niederberger *et al*, (1984).

4.2 Construction of a *ura3* derivative of RH1207

The experimental protocol demands the introduction of both pME554 and pPH28 into the same strain. In order to maintain selection on the *ARO* plasmid, pPH28, it was therefore necessary to introduce a *ura3* mutation into RH1207. This was achieved by growing RH1207 to late exponential phase and plating onto medium containing 5-fluoro-orotic acid (5-FOA) as well as uracil. Resistance to 5-FOA occurs in *ura3* and, to a lesser degree, in *ura5* mutants, possibly because the enzymes encoded by these genes may lead to the conversion of 5-FOA into 5-fluoro-UMP (Boeke *et al.*, 1984). Resistant colonies are therefore likely to be *ura3*. Colonies selected in this

manner were tested for their requirement for leucine, tryptophan and uracil in the media. Nine colonies which showed auxotrophy for all three nutrients were scored for reversion to uracil prototrophy by culture to mid exponential phase and subsequent transfer to media lacking uracil. The suitability of one such colony which gave least reversion, HK1, was also tested for its ability to be transformed with pME554 and pPH28. These plasmids gave transformants which showed prototrophy with respect to tryptophan and uracil respectively. This suggests that the genotype of HK1 is *aat2, leu2, trp1, trp2, trp3, trp4, trp5, ura3*.

4.3 Transformation of *trp* strains with *TRP* and *ARO* plasmids

Attempts were made to transform RH1207 and HK1 with various combinations of the plasmids, pPH28, pPH30 and pPH31 and pME554 or a similar plasmid, pME559, which harbours an allele of *TRP2* (*TRP2_{br}*) which shows resistance to feedback inhibition by tryptophan (Prasad *et al.*, 1987).

RH1207 was successfully transformed with pME554 using selection on media lacking tryptophan. The transformation was most efficient when phenylalanine and tyrosine were included in the media. Attempts to transform with pME559 were unsuccessful, using different media such as SC medium and MV medium + LV (100 µg ml⁻¹) with concentrations of tyrosine and phenylalanine varying between 0 and 100 µg ml⁻¹. This is in accordance with reports which emphasise the instability of pME559 even when grown in the presence of phenylalanine and tyrosine (P. Niederberger, personal communication). Two mechanisms whereby phenylalanine and tyrosine may stabilise transformants containing a multi-copy *TRP* plasmid are possible. Firstly, phenylalanine and tyrosine together inhibit both isozymes of DAHP synthase, the first enzyme of the shikimate pathway. This may result in a reduced flux within the pathway, leading to partial starvation for tryptophan. Cells bearing a multi-copy *TRP* plasmid may have a growth advantage over cells which have a low copy number or lack the plasmid altogether. Secondly, decreased flux to tryptophan by the same mechanism may lead to insufficient excretion of tryptophan to support the growth of

cells which have lost the plasmid at mitosis. In the absence of phenylalanine and tyrosine cells containing low copy plasmid or lacking plasmid altogether may have a growth advantage over cells containing many copies of the plasmid, especially when tryptophan is present in the medium.

HK1 was transformed with pPH28, pPH30 and pPH31 under Ura^r selection. This confirmed that HK1 contained a *ura3* mutation. It was also transformed with pME554 using media lacking tryptophan. Attempts to transform with pME559 were unsuccessful.

Strains containing both an *ARO* plasmid and a *TRP* plasmid were generated by transforming HK1/pME554 with the *ARO* plasmids, pPH28, pPH30 and pPH31, using medium lacking uracil and tryptophan, to yield the transformants designated HK1/pME554/pPH28 etc.

Prior to further analysis, the plasmids were rescued from transformed strains (HK1/pME554, HK1/pPH28 and HK1/pME554/pPH28) by transformation of competent *E. coli* with plasmid preparations from cultures. as described in Section 2.5.6. Distinctive patterns of restriction fragments from plasmid preparations from transformants showed that pME554 had been rescued from HK1/pME554 and HK1/pME554/pPH28 and that pPH28 was rescued from both HK1/pPH28 and HK1/pME554/pPH28

4.4 Enzyme activities in host strains and transformants

The ability of pME554 and pPH28 to increase the activity of enzymes encoded by genes cloned on these plasmids is clearly important for the experimental strategy implicit in the universal method. This section reports upon the various enzyme activities obtained in different strains/plasmid pairings and in different media. Strains were grown to an OD₅₄₆ of about 1 and cells permeabilised as described in Chapter 2. Specific calibrated assays and protein determinations on these cell preparations allowed the determination of specific activities.

4.4.1 Overexpression of enzymes of the tryptophan pathway

Two assays were performed to assess the degree to which enzymes of the tryptophan pathway may be overexpressed in strains bearing the multi-copy plasmid, pME554.

Preliminary results were presented in section 4.1.2.

Anthranilate synthase

The activities of anthranilate synthase in various strains are summarised in Table 4-3. RH1207, HK1 and HK1/pPH28 showed no detectable activity, consistent with a null mutation in the *TRP2* and *TRP3* genes. The 'wild-type' strain, RH805, gave an activity of $0.07 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Cells transformed with pME554 and cells transformed with both pME554 and pPH28, pPH30 or pPH31 all gave anthranilate synthase activities which were larger than wild-type by factors of about 40 - 60. This is in reasonable agreement with the results of Niederberger and colleagues who achieved enzyme multipliers of between 19 and 50 (Niederberger *et al.*, 1984; Prasad *et al.*, 1987; Niederberger *et al.*, 1992) when anthranilate synthase activity was overexpressed on pME554 and pME559. Thus, pME554 clearly encodes *TRP2* and *TRP3* and is replicated at high copy number.

Tryptophan synthase

Similar results are obtained with assays of tryptophan synthase activity as shown in Table 4-3. RH1207 and HK1 strains and those transformed with the *ARO* plasmid pPH28 gave no detectable activity. The 'wild-type' strain, RH805 gave a specific activity of $1.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, while the strains transformed with pME554 gave greatly increased specific activities of between 25 and 40 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, representing enzyme multipliers of between about 21 and 36.

General control

The activities of both anthranilate synthase and tryptophan synthase in RH805 were unchanged when cells were grown in MV medium. Thus the phenomenon of 'general control', where some biosynthetic enzymes are derepressed under starvation conditions, was not observed. The fact that the activity of anthranilate synthase and tryptophan synthase in the *gcn4* strain, RH1380/pPH28, did not change either, was

Strain	Plasmid	Media	Anthranilate synthase	Tryptophan synthase
			nmol · min ⁻¹ · mg protein ⁻¹	
RH805		MV	0.06	1.2
RH805		MV + LVFY(100)	0.07	1.1
RH1207		MV + LVFY(100) + W(40)	0.00	0.1
HK1		MV + LVFY(100) + UW(40)	0.01	0.0
RH1207	pME554	MV + LVFY(100)	3.60	24.7
HK1	pME554	MV + LVFY(100) + U(40)	2.82	39.9
HK1	pPH28	MV + LVFY(100) + W(40)	0.00	0.0
HK1	pME554/pPH28	MV + LVFY(100)	2.80	38.0
RH1380	pPH28	MV	0.06	1.4
RH1380	pPH28	MV + LVFY(100)	0.08	1.2

Table 4.3 Activities of anthranilate synthase and tryptophan synthase

therefore not significant. The failure to find the expected increases in enzyme activity when strains were grown in MV is not understood. It is possible that effects may be observed at higher cell densities.

4.4.2. Overexpression of enzymes of the shikimate pathway

The expression of enzymes of the shikimate pathway in untransformed and transformed cells was also measured. In this case, it was important to show that all four cloned genes were expressed at levels comparable with their chromosomal homologues. Thus, the specific activities of DAHP synthase (both phenylalanine inhibitable and tyrosine inhibitable, encoded by *ARO3* and *ARO4*, respectively) and chorismate synthase (encoded by *ARO2*) were determined. *ARO1* was investigated by the assay of SDH activity only, which represents the third activity of the penta-functional *AROM* complex.

DAHP synthase

Activities of DAHP synthase are shown in Table 4-4. Strains which contain null mutations in the *ARO4* or in both *ARO3* and *ARO4* genes show appropriately reduced activities compared to the wild-type activities. Thus, strain RH1220 which contains null mutations in both genes shows no detectable activity, whilst strain RH1380 which contains an *aro4* null mutation shows reduced activity compared to those strains, RH805, RH1207 and HK1, carrying the wild-type allele.

In order to determine the contribution of DAHP synthase encoded by *ARO3* and/or *ARO4* genes, the assays are performed in the presence of 1mM phenylalanine or 3mM tyrosine in order to inhibit the activities of the *ARO3* and *ARO4* encoded DAHP synthases, respectively. It was not possible to find concentrations of tyrosine and phenylalanine that gave complete but specific inhibition of *ARO4* and *ARO3* activities, respectively, such that addition of the two separate activities resulted in the activity obtained in the absence of both phenylalanine and tyrosine. Indeed, the use of both tyrosine and phenylalanine in the assay did not abolish activity altogether. Together, these data suggest that there was incomplete inhibition of individual activities and/or

Strain	Plasmid	Media	DAHP synthase activity nmol · min ⁻¹ · mg protein ⁻¹		
			phe-inhib ^a (<i>ARO3</i>)	tyr-inhib ^b (<i>ARO4</i>)	phe-inhibited + tyr-inhibited
RH805		MV	145	184	259
RH805		MV + LVFY(100)	238	158	280
RH1220		MV + LVFY(100) + W(40)	0	0	0
RH1220	pPH22	MV + LV(100)	323	39	228
RH1220	pPH23	MV + LV(100)	247	110	369
RH1220	pPH28	MV + LV(100)	524	264	701
RH1380	pPH22	MV + LV(100)	170	46	197
RH1380	pPH23	MV + LV(100)	174	281	473
RH1380	pPH28	MV	515	507	952
RH1380	pPH28	MV + LVFY(100)	1039	668	1369
HK1		MV + LV(100) + UW(40)	123	220	283
HK1	pPH28	MV + LVFY(100) + W(40)	553	369	615
HK1	pME554/pPH28	MV + LVFY(100)	786	416	818
HK1	pME554/pPH31	MV + LVFY(100)	197	165	304

Table 4.4 Overexpression of *ARO3* and *ARO4* encoding DAHP synthase

The activity of DAHP synthase is assayed in three ways, as described in Chapter 2. ^a Activity of the *ARO3* gene product. The assay mixture includes the addition of tyrosine in order to inhibit the activity of the tyrosine inhibited DAHP synthase. ^b Activity of the *ARO4* gene product. The assay mixture includes the addition of phenylalanine in order to inhibit the activity of the phenylalanine inhibited DAHP synthase. ^c Activity of total DAHP synthase activity. No phenylalanine or tyrosine added to the assay mixture.

partial inhibition of the other activity. The relatively high errors incurred during this assay may also contribute to non-additivity of the component activities.

Within these limitations, however, the results obtained were consistent with those predicted from the particular mutations of the strains. Thus, the strains RH805, RH1207 and HK1 showed both phenylalanine and tyrosine-inhibitable activities, whereas RH1380 showed low *ARO4* activity and RH1220 showed no detectable activity at all.

When strains were transformed with the *ARO* plasmids, pPH22, 23 and 28, there were increases in the phenylalanine-inhibited DAHP synthase activities (*ARO3*), as expected, while strains transformed with pPH23 and 28 also showed an increase in the tyrosine-inhibited DAHP synthase (*ARO4*) relative to untransformed strains. This confirmed that pPH22, 23 and 28 carry an expressed copy of the *ARO3* gene while pPH23 and 28 carry, in addition, an expressed copy of the *ARO4* gene. There was, however, no increase in activity when HK1 was transformed with pPH31, confirming that the increases in DAHP synthase activities are not caused by transformation of a similar plasmid which does not bear copies of either *ARO3* or *ARO4*.

The enzyme multipliers in HK1/pME554/pPH28 relative to RH805 grown on a similar medium were 2.9 for the total DAHP synthase activity and 3.3 and 2.6 for the phenylalanine and tyrosine inhibited isoenzymes, respectively.

As with enzymes of the tryptophan pathway, there was no evidence of derepression when strains were grown in MV medium. Furthermore, the phenylalanine-inhibitable enzyme (encoded by *ARO3*) was detected in the *gcn4* mutant, RH1380, in all growth media. This is in contrast to the results of Paravicini *et al.* (1989a) who showed that basal expression of *ARO3* is dependent upon GCN4 under non-derepressing growth conditions.

Shikimate dehydrogenase

The activities of SDH in various strains are shown in Table 4-5. The strains carrying an *aro1* mutation showed various activities. Strains RH1181 and RH1182 both gave no detectable activity. In contrast, RH1129 gave normal activity.

Strains transformed with pPH28, 30 and 31 show increased activities of this enzyme. The factor by which the enzyme is overexpressed varies between 2 and 7 depending on the strain and the particular transformant. Strains transformed with plasmids pPH20, 22 and 23 did not show increased SDH activity (data not shown)

As with all the enzymes assayed, no increase in activities were noted in RH805 when grown in MV compared to growth in richer media.

Chorismate synthase activity

The results obtained in chorismate synthase assays are shown in Table 4-6. They show similar trends as described for DAHP synthase and SDH. The *aro2* mutant RH1610 is defined as having no activity and did not give a significantly greater value than assays stopped at zero time. RH805, RH1207, HK1 and all transformants bearing only pME554 gave activities between 23 and 46 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. However HK1 transformed with pPH28 or pPH30 gave values of between 76 and 102 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. These increases in chorismate synthase activity suggest that chorismate synthase is expressed from these plasmids. The transformant containing pPH31 (bearing the *ARO1* gene only) gave a value of 40 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ which confirms that the overexpression is not a general effect of transformation with a *URA3* plasmid.

As before, no increases in activities were noted in RH805 when grown on MV or richer media.

4.5 Discussion and summary

The data reported in this chapter support both the genotypic designations of the strains used in experiments to be reported in the following chapters, and the identity

Strain	Plasmid	Media	Shikimate dehydrogenase
			$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ protein^{-1}
RH805		MV	30.8
RH805		MV + LV(100)	35.1
RH805		MV + LVFY(100)	37.5
RH1207	pME554	MV + LV(100) + W(40)	28.2
RH1207		MV + LVFY(100)	30.2
RH1129	pPH28	MV + LVWAURKHMT + FY ^a	46.5
RH1129		MV + LVWAURKHMT	63.9
RH1181	pPH28	MV + LVWAURKHMT + FY ^a	0.0
RH1181		MV + LVWAURKHMT	218.6
RH1182	pPH28	MV + LVWAURKHMT + FY ^a	0.0
RH1182		MV + LVWAURKHMT	202.5
HK1		MV + LV(100) + UW(40)	27.7
HK1	pME554	MV + LVFY(100) + U(40)	32.2
HK1	pPH28	MV + LV(100) + W(40)	99.0
HK1	pPH30	MV + LV(100) + W(40)	103.2
HK1	pPH31	MV + LV(100) + W(40)	204.2
HK1	pME554/ pPH28	MV + LVFY(100)	189.3

Table 4.5 Shikimate dehydrogenase activities

Strain	Plasmid	Media	Chorismate synthase
			$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ protein^{-1}
RH805		MV	34
RH805		MV + LVFY(100)	45
RH1207	pME554	MV + LVFY(100)	35
RH1207			23
RH1610	pPH28		0
RH1610			65
HK1	pME554	MV + LVFY(100) + U(40)	46
HK1			28
HK1	pPH28	MV + LVFY(100) + W(40)	93
HK1	pME554/pPH28	MV + LVFY(100)	102
HK1	pME554/pPH30	MV + LVFY(100)	76
HK1	pME554/pPH31	MV + LVFY(100)	40

Table 4.6 Chorismate synthase activities

of the plasmid constructions used. Thus pME554 gave overexpression of the enzymes of the tryptophan pathway by a large factor, while plasmid pPH28 gave a modest overexpression of the enzymes of the shikimate pathway. Together, these plasmids would appear to be adapted to the requirements of the universal method, as defined in Chapter 1.

The measured activities of anthranilate synthase and tryptophan synthase suggest a reliable overexpression of these enzymes from pME554 by factors of between 20 and 60. Because of previous work (Niederberger *et al.*, 1984; Prasad *et al.*, 1987; Niederberger *et al.*, 1992) demonstrating the overexpression of all five enzymes from this plasmid by similar factors, further work conducted here assumes that the activities of the other three enzymes, phosphoribosyl-anthranilate transferase, phosphoribosyl-anthranilate isomerase and indolyl glycerophosphate synthase are raised by factors of an approximately similar magnitude. The two enzyme assays conducted here are thus taken to be representative of the overexpression of all five activities from the *TRP* plasmid, pME554.

For the *ARO1-4* genes, the data in this (and the previous) chapter confirm that pPH28 carries a functional copy of each and that they are expressed, giving modest (two to seven-fold) increases in activity. There is one reservation. The constituent activities of the penta-functional *AROM* complex have not been independently measured. However, SDH is the final (C-terminal) activity on the complex (Duncan *et al.*, 1987). Therefore, the demonstration of this activity supports the assumption that the entire protein is expressed. In strains transformed with pPH28, the increases in DAHP synthase and chorismate synthase activity were less than those of SDH. Also, there was considerable variation between different strain/plasmid combinations. This may reflect a variation in the number of plasmids carried by a particular cell. Individual colonies may be expected to bear one or a low number of plasmids. Alternatively, the pattern of expression from the plasmid-borne gene may be altered with respect to the chromosomal gene. This may lead to increased or decreased expression which may depend upon growth conditions. In the cases presented here, and assuming that the plasmids are present in single copy, the plasmid-borne genes appear, in general but

particularly in the case of SDH, to give rather greater levels of expression than do the chromosomal homologues. If this were the case, then this situation may arise in two ways. Firstly, there may be structural differences between chromosomal and plasmid DNA. Secondly, the role of 5' and 3' flanking regions distal to those cloned has not been characterised. Both of these factors may lead to differential patterns of expression and regulation.

It is noteworthy that neither the chromosomal nor plasmid borne copies of the genes of the shikimate and tryptophan pathways showed derepression on MV medium relative to their basal expression on richer media containing other nutrients and amino acids. Unfortunately therefore, it was not possible to confirm that the plasmid-borne genes were able to show GCN4-dependent transcriptional activation. It is likely that further experiments using higher cell densities and/or the use of tryptophan analogues would allow this analysis. Nevertheless, with the analysis of the *gcn4* mutant, RH1380, it was possible to confirm that significant *ARO3*-dependent DAHP synthase activity was obtained in the absence of GCN4.

Having demonstrated that pME554 and pPH28 give increases in activities which were approximately as expected (and required), in subsequent experiments only anthranilate synthase and SDH activities were used to monitor the presence or absence of the plasmids and to reflect the likely activities of the other enzymes of the tryptophan and shikimate pathways.

Before describing the effect of these plasmids upon the concentrations and fluxes in the aromatic amino acid biosynthetic pathways, the stability of the plasmids and enzymes activities dependent upon them are considered in the next chapter.

Chapter 5

Plasmid stability and enzyme activity

Chapters 3 and 4 described the construction of a single-copy plasmid, pPH28, which bears the four genes encoding enzymes of the shikimate pathway, and the characterisation of both this plasmid and pME554, a multi-copy plasmid bearing the five genes encoding enzymes of the tryptophan pathway, by complementation of corresponding mutations and the determination of specific enzyme activities. It was confirmed that pPH28 increased the specific activities of the enzymes of the shikimate pathway by factors of 2-5, relative to the reference strains and that pME554 increased specific activities of the tryptophan pathway by factors of between 20 and 70.

The experimental analysis of the relationship between enzyme activity and the production of the aromatic amino acids tryptophan, phenylalanine and tyrosine demands that these enzyme levels should be maintained for the duration of culture.

This chapter describes some initial investigations which were undertaken to evaluate the stability of the *TRP* and *ARO* plasmids and the overexpression of enzymes encoded by these plasmids under different culture conditions.

Previous work (Fantes *et al.*, 1976; Miozzari *et al.*, 1978a; Prasad *et al.*, 1987) and pilot studies during this project suggested that wild-type cells contain very low concentrations of tryptophan. Because of the limitations in the sensitivity of the tryptophan assay employed here, it was therefore desirable to delay flux estimations as late as possible during culture. However, some partial or complete loss of plasmid may occur through variation in copy number or expression levels, unequal segregation (including random drift) at mitosis, or loss of selection for the plasmid as tryptophan is excreted into the medium. In order to make an informed decision as to the optimum time at which tryptophan, tyrosine and phenylalanine fluxes should be measured, it was therefore necessary to evaluate

- the stability of the plasmid over time

- the variation in enzyme activity over time

These experiments were carried out in selective and non-selective media. In addition, because of previous work which suggests that the presence of phenylalanine and tyrosine in the medium is important for the stability of the *TRP* plasmids (Prasad, *et al.*, 1987), experiments were undertaken in both the presence and absence of these two amino acids.

The transformed strains used for these experiments were

- HK1/pME554
- HK1/pPH28
- HK1/pME554/pPH28

Cultures were maintained in exponential phase ($OD_{546} < 1.5$) by daily sub-culture, over a period of four days. OD_{546} was monitored and cell samples were appropriately diluted in MV + LV(100) medium and plated on selective and non-selective media. Cells were also collected for enzyme activities and protein determination. The details of the experimental protocol are given in Chapter 2. For selective and non-selective conditions of culture, HK1/pME554 was grown without and with tryptophan ($40 \mu\text{g ml}^{-1}$), HK1/pPH28 was grown without and with uracil ($40 \mu\text{g ml}^{-1}$) and the double transformant, HK1/pME554/pPH28 was grown under four conditions; (i) both uracil and tryptophan were contained in the medium (no selection), (ii) uracil or tryptophan were present in the medium (single selection) and (iii) both uracil and tryptophan were omitted (double selection).

5.1 Stability of pME554 and pPH28 in media lacking phenylalanine and tyrosine (MV + LV)

The strains were cultured in MV medium containing leucine and valine at $100 \mu\text{g ml}^{-1}$ (MV + LV(100)). HK1/pME554 required uracil added ($40 \mu\text{g ml}^{-1}$) while HK1/pPH28 required tryptophan added ($40 \mu\text{g ml}^{-1}$) for growth. HK1/pME554/pPH28, the 'double' transformant required no addition to MV +

LV(100). The percentages of all cells growing on the various plates are plotted graphically in Figures 5-1, 5-2 and 5-3.

5.1.1 Stability of the *TRP* plasmid, pME554

The stability of pME554, is represented in Figure 5-1. There was no apparent loss in cells bearing pME554 from either HK1/pME554 or in HK1/pME554/pPH28 when selection (media without tryptophan) was enforced daily, despite considerable variation from day to day. Estimates of prototrophic cells lay between 64% and 103% of total cells. The variation did not seem to be correlated with the OD₅₄₆ of cells, or upon the size of inoculum for the following day's culture, nor upon the dilution used to plate out samples (data not shown). The variation between triplicate plates and average count was usually less than 10%, but was occasionally higher. This variation may be large enough to explain the discrepancy between the proportion of Trp⁺ cells found on successive days under selective conditions.

When selection is relaxed, by addition of tryptophan in the medium, the percentage of Trp⁺ cells drops away rapidly with a half-life of approximately 24 hours. Figure 5-1 shows that the rate of loss of pME554 is similar in both the single transformant and in the double transformant, regardless of selection on pPH28. The semi-logarithmic plots show a constant rate of loss of plasmid from the cells. The gradient allows an estimation of 0.07 generation⁻¹ to be made for the mitotic instability (see Table 5-1A), as described in Chapter 2. Since this coefficient is similar for the single and the double transformant under both culture conditions without tryptophan selection, this result provides no evidence that the presence of the *ARO* plasmid, pPH28, stabilises the maintenance of the *TRP* plasmid, pME554.

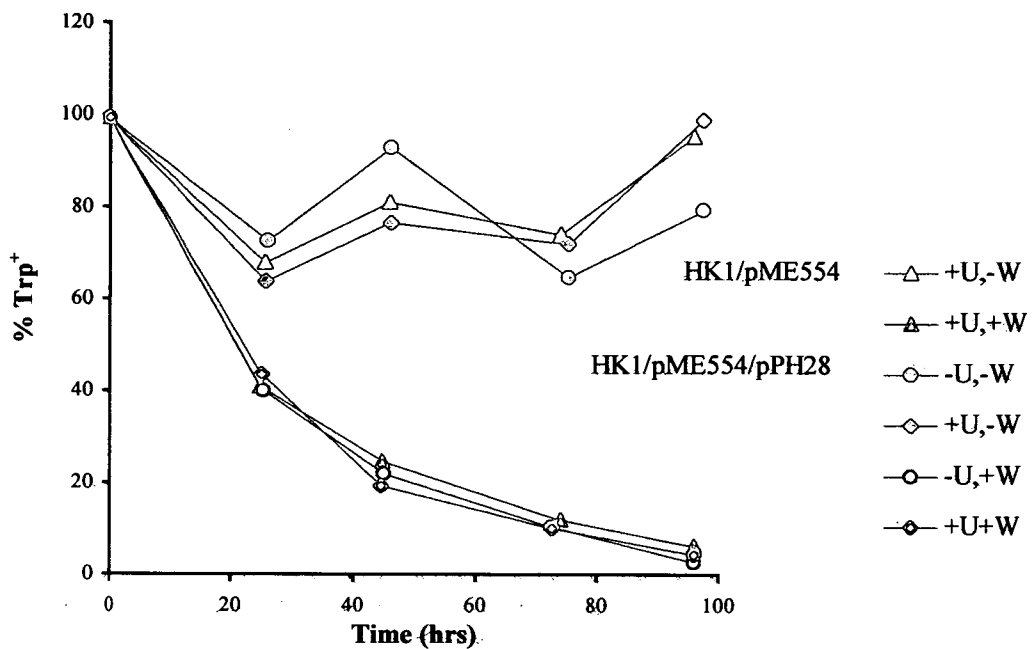
5.1.2 Stability of the *ARO* plasmid, pPH28

The stability of pPH28 is illustrated in Figure 5-2. The results show, complete stability of the plasmid under selective conditions (where uracil is omitted from the medium). In non-selective culture conditions, where uracil is included at 40 µg ml⁻¹, plasmid is progressively lost though at a significantly lower rate than the multicopy

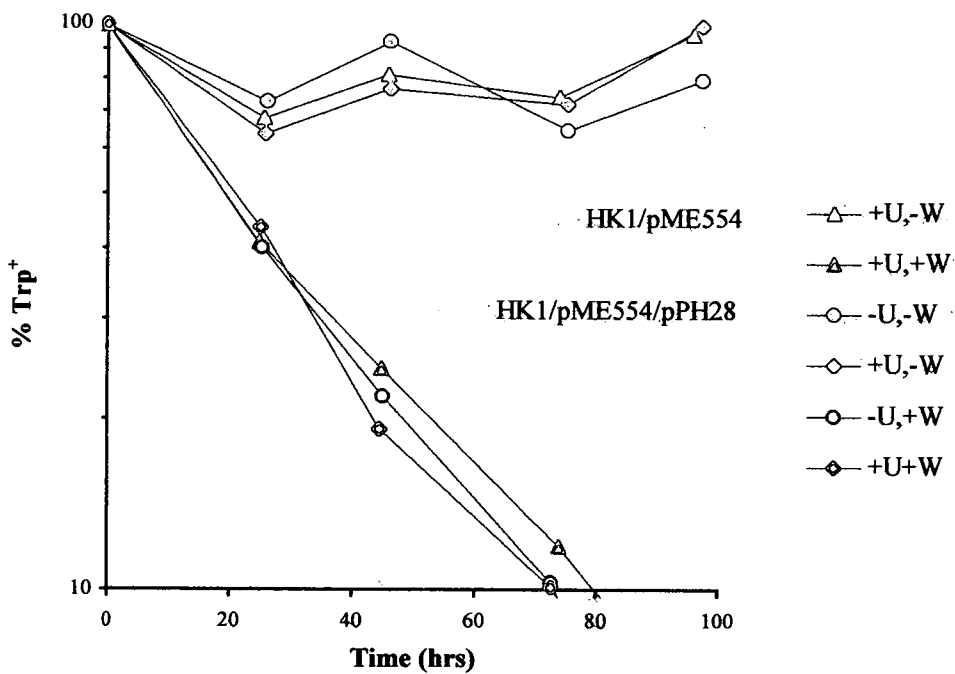
**Figure 5-1 Stability of pME554 in HK1/pME554 and
HK1/pME554/pPH28 in MV+ LV(100)**

A. Linear plot B. Semi-logarithmic plot

A



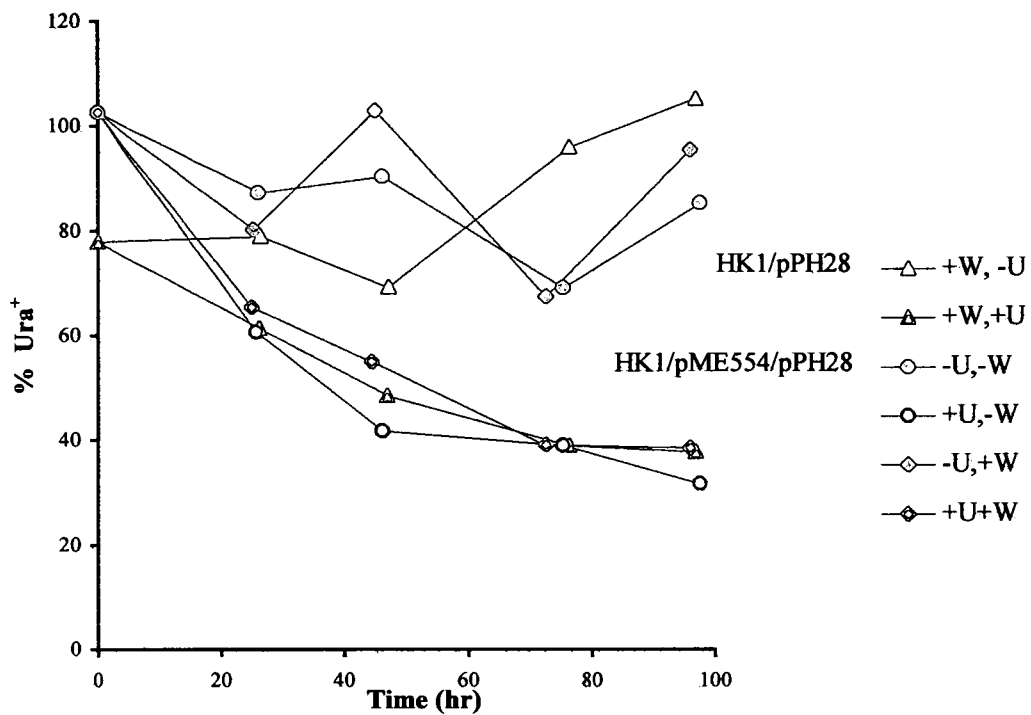
B



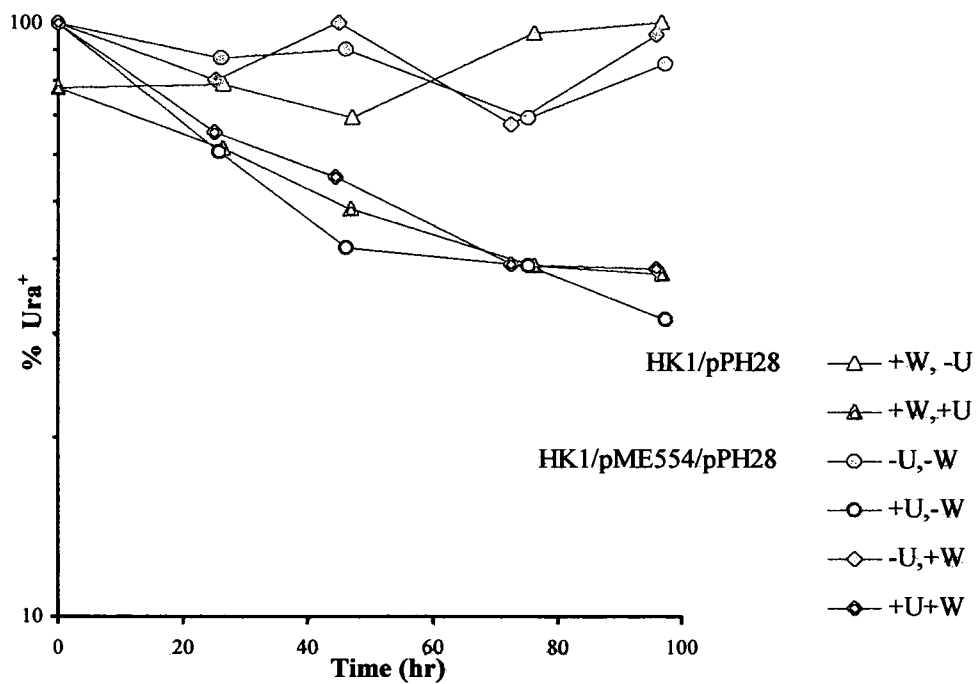
**Figure 5-2 Stability of pPH28 in HK1/pPH28 and
HK1/pME554/pPH28 in MV + LV(100)**

