THE GENETIC BASIS OF HYPER-SYNTHESIS OF β-GALACTOSIDASE[1]

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CONTINUED growth of a bacterial population in a chemostat, where growth rate is limited by the concentration of some required nutrilite, is marked by the appearance of "fitter and fitter" strains (NOVICK and SZILARD 1950; NOVICK 1958). When lactose is used as the factor limiting growth rate of *Escherichia coli*, the fitter strains have higher and higher levels of β-galactosidase, an enzyme needed for lactose utilization. In these "hyper" producing bacteria, β-galactosidase is made up to four times as fast as in normal strains (NOVICK 1961; HORIUCHI, TOMIZAWA, and NOVICK 1962; NOVICK and HORIUCHI 1961). Furthermore, more recent experiments (HORIUCHI and NOVICK, unpublished) have shown that these hyper-synthesizing strains make a second enzyme, thiogalactoside transacetylase (ZABIN, KEPES, and MONOD 1962), also involved in lactose utilization, at two to three times the rate observed in normal strains.

This report is concerned with the genetic basis for the exceptional rate of β-galactosidase production by hyper-synthesizing strains. Much of our present understanding of the genetic basis of the control of protein synthesis comes from the well-known work of JACOB and MONOD and their colleagues on β-galactosidase synthesis (JACOB and MONOD 1961). In their model there are four genes, called *i*, *o*, *z*, and *y*. Gene *z* provides the information for the structure of β-galactosidase and gene *y* for the presence of galactoside permease. The enzyme thiogalactoside transacetylase is determined either by *y* or by a gene closely linked to it. The gene *i* furnishes a substance (repressor) which represses the formation of these enzymes, a repression which is relieved in the presence of an appropriate inducer, usually an added galactoside. The *o* gene, through its response to the repressor, affects at once the functioning of both *z* and *y*, which are thus part of the same operon. Wild-type strains i^+ (inducible) make β-galactosidase at less than 0.1 percent of the maximum rate when grown in medium lacking inducer, while i^- (constitutive) mutants make the enzyme at maximum rate even when grown in the absence of inducer. Alleles at the *o* locus include o^o, which prevents its associated *z* and *y* genes from making β-galactosidase or permease under any condition, and o^c, which, because of its resistance to the action of repressor, causes production of enzyme even in the absence of inducer. In

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order for a given phenotype to be scored as *lac*$^+$, the alleles for *y* and *z* must be +, *o* must be o^+ or o^c, and *i* may be − or +.

MATERIALS AND METHODS

Bacterial and viral strains: All of the present experiments were performed with the strains of *E. coli* K-12 listed in Table 1. For strains derived during the course of the present work, genotypes are assigned on the basis of evidence supplied in the experiments to follow. The Hfr strain E104 was chosen as a typical hyper organism. To isolate this strain a chemostat was inoculated with E102, an inducible Hfr, and the bacteria were grown at a generation time of ten hours, with lactose as the limiting growth factor, for several hundred generations. Early in the history of this population, typical constitutive bacteria became predominant (e.g., E106). Subsequently, the level of β-galactosidase of the population began to rise until after about two hundred generations a plateau three or four times the standard value (e.g., that of E106) was reached. From such a population, E104 was isolated. When E104, grown in lactate minimal medium, is plated on eosin-methylene blue (EMB) lactose agar plates, only 10^{-4} of the bacteria yield colonies. About one percent of this fraction are z^- and do not revert. One of these was taken as strain E111. Apparently E111 also lacks galactoside permease since it cannot be induced to accumulate appreciable quantities of ^{14}C-TMG (thiomethyl-β-D-galactoside). These observations are consistent with E111 having a deletion in the *lac* region.

(This interpretation was strengthened by experiments with strain E52, an F$^-$ strain obtained from a cross between E111 and W1–4 and believed to have the same defect in the *lac* region as E111. Phage P1 was grown on W2241 ($i^+o^+z^+y^-$) and was used to infect the strains E52, 2.320 ($i^-o^cz^+y^+$) and 2.340 ($i^-o^+z^-y^+$). No *lac*$^+$ ($o^+z^+y^+$) transductants were observed when E52 was the recipient, while numerous *lac*$^+$ transductants were obtained with 2.320 and 2.340 as recipients. When P1, grown on an $i^+o^+z^+y^+$ strain, was used to infect E52 under similar conditions, *lac*$^+$ transductants were obtained. The 100 transductants tested were found to be inducible. [The transduction frequency for E52 is ten to a hundred times lower than for standard *lac*$^-$ bacteria.])

Mating conditions: Mating of the bacteria was performed by the method described by PARDEE, JACOB, and MONOD (1959) using penassay broth as the mating medium. The blender technique of WOLLMAN and JACOB (1955) was sometimes used to interrupt matings.

