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Mutations Partially Inactivating the Lactose Repressor of *Escherichia coli*

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After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 133 independent mutants of a haploid strain of *Escherichia coli* able to use phenyl-β-galactoside as a carbon source were obtained. The galactoside was specific in selecting for mutants with increases in their uninduced levels of β-galactosidase. Virtually all mutants (37 in a subsample of 38) carried mutations in the *lac* repressor gene. There were two classes of repressor mutants. As well as the commonly identified class of mutants with completely inactivated repressors, there was a frequent class of mutants (21/37) whose repressors were partially inactivated. Most of these (15/21) repressed β-galactosidase synthesis 4 to 50 times less than wild type, but were more numerous in the lower part of this range. Their β-galactosidase was inducible to levels characteristic of the parent strain. The repressor activities were diverse and stably expressed under routine growth conditions. The decreased activity did not result from the formation of temperature-sensitive repressors. None of the mutants with completely inactivated repressors appeared to carry UAG or UGA chain-terminating codons. On the assumption that the partially defective repressor mutants carried missense mutations, it is argued that missense mutations in the *lac* repressor gene modify the repressor's affinity for the operator with high probability. An explanation is proposed for the apparent sensitivity of this repressor function to partial inactivation as the result of amino acid substitutions.

The lactose repressor protein of *Escherichia coli* represses the expression of the genes of the *lac* operon by binding directly to the deoxyribonucleic acid (DNA) of the *lac* operator (8). Ribonucleic acid (RNA) synthesis, which is initiated at the *lac* promoter and proceeds through the operator into the β-galactosidase gene, is blocked when the repressor is bound to the operator (4, 10, 11, 18). The gene for the repressor protein lies adjacent to the promoter end of the operon and is transcribed separately from the *lac* genes (17). In repressor mutants of the commonly observed i^- completely defective type, the operator-binding function of the repressor tends to be eliminated. The *lac* enzyme synthesis of such mutants is derepressed 500- to 1,000-fold above the low constitutive level of the wild-type strain. Mutants of this type have been obtained by a variety of selection devices (5, 13, 14, 33). However, there are strong indications that these mutants do not represent the full spectrum of effects which mutation can induce in the operator-binding activity of the repressor. For example, Jayaraman et al. (12) isolated two $i^{-\text{defective}}$ repressor mutants which retained repressor function although the operator binding of their repressors was partially inactivated.

Perutz and Lehmann (22) showed that hemoglobin could be inactivated by amino acid substitutions at key sites in the molecule such as those forming nonpolar contacts with the heme group. On the other hand, there were many sites, particularly on the surface of the molecule, where a wide range of substitutions could be tolerated without an apparent impairment of function. The analysis of base substitution mutations affecting the proteins coded for by the *hisC* gene of *Salmonella* (32) and *lacZ* of *E. coli* (15) indicated that missense mutations rarely inactivated the proteins, at least sufficiently to allow mutants to be detected. It is possible that amino acid substitutions in these proteins normally lead to changes in activity which are indistinguishable from wild type on selection or screening plates; but it is also possible that a large proportion of substitutions

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have no effect on function. By analogy, mutations in the repressor gene which partially inactivated the repressor function could be expected, for either of these reasons, to be rare compared with those leading to severe inactivation. This hypothesis was tested by examining the *lac* repressor activities of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced mutants of *E. coli* which were capable of using phenyl-β-D-galactoside (PG) as a carbon source. Smith and Sadler (28) have reported the use of this galactoside to select mutants which synthesize the *lac* proteins constitutively. In the selection described in this paper, a large proportion of the repressor mutants formed partially defective repressors. An analysis of the characteristics and frequency of this class of repressor mutant is presented.

MATERIALS AND METHODS

Parent strain. Mutants were obtained in an *E. coli* K-12 strain, W, which was wild-type for *lac I* and the genes of the *lac* operon. The strain did not suppress nonsense mutations in *lac Z* when the mutations were transduced into the strain by means of P1kc lysates as described by Lennox (16).

Media. Minimal and complete liquid and agar media were as described by Pardee et al. (21).

Mutagenesis. NTG was added at a concentration of 2 mg/ml to log-phase cells of strain W and resuspended in the cold in 0.5 ml of 0.2 M acetate buffer (pH 5.0). The culture was put at 37 C for 1 h, washed in the cold, resuspended in minimal medium, and plated at suitable dilutions on minimal agar plates containing 0.1% PG (wt/vol) as sole carbon source.

