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Characterization of Fusions Between the *lac* Operon and the *ilv* Gene Cluster in *Escherichia coli*: *ilvC-lac* Fusions

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By means of the general procedure of Casadaban (J. Mol. Biol. **104:** 541-556, 1976), the *lac* genes carried on a lambda-Mu-1 hybrid phage were inserted into a temperature-inducible Mu-1 prophage that had earlier been inserted into a site near the beginning of the *ilvC* gene of *Escherichia coli* strain K-12. Selection of temperature-resistant derivatives of the lysogen resulted in a fusion of the *lac* genes to a region of deoxyribonucleic acid that is transcribed under the control of the *ilvC* regulatory elements. A strain bearing the fusion was shown to be inducible for β-galactosidase by acetohydroxybutyrate, a natural inducer of acetohydroxy acid isomeroreductase. Induction of the lysogen by mitomycin C led to the isolation of a plaque-forming lambda derivative carrying this *ilvC-lac* fusion.

In *Escherichia coli* K-12, the four steps in valine biosynthesis are catalyzed by enzymes that catalyze four analogous steps in isoleucine biosynthesis. For isoleucine biosynthesis, an additional enzyme, threonine deaminase, is required to form the first intermediate in the pathway (Fig. 1). The formation of this enzyme and three of the enzymes common to both pathways is controlled by a complex, multivalent repression mechanism (6). In contrast, the formation of one of the common enzymes, acetohydroxy acid isomeroreductase, is controlled by substrate induction rather than by end-product repression (2).

As Fig. 1 shows, the gene specifying the isomeroreductase, *ilvC*, lies between the two repressible transcriptional units of the *ilv* cluster, the *ilvEDA* operon and the *ilvB* gene. That its regulation is completely independent of the multivalent repression affecting the repressible enzymes is shown by the fact that it can be induced by the addition of acetohydroxybutyrate to an L-broth culture when the other enzymes are strongly repressed, whereas it can remain uninduced when the other enzymes are highly derepressed (11).

A study of the elements involved in the regulation of the *ilvC* gene has suffered from the lack of known regulatory mutations specifically affecting *ilvC* gene expression. No such mutations have been described probably because no growth condition is known that would distinguish between constitutive isomeroreductase mutants and wild-type cells or between noninducible mutants and those producing inactive isomeroreductase. This difficulty has now been overcome by applying the general procedure developed by Casadaban (4) that allows the fusion of the *lac* operon to nearly any regulatory region on the *E. coli* chromosome. By means of this procedure, we have been able to isolate mutants containing fusions that place the *lacZ* and *Y* genes under control of the *ilvC* regulatory region. From one such mutant, it has been possible to isolate a plaque-forming transducing phage carrying the *ilvC-lac* fusion.

MATERIALS AND METHODS

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

Media and growth conditions. The medium of Davis and Mingioli (5), modified by the omission of citrate and increasing 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, except for valine, which was 100 μg/ml; and all vitamins, 1 μg/ml. L-broth was used as the rich medium (3). SB medium was used for the preparation of Mu lysates (7). For solid media, agar (Difco) at a final concentration of 1.5% was added to the above media. X-gal plates were prepared from minimal glucose agar containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml.

Mutagenesis. Mutagenesis with the bacteriophage Mu-1 *cts62K1010* was performed by adding Mu *cts* at a multiplicity of infection of 1 to an L-broth-grown culture of the strain to be mutagenized that had been suspended in 5 mM $MgSO_4$-$CaCl_2$. Since a temperature-sensitive Mu was employed, absorption was carried out for 15 min at 30°C. After absorption, the Mu-treated cells were suspended in an appropriately sup-

