NEW MAP LOCATION OF *ilvO* IN *ESCHERICHIA COLI*[1]

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ABSTRACT

Mutations in *ilvO*, the operator site for operon A of the *ilv* region of *E. coli* K-12, are reported to cause a *cis* dominant derepression of the operon A gene products and to map between *ilvC* and *ilvA*. Studies reported here demonstrate that the F25 episome which does not carry the region between *ilvC* and *ilvA* but only *ilvE*, *ilvD* and a part of *ilvA*, can transfer *ilvO* genetic material. Also, genes of operon A on the F25 episome respond normally to a derepression signal. It is proposed that *ilvO* is on the F25 episome. Five-point crosses demonstrate that mutations in *ilvO* map nearer to *ilvE* than to *ilvA*. Considering this and other evidence, it is proposed that the gene order in the *ilv* region is CADEO, not COADE, with transcription and translation in operon A being from *ilvE* to *ilvA* not *ilvA* to *ilvE*. It is confirmed that mutations in *ilvO* cause a *cis* dominant derepression of the operon A gene products but it is also noted that mutations in *ilvO* lead to the appearance of an AHAS activity more resistant to inhibition by valine than that of the present O+ strain.

THE structural genes for the enzymes of isoleucine-valine biosynthesis are clustered at 75′ on the *E. coli* genome as depicted in Figure 1. The genes *ilvA*, *ilvD*, and *ilvE*, specifying threonine deaminase [EC 4.2.1.16, L-threonine hydro-

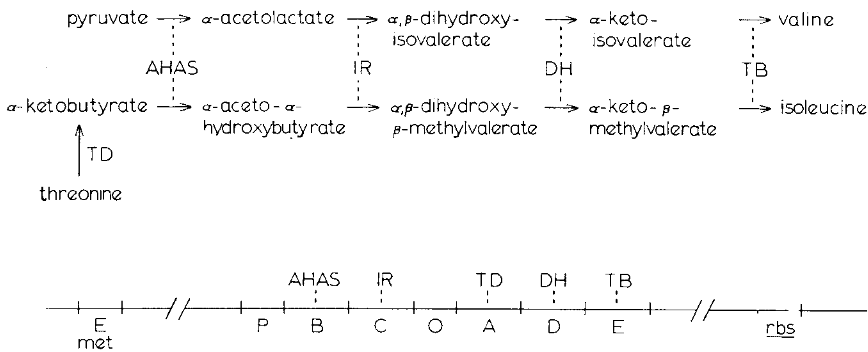

FIGURE 1.—Biosynthetic pathway for isoleucine and valine. The enzymes catalyzing each reaction are abbreviated as follows: TD, threonine deaminase; AHAS, valine-inhibited aceto-hydroxy acid synthetase; IR, acetohydroxy acid isomeroreductase; DH, dihydroxyacid dehydrase; TB, transaminase B. Genetic designations are as given in TAYLOR and TROTTER (1972).

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lyase (deaminating)], dihydroxyacid dehydrase (EC 4.2.1. 9, 2,3-dihydroxyacid hydro-lyase) and transaminase B (EC 2.6.1.42, branched-chain-amino-acid: 2-oxoglutarate aminotransferase) respectively, appear to constitute an operon, designated operon A (RAMAKRISHNAN and ADELBERG 1964). The proposed operator for operon A is *ilvO* and mutations at this site are reported to cause a *cis* dominant derepression of the operon A gene products (RAMAKRISHNAN and ADELBERG 1964, 1965a). Operon A is multivalently repressed by isoleucine, leucine and valine (DWYER and UMBARGER 1968; FREUNDLICH, BURNS and UMBARGER 1962). The *ilvB* gene product, an acetohydroxy acid synthetase [EC 4.1.3.18, acetolactate pyruvate lyase (carboxylating)] is repressed by valine and leucine (DWYER and UMBARGER 1968) and is under the control of operator *ilvP* (RAMAKRISHNAN and ADELBERG 1965a, 1965b). The *ilvC* gene product, isomeroreductase, [EC 1.1.1.86, 3-alkyl-2, 3-dihydroxy-acid: $NADP^+$ oxidoreductase (isomerizing)] is induced by its substrates, α-acetolactate and α-acetohydroxybutyrate (ARFIN, RATZKIN and UMBARGER 1969).

It has been proposed that threonine deaminase (TD) is not only a biosynthetic enzyme but is, in addition, a regulatory protein (repressor or activator) for the genes of the *ilv* locus (HATFIELD and BURNS 1970). There is physical, genetic and physiologic evidence to support this dual role for threonine deaminase (CALHOUN and HATFIELD 1973; HATFIELD and BURNS 1970; LEVINTHAL *et al.* 1973; WASMUTH and UMBARGER 1974; WASMUTH, UMBARGER and DEMPSEY 1973). However, if TD (Figure 1) is involved in the control of expression of genes of the *ilv* region, its role is apparently not an obligatory one, for a mutant bearing a deletion of *ilvA* shows normal control of the remaining, intact *ilvB* and *ilvE* genes (KLINE et al. 1974).

AHAS, the first enzyme in the pathway to valine and the first shared enzyme in isoleucine-valine biosynthesis is inhibited by valine (UMBARGER and BROWN 1958). The growth of wild-type *E. coli* K-12, unlike that of related organisms (*E. coli* W, *E. coli* B, *S. typhimurium*), is inhibited by 10^{-3} M valine. Considerable evidence implicates the extreme sensitivity of AHAS to feedback inhibition by valine in *E. coli* K-12 relative to these other organisms as the source of this phenomenon (LEAVITT and UMBARGER 1962; BLATT, PLEDGER and UMBARGER 1972; O'NEILL and FREUNDLICH 1972; RAMAKRISHNAN and ADELBERG 1965a; UMBARGER and BROWN 1955; UMBARGER and FREUNDLICH 1965). In effect, feeding valine to *E. coli* K-12 causes it to starve for isoleucine. Indeed, *E. coli* K-12 may only have a species of AHAS sensitive to inhibition by valine whereas *S. typhimurium*, *E. coli* B, and *E. coli* W may have at least two species of AHAS, one sensitive to and one resistant to such inhibition (BLATT, PLEDGER and UMBARGER 1972; O'NEILL and FREUNDLICH 1972).

Among the spontaneous mutants of *E. coli* K-12 resistant to growth inhibition by valine are mutants bearing lesions in the *ilvO* region (RAMAKRISHNAN and ADELBERG 1964). *IlvO*$^-$-bearing strains have derepressed levels of the operon A gene products TD, DH, and TB (RAMAKRISHNAN and ADELBERG 1964). The original isolators of these mutants report no alternation from wild type in either the level of AHAS activity or the sensitivity of this activity to valine (RAMA-

KRISHNAN and ADELBERG 1964). Nonetheless, these mutants are resistant to growth inhibition by even high levels (10^{-2} M) of valine. (RAMAKRISHNAN and ADELBERG 1964). The origin of this resistance is unclear.

The map position of the *ilvO*⁻ mutations is controversial. There are contradictory reports of *ilvO* being between *ilvC* and *ilvA* (RAMAKRISHNAN and ADELBERG 1965b; PLEDGER and UMBARGER 1973b), and being outside of an apparent deletion for that same region (KLINE *et al.* 1974).

A number of years ago, we began studies to determine if the *ilvO* locus was distinct from the first gene of operon A, *ilvA*. At the time, both *ilvO* and *ilvA* looked as if they might be regulatory loci and they had not been definitively separated. The results of these studies are presented here because they help characterize the *ilvO*⁻ mutations as to their location and their effect.

MATERIALS AND METHODS

Media

Saline was 0.85% NaCl. *A buffer*: (DAVIS and MINGIOLI 1950) was 7.0 gm KH_2PO_4, 3.0 gm K_2HPO_4, 0.5 gm trisodium citrate·$3H_2O$, 0.1 gm $MgSO_4 \cdot 7H_2O$ and 1.0 gm $(NH_4)_2SO_4$ in 1000 ml H_2O. *A medium*: *A* buffer plus 0.4% sugar and 1 μg/ml thiamine. Amino acids were added to a final concentration of 50 μg/ml except for valine which was used at 100 μg/ml. α-ketoisovalerate and α-ketobutyrate were added at 100 μg/ml. All solutions were autoclaved separately. *LB*: 10 gm tryptone, 5 gm yeast extract, and 1 ml IN NaOH in 1000 ml H_2O. *P broth*: Difco Penassay broth (Difco Laboratories, 1953), 1.5 gm Bacto beef extract, 1.5 gm yeast extract, 5.0 gm peptone, 1.0 gm dextrose, 3.5 gm NaCl, 3.68 gm K_2HPO_4 and 1.32 gm KH_2PO_4 in 1000 ml H_2O. Media were solidified when necessary by addition of sterile 3% agar solution to bring the final concentration of agar to 1.5%.

Strains

A table of strains used in these studies is provided (Table 1). Discussion of the origin and construction of these strains can be found in COHEN 1975.

Penicillin selection of mutants:

Penicillin selection followed the technique of IACCARINO and BERG (1971), with the modification that the method was cyclic as introduced by LUBIN (1962).

Test for the rec⁻ trait:

The *rec*⁻ trait was tested for by UV sensitivity as described by CLARK and MARGULIES (1965). Strains carrying the *rec*⁻ trait were retested before use in any strain construction or experiment.

