VARIATIONS IN ENZYME PRODUCTION AS A FUNCTION OF THE PHYSIOLOGICAL STATE OF BACTERIA

by

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SUMMARY

As an approach to the analysis of the influence of environment on enzyme synthesis by bacteria, a study has been made of the effect of changes in gene frequency distribution on the constitutive synthesis of penicillinase and tryptophan synthetase B in *Bacillus licheniformis* ATCC 9945A. The gene frequency has been varied by alteration of growth rate and by growth of a *thy* strain on low concentrations of thymine, and the variation measured by comparing the ratios of *ade* and *trp* genes in DNA by transformation. Evidence had been found for a nuclease activity present in the transformation systems which is important in governing the number of transformants obtainable from a given amount of DNA. Analysis of the relationship between growth rate and gene frequency distribution has suggested that the time taken for a round of replication in *B. licheniformis* is about 40 minutes and that DNA synthesis is discontinuous at low growth rates (doubling times longer than 1 hr.). The gene-enzyme relationships and regulation of tryptophan biosynthesis in *B. licheniformis* have been examined and found to be similar to those in *Bacillus subtilis* and *Escherichia coli*, and constitutive mutants resistant to 5-methyl-tryptophan which it is postulated are mutant in tryptophanyl t RNA or its activating enzyme have been isolated. A correlation between the levels of tryptophan synthetase B and penicillinase and the frequency of

the *trp* and *pen* genes relative to all genes (the overall relative frequency) was observed when the gene frequency distribution was varied by changing the growth rate but not when it was altered by growth on low thymine concentrations.

C O N T E N T S

		Page
CHAPTER 1	INTRODUCTION	
1.1	General Remarks	1
1.2	Background to the Project	1
1.3	Control of Macromolecular Synthesis	5
	a. Protein synthesis	5
	b. RNA synthesis	10
	c. DNA synthesis	15
1.4	Analysis of the Problem	23
1.5	Experimental Approach	24
1.6	The System	25
	a. *Bacillus licheniformis*	25
	b. Penicillinase	26
	c. Tryptophan synthetase	28
	d. Transformation	31
CHAPTER 2	MATERIALS AND METHODS	
2.1	Materials	35
	a. Chemicals	35
	b. Media	36
2.2	Organisms	40
2.3	Transformation	42
	a. Isolation of DNA	42
	b. Procedure	44
	c. Scoring of transformants	45
2.4	Penicillinase assay	46
	a. Introduction	46
	b. Reagents	46
	c. Procedure	46
	d. Plate test	47

Page

2.5 Tryptophan Synthetase Assay 48
a. Introduction 48
b. Reagents 48
c. Procedure 48

2.6 Estimation of Protein 49
a. Introduction 49
b. Procedure 49

2.7 Isolation of Mutants 50

2.8 Growth Methods 51

CHAPTER 3 EXPERIMENTAL

3.1 Isolation of a Strain Constitutive for Tryptophan Synthetase B and Penicillinase 53
a. Introduction 53
b. Sensitivity of B. licheniformis to 5-methyl-tryptophan 53
c. Selection 54
d. Test for Constitutivity 54
e. Excretion of Tryptophan 56

3.2 Attempts to Isolate further Constitutive Mutants 57
a. Introduction 57
b. Histidine Enzymes 57
c. Arginine Enzymes 60

3.3 Growth Experiments 61
a. Introduction 61
b. Development of Media and Growth Conditions 62
c. Variation of Constitutive Enzyme Synthesis with Growth Rate 64
d. Samples for Measurement of Relative Gene Frequencies 65
e. Distribution of Penicillinase between Supernatant and Cell Bound Forms 65

		Page
3.3	Measurement of Relative Gene Frequencies	67
	a. Introduction	67
	b. Difficulties with the Transformation System	68
	b. Construction of a Doubly Marked Recipient Strain	70
	c. Age of Maximum Competence	73
	d. DNA Concentration and Transformation	74
	e. Measurement of ade/trp Ratio	77
	f. Titration of Recipient Culture	80
	g. Competition Experiment with Heterologous DNA	83
3.4	Variation of Gene Frequency Distribution by Growth on Low Concentrations of Thymine	84
	a. Introduction	84
	b. Isolation of a Low Thymine Requiring Strain	85
	c. Growth Experiments	86
	d. Penicillinase Synthesis	87
	e. Thymine Concentration and Gene Frequency Distribution	89
CHAPTER 4	ANALYSIS OF RESULTS AND DISCUSSION	
4.1	Tryptophan Biosynthesis in B. licheniformis	93
	a. Genes Involved	93
	b. Control	94
4.2	Variation in Enzyme Synthesis with Growth Rate	96
4.3	The Change in Relative Gene Frequencies with Growth Rate	97
	a. The Measurement System	97
	b. Results of Gene Frequency Transformations	99
4.4	DNA Replication in B. licheniformis	102

Page

4.5 The Variation of Gene Frequency Distribution by Thymine Concentration 105

a. The Strain 105

b. Influence of Thymine Concentration on Growth and Replication 105

4.6 The Correlation of Constitutive Enzyme Synthesis with Relative Gene Frequency 106

4.7 The Control of Ribosomal Protein Synthesis 110

4.8 Final Conclusions 111

REFERENCES 113

CHAPTER 1

INTRODUCTION

1.1 The panel set up by the Science Research Council (S.R.C.) to study the field of enzyme chemistry stressed in their report (Enzyme Chemistry and Technology 1969) the present and likely future importance of enzymes in medicine and industry, and recommended that the S.R.C. should support the investigation of the isolation, structure function, and uses of enzymes. Bacteria are potentially an important source of enzymes both for industry and for the study of enzymic structure and function, where the possibility of obtaining the same enzyme with altered specificity and activity by mutation makes them particularly suitable. Therefore the study of the isolation of enzymes should include the development of methods for obtaining increased yields of enzymes from bacteria.

1.2 Traditional approaches to the problem of increasing yields of bacterial enzymes and other products in fermentation processes have involved, on the one hand, screening the effects of varying each of the growth parameters in an attempt to optimise them all with respect to product formation, and, on the other hand, the technique of progressive random mutagenesis and screening known as 'forward mutation' (Alikhanian 1970). These two approaches will be referred to as environmental

and mutational, respectively. Work in the field of Molecular Biology over the past two decades has led to an expansion of our knowledge of the mechanisms by which bacteria alter the pattern of their macromolecular synthesis in response to their environment. It is now relevant to ask the question, 'In the production of enzymes and other macromolecules from bacteria, to what extent can tradition be replaced by rational approaches based on this knowledge?' More precisely, can one now predict the optimum growth conditions and also predict which genes should contain what type of mutations in order to maximise the synthesis of a particular enzyme or other macromolecule? From the genetic standpoint the answer to this question is 'yes'; the mutation to constitutivity (see Section 1.3.a) brought about by a mutation in a regulatory gene is an accepted method for obtaining increased synthesis of inducible enzymes. However, it is probable that this type of mutation is the first obtained in the 'traditional mutational approach' and does not constitute a great advance. However, the potential of the genetic construction of strains employing recombination (genetic engineering) based on knowledge of the genetics of the organism and regulation of the required product, has been demonstrated by Gilbert and Muller-Hill (1970). They obtained a great increase in the synthesis of the lac repressor by attaching the appropriate structural gene to partially defective temperate bacteriophage which allowed the

production of many copies of this gene. It is unlikely that this could have been achieved by the mutational approach.

From the environmental standpoint the question is far more complex as one is considering a large number of interactions between environmental and cell parameters. Thus the question becomes whether one can group parameters sufficiently to give a simple model which can be used to make predictions concerning the influence of the environment on the yield of a required enzyme. In the study of the control of DNA and RNA synthesis it has been found that environmental parameters can be grouped together and expressed in terms of the growth rate which they support (Maaloe and Kjeldgaard 1966). Under conditions of balanced growth, i.e. as defined by Maaloe and Kjeldgaard (1966), growth rate represents the total integrated response of the cell to its environment. As a result by reading 'growth rate' for 'all environmental parameters' one is ignoring specific interactions which are important in the control of the synthesis of enzymes. If one is to use this generalisation to derive a relationship to predict the influence of the environment on enzyme synthesis one is limited to deriving one relationship per enzyme which takes account of specific interactions, or to constructing a relationship in which specific interactions are ignored, and which relates the changes in enzyme synthesis to behaviour of the other control systems in the cell in their response to the environment.

In the latter case one is considering what can be called the general mode of variation of enzyme synthesis.

The object of the work in this thesis has been to investigate the influence of changes brought about by one of these other control systems upon the general mode of variation enzyme synthesis. The control system chosen was that of DNA replication and the reason for considering that this system of control might influence enzyme synthesis stems from the work of Yoshikawa and Sueoka (1963). They have demonstrated (as will be discussed in Section 1.3.c) that as a result of the polarity of DNA replication and the nature of the coupling between DNA replication and cell division, the relative frequency of a gene depends on its chromosomal location and the overall gene frequency distribution varies as a function of growth rate. If the frequency of a gene relative to all genes changes with growth rate, then the amount of enzyme specified by that gene relative to the total protein synthesised should vary in a manner characteristic of the gene's position on the chromosome. This work aims to evaluate the significance of such a variation relative to that produced by other general and specific influences on enzyme synthesis, and a general review of the mechanisms of control of the synthesis of protein RNA and DNA will now be given followed by a discussion of the experimental approach adopted, and a brief survey of the systems used in the experimental work.

1.3 Control Systems in Macromolecular Synthesis

1.3.a The Control of Protein Synthesis

1.3.a (i) Introduction

Five types of systems controlling protein synthesis can be defined at the metabolic level; these are (1) repression by end product feedback which is found in the regulation of biosynthetic enzymes (Cohen 1965; Monod and Cohen-Bazire 1953); (2) induction as seen in the regulation of degradative enzymes; (3) catabolite repression (Magâsanik 1961); (4) the total switch which is seen in the switch over of a cell to a new form of metabolism as in sporulation and (5) the control which operates on ribosomal protein synthesis. Types (1) and (2) are the mechanisms of specific interactions between individual components of the environment and individual genes or small groups of genes (operons), and as such can be grouped together. They can also be grouped on the basis of their mechanisms at the molecular level, which will be discussed briefly in order to establish the nature of mutations which override this type of control for specific genes. Types (3) and (4) are semispecific mechanisms in that they influence the expression of large groups of genes but not of all genes and they can be grouped on the basis of their mechanisms at the molecular level, which recent research (Silverstone et al. 1969; Losick, Shorenstein and Sonenshein 1970) suggests is in both cases by the alteration of the affinity of RNA polymerase for its binding site on its

DNA template. Little is known of the system controlling the synthesis of ribosomal protein; however, since ribosomal RNA and protein should be synthesised in stoichiometric amounts it is likely that both are under the same system of control. For this reason, type (5) will be discussed further with the control of RNA synthesis.

1.3.a (ii) Induction and Repression

From their studies of the genes controlling β-galactosidase synthesis in E. coli, Jacob and Monod (1961) proposed the operon model for induction. The operon was defined as a linear array of genes, which comprised structural genes for coordinately controlled enzymes and a regulatory gene called the operator (O gene). Also specified was a gene called the i gene which coded for an allosteric protein called the repressor which had the property of being able to interact with the operator to prevent transcription of the operon and thus give rise to negative control. However, it could also interact with the inducer to give a form unable to interact with the operator and thus bring about induction. Studies with the tryptophan operon in E. coli (Yanofsky 1967) showed that this model could also be applied to repression, with the modification that the end-product or some derivative of it acted as co-repressor and interacted with the repressor to block transcription.

Following the study of a number of further systems of induction and repression, and the further study of the

individual components of the β-galactosidase system, the operon model has become accepted as the universal explanation for induction and repression in bacteria. However, studies on the L-arabinose system in E. coli have shown there are possible exceptions to this universality (Englesberg, Irr, Power and Lee 1965). In this system a C gene product has been defined which is required for the induction of the enzymes of arabinose catabolism by arabinose. This system has been said to be under positive control and the C gene product is the converse of the i gene product of negative control systems.

In negatively controlled operons, mutants which override the control system, i.e. give constitutive synthesis of the structural gene products, are of two types. The first type are mutants in the i gene which cause the repressor to lose its ability to interact with the operator. The second are operator mutations which also prevent the repressor operator interaction.

1.3.a (iii) Catabolite Repression

Catabolite repression is the term coined by Magasanik (1961) for the repression of inducible enzymes by the level of metabolites. It is considered to be the mechanism whereby certain substrates are used preferentially as manifest in diauxic growth curves on mixed substrates (Monod 1947). Glucose is a rapidly metabolised substrate which exerts strong catabolite

repression, the inhibitory effect of glucose on the synthesis of inducible enzymes having been recognised for many years (for review see Gale 1943; Neidhardt and Magasanik 1956). However, the explanation of catabolite repression put forward by Magasanik (1961) proposes that it is specific not just to glucose but to a range of rapidly metabolised carbon sources, the metabolism of which yields large pools of intermediates, one of which effects the repression. As regards the mechanism, some insight has been gained from recent work on the lac operon of E. coli. In their analysis of mutants which gave low basal and induced levels of the lac enzymes, Ippen et al. (1968) defined a genetic region which they called the promotor and postulated as the binding site for RNA polymerase. Silverstone et al. have found that certain deletion mutants in the promotor region have a lowered sensitivity to catabolite repression and thus have implicated the interaction of polymerase and promotor as the control point for catabolite repression. Other workers approaching the problem from the biochemical standpoint (Perlman, de Combrugghe and Pastan 1969) have found that cyclic AMP can reverse catabolite repression and Emmer, de Combrugghe, Pastan and Perlman (1970) have isolated a protein which binds cyclic AMP and is required for the in vitro transcription of catabolite sensitive operons. Zubay, Schwartz and Beckwith (1970) have defined a similar function genetically which they have called the CAP factor. The current model for catabolite repression

is that cyclic AMP plus CAP factor facilitates the binding of RNA polymerase to the promotors of catabolite sensitive operons and thus permits transcription. The degree of catabolite repression reflects the size of the pool of cyclic AMP. The coupling between the level of catabolites and the size of the pool of cyclic AMP is not yet understood.

The influence of catabolite repression on enzyme synthesis during growth has been studied by Clarke, Holdsworth and Lilly (1968). They studied the constitutive synthesis of amidase in the presence of succinate (a strong agent of catabolite repression) in a chemostat and found that the level of amidase synthesis increased greatly as the dilution rate was taken below 0.4. The experiment was repeated using a mutant in which sensitivity to catabolite repression was reduced and the rise in amidase synthesis occurred at a higher dilution rate confirming that the rise was due to the relaxation of catabolite repression. These findings suggest that the level of catabolite repression itself varies with growth rate with the greatest repression occurring at the fastest growth rates.

1.3.a (iv) The Total Switch

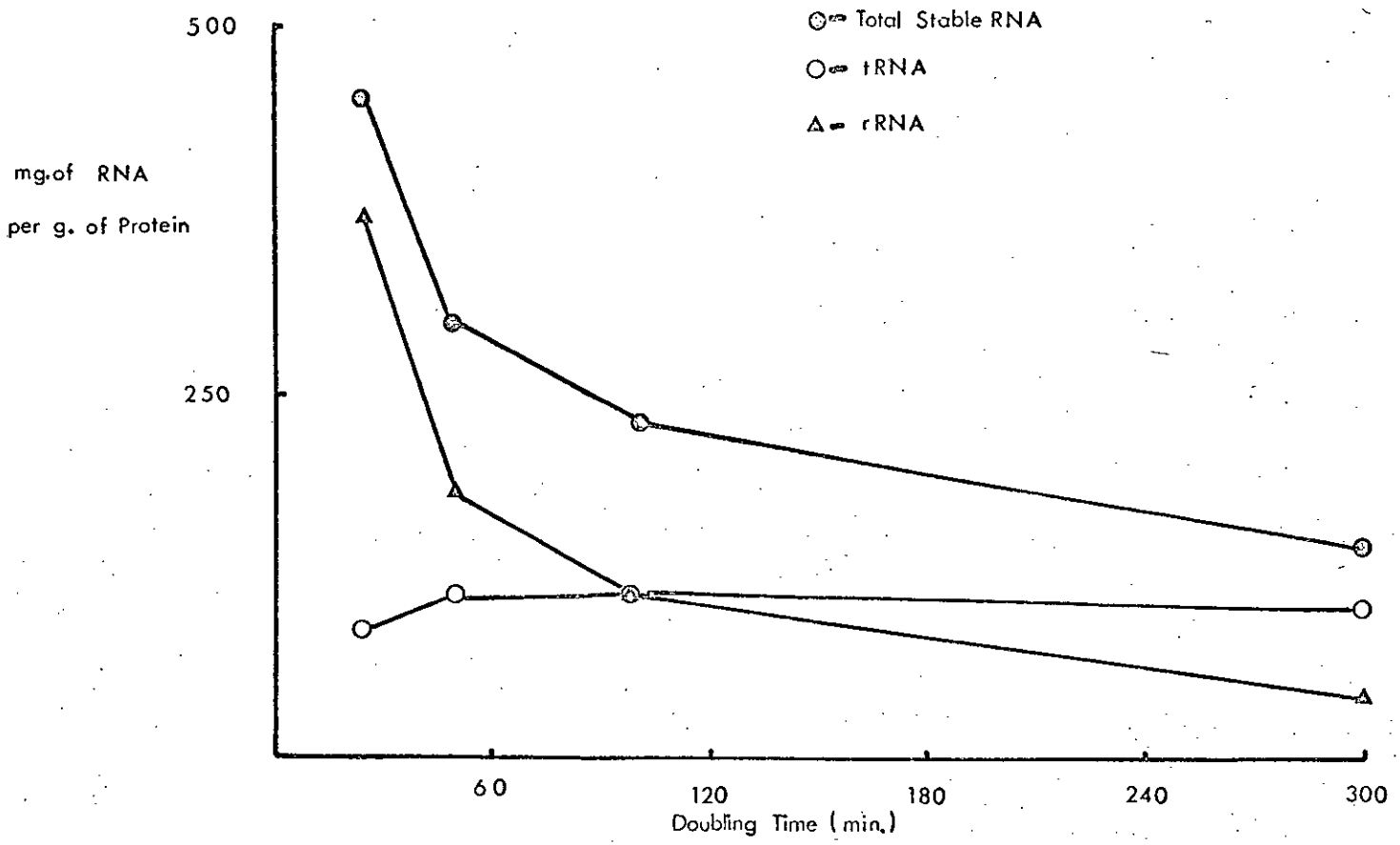
This mechanism is involved in the transition to non-growing states as in sporulation and bacteriophage infection and therefore is unlikely to influence the variation of enzyme synthesis with growth rate. Thus a

discussion of the total switch is not of relevance to this thesis. However, it should be pointed out that some enzymes formed as a result of phage infection are of use (e.g. polynucleotide kinase from T_4 infected E. coli B (Richardson 1965; Novogrodsky and Hurwitz 1966) which is used to label 5′ ends of polynucleotide chains) and that genetic engineering has been used to increase the yields of such enzymes (Hughes and Murray, unpublished).

1.3.b (i) Control of RNA Synthesis

It is first necessary to distinguish between the synthesis of stable and unstable RNA. In the case of stable RNA, which comprises ribosomal (rRNA) and transfer (tRNA) the amount present in the cell represents the amount synthesised. In contrast, the cell content of unstable (messenger (mRNA)) is a function of its rates of synthesis and breakdown. Thus the estimation of the rate of synthesis of mRNA is difficult. For thisreason in the study of RNA synthesis most attention has been paid to the stable components, in particular rRNA. The work of Maaloe and Kjeldgaard (1966) on the variation of the relative amounts of stable RNA and protein synthesised with growth rate (Fig. 1.1) led them to the conclusion that the rate of translation by individual ribosomes is constant and independent of growth rate, and that the cell content of rRNA is adjusted in order to maintain the maximum rate of protein synthesis permitted by the supply

Figure 1.1. The Variation in the Cell Content of Stable RNA Species With Growth Rate. From the Data of Maaloe and Kjeldgaard (1966).



of amino acids. The great increase in the rRNA-protein ratio observed when the growth rate is increased is the cell's method of increasing the overall rate of translation* by individual units. Thus the number of ribosomes per cell sets an upper limit on the rate of protein synthesis and it would appear unnecessary for more mRNA to be synthesised than can be saturated by the available ribosomes. This raises the question whether rRNA and mRNA are controlled independently or coordinately. Before these possibilities are discussed further it is important to emphasise that the supply of intermediates cannot be implicated in the control mechanism, since Maaloe and Kjeldgaard (1966) have shown that in a shift between a medium giving a slow growth rate and one giving a fast growth rate there is an instantaneous increase in the rate of RNA synthesis which indicates a reserve supply of polymerase and precursors. This raises the further question of whether the reserve RNA polymerase is under some inactivation mechanism or whether the templates for rRNA and mRNA are under a repression mechanism. Sufficient evidence is not available to distinguish between these possibilities; however, recently Travers, Kamen and Shleif (1970) have discovered a factor psi R which when added to in vitro systems stimulates the RNA polymerase in the transcription of rRNA templates. This indicates that the template specificity of RNA polymerase may be involved in the distribution of RNA synthesis between the stable and unstable species and thus supports

* by increasing the number of translating units rather than by increasing the rate of translation

the non-coordinate control argument. The difficulty in deciding whether the various species of RNA are co-ordinately or independently controlled stems not only from the difficulty in measuring the rate of synthesis of mRNA but from the inability of workers to agree on whether conditions which block rRNA synthesis also block mRNA synthesis (Edlin and Broda 1968).

1.3.b (ii) The Control Loop for rRNA Synthesis

At the metabolic level the synthesis of rRNA appears to be coupled to the growth rate via the pools of amino acids. Evidence for this and the construction of a model control loop has come from the study of the genetics and physiology of the 'rel' system in E. coli. It was found by Sands and Robertsen (1952) that when auxotrophs of E. coli were starved for a required amino acid rRNA synthesis immediately stopped. However, it was later found that if chloramphenicol was added at the same time as starvation the effect was not observed. Strains have been isolated in which rRNA synthesis is not sensitive to amino acid starvation (Stent and Brenner 1961). These strains were called relaxed and were mutant in a locus designated 'rel' which was considered to be involved in the regulation of RNA synthesis. The wild type (rel^+) was said to be stringent. Possible explanations of stringency are that amino acid starvation prevents the synthesis of a product required for rRNA synthesis or that it causes accumulation of an inhibitor of RNA

synthesis. The demonstration that chloramphenicol reversed the effect of amino acid starvation suggests that the synthesis of protein, be it activator or repressor, is not involved and implies that it is the accumulation of the receptor for the incoming amino acid, or the depletion of the receptor-amino acid complex in the absence of that amino acid, which is responsible for stringency. For this reason the possibility that transfer RNAs form the link between the amino acids and the control of RNA synthesis was put forward (Stent and Brenner 1961). A body of evidence (see Edlin and Broda 1968) now suggests that all the amino acyl tRNAs are required for rRNA synthesis although it is unclear whether the mechanism is one of negative control by a single species of uncharged tRNA, or one of positive control in which all species of activated tRNA are required for rRNA synthesis. Despite this a control loop can be described in which the level of uncharged tRNA (which is a function of the difference in rates of the supply and incorporation into protein of the amino acids) controls the level of rRNA and thus the supply of ribosomes which mediate this incorporation.

