REVERSION INSTABILITY OF AN EXTREME POLAR MUTANT OF THE GALACTOSE OPERON¹

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THE mutation *gal-3* of the galactose operon of *Escherichia coli* (MORSE, LEDERBERG and LEDERBERG 1956) has been shown to be a mutant of the extreme polar type (KALCKAR, KURAHASHI and JORDAN 1959; SOFER 1961), making the cells deficient for the three enzymes of the operon. The extreme polar mutants (formerly called O⁰) were thought to be changes of the operator region but are now believed to be nonsense mutations in the structural gene proximal to the operator region (BECKWITH 1964). The *gal-3* mutation has been mapped genetically (ADLER and KAISER 1963) and located in haploid cells either adjacent to or in the gene for the uridine diphosphogalactose epimerase. It is the purpose here to report an unusual aspect of *gal-3*, its ability to give rise to reversions which yield Gal⁻ with high frequency, such that they resemble the heterogenotes produced by lambda transduction. Mutants with similar properties have been noted in the *lac* operon of *E. coli* (COOK and LEDERBERG 1962; SCHWARTZ 1963, 1964, 1965). Preliminary reports of this study have appeared previously (MORSE 1963a, 1965).

MATERIALS AND METHODS

The mutant studied, W2236, is a derivative of W892, a culture previously described (MORSE, LEDERBERG and LEDERBERG 1956), and has the phenotype F⁻ Thr⁻ Leu⁻ Thi⁻ Lac⁻ Gal⁻ (λ). The galactose mutation of this strain occurred spontaneously and was isolated on Eosin Methylene Blue (EMB) agar.

Liquid cultures were grown either in Difco penassay broth or tryptone broth. Scoring for ability to utilize carbohydrates and the isolation of revertants was made on either cholate neutral red medium (C medium, MORSE and ALIRE 1958) or upon EMB medium containing the appropriate carbohydrates. Quantitative measurement of the low reversion rates was accomplished using platings on minimal galactose agar, and for the high rates of instability C medium with galactose was employed.

Extracts for the assay of cellular enzymes were prepared as described previously (MORSE 1963c) using freezing and thawing in the presence of lysozyme. Enzyme assays were performed according to described procedures or modifications: galactokinase was assayed by measuring acid formation with bromthymol blue (SOLS and CRANE 1954), or in the buffer system with cresol red (DARROW and COLOWICK 1962) and relating the rates of acid production to that by wild-type cells. The kinase assays were read in a Beckman DB spectrophotometer using a blank without galactose. The results were recorded on a Photovolt Model 43 recorder. Uridyl phosphogalactose

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transferase was assayed according to the procedure of KALCKAR *et al.* (1959), which employs coupling to glucose-6-phosphate dehydrogenase. The transferase reaction was started by adding galactose-1-phosphate. The progress of the reaction, reduction of TPN, was recorded in the DB using air as a blank. Uridine diphosphogalactose epimerase was assayed according to the one-step procedure of KALCKAR *et al.* (1959), employing uridine diphosphoglucose dehydrogenase from yeast. The reduction of DPN was recorded with the above equipment using air as a blank. Preliminary experiments had indicated that spontaneous reduction of TPN, DPN in the transferase and epimerase assays was so slow as to not require correction. Reagents for the above assays were obtained from the Sigma Chemical Company. Enzyme induction was accomplished, where necessary, by growth in the presence of galactose, using fresh growth in the presence of 0.5% galactose for 2.5 hours as a standard procedure.

