Mutations in *Escherichia coli* that Relieve Catabolite Repression of Tryptophanase Synthesis. Mutations Distant from the Tryptophanase Gene

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(Received 8 May 1975; revised 11 June 1975)

SUMMARY

Two mutants are described in which the synthesis of tryptophanase is unusually insensitive to catabolite repression. Neither mutation is linked by transduction to the tryptophanase structural gene, neither mutation renders the synthesis of β -galactosidase insensitive to catabolite repression, and the mutations do not permit tryptophanase to be synthesized in strains deficient in adenyl cyclase. During growth in glucose-minimal medium the mutants maintained a similar intracellular concentration of cyclic AMP to their wild-type parent; but since in the wild type the concentration of cyclic AMP was the same in glycerol-minimal medium as in glucose-minimal medium, it is doubtful whether catabolite repression is mediated by measurable changes in the concentration of this nucleotide.

INTRODUCTION

Although the catabolite-sensitive operons of *Escherichia coli* have in common the fact that their expression depends on the cyclic AMP-catabolite-sensitive gene activator (c-AMP-CGA) protein system, the extent of this dependence differs from one operon to another (Lis & Schleif, 1973). One enzyme whose synthesis is extremely sensitive to catabolite repression is tryptophanase: whereas growth in glucose-minimal medium represses expression of *lac* only about 40 % compared with growth in glycerol-minimal medium, the comparable figure for *tna* (the structural gene for tryptophanase) is about 95 % (Peck, Markey & Yudkin, 1971). Therefore, selection for the ability to express *tna* at a high rate in the presence of glucose might result in the isolation of mutants whose metabolism of c-AMP is altered so that they maintain a comparatively high concentration of the nucleotide in glucose-containing medium. This paper shows that it is easy to isolate mutants whose synthesis of tryptophanase is resistant to catabolite repression, but that the intracellular concentration of c-AMP in glucose-grown cultures of two such mutants is no greater than in an isogenic wild-type strain.

METHODS

Genetic symbols. *alt*: a mutation permitting the expression of catabolite-sensitive genes in the absence of c-AMP. *cya*: the structural gene for adenyl cyclase. *cysB*: one of the genes necessary for the synthesis of cysteine. *glp*: the loci necessary for the fermentation of glycerol. *ilv*: the locus necessary for the synthesis of isoleucine and valine. *lac*: the locus necessary for the fermentation of lactose. *metB*, *metE*: two of the genes necessary for the synthesis of methionine. *strA*: one of the genes determining sensitivity to streptomycin. *tna*: the

structural gene for tryptophanase. *tonB*: one of the genes determining sensitivity to phage T1. *trp*: the locus necessary for the synthesis of tryptophan. Val^R: valine resistance.

Media. L-broth contained 1 % tryptone and 0.5 % yeast extract (both Difco), 0.5 % NaCl, 0.1 % glucose and 0.003 % cystine hydrochloride. L-agar was the same medium solidified with 1 % agar and containing CaCl₂ (2.5 mM final concentration). L-soft agar contained 0.8 % nutrient broth (Difco), 0.5 % NaCl and 0.65 % agar. The minimal medium of Vogel & Bonner (1956) was supplemented with 1 µg thiamine/ml, and a carbon source (glucose, glycerol or lactose) was added to give 0.2 % final concentration; glucose was the carbon source except where otherwise specified. Solid minimal media contained 1.5 % (w/v) agar. Other supplements were added, when needed, at the following concentrations (µg/ml): L-cystine hydrochloride, 50; L-methionine, 20; indole, 10; L-tryptophan, 50; indole, 2 plus DL-5-methyltryptophan, 50; L-valine, 50; acid casein hydrolysate, 5000; sodium gluconate, 2000.

Preparation of transducing lysates. To 2.5 ml of molten L-soft agar were added 10⁸ plaque-forming units (p.f.u.) of a clear mutant of phage P1 kindly given by Dr N. C. Franklin, and 0.1 ml of an overnight L-broth culture of bacteria. The mixture was poured on to a warm L-agar plate and incubated at 37 °C for 6 h. Cold L-broth (4 ml) was gently pipetted on to the surface, and the plate kept at 4 °C overnight. The liquid was removed, clarified in a bench-top centrifuge, and kept over chloroform.