1.3.b (iii) The Control of Ribosomal Protein Synthesis and its Importance in Enzyme Synthesis

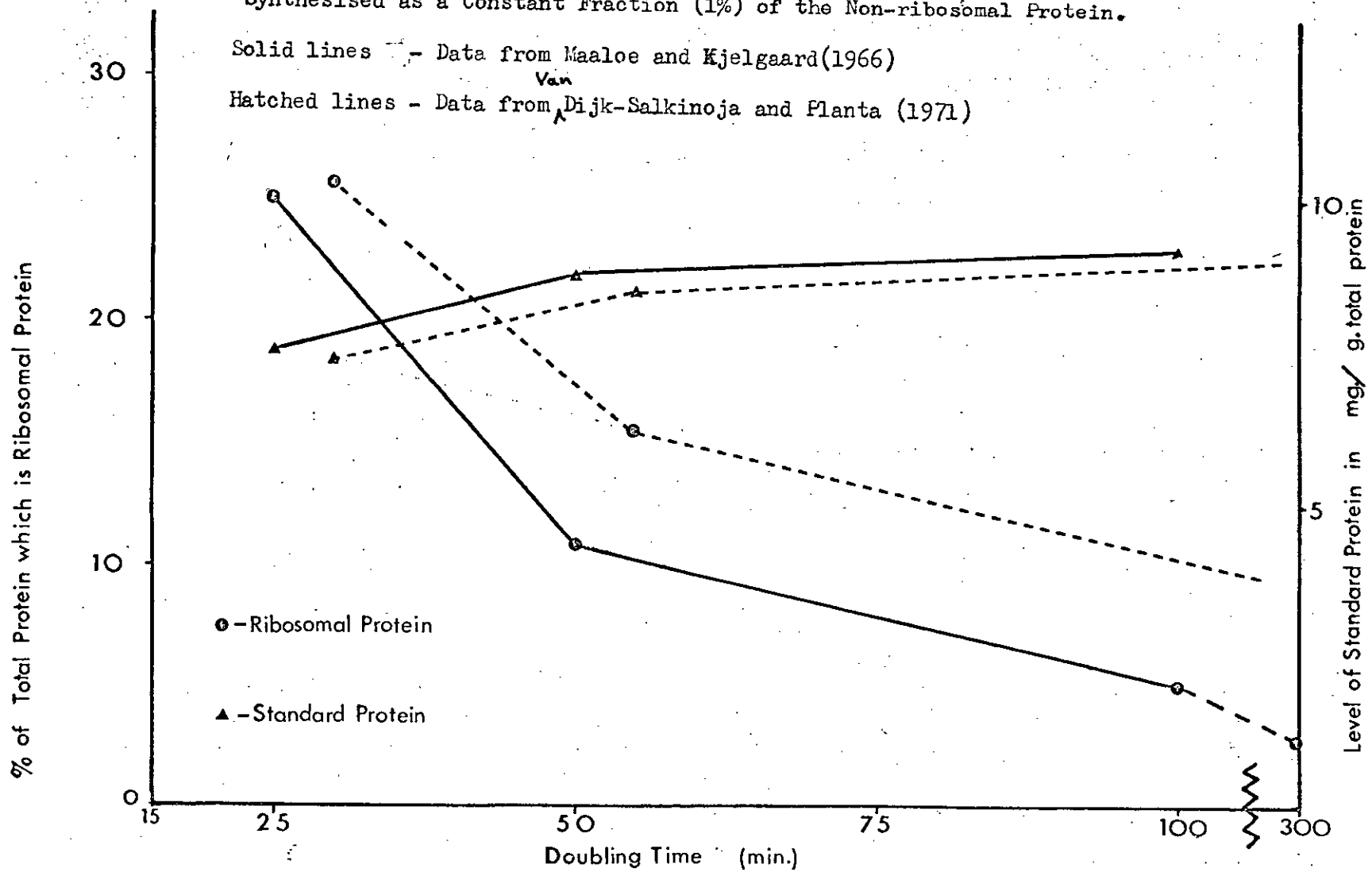
There is evidence that the synthesis of rRNA and mRNA for ribosomal protein are under coordinate control. This comes from experiments carried out on stringent and

relaxed strains in which the distribution of the small amount of protein synthesis obtained during amino acid starvation between ribosomal protein and total protein was measured. (These experiments were reported by Edlin and Broda (1968)). Since stoichiometric quantities of rRNA and r protein are required for construction of ribosomes there is a strong teleological argument for co-ordinate control of these components. If the amounts of ribosomal protein synthesised per cell parallel the amounts of rRNA synthesised, changes in this might also affect the apparent levels of enzyme synthesis. From the number of ribosomes per cell at different growth rates one can obtain information on the influence of growth rate on the synthesis of ribosomal protein. Figure 1.2 shows the percentage of total protein which is ribosomal protein as a function of growth rate, calculated from the data of Kjeldgaard and Maaloe (1966). It can be seen that ribosomal protein can vary from 4% to 25% of the total protein. The effect of this variation upon the level of a standard protein which is synthesised as a constant fraction (1%) of the non-ribosomal protein is also shown in Figure 1.2. This curve shows a significant change over the range of doubling times from 25 min. to 100 min. which must be taken into account when correlations are made between other factors and the variation of enzyme synthesis with growth rate. The effect observed in the curve for the standard protein will be referred to as the diluting effect of ribosomal protein.

Figure 1.2. The Cell Content of Ribosomal Protein as a Function of Growth Rate and Its Influence on the Concentration of a Protein (standard protein) which is Synthesised as a Constant Fraction (1%) of the Non-ribosomal Protein.

Solid lines - Data from Maaloe and Kjelgaard (1966)

Hatched lines - Data from Van Dijk-Salkinoja and Planta (1971)



Recently the influence of growth rate on ribosomal protein and RNA synthesis has been studied by Van Dijk-Salkinoja and R.J. Planta (1971) in B. licheniformis (the organism used in this investigation). Their results agree quite closely with those of Maaloe and Kjeldgaard (1966) and are shown in Figure 1.2. They are used in further analysis in Sections 4.6 and 4.7.

1.3.c The Control of DNA Synthesis and Chromosome Replication

The system controlling DNA synthesis ensures (a) that the rate of DNA synthesis is sufficient to complete the synthesis of one new genome per generation and (b) that one complete genome in the form of a single chromosome is distributed to each daughter cell at division. The control system has a component which adjusts the rate of synthesis to the growth rate and a distribution component which is manifest as a coupling between DNA replication and division. This duality has resulted in investigators adopting two approaches to the study of this control system. One has been to study the effects of agents on the coupling between DNA replication and division and the other has been to study the kinetics of the replication process. The studies of Cairns (1963), Meselson and Stahl (1958) and Sueoka and Yoshikawa (1963) fall into the latter category and together support the model of sequential and multifork replication which is central to this thesis.

Cairns (1963) developed an autoradiographic technique

which enabled him to show (1) that the bacterial chromosome was replicated as a single structure and (2) that at a doubling time of 30 minutes the rate of extension of the polynucleotide chain was not sufficient to account for all the DNA which has to be synthesised in a cell cycle, assuming that there was one replicating point per genome. Meselson and Stahl (1958) showed by density labelling techniques that DNA replication is semi-conservative and also that in a cell cycle at least 90% of the DNA becomes half heavy and none fully heavy which implied that the chromosome is replicated by a single replicating complex (the replicating point), travelling sequentially along the chromosome once per generation, always starting at a fixed point. Yoshikawa and Sueoka (1963) constructed a theoretical relationship between the relative frequency of a gene and its position on the chromosome based on the above implications and the cell age distribution function of Powell (1956). They tested the relationship in Bacillus subtilis by comparing the frequencies of genes in various positions on the chromosome in DNA extracted from cells at various points on a growth curve by transformation. Their theoretical relationship, called the gene frequency distribution function, took the form $p(X) = 2^{(1-x)}$* where 'p(X)' is the frequency of a gene in a population relative to the frequency of chromosome termini and 'x' is the fractional distance of the gene from the chromosome origin (the chromosome origin is defined as the first point on the

* Equation 1.

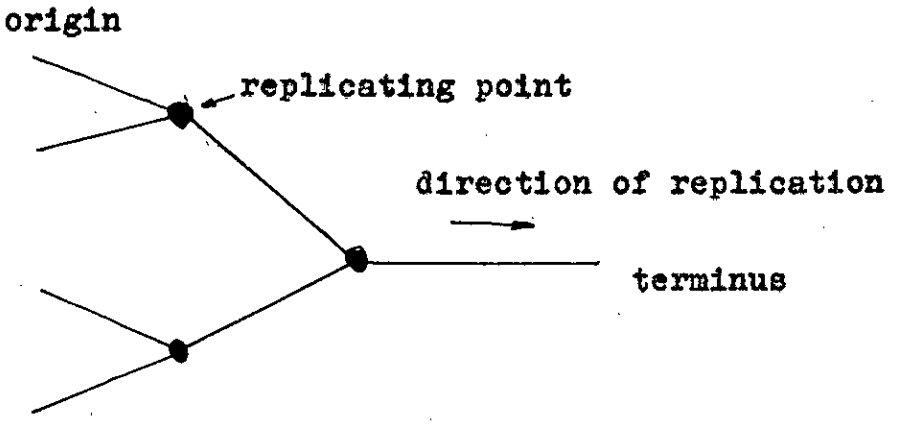
chromosome to be replicated). This function assumed that DNA replication was continuous, occupied the entire cell cycle and was sequential, i.e. started at a fixed point (the origin) and progressed in one direction along the chromosome to the terminus.

They found that if the gene frequencies obtained with DNA from exponential phase were normalised against those obtained with DNA from the stationary phase and inserted in equation (1), a map of the chromosome could be constructed which matched well with linkage data. This justified the assumptions made in the gene frequency distribution function and also the assumption made in using the stationary phase gene frequency to normalise figures obtained in transformation, which was that stationary phase cells contain only completed chromosomes and thus have a uniform gene frequency distribution.

Oishi, Yoshikawa and Sueoka (1964), by observing the gene frequency distribution during the outgrowth of spores on rich media, discovered that the ratio of frequencies of genes at the origin to genes at the terminus, i.e. the ratio of ends which equation (1) predicts should be 2, could rise to 4. This led them to postulate the theory of dichotomous replication which proposes that when a high rate of DNA synthesis is required the chromosome may be replicated by more than one replicating point.

This inferred that a second round of chromosome replication may be initiated before the previous round has finished. Oishi et al. (1964) envisaged a simultaneous

initiation of replication on both daughter strands of the previous unfinished round of replication and proposed that the replicating chromosome could have a dichotomously branched structure as shown below:



Schaechter, Bentzon and Maaloe (1959) found that when cells at low growth rates were given a pulse lasting 10% of a generation time of isotopically labelled thymine, only 80% of all the cells incorporated the label into DNA. This showed that DNA was only being actively synthesised during part of the cell cycle which would vary between 60% and 70% depending on where in the cell cycle the break in DNA synthesis occurred.

Sueoka and Yoshikawa (1965) took account of these findings in modifying the gene distribution function to include a power 'n', the average number of replicating points per chromosome and give a 'generalised equation of chromosome replication':

$$p(X) = 2^{n(1-x)} \qquad (2)$$

In order to maintain a steady state of chromosomes per cell in a population it was clear that there must be one

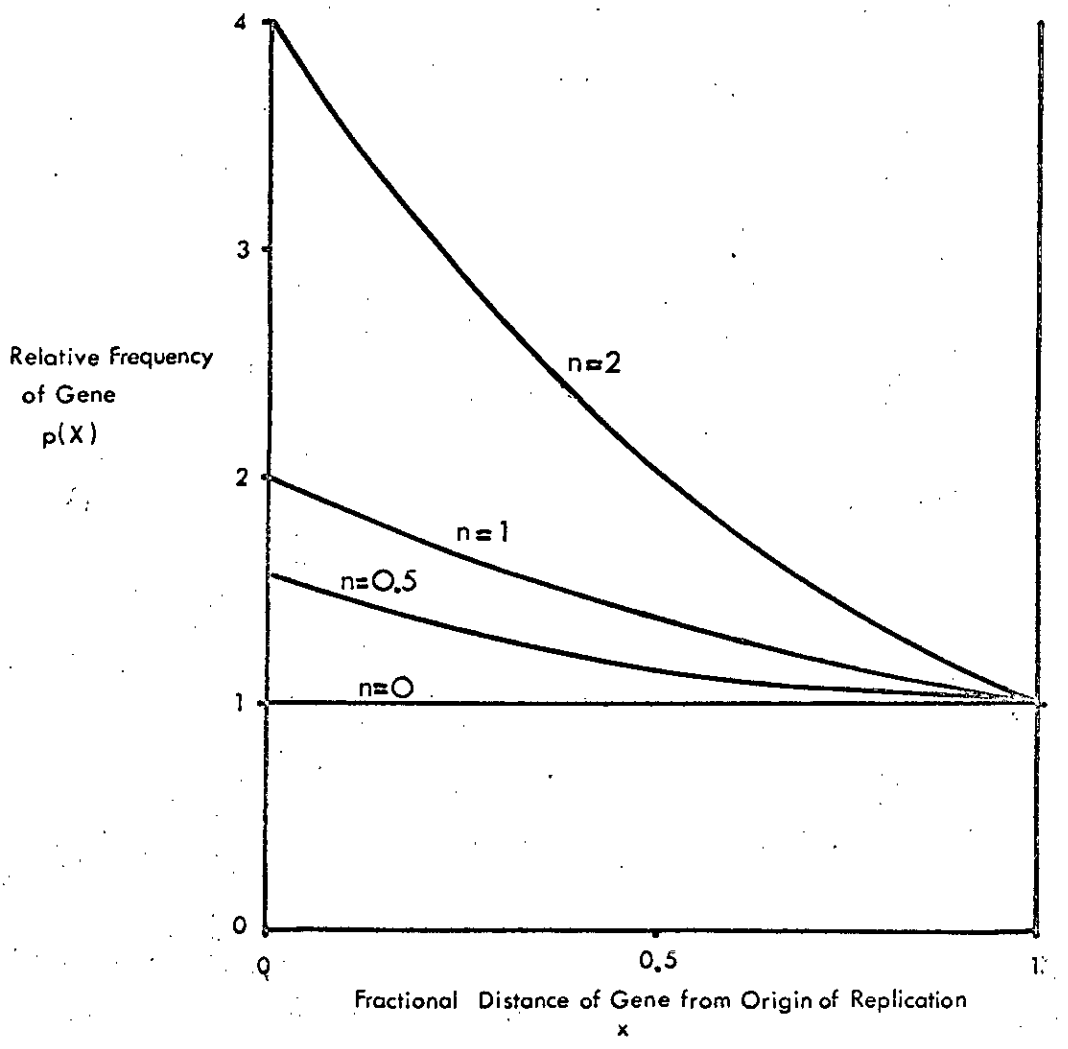


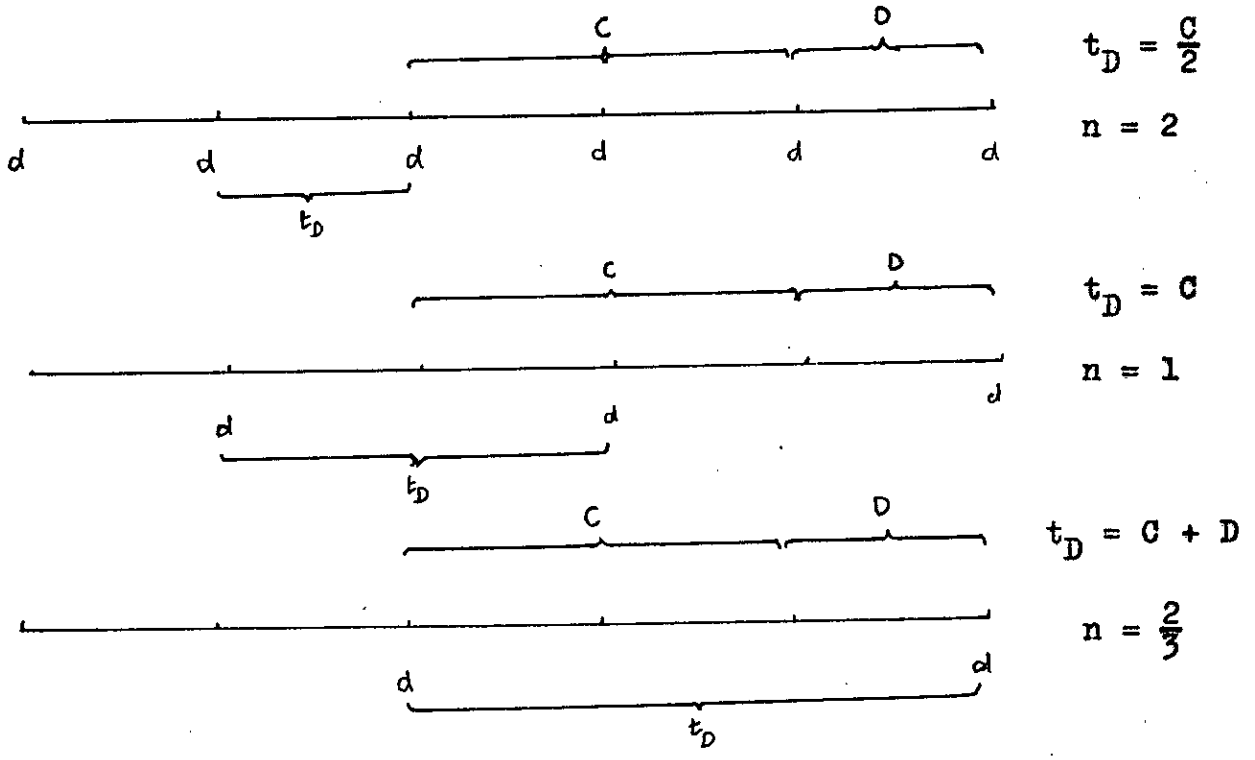
Figure 1.3. A Graph of $p(X) = 2^{n(1-x)}$ for Different Values of n. This Shows How the Gene Frequency Distribution Changes with Growth Rate.

$$n = \frac{\text{Replication Time } (t_R)}{\text{Doubling Time } (t_D)}$$

initiation event per cell cycle. Thus, in terms of time the distance between sets of replicating points, is equal to the generation time. Sueoka and Yoshikawa (1965) concluded that (when $n \geqslant 1$) n is equal to the ratio of the growth rate to the replication rate $(\frac{R}{u})$ (or $\frac{t_R}{t_D}$) provided that R is constant independent of growth rate. When n has a value of less than 1 n is equivalent to 1- the fraction of the cell cycle in which no DNA synthesis occurs. The work of Lark (1966) on the recovery of cells after thymine starvation and on cells growing at different rates support this theoretical analysis and the proposition that R is a constant except at low growth rates. Figure 13 shows a representation of equation (2) for various values of n, in which the x axis is a linear representation of the chromosome. The area under each curve represents the total number of genes at that value of n.

From data on the amounts of DNA per cell at different growth rates and the synthesis of DNA in synchronous populations at various growth rates in E. coli, Helmstetter and Cooper (1968) extended this analysis and constructed a model to show how the periodicity of initiation of replication is coupled to the periodicity of cell division. Their model breaks the cell cycle down into two periods: 'C', the time taken for the replicating point to traverse the genome, and 'D', the time between the completion of a round of replication and a division, which in E. coli B/r are 40 and 20 minutes respectively. For the range of

doubling time from 20 to 60 minutes C and D are constant. The model is: (1) that cells with a doubling time of C have no gap in DNA synthesis and no dichotomy, i.e. n = 1 and the round of replication which stimulates a division was initiated C + D minutes earlier; (2) as the doubling time is decreased from C this initiation event becomes earlier and earlier in the previous cell cycle, replication being dichotomous and (3) as the doubling time is made greater than C there is a gap in DNA synthesis with the initiation event happening later and later in the previous cell cycle until when the doubling time = C + D it occurs immediately after division in the same cell cycle. The model is shown schematically below:



d denotes a division

Since it is likely that when the doubling time is greater than 60 minutes C is no longer constant (Lark 1966) both the coupling of division and replication and the variation of gene frequency with growth rate cannot be predicted from this model. However, this model is useful for making predictions concerning the relative frequencies of genes at doubling times of less than 60 minutes provided that B. subtilis and E. coli do not differ in their pattern of chromosome replication. It also explains the apparent anomaly between the work of Cairns (1963) and Meselson and Stahl (1958); Cairns worked with a doubling time of 30' and a C of 40', so that the extent of labelling by a single replicating point would not be sufficient to label a complete chromosome in one generation.

Studies on the action of agents which specifically block cell division or replication on replication and division have yielded further information on the coupling of these processes. Starvation of thymine requiring auxotrophs of E. coli has been shown to stop DNA synthesis but not growth. However, during thymine starvation division ceases and the cells form filaments (Donachie, Hobbs and Masters 1968). This demonstrates that the completion of a round of replication is a necessary prerequisite for division. If after a period of thymine starvation thymine is added back to the culture there follows a rapid DNA synthesis period which continues until the DNA/mass ratio of the culture reaches the steady state

which it had before starvation (Donachie *et al.* 1968). During this time, rapid division also occurs until the steady state cell size is again reached. This experiment demonstrates the ability of the cell to recover from a large perturbation in its DNA content and emphasises the close coupling between the DNA replication and division. Similar experiments where DNA synthesis was inhibited by nalidixic acid gave the same result except that the post-inhibition rate of DNA synthesis was even higher than after thymine starvation. These experiments suggest that the cell size/DNA ratio may be involved in the control mechanism for DNA synthesis and models based on this idea have been put forward (Pritchard, Barth and Collins 1969).

During prevention of cell growth by starvation for a required amino acid or by inhibition of protein synthesis by drugs, e.g. chloramphenicol, the rate of DNA synthesis was found to fall progressively and reached zero after 1 - 1½ generations (see Maaloe and Kjeldgaard 1966). The net synthesis of DNA during this period for cells with one growing point (*E. coli* on glucose minimal medium) was about 40% of that present prior to starvation, and during this period progressively more cells became resistant to the thymineless death if starved of thymine. Apparently in the absence of protein synthesis no further rounds of DNA replication are initiated and the already initiated rounds are completed. The 40% residual DNA synthesis agrees well with that predicted by the Yoshikawa-Sueoka

age distribution function for chromosomes. This interpretation has been supported by the results of density labelling of cultures after re-initiation of protein synthesis (Pritchard and Lark 1964).

To sum up, the analysis of Sueoka and Yoshikawa (1965) and Helmstetter and Cooper (1968) give an integrated model which may be used to predict the influence of growth rate on the relative frequency of a gene, central to which is the constant rate of replication. Thymine has been shown to be important in regulating the activity of the replicating point (this will be discussed later in Section 3.4.a).

1.4 Analysis of the Problem

From this review of the control of protein DNA and RNA synthesis it is clear that at different growth rates the synthesis of an enzyme will be influenced not only by the relative gene frequency but also by the diluting effect of ribosomal protein, by the redistribution of protein synthesis amongst other genes as a result of their specific interactions with the environment and by any specific controlling system which acts on the enzyme itself. Quantitative data for the effect of relative gene frequency on enzyme yield cannot be obtained unless these latter effects can be measured or fixed. It is possible to ignore the specific controlling systems acting on the enzyme itself by studying the constitutive synthesis of a non-catabolite repressible enzyme. It is not possible

to measure the detailed distribution of protein synthesis at different growth rates and still less possible to fix it. For this reason two approaches to the problem have been adopted. One was to study the variation in enzyme yield with growth rate as a function of the position of the gene coding for it, on the chromosome. The second approach was to maintain a constant growth rate and to vary gene frequency in order to measure directly its influence on enzyme yield. Combining the results from the two approaches it should be possible to evaluate the relative importance of changes in gene frequency and the distribution of enzyme synthesis with growth rate in varying constitutive enzyme synthesis.

1.5 The Experimental Approach to the Investigation

The approach adopted was as follows:

(1) To isolate mutants of B. licheniformis constitutive for the synthesis of several enzymes.

(2) To isolate mutants of a strain of B. licheniformis which could act as a recipient in transformation, marked in a gene of known location and also in the gene's coding for the enzymes for which constitutive mutants were obtained in (1).

