GENETIC INSTABILITY IN AUXOTROPHS OF *SALMONELLA TYPHIMURIUM* REQUIRING CYSTEINE OR METHIONINE AND RESISTANT TO INHIBITION BY 1,2,4-TRIAZOLE

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ABSTRACT

Triazole-resistant (Trz^r) derivatives of six cysteine- or methionine-requiring (Cym$^-$) mutants of *Salmonella typhimurium* were isolated. Some of the derivatives of each mutant (CTS) were prototrophic, *i.e.*, Cym$^-$ was suppressed. In every case suppression was initially unstable, Cym$^-$ auxotrophs being segregated at high frequency, although Trz^r was stable. After several subcultures on selective medium, CTS strains were classified as either persistently unstable or stabilized. The unstable strains segregated Cym$^-$ auxotrophs at frequencies of 50–70%, whereas the stabilized strains segregated at frequencies of less than 1%. All suppressed strains had a stable Trz^r marker co-transducible with *cysA*. However, there was a correlation between the class of CTS strain and Cym$^-$ phenotype. The stabilized strains were Cym$^+$, whereas the unstable strains were Cym$^-$. Acriflavin and ethidium bromide increased segregation in the unstable strains, suggesting the involvement of a plasmid. The stabilized strains were refractory to the curing agents. There was no detectable change in the quantity or quality of the *S. typhimurium* cryptic plasmid. The Trz^r phenotype of the CTS strains suggested that Trz^r mutations were of the stable TrzA type. It is suggested that correction of the Cym$^-$ lesions in CTS strains results from an insertion within the *cysCDHIJ* gene cluster of a DNA species originating in the *cysALKptsHI* region of the *S. typhimurium* chromosome.

A class of auxotrophs comprising 19 mutants of *Salmonella typhimurium*, which require either cysteine or methionine for growth (Cym$^-$) have been described (QURESHI, SMITH and KINGSMAN 1975). Cym$^-$ mutants map within the *cys* genes, and there are one or more Cym$^-$ mutants that map in each of the *cysA*, *C*, *D*, *G*, *H*, *I* and *J* genes. They are alleles of the *cys* genes and are deficient in the relevant enzymes of the cysteine biosynthetic pathway. The distribu-

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TABLE 1

Summary of relationships between Cym and cys *mutants*

Group and subgroup	Mutant numbers	Map location	Enzyme deficiencies	*cys* mutant similarity
CymAa CymAb	1,7 2,13,19	*cysC* and *D* junction	PAPS* synthetase	*cysC* *cysD*
CymBa	3,4	Within *cysH*	PAPS reductase	*cysH*
CymBb	5,8,16	Within *cysI*	Sulphite reductase	*cysI*
CymBc	6,15	Within *cysJ*	Sulphite reductase	*cysJ*
CymCa CymCb	12,14 9,10,11	Within *cysA*	Sulphate permease	*cysA*
CymD	17	Within or close to *cysG*	Sulphite reductase	*cysG*

* PAPS = ATP-adenylsulphate 5¹-phosphotransferase and ATP sulphate adenylyl transferase

tion of the 19 Cym mutants with respect to the *cys* genes is shown in Table 1. To explain the response of Cym mutants to methionine, it was suggested that (a) they may be leaky *cys* mutants, growth on methionine being due to the sparing of endogenous cysteine by exogenous methionine, or (b) that they are defective in regulatory regions of the *cys* genes, growth on methionine being due to some unknown regulatory effect of methionine on cysteine biosynthesis. In order to test these possibilities further, the effect of *cys* "regulatory" mutations on the Cym⁻ phenotype was investigated. One such group of mutations is comprised of those that result in resistance to inhibition by 1,2,4-triazole (Trz^r). In these, the cysteine pathway inducer *O*-acetylserine, is no longer converted to triazole alanine in the presence of 1,2,4-triazole (HULANICKA, KLOPOTOWSKI and SMITH 1972; JONES-MORTIMER 1968; HULANICKA and KLOPOTOWSKI 1972; KREDICH, FOOTE and HULANICKA 1975).

Spontaneous Trz^r mutants of *S. typhimurium* fall into two groups, TrzA and TrzB, on the basis of their stability and linkage relations (HULANICKA and KLOPOTOWSKI 1972). Both TrzA and TrzB mutants were shown to be linked to *cysA* by P22-mediated transduction and to be deficient in the *cysK* enzyme *O*-acetylserine sulphydrylase A (HULANICKA, KREDICH and TREIMAN 1974; KREDICH, FOOTE and HULANICKA 1975), but they are prototrophic since they possess a second sulphydrylase activity. TrzA mutants arise from stable, structural mutations in the *cysK* gene (HULANICKA, KREDICH and TREIMAN 1974). In contrast, TrzB mutants are unstable and segregate Trz^s cells, initially at frequencies of 12–30%, subsequently stabilizing after several subcultures at about 1%. The *S. typhimurium* LT2 cryptic plasmid (SPRATT, ROWBURY and MEY-

NELL 1973) must be present for stabilization (HULANICKA and BAGDASARIAN, unpublished data). Also, TrzB mutations do not appear closely linked to *cysA* in conjugational crosses, and when used as donors to transduce *cysA* mutants to prototrophy, they do so at a frequency seven-fold higher than wild type (HULANIKA, unpublished data). These and other observations (CORDARO and ROSEMAN 1972) have led to the proposal that in TrzB mutants part of the *cysALKptsHI* region of the *S. typhimurium* chromosome, including *cysK*, may excise to form a plasmid that may replicate, at least to a limited extent, autonomously in the cytoplasm. In this situation the *cysK* gene would be inactive, presumably due to disruption during the excision event. The initial instabilityof TrzB mutants could be explained by the reversible reintegration of the "*cysALKptsHI*-episome," leading to the re-establishment of the integrity of the *cysK* gene. Stabilization is thought to occur by recombination between the excised *cysALKptsHI* region and the LT2 cryptic plasmid.

