JOURNAL OF BACTERIOLOGY, Feb. 1971, p. 527-537
Vol. 105, No. 2

Printed in U.S.A.

Isoleucine Auxotrophy as a Consequence of a Mutationally Altered Isoleucyl-Transfer Ribonucleic Acid Synthetase

MAURIZIO IACCARINO AND PAUL BERG

International Laboratory of Genetics and Biophysics, Via Marconi 10, 80125 Naples, Italy, and Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Received for publication 10 August 1970

Among mutants which require isoleucine, but not valine, for growth, we have found two distinguishable classes. One is defective in the biosynthetic enzyme threonine deaminase (L-threonine hydro-lyase, deaminating, EC 4.2.1.16) and the other has an altered isoleucyl transfer ribonucleic acid (tRNA) synthetase [L-isoleucine: soluble RNA ligase (adenosine monophosphate), EC 6.1.1.5]. The mutation which affects *ileS*, the structural gene for isoleucyl-tRNA synthetase, is located between *thr* and *pyrA* at 0 min on the map of the *Escherichia coli* chromosome. This mutationally altered isoleucyl-tRNA synthetase has an apparent K_m for isoleucine ($\sim$1 mM) 300-fold higher than that of the enzyme from wild type; on the other hand, the apparent V_{max} is altered only slightly. When the mutationally altered *ileS* allele was introduced into a strain which overproduces isoleucine, the resulting strain could grow without addition of isoleucine. We conclude that the normal intracellular isoleucine level is not high enough to allow efficient charging to $tRNA^{Ile}$ by the mutant enzyme because of the K_m defect. A consequence of the alteration in isoleucyl-tRNA synthetase was a fourfold derepression of the enzymes responsible for isoleucine biosynthesis. Thus, a functional isoleucyl-tRNA synthetase is needed for isoleucine to act as a regulator of its own biosynthesis.

Isoleucyl-transfer ribonucleic acid (tRNA) synthetase (IRS) has provided a useful model for the study of the structure (1-3, 21) and function (2, 3, 31, 48, 49; *see also* 26) of an aminoacyl-tRNA synthetase. All of this work was carried out with purified enzyme isolated from wild-type strains of *Escherichia coli*. In this investigation, we set out to isolate mutants of *E. coli* with a defective IRS so as to (i) provide proteins with defined modifications which could be used to expand the scope of our structure-function studies and (ii) explore the consequences of such mutations on the regulatory mechanism for isoleucine biosynthesis.

When we began, it was known (9, 47) that mutations affecting the function of valyl-tRNA synthetase [L-valine: soluble RNA ligase (adenosine monophosphate, AMP), EC 6.1.1.9] cause derepression of the enzymes synthesizing isoleucine and valine; since then, Blatt and Umbarger (Bacteriol. Proc., p. 136, 1970) have isolated *Salmonella typhimurium* strains which appear to have an altered IRS. Such strains are partially derepressed for the isoleucine biosynthetic enzymes. In addition, a mutant of *E. coli* K-12 has been described (40) which has an altered IRS, although it appears that in this strain the phenotype is due to two different mutations (Coker and Umbarger, Bacteriol. Proc., p. 135, 1970).

To select for mutants with a defective IRS, we adopted the same strategy Folk and Berg (10) used for isolating glycyl-tRNA synthetase [glycine: soluble RNA ligase (AMP), EC 6.1.1.e] mutants; that is, we screened isoleucine auxotrophs for those with defective IRS. The premise is that mutants in which the K_m of IRS for isoleucine has been increased to the point where charging of $tRNA^{Ile}$ becomes limiting at the normal endogenous levels of isoleucine will grow only in medium supplemented with isoleucine. Because most of the enzymes required for isoleucine biosynthesis are also required for the synthesis of valine (*see* 45), only mutations affecting threonine deaminase or IRS should cause a re-

quirement solely for isoleucine. This expectation was realized, and in this paper we describe the properties of such mutants.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains used and Fig. 1 indicates the map order of the relevant markers.

PB154 is a derivative of *E. coli* K-12 W3110 described by Hill et al. (18). AT739 and AT2459 were kindly provided by A. L. Taylor. AB1206 (F'14 donor) was described by Ramakrishnan and Adelberg (33). JF281 is a derivative of PB154 obtained from John Foulds. KLF4 (F'4 donor) is a *recA* merodiploid obtained from John Foulds and described by Low (27).

MI153 was prepared in the following way: a spontaneous *thy* mutant of MI1 was isolated after growth in minimal medium containing 20 μg of trimethoprim per ml and 200 μg of thymidine per ml, followed by growth in minimal medium containing 20 μg of trimethoprim per ml and 20 μg of thymidine per ml (38). (Trimethoprim was a gift from G. Hitchings, Burroughs Wellcome & Co., White Plains, N.Y.) A *recA56* mutation was introduced in this strain by mating with JC5088 (kindly supplied by John Clark). More than 40% of the isolated *thy*$^+$ recombinants contained the *recA56* mutation, as indicated by their increased sensitivity to ultraviolet (UV) light. One *recA56* derivative of MI1 was purified and mated with KLF4; selection was made for Ile$^+$, thus obtaining a stable merodiploid with both an *ileS*$^-$ and an *ileS*$^+$ allele. Presence of the episome in this strain is shown by its ability to donate it to a *leu*$^-$ strain by mating.

MI154 and MI158 were obtained by transducing AT739 to Thr$^+$ with P1 grown on MI1. Among the *thr*$^+$ transductants (which we purified on plates containing isoleucine), one was Ile$^+$ (MI158) and had normal IRS; the other was Ile (MI154) and had 3% IRS activity in the in vitro assay as compared with MI158.

MI159 is a *ilv* auxotroph prepared by nitrosoguanidine mutagenesis and penicillin selection from AT739. The mutation is probably not in *ilvA* or *ilvB*, because threonine deaminase and acetolactate synthetase activities of this strain appear to be normal; *ilvE* is not likely because a mutation in this gene causes a requirement for isoleucine, valine, and leucine, whereas MI159 is an isoleucine and valine auxotroph. MI160 and MI161 were obtained by transducing *thr*$^+$ into MI159 with P1 grown on MI1. Among the purified *thr*+ transductants, some were *pyrA*$^+$ and others were *pyrA*$^-$; among those that were *pyrA*$^-$, there were some isolates which grew slowly on nutrient broth plates—a behavior characteristic of MI1. Two *thr*+ *pyrA*− isolates were chosen: one (MI160) grew at the normal rate (similar to MI159) on a nutrient broth plate and had normal IRS activity; the other (MI161) grew more slowly than MI160 on the nutrient broth plate (but normally on minimal plates supplemented with isoleucine) and had reduced IRS activity in the in vitro assay (3%) as compared with MI160.

AW206 is a valine-resistant mutant isolated by Alina Wiater from AT739. The isolation procedure was that reported by Glover (15), and after purification the mutants were checked for the level of valine resistance on plates as described by Glover. Since AW206 grows on a medium supplemented with 5 mg of valine/ml, the valine-resistant mutation is probably not linked to *thr* or *leu* (15); it will be shown below that it is closely linked to *ilv*.

