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Negative Control of the Galactose Operon in *E. coli*

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Summary. Non-inducible mutants have been isolated which synthesize the three galactose enzymes with the basal rate both in the absence and in the presence of inducers. These mutations are closely linked to the lysA gene, as are the constitutive mutations in the regulator gene first described by BUTTIN (1963).

The non-inducible mutants are Gal^- on EMB gal plates. Revertants to the Gal^+ phenotpye are constitutive. Heterozygotes have been prepared at the locus of the regulator gene (*galR*), and dominance studies involving the different alleles at this locus have been carried out. The non-inducible mutations are dominant over the wildtype, and this in turn is dominant over constitutive mutations in the *galR* gene.

Starting from the non-inducible mutations, deletions have been isolated, which extend from the *galR* gene into the *lysA* gene. These are constitutive.

The behavior of the non-inducible mutations and of the deletions are strong arguments for negative control of the galactose operon.

The galactose operon in *E. coli* was among the first operons studied, and constitutive mutations at a regulator gene as well as at an operator locus were described by BUTTIN (1963a, b). Dominance studies at the regulator gene were briefly mentioned in a paper by ADHYA and ECHOLS (1966). These findings suggested that the galactose operon is controlled negatively. The recent discovery of positive control in inducible operons and the unexpected dominance relationships of alleles at the regulator locus of the arabinose operon (SHEPPARD and ENGLESBERG, 1967) has cast some doubt on the interpretation of such dominance studies. It was therefore thought to be desirable to look for additional evidence for the type of control exerted over the galactose operon. In the present paper we describe the isolation and characterization of non-inducible mutations in the regulator gene of the galactose operon, and of deletions deleting at least part of this regulator gene.

Materials and Methods

Media were as described by LENGELER (1966) and by PFEIFER, and OELLERMANN (1967). Essentially glucose-free Galactose (Sigma) was used for M9gal[1] plates.

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[1] *Abbreviations used: gal* galactose, *glu* glucose, *LT* tryptone broth, *NB* nutrient broth, *M9* minimal medium, *EMB* eosine-methylene blue, *TTC* triphenyl-tetrazolium chloride, *lys* lysine, *thy* thymine, *thi* thiamine, *thr* threonine, *leu* leucine, *pyr* pyrimidine, *str* streptomycin, *udpg* uridine diphosphoglucose, *TMG* thio-methyl-β-D-galactopyranoside.

Genetic symbols: Genotypes are used with small, phenotypes with capital letters in the abbreviations. The superscript$^+$ over a genetic symbol indicates the wildtype, no superscript indicates the mutated allele, usually a requirement. $T6^r$ is resistance to phage T6, str^r is resistance to streptomycine, galR is the regulator gene for the galactose operon. F^+ indicates the presence of the sex factor.

Enzymes: epimerase E.C.5.1.3.2 UDPglucose-4-epimerase, transferase E.C.2.7.7.10. UTP: α-D-galactose-1-phosphate uridyl transferase, kinase E.C.2.7.1.6. ATP: D-galactose phosphotransferase.

Chemicals. The following substances were purchased from the companies mentioned in parenthesis: Thymine (Bayer, Leverkusen), diaminopimelic acid (Fluka AG, Basel), other amino acids (Mann Research Lab, New York, N.Y.), acridine orange (Chroma Gesellschaft, Stuttgart-Untertürkheim). Other chemicals were as described in SAEDLER and STARLINGER (1967a, b).

Bacteria and Phages. The strains used in this study are listed in Table 1. Other strains derived therefrom are described in the text.

Table 1. *Bacteria and phages*

Strain	Description	Source
W8	*E. coli K12* prototroph	J. WEIGLE
M28	*U95 udpg*, his (SHEDLOVSKY and BRENNER, 1963) carrying *F′8* (HIROTA and SNEATH, 1961)	J. SHAPIRO
Fi25	F^- *lys* derived from *Hfr H* by mutagenesis and selection	this study
L47	*E. coli K12*, F^-, *lys*, *thy*, $T6^r$. The strain is derived from *CR34* (GROSS and CARO, 1966), by mutagenesis and selection	W. VIELMETTER
$galR_2^-$	galactose-constitutive derivative of *Hfr H*	this study
3852	*E. coli C600*, *thr*, *leu*, *thi*, *pyr*, $T1^r$, str^r, carrying *F′15* (ISHIBASHI et al., 1964)	A. EISENSTARK
P1	*P1 kc* (LENNOX, 1955)	U. HENNING
f_r	male-specific RNA phage	H. HOFFMANN-BERLING

Curing of F^+ Strains with Acridine Orange. Cells were inoculated into NB adjusted to pH 7.6, containing 20 µg/ml acridine orange. The initial titer was 10^3/ml. The culture was incubated at 37° with aeration and grown to saturation. Single cells were isolated on solid media and tested for the absence of the *F* particle by plating the f_r phage on them. Failure to yield plaques was used as indication of the absence of the *F*-factor.

