ISOLEUCINE-VALINE REQUIRING MUTANTS OF SALMONELLA TYPHIMURIUM[1]

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THE initial reports on the mutant strains of *Salmonella typhimurium* that require both isoleucine and valine for growth (*ilva* strains) were published concomitantly by GLANVILLE and DEMEREC (1960) and WAGNER and BERGQUIST (1960). These studies showed that although the available *ilva* strains could be divided into four complementation groups (*A*, *B*, *C* and *D*) on the basis of abortive transduction results, only three loci, as determined by enzymatic analysis, were involved. These results are illustrated in Figure 1. Both the *ilvaA* strains and the single *ilvaD* strain are deficient in reductoisomerase. Various attempts to establish definite biochemical differences between these two groups have not been successful (ARMSTRONG and WAGNER 1962), and it is currently thought that the *ilvaA* and *ilvaD* designations may represent complementing subunits within the locus that controls the production of the reductoisomerase. A detailed discusson of complementation within a locus is found in the report by AMES and HARTMAN (1962) on the genetics of histidine biosynthesis in *S. typhimurium.*

The transduction studies further showed that the tranducing fragment bearing the *ilva* loci also contains the locus (*ileA*) that controls the production of α-ketobutyrate, a precursor of isoleucine (Figure 1).

Since the appearance of these reports a number of new *ilva* strains have been isolated by DR. M. DEMEREC and his associates, and the present publication is

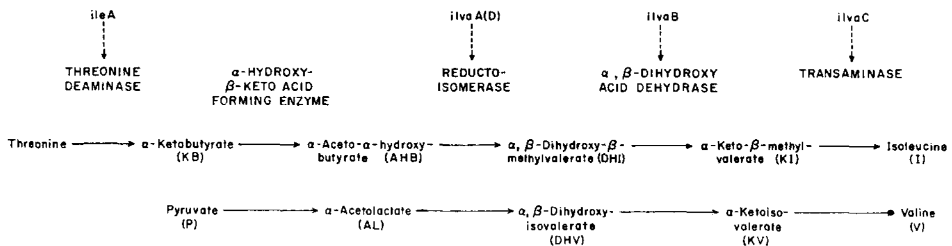

FIGURE 1.—Pathway for the synthesis of valine and isoleucine, including the known loci in Salmonella that control the production of the enzymes involved.

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concerned with genetic and biochemical analyses of these strains, as well as with an extended examination of the 14 original strains. A total of 47 *ilva* strains was used in this study.

MATERIALS AND METHODS

The *ilva* and *ileA* strains of *S. typhimurium* were kindly supplied by DR. M. DEMEREC, Brookhaven National Laboratory. All of the strains were obtained from wild-type LT-2 or LT-7; more detailed information on the individual *ilva* strains is listed in Table 1. The temperate bacteriophage P-22 (ZINDER and LEDERBERG 1952) was used.

The minimal medium of DAVIS, as described by LEDERBERG (1950), was employed throughout the study. Single-enriched minimal medium was prepared by adding 1 percent (v/v) reconstituted Difco nutrient broth to minimal medium. For the preparation of plates, 1.5 percent Difco Bacto-agar was added to the medium.

For the complete (stable) transduction experiments the recipient strain, grown overnight in an inoculum of nutrient broth, was mixed with an equal volume of the phage grown on the donor strain. The ratio of phage to bacteria (multiplicity of infection) was 5:1. The mixture was incubated 5 min at 37°, then 0.1 ml aliquots were spread on each of three minimal agar plates, which were incubated 24 to 48 hr at 37° before the prototrophic recombinants were counted. As controls, the recipient strains were tested against phage grown on the recipient strain itself (homologous phage) and buffer, i.e. no infection.

Reciprocal or joint transduction was carried out according to the complete transduction procedure described above. For these studies the minimal agar was supplemented with the addendum that allowed for the growth of donor recombinants, as well as wild-type recombinants.

In testing for "selfers" the complete transduction procedure was employed, and the individual mutant strains were tested against buffer (detection of back mutations) and homologous phage preparations. In this particular study, however, the multiplicity of infection was increased to 10 to 20, and 0.2 ml aliquots were spread on each of five single-enriched minimal agar plates.