Reagents and media. ^{14}C-L-isoleucine was purchased from New England Nuclear Corp.; D,L-isoleucine (50% L-isoleucine plus 50% D-alloisoleucine), from General Biochemicals, Chagrin Falls, Ohio; L-isoleucine, from Nutritional Biochemicals Corp. or Sigma Chemical Co.; *E. coli* B tRNA, from Schwarz BioResearch Inc.; and acetoin (3-hydroxy-2-butanone), from Aldrich Chemical Co., Inc.

The minimal medium was that described by Vogel and Bonner (46). Usual supplements, when required, were 0.4% glucose, 25 μg of L-tryptophan per ml, 100 μg of L-arginine per ml, 50 μg of other L-amino acids

TABLE 1. *Bacterial strains*[a]

Strain no.	*ileS*	*ilv*	Other markers
PB154	+	+	*argH, trpA36*, F$^-$
MI1	*ileS1*	+	*argH, trpA36*, F$^-$
AT739	+	+	*thr-10, pyrA53, thi-1*, (λ)$^-$, Hfr-H
MI154	*ileS1*	+	*pyrA53, thi-1*, (λ)$^-$, Hfr-H; Thr$^+$ transductant of AT739
MI158	+	+	*pyrA53, thi-1*, (λ)$^-$, Hfr-H; Thr$^+$ transductant of AT739
MI159	+	−	*thr-10, pyrA53, thi-1*, (λ)$^-$, Hfr-H; from At739 by nitrosoguanidine
MI160	+	−	*pyrA53, thi-1*, (λ)$^-$, Hfr-H; Thr$^+$ transductant of MI159
MI161	*ileS1*	−	*pyrA53, thi-1*, (λ)$^-$, Hfr-H; Thr$^+$ transductant of MI159
AW206	+	+	*thr-10, pyrA53, thi-1*, (λ)$^-$, Hfr-H, valine-resistant (*ilv0* ?); spontaneous from AT739
AT2459	+	+	*serB22, thi-1*, (λ)$^-$, F$^+$
AB1206	+	+	*his, pro, thi, str^r*, F'14
JF281	+	−	*argH, trpA36, his*, F$^-$
KLF4	+	+	*thr, leu, thi, recA13, pro, arg, his, str^r*, F'4, thr$^+$ leu$^+$ pro$^+$
Hfr-H	+	+	*metB, trpA36, str^s, tsx^s*
Hfr-C	+	+	*metB, trpA36, RC^{rel}*
MI153	*ileS1*	+	*argH, trpA36, recA56*, F'4 *ileS*$^+$
JC5088	+	−	*thr, thi, str^s, recA56*, Hfr (KL16 origin)

[a] The symbols for genetic markers are those used by Taylor and Trotter (42); *ileS* is the gene coding for isoleucyl-tRNA synthetase.

per ml, 50 μg of nucleosides per ml, and 10 μg of thiamine per ml.

L-broth was described by Lennox (25).

Isolation of mutants. Isoleucine-requiring mutants (*ile*⁻) were isolated by UV mutagenesis and penicillin selection (16). The isolation was carried out at 30 C so as not to lose temperature-sensitive mutants. PB154 was grown in minimal medium supplemented with 0.2% glucose, 100 μg of arginine per ml, and 10 μg of tryptophan per ml; this suspension (3×10^8 cells/ml) was irradiated with UV light to yield 8×10^4 survivors per ml. It was then diluted 25-fold in minimal medium supplemented with 0.2% glucose, 100 μg of arginine per ml, 10 μg of tryptophan per ml, 5 mg of D,L-isoleucine per ml, 100 μg of valine per ml, and 100 μg of proline per ml; this suspension was incubated in a rotary shaker at 30 C until full growth. The cells were then washed and resuspended at a concentration of 10^8 cells/ml in minimal medium supplemented with 0.2% glucose, 100 μg of arginine per ml, and 10 μg of tryptophan per ml, and were incubated at 37 C. When a doubling (A_{590}) was obtained, 2,000 units of penicillin per ml was added, and, after 200 min of incubation at 37 C, the cells were washed and spread on plates containing tryptone supplemented with yeast extract and 5 mg of D,L-isoleucine per ml. The plates were incubated at 30 C, and the colonies that appeared were picked with toothpicks and stabbed into appropriately supplemented minimal plates to detect isoleucine-requiring (*ile*⁻), isoleucine- and valine-requiring (*ilv*⁻), and proline-requiring (*pro*⁻) mutants. Among 180 colonies, 5% were Ile⁻, none was Ilv⁻, and 25% were Pro⁻. Since in a preliminary experiment we learned that some of the Ile⁻ strains had low IRS activity, this class was concentrated upon and a large number of Ile− colonies were purified twice by single-colony isolation.

Transduction. Transductions were performed with P1kc prepared by the confluent lysis technique, by use of a modification of the procedure of Lennox (25) as described by Hill et al. (18).

Preparation of cell extracts. Bacteria grown under the desired conditions were harvested by centrifugation at 4 C, washed once with 0.15 M NaCl, and then frozen as a pellet at −20 C.

To 1 g of frozen cell pellet, 5 ml of the appropriate extraction solution was added, together with acid-washed glass beads; the suspension was mixed on a Vortex mixer. The suspension was treated for 2 min with a Mullard or MSE sonic oscillator, or for 15 sec with a Branson Sonifier, and then was centrifuged for 20 min at 15,000 rev/min in a Sorvall SS-34 rotor. The extracts contained between 15 and 20 mg of protein per ml. Proteins were determined by the method of Lowry et al. (28) or by the method of Groves (17), with crystalline bovine plasma albumin as a standard. These two methods gave equal values.

Different extraction solutions were used; they are described below under Enzyme assays.

Enzyme assays. All reaction mixtures were incubated with different amounts of extract to establish that the amount of product found was proportional to the amount of extract added.

IRS activity was assayed by measuring the rate of formation of ^{14}C-ile-tRNA according to the procedure of Calendar and Berg (5). The extraction solution was

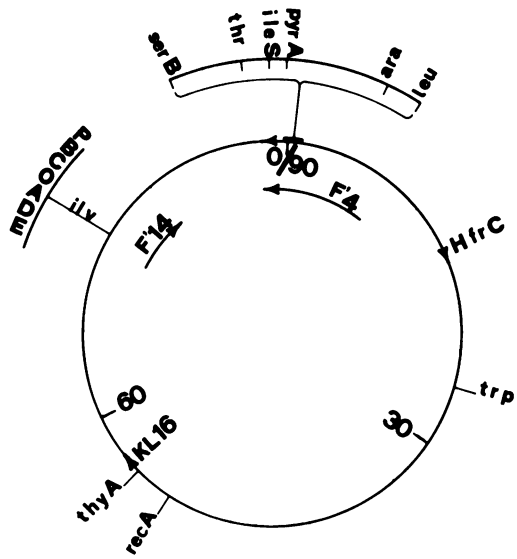

FIG. 1. *Order of markers relevant to this work (42).*

100 mM potassium phosphate (*p*H 7.0), containing 10 mM mercaptoethanol. The reaction mixture (0.5 ml) contained 100 mM sodium cacodylate (*p*H 7.0), 1 mM adenosine triphosphate (ATP), 10 mM $MgCl_2$, 10 mM KCl, 4 mM reduced glutathione, 200 μg of bovine plasma albumin per ml, 0.2 mM ^{14}C-L-isoleucine (10,000 counts per min per nmole), and 25 to 30 absorbance units of tRNA (at 260 nm). The IRS activity is expressed as nanomoles of ile-tRNA formed per 10 min per milligram of protein at 37 C.

