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Mapping of *ilvO* Loci of *Escherichia coli* K-12 with Bacteriophage λd*ilv*

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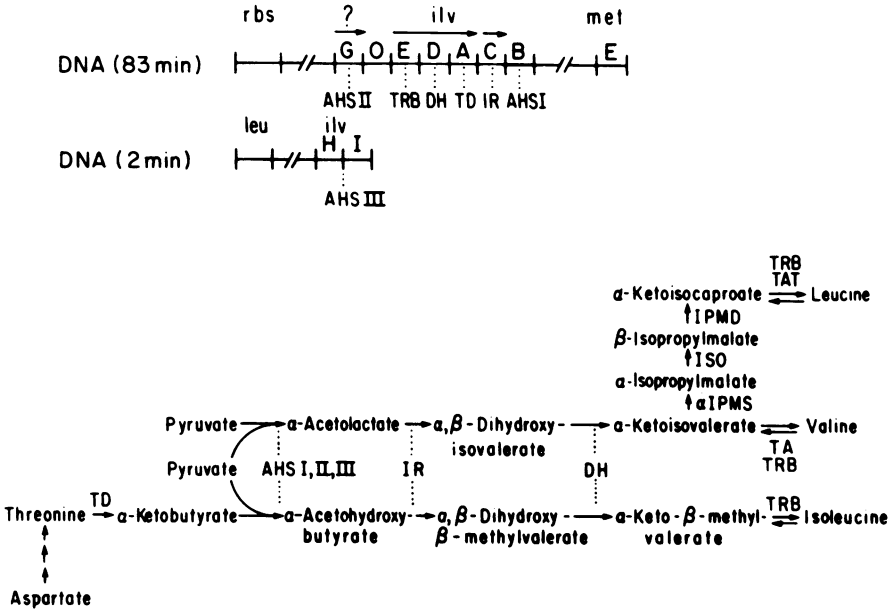
A set of λd*ilv* phage have been used in a deletion mapping procedure to determine the location of two previously characterized *ilvO* alleles. In contrast to earlier conclusions derived from three-factor crosses and episome-shortening techniques with phage P1, the order found is *ilvG-ilvO-ilvEDA*. A three-factor cross with phage P1 is described that is not consistent with this location for an *ilvO* allele. Further analysis of this particular three-factor cross revealed that an artifact attributable to a mutual syntrophism had skewed the apparent frequency of inheritance of the *ilvO* locus. The role of mutual syntrophism is discussed as a source of mapping errors for the *ilvO* locus. The value of this set of λd*ilv* phage and this mapping procedure for obtaining comparatively unambiguous data on the locations of the *ilv* structural and regulatory genes is demonstrated.

Studies of genetic regulatory phenomena are, of necessity, highly dependent upon accurate information concerning the chromosomal order of structural and regulatory genes. Although several methods have been used for fine structure mapping, deletion techniques are the simplest to conduct and are the most reliable. However, deletion mapping procedures require either a standard set of strains with known deletions or, alternatively, a set of specialized transducing phage or plasmids that carry appropriately overlapping segments of DNA derived from the host chromosome. A set of λd*ilv* phage that carry such overlapping segments of bacterial DNA derived from the *ilv* cluster at 83 min (Fig. 1) on the *Escherichia coli* K-12 genome have recently been described (M. Baez, D. W. Patin, and D. H. Calhoun, Mol. Gen. Genet., in press).

Considerable confusion has existed about the function and location or locations of mutations giving the *ilvO* phenotype. The *ilvO* gene (or genes) was originally thought to be an operator site, but there is presently no clear indication of the actual nature of the *ilvO* locus (or loci). The *ilvO* alleles included in the present study are the *ilvO264* mutation originally described by Ramakrishnan and Adelberg (9-11) and the *ilvO468* mutation first described by Pledger and Umbarger (4, 8, 12). These two *ilvO* alleles, like all others described at that time (see Discussion for more details), were mapped between *ilvA* and *ilvC*. The first conflicting evidence for this location of *ilvO* came in 1974 from the experiments of Kline et al. (4). These authors presented suggestive data that were interpreted to indicate that *ilvO* was situated outside of the Δ(*ilvDAC*)*115* region, on the *metE* side of *ilvC* (see Fig. 1). The data of Cohen and Jones (1) in 1976 rigorously demonstrated that two *ilvO* alleles were definitely situated somewhere in the region between *ilvE* and *rbs* (see Fig. 1). Although their results did not permit a determination of the position of these two *ilvO* alleles within the large *ilvE-rbs* region, they assumed that these loci were proximal or contiguous to *ilvE*. Unfortunately, the two *ilvO* alleles mapped by Cohen and Jones (see Discussion) had not been previously mapped by any investigators; for this reason, the possibility of multiple loci that can mutate to give the *ilvO* phenotype was suggested (12).