In this paper we describe the effects of the isolation of Trz^r derivatives of Cym^- mutants on the Cym^- phenotype. Some of the Cym^- Trz^r double mutants were prototrophic, apparently due to suppression of Cym. This suppression was unstable, although no instability of Trz^r was observed. We suggest that integration of the "*cysALKptsHI*-episome" at or near the Cym^- site of mutation may be responsible for the unstable suppression of the Cym phenotype in some Cym Trz^r double mutants.

MATERIALS AND METHODS

Organisms and media: All strains (Table 2) were derived from *S. typhimurium* LT2. Nutrient broth and agar media (NB and NA) and minimal liquid and agar media (MM and MA) were those of SMITH (1961). Amino acid supplements were at a fixed concentration of 20 μg/ml, *O*-acetylserine at 50 μg/ml and 1,2,4-triazole at 2.5 μg/ml. Pennassay broth (PAB) at pH 7.6 was used in the plasmid-curing experiments. The incubation temperature for any particular medium is given in parenthesis after the abbreviation for that medium.

Isolation of Trz^r derivative of Cym^- mutants: An overnight NB (37°) culture of a Cym^- mutant was washed once in MM, samples spread onto MA + triazole and incubated at 25° for three days, after which resistant colonies were picked and tested. Temperature-sensitive Cym^- mutants were used since cysteine and methionine reverse the inhibitory effect of triazole.

Genetic crosses: P22.L4 (SMITH and LEVINE 1967) was used in transduction crosses according to the method of SMITH and CHILDS (1966). In conjugation crosses, 1 ml and 0.5 ml of stationary phase NB cultures of recipient and donor, respectively, were mixed, 0.1 ml of fresh NB added and incubation continued at 37° for one hr without shaking, after which 0.1 ml samples of a 10^{-1} dilution were plated onto selective medium.

Standard segregation assay: Prototrophic Cym Trz^r double mutants segregated temperature-sensitive (ts) auxotrophs with a Cym^- phenotype indistinguishable from the parent Cym^- mutant (see RESULTS). The segregation frequency (Sf) was the percentage of a population unable to grow on MA (37°). The population was diluted to yield a countable number of colonies when a sample was spread onto MA. After incubation for two days at 37°, the number of prototrophic colonies (x) were counted. Incubation was continued for a further three days at 25°. The number of "new" colonies (y) appearing were ts auxotrophic segregants, thus $Sf = y/x+y$. This method ensured that plating errors were irrelevant and segregants are readily identified. In a reconstruction experiment in which the Sf of ten independent, segregating populations was measured, $(x+y)$ in each case showed no significant difference from the viable count on NA. Each segregation frequency value given is for at least 300 colonies.

TABLE 2

*Bacterial strains**

Strain	Additional characters	Source
CTS21†	unstable prototrophy	CymAa2
CTS71	stabilized prototrophy	CymAa7
CTS194	stabilized prototrophy	CymAb19
CTS43	stabilized prototrophy	CymBa4
CTS84	stabilized prototrophy	CymBb8
CTS151	unstable prototrophy	CymBc15
CymAa2	ts	M.A.Q.
CymAa7	ts	K.E.S.
CymAb19	ts	K.E.S.
CymBa4	ts	M.A.Q.
CymBb8	ts	K.E.S.
CymBc15	ts	K.E.S.
CymAa2Trz1	ts	CymAa2
CymAa7Trz6	ts	CymAa7
CymAb19Trz9	ts	CymAb19
CymBa4Trz10	ts	CymBa4
CymBb8Trz11	ts	CymBb8
CymBc15Trz51	ts	CymBc15
cysA20	dl	K.E.S.
cysCD519	dl	K.E.S.
cysCD519 F' lac		M.C.J.M.
cysD505		K.E.S.
cysI68	dl	K.E.S.
Trz110		Wild-type
TrzB17	unstable for Trz^r	M.D.H.
aroB57		K.E.S.

* All strains are derivatives of *Salmonella typhimurium* LT-2.

† The designation CTS is for Cym Trz^r-suppressed in which the Cym phenotype is suppressed. The resultant prototrophy is unstable to greater or lesser extent.

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ts = temperature sensitive; dl = deletion.

Assay of O-acetylserine sulphydrylase: MM + L-djenkolate (125 μg/ml) was used to ensure derepression of the enzyme. Ten ml quantities of this medium were inoculated with 0.5 ml of a stationary phase culture grown in the same medium, and were incubated on an orbital shaker. The bacteria were harvested at an O.D. 650 of 0.6–0.7 measured in a Unicam SP500 spectrophotometer (1 cm light path), washed twice in saline and finally resuspended in 10 ml of 0.2 M Tris-HCl (pH 7.2) containing 1 mM EDTA. Toluene was added to a final concentration of 1% (v/v), the suspensions incubated with vigorous shaking at 37° for 30 min and used immediately. The assay method of BECKER, KREDICH and TOMKINS (1969) was used except that the color in the reaction mixture was found to be stable for 30 min; absorbance was measured at 540 nm.

Alkaline sucrose gradient: The method was based on that of SPRATT, ROWBURY and MEYNELL (1973). Bacteria were grown for 12–18 hr in 5 ml of 25% NB in MM, centrifuged at 800 × g for 15 min and resuspended in the same volume of the same medium containing 165 μg/ml uridine and 8 μCi/ml ^{3}H(methyl) thymidine. Incubation was continued for four hr, the cultures

cooled on ice, washed three times in cold 0.05 M Tris-HCl (pH 8.0) and finally resuspended in 1 ml of TES buffer (0.05 M Tris-HCl; 0.02 M EDTA; 0.05 NaCl; pH 8.0). To this suspension was added slowly (one drop/15 sec), with gentle shaking, 0.5 ml of a 1% (w/v) solution of sodium lauroyl sarcosinate in 0.8 M NaOH. After lysis a small quantity (less than 0.1 ml) of silicone antifoam was added, the lysate sheared by vortexing for one min (FREIFELDER, FOLKMANIS and KIRSCHNER 1971) and 0.2 ml of the sheared lysate layered onto an 8 ml linear 5–20% alkaline sucrose gradient, which was centrifuged at 20° for 45 min in an MSE 3 × 25 ml swing-out rotor, at 30,000 rpm. About 40 fractions each of ten drops were collected on 2 cm squares of Whatman 120 paper and processed by the batch method of BYFIELD and SCHERBAUM (1966), and the papers were counted in a Phillip's liquid scintillation analyzer.

