REVERTANT INSTABILITY IN *ESCHERICHIA COLI*.

I. MUTAGEN AND ALLELE SPECIFICITY¹

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A variety of suppressor genes in *Escherichia coli* and *Salmonella typhimurium* have been shown to be genetically unstable (LEDERBERG 1952; DAWSON and SMITH-KEARY 1963; HILL 1963; SCHWARTZ 1963, 1964, 1965; BRODY and YANOF-SKY 1965).

A suppressor of *lac* *z*₁₈, USRev 1 is extremely unstable since after growth to stationary phase in minimal-lactose medium nearly all the colonies screened on eosin methylene blue lactose agar are lactose-variegated or Lac⁻ (SCHWARTZ 1963). The USRev 1 mutation cannot be mapped by its time of entry, since in Hfr × F⁻ crosses it is transferred at low frequency very soon after mating and is not linked to chromosomal genes (SCHWARTZ 1965). Stable Lac⁺ clones appear in cultures of *lac* *z*₁₈ USRev 1 at low frequency. This alteration is reversible. The stable reversions have characteristics of chromosomal genes—linkage to chromosomal markers, high frequency transfer and definite time of entry (SCHWARTZ 1965). Two independently isolated stable Lac⁺ derivatives of the unstable strain were mapped at different chromosomal sites (SCHWARTZ 1965). Since this suppressor can be stabilized by integration at different chromosomal sites, and can also undergo deintegration, becoming unstable and resembling the original unstable strain, it has characteristics ascribed to episomes (JACOB and WOLLMAN 1961).

Unstable suppressors may represent genetic alterations induced by episomic or controlling elements rather than the simple base-pair changes proposed for mutation (FREESE 1959); we therefore examined various *lac* alleles for their capacity to produce unstable revertants after treatment with mutagens having different specificities. To distinguish between the possible effects of allele *vs.* strain specificity, particular *lac* alleles were transduced into different strains and induced revertants were tested for stability. If unstable reversion resulted from detachment of an episome from a suppressor locus (HILL 1963), we would then expect instability to be dependent upon the strain studied rather than the nature of the primary *lac* allele. In addition, the allelic identity of independently isolated unstable suppressors was investigated.

MATERIALS AND METHODS

The bacterial strains employed are all derivatives of *E. coli* K-12 (Table 1). Media, culture

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TABLE 1

*Strains**

Strain	Origin or derivation	Reference
3300 <i>lac</i> ₁₈	Spontaneous <i>lac</i> z from 3300	SCHWARTZ 1963
3300 <i>lac</i> z _A	UV induced <i>lac</i> z from 3300	SCHWARTZ 1963
3300 <i>lac</i> z _E	UV induced <i>lac</i> z from 3300	SCHWARTZ 1963
3300 <i>lac</i> z _G	UV induced <i>lac</i> z from 3300	SCHWARTZ 1963
3300 <i>lac</i> z _{M1}	EMS induced <i>lac</i> z from 3300	SCHWARTZ 1963
Y2155	<i>lac</i> ₁₈ , multiple auxotroph	SCHWARTZ 1965
AB707 <i>lac</i> ₁₈	AB707 F ⁻ protroph	
AB259 <i>lac</i> ₁₈	AB259, Hfr Hayes 3000	SCHWARTZ 1965
3000 <i>lac</i> z ₆	EMS induced <i>lac</i> z from AB259	SCHWARTZ 1964; 1965
W1895 <i>lac</i> ₁₈	Hfr Cavalli	SCHWARTZ 1965
AB674 <i>lac</i> ₁₈	Hfr Reeves R1	SCHWARTZ 1965
AB264 <i>lac</i> ₁₈	Wild type F ⁺ K-12	
3000 <i>lac</i> ₁₈ USRev 1	EMS induced nonfeeder-type revertant of 3300 <i>lac</i> ₁₈	SCHWARTZ 1963; 1965
AB2271 <i>lac</i> ₁₈ <i>ilvD132</i>	Recombinant derived from AB2271	
AB2291 <i>lac</i> ₁₈ <i>ilv188 metE46</i>	Recombinant derived from AB2291	
AB2271 <i>lac</i> ₁₈ <i>ilvD132</i>	EMS induced <i>ile</i> from 3300 <i>lac</i> ₁₈	

* Nomenclature and gene symbols follow DEMEREC, ADELBERG, CLARK and HARTMAN (1966) and TAYLOR and THOMAN (1964). Mutations of the *lac* operon are designated according to the conventions of JACOB and MONOD (1961). *lac*⁺ and *lac* indicate wild-type and mutant genotypes; Lac⁺ and Lac⁻ represent wild-type and mutant phenotypes. *ilv* and *metE* result in requirements for isoleucine-valine and methionine or vitamin B₁₂. EMS- ethyl methanesulfonate, UV- ultra-violet light.