(3) To use the strain derived in (1) to study the constitutive synthesis of the enzymes during balanced growth as defined by Maaloe and Kjeldgaard (1969) at different growth rates, and at the same time to study the changes in relative frequency of the genes

coding for these enzymes with growth rate by transformation using the strains derived in (2). This should demonstrate the influence of the gene's position on the pattern of variation.

(4) To find ways of altering the gene frequency distribution other than growth rate and to use these to study the influence of relative gene frequency on enzyme synthesis.

1.6 The System

The experimental system to be described in the methods and results sections can be broken into the following components: the organism, Bacillus licheniformis; the enzymes, penicillinase and tryptophan synthetase; the system used for measuring gene frequencies; transformation.

1.6.a Bacillus licheniformis

This organism has been under investigation in Edinburgh for some time, particularly with respect to the structure of penicillinase and the control of its synthesis. The choice of B. licheniformis was based on (1) its present importance to the Department for the production of penicillinase (methods have been devised for the large-scale production of penicillinase from this organism (Hughes, Meadway, Thompson and Collins, in preparation)); (2) on the hope that this investigation might complement the academic work on B. licheniformis

in progress in the Department (several mutants derived in this investigation have been of great use to other members of the Department); (3) on the considerable stock of mutants available and (4) on the availability of a transformation system (Thorne and Stull 1966).

The genetic map of B. licheniformis 9945 A (Tyeryar, Lawton and MacQuillan 1968) constructed by gene frequency analysis and confirmed by transduction shows a great similarity to that of B. subtilis (Dubnau, Goldthwaite, Smith and Marmur 1967) (see Appendix 1). In addition, streptomycin resistance markers can be transformed between B. subtilis and B. licheniformis (Dubnau, Smith, Morell and Marmur 1965), and that some B. subtilis transducing phages can also transduce B. licheniformis (Taylor and Thorne 1963; Tyeryar et al. 1968). Based on these criteria it is believed that B. subtilis and B. licheniformis are closely related organisms.

1.6.b Penicillinase

Penicillinase (penicillin β-lactam amido hydrolase E C 3.5.2.6.) is found in a wide range of bacteria, including Gram negative species such as Enterobacteriaceae and Pseudomonas, and Gram positive species such as Bacillus and Staphylococcus. The properties of the penicillinases from these sources have been reviewed by Citri and Pollock (1966).

The penicillinase of B. licheniformis has been shown to be inducible (Pollock 1952) and to have an induction

response which differs greatly from other characterised enzyme induction systems, in that the maximum rate of induced synthesis is obtained only after 20 minutes, after initial contact with the inducer (Davies 1969). The penicillinases synthesised by strains of B. licheniformis fall into two classes (Pollock 1965), distinguishable by their enzymic and immunological properties and amino acid sequence (Ambler, Meadway and Thatcher, unpublished). One class is produced by strains of 749 and ATCC9945A of which the 749 enzyme has been sequenced (Ambler and Meadway 1969) and the other is typified by the enzyme of strain 6346. B. licheniformis penicillinases are exo-enzymes in liquid culture, the enzyme being about equally distributed between culture fluid (released form) and cell surface (cell bound form) (Kushner and Pollock 1961).

The genetics of the induction system have been investigated in B. licheniformis 9945A (Sherratt and Collins, in preparation). Regulatory and structural gene mutants were mapped by transformation and the two genes orientated with respect to the amino acid sequence of the enzyme. It was shown that a regulatory gene is adjacent or close to the end of the structural gene coding for the C terminus of the protein. Inability to construct merodiploid strains has prevented a dominance study of regulatory mutants but studies of their reversion (Sherratt, (Ph.D.) suggested that the system is one of negative control. He further suggested the possibility

that the repressor is synthesised by read through from the structural gene, i.e. from a polycistonic messenger RNA from which the enzyme is first translated. Thus the repressor could control its own synthesis. Other mutations affecting inducibility have been characterised and found to be genetically unlinked to the structural gene. It has been reasoned that these genes are involved with the fact that the inducer (penicillin) does not enter the cell (Davies 1969) and may act to transfer the induction stimulus into the cytoplasm.

Mutants of B. licheniformis 9945A synthesising penicillinase constitutively were first isolated by Fleming (unpublished). These mutants gave more than the fully induced level of synthesis and were unaffected by presence or absence of inducer. One of these mutants, 9945AC1, was used in this investigation. The mutation (C1) has been shown (Sherratt, Ph.D.) to be in the regulatory gene linked to the penicillinase structural gene.

1.6.c Tryptophan Synthetase

The pathway of tryptophan biosynthesis in E. coli was elucidated by Yanofsky (see reviews by Yanofsky 1960; 1967) from a study of the growth requirements and accumulations of tryptophan requiring auxotrophs (trp mutants). This approach established the gene-enzyme relationships for the five enzymes concerned in the conversion of anthranilic acid to tryptophan, and the

genes were mapped by transduction (Yanofsky and Lennox 1959; see review by Yanofsky 1967). It has been shown that these genes form the operon which is transcribed from an operator gene next to the E gene (Hiraga 1968).

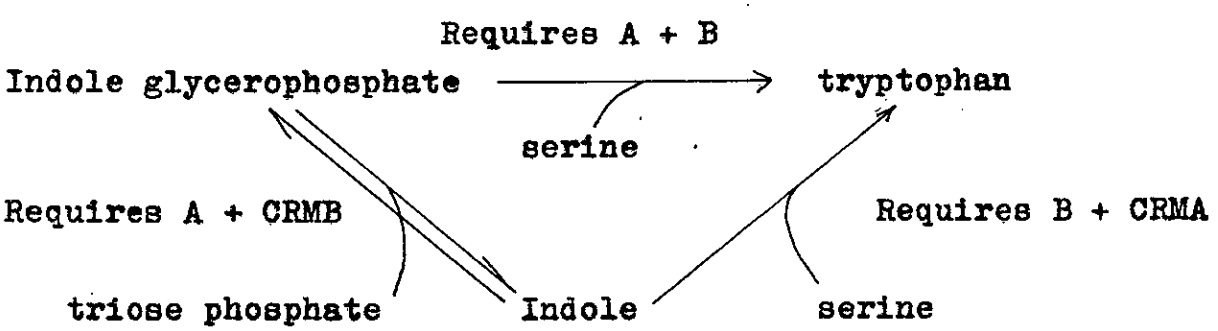
Tryptophan synthetase was the name given to the enzyme concerned in the conversion of indole, or indole-glycerophosphate to tryptophan, by addition of an alanyl group from serine, and it is coded for by the A and B genes (Crawford and Yanofsky 1958; Yanofsky 1959). The reactions involved in this biosynthetic step are:

(1) Indole + L serine ⟶ L tryptophan
+ pyridoxal phosphate

(2) Indole glycerophosphate ⟶ indole + triosephosphate

(3) Indole glycerophosphate + L serine ⟶ L tryptophan
+ triosephosphate + pyridoxal phosphate

Both A and B gene products (A and B proteins) are required for each of the three reactions but, while active B protein is absolutely required for reaction (1), reaction (2) requires only immunologically cross reacting B protein (CRMB) (Yanofsky 1959). Also, only mutants in gene A which produce CRMA protein are active in reaction (1). The formation of tryptophan from IGP and indole can be shown as:



From this diagram the reasons for the growth requirements and accumulations of A and B mutants are clarified. A mutants accumulate indoleglycerolphosphate and will grow on indole if they have CRMA, and B mutants accumulate indole if they have CRMB, IGP if they have not, and will not grow on indole.

The gene enzyme relationships for biosynthesis of tryptophan have been studied in B. subtilis (Carlton 1957; Anagnostopoulos and Crawford 1967) and shown to be identical with those of E. coli except that the indole-glycerolphosphate synthetase (C gene activity) is split into two components: N-5 phosphoribosyl anthranilic acid isomerase, and indoleglycerophosphate synthetase. The genes map close to tyr 1 and lie in the same order as in E. coli, as has been found by transformation using three point crosses.

1.6.c (ii) Regulation of Tryptophan Biosynthesis

That the synthesis of tryptophan synthetase is under repression control by tryptophan was demonstrated by Monod and Cohen-Bazire (1953) in Aerobacter aerogenes. Subsequently Yanofsky and co-workers have shown (Yanofsky 1960) that the formation of all the enzymes of tryptophan biosynthesis is sensitive to the level of available tryptophan. The operon has since been shown to be under the negative control of a repressor coded for by a gene Rtrp which does not map close to the trp operon (Cohen and Jacob 1959). Tryptophan or some metabolic derivative

of tryptophan acts as a co-repressor in the feed back control loop.

Mutants in gene Rtrp were obtained by selection of mutants resistant to the tryptophan analogue 5-methyl tryptophan (Cohen and Jacob 1959). These mutants were shown to have raised levels of the trp operon enzymes and were unaffected by exogenous tryptophan. Similar mutants have been found in B. subtilis (Zamenhof and Heldenmuth 1964; Meduski and Zamenhof 1969) which cross feed trp strains (i.e. overproduce tryptophan) but the mutation has not yet been mapped or classified.

The biosynthesis of tryptophan in B. licheniformis has not been studied. At the commencement of this project the only information available was from Sherratt (Ph.D.) who isolated and classified a trp mutant which corresponded to E. coli mutants blocked in anthranilate synthetase and which he showed was linked to tyrl.

1.6.d Transformation and its Use in the Measurement of Relative Gene Frequencies

Since the discovery of transformation in pneumococcus by Griffiths in 1928, followed by the demonstration that DNA was the transforming principle (Avery, McCleod and McCarty 1944) and the demonstration of recombination in transformation (Ephrusi Taylor 1951a, b), transformation has been developed as a genetic tool for numerous organisms. At the same time the physiology of the transformation process itself has been investigated since it

could provide insight into the methods of uptake of large molecules, the mechanisms of recombination and the relative significance of transformation in the evolution of different transformable organisms (see review, Eriksson 1970).

The use of transformation as an assay tool for measuring the relative frequency of particular genes in a sample of DNA is predicated on the findings of Googdal and Heriot (1957) and Lerman and Tolmach (1957), who showed that the number of transformants formed was proportional to the amount of DNA incorporated and that over a range of concentrations the number of transformants for a particular marker was directly proportioned to the concentration of DNA used.

This suggests that the production of a transformant depends on the interaction of a single bacterium with a single unit of DNA carrying the gene to repair the marker under test. In practice it has been found that the number of transformants obtainable from a given amount of DNA varies greatly with the marker under test, i.e. markers vary in their efficiency of transformation (see Eriksson 1970). Thus in the estimation of the relative frequency of genes in a sample of DNA from the relative frequency of transformants of markers in the genes, one must normalise for the relative efficiencies of transformation of the two markers by comparison with the relative numbers of transformants given by a DNA in which all genes are equally frequent, i.e. DNA prepared from a population

which contained only completed chromosomes. For this purpose, in the case of B. subtilis and B. licheniformis (Yoshikawa and Sueoka 1963; Tyeryar et al. 1968), DNA from stationary phase cultures and spores has been used.

The sequence of events in the uptake and integration of transforming DNA by B. subtilis is of interest with respect to the understanding of the relationship between the number of genes used and the number of transformants obtained.

The transforming DNA interacts first with a site on the cell surface to form a reversible DNA cell complex which is DNAse sensitive. Thus the DNA can pass into a second irreversible complex which is DNAse insensitive (see review, Eriksson 1970) probably through a nuclease which destroys one strand of the DNA. The final step involves the integration of the transforming DNA into the recipient chromosome. Experiments on the temporal variation in the efficiency of transformation by different markers in B. subtilis (Eriksson and Braun 1968) and B. licheniformis (Sherratt and Collins 1968) suggest that the integration may depend on replication of the recipient chromosome, integration occurring at or near the replicating point. This conclusion requires the assumption that the transformation procedure brought about a synchrony of replication of the recipient chromosome. Thus one may speculate the number of transformants for a particular marker obtained from a DNA sample containing a set number of copies of the gene will depend on the number of

receptor sites available on competent cells, the DNAse activity in the recipient culture and the kinetics of transfer of absorbed DNA molecules to the DNAse resistant state and thence into the recipient chromosome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.a Chemicals

All chemicals were of analar grade obtained from B.D.H. (Poole, England) with the following exceptions:

Chemical	Supplier
Sodium-L-glutamate	Ajinomoto (Japan)
Glycine	Cambrian Chemical Co.
L-aspartic acid	Do.
Other amino acids	Sigma Chemical Co.
Canavanine	Do.
DL 5-methyl tryptophan	Do.
∝N. acetyl histidine	Do.
2-thiozoyl alanine	Calbiochem, Los Angeles, U.S.A.
Indole	Sigma Chemical Co.
Benzyl penicillin	Glaxo Ltd.
Adenine sulphate	Sigma Chemical Co.
Thymine	Do.
Polyvinyl alcohol	Shawinigan Ltd. (London)
Agar	Davis Gelatin Co. (N.Z.) Ltd. (New Zealand)
Casamino acids	Difco Laboratories (Michigan, U.S.A.)
Tryptone	Do.
Yeast extract	Do.
Potato extract	Do.
Casitone	Do.

Chemical	Supplier
N-methyl-N^1-nitroso-N-nitrosoguanidine (NG)	Sigma Chemical Co.
Ethyl methane sulphonate (EMS)	Eastman Kodak Co.

2.1.b Media

All media were steam sterilised at 15 lb./sq.in. for 15 min. unless otherwise stated.

(i) Components made up and sterilised separately

(a) Minimal basal salts (Double strength) (2MBS) — g./l.

K_2HPO_4	29.3
KH_2PO_4	11.0
$(NH_4)_2SO_4$	4.0
Sodium citrate $2H_2O$	6.0
$MgSO_4.7H_2O$	0.44

(b) Iron and Manganese (Fe/Mn)

$FeCl_3.6H_2O$	16
$MnSO_4.H_2O$	0.2

HCl added to give a pH less than 1

(c) Calcium solution ($CaCl_2$)

$CaCl_2.2H_2O$	60

(d) Thiamine

Thiamine HCl	10

(e) Agar (Double strength)

Agar	40
Polyvinyl alcohol	15

	g./l.
(f) L-broth, pH 7.2	
Tryptone	10
Yeast extract	5
NaCl	10
(g) Glutamate	
NaL glutamate	100
(h) Sugars	20
(i) Concentrated amino acids (conc. AA)	
Na-L glutamate	25
Glycine	12.5
L aspartic acid	7.5
(j) Casein hydrolysate (ACH)	
Casamino acids	100
(k) Nutrient broth (Double strength) (2NB)	16
Nutrient broth	
(l) $MnSO_4$ solution	
$MnSO_4.4H_2O$	2
(m) Collins Special Salts	
$MgSO_4.7H_2O$	125
$FeSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.5
$MnSO_4.4H_2O$	0.05
$CuSO_4.5H_2O$	0.005
$K_2Cr_2O_7$	0.001

2.1.b (ii) Liquid media

	Amounts to make up 1 l. (approx.)
Medium L	
L-broth	500 ml.
2MBS	500 ml.
Fe/Mn	0.4 ml.
Thiamine	0.4 ml.
Medium A	
ACH	100 ml.
2MBS	500 ml.
Fe/Mn	0.4 ml.
Thiamine	0.4 ml.
Water	400 ml.
Medium G	
Glucose	100 ml.
2MBS	500 ml.
Fe/Mn	0.4 ml.
Thiamine	0.4 ml.
Water	400 ml.
Medium X	
Glutamate	100 ml.
2MBS	500 ml.
Fe/Mn	0.4 ml.
Thiamine	0.4 ml.
Water	400 ml.

BLSG

2NB	500	ml.
2MBS	500	ml.
Glycerol	40	ml.
$CaCl_2$	4	ml.
Fe/Mn	0.2	ml.

Transformation medium (TM)

2MBS	500	ml.
Water	500	ml.
Glucose	25	ml.
$MnSO_4$	6.25	ml.

CHS

Casamino acids	10	g.
KH_2PO_4	2.72	g.
Collins special salts	1	ml.
Water	1	l.

Sporulation medium (SS)

Potato extract	10	g.
Casitone	10	g.
Yeast extract	2	g.
Collins special salts	2	ml.
Water	1	l.

2.1.b (iii) Solid media

All were dispensed in 25 ml. amounts into sterile petri-dishes.

	Amounts for 1 litre (approx.)
Minimal	
Agar	500 ml.
2MBS	500 ml.
Glucose	20 ml.
Fe/Mn	0.5 ml.
AA solid	
Agar	500 ml.
Glucose	5 ml.
2MBS	500 ml.
Fe/Mn	0.5 ml.
Thiamine	0.5 ml.
conc. AA	20 ml.

Supplements, unless specified otherwise, were added at a level of 50 ug./ml.

2.2 Organisms

The choice of *B. licheniformis* ATCC9945A was based on its ability to act as a recipient in genetic transformation (Thorne and Stull 1966). This made possible the transfer of mutant alleles in the construction of complex strains for growth experiments, as in the construction of strain SH W*tlr*C1 (Section 3.4.b). The *pep* allele confers recipient ability and colonies of *pep* strains are small, hard and red. Colonies of *pep*$^+$ strains (used in growth experiments) are large by comparison and mucoid. Strain 749 was used for the

isolation of *trp* mutations which after classification were transferred into the *7d* derivative of *9945A*. Table 2.1 gives a description of all the strains used in this investigation. In the description of genotypes the convention of Demerec, Adelberg and Clark (1966) has been adhered to with the exception of the mutation to adenine requirement which has been written as *ade* rather than *pur* for easier association with its phenotype. Other genotypic designations are given below:

inducible synthesis of penicillinase	*pen* i^+ p^+
constitutive do.	*pen* i^- p^+
resistance to 5-methyl tryptophan	*mtr*
sensitivity to histidine	*Hs*
ability to grow on low concentrations of thymine	*tlr*

Strains derived in the course of this investigation are included in Table 2.1 and have the prefix *SH*. All strains were stored as spore stocks in distilled water made by the method described by Hughes, Meadway, Thompson and Collins (in preparation).

Table 2.1

Strain Designation	Genotype	Derived from	Source
9945A Cl	*peni*$^-$ p^+	ATCC 9945A	
M17	*pep ade 1*	Do.	Thorne
7d	*pep ade 1 tyr 1*	*M17* by NG	Sherratt and Collins
2N	*pep ade 1 trp E1*	Do.	Do.
8g	*pep ade 1 arg C1*	Do.	Do.

Strain Designation	Genotype	Derived from	Source
749	wild type		
SH mtr 1	pen i^- p^+ mtr 1	9945A Cl by UV	
SH trp 1	trp B1	749 by NG	
SH trp 2	trp	Do.	
SH trp 3	trp	Do.	
SH trp B1r	trp B1 ade 1 pep	7d by transformation from SH trp 1	
8a	ade 1 pep ilv D1	M17	Sherratt and Collins
8a T1	ade 1 pep ilv D1 thy	8a by aminopterin selection	Do.
SH 8a T t1r	ade 1 pep ilv D1 thy t1r	8a T1 by selection on low thymine	
SH 8 t1r	ade 1 pep thy t1r pen i^- p^+	SH 8a T t1r by transformation	
SH W t1r C1	thy t1r pen i^- p^+	SH 8 t1r by transformation	
His 1	his	749 by NG	Collins
SH Hs 1	tyr	by EMS on 9945A C1	

by NG = by NG mutagenesis
by UV = by UV do.
by EMS = by EMS do.

2.3 Transformation

2.3.a The Isolation of DNA

(i) From cells

A scaled down version of the Marmur (1961) procedure was used. Cell pellets (0.5 g. as opposed to 5 g. recommended by Marmur) were resuspended in 5 ml. of

saline EDTA (Marmur) to which was added 0.1 ml. of sodium dodecyl sulphate (25%) solution. The suspension was then incubated at 60°C for 10 min. followed by rapid cooling to 4°C. If this procedure did not produce complete lysis two rounds of freezing and thawing were applied. Thereafter, the standard Marmur procedure was followed for one round of deproteinisation. The ethanol precipitated DNA was redissolved in 2M NaCl and stored at 4°C until diluted (again in 2M NaCl) for use in transformation. The UV spectra of DNA samples prepared in this way read in a Unicam SP800 spectrophotometer gave characteristic curves for DNA with maxima at 260 nm. and minima between 230 and 233 nm.

2.3.a (ii) From spores

A 50 l. batch of spores of SH mtr 1 was grown in a Biotec FM50 fermenter, on SS medium over a period of 9 days. Daily checks were made for contamination and the spores were harvested in an Alfa Laval Lab 102 B-20 continuous flow centrifuge. The spore pellet was transferred to 2 x 250 ml. centrifuge bottles and was washed and re-centrifuged (MSE, HS 18, 6 x 250 rotor 4,000 rpm 10 min.) to remove all debris. Washing with distilled water and re-centrifuging was repeated 20 times, by which time the spore pellet was readily resuspendable and this was accepted as a criterion of the removal of all bacterial debris. The spores were then resuspended in an equal volume of saline EDTA (Marmur 1961) and 5 ml.

of this suspension were transferred by syringe to a 5 ml. capacity Hughes Press (Hughes 1951) which had been pre-cooled to -30°C. The spores were extruded from the press at -30°C, pressure being supplied by a fly press. The viscous paste of broken and unbroken spores was diluted with 5 ml. of saline EDTA and heated at 60°C with 0.1 ml. of a 25% solution of SDS for 10 min. Thereafter the normal Marmur procedure was followed. The spore DNA in 2M NaCl was sterilised by filtration (Millipore filter grade HAWP).

The mechanical method of spore disruption was chosen because it was thought that the long-term incubation with lysozyme required in the chemical method (Tabatabai and Walker 1967) could possibly allow germination and the accumulation of part replicated DNA in the extract.

2.3.a (iii) DNA estimation

Each extract of DNA was assayed in terms of its transforming activity rather than by measurement of its absolute concentration.

When knowledge of the absolute concentration was required the Burton diphenylamine assay was used (Burton 1956).

2.3.b Transformation Procedure

The transformation conditions used were those described by Thorne and Stull (1969) as modified by Sherratt (Ph.D.). Recipient cultures were grown in

250 ml. Erlenmeyer flasks containing 25 ml. of BLSG medium from a 0.1 ml. spore inoculum (ca. 10^6 spores) at 37°C. At the time of maximum competence (determined experimentally) the recipient was diluted into transformation medium (TM) (the influence of dilution factor on transformation is reported in Section 3.3.f). Aliquots (1-5 ml.) of the diluted recipient were added to test-tubes (1.8 x 15 cm.) containing suitable DNAs in 2M NaCl (5% of the volume of recipient culture). The tubes were incubated at 37°C on a reciprocal shaker (5 cm. throw, 1-6 hz.), and after two hours were transferred to ice prior to plating out. A control tube was run for each transformation (i.e. no DNA added) to check for reversion of the recipient.

2.3.c Scoring of Transformants

Transformants were scored by plating the transformed culture on selective media. AA solid medium was used supplemented so as to select for the desired transformant class, e.g. if the recipient was *ade tyr* and *tyr*$^+$ transformants were to be scored the plates were supplemented with adenine. Plates were incubated for 48 hr. and the transformant colonies then counted. The plating technique originally adopted was to spread 0.1 ml. of transformed culture on the surface of a dried plate of solid medium with a glass rod until the surface of the plate was again dry. The drawbacks of this method and the improvements made on it are discussed in Section 3.3.b.

2.4 The Penicillinase Assay

2.4.a Introduction

Penicillinase activities were measured by the Perret assay (Perret 1956) as modified by Collins (unpublished).

Penicillinase, E.C. 3.5.2.6., catalyses the conversion of penicillin to penicilloic acid by hydrolysis of the amide bond of the β-lactam ring of penicillin. Use is made of the fact that penicilloic acid can reduce 8 equivalents of iodine to estimate the rate of formation of penicilloic acid in the Perret assay. The incubation of the sample with penicillin is stopped by addition of iodine in acetate buffer pH 4.2 and the amount of unreduced iodine estimated spectrophotometrically.