RESULTS

Preliminary observations: Trz^r derivatives of the six ts Cym$^-$ mutants CymAb2, Aa7, Ab19, Ba4, Ba8 and Bc15 were isolated. The Cym$^-$ and Trz^r phenotypes of the double mutants were then tested by streaking single colonies onto MA (37°), MA(25°), MA + cysteine (37°), MA + methionine (37°), and MA + triazole (25°). In all cases growth on MA (25°), MA + cysteine or methionine (37°) and MA + triazole (25°) was normal, suggesting that the Trz^r mutation had no effect on the growth responses of Cym$^-$ mutants to cysteine or methionine or on their growth on unsupplemented MM at the permissive temperature. Some Trz^r derivatives of each Cym$^-$ mutant grew on MA (37°) suggesting suppression of the Cym$^-$ phenotype by Trz^r; however, this growth was abnormal since streaks on this medium did not yield confluent growth after incubation, but comprised relatively few full-sized (2–3 mm diameter) isolated colonies. Single colonies from each of these unstable streaks were picked and subcultured on MA (37°) two to four times. During these subcultures the facility for unstable growth was initially retained, but after two to four subcultures some strains stabilized, giving growth on MA (37°) that was indistinguishable from that of wild type. The ability to stabilize was a characteristic of the Cym$^-$ mutant from which the particular Trz^r derivative was isolated. Those from CymAa7, CymAb19, CymBa4 and CymBb8 yielded relatively stable "prototrophs," whereas those from CymAa2 and CymBc15 remained persistently unstable and have retained this characteristic during subculturing over a period of two years.

Strains that exhibit stable or unstable suppression of Cym$^-$, were designated CTS (Cym$^-$ Trz^r suppressed) as distinct from Cym$^-$ Trz^r double mutants in which no suppression was observed. Table 2 lists the CTS strains used in this study, together with their origins.

The prototrophy of CTS strains may have been due to reversion of the Cym$^-$ mutation. This is unlikely for the unstable strains CTS21 and 151 since normal revertants should be stable. It is possible, however, that during subculture on MA (37°) of CTS71, 194, 43 and 84 revertants were selected from a mixed population of Cym$^+$ Trz^r and Cym$^-$ bacteria. This seems unlikely since no apparent instability of Trz^r was observed in preliminary streak tests on MA + triazole (25°). Another possibility is that when Cym$^-$Trz^r mutants were tested on MA (37°), the few prototrophic colonies that appeared may have been due to normal

reversion of the Cym⁻ site, suggesting that isolation of Trz^r derivatives is essentially irrelevant. Again this seems unlikely since 20 single colonies of each Cym⁻ mutant produced no revertants when tested in the same way as the CTS strains. The frequency of prototrophs in newly isolated CTS strains was 10^{-3} to 10^{-4}, whereas reversion frequencies of Cym⁻ mutants are less than 10^{-8} (QURESHI 1971). Also Cym Trz^r mutants such as CymAa2Trz1 or CymAa7Trz6, in which no suppression was observed, did not produce revertants when single colonies were picked and streaked onto MA (37°). Lastly, CTS strains may be prototrophic due to some metabolic function of the high concentrations of endogenous *O*-acetylserine in Trz^r mutants (HULANICKA and KLOPOTOWSKI 1972). This was tested by examining growth of the six Cym⁻ mutants used on MA + *O*-acetylserine (50 μg/ml) (37°). No growth was observed despite the fact that *O*-acetylserine is efficiently taken up by *S. typhimurium* (HULANICKA and KLOPOTOWSKI 1972).

It will also be shown that, while CTS71, 194, 43 and 84 are classified as stable for the purposes of this paper, all except CTS194 did show low levels of segregation of the original Cym phenotype (Table 3). We therefore consider that the prototrophy in all six CTS strains is not due to normal reversion. Rather, the isolation of some Trz^r derivatives of Cym⁻ mutants suppresses the Cym⁻ phenotype by the same mechanism in each case, the relative stabilities being a function of the allele concerned.

Measurement of instability in CTS strains: The standard segregation assay was used. Ten clones from each of the six representative strains (Table 2) and single ones from wild type and Trz110 as controls were grown overnight in NB, and the segregation frequency values from the resulting cultures were determined (Table 3). The strains initially classified as unstable (CTS21 and 151) showed relatively high segregation frequencies, whereas those classified as stabilized (CTS71, 194, 43 and 84) showed little or no segregation. For the persistently unstable strains, there was also a high between-clone variation in segregation frequency.

TABLE 3

Segregation frequencies in CTS strains

Strain	No. of clones segregating	Mean Sf (%)	Standard deviation
CTS21	10	48	26.5
CTS71	1	0.02	nc
CTS194	0	0	nc
CTS43	3	0.04	nc
CTS84	2	0.3	nc
CTS151	10	69	22.6
Wild type	0	0	0
Trz110	0	0	0

Sf = segregation frequency; nc = not calculated.

Two segregants from each segregating clone were tested and shown to have the Cym⁻ phenotype, but all colonies, both prototrophs and the auxotrophic segregants, were resistant to triazole. Thus, although Trz^r mutation was necessary for the stable or unstable suppression of Cym⁻, subsequent segregation of auxotroph was not associated with loss of triazole resistance.