A. Linear plot B. Semi-logarithmic plot

A



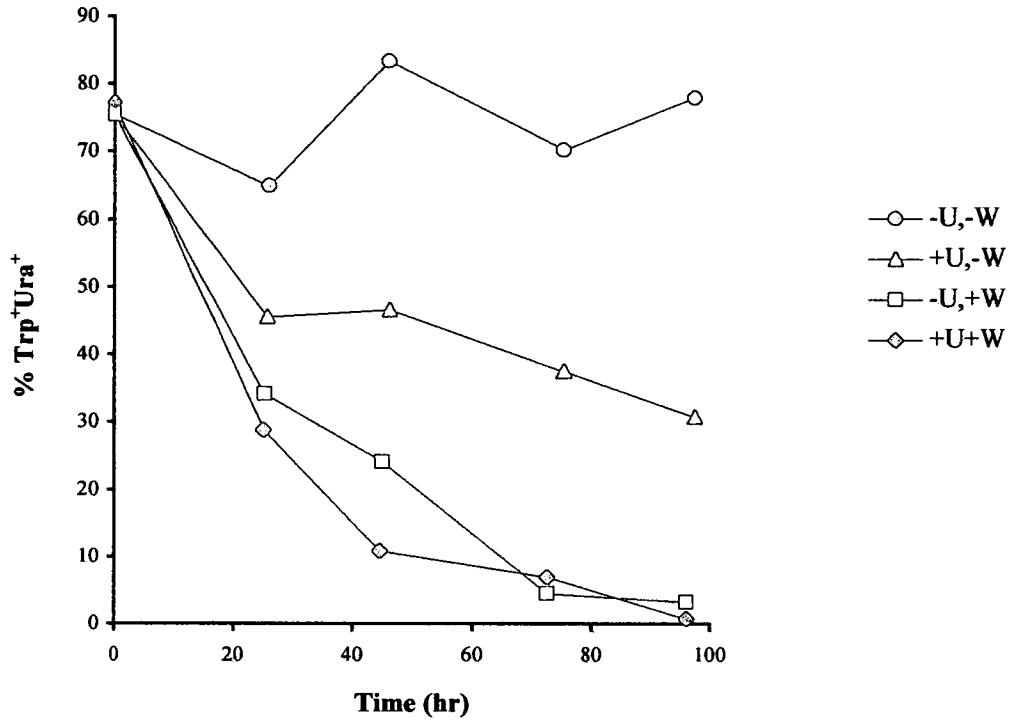
B



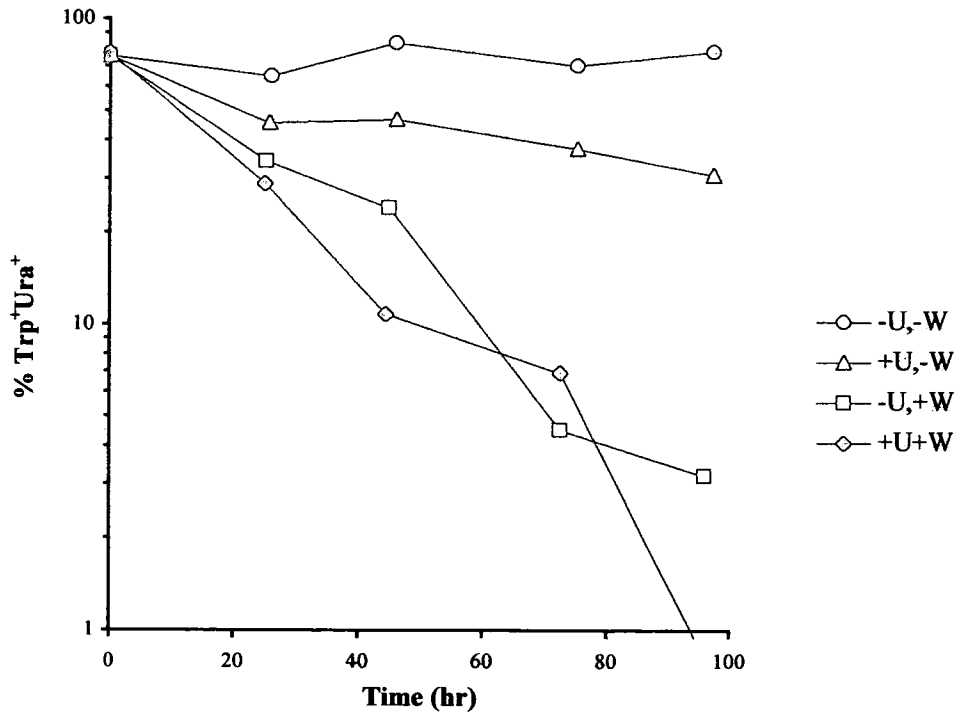
**Figure 5-3 Stability of pME554/pPH28 in HK1/pME554/pPH28
in MV + LV(100)**

A. Linear plot B. Semi-logarithmic plot

A



B



A			
Host strain for pME554	HK1/pME554	HK1/pME554/pPH28	
Non-selective medium used	MV + LV + W + U	MV + LV + W - U	MV + LV + W + U
Mitotic instability	0.07	0.07	0.07

B			
Host strain for pPH28	HK1/pPH28	HK1/pME554/pPH28	
Non-selective medium used	MV + LV + W + U	MV + LV - W + U	MV + LV + W + U
Mitotic instability	0.02	0.02	0.02

C			
Host strain for both plasmids	HK1/pPH28/pPH28		
Non-selective medium used	MV + LV + W - U	MV + LV - W + U	MV + LV + W + U
‘Mitotic instability’	0.08	0.03	0.08

Table 5-1 Mitotic stability of pME554 and pPH28 in single and double transformants in non-selective media lacking phenylalanine and tyrosine

A. pME554 in single and double transformants, B. pPH28 in single and double transformants, C. Loss of double prototrophy in the double transformant when grown upon 3 different types of non-selective media

plasmid, pME554. The semi-logarithmic plot shown in Figure 5-2 allows an estimation of $0.02 \text{ generation}^{-1}$ to be made for mitotic instability (Table 5-1B)

These results show that the centromeric *ARO* plasmid, pPH28 is more stable than the multi-copy *TRP* plasmid, pME554, as expected. The level of stability for pRS316, using the *URA3* marker was $0.01 \text{ generation}^{-1}$ (Sikorski and Hieter, 1989). Loss might occur because of genetic instability within the plasmid itself. Possible defects may occur in the replication of the plasmid prior to mitosis or the segregation of the plasmid between the mother and daughter cells. There is also the possibility of phenotypic effects, whereby cells carrying the plasmid may have a lower growth rate than that of untransformed cells. This factor is thought to be present here in the case of the single transformant, HK1/pPH28 reverting to untransformed cells, HK1. The observed growth rate of single transformants was significantly lower than the wild-type cells, RH805, the *trp1-5* strain, RH1207 and its *ura3* derivative, HK1 as discussed in Chapter 4. The difference in growth rate would certainly contribute to a decrease in the observed stability of this plasmid, compared to the stability of a similar centromeric plasmid which does not lead to such a reduction in growth rate. The possibility that it is the loss of plasmid which contributes to the slow growth rate in selective media may be discounted by the observation that the rate of growth of the single transformed cells is no different under selective conditions (media lacking uracil) than it is under non-selective conditions (media containing uracil), when grown over a short time intervals where loss of plasmid is not sufficient to alter the observed growth rate.

5.1.3 Stability of the Trp^+ , Ura^+ phenotype (pME554 + pPH28)

The stability of both plasmids together is determined by plating double transformants upon medium lacking both uracil and tryptophan. This is repeated for all four media used for the double transformant.

The data are illustrated graphically in Figure 5-3. As expected, the two plasmids were both stable under selective conditions (both tryptophan and uracil lacking). When the double transformant was grown in the presence of tryptophan, then the loss of uracil

+ tryptophan prototrophy closely followed the loss of pME554. Again the semi-logarithmic plot gives an estimate of the rate of loss of prototrophy. The value obtained, $0.08 \text{ generation}^{-1}$ (Table 5-1C) is similar to the value obtained for the loss of pME554 in both the single transformant, HK1/pME554 and in the double transformant (independent of the *ARO* plasmid). Similarly, the rate of loss of uracil + tryptophan prototrophy in the presence of uracil only closely matches that obtained for the loss of uracil prototrophy alone in both the single transformant, HK1/pPH28 and in the double transformant (independent of the *TRP* plasmid). The value of 'mitotic instability' in this case was $0.03 \text{ generation}^{-1}$, which is slightly greater than that for the loss of pPH28 from the single transformant and the double transformant (see Table 5-1).

Together, these results support the view that the two plasmids are maintained and lost independently of each other.

Finally, the rate of loss of double prototrophy in the presence of both tryptophan and uracil, $0.08 \text{ generation}^{-1}$, matches the loss of pME554 alone, as expected.

5.1.4 Enzyme activities

The data presented in this chapter so far, of course, give no indication of either the copy number of the plasmid within prototrophic cells nor of the level of expression occurring from the plasmid-borne genes. To evaluate these questions, the activities of anthranilate synthase and SDH were assayed on each day. The results are illustrated graphically in Figure 5-4 and 5-5

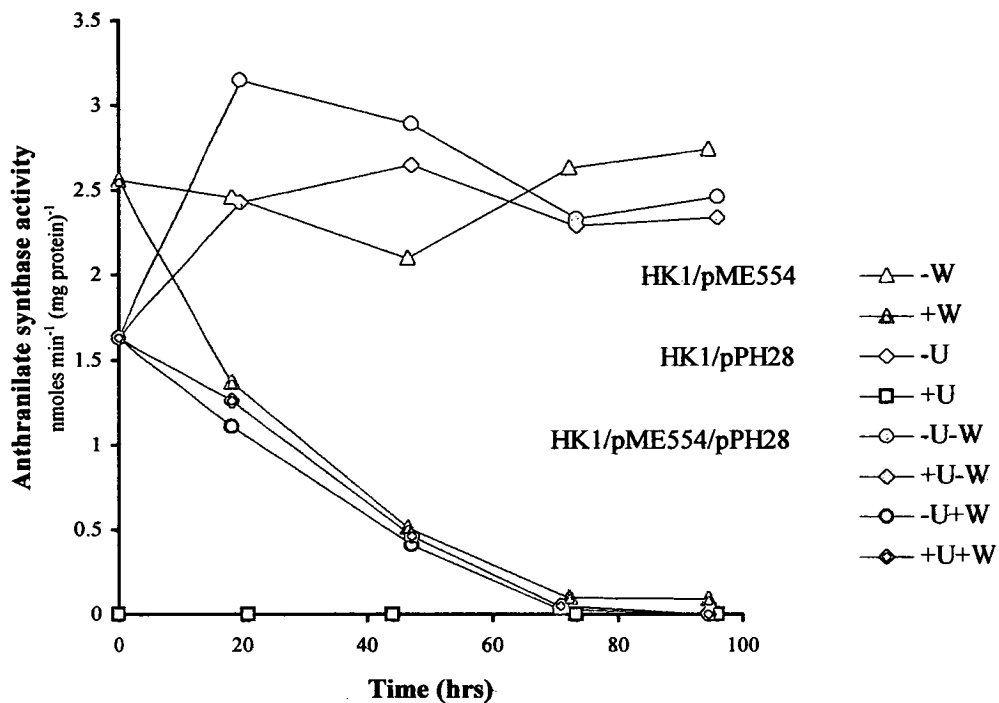
Anthranilate synthase

Figure 5-4 shows the changes in anthranilate synthase activity over time in all cultures. It shows that the level of anthranilate synthase activity parallels the loss of plasmid pME554 (Figure 5-1) in all cultures. Thus during culture in media lacking tryptophan, there is no apparent loss in enzyme activity over time. This suggests that the copy number of the plasmid is also being maintained under selective conditions. Moreover, during relaxation of selection, there is a similar parallel between the loss of

Figure 5-4 Anthranilate synthase activity in single and double transformants +/- selection in MV + LV

A. Linear plots. Note that the activities of HK1/pPH28 was zero in both media at all time points, since this strain has quintuple *trp* mutations and requires tryptophan in the medium. B. Semi-logarithmic plots (HK1/pPH28 results omitted because activities were zero).

A



B

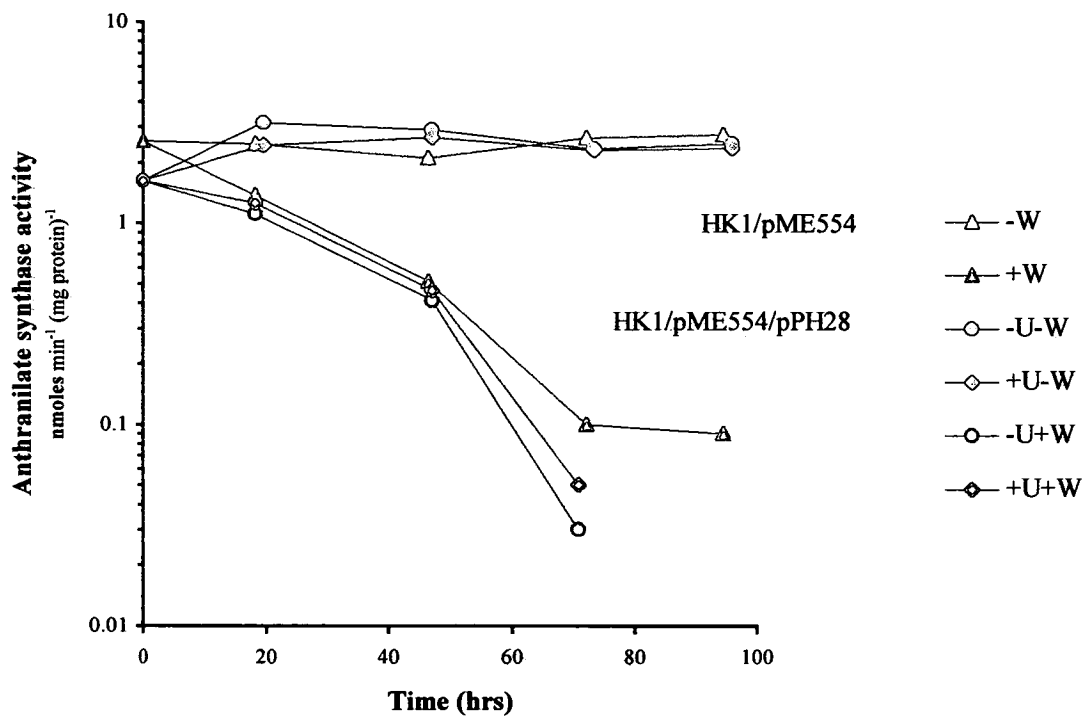
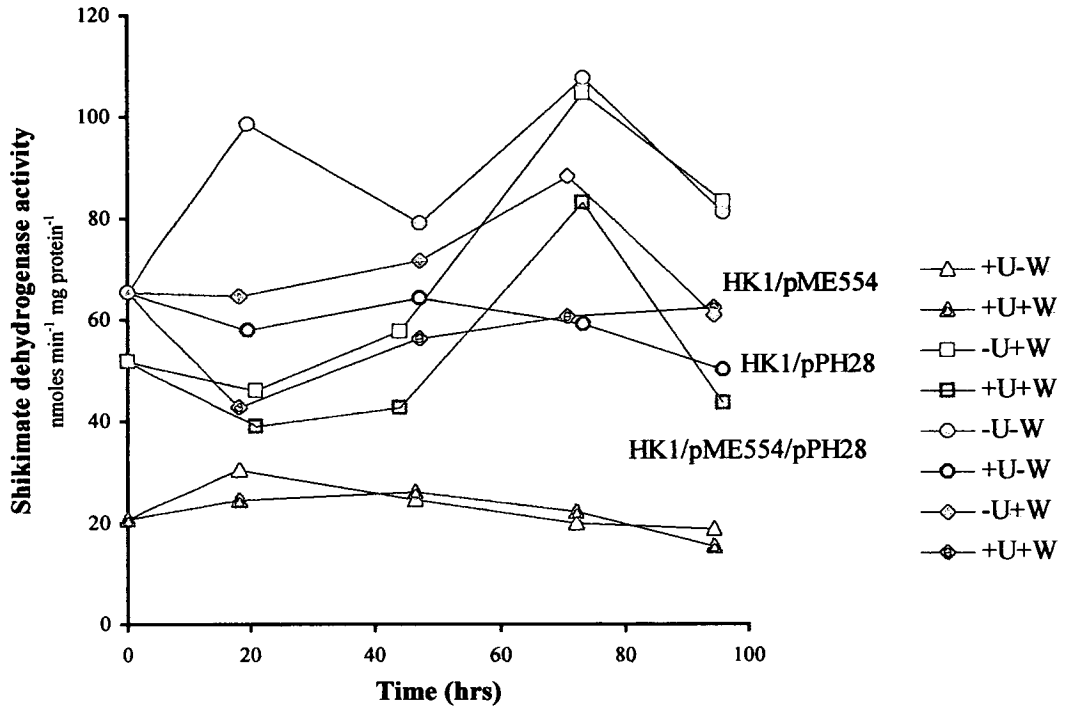
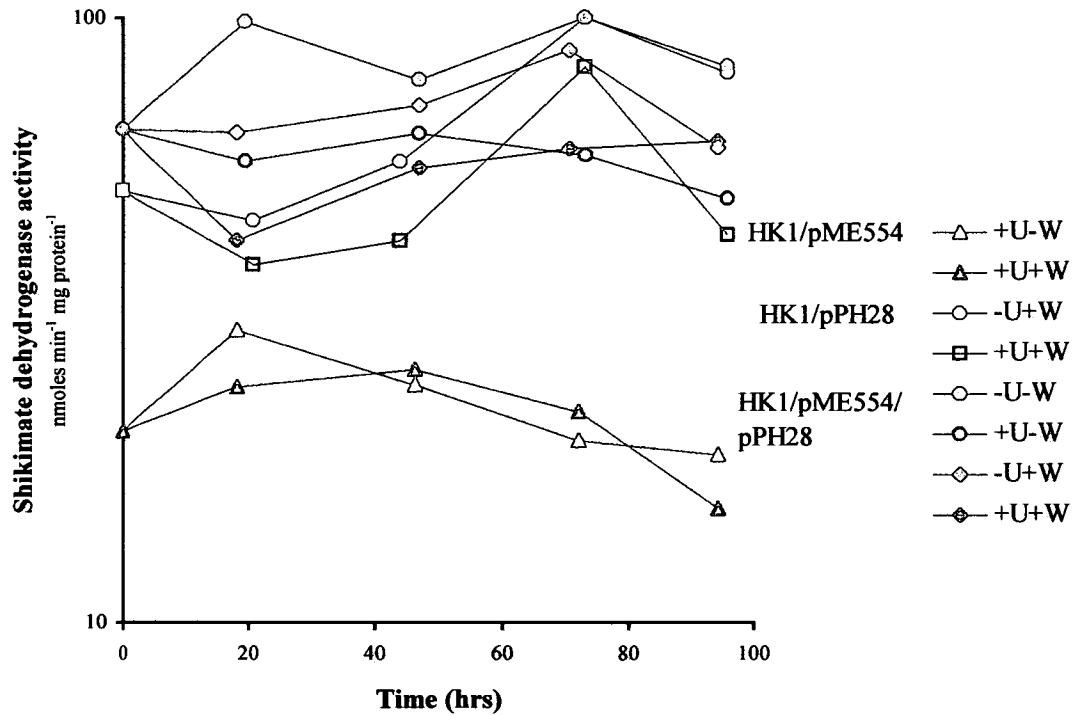


Figure 5-5 Shikimate dehydrogenase activity in single and double transformants +/- selection in MV + LV

A. Linear plots. B. Semi-logarithmic plots.

A**B**

tryptophan prototrophy and anthranilate synthase activity. Comparing the semi-logarithmic plot for anthranilate synthase activity and loss of tryptophan prototrophy gives an indication of the degree of correspondence between the two processes. Enzyme activity loss over the first 48 hours appears to be at a similar rate to the loss of prototrophy (0.08/generation). Therefore, to a first approximation, the average copy number may remain constant over this phase of the culture. After 48 hours however, anthranilate synthase activity appears to be lost at a faster rate than prototrophy. It might be concluded that copy number of pME554 diminishes from this point. However, since the levels of anthranilate synthase activity are very low, there is an increase in the errors which may be assigned to these measurements. A resolution of this point requires a further study using more samples to reduce error in prototrophy estimations and more frequent sampling for enzyme determinations.

Shikimate dehydrogenase

The changes over time in the activity of SDH, encoded by *ARO2*, are shown in Figure 5-5. Firstly, it can be seen that strains HK1/pPH28 and HK1/pME554/pPH28 show increased levels of SDH relative to the strain (HK1/pME554) not transformed with pPH28. The factor by which enzyme levels are increased range from 2-5.

Under selective conditions, there is no observed reduction in SDH activity over time, as expected. This result suggests that the copy number of pPH28 remains stable and that the level of expression from the plasmid-borne gene remains constant. Neither was there any change in SDH activity over time in the strain HK1/pME554. This was as expected, since expression occurs entirely from the chromosomal gene. On the other hand when selection was relaxed in strains bearing pPH28, a different pattern emerged from that observed with pME554 and anthranilate synthase activity. No significant loss of SDH activity was observed as the strains lost plasmid over time. It is impossible to ascertain the contribution of factors such as differential instability of *URA3* and *ARO1* mRNA or the corresponding proteins, if indeed, the result were repeatable. Measurement of other enzymes encoded by the other genes on pPH28 might resolve this question.

5.1.5 Summary

Single and double transformants bearing pME554 and/or pPH28 were cultured under all relevant selective and non-selective conditions.

The results show

- that the plasmids were retained under selective conditions.
- that the multi-copy *TRP* plasmid, pME554, was lost when selection was relaxed: mitotic instability was estimated at about 0.07 - 0.08 generation⁻¹.
- that the single-copy centromeric *ARO* plasmid, pPH28, was lost at a slower rate: instability was estimated at about 0.02 generation⁻¹.
- that anthranilate synthase activity matches the loss of plasmid very closely, at least over the first two days. This suggests that copy number for pME554 is maintained.

5.2 Stability of pME554 and pPH28 in media containing phenylalanine and tyrosine (MV + LVFY)

The previous work of Niederberger and colleagues showed that the stability of *TRP* plasmids was improved in media containing phenylalanine and tyrosine, even under selective conditions. Since it was deemed highly desirable to be able to measure fluxes to tyrosine and phenylalanine in order to evaluate the universal method, it was important to evaluate if the omission of phenylalanine and tyrosine from the media would lead either to unacceptable losses in stability or to losses in the enzyme activities encoded by the plasmids. The rate of loss of the multi-copy *TRP* plasmid, pME554, and of anthranilate synthase activity was therefore of particular interest.

The protocol for this series of cultures is identical to that for the previous section 5.1 except that phenylalanine and tyrosine were added to all media at a concentration of 100 µg ml⁻¹.

The results of the plate counts expressed as percentage of total cells possessing prototrophy for uracil and/or tryptophan are shown graphically in Figures 5-6, 5-7 and 5-8.

5.2.1 Stability of the *TRP* plasmid, pME554 in MV + LVFY

The data relating to the loss of pME554 are shown in Figure 5-6. Under selective conditions, the plasmid is maintained. As with media lacking phenylalanine and tyrosine, although there is considerable variability in the percentage of cells with tryptophan prototrophy, there appears to be no downward trend. When selection is relaxed, a progressive loss of prototrophy occurs after an apparent initial 'lag' phase in the first 24 hours. As shown in Table 5-2, the rates of loss in the case of both the single transformant and of the double transformant lacking selection of both uracil and tryptophan prototrophy are similar (0.08 and 0.07 generation⁻¹, respectively) to that for the loss of plasmid in media lacking phenylalanine and tyrosine. For the plasmid in the double transformant under conditions of selection for the *ARO* plasmid, pPH28 the rate of loss was 0.06 . This may represent a small reduction in the rate of loss of plasmid but further experiments would be needed to achieve results showing statistical significance. If the result were confirmed, it may provide evidence that the presence of the *ARO* plasmid overexpressing the enzymes of the shikimate pathway was able, under some conditions, to stabilise the multi-copy *TRP* plasmid. An apparent improvement in the stability of pME554 in the double transformant under uracil selection could arise through the slower growth rate observed with HK1/pPH28. This could allow an increase in proportions of cells containing pME554 over those which do not.

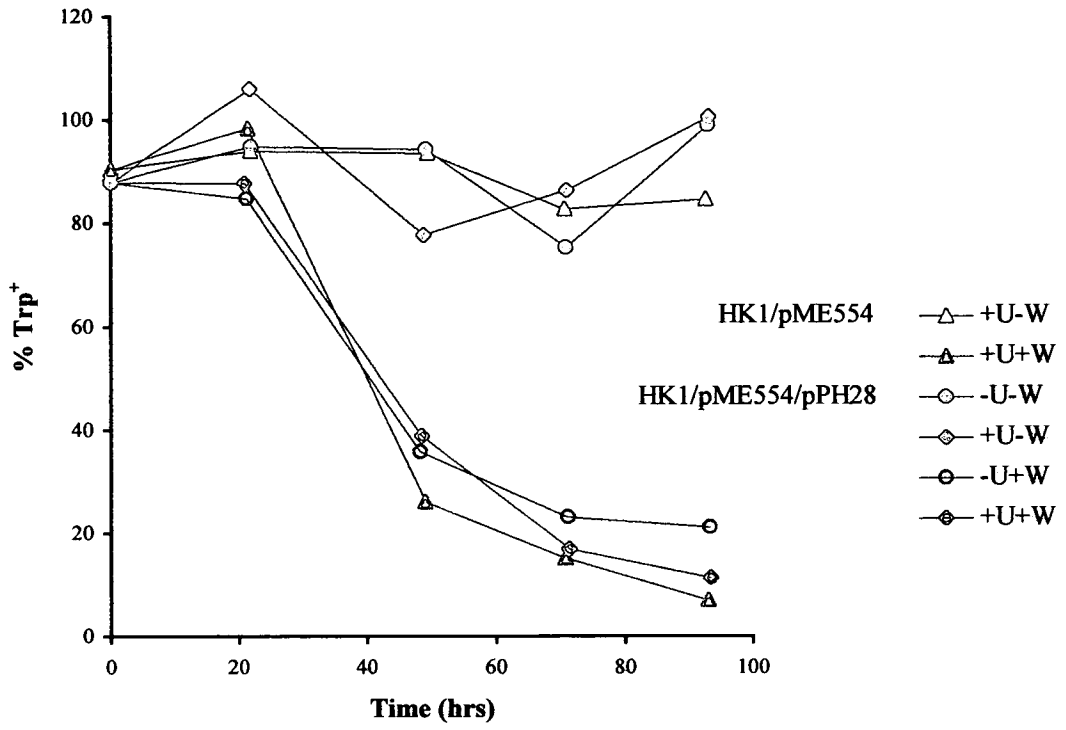
5.2.2 Stability of the *ARO* plasmid, pPH28, in MV + LVFY

Graphs showing the loss of uracil prototrophy are shown in Figure 5-7. There was no apparent loss of pPH28 during uracil selection, as expected. When selection was relaxed however, plasmid was lost progressively. The rates of plasmid loss per generation are shown in Table 5-2. The rates of loss of pPH28, 0.02 generation⁻¹, obtained in the single transformant and in the double transformant grown under

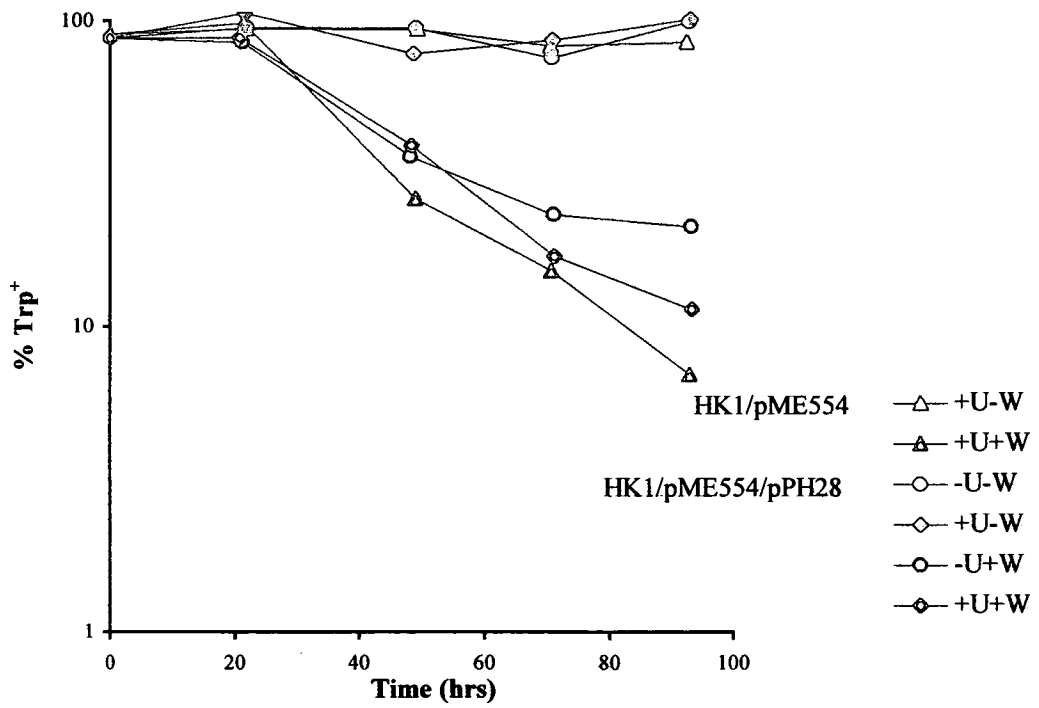
**Figure 5-6 Stability of pME554 in HK1/pME554 and
HK1/pME554/pPH28 in MV + LVFY(100)**

