Isolation and Characterization of *cysK* Mutants of *Escherichia coli* K12

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cysK mutants, deficient in *O*-acetylserine sulphydrylase A [*O*-acetyl-L-serine acetate-lyase (adding hydrogen-sulphide); EC 4.2.99.8], were isolated as strains resistant to selenite or giving a black colour reaction on bismuth citrate indicator medium. All were resistant to the inhibitor 1,2,4-triazole. Four independent mutants were found which possessed lowered levels of *O*-acetylserine sulphydrylase activity and also partially constitutive levels of NADPH-sulphite reductase [hydrogen-sulphide:$NADP^+$ oxidoreductase; EC 1.8.1.2]. Strains containing both a *cysE* mutation and a *cysK* mutation lacked the constitutive levels of NADPH-sulphite reductase showing that these levels were due to the *in vivo* concentration of the inducer, *O*-acetylserine. The *cysK* locus was found to be 81 % cotransducible with the *ptsI* gene.

INTRODUCTION

In *Escherichia coli* K12 and *Salmonella typhimurium* the cysteine biosynthetic pathway consists of two converging branches, the final step of which involves the reaction of *O*-acetyl-L-serine and sulphide to give L-cysteine (Jones-Mortimer, Wheldrake & Pasternak, 1968; Kredich & Tomkins, 1966). On the sulphur branch, sulphate is activated via adenosine 5′-phosphosulphate to form 3′-phosphoadenosine 5′-phosphosulphate which is reduced to sulphite and then to sulphide (Dreyfuss & Monty, 1963; Pasternak *et al.*, 1965). The enzyme NADPH-sulphite reductase [hydrogen-sulphide:$NADP^+$ oxidoreductase; EC 1.8.1.2] catalyses the reduction of sulphite to sulphide (Siegel, Murphy & Kamin, 1973; Siegel & Davis, 1974; Siegel & Kamin, 1971). Serine transacetylase [acetyl-CoA:L-serine *O*-acetyltransferase; EC 2.3.1.30], the enzyme of the carbon branch which converts serine and acetyl-coenzyme A into *O*-acetylserine, is coded for by the *cysE* gene in both organisms (Jones-Mortimer *et al.*, 1968; Sanderson, 1972). In *S. typhimurium* the structural gene for one of the two final enzymes in the pathway, *O*-acetylserine sulphydrylase A [*O*-acetyl-L-serine acetate-lyase (adding hydrogen-sulphide); EC 4.2.99.8], has been designated *cysK* (Hulanicka, Kredich & Treiman, 1974). Jones-Mortimer (1968) has shown that three conditions must be satisfied for the enzymes of the sulphur branch to be synthesized: the intracellular concentration of cysteine must be low to avoid repression; a wild-type allele of the *cysB* regulatory gene must be present in the cell; and the inducer, *O*-acetylserine, must also be present.

cysK mutants of *S. typhimurium*, which have been isolated as strains resistant to the growth inhibitor 1,2,4-triazole, possess low levels of *O*-acetylserine sulphydrylase activity while remaining prototrophic for cysteine (Hulanicka *et al.*, 1974). The prototrophy of these strains is apparently due to the presence of a second *O*-acetylserine sulphydrylase which contributes only a small amount of the total activity of wild-type extracts (Becker &

Tomkins, 1969; Hulanicka *et al.*, 1974). Kredich, Foote & Hulanicka (1975) have shown that *O*-acetylserine sulphydrylase A catalyses a triazolylase reaction between *O*-acetylserine and 1,2,4-triazole, giving 1,2,4-triazole-1-alanine as a product. They have also shown that 1,2,4-triazole lowers the activities of several of the enzymes necessary for sulphate reduction. Thus, inhibition of growth appears to be caused by cysteine starvation due to a decreased availability of the cysteine precursors, *O*-acetylserine and sulphide. Resistance to 1,2,4-triazole can arise from mutations leading to a preferential loss of triazolylase activity over sulphydrylase activity or from mutations which diminish both activities. They have proposed a model which suggests that there could be an accumulation of *O*-acetylserine in *cysK* mutants of the second type. One can predict from this model that such *cysK* mutants, when grown on sulphate, should have elevated levels of enzymes of the sulphur branch of the pathway and that the introduction of a mutation into the *cysE* gene should abolish these elevated levels.

