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Unstable Mutations That Relieve Catabolite Repression of Tryptophanase Synthesis by *Escherichia coli*

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From strains of *Escherichia coli* that carry deletions of the *trp* region, five different mutants were isolated that were capable of synthesizing tryptophanase at unusually high rates in conditions of severe catabolite repression. Notwithstanding the comparative insensitivity to catabolite repression, the rates of tryptophanase synthesis in the mutants were greatly diminished by the introduction of a defective gene for adenyl cyclase. Each of the mutants segregated variants of the parental type. The results of genetic analysis appear to be consistent with the suggestion that the mutants arose by duplication of the tryptophanase gene.

Although the synthesis of tryptophanase in *Escherichia coli* is exquisitely sensitive to catabolite repression, it is possible to select mutants in which catabolite repression of this enzyme is substantially diminished. Two different types of such mutants have been described. In the first type, the mutation that leads to relief of repression is not linked by transduction to the tryptophanase structural gene (10). In the second type, the mutation is extremely closely linked to the structural gene and is probably an alteration in the tryptophanase promoter (9). I now describe a third type mutant in which catabolite repression of tryptophanase synthesis is relieved. These mutants are unstable; they segregate variants that are indistinguishable from their parents.

MATERIALS AND METHODS

Bacteria. The organisms were all derivatives of *E. coli* K-12 strain W3110. Strain MY564 (*trpAE1*) and strain MY571 (*trpAC9*) are from the collection of C. Yanofsky. Strain MY281 (*trpAC9 tna* Val^r) was described previously (10).

Media and genetic techniques. Media and genetic techniques were as described by Yudkin (10). Transduction of strains to *cya*$^-$ was done (10) with phage P1 grown on strain MY267 (*cya*$^-$ Val^r). Transductants selected for valine resistance were screened for inability to use glycerol.

Growth of organisms for enzyme assays. Since the strains described here are unstable, it was necessary to maintain selection for retention of the mutation. The mutant strains were therefore grown overnight at 37°C in glucose-minimal medium containing indole and 5-methyltryptophan (10). In the morning, the cells were harvested and suspended at about 2.5×10^7/ml in warm minimal medium with tryptophan, supplemented with either glycerol or glucose. The cultures were allowed to grow for two generations at 37°C before sampling for enzyme assay was begun. Assays were performed on samples taken over a 2-h period of exponential growth as described previously (10). The differential rate of enzyme synthesis was constant during this period.

RESULTS

Isolation of mutants. *E. coli* normally converts indole to tryptophan through the action of tryptophan synthetase, an enzyme whose synthesis is directed by genes of the *trp* operon. In mutants carrying a deletion of these genes, indole can be used as a source of tryptophan only if the bacteria can make sufficient quantities of an alternative enzyme, tryptophanase. The synthesis of tryptophanase is induced by 5-methyltryptophan and is severely repressed by growth on glucose or other substrates that cause catabolite repression. Most *trp*-deleted strains cannot grow on minimal-salts medium with indole and 5-methyltryptophan if glucose is the carbon source, but they can grow when glycerol replaces the glucose (10).

When cells from a broth culture of strain MY564 (which carries a deletion of all the *trp* structural genes) were washed and plated on indole–5-methyltryptophan agar with glucose and gluconate, mutants (about 5 to $10/10^9$ cells plated) arose after 2 days at 37°C. (In this selection, the use of gluconate, which causes very severe catabolite repression, eliminates mutants of the type described previously [10].) Several mutants isolated in this way were studied; their properties are indistinguishable, and the description that follows refers to one of them, strain MY285.

Genetic characterization of strain MY285. The mutant makes colonies in 2 days at 37°C on

indole-5-methyltryptophan agar supplemented with glucose or with glucose plus gluconate; it does not grow on glucose-indole agar without 5-methyltryptophan, a medium on which the synthesis of tryptophanase is not induced.

The mutation that allows tryptophanase to be synthesized in the presence of glucose plus gluconate is unstable, as is shown by the following results. Seven colonies of strain MY285 were grown overnight in broth; the cells were diluted and plated on nutrient agar, and the plates were replicated to indole-5-methyltryptophan agar with glucose plus gluconate. About 5 to 7% of the colonies on the nutrient agar were unable to grow on the latter medium, even though they retained the ability to grow on indole-5-methyltryptophan agar with glycerol as the carbon source, and were therefore *tna*$^+$. When colonies, from this experiment, that had not lost the ability to grow on indole-5-methyltryptophan with glucose plus gluconate were again grown in broth and tested in the same way, they once more segregated variants that could not grow on this agar. On the other hand, the variants that had lost the ability to grow on indole-5-methyltryptophan agar with glucose plus gluconate did not regain it after further growth in broth.