Transduction. About 1 × 10⁸ p.f.u. of phage P1 were added to 1 ml of a late-exponential L-broth culture of bacteria together with 2.5 mM-CaCl₂. The mixture was incubated at 37 °C for 20 min. The bacteria were harvested by centrifugation, washed twice by centrifugation from 100 mM-sodium citrate pH 7.0, resuspended in citrate and plated on selective media.

Temperature. The organisms were grown at 37 °C.

Bacteria. Table 1 lists the strains of *Escherichia coli* K12 used. Strain MY235 was constructed as follows: phage P1 grown on strain MY525 was used to transduce strain MY534 to Cys⁺ on indole-minimal plates; the resulting *cysB*⁺ *trpE9829* strain was transduced with phage P1 grown on strain MY519, and *tonB* recombinants were selected (Gottesman & Beckwith, 1969). Those *tonB* recombinants that carried the *amber* mutation *trpE9829* were recognized by their ability to grow on indole-minimal agar only when suppressed by *φ8opsu3*⁺ (kindly given by Dr C. Yanofsky). The presence of *trpE*⁻ *D*⁺ *C*⁺ *B*⁺ *A*^{del} was confirmed by streaking on acid casein hydrolysate agar spread with *φ8optpr190* carrying various *trp* point mutations (Jackson & Yanofsky, 1972). *cya*⁻ strains were constructed by transducing recipient strains to valine resistance with phage P1 grown on strain MY267 and replicating to minimal agar with lactose or glycerol as sole source of carbon. *tna*⁻ strains were constructed by transducing recipient strains to valine resistance with phage P1 grown on strain MY600. Transductants were purified and grown overnight in L-broth. The addition of Ehrlich's *p*-dimethylaminobenzaldehyde reagent showed whether or not indole (made by Tna⁺ but not Tna⁻ strains) was present.

Differential rates of enzyme synthesis. The bacteria were grown from a small inoculum with shaking in tryptophan-minimal medium with glycerol or glucose, or in L-broth with sodium gluconate. The cultures were diluted with fresh warm medium to a density of about 10 to 15 µg protein/ml and shaken until they had at least doubled. Induction of β-galactosidase, sampling, and estimation of bacterial protein and of β-galactosidase have been described (Yudkin, 1969). The estimation of tryptophanase followed the procedure described by Peck *et al.* (1971), except that the bacteria were washed twice and were not treated with toluene before the enzyme was assayed. The differential rate of enzyme synthesis is the

Table 1. *Strains of E. coli used*

Strain	Genetic characteristics	Source
MY537	F ⁻ prototroph (w3110)	C. Yanofsky
MY534	F ⁻ <i>cysB trp^{del} AEr2</i>	C. Yanofsky
MY525	F ⁻ <i>trpE9829am</i>	C. Yanofsky
MY519	F ⁻ <i>trp^{del} A905-tonB^{del}</i>	C. Yanofsky
MY235	F ⁻ <i>trpE9829trp^{del} A905-tonB^{del}</i>	See Methods
MY245	F ⁻ <i>trp^{del} AEr2</i>	Strain MY534 transduced with phage P1 grown on MY537
MY247	F ⁻ prototroph	Strain MY245 transduced with phage P1 grown on MY537
MY600	F ⁻ Val ^R <i>tna2 metE</i>	E. Murgola
MY617	F ⁻ <i>cya855 metB strA</i>	J. Scaife
MY267	F ⁻ Val ^R <i>cya855 metB strA</i>	Strain MY617 transduced with phage P1 grown on MY600
MY571	F ⁻ <i>trp^{del} AC9</i>	C. Yanofsky
MY236	F ⁻ <i>trpE9829trp^{del} A905-tonB^{del}</i> catabolite-resistant	See Results
MY242	F ⁻ <i>trp^{del} AEr2</i> catabolite-resistant	See Results

gradient of a graph in which units of enzyme activity (Yudkin, 1969) are plotted against bacterial protein. Each figure quoted is the mean of at least three estimates which differed by no more than $\pm 10\%$.

