JOURNAL OF BACTERIOLOGY, Dec. 1981, p. 998-1001
0021-9193/81/120998-04$02.00/0

Vol. 148, No. 3

Valine-Resistant *Escherichia coli* K-12 Strains with Mutations in the *ilvB* Operon

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Received 5 June 1981/Accepted 5 August 1981

Escherichia coli K-12 mutants resistant to growth inhibition by valine were isolated. These strains contained mutations in the *ilvB* operon effecting either the regulation of acetohydroxy acid synthase I or the sensitivity of the enzyme to end product inhibition by valine.

The growth of *Escherichia coli* K-12, in contrast to other enteric bacteria, is blocked by valine (1). Growth inhibition is overcome by isoleucine and is caused by the sensitivity to valine of the acetohydroxy acid synthase isozymes (EC 4.1.3.18; acetolactate synthase) expressed in this strain (7). These enzymes catalyze the initial step in the biosynthesis of valine and the second reaction in isoleucine formation (18). The isolation and subsequent characterization of mutants of *E. coli* K-12 resistant to growth inhibition by valine have been instrumental in developing an understanding of the nature and expression of the *ilv* genes (4). A number of valine-resistant mutants were isolated by Adelberg and co-workers and were used to tentatively identify the regulatory and structural sites for the *ilvB* gene (11, 12). This gene codes for acetohydroxy acid synthase isozyme I. The *ilvB* gene was thought to be located within the *ilv* gene cluster at 84.1 min (13). However, it has recently been shown to map at 81.5 min, between *uhp* and *dnaA* (10). Further characterization of the *ilvB* gene and its product has been hampered by the unavailability (10, 17) of the valine-resistant mutants originally described by Adelberg and co-workers. We report here the isolation and initial characterization of these types of mutants.

Strain PS1432 [*rbs-115 bgl32 trpR thi-1* Δ(*ara-leu-ilvHI*)*863*] was used for the isolation of valine-resistant mutants in *ilvB*. This strain has a deletion in *ilvHI* and effectively expresses only the acetohydroxy acid synthase coded for by *ilvB* (10). Cells were grown to stationary phase in L broth (8), centrifuged, and washed in minimal medium (2). The cells were concentrated eightfold in minimal medium, and 3×10^9 cells were spread on minimal glucose agar plates containing L-leucine (50 μg/ml), thiamine (5 μg/ml), and L-valine at final concentrations of 6, 24, or 60 μg/ml. Each plate had a 2.5-mm filter paper disk placed in the center containing either a small crystal of 2-aminopurine, 0.1 ml of a 100% solution of ethyl methane sulfonate, or 0.1 ml of sterile water. The plates were incubated at 37°C. Colonies (between 10 and 200) appeared on all of the plates after 24 to 48 h. Five to ten colonies from each plate were pooled and grown in L broth, and phage P1 lysates were prepared (15). These phage lysates were used to transduce strain PS1582 [*thi-1 argE3 uhp2 his4 pyrE* Δ(*ara-leu-ilvHI*)*863*] to either *uhp*$^+$ or *pyrE*$^+$. The resultant transduced colonies were replica plated to minimal agar plus L-valine (6 μg/ml). Those transductants which were *uhp*$^+$ or *pyrE*$^+$ and valine resistant were assumed to contain mutations near or at the *ilvB* locus since *uhp* and *pyrE* are cotransducible with *ilvB* (10). A number of these strains were chosen for further study. The above procedure was used to insure the isolation of valine-resistant mutants in *ilvB* since most valine-resistant strains are not altered in this locus (4).

The extent of resistance to growth inhibition by valine was determined in liquid medium. The strains were grown overnight (18 h) in minimal glucose medium with shaking at 37°C. In the morning, they were centrifuged, washed, and innoculated at a concentration of 2×10^6 cells into minimal glucose medium containing increasing amounts of glycyl L-valine. All of the mutants showed some valine resistance as compared with the parent strain (Table 1). Resistance varied from growth in 2 μg to 1 mg of glycyl valine per ml. None of the mutants grew in minimal medium containing 3 mg of glycyl valine per ml.

Table 1 shows the pattern of valine inhibition of acetohydroxy acid synthase measured in crude extracts of each of the mutants. The enzyme in five of the seven strains was less sensitive to valine than that in the parent strain. The decreased sensitivity of acetohydroxy acid syn-

TABLE 1. *Effect of valine on growth and on acetohydroxy acid synthase activity*

Strain	Selection[a]	Highest valine concn allowing growth[b] (µg/ml)	Valine necessary for 50% inhibition[c] (µg/ml)
PS1582	Parent	0	18
MF2324	EMS	1000	NI[d]
MF2323	AP	8	480
MF2322	AP	8	600
MF2321	Spontaneous	2	30[e]
MF2320	EMS	500	NI
MF52	EMS	2	16
MF34	Spontaneous	2	18

[a] The selection procedure is described in the text. EMS (ethyl methane sulfonate) and AP (2-aminopurine) were mutagens used in the mutant selection. Spontaneous indicates that no mutagen was used.

