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Regulation of Expression of the *ilvB* Operon in *Salmonella typhimurium*

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The *ilvB* gene of *Salmonella typhimurium* encodes the valine-sensitive form of acetohydroxy acid synthase, acetohydroxy acid synthase I, which catalyzes the first step in the parallel biosynthesis of isoleucine and valine. Although nearly all of the other genes involved in this pathway are clustered at minute 83, *ilvB* was found to lie at minute 80.5. Expression of *ilvB* was shown to be nearly completely repressed by the end products leucine and valine. Studies in which we used strains with mutations in *cya* (adenylate cyclase) and *crp* (cAMP receptor protein) demonstrated that synthesis of acetohydroxy acid synthase I is enhanced by the cAMP-cAMP receptor protein complex. Although no stimulation was achieved by growth on poor carbon sources, introduction of *crp* on a multicopy plasmid led to markedly increased expression. Strains of *S. typhimurium* lacking valine-resistant acetohydroxy acid synthase II (*ilvG*) are like *Escherichia coli* K-12 in that they are not able to grow in the presence of L-valine owing to a conditional isoleucine auxotrophy. The valine toxicity of these *ilvG* mutants of *S. typhimurium* was overcome by increasing the level of acetohydroxy acid synthase I. Enzyme activity could be elevated either by maximally derepressing expression with severe leucine limitation, by introduction of either *ilvB* or *crp* on a multicopy plasmid, or by the presence of the *ilv-513* mutation. This mutation, which is closely linked to genes encoding the phosphoenol pyruvate:sugar phosphotransferase system (*pts*), causes highly elevated expression of *ilvB* that is refractory to repression by leucine and valine, as is the major *ilv* operon. The response of *ilvB* to the cAMP-cAMP receptor protein complex was not affected by this lesion. Data obtained by using this mutant led us to propose that the two modes of regulation act independently. We also present some evidence which suggests that *ilvB* expression may be affected by the phosphoenol pyruvate:sugar phosphotransferase system.

The *ilvB* gene of *Salmonella typhimurium* specifies one of the isozymes of acetohydroxy acid synthase (AHAS), AHAS I, which catalyzes the first step in parallel pathways leading to the biosynthesis of isoleucine and valine. Although much work has been done to elucidate the mechanism of regulation of this gene in *Escherichia coli* K-12, less information is available concerning its expression in *S. typhimurium*. Therefore, we carried out studies to determine the mode of regulation of this gene in *S. typhimurium* based on the information available from studies done on *E. coli*.

At least three factors have been described that influence the level of AHAS I activity in cells. First, in both *E. coli* and *S. typhimurium*, the enzyme is subject to feedback inhibition by valine (2, 29). Second, enzyme synthesis in both species is repressed by leucine and valine (15, 40); sequence data from *E. coli* demonstrate that this repression is mediated by attenuation (18, 21). Third, the synthesis of AHAS I in *E. coli* is stimulated by elevated levels of cAMP associated with the cAMP receptor protein (CRP) (46). This mode of regulation is highly unusual for an enzyme that is apparently only involved in biosynthesis. In this paper we present results obtained with strains of *S. typhimurium* that have mutations affecting the synthesis of cAMP (*cya*) or the receptor protein (*crp*) which indicate that a similar form of regulation exists in this species.

Strains of *S. typhimurium* that are mutated in *ilvG* and therefore express only the valine-sensitive isozyme of acetohydroxy acid synthase, AHAS I, are analogous to *E. coli* K-12 in that they are not able to grow on valine-containing media. The valine present inhibits the formation of the isoleucine precursor α-aceto-α-hydroxybutyrate, and thus the cells are starved for isoleucine (40). We found that valine toxicity of these *ilvG* mutants can be overcome by raising the level of AHAS I. A similar finding has not been reported for *E. coli* K-12. We also describe a mutant which lacks valine-insensitive AHAS II but is able to grow in the presence of high concentrations of valine. Expression of AHAS I in this strain is elevated and refractory to repression by leucine and valine, as is the major *ilv* operon.

MATERIALS AND METHODS

Bacterial strains and culture media. All of the strains used in this study are derivatives of *S. typhimurium* LT-2 (Table 1). All of the plasmids are derivatives of pBR322. The minimal medium used was that of Davis and Mingioli (13) modified by omitting the citrate and raising the glucose concentration to 0.5% (wt/vol). Solid medium was prepared by adding 1.5% (wt/vol) agar (Difco Laboratories). The concentrations of potassium phosphate and ammonium sulfate were doubled to increase the buffering capacity of the medium when cultures were used for preparation of cell extracts. The minimal medium used for growth of *ilvG* mutants was supplemented with 20 mg of calcium pantothenate per liter (42).

Indicator plates containing nutrient agar (Difco), 1 g of NaCl per liter, 0.2% glycerol, 0.2% rhamnose, and 50 mg of 2,3,5-triphenyltetrazolium chloride per liter were used in the detection of adenylate cyclase (*cya*) and CRP (*crp*) mutants (36).

Strain construction. P22-mediated transduction was performed by the method described by Margolin (34), except that the HT105/4 mutant derived by Schmieger (44) was used.

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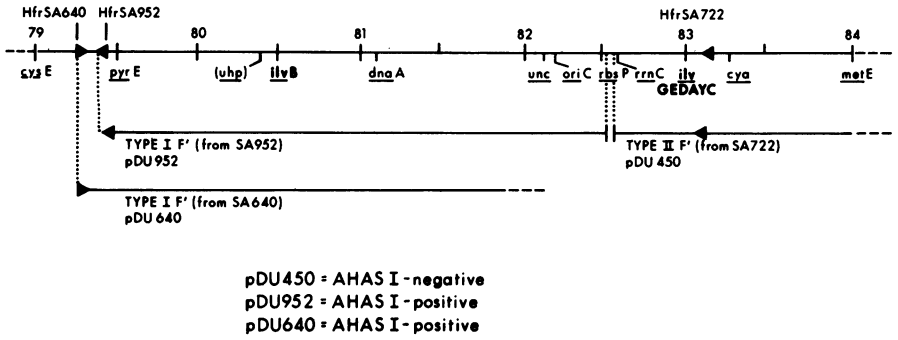

FIG. 1. Genetic map location of *ilvB*.