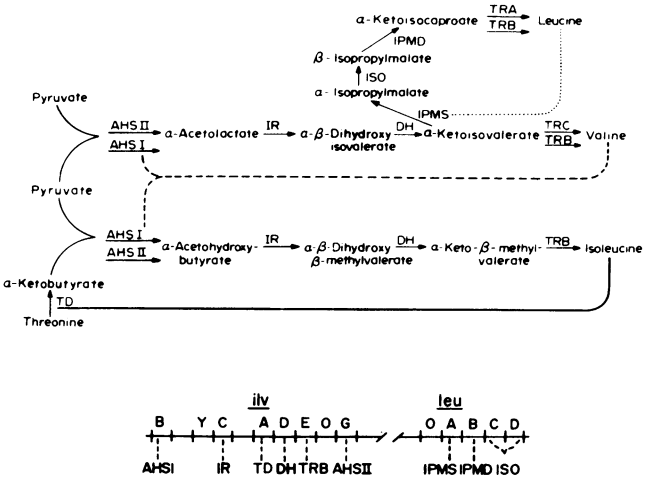

FIG. 1. *Biosynthesis of isoleucine, valine, and leucine. The enzymes catalyzing the indicated steps are abbreviated and the corresponding structural genes (where known) are indicated in parentheses as follows: TD (ilvA), threonine deaminase; AHSI (ilvB), end-product-inhibited acetohydroxy acid synthase; AHSII (ilvG), end-product-noninhibited acetohydroxy acid synthase; IR (ilvC), acetohydroxy acid isomeroreductase; DH (ilvD), dehydroxy acid dehydrase; TRA, transaminase A; TRB (ilvE), transaminase B; TRC, transaminase C; IPMS (leuA), α-isopropylmalate synthase; ISO (leuCD), isopropylmalate isomerase; IPMD (leuB), β-isopropylmalate dehydrogenase. Gene ilvO is a locus that, upon mutation from the wild-type state, provides a cis-acting regulatory element needed for ilvG expression and enhanced ilvEDA expression. Gene ilvY specifies an element needed for induction of ilvC by acetohydroxy acids. The mapping of ilvO, ilvG, and ilvY will be reported at a later date (J. M. Smith and H. E. Umbarger, in preparation).*

TABLE 1. *Bacterial strain list*

Strain	Sex	Genotype	Source or reference
CSH7	F⁻	*lacY thi strA*	Cold Spring Harbor Laboratory
CSH26	F⁻	*ara Δ(lac-pro) thi*	Cold Spring Harbor Laboratory
CU520	F⁻	*rbs-221 metE201 leu-455 galT12*	13
CU544	F⁻	*ilvC44 rbs-221 leu-455 galT12*	P1-mediated transduction of CU520 with AB1412 as donor
CU558	F⁻	*rbs-221 metE201 leu-455 galT12 rpsL*	P1-mediated transduction of CU520 and CSH7 as donor
CU710	F⁻	*ara Δ(lac-pro) thi ilvC2083*::Mu-1 *cts62K1010*	This paper
CU711	F⁻	*ara Δ(lac-pro) thi ilvC2083*::Mu-1 *cts62K1010*::λpl(209)	This paper
CU712	F⁻	*ara Δ(lac-pro) thi ilvC2083*::Mu-1 *cts62K1010*::λp123(209)	This paper
CU713	F⁻	*ara Δ(lac-pro) thi ilvC2083*::λpl(209)	This paper
CU748	F⁻	*ara nalA Δ(lac-pro) thi ilvC2083*::-Mu-1 *cts62K1010*	Diethyl sulfate mutagenesis of CU710
AB1412		*ilvC44 gal-6 his-1 thi-1 argH1 metB1 mlt-2 xyl-7 malA1 rpsL ara-13 tonA2 lac*	10

TABLE 2. *Phage list*[a]

Phage	Genotype	Source or reference
Mu-1	*cts62 Kam1010*	M. Howe (7)
Mu-1	*c25*	M. Howe
λpl(209)	*lacA?ZYO'-Δw209-trp'AB'*::(+Mu')	M. J. Casadaban (4)
λp123(209)	*lacA?ZYO'-Δw209-trp'ABCDE?'*::(-Mu')	M. J. Casadaban (4)
λh80(*int*)Δ-9		M. J. Casadaban
λp*ilv-lac-1*	*lacA?ZYO' ilvC2083*[a]	This paper

[a] Whether the Mu-excision event either added to or subtracted from the DNA in the *lacO'-Δw209-trp'AB'* region of the original λpl(209) phage has not been established.

plemented minimal medium and incubated at 30°C to allow segregation of the mutants. Muc25 (a clear plaque mutant) was sometimes added at a multiplicity of infection of 1 to kill any nonlysogens that may have escaped the first Mu infection.

