JOURNAL OF BACTERIOLOGY
Vol. 87, No. 5, pp. 1051–1055 May, 1964

TRYPTOPHAN- AND INDOLE-EXCRETING PROTOTROPHIC MUTANT OF *ESCHERICHIA COLI*[1]

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Received for publication 20 November 1963

ABSTRACT

LIM, P. G. (Massachusetts Institute of Technology, Cambridge), AND R. I. MATELES. Tryptophan- and indole-excreting prototrophic mutant of *Escherichia coli*. J. Bacteriol. **87**:1051–1055. 1964.—A mutant of *Escherichia coli* K-12, capable of excreting 350 mg of indole and 50 mg of tryptophan per liter when grown on minimal medium, was found to have a level of 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP) synthetase 60% higher than the parent, and to have a 10- to 15-fold elevation of the levels of enzymes in the tryptophan branch of the pathway for aromatic amino acid biosynthesis. Contrary to what previous investigators found in *E. coli* W, the presence of a tyrosine-repressible component of DAHP synthetase sensitive to end-product inhibition by tyrosine could not be demonstrated in either strain K-12 or the mutant. The mutant strain is an example of a microorganism which excretes biosynthetic end products solely because of genetic derepression, as opposed to most previously reported amino acid accumulators which require a combination of genetic and physiological manipulation to achieve derepression.

We previously reported (Lim and Mateles, 1963) the isolation of a prototrophic mutant of *Escherichia coli* capable of excreting millimolar amounts of indole and L-tryptophan when grown on a glucose (or glycerol)-salts minimal medium. Because all previously described bacterial mutants capable of excreting other amino acids in similar or greater concentration (as a result of de novo synthesis rather than conversion of an intermediate) are either vitamin- (Kimura, 1963; Shiio, Otsuka, and Katsuya, 1963; Veldkamp, van den Berg, and Zevenhuizen, 1963) or amino acid-dependent (Huang, 1961*a*, *b*, *c*; Nakayama, Kitada, and Kinoshita, 1961; Kinoshita, Nakayama, and Udaka, 1957; Abe, 1962; Huang, 1963), we thought that an investigation into the enzymatic basis for this excretion would be of interest.

MATERIALS AND METHODS

The parent culture from which the various mutants were obtained was a wild-strain *E. coli* K-12. Minimal medium M63 (Pardee, Jacob, and Monod, 1959) containing 0.2% glucose was used for growth and incubation; in some cases, glycerol replaced the glucose. Other additives were autoclaved or filtered through a Millipore filter separately and added aseptically to the minimal medium. All cultures were shaken on a rotary shaker at 37 C. Growth was estimated by turbidity measurements with a Klett-Summerson colorimeter equipped with a 640-mμ (red) filter.

Cell extracts were prepared by centrifuging an overnight culture (about 200 Klett units), washing once with 0.9% NaCl or 0.04 M KH_2PO_4 (pH 7.4), and resuspending in the buffer appropriate for the enzyme assay. All subsequent operations were carried out at 1 to 5 C. The cell suspension was sonically treated for 7 min in a Raytheon 10-kc oscillator, and was centrifuged at 120,000 $\times$ *g* for 30 min to remove cell debris. The resulting clear supernatant fluid was used directly for most enzyme assays, but was further fractionated with ammonium sulfate and dialyzed before being used for the assay of anthranilate ribonucleotide synthetase (anthranilate phosphoribosyltransferase).

Tryptophan synthetase and tryptophanase were assayed according to the procedure of Lester and Yanofsky (1961) on whole cells or cell-free extracts as indicated. The tryptophan-synthetase assay was modified in that tryptophan production was followed (Dickman and Crockett,

[1] Contribution No. 580 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge. Reported in part at the 145th meeting of the American Chemical Society, New York, N.Y., 8–13 September 1963.

