Polarity of the *cysJIH* Operon of *Salmonella typhimurium*

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SUMMARY

Certain point and deletion mutants with lesions in the *cysJ* gene of *Salmonella typhimurium* have low levels of enzymes coded by the *cysI* and *cysH* genes. These results support the hypothesis that an operon exists comprising genes *cysJ*, *I* and *H* which is transcribed in the direction from *cysJ* to *H*. The nearby *cysC* and *cysD* genes do not form part of this *cysJIH* operon.

INTRODUCTION

In *Salmonella typhimurium*, the genes concerned with cysteine biosynthesis map at a number of widely separated regions of the linkage map (Sanderson, 1972; Hulanicka, Kredich & Treiman, 1974). One such region comprises a cluster of structural genes for the enzymes which convert sulphate to sulphide. Figure 1 shows the order of genes in this cluster (Mizobuchi, Demerec & Gillespie, 1962). The enzymic activities of the products of genes *cysC*, *D* and *H* were established by Dreyfuss & Monty (1963) and of genes *cysI* and *J* by Siegel & Kamin (1971) (see Fig. 1). The orientation of the cluster with respect to nearby genes is *thyA argB cysC(DHI)J* (Jones-Mortimer, 1973).

It is postulated that genes *cysJ*, *I* and *H* form an operon with a promoter at the *cysJ* gene end. Results which support this hypothesis are presented. Some of these results have been the subject of a preliminary report (Loughlin, 1974).

METHODS

Organisms. The prototrophic strain *S. typhimurium* LT2 and cysteine auxotrophs of *Salmonella typhimurium* were obtained from K. E. Sanderson or the late M. Demerec and have been described previously (Mizobuchi *et al.* 1962; Demerec, Gillespie & Mizobuchi, 1963). The salmonella phage P22 was obtained from B. W. Holloway.

Chemicals. Glutathione, NADPH, NADP, glucose 6-phosphate, FAD, glucose 6-phosphate dehydrogenase (yeast) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were obtained from Sigma, and cytochrome *c* (equine heart) from Calbiochem. ATP and methyl viologen were purchased from BDH, and *N*, *N*-dimethyl-*p*-phenylenediamine sulphate from Carlo Erba, Milan, Italy.

Media. Media used were: storage agar (Hartman, Loper & Šerman, 1960), cysteine nutrient agar (0.8 % Bacto nutrient broth, Difco; 0.2 mM-L-cysteine.HCl; 1.2 % agar), and L broth (Lennox, 1955). The liquid minimal medium used was medium E (Vogel & Bonner, 1956), modified by the replacement of MgSO₄ by MgCl₂ and supplemented with glutathione (0.2 mM) and glucose (0.25 %). Solid minimal medium was that of Davis & Mingioli

Fig. 1. The sulphur branch of the pathway of cysteine biosynthesis in *S. typhimurium*: the pathway of conversion of sulphate to sulphide and the relationship of the enzymes concerned to the cluster of structural genes. APS, adenylylsulphate; PAPS, 3'-phosphoadenylylsulphate; MVH, reduced methyl viologen; ATP sulphurylase, ATP: sulphate adenylyl transferase (EC.2.7.7.5); APS kinase, ATP: adenylylsulphate 3'-phosphotransferase (EC.2.7.1.25); PAPS reductase, NADPH: PAPS oxidoreductase. The lesions in the deletion mutants are indicated by rectangles below the linkage map (after Demerec *et al.* 1963). The dotted parts of these rectangles may or may not be deleted.

(1950), similarly modified by the replacement of sulphate salts and supplemented with glucose (0.2 %) and either cystine (0.42 mM, Kredich, 1971) or Na₂SO₄ (8.5 mM).

Transduction. Recipient cultures were grown in L broth by shaking overnight at 37 °C. The cultures were centrifuged and resuspended in one-tenth vol. of T2 buffer (Hershey & Chase, 1952). L broth lysates of P22 were diluted with T2 buffer and adsorption mixtures prepared from 0.1 ml resuspended cells and 0.9 ml diluted phage suspension to give a multiplicity of about 5. After allowing each mixture to adsorb at 37 °C for 5 min, five selection plates were each spread with 0.1 ml.

