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Method of Isolation of Cysteine Constitutive Mutants of the Cysteine Regulon in *Salmonella typhimurium*

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Summary. A method for selection of constitutive *cysB* mutation is described which takes advantage of the resistance of cysteine constitutive mutants to 1,2,4-triazole. Since *cysM cysK* double mutants are cysteine auxotrophs, by selecting for triazole resistance in *cysM* strains, mutants arising under this condition also should be constitutive for cysteine biosynthesis. Genetic analysis of mutants isolated by this technique showed that their mutational sites are located in the *cysB* region. Biochemical assays of cysteine enzymes, sulphite reductase and O-acetylserine sulfhydrylase of the mutants showed the derepressed level of these enzymes and the lack or slight repression by l-cysteine.

Introduction

The final step in the *de novo* biosynthesis of L-cysteine in *Salmonella typhimurium* involves the sulfhydrylation of O-acetyl-L-serine, which reaction is catalyzed by either of two enzymes: O-acetyl serine sulfhydrylase-A (Becker, Kredich and Tomkins, 1969) coded for by the *cysK* gene (Hulanicka, et al., 1974); and *O*-acetylserine sulfhydrylase-B (Becker and Tomkins, 1969) coded for by the *cysM* locus (Hulanicka, Hallquist, Kredich and Mojica-A, unpublished results). Therefore, although cysteine auxotrophy occurs in strains lacking both enzymes, mutations in either *cysK* alone or in *cysM* alone do not lead to a cysteine requirement.

Ordinarily *O*-acetylserine sulfhydrylase-A comprises most of the sulfhydrylase activity in wild type cells and can also catalyze the reaction of 1,2,4-triazole with *O*-acetyl-L-serine to give 1,2,4-triazole-1-*L*-alanine (Kredich et al., 1975). This reaction results in the consumption of *O*-acetyl-*L*-serine in the presence of 1,2,4-triazole with a resultant inhibition of growth which can be overcome by exogenous cysteine. The utilization of *O*-acetyl-*L*-serine in the triazolylase deprives the cell not only of this precursor of the carbon skeleton of cysteine, but also of the inducer of the enzymes necessary for reduction of sulphate to sulphide (Kredich, 1971). Therefore, triazole inhibition is also readily overcome by exogenous sulphide. Mutant strains lacking *O*-acetyl serine sulfhydrylase-A are essentially free of triazolylase activity and are resistant to 1,2,4-triazole, while *cysM* mutants, lacking *O*-acetylserine sulfhydrylase-B are still sensitive to this inhibitor.

Two other types of 1,2,4-triazole resistant mutations have been described. The first is due to a *cysE* promoter-up mutation (Hulanicka and Kredich, 1976) which results in the overproduction of *O*-acetyl-*L*-serine; the other is the prototrophic mutant *cysB1352* (Cheney and Kredich, 1975) which constitutively expresses the enzymes necessary for sulphate reduction even in the absence of *O*-acetyl-L-serine. So far, no method for the selection of constitutive *cysB* mutations has been described. In this paper, we report a new method of isolation of such mutants, which takes advantage of the fact that *cysK cysM* double mutants are usually cysteine auxotrophs. Thus, by selecting for triazole resistance in a *cysM* strain, isolates are much more likely to carry $cysB^c$ or *cysE* promoter-up mutations than $cysK^-$ alleles.

Material and Methods

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

Media and Culture Conditions. The minimal medium used was the medium C of Vogel and Bonner (1965) in which $MgSO_4$ was replaced by equimolar amounts of $MgCl_2$, and the sulphur source

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Table 1. Designation and derivation of strain