RESULTS

The mutant *gal-3* reverts to the Gal⁺ phenotype at a rate of 3.7×10^{-8} /bacterium/division, which is a rate about 20 times higher than that of any galactose mutant previously studied (MORSE 1963b). The revertants are largely of two phenotypes on C medium with galactose: (1) raised colonies with white borders, intermediate in red color; (2) flat-spreading colonies with deep red coloration and no white border to the colonies. The first of these phenotypes, called R1, is produced from the Gal⁻ parent at a probability about 1/25 that of the second phenotype (Table 1). Many revertants of the R1 phenotype are characterized by being unstable and yielding Gal⁻ at a high rate (Table 2). This rate is about 10^4 -fold the rate from Gal⁻ to Gal⁺ and results in cultures containing 1 to 2% Gal⁻. The instability appears an inherent characteristic of all the cells in the

TABLE 1
Reversion of Gal₃⁻ to Gal⁺

Culture	Number of cells plated $\times 10^8$	Total revertants	R1 phenotype	Other phenotypes
1	4.4	437	89	348
2	4.8	123	39	84
3	4.8	192	36	156
4	3.2	669	123	546
5	7.2	297	114	183
6	3.2	91	23	51
7	6.0	120	25	95
8	4.4	47	8	39
9	3.6	247	105	142
10	4.8	30	5	25
	8.6 mean	153 median	38 median	119 median

A protophic derivative of W2236 was divided into ten independent subclones, and, starting with small inocula, minimal glucose cultures were grown. Measured samples, assayed for cell numbers by separate platings on nutrient agar of these subclones were plated on minimal galactose agar to score for Gal⁺ revertants. The revertants obtained after 5 days growth at 37°C were of two kinds, small colonies and large colonies. Colonies from the platings of each subclone were restreaked on C medium with galactose to classify the revertants as to whether they were the R1 phenotype or its alternatives (see text). 0.57 of the large colonies were R1; 0.78 of the small were the alternative. From this distribution in the C-medium streakings the revertants were partitioned into the two classes. The mutation rates to the two phenotypes were estimated from the median of the distribution (LEA and COULSON 1949) and the mean number of bacterial divisions. The rates were: R1 phenotype, 1.2×10^{-8} /bacterium/division; other phenotypes, 3.0×10^{-8} /bacterium/division. Total reversion, 3.7×10^{-8} /bacterium/division. Instability appears to be restricted to the R1 phenotype, but not all R1-appearing colonies are unstable.

TABLE 2

Instability of three independent revertants of W2236

Culture	Revertant 1		Revertant 2		Revertant 3	
	Gal ⁺	Gal ⁻	Gal ⁺	Gal ⁻	Gal ⁺	Gal ⁻
1	7158	21	2267	39	4284	24
2	5910	51	1745	59	3447	30
3	4860	60	1537	51	2955	24
4	3774	24	1719	51	3297	18
5	7722	21	1726	58	3150	30
6	6213	21	1887	53	4746	42
7	5301	33	1319	41	5637	18
8	4536	54	1829	70	3810	12
9	6498	72	1920	46	4761	6
10	4155	78	2052	42	2205	24
11		..	2029	54	2391	27
12		..	2015	75	3393	36
Mean divisions	5656	..	1890	..	3702	..
Median		42		52		24
Mean number						
mutations	11.4 ± 2.9		13.5 ± 2.4		7.3 ± 1.5	
Mutation rate	2.0 × 10 ⁻³		7.1 × 10 ⁻³		2.0 × 10 ⁻³	

Cultures of 0.3 ml volume were started from small inocula containing no Gal⁻ and incubated about 3 hr. Samples of 0.05 ml were then plated on cholate galactose agar. After 24 hr at 37°C the number of Gal⁺ and Gal⁻ on these plates were counted and the content of the small cultures was calculated. The mean number of mutational events was computed using the method of the median, and the mutation rates (probability per bacterium per division of the change from Gal⁺ to Gal⁻) were obtained by dividing mean number of divisions.

unstable revertant culture, for in a series of restreakings of such a culture, all the colonies, either in the first restreaking, or via their progeny in a subsequent restreaking, were found capable of yielding Gal⁻. The incidence of stable Gal⁺ cells in the revertant clones must be the order of one in 1000 or less.

