Mutants of *Salmonella typhimurium* Responding to Cysteine or Methionine: Their Nature and Possible Role in the Regulation of Cysteine Biosynthesis

By M. A. QURESHI,* D. A. SMITH AND A. J. KINGSMAN

Genetics Department, Birmingham University, Birmingham B15 2TT

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SUMMARY

Nineteen mutants of *Salmonella typhimurium* responding to either cysteine or methionine (cym) have been identified amongst cysteine (*cys*) and methionine (*met*) auxotrophs. Their growth responses to known intermediates in the related pathways of cysteine and methionine biosynthesis and complementation patterns in abortive transduction tests divided the mutants into six groups. Results of conjugation, cotransduction and deletion mapping experiments substantiated these groups, each of which carried a lesion within known *cys* genes. Enzyme assays on cym mutants from five of the six groups confirmed their *cys* gene deficiencies. Growth response and enzyme assay data were not consistent with cym mutants being leaky *cys* mutants (spared by methionine). None of eight cym mutants tested were able to convert [³⁵S]methionine into [³⁵S]cysteine. Selenate specifically inhibits the early enzymes of cysteine synthesis. In cym mutants this inhibition was relieved by cysteine but not by methionine, indicating that cym mutants require active *cys* enzymes for growth on methionine. There was evidence that methionine stimulated *in vivo* activity of *cys* enzymes in a cym mutant. Resistance to inhibition by 1,2,4-triazole results in reduced levels of the *O*-acetyl serine sulphydrylase. In cym mutants triazole resistance gave unstable suppression of the cym phenotype. Cym mutants may result from mutation in regulatory regions common to each of the *cys* genes, with the precise role of methionine as yet unknown.

INTRODUCTION

The biochemical and genetical aspects of the nature and regulation of cysteine and methionine synthesis in *Escherichia coli* and *Salmonella typhimurium* have been reviewed (Smith, 1971), and additions in relation to methionine synthesis in *E. coli* were recently made by Ahmed (1973). The two metabolic pathways are interrelated (Fig. 1) and both involve structural genes which, although showing some clustering, are mainly scattered around the genetic maps (Fig. 2). End-product repression control operates in both pathways. Induction of cysteine enzymes by *O*-acetyl serine (Fig. 1) is well established (Kredich, 1971) and an interesting parallel role of methionine precursors in relation to methionine synthesis has been proposed (Whitehouse & Smith, 1973). Synthesis of cysteine is under positive control whereas that of methionine is negative. Although regulatory mutants for both systems exist, the nature of the regulatory proteins and their effectors are still incompletely understood. There is now a strong implication that S-adenosylmethionine is the methionine corepressor (Hobson, 1974) and that both vitamin B₁₂ (Whitehouse & Smith, 1973) and at least one of the enzymes involved in homocysteine methylation (Whitehouse & Smith, 1974) are concerned in methionine regulation.

* Present address: 48 Shah Din Scheme, Ichhra More, Lahore, Pakistan.

Fig. 1

Fig. 2

Fig. 1. S-amino acid synthesis in *Salmonella typhimurium*. *cysA*, sulphate permease; *cysC* and *cysD*, ATP-adenylsulphate 5'-phosphotransferase and ATP sulphate adenylyl transferase (ATP sulphurylase), respectively (these enzymes together constitute PAPS synthetase); *cysE*, serine transacetylase; *cysH*, adenosine 3'-phosphate 5'-sulphatophosphate reductase (PAPS reductase); *cysG,I,J*, hydrogen sulphide: NADP oxidoreductase (sulphite reductase); *metA*, homoserine-*O*-transsuccinylase; *metB*, cystathionine- γ -synthetase; *metC*, β -cystathionase; *metE*, *N*⁵-methyl-tetrahydropteroyl-triglutamate-homocysteine transmethylase (cobalamin-independent methylase); *metF*, *N*⁵*N*¹⁰-methyltetrahydrofolate reductase (tetrahydrofolate reductase). OAS, *O*-acetyl-L-serine; Me, methionine.

Fig. 2. Abbreviated linkage map of *Salmonella typhimurium* (after Sanderson, 1972). Mutant symbols as in Fig. 1; *trz*, triazole resistance. Numbers indicate time intervals in min.

During the course of extensive work with many different auxotrophs of *S. typhimurium* initiated in the late Dr M. Demerec's laboratory at Cold Spring Harbor in the mid-1950s, mutants responding equally well to either cysteine or methionine were isolated but not investigated. Qureshi (1968, 1971) recognized similar mutants amongst newly isolated methionine auxotrophs of the same organism, and initiated further studies of them in an attempt to shed light on the general regulation of S-amino acid metabolism. This paper reports physiological, genetical and enzymological experiments with nineteen cysteine/methionine (cym) auxotrophs which implicate methionine in the regulation of the synthesis of all known cysteine enzymes. Mutations conferring resistance to inhibition by 1,2,4-triazole, which probably interferes with induction of the cysteine enzymes (Hulanicka, Klotkowski & Smith, 1972; Jones-Mortimer, 1968; Hulanicka & Klotkowski, 1972) suppress cym mutants. The stability of this suppression is variable and its nature not only relates to cysteine gene regulation but also to plasmid involvement in suppression (Kingsman, unpublished) probably extending beyond the cysteine system (I. M. Packer, personal communication).

METHODS

Organisms and materials. *Salmonella typhimurium* LT2 was the wild-type bacterium from which all the mutants used (Table 1) were derived. The phenotypic designation cym for the cysteine or methionine mutants may not be permanent: it is retained in this paper for clarity. Nutrient broth and agar media (NB and NA), and minimal liquid and agar media, (MM and MA) were those of Smith (1961), except that in sulphur-free minimal medium [MM(sf)]