Transduction techniques: Transduction of P1 was performed by the method of LURIA, ADAMS, and TING (1960) and of LENNOX (1955).

Culture methods and β-galactosidase assay: Media for cultivation and the details of the β-galactosidase assay have been described in a previous report (HORIUCHI, TOMIZAWA, and NOVICK 1962). Enzyme activities are expressed in units where 1.0 represents the level present in a fully-induced or constitutive normal strain. In some cases, plates containing 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC) (ZAMENHOF 1961), an alternative medium to EMB lactose, were used for selection or characterization of lactose-negative bacteria. On these plates

TABLE 1

*Characteristics of the bacterial strains**

Strain	*lac*	*i*	*o*	*z*	*y*	Remarks	*pro*	*TL*	B_1	*meth*	*T6*	λ	*str*	Sex	Origin	
CS101	+	+	+	+	+		+	+	—	—	S	(λ)	S	Hfr		Ref. 1†
E102	+	+	+	+	+		+	+	+	—	S	(λ)	S	Hfr	CS101	2
E103	+	+?	+	+	+	temperature-sensitive "*i*"	+	+	+	—	S	(λ)	S	Hfr	E102	3
E104	+	—	+	+	+	hyper	+	+	+	—	S	(λ)	S	Hfr	E102	2
E106	+	—	+	+	+		+	+	+	—	S	(λ)	S	Hfr	E103	2
E111	—	—	..	—	—	*lac* deletion?	+	+	+	—	S	(λ)	S	Hfr	E104	.
E16	+	—	+	+	+	hyper	+	+	—	+	R	R	R	F⁻	E104 × W1–4	2
E17	+	+/—	+	+	+/—	hyper anomalous	+	+	—	+	R	R	R	F⁻	E104 × W1–4	.
E18	+	+/—	+	+	+	hyper anomalous	+	+	—	+	S	R	R	F⁻	E104 × W1–4	.
E31	+	—	+	+	+	hyper	+	+	—	+	R	(λ)	S	F⁻	E104 × K7	.
E52	—	—	..	—	—	*lac* deletion?	+	+	—	+	S	R	R	F⁻	E111 × W1–4R	.
E269	+	+/—	+	+	+/—	hyper anomalous	+/—	+	—	+	S/R	R	R	F⁻	E106 × W1–4	.
P4x-6	+	+	+	+	+		+	+	+	—	S	(λ)	S	Hfr		4
E149	+	—	+	+	+		+	+	+	—	S	(λ)	S	Hfr	P4 × –6	.
E150	+	—	+	+	+	hyper	+	+	+	—	S	(λ)	S	Hfr	E149	.
W3747	+	+	+	+	+	F–$i^+z^+y^+$	+	+	+	—	R	(λ)	S	F⁺		5
W1–4	—	+	+	+	—		—	+	—	+	R	R	R	F⁻		6
W1–4R	+	+	+	+	+		—	+	—	+	R	R	R	F⁻	W1–4	.
K3	—	+	+	+	—		+	—	—	+	S	(λ)	R	F⁻		7
K7	—	+	+	+	—		+	—	—	+	S	(λ)	S	F⁻		7
2.0S0	—	+	+	—	+?		+	+	—	+	S	R	R	F⁻		8
2.340	—	—	+	—	+		+	+	—	+	S	R	R	F⁻		8
2.320	—	—	0⁰	+	+		+	+	—	+	S	S	R	F⁻		8
W2241	—	+	+	+	—		+	+	+	+	S	(λ)	R	F⁺		9

* Abbreviations: *pro*, *meth*, *T*, *L* and B_1 refer to ability to make proline, methionine, threonine, leucine and thiamine, respectively; R or S refer to resistance or sensitivity, respectively, to *T6*, virulent λ or streptomycin (*str*) (100μg/ml). (λ) denotes lysogenic for λ.

† References: (1) CAVALLI (1950); (2) HORIUCHI *et al.* (1962); (3) HORIUCHI, HORIUCHI, and NOVICK (1961); HORIUCHI and NOVICK (1961); (4) isolated by ADELBERG; (5) HIROTA and SNEATH (1961); (6) TOMIZAWA (1960); (7) received from LENNOX; (8) isolated by MONOD, received from S. E. LURIA; LURIA *et al.* (1960); (9) isolated by E. LEDERBERG.

lac$^+$ colonies of the strains described in Table 1 are whitish or slightly pinkish in color, while *lac*$^-$ colonies are usually dark red (z^- and i^+y^-) or dark pink (i^-y^-).

