Genetical and Biochemical Investigations on the Regulation of Cysteine Biosynthesis in *Escherichia coli*

A thesis presented to the Faculty of Biological and Agricultural Sciences of the University of Oxford in partial fulfilment of the requirements for the degree of Doctor of Philosophy

by

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ABSTRACT

This thesis is concerned with the mechanism by which the enteric bacterium Escherichia coli regulates its synthesis of the amino acid cysteine. The structural genes for the enzymes of the pathway are located at various positions around the chromosomes of both this organism and the closely related Salmonella typhimurium. Mutations in the cys B region of both organisms cause a deficiency of the cysteine biosynthetic enzymes. We concentrated our efforts on this region in E.coli. Previous work had shown that cys B mutations in E.coli were recessive to wild type and a scheme of positive control of cysteine biosynthesis via the cys B gene product(s) had been proposed. Genetical and nutritional analysis of the cys B region in Salmonella had indicated that it was complex, possibly containing three genes.

Optimal conditions were found for a method of directed mutagenesis in E.coli using mutagenised transducing phage P1. This technique was then used to isolate 60 point mutants in the cys B region. Nutritional and genetical analyses confirmed that they lay in cys B.

We isolated in addition a number of deletions extending into the cys B region of an E.coli F-prime carrying the cys B and trp genes which had been inserted into S.typhimurium. These were used to construct a map and thus to order the point mutations into 16 deletion groups. Point mutations mapping at the extreme ends of the region were unable to complement one another. This result shows that there is only one gene in the cys B region. Nutritional data correlates with this conclusion.

Some of the point mutants are temperature sensitive and in others the

mutations are suppressed by the amber suppressor sup F: the gene therefore codes for a protein.

Deletions ending in cys B and trp were isolated. We measured the rate of synthesis of enzymes of the tryptophan operon in strains carrying such deletions. In none of them was the expression of trp brought under cysteine-mediated control. We tentatively concluded that the cys B gene is transcribed in the direction trp —> cys or, if it is transcribed in the direction cys —> trp it is transcribed at a low, constant rate.

We also sought constitutive mutations mapping in the cys B region. We isolated several mutants constitutive for at least part of the pathway: though the mutations conferring this phenotype were associated with the cys B region, they were unstable and their nature could not be elucidated.

The results obtained in this thesis, together with recent work locating a partial constitutive mutation within the cys B region of Salmonella are strong evidence that cysteine biosynthesis in both organisms is indeed under positive control mediated via the cys B gene product.

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GLOSSARY AND ABBREVIATIONS

ACH	Acid hydrolysed casein
APS	Adenosine 5'-phosphosulphate
cfu	Colony forming units
EMS	Ethyl methane sulphonate
mRNA	Messenger ribonucleic acid
$NADPH_2$	Reduced nicotinamide adenine dinucleotide phosphate
NTG	N-meihyl-N'-nitro-N-nitroso-guanidine
OAS	O-acetylserine
PAPS	3'-phospho adenosine 5'-phosphosulphate
RNA	Ribonucleic acid
pfu	Plaque forming unit
t-RNA	Transfer ribonucleic acid
TRZ	1-2-4 triazole

Enzymes

Trivial Name	Systematic name	Number
ATP sulphurylase	ATP: sulphate adenylyl transferase	2.7.7.4
APS kinase	ATP: adenylsulphate 3'-phosphotransferase	2.7.1.25
PAPS reductase	-	
Sulphite reductase	Hydrogen sulphide: NADP oxidoreductase	1.8.1.2
OAS sulphydrase	O-Acetylserine acetate lyase (adding hydrogen sulphide)	4.2.99.8
Serine transacetylase	Acetyl CoA: L-serine O-acetyl transferase	2.3.1.30
Tryptophan synthetase	L-Serine hydro-lyase (adding indole)	4.2.1.20
Anthranilate synthetase	Chorismate pyruvate-lyase (amino accepting)	4.1.3.27

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Chapter 1

Introduction

1.1 Historical Introduction

The study of the regulation of the synthesis of proteins is one of the major successes of modern "molecular" biology. This is not to say, though, that this line of research is exclusively a recent one. One of the earliest recognised characteristics of microbes was their adaptability (Wortman, 1882). It was gradually realised that there were at least two mechanisms implicated in this adaptability: mutation-selection, and enzyme induction.

The first mechanism was demonstrated by the work of Massini (1907) on the adaptation of E.coli mutabile to growth on lactose, showing that this occurred in a very small fraction of the original population. Lewis (1934) showed that this low frequency was in the range of 10^{-6}, the value which we now consider the typical frequency of a spontaneous mutation in a single gene.

The second mechanism was more difficult to demonstrate. Though, with hindsight, one can look through much of the early work on microbial adaptation and conclude that the adaptation recorded occurred under conditions, or over periods of time, in which mutation-selection was unlikely (for a review, see Monod, 1947), good experimental evidence that adaptation could occur without the selection of mutants was furnished only by its demonstration in the absence of cell division (Stephenson and Stickland, 1933; Stephenson and Yudkin, 1936). In addition, it was shown that the inhibition by glucose of the galactose-mediated induction of galactose-degrading enzymes in cultures of yeast was a result of inhibition of enzyme induction, and not of selection against mutants capable of degrading galactose.

These observations were confirmed and extended by the studies of Monod (1942, 1947). Having confirmed the occurrence of enzyme induction, Monod went on to study its mechanism.

The main problems to which he sought answers were:

1. Whether the appearance of enzyme after addition of inducer was a result of de novo synthesis of protein or of conversion of an inactive precursor to active enzyme (e.g. like trypsin).

2. Whether inducing molecules had to be substrates of the enzymes they induced.

3. Whether the inducing molecule had to be capable of supporting growth to cause enzyme induction.

These problems were obviously interrelated; since the bulk of work to that time had been performed on catabolic systems, i.e. those systems which yielded energy for growth, the observed kinetics of enzyme induction were unable to give unequivocal answers to any of these problems (Monod, 1947; Kacser, 1953).

That induced enzymes are synthesised de novo was shown by radiochemical methods (Hogness, Cohn and Monod, 1955). The main approach to solving the other problems relied upon divorcing induction of the enzyme from the metabolic changes that followed its appearance. This separation was achieved in two ways: the use of the differential plot (Cohn and Monod, 1953), and the systematic search for gratuitous inducers and gratuitous substrates of enzyme systems (Monod, Cohen-Bazire and Cohn, 1951).

This latter approach was not original (Spiegelman, cited by Monod, 1947) but it could be applied most readily to the system of lactose catabolism in E.coli,

enabling the inducibility of this system to be separated unequivocally from cell permeability, growth and metabolic effects of the inducer. The discovery of gratuitous inducers also simplified the genetical analysis and made it more rigorous. As a result the *lac* system of *E.coli* is even now by far the best characterised of regulation in any organism.

Many of our basic concepts about the regulation of enzyme synthesis in bacteria arose from this work. Consequently it will be helpful to describe its chief characteristics here, even though, as we shall see, there are profound differences between it and cysteine biosynthesis.

1.2 The *lac* Operon in *E.coli*

The genes concerned in the utilisation of lactose map together at 9 minutes on the standard genetic map of *E.coli* (Taylor and Trotter, 1972).

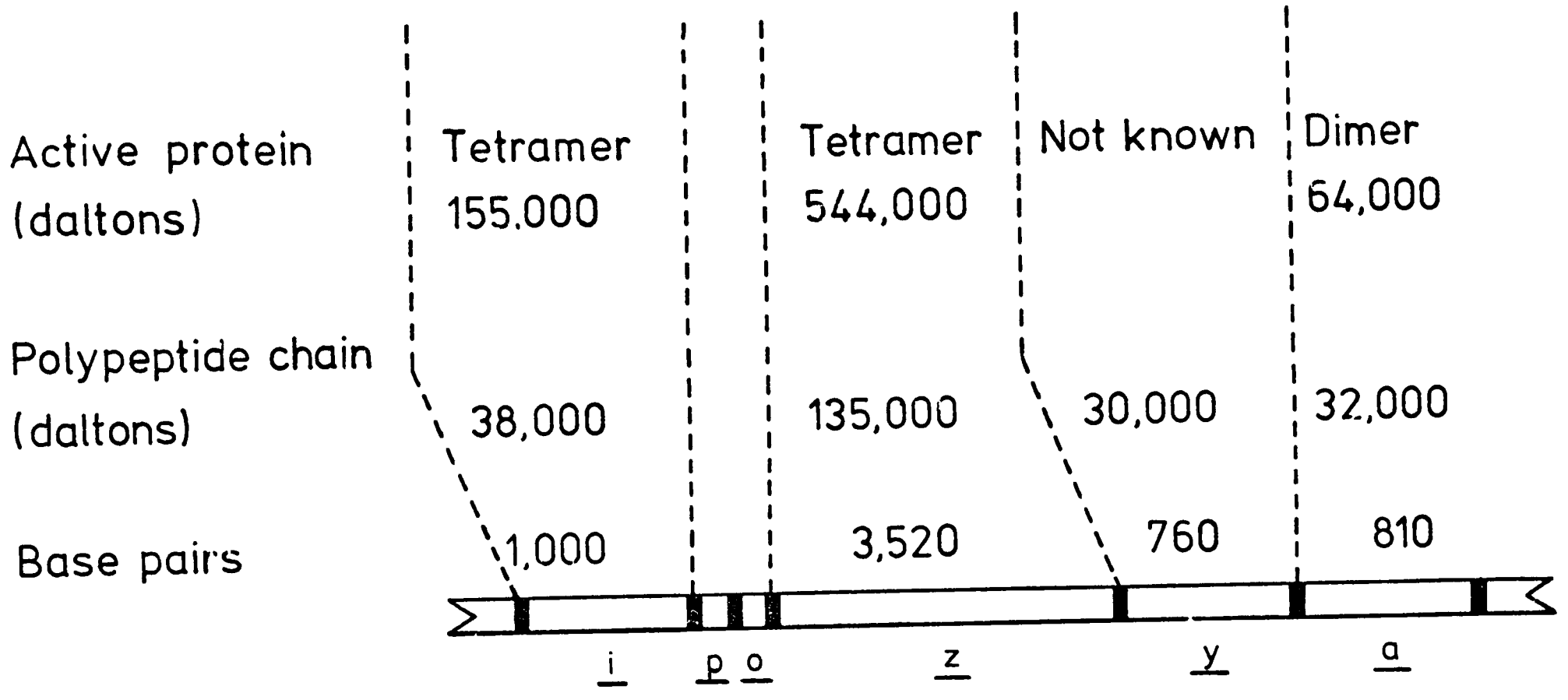
There are three structural genes, named *lac Z Y A*, which code respectively for the proteins β-galactosidase, galactoside permease and thiogalactoside transacetylase. These genes map adjacent to one another; adjacent to the *Z* gene are mapped two regulatory regions designated *lac P* ("the promoter") and *lac O* ("the operator"). Near to these but separate from them is a region called *lac I* (for "inducibility").

A map of the cluster is given in Fig. 1.a.

The three proteins coded for by the structural genes are present in very small amounts in cells grown in the absence of inducer. This is the so-called 'basal level' of synthesis. When cells are grown on lactose or in the presence of any inducing compound the levels of all three proteins rise to the same degree, i.e. induction is "co-ordinate". The fully induced rate is some

Fig. 1a The Lactose Operon of *E. coli*

after Lewin (1974)


Active protein (daltons)
Tetramer 155.000
Tetramer 544,000
Not known
Dimer 64,000
Polypeptide chain (daltons)
38,000
135,000
30,000
32,000
Base pairs
1,000
3,520
760
810
i
p
o
z
y
a

1000 times the basal level. This finding implies that the three structural genes are controlled as a unit. Polarity data (Zipser, 1970) indicate that transcription starts from the lac Z end of the cluster and ends past lac A; this direction is confirmed by colinearity studies of the Z gene and its product (Zabin and Fowler, 1970). Transcription of the genes can be measured indirectly by assay of the gene products and more directly by hybridisation of the lac mRNA. These measurements show that changes in the rate of synthesis of the mRNA and the proteins always occur in parallel: therefore regulation occurs at the level of transcription (Contesse, Crepin and Gros, 1970).

Mutations in lac Z or lac Y confer a Lac^- phenotype. Mutations in lac A have no apparent phenotypic effect. Most mutations in lac P reduce the rate of transcription of the structural genes under both repressing and derepressing conditions.

By contrast most mutations in lac I and lac O render transcription constitutive; i.e. transcription of the structural genes occurs in the absence of inducer. These mutations can be differentiated biochemically and genetically. lac I^- constitutives usually possess completely derepressed levels of the lac enzymes. lac O^c usually possess lower levels. Another most important difference was shown by the experiment of Pardee, Jacob and Monod (1959). They followed the kinetics of β-galactosidase synthesis in the absence of inducer in reciprocal Hfr/F^- crosses, using parental strains which were lac I^+ Z^+ (able to make β-galactoside only in the presence of inducer) and lac I^- Z^- (constitutive but unable to make active enzyme).

In the cross Hfr I^- Z^-/F^- I^+ Z^+ no enzyme was formed by the merozygotes.

In the cross Hfr $I^+ Z^+ / F^- I^- Z^-$ the merozygotes were able to make β-galactosidase at the fully derepressed rate just as if the Z^+ gene alone had been transferred. This synthesis stopped after 60 minutes but recommenced upon the addition of inducer.

This experiment indicated that the difference in expression observed depended on the cytoplasm of the recipient; I^+ is dominant to I^- and it also appears that the I^+ allele acts via a cytoplasmic effector.

These experiments were repeated in stable merodiploids. The results confirmed the *trans* dominance of I^+ over I^-; i.e. in the diploids $F' I^- Z^+ / F^- I^+ Z^-$ and $F' I^+ Z^- / F^- I^- Z^+$ the synthesis of β-galactosidase is inducible. Similar studies showed that O^c mutations affected only the structural genes adjacent to them i.e. they are *cis* dominant.

Since the vast majority of mutations in *lac* I confer constitutivity it follows that an intact I gene is needed to prevent transcription and that *lac* is a system under negative control.

There are two possible ways in which the I gene product might act. It might act enzymically by destroying an endogenous inducer. Alternatively it might act non-enzymically by blocking the transcription of the structural genes; this block could be removed by interaction of the gene product with an inducer molecule.

That the second hypothesis, the 'repressor' hypothesis, is correct was supported initially by the isolation of mutations mapping within the I gene that prevented the organisms from being derepressed for the structural genes: I^s mutations (Willson, Perrin, Cohn, Jacob and Monod, 1964). Genetic evidence showed that the gene product was a protein (Bourgeois, Cohn and Orgel, 1965).

The most rigorous proof of the repressor hypothesis was its isolation after a search for a molecule with the predicted properties (Gilbert and Muller-Hill, 1966). The isolated repressor is a protein; it is able to bind to DNA isolated from the lac region and has a lower affinity for lac DNA carrying an O^c mutation; it is able to bind inducers and upon doing so acquires a lower affinity for lac DNA; it can be isolated from I^+ but not from I^- strains (Gilbert and Muller-Hill, 1970).

Thus the model accounts very neatly for all the data cited; I^- mutants contain those mutations in lac I which cause loss of affinity of the repressor for the operator; I^s mutations are those mutations which do not affect this property but affect only the ability to bind inducer or undergo the conformational change necessary for dissociation of the repressor from the operator. A prediction from this is that I^s mutations will be relatively infrequent and map in only a small portion of the I gene. This prediction has been tested and confirmed (Pfahl, Stockter and Gronenbaum, 1974). The model also accounts for the dominance relationships of I^+ over I^- or I^s over I^+ mutations.

Several of the other regulatory systems studied simultaneously with lac showed certain similarities. Phage lysogeny (Luria, Fraser, Adams and Burrous, 1958) and tryptophan biosynthesis (Cohen and Jacob, 1959) both appeared to be under negative control. Other catabolic pathways such as that of galactose degradation (Morse, Lederberg and Lederberg, 1956) and anabolic pathways such as those of tryptophan (Demerec and Hartman, 1956) and histidine (Hartman, 1956) biosynthesis showed extensive clustering of the structural genes. The fact that all these pathways could at first sight be accommodated within the same theory - the operon theory (Jacob and Monod, 1961) - encouraged research into other enterobacterial regulatory systems which showed obvious differences from them.

Among such systems was the regulation of cysteine biosynthesis; the subject of this thesis.

1.3 The Pathway of Cysteine Biosynthesis in E.coli and S.typhimurium

The evidence for the pathway comes from nutritional, radiochemical, biochemical and genetical studies. Prototrophic strains of E.coli and S.typhimurium can utilise sulphate for the synthesis of cysteine. Certain mutants unable to do this can utilise more reduced forms of inorganic sulphur such as sulphite, thiosulphate and sulphide (Lampen, Roepke and Jones, 1947, Clowes, 1958a). This evidence indicates that sulphate is reduced before incorporation into the carbon skeleton. This conclusion is supported by radiochemical investigations which also identify the carbon skeleton as being derived from serine (Roberts, Abelson, Cowie, Bolton and Britten, 1955).

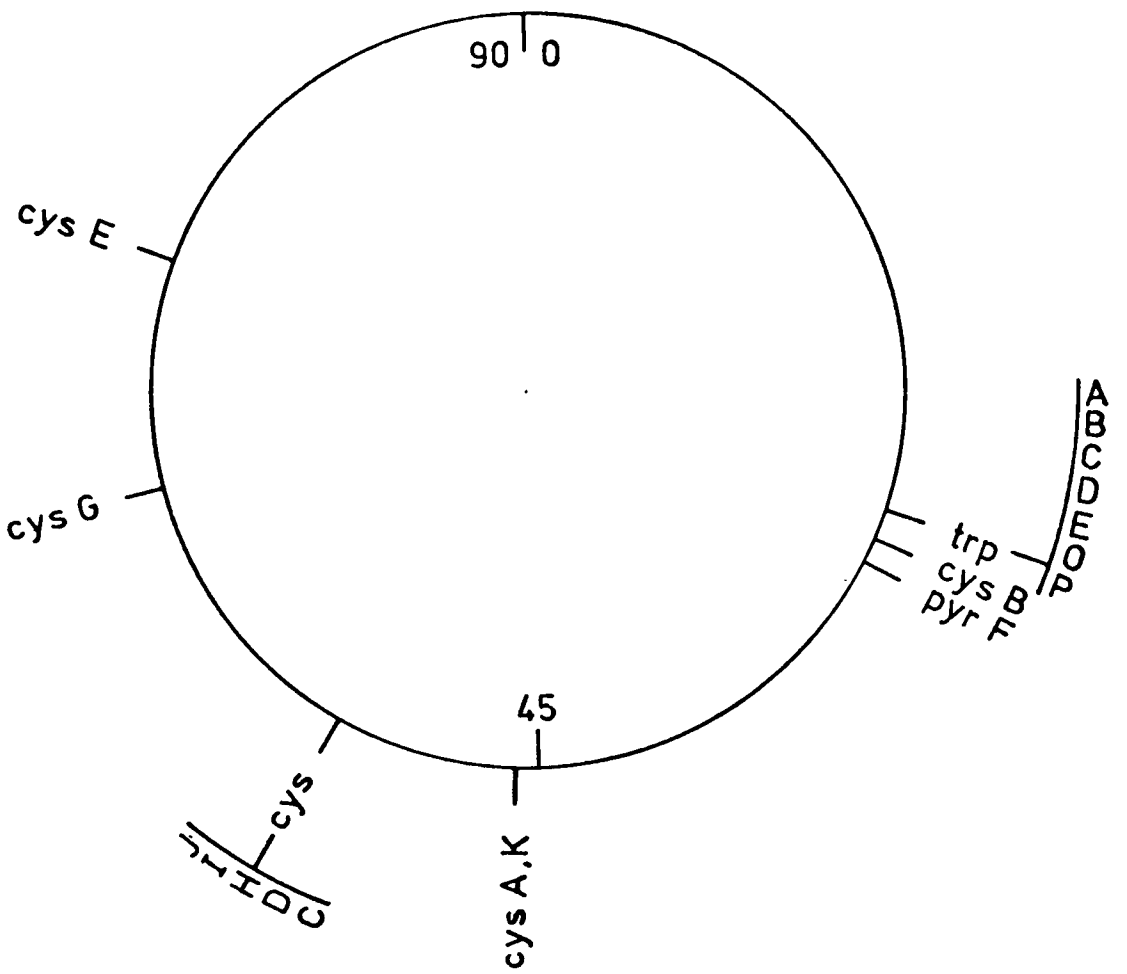
Mutations that affect cysteine biosynthesis or its regulation have been mapped into 11 genes, cys A B C D E G H I J K L. These are distributed into five regions around the chromosomes of both E.coli and S.typhimurium, in similar locations in the two organisms but not always in similar orientations (Fig. 1b). For the purposes of all future discussion, this genetical homology will be assumed to extend to the biochemical level except where there is evidence against this view.

Mutations in cys C D G H I or J lead to auxotrophy for cysteine. Biochemical analysis of these mutants reveals that they each lack one of the enzymes of the cysteine biosynthetic pathway; cys C mutants lack APS kinase; cys D, ATP sulphurylase; cys H, PAPS reductase, and cys G I and J all lack sulphite reductase (Dreyfuss and Monty, 1963; Jones Mortimer, 1968a). These results

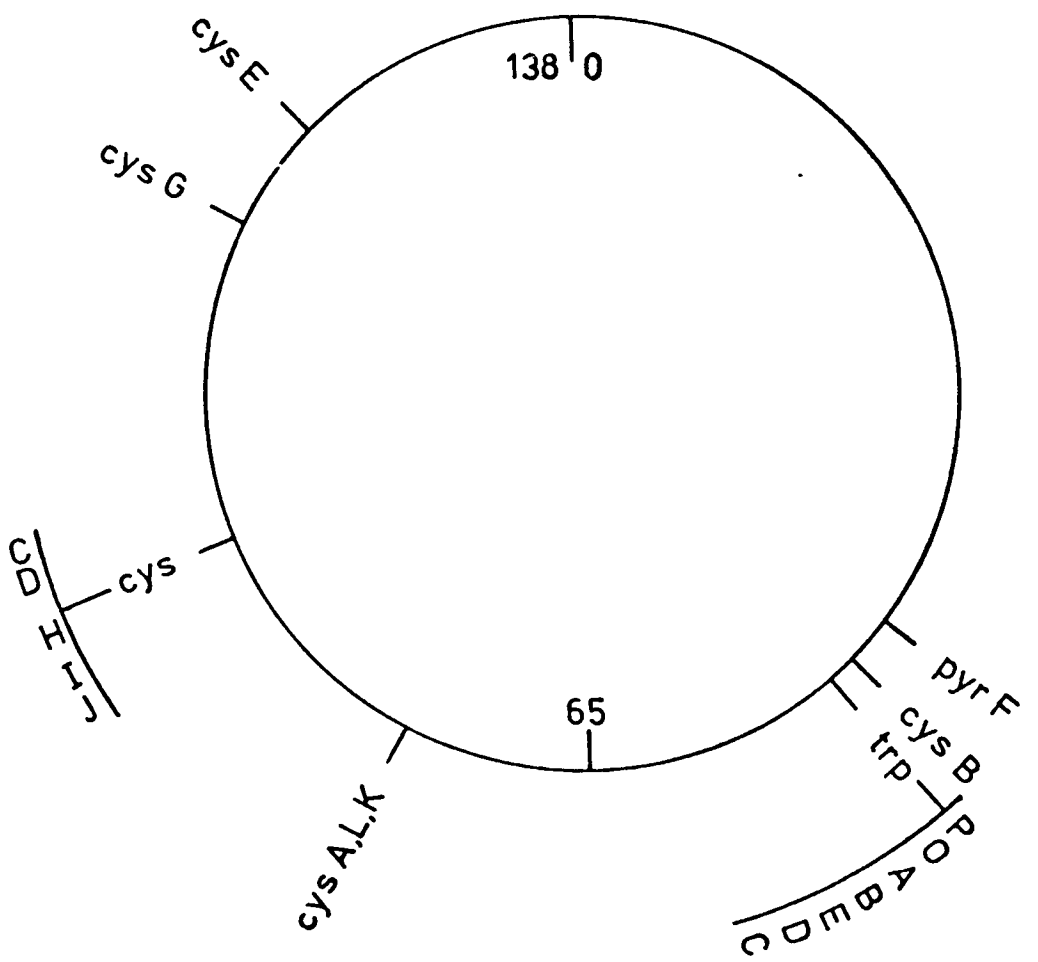
Fig. 1b The Position of the *cys* Genes in *E.coli* and *S. typhimurium*

compiled from Taylor and Trotter (1972)
Sanderson (1972)
Jones-Mortimer (1973)

E. coli K-12



Salmonella typhimurium LT2



are confirmed by nutritional analysis, i.e. cys C D H are able to utilise sulphite for cysteine synthesis, but cys G I J can use only sulphide or thiosulphate, (which is probably not an intermediate but may act by being converted into sulphide - Leinweber and Monty, 1963; Roy and Trudinger, 1970, p.253).

It is therefore probable, in spite of the fact that only one of the enzymes, sulphite reductase, has been purified and studied in detail, that these 6 genes are structural.

Mutants in cys A are also cysteine auxotrophs and are deficient in sulphate uptake. This system is quite complex; the one component of it which has been isolated and studied in detail is the periplasmic sulphate binding protein (Pardee, Prestidge, Whipple and Dreyfuss, 1966). This is present in point and deletion cys A mutants, but is found only in small amounts in cys A amber mutants (Ohta, Galsworthy and Pardee, 1971). It is therefore possible that cys A is a region coding for unidentified components of the sulphate uptake system, and that the hitherto unidentified gene coding for the binding protein lies to one side of it and is transcribed after it. The complexity of the sulphate uptake system is also reflected by the fact that the cys A region can be divided into two (Howarth, 1958; Qureshi, Smith and Kingsman, 1975) or three (Mizobuchi, Demerec and Gillespie, 1962) cistrons.

cys K (trz A) mutants are deficient in the last enzyme of the pathway, OAS sulphydrylase; there is good evidence that cys K is the structural gene for this enzyme (Hulanicka, Kredich and Treiman, 1974). These mutants are not cysteine auxotrophs as there is a second enzyme present in the cell which can catalyse the sulphydration of OAS (Becker and Tomkins, 1969).

cys E mutants are cysteine auxotrophs, but they are pleiotropic; they

lack all the enzymes of the cysteine biosynthetic pathway and cannot utilise sulphide. However they can utilise sulphate if they are supplied with exogenous OAS; biochemical analysis of cells from these cultures shows the presence of all the cysteine biosynthetic enzymes except serine transacetylase (Jones-Mortimer, Wheldrake and Pasternak, 1968; Kredich, 1971). In this respect, then, cys E mutants appear pleiotropic for a trivial cause; they will be discussed further in the next section. Leaky cys E mutants contain low levels of serine transacetylase (Hulanicka, Klopotowski and Smith, 1972) and a point mutation in the cys E region alters the physical and chemical behaviour of the isolated enzyme (Becker and Tomkins, 1969). Thus there is little doubt that serine transacetylase is coded for by a gene, or more than one gene, in the cys E region. cys E can be divided into two cistrons, cys E a and cys E b. The point mutant referred to above is a cys E b mutant. However, it is not clear how many different polypeptide chains there are in a molecule of serine transacetylase (Kredich, Becker and Tomkins, 1969).

