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Constitutive Mutation of *cysJIH* Operon in a *cysB* Deletion Strain of *Salmonella typhimurium*

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Summary. In a *cysB* deletion strain a new mutation, denoted *cys-2332* was isolated, which causes the constitutive expression of the *cysJIH* operon. *cys-2332* is closely linked to *cysJIH* and presumably is located in the initiator region of this operon, rendering its expression independent of the *cysB* gene product and the internal inducer *O*-acetyl-L-serine. The presence of sulfite reductase (encoded by *cysI* and cysJ) activity in a *cysB*$^-$ *cys-2332* double mutant indicates that *cysG*, which is not linked to *cysJIH* but is required for the synthesis of the sulfite reductase co-factor siroheme, is not controlled by *cysB*.

Introduction

The *cysB* region in *S. typhimurium* and in *E. coli* is involved in the positive regulation of the cysteine regulon (Spencer et al., 1967; Jones and Mortimer, 1968; Kredich, 1971). This locus controls the expression of all other cysteine genes, except for *cysE* (Kredich, 1971) and *cysG* (this paper), and is located apart from all of them. *cysB* auxotrophs generally show low or unmeasurable levels of cysteine enzymes except for serine transacetylase, specified by the *cysE* gene. This enzyme catalyzes the synthesis of *O*-acetyl-L-serine, which is an immediate precursor of cysteine and the inducer of the cysteine biosynthetic pathway. Consequently *cysE* mutants are pleiotropic and, unlike wild type, cannot be depressed for cysteine enzymes by growth on poor sulfur sources.

Several recent reports have helped to extend our knowledge concerning the regulation of cysteine biosynthesis. The isolation of amber mutants in the *cysB* gene of *E. coli* has provided evidence that the product of this gene is a protein (Tully and Yudkin; 1975). Fimmel and Loughlin described the isolation of a λd*cysJIHD* transducing bacteriophage (Fimmell and Loughlin, 1977) and using this phage in DNA-RNA hybrydization assays for cys specific messenger RNA, they showed in intact bacteria that cysteine biosynthesis is controlled at the transcriptional level by the inducer *O*-acetyl-L-serine, the *cysB* protein and cysteine. Recently, a method has been described for the isolation of pleiotropic constitutive mutants mapping within the *cysB* gene (Sledziewska and Hulanicka, 1978), and an analysis of merodiploids of the *cysB* region demonstrated dominance of constitutive mutant alleles over wild type (Jagura, Hulanicka and Kredich, 1978). This communication reports the isolation of a constitutive mutant in the *cysJIH operon in a cysB* deletion strain, and thus provides additional evidence for the positive regulation of the cysteine regulon.

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Materials and Methods

Organisms. All bacterial strains used were derivatives of *S. typhimurium LT2* (Table 1).

Media and Culture Conditions. The minimal medium E of Vogel and Bonner (1956) in which $MgSO_4$ was replaced by an equimolar amount of $MgCl_2$ (Hulanicka et al., 1972) was used. Either 0.1 mM L-cysteine, 0.5 mM L-djenkolic acid, 1 mM L-cysteine sulfinic acid, 1 mM $NaHSO_3$ or 0.01 mM $Na_2S \cdot 9H_2O$ were used as the sulfur source as indicated. The medium was supplemented with 0.5% glucose and with 0.2 mM uracil or 0.1 mM of appropriate amino acids when required. Solid media were prepared by addition of agar to a final concentration of 1.5%. Recipient bacteria for transductional crosses were grown in nutrient broth (0.8% dry nutrient broth and 0.4% NaCl). Except where specified, all liquid cultures were grown with rotary shaking at 37° C. Cell densities were estimated by measuring turbidity at 650 nm. Bacteria for enzyme assays were harvested from exponentially growing cultures by centrifugation.

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Table 1. Designations and derivations of strain

Strain	Genotype	Source or method of construction
TK1000	wild type	N.D. Zinder
TK1415	*cysCD519*	K.E. Sanderson
TK1514	*cysG382*	K.E. Sanderson
TK1536	*aroB74*	K.E. Sanderson
TK1472	*cysE11*	K.E. Sanderson
TK2172	*cysJ266*	N.M. Kredich
DW363	*leu500 (supX cysB1763) pyrF146*	N.M. Kredich
TK2167	*leu500 (supX cysB1763) pyrF146 cys-2332*	mutation in strain DW363
TK2168	*cysJ226*$^+$ *cys-2332*	transductant from TK2167 lysate × *cysJ226*
TK2169	*cysCD519*$^+$ *cys-2332*	transductant from TK2167 lysate × TK1415
TK2170	*cysCD519*$^+$ *cys-2332 cysG2333*	NG-induced mutation in strain TK2169
TK2171	*cysCD519*$^+$ *cys-2332 cysE2334*	NG-induced mutation in strain TK2169
TK2173	*cysCD519*$^+$ *cys-2332 cysG2333*$^+$	transductant from TK1000 lysate × TK2170
TK2174	*leu500 supX*$^+$ *cysB1763*$^+$ *pyrF146 cys-2332*	transductant from TK1000 lysate × TK2167