Complementation studies (abortive transduction) were carried out according to the sectored-plate procedure described by MIZOBUCHI, DEMEREC and GILLESPIE (1962) and the method used by MARGOLIN (1963).

One method used for the determination of point or single-site mutations was the diethylsulfate (DES) test described and discussed by EISENSTARK and ROSNER (1964).

TABLE 1

Classification and origin of isoleucine-valine requiring strains of S. typhimurium*

Origin	*ilvaA* (Reductoisomerase-deficient)	*ilvaB* (Dehydrase-deficient)	*ilvaC* (Transaminase-deficient)
I. Spontaneous			
	A. LT-2: *8, 28, 29*[a]	A. LT-2: *6, 7, 9, 10*	LT-2: *13*[a], *16*[a]
	B. LT-7: *12, 14*[b], *19*[b], *27*	B. LT-7: *15*[b], *18*[b]	
II. 2-NH_2 purine			
	LT-2: *31, 33, 34, 35, 36, 39, 42, 43, 44, 46, 48, 50, 51*[d], *63, 66*	LT-2: *30, 37, 38, 40, 41, 47, 49, 62, 64, 65, 67*	
III. Diethyl sulfate			
	LT-2: *57*	LT-2: *56, 58*	
IV. Ultraviolet light			
	A. LT-2: *52*		
	B. LT-7: *54*[c], *55*[c]		

* The strains numbered *6* through *27* comprise the original collection of *ilva* mutants. Strain *ilvaD-27* is listed among the *ilvaA* mutants. [a]LT-7 proline fragment in parent LT-2. [b]LT-2 proline fragment in parent LT-7. [c]LT-7 Salmonella × *E. coli* hybrid. [d]Also requires cysteine for growth.

Auxanography was carried out as described by WAGNER and BERGQUIST (1960), except that single-enriched minimal agar plates were used instead of minimal; the utilization of the dihydroxy acid precursors of isoleucine and valine is more pronounced on the single-enriched medium.

Reductoisomerase activity was determined as described by ARMSTRONG and WAGNER (1961), and α, and β-dihydroxy acid dehydrase activity as described by WAGNER, BERQUIST, and KARP (1958). Abbreviations are: AHB = α-aceto-α-hydroxybutyrate; DHI = α,β-dihydroxy-β-methylvalerate; DHV = α,β-dihydroxyisovalerate.

RESULTS

The *ilva* stock strains are listed in Table 1. The primary classification refers to the nature of the biochemical block; this classification is subdivided to include the parent strain (LT-2 or LT-7) and origin of each mutant strain.

The mutated locus in each of the strains has been analyzed by methods that include growth, transduction and enzymatic studies. A small portion of these data are shown in Tables 2 and 3. Six *ilva* strains isolated since the reports by GLANVILLE and DEMEREC (1960) and WAGNER and BERGQUIST (1960) were tested by transduction procedures with known *ilva* strains (Table 2) and by assay of cell-free preparations for reductoisomerase and α, β-dihydroxy acid dehydrase activities (Table 3). By both criteria the six strains classify as *ilvaB*. Auxanographic

TABLE 2

Frequency of recombination (stable transduction) between ilva *mutant strains. Total number of colonies on three minimal agar plates*

Recipient	Control	Wild	Donor					
			ilvaB-30	*ilvaB-38*	*ilvaB-40*	*ilvaB-41*	*ilvaB-47*	*ilvaB-49*
ilvaA-14	0	948	201	204	165	113	219	263
ilvaA-8	0	367	108	150	75	104	154	100
ilvaD-27	2	307	100	153	67	70	122	81
ilvaB-6	0	485	5	4	5	8	3	7
ilvaB-10	0	834	27	27	6	20	28	17
ilvaC-13	0	547	353	519	335	279	256	325