Mutagenesis by diethyl sulfate was performed with cells from a fresh, fully grown L-broth culture suspended in one-half the growth volume of 1 M tris(hydroxymethyl)aminomethane, pH 7.5. A 25-μl portion of diethyl sulfate was added to 0.2 ml of the cell suspension. After incubation with shaking at 37°C for 5 to 10 min, the mutagenized cells were washed twice with minimal medium without carbon source. The mutagenized cells were then suspended in 5 ml of supplemented minimal medium for segregation of mutants.

Transduction. The P1*cmclr*100 bacteriophage was used for generalized transductions. P1 transductions were performed by the method of Rosner (12). Specialized transductions with lambda phages were performed by the method of Shimada et al. (13).

Preparation of phage lysates. P1*cm* lysates were prepared by heat induction (12). Lambda lysates were prepared by lytic growth on strain CSH26. Mitomycin C at a final concentration of 1 μg/ml was added after infection for the lytic growth of λ*pilv-lac-1*. After lysis was complete, the lysates were treated with chloroform and clarified by centrifugation. The lysates were stored in the cold in 0.01 M $MgSO_4$ over chloroform. Mu lysates were prepared and stored as described by Howe (7). Non-temperature-sensitive lambda lysogens were induced by adding mitomycin C at a final concentration of 1 μg/ml to an early log-phase culture of the lysogen. Incubation was continued at 37°C until lysis had occurred and the resulting lysate had been treated as above.

Enzyme assays. Preparation of crude extracts and assay of the isoleucine and valine biosynthetic enzymes were performed as described previously by Smith et al. (14). The enzymatic activity of β-galactosidase was assayed by the method of Zubay et al. (17). External induction of the *ilvC* gene with acetohydroxybutyrate was performed by the method of Ratzkin et al. (11). The method of Lowry et al. (9) was used for the determination of protein in crude extracts. Enzyme specific activities are expressed as nanomoles per minute per milligram of protein.

Chemicals. All reagents used were of highest purity commercially available. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (x-gal) was purchased from either Vega-Fox (Tucson, Ariz.) or Sigma (St. Louis, Mo.). The ethyl, acetoxy ester of acetohydroxybutyrate was purchased from the Edinboro Foundation (Edinboro, Pa.). Streptomycin was obtained from Calbiochem (San Diego, Calif.). All other chemicals were purchased from Sigma.

Ampicillin enrichment. Ampicillin counterselection as described previously was employed for the enrichment of auxotrophs (14).

RESULTS

Isolation of Mu-induced *ilvC* auxotrophs. Since the application of the Casadaban method (4) to isolate *lac* fusions requires the isolation of Mu-induced auxotrophs, strain CSH26 was mutagenized with Mu-1 *cts62K*1010 as described above. After two ampicillin counterselections at 30°C, the mutagenized culture was plated on minimal agar supplemented with suboptimal quantities of isoleucine and valine. Small colonies were scored for their Ilv phenotype and temperature sensitivity. The strains that were Ilv^- and temperature sensitive were then examined for the sites of their presumed Mu insertions by observing growth on petri plates containing various isoleucine and valine biosynthetic intermediates. One of those that was identified as an *ilvC* mutant (i.e., by its growth on dihydroxy acids) was retained as strain CU710.

Isolation of λp1(209) and λp123(209) lysogens. λp1(209) and λp123(209) lysogens of a strain (CU710) containing Mu in *ilvC* were isolated by the method of Casadaban (4). Droplets containing the lysates were applied to a lawn of the strain. After incubation for 12 h at 30°C, cells from the center of each turbid plaque were purified by single-colony isolation on L-agar plates seeded with λh80Δ*int*9 to kill any nonlysogens. A presumed lysogen carrying λp1(209) was designated CU711 and one carrying λp123(209) was designated CU712. Both were examined for their capacity to give rise to *ilv-lac* fusions.