NG – *N*-Methyl-*N'*-nitro-N-nitrosoguanidine

Genetic Methods. Phage P22 transduction was carried out as described previously (Hulanicka et al., 1972), using the P22 L4 mutant (Smith and Levine, 1967). Strains were mutagenized according to the procedure of Hulanicka et al. (1972), and penicillin selection was done according to Roth (1970).

Enzyme Assays. *O*-Acetylserine sulphydrylase and 3'-phosphoadenosine-5'-phosphosulphate reductase (PAPS reductase) activities were determined as described previously (Kredich, 1971). Sulfite reductase was assayed in cell free extracts by the method of Vito and Dreyfuss (1964). For the assay of sulfite reductase activity in intact cells, one ml of an overnight stationary culture in nutrient broth containing 0.1 mM cystine was diluted with 4 ml of the same medium and incubated at 37° C with rotary shaking for two hours. This culture was then quickly cooled, centrifuged, and the cells were washed once with 0.15 M saline and resuspended in 2.5 ml of 0.15 M saline. One ml of this inoculum was diluted with 1 ml of 2 × concentrated medium E (Vogel and Bonner, 1956) containing 1 mM $NaHSO_3$. The tube was then closed with a plastic cap and placed at 37° C for 40 min, following which sulfide was determined by the method of Siegel (1965). This method is a screening procedure, giving only qualitative data.

Chemicals. *O*-Acetyl-L-serine was prepared by the method of Sakami and Toennies (1942). Other chemicals were commercial products of reagent grade.

Results

Isolation and Characterization of Constitutive Mutants in the cysJIH Operon

The strain DW363 [*leu500*△(*supX, cysB1763*) *pyrF146*] carrying a deletion in *cysB* was plated on minimal plates containing 0.2 mM uracil and either 1 mM L-cysteine sulfinic acid (CSA) or 1 mM $NAHSO_3$. After 3-4 days of incubation at 37° C a few mutant colonies appeared on the plates. These were purified by repeated cloning, and their ability to grow on CSA and sulfite was determined by replica plating. The ability of these mutants to grow on sulfite suggests that they have regained sulfite reductase activity. Therefore, the activity of this enzyme was assayed in cell-free extracts of bacteria grown both on L-djenkolic acid and on L-cysteine. All examined mutants had appreciable levels of sulfite reductase on both sulfur sources. Apparently, genes coding for the protein components of sulfite reductase are no longer under the control of *cysB* in these strains and are not repressible by L-cysteine.

Genetic Mapping

Sulfite reductase activity depends on the expression of three genes *cysG, cysJ, cysI.* The genes *cysJ* and *cysI* code for protein components of sulfite reductase and the *cysG* product is required for synthesis of the sulfite reductase prosthetic group – siroheme (Murphy et al., 1973). Two out of these three genes belong to the *cysJIH* operon which is closely linked with the *cysC* and *cysD* genes. The third gene *cysG* is not linked to *cysD* but is 50% contransduced with *aroB* by P22. Mapping of the isolated mutants, designated CSA, was performed by P22 mediated transduction. Phage lysates prepared on five mutants were used as donors in crosses with recipient strains *cysJ226*, *cysCD519* and *aroB74*, and prototrophic recombinants were selected on minimal glucose plates. The presence of a mutation responsible for the CSA phenotype in transductants could be scored only biochemically by assaying sulfite reductase activity. Because of the large number of colonies to be tested a modification of the sulfite reductase assay was developed. This enzyme was assayed in strains grown on L-cystine, which represses sulfite reductase in wild type but not in CSA transductants. Of the five CSA mutants tested, only *cys-2332* showed linkage with *cysJ*, and this mutant was used in further experiments. The other mutants showing constitutive sulfite reductase expression but not linked to *cysJ* will be the subject of future work. All ten CysJ$^+$ and four out of six CysCD$^+$ transductants using the *cysB1763 cys-2332* strain as donor showed constitutive levels of sulfite reductase, indicating that the unregulated sulfite reductase activity found in the *cysB1763 cys-2332* strain is caused by a mutation located in or very close to *cysJ*. The very presence of sulfite reductase in the *cysB1763 cys-2332* strain suggests that *cysG* is transcribed even in the absence of the *cysB* gene