We report here the isolation and characterization of four *cysK* mutants which possess elevated levels of NADPH-sulphite reductase. *cysE cysK* double mutants have been constructed which lack these elevated levels of NADPH-sulphite reductase. These observations lend further support to the model of Kredich *et al.* (1975).

METHODS

Chemicals. Glutathione, NADPH, Tris, 1,2,4-triazole, DL-diaminopimelic acid (DAP), FAD, and 5-fluorouracil were obtained from Sigma. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. *O*-Acetylserine was synthesized by the method of Sakami & Toennies (1942). Ammonium bismuth citrate was purchased from Merck. Sodium selenite was from BDH.

Media. The nutrient medium used was L-broth (Lennox, 1955). The minimal medium used was medium E of Vogel & Bonner (1956) modified by the replacement of $MgSO_4$ by $MgCl_2$ and supplemented with glucose (0·2%, w/v), amino acids (40 μg ml^{-1}), thiamin (4 μg ml^{-1}), DAP (50 μg ml^{-1}) and other required growth factors. All solidified media contained 1·5% (w/v) agar. Bacteria for enzyme assays were grown in enriched liquid minimal medium which contained glucose (0·2%), a cysteine-free supplement of 19 amino acids (each at 30 μg ml^{-1}) and the required vitamins. Cystine was added as described by Kredich (1971). Sulphur compounds were added as indicated. Glutathione and *O*-acetylserine were filter-sterilized. 5-Fluorouracil was added at 2·5 μg ml^{-1}.

Bismuth citrate-enriched minimal detection medium contained ammonium bismuth citrate (0·15%, w/v) and yeast extract (0·02%, w/v). Selenite minimal medium contained 0·25 mM-sodium selenite and 1 mM-L-methionine. Triazole minimal medium contained 3 mM-1,2,4-triazole. The sulphur source in these three media was 8·5 mM-Na_2SO_4.

Bacterial strains. All the strains used were derived from *E. coli* K12 and are listed in Table 1.

Genetic techniques. Transductions with the generalized transducing phage P1 were performed according to Miller (1972). Recombinants were screened by replica-plating and single colonies of the required type were selected and isolated.

Mutagenesis of strain PA309 by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) was carried out according to the procedure of Adelberg, Mandel & Chen (1965); 10% of the cells survived a 20 min treatment with 100 μg NTG ml^{-1}. The mutagenized cell suspension was distributed into 20 test tubes and incubated at 37 °C overnight. About 100 cells from each culture were spread on to bismuth citrate indicator medium and selenite medium. Independent mutants, isolated as either black colonies on bismuth citrate agar or as red selenite-resistant colonies, were purified by restreaking on the same medium.

Mapping of the *cysK* locus was carried out by scoring the percentage cotransduction with the *ptsI* locus. The cotransduction frequency of the *dapE* marker with the *ptsI* gene was scored as a control in the same experiment. Positive selection for the *cysK* marker is complicated by a high rate of spontaneous mutation and it was therefore used as the unselected marker in transductional crosses. Growth on mannitol or on glucose was used to select *ptsI*$^+$ transductants and these were replica-plated on to minimal medium containing 1,2,4-triazole to score the cotransduction of the *cysK* marker. The results for the two carbon sources were averaged. Since the *dapE* gene was also an unselected marker, the medium was supplemented with diaminopimelic acid.

Growth and disruption of bacteria. Cultures used for enzyme assays were grown as follows. An overnight L-broth culture (4 ml) was inoculated into 25 ml enriched minimal medium and shaken overnight at 37 °C. A 10 ml portion of this culture was inoculated into 200 ml enriched minimal medium and shaken on a rotary