Table 1. Mutants of *Salmonella typhimurium* LT2

A. Cysteine or methionine (cym) mutants							
Mutant	Cym designation	Previous designation	Other markers	Mode of origin	Other properties	Source*	
cym1	Aa	23	.	NTG	.	M.A.Q.	
cym2	Ab	28	.	NTG	ts	M.A.Q.	
cym3	Ba	55	.	NTG	ts	M.A.Q.	
cym4	Ba	72	.	NTG	ts	M.A.Q.	
cym5	Bb	<i>cys-71</i>	.	sp	.	K.E.S.	
cym6	Bc	<i>cys-72</i>	.	sp	ts	K.E.S.	
cym7	Aa	<i>cys-177</i>	<i>ara-9</i>	sp	ts	K.E.S.	
cym8	Bb	<i>cys-204</i>	<i>ara-9</i>	2AP	ts	K.E.S.	
cym9	Cb	<i>cys-205</i>	<i>ara-9</i>	2AP	.	K.E.S.	
cym10	Cb	<i>cys-286</i>	.	2AP	.	K.E.S.	
cym11	Cb	<i>cys-288</i>	.	2AP	.	K.E.S.	
cym12	Ca	<i>cys-333</i>	.	u.v.	.	K.E.S.	
cym13	Ab	<i>cys-797</i>	.	2AP	.	K.E.S.	
cym14	Ca	<i>cys-798</i>	.	2AP	.	K.E.S.	
cym15	Bc	<i>cys-803</i>	.	2AP	ts	K.E.S.	
cym16	Bb	<i>cys-896</i>	.	2AP	.	K.E.S.	
cym17	D	<i>cys-898</i>	.	2AP	.	K.E.S.	
cym18	Bb	<i>cys-1053</i>	.	5BU	.	K.E.S.	
cym19	Ab	<i>cys-1219</i>	.	NTG	ts	K.E.S.	
B. Other mutants							
Mutant	Other markers	Other properties	Source	Mutant	Other markers	Other properties	Source*
<i>cysA20</i>	.	dl	K.E.S.	<i>cysD505</i>	.	.	K.E.S.
<i>cysA21</i>	.	.	K.E.S.	<i>cysG439</i>	.	.	K.E.S.
<i>cysA22</i>	.	.	K.E.S.	<i>cysH47</i>	<i>gal-24 trp-7</i>	.	K.E.S.
<i>cysA69</i>	.	.	K.E.S.	<i>cysH75</i>	.	dl	K.E.S.
<i>cysA197</i>	<i>ara-9</i>	.	K.E.S.	<i>cysH364</i>	.	dl	K.E.S.
<i>cysA201</i>	<i>ara-9</i>	.	K.E.S.	<i>cysH398</i>	.	dl	K.E.S.
<i>cysA351</i>	.	.	K.E.S.	<i>cysH572</i>	.	dl	K.E.S.
<i>cysA533</i>	.	dl	K.E.S.	<i>cysI51</i>	.	.	K.E.S.
<i>cysC218</i>	<i>ara-9</i>	.	K.E.S.	<i>cysI68</i>	.	dl	K.E.S.
<i>cysC520</i>	.	dl	K.E.S.	<i>cysI295</i>	<i>ara-9</i>	.	K.E.S.
<i>cysC532</i>	.	.	K.E.S.	<i>cysHI36</i>	.	dl	K.E.S.
<i>cysC537</i>	.	dl	K.E.S.	<i>cysHI536</i>	.	dl	K.E.S.
<i>cysCD519</i>	.	dl	K.E.S.	<i>cysJ299</i>	<i>ara-9</i>	.	K.E.S.
<i>cysC700</i>	.	.	K.E.S.	<i>cysJ447</i>	.	.	K.E.S.
<i>cysC710</i>	.	dl	K.E.S.	<i>cysJ538</i>	.	dl	K.E.S.
<i>cysC1021</i>	.	dl	K.E.S.	<i>metA15</i>	.	dl	D.A.S.
<i>cysD220</i>	.	dl	K.E.S.	<i>metF96</i>	.	.	D.A.S.
<i>cysD305</i>	.	.	K.E.S.	trz12	.	.	A.J.K.
				trz110	.	.	A.J.K.

2AP, 2-aminopurine; 5BU, 5-bromouracil; NTG, *N*-nitroso-*N'*-nitro-*N*-nitrosoguanidine; sp, spontaneous; u.v., ultraviolet light; dl, deletion; ts, temperature-sensitive; *cys*, *met*, and *trp*, genes involved in cysteine, methionine and tryptophan biosynthesis; *ara* and *gal*, genes involved in utilization of arabinose and galactose; *trz*, triazole resistance.

* A.J.K. (Mr A. J. Kingsman), M.A.Q. (Dr M. A. Qureshi) and D.A.S. (Dr D. A. Smith) of Birmingham University, and K.E.S. (Dr K. E. Sanderson) of the Department of Biology, The University, Calgary, Alberta, Canada.

0.81 g NH_4Cl and 0.04 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were added to each litre instead of K_2SO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Media supplementation, culture and subculture procedures, dilutions, assays, and routine testing and storage of all bacterial strains were also those of the same author. Concentrations of cysteine and methionine precursors and related compounds used were: sulphite and sulphide, 10 μg sulphur/ml; thiosulphate, 10 μg and 1 mg sulphur/ml (Clowes, 1958); cysteine, L-allo-cystathionine, L-homocysteine thiolactone HCl and L-methionine, 20 μg /ml; vitamin B_{12} , 1 μg /ml; djnkolic acid, 38.1 μg /ml.

[^{35}S]sulphate (carrier-free) and [^{35}S]methionine were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Phage P22 and, less frequently, its non-lysogenizing derivative L4 (Smith & Levine, 1967) were used for transduction. The preparation and storage of phage lysates were as described by Smith (1961) except that lysates were occasionally prepared from confluent lysed lawns of bacteria instead of liquid cultures. All incubations were at 37 °C unless otherwise stated.

Mutagenesis. Overnight NB cultures of wild-type bacteria were washed and resuspended in the same volume of MM, diluted 1:20 in MM + *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and then incubated for 30 min (after Eisenstark, Eisenstark & van Sickle, 1965). These suspensions (0.3 to 0.5 % survival) were diluted 10^{-3} and 10^{-4} in saline (0.85 %, w/v, NaCl) and appropriate volumes plated on to MA + 0.02 % (w/w) NB and NA. After 48 h incubation small colonies were picked from both enriched MA and NA plates and subcultured on to NA. These subcultures and the remaining large colonies from the original NA plates were replicated to MA and MA + methionine. Apparent methionine auxotrophs were subcultured to give single colonies whose phenotype was rechecked by similar replication before the establishment of triplicate stock subcultures from a single colony. Forty-six stable methionine auxotrophs were derived from enriched MA, and 24 from NA.

Growth responses. Initially, growth responses and the heat sensitivity of mutants were determined on solid media. Overnight NB cultures were washed in saline and loopfuls streaked on to each of three MA or appropriately supplemented MA plates. Single plates were then incubated at 25, 37 and 42 °C and growth responses observed during at least 48 h. If growth of a mutant at 25 °C was similar to that of wild type at the same temperature and clearly less than that of wild type at 37 or 42 °C, it was designated heat sensitive (Smith & Childs, 1966).

