A Search for a General Phenomenon of Adaptive Mutability

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Manuscript received January 2, 1996
Accepted for publication March 14, 1996

ABSTRACT

The most prominent systems for the study of adaptive mutability depend on the specialized activities of genetic elements like bacteriophage Mu and the F plasmid. Searching for general adaptive mutability, we have investigated the behavior of *Salmonella typhimurium* strains with chromosomal *lacZ* mutations. We have studied 30 revertible nonsense, missense, frameshift, and insertion alleles. One-third of the mutants produced ≥10 late revertant colonies (appearing three to seven days after plating on selective medium). For the prolific mutants, the number of late revertants showed rank correlation with the residual β-galactosidase activity; for the same mutants, revertant number showed no correlation with the nonselective reversion rate (from fluctuation tests). Leaky mutants, which grew slowly on selective medium, produced late revertants whereas tight nongrowing mutants generally did not produce late revertants. However, the number of late revertants was not proportional to residual growth. Using total residual growth and the nonselective reversion rate, the expected number of late revertants was calculated. For several leaky mutants, the observed revertant number exceeded the expected number. We suggest that excess late revertants from these mutants arise from general adaptive mutability available to any chromosomal gene.

THE orthodox neo-Darwinian view of mutability postulates that all mutations occur without regard to their fitness value; in this sense they are "random". This view excludes the notion of "adaptive" mutability, the preferential production of fitness-enhancing mutations. Classical experiments demonstrated that some mutability is random (LURIA and DELBRÜCK 1943; LEDERBERG and LEDERBERG 1952). However, DELBRÜCK (1946), SHAPIRO (1984), and CAIRNS *et al.* (1988) have pointed out the lack of evidence to exclude the possibility that another fraction of total mutability is adaptive.

Reviving this question, SHAPIRO (1984), CAIRNS *et al.* (1988), and CAIRNS and FOSTER (1991) described experimental systems that appeared to demonstrate adaptive mutability. However, the participation of complex genetic elements like bacteriophage Mu and the F plasmid has complicated these prominent bacterial examples. In these systems, the locus under selection shows increased mutability during starvation because it is experimentally coupled to the transposase-dependent excision of Mu in one case (SHAPIRO and LEACH 1990; MAENHAUT-MICHEL and SHAPIRO 1994; FOSTER and CAIRNS 1994) or mutationally derepressed transfer functions of F in the other (FOSTER and TRIMARCHI 1995; GALITSKI and ROTH 1995; PETERS and BENSON 1995; RADICELLA *et al.* 1995a). While these systems have provided examples of adaptive mutability, their singular complexity makes one suspect that they may be examples of limited generality. The evolutionary relevance of these novel systems is the subject of lively debate (BRIDGES 1995a; CAIRNS 1995; LENSKI and SNIEGOWSKI 1995; RADICELLA *et al.* 1995b; ROTH and GALITSKI 1995; SHAPIRO 1995a,b).

Because of the great attention received by the F-based system, some of its most striking characteristics have become associated with adaptive mutability. In particular, mutability of nongrowing cells (CAIRNS and FOSTER 1991; FOSTER 1994) and dependence on homologous recombination functions (CAIRNS and FOSTER 1991; HARRIS *et al.* 1994) are cited. Neither of these properties is, *a priori*, a characteristic of adaptive mutability. We suggest that adaptive mutability should be a more general phenomenon. It should be easily detectable without resorting to complex genetic systems that require transposon or conjugation functions, and it should operate under naturally relevant conditions of slow growth, not only in nongrowing cells.

The possibility of adaptive mutability has been investigated in various bacterial and yeast systems (reviewed in FOSTER 1991, 1992, 1993; HALL 1992; STAHL 1992; LENSKI and MITTLER 1993; ROSENBERG 1994; SYMONDS 1994; BRIDGES 1995b). Since organisms typically exist under relatively adverse or selective conditions, the potential importance of adaptive mutability is great. Most of what we know of mutational mechanisms involves the study of microorganisms growing rapidly under favorable conditions. A thorough investigation of mutational mechanisms under conditions of strong selection and slow growth may reveal new aspects of mutability with evolutionary implications.

We have undertaken a systematic study of the rever-

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sion of a collection of strains carrying various mutant alleles of a single gene, *lacZ*. In this collection, the locus under selection is chromosomally situated; reversion events are not obviously associated with the specialized activities of any extraneous genetic element. The goal was to correlate various characteristics of mutant strains with their ability to revert under selection. We found that leaky *lacZ* mutants show reversion under selection whereas tight nongrowing mutants generally show no late reversion. Late reversion of leaky chromosomal *lacZ* mutants does not require homologous recombination functions. This behavior is expected if there is no adaptive mutability. However, many of these strains produce an excess of revertants under selective conditions compared with the number expected based on observed growth and nonselective reversion rates. We suggest that these findings reveal a general phenomenon of adaptive mutability.

MATERIALS AND METHODS

Bacterial strains and growth media: All bacterial strains used in this study (Table 1) are derivatives of *Salmonella typhimurium* LT2.

The defined minimal medium was NCE salts (BERKOWITZ *et al.* 1968) with 0.2% of the appropriate carbon source plus auxotrophic supplements at final concentrations recommended by DAVIS *et al.* (1980). The complex medium was nutrient broth, NB, (8 g/liter, Difco Laboratories) with added NaCl (5 g/liter). Solid media contained BBL agar at 1.5%. MacConkey agar was obtained from Difco laboratories and prepared according to the manufacturer's instructions. Final concentrations of antibiotics in complex medium were as follows: tetracycline hydrochloride, Tc, 20 μg/ml; and kanamycin sulfate, Km, 50 μg/ml. The β-galactosidase chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), was added to a final concentration of 25 μg/ml. The nonmetabolizable inducer of the *lac* operon, isopropyl-β-D-thiogalactopyranoside (IPTG), was used at a final concentration of 1 mM. All incubations were at 37° except as noted. Sterile 0.85% NaCl (saline) was used to dilute cultures except as noted.

Transductional crosses, mediated by the phage mutant P22 HT105/1 *int-201* (SCHMIEGER 1971), were performed as described by ROTH (1970a). Recombination-deficient derivatives of *lacZ* mutants were constructed by first introducing the *srl-203*::Tn*10*dCm mutation by transduction; the linked *recA1* mutation was cotransduced into these strains selecting a Srl^+ phenotype on NCE sorbitol histidine plates.

Isolation of lac mutants: To reference strain SGSC180 (*ara-9*) an insertion of MudF(*lacI*$^+$ *Z*$^+$ *Y*$^+$ *A*$^+$ Km^R) in the *hisC* gene was introduced by transduction, resulting in strain TT18519 (*ara-9 hisC10081*::MudF). The MudF insertion of this strain is oriented such that *lac* operon transcription is opposite that of the *his* operon (data not shown; Figure 1). This element is unable to catalyze its own transposition or excision since it lacks all Mu genes, including the Mu *A* and *B* transposase genes.

