ISOLEUCINE-VALINE REQUIRING MUTANTS OF *SALMONELLA TYPHIMURIUM* II. STRAINS DEFICIENT IN DIHYDROXYACID DEHYDRATASE ACTIVITY[1]

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AMONG the available isoleucine-valine requiring strains of *Salmonella typhimurium* (termed *ilv*) are a number of mutants deficient in α, β-dihydroxyacid dehydratase[4] activity (ARMSTRONG and WAGNER 1964). The production of this enzyme is under the control of the *ilvD* gene (WAGNER and BERGQUIST 1960). This report is concerned with transductional analyses that have been carried out using the *ilvD* strains to obtain information on the arrangement of their mutation sites within the *ilvD* locus. Two-point ratio tests (cotransduction and recombination index tests) were the genetic techniques used. Also included in the report are enzymatic data obtained on certain of the *ilvD* strains.

MATERIALS AND METHODS

The loci designations for the isoleucine-valine mutants of *Escherichia coli* have recently been revised (RAMAKRISHNAN and ADELBERG 1965) and to assure uniformity of nomenclature in the literature, the loci designations for the *ilv* strains of *S. typhimurium* have been changed accordingly. The revised Salmonella designations are presented in Table 1. Because of the change in the three-letter locus designation of *ile* strains (to *ilv*), the allele numbers of the former *ile* strains have also been changed to comply with the proposal that there should be no duplication of allele numbers for a specific three-letter designation (DEMEREC, ADELBERG, CLARK and HARTMAN 1966).

TABLE 1

Revised designations for ilv *loci in* S. typhimurium

Locus designation		Enzyme governed
This paper	Previous paper (ARMSTRONG and WAGNER 1964)	
ilvA	*ile*	Threonine dehydratase
ilvC	*ilvA*	Reductoisomerase
ilvD	*ilvB*	α,β-Dihydroxyacid dehydratase
ilvE	*ilvC*	Transaminase B

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[4] Previously described under the trivial name: α,β-dihydroxyacid dehydrase (2,3-dihydroxyacid hydro-lyase E. C. 4.2.1.9.)

Therefore, mutants previously designated *ile*-1 to -100 are now identified as *ilv*-101 to -200, e.g. *ile*-18 is now designated *ilvA*118.

All mutant strains were obtained from the collection of the late Dr. M. Demerec and are derivatives of wild-type strains LT2 and LT7. The following *ilvD* strains were used in the study: 6, 7, 9, 10, 15, 18, 37, 38, 40, 41, 47, 49, 56, 58, 65, 67, 71 argF118, 73, 74, 81 and 82. Strain Val-8, shown to be an *ilvD* mutant that requires both isoleucine and valine for growth (Elliott and Armstrong 1966), was also included in the analysis. Strains *ilvD*7, 10 and 18 are considered to be multisite mutants, and *ilvD*40, 41 and 49 have been found to be "selfer" mutants (Armstrong and Wagner 1964).

Cotransduction tests: The procedures used can be found in Armstrong and Wagner (1964). Two valine-requiring strains (Val–12 and –14), each possessing a mutated site within the *ilvD* locus (Elliott and Armstrong 1966), were used as donors in intragenic cotransduction tests. Strain Val–12 can grow on a valine supplement at room temperature (29°) but requires isoleucine and valine when grown at 37°. Strain Val–14 was also found to grow better at 29° on a valine supplement. In these studies the transduction mixtures were plated onto minimal-medium agar plates containing 10 μg L-valine per ml. The plates were incubated 24 hrs at 37° after which time the wild-type recombinants were counted. The plates were left an additional 48 hrs at room temperature before the donor-type recombinants were counted. Approximately 1600 donor-type recombinants were picked at random from the test plates and tested by growth assay to verify the validity of the scoring procedure.

For intergenic cotransduction testing, *ilvA*118 and *ilvE*13 were used as donors in crosses with *ilvD* strains. Both of the donor strains can grow with only an isoleucine supplement. In the tests with *ilvA*118 the transduction mixture was spread onto minimal-medium agar plates containing 2.5 μg L-isoleucine per ml. After 24 hr incubation at 37°, the recombinants were replica plated onto minimal-medium agar to allow identification of the wild-type recombinants. With *ilvE*13 as donor, the test plates contained 0.025 μg L-isoleucine per ml, and the wild- and donor-type recombinants were scored by difference in colony size after 48 hrs of incubation at 37°.

