Positive Control of Sulphate Reduction in *Escherichia coli*

THE NATURE OF THE PLEIOTROPIC CYSTEINELESS MUTANTS OF *E. COLI* K12

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1. The function of the wild-type alleles of the pleiotropic mutants *cysB* and *cysE* of *Escherichia coli* was investigated. 2. The wild-type allele *cysB*⁺ is dominant to the mutant allele *cysB* in stable and transient heterozygotes. 3. The wild-type allele *cysE*⁺ is dominant to the mutant allele *cysE*, as predicted. 4. Sulphur-starved cultures of *cysB* or *cysE* strains contain less than 0.2 nmole of free cysteine/mg. dry wt. 5. Complementation *in vitro* is not observed between extracts of *cysB* mutants and mutants lacking sulphite reductase only. 6. A scheme, involving positive control of the enzymes of sulphate activation and reduction, is suggested to account for the control of cysteine biosynthesis.

Two classes of *Escherichia coli* mutant, *cysB* and *cysE*, are of special interest, as they lack all or almost all the enzymes required for sulphate activation and reduction (Jones-Mortimer, 1968b), though the map of the cysteine loci (Jones-Mortimer, 1968b) shows that neither of these types of mutant can have deletions of all the structural genes corresponding to these enzymes. How then do their mutations exert their pleiotropic effect?

Pleiotropic mutants might in theory arise in any of the ways listed in Table 1, and the predicted properties of these types of mutants should allow them to be distinguished.

To determine which of these explanations is correct, four types of experiment were carried out. First, the dominance of the *cysB*⁺ and *cysE*⁺ alleles was investigated. Both *cysB* and *cysE* mutations were found to be recessive to the wild-type alleles; hence it was unnecessary to attempt to distinguish further between mutations of types 1, 3 and 5 (Table 1). Secondly, the size of the cysteine pool in *cysB* and *cysE* mutants that had been starved of sulphur was investigated. Thirdly, an attempt was made to demonstrate complementation *in vitro* between extracts of *cysB* or *cysE* mutants and mutants lacking only one enzyme. Fourthly, revertants of the *cysB* locus were tested for constitutive synthesis of PAPS† synthetase.

A preliminary account of these experiments has been published (Jones-Mortimer, 1968a).

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† Abbreviation: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

MATERIALS AND METHODS

Organisms. *E. coli* K12 Broda 10 (Hfr met; streptomycin-sensitive) was kindly supplied by Professor W. Hayes. *E. coli* C3000 (*thi*) and the male-specific bacteriophage MS2 were kindly supplied by Dr M. R. Lunt. Other strains of *E. coli* K12 are described in the preceding paper (Jones-Mortimer, 1968b).

Construction of hybrid strains. (a) Strain JM78. An overnight (stationary-phase) broth culture (0.5 ml.; about 5×10^8 cells) of strain YS-57 was diluted with 4 ml. of fresh broth, and mated with about 5×10^7 cells of an early-exponential-phase culture of strain Broda 1 for 90 min. A portion of the mixture was diluted 100-fold with iso-osmotic phosphate buffer and blended for 1 min. Portions (0.1 ml.) of this cell suspension were plated on minimal medium supplemented with proline. The plates were incubated at 36° for about 40 hr. Histidine-positive colonies were picked, and screened for proline requirement and streptomycin sensitivity. A selected *pro str*^s colony (strain JM78) was shown to be able to restore strain JM 63 (*cysB*) to cyst(e)ine independence.

(b) Strain JM99. Strain JM73 was mated with strain Broda 10 (Hayes, 1957). Mating was interrupted after 5 hr. by blending the cells with a Whirlimixer, and samples were plated on minimal medium supplemented with threonine, leucine, tryptophan, cystine, arginine and thiamine, to select for *his*⁺ recombinants. The *his*⁺ clones were replicated on to plates supplemented with threonine, leucine, tryptophan, arginine and thiamine (with sulphate as the sulphur source) to determine which of the *his*⁺ recombinants were *cys*, and in duplicate on to lawns of about 10^8 cells of strain 712 on minimal-medium plates supplemented with tryptophan and histidine to determine which of the recombinants were Hfr (cf. Taylor & Adelberg, 1960). A single colony (strain JM99) was reisolated from one of the Hfr *cys* clones.