Mutant Lac^- derivatives of strain TT18519 were isolated after mutagenesis with either DES (diethyl sulfate) or ICR-191 [2-chloro-6-methoxy-9-[3-(2-chloroethyl) aminopropylamino] acridine dihydrochloride]. These mutageneses were carried out as described by ROTH (1970a). Insertion mutations were made using Tn*10*dTc, the transposition-defective Tn*10*-derived element No. 11 of WAY *et al.* (1984). Methods for use of the Tn*10*dTc element were those of KLECKNER *et al.* (1991). Independent Lac^- mutants were isolated after each mutagenesis by looking for white mutant colonies among the red colonies on MacConkey agar plates. Each *lac* mutation was transductionally backcrossed to the unmutagenized parent strain SGSC180 at least once by selecting the kanamycin resistance of the MudF element on NB Km plates. Transductants from these backcrosses were saved for further study. For all mutants used, the Lac^- phenotype was 100% linked to the kanamycin resistance determinant. This procedure ensured that only *lacZ* or *lacY* mutants were saved and that other unlinked mutations were not present in the isolates saved for further study.

Qualitative β-galactosidase assay: To classify *lac* mutants as either *lacZ* or *lacY* types, qualitative assays of β-galactosidase activity were performed. Mutants of *lacZ* have little or no β-galactosidase activity whereas *lacY* mutants have wild-type levels of β-galactosidase activity. Overnight cultures of strains were grown in NCE glycerol histidine IPTG medium. To 0.9 ml of Z buffer (MILLER 1972), 50 μl of $CHCl_3$ and 0.1 ml of overnight culture were added. These mixtures were vortex mixed for 10 sec and incubated at room temperature for 10 min. Reactions were started by adding 0.2 ml of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG). After 10 min at room temperature, the development of yellow color (nitrophenol production) was noted. Assays of *lacY* mutants produce strong yellow color whereas assays of *lacZ* mutants produce little or no yellow color. The *lac* operon structure (*lacZYA*) prevents mistaken interpretations due to polarity—*lacZ* is the first gene of the operon.

Classification of DES-induced mutations: Mutants isolated after DES mutagenesis were classified by testing for informational suppression. The *lacZ* mutations were introduced to suppressor strains by transduction crosses selecting the Km^R determinant of the MudF element on MacConkey Km plates. Transductant colonies were either white like their donor parents (unsuppressed) or red (suppressed). The suppressors (Table 1) included: four amber (UAG) suppressor tRNAs (*supD*, *supE*, *supF*, and *supJ*), three ochre (UAA) suppressor tRNAs (*tyrU*, *supC*, and *supG*), a recessive opal (UGA) suppressor (*supK*), as well as a missense suppressor, *sumA*, that suppresses ~25% of all missense mutations (WHITFIELD *et al.* 1966; K. HUGHES, L. MIESEL, M. MESERVY, and E. ALTMAN, personal communications). Strains classified as amber mutants were suppressed by all of the amber and ochre suppressors and by no others. Recall that ochre tRNA suppressors suppress both ochre (UAA) and amber (UAG) mutations whereas amber tRNA suppressors suppress only amber mutations. No ochre mutations were found in our set. Mutations classified as opal were suppressed by only the *supK* suppressor. The missense suppressor, *sumA*, suppressed one allele, *lacZ479*m. Four other DES-induced mutations were not suppressed by any of these suppressors. These four, as well as *lacZ479*m, were found to be nonpolar (see below) and thus are likely to be missense alleles. All DES-induced mutations were checked for temperature sensitivity of growth on NCE lactose Xgal histidine plates. No temperature sensitivity was found at 30, 37, and 42°. This spectrum of DES-induced mutations coincides closely with that reported previously (LANGRIDGE and CAMPBELL 1969) after mutagenesis of *lacZ* with MNNG (N-methyl-N′-nitro-N-nitrosoguanidine, another alkylating agent). All the *lacZ* alleles of Table 1, other than the frameshifts and the Tn*10*dTc insertions, were induced by DES.

Classification of mutations induced by ICR-191: Mutants isolated after treatment with ICR-191 (a DNA-intercalating acridine compound) were most likely to be frameshift mutants (AMES and WHITFIELD 1966; BRAMMAR *et al.* 1967). Mutants that were induced to revert to Lac^+ by ICR-191 were

