RECOMBINATION STUDIES OF LACTOSE NONFERMENTING MUTANTS OF ESCHERICHIA COLI K-12[1,2]

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Received May 15, 1962

THE fermentation of lactose is a diagnostic character of *Escherichia coli;* however, nonfermenting mutants can readily be obtained. These mutants may show losses or changes in the accumulation of lactose or other β-galactosides in the cell, or in the formation or activity of the enzyme, β-galactosidase, which splits lactose to the constituent monosaccharides. Such mutants have played an important part in the analysis of gene action (JACOB and MONOD 1961).

A comprehensive model of the action of the *Lac* region has been formulated by JACOB, PERRIN, SANCHEZ and MONOD (1960) (see Figure 1). The *Lac* region is an "operon" comprised of four elements:

y, responsible for the structure of the relevant permease;

z, controlling the structure of β-galactosidase;

i, which determines whether the permease and enzyme will be produced only in the presence of an inducer (i^+), or synthesized constitutively (i^-);

o, the operator coordinating the other elements of the region.

The first lactose mutants analyzed in this laboratory were designated Lac_1–Lac_7 without regard to their specific physiological effects (LEDERBERG, LEDERBERG, ZINDER and LIVELY 1951). Lac_1 and Lac_4 were the most thoroughly investigated. Crossing analysis (J. LEDERBERG 1947; CAVALLI-SFORZA and JINKS 1956; WOLLMAN, JACOB and HAYES 1956; HARTMAN 1957) located Lac_1 between V_1 and V_6, loci controlling resistance to the phages T_1 and T_6, respectively (Figure 1). On the basis of cis-trans tests of heterozygous diploids, several recombinationally distinct mutations were classified in the same cistron in Lac_1 (LEDERBERG *et al.* 1951). Others, which did not recombine, could be distinguished by their reverse-mutability (E. M. LEDERBERG 1952). Lac_1 and Lac_4 were closely linked, but complemented each other (LEDERBERG *et al.* 1951).

In the early studies (LEDERBERG *et al.* 1951), Lac_2 and Lac_4 were found to lack β-galactosidase, i.e. to have a z^- defect. However, Lac_1 showed appreciable levels

[1] This work supported by grant G6411 of the National Science Foundation, and grants 2G295 and C4496 of the National Institutes of Health, United States Public Health Service, Washington, D.C.

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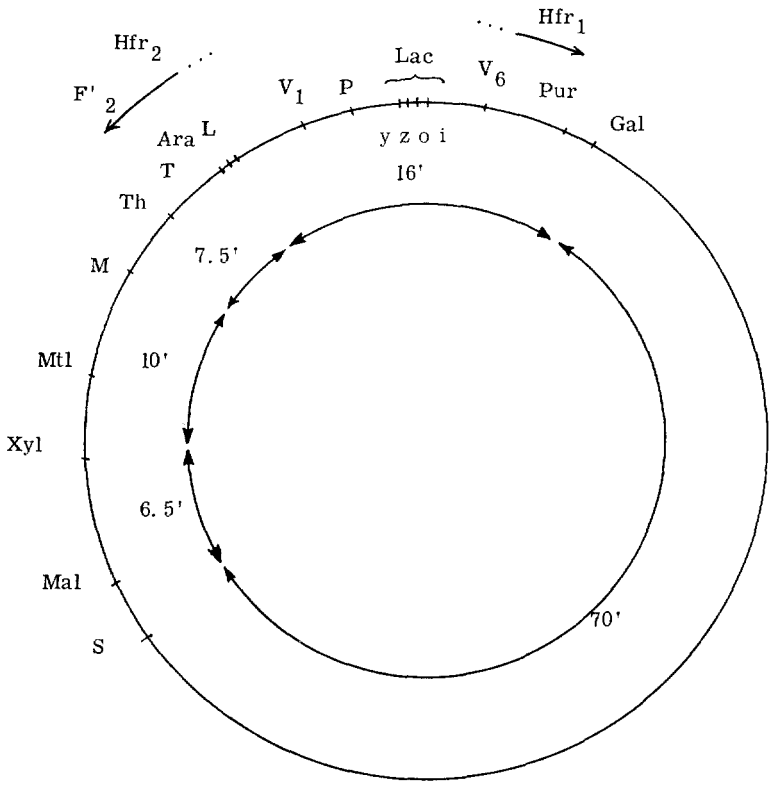

FIGURE 1.—Genetic map of *E. coli*. Distance between markers is expressed in minutes. (Adapted from TAYLOR and ADELBERG 1960; and JACOB and MONOD 1961.)

of the enzyme. This anomaly appeared to be clarified by the discovery of the permease system for the entry of β-galactosides, Lac_1 being classified as a y^- defect (RICKENBERG, COHEN, BUTTIN and MONOD 1956; ROTMAN 1958).

Lac_3 (LEDERBERG *et al.* 1951) affects the fermentation of glucose and maltose, as well as lactose. Both its physiology and genetics remain obscure (PARDEE, JACOB and MONOD 1959). The same is true of Lac_5, which fails to split maltose or to ferment gluconate. Lac_7 is recorded as producing "reduced but significant amounts" of both β-galactosidase and permease (PARDEE *et al.* 1959).

PARDEE *et al.* (1959) located ten y^- mutations clustered at the left of the *Lac* region toward *P*, and several z^- mutations to the right of *y*. They could order eight of the z^- mutations in relation to each other and an i^- mutation by three-point crosses between pairs of z^- mutants, one member of each pair also being i^-. The z^+ progeny were scored for the presence of i^-. Although the method is sound if all the z^- mutations lie on one side of *i*, there was one exception which could not be mapped by this procedure. In addition, the method is highly laborious at best, and results can be obscured by high rates of coincidence. Deletion analysis (MCCLINTOCK 1944; BENZER 1959), because of its greater efficiency, should permit ordering much larger numbers of mutations, as well as providing an

independent estimate of their order to compare with more conventional types of mapping.

This paper describes an attempt to map a series of *Lac* mutations by deletion analysis. The procedure entails the isolation of a series of overlapping deletions, used as testers against point mutations to be mapped.

Terms and Abbreviations

The following terms and abbreviations will be used.

ONPG *o*-nitrophenyl-β-D-galactopyranoside

TMG methyl-β-D-thiogalactopyranoside

TDG β-D-galactosyl-β-D-thiogalactopyranoside

Permease refers to galactoside permease.