TABLE 3

Reductoisomerase and dehydrase activities of ilvaB *Salmonella strains*

Strain	Specific activity—μmoles/hr/mg		
	Reductoisomerase AHB*	Dehydrase DHI	DHV
LT-2 (wild type)	12.0	6.0	9.0
ilvaB-30	17.4	0	0
ilvaB-38	18.3	0	0
ilvaB-40	21.0	0.2	0.3
ilvaB-41	12.0	0	0
ilvaB-47	12.0	0	0
ilvaB-49	17.0	0	0

* For abbreviations and references to assay procedures see METHODS.

data support this designation. All the *ilva* strains numbered through 55 have been analyzed by transduction, enzymatic and auxanographic techniques; the remaining nine *ilva* strains (*56–67*) lack enzymatic analysis. Enzymatic data on the *ilvaA* strains have been published by ARMSTRONG and WAGNER (1962). Thus, the collection of 47 *ilva* strains contains 26 reductoisomerase-deficient (*ilvaA*), 19 dehydrase-deficient (*ilvaB*) and 2 transaminase-deficient (*ilvaC*) strains. The low number of *ilvaC* strains may be due to the fact that these strains are capable of utilizing isoleucine alone as a growth supplement (GLANVILLE and DEMEREC 1960; WAGNER and BERGQUIST 1960) and, hence, would be more difficult to detect in a routine screening program for *ilva* strains.

Changes in the classification of two of the strains have been made. Strain *ilvaA-22*, as reported by GLANVILLE and DEMEREC (1960), was found on subsequent investigation to be an *ileA* (threonine deaminase-deficient) strain and has been labelled *ileA-12*. The culture of *ilvaA-37*, as reported by ARMSTRONG and WAGNER (1962), was discovered to be mislabelled and a new culture obtained from DR. M. DEMEREC proves to be an *ilvaB*. All other previously reported strains retain their original designations.

Five of the *ilva* strains show no spontaneous or chemically induced reversion to prototrophy and are considered to be multisite mutations. These strains are *ilvaA-8*, *ilvaB-7*, *ilvaB-10*, *ilvaB-18* and *ilvaC-16*; all were obtained as spontaneous mutations. Six of the *ilvaA* point mutants (*ilvaA-29*, *-31*, *-35*, *-36*, *-42* and *-46*) have been shown by transduction studies to contain mutation sites that are covered by the multisite region of *ilvaA-8*. None of the *ilvaB* point mutations falls within the mutated regions of the three multisite *ilvaB* strains; however, two of the latter strains (*ilvaB-10* and *-18*) are considered to possess overlapping mutated regions since no transduction is noted between them. The multisite region of *ilvaC-16* does not cover the mutated site of *ilvaC-13* (See DISCUSSION).

Strain *ilvaD-27* is capable of suboptimal growth on a valine supplement (GLANVILLE and DEMEREC 1960; WAGNER and BERGQUIST 1960), and recent growth studies provide evidence that *ilvaA-28* also exhibits this property. Strain *ilvaA-12* has been observed to utilize valine alone as a growth supplement, but this observation is not always reproducible with this particular strain.

Abortive transduction studies were carried out to verify the complementation results obtained by GLANVILLE and DEMEREC (1960). Minimal medium enriched with an amino acid supplement (containing 20 μg of the L-form of each amino acid and lacking isoleucine and valine) was found to be the most suitable medium for detection of intergenic complementation. Single-enriched medium with or without various supplements proved unsatisfactory because growth of many of the recipient strains interfered with the observations. Complementation was observed between the three loci *ilvaA*, *ilvaB* and *ilvaC*. Studies with *ilvaD-27* showed that meaningful results could be obtained only when this strain was used as a donor (as a recipient the background growth is heavy). Complementation is noted between *ilvaD-27* and the *ilvaB* and *ilvaC* strains after 24 hours incubation; no abortive transduction, however, has been observed with the *ilvaA* strains. In tests with *ilvaA* strains, minute colonies are noted after 30 to 36 hours incubation (and occasionally at 24 hours on a sectored plate), but additional incubation

of the plates (72 to 96 hours) reveals that these colonies are not abortives but donors.