The Gal⁻ issuing from the unstable revertants repeat the phenomenon shown by the *gal-3* culture of yielding, at approximately the same frequency, Gal⁺ revertants which yield Gal⁻ at a high rate. These Gal⁻ are similar to *gal-3* also, and the entire process has been repeated through 12 cycles of reversion followed by subsequent instability of the Gal⁺ produced. At each step the unstable Gal⁺ are produced at a rate of about 10⁻⁸ per bacterium per division, and the Gal⁻ are produced at a rate of about 10⁻³ per bacterium per division.

Biochemical and genetic analyses: The parent *gal-3* and a sequence of six cultures (3 Gal⁺ revertants and 3 Gal⁻ issuing from them) were examined for their ability to produce galactokinase, transferase and epimerase with and without prior induction with galactose (Table 3). All of the Gal⁺ (unstable) produced all three enzymes constitutively, but at levels less than that of the fully induced Gal⁺ reference culture. The synthesis in these strains has therefore been derepressed. The Gal⁻ produced by the unstable revertants appear biochemically similar to the W2236 parent culture in that they produce little of each of the three enzymes in the galactose pathway.

The mutations to the Gal⁺ phenotype, because of the constitutive production

TABLE 3

Enzymatic activity in extracts of the Gal₃⁻ culture and six cultures derived sequentially from it

Cultures	Gal phenotype	Kinase*		Transferase†		Epimerase‡	
		noninduced	induced	noninduced	induced	noninduced	induced
W3110	+	...	2.8, 2.1, 2.8, 3.8	...	4.6, 4.7, 3.2	..	6.9, 10.2
W2236	—	0.22	<2.26	0.35	0.0	1.1	0.27
R2	+	2.3, 0.88	1.2, 1.9	2.0, 1.9	3.2, 4.9	14.7	15.7
SR2	—	...	0.0	0.24	0.13, 0.08	1.0	0.0, 0.67
RSR2	+	0.89	1.2	2.6	2.8	5.0	7.6
SRSR2	—	...	0.08	...	0.01	..	0.5
RSRSR2	+	2.0, 1.4	2.5	3.8, 2.6	5.1	4.1	12.7
SRSRSR2	—	0.48	0.08	0.17	0.01	0.65	0.43

* The results of the kinase assay are given in optical density units per hr per mg protein.

† μ moles TPN reduced per hr per mg protein.‡ μ moles DPN reduced per hr per mg protein.Results for W3110 are given for reference. The cultures shown were derived in a sequential manner from W2236; 2236 $\rightarrow$ R2 $\rightarrow$ SR2 $\rightarrow$ RSR2 $\rightarrow$ SRSR2 $\rightarrow$ RSRSR2 $\rightarrow$ SRSRSR2, by reverting the Gal⁻ on cholate galactose medium and by selecting Gal⁻ issuing from the unstable revertants.

of enzymes, could be the consequence of either a change at the site of repressor action (operator region) or in the repressor itself. The first of these changes could result in a lack of sensitivity to repression, the second a repressor which fails to repress. The genetic location of the above changes would be at quite different places, the operator region being a portion of the *gal* operon and the regulator gene for this operon being located near the gene affecting lysine synthesis, some 38 minutes (clockwise) from the site of its activity (BUTIN 1963a, b; TAYLOR and THOMAN 1964).

The site of the changes to constitutive production of enzyme shown by the Gal⁺ cultures in Table 2 can be localized by making transductions from them

TABLE 4

Enzyme activity in extracts of transductants formed between the unstable revertants and a k⁻ recipient culture

Donor	Transductant											
	Kinase						Transferase					
	1	2	3	4	5	6	1	2	3	4	5	6
R1*	2.6	2.9	0.17	1.7	3.0	..	1.7	1.6	0.4	1.4	2.7	..
R2*	3.1	2.0	3.6	3.4	2.6	..	3.6	2.0	4.3	3.5	3.0	..
R3*	3.5	2.3	0.2	2.6	1.7	..	4.2	2.0	..	2.4	2.3	..
RSR2 ⁺	2.3	0.66	1.7	..	..	..	..	0.24	0.85	..	..	..
RSRSR2 ⁺	0.88	7.1	..	3.3	2.9	4.4	1.0	17.5	4.3	5.4	2.8	5.0

* R1, R2, R3 are independent reversioners derived from W2236.