Genetic loci:

Nutritional requirements ($^-$ indicates dependence)

M, methionine; *P*, proline; *L*, leucine

T, threonine; *Th*, thiamine; *Ad*, adenine

Pur, purines (optimal growth with thiamine and adenine or guanine)

Fermentation markers ($^-$ indicates inability to ferment)

Ara, arabinose; *Gal*, galactose; *Lac*, lactose

Mal, maltose; *Mtl*, mannitol; *Xyl*, xylose

Resistance markers (^{r} indicates resistance)

V_1 phage T_1; V_6 phage T_6; *S* streptomycin

Sexual compatibility characteristics

F^- female, or recipient; F^+ infective, wild-type male

F′ infective male showing high frequency of recombination (JACOB and ADELBERG 1959).

F'_2 injects chromosome in the order Ara_2 Lac_1 Gal_2. (HIROTA and SNEATH 1961). Hfr male showing high frequency of recombination, not infective

Hfr_1 (CAVALLI-SFORZA 1950) injects chromosome in the order *Pur*, or V_6, *Lac P L T*.

Hfr_2 (HAYES 1953) injects chromosome in the order *T L Lac Gal* (SKAAR and GAREN 1956).

The only infertile combination is $F^- \times F^-$.

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

Steps in the program: 1. Isolation of a series of Lac^- mutants. 2. Systematic intercrossing, screening (qualitatively, for the most part) for the occurrence of Lac^+ recombinants. 3. Tabulation of results to: a. distinguish the deletions from point mutations b. map the point mutations within specified deletions.

Media: The media employed included Davis minimal medium, complete medium (YZ broth), minimal eosin-methylene blue medium without succinate (EM), and complete eosin-methylene blue medium (EMB). These are defined in J. LEDERBERG (1950a) as A, B, C, and D, respectively.

Supplemented Davis minimal agar contained the amino acids at double the

concentration specified in J. LEDERBERG (1950a). 1.6 percent agar and 0.5 percent glucose were used.

0.2 percent sugar was routinely added to Davis minimal broth after autoclaving.

200 μg per ml of streptomycin sulfate was incorporated when necessary.

Penassay broth, or Difco Bacto Antibiotic medium 3, was employed as a standard complete liquid medium.

Cultures were routinely incubated at 37°C. Those in liquid media were generally not aerated.

Cultures were stored at room temperature in corked stab tubes containing 0.8 percent Difco nutrient broth and 0.8 percent Difco Bacto agar.

Strains: Most of the *Lac* mutations studied were induced by ultraviolet irradiation. The parent strain, W3787 (Hfr_1 Lac^+ M^- P^- V_6^r), was streaked out on EMB-lactose agar. Several Lac^+ single colonies were isolated to Penassay broth, and incubated overnight. Samples of 0.1 ml were spread on plates of EMB-lactose agar, a maximum of 20 plates from each isolate. The plates were then irradiated with ultraviolet at a dose that would leave 100 to 300 colony formers per plate. Mutants were identified by their pale color on the EMB-lactose. They were purified by at least two successive single colony isolations on EMB-lactose agar.

Nineteen Lac^- mutants were isolated during experiments with nitrous acid. Concentrations of 0.025 M or 0.05 M of KNO_2 in citrate or acetate buffer at pH values between 4 and 6 were used. It is questionable, however, how many of the mutations the nitrous acid induced, as six mutants appeared in the citrate control experiments.

Lac11D3 and *Lac14D7* were collected by spreading Lac^- stocks having the mutable markers, *Lac11* and *Lac14*, respectively, on EMB-lactose agar, and choosing Lac^- colonies that had no secondary Lac^+ colonies after three to four days incubation.

Each mutation, including those previously described at the loci Lac_1 through Lac_7, was arbitrarily assigned an identifying *Lac* number. When possible, the same number used as the locus designation was employed as the *Lac* number, e.g. Lac_2 and *Lac2* refer to the same mutation. When more than one mutation had been described at the same locus, however, each was renumbered (COOK 1958).

For the study of recombination patterns, F^- Lac^- prototrophs were derived from the Hfr_1 P^- M^- V_6^r Lac^- mutants by crossing them with either Y10 (F^- T^- L^- Th^-), or its streptomycin-resistant offspring, W1394.

In some cases, stocks having particular *Lac* markers in different genetic backgrounds were necessary. These stocks and their manufacture will be described in connection with the experiment for which they were used.

Table 1 lists some of the more important foundation stocks.

Crosses: Simplified procedures were required to screen Hfr_1 Lac^- × F^- Lac^- crosses for the formation of Lac^+ recombinants.

Mixed cultures: one-half ml samples of overnight broth cultures of each parent

TABLE 1

List of some foundation stocks

Y10	F⁻ *T⁻ L⁻ Th⁻*	W4506	F⁻ *Pur⁻ V₆ʳ*
W1394	F⁻ *T⁻ L⁻ Th⁻ Sʳ*	W4607	F′₂ *Gal*$_{i}$
W1895	Hfr$_1$ *M⁻*	W4983	F⁻ *M⁻ V₆ʳ Sʳ Lac59* Suppressor+
W2612	F⁻ *T⁻ L⁻Th⁻ Sʳ Gal⁻*	W4984	F′₂ *M⁻ V₆ʳ Sʳ Lac59* Suppressor+
W3787	Hfr$_1$ *P⁻ M⁻ V₆ʳ*		

were mixed in five ml of Penassay broth, incubated for two to three hours, then samples spread on selective media.

Plate crosses: 10^{-3} to 10^{-1} ml of broth cultures were mixed and spread directly on plates of selective medium.

Spot tests: drops of overnight broth cultures were deposited upon plates of suitable selective medium with a large platinum loop, or a fine-tipped inoculating pipette, and allowed to dry on the agar. Each spot was then inoculated with a drop of the other parent culture. A loop contained approximately 5×10^{-3} ml, or 5×10^6 cells, while a drop from a pipette was ten times larger.

Infection of F⁻ with F′: To infect an F⁻ strain with F′ (e.g. F′$_2$), overnight cultures were grown in Penassay broth of the F⁻ to be infected, and of an F′ strain which differed from it by at least one fermentation marker. One ml of the F′, and 0.1 ml of the F⁻ were then mixed in five ml fresh Penassay broth, and incubated overnight. The mixture was streaked out on medium which would distinguish the two stocks. Appropriate colonies were picked to broth and tested for fertility by spot testing against an F⁻ with an auxotrophic marker in the region the F′ transfers with high frequency. This procedure is essentially the same as that for the transmission of F (LEDERBERG, CAVALLI and LEDERBERG 1952). Under the conditions described, at least 50 percent of the F⁻ culture becomes F′ (HIROTA, unpublished).

T_6 resistance: Strains were tested for resistance by the cross-streaking method (DEMEREC and FANO 1945) on EMB-agar without sugar. Bacteria were inoculated either by a loop from broth cultures, or by replica plating (LEDERBERG and LEDERBERG 1952).

ONPG tests: β-galactosidase activity was tested for on agar and in broth (J. LEDERBERG, 1950b).