Strain	Genotype	Source or method of construction
TK 1000	wild type *pyrE125*	E.D. Zinder P.E. Hartman
SB 1690	*trpB223 ptsI34*	C. Cordaro
DW 221	*ara9 hisC340 thy trpA160 pyrF146* *trpC109*	N.M. Kredich K.E. Sanderson
TK 2013	*trpC109 trzB cysM2362*	D. Hulanicka
TK 2021	*trpC109 cysM2362*	triazole sensitive revertant of TK 2013
DW 25	*cysB1352*	N.M. Kredich
DW 379	*trpC109 trzB cysM1771*	D. Hulanicka
DW 385	*trpC109 cysM1771*	triazole sensitive revertant of DW 379
TK 2119	*trpC109 cysM2362 cysB^c2346*	DES-induced mutation in strain TK 2021
TK 2120	*ara9 hisC340 thy cysB^c2346*	Transductant from TK 2119 lysate × DW 221
TK 2108	*trpC109 cysM2362 cysB^c2341*	DES-induced mutation in strain TK 2021
TK 2109	*ara9 hisC340 thy cysB^c2341*	Transductant from TK 2108 lysate × DW 221
TK 2102	*trpC109 cysM2362 cysB^c2337*	DES-induced mutation in strain TK 2021
TK 2103	*ara9 hisC340 thy cysB^c2337*	Transductant from TK 2102 lysate × DW 221
TK 2131	*trpC109 cysM1771 cysB^c2351*	DES-induced mutation in strain DW 385
TK 2132	*ara9 hicC340 thy cysB^c2351*	Transductant from TK 2131 lysate × DW 221
TK 2159	*trpC109 cysM1771 cysB^c2360*	DES-induced mutation in strain DW 385
TK 2160	*ara9 hisC340 thy cysB^c2360*	Transductant from TK 2150 lysate × DW 221
TK 2165	*trpC109 cysM1771 cysB^c2361*	DES-induced mutation in strain DW 385
TK 2166	*ara9 hisC340 thy cysB^c2361*	Transductant from TK 2165 lysate × DW 221

was provided as either 0.1 mM L-cysteine, 0.5 mM L-djenkolic acid or 0.1 mM Na_2SO_4 (Hulanicka and Kłopotowski, 1972). Supplements consisted of 0.5% glucose and, when appropriate for the growth of auxotrophs, 0.2 mM uracil and 0.1 mM of the required amino acid.

Solid media were prepared by the addition of agar and additional glucose to final concentrations of 1.5% and 2.0%, respectively. The recipient bacteria in transduction were grown in nutrient broth.

Except where specifically noted, all liquid cultures were grown with rotary shaking at 37°. Cell densities were estimated by measuring turbidity at 650 nm. Bacteria for enzyme studies were harvested from exponentially growing cultures by centrifugation.

Genetic Methods. Phage P22 transduction was performed as described previously (Hulanicka and Kłopotowski, 1972), with the use of the L4 strain (Smith and Levine, 1967). Resistance to 1,2,4-triazole was scored by replicating colonies onto minimal plates containing 10 mM of this inhibitor. Resistance to the second inhibitor of sulphate metabolism, sodium selenate, was scored by replicating recombinants onto minimal agar plates containing 1 mM of this compound.

Enzyme Studies. O-acetylserine sulfhydrylase was determined as described previously (Kredich et al., 1969). Sulphite reductase was assayed by the method of de Vito and Dreyfuss (1964). Protein was determined by the biuret method (Gornall et al., 1949) using bovine albumin as a standard.

Chemicals. O-Acetyl-L-serine was prepared by the method of Sakami and Toennies (1942). 1,2,4-triazole was obtained from Sigma, and other chemicals were commercial products of reagent grade.

Results

Isolation of Mutants. The *cysM* mutants were plated on 1,2,4-triazole solid agar media with paper discs containing one drop of DES. Colonies formed after 3–4 days of incubation at 37° were purified and the phenotypes were checked by replica plating. It is known that the constitutive mutant *cysB1352* excretes sulphide and can feed wild type on triazole and selenate plates (Cheney and Kredich, 1975). Because of this property trz^+ colonies might be scored as trz^-. However, colonies of the wild type strain can be distinguished from the constitutive ones by their color on 1 mM selenate plates, where constitutive mutants are orange, whereas wild type colonies appear white.

