NATURE OF ETHYL METHANESULFONATE INDUCED REVERSIONS OF *lac*⁻ MUTANTS OF ESCHERICHIA COLI[1,2]

NORMAN M. SCHWARTZ[3]

Department of Microbiology, The University of Chicago, Chicago, Illinois

Received May 20, 1963

PRIOR to the proposals of FREESE (1959a, b, c) that particular mutagenic agents induce specific alterations of deoxyribonucleic acid (DNA), WESTERGAARD (1957) stated that "the back mutation pattern of a given mutant gene, as defined by its response to various physical and chemical mutagens, depends upon how the gene was originally damaged." This report is concerned with the reversion patterns of ethyl methanesulfonate (EMS) and ultraviolet (UV) induced *lac*⁻ mutants of *Escherichia coli* and with the genetics of the back mutants induced. A series of allele-specific suppressors of one *lac*⁻ mutation has been localized to the *pro lac* region of the chromosome by conjugation and transduction experiments.

MATERIALS AND METHODS

The following gene symbols are used in this paper: *thr*–threonine, *leu*–leucine, *pro*–proline, *lac*–lactose, *pur*–purine (closely linked to *lac*) *gal*–galactose, *try*–tryptophan, *met*–methionine, *str*–streptomycin and P1–phage P1kc. The genes of the *lac* segment, *y*, *z*, *o* and *i* are used as suggested by JACOB and MONOD (1961a). Superscript (−) and (+) indicate mutant and wild-type alleles respectively, except that *lac*⁺ indicates the gene while lac⁺ is used to indicate phenotype only. All lactose utilizing revertants of *lac*⁻ mutants are designated lac⁺. Superscript (*r*) and (*s*) indicate resistance and sensitivity to streptomycin or phage.

EMS and UV induced *lac*⁻ mutants were isolated from two constitutive (i^-) strains of *E. coli;* K–12 3.300 provided by DR. S. E. LURIA and ML 308 obtained from DR. D. B. COWIE. *E. coli* K–12 3.310 ($y^+ z^- o^+ i^-$) was obtained from DR. LURIA, K–12 2.340 ($y^+ z^- o^+ i^-$) from DR. A. B. PARDEE, and 2.320 ($y^+ z^+ o^0 i^-$) from DR. F. JACOB. The properties of these three strains have been described by FRANKLIN and LURIA (1961). K–12 strain X148 (F^- *leu*⁻ *lac*⁻ ($y^+ z_2^- o^+ i^+$) *pur*⁻ *gal*⁻ *try*⁻ *str*^{r}) was provided by DR. R. CURTISS and was derived (CURTISS 1962) from strain W945 originally supplied by DR. M. L. MORSE. None of the K–12

[1] Supported in part by funds from the National Science Foundation and from the National Institutes of Health.

[2] Taken in part from a thesis submitted to the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Predoctoral Research Fellow, Public Health Service. Present address: Department of Microbiology, Yale University, New Haven, Connecticut.

strains used was lysogenic for phage lambda. Transducing phage P1kc was obtained from DR. C. YANOFSKY.

Medium E (VOGEL and BONNER 1956) with 0.3 percent (v/v) glycerol was used as a minimal medium; the salt solution without added carbon source was used for washing and resuspending bacterial pellets and as a diluent. EMB-lactose agar (LURIA, ADAMS, and TING 1960) was used to test lactose fermenting ability. Minimal EMB-lactose medium was prepared by adding 0.4g eosin Y, 0.065g methylene blue, 5g sodium succinate and 10g lactose to 1 liter of medium E; other required growth factors were added as indicated below. The E salt-solution, lactose, and succinate were sterilized separately.

Lactose utilizing revertants of *lac*$^-$ mutants were scored and selected on minimal medium hardened with 1.5 percent agar and containing 1 percent filter sterilized lactose as the sole carbon source. Numbers of viable *lac*$^-$ organisms were determined on minimal (glycerol) agar. Medium E was supplemented with 20 μg/ml of the L-isomer of the required amino acids, 20 μg/ml of adenine and 200 μg/ml of streptomycin sulfate as necessary for the selection and purification of recombinants. Media were supplemented with 10 μg/ml of thiamine hydrochloride when necessary. All plating media were solidified by the addition of 1.5 percent Difco Bacto-Agar. The agar and required growth factors, salts, and carbon source were sterilized separately.

Isolation of lac$^-$ *mutants:* Exponential *lac*$^+$ cultures containing about 2×10^8 bacteria/ml in minimal-lactose medium were prepared starting from inocula of about 200 organisms/ml. The cultures were collected by centrifugation, washed and resuspended to the original volume in "E" salts. Organisms were treated with ethyl methanesulfonate (EMS) obtained from Eastman Organic Chemicals by adding 0.1 ml of vacuum distilled EMS to 10 ml of bacterial suspension to give a final concentration of mutagen of about 0.1 M. The mixture was aerated at 37°C for one hour. About 20 percent of the organisms survived the treatment. Organisms were treated with ultraviolet light (UV) by irradiating in 100×15 mm Petri dish bottoms for 60 seconds at a distance of 48 cm from a 15 watt germicidal lamp. About 10 percent of the bacteria survived UV treatment. EMS or UV treated bacterial suspensions were diluted immediately after treatment and spread on EMB-lactose agar to give about 200 colonies per plate. Colorless or pinkish color variant colonies and sectored colonies were purified by two single colony isolations on EMB lactose medium. Stable variant strains producing no, or very few, EMB color positive papillae after 72 hours incubation at 37°C were then tested for their ability to grow on minimal–1 percent lactose, and minimal–0.5 percent glucose agar. Strains which grew on the latter medium but failed to grow with lactose as a sole carbon source after five days incubation at 37° were classified as *lac*$^-$. Most of the *lac*$^-$ mutants obtained were induced by the mutagenic treatment since the frequency of spontaneous *lac*$^-$ mutants was about 0.002 percent compared to induced frequencies of about 0.1 percent.

Treatments to induce reversion: lac$^-$ mutants which reverted with a spontaneous frequency of about 10^{-8} were treated with EMS, UV and with 2-aminopurine (AP). Stationary phase cultures grown 18 to 24 hours at 37°C with aera-

tion in minimal-glycerol medium were harvested by centrifugation, washed and resuspended in E salts at a concentration of about 10^9 bacteria/ml. The suspensions were either treated with UV for 20 sec or incubated with 0.1 M EMS for 30 min. About 70 percent of the bacteria survived this UV treatment; 80 percent survived EMS treatment. Organisms were treated with aminopurine by dissolving 5 mg of 2-aminopurine nitrate (AP) in 9 ml of E-glycerol medium which was then inoculated with 1 ml of bacterial suspension; the mixtures were incubated with aeration at 37°C for 3 hours. After treatment with AP or EMS the bacteria were washed and resuspended twice in E salts before incubation in growth medium. UV or EMS treated bacteria were incubated in minimal-glycerol medium for 3 hours at 37°C with aeration to permit phenotypic expression of the lac^+ character before plating on minimal-lactose medium. The plates were incubated for 5 days at 37°C at which time the revertant colonies had reached a constant number. No significant difference in reversion frequency was observed when bacteria were plated directly, without post-treatment incubation in minimal-glycerol medium. All the lac^- strains, including the lac^{del} strains, form visible microcolonies when plated at high dilution on minimal-lactose agar.

Genetic studies: Transductions were performed with P1kc as described by LENNOX (1955). Lysates were prepared by the plate method through two rounds of infection on the donor strain in order to avoid inclusion of any unadsorbed phages in the lysate. Conjugation mixtures were prepared by combining non-aerated exponential phase cultures of the donor strain with aerated exponential phase recipient bacteria. Ten ml of the mating mixture were allowed to stand in a 250 ml Erlenmeyer flask for 2 hours at 37°C. Mating mixtures contained about 10^7 donor and 2×10^8 recipient bacteria per ml. Either Penassay or L-broth (LENNOX 1955) were used to prepare cultures for conjugation experiments and as a mating medium Similar results were obtained with either medium. Recombinant clones were purified by two successive single-colony isolations on the primary selection medium. The purified recombinants were then picked and streaked or replicated onto appropriate media to score for unselected markers.