More precise measurements were made in liquid culture. Overnight cultures in MM + methionine or djnkolic acid were diluted 1:5 (v/v) into fresh medium and incubated for 90 min. Bacteria were then washed twice, resuspended in either MM or MM(sf), and appropriate volumes added to 50 ml prewarmed test media in 250 ml conical flasks to give an E_{650} of 0.05 to 0.06 measured in a Unicam SP500 spectrophotometer, using a glass cuvette of 1 cm light pathway. During continued incubation, samples were removed every 30 to 60 min and their turbidity recorded. Extinction was plotted on a log-linear scale against time of sampling. Mean generation times were deduced from the graphs as the time required for a twofold increase in *E*.

Transduction. The whole-plate technique to select recombinants for replication analysis was that of Smith (1961). To detect complete and abortive transductants when large numbers of pairs of auxotrophs were crossed, the spot method of Hartman, Hartman & Šerman (1960) as modified by Smith & Childs (1966) was used. In the cotransduction experiments all selective plates (except those for crosses in which the donor was cymCb11) were incubated for 48 h and replicated to selective plates which were incubated for 10 or for 16 to 20 h, depending upon the *cys* recipient. With cymCb11 strains, recombinants of the donor phenotype could be detected as small colonies after 72 h incubation of the selective plates; replica plating was unnecessary.

Table 2. Mean generation times of *cym* and other mutants in sulphur-free minimal medium at 37 °C with and without cysteine or methionine

Mutant	Generation time (min)		
	MM(sf)	MM(sf)+ cysteine	MM(sf)+ methionine
<i>cysCD519</i>	α	82.5	α
<i>metF96</i>	α	α	39
<i>cym2</i>	α	57	54
<i>cym3</i>	360	60	150
<i>cym4</i>	489	66	156
<i>cym5</i>	345	75	177
<i>cym11</i>	420	66	123
<i>cym12</i>	414	63	132
<i>cym15</i>	106	73	51
<i>cym18</i>	α	61	81
<i>cym19</i>	α	74	78

MM(sf), sulphur-free minimal medium; α , generation time too long to calculate.

Protein hydrolysates and chromatography. Protein hydrolysates were prepared according to the method of Moore & Stein (1963). Descending one- or two-dimensional chromatography involved the use of solvents A (ethanol, 2-methyl-propan-2-ol, aqueous ammonia and water) and B (2-methyl-propan-2-ol, formic acid and water) of Margolis & Mandl (1958), and solvents C (*n*-butanol, glacial acetic acid and water) and D (*n*-butanol, pyridine and water) of Smith (1958). Whatman 3 MM paper was used, and was dipped in 0.2 % ninhydrin in ethanol and dried at 100 °C for 5 min to detect the amino acid spots. The colour of these spots was stabilized by spraying with 3 % cupric nitrate in ethanol.

Detection and assay of radioactivity. Radioactive spots were located on chromatographic or electrophoretic strips with a Packard radiochromatogram scanner model 7200 (efficiency 10 %) at a scanning rate of 2 to 5 cm/min, a time constant of 10 to 100 s and a slit width of 2.5 mm. Radioactivity was determined in a Nuclear Chicago 720 liquid scintillator (efficiency 60 %). The scintillating liquid contained 4.0 g 2,5-diphenyloxazole and 0.12 g 1,4-di [2-(5-phenyloxazolyl)] benzene/l toluene.

Preparation of cell-free extracts. Overnight NB or appropriately supplemented MM cultures of bacteria were washed and resuspended in the same media, diluted 1:100 or 1:40 in 500 ml MM(sf)+djenkolic acid, and incubated with aeration to mid or late exponential phase to give derepression of cysteine enzymes. Bacteria were sedimented at 4 °C, washed in 50 ml of 0.1 M-tris-HCl buffer pH 8.0+0.005 M-EDTA, and resuspended in 3 ml of the same buffer (30 to 60 mg dry wt/ml), chilled and sonicated in two 15 s bursts with an MSE 100 W ultrasonic disintegrator ($\frac{3}{8}$ in probe, 8 μ m peak to peak). The crude extract was centrifuged at 30000 *g* for 15 min and the supernatant kept in ice for enzyme assays. The method of Lowry *et al.* (1951) was used for protein determinations.

Enzyme assays. The method of Pasternak (1962) was used for 3'-phospho-adenosine 5'-sulphatophosphate (PAPS) synthetase, that of Ellis (1964) or Dreyfuss & Monty (1963) for sulphite reductase, and the filter method of Hulanicka (1972) for sulphate permease.

Fig. 3. Growth curves of *cym* mutants in sulphur-free minimal medium at 37 °C with and without added cysteine or methionine. ●, MM(sf); ▲, MM(sf)+cysteine; ■, MM(sf)+methionine. (a) *Cym2*, (b) *cym3*, (c) *cym15*.

Fig. 4. Growth curves of a cysteine and a methionine mutant in sulphur-free minimal medium at 37 °C with and without added cysteine or methionine. Symbols as in Fig. 3. (a) *cysCD519*, (b) *metF96*.

RESULTS

Isolation of cysteine or methionine (cym) mutants

All 19 mutants (Table 1A) were either spontaneous or induced with NTG, 2-aminopurine (2AP), 5-bromouracil (5BU) or ultraviolet irradiation. Of 70 stable methionine auxotrophs induced with NTG, four (*cym1*–4 inclusive) grew equally well on MA+cysteine or methionine. The other 15 similar mutants (*cym5*–19) were identified amongst 28 cysteine (*cys*) mutants reported also to utilize methionine which were obtained from Dr K. E. Sanderson, Department of Biology, University of Calgary, Alberta, Canada. Eight mutants (*cym2*, 3, 4, 6, 7, 8, 15 and 19) were temperature-sensitive (*ts*).

The homogeneity of all mutant cultures was tested by spreading dilutions of overnight NB cultures of each mutant on to NA, incubating overnight and testing each of 25 randomly chosen single colonies for their growth responses to cysteine and methionine. Bacteria from all colonies of each *cym* mutant grew equally well on both amino acids, making it unlikely the *cym* phenotype resulted from mixtures of cysteine (*cys*) and methionine (*met*) mutants.