Strains with AB-prefixes are derivatives of strains in the collection maintained by DR. E. A. ADELBERG.

methods and genetic techniques have been described previously (SCHWARTZ 1963, 1964, 1965). In exception to usual procedures, all glassware was both washed and rinsed in tap water only. Media were prepared in tap water demineralized by a Barnstead standard (0802) cartridge. These procedures produced no obvious effects, and the results and general features (growth rates, viable counts, mutant and recombinant frequencies) of previous experiments (SCHWARTZ 1965) were reproducible. To test stability, lactose-utilizing revertants were picked from minimal-lactose selection plates and streaked onto similar medium. A single well isolated colony was then re-streaked as above. The second colony-isolate was then streaked on eosin methylene blue (EMB) lactose agar. Each revertant scored as unstable produced at least 10% lactose-variegated or lactose-negative colonies on EMB indicator medium (see Figure 1).

RESULTS

"Feeder"-type Lac⁺ revertant colonies of *lac* strains give rise to halos of *lac* satellite background growth on minimal-lactose agar and have genetic properties expected of true back-mutants. On the other hand, "nonfeeder"-type revertants fail to support satellite growth, and prove to be suppressed mutants (SCHWARTZ 1963). An unstable suppressor of *lac*₁₈, USRev 1, behaving like an episome (SCHWARTZ 1965) was isolated as a nonfeeder-type revertant of strain 3300 *lac*₁₈, after treatment with ethyl methanesulfonate (EMS) (SCHWARTZ 1963).

In order to determine if this *lac*₁₈ strain still produces unstable revertants, it was treated with EMS and the induced revertants were characterized and tested for stability. (This strain has been transferred bimonthly and occasionally reiso-

TABLE 2

Stability of EMS induced revertants of strain 3300 lac₁₈

Colony type	Frequency	Number of revertants tested	Number of unstable revertants
Large feeder	.37	102	0
Large nonfeeder and medium	.52	116	56
Small	.07	97	0
Minute	.04	4*	0

* See text for explanation.

About 80% of the bacteria survived treatment. It is likely that revertants studied were mutagen induced rather than spontaneous. EMS treated cultures produced about 165 revertant colonies per plate compared to 4 in control platings.

lated from single colonies during a 4 year period since its unstable revertant was originally observed.) Revertant colonies induced by EMS were classified into four types, occurring with the frequencies shown in Table 2. Large nonfeeder and medium sized colonies are scored as a single class, since those which are of medium size after 48 hours invariably appear as large nonfeeders 24 hours later. On the contrary, small and minute-type revertants retain their original phenotypes. Approximately 100 EMS-induced revertants of each phenotypic class (except the minute revertants), were purified and tested for stability on EMB lactose agar (Table 2). About one half of the large nonfeeder and medium sized revertant colonies tested were unstable. In contrast, none out of either 102 large feeder-type colonies or 97 small-colony types were unstable. Seventeen of 21 minute-colony revertants grew too slowly for characterization even on EMB lactose agar (where the capacity to utilize lactose is not required for rapid growth). After 72 hours on EMB medium, colonies of these minute revertants were outgrown by Lac⁻ secondary reversions as a result of their selective advantage over the original Lac⁺ cells plated. The remaining four minute-colony revertants were stable Lac⁺.

A variety of unstable phenotypes was observed while testing revertant stability on EMB lactose agar. Each of these phenotypes was maintained after repeated subculture on minimal lactose medium and served as a marker for recognizing unstable revertants of independent origin. For example, whereas about 15% of USRev 30 colonies are Lac-variegated, 100% of USRev 84 colonies appear variegated (Figure 1). USRev 18 appears heterogeneous, producing both Lac-variegated and entire Lac⁺ colonies (Figure 1). (All of ten entire-Lac⁺ clones of strain USRev 18 that were tested produced both Lac-variegated and Lac⁺ entire colonies in a proportion similar to that obtained with the original unstable strain). In addition to the degree of sectoring, phenotype distinction between USRev 30 and USRev 84 can be made on the basis that Lac⁻ sectors of the former arise from peripheral regions of colonies, while those of the latter arise from the centers of colonies (Figure 1). Differences in the intensity of the Lac⁺ color reaction occurred as well. With the use of these criteria, seven different unstable phenotypes were observed. Phenotypes characteristic of USRev 1 (SCHWARTZ 1963, 1965) and USRev 84 occurred most frequently.