Isolation of a strain with a fusion placing the *lac* genes under control of the *ilvC* regulatory region. Heavy suspensions of the two lysogens described above were incubated on minimal agar plates containing lactose as carbon source, repressing concentrations of leucine, valine, and isoleucine and ca. 6 mM acetohydroxybutyrate. The plates were incubated at 42°C for 12 h and then at 37°C until colonies appeared. Since the *ilvC* product is substrate induced, acetohydroxybutyrate was required to provide for a high-level transcription initiated from the *ilvC* promoter. Incubation at 42°C was necessary to induce Mu phage in those cells in which fusion events deleting the phage had not occurred. This step reduced the background from Lac^- cells using proline as a carbon source. (Proline was included since the lysogens contained *pro-lac* deletions.)

Since the selection was for any event that allowed the expression of the *lac* genes, the colonies that appeared were then screened for expression of the *lac* genes associated with the *ilvC* gene regulatory signal. The initial screening was for those strains that could ferment lactose on a McConkey plate only in the presence of acetohydroxybutyrate. Only strain CU711, which was the lysogen bearing the λp1(209) phage, gave rise to such acetohydroxybutyrate-inducible Lac^+ derivatives.

From the fact that only a λp1(209) lysogen of strain CU710 gave rise to derivatives with an acetohydroxybutyrate-induced Lac$^+$ phenotype, the orientation of the Mu insertion in this strain was deduced to be that shown in Fig. 2. This is termed a (−) orientation. The orientation is designated (−) if the *c* gene on the phage is clockwise with respect to the *S* gene on the *E. coli* chromosome map, as usually represented (1). Figure 2a shows the insertion that is presumed to have occurred during the lysogenization of strain CU710 by λp123(209), and Fig. 2b shows that presumed to have occurred with λp1(209). Figure 2b also shows the deletion that presumably resulted in fusion of the *lac* genes to the *ilvC* regulatory region. However, it has not been determined whether all of the *trp* and Mu deoxyribonucleic acid (DNA) between the *lac* and *ilv* regions of strain CU711 was deleted during the fusion event, as the drawing implies.

Characterization of a fusion placing the *lac* genes under the control of the *ilvC* regulatory region. A fusion strain (CU713) that could only ferment lactose in the presence of acetohydroxybutyrate was chosen for further characterization. If the *lac* genes had been fused to the *ilvC* regulatory region, they should have responded in the same manner physiologically, as does the *ilvC* gene itself. The ability of the *lac* genes to respond to external induction was tested by measuring the induction of the *lac* genes during growth in a rich medium containing acetohydroxybutyrate. The data in Table 3 show that over 100-fold induction was obtained, which is even greater than the induction obtained with the wild-type *ilvC* gene in strain CSH26.

Mapping of the *ilvC-lac* fusion. To locate the *lac* genes inserted in *ilvC* with respect to other *ilvC* markers, genetic crosses were performed. Initially, the *ilvC44* marker was used. Phage grown on strain CU544 (*ilvC44 rbs-221*) was used as the donor and selection was for Ilv$^+$. The Rbs phenotype of the transductants was then tested. The reciprocal cross was also