Mutagenesis with Nitrous Acid. Cells were grown overnight in LT, centrifuged and washed. Mutagenesis was carried out by incubation in 0.4 M acetate buffer, pH 4.6, containing nitrous acid at a final concentration of 0.025 M. The cell density during this incubation was around 10^9/ml, and about 10^{-3} of these survive. The incubation was terminated by 1:10 dilution into M9. The cells were then centrifuged and resuspended in M9 containing necessary supplements and glucose. The cells were incubated in this medium for a period of time differing in different experiments to allow the expression of the mutations. The cells were then plated on M9 plates with galactose as the carbon source and appropriate supplements.

Complementation Tests with Fgal Particles were as described by JORDAN, SAEDLER, and STARLINGER (1967).

Transduction Experiments with Phage P1 were as described by LENNOX (1955).

Reversion Tests to the Gal^+ Phenotype were as described by JORDAN, SAEDLER, and STARLINGER (1967).

Color Test for Constitutivity (modified after LIN et al., 1960). Cells to be tested were plated on black membrane filters with 2.5 ml of soft agar containing no nutrients. After solidification, the agar layer was overlaid with another layer of the same agar without cells. This prevents the development of colonies of different size which happens if some colonies grow on the surface and some within the soft agar layer. 5000 colonies can be plated in this way on one filter the size of a conventional Petri dish. (MESSER, and VIELMETTER, 1965).

The filters were incubated at 37° on M9glu plates with appropriate supplements for 24 hours. At this time the colonies had a diameter of between 0.5 and 1 mm. The filters were then transferred to plates without a carbon source and starved for 5 hours at 37°. After

starvation, the filters were overlaid on the same plates with 2 ml of soft agar containing 100 mg/ml D-galactose, 10 mg/ml TTC and 5 mg/ml of chloramphenicol. The plates were incubated for another 60 min at 37°. Constitutive colonies are bright red after this period while inducible colonies stay white. The color is stable for at least 24 hours at room temperature.

Enzyme Assays were as described by SAEDLER and STARLINGER (1967).

Results

1. Isolation of Non-inducible Mutations in the gal R Gene

Gal$^+$ *udpg* cells are sensitive to galactose, presumably because they cannot metabolize the galactose-1-phosphate synthesized by galactokinase. Any mutation which makes the cells unable to synthesize galactokinase makes the cell resistant to galactose. Most of these mutations occur in the gal operon. By making the cells diploid for this operon, these events are eliminated or greatly reduced. Under these circumstances, mutations outside the galactose operon, but making the cells unable to synthesize galactokinase, should be found preferentially among the galactose-resistant cells. Among these, non-inducible mutations of the *gal R* gene (*gal R*^{s} mutations) should be found, if they exist at all. The positions of the relevant genes are shown in Fig. 1.