2.4.b Reagents

A. Iodine (.16 M in KI (1.2 M)).

B. Acetate buffer 2 M, pH 4.2.

C. Penicillin:

600 mg. of sodium penicillin G in 0.1 M phosphate buffer pH 7.

D. Iodine acetate:

5 ml. reagent A plus 95 ml. reagent B.

2.4.c Procedure

To each of two tubes was added 2.5 ml. of penicillin solution (C) and the tubes incubated at 30°C for 2 min. before 0.1 ml. of sample was added to one of them. Incubation was continued for a time, calculated from the

expected activity of sample, to give a 50% reduction of the iodine. Reagent D (5 ml.) was then added to both tubes and 0.1 ml. of sample added to the blank. Incubation was continued for a further 10 min. after which the Δ OD at 499 nm. between blank and sample was read in a Unicam SP600 spectrophotometer. The activity of the sample was calculated as follows:

Units per ml. = Δ OD at 499 nm. x total volume of assay x factor to convert time to 1 hr. x factor to bring sample volume to 1 ml.

2.4.d Penicillinase Plate Test

A method for the detection of penicillinase production by colonies on solid media was designed by Sherratt and Collins (unpublished).

A $\frac{1}{4}$ dilution of reagent A containing sodium tetraborate (1 mg./ml.) was used to stain plates of solid media for 1 min. This reagent produced a strong blue colour with the PVA incorporated in the solid medium. The plates were then developed by flooding with penicillin solution (10 mg./ml.). Penicillin producing colonies formed clear halos in the blue colour by the interaction of penicilloic acid with the iodine. Using this test it was found easy to distinguish between colonies with the basal inducible level of penicillinase and colonies with the constitutive level.

2.5 The Tryptophan Synthetase Assay

2.5.a Introduction

This assay was based on that described by Whitt and Carlton (1968). The conversion of indole plus serine to tryptophan in the presence of pyridoxal phosphate is measured by the disappearance of indole.

2.5.b Reagents

A. Substrate mixture

10 ml. of substrate mixture contained:

0.5 ml. 1.0 M potassium phosphate buffer

1.0 ml. indole solution (350 ug./ml.)

4.0 ml. serine solution (20 mg./ml.)

0.2 ml. pyridoxal phosphate (740 ug./ml.)

4.3 ml. H_2O (distilled)

This reagent was made up afresh for each set of assays and the components stored at $4^{\circ}C$.

B. Indole reagent (PDMAB reagent)

Paradimethylaminobenzaldehyde	36 g.
Ethanol	800 ml.
conc. HCl	180 ml.

2.5.c Assay Procedure

Samples of culture (10 ml. or 5 ml.) were harvested by centrifugation (Sorval RC2 8 x 30 ml. rotor, 10,000 rpm, 10 min.) in Corex 15 ml. tubes. The supernatant was discarded and the cell pellets stored frozen at $-20^{\circ}C$ still in the Corex tubes. Prior to assay the tubes

were placed for 3 min. in a 35°C water bath to thaw the pellet. Then 1 ml. of substrate mixture was added and thoroughly mixed to resuspend the pellet. After 10 min. of incubation the reaction was stopped by addition of 0.1 ml. M NaOH. The residual indole was extracted by shaking with 4 ml. of toluene. The extract (1 ml.) was then added to 4 ml. of ethanol plus 1 ml. of indole reagent and mixed. The mixture was left for 30 min. before its OD 540 nm. was read against a zero blank containing no indole. The amount of indole used up was found by subtracting this OD from the OD of a reference which was prepared in the same way as the assay except that the reaction was stopped at time zero.

A unit of activity was defined as that giving an uptake of 1 m umole. of indole per min. Activities were thus calculated as follows:

$$\frac{\Delta \text{OD between sample and reference} \times \text{substrate volume} \times 30}{\text{OD of reference} \times \text{time (min.)} \times \text{sample volume}} = \text{units/ml.}$$

2.6 The Estimation of Protein

2.6.a Introduction

The total protein in cultures was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) on trichloracetic acid precipitates (TCA precipitates) of whole cultures.

2.6.b Procedure

Duplicate 10 ml. samples of culture were added to 15 ml. Corex tubes containing 3 ml. of 30% TCA. The

tubes were stored overnight at 4°C and then the TCA precipitates were spun down in the Sorval RC2 centrifuge (8 x 30 ml. rotor 10,000 rpm, 5 min.). The supernatants were then drawn off very carefully (to avoid losing any precipitate). Ten per cent TCA (3 ml.) was then added to each tube and the precipitates resuspended, and again spun down in the Sorval. The supernatants were again drawn off with great care and discarded. The precipitates were then resuspended in 1 ml. N NaOH and incubated at 37°C for two hours with the tubes sealed with Parafilm (Gallenkamp Ltd.). If this treatment was unsuccessful in dissolving the precipitate the tubes were then incubated at 60°C for 20 min. A 0.4 ml. aliquot of each dissolved precipitate was then added to 4 ml. of Lowry solution C and thereafter the normal Lowry procedure was followed. Lysozyme (hen's egg white) was used as a standard to calibrate this system.

In recovery experiments with lysozyme the above procedure for recovering and redissolving TCA precipitates was found not to result in any loss of protein.

2.7 Isolation of Mutants

A standard method of mutagenesis by N-methyl-N-nitro-N-nitrosguanidine (NG) devised by J.F. Collins in this laboratory was used in the isolation of auxotrophic mutants.

An overnight culture of B. licheniformis in L-broth was diluted into minimal medium 10 fold, and a freshly

prepared solution of NG (1 mg./ml.) added to a final concentration of 2 ug./ml. Mutagenesis was allowed to run for 20 min. before the culture was diluted 1 in 10 into fresh L-broth and grown up to a density of 10^9 cells/ml. (OD 1.0). The culture was then diluted and plated out on plates supplemented to allow growth of the desired auxotroph, so as to give 100-200 colonies per plate. Mutants were screened for by replica plating.

2.8 Growth Methods

Cultures were routinely grown in Erlenmeyer flasks containing 10% of their volume of medium. Flasks were shaken on a reciprocal shaker (1.6 hz., 5 cm. throw) to aerate cultures. It has been shown (Hughes et al., in preparation) that this level of aeration is able to support growth to an OD of 3.0 without oxygen limitation so it was assumed that in growth experiments in which growth was never allowed to go beyond 0.2 OD units there was no chance of oxygen limitation.

Growth was monitored by optical density measurement at 675 nm. in a Unicam SP600 spectrophotometer. When the abbreviation OD is used in the text, it refers to the optical density at 675 nm. in a 1 cm. pathlength cell. If the measurement was made at any other pathlength or wavelength this is quoted.

The relationship between specific growth rate (μ) and doubling time (t_D) of $\mu = \frac{1 \text{ n. } 2}{t_D}$ has been used throughout for interconversion of these terms. Doubling

time has been most commonly used and it should be remembered that since OD was used for measuring growth this is the mass doubling time.

Cultures were routinely grown at 37°C.

CHAPTER 3

EXPERIMENTAL

3.1 Isolation of a Mutant Strain Constitutive for Tryptophan Synthetase B and Penicillinase Synthesis

3.1.a Introduction

A strain resistant to the tryptophan analogue 5-methyl tryptophan (5MT) was selected following studies on the sensitivity of *B. licheniformis* to this anti-metabolite.

3.1.b Sensitivity of *B. licheniformis* to 5-methyl tryptophan

5MT at a level of 160 ug./ml. was found not to influence the growth rate of *9945A Cl* in X medium. Also when vegetative cells grown on medium A were harvested by filtration (Millipore filter type HAWP) resuspended in MBS and plated out at 10^6 cells per plate on minimal solid medium containing 5MT (100 ug./ml.), confluent growth was obtained after 2 days of incubation. Thus *B. licheniformis* is highly resistant to 5MT compared with *E. coli* which is sensitive to 10 ug./ml. (Hiraga 1969). It was thought that vegetative cells might owe their resistance to pools of tryptophan and that the germination of spores might be sensitive to 5MT. This idea was tested as follows: 0.1 ml. of a suspension *9945A Cl* spores were irradiated (UV 480 ergs./mm.^{2}) and plated out at 2 x 10^6 spores per plate on minimal medium

containing 5MT (100 ug./ml.). After 2 days of incubation at 37°C the plates were examined and found to have about 20 colonies per plate on a thin lawn of background growth. Thus it was reasoned that spore germination is sensitive to 5MT and that the colonies appearing could be 5MT resistant mutants.

3.1.c Selection

The procedure used in the test above was repeated using 200 ug./ml. of 5MT and the colonies which appeared after 2 days of incubation were picked (using sterile toothpicks) and streaked for single colonies on solid minimal medium containing 5MT (200 ug./ml.). A single colony of each mutant and the wild type was then patched on solid minimal medium containing 5MT (200 ug./ml.) supplemented with adenine, which had been seeded with a lawn of spores of strain 2N. Following overnight incubation 4 out of 10 of the mutants gave large halos (a result of cross feeding tryptophan to 2N); the 6 others and the wild type gave no halos. The halo-producing colonies were assumed to be mutant in the regulation of tryptophan biosynthesis so as to over-produce tryptophan and crossfeed 2N.

3.1.d Test for Constitutivity of Tryptophan Synthetase B

Two of the mutants isolated above were designated SH mtr 1 and SH mtr 2 and tested as follows.

Cultures (10 ml.) of 9945A Cl, SH mtr 1 and SH mtr 2 were grown overnight on X medium and each diluted 20 fold into a set of three flasks containing 25 ml. volumes of X medium. To one flask in each set was added 5MT (200 ug./ml.) and to another tryptophan (50 ug./ml.). Growth was followed for 6 hours, and it was found that all the cultures had 90 min. doubling times. Samples (5 ml.) were taken for tryptophan synthetase B assay and stored frozen. The results of these assays are shown in Table 3.1 below.

Table 3.1

Flask	Strain	Supplement	Tryptophan Synthetase B activity u per ml./OD
1	9945A Cl	-	0.041
2	"	5MT	0.045
3	"	tryptophan	0.0040
1	SH mtr 1	-	1.31
2	"	5MT	1.26
3	"	tryptophan	1.27
1	SH mtr 2	-	0.97
2	"	5MT	1.25
3	"	tryptophan	1.20

Thus it can be seen that the synthesis of tryptophan synthetase B by the wild type is repressed by tryptophan but not by 5MT. This unexpected result may help to explain the high level resistance of growing cells of B. licheniformis to this analogue. SH mtr 2 gave

30 fold higher levels of activity than the physiologically derepressed wild type and was not repressed by tryptophan, suggesting that this was a derepressed mutant and that the wild type was not fully derepressed when growing on minimal media. However, SH mtr 2 gave a slightly increased level of synthesis in the presence of 5MT and tryptophan. For this reason SH mtr 1, which gave constant levels of activity regardless of the presence of tryptophan or 5MT, and was therefore assumed to be constitutive for tryptophan synthetase B synthesis, was chosen as the test organism for growth experiments.

3.1.e Excretion of Tryptophan by SH mtr 1

Since SH mtr 1 showed a high level of derepression for tryptophan synthetase B and cross fed tryptophan on solid media the possibility of this strain being of commercial importance for the production of tryptophan was tested.

SH mtr 1 was grown in 25 ml. of G medium and samples taken during growth and stationary phase to measure the concentration of tryptophan in the culture supernatant. Samples were harvested by centrifugation (Sorval RC2 8 x 30 ml. rotor 10,000 rpm, 10 min.) and the supernatant given to Dr R.P. Ambler for amino acid analysis. He was unable to detect tryptophan in any of the samples by a method which would have detected a level of 75 ug./ml. It was concluded that if SH mtr 1 does excrete tryptophan when grown in liquid culture it does not exceed the

capacity of derepressed mutants of E. coli to do so (Sahn and Zahnen 1970) and is almost certainly not of commercial importance.

3.2 Attempts to Isolate Further Constitutive Mutants

3.2.a Introduction

Unsuccessful attempts were made to isolate mutants constitutive for histidine and arginine biosynthetic enzymes. These will be briefly reported since they provide information on the physiology of B. licheniformis. The choice of these enzymes was based on the position of his and arg mutations on the B. subtilis and B. licheniformis chromosome maps (Appendix 1). A his marker is close to the origin of replication in B. licheniformis and an arg marker about in the middle of the map. The ability to assay these enzymes in the constitutive state would have extended greatly the scope of this project.

3.2.b Histidine Biosynthetic Enzymes

The histidine analogue and antimetabolite 1,2,4-triazole-3-alanine (TRA) has been shown to repress histidine biosynthesis in Salmonella typhimurium (Levin and Hartman 1963) and has been used to isolate resistant mutants constitutive for histidine synthesising enzymes (Roth, Anton and Hartman 1966). Also the histidine analogue 2-thiazoyl alanine (THA) has been shown to cause false feedback inhibition of the first enzyme of histidine biosynthesis (Sheppard 1964). It was decided to test the

sensitivity of B. licheniformis to these analogues. Unfortunately TRA is not produced commercially and is totally unavailable (H. Whifield, personal communication). However, THA was obtained and it was hoped that the germination of spores might be sensitive to this compound and that resistant mutants might be obtained which were derepressed for the histidine biosynthetic enzymes analogous to the 5MT resistant mutants. However, the germination of spores was found to be insensitive to concentrations as high as 800 ug./ml. so this approach was dropped.

A second approach was attempted based on the finding in B. subtilis that tyrosine bradytrophs (tyr mutants which grow slowly in the absence of tyrosine) blocked at prephenate dehydrogenase were sensitive to histidine (Chapman and Nester 1968). Revertants to histidine insensitivity were found by them to be derepressed for both aromatic amino acid and histidine biosynthetic enzymes. First the histidine sensitivity of 7d, a known tyrosine bradytroph was tested. Growth of this strain in the absence of tyrosine was uninhibited by histidine. Therefore it was concluded that either the regulation of the histidine and aromatic amino acid pathways in B. licheniformis is not linked as in B. subtilis or the lesion in 7d is not at prephenate dehydrogenase. An attempt was then made to isolate a histidine sensitive tyr bradytroph in 9945A C1. Mutagenesis by the action of ethylmethane sulphonate (EMS) on spores (~~Thorne~~ Spizizen,

personal communication) was chosen as it offered a high frequency of mutagenesis and thus avoided the problem of how to use normal enrichment techniques to isolate bradytrophs. Also it was felt that a mutagenised spore stock would be a more convenient basis for screening than a frozen culture of cells.

Procedure

A 50% dilution of spore stock (5 ml. - ca. 8×10^7 spores) of 9945A C1 was incubated for 15 min. at 70°C and then cooled. EMS (0.25 ml.) was added to give a mixture equivalent to 0.5 M and the mixture incubated at 30°C overnight. The viable count of spores was then measured and found to have fallen by 99.9%. The spores were then plated on AA solid medium supplemented with tyrosine so as to give 200 colonies per plate, incubated for 2 days at 37°C and then left at room temperature for the colonies to dry (so that they could be replicated). About 10% of the colonies had altered morphology suggesting a high level of mutagenesis had been obtained. The plates were replicated onto AA solid medium unsupplemented, or supplemented with histidine or with tyrosine, and incubated for 18 hours. The replicas were then screened for mutants which grew well on tyrosine, less well without supplementation and not at all on histidine. The screening of 4,000 colonies gave 20 possible mutants which were re-examined by patching on unsupplemented, and tyrosine and histidine supplemented plates. One of the possible mutants again showed the desired phenotype

and was purified and designated SH Hs 1. Unfortunately the growth of this strain was good enough to make the finding of revertants on histidine supplemented plates impossible. Higher concentrations of histidine were tried but found not to be any more effective in inhibiting the growth of SH Hs 1. When grown on tyrosine colonies were white but when grown without supplement or with histidine the colonies had a strong brown colour. It was thought that the brown colour was an intermediate or breakdown product of an intermediate of tyrosine biosynthesis the synthesis of which was repressed by tyrosine. However, if the repression control of tyrosine and histidine were common as in B. subtilis (Chapman and Nester 1968) one would have expected colonies to be white on histidine. Thus SH Hs 1 was considered unlikely to be the type of mutant required and therefore work on it was dropped.

Time did not allow further screening for tyrosine bradytrophs.

3.2.c Arginine Biosynthetic Enzymes

The arginine analogue canavanine has been used to obtain constitutive mutants for arginine synthesising enzymes in E. coli (Maas 1961). However, the growth of cells and the germination of spores of B. licheniformis was found to be insensitive to this analogue even when it was placed as solid on a minimal plate.

Baumberg (1970) has published a method for the

selection of arginine regulatory mutants in E. coli, which depends on the action of acetyl ornithinase on ∝-N-acetyl histidine (NAcH) which releases free histidine. His mutants of E. coli will grow on this compound but not in the presence of arginine, ornithine or citrulline which repress the synthesis of acetyl-ornithinase. Mutants insensitive to arginine, ornithine and citruline were found to be altered in the regulation of arginine biosynthesis.

This method was tried for B. licheniformis on AA solid medium containing NAcH (20 ug./ml.) but it was found that although a his mutant (His 1) would grow on NAcH growth was not inhibited by arginine, citrulline or ornithine (100 ug./ml.). The NAcH was tested by amino acid analysis (with the help of Dr R.P. Ambler) and found to contain less than 0.25% free histidine which was not sufficient to account for the growth observed. It was concluded that in B. licheniformis some other acetylase can release histidine from NAcH and that the method of Baumberg cannot be applied.

3.3' Growth Experiments

3.3'.a Introduction

In this section experiments are described which were designed to measure the levels of penicillinase and tryptophan synthetase B in terms of enzyme activity/total protein synthesised, in balanced growth, at a range of growth rates, and also to take samples for the analysis

of the gene frequency distributions at these growth rates.

3'
3.2.b Development of Media and Growth Conditions

The decision was made to use carbon and energy sources to control growth rate, i.e. internal bottlenecks in metabolism (Ierusalemsky 1967) rather than the chemostat. This decision was based on the following considerations:

1) Batch growth experiments are simple compared with the mechanical complexity of the chemostat.
2) There is a danger of mutant selection during long periods of growth in the chemostat. The load of two constitutive mutations in a strain (such as SH mtr 1) might give a selective advantage to revertants to wild type resulting in a gradual loss of the desired strain from the chemostat.
3) Designing a limiting condition for the chemostat which would allow doubling times from 30 to 90 min. (the chosen range of doubling times) promised to be difficult.
4) Balanced batch growth as defined by Maaloe and Kjeldgaard (1966), with the population density kept low enough (below 2 x 10^8 cells/ml.) not to substantially alter the concentration of any component of the medium, provides an acceptable steady state from which to make observations.

A set of preliminary experiments were carried out (Table 3.2) to establish, 1) a series of media with

Table 3.2

Medium	Experiment no							
	1		2		3		4	
	t_D	pen'ase* activity	t_D	pen'ase* activity	t_D	pen'ase* activity	t_D	pen'ase * activity
L broth	28		30	774	28	695		changed to L
Pen assay Broth					28	617		
CHS			48	1015	49	1050		changed to A
A							48	1025
L							31	690
X	90		90	1340	90	1425	87	1316
G	NOT TESTED IN PRELIMINARY EXPERIMENTS							

* units per mg total protein

different carbon sources, 2) a standard growth procedure and 3) to work out suitable sampling methods. The choice of media was based on the work of Maaloe and Kjeldgaard (1966) which gave an indication of the relationship between growth rate and carbon source which could be expected. Penassay broth (Difco) was tested and found to give a growth rate and penicillinase activities close to L-broth and L-medium; also it contained TCA precipitable material which interfered with protein assays, therefore it was not considered further.

Based on the preliminary experiments the media chosen were L, A, G and X. These media gave doubling times from 30 to 90 min. and had a common salt base (MBS). This was intended to permit investigation of transitions between steady states of growth if results suggested that this might be interesting. In preliminary tests with L-broth and CHS, clumping of cells at low cell densities was observed, whereas in the glutamate MBS medium (X) clumping was not observed. Thus it was possible that MBS could prevent clumping. However, this was found not to be the case and it is likely that clumping is a phenomenon of high growth rates at low cell densities in B. licheniformis.

The growth procedure for the measurement of steady state growth rates was as follows.

A loopful of spores of SH mtr 1 was added to 10 ml. of medium X and allowed to grow to an OD of 1.0. Then

this culture was diluted $\frac{1}{250}$ into 25 ml. of L and A media and 1 in 100 into G and X media. Growth was followed by OD when measurable. On reaching an OD of between 0.1 and 0.2 each culture was again diluted $\frac{1}{100}$ into fresh pre-warmed (to 37°C) media and growth again followed. This sequential dilution allowed growth to be followed for 12 generations after the shift to the 4 media. In the case of X medium the second dilution was lowered to $\frac{1}{20}$ to shorten the time taken for the experiment. This was thought permissible since the original culture was already in steady state on X medium. The linearity of plots of log OD against time were taken as evidence of steady state growth and the growth rates were determined from the slopes of these plots. Growth rates on the 4 media are shown below.

Table 3.3

Medium	Carbon/energy source	Mass doubling time (min.)
L	L-broth	30
A	Casamino acids	45
G	Glucose	60
X	Sodium L-glutamate	90

3.2.c The Variation in Constitutive Enzyme Synthesis with Growth Rate

In growth experiments carried out as described in Section 3.2.b samples were taken for enzyme assay after the second dilution at ODs between 0.1 and 0.2. Duplicate 1 ml. samples were taken and stored frozen for

penicillinase assays. Simultaneously 4 x 10 ml. samples were harvested for tryptophan synthetase B assays and 2 x 10 ml. samples were added to 3 ml. TCA (20%) for protein estimation.

Results of these experiments are shown in Figures 3.1 and 3.2.

3.2.d Samples for Measurement of Relative Gene Frequencies

The experiment described in Section 3.2.b was repeated using 250 ml. cultures. The entire 250 ml. cultures at ODs between 0.1 and 0.2 were harvested by pouring onto 100 g. of crushed ice (to lower the temperature and stop further DNA synthesis) and centrifugation (M.S.E. HS18 8 x 250 ml. rotor, 8000 revs./min., 10 min.). The cell pellet was stored frozen until DNA extraction was carried out. The extracted DNA was stored in NaCl (2 M) at 4°C and each DNA preparation was named according to the medium it was produced on, i.e. DNA L; DNA A; DNA G; DNA X.