Genetic methods

Tests of donor ability and complementation, used primarily to characterize the F25 and F25-1 episomes, when done on plates, followed the procedures of WECHSLER and ADELBERG (1969). For complementation tests, the F⁻ recipient was always *recA56* although recombination could be distinguished from complementation with a *rec*+ recipient.

Matings in which the donor carried an episome were performed on the model of the method of MARSH and DUGGAN (1972). Donor and recipient in exponential growth at 37° at titers of 1×10^8 and 3×10^8 cells/ml respectively in *A* medium were mixed in equal volume. Incubation was for 60 minutes, stationary, at 37°. Mating was terminated by vigorous swirling on a vortex mixer, and the cells were washed in cold *A* buffer. Appropriate dilutions were spread on selective media or at selective temperature. Counterselection of the donor was always by nutritional means. Controls always included the plating of unexposed donor and recipient cultures to

TABLE 1

*Bacterial strains**

Strain	Sex	Genotype	Origin
AB673	HfrP10	point of origin in *malB thr-19 leu-30 malB16 lac-21 thi-20*	CGSC (JACOB and WOLLMAN 1957; SCHWARTZ 1966)
BC200	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilv⁻*	p s on AB673
BC230	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 rbs-215*	trans. of BC200 (CU16)
BC201–220	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvAts1–20* respectively (20 strains)	trans. of BC230 (BC1–20)
BC231	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvC7*	trans. of BC230 (AB1203)
BC232	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvE12*	trans. of BC230 (AB2070)
BC240	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvO268 ilvAts11*	trans. of BC230 (BC150)
BC241	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvO269 ilvAts11*	trans. of BC230 (BC151)
BC242	Hfr	*thr-19 leu-30 malB16 lac-21 thi-20 ilvO269 ilvAts6*	trans. of BC230 (BC152)
JC5088	Hfr	point of origin KL16 between *serA* and *lysA recA56 thr-300 ilv-318 spc-300* sm^s	A. J. CLARK (CLARK 1967; WILLETTS, CLARK and LOW 1969)
AB1206	F′	F′14 (▶ *ilvE argH* →)/*thi-1 hisG4 proA2 lacY1 galK2 thr-3 str-8* chromosomal deletion corresponding to F′14	CGSC (PITTARD and ADELBERG 1963; PITTARD, LOUTIT and ADELBERG 1963; PITTARD and ADELBERG 1964; GLANSDORFF 1967)
AB1009	F′	F′14 *ilvO268*/chromosome as for AB1206	CGSC (RAMAKRISHNAN and ADELBERG 1964)
AB1013	F′	F′14 *ilvO269*/chromosome as for AB1206	CGSC (RAMAKRISHNAN and ADELBERG 1964)
AB3519	F′	F′25 (▶ *ilvE ilvD* →)/*thi-1 ilvD188 hisG4 trp-3 proA2 mtl-1 malA1 ara-9 galK2 lacY1* or *lacZ4 ton-1 tsx-3 sup-48 str-8* or *9*	CGSC (WECHSLER and ADELBERG 1969)
AB3526	F′	F′25–1 (originally F219) (▶ *ilvE ilvD* →) *ilvE316/thi-1 ilvE316 hisG4 trp-3 mtl-1 malA1 lacZ13 str-8* or *9 ton-1? tsx-3* λ^R λ^-	CGSC (WECHSLER and ADELBERG 1969)
BC270	F′	F′25/BC71	conj. BC280×BC71
BC271	F′	F′25/BC71 but *leu⁻*	p s on BC270
BC273	F′	F′14 *ilvO268*/BC71	conj. AB1009×BC71
BC274	F′	F′14 *ilvO269*/BC71	conj. AB1013×BC71
BC275	F′	F′14 *ilvO268 ilvE⁻*/BC71	p s on BC273
BC276	F′	F′14 *ilvO269 ilvE⁻*/BC71	p s on BC274
BC280	F′	F′25/BC80	conj. AB3519×BC80
BC281	F′	F′25–1/BC80	conj. AB3526×BC80
BC282	F′	F′14/BC80	conj. AB1206×BC80
BC283	F′	F′14 *ilvO268*/BC80	conj. AB1009×BC80
BC284	F′	F′14 *ilvO269*/BC80	conj. AB1013×BC80

TABLE 1—Continued

Strain	Sex	Genotype	Origin
BC285	F′	F′14 *ilvO268* $ilvE^-$/BC80	conj. BC275×BC80
BC286	F′	F′14 *ilvO269* $ilvE^-$/BC80	conj. BC276×BC80
DB1032	F^-	$araA^-$ xyl^- $glpT^-$ gal^- $strA^-$	(BOTSTEIN and JONES 1969)
BC1–20	F^-	$araA^-$ xyl^- $glpT^-$ gal^- $strA^-$ *ilvAts1–20* respectively (20 strains)	p s on DB1032
BC30	F^-	$araA^-$ xyl^- $glpT^-$ gal^- $strA^-$ $ilvA^-$	p s on DB1032
BC40	F^-	$araA^-$ xyl^- $glpT^-$ gal^- $strA^-$ *ilvO268*	trans. of BC30 (AB1009)
BC41	F^-	$araA^-$ xyl^- $glpT^-$ gal^- $strA^-$ *ilvO269*	trans. of BC30 (AB1013)
AB1203	F^-	*ilvC7 argE3 thi-1 xyl-5 galK2 lacY1 lacZ4 tfr-3 tsx-6 sup-17*	CGSC (PITTARD and ADELBERG 1964; RAMAKRISHNAN and ADELBERG 1965b)
BC50	F^-	As for AB1203 with *recA56*	rec^- derivative of AB1203
AB1255	F^-	*ilvA201 argH1 metB1 his-1 thi-1 xyl-7 malA1 tsx-5 str-8* or *9* or *17 sup-48*? λ^R λ^-?	CGSC (PITTARD and ADELBERG 1964; RAMAKRISHNAN and ADELBERG 1965a,b; WECHSLER and ADELBERG 1969)
BC60	F^-	As for AB1255 with *recA56*	rec^- derivative of AB1255
BC61	F^-	As for AB1255 but arg^+	trans. of AB1255 (DB1032)
BC62	F^-	As for BC61 with *recA56*	rec^- derivative of BC61
AB2070	F^-	*ilvE12 metE46 hisG4 trp-3 proA2 thi-1 mtl-1 malA1 ara-9 galK2 lacY1* or *lacZ4 tsx-3 str-8* or *9 ton-1* λ^R λ^- *sup-36*	CGSC (EGGERTSON and ADELBERG 1965; WECHSLER and ADELBERG 1969)
BC70	F^-	As for AB2070 but his^+	trans. of AB2070 (DB1032)
BC71	F^-	*ilvE12 metE46 trp-3 proA2 thi-1 mtl-1 malA1 ara-9 galK2 lacY1* or *lacZ4 tsx-3 str-8* or *9 ton-1* λ^R λ^-*sup-36 recA56*	rec^- derivative of BC70
BC72	F^-	As for AB2070 but trp^+ pro^+	trans. of AB2070 (two steps) (DB1032)
BC73	F^-	As for BC72 but *recA56*	rec^- derivative of BC72
AB1472	F^-	*ilvD16 argH1 metB1 thi-1 malA1 gal-6*? *lacY1*? *str-8* or *9* or *17 sup-48* λ^R λ^-	CGSC (RAMAKRISHNAN and ADELBERG 1965b)
BC80	F^-	*ilvD16 argH1 metB1 thi-1 malA1 gal-6*? *lacY1*? *str-8* or *9* or *17* λ^R λ^- *recA56*	rec^- derivative of AB1472
BC81	F^-	As for AB1472 but arg^+	trans. of AB1472 (DB1032)
BC82	F^-	As for BC81 with *recA56*	rec^- derivative of BC81
CU16	F^-	*metE200 rbs-215* gal^-	(PLEDGER and UMBARGER 1973a,b)
BC100	F^-	*metE200 rbs-215* gal^- ilv^-	p s on CU16
BC101–120	F^-	*metE200 rbs-215* gal^- *ilvAts1–20* respectively (20 strains)	trans. of BC100 (BC1–20)
BC150	F^-	*ilvO268 ilvAst11 metE200* gal^-	trans. of BC111 (BC40)

TABLE 1—Continued

Strain	Sex	Genotype	Origin
BC151	F⁻	*ilvO269 ilvAts11 metE200 gal⁻*	trans. of BC111 (BC41)
BC152	F⁻	*ilvO269 ilvAts6 metE200 gal⁻*	trans. of BC106 (BC41)
BC160	F⁻	*metE200 rbs-215 ilvO268 ilvAts11 gal⁻*	trans. of BC100 (BC150)
BC161	F⁻	*metE200 rbs-215 ilvO269 ilvAts11 gal⁻*	trans. of BC100 (BC151)
BC162	F⁻	*metE200 rbs-215 ilvO269 ilvAts6 gal⁻*	trans. of BC100 (BC152)

* CGSC indicates the strain was obtained from the Coli Genetics Stock Center, Yale University. Designations are: p s, penicillin selection; trans., transduction with phage P1 (donor strain given in parentheses); conj., conjugation.

selective plates. Colonies were counted and/or picked at two days. Recombination frequencies were calculated as the number of recombinants arising per donor cell in the initial mating mixture. Reversion rates are given as revertants per bacteria plated.