† The first (RSR2) and second (RSRSR2) reversioners derived sequential from R2.

Extracts of transductants derived via low frequency transducing lambda preparations of the revertants were analyzed for galactokinase and transferase. The cultures were not induced. The values given indicate constitutive formation of kinase and transferase by the heterogenotes in most cases. The exceptions are not explained. Many heterogenotes formed between RSR2 and the k⁻ recipient were not phenotypically Gal⁺, but Gal⁻ papillating. The basis for this not understood. Transductions from a repressed Gal⁺ culture to the k⁻ recipient did not produce kinase (<0.00 optical density units/hr/mg protein) or transferase (0.05 μ moles TPN reduced per hour per mg protein) constitutively.

with phage lambda which is restricted to transducing genes in the vicinity of the *gal* region. Thus any changes to derepressed enzyme production as a consequence of a change in the regulator gene will not be transducible via lambda. Transductions were made via lambda from the cultures to a kinaseless transferaseless (*k⁻t⁻*) recipient synthesized by recombination, and the resulting heterogenotes were examined for ability to produce kinase, transferase and epimerase constitutively. The results, shown in Table 4, indicate that in each case transductants were formed which produced the three enzymes constitutively. Transductants formed by transducing from a repressed wild-type culture of the same *k⁻t⁻* recipient did not produce kinase or transferase constitutively. Thus the site of the change to derepressed production of the enzymes of the *gal* operon is in the material transduced by lambda and presumed to be in the operator region.

The *Gal⁻* mutants shown in Table 3 produce little of the three enzymes necessary for galactose utilization, and they resemble the polar mutant parent of the series. Genetic identity with the parent, and among themselves, was tested via low frequency transduction with the phage lambda. The results indicated that there are no differences between the polar mutants, as detected by the formation of wild-type transductants. However, it should be noted that in low frequency transduction there are only the order of several thousand transducing particles per ml, and that the revertibility of the polarity mutants is rather high, such that the formation of a small number of wild-type recombinants would not be detected. The identity of polarity mutants will be examined in future experiments in a sequence derived in an Hfr strain.

Growth studies: The unstable *lac* suppressor mutation studied by SCHWARTZ (1964) produced a profound change in the cell, and resulted in a change in the generation time of the cells from 35 to 63 minutes in nutrient broth. The changes being dealt with here are not of such a profound nature since the growth curves of the parent and of some derivatives (4 *Gal⁺* and 4 *Gal⁻*, derived sequentially) in nutrient broth indicate identical generation times.

Affect of acridine: Acridines have been shown in some instances to cause the loss from the cell of episomes, for example, of the F factor from *E. coli* (HIROTA 1960). In the case of *lac* instability studied by SCHWARTZ, because of the action of acridine in accelerating the formation of stable colonies, it was concluded that the suppressor was located on an episome. In the case of the unstable *Gal⁺* revertants, there appears to be no action by 9-amino acridine on instability (Table 5), and thus it appears that in this case the reversion is not associated with an episome acted upon by acridines. In addition, mixtures of the unstable *F⁻ Gal⁺* with an *F⁻ Gal⁻ Str^r* failed to show transfer of the *Gal⁺* character to the *F⁻ Gal⁻ Str^r* tester strain.