3.2.e Demonstration that the Penicillinase Levels assayed in Whole Cultures did not reflect Differential Release

(i) Introduction

There is no evidence that the cell bound and released forms of penicillinase have the same activity per molecule in the assay system. Therefore it was necessary to examine the distribution of activity between the cell

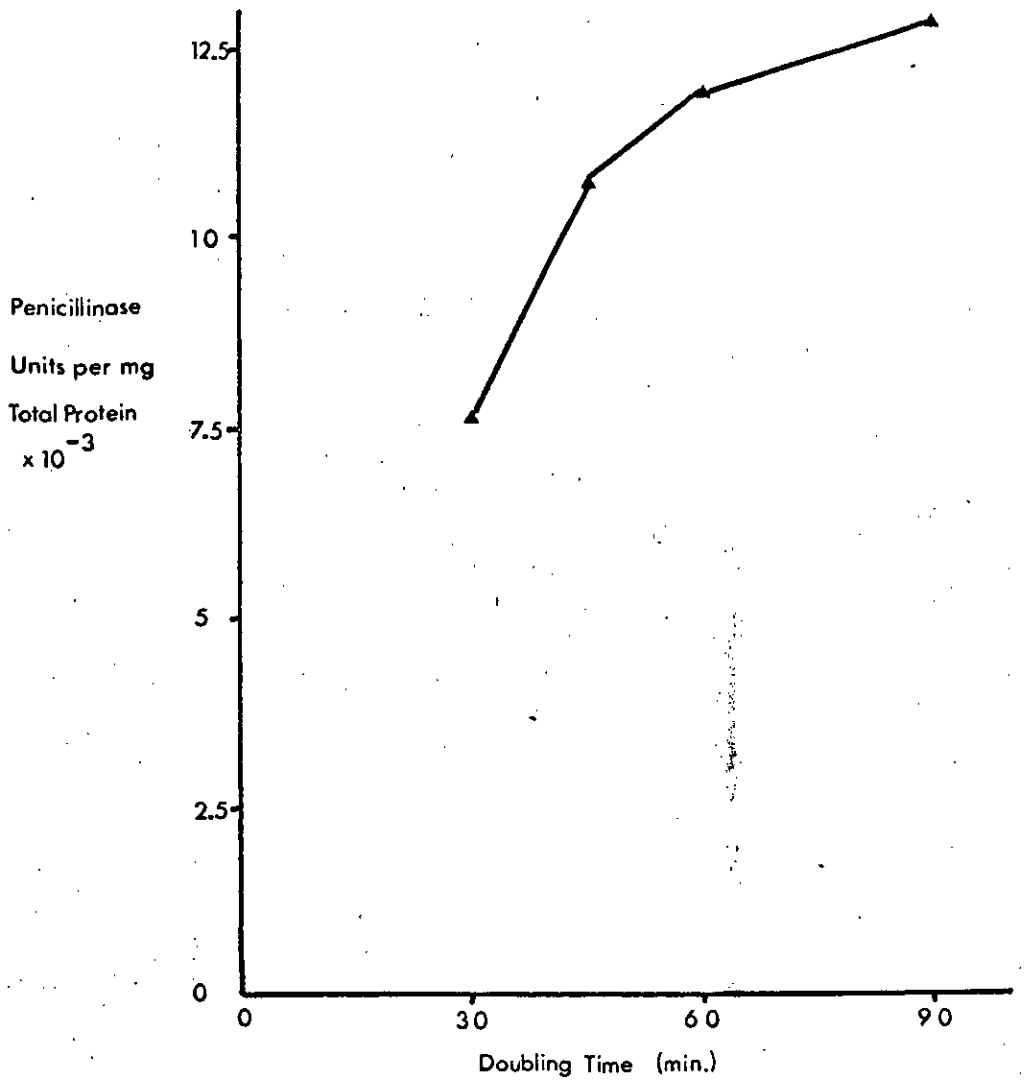


Figure 3.1. The Variation in Constitutive Penicillinase Synthesis with Growth Rate.

Penicillinase activity and total protein concentration were measured in samples taken from steady state cultures of SHmtrl on carbon sources giving doubling times of 30, 45, 60, and 90min..

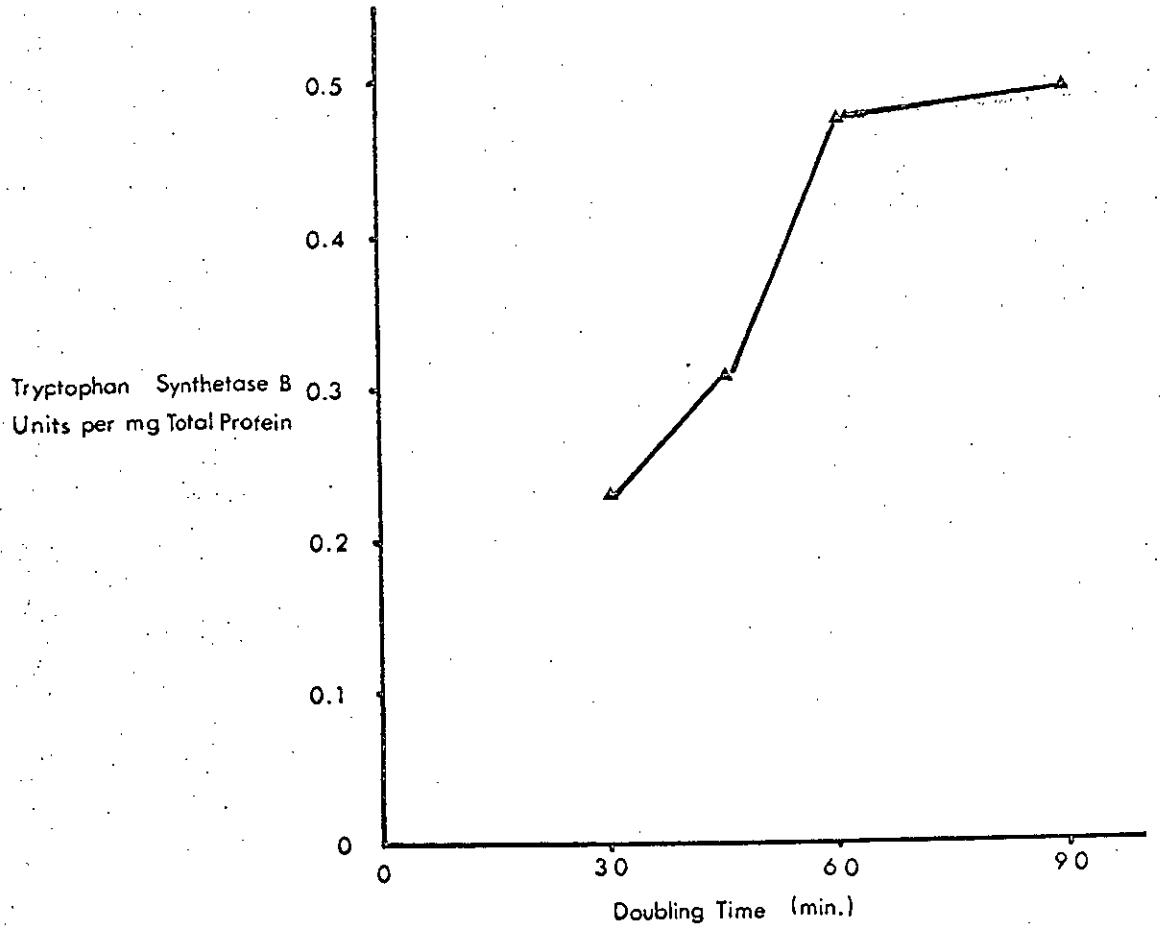


Figure 3.2. The Variation in Constitutive Synthesis of Tryptophan SynthetaseB with Growth Rate.

Tryptophan synthetaseB activities and total protein concentrations were measured in samples taken from steady state cultures of SHmtrl in media giving doubling times of 30, 45, 60, and 90,min..

bound and released forms to check that the apparent alterations in synthesis of penicillinase with growth rate were not merely reflections of different rates of release in the different environments.

3.2.e (ii) Procedure

Samples taken in growth experiments with SH mtr 1 were assayed for total penicillinase and then centrifuged (M.S.E., HS 18, 8 x 30 ml. rotor, 10,000 revs./min., 5 min.). The supernatant was then assayed and the percentage of total activity released calculated.

3.2.e (iii) Results

Medium	Percentage of total activity released
L	31.8
A	31.6
X	27.2

3.2.e (iv) Conclusion

It is clear that the distribution of penicillinase between the bound and released form does not vary significantly with medium and cannot account for the apparent alterations in synthesis in growth experiments.

3.3 The Measurement of Relative Gene Frequencies by Transformation

3.3.a Introduction

The original goal in these experiments was to find the frequencies of the *pen* and *trp* genes relative to the *ade* gene for each of the growth rates and use the theoretical relationships of Sueoka and Yoshikawa to (1) evaluate the influence of growth rate on gene frequency distribution, (2) calculate the rate of replication in *B. licheniformis* and (3) confirm the map of Tyeryar *et al.* (1968). The *ade* marker was taken as reference marker since it defines the origin of the Tyeryar map (Appendix 1). As difficulties were encountered with the transformation system this goal was narrowed and only the relative frequencies of the *ade* and *trp* genes were measured, and used in conjunction with the Tyeryar map to construct gene frequency distributions. This necessitated reference to unpublished information from Sherratt and Collins concerning the map location of the *pen* gene. They have shown that *pen* is linked to *ilv* and have since found a mutant in which there is high linkage between *ilv* and *met* which they conclude is the result of a small deletion. Thus they assign *pen* a position close to *met* which is the marker closest to the terminus on the Tyeryar map (Appendix 1).

3.3.b Difficulties with the Transformation System and their Effect on the Development of the Investigation

(i) Plating

The determination of relative numbers of transformants requires an accurate plating method. The standard method of plating gave a variation in the region of 50% in the number of transformants per plate and also gave a very uneven distribution of transformants on the plate making colonies in dense regions difficult to count. Therefore a pour plate method was developed which used a normal AA solid plate supplemented to select for the desired transformants, onto which was poured 2.5 ml. of 0.7% water agar containing an appropriate dilution of the transformed culture. This procedure gave evenly distributed colonies, a variability of about 10% and was exceedingly quick compared with the spreading technique. It was found that it was best to maintain molten water agar at 45-47°C. Above 50°C there was a loss of transformants and below 45°C there was danger of the agar starting to set during mixing and pouring.

(ii) The Variation in Levels of Competence of Recipient Cultures

The number of transformants obtained from a given amount of DNA was found to vary a great deal from experiment to experiment for all strains. This gave rise to an obvious problem in judging the dilution of transformants for counting on plates. A possible solution to

this problem would have been to store competent cultures in liquid nitrogen and always use the same culture as is routine in the B. subtilis system but Sherratt (Ph.D. Thesis) showed that although viability was maintained by B. licheniformis, competence was lost during this treatment. Consequently an attempt was made to cut down the variation in competence by always using the same batch of 2MBS and 2NB, always adding the components of BLSG in the same order and always using 25 ml. cultures in 250 ml. flasks. Despite this standardisation it was found that the level of competence still varied over a 2-3 fold range which still raised problems with respect to plating dilutions since it was desired to count at least 2000 transformants per marker. It was thought that a way round this problem might be to carry out transformations over a range of recipient concentrations (assuming that the level of competence reflects the concentration of recipient cells) and find the maximum number of transformants for a particular marker obtainable from a given amount of DNA. Experiments designed to test this possibility are described in Section 3.3.f. Although plotting the number of transformants obtained against the concentration of recipient cells gave a plateau this was found not to represent the maximum number of transformants obtainable from the fixed amount of DNA. However, the shapes of the curves obtained gave an indication that the source of the variation was a variable nuclease activity in recipient cultures. This is discussed in Section 4.

This knowledge did not help to cure the variation and thus it maintained its status as an unavoidable hazard in the study of B. licheniformis.

(iii) The Low Transformability of the ilva Marker

It was originally intended to establish the relative frequencies of the ade and ilv D markers and use this as a measure of the relative frequency of the pen gene which is 5% linked to ilva D. However, it was not found possible to obtain high enough frequencies of transformation for ilva D using the strain 8a.

The possibility of scoring pen^+ transformants using a strain which was ade pen i^- p^- without selection was investigated but it was not found possible to score reliably pen^+ transformants. This was due to the variation in colony size obtained by plating at the cell densities required to screen for them. Thus the theoretical location of the pen gene in conjunction with the ade/trp relative frequencies was used to examine the variation in relative frequency of the pen gene with growth rate.

3.3.b The Construction of a Doubly Marked (ade, trp) Recipient Strain

A series of 6 trp mutations were made in strain 749 by NG mutagenesis and classified by crossfeeding and accumulation of intermediates when grown on limiting tryptophan. Cultures (10 ml.) were grown in X medium

(+ 2 ug./ml. tryptophan) to stationary phase and culture supernatants tested for presence of indole (with PDMAB reagent - see Section 2), indole glycerol phosphate (with ferric chloride reagent, Yanofsky 1956) or fluorescent intermediates.

Mutant 1 (a probable B mutant), mutant 2 (not a B or A mutant) and mutant 3 (a D or C mutant) were tested for tryptophan synthetase B activity. 100 ml. cultures were grown on X medium supplemented with 4 ug./ml. of tryptophan, to stationary phase and 10 ml. samples taken for tryptophan synthetase B assay. It was found that mutants 1 and 2 had no tryptophan synthetase B activity but mutant 3 had 0.186 units/OD, thus confirming that the growth procedure would have derepressed tryptophan synthetase activity in mutants 1 and 2 had they contained a good trp B gene. Thus mutant 1 was confirmed as deficient in tryptophan synthetase activity and classified as a trp B mutant, and its trp allele designated trp B1.

These results are summarised in the table below.

Table 3.4

Mutant	Growth on indole	Accumulated Indole	Accumulated IGP	intermediate Fluorescence	Tryptophan synthetase B activity
1	-	+	-	-	0
2	-	-	-	+	0
3	+	-	-	+	0.1867 u/OD
4	+				
5	+		Not tested		
6	+				

The trp B1 allele was transferred to a recipient strain by transformation. DNA from 749 trp B1 was used to transform 7d (ade 1 tyr 1) and tyr$^+$ transformants selected. The trp genotype of tyr$^+$ transformants was found by replica plating and a trp transformant isolated and designated SH trp B1r. This strain was used for the measurement of relative gene frequencies. At the same time the linkage between tyr 1 and trp B1 was estimated by comparing the frequencies of tyr$^+$ trp$^+$ and tyr$^+$ trp$^-$ transformants. Also the linkage between tyr 1 and trp 2 and trp 3 was estimated. The frequency of co-transformation of pep and tyr (the recipient character) which is not linked to tyr (Sherratt, Ph.D. Thesis, Edinburgh) was measured in order to check that the tyr trp linkage was not being distorted by pseudolinkage as a result of saturating DNA. The results of these experiments are shown in Table 3.5 below.

Table 3.5

Donor	B tyr$^+$ transformants per ml.	A tyr$^+$ trp$^-$ transformants per ml.	A/B x 100% tyr trp linkage	tyr$^+$ pep$^+$ transformants per ml.
SH749 trp B1	7330	4830	60%	80
SH749 trp B2	9640	6040	62%	22
SH749 trp B3	620	270	44%	0

These data correspond well with those of Sherratt (Ph.D.) who found that tyr 1 was about 60% linked to a trp E mutation suggesting that the trp genes of B. licheniformis form a closely linked group.

3.3.c Determination of the Age of Maximum Competence of Recipient Cultures

Samples were taken at 60 min. intervals from a recipient culture of SH trp Blr starting after 12 hours of incubation. The samples were used in 90 min. transformations at a 1/20 dilution with DNA from SH mtr 1. Data from the experiment are tabulated below.

Table 3.6

Age of Culture	*ade*$^+$ transformants per ml.	*trp*$^+$ transformants per ml.
12.5 hr.	20	210
13.5 hr.	3050	6000
14.5 hr.	630	1440
15.5 hr.	3100	113
16.5 hr.	1720	42
17.5 hr.	1675	30

It was established that a 13.5 hr. incubation gave the highest level of competence for transforming the *ade* and *trp* markers, although there was a second peak of competence for *ade* transformation at 15.5 hours. This suggested that different populations of competent cells are responsible for the transformation of the *ade* and *trp* markers.

The above experiment was repeated for strain SH8T1r (ilv) in the construction of the low thymine requiring prototroph SHWT1rc1. Data from this experiment are shown below.

Table 3.7

Age of Recipient Culture	*ilv*$^+$ transformants per ml.
16.2 hr.	0
17.2 hr.	90
17.5 hr.	20
19.5 hr.	100
20.5 hr.	250
21.5 hr.	590
23.0 hr.	1170

Thus in the case of SH8T tlr competence develops late and transformations using this strain were carried out after 23 hr. of incubation of the recipient culture. These experiments emphasise the difference in behaviour of recipient strains.

3.3.d The Influence of DNA Concentration on Transformation

(i) Introduction

In order to draw conclusions concerning the relative number of copies of a pair of genes in a sample of DNA, from the relative frequencies of transformation of markers in these genes, it is first necessary to demonstrate that the number of transformants obtained in the system is proportional to DNA concentration. In other words care must be taken to ensure that the concentration of DNA used is non-saturating. The danger of saturation to relative gene frequency measurement is that if different competent populations exist in the recipient for the two markers

(as was suggested in Section 3.3.c and by Sherratt and Collins (1968)), saturation may be reached for one marker but not for the other and thus for one gene one would be measuring the maximum ability of the relevant competent population to take up that gene and for the other the relative concentration of that gene in the DNA.

Therefore, a set of transformations were carried out to determine the concentrations of each of the DNAs which should be used in order to avoid saturation in the measurement of the relative frequencies of the ade and trp genes.

3.3.d (ii) Procedure

A 13.5 hr. culture of SH trp Blr was diluted 10 fold into TM and used in 2 ml. transformations with serial dilutions of the DNAs as shown in Table 3.8. This table contains the results of two such experiments.

Table 3.8

Donor DNA	Dilution	*ade*$^+$ transformants per ml.	*trp*$^+$ transformants per ml.
L	0	83,680	22,640
L	$\frac{1}{10}$	7,984	2,752
L	$\frac{1}{100}$	784	261
Spore	$\frac{1}{10}$	1,010	1,157
Spore	$\frac{1}{100}$	88	117
X	$\frac{1}{10}$	388	154
Spore	0	11,092	7,704
Spore	$\frac{1}{10}$	1,242	1,200
Spore	$\frac{1}{100}$	100	152
G	$\frac{1}{10}$	12,948	7,200
G	$\frac{1}{100}$	1,413	850
A	$\frac{1}{10}$	4,624	2,676

(iii) Conclusions

A log log plot of DNA dilution versus transformants per ml. is shown in Figure 33 for DNA L and DNA spore. The two curves for *ade*$^+$ transformants show proportionality over the entire range of DNA concentrations. However, the *trp*$^+$ curves were slightly less convincing at the highest DNA concentrations and therefore it was decided that to avoid the risk of exceeding the proportional range the DNAs should be diluted to give transforming activities close to that of the 10 fold dilution of DNA L for the *ade* marker.

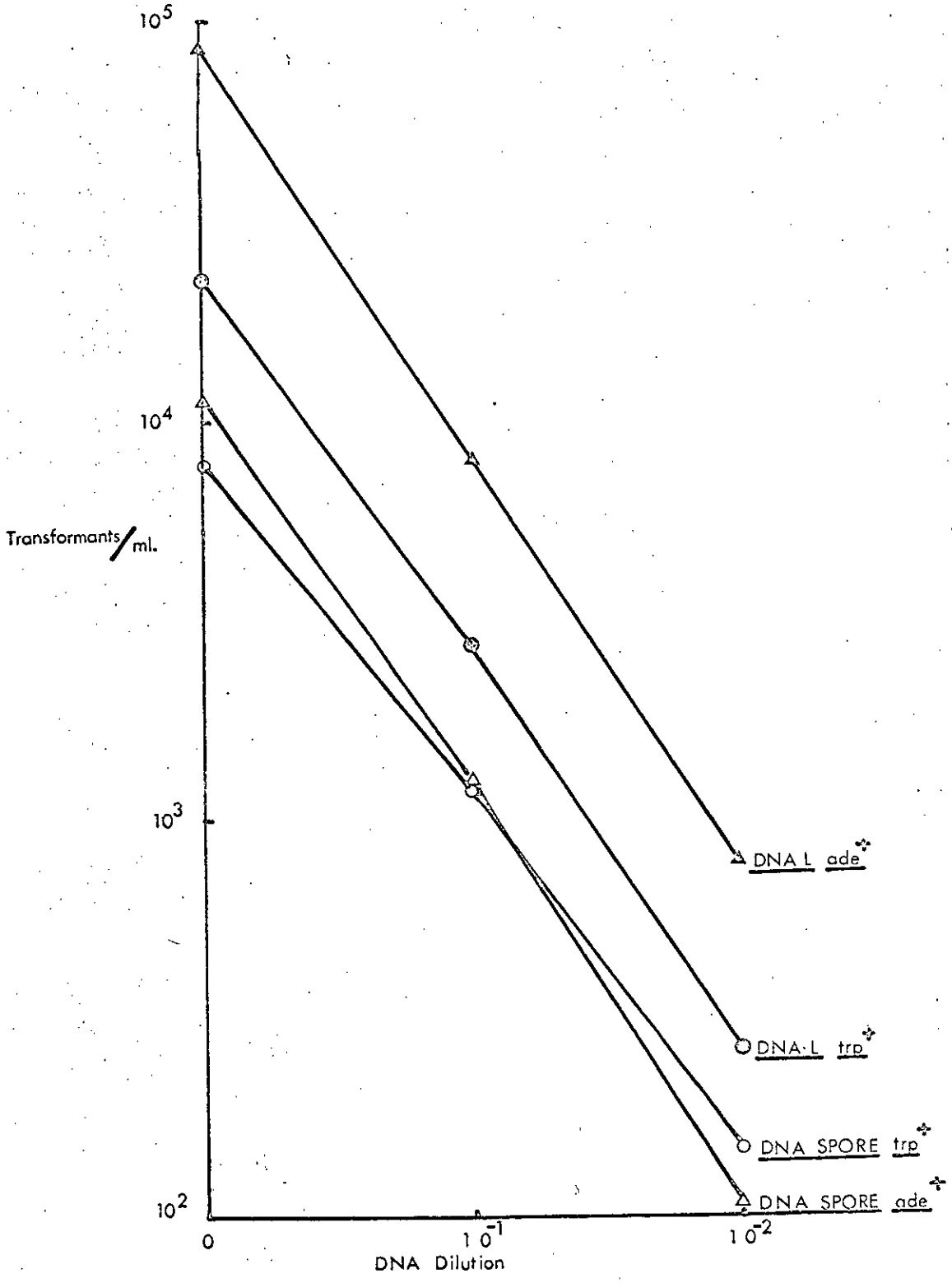


Figure 3.3. The influence of DNA Concentration on Transformation

Ten fold serial dilutions of DNA L and DNA SPORE were used in 2hr. transformations . The recipient was a 13.5hr. culture of trpB1r and ade+ and trp+ transformants were both scored.

3.3.e Transformations to Measure the Relative Frequencies of the *ade* and *trp* genes

3.3.e (i) Procedure

DNA L x 10^{-1} (i.e. diluted $\frac{1}{10}$), *DNA A* x 10^{-1}, *DNA G* x 10^{-1}, *DNA X*, *DNA spore* and *DNA spore* x 10^{-1} were used to transform a 13.5 hr. culture of *SH trp Blr* which was diluted $\frac{1}{10}$ into TM. Each transformed culture was diluted by a factor calculated from the transforming activity of the DNA as measured in the previous section.

3.3.e (ii) Results

The average number of transformants per plate and the total number of transformants counted for each marker are shown in Table 3.9. Table 3.10 contains the results of a repeat experiment using *DNA L* x 10^{-1}, *DNA X* and *DNA spore* x 10^{-1}. In this experiment the recipient was diluted 1.5/10 by error. In the last column of each table the ratio of *ade*$^+$ to *trp*$^+$ transformants has been calculated. In order to calculate the relative frequencies of the *ade* and *trp* genes from these data it is necessary to divide the *ade*$^+$/*trp*$^+$ ratio for each DNA with that found for *DNA spore* in order to normalise for the different efficiencies of transformation of the *ade* and *trp* markers. However, in Table 3.9 two values are shown for *DNA spore* depending on the concentration used. This may be explained by the observation that in Figure 3.3 the frequency of transformants for the *trp* marker in *DNA spore* was less satisfactorily proportional to DNA

Table 3.9. Results of transformation to measure the relative frequency of the trp and ade genes in the DNAs obtained in growth experiments (Section 3.2).