The order of the known loci on the *ilva* transducing fragment was determined by reciprocal (joint) transduction. The number of transductants that have lost the requirement of the recipient bacteria and gained that of the donor (donor recombinants) was compared to the number of single transductants (wild-type recombinants) obtained. The higher the frequency of donor recombinants the closer the two loci involved are presumed to be on the linkage map. The success of this procedure relies on the ability to identify both wild-type and donor recombinants and to select a medium that allows for growth of donors without interference from the recipient strains. Advantage was taken of the fact that *ilvaD-27* grows suboptimally on a valine supplement and that *ileA* and *ilvaC* strains require only an isoleucine supplement for growth; strains of these types, therefore, served as the donor strains. The results are listed in Tables 4, 5, and 6, and the combined data furnished evidence that the order of the loci is: *ilvaA-ileA-ilvaB-ilvaC*. Studies are currently underway to determine more precisely the linear arrangement of the individual *ilva* mutation sites.

TABLE 4

Frequency of joint transduction of ilva *markers, using* ilvaC-16 *as donor*
Order of loci: *ilvaA-ilvaB-ilvaC*

Recipient strain	Total number of recombinants	Percent donor	Recipient strain	Total number of recombinants	Percent donor
ilvaA-14	2607	38.0	*ilvaB-18*	2639	65.9
ilvaA-29	2643	44.3	*ilvaB-10*	2559	67.1
ilvaA-8	2608	46.1	*ilvaB-67*	2319	78.4
ilvaA-35	2428	47.0	*ilvaB-9*	2809	82.4

Transduction mixture was plated onto minimal medium agar containing 0.1 μg L-isoleucine per ml. Wild-type and donor recombinants were identified by colony size after the plates were incubated at 37° for 24 hr, then allowed to remain at room temperature for an additional 48 hr.

TABLE 5

Frequency of joint transduction of ileA *and* ilva *markers using* ileA *strains as donors*
Order of loci: *ilvaA-ileA-ilvaB*

Recipient strain	*ileA-6*		*ileA-14*		*ileA-18*		*ileA-12*	
	Total*	Percent donor	Total	Percent donor	Total	Percent donor	Total	Percent donor
ilvaA-14	1423	52.4	2052	56.5	1567	52.7	2055	64.0
ilvaA-8	1542	58.1	2037	59.0	1529	54.5	2066	70.5
ilvaA-35	1579	61.8	2079	60.1	1505	55.3	2130	75.0
ilvaB-18	1437	68.1	1893	70.2	1603	79.2	2254	63.9
ilvaB-10	1920	64.8	2104	65.4	1458	76.4	1962	77.6
ilvaB-6	1541	60.8	2055	63.4	1482	67.1	1956	75.5
ilvaB-67	1308	53.3	2027	53.8	1663	59.0	2008	63.7

* Total number of recombinants.
Selection was made for wild-type and donor-type recombinants on minimal agar medium containing 5 μg L-isoleucine per ml. The transductants were replica plated onto minimal agar medium to identify the two classes.

TABLE 6

Frequency of joint transduction of ileA *and* ilva *markers, using* ilvaD-27 *as donor*
Order of loci: *ilvaA-ileA-ilvaB-ilvaC*

Recipient strain	Total number of recombinants	Percent donor	Recipient strain	Total number of recombinants	Percent donor
ilvaA-14	1920	89.9	*ilvaB-18*	2038	53.3
ilvaA-35	1936	95.9	*ilvaB-10*	1942	51.5
ilvaA-8	2218	96.0	*ilvaB-6*	2013	50.9
ilvaA-29	1981	97.5	*ilvaB-9*	1938	43.0
ileA-12	1919	69.6	*ilvaC-13*	1954	15.7
ileA-18	2002	58.4	*ilvaC-16*	2166	15.4

Transduction mixture was plated onto minimal medium agar containing 10 μg glycyl-L-valine per ml. Wild-type recombinants were counted after 24 hr incubation at 37°, and the donor-type recombinants were counted after an additional incubation period of 48 hr at room temperature.