Threonine deaminase was assayed by measuring the rate of α-ketobutyrate formation according to the "direct procedure" of Friedemann and Haugen (14) as described by E. A. Adelberg (*personal communication*). The extraction solution was 50 mM potassium phosphate (*p*H 7.4) containing 0.1 mM L-isoleucine, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM dithiothreitol (4). Threonine deaminase activity in this extraction solution is higher than 85% of the initial value after 5 hr at 0 C; usually the activity was measured within 0.5 hr after sonic treatment. The reaction mixture (1 ml) contained 0.1 mM pyridoxal-5-phosphate, 20 mM NH_4Cl, 100 mM tris(hydroxymethyl)aminomethane (Tris), *p*H 8.0, 0.5 mM dithiothreitol, and 20 mM L-threonine (omitted in the control tubes). This mixture was incubated with appropriate amounts of extract for 10 min at 37 C, and the reaction was terminated by the addition of 1 ml of dinitrophenylhydrazine (0.1% in 2 N HCl). After 15 min at room temperature, 4 ml of 1.25 N NaOH was added, and the A_{520} was measured after 10 more min at room temperature. Sodium pyruvate was used as a standard. The activity is expressed as nanomoles of keto acid formed per minute per milligram of protein.

Acetolactate synthetase (condensing enzyme) activity was assayed by determining the rate of acetolactate formation according to Størmer and Umbarger (39). The extraction solution was 100 mM potassium phos-

phate, *p*H 8.0. The activity is expressed as nanomoles of acetoin formed per minute per milligram of protein.

Transaminase B (L-isoleucine: 2-oxoglutarate aminotransferase, EC 2.6.1.e) activity was assayed by determining the rate of α-ketoisovalerate formed (Adelberg, *personal communication*). The extraction solution was 50 mM Tris, *p*H 7.8; the reaction mixture (1 ml) contained 0.1 mM pyridoxal-5-phosphate, 200 mM Tris, *p*H 7.8, 25 mM neutralized α-ketoglutaric acid, and 50 mM L-valine (omitted in the control tubes). This mixture was incubated with appropriate amounts of extract for 15 min at 37 C, and the reaction was terminated by adding 2.5 ml of dinitrophenylhydrazine (0.3% in 2 N HCl) and 2 ml of toluene. The tubes were then mixed for a few min on a Vortex mixer and centrifuged; 1 ml of the toluene layer was added to 5 ml of 10% Na_2CO_3. The toluene was extracted by mixing on a vortex and, after centrifugation, 3 ml of the Na_2CO_3 layer was mixed with 3 ml of 1.5 N NaOH. After 10 min at room temperature, the A_{520} was measured. Sodium pyruvate was used as a standard. The activity is expressed as nanomoles of keto acid formed per minute per milligram of protein. Although we obtained a straight line by plotting the concentration of added sodium pyruvate versus A_{520}, this is not the case when the amount of extract is plotted versus the amount of product formed; however, the points fall on a straight line if the square of extract concentration is used, as shown in Fig. 2. When points were averaged by means of this plot, the values we obtained for specific activity were not very different from those found in the literature (see, for example, 8 or 34), and were reproducible for a given strain. Since different strains can be compared in this way (see Table 4), we did not investigate this point further.

RESULTS

Genetic characterization of Ile⁻ mutants. Thirty Ile⁻ colonies (which will be called MI1 to MI30) were purified, and some of their characteristics were analyzed. All grew on minimal medium supplemented with isoleucine, and all grew at 37 and 42 C; no growth was observed in the absence of isoleucine. The isoleucine requirement of the 30 strains considered might be due to a mutation in different genes. To find out how many genes had been affected to account for the Ile⁻ phenotype, transduction from one Ile⁻ into another and selection for Ile⁺ recombinants were carried out: if donor and recipient strains are mutant in the same gene, the frequency of Ile⁺ transductants should be extremely low; if, however, donor and recipient strains are altered in different genes, there should be a normal frequency of transductants (200 to 500 under the conditions used). When a P1kc phage lysate prepared on MI1 was used to transduce all other strains, Ile⁺ transductants were obtained only with MI2 and MI5; this indicates that MI1, MI3, MI4, and MI6 to MI30 are mutated in one gene, and MI2 and MI5 are mutated in another gene(s). Another P1 lysate was prepared on MI2 and used to transduce the other strains. Ile⁺ transductants were obtained with all strains except MI5. We conclude that there are two genetically different isoleucine-requiring strains in our collection; MI2 and MI5 form one class and all of the others are in another.

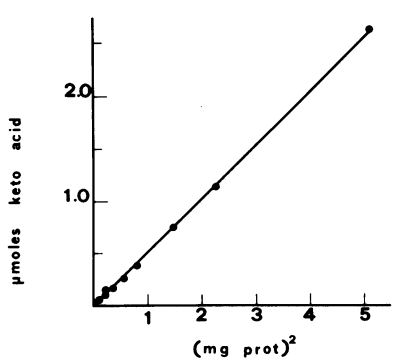

FIG. 2. *Transaminase B activity as a function of protein concentration; on the abscissa is plotted the square of the amount of protein used in each assay tube (see Materials and Methods).*

Among the cluster of *ilv* genes at 74 min on the map of *E. coli* (42), only a mutation in *ilvA*, the gene for threonine deaminase, can cause a requirement for isoleucine alone; mutations in other genes of the *ilv* cluster cause a mutational requirement for isoleucine and valine and in one case (*ilvE*) for isoleucine, valine, and leucine. To determine whether our Ile⁻ isolates were mutant in *ilvA*, MI1 and MI2 were mated with the F'14 donor AB1206, and Arg⁺ recombinants were selected as an indication of episome transfer. Since AB1206 transfers the chromosome at a barely detectable rate (less than 0.001% for any marker; 32), if the mutation responsible for the Ile⁻ phenotype maps in the region covered by the episome most of the Arg⁺ should become Ile⁺; if the mutation causing Ile⁻ maps outside the episome region, the Arg⁺ recombinants should remain Ile⁻. After mating, among 48 Arg⁺ colonies which were checked for their isoleucine requirement, 94% were also Ile⁺ when MI2 was the recipient whereas none was Ile⁺ when MI1 was the recipient. We conclude, therefore, that the MI2 mutation maps in the region covered by F'14, whereas the MI1 mutation lies outside this segment.

To determine whether the MI2 mutation was within the *ilv* cluster, we transduced an *ilv* strain (JF281) with lysates of MI1 and MI2, and Ilv⁺ transductants were selected on plates containing isoleucine but no valine. All (47 of 47) of the valine-independent transductants were also isoleucine-independent; that is, they were Ilv⁺ and Ile⁺ when the P1 was prepared on MI1; however, with

MI2 as donor none of the valine-independent transducants (0 of 48) was also isoleucine-independent. From these data, together with in vitro assays of threonine deaminase (see below), we conclude that MI2 is a mutant in the *ilvA* gene.

