JOURNAL OF BACTERIOLOGY, Sept. 1979, p. 1014-1020
0021-9193/79/09-1014/07 $02.00/0

Vol. 139, No. 3

Positive Control of *ilvC* Expression in *Escherichia coli* K-12; Identification and Mapping of Regulatory Gene *ilvY*

MARTIN D. WATSON, JADWIGA WILD,† AND H. E. UMBARGER*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received for publication 27 March 1979

The construction of a plasmid carrying the *ilvC::lacZ* fusion is described. This plasmid provides a convenient source of template deoxyribonucleic acid for use in an in vitro protein-synthesizing system. We screened strains deleted in regions of the *ilv* cluster for their ability to support *ilvC*-dependent β-galactosidase synthesis. The fact that two deletions prevented β-galactosidase production indicated that *ilvC* expression is under positive control. By use of plasmids carrying the positive-control factor structural gene *ilvY*, we were able to restore protein-synthesizing ability to these strains. These plasmids also enabled us to map *ilvY* between *ilvA* and *ilvC*.

In *Escherichia coli* four enzymes form valine from pyruvate. The same four enzymes also produce isoleucine from α-ketobutyrate, which in turn is produced by a fifth enzyme, threonine deaminase. Genes specifying the isoleucine-valine biosynthetic enzymes form a cluster at 83 min on the *E. coli* K-12 genetic map (3). *ilvE*, *ilvD*, and *ilvA* form an operon under multivalent control by isoleucine, valine, and leucine. *ilvG* specifies the valine-resistant isoenzyme of acetohydroxy acid synthase and is only expressed in *ilvO* mutants. *ilvO* lies between *ilvE* and *ilvG* and is thought to be a regulatory locus for *ilvEDA* (20). The acetohydroxy acid synthases that are expressed in *ilvO*$^+$ strains are specified by the *ilvHI* and *ilvB* genes which are unlinked to the *ilv* cluster. All of the above-mentioned structural genes are repressible by the end products of branched-chain amino acid biosynthesis.

The remaining structural gene, *ilvC*, specifies isomeroreductase and is unusual in being induced by its substrates, acetohydroxybutyrate and acetolactate (2). The regulation of *ilvC* is completely independent of multivalent repression (17). To date, no regulatory mutations affecting *ilvC* expression have been described. An *ilvY* locus, postulated to have a positive-control role in induction of the *ilvC* gene, was thought to be altered by the *ilvY466* mutation (16). However, the abnormal phenotype has since been shown to be due to at least two mutations, *ilvA466* and *ilvC2004* (John Smith, personal communication).

To aid our understanding of the *ilvC* regulatory mechanisms, an *ilvC::lacZ* fusion strain was prepared by the technique of Casadaban (6) and Smith and Umbarger (22). From this strain, the λp*ilvC-lac*-1 phage was derived (24). This phage provided efficient template DNA for *ilvC*-directed synthesis of β-galactosidase in an in vitro protein-synthesizing system. Using this and a modified template, we demonstrate that *ilvC* is under positive control and that the gene (*ilvY*) specifying the positive-control factor υ (upsilon protein) is located between *ilvC* and *ilvA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used were derivatives of *E. coli* K-12. The bacterial strains used and their sources are listed in Table 1. The plasmids used and their sources are listed in Table 2.

Media and growth conditions. The medium of Davis and Mingioli (8), modified by an omission of citrate and an increase in the glucose concentration to 0.5%, was used as a minimal growth medium. Necessary supplements were added in the following final concentrations: all amino acids, 50 μg/ml; and all vitamins, 1 μg/ml. L-broth was used as the rich medium (4). For solid media, agar (Difco Laboratories) at a final concentration of 1.5% was added to the above-mentioned media. Antibiotics were used at final concentrations of: ampicillin (Ap), 50 μg/ml; kanamycin (Km), 100 μg/ml; and tetracycline (Tc), 10 μg/ml.

Transduction. Bacteriophage P1*cmclr*100 was used for generalized transductions. P1*cm* lysates were prepared by heat induction and used in transductions by the method of Rosner (18).

Enzyme assays. Cells were grown in minimal media with limiting valine. Growth conditions, preparation of cell extracts, and enzyme assays were according to Smith et al. (20). Enzyme activities are expressed as nanomoles of product formed or substrate used per minute per milligram of protein.

Isolation of plasmid DNA. Plasmid DNA for use

† Present address: Instytut Biochemii i Biofizyki PAN, 02-532 Warsaw, Poland.