The medium for tests on agar was Davis minimal supplemented with 0.8 percent NZ Case (Sheffield Chemical Co., Norwich, Conn.) and either 0.5 percent glucose (noninducing medium) or 0.5 percent lactose and 0.5 percent glycerol (inducing medium). Small drops of the strains to be tested were put on the plates and incubated overnight, then exposed for one to two hours to chloroform vapor. The plates were righted, and each colony flooded with a drop of a solution of one mg per ml of ONPG in 0.05 M sodium phosphate buffer, pH 7.5. Tests were read when the yellow color developed in control areas. In general, the presence of revertants in the cultures was looked for by spotting them on EMB-lactose or

EM-lactose at the same time that they were inoculated onto the test medium.

Strains not giving positive reactions on agar with lactose as an inducer, were grown in YZ broth with 10^{-3} M of TMG. The cultures were centrifuged and the pellets resuspended in 0.05 M sodium phosphate buffer, pH 7.5. A few drops of toluene were shaken with the cells. An ONPG solution was added to give a final concentration of 3×10^{-4} M. The mixtures were incubated at 37°C for ten minutes and then compared to the control, W3787, by eye.

Permease tests: Single colonies from streakings on EMB-lactose were inoculated into YZ broth containing 10^{-3} M TMG. (DR. MELVIN COHN kindly supplied the thiogalactosides, together with advice on their use.) After eight to ten hours' growth at 30°C with shaking, the cultures were diluted 1 to 4 with fresh medium of the same composition. After 60 minutes incubation at 37°C with shaking, the cultures were centrifuged ten minutes in the cold at 17,300 × *g*, and washed with five ml of cold Davis minimal containing 10^{-4} M glucose, and 10^{-3} M mercaptoethanol. The pellets were resuspended in the same medium. Cell densities were adjusted to $2–5 \times 10^8$ viable units per ml. Drops of the cell suspensions were spotted on EMB-lactose for reversion controls. One ml of each cell suspension was added to 0.1 ml of 10^{-2} M cold TMG containing enough C^{14} labeled TMG to give 3×10^6 counts per minute per ml. After 15 minutes incubation with shaking at 37°C, the suspensions were filtered through Millipore membranes (HA), and washed with ten ml of cold Davis minimal into which 10^{-4} M glucose, and 10^{-3} M mercaptoethanol had been incorporated. Each filter was glued to a planchet with rubber cement, dried and counted in a windowless gas flow counter for a period of two minutes.

EXPERIMENTAL RESULTS

An Hfr_1 P^- M^- $V_6{}^r$ stock of K-12 was chosen to provide the source of *Lac*$^-$ mutants because Hfr_1 transfers the *Lac* region with high frequency (SKAAR and GAREN 1956), and because *P* and V_6 are the outside markers closest to *Lac* (Figure 1). From each Hfr *Lac*$^-$ mutant stock, a corresponding F^- *Lac*$^-$ prototroph was derived by crossing. Matings were then made between different Hfr and F^- stocks on EM-lactose medium, and each cross scored for the production of *Lac*$^+$ prototroph recombinants. Mutants were also inoculated onto EMB-maltose, EMB-glucose, and EMB-galactose to test for the fermentation of these sugars. Results of the recombination tests suggested the following grouping for the *Lac* mutations studied.

(1) Short deletions: A deletion is defined as a mutant which fails to recombine with two or more recombinationally distinct mutants. When tested for reversion by spreading 10^8 cells from fresh broth cultures on each of ten EMB-lactose plates, no *Lac*$^+$ colonies or papillae appeared even after seven days' incubation.

(2) Long deletions: These have not been observed to yield *Lac*$^+$ progeny with any of the other *Lac* mutations (with the possible exception of the *Nr* group, see below). When tested for reversion as outlined above, they also produced no *Lac*$^+$ clones.

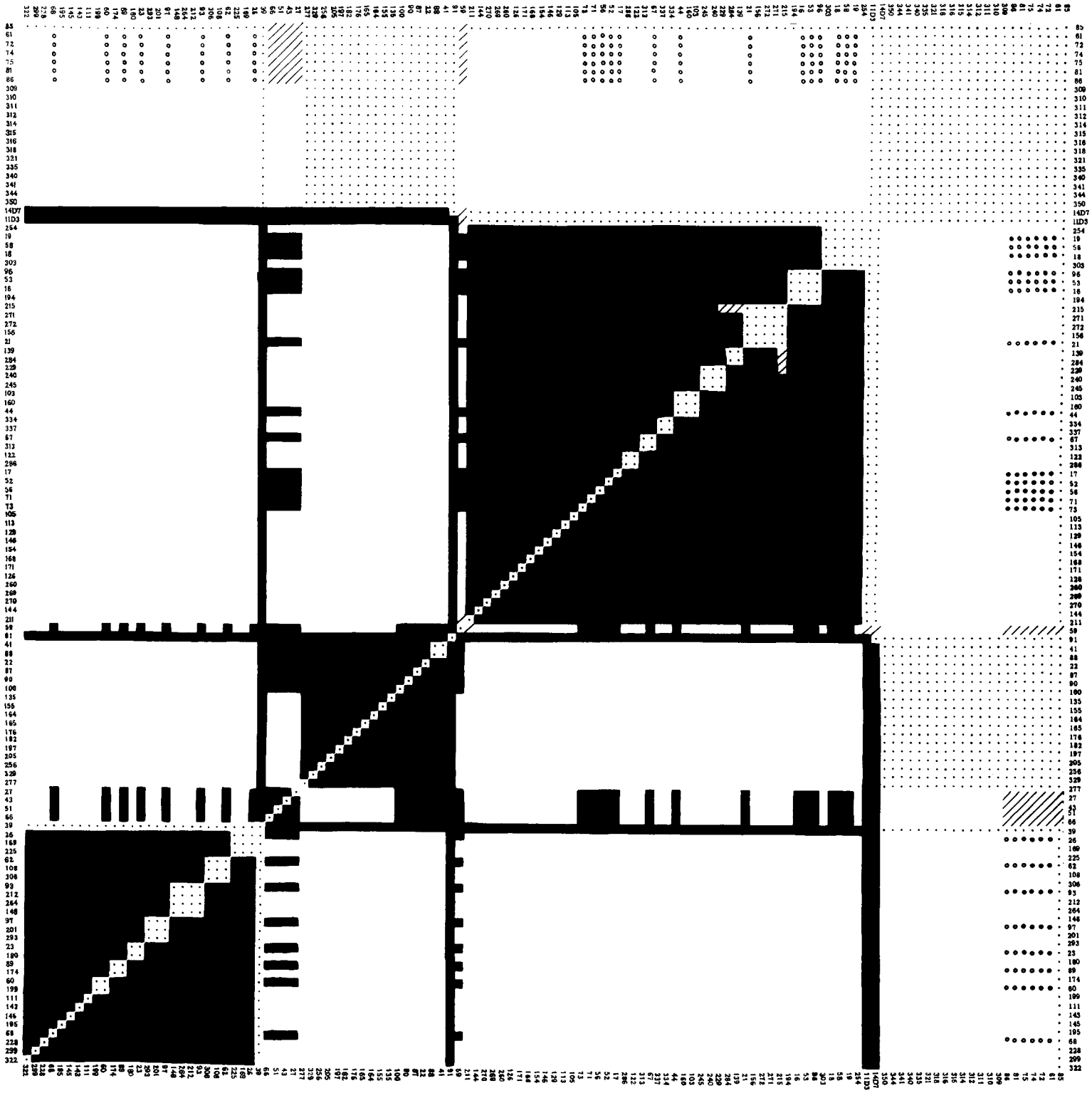

FIGURE 2.—Recombination among *Lac*⁻ mutants.