For the experiments detailed in section (b) of RESULTS wherein donors carried the F25 episome and recipients were *ilv⁻* strains, recombination rates appeared (as given in that section) in the range of 10^{-2} and $1\text{–}7 \times 10^{-5}$. Rates for certain crosses were zero or indistinguishable above the background of revertants. The reversion rate for the donor strains was insignificant at the concentrations of cells plated. The reversion rate for the recipients was between 8×10^{-8} and 1×10^{-8}. Given the recipient-to-donor ratio of 3/1, this means that recombination rates below roughly 10^{-7} could not be distinguished above the background of revertants.

For the matings described in section (c) of RESULTS donors carrying the F25 episome were crossed with *metE⁻ ilvO⁻ ilvAts rbs⁻* recipient strains with selection for *ilvA+* clones and subsequent scoring of the *ilvO⁻* marker. For these matings, dilutions were made so that the matings would yield 50–100 *ilv+* recombinants per selective plate. Controls included plating donor and recipient on selective plates in numbers equal to and twice that expected on the experimental plates. Recombinants underwent two successive single colony isolations on LB plates before being tested for the *met⁻* and *rbs⁻* markers as well as the presence or absence of the temperature conditional requirement for isoleucine and sensitivity or resistance to valine. This isolation was required because of the background of *ilvO⁻* (valine resistant) strains on the selective plate. Recombination rates in these studies were 6×10^{-5} while reversion rates were $4\text{–}6 \times 10^{-8}$. In practice, as seen on the plates and consistent with the donor-to-recipient ratio as given, this meant a recombinant-to-revertant ratio of about 200/1. The spontaneous revertants mentioned in section (c) of RESULTS were obtained by spreading roughly 10^8 of the recipients from the log phase culture, also used for withdrawal of a sample for mating, on a selective plate after washing in *A* buffer.

When matings were done with Hfr donors to map the various *ilvA* and *ilvO* markers, the techniques of SADLER and SMITH (1971); CURTISS (1969); and CURTISS *et al.* (1968) were employed. Donor and recipient from *P* broth overnights were diluted 1/500 and 1/100 respectively in fresh *P* broth. Each was incubated shaking at 37°. Donor and recipient were mixed in a 1/10 ratio when the donor reached a titer of $1\text{–}2 \times 10^8$ cells/ml in exponential growth. The recipient was initially at $2\text{–}3 \times 10^8$ cells/ml in the mating mixture. After 30–40 minutes of stationary incubation at 37°, the mating mixture was gently diluted five-fold with warm *P* broth. For the four-point crosses described in section (e) of RESULTS, three more hours of stationary incubation at 37° were then allowed. For the five-point crosses of section (f) of RESULTS two more hours of stationary incubation at 37° were then allowed. At the end of this time, the mating mixtures underwent one minute of vigorous shaking, followed by 30 minutes incubation, shaking at high speed in a water bath at 37°. They were then washed and resuspended in *A* buffer. Appropriate dilutions were spread on selective plates. In each case controls included separately spreading unexposed donors and recipients on selective plates in numbers equal to and twice that expected from the mating mixture.

Reversion rates, expressed as revertants per recipient plated, were in the range of 10^{-7} to 10^{-8} for the four-point crosses of section (e) of RESULTS. In the five-point crosses, the reversion rate of strains BC160 and BC161 (both *ilvO^c ilvAts11*) was $5 \times 10^{-8} — 1 \times 10^{-7}$ while the reversion rate of strain BC162 (*ilvO^c ilvAts6*) was $9 \times 10^{-8} — 2 \times 10^{-7}$. Recombination rates, the number of recombinants arising per donor cell in the initial mating mixture, were also calculated. For the four-point crosses, in which recombination was required between two *ilvA* mutations, recombination rates were $3 \times 10^{-6} — 4 \times 10^{-5}$. For the five-point crosses in which recombination was required between an *ilvE* mutation (in the Hfr donor) and an *ilvA* mutation (in the F^- recipient), the recombination rate was $1–2 \times 10^{-4}$. For the five-point crosses in which recombination was required between an *ilvC* mutation (in the Hfr donor) and an *ilvA* mutation (in the F^- recipient), the recombination rate was $3–7 \times 10^{-5}$. In a test cross to yield *ilvA*+ recombinants with an *ilv*+ Hfr donor strain and an *ilvA*- F^- recipient using the mating technique given above, the recombination rate was $1–3 \times 10^{-3}$.

Enzyme assays

Techniques and procedures used in the preparation of cell extracts were those of GUARDIOLA and IACCARINO (1971) and IACCARINO and BERG (1971). Threonine deaminase and transaminase B were assayed by determining the rate of α-ketobutyrate and α-ketoisovalerate formation, respectively, according to the procedures of IACCARINO and BERG (1971). Acetohydroxy acid synthetase was assayed by determining the rate of acetolactate formation according to the method of STØRMER and UMBARGER (1964). All activities are expressed as nanomoles of product formed per minute per milligram of protein.

Reagents

Nearly all biological reagents (amino acids, α keto acids, sugars, cofactors, acridine orange, glycl-valine, dithiothreitol, Tris, and bovine serum albumin) were from Sigma Chemical Co. with exceptions as follows: Glucose, tryptone, and yeast extract were from Difco Laboratories Inc. Disodium (ethylenedinitrilo) tetraacetate (EDTA) was from J. T. Baker Chemical Co. Acetone was from Aldrich Chemical Co., Inc. Penicillin G was from E. R. Squibb and Sons.

RESULTS

a) *The* ilvAts *mutations*

Twenty mutants with twenty independent mutations in *ilvA* leading to a temperature-sensitive TD were chosen by penicillin selection following UV mutagenesis. These mutations (*ilvAts*1–20) lead to the following temperature-conditional phenotype: Ilv prototrophy at 30° and an alternate isoleucine or α-ketobutyrate requirement at 42°. The most leftward and rightward of these *ilvA* mutations were to be determined for ordering with respect to the *ilvO*- mutations so that *ilvO* could be mapped inside or outside of *ilvA*.

b) *Preliminary mapping of the* ilvAts *mutation with episomes F25 and F25–1*

Episomes are known which carry only part of the genetic material of the *ilv* locus. They are produced (PITTARD and ADELBERG 1963; RAMAKRISHNAN and ADELBERG 1965b) in P1 mediated transductions of an *ilv*- recipient-to-Ilv prototrophy wherein the donor bears the F14 episome, which carries the *ilv* locus quite close to the sex factor (PITTARD, LOUTIT and ADELBERG 1963; ROSNER, KASS and YARMOLINSKY 1968). The order of the *ilv* genetic material on these episomes, including the F25 and F25–1 episomes used in these studies, appears to be the same as that of the wild-type *E. coli* K-12 (LEE *et al.* 1974; MARSH and DUGGAN

1972) and, therefore, these episomes have been used for mapping studies in the *ilv* region by a technique akin to deletion mapping (MARSH and DUGGAN 1972; RAMAKRISHNAN and ADELBERG 1965b).

Two episomes (F25 and F25–1, as mentioned) were obtained which carry only *ilvE*, *ilvD*, and part of *ilvA* (MARSH and DUGGAN 1972; PITTARD and ADELBERG 1963; WECHSLER and ADELBERG 1969; LEE *et al.* 1974) for use in crosses to give a preliminary ordering of the *ilvAts* mutations. In crosses in which the donor bears the F25 or F25–1 episome and is *recA*$^-$ and the F$^-$ recipient is *ilv*$^-$, *ilv*$^+$ recombinants will appear only when the *ilv*$^-$ lesion of the recipient is in a region carried by the episome. The results of such crosses between F25 or F25–1 bearing donors and *ilvAts* as well as *ilvD*$^-$ and *ilvC*$^-$ bearing recipients with selection of *ilv*$^+$ recombinants are presented in Table 2.

The cross involving the *ilvD*$^-$ recipient shows recombination while the cross involving the *ilvC*$^-$ recipient does not. In other words, *ilvD*$^+$ is carried on the episomes, *ilvC*$^+$ is not. Crosses with recipients bearing the *ilvAts2* and *ilvAts11* lesions yield recombinants while crosses involving recipients bearing the other *ilvAts* mutations do not. This last fact implies that *ilvAts2* and *ilvAts11* are the most rightward of the *ilvAts* mutations and lie within the region carried by the episome. The crosses described are diagrammed in Figure 2, which incorporates the data of Table 2, as well as Tables 5 and 6, for positioning the mutations in *ilvA*.

c) *Mapping* ilvO *by the use of episome F25*

It is apparent that the same episomes which were used to preliminarily order the *ilvAts* mutations can be used to approximately map the *ilvO*$^-$ mutations by determining whether these episomes carry *ilvO*$^+$ genetic material. If the episomes do carry *ilvO* genetic material, then *ilvO* must lie in *ilvA* or in a region to the

TABLE 2

Results of matings to order the ilvAts *mutations using episome carrying donors*

F$^-$ recipient	Relevant genotype	Recombination rate* with donor BC280	BC281
BC1	*ilvAts1*	......	......
BC2	*ilvAts2*	3×10^{-5}	1×10^{-5}
BC3–BC10	*ilvAts3–10* respectively	......	......
BC11	*ilvAts11*	7×10^{-5}	3×10^{-5}
BC12–20	*ilvAts12–20* respectively	......	......
BC81	*ilvD16*	2×10^{-2}	2×10^{-2}
AB1203	*ilvC7*	......	......

