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Properties and Regulation of the β-D-Galactosidase in *Shigella dysenteriae* and in *Escherichia coli-Shigella dysenteriae* Hybrids

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ABSTRACT

SARKAR, S. (Massachusetts Institute of Technology, Cambridge). Properties and regulation of the β-D-galactosidase in *Shigella dysenteriae* and in *Escherichia coli-Shigella dysenteriae* hybrids. J. Bacteriol. **91**:1477–1488. 1966.—*Shigella dysenteriae* strain 60 has a β-D-galactosidase related to that of *Escherichia coli* but more heat-sensitive and with a turnover number about 10 times lower. Hybridization by transduction produces strains with enzymes of intermediate properties by recombination within the *z* gene. Both *E. coli* and *S. dysenteriae* have a regulatory i^+ gene. Recombination between i^- mutants of the two organisms leads to restoration of the i^+ genotype. In *S. dysenteriae* 60, most of the i^- mutants are subject to genetic suppression by suppressor mutations at unlinked loci. The effect of these suppressors on the products of the suppressed i^- genes is discussed.

Lactose utilization by *Escherichia coli* depends on the participation of β-D-galactosidase and galactoside permease, whose inducibility is coordinately controlled by a common repressor. Three closely linked loci control, respectively, inducibility (*i* locus), galactosidase (*z* locus), and permease (*y* locus). An additional enzyme, galactoside transacetylase, is also determined by a portion of the *lac* operon (7).

The ability to utilize lactose, characteristically present in *Escherichia* and *Aerobacter* and absent in most bacteria of the genera *Shigella*, *Salmonella*, and *Proteus*, has been used as a major criterion for classification of enteric bacteria. The availability of means to produce hybrids between *E. coli* and various strains of *Shigella* either by transduction (8) or by mating (12) has made it possible to analyze the genetic basis of the differences in lactose-utilizing capacity of these strains (5, 9, 11). The strains of *S. dysenteriae* that have been analyzed have no permease and varying levels of galactosidase (5, 9, 11, 16). All strains tested contain the i^+ locus (2, 5). Genetic recombination by transduction indicates that the *z* locus of *S. dysenteriae* is homologous to that of *E. coli*. The z^+ recombinants derived by transduction from a *S. dysenteriae* donor to an *E. coli* z^- recipient often have levels of galactosidase activity intermediate between that of a normal *E. coli* strain and that of the *Shigella* parent (5). The *y* locus appears to be missing in the *S. dysenteriae* strains since no lac^+ recombinants are produced in crosses between them and any y^- mutants of *E. coli*.

The present paper concerns a study of the properties and genetic determination of the galactosidase in *S. dysenteriae* strain 60 and in a series of *Shigella-E. coli* hybrids. The regulatory system (*i* locus) in this group of organisms is also described. In a preliminary communication (20), the finding of *su* mutations which suppress the effects of mutations i^- to constitutive synthesis of β-D-galactosidase in *S. dysenteriae* strain 60 was reported. The *su* mutations are not cotransduced by phage P1 with the genetic region controlling lactose utilization. The repressors produced by the combination of *su* mutations with their respective i^- mutations can, in partially diploid strains, repress the function of a *lac* operon in the *trans* configuration. Here we summarize the results of a genetic analysis of suppressor-bearing strains and of functional studies on the repressors produced in these strains.

MATERIALS AND METHODS

Most of the strains, media, and procedures employed in this work have been described previously (5).

Preparation of cultures and cell extracts. For measurement of galactosidase activity, cells were grown at

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the desired temperature in tris(hydroxymethyl)-aminomethane (Tris)-glycerol-amino acid medium with or without added inducer [10^{-3} M methyl-β-D-thiogalactoside (TMG) or 2×10^{-4} M isopropyl-β-D-thiogalactoside (IPTG), both compounds from Mann Laboratories, New York, N.Y.]. For routine assays, samples of culture diluted in PM2 buffer (0.1 M NaH_2PO_4, Na_2HPO_4; 0.001 M $MgSO_4$; 0.002 M $MnSO_4$; 0.1 M β-mercaptoethanol; *p*H 7.0) received 1 small drop of 1% sodium deoxycholate and 1 drop of toluene, and were shaken at 37 C for 30 min in stoppered tubes before being assayed for galactosidase.

The optical density of the cultures was measured in a Zeiss spectrophotometer at a wavelength of 500 mμ. One OD_{500} unit corresponds to approximately 5.6×10^8 cells per milliliter for *E. coli* and 3×10^8 cells per milliliter for *S. dysenteriae*. Specific activities are given in enzyme units per unit OD_{500}.

For preparation of concentrated bacterial extracts and for enzyme purification, bacteria were grown at 30 C either in 1-liter cultures in shaken 2-liter flasks or in 15-liter quantities in aerated 20-liter carboys. When the optical density reached 0.8 to 1.0, the cells were collected, washed, and suspended in precooled MP2 buffer at 4 C. Approximately 1 g (wet weight) of cells per liter was obtained. Extracts were prepared by sonic oscillation or by alumina grinding. The protein content of the cell extracts was estimated from nomograph values (22) for optical densities at 260 and 280 mμ. For more accurate estimation, the protein content was determined according to Lowry et al. (10).

Assay of galactosidase. Galactosidase activity was measured by the rate of hydrolysis of orthonitrophenyl-β-D-galactoside (ONPG; Mann Laboratories). One unit of enzyme activity is the amount of enzyme which hydrolyzes 1 mμmole of ONPG in 1 min at 28 C at *p*H 7.0 in PM2 buffer. The assay was run kinetically when needed to determine the affinity constants of the enzyme for various substrates and inhibitors.

Partial purification of galactosidase. Purification was done by a procedure (recommended by David Perrin) which consists of precipitation in ammonium sulfate at 35% saturation, dissolution of the precipitate in Tris buffer (0.1 M Tris Sigma 121, 0.01 M β-mercaptoethanol, 0.01 M $MgCl_2$, 0.0005 M $MnCl_2$, *p*H adjusted to 7.7 with glacial acetic acid), absorption on a diethylaminoethyl (DEAE) cellulose column, and elution by a linear gradient of sodium chloride (0.5 to 2.0%).

Serum precipitation reaction. The serum precipitation reaction was performed as described by Horiuchi et al. (6).

Sedimentation in sucrose gradient. Sedimentation in a sucrose gradient was performed according to Martin and Ames (13).

Thermal inactivation. Thermal inactivation of galactosidase was tested in PM2 buffer. The enzyme preparations were diluted into a buffer preheated in a bath kept at ±0.1 C of the desired temperature. At intervals, samples were diluted into cold buffer for assay.

Transduction by phage P1. The transduction experiments were carried out as described by Luria et al. (11). P1*dl*60 is the designation of the transducing particles present in high frequency transduction (HFT) lysates and carrying the *lac* region of Sh 60 (5).

Bacterial strains. The mutational sites of various *lac*$^+$ mutants of *E. coli* K-12 used are indicated in Fig. 1. Most of these mutants were originally received through the courtesy of F. Jacob, E. M. Lederberg, and J. Monod. *S. dysenteriae* strain 60, originally obtained from L. Barksdale's laboratory, has been described by Li et al. (9) and by Franklin and Luria (5); this strain is *lac*$^-$, $i^+z^+y^{del}$. Heterogenote strains are indicated as *i z*/ ex *i z*, where the first pair of symbols refers to chromosomal genes (endogenote) and the pair of symbols following ex (exogenote) refers to the genes in the P1*dl* prophage or in the F′ episome.