A. Linear plot B. Semi-logarithmic plot

A



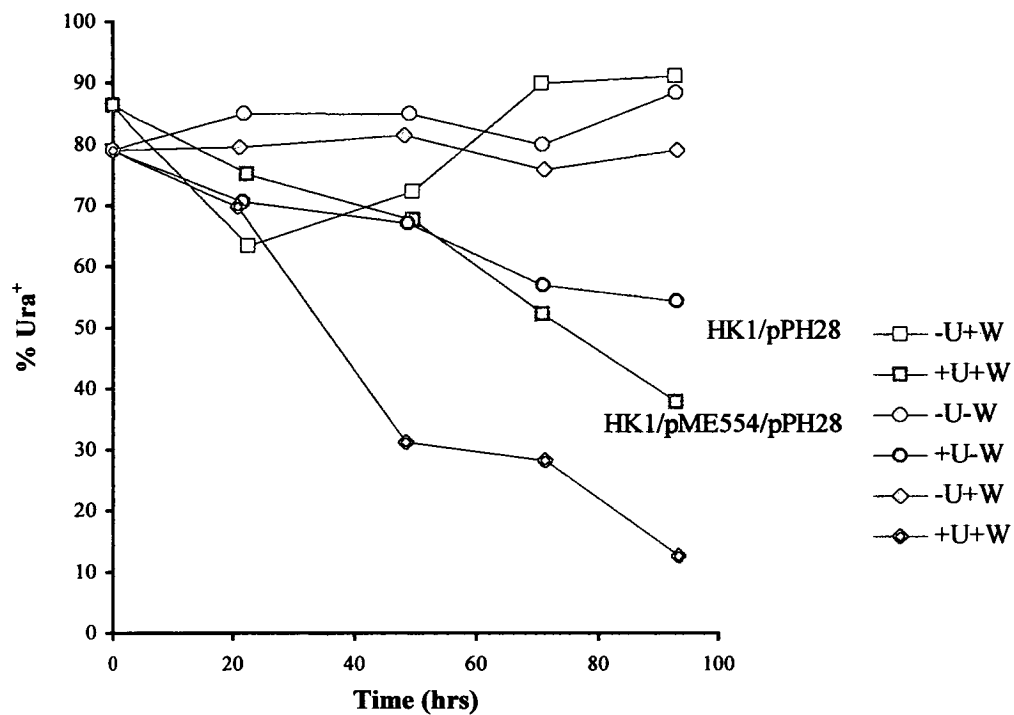
B



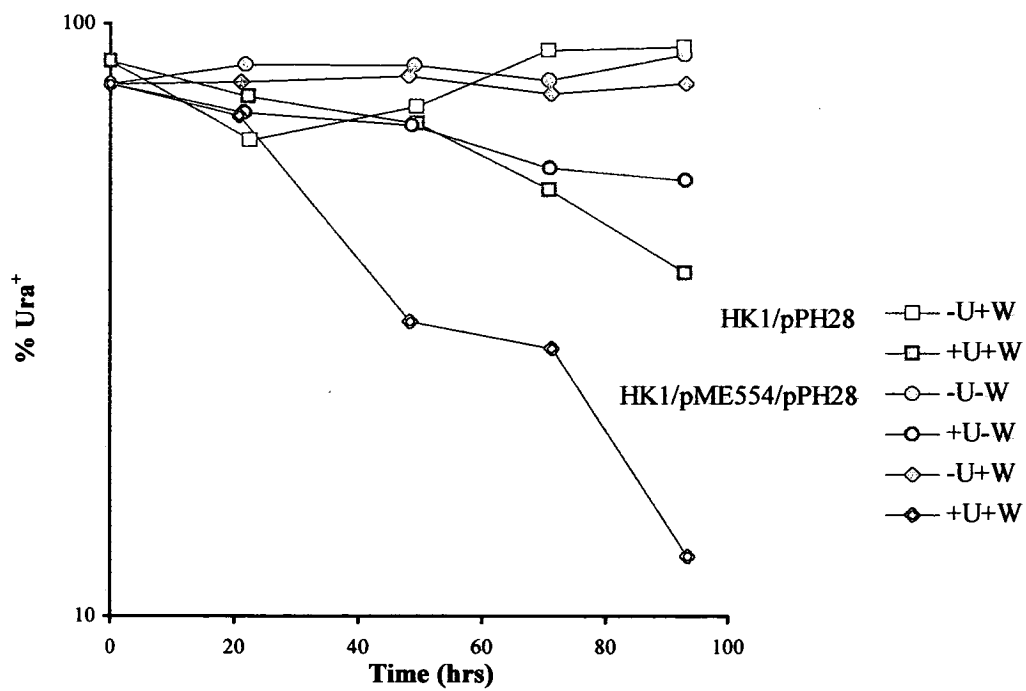
**Figure 5-7 Stability of pPH28 in HK1/pPH28 and
HK1/pME554/pPH28 in MV + LVFY(100)**

A. Linear plot B. Semi-logarithmic plot

A

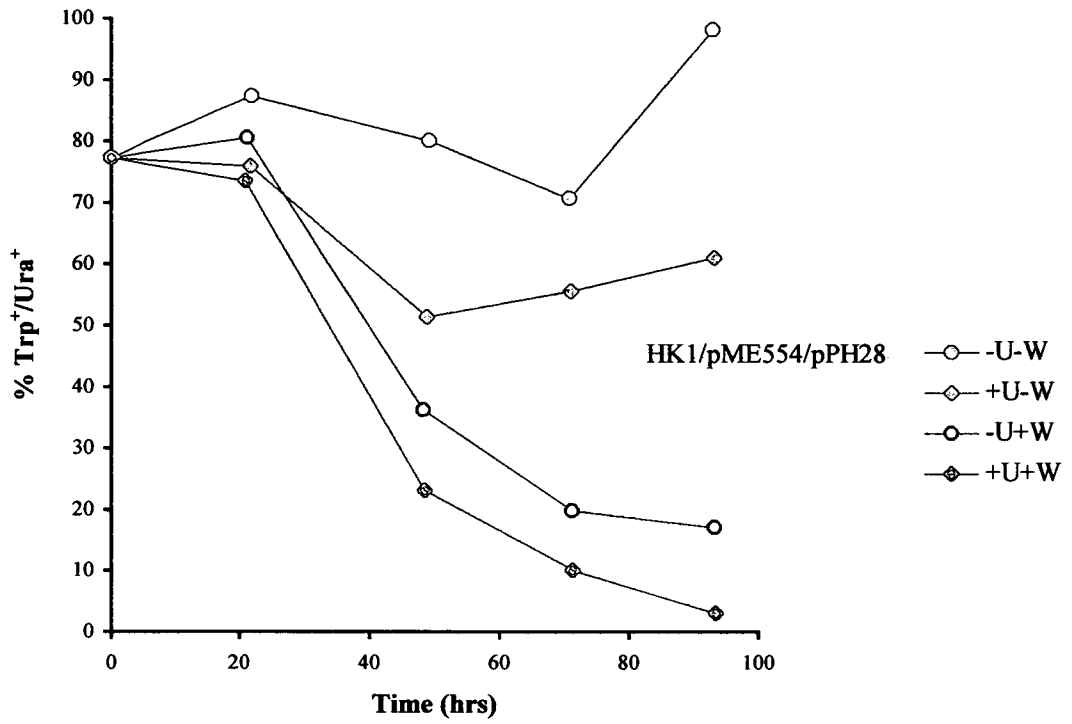
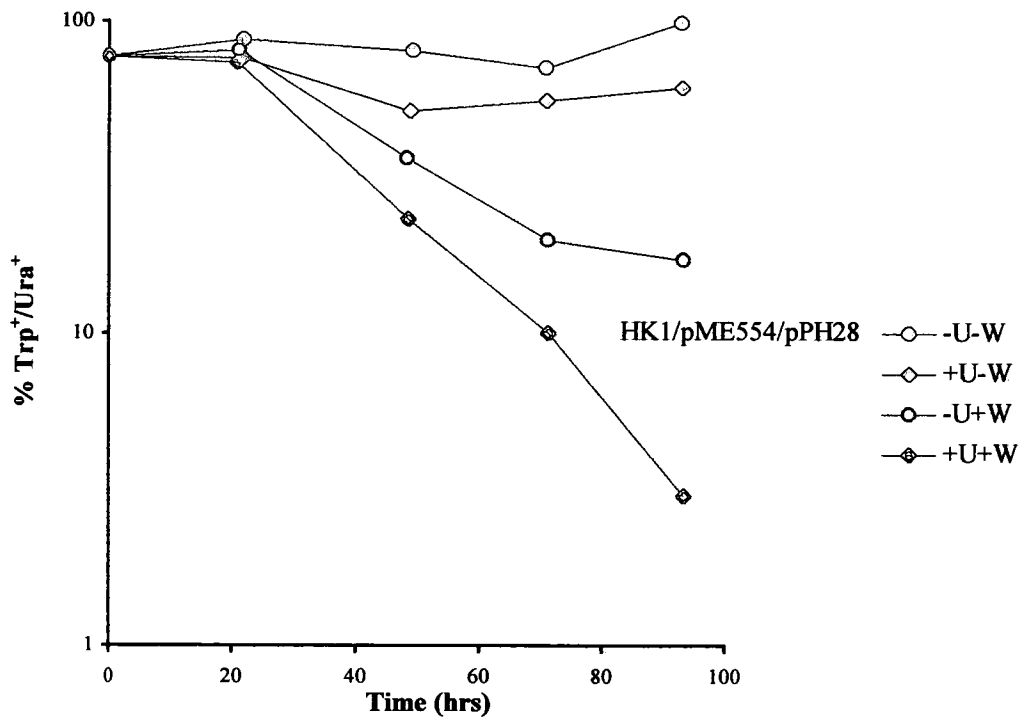


B



**Figure 5-8 Stability of pME554/pPH28 in HK1/pME554/pPH28
in MV + LVFY(100)**

A. Linear plot B. Semi-logarithmic plot

A**B**

A

Host strain for pME554	HK1/pME554	HK1/pME554/pPH28	
Non-selective medium used	MV+LVFY + W + U	MV+LVFY + W - U	MV+LVFY + W + U
Mitotic instability	0.08	0.06	0.07

B

Host strain for pPH28	HK1/pPH28	HK1/pME554/pPH28	
Non-selective medium used	MV+LVFY + W + U	MV+LVFY - W + U	MV+LVFY + W + U
Mitotic instability	0.02	0.02	0.05

C

Host strain for both plasmids	HK1/pPH28/pPH28		
Non-selective medium used	MV+LVFY + W - U	MV+LVFY - W + U	MV+LVFY + W + U
'Mitotic instability'	0.06	0.01	0.10

Table 5-2 Mitotic stability of pME554 and pPH28 in single and double transformants in non-selective media containing phenylalanine and tyrosine

A. pME554 in single and double transformants, B. pPH28 in single and double transformants, C. Loss of double prototrophy in the double transformant when grown upon 3 different types of non-selective media

tryptophan selection is similar to the result obtained for the stability in media lacking phenylalanine and tyrosine. However, there appears to be an increase in instability of pPH28 under conditions where the selection of pME554 is also relaxed. The instability for pPH28 under these conditions was 0.05 which is about half the value obtained for the loss of pME554. This result seems to complement the increased stability of pME554 in the double transformant when selection for pPH28 was maintained and suggests that there is an interaction between the two plasmids such that maintenance of selection on pPH28 increases the stability of pME554 and relaxing the selection on pME554 decreases the stability of pPH28. However, the results may also be explained by the fact that the growth rate of HK1/pPH28 is significantly lower than both HK1/pME554 and HK1/pME554/pPH28. This means that cells losing pME554 from the double transformant become slower growing and therefore are outgrown by cells containing both plasmids, pME554 and no plasmids. With both uracil and tryptophan in the media, when the percentage of Trp⁺, Ura⁺ cells are diminishing, and the Trp⁻, Ura⁺ cells are slow growing, the proportion of Ura⁺ cells may fall particularly rapidly.

5.2.3 Stability of the Trp⁺, Ura⁺ phenotype (pME554 + pPH28)

Graphs showing the trends in these data are shown in Figure 5-8.

This shows an apparent lag phase of at least 24 hours prior to loss of plasmid. Thereafter, the rates of loss of either/both plasmid(s) (as represented by the loss of the Trp⁺, Ura⁺ phenotype) calculated from the semi-logarithmic plots are given in Table 5-2. The rate for the loss of either plasmid when uracil selection is relaxed, 0.01 generation⁻¹, is similar to the loss of pPH28 from the double transformant under the same conditions. It is therefore likely that the value for the rate of loss of the Trp⁺, Ura⁺ phenotype is entirely accounted for by the loss of pPH28. Similarly, when tryptophan selection is relaxed, the loss of either plasmid, 0.06 generation⁻¹, is similar to the loss of pME554 from the double transformant under the same growth conditions. It is therefore probable that the value of the rate of loss of the Trp⁺, Ura⁺ phenotype is entirely accounted for by the loss of pME554. When selection for both plasmids is relaxed, the loss of the Trp⁺, Ura⁺ phenotype appears to increase to 0.10

generation⁻¹, close to the maximum rate for loss of pME554 alone. The probable reason for this, as discussed previously, is the possible underestimate of colonies on plates containing neither uracil nor tryptophan because of the slower growth of cells containing pPH28 alone.

5.2.4 Enzyme activities

In order to explore the maintenance of enzyme activities during culture and to evaluate whether copy number changed during loss of plasmid, the activities of anthranilate synthase, encoded on pME554 and SDH, encoded by pPH28 were assayed. The results are shown in Figure 5-9 and 5-10

Anthranilate synthase

Anthranilate synthase activities are maintained at high levels in cultures in which selection for pME554 is enforced by the absence of tryptophan in the growth medium. There appears to be a consistent increase in activity after Day 0. This may result from an underestimation of anthranilic acid at Day 0

When selection for pME554 was relaxed, the enzyme activity dropped at a similar rate to that of loss of prototrophy. As in the previous experiment, this suggests that copy number is maintained during the experiment.

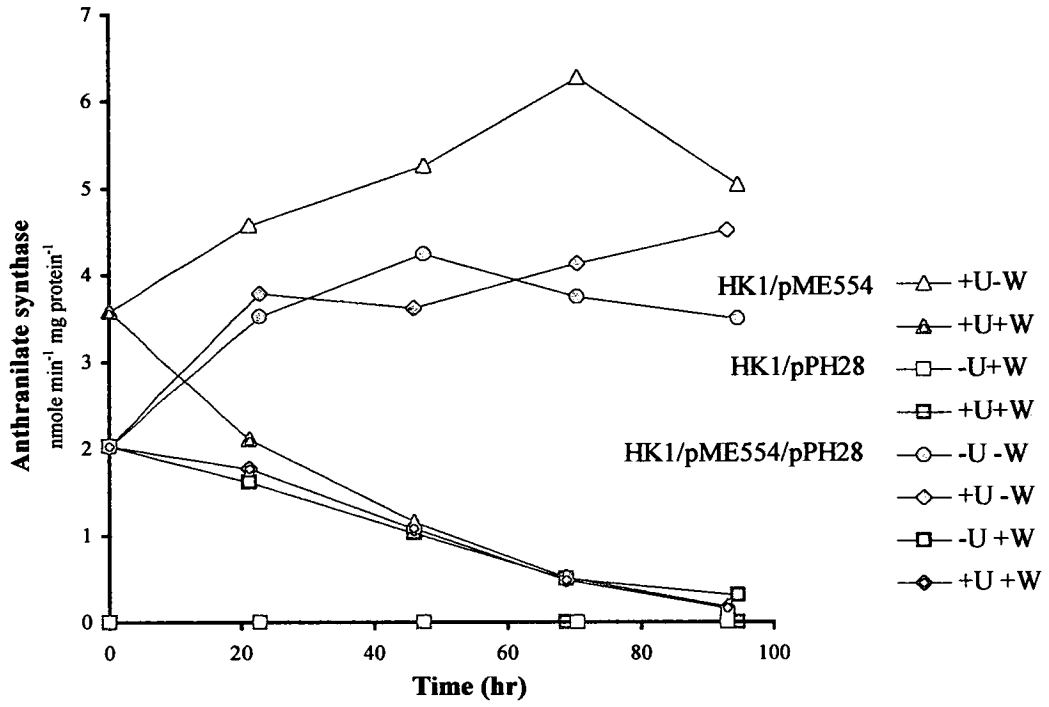
Shikimate dehydrogenase

Estimates of SDH activity in these cultures are shown in Figure 5-10. These data show that SDH activities were not reduced over time, regardless of the presence or absence of uracil in the media. These results therefore show the same pattern as in the previous experiment where tyrosine and phenylalanine are omitted from the media. The possible explanations for this phenomenon are similar. They include the possibility of high enzyme stability or maintained synthesis of protein from stable mRNA in cells which no longer carry pPH28. This stability would have to be significantly greater than that of the *URA3* gene product, orotidine-5'-phosphate decarboxylase. However, further experiments are required to substantiate this claim and to investigate the factors involved.

Figure 5-9 Anthranilate synthase (specific activity) in single and double transformants +/- selection in MV + LVFY

A. Linear plots. Note that the activities of HK1/pPH28 was zero in both media at all time points, since this strain has quintuple *trp* mutations and requires tryptophan in the medium. B. Semi-logarithmic plots (HK1/pPH28 results omitted because activities were zero).

A



B

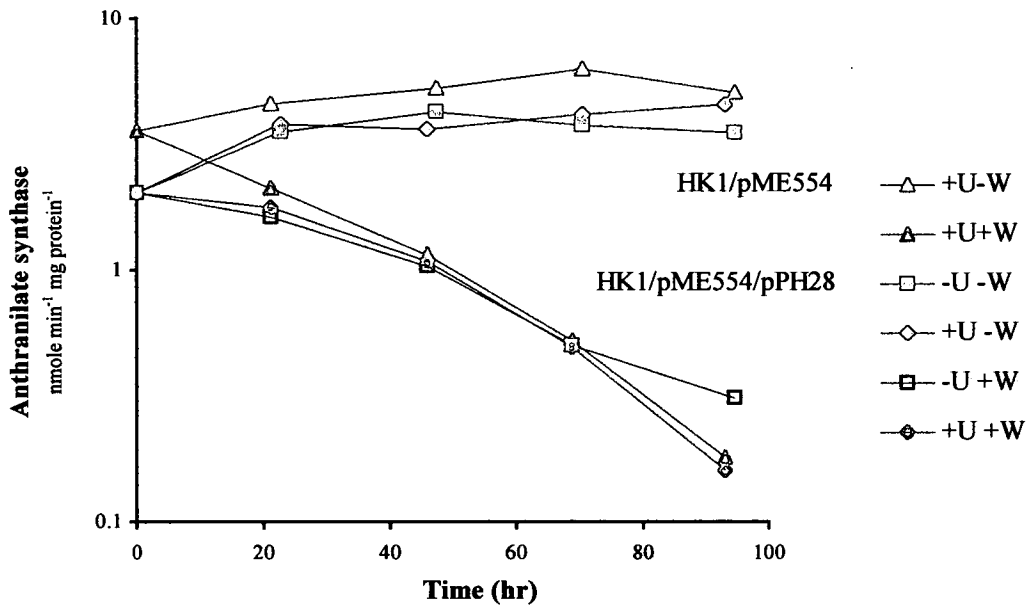
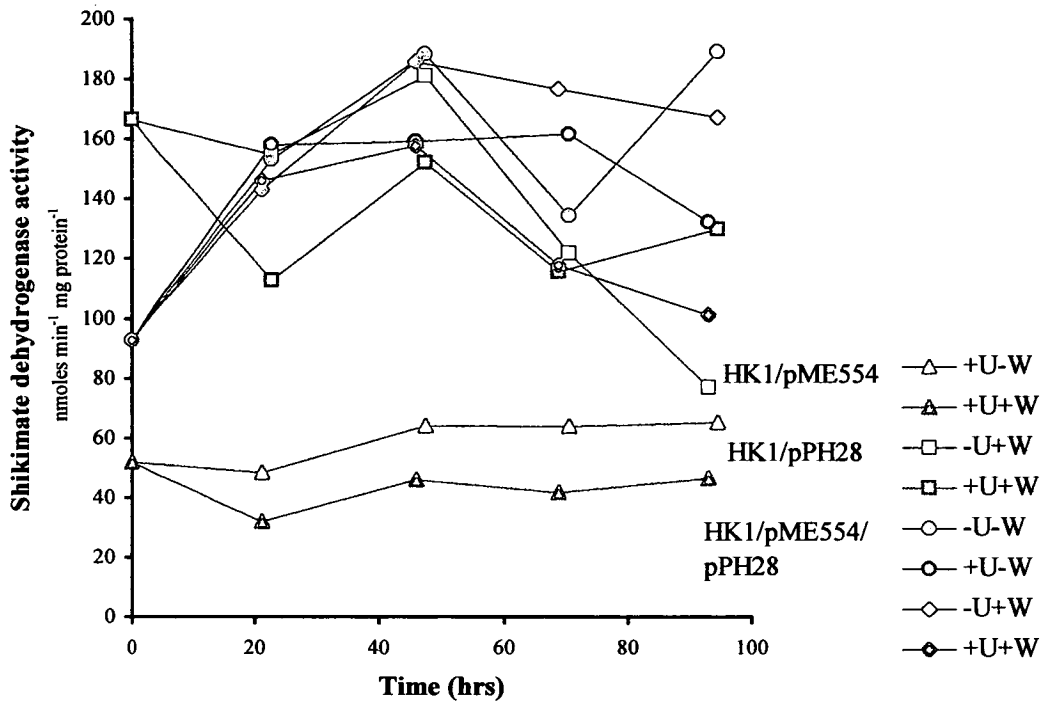


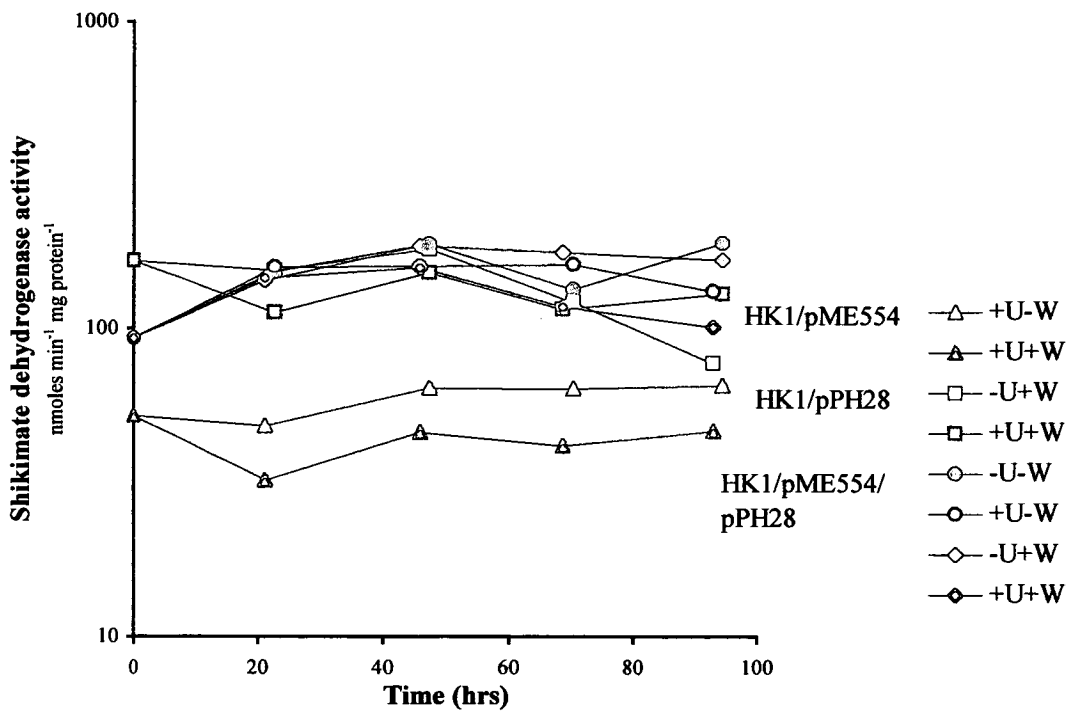
Figure 5-10 Shikimate dehydrogenase (specific activity) in single and double transformants +/- selection in MV + LVFY

A. Linear plots. B. Semi-logarithmic plots.

A



B



5.2.5 Summary

Single and double transformants bearing pME554 and/or pPH28 were cultured under all relevant selective and non-selective conditions in media containing LVFY.

The results show

- that both plasmids are retained under selective conditions.
- that the multi-copy *TRP* plasmid, pME554, is lost when selection is relaxed: mitotic instability was estimated at about 0.06 - 0.08 generation⁻¹.
- that the centromeric *ARO* plasmid, pPH28, was lost when selection was relaxed, but at a slower rate than the *TRP* plasmid: instability was estimated at about 0.02 - 0.05 generation⁻¹.
- that anthranilate synthase activity matches the loss of pME554 very closely, at least over the first two days. This suggests that copy number is maintained. Enzyme activity continued to drop at an increased rate.
- that there may be an interaction between the two plasmids under some conditions, such that the stability of pME554 is increased when selection is maintained upon pPH28.

5.3 Discussion

Before discussing the results in more detail, there are a number of reservations which need to be made concerning the underlying assumptions and limitations of these experiments.

Assumptions and limitations

The data on loss of prototrophy are assumed to give information on the percentage of cells carrying at least one copy of the plasmid in question. This data can be used to calculate the mitotic instability of the plasmid. However, this assumes that the level of selection is constant during the experiment. This is not necessarily so, since a strict

selection is only applied once a day. Neither is there control over the concentration of the selective nutrient within the medium at other times. The nutrient (*e.g.* tryptophan) may be released by excretion or by lysis of cells. Furthermore the growth rate of prototrophic and auxotrophic strains may differ in permissive cultures as well as selective ones. If the loss of one plasmid from the double transformant gives a single transformant which grows more slowly than the other strains, then this will tend to lead to an underestimate for the rate of loss of the first plasmid. This situation applies here. HK1/pPH28 has a significantly lower growth rate than both HK1/pME554 and HK1/pME554/pPH28 under both selective and permissive conditions. Finally, the observed rate of loss of plasmid will depend upon how many times the resulting strain may divide once the auxotrophic marker/plasmid has been lost. This will depend upon factors such as stability of the protein encoded by the auxotrophic marker or its corresponding mRNA, and the complex relationship between the activity of the selective marker (*e.g.* *URA3* gene product), flux in the particular pathway (biosynthesis of pyrimidines) and growth rate. This relationship will not be obvious, *a priori*. Generally, therefore, the cultures are, over time, ill-defined with respect to medium composition, strain composition, homogeneity of growth and detailed dynamic physiology. Although a more rigorous analysis is possible in a series of experiments using chemostats, which at least overcomes the variability of the medium over time, the problem of defining an 'intrinsic' instability is probably intractable.

For most purposes however, it is the estimation of stability in batch culture, and in this project, the resulting stability of enzyme activity, which is important.

The level of enzyme activity within the cell samples has been assumed to reflect the level of expression and hence, the plasmid copy number. A number of other factors may be involved however. For instance, a clear result of both experiments was the maintenance of SDH activity whilst the corresponding *Ura*⁺ prototrophy was decreasing. This can be interpreted in different ways. The copy number in those cells carrying the plasmid may increase at a rate sufficient to offset the loss of plasmid in other cells. Secondly, the stability of the enzyme and/or its mRNA may, at least partially, offset the loss of SDH activity caused by loss of plasmid. Finally, some

other level of regulation of expression may be operating to maintain enzyme activity in the culture as a whole. A number of approaches may be used to clarify this question. For example, the half-life of SDH and/or its mRNA may be estimated by inhibiting protein synthesis and RNA synthesis and compared with that for anthranilate synthase. Alternatively, the demonstration that other *ARO* enzymes, such as chorismate synthase, lose activity in line with the loss of plasmid would support the 'stability' argument. It should also be emphasised that there is a relatively small increase in activity of enzymes of the shikimate pathway relative to a wild-type (chromosomal) background (as opposed to the tryptophan pathway). Both of these factors make the empirical relationship between loss of plasmid and enzyme activity more vulnerable to error and variability.

5.4 Conclusions

Whilst bearing in mind the limitations of the data and the problematic relationships between such factors as strain and media composition, enzyme activity, growth rates etc., the following conclusions are suggested by the results.

Both the plasmids, pME554 and pPH28, are stable under selective conditions. Under permissive conditions, pME554 was lost from both the single and the double transformant with a frequency of between 0.06 and 0.10 generation⁻¹. There was some evidence that the rate of loss from the double transformant grown with selection for pPH28, was reduced to about 0.06 generation⁻¹ in media which contained phenylalanine and tyrosine. This was interpreted either in terms of stability being conferred by pPH28, for example, through metabolic fitness, or, more likely, by the poor growth rate of HK1/pPH28. Under permissive conditions, the stability of pPH28 was higher. However the values for mitotic instability obtained, ranging from 0.01 to 0.05, were higher than expected for a centromeric plasmid. The loss of pPH28 from the double transformant was particularly high, 0.05, when selection for pME554 was also relaxed in media containing phenylalanine and tyrosine. The reasons for these results are likely to include the poor growth rate of the single

transformant, HK1/pPH28, compared to the other strains, HK1, HK1/pME554 and HK1/pME554/pPH28.

The loss of anthranilate synthase activity corresponded with the loss of plasmid pME554 in all cases, although there was some evidence that the loss of activity was progressive, with loss of activity being slower at first and then increasing relative to the loss of plasmid. A loss of SDH activity, on the other hand, was not detected under permissive conditions.

There was little evidence that phenylalanine and tyrosine in the media contributed to the stability of either plasmid.

Chapter 6

Aromatic amino acid production and flux analysis

The previous three chapters describe the construction and characterisation of a single-copy plasmid, pPH28, which bears a copy of each of the genes (*ARO1-4*) encoding the enzymes of the shikimate pathway. Chapter 4 described the effect of this plasmid upon the specific activity of these enzymes in transformed strains. The enzyme multipliers obtained in various strains generally ranged from 2-5. Similarly, a multi-copy plasmid, pME554, constructed by Niederberger and colleagues (Niederberger *et al.* 1984) and bearing a copy of each of the genes (*TRP1-5*) encoding enzymes of the tryptophan pathway, gave enzyme multipliers for anthranilate synthase and tryptophan synthase of between 20 and 70. A double transformant, HK1/pME554/pPH28, gave corresponding levels of overexpression from both plasmids. In Chapter 5 the stabilities of both plasmids and the specific activities of the enzymes encoded, were characterised in single and double transformants and in various selective and non-selective media. These data suggested that expression from these plasmids would be maintained at high levels over a period of 24 hours when grown in selective media, at least during exponential phase..