The genetic composition of CTS strains: If stable or unstable suppression of Cym⁻ were a consequence of Trz^r mutations, then all CTS strains should be Cym⁻ Trz^r, where presumably Cym⁻ is suppressed and Trz^r is the suppressor. All CTS strains were derived from Cym⁻ mutants located in the *cysCDHIJ* region (minute 90) of the *S. typhimurium* chromosome (QURESHI, SMITH and KINGSMAN 1975), but all known Trz^r mutants are linked to *cysA* (minute 76) (HULANICKA and KLOPOTOWSKI 1972). Therefore, Cym⁻ and Trz^r in CTS strains should be readily detectable and separable in transduction crosses. Also in

TABLE 4

The presence of a Cym and Trz^r mutation in CTS strains

Donor	Recipient	(a) Cym⁻ mutations *cys*⁺ recombinants No.	% Cym⁻	(b) Trz^r mutations *cys*⁺ recombinants* No.	% Trz^r
CTS21	*cysD505*	200	84	200	72
CymAa2		200	87.5	200	0
CymA2 Trz-1†		200	91	200	60
CTS71	*cysCD519*	250	0	300	57
CymAa7		300	92.5	300	0
CymA7 Trz-6†		200	98	300	69
CTS194		300	0	300	55
CymAb19		300	99.5	300	0
CymA19 Trz-9†		300	96	247	61
CTS43		250	0	300	60
CymBa4		250	93	300	0
CymBa4 Trz-10†		250	94	143	49
CTS84	*cysI68*	186	5.7	131	45
CymBb8		189	88.5	170	0
CymBb8 Trz-11†		200	89	300	52
CTS151		300	91.5	250	71
CymBc15		300	98	300	0
CymBc15 Trz		300	99.5	300	65
Wild-type	*cysCD519*	160	0		
	cysD505	200	0	199	0
	cysI68	173	0		
Trz110	*cysCD519*	175	0		
	cysD505	200	0	200	59
	cysI68	147	0		

* Recipient in all crosses *cysA20*.
† These strains do not show suppression of the Cym phenotype.

crosses homologous for a given Cym$^-$ mutation with a CTS strain as donor, all prototrophic transductants should be resistant to triazole.

The presence of Cym$^-$ in CTS strains: P22.14 lysates of all CTS strains (Table 2) were used in whole-plate traansduction crosses with the appropriate recipients (Table 4). In addition, lysates of the original Cym$^-$ mutants and Cym$^-$Trz^r double mutants that had never shown any apparent suppression were used as controls. Recipients had deletion mutations either closely linked to or covering the Cym$^-$ site involved for each cross (see QURESHI, SMITH and KINGSMAN 1965, Figure 5). Recombinants were selected on MA + methionine (37°) and their Cym$^-$ phenotype tested subsequently.

Our results (Table 4a) show that there was a correlation between whether or not a CTS strain had stabilized and the presence or absence of the appropriate Cym$^-$ marker. Thus, in crosses in which the persistently unstable CTS21 and 151 were donors, the Cym$^-$ markers could be detected in recombinants at about the same frequency as when the parent CymAa2 and CymBc15 strains, respectively, were donors. By contrast, in crosses in which the four stabilized strains were donors, Cym$^-$ markers either could not be detected among recombinants (CTS71, 194 and 43) or occurred at a low frequency (CTS84).

We concluded that CTS21 and 151 contain the original CymAa2 and Bc15 mutations, respectively, and that the absence (or near absence, in the case of CTS84) of the relevant Cym$^-$ markers in the stabilized strains means that these markers are indeed absent and that the strains are Cym+.

The presence of Trz^r in CTS strains: The same donor lysates were also used in crosses with *cys*A20 as recipient since the TrzA and TrzB markers are about 60% co-transduced with this marker (HULANICKA and KLOPOTOWSKI 1972). Recombinants were scored for resistance to triazole, and the data (Table 4b) show that Trz^r was detected in crosses with both CTS strains and Cym Trz^r mutants that had never shown any suppression as donors at frequencies comparable to that using Trz110 as donor. These co-transduction frequencies were similar to those of HULANICKA and KLOPOTOWSKI (1972) for TrzA mutants. Thus, all CTS strains and all Cym$^-$ Trz^r double mutants that do not exhibit suppression carry a Trz^r marker.

Tests for the presence of a transducible Cym$^+$ genetic element: In Cym$^-$ × CTS croses in which the donor was derived from the Cym$^-$ strain used as recipient, prototropic transductants were obtained only when stabilized CTS strains were donors. None of these prototrophs were resistant to triazole. This is consistent with the unstable CTS strains being Cym$^-$ Trz^r and stabilized strains, Cym$^+$ Trz^r.

The genetics of the suppression of Cym$^-$ in CTS strains is complex. Both Cym$^-$ and Trz^r are present in the unstable strains TCS21 and 151, yet no prototrophic recombinants were obtained in crosses of the type Cym$^-$ × CTS (donor) that were homologous for Cym. Since Trz^r markers of CTS21 and 151 are efficiently transduced to recipients (Table 4b), Trz^r alone does not suppress Cym$^-$. This suggests that another (possibly unstable) component of the system is involved

that cannot be transduced from CTS21 and 151. This is concordant with the instability of prototrophy in CTS strains not being associated with instability of triazole resistance. In the stabilized strains CTS71, 194, 43 and 84, which are Cym$^+$ Trz^r, it could be that an unstable component directly responsible for suppression of Cym$^-$ is stabilized and closely linked to Cym, making them Cym$^+$. This would explain how prototrophic recombinants can be obtained in transduction crosses with Cym$^-$ mutants when stabilized strains are used as donors. Since none of the prototrophic recombinants were resistant to triazole, prototrophy is dissociated from Trz^r.

The nature of the instability of CTS strains: Phenotypic instability in bacteria is well known (NOVICK 1969). In most cases it results from either a tandem duplication of the chromosome, causing frequent looping out of fragments between the duplicated regions (HILL *et al.* 1969; JACKSON and YANOFSKY 1973) or the involvement of a plasmid (AMES, HARTMAN and JACOB 1963; LEVINTHAL and YEH 1972). Efficient plasmid segregation to daughter cells is frequently disrupted by intercalating dyes, such as acridine orange (HIROTA 1960) and ethidium bromide (KOZAK, RATCHERT-TZPIL and DOBRANSKI 1974); these agents have little effect on recombination between tandem duplications except perhaps to reduce looping out by inhibiting exonuclease V (BROWN 1973). Thus the existence of a plasmid responsible for the prototrophy of CTS strains was examined by attempting to "cure" them of their prototrophy with acriflavine and ethidium bromide.