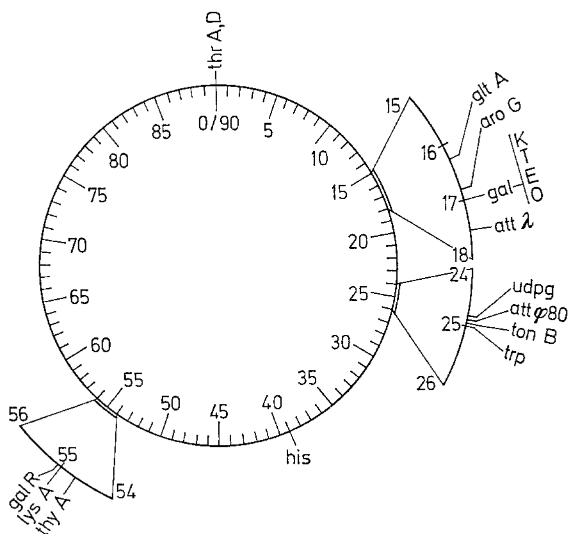

Fig. 1. Chromosome map of *E. coli*. The map has been abstracted from the extensive map described by TAYLOR and TROTTER (1967). The letters indicating single galactose genes designate operator (*O*), epimerase (*E*), transferase (*T*), and kinase (*K*). The attachment site of phage λ is designated att λ, the attachment site of phage ∅*80* is designated att ∅*80*. Other genes mentioned are *thrA*, *D* (threonine biosynthesis), *gltA* (citrate synthase), *aroG* (biosynthesis of aromatic compounds), *udpg* (UDPG pyrophosphorylase), *tonB* (resistance to phage Tl), *trp* (tryptophan biosynthesis), *his* (histidine biosynthesis), *thyA* (thymine biosynthesis), *lysA* (lysine biosynthesis), and *galR* (regulator gene of the galactose operon). The latter two genes have been placed in closer proximity than given by TAYLOR and TROTTER, as is indicated by our co-transduction experiments. The relative order of *lysA* and *galR* is not known with certainty

4×10^5 cells of strain *M28* were plated on EMB gal plates either directly or after uv irradiation to a survival of 10^{-3}. Dark revertants to *udpg*+ and large white colonies of galactose-resistant cells grew after incubation of the plates at 37° for 24 hour. The white colonies were picked and purified by restreaking. The presence of an intact *gal* operon on the *F8* particle carried by *M28* was verified by restreaking against known mutations in the *gal* operon. Two mutants, G_{18} and G_{59}, were finally found, which complemented all three galactose genes. Enzyme tests showed that they made all three galactose enzymes at low levels and were not inducible by D-fucose (Table 2).

Table 2. *Characteristics of mutants G18 and G59*

Strain	Phenotype on EMB gal	Complementation of e⁻, t⁻, and k⁻	Enzyme		
			Epimerase	Transferase	Kinase
M28	*Gal*⁻, galactose sensitive	+	100	100	100
G18	*Gal*⁻, galactose resistant	+	11	24	24
G59	*Gal*⁻, galactose resistant	+	19	12	16

Complementation was tested by cross-streaking against known *gal*-mutants on EMB gal and observing massive *Gal*+ growth after 24 hours.

Enzyme was measured in extracts prepared from cells grown in the presence of D-fucose, as described in *Methods*. The values observed with strain *M28* were set equal to 100.

2. *Linkage of the Isolated Mutations to lysA and thyA*

In order to test the properties of the isolated mutations, they had to be separated from the *udpg* mutation. This was accomplished by growing them on TTC gal plates. On these plates, red colonies could be detected after incubation for 48 hours at 37°. These were purified and restreaked on EMB gal plates where they were *Gal*⁻. While the original mutants did not revert to a *Gal*+ phenotype on EMG gal plates, these isolates reverted readily to the *Gal*+ phenotype. It was assumed that they had lost the mutation in the *udpg* gene by reversion.

P1 lysates were grown on these revertants and used to transduce strain *Fi25* to *Lys*+ phenotype. Transductants were picked and tested for their *Gal* phenotype. More than 95% of the *Lys*+ cells were *Gal*⁻ as can be seen from Table 3. This indicates a close linkage of our mutations to lysA, as was expected, if the mutation had occured in the *galR* gene described by BUTTIN (1963a, b). Co-transduction of *galR* with *thyA* is in the order of 50%.

Table 3. *Linkage of $galR^s$ mutations to lysA*

Pl lysate prepared on strain	Number of *Lys*+ colonies tested for Gal phenotype	Number of *Gal*⁻ observed	% cotransduction
$galR^s_{18}$ *lys*+	176	168	95
$galR^s_{59}$ *lys*+	39	36	93

P1-Lysates were prepared on udpg+ revertants of strains *G18* and *G59*, as described in the text. Strain Fi25 was used as recipient. Transduction was carried out as described in *Methods*. *Lys*+ colonies were picked and restreaked on EMBgal. These *lysA*-linked mutations are named $galR^s_{18}$ and $galR^s_{59}$ respectively.

3. *Reversion of the galR^s Mutation to the Gal$^+$ Phenotype*

Five cultures each of mutants *galR*$^s_{18}$ and *galR*$^s_{59}$ were grown and tested for reversion to *Gal*$^+$ phenotype as described in *Methods.* More than 100 revertants of both *galR*$^s_{18}$ and *galR*$^s_{59}$ were tested for constitutivity. The results of the transferase tests on 10 randomly selected revertants are shown in Table 4. They are all constitutive to varying degrees. Most of these mutations leading to *Gal*$^+$ phenotype are closely linked to *lysA*, and are therefore thought to have occured in the *galR* gene. Only around 10% are not linked to the *lysA* locus. These can be co-transduced with the galactose operon and are therefore believed to be mutations of the o^c-type (Fiethen and Starlinger, to be published).