Genetic Mapping of trz^- Mutants

Mapping of the isolated mutants was performed by P22-mediated transduction. The three types of triazole resistant mutants are linked in P22-mediated transduction with known chromosomal makers: the *cysK* gene with the *pts* operon, the constitutive mutants *cysB1352* with the *pyrF* locus and the *trp* operon, the *cysE* promoter-up mutation with the *cysE* locus. Phage lysates prepared on the isolated triazole-resistant mutants were used as donors in crosses where the recipient carried the above markers. The transductants were scored for their resistance phenotype. From more than one hundred separated mutants none yielded the triazole resistant type linked with the *cysE* locus. Ten out of hundred triazole-resistant mutants showed linkage with the *pts* operon, which is due to resistance resulting from mutation in the *cysK* gene. Since the double mutant *cysM cysK* is a cysteine auxotroph, the appearance of these ten mutants could be caused either by reversion of the

Table 2. Activity of OASS and SR in wild type, *cysB1352* and in isolated newly *cysB*^{c} mutants grown on different sulfur sources

Pertinent genotype	OASS			SR		
	Sulfur source					
	Djenk	SO_4^{2-}	Cys	Djenk	SO_4^{2-}	Cys
cysB$^+$	100	49±5.5	9±2	100	50±6	8±1.5
cysB^{c}1352	101±1	82±6	67±1	157±17	136±5	115±18
cysB^{c}2346	134±1	139±1	111±10	116±20	106±22	115±10
cysB^{c}2341	90±1	86±1	67±1	75±12	50±11	53±6
cysB^{c}2337	136±1	136±30	137±7	171±8	99±20	121±9
cysB^{c}2351	87±5	82±4	78±3	91±3	74±9	68±1
cysB^{c}2360	91±5	68±2	46±5	130±20	82±10	61±17
cysB^{c}2361	101±5	69±1	49±12	142±11	63±16	58±18

OASS = O-acetylserine sulfhydrylase; SR = sulphite reductase; ± = plus or minus standard error

Enzyme assays were performed on transductants in order to prevent any effects of additional mutations. Enzyme activity expressed as % of mean values of wild type on L-djenkolate

cysM mutation or by the leakiness of the *cysK* mutants formed.

In order to check if the triazole resistant phenotype results from the mutation in *cysB* regulatory gene, *trp*$^-$ and *pyrF*$^-$ markers were transduced to prototrophy. This procedure required the transfer of the whole *cysB* region: which means that one cannot calculate the linkage of the *trz* mutational site with the transduced markers. However, the fine mapping of mutational sites leading to cysteine constitutive mutants is hindered in any event by the ability of strains bearing the allele to feed triazole-sensitive colonies on triazole plates. The remained ninety triazole-resistant mutants showed the linkage with *trpA* and *pyrF* loci, similary to the previously known *cysB1352* mutant. All transductants *trpA*$^+$ *trz*$^-$ *pyrF*$^+$ were also resistant to selenate.

Table 3. Repression index of OASS and SR in wild type and in constitutive cysteine mutants

Pertinent genotype	Mutants repression index[a] (RI)	
	OASS	SR
cysB$^+$	11.1	12.5
cysB^{c}1352	1.51	1.37
cysB^{c}2346	1.21	1.01
cysB^{c}2341	1.35	1.42
cysB^{c}2337	1.00	1.41
cysB^{c}2351	1.12	1.34
cysB^{c}2360	1.98	2.13
cysB^{3}2361	2.06	2.45

[a] Repression Index is the ratio of the enzyme activity of bacteria grown on L-djenkolic acid to the enzyme activity of bacteria cultured on L-cysteine

Biochemical Analysis

In order to avoid any potential confusion due to a possible secondary mutation as well as the effect of the *cysM* mutation all biochemical assays were performed on nonlysogenic transductants which were *pyrF*$^+$ *trp*$^+$ *trz*$^-$. The constitutive mutant *cysB1352* is characterized by the derepressed level of the cysteine biosynthetic enzymes on minimal media (Kredich, 1971; Spencer, 1967) which are not repressed by growth on cysteine. In order to check if our newly isolated mutants have a similar level of cysteine enzymes, we studied the activities of sulphite reductase and *O*-acetylserine sulfhydrylase in cells grown on different sulfur sources. All the enzymes of the reductive part of the pathway are known to respond in parallel to each sulfur source, thus sulphite reductase activity could be considered as representative of the entire reductive pathway.