From the frequency of Ile$^+$ and Ilv$^+$ recombinants obtained during uninterrupted matings of Hfr-H and Hfr-C with both MI1 and MI2, we could tentatively assign the MI1 mutation to the region between 88 and 15 min (passing through zero), or around 50 min (see Fig. 1). Since matings of MI1 with strain KLF4, which contains an episome that covers the region between 88 and 8 min, yields Ile$^+$ merodiploids, we conclude that the MI1 mutation is within this segment.

In Table 2 are shown the results of a transduction analysis in which the contransduction frequency of the Ile$^-$ character with known markers in this segment of the chromosome was measured. The data indicate that the mutation causing the Ile$^-$ phenotype (*ileS*) is closely linked to *pyrA* and is between *pyrA* and *thr* at about 0 min on the map of *E. coli* (42).

Enzyme defect in the Ile$^-$ isolates. Extracts prepared from MI2 and MI5 contained less than 2% of the threonine deaminase activity of the parental cells, PB154. Two Ile$^+$ transductants each of MI2 and MI5 (obtained as described above) contained normal levels of threonine deaminase activity. Thus, one class of Ile$^-$ mutants has an altered *ilvA* gene and a defective threonine deaminase; its requirement for isoleucine but not for valine is what is expected from the biosynthetic pathway for these branched-chain amino acids (34).

Extracts prepared from MI1 and MI3 contained 3 and 6%, respectively, of the IRS activity of the parental cells, PB154; other isolates of this genetic class had levels of IRS activity ranging between 1.5 and 10% of wild-type activity. Assays with a mixture of extracts from MI1 and PB154 gave additive values, indicating that the low activity was not due to an inhibitor in MI1 extracts. Moreover, MI153, a stable merodiploid of MI1, which carries an episome with the wild-type allele of *ileS*, has normal activity, also demonstrating the lack of any inhibitor or cellular inactivator of IRS. Three different Ile$^+$ transductants of MI1 and three of MI3 had normal IRS activities. In several instances when Ile$^-$ transductants were recovered from Ile$^+$ recipients (see, for example, the preparation of MI154), these had the low level of IRS activity. It is apparent, therefore, that in the second class of Ile$^-$ mutants the isoleucine requirement for growth results from a mutation which affects the enzyme activity of IRS. Since isoleucine is the substrate and not the product of IRS action, we assume that the defective IRS has an altered affinity for the amino acid substrate.

This point was studied by measuring the IRS activity as a function of isoleucine concentration to determine whether the mutation affected the K_m or V_{max} (Fig. 3). The apparent V_{max} of the wild type and MI1 was found to be 8.1 and 1.8 units/mg of protein, respectively, and the apparent K_m was found to be 0.003 and ~1 mM, respectively. Thus, the main alteration in the mutant enzyme probably affects the affinity for isoleucine, which is decreased ~300-fold, whereas the turnover number at infinite isoleucine concentration is reduced 4-fold. We cannot exclude, however, the possibility that this small difference is due to a difference in the extractability or stability of the mutant enzyme. Extracts of the strain MI3 had only a slightly reduced apparent V_{max}, 4.34 units/mg, but the K_m was also strikingly increased to about 1 mM.

Characterization of the isoleucine requirement of MI1. In minimal medium supplemented with 50 μg of isoleucine/ml, the two classes of mutants, as exemplified by MI1 and MI2, have essentially the same growth rate as the wild-type

TABLE 2. *Frequency of cotransduction of the isoleucine requirement of MI1 with other markers*[a]

Recipient strain	Marker selected	Frequency of the unselected Ile$^-$ phenotype (%)
AT2459	*serB*$^+$	17 (16/95)
AT739	*thr*$^+$	44 (41/93)
AT739	*pyrA*$^+$	68 (56/82)
AT739	*thr*$^+$ + *pyrA*$^+$	90 (67/74)

[a] Phage P1 grown on MI1 was used. The cotransduction frequency of *ileS* with *ara* is about 2% (S. Lerner, *personal communication*).

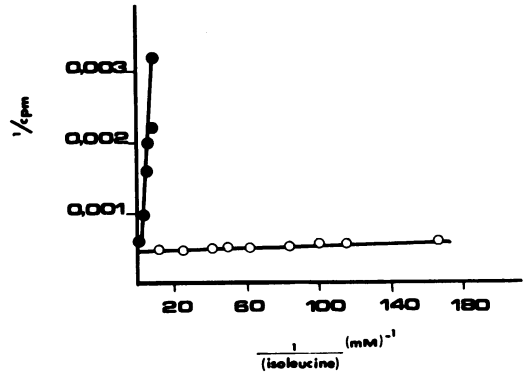

FIG. 3. *Plot of 1/(counts per minute) as a function of 1/S for PB154* (○) *and MI1* (●) *extracts.* K_m *and* V_{max} *are given in the text.*

strain PB154. Figure 4 compares the rate and extent of growth when MI1 and MI2 were cultured in a minimal medium containing 4 μg of isoleucine per ml. MI2 grew at the wild-type growth rate and then growth ceased rather abruptly when the isoleucine in the medium was exhausted; MI1 reproducibly grew somewhat slower at this isoleucine concentration, but growth did not stop sharply and continued slowly (generation time greater than 600 min). The increase in turbidity probably represents an increase in cell number because, when 10^7 cells were seeded onto minimal agar plates lacking isoleucine, a background growth was readily detectable.

Since MI1 contains all of the enzymes needed to synthesize isoleucine (because it carries a wild-type *ilvA* gene), its inability to grow in the absence of added isoleucine suggests that it is the intracellular level of isoleucine which is limiting growth. Inasmuch as the biosynthesis of isoleucine is regulated via negative feedback control (6, 12) by the isoleucine concentration itself, the intracellular level of isoleucine cannot rise appreciably. Thus, if MI1 requires higher concentrations of isoleucine for growth than does the wild type, the only way this requirement can be satisfied is by adding isoleucine to the medium.

Supporting this view is the effect of valine on the growth rate of MI1 (Fig. 5 C). In K-12 strains of *E. coli*, growth is inhibited by valine, and isoleucine reverses this inhibition (41). Figure 5A shows that the addition of increasing quantities of valine to the wild type, growing in the presence of isoleucine, caused a measurable decrease of the growth rate, and that about the same degree of inhibition was obtained with MI2, the biosynthetic mutant (see Fig. 5). However, MI1 was considerably more sensitive to the addition of valine; Fig. 5B shows that the lowest valine concentration tested increased the generation time nearly threefold with MI1 but had no detectable effect on the wild type or the MI2 mutant. This effect of valine probably results from the inhibi-

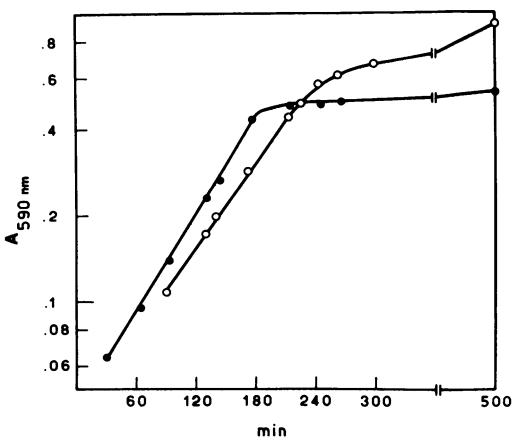

FIG. 4. *Growth curve of MI1* (○) *and MI2* (●) *in minimal medium supplemented with arginine, tryptophan, and 4 μg of* L*-isoleucine per ml.*