TABLE 1. *E. coli strains used*

Strain	Genotype	Source or Reference
AB2944	F⁻ *ilvC285 his-4 trp-3 rpsL*	Duggan and Marsh (12)
CSH26	*ara thi* Δ(*proAB-lac*)	Cold Spring Harbor Laboratory
CU344	F⁻ *ΔilvDAC115 galT12* λ⁻	Kline et al. (10)
CU447	*rbs-221 thi arg trp Δlac*	Smith et al. (21)
CU452	*ΔilvDAC115 thi arg trp Δlac*	Smith et al. (21)
CU482	*rbs-221 leu-454*	Pl transduction of CU1014 with CU829 as donor by John M. Smith
CU483	*ΔilvEDAC2049 leu-455*	Pl transduction of CU482 with CU829 as donor by John M. Smith
CU486	*ilvC2060 thi arg trp Δlac*	Wild et al. (24)
CU504	F⁻ *rbs-221 leu-455 galT12* λ⁻	Smith et al. (20)
CU505	*ΔilvEDAC2049 leu-455 galT12*	Pl transduction of CU504 with CU483 as donor by John M. Smith
CU520	*rbs-221 metE201 leu-455 galT12* λ⁻	Smith et al. (21)
CU527	F⁻ *ΔilvEDAC2049 metE201 leu-455 galT12* λ⁻	Pl transduction of CU520 with CU829 as donor by John M. Smith
CU532	F⁻ *ilvE2050 leu-455 galT12* λ⁻	Smith et al. (21)
CU564	F16 *ilv*⁺/*ΔilvE2050 ara thi* Δ(*proAB-lac*)	Episomal transfer from CU72 to CU505 by John M. Smith
CU577	F⁻ *glmS metE201 leu-455 galT12* λ⁻	Pl transduction of CU527 with E111 as donor by John M. Smith
CU578	F⁻ *ΔilvEDAC2049 glmS leu-455 galT12* λ⁻	Pl transduction of CU577 with CU829 as donor by John M. Smith
CU636	F16 *ilv*⁺/*ΔilvEDAC2049 glmS leu-455 galT12* λ⁻	Episomal transfer from CU578 to CU564 by John M. Smith
CU653	*ΔilvDA2076 ara thi* Δ(*proAB-lac*)	Smith et al. (20)
CU697	*rbs-221 metE201 ara thi* Δ(*proAB-lac*)	Smith et al. (20)
CU713	*ilvC2209*::λpl(209) *ara thi* Δ(*proAB-lac*)	Smith and Umbarger (22). The insertion in this strain was formerly designated *ilvC2083*::λpl(209)
CU723	*ilvC285 ara thi* Δ(*proAB-lac*)	Pl transduction of CU697 with AB2944 as donor by John M. Smith
CU311	HfrH *ilvC2092*::λ*c*I857Δ*am7*Δ*b515*Δ*b519rifam6*	Wild et al. (24)
CU825	*ilvA454 rbs-221 ara thi* Δ(*proAB-lac*)	Pl transduction of CU697 with CU1008 as donor by John Noti
CU827	*ΔilvDAC115 ara thi* Δ(*proAB-lac*)	Pl transduction of CU697 with CU344 as donor by John Noti
CU829	HfrH *ΔilvEDAC2049 thi* Δ(*gal att* λ *bio uvrB*)	Spontaneous survivor of CU811 at 42°C isolated by John M. Smith
CU838	*ΔilvEDAC2049 ara thi* Δ(*proAB-lac*)	Pl transduction of CU697 with CU505 as donor by Timothy D. Leathers
CU902	*ΔilvE2050 thi arg trp Δlac* λ⁻	Pl transduction of CU447 with CU532 as donor
CU903	*ΔilvE2050 thi arg trp Δlac* λ⁺	λ lysogen of CU902
CU906	F16 *ilv*⁺/*ΔilvEDAC2049 ara thi* Δ(*proAB-lac*) λ⁻	Episomal transfer from CU636 to CU838 by John M. Smith
CU918	F16 *ilv*⁺/*ΔilvEDAC2049 ara thi* Δ(*proAB-lac*) λ⁺	λ lysogen of CU906
CU941	F16 *ilvC2209*::λpl(209)/*ΔilvEDAC2049 ara thi* Δ(*proAB-lac*) λ⁺	Pl transduction of CU918 with CU713 as donor
CU968	pMD4/*ilvA454 rbs-221 ara thi* Δ(*proAB-lac*)	Ilv⁺ transformant of CU825 with pMD4 DNA
CU1014	***ilvC462 leu-454***	Wasmuth and Umbarger (23)
E111	F⁻ *glmS galE rpsL*	Wu and Wu (25)

in transformation and restriction analysis was isolated by the method of Humphreys et al. (9). Covalently closed circular plasmid DNA was purified by two cycles of cesium chloride-ethidium bromide centrifugation. The final refractive index of the cesium chloride was 1.392. Ethidium bromide was extracted by cesium chloride-saturated isopropanol. The plasmid DNA was then dialyzed extensively against TES buffer (50 mM Tris-chloride [pH 8.0], 1 mM EDTA, 0.17 M NaCl) for 48 h and then for two changes against 10 mM Tris-acetate, pH 8.0.