Donor DNA	Average transformants per plate		Total number of transformants counted		ade^+/trp^+
	ade^+	trp^+	ade^+	trp^+	
L x 10^{-1}	743	142	2972	1137	5.232
A x 10^{-1}	653	168	2612	672	3.886
G x 10^{-1}	764	289	2293	1147	2.643
X x 10^{-1}	750	311	4501	1870	2.404
Spore	470	216	1882	1083	2.172
Spore x 10^{-1}	346	202	2424	1617	1.714
Table 3.10.					
L x 10^{-1}	760	222	3042	1774	3.44
X	819	436	4911	3490	1.876
Spore x 10^{-1}	425	326	2975	2609	1.303

concentration than for the ade marker. Therefore the mean of the two DNA spore values has been used for calculating relative gene frequencies. The results of this treatment are shown below.

Table 3.11.

DNA	Relative frequency of ade and trp genes
DNA L	2.692
DNA A	2.000
DNA G	1.361
DNA X	1.237

Treated in the same way the data from Table 3.10 give the following results.

Table 3.12

DNA	Relative frequency of *ade* and *trp* genes
DNA L	2.64
DNA X	1.44

A discussion of the significance of the differences in these two sets of results is given in Section 4.3.b.

Conclusion

In the first of these experiments the frequency of transformation for *trp* was much lower than expected and did not permit the counting of 2,000 transformant colonies for this marker at the plating dilutions which had been selected. However, in the second experiment where a higher concentration of recipient cells was used (in error) the frequency of *trp* transformants was far higher. It was concluded that adding more recipient cells to the system could increase the efficiency of transformation for *trp*. It was therefore considered that a titration of the recipient culture against the number of transformants obtained might give a maximum number of transformants obtainable from the fixed amount of DNA, which would not vary from transformation to transformation. For this reason a series of transformations was carried out in which the frequency of *ade*$^+$ and *trp*$^+$ transformants from a given amount of DNA was measured

over a range of dilutions of the recipient culture. Such a system offered the greater accuracy inherent in the measurement of a plateau as opposed to single points.

3.3.f Titration of Recipient Culture to find a Maximum Efficiency of Transformation

3.3.f (i) Results

Three experiments were carried out and are reported in Figures 3.4, 3.5 and 3.6. In each case serial two-fold dilutions were used to give recipient concentrations from 4×10^8 cells/ml. to 6.75×10^6 cells/ml. and the ranges were altered slightly from experiment to experiment.

3.3.f (ii) Conclusions

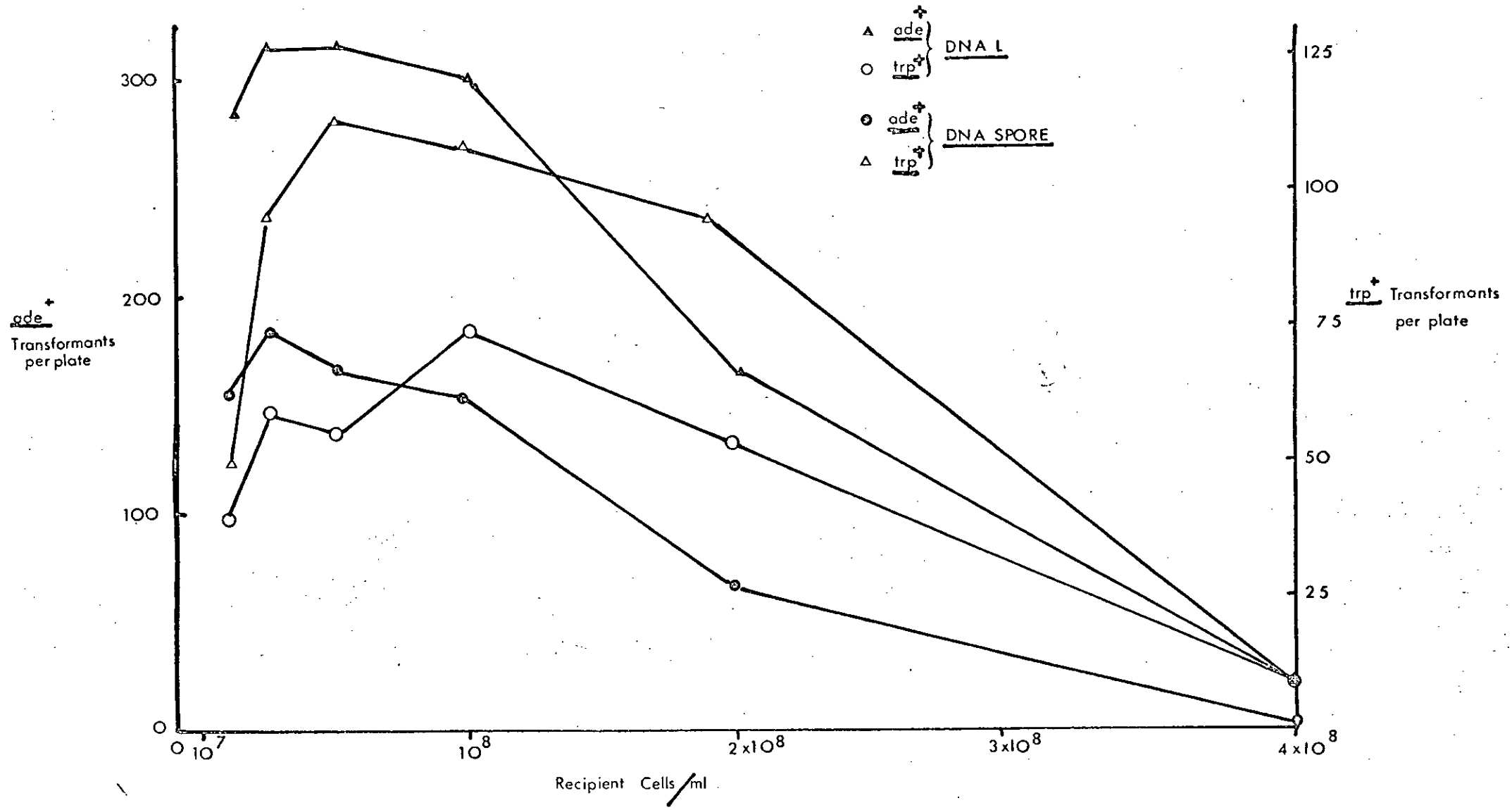
The shapes of the curves shown in Figures 3.4, 3.5 and 3.6 are difficult to interpret and not as expected when the experiments were conceived. Plateaus are discernible but not only do they not occur over the expected range of recipient concentrations (in the $\frac{1}{10}$ to $\frac{1}{2.5}$ region, i.e. 10^8 to 4×10^8 cells/ml.) but their range is different for the two markers and from experiment to experiment. Also the heights of the plateaus for DNA spore vary from experiment to experiment. Thus it is clear that the plateau does not represent a maximum efficiency of transformation. Each of the curves can be broken up into three sectors: (i) the falling sector at high recipient concentrations, (ii) the plateau, and (iii) the rising sector at low recipient concentrations

Legend to Figures 3.4, 3.5 and 3.6.

The Influence of Recipient Cell Concentration on Transformation.

A series of serial two fold dilutions of a 13.5 hr. BLSG culture of SH trp Blr in TM were used in 2 hr. transformations with DNA L (Fig. 3.4), A (Fig. 3.6) or G (Fig. 3.5) and DNA Spore. The transformed cultures were scored for ade^+ and trp^+ transformants.

Figure 3.4. The Influence of Recipient Cell Concentration on Transformation.



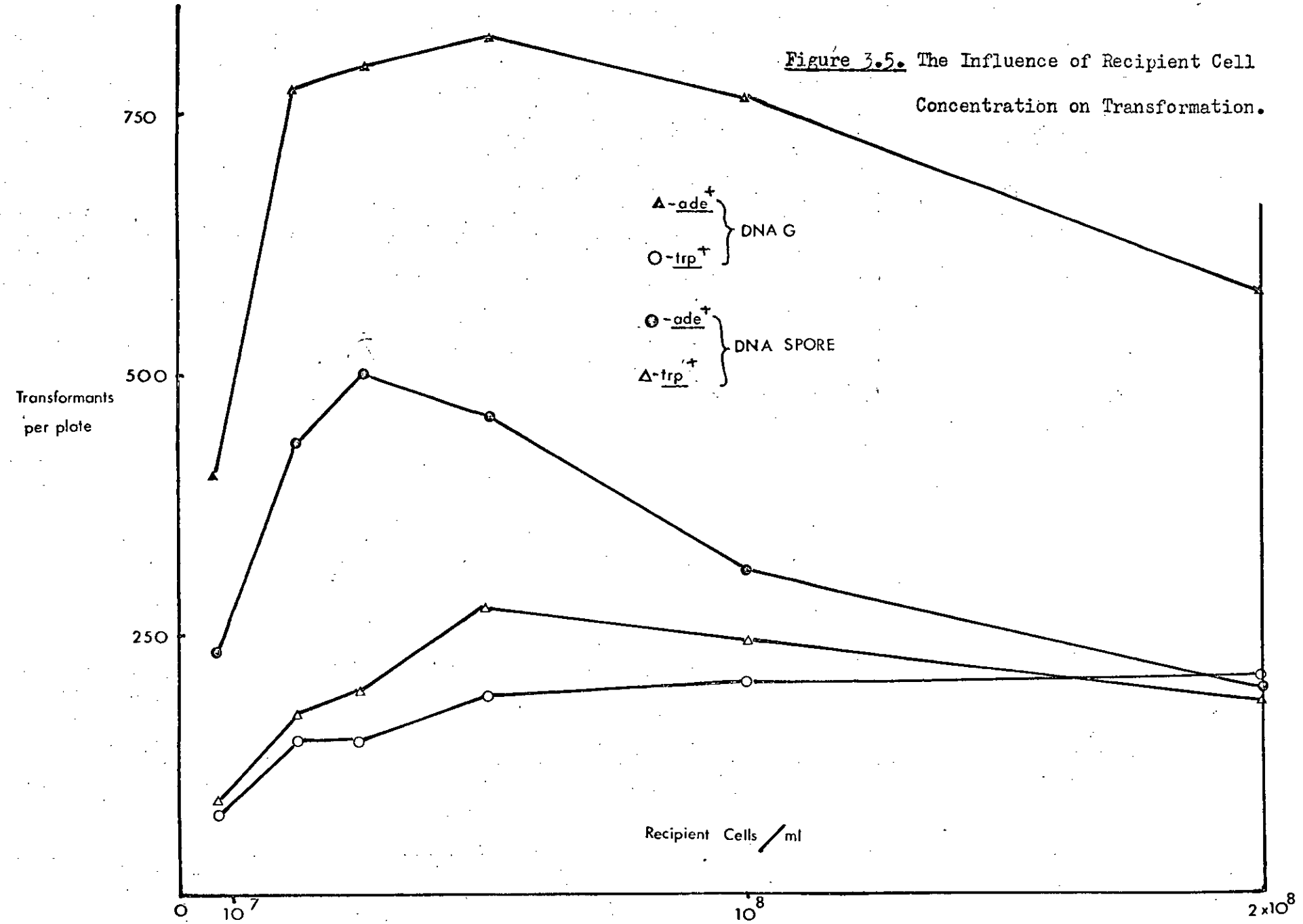
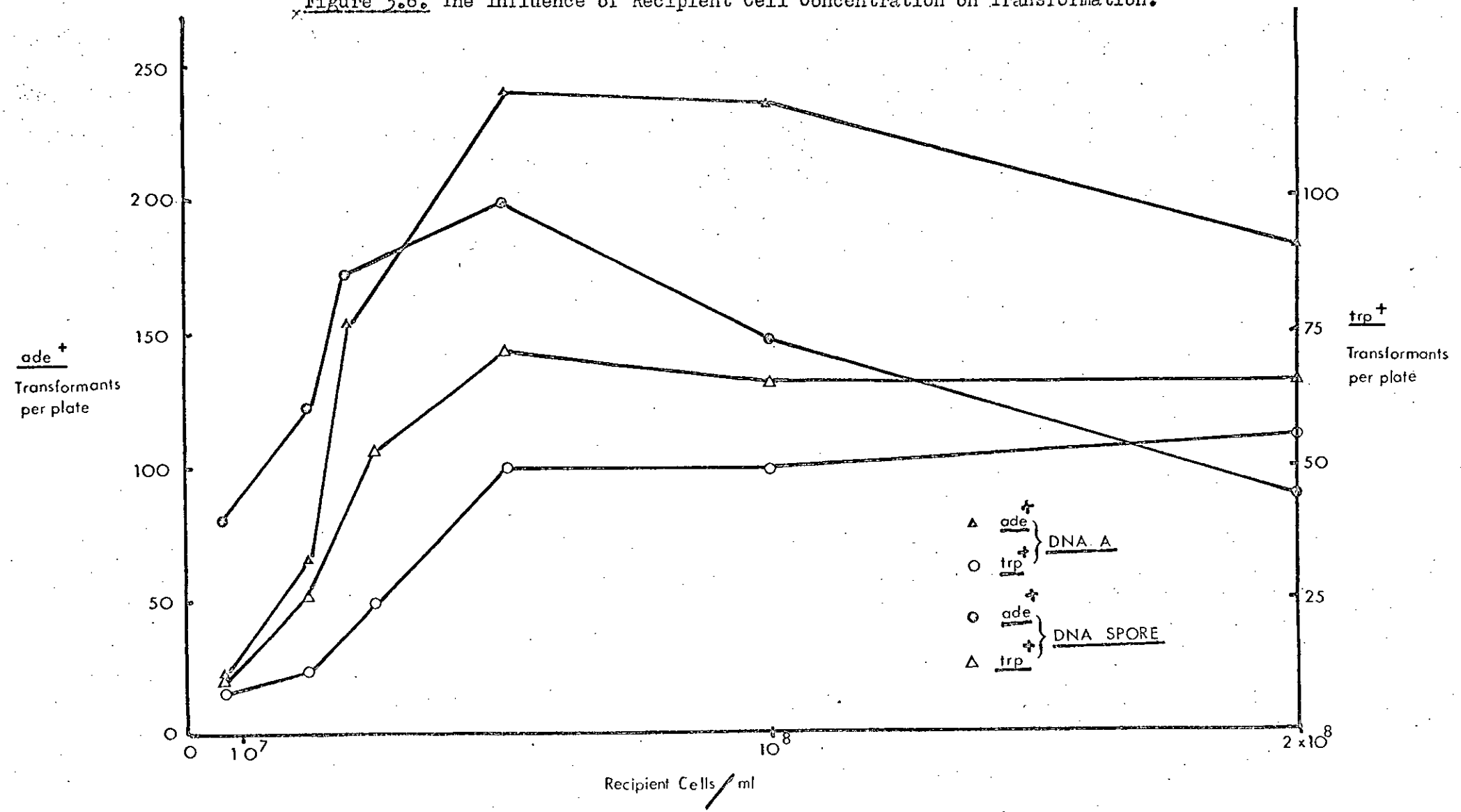


Figure 3.5. The Influence of Recipient Cell Concentration on Transformation.

Figure 3.6. The Influence of Recipient Cell Concentration on Transformation.



where increasing the number of recipients increases the number of transformants. The simplest interpretation of sector (i) is that some component in the BLSG recipient culture has reached a concentration in the transformation at which it inhibits transformation. Sector (iii) could be interpreted as the region in which competent cells are limiting and thus are being titrated against excess DNA. However, this interpretation would demand the curves in this sector to be independent of DNA concentration and to have a strict proportionality between input cells and transformants, neither of which is the case. This problem of interpretation can be avoided by the proposition that a nuclease is present in the transformation system. Thus the input DNA has two possible fates, to be absorbed by a competent cell into a nuclease resistant state or to be broken down by the nuclease. Therefore in sector (iii) the production of transformants would depend on the kinetics of interaction of the input DNA with competent cells and the nuclease. This would also be true on the plateau (sector (iii)) and here it could be imagined that the competition between the nuclease and competent cells is unaffected by their concentration relative to the input DNA. Thus it could be further predicted that the apparent level of competence of recipient cultures is dependent on the ratio of competent cells to nuclease activity. The nuclease proposition will be discussed further in Section 4.3.a.

A further pair of experiments were set up to test the nuclease hypothesis in which various amounts of a heterologous DNA were added to the system. It was thought that adding heterologous DNA might protect the transforming DNA against the action of the nuclease. These experiments are reported in Section 3.3.g.

3.3.f (iii) The Relative Gene Frequencies

Although these experiments were unsuccessful in finding a maximum efficiency of transformation it was considered worthwhile to examine the relative frequencies of *ade*$^+$ and *trp*$^+$ transformants at the plateaus and to calculate the relative gene frequencies. The extent of each plateau was determined by inspection and the frequency of transformants calculated as the mean of all the determined values which fell on the plateau. The results of this analysis are shown in the table below.

Table 3.13

DNA	Average transformants per plate		Ratio *ade*$^+$/*trp*$^+$
	ade$^+$	*trp*$^+$	
L	310 (3)	60 (4)	5.16
Spore	168 (3)	102 (4)	1.64
G	789 (4)	206 (3)	3.84
Spore	469 (4)	183 (3)	2.56
A	239 (2)	52 (4)	4.571
Spore	173 (3)	68 (4)	2.521

(Figures in parenthesis denote the number of points on the plateau used to calculate the mean)

These data were used to calculate the relative frequencies of the *ade* and *trp* genes which are shown in the table below.

Table 3.14

DNA	Relative frequency of *ade* and *trp* genes
DNA L	3.15
DNA G	1.49
DNA A	1.88

These results are discussed in Section 4.3.b.

3.3.g Competition Experiments with Heterologous DNA

DNA for use in these two experiments was extracted from *E. coli* B/r grown on L-broth. Prior to the transformations the concentrations of the transforming DNA (DNA L) and the *E. coli* DNA were measured. Transformations were carried out at two recipient concentrations, i.e. at a $\frac{1}{160}$ dilution (0.675×10^7 cells/ml.) and at a $\frac{1}{40}$ dilution (0.5×10^8 cells/ml.). The two experiments are reported in Figures 3.7 and 3.8.

Conclusions

From the first experiment (Fig. 3.7) it is clear that the general effect of adding heterologous DNA to a transformation is to reduce the frequency of transformation. However, it appears that at a heterologous DNA concentration close to the concentration of the transforming DNA there is a stimulation of transformation at

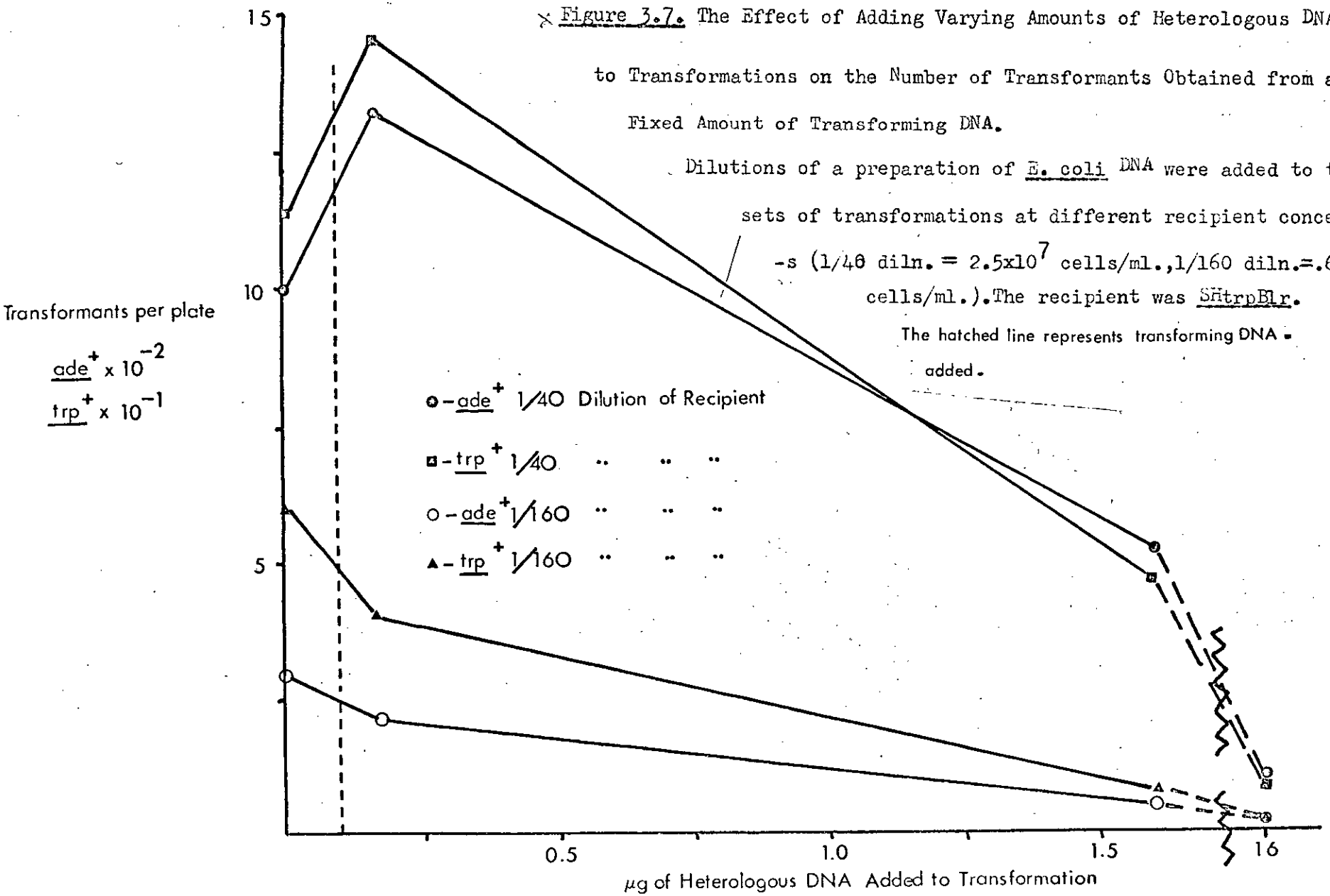


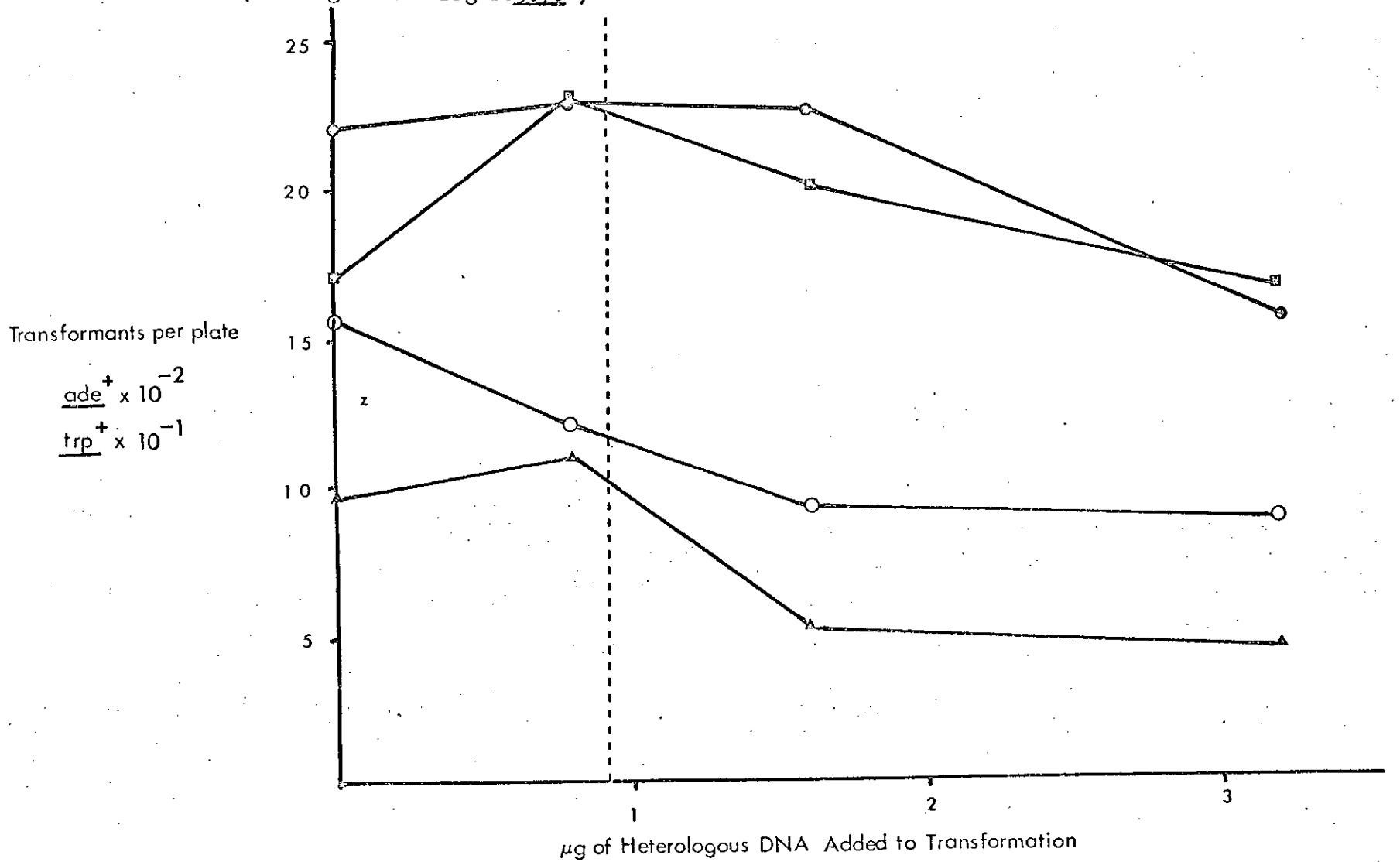
Figure 3.7. The Effect of Adding Varying Amounts of Heterologous DNA to Transformations on the Number of Transformants Obtained from a Fixed Amount of Transforming DNA.