TABLE 1

Bacterial strains

Strain[a]	Genotype[b]
SGSC180 = TR6611	*ara-9*
TT18518	*ara-9 metE205 melB398*
TT18519	*ara-9 hisC10081*::MudF(*lac*$^+$)
TT18520	*ara-9 hisC10081*::MudF(*lacZ479*m)
TT18521	*ara-9 hisC10081*::MudF(*lacZ480*op)
TT18522	*ara-9 hisC10081*::MudF(*lacZ481*am)
TT18523	*ara-9 hisC10081*::MudF(*lacZ482*op)
TT18524	*ara-9 hisC10081*::MudF(*lacZ483*op)
TT18525	*ara-9 hisC10081*::MudF(*lacZ484*am)
TT18526	*ara-9 hisC10081*::MudF(*lacZ485*op)
TT18527	*ara-9 hisC10081*::MudF(*lacZ486*)
TT18528	*ara-9 hisC10081*::MudF(*lacZ487*am)
TT18529	*ara-9 hisC10081*::MudF(*lacZ488*am)
TT18530	*ara-9 hisC10081*::MudF(*lacZ489*am)
TT18531	*ara-9 hisC10081*::MudF(*lacZ492*am)
TT18532	*ara-9 hisC10081*::MudF(*lacZ493*)
TT18533	*ara-9 hisC10081*::MudF(*lacZ494*am)
TT18534	*ara-9 hisC10081*::MudF(*lacZ495*)
TT18535	*ara-9 hisC10081*::MudF(*lacZ497*)
TT18536	*ara-9 hisC10081*::MudF(*lacZ499*am)
TT18537	*ara-9 hisC10081*::MudF(*lacZ500*::Tn*10*dTc)
TT18538	*ara-9 hisC10081*::MudF(*lacZ501*::Tn*10*dTc)
TT18539	*ara-9 hisC10081*::MudF(*lacZ502*::Tn*10*dTc)
TT18540	*ara-9 hisC10081*::MudF(*lacZ503*::Tn*10*dTc)
TT18541	*ara-9 hisC10081*::MudF(*lacZ505*::Tn*10*dTc)
TT18542	*ara-9 hisC10081*::MudF(*lacZ507*::Tn*10*dTc)
TT18543	*ara-9 hisC10081*::MudF(*lacZ511*::Tn*10*dTc)
TT18544	*ara-9 hisC10081*::MudF(*lacZ4632*f+1)
TT18545	*ara-9 hisC10081*::MudF(*lacZ4633*f+1)
TT18546	*ara-9 hisC10081*::MudF(*lacZ4634*f−1)
TT18547	*ara-9 hisC10081*::MudF(*lacZ4635*f−1)
TT18548	*ara-9 hisC10081*::MudF(*lacZ4638*f+1)
TT18549	*ara-9 hisC10081*::MudF(*lacZ4639*f−1)
TT18753	*ara-9 hisC10081*::MudF(*lac*$^+$) *hisG10051*::Tn*10*dTc
TT18754	*ara-9 hisC10081*::MudF(*lacZ489*am) *hisG10051*::Tn*10*dTc
TT18758	*ara-9 hisC10081*::MudF(*lacZ489*am)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT18759	*ara-9 hisC10081*::MudF(*lacZ489*am)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT18760	*ara-9 hisC10081*::MudF(*lacZ489*am)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT18761	*ara-9 hisC10081*::MudF(*lacZ480*op)*hisG10051*::Tn*10*dTc
TT18765	*ara-9 hisC10081*::MudF(*lacZ480*op)*hisG10051*::Tn*10*dTc day 5 Lac$^+$ revertant
TT18766	*ara-9 hisC10081*::MudF(*lacZ480*op)*hisG10051*::Tn*10*dTc day 5 Lac$^+$ revertant
TT18767	*ara-9 hisC10081*::MudF(*lacZ480*op)*hisG10051*::Tn*10*dTc day 5 Lac$^+$ revertant
TT18768	*ara-9 hisC10081*::MudF(*lacZ4638*f+1)*hisG10051*::Tn*10*dTc
TT18772	*ara-9 hisC10081*::MudF(*lacZ4638*f+1)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT18773	*ara-9 hisC10081*::MudF(*lacZ4638*f+1)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT18774	*ara-9 hisC10081*::MudF(*lacZ4638*f+1)*hisG10051*::Tn*10*dTc day 4 Lac$^+$ revertant
TT9167	*leuA414*am *supD10*
TT9168	*leuA414*am *supE20*
TT9169	*leuA414*am *supF30*
TT9170	*leuA414*am *supJ60*
TT2839	*leuA414*am *hisC527*am *zii-614*::Tn*10 tyrU90*
TT4217	*leuA414*am *supG50*
TT13029	*leuA414*am *hisC527*am *zde-605*::Tn*10 supC80*
TR1975	*his01242 hisD6500 supK584*
TT16237	*hisC537 zif-3693*::Tn*10*dTc *sumA10*
TR6625	*strA1*

[a] All strains are derivatives of *Salmonella typhimurium* LT2. Strain SGSC180 was provided by K. SANDERSON and the Salmonella Genetic Stock Center. Strains TT18518–TT18774 were isolated or constructed during the course of this work. The remainder are from the ROTH laboratory collection.

[b] Abbreviated allele descriptors following *lacZ* allele numbers represent the following: am, amber (UAG); op, opal (UGA); f−1, −1 frameshift; f+1, +1 frameshift; m, missense; ::Tn*10*dTc, insertion of a transposition-defective Tn*10*. The *lacZ* mutations lacking descriptors are likely missense alleles. The full genotypes of Lac$^+$ revertant strains are unknown.

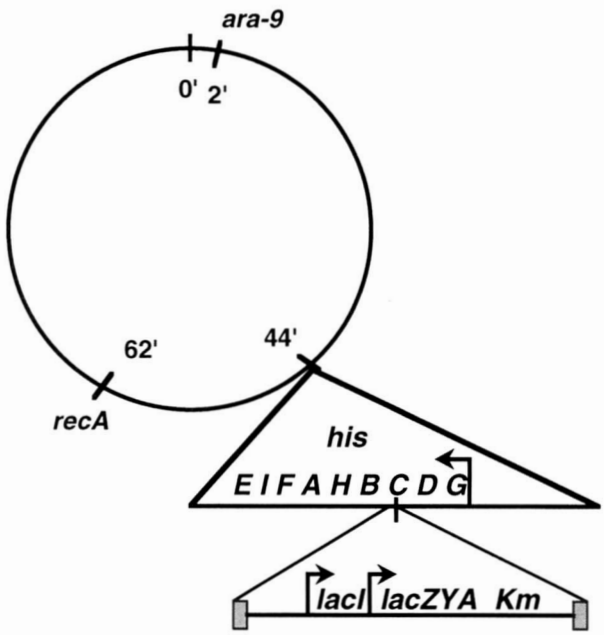

FIGURE 1.—A map of the *Salmonella* chromosome illustrating relevant markers (see Table 1). The structure of the *his* locus interrupted by an insertion of the Mu-derived element, MudF, is magnified. MudF is a transpositionally inert element with Mu ends but no Mu functions. It includes a wild-type *lac* locus and a kanamycin resistance determinant (CHACONAS *et al.* 1981; SONTI 1990). Salmonellae are naturally Lac$^-$; the *lac* genes are derived from *Escherichia coli* K12.

saved for further study. It was also possible to classify frameshift mutations as likely +1 or −1 shifts by screening for an induction of reversion with MNNG. MNNG tends to revert +1 frameshifts but not −1 frameshifts (RIDDLE and ROTH 1970). Two NCE lactose Xgal histidine plates were spread with 0.1-ml aliquots from an overnight culture of each Lac$^-$ strain. On one plate a 20-μl drop of a 1 mg/ml solution of MNNG in citrate buffer (MILLER 1972) was placed in the center. Similarly, a 1 mg/ml solution of ICR-191 in 70% ethanol was applied to the other. Solutions and plates with ICR-191 were handled and incubated in subdued light. Plates were incubated for 3 days and scored for a pronounced induction of revertants around the zone of killing.

Recombinational grouping of Tn*10*dTc insertions: Since Tn*10* elements show insertion site specificity (KLECKNER *et al.* 1979), some independent *lacZ* insertion alleles were likely to have the insertion at the same site. To study only insertions at distinct sites, insertion strains were placed into groups of mutants that failed to recombine with each other to give Lac$^+$ transductants. Insertion mutants were crossed to each other by transduction selecting Lac$^+$ on NCE lactose Xgal histidine plates. For each strain, a cross with a *lac*$^+$ donor and a self-cross served as positive and negative controls respectively. Seven groups of nonrecombining insertions were found; only one member of each recombination group was tested further.

Tests of polarity: Classification of *lacZ* mutations was extended by testing for polarity on the downstream *lacY* gene. The LacY permease can substitute for the MelB melibiose permease (KENNEDY 1970). Accordingly, we crossed the MudF elements from the *lacZ* mutants into a *melB* deletion strain and screened for utilization of melibiose as a sole source of carbon and energy. MudF elements with *lacZ* mutations were crossed into strain TT18518 (*ara-9 metE205 melB398*) selecting Km^R on NB Km plates. For each cross, four transductants were screened for growth on an NCE melibiose histidine methionine plate.