Isolation of constitutive mutants. Bacteria from overnight broth cultures of *S. dysenteriae* strain 60 were washed and resuspended at about 2×10^9 cells per milliliter in Tris minimal medium base with carbon and phosphate sources omitted. A 2-ml sample of suspension received 0.03 ml of ethyl methane sulfonate (Eastman Kodak Co., Rochester, N.Y.), and was incubated for 2 hr at 37 C with aeration. The survivors (about 10^{-3}) were plated on tryptone agar, and the resulting colonies were exposed to toluene for a few minutes, then sprayed with a 0.01 M solution of ONPG. Constitutive colonies are easily recognized by their yellow color. The toluene treatment leaves survivors from which pure constitutive clones can then be isolated. The frequency of i^- mutation among the survivors is about 0.1%. A similar spraying technique was used to score for i^- and i^+ colonies from crosses.

RESULTS

Properties of the galactosidase of S. dysenteriae strain 60. The galactosidase of *E. coli*, here designated as Z-coli (1, 7, 17, 21), was used as a standard for comparison with that of *S. dysenteriae* strain 60, designated as Z-60. Z-coli was prepared from induced cultures of *E. coli* K-12 substrain 3000.

The specific activity of galactosidase in crude extracts or in partially purified preparations of *Shigella* 60 is 10 to 20 times lower than in similar preparations of *E. coli*. Partial purification of the enzyme from the crude extract of two organisms was carried out. The elution patterns of the activities from a DEAE cellulose column were similar. A preparation of Z-coli obtained from the column had a specific activity of 3.36×10^5 units per mg of protein; a similar preparation of Z-60 had an activity of 3.8×10^4 units.

Partially purified preparations containing about 14,400 units of Z-60 were mixed with 2,000 units of Z-coli in a volume of 0.25 ml and layered on a sucrose gradient (5 to 20%). After centrifugation, fractions were collected in buffer, and were assayed for the amount of Z-coli and for total amount of enzyme present according to the

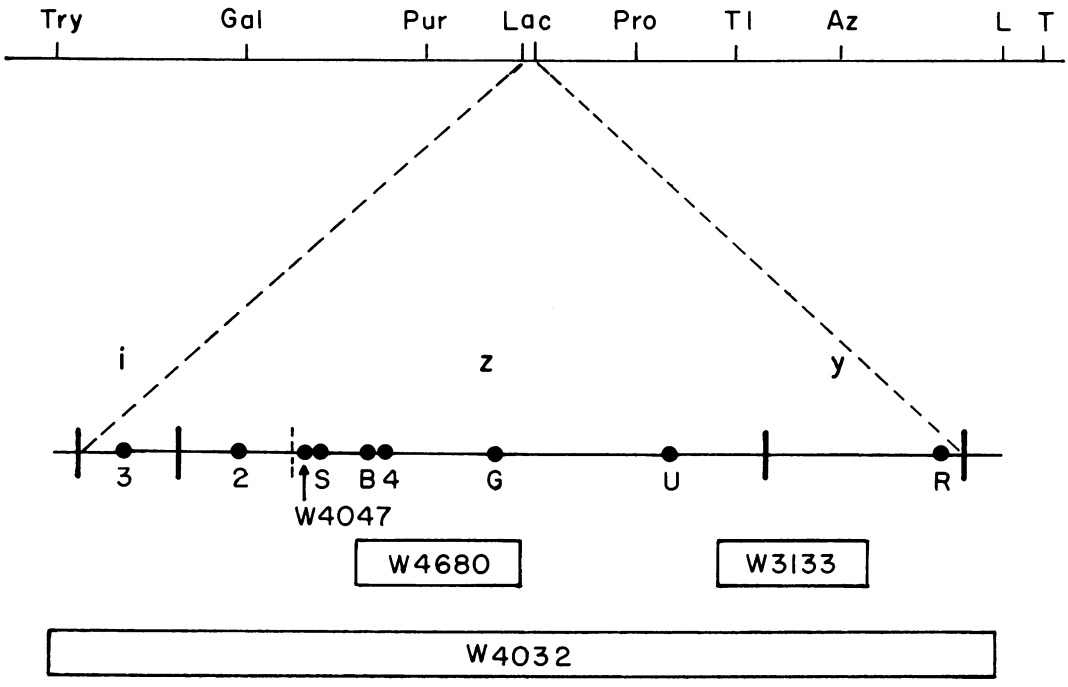

FIG. 1. *Map of the lac region and adjacent loci in Escherichia coli. The rectangles represent multisite mutations or deletions. The mutant sites are mapped according to Jacob and Monod (7) and to additional tests.*

method described below. The results (Fig. 2) indicate single overlapping peaks for both enzymes.

The heat stabilities of Z-60 and Z-coli were compared both in crude and partially purified preparations. The results (Fig. 3) indicate that Z-60 is much more heat-sensitive than Z-coli. The heat sensitivity was made use of in measuring the amount of Z-coli in the presence of an excess of Z-60, for example, in the experiment of Fig. 2. By heating a mixture at 54 C for 10 min, the residual activity measures the amount of Z-coli. Control experiments verified that the heat sensitivities of the two enzymes are not altered in their mixtures.

The rates of hydrolysis of ONPG by crude preparations of Z-60 and Z-coli were compared. K_m values were as follows: with ONPG as substrate, 1.82×10^{-4} M for Z-60 and 1.7×10^{-4} M for Z-coli; with FDG as substrate, 4×10^{-4} M for both Z-60 and Z-coli. Inhibition of hydrolysis by different β-D-galactosides was also measured and found to be quite similar for the two enzymes (Table 1).

The above results suggest that Z-coli and Z-60 are two proteins with qualitatively similar enzymatic activity but differing in heat sensitivity and in specific activity. The lower specific activity of Z-60 as compared with Z-coli might be due

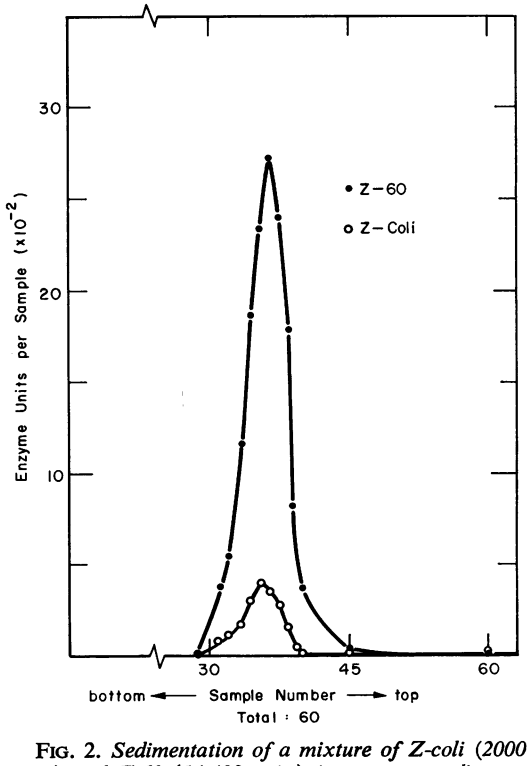

FIG. 2. *Sedimentation of a mixture of Z-coli (2000 units) and Z-60 (14,400 units) in sucrose gradient.*