RESULTS

The ability of the hyper-synthesizing strains to make β-galactosidase at high rates could be the result of (1) a point mutation resulting in more effective functioning of the *lac* genes, or (2) presence of extra, independently functioning, *lac* genes. Extra genes could be associated with the bacterial chromosome or present elsewhere in the cell, e.g., associated with the F episome as in F-*lac* strains (JACOB and ADELBERG 1959; JACOB, PERRIN, SANCHEZ and MONOD 1960). From earlier experiments (NOVICK and HORIUCHI 1961) it appeared that this ability does derive from the presence of extra *lac* genes, and this hypothesis is examined more fully in the following experiments.

Studies using genetic recombination: If a point mutation had occurred, a clear-cut segregation of parental types would occur after conjugation. On the other hand, the demonstration of partial diploids in the recombination experiments, to be described below, indicates the presence of extra sets of genes.

If an Hfr hyper-synthesizing strain does have extra sets of the *lac* genes, it is possible that during conjugation with an F$^-$ strain, some of the extra sets of the *lac* genes from the Hfr parent might become associated with the *lac* region of the F$^-$ chromosome, resulting in recombinants heterozygous for the *lac* genes. If the Hfr parent were $i^-o^+z^+y^+$ (hyper) and the F$^-$ parent were $i^+o^+z^+y^-$ (standard), the expected heterozygotes would be $i^-o^+z^+y^+/i^+o^+z^+y^-$. Such partial heterozygotes (or heterogenotes) would have the unique attribute of being inducible and hyper-synthesizing, inducible because i^+ is dominant over i^- (PARDEE, JACOB, and MONOD 1959), and hyper-synthesizing because of the presence of extra z^+ genes.

This expectation was tested by comparing a cross between the constitutive hyper-synthesizing Hfr strain E104 ($T6^Si^-o^+z^+y^+pro^+meth^-str\text{-}S$ hyper) and the F$^-$ *lac*$^-$ strain W1–4 ($T6^Ri^+o^+z^+y^-pro^-meth^+str\text{-}R$ standard) with a cross between a normal constitutive Hfr, E106 ($T6^Si^-o^+z^+y^+pro^+meth^-str\text{-}S$ standard) and W1–4. A search was made among the recombinants from these crosses for inducible hyper-synthesizing bacteria, and the results are given in Table 2. In Cross I the selective markers were $y^+meth^+str\text{-}R$ and in Cross II they were $y^+pro^+meth^+str\text{-}R$. Since it was necessary to use lactose agar plates to identify y^+ bacteria as *lac*$^+$, the inhibitory effect of lactose on hypersynthesizing bacteria was minimized by growing and mating in penassay broth, a medium containing glucose (HORIUCHI, TOMIZAWA, and NOVICK 1962).

In these crosses, attachment of the Hfr donor strains to the F$^-$ recipients was complete within 20 minutes after mixing. Earliest injection of the *pro*$^+$ gene into W1–4 occurs at 18 minutes; within 100 minutes for the E104 cross and within 70 minutes for E106 injection has occurred for most mating pairs. The number of $pro^+meth^+str\text{-}R$ recombinants in these crosses was 6.0 percent for E104 and 5.5 percent for E106, taking the number of F$^-$ cells at zero time as 100

TABLE 2

Comparison of lac$^+$ *recombinants from crosses with an Hfr hyper strain and with an Hfr normal strain*

	Donor	E104 (Hyper) $T6^S$ $i^-z^+y^+pro^+B_1^+meth^-str\text{-}S$				E106 (Normal) $T6^S$ $i^-z^+y^+pro^+B_1^+meth^-str\text{-}S$			
	Acceptor	W1–4 (Normal) $T6^R$ $i^+z^+y^-pro^-B_1^-meth^+str\text{-}R$				W1–4 (Normal) $T6^R$ $i^+z^+y^-pro^-B_1^-meth^+str\text{-}R$			
		Number of colonies	Hyper percent	*pro*$^-$ percent	$T6^R$ percent	Number of colonies	Hyper percent	*pro*$^-$ percent	$T6^R$ percent
Cross I (Selective markers: $y^+meth^+str\text{-}R$)	Constitutive	283	69	20	13	318	0	13	18
	Anomalous constitutive	22	100	50	73	0	..	..	..
	Inducible	8	0	25	63	10	0	10	50
	Total	313	..	..	..	328	..	..	..
Cross II (Selective markers: $y^+pro^+meth^+str\text{-}R$)	Constitutive	507	54	..	11	584	0	..	8
	Anomalous constitutive	18	100	..	56	1	100	..	0
	Inducible	6	0	..	50	15	0	..	67
	Total	531	..	..	..	600	..	..	..