Clones of CTS strains were grown overnight in MM + 1% NB to select for bacteria with the suppressed Cym$^-$ phenotype. These were used to innoculate 5 ml amounts of PAB, PAB + acriflavine (5 μg/ml) and PAB + ethidium bromide (20 μg/ml), which were incubated at 37° overnight, and their segregation frequencies were determined (Table 5). The segregation frequencies of the unstable strains CTS21 and 151 were increased by acriflavine and, to a lesser extent, by ethidium bromide, but these agents had no such effects on the stabilized strains CTS71, 194, 43 and 84. This suggests that a plasmid-like element may be involved in the suppression of the Cym$^-$ phenotype, at least in the unstable strains. If the same is true of the stabilized strains, the plasmid must be in a state in which it is refractory to curing. However, NOVICK (1969) has warned against confusing increased defective segregation with selection within a population by intercalating drugs. This was tested in reconstruction experiments.

Trz110 and CymAa2Trz1 or CymBc15Trz51, representing suppressed and nonsuppressed bacteria, respectively, were used to simulate segregating populations. Overnight NB cultures were mixed in the ratios 2:1, 1:1 and 1:2 (five replicates of each) and the mixtures grown in PAB with and without curing agents as in the curing experiment. After overnight incubation, the apparent segregation frequencies were measured by the standard segregation assay. Any marked difference between segregation frequencies in the presence or absence of the curing agents would indicate the occurrence of selection. Plasmid-curing treatments did not select for bacteria with the Cym$^-$ phenotypes (Table 6).

TABLE 5

The effect of curing agents on instability in ACTS strains

Strain	Curing agent	No. of clones tested	Mean Sf	Standard error	P*
CTS21	None	30	32.6	3.9	—
	ACR	30	98.4	0.1	0.001
	EB	10	71	3.4	0.001
CTS71, 194, 43 and 84	None	5	0	—	—
	ACR	5	0	—	—
	EB	5	0	—	—
CTS151	None	10	69.2	6.27	—
	ACR	10	97.7	0.1	0.001
	EB	5	94	2	0.05
Wild type	None	2	0	—	—
	ACR	2	0	—	—
	EB	2	0	—	—
CymAa2 and CymBc15	None	2	100	—	—
	ACR	2	100	—	—
	EB	2	100	—	—

Sf = Segregation frequency; ACR = Acriflavin; EB = Ethidium bromide.
* P = Probability that the effect of the curing agent is due to error variance.

TABLE 6

The effect of curing agents on mixed populations of CymTrz^r and Trz^r mutants

Mixtures of strains	Ratio	Curing treatment	Mean Sf	Standard error
CymAa2Trz1:Trz110	2 : 1	None	52	3.8
		ACR	49	3.6
		EB	55	3.7
	1 : 1	None	48	4.6
		ACR	50	4.1
		EB	44	4.0
	1 : 2	None	43	2.9
		ACR	47	4.0
		EB	31	3.9
CymBc15Trz51:Trz110	2 : 1	None	61	3.7
		ACR	57	4.6
		EB	41	4.5
	1 : 1	None	60	4.3
		ACR	59	4.4
		EB	58	4.4
	1 : 1	None	21	3.6
		ACR	27	3.8
		EB	42	3.7

Abbreviations as in Table 5.

Where differences between curing and noncuring conditions were obtained, the selection was against such bacteria and in favor of the prototrophs (Trz110) *i.e.*, the segregation frequency tended to decrease. Thus, the results of the curing experiments strongly suggest involvement of a plasmid-like element in suppression of Cym⁻ in CTS strains.

Attempts to detect a plasmid in CTS strains: The DNA content of CTS strains was examined. Alkaline sodium lauroyl sarcosinate lysates of wild type, CymAa2, CymBb8, CTS21, at various segregation frequencies, and CTS84 were analyzed on 5–20% alkaline sucrose gradients. In all cases the DNA profiles were identical (Figure 1). There was no extra plasmid DNA peak in any of the lysates