[b] The strains were grown overnight (18 h) with shaking at 37°C in 0.4% glucose minimal medium (2) supplemented with L-leucine (50 µg/ml) and thiamine (2 µg/ml). In the morning, the cells were centrifuged, washed once in minimal medium, and inoculated at a concentration of 2×10^6 cells into the supplemented minimal medium containing various amounts of glycyl-L-valine. The cells were grown in 2 ml of medium in test tubes (12 by 75 mm) with shaking at 37°C. Growth was determined after 40 h of incubation.

[c] The strains were grown overnight (18 h) in the minimal medium as described in footnote *b* above, except the glucose concentration was 0.05%. In the morning, additional glucose (0.5%) was added, and the cells were grown for two doublings. Cell extracts were prepared (14), and acetohydroxy acid synthase was determined (16) by the methods described previously.

[d] Acetohydroxy acid synthase was not inhibited (NI) more than 25% by saturating concentrations of valine (7 mg/ml).

[e] Acetohydroxy acid synthase in strain MF2321 was only slightly less sensitive to valine than in the enzyme from the wild type. The growth of MF2321 in the presence of small amounts of valine may reflect a greater resistance in vivo of acetohydroxy acid synthase in this strain.

TABLE 2. *Level of acetohydroxy acid synthase during repression and derepression*[a]

Strain	Growth conditions	Sp act[b]	
		Acetohydroxy acid synthase	Threonine deaminase
PS1582	Repressed	11.7	20.0
	Limiting leucine	121.7	371.7
MF2324	Repressed	15.0	25.0
	Limiting leucine	103.3	298.3
MF2323	Repressed	11.8	33.3
	Limiting leucine	71.9	238.3
MF2322	Repressed	13.3	20.8
	Limiting leucine	128.3	364.5
MF2321	Repressed	13.5	30.0
	Limiting leucine	118.4	378.3
MF2320	Repressed	17.5	26.7
	Limiting leucine	113.5	373.3
MF52	Repressed	213.3	30.0
	Limiting leucine	173.4	366.7
MF34	Repressed	155.0	36.6
	Limiting leucine	120.0	406.6

[a] The cells were grown as described in Table 1, footnotes *b* and *c*, except that L-valine (100 µg/ml) and L-isoleucine (50 µg/ml) were added to the medium. Repressed cultures had 50 µg of L-leucine per ml; limiting leucine cultures had 8 µg of L-leucine per ml. Cell extracts were prepared and acetohydroxy acid synthase was measured as described for Table 1. Threonine deaminase was assayed as previously described (5). Protein was measured by the method of Lowry et al. (9).

[b] Enzyme specific activity is expressed as nanomoles of product formed per milligram of protein per minute.

thase in each of the five strains correlated well with the extent of resistance to growth inhibition of valine. In contrast, acetohydroxy acid synthase from strains MF34 and MF52 was as sensitive as that from the parent strain to valine inhibition. To further analyze the basis for the resistance of these mutants to growth inhibition by valine, we examined the level of acetohydroxy acid synthase in these strains grown under repressing conditions. The data (Table 2) show that this enzyme was 13- to 18-fold higher in strains MF34 and MF52 as compared with the parent strain. The levels of another isoleucine and valine enzyme, threonine deaminase (EC 4.2.1.16; L-threonine hydrolyase [deaminating]), was normal under these conditions, indicating that the mutation specifically affected *ilvB* expression. The mutants less sensitive to feedback inhibition by valine had levels of acetohydroxy acid synthase comparable to that of the parent (Table 2).

To determine whether the mutations conferring valine resistance were located in *ilvB*, we examined the frequency of cotransduction of the valine resistance marker with *ilvB* and with a number of loci closely linked to *ilvB* (Table 3). In all cases, the valine resistance loci were tightly linked to *ilvB* (95 to 100%). In addition, the frequency of cotransduction of these loci with other markers was consistent with the conclusion that the valine resistance mutations are located in either the structural or regulatory region of *ilvB*.