Enzyme studies: β-galactosidase activity was assayed by the method of LEDERBERG (1950) and by the colorimetric method of NOLL and ORLANDO (1961). This latter method is particularly useful for the measurement of low levels of enzyme activity. One unit of galactosidase releases 1 μM of o-nitrophenol/min at pH 7.2 and 37°C. Specific enzyme activity is expressed as enzyme units/mg protein. Protein was measured with the phenol reagent described by SUTHERLAND, CORI, HAYNES, and OLSEN (1949) using crystalline bovine albumin as a protein standard. β-galactosidase was purified by modification of the method of KAMEYAMA and NOVELLI (1962). Galactoside permease activity was assayed with C^{14}-thiomethyl-β-D-galactoside (New England Nuclear) as described by HORIUCHI, TOMIZAWA and NOVICK (1962). Electrophoresis was performed with an EC 470 vertical gel electrophoresis apparatus (EC Apparatus Co.) on 5 percent acrylamide gel. β-galactosidase activity was localized by incubating the gels with o-nitrophenyl-β-D-galactoside. Protein bands were demonstrated by staining with aniline blue-black.

RESULTS

Twenty-three *lac*$^-$ mutants of the *i*$^-$ strain ML 308 and five *lac*$^-$ mutants of strain 3.300 (*i*$^-$) were isolated after EMS treatment. Five *lac*$^-$ mutants of ML 308 and four *lac*$^-$ mutants of 3.300 were isolated after UV treatment. These isolates were selected for reversion studies because they were stable, spontaneously reverted to lac$^+$ at low frequencies, did not grow on minimal-lactose medium and were unable to synthesize normal levels of β-galactosidase. The *lac*$^-$ mutations of each of the 3.300 mutants was localized within the *lac* region by transduction crosses with a *lac* deletion mutant, strain W 1485–1, which was isolated by penicillin selection after UV treatment of W 1485, a K–12 *lac*$^+$ prototroph. No lac$^+$ recombinants were obtained in reciprocal transduction crosses between W 1485–1 and seven of nine *lac*$^-$ derivatives of strain 3.300 or between W 1485–1 and strains 2.340 (*z*$^-$), 3.310 (*z*$^-$) or 2.320 (*o*^{c}). The deleted site of strain W 1485–1 does not span the entire *lac* region since between 20 to 400 transductants were obtained in crosses between W 1485–1 and two of the 3.300 *lac*$^-$ mutants as well as with strain C600 (*y*$^-$). About 35,000 transductants per ml were obtained under comparable conditions when the prototroph 3.300 was used as a donor. All the *lac*$^-$ point mutants synthesized low amounts of β-galactosidase (Table 1) in contrast

TABLE 1

β-galactosidase and permease activities

Strain	Origin	Genotype	β-galactosidase units/mg protein	Constitutive permease: percent of strain 3.300
3.300	LURIA	$y^+z^+o^+i^-$	1.2×10^4	100
3.310	LURIA	$y^+z^-o^+i^-$	115	7
2.340	PARDEE	$y^+z^-o^+i^-$	0.02	18
2.320	JACOB	$y^+z^+o^0i^-$	...	15
lac_A^-	3.300(UV induced)		0.07	...
lac_E^-	3.300(UV induced)		0.05	...
lac_G^-	3.300(UV induced)		0.04	20
lac_H^-	3.300(UV induced)		0.07	...
lac_{M1}^-	3.300(EMS induced)		0.03	120
lac_{M4}^-	3.300(EMS induced)		0.14	...
lac_{M7}^-	3.300(EMS induced)		0.05	...
lac_{M12}^-	3.300(EMS induced)		0.08	...
lac_{IS}^-	3.300(spontaneous)		0.007	15
W1485–1	W1485 (UV)		0*	...

* Strain W1485–1, derived from W1485 ($y^+z^+o^+i^+$), was grown in minimal-glycerol medium containing lactose, 10 mg/ml, to induce β-galactosidase.

Galactosidase assay: All strains were grown in minimal glycerol medium to the stationary phase, and a washed concentrated bacterial suspension of about 5×10^{11} bacteria per ml was suspended in Tris buffer (KAMEYAMA and NOVELLI 1962) and disrupted by sonic oscillation for 10 min at 5°C in a Raytheon 10kc sonic oscillator. The sonicate was used as an enzyme sample; 0.2 ml M/75 o-nitrophenyl-β-D-galactoside was added to 1 ml test sample and incubated at 37°C for a sufficient time to give a satisfactory color intensity. The reaction was halted with perchloric acid (final concentration 0.1M). The sample was centrifuged, and the supernatant obtained extracted twice with 2, 2, 4-trimethylpentane (NOLL and ORLANDO 1961). o-nitrophenol was extracted from the trimethlypentane with M/10 sodium hydroxide and the optical density was determined at 420 mμ in a Bausch and Lomb "Spectronic 20" colorimeter. A boiled bacterial extract was always included as a control to measure any nonenzymatic hydrolysis of the substrate, which was never detected under the conditions employed. Permease assay: C^{14}-thiomethyl-β-D-galactoside, (New England Nuclear Corp., 4.1×10^{12} counts min/mole), was added to 2 ml log phase culture (about 2×10^8 bacteria/ml) to a final concentration of 2.4×10^{-4} M. After 12 min incubation with shaking at 37°C, the suspensions were filtered through Millipore membranes and washed twice with 5 ml cold 0.1M sodium phosphate buffer (pH 7.2). A boiled bacterial culture was treated similarly to measure nonspecific radioactivity. The air-dried membranes were glued to planchets with rubber cement and counted for 2 min in a gas flow counter. Counts/min/O.D.$_{350}$ were corrected for background and nonspecific radioactivity.

to the complete lack of activity in strain W 1485–1 and other *lac*⁻ strains classified as deletion mutants because of their inability to undergo spontaneous or induced reversion.

The galactoside permease activity of the 3.300 *lac*⁻ point mutants was determined to distinguish possible z^- and o^o mutants; o^o mutants have both reduced galactosidase and permease activity whereas some z^- mutants form a galactosidase immunologically related to the normal and have normal permease activity (FRANKLIN and LURIA 1961). Strains lac_G^- and lac_{M4}^- have greatly reduced galactosidase activity and partially reduced permease activity (Table 1). Strain lac_{M1}^- has greatly reduced galactosidase activity but normal permease activity and is therefore probably a z^- mutant. Strains 2.340 (z^-) and 3.310 (z^-) have been reported to synthesize galactosidases which cross react with antibody to normal β-galactosidase (FRANKLIN and LURIA 1961) but have reduced permease activities comparable to that of strain 2.320 (o^-). Therefore it is not possible to decide whether lac_G^- and lac_{M4}^- are z^- or o^o.

Reversion studies: EMS and UV induced *lac*⁻ mutants were treated with either EMS, UV or AP to induce reversion (Table 2). The colony morphology of the revertants obtained was heterogeneous; some large colony, fast growing, revertant types feed the *lac*⁻ background producing halos of satellite growth. Other revertant colony types include large, medium and small sized colonies which are not "feeders" (Figure 1). When synthetic mixtures containing wild-type *lac*⁺ bacteria are plated on minimal-lactose medium with an excess of *lac*⁻ mutant organisms, the wild-type bacteria produce feeder colonies.

Mutagenic treatments which do not induce wild type-like lac⁺ revertants at high frequency are therefore easily detected by this procedure. Although true back mutants should produce feeder-type revertant colonies, other types of genetic alteration (unlinked suppressors and some "primary site" reversions) can restore

TABLE 2

Reversion patterns of ultraviolet and EMS induced lac⁻ *mutants*

Strain	Number of mutants	Origin	Mutagen to induce reversion			
			None	UV	EMS	AP
E. coli ML308	23	EMS	1–29*	1–28	150–830	++
3.300	4	EMS	1–5	2–39 mostly nonfeeder revertant types	2100–3200 mostly nonfeeder revertant types	120–390 feeder and nonfeeder revertant types
E. coli ML308	5	UV	1–15*	430–760	610–1190	....
3.300	4	UV	1–5	170–380 mostly feeder revertant types	170–500 mostly nonfeeder revertant types	5–34 mostly nonfeeder revertant types

* Range of reversion frequency per 10^8.
† Reversion frequencies not determined; nine out of nine mutants tested were induced to revert.