Fluctuation tests: The nonselective reversion rate of each *lacZ* mutant was measured using the fluctuation test of LURIA and DELBRÜCK (1943). From a single colony, an overnight culture was grown in NCE glycerol histidine medium and diluted 10^5-fold in fresh medium. At least 30 0.5-ml aliquots of this dilution were dispensed to tubes and incubated to saturation. To verify the independence of mutants found in the 0.5-ml cultures, the original overnight culture was sampled (0.1 ml) and plated on an NCE lactose Xgal histidine plate. Revertant colonies were counted after 40 hr of incubation. To determine the total number of colony-forming units in the saturated independent cultures, one was chosen at random. Its volume was measured (some was lost due to evaporation). A sample was diluted and plated on nutrient agar plates. To all cultures, 2.5 ml of molten NCE lactose Xgal histidine with 0.7% agar was added. These were mixed, poured onto an NCE lactose Xgal histidine plate, incubated for 40 hr, and scored for the numbers of Lac$^+$ colonies. Since some strains continually produce Lac$^+$ revertants with time, reconstruction control experiments were performed to determine the optimal incubation time to score the number of Lac$^+$ mutants present at the time of plating. This time was 40 hr (data not shown). Mutation rates were calculated using the P_0 method (LURIA and DELBRÜCK 1943) or the method of the median (LEA and COULSON 1949).

Modified quantitative β-galactosidase assays: Assays were performed, and units of β-galactosidase activity were calculated, as described by MILLER (1972). The following modifications were employed to allow the reliable measurement of low activities: (1) Cultures of assayed strains were grown in NCE glycerol histidine with or without IPTG to 100 Klett. The use of glycerol increases induced β-galactosidase levels about sixfold (due to Crp/cAMP control) compared with growth on glucose (data not shown). (2) Higher concentrations of bovine serum albumin and 2-mercaptoethanol were used to stabilize β-galactosidase activity. To Z buffer, we added bovine serum albumin to 100 μg/ml, 2-mercaptoethanol to 200 mM, and sodium dodecyl sulfate to 0.005%. (3) Mixtures of Z buffer and culture were prepared in 15-ml polypropylene screw-cap tubes to prevent evaporation during extended incubations. Cells were permeabilized by vortex mixing with two drops of chloroform per milliliter of mixture. (4) Reactions were started by adding the substrate, ONPG. Reaction times were extended over as much as 3 days to allow sufficient product development. (5) Reactions were monitored by withdrawing 1-ml samples over time, mixing these with 0.42 ml of 1 M Na_2CO_3 in a microcentrifuge tube, centrifuging the sample for 1 min to remove most cellular debris, removing 1 ml of the supernatant to a cuvette, and measuring absorbances of 550 and 420 nm light. Sample clarification eliminates most of the experimental error associated with the correction for bacterial absorbance of 420 nm light.

The ability of the modified assay to reliably measure low activities was tested. A culture of the *lac*$^+$ parent strain, TT18519, was diluted 10^3-, 10^4-, and 10^5-fold before assay. The undiluted culture of strain TT18519 had 5700 units of activity, whereas the dilutions were determined to have 5.5, 0.42, and 0 units, respectively. All assays that required extended (>40 hr) incubation showed a significant loss of activity (even diluted wild-type extracts); thus assays of strains with a very low reported activity are likely to underestimate total activity.

Reversion on selective medium: The ability of *lacZ* mutants to produce late revertants was measured. Independent cultures (started from different single colonies) of test strains were grown to full density in NCE glycerol histidine medium. Cells were pelleted and resuspended in an equal volume of

saline. From each culture, ~5 × 10^8 tester cells (0.1 ml) were spread on a plate of selective medium, NCE lactose Xgal histidine. Histidine was included as an essential nutrient for these auxotrophic strains; it does not provide a carbon or energy source (GUTNICK *et al.* 1969). The chromogenic substrate Xgal was included to facilitate the scoring of Lac$^+$ revertant colonies. Plates were incubated at 37° in loosely closed plastic sleeves to minimize drying. New Lac$^+$ colonies were counted each day for 7 days of incubation. Ten independent cultures were tested for each strain. In some experiments (noted in RESULTS), strains were plated with a fivefold excess of scavenger cells to consume carbon and energy sources other than lactose that might be present in the medium or excreted by Lac$^+$ revertant cells. Scavenger cells of the Lac$^-$ strain SGSC180 (*ara-9*) or strain TR6625 (*strA1*) were prepared in the same way as the tester cells. The strains tested both in the presence and absence of scavengers included TT18520 (*lacZ479*m), TT18521 (*lacZ480*op), TT18522 (*lacZ481*am), TT18523 (*lacZ482*op), TT18524 (*lacZ483*op), TT18525 (*lacZ484*am), TT18526 (*lacZ485*op), TT18527 (*lacZ486*), TT18528 (*lacZ487*am), and TT18529 (*lacZ488*am).

Growth on selective medium: To determine the number of viable Lac$^-$ tester cells per plate over time, agar plugs were taken (avoiding Lac$^+$ colonies) from selective platings described above for reversion experiments. Bacteria on 20 mm^2 agar plugs were suspended in saline by vigorous vortex mixing for 15 sec followed by 15 min of benchtop incubation without agitation and another 15-sec vortex mixing. Dilutions were prepared in saline, spread on nutrient agar plates (with kanamycin for assaying tester strains in the presence of a scavenger strain), and incubated. The numbers of colonies on nutrient agar plates were multiplied by their respective dilution factors and 289 (the ratio of the area of the agar plate to the area of the agar plug) to determine the number of Lac$^-$ tester cells on the selective plates.

RESULTS

Experimental system: We have characterized the reversion and residual β-galactosidase levels of 30 *lacZ* mutants derived from a parental Lac$^+$ derivative of *Salmonella typhimurium* LT2. Salmonellae are naturally Lac$^-$; the *lac* operon was introduced as part of a MudF element inserted in the *hisC* gene (Figure 1). MudF is a transpositionally inert Mu-derived element with Mu ends flanking a wild-type *lac* locus and a kanamycin resistance (Km^R) determinant (CHACONAS *et al.* 1981; SONTI 1990). Since it lacks all Mu genes, including the *A* and *B* transposase genes, it has no transposition activity. None of the Lac$^+$ reversion events occurring in the *his*::MudF system was associated with excision or transposition of Mu; all revertants tested had the original MudF insertion and no others (T. GALITSKI, unpublished results). The *his*::MudF construction was used because its His$^-$ Km^R phenotype facilitates genetic manipulation. The *lac* genes can be transduced to a new strain by selecting Km^R; they can be removed from a strain by selecting His$^+$.