There is evidence, therefore, for each of the genes described above, that they are structural genes.

However some mutations in cys C D H I J, in addition to causing a deficiency of the enzyme assigned to the particular gene, also have a secondary effect in that they cause a deficiency of sulphate uptake (Jones-Mortimer, 1968a; Ohta Galsworthy and Pardee, 1971). This phenomenon cannot be explained by the generally accepted scheme for the regulation of cysteine biosynthesis described below.

1.4 The Regulation of the Cysteine Biosynthetic Pathway

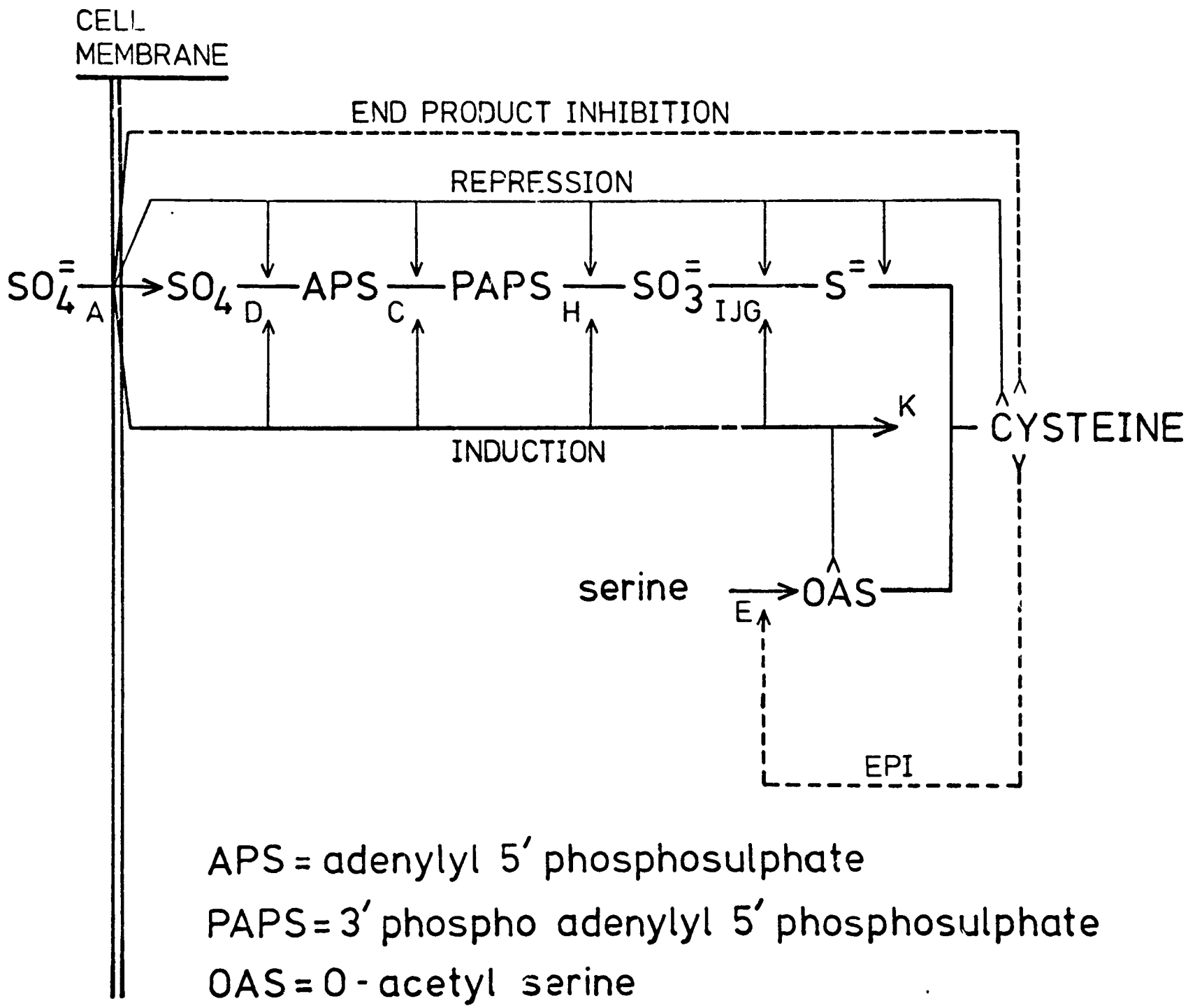
The pathway of cysteine biosynthesis (Fig. 1 c) is therefore a convergent one with two branches of unequal length, one for the production of the carbon skeleton and the other for the production of the reduced sulphur atom.

The first branch contains the serine transacetylase reaction only. The enzyme mediating this reaction is synthesised constitutively (Jones-Mortimer, Wheldrake and Pasternak, 1968; Kredich, 1971). It is regulated by feedback inhibition by cysteine (Kredich, Becker and Tomkins, 1969).

The first 'enzyme' of the second branch, the sulphate uptake system, also appears to be feedback inhibited by cysteine in E.coli, (Ellis, 1964) but not in S.typhimurium (Dreyfuss, 1964; Pardee et al., 1966). This system, the enzymes concerned in the activation and reduction of sulphate, and OAS sulphydrylase are all fully repressed in cells grown on cysteine or sulphide (Jones-Mortimer, Wheldrake and Pasternak, 1968; Kredich, 1971). In cells grown on sulphite, these enzymes are not repressed co-ordinately. Rather, there is a system of 'differential repression' (Pasternak, Ellis, Jones-Mortimer and Crichton, 1965): ATP sulphurylase and APS kinase are almost fully repressed but PAPS reductase (the product of which is sulphite) and sulphite reductase are only partially repressed. This correlates well with the genetic structure of the cys C D H I J cluster; cys C and D are contiguous and are separated from cys H I J by a long 'silent section' (Mizobuchi, Demerec and Gillespie, 1962). cys H appears to be separated from cys I and J by a short silent section, but polarity studies show that it belongs to the same transcriptional unit as the other two genes (Loughlin, 1975). We do not know whether repression in this system is mediated by cysteine or sulphide; though repression does correlate with the size

Fig. 1c The Cysteine Biosynthetic Pathway and its Regulation

from Smith (1971)



APS = adenylyl 5′ phosphosulphate

PAPS = 3′ phospho adenylyl 5′ phosphosulphate

OAS = O - acetyl serine

of the cysteine pool (Wheldrake and Pasternak, 1965), there are enzymes present in the cell that can rapidly interconvert these substances (Kredich, 1971).

The enzymes that are subject to repression by cysteine are also missing in cys E mutants, but appear when the cells are incubated with exogenous OAS (Section 1.3). OAS, therefore, besides being an intermediate in the pathway, also acts as an internal inducer of these enzymes. Addition of OAS to cultures of cys E^+ bacteria growing on sulphate or sulphite also derepresses these enzymes; but this effect is not observed in identical organisms growing on sulphide or cysteine. This indicates that the repression by cysteine is not exerted only as a result of the inhibition of OAS synthesis. It is likely that cysteine represses more directly than this, or that it prevents the uptake of OAS (Kredich, 1971).

That OAS is required for induction of the cysteine biosynthetic enzymes at a concentration above that which is required for the synthesis of cysteine is shown by the study of a 'leaky' cys E mutant; this mutant is unable to grow on sulphate, and hence cannot reduce it, but is able to grow on sulphide, and hence can synthesise enough cysteine from the OAS it produces, provided that is supplied with already reduced sulphur (Hulanicka, Klopotowski and Smith, 1972). This conclusion is confirmed by studies with the inhibitor 1-2-4 triazole (Section 8.1).

The 'cysteine-repressible' cysteine biosynthetic enzymes are also missing in cys B mutants, but these deficiencies cannot be remedied by the addition of OAS, or any other known intermediate of the pathway, to the medium (Jones-Mortimer, 1968b; Kredich, 1971). Similarly, most cys B mutants cannot use inorganic sulphur derivatives for growth other than sulphide (this presumably

occurs because of the 'second' OAS sulphydrylase), though a minority of isolates, at least in Salmonella, can utilise sulphite and high concentrations of thiosulphate (Clowes, 1958a; Mizobuchi, Demerec and Gillespie, 1962; Jones-Mortimer, 1968a).

Thus for derepression of the enzymes of the cysteine biosynthetic pathway a cell requires an appreciable intracellular concentration of OAS, low concentrations of cysteine or sulphide, and an intact cys B gene.

A comparison of the lac and cys systems may be useful at this point. We would expect certain differences; lac is a catabolic system; cys is anabolic, and although we expect catabolic pathways to be controlled by induction and anabolic pathways by repression (Kornberg, 1965), cys is an apparent exception to this rule. There are obvious differences in the genetic organisaiion - not surprisingly, because of the greater complexity of the cys pathway. The most evident difference in the control of the two systems is that in lac the vast majority of pleiotropic mutations lead to constitutivity, but in cys they lead to loss of expression of the regulated structural genes.

There are a number of possible explanations for the phenoiype of cys B mutants which would be compatible with cys B being a gene analogous to lac I; the most obvious one is that cys B^- mutations are analogous to lac I^s. This is the easiest explanation to exclude; cys B^- mutants occur at high frequency, and they are recessive to cys B^+ (Clowes, 1958a; Mizobuchi, Demerec and Gillespie, 1962; Jones-Mortimer, 1968b). Experimental evidence excludes some other explanations compatible with cys being regulated in the same way as lac, and the most likely explanation of all the facts is that cys is positively controlled (Jones-Mortimer, 1968b).

There is a precedent for positive regulation; there is convincing evidence for it in the regulation of arabinose catabolism (Englesberg, 1972). Positive regulation has also been proposed in the regulation of the catabolism of maltose (Hatfield, Hofnung and Schwartz, 1969) and rhamnose (Power, 1967) on the basis of evidence similar to that obtained in cys. This evidence, the isolation of pleiotropic-negative mutations in a particular gene at high frequency, is necessary evidence for positive control, but it is not sufficient. In several inducible systems the initial substrate is not itself the inducer but requires conversion by an enzyme. This is precisely what happens in lac. lac I^+ Z^- Y^+ strains cannot be induced by lactose, because active β-galactosidase is required in the cell to convert lactose to allolactose, the 'true' inducer (Jobe and Bourgeois, 1972). Analogous situations occur in the catabolic systems of glycerol utilisation (Cozzarelli, Freedberg and Lin, 1968) and histidine utilisation (Brill and Magasanik, 1969). In all these cases, in the absence of evidence for the enzymic function of the gene responsible for transforming substrate to inducer, it might have appeared that the gene was a positive control gene. For an analogous reason genes mediating permease activities can also be confused with control genes; this is precisely what happened in the work on the regulation of alkaline phosphatase synthesis (compare Echols, Garen, Garen and Torriani (1961) with Willsky, Bennett and Malamy (1973)).

These considerations lead us to ask: What kind of criteria are sufficient to characterise a system of positive regulation? It is obvious that the criteria chosen will determine the lines of research to be followed. This question has been addressed in two recent reviews (Englesberg and Wilcox, 1974; Beckwith and Rossow, 1974). They are as follows:-

1. The isolation of deletions or nonsense mutations within the putative positive regulatory gene, which result in a pleiotropic negative phenotype and which can be complemented by the intact gene. This is a more rigorous test of positive regulation than the simple isolation of point mutants because the possibility of subunit mixing between partially defective and fully intact activator molecules has been excluded.

2. The exclusion of the regulator gene from the operon(s) it controls.

3. The isolation and mapping within the regulator gene of pleiotropically constitutive mutations.

4. The demonstration of trans dominance of these constitutive mutations over both pleiotropic negative and inducible alleles.

5. The isolation and characterisation of cis-dominant constitutive mutations in the controlled operons as revertants of the pleiotropic-negative mutations and the ability to account for all such revertants with this mechanism.

How do these criteria apply to the regulation of cys?

The first criterion had been partially met; deletion mutations in cys B had been isolated in S. typhimurium (Mukai and Margolin, 1963). They had not been further characterised or tested for dominance.

The second criterion is met by the fact that cys B is distant from the known structural genes (Jones-Mortimer, 1968a; Sanderson, 1972).

Much research had been performed towards meeting the third criterion (see Section 8.1). Partially constitutive mutations mapping in or near cys B had been found in S.typhimurium, as secondary mutations occurring in cys Ea mutants (Spencer, Collins and Monty, 1967; Kredich, 1971). They had not been further

mapped or characterised, thus their significance was not known.

From consideration of the cysteine biosynthetic pathway, we would predict that the fifth criterion would be very difficult to apply. Since there was a second enzyme catalysing the sulphydration of OAS, and since this was not apparently controlled by cys B, there seemed little possibility of selection for constitutive synthesis of OAS sulphydrylase I. Neither could we look for constitutive synthesis of sulphite reductase (i.e. look for revertants of cys B on medium containing sulphite): since cys I and J are not in the same transcriptional unit as cys G it was probable that mutations in both operators would be needed to enable the enzyme to be made constitutively.

We also thought of examining analogues of OAS to see if any were gratuitous inducers of the pathway. This approach did not appear promising: very few such analogues were available; in any case OAS might act as inducer simply by lowering the intracellular pool of sulphide, one of the two likely candidates for the repressor molecule (see above).

Thus we concluded that the best approach to the problem was to isolate and characterise point and deletion mutants in the cys B region, construct a fine structure map and test the dominance of these alleles. We could also look for constitutive mutations in cys B and test their dominance. In this way we could test criteria 1, 3 and 4.

We set out to do this work in E.coli rather than in Salmonella for two reasons.

Virtually all of the chromosome of E.coli can be made diploid using F-primes (Low, 1972). This would simplify the isolation of constitutive mutations (see Section 8.1) and enable us easily to test dominance and perform

complementation analysis.

If cys B did turn out to be the 'true' regulator gene, subsequent analysis e.g. using specialised transducing phage, and in particular isolation and purification of the gene product, would be far easier to achieve in E.coli.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS), mitomycin C, chorismic acid-barium salt (grade II), L-djenkolic acid, cystine, 2-mercaptoethanol, 5-methyl-tryptophan and tris (hydroxymethyl) amino methane ("Tris") were from Sigma.

Acridine orange, sodium dodecyl sulphonate (biochemical grade) sodium selenate, amino acids (L-form) adenine and uracil were from B.D.H.

Reduced nicotinamide adenine dinucleotide (Grade I) was from Boehringer.

1-2-4 Triazole was purchased from both these sources, as was hydroxylamine hydrochloride (A.R.).

Anthranilic acid was from Sigma; it was purified further by repeated crystallisation from ethanol.

All other chemicals were from either B.D.H. or Fisons and of analytical grade where available.

2.1.2 Strains of Bacteria and Bacteriophage

All strains of E.coli used in this work were K-12 derivatives; all strains constructed in this work were derivatives from the reconstructed prototrophic strain 3110 (Bachmann, 1972). They are described in Table 2-i.

All strains of Salmonella typhimurium used in this work were derivatives of the LT-2 strain. They are described in Table 2-ii.

A mutant of P1 that gave clear plaques (i.e. was non lysogenizing) was used throughout this work.

References in the text to "E.coli", "S.typhimurium" and "phage P 1" refer to these particular strains or derivatives thereof.

The specialised transducing phage carrying point mutants in trp were all derivatives of phage Ø 80 pt 190 (Ø 80 hp Trp^+) (Jackson and Yanofsky, 1972). The phage carrying sup F was Ø 80 p sup F (Ø 80 p su_{III}) (Russell, Abelson, Landy, Gefter, Brenner and Smith, 1970). They are listed in Table 2-iii.

2.1.3 Media

Minimal media; four were used.

1. That of Vogel and Bonner (1956) prepared as a 50 x concentrate.

$Mg\ SO_4 \cdot 7\ H_2O$	10 g
Citric acid $\cdot\ H_2O$	100 g
K_2HPO_4	500 g
$NaNH_4PO_4 \cdot 4H_2O$	175 g

dissolved in this order in 600 ml distilled water and made to 1 litre.

Table 2-i Strains of E.coli and E.coli/S.typhimurium Hybrids

No.	Description	Construction
JPT 1	pro cys B-1 trp AE 12 /F' cys B^+ trp^+	Reversion to Trp^+ of MY 576
JPT 6	met cys B trp AE 12 / F' cys B^+ trp^+	JPT 1 X MY 508
JPT 11	Salmonella cys B^Δ sup X^Δ trp AC^Δ chr^Δ leu-500 / F' cys B^+ trp^+	Section 4.2
JPT 14	cys B-14 trp E 9829	Section 3.3
JPT 15	cys B-15 "	"
JPT 16	cys B-16 "	"
JPT 17	cys B-17 "	"
JPT 19	cys B-19 "	"
JPT 20	cys B-20 "	"
JPT 21	cys B-21 "	"
JPT 22	cys B-22 Trp^+	"
JPT 23	cys B-23 trp E 9829	"
JPT 24	trp B 9578 "	Section 4.6.2
JPT 25	cys B-25 "	Section 4.6.3
JPT 26	cys B-26 "	"
JPT 27	cys B-27 "	"
JPT 28	cys B-28 "	"
JPT 29	cys B-29 "	"
JPT 30	Salmonella cys B^Δ sup X^Δ trp AC^Δ chr^Δ leu-500 / F' cys B^Δ sup X^Δ Trp^- Ind^+	Section 7.2

Table 2-i (cont'd)

No.	Description	Construction
JPT 31	cys B-1 trp AE 12 / F' cys B^{Δ} sup X^{Δ} Trp^{-} Ind^{+}	Section 7.2
JPT 32	pyr F trp E 9829	Section 4.6.4
JPT 33-49	cys B 33-49 trp E 9829	Section 4.6.3
JPT 51-53	cys B 51-53 "	"
JPT 54-82	cys B 54-82 "	Section 4.6.5
JPT 83	Salmonella cys B^{Δ} sup X^{Δ} trp AC^{Δ} chr^{Δ} leu 500 / F' 123 pyr F^{+} cys B^{+} trp^{+}	CGSC 4256 X Salmonella 523
JPT 84	pyr F cys B-1 trp^{+}	JPT 32 x Pl ex cys B-1 3110
JPT 86	pyr F 3110	JPT 32 x Pl ex 3110
JPT 87	pro pyr F trp AE 12 str^{R}	MY 509 x Pl ex JPT 86
JPT 89	cys B-54 trp AE^{Δ} ton B	JPT 54; selection for ton B
JPT 91	cys B-54 3110	JPT 54 x Pl ex 3110
JPT 92	cys B-42 3110	JPT 42 x Pl ex 3110
JPT 93	pyr F cys B-54	JPT 32 x Pl ex JPT 91
JPT 94	pyr F cys B-42	JPT x Pl ex JPT 92
JPT 96	pyr F cys B-54 trp AE^{Δ} ton B	JPT 93; selection for ton B
JPT 110	cys B-1 trp AE 12 tna val^{R}	cys B-1 trp AE 12 x Pl ex MY 600
JPT 111	pro C pts 140 bgl B 6 rec A1 str^{R} λ^{-} rel^{-}	Section 8.4.2
JPT 112	pyr F trp AE 12 tna val^{R}	JPT 110 x Pl ex JPT 86
JPT 113	cys B-14 trp AE 12 tna val^{R}	JPT 112 x Pl ex JPT 14

Table 2-i (cont'd)

No.	Description	Construction/Derivation
JPT 115	pyr F trp E 9829 cys A	Section 8.4.3
JPT 116	pyr F trp E 9829 pts^{Δ}	Section 8.4.3
JPT 117	pyr F trp E 9829 cys A / F'198 $cys\ A^{+}$	Section 8.4.3
JPT 118	pyr F trp E 9829 pts^{Δ} / F' 198 pts^{+}	Section 8.4.3
JPT 119	Trz^{R} derivative of JPT 119	Section 8.4.4
JPT 122	pro cys B-39 trp AE 12 str^{R}	JPT 87 x PI ex JPT 39
JPT 124	pyr F trp E 9829 cys K	spontaneous Trz^{R} mutant of JPT 32
JPT 125	cys B-25 trp AE 12 val^{R}	JPT 112 x PI JPT 25
JPT 126	cys B-53 "	" " JPT 53
JPT 127	cys B-56 "	" " JPT 56
JPT 128	cys B-61 "	" " JPT 61
JPT 129	cys B-62 "	" " JPT 62
JPT 130	cys B-81 "	" " JPT 81
JPT 131	pro cys B-39 trp AE 12 str^{R} / F' 123 pyr F^{+} cys B-39 trp^{+}	Section 6.2
trp A 33	trp A 33	from M.D. Yudkin
trp A 905	trp A^{Δ} 905 ton B	"
trp B 9578	trp B 9578	"
trp C 55	trp C 55	"
trp D 1383	trp D 1383	"
trp D 159	trp D 159 (this is known in the literature also as trp D 9778)	"
trp E 5972	trp E 5972	"

Table 2-i (cont'd)

No.	Description	Construction/Derivation
trp E 9829	trp E 9829	from M.D. Yudkin
trp E 9914	trp E 9914	"
trp AE 12	trp AE 12 ton B (this deletion covers the whole trp operon)	"
cys B-1 3110	cys B-1	"
cys B-1 trp AE 12	cys B-1 trp AE 12	"
cys B-1 trp E 9829	cys B-1 trp E 9829	"
MY 303	pyr F cys B-54 trp AE^{Δ} / F' 123 pyr F^{+} sup X^{Δ}-cys B^{Δ}-83 trp^{+}	"
MY 508	met cys B-1 trp AE 12	"
MY 509	pro cys B-1 trp AE 12 str^{R}	"
MY 535	thr $trpR^{-}$ trp^{+}	"
MY 576	pro cys B-1 trp AE 12 / F' cys B^{+} trp B 9578	"
MY 600	met E ina-2 val^{R}	"
1485	Strain W1485 su_{11}^{+} prototrophic	"
thr trp R^{-} trp AE 12	thr trp R^{-} trp AE 12	from W. Brammar
CGSC 4256 (KLF 23/KL 181)	pyr D his-68 trp-45 rec A1 mtl xyl-7 mal gal str^{R}/F' 123 pyr F^{+} cys B^{+} trp^{+}	from the Coli Genetic Stock Centre
CGSC 4344 (F'198/FF7040)	pro C pts-140 bgl B6 rec A1 str^{R} λ^{-} rel^{-} / F' 198 sup N^{+}- nad B^{+}	"

Table 2-i (cont'd)

No.	Description	Construction/Derivation
X 193	pro his trp met pur B pyr F tyr lac str^R	from J.D. Smith
JM 464	Hfr C cys E met B	from Maurice Jones-Mortimer
AT 713	arg A-8 cys C lys A-10 str^R mal A-2 xyl-1 λ^-	from S. Baumberg

Table 2-ii Strains of S.typhimurium

Strain designation	Genotype	Source
LT 2	cys D 234	N.M. Kredich
LT 2	cys J 266	N.M. Kredich
523	cys B^{Δ} sup X^{Δ} trp AC^{Δ} chr $^{\Delta}$ leu-500	M.D. Yudkin

Table 2-iii Derivatives of phage Ø 80 p t 190

The map positions of these mutations are shown in Fig. 7 a

Ø80 pt 190	trp^+
"	trp A 33
"	trp B 9578
"	trp C 782
"	trp D 1383
"	trp D 159
"	trp E 5972
"	trp E 10247
"	trp E 9914
"	trp E 9777

2. Sulphur-free minimal medium (Kredich, 1971)

this is medium I with magnesium sulphate replaced by an equivalent concentration of magnesium chloride.

3. Tris-minimal medium (Hershey, 1955) prepared as a 5 x concentrate

NaCl	27 g
KCl	15 g
NH_4Cl	5.5 g
Tris	60.5 g
$MgCl_2$	0.472 g
KH_2PO_4	0.435 g
Na_2SO_4	0.115 g

dissolved in c 800 ml distilled water. About 35 ml concentrated A.R. HCl was added to bring the pH to 7.4 and the volume was made to 1 litre. This solution required storage over chloroform. After dilution and sterilisation, sterile $CaCl_2$ solution was added to a final concentration of 5×10^{-3}M.

4. M 9 medium (Anderson, 1946)

Solution A	prepared as a 10 x concentrate
Na_2HPO_4 anhydrous	60 g
KH_2PO_4 anhydrous	30 g
NaCl	5 g
NH_4Cl	10 g

dissolved in water and made to 1 litre.

Solution B	prepared as a 100 x concentrate
$MgSO_4 \cdot 7H_2O$	2.5 g
$CaCl_2$ anhydrous	0.11 g

dissolved in water and made to 100 ml.

These minimal media were appropriately diluted with distilled water and 1 ml of 0.01M $FeCl_3$ was added per litre. They were solidified when necessary by the addition of 1.5% w/v agar powder. The agar powders used throughout this work were tested for their inability to support growth of three mutant strains Salmonella cys D-234 and cys J-266 and E.coli cys B-1) in the absence of supplementation with reduced forms of sulphur. They were obtained from Oxoid (Purified agar); Difco; Lab M.

These media were sterilised by autoclaving (121°C for 15 minutes at 15 lb pressure). After this step they were supplemented as necessary with

L-amino acids	at a final concentration of 50 μg/ml
Purines and pyrimidines	20 μg/ml
Indole	10 μg/ml
5-methyl tryptophan	50 μg/ml
Anthranilic acid	2-5 μg/ml
Carbon Source	2 mg/ml

These were made up in aqueous solution at 100 x their final concentration, adding dilute acid or alkali where necessary, to ensure solution, and were sterilised by autoclaving at 115°C for 10 minutes at 10 lb pressure.

The sulphur sources djenkolic acid and cystine were added to media at a final concentration of 0.2 mM, but to liquid media to be used for the growth of cultures for enzyme assay they were added at a final concentration of 1 mM.

Stock solutions of these compounds were made up at a concentration of 100 mM in 1M hydrochloric acid. This acid was then neutralised by the addition of a 1M solution of 'Tris' base. These stock solutions were sterilised by autoclaving at 115°C for 10 minutes at 10 lb pressure.

Undefined media

L. Broth	Difco tryptone	10 g
	Difco Yeast extract	5 g
	NaCl	2.5 g
	glucose	0.5 g
	cysteine hydrochloride	30 mg
	distilled water	1 litre

L. agar was made by the addition of 10 g Difco agar per litre of the above. Both media were further supplemented with $CaCl_2$ at a final concentration of 2.5 mM if they were to be used for the growth of phage P 1.