The indicated mixtures (donor : 5×10^7/ml, acceptor : 1×10^8/ml) were shaken gently for 90 minutes. The arrow indicates the direction and order of gene injection. At 90 minutes, the mating was stopped by blender treatment and the mixture was plated on the selective plates. A strain was classified as hyper if its β-galactosidase content in the fully induced state was two or more times greater than the level found in constitutive normal strains. An anomalous constitutive strain is one in which the β-galactosidase level in the absence of inducer is much higher than in normal inducible strains and in which, in the presence of inducer, the level is increased beyond that found in normal constitutive strains.

percent. Also, it was observed that the time at which injection of the hyper-synthesizing character occurs is roughly the same as for the *lac* gene, i.e., about 13 minutes at 37°.

As can be seen in Table 2, apparently no inducible hyper-synthesizing bacteria were found among several hundred recombinants. However, in the cross with the hyper-synthesizing Hfr parent considerable numbers of a curious class of recombinants were found. These have quite high basal levels of β-galactosidase in the absence of inducer and hyper levels in the presence of inducer. Such strains, called anomalous constitutives, typically have enzyme levels of 0.02 to 0.2 units in the absence of inducer and 1.5 to 3.5 in the presence of inducer [10^{-4}M isopropyl-β-D-thiogalactoside (IPTG)] on a scale where a normal constitutive shows 1.0. With the hyper-synthesizing Hfr parent there were 7.0 percent anomalous constitutives in Cross I and 4.3 percent in Cross II, while with the standard Hfr parent only one anomalous type was found out of 600 recombinants.

When anomalous constitutive strains are streaked on appropriate indicator agar (EMB lactose or TTC lactose), in about half of the cases several percent of *lac*$^-$ colonies are found. These are y^- like the F$^-$ parent. Likewise, several percent of constitutive bacteria are found in an anomalous constitutive population. This segregation for y and for i indicates that the anomalous constitutives are heterozygous at these loci. By analogy, the fact that they have hyper levels of β-galactosidase could be accounted for if they were homozygous for z^+.

It is clear that the anomalous constitutives represent the partial heterozygotes expected on the basis that the hyper Hfr parent has extra sets of the *lac* genes.

In the following sections these partial heterozygotes are the subject of further study.

Heterozygosity of anomalous constitutive strains: It should be noted in Table 2 that there was one anomalous constitutive strain (E269) found in the cross using standard Hfr and F^- strains as parents. Like the anomalous constitutives found so abundantly in crosses involving a hyper-synthesizing Hfr parent, E269 also behaves as a partial diploid, its segregation being similar to that of the aberrant heterozygote strains reported by LEDERBERG (1949). For example, E269 ($T6^S$, lac^+, and pro^+) segregates $T6^R$, pro^-, and y^- as well as i^- and i^+ strains among its progeny at a fairly high rate. Apparently E269 is diploid in the *T6-lac-pro* region, containing the $T6^R i^+ z^+ y^- pro^-$ segment from parent W1–4. The relatively high β-galactosidase level (0.02 to 0.1) of this strain in the absence of inducer can be understood as due to the presence of constitutive segregants (diploid and haploid), and the hyper-synthesis of β-galactosidase (nearly twice that of haploid bacteria) in the presence of inducer can be interpreted to result from the presence of the extra z^+ gene; a similar gene-dosage effect is seen in the increased rate of galactokinase synthesis in gal^+ homogenotic strains (HOROWITZ and NOVICK 1962).

The behavior of the anomalous constitutives from the cross with the hyper-synthesizing parent can be accounted for in the same way. Accordingly, polyploidy in the *lac* region provides extra z^+ genes, which are the basis for the hyper rate of β-galactosidase synthesis in such strains. Heterozygosity makes possible the segregation of i^- bacteria, which would be expected to produce high levels of enzyme in the absence of inducer. Indeed, all of the anomalous constitutive strains summarized in Table 3 segregate i^- bacteria (haploid and diploid) among their progeny to an extent (two to five percent) which fully accounts for their high β-galactosidase levels in the absence of inducer. This heterozygosity for the *i* gene also includes the *y* locus since half of the anomalous constitutives obtained segregate y^- bacteria (haploid and diploid) among their progeny (Types Ia and IIa in Table 3).