The lack, on the transducting fragment, of a known marker that is distinctly different in phenotype from *ilva*, such as the arabinose marker associated with the leucine operon (MARGOLIN 1963), imposes a situation that requires close scrutiny of any joint-transduction results obtained with the *ilva* fragment. There are indications that the relationship between the donor and recipient cells can be an important factor in the results that are obtained. Two examples are presented in the joint transduction data. In Table 6, the *ileA-12* and *-18* results do not reflect the closeness of these two mutant sites, as shown by repeated complete transduction studies (total of 10 to 20 wild-type recombinants on three minimal plates). Examination of the data shows that in individual tests the *ileA-12* results are very consistent, whereas those with *ileA-18* are not (occasionally only 35 percent donors are obtained). This difficulty is evident in tests with other *ileA* strains, and variation of experimental conditions has not yet solved this particular problem. As seen in Table 5, the frequency of donors obtained with *ilvaB-18*, when *ileA-12* is used as the donor, is considerably lower than with *ilvaB-10*. These results with *ilvaB-18* are considered erroneous because the data obtained with this strain in all other studies (Tables 4, 5 and 6) are consistent among themselves and with the complete transduction results that show that the mutation sites in *ilvaB-10* and *-18* overlap. The *ilvaB-18/ileA-12* results are not accountable by an error in distinguishing donor recombinants from the wild-type recombinants or by a delayed appearance of donors, and are still unexplained.

The reports by DEMEREC (1962, 1963) on the selfing phenomenon in *S. typhimurium* prompted an investigation of the *ilva* strains for similar behavior. A portion of the results of this study are presented in Table 7. Seventeen (36 percent) of the *ilva* strains qualify as "selfers" (mutants that give rise to wild-type recombinants in matings with themselves), with the degree of selfing being a characteristic of each mutation. Fourteen of the 17 "selfers" are reductoisomerase-deficient strains, and a majority of these mutants were obtained by 2-aminopurine treatment. The significance of this observation cannot be evaluated since both reductoisomerase-deficient and 2-aminopurine-derived mutants are frequent in the limited number of *ilva* strains available. As illustrated in Table 7, single-

TABLE 7

"Selfers" among ilva *strains of Salmonella. Total number of colonies on five plates*

	Single enriched				Minimal	
	Experiment 1*		Experiment 2			
ilva strain	Control	Homologous phage	Control	Homologous phage	Control	Homologous phage
D-27	16	194	11	112	1	10
A-28	9	69	7	102	0	9
A-33	2	39	0	43	0	1
A-34	0	89	2	67	0	6
A-35	0	10	0	12	.	.
A-36	0	17	3	20	.	.
A-43	4	27	0	25	2	5
A-44	21	70	21	87	6	10
A-50	9	39	17	31	1	5
A-52	3	18	0	23	2	4
A-54	2	17	3	33		
A-55	5	19	8	35	3	6
A-57	1	27	1	12	.	.
A-63	12	59	13	32	.	.
B-40	17	37	22	38	.	.
B-41	6	15	2	15	.	.
B-49	1	11	1	22	.	.

* 1 and 2 represent two separate experiments, each using a different homologous phage preparation. See METHODS for procedure.

enriched medium is necessary for the detection of selfing. DEMEREC (1963) has reported that a point-mutation "selfer" will exhibit selfing not only with homologous phage but also with phage grown on a strain containing a multisite mutation that covers the mutation site in the "selfer." Strain *ilvaA-8* covers the mutation sites of "selfers" *ilvaA-35* and *-36*; when used as a donor with *ilvaA-35* the results were: control = 1, phage = 17, and with *ilvaA-36* as recipient: control = 1, phage = 23. These results are comparable to those obtained with homologous phage.

DISCUSSION

The *ilva* transducing fragment of Salmonella is known to contain four loci that are associated with the synthesis of isoleucine and valine (Figure 1). At the present time none of the available mutants are deficient in the first enzyme of the common pathway (α-hydroxy-β-keto acid forming enzyme) and, consequently, there is no information on the locus that controls the production of this enzyme.