To obtain a Tn*10* transposon closely linked to a gene of interest, the method of Davis et al. (14) was used. Tetracycline-sensitive derivatives were isolated by the ampicillin selection procedure described by Kleckner et al (27).

Bacterial conjugation and isolation of F' *ilvB* factors. The conjugation protocol described by Miller (36) was used. When isolating F' strains from Hfr strains, we used a *recA* recipient (31). F' *ilvB* transconjugants were tested for sensitivity to phage MS2 (36) and loss of *ilvB* by growth under nonselective conditions or by treatment with acridine orange (23).

Preparation of cell extracts for enzyme assays. Cells were routinely grown as 250-ml batch cultures at 37°C with aeration. Cells in mid-log phase were disrupted by sonic oscillation. Extracts were prepared as previously described, except that pyridoxal phosphate was omitted from the resuspension buffer (4).

Enzyme assays. Assays for the following enzymes were performed as previously described: threonine deaminase (7), transaminase B (17), AHAS (45), β-isopropylmalate dehydrogenase (41), homoserine dehydrogenase (12), and histidinol phosphate phosphatase (35). Valine at a concentration of 1 mM was added to the AHAS assay to distinguish the valine-insensitive fraction of the enzyme (AHAS II) from the total AHAS activity detected (2).

All specific activities were expressed as nanomoles of product formed per minute per milligram of protein. Protein concentration was measured by the method of Lowry et al. (32).

Bacterial transformation. Strains of *S. typhimurium* were transformed by the procedure of Lederberg and Cohen (30). All plasmid constructions were done in *E. coli* K-12. These strains were transformed by the method of Mandel and Higa (33). Plasmids were transferred to an *hsdR* mutant of *S. typhimurium*, mutant DU7 (6), prior to transformation of other strains of *S. typhimurium*. This step increased the frequency of transformants by lowering the loss of plasmid DNA by the action of the restriction system of *S. typhimurium* on DNA prepared from a heterologous host.

Plasmid construction. The F' plasmid DNA was isolated by the method of Hansen and Olsen (20). All digestions were performed by the method of Davis et al. (14). *ilvB* plasmid pDU101 was constructed from an *Hin*dIII (Bethesda Research Laboratories) digest of F' pDU640 and was cloned into pBR322 by using T4 DNA ligase (Bethesda Research Laboratories) (47). The original *ilvB* clone was subcloned by using *Eco*RI and *Hin*dIII. Plasmid pDU111 was constructed by digesting both pHA7 and pDU101 with *Eco*RI, mixing the two, and ligating (see Fig. 3). The ligation mixture was used to transform an *ilvG ilvB* double mutant, DU2603. Transformants were selected on minimal medium supplemented with 1 g of valine per liter and ampicillin. Plasmid DNAs were prepared from transformants and digested with *Eco*RI and *Hin*dIII to determine whether they contained all of the *ilvB* information from pDU101, as well as all of pHA7. One such plasmid was isolated, pDU111.

Plasmid DNA was purified by the rapid alkaline lysis method of Birnboim and Doly (3) as modified by Ish-Horowicz and Burke (25) for most manipulations. When plasmid DNA was used for construction of recombinants, it was isolated by separating the plasmid DNA on a cesium chloride gradient as described by Humphreys et al. (24).

Chemicals. Most amino acids, sugars, and antibiotics were purchased from Sigma Chemical Co. β-Isopropylmalate was prepared by the method of Calvo and Gross (8). Technical grade cesium chloride was purchased from Kawecki Chemicals.

RESULTS

Mapping of *ilvB*. Strain DU2616 lacking AHAS activity (*ilvG ilvB*) was isolated following nitrosoguanidine mutagenesis (36) of *ilvG* mutant DU12. This strain was then used in mating experiments to locate *ilvB*. Matings with F' plasmid pDU540 having a mutation in *ilvG* (D. L. Blazey, R. Kim, and R. O. Burns, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, 1453, p. 112) that extended from *rrnC* (minute 82.5) through *metE* (minute 84) yielded no valine-sensitive prototrophs, demonstrating that *ilvB* was not contiguous with the major *ilv* gene cluster (43). Two F' plasmids were isolated that did not contain *ilvG* but did complement the mutant lacking AHAS activity. F' plasmid pDU952 complemented *rbsP* (minute 82.5) and *pyrE* (minute 79.5) and probably contained *oriC* (minute 82) as it was extremely unstable (22). The other F' plasmid, pDU640, was more stable and complemented *pyrE* but not *rbsP*. These results suggested that *ilvB* was located between *pyrE* and *oriC* (Fig. 1).

To locate *ilvB* more precisely, we isolated a strain with a Tn*10* element (*zia-2*) determined to be 22% linked to *ilvB* by P22 cotransduction but not contransducible with *rbsP* or *pyrE*. Isolation of tetracycline-sensitive derivatives from strains containing *zia-2*::Tn*10* via ampicillin selection (27) demonstrated that *ilvB* was closely linked to *uhp*, as 60% of the isolates that lacked AHAS I activity were unable to grow on glucose 6-phosphate as a sole carbon source. Thus, *ilvB* was located at minute 80.5, a position analogous to the position reported for *ilvB* in *E. coli* K-12 (39).