Intracellular concentration of cyclic AMP. The bacteria were grown overnight as described above, diluted with fresh warm medium and shaken for at least two doublings. The bacteria from a measured volume (usually 10 ml) of culture were rapidly collected on a Millipore filter (0.45 μ m pore size) and washed with 5 ml of warm medium (see Wayne & Rosen, 1974). The filter was immediately placed in 1.0 ml of 0.1 M-formic acid, and the mixture heated to 90 °C for 5 min. The formic acid extract was clarified by centrifugation, and measured volumes were added to small test tubes. To another set of test tubes were added known quantities (0.1 to 1.0 pmol) of c-AMP in 0.1 M-formic acid. The extracts and the standards were dried overnight under reduced pressure and the c-AMP was dissolved and assayed in triplicate by the method of Cooper, McPherson & Schofield (1972). The c-AMP in the extracts was thus determined by reference to a standard curve prepared for each batch of samples.

RESULTS

Isolation of mutants

In *E. coli*, tryptophan is made from chorismate by enzymes whose synthesis is directed by the genes of the *trp* operon. The final reaction, which converts indole-3-glycerol phosphate to tryptophan, is catalysed by tryptophan synthetase. Strains carrying mutations in *trp* can use indole as a source of tryptophan, provided that they are capable of synthesizing the β -subunit of tryptophan synthetase at a high differential rate. Strains such as MY235 that synthesize little or no β -subunit, are unable to grow on indole-minimal medium. When a broth culture of strain MY235 was streaked on a glucose-minimal plate containing indole and 5-methyltryptophan, mutants able to grow on this medium were found. One of these mutants was purified and named MY236. Another mutant, MY242, was isolated by streaking strain MY245 (which carries a deletion of all the *trp* structural genes) on a glucose-minimal plate containing indole and 5-methyltryptophan.

Fig. 1. Part of the genetic map of *E. coli*. The numbers below the horizontal line are minutes on the linkage map of Taylor & Trotter (1972). The numbers above the line show the co-transduction frequencies between *tna* and *ilv* and between *cya* and *ilv* (unpublished experiments of D. F. Ward). The valine-resistance marker used in the work reported in this paper maps in *ilv*.

Table 2. *Differential rates of enzyme synthesis in different media*

Strain	Differential rate of tryptophanase synthesis (enzyme units/mg protein)		Differential rate of β -galactosidase synthesis (enzyme units/mg protein)		
	Glycerol-minimal medium	Glucose-minimal medium	Glycerol-minimal medium	Glucose-minimal medium	L-broth plus gluconate
MY247	147	4.3	542	280	46
MY236	241	23	703	542	40
MY242	128	54	477	332	44

Growth of the mutants is dependent on tryptophanase synthesis

Neither MY236 nor MY242 grows on minimal medium containing indole in the absence of 5-methyltryptophan. 5-Methyltryptophan is an inducer of tryptophanase, an enzyme that catalyses the conversion:

The usual function of this enzyme in *E. coli* is believed to be to degrade tryptophan, but the enzyme is also capable of synthesizing tryptophan from indole. That the ability of mutants MY236 and MY242 to grow on glucose-minimal medium containing indole and 5-methyltryptophan is dependent on tryptophanase was proved in the following way. Phage P1 grown on the valine-resistant *tna*⁻ strain MY600 was used to transduce strains MY236 and MY242 to valine resistance. Several dozen valine-resistant transductants were found. Of these, about 20 % were *tna*⁻ (see Fig. 1). The *tna*⁻ transductants had lost the ability to grow on glucose-minimal plates containing indole and 5-methyltryptophan, whereas the *tna*⁺ transductants retained this ability.

Catabolite resistance in the mutants

The above results show that strains lacking tryptophan synthetase β -subunits require tryptophanase to grow on glucose-minimal medium with indole and 5-methyltryptophan. However, the presence of tryptophanase alone is not sufficient, since the *tna*⁺ parental strains from which the mutants MY236 and MY242 are derived do not grow on such a medium.

One possible explanation is that the mutants synthesize tryptophanase at a higher rate than the parents. In glycerol-minimal medium strains MY236 and MY242 made about the same amount of tryptophanase as a wild-type strain (Table 2). In glucose-minimal medium, however, synthesis of tryptophanase by the wild type was very severely repressed whereas repression in the mutants was more moderate, suggesting that the inability of strains MY235 and MY245 to grow on glucose-minimal plates with indole and 5-methyltryptophan is due to catabolite repression. This explanation is borne out by the fact that these strains (as well as all other *trpB* strains tested) grew on glycerol-minimal plates with indole and 5-methyltryptophan.