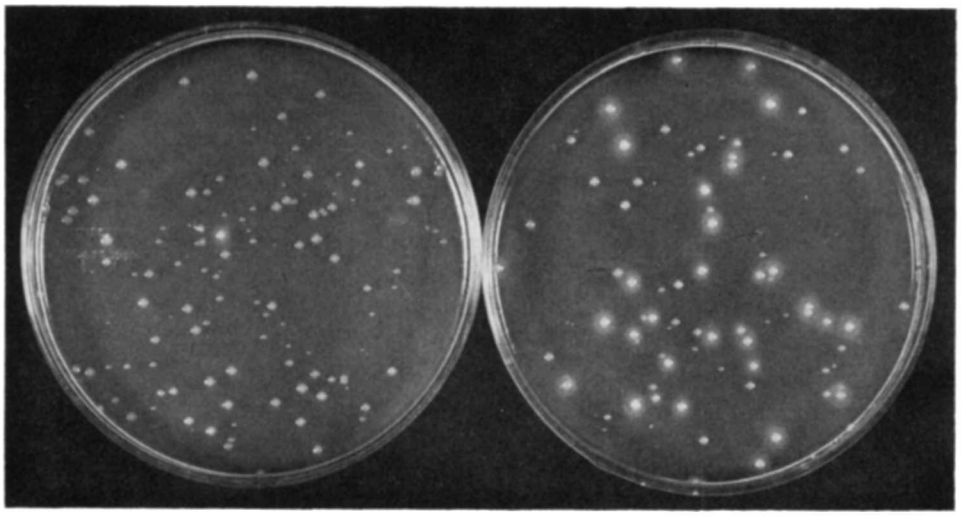

FIGURE 1.—Feeder and nonfeeder-type revertants of strain lac_E^-, (UV induced *lac*$^-$ mutant). Left: EMS treated. Right: UV treated. About 10^8 viable cells were plated on minimal-lactose medium (filter sterilized).

a wild-type phenotype and wild-type levels of enzyme activity (HENNING and YANOFSKY 1962; GAREN and SIDDIQI 1962). Throughout this paper the term "revertant" has been used to indicate derivatives of *lac*$^-$ strains able to utilize lactose as a carbon source, but without any implication as to the type of genetic alteration responsible for the phenotype.

EMS induced mutants had a characteristic reversion pattern which differed from the reversion pattern of UV induced mutants. AP induced feeder-type revertants of 13 EMS mutants tested (Figure 2). EMS did not induce a high frequency of feeder-type revertants with any of 28 EMS induced mutants although many revertants were induced. UV did not induce many revertants of EMS induced mutants and the few that were obtained were of the nonfeeder type. On the other hand, UV induced many feeder-type revertants of UV induced lac$^-$ strains (Figure 1). EMS also induced many revertants of the nine UV induced mutants tested, but most of the revertants were nonfeeder types. AP did not induce many revertants of the four UV induced mutants tested and those which were obtained were nonfeeder types.

Genetic nature of the revertants: Revertants were purified by at least two single colony isolations on EMB-lactose agar and were maintained on minimal-lactose agar. Feeder-type revertants gave EMB color reactions typical of a wild-type *lac*$^+$ strain whereas nonfeeder types gave weaker color reactions. Most nonfeeder-type revertants were unstable and reverted to *lac*$^-$ (Figure 3). The revertant illustrated was so unstable that it could only be maintained on minimal-lactose medium. This instability was not a characteristic of the particular *lac*$^-$ strain from which the revertant was derived since unstable revertants were obtained from many *lac*$^-$ strains. *lac*$^-$ derivatives of unstable revertants retained the mating properties of

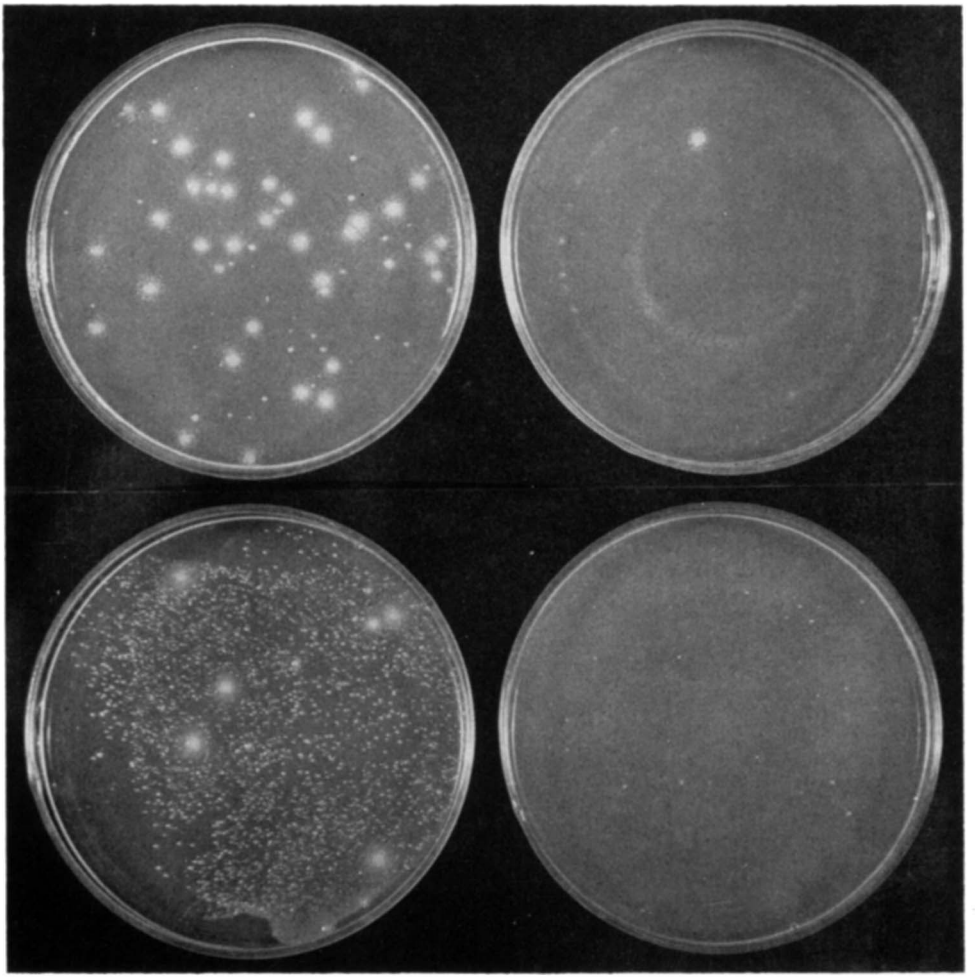

FIGURE 2.—Reversion pattern of strain lac_{GH1}^-, (EMS induced lac^- mutant). Top left: AP induced revertants. Top right: spontaneous revertants. Lower left: EMS induced revertants. Lower right: UV induced revertants. Comparable numbers of viable bacteria were plated, and the plates were incubated for 5 days at 37°C before photography.

the original lac^- mutant indicating that the instability was not due to the loss of a sex factor carrying the reversional mutation. Because very unstable revertants were difficult to maintain, their genetics was not studied.