(3) Point mutations: These are mutants that cannot be recognized as deletions by their recombination patterns (Figure 2). In general, they recombine with every other point mutant. A few have been found which fail to recombine with one another, but react identically with all other testers. Future tests against additional markers could indicate the reclassification of a point mutant as a short deletion. Most of the point mutants in the present classification are revertible, however. The point mutants have been divided into three subgroups on the basis of their recombination with the short deletions, *Lac11D3* and *Lac39*. Group A members recombine with *Lac39*. Group B members recombine with *Lac11D3*. Group C members recombine with both *Lac39* and *Lac11D3*.

(4) *Nr* mutations: These give anomalous reactions with certain other *Lac* mutations. When the F^- parent contains an Nr mutation, and the Hfr the other *Lac* mutation, no Lac^+ progeny are recovered (less than 10^{-7} Lac^+ recombinants per viable unit of the limiting parent). When the Hfr contributes the *Nr* mutation to an F^- bearing the other mutation, Lac^+ progeny appear with a frequency of one per 10^4 cells. Most of the cases observed involved a long deletion as "the other mutation."

In general, the mutants that gave negative fermentation reactions on EMB-glucose, EMB-maltose, or EMB-galactose, grew too well, or produced too much color on EM-lactose to enable the determination of their recombination characteristics by this method. Nine mutants formed pale colonies on all three sugars, and thus resembled Lac_3.

After the mutants had been divided into groups, the members of each group were tested more extensively with each other for recombination. Those not included were too revertible, produced too much color on EM-lactose, or were no longer Hfr. Only a few of the long deletions were investigated. Drop tests were made of all possible combinations within each group, and each series included the standard testers, *Lac85* (a long deletion), *Lac11D3* (a short deletion), *Lac14D7* (a short deletion identical to *Lac11D3* except that it does not recombine with *Lac91*), *Lac39* (a short deletion distinct from both *Lac11D3* and *Lac14D7*), and *Lac91* (the Group C point mutation that distinguishes *Lac11D3* from *Lac14D7*). Each series was repeated at least three times, using overnight broth cultures inoculated from single colonies or directly from storage tubes for comparison. Syntrophy of the $F^- Lac^-$ prototroph with an $F^- M^-$ stock, reversion of the $F^- Lac^-$ prototrophs, and their ability to give Lac^+ progeny with W3787 (Hfr_1 $P^- M^- V_6{}^r$ Lac^+) were looked for in each case by similar drop tests.

The results of all the crosses appear in Figure 2. It can be seen that the tests failed to differentiate among the long deletions. As none of the deletions yielded Lac^+ progeny when crossed with *Lac11D3* or *Lac39*, only one of them, *Lac85*, was tested extensively against individual members of Group A and Group B. It should be noted, however, that the preliminary tests to group the long deletions involved spot tests with two Group A point mutations and two Group B point mutations. In addition, the long deletions *Lac61*, *Lac72*, *Lac74*, *Lac75*, *Lac81*, and *Lac86* were spotted at least once against 14 members of Group A and eight members of Group B. The results were uniformly negative. It was thought, how-

ever, that the Group C mutations might well be scattered along the *Lac* region, with some located at its ends. In this case, they might react differently with the various deletions if the latter varied in length. Fifteen long deletions were drop tested three times with members of Group C, as well as the standard testers. As none of the crosses gave rise to detectable numbers of Lac^+ recombinants, the long deletions all appeared to be identical. Neither could they be used to deduce the linear order of Group C mutations.

There were several sets of mutations, for example, *Lac96*, *Lac53*, *Lac16* and *Lac194*, that failed to recombine with each other. Since members of such a set showed identical recombination patterns in crosses with other stocks, they were considered to be point mutations, recurrences at a single site.

Figure 2 also illustrates the fact that only three short deletions, *Lac39*, *Lac11D3*, and *Lac14D7*, were discerned (possibly four, if *Lac215* is a short deletion; see "Anomalous recombination"). Because of this, BENZER's method of locating point mutations from their behavior with overlapping deletions was not applicable to the system.

Nineteen Lac^- mutants obtained after exposing W3787 to nitrous acid, and six from the citrate buffer controls, were briefly examined for short deletions. Twenty-two of them, or 88 percent, behaved as long deletions, an increase of 46 percent above the proportion obtained with ultraviolet light. The rest were point mutations, one fitting the Group B criteria (but not included in Figure 2), and two, *Lac334* and *Lac337*, belonging to Group A. Five of the long deletions, *Lac335*, *Lac340*, *Lac341*, *Lac344*, and *Lac350*, were included in the tests represented in Figure 2. They appeared to be identical to the long deletions obtained after ultraviolet treatment of W3787.

The first group of Lac^- mutants obtained after ultraviolet irradiation of W3787 included 22, or 37 percent, which behaved as long deletions (Table 2). Seven different Lac^+ single colonies had provided the inocula for the irradiations that

TABLE 2

Incidence of different types of mutations among Lac⁻ *mutants of W3787 (*Hfr_1 P^- M^- $V_6{}^r$*)*

	Series 1 (W4001–W4060)		Series 2 (W5001–W5233)	
	Number	Percent	Number	Percent
Type of mutation				
Long deletion	22	37	100	43
Short deletion	0	0	0	0
Point mutation	23	38	95	41
Group A	10	17	52	22
Group B	7	12	26	11
Group C	6	10	17	7
Anomalous mutations (*Nr*)	5	8	14	6
Not classified				
Not Hfr	1	2	6	3
Revertible, or able to grow on EM-lactose	9	15	11	5
Other	0	0	7	3

furnished the 60 mutants in this series. All seven of them gave rise to at least one deletion mutant. Among the second set of mutants similarly collected, were 100, or 43 percent, deletions. Forty-two of the 48 single colonies from which these 233 mutants were derived contributed deletions. In order to determine whether this same high proportion of *Lac* deletions was characteristic of other K-12 derivatives, three were irradiated with ultraviolet, according to the same procedure used with W3787. Two were F^- prototrophs unrelated to W3787, and one was the Hfr_1 M^- progenitor of W3787. More than 20 mutants were obtained from each, and tested to see whether any were long deletions. None were found. The probability of such a result being due to chance is less than one percent in each case (Table 3), indicating that W3787 has a special propensity to yield long deletion mutants.