Recombination index tests: The double mutant *ilvD*71 *argF*118 was used as the recipient in crosses with *ilvD* mutants and the wild-type strain LT2. Each transduction mixture was spread onto two sets of single-enriched (1% Difco nutrient broth v/v) minimal-medium agar plates. One set contained 20 μg L-arginine per ml (to select for IlvD$^+$ArgF$^-$ recombinants) and the other contained 10 μg L-isoleucine and 20 μg L-valine per ml (IlvD$^-$ArgF$^+$ recombinants). The plates were incubated 24 hrs at 37° before the colonies were counted. The recombination index was calculated by comparing the frequency of the two classes of recombinants obtained in a cross with an *ilvD* mutant with that obtained with LT2, and is derived as follows (Kiritani, Matsuno and Ikeda 1965):

$$\text{Recombination index} = \frac{\text{IlvD}^+\text{ArgF}^-}{\text{IlvD}^-\text{ArgF}^+} \times \frac{1}{\dfrac{\text{IlvD}^+\text{ArgF}^- \text{ (WT)}}{\text{IlvD}^-\text{ArgF}^+ \text{ (WT)}}}$$

In the above expression the recombinants written as (WT) represent those obtained from transduction with a wild-type donor; other recombinants are those from transduction with an *ilvD* donor. The smaller the numerical value of the recombination index, the closer the mutation site of the donor is presumed to be to the *ilvD* mutation site of the recipient.

References to four of the five enzymatic assays utilized can be found in Wagner, Bergquist, Barbee and Kiritani (1964). Transaminase B was assayed according to the method of Dr. H. E. Umbarger (personal communication), as described by Ramakrishnan and Adelberg (1964).

Statistical determination of sample size to obtain a desired precision: The following formula was used to determine the number of colonies necessary to be statistically within 1.0 percent of the true mapping value at the 99 percent level of confidence for the cotransduction tests employing Val–12 and –14 as donors:

$$N = (Z_{1-0.5\alpha}\, \sigma/d)^2$$

N = required sample size; $Z_{1-0.5\alpha}$ = normal deviate taken from a normal distribution table; $\sigma = p(\sigma - p)$; p = proportion; and d = desired precision (Dixon and Massey 1957).

RESULTS

The results of intragenic cotransduction tests using Val-12 and -14 as donors are presented in Table 2. The total number of recombinants listed for each recipient strain represents the cumulative totals from a series of tests. Individual crosses were repeated until the number of recombinants needed for the 99 percent confidence level for a distance within one map unit (arbitrarily set as a one percent wild-type value) was reached. In cotransduction tests the lower the frequency of wild-type recombinants, the closer the mutation site in the recipient strain is presumed to be to that of the donor. Because of the use of two *ilvD* alleles as donors, an arrangement of the *ilvD* sites could be derived from the combined results.

As seen in Table 2, the mutation sites of Val-12 and *ilvD*58 are quite close to each other (0.01% WT) and, therefore, the sites of Val-12 and Val-14 can be presumed to be four percentage units apart (Val-14 × *ilvD*58 = 3.94% WT). A comparison of other results obtained shows that the difference in the frequency of wild types between the Val-12 and -14 tests for a given recipient is approximately four percentage units in the majority of cases. The seven exceptions in which the difference in frequency is not four percentage units are *ilvD*7, 9, 10, 15, 18, 41 and 73. However, if the results with *ilvD*7, 9, 41 and 73 are removed

TABLE 2

Results of cotransduction tests that utilized Val-12 and Val-14 as donors in crosses with ilvD *recipient strains*