Conflicting data have also appeared concerning the location of the *ilvG* gene. The *ilvG* gene codes for a Val^r isozyme of acetohydroxyacid synthase that is expressed only in *ilvO* mutants (2; see legend to Fig. 1). There has been only one report in the literature that presents genetic mapping data for *ilvG* (2), and the order given was *ilvEGDA*. Smith et al. (12) presented no genetic mapping data for *ilvG*, but their studies of polarity led them to propose that *ilvG* did not lie within the *ilvEDA* control unit. Their polarity data did not distinguish between the order *rbs-ilvG-ilvEDA* and *rbs-ilvEDA-ilvG*, although the former order was presented on an apparently arbitrary basis.

The deletion mapping experiments reported here with λd*ilv* phage indicate that for the two *ilvO* alleles studied the order is *ilvG-ilvO-ilvEDA* (Fig. 1). Similar results have recently



BRANCH POINT ENZYME	BRANCHLET	PRIMARY EFFECTORS	REMOTE EFFECTORS
L-Threonine Deaminase (TD)	ILE	ILE	LEU, VAL
α-Acetohydroxyacid Synthase (AHS) I	VAL	(I) VAL	LEU, ILE
α-Acetohydroxyacid Synthase (AHS) II	VAL	(II) ?(VAL^R)	LEU, ILE
α-Acetohydroxyacid Synthase (AHS) III	VAL	(III) VAL	LEU, ILE
α-Isopropylmalate Synthase (α-IPMS)	LEU	LEU	ILE

FIG. 1. *Biosynthesis of leucine, isoleucine, and valine in E. coli K-12. The figure illustrates some of the current information on the biosynthetic steps, gene-enzyme relationships, map locations of structural and regulatory genes, and primary and remote enzyme effectors. The ilv genes located near 83 min are thought to be expressed as four control units: ilvG, ilvEDA, ilvC, and ilvB; the genes at 2 min (leu, ilvHI) are closely linked and may constitute one or two additional control units. The arrows indicate the probable direction of transcription for the ilvG, ilvEDA, and ilvC genes. The ilvG gene is expressed at significant levels only in ilvO mutants. Mutations in ilvO are cis-dominant and result in high levels of ilvG and ilvEDA expression. Control sites postulated to be operator genes for ilvC and ilvB are not shown since they have not been well characterized. The primary and remote effectors are shown for the feedback-regulated enzymes that catalyze the first reaction unique to each branchlet of the pathway. These enzymes are sensitive to inhibition by the corresponding branchlet end product amino acid, except for AHSII, which is Val^r. The remote effectors act at higher concentrations and are inhibitors of enzyme activity except for the reversal by valine of the inhibition of L-threonine deaminase caused by leucine and isoleucine. The action of leucine and isoleucine as remote effectors of AHS activity was observed in crude extracts of wild type (ilvO$^+$), cells that presumably contain a mixture of AHSI and III, but little or no AHSII. The abbreviations given for the enzymes are L-threonine deaminase (TD), α-acetohydroxyacid synthase (AHS), α-acetohydroxyacid isomeroreductase (IR), dihydroxy-acid dehydrase (DH), transaminase B (TRB), one or more transaminases (currently controversial) catalyzing reactions with valine (TA), α-isopropylmalate synthase (α-IPMS), isopropylmalate isomerase (ISO), β-isopropylmalate dehydrogenase (IPMD), and tyrosine aminotransferase (TAT).*

been reported for the *ilvO671* allele isolated in this laboratory (3; Baez et al., in press).

During the course of constructing strains for other studies, a three-factor cross with the generalized transducing phage P1 was performed that yielded ratios of recombinants inconsistent with the established location of the *ilvO671* mutation. This prompted an analysis that clearly demonstrated the existence of a particularly striking cross-feeding phenomenon. This phenomenon can affect the apparent recombination frequencies in genetic analyses if either the donor or recipient strains contain an *ilvO* mutation. The elegant design of the isoleucine-valine con-

trol circuit places special stress upon mapping experiments, and the use of deletion techniques is particularly appropriate for studies of the *ilv* genes.

MATERIALS AND METHODS

Bacteria and bacteriophage. The bacterial strains used are given in Table 1. The λ phages described in this report contain both the cI857 mutation, which codes for a temperature-sensitive phage cI repressor protein, and the *Sam7* mutation, which confers a lysis-defective phenotype. The term λd*ilv* is used here to refer to phage containing these mutations.

Transduction. Techniques used for specialized transduction with λd*ilv* phage and generalized transduction with phage P1CM*clr100* have been described (Baez et al., in press).