Finally, the anomalous constitutives can be classified into at least two main groups (Type I and Type II in Table 3) according to their capacity for making

TABLE 3

Summary of anomalous constitutive strains

	Number of strains tested	β-galactosidase level with no IPTG	β-galactosidase level with IPTG(10^{-4}M)	Frequency of i^- segregants percent	Frequency of y^- segregants percent
Type Ia	18	0.02–0.1	1.5–2	1–5	1–5
Type Ib	17	0.02–0.1	1.5–2	1–5	<0.1
Type IIa	6	0.05–0.2	2.5–4	1–5	1–5
Type IIb	4	0.05–0.2	2.5–4	1–5	<0.1

Colonies of each strain were picked from glucose minimal plates supplemented with appropriate growth factors and resuspended in penassay broth and cultured for two to three generations. A sample of the culture was spread on glucose minimal plates supplemented with appropriate growth factors to allow determination of the fraction of i^- colonies; plating on TTC-lactose or EMB-lactose agar gave the fraction of y^- colonies. Presumably Types Ia and IIa have the $i^+z^+y^-$ genes originating from W1–4 and the $i^-z^+y^+$ extra segment(s) of E104. Types Ib and IIb, on the other hand, although having *i* genes from both parents, appear to have only y^+ genes. *pro* strains have lower specific activities than do pro^+ strains.

β-galactosidase in the presence of inducer. The first comprises strains synthesizing 1.5 to 2.0 times, and the second, 2.5 to 4.0 times as much β-galactosidase as haploid bacteria. Since bacteria diploid for the z^+ gene (E269) synthesize twice as much β-galactosidase as normal haploid bacteria, the strains of Type I presumably are diploid for the z^+ gene, and also for the *i* and *y* genes. By analogy, strains of Type II are presumably of higher ploidy for these genes.

Thus, the findings that anomalous constitutive strains appear in high proportion among the recombinants of the cross with a constitutive hyper-synthesizing Hfr parent and that these strains have hyper levels of enzyme and are heterozygous in the *lac* region support the hypothesis that the hyper-synthesizing strains contain one or more extra sets of *lac* genes.

Some characteristics of the extra segment in hyper strains

(1) The length of the extra segment: None of the anomalous constitutive strains so far tested have shown heterozygosity with respect to the *T6* or *pro* markers, the nearest known markers on either side of the *lac* region, in contrast to E269 which does. This can be seen from the data in Table 4, where the *T6* and *pro* character of the y^+ and y^- offspring of E269 are compared with similar data for y^+ and y^- offspring of some anomalous constitutives ($T6^S$ pro^+). Presumably, the lac^+ strains here are mostly diploid while the lac^- are mostly haploid. No $T6^R$ and pro^- bacteria were observed among the haploid y^- segregants in the case of anomalous constitutives, while about 80 percent of the haploid y^- segregants of E269 were found to be $T6^R$ and/or pro^-. Apparently the extra segments of the hyper-synthesizing strains are fairly short, including little if anything more than the *lac* region.

(2) Recombination in anomalous constitutives: As the extra segments of the anomalous constitutives are derived from the hyper strain E104, they originally had the markers $i^-o^+z^+y^+$ in the *lac* region, while the F^- chromosome originally contained $i^+o^+z^+y^-$. The progeny of such an anomalous constitutive include many kinds of segregants which can be classified as haploid or diploid on the basis of pattern of segregation and rate of β-galactosidase synthesis. If a strain gives no segregation at *i* or *y* and if the maximum rate of enzyme synthesis is 1.0,

TABLE 4

Segregation of T6 *and* pro *markers from E269 and from anomalous constitutive strains*

	Colony type	Colonies tested	$T6^R$ (percent)	pro^- (percent)
Progeny of E269*	lac^+	48	0	2
($T6^S lac^+ pro^+$)	lac^- (y^-)	42	86	76
Progeny of some anomalous	lac^+	200	0	0
constitutives† ($T6^S lac^+ pro^+$)	lac^- (y^-)	189	0	0

Strains were plated on TTC-lactose and EMB-lactose agar. lac^+ colonies were picked, suspended in penassay broth, and grown for about five generations. These cultures were plated on TTC-lactose or EMB-lactose plates. lac^+ and lac^- colonies were picked and tested for sensitivity to T6 and requirement for proline.

* Several lac^+ and lac^- colonies from each of ten independent clones were tested.

† From ten to 30 lac^+ and lac^- colonies from several independent anomalous recombinants ($T6^S lac^+ pro^+$) were tested.

it is assumed to be haploid. A diploid strain is identified as one which makes enzyme at twice the normal rate and which segregates haploid progeny. For example, E17, an anomalous constitutive strain derived from the cross of E104 with W1–4, segregates i^-, i^+, y^-, and y^+ haploid strains. In none of these haploid segregants does the β-galactosidase level exceed 1.0. Since the level of β-galactosidase of E17 never exceeds 2.0 in its fully induced state and since most of the y^- segregants are i^+, the genotype of E17 is probably $i^+z^+y^-/i^-z^+y^+$. The segregants derived from E17 are depicted in Table 5, and it can be seen that nearly all of the segregant types expected are observed. (Such segregation is similar to that found in the case of λdg heterogenotes of the *gal* region [LEDERBERG 1960].)