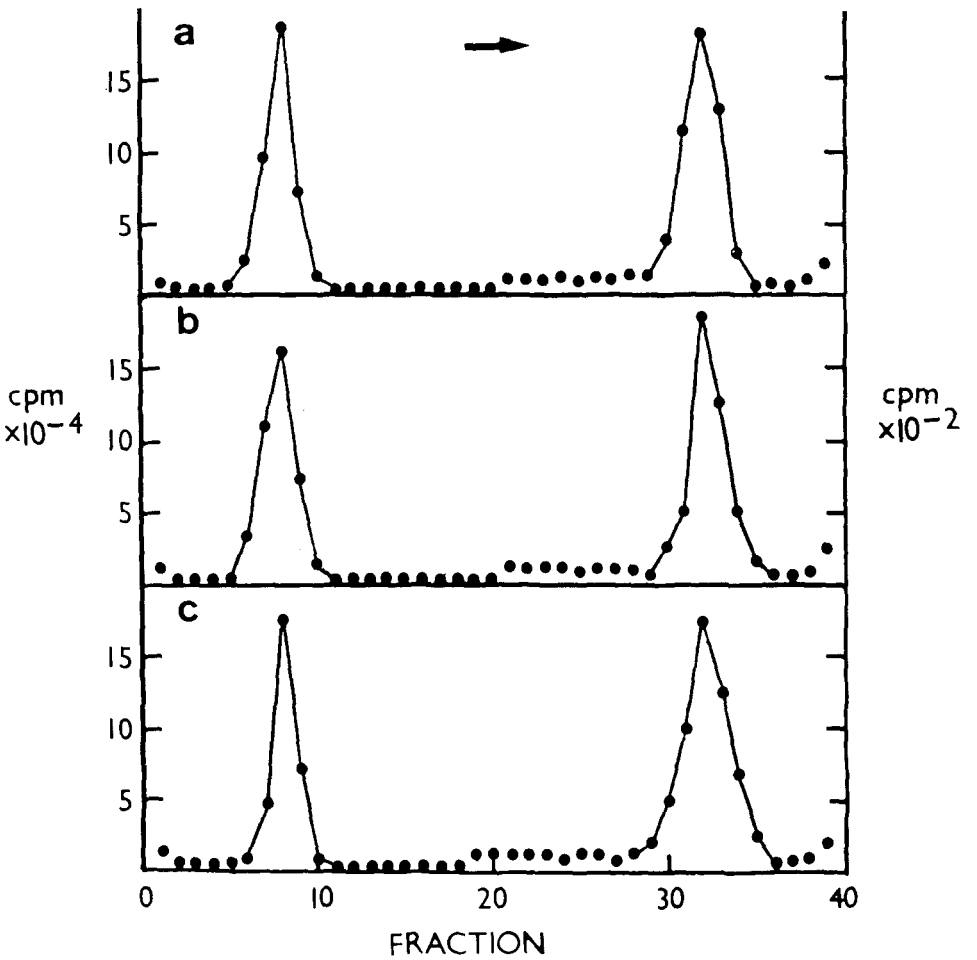

FIGURE 1.—Alkaline sucrose density gradient centrifugation profile of ^{3}H-labelled DNA from, (a) wild type, (b) CymA2, and (c) CTS21. The profile of the DNA from CTS21 was obtained from a culture with a segregation frequency of 40%. Sedimentation is from left to right. The scale changes at fraction 20. In each case the peak at the top of the gradient is chromosomal, open circular and linear plasmid DNA; the peak nearer the bottom is the closed circular form of the *S. typhimurium* crytic plasmid.

of the CTS strains and no change in the quantity or quality of the *S. typhmiurium* LT2 cryptic plasmid. This suggests that a plasmid is not involved in suppression of Cym⁻. However, the technique has limited resolving power. It would not detect (a) a plasmid of molecular weight less than about 2Md (b) a plasmid with a very low copy number or (c) a small change in the cryptic plasmid.

The reversibility of segregation: If an autonomous plasmid highly susceptible to curing with acridine and ethidium bromide suppresses Cym⁻, auxotrophic segregants of CTS strains should not revert to prototrophy, *i.e.*, the loss of prototrophy should be irreversible. To test this, five clones each of CTS21 and 151 and two each of CymAa2Trz-1 and CymBc15Trz-5 as controls in which Cym⁻ was not suppressed, were grown overnight in NB and then plated as in the standard segregation assay. Segregants identified as colonies appearing only after incubation at 25° were picked and tested for their ability to yield prototrophic clones by streaking onto MA (37°). One hundred segregants from each clone of the CTS strains and 20 apparent segregants from those of the controls were tested. All the auxotrophic segregants of CTS21 and 151 yielded prototrophic clones on re-subculture to MA (37°). The test streaks showed unstable growth and the prototrophic clones showed persistant instability over five single-colony subcultures. The fraction of segregants yielding prototrophs varied between clones, but the mean fractions for both CTS21 and 151 were similar (70% and 61% respectively). No prototrophs were obtained from the controls. The stabilized strains, CTS71, 194, 43 and 84, were similarly tested, but due to the very low segregation frequencies only five segregants were tested for CTS71, 43 and 84 and only two for CTS 194. In each case segregation was shown to be reversible and the prototrophs obtained restabilized within two subcultures on MA (37°).

This reversibility would suggest that the loss of a plasmid is not the primary event in the segregation of a auxotroph from a population of CTS prototrophs. Rather, if a plasmid is involved, the primary segregation event is probably a change of state of that plasmid, such as integration or excision, rather than loss. If it is the excision of a plasmid, the reversibility of segregation would presumably be due to reintegration. Since curing agents eliminate autonomous plasmids (HIROTA 1960) fewer segregants from acriflavin-treated CTS strains should yield prototrophic clones when subcultured to MA (37°). To test this, two clones of CTS21 and 151 were grown as before in the presence and absence of acriflavin. Segregants were then tested for their ability to yield prototrophs. Table 7 shows that there was a marked reduction in the reversibility of segregation in CTS21 and 151 after acriflavin treatment.

The nature of Trz^r mutation in CTS strains: Although resistance to triazole was not unstable, it is possible that the instability of the CTS strains could relate to mutations like TrzB (HULANICKA and KLOPOTOWSKI 1972; HULANICKA, unpublished data) whose main properties are:

(1) increased transduction of *cysA* mutants to prototrophy,

(2) reduced linkage of *cysA* and *purC* in conjugation crosses, and

TABLE 7

The effect of acriflavin on the reversibility of segregation in CTS21 and 151

Strain	Clone	ACR	Sf	Segregants tested	% yielding prototrophs on MA(37°)
CTS21	1	—	56	95	79
		+	97	100	37
	2	—	46	100	62
		+	100	100	33
CTS151	1	—	29	100	67
		+	98	80	21
	2	—	86	100	55
		+	93	100	22

Abbreviations as in Table 5.

(3) negligible *O*-acetylserine sulphydrylase-A (OASS-A) activity. These three properties were measured for all the CTS strains and wild type, TrzB17, Trz110 and the nonsuppressed CymAa2Trz1 and CymAa7Trz6 strains as controls (Table 8). The nearly three-fold increase in transduction frequency of *cysA20* to prototrophy obtained with TrzB17 in comparison with wild type as donor was not obtained for any of the CTS or the other Trz^r control strains. Also conjugational linkage of TrzB17 to *cysA20* and *purC7* was very low as compared with Trz110, Trz1 and Trz6. Curiously, linkage of Trz^r of CTS strains to these

TABLE 8

Properties of the Trz^r mutations in CTS strains

Strain	Relative ability* to transduce *cysA20* to prototrophy	Conjugation linkinge (%)† of Trz^r to: *cysA20*	*purC7*	Relative‡ enzyme activity
Wild type	1	0	0	100
TrzB17	2.8	21	3	1.9
CTS21	1.06	67	52	4.3
CTS71	0.98	89	69	38
CTS194	1.0	71	54	26.4
CTS43	1.0	73	52	27.4
CTS84	1.07	77	56	7.0
CTS151	0.97	74	51	26.4
Trz110	1.07	93	77	13.2
CymA2Trz1	nt	91	81	nt
CymA7Trz6	nt	87	81	nt

* The number of transducing particles in each donor lysate was standardized by dividing the number of *cysA*+ transductants by the number of *aroB*+ transductants obtained using *aroB57* as recipient. Each of the resulting values was then divided by a similar value obtained for wild type.