Media and methods of cultivation. The compositions of the media used have been described (3; Baez et al., in press). Growth of cells was monitored with a Klett colorimeter, and viable cell numbers were correlated with turbidity from a standard curve (100 Klett units [no. 54, green filter] equals approximately 7.8×10^8 cells/ml).

RESULTS

Mapping of *ilvO468* and *ilvO264* with λd*ilv* phage. The λd*ilv* phage contain segments of *ilv* DNA in combinations beginning at a common point between *ilvB* and *ilvC* and extending in a counterclockwise direction towards *ilvG* (Fig. 1, Table 2). Isoleucine-valine auxotrophs (*ilvC*, *ilvD*, or *ilvE*) containing the previously characterized *ilvO264* or *ilvO468* alleles were transduced to prototrophy with the set of six λd*ilv* phage. Selection was at 42°C so that nonlysogenic recombinant transductants would be selected. Prototrophic transductants that retain the *ilvO264* or *ilvO468* alleles will be valine resistant. Valine-sensitive (*ilvO*$^+$) transductants can be formed in recombinants only if the donor phage carries an *ilvO*$^+$ gene. The results (Table 2) reveal that both *ilvO264* and *ilvO468* lie between *ilvG* and *ilvE*, since valine-sensitive transductants are formed only with phages λd*ilv58*, λd*ilv37*, and λd*ilv26*. Phage λd*ilv73* carries the entire *ilvD* gene and probably a contiguous portion of the *ilvE* gene (Baez et al., in press), but does not carry *ilvO*, since no valine-sensitive transductants are formed among prototrophic transductants of strains CU627 and CU390 (Table 2).

In the crosses described in Table 2, the presence of valine-sensitive transductants, rather than the frequency of their occurrence, is the significant finding with regard to determination of gene locations. Although the frequency of formation of Val^s transductants varies with the combinations of phage donors and bacterial recipients tested, it is crucial to note that the causes of this variability, and the interpretations placed upon it, are markedly different from those that affect recombination frequencies in traditional three-factor crosses. It has previously been demonstrated, with these and other specialized transducing phages, that the cotransduction frequency of two genes decreases 10- to

TABLE 1. *Bacteria and bacteriophage*

Strain	Genotype	Source or reference
AB1514(CU2)	F$^-$ *ilvE12 ilv-2025*	B. Bachman (7)
CU4	*galT12* λ$^-$	D. Smolin (8)
CU17	F$^-$ *galT12 rbs-215 metE200 ilvA467* λ$^-$	H. E. Umbarger (8)
CU390	F$^-$ *galT12 ilvO264 ilvC2026* λ$^-$	G. W. Hatfield
CU449	F$^-$ *ilvC462 rbs224* λ$^-$	H. E. Umbarger
CU592	F$^+$ *rbs-215 ilvO468 ilvE2066*::Mu-1	H. E. Umbarger (12)
CU627	F$^+$ *rbs-215 ilvO468 ilvD2072*::Mu-1	H. E. Umbarger (12)
CU2501	F$^-$ *rbs-200 ilvO468*	G. W. Hatfield (8)
L22	F$^-$ *thi thr leu ilvC* (introduced by phage Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvAC22*	P. Jørgensen (Baez et al., in press)
L26	F$^-$ *thi thr leu ilvC* (introduced by phage Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvOEDAC26*	P. Jørgensen (Baez et al., in press)
L29	F$^-$ *thi thr leu ilvC* (introduced by Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvC29*	P. Jørgensen (Baez et al., in press)
L37	F$^-$ *thi thr leu ilvC* (introduced by phage Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvGOEDAC37*	P. Jørgensen (Baez et al., in press)
L58	F$^-$ *thi thr leu ilvC* (introduced by phage Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvGOEDAC58*	P. Jørgensen (Baez et al., in press)
L73	F$^-$ *thi thr leu ilvC* (introduced by phage Mu-1) T1^r λcI857*Sam7* λcI857*Sam7*d*ilvDAC73*	P. Jørgensen (Baez et al., in press)
MSR50	F$^-$ *ilvO671 ilv-2025*	This paper, P1·MSR109 × AB1514
MSR109	F$^-$ *ilvA538 gal-8 ilvO671 ilvD670* λ$^-$	(3)
MSR110	F$^-$ *ilvA538 gal-8 ilvO671 ilvE672* λ$^-$	(3)