The *lacZ* mutant set: Independent mutants of the *lacZ* gene, encoding β-galactosidase, were isolated after mutageneses of strain TT18519 (*lac*$^+$) with one of three different mutagens, DES (diethyl sulfate, an alkylating agent), ICR-191 (a frameshift-inducing acridine compound), and Tn*10*dTc (a transposition-defective Tn*10* derivative). Lac$^-$ mutants were isolated by screening for white colonies among the red (Lac$^+$) colonies on MacConkey agar plates. Since MacConkey medium indicates acid production from lactose catabolism but allows both Lac$^+$ and Lac$^-$ colonies to grow, white colonies isolated from MacConkey plates were checked for their ability to use lactose as a sole source of carbon and energy. The chosen mutants failed to grow overnight when patches were replicated to NCE lactose Xgal histidine plates; extremely leaky *lac* mutants were excluded. Transductional backcrosses (selecting the Km^R phenotype of MudF) to strain SGSC180 (*ara-9 his*$^+$) were performed to ensure that the Lac$^-$ phenotype was due to a mutation of the *lac* operon (*lacI, lacZYA*) and to eliminate any unlinked mutations incurred during mutagenesis. One transductant from each cross was saved for further study. Mutants of the *lacA* gene are Lac$^+$; they were systematically excluded. Mutants of the *lacY* gene, encoding lactose permease, were eliminated based on the results of a qualitative β-galactosidase assay (see MATERIALS AND METHODS); *lacY* mutants have wild-type levels of β-galactosidase activity whereas *lacZ* mutants show a loss of activity. Rare mutant *lacI* alleles encoding a noninducible repressor (Lac$^-$) would have been classified in our tests as "polar" missense (most likely) mutations (classifications described below). Since any *lacI* null mutation will suppress these dominant mutations, such strains should show nonselective reversion rates ≥100 times higher than the highest rates observed in our set. We found no mutants resembling this description. Also, nonrevertible *lac* mutants (deletions, double mutations) were excluded. Thus, the mutant set includes only revertible *lacZ* alleles.

Classification of *lacZ* mutations: Various tests were employed to classify *lacZ* mutations without resorting to sequence determination. These tests included screens for informational suppression, induced reversion with different mutagens, polarity on the *lacY* gene, temperature sensitivity, and recombination (see MATERIALS AND METHODS). The resulting classifications are indicated by allele descriptors (Table 1 and Table 2). Mutagenesis with DES gave a distribution of alleles including all the amber (UAG), opal (UGA), and missense types. None of these showed any temperature sensitivity; they had the same Lac$^-$ phenotype at 30, 37, and 42°. Frameshift mutagenesis with ICR-191 produced both +1 and −1 frameshift alleles. Multiple independent insertions of Tn*10*dTc were isolated. Insertions at distinct sites were identified by recombination tests and included in this study. To further classify the *lacZ* alleles, polarity on the downstream *lacY* gene was assessed (see MATERIALS AND METHODS and Table 2). Insertion mutations exhibited strong polarity. Nonsense and frameshift alleles showed a wide spectrum of polarities. This spectrum is known to correlate with position in the *lacZ* gene (reviewed in ZIPSER 1970). Suspected missense alleles showed no polarity.

TABLE 2

Polarity, nonselective reversion rate, late revertants, and residual β-galactosidase activity of *lacZ* mutants

lacZ allele[a]	Polarity on *lacY*[b]	Nonselective reversion rate[c]	Late revertants[d]	Residual β-galactosidase activity[e]
*489*am	Polar	1.4	345 ± 30	13
*482*op	Polar	1.7	85 ± 9	0.83
*480*op	Polar	1.9	72 ± 8	1.3
*483*op	Polar, weak	2.1	71 ± 6	0.37
*485*op	Polar, weak	0.82	61 ± 9	0.57
*4635*f−1	Polar, weak	0.40	40 ± 8	2.3
*4638*f+1	Polar	7.7	30 ± 2	0.09
*4634*f−1	Nonpolar	3.7	17 ± 2	0.11
*492*am	Nonpolar	3.0	17 ± 2	0.13
*4633*f+1	Polar	0.55	11 ± 1	0.81
*4632*f+1	Polar	0.89	9 ± 1	0.17
*488*am	Polar	7.1	6 ± 2	0.08
*487*am	Nonpolar	1.1	6 ± 1	0.28
*4639*f−1	Nonpolar	0.39	6 ± 1	6.7
*494*am	Nonpolar	0.80	5 ± 1	0.09
*484*am	Nonpolar	2.4	3 ± 1	0.05
486	Nonpolar	0.76	3 ± 1	0.45
*481*am	Nonpolar	2.5	2 ± 1	0.16
*499*am	Nonpolar	2.7	2 ± 1	0.53
*479*m	Nonpolar	0.24	2 ± 1	0.08
505::Tn*10*dTc	Polar	0.038	1 ± 1	0
500::Tn*10*dTc	Polar	0.33	1 ± 1	0
495	Nonpolar	0.15	1 ± 1	0.65
493	Nonpolar	0.12	0 ± 1	0.29
497	Nonpolar	0.11	0 ± 0	0.55
501::Tn*10*dTc	Polar	0.20	0 ± 0	0
502::Tn*10*dTc	Polar	0.023	0 ± 0	0
503::Tn*10*dTc	Polar	0.19	0 ± 0	0
507::Tn*10*dTc	Polar	0.19	0 ± 0	0
511::Tn*10*dTc	Polar	0.033	0 ± 0	0

[a] *lacZ* allele number and type. The abbreviations used represent the following: am, amber (UAG); op, opal (UGA); f−1, −1 frameshift; f+1, +1 frameshift; m, missense; ::Tn*10*dTc, insertion of a transposition-defective Tn*10*. The mutations lacking descriptors are likely missense alleles.

[b] Polarity of *lacZ* alleles on *lacY* gene expression was assessed by screening for melibiose utilization in a *melB* deletion background (see MATERIALS AND METHODS).

[c] Reversion rate to Lac^+ (revertants per cell division $\times 10^9$) during growth without selection for Lac^+ (from fluctuation analysis, $n \geq 30$).

[d] Number (mean ± SEM, $n = 10$) of Lac^+ revertant colonies appearing from day 3 through day 7 of incubation on NCE lactose Xgal histidine plates. Error values are the standard error of the mean, SEM = $\sqrt{(\text{var}/n)}$.

[e] Residual β-galactosidase activity in Miller units. Note that the modified assay employed here may underestimate activities <0.4 units (see MATERIALS AND METHODS).