Matings were performed as described in MATERIALS AND METHODS using BC280, carrying episome F25, and BC281, carrying episome F25–1, as donors. F$^-$ recipients bore *ilv*$^-$ lesions as noted. Selection was for *ilv*$^+$ recombinants. Donors were eliminated by nutritional counterselection.

* Figures in the table are recombination rates calculated as the number of *ilv*$^+$ recombinants arising in the mating mixture per donor cell. Blanks in the table indicate that the recombination rate is not discernible against the background rate expected from reversion (see MATERIALS AND METHODS). This implies a recombination rate $\leq 10^{-7}$.

Complete genotypes may be found in Table 1.

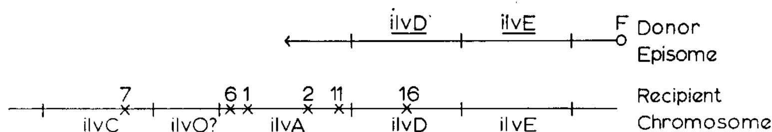

FIGURE 2.—Short episome crosses. In this diagrammatic representation of a cross the donor bears an F25 or F25-1 episome and the recipient bears an *ilv*⁻ mutation. X's on the recipient chromosome represent sites of *ilv*⁻ mutations. The *ilvC7* and *ilvD16* mutations were mapped by others. For positioning of *ilvAts2, 6, 8* and *11* see text.

right of *ilvA*. The donor for these studies was a *recA*⁻ strain bearing episome F25. The recipients were *metE*⁻*rbs*⁻ *ilvAts11* (located in the region of *ilvA* carried by the episome) and carried either *ilvO268* or *ilvO269*, *ilvO*⁻ mutations isolated by RAMAKRISHNAN and ADELBERG (1964), the original isolators of the *ilvO*⁻ class of mutations. Recombinants were selected as *ilvA*⁺ and were scored as *ilvO*⁻ (resistant-to-growth inhibition by valine, Val-r) or *ilvO*⁺ (sensitive-to-growth inhibition by valine, Val-s). Finding *ilvA*⁺ recombinants which are Val-s implies that *ilvO*⁺ genetic material is carried on the episome. The recombinants were also scored for the *metE* and *rbs* markers.

The results of this study, given in Table 3, show that 10–12% of the *ilvA*⁺ recombinants were indeed *ilvO*⁺ (Val-s), implying that *ilvO*⁺ genetic material is present on the F25 episome. Supporting the *ilvA*⁺ *ilvO*⁺ progeny of the cross as true recombinants, not artifactual in presence or frequency is the following information: (1) all recombinants retained the *met*⁻ and *rbs*⁻ markers of the recipient, (2) revertants of neither parent could contribute significantly to the *ilvA*⁺ *ilvO*⁺ class as the reversion rates were too low in each case (see MATERIALS AND METHODS), (3) the F' donors were *met*⁺ *rbs*⁺ and any revertants of this

TABLE 3

Results of matings to determine if ilvO *is carried on the F25 episome*

F⁻ recipient	Relevant genotype*	Total *ilv*⁺ recombinants tested	No. of *ilv*⁺ recombinants Val-s	% of *ilv*⁺ recombinants Val-s
BC160	*ilvAts11 ilvO268*	100	10	10%
BC161	*ilvAts11 ilvO269*	50	6	12%

Matings were performed as described in MATERIALS AND METHODS using BC280, carrying episome F25, as donor and BC160 or BC161 as recipient. Selection was for *ilv*⁺ recombinants at 42°. Donors were eliminated by nutritional counterselection. Recombinants were repurified by two successive single colony isolations on LB. All recombinants tested were phenotypically *ilv*⁺ *met*⁻ *rbs*⁻. Recombinants were scored for valine sensitivity (Val-s) or resistance (Val-r) according to their ability to grow on minimal medium (with methionine) containing 100 μg/ml valine. Val-s strains are those unable to grow on 100 μg/ml valine.

50 spontaneous revertants of BC160 and BC161 to *ilv*⁺ were selected at 42° (see MATERIALS AND METHODS). All were characterized as above and all were phenotypically *ilv*⁺, *met*⁻, *rbs*⁻, and Val-r.

The reversion rates were tested for donor and recipient strains under the conditions employed for the experiment. The recipients yield less than 1 revertant per 200 recombinants; the donors yield revertants at an even lower rate and, therefore, the rate was not precisely determined (see MATERIALS AND METHODS).

* Complete genotypes may be found in Table 1.

episome-bearing parent should have been *met*$^+$ *rbs*$^+$ unlike the recombinants seen, (4) fifty *ilvA*$^+$ revertants were selected from each F$^-$ recipient and all were *ilvO*$^-$, so that *ilvA*$^+$ revertants of the F$^-$ parent could not account for the *ilvA*$^+$ *ilvO*$^+$ recombinants seen.

The results clearly imply that *ilvO* genetic material is carried on the F25 episome. The fact that only 10–12% of the *ilvA*$^+$ recombinants of the above crosses were *ilvO*$^+$ argues that *ilvO* is close to the sex factor of the episome, on one side or the other, such that the distance between the *ilvO*$^-$ and *ilvAts* sites is much greater than the distance between *ilvO*$^-$ and the sex factor. With this configuration, crossover events to excise the *ilvAts* lesion occur most often between the *ilvO*$^-$ and *ilvAts* sites and the recipient remains *ilvO*$^-$. Taken as a whole, the results imply that *ilvO*, mapped by the *ilvO*$^-$ lesion, is in *ilvA* or is between *ilvE* and the sex factor.

d) *Control of genes on the F25 episome*

The results of the last section imply that *ilvO*, the operator region for operon A, is carried on the F25 episome. If this is so, then the genes of operon A on the episome should respond normally to a derepression signal. Arguing the reverse, finding that the genes on the F25 episome do respond normally to a derepression signal would imply that they are under the control of their normal operator. This would indicate that the normal operator for operon A is on the F25 episome.

To test the regulatory response of the genes on the F25 episome, a merodiploid (BC271) was constructed which bore the F25 episome (*ilvE*$^+$ *ilvD*$^+$ *ilvA*$^-$ part of A) and whose chromosome was *ilvE12 ilvD*$^+$ *ilvA*$^+$ *leu*$^-$ *recA56*. For the genes of operon A, this merodiploid was F'E$^+$D$^+$A$^-$ (partial A)/E$^-$D$^+$A$^+$. Thus the only active *ilvE* gene product (TB) was coded for by the *ilvE* gene on the episome, while the only active *ilvA* gene product (TD) was coded for by the *ilvA* gene on the chromosome. (That the chromosomal *ilvE12* allele codes for an inactive TB was confirmed by assays on cell-free extracts of strain BC71, the *ilvE12* F$^-$ parent of BC271). A parallel increase of the TD and TB activities of strain BC271 in response to a derepression signal would imply that the episomal and chromosomal genes are under similar control and, thus, that the normal operator for operon A was on the episome as well as on the chromosome.

Strain BC271 was grown under conditions of leucine excess and leucine limitation and TD and TB activities were measured. The results of this study are presented in Table 4. It can be seen that leucine limitation, as expected, is a powerful derepression signal for the genes of operon A. It can also be seen that the activities of TD and TB are nearly equivalent in their response to this signal. As noted, this argues that the episomal *ilvE* gene is under normal control and is *cis* to its normal operator. Consistent with the genetic results of the last section, these results imply that the operator for operon A (*ilvO*) is on the F25 episome and is not in the region between *ilvA* and *ilvC* as the episome does not carry that region.

e) *Four-point reciprocal crosses to order the* ilvAts *mutations*

To effectively map *ilvO* inside or outside of the *ilvA* gene, boundary mutations for *ilvA* (the most leftward and rightward of the twenty *ilvAts* mutations) were

TABLE 4

Results of the attempted derepression of ilvE *on the F25 episome*

Strain	Relevant genotype in *ily*	Growth condition	Specific activity TD	Specific activity TB
BC271	F'E+D+A- (partial A)	excess leucine*	15±3	11±2
	E-D+A+	limiting leucine†	220±10	170±20

Strain BC271 grown in minimal medium with excess isoleucine, leucine, and valine (50 μg/ml isoleucine, 50 μg/ml leucine, 100 μg/ml valine)* or in minimal medium limited in leucine (50 μg/ml isoleucine, 4 μg/ml leucine, 100 μg/ml valine).† Techniques of growth, harvest, extract preparation and execution of assays are as described in MATERIALS AND METHODS. Activities are nanomoles product/minute/mg protein and are expressed as the mean of the observed values ± the standard deviation calculated from observed values.

determined. This ordering of the twenty *ilvAts* mutations was accomplished by reciprocal four-point crosses as diagrammed in Figure 3 for postulated mutations *ilvA1* and *ilvA2*. The donor in each cross was an Hfr which was *met*+ *ilvAtsX rbs*+. The recipient was a *metE*- *ilvAtsY rbs*- *F*- strain. Selection was for *ilvA*+ recombinants and these recombinants are scored for the outside markers. It is the ratio of *met*+ *rbs*- clones to *met*- *rbs*+ clones among the *ilvA*+ recombinants which implies an order for *ilvAtsX* and *ilvAtsY*. Noting the diagram of Figure 3, if the *ilvA* lesion of the donor is rightward of the *ilvA* lesion of the recipient, then *met*+ *rbs*- clones are more frequent than *met*- *rbs*+ clones among the *ilvA*+ recombinants. If the *ilvA* lesion of the donor is more leftward than that of the recipient, then *met*+ *rbs*- clones are less frequent than *met*- *rbs*+ clones among the *ilvA*+ recombinants. *IlvA*+ recombinants with a parental arrangement of outside markers are due to reversion or are the result of multiple crossover phenomena or nonreciprocal recombination (conversion phenomena) in the region studied. Thus, they are not useful for ordering the inside markers.