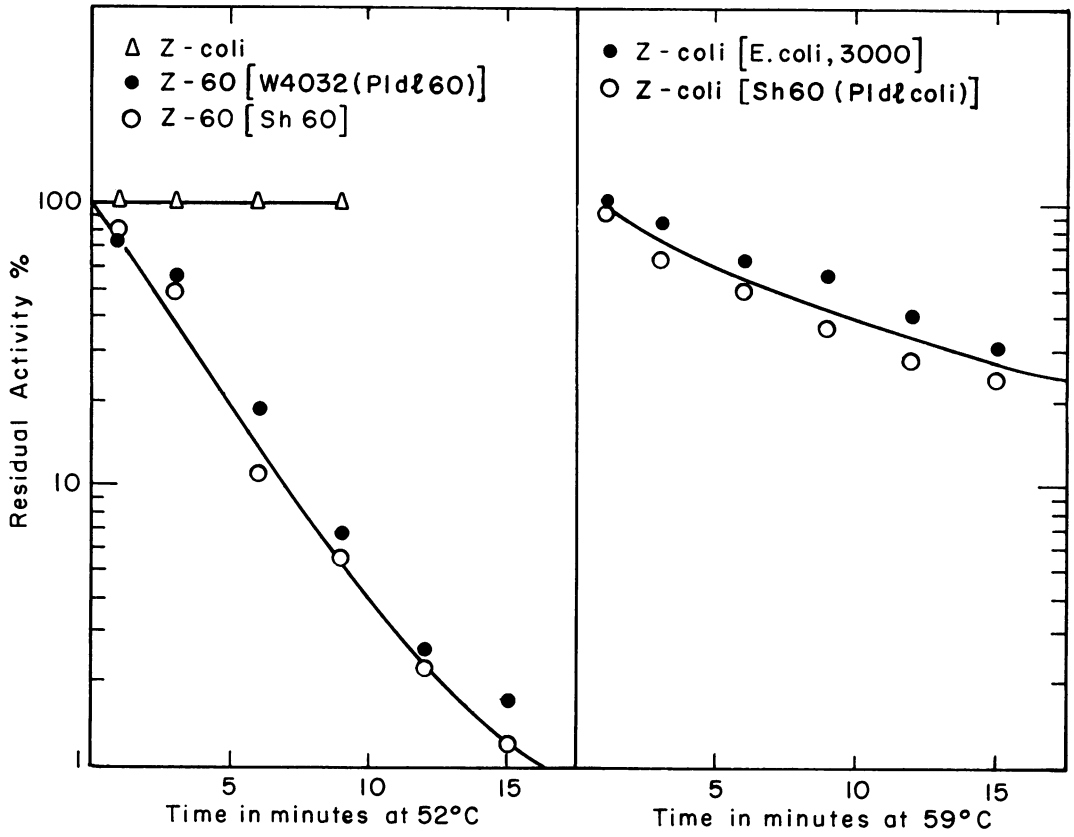

FIG. 3. *Thermal inactivation of Z-60 and Z-coli synthesized in different strains. Z-60 [Sh 60] refers to enzyme extracted from strain Sh 60. Z-60 [W4032(PIdl60)] refers to enzyme extracted from the heterogenote Escherichia coli W4032(PIdl60). Similarly, Z-coli [E. coli 3000] and Z-coli [Sh60(PIdlcoli)] refer to enzyme extracted from E. coli strain 3000 and from the heterogenote Sh 60(PIdlcoli), respectively.*

TABLE 1. *Michaelis constants and inhibition indices for Z-60 and Z-coli*

Inhibitor	Inhibition index*	
	Z-60	Z-coli
IPTG	0.50	0.64
TMG	<0.01	<0.01
Lactose	<0.01	0.018

* Fractional reduction of activity when the inhibitor is present in equimolar amount with the substrate (1.66×10^{-3} M ONPG).

either to a lower activity per enzyme molecule (lower turnover number) or, less likely, to the presence in Z-60 extracts of a 10-fold excess of inactive material.

A choice between these two alternatives was made possible by the use of the fluorogenic substrate fluorescein-di-β-D-galactoside (FDG; 17, 18). These experiments were kindly carried out by B. Rotman. FDG is hydrolyzed with similar kinetics by Z-60 and Z-coli (see Table 1). Hydrolysis of the nonfluorescent substrate FDG in microdrops liberates fluorescein and makes it possible to detect and measure the activity of single molecules of galactosidase. The principle of the method is as follows. An enzyme preparation is diluted in such a way that, with Z-coli, there is less than one molecule of enzyme per droplet on the average (in droplets of a suitably chosen size). The droplets with no enzyme, and those with one or more than one enzyme molecule, can be scored by microscopic measurement of fluorescence after suitable incubation. When the same procedure is applied to a preparation of Z-60 diluted to the same level of enzyme activity, the findings should be similar to those with Z-coli if the Z-60 activity is carried by molecules with the same activity as those of Z-coli. If the Z-60 molecules have a turnover number lower by a

factor *n*, however, the amount of activity carried by 1 molecule of Z-coli will be carried by *n* molecules of Z-60, and the distribution of activities among microdroplets will be very different.

The results of the experiment fit the second alternative. For Z-coli, the distribution is the expected one, with a mixture of droplets with and without fluorescence. For Z-60, the distribution is quite different, with a fairly uniform distribution of low fluorescence in all droplets. It is concluded, therefore, that the Z-60 contains a population of molecules whose individual enzyme activity is much lower than that of Z-coli molecules.

The reactivity of Z-60 and Z-coli with an anti-Z-coli goat serum (a gift from David Perrin) was measured by the precipitation test performed according to Horiuchi et al. (6). The results are presented in Fig. 4. Z-coli gave the normal precipitation curve, with a short inflection from the region of antibody excess to the region of antigen excess. The curve for Z-60 is very different with no definite inflection. This indicates gross differences in precipitability by anti-Z-coli serum between Z-coli and Z-60.

In summary, the results of the above experiments indicate that Z-coli and Z-60 are two proteins of approximately equal size, whose active sites behave similarly towards β-D-galactosides; the two enzymes, however, differ in turnover number, serological specificity, and heat stability. In addition, the synthesis of Z-60 is much more heat-sensitive than that of Z-coli (20). The synthesis of Z-60 is completely inhibited at 41 C, a temperature at which Z-60 itself is stable, whereas Z-coli is made normally in bacteria growing at 41 C.

It should be emphasized that these properties of Z-60 reflect the intrinsic character of the protein determined by the *z* gene, since they are unchanged when Z-60 is synthesized in *E. coli* cells, for example, in a heterogenote *E. coli* W4032 (P1*dl*60) that has a complete deletion of the chromosomal *lac* region and carries the *lac* region of strain 60 as an exogenote. The same is true in heterogenotes of the type *E. coli* 2340(P1dl_{60}), in which the bacterial chromosome carries a *z* gene that produces a material that cross-reacts specifically with Z-coli. Similarly, the galactosidase from strains of Sh 60 (P1*dl* z^+_{coli}) has all the properties of Z-coli (in addition, such strains presumably produce also Z-60).

