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Isolation of Mutants Affecting Tryptophanase Production in *Escherichia coli*

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ABSTRACT

GARTNER, THEODORE K. (University of California, Davis), AND MONICA RILEY. Isolation of mutants affecting tryptophanase production in *Escherichia coli*. J. Bacteriol. 89:313-318. 1965.—Mutants of *Escherichia coli* K-12 were isolated which appear to have suffered an alteration in the regulation system governing tryptophanase synthesis. A novel selection method was used to isolate tryptophanase mutants from tryptophan synthetase deletion mutants. Mutants were obtained which exhibited the following phenotypes either singly or in combination with others: constitutivity and inducibility at 13 C (wild type is not inducible at 13 C), constitutivity and hyperinducibility at 37 C. Mutation to constitutivity at 13 C seems to require two mutational changes of the wild type. Other mutants, presumably structural gene mutations, were isolated which lack tryptophanase activity under all conditions tested.

Tryptophanase, an inducible catabolic enzyme found in *Escherichia coli*, carries out a pyridoxal phosphate-dependent conversion of L-tryptophan to indole, ammonia, and pyruvate (Wood, Gunsalus, and Umbreit, 1947). Recently, the enzyme was highly purified by Burns and DeMoss (1962), and was crystallized by Newton and Snell (1964).

Synthesis of tryptophanase and the inducible tryptophan permease (Burrous and DeMoss, 1963) is presumably controlled by a specific regulation system similar to the system regulating β-galactosidase synthesis (Jacob and Monod, 1961). Tryptophanase is formed in response to the presence of the substrate L-tryptophan or an analogue of the substrate, 5-methyl-DL-tryptophan. Indole is not active as an inducer (Newton and Snell, 1962). Within a few minutes after induction, tryptophanase activity increases at a constant differential rate (Pardee and Prestidge, 1961). Synthesis of the enzyme is repressed under conditions of catabolite repression (McFall and Mandelstam, 1963). To this extent, regulation of tryptophanase formation is similar to regulation of formation of other catabolic enzymes such as β-galactosidase and galactokinase.

However, differences may exist between tryptophanase and β-galactosidase regulation systems. β-Galactosidase can be induced in cultures of *E. coli* growing at 13 C (Marr, Ingraham, and Squires, 1964), whereas tryptophanase is induced only poorly if at all at this temperature (Ng, 1963).

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To study the regulation of tryptophanase formation, we isolated a number of mutants of *E. coli* which appear to be modified in their ability to regulate tryptophanase synthesis. Isolation and preliminary characterization of the mutants is described in this paper, and a genetic analysis of some of the mutants is described in the following paper.

MATERIALS AND METHODS

Media. A minimal medium, M63, was used for cultivation of the bacteria (Pardee, Jacob, and Monod, 1959). Glycerol (0.2%) was used as a carbon source unless otherwise stated. In some experiments, L-tryptophan (0.5%) or glucose (0.2%) replaced glycerol. When appropriate, the following additions were made to the medium: amino acids as growth factors, 20 μg/ml; thiamine, 0.5 μg/ml; 5-methyl-DL-tryptophan, 50 μg/ml; L-tryptophan as inducer, 500 μg/ml; indole in mutant-selection media, 15 μg/ml; indole in cultures used for determination of differential rate of constitutive tryptophanase synthesis, 100 μg/ml. Minimal medium was solidified by the addition of 15 g per liter of agar (Difco). Nutrient agar plates contained 8 g per liter of nutrient broth (Difco) and 10 g per liter of agar.

Strains. All strains used are derivatives of *E. coli* K-12. W1692 was obtained from C. Yanofsky. It is a tryptophan synthetase deletion mutant which requires histidine and proline. A *pro*$^+$ derivative of W1692 called W1692-2 was made by transduction. C600-1 is a B_1^+ (*thi*$^+$) derivative of a streptomycin-resistant *thr*$^+$ *leu*$^+$ revertant of C600 obtained from A. B. Pardee. W3018, obtained from E. Lederberg, is a F$^-$ *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *mal*$^-$ *xyl*$^-$ *mtl*$^-$ strain.