L. soft agar	Bacto-nutrient broth	8 g
	NaCl	5 g
	Difco agar	6.5 g
	cysteine hydrochloride	30 mg
	distilled water	1 litre

Stab agar	Bacto-nutrient broth	8 g
	Difco agar	8 g
	cysteine hydrochloride	30 mg
	distilled water	1 litre

ACH medium — this consists of minimal medium I plus 5 g Difco casamino acids and 30 mg cysteine hydrochloride per litre with glucose as carbon source.

Nutrient agar

Bacto - nutrient broth	8 g
NaCl	5 g
Difco agar	20 g
cysteine hydrochloride	30 mg
distilled water	1 litre

Tryptone agar

Bacto Tryptone	10 g
NaCl	10 g
cysteine hydrochloride	30 mg
Bacto agar	10 g
distilled water	1 litre

Tryptone soft agar as above, but containing only 0.6% w/v agar.

All media were sterilised by autoclaving at 121°C for 15 minutes, except where otherwise specified.

Buffers

100 mM citrate pH 7

trisodium citrate 29.4 g
distilled water 900 ml
pH brought to 7 with about 10 ml of 0.1M citric acid
volume made to 1 litre

100 mM citrate pH 5.5

trisodium citrate 0.1M 15.4 volumes
citric acid 0.1M 4.7 volumes

100 mM phosphate pH 7.0

Na_2HPO_4	0.1M	61 volumes
$Na\ H_2PO_4$	0.1M	39 volumes

The buffers described above were dispensed in 100 ml volumes and sterilised by autoclaving.

2.2 Genetical Methods

2.2.1 Techniques of Generalised Transduction

Growth of Phage P 1

We used the confluent lysis plate method (Swanstrom and Adams, 1951). 10^6 pfu phage P 1 and 0.1 ml of a late exponential L-broth culture were added to 2.5 ml of molten L-soft agar at 45°C. The tubes were mixed and each was poured onto a fresh pre-warmed L-agar plate. The plate was transferred to the 37°C incubator as soon as the top layer had set and incubation was continued for about 6 hours until the bacterial lawn was fully lysed. The surface of the plate was covered with 4 ml cold L-broth and the plate was stored overnight at 4°C. The supernatant fluid was then harvested, clarified by centrifugation in a bench centrifuge and stored over chloroform. Titres of phage suspensions obtained by this method varied from 5×10^9 to 6×10^{10} pfu/ml.

This method was slightly modified when we grew P1 on strains carrying F-primes or unstable mutations. Cultures of these strains were grown in liquid minimal medium 1 while selection was maintained for the presence of the plasmid or mutation. At a density of about 5×10^8 cells/ml, these cultures were centrifuged, washed once with fresh, warm broth and resuspended in the same

volume of fresh warm broth. Incubation was continued until this culture reached a suitable density for the growth of phage (about 2×10^9 cells/ml).

Titration of Phage P 1

The phage suspension was diluted suitably in sterile saline to give about 10^3 pfu/ml. 0.1 ml of this suspension and 0.1 ml of a late exponential broth culture of Shigella dysenteriae were added to 2.5 ml soft agar. This mixture was layered onto L-agar plates as above; but incubation at 37°C was continued overnight.

Transduction with Phage P 1

2×10^8 pfu of phage P 1 grown on the donor were mixed with 2×10^9 cells of the recipient in broth culture in late exponential phase, and one tenth volume of 0.05M $CaCl_2$ was added. This mixture was incubated at 37°C for 20 minutes, and the cells were deposited by centrifugation. These were then washed two or three times by resuspension in and deposition from 3 ml volumes of sterile 100 mM citrate buffer, and finally resuspended in the original volume of citrate buffer and plated on selective media containing citrate.

Phenotypic expression

This was required to introduce ton B trp deletions into recipient strains, as the ton B mutation is recessive. A transduction with P 1 grown on a strain carrying one of these markers was carried out as above except that the final suspension in citrate was diluted 1 in 200 in broth containing citrate. This culture was incubated overnight and then selection was made for recipients carrying ton B mutations.

2.2.2 Techniques of Specialised Transduction

Growth of phage Ø 80 pt 190 and its derivatives.

This was performed in a similar manner to the growth of phage P1 except that all these strains of phage were grown on strain trp AE 12 and that tryptone and tryptone-soft agar replaced L- and L-soft agar.

Titration of phage Ø 80 pt 190 and its derivatives.

This was performed in a similar manner to the titration of P1 phage except that strain 1485 replaced the Shigella and tryptone media replaced L-media as above.

Transduction with derivatives of phage Ø 80 pt 190 and phage Ø 80 p sup F

5×10^8 pfu phage were spread on the surface of an ACH plate. On to this were streaked saline suspensions of the isolates under test, together with controls. The plates were incubated at 37°C and transductants scored after 40-72 h incubation.

2.2.3 Sexduction

Liquid mating

Cultures of both donor and recipient strains of E.coli were grown overnight in broth without shaking. These cultures were diluted into fresh warm broth; the donor was diluted $^1/_{100}$ and incubated without shaking: the recipient was diluted $^1/_{50}$ and incubated with shaking. After 2 h incubation at 37°C, equal volumes of the cultures were mixed and incubated with slow mixing at 37°C for 1 h. The cells from this mixture were deposited by

centrifugation, washed once in saline and plated. The plates were then incubated at 37°C.

Intergeneric crosses between S.typhimurium and E.coli were performed in the same way except that both donor and recipient cultures were grown in appropriately supplemented minimal medium. In crosses between Salmonella sup X donors and E.coli recipients, the selective plates containing lactose as carbon source were incubated overnight at 37°C and subsequently at 42°C. Thus selection was made for the Lac^+ recipient and at the same time the donor was being counter selected by reason of its Leu^- phenotype at 42°C.

Plate mating

Cultures were prepared as for liquid mating, but the culture of recipient was centrifuged to deposit the cells, which were washed in saline and resuspended in saline at ten times the original concentration. 0.05 - 0.1 ml of this suspension was streaked across the diameter of a plate of selective media and allowed to dry. Loopfuls of donor cultures were streaked at right angles across the recipient streak. These streaks were allowed to dry and the plates incubated as above.

2.2.4 'Curing' of Plasmids

Two methods were used; acridine orange was used when the exogenote was required for further genetic testing. Sodium dodecyl sulphate was used when segregation only was to be demonstrated.

Acridine Orange (Hirota, 1960)

L broth was made to the recipe given above, but the pH was adjusted with NaOH to give pH 7.6 after autoclaving. It was dispensed in 5 ml volumes.

A fresh aqueous solution of acridine orange (5 mg/ml) was made and sterilised by boiling for ten minutes. Volumes of this sterile solution were added to five tubes of the broth to give concentrations of 25, 50, 75, 100 and 125 μg/ml. A stationary phase culture of the strain to be cured was diluted 10^{-6} and 0.15 ml of this dilution was added to each of the tubes. These were then incubated with shaking overnight at 37°C. The tube containing the highest concentration of acridine orange allowing growth was diluted and plated on nutrient agar to give isolated colonies. After growth, these colonies were replicated onto plates of the appropriate selective media to demonstrate loss of the plasmid.

Sodium dodecyl sulphate (Salisbury, Hedges and Datta, 1972)

A 10% (w/v) solution of SDS in L broth was made and the final pH adjusted to 7.6. It was sterilised by autoclaving. 0.5 ml of this solution was added to a tube of L broth pH 7.6 and this was then inoculated with 0.1 ml of a stationary phase culture of the strain to be cured. The culture was then incubated at 37°C with shaking for 72 h. A loopful of this culture was streaked onto a plate of nutrient agar to give isolated colonies. After incubation this plate was replicated onto appropriate selective media to detect loss of the plasmid.

2.2.5 Selection of *ton B trp* deletions

0.1 ml of an overnight culture of the strain in which the deletion is desired was mixed with 0.1 ml of a suspension of phage *Ø80 vir* (2×10^{11} pfu/ml) and 0.1 ml of colicin V and B lysate. This mixture was incubated at 37°C for 20 minutes and then spread over the surface of a well-dried plate of nutrient agar. The colonies developing after overnight incubation of this plate at 37°C were then replica plated to selective media to detect Trp^- clones. The

selective plates were also spread with Ø 80 vir and colicin lysate to maintain selection for ton B ciones.

Preparation of a suspension of phage Ø 80 vir

This was prepared in exactly the same way as suspensions of phage Ø 80 pt 190 except that it was grown on strain 1485.

Preparation and Titration of a lysate of colicins V and B

Strain YS-57 was grown in 100 ml L-broth at 37°C with shaking to a density of about 5 x 10^8 cells/ml. A solution containing 100 µg Mitomycin C was then added and incubation with shaking was continued for another 6 h. The cell debris was harvested by centrifugation and resuspended in 10 ml of 0.01M phosphate buffer pH 7 containing 1M NaCl. 0.2 ml of chloroform was then added and the mixture was shaken vigorously and allowed to stand overnight.

The colicin was titrated by mixing 0.1 ml of dilutions of the lysate with 0.1 ml of a late exponential broth culture of strain 3110, adding 1 ml of warm sterile L broth and allowing it to incubate at 37° for 10 minutes. 0.1 ml of this mixture was spread onto a plate of nutrient agar which was then incubated at 37°C for 24 h. The highest dilution which allows the survival of only a few colonies was recorded; a suspension at ten times this concentration was prepared and used in the procedure given above.

2.2.6 Tests for the Accumulation of Indole and Indole Glycerol Phosphate (InG)

Organisms to be tested were grown overnight at 37°C with shaking in minimal medium containing limiting concentrations of tryptophan (4 µg/ml) or

indole (1 μg/ml) to derepress the tryptophan biosynthetic pathway.

Accumulation of indole was detected by the addition of 0.75 ml indole reagent to 0.5 ml of this culture. A red colour developed immediately if indole was present.

Indole Reagent

Dimethyl benzaldehyde 7.2 g was dissolved in 163 ml 95% ethanol. The mixture was chilled to 4°C before adding 36 ml concentrated hydrochloric acid. A.R.

Accumulation of Indole Glycerol Phosphate was shown by the addition of 0.75 ml InG reagent to 0.5 ml of derepressed culture. A pink colour developed within 3 minutes if InG was present.

Indole Glycerol Phosphate Reagent

aqueous $FeCl_3$ 0.5M	1 ml
distilled water	50 ml
concentrated sulphuric acid	30 ml

2.2.7 Mutagenesis

Mutagenesis with EMS

The strain to be mutagenised was grown to late exponential phase in 50 ml L broth. The cells from this culture were deposited and resuspended in 10 ml fresh broth. 3 mls of this suspension were diluted appropriately and plated on selective and non-selective media. To the remaining suspension at 37°C was added 0.1 ml EMS. This mixture was incubated at 37°C with slow shaking. 3 ml samples were taken after 30 and 60 minutes, washed twice, diluted appropriately and plated on non-selective media to determine survival

and to selective media to isolate mutants. 0.05 ml samples were also taken, and used to inoculate broth cultures. These, after overnight incubation at 37°C, were also plated on selective media to isolate mutants.

Mutagenesis with NTG (after Miller, 1972)

The strain to be mutagenised was grown to exponential phase in 5 ml of L broth and the cells were harvested by centrifugation. These were washed twice in citrate buffer pH 5.5 and resuspended in 5 ml of this buffer. NTG was dissolved in citrate buffer 5.5 at a concentration of 1 mg/ml and an appropriate amount of this solution was added to the suspension of organisms. The final concentration of NTG used was determined for each organism, and was that giving 50% survival after 30 minutes incubation at 37°C; it was usually about 50 μg/ml. After incubation under these conditions the suspension was centrifuged and the cells washed once in phosphate buffer pH 7.0. These were then plated in the same way as cells mutagenised in EMS.

2.3 Biochemical Methods

2.3.1 Growth of Organisms for Enzyme Assay and Preparation of Cell Extracts

Stock cultures of haploid strains were prepared in nutrient broth. Stock cultures of diploid strains or strains carrying unstable mutations were prepared in minimal medium 1 containing cystine, 0.2% glucose as carbon source and appropriate supplements to select for the maintenance of the plasmid or mutation.

These cultures were diluted $^{1}/_{100}$ into 5 ml volumes of minimal medium 2 containing supplements and the appropriate sulphur source at a concentration of

1 mM, but with glucose at a growth-limiting concentration of 0.05%. These cultures were incubated at 37°C with shaking.

These in turn were diluted appropriately into 350 ml volumes of the same medium but containing 0.2% glucose, contained in 2 l conical flasks. These cultures were incubated overnight at 37°C in a New Brunswick orbital shaker set at 300 r p m.

The cultures were harvested at a cell density of not more than 5×10^8/ml ($E_{450} \simeq 0.5$). Before harvesiing a sample was taken using aseptic precautions, and plated to detect reversion or contamination in the case of haploid strains, or in the case of diploid strains diluted and plated on non selective medium. This was then replicated to selective media to confirm the presence of the plasmid in all cells in the population.

The cultures were harvested by centrifugation at 6000 g for 20 minutes (6000 rev/min, Sorvall RC-2B centrifuge; GS-3 head). The cells were resuspended in 40 ml of the relevant buffer (0.1M Tris -HCl pH 7.8 for the tryptophan enzymes, 0.1M potassium phosphate pH 7.7 for sulphite reductase) and deposited by centrifugation at 31,000 g for 3 minutes (16000 rev/min, Sorvall RC-2B centrifuge; SS-34 head). This washing was repeated twice more before sonication. For most of the enzyme assays reported in this thesis, the cell pellet was stored frozen overnight at -20°C between the second and third washes (this was shown to have no effect on the subsequent enzyme levels). Finally the pellet of washed cells was resuspended in ten times its own volume of the relevant buffer, (the 'Tris' buffer used for extracts to be assayed for the tryptophan enzymes contained 1 mM 2-mercaptoethanol at this stage), and disrupted by sonication at 0°C (3 x 30 second intervals, MSE MK II sonicator

at maximum output 7μ peak to peak). The sonicated extract was centrifuged at 31,000 g for 30 minutes (16,000 rev/min, Sorvall RC-2, SS-34 head) and then assayed within two hours.

2.3.2 Assay of Anthranilate Synthetase and trp D Gene Product

The anthranilate synthetase reaction in E.coli is catalysed by an enzyme complex consisting of two polypeptide chains specified by the trp E and trp D genes. The trp E gene product "Component I" can catalyse the synthesis of anthranilate from chorismate using ammonia as amino donor, but it requires the presence of the trp D gene product "Component II" to catalyse the same reaction using glutamine as amino donor (Ito, Cox and Yanofsky, 1969). This property can be used as the basis for a sensitive assay of the trp D gene product (Pouwels and van Rotterdam, 1975; Brammar, personal communication).

Anthranilate synthetase was measured according to Ito, Cox and Yanofsky (1969).

The reaction mixture contained

0.2	mM	chorismic acid
4	mM	magnesium acetate
20	mM	L-glutamine
1	mM	2-mercaptoethanol
10	mM	Tris-HCl buffer pH 7.8

and enzyme in a final volume of 3 ml.

Enzyme was added to start the reaction and the formation of anthranilate was followed spectrofluorimetrically (Perkin Elmer MPF-2A instrument and Hitachi QPD 33 recorder; excitation wavelength 322 nm, emission wavelength 397 nm).

One unit of activity is defined as that amount of enzyme which produces 0.1 μmole of anthranilate in 20 min at 37°C. Duplicate determinations agreed

to within 5%.

The trp D gene product was measured by a method similar to that used by Pouwels and van Rotterdam (1975). To 100 µl of extract containing the D enzyme to be measured was added 10 µl of a crude extract of strain trp D 159 containing 0.03 units of anthranilate synthetase component I. This mixture was left at room temperature for 15 minutes to allow the formation of an enzymically active anthranilate synthetase complex and then assayed under the conditions described above. Duplicate determinations agreed to within 7%.

We found that anthranilate synthetase and its individual subunits were not stable to storage at -20°C for long periods, in cell pellets or crude extract, though they were stable to similar storage overnight. Therefore we prepared fresh crude extract containing anthranilate synthetase component I for each batch of trp D enzyme assays.

The preparation of this extract was performed as follows. Strain trp D 159 was grown overnight under derepressing conditions at 37°C, in 350 ml volumes of minimal medium 1 containing 0.05% w/v ACH and 1 µg/ml indole. Incubation of the cultures and extraction of the enzyme were as above. The amount of anthranilate synthetase component I was estimated by an assay similar to that for component II but in the presence of excess component II. (This was prepared as a crude extract of strain trp E 5972 in exactly the same way as that of trp D 159 but was stored for up to two weeks at -20°C.)

2.3.3 Assay of Tryptophan Synthetase B Subunit

This was measured by the method of Smith and Yanofsky (1962)

The reaction mix contained

0.03	mM	pyridoxal phosphate
0.4	mM	indole
80	mM	DL-serine
0.3	ml	of a saturated aqueous solution of NaCl
100	mM	Tris-HCl buffer pH 7.8

and enzyme in 1 ml volumes

After incubation at 37°C for an appropriate time (20 minutes to 20 hours) to give about 50% conversion of indole to tryptophan, the reaction was terminated by the addition of 0.1 ml M.NaOH. The unreacted indole was extracted into 4 ml of toluene and measured in 1 ml of this extract by the addition to it of 3 ml 95% ethanol and 1 ml indole reagent (Section 2.2.6). After mixing and waiting 20 minutes the colour developing was measured spectrophotometrically at 568 nm (Unicam SP 500 instrument).

One enzyme unit is defined as that amount which catalyses the disappearance of 0.1 μmole of indole in 20 minutes at 37°C.

When a short incubation time was used (i.e., less than an hour) duplicate assays differed by no more than 5%.

When a longer incubation time was required, enzyme extracts were assayed in triplicate. These assays differed by no more than 15%. The mean of the three results was taken.

2.3.4 Assay of Sulphite Reductase

This was performed by the method of Siegel and Kamin (1971) with hydroxylamine rather than sulphite as substrate.

The reaction mixture contained

1 mM potassium phosphate buffer pH 7.7
0.2 mM $NADPH_2$
10 mM hydroxylamine
and enzyme
in volume of 3 ml, and was incubated at 30°C.

The rate of $NADPH_2$ oxidation in the absence of substrate was found (this was negligible with grade I $NADPH_2$), and then hydroxylamine was added to start the reaction. The rate of oxidation of $NADPH_2$ was followed spectrophotometrically at 340 nm (Unicam instrument and Servoscribe Is recorder) and the enzyme units were calculated from the linear portion of the reaction.

The unit of activity is defined as that amount of enzyme which catalyses the oxidation of 1 micromole of $NADPH_2$ per minute with hydroxylamine as electron acceptor. These units are equivalent to 5.5 such units determined with sulphite as electron acceptor. Duplicate assays agreed to within 5%.

2.3.5 Estimation of Protein

This was performed by a micro modification of the method of Lowry, Rosebrough, Farr and Randall (1951); bovine serum albumin was used as standard. Duplicate assays agreed to within 5%.

Chapter 3

Directed Mutagenesis in E.coli

3.1 Introduction

Mutations to cysteine auxotrophy may occur in any one of five widely separated areas of the chromosome of E.coli (Smith, 1971). We required only one class of these, those in the cys B area, which maps between the pyr F and trp genes at 27 minutes on the standard genetic map (Taylor and Trotter, 1972). Using conventional techniques of mutagenesis and selection, Demerec's group isolated about 425 cysteine auxotrophs of Salmonella typhimurium, of which only 30 were mutant in cys B (Mizobuchi, Demerec and Gillespie, 1962). In E.coli, of 90 cysteine auxotrophs isolated by similar methods, only 19 were cys B mutants (Jones-Mortimer, 1968a and personal communication). A more specific method of mutagenesis was therefore chosen: the mutagenised phage technique, originally devised for use with phage P22 (Hong and Ames, 1971), modified for use with phage P1 (Murgola and Yanofsky, 1974).

The principle of the technique is that a preparation of a generalised transducing phage is mutagenised in vitro and used to transduce a recipient bacterium which is mutant in a gene near that in which mutations are required. Selection is made for the transduction of this neighbouring gene, and the resulting progeny, which will have incorporated nucleic acid from the mutagenised transducing particles into their genomes, are screened for mutation of the required gene.

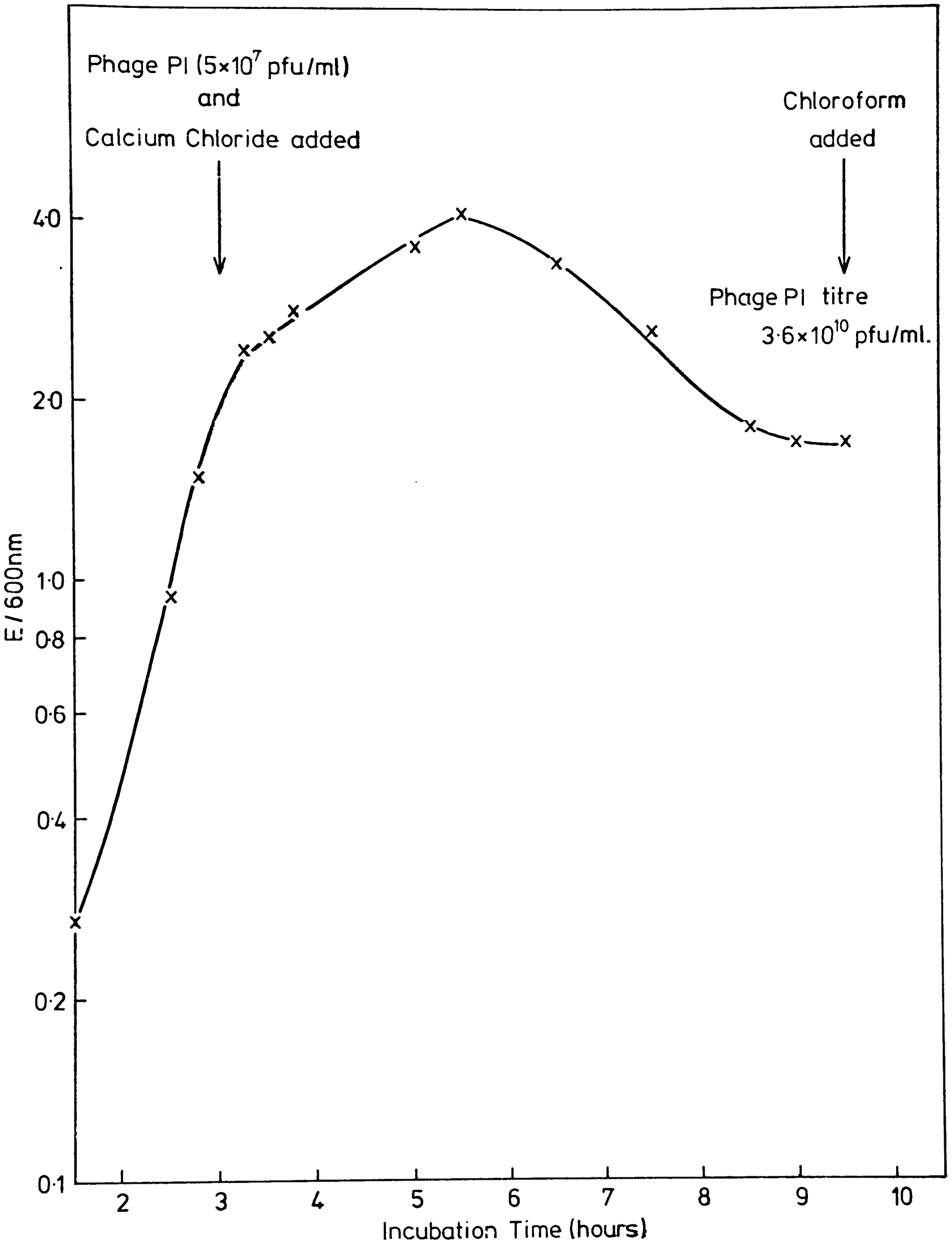
3.2 Bulk Growth of Phage P1

We wanted the cys B mutants that were to be isolated by directed mutagenesis also to carry a well defined neighbouring marker - preferably a suppressible mutation (see Ch. 4). Phage P1 for mutagenesis was therefore grown on the amber mutant trp E 9829 (Yanofsky, Horn, Bonner and Stasiowski, 1971).

Sterile L-broth (50 ml) in a 500 ml flask was inoculated with this strain, and incubated overnight at 37°C with shaking. The following morning, 2 ml of this culture was added to each of twelve 2 l flasks containing 250 ml of warm sterile L broth. The flasks were then incubated in a New Brunswick rotary incubator at 37°C. When the bacterial density had reached 5×10^8/ml (E 600 nm = 2.0), 2.5 ml of a sterile 0.5M solution of calcium chloride was added, together with sufficient P1 phage grown on trp E 9829 to give a multiplicity of infection of 0.1. Incubation was continued and the optical density of the culture was measured at intervals to follow lysis of the bacteria. A typical growth curve is given in Fig. 3 a. When lysis was complete, judged by the optical density becoming constant, 2.5 ml of chloroform was added to each flask. After incubation for a further 30 minutes the phage was harvested as follows.

Cell débris was removed by two centrifugations at 1500 g for 15 minutes at 4°C (GS-3 rotor, 3000 rev/min Sorval RC 2-B centrifuge). The supernatant liquid from the second centrifugation was then centrifuged at 35000 g for 120 minutes at 4°C (21 rotor 19000 rev/min Beckman L centrifuge). The phage was deposited as a translucent pellet; it was resuspended by adding 1-2 ml of fresh L broth and a glass or Teflon covered magnetic follower to each tube, placing on a magnetic stirrer set to the lowest possible speed, and leaving

Fig. 3a The Growth of Phage P1 on Strain trp E 9829


Phage P1 (5×10^7 pfu/ml)
and
Calcium Chloride added
Chloroform
added
Phage P1 titre
$3{\cdot}6\times10^{10}$ pfu/ml.
E/600nm
4·0
2·0
1·0
0·8
0·6
0·4
0·2
0·1
2
3
4
5
6
7
8
9
10
Incubation Time (hours)

overnight at 0-4°C. The small volumes were combined, clarified by centrifugation at 2000 g for five minutes at room temperature in a bench centrifuge and stored over chloroform.

A typical phage recovery is shown in Table 3.i.

3.3 Mutagenesis of Phage P1

We carried out an experiment to find those conditions of mutagenesis which gave a good yield of adequately mutagenised phage particles.

The composition of the solution required for hydroxylamine mutagenesis of P1 phage was known (Scott, 1968; Murgola, personal communication to M.D. Yudkin). The optimum period of mutagenesis was determined as follows.