(c) Strain JM107. A *thr*⁺ *leu*⁺ derivative (strain JM104) was obtained by mating strain JM73 with strain Broda 1

Table 1. *Possible effects of pleiotropic mutations*

Mutant type	Predicted properties
(1) Super-repressor	Dominant to wild-type in heterozygotes
(2) Defective utilization of repressor	Recessive to wild-type; high repressor pool under conditions of sulphur starvation
(3) Excessive production of apo-repressor	Dominant to wild-type
(4) Loss of an activator	Recessive to wild-type; provided that any postulated repressor is an intermediate between sulphate and protein cysteine, its intracellular concentration under conditions of sulphur starvation cannot be greater than that required for complete repression of the enzymes
(5) Enhanced glutathionase activity	Dominant to wild-type (high glutathionase activity)
(6) Loss of a required structural unit	Recessive to wild-type; complementation <i>in vitro</i> ; low repressor pool during sulphur starvation

for 1 hr. and plating on minimal medium supplemented with tryptophan, histidine, cystine, arginine and thiamine. Strain JM104 was mated with strain AB2271 for 5 hr. (cf. construction of strain JM99) and *arg⁺* recombinants were selected on minimal medium supplemented with tryptophan, histidine, cystine, isoleucine and valine. Recombinants were tested for their cystine and isoleucine + valine requirement, and for their ability to ferment xylose. A single colony (strain JM107) was reisolated from a *cys ilv xyl⁺* clone and shown to be Hfr.

Chemicals. DL-Proline was obtained from F. Hoffmann-La Roche et Cie. (Basle, Switzerland), and sodium aspartate from British Drug Houses Ltd. (Poole, Dorset). All other chemicals were as described by Jones-Mortimer (1968b).

Growth of organisms. Solid medium and broth were as described by Jones-Mortimer (1968b). The minimal medium, pH 6.3, was that described by Jones-Mortimer, Wheldrake & Pasternak (1968), and was supplemented with amino acids (40 mg. of the L form/l.) and thiamine hydrochloride (4 mg./l.) when required. Sulphur compounds were added as indicated. Organisms were grown and mated at 36–37°. The number of cells/ml. was determined nephelometrically as described by Pasternak (1962) and by a viable-cell count on complete medium. Bacteriophage MS2 was grown on *E. coli* C3000 by the confluent-lysis plate technique of Swanstrom & Adams (1951). The phage suspension was purified by dialysis against 5 mM-potassium phosphate buffer, pH 7, overnight at 4°. The phage titre was obtained by plating the phage with *E. coli* C3000 on L-broth (Lennox, 1955).

Assay of radioactivity. Samples were counted at infinite dilution in a low-background automatic-sample-changing counter (Isotope Developments Ltd., Beenham, Berks.). The counting efficiency was 5%.

RESULTS

Dominance of the wild-type alleles: design of the experiments

None of the enzymes of the sulphate-reduction pathway is particularly easy to assay. This difficulty can be avoided if the experiment can be designed so that the heterozygotes (but neither parent) can utilize sulphate as the sole sulphur source. [³⁵S]Sulphate may then be included in the

mating mixture, and the ³⁵S will be incorporated into protein only in heterozygotes, and in the recombinant progeny that have inherited the *cys⁺* phenotype from a heterozygote.

Preliminary experiments showed that strain JM78 incorporated ³⁵S from [³⁵S]sulphate into protein in the presence of streptomycin only if the enzymes of the sulphate-reduction pathway were de-repressed before the addition of the antibiotic (cf. Fitzgerald, Bernheim & Fitzgerald, 1948). Therefore, when strain JM78 was used as a donor, mating was effected in medium containing cystine and the cells were transferred to medium containing [³⁵S]sulphate, streptomycin and GSH.

Strains JM78 and JM63 failed to mate in the medium described by Pasternak (1962) supplemented with cystine (and the other amino acids required). The mating medium described by Jones-Mortimer *et al.* (1968) was therefore used; this is essentially the medium described by Pasternak (1962) adjusted to pH 6.3, with sodium DL-aspartate (final concn. 0.2 mg./ml.) added (Fisher, 1957) just before mating.