Growth and disruption of bacteria. Colonies on cysteine nutrient agar were inoculated into 2 ml of L broth and grown with shaking at 37 °C. Cultures were grown and cells disrupted by method A or B below.

Method A. L broth (10 ml) was inoculated with 0.5 ml of an overnight L broth culture and shaken for 7 h at 37 °C. A 5 ml portion of this culture was used to inoculate 75 ml of minimal medium and growth was continued immediately on a rotary shaker overnight at 37 °C. This culture was then added to 1.42 l of minimal medium and grown with aeration at 37 °C. Growth was followed by measuring the turbidity of a 1 to 10 dilution in 0.9 % NaCl at 600 nm in a Unicam SP 600 spectrophotometer. Cells from about 1.2 l of culture were harvested in late log phase and the cell pastes frozen overnight. Frozen cells were disrupted in a modified Hughes press and the preparations mixed with 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA (2.7 ml/g wet wt cell paste).

Method B. Minimal medium (10 ml) was inoculated with 0.5 ml of an overnight L broth culture and shaken overnight at 37 °C. This culture was then added to 150 ml of minimal

medium in a 1 l flask and shaken on a rotary shaker at 37 °C. Turbidity was followed as in method A. Cells were harvested in late log phase and resuspended in 3 ml of 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA and stored at 4 °C overnight. Cells were disrupted using a Branson Model B-12 sonifier fitted with a micro tip.

In both methods, cultures with revertants greater than 0.01 % of the population were rejected. Cell debris was removed by centrifugation at 28000 g for 40 min and the supernatants (crude extracts) were used for enzyme and protein assays.

Preparation of 3'-phosphoadenylyl sulphate (PAPS). A crude extract was prepared from three cultures of *S. typhimurium cysG439* by method A above. Four incubation mixtures, each with a final volume of 250 ml, contained per litre: 10 mmol MgCl₂, 20 mmol Na₂SO₄, 10 mmol sodium ATP pH 7.3, 100 mmol tris-HCl buffer pH 8.5, and 40 ml of crude extract containing 825 mg protein. The reaction in the first incubation mixture was commenced by the addition of extract 45 min before adding the remaining mixtures. Incubation was carried out at 37 °C and 0.5 ml samples were transferred from the first mixture to centrifuge tubes at 30 min intervals. After immersing these tubes in a boiling water bath for 2 min, cooling in ice and centrifuging in the cold, the supernatants were assayed for PAPS (see below) to determine the duration of the incubation. The reaction in the main incubation vessels was stopped after about 4 h by cooling in an ice bath and adding cold 2 M-perchloric acid (37.5 ml/vessel). Subsequent operations were performed at 4 °C. After standing for a short time, the material was centrifuged at 10000 g for 10 min. The supernatant was adjusted to pH 7.7 with 5 M-KOH and then allowed to stand for 20 min before filtering through Whatman No. 1 paper. The filtrate was diluted with 5 vol. H₂O and applied to a 58 cm × 5.5 cm² column of Bio-Rad AG1X4, 200 to 400 mesh, at an average flow rate of 315 ml/h. After application of 200 ml H₂O, a linear gradient (2.0 l, 0 to 1.0 M-NaCl) was applied at a flow rate of 100 ml/h followed by 1.0 M-NaCl. The fractions containing PAPS were mixed with 50 g of acid-washed Nuchar C-N charcoal overnight. The charcoal was separated by filtration on Whatman No. 1 paper, washed with water and the PAPS eluted by stirring with two successive lots (350 ml) of ethanol-H₂O-NH₄OH (12:12:1, by vol.). These eluates were combined and evaporated nearly to dryness using a rotary evaporator and a 45 °C bath. The damp residue was dissolved in 6 ml 0.05 M-tris-HCl buffer pH 8.0 and filtered through various grades of Whatman filter paper and finally through a 0.45 µm Millipore filter. The orange filtrate was adjusted to pH 8.1 using 1 M-tris-HCl buffer pH 8.5 and frozen in several tubes. The yield was 500 µmol PAPS.