Tryptophanase activity. The differential rate of either induced or constitutive tryptophanase synthesis was measured in exponentially growing cultures. Induced synthesis was measured in cultures to which 500 μg/ml of tryptophan were added as inducer. Constitutive synthesis was measured in the absence of added inducer. In the case of the tryptophan synthetaseless strains which are constitutive for tryptophanase, the medium contained 100 μg/ml of indole as a source of tryptophan for growth. Samples were removed from cultures at intervals for measurement of both total cellular protein and activity of tryptophanase. At least five determinations were made on each culture. Increase in cellular protein per milliliter of the growing culture was plotted against increase in units of tryptophanase activity per milliliter of the culture. The slope of the line equals the specific activity of tryptophanase in newly formed protein and represents the differential rate of synthesis of tryptophanase (Monod, Pappenheimer, and Cohen-Bazire, 1952).

Cellular protein was determined by relating the optical density of the culture at 550 mμ, measured with a Zeiss model PMQII spectrophotometer, to milligrams of protein per milliliter by means of a standard curve.

Activity of tryptophanase was measured as follows. Samples (1.0 ml) of the culture were filtered through a Millipore HA nitrocellulose membrane and washed free from growth medium with a phosphate-glutathione buffer (0.1 M potassium phosphate, 0.01 M glutathione, *p*H 7.0). The washed bacteria were resuspended in 1.0 ml of cold phosphate-glutathione buffer, and the cell density was measured at 550 mμ. Samples (0.1 ml) of this suspension were shaken gently with 0.1 ml of toluene, and then stored on ice for no longer than 3 hr before assay of tryptophanase activity. For assay, the sample was preincubated for 5 min at 37 C, and then 0.5 ml of a prewarmed substrate-cofactor-buffer mixture was added. This mixture was prepared fresh daily from a frozen concentrated stock solution and contained 1.0 mg/ml of tryptophan, and 55 μg/ml of pyridoxal phosphate in 0.1 M phosphate buffer (*p*H 7.0). After incubation for 30 min at 37 C, 2.3 ml of cinnamaldehyde reagent were added. The reagent contained 233 mg of 2,4-dimethylcinnamaldehyde, 75 ml of 95% ethyl alcohol, and 10 ml of concentrated HCl (Scott, 1961). Color development was allowed to proceed for 30 min at 37 C, and then optical density of the sample at 625 mμ was measured.

Specific activity of tryptophanase in a culture was measured as the optical density at 625 mμ divided by that at 550 mμ. A value of 1.0 corresponds to 52 units per mg of protein, where the unit of activity is defined as 1 mμmole of indole formed per minute. Duplicate samples agreed within ±5%.

In preliminary experiments and for roughly characterizing different mutant types, a simpler, less accurate assay was used. In this assay, only a single specific activity determination was made on a fully induced or constitutive culture, rather than carrying out a complete determination of the differential rate of synthesis over a period of time. The assay procedure used for these determinations was based on the one described by Pardee and Prestidge (1961), in which the color reagent is a modification of Ehrlich's *p*-diaminobenzaldehyde reagent.

The first procedure described here has the advantage of increasing the stability and activity of tryptophanase in crude extracts through the use of a reducing agent and a preincubation period. Further, since the cinnamaldehyde reagent is more sensitive than Ehrlich's reagent, the reaction can be carried out at indole concentrations which do not inhibit enzyme activity.