This chapter presents a limited set of data obtained from batch cultures of the reference and transformed strains which were sampled for enzyme activity and metabolite concentration determinations. Here, a single time point, near to the end of exponential phase, is used to allow an estimation of the fluxes in the three branches of the shikimate pathway from values of the intracellular, extracellular and total pools of the aromatic amino acids. The flux can be calculated in two ways. Firstly, the difference between two closely spaced time points may give rates of production. Secondly, provided that steady state conditions during exponential growth apply, rates of production may be calculated from concentrations at a single time point and the growth rate. Since it was not possible here, because of small concentrations, to make accurate determinations by differences between closely spaced time points, it was necessary to use the second method. Estimations from cultures close to the end

of exponential phase were adjudged most likely to fulfil assumptions of steady state and to yield reasonable metabolite concentrations with least error.

A more extensive analysis of the full data set is given in Chapter 7. It includes an investigation of changes during the 'diauxic shift' and onset of stationary phase, the excretion and re-uptake of amino acids, and the effect of 'bypassing' anthranilate synthase by feeding cells with anthranilic acid.

6.1 The effects of pME554 and pPH28 on aromatic amino acid production

RH805 (the reference strain), HK1/pME554 and HK1/pME554/pPH28 were cultured in MV+LV(100) (+ U(40) for HK1/pME554). Freshly grown isolated colonies were grown in 10 ml of media (MV+LVFY(100) +/- U(40)) containing phenylalanine and tyrosine (starter culture) prior to transfer to 'pre-cultures', in shake flasks, consisting of 100 ml of the same media but lacking phenylalanine and tyrosine. Cells were pelleted and transferred to 400 ml 'main' cultures which used this same media, as described fully in Chapter 2.

The optical densities of starter cultures were between 0.41 and 1.01, depending upon the strain, at inoculation of 100 ml pre-cultures. These were grown overnight to an OD₅₄₆ of 0.76 - 1.27. At this point, cells were used to inoculate 400 ml cultures at time $t=0$, to an OD₅₄₆ of 0.170.

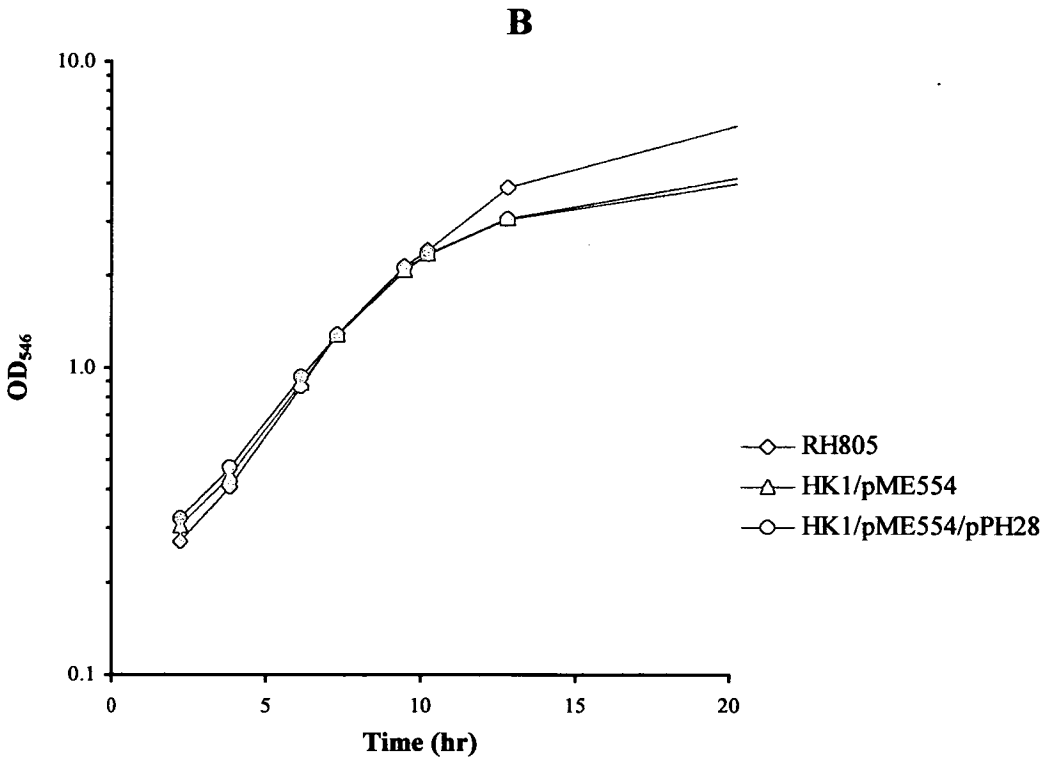
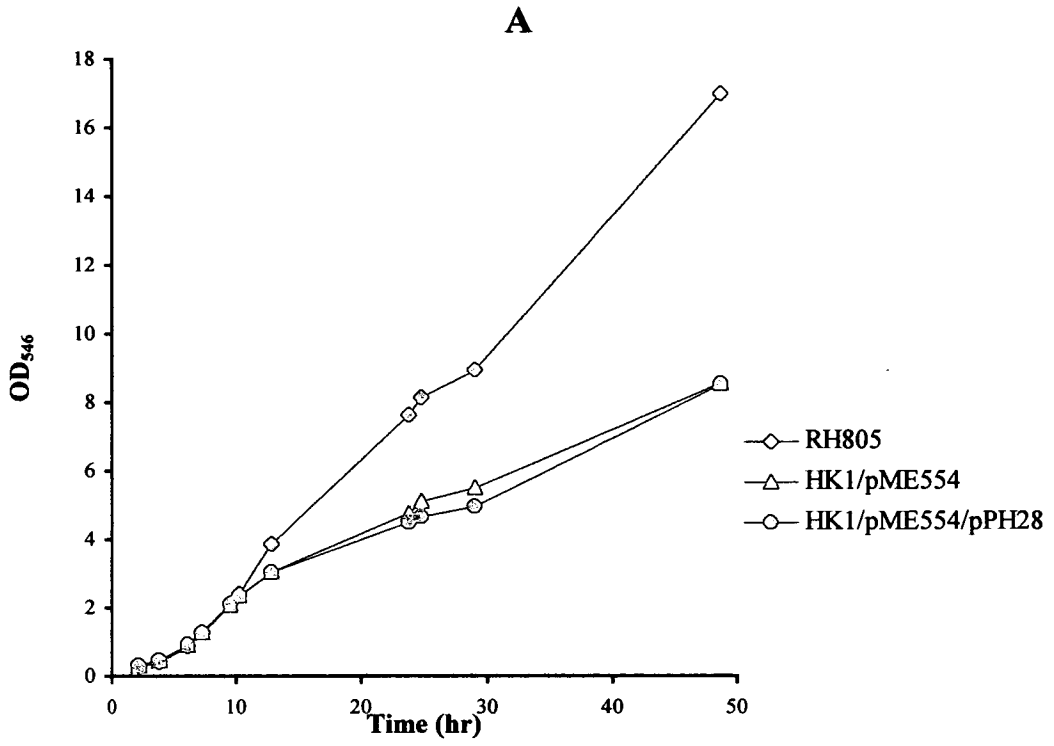
Optical densities were monitored and samples taken at intervals for the determination of enzyme activities, protein concentrations and intracellular, extracellular and total tryptophan concentrations.

6.1.1 Growth of cultures.

The optical densities of the main cultures are plotted in Figure 6-1. The semi-logarithmic plots allow the estimation of growth rates (μ , hr⁻¹) during exponential phase. These were 0.31 for RH805, 0.30 for HK1/pME554 and 0.29 for HK1/pME554/pPH28. The data show evidence of a 'diauxic shift' after about 12

Figure 6-1 Growth of strains on MV + LV(100)

A. The growth of strains on linear scale. **B.** Growth of strains during the first 20 hours only, on a semi-logarithmic plot



hours ('mid-culture'), where cell growth transiently slowed, due to the exhaustion of glucose. This was followed by a subsequent adaptation to respiratory metabolism, during which the growth rate increased again. A discussion of these changes is included in Chapter 7.

The optical densities of the cultures after 10 hours were 2.41 for RH805, 2.35 for HK1/pME554 and 2.33 for HK1/pME554/pPH28. Reference to Figure 6-1 shows that these OD₅₄₆ occurred towards the end of the exponential phase prior to the 'diauxic shift'. The following analysis of enzyme activities and metabolite concentrations concerns samples taken at this time point only. The full data set is given and analysed in Chapter 7.

6.1.2 Enzyme activities

The specific and relative activities of SDH and anthranilate synthase in the three strains after 10 hours are given in Table 6-1. The activity of SDH was raised 2.7-fold in HK1/pME554/pPH28 and anthranilate synthase was overexpressed by a factor of between 38 - 46 in both of the strains transformed with pME554, all relative to activities in RH805. These factors are similar to those reported in the previous chapter and indicate that the copy number of pPH28 and pME554 were within the required, and expected range.

6.1.3 Tryptophan concentrations

The concentrations of intracellular, extracellular and total tryptophan (for a reminder of the terminology used to identify concentrations, please consult Section 2.8.2) at 10 hours are given in Table 6-2, along with the data for phenylalanine and tyrosine. The estimated intracellular concentrations are calculated from internal pools as described in Chapter 2. Also included are the sums of the experimentally determined internal pools and extracellular concentrations, for comparison with the experimentally determined total concentrations. There was usually good agreement (within experimental error) between these two values.

Enzyme activities

Strain	Shikimate dehydrogenase		Anthranilate synthase	
	Specific activity nmol • min ⁻¹ • mg protein ⁻¹	Relative activity	Specific activity nmol • min ⁻¹ • mg protein ⁻¹	Relative activity
RH805	39.5	1.00	0.02	1.0
HK1/pME554	36.3	0.92	0.94	45.7
HK1/pME554/pPH28	107.4	2.72	0.78	38.2

Table 6-1 Enzyme activities

The specific activity of shikimate dehydrogenase and anthranilate synthase in strains RH805, HK1/pME554 and HK1/pME554/pPH28 after 10 hours of culture in MV + LV(100) (+ U(40) for HK1/pME554) are given. Relative activities are calculated with respect to RH805 at this time point, and are therefore dimensionless.

Metabolite	Strain	Intracellular pool		Extracellular pool	Internal + extracellular pool	Total pool measured from culture
		internal pool	intracellular concentration			
Concentration (μM)						
Tryptophan	RH805	0.07	50	0.06	0.13	0.16
	HK1/pME554	0.10	70	0.06	0.15	0.16
	HK1/pME554/pPH28	0.15	110	0.11	0.26	0.25
Phenylalanine	RH805	0.59	410	0.56	1.15	1.10
	HK1/pME554	0.49	350	1.06	1.55	1.48
	HK1/pME554/pPH28	1.30	930	3.85	5.15	5.83
Tyrosine	RH805	0.97	670	1.13	2.10	1.90
	HK1/pME554	0.75	530	1.35	2.09	1.95
	HK1/pME554/pPH28	2.97	2130	3.03	6.00	7.16

Table 6-2 Amino acid concentrations in late exponential phase

This data was obtained from main cultures when the OD_{546} lay between 2.33 and 2.41 (i.e. at the end of exponential phase). The intracellular pools are expressed with respect to culture volume (internal pool) and cell volume (intracellular concentration), as described in Section 2.8.2. The intracellular pool and extracellular pools may be summed to give an estimated total pool, which may be compared with the actual total pool given in the final column.

Intracellular tryptophan

The internal tryptophan pools (*i.e.* concentrations with respect to culture volume, as described in Chapter 2) were 0.07, 0.10 and 0.15 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. The intracellular tryptophan concentrations (*i.e.* concentrations with respect to cell volume, calculated from the internal pool and OD_{546} , as described in Chapter 2) for RH805 (50 μM) was about twice the estimate (20 - 30 μM) previously made (Fantes *et al.*, 1976). pME554 gave an increased value of 70 μM . Subsequent transformation with pPH28 increased the intracellular concentration two-fold to 110 μM .

Extracellular tryptophan

Estimation of excreted tryptophan gave 0.06, 0.06 and 0.11 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. These concentrations are low and are therefore subject to large errors in their determination. However, they suggest that cells in all strains may excrete tryptophan into the media, even at normal intracellular tryptophan concentrations.

Total tryptophan

The total culture concentrations of tryptophan were 0.16, 0.16 and 0.25 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. These values give good agreement with the estimations based on the sum of the independently determined internal pools and extracellular concentrations. Thus, pME554 led to little, if any, increase in tryptophan concentrations, while the additional presence of pPH28 increased the concentration of tryptophan nearly two-fold.

Summary

These results show that overexpression of the tryptophan enzymes, by a factor of about 50, did not greatly increase tryptophan concentrations, if at all, but that the additional overexpression of the shikimate enzymes, by a factor of 2-5, did increase tryptophan concentrations. The resulting pool sizes were nearly twice those of the untransformed strain.

6.1.4 Phenylalanine concentrations

Intracellular phenylalanine

The internal pools of phenylalanine were 0.59, 0.49 and 1.30 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. The corresponding values for the estimated intracellular concentrations are 410, 350 and 930 μM , compared to the value of 600 μM reported by Messenguy *et al.* (1980). Therefore, the overexpression of the tryptophan enzymes gave little change in the concentration of phenylalanine. The additional overexpression of the shikimate enzymes increase concentrations to double that of the reference strain.

Extracellular phenylalanine

The extracellular concentration of phenylalanine increased from 0.56 to 1.06 μM when the tryptophan enzymes were overexpressed and to 3.85 μM when the shikimate enzymes were also overexpressed. As with tryptophan, all strains excreted phenylalanine. However, excretion appears to have increased, relative to RH805, in the single transformant by a small amount, and in the double transformant by a much greater factor.

Total phenylalanine

The values for total phenylalanine concentrations in RH805, HK1/pME554 and HK1/pME554/pPH28 were 1.1, 1.48 and 5.83, respectively. Again, the agreement between the sum of intracellular and extracellular determinations and that of the total concentration is good. The overexpression of the tryptophan enzymes increased the total phenylalanine concentrations within the culture by a small factor. A further small overexpression of the shikimate pathway enzymes led to a large increase in phenylalanine concentrations (5 times that of the reference strain).

Summary

These results indicate that the intracellular concentration of phenylalanine was unchanged when tryptophan enzyme activities were increased. When the shikimate enzymes were also overexpressed, the intracellular pool increased by a factor of about 2. Extracellular and total pools of phenylalanine were increased in both the single

and, especially, the double transformants. Thus both of these strains showed increased excretion of phenylalanine, compared to that of RH805.

6.1.5 Tyrosine concentrations

Intracellular tyrosine

The internal tyrosine pools were 0.97, 0.75 and 2.97 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively, and these may be used to calculate estimated intracellular concentrations of 670, 530 and 2130 μM . The value of 670 μM for RH805 is similar to the value of 500 μM given by Messenguy *et al.* (1980). The data show that intracellular tyrosine concentrations were largely unchanged by the overexpression of the tryptophan enzymes, but were increased more significantly (factor of 3-4), by the simultaneous overexpression of the shikimate enzymes. These factors are quite similar to those obtained with phenylalanine.

Extracellular tyrosine

Values for the extracellular tyrosine concentrations were 1.13, 1.35 and 3.03 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. These results, again, show a very similar pattern to that seen with phenylalanine, pPH28 giving a significant increase in the extracellular pool.

Total tyrosine

The total concentrations of tyrosine within the cultures were 1.90, 1.95 and 7.16 μM for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. As with tryptophan and phenylalanine, there is good agreement between the sums of the internal and extracellular pools and the value for the total pool. The overexpression of enzymes of the tryptophan pathway did not appear to change the total tyrosine pool. The overexpression of the shikimate enzymes resulted in a 3 - 4-fold increase in tyrosine concentration.

Summary

These results indicate that intracellular concentrations of tyrosine were essentially unchanged when tryptophan enzyme activities were increased. When the shikimate

enzymes were also overexpressed, the intracellular, extracellular and total concentrations increased. The results are very similar to those of phenylalanine.

6.1.6 Flux estimations

The estimation of pathway flux may be achieved in two ways. Firstly, appropriate concentrations of metabolites and protein concentrations may be measured at two closely spaced time points. Secondly, if the culture approaches a steady state, one time point will suffice. In this case, the concentrations of the various pools may be multiplied by the growth rate and a factor to account for protein concentration. Both methods require knowledge of the concentration of amino acids in protein. Because the changes in pool size (especially tryptophan) in the present experiment were small, the determination of concentrations in the various pools at closely spaced times was error-prone and adjudged inadvisable. Therefore, the second option was adopted here. The method by which fluxes were calculated is given in Chapter 2. The protein concentrations in cultures measured in the course of this study lay between 150 and 170 mg l⁻¹ at OD₅₄₆ = 1, in contrast to the value of 80 mg ml⁻¹ given by Fantes *et al.* (1976). This discrepancy may be due to differences in spectrophotometric estimation of cell density, calibration of protein assay with different grades of BSA, variations in procedure of protein determination etc. Since there was no strain variation in the protein concentration at OD₅₄₆ = 1, all calculations were carried out using a protein concentration of 160 mg l⁻¹ at OD₅₄₆ = 1.

The fluxes calculated from the amino acid composition of yeast proteins (Cortessa *et al.*, 1995) and the concentrations of amino acids in the intra- and extracellular compartments, as determined above, are shown in Table 6-3. These may be summarised as follows.

- The total flux in the shikimate pathway in all three strains amounted to nearly 3 nmol min⁻¹ mg protein⁻¹. This figure compares closely with the value of Cortessa *et al.* (1995) who calculate the flux through erythrose-4-phosphate at 0.092 mmol hr⁻¹ g dry wt.⁻¹ for growth on glucose. Using their factor of

Metabolite	Strain	Flux nmol • min ⁻¹ • mg protein ⁻¹			% of shikimate flux
		To protein	To metabolite pool (Accumulation rate)	Total	
Tryptophan	RH805	0.31	0.002	0.31	10.4
	HK1/pME554	0.30	0.002	0.30	10.4
	HK1/pME554/pPH28	0.29	0.003	0.29	10.1
Phenylalanine	RH805	1.50	0.015	1.51	50.7
	HK1/pME554	1.44	0.021	1.46	50.7
	HK1/pME554/pPH28	1.39	0.066	1.45	50.5
Tyrosine	RH805	1.13	0.028	1.16	38.9
	HK1/pME554	1.09	0.028	1.12	38.9
	HK1/pME554/pPH28	1.05	0.077	1.13	39.3
Prephenate	RH805	2.63	0.044	2.67	89.6
	HK1/pME554	2.53	0.048	2.58	89.6
	HK1/pME554/pPH28	2.43	0.144	2.58	89.9
Shikimate	RH805	2.94	0.045	2.98	100
	HK1/pME554	2.83	0.050	2.88	100
	HK1/pME554/pPH28	2.72	0.147	2.87	100

Table 6-3 Fluxes in tryptophan, phenylalanine and tyrosine pathways during late log phase

This data was obtained from main cultures when the OD₅₄₆ lay between 2.33 and 2.41 (*i.e.* at the end of exponential phase).

- 0.21 mg dry wt. ml⁻¹ culture at 1 unit OD₅₄₆, this is equivalent to 2.01 nmol • min⁻¹ mg protein⁻¹.
- The total flux to tryptophan in RH805 was 0.31 nmol min⁻¹ mg protein⁻¹.
- The relative proportions of this total flux attributed to the tryptophan, phenylalanine and tyrosine branches in all three strains were approximately 10%, 50% and 40%, respectively, in agreement with the projections made and summarised in Table 1-4.
- The total fluxes to tryptophan, phenylalanine and tyrosine all decreased by a small factor in the single and double transformant, relative to the reference strain. This result occurs largely because of the reduction in the growth rates of the single and double transformants. This decrease is therefore ignored in the present context and analysis given below, although it is recognised that the changes in growth rate may be related to the changes in the tryptophan and shikimate pathways.
- Apart from this factor, there was no significant effect of overexpressing enzymes of the tryptophan pathway, by a large factor, upon the total fluxes to tryptophan, phenylalanine and tyrosine. Subsequent overexpression of the enzymes of the shikimate pathway also gave minimal changes in the fluxes.
- The total flux may be broken down into two components. The flux to protein accounted for a overwhelming proportion of the total flux. For tryptophan, the contribution of flux to protein was about 99% of the total flux. For phenylalanine, the contribution was also about 99% in the reference strain, while for tyrosine, the contribution was about 97%. This, of course, means that even large changes in the flux to free amino acid will result in only small increases in total flux.
- Significant changes in flux to free metabolite did occur. These occurred because of changes in the intracellular and extracellular pools.
- Flux to free tryptophan in RH805 was 0.002 nmol min⁻¹ mg protein⁻¹, which, given the detection limits experienced here is similar to the value of 0.001 obtained with wild-type strain X2180-1A (Miozzari *et al.*, 1977). There was no significant increases in either the single or the double transformant.

- For phenylalanine, there was a small increase in flux to metabolite pool upon transformation with pME554. There was little change in flux to intracellular phenylalanine and a small increase in flux to the extracellular pool. Transformation with pPH28 gave a further increase in flux (both intra- and extracellular pools) which represented a four-fold increase over that of RH805.
- Similarly, there was little change in flux to free tyrosine upon transformation with pME554. An increased flux to the external pool was balanced by a decrease in flux to the internal pool. Additional transformation with pPH28, however, gave a three fold increase in flux relative to RH805.
- The absolute changes in flux to total phenylalanine and tyrosine pools were similar. However these changes have disproportionate effects on total flux to phenylalanine and tyrosine, because flux to tyrosine pool is a greater proportion of total flux to tyrosine than is the case for phenylalanine.

6.2 Discussion

The experimental data presented here, confirm that both pME554 and pPH28 lead to the required and expected overexpression of enzymes of the tryptophan and shikimate pathways. pME554 leads to anthranilate synthase activities which are approximately 50 times that of the reference strain, while pPH28 leads to a three-fold increase in SDH activities. Enzymes encoded by other genes born on the two plasmids were not assayed in this experiment. Chapter 4 described how these other activities were increased by similar factors to anthranilate synthase (for the *TRP* enzymes encoded on pME554) and SDH (for the *ARO* enzymes encoded on pPH28) in various transformed strains grown to an OD_{546} of about 1-1.5 (*i.e.* during mid-exponential phase). There is reason to believe that expression of these genes is regular and predictable.

Control of flux

The effect of overexpressing enzymes of the tryptophan pathway upon the three component fluxes in the shikimate pathway were negligible. Transformation with

pME554 does not significantly change the flux to tryptophan. This is consistent with the results of Niederberger and colleagues who measured flux to free tryptophan using an *aro7* (chorismate mutase) mutant transformed with pME554 and report that tryptophan accumulation was 'very low' during this phase of culture (Prasad *et al.*, 1987). Careful examination of their data suggests that concentrations were close to their limits of detection, as is the case during this study.

These results are highly suggestive of low flux control coefficients for all enzymes of the tryptophan pathway, which is consistent with the results of the tetraploid analysis of Miozzari *et al.* (1978a). Since Metabolic Control Analysis considers the consequences of small changes (strictly, infinitesimal) in enzyme activity upon flux, it is not strictly possible to interpret the present results in the terms of MCA (*e.g.* flux control coefficients). For instance, a large change in enzyme activity may produce a significant change in flux for the initial small changes in enzyme activity, followed by no change (or even, conceivably, a negative change) for the greater proportion of the change in enzyme activity. This implies that the distribution of control may change as the expression of one or more enzymes are modified, and as modulation of the flux occurs. However, the number of enzymes overexpressed together with both the magnitude of the level of overexpression and the minimal changes in flux produced, suggest that most of the control of flux within the tryptophan pathway lies outwith the tryptophan pathway itself, at least within the context of the growth conditions (media, growth rate etc.) and genotype used here. In particular, it must be emphasised that (feedback inhibitable) anthranilate synthase is no more likely to have a significant flux control coefficient than the other enzymes of the pathway.

When enzymes of the shikimate pathway were also increased by a smaller factor of 2 - 5, there was little observable change in fluxes either. This implies that the flux control coefficients for these enzymes are also low.

Control of intracellular concentrations

There were small, possibly insignificant, changes to the internal metabolite pools when the enzymes of the tryptophan pathway were overexpressed by a large factor.

However, there were significant changes to the pools when the enzymes of the shikimate pathway were also overexpressed by a smaller factor. Thus internal pools of tryptophan increased by a factor of 1.5 relative to RH805 and the single transformant. Phenylalanine and tyrosine pools increased by factors of between 3 and 4. This suggests that the control coefficients for tryptophan, phenylalanine and tyrosine concentrations are higher for the shikimate pathway enzymes than for the tryptophan pathway enzymes.

In summary, there appears to be little control of flux to tryptophan (and phenylalanine and tyrosine) by the activity of the enzymes of the tryptophan and shikimate pathways. Instead, the control of flux to tryptophan may be located (i) downstream, in transporter-mediated excretion, degradation of tryptophan, tyrosine and phenylalanine, or in steps involved in protein synthesis, (ii) upstream within the pathways generating the pathway precursors erythrose-4-phosphate and phosphoenol-pyruvate, in enzymes involved in the generation of NADPH, ribose-5-phosphate and ATP or, (iii) elsewhere in the rest of metabolism. In particular, it is emphasised that there is no evidence that the 'regulatory' enzymes, DAHP synthase and anthranilate synthase have significant control coefficients.

Control and regulation of metabolite pools

The changes in the intracellular concentrations were small, even after large increases in the activity of the enzymes of the tryptophan pathway. This result suggests that the intracellular pools are highly regulated. The question of control, as defined by MCA, was considered in the foregoing paragraphs. However, it will have become obvious, that this does not necessarily illuminate the mechanisms involved in the behaviour of this pathway. This is a problem of regulation.

This regulation may simply be a result of the buffering of the metabolic pathway mediated by an interaction between the rates of variably-saturated enzymes (whose kinetic parameters *in vivo* are not well characterised) and changes in intermediate metabolite pools which may be complex. For instance, the increase of an enzyme which is close to saturation by its substrates, is unlikely to increase the concentrations

of downstream metabolites. In this case, though, we would expect such an enzyme to exhibit a significant control on flux. Alternatively, there may be an important role for the regulation of tryptophan concentration through the feedback inhibition of anthranilate synthase by tryptophan. This inhibition does not imply a high flux control coefficient for tryptophan synthase. On the contrary, as discussed above, the fact that the overall activity of the enzyme changes by a factor of 50, far in excess of that of the flux change, argues in favour of a rather small control coefficient. Furthermore, a theoretical analysis shows that the effect of introducing modulation of activity by introduction of a feedback mechanism is to **reduce** the flux control coefficient control of that enzyme. This may be illustrated by reconsidering the effects of a feedback-resistant anthranilate synthase, first discussed in section 1.3. The changes which may be considered are (i) the intracellular concentrations of tryptophan, (ii) the flux to tryptophan and (iii) the new control coefficient of the enzyme. Fantès *et al.* (1976) report that two feedback resistant mutants, RH597 and RH598, give 50-fold increases in internal tryptophan pools, while Kradolfer *et al.* (1982) used RH511 which gave a 100-fold increase. On the assumption that excretion is negligible and that the growth rates are unaltered, it is possible to predict that the overall flux to free tryptophan will be about $0.1 - 0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. In fact, this value is in good agreement with the data of Miozzari *et al.* (1978a) and Kradolfer *et al.* (1982) who estimated tryptophan accumulation in RH511 at 0.17 and $0.13 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, respectively. If the flux to tryptophan in protein is included, the total tryptophan flux is predicted to be $0.4 - 0.5 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. This is in close agreement with the flux determined ($0.43 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) in wild-type strains using exogenous anthranilic acid to avoid feedback inhibition of anthranilate synthase (Niederberger *et al.*, 1992). This shows that feedback resistance of anthranilate synthase results in 50 - 100-fold increases in intracellular tryptophan concentrations and a total flux increase of about 40%. These data address the first two questions posed above. Thus, feedback inhibition of anthranilate synthase is essential for the regulation of tryptophan concentrations observed in wild-type cells. The regulation of flux is less decisive. The question regarding the new flux control coefficient must rest upon the work of Prasad *et al.* (1987) who show that the flux in strain RH1207 when

transformed with pME559 (bearing all five *TRP* genes, including *TRP2_{fbt}*) increases to values between 3.1 and 3.8, representing about a 6 - 9-fold increase relative to an untransformed feedback-resistant mutant. Clearly, this represents a much greater increase compared to the experiments conducted in this study, and suggests that significant flux control has shifted to anthranilate synthase. These results are consistent with the idea that the change to a feedback-resistant allele has increased the control coefficient for this enzyme. It is worth emphasising the point that changing the feedback networks of metabolic pathways may not necessarily change fluxes, but may change the distribution of control such that changes in flux become easier to effect by overexpressing enzymes. A corollary to this principle is that the evolution of feedback mechanisms may fulfil two functions. Firstly, they allow regulation of metabolite concentrations and secondly, they may distribute flux control more evenly.

The explanations for the limited decreases in phenylalanine and tyrosine concentrations when enzymes of the tryptophan pathway are overexpressed by such large factors may involve a number of similar factors to those described for tryptophan. The concentration of chorismic acid may be regulated itself by general 'buffering' of the system, as discussed above, and by the feedback inhibition of anthranilate synthase. This may interact with other factors such as the regulation of chorismate mutase activity by tryptophan (activation) and tyrosine (inhibition) and the regulation of the two isoenzymes of DAHP synthase, the first enzyme of the shikimate pathway. The effect of these factors and their interactions cannot easily be evaluated because the change in chorismic acid concentrations was not quantified and because the *in vivo* regulation of chorismate mutase and DAHP synthase activities and their subsequent effect on phenylalanine and tyrosine concentrations cannot be defined without more thorough investigation, involving the characterisation of metabolite concentrations and the effects of regulatory mutants. In addition, it is necessary, as with all regulatory mechanisms, to co-ordinate an investigation of the *in vivo* kinetics of the enzymes with mathematical modelling of the system.

Both tryptophan and phenylalanine intracellular pools doubled (with respect to RH805) while the increase for tyrosine was somewhat larger (three-fold) when the

enzymes of the shikimate pathway was overexpressed. It is likely that this resulted in an increase in chorismic acid concentrations which, as a common substrate, may compensate the feedback inhibitions of chorismate mutase by increased intracellular concentrations of tyrosine and of anthranilate synthase activity by increased tryptophan concentration, all occurring within a context of maintained total flux. Also, there may be a role for the two feed-back inhibited isoenzymes of DAHP synthase in regulating these concentrations.

Aromatic amino acid excretion

All strains appeared to show some excretion even when intracellular concentrations were at or below those obtained with RH805. This phenomenon is discussed below. However, it begs the question of the possible mechanisms by which this process occurs. For instance, how specific is the excretion? Does it occur through passive diffusion? Are the aromatic amino acid transporters, encoded by *TAT1* and *TAT2* involved, or are these proteins, as often suggested, strictly uni-directional in favour of uptake of amino acids into the cell (for example, see Opekarova *et al.*, 1993). Is there 'leakage' of some metabolites into secretory vesicles, perhaps during the insertion of membrane proteins and fusion of vesicles during the secretory process or loss of metabolites during cell division.

Net excretion may arise when the export process is greater than uptake through active proton/amino acid symporters. Alternatively transporters may mediate an equilibrium. Thus the external concentration of amino acids may depend upon their intracellular concentrations and the relative concentrations of intracellular and extracellular protons. This could be modulated by an electrogenic potential. Also there may be kinetic constraints. For instance, Kotyk and others report that the activity of the membrane H⁺/ATP-ase affects the uptake of amino acids, while the external bulk pH does not (Kotyk and Dvorakova, 1992; Kotyk, 1994). This suggests that the local proton concentration in the vicinity of the transporter may be critical, and that this is kinetically controlled. This increases the possibility that at higher intracellular concentrations of amino acids, or when active proton concentrations at the

extracellular domain are low, transporters may facilitate the specific excretion of amino acids.