Prevalence of late reversion: Late reversion was not a universal phenomenon. This has been reported previously by HALL (1991), studying active-site frameshift and missense alleles of *lacZ* (CUPPLES and MILLER 1989; CUPPLES *et al.* 1990), and by FOSTER (1993), studying 20 ICR-191-induced *lacZ* and *ara* mutations (probably frameshifts). Among our set of 30 revertible *lacZ* mutants, only a minority showed a significant number of late revertants first appearing between 3 and 7 days of incubation under selective conditions. The panel of *lacZ* mutants was screened for the accumulation of Lac^+ revertants during a week of incubation on selective medium. Independent cultures were grown nonselectively in NCE glycerol histidine medium, pelleted, and resuspended in saline. About 5×10^8 cells were spread on plates of selective medium, NCE lactose Xgal histidine. New Lac^+ revertant colonies were counted on each of 7 days of incubation. All mutants showed some reversion during nonselective growth preceding selective plating; revertants present at the time of plating produce macroscopic colonies observable on day 2. However, most *lacZ* mutants (20 of 30) showed zero or very few (<10) additional revertants appearing after day 2. Only 10 of 30 mutants produced a noteworthy number of late

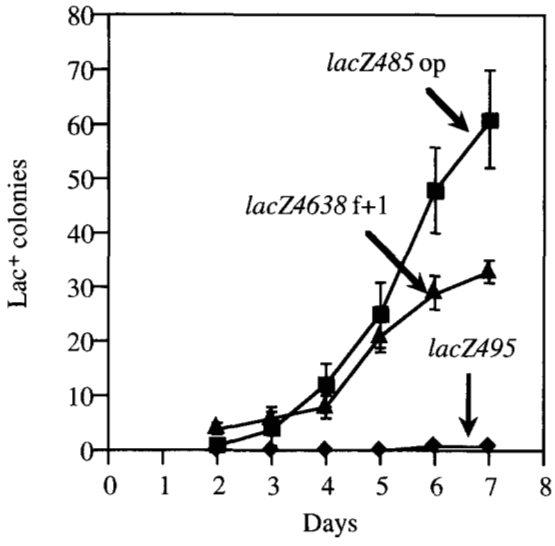

FIGURE 2.—Reversion kinetics on minimal lactose medium. Strains grown to saturation in NCE glycerol histidine medium were pelleted and resuspended in saline. About 5×10^8 cells were spread on NCE lactose Xgal histidine plates and incubated at 37°. Every day, new Lac^+ colonies were counted. Data points are mean ± SEM, $n = 10$. The strains represented are TT18526 (*lacZ485*op, ■), TT18548 (*lacZ4638*f+1, ▲), and TT18534 (*lacZ495*, ◆).

revertants (first appearing on day 3 through day 7). The average total number of late revertants from these 10 strains ranged from 11 to 345. Table 2 lists the *lacZ* mutants in descending order of their late revertant totals.

The reversion kinetics of some representative strains are plotted in Figure 2. Most strains, like TT18534 (*lacZ495*), showed no accumulation of revertants with time. Others like TT18548 (*lacZ4638*f+1), and TT18526 (*lacZ485*op), produced intermediate or high numbers of revertants during a week of selective incubation. Among the 10 mutants showing ≥10 late revertants, reversion kinetics varied. Seven of these 10 strains showed increasing rates of revertant accumulation early, followed by decreasing rates later [TT18526 (*lacZ485*op, Figure 2); TT18548 (*lacZ4638*f+1, Figure 2); TT18530 (*lacZ489*am, Figure 4); TT18524 (*lacZ483*op, Figure 4); TT18523 (*lacZ482*op); TT18521 (*lacZ480*op); TT18545 (*lacZ4633*f+1)]. Three exceptional strains were observed. Strain TT18546 (*lacZ4634*f−1) accumulated revertants at a constant rate. Strain TT18547 (*lacZ4635*f−1) showed exponential reversion kinetics. One strain, TT18531 (*lacZ492*am), produced a wave of late revertants (94% of the total) during the final day of the experiment.

Late reversion occurring under selective conditions: We tested whether late-appearing Lac^+ revertants were preexisting or arose under selective conditions. It was possible that revertants appearing after day 2 were present at the time of selective plating but took longer than 2 days to produce macroscopic colonies. If the late appearance of a colony were due to slow growth, this should be apparent in a reconstruction experiment in which revertant cells are seeded and incubated selectively with a lawn of their nonrevertant parent strain. Preexisting slow-growing revertant strains would require the same time to form colonies on selective medium as they did at their original isolation; strains that attained a fully revertant genotype after selective plating would require less time to form a colony.

Three independent late revertants from each of three strains, TT18521 (*lacZ480*op), TT18530 (*lacZ489*am), and TT18548 (*lacZ4638*f+1), were picked from selective plates and purified nonselectively on NB Xgal IPTG plates. To these, as well as their Lac^- parents and the $lacZ^+$ grandparent, the *hisG10051*::Tn*10*dTc mutation was added by transduction to serve as a marker for use in the reconstruction experiment. This *hisG* marker was chosen because it would introduce tetracycline resistance while disrupting an operon that is already disrupted (Figure 1); thus, no unnecessary phenotypes are introduced. About 30–50 of these marked revertant cells were seeded in a suspension of their Lac^- parent prepared as described above. Aliquots of these mixtures were spread and incubated on NB Tc plates to determine the approximate numbers of seeded Tc^R cells (data not shown) and on NCE lactose Xgal histidine plates to observe the time required for marked Lac^+ revertant cells to form colonies. Each day, new revertants were picked and their phenotypes, including sensitivity to tetracycline, were scored (Table 3). The nine late revertants had originally appeared after 4 or 5 days of incubation on selective plates. For eight of the late revertants Tc^R Lac^+ colonies appeared 2 or 3 days earlier in the reconstruction experiment.

We concluded that most late revertants acquired their revertant genotype under selective conditions; they were not preexisting slow-growing revertants. The one exceptional late revertant, strain TT18774, derived from strain TT18548 (*lacZ4638*f+1), was originally isolated on day 4 but took 5 days to appear when seeded in a lawn of its parent. This revertant was either preexisting in the original experiment or perhaps it incurred an unrelated mutation between its isolation and subsequent testing. Control experiments in which Tc^R parental Lac^- cells were seeded in a lawn of Tc^S parental Lac^- cells showed no Tc^R Lac^+ colonies. Like most of the revertants, seeded $lacZ^+$ Tc^R cells produced Lac^+ colonies on day 2 (data not shown). All the Tc^R marked strains used in these reconstruction experiments grew at normal rates in NCE glycerol histidine medium (data not shown).