Tests for β-galactosidase

All of the mutants, and most of their F^- *Lac*$^-$ prototroph derivatives, were qualitatively screened for the production of active β-galactosidase. Cultures were first tested as colonies on agar, with 0.5 percent lactose as inducer. All members of Group A responded positively. *Lac11D3*, the short deletion which covers the segment to which these mutations belong, did not hydrolyze ONPG in these conditions. All of the long deletions tested, as well as the short deletions, *Lac14D7* and *Lac39*, failed to give positive reactions. Group B and C point mutations in general gave negative responses. The exceptions were *Lac22*, *Lac155*, and *Lac277*, all of Group C. In each of these cases the reaction was less pronounced than that of the *Lac*$^+$ control, W3787. Table 4 illustrates some of the results.

Because lactose is considered a poor inducer for bacteria defective in permease, cultures which showed no activity on agar with lactose induction were tested in broth with TMG as inducer (Table 4). *Lac85*, *Lac11D3*, *Lac14D7*, *Lac39*, and most of the members of Groups B and C still failed to give detectable reactions after ten minutes' incubation with ONPG. (Some did show color after 24 hours). Three mutants which had previously been negative, *Lac60*, and *Lac299* of Group

TABLE 3

Incidence of long deletions among Lac$^-$ *mutants of four* E. coli *K-12 stocks*

Source	Number of independent long deletions	Number of mutants not deletions	Total
W3787 (1st series)	7	38	45
W3787 (2nd series)	42	133	175
W4955	0	23	23
W3100	0	30	30
W1895	0	27	27
W3787 (1st series) compared to W3787 (2nd series) Observed $\chi^2 = 1.37$		P = 20–30 percent	
W3787 (pooled) compared to W4955		P = 0.42 percent	
W 3787 (pooled) compared to W3100		P = 0.09 percent	
W3787 (pooled) compared to W1895		P = 0.17 percent	

TABLE 4

Examples of results of tests for β-galactosidase and permease

Lac designation	Reaction with ONPG: Lactose induced	Reaction with ONPG: TMG induced	TMG accumulation (Percent of control) (*Lac39*)
Long deletions			
Lac72	—	not tested	11
Lac85	—	—	4
Short deletions			
Lac11D3	—	—	4
Lac39	—	—	100
Point mutations			
Group A			
Lac17	+	not tested	4
Lac21	+	not tested	1
Lac129	+	not tested	15
Lac269	+	not tested	1
Group B			
Lac60	—	±	144
Lac89	—	—	14
Lac97	—	—	39
Lac212	—	—	13
Lac264	—	—	6
Lac299	—	±	132
Group C			
Lac22	±	±	16
Lac88	—	±	13
Lac155	±	±	18
Lac165	—	—	5
Lac256	—	—	90
Lac277	±	—	32
Nr Group			
Lac43	—	—	12
Lac51	—	±	11
Lac59	+	+	11
Lac66	—	—	12

B, and *Lac88* of Group C, responded weakly. On the other hand, one mutant of Group C, *Lac277*, would have been classified as negative on TMG, but produced slight but perceptible color on lactose. *Lac22* and *Lac155* reacted weakly in the presence of either inducer.

Tests for permease

Each series of tests included W4991, which has the original Lac_4 marker (*Lac39*). RICKENBERG *et al.* (1956) and ROTMAN (1959) have shown that another strain (W2244) with the same *Lac*⁻ mutation has the capacity to accumulate TMG after induction with the same compound. Twelve separate determinations on induced cultures of W4991 averaged 1788 cpm (counts per minute) per ml of culture (corrected for 81 cpm nonspecific radioactivity). Using 250 μg per 10^9 cells for the dry weight of *E. coli* (ROBERTS *et al.* 1955),this is equivalent to

82 μ moles of TMG accumulated per gram dry weight of cells. The range was 30 to 142 μ moles per gram dry weight of bacteria. Although these values compare with those in the literature, the wide range found here made it difficult to establish limits for classifying mutants as RICKENBERG *et al.* (1956) have done. Some general observations may be made regarding the results, however.

Group A mutants showed the least amount of accumulation of TMG, and the greatest uniformity of responses within a group. *Lac11D3* also showed reduced accumulation (Table 4).

It was expected that all Group B mutants would accumulate TMG, since *Lac39* does. As Table 4 illustrates, however, responses were extremely heterogeneous, with some mutants showing no more accumulation than members of Group A. *Lac89*, *Lac264*, and *Lac212*, which gave low values, were tested for reversion by spreading EMB-lactose plates with 0.1 ml of overnight Penassay broth cultures and incubating them for several days. *Lac*$^+$ papillae appeared on all the plates, indicating that the markers tested are probably not short deletions.

Group C mutants also showed heterogeneous responses (Table 4). Since there is no single deletion that spans the Group C mutations as *Lac39* does the Group B members, such a result was not so surprising.

Seven long deletions were examined. All were negative in accumulation tests. In general, their values were slightly higher than those of the Group A mutants. The highest value was registered by *Lac72*, reported elsewhere (LURIA, ADAMS and TING 1960) to be $i^- y^- z^-$.

Anomalous recombination

Although in general, a cross in which one of the parents contained a long *Lac* deletion produced no *Lac*$^+$ progeny unless the other parent was *Lac*$^+$, there were some peculiar exceptions. As mentioned before, a small number of mutations gave about 10^{-4} (one per 10^4 cells of the limiting parent) recombinants on EM-lactose when the Hfr was crossed to an F$^-$ carrying a long *Lac* deletion. In contrast, when the Hfr transferred the deletion, less than 10^{-7} *Lac*$^+$ offspring were recovered. Of the 293 mutants of W3787, 19 showed this property, and so did *Lac2*. Crosses of these mutations with point mutations revealed no anomalies, as all of them produced *Lac*$^+$ recombinants.