Instability is characteristic of duplications of genetic material (1, 5, 6). The results with strain MY285 could perhaps be explained by supposing that the tryptophanase gene has been duplicated and that the duplicate copy is now read from a promoter that permits it to be expressed in conditions that produce strong catabolite repression. The results of subsequent experiments are consistent with this idea.

In wild-type strains of *E. coli*, the tryptophanase structural gene is cotransducible with a valine resistance marker located in the *ilv* gene cluster; when a *tna*$^-$ Val^r strain is transduced with phage P1 grown on a *tna*$^+$ Val^s strain, about 20% of the Tna$^+$ transductants are Val^s (10). If the ability of strain MY285 to express *tna* in the presence of glucose plus gluconate is due to a translocated duplicate *tna* gene, one would expect this gene to be either less distant or more distant from *ilv* than the normal *tna* gene, so that there should be a corresponding change in cotransduction frequency. Accordingly, phage P1 grown on strain MY285 was used to transduce strain MY281 (*trp*^{del} *tna*$^-$ Val^r) to Tna$^+$ on indole-5-methyltryptophan medium supplemented with glucose plus gluconate. Of the 1,581 transductants tested, only 8 (0.5%) proved to be valine sensitive.

A reasonable conclusion is that strain MY285 contains a duplicate *tna* gene, which is attached to a catabolite-insensitive promoter and is more distant from *ilv* than the original *tna* gene. The conclusion that a duplicate *tna* gene is present was also consistent with additional results from the experiment in which phage P1 grown on strain MY285 was used to transduce strain MY281. Transductants selected on indole-5-methyltryptophan agar supplemented with glycerol (on which transductants can grow if they acquire any *tna*$^+$ gene) were nearly twice as numerous as those selected on indole-5-methyltryptophan agar supplemented with glucose plus gluconate (on which transductants can grow only if they acquire a *tna*$^+$ gene whose expression is resistant to catabolite repression). (The actual figures were 2,300 glycerol-selected transductants per ml of transduction mixture, and 1,370 glucose-gluconate-selected transductants per ml.) Of the glycerol-selected transductants, 6% were valine sensitive, and 55% were capable of growth on indole-5-methyltryptophan agar supplemented with glucose plus gluconate. Six colonies of the latter class were tested for stability as described above: all proved to be unstable, segregating variants that were still Tna$^+$ but no longer capable of growing on indole-5-methyltryptophan agar supplemented with glucose plus gluconate. The same instability was noted in the transductants selected on medium containing glucose plus gluconate.

By contrast, phage P1 grown on strain MY564, the parent of strain MY285, was capable of transducing strain MY281 to growth on indole-5-methyltryptophan agar if supplemented with glycerol but not if supplemented with glucose plus gluconate. Transductants obtained when strain MY564 was the donor failed to grow when streaked to the medium with glucose plus gluconate, so that they appeared to have acquired only a wild-type *tna*$^+$ gene.

Biochemical studies of strain MY285. Table 1 presents the rates of tryptophanase synthesis by strain MY285 and by its parent, strain MY564, in glycerol-minimal and in glucose-minimal medium. If the conclusion reached

TABLE 1. *Differential rate of tryptophanase synthesis in strains MY564 and MY285*

Strain	Enzyme units/mg of protein[a]	
	Glycerol	Glucose
MY564	127	10
MY285	181	47

[a] Cells were grown in minimal medium supplemented with tryptophan and the carbon source shown. The differential rate of enzyme synthesis was derived by measuring the enzyme content and total cell protein in several samples taken over a 2-h period of exponential growth (10).

above, that strain MY285 contains a duplicate *tna* gene, is correct, we may roughly estimate the contribution of this duplicate gene to the total rates of enzyme synthesis in strain MY285 by subtracting the rates of enzyme synthesis in strain MY564 from those in strain MY285. Thus, the putative duplicated gene appears to be responsible for the synthesis of about 54 units of enzyme per mg of protein in glycerol-minimal medium and about 37 units of enzyme per mg of protein in glucose-minimal medium. If expression were completely indifferent to catabolite repression, one would expect these figures to be identical. It is possible, therefore, that expression of this gene has some sensitivity to catabolite repression. On the other hand, since the discrepancy between the two figures is quite small, and since one of them is obtained as the difference between two fairly large numbers, it may be that the rate of expression is, in fact, the same in glycerol-minimal and in glucose-minimal medium.