XG indicator plate tests. Patches of mutants were replica-plated and grown to saturation on minimal glucose agar containing the noninducing chromogenic substrate, 5-chloro-4-bromo-3-indolyl-β-D-galactoside (XG) (Cyclochemical Corp.). This galactoside releases a blue dye on hydrolysis. When it was included in agar at appropriate concentrations (0.001 to 0.004%), the intensity of the blue color increased with the level of β-galactosidase produced in the cells of a patch (6).

β-Galactosidase assays. Overnight broth cultures were diluted in tubes containing 5 ml of 0.2% glycerol minimal medium and shaken at 37 C. Induced cultures were grown in the presence of 1 mM isopropyl-thio-β-D-galactoside (IPTG). Cultures were collected in the exponential phase of growth after the cells had undergone at least 10 divisions. Assays were made on 1 ml of an appropriate dilution of the culture by the method of Pardee et al. (21). Units of specific activity are expressed as nanomoles of ONPG hydrolyzed per minute per unit of cell mass. A unit of cell mass is contained in 1 ml of culture normalized to an optical density of 1.0 at 550 nm. Optical densities were read in a Zeiss PMQ II spectrophotometer.

Recessivity tests. The F' *lac* episome used in these tests carried the $i^{s}694$ allele (33). The operator and repressor alleles, $o^{c}40$, $o^{c}584$, $i^{-}3$, and $i^{-d}198$ (6) served as controls after their transduction into strain W.

A donor strain carrying the F' $lacI^{s}$ episome was mated to test strains under conditions giving at least 50% episome transfer. Cells receiving the episome formed Lac^{+}, Lac^{-}, or Lac$^{\pm}$ colonies on lactose tetrazolium plates depending on whether they carried, respectively, *cis*-dominant operator, recessive repressor or partially dominant (i^{-d}) repressor alleles.

Suppression tests. The *lac* genes of the test strains were transduced by means of P1kc lysates into: (i) UAG suppressor strain, RV/E16, carrying an F' *lac* episome in which *lac I*, *lac P*, *lac O*, and the amino terminal region of *lac Z* were deleted; (ii) UGA suppressor strain, CAJ 64 (27). UAG repressor mutants, A15 and A84 (19), were used as controls.

RESULTS

Selection of mutants. Samples of cultures of strain W, treated with NTG to a survival of 22%, were spread on minimal agar supplemented with 0.1% PG. After an incubation period of 72 h at 37 C, 133 colonies were picked from random positions on the plates without regard to colony size and were purified by restreaking on PG selective medium. Because cell division was not permitted before plating, the mutants must have been independent in origin. The frequency of mutants in the mutagen-treated cultures was some 20 to 30 times greater than in the untreated controls. Mutations which were spontaneous in origin would thus have occurred in few members of the sample.

Plate tests. The mutants were screened for increases in their constitutive levels of β-galactosidase, both qualitatively on XG indicator plates and by assays of the enzymes of a subsample of mutants. A classification of the mutants was made on 0.001% XG plates on which the mutants were scored 1 to 4 depending on the intensity of blue color which developed (Table 1). Mutants scoring 3 or more were designated *HC* and those scoring less than 3 were designated *LC*.

In further tests on XG plates, the uninduced levels of β-galactosidase of all *LC* mutants were higher than that of wild type, but were heterogeneous with respect to the level of their constitutivity. On 0.001% XG plates, 21 of the *LC* mutants scored 1 and could not be distinguished from the parent. On plates with 0.004% XG, all but 7 of the 21 mutants were a darker blue than the parent; however, single colonies of these 7 mutants were bluer than the parent strain on these plates. All *LC* mutants appeared to be fully inducible as they were a uniform dark blue, indistinguishable from the parent on XG

plates containing 1 mM IPTG. On the other hand, the scores of *HC* mutants tended to be uniform on plates of different XG composition. The incorporation of 1 mM IPTG in these plates did not increase their blue coloration.

β-Galactosidase assays. The constitutive and induced β-galactosidase activities of the first 38 mutants from the plate tests were determined (Table 2). With three exceptions, all *HC* mutants were completely constitutive. Their β-galactosidase levels were high and did not increase in induced cultures. All *LC* mutants were partially constitutive. Their induced levels of enzyme tended to be slightly in excess of those of induced cultures of the parent. In this analysis, 22/38 (55%) mutants were partially constitutive for β-galactosidase synthesis.