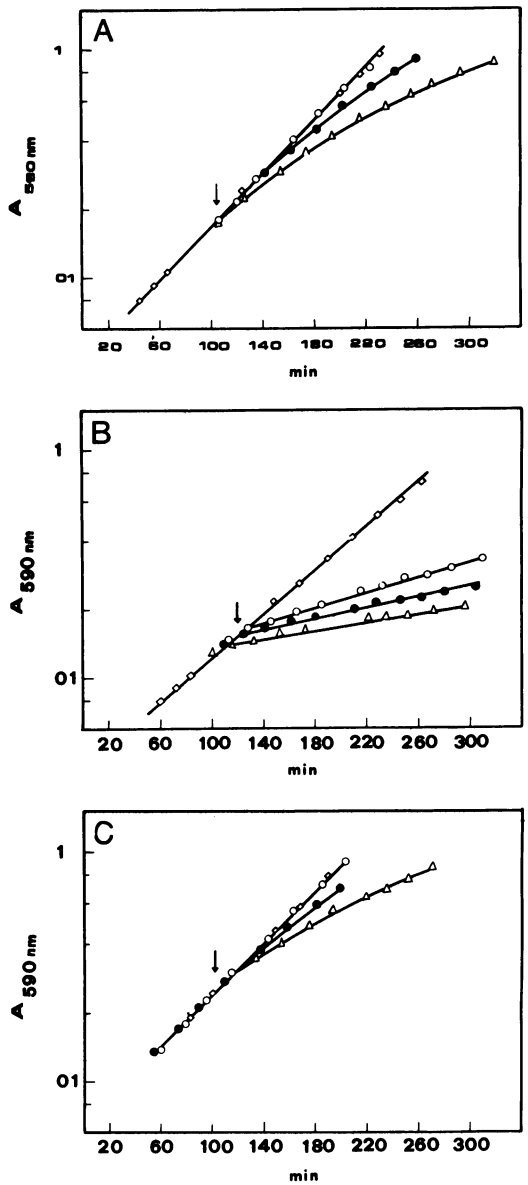

FIG. 5. *Growth curve of PB154* (*A*), *MI1* (*B*), *and MI2* (*C*) *in minimal medium supplemented with arginine, tryptophan, and 10 μg of* L*-isoleucine per ml.* (□) *No other addition;* (○) *0.75 mg of* L*-valine per ml (final concentration);* (●) *1.5 mg of* L*-valine per ml;* (△) *3 mg of* L*-valine per ml.*

tion of isoleucine entry into the cells (since valine and isoleucine show common transport systems; 44), or from inhibition of isoleucine synthesis by the biosynthetic pathway (since valine inhibits the first common step of isoleucine and valine biosynthesis; 24), or from a combination of these effects. Regardless of the mechanism by which the intracellular pool of isoleucine is reduced, it is clear that the growth of MI1 is considerably more sensitive to this reduction than is the wild type or MI2.

If the growth of MI1 is, in fact, limited by the low level of intracellular isoleucine, the requirement for added isoleucine should disappear if the endogenously produced isoleucine can be increased. Accordingly, we examined the growth requirement of strains which contain the *ileS* mutation of MI1 but which overproduce isoleucine as a consequence of derepression of threonine deaminase. Strain AW206 is derepressed for the formation of threonine deaminase (specific activity of 210 units/mg compared with a wild-type activity of 30 units/mg) because of a mutation linked to the *ilv* sequence (see below); it is resistant to the growth inhibition of even 5 mg of valine/ml, presumably as a consequence of the overproduction of isoleucine. The *ileS* mutation of MI1 can be introduced into AW206 by selecting for Thr$^+$ transductants by use of P1 grown on MI1. Thr$^+$ transductants were selected on minimal plates containing the appropriate supplements plus isoleucine, and 23 clones were purified by single-colony isolations (Table 3).

Based on the linkage of *ileS* to *thr* (see Fig. 1 and Table 2), a high proportion of the Thr$^+$ transductants should also have received the *ileS* marker from the donor and thereby become Ile$^-$. Moreover, about 90% of the transductants which have received both *thr*$^+$ and *pyrA*$^+$ should be *ileS* mutants and be phenotypically Ile$^-$. Although this was not strictly so, we noted two classes of Thr$^+$ recombinants: one grows rapidly on minimal medium and is resistant to valine (4 mg/ml); the other grows slowly in the absence of isoleucine (no colonies are visible after 20 hr at 37 C, but by 48 hr colonies can easily be seen) and fails to grow in the presence of valine (Table 3). Thus, when the *thr* marker is transduced from MI1, 11 of 23 Thr$^+$ recombinants become valine-sensitive and grow slowly in the absence of isoleucine. Among those transductants which received both *thr*$^+$ and *pyrA*$^+$, the frequency of this phenotype was even higher (7 of 10). The correlation of the new phenotype with the expected frequency of transduction of *ileS* is unmistakable.

Extracts of three of the valine-sensitive transductants (isolates 1, 14, and 18) were tested for their IRS activity and were found to have the reduced activity characteristic of the MI1 mutant. Similar assays of extracts from three valine-resistant strains (isolates 2, 3, and 4) showed normal wild-type activity. Isolate 18 grew normally in medium containing isoleucine (generation time, 65 min) but, in contrast to MI1, it grew slowly in the absence of isoleucine (generation time, 158 min). Although isolate 18 is phenotypically valine-sensitive, it still contains the original mutation conferring valine resistance; when P1 phage, grown on isolate 18, transduces an Ilv$^-$ strain (JF281) to Ilv$^+$, all of 80 Ilv$^+$ transductants are also valine-resistant [the mutation conferring valine-resistance to AW206 is presumably an operator-constitutive mutation, *ilvO* (35)].

TABLE 3. *Phenotypic expression of the ileS mutation in strains overproducing isoleucine*[a]

Transductant	PyrA phenotype	Rapid growth on minimal medium[b]	Growth on valine[c]
1	−	−	−
2	−	+	+
3	−	+	+
4	−	+	+
5	+	−	−
6	−	+	+
7	−	+	+
8	+	−	−
9	+	+	+
10	−	+	+
11	+	−	±
12	−	+	+
13	+	−	±
14	−	−	−
15	+	−	−
16	−	−	−
17	+	+	+
18	−	−	−
19	−	+	+
20	−	+	+
21	+	−	−
22	+	−	−
23	+	+	±

[a] AW206 (*thr*, valine-resistant, *pyrA*) was transduced to Thr$^+$ with P1 grown on MI1, and transductants were selected on plates containing isoleucine and other necessary growth factors; 23 transductants were purified twice by single-colony isolation and then their growth was tested by streaking on appropriate plates.

[b] Some of the strains showed normal-size colonies (+) after 20 hr of incubation at 37 C on minimal plates supplemented with necessary growth factors but no isoleucine; others (−) were almost invisible at that time, but 1 day later colonies were of normal size.

[c] Minimal plates containing arginine, uridine, and thiamine were supplemented with 4 mg of L-valine/ml (the parental strain grows on these plates when supplemented with threonine).