Summary and conclusion

In summary, the flux through all the three major branches of the shikimate pathway are unresponsive to changes in the activities of all of the enzymes of the tryptophan and shikimate pathway. Insignificant changes in concentrations of tryptophan, phenylalanine and tyrosine occur following the overexpression of the tryptophan pathway, whereas larger changes were observed (with respect to enzyme multiplier) when enzymes of the shikimate pathway were increased. The perspective offered here is that the buffering of metabolic pathways by non-saturated enzyme kinetics and feedback inhibition of anthranilate synthase (also possibly, chorismate mutase and DAHP synthase) activity mediates regulation of metabolite concentration, at least within a context where flux is maintained. This should not imply that flux is controlled in the same way, since feedback inhibition of enzyme activity (for example, anthranilate synthase) acts to reduce the enzyme's flux control coefficient. Thus the use of a feedback resistant allele is predicted to increase the intracellular concentration of tryptophan, and to increase the enzyme's flux control coefficient such that overexpression of this allele may now lead to a significant change in flux. Finally, the use of transport processes to reduce the intracellular concentrations of metabolites requires a characterisation of the mechanisms and kinetics of excretion and uptake, and an investigation of changes following the overexpression or deletion of transporters encoded by *TAT1* and/or *TAT2*.

Chapter 7

Further analysis

The previous chapter outlined the effects of overexpressing enzymes of the tryptophan and shikimate pathways upon the concentrations of tryptophan, phenylalanine and tyrosine and upon the fluxes through the pathways, at a single time-point toward the end of the exponential phase of batch culture. Flux changes in the pathways were negligible although there were significant changes in the intracellular and extracellular concentrations of the aromatic amino acids. In this respect the overexpression of enzymes of the shikimate pathway by a factor of 3 gave larger changes than a fifty-fold overexpression of the enzymes of the tryptophan pathway.

This chapter gives a more detailed account of the measurements made at different time points during the growth of the strains. It also describes an experiment performed to evaluate the effect of feeding cells on anthranilic acid.

7.1 Growth on MV + LV(100)

The following data are taken from the same culture as that described in Chapter 6. Three other time points are considered; at about 2.5, 24 and 48 hours after the inoculation of the cultures. The growth curves were given in Figure 6-1. Even though determinations of cell density were not made consistently through the course of the cultures, Figure 6-1 shows clear evidence of a transient slowing of growth rate between 10 and 20 hours followed by a further increase. This behaviour is the diauxic shift which occurs as glucose becomes exhausted and as cells change from fermentative to respiratory metabolism, provided that cultures are well aerated. Important changes in the pattern of gene expression are known to occur during this phase.

Reproducibility of results

As explained in Chapter 6, the estimations of fluxes are insensitive to errors made in the determination of metabolite concentrations, because the contributions of

accumulation rates are small compared to the fluxes to protein. On the other hand, a discussion of the effects of pME554 and pPH28 upon metabolite pools should take account of likely errors, reproducibility and variation. Unfortunately, a rigorous assessment of standard errors in determinations was beyond the scope of this study. In particular, an evaluation of the variation in results between similar experiments was difficult.

Replicate determinations of amino acid concentrations rarely differed by more than 20%. If different dilutions of samples were used, this factor was, on occasions, larger. It has been emphasised however that the difference between experimental determinations of internal and external pools were usually close to the experimental values for external pools.

A number of pilot, aborted and repeat experiments were performed. This allowed some discrimination between those phenomena which appeared repeatable and those which were less so. Naturally, those phenomena which concerned me in this and the previous chapter, are of the former kind. It is appreciated that a rigorous confirmation of these would require further extensive experimental work.

7.1.1 Enzyme activities

The specific activities of SDH and anthranilate synthase in the three strains are shown in Figure 7-1.

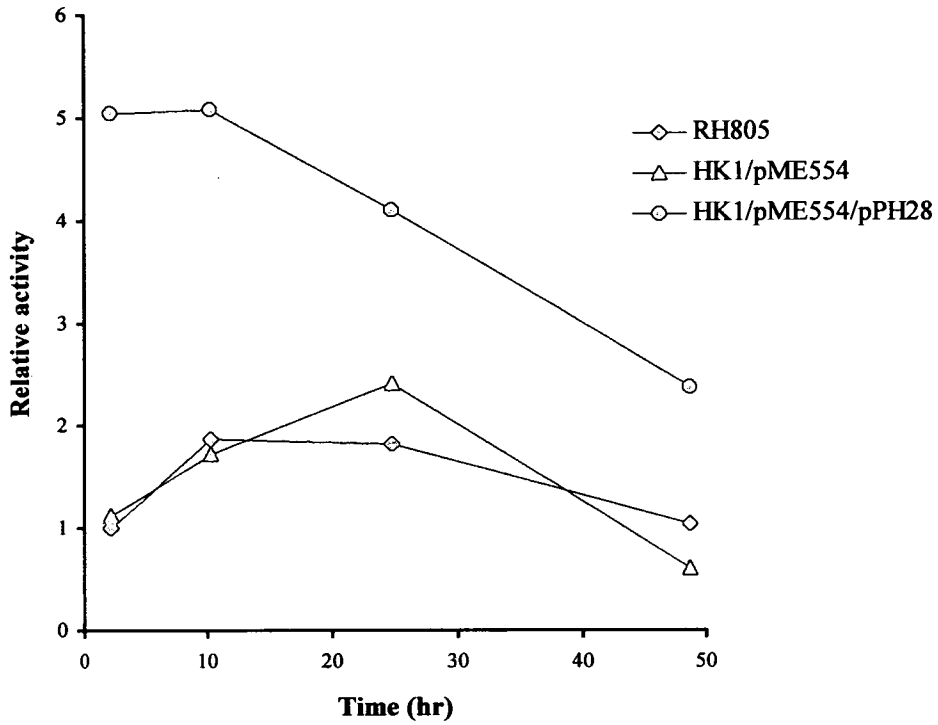
Shikimate dehydrogenase

As expected, the specific activity in the double transformant remained significantly higher than in both RH805 and HK1/pME554 throughout culture. SDH activities (relative to the initial activity for RH805) varied between 1 - 1.9, 0.6 - 2.4 and 2.4 - 5.1 for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. The specific activity of the strain transformed with pPH28 was greater than the other two strains at all time points by a factor of between 2 and 5. The level of overexpression achieved from the plasmid was thus in line with the results described in Chapter 5. SDH activities of RH805 and HK1/pME554 were similar. However, the values suggest

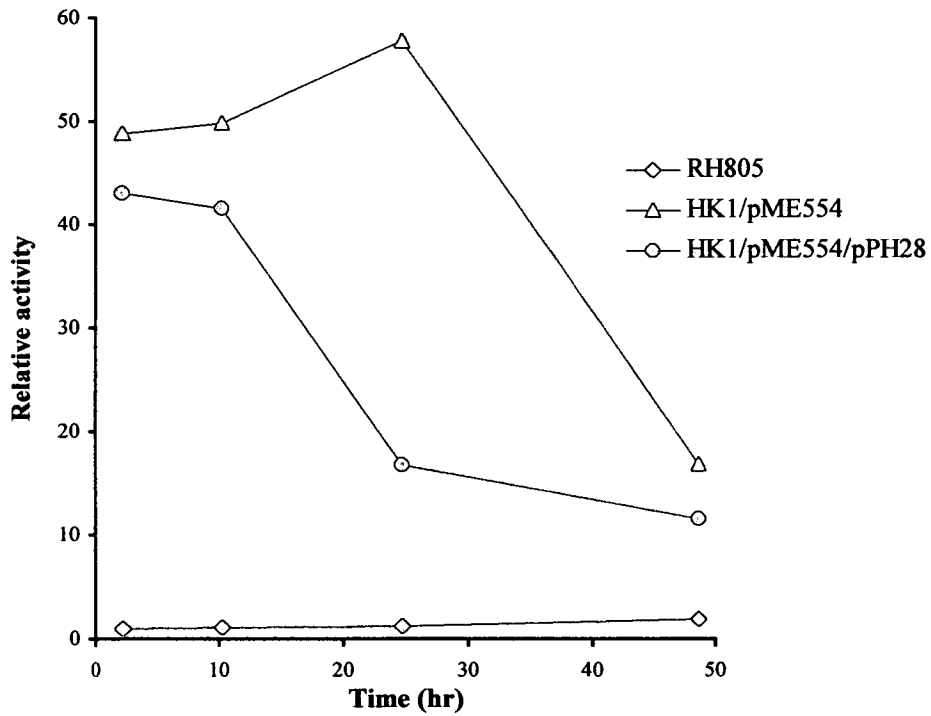
Figure 7-1 Relative specific enzyme activities

The specific activities of shikimate dehydrogenase (A) and anthranilate synthase (B) are expressed relative to the initial activities of the reference strain (RH805) which were 21.1 and 0.02 nmol • min⁻¹ • mg protein⁻¹, respectively.

A. Shikimate dehydrogenase



B. Anthranilate synthase



that in RH805 and HK1/pME554 there was a gradual increase in activity for 24 hours before a decline to initial levels. HK1/pME554/pPH28, on the other hand showed a steady decline throughout the culture, although after 48 hours, its activity was still over double that of the other two strains.

Possible explanations for the transient increases in activity include the possibility of increased gene expression of biosynthetic enzymes during the later stages of exponential growth, mediated by the transcriptional activator, GCN4, during starvation of specific amino acids. However, this is not considered likely; firstly because anthranilate synthase activities (also subject to GCN4 control) did not appear to show a similar trend (but see below) and secondly because cells were probably not starved for a specific amino acid, at this stage during culture. SDH may instead be subject to more specific control. In this context, it may be relevant that the double transformant HK1/pME554/pPH28 does not show the same behaviour, *i.e.* there is no observed increase in activity at any time during culture. Finally, chromosomal genes and plasmid-borne genes may show different patterns of expression. This may result from the influence of enhancer binding sequences at distant sites upstream of the normal promoter regions, or from the effect of chromatin structure, which includes patterns of nucleosomes and histone binding.

Since all three strains showed a decline in the activities over the final 24 hour period, this loss in HK1/pME554/pPH28 is unlikely to result from a significant loss of plasmid, pPH28. It is possible that some of the loss is due to a change in the pattern of gene expression during the diauxic shift, when up-regulation of some genes, for instance those encoding the respiratory enzymes, may be expected. Alternatively, there may be specific down-regulation of genes of the shikimate pathway, or increased mRNA turnover in response to higher intracellular concentrations of the aromatic amino acids.

Anthranilate synthase

Figure 7-1B shows that the two strains bearing the plasmid pME554 gave greatly increased anthranilate synthase activities over the entire time course. Relative

anthranilate synthase activities varied between 1 - 1.9, 16.8 - 57.7 and 11.5 - 43.1 for RH805, HK1/pME554 and HK1/pME554/pPH28, respectively. The level of overexpression obtained from pME554 was about 6-50 times that of the reference strain. These results are in line with previous estimations of activities in transformed and untransformed strains.

However, there was a marked loss of activity in the transformants in the later stages. For the single transformant, HK1/pME554, this loss occurred between 24 and 48 hours, while for the double transformant, the loss began earlier, at some point between 10 and 24 hours. By 48 hours these strains had specific activities of between 10 and 20 times that of RH805. Part of this loss may have been due to the loss of plasmid when the concentration of tryptophan within the medium was increasing, leading to a possible loss of selection. However, this is unlikely to play a major role, because the loss occurs over a period of only two to four cell divisions. Also, the stability data given in Chapter 5 do not easily sustain an argument for this rate of loss. Another possible explanation involves a differential change in the pattern of gene expression from the chromosomal and plasmid-borne copies of the gene.

The anthranilate synthase activity of RH805 appeared to increase during the culture (by a factor of nearly 2 by 48 hours). It is possible that this was due to upregulation of GCN4 as nutrients became depleted at very high cell densities. This may not be observed with HK1/pME554 and HK1/pME554/pPH28 because by 48 hours, the optical density of the two transformed strains were approximately half that of RH805. Therefore, nutrient depletion may not have been so advanced in the transformed strains.

An investigation of the factors involved in the maintenance and change of enzyme activity in these experiments would require a close study of the time course of specific activity and plasmid stability and their dependence upon different growth conditions such as nutrient concentration, in different strains including, for example, a *gcn4* mutant. An analysis of other enzyme activities both within and outwith the pathway would be required to resolve the question.

In conclusion, estimation of enzyme activity confirms that the two plasmids gave the required and the expected levels of SDH and anthranilate synthase activities within the three strains. Since the activities of the other enzymes of the shikimate and tryptophan pathways in these strains were previously demonstrated (Chapter 5) to be overexpressed by broadly similar factors during exponential phase, it is assumed that this is the case, also, in this experiment. The changes in these enzyme activities during later phases of the cultures are unknown.

7.1.2 Tryptophan concentrations

The intracellular pools of tryptophan are shown in Figure 7-2 and the extracellular and total concentrations in Figure 7-3.

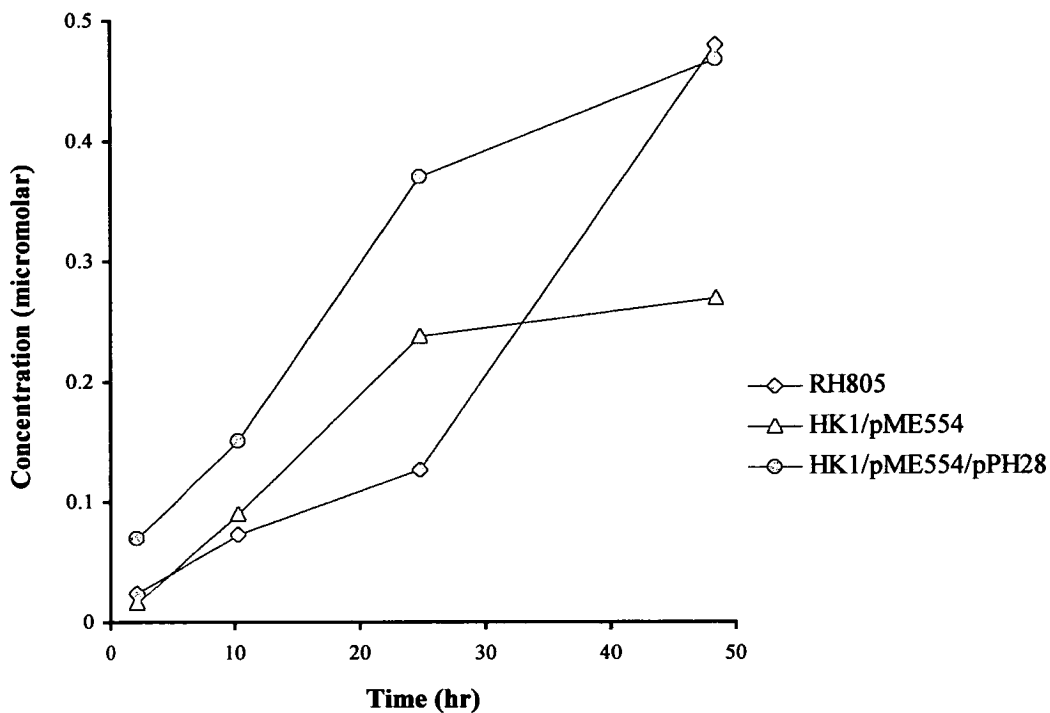
The internal pools showed a steady rise with time. When these are plotted relative to cell volume (Figure 7-2B) the calculated intracellular concentrations show approximate constancy, with the exception of the initial time point, for which low measured concentrations (low cell density) increased the probability of large errors. Despite this reservation, pME554 gave a small increase in intracellular concentration, and the double transformant gave a further and more significant increase.

The extracellular pools (figure 7-3A) showed a different picture. Large differences between the three strains are evident after 24 hours only. The extracellular concentration of the single transformant is increased by a large factor relative to RH805 (almost 10-fold). The double transformant gave an increase over the single transformant by a further factor of six (*i.e.* 60 - fold). Following these increases, the extracellular concentrations fell to nearly zero after 48 hours. Thus, it appears that the transformed strains, particularly the double transformant, excreted tryptophan, toward the end of exponential phase or at onset of the diauxic shift. Furthermore, the results suggest that this tryptophan was then taken up from the medium as cells progressed toward stationary phase. It should be noted here that the large increases in excretion in HK1/pME554 and HK1/pME554/pPH28 occurred despite relatively small changes in intracellular concentrations. However, if raised internal

Figure 7-2 Intracellular tryptophan

A. The internal pools of tryptophan are expressed as concentrations with reference to the culture volume. B. The intracellular tryptophan concentrations are expressed with respect to cell volume as described in Chapter 2.

A. Internal tryptophan pool



B. Intracellular tryptophan concentration

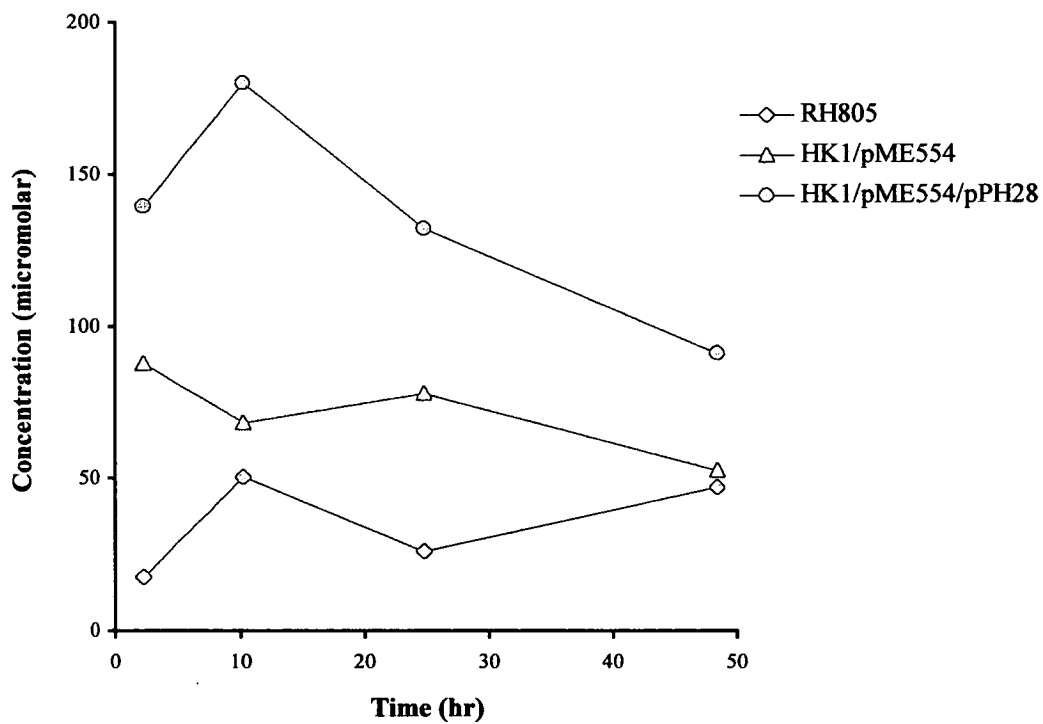
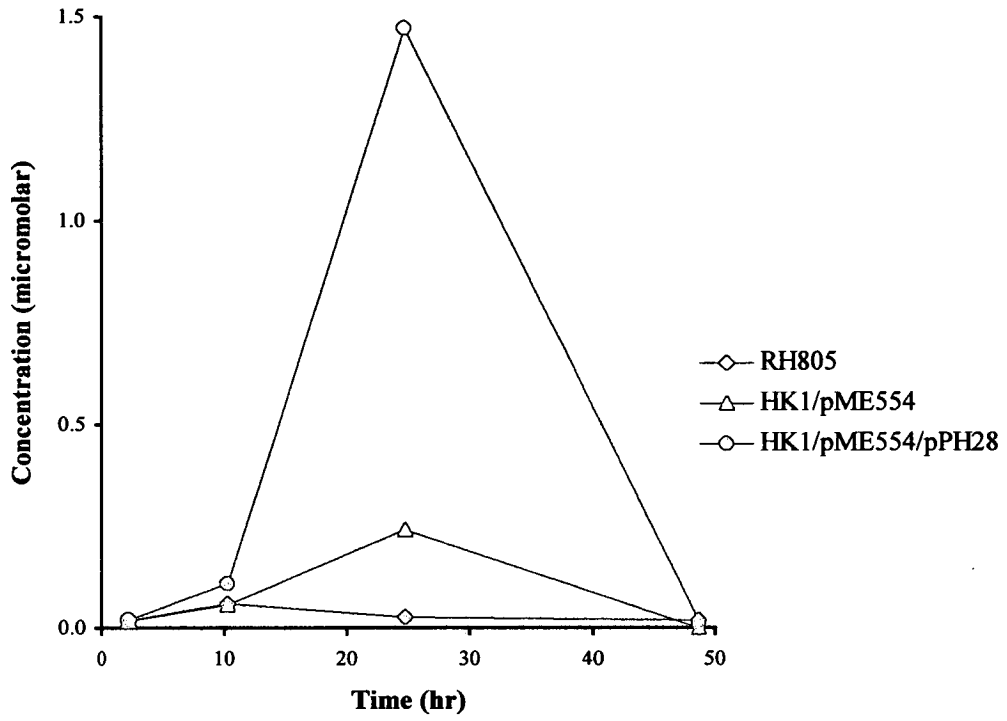


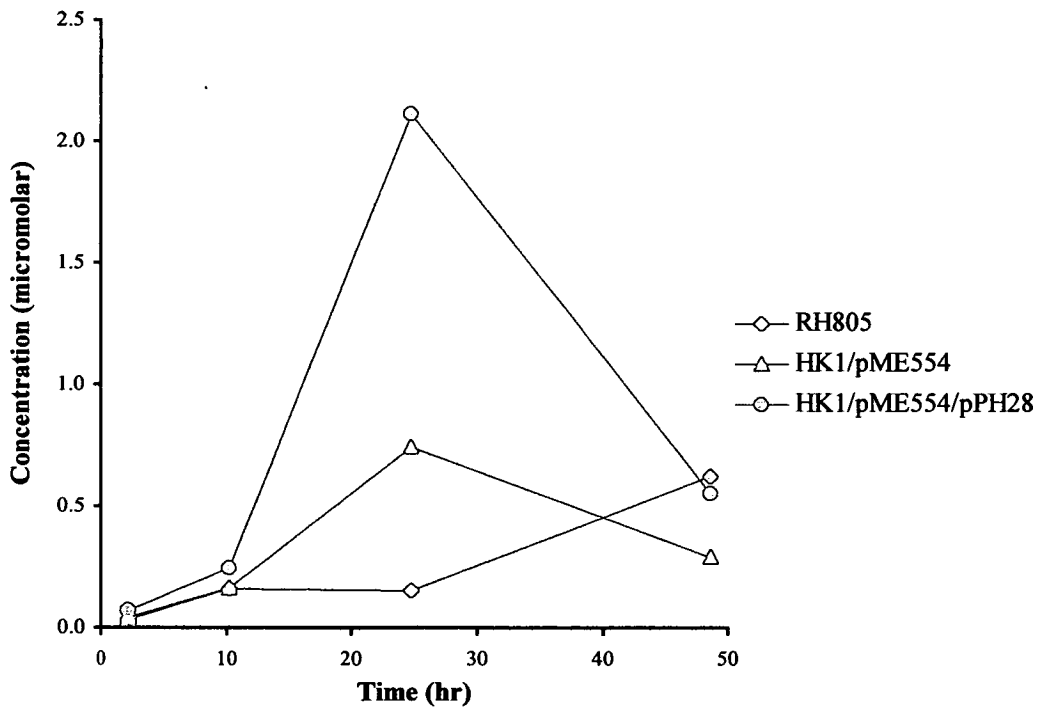
Figure 7-3 Extracellular and total tryptophan

A. The concentrations of tryptophan within the supernatant are plotted. B. The total tryptophan concentrations within the culture are plotted.

A. Extracellular tryptophan pool



B. Total tryptophan pool



concentrations lead to increased excretion, then excreted tryptophan may accumulate in the media.

The data on total tryptophan concentrations (Figure 7-3B) gave similar trends to the extracellular data, and reasonable agreement with the values calculated from the independent determination of intracellular and extracellular concentrations. Thus RH805 gave a steady increase in tryptophan concentrations over the course of the culture, while both the transformants give maximum concentrations after 24 hours. At this point the concentrations are much higher than for RH805 (5 - 15-fold) which is largely a reflection of the contribution of extracellular concentrations. At 24 and 48 hours, there is some discrepancy between calculated and experimentally determined total concentrations. Specifically, the experimental values are higher by a factor of about 1.3-1.5 than those calculated. This may be due to a systematic under-estimation of intracellular concentrations at high OD₅₄₆ or, conversely, an over-estimation of the experimental total concentrations. The former explanation is favoured here. The procedure used for extracting intracellular pools on filters, using TCA, at high OD₅₄₆ is considered more likely to lead to poor extraction of amino acids, than the possibility that total concentrations are over-estimated using a protocol similar to that used for the determination of extracellular pool.

7.1.3 Phenylalanine concentrations

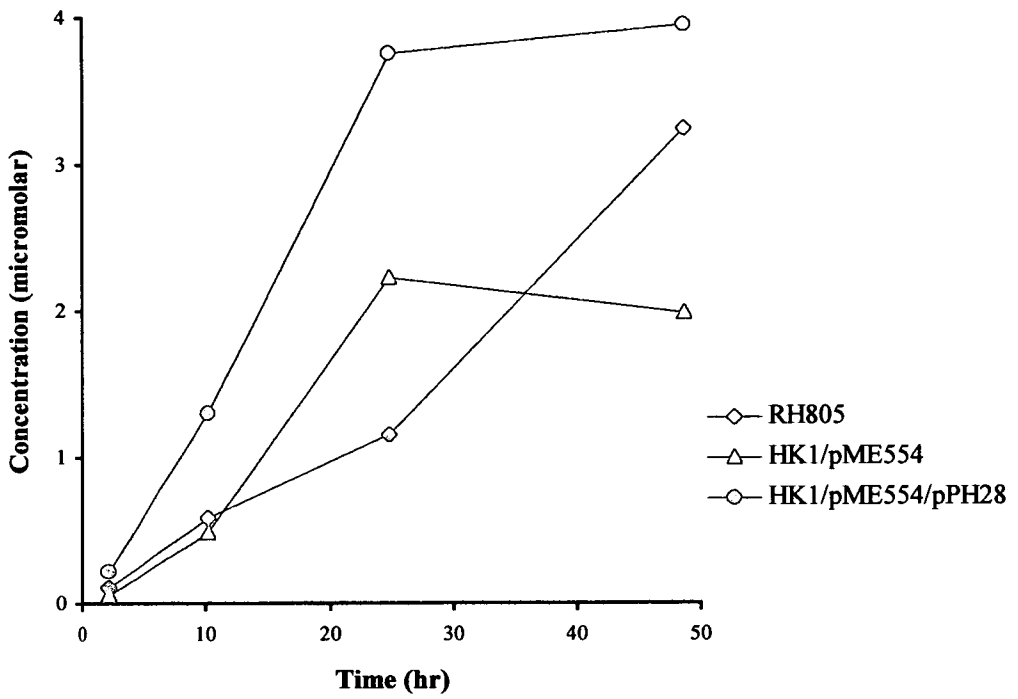
The values for intracellular pools are given in Figure 7-4 and the extracellular and total concentrations are given in Figure 7-5.

The intracellular pool of phenylalanine show trends which are similar to those of tryptophan, except that the values are considerably larger. There is a steady increase in internal pools (Figure 7-4A) as biomass increases. Intracellular concentrations (Figure 7-4B) are fairly constant, with the double transformant giving values 2-3 times those of RH805. The single transformant gave similar concentrations to RH805, except at 24 hours. At this time point HK1/pME554 and HK1/pME554/pPH28 both gave rather large increases in intracellular concentrations, which paralleled the results obtained for tryptophan.

Figure 7-4 Intracellular phenylalanine

A. The internal pools of phenylalanine are expressed as concentrations with respect to the culture volume. B. The intracellular phenylalanine concentrations are expressed with respect to cell volume as described in Chapter 2.