The distribution of the noninduced β-galactosidase of mutants in the subsample (Fig. 1) revealed a major discontinuity between the partially and the completely constitutive mutants. The constitutive β-galactosidase activity of the bulk of the partially constitutive strains fell in a narrow band with values from 4 to 50 times that of the basal level of the parent. Within this band, the distribution of the enzyme levels was skewed towards the lower part of the range (Fig. 2).

Identification of repressor mutants. Of the 38 mutants selected for intensive study, 37 carried mutations which were recessive to the i^s694 repressor allele (33) and were therefore classed as *lac* repressor mutants. The mutation of strain *LC*13 was dominant to the i^s allele and was ascribed to the operator. No partially dominant repressor alleles were detected. Mapping studies have confirmed the allocation of the mutations of all *LC* strains, except *LC*13, to sites within the repressor gene (B. Shineberg, unpublished data).

Diversity and stability in the expression of the LC repressors. The rankings of the *LC* mutants by their constitutive β-galactosidase activities tended not to change in independently made assays at 37 C (Table 3). In two

TABLE 1. *Color scores[a] of mutants on 0.001% XG indicator plates at 37 C*

Sample batch	Number of mutants scoring		
	1-2	3	4
1	10	10	1
2	12	9	1
3	8	14	1
4	11	7	3
5	7	6	2
6	14	3	3
7	8	3	0

[a] Scoring system: 1, white; 2, very light blue; 3, light blue; 4, medium blue. Strain W scored 1.

TABLE 2. *Mean specific activities[a] of β-galactosidase of duplicate cultures of uninduced and induced mutants*

Mutant	Non-induced	Induced	Mutant	Non-induced	Induced
*LC*1	12	1,300	*HC*1	1,400	1,200
2	830	1,300	2	1,800	1,400
3	32	1,400	3[b]	28	1,200
4	96	1,700	4	1,200	1,300
5	86	1,500	5	690	1,200
6	14	1,700	6	1,300	NT[c]
7	36	1,500	7	1,300	1,300
8	11	1,400	8	1,200	1,100
10	400	1,000	9	1,800	1,200
11	130	1,400	11	560	NT
12	24	1,200	12	1,300	NT
13	100	1,900	13	1,600	1,500
14	29	1,300	14	1,200	NT
15	41	1,200	15	1,400	NT
16	88	1,200	16	1,300	1,200
17	24	1,300	17	1,300	1,200
18	100	1,000	18	1,200	1,100
19	790	1,300	19	1,200	1,400
20	240	1,200	20	1,300	1,200
W	2.6	1,100			

[a] Units of specific activity are defined in Materials and Methods. The mean of the deviation of duplicate assays from listed values was 9%.

[b] Mutant was found to be misclassified by the original XG indicator plate tests.

[c] NT, Not tested.

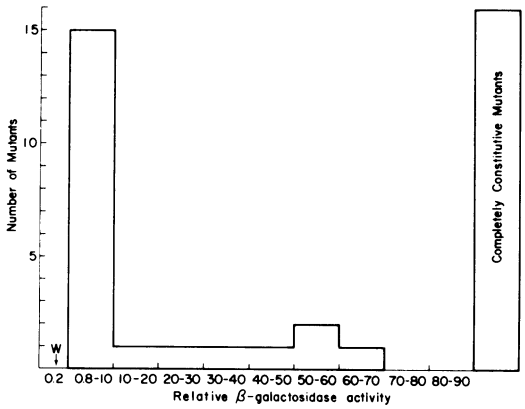

FIG. 1. *The distribution of the activities of the constitutively formed β-galactosidase of a subsample of 38 mutants. The parent W has a value of 0.2. The activities are expressed as a percentage of the mean of the activities of mutants with completely defective repressors.*