Recombination between the z genes of S. dysenteriae 60 and E. coli. S. dysenteriae 60 behaves as a typical $i^+z^+y^{del}$ strain. Preliminary analysis of *lac*$^+$ recombinants obtained by P1-transduction from strain 60 to several z^- mutants of *E. coli* K-12 (5) have shown that the galactosidase of a recombinant may differ from both Z-60 and Z-coli. A further genetic analysis was carried out in an attempt to clarify the genetic differences between the two homologous *z* loci.

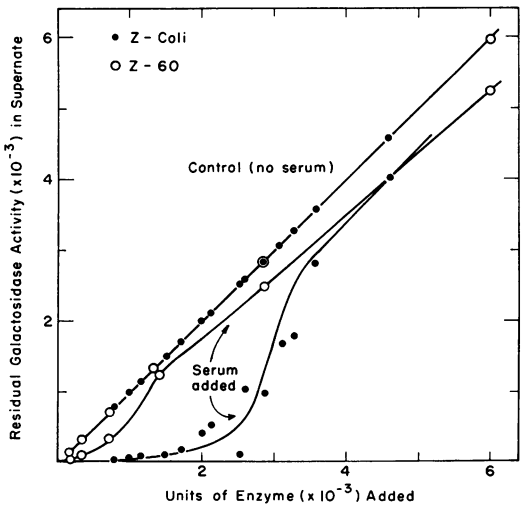

FIG. 4. *Serological titration of Z-coli and Z-60 with anti-Z-coli serum.*

The map of the z^- mutants used is shown in Fig. 1. They include both point mutations and deletions, identified as such by their failure to revert and their inability to give *lac*$^+$ recombinants with certain sets of point mutants (3). The relation of the deletions to the set of point mutants used in the present work was checked by using the deletion strains as recipient for transduction by P1 lysates grown on the point mutants.

After transduction of various *E. coli* z^- mutants by lysates containing P1dl_{60}, which carries the z^+_{60} gene, transductants were isolated from EMB-lactose agar transduction plates, and those with an integrated *lac*$^+$ chromosomal region were selected (5). The integrated transductants are those that do not segregate *lac*$^-$ progeny, do not yield lysates transducing at high frequency, and are either P1-sensitive or normally P1-lysogenic. The integrated transductants must have a hybrid *lac*$^+$ operon, since at least part of the *z* gene must have come from the transducing phage, hence from Sh 60, whereas the *y* gene is from the *E. coli* recipient. In agreement with previous results (5), no *lac*$^+$ transductants were found when the recipient was *E. coli* strain W3133, which has a deletion reaching into the *y* locus.

The galactosidase activity levels and heat stability of the enzyme in a series of integrated *lac*$^+$ transductants derived from different *E. coli* z^- recipients were measured on crude extracts of cultures grown with 10^{-4} M IPTG (Table 2). In a majority of the transductants, enzyme levels and heat sensitivity were like those of strain 60, an

TABLE 2. *Activity and heat stability of galactosidases in lac+ recombinants from transduction with P1dl60 to various Escherichia coli recipients*

Strain	Galactosidase activity level as per cent of activity in wild-type *Escherichia coli*		Per cent residual enzyme activity after heating at 54 C for 10 min
	No. of clones	Level	
E. coli 3000...		(100)	88–100
W4047...	4	4–8	2/4: 100; 2/4: <1
	7	10–20	2/7: 100; 5/7: <1
	8	25–45	5/8: 100; 3/8: <1
	6	70–100	5/6: 65–100; 1/6: <1
30B0.....	12	4–8	12/12: <1
30B0*....	8	30	8/8: <1
	4	30	4/4: <1
	7	65	6/7: 42–100; 1/7: <1
20G0.....	22	4	22/22: <1
	1	12	1/1: <1
	2	30	2/2: <1
30U0.....	27	2–4	27/27: <1
	1	18.5	100
	2	40	56, 100
W4680...	14	4–8	14/14: <1
W4680†..	11	2–4	11/11: <1
	1	7	1/1: <1
	3	14	3/3: 50
	3	30–45	3/3: 7–50

* *Lac*+ transductants selected at 43 C.

† Transducing lysate treated with ultraviolet light to increase recombination.

indication that most or all of the z^+ locus from strain 60 was present. Exceptions were found, especially among transductants derived from W4047, whose mutational site is near the operator side of the *z* locus. Here a majority of the transductants had enzyme levels intermediate between those of strain 60 and *E. coli*. A few transductants of such intermediate classes were found with the other recipients such as 2320, 2340, and 20S0 (5). Note in Table 2 that there is little correlation between heat sensitivity and enzyme level among these recombinants. Thus, even in strains with almost as much galactosidase activity as *E. coli* z^+, the enzyme may be as heat-sensitive as that of *Shigella* 60.

A particularly interesting group of transductants are those obtained from recipient strain W4680, which has a deletion reaching near the W4047 mutant site (see Fig. 1). Thus, the transductants must have received at least that part of the z^+_{60} locus whose homologue is missing in the recipient. All transductants obtained in the usual fashion had enzyme resembling Z-60. Ultraviolet treatment of the transducing lysate, which presumably increases recombination frequency, yields a few recombinants with enzyme in larger amounts and of higher heat stability.

With recipient strain 30B0, whose mutant site is supposedly located between those of W4047 and 2340, 12 of 12 transductants resembled strain 60 in their galactosidase. Whether this behavior is accidental or due to some pairing anomaly is unknown. Hybrid strains with intermediate enzyme levels could, however, be obtained from this transduction by a selective method (4) which consists of plating the P1-transduced bacteria on minimal-lactose agar and incubating at 43 C. The hybrids that grow are those whose enzyme can be synthesized at this temperature, at which no Z-60 is made (20). Of 19 such hybrids, 6 had heat-stable galactosidase (see Table 2).

The properties of the galactosidases of the recombinants were maintained when these strains were used as donors for transduction of the *lac*+ properties to an *E. coli* lac^{del} recipient that lacks the whole *lac* region; hence, the peculiarities of the galactosidase appear to reflect directly those of the *z* gene itself. Some tests with the fluorogenic substrate FDG, carried out by B. Rotman, confirmed the molecular basis of the enzyme levels found in the hybrid strains. Serological tests on extracts from two hybrid strains derived from W4680, one with galactosidase levels like Sh 60, the other with 45% as much enzyme as *E. coli*, showed a low reactivity with anti-Z-coli serum, similar to that of Z-60.

Recombination within the *z* locus per se does not lead to production of unusual galactosidases; the activity levels and heat sensitivities of *lac*+ recombinants obtained by cross-transduction between three pairs of z^- strains of *E. coli* all (13 of 13) showed enzyme levels and heat stability comparable to that of *E. coli* z^+ wild type. Instead, revertants from *E. coli* z^- point mutants (Table 3) exhibit a whole range of galactosidase levels between that of *E. coli* wild type and the very low level of Sh 60. Likewise, the heat stability of the revertant enzyme is often lower than that of *E. coli*, although generally not as low as that of Z-60.