Table 1. *Escherichia coli* K12 *strains*

Strain	Sex	Description*	Source or derivation
AT978	HfrKL16	*thi-1, rel-1, dapE9*, (λ^-)	K. D. Brown
FF8028	F^-	*ptsI28, proC*	W. Epstein
JM70	F^-	*cysE, mtl-2*†	M. C. Jones-Mortimer
PA309	F^-	*mtl-2*†	M. C. Jones-Mortimer
RC703	F^-	Wild-type	M. C. Jones-Mortimer
RL103	F^-	*cysE*†	RC703 → JM70 (Mtl^+)‡
RL162	F^-	*cysK4, mtl-2*†	NTG-induced mutant of PA309 screened for black colour reaction on bismuth citrate medium
RL163	F^-	*cysK7, mtl-2*†	NTG-induced mutant of PA309 selected for resistance to selenite
RL164	F^-	*cysK8, mtl-2*†	As for RL163
RL165	F^-	*cysK11, mtl-2*†	Spontaneous selenite-resistant mutant of PA309
RL166	F^-	*cysK11, mtl-2, upp, dapE9*†	RL167 × RL165 (5-fluorouracil resistance)‡
RL167	HfrKL16	*thi-1, rel-1, dapE9, upp*, (λ^-)	This work; spontaneous 5-fluorouracil-resistant mutant of AT978
RL171	F^-	*cysK4, cysE*†	RL103 → RL162 (Mtl^+)‡
RL172	F^-	*cysK7, cysE*†	RL103 → RL163 (Mtl^+)‡
RL173	F^-	*cysK8, cysE*†	RL103 → RL164 (Mtl^+)‡
RL174	F^-	*cysK11, cysE*†	RL103 → RL165 (Mtl^+)‡

* Mutant allele abbreviations according to Bachmann, Low & Taylor (1976).

† Other markers: *thr-1, leu-6, trp-1, his-1, argH1, thi-1, xyl-7, ara-13, gal-6, lacY1, tonA2, malA1, str-9, supE*, (λ^-).

‡ Gene transfer: →, P1 transduction; ×, conjugation. The basis of selection is given in parentheses.

shaker at 37 °C. Growth was followed by measuring the turbidity of a 1 in 10 dilution in 0·9% (w/v) NaCl a 600 nm in a Unicam SP600 spectrophotometer. Cells were harvested in the late-exponential phase at 4 °C, resuspended in 3 ml cold 0·1 M-potassium phosphate buffer (pH 7·7) containing 1 mM-EDTA, and stored at 4 °C overnight. Cultures were checked for contamination by testing their phenotypic characteristics. Cells were disrupted using a Branson model B-12 sonifier fitted with a microtip. Cell debris was removed by centrifuging at 28000 *g* for 40 min and the supernatants (crude extracts) were used for enzyme and protein assays. FAD was added to the extracts to a concentration of 5 μM in order to stabilize NADPH-sulphite reductase.

NADPH-sulphite reductase assay. This activity was followed by measuring the initial rate of NADPH oxidation at 340 nm in a Cary spectrophotometer. Cuvettes contained (in 1·0 ml): 200 μM-NADPH, 50 mM-potassium phosphate buffer (pH 7·7), 600 μM-Na_2SO_3, 100 μM-EDTA (added in phosphate buffer), and 0·2 ml crude extract. A control lacking sulphite was run for each assay. All extracts were assayed in duplicate and corrected for any activity present in the control.

O-Acetylserine sulphydrylase assay. The incubation mixture (in 1·0 ml in small stoppered tubes) had a final pH of 7·5 and contained: 10 mM-Tris/HCl buffer (pH 8·0), 60 μM-EDTA (added in Tris/HCl buffer), 20 mM-HCl, 20 mM-Na_2S, 30 mM-*O*-acetylserine, and 0·1 ml crude extract [diluted as required in 0·1 M-phosphate buffer (pH 7·7) containing 1 mM-EDTA]. The reaction was started by the addition of crude extract and the incubation was carried out for 30 min at 37 °C. The reaction was stopped by the addition of 0·4 ml 7·5% HPO_3. One drop of octanol and approximately 15 mg NaCl were added, and nitrogen was bubbled through for 10 min to remove the remaining sulphide. The solution was then filtered through 4·25 cm Whatman no. 1 filter paper, and 0·6 ml filtrate was added to 2·0 ml 0·1 M-Tris, 1·0 ml water and 0·05 ml Ellman's reagent (Ellman, 1959). The absorbance was read at 412 nm and enzyme activities were calculated assuming an extinction coefficient of ϵ_{412} = 13600 M^{-1} cm^{-1}. All extracts were assayed in duplicate.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Isolation of cysK mutants

Mutants were isolated on indicator media as described in Methods and in Table 1. Each of the mutants was prototrophic and gave black colonies on bismuth citrate agar (wild-type colonies appear white), red selenite-resistant colonies on selenite agar (growth of wild-type colonies is inhibited on this medium), and each was resistant to 3 mM-1,2,4-triazole.