Possible correlations with late reversion: The various classes of mutants (nonsense, missense, etc.) were nonrandomly distributed with respect to numbers of late revertants (Table 2). Among the insertion alleles, all seven showed essentially no late revertants. All mutants showing 10 or more late revertants were of the nonsense or frameshift classes. Note that all four of the studied

TABLE 3

Time of Lac$^+$ colony appearance from seeded late revertant cells

Lac$^-$ lawn strain	Seeded Tc^R Lac$^+$ cells[a]	New Lac$^+$ Tc^R colonies[b]			
		Day 2	Day 3	Day 4	Day 5
*lacZ489*am	*lacZ489*am day 4 revertant, TT18758	34	9	0	ND
*lacZ489*am	*lacZ489*am day 4 revertant, TT18759	33	0	0	ND
*lacZ489*am	*lacZ489*am day 4 revertant, TT18760	36	3	0	ND
*lacZ480*op	*lacZ480*op day 5 revertant, TT18765	33	0	0	0
*lacZ480*op	*lacZ480*op day 5 revertant, TT18766	0	29	0	0
*lacZ480*op	*lacZ480*op day 5 revertant, TT18767	0	34	1	0
*lacZ4638*f+1	*lacZ4638*f+1 day 4 revertant, TT18772	59	0	0	0
*lacZ4638*f+1	*lacZ4638*f+1 day 4 revertant, TT18773	35	0	0	0
*lacZ4638*f+1	*lacZ4638*f+1 day 4 revertant, TT18774	0	0	0	31

[a] Independent late revertant strains were marked with the *hisG10051*::Tn*10*dTc insertion conferring tetracycline resistance. Control strains (not shown; see Table 1 for complete genotypes; see text for results) included: TT18753 (*lac*$^+$ *hisG10051*::Tn*10*dTc), TT18754 (*lacZ489*am *hisG10051*::Tn*10*dTc), TT18761 (*lacZ480*op *hisG10051*::Tn*10*dTc), and TT18768 (*lacZ4638*f+1 *hisG10051*::Tn*10*dTc).

[b] Lawns of *lacZ* mutant strains seeded with ~30–50 Tc^R revertant cells were spread on NCE lactose Xgal histidine plates and incubated. Each day, new Lac$^+$ colonies were counted and their sensitivity to tetracycline was scored. The daily counts of Lac$^+$ Tc^R colonies are tabulated; ND, no data.

opal (UGA) mutants are among the top five producers of late revertants. These observations suggested two likely explanations for the differences in late reversion. One possibility was that the differences in late reversion reflect differences in nonselective reversion rates dictated by each mutation's particular sequence change. For example, nonselective reversion rates of Tn*10* insertion mutations are relatively low (Table 2). In this case, the low nonselective reversion rate to Lac$^+$ would predict few late revertants. Alternatively, the fact that insertion mutations are usually null alleles whereas opal mutations are notoriously leaky in *S. typhimurium* (ROTH 1970b) suggested that the mutants' residual β-galactosidase activities might correlate well with their numbers of late revertants.

Correlation of late reversion with residual β-galactosidase activity but not with nonselective reversion rate: For all the *lacZ* mutants, we compared the number of late revertants with the reversion rate during nonselective growth and to the residual β-galactosidase activity (Table 2). Reversion rates during nonselective growth were determined in standard fluctuation tests (see MATERIALS AND METHODS). A 300-fold range of rates was observed. Insertion alleles had the lowest reversion rates; other types varied. Residual β-galactosidase activities were measured in a modified assay based on that of MILLER (1972). This modified assay was designed to measure tiny fractions (less than $1/10^4$) of the wild-type levels of activity (see MATERIALS AND METHODS). The residual activities ranged from 0 units to 13 units. All of the insertions were null alleles; they had no detectable activity. All of the other alleles showed some residual activity.

The number of late revertants showed a correlation with residual β-galactosidase activity but not with nonselective reversion rate. HALL (1991) has also reported a lack of correlation between late reversion rate and nonselective reversion rate of *lacZ* mutants. These parameters were compared using a standard coefficient of correlation as well as the Spearmann rank-correlation test. The standard coefficient of correlation between the mean numbers of late revertants and the nonselective reversion rates was close to zero, $r = 0.07$. The standard coefficient of correlation between the mean numbers of late revertants and the residual β-galactosidase activities was high, $r = 0.81$. These results indicated that late revertant totals correlate with residual enzyme activity but not with the nonselective revertibility of a *lacZ* mutant. However, the high correlation with residual enzyme activity depended on a disproportionate contribution from a single mutant, TT18530 (*lacZ489*am, Table 2), which showed the most late revertants and the highest residual β-galactosidase activity. Leaving out this extreme example and considering only the other mutants that show ≥10 late revertants, the coefficient of correlation was still positive, but much lower ($r = 0.29$). Using this restricted data set, late revertant totals still had no correlation ($r = -0.14$) with nonselective reversion rate.

To further test these correlations, we applied the Spearmann rank correlation test. This test is insensitive to the magnitude of extreme values and provides the probability that two parameters are independent. Since the many mutants with few late revertants cannot be meaningfully ranked, we tested all mutants showing ≥10 late revertants. For nonselective reversion rate and number of late revertants, there was no correlation ($r_s = -0.14$); the probability that these parameters are unrelated is high ($P = 0.66$). However, for residual β-galactosidase activity and number of late revertants,

there was a modest positive correlation with late revertant number (r_s = 0.60); the probability that these parameters are independent is low (P = 0.037).

Whereas there is no strict proportionality between residual β-galactosidase activity and number of late revertants, a trend linking these observations is evident (Table 2). Strains that showed high residual β-galactosidase activity tended to produce significant numbers of late revertants. One exception to this is strain TT18549 (*lacZ4639*f−1), which had 6.7 units of activity but showed only six late revertants. Apparently, high residual activity does not guarantee high numbers of late revertants. Without exception, strains with no detectable activity produced no late revertants. Note that low residual activities can allow late reversion, *e.g.*, TT18548 (*lacZ4638*f+1), TT18546 (*lacZ4634*f−1), and TT18531 (*lacZ492*am).

Growth and reversion on selective medium: Since late reversion requires residual β-galactosidase activity, we considered the possibility that mutations were accumulating during growth on the selective medium. This was tested by measuring growth of a subset of mutants on NCE lactose Xgal histidine plates (see MATERIALS AND METHODS). This subset of mutants included six of the 10 mutants producing ≥10 late revertants and a representative few producing <10. Cells were prepared and plated as in the reversion experiments; the nonrevertant Lac$^-$ populations on these plates were sampled and counted each day during a week of incubation. The numbers of Lac$^-$ cells per plate were calculated (Figure 3). For these strains, residual β-galactosidase activity and growth on selective medium (quantified as cell yield) showed a high rank correlation (r_s = 0.82) and a low probability of independence (P = 0.005). As expected, assayed residual activity predicts residual growth.