Hydroxylamine hydrochloride 1.55 g was dissolved in 40 ml of cold L broth. To this mixture was added 1 ml of 0.5M $CaCl_2$ and 1 ml of 0.1M sodium EDTA. The pH of this solution was adjusted to 6 by the careful addition of 10N NaOH and the final volume made to 50 ml with more cold L broth (final concentrations: hydroxylamine 450 mM, $CaCl_2$ 10 mM, EDTA 2 mM). The solution was sterilized by passage through a Millipore filter grade H A. Calcium ions are required to stabilize phage P I (Scott, 1968); EDTA inhibits phage inactivation by a non mutagenic reaction with hydroxylamine or its oxidation products (Tessman, 1968).

The solution was warmed to 37°C, and 5 ml of a suspension of P 1 phage containing about 5×10^{11} pfu/ml were added. The mixture was incubated at 37°C for up to 24 hours with occasional mixing. A sample was taken immediately for the determination of pfu, and then further samples were taken at 8, 12, 18 and 24 hours. Each of the last four samples was added to 25% of

Table 3 - i Purification of Phage P 1

Step	Volume (ml)	Titre (pfu/ml)	Total (pfu)	Yield (%)
culture	3,000	9.8×10^{10}	2.9×10^{14}	100
supernatant of 1st slow centrifugation	2,265	9.7×10^{10}	2.2×10^{14}	74
supernatant of 2nd slow centrifugation	2,040	9.8×10^{10}	2.0×10^{14}	68
after deposition by high speed centrifugation and resuspension	68	1.5×10^{12}	1.0×10^{14}	35

its own volume of frozen L broth containing 10 mM $CaCl_2$ and 2 mM EDTA and centrifuged immediately at either 60,000 g for 45 minutes (8 and 12 h samples) or at 65,000 g for 1 h (18 and 24 h samples) at 4°C. The supernatant fluid was discarded and the pelleted phage was resuspended in 1 ml of fresh L broth containing $CaCl_2$ and EDTA and stored over chloroform at 4°C.

The number of surviving plaque-forming and transducing particles was determined for these samples, and the transducing frequency was calculated. The results are given in Fig. 3 b.

The proportion of transducing particles carrying mutations in the cys B gene was found in the following manner. E.coli trp B 9578 was transduced with phage from each of the four later samples, and the transduction mixture was spread on plates of minimal medium containing ACH and indole to give about 300 transductants per plate. These plates were incubated overnight at 30°C to give small colonies (1-2 mM diameter). They were then replica plated to indole-minimal medium with and without cystine. These plates were incubated overnight at 41°C and examined to detect colonies able to grow at this temperature only in the presence of cystine. The results are given in Table 3.ii.

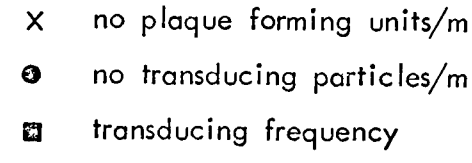
The number of plaque forming units decreased exponentially with time during mutagenesis. The kinetics of this process are similar to those observed by Hong and Ames (1971) and Murgola and Yanofsky (1974). The number of transducing particles also decreased exponentially at the same rate; the transducing frequency is thus constant.

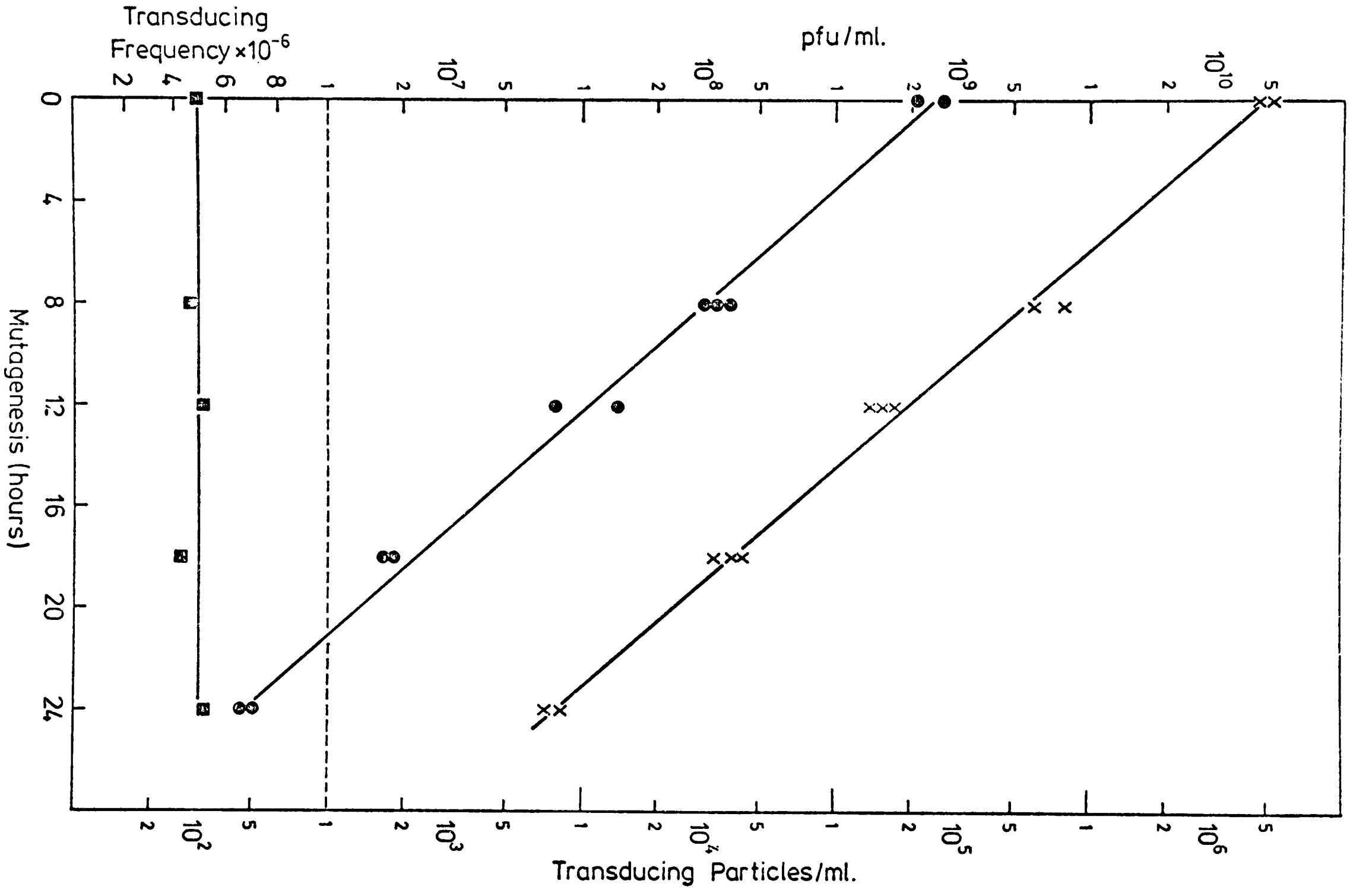
The incidence of cysteine auxotrophs remained more or less constant and independent of the length of mutagenesis of the phage from which they were derived (although the number of mutants recovered is very small and there might

Table 3 - ii Effect of Mutagenesis of Phage P1 upon the Incidence of Mutations in Transductants

Length of Mutagenesis of Phage (hr)	No colonies replicated	No. of Cys^- mutants identified	Incidence of Mutation
8	9.8×10^3	3	3.25×10^{-4}
12	1.23×10^4	1	8.1×10^{-5}
18	6.47×10^3	2	3.1×10^{-4}
24	6.47×10^3	4	6.2×10^{-4}

Fig. 3 b The Survival of Phage P1 During Mutagenesis with Hydroxylamine




Transducing Frequency ×10⁻⁶
2
4
6
8
1
pfu/ml.
10⁷
10⁸
10⁹
10¹⁰
Mutagenesis (hours)
0
4
8
12
16
20
24
10²
10³
10⁴
10⁵
10⁶
Transducing Particles/ml.

be considerable statistical fluctuation). This raised the possibility that transduction itself was the mutagenic event rather than mutagenesis of the transducing phage. Hong and Ames (1971) observed that phage P22 mutagenised to survivals of 5% and 0.03% gave a similar proportion of transductants that carried mutations. They did not examine whether transduction with unmutagenised phage was mutagenic.

However, this is a real possibility; any process involving recombination can be mutagenic (Witkin, 1969). Transduction in Salmonella often increases the frequency of reversion of certain auxotrophic markers ("selfing"), even when the transducing particles contain chromosomal material carrying a deletion of the relevant region (Demerec, 1963). Transformation can also be mutagenic; some of the mutations are closely linked to the transformed marker (Yoshikawa, 1966).

We excluded this possibility by transducing a culture of trp 3 9578 to trp B^+ exactly as described above but with non mutagenised phage grown on trp E 9829. Of 96,460 transductants so obtained and examined for cysteine auxotrophy, not one was found to require cysteine.

It follows that mutagenesis of the phage before transduction is required to cause linked mutations in the progeny.

Another possible explanation for the constant incidence of mutations is that hydroxylamine not only causes transitions but also produces breaks in the transducing DNA (or damages it in such a way that it is cleaved by the recipient), with the result that the amount of mutagenesis per unit length of transducing DNA increases, but the length of transducing fragment becomes shorter. If this were so, we would expect that with increasing mutagenesis of phage, the co transduction frequency of linked markers might decrease.

This prediction was easily tested. We took some of the master plates of

ACH-indole medium on which were transductants derived from the mutagenized and non-mutagenized phage and replicated them to identical medium and to ACH plates without indole; on the latter plates only tryptophan prototrophs could grow i.e. those transductants that had received the trp B^+ allele from the phage, but had not had their trp E^+ allele displaced by the incoming phage.

These results are given in Table 3.iii. They show clearly that with increasing mutagenesis of the phage, the linkage between trp B and trp E does decrease.

We did not pursue this line of investigation further, but decided that since mutagenesis of phage for eight hours gave about the same incidence of mutations per viable particle as at twenty four hours but with much greater survival of phage, we would in future mutagenise the phage for this length of time.

The cysteine-requiring mutants isolated during this work were numbered JPT 14 to 23 inclusive. JPT 18 reverted at high frequency, and was not used in subsequent work. JPT 22 was a tryptophan prototroph, and was used only in work described in Chapter 5.

Table 3 - iii Effect of Mutagenesis of Phage P1 upon Recombination Frequency between trp B and trp E

Length of Mutagenesis of Phage (hr)	No. cols ACH indole	No. cols ACH	% Prototrophs
0	606	145	24
8	655	200	32.8
12	615	224	36.4
18	332	160	47
24	263	150	57

Chapter 4

Isolation of Deletions and Point Mutations in the cys B Region of E.coli; Construction of a Fine Structure Map

4.1 Introduction

With ten presumptive cys B mutants (see Chapter 3), we looked for a method with which to map them. We decided to use deletion mapping: fine structure mapping with three factor crosses can give anomalous results (Beckwith, 1970) and had indeed done so in previous work on this region in S.typhimurium (Clowes, 1968b; Mizobuchi et al., 1962).

We decided to isolate the deletions on a plasmid, to facilitate mapping and possibly increase the incidence of deletions. Initially we used the F'trp plasmid (Frédéricq, 1969) which carries the cys B region, the tryptophan cluster and genes mediating resistance to phage T1 and directing production of colicins V and B.

Deletions of the cys B and tryptophan genes had been previously isolated in S.typhimurium and had been used extensively to map the trp operon (e.g. Margolin and Bauerle, 1966). We wondered if a similar approach could be used to isolate similar deletions in a plasmid from E.coli. There was much information to encourage us in this attempt.

There is considerable homology between E.coli and S.typhimurium (Sanderson, 1971) and this homology had been studied extensively in the pyr F cys B trp regions of the two organisms (Ino and Demerec, 1968; Mojica-a and Middleton, 1972). Hybrids in which E.coli cys and trp genes had displaced the homologous genes in Salmonella were Cys^+ Trp^+ (Ino and Demerec, 1968).

Conditions had been found in which intergeneric matings could occur at high frequency (Middleton and Mojica-a, 1971).

We decided to try to insert the E.coli F'trp plasmid into a Salmonella in which the cys B - trp region had been deleted and to try then to delete these genes in turn from the plasmid. In order to describe the practical details of this approach, we must first consider the phenotypic effects of deletions in the cys B - trp region of S.typhimurium.

In the leucine operon of S.typhimurium there is a mutation (possibly in the promoter) named leu - 500 (Mukai and Margolin, 1963). Strains carrying this mutation are leucine auxotrophs, but they may revert to Leu^+ in two different ways. The first is by reversion of the original mutation. The second is by suppression; the mechanism by which the original mutation is suppressed is not clear, but it involves the inactivation of a gene sup X which maps between cys B and trp. These two classes of revertants may easily be distinguished; on agar plates lacking leucine the former grow to give large colonies (> 5 mm diameter) after 48 hours incubation at 37°C, at which time the latter have grown to give only very small colonies (0.5 mm). Furthermore the sup X suppression of leucine auxotrophy does not occur at 42°C (Dubnau, Lenny and Margolin, 1973). Mukai and Margolin (1963) found that some spontaneous sup X revertants of leu - 500 were either Cys^- or Trp^- or in a few cases both Cys^- and Trp^-; the sup X gene was later located between cys B and the trp cluster.

We wished to see if the known homology between E.coli and Salmonella was so close that E.coli would possess a gene homologous to the sup X gene of S.typhimurium. This could easily be found by transferring the E.coli F'trp to a Salmonella carrying a sup X deletion extending into both cys B and trp, selecting

for both cysteine and tryptophan prototrophy and seeing whether the merodiploid thus formed was a leucine auxotroph or not. If it turned out to be Leu$^-$, one would infer that there is a region on the F'trp that complements the chromosomal sup X deletion of the Salmonella.

4.2 Demonstration of the sup X Gene in E.coli

Strain JPT 6 and Salmonella 523 were grown and mated in appropriately supplemented minimal medium (see Materials and Methods) and the resulting mixture was washed and plated on minimal agar supplemented with leucine. After 48 hours of incubation at 37°C many colonies appeared on these plates, but not on similar ones inoculated with donor or recipient alone.

10 such colonies were taken and purified. Their phenotypes were as follows: they were Cys$^+$ Trp$^+$, but segregated Cys$^-$ Trp$^-$ clones at high frequency after growth in media containing acridine orange. They were Lac$^-$, hence were not Met$^+$ revertants of strain JPT 6. This conclusion was reinforced by the fact that they were Leu$^-$, but that the Cys$^-$ Trp$^-$ segregants were Leu$^+$.

These observations show that the progeny of this cross were merodiploids of Salmonella 523 carrying the F'trp plasmid and that this plasmid carries a gene homologous to the sup X gene of Salmonella. One of these progeny was purified and numbered JPT 11.

Later we found that Somerville (1966) had already made such a diploid in the course of his work on tryptophan biosynthesis.

4.3 Isolation of sup X Deletions of the cys and trp Genes

Twenty four independent clones of JPT 11 were grown overnight at 37°C in liquid minimal medium containing cystine, tryptophan and leucine with glucose as carbon source. Cells from these cultures were washed and plated on minimal agar containing cystine and tryptophan, which were then incubated for two days at 37°C.

All plates yielded a mixture of two colonial types; large colonies of about 5 mm diameter and small colonies of about 1 mm diameter. They were replica plated to four further plates:

to the same medium
to minimal agar containing cystine
to minimal agar containing tryptophan
to minimal agar

These were in turn incubated overnight at 37°C.

By comparison of these plates it was possible to observe that all the large colonies were Cys^+ and Trp^+ as well as Leu^+, but that the majority of the small colonies from each plate failed to replicate to at least one of the further replicas.

We purified one colony that required only cysteine from plates of each of the twenty four cultures and one colony that required only tryptophan from one plate. These isolates were plate mated with strain trp A 33 and cys B-1 on M9 lactose minimal agar: this showed that all the Cys^- Trp^+ derivatives of JPT 11 were able to transfer Trp^+ but not Cys^+ under these conditions. Similarly the Cys^+ Trp^- derivative could transfer Cys^+ but not Trp^+.

All isolates segregated Cys^- Trp^- colonies at high frequency after growth in media containing acridine orange. All isolates and segregants were able to

grow on minimal medium appropriately supplemented with cystine or tryptophan or both at 37°C, but did not do so at 42°C unless the medium was further supplemented with leucine. None of the Cys^- isolates ever reverted to Cys^+.

This demonstrated that the isolates were sup X derivatives of JPT 11 in which either the cys or trp genes had been inactivated by deletion. The Cys^- sup X derivatives of strain JPT 11 were numbered Δ1 - Δ23 inclusive.

4.4 Location of the End-points of the Deletions in sup X Cys^- Derivatives of JPT 11

The next step was to find out if the deletions in the Cys^- sup X derivatives extended through the entire cys B gene or only through part of it. In the latter case we would expect to observe recombination between some of the deleted plasmids and some point mutations in cys B.

The full characterisation of the cysteine-requiring mutants isolated in Section 3.3 will be given in Chapters 5 and 6. At this stage of the work, it was inferred that they were indeed cys B mutants from their method of isolation and from the fact that all of them (except for JPT18 and 22 which were not tested) were complemented by the cys B^+ gene carried on the F'trp plasmid.

This was shown as follows; strain JPT 11 (carrying F'cys^+ trp^+) was grown and mated with each of the 8 point mutants, each of which is also Trp^-, in appropriately supplemented minimal medium. Dilutions of this mixture were spread onto plates of M9 minimal medium containing cystine with lactose as carbon source. After 48 hours incubation (see Materials and Methods) plates containing about 100 Trp^+ colonies were replicated to plates of the same medium with and without cystine. After incubation overnight at 37°C (41°C in the case

of JPT 21), it was evident that all Trp^+ colonies were Cys^+ as well. That this was not always a result of recombination was shown by purifying representative colonies and attempting to 'cure' them of the plasmid (see Materials and Methods). This was possible in at least one representative colony from each of the original Cys^- Trp^- mutants: all colonies that did become Trp^- after curing became Cys^- simultaneously (and vice versa). These results show that each Cys^- mutant can be complemented by a plasmid carrying an intact cys B^+ gene.

The question then to be considered was; Could some of the deleted plasmids give recombinants with some of these point mutants?

This was answered in the following way. We plate mated each of the deleted merodiploids with each of seven Cys^- point mutants and with strain cys B-1 trp E 9829 on M9 minimal agar containing cystine with lactose as carbon source. We purified one Trp^+ diploid from each mating, grew it to saturation in similar liquid medium, centrifuged and washed the cells and plated them on the same agar medium without cystine. The results are given in Table 4-i.

They show that deletions 3 and 4 do recombine with some of the point mutations. They divide the Cys^- mutants into three classes: those that recombine with both deletions, those that recombine with deletion 3 only and those that recombine with neither.

We assume that the sup X gene maps between the cys B region and the trp cluster on the F'trp just as it does in Salmonella typhimurium (Mukai and Margolin, 1963); that this is indeed so is indicated by our isolation of both sup X Cys^- Trp^+ and sup X Cys^+ Trp^- deletions (Section 4.3). Further, we assume that only one event has occurred to delete simultaneously part of the cys B region and the sup X gene. Given these assumptions, we may conclude

Table 4 - i Recombination between Putative Deletion and Point Mutations in cys B

Donor		Recipients: none	cys B-1 trp E 9829	JPT 14	15	16	17	19	20	23
JPT 11	Δ1	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0
	3	0	179	0	c10^3	c10^3	c10^3	542	c10^3	700
	4	0	0	0	100	205	205	1	90	0
	5 12 19	0	0	0	0	0	0	0	0	0
	6 13 20									
	7 14 21									
	8 15 22									
	9 16 23									
	10 17									
	11 18									
none (0.1 ml of an overnight culture of each recipient plated on M9 lac trp)			0	0	0	0	0	0	0	0

that the cys marker in JPT 14, which recombines with neither of these deletions, lies towards the right hand end of the cys B region which has been deleted in both; that cys B-1, and the mutations present in JPT 17, 19, 23 lie in that region deleted in deletion 4 but not in 3; and that the mutations present in JPT 15, 16, 20 lie towards the left hand end (i.e. sup X-distal) of the cys B region which has been left intact in both deletions. A map of this interpretation is given in Fig. 4 a. Repetition of this experiment gave quantitatively similar results. From this point the Cys$^-$ markers present in JPT 14-23 will be termed cys B-14 to cys B-23.

These self-consistent results encouraged us to look for more deleted plasmids and cys B point mutants to both extend and refine our map.

4.5 Isolation of More Deletions of the cys B Gene

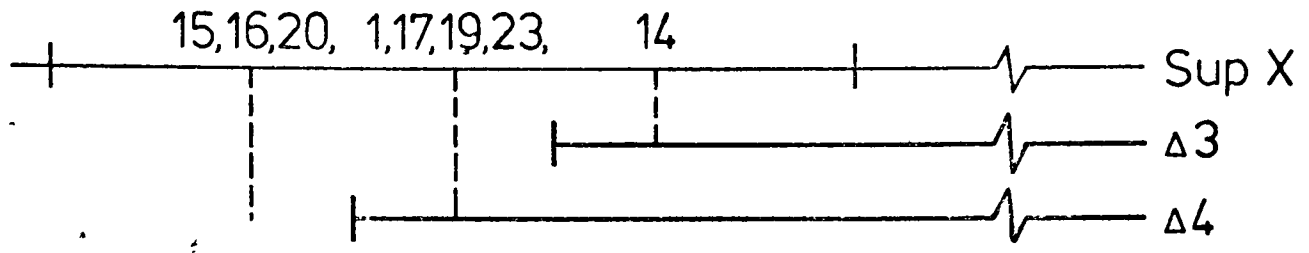
A large fraction of sup X deletions eliminate the whole of cys B and are thus of no use for mapping. We wished to retain only those deletions which left intact a part of the cys B region.

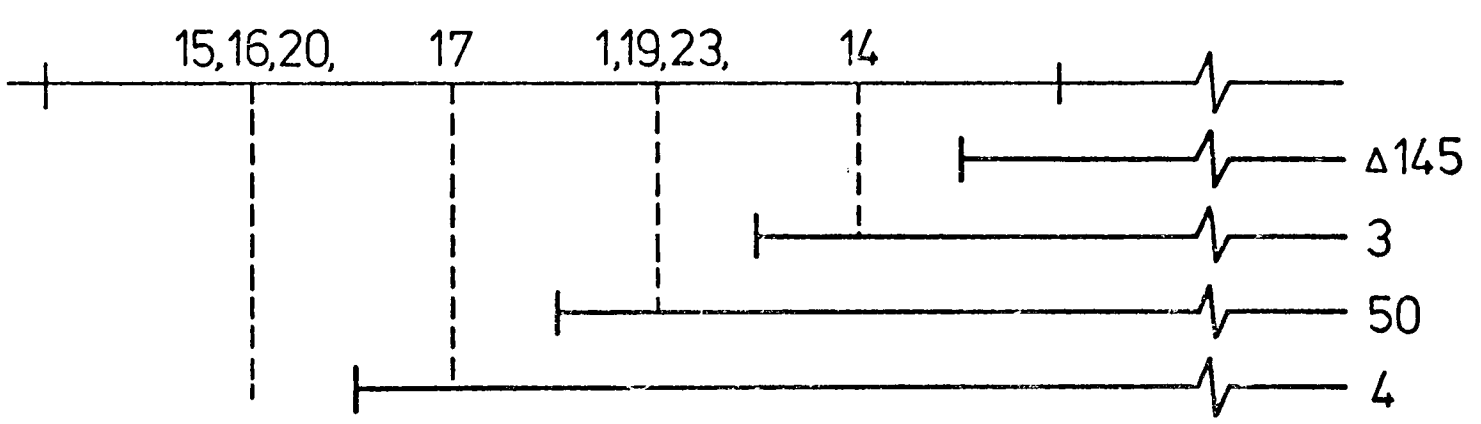
Our strategy was to isolate a number of sup X Cys$^-$ derivatives of JPT 11; mate them with the presumptively most left-hand cys B point mutation; find that derivative which did recombine with it, but at the lowest frequency (hence that which carried the presumptively longest deletion ending within cys B), and use it in turn to screen the next batch of point mutants in cys B. That point mutant which recombined best with it would be presumptively even more to the left.

This method was a sort of successive approximation to finding the most sup X distal mutation in the gene; its success depended on our ability to

Fig. 4 a. First Stage Map of the cys B Region

Fig. 4 b. Second Stage Map of the cys B Region


15,16,20,
1,17,19,23,
14
Sup X
Δ3
Δ4


15,16,20,
17
1,19,23,
14
Δ145
3
50
4

'saturate' the gene with point mutations and to isolate a sufficient number of sup X Cys$^-$ derivatives of JPT11, and also on the assumption that the point mutation that gave the highest frequency of recombination with a given deleted plasmid was the most distant from the end of the deletion. This last assumption we knew not to hold true in all cases; as events proved, our scepticism was justified.

On the basis of the highest recombination frequencies with deletions 3 and 4, cys B-16 appeared to be the most left hand of our cys B mutations. We isolated 140 sup X - Cys$^-$ derivatives of JPT11 in the same manner as in Section 4.3 but with a minor technical modification. We knew that the majority of sup X colonies selected from JPT11 on cystine-minimal agar were Cys$^-$; rather than replicate the entire plate, we picked five sup X colonies from each selection plate using sterile toothpicks and gridded them to another plate of the same medium. Up to fifty colonies were gridded per secondary plate, which after incubation overnight at 37°C was replicated to plates of minimal medium with and without cystine to identify Cys$^-$ clones. Only one such isolate per original plate was purified and used in subsequent work.

These 140 new derivatives of JPT11 were mated with JPT 16, and the progeny were examined for Cys$^+$ recombinants exactly as in Section 4.4. Only 2 isolates gave recombinants, deletions 50 and 145. These were in turn mated with the other seven cys B point mutants.

The results are given in Table 4-ii and the resultant map in Fig. 4 b. They show that neither of these new deletions separated the most left hand group of point mutations. This result could be explained in several ways.

Table 4 - ii Recombination between Putative Deletion and Point Mutations in cys B

		Recipients							
Donor		JPT20	16	15	17	23	19	cys B-1	JPT 14
JPT 11	Δ145	+	+	+	+	+	+	+	20 cols
	3	+	+	+	+	+	+	+	-
	50	+	+	+	+	-	-	-	-
	4	+	+	+	-	-	-	-	-

1. cys B 15, 16 and 20 could be so close together that the probability of finding a deletion ending between them is very low.

2. Deletions might not end at random within the gene (Pfeifer, Hirsh, Bergman and Hamaoui, 1974) so that these three point mutations might lie in a region in which deletions would end infrequently.

3. cys B-16 might lie to the right of the other two mutations and yet still recombine at a higher frequency with deletion 4.