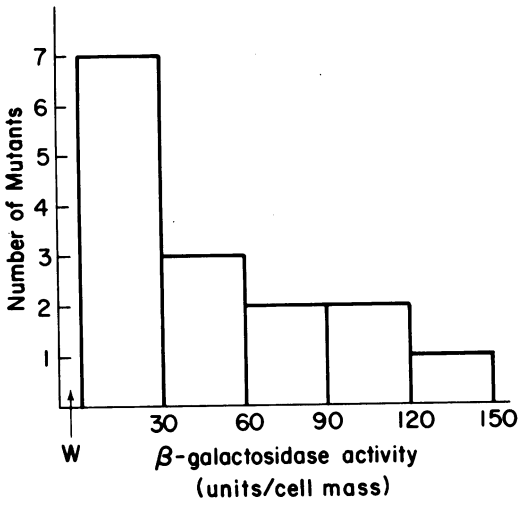

FIG. 2. *The distribution of the β-galactosidase activities of mutants with a 4- to 50-fold reduction in repressor activity. Units of specific activity are defined in Materials and Methods.*

TABLE 3. *Comparison of ranks of LC mutants in two assays of constitutive β-galactosidase activities*[a]

Mutant	Sp act		Ranks[b]	
	Assay 1	Assay 2	Assay 1	Assay 2
*LC*1	16	12	3	3
2	550	830	18	19
3	35	32	6	7
4	180	96	15	12
5	99	86	12	10
6	22	22	5	4
7	49	36	8	8
8	12	11	2	2
10	340	400	17	17
11	170	130	14	15
12	NT[c]	22	NT	NT
13	150	100	13	14
14	39	29	7	6
15	55	40	9	9
16	83	88	10	11
17	19	24	4	5
18	93	100	11	13
19	600	800	19	18
20	200	240	16	16
W	1.6	2.6	1	1
W induced	900	1,100		

[a] Units of specific activity are defined in Materials and Methods. The activities of assay 2 are taken from Table 2.

[b] Rank correlation coefficients (r): 19 strains, r = 0.98; strains ranking 10 or less, r = 0.96. The probability of obtaining these values of r by chance is less than 0.01%.

[c] NT, Not tested.

assays the rank correlation coefficients determined by the methods of Snedecor (29) were 0.98 in the case of all *LC* mutants, and 0.96 in the case of the 10 mutants with the lowest levels of constitutivity.

Plate tests of patches of mutants growing on XG indicator plates at temperatures down to 21 C revealed that temperature sensitivity was not responsible for the constitutivity of the partially defective repressor mutants. This was confirmed by determining the β-galactosidase activities of a sample of 7 mutants growing at 21 C. The constitutivity of all mutants was preserved at this temperature and, moreover, the ranks of the mutants with respect to their levels of constitutivity at 37 C were maintained (Table 4). The partially defective repressors appeared therefore to be genetically diverse and stable in their expression.

Bias of the sample. The question of how far the genetic diversity within the sample represented the full range of possible variation in repressor activity could not be answered directly. Insight into the matter was obtained, however, by a determination of the growth rates in PG medium of a selection of mutants which had similar growth rates in minimal succinate medium (Table 5).

With the exception of mutants *LC*1 and *LC*3, whose basal enzyme levels were closest to that of strain W, the growth rates of the mutants were similar. The slow growth rates of the *LC*1 and *LC*3 strains indicated that mutants of this class would be at a disadvantage, compared with mutants of higher levels of constitutivity, when growing on PG. Furthermore, *LC*1, with a lower basal enzyme level than *LC*3, grew slowest in PG medium. This suggests that selection for growth on PG would have biased the sample against mutants with very low levels of con-

TABLE 4. *Specific activities*[a] *of β-galactosidase of seven mutants grown at 21 C*

Mutant	Sp act 21 C	Constitutivity ranking at 37 C
*LC*1	6.4	1
*LC*7	57	2
*LC*5	130	3
*LC*11	180	4
*LC*10	410	5
*LC*19	590	6
*HC*4	1200	7
W	3.5	

[a] Units of specific activity are defined in Materials and Methods.

stitutivity. Therefore, although mutants with higher repressor activities than *LC*3 formed about 20% of the sample (Table 2), it is unlikely that they were over-represented. On the other hand, above a certain level of constitutivity, there appeared to be no difference among mutants in their ability to grow on PG, and thus it is improbable that the higher level constitutives were under-represented in the sample.

Tests for UAG and UGA mutations in the sample. Table 6 lists the constitutive and induced β-galactosidase activities of UAG and UGA suppressor strains carrying the *lac* genes of the completely constitutive mutants. Because no restoration of an inducible phenotype was detected, UAG and UGA mutations were concluded not to be present in any of the repressor genes. The characteristic basal β-galactosidase levels of strains W and HC 11, and also the suppression of control UAG mutations, A15 and A84, were detectable in their new backgrounds.