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1956). Anthranilate ribonucleotide synthetase was assayed according to the procedure of Smith and Yanofsky (1962), which was modified in that the incubation mixture contained 0.044 rather than 0.22 μmoles of sodium anthranilate. The activities of 5-dehydroshikimic reductase and 5-dehydroquinase were measured by the procedure of Davis, Gilvarg, and Mitsuhashi (1955). Purified glutathione reductase (0.56 enzyme unit per assay) was substituted for the guinea pig liver extract in the dehydroshikimic reductase assay. The substrate for the dehydroquinase assay was the culture filtrate of *E. coli* 83-1. 3-Deoxy-D-*arabino*-heptulosonic acid-7-phosphate (DAHP) synthetase was assayed according to the procedure of Sprinson, Srinivasan, and Katagiri (1962). The protein content of cell suspensions and extracts was determined according to the method of Lowry et al. (1951), with crystalline bovine serum albumin as a standard.

The biochemicals used in this investigation were obtained from commercial sources and used directly, except that the anthranilic acid used for enzyme assay was twice recrystallized from the chemically pure product obtained from Distillation Products Industries, Rochester, N.Y.

The intracellular concentrations of phenylalanine and tyrosine were determined by boiling washed cells in distilled water for 10 min, centrifuging, and assaying the centrifugate for phenylalanine and tyrosine microbiologically (Mandelstam, 1958). The growth of *Leuconostoc mesenteroides* P-60 in Difco Phenylalanine or Tyrosine Assay Medium (measured turbidimetrically after 24 hr of growth) was used as an indication of the quantity of amino acid present (Steele et al., 1949).

RESULTS

A summary of the procedure used for the selection of the mutants, and of their tryptophan synthetase levels, is presented in Table 1. The purpose of the terminal selection step which resulted in the isolation of strain E971 was to obtain a tryptophanase-negative strain. Strain E971, however, proved to have substantial tryptophanase activity, although the tryptophan synthetase-tryptophanase ratio was greater than that of strain E9. When an overnight culture of strain E971 was centrifuged and resuspended in M63 medium containing 1% glycerol instead of glucose, approximately 350 mg of indole and 50 mg of tryptophan per liter accumulated during 9 hr. During this time, the culture density increased from about 200 to about 380 Klett units.

Table 2 presents the results of assays of some of the enzymes involved in the biosynthesis of the aromatic amino acids in strain E971 and in the parent strain K-12. The enzymes in the pathway after the branch point (anthranilate ribonucleotide synthetase and tryptophan synthetase) are constitutive in strain E971 and are elevated about

TABLE 1. *Criteria for the selection of mutants and the tryptophan synthetase levels of the mutants*

Parent organism	Mutagen	Criterion of selection	Mutant obtained	Tryptophan synthetase (units per 300 KU)*
K-12 wild type	—	—	—	1.1
K-12 wild type	Ultraviolet	Resistance to 5-methyltryptophan (10 mg/liter)	R7	4.9
R7	Ultraviolet	Resistance to anthranilic acid (300 mg/liter)	R7A9	5.0
R7A9	Ethyl methane sulfonate	Ability to feed a strict tryptophan auxotroph	E9	8.5
E9	Ethyl methane sulfonate	Inability to use tryptophan as sole nitrogen source	E971	10.8

* Assayed on whole cells; 1 unit of enzyme is defined as that amount which will catalyze the formation of 0.1 μmole of tryptophan in 30 min under the assay conditions employed. For purposes of comparison, the observed values of tryptophan production were converted to those expected for 1.0 ml of cell suspension having a turbidity of 300 Klett units (KU).

TABLE 2. *Levels of enzymes involved in the biosynthesis of tryptophan in strains K-12 and E971 grown under unrepressed and in repressed conditions**

Enzyme	In M63 (unrepressed)			In M63 + aromatics (repressed)†		
	K-12	E971	E971/K-12	K-12	E971	E971/K-12
Tryptophan synthetase	0.15	1.9	13	0.03	1.7	55
Anthranilate ribonucleotide synthetase‡	0.014	0.2	15	0.004	0.16	40
Dehydroshikimic reductase	1.0	1.1	0.9	0.84	0.72	0.86
Dehydroquinase	1.3	1.3	1	1.4	1.2	0.85
DAHP synthetase	2.2	3.7	1.6	1.1	1.3	1.2

* The units are μmoles/mg of protein (30 min).