If this last explanation were correct, it would follow that there might be some deletions which would recombine with cys B-15 and 20 but not with cys B-16. However, rather than repeat the screening of this batch of deleted plasmids with the other two point mutations, we turned to isolating more point mutants.

4.6 The Isolation of More Point Mutations in the cys B Region

4.6.1 The Necessity for Additional Recipients for Transduction with Mutagenized Phage

One of the point mutants we had isolated in Section 3.3 was a tryptophan prototroph; it therefore could not be mapped by the method described above. It arose by crossing over occurring in the interval between the trp B and trp E genes. This occurrence might require us to discard about a third of the mutants we would isolate (Table 3-iii).

This problem could be avoided by the construction of a recipient for transduction with mutagenised phage, which would carry the trp E 9829 marker. Donor and recipient would then both carry this marker and consequently so would the progeny. Construction of a trp B trp E double mutant (JPT 24) is detailed below.

We also constructed another recipient for transduction with mutagenized phage, which eliminated the possibility of isolating Trp$^+$ transductants and also overcame another possible problem. It was conceivable that selection of cys B mutants from transductants of a trp strain might enrich for mutations in the trp-proximal part of the region. We decided therefore to look for cys B mutants among the progeny of a pyr F strain transduced to pyr F$^+$ with mutagenised phage. pyr F lies on the trp-distal side of cys B and is cotransduced with cys B about 60% (Signer, Brenner and Beckwith, 1965). Construction of a pyr F trp E 9829 strain (JPT 32) is given below.

4.6.2 Construction of a Strain Carrying a Mutation in the trp B Gene and trp E 9829 (JPT 24)

A strain carrying both trp B 9578 and trp E 9829 was constructed in the following manner. Phage grown on cys B$^+$ trp B 9578 was used to transduce cys B-1 trp E 9829 to cys B$^+$; selection was made on minimal agar plates containing tryptophan . The colonies growing on these plates were replica plated to the same medium and to minimal agar containing indole. Colonies that did not grow on the latter medium, and which were therefore trp B, were purified and tested for the presence of the trp E marker by two different methods.

First, each isolate was tested for recombination with phage Ø 80 pt trp E 9777. This marker is very close to trp E 9829: recombination between them is rare. Secondly, each isolate was tested for the ability to accumulate Indole Glycerol Phosphate (InG). trp B single mutants do so, but double mutants, the second mutation in which occurs earlier on in the pathway than trp B, are not able to do so.

Of 122 Trp^- Ind^- progeny from the transduction cross, 2 neither recombined with the trp E 9777 marker present in the transducing phage nor accumulated InG. One of them was chosen for further study. That it contained both trp B 9578 and trp E 9829 was shown by growing phage P1 on it and transducing cys B 3110 to Cys^+ on minimal agar containing tryptophan . 87 colonies from this cross were streaked to plates of:

1. the same medium
2. Minimal medium containing ACH
3. Minimal medium containing ACH and indole
4. Minimal medium containing ACH and spread with phage

   Ø80 pt trp E 9777

24 isolates grew on all four media, hence were tryptophan prototrophs .

17 isolates grew on plates 1 and 3 only; these were presumed to contain the trp E 9829 marker only. That this was so, was shown by the fact that all these isolates could grow on ACH plates spread with phage Ø 80 p sup F.

4 isolates grew on plates 1 and 4 only; they possessed the trp B 9578 mutation only. This was shown by their not giving recombinants when transduced with phage Ø 80 pt trp B 9578, but doing so when this phage carried either the trp C 782 or trp A 33 markers.

42 isolates grew on plate 1 only. They were assumed to have acquired both trp markers.

These data clearly show that the isolate, on which the phage P 1 had been grown, carried both trp B 9578 and trp E 9829. It was numbered JPT 24.

4.6.3 Isolation of Point Mutants in cys B from JPT 24

Phage P1 was grown on strain trp E 9829 and mutagenised as in Section 3.3 except that it was treated with hydroxylamine for nine hours (to 10% survival). This preparation was used to transduce JPT 24 to growth on indole, and the transductants were screened for cysteine auxotrophy exactly as described in the same Section. Of 33, 280 transductants, 26 were cysteine auxotrophs. They were numbered JPT 25 to 29 and JPT 33 to 53. JPT 50 reverted at high frequency and was not used in subsequent work.

4.6.4 Construction of a Strain Carrying a Mutation in the pyr F Gene and trp E 9829 (JPT 32)

Phage P1 was grown on strain X 193 (pro his trp met pur B pyr B tyr lac) and used to transduce strain cys B-1 trp E 9829. Selection was made for Cys^+ on minimal agar plates containing uracil and tryptophan; the colonies growing on these plates were replicated to plates of the same medium and of minimal agar containing uracil or tryptophan alone. Ten isolates which grew only on the first of these media were purified and tested for the presence of the trp E 9829 mutation, by streaking on plates of the following media.

1. Minimal medium containing ACH, uracil and tryptophan
2. Minimal medium containing ACH and uracil
3. Minimal medium containing ACH and uracil, spread with

   Ø 80 p sup F

These plates were incubated at 37°C. All ten isolates grew on 1 after overnight incubation. After 40 h incubation 6 isolates grew on 3, but none on 2. One isolate which possessed a trp marker suppressible by sup F was numbered JPT 32.

Testing that the trp marker was sup F-suppressible was necessary as both donor and recipient carried trp markers and were Ind^+. but the donor trp marker was not sup F-suppressible, in contrast to trp E 9829.

4.6.5 Isolation of Point Mutants in cys B from JPT 32

Another batch of P i was grown on strain trp E 9829 and mutagenised as in Section 4.6.3. It was used to transduce JPT 32 to Ura^+ on minimal agar plates containing cystine and indole. Incubation of these plates and identification of Cys^- transductants was as given in Section 3.3. Of 45,325 transductants, 29 were cysteine auxotrophs. They were numbered JPT 54 to 82 inclusive.

4.7 Identification of the most sup X-Distal of the Point Mutations in cys B

In order to pursue the mapping, the first part of which we described in Sections 4.4 and 4.5, we wished to find the most left hand (i.e. sup X-distal) of the point mutations. We had already shown that the longest partial cys B deletion so far mapped was deletion 4; therefore we plate mated JPT 11 Δ 4 with all 54 new cys B point mutations as in Section 4.4 and looked to see whether Cys^+ recombinants occurred among the diploid progeny.

We were able to detect them in the Trp^+ progeny of 23 of the cys B point mutants, JPT 25, 35, 40, 42, 43, 45, 48, 53, 54, 55, 56, 61, 62, 65, 68, 69, 70, 71, 75, 79, 80, 81, 82; this indicates that the point mutations in these strains lie in the region left intact in deletion 4, i.e. they lie towards the left hand, sup X-distal, end of the region.

JPT 42, 54, 62 recombined with deletion 4 at a much higher frequency

than did the other eighteen mutants, thus suggesting that they probably carried the most sup X-distal point mutations.

4.8 Screening of More sup X Cys⁻ Derivatives of JPT 11 with the Presumptively Most sup X-distal Point Mutations in cys B

These three point mutants were in turn mated with 270 sup X Cys⁻ derivatives of JPT 11 (JPT 11 Δ24 - 49, 51 - 144, 146 - 163, from Section 4.5 and new isolates Δ 164 - 333 from Section 7). Deletions 67, 125, 167, 169, 183, 198, 209, 245 gave Cys⁺ recombinants in diploids obtained from crosses with all three recipients. Deletions 32, 115, 120, 122, 123, 175, 179, 241, 258 gave Cys⁺ recombinants in crosses with JPT 54 and 62 only. These results unequivocally place cys B-54 and 62 to the left of cys B-42. Since the deletions up to Δ 163 had been tested against JPT 16, but had not given recombinants with it, it follows that cys B-42, 54, 62 lie to the left of cys B-16.

We had still to establish that cys B-54 and 62 were indeed the most left hand of the point mutations. That they were likely to be so was indicated first by their mapping position relative to the point mutations cys B-16 and 42 and secondly by the fact that they recombined with so many deletions. If they were indeed so, one might expect that one or more of the deleted plasmids which recombine with them might not recombine with any of the other point mutations. In order to find such deletions it was not necessary to mate all the deletion mutants with every one of the point mutations. Instead we decided to use the point mutations that were already partially mapped to divide into groups the 21 deletions that had been shown to end within cys B. These groups of deletions could be used in turn to make a preliminary map of the remaining point mutants.

Any point mutation that recombines with a member of a particular group of deletions will recombine with all the members of groups which contain deletions ending more sup X-proximally. It need not therefore be tested against them, but only against the other deletions in the same group and against deletions known to end more sup X-distally.

4.9 Construction of a Preliminary Map of the cys B Region

All 21 deletions that ended within cys B were mated with strain cys B-1 trp E 9829, JPT 14, 15, 16, 17, 20, 42 and 54, and the diploid progeny screened for Cys^+ recombinants exactly as in Section 4.4 The results are given in Table 4-iii and the resultant map in Fig. 4 c.

They agree with the results obtained earlier (Sections 4.4, 4.5, 4.8) except that cys B-15 and 20 are separated from cys B-16 and lie to the left of both it and cys B-42. They divide the deletions into seven groups.

4.10 Construction of the Final Map of the cys B Region

We already knew which point mutations recombined with deletion 4 (Section 4.7). Those that did not were mapped against deletion group 2 (JPT 11 Δ3 and Δ167). There were three possible results to this experiment:

1. The point mutation in question recombined with both deletions. In this case they were then mapped against deletion group 3 (JPT 11 Δ50, Δ198, Δ245)

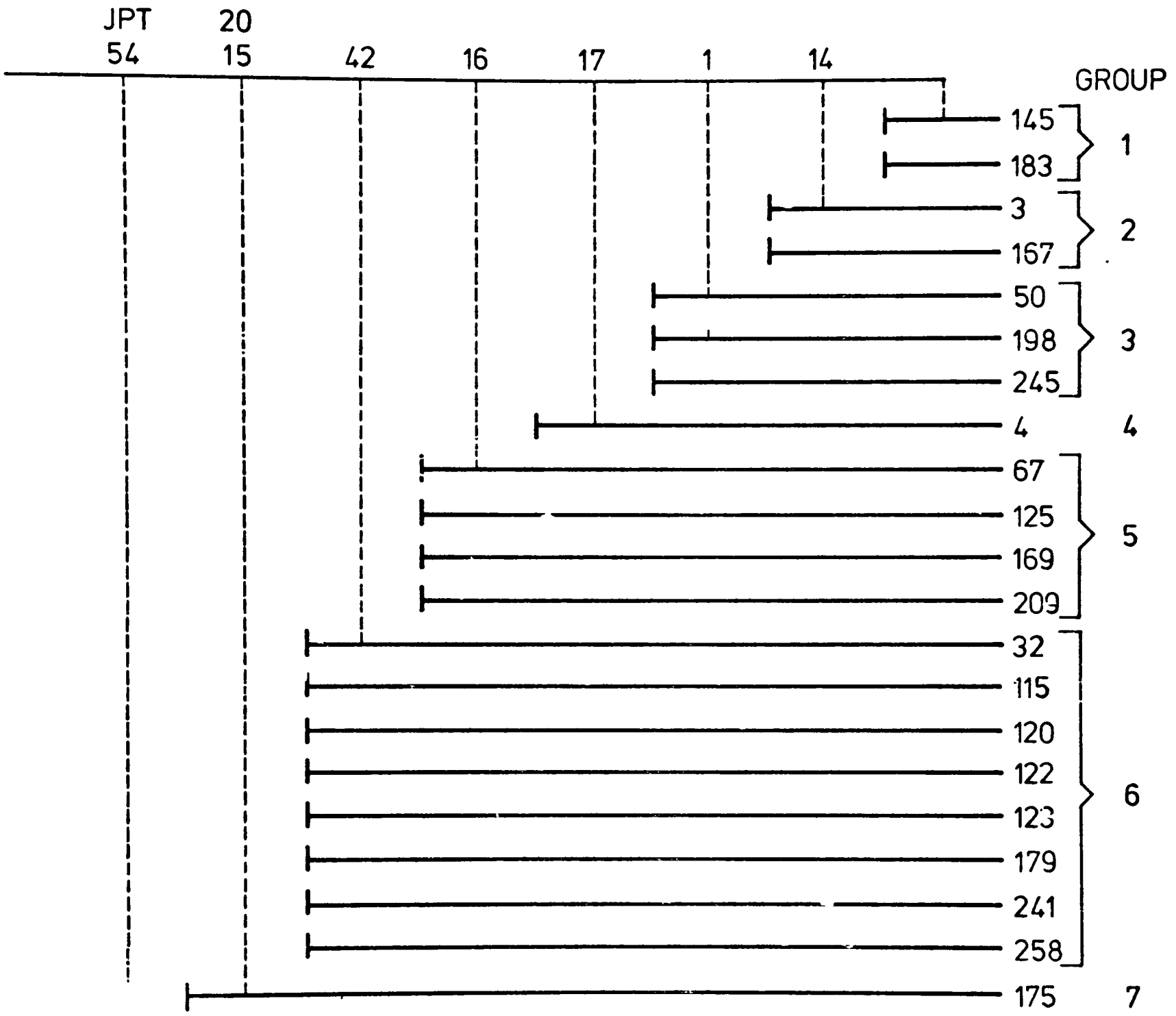
2. The point mutation recombined with neither deletion. In this case it was mapped against deletion group 1 (JPT 11 Δ145 and Δ183)

Table 4 - iii Recombination between Deletion and Point Mutations in cys B

cys B - Third Stage

		Recipients							
Donors	JPT	54	15	20	42	16	17	cys B-1 9829	14
JPT 11Δ	145	+	+	+	+	+	+	+	+
	183	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	-
	167	+	+	+	+	+	+	+	-
	50	+	+	+	+	+	+	-	-
	198	+	+	+	+	+	+	-	-
	245	+	+	+	+	+	+	-	-
	4	+	+	+	+	+	-	-	-
	67	+	+	+	+	-	-	-	-
	125	+	+	+	+	-	-	-	-
	169	+	+	+	+	-	-	-	-
	209	+	+	+	+	-	-	-	-
	32	+	+	+	-	-	-	-	-
	115	+	+	+	-	-	-	-	-
	120	+	+	+	-	-	-	-	-
	122	+	+	+	-	-	-	-	-
	123	+	+	+	-	-	-	-	-
	179	+	+	+	-	-	-	-	-
	241	+	+	+	-	-	-	-	-
	258	+	+	+	-	-	-	-	-
	175	+	-	-	-	-	-	-	-

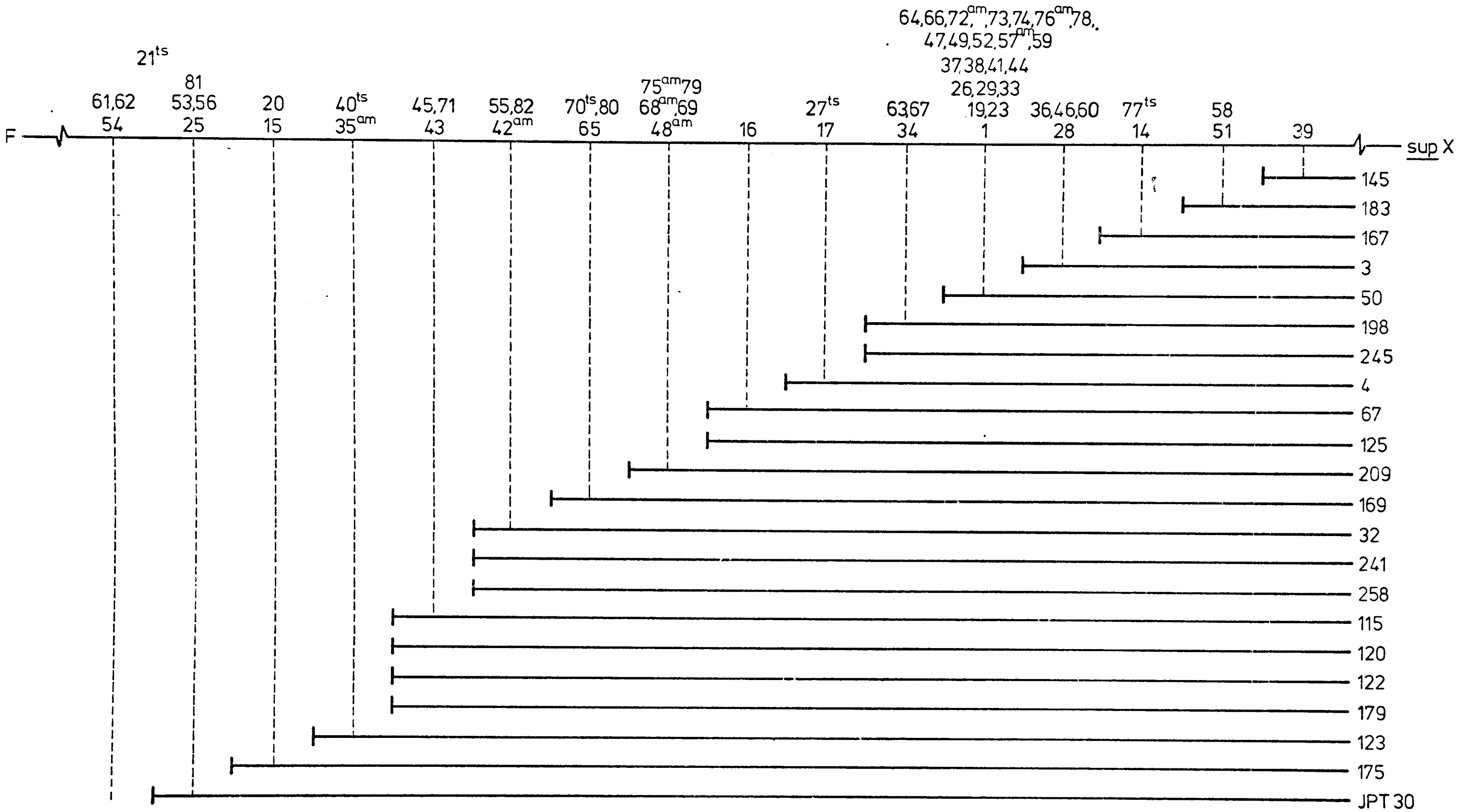
Fig. 4 c Third Stage Map of the cys B Region in E.coli


JPT
54
20
15
42
16
17
1
14
GROUP
145
183
1
3
167
2
50
198
245
3
4
4
67
125
169
209
5
32
115
120
122
123
179
241
258
6
175
7

3. Any point mutation which recombined with deletion 167 but not with deletion 3 was not mapped further.

Those point mutations which did recombine with deletion 4 were mapped in an analogous manner to the above:- first with deletion group 6 (JPT 11 Δ32, 115, 120, 122, 123, 179, 241, 258) and if necessary subsequently with either deletion group 5 (JPT 11 Δ67, 125, 169, 209) or deletion group 7 (JPT 11 Δ 175). The results, the final map, are given in Table 4-iv and Fig. 4 d. Also in Fig. 4 d are given the results of a subsequent mapping of another deletion, JPT 30 (Section 7.3).

Fig. 4 d. The Final Map of the *cys B* Region in *E.coli*


F
21ts
81
61,62
54
53,56
25
20
15
40ts
35am
45,71
43
55,82
42am
70ts,80
65
75am79
68am,69
48am
16
27ts
17
64,66,72am,73,74,76am,78,
47,49,52,57am,59
37,38,41,44
26,29,33
63,67
34
19,23
1
36,46,60
28
77ts
14
58
51
39
sup X
145
183
167
3
50
198
245
4
67
125
209
169
32
241
258
115
120
122
179
123
175
JPT 30

Chapter 5

Characterisation of Point Mutants in the cys B Region of E.coli

5.1 Nutritional Analysis of cys B Mutants of E.coli

We believed that the mutations leading to cysteine auxotrophy which we had isolated and mapped in the work described in Chapter 4 lay in the cys B region. They had been isolated after site specific mutagenesis, they were complemented by a gene present on the F'trp plasmid and they had been mapped to either side of the cys B mutation isolated and characterised earlier (Yanofsky and Lennox, 1959).

We wished to see if they also behaved nutritionally like the cys B mutants in S.typhimurium (Section 1.4). We grew cultures of all the mutants in liquid minimal medium containing cystine and indole, harvested the cells by centrifugation, washed them and resuspended them in sterile saline, and streaked them on plates of sulphur-free minimal medium containing indole and supplemented with sources of inorganic sulphur.

These media were made as follows: minimal agar 2 made with Oxoid purified agar was melted and cooled. Glucose and indole were added in the usual way and the medium was cooled to 50°C. Solutions of sodium sulphate, sulphite, thiosulphate, sulphide and methionine, sterilised by membrane filtration, were then added to final concentrations of 0.2 mM. In addition, plates were made containing thiosulphate at a final concentration of 20 mM. The plates were allowed to set and inoculated the same day.

The results are given in Table 5-i; they are identical with those of Jones-Mortimer (1968a). Those mutants which are not temperature sensitive give

Table 5-i Nutritional analysis of non-temperature sensitive cys B mutants of E.coli

The media was prepared and inoculated as given in the text. Results were scored after 40 h incubaiion at 37°C.

++ indicates good growth,

+ poor growth,

f residual growth,

- no growth.

	sulphate	sulphite	thiosulphate 0.2 mM	thiosulphate 20 mM	sulphide	methionine
cys B- 1	f	-	-	++	+	-
14	-	-	-	++	+	-
15	-	-	-	++	+	-
16	-	-	-	++	+	-
17	-	-	-	++	+	-
19	-	-	-	++	+	-
20	-	-	-	++	+	-
23	-	-	-	++	+	-
25	-	-	-	++	+	-
26	-	-	-	++	+	-
28	-	-	-	++	+	-
29	-	-	-	++	+	-
33	-	-	-	++	+	-
34	-	-	-	++	+	-
35	-	-	-	++	+	-
36	-	-	-	++	+	-
37	-	-	-	++	+	-
38	-	-	-	++	+	-
39	-	-	-	++	+	-
41	-	-	-	++	+	-
42	-	-	-	++	+	-
43	-	-	-	++	+	-
44	-	-	-	++	+	-
45	-	-	-	++	+	-
46	-	-	-	++	+	-
47	-	-	-	++	+	-
48	-	-	-	++	+	-
49	-	-	-	++	+	-
51	-	-	-	++	+	-
52	-	-	-	++	+	-
53	-	-	-	++	+	-
54	f	-	-	++	+	-
55	-	-	-	++	+	-

Table 5-i (cont'd)

	sulphate	sulphite	thiosulphate 0.2 mM	thiosulphate 20 mM	sulphide	methionine
56	f	-	-	++	+	-
57	-	-	-	++	+	-
58	-	-	-	++	+	-
59	-	-	-	++	+	-
60	-	-	-	++	+	-
61	-	-	-	++	+	-
62	f	-	-	++	+	-
63	-	-	-	++	+	-
64	-	-	-	++	+	-
65	-	-	-	++	+	-
66	-	-	-	++	+	-
67	-	-	-	++	+	-
68	-	-	-	++	+	-
69	-	-	-	++	+	-
71	-	-	-	++	+	-
72	-	-	-	++	+	-
73	-	-	-	++	+	-
74	-	-	-	++	+	-
75	-	-	-	++	+	-
76	-	-	-	++	+	-
78	-	-	-	++	+	-
79	-	-	-	++	+	-
80	-	-	-	++	+	-
81	f	-	-	++	+	-
82	-	-	-	++	+	-

results similar to those of Clowes (1958a), and Mizobuchi et al (1962). The results are not identical, however. None of the E.coli mutants utilised sulphite or low concentrations of thiosulphate whereas a minority of mutants in Salmonella are able to do so. In contrast a minority of mutants of E.coli showed only residual growth on medium containing sulphate. This did not continue on further incubation at 37°C, thus was not a consequence of reversion. It occurred to a similar extent when the same organisms were tested at different temperatures (30°C and 41°C).

5.2 Temperature Sensitive Mutations in cys B

Six of the mutants were temperature-sensitive; cys B-77 was a cysteine prototroph at 30°C but not at 37°C or 41°C; cys B-70 was a cysteine prototroph at 30°C, grew only slowly in the absence of cystine at 37°C and not at all at 41°C. cys B-21, 22, 27 and 40 were cysteine prototrophs at 30°C and 37°C but not at 41°C. Growth could occur at temperatures above that permitting growth on sulphate providing that the medium was supplemented with more reduced forms of sulphur; the more reduced the form of sulphur, the more it appeared to allow growth at restricting temperatures (Table 5-ii). Methionine also allowed growth, presumably because it spares cysteine (p. 335 of Roberts et al., 1955).

These results suggested another possible line of investigation. If cys B does in fact code for an activator molecule in the manner proposed by Jones-Mortimer (1968b), these temperature-sensitive mutants could possess either temperature-sensitive synthesis of a normal activator, or normal synthesis of a temperature-sensitive activator. Since all the mutations concerned had been

Table 5-ii Nutritional analysis of temperature sensitive cys B mutants of E.coli

These results were obtained in exactly the same manner as those given in Table 5-i. Growth was scored after 40 h incubation at the temperature indicated.

isolate no.	temperature	sulphate	sulphite	thiosulphate 0.2 mM	thiosulphate 20 mM	sulphide	methionine
21,22	37	++	++	++	++	+	++
27,40	41	-	±	++	++	+	++
70	30	++	NT	NT	NT	NT	NT
	37	±	+	++	++	+	++
	41	-	±	++	++	+	++
77	30	++	NT	NT	NT	NT	NT
	37	-	±	++	++	+	+
	41	-	-	+	++	+	±

mapped within the gene, the first possibility was unlikely. A prediction of the second possibility was that it would be possible, by varying the temperature of growth, to change the affinity of the activator for the different initiator regions (and hence vary the rate of transcription of the structural genes) independently of the concentration of activating or repressing metabolites. This type of approach had yielded valuable information on the regulction of the lac operon (Sadler and Novick, 1965) and of the glp regulon (Freedberg and Lin, 1973).

We therefore performed temperature shift experiments. We grew the mutants overnight (at 30°C) in liquid minimal medium 2 containing sulphate and indole. The next morning we diluted these cultures to give $E_{450} \simeq 0.02$ into flasks of the same medium and incubated these in a shaking water bath at 30°C. When these cultures had reached $E_{450} = 0.2$ and were growing exponentially, we transferred them to a similar water bath at 41°C. Equilibration of the cultures to the higher temperature occurred within one minute. When the cultures had stopped growing at this higher temperature, we returned them to the 30°C water bath.