† Conjugation crosses were mediated by F*lac* in each of the test strains. Linkage values were based on analysis of not less than 150 exconjugants.

‡ Relative *O*-acetylserine sulphydrylase A activity is expressed as a percentage of that of wild type whose derepressed specific activity was 5.3 units/mg protein.

nt = not tested.

markers was intermediate between that for TrzB17 and the stable TrzA-like Trz110, Trz1 and Trz6. OASS-A levels in CTS strains varied from 4.3% to 38% of wild type. All TrzB mutants have very low levels of this enzyme (HULANICKA, unpublished data and Table 8), suggesting that the Trz^r mutations in the CTS strains are similar to Trz110 (13.2% of wild-type activity). This means they resemble TrzA, since HULANICKA, KREDICH and TREIMAN (1974) showed that TrzA mutants may have as much as 20% of wild-type activity. There is, thus, no strong evidence that the Trz^r mutations in the CTS strains are of the TrzB type.

DISCUSSION

In some cases the isolation of Trz^r derivatives of a Cym^- mutants results in suppression of the Cym^- phenotype that is initially unstable, although resistance to triazole remains stable. Upon subculture of these (CTS) strains, the suppression may stabilize, dependent upon the Cym^- mutant. Stabilized strains are Cym^+ Trz^r, and they segregate Cym^- auxotrophs with frequencies of less than 1%; their phenotype is unaffected by plasmid-curing agents. Persistently unstable strains are Cym^- Trz^r, and they segregate auxotrophs whose frequency is increased by treatment with these agents, which suggests that a plasmid-like element may suppress Cym^- in these strains, although no new DNA species was detected in alkaline sucrose gradients (Figure 1). Segregated auxotrophs can revert to prototrophy, so that if a plasmid-like element is involved, then the change from prototrophy to auxotrophy reflects a reversible change of state of the element. The phenotypic instability of CTS strains is dissimilar from that of TrzB mutants.

A working hypothesis to explain these findings that permits a number of simple and testable predictions is proposed. In Figure 2 the various states of a *cys* gene containing a Cym^- mutation and the *cysALKptsHI* region of the *S. typhimurium* chromosome are indicated. Figure 2A represents the genotype of a Cym^- mutant, with its mutation located at one end of either the *cysC*, *D*, *H*, *I* or *J* gene (QURESHI, SMITH and KINGSMAN 1975) and a wild-type *cysALKptsHI* region. It is assumed that there is a sequence of DNA bases common to each gene in the *cysCDHIJ* cluster and the *cysALKptsHI* region, (this could be a regulatory region common to all *cys* genes, recognizing a common regulatory signal), and that mutation in this region results in the Cym^- phenotype.

CORDARO and ROSEMAN (1972) and HULANICKA (unpublished data) have shown that the *cysALKptsHI* region may be unstable and subject to frequent "looping out" events (SCAIFE 1967) producing deletions in the chromosome and plasmid-like elements in the cytoplasm. It is suggested that when Trz^r derivatives of Cym^- mutants are isolated, such a looping out event occurs, destroying the integrity of the *cysK* gene and leading to the formation of a closed circle of DNA (Figure 2B). We will refer to this DNA species as the Cym^--suppressor-plasmid (CSP). Since CSP carries a DNA sequence common to *cys* genes, it may pair with the homologous sequence in the *cys* gene in which the Cym^- mutation is located (Figure 2C). A single recombination event could then result in the

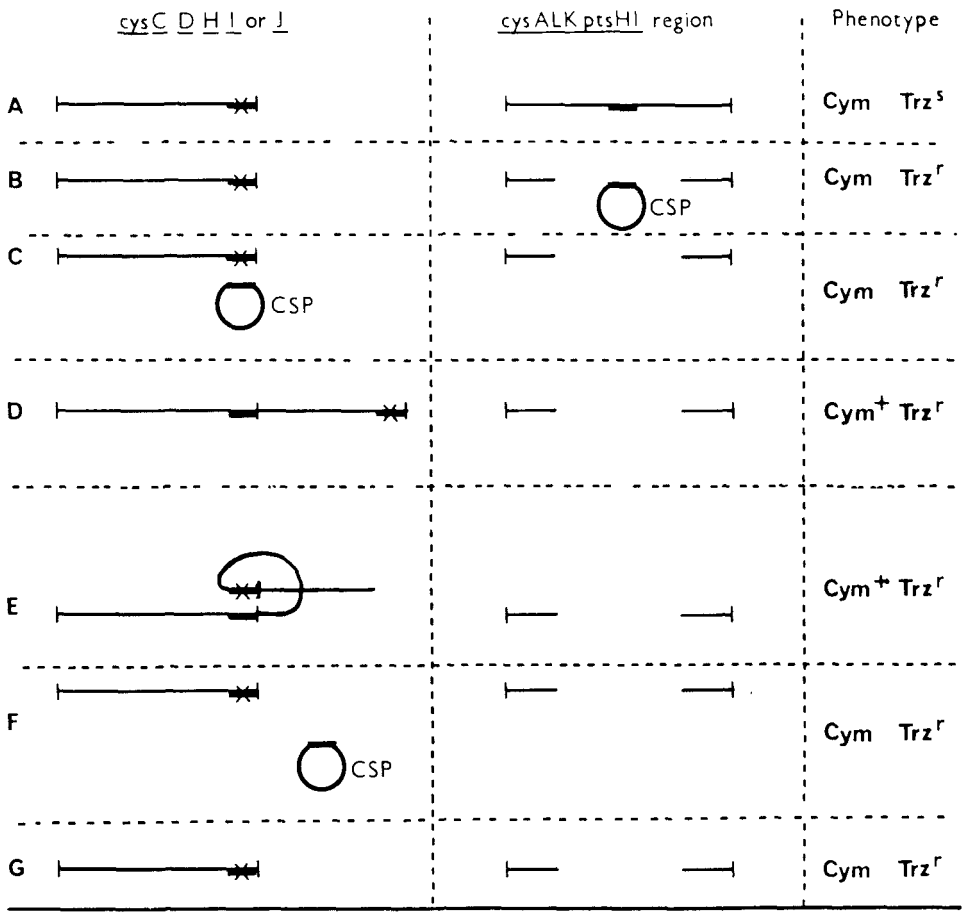

FIGURE 2.—A diagram of the postulated events involved in unstable suppression of the Cym^{-} phenotype in CTS strains. The thick line represents a DNA base sequence common to each of the genes in the *cysCDHIJ* cluster and to the*cysALKptsHI* region. The Cym^{-} mutation is represented by X. See text for explanation.