The results of these experiments are presented in Table 2. The enzyme activities listed in this Table are averages of several independent experiments. Assays of the same cell free extract were very reproducible, but enzyme activities varied in the different cultures grown on the same sulfur source. A similar variability has been observed by other authors (Kredich, 1971; Borum and Monty, 1976). Therefore wild type strains were grown in parallel on different sulfur sources and the enzyme activity was expressed as the percentage of wild type activity on L-djenkolic acid. Levels of both enzymes (sulphite reductase and O-acetylserine sulfhydrylase) in the newly isolated mutants were nearly the same for cultures grown on the different sulfur sources, as was observed in the case of

the constitutive cysteine mutant *cysB1352*. The lack of regulation of cysteine biosynthetic enzymes of our mutants and the previously known *cysB1352* is more evident if we compare the repression index of constitutive mutants and of the wild type (Table 3). The repression index is the ratio of enzyme activity of the derepressed culture (grown on L-djenkolic acid) to the enzyme activity of the repressed culture (grown on the L-cystine).

The value of the repression index for both sulphite reductase and O-acetylserine sulfhydrylase for wild type is 10–11 whereas that for the constitutive mutants is 1.0–2.0

Discussion

It has been shown previously that the *cysB* region in *S. typhimurium* and *E. coli* exerts control in a positive manner over the enzymes concerned with the biosynthesis of L-cysteine (Spencer et al., 1967; Jones Mortimer, 1968; Kredich, 1971). Mutations leading either to loss of expression or to constitutivity are both found in the *cysB* region. It seems probable that this gene codes for an effector molecule. Isolation of amber mutants in *E. coli* has proved that this effector molecule is a protein (Tully and Yudkin, 1975). Englesberg and Wilcox in their review article described the criteria for defining a system as positively regulated (Englesberg and Wilcox, 1975).

They are as follows:

1. The isolation of deletion or nonsense mutations within the putative positive regulatory gene, this resulting in a pleiotropic negative phenotype.
2. The exclusion of the regulator gene from the operon which it controls.
3. The isolation and mapping within the regulator gene of pleiotropically constitutive mutants.
4. The demonstration of transdominance of these constitutive mutants over their pleiotropic negative and inducible alleles.
5. The isolation and characterization of cis-dominant constitutive mutants in the controlled operons as revertants of the pleiotropic negative ones.

How do these criteria apply to the cysteine regulon?

The first and second criteria are fullfilled, deletion and nonsense mutations have been isolated (Cheney and Kredich, 1975; Tully and Yudkin, 1975). The *cysB* gene is not adjacent to the known cysteine structural genes (Sanderson, 1972). This paper aimed at checking the third criterion. Partially constitutive mutations mapping in the *cysB* region have been found in *S. typhimurium* as secondary mutations occuring in a *cysE* mutant (Spencer et al., 1967; Kredich, 1971). So far no method to select for constitutive mutations has been available. In our method, we relied on the fact that *cysK cysM* double mutants are cysteine auxotrophs and on the prediction that constitutive *cysB* mutants should be resistant to triazole. Thus the mutants expected by plating *cysM* strains on triazole plates could be only constitutive mutants or mutants with an promoter-up in *cysE*. The constitutive mutations we isolated showed the same properties as the previously known and well characterized constitutive mutant *cysB1352*:

a) they map in the *cysB* region;

b) they are cross resistant to selenate;

c) they have increased levels of cysteine enzymes, which are only slightly, if not all, repressed by cysteine. The repression index of both enzymes is 1–2 the same as for the *cysB1352* mutant.

This method allows the isolation of large numbers of *cysB* constitutive mutants which could facilitate future studies on the nature of *cysB* gene and its product.

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