Reciprocal four-point crosses as described were performed to find the most leftward and rightward of the twenty *ilvAts* mutations. The crosses showed *ilvAts6* and *ilvAts11* to be, respectively, the most leftward and rightward of the twenty *ilvAts* mutations. The results of all crosses involving *ilvAts6* are given in Table 5. It can be seen that in any cross in which *ilvAts6* is in the donor, the *met*+ *rbs*- clones are less frequent than the *met*- *rbs*+ clones among the *ilvA*+ recombinants. The opposite ratio always holds in the reciprocal cross in which *ilvAts6* is in the recipient. These results are what one would expect in mapping

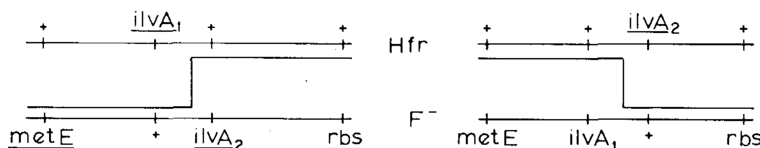

FIGURE 3.—Diagrammatic representation of reciprocal four-point crosses to order two *ilvA* mutations. *ilvA*+ recombinants are selected. Only the crossover event yielding an *ilvA*+ recombinant is diagrammed. A second crossover event necessary to incorporate donor genetic material is not diagrammed.

TABLE 5

Results of the reciprocal four-point crosses involving ilvAts6

Matings* Hfr × F-	Total *ilvA*+ recombinants	Of total *ilvA*+ recombinants, those:			
		met+ *rbs*+	*met*+ *rbs*-	*met*- *rbs*+	*met*- *rbs*-
BC206 × BC101	46	10	9	12	15
BC201 × BC106	39	4	24	1	10
BC206 × BC102	32	6	5	13	8
BC202 × BC106	43	6	19	2	16
BC206 × BC103	24	0	5	15	4
BC203 × BC106	19	3	8	2	6
BC206 × BC104	22	1	6	10	5
BC204 × BC106	24	2	14	2	6
BC206 × BC105	27	3	5	13	6
BC205 × BC106	40	8	17	4	11
BC206 × BC107	50	9	8	21	12
BC207 × BC106	41	11	18	2	10
BC206 × BC108	46	8	5	24	9
BC208 × BC106	52	5	38	5	4
BC206 × BC109	21	2	4	9	6
BC209 × BC106	41	4	19	1	17
BC206 × BC110	24	3	3	8	11
BC210 × BC106	21	4	7	2	8
BC206 × BC111	60	2	5	20	33
BC211 × BC106	40	7	19	1	13
BC206 × BC112	25	6	2	10	7
BC212 × BC106	18	5	5	1	7
BC206 × BC113	49	14	7	16	12
BC213 × BC106	50	12	20	2	16
BC206 × BC114	36	6	6	15	9
BC214 × BC106	33	5	17	3	8
BC206 × BC115	27	5	3	13	6
BC215 × BC106	23	5	10	2	6
BC206 × BC116	21	1	5	10	5
BC216 × BC106	21	4	12	0	5
BC206 × BC117	43	5	8	20	10
BC217 × BC106	20	5	9	1	5
BC206 × BC118	29	4	3	14	8
BC218 × BC106	43	4	18	2	19
BC206 × BC119	24	0	5	12	7
BC219 × BC106	22	3	11	1	7
BC206 × BC120	19	3	4	7	5
BC220 × BC106	20	2	10	1	8

Techniques for mating as well as recombination and reversion rates are as in MATERIALS AND METHODS. Selection was for *ilvA*+ recombinants at 42°. Recombinants are picked and scored for the *met* and *rbs* markers.

* Note that in all cases the Hfr is an Hfr P10 with genotype *metE*+ *ilvAtsX rbs*+ and the F- is *metE200 ilvAtsY rbs-215*. Further genotype on specific strains is in Table 1.

The last two digits in each strain number are identical to the numerical designation of the *ilvAts* mutation carried by the strain. Thus BC106 and BC206 bear *ilvAts6*.

TABLE 6

Results of reciprocal four-point crosses involving ilvAts11

Matings* Hfr × F-	Total *ilvA*+ recombinants	Of total *ilvA*+ recombinants, those: *met*+ *rbs*+	*met*+ *rbs*-	*met*- *rbs*+	*met*- *rbs*-
BC211 × BC101	33	4	20	1	8
BC201 × BC111	21	2	4	11	4
BC211 × BC102	46	9	13	1	23
BC202 × BC111	49	10	5	14	20
BC211 × BC103	25	1	19	3	2
BC203 × BC111	29	1	4	18	6
BC211 × BC104	27	0	20	1	6
BC204 × BC111	34	2	6	21	5
BC211 × BC105	39	1	26	4	8
BC205 × BC111	42	4	7	22	9
BC211 × BC107	22	4	11	1	6
BC207 × BC111	40	9	5	16	10
BC211 × BC108	27	8	19	0	0
BC208 × BC111	52	10	5	22	15
BC211 × BC109	32	6	10	2	10
BC209 × BC111	32	5	7	12	8
BC211 × BC110	38	10	19	1	8
BC210 × BC111	26	4	4	12	6
BC211 × BC112	24	4	11	1	8
BC212 × BC111	23	3	5	9	6
BC211 × BC113	27	5	12	1	9
BC213 × BC111	31	1	5	15	10
BC211 × BC114	21	3	12	1	5
BC214 × BC111	30	3	6	17	4
BC211 × BC115	37	6	20	1	10
BC215 × BC111	18	2	2	12	2
BC211 × BC116	44	4	25	2	13
BC216 × BC111	34	3	7	17	7
BC211 × BC117	31	1	20	1	9
BC217 × BC111	36	4	8	20	4
BC211 × BC118	29	4	18	1	7
BC218 × BC111	20	2	4	9	5
BC211 × BC119	27	4	15	2	11
BC219 × BC111	28	5	6	11	6
BC211 × BC120	33	5	18	0	10
BC220 × BC111	23	2	5	9	7

Techniques for mating as well as recombination and reversion rates are as in MATERIALS AND METHODS. Selection was for *ilvA*+ recombinants at 42°. Recombinants are picked and scored for the *met* and *rbs* markers.

* Note that in all cases the Hfr is an Hfr P10 with genotype *metE*+ *ilvAtsX rbs*+ and the F- is *metE200 ilvAtsY rbs-215*. Further genotype on specific strains is in Table 1.

The last two digits in each strain number are identical to the numerical designation of the *ilvAts* mutation carried by the strain. Thus BC111 and BC211 bear *ilvAts11*.

the most leftward *ilvAts* mutation (see Figure 3). Similarly the results of all crosses involving *ilvAts11* are given in Table 6. That the results indeed establish *ilvAts11* as the most rightward of the *ilvAts* mutations can be verified by examining the data of Table 6 with reference to Figure 3.

The mutants bearing the *ilvAts* boundary mutations (*ilvAts6* and *ilvAts11*) are phenotypically temperature-sensitive mutants *in vivo*. Cell-free extracts of these mutants grown and assayed at the restrictive temperature (42°) show no TD activity *in vitro*. Cell-free extracts of wild-type strains grown and assayed at 42° show considerable TD activity (data not shown).

f) *Five-point crosses to map* ilvO⁻

Having established boundary mutations for *ilvA*, it was possible to map *ilvO* relative to *ilvA*. Five-point crosses as diagrammed in Figure 4 were performed for this purpose. (Four-point crosses in which the inside markers were *ilvO*⁻ and *ilvAts* with outside markers *met*⁻ and *rbs*⁻ and selection for *ilvA*+ *ilvO*⁻ recombinants were not employed because the phenotypes of *ilvAts ilvO*⁻ and *ilvA*+ *ilvO*⁻ strains were not distinguishable in the presence of valine) (COHEN 1975). In each cross, the Hfr donor was *metE*+ *ilvE*⁻, or *ilvC*⁻, *ilvO*+, and *rbs*+ while the F⁻ recipient was *metE*-, *ilvAts6* or *ilvAts11*, *ilvO*⁻, and *rbs*⁻. Selection was for *ilvA*+ *ilvE*+ recombinants in one case (A of Figure 4) and for *ilvA*+ *ilvC*+ recombinants in the other (B of Figure 4). Among such recombinants of each mating, that class containing a high frequency of clones produced by crossover events between the *ilvAts* and *ilvE*⁻ lesions or the *ilvC*⁻ and *ilvAts* lesions and no other crossover events in the region *metE* to *rbs* can be identified. This is done