pMD4 plasmid DNA for use as a template in in vitro protein synthesis was prepared by chloramphenicol amplification, using the method of Sidikaro and Nomura (19). Ethidium bromide was removed and the DNA was dialyzed as described above.

S-30 extract preparation. The preparation of S-30 extracts was initially that of Wild et al. (24). In later experiments the following modifications were made. Cells were grown to a final absorbancy at 660 nm of 1.8. Buffer III was used throughout, and cells were lysed at the lower pressure of 6,500 lb/in^2.

In vitro protein synthesis. In vitro protein synthesis and β-galactosidase assays were performed as described by Wild et al. (24).

Restriction endonuclease cleavage analysis. All restriction endonucleases were purchased from New England BioLabs. Endonuclease restriction digestion was performed as described by Meagher et al. (14). Agarose gel electrophoresis was performed by the method of D. Finnegan (personal communication). To make the horizontal gel (0.6 by 19 by 11.5 cm), 125 ml of 0.7% agarose in Tris-borate buffer (0.7 M Tris base, 0.7 M boric acid, 25 mM EDTA; pH 8.0) was used. Samples (25 μl) containing 10% glycerol plus trace amounts of bromophenol blue and orange G were allowed to enter the gel at 60 V for 20 min. The voltage was then reduced to 30 V, and electrophoresis continued at constant voltage until the orange G dye reached the end of the gel. The gel was photographed under short-wavelength UV illumination, using Tri-X Pan film with a 4x red filter. The film was developed for 9.5 min in Microdol-X.

Ligation of restricted DNA. Restricted DNA was

ligated by the method of Murray et al. (15).

Transformation of DNA. *E. coli* K-12 was made competent and transformed with DNA by the method of Lederberg and Cohen (11).

Construction of pGMM52. pGMM52 was constructed by G. McCorkle by partial digestion of pGMM201 with *Eco*RI followed by religation. The ligation mixture was used to transform cells to tetracycline resistance, thereby selecting for retention of the large *Eco*RI fragment containing the *tet* genes, the origin of replication, and *ilvA*, represented by fragments fA and fF in Fig. 1 and 2. pGMM52 was isolated by screening transformants for plasmid DNA and identifying an isolate that also retained fB (Fig. 1 and 2) but had lost fragments fC, fD, and fE of chromosomal DNA and fragment fG of pBR322 adjacent to fD.

Construction of pMD4. Recently, difficulties were encountered in routinely preparing pure large-scale lysates of λ*pilvC-lac*-1 phage. To circumvent this problem, we decided to clone the *ilvC::lacZ* fusion

TABLE 2. *Plasmids used*

Designation	Description	Source or reference
pBR322	Wild-type plasmid *bla*$^+$ *tet*$^+$	Bolivar et al. (5)
pGMM150	pBR322Ω8[0.375kb:K-12 *ilvC* −5.88–1.69kb(−)][a]	*Bgl*II fragment of K-12 chromosome carrying *ilvC* inserted in *Bam*HI site of pBR322 by George M. McCorkle
pGMM151	pBR322Ω9[0.375kb:K-12 *ilvC* −5.88–1.69kb(+)]	Same fragment as in pGMM150 but in opposite orientation
pGMM201	pBR322Ω10[3.612kb:λ*h80dilv ilvAYC* −0.27–4.35kb(+)]	Formerly pGM201, McCorkle et al. (13)
pGMM52	pGMM201Δ1[*tet-ilvC* 6.57–8.98kb]	Digestion of pGMM201 by *Eco*RI and religation by George M. McCorkle
pMD4	pBR322Ω31[0.029kb:K-12 *ilvAC2203*::λp1(209) 4.8(K-12)–44.5(λ)kb][b]	*Hin*dIII fragment of F16 carrying *ilvC2203*::λp1(209) inserted in *Hin*dIII site of pBR322
pMD7	RP4Ω1[34.7kb:K-12 *ilvAC2203*::λp1(209) 4.8(K-12)–44.5(λ)kb]	*Hin*dIII fragment from pMD4 inserted in *Hin*dIII site of RP4
pMD8	pBR322Ω33[0.375kb:λ*h80dilv ilvEDA* 1.69–12.25kb]	*Bgl*II fragment of λ*h80dilv* inserted in *Bam*HI site of pBR322
RP4	Wild-type, broad-host-range plasmid *bla*$^+$ *tet*$^+$ Km^r	Datta et al. (7)