Table 2. Activities of sulfite reductase, PAPS reductase and O-acetylserine sulphydrylase after growth on different sulphur sources

Strain	Relevant genotype	Sulfite reductase (nmol/min/mg)			O-Acetylserine sulphydrylase (μmol/min/mg)			PAPS reductase (nmol/min/mg)	
		L-djenkolic acid	SO_4^{2-}	cystine	L-djenkolic acid	SO_4^{2-}	cystine	L-djenkolic acid	cystine
TK100	*cysB*$^+$	170	80	0	20	6	0.4	0.59	–
DW363	*cysB1763*	0	–	0	0.3	0.4	0.2	–	–
TK2167	*cysB1763 cys-2332*	56	71	69	0.2	0.3	0.2	–	0.28
TK2174	*cysB1763*$^+$ *cys-2332*	65	72	71	17	7	0.2	–	–
TK2169	*cysCD519*$^+$ *cys-2332*	71	73	70	18	6	0.4	–	–
TK2168	*cysJ226*$^+$ *cys-2332*	53	84	74	18	6	0.3	–	–

PAPS; 3′-phosphoadenosine-5′-phosphosulfate

product. These results suggest that the mutation *cys-2332* lies in the initiator region of the *cys-JIH* operon, and allows expression of *cysJ* and *cysI* in the absence of the *cysB* regulatory protein.

Biochemical Analyses

Sulfite reductase, PAPS reductase and *O*-acetylserine sulphydrylase were assayed in strains bearing *cys-2332*. Bacteria were grown on various sulfur sources and enzyme activities were determined in cell free extracts. The results of these experiments are presented in Table 2. In the *cysB*$^+$ strain cysteine biosynthetic enzymes are derepressed by growth on the poor sulfur source L-djenkolate or repressed by growth on L-cysteine (Kredich, 1971). As expected, strain *cysB1763* was found to have unmeasurable levels of sulfite reductase and a very small amount of *O*-acetylserine sulphydrylase. However, the specific activity of sulfite reductase found in the double mutant *cysB1763 cys-2332* was approximately equal to that of sulfate-grown wild type and independent of the sulfur source used for growth. The results obtained with the *cysB*$^+$ *cys-2332* strain were virtually identical to those found in the double mutant. Furthermore, PAPS reductase activity in L-cysteine grown *cysB1763 cys-2332* was approximately one-half that of L-djenkolate grown wild type. Since this enzyme is known to be repressed to unmeasurable levels by growth of wild type on L-cysteine and under any conditions of growth in all *cysB*$^-$ mutants examined, this result indicates that *cys-2332* causes the constitutive expression of the structural gene for PAPS reductase, *cysH*. Although the mutation *cys-2332* affects the expression of sulfite reductase genes, it has no effect on *O*-acetylserine sulphydrylase levels (Table 2). In order to exclude any potential confusion due to a possible secondary mutation, sulfite reductase activity was assayed in strains into which the *cys-2332* mutation was introduced by P22 mediated transduction. Both *cysCD*$^+$ *cys-2332* and *cysJ*$^+$ *cys-2332* transductants showed the same constitutive levels of sulfite reductase as were found in the double mutant. In further experiments K_m values and the effects of KCN on sulfite reductase activities were examined. The sulfite reductase activities of wild type and the *cys-2332* strain were completely inhibited by 50 μM KCN and the K_m values for sulfite were equal to 1.25×10^{-5} M in extracts of both strains. This values is consistent with that reported by Siegel (1964). Therefore, it is not likely that the phenotype of the *cys-2332* strain is due to an altered sulfite reductase.

Growth Experiments

Growth rates of the double mutant *cysB1763 cys-2332* on various sulfur sources were measured. This mutant grows at the same rate as wild type on L-cystine and L-djenkolate. However the generation time of the double mutant on sulfite is longer (120 min) than that of wild type and *cysB*$^+$ *cys-2332* (60 min). We also found that exogenous sulfide cannot support the growth ot the double mutant, suggesting that only endogenously formed sulfide, probably bound to some component of the sulfate reductive system, can be used as a substrate for the sulphydrylation of O-acetylserine as proposed by Tsang and Schiff (1976).