Even those revertants of the nonfeeder type which could be analyzed were somewhat unstable; several variegated colonies were always observed when approximately 1000 colonies were screened on EMB-lactose agar. The weak EMB color reaction and nonfeeder colony morphology on minimal-lactose medium was maintained after repeated transfer on nonselective media. Revertant stocks were maintained on minimal-lactose slants and periodically reisolated from a single colony on EMB-lactose agar. Nonfeeder-type reverants have lower β-galactosidase

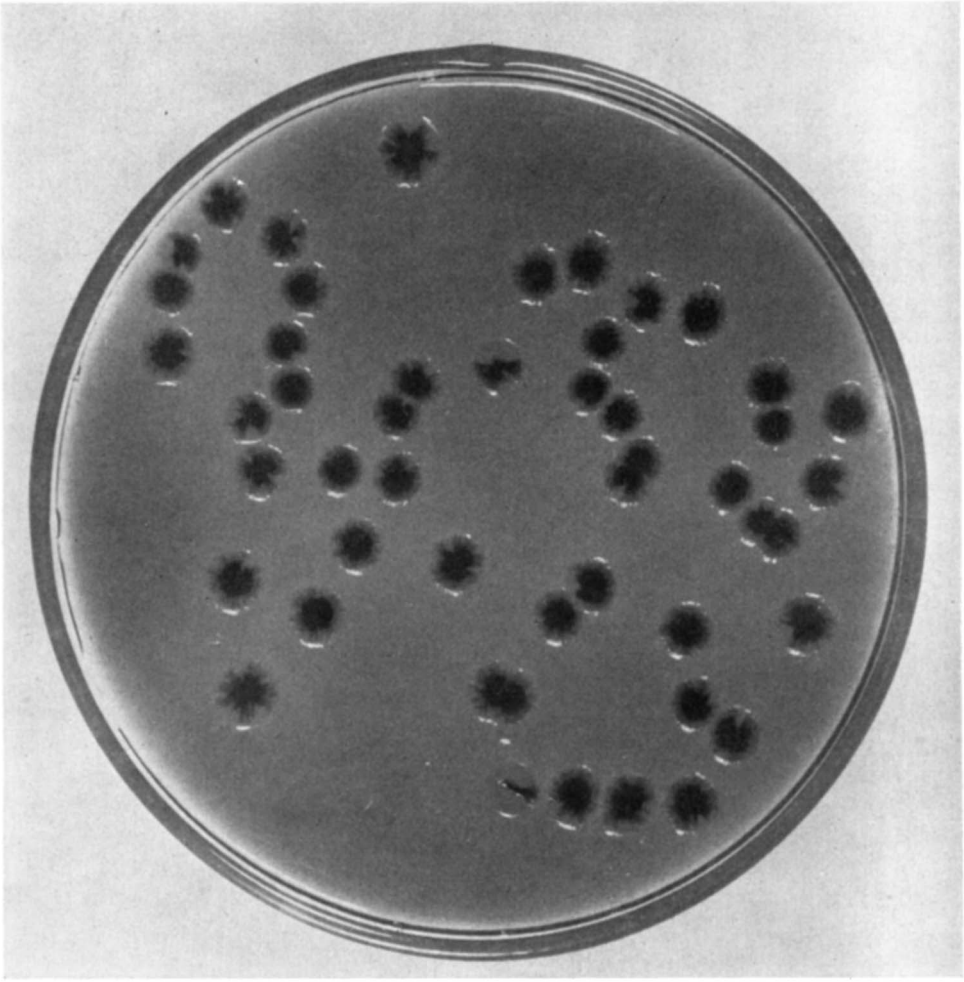

FIGURE 3.—Colonies of an extremely unstable nonfeeder-type revertant on EMB-lactose agar. An EMS induced nonfeeder-type revertant of strain lac_{18}^- (spontaneous *lac*$^-$ mutant) was grown to the stationary phase in minimal-lactose liquid medium, diluted and plated. The plate was incubated for 48 hrs at 37°C before photography.

activity than do either the wild-type strain or feeder-type revertants (Table 3). This lower activity can not result from the presence of *lac*$^-$ cells produced from the unstable revertants.

Transducing phage P1kc prepared on different revertant types was employed as a donor in crosses with *lac*$^-$ mutants to test the genetic nature of the revertants. If the reversions are due to (allele specific) suppressors and if the suppressor is not linked to the *lac* locus, very few lactose utilizing transductants should be obtained in crosses with nonallelic *lac*$^-$ mutants. Any transductants obtained should equal in number the few typically wild type-like feeder recombinants expected by recombination of two *lac*$^-$ alleles.

TABLE 3

β-galactosidase activities of feeder and nonfeeder type revertants of strain lac_G^-

	Strain	Colony type	Enzyme units per mg protein
Revertant	1	nonfeeder	700
Revertant	2	nonfeeder	1,000
Revertant	3	feeder	11,800
Revertant	4	feeder	12,200
3.300 (i^-z^+)		. . . .	12,000

Each of the strains was grown in minimal-glycerol medium, and the level of β-galactosidase activity was determined as described in the legend to Table 1.

P1 prepared on an EMS induced nonfeeder revertant of strain lac_G^- did not give lactose utilizing transductants of nonallelic lac^- mutants in excess of the number expected (Table 4). Nonfeeder-type transductants were obtained in a cross employing P1 prepared on a nonfeeder-type revertant of strain lac_G^- when strain lac_G^- was the recipient (Table 4). P1 prepared on the wild-type strain or on a UV induced feeder-type revertant of strain lac_G^- gave large numbers of lac^+ transductants with each of the three lac^- recipients tested.

The allele specificity of two EMS induced nonfeeder-type revertants of each of three different UV induced lac^- mutants was tested in a similar manner. Five different lac^- mutants and the original strain from which the revertants were derived were used as recipient strains. In each case, the nonfeeder reversional mutations were allele specific. Similar results were obtained in crosses employing

TABLE 4

Transductional analysis of revertants

	lac^+ transductants per ml				
	Recipient strains				
Donor	2.340 (z^- i^-)	3.310 (z^- i^-)	lac_G^-	lac_{M1}^-	W1485–1 (lac^{del})
3.300 (z^+ i^-)	1.3×10^4	2.8×10^4	3.4×10^4	. . .	1.9×10^4
lac_G^-	2.2×10^1	5.5×10^1	0	. . .	0
G(E1)R* (nonfeeder)	2.1×10^1	3.4×10^1	3.1×10^4†	. . .	0
G(U2)R (feeder)	1.3×10^4	2.2×10^4	2.6×10^4	. . .	. . .
lac_{M1}^-	1.6×10^2	2.1×10^2	3.0×10^2	0	0
M1(E3)R (nonfeeder)	1.3×10^2	2.1×10^2	2.4×10^2	2.4×10^4†	0
M1(U4)R (nonfeeder)	1.1×10^2	2.0×10^2	2.6×10^2	3.0×10^4†	0
M1(AP3)R (feeder)	4.5×10^4	5.1×10^4	4.9×10^4	5.0×10^4	3.4×10^4

* G(E1)R: EMS induced nonfeeder-type revertant of strain lac_G^-.
G(U2)R: UV induced feeder-type revertant of strain lac_G^-.
M1(E3)R: EMS induced nonfeeder-type revertant of strain lac_{M1}^-.
M1(U4)R: UV induced nonfeeder-type revertant of strain lac_{M1}^-.
M1(AP3)R: AP induced feeder-type revertant of strain lac_{M1}^-.

† Nonfeeder-type transductants. All other transductants were of the feeder type.

P1kc derived from each of the indicated donor strains was added to L-broth log phase cultures of each of the indicated recipient strains (about 2×10^8 bacteria per ml, containing 2.5×10^{-3}M $CaCl_2$, at a multiplicity of 3. After shaking at 37°C for 30 min, the bacteria were collected by centrifugation, washed, resuspended, diluted as necessary and plated on minimal-lactose medium. The indicated number of transductants is corrected for spontaneous lac+ reversion as was determined in control platings.

phage derived from nonfeeder-type revertants of an EMS induced mutant (Table 4). It was therefore concluded that the reversion was due to an unlinked suppressor-type mutation.