O-Acetylserine sulphydrylase activity of cysK strains

The four *cysK* mutants RL162, RL163, RL164 and RL165 had lowered levels of *O*-acetylserine sulphydrylase activity; these were approximately 5% of that found in the parent strain PA309 (Table 2).

NADPH-sulphite reductase activity of cysK strains

In order to isolate *cysK* mutants with both lowered triazolylase and sulphydrylase activity, the mutants isolated on the indicator media were screened for constitutive levels of NADPH-sulphite reductase. This was carried out on the basis that high concentrations of *O*-acetylserine would be expected to accumulate before the lowered sulphydrylase reaction, causing induction of the enzymes of the sulphur branch of the pathway. The results for four mutants (RL162, RL163, RL164 and RL165) which had partially constitutive levels of NADPH-sulphite reductase are shown in Table 2. Five out of 12 mutants isolated by these techniques possessed these characteristics. None out of 14 isolated as being directly resistant to 1,2,4-triazole possessed NADPH-sulphite reductase levels significantly above that of the parent strain.

To demonstrate that the partially constitutive levels of NADPH-sulphite reductase were dependent on the *O*-acetylserine concentration *in vivo*, a *cysE* mutation was introduced by transduction into the four constitutive mutants. Since the *cysE* gene codes for serine transacetylase, the enzyme responsible for producing *O*-acetylserine, this effectively blocked the production of *O*-acetylserine in the four transductants (RL171, RL172, RL173 and RL174). As shown in Table 2, the NADPH-sulphite reductase levels in these *cysK cysE* strains were much lower than in their *cysK cysE*$^+$ parents. Thus an elevated *O*-acetylserine concentration *in vivo* is responsible for the induction of NADPH-sulphite reductase in these mutants.

To check that the four *cysK cysE* transductants still possessed a mutant *cysK* gene, they were transduced back to *cysE*$^+$. This was necessary since *cysE* mutants require cysteine for growth, which overcomes the inhibition by 1,2,4-triazole (Hulanicka, Klopotowski & Smith, 1972). All the *cysK cysE*$^+$ transductants were resistant to 1,2,4-triazole, indicating that a defective *cysK* gene was still present.

Of two of the constitutive mutants tested, strains RL162 and RL163, both were found to have NADPH-sulphite reductase levels which were repressible by cystine when grown on this sulphur source (Table 2).

Mapping the cysK mutation

We found that the *cysK* gene was cotransducible with the *ptsI* gene. Strain FF8028 was transduced to *ptsI*$^+$ using P1 grown on strain RL166 as the donor. Of 446 *ptsI*$^+$ colonies selected on glucose plates, 75% were resistant to 1,2,4-triazole (*cysK*) and 12% were *dapE*. Similarly, of 559 *ptsI*$^+$ colonies selected on mannitol plates, 86% were *cysK* and 7% *dapE*. Thus the *cysK* and *dapE* loci were found to be 81% and 9% cotransducible respectively with the *ptsI* locus. The *cysK* locus therefore maps at approximately 52 min on the *E. coli* linkage map (Bachmann, Low & Taylor, 1976).

Table 2. *Specific activities of enzymes of the cysteine biosynthetic pathway in cysK mutants and their parent strains*

Strains were grown with 8·5 mM-sulphate or 0·4 mM-glutathione (GSH) or 0·4 mM-cystine as sulphur source. In some experiments, 2 mM-*O*-acetylserine was added to the medium. Enzyme assays were carried out as described in Methods: *O*-acetylserine sulphydrylase activities are expressed as μmol cysteine formed (mg protein)$^{-1}$ h^{-1}, and NADPH-sulphite reductase activities as nmol NADPH oxidized (mg protein)$^{-1}$ min^{-1}.