The hypothesis proposed to account for this phenomenon is that in Y10 and its *S*^{r} derivative, W1394, there is a factor, or factors, which may be called a suppressor, to which certain of the *Lac* mutations are sensitive. Bacteria which have both the suppressor and one of the *Lac* mutations which responds to it, are able to grow on EM-lactose, and to produce dark colonies on EMB-lactose. Bacteria which have only the *Lac*$^-$ mutation, but not the suppressor, do not grow on EM-lactose, and produce only white or pale pink colonies on EMB-lactose. As the suppressor does not noticeably affect the expression of the majority of the *Lac* mutations, especially the long deletions, there may be F$^-$ *Lac*$^-$ prototroph strains derived from crosses of the Hfr *Lac*$^-$ mutants and Y10 or W1394, which include the suppressor. If the suppressor is located on that portion of the genetic map

between *M* and the end of the chromosome segment donated by Hfr_1 (on the *M*, *S*, *Gal* . . . segment), the number of such strains may be quite large, as the nature of the crosses precluded the Hfr parent from contributing the alleles in this region very frequently (RICHTER 1957).

Accordingly, if an F^- *Lac*$^-$ having both a long deletion and the suppressor is crossed with an Hfr having a mutation sensitive to the suppressor, *Lac*$^+$ progeny will arise which include the suppressor and the sensitive mutation. In the reverse cross, the F^- parent has no suppressor, only the suppressor-sensitive mutation (otherwise it would not be *Lac*$^-$), while the Hfr has the deletion and no suppressor. No *Lac*$^+$ offspring appear since the deletion affects some or all of the genetic material altered by the suppressor-sensitive mutations.

Testable predictions based upon this theory include: (1) at least two types of recombinants able to grow on EM-lactose should occur in a cross between Y10 or W1394 and an Hfr with a suppressor-sensitive mutation, a true *Lac*$^+$, and a suppressed *Lac*$^-$; (2) *Lac*$^+$ recombinants from crosses involving a deletion and another *Lac*$^-$ mutation should contain both the *Lac*$^-$ mutation and the suppressor, i.e. *Lac*$^-$ progeny should be recoverable from a cross between such *Lac*$^+$ recombinants and a *Lac*$^+$ strain which has no suppressor.

Evidence for a suppressor

In the experiments to be described, *Lac59* and *Lac66* were chosen as examples of mutations participating in the anomalous reactions. *Lac85* represented a typical long deletion, and *Lac89* a normal point mutation.

Usually from a cross involving W1394 (F^- *T*$^-$*L*$^-$ *Th*$^-$ *S*^{r}) and a *Lac*$^-$ mutant of W3787 (Hfr_1 *P*$^-$ *Lac*$^+$ *M*$^-$ $V_6{}^r$), a large number of the recombinants able to grow on Davis minimal plus streptomycin with or without proline, were *Lac*$^-$. For example, if the Hfr was *Lac85*, or *Lac89*, more than 50 percent of the recombinants were *Lac*$^-$ (Table 5). In addition, most of them were $V_6{}^r$. When the Hfr was *Lac59*, however, less than one percent of the recovered progeny were *Lac*$^-$. Instead, about 50 percent were phenotypically intermediate between the *Lac*$^+$ F^- and the *Lac*$^-$ Hfr. Most of the intermediates, *Lac*$^\pm$, were $V_6{}^r$ (Table 5).

To select recombinants which received the terminal portion of the chromosome from the Hfr, a *Gal*$^-$ derivative of W1394 (W2612) was used as the recipient. If the cross was plated on EM-galactose, or EM-galactose plus methionine (Figure 3), the proportion of *Lac*$^-$ progeny approached 30 percent.

TABLE 5

Incidence of different types of recombinants from crosses of W1394 (F^- T^- L^- Th^- S^r) *with various* Hfr_1 Lac^- P^- M^- $V_6{}^r$ *stocks. Selection on Davis minimal plus proline*

	Lac$^-$		*Lac*$^+$		*Lac*$^\pm$		Total number examined
	$V_6{}^r$	$V_6{}^s$	$V_6{}^r$	$V_6{}^s$	$V_6{}^r$	$V_6{}^s$	
W1394 × W4045 (*Lac85*)	45	5	4	34	0	0	88
W1394 × W4049 (*Lac89*)	53	5	6	28	0	0	92
W1394 × W4019 (*Lac59*)	0	0	2	33	57	3	95

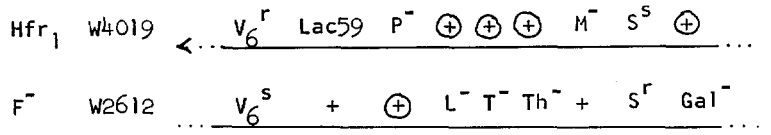

FIGURE 3.—Diagram of W4019 × W2612 plated on EM-galactose + methionine. Circles indicate selected markers. If the same cross is plated on EM-galactose, the M^+ from the F⁻ will also be selected.

This supported the idea that the Lac⁻ phenotype was associated with a factor on the distal portion of the Hfr chromosome. If this factor was the (inactive) allele of a suppressor contained in W1394, the intermediate phenotype might characterize recombinants which received *Lac59* from the male, but retained the suppressor from the female. If this be so, *Lac*⁻ offspring should appear in a mating of a clone of intermediate phenotype with a *Lac*⁺ which does not have the suppressor.

W4506 (F⁻ *Pur*⁻) gave *Lac*⁻, but no *Lac*± recombinants in crosses with W4019 (Hfr$_1$ *Lac59 P*⁻ *M*⁻ V_6^r). In interrupted mating experiments (WOLLMAN and JACOB 1955) *Lac*⁻ appeared to enter about 17 minutes after the start of mating, and about 15 minutes after the entry of the *Pur* marker, as expected if the *Lac*⁻ phenotype depended upon a factor in the region between V_6 and *P*. W4506 could thus be used as an indicator for the presence of *Lac59* in *Lac*± recombinants.

From a mating of W1394 (F⁻ *T*⁻ *L*⁻ *Th*⁻ S^r) with W4019 (Hfr$_1$ *Lac59 P*⁻ *M*⁻ V_6^r) an F⁻ *Lac*± *M*⁻ S^r (W4983) was chosen. After purification on EMB-lactose agar, it was infected with F′$_2$ by growth in mixed culture with W4607 (F′$_2$ *Gal$_6$*). The repurified F′$_2$ *Lac*± *M*⁻ S^r (W4984) was crossed with W4506 and selection made on Davis minimal (Figure 4). Of 94 colonies picked and tested, 32 were *Lac*⁺ (like W4506), 42 were *Lac*± (like W4984), and 20 were *Lac*⁻. Accordingly, W1394 may be said to be *Lac*⁺ *Su*⁺, W4019 to be *Lac59 Su*⁻, and the intermediate type to be *Lac59 Su*⁺. The phenotype of the other possible recombinant, *Lac*⁺ *Su*⁻, is expected to be *Lac*⁺, like W1394.

