REGULATION OF ISOLEUCINE-VALINE BIOSYNTHESIS IN *PSEUDOMONAS AERUGINOSA*. I. CHARACTERISATION AND MAPPING OF MUTANTS

M. G. MARINUS AND J. S. LOUTIT

Department of Microbiology, University of Otago Medical School, Dunedin, New Zealand

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GENE loci controlling enzymes in certain biosynthetic pathways in *Escherichia coli* and *Salmonella typhimurium* have been shown to be clustered on the chromosome (AMES and MARTIN 1964; DEMEREC 1964). The gene loci in some of these clusters are coordinately controlled from an operator locus situated at one end of the cluster. Such a unit of coordinate expression is called an operon (JACOB and MONOD 1961).

Little is known about the regulation of gene loci specifying enzymes in biosynthetic pathways of other genera but preliminary reports on *Pseudomonas aeruginosa* suggest that this species may be different from the enterobacteria in its regulatory mechanisms. FARGIE and HOLLOWAY (1965) reported that there was no clustering of phenotypically related genes in *P. aeruginosa* and HORVATH, VARGA and SZENTIRMAI (1964) suggested that the regulation of enzymes involved in isoleucine-valine biosynthesis differed from *E. coli*. These observations are particularly interesting since the genus Pseudomonas has been placed in a separate taxonomic order from the enterobacteria (BREED, MURRAY and SMITH 1957) and is likely to be genetically different from a comparison of the guanosine–cytosine content of their respective deoxyribonucleic acids (MARMUR, FALKOW and MANDEL, 1963).

For these reasons, it seemed of interest to investigate further the report of PEARCE and LOUTIT (1965) that two genes of the isoleucine-valine pathway were linked and to determine whether these linked genes constitute a unit of coordinate expression. The present paper extends the characterization and mapping of mutants in the isoleucine-valine pathway in *P. aeruginosa* as a preliminary step to a study of regulation.

MATERIALS AND METHODS

Bacterial strains and bacteriophages: The designations for the isoleucine-valine loci have recently been revised (RAMAKRISHNAN and ADELBERG, 1965) and, to ensure uniformity, the designations of the isoleucine-valine loci of *P. aeruginosa* used by PEARCE and LOUTIT (1965) have been changed (Table 1). In addition, the proposals of DEMEREC, ADELBERG, CLARK and HARTMAN (1966) have been adopted and all strains in this laboratory have been prefixed with the letters OT. With this nomenclature, a strain described as *ilva-36* by PEARCE and LOUTIT (1965) will now be designated OT536 (*ilv-36*). It should also be noted that the designation *val* signifies an absolute requirement for this amino acid and not sensitivity or resistance.

TABLE 1

Revised designations of isoleucine-valine loci

Locus designation		
Previous*	Present	Enzyme specified
ilvA†	*ilvB*	Acetohydroxy acid synthetase
ilvB	*ilvC*	Reductoisomerase
ilvC	*ilvD*	Dihydroxy acid dehydratase
ilvD	*ilvE*	Transaminase

* PEARCE and LOUTIT (1965).
† *ilv*=isoleucine plus valine requirement.

Wild-type *P. aeruginosa*, strain 78, and its isoleucine-valine derivatives, OT501–OT565, have been described by PEARCE and LOUTIT (1965). Strains OT567 (*ilv-67*)–OT595(*ilv-95*) were isolated subsequently using the same methods except that ampicillin was substituted for benzylpenicillin. The *P. aeruginosa* strain 1 derivatives OT19(*ilv-101*)–OT36(*ilv-135*) were supplied by DR. M. D. COOKE. Strain 1 derivatives which were used in conjugation studies are shown with their genotypes in Table 2.

The transducing system for *P. aeruginosa* strain 78 has been described by PEARCE and LOUTIT (1965) and phage F116, kindly supplied by DR. B. W. HOLLOWAY, was used in transduction experiments with strain 1.

Media: These have been described previously (PEARCE and LOUTIT, 1965) and were used with the following modifications: (1) no enrichment was used in the minimal medium and (2) 0.3% instead of 0.6% agar was used in the soft agars for the preparation of bacteriophage.