[a] The chromosomal regions near the *ilv* genes have been given coordinates based upon a zero point at the site of the φ80 DNA-*ilv* DNA junction in λ*h80dilv* (almost precisely at the terminus of the *ilvC* gene) (13). Positive coordinates are thus to the *rbs* side of *ilvC*, and negative coordinates are to the *metE* side of *ilvC* (or in the left arm of φ80). (+) or (−) indicates that the orientations of the plasmid and bacterial genes, with respect to each other, are the same as or opposite to, respectively, the way they are usually represented on their genetic maps.

[b] The fragment inserted at the *Hin*dIII site in the *tet* gene of pBR322 contained DNA extending from the *Hin*dIII site at 4.5 kb in *ilvD* through the *ilvC-lac* fusion to the λ *Hin*dIII site which is designated 44.5 kb on the λ vegetative map.

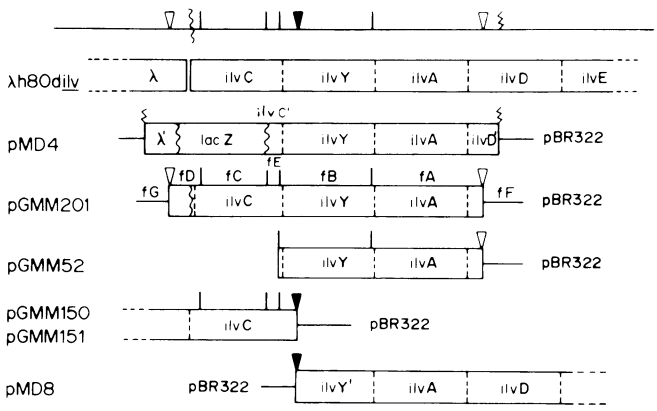

FIG. 1. *Physical map of the ilvC-ilvD region. The figures are not drawn to scale. The structural gene extremities ( ) are shown as delineated by restriction endonuclease sites only. pGMM201, pGMM52, and pMD8 were all derived from λh80dilv. pGMM150 and pGMM151 were derived from chromosomal DNA (G. M. McCorkle and H. E. Umbarger, manuscript in preparation). The derivation of pMD4 is described in the text. Restriction sites are: EcoRI (|), HindIII ( ), PstI (▽), and BglII (▼). Fusion sites between DNA of different origins are marked by: ⅋.*

onto a plasmid and use this as a source of template DNA. To ensure that all of the *ilvC* operator-promoter region was cloned along with *lacZ*, we chose to clone the *ilvC*::*lacZ* fusion from chromosomal DNA of strain CU713 rather than λp*ilvC-lac*-1. This procedure had the secondary advantage of cloning *ilvA*$^+$ along with the fusion and thus provided a powerful means of selection. The restriction endonuclease *Hin*dIII does not cleave DNA at any sites in *ilvA* or *ilvC* (13) or in *lac* (1); so, given the order λ-*lac-ilvC'-ilvA* in strain CU713 (22), a *Hin*dIII fragment will contain all of the *ilvC*::*lacZ* fusion, including the *ilvC* control region.

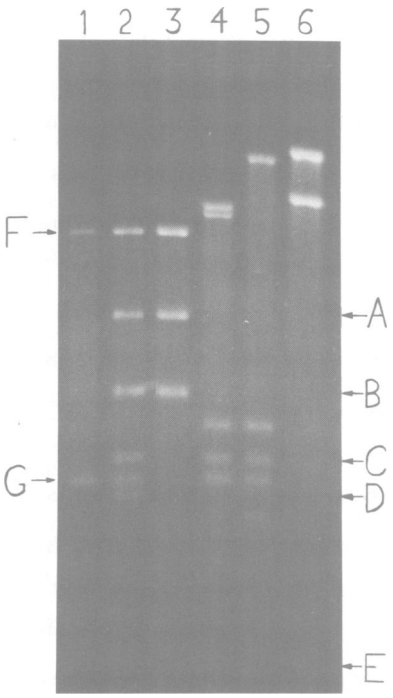

FIG. 2. *Restriction analysis of ilv plasmids. Plasmids were restricted with EcoRI plus PstI (pBR322, track 1; pGMM201, track 2; and pGMM52, track 3) or EcoRI alone (pGMM150, track 4; pGMM151, track 5; pMD8, track 6) for 2 h at 37°C and separated by electrophoresis on a 7% agarose gel. The band designated E could be seen on the negative in tracks 2, 4, and 5 but is not reproduced in the print.*