Assay of PAPS. Phenolsulphokinase was prepared from lamb liver by the method of Gregory & Lipmann (1957) with the modification that 10 mM-2-mercaptoethanol was included in the buffers. The preparation was stored frozen in small lots. Freshly thawed lots were used in the assay and any excess discarded. PAPS was assayed by the method of Robbins (1963) except that cysteine was replaced by freshly diluted 2-mercaptoethanol.

NADPH-cytochrome c reductase assay. This activity was followed spectrophotometrically at 20 °C by measuring cytochrome c reduction at 550.5 nm. Cuvettes contained (in a final volume of 1.0 ml): 0.1 ml of 0.5 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA, 0.2 mM-NADPH, 0.023 mM-cytochrome c, and up to 0.1 ml of extract diluted with 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA. Duplicates varied by no more than ± 8 % from the mean value.

Reduced methyl viologen-sulphite reductase assay. Methyl viologen was reduced in the apparatus shown in Fig. 2. Commercial oxygen-free nitrogen was purified by bubbling through alkaline sodium dithionite containing anthroquinone-2-sulphonic acid (Vogel, 1961) and a solution of methyl viologen maintained in the reduced state by acid-washed

Fig. 2. Reduced methyl viologen apparatus. Methyl viologen is reduced with zinc (see Methods) in the reduction vessel (A) which is connected to the remainder of the apparatus by polyvinyl chloride tubing (B). The MVH solution is passed through the sintered glass filter (C) to the reservoir (D) by inverting the reduction vessel. A sample of MVH solution is measured by passing through the T bore stopcock (E) to tube F and the burette G. This sample is then transferred to the incubation tube (J) through the adaptor (I) which is connected to the vessel and apparatus by ground glass joints (H).

zinc. A solution of methyl viologen was added to acid-washed granulated zinc in the reduction vessel and stirred. The apparatus was evacuated and refilled with purified N_2 four times. After stirring the reduction vessel contents for 35 min, the reduced methyl viologen (MVH) was transferred to the reservoir through the filter. After flushing the burette with the MVH solution, MVH was titrated by adding to 2 ml of acidified 2 mM- $KMnO_4$. The extent of reduction of the dye ranged from 60 to 80 % between different batches. Incubation tubes containing extract, buffer and sulphite were connected, using adaptors, to the apparatus, evacuated twice and the MVH solution added. The stopcock on the adaptor was then closed, and the tubes incubated at 37 °C for 15 min with the adaptor still attached. Incubation mixtures contained (in a final volume of 2.5 ml): extract diluted in 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA, 0.8 mM- Na_2SO_3 , and up to 2 ml of the MVH solution. The reaction was stopped by oxidizing the MVH. The adaptor was replaced by a stopper and the oxygen thus admitted was used to oxidize the MVH by vortexing. Sulphide was determined by the method of Siegel (1965) with the following modifications: 0.2 ml of the *N,N*-dimethyl-*p*-phenylene diamine reagent was added, followed immediately by the addition of 0.2 ml of the ferric chloride reagent. After 20 min, 0.5 ml ethanol was added and insoluble material removed by centrifuging at 28000 *g* for 20 min. Variation of the MVH concentration showed that optimal sulphide production was obtained with 1.5 ml MVH solution (75 % reduced) per tube, prepared from 20 mM-methyl viologen. Addition of 2.0 ml MVH solution/tube gave 90 % of the optimum value. At this level of MVH, sulphide production at low enzyme concentrations was up to 10 % less than that expected from data obtained at high enzyme concentrations, assuming linearity. The addition of 10 μ mol of zinc acetate to the incubation mixtures inhibited sulphide production by 30 %. Since batches

of MVH varied in their extents of reduction and probably their zinc concentrations, a wild-type extract was assayed with each batch of MVH. Each extract was assayed in duplicate at two protein concentrations. Standard deviations derived from these four values were less than 10 % of the mean wild-type value and less than 3 % of the mean wild-type value for extracts with less than 10 % of the wild-type specific activity.