The growth of these mutants in this experiment is given in Figure 5 a. It may be seen that even the most temperature-sensitive mutant, JPT 77, takes more than three hours to stop growth after the shift to 41°C; the least temperature-sensitive mutants JPT 21 and 22 were still able to grow slowly after five hours at this temperature. Those mutants which eventually did stop growing at 41°C failed to recommence growth at 30°C even after 16 h incubation at this temperature. This was not a result of the inviability of the organisms, at least in the case of JPT 27, as in a subsequent experiment we found that after exposure to the restrictive temperature all the organisms could still form colonies

Fig. 5 a Responses of Temperature Sensitive *cys B* Mutants to Changes in Temperature of Incubation

▲ JPT 21

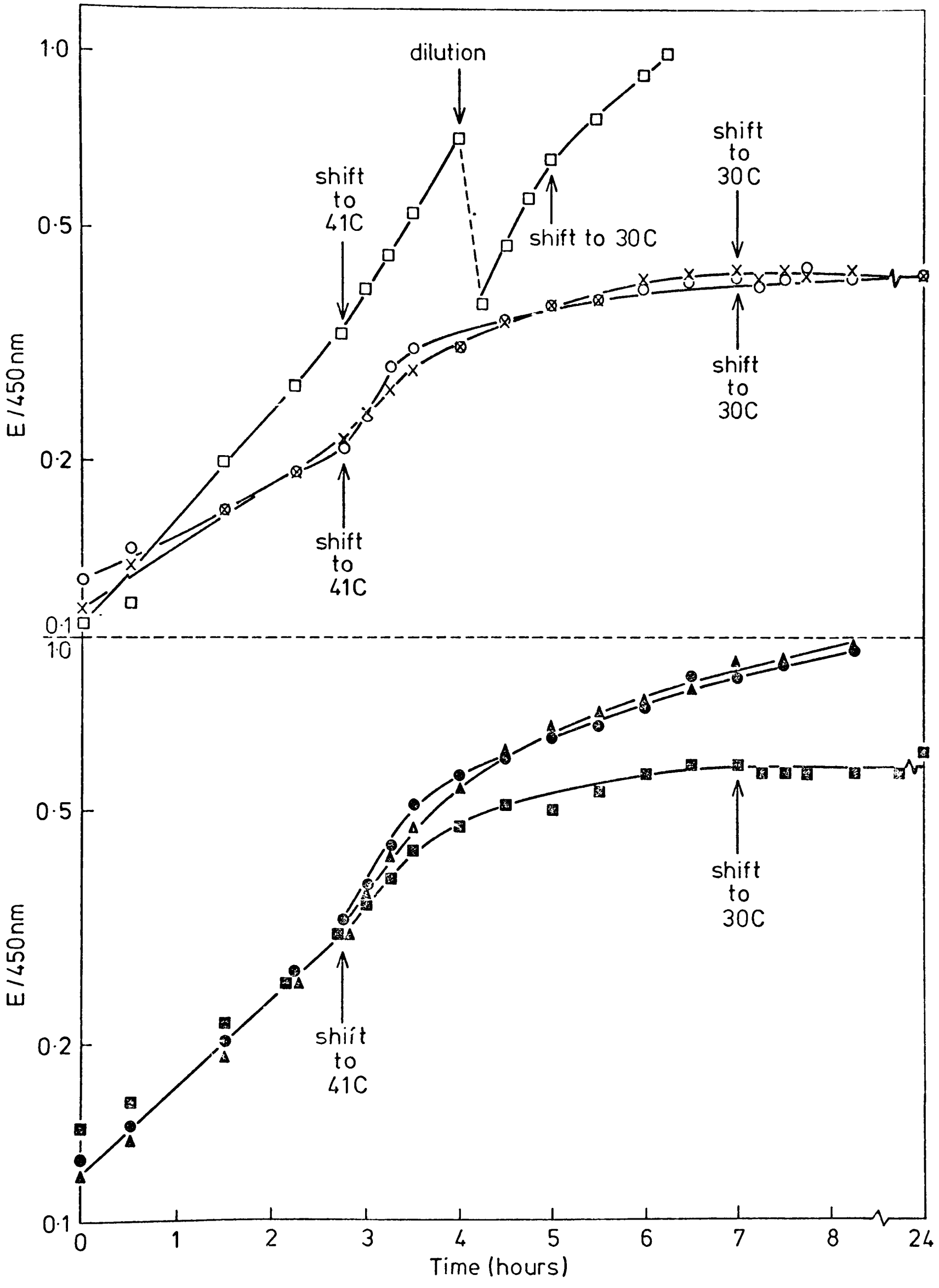
◐ JPT 22

◪ JPT 27

× JPT 40

○ JPT 77

□ *cys* B^+ *trp E* 9829


1·0
0·5
0·2
0·1
E / 450 nm
dilution
shift to 41C
shift to 30C
shift to 30C
shift to 41C
shift to 30C
1·0
0·5
0·2
0·1
E / 450 nm
shift to 41C
shift to 30C
0
1
2
3
4
5
6
7
8
24
Time (hours)

on nutrient agar and the culture could grow if supplied with cystine (see Fig. 5 b). Because of these complications in the experiments, we did not continue this line of investigation.

5.3 Amber Mutations in cys B

The existence of temperature-sensitive cys B mutations implied that cys B might code for a protein - though at least two temperature-sensitive mutations have been located in a gene coding for t-RNA (Smith, Barnett, Brenner and Russell, 1970). We looked to see if any of our cys B point mutations could be suppressed by a well characterised suppressor mutation. Certain suppressor mutations lie in genes coding for species of t-RNA and each causes the production of molecules of transfer RNA with an altered anticodon that is complementary to a chain terminating codon. This has the result that instead of chain termination occurring, an amino acid is inserted and translation is continued past this point; the protein thus formed may be at least partially functional. It is evident that this mechanism can only operate at the level of translation of m-RNA into protein; hence identification of such a suppressible mutation in a gene indicates unequivocally that the product of that gene is a protein (Weigert, Galluci, Lanka and Garen, 1966).

It was easy to see whether any of the cys B mutants mapped in Chapter 4 were suppressible. The method of their construction was such that it ensured that they possessed trp E 9829, an amber mutation which is suppressed well by sup F (Yanofsky et al., 1971). sup F is a suppressor mutation of the type described above; strains carrying this mutation possess a mutant tyrosine t-RNA (Goodman, Abelson, Landy, Brenner and Smith, 1968); this is able to cause

Fig. 5 b Growth and Survival of a Temperature Sensitive cys B Mutant (JPT 27) During Changes in Temperature of Incubation

Culture No.		Organism	Sulphur source
1	●——●	cys B^+ trp E 9829	sulphate
2	▲——▲	JPT 27	cystine
3	□——□	JPT 27	sulphate

These cultures were incubated at 30°C. After 5.5 h of such incubation, they were transferred to 41°C.

at point A culture no. 1 was diluted 1 in 3 in fresh warm minimal medium

at point B culture no. 2 was diluted 1 in 3 in fresh warm minimal medium containing cystine (0.2 mM)

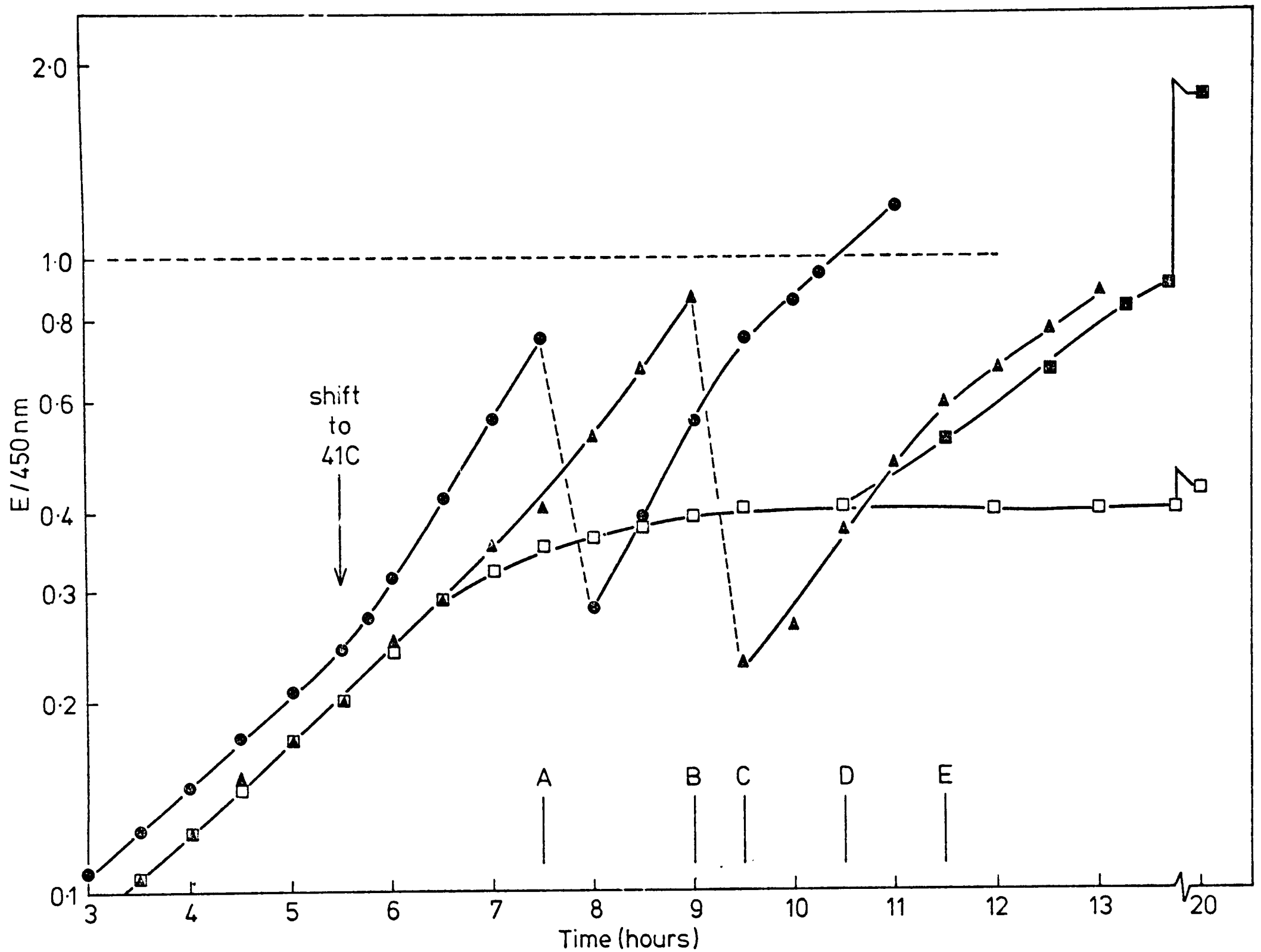
at point C cultures no. 1 and no. 3 were shifted back to 30°C

at point D culture no. 3 was divided into two: to one half cystine was added to a final concentration of 0.2 mM ■——■

at point E culture no. 2 was shifted back to 30°C

samples were taken from flask no. 3 for viable count

at 5.5 h incubation = 2.0×10^8 cfu/ml
at 9.5 h incubation = 4.9×10^8 cfu/ml
at 20 h incubation = 4.7×10^8 cfu/ml


2·0
1·0
0·8
0·6
0·4
0·3
0·2
0·1
E/450nm
shift
to
41C
A
B
C
D
E
3
4
5
6
7
8
9
10
11
12
13
20
Time (hours)

the insertion of tyrosine at a UAG (amber) codon at high efficiency (Weigert et al., 1966).

We grew overnight broth cultures of each point mutant and transduced them to Trp^+ with phage Ø80 p sup F as described in Materials and Methods. Four isolated transductants from each strain suppressed to Trp^+ were then streaked to ACH medium without phage both to check for stability of the lysogeny and to purify. This process was repeated until one stable pure lysogen was obtained from each strain. We then replica plated these purified isolates to minimal medium with and without cystine. All the lysogens could grow on minimal medium containing cystine; only eight of them could grow on minimal medium without cystine (lysogens of JPT 35, 42, 48, 57, 68, 72, 75, 76).

These results suggested that presence of Ø 80 p sup F suppresses the cys mutations in these strains and renders them Cys^+. To exclude the possibility that growth on minimal medium was a result of reversion of the cys mutations, we tried to recover the original Cys^- phenotype by curing the lysogen of its phage. We could identify 'cured' strains as they would become Trp^-; strains that had been rendered Cys^+ by suppression would simultaneously become Cys^-, but Cys^+ revertants would remain Cys^+.

We attempted to cure all eight Cys^+ Trp^+ lysogens of their phage by growth in media containing acridine orange (Materials and Methods), diluting and plating these cultures to nutrient agar and replicating these plates to plates of ACH medium with and without indole to detect Trp^- segregants. We were able to isolate these only from the lysogens of JPT 57, 68 and 72 in spite of many attempts and even after increasing the temperature of growth in acridine orange to 41°C. The Trp^- segregants from all three of these strains proved to be also

Cys^-. That this Cys^- phenotype was indeed due to the original sup F suppressible mutation, was shown by relysogenising these transductants with phage Ø 80p sup F and showing that they once again became Trp^+ and Cys^+.

5.4 Discussion

The nutritional analysis of the mutants we had isolated in E.coli gave results slightly different from those obtained in similar work on S.typhimurium; in particular, the E.coli mutants behaved as a more homogeneous group than the cys B mutants in S.typhimurium. The possible reasons for this will be discussed in Section 6.4. The differences between the overall nutritional behaviour of our mutants in E.coli and the Salmonella cys B mutants can probably be explained by the slight differences in experimental methods and the known instability of sulphur compounds (Postgate, 1963). We should point out that there are discrepancies between the results of similar analyses performed by different workers on the mutants in Salmonella (Demerec, Blomstrand and Demerec, 1955; Clowes, 1958a; Mizobuchi et al., 1962). We therefore think that the similarities between the mutants in the two organisms are sufficiently great to confirm the identification made on genetic grounds that our mutants lie in cys B.

The large intracellular pools of reduced sulphur present in E.coli (p. 330 et seq of Roberts et al., 1955) probably explain the long continuation of growth of the temperature sensitive mutants at the non-permissive temperature. We cannot explain why the mutants are then unable to resume growth at the permissive temperature. Possibly the product of the cys B gene is unstable at the higher temperature or becomes susceptible to degradation (it is perhaps relevant to note

that the ara C protein is extremely unstable in vitro (Wilcox, Clementson, Cleary and Englesberg, 1974)).

We may conclude from the isolation of temperature sensitive and suppressible mutations in cys B that the cys B gene product is a protein (cf. Bourgeois, Cohn and Orgel, 1965; Morse and Yanofsky, 1969).

Chapter 6

Complementation Analysis of the cys B Region; Estimation of its Size

6.1 Introduction

The mutants we had isolated and mapped in the work described in Chapter 4 appeared, by nutritional criteria, to be an homogeneous group. We therefore wished to see if all the mutations lay in the same gene or in more than one. This could be easily done by complementation analysis, by constructing diploids that have two copies of the cys B region each containing different cys B mutations. If there is only one gene in the cys B region, all such merodiploids will contain both mutations in the same functional unit and will have the same phenotypes as that of the haploids from which they were derived. In contrast, if there is more than one gene in the cys B region some of the merodiploids will contain one of the cys B mutations in one of the functional units and the other mutation in a different one; these merodiploids will thus contain one intact copy of each gene and will thus be phenotypically Cys^+.

We had already obtained a linear map of the cys B region and decided first to test for complementation between the mutations mapping at each end of the region. If complementation did not occur between these, they belonged to the same gene and so then would all other mutations mapping between them.

6.2 Complementation Analysis of the cys B Region

The most trp proximal point mutation cys B-39 was transduced onto the plasmid F'123 which carries pyr F, cys B trp and ton B by a two stage procedure. First strain MY303 (pyr F cys B-54 trp AE$^{\Delta}$ ton B / F' pyr F^{+} sup X -cys B$^{\Delta}$ -83 trp^{+} ton B^{+}) was transduced with phage P1 grown on strain trp A$^{\Delta}$905 ton B. The transduction mixture was diluted into liquid minimal medium containing tryptophan; this culture was incubated overnight at 37°C with shaking. It was then treated with colicin and phage Ø80 vir to select ton B mutants (Materials and Methods) and the resulting mixture was washed and plated on minimal agar containing tryptophan and spread with phage Ø80 vir. These plates were incubated at 37°C for 48 h and then were replicated to plates of the same medium and to minimal agar. The vast majority of colonies were Trp^{-}. Among these we expected clones of genotype pyr F cys B-54 trp AE$^{\Delta}$ ton B / F' pyr F^{+} cys B^{+} trp A$^{\Delta}$905 ton B. These could be recognised by their ability to donate Cys^{+} and Ind^{+}. Accordingly we mated twelve clones with JPT 122 (pro cys B-39 trp AE12 ton B str^{R}) selecting for transfer of the plasmid on minimal agar containing proline, indole and streptomycin. We recovered Cys^{+} Ind^{+} progeny from four of these crosses. These progeny were purified and tested. They segregated Cys^{-} Ind^{-} clones after growth in media containing acridine orange and grew very poorly on M9 minimal medium containing the appropriate supplements (this implies that they were homozygotes for ton B - cf. Section 7.5). These isolates were also able to transfer Cys^{+} and Ind^{+} but not Trp^{+} to strain cys B-1 trp AE12. These characteristics show that their genotype was pro cys B-39 trp AE12 ton B / F' pyr^{+} cys B^{+} trp A$^{\Delta}$905 ton B.

One such clone was transduced with phage P1 grown on JPT 39. Selection

was made for Trp^+ on minimal medium containing proline and cystine. Cys^- progeny from this cross were identified by replication to minimal medium containing proline. One such colony was purified and tested. It was Pro^- Cys^- Trp^+ str^R but segregated Trp^- colonies after growth in medium containing acridine orange. It was able to transfer Trp^+ but not Cys^+ at high frequency to cys B-1 trp AE 12 and Ura^+ to JPT 32 (pyr F trp E 9829). It was thus pro cys B-39 trp AE 12 str^R/F' pyr F^+ cys B trp^+. It was numbered JPT 131. The cys B mutation on the plasmid must have been cys B-39, from the method of construction. The diploid reverted to Cys^+ at a similar frequency to the haploid strain JPT 122; furthermore, very occasional recombinants were found among the diploids made by mating this diploid with strain cys B-1 trp AE 12.

In order to see whether cys B-39 complemented mutants carrying lesions at the extreme trp distal end of the cys B region, we mated strain JPT 131 with strains JPT 89, 128 and 129 (carrying cys B-54, 61, 62 respectively) in appropriately supplemented minimal media. As a control we mated these recipients with strain JPT 83 (Salmonella 523/F'123 pyr F^+ cys B^+ trp^+) in exactly the same way. These mixtures were diluted and plated on M9 minimal agar containing cystine with lactose as carbon source (and valine in the cases of the matings with JPT 128 and 129). At least one hundred colonies arising from these plates were replica plated to the same medium with and without cystine. All colonies from the matings with JPT 83 grew equally well on both media; after incubation overnight colonies grown in the presence or absence of cystine were of about 2-3 mm diameter. We may conclude that the cys B^+ gene on the plasmid complements, as expected, the mutations cys B-54, 61 and 62.

After incubation overnight the replicated colonies from the matings with JPT 131 had grown well only on the medium containing cystine. On the plates lacking cystine, only a few colonies had grown at all, and those showed slight growth only. After further incubation (48 h) the colonies on medium containing cystine had grown to 5-6 mm diameter; on the medium lacking cystine, only 60% of the colonies had grown to about 2-3 mm diameter. These results are characteristic of recombination; since during this experiment we maintained selection for diploidy by omitting tryptophan from the plates, they exclude the possibility of complementation between the mutations cys B-39 and cys B-54, 61, 62 (cf. Power, 1967). Thus we may safely conclude that they lie in the same complementation group.

6.3 Size of the cys B Gene

This was estimated by reciprocal two factor transduction crosses between strains carrying mutations mapping towards the ends of the cys B gene. Phage P1 was grown on JPT 91 and 92 and used to transduce JPT 14 and 39 to Trp^+; selection was made on minimal agar plates containing cystine. The colonies growing on these plates after incubation at 37°C for 40 h were replicated to plates of the same medium and of minimal agar. In this way the proportion of cysteine prototrophs arising from the cross was found. This proportion was adjusted for the percentage cotransduction between trp E 9829 and the cys B region.

In an analogous manner Phage P1 grown on JPT 14 and 39 was used to transduce JPT 93 and 94 to Ura^+; selection was made on minimal agar plates containing cystine and indole. These transductants were replicated to plates of

the same medium and to minimal agar containing indole. Again the percentage of Cys^+ recombinants was found and corrected for the cotransduction frequency of pyr F and cys B. The results are given in Table 6-i.

The results are reasonably consistent. The most interesting results are those of crosses 3 and 9 between cys B-39, the most trp proximal, and cys B-54, a member of the most trp-distal group, which indicate that the size of the cys B gene is between 5 and 7 map units.

These values can be used as a basis for the estimation of the physical size of the cys B gene. A comparison has been made of the genetic size of four of the trp genes with the size of the products of the same genes. This work was performed with strains of bacteria closely related to ours, with the same phage and the same techniques (Yanofsky et al., 1971). The quite reproducible figure of about 220 nucleotide pairs per map unit obtained by Yanofsky may thus be extended to our work on cys B. This gives a physical size of the cys B gene as about 1100 to 1500 nucleotide pairs.

Although this estimate depends upon a number of assumptions which have not been proved, it seems likely that the cys B gene is of about the same size as one of the trp structural genes and perhaps a little larger than the lac I gene.

6.4 Discussion

The results of the work described in this chapter taken together with those described in the preceding one, indicate that the cys B region of E.coli contains a single gene of moderate size, mutations in which exhibit a consistent phenotype.

This account differs somewhat from that put forward by Mizobuchi et al

Table 6-i Recombination Frequencies Between Mutations in cys B

Cross	Donor		Recipient		No. Cys^+ recombinants detected	No. Trp^+ or Ura^+ transductants scored	% Recombination	Correction 100/	Map units
	Strain	Mutation	Strain	Mutation					
1	3110		cys B trp E 9829	cys B-1	249	455	55		
2	JPT 91	cys B-54	JPT 14	cys B-14	30	970	3.1	55	5.65
3	JPT 91	cys B-54	JPT 39	cys B-39	45	1260	3.6	55	6.5
4	JPT 92	cys B-42	JPT 14	cys B-14	13	756	1.7	55	3.1
5	JPT 92	cys B-42	JPT 39	cys B-39	18	793	2.3	55	4.2
6	3110		JPT 84	cys B-1	334	386	86.5		
7	JPT 14	cys B-14	JPT 93	cys B-54	22	703	3.1	86.5	3.65
8	JPT 14	cys B-14	JPT 94	cys B-42	23	844	2.7	86.5	3.15
9	JPT 39	cys B-39	JPT 93	cys B-54	46	937	4.9	86.5	5.65
10	JPT 39	cys B-39	JPT 94	cys B-42	27	924	3.0	86.5	3.5

(1962) to describe the homologous region in S.typhimurium. These workers divided the region into three complementation groups on the basis of analysis by abortive transduction and three factor transduction crosses. Mutants mapping in the central complementation group cys Bb had more stringent nutritional requirements than those mapping in the complementation groups on either side, cys Ba and Bc. Yet the cys B genes in E.coli and S.typhimurium are homologous both genetically and functionally (Ino and Demerec, 1968; Section 4.2). How may the differences between our results and those of Mizobuchi et al (1962) be explained?

One possibility is that though the mutations are in the homologous gene, the mutations isolated in E.coli are different in character from those in S.typhimurium. This might at first glance seem quite likely, as the mutations described here were all induced with the mutagen hydroxylamine. The mode of action of this mutagen is well documented (Freese, Bautz and Bautz-Freese, 1961; Fraenkel-Conrat and Singer, 1973); it induces predominantly G-C to A-T transitions. In contrast, the cys B mutants in Salmonella were isolated after induction with UV and other mutagens and also include spontaneous mutants. However, even if this possibility is accepted, we might expect that the E.coli mutants would on the whole be 'leakier' phenotypically than the similar mutants in S.typhimurium; transitions will in general cause smaller differences in the amino-acid sequence of a polypeptide chain than will other types of mutation. Rather the opposite of this expectation is observed (Section 5.2).

We should point out a discrepancy between the genetic results published on the cys B region in Salmonella (Clowes, 1958b; Mizobuchi et al., 1962). The latter workers were able to demonstrate abortive transduction between

members of groups cys Ba, Bb and Bc; the former was not able to do so. The latter workers ordered the cys Ba and cys Bb mutants on their map only by three factor crosses which showed that cys Ba and Bb mutants lie trp proximal to cys Bb-12 apparently the most trp-distal cys Bb mutation. In contrast Clowes shows by means of a three factor cross (his Table 6, number 5), that cys B-14 (a cys Bb mutant) maps trp proximally to cys B-18 (a cys Ba mutant) so that one cys Bb mutant is separated from the main cluster of cys Bb mutants by at least one Ba mutant. The classification of these two mutants is very likely to be correct; both papers agree on their nutritional classifications; these agree with biochemical analyses (Table VII of Kredich, 1971). Thus if there is more than one complementation group corresponding to the different nutritional classes, there would have to be at least four complementation groups.

In fact, the only evidence for complementation is the suggestion that abortive transduction can occur between different cys B mutations. It is perhaps pertinent to say that we have observed that some of the cys B mutants in E.coli can produce very small colonies on medium containing sulphate as sole source of sulphur (Section 5.2). In the light of the results of Clowes and of our own results, we tend to the view that the cys B regions of both organisms contain a single gene.

Chapter 7

Deletions Fusing the cys B Gene with the trp Operon

"There's many a slip twixt cys and trp"

7.1 Introduction

There are several possible schemes of regulation of the cysteine biosynthetic pathway, other than positive control, which are compatible with the evidence. For example cys B might code for an enzyme which converts OAS into the 'true' inducer. This suggestion is attractive for several reasons; it is almost as simple as positive control; there are several precedents for it (Section 1.4); and it could be extended to account both for the inability of exogenous OAS to derepress cultures grown on cysteine or sulphide (these compounds could repress or inhibit the conversion step) and for the occurrence of the partial constitutive mutations mapping near cys B (these could be cys B operator-constitutives). A scheme is also possible in which the cys B gene product has both enzymic and regulatory functions ("autogenous regulation" - Goldberger, 1974).

All possible hypotheses, however, can be allocated into one of two classes. The first contains those schemes in which cys B is a 'classical' regulator gene (Spencer et al., 1967). The second contains those schemes in which the cys B gene product has a structural or enzymic function.

We can then ask the questions:

1. At what rate is cys B transcribed?
2. Is the rate of transcription constant or is transcription of cys B itself regulated?

A constant and low rate of transcription will probably be characteristic of the first group. A regulated and higher maximal rate of transcription will probably be characteristic of the second.