The *ilvA-ilvC*::*lac* fragment was isolated from F'16*ilvC*::*lac* DNA (CU941) and cloned into pBR322, using *Hin*dIII. It was identified by transforming strain CU825 (*ilvA454*) to Ilv$^+$. After purification, all Ilv$^+$ Ap^r transformants were found to be Lac$^+$ Tc^s. One such clone was kept, strain CU968, and the plasmid DNA (designated pMD4) was isolated after chloramphenicol amplification. Restriction analysis of pMD4 shows that only one large fragment was cloned into pBR322. This plasmid DNA was used as the template for all subsequent in vitro protein synthesis experiments.

RESULTS

_ilv_C-directed β-galactosidase formation by S-30 extracts prepared from several *E. coli* strains. Several strains of *E. coli* were used for the preparation of S-30 extracts and tested for the capacity to support β-galactosidase synthesis in the presence of acetohydroxybutyrate and the *ilvC-lac* template. Extracts prepared either from strains bearing presumed point mutations in *ilvE*, *ilvA*, or *ilvC* or from strains bearing intact *ilv* regions exhibited an acetohydroxy butyrate-induced formation of β-galactosidase when DNA bearing the *ilvC-lac* fusion was the template (Table 3). Although there were differences between the synthetic capacities of the various extracts, they were not specifically related to the nature of the template, since the extracts varied in their responses to the *lacP* template as well.

In contrast, when the S-30 extracts were pre-

TABLE 3. *In vitro protein synthesis of β-galactosidase*

Strain used for S-30 extract	*ilv* genotype	β-Galactosidase formed[a]		
		lacP directed[b]	*ilv-lac* directed	
			Acetohydroxybutyrate present	Acetohydroxybutyrate absent
CU446[c]	*ilv*$^+$	1.15	0.017[d]	0.005
CU903	*ilvE2050*	2.37	0.012[e]	0.004
CU486	Δ*ilvC2060*	1.35	0.056[d]	0.007
CU723	*ilvC285*	1.19	0.010[e]	0.002
CU653	Δ*ilvDA2076*	0.72	0.011[d]	0.002
CU827	Δ*ilvDAC115*	1.21	0.004[e]	0.004
CU452	Δ*ilvDAC115*	0.82	0.002[d]	0.001
CU838	Δ*ilvFDAC2049*	1.09	0.002[e]	0.001

[a] Change in absorbance (at 420 nm) per hour per milligram of protein.
[b] λ*h80c*I857*St*68d*lacP* DNA as template.
[c] CU446 is the *rbs*$^+$ parent of CU447 (21).
[d] λp*ilv-lac*-1 DNA as template.
[e] pMD4 DNA as template.

pared from strains bearing either the *ilvDAC115* deletion (strains CU452 and CU827) or the *ilvEDAC2049* deletion (strain CU838), acetohydroxybutyrate did not induce β-galactosidase formation. That the failure to exhibit *ilvC*-directed β-galactosidase synthesis is due to the *ilvDAC115* deletion itself is shown by the fact that the effect was demonstrated in two different genetic backgrounds. Furthermore, a strain bearing a deletion in the *ilvA* genetic material (*ilvA454* [CU825] [24]), one in which at least parts of both *ilvD* and *ilvA* are deleted (*ilvDA2076* [CU653]), and one in which part of the *ilvC* gene has been deleted (*ilvC2060* [CU486]) all exhibit an acetohydroxybutyrate-dependent formation of β-galactosidase in the presence of an *ilvC-lac* template.

Thus, strains CU827 and CU838 both lack a factor required for the acetohydroxybutyrate-dependent expression of *ilvC*-directed β-galactosidase that is found in isogenic strains carrying *ilv* point mutations. This by definition is a positive-control factor and shows that expression of *ilvC* is under positive control.