Effect of elevated levels of the *ilvB* gene on cell growth. The *ilvB* gene was cloned by standard procedures with *Eco*RI and *Hin*dIII digests of pBR322 and F' *ilvB* DNA from pDU640. We isolated a plasmid (pDU101) that complement-

TABLE 1. List of strains and plasmids

Strain or plasmid	Genotype	Source
S. typhimurium		
DU12	*ilvG236 ara gal pan-187*	D. Primerano
DU503	*ilvG236 ara-9*	Laboratory collection
DU513	*ilvG236 ilv-513 ara-9*	Spontaneous Val^r derivative of DU503
DU521	*ilvG236 gal leu-124 ilv-513*	HT105/4(LeU124):DU513[a]
DU608	Δ(*ilvGEDA236*)*691*	Laboratory collection
DU2636	*ilvG236 zia-2*::Tn*10*	Tet hop into DU2616
DU2603	*ilvG236 ilvB1 zia-2*::Tn*10*	HT105/4(DU2636):DU2616
DU2613	*ilvG236 ilv-513 ilvB1 zia-2*::Tn*10*	HT105/4(DU2603):DU513
DU2616	*ilvG236 ilvB1 ara-9 gal pan-187*	MNNG mutagenesis of DU12[b]
DU5586	*leu-500 supX-22 ara-9 ilvG236*	Laboratory collection
DU8800	*cysA crp zhb*::Tn*10*	Tet hop into NCR114
DU8811	*ilvG236 cya-961 zid-62*::Tn*10*	HT105/4(TT2104):DU503
DU8812	*ilvG236 crp zhb*::Tn*10*	HT105/4(DU8800):DU503
DU8821	*ilvG236 ilv-513 cya-961 zid62*::Tn*10*	HT105/4(TT2104):DU513
DU8822	*ilvG236 ilv-513 crp zhb*::Tn*10*	HT105/4(DU8800):DU513
NCR114	*crp cysA*	W. Dobrogosz
SA640	Hfr K2-2 *serA150*	K. Sanderson
SA722	Hfr K10 *serA150 pur-268*	K. Sanderson
SA952	Hfr K14 Δ(*leuBCD*)*39 ara-7*	K. Sanderson
TT2104	*argI539 proAB147 trp-130 zid-62*::Tn*10 cya-961*	J. Roth
DU5523	*cysK* Δ(*ptsPHI*)*42 trpB223 ilvG236 zid-64*::Tn*10*	Laboratory collection
DU5524	*pts-181 trpB223 ilvG236 zid-64*::Tn*10*	Laboratory collection
DU5525	*ptsI* Δ*crr-167 trpB223 ilvG236 zid-64*::Tn*10*	Laboratory collection
DU5526	*ptsH196 trpB223 ilvG236 zid-64*::Tn*10*	Laboratory collection
F′		
pDU450	F′ *ilvGEDA* from SA722	D. Blazey
pDU640	F′ *ilvB pyrE* from SA640	This paper
pDU952	F′ *ilvB oriC rbsP* from SA952	This paper
pBR322 derivatives		
pDU101	*ilvB*	From pDU640
pDU111	*ilvB crp* (*E. coli*)	pDU101 + pHA7
pHA7	*crp* (*E. coli*)	Aiba et al.[c]

[a] The HT105/4 lysate was made on Leu-124 and was used to transduce strain DU513.
[b] MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.
[c] See reference 1.

ed a mutant devoid of AHAS activity (mutant DU2603). The AHAS activity present in these transformants was inhibited 85% by valine, the same sensitivity that is found in *ilvG* mutants. The expression of the cloned *ilvB* gene in transformants of mutant DU2603 was reponsive to regulation by leucine and valine (Table 2), a property that indicates that the regulatory region was present on the plasmid. However, transformants of double mutant DU2603 and *ilvG* mutant DU503 were able to grow in concentrations of valine as high a 1 g/liter. This valine resistance was attributed to the high level of AHAS I in these organisms.

Role of leucine and valine in the regulation of *ilvB*. We attempted to physiologically raise the level of AHAS I activity to a point that would permit the growth of an *ilvG* mutant in media supplemented with 1 g of valine per liter. Strain DU5586 (*ilvG236 leu-500 supX*) was used since it is intrinsically limited for leucine biosynthesis owing to a mutation in the *leu* regulatory region (*leu-500*) that is partially suppressed by the presence of *supX* (37). When this strain was plated onto minimal medium supplemented with 1 g of valine per liter, it grew. The level of AHAS I measured in crude extracts was nearly identical to the level seen in *ilvG* mutants containing *ilvB* on a multicopy plasmid (Table 2). If, however, in addition to valine, leucine was added to the medium, strain DU5586 no longer grew. This inability to grow was the result of lowered AHAS I levels, as shown by the very low specific activity of this enzyme in strain DU5586 cultures grown under repressing conditions (Table 2).

The wide range of AHAS I activities observed in response to the availability of leucine led us to study in greater detail how the end products leucine and valine modulate the expression of *ilvB*. The repression of *ilvB* by leucine was found to require the cognate tRNA molecules, as is true for all amino acid biosynthetic operons regulated by attenuation. No repression of *ilvB* was observed in strains with an altered leucyl-tRNA due to a *hisT* lesion when these strains were grown in the presence of excess branched-chain amino acids; the specific activity of AHAS I measured in the presence or absence of the branched-chain amino acids was 105 nmol/min per mg.