We conclude from this experiment that the slow growth rate in the absence of isoleucine, the valine sensitivity, and the altered IRS activity in the extracts are all due to the introduction of the *ileS* mutation into AW206. Thus, if the intracellular pool of isoleucine is elevated, the effect of the *ileS* mutation is partially overcome. However, since the cell is now very dependent on the biosynthetic supply of isoleucine, it is valine-sensitive even though it carries a mutation which confers valine-resistance to an *ileS*$^+$ strain.

Effect of the ileS mutation on isoleucine biosynthetic enzymes. Extracts of MI1, grown in isoleucine-supplemented minimal medium have three- to fourfold higher activity of threnonine deaminase activity than does the wild type. Does the *ileS* mutation alter the normal regulation of the *ilvA* gene? To answer this, we compared two isogenic strains which differed only by the region including the *ileS* gene (see Table 1 and methods for preparation of strains MI154 and 158); MI154 requires isoleucine for growth and has the low IRS activity characteristic of MI1, whereas MI158 is prototrophic and has normal IRS activity.

Table 4 records the activities of three enzymes involved in isoleucine biosynthesis in extracts of MI154 and MI158. MI154 had about a fourfold higher activity for threonine deaminase and transaminase B than did the isogenic *ileS*$^+$ strain, MI158, but the acetolactate synthetase activity was, if anything, somewhat reduced. Although the two remaining biosynthetic enzymes, dihydroxyacid dehydrase (2,3-dihydroxyacid hydrolyase, EC 4.2.1.9) and acetohydroxy acid isomero reductase activities were not assayed, the pattern of derepression was similar to that found after starvation of isoleucine auxotrophs (8), and in mutants which have an altered IRS leading to resistance to the isoleucine analogue thiaisoleucine (40). The increased synthesis of threonine deaminase and transaminase B in the *ileS* mutant growing in low isoleucine concentrations was repressed by excess isoleucine. Although we cannot rule out the possibility that our *ileS* mutants have a second, closely linked mutation which causes partial derepression of the operon containing *ilvA* or *ilvE*, we are inclined to attribute the derepression to the defect in IRS itself. Glover (15) has, in fact, described a mutation which confers valine resistance and which maps between *thr* and *leu*, but it is not known whether this affects IRS and whether the valine resistance results from the consequent derepression of isoleucine biosynthesis. This question is presently under study.

Although the *ileS* mutation causes a fourfold derepression of threonine deaminase and transaminase B (and presumably the other enzyme of that operon as well), this group of enzymes can be derepressed to a considerably greater extent. Dwyer and Umbarger (8) showed that, when an organism which requires isoleucine and valine for growth was grown on limiting isoleucine in a chemostat, the level of threonine deaminase rose about eightfold over that of the wild type growing in minimal medium. In the case of the putative *ilvO* operator mutation, which leads to valine-resistance (AW206), there is approximately a 7-fold derepression, but when it is combined with the MI1 *ileS* mutation the net derepression of threonine deaminase is about 35-fold (1,080 units/mg). Conceivably, maximal derepression of threonine deaminase in the *ileS* mutant is prevented by the presence of isoleucine added to the medium to support growth. Accordingly, we compared the change in threonine deaminase level in *ileS* and *ilv* mutants when each runs out of isoleucine.

When MI160 (*ileS*$^+$ *ilv*) was grown in a minimal medium containing all of its required supplements, but limiting isoleucine (6 μg/ml), there was an increase in the specific activity of threonine deaminase immediately before the slow-down in growth (Fig. 6A). It is not clear why the increase is only twofold, but quite possibly it is caused by a limitation in the ability to synthesize proteins [the sevenfold derepression found by Dwyer and Umbarger (8) was achieved by isoleucine limitation in a chemostat]. When MI154 (*ileS ilv*$^+$) was subjected to a similar isoleucine depletion, there was only a slight further derepression above the already elevated value (Fig. 6B), but in this case isoleucine continued to be synthesized. We therefore tested an *ileS* mu-

TABLE 4. *Activity of some isoleucine biosynthetic enzymes in extracts from MI158 (ileS*$^+$*) and MI154 (ileS)*[a]

Strain	Isoleucine concn during growth (μg/ml)	Threonine deaminase	Transaminase B	Acetolactate synthetase
MI158 (*ileS*$^+$)	10	32 (1)	13 (1)	53 (1)
MI158 (*ileS*$^+$)	2,500	54 (1.7)	16 (1.2)	120 (2.2)
MI154 (*ileS*)	10	131 (4.1)	46 (3.5)	25.5 (0.48)
MI154 (*ileS*)	2,500	56 (1.7)	19 (1.5)	43 (0.81)

[a] The units of activity are those mentioned in Materials and Methods, and the numbers in parentheses represent the fold increase over that of the *ileS*$^+$ strain grown in the presence of 10 μg of isoleucine per ml.

tant which is also unable to synthesize isoleucine (MI161, *ileS ilv*). When this strain was grown in the presence of an optimal concentration of isoleucine and valine (50 and 100 μg/ml, respectively), the specific activity of threonine deaminase was equal to that of MI154 (140 units/mg), but when the isoleucine concentration was decreased to 6 μg/ml the growth rate decreased and the threonine deaminase level rose further to 320 units/mg. Thus, the derepression caused by the *ileS* mutation can be accentuated by starvation for isoleucine.

DISCUSSION

Isoleucine requirement and the mutation in isoleucyl-tRNA synthetase. In this paper, we have reported the isolation of an isoleucine auxotroph which differs genetically and phenotypically from mutants altered in the *ilvA* gene. Enzymatic assays on extracts of this new mutant, as well as on extracts of Ile$^+$ transductants, showed that the mutation affects the enzymatic activity of IRS. As a result of the alteration, the enzyme has a decreased affinity for isoleucine. We believe that as a consequence of the mutation the synthesis of isoleucyl-tRNA is limited at the normal intracellular isoleucine concentration and the cell cannot grow. Growth can occur, however, if the pool of isoleucine is increased by adding this amino acid to the medium.

The finding of the IRS mutants shows that there is in *E. coli* either a single IRS which esterifies the three different tRNA^{Ile} chains (50), as suggested by the data of Baldwin and Berg (2), or that the enzyme which has been mutationally altered carries out some other indispensable function.

The genetic location of the IRS gene confirms the pattern which is emerging in *E. coli*, that aminoacyl-tRNA synthetases are neither located close to the genes coding for the specific amino acid biosynthetic enzymes nor are they all clustered in one region of the chromosome (30).