Genetic location of the gene for the positive-control factor. The data presented above show that the gene specific for the positive-control factor (*ilvY*) is deleted in strains CU827 (Δ*ilvDAC115*) and CU838 (Δ*ilv-2049*). Preliminary mapping has determined that *ilvDAC115* probably deletes the entire *ilvC* gene and extends into *ilvD*. The *ilv-2049* deletion starts within *ilvC* and extends to *ilvG* (J. Noti and A. Biel, unpublished data). As both deletions destroy the *ilvY* function, the *ilvY* gene should be located in the *ilvC-ilvD* region. That the *ilvY* function is not a regulatory activity of either the *ilvA* gene or the *ilvC* gene itself is indicated by the fact that deletions in either *ilvA* or *ilvC* do not destroy *ilvY* function (Table 3), nor did the insertion of λ and *lac* into *ilvC* of the original fusion strain (22). A more precise location of *ilvY* was achieved by transforming strain CU827 with plasmids carrying portions of the *ilv* cluster and by examining the transformants for restoration of *ilvY* activity. The restoration of the *ilvY* function was accomplished by making S-30 extracts and testing for in vitro acetohydroxybutyrate-induced β-galactosidase synthesis.

pGMM201 carries the *Pst*I fragment of *ilv* DNA which carries part of *ilvD* and all of *ilvC* (Fig. 2) (13). This plasmid produces threonine deaminase (*ilvA*$^+$) and isomeroreductase (*ilvC*$^+$) and restores acetohydroxybutyrate-dependent inducibility to strain CU827 (*ilvY*$^-$) (Table 4). This confirms our prediction that *ilvY* lies in the *ilvA-ilvC* region.

pGMM52 is an *Eco*RI-shortened derivative of pGMM201. It retains *ilvA*$^+$ but loses isomeroreductase activity (*ilvC*). It also retains acetohydroxybutyrate inducibility (*ilvY*$^+$). From this result, we conclude that *ilvY* must lie either between *ilvC* and *ilvA* or between *ilvA* and *ilvD*. pMD8 carries the *Bgl*II fragment, which extends from between *ilvA* and *ilvC* rightwards to a point beyond *ilv* in λ*h80dilv* DNA. It produces threonine deaminase (*ilvA*$^+$) and dehydrase (*ilvD*$^+$) but does not restore inducibility to strain CU827 (*ilvY*). Therefore, *ilvY* must lie between *ilvA* and *ilvC*.

pGMM150 and pGMM151 both carry the *Bgl*II fragment complementary to that in pMD8. It extends from between *ilvA* and *ilvC* to beyond *ilv* and beyond the phage-bacterial junction in λ*h80dilv*. These two plasmids have the fragment inserted into the vector pBR322 in opposite orientations. They produce isomeroreductase (*ilvC*$^+$) but do not restore inducibility (*ilvY*) (Table 4). This eliminates any possibility of *ilvY* being to the left of *ilvC*.

TABLE 4. *Effect of ilv recombinant plasmids on the properties of strain CU827 ΔilvDAC115*

Plasmid	Sp act[a]					In vitro β-galactosidase synthesis[b]		
				Acetohydroxy acid synthase (*ilvB ilvH*)		*ilv-lac* directed		
	Threonine deaminase (*ilvA*)	Dihydroxy acid dehydrase (*ilvD*)	Acetohydroxy acid isomeroreductase (*ilvC*)	+Valine[c]	−Valine	*lacP* directed	Acetohydroxybutyrate present	Acetohydroxybutyrate absent
None	0.0	0.0	0.0	17.8	129.4	0.454	0.0	0.003
pGMM201	24.3	0.0	768	5.5	55.7	2.256	0.050	0.009
pGMM52	45.8	0.0	0.0	13.3	127.1	0.376	0.046	0.0
pGMM150	0.0	0.0	39.5	12.8	136.4	0.667	0.001	0.006
pGMM151	0.0	1.4	93.9	12.8	154.4	1.547	0.0	0.0
pMD8	>2,900	310	0.0	10.2	141.5	0.354	0.002	0.003

[a] Nanomoles per minute per milligram of protein in extracts of cells grown with limiting valine.
[b] Change in absorbance (at 420 nm) per hour per milligram of protein.
[c] Enzyme assayed in the presence of 1 mM L-valine.

Two important observations can be made regarding these results. pGMM150 and pGMM151 produce isomeroreductase but at an extremely low level. This low level is due to the lack of *ilvY*, which is necessary for efficient expression of *ilvC*. pGMM201, which has both *ilvC* and *ilvY*, produces isomeroreductase at a very high level. The lack of *ilvY* expression from pMD8, pGMM150, or pGMM151 indicates that the *Bgl*II site between *ilvA* and *ilvC* must lie within *ilvY*.