F⁺ heterozygotes of the *cysB* gene. Strains JM78 and JM63 were grown overnight on supplemented mating medium with cystine (100 mg./l.) as the sulphur source, and subcultured by diluting them 1:50 in fresh medium. When in early exponential phase, 9.1×10^8 cells of strain JM63 were mixed with 2.35×10^8 cells of strain JM78 in a total volume of 15 ml., and incubated at 37° with gentle shaking. A corresponding number of cells of each strain was diluted to 15 ml. with fresh medium, and incubated in parallel. After 77 min., the cells from each culture were transferred (by the Millipore-filter technique of Kjeldgaard, Maaløe & Schaechter, 1958) to 15 ml. of fresh medium containing no cystine, but with [³⁵S]sulphate (60 µC, 1.5 µmoles) and streptomycin (100 µg./ml.), and blended for 1 min. The cultures were then reincubated as before. At 15 or 20 min intervals, portions (0.5 ml.) from each culture were pipetted into 5 ml. of 10% (w/v)

Fig. 1. Incorporation of ^{35}S (from $[^{35}\text{S}]$ sulphate) into trichloroacetic acid-insoluble material by F' heterozygotes of the *cysB* gene. Strains JM63 (F^- *cysB*, streptomycin-resistant) and JM78 (F' *cysB* $^+$, streptomycin-sensitive) were mated in supplemented minimal medium with cystine as the sulphur source for 77 min., and then transferred (indicated by the arrows) to medium containing streptomycin and $[^{35}\text{S}]$ sulphate as the sulphur source. ●, *cysB* $^+$ heterozygotes; ○, radioactivity incorporated.

trichloroacetic acid-0.1M-sodium sulphate, and portions (0.05 ml.) were removed from the mating flask, diluted 1:100 with iso-osmotic phosphate buffer, blended and further diluted 1:100, and 0.1 ml. of the suspension was plated on supplemented minimal medium with sulphate as the sulphur source. The trichloroacetic acid-precipitated protein was collected on Millipore filters, which were washed with 5 and 10 ml. of the precipitant, stuck on to planchets and dried. The radioactivity incorporated into the precipitated protein was measured and the sum of the radioactivity incorporated by the male and female cultures separately was subtracted from the radioactivity incorporated by the mating mixture. This difference was assumed to represent the amount of radioactivity incorporated by the zygotes. This value, together with the number of *cys* $^+$ *pro* $^+$ cells in the mating medium, is plotted as a function of time in Fig. 1.

Since incorporation of ^{35}S from $[^{35}\text{S}]$ sulphate starts within 20 min. of the step-down, this experiment indicates that *cysB* $^+$ could be dominant to *cysB*. It is, however, not a conclusive result because, first, Markovitz & Rosenbaum (1965) have shown that in the production of cell-wall material any allele on an episome may be dominant to any chromosomal allele, a phenomenon that may be widespread and, secondly, it is possible that recombination and segregation are taking place.

Transient heterozygotes of the cysB gene. To obviate the objection against F' heterozygotes, a

Fig. 2. Incorporation of ^{35}S (from $[^{35}\text{S}]$ sulphate) into trichloroacetic acid-insoluble material by transient heterozygotes of the *cysB* gene. Strains JM63 (F^- *cysB*) and JM99 (Hfr *cysP*) were mated in supplemented minimal medium with GSH and $[^{35}\text{S}]$ sulphate as the sulphur sources. ●, *cysB* $^+$ recombinants; ○, radioactivity incorporated.

similar cross was carried out with an Hfr strain that transfers the *cysB* $^+$ locus with high frequency. In these transient heterozygotes the number of *cysB* $^+$ genes per F^- cell is unlikely to exceed unity until after recombination and segregation.