Growth characteristics in liquid culture

It was desirable to establish more firmly the phenotype of the *cym* mutants and to distinguish them from *cys* and *met* mutants. The growth responses and generation times of 9 *cym* mutants (*cym2*, 3, 4, 5, 11, 12, 15, 18 and 19) during 6 to 7 h incubation in MM(sf) at 37 °C were compared with those of *cysCD519* and *metF96* mutants grown under similar conditions. From the generation times of all the mutants (Table 2), and the growth response

curves of representative cym mutants 2, 3 and 15 (Fig. 3) and *cysCD519* and *metF96* (Fig. 4) it can be seen that:

(1) There is always a lag of 1 to 3 h in growth response to cysteine but no lag with respect to methionine. Cysteine inhibits the growth of wild type in MM(sf) for about the same time as this lag (Qureshi, 1971). It may be that cysteine similarly affects cym mutants by, for example, inhibiting either the release of repression of dihydro-orotic acid dehydrogenase (Tonomura & Novelli, 1960), the activity of homoserine dehydrogenase (Datta, 1967), the synthesis of leucine, isoleucine, valine and threonine (Nagy, Kari & Hernádi, 1969), or catalase and respiratory chain function (Bhuvaneswaran, Sreenivasan & Rege, 1964).

(2) All cym mutants are clearly different from *cysCD519* and *metF96* in that they respond to both cysteine and methionine instead of one of these compounds.

(3) Growth response patterns and leakiness vary between cym mutants but three basic types are recognizable (Table 2): (i) cym2, 18 and 19 – similar response to cysteine and methionine, not leaky (Fig. 3*a*); (ii) cym3, 4, 5,¹ 11 and 12 – better growth response to cysteine than to methionine, slightly leaky (Fig. 3*b*); (iii) cym15 – similar growth response to both cysteine and methionine, leaky (Fig. 3*c*).

Growth responses to cysteine and methionine precursors

To clarify the phenotype of the cym mutants, their growth responses on MA+ either sulphite, thiosulphate (high and low concentration), sulphide, cysteine, cystathionine, homocysteine, vitamin B₁₂ or methionine were compared with wild type at both 37 and 25 °C. At 37 °C all cym mutants responded equally well to thiosulphate, sulphide, cysteine, cystathionine, homocysteine and methionine. None grew on vitamin B₁₂. In contrast, incubation at 25 °C resulted in much heterogeneity and no clear pattern of growth response amongst the 11 mutants which were not ts.

In similar experiments in MM at 37 °C, care was taken to ensure the stability of the sulphur-containing compounds used (Postgate, 1963). Fresh solutions of sulphite, thiosulphate and sulphide were always used. Supplemented liquid minimal media with and without bacteria were incubated for the full period of the experiments, after which retention of pH values 7.0 to 7.3 was shown, and the presence of each of the inorganic salts confirmed by the quantitative methods of Feigl (1947). As additional controls the cultures of single representative *cys* and *met* mutants, each with different patterns of growth responses to either the cysteine or the methionine precursors, were included. All of the *cys* and *met* mutants gave growth responses in accordance with their phenotypes. The cym mutants behaved as in the solid media experiments except that: (i) only seven mutants (cym5, 6, 8, 15, 16, 17 and 18) failed to grow on sulphite whereas the remaining 12 grew well; (ii) growth responses to thiosulphate were variable, particularly at the lower concentration, both between mutants and between experiments (an unreliability also encountered by Hockenhull, 1949).

The only conclusion permissible is that some cym mutants appear to be blocked before sulphite on the cysteine pathway (Fig. 1) and may be deficient in the *cysC*, *D*, or *H* gene products and others after it with probable deficiencies in the *cysI*, *J* or *G* products. If this is so, are cym mutants different from comparable *cys* mutants? For example, could their growth responses to methionine and its precursors be due to highly efficient utilization of small amounts of cysteine present as impurities in these compounds? To test this, the growth responses in liquid culture of cym2 and *cysC700* mutants to cysteine, cystathionine, homocysteine and methionine, each at concentrations of 0.05, 0.5, 1.0, 5.0 and 10 µg/ml, were compared. As a control, the *metA15* mutant which is able to utilize both cystathionine

and homocysteine, was included. The minimum concentration of cysteine to support the maximum growth of both *cym2* and *cysC700* mutants was the same (10 µg/ml). The same concentrations of methionine (5 µg/ml) and cystathionine and homocysteine (10 µg/ml) elicited maximum growth responses from both *cym2* and *metA15*. *cysC700* failed to respond to methionine and its precursors and *metA15* to cysteine at any concentration. Thus *cym2* did not utilize cysteine more efficiently than the *cysC700* mutants. Its growth response pattern to methionine precursors was the same as *metA15* and there was no evidence of cysteine impurities in these precursors. The growth distinction between the *cym* phenotype and those of similar *cys* or *met* mutants was upheld.

Linkage and complementation analysis of cym mutants

The similar phenotypes of the *cym* mutants and their apparent relationship to the clustered *cysC* and *D* or *G*, *I* and *J* genes suggested that some or all *cym* mutants could be closely linked to each other and possibly possess similar functional deficiencies. In a series of reciprocal spot transduction experiments between all *cym* mutants selecting wild-type recombinants on MA using wild-type donor phage as a standard, the relative frequency of complete transductants and the presence or absence of abortive transductants (complementation) between *cym* mutants was determined. A yield of 60 to 200 recombinants/spot was assumed to indicate no linkage and 0 to 60/spot to reflect linkage. Failure to recombine (an indication of identical site mutation or a deletion) or lack of complementation in a cross was checked by repeating the cross three times in both directions.

On the basis of recombination frequencies the *cym* mutants fell into four groups, A, B, C and D, with low frequencies of recombination between members of the same group and a high frequency between members of different groups. All mutants within groups recombined with each other. Within groups B and C, subgroups were apparent in which 0 to 30 (often < 15) recombinants per spot were obtained; there were three *cymB* subgroups (a, b and c), and two *cymC* subgroups (a and b). The *cym* mutants in these groups and subgroups and the complementation patterns within them were: *cymA* (1, 2, 7, 13 and 19) – 1 and 2, 2 and 19, and 13 and 19 failed to complement but all other combinations did, so that no clear pattern emerged; *cymBa* (3, 4), b (5, 8 and 16) and c (6, 15 and 18) – mutants within subgroups a and c failed to complement each other but those of group b did, and complementation always occurred between mutants of different subgroups; *cymCa* (12, 14) and b (9, 10, 11) – there was no complementation between mutants of the same subgroup but it did occur between those of different subgroups; *cymD* (17) – this mutant complemented all other *cym* mutants.

Map locations

The results of preliminary conjugation experiments with five different Hfr strains (Qureshi, 1968) indicated that *cymA* and *B* mutations were located between 50 and 100 min, *cymC* between 50 and 80 min, and *cymD* elsewhere – probably beyond 100 min (Fig. 2).