Physical location of *ilvY*. To locate *ilvY* on the *ilv* physical map, plasmids were digested with *Pst*I and *Eco*RI endonucleases and compared after electrophoresis on agarose gels. pBR322 (Fig. 2, track 1) yielded two fragments (fF and fG) upon digestion with *Pst*I plus *Eco*RI. These two fragments were also found in pGMM201 (Fig. 2, track 2), which has the *ilvAYC* fragment cloned into the *Pst*I site of pBR322. pGMM201 yielded five *Eco*RI *ilv* fragments, two of which (fA and fB) are present in pGMM52 (Fig. 2, track 3). fA and fB together carry the complete coding sequences for *ilvA* and *ilvY*, with fA specifying all of *ilvA* (G. McCorkle, personal communication). pGMM150 and pGMM151 (tracks 4 and 5, Fig. 2) have fragments fC and fE found in pGMM201. Fragments fC and fE carry part of *ilvC*; fD in pGMM201 and a different fragment in pGMM150 and pGMM151 carry the remainder of *ilvC*. This difference is due to the separate derivation of *ilv* DNA in these plasmids. The *Bgl*II site used to construct pGMM150, pGMM151, and pMD8 lies within fB, close to the adjacent fragment, fE. pMD8 shows fA but not fB upon digestion with *Pst*I plus *Eco*RI (data not shown). This *Bgl*II site is within *ilvY* and must be close to one end of the coding sequence. It therefore seems probable that fE carries much of the *ilvC* control region and will be the subject of further investigation.

pMD4. Analysis of the results presented above shows that the *ilvC-lac* template DNA, pMD4, must carry *ilvY* as it also carries *ilvA*. Therefore, this DNA should show normal control of the *ilvC-lac* fusion in the *ilvY* strains CU827 and CU838. To overcome problems of plasmid copy number, the *Hin*dIII *ilvC-lac* fusion fragment was cloned into the large low-copy-number plasmid RP4, which has only a single *Hin*dIII site. The resultant recombinant plasmid was called pMD7. Strains CU827 and CU838 were transformed with pMD7, and Ap^r Tc^r Km^s clones were tested for inducibility on lactose MacConkey agar. Strains CU827/pMD7 and CU838/pMD7 were Lac^+ only in the presence of the inducer acetohydroxybutyrate. These experiments provide final in vivo proof that *ilvY* indeed lies between *ilvA* and *ilvC*.

DISCUSSION

Further study of the mechanism by which acetohydroxy acids induce β-galactosidase in an in vitro coupled transcription-translation system with DNA containing the *lac* genes under control of the *ilvC* promoter has provided genetic evidence for the obligatory involvement of a positive-control element. This involvement was revealed when it was observed that S-30 extracts derived from two strains of *E. coli* containing deletions in the *ilvA-ilvC* region were unable to form β-galactosidase when *ilvC-lac* DNA was the template. That the genetic deficiency might be between *ilvA* and *ilvC* was indicated by the finding that three smaller deletions (one extending from the *ilvE* gene only into, but not through, *ilvA*; another deleting only part of *ilvA*; and the third deleting only part of *ilvC*) all allowed retention of the capacity to support acetohydroxybutyrate-dependent synthesis of β-galactosidase. Final proof of the location of the gene specifying the positive-control element was shown by the fact that only plasmids containing the entire region between *ilvA* and *ilvC* were able to restore the capacity to form the positive factor to the *ilvDAC* strain.

The gene specifying positive control has been designated *ilvY*, and the positive-control element itself is referred to as upsilon (υ). Preliminary experiments indicate that upsilon is a multimeric protein with approximately 35,000-dalton subunits.

Of some interest, but not explained, is the fact that thus far no evidence has been obtained for the in vitro synthesis of upsilon itself. All of the templates containing the *ilvC-lac* fusion also contained *ilvY*. If upsilon had itself been formed in vitro, the appearance of β-galactosidase should have been delayed. The reason for this failure is being explored.

In the course of examining the properties of the cloned fragments of the *ilv* cluster in vivo, we found that strains containing the *ilvC-lac* fusion grew on lactose minimal agar supplemented with isoleucine and valine. Thus, even when acetohydroxy acid synthase was both repressed and inhibited, there was sufficient acetohydroxy acid formed to allow induction. In contrast, on a rich medium like MacConkey lactose agar, exogenous acetohydroxybutyrate had to be added to produce the Lac^+ phenotype. A merodiploid of the *ilvC::lac/ilvC*$^+$ type grew prototrophically on lactose minimal medium but was Lac^- on MacConkey lactose agar even in the presence of exogenous acetohydroxybutyr-

ate. The probable reason for this paradoxical behavior is that the isomeroreductase formed by the intact *ilvC*$^+$ gene (and induced by acetohydroxybutyrate) removed the inducer and also prevented β-galactosidase induction. *ilvC*::*lac*/*ilvC* merodiploids are Lac$^+$ on MacConkey agar.