DISCUSSION

The binding of the repressor to the *lac* operator represses the synthesis of β-galactosidase apparently by blocking the transcription of the *lac* genes (4). Thus, the constitutive levels of β-galactosidase in the repressor mutants may be used as a measure of their operator-binding activity (7, 8, 28). Repressor mutants in which this activity was impaired but not eliminated occurred with a frequency of 57% (21/37) in a sample of mutants selected for at least partial constitutivity. The majority of the partially defective repressors (15/21 or 71%) inhibited β-galactosidase synthesis 4 to 50 times less effectively than the wild type, and their distribution was skewed towards the lower part of this range. Thus, on this scale, the activities of the mutant repressors ranged between 2 and 25% of the activity of the wild-type repressor. Levels of partial repression tended to be stably expressed under routine conditions of culture.

TABLE 5. *Generation times, in minutes, of mutants growing in 0.1% phenyl-β-galactoside medium at 37 C*

Mutant	Noninduced	Induced[a]	Constitutivity[b] rank at 37 C
*LC*1	132	63	1
*LC*3	85		2
*LC*5	66		3
*LC*2	70		5
*LC*13	60		4
*HC*4	60		6
W		63	

[a] Induced cultures were grown in the presence of 1 mM IPTG.

[b] The ranks are taken from the data of Table 2.

TABLE 6. *Mean specific activities[a] of β-galactosidase of duplicate cultures of UAG and UGA suppressor strains carrying the lac genes of constitutive mutants*

Mutant	Sp act			
	UAG suppressor strain		UGA suppressor strain	
	Non-induced	Induced	Non-induced	Induced
*HC*1	600	540	1,500	1,200
2	650	680	1,300	1,300
4	950	620	1,100	900
6	870	690	1,200	1,100
7	690	660	1,200	1,100
8	700	710	1,300	1,300
9	680	540	1,400	1,300
12	720	520	1,400	1,100
13	770	660	1,500	1,300
14	770	680	1,300	1,100
15	820	650	1,400	1,100
16	820	800	1,300	1,200
17	750	760	1,100	1,200
18	840	820	1,200	1,100
19	870	840	1,400	1,200
20	860	810	1,500	1,400
*HC*11	540	620	950	1,100
W	5.2	850	5.0	1,000
A15	190	770	1,500	1,500
A84	180	700	1,600	1,400

[a] Specific activities are defined in Materials and Methods. The mean of the deviation of duplicate assays from listed values was 10%.

The activities of the mutant repressors varied across a 100-fold instead of a theoretically possible 500-fold range corresponding to the activities of the wild-type repressor in uninduced and induced cultures. The failure to detect defective repressors whose activities were up to four times less than that of the wild type on this scale was probably due to the method of selection rather than to an absolute discontinuity at this point. The rapid decrease in the growth rate of mutants on PG medium as their repressor activities increased towards the lower end of the range (Table 5) suggests that beyond a given point repressor activity would prevent the growth of mutants. This interpretation is strengthened by a consideration of the selective function of PG. Although this galactoside inhibits the induction of the *lac* operon (24), the transgalactosidation products arising from its hydrolysis in uninduced cultures would be expected to act as inducers (3). Its role in the selection of repressor mutants is thus likely to

hinge on the relative cellular concentrations of PG and its transgalactosidation products. Mutant strains having a constitutive level of β-galactosidase below a certain threshold would not be able to produce sufficient inducer by hydrolysis to counteract the galactoside's effect in antagonizing induction. They would be unable to use it as a carbon source and therefore would be lost from the sample.

Above a certain level of constitutivity, on the other hand, there was no indication of any differences in the abilities of the mutants to grow on the substrate. For example, a mutant such as *LC*5, with a level of constitutivity of 6% of the derepressed enzyme level, grew equally as well on the selective medium as the completely constitutive mutants (Table 5). Therefore, it is unlikely that the high level constitutive mutants were under-represented in the sample. The discontinuity between the distributions of the partially and completely defective repressor mutants (Fig. 1) would thus appear not to be an artifact of the selection.