Roth and Ames (36) reported the isolation of some analogue-resistant mutants of *S. typhimurium* which were altered in histidyl-tRNA synthetase. This enzyme had a decreased affinity for the substrate histidine and, as a consequence, the mutant bacteria needed histidine to grow at a normal rate in minimal medium. Nass and Neidhardt (Bacteriol. Proc., p. 87, 1966) made similar observations with a histidine-requiring mutant of *E. coli*. Several other cases have been described in which a requirement for an amino acid for normal growth results from mutational alteration of an aminoacyl-tRNA synthetase: glycine (10, 11), tryptophan (7, 19, 22, 23), arginine (20), tyrosine (37), and methionine (S. Lerner and P. Berg, *unpublished data*). All of these mutants are

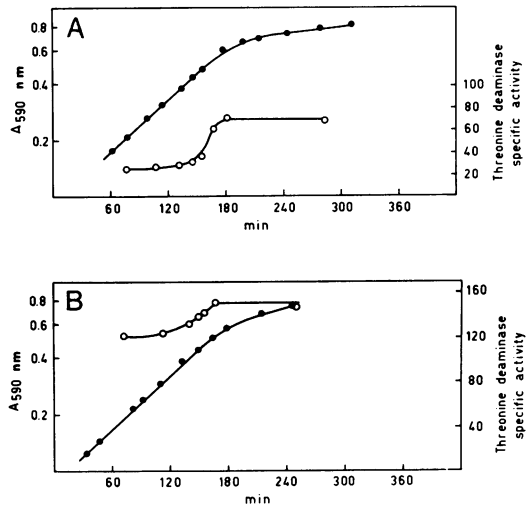

FIG. 6. *Increase in threonine deaminase activity accompanying isoleucine starvation. Cells were grown in minimal medium supplemented with arginine, uridine, thiamine, 100 μg of valine per ml, and 6 μg of isoleucine per ml. Samples were centrifuged at different times, and the threonine deaminase activity was measured in extracts of the cells. (A) MI160 (ileS$^+$, ilv); (B) MI154 (ileS, ilv$^+$). (○) Threonine deaminase specific activity; (●) cell turbidity.*

phenotypically auxotrophic for an amino acid, even though the biosynthesis of that amino acid is normal. In each case, an alteration in the K_m of the specific aminoacyl-tRNA synthetase has been demonstrated.

Regulatory role of IRS. One of the consequences of the alteration in IRS is a fourfold derepression of the isoleucine biosynthetic enzymes. This derepressed phenotype cotransduces with the mutant *ileS* gene. Similar mutants have been isolated from *S. typhimurium* by Blatt and Umbarger (Bacteriol. Proc., p. 136, 1970) and from *E. coli* K-12 by Szentirmai, Szentirmai, and Umbarger (40), and these mutants also show derepression of biosynthetic enzymes. In the latter case, however, evidence has been presented by Coker and Umbarger (Bacterial. Proc., p. 135, 1970) that the phenotype of these mutants is due to two different mutations. It appears, therefore, that isoleucine itself cannot serve as the repressor, but that repression requires some interaction between isoleucine and IRS. This could be (i) the product of the IRS reaction, isoleucyl-tRNA, (ii) some product derived from isoleucyl-tRNA, (iii) a complex of IRS and isoleucine, or (iv) some combination of these. In any case, IRS appears to be needed for the formation of the active repressor in the same way that valyl-tRNA synthetase (9, 47), histidyl-tRNA synthetase [L-histidine: soluble RNA ligase (AMP), EC 6.1.1.; 36;

Nass and Neidhardt, Bacteriol. Proc., p. 87, 1966], tryptophanyl-tRNA synthetase [L-tryptophan: soluble RNA ligase (AMP), EC 6.1.1.2; 22], and leucyl-tRNA synthetase [L-leucine: soluble RNA ligase (AMP), EC 6.1.1.4; Calvo, quoted in 43] are needed to regulate their respective biosynthetic pathways. The elevated levels of threonine deaminase in the IRS mutant can be repressed by an excess of isoleucine, in the same way as was found with the histidyl-tRNA synthetase mutant (36).

The IRS mutants could be isolated because they fail to grow in the absence of isoleucine. If the particular mutations had caused extensive derepression of isoleucine biosynthesis, we would never have recovered them since such mutants would have been killed during the penicillin selection. This can be predicted from the fact that *ileS* mutants, which also overproduce isoleucine as a result of a second mutation in the *ilv* operator, grow in the absence of isoleucine. Very likely, then, our isolation procedure selected *ileS* mutants that could not be extensively derepressed; therefore, it is possible that mutations at another site(s) in the same gene would result in greater derepression.

It has been reported that, for complete repression of the isoleucine-valine biosynthetic enzymes, isoleucine, valine, and leucine (12), as well as pantothenic acid (Freundlich and Umbarger, Bacteriol. Proc., p. 126, 1963), are needed. Since it seems that isoleucine, valine, and leucine need to interact with their specific aminoacyl-tRNA synthetases in order to function in repression, it would be interesting to know whether an analogous interaction is required for pantothenic acid. Although there is in this case no tRNA involvement, the reaction catalyzed by pantothenic acid synthetase [L-pantoate: β-alanine ligase (AMP), EC 6.3.2.1] is quite similar to the activation of amino acids, and it would be interesting to know whether in pantothenic acid-requiring mutants derepression is caused by the lack of pantothenic acid per se. Indeed, a mutant in pantothenic acid synthetase has been described by Maas and Davis (29), but, as far as we know, the level of the isoleucine biosynthetic enzymes has not been determined in extracts of this mutant.

ACKNOWLEDGMENTS

We thank Sondra Schlesinger and Milton Schlesinger for very useful discussions; A. L. Taylor, J. Foulds, and J. C. Clark for providing bacterial strains; G. Hitchings for a gift of trimethoprim; and Gerda Treiber for skillful assistance in some experiments.