In summary, hybridization within the *lac* operon between Sh 60 and *E. coli* yields strains that exhibit a range of galactosidase types. Although many recombinants probably receive almost the whole z^+ locus of Sh 60, since their enzyme resembles Z-60 in all properties examined, a number of recombinants appear to have hybrid *z* loci. Very few recombinants have enzyme re-

sembling *E. coli*, and these are found only among those derived from the *E. coli* recipients whose mutant sites are not too far from the operator site, so that a functional z^+ gene can be formed by incorporating only a small part of the Sh 60 locus. None of the recombinants derived from mutant strains 20G0, 30U0, or W4680 has enzyme like Z-coli.

It is important to note that the correlation between heat sensitivity of the galactosidase and its amount is not very close, except among those recombinants that have received the full z^+ gene from *Shigella* 60.

The results as a whole can be interpreted by assuming that the *z* genes of *E. coli* and *S. dysenteriae* are homologous genetic regions, with several genetic differences that affect the activity level as well as the heat stability of the enzyme. The sites of these differences can be reassorted by recombination to yield a spectrum of loci of different structure, with a corresponding spectrum of enzyme types. Probably none of the critical differences between Z-coli and Z-60 is located between the operator end of the locus and the mutant site of W4047, since several recombinants derived from this mutant resemble *E. coli* wild type. On the other hand, at least some of the site differences between the two loci reside within the region deleted in mutant W4680.

The i locus and repressibility. The lactose operons of Sh 60 and *E. coli* are both sensitive to repression determined by the inducibility gene *i* of either Sh 60 or *E. coli*. Heterogenote strains of Sh 60 $i^+_{60}z^+_{60}/\text{ex}\ i^-_{coli}z^+_{coli}$, carrying the *lac* region from *E. coli*, exhibited about the same degree of repression as comparable strains of *E. coli*, $i^+_{coli}z^-_{coli}/\text{ex}\ i^-_{60}z^+_{60}$.

The i^+ strains of *E. coli* cells grown at 43 C remain fully inducible, indicating that the repressibility is stable at this high growth temperature. To test the stability of the i^-_{60} product at 43 C, triploid strains of *E. coli* were used, because Sh 60 does not grow at this temperature. The strains used are derivatives of W4032 and carry two exogenotes: $lac^{del}/\text{ex}_{60}i^+z^+y^{del}/\text{ex}_{coli}i^-z^+y^+$. It should be recalled that the synthesis of active Z-60 is inhibited at 43 C; hence, the *coli* exogenote provided the means to measure galactosidase synthesis. If the repressor determined by the i^+_{60} gene were heat-sensitive, the strain might be phenotypically constitutive at 43 C. The results shown in Table 4 indicate that repressibility in Sh 60 as in *E. coli* is heat-stable. In fact, no noticeable difference between the i^+ loci of the two organisms was found.

Constitutive mutants and semiconstitutive variants. Three constitutive mutants, designated 60A, 60B, and 60C, were isolated after mutagenic treatment of *S. dysenteriae* strain 60. Strain 60B is stably constitutive, the uninduced enzyme level being similar to the fully induced level of Sh 60 (Table 5).

The other two constitutive strains, 60A and 60C, upon subculture change rapidly to strains which are "semiconstitutive," that is, whose uninduced cultures have levels of galactosidase activity 5 to 10% the levels of the induced cultures (Table 5).

A culture of 60A that was rapidly changing to semiconstitutivity was streaked out, and 8 iso-

TABLE 3. *Activity and heat stability of the galactosidases of Escherichia coli z^+ revertants*

Strain	Cause of reversion	Level of galactosidase activity as per cent of activity in wild-type *E. coli*		Per cent residual enzyme activity after heating at 54 C for 10 min
		No. of clones	Level	
E. coli 3000			(100)	88–100
20S0	Spontaneous	6	100	6/6: 54–89
	HNO_2	2	100	2/2: 58–76
20G0	Spontaneous	5	100	3/5: <1–7; 2/5: 50
		2	3	2/2: 50
	HNO_2	5	10	5/5: 1–25
2340	Spontaneous	7	100	3/7: 6–15; 4/7: 61–76
		1	50	36
		1	10	2
30U0	Spontaneous	1	100	50
		1	5	87

TABLE 4. *Heat stability of repressor synthesis in Escherichia coli lac⁺ recombinants carrying the i⁺ locus from Sh60*

Genotype	Clone	Inducer	Galactosidase activity in units per 1 unit of cell mass	
			Cells grown at 43 C	Cells grown at 37 C
E. coli 3000 $i^+z^+y^+$		−	1.4	1.3
		+	1,195	1,480
E. coli W4032(P1*dl*60; P1*dl* $i^-z^+y^+$), double heterogenote, $lac^{del}/ex_{60}i^+z^+y^{del}/ex_{coli}i^-z^+y^+$	1	−	2.0	1.2
		+	1,470	1,520
	2	−	3.3	1.5
		+	1,480	1,532
	3	−	3.0	2.2
		+	1,300	1,485

TABLE 5. *Galactosidase levels in mutant strains of Sh 60**

Shigella dysenteriae strain	Galactosidase activity in per cent of the induced wild-type value		Probable genotype
	Without inducer	With 5×10^{-4} M IPTG	
60	0.14	(100)	$i^+z^+_{60}$
60B	120	104	$i_B^-z^+_{60}$
60A	30; 12†	66	$i_A^-z^+_{60}$
60A-M	5.6	116	$i_A^-z^+_{60}$ su_m
60C	30.2; 4.7†	88	$i_C^-z^+_{60}$
60C-N	5.6	68	$i_Cz^+_{60}$ su_n

* All values are for cultures grown at 28 C.

† All cultures of strains 60A and 60C contain variable amounts of bacteria 60A-M (or 60C-N). Hence, the noninduced enzyme levels are poorly reproducible.

lated clones were tested for induced and uninduced levels of galactosidase activity. Heterogeneity was seen among the clones tested, two of eight being fully constitutive and the others semiconstitutive. Thirty independent isolates from a semiconstitutive clone were all semiconstitutive. This finding excluded the possibility that the changing culture was accumulating fully inducible revertants and suggested that a new genotype characterized by semiconstitutivity became established. The semiconstitutive derivative from strain 60A was called 60A-M; that from 60C was called 60 C-N (Table 5). The reason for the rapid accumulation of the semiconstitutive variants 60A-M and 60C-N from their constitutive parents has not been clarified.

Mapping the i⁻ mutations. The relative positions of the constitutive mutations in 60A, 60B, and 60C, and their location with respect to the *z* locus were determined as follows.

Heterogenotes were prepared by transduction of lac^+ to *E. coli* strain 2340, $i_3^-z^-y^+$, by use of phage P1 grown separately on strains 60, 60A-M, 60B, 60C-N. High-frequency transduction lysates obtained from these heterogenotes contain transducing phage P1*dl60* with whatever *i* locus had been present in the original *Shigella* donor (5). These lysates were used to transduce lac^+ to *E. coli* strain 3320, $i_3^-z_2^-y^+$. The mutation z_2^- is an o^0 mutation (7), a polarity mutant which causes the simultaneous loss of both *z* and *y* functions. Strains 2340 and 3320 are isoallelic for the constitutive mutation i_3^-. In transductions to strain 3320, most lac^+ transductants are of the integrated type (5), in which the function of the y^+ gene of the *lac* operon in the chromosome has been restored by the replacement of the z_2^- mutant site with its homologous site from the z^+ gene from P1*dl60*. Recombination between the $i^-z^+y^{del}$ region from Sh 60 and the $i^-z_2^-y^+$ region from *E. coli* provides an opportunity for recombination between i^- mutations at different sites to yield i^+ recombinants.