Recipient strain	Val-12		Val-14	
	Total number of recombinants	Percent wild type	Total number of recombinants	Percent wild type
(*ilvD*9)	11715	30.76	13982	15.62
*ilvD*67	18321	22.42	15969	15.83
*ilvD*47	12559	18.89	12349	15.26
(*ilvD*41)	10003	14.36	9255	12.89
*ilvD*15	12665	18.74	8734	10.99
(*ilvD*7)	10436	11.47	8168	5.55
*ilvD*81	6663	10.40	6377	7.35
*ilvD*56	9312	10.34	5757	6.54
*ilvD*65	8342	8.98	6467	5.98
Val-8	6453	8.34	3421	5.14
*ilvD*6	6275	8.10	4341	4.81
*ilvD*82	7771	7.89	4155	4.02
(*ilvD*73)	4321	6.50	3956	4.75
*ilvD*37	4843	6.57	3157	3.08
*ilvD*38	3146	6.13	1655	1.87
*ilvD*49	3585	5.75	2944	1.60
*ilvD*74	3950	2.05	2663 Val-14	2.18
*ilvD*58	3763 Val-12	0.01	4446	3.94
*ilvD*10	4502	3.78	12101	12.91
*ilvD*18	5980	4.75	11459	13.88
*ilvD*40	8566	15.07	10878	19.28

from consideration, the same order for the remainder of the sites can be derived from both the Val-12 and -14 results (Table 2). The following comments can be made concerning the data on the seven mutants whose sites could not be ordered with certainty as a result of this study. (1) Strain *ilvD*9 behaved erratically in both the Val-12 and -14 studies. The frequency of wild types varied from 24–43 percent with Val-12 as donor and 6–20 percent with Val-14. The range in both cases is much greater than that encountered with other strains. (2) Inconsistency in results was also observed with *ilvD*41 tests. The frequency of wild types varied from test to test from approximately 10 to 16 percent with both donors. This fluctuation could account for the lack of more definitive data on this strain. It should be noted, however, that the results with *ilvD*15 (closely linked to *ilvD*41) display a 7.8 percent wild-type difference between the Val-12 and -14 results. Examination of results of individual tests does not reveal any specific problems or peculiarities associated with *ilvD*15, such as were found with *ilvD*41, and the observed variation is unexplained. (3) The background growth that occurred in tests with *ilvD*73 could have resulted in counts that were less objective than would have been desired and, hence, could account for the discrepancies observed. (4) In the case of *ilvD*7, results with the Val-12 tests are considered to be more valid than those obtained with Val-14 because in two-point crosses between *ilvD*7 and 81 no wild-type recombinants have been observed. This observation is compatible with the Val-12 results. Strains *ilvD*7, 10 and 18 are considered to be multisite mutants (no spontaneous or chemically-induced reversion). It is not unusual for multisite mutants to show inconclusive results in cotransduction tests (ARMSTRONG 1967). Both *ilvD*10 and 18 do not map in the same position with each of the donors but because there are no other mutation sites in the region near them, the inconsistency does not affect the order determined.

Although the results with *ilvD*7, 9, 41 and 73 do not allow for a distinct ordering, they do permit an assignment of the individual mutation sites to a region of the locus.

In an attempt to verify the order of sites obtained by results of cotransduction tests, recombination index tests were carried out. For these studies, the double mutant *ilvD*71 *argF*118 was used as recipient in crosses with *ilvD* strains. A portion of the data is presented in Table 3. In this test 15 *ilvD* strains were utilized. As seen in the table the mutation site of *ilvD*49 is very close to the *ilv* site of *ilvD*71 *argF*118. Similar results were obtained with *ilvD*49 in other tests. For the most part the order of sites obtained using recombination index tests can be arranged to correspond to that obtained by cotransduction. The only strains that fail to fit the cotransduction order are *ilvD*7, 41 and 73 (and 9, not shown). These are the same strains that provided inconclusive results in cotransduction. Other tests using fewer *ilvD* strains, as well as smaller tests using all strains, revealed the same order of sites as presented in Table 3.

Although the intragenic tests allow for an arrangement of sites to be made, the results provide no information concerning the orientation of this order with respect to adjacent loci. The *ilvD* locus is located between the *ilvA* and *E* loci (ARMSTRONG and WAGNER 1964). Mutants of both of the latter loci can grow

TABLE 3

Results of recombination index test utilizing ilvD*71* argF*118 as recipient and* ilvD *strains as donors*