Strains JM99 (Hfr *cysP*) and JM63 (F^- *cysB*) were grown separately overnight on supplemented mating medium with GSH (0.125 mM) as the sulphur source, and subcultured by diluting them 1:25 in fresh medium. When in early exponential phase, 9.3×10^8 cells of strain JM63 were mixed with 2.95×10^8 cells of strain JM99 in a total volume of 15 ml. of mating medium + GSH, to which sodium $[^{35}\text{S}]$ sulphate (60 μC , 1.5 μmoles) was added. Corresponding numbers of cells of each culture were diluted with fresh medium to 15 ml., and incubated with equal quantities of $[^{35}\text{S}]$ sulphate in parallel. Samples were taken at 20 min. intervals to determine the amount of radioactivity incorporated into protein and the number of recombinants, as in the previous experiment. The results of this experiment are plotted in Fig. 2.

These results show that the *cysB* female strain acquires the ability to incorporate ^{35}S from $[^{35}\text{S}]$ sulphate within 70 min. of the beginning of mating, and within 40 min. (at most) of the transfer of the *cysB* $^+$ gene. This result is not compatible with the hypothesis that recombination and segregation are necessary for the expression of the *cysB* $^+$ allele, as the generation time of strain PA 309 in this medium with sulphate as the sulphur source is 105 min. and the zygotes do not grow any faster in the GSH-sulphate mixture. It therefore appears that *cysB* $^+$ is dominant to *cysB*.

Fig. 3. Effect of male-specific bacteriophage MS2 on the formation of transient heterozygotes of the *cysB* gene and on the incorporation of ^{35}S (from $[^{35}\text{S}]$ sulphate) into trichloroacetic acid-insoluble material. Strains JM14 (F^- *cysB*) and JM99 (Hfr *cysP*) were mated in supplemented minimal medium with GSH and $[^{35}\text{S}]$ sulphate as the sulphur sources. Bacteriophage MS2 was added 90 min. after the beginning of mating (indicated by the arrow). ●, *cysB* $^+$ recombinants; O, radioactivity incorporated.

Fig. 4. Incorporation of ^{35}S (from $[^{35}\text{S}]$ sulphate) into trichloroacetic acid-insoluble material by transient heterozygotes of the *cysE* gene. Strains JM15 (F^- *cysE*) and JM107 (Hfr *cysP*) were mated in minimal medium with GSH and $[^{35}\text{S}]$ sulphate as the sulphur sources. ●, *cysE* $^+$ recombinants; O, radioactivity incorporated.

In an attempt to confirm this result, this experiment was repeated by using the same male strain with two other *cysB* mutants, strains JM13 and JM14. Heterozygotes of these strains showed no ^{35}S uptake until 3 hr. after the beginning of mating, and even then it was considerably less than that shown by the heterozygotes of strain JM63. This result could be interpreted in two ways: either the *cysB* $^+$ allele of strain JM99 is recessive to the *cysB* alleles of strains JM13 and JM14, or the *cysB* $^+$ allele functions less effectively in strains JM13 and JM14 than it does in strain JM63.

To distinguish between these possibilities, an experiment was carried out to determine whether the heterozygotes begin to utilize $[^{35}\text{S}]$ sulphate before or after segregation of the *cysB* $^+$ allele.

Strains JM14 (F^- *cysB*) and JM99 (Hfr *cysP*) were grown separately overnight in supplemented mating medium with cystine as the sulphur source, harvested, washed, resuspended in buffer and inoculated into supplemented minimal medium with GSH as the sulphur source. When in early exponential phase, 4.5×10^8 cells of strain JM14 were mixed with 3.0×10^7 cells of strain JM99 in 10 ml. of supplemented mating medium + GSH, to which sodium $[^{35}\text{S}]$ sulphate ($40 \mu\text{C}$, $1 \mu\text{mole}$) was added. The male and female strains were also cultured separately as controls, as in the previous experiments. Samples were taken as before to determine the incorporation of ^{35}S and the number of recombinants. To each flask, after 90 min., 1 ml. of a suspension of bacteriophage MS2 (about

2.85×10^{11} phages) in dilute buffer was added, together with calcium chloride (final concentration 2 mM). Sampling was continued for a further $5\frac{1}{2}$ hr.