Isolation of mutants lacking tryptophanase activity. The penicillin method described by Davis (1948) was used to isolate mutants lacking tryptophanase activity. Cells growing exponentially in minimal medium were irradiated with ultraviolet light to give 99.9% loss of viability. About 10^9 irradiated cells were transferred to fresh minimal medium and incubated for 24 hr to allow phenotypic expression of mutated genes. About 10^8 of these cells were inoculated into minimal medium containing 0.5% tryptophan as the only carbon source. After incubation at 37 C for 4 hr, penicillin G was added to a final concentration of 2×10^3 units per ml. Selection was allowed to proceed for 48 hr before screening for mutants. A portion of the selection-flask contents was spread on nutrient agar plates; after incubation, single colonies were picked and inoculated into tubes of minimal glycerol medium containing 10 μg/ml of tryptophan. After incubation for 24 hr, the tubes were assayed for the presence of indole. Tubes in which growth had occurred but no indole had been produced contained bacteria lacking tryptophanase activity.

Selection and isolation of mutants altered in tryptophanase activity. Two methods were used to isolate mutants constitutive for tryptophanase at 37 C. The first (Ng and Gartner, 1963) was based on a modification of the limiting nutrient technique of Novick and Horiuchi (1961). Tryptophan was fed slowly at subinducing concentrations to a culture growing at 37 C. This procedure is referred to as method A.

An alternate procedure, method B, permits selection of constitutive mutants on solid as well as liquid medium, providing the parental strain used is a tryptophan auxotroph. The tryptophan synthetaseless parental strain, W1692-2, was incubated at 37 C in either solid or liquid minimal medium containing 15 μg/ml of indole as a source of tryptophan for 1 to 2 weeks. Only mutants constitutive for tryptophanase can convert the indole into the tryptophan needed for growth.

Method C, used for selection of mutants inducible for tryptophanase at 13 C, has been described briefly elsewhere (Ng and Gartner, 1963). Uninduced parental bacteria were inoculated into

minimal medium with 0.5% tryptophan as the only carbon source and incubated at 13 C for 6 to 10 weeks. Single colonies obtained from samples of the culture were then tested individually for mutant phenotypes.

Mutants able to form tryptophanase at 13 C can be isolated from strain W1692-2 by a procedure analogous to that used for isolation of mutants constitutive at 37 C. In method D, tryptophan synthetaseless parental bacteria were incubated at 13 C in medium containing indole as a source of tryptophan and 5-methyltryptophan as an inducer of tryptophanase.

Mutants constitutive for tryptophanase at 13 C were selected by method E. Selection was exerted as in method D, except that the inducer 5-methyltryptophan was omitted from the medium.

RESULTS

Tryptophanase-negative mutants. Two tryptophanase-negative mutants were isolated by the pencillin technique. One from strain W3108 is called GR-107; the other, from C600-1, is called GR-103. No tryptophanase activity was detectable in either mutant whether grown with or without tryptophan at 13 or 37 C. These mutants also lack the inducible tryptophan synthetase activity attributed to the tryptophanase protein (Newton, *personal communication*). Both mutants are, therefore, tentatively considered to be structural gene mutations, although there is no conclusive evidence for this interpretation at present.

Mutants constitutive for tryptophanase at 37 C. Mutants isolated by method A were found to be partially rather than completely constitutive at 37 C, having only 5 to 50% of the tryptophanase activity of the fully induced wild-type parental strain (Ng and Gartner, 1963). Most of the mutants responded to the addition of inducer and achieved a specific activity as great as five times that of a fully induced wild type. We shall refer to the last property as hyperinducibility. None of the method A mutants tested was inducible at 13 C.

Method B mutants also showed different degrees of constitutivity at 37 C (Table 1). For example, mutants GR-5 and GR-6, isolated independently in liquid media, are partial constitutives, having uninduced tryptophanase specific activities about half that of induced wild type. Other mutants, GR-8 and GR-9, isolated on solid media, are full constitutives whose specific activity when uninduced is at least as great as that of induced wild type. All mutants tested responded to the addition of inducer. Induced cultures of mutants GR-5 and GR-6 achieved specific activities about the same as induced wild type. Induced cultures of GR-8 and GR-9 exhibited hyperinducibility; the specific activity achieved is 1.5 to 1.7 times induced wild-type activity.