Strain and description	Sulphur source	*O*-Acetylserine	Enzyme specific activity: *O*-Acetylserine sulphydrylase	Enzyme specific activity: NADPH-sulphite reductase
PA309 (*cysK*$^+$)	SO_4^{2-}	−	411·3	3·7
PA309 (*cysK*$^+$)	GSH	+	ND	38·2
RL162 (*cysK*)	SO_4^{2-}	−	19·0	16·8
RL163 (*cysK*)	SO_4^{2-}	−	33·2	21·8
RL164 (*cysK*)	SO_4^{2-}	−	23·9	11·2
RL165 (*cysK*)	SO_4^{2-}	−	16·4	27·2
RL162 (*cysK*)	Cystine	−	ND	<0·5
RL163 (*cysK*)	Cystine	−	ND	<0·5
RL103 (*cysK*$^+$ *cysE*)	GSH	−	ND	1·3
RL103 (*cysK*$^+$ *cysE*)	GSH	+	ND	30·5
RL171 (*cysK cysE*)	GSH	−	ND	4·4
RL172 (*cysK cysE*)	GSH	−	ND	2·9
RL173 (*cysK cysE*)	GSH	−	ND	3·6
RL174 (*cysK cysE*)	GSH	−	ND	4·3

+, Present; −, absent; ND, Not determined.

DISCUSSION

The isolation of *cysK* mutants of *E. coli* yielded some strains which possessed both lowered levels of *O*-acetylserine sulphydrylase activity and partially constitutive levels of NADPH-sulphite reductase. That this elevated NADPH-sulphite reductase level is dependent on a high *O*-acetylserine concentration *in vivo* was demonstrated by the observation that the introduction of a *cysE* mutation into the pathway of *O*-acetylserine biosynthesis resulted in a significant lowering of the activity of NADPH-sulphite reductase in these mutants. Of the two strains assayed for repression of NADPH-sulphite reductase by cystine, this enzyme was found to be repressible in both. These observations support the hypothesis that in these *cysK* mutants elevated levels of *O*-acetylserine accumulate before the lowered sulphydrylase reaction causing induction of NADPH-sulphite reductase. Concomitantly, if a decrease in the intracellular level of cysteine occurred as a result of the lowered sulphydrylase activity, conditions for derepression of NADPH-sulphite reductase would result.

Hulanicka *et al.* (1974) have reported that *S. typhimurium cysK* mutants grown on djenkolic acid as the sulphur source, possess the same derepressed levels as the wild-type strain for several enzymes of the sulphur branch of the pathway. The results reported in this paper show that NADPH-sulphite reductase levels in *E. coli cysK* mutants are higher than in the parental strain when grown on sulphate which leads to partial repression of these enzymes in the wild type.

Only some *cysK* mutants have elevated NADPH-sulphite reductase levels. A possible explanation for the *cysK* mutants which possess normal levels of NADPH-sulphite reductase is that there is a preferential loss of triazolylase activity over sulphydrylase activity, with the result that *O*-acetylserine does not accumulate but is metabolized to form cysteine.

Although the basis for the 1,2,4-triazole resistance of *cysK* strains has been well documented (Kredich *et al.*, 1975), the basis for selenite resistance and the black colour reaction on bismuth citrate indicator medium is less well understood. Selenite, an analogue of sulphite, has been shown to inhibit the growth of *E. coli* (Scala & Williams, 1962), while

E. coli grown on media containing sodium selenite will reduce the selenite to elemental selenium and give the culture a brick-red colour (Gerrard, Telford & Williams, 1974). In *S. typhimurium*, a selenite-resistant mutant has been found to map at the locus for 1,2,4-triazole resistance (Hulanicka *et al.*, 1974).

The *cysK* mutation in *S. typhimurium* (originally called the *trzA* mutation because it conferred resistance to 1,2,4-triazole) maps at a point very close to the *ptsI* locus (Cordaro & Roseman, 1972). Our results indicate that it also maps very close to this gene in *E. coli*. The cotransduction frequency of 9% for the control experiment between the *dapE* and *ptsI* loci agrees with the value of 8% reported by Bukhari & Taylor (1971).

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