The results of these tests are shown in Table 6. When the lysate contained P1dl_{60C}, carrying the i_C^- mutation, 17% of the lac^+ recombinants were i^+. With lysates of P1dl_{60A}, the i^+ recombinants were 2%. With P1dl_{60B}, no i^+ was found among 47 transductants. The results can be interpreted by assuming that, in order to generate an $i^+z^+y^+$ region, one crossover must occur within the *z* locus and another between the two i^- sites of donor and recipient. The results indicate that i_3^-, i_A^-, and i_C^- are mutations at different sites within homologous *i* loci of *E. coli* and Sh 60. The i_B^- site may be the same as i_A^- or close to it. The results suggest the map shown in Fig. 5, in which site i_C is located on the opposite side of i_3 with respect to z_2 (order $i_Ci_3z_2$). Site i_A^- may be either very close to i_3^- or may be located between

TABLE 6. *Frequency of recombinants lac^+i^+ after transduction of lac^+ to strain 3320, $i^-z_2^-$, with P1dl60 containing various i alleles*

HFT lysate from	*Lac* region in donor phage	(a) Total no. of clones tested	(b) No. of i^+z^+ recombinants	(b)/(a)
2340 (P1*dl*60A)	$i_A^-z^+$	100	2	0.02
2340 (P1*dl*60B)	$i_B^-z^+$	47	0	<0.02
2340 (P1*dl*60C)	$i_C^-z^+$	75	13	0.17
2340 (P1*dl*60)	i^+z^+	12	2	0.16

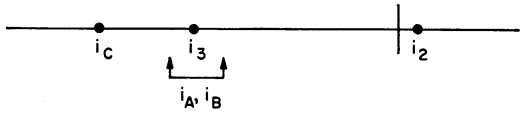

FIG. 5. *Tentative map of the relative positions of the constitutive mutations in strains 60A, 60B, and 60C, and their locations with respect to the z locus.*

i_3^- and z_2 (double crossover needed to produce i^+o^+). Similar considerations apply to i_B^-.

Thus, the *i* locus of Sh 60 appears to consist of a single cistron homologous to the *i* cistron of *E. coli*, and similarly located adjacent to the operator end of the *lac* operon.

Suppression of constitutivity in mutants and heterogenotes. Semiconstitutive strains Sh 60 A-M and C-N arise spontaneously in cultures of Sh 60 A and 60 C. Transduction experiments show that semiconstitutivity is due to suppressor mutations (su_m or su_n) unlinked to *i* locus, since suppression is not cotransduced with the *lac* markers of the Sh 60 strains (20). For instance, after transduction of lac^+ from Sh 60 A-M to *E. coli* i^-z^-, all heterogenotes $i_3^-z^-_{coli}$/ex $i_A^-z^+_{60}$ and all integrated recombinants i^-z^+ are fully constitutive. Moreover, a *Shigella* heterogenote of the genetic constitution $i_B^-z^+_{60}/i_A^-z^+_{coli}$ was found to be constitutive. (This strain, unlike other heterogenotes of *E. coli* or *Shigella*, is rather unstable and readily loses the exogenote.) Thus, the mutation responsible for suppression of constitutivity is readily separable from the inducibility locus.

Although the uninduced enzyme level in the semiconstitutive strains is 40 to 100 times higher than in the i^+ strains, the repressor formed as a result of suppression retains its ability to respond to the inducer IPTG and also its affinity for the operator site of the *lac* operon. The heterogenotes Sh 60 A-M/ex $i_3^-z^+_{coli}$ and Sh 60 C-N/ex $i_3^-z^+_{coli}$ are suppressed almost to the same extent as are 60 A-M and 60 C-N strains (see Tables 5 and 7); in all these strains, the semiconstitutive enzyme level is about 5 to 10% of the fully induced level. Since suppressibility is dominant in *trans* configuration, the suppressed products must have comparable effect on both operator sites. In addition, the response of these products to inducer appears not to have been altered, because these strains require the same concentration of inducer for full induction as do the wild type and the heterogenote strain 60 A-M/ex $i^+z^+_{coli}$ (see Table 7).

Temperature dependence of the suppressor function. The partial constitutivity of strains 60 A-M and 60 C-N is supposedly due to suppressor mutations su_m and su_n, which in combination with i_A^- and i_C^-, respectively, restore partial repression of the *lac* operon. The incomplete repression, 90 to 95% instead of over 99%, in the wild type (see Tables 5 and 7) might be due either to a lower amount of repressor or to a different, less efficient repressor than the product of the wild-type i^+ gene.

Repressibility in wild-type Sh 60 is stable at 39 C. If the suppression of constitutivity is due to production of an altered repressor, then its heat stability might be changed, whereas changes in repressor concentration should not affect its heat stability.

Heterogenote *Shigella* strains carrying an $i^-z^+y^+$ region from *E. coli* (see Table 7) were used to study the heat stability of repressor because the galactosidase of *E. coli* is produced normally even at high growth temperatures. These strains were grown at 28 C or at 39 C without inducer to compare the heat sensitivity of the repressor functions.

Strains Sh 60 A-M(P1*dl* $i_3^-z^+_{coli}$) and Sh 60 C-N(P1*dl* $i_3^-z^+_{coli}$) grow at about the same rate at 28 or 39 C, with a doubling time of 2.5 hr. The uninduced differential rate of galactosidase synthesis in the heterogenote strain derived from Sh 60 A-M is the same at 28 and 39 C. The uninduced rate for the heterogenote strain from Sh 60 C-N, in contrast, responds strongly to temperature changes (Fig. 6). When this strain is grown at 28 C without inducer, the rate of galactosidase synthesis is very low (Fig. 6, curve C). Within a few hours of growth at 39 C the rate of uninduced synthesis (Fig. 6, curve B) becomes equal to the fully induced rate (Fig. 6, curve A). When the uninduced culture is returned to 28 C, the fully induced rate remains undiminished for about 30 min and then tapers off to the characteristic low rate. It takes about 3 generations for almost complete derepression and 0.6 generation for the return to the partially repressed state.

The appearance of repression after returning the uninduced culture to 28 C resembles the kinetics observed in newly formed merozygotes following the introduction of i^+z^+ loci into constitutive (i^-z^-) recipients (15).