Relationship to amber mutation suppressors: Many of the threonine, leucine, thiamine requiring strains of *E. coli* K-12 contain suppressors which permit the growth of amber mutants of T4 (EPSTEIN *et al.* 1963). A sequence of seven *Gal⁺* revertants and seven *Gal⁻* derived from them, and the parental W2236 were used to assay amber mutants 17 and 130. CR63 was used as an assay control. All the sequence of cultures permitted the growth of the amber mutants, with efficiencies

TABLE 5

The lack of effect of acridine on the production of Gal⁻ by an unstable revertant

Culture	Without acridine		With acridine	
	Gal ⁺	Gal ⁻	Gal ⁺	Gal ⁻
1	2267	39	...	46
2	1745	59	589	25
3	1537	51	623	17
4	1719	51	636	33
5	1726	58	437	22
6	1887	53	667	18
7	1319	41	857	34
8	1829	46	718	17
9	1920	46	764	17
10	2052	42	838	28
11	2029	54	881	41
12	2015	75	...	...
Mean divisions	1890		820	
Median number Gal ⁻		52		25
Mean number mutations		13.5 ± 2.4		7.0 ± 1.8
Mutation rate		7.1 × 10 ⁻³		8.5 × 10 ⁻³

The technic was the same as in Table 2 except that 9-amino acridine ($2 \times 10^{-4}M$ final concentration) was added to one set of cultures. The acridine slowed the growth rate such that 0.43 as many divisions occurred in its presence as in its absence. No effect was observed in other experiments at lower concentrations of acridine.

approximating 0.3 to 0.9 that of CR63 and with no apparent significant variation between cultures. It is concluded that the changes in the galactose operon being observed are not related to the presence or absence of the amber suppressors. Likewise several T4rII mutants were assayed on lambda lysogenic derivatives of some of the Gal⁺ and Gal⁻ and all failed to grow.

Miscellaneous: The parental strain was *F⁻ Lac⁻ Thr⁻ Leu⁻ Thi⁻* (λ). In this study it was necessary to make a number of derivative strains involving changes in these characters. During the derivation of these strains the following was noted: (1) curing the parental strain of lambda did not change the ability of the strain to form unstable revertants or the rate of instability of these revertants; (2) lysogeny with lambda of an unstable revertant derived in a lambda-sensitive strain did not affect its stability; (3) addition of the F factor did not change the ability of the strain to give rise to unstable revertants; (4) an Hfr strain isolated from an unstable revertant was as unstable as F⁻ or F⁺ unstable revertants; (5) reversion of the *lac*, *thr*, *leu* markers was not accelerated in a strain unstable for the Gal⁺ character; (6) an unstable revertant did not produce auxotrophic mutants at a high rate.

DISCUSSION

The results presented show that the phenotype of a polar mutant of the galactose operon can be reverted in at least two ways, one of which results in an unstable Gal⁺ phenotype. Analysis of the unstable Gal⁺ shows that they produce

enzymes of the galactose operon constitutively, and that the site of the reversion is near the *gal* region.

The nature of the reversion to Gal⁺ remains to be demonstrated, but several models can be proposed and excluded. For example, a model dependent upon the existence of a mutator gene in the culture (TREFFERS, SPINELLI and BELSER 1954; SKAAR 1956) can be made. Against this proposal is the stability of the other genetic characteristics of the strain (*thr*, *leu*, *lac*, λ , and the parental *gal-3* itself) which is the same order as other strains in the laboratory, and there is no evidence indicating that this strain mutates to auxotrophy at a higher rate than any other strain. An explanation based on an "error-prone" DNA replicating enzyme is also excluded by the genetic stability of this strain.

The presence or absence of other factors, considered episomal, in the cell can also be excluded as an explanation. The presence of the bacteriophage lambda is not relevant since the basic phenomenon, i.e., instability of a class of revertants, can be repeated in *gal-3* derivatives which have been "cured" of lambda lysogeny. Thus, an explanation based on transductions to self by phage lambda, an idea which prompted the study of this extreme polar mutant initially, cannot be employed here.

The fact that the F factor can be varied also eliminates another explanation. It has been proposed (NAGATA 1963) and that in F⁻ cells, chromosomal replication commences at sites chosen at random, where in Hfr cells, replication commences at only one site, that of the integrated F factor. If the *gal* region of the unstable culture contains some rearrangement such that the replication enzyme, commencing once in a thousand times at the *gal* region, is caused to make errors, then instability might be apparent. Instability would disappear if a fixed site of replication was introduced into the cell. This can be accomplished, according to NAGATA, by introducing an integrated F factor into the chromosome. An Hfr derivative which transfers methionine as an early marker was obtained in W2236, and the stability of its R1 revertants examined. These revertants were in no way different from R1 revertants of W2236 itself. Thus an explanation based on this model is not possible.