To see whether the relief of catabolite repression in mutants MY236 and MY242 was specific to tryptophanase synthesis, I measured the rate of induced β -galactosidase synthesis in these mutants and in the wild-type control strain. Table 2 shows that the extent of repression caused by growth in glucose-minimal medium was similar in the mutants and the wild type. But with tryptophanase synthesis, the catabolite repression that is found in the wild type but relieved in the mutants is very severe; one might argue that with β -galactosidase synthesis too it would be only a very severe catabolite repression that would be relieved in the mutants. However, when each strain was grown in L-broth with gluconate (a medium that represses β -galactosidase synthesis by more than 90 % in the wild type) there was still no relief of catabolite repression in the mutant strains (Table 2).

Strains MY236 and MY242 are not tna promoter mutants

The above results show that in the two mutants the synthesis of tryptophanase, but not of β -galactosidase, is rather resistant to catabolite repression. Resistance to catabolite repression is characteristic of promoter mutations in the *glp* system (Berman-Kurtz, Lin & Richey, 1971) and the *lac* system (Silverstone *et al.* 1969; Yudkin, 1971; Arditti, Grodzicker & Beckwith, 1973); one obvious possibility therefore is that strains MY236 and MY242 carry promoter mutations in the *tna* system.

To examine this possibility, I grew phage P1 on the valine-resistant derivatives of MY236 and MY242 described above, used the phage to transduce the *trp*-deleted strain MY571 to valine resistance on glucose-minimal plates containing tryptophan, and replicated the transductants to glucose-minimal plates with indole and 5-methyltryptophan. [Strain MY571 cannot normally grow on minimal plates with indole as a source of tryptophan; like other *trpB* strains (see above) it can grow on indole-5-methyltryptophan plates with glycerol as a carbon source, using the induced tryptophanase to synthesize tryptophan from indole, but when glucose replaces glycerol its synthesis of tryptophanase is so repressed that it cannot grow on indole-5-methyltryptophan plates.] If the mutations in strain MY236 and MY242 were in the *tna* promoter, one would expect about 20 % of the valine-resistant transductants that had been selected on glucose-minimal plates with tryptophan to be able to grow when replicated to glucose-minimal plates with indole and 5-methyltryptophan. In fact all of the valine-resistant transductants from strains MY236 and MY242 grew when replicated to such plates.

This surprising result suggests that the mutations in strains MY236 and MY242 are extremely closely linked to *ilv*, the locus in which valine-resistance lies. However, when some of the transductants were streaked on indole-5-methyltryptophan plates with either glycerol or glucose as carbon source, they were all found to grow well on the glycerol-minimal plates, whereas growth on glucose-minimal plates showed the presence of several secondary mutants against a non-growing background. It appears that in strain MY571 – and the same is true of all *trpB* strains tested – mutations that relieve catabolite repression of tryptophanase occur very frequently. As a result, any colony will grow when replicated to a glucose-minimal plate with indole and 5-methyltryptophan, because mutations permitting the synthesis of tryptophanase in the presence of glucose will have accumulated during the growth of the original colony. To avoid these false positives it is necessary, instead of replicating, to pick colonies and streak them on to glycerol-minimal and glucose-minimal plates containing indole and 5-methyltryptophan. Valine-resistant transductants from strains MY236 and MY242 (50 from each) were tested in this way and none carried the mutation from the donor; it follows that neither mutation is closely linked to *tna*.

Table 3. *Intracellular concentrations of c-AMP*

Strain	Intracellular concns of c-AMP* (pmol/mg protein)	
	Glycerol-minimal medium	Glucose-minimal medium
MY247	37.1, 39.3, 10.0, 16.6, 11.1, 23.1, 16.6 (22.0)	10.4, 20.1, 13.8, 17.0, 30.3, 24.6, 45.2 (23.1)
MY236	6.6, 5.3, 8.5, 4.1 (6.1)	15.2, 29.8, 16.3, 11.1 (18.1)
MY242	7.5, 28.6, 8.1, 9.0, 17.5, 11.1 (13.6)	46.7, 38.4, 21.3, 17.5, 32.4, 5.8, 12.6 (25.0)

* The mean values are given in parentheses.