The assignment of genotypes can be examined by testing the strains for their sensitivity to inhibition by lactose. As noted earlier, a constitutive hyper-synthesizing strain fails to give colonies on lactose agar except when pre-grown on glucose. If the strain is y^- or contains an i^+ allele then it is insensitive to lactose. As can be seen in Table 5, the assignment of genotypes is also consistent on this basis.

Apparently recombination does takes place among the extra sets of *lac* genes since the segregant types shown in Table 5 include not only the parental *lac* genotypes but recombinants as well. Such recombination frequently resembles "gene conversion" (MITCHELL 1955) since strains heterozygous for two characters (e.g., *i* and *y*) give rise to offspring homozygous for one and heterozygous for the other.

Segregation of stable haploid strains through loss of the extra gene sets is relatively frequent. This accounts for the observed presence of a large proportion of constitutive normal strains in a culture of a constitutive hyper-synthesizing strain.

(3) Absence of independent transfer: It is possible for the *lac* region, as well as other small chromosomal segments, to become associated with the sex factor (F) and be transferred into F^- bacteria with high frequency upon conjugation (JACOB and ADELBERG 1959). An F^+ strain having *lac* genes associated with the F episome makes β-galactosidase at higher rates than wild type strains (JACOB *et al.* 1960), presumably because it has extra sets of *lac* genes.

To test whether such an association of *lac* genes with the F episome is the basis for hyper β-galactosidase activity in the strains described here, the present hyper strains were compared with a known F-*lac* strain with regard to (1) the rate of transfer of the *lac* genes by conjugation, and (2) the correlation of transfer of the *lac* character with transfer of F factor in conjugation.

For comparison of the rate of transfer of *lac* genes, the strains P4x-6 (an Hfr which transfers *pro* very early after contact with an F^- and which transfers *lac* very late (two hours)), E150 (a constitutive hyper Hfr derived from P4x-6 by selection at limiting lactose in the chemostat), and W3747 (an F-*lac* strain) were used. As shown in Table 6, both P4x-6 and E150 transfer pro^+, T^+ and L^+ markers efficiently, i.e., both are clearly Hfr, while the F-*lac* strain gives no detectable recombinants for these markers. On the other hand, when selection was made for the lac^+ character (in this case y^+), the F-*lac* is found to give very

TABLE 5

Segregants of E17 (an anomalous constitutive)

Type	Assumed genotype*	β-galactosidase level observed†		EMB phenotype‡	Segregants observed in the progeny§	Inhibition by lactose
		Uninduced	Induced			
(E17)	$\frac{-++}{++-}$ or $\frac{+\,+-}{-++}$	0.02–0.1	~2	+	i^+, i^- y^-	insensitive
A	$\frac{-+-}{++-}$ or $\frac{+\,+-}{-+-}$	0.02–0.1	~2	−	i^+, i^- no y^+	insensitive
B	$\frac{-++}{-+-}$ or $\frac{-+-}{-++}$	~2	~2	+	no i^+ y^-	sensitive
C	$\frac{+++}{-++}$ or $\frac{-++}{+++}$	0.02–0.1	~2	+	i^+, i^- no y^-	insensitive
D	$\frac{-++}{-++}$	~2	~2	+	no i^+, no y^- type G	sensitive
E	$\frac{-+-}{-+-}$	~2	~2	−	no i^+, no y^+ type H	insensitive
F	+++	<0.001	~1	+	no i^- no y^-	insensitive
G	−++	~1	~1	+	no i^+ no y^-	insensitive
H	−+−	~1	~1	−	no i^+ no y^+	insensitive
I	++−	<0.001	~1	−	no i^- no y^+	insensitive

* Genotype is for genes *izy*, + + + meaning $i^+z^+y^+$. E17 is classified as Type Ia in Table 3.
† To induce y^+ and y^- bacteria, 10^{-4}M and 10^{-6}M of IPTG were used, respectively.
‡ Colony type on EMB-lactose agar.
§ TTC-lactose or EMB-lactose agar were employed to detect the presence of y^- bacteria. To detect i^- or i^+ segregants, in most cases 50 to 100 colonies from a glucose minimal plate were picked and tested for level of β-galactosidase with and/or without inducer.

efficient transfer of *lac*$^+$, while P4x-6 and E150 give barely detectable transfer. Thus, E150 acts like the Hfr strain from which it was derived rather than like an F-*lac* strain.

To rule out the possibility that *lac*$^+$ recombinants were formed in the E150 cross but were subsequently lost on lactose-containing media used to score *lac*$^+$, this cross was repeated using a donor/acceptor ratio of 10:1, plating on streptomycin agar both with and without lactose. No fewer *str-R* colonies were found in the presence of lactose, thus proving that no appreciable numbers of lactose-sensitive *lac*$^+$ recombinants are formed.