One way of answering these questions is to fuse the cys B gene to another well defined gene of known function and follow the rate of expression of the former by measuring that of the second. The phenomenon of gene fusion was found during work on lac (Jacob, Ullman and Monod, 1965). Certain mutants bearing deletions extending through the lac regulatory genes into lac Z were found to have the synthesis of galactoside permease subject to repression by purines, and insensitive to the usual inducers; it was subsequently found that the other ends of the deletions were in the gene pur E. This approach has been extended to other systems, for example trp (Beckwith, 1970; Reznikoff and Thornton, 1972), ara (Kessler and Englesberg, 1969), and bio (Ketner and Campbell, 1974).

In order to use this approach we required a well characterised 'reference' gene to which cys B could be fused, preferably coding for a well characterised and easily measured product. We would require a method for generating the deletions and also require that such deletions would not in addition delete genes of essential function. Finally we would require that there would be a reasonable change of cys B being transcribed in the same direction as the reference gene.

Our first requirement was satisfied by the proximity of the trp operon (Section 6.3). Our second and third requirements were also met (Section 4.3; Mukai and Margolin, 1963; Spudich, Horn and Yanofsky, 1970). Finally we considered the last requirement; the probability that cys B was transcribed in the same direction as the trp operon depended in part on the number of genes there

were in the cys B region. (At this time the work described in Section 6.2 had not been completed.) If there were three as suggested in work on Salmonella (Mizobuchi et al., 1962) they could be transcribed divergently, as in arg (Jacoby, 1972) and bio (Gutra, Saturen and Szybalski, 1971), or all in the same direction. In the former case at least one of the genes would be transcribed in the same direction as the trp operon. In the latter case, the probability of cys B and trp being transcribed in the same direction would be the same as if there were only one gene; on mathematical considerations this would be 0.5, but on genetical considerations somewhat greater. (More genes are transcribed in the same direction as trp i.e. anticlockwise on the standard map of E.coli than are transcribed in the opposite direction (Taylor and Trotter, 1972).)

We therefore set out to isolate cys - trp fusions on the F'trp plasmid. The only bacterial genes known to be carried on this plasmid are cys B, trp and ton B (Fredéricq, 1969). We had isolated sup X cys B deletions in the hybrid merodiploid while maintaining selection of Trp^+ and similarly sup X - trp deletions while maintaining selection for Cys^+ (Section 4.3). At first sight it would appear difficult to distinguish between cys B- sup X - trp deletions and plasmid-free segregants. In this respect, a particular property of the trp operon proved very useful.

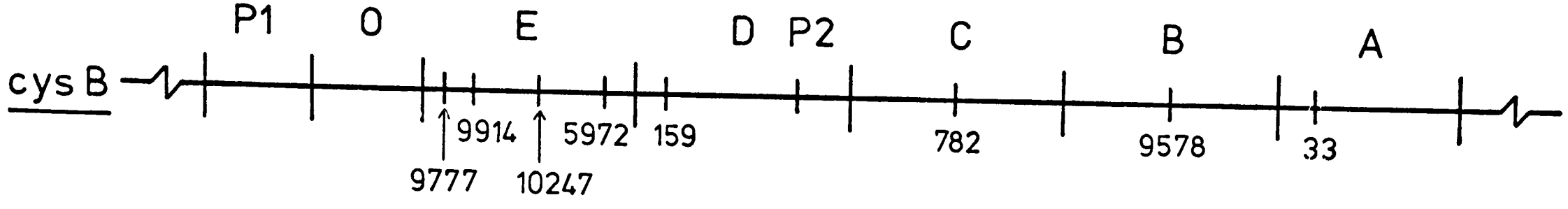
In the trp operon of both E.coli and S.typhimurium there is a second promoter near the operator-distal end of the gene coding for component II of the anthranilate synthetase enzyme complex (Margolin, 1971; see Fig. 7 a). This promotes sufficient transcription of the tryptophan synthetase genes to allow the organism to make tryptophan from indole and hence to grow on medium containing

Fig. 7 a The trp operons of E.coli and S.typhimurium

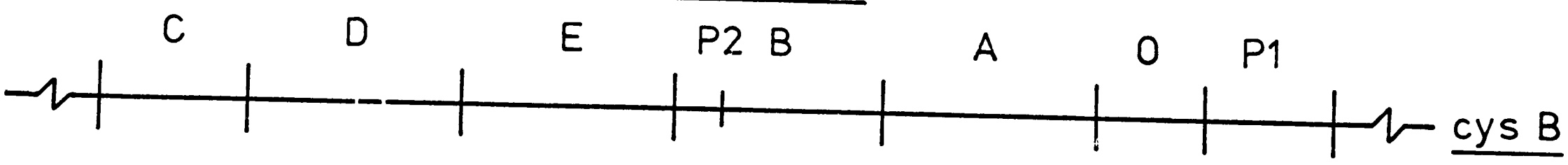
(after Margolin, 1971)

O	the operator
P 1	the promoter associated with the operator
P 2	the low efficiency internal promoter
AS I	anthranilate synthetase component I
AS II	anthranilate synthetase component II
PRT	phosphoribosyl transferase
PRAI	phosphoribosyl anthranilate isomerase
INGPS	indole glycerol phosphate synthetase
TS β	tryptophan synthetase β chain
TS α	tryptophan synthetase α chain

E.coli



S.typhimurium



indole, whether or not it possesses an active 'true' promoter-operator region. Thus, if we selected sup X deletions on minimal agar containing a source of cysteine and indole, the colonies that appeared must have at least an intact trp B gene. In addition they might contain the rest of the trp operon and hence be Trp^+, or, if they were Trp^-, they would either have to contain the second promoter (and hence the trp C gene), or have the intact trp B gene fused to a new promoter. Among such Ind^+ Trp^- derivatives there might well be the required fusions.

How could we further identify such fusions? The exhaustive approach would be to collect all the sup X Cys^- Trp^- Ind^+ derivatives, discard all those in which the deletions did not end in cys B, map the other end of the deletions in the strains remaining and then analyse the expression of the trp genes of these strains. This approach required a map of cys B, so that the cys B end of the deletions could be mapped; we therefore delayed it until the work described in Chapter 4 had been completed.

An alternative approach was to assume for experimental purposes that there might be a change of expression of cys B when cells were repressed or derepressed for cysteine biosynthesis, and that in a fusion of the type for which we were searching the rate of expression of trp would depend on this repression or derepression. We therefore looked to see whether among sup X Cys^- Trp^- Ind^+ derivatives there were some that exhibited a change of Trp phenotype in response to a change in cysteine repression.

7.2 Isolation of a Strain Carrying a Putative cys B - trp Fusion (JPT 30)

Twenty-four tubes of L-broth were inoculated with individual colonies of strain JPT 11. These cultures were incubated overnight at 37°C with shaking, and the cells were harvested, washed, and plated on minimal agar 2 containing cystine and indole and minimal agar 2 containing djenkolate and indole. These plates were incubated at 37°C for 48 h (media with cystine) or 72 h (media with djenkolate). Five sup X colonies were taken from each plate and gridded to plates of the same medium. These were incubated and then replicated to plates of the same medium, the same medium without indole, and indole-minimal agar.

In this way, we found six Cys^- Trp^- Ind^+ clones by selection on cystine-indole medium and four similar clones on djenkolate-indole medium. In addition from this experiment we isolated 24 Cys^- Trp^+ clones; these were used in the work described in Section 4.8. The Cys^- Trp^- Ind^+ derivatives were purified on the same medium on which they were isolated and streaked to plates of the following media

1.	minimal medium 2 containing	cystine and indole
2.	" " " "	djenkolate and indole
3.	" " " "	cystine and anthranilate
4.	" " " "	djenkolate and anthranilate
5.	" " " "	cystine
6.	" " " "	djenkolate

Growth was scored after incubation at 37°C for 40 h (media containing cystine) or 72 h (media containing djenkolate). All isolates from media containing cystine and indole grew on media containing djenkolate and indole,

and vice versa. One of the isolates from medium containing cystine and indole was able to grow on djenkolate-containing medium in the absence of indole but not on cys-min medium without indole. This was not a result of residual growth; streaking of colonies growing on djenkolate minimal agar to plates of the same medium with and without indole gave colonies of similar size on both media after incubation. This isolate was numbered JPT 30. The other sup X Cys^- Trp^- Ind^+ derivatives of JPT 11 were kept; they will be described further in Section 7.5.

7.3 Genetic Analysis of JPT 30

Strain JPT 30 was Lac^- Cys^- Trp^- Ind^+ Leu^+ at 37° but Leu^- at 41°C. After growth in media containing acridine orange, it segregated clones which were Lac^- Cys^- Trp^- Ind^- Leu^+ at 37°C but Leu^- at 41°C. JPT 30 was mated with strain cys B-1 trp AE 12 on M9 medium containing cystine and indole with lactose as carbon source. We recovered progeny from this cross which were Lac^+ Cys^- Trp^- Ind^+. One such isolate was purified and numbered JPT 31. It too could grow on minimal medium 2 without indole, provided that djenkolate rather than cys was the source of sulphur, after incubation at 37°C for 90 h. It segregated Lac^+ Cys^- Trp^- Ind^+ clones after growth in media containing acridine orange. These segregants were unable to grow on djenkolate minimal medium.

These results not only prove the genotype of these isolates but also show that their ability to grow on djenkolate-minimal medium without indole is a property associated with the plasmid.

We studied the location of the trp end of the deletion present in JPT 31

by using derivatives of phage Ø80 pt 190 carrying well characterised point mutations in the trp genes (Fig 7a). We concentrated on the E gene; as both strains JPT 30 and 31 were tryptophan prototrophs under certain conditions, we expected that either the E gene was genetically intact or the deletion ended just inside the gene so that transcription of the gene gave an enzymically active product. The results are given in Table 7-i.

They confirmed our expectations. trp E 9777 and trp E 9914, the most operator proximal trp E mutations, did indeed recombine with JPT 31. Thus if the deletion does extend into the trp E gene, it stops short of these mutations. We then grew phage P1 on JPT 31 and used this preparation to transduce trp E 9914, trp E 9829 and trp E 5972 to Trp^+. This showed that the deletion recombined at quite a high frequency with trp E 9914 and at a greater frequency with the other two trp E recipients. This result tends to locate the end of the deletion present in JPT 31 outside the trp E gene.

Did the deletion end in the trp promoter or operator? We had no promoter or operator mutants mapping in this region; thus we could not answer this question genetically. We therefore tested growth in the presence of the inhibitor 5-methyltryptophan. This compound inhibits growth by two mechanisms. It acts as a false co-repressor of the tryptophan operon and as a false feedback-inhibitor of anthranilate synthetase (for a review, see Margolin, 1971).

Resistance to 5-methyltryptophan can be caused by mutation in any of four different sites: trp R - these result in constitutive expression of the trp operon; trp O - these result in cis-dominant constitutive expression of the trp operon; trp E - these result in a feedback-insensitive anthranilate synthetase; mtr - these probably cause an inability to take up the inhibitor (Hiraga, 1969).

Table 7-i Frequency of Recombinants occurring in Transductions of strain JPT 31 with derivatives of phage Ø 80 pt 190 carrying point mutants in the trp operon

		Ø 80 pt 190				
		trp E 9914	E 9777	E 10247	E 5972	D 159
	JPT 31	±	++	++	++	+++
STRAINS	trp E 9914	0	+	++	++	+++
	trp E 9829	++	±	++	++	+++
	trp E 5972	++	++	++	0	+++
	trp D 159	++	++	++	++	0
	cys B-1 trp AE 12	0	0	0	0	0

Thus we reasoned that if the operator region were intact in JPT 30 and 31, 5-methyltryptophan could interact with the trp R gene product and prevent transcription of the trp operon. In this case growth of these organisms would be prevented by the incorporation of 5-methyltryptophan into the medium. Accordingly these strains together with appropriate controls were streaked to plates of djenkolate minimal medium with and without 5-methyltryptophan. The results are given in Table 7-ii.

They show that though the organisms under test do grow in the presence of 5-methyltryptophan, they do so much less well than a trp R^- strain. Was this inhibition mediated via the trp R gene product? We mated JPT 30 with strain thr trp R^- trp AE 12 in exactly the same way as described above. After purification of this strain, a trp R^- homologue of JPT 31, it was tested for its sensitivity to 5-methyltryptophan. It was just as sensitive as JPT 31; this suggests that it is not the transcription of trp that is affected by the inhibitor but rather the trp E gene product that is inhibited. If this interpretation is correct, the operator region is not intact. We can not show whether the deletion stops in or past the operator region.

The location of the cys end of the deletion present in JPT 31 was determined as follows; in order to test whether the deletion on the plasmid covered the cys B-1 mutation, we grew the diploid strain JPT 31 to stationary phase in liquid minimal medium with cystine and indole, harvested the cells, washed them and plated them on minimal agar plates containing tryptophan. Similarly we grew cultures of the corresponding haploid strain cys B-1 trp AE 12 in liquid minimal medium containing cystine and tryptophan, and harvested and plated the cells in exactly the same way. After incubation of these plates we

Table 7-ii Sensitivity of Strains of *E.coli* to 5-methyltryptophan

Strain		medium djenkolate minimal	djenkolate minimal + 5-methyltryptophan
JPT 30		++	poor
JPT 31		++	poor
JPT 11	*	++	0
MY 535 (*trp* R^- trp^+)	*	++	++
F'*trp* from JPT 30 in *thr trp* R^- *trp AE* 12	*	++	poor

* media appropriately supplemented with leucine or threonine

scored the number of Cys^+ revertants. These arose at a similar frequency, (JPT 31 2×10^{-8}, cys B-1 trp AE 12 4.3×10^{-8}); this indicates that the deletion in JPT 31 extends past cys B-1.

We performed a similar mating of JPT 30 with JPT 89 (cys B-54 trp AE^{Δ}) and again compared the frequency of occurrence of Cys^+ derivatives with that of the haploid strain. In this case we recovered numerous Cys^+ recombinants from the diploid but not from the haploid strain. This indicates that the deletion in JPT 31 ends inside the cys B gene. Furthermore we mated JPT 30 with strains JPT 125-30 inclusive, which are trp AE 12 val R mutants bearing mutations belonging to the extreme left hand deletion group (cys B-25, 53, 56, 61, 62, 81) in exactly the same way. The results showed that in addition to recombining with cys B-54, the deleted plasmid present in JPT 30 recombines with cys B-61 and 62 but not with the other 4 mutations.

7.4 Biochemical Analysis of Strains JPT 30 and 31

These strains are able to grow on djenkolate minimal but not on cystine minimal medium (Section 7.2). This ability might possibly reflect transcription that starts in cys B and continues through the trp structural genes but which is repressed by cystine. If this idea is correct, it follows that in strains JPT 30 and 31, we should be able to measure a substantial difference in the levels of the trp enzymes according to whether the cells are grown in conditions which repress or derepress cysteine biosynthesis.

Accordingly, JPT 31 was grown in minimal medium 2 containing tryptophan and either djenkolate or cystine as sulphur source. The specific activities of the enzymes anthranilate synthetase and tryptophan synthetase β subunit were measured

(see Materials and Methods). The results are given in Table 7-iii.

They show that the amount of anthranilate synthetase made by strain JPT 31 when grown in cystine-containing medium is very small; about one sixth of the level in the fully repressed haploid strain. This is consistent with the results of Section 7.3, which indicate that trp P1 has been inactivated. Strain JPT 31 does produce more anthranilate synthetase when it is grown in djenkolate medium than when it is grown in cystine medium, but only about 60% more. The increase may be sufficient to allow growth on djenkolate minimal medium (cf. Scaife and Beckwith, 1966), but it is much smaller than the increase found on derepression of known enzymes in the cysteine biosynthetic pathway (Kredich, 1971). It is thus unlikely to be a consequence of regulated transcription of cys B continuing through trp. The levels of tryptophan synthetase-β will be discussed in Section 7.7.

7.5 The Isolation and Genetic Characterisation of More Deletions in the cys B-trp Region of E.coli

While we characterised the deletion present in JPT 30, we continued to search for further deletions fusing cys B to trp. We isolated 590 more sup X Cys^- Trp^- Ind^+ derivatives of JPT 11 in the way described in Section 7.2. (In the course of isolating the first batch of about 150 of these deletions, we also isolated 145 sup X Cys^- Trp^+ derivatives. These were used for mapping (Section 4.8).) None of this first batch of Cys^- Trp^- Ind^+ deletions grew when replica plated to plates of djenkolate-minimal agar containing anthranilate.

We then modified our selection procedures; the approach we had used previously would isolate only those deletions which permitted transcription of

Table 7-iii The Specific Activities of the Enzymes Anthranilate Synthetase and Tryptophan Synthetase β in Cultures of JPT 31 Grown in Conditions which Repress and Derepress Cysteine Biosynthesis

	Medium	Cystine plus Tryptophan		Djenkolate plus Tryptophan	
		Asase	TS - β	Asase	TS - β
		milliunits/mg protein (number of determinations)		milliunits/mg protein (number of determinations)	
STRAINS	JPT 31	4.6 (3)	600 (2)	7.2 (3)	3100 (2)
	JPT 1	142 (5)	2610 (4)	77 (5)	1010 (4)
	3110	30 (2)	ND	ND	ND

the trp B gene at a rate sufficiently high to allow growth on indole i.e. those ending operator proximal to trp P2 and those in which the other end of the deletion ended in a gene with an adequate level of transcription. Only if we could devise a method for maintaining the F'trp in the hybrid merodiploid would we be able to isolate deletions extending further into trp.

Since ton B was the only other bacterial gene known to be carried on this plasmid besides cys B and trp, we looked to see if it would be possible to select positively for ton B^+. The ton B gene product mediates sensitivity to phages T1, Ø80 and colicins V and B. It is therefore easy to select for ton B (Gottesman and Beckwith, 1969). Such mutants show another phenotypic effect: they require for growth on minimal agar both additional iron and the inclusion of citrate in the medium. Some sup X Trp^- Salmonellae were found to have the same requirements - "chr mutants" (Mukai and Margolin, 1963); these are presumably those strains in which the deletion extends into the gene homologous to ton B. Work in Salmonella showed that the additional growth requirements in effect reverse an inhibition of growth of these strains by chromic ion (Corwin Fanning, Feldman and Margolin, 1966). Further work showed that both E.coli and S.typhimurium possess two uptake systems for ferric ion: one functions only in the presence of citrate; the other is citrate independent. This latter system is defective in ton B strains. In the absence of citrate, growth of these mutants can occur only in the presence of concentrations of iron 10 times that required by ton B^+ strains, but this residual ability is inhibited by chromic ions. These act either by competitive inhibition or by the formation of Cr^{+++} / Fe^{+++} ion complexes (Wang and Newton, 1969).

We attempted to select for the maintenance and transfer of the ton B^+

allele between strains of S.typhimurium and E.coli, on minimal agar media lacking citrate (M9 and 'tris' media). This did not succeed as ton B and chr strains of bacteria could grow slowly on these media. However they did not grow at all on M9 minimal agar containing 5 mM chromic chloride, whereas all ton B^+ and chr^+ strains were able to do so. Furthermore we were able to select for transfer of the ton B^+ alleles between strains of Salmonella and E.coli using this medium.

We then made use of this system to attempt the isolation of deletions fusing cys B and trp. We isolated 580 Cys^- Trp^- ton B^+ derivatives of JPT 11, by selection for growth on M9 minimal agar lacking leucine but containing cystine, tryptophan and chromic chloride.

All the derivatives of JPT 11 isolated by these two procedures were then plate mated with JPT 89; selection was made for transfer of ton B^+ on M9 minimal medium containing cystine, tryptophan and chromic chloride with lactose as carbon source. We purified the Lac^+ progeny from these crosses and looked to see how many gave Cys^+ recombinants when replicated to the same medium lacking cystine; i.e. how many possessed sup X cys B deletions which stopped within the gene (short of cys B-54).

Only seven did so. The Salmonella strains harbouring these sup X Cys^- ton B^+ plasmids were numbered JPT 11 Δ 383, 393, 406, 413, 415, 450, 451. The first three of these strains had been isolated as Ind^+; the other four had been isolated as ton B^+; of these only JPT 11Δ415 was Ind^+.

We studied the trp end of the deletions present in these strains after transfer into the cys B-1 trp AE 12 background, exactly as in Section 7.3. The results of the analysis with Ø80 trp phage are given in Table 7-iv. They are

Table 7-iv Frequency of Recombination Occurring in Transductions of Strains Bearing cys B-trp Deletions with Derivatives of Phage Ø 80 pt 190 Carrying Point Mutations in the trp Operon

Deletion from JPT 11 Δ F'trp sup X	trp E 9914	E 9777	E 10247	E 5972	D 159	D 1383	C 782	B 9578	A 33
383	-	-	+	++	++	++	++	++	++
393	-	-	-	-	-	++	++	++	++
406	-	-	+	++	++	++	++	++	++
413	-	-	-	-	-	-	-	++	++
415	-	-	-	++	++	++	++	++	++
450	-	ND	ND	-	-	-	-	-	-
451	-	ND	ND	-	-	-	-	-	-

consistent with the Trp phenotypes of these strains. Since the deletions carried on F'trp sup X Δ 450 and 451 extended past trp A 33, they were discarded.

The cys B ends of the remaining deletions were located in relation to cys B-1 in exactly the same way as in Section 7.3 (but in the case of F'trp sup X Δ 413 maintaining selection for ton B^+ rather than for trp B^+). Only F'trp sup X Δ 406 recombined with this marker. However this plasmid did not give recombinants with the cys B-14 mutation present in JPT 113. These findings locate the cys B ends of the deletions in F'trp sup X Δ 383, 393, 413, 415 between cys B-1 and cys B-54 and that of the deletion in F'trp sup X Δ 406 between cys B-14 and cys B-1.

7.6 Biochemical Analyses of Strains Carrying Plasmids Containing Deletions Ending in cys B and trp

These were carried out as in Section 7.4 except that component II of anthranilate synthetase was measured (i.e. the product of the trp D gene) in place of the anthranilate synthetase complex. The results are given in Tables 7-v and 7-vi.

All the strains tested show very low levels of component II (3 to 7 x the background) except Δ 393 which shows no activity at all. This is consistent with the results of the genetic analysis of this strain which shows the sup X deletion entering the trp D gene, but leaving trp P2 intact. All three strains which do produce measurable levels of component II produce 30-100% more when grown in medium containing djenkolate than when grown in medium containing cystine. Similarly, the strain in which trp P2 has been deleted (Δ 413) shows low but measurable amounts of tryptophan synthetase-B, and these levels are also 50%

Table 7-v The Specific Activities of Component II of Anthranilate Synthetase in Strains Carrying Putative cys B-trp Fusions Grown in Conditions which Repress and Derepress Cysteine Biosynthesis

F'trp sup X Δ	background strain	Medium: Cystine plus Tryptophan	Medium: Djenkolate plus Tryptophan
		milliunits/mg protein (number of determinations)	
383	JPT 110	0.45 (2)	0.6 (2)
393	JPT 110	<0.1 (1)	<0.1 (1)
406	JPT 113	0.3 (1)	0.7 (1)
415	JPT 110	0.47 (2)	0.6 (2)

Table 7-vi The Specific Activities of Tryptophan Synthetase β-Subunit in Strains Carrying Putative cys B - trp Fusions Grown in Conditions which Repress and Derepress Cysteine Biosynthesis

F trp' sup X Δ	background strain	Cystine plus Tryptophan	Djenkolate plus Tryptophan
		milliunits/mg protein (number of determinations)	
383	JPT 110	580 (1)	2200 (1)
393	JPT 110	400 (1)	1600 (1)
413	JPT 110	85 (4)	124 (4)

higher in djenkolate-than in cystine-grown cells.

7.7 Discussion

We have been able to measure relevant trp enzymes in five strains in which there are deletions beginning in cys B and ending within the trp operon. In all these strains there is more enzyme in djenkolate grown than in cystine grown cells. Is this difference a reflection of transcription starting in cys B?

The discussion on the quantitative aspect of this difference in JPT 31 (Section 7.4) applies equally to the strains analysed in Section 7.6. However in addition these latter strains contain much lower levels of enzymes. In agreement with this, they do not exhibit the expected phenotypes. (Strains carrying deletions 383, 393, 415 do not grow on medium containing djenkolate and anthranilate and strains carrying deletion 413 do not grow on medium containing djenkolate and indole.) These low levels of trp enzymes may well be due to their principal difference from JPT 31; since their deletions end within structural genes of the trp operon, they unequivocally lack all its regulatory regions (except trp P2). Recent work (Bertrand, Korn, Lee, Platt, Squires, Squires and Yanofsky, 1975; Pouwels and van Rotterdam, 1975) show that in addition to trp P1 and trp O there is another region between trp O and trp E, mutations in which have regulatory properties. The deletion in JPT 31 might end in this region. An alternative explanation is that, as we would expect only one third of gene fusions to end 'in phase', it may be that the deletion in JPT 31 is the only one that does so.

Our observations that levels of tryptophan synthetase B in strains carrying deletions 383 and 393 are about 4 x higher in djenkolate-grown than in cystine-

grown cells is strong evidence against the possibility that altered transcription from cys B is responsible for the differences in enzyme levels in fusions grown in conditions which repress and derepress cysteine biosynthesis. In these strains trp P2 is intact; more than 90% of the transcription of the trp B gene starts at this site. It therefore seems more likely that changes in cysteine repression can affect the expression of the trp operon but by physiological means rather than by changes in the rate of read-through transcription from cys B.

We can make no firm conclusions about either the direction or the rate of transcription of cys B from the data presented in this Chapter. Since we expect only one third of all fusions to end 'in phase' we should have to isolate 8 such fusions (or 12) to obtain a 95% (or 99%) confidence level of not having missed such a deletion.

Chapter 8

Search for Mutants in E.coli Constitutive for the Synthesis of the Enzymes of the Cysteine Biosynthetic Pathway

8.1 Introduction

If cys B is the true 'positive' regulatory gene of the cysteine biosynthetic pathway, we should be able to locate within it mutations leading to constitutive synthesis of the enzymes of the pathway as well as mutations leading to their loss. Mutations in S.typhimurium which lead to partial constitutivity of the pathway have been found but their significance is not clear (Section 1.4).

We therefore set out to look for single-step mutants of E.coli constitutive in cysteine biosynthesis. Other workers had attempted this without success, which was not altogether surprising; if cys B did indeed code for an activator, like that coded for by ara C, we would predict that constitutive mutations in such a system would be analogous to lac I^s mutations and thus likely to be rare (Section 1.2). This prediction has been tested and confirmed recently in ara (Nathanson and Schleif, 1975).

The most common approach used to isolate mutants constitutive in a biosynthetic pathway is to look among mutants resistant to inhibitory analogues of metabolites of the pathway. This approach had been successful in work on the regulation of the biosynthesis of tryptophan (Section 7. 3) arginine (Maas, 1961) and histidine (Brenner and Ames, 1971). Ideally the inhibitor used should be a non-metabolisable false repressor or end product inhibitor; this would enable repressibility to be separated from metabolism (cf. Section 1.1), but selection for resistance to inhibitors with other modes of action (e.g.

competitive inhibitors) or even with unknown modes of action (e.g. fucose in arabinose catabolism - Englesberg, Irr, Power and Lee, 1965) can select for increased levels of enzyme and thus for constitutivity.