PAPS reductase assay (NADPH:PAPS oxidoreductase). The method used was that described by Kredich (1971) with minor modifications. Incubation mixtures contained (in a final volume of 1.5 ml): 0.2 ml 0.5 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA, 5 μ mol glucose 6-phosphate, 0.1 μ mol NADP, 0.17 units of glucose 6-phosphate dehydrogenase, 15 nmol FAD, 5.5 μ g highly purified sulphite reductase (from *S. typhimurium* LT2, 0.49 μ mol sulphide formed/mg protein/min), crude extract containing 2 to 4 mg of protein diluted into 0.5 ml of 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA, and 0.45 ml 50 mM-tris-HCl buffer pH 8.0 containing 0.44 μ mol of PAPS. Incubation was carried out in stoppered tubes at 37 °C for 15 min. Sulphide was determined as described above for the MVH reductase assay, using 0.2 ml of each reagent per tube. Each extract was assayed at two protein concentrations. Active extracts showed large differences, the higher protein concentration showing the higher specific activity. Thus the results are regarded as semi-quantitative even though duplicates varied by no more than ± 10 % from the mean value.

Protein determination. The protein in crude extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

In preliminary experiments, an extract of the deletion mutant *cysJ538* was found to have a very low level of MVH-sulphite reductase. To explain this result it was proposed that genes *cysJ* and *I* form an operon in which the *cysJ* gene is transcribed before the *cysI* gene. This implies the existence of a promoter at the right end of the *cysJ* gene as the map is drawn in Fig. 1. This promoter would be missing in the deletion mutant *cysJ538*.

Extracts of the deletion mutant *cys536* which would be predicted to have wild-type levels of NADPH-cytochrome *c* reductase were found to have very low levels of this activity. The paper by Demerec *et al.* (1963) refers to this mutant in some places as *HIJ536* and in other places as *HIJ536*. To clarify this situation, transduction experiments were performed which showed that deletion 536 could yield wild-type recombinants with some *cysJ* point mutants (*cysJ494*, *J275*) but not others (*cysJ389*, *J317*). It is concluded that the deletion extends into the *cysJ* gene. This confirms the designation of 536 as *HIJ536* in the records of the Salmonella Genetics Stock Centre (Sanderson, personal communication) and accounts for the low level of NADPH-cytochrome *c* reductase activity in extracts of this mutant.

To determine whether the *cysH* gene is part of the *cysJII* operon, extracts of selected deletion mutants were assayed for PAPS reductase as well as MVH-sulphite and NADPH-cytochrome *c* reductases. The results are shown in Table 1. The finding that deletion mutant *cysJ538* also lacks PAPS reductase indicates that gene *cysH* is part of the *cysJII* operon. If the *cysJ* gene is the first gene in the *cysJIIH* operon, nonsense mutants in the *cysJ* gene would be expected to have polar effects, namely lowered expression of the *cysI* and *H* genes. To search for such polar mutants, extracts were prepared by method B (Methods) from nearly all of the available *cysJ* point mutants (Demerec *et al.* 1963) and assayed for MVH-sulphite reductase activity (2.0 ml of MVH solution prepared from 10 mM-methyl viologen was added to vessels containing enzyme diluted into 0.2 ml of 0.1 M-potassium phosphate buffer pH 7.7

Table 1. *Relative enzyme levels in mutant extracts*

Enzyme levels are expressed as a percentage of the specific activity of the wild-type extract.