As a control to verify that hyper-synthesizing bacteria are able to transfer an F-*lac* segment when they actually are F-*lac*, a number of hyper-synthesizing

TABLE 6

*Comparison of transfer of markers by an Hfr hyper strain and by an F-*lac *strain*

Donor	Acceptor	Frequency *pro⁺meth⁺str-R*	Frequency *y⁺str-R*	Frequency *T⁺L⁺meth⁺str-R*	Frequency *y⁺str-R*
P4x-6 (*meth⁻str-S i⁺z⁺y⁺*; Hfr, normal)	W1-4 (F⁻*pro⁻str-R i⁺z⁺y⁻*)	18.0	<0.2	..	..
	K-3 (F⁻ *T⁻L⁻str-R i⁺z⁺y⁻*)	...	..	1.5	<0.1
E150 (*meth⁻str-S i⁻z⁺y⁺*; Hfr, hyper)	W1-4	17.7	<0.2	..	..
	K-3	...	..	1.0	<0.1
W3747 (*meth⁻str-S i⁺z⁺y⁺/F⁻i⁺z⁺y⁺*; F-*lac*, hyper)	W1-4	<0.1	70.0	..	..
	K-3	...	...	<0.1	91.5

Evidence showing that hyper character is not due to the presence of F-*lac* episomes. The indicated mixture (donor: 2×10^8/ml, acceptor: 2×10^7/ml) were shaken gently for 80 minutes before dilution and plating. For expression of recombinant frequency, the number of *str-R* bacteria in the mixture present at 80 minutes is taken as 100.

recombinants obtained from crossing an Hfr hyper strain with an F⁻ normal strain were infected with F-*lac* episome through contact with W3747. The resulting F-*lac* hyper strains gave very efficient transfer of z^+y^+, while the hyper recombinants (presumably F⁻) gave no detectable transfer. As a further control, the inability of hyper-synthesizing bacteria to transfer z^+ genes was demonstrated with z^-y^+F⁻ acceptor strains (2.340 and 2.0S0). Here too, no detectable transfer from any hyper recombinant was observed, while z^+y^- bacteria carrying the F-z^+y^- episome gave efficient transfer. Also, a hyper recombinant infected with wild type F cannot transfer at a high rate either z^+ or z^+y^+ to a *lac*⁻ F⁻ recipient.

Furthermore it could be shown that the hyper-synthesizing recombinants are in fact F⁻, *i.e.*, they accept F-*lac*. When constitutive hyper recombinants (e.g., E16: *i*⁻ and *str*-R) were placed in contact with the F-*lac* strain W3747 (*i*⁺ and *str-S* and plated on streptomycin-containing medium, 53 out of 70 colonies were found to be inducible F-diploids (*i*⁺ being dominant over *i*⁻). (Some of these inducible strains have a fairly high basal β-galactosidase level because of the presence of constitutive segregants.) Similarly, W3747 can transfer F-*lac* very efficiently to *lac*⁻ segregants (including partial diploid or polyploid strains) of anomalous constitutives. On the other hand, W3747 does not transfer F-*lac* at significant rates to *lac*⁻ bacteria that are F⁺ or Hfr. Thus, it can be concluded that the hyper-synthesizing recombinants are F⁻.

It is also interesting to note that while the F-*lac* episome can be eliminated by treatment with an acridine-orange (HIROTA and SNEATH 1961), the extra segments of hyper-synthesizing bacteria (e.g., E16 and E18), however, could not be eliminated by this dye.

(4) Location of the extra-chromosomal segment: Co-transducibility with chromosomal markers by P1: The hyper-synthesis character, like many other

bacterial traits, can be transduced by phage P1 (NOVICK and HORIUCHI 1961; HORIUCHI *et al.* 1962), and this phenomenon can be used to locate more exactly the extra sets of *lac* genes present in hyper bacteria. [The frequency of y^+ transductants of an $i^+z^+y^-$ strain by P1 grown on a constitutive hyper strain is roughly equal to that of P1 grown on a constitutive normal strain. However, y^+ transductants after successive reisolations on plates containing lactose (to purify the transductants of parental and other clones) do not show the hyper character. This probably results from the selection of normal haploid segregants from the original hyper transductants.]

To demonstrate such transduction one must consider the possibility that hyper transductants might result from the presence of several P1*dl* prophages (LURIA *et al.* 1960) rather than from direct transfer from the donor hyper strain. In fact, bacteria carrying several λ*dg* prophages have been found to produce several times as much galactokinase as normal *gal*$^+$ strains (E. B. HOROWITZ, personal communication). This possibility can be discounted if the transductants are sensitive to phage P1, a strong indication that they lack P1*dl* prophage.