Transduction procedure: The technique of PEARCE and LOUTIT (1965) was used except that the recipient culture was centrifuged and resuspended in minimal medium before use. When large numbers of mutants were to be tested, a drop method was used. Four drops of an overnight

TABLE 2

Genotype and derivation of strains used in conjugation studies

Strain	Genotype	FP	Derivation
OT1	*trp-1 str*^{r}	+	HOLLOWAY, 1965
OT2	*leu-1 str*^{s}	—	HOLLOWAY, 1965
OT8	*trp-1 ade-2*	+	Mutant from OT1
OT11	*leu-1 pro-1*	—	Mutant from OT2
OT12	*leu-1 met-2*	—	Mutant from OT2
OT15	prototrophic *str*^{s}	+	Recombinant OT8 × OT54
OT19	*trp-1 ilvD101*	+	Mutant from OT1
OT30	*trp-1 ilvB112**	+	Mutant from OT1
OT46	*leu-1 ilvD101 str*^{r}	—	Recombinant OT19 × OT11
OT47	*leu-1 ilvB112 str*^{r}	—	Recombinant OT30 × OT11
OT51	*pro-1 str*^{s}	+	Recombinant OT8 × OT60
OT54	*leu-1 lys-1*	—	Mutant from OT2
OT60	*leu-1 pro-1*	—	Mutant from OT2
OT63	*leu-1 met-2*	—	Mutant from OT2
OT109	*leu-1 ilvB112 pro-5 str*^{r}	—	Mutant from OT47

Symbols used for genetic markers: *ade*=adenine; *leu*=leucine; *lys*=lysine; *ilv*=isoleucine plus valine; *met*=methionine; *pro*=proline; *str*=streptomycin (sensitivity or resistance); *trp*=tryptophan.
* Previously described as *ilvA12* (LOUTIT, PEARCE and MARINUS 1968).

culture of the mutant were placed separately on a minimal agar plate. To successive drops were added phage lysates from mutants of each functional group. The plates were incubated for two days and scored for the presence or absence of recombinants.

Ratio test: Bacteriophage was grown on OT500 (*val-1*) and this was used to infect *ilvB* mutants as well as representatives of the other groups. The mixture was plated on minimal agar supplemented with 50 μg/ml D,L-valine allowing only wild type and valine-requiring strains to grow. The ratio of wild-type recombinants to the total number of recombinants was determined by patching 320 from each cross onto minimal agar and incubating for a further 24 hr.

Auxanography and cross-feeding: The procedures for auxanography have been previously described (PEARCE and LOUTIT, 1965). To test for cross-feeding, an overnight broth culture of the mutant to be tested was centrifuged and resuspended in the same volume of minimal medium. A volume containing 10^8 cells was added to 3 ml 0.6% soft agar and poured on a minimal agar plate. After the agar had set, drops containing about 10^6 cells of reference strains, one from each functional group, were spotted on the plate. Reciprocal experiments, in which reference strains were incorporated in soft agar and the test strains spotted on the agar plate, were also carried out. The plates were incubated for 3–5 days at 37°C and examined for growth.

Conjugation procedures: The methods of LOUTIT and MARINUS (1968) were used except that, for the kinetic experiments, the cells were diluted 10^{-2} rather than 10^{-3} after the five min allowed for pairing. For estimation of the *ilvB112*+ recombinants, the samples were diluted a further 10^{-1} before plating.

Preparation of cell free extracts: The cultures were grown on minimal agar supplemented with 200 μg/ml of isoleucine and valine, in plastic instrument trays (20cm × 15cm). After 18 hrs incubation the cells were harvested in 0.1 M phosphate buffer (pH 8.0) and washed twice. This suspension was subjected to sonic oscillation, at 2°C. Initially, a 9kc Raytheon Magnetostriction apparatus was used, but in later studies a Soniprobe (Dawe Instruments, London) was employed. The sonicated extract was centrifuged at 12,000 × *g* for 15 min in a Servall SS-1 centrifuge or a Beckman Model L Ultracentrifuge. The supernatant was diluted with 0.1 M phosphate (pH 8.0) to obtain 8–10 mg protein per ml and was used immediately for enzyme assays. Protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Enzyme Assays: Acetohydroxy acid synthetase (AHS) activity in cell free extracts was measured by the method of UMBARGER and BROWN (1958) modified by the inclusion of flavine adenine dinucleotide (STØRMER and UMBARGER 1964). Assays were carried out at pH 8.0 where maximum activity occurred. AHS activity from toluene-treated cells was determined by the method of HALPERN and UMBARGER (1959), with the inclusion of flavine adenine dinucleotide.