TABLE 3. *Cotransduction frequency of valine resistance markers with ilvB and other loci near ilvB*[a]

Transducing phage (donor strain)	Selection for *uhp*$^+$ (%V^r)	Selection for *pyrE*$^+$ (%V^r)	Selection[b] for *ilv*$^+$ (*ilvB*$^+$) (%V^r)
MF2324	91	30	95
MF2322		32	98
MF52	72		100

[a] Phage P1 lysates of the valine-resistant strains were prepared, and transduction experiments were carried out by methods previously described (15). The recipient strain was either PS1582 [*thi-1 argE3 uhp2 pyrE his4* Δ(*ara-leu-ilvHI*)*863*] (10) or PS1481 [*ilvB805* Δ(*ara leu ilvHI*)*863*] (10). The *uhp*$^+$ transductants were selected on supplemented minimal agar with glucose 6-phosphate (0.1%) as a carbon source. The *pyrE*$^+$ transductants were selected on agar lacking uracil and cytosine. The recipient strain PS1481 does not express any of the acetohydroxy acid synthase isozymes and is therefore an *ilv* mutant (6). The *ilv*$^+$ (*ilvB*$^+$) transductants were selected on supplemented minimal agar lacking isoleucine and valine. Since the donor strains are *ilvHI* and do not express *ilvG*, only *ilvB*$^+$ transductants will be *ilv*$^+$. The resultant transduced colonies were replica plated to agar plates containing glycyl-L-valine (5 μg/ml). The frequency of cotransduction is expressed as the percentage of colonies which received both the selected marker and the valine resistant mutation (%V^r).

[b] In all cases, the number of transductants used to compute the cotransduction frequencies was at least 300. The cotransduction frequency between *ilvB* and *uhp* is 85 to 90%, and that between *ilvB* and *pyrE* is 35% (10).

We utilized a multicopy plasmid containing the *ilvB* gene (T. Newman, P. Friden, and M. Freundlich, unpublished data) to study the nature of the dominance of the constitutive *ilvB* mutation in strain MF52 (Table 4). When this plasmid was transferred to strain MF2344 (MF52 *recA*), acetohydroxy acid synthase activity was equivalent to the sum of the activity found in strain MF52 and the activity of the *ilvB* genes on the plasmid (specific activity, 316). Thus, the mutation causing constitutive expression of *ilvB* on the bacterial chromosome did not effect the activity of *ilvB* on the plasmid. However, when the clone was transferred to strain MF2348 (MF550 *recA*) that contains constitutive *ilvB* expression because of a mutation unlinked to *ilvB* (T. Newman and M. Freundlich, unpublished data), the level of acetohydroxy acid synthase was that expected if the mutation affected *ilvB* expression on the bacterial chromosome and on the plasmid (specific activity, 8,100). These data indicate that *ilvB* is derepressed when it is *cis* but not *trans* to the constitutive locus in strain MF52. The results strongly suggest that the mutation in MF52 is in the regulatory region (operator or attenuator) of the *ilvB* operon.

TABLE 4. *cis dominance of the constitutive acetohydroxy synthase activity in strain MF52*[a]

Strain[b]	Sp act[a] of acetohydroxy acid synthetase
PS1596	0.8
MF2358(pTCN12/PS1596)	150
MF2334(MF52 *recA*)	170
MF2359(pTCN12/MF2334)	316
MF2348(MF550 *recA*)	425
MF2361(pTCN12/MF2348)	8,100

[a] The cells were grown under repressed conditions, and acetohydroxy acid synthase was measured as described for Table 2.

[b] Strain PS1596 has a deletion in *ilvHI* and a Mu insertion in *ilvB* (10). The plasmid pTCN12 was constructed from pBR322 and an F′ factor containing *ilvB*. This plasmid contains a 3.2-kilobase piece of *E. coli* K-12 DNA which includes the *ilvB* gene and its regulatory region (Newman, Friden, and Freundlich, unpublished data). Strain MF550 has a mutation that causes constitutive *ilvB* expression. The site of this mutation is linked to *valS* near 96 min, approximately 15 min from the location of *ilvB* (Newman, unpublished data). The plasmid copy number was estimated by isolating plasmid and chromosomal DNAs from each of the strains and separating them by electrophoresis in 0.7% agarose (3). DNA concentration was estimated by using a Joyce-Loebl microdensitometer model 3CS. The plasmid DNA concentration varied no more than 50% among the strains.

The valine-resistant strains characterized in this report appear to contain mutations in either the structural or regulatory regions of *ilvB*. These mutants are probably equivalent to the strains described by Adelberg and co-workers (11, 12), which were either incorrectly characterized (10) or subsequently lost (17).

We thank J. Hollander for excellent technical assistance and R. Grasso for help in enzyme assays.

This work was supported by Public Health Service grant GM1715212 from the National Institute of General Medical Sciences.

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