The *cysC, D, H, I, J* cluster of genes (Fig. 2) is located at 90 min and as *cymA* and *B* mutants resembled mutants of these genes phenotypically, the frequency of recombination in crosses involving representative *cys* and *cym* mutants was compared with that obtained with the wild-type donor in a series of spot transduction experiments. Recombination to prototrophy between *cymA* and *cysC* and *D*, *cymBa* and *cysH*, *cymBb* and *cysJ*, and *cymBc* and *cysI* was never greater than 10 % of that with the wild-type marker, indicating linkage in each case. Linkage of *cymC* mutant with *cysA* (88 min) was similarly established and *cymD*

Table 3. Cotransduction of *cym* and *cys* mutants

Donor	Recipient	Cotransduction (%)	Donor	Recipient	Cotransduction (%)
cymA2	<i>cysC523</i>	57.4	cymBb8	<i>cysJ299</i>	92
	<i>cysC218</i>	96.6		<i>cysI68</i>	88.5
	<i>cysC700</i>	94.2	cymBc6	<i>cysI295</i>	93
	<i>cysD220</i>	97.8		<i>cysJ299</i>	73
	<i>cysD505</i>	79.1			
cymBa4	<i>cysH182</i>	96.5	cymCa12	<i>cysA69</i>	25.9
	<i>cysI51</i>	92.0	cymCb11	<i>cysA69</i>	70.4
	<i>cysJ447</i>	68.6	cymD17	<i>cysG439</i>	92.5

Fig. 5. Map location of *cymA*, Ba, Bb and Bc mutants in the *cysCDHIJ* region of *Salmonella typhimurium* (after Demerec *et al.* 1963). Cym mutants are in roman type. S. Sec., silent section.

mutants also yielded few recombinants with both *cysE* and *G* mutants located at 110 to 117 min (see Fig. 2).

More precise mapping of cym mutants was achieved by measuring cotransduction with the various *cys* mutants. Optimum conditions for the detection of colonies with the cym donor phenotype of a growth response to methionine were first established and then large numbers of cotransduction crosses carried out. Never less than 150 and often more than 1000 recombinants were screened from each cross. Individual mutants within cym groups A and D and the subgroups of groups B and C gave similar cotransduction frequencies, so that only data for representative donors are recorded (Table 3). Thus cymA mutants are close to the junction of the *cysC* and *D* genes (Fig. 5), and cymBa mutants are confirmed as being closest to *cysH* or *I*, as are cymBb to *cysJ* and cymBc to *cysI*. CymC linkage to *cysA* is also confirmed, although the lesions in cymCb mutants are closer to *cysA69* than cymCa. Linkage of cymD to *cysG* was clearly indicated; there was no cotransduction with *cysE*.

Finally, some additional information emerged from a series of reciprocal spot transduction crosses between relevant *cym* mutants and deletions of the *cysC*, *D*, *H*, *I*, *J* and *A* genes. There was recombination between all five *cymA* mutants and *cysC* deletions 537, 520 and 710, but not with *cysCD519* (Fig. 5), a result compatible with their location at the right end of the *cysC* gene or the left end of *cysD*. On the basis of the recombination profiles of *cymB* mutants with *cys* deleted mutants (Table 4) it is concluded that: (i) both *cymBa* mutants are

Table 4. *Recombination between cymB mutants and cys deletions*

Cym mutants	cys deletions							
	<i>D305</i>	<i>HI36</i>	<i>HI536</i>	<i>H398</i>	<i>H364</i>	<i>H572</i>	<i>I68</i>	<i>J538</i>
cymBa3 and 4	—	—	—	+	+	+	+	+
cymBb5, 8 and 16	—	+	+	+	+	+	+	—
cymBb18	—	+	—	+	+	+	+	+
cymBc6	—	—	—	+	+	+	—	+
cymBc15	—	—	—	+	+	+	+	+

likely to be located at the right end of *cysH* (or possibly in the adjacent silent section); (ii) cymBb5, 8 and 16 are probably at the right end of *cysJ*, but as *cysBb18* recombines with *cysJ538* and fails to recombine with *cysHI536*, it is likely to be at the left end of *cysJ* or right end of *cysI*; (iii) both cymBc mutants are obviously located in the *cysHI* region with cymBc6 within the middle region of the *cysI* gene covered by *cysI68*.

None of the cymCa or b mutations recombined with the two deletions *cysA20* and *533* thought to cover the whole *cysA* gene (Demerec, Gillespie & Mizobuchi, 1963; Dreyfuss, 1964), suggesting their location within it.

Functional deficiencies

Since each cym mutant was located within or close to a *cys* gene, it was of interest to determine whether the difference in phenotype between the mutants was associated with possession of the same or different functional deficiencies. This promoted a series of complementation tests and enzyme assays.

Complementation between cym and cys mutants. The spot transduction method was used and the criteria of lack of complementation between any pair of mutants were the same as those used previously. In reciprocal crosses between the five cymA, ten *cysC* and five *cysD* mutants it first became apparent that, in contrast to the results of Mizobuchi, Demerec & Gillespie (1962), *cysC* mutants fell clearly into two complementation groups. Furthermore, not only did two cymA mutants (A1 and 7 – subgroup a) complement all *cysD* mutants and three (2, 13 and 19 – subgroup b) fail to complement *cysD* mutants, but cymAa also complemented one group of the *cysC* mutants but not the other. It was concluded that cymAa mutants had the same defect as some *cysD* mutants and cymAb mutants the same defect as all *cysD* mutants tested.

Similar reciprocal crosses between four *cysH*, three *cysI*, three *cysJ*, and all cymBa, B, and C mutants were carried out. In accordance with Demerec *et al.* (1963), no complementation between *cys* mutants of the same group occurred. There was also no complementation between both cymBa mutants and all *cysH* mutants, all cymBb mutants (except cymBb18) and the *cysJ* mutants, and both cymBc (and cymBb18) and *cysI* mutants. Abortive transductants were detected in all other crosses. Thus cymBa and *cysH* mutants had the same functional defect as did all cymBc, cymBb18 and *cysI* mutants, and four cymBb and *cysJ* mutants. It was interesting that cymBb18 also mapped away from the other three cymBb mutants. Mizobuchi *et al.* (1962) reported the existence of three *cysA* cistrons *a*, *b* and *c*. The similarity between cymCa and b and *cysAa*, *b* and *c* defects was tested in further reciprocal crosses between *cysAa201*, *Ab21*, *Ac22*, *Ac197* and *Ac351*, and all five cymC mutants. The distinction between *cysAa* and *Ab* was not sustained in that no complementation occurred between the representative mutants, but whereas cymCb complemented all *cysA* mutants, cymCa only complemented *cysAc* mutants. It can thus be stated that both

Table 5. *Enzyme deficiencies of cym mutants*

Growth of all mutants was in MM(sf)+djenkolic acid.