The abortive-transduction results obtained in this study show that intergenic complementation distinguishes three separate loci for the isoleucine-valine requiring strains: *ilvaA, ilvaB* and *ilvaC*, as originally proposed by DEMEREC *et al.* (1958). The complementation data also support the biochemical findings and the suggestion made by ARMSTRONG and WAGNER (1962) that the single *ilvaD*

strain is an *ilvaA* mutant. The designation "*ilvaD*" was given to this strain primarily on the basis of abortive transduction results with the other three *ilva* loci. In this study, a close examination of the minute colonies observed in *ilvaD-27* (donor) × *ilvaA* tests reveals that the colonies are not the result of abortive transduction but, rather, are donor recombinants. Thus, the data do not distinguish *ilvaD* as a separate locus from *ilvaA*.

The suboptimal growth of *ilvaD-27* on a valine supplement is explainable if the strain possesses minimal reductoisomerase activity. *In vitro* data on this enzyme show that the isoleucine precursor is tenfold more active in the assay than the precursor of valine (ARMSTRONG and WAGNER 1961) and, hence, minimal activity in a mutant could allow the production of amounts of isoleucine that permit growth if valine is furnished. Recently, partial revertants have been obtained from *ilvaA-42*; these revertants, like *D-27*, grow suboptimally on a valine supplement. It appears, therefore, that suboptimal growth on a valine supplement may be a feature of certain *ilvaA* strains.

On the basis of available biochemical and genetic data, it is also unlikely that the *ilvaA* and *ilvaD* designations represent complementing subunits within the *ilvaA* locus (intragenic complementation); however, this possibility cannot be ruled out until further testing is done.

The order of the loci, as proposed by GLANVILLE and DEMEREC (1960), is *ileA-ilvaA-ilvaB-ilvaC*. The order as derived from this study differs in that the *ileA* locus is located between *ilvaA* and *ilvaB*. This latter order can be obtained from the data presented by GLANVILLE and DEMEREC (1960) if the strain listed in their report as "*ilvaA-7*" is, in reality, *ilvaB-7*. This suggestion is offered because the *ilva* mutant numbered "7" is an *ilvaB*. Studies on the regulatory mechanisms of the pathway in Salmonella by FREUNDLICH and UMBARGER (1963) furnish evidence that the loci controlling the production of threonine deaminase (*ileA*) and α, β-dihydroxy acid dehydrase (*ilvaB*) (but not the other loci concerned with the pathway) are contained in the same operon. The newly proposed order (*ilvaA-ileA-ilvaB-ilvaC*) agrees with these conclusions.

The selfing phenomenon, as described by DEMEREC (1962), is also observed in a large proportion of the *ilva* strains. An adequate analysis and discussion of this phenomenon can be found in the report published by DEMEREC (1963).

As mentioned previously, five of the *ilva* strains test as multisite mutants. The evidence obtained on these strains suggests that none of these multisite regions are extensive, i.e. they are "short multisites." More *ilva* strains will be required before a more accurate evaluation can be made of each of the multisite regions. The six *ilvaA* strains containing point mutations that fall within the multisite region of *ilvaA-8* have been shown by transduction to be closely linked. Four of these six strains (*ilvaA-35*, *-36*, *-42* and *-46*) are among the six *ilvaA* strains that were previously shown to possess crossreacting material (CRM) when tested with immune serum prepared against wild-type reductoisomerase (ARMSTRONG and WAGNER 1962). Strain *ilvaA-8* does not produce CRM. Hence, within the mutated region of *ilvaA-8*, mutations can occur that affect the enzymatic but not the serological properties of the reductoisomerase.

A study is underway to determine the linear arrangement of the mutation

sites in all of the *ilva* strains. Most of the mutation sites in *ilvaA* strains are mapping as a cluster in the *ilvaA-8* and *ilvaD-27* region of the locus (at least 95 percent donors in joint transduction tests with *ilvaD-27*). It is anticipated that additional information will be provided concerning the *ilva* transducing fragment, as well as the nature of the various mutations that can occur within the loci involved.

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SUMMARY

Transduction studies on 47 isoleucine-valine requiring strains of Salmonella support the biochemical evidence for three separate *ilva* loci (*A*, *B* and *C*). On the basis of joint transduction results the order of the four known loci on the *ilva* transducing fragment is *ilvaA-ileA-ilvaB-ilvaC*.

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