The kinetics of repression of *ilvB* were compared with those of *ilvGEDA*, an operon known to be regulated by attenuation (28, 38). These studies were carried out by growing strain DU5586 (*ilvG236 leu-500 supX*) in the absence of leucine to obtain maximal derepression, until the culture reached early log phase. At this time a sample was removed for enzyme assays, and repressing amounts of the branched-

TABLE 2. Specific activities of AHAS I in transformants of strains with mutations in *ilvG*

Strain and plasmid	Sp act (nmol/min per mg of protein) in:[a]	
	Pan medium[b]	PLIV medium[c]
DU503 *ilvG236*	197	4
DU503(pHA7 *crp*)	885	206
DU503(pDU101 *ilvB*)	998	237
DU503(pDU111 *ilvB crp*)	1,274	514
DU2603*ilvG236 ilvB1*(pHA7)	0	0
DU2603(pDU101)	862	219
DU2603(pDU111)	1,823	388
DU8812 *ilvG236 crp*	18.5	0.1
DU8812(pHA7)	1,308	113
DU8812(pDU101)	62.5	22.0
DU8812(pDU111)	1,035	138
DU5586 *leu500 supX ilvG236*	1,064	37

[a] Ampicillin (50 mg/liter) was added to the medium when plasmid-containing strains were grown.

[b] Minimal medium supplemented with 20 mg of calcium pantothenate per liter.

[c] Minimal medium supplemented with 20 mg of calcium pantothenate per liter, 100 mg of leucine per liter, 50 mg of isoleucine per liter, and 100 mg of valine per liter.

chain amino acids were added to the remainder of the culture. The levels of threonine deaminase (*ilvA*), transaminase B (*ilvE*), and AHAS I (*ilvB*) were measured in samples that were removed periodically until the culture reached stationary phase. As Fig. 2A shows, all three enzymes followed the same time course of repression; however, AHAS I repression was more nearly complete, exhibiting the greatest change in specific activity from the derepressed state to the repressed state. A determination of the extent of enzyme synthesis after imposition of repressing conditions suggested that synthesis of AHAS I very nearly ceased, whereas synthesis of threonine deaminase and transaminase B continued (Fig. 2B). These results suggested that *ilvB* expression was very tightly regulated.

Effect of the cAMP-CRP complex on the expression of *ilvB*. It was reported by Whitlow and Polglase (48) that growth of *E. coli* K-12 on carbon sources that were subject to catabolite repression allowed this strain to grow in the presence of valine owing to the high level of activity of AHAS I present, a result analogous to the results described above. We wanted to determine whether the same phenomenom of carbon source-induced valine resistance was possible in *S. typhimurium*. To test this possibility, we grew *ilvG* mutant DU503 on carbon sources that differed in ability to elicit catabolite

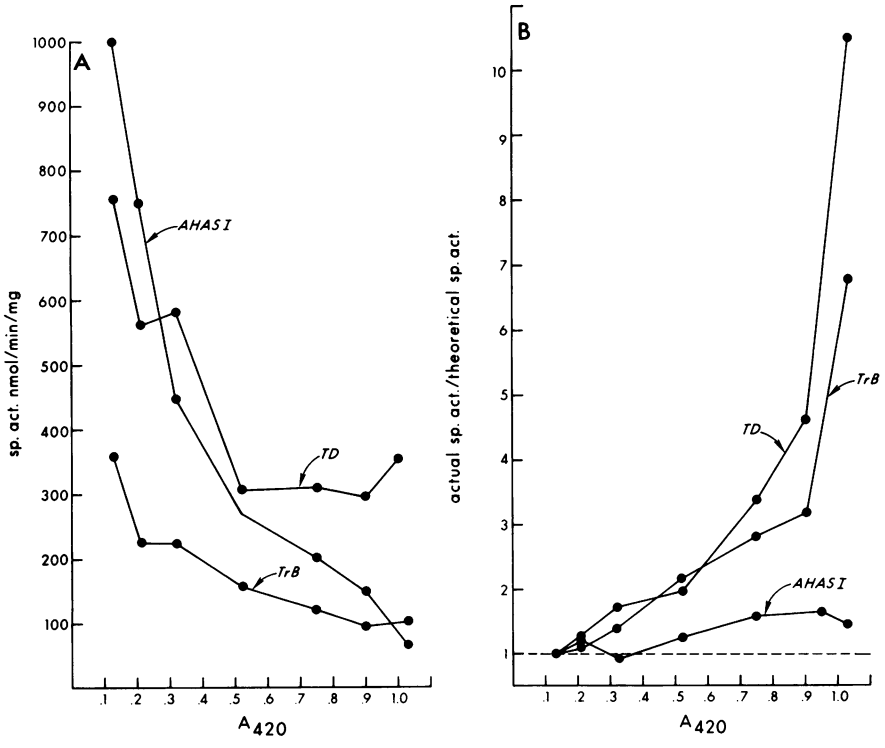

FIG. 2. Kinetics of repression of the isoleucine and valine biosynthetic enzymes in strain DU5586 (*ilvG236 leu-500 supX*). The enzymes assayed were threonine deaminase (TD), the product of *ilvA*, transaminase B (TrB), the product of *ilvE*, and AHAS I, the product of *ilvB*. (A) Absorbance versus specific activity. Strain DU5586 was grown on minimal medium until the culture reached early log phase. Repressing conditions were then imposed by the addition of 50 mg of isoleucine per liter, 100 mg of leucine per liter, and 100 mg of valine per liter. Samples were removed when the culture reached the levels of absorbance at 420 nm (A_{420}) indicated, and the enzymes were assayed. The first sample was taken prior to the addition of the amino acids and represents the derepressed level of enzyme expression. (B) Absorbance of the repressed culture versus ratio of actual specific activity to theoretical specific activity. The theoretical specific activity was determined by dividing the activity per milliliter obtained in the derepressed crude extract by the protein concentration of the crude extract at a given time point, thus assuming that none of the newly synthesized protein was present as the enzyme. If repression were complete, the data points would lie on the dashed line.