The derepression of enzyme synthesis observed at 39 C in the heterogenotes Sh 60 C-N(P1*dl*

TABLE 7. *Galactosidase levels in heterogenotes of Sh 60 strains**

Strain	Galactosidase activity in per cent of the induced level in *Escherichia coli* 3000		Probable genotype
	Without inducer	With 5×10^{-4} M IPTG	
E. coli 3000	0.06	(100)	$i^+z^+_{coli}$
E. coli 3300	94	66	$i_3^-z^+_{coli}$
Shigella dysenteriae 60(P1*dl*$_{3300}$)	0.06	95	$i^+z^+_{60}$/ex $i_3^-z^+_{coli}$
S. dysenteriae 60B(P1*dl*$_{3300}$)	96	92	$i_B^-z^+_{60}$/ex $i_3^-z^+_{coli}$
S. dysenteriae 60A-M(P1*dl*$_{3300}$)	10.8	98	$i_A^-z^+_{60}$ su_m/ex $i_3^-z^+_{coli}$
S. dysenteriae 60A-M(P1*dl*$_{3000}$)	0.15	80	$i_A^-z^+_{60}$ su_m/ex $i^+z^+_{coli}$
S. dysenteriae 60C-N(P1*dl*$_{3300}$)	10.1	99	$i_C^-z^+_{60}$ su_n/ex $i_3^-z^+_{coli}$
S. dysenteriae 60C-N(F'*lac*)	9.8	92	$i_C^-z^+_{60}$ su_n/ex $i_A^-z^+_{coli}$

* All values are for cultures grown at 28 C, the fully induced level of enzyme activity in wild-type *E. coli* being taken as 100%.

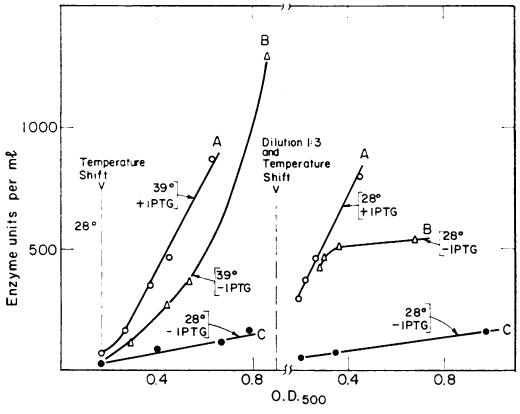

FIG. 6. *Differential rate of enzyme synthesis by strain 60C-N/ex* i_3^- z^+_{coli}. *The heterogenote was grown at 28 C without inducer (C). One portion of this culture was divided into two flasks at 39 C, one containing* 5×10^{-4} M *IPTG (A), the other without inducer (B). After about 2 generations at 39 C, all cultures were diluted 1:3 in precooled medium at 28 C and incubated further at this temperature.*

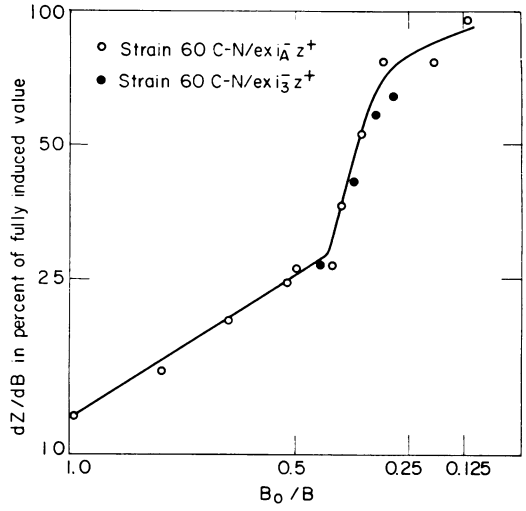

FIG. 7. *Two heterogenote strains derived from 60C-N were grown at 28 C, then shifted to 39 C. No inducer was added. The differential rate of enzyme synthesis dZ/dB was measured during growth at 39 C. The values of dZ/dB, expressed as percentages of the fully induced differential rate, are plotted on the ordinate. The abscissa shows the ratio* B_0/B, *where* B_0 *is the* OD_{500} *of the culture at the time of shift from 28 C to 39 C and B is the* OD_{500} *after various times of growth at 39 C [see Novick et al. (14)].*

$\cdot i_3^-z^+_{coli}$) is analyzed further in Fig. 7. The rate of derepression following the temperature rise is slow in the first generation, then it becomes fast rather abruptly. Assuming that the repressor is no longer made at 39 C, the repressor concentration at any one time should be a function of its half-life and of dilution due to growth. The results show that derepression becomes suddenly accelerated in the second generation and is practically complete after 3 generations.

Suppressor specificity. An experiment similar to the one described in Fig. 7 was done with another heterogenote strain, 60 C-N(P1*dl* $i_A^-z^+y^+$). The purpose of this experiment was to test the effect of the suppressor su_n on the i_A^- mutation. If su_m and su_n were identical, then su_n should suppress i_A^-, generating a partially repressed condition stable during growth at 39 C, as in strain 60 A-M. The results, included in Fig. 7, show that the kinetics of derepression in the heterozygote 60 C-N(P1 $i_A^-z^+y^+$) followed the same pattern as in strain 60 C-N. This indicates that su_m and su_n are different mutations: the combination $su_mi_A^-$ gives a heat-stable partial repression; $su_ni_A^-$ gives either no repressor or a heat-labile one.

The study of the specificity of suppressor action has not been extended beyond this test. It has not been possible to combine the suppressors su_m and su_n with i^- alleles other than those originally associated with them; for example, it has not yet been possible to produce strains of genetic constitution $su_m i_B^- z^+$. Likewise, it has not yet been possible to test the effect of the su_m and su_n mutations on mutant alleles of other genetic systems known to be susceptible to suppressors. The most promising test, done with rII mutants of bacteriophage T4, depends on the bacterial host being lysogenic for phage λ. *S. dysenteriae* strain 60 is naturally resistant to bacteriophage λ; hence, these tests cannot be performed on presently available strains. Efforts to introduce λ lysogeny into Sh 60 failed. It was possible to transfer the *su* markers from *Shigella* 60 into *E. coli* recipients by mating, but all strains isolated proved to be very unstable heterogenotes.

The task of determining the nature of this type of suppressor mutations would have been simplified, if one could obtain such mutations in *E. coli*. Search for suppressors for several independently isolated i^- mutants of *E. coli* was without success. Likewise, when the i_A^-, i_B^-, and i_C^- markers from Sh 60 were introduced by transduction into the *E. coli* chromosome to generate *E. coli* strains $i_A^- z^+$, $i_B^- z^+$, $i_C^- z^+$, respectively, none of these these strains developed partial inducibility even after many repeated subcultures.

DISCUSSION

The molecular weight of galactosidase of the *Shigella* 60 strain is apparently the same as that of the *E. coli* galactosidase, whereas the specific activity of the *Shigella* enzyme is lower by almost an order of magnitude. In bacteria containing hybrid z^+ genes from *Shigella-E. coli* crosses, the specific activity of the enzyme is anywhere between the values found in the two parents. These results suggest that the lower activity of the *Shigella* enzyme is probably due to a cumulative effect of mutations at several sites of the structural gene, which in recombinants are assorted in various ways. These mutations appear to affect the properties of the enzyme protein—*p*H and heat stability, heat sensitivity of enzyme synthesis, serological reactivity—without altering one of the important properties of the enzyme, namely, the K_m for its substrates. The Sh 60 strain is permeaseless because of an apparent deletion in the *y* region and cannot, therefore, utilize lactose effectively. It is not clear what kind of selective pressure may have caused the product of the *z* gene to retain its affinity for substrate and its molecular integrity.