† The "aromatics" consist of: L-phenylalanine, 200 mg per liter; L-tyrosine, 100 mg per liter; L-tryptophan, 50 mg per liter; *p*-aminobenzoic acid, 1 mg per liter; *p*-hydroxybenzoic acid, 1 mg per liter.

‡ Assayed on partially purified extracts.

10- to 15-fold, as compared to strain K-12. The first enzyme in the common pathway for the aromatic amino acids (DAHP synthetase) was elevated about 60% in strain E971. The levels of two other enzymes in the common pathway (5-dehydroshikimic reductase and 5-dehydroquinase) were unaffected by the mutations, and are probably not under the control of the gene regulating DAHP synthetase (Brown and Doy, 1963).

Smith et al. (1962) reported that the DAHP synthetase in derivatives of *E. coli* W was composed of several distinct proteins. One fraction was repressible by phenylalanine and subject to feedback inhibition by phenylalanine. Another fraction was repressible by tyrosine and subject to feedback inhibition by tyrosine. A third residual fraction may have existed but was not unequivocally demonstrated. Because of the possibility that the 60% increase in DAHP synthetase in E971 may have reflected an increase in the residual insensitive portion of the enzyme, assays of this enzyme were performed on strain E971 and the parent strain K-12. Table 3 reports the results of an experiment to determine the sensitivity to feedback inhibition of the enzyme produced when the cells were grown on minimal medium (no exogenous amino acids added). Both organisms were inhibited to about the same extent (Table 3). In addition, it was found that tyrosine exerted no inhibitory effect on the DAHP synthetase. Even when strain K-12 was grown on minimal medium supplemented with phenylalanine (conditions which should fully repress the phenylalanine-sensitive portion of the enzyme and lead to production of a tyrosine-sensitive portion), no portion sensitive to tyrosine inhibition could be demonstrated (Table 4). Because the tryptophan portion of the pathway in strain E971 is no longer under effective end-product control by tryptophan, the effect of tyrosine and phenylalanine inhibition could be demonstrated in growing cells by their effect on indole excretion. This effect necessarily reflects inhibition of the

TABLE 3. *Inhibition of DAHP synthetase by phenylalanine and tyrosine*

Additive during enzyme assay*	Concn	Specific activity (units per mg of protein)*		Per cent inhibition	
		K-12, w.t.	E971	K-12, w.t.	E971
	mM				
None	—	2.7	3.9	0	0
Phenylalanine + tyrosine	0.20	0.2	0.5	93	88
Phenylalanine + tyrosine	0.03	0.8	1.5	69	63
Phenylalanine	0.20	0.3	0.5	91	88
Phenylalanine	0.03	0.9	1.4	68	64
Tyrosine	0.20	2.5	4.0	6	0
Tyrosine	0.03	2.7	4.0	0	0

* A unit of enzyme is defined as that amount of enzyme which will form 1 μmole of DAHP in 30 min under the conditions of the assay. The abbreviation w.t. represents wild type.

TABLE 4. *Insensitivity to tyrosine inhibition of DAHP synthetase in phenylalanine-repressed cells of Escherichia coli K-12**

Additive during enzyme assay	Concn	Specific activity (units per mg of protein)	Per cent inhibition
	mM		
None		1.1 (2.8)	0 (0)†
Phenylalanine	0.20	0.2	82 (91)
Phenylalanine	0.03	0.6	51 (68)
Tyrosine	0.20	1.2	0 (6)
Tyrosine	0.03	1.2	0 (0)

* Cells were grown in minimal medium supplemented with 1 mM of L-phenylalanine.