TABLE 3. Specific activities of isoleucine and valine biosynthetic enzymes and β-isopropylmalate dehydrogenase in strain DU503 and its *cya* and *crp* derivatives

Growth conditions	Sp act (nmol/min per mg of protein)											
	Strain DU503				Strain DU8811 *cya*				Strain DU8812 *crp*			
	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase
Pan[a]	1,247	856	141.0	126	962	621	27.0	105	1,080	832	19.5	134
Pan + cAMP[b]	786	527	62.0	98	539	284	141.0	104	472	546	43.5	116
LIV[c]	371	231	2.5	ND[d]	452	283	0.5	ND	958	490	0.1	ND
LIV + cAMP[e]	193	150	1.5	ND	164	128	1.0	ND	947	483	0.1	ND

[a] Minimal medium supplemented with 20 mg of calcium pantothenate per liter.
[b] Minimal medium supplemented with 20 mg of calcium pantothenate per liter and 5 mM cAMP.
[c] Minimal medium supplemented with 100 mg of leucine per liter, 50 mg of isoleucine per liter, and 100 mg of valine per liter.
[d] ND, Not determined.
[e] Minimal medium supplemented with 100 mg of leucine per liter, 50 mg of isoleucine per liter, 100 mg of valine per liter, and 5 mM cAMP.

repression. No correlation was evident between the level of *ilvB* expression and the extent to which each carbon source allowed rapid growth and, hence, generated catabolite repression. In addition, none of these carbon sources allowed growth in the presence of valine.

However, when a mutation in *cya* or *crp* was introduced into the strain, there was a dramatic reduction in *ilvB* expression (Table 3). This suggests that, as in *E. coli*, AHAS I synthesis in *S. typhimurium* was sensitive to the cAMP-CRP complex. The addition of cAMP restored the level of AHAS I activity in *cya* strain DU8811 but had no effect on *crp* derivative DU8812, indicating that cAMP was acting through CRP.

An interesting finding during these experiments was the reduction of AHAS I activity when cAMP was added to a strain that responds normally to catabolite repression (strain DU503) (Table 3). The reason for this decline was not clear. Little effect on the expression of the other isoleucine and valine biosynthetic enzymes was observed, although the enzyme activities did decrease slightly upon the addition of cAMP to the medium. Expression of *ilvB* remained subject to repression by the branched-chain amino acids in the presence of the *cya* and *crp* mutations.

Since we were unable to obtain an increase in AHAS I activity sufficient to allow growth of an *ilvG* mutant on valine by raising the level of cAMP-CRP physiologically, we tried another method to increase the concentration of CRP in the cells. *ilvG* mutant DU503 was transformed with a multicopy plasmid containing the *crp* gene, pHA7 (1). The resulting transformants exhibited elevated expression of *ilvB* nearly equivalent to the levels seen when the same strain harbored *ilvB* plasmid pDU101 (Table 2). Furthermore, the cells transformed with the *crp* plasmid were able to grow in the presence of 1 g of valine per liter. (This effect was found to be cAMP dependent.) These results suggested that haploid levels of CRP did not allow maximal expression of *ilvB* in *S. typhimurium*.

In an attempt to determine the concentration of CRP needed to allow maximal *ilvB* expression, we constructed a pBR322 derivative, pDU111, that contained both *ilvB* and *crp* (Fig. 3). (It should be noted that the cloned *ilvB* gene was responsive to enhancement by CRP; very low levels of AHAS activity were detected when *ilvB* plasmid pDU101 was present in a strain containing a mutation in *crp* [strain DU8812] [Table 2].) The presence of both genes, *ilvB* and *crp*, on the plasmid allowed higher levels of AHAS I activity than when either gene was present on the plasmid alone (Table 2). However, the increase in expression was at best twofold, or equivalent to the sum of the activities measured in a strain containing the *ilvB* plasmid (strain DU2603/pDU101) and a strain containing the *crp* plasmid (strain DU503/pHA7). Thus, these results suggested that the increased level of CRP due to the plasmid was only great enough to maximally induce one copy of *ilvB*.

Isolation and characterization of strain DU513, a regulatory mutant of *ilvB*. Mutants that exhibited aberrant regulation of *ilvB* expression were obtained by plating 10^8 cells of valine-sensitive *ilvG* mutant DU503 on minimal glucose medium containing 1 g of valine per liter and selecting for valine-resistant colonies. Such colonies appeared at a frequency of 10^{-6}. Surprisingly, all of the isolates examined had the same phenotype; all constitutively overproduced valine-sensitive AHAS. One strain, strain DU513, was kept for further study. Expression of AHAS I in this strain was totally refractory to repression by leucine and valine, as indicated by the equivalent level of activity present in a leucine-requiring variant, strain DU512, grown either in a chemostat

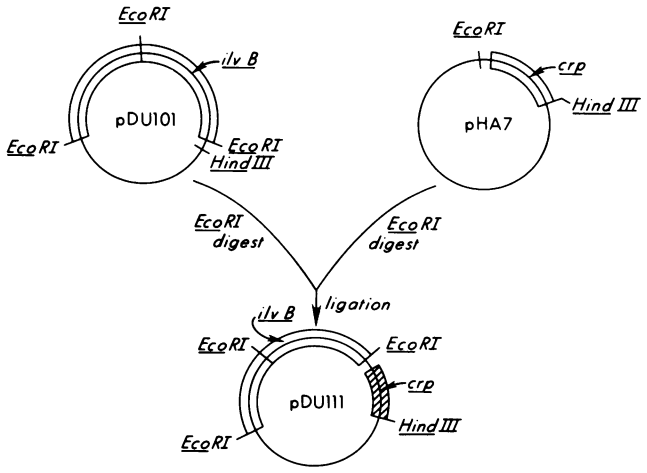

FIG. 3. Construction of pDU111. Both pDU101 and pHA7 were digested with *Eco*RI and ligated. The *crp* gene was transcribed from a plasmid promoter near the *Eco*RI site (1).