TABLE 2. *Mapping of ilvO468 and ilvO264 with λdilv phage*

Cells	Phage donor and *ilv* genes present in phage[a]					
	λd*ilv58* (GOEDAC)	λd*ilv37* (GOEDAC)	λd*ilv26* (OEDAC)	λd*ilv73* (DAC)	λd*ilv22* (AC)	λd*ilv29* (C)
CU592 (*ilvO468 ilvE2066*::Mu-1)	54/100 (1.7×10^5)	3/97 (2.1×10^3)	1/147 (2.0×10^3)	NT (0)	NT NT	NT NT
CU627 (*ilvO468 ilvD2072*::Mu-1)	3/147 (2.4×10^5)	3/100 (1.5×10^4)	0/150 (2.5×10^4)	0/97 (1.8×10^4)	NT NT	NT NT
CU390 (*ilvO264 ilvC2026*)	17/177 (1.0×10^7)	2/98 (2.4×10^5)	1/99 (7.0×10^6)	0/96 (5.0×10^6)	0/98 (1.4×10^6)	0/100 (6.0×10^6)

[a] The number of transductants formed with each phage donor is given in parentheses. The ratio given for each cross represents: (number of valine-sensitive transductants)/(number of transductants tested). NT, Not tested. See text for details.

1,000-fold when one of the genes contains the termination point of bacterial DNA within the phage (see Baez et al. [in press] for discussion). Thus, it can be seen from the data in Table 2 that phage λd*ilv26* contains bacterial DNA that terminates very close to or within *ilvO*. For example, with strain CU592 as recipient, the cotransduction frequency of *ilvE2066*::Mu-1 with *ilvO468* decreased from 54 of 100 (54%) with phage λd*ilv58*, and 3 of 97 (3%) with phage λd*ilv37*, to 1 of 147 (0.7%) with phage λd*ilv26*. The transduction of single genes in these crosses is not as dramatically affected by the position of the termination point of bacterial DNA in the phage. For example, compare the total number of prototrophic transductants (indicated in parentheses in Table 2) obtained when strain CU627 was used as recipient with phages λd*ilv58*, *-37*, *-26*, and *-73* as donors. The increasing recombination frequencies between the selected (*ilv* prototrophy) and nonselected (*ilvO*) markers observed with donor phages λd*ilv26*, λd*ilv37*, and λd*ilv58*, respectively, are due to increasing lengths of bacterial DNA present in the phage extending from *ilvO* in a counterclockwise direction (towards *rbs*, see Fig. 1).

The reduced recombination frequency observed with strains CU627 (*ilvD2072*::Mu-1) and CU390 (*ilvC2062*) as recipients, compared to that observed with strain CU592 (*ilvE2066*::Mu-1) as recipient, is due to the increased distance between the selected and nonselected loci. The presence of phage Mu-1 generates a further decrease in recombination frequency by increasing the genetic distance to *ilvO*. For example, compare the recombination frequencies observed with phage λd*ilv58* as donor with the three recipients tested (strains CU592, CU627, and CU390, Table 2). The high recombination frequency (54 of 100, 54%) with strain CU592 (*ilvE2066*::Mu-1) as recipient was reduced dramatically (3 of 147, 2%) with strain CU627 (*ilvD2072*::Mu-1) on account of the increased genetic distance to the *ilvD2072*::Mu-1 lesion (compared to *ilvE2066*::Mu-1). The intermediate recombination frequency (17 of 177, 10%) observed with strain CU390 (*ilvC2066*), containing a point mutation rather than a phage Mu-1 insertion, reflects the distance between *ilvO* and *ilvC* in the absence of the Mu-1 phage. The recombination frequencies observed with phage λd*ilv37* with strains CU592 and CU527 as recipients were similar (3 of 97, 3%, and 3 of 100, 3%, respectively), in contrast to the markedly different recombination frequencies observed with phage λd*ilv58* and these same bacterial recipients (54 of 100, 54%, and 3 of 147, 2%, respectively). These effects could result from very small differences in the lengths of bacterial DNA in phages λd*ilv58* and λd*ilv37* extending towards *rbs*, and the consequent critical effects (mentioned above) upon the cotransduction of two markers in these crosses.

As previously noted, the frequencies of recombination in these crosses do not provide the basis for deducing the genes present in the λd*ilv* phage and the determination of gene order. It is, of course, necessary to test sufficient numbers of prototrophic transductants to obtain even rarely occurring *ilvO*$^+$ recombinants. It is obviously not necessary, however, to screen thousands of prototrophic transductants for the Val^s (*ilvO*$^+$) phenotype, since *ilvO*$^+$ recombinants are readily detected by screening far fewer transductants even when the termination point of bacterial DNA is near or within the *ilvO* gene, as with phage λd*ilv26*. Thus, this mapping procedure, which has previously been successfully applied to other *E. coli* genes, also provides a simple and relatively unambiguous technique for mapping the *ilv* genes.

The data in Table 2 serve to confirm the locations of the structural gene defects in strains CU592 and CU390. It can be seen, for example, that phage λd*ilvDAC73* gave 1.8×10^4 prototrophic transductants with strain CU627 (*ilvD2072*::Mu-1), but none with strain CU592 (*ilvE2066*::Mu-1). These results illustrate the

ease and decisiveness of this technique compared to the analysis of recombination frequencies in traditional three-factor crosses.