TABLE 1. *Tryptophanase activity in Escherichia coli mutants*

Strain designation	Tryptophanase specific activity* (units/mg of protein)			
	13 C (*10 C*)†		37 C	
	With inducer	Without inducer	With inducer	Without inducer
C600-1....	3.5 (*0.7*)†	70 (*18*)	6.5	215
GR-101..	65 (*12*)	400 (*195*)	20	650
W1692-2...	IS‡	0.7	2.5	160
GR-5....	IS	IS	90	140
GR-6....	ND‡	ND	70	170
GR-8....	ND	ND	220	280
GR-9....	ND	ND	160	240
GR-11...	IS	60	50	ND
GR-10...	IS	50	70	200
GR-32...	IS	IS	280	280
GR-27...	160	240	150	250
GR-28...	50	180	55	350

* The values recorded are maximal specific activities achieved by cultures growing at steady-state conditions in a minimal medium containing glycerol as a carbon source.

† Roman type face numerals are values for specific activities determined at 13 C; italicized numerals are values determined at 10 C.

‡ Abbreviations used: ND = not determined; IS = insignificant levels of tryptophanase as judged by failure of the strain to grow in medium containing indole as a source of tryptophan at the appropriate temperature (13 or 37 C) with 5-methyltryptophan added as the inducer when appropriate.

Mutants inducible for tryptophanase at 13 C. Selection of a mutant of *E. coli* strain C600-1 by method C has been reported previously (Ng and Gartner, 1963). Mutant C600-1T, now renamed GR-101, was studied in some detail; it shows the following properties not possessed by the parental strain, C600-1: partial constitutivity and hyperinducibility at 37 C, partial constitutivity and inducibility at low temperature.

At 37 C, the parental strain has very little tryptophanase activity when grown in the absence of inducer, whereas the mutant has about 10% of fully induced wild-type activity. When grown in the presence of inducer, the mutant has about three times the fully induced wild-type activity (Table 1). Kinetics of induction of the parental strain and the mutant at 37 C were compared (Fig. 1). Although maximal rate of tryptophanase synthesis is achieved in the wild type soon after addition of inducer, the maximal rate is achieved

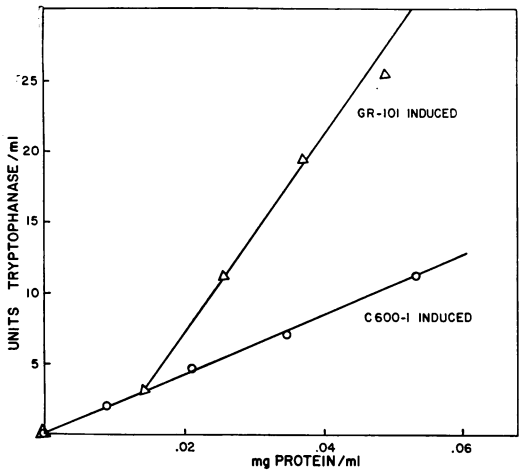

FIG. 1. *Kinetics of induction of strain C600-1 and its derivative, strain GR-101. Cultures growing exponentially at 37 C in minimal medium were induced, when they achieved a protein concentration of about 20 μg/ml, by the addition of a small volume of tryptophan to give a final concentration of 500 μg/ml in the culture. Samples of the culture were removed at intervals to determine the increase in cellular protein and increase in tryptophanase per milliliter. Symbols: △ = GR-101, induced; ○ = C600-1, induced.*