The high frequency of the low level constitutive repressor mutants was unexpected. Although mutants with partial repressor activity have been identified (12), the class has not been recognized as a common product of mutation in the repressor gene. Furthermore, the incidence of mutant proteins with a partially but not drastically inactivated function appears to be rare among proteins other than the repressor. Missense mutants in *lacZ* with 5 to 40% of wild-type β-galactosidase activity were found to be rare compared with mutants of a lower activity and clustered into the lower part of the range (J. B. Langridge, personal communication).

Although there is no direct proof that the mutations giving rise to the partially defective repressors were missense, two considerations make it likely that they were of this type. The mutagen, NTG, used in their induction produces base substitutions. Although it can induce large deletions, cases of its induction of small deletions or frameshifts are unknown in spite of its extensive use as a mutagen (7, 15, 32). Furthermore, the stability of the expression of the repressors of the low level constitutive mutants suggests that the proteins had undergone only minor structural changes such as could arise from amino acid substitutions.

Alternatively, the partial defectiveness could be due to mutations which caused a decrease in the number of repressor molecules. This possibility cannot be ruled out. Mutations in the repressor promoter (17) would have this effect without altering the structure of the protein. However, because a promoter site appears to be small, such mutations would probably be rare compared with mutations occurring within the repressor gene. Moreover, the mutations of the *LC* strains appeared not to be restricted to the end of the gene containing the promoter (B. Shineberg, unpublished data).

The apparent frequency with which an amino acid substitution partially inactivates the operator-binding activity of the repressor seems to be high. The expected frequency of nonsense codons among all base substitutions altering the amino acid sequence of the protein may be calculated from the genetic code and the amino acid composition of the *lac* repressor as given by Müller-Hill et al. (20). These calculations give an expected frequency of 95% mutations leading to amino acid replacements compared with 3% leading to UAG and UGA codons. Of those repressor mutants examined, the observed ratio of mutations partially inactivating the repressor to UAG and UGA codons was found to be 21:0, a ratio which does not depart significantly from expectation. The failure to find nonsense mutants was not unexpected. Bourgeois et al. (1) found that 3% of 850 spontaneously arising *lac* constitutive mutants had UAG mutations in the *lac* repressor gene. Müller-Hill (19) detected 1% UAG repressor mutations in a sample of 187 NTG and 2-aminopurine induced *lac* constitutives. These frequencies are consistent with the view that the operator-binding activity of the repressor protein is highly sensitive to amino acid replacement.

In considering explanations for the apparent sensitivity of the repressor to partial inactivation as the result of missense mutations, it is important to emphasize that such sensitivity may be a reflection of the PG selection rather than any difference between the repressor and other proteins. On the other hand, it is possible that such sensitivity is not characteristic of all protein functions. For example, the functioning of small substrate-binding sites, such as the lactose-binding sites of β-galactosidase, and the inducer-binding sites of the *lac* repressor, may not be sensitive to many amino acid substitutions in regions of the polypeptide which do not form the sites.

It is proposed that the sensitivity in the case of the repressor stems from factors associated with the large size of the operator as a substrate for the repressor. The *lac* operator consists of 21 base pairs (10). Native DNA is required to bind the repressor and only one repressor molecule is involved in the binding (8, 23, 24). The base

sequence of operator DNA is bilaterally symmetrical about a central point of reflection (10). Thus, it is probable that the operator provides sites for binding to two repressor monomers, although the repressor binds only in its tetrameric form (2). A similarly symmetrical arrangement of at least 16 mutable operator sites has been detected by mapping studies (26). If the double helix structure of the operator DNA is conserved during repressor binding, the dimensions of the operator would be equivalent to at least that of a small protein (about 7 by 2 nm). The repressor-operator interaction would thus occur across a complex interface. It is possible that a degree of independence exists in the functioning of the components of the interface, so that an alteration in one region of the interface, due to a structural alteration in the repressor, would modify the operator-binding function without eliminating it. One would predict that a series of residue sites exists in the repressor polypeptide where an amino acid substitution would drastically affect the binding activity (31). However, there should be other sites where a substitution will modify repressor activity less severely, and it is possible that alterations at sites not directly involved in the binding, but producing small changes in the conformation of the protein, may also modify interactions occurring at the operator-repressor interface. Furthermore, the binding of the repressor depends partly on electrostatic forces (24, 25), and consequently amino acid substitutions affecting the net charge of the protein may alter its affinity for the operator.

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