An anomalous result for the location of *ilvO671* in a three-factor cross with phage P1. In order to construct a strain containing the *ilvO671* allele in the absence of the *ilvA538* mutation, phage P1 was grown on strain MSR109 (*ilvO671 ilvD670 ilvA538*) and used to transduce strain AB1514 (*ilvE12*) to prototrophy (Fig. 2). The majority of transductants from such a cross should retain the *ilvO671* marker, but should be *ilvA538*$^+$. After 2 days of incubation on selective media (minimal medium without leucine, isoleucine, and valine), colonies of two distinct size classes were present. The smaller colonies constituted approximately 81% of the total transductants formed in the cross. When tested for growth on minimal medium in the presence and absence of valine in order to score for the presence of the *ilvO671* locus, most of the large colonies tested were Val^r (45 of 48, 94%), but none of the small colonies tested was Val^r (0 of 50, 0%). The presence of such a high percentage of valine-sensitive transductants was unexpected and is not consistent with previous data for the relative map locations of *ilvO671* and *ilvE* (3; Baez et al., in press). Two transductants of each type (Val^s and Val^r) were streaked for single-colony isolation on unsupplemented minimal media for further study.

After three clonings by single-colony isolation, both of the Val^r transductants retained their original properties. These strains were found, as expected, to constitutively express the *ilvA*$^+$ gene product as judged by the specific activity

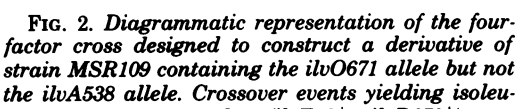


FIG. 2. *Diagrammatic representation of the four-factor cross designed to construct a derivative of strain MSR109 containing the ilvO671 allele but not the ilvA538 allele. Crossover events yielding isoleucine-valine prototrophs (ilvE12*$^+$ *ilvD670*$^+$*) are shown. Crossover events necessary for the incorporation of other combinations of the nonselected ilvO671 and ilvA538 alleles are not shown. The majority of the transductants were expected to be prototrophs that are Val*^{r} *(i.e., ilvO671), but Val*^{s} *"transductants" were more numerous (80%) on the selection plates. The high frequency of Val*^{s} *"transductants" was subsequently shown to be the result of an artifact due to colony formation by a reciprocally syntrophic pair consisting of strain AB1514 auxotrophic cells and ilvD670 auxotrophs formed by transduction of strain AB1514. The syntrophic pair grows well on unsupplemented minimal agar plates, but not on minimal agar plates containing valine. See text for details.*

and sensitivity of L-threonine deaminase to inhibition by isoleucine. Attempts at cloning the two Val^s transductants revealed that these "transductants," while producing apparently normal growth on minimal plates in the initial part of the streak where cell density was high, were not capable of forming isolated single colonies. Growth was irregular on cloning plates in areas containing a lower cell density. When cells taken from several of these areas of lower cell density were tested, no growth occurred for about 50% of the areas tested on unsupplemented minimal agar plates, and none grew on plates containing valine. However, normal growth occurred in every case on plates supplemented with leucine, isoleucine, and valine. This result suggested that the Val^s "transductants" were comprised of a mixture of auxotrophic cell types that exhibited a mutual syntrophism.

The possibility was considered that transductants with the donor *ilv* genotype (*ilvO671 ilvD670 ilvA538*) might have exhibited syntrophism with cells of the untransduced recipient strain AB1514 (*ilvE12*) present in large numbers on the selection plate. Transductants with the strain MSR109 *ilv* genotype would be expected to occur more frequently than the *ilvE12*$^+$ *ilvD670*$^+$ genotype for which the selection was intended.

This possibility was tested by cloning Val^s "transductants" on plates containing leucine, isoleucine, and valine, and testing isolated colonies for growth on minimal plates supplemented with valine plus α-keto-β-methylvalerate. Strain MSR109, but not strain AB1514, grows with these supplements, and therefore this test serves to distinguish between the putative members of the possible syntrophic pair. When two Val^s "transductants" were tested in this way, 16 of 20 and 15 of 20 of the colonies formed on medium supplemented with leucine, isoleucine, and valine grew on plates containing valine plus α-keto-β-methylvalerate. All colonies tested grew on minimal agar plates supplemented with leucine, isoleucine, and valine, but none grew on unsupplemented minimal medium. All of these colonies grew on plates containing galactose as carbon source, confirming that all are derived from strain AB1514 (*gal*$^+$). The donor strain MSR109 is *gal* and cannot utilize galactose as a carbon source. Thus, these results demonstrate that the valine-sensitive "transductants" selected as isoleucine-valine prototrophs are comprised of a mixture of two (or more) cell types, both of which require isoleucine and valine for growth. Phenotypically, this syntrophic pair grows on minimal medium, but not on minimal medium containing valine.