To investigate this point further, I transduced *cya*$^-$ (a defective adenyl cyclase gene) into strain MY285. In *cya*$^-$ strains, a wild-type *tna* gene is not expressed at all (10), so, if the expression of the presumptive duplicated *tna* gene were truly unaffected by catabolite repression, the *cya*$^-$ derivative of strain MY285 would make as much tryptophanase as this gene alone makes in the original *cya*$^+$ strain, MY285. In fact, the *cya*$^-$ derivative of strain MY285, grown in glucose-minimal medium, made only 6 units of enzyme per mg of protein, suggesting that the mutant *tna* gene is sensitive in some degree to catabolite repression. However, the *cya*$^-$ derivative of strain MY285 can still make colonies on glucose-indole-5-methyltryptophan agar.

Additional mutants. Further mutants independently isolated from strain MY564 were indistinguishable from strain MY285. To isolate different mutants, I used as parent another *trp*-deleted strain, MY571. Four mutants, derived from this strain by selection on indole-5-methyltryptophan agar with glucose plus gluconate, and named MY289, MY290, MY291, and MY292, were studied by techniques similar to those used for MY285.

That each mutant is unstable was shown in the following way. Cultures were grown overnight in broth at 37°C, diluted, and plated on nutrient agar. Of the colonies that appeared, 1 to 4% were unable to grow on glucose-indole-5-methyltryptophan agar.

Phage P1 was grown on each of the mutants and used to transduce strain MY281 (*trp*^{del} *tna* Val^r) to grow on indole-5-methyltryptophan agar in the presence of glucose plus gluconate. This ability (presumed to be due to a duplicated tryptophanase gene) was cotransduced with valine sensitivity less than 0.5% in strains MY289 and MY291, 1% in strain MY290, and 2.5% in strain MY292.

Table 2 presents the rates of tryptophanase synthesis in the parental strain MY571, in the mutants, and in *cya*$^-$ derivatives of these strains. *cya*$^-$ derivatives of strains MY291 and MY292 were able to grow on glucose-indole-5-methyltryptophan agar; *cya*$^-$ derivatives of strains MY289 and MY290 were not.

DISCUSSION

Strains carrying deletions of the *trp* operon can grow on indole-5-methyltryptophan agar when glycerol is the carbon source but (generally) not when glucose is. I suggested previously (10) that in glucose medium the differential rate of synthesis of tryptophanase is too low to allow sufficiently rapid conversion of indole to tryptophan. However, the present results show that whether a strain grows on glucose-indole-5-methyltryptophan agar is not dependent only on the differential rate of tryptophanase synthesis in glucose-minimal medium. The *cya*$^-$ derivative of strain MY285, which synthesizes 6 units of enzyme per mg of protein in glucose-minimal medium, can make colonies

TABLE 2. *Differential rate of tryptophanase synthesis in strain MY571 and its derivatives*

Strain	Enzyme units/mg of protein[a]				
	cya$^+$ background			*cya*$^-$ background	
	Glycerol	Glucose	Difference in mutant	Glucose	Difference in mutant
MY571	173	13		0	
MY289	156	100	87	0	0
MY290	202	84	71	0	0
MY291	185	133	120	18	18
MY292	226	156	143	20	20

[a] Cells were grown in minimal medium supplemented with tryptophan and the carbon source shown.

on glucose-indole-5-methyltryptophan agar, but the *cya*$^+$ strains MY564 and MY571, which make tryptophanase in glucose-minimal medium at a higher differential rate, cannot. The ability of another *cya*$^-$ *trp*-deleted strain to grow in indole-5-methyltryptophan medium with glucose as the carbon source, while making tryptophanase at a low differential rate, was noted by Ward and Yudkin (9).