Table 4
Constitutivity of Gal$^+$ revertants of gal R$^s_{18}$

Strain	Transferase in uninduced cells
Reversion	
1	44
2	74
3	72
4	16
5	73
6	68
7	65
8	98
9	75
10	58
H61	4
galR$^-_2$	100

Revertants to the *Gal*$^+$ phenotype were isolated from *galR*$^s_{18}$ on EMBgal plates. *H61* is a *galR*$^+$ wildtype strain, *galR*$^-_2$ is a constitutive mutant derived from it. All strains were grown in LT + 5×10^{-3}M TMG, which inhibits internal induction (Buttin, 1963a), and transferase was assayed as described in *Methods.* The value observed with *galR*$^-_2$ was arbitrarily set equal to 100.

4. *Dominance Studies at the galR Locus*

In order to study the dominance relationships of various *galR* alleles, heterozygotes were prepared. This was done with a reisolate of *F'15,* originally described by Ishibashi et al. (1964), and kindly given to us by Dr. Eisenstark. *F'15* carries both *lysA*$^+$ and *thyA*$^+$. In order to use the latter marker for the selection of heterozygotes, the *galR* alleles to be studied had to be transferred into a *thyA* background. This was done by growing *P1* lysates on the *galR* strains, and using these lysates for the transduction of strain L 47 (lysA thyA T6^r) to Lys$^+$ phenotype. Nearly all transductants carry the *galR* allele of the donor, due to the close linkage of *galR* and *lysA*, and about 50% of these are still *Thy*$^-$, since the *thyA* locus is only loosely linked to *lysA* and *galR*. *Thy*$^-$ cells were used for the production of heterozygotes, after the appropriate genotype at the *galR* locus had been verified by plating on EMB gal in the case of the *galR*^{s} mutants, and by using the color test for constitutivity in case of the *galR*$^-$ mutants.

The *galR* thyA strains were mated with the donor of *F'15,* and recombinants were selected on: M9glu+thi plates. The presumed heterozygotes were purified and tested for heterozygosity in the following way:

a) phage f_r was plated on them to show the male character of the strain.

b) *T6* resistance was tested and taken as evidence that the strain was derived from the recipient.

c) Each heterozygote was cured by acridine orange and six single cell isolates were tested for the absence of the *F* particle with phage f_r. All but one of the

f_r-resistant strains became, at the same time, *Thy*⁻. The latter was presumably a recombinant and was not further used.

d) The presence of the $galR^+$ allele in the heterozygotes of the type $galR^s/galR^+$ which were phenotypically Gal^- was shown by growing *P1* lysates on these strains and using them for the transduction of $galR^s_{18}$ $thyA^-$ to the Thy^+ phenotype. Due to the linkage of *thyA* and *galR*, about half of these transductants were found to be Gal^+.

In the case of $galR^s_{18}/galR^+$ this finding is proof of the presence of the $galR^+$ allele in the heterozygote. In case of $galR^s_{59}/galR^+$, recombination within the *galR* gene could lead to the appearance of $galR^+$ recombinants, but since Gal^+ cells were found in the same proportion among the Thy^+ transductants as in the case of $galR^s_{18}/galR^+$, this is believed to be highly unlikely.

e) The presence of the $galR^-$ allele in the $galR^-/galR^+$ heterozygotes was verified by using the color test for constitutivity on the heterozygotes and on cultures grown from these in the presence of acridine orange. The original heterozygotes were white, and red colonies were found only at a frequency of around 10^{-3}. After treatment with acridine orange, the frequency of red colonies was between 0.5 and 0.9. This is a strong indication of the curability of the inducible phenotype together with the *F* particle.