The Effects of cysE and cysG Mutations on Sulfite Reductase Activity in cys-2332 Strains

The activity of sulfite reductase in *cys-2332* strain carrying a deletion in the *cysB* regulatory gene indicates that *cys-2332* allows transcription of sulfite reductase genes in the absence of the *cysB* activator protein. It was of interest to determine whether the presence of the inducer of the cysteine regulon, *O*-acetyl-L-serine, is also dispensable for sulfite reductase expression in a *cys-2332* strain. The strain *cysE2334*

Table 3. Effects of *cysE* and *cysG* mutations on sulfite reductase activity

Strain	Relevant genotype	Sulfite reductase nmol/min/mg			O-Acetylserine sulphydrylase μmol/min/mg		
		Nutrient broth	L-djenkolic acid	Cystine	Nutrient broth	L-djenkolic acid	cystine
TK1000	*cysB*$^+$	29		0	5		0.5
TK1472	*cysE11*	0		0	0		0
TK2171	*cys-2332 cysE2334*	57		48	0		0
TK1514	*cysG382*	–	0	0	–	17	0.3
TK2170	*cys-2332 cysG2333*	–	0	0	–	15	0.5
TK2173	*cys-2332 cysG2333*$^+$	–	70	64	–	18	0.4

cysE mutants were grown on nutrient broth because of the very poor growth on L-djenkolic acid

cys-2332, which is a non-reverting cysteine auxotroph lacking any serine transacetylase activity, was grown on nutrient broth, and the level of sulfite reductase in crude extracts of this mutant was assayed. The results presented in Table 3 show that the mutation *cys-2332* renders expression of *cysJI* genes independent not only of the product of *cysB* but also of the presence of the internal inducer *O*-acetyl-L-serine.

The fact that sulfite reductase activity is present in the *cysB1763 cys-2332* strain indicates that the *cysG* gene, which is involved in the synthesis of the sulfite reductase co-factor, siroheme, is expressed even in the absence of a *cysB*$^+$ gene product or that there may be another electron carrier which can substitute for siroheme in the *cys-2332* strain. To exclude the latter possibility the cysteine auxotroph *cysG2333 cys-2332* was obtained by mutagenesis of a *cys-2332* strain. The *cysG* genotype of this double mutant was established by P22 transduction using an *aroB74* strain as recipient. The lack of sulfite reductase activity in *cysG2333 cys-2332* (Table 3) shows that the product of the *cysG* gene is indispensible for sulfite reductase activity.

Discussion

A mutant with constitutive synthesis of sulfite reductase and PAPS reductase was isolated in a strain carrying a deletion in the *cysB* gene. This new mutation, denoted *cys-2332*, maps in or very close to the *cysJ* gene and renders the expression of the *cysJ, cysI* and *cysH* genes independent of the *cysB* gene product. The specific activity of sulfite reductase in *cys-2332* strains is independent of the sulfur source used for growth and equal to the activity in wild type grown on sulfate (Table 2). Introduction of a *cysG* mutation into the *cys-2332* strain (Table 3) proved that siroheme is necessary for sulfite reductase activity. Thereore the most likely explanation for the sulfite reductase activity found in the double mutant *cysB1763 cys-2332* is that the *cysG* gene is not controlled by the *cysB* regulatory gene. Furthermore, by introducing a *cysE* mutation into the *cys-2332* strain we have shown that the expression of *cysJ* and *cysI* genes proceeds in this double mutant without the internal inducer *O*-acetyl-L-serine.

Since both *cysJ* and *cysI* must be expressed for sulfite reductase activity, and since PAPS reductase is expressed at a high level in a L-cystine grown culture of a *cysB*$^-$ *cys-2332* strain, it seems likely that *cys-2332* is a mutation of the regulatory region of the *cysJIH* operon (Reznikoff, 1974). In the case of negatively controlled operons such mutants are described as operator constitutive mutants. When a operon is regulated positively, such mutants are denoted as initiator constitutive.

Similar type mutations have been obtained in an initiator site of the arabinose operon in strains carrying deletions of the *araC* regulatory gene (Gielow et al., 1971). These *araI*^{c} strains show constitutive expression of the *araOIBAD* operon and are not further inducible by L-arabinose. Recently we reported the isolation of a constitutive mutant of the *cysK* gene. (Wiater and Hulanicka, 1978). The expression of *cysK* in this mutant was independent of the *cysB* product and of the sulfur source used for growth.

The presence of sulfite reductase activity in a *cysB* strain eliminates an alternative model in which the *cysB* protein converts *O*-acetyl-L-serine to a true inducer, which in turn inactivates a repressor produced by unknown regulatory gene, since one would expect to find mutants lacking repressor. Such mutants should show constitutivity for all cysteine genes controlled by *cysB* whereas the *cys-2332* mutation affects a transcription of only one operon, *cysJIH*.

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