The deletion W 1485–1 does not give recombinants with many of the *lac*$^-$ point mutants and it must therefore "cover" these mutant sites. The deletion can therefore be used to determine whether reversions of these *lac*$^-$ mutants are within the *lac* region; in order to obtain lac$^+$ transductants in crosses between revertants of strains such as lac_{M1}^- or lac_G^- and W 1485–1, both the primary mutation and its reversional alteration must be linked closely enough to permit their joint transduction. Phage prepared on either lac_{M1}^- or its nonfeeder type revertants did not give any lac$^+$ transductants of W 1485–1. P1 derived from a feeder-type revertant of lac_{M1}^- gave many wild-type-like lactose utilizing transductants of W 1485–1 (Table 4). Transducing phage derived from three nonfeeder-type revertants of lac_G^- or from lac_G^- itself did not give lac$^+$ transductants of W 1485–1 in contrast to phage derived from three feeder-type revertants of lac_G^- or from a z^+ strain. These results support the hypothesis that the genetic alteration restoring β-galactosidase activity and lactose utilizing ability to (some) nonfeeder revertants is a suppressor mutation which is not closely linked to the *lac* locus.

The genetic site of one suppressor of lac_G^- was determined by conjugation analysis. Strain X148 (see above, MATERIALS AND METHODS) was used to construct a multiply marked F$^-$ strain bearing the lac_G^- mutation (Table 5). Strain 13, requiring proline, was isolated from X148 by UV irradiation and penicillin selection. Strain 13–6, *gal*$^+$ was isolated from a cross of strain 13 with P1 prepared on wild type. (The *gal*$^+$ gene was introduced to prevent possible alteration of the lactose utilizing properties of the strain.) The lac_G^- mutation was inserted into strain 13–6 by selection of a *pro*$^+$ transductant from a cross of 13–6 times P1 derived from lac_G^-. Joint transduction of *pro* and *lac* has been demonstrated with four independently isolated *pro*$^-$ derivatives of X148. The new strain, 13–6–3, bears the lac_G^- mutation since: (a) its *lac*$^-$ to lac$^+$ reversion pattern is characteristic of lac_G^- rather than of lac_2^-, (b) it does not give *lac*$^+$ recombinants in crosses with lac_G^- but does recombine with lac_2^- to give *lac*$^+$ and (c) suppressed *lac*$^-$ transductants can be obtained from crosses of P1 derived from nonfeeder-type lac_G^- revertants with 13–6–3 and are not obtained from crosses with 13–6. In addition lac$^+$ revertants of 13–6–3 are constitutive for galactosidase as are revertants of lac_G^- in contrast to lac$^+$ revertants of X148 which are inducible.

TABLE 5

Construction of strain K4: F$^-$thr$^-$leu$^-$pro$^-$$lac_G^-$ pur$^-$gal$^+$try$^-$met$^-$str^r

Parent strain X148 (CURTISS 1962): F$^-$ *leu*$^-$*lac*$^-$(y^+ z_2^- o^+ i^+) *pur*$^-$*gal*$^-$*try*$^-$*str*^{r}
(1) UV, penicillin selection: *pro*$^-$, strain 13
(2) strain 13 × P1kc (3.300): *gal*$^+$ P1^s, strain 13–6
(3) strain 13–6 × P1kc (lac_G^- *pro*$^+$): *pro*$^+$ lac_G^- P1^s, strain 13–6–3
(4) UV, penicillin selection (3 times): F$^-$ *thr*$^-$*leu*$^-$*pro*$^-$$lac_G^-$*pur*$^-$*gal*$^+$*try*$^-$, *met*$^-$*str*^{r}, strain K4

Each of the transductants selected in the construction of strain 13–6–3 was stable and P1 sensitive. *thr*$^-$, *pro*$^-$ and *met*$^-$ markers were added to 13–6–3 by successive application of the UV-penicillin selection technique giving strain K4 (Table 5). Strain K4 serves as a recipient for joint *pro*$^+$ *lac*$^+$ transduction in crosses with P1 derived from a *pro*$^+$ *lac*$^+$ strain.

An EMS induced nonfeeder revertant of *lac*$_G^-$, strain G (E13)R, presumed to be a suppressed *lac*$^-$ mutant, *lac*$_G^-$ *su-lac*-E13, on the basis of its allele specificity (Table 4) was mated with K4, and lactose utilizing *str*^{r} recombinants were selected on minimal-lactose medium containing streptomycin and supplemented with the auxotrophic growth requirements. Many small-colony, lactose utilizing, recombinants were obtained; 206 of these recombinants were purified by two successive single colony isolates on the primary selection medium. The genetic constitution of the recombinants with respect to the unselected markers (*thr*, *leu*, *pro*, *pur*, *try* and *met*) was determined by replica plating. One hundred percent of the suppressed *lac*$^-$ *str*^{r} recombinants also inherited the *pro*$^+$ allele of the male parent, 96 percent the *pur*$^+$ allele, 29 percent *try*$^+$, 14 percent *thr*$^+$ *leu*$^+$ and 1 percent *met*$^+$.

In order for any recombinant type to grow on the selection medium; it must inherit the *su-lac* gene from the male parent. The relative location of the suppressor should be indicated by the fraction of suppressed *lac*$^-$ recombinants which have also inherited one of the nonselected markers. The results indicate that the suppressor is more closely linked to *pro* and *pur* than to *thr*, *leu*, *try*, or *met*. This conclusion is supported by the results of a similar cross. Strain *lac*$_G^-$ *su-lac*-E13 was mated with strain K4 and aliquots of the diluted mating mixture were plated on media with either glucose or lactose as carbon source and made selective for each of the five different prototrophic recombinant types (Table 6). The numbers of each determined on selective glucose medium indicates the relative frequency at which the different wild-type alleles are inherited by the recipient. In order for a recombinant to grow on the selective lactose medium, both the *su-lac* gene and one of the prototrophic alleles of the male parent must be inherited. The results again indicate that the *su-lac*-E13 gene is more closely linked to *pro* and *pur* than to *thr*, *leu*, *try* or *met*.

TABLE 6

Linkage of su-lac-*E13 to the markers* thr$^+$ leu$^+$, pro$^+$, pur$^+$, try$^+$, *and* met$^+$

Selected prototrophic marker	[1] Number of prototrophic *su-lac*-E13 *str*^{r} recombinants per ml (lactose as sole carbon source)	[2] Number of prototrophic *str*^{r} recombinants per ml (glucose as carbon source)	Ratio 1/2
thr$^+$ *leu*$^+$	1,315	12,250	0.11
pro$^+$	23,250	16,300	1.4
pur$^+$	17,000	27,000	0.63
try$^+$	6,375	36,625	0.17
met$^+$	60	1,575	0.03

F$^+$ strain *lac*$_G^-$ *su-lac*-E13 (*str*^{s}) was mated with F$^-$ strain K4 *lac*$_G^-$ (*str*^{r}) for 120 min and aliquots of the diluted mating mixture were plated on minimal streptomycin agar, selective for the indicated prototrophic markers, and containing either [1]lactose or [2]glucose as carbon source.

Since both *pro* and *pur* are closely linked to *lac* it seemed likely that this suppressor was linked to the *lac* region. Cotransduction of *pro* and *lac* can be obtained in four different pro^- lac^- strains derived from X148 (Table 7). A peculiar feature of this transduction is that the apparent linkage of *pro* to *lac* is influenced by the primary selection medium. All the transductants selected for lac^+ are pro^+, but only about 40 percent of the transductants selected for pro^+ are lac^+ (Table 7). Transducing phage prepared on five different nonfeeder revertants lac_G^- were used as donors in a transduction cross with K4 (pro^- lac_G^-). Four of the five strains were classified as suppressed lac^- on the basis of the allele specificity of the reversional mutation (Table 4). The suppressed lac^- transductants obtained by selection on minimal-lactose medium supplemented so as to satisfy all the auxotrophic requirements of K4 were replicated to the same medium but lacking proline to determine whether pro^+ and a lac^- suppressor could be cotransduced. The *su-lac*-E13 gene is closely linked to *pro* as determined by recombination following conjugation (see above). In each cross 100 percent of the suppressed lac^- transductants were pro^+ (Table 8). The five independently isolated suppressors must therefore map close to *pro*, since only a small portion of a bacterial genome can be incorporated into, and transduced by, a P1 phage.