The mutants described in this paper were isolated by a selection procedure that required the synthesis of tryptophanase to escape from severe catabolite repression. The suggestion that they may contain duplications of the tryptophanase gene seems to account reasonably well for the following observations. (i) The mutants are unstable, segregating variants which, although remaining Tna$^+$, can no longer grow on indole-5-methyltryptophan with glucose plus gluconate. (ii) Transductants obtained by using the mutants as donors and a *tna*$^-$ strain as the recipient and selecting for Tna$^+$ are of two classes, which are obtained in roughly equal numbers: one class resembles the unstable, catabolite-insensitive mutant donors, and the other resembles the stable, catabolite-sensitive segregants to which these mutants give rise. Duplications of genetic material are well known both in procaryotes and in eucaryotes (2, 3), and their comparatively frequent occurrence has suggested that they may be of evolutionary significance.

If it is accepted, for the purposes of discussion, that the mutation involves duplication of the tryptophanase gene, we may ask where the new copy of the gene is. Analysis of the transductants that had acquired the mutation suggested that the new copy was some distance from the original *tna* gene (cf., duplications of the *trp* genes [6] and the *arg* genes [4] of *E. coli*) but still weakly linked to the *ilv* locus. Therefore, we may suggest that, if there has been a duplication, the duplicated gene has been translocated to a new position several genes further away from the *ilv* locus. An alternative possibility is that a tryptophanase gene has been acquired by a plasmid; however, treatment of the strains with acridine orange did not increase the rate of segregation (data not shown).

Estimates of the rate of expression of the presumed duplicated tryptophanase gene are slightly uncertain because it is possible that the original tryptophanase gene is being expressed simultaneously. However, in *cya*$^-$ strains we can be reasonably sure that all of the tryptophanase is synthesized under the direction of the new gene, since we know that the wild-type gene produces no detectable enzyme in a *cya*$^-$ background. In *cya*$^+$ strains we can make what is probably a quite accurate estimate of the contribution of the mutant gene when the cells are grown in glucose, by subtracting from the total quantity of enzyme synthesized the small amount of enzyme made when the wild-type *cya*$^+$ parent is grown in glucose.

The resulting estimates of the rate of expression of the putative duplicated gene (Tables 1 and 2) show that in each case expression is greatly enhanced by the presence of *cya*$^+$; indeed, in strains MY289 and MY290 the gene is not expressed at all in a *cya*$^-$ background. We must conclude, therefore, that the gene is subject to catabolite repression. On the other hand, its rate of expression in a *cya*$^+$ background when the strain is grown in glucose-minimal medium is always much higher (11-fold higher in strain MY292) than that of the wild-type gene. It would be consistent with these results to suggest that a tryptophanase gene, after duplication, has been detached from its own promoter and fused in the five strains to five different promoters, each of which shows a different characteristic response to catabolite repression. Such an explanation would strain credulity; moreover, it would not account for the fact that tryptophanase synthesis in the mutants is still dependent on induction by indole-5-methyltryptophan.

It would appear more likely that the tryptophanase gene and its controlling elements have together been duplicated, and that the unit has been fused to catabolite-insensitive promoters from which transcriptional "read-through" is occurring (8). The rate of expression in each strain would then reflect both transcription initiated at the normal promoter and read-through from the foreign promoter, this read-through being modulated when the ribonucleic acid polymerase reaches the controlling elements of the tryptophanase gene. For instance, in the *cya*$^-$ derivatives of strains MY289 and MY290, the tryptophanase promoter might perhaps remain so stubbornly closed that read-through from the foreign promoter is aborted. In addition, the nucleotide sequence near the point of fusion might inhibit translation of the tryptophanase messenger ribonucleic acid (7). An alternative possibility is that the duplication/translocation event might itself give rise to a new promoter from which the duplicated gene could be read. Promoters thus generated might differ in their sensitivity to catabolite repression.

The contribution of the putative duplicated gene to the rate of tryptophanase synthesis in *cya*$^+$ strains grown in glycerol cannot be so accurately determined, because the original

copy of the tryptophanase gene is presumably being expressed at a rapid rate. However, the rate of tryptophanase synthesis in the mutants of strain MY571 during growth in glycerol is in no case more than 30% higher than in the parental strain; one would perhaps expect a strain carrying a duplication of the tryptophanase gene to make approximately twice as much enzyme as the parent with a single copy of the gene. These results perhaps suggest that there is some overall limit set on the maximum rate of tryptophanase synthesis. Studies now in hand with strains diploid and triploid for the tryptophanase gene should show whether this suggestion is correct.

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