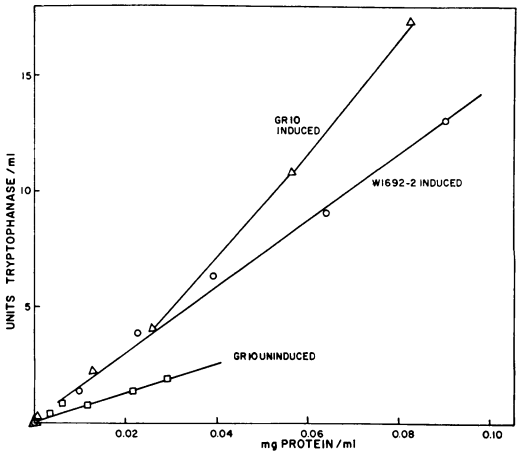

FIG. 2. *Kinetics of induction of strain GR-10, derivative of strain W1692-2. Strains W1692-2 and GR-10 were grown at 37 C on minimal medium supplemented with 20 μg/ml of histidine. The W1692-2 culture was further supplemented with 500 μg/ml of tryptophan and was therefore fully induced. The GR-10 culture was supplemented with 100 μg/ml of indole. When the cultures reached a concentration of about 20 μg/ml of protein, the GR-10 culture was divided in two. One sample was induced by the addition of tryptophan to a final concentration of 500 μg/ml. The other sample was not induced. Immediately, samples were removed from the three cultures at intervals to determine the increase in cellular protein and in tryptophanase activity per milliliter. Symbols: △ = GR-10, induced; □ = GR-10, uninduced; ○ = W1692-2, induced.*

by the mutant only after a lag, as if synthesis occurred at the wild-type rate for a period after inducer had been added and then increased only after an additional condition had been attained.

At a low temperature (10 C), the parental strain has virtually no tryptophanase activity when grown without inducer, and only low activity when grown with inducer. The mutant, however, does have tryptophanase activity when grown without inducer, and achieves an activity when grown with inducer that is about equal to the activity of the wild type induced at 37 C (Table 1).

Note that the parental strain C600-1 forms some tryptophanase at 13 C, but much less at 10 C, whereas the parental strain W1692 forms very little tryptophanase at 13 C. This difference may be related to differences in the growth rates of these two strains at low temperature. The specific low temperature at which growth ceases to be proportional to temperature (lower limit of the range of constant μ; Ng, Ingraham, and Marr, 1962) varies from strain to strain (Ng, 1963).

TABLE 2. *Selection of mutants inducible for tryptophanase at 13 C*

Isolation conditions*				Mutant designation	Phenotype		
Medium	C source	Tryptophan source	Inducer		Inducible at 13 C	Constitutive at 37 C	Hyper-inducible at 37 C
Liquid	Tryptophan	Tryptophan	Tryptophan	GR-101	+	+	+
Solid	Tryptophan	Tryptophan	Tryptophan	GR-11	+	+	ND†
Liquid	Glycerol	Indole	5-MT	GR-10	+	+	+
Solid	Glycerol	Indole	5-MT	GR-12	+	−	ND

* All cultures were incubated at 13 C.
† Not determined.

The growth rates of strains C600 and W1692 have been measured as a function of temperature. For strain C600, 13 C was found to fall within the range of constant μ, whereas 10 C is below it; for strain W1692, 13 C is below the range of constant μ. Therefore, failure of wild type to produce tryptophanase at a low temperature does not appear to be a function of the absolute temperature, but rather appears to be a function of the physiology of bacteria growing at temperatures below the constant μ range.

Mutants inducible at low temperature were isolated from the tryptophan synthetase deletion strain W1692-2 by both methods C and D (Tables 1 and 2). Kinetics of induction of mutant GR-10 are similar to those observed for mutant GR-101 (Fig. 2).

Initial observations suggested that mutation to 13 C inducibility was frequently accompanied by mutation to 37 C constitutivity. For this reason, selection for mutants having both properties was used for convenience. To determine whether these changes always occur together, a selection was carried out on solid instead of in liquid medium; 10 of the resulting colonies, called GR-12 to GR-21, were picked and purified. All grew at 13 C in the presence of inducer, but none grew at either 13 or 37 C in the same medium lacking inducer. Therefore, mutation to inducibility at 13 C can occur independently of mutation to constitutivity at either 13 or 37 C.