β-galactosidases produced by suppressed mutants: Suppressed lac_G^- mutants produced galactosidases which differ qualitatively from the wild-type enzyme.

TABLE 7

Cotransduction of pro *and* lac *by P1kc derived from strain 3.300*

(1) lac^+ transductants selected on lactose agar:

Recipient strain*	Percent pro^+	Number tested
(a) 13–6 (pro^- z_2^-)	100	10,000
(b) 156–5 (pro^- z_2^-)	100	422
(c) K4 (pro^- lac_G^-)	100	267
(d) 158–1 (pro^- z_2^-)	100	130

(2) pro^+ transductants selected on EMB-lactose-succinate agar lacking proline:

Recipient strain*	Percent lac^+ or *lac*-variegated	Number screened
(a) 13–6 (pro^- z_2^-)	35	435
(b) 156–5 (pro^- z_2^-)	42	298
(c) K4 (pro^- lac_G^-)	45	304
(d) 158–1 (pro^- z_2^-)	39	193

(3) pro^+ transductants selected on glucose agar lacking proline:

Recipient strain*	Percent lac^+	Number tested
(a) 13–6 (pro^- z_2^-)	43	581
(b) 156–5 (pro^- z_2^-)	38	321
(c) K4 (pro^- lac_G^-)	41	242
(d) 158–1 (pro^- z_2^-)	45	146

* Recipient strains 13–6, 156–5, K4 and 158–1 are four independently isolated proline requiring derivatives of strain X148.

(1) lac^+ transductants were selected on lactose agar containing all of the growth factors required by the recipient strains. The selected lac^+ transductants were then replicated to minimal-lactose medium lacking proline to determine the percent pro^+ among the lac^+ recombinants. (2) pro^+ transductants were selected on minimal-EMB-lactose-succinate agar and the percent lac^+ or *lac*-variegated was determined visually. (3) pro^+ transductants were selected on glucose (0.5 percent) agar containing all of the growth factors required by the recipient strains, but lacking proline. The selected pro^+ transductants were then replicated to minimal-lactose agar lacking proline to determine the percent lac^+.

At least ten transductants from each cross were purified through two successive single-colony isolates and then tested on suitable indicator medium to confirm nutritional characteristics or lactose fermenting ability.

TABLE 8

Cotransduction of suppressors of lac_G^- *and* pro^+ *by P1kc derived from suppressed* lac_G^- *mutants*

Origin of lysate	Percent *pro*+ among suppressed *lac*- transductants	Number tested
lac_G^- *su-lac*-E1	100	363
lac_G^- *su-lac*-E4	100	391
lac_G^- *su-lac*-E5	100	361
lac_G^- *su-lac*-E6	100	440
lac_G^- *su-lac*-E13	100	419
3.300 (wild type)	100	498

Phage derived from each of the indicated strains was crossed with strain K4 (*pro*- lac_G^-). The suppressed lac_G^- transductants obtained on lactose medium supplemented with threonine, leucine, proline, adenine, tryptophan and methionine were replicated to this same medium lacking proline.

Strain lac_G^- *su-lac*-E1 produces an enzyme which is more heat stable than that of the wild type (Figure 4). The enzyme produced by lac_G^- *su-lac*-E13 is rapidly inactivated in the 0.1M Tris-borate-EDTA buffer, pH 9, used for electrophoresis in contrast to the wild-type ($i^- z^+$) enzyme and to the enzymes produced by three

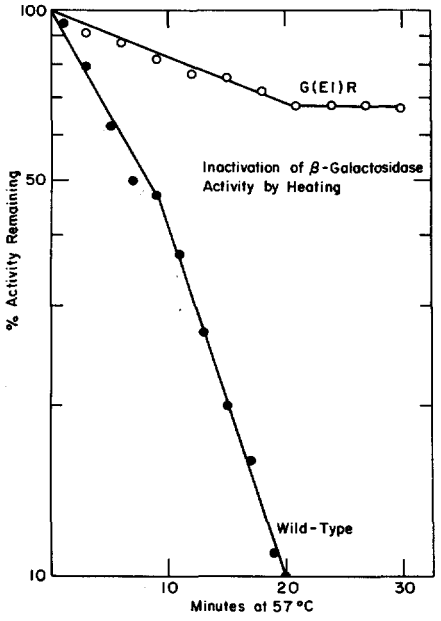

FIGURE 4.—Inactivation of β-galactosidase activity by heat. Sonic extracts of strain 3.300 ($i^- z^+$) and strain G(E1)R (an EMS induced nonfeeder-type revertant of strain lac_G^-) were partially purified (20 to 30-fold increase in specific β-galactosidase activity) by the method of KAMEYAMA and NOVELLI (1962). The preparations were diluted 100-fold in Tris-buffer, pH 7.2, (KAMEYAMA and NOVELLI 1962) and placed in a water bath at 57°C. Aliquots were removed at intervals and diluted tenfold into buffer of the same composition at 0°C. Samples of the wild preparation were diluted another tenfold before assay. β-galactosidase activity was assayed with o-nitrophenyl-β-D-galactoside (LEDERBERG 1950).

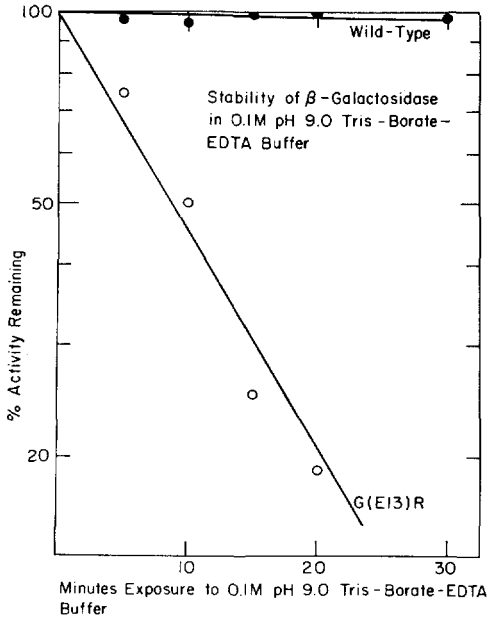

FIGURE 5.—Stability of β-galactosidase in 0.1 M Tris-Borate-EDTA buffer, pH 9.0. Partially purified extracts of strains 3.300(i^-z^+) and G(E13)R, an EMS induced nonfeeder type revertant of strain lac_G^-, were diluted at 0°C tenfold into the buffer employed for electrophoresis. The buffer contains per liter; 12.1g Tris(hydroxymethyl)aminomethane, 0.54g disodium ethylenediaminetetraacetate·$2H_2O$ and 0.76g boric acid. Aliquots were removed after varying periods of time and diluted 100-fold into the standard buffer (KAMAYAMA and NOVELLI 1962) at 0°C. β-galactosidase activity was determined with o-nitrophenyl-β-D-galactoside (LEDERBERG 1950).

other suppressed mutants (Figure 5). Figure 6 shows a gel stained with aniline blue-black after electrophoresis and containing extracts of the following strains: 3.300, W 1485–1, lac_{M1}^-, lac_G^- *su-lac*-E1 and lac_G^- *su-lac*-E4. The protein band most probably corresponding to β-galactosidase can be deduced from the genotype of the strains used. The deduction was supported by comparison of the pattern with that of gels incubated with o-nitrophenyl-β-D-galactoside. In other experiments, it was shown that the enzymes of four suppressed mutants differed slightly from each other and from the wild type in electrophoretic mobility, but the resolution of the method was not good. The gel stained with aniline blue-black showed that a dense band present in extracts of the wild-type strain was lacking in the three lac^- mutants (Figure 6); a new band of greatly altered mobility was present in extracts of the deletion mutant W 1485–1.