Direct demonstration of cross-feeding

between isoleucine-valine auxotrophic pairs. The possibility that cross-feeding could occur between strains MSR109 and AB1514 was tested directly. In addition, several other combinations of *ilv* mutants were tested at the same time. The pairs tested are shown in Table 3. Cells of each strain were grown by procedures used for transductional crosses and were spread to plates with and without valine. The plates were allowed to dry, and then they were patched with a short (3 to 4 cm) streak with cells to be tested (eight patches per plate). The plates were incubated at 30°C and examined for growth for 3 days. The appropriate control plates were included. The effects of patching a wild-type strain (CU4) and two *ilvO* mutant prototrophs were also tested. Since strain CU17 also requires methionine for growth, plates with and without methionine were included.

The expected syntrophism between strains MSR109 and AB1514 was observed (Table 3, Fig. 3). In addition, syntrophism was also apparent with other pairs of strains. It was also found that when the valine-sensitive wild-type strain CU4 was spread to plates containing valine, growth occurred where strain MSR110 was patched (Table 3, Fig. 3).

An unexpected effect of supplementation with methionine, the additional growth requirement of strain CU17, was observed. Significant cross-feeding was apparent between strains CU17 and AB1514 (Fig. 2), and between strains CU17 and CU449, even in the absence of methionine, and supplementation with methionine significantly

TABLE 3. *Syntrophism between ilv auxotrophs*

Cells surface-spread[a]	Plate tested[b]	Growth at area patched with[c]:							
		MSR109	MSR110	AB1514	CU17	CU449	CU4	CU2501	MSR50
MSR109 (*ilvA538 ilvO671 ilvD670*)	MIN		+	+++	+	++	+++	+++	+++
	MIN + MET		+	+++	+	++	+++	+++	+++
	VAL							+++	+++
	VAL + MET							+++	+++
MSR110 (*ilvA538 ilvO671 ilvE672*)	MIN	+			+	+	+++	+++	+++
	MIN + MET	+			+	+	+++	+++	+++
	VAL						+	+++	+++
	VAL + MET						+	+++	+++
AB1514 (*ilvE12*)	MIN	+++			+++	+++	+++	+++	+++
	MIN + MET	+++			+	+++	+++	+++	+++
	VAL							+++	+++
	VAL + MET							+++	+++
CU17 (*ilvA467 metE200*)	MIN		++	+++		++	+++	+++	+++
	MIN + MET		+++			+	+++	+++	+++
	VAL							+++	+++
	VAL + MET							+++	+++
CU449 (*ilvC462*)	MIN		+++	+++	++		+++	+++	+++
	MIN + MET		+++	+++			+++	+++	+++
	VAL							+++	+++
	VAL + MET							+++	+++
CU4 (*ilv*$^+$)	VAL		+++					+++	+++
	VAL + MET		+++					+++	+++
None	MIN						+++	+++	+++
	MIN + MET						+++	+++	+++
	VAL							+++	+++
	VAL + MET							+++	+++

[a] Approximately 5×10^7 cells were surface-spread and allowed to dry before patching.

[b] The plates contained unsupplemented minimal medium (MIN) or minimal medium supplemented as indicated with 1 mM valine (VAL) or methionine at 50 μg/ml (MET).

[c] Cells were patched as indicated and scored for growth after incubation for 3 days at 30°C. Strains CU2501 and MSR50 are valine-resistant prototrophs containing the *ilvO468* and *ilvO671* alleles, respectively. The designations +, ++, and +++ indicate the extent of growth apparent where cells were patched to the surface of the plates. A photograph of the plates surface-spread with no cells, strain AB1514, and strain CU4, is shown in Fig. 3.

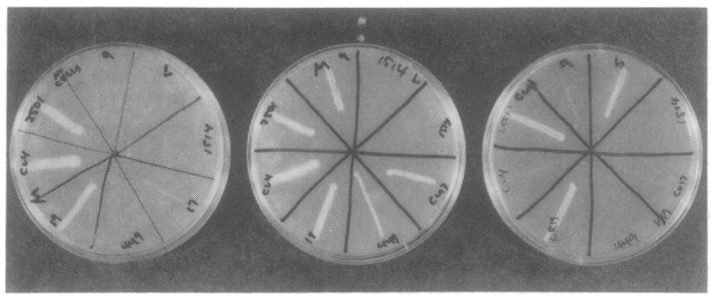


FIG. 3. *Syntrophism observed between isoleucine-valine auxotrophs. These plates were surface-spread with cells, and then patches were applied in short streaks with a flat toothpick. Each plate was patched with eight strains. Beginning at "one o'clock" and proceeding clockwise, these are: MSR110, AB1514, CU17, CU449, MSR50, CU4, CU2501, and MSR109. The plate on the left is a control plate (unsupplemented minimal medium) to which no cells were surface-spread. The center plate is unsupplemented minimal medium surface-spread with strain AB1514, and the plate on the right is a plate supplemented with valine and methionine that was surface-spread with strain CU4. Spontaneously occurring Val^r derivatives of strain CU4 can be seen on this plate as colonies appearing in the lawn of cells that were surface-spread. The photograph was taken after the plates had been incubated for 3 days at 30°C. The diagonal lines divide the plate into segments, each containing the patch of one of the eight strains tested.*