ACKNOWLEDGMENT

This work was supported by Public Health Service research grant GM12522 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Allet, B., and A. I. Bukhari.** 1975. Analysis of bacteriophage Mu and λ-Mu hybrid DNAs by specific endonucleases. J. Mol. Biol. **92**:529–540.
2. **Arfin, S. M., B. Ratzkin, and H. E. Umbarger.** 1969. The metabolism of valine and isoleucine in *Escherichia coli*. XVII. The role of induction in the derepression of acetohydroxy acid isomeroreductase. Biochem. Biophys. Res. Commun. **37**:902–908.
3. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
4. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**:293–300.
5. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloned vehicles. II. A multiple cloning system. Gene **2**:95–113.
6. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**: 541–556.
7. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Syke, and M. H. Richmond.** 1971. Properties of an R-factor from *Pseudomonas aeruginosa*. J. Bacteriol. **108**:1744–1749.
8. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
9. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta **383**:457–463.
10. **Kline, E. L., C. S. Brown, W. G. Coleman, Jr., and H. E. Umbarger.** 1974. Regulation of isoleucine-valine biosynthesis in an *ilvDAC* deletion strain of *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **57**: 1144–1151.
11. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**:1072–1074.
12. **Marsh, N. J., and D. E. Duggan.** 1972. Ordering of mutant sites in the isoleucine-valine genes of *Escherichia coli* by use of merogenotes derived from F14: a new procedure for fine structure mapping. J. Bacteriol. **109**:730–740.
13. **McCorkle, G. M., T. D. Leathers, and H. E. Umbarger.** 1978. Physical organization of the *ilvEDAC* genes of *Escherichia coli* strain K-12. Proc. Natl. Acad. Sci. U.S.A. **75**:89–93.
14. **Meagher, R. B., R. J. Shepherd, and H. W. Boyer.** 1977. The structure of cauliflower mosaic virus. I. A restriction endonuclease map of cauliflower mosaic virus DNA. Virology **80**:362–375.
15. **Murray, N. E., W. J. Brammar, and K. A. Murray.** 1977. Lamboid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:53–61.
16. **Pledger, W. J., and H. E. Umbarger.** 1973. Isoleucine and valine metabolism in *Escherichia coli*. XXII. A pleiotropic mutation affecting induction of isomeroreductase activity. J. Bacteriol. **114**:195–207.
17. **Ratzkin, B., S. M. Arfin, and H. E. Umbarger.** 1972. Isoleucine and valine metabolism in *Escherichia coli*. XVIII. The induction of acetohydroxy isomeroreductase. J. Bacteriol. **112**:131–141.
18. **Rosner, J. L.** 1972. Formation, induction and curing of bacteriophage P1 lysogens. Virology **48**:679–689.
19. **Sidikaro, J., and M. Nomura.** 1975. *In vitro* synthesis of the E3 immunity protein directed by Col E3 plasmid deoxyribonucleic acid. J. Biol. Chem. **250**:1123–1131.
20. **Smith, J. M., F. J. Smith, and H. E. Umbarger.** 1979. Mutations affecting the formation of acetohydroxy acid synthase II in *Escherichia coli* K-12. Mol. Gen. Genet. **169**:299–314.
21. **Smith, J. M., D. E. Smolin, and H. E. Umbarger.** 1976. Polarity and the regulation of the *ilv* gene cluster in *Escherichia coli* K-12. Mol. Gen. Genet. **148**:111–124.
22. **Smith, J. M., and H. E. Umbarger.** 1977. Characterization of fusions between the *lac* operon and the *ilv* gene cluster in *Escherichia coli*: *ilvC*-*lac* fusions. J. Bacteriol. **132**:870–875.
23. **Wasmuth, J. J., and H. E. Umbarger.** 1973. Participation of branched-chain amino acid analogues in multivalent repression. J. Bacteriol. **116**:562–570.
24. **Wild, J., J. M. Smith, and H. E. Umbarger.** 1977. In vitro synthesis of β-galactosidase with *ilv*-*lac* fusion deoxyribonucleic acid as template. J. Bacteriol. **132**: 876–883.
25. **Wu, H. C., and T. C. Wu.** 1971. Isolation and characterization of a glucosamine-requiring mutant of *Escherichia coli* K-12 defective in glucosamine-6-phosphate synthetase. J. Bacteriol. **105**:455–466.