Threonine dehydratase (E.C. 4.2.1.16) activity was measured by the method of UMBARGER and BROWN (1957). Reductoisomerase activity was determined by the method of UMBARGER, BROWN and EYRING (1961). No difference in reaction rate was noted whether acetolactic or acetohydroxy butyric acid was used as substrate. Dihydroxy acid dehydratase (E.C. 4.2.1.9) activity was initially measured by the method of MYERS (1961). After some modification the reaction mixture contained, in 1 ml; 100 μmoles Tris-HCl, pH 7.8; 10 μmoles magnesium chloride; 5 μmoles α, β-dihydroxy-β-methylvaleric acid and 0.8–1.0 mg protein. After ten min incubation, 0.1 ml 50% trichloroacetic acid was added to stop the reaction. The protein was removed by centrifugation and the amount of keto acid in the supernatant was determined by the method of FRIEDEMANN and HAUGEN (1943). Transaminase activity was determined by the method of UMBARGER as cited by RAMAKRISHNAN and ADELBERG (1964).

All specific activities are given as μmoles of product formed per hr per mg protein.

Chemicals: α, β-Dihydroxy-β-methylvaleric acid, acetolactic acid and acetohydroxybutyric acid were kindly provided by DR. R. P. WAGNER. Additional α, β-dihydroxy-β-methylvaleric acid was synthesised by the method of SJOLANDER, FOLKERS, ADELBERG and TATUM (1954).

RESULTS

Characterisation of Mutants—Grouping of the original strains by auxanography, cross-feeding and transduction: The 56 isoleucine-valine mutants origi-

nally described by PEARCE and LOUTIT (1965) were reexamined. The results were similar except that ten strains have been reclassified as follows: OT511, OT527, OT542, OT560 and OT561 are now grouped as *ilvB* mutants; OT538, OT547, OT548, OT556 and OT557 are now grouped as *ilvD* mutants. The regrouping was supported by the enzyme assays described below and it is particularly interesting that OT538 and OT556 were not deficient in the transaminase enzyme as previously reported.

In addition to the regrouping, a number of other differences was noticed. OT529 (*ilvB29*) was the only mutant to respond to acetolactate and isoleucine. OT502 still could not be placed by cross-feeding and auxanography but it failed to produce recombinants with phage lysates from OT501 (*ilvD1*) and was classified as *ilvD2*.

Two other strains should be mentioned because they showed either a partial or a complete response to valine alone. OT527 (*ilvB27*) had a partial response apparent only on plates and not in liquid minimal medium supplemented with valine. OT500 (*val-1*) had an absolute requirement for valine which could not be replaced by acetolactate, dihydroxyisovalerate or ketoisovalerate. The strain was not described previously.

Grouping of new strains: Strain 78 mutants were readily separated using transduction with phage grown on an *ilvB* and an *ilvD* mutant and assaying all strains for the presence of the enzyme acetohydroxy acid synthetase. The strain 1 mutants were separated by transduction with phage grown on an *ilvB* and an *ilvD* mutant, by auxanography and by cross-feeding. The stable strain 78 mutants were placed in three functional groups (Table 3), whilst the strain 1 derivatives were placed in two groups (Table 4).

Assay of isoleucine-valine enzymes in selected auxotrophs: The enzyme activities in cell free extracts from a selected set of mutants are shown in Table 5. The data confirm the auxanographic, cross-feeding and transduction results; *ilvB* mutants lacked the acetohydroxy acid synthetase, *ilvC* mutants the reductoisomerase and *ilvD* the dihydroxy acid dehydratase. Three strains were of particular interest because they showed pleiotropic effects. OT500 (*val-1*) had an

TABLE 3

Separation of mutants OT567–595 into functional groups by transduction and acetohydroxy acid synthetase assays*

Producing recombinants with lysates of mutants†		Acetohydroxy acid synthetase‡					
ilvB	*ilvD*	Present			Absent		
		579	581	583	567	568	569
—	+	589	590	593	572	575	576
		594	595	. . .	577	578	588
		. . .	. . .	. . .	591	. . .	. . .
+	—	574	582	. . .	. . .	. . .	. . .

* Some of these mutants were unstable and could not be classified.
† Phage B3 lysates were prepared on OT501 (*ilvD1*) and OT509 (*ilvB9*).
‡ The enzyme assays were carried out on toluenized cells.