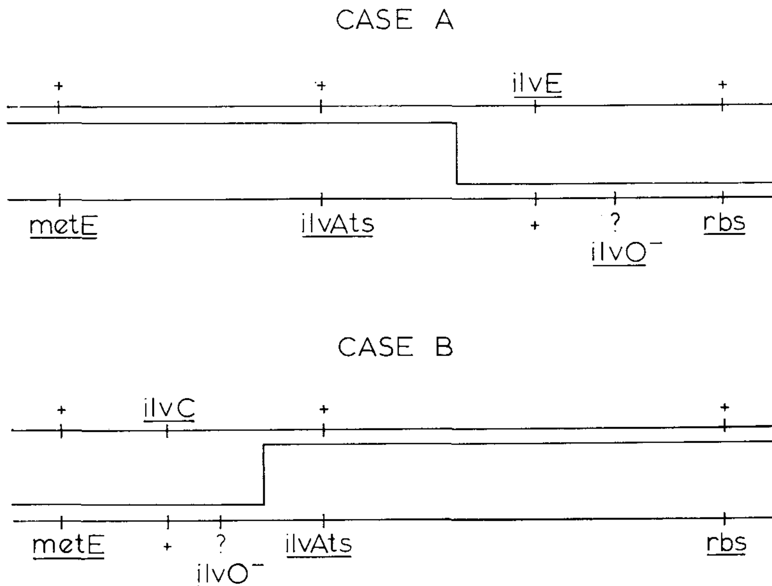

FIGURE 4.—Diagrammatic representation of five-point crosses to map *ilvO*⁻ mutations. In case A selection is for *ilvA*+ *ilvE*+ recombinants. In case B, selection is for *ilvC*+ *ilvA*+ recombinants.

by scoring for the outside markers. Thus in case A of Figure 4 (Hfr bears *ilvE*⁻, F⁻ bears *ilvAts*), the class of *ilvA*⁺ *ilvE*⁺ recombinants which are *metE*⁺ and *rbs*⁻ should consist primarily of clones generated by crossover events between the *ilvAts* and *ilvE*⁻ mutations and no other crossover events in the regions between *metE*⁻ and *rbs*⁻. Other arrangements of outside markers are the result of multiple crossover events or nonreciprocal recombination in the region studied. Similarly in case B of Figure 4 (Hfr bears *ilvC*⁻, F⁻ bears *ilvAts*), the class of *ilvC*⁺ *ilvA*⁺ recombinants which are *metE*⁻ *rbs*⁺ should consist primarily of clones generated by crossover events between the *ilvC*⁻ and *ilvAts* lesions and no other events in the region from *metE*⁻ *to rbs*⁻. Scoring these classes of recombinants generated by single crossover events in the region studied as either *ilvO*⁺ (Val-s) or *ilvO*⁻ (Val-r) will yield the following:

1) For the cross of Figure 4 case A (Hfr bears *ilvE*⁻), if *ilvO*⁻ is near *ilvE* as diagrammed, then among *ilvA*⁺ *ilvE*⁺ recombinants which have the outside marker configuration *metE*⁺ *rbs*⁻, the ratio of *ilvO*⁺ (Val-s) to *ilvO*⁻ (Val-r) clones (O⁺/O⁻ ratio) should be very low. As *ilvO*⁻ approaches *ilvAts* this ratio rises and if *ilvO*⁻ is leftward of *ilvAts*, the O⁺/O⁻ ratio should be high (certainly greater than 1).

2) For the cross of Figure 4 case B (Hfr bears *ilvC*⁻), if *ilvO*⁻ is near *ilvC* as diagrammed, then among *ilvC*⁺*ilvA*⁺ recombinants which have the outside marker configuration *metE*⁻ *rbs*⁺, the O⁺/O⁻ ratio should be low. As *ilvO*⁻ approaches *ilvAts* this ratio rises and if *ilvO*⁻ is rightward of *ilvAts*, the O⁺/O⁻ ratio should be high.

These crosses were done as described and the results are presented in Table 7. Crosses A, B, and C of Table 7 correspond to case A of Figure 4. The Hfr bore *ilvE*⁻, the recipients were *metE*⁻ *ilvAts ilvO*⁻ *rbs*⁻. The right-most *ilvAts*, *ilvAts11*, was present in crosses A and B. In the former cross the *ilvO*⁻ was *ilvO268*, in the latter cross *ilvO269*. In cross C *ilvO269* was again present in the recipient, along with the leftmost *ilvAts*, *ilvAts6*. For crosses A, B and C, the class of *ilvA*⁺*ilvE*⁺ recombinants arising from single crossover events should have the outside marker alignment *metE*⁺*rbs*⁻. The O⁺/O⁻ ratios for these *metE*⁺ *rbs*⁻ recombinants were O/31, O/30 and O/37 for crosses A, B, and C respectively. These are the expected ratios if *ilvO* maps very close to *ilvE*, certainly much closer to *ilvE* than to *ilvA*. This low ratio of O⁺/O⁻ was obtained irrespective of which *ilvAts* mutation or which *ilvO* mutation was present in the recipient.

Crosses D, E and F correspond to case B of Figure 4. The Hfr bore *ilvC*⁻. In crosses D and E the *ilvAts* mutation present in the recipient was the rightmost *ilvAts11*. In cross D, the operator mutation present in the recipient was *ilvO268*, in cross E it was *ilvO269*. Cross F differed from cross E in that the leftmost *ilvAts6* was present in the recipient. For crosses D, E, and F the class of *ilvA*⁺ *ilvC*⁺ recombinants arising from single crossover events should have had the outside marker combination *metE*⁻ *rbs*⁺. The O⁺/O⁻ ratios for these *metE*⁻ *rbs*⁺ recombinants were 24/8, 28/0, and 22/4 for crosses D, E and F respectively. These high O⁺/O⁻ ratios are the expected ratios if *ilvO* is located to the right of

TABLE 7

Results of five-point crosses to position ilvO⁻ *in the* ilv *region*

Class of outside markers	Total *ilvA*+ *ilvE*+ recombinants in class	Of *ilvA*+ *ilvE*+ recombinants those:	
		Val-r	Val-s
A: BC232 × BC160			
met+ *rbs*+	8	8	0
met+ *rbs*−	31	31	0
met− *rbs*+	3	3	0
met− *rbs*−	22	22	0
B: BC232 × BC161			
met+ *rbs*+	27	27	0
met+ *rbs*−	30	30	0
met− *rbs*+	8	8	0
met− *rbs*−	34	34	0
C: BC232 × BC162			
met+ *rbs*+	17	17	0
met+ *rbs*−	37	37	0
met− *rbs*+	6	6	0
met− *rbs*−	18	18	0

Class of outside markers	Total *ilvC*+ *ilvA*+ recombinants in class	Of *ilvC*+ *ilvA*+ recombinants those:	
		Val-r	Val-s
D: BC231 × BC160			
met+ *rbs*+	7	0	7
met+ *rbs*−	9	1	8
met− *rbs*+	32	8	24
met− *rbs*−	36	21	15
E: BC231 × BC161			
met+ *rbs*+	9	0	9
met+ *rbs*−	8	3	5
met− *rbs*+	28	0	28
met− *rbs*−	35	23	12
F: BC231 × BC162			
met+ *rbs*+	31	15	16
met+ *rbs*−	15	10	5
met− *rbs*+	26	4	22
met− *rbs*−	27	27	0

BC160: F− *metE200 ilvAts11 ilvO268 rbs-215*
BC161: F− *metE200 ilvAts11 ilvO269 rbs-215*
BC162: F− *metE200 ilvAts6 ilvO269 rbs-215*
BC231: Hfr *metE*+ *ilcC7 ilvO*+ *rbs*+
BC232: Hfr *metE*+ *ilvE12 ilvO*+ *rbs*+

Matings were performed as described in MATERIALS AND METHODS. Selection was for *ilvA*+ *ilvC*+ or *E*+ recombinants at 42°. Recombinants were repurified by two successive single colony isolations on LB. Repurified recombinants were all *ilvA*+, *C*+ and *E*+. They were scored for the *met* and *rbs* markers as well as for valine sensitivity (Val-s) or resistance (Val-r). Val-r strains grow on 100 μg/ml valine (without isoleucine) as do their F− *ilvO*− parents.

ilvA. This high ratio of O^+ to O^- was obtained irrespective of which *ilvAts* or which *ilvO*$^-$ was present in the recipient.

The results of all six crosses are consistent with the expectations if *ilvO*$^-$ maps near the *ilvE*$^-$ site, well to the right of the most rightward *ilvAts* mutation, *ilvAts11*. Indeed if one examines the results of crosses A, B, and C (case A of Figure 4), one finds no *ilvO*$^+$ recombinants among the *ilvA*$^+$ *ilvE*$^+$ recombinants, irrespective of the outside marker constitution of the recombinants. The exclusion of the *ilvE*$^-$ and *ilvA*$^-$ lesions from the recombinants (the only requirement of the selection) seems to lead to inclusion of *ilvO*$^-$ in the recombinants. That it is the exclusion of *ilvE*$^-$, rather than the exclusion of *ilvA*$^-$, which leads to the inclusion of *ilvO*$^-$ in the recombinants, can be seen from the results of crosses D, E, and F. Exclusion of *ilvA*$^-$ and *ilvC*$^-$ in crosses D, E, and F does not always lead to inclusion of *ilvO*$^-$ in the recombinants, for *ilvO*$^+$ recombinants are recovered in these crosses. These are the expected results if *ilvO*$^-$ maps rather close to the *ilvE*$^-$ lesion in each case (*ilvO268* or *ilvO269*), certainly much closer to the *ilvE*$^-$ lesion than to either *ilvAts* or to the *rbs*$^-$ mutation.