The results (Fig. 3) show that the addition of bacteriophage MS2 prevents further mating in the culture, and that after the addition of bacteriophage MS2 the number of recombinants does not increase significantly for a further $3\frac{1}{2}$ hr. The generation time of strain 703 (from which strain JM14 is derived) in this medium is about 75 min. Segregation in a bacterial heterozygote takes place one generation before the segregants start to increase in number. Therefore until about 4 hr. after the beginning of mating there can be no female cells that are homozygous for the *cysB* $^+$ gene. Since there is an appreciable uptake of ^{35}S by 4 hr. after mating, the *cysB* $^+$ allele must be dominant to the *cysB* allele in these heterozygotes also.

Since no *cysB* mutant was found (Jones-Mortimer, 1968b) that did not permit the expression of the *cysB* $^+$ episomal gene, it was assumed that in all cases *cysB* $^+$ is dominant to *cysB*.

Transient heterozygotes of the cysE gene. Exponential-phase cultures of strains JM15 (F^- *cysE*) and JM107 (Hfr *cysP*) (utilizing GSH as the sole sulphur source) were obtained as in the previous experiment. About 1.3×10^9 cells of strain JM15 were mixed with 2.9×10^8 cells of strain JM107, in 15 ml. of supplemented mating medium + GSH, containing sodium $[^{35}\text{S}]$ sulphate ($60 \mu\text{C}$, $1.5 \mu\text{moles}$). Control cultures of the male and female strains separately were grown as in the previous experiments. Samples were taken at 20 min. intervals to

measure the incorporation of ^{35}S into protein and the number of recombinants. The results (Fig. 4) show that ^{35}S incorporation starts within 1 hr. after the beginning of mating. Therefore the *cysE*⁺ allele is dominant to the *cysE* allele.

Measurement of the intracellular cysteine concentration

It has been shown by Wheldrake (1967) that the degree of repression of the enzymes of sulphate reduction can be correlated with the intracellular cysteine concentration.

Recessive pleiotropic mutants might have abnormal repressor pools under conditions of sulphur starvation (Table 1). An attempt was therefore made to measure the internal cysteine concentration in cells of strains JM14 (*cysB*) and JM15 (*cysE*) by the [^{14}C]N-ethylmaleimide technique of Wheldrake (1967).

No radioactivity could be detected in the area of the chromatogram where the N-ethylmaleimide derivative of cysteine is known to run. All radioactive areas in the chromatogram of the extract of strain JM14 (*cysB*) were shown to be due to the degradation of the N-ethylmaleimide. The extract of strain JM15 (*cysE*) showed one extra radioactive area, corresponding to the N-ethylmaleimide derivative of GSH. [Strain JM15 (*cysE*) does not grow well with GSH as the sulphur source.]

From these results it was concluded that during sulphur starvation neither *cysB* nor *cysE* mutants contained significant quantities of free cysteine or other thiols (i.e. they contained less than 0.2 nmole/mg. dry wt.), and it was therefore assumed that neither type of mutant had a large pool of a small-molecule repressor under the conditions of the experiment.

Complementation in vitro

Extracts (2–4 mg. of protein) of strains JM37 (*cysB*), JM38 (*cysB*), JM39 (*cysE*) and 13A (*cysB*), grown with GSH as the sulphur source, were mixed with extracts (4 mg. of protein) of strains JM40 and 11B, and the mixtures were assayed for sulphite reductase. None of the mixtures showed any detectable sulphite reductase activity (< 10 mmoles of sulphide formed in 30 min.). Under the conditions of this assay extracts of the wild-type produce about 200 mmoles of sulphide/mg. of protein. These mutants show no complementation *in vitro*.

*Revertants of the *cysB* locus*

Isolation of strains. Strain JM63 was grown overnight in broth, diluted 1:100 with minimal medium, and 0.1 ml. of this cell suspension was

inoculated into 5 ml. of supplemented minimal medium with sulphate as the sulphur source. Any culture that had not grown after 48 hr. was re-inoculated until 20 out of 46 tubes produced revertants. These were shown not to be prototrophic contaminants as they grew on supplemented but not on unsupplemented minimal-medium plates.