TABLE 4. Specific activities of AHAS I and threonine deaminase in strains DU521 *ilv-513 leu-124* and DU608 Δ(*ilvGEDA236*)*691*

Strain	Growth conditions	Sp act (nmol/min per mg of protein)	
		AHAS I	Threonine deaminase
DU521 *ilv-513 leu-124*	Limiting leucine[a]	2,451	1,174
	LIV[b]	2,145	424
DU608 Δ(*ilvGEDA236*)*691*	Limiting leucine[c]	895	
	LIV[b]	1.5	

[a] Limiting leucine conditions were imposed by growth in a chemostat.
[b] The culture was grown in the presence of 100 mg of leucine per liter, 50 mg of isoleucine per liter, and 100 mg of valine per liter.
[c] The culture was grown in the presence of 50 mg of isoleucine per liter and 25 mg of glycyl-L-valine per liter.

TABLE 6. Specific activities of homoserine dehydrogenase and histidinol phosphate phosphatase in strains DU503 and DU513

Strain	Growth conditions[a]	Sp act (nmol/min per mg of protein)	
		Homoserine dehydrogenase	Histidinol phosphate phosphatase
DU503 *ilvG236*	Pan[a]	16.49	3.20
	LIV + His + Thr[b]	2.38	1.98
DU513 *ilvG236*	Pan[a]	2.91	4.49
ilv-513	LIV + His + Thr[b]	1.83	2.91
LT-2	Minimal medium	3.67	

[a] See Table 3, footnote *a*.
[b] Minimal medium supplemented with 100 mg of leucine per liter, 50 mg of isoleucine per liter, 100 mg of valine per liter, 40 mg of histidine per liter, and 40 mg of threonine per liter.

under limiting leucine conditions or under repressing conditions (Table 4). Thus, once again high levels of AHAS I overcame valine toxicity. The mutation causing this phenotype was designated *ilv-513*. Transductional analysis revealed that the *ilv-513* lesion was not cotransducible with the *ilvB* structural gene and, therefore, was not in the promoter or attenuator region.

Upon assaying other enzymes in the *ilv* pathway, threonine deaminase (*ilvA*) and transaminase B (*ilvE*), as well as β-isopropylmalate dehydrogenase (*leuB*), in crude extracts of valine-resistant strain DU513, we found that the expression of these enzymes was also no longer repressed by the branched-chain amino acids (compare Table 3, strain DU503, and Table 5, strain DU513). These observations led us to ask whether the *ilv-513* mutation might be acting as a general anti-terminator for attenuated systems. To test this hypothesis, the levels of expression of two attenuated genes, *hisB* and *thrA*, not directly involved in the biosynthesis of the branched-chain amino acids, were assayed in valine-resistant strain DU513. The results argued against this hypothesis since both genes were expressed at normal levels and responded to repression in strain DU513 (Table 6). (The high level of homoserine dehydrogenase in strain DU503 reflected the isoleucine limitation in that strain [42].) Furthermore, the data in Table 4 indicate that the level of threonine deaminase could be repressed in an *ilv-513*-bearing strain, strain DU512 (*ilv-513 ilvG236 leu-124*), although it was not repressible in the parent strain, strain DU513. The reason for the discrepancy in the repressibility of *ilvA* in a valine-resistant strain that has a mutation in the *leu* operon and in a strain that does not was not at all clear.

Effect of *ilv-513* on cAMP-CRP regulation of *ilvB*. Since the expression of *ilvB* is mediated by both the presence of the end products leucine and valine and the cAMP-CRP complex, we carried out studies to investigate whether the *ilv-513* lesion affected this latter mode of regulation as well. We found that introduction of either a *cya* or *crp* mutation into valine-resistant strain DU513 lowered the level of activity of AHAS I (Table 5), as it had done in valine-sensitive strain DU503, and that the addition of cAMP to *cya ilv-513* double mutant DU8821 restored the activity of AHAS I to nearly wild-type levels. The *ilvB* expression in these *cya* and *crp* strains, however, was still refractory to repression by leucine and valine. Again, as in valine-sensitive strain DU503, the introduction of *crp* plasmid pHA7 into valine-resistant strain DU513 and its *crp* derivative, strain DU8822, led to increased expression of *ilvB* (Table 7). This expression was also refractory to repression by the branched-chain amino acids (Table 7). These results demonstrated that the *ilv-513* mutation did not affect the cAMP-CRP-mediated regulation of *ilvB* and supported the notion that the regulation mediated by the cAMP-CRP complex was acting independently of that mediated by leucine and valine.

Mapping of the *ilv-513* mutation. In an attempt to identify the target of the *ilv-513* lesion, we tested the sensitivity of valine-resistant strain DU513 to a number of antibiotics. The most striking observation which we obtained was the increased resistance of this mutant to fosfomycin. It grew in the presence of 75 mg of fosfomycin per liter when the inducer glucose 6-phosphate was present, whereas the growth of the valine-sensitive strain was inhibited by as little as 7.5 mg/liter. An increase in resistance to fosfomycin can arise as a result of mutations in a number of systems, including glucose-6-phosphate uptake, the cAMP-CRP complex, or the phosphoenol pyruvate-dependent sugar phos-

TABLE 5. Specific activities of isoleucine and valine biosynthetic enzymes and β-isopropylmalate dehydrogenase in strain DU513 and its *cya* and *crp* derivatives

Growth conditions	Sp act (nmol/min per mg of protein)											
	Strain DU513				Strain DU8821 *cya*				Strain DU8822 *crp*			
	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase	Threonine deaminase	Transaminase B	AHAS I	β-Isopropylmalate dehydrogenase
Pan	2,052	1,389	1,258	197	3,044	1,401	75	49	3,771	1,809	271	35
Pan + cAMP	1,109	855	1,221	194	1,923	955	457	19	2,654	1,682	217	21
LIV	2,815	1,101	1,616	132	2,238	959	580	11	3,777	1,697	293	18
LIV + cAMP	1,814	966	1,581	309	2,153	884	1,370	11	3,636	1,720	244	12

[a] See Table 3 footnotes.