These results imply, then, that *ilvO* is very near *ilvE* and certainly much closer to *ilvE* than it is to *ilvA*. If *ilvO* is the operator for operon A, this finding is more consistent with the gene order ADEO than OADE in operon A. As reported and noted previously in this paper, the gene order ADEO, but not OADE, is also consistent with the results of genetic and physiologic studies using the F25 episomes.

g) *Biochemical effects of the* ilvO$^-$ *mutation*

In addition to genetic studies of the location of the *ilvO*$^-$ mutation, biochemical studies were performed to reinvestigate the effects of this lesion. Appropriate enzyme activities in cell-free extracts were compared between strains bearing the *ilvO*$^-$ mutation and their wild-type parents. This comparison was made using the original strains in which RAMAKRISHNAN and ADELBERG isolated the *ilvO268* and *ilvO269* mutations used in these studies (RAMAKRISHNAN and ADELBERG 1964). TD and TB activities were measured to gauge control of operon A. AHAS activity was measured to gauge control of an *ilv* structural gene outside of operon A. In addition, the sensitivity of AHAS to feedback inhibition by valine was determined as a decrease in that sensitivity is known to cause a Val-r phenotype. Expectations, drawn from the reports of the original isolators of the *ilvO*$^-$ mutants, were that TD and TB activity would be derepressed but that AHAS activity and sensitivity to valine would be unchanged in the *ilvO*$^-$ strains relative to the *ilvO*$^+$ strains.

The results of these assays on cell-free extracts of the *ilvO*$^-$ and *ilvO*$^+$ bearing strains are collected in Table 8. They show that the gene products of operon A, TD and TB, were indeed derepressed 4–7-fold in the *ilvO*$^-$ strains while the specific activity of AHAS was comparable to that of the wild-type parent as expected. In addition, they show that the sensitivity of AHAS to feedback inhibition by valine was significantly lower in the strains bearing *ilvO*$^-$ than in the parental *ilvO*$^+$ strains.

TABLE 8

Results of enzyme assays on ilvO⁻ *and their parental* ilvO⁺ *strains*

Strain	Relevant* genotype in *ilv*	Specific activity TD	TB	AHAS	% inhibition AHAS by 1 mM valine	P†
AB1206	$B^+O^+A^+E^+$	16±2	17±3	22±7	78±4	
AB1009	$B^+O^-A^+E^+$	68±10	73±4	17±3	48±11	<0.01
AB1013	$B^+O^-A^+E^+$	110±10	100±9	19±3	45±7	<0.001

Cells are grown in minimal medium with repressing concentrations of isoleucine, leucine, and valine (50 μg/ml, 50 μg/ml, and 100 μg/ml respectively). Techniques of growth, harvest, preparation of extracts and execution of assays are as in MATERIALS AND METHODS. Activities are nanomoles product/minute/mg protein and are expressed as the mean of the observed values ± the standard deviation calculated from observed values. % inhibition of AHAS by valine is calculated as the ratio: the difference in AHAS activity with and without 1 mM valine in the assay system/AHAS activity without valine in the assay system. It is expressed as a percent, and the mean of observed values ± the standard deviation calculated from observed values is recorded in the table.

* AB1009 is *ilvO268*; AB1013 is *ilvO269*. Complete genotypes are in Table 1.

† P is the probability of a value of T ≥ that observed in a two tailed T test comparing the mean % inhibition of AHAS by valine in extracts of one strain to the mean % inhibition of AHAS by valine in extracts of AB1206 on the hypothesis that the obtained means are from populations with equivalent means.

h) *cis/trans tests of* ilvO⁻ *action*

If the *ilvO⁻* mutations are operator mutations, then their effects should be *cis*-dominant. Dominance or recessiveness and *cis* or *trans* action of the *ilvO⁻* lesions were determined. Merodiploids for the *ilv* region were constructed, all bearing F14 episomes and the chromosome of BC80 (which is *recA56* to prevent exchange of genetic material between episome and chromosome). These strains fall into three classes, as follows, for the *ilv* region:

1) $F'B^+C^+O^+A^+D^+E^+/B^+C^+O^+A^+D^-E^+$ strains
2) $F'B^+C^+O^-A^+D^+E^+/B^+C^+O^+A^+D^-E^+$ strains
3) $F'B^+C^+O^-A^+D^+E^-/B^+C^+O^+A^+D^-E^+$ strains

Thus, in each case, the chromosomes were *ilvD⁻* but otherwise *ilv⁺* while the episomes were either (1) *ilv⁺*, (2) *ilvo⁻* and otherwise *ilv⁺*, or (3) *ilvO⁻* and *ilvE⁻* but otherwise *ilv⁺*. If the *ilvO⁻* mutation is dominant, the gene products of operon A should be derepressed in merodiploids heterozygous for *ilvO⁻* relative to merodiploids homozygous for *ilvO⁺*. If the mutation acts only *cis*, then the TD activity of strains of class (3) will be derepressed but the TB activity will not, as the only structural gene, *ilvE⁺*, for active TB is *cis* to *ilvO⁺*, *trans* to *ilvO⁻*. AHAS activity was also measured in cell-free extracts of these strains as was sensitivity of that activity to inhibition by valine to determine if the effect of the *ilvO⁻* mutation on this sensitivity was dominant or recessive.

The results of assays on these merodiploids bearing either the *ilvO268* or *ilvo269* mutations are presented in Table 9. The effect of each *ilvO⁻* lesion was dominant as *ilvO⁻/ilvO⁺* merodiploids were derepressed for the gene products of operon A. In addition, the effect of *ilvO⁻* was only *cis* acting as the TB activity of *ilvO⁻ ilvE⁻/ilvO⁺ ilvE⁺* merodiploids was at the haploid, repressed level.

TABLE 9

Results of cis-trans *tests of* ilvO^c *activity*

Strain	Relevant* genotype in *ilv*	TD (A)	Specific activity TB (E)	AHAS (B)	% inhibition AHAS by 1 mM valine	P†
BC80	B+O+A+E+	18±3	8.2±3	34±5	79±2	
BC280	F'B+O+A+E+ / B+O+A+E+	28±4	19±3	32±5	83±3	
BC283	F'B+O- A+E+ / B+O+A+E+	140±10	66±4	41±9	67±4	<0.01
BC284	F'B+O- A+E+ / B+O+A+E+	180±9	69±4	36±5	70±3	<0.01
BC285	F'B+O- A+E- / B+O+A+E+	220±20	7.7±2	31±5	68±3	<0.01
BC286	F'B+O- A+E- / B+O+A+E+	190±10	9.5±2	41±7	68±3	<0.01

Cells are grown in minimal medium with repressing concentrations of isoleucine, leucine, and valine (50 μg/ml, 50 μg/ml, and 100 μg/ml respectively). Techniques of growth, harvest, preparation of extracts and execution of assays are as in MATERIALS AND METHODS. Activities are nanomoles product/minute/mg protein and are expressed as the mean of the observed value ± the standard deviation calculated from observed values. % inhibition of AHAS by valine is calculated as the ratio: the difference in AHAS activity with and without 1 mM valine in the assay system/ AHAS activity without valine in the assay system. It is expressed as a percent, and the mean of observed values ± the standard deviation calculated from observed values is recorded in the table.

* BC280, BC283, BC284, BC285, and BC286 are merodiploid strains whose chromosomes are equivalent to the haploid BC80. BC283 and BC285 bear *ilvO268* on their episome. BC284 and BC286 bear *ilvO269* on their episome. Complete genotypes are in Table 1.

† P is the probability of a value of T ≥ that observed in a two tailed T test comparing the mean % inhibition of AHAS by valine in extracts of one strain to the mean % inhibition of AHAS by valine in extracts of BC280 on the hypothesis that the obtained means are from populations with equivalent means.

The effect of each *ilvO*$^-$ lesion on the sensitivity of AHAS to inhibition by valine persisted in the *ilvO*$^-$/*ilvO*$^+$ merodiploids.

t tests were performed which demonstrate that the sensitivity of AHAS to inhibition by valine was lower in the heterozygous *ilvO*$^-$/*ilvO*$^+$ merodiploids than in the *ilvO*$^+$ homozygotes (see Table 9). In addition, similar t tests demonstrate that the sensitivity of AHAS to inhibition by valine was higher in the heterozygous *ilvO*$^-$/*ilvO*$^+$ merodiploids than it was in the *ilvO*$^-$ homozygotes (specific results not given). All of these differences are significant with p's <0.05. Thus the effect of *ilvO*$^-$ on the sensitivity of AHAS to inhibition by valine is codominant.

DISCUSSION

The results of studies reported in this paper imply that the *ilvO* region is carried on the F25 episome. Specifically, the F25 episome can serve as a donor of *ilvO*$^+$ genetic material and the *ilvE* gene of operon A on the F25 episome responds normally to a derepression signal (leucine limitation) as if it were *cis* to its normal operator. In addition, five-point crosses indicate that *ilvO*$^-$ lesions map

near *ilvE* and not near *ilvA*. All of these results are consistent with a map order CADEO in *ilv* (with operon A transcribed and translated from E to A) and are inconsistent with the standard map order COADE (with operon A transcribed and translated from A to E).