Donor strain	IlvD+ ArgF- recombinants	IlvD- ArgF+ recombinants	Recombination index
LT2	1879	1683	....
ilvD67	442	3776	0.105
ilvD41	500	4517	0.099
ilvD47	230	2217	0.093
ilvD81	128	3027	0.038
ilvD56	12	378	0.029
ilvD7	66	2463	0.027
ilvD65	50	2332	0.021
ilvD73	44	2893	0.014
ilvD6	25	1776	0.013
ilvD82	24	2525	0.012
ilvD37	8	1179	0.007
ilvD49	0	1055	0.000
ilvD74	97	2901	0.029
ilvD10	219	2597	0.076
ilvD40	646	4833	0.119

on an isoleucine supplement and, hence, can serve as donors in intergenic cotransduction tests with *ilvD* strains. Therefore, selected *ilvD* strains were tested, and the results are presented in Table 4. The recipient strains are listed according to the order derived from intragenic testing. The results are more qualitative than those of intragenic studies but nevertheless, they show that the sites of *ilvD67*

TABLE 4

Results of cotransduction tests that utilized ilvA*118 and* ilvE*13 as donors in crosses with selected* ilvD *strains*

Donor strain	Recipient strain	Total number*	Percent wild type
ilvA118	*ilvD40*	2298	23.8
	ilvD18	4234	18.1
	ilvD74	3965	16.1
	ilvD6	3476	28.5
	ilvD15	5693	30.4
	ilvD47	6233	32.0
	ilvD67	6295	58.3
ilvE13	*ilvD67*	5803	8.4
	ilvD15	6060	5.7
	ilvD6	4979	5.8
	ilvD74	2493	6.9
	ilvD18	8271	27.1
	ilvD40	6563	20.4

* Total number of recombinants analyzed.

and 47 are the most distal from that of *ilvA*118 and those of *ilvD*18 and 40 from that of *ilvE*13. These data provide evidence for the orientation of the proposed arrangement.

The interesting feature of the results presented in Table 4 is that the sites most proximal to the site in the donor strain (as judged by intragenic testing) do not show this pattern in intergenic tests. In the case of *ilvA*118, *ilvD*18 and 40 do not map as would be expected and the same is true of *ilvD*15 and 67 in the *ilvE*13 tests. This phenomenon has also been observed when *ilvC* mutants are crossed with *ilvA* strains (LESLIE and ARMSTRONG, unpublished data).

The findings of FREUNDLICH and UMBARGER (1963) that the *ilvA* and *D* genes (responsible for the production of threonine dehydratase[5] and dihydroxyacid dehydratase, respectively) are contained in the same operon prompted an investigation for polarity mutants among the available mutants for these loci. For these studies enzymatic analyses were carried out on cell-free extracts prepared on each mutant. All 13 *ilvA* strains tested were found to lack threonine dehydratase and to possess wild-type levels of dihydroxyacid dehydratase activity. Twenty-six *ilvD* strains were also examined. Twenty-three strains were deficient only in dihydroxyacid dehydratase activity; however, three strains were found to lack threonine dehydratase activity as well. A portion of the data obtained on the latter strains (*ilvD*7, 40 and 47) is presented in Table 5. For these experiments five *ilvD* strains were grown under conditions that favor derepression of the enzymes of the *ilv* pathway. When *ilvD*6 and 18 were assayed for the five enzymatic activities associated with the pathway, only dihydroxyacid dehydratase activity was lacking; the other four activities were present in derepressed amounts (Table 5). These are the results expected from analysis of nonpolar mutants. However, analysis of *ilvD*7, 40 and 47 showed that in these strains, both activities associated with the operon were essentially lacking. These results suggest that these strains are polarity mutants. Spontaneous revertants of *ilvD*40 and 47

TABLE 5

Enzymatic analysis of ilvD *mutants*

Mutant strain*	Specific activity (μM per hr per mg) Condensing	Reductoisomerase	Threonine dehydratase	Dihydroxyacid dehydratase	Transaminase
Non-polar					
*ilvD*6	7.5	40.0	73.3	0	9.5
*ilvD*18	3.6	48.0	108	0	14.1
Polar					
*ilvD*7	3.0	29.4	1.2	0	8.0
*ilvD*47	3.2	30.0	2.6	0	12.6
*ilvD*40	2.4	31.4	(0.1)	0	6.8

* All strains were grown in minimal medium containing 10 μg L-isoleucine, 50 μg L-leucine and 100 μg glycyl-L-valine per ml, i.e., a limiting amount of one (isoleucine) of three end products. After the cultures had remained in stationary phase for 1 hr, the cells were harvested and cell-free extracts were prepared by the use of sonic disruption.