Dilutions of a preparation of *E. coli* DNA were added to two sets of transformations at different recipient concentrations (1/40 diln. = 2.5×10^7 cells/ml., 1/160 diln. = $.625 \times 10^7$ cells/ml.). The recipient was SHtrpBlr.

The hatched line represents transforming DNA added.

Figure 3.8. The Effect of Heterologous DNA on Transformation.

(For legend see figure 3.7.)



the higher recipient concentration. The second experiment was designed to further investigate this effect by examining the influence of heterologous DNA concentration over a narrower range close to the transforming DNA concentration. In this experiment (Fig. 3.8) the stimulatory effect was less marked in the case of *ade*$^+$ transformants but was present for *trp*$^+$ transformants at both cell concentrations. These two experiments suggest that heterologous DNA can have a protective effect on transforming DNA when present at a similar concentration to the transforming DNA. This result supports the nuclease hypothesis.

3.4 Variation of Gene Frequency Distribution by Growth on Low Concentrations of Thymine

3.4.a Introduction

From studies on the cellular DNA content during steady state growth and in transitions between steady states, Pritchard and Zaritsky (1970) concluded that in *E. coli* 15T$^-$ (a *thy* auxotroph which will grow on concentrations of thymine down to 0.25 ug./ml., i.e. as a low thymine requirer) the DNA replication time (t_R) can be influenced by the concentration of thymine. This suggested a method for altering the gene frequency distribution at constant growth rate as can be seen by substituting different values of t_R in the generalised equation of chromosome replication. This would allow a simple direct assessment of the influence of relative

gene frequency on gene expression. It was decided to see whether thy mutants of B. licheniformis would behave like E. coli 15T⁻. The first step in this investigation was to isolate thymine requiring strains of B. licheniformis which would grow on low concentrations of thymine and which were constitutive for penicillinase synthesis. The second step was to investigate the influence of thymine concentration first on the growth of the strain, second on the level of constitutive synthesis of penicillinase and third on the gene frequency distribution in this strain.

3.4.b Isolation of a Low Thymine Requiring Strain Constitutive for Penicillinase Synthesis

A low thymine requiring recipient strain was isolated starting from strain 8aT1. This strain was isolated by the method of Okeda, Homma and Sonohara (1962) and was found to require at least 15 ug./ml. of thymine for growth (Sherratt, Ph.D.). However, when a spore stock of this strain was plated out on 1 ug./ml. of thymine a high frequency of low thymine requiring mutants was obtained (ca. 1 in 10^4). A possible explanation for this is that low requiring mutants were selected for during the manufacture of the spores. The sporulation medium was supplemented with 20 ug./ml. of thymine and it is possible that this concentration fell enough to select low requirers during spore growth. Several low thymine requirers (designated SH8aT tlr 1...) were purified and

their growth response to thymine rechecked. One of them (SH8aT tlr 1) was then transformed to prototrophy for ade and ilv and to pen i^- and pep^+. This was done in two steps. First SH8aT tlr 1 was transformed with pen i^- p^+ prototrophic DNA with selection for ilv^+ transformants. These were replicated and scored for penicillinase phenotype. Penicillinase constitutives were found at a frequency of 0.5% which was 10 fold lower than expected (Sherratt (Ph.D.) reported 5% linkage between ilv and pen) but this may be explained by the low level of transformation obtained for the ilv marker with this recipient. The second step was to transform SH8aT tlr ilv^+ pen i^- (designated SH8T tlr) with wild type DNA selecting for ade^+ transformants and to screen these for the pep^+ phenotype. In all these transformations both the BLSG and TM were supplemented with thymine (10 ug./ml.). The final strain was designated SH W tlrCl and was used in the following growth experiments.

3.4.c Growth Experiments with SH W tlrCl

3.4.c (i) Introduction

Preliminary experiments with SH W tlrCl were designed to find the range of concentrations of thymine over which it would grow in liquid culture at normal growth rates. Medium A was chosen for growth experiments since in growth experiments with SH mtr 1 it gave a doubling time close to the replication time found in E. coli and as such was expected to give large changes in the gene frequency

distribution if the replication time was altered by changes in thymine concentration.

3.4.c (ii) Procedure

A culture of SHW tlrC1 λ was grown up from spores on medium A supplemented with thymine (5 ug./ml.) to an OD of 0.2 and diluted 200 fold into a series of flasks containing 25 ml. of medium A plus 2, 1, 0.75 and 0.5 ug./ml. of thymine. SHW tlrC1 λ grows at the normal rate on medium A on thymine concentrations down to 2 ug./ml. but at 1 ug./ml. the growth rate falls to give a doubling time of 75 min. and below 1 ug./ml. the growth rate falls off drastically. For this reason the region above 1 ug./ml. was chosen for further study.

3.4.d Penicillinase Synthesis during Growth on a Range of Thymine Concentrations

From samples taken in the previous experiment at ODs of less than 0.2 it was found that penicillinase activity (when measured as units per ml./OD) was independent of thymine concentration as shown below.

Table 3.15

Thymine concentration	Doubling time	Penicillinase activity units per ml./OD
2 ug./ml.	48 min.	1435
1 ug./ml.	75 min.	1500
0.75 ug./ml.	200 min.	1520

Working backwards from the expected results of the investigation, which was that there should be a correlation between relative gene frequency and the level of enzyme synthesis, this result tended to suggest that growth on thymine concentrations over the range 2 ug./ml. to 0.75 ug./ml. did not influence the gene frequency distribution. However, another interpretation was that over this range the maximum number of replicating points possible had already been induced and the only way in which the cell could keep DNA replication in pace with division was to lower its growth rate. Thus the gene frequency distribution would not vary over this range of concentrations. In order to study a range of concentrations over which the gene frequency did alter it was decided to try a higher upper concentration. A repeat experiment was carried out at 20 ug./ml., 2 ug./ml. and 1 ug./ml. In this case a second $\frac{1}{100}$ dilution was carried out to permit growth to be followed for 12 generations. Also the starting culture was grown on 20 ug./ml. of thymine and extra samples were taken for DNA extraction (for measurement of relative gene frequencies) and protein assays. Table 3.16 gives the results of penicillinase assays.

Table 3.16

Concentration of thymine	Doubling time	Penicillinase units/mg. total protein
20 ug./ml.	43 min.	970
2 ug./ml.	43 min.	1,025
1 ug./ml.	60 min.	1,023

Again there was no significant change in penicillinase synthesis and the level in terms of units per mg. total protein was close to that found for SH mtr 1 on medium A. This suggested that the gene frequency distribution was in fact not varying over this range either and was close to that of a thy^+ culture. Thus it seemed likely at this stage that strain SHW tlrCl 1 did not behave like E. coli $15T^-$ with respect to thymine concentration. The influence of thymine concentration on the gene frequency was next measured as a final test of this.

3.4.e The Influence of Thymine Concentration on Gene Frequency Distribution in SHW tlrCl 1

3.4.e (i) DNA Extraction

The standard DNA extraction procedure was found to be ineffective for strain SHW tlrCl 1 because the cells did not lyse. The lysis procedure was therefore modified as follows. The suspension of cells in saline EDTA was first heated at 60°C for 10 min. to inactivate nucleases and the replicating system, then cooled and incubated with lysozyme (500 ug./ml.) at 37°C for 10 min. This was followed by the normal heating at 60°C with SDS.

At the ethanol precipitation stage it was found difficult to extract DNA threads in the normal way from the 2 ug./ml. preparation and impossible from the 1 ug./ml. preparation. However, it was found in the latter case that on standing overnight, a gelatinous precipitate was formed in the preparation. This was

redissolved in 2 N NaCl and its UV spectrum read over the range 300 nm. to 200 nm. A curve uncharacteristic of normal DNA preparations was obtained. It was thought that this spectrum was possibly due to SDS which had co-precipitated with the DNA and for this reason the solution was dialysed against 2 N NaCl overnight. Following dialysis the solution gave a spectrum identical with normal transforming DNA preparations, and was found to be satisfactory in transformation. The three DNAs were designated DNA 20, DNA 2 and DNA 1 where the figure represents the thymine concentration in the culture from which they were extracted.

3.4.e (ii) Transformations

Following a test of the transforming activities of DNAs 20, 2 and 1 they were diluted 10 fold for gene frequency transformations. Two transformations were carried out at 1/20 recipient dilutions. In the first DNA A was used as standard and in the second DNAs L, A and Spore were compared with 20, 2 and 1.

Results are shown in Table 3.17.

Table 3.17. Results of transformations to measure the relative frequency of *ade* and *trp* genes in DNAs produced on different concentrations of thymine.

Donor DNA	Average transformants per plate		Total number of transformants counted		Ratio of transformants
	ade$^+$	*trp*$^+$	*ade*$^+$	*trp*$^+$	*ade*$^+$/*trp*$^+$
DNA 20	209	53	836	319	3.93
DNA 2	299	57	1195	342	5.24
DNA 1	293	32	1170	193	9.09
DNA A	411	83	1642	413	4.97
DNA 20	286	146	1143	874	1.96
DNA 2	298	123	1195	615	2.43
DNA 1	277	88	1107	527	3.16
DNA Spore	358	299	2148	1794	1.19
DNA L	799	258	3194	1548	3.09
DNA A	566	266	2265	1596	2.13

The data from the second experiment when normalised by the DNA Spore *ade*$^+$/*trp*$^+$ ratio give the following relative gene frequencies.

Table 3.18.

DNA	Relative frequency of *ade* and *trp* genes
DNA 20	1.63
DNA 2	2.03
DNA 1	2.64
DNA L	2.59
DNA A	1.78

The relative gene frequency for DNA A in Table 3.18 was then used to normalise the relative transformation frequencies from the first experiment, to give the results in Table 3.19 below.

Table 3.19

DNA	Relative frequency of *ade* and *trp* genes
DNA 20	1.41
DNA 2	1.88
DNA 1	3.26

Inspection of these results reveals that growth on a range of thymine concentrations can be used to vary the gene frequency distribution in strain SHW tlrCl L and thus they contradict the suggestions made in Section 3.4.c. The extent to which this contradiction affects the general principle of the influence of relative gene frequency on enzyme synthesis is discussed further in Section 4.5.

CHAPTER 4

ANALYSIS OF RESULTS AND DISCUSSION

4.1 Tryptophan Biosynthesis in B. licheniformis

4.1.a Genes involved in Tryptophan Biosynthesis

In the isolation of a doubly marked recipient (ade trp) strain the properties of three trp auxotrophs were studied. Since then a series of 26 trp mutants have been isolated by A.F. Taylor (under the direction of J.F. Collins and myself), which, on the basis of accumulation of intermediates, contains representatives of each of the structural gene mutant classes obtainable in E. coli. Mutant trp B1 behaved as a B mutant in every respect but trp 2 behaved like a B mutant as far as enzyme activity and growth requirement were concerned but accumulated a fluorescent intermediate characteristic of C and D type mutants. This mutant was revertible and therefore could not be a deletion. It was considered, therefore, that mutant trp 2 was likely to be either a polar mutant (Jacob and Monod 1961b) which prevented the proper expression of both the B and C or D gene, or a mutation in some regulatory gene common to B and C or D genes (but not to the E gene which must be active for the accumulation of a fluorescent intermediate (Whitt and Carlton 1968)). Whichever of these possibilities is correct, mutant trp 2 provides evidence for co-ordinate control of tryptophan synthesising enzymes. The former possibility suggests that the trp genes form

an operon. Mutant trp 3 had not lost tryptophan synthetase B activity and accumulated a fluorescent intermediate. Thus it can be classed as a C or D mutant.

In transformation these three mutants were highly linked to tyr 1 and this together with data from Sherratt that trp E is highly linked to tyr, and the information on mutant classes from A. Taylor, suggests a general similarity in the gene enzyme relationships of B. licheniformis and B. subtilis.

4.1.b Control of Tryptophan Biosynthesis in B. licheniformis

In the 5MT resistant strain (SH mtr 1) only the constitutivity of the trp B gene has been demonstrated and for the wild type only the repression of the trp B gene by tryptophan has been demonstrated. Thus to extrapolate from the regulation of tryptophan synthetase B to the regulation of tryptophan biosynthesis requires the assumption of coordinate control of the tryptophan synthesising enzymes. There is no evidence that this is not the case and there is preliminary evidence of the existence of an operon (Section 4.1) so this assumption will be made. The synthesis of tryptophan appears to be different from that in E. coli and B. subtilis in its sensitivity to 5MT. This analogue does not repress the synthesis of tryptophan synthetase B in the wild type. The question thus arises: how did 5MT select for

derepressed mutants? It has been shown in B. subtilis (Jensen 1969) that 5MT acts as an inhibitor of anthranilate synthetase. However, it is difficult to see how a mutation to constitutivity would overcome this inhibition since it would be likely to cause full derepression itself. A possible explanation of the action of 5MT is that it cannot enter cells growing in liquid culture and thus cannot influence tryptophan biosynthesis. However, it is possible that during spore germination 5MT can enter the cell and then act as a co-repressor. A constitutive mutant overcomes the consequent repression of tryptophan synthesis and is able to grow. A further possibility stems from the work of Barlati and Ciferri (1970) who showed that during tryptophan starvation of a trp auxotroph of B. subtilis 5MT is incorporated into protein. It is possible that during spore germination a stage is passed through in which pools of tryptophan are sufficiently low to mimic tryptophan starvation and allow 5MT into protein, with toxic effects. Mutants with reduced ability to incorporate 5MT would be resistant to such toxic effects. Sites which could be altered in these mutants are the tryptophanyl tRNA activating enzyme, in an analogous way to mutants resistant to p fluorophenylalanine (Fangman and Neidhardt 1964) or the tryptophanyl tRNA itself. If the loaded tRNA itself is invoked as the co-repressor it is possible to reason that some of these mutants would also have lost their

ability to repress the biosynthesis of tryptophan. This could help to explain the two types of 5MT resistant mutants obtained, i.e. those which excreted tryptophan and those which did not, the first type possibly being altered in the tRNA and the second in the activating enzyme. It would also be possible for activating enzyme mutants to be derepressed (Roth and Ames 1966). A precedent for this regulation mechanism and these types of mutations to constitutivity exists in the histidine system in Salmonella typhimurium (Roth, Anton and Hartman 1966; Sibert, Fink and Ames 1966).

4.2 The Variation of Enzyme Synthesis with Growth Rate

The variation in penicillinase and tryptophan synthetase B specific activities with growth rate has been assumed to represent the variation in their rates of synthesis relative to total protein. This assumption rests on the demonstration that the cultures from which samples were taken for assay were in steady state. The evidence supporting a steady state is (i) the constant growth rate obtained on each of the media by successive dilution and (ii) the reasonably constant levels of penicillinase activity from experiment to experiment on each of the media.

The pattern of variation with growth rate was that expected for genes close to the terminus of replication from the theory of Sueoka and Yoshikawa (1965). Unfortunately no genes in other locations were studied

and it was not possible to show that the specific activity of the product of a gene close to the origin of replication increases with growth rate. It is not possible to conclude that the fall in the relative rates of synthesis of penicillinase and tryptophan synthetase as the growth rate was increased was causally related to the decrease in relative frequency of the trp and pen genes even if there was a correlation between them. However, the variation is too great to be explained simply by the diluting effect of ribosomal proteins. This can be seen by comparing the curves shown in Figures 2.2, 3.2 and 3.3. The correlation of relative gene frequency with relative enzyme synthesis will be further discussed in Section 4.5.

4.3 The Change in Relative Gene Frequencies with Growth Rate

4.3.a The Measurement System

In the course of an attempt to optimise the transformation system for the measurement of the relative frequencies of ade and trp genes, some interesting data on the transformation process itself in B. licheniformis have been obtained. Studies on the influence of the concentration of recipient cells on transformation have suggested the presence of a nuclease activity in recipient cultures which influences the probability of a given piece of DNA giving rise to a transformant. The results of competition experiments with heterologous DNA

support the nuclease hypothesis. At low recipient concentrations the heterologous DNA reduced the efficiency of transformation by competing for competent cells which were limiting, and thus protection of the transforming DNA which was relatively in excess was not observed. At higher recipient concentrations (on the "plateau") where the competition between cells and nuclease was independent of recipient cell concentration it is reasoned that recipient cells were relatively in excess so that sparing effect of the addition of heterologous DNA on the transforming DNA increased the frequency of transformation at low heterologous DNA concentrations. As the concentration of heterologous DNA was increased the excess of competent cells was progressively diminished resulting eventually in an inhibition of transformation, as observed at lower recipient concentrations. A more precise understanding of the "plateaus" and the protective effect of heterologous DNA would require a study of the kinetics of the nuclease activity, and some method of isolating competent cells so that the kinetics of interaction of competent cells with transforming DNA could be studied.

The presence of a nuclease may influence the measurement of relative gene frequencies. If one considers the two extreme DNAs, i.e. DNA L and DNA Spore, which were likely to have the largest difference in the relative frequencies of ade and trp genes according to the theory of Sueoka and Yoshikawa (1965) one can see

(Fig. 1.3) that in the case of DNA L far more DNA per trp gene was added to the transformation than was in the case of DNA Spore. It might be expected that the ade/trp ratios obtained for DNA L might be underestimates of the actual frequencies in the DNA, owing to the extra protection of the trp gene against nuclease. This would be true also but to a decreasing extent in DNAs A, G and X respectively. Confirmation of this interpretation would need the determination of the gene frequency distribution in some other way, e.g. by measuring the residual DNA synthesis after chloramphenicol treatment (see Section 1.3.d). This problem might have been avoided by adding an excess of either a heterologous DNA or DNA from an ade trp strain of B. licheniformis to each of the DNAs to cancel out possible differences in DNA concentration and marker protection effects, but at the same time this would have given rise to problems by lowering the absolute frequencies of transformation.

4.3.b The Results of Gene Frequency Transformations

In Table 4.1 the normalised ade/trp ratios obtained in the major transformations are assembled. Mean values and variances (σ^2) have been calculated for each of the DNAs. Sueoka and Yoshikawa (1965) have calculated the expected variances for gene frequency analysis by transformation for various sample sizes (numbers of colonies counted per marker) and the

Table 4.1. Summary of transformations to measure relative gene frequencies.

Trans-formation	Normalised ade/trp ratios = B						Mean	Observed (σ^2) variance
	1	2	3	4	5	6		
DNA								
L	2.692	2.64	3.1470	2.632			2.76	0.045
A	2.00		1.884	1.78			1.89	0.0076
G	1.3609		1.49				1.425	0.0042
X	1.237	1.44					1.34	0.010
20					1.41	1.63	1.52	0.096
2					1.88	2.03	1.955	0.0056
1					3.26	2.64	2.95	0.012

relationships which they used can be used to calculate the expected variances for the various ade/trp ratios determined in the gene frequency transformations reported in Table 4.1. These are shown below in Table 4.2.

Table 4.2

Number of colonies counted	Expected variances (σ^2) for values of B*		
	B = 1.5	B = 2	B = 2.7
100	0.101	0.2	0.41
500	0.0202	0.04	0.082
1000	0.0101	0.02	0.041
2000	0.005	0.01	0.025

* B is the ratio of origins to termini; in this case since ade and trp are original and terminal markers respectively B = ade/trp ratio.

Comparing Tables 4.1 and 4.2 the actual variances for all of the DNAs fall within or very close to the values predicted by the formula of Sueoka and Yoshikawa (1963) for 1,000 colonies, with the exception of DNA 1, the variance of which is close to that expected for the counting of 500 colonies. However, in transformation 5 less than 500 colonies were counted and for this reason in the discussion of relative gene frequencies the results of this transformation have been ignored. In conclusion, the results obtained from gene frequency transformations fall within the expected range of variability, and since averaging the results for the two different methods of estimating relative gene frequencies

(single points and the "plateau") did not produce variances outside the expected range these methods are both acceptable.

The change in the ade/trp ratio with growth rate will be related to DNA replication in B. licheniformis and also to the change of gene frequency distribution in an attempt to correlate this with changes in enzyme synthesis.

4.4 DNA Replication in B. licheniformis

The mean ade/trp ratios in Table 4.1 have been analysed using the Sueoka and Yoshikawa (1965) generalised equation of chromosome replication, to find the replication time in B. licheniformis and to establish whether it is constant or related to growth rate. Since the ade and trp genes demark the ends of the chromosome this equation simplifies to (ade/trp) = 2^n, which permits the calculation of the replication time from the relationship $n = \frac{t_R}{t_D}$ (Section 1.3.c). This analysis is shown in Table 4.3. Some unexpected results were observed. First, in the case of DNAs G and X the replication time was expected to be longer or the same as for DNAs A and L, whereas lower replication times were actually observed. However, from the calculated expected variances it can be shown that all the replication times fall within two predicted standard deviations (94% confidence limits) of one another, two standard

Table 4.3. Replication times compared with doubling times.

DNA	B	n*	Replication time in minutes	Doubling time in minutes
L	2.76	1.486	44.7	30
A	1.89	0.919	41.4	45
G	1.43	0.512	30.6	60
X	1.34	0.423	38.9	90
20	1.63	0.706	31.8	45
2	2.03	1.008	45.38	45
1	2.64	1.405	84.3	60

* n is calculated from $\frac{\ln B}{\ln 2}$

Replication time is calculated from

$$n = \frac{\text{replication time}}{\text{doubling time}}$$

deviations being about 10 min. for DNA G and DNA L. Two theoretical standard deviations were used as confidence limits in the construction of the Tyeryar map (Tyeryar et al. 1968). The data from transformation cannot strictly confirm whether the replication time is constant or not. However, if the observed replication times are interpreted in terms of replication in other organisms, and the value for the replication time is calculated from the data of Tyeryar et al. (1968) it is unlikely that the replication time at a doubling time of 60 min. is as low as 30.6 min. The result for a doubling time of 30 min. suggests that multiple replicating points are required at this growth rate, which in turn suggests that a

replicating time of 30 min. is unlikely. Thus it is likely that the calculated replicating times which cluster around 40 min. do reflect a constant replication time. Despite this uncertainty, these results suggest strongly that DNA synthesis is discontinuous at doubling times of 60 and 90 min.