integration of CSP and the correction of the Cym^{-} lesion to give a prototroph (Figure 2D). There would now exist two *cys* gene DNA sequences in tandem. This could lead to instability, since these homologous regions would lead to the excision of CSP (HILL *et al.*, 1969) with consequent reinstatement of the Cym^{-} region in the *cys* gene (Figure 2E and F). Such a bacterium would revert to auxotrophy and appear as a segregant. Clearly integration may recur, and the system would exist in a state of integration-excision equilibrium comparable to that between F^{+} and Hfr (RICHTER 1961). When in the extrachromosomal state, CSP may be lost (Figure 2G).

Most of the properties of the CTS strains are consistent with this hypothesis. Thus, some of the Trz^{r} derivatives of the six Cym mutants were double mutants initially in which a CSP had been formed by excision of part of the *cysALKptsHI*

region. Subsequent alternation between integrated (suppressing) and excised (nonsuppressing) states would account for the unstable prototrophy of the CTS strains. The segregation frequencies in CTS strains are comparable to those in which tandem duplications have been shown to cause genetic instability (JACKSON and YANOFSKY 1973; MARGOLIN and BAUERLE 1966; HILL *et al.*, 1969; AUDIT and ANAGNOSTOPOULOS 1972; BEEFTINICK, CUNIN and GLANSDORF 1974). The difference between the stabilized and the persistently unstable CTS strains is difficult to explain. In the cases of CymAa2 and Bc15, the occurrence of CSP in the integrated and excised states may be more or less equally probable, whereas for CymAb19, Ba4 and Bb6 the equilibrium is in favour of the integrated state. It could be that the nature of the site of integration of CSP (*i.e.*, Cym^-) determines the stability of the integrated state.

Concerning the genetics of the CTS strains, when CSP is integrated and Cym^- suppressed, the bacteria are genetically Cym^+ Trz^r; when it is excised and Cym^- not suppressed, they are Cym^- Trz^r. Thus, in the case of CTS21 and 151, the CymA2 and Bc15 markers, respectively, were detected at frequencies similar to those using unsuppressed strains as donors (Table 4A), presumably because of the high frequency of auxotrophic (Cym^-) segregants in the population, particularly after three passages through unselective medium involved in the preparation of donor phage lysates. In contrast, populations of the stabilized strains CTS71, 194, 43 and 84, would contain few segregants; in most of the bacteria CSP would be integrated at Cym^- and they would be genetically Cym^+. This was the case except for CTS84, from which transduction of CymBb8 was detected at a frequency lower than that of control strains (Table 4A). CTS84 appeared stablized in streak tests, but segregated auxotrophs at frequencies slightly higher than other stabilized strains. Thus, the low frequency at which the CymBb8 marker was transduced probably resulted from the presence in the lysate population of a small number of segregants with CSP excised, thus being genetically Cym^-. If bacteria of stabilized strains were mainly Cym^+ and those of the persistently unstable strains Cym^-, this would also explain why only donor lysates grown on the stabilized strains could transduce homologous Cym^- mutants to prototrophy.

All the CTS strains possessed a Trz^r marker (Table 4b), and in transduction crosses $Cym^- \times$ CTS (homologous for Cym^-) when prototrophs were obtained they were not resistant to triazole. This implies that once CSP has been formed, Trz^r would be unnecessary for the suppression of Cym, which was dependent upon interaction between CSP and the Cym site.

The curing of Cym^- suppression in CTS21 and 151 by acriflavin and ethidium bromide (Tables 5 and 6) suggests that CSP replication is affected by these agents; increase in segregation frequency would result from the removal of autonomous CSP from one side of the integration-excision reversible reaction causing a shift in the equilibrium away from integration. Acriflavin and ethidium bromide are unlikely to affect stabilized strains since, like F in an Hfr strain (HIROTA 1960), CSP would be refractory to curing in the integrated state.

Finally, the reversibility of segregation suggests the reintegration of CSP at the appropriate Cym site.

Although this model explains most of the observations, an alternative should be considered. It is possible that the *cys* gene in which a particular Cym$^-$ mutation maps may escape the effect of the Cym$^-$ mutation by duplication. The *cys* gene would then come under the control of a different promoter (JACKSON and YANOFSKY 1973; LEVINTHAL and YEH 1972). Such a duplication would be highly unstable (HILL *et al.* 1969; AUDIT and ANAGNOSTOPOULOS 1972). While definitive evidence awaits a physical analysis of the *cysCDHIJ* region of CTS strains, we find the duplication model to be less attractive than that shown in Figure 2. First, it is hard to accommodate the requirement of the isolation of Trz^r mutants for suppression unless disruption of *cysK* during duplication is invoked. This would imply a large duplication involving the region between *cysCDHIJ* and *cysALKptsHI*, which seems unlikely since genetic disruption of the *cysCDHIJ* region in CTS strains is limited to a 9kb insertion (KINGSMAN 1977). Second, it is unlikely that the restored activity of the suppressed mutants is under the control of a new promoter, since all activities are regulated by cysteine (KINGSMAN and SMITH 1978). Third, the suppression of Cym$^-$ in CTS strains is *recA* dependent (KINGSMAN and SMITH 1978). However, this is perhaps a less strong point since, although the formation of duplications has been reported as *recA* independent (BEEFTINCK, CUNIN and GLANSDORF 1974; FRANKLIN 1971), there has also been a report of *recA* dependence (BASU and MARGOLIN, 1973).

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