Two protein bands were restored in extracts of two suppressed mutants (Figure 6). The slower moving of the two was enzymatically active but was less mobile than the wild-type enzyme. Two enzymatically active bands were resolved when mixtures of wild-type and lac_G^- *su-lac*-E4 extracts were subjected to electrophoresis. Such results indicate that these closely linked suppressors function by restoring an active enzyme which differs from that produced by the wild type.

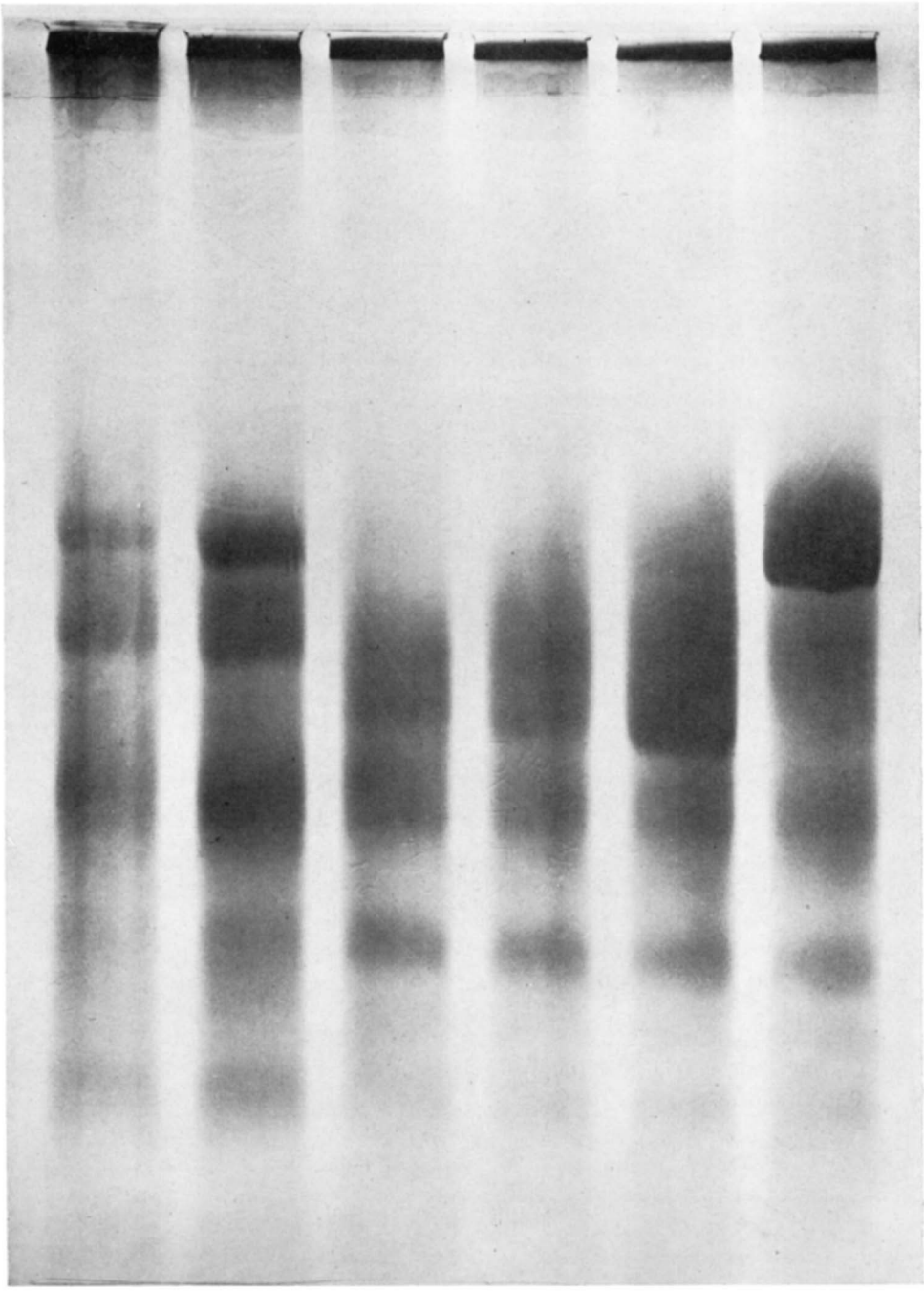

FIGURE 6.—Vertical acrylamide gel electrophoresis of partially purified extracts of *lac*$^-$ mutants. Twenty-μl samples (8mg protein per ml) of partially purified extracts of the following strains were applied, from right to left: 3.300 (i^- z^+), W1485–1 *lac* deletion mutant, $lac_{M1}{}^-$, $lac_G{}^-$, $lac_G{}^-$ *su-lac*-E1 and $lac_G{}^-$ *su-lac*-E4. The gel was stained with aniline blue black for the localization of protein. The origin, is indicated by dense bands representing protein immobile in the field.

The β-galactosidases of two feeder-type revertants of lac_G^- were indistinguishable in mobility or physical properties from the wild-type enzyme.

DISCUSSION

Mutagen specificity: EMS induced mutants have characteristic reversion patterns differing from those typical of UV induced mutants. If it is assumed that some of the wild type-like feeder revertants obtained from *lac*⁻ mutants are true back mutants (HENNING and YANOFSKY 1962), it is possible to offer a logical explanation of the observed mutagen specificity.

Base analog inducible mutants are the result of transitions (FREESE 1959c; CHAMPE and BENZER 1962). Since AP induced feeder-type revertants of EMS induced mutants, EMS must induce transitions. However, since EMS induced mutants are not efficiently induced to give wild type-like revertants by EMS it can be concluded that EMS preferentially induces one particular transition.

UV does not induce many revertants of EMS induced mutants, and the few that are obtained are not phenotypically wild-type. Therefore UV does not induce true reversion of transitions induced in the first instance by EMS. UV did induce many wild type-like revertants of the nine UV induced mutants tested. Aminopurine did not induce a significant frequency of wild type-like revertants of the four UV mutants tested. Although EMS induced many revertants of UV induced mutants most of the revertants were not phenotypically wild-type. If EMS induces transitions, UV induced mutants must represent nontransition mutations.

EMS reacts mainly with guanine residues in the DNA but the mechanism by which mutations are induced is not completely known. BAUTZ and FREESE (1960) have proposed that ethylating agents induce mutations as a consequence of the depurination of alkyl guanine from DNA. Their hypothesis predicts that both transition and non-transition mutations should be obtained after ethylation and E. B. FREESE (1961) has reported that about one third of the mutants of bacteriophage T4 induced by ethyl ethanesulfonate were of the non-transition type. Treatment of phage at low pH and high temperature also yielded mutants with characteristics similar to those induced by ethylation (*loc. cit.*). This treatment is presumed to induce mutation by depurination mechanisms (FREESE 1959a).

The major reaction product of nucleic acids with monofunctional alkylating agents both *in vitro* and *in vivo* is a 7-N-alkylguanine, and LAWLEY and BROOKES (1961) have proposed that the alkylguanine has altered pairing properties while still attached to the nucleic acid permitting pairing with thymine as well as with cytosine. Alkylating agents would therefore be expected to induce G:C to A:T transitions as a consequence of pairing errors. Some reaction does occur between adenine and ethylating agents *in vitro* (PAL 1962), and KRIEG (1963) has proposed that EMS can induce both G:C to A:T and A:T to G:C transitions but that the frequency of the former transition is likely to exceed that of the latter by two orders of magnitude. In the experiments reported here, 28 EMS induced *lac*⁻ mutants were not efficiently induced to revert to a wild-type phenotype by EMS although in some cases a small number of wild type-like revertants were obtained

against a background of many nonfeeder-type mutants. This specificity is most easily interpreted on the basis of KRIEG's modification of the hypothesis of LAWLEY and BROOKES.

Genetics of the revertants: On the basis of the allele specificity in transduction crosses of the nonfeeder-type revertants it can be concluded that the reversion is due to an unlinked suppressor mutation. However, when suppressed *lac*$^-$ mutants were crossed in transduction or conjugation experiments with strains bearing a suppressible *lac*$^-$ allele, the suppressors were found to map close to the *pro lac* region. The suppressors map closer to *pro* than to *lac*, since they are co-transduced with *pro* and not with *lac*.