TABLE 4

Separation of mutants OT19–36 by transduction, nutritional responses and cross-feeding

Producing recombinants with lysates of mutants*		Mutant response to valine and DHI†							Feeding group‡
ilvB	*ilvD*	No growth				Growth			
—	+	..	..	..	..	20	27	29	I
		..	..	..	..	30	31	33	
		19	21	22	23	..	..	..	
+	—	24	25	26	28	..	..	..	II
		32	34	35	36	..	..	..	

* Phage F116 lysates were prepared on OT19 (*ilvD101*) and OT30 (*ilvB112*).
† DHI = α, β-dihydroxy-β-methylvaleric acid.
‡ Members of group II fed those of group I.

absolute valine requirement as well as a feedback-resistant acetohydroxy acid synthetase (MARINUS and LOUTIT, 1969). OT527 (*ilvB27*) had low acetohydroxy acid synthetase activity as well as a partial requirement for valine. The reducto-isomerase enzyme in these two strains differed from all other strains in that it

TABLE 5

The levels of isoleucine-valine biosynthetic enzymes in wild type and various mutants of strain 78

Strain	Specific activity					
	TD	AHS	RI		DHD	TR
OT499 (wild type)	10.2	2.8	1.8		0.45	0.67
OT509 (*ilvB9*)	10.8	ND*	1.8		0.53	0.50
OT529 (*ilvB29*)	10.5	ND*	2.0		...	0.95
OT543 (*ilv43*)	11.3	ND*	2.1		...	0.68
OT513 (*ilvC13*)	9.8	2.5	ND*		0.08	0.50
OT551 (*ilvC51*)	9.2	2.5	ND*		...	0.84
OT508 (*ilvC8*)	8.1	1.8	ND*		...	0.78
OT501 (*ilvD1*)	10.0	2.6	2.9		ND	0.95
OT538 (*ilvD38*)	..	..	..		ND*	0.70
OT548 (*ilvD48*)	..	..	..		ND*	0.70
OT556 (*ilvD56*)	..	..	..		ND*	0.74
OT502 (*ilvD2*)	8.4	2.2	2.0		ND*	0.76
OT517 (*ilvC17*)	11.3	11.5	ND*		0.45	0.66
			AL†	AHB†		
OT500 (*val-1*)	10.0	2.5	0.29	1.8	0.66	0.78
OT527 (*ilvB27*)	8.0	0.3	0.18	1.8	0.57	0.44

TD = Threonine dehydratase
AHS = Acetohydroxy acid synthetase
RI = Reductoisomerase
DHD = Dihydroxy acid dehydratase
TR = Ketoisovalerate-valine transaminase
* Not detected
† AL = α-acetolactic acid
AHB = α-aceto-β-hydroxybutyric acid
RI assays were done with two substrates, AL and AHB. For most strains the results were identical and only one figure has been recorded. For OT500 and OT527 figures for both substrates have been included.

exhibited wild-type activity when tested with the isoleucine precursor (acetohydroxybutyrate) but only one tenth of the wild-type activity when tested with the valine precursor (acetolactate). Probably the absolute valine requirement of OT500 and the partial requirement of OT527 can be accounted for by this observation. Because of overproduction of isoleucine, valine must be supplied since the amino acids must be present in equal proportions for optimal growth. The third strain OT517 (*ilv-17*) showed no detectable reductoisomerase but had extremely high levels of acetohydroxy acid synthetase.

Genetic mapping—Ratio tests: Since transduction-mediated, two-factor reciprocal crosses could not be used to obtain the order of mutations within the genes (PEARCE and LOUTIT 1965; MARINUS, unpublished data), a ratio test was devised using the *val-1* marker which was closely linked to *ilvB* and *ilvC*.

The coefficient of independent integration ($\bar{p}$) was determined (HARTMAN, LOPER and SERMAN 1960) for each *ilvB* and *ilvC* mutant, and this should provide a direct measure of the distance between the *ilv* site and the *val-1* marker. The results showed that all but one of the mutations in the *ilvB* locus mapped in a region of $\bar{p} = 0.360$–0.175 and this allowed the sites to be ordered (Table 6). The mutations in the *ilvC* locus occupied the region $\bar{p} = 0.175$–0.040 but they could not be ordered as the position of the *val-1* site in the *ilvC* gene is unknown. The results do suggest, however, that the *ilvB* and *ilvC* genes are contiguous.