Table 5. *Dominance studies at the galR locus*

Strain	Phenotype on EMB gal plates	Epimerase		Transferase		Kinase	
		TMG	D-fucose	TMG	D-fucose	TMG	D-fucose
$galR^+$	+	5	76	4	190	3	107
$galR^-_2$	+	100	157	100	130	100	160
$galR^-_2/galR^+$	+	7	122	4	200	6	210
$galR^s_{18}$	—	6	12	8	20	5	16
$galR^s_{18}/galR^+$	—	4	12	6	18	1	11
$galR^s_{59}$	—	7	8	4	5	2	4
$galR^s_{59}/galR^+$	—	4	9	4	6	3	6
$galR^s_{59}R^-_4$	+	126	157	176	150	170	200
$galR^s_{59}R^-_4/galR^+$	+	8	96	3	153	4	162

The cells were grown in LT with either 10^{-3} M TMG or 10^{-3} M D-fucose. The cultures were assayed for heterozygosity, as described in the text, and aliquots of them were stored frozen for the enzyme assays, as described in *Methods*. In the case of $galR^s_{59}/galR^+$, 10 independently isolated heterozygotes were grown, tested, and assayed for enzyme content. For this strain, the values in the table are averages. The other strains were grown twice, and in most cases the enzyme assays and microbiological tests were done on both cultures. All enzyme assays were done in duplicate. The values obtained with strain $galR^-_2$ in TMG were arbitrarily set equal to 100. TMG is used as an inhibitor of internal induction, D-fucose is used as inducer. $galR^s_{59}R^-_4$ is a revertant of $galR^s_{59}$ to the Gal^+ phenotype.

The results of enzymatic analyses of these strains grown both in the presence of TMG or of D-fucose are presented in Table 5. From these results it is seen that $galR^s$ is dominant over $galR^+$ and that $galR^+$ is dominant over $galR^-$ and the Gal^+ revertants of $galR^s$.

5. Isolation of Deletions of the galR Gene

Since the reversion of the *galR^s* mutants to the *Gal⁺* phenotype offers a positive selection for the loss of function of the *galR* gene, it was tried, whether this could be used to isolate deletions extending from the *galR* gene to one of the adjacent genes.

Cells of strain $galR^s_{18}$ were mutagenized with nitrous acid, as described in *Methods*, and plated on minimal gal plates supplemented with lysine, arginine, cysteine, diaminopimelic acid, and thymine. Mutation in genes in the neighborhood of *galR* can lead to a requirement for these substances. Seven out of 336 Gal^+ revertants tested had, at the same time, acquired a lysine requirement. None of the revertants required any other of the metabolites mentioned above. Among 200 spontaneous Gal^+ revertants of $galR^s_{18}$ no Lys^- cells were found. The seven mutants were found to be *gal* constitutive. Data on three of them are shown in Table 6.

Table 6. *Studies with galR deletions*

Strain	Lys^+ revertants per 10^{10} cells	Epimerase		Transferase		Kinase	
		TMG	D-fucose	TMG	D-fucose	TMG	D-fucose
Δ 2	0	95	68	105	123	76	113
Δ 3	0	95	108	115	158	81	174
Δ 4	0	95	108	109	174	96	183
$galR^-_2$	—	100	92	100	168	100	179
$galR^+$	—	6	108	4	124	3	118

The $galR^+$ strain is *W8*. Cells were grown and enzymes assayed as described in *Methods*. Growth medium was LT with 10^{-3}M TMG or D-fucose respectively. The values obtained with strain $galR^-_2$ grown in the presence of TMG are arbitrarily set equal to 100. TMG is used to inhibit internal induction, D-fucose is used as inducer.

Of each of the deletions strains 10^{10} cells were plated on minimal gal plates lacking lysine, and no revertants to the Lys^+ phenotype were detected.

Thus it is very likely that the mutation leading to the Lys^- phenotype is a deletion. Since these Lys^- mutations occur at a frequency of around 2% among nitrous acid induced Gal^+ reversions of *galR^s* mutations, they cannot have originated by an independent event. The most probable explanation is therefore that the mutation is a deletion extending from the *galR* gene into the *lysA* gene.

Discussion

Studies on the control operating in the galactose operon in *E. coli* were first described by Buttin (1963a, b), who isolated mutations in the *galR* gene leading to constitutivity. From this result he concluded that the control in the galactose operon is similar to the control in the lactose operon. This finding was extended by Adhya and Echols (1966), who described the dominance of $galR^+$ over $galR^-$. Similar experiments in the lactose operon have been interpreted to mean that the product of the regulator gene is a repressor and that the phenotype of constitutive mutants results from loss of functional repressor (Jacob and Monod, 1961).