A. PAPS-synthetase and sulphite reductase activities in the supernatant fraction of cymA, Ba, Bb and D mutants

Strains	PAPS synthetase		Sulphite reductase	
	Specific activity (nmol/mg protein/min)	Relative specific activity	Specific activity (nmol/mg protein/min)	Relative specific activity
Wild type	199.7	100	13.3	100
cymAa1	0	0	8.7	65.6
cymAb2	0	0	10.4	78.1
cymAa7	0	0	11.2	84.2
cymAb19	0	0	21.5	161.6
<i>cysC1021</i> *	188.8	94.5	14.4	108.2
<i>cysD220</i>	0	0	11.9	89.4
Wild type	104.0	100	25.4	100
cymBa3	181.2	174.2	25.9	101.9
cymBa4	337.5	324.5	34.5	135.8
<i>cysH75</i>	104.0	100	19.7	77.5
Wild type	144.0	100	27.8	100
cymBb5	327.9	227.7	0	0
cymBb8	374.9	26.03	0	0
cymBb16	828.9	575.6	0	0
cymBb18	820.9	570.6	0	0
<i>cysJ299</i>	101.4	70.4	0	0
Wild type	87.5	100	31.5	100
cymBc6	471.5	538.8	0.25	0.79
cymBc15	108.1	123.5	0	0
<i>cysI1295</i>	256.9	293.6	0.80	2.51
Wild type†	NT	NT	42	100
cymD17†	NT	NT	0	0
<i>cysG439</i> †	NT	NT	0	0

B. Sulphate permease activity in cymCa and Cb mutants

	Specific activity (pmol/min/mg dry wt)	Relative specific activity
Wild type	167	100
cymCa12	3.3	2.0
cymCa14	3.0	1.8
cymCb9	0.3	0.2
cymCb10	3.7	2.2
cymCb11	4.85	2.9
<i>cysA20</i>	2.5	1.5
<i>cysA21</i>	9.3	5.6

* APS not PAPS made.

† Sulphite reductase assay by the rate of sulphite-dependent NADPH oxidation (Dreyfuss & Monty, 1963), instead of by sulphide formation (Ellis, 1964) as for all other strains.

NT, Not tested.

Table 6. *Summary of relationships between cym and cys mutants*

Group and subgroup	Mutant numbers	Basis of subgroups	Map location	Enzyme deficiencies	<i>cys</i> mutant similarity
cymAa cymAb	1, 7 2, 13, 19	No complementation with <i>cysC</i> or <i>D</i> mutants	<i>cysC</i> and <i>D</i> junction	PAPS synthetase	<i>cysC</i> <i>cysD</i>
cymBa	3, 4	Recombination and complementation	Within <i>cysH</i>	Sulphite reductase	<i>cysH</i>
cymBb	5, 8, 16, 18*	Recombination and complementation	Within <i>cysI</i>	Sulphite reductase	<i>cysI</i>
cymBc	6, 15	Recombination and complementation	Within <i>cysJ</i>	Sulphite reductase	<i>cysJ</i>
cymCa cymCb	12, 14 9, 10, 11V	Recombination and complementation with <i>cysA</i> mutants	Within <i>cysA</i>	Sulphate permease	<i>cysA</i>
cymD	17		Within or close to <i>cysG</i>	Sulphite reductase	<i>cysG</i>

* This mutant is anomalous in that it maps within the *cysI* gene.

cymCa and b mutants have a different functional deficiency from *cysAc* mutants and that cymCb are also functionally different from *cysAa* or *b* mutants.

Enzyme deficiencies of cym mutants. CymA, B and D mutants were likely to have deficiencies in cysteine biosynthesis at some stage in the conversion of intracellular sulphate to sulphide (Fig. 1), and cymC mutants in the uptake of sulphate. Thus assays of the activities of PAPS synthetase (the product of *cysC* and *D*) and sulphite reductase (*cysI*, *J* and *G*) in cymAa and b, Ba, b and c, and cymD strains, and of sulphate permease (*cysA*) in cymCa and b mutants were carried out. The cym mutants were assayed group by group together with wild type and relevant *cys* mutants in duplicate. All bacteria were grown in sulphur-free minimal medium [MA(sf)]+djenkolic acid to ensure derepression. For cymA and B mutants the sulphite reductase assay used was that of Ellis (1964). Later, and exclusively for the cymD mutant, the more sensitive assay of Dreyfuss & Monty (1963) was used.

From Table 5, all cymA mutants completely lack PAPS synthetase like *cysD* mutants; mutants cymBb, cymBc and cymD lack or have much reduced levels of sulphite reductase like *cysJ*, *I* and *G* mutants respectively, and cymC mutants have reduced levels of sulphate permease like *cysA* mutants. There is no evidence that cym mutants possess leaky deficiencies for *cys* enzymes. The elevated levels of PAPS synthetase in cymBa, Bb and Bc mutants lacking sulphite reductase could be due to derepression, even super-derepression, in the absence of cysteine (Dreyfuss, 1964; Pardee & Prestidge, 1966; Dreyfuss & Monty, 1963) and induction by non-utilized *O*-acetylserine (Jones-Mortimer, 1968), but it is strange that derepression of sulphite reductase does not occur in cymA mutants lacking PAPS synthetase, for similar intracellular levels of cysteine and *O*-acetylserine would be anticipated.

The nature of cym mutants

From a summary of all the results described so far (Table 6) the very close similarity between cym and *cys* mutants is apparent. To all intents cym mutants are *cys* mutants, except that they are able to use methionine and its precursors for growth whereas *cys* mutants cannot. The rest of this paper is concerned with attempts to explain this difference.

Possible interconversion of methionine and cysteine. As long ago as 1947, Lampen, Roepke

and Jones reported the growth of a sulphite-less mutant of *E. coli* on methionine as its sole source of sulphur. Also, growth of *Torulopsis utilis* in the presence of exogenous [^{35}S]-methionine resulted in the appearance of intracellular [^{35}S]cysteine (Roberts *et al.* 1955), and the breakdown of the methionine precursor, cystathionine, in various fungi by cystathionine- γ -synthetase (which normally catalyses its synthesis from cysteine) in the absence of cysteine has been reported (Flavin, Delavier-Klutchko & Slaughter, 1964; Delavier-Klutchko & Flavin, 1965). These results all support the conversion of methionine to cysteine in these organisms. Could such a conversion be occurring in cym mutants of *S. typhimurium*? If so, then the radioactivity of [^{35}S]methionine in their growth medium should be incorporated into the cysteine of their protein.