The presence of recombination indicates the existence of extensive genetic homology between the structural *z* genes of *E. coli* and *S. dysenteriae*, implying a common ancestry. This view is strengthened by the finding with the i^- mutants, which show that the linkage relations of the loci are the same. The similarity of the linkage relationships of other markers was shown earlier by Luria and Burrous (12).

The inducibility locus i^+ of *S. dysenteriae* 60 is probably identical to that of *E. coli*. The i^+ recombinants from any two i^- mutants, one from *Shigella* and another from *E. coli*, have full repressor activity. The inducibility gene product of the two strains and of the i^+ recombinants are all equally heat-stable. The recent finding of a repressor-insensitive (o^c) mutation in *Shigella* 60 (I. Hertman, *personal communication*), together with dominance tests on various heterogenote strains, indicate the similarity of regulatory mechanism of the lactose operon. As in *E. coli*, only one locus controls the inducibility in *S. dysenteriae* 60 and the noncomplementability of mutants at this locus suggests that it consists of a single cistron.

Constitutivity in *Shigella* 60 is often suppressed by other mutations unlinked to the *lac* operon. This feature of the regulatory mechanism has not yet been observed in *E. coli*. Two different suppressor mutations have been characterized, each specific for a different i^- mutation. In one of the suppressed strains, the synthesis of repressor is heat-sensitive; in cells growing without inducer at 28 C, the galactosidase level is 5% of the induced level, whereas at 39 C a full level of enzyme is synthesized. When the suppressible i^- alleles are transferred from *Shigella* 60 to *E. coli*, no suppressible strains appear.

The nature of these suppressor mutations is not clear. Although adequate tests for allele specificity (24) have not been performed, it seems doubtful that these mutations belong to the class of those that restore sense to mis-sense or nonsense codons. Two of three i^- mutants described above were suppressible by two different suppressors, which arose spontaneously in *Shigella*. I. Hertman (*personal communication*) has found 11 suppressible mutants among 17 additional i^- mutants of *Shigella* 60. A functional bypass for the i^+ product to restore repression is improbable, since the specific inducers counteract the effect of the suppressed product in the same way as they affect the normal i^+ product. A possible alternative is that an inactive repressor can be functionally altered and activated by some substances, the production or accumulation of which is brought about by the suppressor mutations. That the *lac* operon repressor can be functionally modified by

small molecules such as the inducers was shown in recent studies (19, 23).

The additional system of regulation conferred to *S. dysenteriae* 60 by the suppressor may provide some selective advantage. As a consequence of the loss of the *y* locus, the function of the *z* gene, that is, the production of galactosidase, is rendered useless at least under normal circumstances, since lactose can hardly enter in bacterial cells without the specific transport system. As long as the *z* gene is functional, the *i* locus becomes especially valuable, since repression prevents wasteful biosynthesis of an enzyme which has lost its physiological role. Thus, selective pressure to maintain the functional integrity of z^+ is to some extent relaxed, but selection continues to operate in preserving the i^+ function, and favors additional safety devices such as the suppressor mutations.

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LITERATURE CITED

1. COHN, M. 1957. Contribution of studies on the β-galactosidase of *Escherichia coli* to our understanding of enzyme synthesis. Bacteriol. Rev. **21**:140–168.
2. COHN, M., E. LENNOX, AND S. SPIEGELMAN. 1960. On the behaviour of the *E. coli* P_2-"β-galactosidase system" introduced into *Shigella dysenteriae*. Biochim. Biophys. Acta **39**:255–266.
3. COOK, A., AND J. LEDERBERG. 1962. Recombination studies of lactose nonfermenting mutants of *Escherichia coli* K-12. Genetics **47**:1335–1353.
4. FRANKLIN, N. C., AND B. D. HOWARD. 1965. Double heterogenotes formed by P1-mediated transduction of *lac* genes in *Escherichia coli*. Virology **25**:98–110.
5. FRANKLIN, N., AND S. E. LURIA. 1961. Transduction by bacteriophage P1 and the properties of the *lac* genetic region in *E. coli* and *S. dysenteriae*. Virology **15**:299–311.
6. HORIUCHI, T., J. TOMIZAWA, AND A. NOVICK. 1962. Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochim. Biophys. Acta **55**:152–163.
7. JACOB, F., AND J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
8. LENNOX, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
9. LI, K., L. BARKSDALE, AND L. GARMISE. 1961. Phenotypic alterations associated with the bacteriophage carrier state of *Shigella dysenteriae*. J. Gen. Microbiol. **24**:355–367.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. LURIA, S. E., J. N. ADAMS, AND R. C. TING. 1960. Transduction of lactose utilizing ability among strains of *E. coli* and *S. dysenteriae* and the properties of the phage transducing particles. Virology **12**:348–390.
12. LURIA, S. E., AND J. W. BURROUS. 1957. Hybridization between *Escherichia coli* and Shigella. J. Bacteriol. **74**:461–476.
13. MARTIN, R. G., AND B. N. AMES. 1961. A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
14. NOVICK, A., E. S. LENNOX, AND F. JACOB. 1963. Relationship between rate of enzyme synthesis and repressor level. Cold Spring Harbor Symp. Quant. Biol. **28**:397–401.
15. PARDEE, A. B., F. JACOB, AND J. MONOD. 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J. Mol. Biol. **1**:165–178.
16. RICKENBERG, H. V. 1960. Occurrence of β-galactosidase in the genus *Shigella*. J. Bacteriol. **80**:421–422.
17. ROTMAN, B. 1961. Measurement of activity of single molecules of β-D-galactosidase. Proc. Natl. Acad. Sci. U.S. **47**:1981–1991.
18. ROTMAN, B., J. A. ZDERIC, AND M. EDELSTEIN. 1963. Fluorogenic substrates for β-D-galactosidases and phosphatases derived from fluorescein (3,6-dihydroxyfluoran) and its monomethyl ether. Proc. Natl. Acad. Sci. U.S. **50**:1–6.
19. SADLER, J. R., AND A. NOVICK. 1965. The properties of repressor and the kinetics of its action. J. Mol. Biol. **12**:305–327.
20. SARKAR, S., AND S. E. LURIA. 1963. Regulation of biosynthesis of a heat-sensitive β-D-galactosidase. Biochim. Biophys. Acta **68**:505–508.
21. WALLENFELS, K., AND P. O. MALHOTRA. 1960. "β-Galactosidase," p. 409–430. *In* P. D. Boyer, H. Lardy, and K. Myrbäck [ed.], The enzymes, vol. 4. Academic Press, Inc., New York.
22. WARBURG, O., AND W. CHRISTIAN. 1941. Isolierung und Kristallisation des Gärungsfermente Enolase (a nomograph constructed by E. Adams and distributed by California Corporation for Biochemical Research). Biochem. Z. **310**:384.
23. WILLSON, C., D. PERRIN, M. COHN, F. JACOB, AND J. MONOD. 1964. Noninducible mutants of the regulator gene in the "lactose" system of *Escherichia coli*. J. Mol. Biol. **8**:305–327.
24. YANOFSKY, C., AND P. ST. LAWRENCE. 1960. Gene action. Ann. Rev. Microbiol. **14**:311–340.