A. Internal phenylalanine pool



B. Intracellular phenylalanine concentration

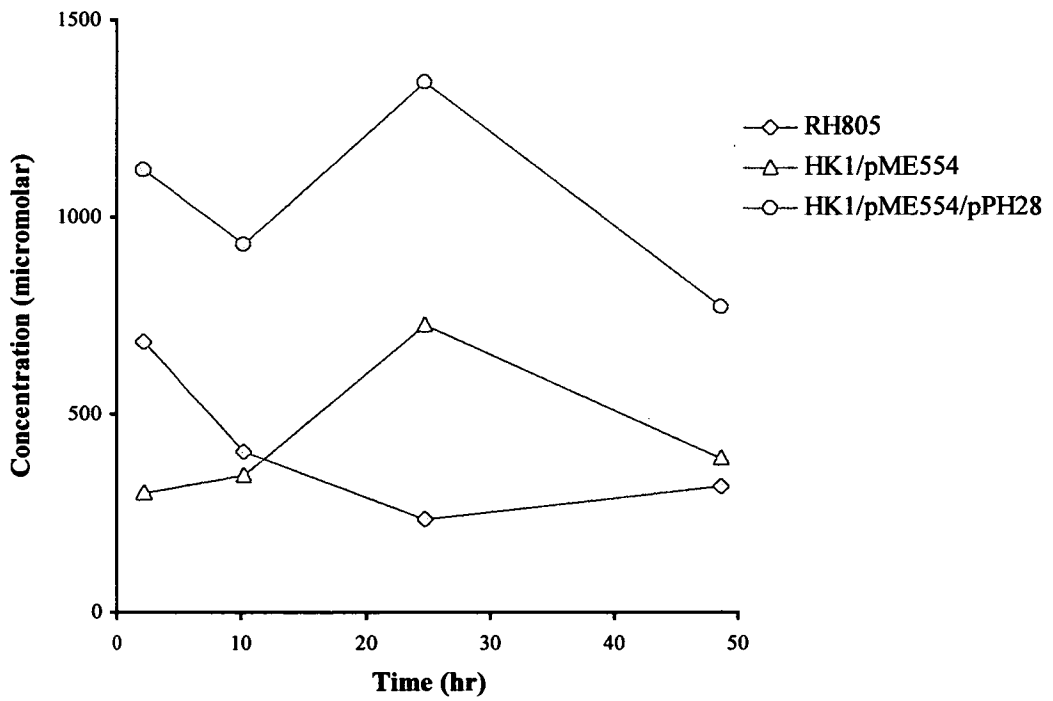
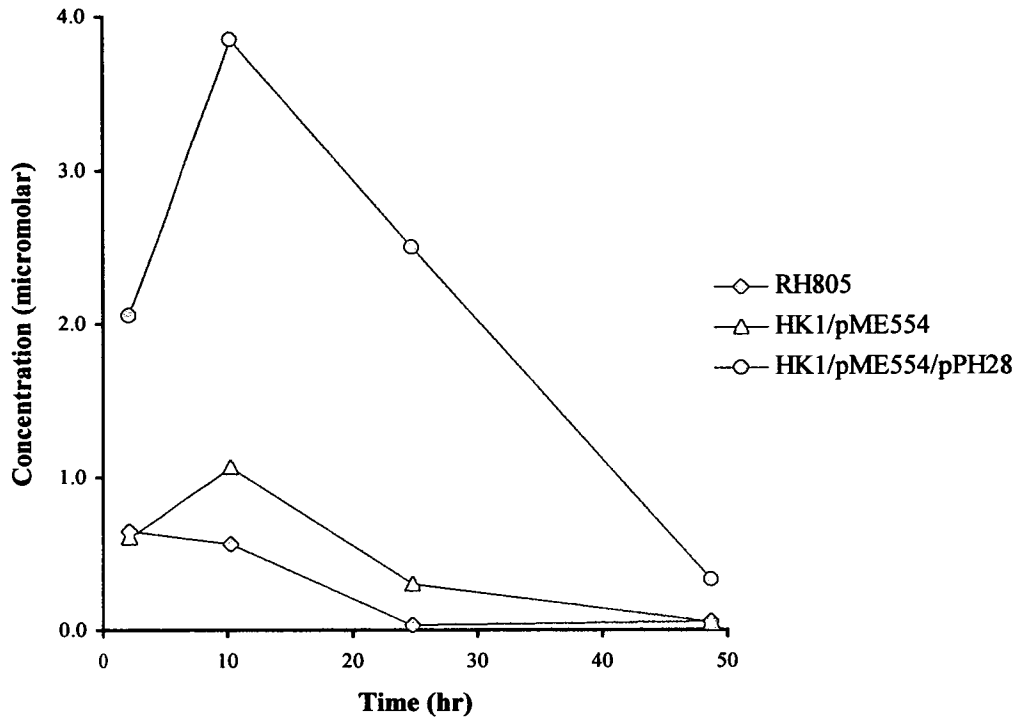


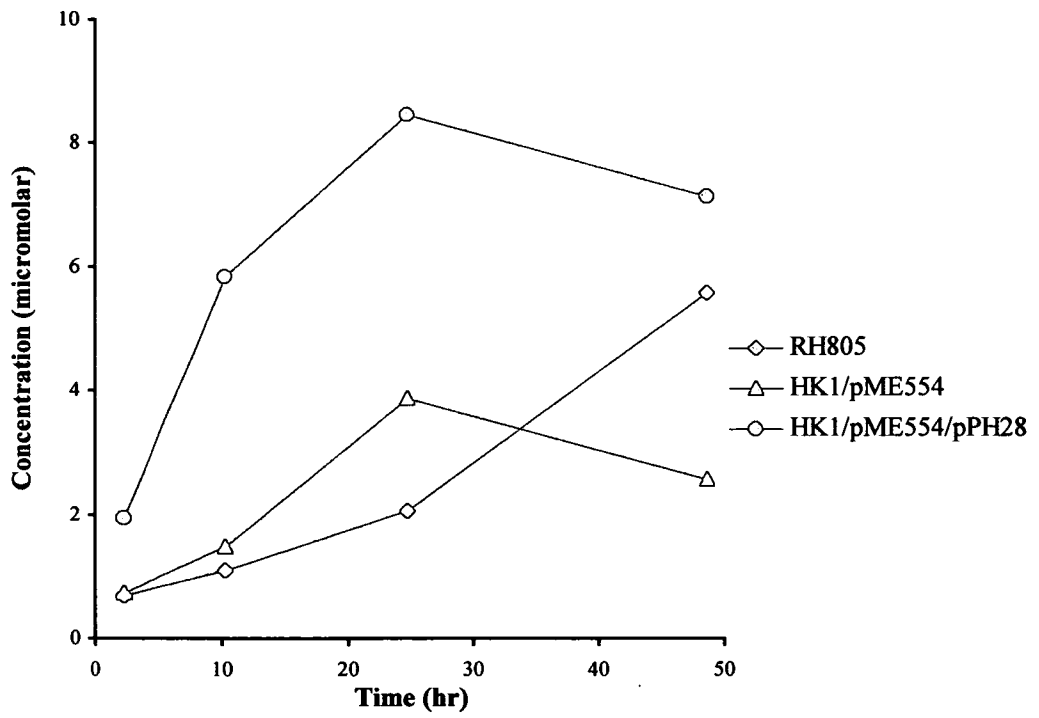
Figure 7-5 Extracellular and total phenylalanine

A. The concentrations of phenylalanine within the supernatant are plotted. B. The total phenylalanine concentrations within the culture are plotted.

A. Extracellular phenylalanine pool



B. Total phenylalanine pool



Extracellular concentrations of phenylalanine (Figure 7-5A) were much greater in the double transformant. At 10 hours, the concentration was about 4 times that of RH805 and HK1/pME554 and at 24 hours the factor was about 8. In all three strains, maximum phenylalanine concentrations were obtained after 10 hours. After this, extracellular concentrations fell, presumably due to re-uptake by the cells.

The total pools (Figure 7-5B) generally reflected the component pools. As with tryptophan, there was a similar discrepancy between calculated and experimentally determined figures at 12 and 24 hours. Again, a systematic under-estimation of internal pools would account for the results.

Total pools rose in the transformants to a maximum at 24 hours. Because the two component pools showed maximum values at different times (24 - 48 hours for intracellular and 12 hours for extracellular), the time course for total concentration shows a less pronounced peak than those observed for tryptophan and tyrosine. As with tryptophan, RH805 showed steady accumulation of phenylalanine throughout the culture.

The distinctive feature of phenylalanine production, compared to tryptophan and tyrosine production (see below), is the time of excretion of phenylalanine into the medium. For phenylalanine, maximum intracellular concentrations appear later than that of the extracellular concentration, whereas the reverse is true for tryptophan and tyrosine. Indeed, significant excretion had already taken place at 2.5 hours. This suggests that excretion may not be solely dependent upon intracellular concentration.

7.1.4 Tyrosine concentrations

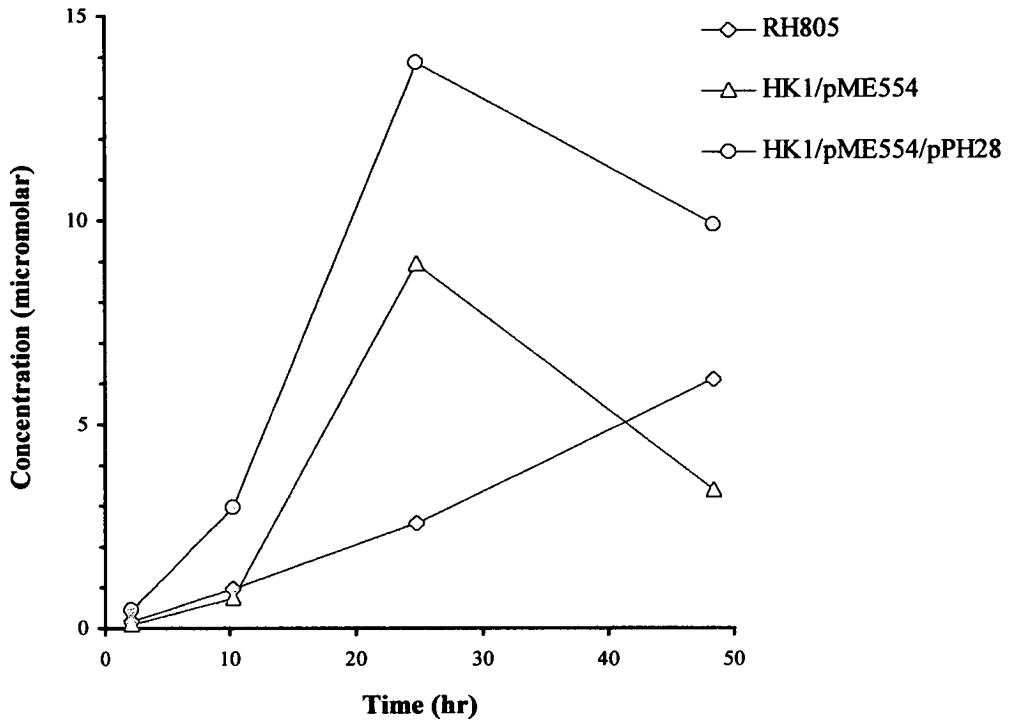
The values for intracellular pools are given in Figure 7-6 and the extracellular and total concentrations are given in Figure 7-7.

The intracellular concentrations of tyrosine showed broadly similar trends to those of phenylalanine. However, the absolute values were about 3 - 4 times greater and also

Figure 7-6 Intracellular tyrosine

A. The internal pools of tyrosine are expressed as concentrations with reference to the culture volume. B. The intracellular tyrosine concentrations are expressed with respect to cell volume as described in Chapter 2.

A. Internal tyrosine pool



B. Intracellular tyrosine concentration

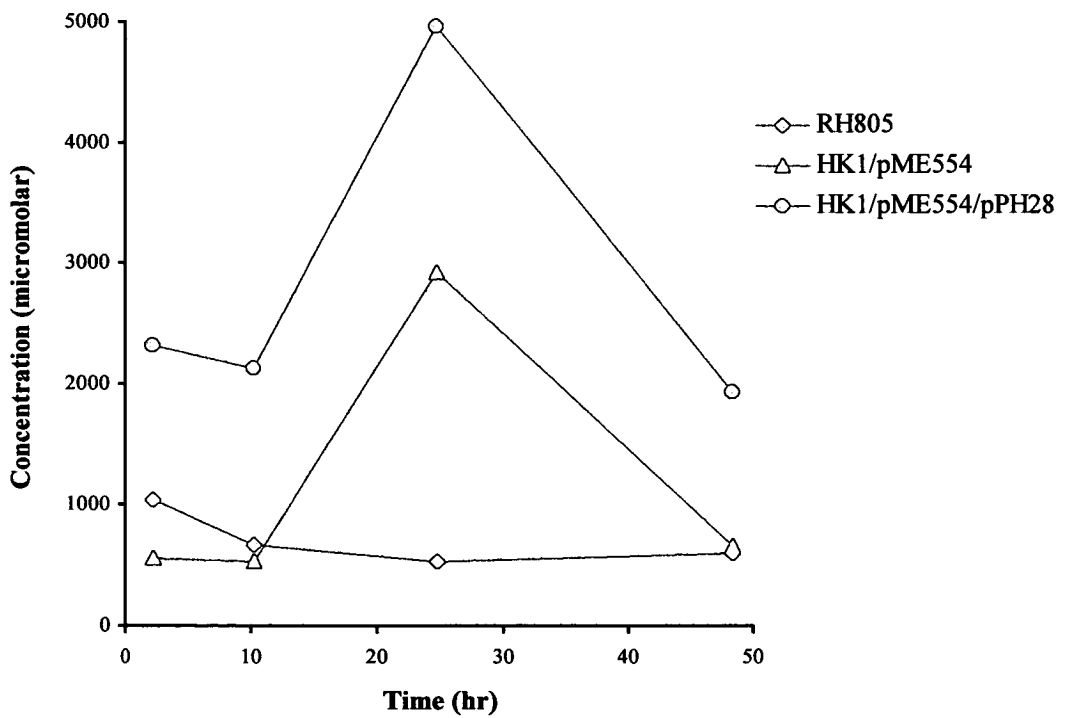
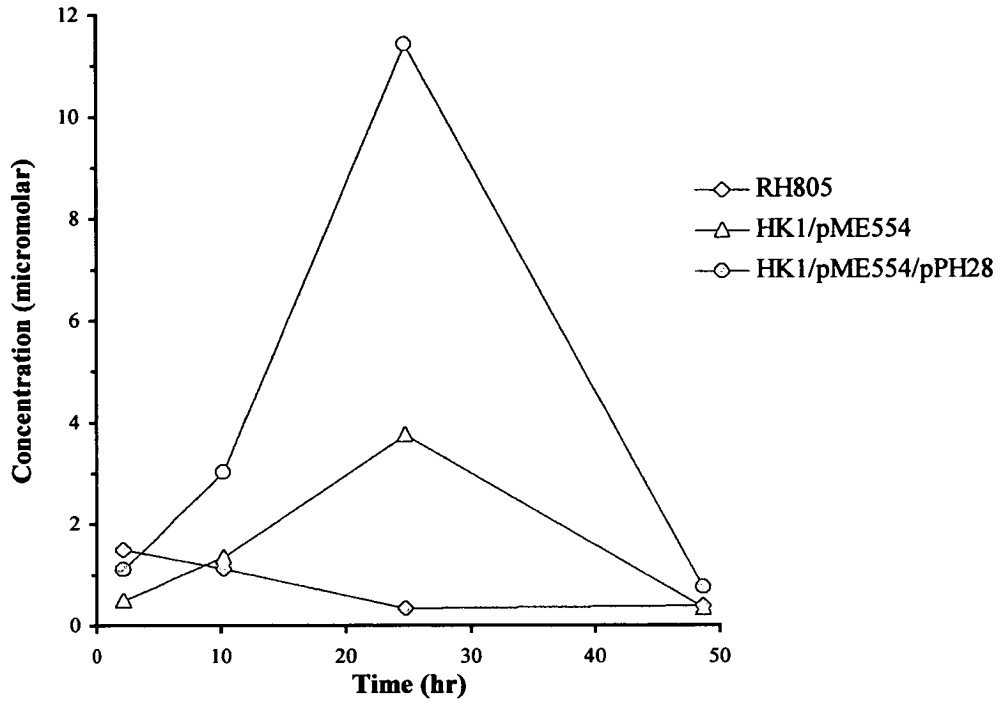


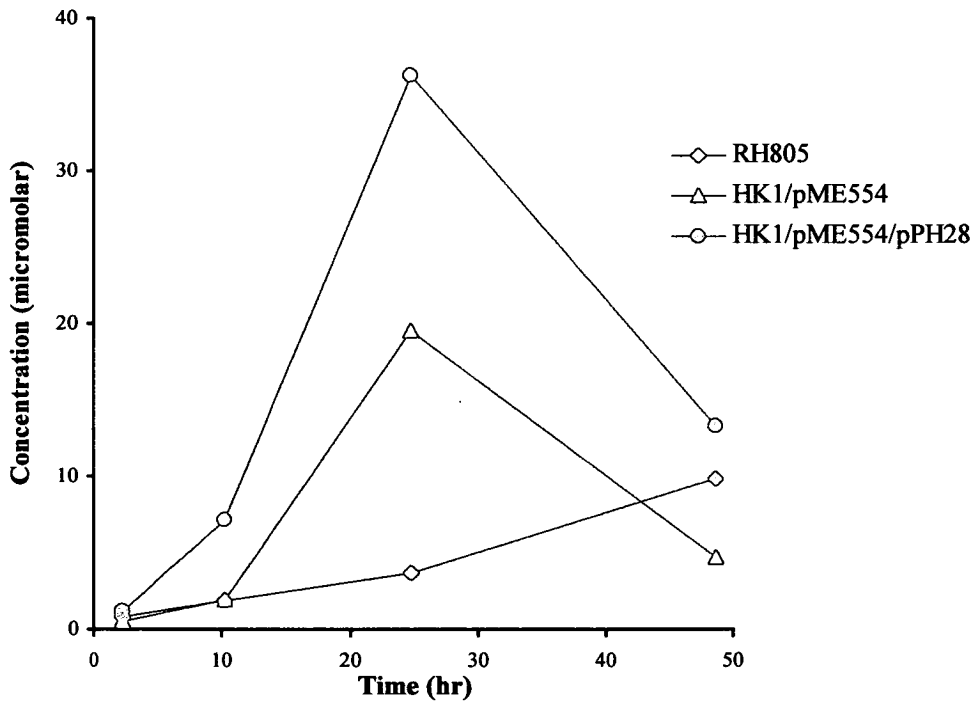
Figure 7-7 Extracellular and total tyrosine

A. The concentrations of tyrosine within the supernatant are plotted. B. The total tyrosine concentrations within the culture are plotted.

A. Extracellular tyrosine pool



B. Total tyrosine pool



there was a sharper increase in intracellular concentrations (Figure 7-6B) at 24 hours for the transformants HK1/pME554 and HK1/pME554/pPH28.

The extracellular concentrations of tyrosine in the transformants also showed a rather dramatic increase at 24 hours (Figure 7-7A) and thereafter a decay to zero at 48 hours, indicating significant excretion and re-uptake. Unlike phenylalanine, this is consistent with the dependence of excretion upon intracellular concentrations.

The total concentrations of tyrosine (Figure 7-7B) follow the patterns set out for tryptophan and phenylalanine. They show reasonable agreement with values calculated on the basis of the experimentally determined intracellular and extracellular pools, but exhibit a similar discrepancy at 24 and 48 hours as that observed for tryptophan and phenylalanine.

7.1.5 Summary

The complex set of data presented in the preceding sections illustrates the following trends which were also observed in other similar experiments performed in the context of this project. The intracellular concentrations of tryptophan were increased by overexpression of the enzymes of the tryptophan pathway and, additionally, by overexpression of enzymes of the shikimate pathway throughout the time course. The appearance of tryptophan in the medium around the onset of the diauxic shift was also greatly increased by both manipulations. These trends were even more marked in the case of phenylalanine and tyrosine, and particularly marked in the double transformant. This constitutes some evidence of excretion of amino acids towards the end of exponential phase followed by a fairly rapid re-uptake from the medium.

It is also worth emphasising another interesting result. In RH805 the internal pools and total pools for tryptophan, phenylalanine and tyrosine all increased steadily throughout the culture. In contrast there was a decrease (or marked levelling off) of internal pools in both the single and double transformants between 24 and 50 hours. For the total pools, there was a decrease in all three amino acids in the transformants between these time point. Indeed, the total pool for all three amino acids was actually

higher in RH805 than in the single transformant at the final time point. This may be a function of the higher cell densities achieved in the untransformed strain.

7.1.6 Discussion

Dynamics of aromatic amino acid production

The results presented in the above sections reinforce some of the conclusions drawn in the previous chapter which considered data from a single time-point (10 hours) towards the end of exponential phase. Most importantly, the total pools of tryptophan, phenylalanine and tyrosine were increased significantly when enzymes of both the shikimate and tryptophan pathways are overexpressed. It is important to stress that although the changes, particularly after 24 hours, were quite dramatic, the effect on flux is still minimal, because of the disproportionate contribution of flux to protein, as discussed fully in Chapter 6.

On the other hand, there were some results which emerged here which contrast with those of Chapter 6. Data from samples taken at 10 hours (Chapter 6) showed that HK1/pME554 had concentrations of tryptophan, phenylalanine and tyrosine which were not significantly different to those obtained with RH805. The implications of this result was discussed in the previous chapter. After 24 hours however, the intracellular concentrations and the extracellular pools of tryptophan, phenylalanine and tyrosine were both increased by a significant factor in HK1/pME554 and by a larger factor in the double transformant. Thus, the more general result presented in this chapter, is that overexpression of enzymes of the tryptophan pathway actually increases the concentrations of phenylalanine and tyrosine, especially at 24 hours. How may we account for these results?

One possibility concerns the interaction of tryptophan and tyrosine concentrations in modulating the activity of chorismate mutase, the first enzyme of the prephenate pathway. This enzyme is inhibited by tyrosine and, effectively, activated by tryptophan, as explained in section 1.2.6. Regardless of the change in chorismic acid concentrations, an activation of chorismate mutase by an increased concentration of

tryptophan is capable of increasing concentrations of tyrosine and phenylalanine, within a context of no change in fluxes.

Another possibility concerns the possible role of phosphoribosyl isomerase, encoded by *TRP1*. pME554 contains a form of this gene, *TRP1d*, which has a defective promoter, such that the specific activity of the gene product, phosphoribosyl-anthranilate isomerase, does not reach the same levels of overexpression as the other enzymes encoded upon the plasmid. The experiments conducted by Niederberger and others (Niederberger *et al.*, 1992) show that this does not seem to have a detrimental effect on the rate of flux through the pathway when cells are fed on anthranilic acid. However, it is possible that reduced overexpression of this enzyme could lead to increases in metabolite pools upstream and decreases in metabolite pools downstream. This may have the effect of increasing the concentration of chorismic acid and of decreasing the concentration of tryptophan, both relative to those levels which would be obtained in strains carrying a *TRP1* gene with the wild-type promoter. Thus, it is still possible that chorismic acid concentrations had increased in HK1/pME554 relative to RH805. In this case, tyrosine and phenylalanine concentrations could increase, when flux is maintained, independently of any effect of an increase in tryptophan concentrations.

A third possibility is that increased tryptophan concentrations may up-regulate the expression of the genes encoding enzymes of the prephenate pathway, though the adaptive significance of this is not clear.

The first two possibilities could be resolved by investigating the effect of replacing *TRP1d* by *TRP1* on pME554, and by determining chorismic acid concentrations.. This is a non-trivial change requiring a considerable investment of time and could not be attempted in the present study.

Alternatively, a study of the relationship between tryptophan concentration and phenylalanine and tyrosine concentrations, using anthranilic acid as an exogenous substrate for the tryptophan pathway might illuminate the problem. Unfortunately, the effect of feeding cells on anthranilic acid will not only increase tryptophan

concentrations, but will also tend to increase the intracellular chorismic acid concentration. Therefore, any increase in phenylalanine and tyrosine concentrations may result directly from the increase in chorismic acid concentration or from the 'activation' of chorismate mutase. Nevertheless, partly because a large increase in tryptophan concentration would give some assurance that enzymes other than anthranilate synthase were overexpressed, an experiment involving feeding strains on exogenous anthranilic acid was undertaken. The results of this experiment are given below, in section 7.2

7.2 Cultures fed with exogenous anthranilic acid

400 ml cultures of both RH805 and HK1/pME554 were generated from freshly isolated colonies, using 10 ml starter cultures in MV + LVFY (+ U (40) for HK1/pME554) and 100 ml pre-cultures in media lacking phenylalanine and tyrosine. Cells were pelleted from the pre-cultures and washed before resuspension in medium. This inoculum was split equally between two identical main cultures, one to be grown with the addition of anthranilic acid ('fed') and one without ('unfed'). Anthranilic acid was added at intervals (see below) to a concentration of 20 μ M, using a sterile 20 mM stock solution. Optical densities at 546 nm were monitored during the growth of the cultures, and samples were taken at intervals for the analysis of anthranilate synthase and SDH activity, and for the determination of concentrations of tryptophan, phenylalanine and tyrosine.

Aliquots of anthranilic acid were added at hourly intervals from 3.25 hr to 9.25 hr and from 23.5 hr to 27.5 hr, inclusive. This schedule therefore involved two periods of addition; one during the exponential phase and one closer to the diauxic shift, separated by a period of 'recovery'. A final period of recovery was included. These were included to detect re-uptake of amino acids from the large extracellular pools which were assumed would develop during 'feeding'.

Samples for amino acid analysis were taken after 2.7, 6.25, 8.5, 23.5, 27.5 and 51.5 hours. For clarity, these times are given subsequently, to the nearest hour.

Comparison of the sampling times with feeding times shows that a sample was taken

some time after inoculation but prior to the initiation of feeding, and two samples taken during the first period of feeding. After the first recovery period (over-night), a sample was taken prior to the start of the second period of feeding, and another during feeding. A final sample was taken after another overnight recovery period.

In the previous experiment, there was good agreement between the estimated total concentration (calculated by adding the experimentally determined intra- and extracellular concentrations) and the experimentally determined concentration. For this reason, in this experiment, the extracellular concentrations were estimated by subtracting the intracellular values from the corresponding total concentrations.

7.2.1 Growth of cultures

The optical densities of the four cultures at 546 nm are illustrated in Figure 7-8. RH805 grew slightly quicker ($\mu = 0.31 \text{ hr}^{-1}$) than HK1/pME554 ($\mu = 0.29 \text{ hr}^{-1}$) during exponential phase which ended at about 9 hours. The addition of anthranilic acid made little difference to the growth rate of RH805. For HK1/pME554, it appears that the feeding of anthranilic acid slightly decreased the growth rate after 4-5 hours. This inhibitory effect may have arisen from the rapid and large increase in intracellular tryptophan concentration (or intermediates of the tryptophan pathway), or because of effects at the cell membrane connected with the rapid excretion of tryptophan (see below).

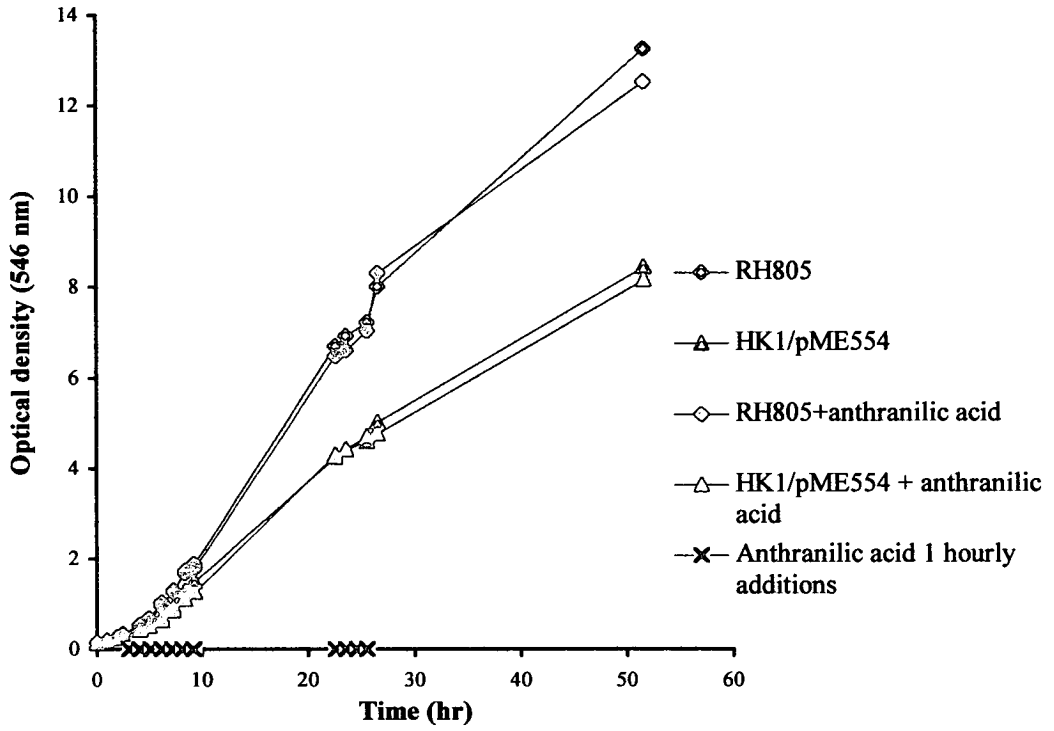
7.2.2 Enzyme activity

The activities of SDH and anthranilate synthase in the four cultures after 15 hours and 36 hours are given in Table 7-1. These data show that the activity of SDH varied between 21.4 and 39.4 which is within the normal range of activities for a chromosomal copy of the gene *ARO1*. These activities, relative to RH805 ('unfed') at 15 hours, varied between 0.7 and 1.3. There appeared to be no distinct difference between the activities of RH805 and HK1/pME554, nor between cells grown in the absence and presence of anthranilic acid. There were no large changes between the activities at the two time points.

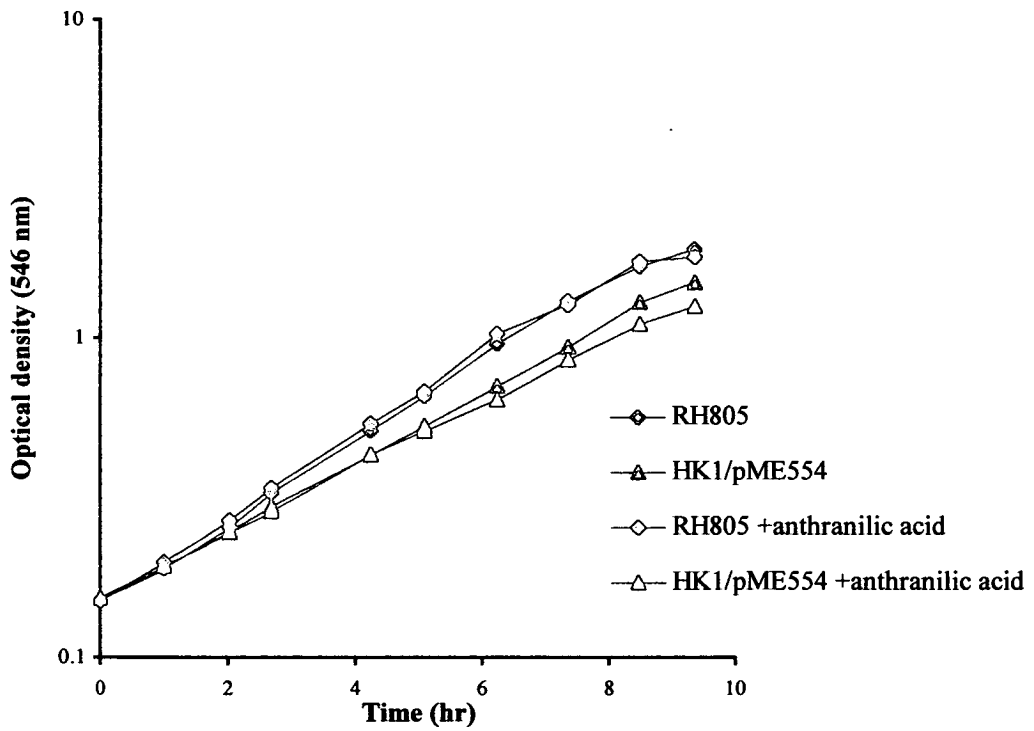
Figure 7-8 Growth of cultures

The growth of RH805 and HK1/pME554, +/- anthranilic acid are represented by changes in optical density at 546 nm. A Linear plot, showing times at which anthranilic acid was added. B Semi-logarithmic plot over the first ten hours, when growth appeared to be exponential.

A. Growth of cultures



B. Growth of cultures



Strain		Shikimate dehydrogenase		Anthranilate synthase	
		Specific activity (nmol · min ⁻¹ · mg protein ⁻¹)	Relative activity	Specific activity (nmol · min ⁻¹ · mg protein ⁻¹)	Relative activity
RH805	14.7 hr	30.0	1.0	0.03	1.0
	35.5 hr	22.1	0.7	0.02	0.8
HK1/pME554	14.7 hr	21.4	0.7	1.37	54.9
	35.5 hr	39.4	1.3	1.23	49.3
RH805 + anthranilic acid	14.7 hr	21.9	0.7	0.02	0.9
	35.5 hr	27.7	0.9	0.03	1.0
HK1/pME554 + anthranilic acid	14.7 hr	24.2	0.8	1.17	46.6
	35.5 hr	36.8	1.2	0.96	38.2

Table 7-1 Enzyme activities

The specific activity of shikimate dehydrogenase and anthranilate synthase in strains RH805, HK1/pME554 after 14.7 and 35.5 hours of culture in MV + LV(100) +/- anthranilic acid are given. Relative activities are calculated with respect to RH805 after 14.7 hours.

The anthranilate synthase activities clearly shows that HK1/pME554 ('fed' and 'unfed') overexpresses this enzyme by factors of between 38 and 54, relative to RH805 ('unfed') at 15 hours. The relative activities in RH805 varied between 0.8 and 1.0.

These data show that expression of these two enzymes from chromosomal and/or plasmid borne genes was within the range obtained in the previous experiments.