The results obtained with the low thymine requiring strain show the expected dependence of replication time on thymine concentration. However, the replication time for growth on 20 ug./ml. of thymine is lower than expected (about 30 min.), since the replication time of a thy$^+$ strain on the same medium was 40 min. However, it is possible that the low thymine requirer has an altered control of its thymine pools which permits it to maintain larger pools of thymine, than the wild type when grown on 20 ug. thymine/ml. It is possible that this would permit a shorter replication time in the mutant than in the wild type. However, it is also possible that the slower replication time is more apparent than real since the 94% confidence limits of a 30 min. replication time (20 ug./ml. thymine) and a 40 min. replication time (2 ug./ml. thymine) do overlap. The behaviour of the low thymine requirer is considered further in Section 4.5.

4.5 The Variation of Gene Frequency Distribution by Thymine Concentration in the Low Thymine Requiring Strain

4.5.a The Strain

The mutation to thymine requirement has been shown to require two mutations in B. subtilis (Anagnostopoulos and Schneider Champagne 1966). Such double mutants required 4 ug./ml. of thymine for growth and were isolated by the method of Okada et al. (1962). The original thy strain of B. licheniformis was isolated by this method but has not been shown to be a double mutant. Thus it is not possible to say whether the mutation to low thymine requirement in the isolation of SH 8a T tlr was a mutation analogous to the lesions in deoxyribo-aldolase or deoxyribomutase found in E. coli (Alikhanian et al. 1966; Beacham, Eisenstark, Barth and Pritchard 1968) or a reversion in one of two mutant sites analogous to those in B. subtilis or a third mutation in an already double mutant. Attempts to classify this strain further were unsuccessful as the purified high thymine requiring strain could not be transformed.

4.5.b The Influence of Thymine Concentration on Growth and Replication

Deutch and Pauling (1971) have found that at concentrations of thymine below 2 ug./ml. the growth rate (as measured by the rate of increase of viable count) and rate of DNA synthesis in E. coli $15T^-$ decreased, in a

similar way to that observed in B. licheniformis. In B. subtilis (thy) the influence of thymine concentration on growth and DNA replication has been studied by Ephrati-Elizur and Borenstein (1971). They have shown that the mass doubling time in "enriched medium" (very similar to medium A) is independent of thymine concentration down to 1.0 ug./ml. and that at 0.5 ug./ml. the doubling time is only slightly reduced (from 42 to 48 min.). The rate of replication was found to fall with thymine concentration and t_R was 86 min. at 5 ug./ml., 110 min. at 2 ug./ml. and 148 to 123 min. at 1 ug./ml. Thus B. licheniformis SH WT lrc 1 shows similarity in its behaviour to both E. coli 15T$^-$ and B. subtilis although it should be noted that the replicating times in B. subtilis are far longer than in B. licheniformis. An understanding of this difference will require a genetic analysis of SH WT lrc 1.

4.6 The Correlation of Constitutive Enzyme Synthesis with Relative Gene Frequency

In order to consider this correlation it is first necessary to calculate from the relative ade/trp gene frequencies for each of the DNAs, the frequencies of the trp and pen genes (it is assumed that these are the same as one another) relative to the sum of all genes (i.e. the overall relative frequency (ORF)). As in the calculation of replication times, this requires the assumption that ade and trp are at either end of the

chromosome. It also requires the integration of the equation of chromosome replication from x = 0 to x = 1 (in order to sum the frequencies of all the genes on the chromosome) for each value of n (each growth rate) in order to find the size of the gene pool per chromosome terminus. This integral has been worked out by Sueoka and Yoshikawa (1965) for the purpose of calculating the amount of DNA per chromosome at different growth rates and it is given by the expression:

$$\frac{2^n - 1}{n} \cdot \frac{1}{\ln 2}$$

This expression gives the size of the gene pool per chromosome terminus (therefore per trp or pen gene) in arbitrary unit genes where a unit gene is equivalent to a complete non-replicating chromosome. The ORF of the trp and pen genes is given by the reciprocal of this expression. This has been calculated for the values of n in Table 4.3 and plotted against the corresponding doubling times in Figure 4.1. Also plotted for comparison are the curves for penicillinase synthesis and tryptophan synthetase B synthesis from Figures 3.1 and 3.2, normalised at the 90 min. doubling time. Comparing the plots of enzyme per total protein and genes per unit gene it can be seen that while the shapes of the curves are similar, the change in enzyme synthesis with growth rate is too large to be explained by the change in ribosomal protein synthesis with growth rate (shown in Fig. 1.2). This possibility has been tested

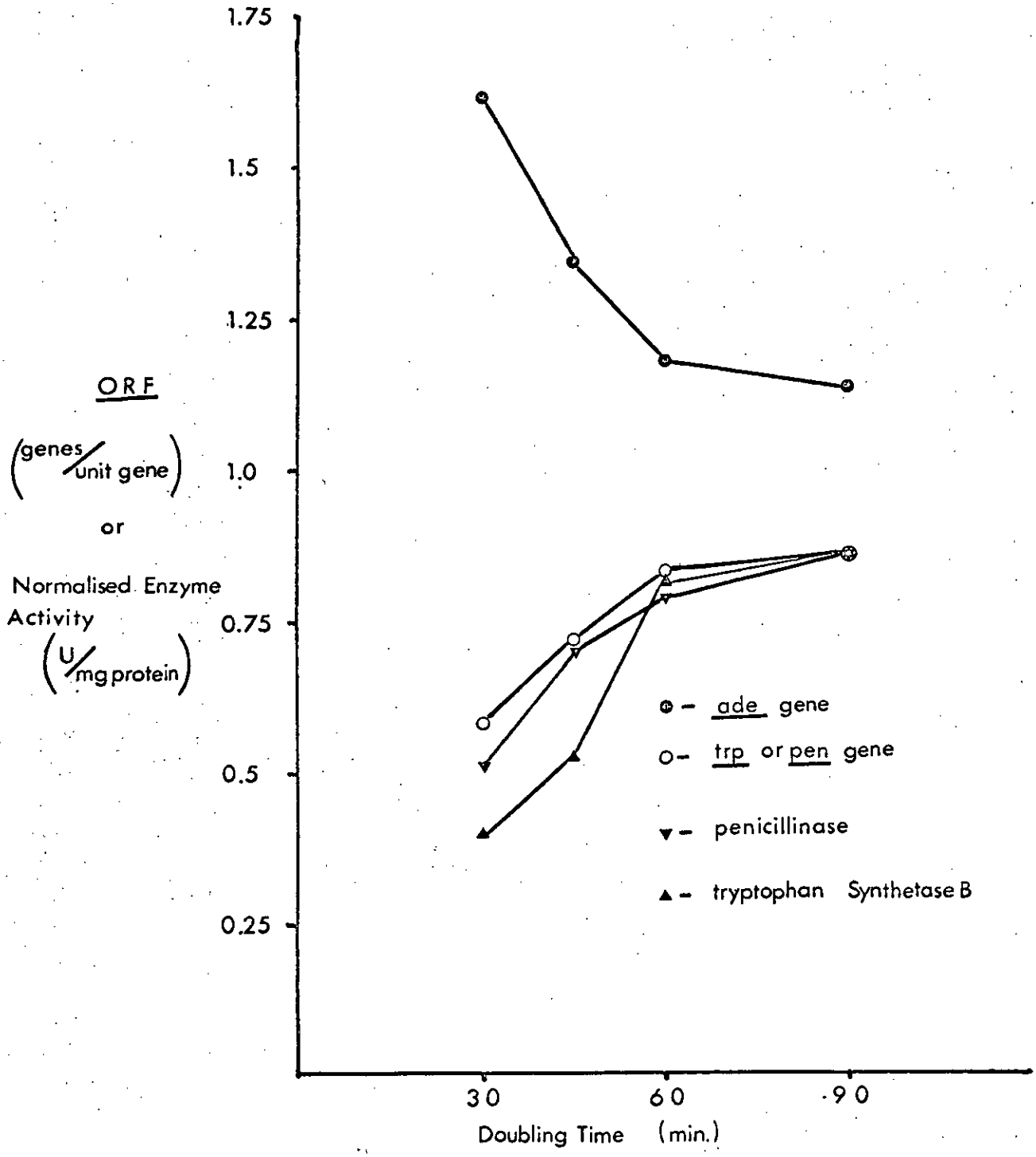


Figure 4.1. The Overall Relative Frequencies (ORFs) of the trp, pen and ade Genes as a Function of Growth Rate, Compared with the Normalised activities of Tryptophan SynthetaseB and Penicillinase.

by dividing the enzyme per total protein by the genes per unit gene to obtain a measure of the "efficiency" of gene expression at the various growth rates. The resultant values have been normalised to 1 at the 90 min. doubling time and are plotted against growth rate in Figure 4.2. Also shown is the curve for a standard protein taken from Figure 1.2 which represents the change in efficiency of gene expression with growth rate which results from the change in ribosomal protein synthesis. This figure emphasises the difference in behaviour of tryptophan synthetase B and penicillinase with tryptophan synthetase B showing much lower efficiencies of expression at high growth rates than either penicillinase or the standard protein. When this project was conceived it was thought that at fast growth rates in rich media the number of different genes expressed would be lower than at slow growth rates (as a result of the repression of many biosynthetic enzymes) and that in consequence constitutive enzymes should form a higher proportion of the non-ribosomal protein. This effect was expected to compensate for the diluting effect of ribosomal protein. On this model the curve for penicillinase (in Fig. 4.2) is what would be expected, since the efficiency of expression is higher at high growth rates than predicted by compounding the influences of ORF and ribosomal protein synthesis. The low efficiency of expression of the <u>trp</u> gene at fast growth rates needs to be explained. Possible

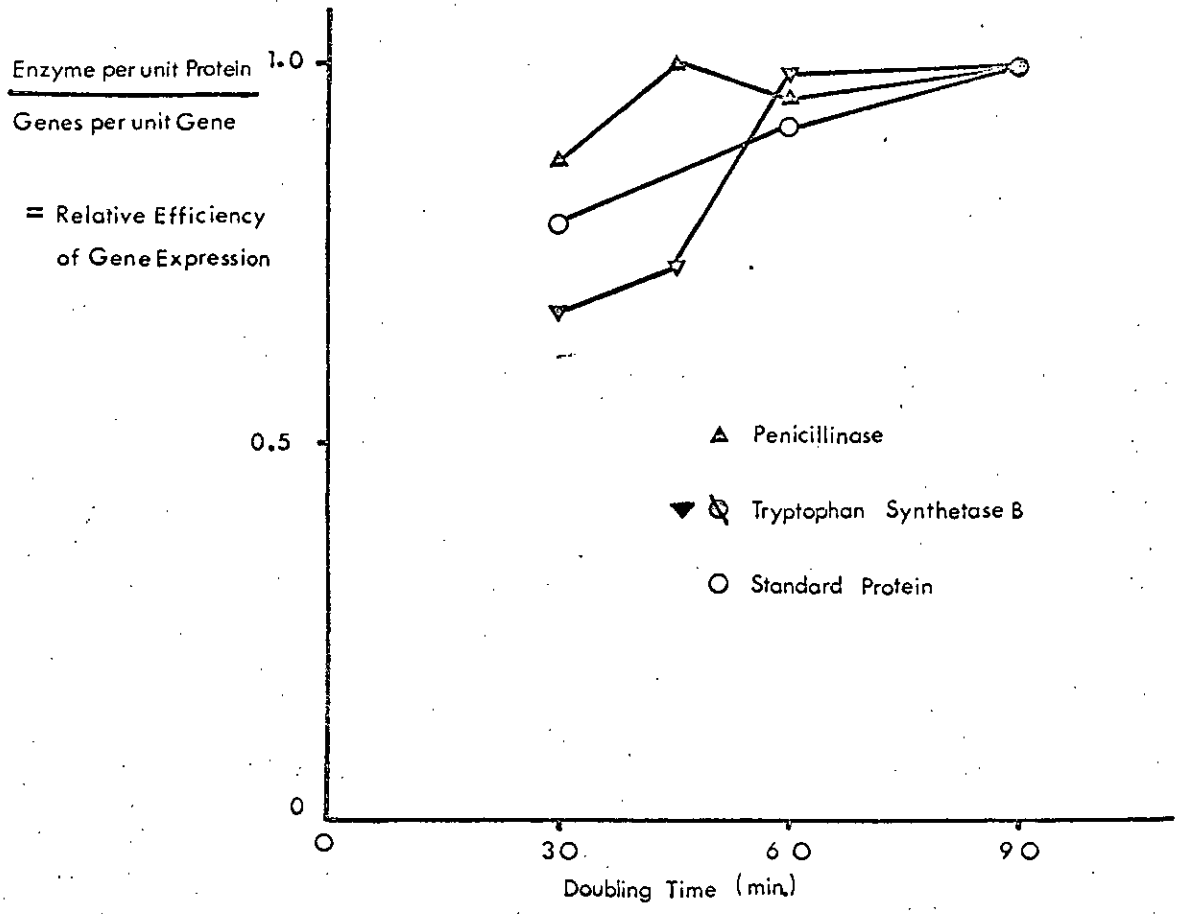


Figure 4.2. A Comparison of Relative Efficiencies of Expression of the trp and pen Genes with the Theoretical Curve for the 'Standard Protein' Which Takes Account of the Diluting Effect of Ribosomal Protein (from figure 1.2.).

The comparison is made over the range of doubling times from 30 to 90min. and the curves are normalised at unit efficiency at the 90min. doubling time.

explanations are (1) that the low level of expression is only an apparent low level of expression due to the instability of the enzyme at high growth rates or due to inhibition of its activity by some component of fast grown cells and (2) that there is a phenomenon similar to catabolite repression which acts on biosynthetic genes and which is manifest at high growth rates.

The most puzzling result is the lack of correlation between relative gene frequency and penicillinase synthesis in the low thymine requiring strain. If the interpretation of the results of growth rate experiments is correct then the relative gene frequencies measured in the DNA from low thymine experiments might have been in error; one possibility for this would be that the trp marker was unstable at low thymine concentrations. The suggestion that the pen gene is carried on a plasmid might also be put forward since it has not been formally shown that pen is a chromosomal marker. One could imagine that in normally growing cells the plasmid is replicated at the same time as the chromosome terminus but that at low thymine concentrations the plasmid is replicated at the middle of the chromosome replication cycle giving a fairly constant relative gene frequency. However, this is a highly speculative argument and further studies on the physiology of growth at low thymine concentrations must be undertaken before a proper understanding of the results obtained with the low thymine requiring strain can be gained. These

studies should include examination of the variation in cell DNA content with thymine concentration (as a check on the gene frequency distributions established by transformation), the assay of other constitutive enzyme levels at low thymine concentrations (first of all tryptophan synthetase B) and the study of the effect of thymine concentration on the cell ribosome content.

4.7 The Control of Ribosomal Protein Synthesis

It has been noted that in *B. subtilis* markers for resistance to streptomycin and related antibiotics map close to the origin of replication and it has been shown (Smith, Goldthwaite and Dubnau 1969) that these markers are in genes coding for ribosomal protein. It has not escaped my attention nor that of other workers (Van Dijk-Salkinoja and Planta 1971; Sueoka *et al.* 1970) that the change in overall relative frequency of genes with growth rate might be responsible for the changes in ribosomal ~~and~~ protein synthesis shown in Figures 1.1 and 1.2. In order to evaluate this idea the ORF of the *ade* gene has been plotted against growth rate in Figure 4.3 and compared with the curve for ribosomal protein synthesis from Figure 1.2. From this comparison it is clear that the change in ORF of the ribosomal genes with growth rate is not sufficient to account for the variation in synthesis of ribosomal protein. This confirms that in *B. licheniformis* some other mechanism is controlling ribosomal protein

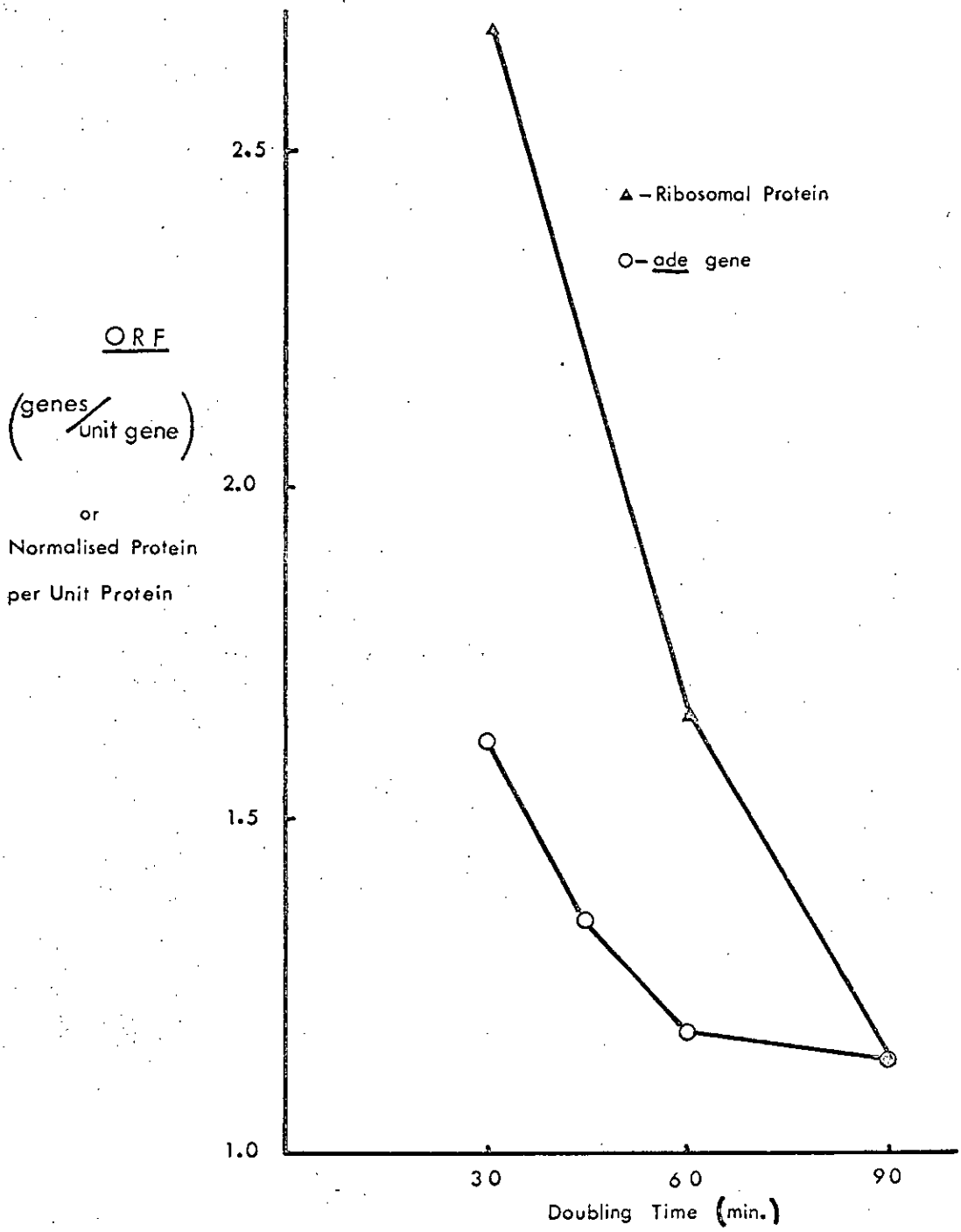


Figure 4.3. A Comparison of the Overall Relative Frequency (ORF) of a Gene Near to the Origin of Replication with the Cell Content of Ribosomal Protein (from the data of Van Dijk-Salkinoja and Planta 1971) over the Range of Growth Rates from 30 to 90min..

The ade gene has been used as a gene near the origin and the curve for ribosomal protein has been normalised to match the ade gene curve at the 90min. doubling time.

synthesis although it is likely that the location of the ribosomal genes close to the origin does help to amplify their expression at high growth rates.

4.8 Final Conclusions

It is clear that the ability to study the constitutive synthesis of enzymes which map close to the origin would have helped greatly in the analysis of the influence of relative gene frequency on enzyme synthesis.

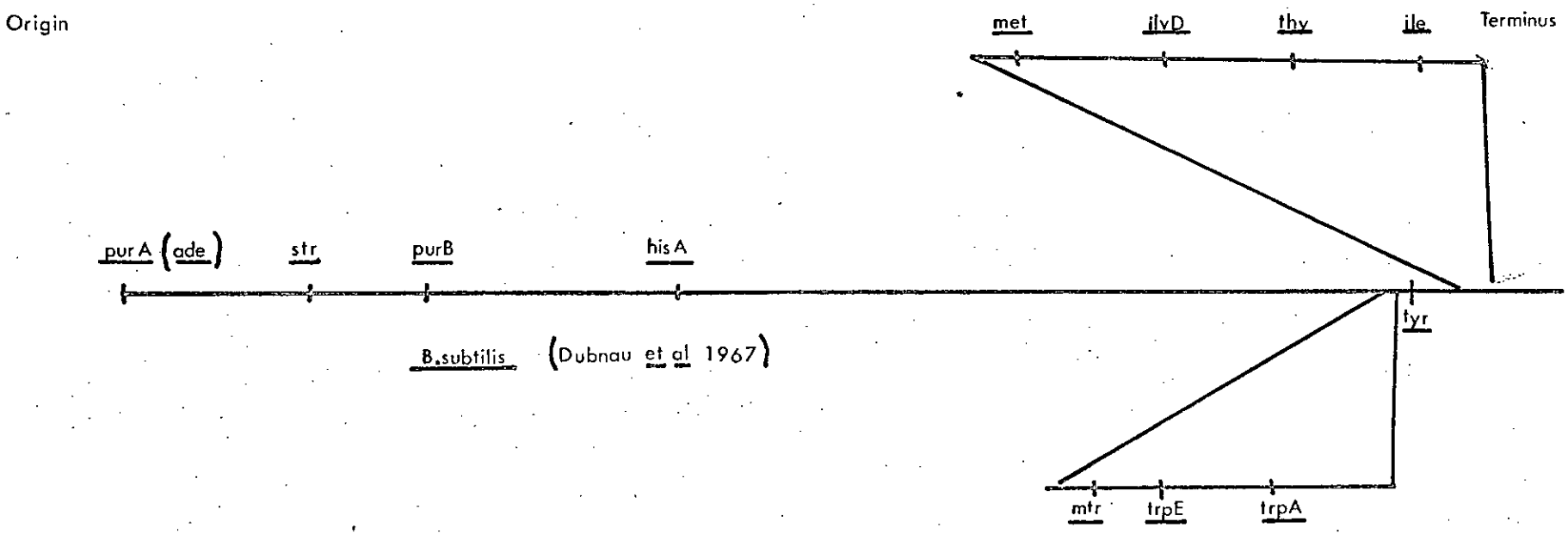
A more promising approach to this project might have been to study one constitutive enzyme in different chromosomal locations. This would require a system for transposing sets of genes around the chromosome. Techniques for this are now available in *E. coli* based on the directed transposition technique of Beckwith and Signer (1966), and a study of the inducible levels of β-galactosidase in strains in which the *lac* genes have been transposed is now being undertaken by Donachie (personal communication). The results of this study should prove interesting when compared with the results presented in this thesis.

The overall implications of the results of this investigation with respect to the large scale production of enzymes are that for genes near the terminus the highest yields are to be had at slow growth rates and that although the enzyme yield for a gene near the ~~terminus~~ origin might be expected to be greater at fast growth rates the diluting

effect of ribosomal protein and non-specific repression mechanisms may cancel out the effect of relative gene frequency.

Appendix 1. Chromosome Maps of B. licheniformis and B. subtilis .

B. licheniformis (Tyeryar et al 1968)



B. subtilis (Dubnau et al 1967)

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