Instabilities associated with the presence of an episome, or an episome to which was attached a duplication, have been proposed as an explanation for phenomena similar in some respects to that shown by *gal-3* (DAWSON and SMITH-KEARY 1963; AMES, HARTMAN and JACOB 1963; SMITH-KEARY and DAWSON 1964; SCHWARTZ 1965). In at least one of these cases (SCHWARTZ 1965) acridine orange was found to affect stability. No effect of 9-amino acridine on the rate of production of Gal⁻ from the unstable Gal⁺ was noted in the present study.

The high frequency of instability (10^{-3} /bacterium/division) might be more easily explained on the basis of a recombinational event rather than mutation. A model based upon recombination between duplicated segments similar to the case of Bar eyes in *Drosophila* (STURTEVANT 1925) can be considered. The event that formed the *gal-3* mutation was a duplication of some material of the *gal* operon. The duplicated material was either mutant or the duplication itself produced the mutant phenotype. The unstable revertants represent change in one of

the duplicated segments causing reversion of phenotype. During growth of the cells with a single reverted segment there is recombination between segments to yield the parent mutant type with two nonaltered segments (*gal-3* phenotype) and a reciprocal recombinant type with two altered (reverted) segments.

The mutant type with two reverted segments might be more strongly wild in character than the R1 revertant. Such a model could explain the observations if it be argued that the non-R1 revertants represent the recombinant with two reverted segments. However, the formation of the parental *gal-3* phenotype in the unstable R1 revertants would always be accompanied by the formation of a stable non-R1 clone. The results on stability indicate that the frequency of occurrence of stable Gal⁺ in the unstable clones is less than 1-1000, an argument against this model. However, the model assumes equivalent viability for the recombinants, and if this not be the case then the difficulty can be circumvented. A corollary of this model is that the *gal-3* mutation itself is unstable and produces some other phenotype. In the absence of knowledge of this phenotype, it might be difficult to detect. However, there is some evidence for instability in W2236 (see below).

The presence of duplications may possibly be detected in experiments in progress which are measuring the cotransduction frequency of mutations on the outside of the *gal* region. The "stretching" between outside mutations introduced by the duplicated genetic material present might result in lowered cotransduction frequencies. In addition, if the basis for the instability is a recombinational event, introduction of the *rec⁻* gene (CLARK and MARGULIES 1965), which prevents recombination, into the unstable clone might cause a decrease in instability. It may be possible to test this hypothesis if a *rec⁻* gene can be introduced into W2236 or into one of the unstable cultures.

During the later stages of this study, W2236 and some of the reversions of W2236 were examined accidentally at 42.5°C. It appears that under these conditions mutation to and from isoleucine requirement at a rapid rate can be demonstrated in some of the cultures studied. These results suggest the presence of a suppressor or regulator gene for threonine deaminase in the vicinity of the *gal* operon. Independent results suggesting this have been presented (EGGERTSSON and ADELBERG 1965). The relationship of the threonine deaminase suppressor-gene locus to the phenomena described above remains to be clarified.

Note added in proof: HILL and ECHOLS (1966) also note instability of *gal-3* revertants.

SUMMARY

One class of revertants of an extreme polar mutant of the galactose operon is characterized by instability. The change to this revertant phenotype occurs at a frequency of 1.2×10^{-9} per bacterium per division, while the reverse change (Gal⁺ → Gal⁻) occurs at about a frequency of 10^{-3} . The unstable reversions produce galactokinase, transferase and epimerase constitutively. The site of the reversion to Gal⁺ is located in the region of the *E. coli* chromosome transduced by the phage lambda, since heterogenotes formed by transducing from the revertants produce the enzymes constitutively. Several models are considered in an attempt to explain the phenomena.

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