There were two inhibitors that looked as if they might be useful in our search for constitutivity of cysteine biosynthesis; selenate ion and 1-2-4 triazole.

Selenate and Group VI anions (chromate, tungstate, molybdate and vanadate) have been studied extensively as inhibitors of ATP sulphurylase (Bandurski, Wilson and Squires, 1956). They all compete for the active site of the enzyme with sulphate, which they resemble closely, but the products of the reaction are unstable; thus, in the presence of the enzyme they catalyse degradation of ATP. Pasternak (1962) showed that selenate inhibited the growth of E.coli in medium containing sulphate, but that cysteine prevented this inhibition. He suggested that selenate inhibits growth by the mechanism described above; its reversal depends upon the repression by cysteine of the synthesis of ATP sulphurylase. If this were so, one might expect that mutants constitutive for cysteine biosynthesis would be sensitive to selenate in the presence of cysteine. A search for mutants with this phenotype was unsuccessful (Jones-Mortimer, D.Phil. thesis, Oxford, 1968).

However, Pasternak's paper showed that the inhibition of growth by selenate could also be prevented by the addition of glutathione to the medium. We found that E.coli could indeed grow on minimal medium 2 containing 2 mM selenate when glutathione or djenkolic acid were sulphur sources but not when sulphate (1 mM) was the sulphur source. Since growth on glutathione or djenkolic acid derepresses the cysteine biosynthetic pathway, we concluded that selenate inhibited growth, not by causing the futile degradation of ATP, but

rather as a result of inhibiting cysteine biosynthesis, possibly by acting as a competitive inhibitor of sulphate uptake or activation; thus it was likely that mutants constitutive for cysteine biosynthesis could be resistant to selenate but remain prototrophic. Hulanicka (1972) showed that a partial constitutive mutant of Salmonella had just this phenotype, but that mutants selected as being resistant to selenate, though possessing partially derepressed levels of sulphite reductase, mapped not in cys B but near to cys A. Hulanicka named this locus cys L and concluded that it coded for a component of the sulphate uptake system.

The work with 1-2-4 triazole was carried out exclusively on S.typhimurium. This organism is inhibited by triazole but this inhibition, like that of selenate, can be reversed by cysteine. It can also be reversed by methionine, serine and OAS (Boguslawski, Walczak and Klopotowski, 1967; Hulanicka et al., 1972). Later work demonstrated that OAS sulphydrylase catalysed a reaction between triazole and OAS; the inhibition of growth was a result of the depletion of the OAS pools of the cells (Kredich, Foote and Hulanicka, 1975). Mutants resistant to triazole are common; they have low levels of OAS-sulphydrylase I, and the mutations map in cys K (trz A) or are unstable (trz B). Though the partially constitutive mutant mentioned above was resistant to triazole, none of the mutants selected for resistance to triazole could be located near cys B (Hulanicka and Klopotowski, 1972). It seemed very likely that, if a mutation could occur which conferred resistance to these analogues and simultaneously rendered the organism constitutive, the chances of isolating such a mutant would be diminished by its low frequency of occurrence in comparison with the occurrence of other mutations leading to resistance to the analogues.

We set out first to find if E.coli was sensitive to triazole, and then to see if any of our progeny selectively mutagenised in the cys B region were resistant to selenate or triazole or both.

8.2 The Sensitivity of E.coli to 1-2-4 Triazole

This was shown by the following experiment, similar to that of Boguslawski et al (1967).

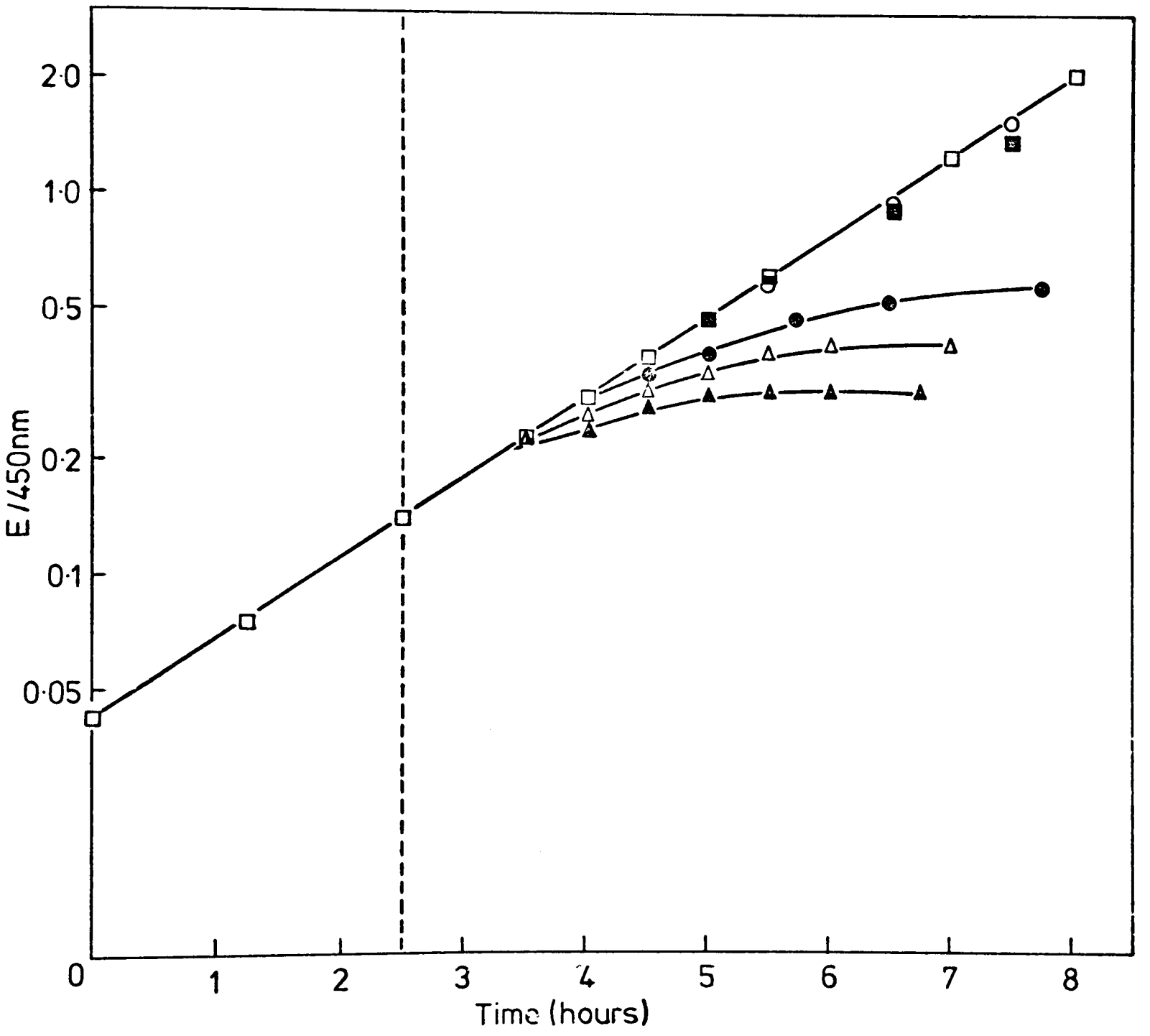
Sterile warm minimal medium 1 (200 ml in a 1 l flask) was inoculated with 2 ml of an overnight culture of strain 3110 in the same medium, and incubated with shaking at 37°C. Samples were taken at intervals for the measurement of E_{450}. After several generations of exponential growth, the culture was distributed into six pre-warmed sterile flasks containing the following compounds. The first flask contained triazole alone; the second triazole and glycine; the third triazole and serine; the fourth triazole and cysteine; the fifth triazole and methionine. In all flasks the concentration of triazole was 2 mM; that of the amino acid was 0.1 mM. The sixth flask was a control without additions. All flasks were incubated at 37°C and samples were withdrawn for measurement of E_{450}. The results are given in Fig. 8 a (compare with Fig. 2 of Boguslawski et al., 1967).

They show that this strain of E.coli is inhibited by triazole and that this inhibition is reversed and potentiated by the same compounds as in S.typhimurium. If anything E.coli appears more sensitive than Salmonella as inhibition occurs more rapidly.

Triazole was added to the media used in subsequent experiments at a final concentration of 10 mM. This was achieved by adding a 1M solution of

Fig. 8 a The inhibition of the growth of E.coli by 1-2-4 triazole, its potentiation by glycine and its reversal by cysteine, methionine and serine

	Triazole	Additions
□	none	none
Δ	2 mM	none
▲	2 mM	glycine 0.1 mM
●	2 mM	serine 0.1 mM
O	2 mM	cysteine 0.1 mM
■	2 mM	methionine 0.1 mM


2·0
1·0
0·5
0·2
0·1
0·05
0
E /450nm
1
2
3
4
5
6
7
8
Time (hours)

1-2-4 triazole, sterilised by autoclaving (115°C, 10 minutes at 10 lb pressure), to the already sterilised medium. Solutions of sodium selenate, (200 mM) were prepared, sterilised and added to the medium in the same manner, to give a final concentration of 2 mM.

8.3 Search for Constitutive Mutations in cys B - first attempt

In Section 4.6 we described how strains JPT 24 and 32 were transduced with mutagenised phage P1 to Ind^+ and Ura^+ respectively, and the transductants replicated to plates for detecting cysteine auxotrophy. In an attempt to detect constitutive strains with mutations in cys B, we also replicated the master plates to plates of minimal medium containing indole and either triazole or sodium selenate.

We replica plated a total of 18,000 colonies. Of these, 129 replicated to medium containing triazole and 47 to medium containing selenate. We were most interested in the 23 colonies that apparently replicated to both media.

These were taken from the selective plates and streaked to minimal medium containing indole and selenate or triazole and to the same medium without inhibitor. None of the colonies arising on the triazole replica plates grew on medium containing selenate. Similarly, none of the colonies arising on the replica plates containing selenate could grow on medium with triazole. This demonstrates that the resistance to these inhibitors arose from independent mutations.

In spite of this discouraging result we examined ten selenate-resistant and ten triazole-resistant clones to see if the mutation conferring resistance lay in or near cys B; the procedure was as follows:

We knew that if the marker were indeed located in this region, it should be about 55% co transducible with trp (Section 6.3). Transduction of strains containing such a marker to Trp^+ with phage grown on a Trp^+ Cys^+ strain known to be sensitive to selenate and triazole should then cause the displacement of the mutation conferring resistance by the incoming 'sensitive' allele in about half the progeny.

All the strains in question possessed the trp E 9829 mutation. They were purified on indole minimal medium, checking for the maintenance of their resistance, and transduced to Trp^+ with phage P1 grown on strain 3110. Progeny were selected for their ability to grow on minimal medium. At least 100 transductants derived from each original resistant strain were replicated to the same medium with and without the relevant inhibitor. All colonies were able to grow equally well on both media.

We may conclude from this result that in spite of the directed mutagenesis, none of the resistant mutants we isolated were the result of mutations in the cys B region. Since in the 18,000 transductants derived from mutagenised phage, we had isolated 11 cysteine auxotrophs, we concluded that mutations to constitutivity in cys B, if they occur at all, occur at a frequency of less than one tenth the frequency of mutations to cysteine auxotrophy.

8.4 Search for Constitutive Mutations in cys B - second attempt

8.4.1 Rationale

We thought it likely that the triazole - and selenate - resistant mutants encountered in the work described above were cys K and cys L mutants. How could we reduce the incidence of these mutations?

We knew that cys K and cys L mapped close together in S.typhimurium (Hulanicka and Kloporowski, 1972), and that cys L was recessive to cys L^+. We thought that it was possible that cys K would also be recessive to cys K^+; if this were so, and if we could construct a strain diploid for both genes, triazole resistance and selenate resistance as a result of mutations in cys K and cys L would be extremely rare in such a strain.

8.4.2 Demonstration of the Presence of the Genes cys K and cys L on the Plasmid F'198

To construct such a diploid, we required a plasmid that carried the region of the E.coli chromosome from about 45 to 48 minutes on the standard map (Taylor and Trotter, 1972). We chose plasmid F'198, which carries the genes sup N, pts and nad B; these map at 45, 46, and 49 minutes respectively. We showed that this plasmid did indeed carry the genes cys K and cys L by the following procedure.

Strain CGSC 4344 (pro C pts 140 bgl B rec A str^R / F'198) was grown in medic containing acridine orange to remove the plasmid. The haploid was identified by its ability to grow on minimal medium containing proline with lactate as carbon source but not on similar medium with mannitol as carbon source (pts mutants are deficient in the PEP dependent phosphotransferase system and are unable to take up hexitols). This strain was purified and numbered JPT 111. This strain, together with its diploid parent was checked for triazole - and selenate - sensitivity by streaking to lactate-minimal medium containing proline and triazole or selenate in the first case and mannitol-minimal medium containing proline and triazole or selenate in the second. Both strains were sensitive to both inhibitors.

Cultures of these strains were grown in sterile liquid minimal medium containing proline and appropriately either lactate or mannitol as carbon source (100 ml volumes in 1 l flasks) at 37°C overnight with shaking. The cells collected by centrifugation from these cultures were washed in saline and finally resuspended in 2.5 ml volumes of saline. Portions (0.1 ml) of these suspensions were diluted and plated on media identical to that in which the cells had been grown, but solidified with agar. The rest of the suspension was spread onto plates of similar medium but containing either triazole or selenate. These plates were then incubated at 37°C for 48 h.

We observed that in JPT 111 mutants to triazole- and selenate-resistance arose at frequencies of 5 per 10^7 cells plated and 2 per 10^7 cells plated respectively. We did not observe any triazole- or selenate-resistant clones arising from the plates spread with CGSC 4344 (about 10^{11} cells plated). We can account for the absence of selenate resistant clones in the latter strain (Hulanicka and Klopotowski, 1972); it is extremely likely that a similar mechanism prevents mutation to triazole resistance i.e. the plasmid carries the cys K gene, that cys K mutations are recessive to cys K^+, and therefore a mutation in both cys K genes is required to permit the diploid strain to become resistant to triazole by this mechanism.

8.4.3 Construction of Strains of E.coli Diploid for the cys K and cys L Genes (JPT 117 and 118)

Strain CGSC 4344 is rec A, and was therefore unsuitable as a parent strain for the work to be described below. We required a strain of E.coli which was diploid for the cys A - L - K region, which was cys B^+ and which carried mutations near this gene. We constructed two such strains.

Construction of a Strain pyr F trp E 9829 cys A/F'198 (JPT 117)

A cys A mutation was isolated in strain JPT 32 (pyr F trp E 9829) by selection for resistance to chromate (Pardee et al., 1966). A culture of strain strain JPT 32 in minimal medium 2 containing uracil and indole, with djenkolate as sulphur source was grown overnight at 37°C with shaking. Cells from this culture were harvested, washed, diluted appropriately and spread onto plates of the same medium with and without 0.25 mM potassium chromate. After incubation of these plates at 37°C for 72 h, colonies grew on the plates containing chromate at 1×10^{-7} of the frequency of colonies on the same medium without chromate. Several of these resistant colonies were purified and tested nutritionally. They grew on minimal medium 2 containing djenkolate, cystine or sulphite as sulphur source but not on similar medium containing sulphate or thiosulphate. They all required uracil and indole. This phenotype is that expected of a cys A mutant of JPT 32. One such isolate was numbered JPT 115.

This isolate was mated with CGSC 4344 (see Materials and Methods) and selection made for Cys^+ on minimal medium containing uracil and indole, but not proline. Colonies arose on these plates but not on similar plates spread with donor or recipient alone. One such isolate was purified and tested: it was Ura^- Ind^- Cys^+ but segregated Cys^- clones. It was thus pyr F trp E 9829 cys A/F'198 cys A^+. It was numbered JPT 117.

Construction of a Strain pyr F trp E 9829 pts^Δ/F'198 (JPT 118)

The pts mutation was isolated in strain JPT 32 by selection for resistance to the antibiotic phosphonomycin (Roseman, 1972). A culture of this strain in broth was grown overnight and the cells were harvested, diluted and plated on

nutrient agar containing 125 μg of the drug per ml. One plate containing about 100 colonies was replicated to plates of minimal medium lacking the drug and containing uracil, indole and either lactate or mannitol as a carbon source. After incubation of these plates overnight at 37°C 6 colonies were observed to be able to grow only on the plates containing lactate. These were purified and checked. All proved to be Ura$^-$ Ind$^-$ and able to grow well on appropriately supplemented minimal medium with lactate as carbon source. Five of these isolates were unable to grow on similar medium with mannitol (or other hexitols) as carbon source but grew slowly on similar medium with glucose as carbon source. The sixth isolate was unable to utilise hexitols and in addition was unable to utilise glucose. The probable explanation for this is that this isolate contains a deletion of the pts gene which extends into crr (similar deletions have been reported in S.typhimurium - Cordaro and Roseman, 1972). This is supported by the observation that it did not revert to ability to utilise glucose (and see below). This isolate was numbered JPT 116.

The F'198 plasmid was introduced into this strain by a mating with CGSC 4344 exactly as described in the previous section. The pts$^+$ progeny were Ura$^-$ Ind$^-$ and segregated progeny which could grow on media containing lactate as carbon source, but not with glucose or mannitol.

The fact that the introduction of the plasmid into JPT 116 conferred simultaneously the ability to grow with glucose or mannitol as carbon source supports our explanation of the nature of the pts mutation in this strain. One pts$^+$ derivative of JPT 116 from this cross was purified and numbered JPT 118.

8.4.4 A Selenate and Triazole Resistant Derivative of JPT 117 (JPT 119)

The frequencies of mutation to selenate - and triazole - resistance were tested in strains JPT 32, 116, 117, 118 in a similar manner to that given above (Section 8.4.2). As described in that Section the haploid strains mutated to resistance at frequencies of approximately 10^{-7}. Spontaneous mutation to resistance did not occur in JPT 118. We recovered one resistant isolate only of JPT 117 from medium containing triazole (7×10^{10} cells plated). This was purified by streaking on the same medium and numbered JPT 119.

8.4.5 Characterisation of JPT 119

Its phenotype was as follows; it was Ura^- Ind^-; it grew well on minimal medium containing triazole, slowly on minimal medium containing selenate, and not at all on minimal medium containing chromate (0.25 mM). It segregated Cys^- clones at high frequency after growth in media containing acridine orange.

We grew cultures of this isolate with djenkolate or cystine as sulphur source in order to obtain cells derepressed or repressed for the cysteine biosynthetic pathway. We also grew the strains from which it was derived and other relevant controls under the same conditions and measured the levels of sulphite reductase in cells from such cultures. The results are given in Table 8-i.

They show clearly that cells of JPT 119 contain 4-5 times as much sulphite reductase as do the strains from which it was derived, when compared under repressing conditions, but that they can be derepressed only to the same extent. JPT 119 therefore appears to be constitutive for at least part of the cysteine biosynthetic pathway.

Table 8-i Levels of Sulphite Reductase in Mutants of E.coli

	Cystine as sulphur source		Djenkolate as sulphur source	
Strain	Sulphite reductase milli units/mg protein	number of estimations	Sulphite reductase milli units/mg protein	number of estimations
JPT 32	6.5	4	96	3
JPT 115	6.4	1	76	1
JPT 117	6.0	3	86	1
JPT 124	6.0	2	77	1
JPT 119	28	4	82	4

We attempted to locate the mutation conferring this constitutivity by transduction, as in Section 8.3. During this procedure, we discovered two further characteristics of this mutation; first, that it was unstable and second, that the strain harbouring it, when streaked side by side with cells of triazole-sensitive strains on medium containing triazole, could allow these latter strains to grow.

We transduced JPT 119 to Ura^+ Ind^+ with phage grown on strain 3110, selecting on minimal medium. Of 110 such colonies replicated to the same medium with and without triazole, only five proved to be triazole resistant. This indicates that the mutation is associated with the cys B region. At the same time, however, we also diluted and plated cells that had been grown up for this transduction but not treated with phage P1, on minimal agar containing uracil and indole. Replication of 91 such colonies to the same medium with and without triazole showed that three of them had lost triazole resistance, and therefore that the mutation is unstable.

When the plates containing both triazole resistant and triazole sensitive colonies were incubated for more than 14 h, we found that all the colonies started to grow on medium containing triazole, due to cross feeding. This we showed to be a result of the over-production of sulphide by JPT 119, as this organism could cross feed strains AT 713 (cys C), Salmonella D-234 and J-266 and all cys B strains tested, but not JM 464 (cys E).

Phage P1 grown on JPT 119 was used to transduce cys B recipients (cys B-1, 14, 39, 42 and 54) to Cys^+. On streaking such transductants to medium with and without triazole, we found that 50-70% of them were resistant to triazole. All such triazole-resistant clones tested produced sulphide as

judged by their ability to cross-feed cysteine auxotrophs. Purification of these triazole resistant transductants was difficult because of their instability; they segregated both Cys^+ triazole sensitive and Cys^- clones. In contrast, those Cys^+ transductants that were initially triazole sensitive remained triazole sensitive and did not segregate Cys^- clones. Cultures of five purified Cys^+ triazole-resistant transductants and five triazole sensitive transductants were grown in minimal medium 2 with cystine as sulphur source; the cells were harvested and their levels of sulphite reductase measured. These are given in Table 8-ii.

8.4.6 Isolation of More Triazole-resistant Derivatives of Strains of E.coli Diploid for the Genes cys K and cys L.

We isolated more triazole resistant clones from the diploid strains after mutagenesis with EMS and NTG in the hope of finding mutants with a similar phenotype but which were, however, stable. We isolated 5 strains from JPT 117 and 4 from JPT 118. All proved to be unstable and were not studied further.

8.5 Discussion

The precise nature of the mutants isolated in the work described in this Chapter is not clear. It is difficult to make a rigorous comparison, from the data published, between them and the mutations (cys K and trz B) which have been isolated in S.typhimurium and which also confer resistance to triazole.

In that they are unstable, they resemble trz B mutants. Though trz B mutants have not hitherto been isolated in E.coli (Section 8.3; Hulanicka, personal communication), we should point out that haploid strains only have been tested. In this respect, it would be interesting to know whether trz B

Table 8-ii Levels of Sulphite Reductase in Triazole-Resistant and Triazole-Sensitive Transductants derived from JPT 119 as donor

Recipient	Triazole sensitive Sulphite reductase (milli units/mg protein)	Triazole resistant Sulphite reductase (milli units/mg protein)
JPT 92	5.4	30
JPT 92	5.8	28
JPT 92	5	28
JPT 39	5.2	26
JPT 39	5	30

All cultures were grown with cystine as sole source of sulphur

mutations occur in strains of S.typhimurium that have been cured of the LT-2 'silent' plasmid.

They are clearly not cys K (trz A) mutants, as the occurrence of cys K mutants was prevented during their isolation; in addition cys K mutants are stable, are characterised by only slight derepression of the cysteine enzymes and are selenate-sensitive (Hulanicka and Klopotowski, 1972). Though cys K mutations can interact with cym mutations which map in certain cys genes, no cym mutations have been found to map in cys B (Qureshi et al., 1975). Since trz B mutants possess the same enzyme deficiency as cys K mutants (Hulanicka et al., 1974) and presumably are thus resistant to triazole for the same reason, it is likely that trz B mutants are only slightly derepressed for cysteine biosynthesis; if this is true they will consequently differ from our partially constitutive mutants.

In this respect, it would have been valuable to have measured the level of OAS-sulphydrylase in these mutants, as well as to have continued the genetic analysis. However though these mutants are interesting, and will repay further investigation, they do not appear to be 'simple' constitutives of the ara C^c type (and therefore analogous to lac I^s). They might, however, possess multiple copies of a 'normal' cys B gene (either a tandem duplication or a duplicate integrated into a plasmid). This idea would be consistent with the results of the transductional analysis. We can envisage that a strain in which over-production of a normal activator occurs could possess a constitutive phenotype (cf. Muller-Hill, Crapo and Gilbert, 1968).

Concluding Discussion

In this we shall discuss the significance of our experimental results in relation to both the criteria required for the identification of a positive control gene (Section 1.4) and recent publications on the cys B region in Salmonella (Cheney and Kredich, 1975; Borum and Monty, 1976).

We have found that the cys B^+ allele can complement all amber mutations identified within the cys B gene of E.coli (Section 4.4), as well as a deletion extending into the cys B region of Salmonella (Section 4.2). This therefore confirms and extends the work of Jones Mortimer (1968b), and excludes the possibility that the dominance of cys B^+ over cys B is a consequence of subunit mixing.

Though we did not succeed in isolating constitutive mutants within the cys B gene of E.coli, one partial constitutive cysteine mutation has been located within the cys B region of Salmonella by deletion mapping in a manner similar to ours (Cheney and Kredich, 1975). Its location correlates with reversion studies on cys B mutants (Borum and Monty, 1976).

It is interesting to note that Cheney and Kredich were unable to demonstrate abortive transduction between mutations previously characterised as members of the classes cys Ba Bb and Bc; furthermore they located cys Bb mutations to either side of cys Ba and cys Bc mutants (cf. Section 6.4).

These results together with our demonstration that the cys B region in E.coli contains only one gene (Section 6.2) and that this gene is functionally as well as genetically homologous with the cys B region in Salmonella (Section 4.2) makes it almost certain that the mutations leading to pleiotropic loss of expression and to constitutivity of expression of the cys structural genes both

reside in the same gene. Since the RNA made from this gene is translated (Section 5.3), this control is mediated via the protein product of the cys B gene.

In Section 1.4 we proposed criteria for defining positive regulatory genes:

1. The isolation of deletion mutants or nonsense mutations within the gene which give the pleiotropic negative phenotype and which can be complemented by the intact gene.

2. The exclusion of the regulator gene from the operon(s) it controls.

3. The isolation and mapping within the regulatory gene of pleiotropically constitutive mutations.

4. The demonstration of trans dominance over these constitutive mutations over both pleiotropic negative and inducible alleles.

5. The isolation and characterisation of cis dominant constitutive mutations in the controlled operons as revertants of the pleiotropic negative mutations and the ability to account for all such revertants with this mechanism.

The cys B gene now fulfils the first three of these criteria and is therefore very likely to be a positive regulatory gene. However further work is required to prove this hypothesis. We can predict that the fourth criterion, though perhaps not the fifth, will be easy to test. On the other hand, now that a specialised transducing phage carrying the cys B gene of E.coli has been isolated (Brammar, personal communication) experiments to provide the most rigorous test of such a hypothesis, the isolation of the cys B gene product, are now possible. These are already in progress (Mascarenhas, personal communication).

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