Mutagenic specificity: The type of mutational alteration responsible for un-

FIGURE 1.—Colonies of unstable nonfeeder-type revertants on EMB-lactose agar. EMS induced nonfeeder-type revertants of strain 3300 *lac*₁₈ were grown to stationary phase in minimal-lactose broth, diluted and plated. The plates were incubated for 24 hours at 37°C before photography. Top left—USRev 84, top right—USRev 30, bottom left—USRev 58, bottom right—USRev 18.

stable reversions (e.g. transition, nontransition, base-pair deletion) was determined by treating strain 3300 *lac*₁₈ with 2-aminopurine (AP), methyl methane-sulfonate (MMS) and ultraviolet light (UV) to induce Lac⁺ revertants. Since only the EMS-induced large nonfeeder colonies and medium sized revertant colonies were unstable (Table 2), only these revertant phenotypes were tested for stability. Spontaneous revertants of similar phenotype were also isolated and tested. Many large nonfeeder and medium sized revertant colonies were obtained

TABLE 3

*Phenotype of spontaneous and induced revertants of strain 3300 lac*₁₈

Origin	Number of revertants scored	Fraction of revertant colonies classified as:			
		Large feeder	Large nonfeeder and medium size	Small	Minute
Spontaneous	207	.70	.16	.07	.08
2-Aminopurine	1232	.47	.26	.19	.07
Methyl methanesulfonate	891	.12	.63	.20	.05
Ultraviolet light	1517	.73	.16	.08	.03

MMS-induced revertants: Cells were treated with 0.1 M MMS (Eastman Organic Chemicals) in the same manner used for EMS. Plates were scored after 72 hours at 37°C. The following Lac⁺ reversion frequencies were obtained per 10⁸ viable *lac*₁₈ plated: Spontaneous, 2; AP, 62; MMS, 79; UV, 95.

TABLE 4

Stability of spontaneous and induced revertants of strain 3300 lac_{IS}

Origin	Number of nonfeeder revertants tested	Number of unstable revertants
Spontaneous	125	0
2-Aminopurine	112	0
Methyl methanesulfonate	108	1*
Ultraviolet light	102	1*

* Unstable phenotype similar to that described for USRev 1 (SCHWARTZ 1963; 1965).

from both the mutagen treated and nontreated cultures (Table 3). None of the AP-induced or spontaneous revertants tested were unstable; one MMS-induced and one UV-induced revertant were unstable (Table 4). It can therefore be concluded that of the agents tested, EMS is most efficient for the induction of unstable-type revertants of *lac_{IS}*.

Allele specificity: Experiments were next performed to determine whether EMS could induce unstable reversions of *lac* alleles other than *lac_{IS}*. The *lac* mutations *z_A*, *z_E*, *z_G*, *z_{M1}* (each in the genetic background of the *lac⁺* parent of *lac_{IS}*, strain 3300) and *z₆* (derived from strain 3000), were chosen for study. It was of special significance to test this *lac z₆* derivative of 3000 because, unlike 19 other *lac* point mutations (including *z_A*, *z_E*, *z_G* and *z_{M1}*), it was suppressed by USRev 1 to yield an unstable Lac⁺ phenotype (SCHWARTZ 1965). EMS-induced nonfeeder revertants from *lac z* mutants A, E, G, M1 and 6 were tested for instability. No unstable revertants were found among 108 to 189 revertants tested for each of the five strains. It may, therefore, be concluded that revertant instability is a characteristic peculiar to the allele, *lac_{IS}*.

Strain nonspecificity: Various strains carrying *lac_{IS}* and *lac z₆* were constructed and then treated with EMS to determine whether nonfeeder-type Lac-unstable revertants were induced. Strains bearing *lac_{IS}* were prepared by phage P1-mediated cotransduction of *proC⁺ lac_{IS}* into *proC lac⁺* derivatives of strains 13-6, AB707, AB259, W1895, AB674 and AB264. The strains constructed all carry *lac z_{IS}* and are referred to as Y21S5, AB707 *lac_{IS}*, AB259 *lac_{IS}*, W1895 *lac_{IS}*, AB674 *lac_{IS}* and AB264 *lac_{IS}*, respectively (Table 1). By the same method a *lac z₆* transductant of a *proC* derivative of 3300 *lac_{IS}* was prepared. As expected if the induction of unstable revertants was allele specific but strain nonspecific, many unstable revertants of *lac_{IS}* derivatives and no unstable revertants of the 3300 *lac z₆* strain were found (Table 5).