† Figures in parentheses refer to the specific activity and per cent inhibition of extracts of cells grown without phenylalanine supplementation.

TABLE 5. *Inhibition of indole excretion during growth of strain E971*

Medium additive*	Indole excretion rate†
None	0.23
Tyrosine	0.26
Tyrosine + phenylalanine	<0.01
Phenylalanine	<0.01

* Cells were grown in M63 + glucose, centrifuged, and resuspended in M63 + 1% glycerol to which the additions were made. All of the additives had a concentration of 0.2 mM.

† Expressed as μmole/mg (dry weight) per hr.

TABLE 6. *Concentration of intracellular phenylalanine and tyrosine in strains E971 and K-12**

Amino acid	Strain E971	Strain K-12
Tyrosine	2.32	1.27
Phenylalanine	4.55	4.55

* Expressed as mμmoles/mg of dry cells.

DAHP synthetase in the common pathway and confirms the enzyme assays (Table 5).

The levels of intracellular tyrosine and phenylalanine in strains K-12 and E971 are shown in Table 6. On the assumption that 75% of the cell fluid is accessible to amino acids and that the dry weight is 25% of the wet weight (Roberts et al., 1957), the concentration of endogenous phenylalanine reported in Table 6 is approximately 2 mM.

DISCUSSION

Strain E971 appears to differ from the parent in two major ways. The enzymes in the tryptophan branch of the pathway are elevated in level and are not repressible, and the level of DAHP synthetase is about 60% higher than that of the parent.

Although the parallel derepression observed for two of the enzymes in the tryptophan branch of the pathway (Table 2) was reported to be the result of a single mutation (Cohen and Jacob, 1959), the evidence of Table 2 does not bear on this point because strain E971 is the product of several successive mutations.

Both Smith et al. (1962) and Brown and Doy (1963) reported that mutants derived from *E. coli* W were capable of synthesizing a tyrosine-repressible DAHP synthetase component sensitive to end-product inhibition by tyrosine. The evidence reported here indicates that strain K-12 and its derivative, strain E971, do not synthesize such a component when grown on minimal medium. Even when strain K-12 was stressed by growth in phenylalanine-supplemented medium, which would be expected to repress and inhibit the phenylalanine-sensitive portion of the DAHP synthetase, it was not possible to demonstrate a tyrosine-sensitive component. Whether this is because strain K-12 is incapable of synthesizing a tyrosine-sensitive component under any condition, or is fully repressed by the endogenous tyrosine, remains to be determined. This point could be resolved by the use of a tyrosine-dependent auxotroph of strain K-12.

An interesting observation was the high intracellular concentration of endogenous phenylalanine. Because maximal inhibition of the DAHP synthetase (about 90%) occurs at about 0.2 mM phenylalanine and the intracellular concentration is much higher, the implication is that the DAHP synthetase in the cells grown on minimal medium is functioning at only 10% of its capacity because of this inhibition. It is entirely possible, however, that this implication is erroneous and that the enzyme is not actually exposed to such a high concentration of free phenylalanine because the phenylalanine is in some way "bound" and is released by the extraction procedure used.

It is clear that strain E971 is an example of a

prototrophic mutant which has been genetically modified so as to over-produce a biosynthetic end product or its immediate degradation product. This is in contrast to the many vitamin- or amino acid-dependent amino acid-accumulating bacteria in which the derepression is obtained by a combination of genetic and physiological manipulation. A possible advantage of using prototrophic mutants for amino acid production is that it is not necessary to limit growth (to achieve derepression), and higher cell densities of specific activity equal to that of growth and factor-limited auxotrophic cells may be obtained.

ACKNOWLEDGMENTS

We would like to thank Boris Magasanik and Louis C. Smith of the Biology Department, Massachusetts Institute of Technology, for many useful suggestions. This investigation was supported in part by a grant from the Nutrition Foundation.

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