Exponential-phase cultures of cymAa1, Ab2, Ba4, Bb18, Bc15, Ca12, Cb11 and D17 mutants and of wild type in MM + methionine were transferred to MM(sf) + [^{35}S]methionine, incubation was continued for 4 h and protein hydrolysates were made. All preparations were subjected to two- and one-dimensional chromatography using solvents A, B, C and D. Only one radioactive spot was seen after two-way descending chromatography using either solvents A or B, so all four solvents were used for one-way descending chromatography, the chromatograms cut into a number of strips 5 cm wide and the radioactive spots detected with a radiochromatogram scanner. The R-proline of the spots for solvents A and B and the R_F values of the same spot for C and D were then calculated.

All the radioactive spots were associated with methionine sulphone (R-proline 0.69 to 0.72 and 0.45 to 0.48, in solvents A and B; R_F 0.22 to 0.23 and 0.31 to 0.32, in solvents C and D). To confirm this, all spots were eluted and re-chromatographed; the same R_F values were obtained. Although a spot equivalent to cysteic acid was always detected from all extracts following development with ninhydrin and preservation with copper nitrate solution, it was never radioactive. Methionine appeared not to be converted to cysteine by cym mutants via a reversal of the synthesis of cystathionine from *O*-succinylhomoserine.

Requirement for the activity of cysteine enzymes in cym mutants responding to methionine. If in the presence of methionine normal synthesis of cysteine from inorganic sulphur occurs (Fig. 1), involvement of sulphate activating and reducing enzymes is implied, perhaps utilizing small amounts of sulphate impurity in MM(sf). ATP sulphurylase is specifically inhibited by selenate (Wilson & Bandurski, 1958; Hilz, Kittler & Knappe, 1959; Pasternak, 1962), so that if activity of this enzyme in cym mutants is required for their growth response to methionine, then methionine should not reverse inhibition by selenate. The cell yields, by weight, of wild type, *cysCD519* and *metF96* mutants were compared with those of ten representative cym mutants after 7 h growth on MM(sf), MM(sf) + cysteine and MM(sf) + methionine in the presence or absence of 2 mM-selenate. Wild-type responses were also measured in MM, which contains sulphate (Table 7).

Although the results varied between strains in terms of individual growth responses to cysteine or methionine, they were consistent with previous data (Figs. 3 and 4, and Table 2) and indicated clearly that for all strains cysteine reversed inhibition by selenate but that methionine did not. The possibility that methionine + selenate formed an inhibitory complex with a different effect from selenate alone was excluded when the growth response of wild type in MM(sf) + cysteine was shown to be virtually unaffected by the addition of methionine + selenate early in the exponential phase. Thus non-reversal of the methionine biosynthetic pathway in cym mutants is upheld, and their requirement for activity of their cysteine biosynthetic enzymes in the presence of methionine substantiated.

In vivo stimulation by methionine of cysteine enzyme activities in cym mutants. Cysteine enzyme activity in cym mutants presumably involved uptake and activation of sulphate

Table 7. Increase in mass of wild type, *cym* mutants and other auxotrophs after growth in sulphur-free minimal medium with and without cysteine or methionine and in the presence or absence of selenate (2 mM)

	Increase in weight* (mg dry wt/ml cells)					
	MM(sf)		MM(sf) + cysteine		MM(sf) + methionine	
	Selenate absent	Selenate present	Selenate absent	Selenate present	Selenate absent	Selenate present
Wild type	0.125	0.0007	0.650	0.315	0.460	0.010
Wild type†	0.725	0.015	0.650	0.280	0.725	0.017
<i>cymA2</i>	0.010	0.005	0.325	0.237	0.500	0.010
<i>cymA19</i>	0.007	0	0.112	0.232	0.360	0.010
<i>cymBa3</i>	0.017	0.010	0.505	0.275	0.112	0.012
<i>cymBa4</i>	0.012	0.004	0.460	0.277	0.107	0.009
<i>cymBb5</i>	0.023	0.010	0.193	0.205	0.083	0.016
<i>cymBb18</i>	0.009	0.005	0.316	0.242	0.184	0.007
<i>cymBc6</i>	0.062	0.007	0.112	0.305	0.307	0.012
<i>cymBc15</i>	0.065	0.010	0.328	0.430	0.278	0.015
<i>cymCa12</i>	0.015	0.005	0.273	0.280	0.178	0.007
<i>cymCb11</i>	0.016	0.003	0.204	0.233	0.181	0.010
<i>cysCD519</i>	0.015	0	0.225	0.152	0.021	0.007
<i>metF96</i>	0	0.004	0.004	0.004	0.469	0.014

* Final dry wt minus initial dry wt (in mg/ml).

† Media also contained SO_4^{2-} (10 $\mu\text{g S/ml}$).

present as an impurity in MM(sf) which must in some way be stimulated by methionine.

Could the cysteine enzymes of *cym* mutants still be functioning *in vivo* in the presence of methionine? If so, it should be possible to stimulate with methionine the incorporation of radioactivity from extracellular $^{35}\text{SO}_4^{2-}$ into protein. Exponential-phase cultures of wild type and *cymA2* grown in MM+methionine were washed twice in MM(sf) medium and transferred to MM(sf) + $^{35}\text{SO}_4^{2-}$ (0.85 mM; 0.2 $\mu\text{Ci/ml}$) in either the presence or absence of methionine, and incubation was continued for 3 h. Some samples were collected on Millipore filters, with a layer of Celite (Dreyfuss, 1964), either directly or after treatment with 50 % TCA in MM(sf) + SO_4^{2-} , and washed with either MM(sf) + SO_4^{2-} or 5 % TCA in MM(sf) + SO_4^{2-} as appropriate, before their radioactivity was measured. Other samples were treated with 50 % TCA, washed twice in 5 % TCA, the protein contents oxidized (Margolis & Mandl, 1958) overnight and hydrolysates subjected to one-way descending chromatography in solvent C.

As the amount of radioactivity in whole cells and TCA-insoluble fractions of wild type and *cymA2* bacteria grown in the presence of methionine were almost the same (Table 8), methionine enabled the *cym* mutant to utilize exogenous sulphate for growth. In addition, the chromatograms of extracts of wild type growing in MM(sf) + $^{35}\text{SO}_4^{2-}$ without methionine gave two radioactive spots corresponding to cysteic acid and methionine sulphone, the oxidized forms of cysteine and methionine respectively. By contrast, extracts of both wild type and *cymA2* grown in the presence of methionine gave one spot corresponding to cysteic acid, which is compatible with methionine stimulating intracellular reduction of sulphate to sulphide and its subsequent conversion to cysteine in *cymA2*.