7.2.3 Tryptophan concentrations

Intracellular tryptophan

The internal pools and intracellular concentrations of tryptophan, in the four cultures are given in Figure 7-9A,B.

Because of the scale of the vertical axis in Figure 7-9, the values obtained for RH805 and HK1/pME554 grown in the **absence** of anthranilic acid ('unfed') are not clearly visible but were similar to those obtained from the previous experiment, in section 7.1.3. The following features (obtained for 'unfed' cultures) are common to both experiments.

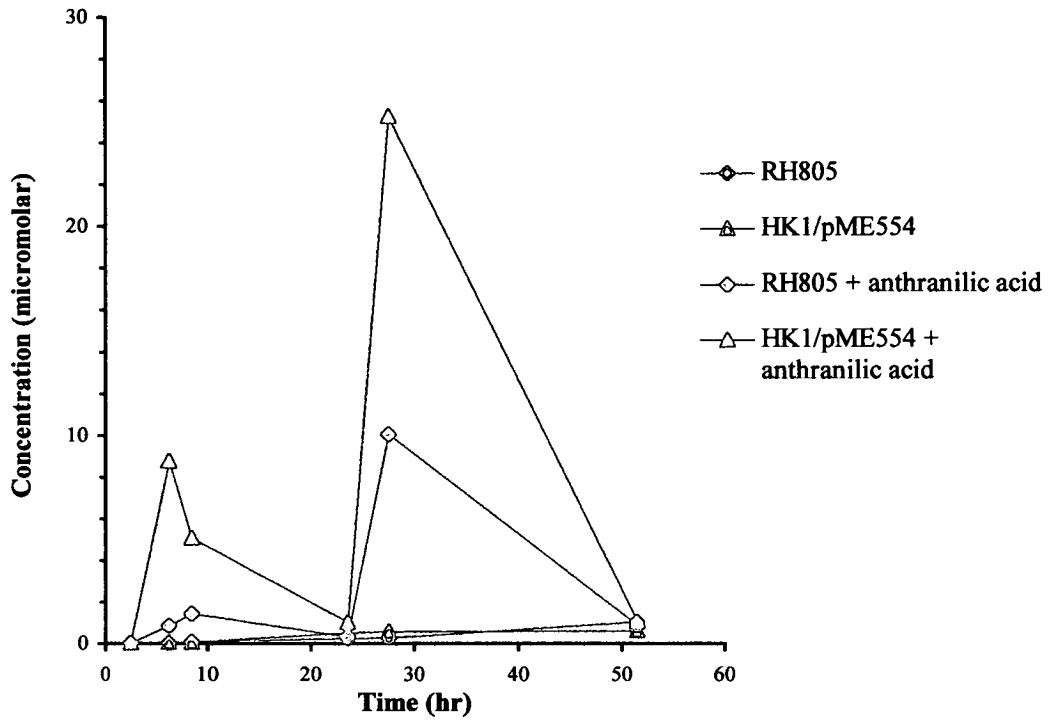
The internal pools of tryptophan in RH805 rose steadily through the course of the culture, whereas HK1/pME554 gave higher values (3-fold increase relative to RH805) during mid-culture, which then levelled off after 24 hours. This was only partly accounted for by the increased OD_{546} in RH805, because by 50 hours, the intracellular concentration in RH805 was greater than that of HK1/pME554. At other time points, the intracellular concentrations of HK1/pME554 were only slightly greater than for RH805 (less than 2-fold)

When the strains were cultured in the presence of anthranilic acid ('fed'), there were very large changes in intracellular concentrations of tryptophan. Increases were specific for times when anthranilic acid was being added to the cultures, or soon after. Thus, concentrations showed marked rises from 7 - 9 hours and at 28 hours, but not at 3, 24 and 52 hours. For RH805, intracellular concentrations reached 1.4 mM at 6 - 9 hours and 2.3 mM after 28 hours, representing a 20 - 40-fold increase in

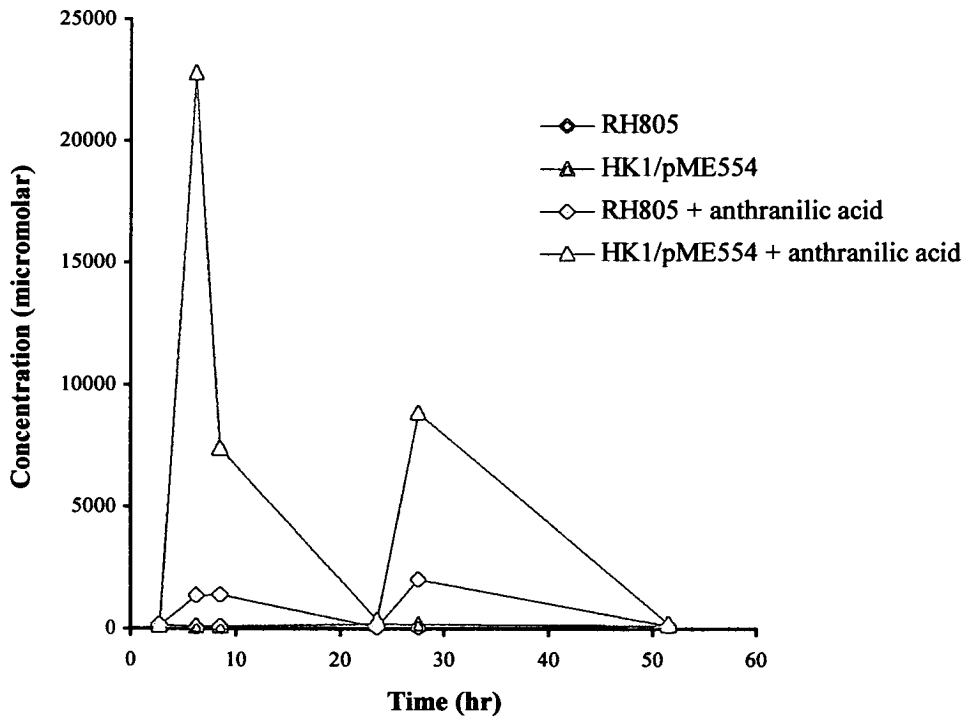
Figure 7-9 Intracellular tryptophan

A. The internal pools of tryptophan are expressed as concentrations with reference to the culture volume. B. The intracellular tryptophan concentrations are expressed as concentrations within the cell based upon the equations given in Chapter 2.

A. Internal tryptophan pool



B. Intracellular concentration of tryptophan



concentration. For HK1/pME554, the corresponding concentrations were 22.8 mM at 7 hours and 8.8 mM at 28 hours, representing increases in tryptophan concentrations by a factor of 40 - 300.

Direct comparison of 'fed' RH805 and HK1/pME554 shows that overexpression of the tryptophan enzymes leads to a 4 - 16-fold increase in intracellular tryptophan concentrations, under this particular feeding regimen of anthranilic acid.

Total tryptophan

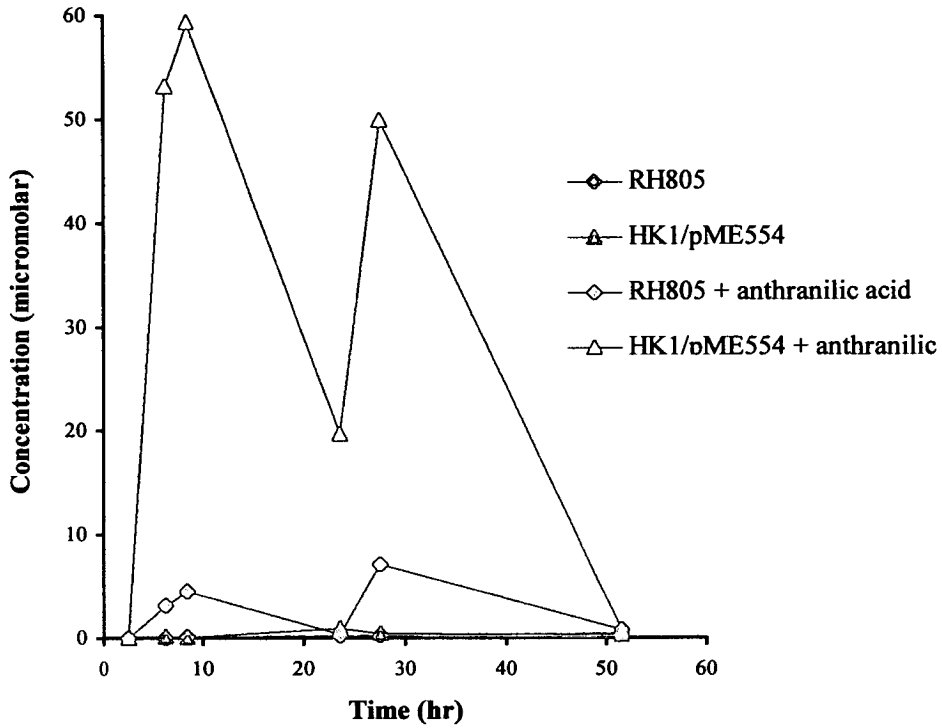
The values of the experimentally determined total concentrations are given in Figure 7-10A. Again, values for RH805 and HK1/pME554 grown in the absence of anthranilic acid are not clearly illustrated because of the scale of the vertical axes. However, they gave similar results to those reported for the previous experiment. Thus, RH805 showed a steady accumulation of tryptophan over the course of the experiment while HK1/pME554 gave an increased accumulation of tryptophan at only 24 and 28 hours, when the total concentration was 2-5 times that of RH805. From 24-50 hours, HK1/pME554 showed a net loss of free tryptophan. The absolute values obtained here were in the same range as those reported from the previous experiment.

With the addition of exogenous anthranilic acid, both RH805 and HK1/pME554 showed very large increases in total tryptophan pools, followed by rapid loss during the 'recovery' periods. RH805 gave 3.1 and 4.8 μM tryptophan at 7 and 9 hours, respectively; a 25-fold increase over 'unfed' RH805. At 28 hours there was 7.1 μM tryptophan; which was a 30-fold increase. However, at 24 hours, prior to the initiation of the second phase of 'feeding', the tryptophan concentration was 0.3 μM , which was almost the same as that of 'unfed' RH805. HK1/pME554 gave an even higher concentration of free tryptophan; 53, 59 and 50 μM at 7, 9 and 28 hours respectively, representing a 600 - 700-fold increase in concentration, relative to the 'unfed' strain. At 24 hours, after a 12 hour recovery period, the concentration had dropped to 20 μM , still a 20-fold increase over the 'unfed' cells.

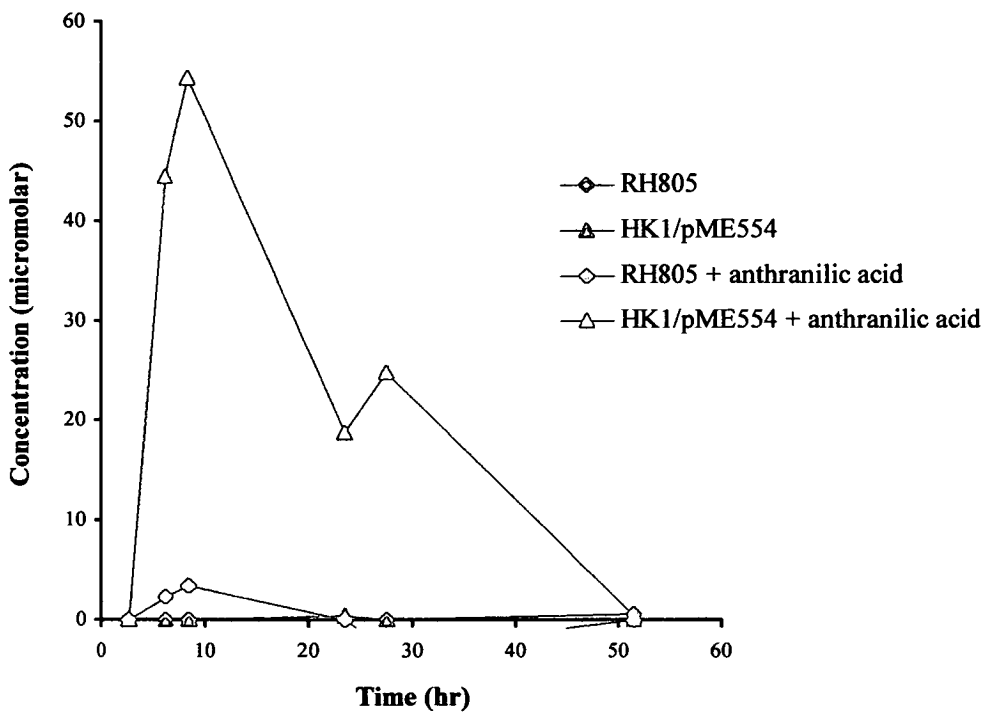
Figure 7-10 Total and estimated extracellular tryptophan

A. Total tryptophan. B. The extracellular tryptophan is calculated from the values determined for total and internal pools.

A. Total tryptophan pool



B. Estimated extracellular tryptophan pool



Extracellular tryptophan

The calculated values (see section 7.2) are plotted in Figure 7.10 (B).

In 'unfed' strains, the calculated extracellular concentrations are low, (less than 0.07 μM), except for HK1/pME554 at 24 hours, when the concentration was calculated to be 0.42 μM . This result is in good agreement with the previous experiment, where a definite peak in excreted tryptophan was obtained in the single (and double) transformant during mid-culture.

The concentrations calculated for the 'fed' strains are as follows. RH805 gave elevated values at times when the internal concentrations of tryptophan were highest; 2.3 and 3.4 μM at 6 and 9 hours. It is not possible to give a valid factor by which these values exceed those of the 'unfed' strains, because of the very low values obtained in RH805 ('unfed') which therefore incur unacceptably large errors. At 28 hours, when the intracellular concentration of this strain was at its maximum value, the estimated extracellular concentration was -2.9 μM . In order to investigate this value, determinations of concentrations in samples taken at this time-point were repeated many times. Negative values for the estimated extracellular concentration were always obtained. The corresponding calculations for tyrosine and phenylalanine gave large positive values. Hence, it must be concluded that the extracellular tryptophan concentration was probably quite low and that the negative concentration arose because of an underestimation of tryptophan in the 'total' sample, an over-estimation of tryptophan in the 'intracellular' sample, or both of these. The calculated concentrations at other time-points did not differ from those of 'unfed' RH805.

For HK1/pME554, the calculated extracellular tryptophan concentrations were much higher. Values of 44.4, 54.3, 18.7 and 24.7 μM were obtained at 7, 9, 24 and 28 hours, respectively. The values at 3 hours (before feeding) and 52 hours (after feeding) are low and not significantly different from those of the 'unfed' culture.

These results show that excretion of tryptophan is greatly increased in 'fed' strains by a large factor compared to 'unfed' strains. For instance, at 24 hours there was a 40-

fold increase. Furthermore, the results show that tryptophan is rapidly taken up from the supernatant, during both recovery periods.

Summary

- pME554 increases tryptophan production especially during mid-culture. This confirms the results of the previous experiment.
- Exogenous anthranilic acid increased the intracellular concentrations, and total concentrations in RH805 and HK1/pME554 during both periods of feeding.
- Increased excretion of tryptophan accompanied these changes (except in the case of RH805, during the second feeding period).
- in 'fed' cultures, pME554 gave a 12 - 17-fold increase in total tryptophan concentrations during exponential phase compared to RH805.

The fact that HK1/pME554 gives such a large amplification of tryptophan accumulation when exogenous anthranilic acid is supplied, compared to RH805, strongly suggests that some, if not all, of the other enzymes of the tryptophan pathway are being overexpressed, in addition to the two enzymes (anthranilate synthase and tryptophan synthase) which have been assayed in this study.

This experiment also suggests that anthranilate synthase's sensitivity to feedback inhibition by tryptophan plays a role in the regulation of intracellular concentration. Of course, it does not constitute rigorous evidence, because the internal concentration of anthranilic acid is likely to be much higher than when exogenous anthranilic acid is not supplied. Instead, a proper evaluation of the role of feedback inhibition requires the use of a feedback insensitive anthranilate synthase mutant.

7.2.4 Phenylalanine concentrations

Intracellular phenylalanine

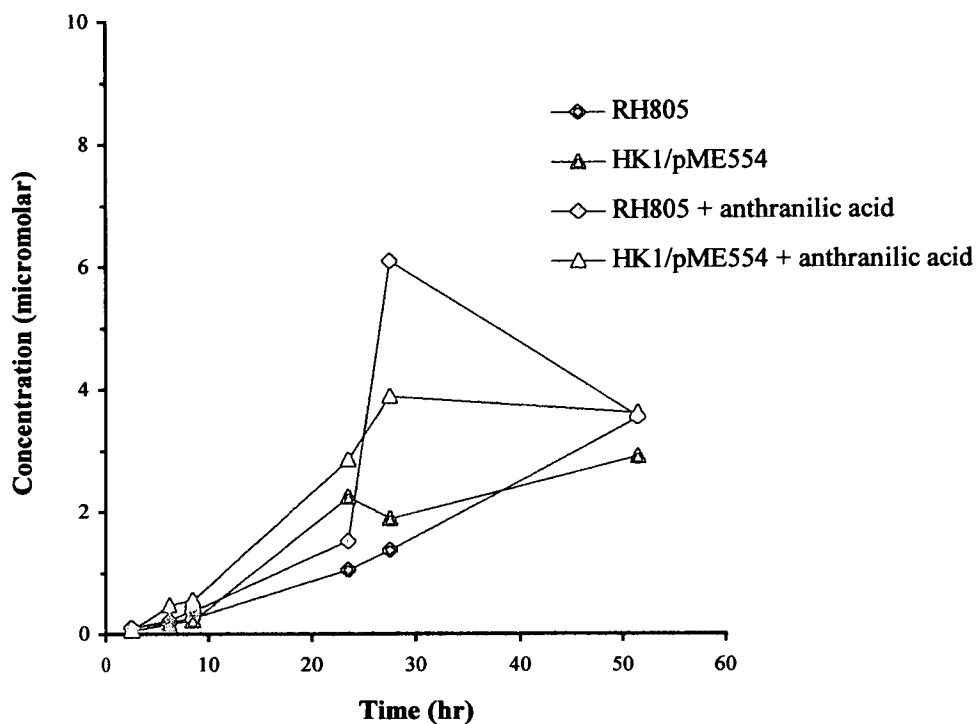
The intracellular concentrations of phenylalanine are illustrated in Figure 7-11.

For RH805 and HK1/pME554 grown in the absence of anthranilic acid, the observed concentrations were very similar to those obtained in the previous experiment. Thus

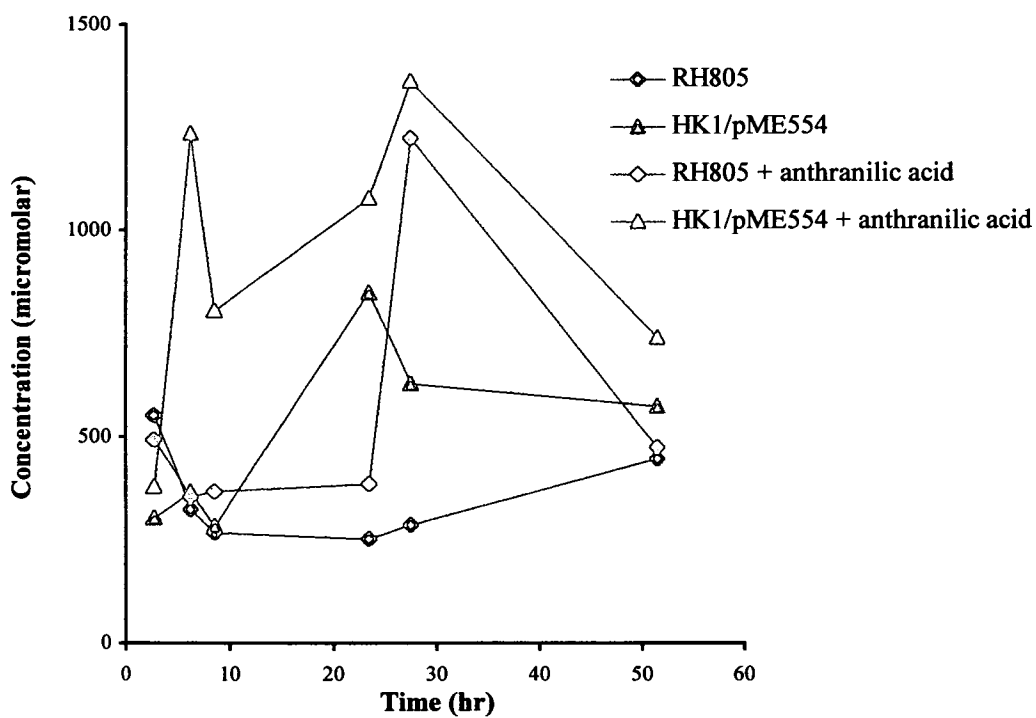
Figure 7-11 Intracellular phenylalanine

A. The internal pools of phenylalanine are expressed as concentrations with reference to the culture volume. B. The intracellular phenylalanine concentrations are expressed with respect to cell volume as described in Chapter 2.

A. Internal phenylalanine pool



B. Intracellular concentration of phenylalanine



in RH805, internal phenylalanine pools increased steadily over time in RH805, resulting in a constant intracellular concentration of about 250 - 450 μM .

In HK1/pME554, the intracellular concentrations were slightly greater than those of RH805, except for a large increase to 850 and 630 μM after 24 and 28 hours, respectively, when the OD_{546} were 4.4 - 5.0.

When fed with anthranilic acid, the cultures showed increased phenylalanine concentrations. RH805 showed slight increases in intracellular concentrations at most time points. For instance, there were 10%, 37%, 53% and 6% increases at 6, 8, 24 and 52 hours, respectively. However, at 28 hours, there was a very large increase of 320%. At this point, the intracellular concentration (1,220 μM) was about double that of the strain transformed with pME554 (unfed) (630 μM). This point appears toward the end of the second phase of feeding with anthranilic acid. For HK1/pME554, feeding with anthranilic acid increased intracellular phenylalanine concentrations by over 100% after 6, 9 and 28 hours. In contrast, the increases (with respect to the unfed transformant) appeared to be less marked after the two periods when feeding was relaxed (24 and 52 hours). The observed profile therefore shows two definite peaks of intracellular concentration which appear during the 'feeding' periods.

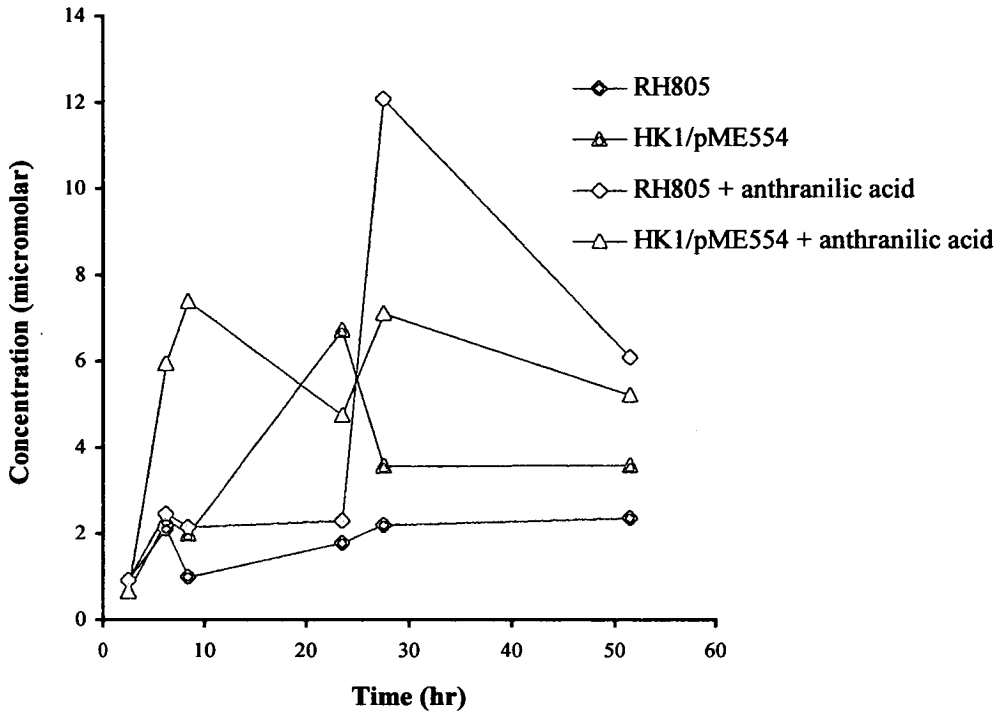
Total phenylalanine

The total concentrations of phenylalanine are illustrated in Figure 7-12A. These data showed similar patterns observed with the intracellular concentrations. Thus, an increase in total concentration was observed in HK1/pME554 ('fed') between 3 and 9 hours, during the first period of feeding, whilst no significant increase was noted in RH805 or in HK1/pME554 ('unfed'). In this culture, there was also a subsequent rise during the second period of feeding. In contrast the 'unfed' HK1/pME554 showed an increase from 9 hours to 24 hours which was followed by a subsequent decrease, in line with the previous experiment. RH805 ('fed') showed a hugely increased concentration during the second period of feeding, before dropping back to former levels.

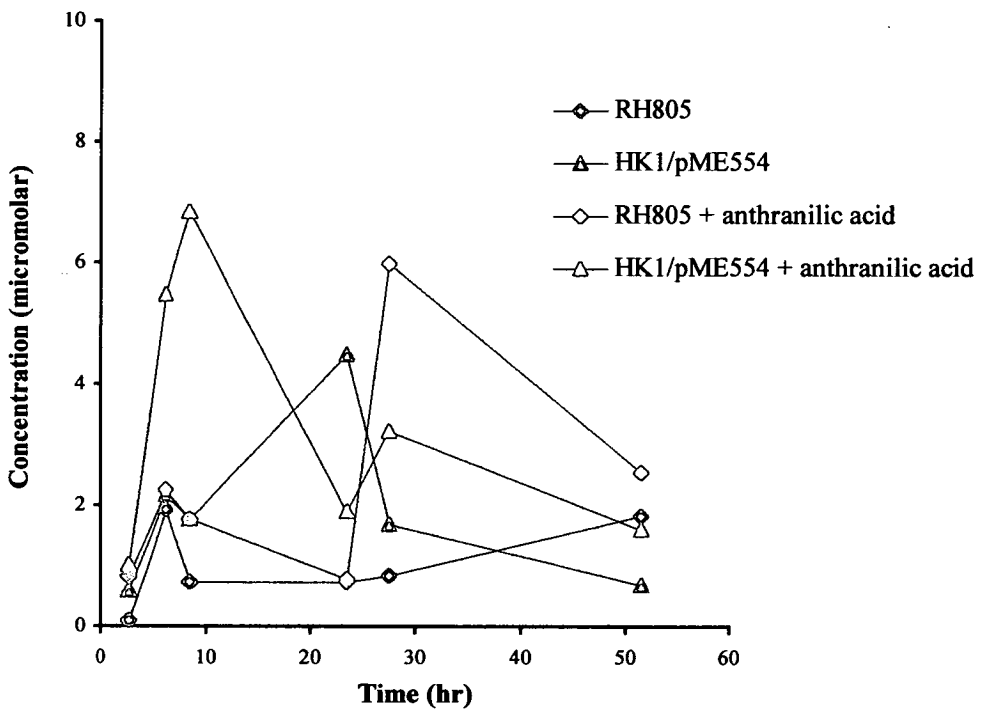
Figure 7-12 Total and estimated extracellular phenylalanine

A. Total phenylalanine. B. The extracellular phenylalanine is calculated from the values determined for total and internal pools.

A. Total phenylalanine pool



B. Estimated extracellular phenylalanine pool



Extracellular phenylalanine

These data are given in Figure 7-12B. In the 'unfed' strains, there was an increase in the calculated extracellular phenylalanine from HK1/pME554 compared to RH805 and this difference was particularly marked at 24 hours, somewhat later than in the previous experiment where the excretion of phenylalanine was observed to occur earlier than the excretion of tyrosine. This may result from the sharp increase (and subsequent decrease) which appeared at this time point, and leads to a high sensitivity of phenylalanine concentration upon rather small changes in time (or OD_{546}). In the two 'fed' strains there was a clear, and striking result; increased extracellular concentrations were expected to occur in two separate waves, corresponding to the periods of feeding on anthranilic acid. During the two subsequent 'recovery' phases, the extracellular concentrations dropped, suggesting re-uptake of phenylalanine into the cells.

Summary

- For 'unfed' cells, the results of the previous experiment were confirmed. Of particular interest here, is the peak concentration which occurred at 24 hours in HK1/pME554.
- Anthranilic acid increased intracellular concentrations of phenylalanine at all stages, in both HK1/pME554 and in RH805, but big rises occurred during both feeding periods for HK1/pME554 but only the second period with RH805.
- Total concentrations of phenylalanine and extracellular concentrations followed a similar pattern

When attempting to account for these results (and those of tyrosine and, to a lesser extent, tryptophan) it is important to appreciate that during the period from 9 hours to 28 hours, two separate factors appear to operate. One is the transient increases in intracellular and extracellular concentrations which accompany transformation with pME554 and which were also observed in the previous experiment. This occurred between 9 and 24 hours ($OD_{546} = 1.29 - 4.41$) in this experiment and between 10 and 25 hours in the previous experiment ($OD_{546} = 2.35 - 5.10$). The second factor, in the

present experiment, is the increased concentrations which appear to be contingent upon anthranilic acid. Thus HK1/pME554 showed two increases in intracellular phenylalanine concentration at 7 and 28 hours while both 'fed' strains showed increased intracellular phenylalanine concentrations at 28 hours after the second phase of 'feeding'.

The results suggest a number of questions which need to be addressed. Why do transformed strains (pME554) show higher intracellular concentrations of phenylalanine than RH805 in mid-culture? Why does feeding of anthranilic acid increase the intracellular phenylalanine concentrations (in both RH805 and HK1/pME554), particularly during the feeding period? Finally, what factors are involved in the particularly abrupt increase in RH805 (fed) between 24 and 28 hours. These questions are discussed in section 7.2.6 below.