It seemed possible that the gain in ability to synthesize active tryptophanase at 13 C exhibited by the mutant GR-101, inducible at 13 C, was due to a gain in the stability of the enzyme

TABLE 3. *Tryptophanase activity of mutant and wild type at 13 and 37 C**

Strain	Specific activity of tryptophanase (units/mg of protein)		Specific activity at 37C / Specific activity at 13 C
	At 13 C	At 37 C	
GR-101	92	690	7.5
C600-1	28	224	8.0

* Cultures of strains C600-1 and GR-101 were grown at 37 C in minimal medium containing 500 μg/ml of tryptophan. Samples were filtered, washed, and resuspended in glutathione-phosphate buffer at 4 C and treated with toluene. Duplicate 0.1-ml samples at appropriate cell densities were placed at 13 and 37 C. After temperature equilibration, tryptophan and pyridoxal phosphate were added. After 30 min, the reaction was terminated and the amount of indole produced was determined by the addition of dimethylcinnamaldehyde.

TABLE 4. *Selection of mutants constitutive for tryptophanase at 13 C*

Medium	Parental strain	Mutant designation	Phenotype*		
			13 C con	37 C con	37 C hyper
Liquid	W1692	None obtained			
Solid	W1692	None obtained			
Liquid	GR-10	GR-27	+	+	+
		GR-28	+	+	+
Solid	GR-10	GR-29-31	+	+	ND
Liquid	GR-32	GR-33	+	ND	ND
Solid	GR-32	GR-36	+	ND	ND

* Abbreviations: con = constitituve; hyper = hyperinducible; ND = not determined.

at this temperature. To test this hypothesis, stability of the enzyme in toluene-treated suspensions of mutant GR-101 and the parental strain C600-1 was compared at both 13 and 37 C (Table 3). The ratio of the activities at the two temperatures is about the same for the mutant as for the wild type, indicating that the mutation to inducibility at 13 C does not involve synthesis of an altered tryptophanase which has greater stability at 13 C.

Mutants constitutive at 13 C. Efforts to isolate mutants constitutive for tryptophanase at 13 C from the tryptophan synthetaseless strain W1692-2 by method E were not successful.

However, isolation of bacteria constitutive at 13 C proved possible when selection was attempted not from the parental W1692-2 but from mutant derivatives of the strain which were constitutive at 37 C. When either the mutant strain GR-10, constitutive at 37 C and inducible at 13 C, or mutant GR-32, constitutive at 37 C but not inducible at 13 C, was used as a parental strain, mutants constitutive at 13 C were isolated (Table 4).

DISCUSSION

Mutants altered with respect to their ability to produce tryptophanase have been isolated by a variety of selective techniques. The method used to isolate constitutive mutants on solid medium was based on the work of Newton and Snell (1962, 1964). Tryptophan synthetaseless bacteria can convert indole and serine into the tryptophan needed for growth via tryptophanase. Since indole is not an inducer of tryptophanase, only bacteria forming this enzyme constitutively are able to grow on medium in which indole is supplied as the source of tryptophan.

The mutants isolated have a variety of phenotypes with respect to tryptophanase production: inducibility or partial or complete constitutivity at low temperature (10 or 13 C), partial or complete constitutivity or hyperinducibility at 37 C. Mutation to constitutivity at low temperature seems to require two separate genetic alterations of the wild type. Mutation to both hyperinducibility and constitutivity at 37 C may result from a single mutational change.

These mutants are assumed to have suffered genetic changes in the system regulating tryptophanase production. Alternative explanations are possible, and two of these have been eliminated. Mutation from the phenotypic negativity of wild type at low temperature to inducibility at low temperature does not involve a change in the tryptophanase protein of mutant GR-101 which leads to increased activity at low temperature. Also, this mutational change probably does not involve the tryptophan permease, since high concentrations of tryptophan were present in media used to test for inducibility of wild type at low temperature, ensuring adequate internal concentrations of the inducer.

As a working hypothesis, then, these mutants are considered to be regulation mutants. Some of the mutants appear to differ from wild type in the sensitivity of some part of the tryptophanase regulation system to temperature change. A preliminary genetic analysis of certain of these mutants is described in the accompanying paper.

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