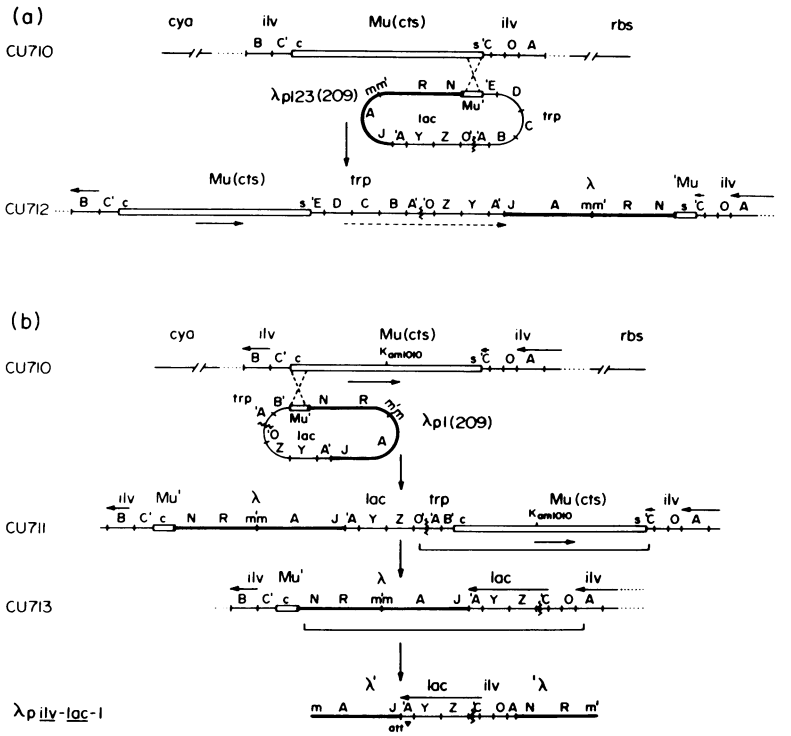

FIG. 2. *Isolation of ilvC-lac fusions and a lambda phage carrying an ilvC-lac fusion. (a) The lysogenization of strain CU710 with the λp123(209) phage, as described in the text, did not lead to the isolation of ilvC-lac fusions since the direction of transcription for the inserted lac genes in strain CU712 is opposite that of the ilvC gene. (b) The lysogenization of strain CU710 with the λp1(209) phage resulted in strain CU711 where the lac genes are inserted in the correct orientation with respect to the ilvC gene. Deletion of the Mu cts phage in strain CU711 resulted in fusion events that placed the lac genes under ilvC control. A specialized transducing phage carrying an ilvC-lac fusion was isolated after induction of strain CU713 with mitomycin C as described in the text.*

performed. The data in Table 4 show that the gene order is *ilvC44-lac-rbs-221*. Since *ilvC44* is the *ilvC* mutation most proximal to *ilvA* available to us (10), we could not further localize the insertion point of the *lac* genes. All other *ilvC* mutants tested also bore lesions on the *ilvA* distal side of the *lac* insertion (data not shown). We conclude therefore that there is very little, if any, of the *ilvC* structural gene remaining on the *ilvA* side of the *lac* region.

Isolation of a λ phage carrying the *ilvC* fusion. Deletions that place the *lac* genes under the control of other promoters should not affect the λ prophage inserted in the *ilvC* gene since the prophage lies distal to the *lac* genes (Fig. 2). Thus, the isolation of a λ phage carrying the *lac* genes fused to the *ilvC* regulatory region should be feasible. Since the λ phage employed here is deleted for the phage *att* site, it was desirable to isolate the transducing phage as a plaque former.

A mixed lysate was prepared from strain CU713 containing the *ilvC-lac* fusion by induction of the lambda phage with mitomycin C, as described above. To distinguish the phages carrying the *ilvC-lac* fusion from the other phages, the lysate obtained was titrated on strain CSH26 on x-gal plates in the presence of 6 mM acetohydroxybutyrate. Several blue plaques that appeared on the x-gal plates were purified by single plaque isolation on the same host until the lysate obtained was of one phage type.

One such phage obtained was designated λp*ilv-lac-1* and retained for further characterization. In the absence of acetohydroxybutyrate, λp*ilv-lac-1* plaques with strain CSH26 on x-gal plates were almost colorless. Thus, the phage is assumed to carry *lac* genes that are under the control of the *ilvC* promoter region. The lower part of Fig. 2b shows the excision event that is assumed to have led to the formation of λp*ilv-lac-1*.

TABLE 3. *Induction by acetohydroxybutyrate*[a]

Strain	Activity determined	Sp act	
		Noninduced	Induced
CSH26	Isomeroreductase	0.67	17.3
CU713	β-Galactosidase	3.3	345

[a] Cells were grown in L-broth at 37°C. Induced cells of strain CSH26 were harvested 25 min after induction, and those of strain CU713 were harvested 15 min after induction.