Cotransduction of USRev 1, 18, 30 and 84 with ilv or ile: The genetic determinant of instability, USRev 1, is not linked to *lac_{IS}* or transferred with the bacterial chromosome during conjugation. This has been demonstrated from the results of transduction and conjugation experiments reported previously (SCHWARTZ 1965). Furthermore, these experiments demonstrated that USRev 1 was carried on an extrachromosomal element capable of the classical transitions characteristic of episomes.

TABLE 5

Revertant instability—strain nonspecificity

Strain	Number of EMS induced nonfeeder revertants tested	Number of unstable revertants
AB707 <i>lac</i> ₁₈	24	9
Y21S5	24	15
AB259 <i>lac</i> ₁₈	24	12
W1895 <i>lac</i> ₁₈	24	14
AB674 <i>lac</i> ₁₈	24	13
AB264 <i>lac</i> ₁₈	24	11
3300 <i>lac</i> <i>z</i> ₆	212	0

It was therefore surprising to isolate one lactose-utilizing transductant selected from a cross of phage P1 grown on *lac*₁₈ USRev 1 with strain AB2271 *lac*₁₈ *ilvD132* as recipient, that had inherited unselected *ilvD132*⁺. The possibility of non-specific suppression rather than cotransduction was ruled out by the finding that USRev 1 was also cotransduced with *ilv188*, two different *ile* alleles, or *metE*. It is therefore apparent that USRev 1 is linked to these markers, which in turn are closely linked in the bacterial chromosome (TAYLOR and THOMAN 1964).

USRevs 18, 30 and 84 are phenotypically distinguishable from each other and from USRev 1. Cotransduction with *ile* would test whether these represent different alterations of the same gene. Each of these three unstable reversions and USRev 1 are cotransduced with *ile*⁺ at high frequency (Table 6). The nature and relation of the linkage of USRev 1 to these markers and to instability will be reported elsewhere (SCHWARTZ 1967).

Acridine orange curability and instability: Acridine orange (AO) cures F⁺ males of sex factor (HIROTA 1960) and enhances segregation of USRev 1 from *lac*₁₈ (SCHWARTZ 1965). To determine if revertant instability is a persistent characteristic peculiar to *lac*₁₈, AB674 *lac*₁₈ was treated with EMS and an USRev 1-type strain isolated. This unstable revertant was treated by growing 18 hours in nutrient broth containing 50 µg/ml acridine orange at 37°C in the dark. This treatment produced a 12-fold increase over the control in the frequency of Lac⁻

TABLE 6

Cotransduction of USRev and ile

Selected marker	Number of Lac ⁺ transductants tested	Percent Ile ⁺
USRev 1	220	70
USRev 18	88	68
USRev 30	88	72
USRev 84	88	74

ile⁺ *lac*₁₈ P1 donors: USRev 1, USRev 18, USRev 30, USRev 84.

ile *lac*₁₈ recipient: 3300 *ile*26.

Several Lac⁺ and Lac⁺ Ile⁺ recombinants from each cross gave Lac-unstable phenotypes on EMB lactose agar after purification.

segregants. (The higher frequency of Lac⁻ segregants found in the AO treated culture does not appear to represent a chance event. Acridine treatment has induced increases of Lac⁻ segregation frequency in all of 57 similar experiments involving five USRev 1 carrying strains.) One Lac⁻ segregant from the AO and control cultures was then treated with EMS and plated for lactose-utilizing revertants. The frequency of unstable revertants amongst 20 nonfeeder-type colonies of each strain was very similar, .50 (AO) and .45 (control). This cycle of AO treatment, isolation of Lac⁻ segregants, followed by EMS treatment to induce reversion to Lac⁺, was repeated once again. There was no difference in the ability of these *lac*₁₈ segregants, as compared to the original 3300 *lac*₁₈ strain, to yield unstable nonfeeder-type revertants. It is therefore concluded that *lac*₁₈ yields unstable reversions, unaffected by spontaneous or acridine orange induced loss of the USRev 1 characteristic.