All of the *pro* mutations studied here are co-transduced with *lac* at high frequency. Experiments performed by A. J. PITTARD (personal communication) demonstrate that the *pro*$^-$ mutation of strain 13 differs from another *pro*$^-$ mutation carried by AB1157 (DEWITT and ADELBERG 1962) in that it is transferred by Hfr strain P4X–6 as a terminal marker linked to *lac* and sex factor, and in cross-feeding tests strain 13 excretes a compound which supports the growth of AB1157. The *pro*$^-$ mutation of AB1157 is not co-transduced with *lac* and is transferred by strain P4X–6 as an early marker. It is likely that all *pro*$^-$ mutations which can be transduced jointly with *lac* are of the type carried by strain 13.

E. M. LEDERBERG (1952) reported on revertants of *lac*$^-$ which utilized lactose more slowly than the wild-type strain and which were genetically unstable, reverting to *lac*$^-$ at high frequency. The reversion of one such strain was attributed to a suppressor mutation linked to the locus determining resistance to T6 bacteriophage which, in turn, maps close to the *lac* region. A suppressor mutation which is both closely linked to the primary mutation and also allele-specific in transduction crosses has been observed in Salmonella (SMITH-KEARY 1960).

Two revertant strains of lac_G^- give galactosidases qualitatively different in physical properties both from the wild-type enzyme and from each other (Figures 4, 5). This implies that the suppressor mutations result in structural changes in the enzyme. The data imply that the original lac_G^- mutation was located in the *z* region even though the strain was found to have decreased permease activity and might therefore have been classified as an o^o type mutation. However, it is possible that the attempted distinction between *o* and *z* loci is not necessary since recent experiments (JACOB and MONOD 1961b; PARDEE and BECKWIRTH 1962) demonstrate that the *o* locus can provide both regulatory and structural information.

SUMMARY

Twenty-three ethyl methanesulfonate induced and nine ultraviolet light induced *lac*$^-$ mutants with greatly reduced levels of β-galactosidase activity were isolated. EMS mutants were induced to revert to a wild type-like phenotype by aminopurine (AP) but not by UV or EMS. EMS induces transitions, with one class of mutation, probably G:C to A:T being preferentially induced. UV induced mutants are induced to revert to a wild type-like phenotype by UV but not by

EMS or AP. UV therefore appears to induce mostly nontransition type mutations.

"Feeder" revertants of *lac*⁻ mutants behave like the wild type in transduction tests, in their β-galactosidase activity and in the electrophoretic behavior of the revertant enzyme: "Nonfeeder" revertants are all more or less genetically unstable, produce less β-galactosidase and are allele specific in transduction crosses. They therefore appear to be suppressor-type reversions. The β-galactosidases synthesized by "nonfeeder" revertants differ qualitatively from the wild-type enzyme.

When a suppressed *lac*⁻ mutant is crossed with a strain bearing the suppressible *lac*⁻ allele, the suppressor mutation is found to map close to the *pro lac* region of the chromosome. One of the suppressors studied was more closely linked to *pro* and *pur* than to *try*, *thr*, *leu*, or *met*. Five different *lac*⁻ suppressors were jointly transduced with *pro*, indicating linkage with the *pro lac* region.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the advice and guidance of DR. BERNARD S. STRAUSS and his help in the preparation of this manuscript. I am indebted to DR. ROY CURTISS for many helpful discussions and for the provision of bacterial strains, and to DR. JOHN L. HUBBY for his advice in the use of the electrophoresis apparatus.

LITERATURE CITED

BAUTZ, E., and E. FREESE, 1960 On the mutagenic effect of alkylating agents. Proc. Natl. Acad. Sci. U.S. **46**: 1585–1594.

CHAMPE, S. P., and S. BENZER, 1962 Reversal of mutant phenotype by 5-fluorouracil: an approach to nucleotide sequences in messenger-RNA. Proc. Natl. Acad. Sci. U.S. **48**: 532–546.

CURTISS, R., 1962 Studies on the genetics of *Escherichia coli*. Ph.D. dissertation. The University of Chicago.

DEWITT, S. K., and E. A. ADELBERG, 1962 The occurrence of a genetic transposition in a strain of *Escherichia coli*. Genetics **47**: 577–585.

FRANKLIN, N. C., and S. E. LURIA, 1961 Transduction by bacteriophage P1 and the properties of the *lac* genetic region in *E. coli* and *S. dysenteriae*. Virology **15**: 299–311.

FREESE, E., 1959a On the molecular explanation of spontaneous and induced mutations. Brookhaven Symp. Biol. **12**: 63–75.

1959b The difference between spontaneous and base analogue induced mutations of phage T4. Proc. Natl. Acad. Sci. U.S. **45**: 622–633.

1959c The specific mutagenic effect of base analogues on phage T4. J. Mol. Biol. **1**: 87–105.

FREESE, E. B., 1961 Transition and transversions induced by depurinating agents. Proc. Natl. Acad. Sci. U.S. **47**: 540–545.

GAREN, A., and O. H. SIDDIQI, 1962 Suppression of mutations in the alkaline phosphatase structural gene of *E. coli*. Proc. Natl. Acad. Sci. U.S. **48**: 1121–1127.

HENNING, U., and C. YANOFSKY, 1962 Amino acid replacements associated with reversion and recombination within the *A* gene. Proc. Natl. Acad. Sci. U.S. **48**: 1497–1504.

HORIUCHI, T., J. TOMIZAWA, and A. NOVICK, 1962 Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochim. Biophys. Acta **55**: 152–163.

JACOB, F., and J. MONOD, 1961a Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**: 318–356.

1961b On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol. **26**: 193–211.

KAMEYAMA, T., and G. D. NOVELLI, 1962 Effect of ultraviolet inactivation and photoreactivation on the induced synthesis of β-galactosidase by *Escherichia coli*. Arch. Biochem. Biophys. **97**: 529–537.

KRIEG, D. R., 1963 Ethyl methanesulfonate-induced reversion of bacteriophage T4*r*II mutants. Genetics **48**: 561–580.

LAWLEY, P. D., and P. BROOKES, 1961 Acidic dissociation of 7:9-dialkylguanines and its possible relation to mutagenic properties of alkylating agents. Nature **192**: 1081–1082.

LEDERBERG, E. M., 1952 Allelic relationships and reverse mutation in *E. coli*. Genetics **37**: 469–483.

LEDERBERG, J., 1950 The beta-D-galactosidase of *Escherichia coli*, strain K-12. J. Bacteriol. **60**: 381–392.

LENNOX, E. S., 1955 Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**: 190–206.

LURIA, S. E., J. N. ADAMS, and R. C. TING, 1960 Transduction of lactose-utilizing ability among strains of *E. coli* and *S. dysenteriae* and the properties of the transducing phage particles. Virology **12**: 348–390.

NOLL, H., and J. ORLANDO, 1961 An ultrasensitive assay of β-galactosidase with C^{14}-labeled o-nitrophenyl-β-D-galactoside. Analyt. Biochem. **2**: 205–215.

PAL, B. C., 1962 Studies on the alkylation of purines and pyrimidines. Biochem. **1**: 558–563.

PARDEE, A. B., and J. R. BECKWIRTH, 1962 Genetic determination of constitutive enzyme levels. Biochim. Biophys. Acta **60**: 452–454.

SMITH-KEARY, P. F., 1960 A suppressor of leucineless in *Salmonella typhimurium*. Heredity **14**: 61–71.

SUTHERLAND, E. W., C. F. CORI, R. HAYNES, and N. S. OLSEN, 1949 Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. **180**: 825–837.

VOGEL, H. J., and D. M. BONNER, 1956 A convenient growth medium for *E. coli* and some other microorganisms (Medium E). Microbial Genetics Bull. **13**: 43–44.

WESTERGAARD, M., 1957 Chemical mutagenesis in relation to the concept of the gene. Experientia **13**: 224–234.