inhibited this syntrophic response. These findings suggest that methionine is excreted by strains AB1514 and CU449 on unsupplemented minimal agar plates in quantities sufficient to supply the growth requirement of strain CU17. It further suggests that the methionine present in the plates (50 μg/ml) prevents excretion of precursors of isoleucine and valine that are excreted in its absence. However, if this is the case, then it is not obvious why supplementation with methionine exhibited no apparent inhibitory effect upon the other cross-feeding pairs. Possibly the effect of methionine is related to the particular auxotrophic or regulatory mutations present. Plates spread with strain CU17 produced significantly more cross-feeding with patches of strain MSR110 in the presence of methionine than in its absence (Table 3).

The presence of a syntrophic effect will depend upon the level of precursors accumulated, their permeability, and their stability. The absence of normal repression control (*ilvO671 ilvO468*) and the presence of a feedback hypersensitive L-threonine deaminase (*ilvA538*) would also be expected to influence the cross-feeding response. In the example analyzed in detail (strain MSR109 with strain AB1514), the cross-feeding response was reciprocal, not unidirectional. In every pair examined (Table 3) growth was confined to the area of the patch, and there were no "halos" of growth surrounding the patch (see Fig. 3). This suggests that most or all of the syntrophic pairs are at least partly reciprocal. The identity of the excreted metabolites can be deduced by examination of the missing enzymes in each member of the pair. Similar responses were observed, with slight quantitative differences, when the two members of each pair were tested together with each being present, in turn, in the lawn and patch (Table 3), as expected for mutual cross-feeding. The most pronounced cross-feeding was observed with strain AB1514 (*ilvE12*) in combination with strain MSR109 (*ilvD670*), CU17 (*ilvA467*), or CU449 (*ilvC462*). Weaker cross-feeding was observed with (i) strain MSR110 (*ilvE672*) in combination with these same three strains, i.e., MSR109 (*ilvD670*), CU17 (*ilvA467*), or CU449 (*ilvC462*), and (ii) strain CU17 (*ilvA467*) with strain CU449 (*ilvC462*). Cross-feeding was slight or absent in the other two pairs tested: MSR109 (*ilvD670*) with CU17 (*ilvA467*) or with CU449 (*ilvC462*). Thus, strong cross-feeding responses are most closely correlated with transaminase B (*ilvE*) mutants and L-threonine deaminase (*ilvA*) mutants, which accumulate precursors (α-keto-β-methylvalerate and α-ketobutyrate, respectively) that are known to be relatively stable and quite permeable to auxotrophic cells. The two *ilvE* mutants tested, AB1514 (*ilvE12*) and MSR110 (*ilvE672*), differ in at least three regulatory loci: *ilv-2025*, leading to the loss of repressibility of transaminase C; *ilvA538*, which codes for a feedback hypersensitive L-threonine deaminase and affects the expression of several *ilv* genes; and *ilvO671*, which leads to derepression of the *ilvEDA* genes, suppresses the genetic regulatory effects of the *ilvA538* allele, and confers a Val^r phenotype (in prototrophs). These differences in regulatory alleles probably account for the more pronounced cross-feeding observed with strain AB1514 when paired with strains MSR109, CU17, and CU449, compared to strain MSR110 paired with these same three

strains (Table 2; also see above). On the other hand, strain MSR110 (*ilvE672 ilvO671 ilvA538*), but not strain AB1514 (*ilvE12 ilv-2025*), produced a Val^r syntrophic pair with the wild-type (*ilv*$^+$) Val^s strain CU4 on plates containing valine (Table 3, Fig. 3). The Val^r phenotype of the pair containing strain MSR110 with strain CU4 probably is due to overproduction and excretion of α-keto-β-methylvalerate by strain MSR110; uptake of this compound should promote the overproduction and excretion of isoleucine by strain CU4. Strain MSR110 would be expected to produce extremely high levels of α-keto-β-methylvalerate since (i) it is fully derepressed for the enzymes catalyzing the formation of this precursor (as a result of the effects of the *ilvO671* allele), and (ii) the end product, isoleucine, does not cause inhibition of L-threonine deaminase, a major rate-limiting step, since no isoleucine is produced within these cells. Also, strain CU4 would be expected to convert the α-keto-β-methylvalerate to isoleucine very efficiently, since this precursor bypasses the sites of allosteric regulation of L-threonine deaminase and α-acetohydroxyacid synthase (see Fig. 1). The failure of strain MSR109 (*ilvD670 ilvO671 ilvA538*) to produce a syntrophic pair with strain CU4 on plates containing valine probably reflects the decreased stability or permeability of α,β-dihydroxy-β-methylvalerate, which probably is produced at high levels within these cells.