The proportion of *Lac*⁻ among offspring of the W4984 × W4506 cross may argue that the suppressor is fairly close to *Pur*. Unfortunately, the cross of W2612 × W4019 did not readily permit locating the suppressor. There appeared to be interaction between *Gal* and some factor near *S*, so that when selection was made for *Gal*⁺ S^r prototrophs on EM-galactose plus streptomycin, the colonies which grew were not *Gal*⁺ on EMB-galactose, but of intermediate phenotype. Since a few S^r *Gal*⁺ prototrophs (about five percent) have been obtained, the

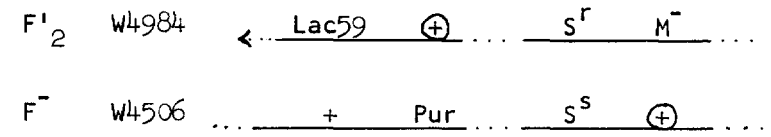

FIGURE 4.—Diagram of W4984 × W4506 plated on Davis minimal. Circles indicate selected markers. Note that the direction of transfer of F′$_2$ is opposite to that of Hfr$_1$, and that the point of origin of F′$_2$ is just before *L*. The distance between *Pur* and *S* is very great (cf. Figure 1).

factor cannot be S^r itself. Since Lac$^+$, Lac$^\pm$, and Lac$^-$ phenotypes have all been observed among the *Gal*$^+$ offspring, it seems unlikely that *Lac59* or its suppressor is to blame.

The most important prediction of the suppressor idea is that anomalous lactose positive progeny contain a *Lac*$^-$ mutation and the suppressor. Lactose positive phenotypes were tested by infecting them with F′$_2$ and mating them with an S^r derivative of W4506 (F$^-$ *Pur*$^-$). None of the progeny of control crosses, W4145 (F$^-$ *Lac85*) × W3787 (Hfr$_1$ *Lac*$^+$ *P*$^-$ *M*$^-$ V_6^r), or W3133 (F$^-$ *Lac11D3*) × W4991 (Hfr$_1$ *Lac39 P*$^-$ *M*$^-$ V_6^r), sired anything but *Lac*$^+$. All of the progeny tested from anomalous crosses, nine from W4019 (Hfr$_1$ *Lac59 P*$^-$ *M*$^-$ V_6^r) × W4145 and ten from W4026 (Hfr$_1$ *Lac66 P*$^-$ *M*$^-$ V_6^r) × W4145, produced *Lac*$^-$ recombinants with the *Lac*$^+$ F$^-$. In other words, the results substantiated the hypothesis.

Clones whose genotype included a suppressor and *Lac59* or *Lac66* were unstable. Colonies formed by them on EMB-lactose often contained lighter or darker colored sectors. In the case of *Lac59*, the colonies darkened gradually over a period of two days or so, and sectors could be detected with certainty only after about 36 hours at 37°C. Colonies from cultures having *Lac66* and its suppressor, however, darkened rapidly. Sectors appeared by 24 hours and then were obscured by darkening of the entire colony. Occasionally, whole colonies were found having either a Lac$^-$ phenotype, or a very dark color almost like *Lac*$^+$. *Lac*$^-$ sectors and segregants appeared in F$^-$ as well as F′$_2$ cultures with a frequency of approximately one per 1000 viable units. These lactose negative sectors appear to contain new suppressor-resistant *Lac*$^-$ mutants, which behave like long deletions. At least they failed to recombine with *Lac11D3*, *Lac39*, and 16 different point mutants from Groups B and C. They must also have had the suppressor, as they gave positive recombinants with both *Lac59* and *Lac66*. When recovered from recombinants of crosses with W4145 (F$^-$ *Lac85*) as the recipient, such *Lac*$^-$ behaved like *Lac85* in the tests employed, but when they occurred in clones derived from *Lac*$^+$ recipients, they resembled neither parental marker.

From the foregoing, it is evident that W4019 (Hfr$_1$ *Lac59 P*$^-$ *M*$^-$ V_6^r) typifies a series of Hfr mutants having *Lac*$^-$ mutations which are sensitive to a suppressor carried by W1394. It is not known whether W1394 has one suppressor which is active upon all of the *Nr* mutations, or whether there exists a series of suppressors, each one specific for a particular mutation. In connection with this, *Lac215* can be noted (Figure 2). This mutation does not recombine or behave abnormally with the suppressor-carrying long deletion stocks. It does give a nonreciprocal pattern of recombination with *Lac139*, *Lac284*, *Lac229*, however. This suggests that the F$^-$ *Lac215* derivative of W1394 is carrying a suppressor of different specificity from the one characteristic of the long deletion stocks.

Other characteristics of suppressor-sensitive Lac *mutations*

The fact that W4506 (F$^-$ *Pur*$^-$) behaves normally with the sensitive mutations enables one to use it to obtain F$^-$ *Lac*$^-$ prototrophs for recombination tests. Such derivatives were made for *Lac85*, *Lac61*, *Lac72*, *Lac74*, *Lac81*, *Lac86* (all long deletions), *Lac11D3*, and *Lac39* (both short deletions), and *Lac59* (an *Nr* muta-

tion). These were drop tested on EM-lactose with the suppressor-sensitive mutations, *Lac2*, *Lac43*, *Lac45*, *Lac51*, *Lac59*, and *Lac66*. Figure 5 presents the results. The lesions in the *Lac* region seem to be located within the area covered by the long deletions, with *Lac45* and *Lac59* at the same site within the *Lac11D3* segment. The other suppressor-sensitive mutations act as different Group C mutations, recombining with both *Lac11D3* and *Lac39*, but not with the long deletions. Preliminary examination of other *Nr* mutations suggested that three of them, *Lac230*, *Lac231*, and *Lac237*, might occupy the same site as *Lac2*. Tests were not rigorous enough to warrant including the results in Figure 5.

A derivative of W4506 carrying *Lac215* was also made, and tested against *Lac139*, *Lac284*, and *Lac229* (cf. Figure 2). No recombinants appeared in any of the three crosses, indicating that *Lac215* overlaps these other mutations.

Tests for β-galactosidase and permease were made of some of the *Nr* mutants. *Lac45* and *Lac59* both gave positive reactions with ONPG. *Lac2*, *Lac27*, *Lac43*, *Lac51*, and *Lac66* were all negative. All of the mutants tested, *Lac43*, *Lac51*, *Lac59*, and *Lac66*, showed reduced accumulation of TMG (Table 4). *Lac2* was not tested, but according to the results of RICKENBERG *et al.* (1956), it should be permease positive.

DISCUSSION

Studies of the problem of coding proteins from nucleic acids necessitate ordering large numbers of mutations. For this, the most efficient method employs overlapping genetic deletions (BENZER 1959). For the procedure, however, a suitable set of overlapping deletions represents a *sine qua non*. Plainly, the *Lac* mutations studied here do not meet this requirement. Deletions obtained from W3787 were all too large to be of use in mapping. That they did not recombine with any of the point mutations, together with the fact that those tested were deficient in both β-galactosidase and permease means they are probably $y^- z^-$. LURIA, ADAMS and TING (1960) used one of them, Lac72, and reported it to be $i^- y^- z^-$. On the basis of the gene order given by JACOB *et al.* (1960), one might also expect it to include *o*, between *i* and *z*.