Inhibition by triazole and the cym phenotype. 1,2,4-triazole inhibits the growth of wild-type *S. typhimurium* in MM, this effect being reversed by cysteine, methionine or serine (Hulanicka *et al.* 1972). These authors presented evidence that triazole interferes with the induction of the cysteine biosynthetic enzymes by *O*-acetyl serine (OAS) involving the *cysB*

Table 8. Amount of radioactivity incorporated from $^{35}\text{SO}_4^{2-}$ into protein in wild-type and *cymA2* bacteria in the presence and absence of methionine

Strains	Growth media	Fractions	c.p.m./mg
Wild type	MM(sf) + $^{35}\text{SO}_4^{2-}$	WC†	37 815
		TCA	31 800
	MM(sf) + $^{35}\text{SO}_4^{2-}$ + methionine	WC	13 340
		TCA	9 149
<i>cymA2</i>	MM(sf) + $^{35}\text{SO}_4^{2-}$ *	WC	5 979
		TCA	4 966
	MM(sf) + $^{35}\text{SO}_4^{2-}$ + methionine	WC	12 215
		TCA	9 403

* *CymA2* did not grow at all in this medium.

† WC, whole cells; TCA, TCA-insoluble precipitate.

gene product (Jones-Mortimer, 1968). Triazole resistant (*trz^r*) mutants have been isolated in which OAS-sulphydrylase is depleted, with a consequent elevation in intracellular OAS levels, and which possess derepressed, but repressible, levels of the sulphate activation and reduction enzymes (Hulanicka & Klopotoski, 1972).

The effect of triazole on the wild type is similar to the effect of a *cym* mutation, and so it was of interest to determine the effect of *trz^r* mutations on the *cym* phenotype. *Trz^r* derivatives of the temperature-sensitive *cymA2* mutant were isolated by spreading washed overnight NB cultures on to MA + triazole (10 mM), incubating at the permissive temperature of 25 °C and picking colonies after three days. Spontaneous *cymA2 trz^r* double mutants arose at a frequency of 10^{-6} . The genotype of these strains was confirmed by transducing both the *cymA2* and the *trz^r* markers from the double mutant into *cysD505* and *cysA21*. The frequency of cotransduction of *cymA2* and *cysD⁺* (84 %) and of the *trz^r* lesion and *cysA⁺* (72 %) were virtually identical to that obtained using the singly marked strains *cymA2* and *trz110* as donors. The phenotype of the double mutants was determined by testing growth on MA, MA + cysteine, and MA + methionine at 37 °C. Of 17 examined, all grew well on MA + cysteine or methionine, and six grew on unsupplemented MA, suggesting that in these few at least the *trz^r* mutation was suppressing the *cym* mutation. However, the growth of the apparently suppressed strains on MA was not normal. High density inoculum streaks on to MA (37 °C) produced relatively few isolated colonies of normal size (3 to 5 mm); when these were similarly subcultured to MA, MA + cysteine, and MA + methionine, this pattern was again observed on MA but growth was normal on the supplemented media. It would seem that the suppression is unstable, and there is now further evidence to support this (Kingsman, unpublished).

DISCUSSION

The phenotypic distinction between *cym* mutants and *cys* or *met* mutants seems very clear. Growth responses to methionine and cysteine (Table 2 and Figs. 3 and 4) and their precursors (Fig. 3a) support this and also relate *cym* mutants to the established pathways of S-amino acid metabolism in *S. typhimurium* (Fig. 1). The division of the *cym* mutants into subgroups (Table 6) is based on: (i) their ability or inability to utilize sulphite as an alternative to cysteine; (ii) their complementation patterns both with each other and with *cys* mutants; (iii) the mapping data available (Tables 3, 4), which is virtually complete in cotransduction terms but which could be improved if further *cys* deletions were available; (iv) the

enzyme assays that have been carried out (Table 5), although there is a requirement for PAPS reductase assays (Fig. 1) particularly to define precisely the nature of *cymB* mutants. There is a consistency in these data which indicates firmly that each *cym* mutant is deficient in a cysteine enzyme and that methionine relieves that deficiency. Since there is no evidence of conversion of methionine to cysteine in *cym* mutants, they may be leaky and methionine spares intracellular cysteine, or methionine may restore their *cys* enzyme activity, presumably via a component of the wild-type cysteine regulatory system.

Leakiness is unlikely on a number of counts. Although growth in MM varied between *cym* mutants (Fig. 3), the increase in turbidity of cultures of some strains such as *cymA1* or *B3* during 7 h incubation indicated virtually no cell division. Also, the respective *cys* enzyme activities in *cymA*, *Bb*, *Bc*, *D* and *C* mutants were either undetectable or very low, and certainly were very similar to those in equivalent non-leaky *cys* mutants (Table 5). This is also true for sulphite reductase levels in *cymBb* mutants assayed in toluenized bacteria. Finally, sparing by methionine is likely to be partially or completely dependent upon *metJ*-mediated repression of methionine enzymes (Chater, 1970) and if it is a significant component of the response to methionine, *cym metJ* double mutants derepressed for methionine synthesis should require a greater minimum amount of methionine to give maximum growth. In fact there was no significant difference between *cymA2* and *cymA2 metJ* mutants in their growth response to methionine.

Direct evidence for the restoration of *cys* enzyme activity is sparse. Clearly the S-assimilation pathway is required during the growth of *cym* mutants on methionine (Table 7). However, no methionine-mediated restoration of *cys* enzyme activity in *cym* mutants has been observed either *in vitro* (cell-free extracts) or *in situ* (toluenized bacteria). *In vivo*, S-assimilation is stimulated by methionine (Table 8). These anomalies may be due to instability of methionine-restored enzyme activity during the assay procedures. If enzyme activity in *cym* mutants is restored, it implies deficiencies in their *cys* gene expression at the transcriptional or translational level since it seems unlikely that methionine could restore *cys* enzyme activity at a post-translational level. Support for this comes from the location of most of the *cym* mutations at the ends of the *cys* genes (Fig. 5) and from the apparent polarity of *cymA1* and *A7* (Table 5).

The unstable suppression by some *trz^r* mutations of the phenotype of the *cymA2* mutant (and of other *cym* mutants – Kingsman, unpublished) is another interesting property. Recent findings suggest that plasmid formation is involved in some cases of mutation to *trz^r* (Dr D. Hulanicka, personal communication) and that integration of this plasmid at or near the sites of the *cym* mutations is responsible for the suppression (Kingsman, unpublished). Similar integrative events have been found for the *galOP* region of *E. coli* (Saedler *et al.* 1974).

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