TABLE 7. Specific activities of AHAS I in transformants of strains containing the *ilv-513* mutation

Strain and plasmid	Sp act (nmol/min per mg of protein) in:	
	Pan medium[a]	PLIV medium
DU513(*ilvG236 ilv-513*)	1,164	1,460
DU513(pDU101 *ilvB*)	9,221	6,947
DU513(pHA7 *crp*)	9,713	6,121
DU8822(*ilvG236 crp ilv-513*)	352	359
DU8822(pDU101)	1,618	1,467
DU8822(pHA7)	6,042	1,890

[a] The media used are described in the footnotes to Table 2.

photransferase system (PTS) (10). We favored the latter as the site of alteration in the valine-resistant strain since we had observed that this strain grew normally on glucose 6-phosphate and appeared to be unaltered in cAMP-CRP regulation of *ilvB*.

Therefore, a transductional analysis, was carried out to determine the linkage of *ilv-513* to *pts* (Table 8). Each of four *ilvG236 pts* mutants was infected with a P22 lysate grown on valine-resistant strain DU513. Transductants were selected either for their ability to grow on mannitol as a sole carbon source, (*pts*$^+$) (16), or for valine resistance. Cotransductional frequencies demonstrated that *jlv-513* was closely linked to *pts* and that the gene order was most likely *ptsHI crr ilv-513*. However, the degree of linkage was not reciprocal; when the primary selection was done on minimal medium supplemented with valine, the cotransductional frequencies were greater. We also noted that the introduction of *ilv-513* into two of the *pts* mutants, strains DU5526 (*ptsH*) and DU5525 (*ptsIcrr*), always led to correction of the *pts* lesion, possibly suggesting that the gene products interact with one another.

Another set of observations suggested a possible interlock between AHAS I and the PTS. This view was based on the demonstration that one substrate of AHAS I, pyruvate, is the product of the first step in the PTS pathway and the other AHAS I substrate, α-ketobutyrate, is an inhibitor of this reaction (16). We have observed that introduction of the *crp* lesion into an *ilvG* mutant which accumulates α-ketobutyrate (42) greatly retarded its growth and that the *crp* mutation was extremely unstable in this background. Such a result was not obtained when the *crp* lesion was introduced into the valine-resistant strain which did not accumulate α-ketobutyrate. Therefore, it seemed plausible that the presence of the *crp* lesion in the *ilvG* mutant might exacerbate the possible inhibition of PTS by α-ketobutyrate, causing the growth rate to diminish. To test this hypothesis, a *crp* mutant deleted in the *ilvGEDA* operon and therefore unable to synthesize α-ketobutyrate was grown in the presence of this metabolite. After several passages this strain lost its Crp phenotype and the *crp* mutation could not be retrieved from these revertants, mimicking the results obtained with the *ilvG crp* mutant.

TABLE 8. Linkage of *pts* to *ilv-513* by P22 cotransduction

Recipient	Donor	Selected marker	Unselected marker	% Cotransduction
DU5524 Δ*ptsI ilvG*	DU513	Mtl[a]	Val^r	21
		Val^r	Mtl	85
DU5523 Δ*ptsPHI ilvG*	DU513	Mtl	Val^r	28
		Val^r	Mtl	65
DU5525 Δ*ptsI crr ilvG*	DU513	Mtl	Val^r	43
		Val^r	Mtl	100
DU5526 *ptsH ilvG*	DU513	Mtl	Val^r	ND[b]
		Val^r	Mtl	100

[a] Growth on mannitol was used as selection for *pts*$^+$.
[b] ND, Not determined.

***Cis-trans* analysis of the *ilv-513* mutation.** We expected from its distal location in relation to *ilvB* that the lesion causing valine resistance could act in *trans*. Valine-resistant strain DU513 and its *ilvB* and *crp* derivatives, strains DU2613 and DU8822, were transformed with the *ilvB* plasmid pDU101 to determine whether this was the case. We found that the percent increase in *ilvB* expression in the valine-resistant strain that had been transformed was equivalent to that observed in the valine-sensitive strain harboring the same plasmid (Tables 2 and 7). Furthermore, the expression of *ilvB* by the plasmid in the valine-resistant background was not repressed by the branched-chain amino acids. (The slight decrease in activity seen under repressing conditions may reflect copy number differences.) These results suggested that the *ilv-513* mutation could act in *trans*.

DISCUSSION

The results presented above indicate that the regulation of the *ilvB* operon in *S. typhimurium* is complex. Although immediately involved in the biosynthesis of the branched-chain amino acids, this gene is not part of the major *ilv* operon, nor is it closely linked to it. Synthesis of the gene product AHAS I is repressed by the end products leucine and valine, is enhanced by the cAMP-CRP complex, and is possibly modulated by the PTS.