DISCUSSION

Ethyl methanesulfonate is the most efficient of the agents (MMS, AP, UV, EMS) tested for the induction of unstable Lac⁺ reversions of *lac* *z*₁₈. EMS presumably induces transitions and transversions as a consequence of depurination of 7-ethylguanine from deoxyribonucleic acid (FREESE 1963), and G-C to A-T transitions as a consequence of errors in base pairing by 7-alkylguanine (LAWLEY and BROOKES 1961). It is unlikely that transitions are responsible for unstable reversions, since none out of 125 AP induced nonfeeder revertants were unstable. One of 108 MMS induced, and one of 102 UV induced nonfeeder revertants are unstable. It is improbable these two unstable revertants are spontaneous since (1) none of 125 spontaneous revertants was unstable, and (2) both MMS and UV increased the Lac⁺ reversion frequency 80-fold above the spontaneous frequency. MMS probably induces transitions as a consequence of pairing errors by 7-methylguanine (FREESE 1963). UV is presumed to induce about equal numbers of transition and small addition and deletion *rII* mutations of T4 (DRAKE 1963). The most likely cause of unstable reversions therefore appears to be depurination, since this reaction is most effectively induced by ethylating agents (BAUTZ and FREESE 1960).

Recent explanations and ideas on the induction of unstable suppressors (HILL 1963; DAWSON and SMITH-KEARY 1963) have involved episome transitions rather than base pair transitions and transversions used to explain mutation (FREESE 1963). The former explanations are either related to or derived from the mutator control system in maize (McCLINTOCK 1961). In this system a structural gene is inactivated by the attachment of an "operator" or controlling element. Under the influence of a second element, the "mutator" or "regulator," the operator turns off or moves away from the structural gene thereby restoring its wild-type activity. (The terms, operator and regulator, as used here and in the discussion that follows should be distinguished from the operator and regulator of JACOB and MONOD (1961).) The suppressor instability studied here can also be explained in these terms. If *lac* *z*₁₈ is a structural-gene-operator complex, USRev 1, 18, 30 and 84 represent alterations of the mutator which affect *lac*-operator

affinity. As expected, *lac z₁₈* is transferable to other strains from which unstable revertants can be isolated as a result of the action of EMS on the regulator. Since *lac z₆* is reactivated by USRev 1, it should also be a operator-inactivated structural gene. On the other hand, *z_A*, *z_E*, *z_G* and *z_{M1}* are not turned on by USRev 1 and may not be operator-inactivated genes. None of the EMS induced nonfeeder Lac⁺ revertants of these four mutants are unstable. A result which is not easily explained is the failure to find EMS induced unstable revertants of *lac z₆* (out of 401 that have been tested). This might be explained by the existence of "hot spots" for stable *lac z₆*⁺ reversions.

It may be possible to isolate induced or spontaneous stable alterations of the mutator locus which are linked to *ilv* and *metE* as are USRevs 1, 18, 30 and 84. An alteration responsible for the conversion of USRev 1 to one stable form did actually map in the *ilv metE* region (SCHWARTZ 1965). An interesting and perhaps related finding is the observation that stable "mutator induced streptomycin resistance" maps somewhere between *str* (the genes for ordinary streptomycin resistance) and *thr* (GUNDERSEN 1963), which places it near the *ilv metE* region.

We have shown that unstable reversions of *lac z₁₈* are produced by an episome-like agent (SCHWARTZ 1965) and have presented evidence for linkage of USRev 1 (and three other unstable reversions) to the *ile ilv metE* region of the chromosome. These results are not incompatible, since it is also known that these unstable revertants (and unstable derivatives obtained by transduction) are partially diploid *lac z₁₈ ile ilv metE/ ile ilv metE* USRev 1 (SCHWARTZ 1967).

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SUMMARY

Ethyl methanesulfonate induces unstable Lac⁺ revertants of one of six *lac z* mutants. One half of EMS-induced "nonfeeder-type" Lac⁺ revertants of *z₁₈* are extremely unstable. No spontaneous or 2-aminopurine induced, and less than 1% of ultraviolet light and methyl methanesulfonate induced Lac⁺ revertants of *z₁₈*, are unstable. Revertant instability is probably a consequence of depurination caused by ethylation. The capacity to yield unstable Lac⁺ reversions is a characteristic of *z₁₈*, transferred with it into strains constructed by transduction. —Seven different unstable Lac⁺ phenotypes are obtained. Four unstable reversions tested are cotransduced with *ile* by phage P1.

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