[5] Previously described under the trivial name: threonine deaminase (L-threonine hydro-lyase (deaminating) E. C. 4.2.1.16).

*MULTISITE MUTANT
SSELFER MUTANT
PPOLARITY MUTANT

FIGURE 1.—Arrangement of mutation sites within the *ilvD* locus as derived from cotransduction results. Alleles for which a definite placement could not be ascertained are presented in parentheses.

(*ilvD7* shows no reversion to prototrophy) were found to possess wild-type levels of both the activities missing in the parent mutant strain. Similar evidence for polarity mutants has been reported by FREUNDLICH and UMBARGER (1963).

DISCUSSION

An arrangement of 24 mutational sites within the *ilvD* locus of *S. typhimurium* has been derived from the results of transductional analysis and is presented in Figure 1. The order depicted is based on data obtained from intragenic cotransduction tests that utilized two valine-requiring strains as donors (Table 2) and is supported by evidence obtained from recombination index studies (Table 3). The strains enclosed in parentheses are those for which a definite position for the mutation site could not be assigned, and their placement in the arrangement indicates the region in which they occur. The orientation of the order of sites with respect to *ilvA* and *E* loci was determined by intergenic transduction studies (Table 4). The multisite, polarity and selfer (ARMSTRONG and WAGNER 1964) mutants have been identified. Fine structure mapping by the use of two-point crosses is subject to erroneous conclusions because of certain phenomena, such as specific allele effects upon recombination frequencies (HARTMAN, LOPER, and ŠERMAN 1960) and, hence, the order of sites determined in this study is presented as a proposed arrangement rather than as a map.

The study provided an opportunity to examine closely the usefulness of intra- and intergenic cotransduction. The use of three *ilvD* alleles in tests with other *ilvD* strains (Tables 2 and 3) allowed for a comparison of several sets of data obtained by intragenic testing, and the good agreement among the results encourages the use of the techniques utilized. For a large majority of the strains, the results were remarkably reproducible from test to test; this was especially true of strains whose mutation sites were more closely linked to that of the donor (to a wild-type frequency of 10 percent). Whereas intragenic testing produced satisfactory results, intergenic testing did not. The results presented in Table 4 represent some of the uncertainties encountered in intergenic cotransduction. In this

laboratory the usefulness of intergenic cotransduction has been limited to determining the orientation of order of sites (Table 4) and to the placement of loci with respect to one another (ARMSTRONG and WAGNER 1964).

Enzymatic analysis of the *ilvD* strains shows that in three of the mutants both enzymatic activities associated with the operon containing the *ilvA* and *D* genes are lacking. These results suggest that these strains represent strongly polar mutants. As seen in Figure 1, the three mutation sites involved are not clustered within the locus but, rather, are spaced throughout the order of sites. Neither the frameshift nor nonsense test have been carried out on these strains. Because of the proximity of the *ilvD*40 site to the *ilvA* locus, there is the possibility that the lack of the two enzyme activities in this strain represents pseudopolarity (BAUERLE and MARGOLIN 1966). However, no evidence has been obtained to date that such is the case for *ilvD*40 or the other two mutants. Based on information gained about polarity mutants (AMES and HARTMAN 1963) and on data presented in this report, it is likely that a polycistronic message is produced for threonine dehydratase and dihydroxyacid dehydratase and that the polarity for the reading of the message is from *ilvD* to *ilvA*. The assumption can also be made that the operator region of the operon is associated with the *ilvD* gene. Studies on the *ilv* genes in *E. coli* (RAMAKRISHNAN and ADELBERG 1965a,b) provide evidence that in this organism the *ilvA*, *D* and *E* loci are contained in the same operon and that the order of transcription for the operon is: *ilvA ilvD ilvE*. Thus, it would appear that although *E. coli* and *S. typhimurium* are very similar genetically, the regulatory mechanisms for the expression of the *ilv* genes may not be identical. However, because of the lack of more definitive data on *S. typhimurium*, any implications concerning dissimilarity between the two organisms must rely on future studies for confirmation.

SUMMARY

By the use of cotransduction and recombination index tests an order for 24 mutation sites within the *ilvD* locus of *S. typhimurium* has been derived. Enzymatic data on the available *ilvD* strains provide evidence for three polarity mutants.

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