The experiments reported in this paper were supported by a Postdoctoral Fellowship from the Jane Coffin Childs Memorial Fund for Medical Research to M. I. at Stanford University, where this work was initiated, and later by a Research Grant from the same Fund. They were also supported by Public Health Service research grants GM 13235 and GM 196 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Arndt, D. J., and P. Berg. 1970. Isoleucyl transfer ribonucleic acid synthetase is a single polypeptide chain. J. Biol. Chem. **245**:665-667.
2. Baldwin, A. N., and P. Berg. 1966. Purification and properties of isoleucyl ribonucleic acid synthetase from *Escherichia coli*. J. Biol. Chem. **241**:831-838.
3. Baldwin, A. N., and P. Berg. 1966. Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase. J. Biol. Chem. **241**:839-845.
4. Burns, R. O., and M. H. Zarlengo. 1968. Threonine deaminase from *Salmonella typhimurium*. I. Purification and properties. J. Biol. Chem. **243**:178-185.
5. Calendar, R., and P. Berg. 1966. Tyrosyl tRNA synthetase from *Escherichia coli* B, p. 384-399. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper and Row, New York.
6. Changeux, J. 1963. Allosteric interactions on biosynthetic L-threonine deaminase from *E. coli* K-12. Cold Spring Harbor Symp. Quant. Biol. **28**:497-504.
7. Doolittle, W. F., and C. Yanofsky. 1968. Mutants of *Escherichia coli* with an altered tryptophanyl-transfer ribonucleic acid synthetase. J. Bacteriol. **95**:1283-1294.
8. Dwyer, S. B., and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XVI. Pattern of multivalent repression in strain K-12. J. Bacteriol. **95**:1680-1684.
9. Eidlic, L., and F. C. Neidhardt. 1965. Role of valyl-sRNA synthetase in enzyme repression. Proc. Nat. Acad. Sci. U.S.A. **53**:539-543.
10. Folk, W. R., and P. Berg. 1970. Isolation and partial characterization of *Escherichia coli* mutants with altered glycyl transfer ribonucleic acid synthetase. J. Bacteriol. **102**:193-203.
11. Folk, W. R., and P. Berg. 1970. Characterization of altered forms of glycyl transfer ribonucleic acid synthetase and the effects of such alterations on aminoacyl transfer ribonucleic acid synthesis in vivo. J. Bacteriol. **102**:204-212.
12. Freundlich, M., R. O. Burns, and H. E. Umbarger. 1962. Control of isoleucine, valine and leucine biosynthesis. I. Multi-valent repression. Proc. Nat. Acad. Sci. U.S.A. **48**:1804-1808.
13. Freundlich, M., and H. E. Umbarger. 1963. The effects of analogues of threonine and isoleucine on the properties of threonine deaminase. Cold Spring Harbor Symp. Quant. Biol. **28**:505-511.
14. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**: 415-442.
15. Glover, S. W. 1962. Valine resistant mutants of *Escherichia coli* K-12. Genet. Res. **3**:448-460.
16. Gorini, L., and H. Kaufman. 1960. Selecting bacterial mutants by the penicillin method. Science **131**:604-605.
17. Groves, W. E., F. C. Davis, Jr., and B. Sells. 1968. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal. Biochem. **22**:195-210.
18. Hill, C. W., J. Foulds, L. Soll, and P. Berg. 1969. Instability of a missense suppressor resulting from a duplication of genetic material. J. Mol. Biol. **39**:563-581.
19. Hiraga, S., K. Ito, K. Hamada, and T. Yura. 1967. A new regulatory gene for the tryptophan operon of *Escherichia coli*. Biochem. Biophys. Res. Commun. **26**:522-527.
20. Hirshfield, I. N., R. De Deken, P. C. Horn, D. A. Hopwood, and W. K. Maas. 1968. Studies on the mechanism of repression of arginine biosynthesis in *Escherichia coli*. III. Repression of enzymes of arginine biosynthesis in arginyl-tRNA synthetase mutants. J. Mol. Biol. **35**:83-93.
21. Iaccarino, M., and P. Berg. 1969. Requirement of sulphydryl groups for the catalytic and tRNA recognition functions of isoleucyl-tRNA synthetase. J. Mol. Biol. **42**:151-169.
22. Ito, K., S. Hiraga, and T. Yura. 1969. Tryptophanyl transfer RNA synthetase and expression of the tryptophan operon in the *trpS* mutants of *Escherichia coli*. Genetics **61**:521-538.
23. Kano, Y., A. Matsushiro, and Y. Shimura. 1968. Isolation of the novel regulatory mutants of the tryptophan biosynthetic system in *Escherichia coli*. Mol. Gen. Genet. **102**:15-26.
24. Leavitt, R. I., and H. E. Umbarger. 1962. Isoleucine and valine metabolism in *Escherichia coli*. XI. Valine inhibition of the growth of *Escherichia coli* strain K-12. J. Bacteriol. **83**:624-630.

25. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190-206.
26. Loftfield, R. B. 1970. The aminoacylation of transfer ribonucleic acid, p. 1-35. *In* E. McConkey (ed.), Protein synthesis, vol. 1. Marcel Dekker, New York.
27. Low, B. 1968. Formation of merodiploids in matings with a class of *rec*⁻ recipient strains of *Escherichia coli* K-12. Proc. Nat. Acad. Sci. U.S.A. **60**:160-167.
28. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
29. Maas, N. K., and B. D. Davis. 1952. Production of an altered pantothenate-synthesizing enzyme by a temperature-sensitive mutant of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **38**:785-797.
30. Neidhardt, F. C. 1966. Roles of amino acid activating enzymes in cellular physiology. Bacteriol. Rev. **30**:701-719.
31. Norris, A. T., and P. Berg. 1964. Mechanism of aminoacyl RNA synthesis: studies with isolated aminoacyl adenylate complexes of isoleucyl RNA synthetase. Proc. Nat. Acad. Sci. U.S.A. **52**:330-337.
32. Pittard, J., J. S. Loutit, and E. A. Adelberg. 1963. Gene transfer by F' strains of *Escherichia coli* K-12. I. Delay in initiation of chromosome transfer. J. Bacteriol. **85**:1394-1401.
33. Ramakrishnan, T., and E. A. Adelberg. 1964. Regulatory mechanisms in the biosynthesis of isoleucine and valine. I. Genetic derepression of enzyme formation. J. Bacteriol. **87**:566-573.
34. Ramakrishnan, T., and E. A. Adelberg. 1965. Regulatory mechanisms in the biosynthesis of isoleucine and valine. II. Identification of two operator genes. J. Bacteriol. **89**:654-660.
35. Ramakrishnan, T., and E. A. Adelberg. 1965. Regulatory mechanisms in the biosynthesis of isoleucine and valine. III. Map order of the structural genes and operator genes. J. Bacteriol. **89**:661-664.
36. Roth, J. R., and B. N. Ames. 1966. Histidine regulatory mutants in *Salmonella typhimurium*. II. Histidine regulatory mutants having altered histidyl-tRNA synthetase. J. Mol. Biol. **22**:325-334.
37. Schlesinger, S., and E. W. Nester. 1969. Mutants of *Escherichia coli* with an altered tyrosyl-transfer ribonucleic acid synthetase. J. Bacteriol. **100**:167-175.
38. Stacey, K. A., and E. Simson. 1965. Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. J. Bacteriol. **90**:554-555.
39. Stormer, F. C., and H. E. Umbarger. 1964. The requirement for flavine adenine dinucleotide in the formation of acetolactate by *Salmonella typhimurium* extracts. Biochem. Biophys. Res. Commun. **17**:587-592.
40. Szentirmai, A., M. Szentirmai, and H. E. Umbarger. 1968. Isoleucine and valine metabolism of *Escherichia coli*. XV. Biochemical properties of mutants resistant to thiaisoleucine. J. Bacteriol. **95**:1672-1679.
41. Tatum, E. L. 1946. Biochemical mutations in bacteria. Cold Spring Harbor Symp. Quant. Biol. **11**:278-283.
42. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. **31**:332-353.
43. Umbarger, H. E. 1969. Regulation of amino acid metabolism. Annu. Rev. Biochem. **38**:323-370.
44. Umbarger, H. E., and B. Brown. 1955. Isoleucine and valine metabolism in *Escherichia coli*. VI. Antagonism between isoleucine and valine. J. Bacteriol. **70**:241-248.
45. Umbarger, H. E., and B. D. Davis. 1962. Pathways of amino acid biosynthesis, p. 167-251. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 3. Academic Press Inc., New York.
46. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. **218**:97-106.
47. Yaniv, M., and F. Gros. 1967. Studies on temperature conditional mutants of *E. coli* with a modified aminoacyl RNA synthetase, p. 157-178. *In* D. Shugar (ed.), Genetic elements. Academic Press Inc., New York.
48. Yarus, M., and P. Berg. 1967. Recognition of tRNA by aminoacyl tRNA synthetases. J. Mol. Biol. **28**:479-490.
49. Yarus, M., and P. Berg. 1969. Recognition of tRNA by isoleucyl-tRNA synthetase. Effect of substrates on the dynamics of tRNA; enzyme interaction. J. Mol. Biol. **42**:171-189.
50. Yegian, C. D., and G. S. Stent. 1969. Differential aminoacylation of three species of isoleucine transfer RNA from *Escherichia coli*. J. Mol. Biol. **39**:59-71.