It is most probable that the repression caused by the end products leucine and valine is mediated by attenuation in *S. typhimurium*. This assumption is based on analogy to the *E. coli* system (18, 21), as well as the lack of repression which we observed in *hisT* mutants of *S. typhimurium*. The degree of repression achieved by attenuation was found to be large. The kinetics of repression presented above indicate that the attenuation of *ilvB* is much more efficient than the attenuation of the major *ilv* operon. It appears that the repression of AHAS I synthesis is nearly complete.

The expression of *ilvB* in *S. typhimurium* is enhanced by the cAMP-CRP complex, as was found to be the case in *E. coli* (46, 48). This is clear from the lower level of AHAS I activity measured in *cya* and *crp* mutants of *S. typhimurium* and by the rise in AHAS I activity seen when *crp* was present on a multicopy plasmid. (It should be pointed out that no such rise in β-galactosidase activity was reported when a similar *crp* plasmid was present in *E. coli* [26].) However, differences do exist concerning the degree to which this enhancement of *ilvB* expression operates in the two species. Whereas a rise in AHAS I activity in *E. coli* was observed in response to growth on carbon sources that lack the ability to cause catabolite repression (46), no such increase in AHAS I activity was observed in *S. typhimurium*; nor could a poor carbon source permit growth of an *ilvG* mutant in the presence of valine, as it has been shown to do in *E. coli* K-12 (48). Other workers have demonstrated in vitro that CRP binds to the *ilvB* promoter DNA of *E. coli* (P. Friden and M. Freundlich, Fed. Proc. 42:2041, 1983). Therefore, it is possible that the binding of CRP to the *ilvB* promoter in *S. typhimurium* is weaker than that in *E. coli*.

We suggest from our results that the expression of *ilvB* in response to attenuation (leucine-valine) is independent of

that attributable to the cAMP-CRP complex. The introduction of a *cya* or *crp* mutation does not affect the repression of *ilvB* by leucine and valine, nor does the presence of *crp* on a multicopy plasmid. Furthermore, valine-resistant strain DU513, which is refractory to repression by leucine and valine, shows normal cAMP-CRP regulation. This is apparent from the decrease in gene expression upon introduction of a *cya* or *crp* mutation and the increased expression upon introduction of a *crp*-containing plasmid. In addition, these alterations in the concentration of the CRP in the cell did not restore the response to leucine-valine repression.

Strains of *S. typhimurium* having a mutation in *ilvG* are like *E. coli* K-12 in that they are not able to grow in the presence of valine because they cannot synthesize isoleucine (40). We demonstrated that the valine toxicity of *ilvG* mutants of *S. typhimurium* could be overcome by raising the level of AHAS I. This increase was achieved in the following ways: (i) cells were grown under conditions that caused extreme leucine limitation; (ii) *ilvB* was introduced on a multicopy plasmid; (iii) a multicopy plasmid containing *crp* was introduced; and (iv) the *ilv-513* mutation was present. Since in all of these instances 15% of the AHAS I activity was detected even when 1 mM valine was added to the reaction mixture, it is possible that this fraction of activity is great enough to allow isoleucine synthesis when the enzyme is present at greatly elevated levels.

The cause of the increase in *ilvB* expression when the *ilv-513* mutation is present is not clear. The lack of repression by the branched-chain amino acids of *ilvB* as well as *ilvGEDA* suggests that the mutation might affect one of the cognate tRNA molecules, in particular the leucyl-tRNA gene, as expression of the *leu* operon is also affected. There are several arguments which can be raised against this possibility. First, the map location of the mutation does not coincide with any of the described leucyl-tRNA structural genes (9) or with the leucyl-tRNA synthetase gene (43). Second, *E. coli* K-12 strains mutant in leucyl- or valyl-tRNA have not been reported to be valine resistant. (Similarly, the introduction of *hisT* into an *ilvG* mutant did not allow its growth on valine.) Third, repression of threonine deaminase was evident in a strain harboring the valine resistance mutation when the *leu-124* lesion was present, yet *ilvB* expression was still refractory to repression. Fourth, the generation time of the valine-resistant strain was not affected by the addition of the branched-chain amino acids (data not shown). Fifth, the inability to obtain the valine-resistant lesion in certain PTS-deficient strains is not at all indicative of a tRNA mutant.

The close linkage of *ilv-513* to *pts*, the inability to obtain the mutation in *ptsH* or *ptsIcrr* strains, the increased resistance to fosfomycin, and the increased stability of the *crp* lesion in the valine-resistant strain compared with the valine-sensitive parent all suggested that the phenotype observed in *ilv-513* mutants may reflect an alteration in PTS action. It is possible that the selective pressure being exerted by plating the *ilvG* mutant onto valine-containing medium is not starvation for isoleucine but rather a need to rid the cell of excess α-ketobutyrate that is accumulating as a result of the inhibition of AHAS I by valine. Daniel et al (11) have identified α-ketobutyrate as a putative alarmone whose primary target is the PTS. In this regard it should be noted that although we were able to isolate a large number of these valine-resistant colonies when the selection was done on glucose, no such revertants were obtained when glucose 6-phosphate, a non-PTS sugar, was used as the carbon source. This observation, along with the finding of other workers that growth of *E. coli* on glucose is inhibited by the addition of α-ketobutyrate but growth on glucose 6-phosphate is not (11), supports the hypothesis presented above. One pathway by which cells can lower the level of α-ketobutyrate is to increase the level of activity of AHAS I. Components of the PTS complex have been shown to affect initiation of transcription at operons that are catabolite repressible, and this effect is most pronounced when cAMP-CRP is limiting (19). The *ilvB* operon is such an operon. This conclusion is based on our evidence that haploid levels of CRP are not sufficient to fully enhance AHAS I synthesis. Precisely what is altered by the *ilv-513* lesion and how it overrides attenuation of both the *ilvB* and *ilvGEDA* operons is not clear at the present time. Work is currently being done to answer these questions.

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