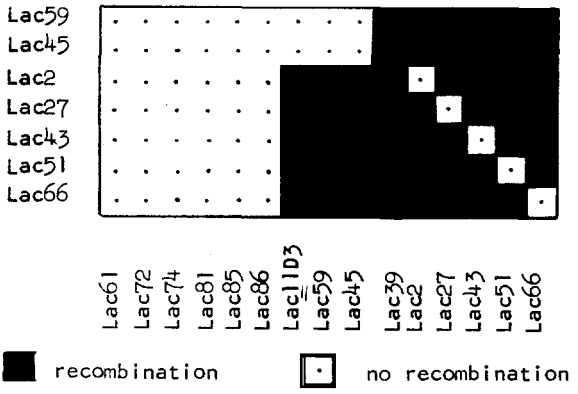

FIGURE 5.—Recombination of suppressor-sensitive mutations.

The unusually high frequency of deletions encountered among the derivatives of W3787 probably indicates the presence of a structural abnormality which predisposes to large deletions, and may also make short deletions correspondingly rare. Generally, deletion mutations occur more rarely than point mutations, but at least two cases have been reported of loci unusually susceptible to deletions: the region controlling T_1 resistance in *Escherichia coli* B (GOTS, KOH and HUNT 1954; YANOFSKY and LENNOX 1959) and the *CysC* locus in Salmonella (DEMEREC 1960; DEMEREC *et al.* 1960). In the present case, the aberration does not seem to be a property of W1895, the Hfr_1 M^- progenitor of W3787; therefore, a more rigorous comparison of the two stocks might lead to an elucidation of the nature of the aberration.

The three short deletions available (*Lac11D3*, *Lac14D7*, and *Lac39*) were not obtained from W3787. *Lac11D3* closely resembles *Lac14D7*, although they were stable (*Lac*$^-$) derivatives of two, mutable Lac_1 mutations, *Lac11* and *Lac14*, respectively. The distinction between them is that *Lac14D7* does not recombine with *Lac91*, while *Lac11D3* does. *Lac91* recombines with both of the original Lac_1 mutations. *Lac14* is so revertible that it has not been possible to determine whether it occupies the same site as *Lac11*, but the two mutations originated in different stocks, and have different reversion rates. Two other stable derivatives of *Lac11*, and one of *Lac14*, had previously been tested (COOK 1958) and found to be long deletions indistinguishable from each other or from other long deletions, such as *Lac85*.

Group A point mutations failed to recombine with *Lac11D3*. As this is a two-step mutation, and the two events need not have altered contiguous nucleotide sequences, it would not have been surprising to find two sorts of mutations in Group A, particularly since *Lac11D3* itself decreased β-galactosidase activity as well as permease activity. All members of Group A, however, showed reduced permease activity, but made perceptible amounts of β-galactosidase.

In addition to being derived from a Lac_1 mutant, *Lac11D3* does not recombine with other Lac_1 mutants tested (e.g. *Lac12* from W2241, cited by RICKENBERG *et al.* 1956). In many cases the reversion rate of Lac_1 mutants approaches the expected rate of recombination between them, making interpretation difficult. However, it is fairly certain that *Lac12* fits into Group A.

Lac39 is the Lac_4 prototype (LEDERBERG *et al.* 1951; RICKENBERG *et al.* 1956). Because it probably resulted from a single event, the deleted segment should span an uninterrupted sequence of point mutations. Like *Lac39* ($i^+ y^+ z^-$), these mutants should fail to make active β-galactosidase, but should have an intact permease mechanism. Paradoxically, several mutants of Group B, located at different sites, lack β-galactosidase, and also have defective permease systems. JACOB and MONOD (1961) have described point mutations having reduced permease capacity and lacking β-galactosidase. They have not reported a case where such mutants fail to recombine with a deletion having one of the activities missing in the point mutants.

Of the original seven *Lac* loci, then, Lac_1 and Lac_4 can be most easily related to the present system. The mutation at Lac_2 belongs to the group of mutations

showing anomalous recombination. On the basis that such recombination is due to a suppressor at a different locus, and not to crossing-over within the *Lac* region, Lac_2 can be said to be in the *Lac* region. The finding of PARDEE *et al.* (1959), that phage 363 can transduce it jointly with other *Lac* mutations agrees with this. Lac_3, Lac_5, and Lac_7 mutants grow too well on EM-lactose to tell by this method whether they recombine with the long deletions or not, and thus whether they are in the *Lac* region. The same was true of several mutants of W3787 with the traditional Lac_3 phenotype.

While the original aim of comprehensive mapping of the *Lac* region has not been accomplished, the deletion stocks offer useful material for the study of the activity of the *Lac* gene; for example, the behavior of deletions in transductional heterozygotes (LURIA, ADAMS and TING 1960; REVEL, LURIA and ROTMAN 1961; REVEL and LURIA 1961; REVEL, LURIA and YOUNG 1961), or the discrepancy between the biochemical properties of *Lac39* and the point mutations it spans.

SUMMARY

Two hundred ninety-three *Lac*$^-$ mutants were collected after ultraviolet irradiation of a stock of *Escherichia coli* K-12. Stocks for recombination analysis were derived from them by appropriate matings. Drops of male and female cultures carrying the mutations were paired on minimal lactose agar and scored for the production of *Lac*$^+$ prototrophic recombinants. All of the mutations had altered sites in one region located between the markers *P* and V_6. Thirty percent were long deletions. As the long deletions did not recombine with any of the point mutations tested, they were indistinguishable from each other. The proportion of deletions was significantly higher among *Lac*$^-$ derivatives of one stock of K-12 (W3787) than among *Lac*$^-$ derivatives of any of the other three stocks tested.

The point mutations formed three groups on the basis of recombination with two short deletions. Mutants belonging to one group all had active β-galactosidase, but accumulated TMG only weakly. In these properties they resembled Lac_1 or y^- mutants (RICKENBERG *et al.* 1956). Both the other two groups included some mutants with reduced β-galactosidase activity, but normal TMG accumulation, and some with both the β-galactosidase activity and TMG accumulating ability diminished. A short deletion that retained the capacity to accumulate TMG spanned several of the point mutations which changed both functions.

A fourth group of point mutations was found to be sensitive to the actions of a suppressor factor, or factors, present in certain K-12 stocks.

As only three short deletions were available, no unique sequence for point mutations could be obtained by the method of overlapping deletions.

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