Tryptophanase synthesis in the mutants is dependent on c-AMP

Valine resistance can be co-transduced not only with *tna* but also with *cya*, the gene that directs the synthesis of adenylyl cyclase (see Fig. 1). The results described above exclude the possibility that the mutations in strains MY236 or MY242 are in the *cya* gene. It is possible that the effect of the mutations is to render tryptophanase synthesis altogether independent of the c-AMP-CGA protein system; Silverstone, Goman & Scaife (1972) described a mutation, *alt*, that permits the expression of catabolite-sensitive operons in mutants that lack either adenylyl cyclase or the CGA protein. To test this idea, I transduced *cya*⁻ into strains MY236 and MY242, and tried to measure tryptophanase synthesis in the *cya*⁻ transductants. The enzyme was undetectable (rate of synthesis < 0.1 % of that in the wild type); moreover the *cya*⁻ derivatives of strains MY236 and MY242 failed to grow on glucose-minimal plates with indole and 5-methyltryptophan.

Intracellular concentration of c-AMP in wild-type and mutant strains

Since these results prove that expression of *tna* in the mutants is still dependent on c-AMP, a possible explanation for the relief of catabolite repression is that the mutants maintain a higher intracellular concentration of c-AMP in glucose-minimal medium than the wild type does. The results presented in Table 3 belie this idea. Not only was the concentration of c-AMP in glucose-grown cultures much the same for the two mutant strains as for the wild type, but in fact the concentration of c-AMP in the wild type was not consistently lower in glucose-minimal medium than in glycerol-minimal medium. [Results of measurements on cells grown in glucose with gluconate (not shown) were also not significantly different.] There is some indication that in mutant MY236 glucose-grown cells contain more c-AMP than glycerol-grown cells – contrast the rates of tryptophanase synthesis shown in Table 2 – but the most striking feature of the measurements is their variability.

DISCUSSION

For the wild type, the results confirm previous reports that the synthesis of tryptophanase is far more sensitive to catabolite repression than the synthesis of β -galactosidase (Peck *et al.* 1971). It might be that the rate of expression of *tna* responds extremely sharply to small changes in the concentration of c-AMP whereas the rate of expression of *lac* responds less sensitively. However, direct measurements of the intracellular concentration of c-AMP show that there is no clear relationship between this concentration and the rate of tryptophanase synthesis, and Buettner, Spitz & Rickenberg (1973) and Wayne & Rosen (1974) have also reported that there is no consistent correlation between the intracellular concentration of c-AMP and the extent of catabolite repression. There is the possibility that the measurement

of total intracellular c-AMP does not reveal the concentration of c-AMP available to act as an effector in the expression of catabolite-sensitive genes. Alternatively, changes in the concentration of c-AMP may not in fact be the means by which the organism mediates catabolite repression (see Ullmann, 1974). For example, the results of Bernlohr, Haddox & Goldberg (1974) suggest that the concentration of cyclic GMP (c-GMP) changes, in response to different conditions of bacterial growth, in an opposite direction to that of c-AMP, and Artman & Werthamer (1974) showed that c-GMP inhibits the synthesis of both β -galactosidase and tryptophanase in growing wild-type *E. coli*.

The nature of the mutants is uncertain. They are not in the tryptophanase promoter, as they do not map near *tna*, and they are not of the *alt* type, as expression of catabolite-sensitive genes still depends on *cya*⁺. Since they fail to relieve catabolite repression of *lac*, it may be that they do not intervene in the central mechanism of catabolite repression but are specific to *tna*. That argument is far from conclusive because growth in extremely rich medium is necessary to repress *lac* severely, and the fact that the mutations fail to relieve catabolite repression in such conditions does not prove that they do not affect the mechanism of catabolite repression under much milder conditions. If they do have some general effect on catabolite repression, it is evidently premature to speculate on their mode of action. If they have a specific effect on the expression of *tna*, it may be connected with the regulatory locus for tryptophanase synthesis that is not linked to the *tna* structural gene (Gartner & Riley, 1965).

I am grateful to Dr N. C. Franklin, Dr E. J. Murgola, Dr J. Scaife and Dr C. Yanofsky for gifts of strains, and to Mr L. Turley for capable and conscientious technical assistance.

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