This reasoning cannot be accepted, however, in the light of the findings obtained by Englesberg and his co-workers in the arabinose operon of *E. coli*

(SHEPPARD and ENGLESBERG, 1967). The arabinose enzymes are inducible. Mutations are known in the *araC* gene which lead to constitutivity. These mutations are recessive to the wildtype allele of the *araC* gene. However, deletions of the *araC* gene do not lead to the constitutive phenotype, but to the simultaneous loss of the expression of all arabinose genes. This expression can be restored by a functional *araC* gene in position trans. These results were interpreted by the authors as indicating positive control. Dominance relationships between constitutive and inducible alleles of a regulator gene have therefore to be interpreted with caution.

We have therefore tried to obtain additional evidence for the type of control operating in the galactose operon. Such evidence is found by the isolation of deletions of part of the *galR* gene. These deletions are constitutive and produce the enzyme to at least the same extent as does the wildtype. We consider this as strong argument for the negative type of control.

It should be pointed out, however, that our argument rests on the lack of functional gene product in the deletions. Since we were not yet able to measure the extent of the deletions within the *galR* gene, we cannot say with certainty that no such product is being made. It would be desirable to isolate deletions which abolish the whole *galR* gene.

Additional evidence for negative control comes from the behavior of the *galR*^{s} mutants. These must produce a functional gene product, since they are dominant over *galR*$^+$ in heterozygotes. This cannot be ascribed to the cis-type of dominance exerted by operator mutations, since *galR* is so far away from the galactose operon on the *E. coli* chromosome that its action must be mediated through the cytoplasm. The phenotype of the *galR*^{s} mutations is lost by secondary mutations which occur at a frequency indicative of forward mutation rather than of backmutation. These mutations are constitutive. Though it would be possible to explain this on the basis of other assumptions, the simplest hypothesis is that the product of the *galR* gene is really a repressor which in the *galR*^{s} mutant has lost its affinity to the inducer, and which upon secondary mutation at any of several sites in the *galR* gene loses its function completely. The assumption of the loss of function is supported by the finding that the secondary mutations occuring in the *galR* gene are recessive to the *galR*$^+$ allele.

SHAPIRO (1967) has also described a mutation which on the basis of preliminary mapping data was assumed to be of the *galR*^{s} type. He also described the *Gal*$^-$ phenotype, the high frequency of reversion to *Gal*$^+$, and the constitutivity of three revertants tested.

This discussion only concerns the role of the *galR* gene in the regulation of the galactose operon, and does not exclude the possibility that other genetic factors are also participating in this control. In this respect it is worth mentioning that all constitutive mutants investigated so far can still be induced by a factor of around two by D-fucose. This effect had already been observed by BUTTIN (1963) and by SHAPIRO (1967). While it was possible to assume in their cases that the function of the *galR* gene had not been abolished completely, this is harder to maintain in the case of our deletions. The possibility of another factor participating in the control of the galactose operon has to be taken into consideration. We do not yet know, whether the observation that the specific activity of the galactose enzymes increases sharply at the end of the exponential growth

period even at saturating inducer concentrations (JORDAN, SAEDLER, LENGELER, and STARLINGER, 1967) has anything to do with the residual inducibility of the constitutive mutants. A situation, where more than one gene takes part in the regulation of another gene has been described by GAREN and ECHOLS (1962a, b).

The deletions of the *galR* gene were found after mutagenesis with nitrous acid in two independent experiments. No such deletions were recovered in a comparative sample of spontaneous mutations. While our statistics are not yet sufficient to draw firm conclusions on this observation, they may indicate that nitrous acid is active in producing deletions in bacteria. A similar observation was made by BECKWITH, SIGNER, and EPSTEIN (1966).

As the result of this study, we now know three operons which according to presently available genetic evidence are under negative control. These are the lactose operon (WILLSON et al., 1964, BECKWITH, SIGNER, and EPSTEIN, 1966), the L-α-glycerophosphate system (COZZARELLI, FREEDBERG, and LIN, 1968), and the galactose operon. Positive control has been firmly established in the arabinose operon (SHEPPARD and ENGLESBERG, 1967), and it is strongly indicated in the rhamnose operon (POWER, 1967). Indications for positive control are also found in the system for alkaline phosphatase (GAREN and ECHOLS, 1962a, b) and maltose (SCHWARTZ, 1966), and in the galactose system in yeast (DOUGLAS and HAWTHORNE, 1966). The reason why inducible operons can be controlled either negatively or positively remains to be elucidated.

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