Two strains were of particular interest regarding the map positions of their mutational sites. Biochemically strain OT517 had derepressed levels of AHS and no detectable RI but the mutational site mapped outside the *ilvB* gene ($\bar{p} = 0.390$). Strain OT560 (*ilvB6O*) apparently bore a lesion in the *ilvC* gene ($\bar{p} = 0.122$) but biochemically behaved like an *ilvB* mutant. This latter strain produced very few recombinants in genetic crosses which may account for the discrepancy.

TABLE 6

Probability of independent integration ($\bar{p}$) *of various* ilv *mutations*

Strain	$\bar{p}$	Strain	$\bar{p}$
OT501 (*ilvD1*)	1.0		
		OT576 (*ilvB76*)	0.234
OT517 (*ilv-17*)	0.390	OT575 (*ilvB75*)	0.225
		OT541 (*ilvB41*)	0.216
OT511 (*ilvB11*)	0.360	OT558 (*ilvB58*)	0.212
OT569 (*ilvB69*)	0.294	OT509 (*ilvB9*)	0.184
OT567 (*ilvB67*)	0.293	OT568 (*ilvB68*)	0.181
OT591 (*ilvB91*)	0.279	OT529 (*ilvB29*)	0.175
OT553 (*ilvB53*)	0.269		
OT561 (*ilvB61*)	0.264	OT560 (*ilvB60*)	0.122
OT542 (*ilvB42*)	0.244		
OT543 (*ilvB43*)	0.244	OT581 (*ilvC81*)	0.175
OT578 (*ilvB78*)	0.238	OT563 (*ilvC63*)	0.040

Each strain in the Table was transduced with a phage lysate prepared from OT500 and from 320 of the resulting transductants, the probability of independent integration was calculated. Only two *ilvC* mutants have been included, OT563 with the lowest $\bar{p}$ and OT581 with the highest.

TABLE 7

The linkage of ilvB112 *and* ilvD101 *to various other markers*

Cross	Selected marker	Unselected marker	Linkage (percent)
OT51 × OT46	*ilvD101*	*pro-1*	48 (240)*
OT19 × OT11	*pro-1*	*ilvD101*	63 (231)
OT48 × OT46	*ilvD101*	*met-2*	68 (78)
OT19 × OT12	*met-2*	*ilvD101*	77 (153)
OT30 × OT12	*met-2*	*ilvB112*	4 (400)
OT30 × OT11	*pro-1*	*ilvB112*	4 (400)
OT51 × OT46	*ilvD101*	*str*^{s}	0 (100)
OT51 × OT47	*ilvB112*	*str*^{s}	0 (100)

* The figures in parenthesis indicate the number of recombinants examined.

Linkage analysis using conjugation: In preliminary experiments, strains carrying the *ilvB112* and *ilvD101* markers were mated with strains carrying other markers. The results in Table 7 showed that the *ilvD* marker was closely linked to *pro-1* and *met-2*, and reciprocal crosses showed that the probable order was *ilvD101–met-2–pro-1*. Neither *ilv* marker was closely linked to the streptomycin locus. The *ilvB* marker was only 4% linked to *met-2* and *pro-1*, and mapped in a different region of the chromosome which was transferred proximally with relatively high frequency during conjugation.

Time of entry studies: The methods of LOUTIT and MARINUS (1968) had to be modified because of the low number of recombinants obtained when working with the more distal markers. It was demonstrated that the decreased dilution had no effect on the times of entry of the markers dealt with in the previous paper although the shape of the curves changed from a straight line to a parabolic curve.

With the strains available it was not possible to measure directly the distance between the two *ilv* markers but, since *ilvD* was known to be closely linked to *pro-1* (Table 1) and to be proximal to it, a reasonable estimate could be obtained by measuring the distance between *ilvB* and *pro-1*. In fact the distance between *ilvB112* and *pro-5* was measured since it was shown by transduction that *pro-1* and *pro-5* belong to the same proline locus (LOUTIT, unpublished data). The results are shown in Figure 1 and the distance between these markers can be seen to be 24–25 minutes. The distance between *ilvB* and *ilvD* therefore must be less than but close to 25 minutes.