Extract	PAPS reductase	MVH- sulphite reductase	NADPH- cytochrome <i>c</i> reductase
Wild-type			
LT2	100	100	100
Deletion mutants			
<i>cysHI36</i>	1	3	94
<i>cysI68</i>	24	1	106
<i>cysH398</i>	1	140	104
<i>cysHIJ536</i>	1	2	3
<i>cysJ538</i>	1	1	3
Selected <i>cysJ</i> mutants			
<i>cysJ148</i>	5	4	3
<i>cysJ223</i>	3	2	3
<i>cysJ304</i>	9	6	3
<i>cysJ630</i>	4	4	3

Extracts were prepared by method A (Methods). In the assays for MVH-sulphite reductase, 1.5 ml of MVH solution prepared from 20 mM-methyl viologen was added to vessels containing enzyme diluted into 0.5 ml 0.1 M-potassium phosphate buffer pH 7.7 containing 1 mM-EDTA. Other details are given in Methods. The specific activities of the LT2 extract (wild-type) used as a control for the deletion mutants were: PAPS reductase and MVH-sulphite reductase, 1.5 and 36 nmol sulphide formed/mg protein/min, respectively; NADH-cytochrome *c* reductase, 570 nmol cytochrome *c* reduced/mg protein/min. The specific activities of the LT2 extract used as a control for the selected *cysJ* mutant extracts were: PAPS reductase and MVH-sulphite reductase, 1.3 and 23 nmol sulphide/mg protein/min, respectively; NADPH-cytochrome *c* reductase, 530 nmol cytochrome *c* reduced/mg protein/min.

containing 1 mM-EDTA; other details are given in Methods). The extracts exhibited a wide range of activity from 0 % to slightly more than 100 % of the wild-type specific activity. *CysJ* point mutants having low levels of activity (between 0 and 10 % of wild-type) were tested for reversion by NTG (Berkowitz *et al.* 1968). Those which could be reverted were selected for enzyme assays. Table 1 shows the enzyme levels of these selected *cysJ* point mutants. It is clear that all of these selected *cysJ* point mutants are polar, having very low levels of the *cysI* and *H* gene products.

DISCUSSION

The results of enzyme assays of extracts of the deletion mutant *cysJ538* and the selected *cysJ* point mutants (Table 1) show that these mutants have polar effects. From the direction of these polar effects it is proposed that genes *cysJ*, *I* and *H* form an operon which is transcribed from the *cysJ* gene end. This implies the existence of a promoter near the commencement of the *cysJ* gene. Such a promoter would be missing from the deletion mutant *cysJ538*, thus accounting for the observed polarity. The point mutants listed in Table 1 are polar and are revertible by NTG. It is thus probable that they are nonsense mutants. This would explain their polarity since in other operons nonsense mutants which map in the first gene of an operon can exhibit polarity (Beckwith, 1964; Brenner & Beckwith, 1965; Newton, Beckwith, Zipser & Brenner, 1965).

That the operon is not transcribed in the direction from *cysH* to *J* is indicated by the results of assays of the deletion mutant *cysHI36*. Extracts of this mutant have high levels of NADPH-cytochrome *c* reductase (Table 1). This result is the one expected if polarity is from *cysJ* to *cysH*. The ditto deletion mutant *cys431* (Demerec *et al.* 1963; Itikawa & Demerec, 1967), like *J538*, would be expected to lack the promoter for the *cysJI* operon (see

Fig. 1). An extract of *cys431* has been shown to possess ATP sulphurylase and APS kinase activities (Kredich, 1971). This indicates that genes *cysC* and *D* are not part of the *cysJIIH* operon.

On mixing extracts of *cys168* with extracts of *cysJ266*, reconstitution of up to 50 % of the wild-type NADPH-sulphite reductase activity has been observed (Siegel *et al.* 1971). By contrast, previous experiments, using extracts of *cys168* and *cysJ538*, showed that less than 1 % of the wild-type NADPH-sulphite reductase activity was formed (Henderson & Loughlin, 1968). This difference can now be explained by the polarity exhibited in extracts of *cysJ538* which contain very low levels of MVH-sulphite reductase activity (1 %; Table 1). In the survey of *cysJ* mutants (see Results) *cysJ266* was found to have 15 % of the wild-type MVH-sulphite reductase activity. This level is probably sufficient to account for the results of mixing *cysJ266* extracts with extracts of *cys168*.

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