The demonstration of transduction of the hyper-synthesis character can be made more convincing by use of the anomalous constitutive strain E17 ($i^+z^+y^-/i^-z^+y^+$) as the donor and E52 (z^-y^-, apparently a deletion) as the acceptor. The finding that all of the hyper transductants obtained in such a transduction are anomalous constitutives like E17 would provide direct evidence for the co-transduction of the extra sets of *lac* genes in a hyper-synthesizing strain. Such transductants would segregate *lac*$^-$ strains which are z^+y^- rather than z^-y^- like E52.

Such an experiment was performed and 11 *lac*$^+$ transductants were obtained. Of these, eight were found to be of the anomalous constitutive type and three to be standard constitutives. As anticipated, all of the anomalous constitutive transductants segregated i^- and i^+ and y^- and y^+ strains exactly as E17 and never any z^- as E52. Furthermore all eight were sensitive to phage P1, ruling out the presence of P1*dl* prophages.

The transduction of multiple sets of *lac* genes by a single phage particle points up another difference between the present hyper strains and the F-*lac* strains. In the latter case a single phage particle can carry *lac* genes from the chromosome or from the F episome but not from both at once (F. JACOB, personal communication).

(5) Affinity for the bacterial chromosome: The type of recombinants in a cross between a hyper-synthesizing strain and a normal strain (such as Cross I of Table 2, hyper Hfr $T6^Si^-z^+y^+pro^+str\text{-}S$ by F$^-$ $T6^Ri^+z^+y^-pro^-str\text{-}R$) gives an indication of the tightness of attachment to the chromosome of the extra *lac* genes in the hyper strain. If the binding were weak, one would expect a high percentage of the *lac*$^+$ recombinants to be anomalous constitutives of the type $i^+z^+y^-/i^-z^+y^+$ since these could form by association of the extra *lac* genes from the Hfr with the F$^-$ chromosome. Also, these would be $T6^R$, *pro*$^-$ and *str-R* since these markers would derive from the F$^-$ chromosome. Among the observed recombinants, however, the number of anomalous constitutives is less than ten

percent of the total *lac*$^+$ recombinants; the type *T6*^{R}*i*$^+$*z*$^+$*y*$^-$*pro*$^-$*str-R*/*i*$^-$*z*$^+$*y*$^+$ was found in only two of 22 anomalous constitutives.

It thus appears that the affinity of the extra *lac* segments to the *lac* region is fairly strong, detachment occurring apparently only when recombination takes place near the *lac* region, i.e., between the markers *T6* and *pro*.

DISCUSSION

Hyper strains are apparently able to make β-galactosidase at higher rates than normal strains because they have extra copies of the *lac* genes relative to all other genes. The extra *lac* genes are present in segments which include little, if anything, more than the *lac* region. Furthermore, all of the sets of *lac* genes present in a hyper-synthesizing strain are closely linked with the *lac* locus of the bacterial chromosome since they can be transferred with it in genetic conjugation and since they can be co-transduced by a single phage particle.

Beyond this little is known about the structure of these partial polyploids. The association of the extra *lac* segments with the bacterial chromosome is reminiscent of the extra bacterial genes in a λ*dg* heterogenote (LEDERBERG 1960). In both cases, the extra genes are lost at rates of the order of 10^{-3} per generation (segregant frequencies of 10^{-2}). Also, in both cases there is considerable genetic recombination, frequently of a nonreciprocal character. While the extra bacterial genes in a λ*dg* heterogenote are maintained by the mechanism which presumably normally holds an extra prophage, no such mechanism is apparent for the hyper-synthesizing strains.

Likewise, nothing is known about the nature of the event giving rise to polyploidy for the *lac* genes. Conceivably, the partial polyploids might be the result of some kind of mating. This could occur since the Hfr strain used for selection of the hyper strains might mate with an F$^-$ phenocopy. Such a mechanism, however, does not readily account for the restriction of polyploidy to the *lac* region. Alternatively, one could imagine some mutational mechanism to account for the origin of the hyper-synthesizing strains. The fact remains, however, that there is a mechanism for the production of partial polyploids such as are found in the present case.

SUMMARY

The genetic basis of increased β-galactosidase synthesis in strains of *Escherichia coli* (hyper-synthesizing strains), which can produce β-galactosidase at rates up to four times that of wild-type strains, has been studied. Extra segments carrying the *lac* region were shown to exist in these strains and to account for the hyper-synthesis of β-galactosidase. The extra segments do not extend to the neighboring *T6* and *pro* markers, and are not associated with the sex factor. They can be transduced jointly with the *lac* region of the bacterial chromosome by a single transducing phage (P1), and appear to be fairly tightly associated with the *lac* region of the bacterial chromosome.

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