Test for constitutive synthesis of PAPS synthetase. The 20 revertants of strain JM63 were grown overnight in 10 ml. of supplemented minimal medium with limiting glucose (0.075%) and cystine as the sulphur source. Strain PA309 was grown in similar medium (but with sulphate as the sulphur source) as a control. The cells were harvested, washed with 5 ml. of sulphur-free medium and re-harvested. The cell pellet was resuspended in the liquid that failed to drain out of the tube, and 1 drop of toluene was added. The toluene-lysed cells were assayed for PAPS synthetase. Only the sulphate-grown cells of strain PA309 showed any PAPS synthetase activity.

This experiment would have demonstrated constitutive synthesis of the enzymes of sulphate activation in the revertants, but failed to do so. Though the result may be interpreted in a number of ways, it is further evidence against the super-repressor hypothesis of the *cysB* mutation.

DISCUSSION

These results rule out all the hypotheses proposed for the explanation of the pleiotropic mutants of the cysteine biosynthetic pathway, except that of the activator gene. But Jones-Mortimer *et al.* (1968) showed that, for the *cysE* gene, it is not the polynucleotide or polypeptide coded for by the gene that acts as an activator, but O-acetylserine, the product of the reaction catalysed by serine transacetylase (the product of the *cysE* gene).

Scheme for the control of cysteine biosynthesis

From the results obtained, and from those of Ellis (1964, 1966), Kredich & Tomkins (1966) and Jones-Mortimer *et al.* (1968), it is possible to deduce a scheme for the control of cysteine biosynthesis in *E. coli* (Scheme 1).

Ellis (1964, 1966) presented evidence that was interpreted to mean that there was feedback inhibition of the sulphate uptake mechanism by cysteine. This feedback inhibition might not be direct: Dreyfuss & Pardee (1966) suggested that, in *Salmonella typhimurium*, the true inhibitor is PAPS. C. A. Pasternak (personal communication) has not been able to demonstrate feedback inhibition of PAPS reductase in extracts of *E. coli*, though such an inhibition must be postulated if

Scheme 1. Control of cysteine biosynthesis in *E. coli*. Cysteine represses enzymes (1)–(5) and (7). Cysteine is a feedback inhibitor of enzyme (6) (Kredich & Tomkins, 1966) and of sulphate permease (enzyme 1) (Ellis, 1966). *O*-Acetylserine induces enzymes (1)–(3) and (5). The *cys B*⁺ allele is required for the production of enzymes (1)–(5). Abbreviations: APS, adenosine 5'-sulphatophosphate; OAS, *O*-acetylserine. —→, Repression; ----→, induction; -.-.-.-→, feedback inhibition.

PAPS is the inhibitor of sulphate uptake in *E. coli*. Kredich & Tomkins (1966) demonstrated feedback inhibition of serine transacetylase by cysteine. These results are compatible with the generalizations of Monod, Changeux & Jacob (1963).

The feedback inhibition of serine transacetylase affects the synthesis of the enzymes of sulphate activation and reduction: if the synthesis of the inducer is prevented, induction cannot take place. The presence of *O*-acetylserine is, however, not a sufficient condition for the synthesis of the enzymes of the pathway. The intracellular cysteine concentration must be below a certain critical value and the wild-type allele of the *cys B* gene must be present (Jones-Mortimer *et al.* 1968). It is possible that cysteine prevents the entry of *O*-acetylserine into the cell, and that the product of the *cys B*⁺ gene converts *O*-acetylserine into the true low-molecular-weight inducer. A less complex hypothesis is that *O*-acetylserine and the product of the *cys B*⁺ gene are involved in the positive control of the regulon, resembling in their functions arabinose and the *ara C*⁺ gene (Sheppard & Englesberg, 1966). Further, such a positive-control model provides a simple explanation for the apparent pleiotropic negative phenotype of the *cys E* mutants (that they are unable to make the inducer of the enzymes of the pathway). A negative-control model would predict that mutants lacking the ability to make the inducer of the regulon become de-repressed under conditions when the low-molecular-weight repressor is depleted. A somewhat similar scheme for the control of choline sulphatase, arylsulphatase and sulphate permease in *Neurospora crassa* has been proposed by Marzluf & Metznerberg (1968).

The scheme proposed for the regulation of the pathway of cysteine biosynthesis is an elaboration of the theoretical scheme 1(b) proposed by Newell &

Tucker (1966) for the control of converging branched pathways.

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