The syntrophic effects observed (Table 2) are undoubtedly complex and probably depend upon many genetic and physiological components. Regardless of the mechanisms involved, the phenomenon clearly exists and could easily confound the interpretation of genetic analyses that depend upon recombination frequencies. This problem is particularly apparent when mapping valine-resistance determinants linked to the *ilv* genes at 83 min, and effects of this type may provide an explanation for some of the conflicting data previously obtained for the genetic location of the *ilvO* locus.

Reconstruction experiment with strains AB1514 and MSR109. The experiments described above suggest that cells of the MSR109 genotype are capable of colony formation on unsupplemented minimal agar plates surface-spread with cells of strain AB1514. The plating efficiency of strain MSR109 on unsupplemented minimal agar plates surface-spread with 5×10^7 cells of strain AB1514 was found to be 37% of the efficiency of plating observed on minimal agar plates supplemented with leucine, isoleucine, and valine. The appropriate controls demonstrated that colony formation was not attributable to reversion of either of the auxotrophic strains.

DISCUSSION

The results reported here indicate that two previously characterized *ilvO* alleles, *ilvO264* and *ilvO468*, are located between *ilvG* and *ilvE*, rather than between *ilvA* and *ilvC* as originally reported. In addition, an unexpected artifact is documented that can account for erroneous interpretations of three-factor crosses involving *ilvO* loci.

It seems appropriate at this time to scrutinize carefully previous reports indicating that *ilvO* alleles are located between *ilvA* and *ilvC*. Ramakrishnan and Adelberg (9–11) isolated five *ilvO* mutations: *ilvO264, ilvO266, ilvO267, ilvO268,* and *ilvO269*. Only the *ilvO264* allele was used for genetic mapping studies by these investigators, however, and the order reported was *ilvA-ilvO-ilvC*. Cohen and Jones (1) reported results for the *ilvO268* and *ilvO269* alleles consistent with a location between *rbs* and *ilvE*. Kline et al. (4) obtained results indicating that the *ilvO264* and *ilvO266* alleles lie outside of the Δ(*ilvDAC*)*115* deletion. The results in the present study confirm the conclusion of Kline et al. (4) for *ilvO264* and indicate that the order is *ilvG-ilvO264-ilvEDAC*. Thus, it is unlikely that any of the *ilvO* alleles isolated by Ramakrishnan and Adelberg actually lie between *ilvA* and *ilvC*, as was originally reported.

The *ilvO468* and *ilvO469* alleles were isolated by J. Jackson (cited in 8), and *ilvO468* was originally reported to be situated between *ilvA* and *ilvC*. The results of Kline et al. (4) raised questions about this location, and the experiments reported here indicate the order *ilvG-ilvO468-ilvEDAC*. Thus, the data presently available do not support a location for these *ilvO* alleles between *ilvA* and *ilvC*.

Two other independently isolated alleles conferring the *ilvO* phenotype have been located in three-factor crosses with phage P1 between *ilvA* and *ilvC*. The mapping of the *ilvO603* allele, described by Favre et al. (2), is not inconsistent with the order *ilvG-ilvO603-ilvEDA*, although further studies would be helpful to interpret some anomalous results reported for crosses involving this allele. The *vlr-2005* mutation described by Levinthal et al. (5) is phenotypically indistinguishable from the *ilvO671* allele (Baez et al., in press), and both were isolated by selecting for suppressors of the *ilvA538* mutation. Although the three-factor crosses described for *vlr-2005* appear to be straightforward in their interpretation, further studies are clearly indicated in light of more recent findings.

The preponderance of evidence clearly indicates that *ilvO* lies between *ilvG* and *ilvE*. It cannot be rigorously excluded, of course, that

mutations conferring the *ilvO* phenotype might exist between *ilvA* and *ilvC*. One interesting possibility that remains to be fully explored is the existence of *ilvA* mutations that could mimic the *ilvO* phenotype (e.g., *vlr-2005*). Several *ilvA* mutations have been described that affect the repression control of the *ilv* genes, but none has been isolated to date that confers the valine-resistance phenotype characteristic of *ilvO* mutations.

The order reported here of *ilvG-ilvO-ilvEDA* has also been recently observed by J. M. Smith (unpublished data cited in 6).

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