DISCUSSION

Ninety three isoleucine-valine mutants of *P. aeruginosa* strain 1 and strain 78 have been placed in three functional groups. Strains with lesions in the *ilvB* locus lacked acetohydroxy acid synthetase and it is interesting that one-step mutants blocked in this enzyme can be obtained so readily. At the time that a preliminary report was made (MARINUS and LOUTIT 1966) there were no other reports of such isolations but since then they have been demonstrated in *Staphylococcus aureus* by SMITH and PATTEE (1967). Mutants with lesions in the *ilvC* locus

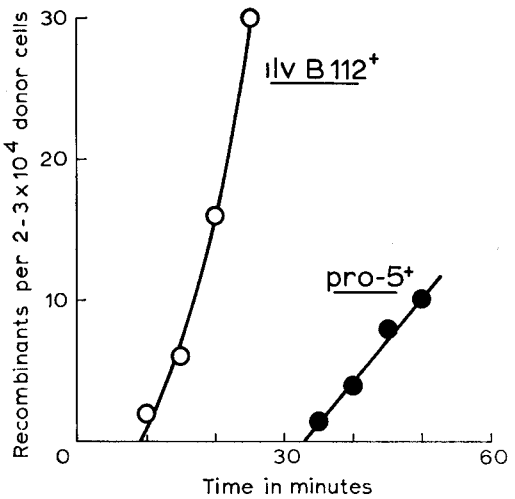

FIGURE 1.—Time of entry kinetics of *ilvB112* and *pro-5* markers. Pairing was allowed for 5 minutes after which the cells were diluted 10^{-2} in fresh medium. The time is measured from the first mixing of the cells.

lacked reductoisomerase and those in the *ilvD* locus were deficient in the dihydroxy acid dehydratase enzyme. One further interesting point about these groups is that it has not been possible to isolate a transaminase mutant or one lacking the threonine dehydratase. These latter mutants in particular have been readily isolated in other species investigated. This suggests that the catabolic L-threonine-L-serine deaminase (WOOD and GUNSALUS 1949) may take over the role of the biosynthetic enzyme if the latter is nonfunctional. For the transaminase step a similar situation may exist as an extract from *P. aeruginosa* was shown to transaminate a variety of amino acids, including isoleucine and valine, to keto acids (NORTON and SOKATCH 1966). Whether more than one transaminase was present or whether a nonspecific transaminase was acting remains to be seen.

Linkage studies have shown that the *ilvB* and *ilvC* gene loci are contiguous and the conjugation system has allowed a quantitative estimate of the distance between the linked loci and *ilvD*. There is no doubt that they are well separated on the chromosome in *P. aeruginosa*. In this respect the species is like *Bacillus subtilis* (BARAT, ANAGNOSTOPOULOS and SCHNEIDER 1965).

One other class of mutants should be mentioned. These are the ones responding either completely or partially to valine which have been shown to have a partial block at the reductoisomerase step. Such mutants have also been isolated in *E. coli* (UMBARGER, BROWN and EYRING 1960) and in *Salmonella typhimurium* (WAGNER and BERGQUIST 1960). The strain with the absolute requirement (OT500) for valine has been extremely useful because it has allowed the development of a ratio test for mapping the sites in the *ilvB* gene. A similar method has recently been used by ELLIOT and ARMSTRONG (1968) to map the sites in the *ilvD* gene of *S. typhimurium*. The ratio tests also showed that the mutational site

in OT517 (*ilv-17*) mapped outside but close to, the *ilvB* gene. It was also shown that this strain produced large amounts of acetohydroxy acid synthetase but no reductoisomerase. The strain, therefore, has some of the properties expected of an operator-constitutive mutant and will be dealt with more fully in a subsequent paper. Only one strain (OT560) gave anomalous results when its biochemical properties and its position on the chromosome were compared. It is probable, however, that some aberration is present with regard to the behaviour of this strain in genetic crosses.

It is obvious that *P. aeruginosa* differs from *E. coli* in a number of ways in the isoleucine-valine pathway and some further aspects now need to be investigated. We need to know whether the clustered genes constitute a unit of coordinate expression, whether synthesis of all isoleucine-valine enzymes is subject to multivalent repression and whether the early enzymes in the pathway are subject to feedback inhibition. These questions will be the subject of a further communication.

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SUMMARY

Ninety-three isoleucine-valine mutants of *P. aeruginosa* strains 1 and 78 were characterised into three functional groups. The group with lesions in the *ilvB* locus lacked acetohydroxy acid synthetase; in the *ilvC* locus, reductoisomerase and the *ilvD* locus, dihydroxy acid dehydratase. The *ilvB* and *ilvC* loci were contiguous but the ilvD locus mapped at a site almost 25 minutes from the linked loci, as determined by conjugation. A tentative fine-structure map of the *ilvB* gene was constructed by co-transduction of mutants and a valine-requiring marker.

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