7.2.5 Tyrosine concentrations

Intracellular tyrosine

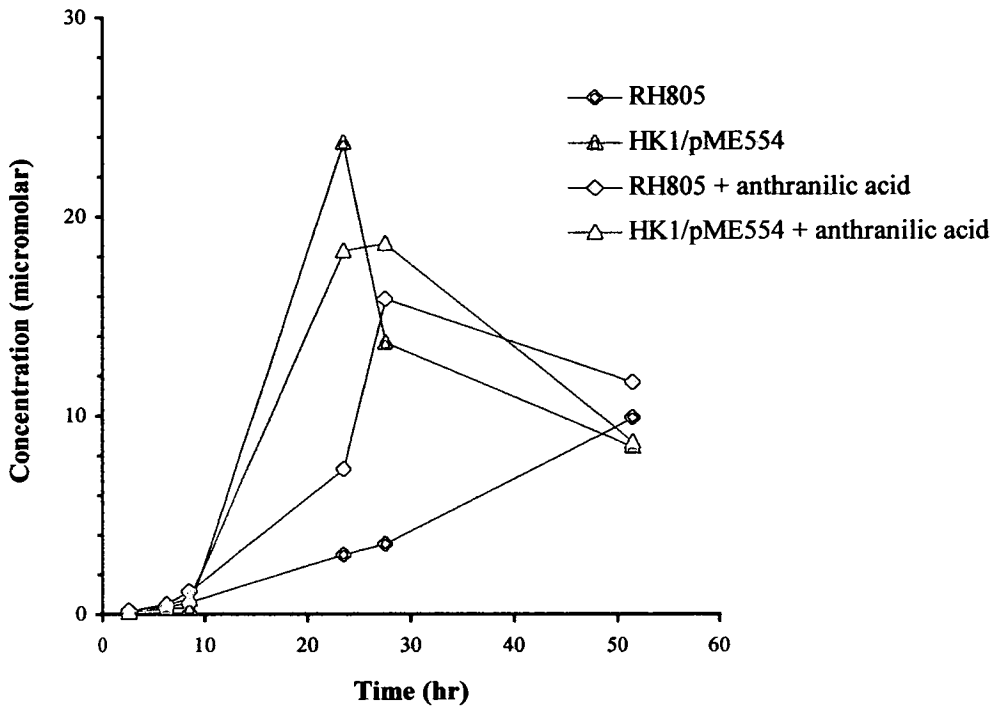
The results of the determination of intracellular pools of tyrosine are given in Figure 7-13. The data for 'unfed' strains are similar to those obtained in the previous experiment. Thus, intracellular concentrations of tyrosine in HK1/pME554 are very similar to those of RH805 except at 24 (and 27) hours when a large increase was observed. In this experiment, the increase was over 10-fold, compared to about six-fold in the previous experiment. This discrepancy may occur because of the rather sharp rise (and subsequent decrease) which probably occurs around this time point, as discussed above.

The effect of feeding anthranilic acid upon the values of intracellular tyrosine concentrations was rather small, in comparison to the phenylalanine concentrations. Firstly, there was only a small initial increase in HK1/pME554 during the first period of feeding, as was the case with phenylalanine. But there was only a small effect during the second period. Thus, RH805 showed an increase by 28 hours, while for HK1/pME554 the drop in concentration was lower for the 'fed' cells, compared to

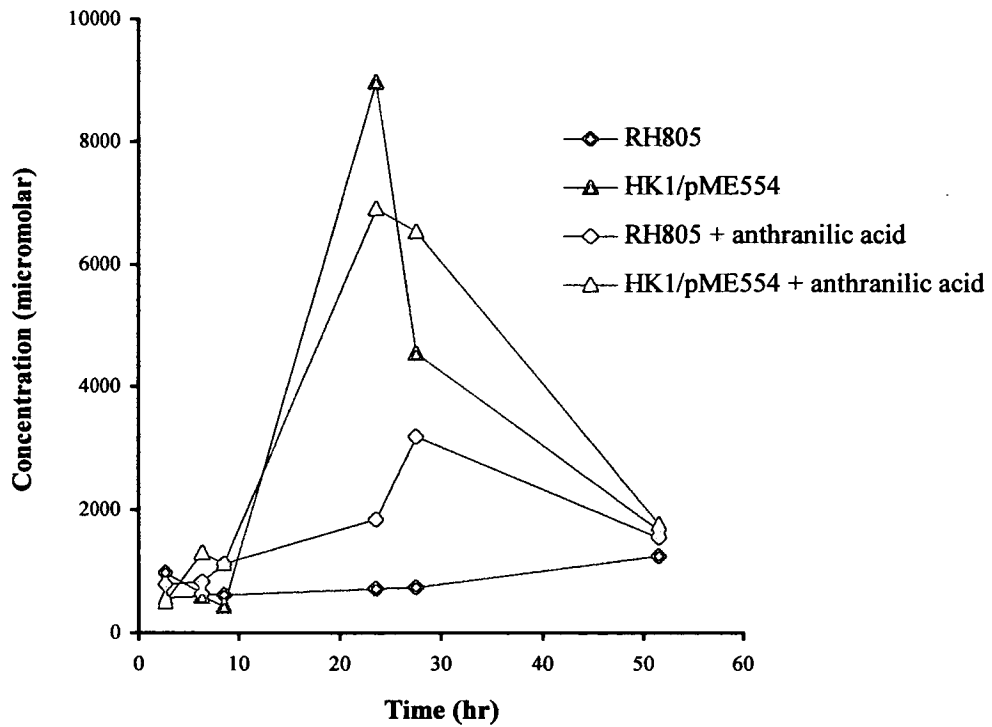
Figure 7-13 Intracellular tyrosine

A. The internal pools of tyrosine are expressed as concentrations with respect to the culture volume. B. The intracellular tyrosine concentrations are expressed with respect to cell volume as described in Chapter 2.

A. Internal tyrosine pool



B. Intracellular concentration of tyrosine



that of the 'unfed' cells. In all cases, the intracellular concentrations returned to the 'normal' levels.

Total tyrosine

The total concentrations of free tyrosine (Figure 7-14A) generally reflected the values obtained for the intracellular concentrations, with the exception of 'fed' RH805. Thus the transformed strain, HK1/pME554 ('fed' and 'unfed') showed large increases in tyrosine concentrations at the 24 (and maintained at the 27) hour time point, confirming the result of the previous experiment. The surprising result was the large increase observed in the 'fed' RH805 which occurred between the 24 and 28 hour sample. This increase is thus likely to be a response to feeding on anthranilic acid. This result paralleled that obtained for phenylalanine. However, another distinctive result for phenylalanine, namely the increases which occurred for HK1/pME554 during both periods of feeding, was not observed for tyrosine.

Extracellular tyrosine

The calculated extracellular concentrations of tyrosine show large increases by 24 hours in HK1/pME554 ('fed' and 'unfed'). RH805 ('fed') showed a massive increase during the second period of feeding.. In all cultures, the concentrations of extracellular tyrosine were predicted to fall back to low levels by 52 hours.

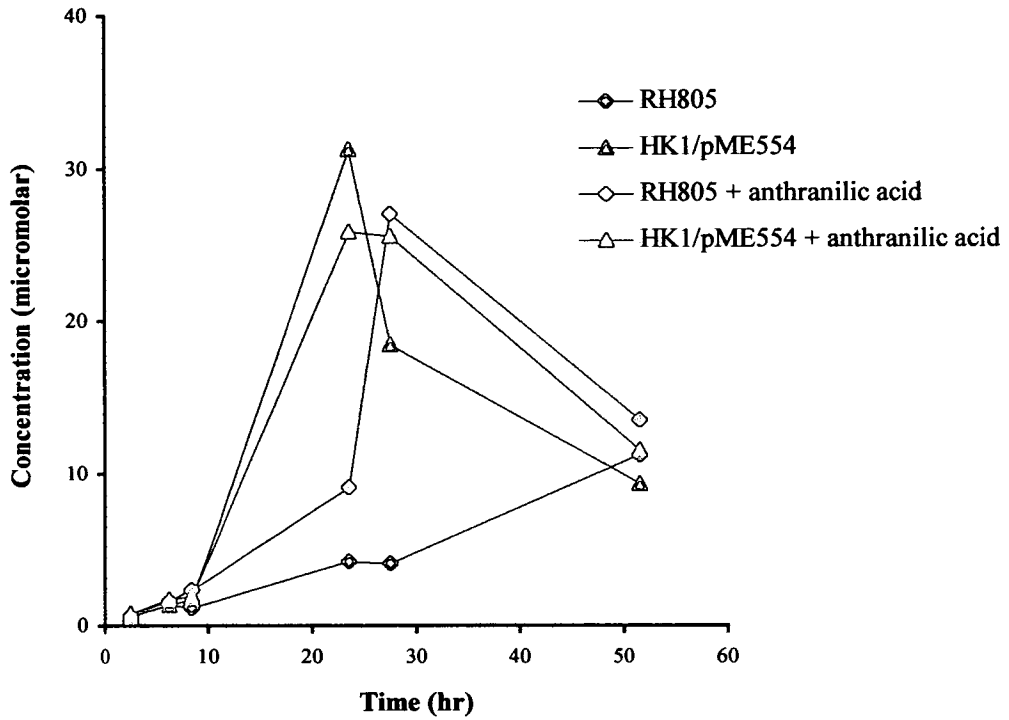
Summary

- In the 'unfed' strains, intracellular tyrosine concentrations peaked at 24 hours in HK1/pME554, before returning to previous concentrations, whereas RH805 maintains a more constant concentration. This result confirms those of the previous experiment.
- The addition of anthranilic acid had little effect on intracellular concentrations during the first period (exponential phase). During the second feeding period, the concentration in RH805 increased slightly and there was a small decrease in the (normal) drop in concentration in HK1/pME554.
- The total concentrations largely reflected the effect of the intracellular concentrations. However, the second period of anthranilate acid addition

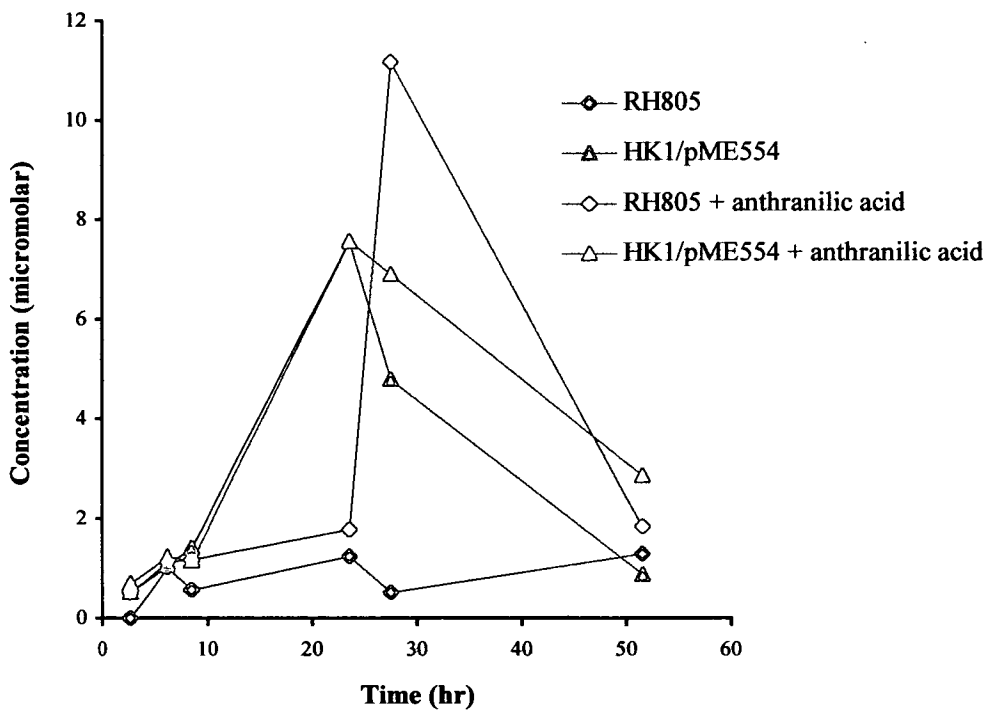
Figure 7-14 Total and estimated extracellular tyrosine

A. Total tyrosine. B. The extracellular tyrosine is calculated from the values determined for total and internal pools.

A. Total tyrosine pool



B. Estimated extracellular tyrosine pool



- greatly increased the total concentration in RH805 (at similar levels to those of HK1/pME554).
- The calculated extracellular concentrations followed a similar pattern. Feeding only slightly increased extracellular concentrations, relative to ‘unfed’ culture at 28 hours. For RH805, however, there is a very large predicted appearance of tyrosine into the medium, during the second feeding period.

7.2.6 Discussion

Before discussing the effects of adding exogenous anthranilic acid upon the production of tryptophan, phenylalanine and tyrosine, some comments on the results from the ‘unfed’ control cultures are required. The results obtained here confirmed the findings of the previous experiment, reported in section 7.1. In particular, there was a striking increase in intracellular and extracellular concentrations of tryptophan, phenylalanine and tyrosine which appeared during mid-culture, in strains transformed with pME554. This peak occurs somewhere between 9 and 28 hours; *i.e.* between OD₅₄₆ of 1 - 5. Because of the limited number of data points, and because of the possible rapidity with which concentrations may rise (and fall), it is not possible to define accurately the growth phase where this increase occurs, nor is it possible to be absolutely sure that a corresponding rise in concentrations does not occur in RH805. Nevertheless, the fact that no evidence of a rise has been detected in any of the three metabolites in three (including ‘fed’ RH805) different cultures reported here, nor in other ‘pilot’ experiments, strongly argues against this. Clearly, further experiments using more closely spaced time-points are required to confirm this presupposition and, more interestingly, to characterise the dynamics of the production for all three amino acids. For instance, in the light of the possibility that maximum phenylalanine excretion might occur before that of tryptophan and tyrosine (see Figure 7-5A), it would be helpful to know if the increase in one amino acid was contingent upon the change in another. Furthermore, it might be important to know if these phenomena are related to a specific growth phase or to cell density. In this case, an experiment which shifts cells into fresh medium at high cell densities may resolve this question.

It is necessary to offer some speculation about the reasons for the phenomenon as it stands. It is possible that these mid-culture changes are associated with increased internal tryptophan concentrations. This might be mediated by two mechanisms, as was discussed in section 7.1.6. Firstly, increased tryptophan concentrations may activate the enzyme chorismate mutase. This may increase both phenylalanine and tyrosine concentrations, in the context of constant flux. Secondly, increased tryptophan concentrations may inhibit anthranilate synthase, leading to an increase in chorismic acid concentrations, which may allow phenylalanine and tyrosine concentrations to rise. The evidence that one or other of these mechanisms may operate is not clear. Thus, feeding anthranilic acid to RH805 and HK1/pME554 between 3 - 10 hours and between 23 - 28 hours is associated with transient increases in intracellular and extracellular phenylalanine and, possibly, tyrosine (see Figures 7-11B, 7-12A, 7-13B and 7-14A). The presence or absence of effects are summarised in Table 7-2. The effect upon phenylalanine concentrations is large for both internal and external concentrations (Figure 7-11B, 7-12A) except during the first feeding period in RH805. There are similar effects with tyrosine except that extracellular concentrations are not increased in the double transformant, probably because the concentrations are already so high (Figures 7-13B and 7-14A). These data suggest that tryptophan over-production may have the predicted effect but that this is modified by differential regulation of phenylalanine and tyrosine. Despite this demonstration that tryptophan may regulate the concentrations of phenylalanine and, to a lesser degree, tyrosine, it must remain doubtful that this effect is responsible for the 'mid-culture' peaks in 'unfed' HK1/pME554 seen in both experiments. Thus the rise in tryptophan concentration under these circumstances is so much smaller (2-fold relative to 'unfed' RH805) than those resulting from the exogenous addition of anthranilic acid. Also, it is predicted that feeding anthranilic acid to HK1/pME554 will have a greater effect than for RH805, since tryptophan concentrations are increased by a larger factor in the transformant. The data do not fully support this hypothesis. Although the data in respect of the production of phenylalanine during the first period of feeding are consistent with this view, the situation is reversed during the second period, where RH805 shows a particularly rapid increase in

Strain	Time of sample	Phenylalanine		Tyrosine	
		Intracellular	Extracellular	Intracellular	Extracellular
RH805	7-9 hours	-	+/-	-	-
	24-28 hours	+	+	+	+
HK1/pME554	7-9 hours	+	+	+	-
	24-28 hours	+	+	+/-	-

Table 7-2 The effect of Anthranilic acid on phenylalanine and tyrosine concentrations

The degree to which anthranilic acid appears to increase intracellular and extracellular concentrations of phenylalanine and tyrosine in RH805 and HK1/pME554 during two time intervals is shown. ‘-’ indicates no apparent change, ‘+’ indicates a definite increase and ‘+/-’ indicates a doubtful or small increase.

phenylalanine production. In any event, explanations must be sought for the rather transient increase in tryptophan concentration itself in transformed cells.

If, as seems likely, the increase in tryptophan concentration resulting from transformation by pME554 is not wholly responsible for the rather large increases in phenylalanine and tyrosine concentrations during mid-culture in the absence of anthranilic acid, then other reasons must be sought. It is tempting to speculate that these effects are coincident with changes occurring during the diauxic shift. During this period, cells are adapting to low concentrations of glucose and are shifting from a fermentative to a respiratory mode of metabolism. This requires a shift in the pattern of gene expression. For instance, enzymes specific to glycolysis may be down-regulated, while those specific to respiratory and gluconeogenic pathways are up-regulated. Similarly, and possibly significantly, the flux through amino acid biosynthetic pathways is likely to be less because of the reduction in the growth rate which reduces the requirement for amino acids in protein, and may lead to a transient over-supply of amino acids if flux cannot be regulated rapidly without changing enzyme expression levels. It seems not unlikely, therefore, that a transient increase in amino acids arises during this period of adjustment. A number of predictions follow from this analysis. For instance, the appearance of peaks in the intracellular concentrations of the aromatic amino acid are expected to arise as the growth rate begins to drop. Also, similar increases in the intracellular concentrations of other amino acids are expected. In contrast, the timing of the subsequent 're-adjustment' of amino acid concentrations may be less significant. It may occur as a response to an increase in growth rate after the diauxic shift. Alternatively, it may occur earlier in response to an adaptive change in patterns of gene expression. However, this analysis does not explain the fact that the untransformed strain, RH805, does not show the phenomenon. Because of this fact, the differences in the growth curves obtained for RH805 and HK1/pME554 may be crucial. For example, HK1/pME554 grows slower than RH805 after the end of exponential phase and, indeed, may not grow to such a high density. This suggests that HK1/pME554 may be less able to adapt during the diauxic shift. This may be connected with a loss in this strain's ability to regulate the concentrations of aromatic amino acids. Clearly, experiments which involve a more

thorough investigation of the time-course of aromatic amino acid concentrations and its relation to cell density, growth rate and pattern of gene expression during the diauxic shift are required to illuminate these questions.

Chapter 8

Discussion

This final discussion briefly summarises the main findings of the present study. It attempts to place these results in the context of the original objectives of the project and discusses their implications with respect to an understanding of metabolic regulation. The biotechnological implications are considered with special reference to recent attempts both to provide a theoretical basis for pathway manipulation and experimental work on the system of aromatic amino acid biosynthesis. Finally, some thoughts about how future work in this area should be taken forward are presented.

8.1 Summary of the results

In order to apply the universal method to the problem of tryptophan over-production, a plasmid, pPH28 has been constructed which overexpresses enzymes of the shikimate pathway by a small factor. It has been introduced into an appropriate host strain, HK1, along with a multi-copy plasmid, pME554, which overexpresses the enzymes of the tryptophan pathway by a much larger factor. The effects of these changes were remarkably small under the conditions tested.

Fluxes to all three aromatic amino acids were effectively unchanged. During exponential growth when steady state conditions were most likely to prevail, there were, however, some changes to amino acid pools, especially those of phenylalanine and tyrosine. Nevertheless, these changes were very limited. When pME554 alone was present there were fairly insignificant changes to tryptophan, phenylalanine and tyrosine, while all three were slightly increased when both plasmids were present. These results were interpreted as suggesting that the flux and metabolite control coefficients of the enzymes within these pathways are very small. The levels of tryptophan, in particular, are highly regulated and this was discussed in terms of the role of feedback inhibition of anthranilate synthase by tryptophan. The data of Niederberger and others was used to discuss how feedback resistance has the effect of reducing the control coefficient of the enzyme. Thus removing feedback resistance

not only has the effect of allowing the metabolite pool to increase, but also allows greater amplification of flux following overexpression of the enzyme (Prasad *et al.*, 1987).

The concentrations of tryptophan, phenylalanine and tyrosine increased quite dramatically during the intermediate phase of batch culture of transformants containing pME554 and, especially, those containing both plasmids. This corresponded to the onset of the diauxic shift. The increases were largely accounted for by an increase in the extracellular pool. There was therefore evidence of excretion of all three amino acids during this phase which was subsequently followed by re-uptake of amino acids during respiratory growth phase.

8.2 Evaluation of the project's objectives

The objectives set at the beginning of the project and given in Chapter 1 were

- to apply the universal method to tryptophan biosynthesis in yeast
- to achieve increases in tryptophan production
- to reduce changes in other closely-related pathways
- to test the validity of 'the universal method'
- to understand what factors may limit the effectiveness and applicability of the method

The universal method has been applied, at least in a limited form (see below section 8.3). As explained above, significant increases in tryptophan production were not observed (except transiently during the diauxic shift). The third objective was founded on a false premise; *i.e.* it was assumed that the overexpression of the tryptophan enzymes would lead to a reduction in the flux to phenylalanine and tyrosine, or at least to a reduction in their pools. This was found not to be the case. Indeed, during the diauxic shift, phenylalanine and tyrosine were found at higher concentrations. Also, the changes in phenylalanine and tyrosine concentrations upon transformation with pPH28 were actually greater than those obtained for tryptophan. The evaluation of the final two objectives is given below in the next section.

8.3 Evaluation of the universal method

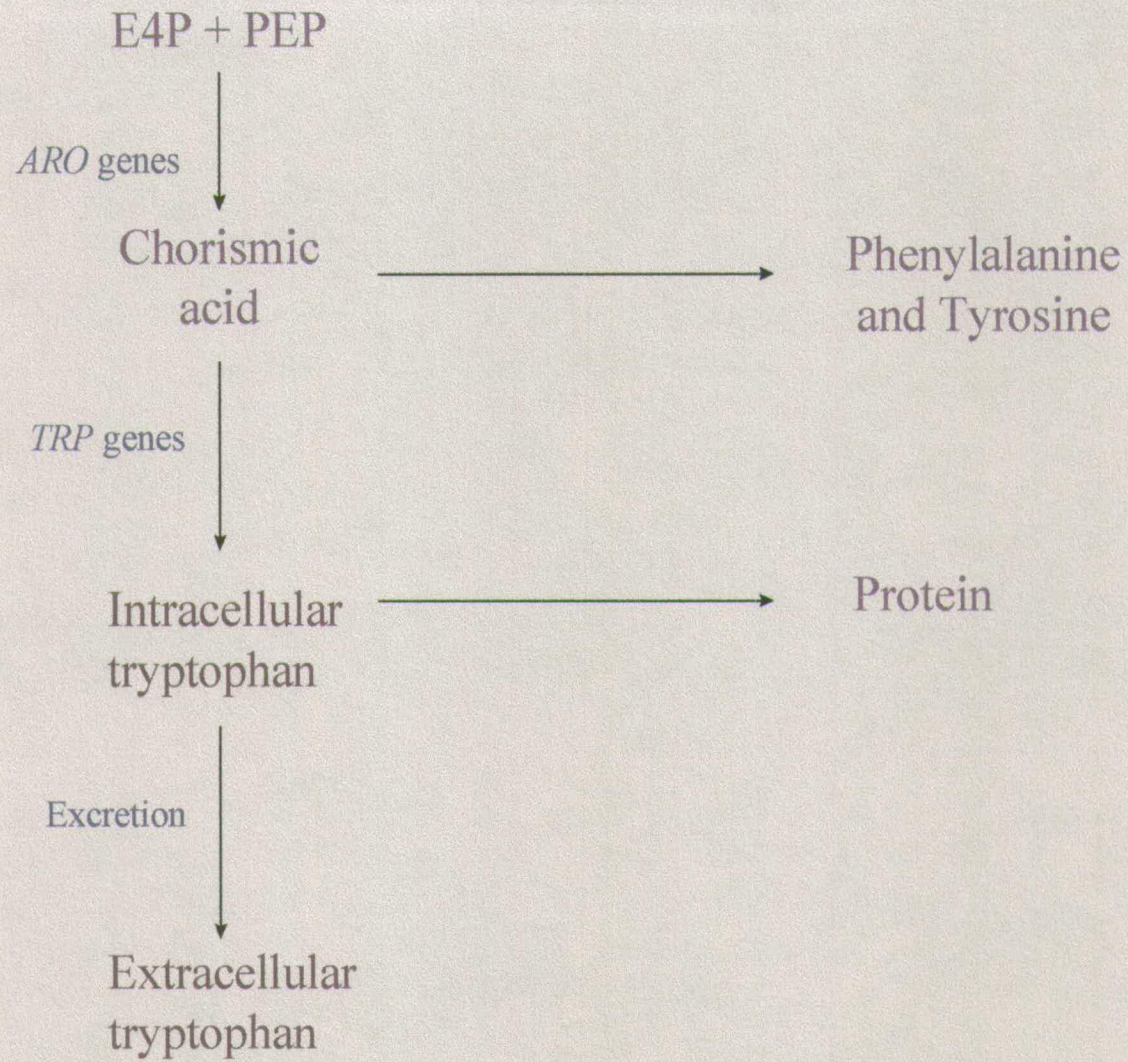
The implementation of the changes outlined in Chapter 1 has resulted in an insignificant change to flux to tryptophan. Therefore, experimental support for the universal method has not been obtained.

However, there are a number of important factors which suggest that the universal method has not been properly applied to this system. Most importantly, the full pathway has not been considered. Other fluxes from tryptophan were ignored. Also, because there remains the need to maintain internal tryptophan pools at unchanged concentrations, excess tryptophan should be excreted from the cell into the medium. If this is the case, then another branch point metabolite (tryptophan) appears (Figure 8-1). This creates two new pathway sections. Excretion to extracellular tryptophan and incorporation to protein. If the universal method is applied to these sections (in a comparable way to its application at the branch-point metabolite, chorismic acid) then excretion would have to be increased by a factor of about, say, 2500 for the full effects of the present system to be realised. Alternatively, a more realistic increase in the excretion step would require a much smaller overexpression of the tryptophan pathway enzymes than was achieved here, and a negligible increase in the shikimate pathway enzymes.

The question of increasing tryptophan excretion may, however, be problematic as discussed earlier in Chapter 6. If excretion depends upon the specific transporters, *TAT1* and *TAT2*, the intracellular concentration of tryptophan required to give significant net excretion may be significantly higher than the normal tryptophan concentration, which defeats the purpose of the exercise and, in any case, is likely to limit the flux to small increases. The most practical way to achieve increased excretion is to use the feedback resistant allele encoding anthranilate synthase, *TRP2_{br}*, which allows the pathway to function at very high intracellular concentrations of tryptophan and, thereby, leads to increased excretion (Prasad *et al.*, 1987; Kradolfer *et al.*, 1982). This implies that a serious evaluation of the universal method is only possible by comparing the changes in flux in a *TRP2_{br}* host strain,

Figure 8-1 The tryptophan pathway, branch pathways and branch-point metabolites

E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate



before and after transformation with pME559 (which bears the same allele as well as a copy of the other tryptophan genes) and pPH28. Unfortunately, no stable transformant bearing pME559 was obtained. Even if the universal method were successfully employed in this modified system, the applicability of the method would be restricted. One of the claims of the method negates the need to understand or modify the regulatory mechanisms operating within the pathway of interest. In the case of pathways which are highly regulated by their end-product, this problem must be specifically addressed. This thesis has therefore clarified the constraints which may operate to limit the applicability of the method.

8.4 Metabolic engineering with specific reference to aromatic amino acid biosynthesis.

The approach adopted in this thesis is not the only one used in attempts to increase production through aromatic amino acid biosynthesis. An examination of these others is revealing.

For instance, Flores *et al* (1996) describe the manipulation of a number of factors using *E. coli* as a model system. Firstly, they identify PEP production as a limiting factor. Since the phosphotransferase system (PTS) uses PEP to transport glucose into the cell, they generate a PTS⁻ derivative of their chosen strain, which can still utilise glucose (using a galactose transporter of reduced specificity). In addition, they also overexpress a feedback insensitive DAHP synthase from a plasmid and measure the DAHP excreted into the culture supernatant. Their data show that excretion of DAHP is approximately doubled in the PTS⁻ strain. They also overexpress a transketolase from the same plasmid in order to increase the supply of E4P. This also has the effect of doubling DAHP excretion in both the PTS⁺ and PTS⁻ strains.

A number of comments may be made. These experiments were performed in strains which already overproduced tryptophan (unpublished work). It is difficult to make claims about the generality of the data without knowing the genotype of their strains. Secondly, the use of a feedback resistant allele of DAHP synthase may change the structure of control within the pathway. Thirdly, and most importantly, the results tell

us nothing about the effect of these changes upon flux to chorismate and beyond. They only report on possible increases in DAHP excretion. What the results do suggest is the idea that many factors may contribute to the control of metabolite concentration. This important point was not made explicit. What were the effects upon tryptophan production? It would seem certain that significant increases in tryptophan would have been reported. Therefore it is tempting to suppose that very modest increases were obtained, if at all.

Katsumata and Ikeda (1993) studied tryptophan overproduction in *Corynebacterium glutamicum*. Their starting strain bears a mutation in chorismate mutase giving auxotrophy for phenylalanine and tyrosine. It was also derepressed for tryptophan. It excreted tryptophan into the medium to a concentration of 28 g l⁻¹. They assumed that DAHP synthase was rate-limiting because they could not detect tryptophan pathway intermediates in supernatants. However, when DAHP synthase was overexpressed (by a factor of 8), tryptophan production increased by only 10%. Since they now detected chorismate in the medium, they assumed that anthranilate synthase was now rate-limiting. However when this and the other tryptophan enzymes were over-expressed by a factor of about 10 no increase in tryptophan production was noted. A modest increase (about 25%) was only obtained when anthranilate synthase and anthranilate phosphoribosyltransferase were made insensitive to feedback inhibition by tryptophan.

Despite the fact that the starting strain produced enormous amounts of tryptophan even before manipulation, data such as these as well as those presented in this thesis may convince us that control of metabolite concentration and control of flux may be (and probably usually are) distributed across many metabolic reactions, that simple efforts to manipulate fluxes are rarely successful, and that success will most easily be gained by (i) identification of those steps which do exert control by experiment rather than argument based upon inappropriate theory, (ii) manipulation of many steps or (iii) manipulation of the mechanisms which operate to regulate the concentrations of useful metabolites.

8.5 Future directions

Despite the limitations of the Universal Method illustrated here, it would be interesting to repeat the same manipulations substituting a feedback-insensitive anthranilate synthase allele in the *TRP* plasmid and in the host strain. The results of Prasad *et al.* (1987) suggest that the flux multipliers obtained would be greater than obtained with pME554. Additionally, the role of the aromatic amino acid transporters *TAT1* and *TAT2* in mediating excretion and a further increase in tryptophan production could be investigated by overexpressing these genes.

Our ability to predictively manipulate microbiological growth in order to achieve a given output is likely to depend upon a matching of mathematical modelling with observed physiology. This in turn may depend upon our theoretical understanding of pathway regulation but will also involve validation of models against their real behaviour. Thus, there is a need to determine the kinetic parameters operating within a pathway and to test these against the actual behaviour of the pathway, possibly under 'steady state' conditions within a chemostat. The tryptophan pathway is reasonably well characterised in different micro-organisms and has real industrial potential. It would provide a good model system for further kinetic characterisation and modelling.

The problems of modelling other modes of culture more relevant to industrial needs (*e.g.* batch mode) are, of course, more complex. The rapid increases in excretion of tryptophan, phenylalanine and tyrosine toward the end of exponential phase and their subsequent re-uptake from the medium are interesting phenomena which are now amenable for study. A more precise characterisation of their dynamics and their contingencies could be undertaken in order to better understand the transitions in physiological state during the diauxic shift.

It is interesting to speculate that any future exploitation of biotechnology may be limited by our lack of understanding of microbiological physiology as much as our ability to manipulate gene expression at will.

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