DISCUSSION

The general method of Casadaban (4) for isolating *lac* gene fusions to the promoters of any nonessential gene has opened many new areas of investigation in the study of gene regulation. We have used this procedure for the generation of fusions linking the *lac* genes to the *ilvC* promoter. Strain CU713 is the result of such a fusion event.

With this strain, the isolation of *ilvC* regulatory mutants should be made possible. In *E. coli*, there has been no means for the identification of *ilvC* regulatory mutants other than screening randomly selected isolates for an inducer-independent formation of isomeroreductase. Since only one gene product, the isomeroreductase itself, is known to be induced by the acetohydroxy acids, it is also difficult to distinguish between noninducible mutants and those that contain lesions in the *ilvC* structural gene. One strain that might provide an example of a noninducible mutant was described by Ratzkin et al. (11). This strain forms a very low level of what appears to be normal isomeroreductase, but the enzyme level is not increased by induction. The strain was derived from *E. coli* strain W and so has not been studied genetically.

We have begun to employ strain CU713 for the isolation of putative *ilvC* regulatory mutants by selecting for the expression of β-galactosidase under conditions that do not induce the *ilvC* gene. Similarly, we have selected mutants of CU713 that have lost the capacity to induce the *lac* operon even in the presence of acetohydroxybutyrate. The in vivo and in vitro analysis of these regulatory mutants will be reported in a later paper (J. M. Smith, J. Wild, and H. E. Umbarger, in preparation). This analysis should lead to a greater understanding of how the *ilvC* gene is regulated.

The isolation of a phage (λp*ilv-lac-1*) containing the *ilvC-lac* fusion from strain CU713 makes possible the in vitro synthesis of β-galactosidase under the control of the *ilvC* promoter. The following paper describes a study of this in vitro system (16). The use of this fusion DNA in an in vitro protein synthesizing system has consid-

TABLE 4. *Genetic analysis of the ilv-lac fusion*

Donor	Recipient	Selected genotype	Phenotype scored	% Donor phenotype	Probable order
CU544 *ilvC44 rbs-221*	CU713 *ilvC::lac-2083*	*ilv*$^+$	Rbs	84 (42/50) Rbs$^-$	*ilvC44 ilvC-lac-2083 rbs-221*
CU713 *ilvC::lac-2083*	CU544 *ilvC44 rbs-221*	*ilv*$^+$	Rbs	13 (14/110) Rbs$^+$	*ilvC44 ilvC-lac-2083 rbs-221*

erable advantage over the use of wild-type *ilvC* DNA. Although both the isomeroreductase and β-galactosidase are stable enough to allow a prolonged assay period when the in vitro synthesis has yielded only a small amount of product, the turnover number of the isomeroreductase with the best substrate is such that a radioactive assay is required. Furthermore, the radioactive product must be chromatographically separated from the substrate and its spontaneous breakdown products. Whereas such experiments have been performed, the manipulations do not lend themselves to convenient routine use (J. Wild, J. M. Smith, and H. E. Umbarger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K161, p. 213).

The analysis of the fusion strain and the lambda transducing phage carrying the fusion allows an independent deduction to be made regarding the direction of transcription of the *ilvC* gene. The mapping of the site of insertion into *ilvC* revealed that the *lac* genes have been inserted at the *ilvA* proximal end of the *ilvC* gene (Table 4). Indeed, the original Mu insertion used to generate the fusion has been found to be the most *ilvA* proximal lesion in *ilvC* that we have encountered. Whether the insertion is in the structural gene itself or in a DNA region specifying a leader sequence for *ilvC* messenger ribonucleic acid is not known. The regulation of the inserted *lac* genes by acetohydroxybutyrate is evidence that the *ilvC* regulatory region is proximal to *ilvA*. Thus, the conclusion that the control region of the *ilvC* gene lies closest to *ilvA* and that the direction of transcription is clockwise on the *E. coli* chromosome is in agreement with the evidence for the clockwise transcription of the *ilvEDA* operon reported earlier (14), since it had been shown earlier by hybridization to the separated strands of λh80d*ilv* that both *ilvC* and *ilvEDA* are transcribed off the same strand (8, 15).

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