The standard map for the *ilv* region in *E. coli* K-12 arose from the studies of RAMAKRISHNAN and ADELBERG (1965b). Those authors mapped *ilvO* by generating shortened episomes (like F25) in transductions with an F14-bearing donor and an *ilvA*⁻ recipient. The donor was *ilvO*⁻ (Val-r) but otherwise *ilv*⁺. They characterized the genes carried on these shortened episomes by replica-plating donors bearing the episomes onto lawns of *ilv*⁻ or *ilvO*+ recipients and selecting for *ilv*⁺ or Val-r (*ilvO*⁻) recombinants. Doing so, they found that donors of those episomes able to effectively transfer the Val-r trait could also yield *ilv*⁺ recombinants with recipients bearing *ilvA*⁻, *ilvD*⁻ and *ilvE*⁻ lesions and, sometimes, *ilvC*⁻ lesions as well. Some matings with donors of these shortened episomes could yield *ilv*⁺ recombinants when recipients had *ilvA*⁻, *ilvD*⁻, and *ilvE*⁻ lesions while the donors were not apparently able to transfer the Val-r trait. These findings implied that *ilvO* was between *ilvA* and *ilvC*. However, this replica-plating technique does not require recombination (crossover events that transfer *ilv* genetic material from episome to chromosome) for the detection of genes on the episome. With this technique, the presence of these genes is probably most efficiently detected by complementation, not recombination. If *ilvO*⁻ is near the sex factor of these F14-derived episomes, as studies reported here would imply, then crossover events which incorporate *ilvO*⁻ carried on the episome of the donor into the chromosome of the recipient will occur with low frequency. Thus, efficient transfer of the Val-r trait may occur only if the episome carries *ilvO*⁻ and whatever genes of operon A are necessary *cis* to *ilvO*⁻ to produce a Val-r phenotype. For example, RAMAKRISHNAN and ADELBERG (1965a) noted that *ilvA*⁺ was needed *cis* to *ilvO*⁻ for a strain to be Val-r. If this is so, then an episome-bearing donor must contain not only *ilvO*⁻ but all of operon A through a complete *ilvA* gene on its episome before it can serve as an efficient donor of the Val-r trait by replica plating. Thus *ilvO*⁻ might appear to be between *ilvC* and *ilvA* by this replica-plating mapping technique when it was truly between *ilvE* and the F14 sex factor as is consistent with results reported here.

At present, there are further data on the location of *ilvO*. PLEDGER and UMBARGER (1973b) interpreted the results of three-point crosses they performed as indicating that *ilvO* lay between *ilvC* and *ilvA*. However, deriving this order required comparisons between results of transductions involving somewhat different regions of the chromosome and different markers. In addition, three-point crosses do not allow one to clearly define the location of crossover events and eliminate from consideration the recombinants which are the result of multiple crossover phenomena. Interestingly, more recent results of KLINE *et al.* 1974) demonstrate that *ilvO*⁻ lesions lie outside of an apparent deletion (*ilvDAC115*) which includes all of the *ilvA* gene and at least parts of the *ilvD* and *ilvC* genes to each side, implying that *ilvO*⁻ cannot be in the region between *ilvC* and *ilvA*. These same authors have shown that in a mutant bearing the *ilvDAC115* de-

letion, which lacks the region between *ilvC* and *ilvA*, the intact *ilvE* gene remains under normal control. This implies that *ilvE* is still *cis* to its own operator in the *ilvDAC115* mutant, and, thus, that the operator cannot be between *ilvC* and *ilvA*. Thus, studies using the *ilvDAC115* deletion are clearly consistent with the gene order CADEO in *ilv* and are not consistent with the gene order COADE.

The direction of transcription and translation of operon A could probably best be determined by polarity studies. Unfortunately, no extensive studies of this kind have been done in *ilv*. However, WECHSLER and ADELBERG (1969) report on two polar mutations in *ilv*. One mutant, bearing an *ilvE* amber mutation, has 0% of the TB, 30% of the DH and 105% of the TD activity of its wild-type parent. Another mutant, bearing an *ilvD* ochre mutation, has 128% of the TB, 0% of the DH, and 40% of the TD activity of its wild-type parent. The phenomena seen in these strains are clearly consistent with polarity in the direction *ilvE* to *ilvA* and, thus, with transcription and translation of operon A from *ilvE* to *ilvA* with an operator adjacent to *ilvE*.

The results of biochemical studies presented in this paper demonstrate that the *ilvO*⁻ lesion behaves like an operator mutation for operon A of the *ilv* region in that it causes a *cis*-dominant derepression of the operon A gene products and only these products. This effect was also noted by the original isolators of these *ilvO*⁻-bearing stains (RAMAKRISHNAN and ADELBERG 1965a).

In addition, the *ilvO*⁻ lesion leads to a decrease in the sensitivity of AHAS activity to inhibition by valine. The trivial explanation for this phenomenon would be that derepressing operon A leads directly to the noted alteration in AHAS sensitivity to valine. However, in an experiment not reported in the RESULTS section of this paper, operon A of an *ilvO*⁺ strain was derepressed tenfold by growth of the strain on 10^{-2} M valine. No change was noted in the sensitivity of AHAS to valine in cell-free extracts of the derepressed relative to a repressed culture. This argues against the trivial explanation for the decrease in sensitivity of AHAS to valine in *ilvO*⁻ strains. One should note, however, that in an *ilvO*⁻ strain the isoleucine pool will be high, due to the derepressed level of TD. Oppositely, in a culture of an *ilvO*⁺ strain whose growth is being inhibited by valine (which starves the culture for isoleucine), valine pools will be high, but isoleucine pools will be low. AHAS is known to interact with isoleucine and valine and it is possible that AHAS has more than one stable configuration, with one configuration valine-sensitive and one configuration valine-resistant. If this were so, and if the configuration of AHAS depended on ligand pools at the time of its translation and maturation, then derepression of operon A through growth inhibition by valine would not be equivalent to derepression of operon A by an *ilvO*⁻ mutation. Thus, the insensitivity of AHAS to inhibition by valine in an *ilvO*⁻ strain might still simply be secondary to the derepression of operon A caused by the *ilvO*⁻ lesion and the attendant effects of that derepression.

There is at present a more satisfying explanation for the appearance in an *ilvO*⁻ strain of AHAS activity resistant to inhibition by valine. Several recent studies have shown that *S. typhimurium*, *E. coli* B and *E. coli* W have at least two AHAS activities (BLATT, PLEDGER and UMBARGER 1972; O'NEILL and

FREUNDLICH 1972). One such activity is valine-sensitive (AHAS-s) and one is valine-resistant (AHAS-r). The structural gene for the AHAS-r activity of *S. typhimurium*, called *ilvG*, appears to map between *ilvD* and *ilvE* (O'NEILL and FREUNDLICH 1972, 1973). (As in *E. coli* K-12, the gene order for the *ilv* region in *S. typhimurium* is CADE (SANDERSON 1972) where structural gene-enzyme relationships are as in Figure 1.) Recently, other authors have reported finding, as these studies have found, AHAS activity resistant to inhibition by valine in *ilvO⁻* strains of *E. coli* K-12 (GUARDIOLA, DE FELICE and IACCARINO 1974). Indeed, these authors report that the structural gene for this activity, again designated *ilvG*, is between *ilvD* and *ilvE* in *E. coli* K-12 as it is in *S. typhimurium* (GUARDIOLA, DE FELICE and IACCARINO 1974). The existence of such a gene in operon A would clearly help explain the insensitivity of AHAS activity to inhibition by valine in cell-free extracts of *ilvO⁻*-bearing mutants and, thereby, would help explain the Val-r phenotype of these *ilvO⁻* mutants. It is fair to ask why the original isolators of the *ilvO⁻* bearing mutants did not see this valine-resistant AHAS activity in cell-free extracts of their *ilvO⁻* strains. There may have been two contributing factors as follows:

(1) The original isolators of the *ilvO⁻* strains used 10^{-4} M valine for inhibition of AHAS in their assays (RAMAKRISHNAN and ADELBERG 1964) unlike the 10^{-3} M valine used in the studies reported here and elsewhere.

(2) The original isolators of the *ilvO⁻* strains used an assay system for AHAS activity (RAMAKRISHNAN and ADELBERG 1964) which did not include FAD or any source of FAD (e.g., boiled extract of yeast or bacteria) and FAD has been reported to be necessary to render AHAS fully sensitive to valine *in vitro* (BAUERLE *et al.* 1964).

Thus, the use of lower levels of valine and the failure to include FAD in assays of AHAS activity may have masked the difference in sensitivity of AHAS to valine in cell free extracts of *ilvO⁻ versus ilvO⁺* strains by reducing the measured sensitivity of the wild-type AHAS.

It is still fair to ask why, if there is a structural gene for an AHAS activity resistant to valine in operon A, one does not see this AHAS-r activity when derepressing operon A through means other than an *ilvO⁻* mutation? This question cannot be answered until more is known about the status of *ilvG* and the *ilvG* gene product in both *ilvO⁻* and wild-type strains.

In conclusion, the results presented in this paper and the results of others discussed above suggest that the gene order COADE for the *ilv* region of *E. coli* K-12 is not correct. Rather, they suggest that *ilvO* is adjacent to ilvE which implies a gene order CADEO in the *ilv* region with transcription and translation of operon A from *ilvE* to *ilvA*. The results also suggest that the effect of the *ilvO⁻* lesions is a *cis*-dominant derepression of operon A; an effect consistent with their being mutations in the operator region of operon A. In addition, *ilvO⁻* lesions may lead to the appearance of an AHAS activity resistant to inhibition by valine. This activity may explain the Val-r phenotype of strains bearing *ilvO⁻*. The AHAS-r activity most probably relates to the *ilvG* gene of operon A but the nature and status of this gene and its product in wild-type and *ilvO⁻*-bearing strains is as yet unclear.

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