Among the subset of strains tested, *lacZ* mutants that grew on minimal lactose medium also produced late revertants. Two of three tested mutants that grew very little produced no late revertants. The exception that showed little growth, strain TT18531 (*lacZ492*am), also exhibited exceptional reversion kinetics; its 17 late revertants appeared in a final-day burst. A rank correlation test confirmed the significance of the association of growth (cell yield) and number of late revertants (see Figure 3; calculations excluded data involving scavengers). This test showed a modest positive correlation (r_s = 0.67) and a low probability of independence (P = 0.029). These results extend the coincidence between ability to metabolize lactose (as measured by β-galactosidase assays and growth assays) and late reversion. Note, however, there was not a strict proportionality between rate of growth or cell yield and numbers of late revertants (Figure 3).

Since crossfeeding of Lac$^-$ cells by revertants could contribute to growth and revertant frequencies, the effect of scavenger cells on late reversion of 10 *lacZ* mutants (listed in MATERIALS AND METHODS) was tested. The scavenger strains were naturally Lac$^-$ *S. typhimurium* strains that do not mutate to Lac$^+$; they were included to deplete any nonlactose carbon sources either in the growth medium or excreted by Lac$^+$ revertant colonies. Strains were prepared and plated as described above except that a fivefold excess of scavenger cells was included. The addition of scavengers reduced late revertant numbers by approximately one half for all 10 strains (data not shown). This reduction was uniform; the fundamental observations (rankings, correlations, *etc.*) were not changed by the inclusion of scavenger cells.

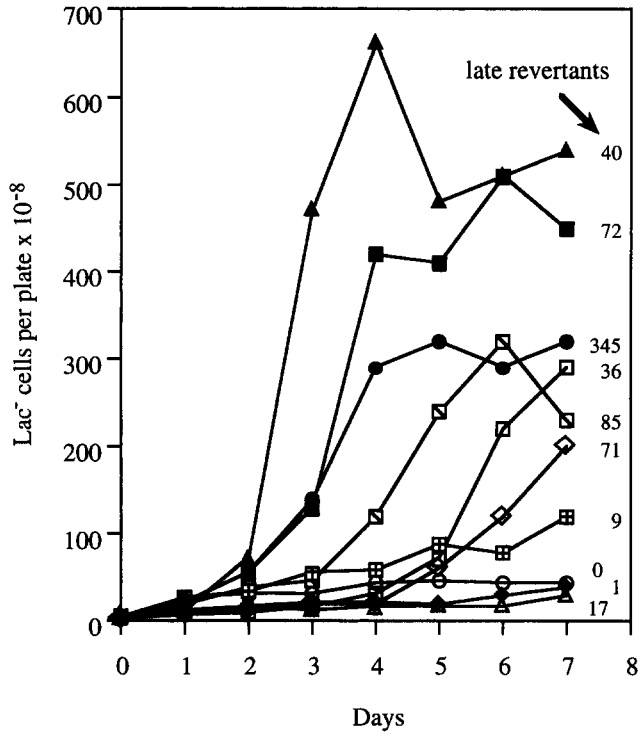

FIGURE 3.—Growth on minimal lactose medium. From platings described in Figure 2 (initial populations were ~5 × 10^8 cells), agar plugs were taken avoiding Lac$^+$ colonies. Bacteria were suspended in saline, diluted, and spread on nutrient agar plates to determine the number of Lac$^-$ cells per plate. Data points are the mean of three or more independent determinations; error bars were omitted for clarity. The strains represented are: TT18521 (*lacZ480*op, ■), TT18521 with a fivefold excess of scavenger strain TR6625 (*lacZ480*op with *strA1*, □), TT18547 (*lacZ4635*f−1, ▲), TT18530 (*lacZ489*am, ●), TT18523 (*lacZ482*op, ⧅), TT18524 (*lacZ483*op, ◇), TT18544 (*lacZ4632*f+1, ⊞), TT18539 (*lacZ502*::Tn*10*dTc, ○), TT18534 (*lacZ495*, ♦), and TT18531 (*lacZ492*am, △). The number at the end of each curve indicates the number of late revertants produced by the strain in reversion tests described in MATERIALS AND METHODS and Figure 2.

For one strain, TT18521 (*lacZ480*op), the effect of scavenger cells on plate growth was also determined. Though this strain grew in spite of excess scavengers, a reduction in cell yield was observed. This reduced yield was due mainly to a prolonged initial lag (Figure 3). After the lag phase, strain TT18521 grew at a rate

TABLE 4

Observed and expected numbers of late Lac$^+$ revertants

lacZ allele	Cell divisions $\times 10^{-9}$ [a]	Revertants expected[b]	Revertants observed[c]	S (obs/exp)[d]
*489*am	32 ± 1	45 ± 2	345 ± 30	7.7
*483*op	6.0 ± 1.4	13 ± 3	71 ± 6	5.5
*492*am	1.6 ± 0.3	5 ± 1	17 ± 2	3
*482*op	24 ± 1	41 ± 2	85 ± 9	2.1
*4635*f−1	47 ± 6	19 ± 2	40 ± 8	2.1
*480*op	41 ± 6	78 ± 12	72 ± 8	0.92
*480*op + scavengers[e]	6.8 ± 2.5	13 ± 5	39 ± 5	3.0
495	1.8 ± 0.2	0.3 ± 0.1	1 ± 1	3
*4632*f+1	8.3 ± 0.5	7 ± 1	9 ± 1	1
502::Tn*10*dTc	4.0 ± 0.5	0.1 ± 0.1	0 ± 0	0

[a] The number of viable cells present on selection plates on day 5 less the number of viable cells originally plated. This number is equivalent to the number of cell divisions occurring on selection plates through day 5. Values are mean ± SEM ($n \geq 3$).

[b] The product of the nonselective reversion rate (revertants per cell division) observed in fluctuation tests (Table 2) and the number of cell divisions on selective plates.

[c] The observed number of late revertants (from Table 2).

[d] The coefficient of coincidence of the observed and expected numbers of late revertants.

[e] The effect of the presence of a fivefold excess of scavenger cells (strain TR6625) on growth and reversion under selection.

of one doubling per day on NCE lactose Xgal histidine medium in the absence of scavenger cells, or 0.9 doublings per day in the presence of scavenger cells. This growth rate was typical of strains producing high numbers of late revertants (Figure 3). Previously, we have shown that our minimal lactose medium allows two or three generations of growth (a four- to eightfold increase) within the first 24 hours of incubation. This is probably due to small amounts of carbon sources other than lactose in the medium. After this first day, growth depends on ability to utilize lactose (GALITSKI and ROTH 1995). Thus, the decrease in the yield of *lacZ480*op tester cells (and the associated decrease in revertant frequency) is probably due to competition with scavenger cells for nonlactose carbon sources in fresh selective medium. Scavengers decrease the nonselectively grown cell populations but do not affect lactose metabolism on the selective medium.

An accounting of revertants: Thus far, the results were as one might predict if there were no adaptive mutability. A null hypothesis would state that the same mutations are occurring randomly at the same rates regardless of selection; populations that grow on selective plates have more opportunities to accumulate revertants (LENSKI and MITTLER 1993). This hypothesis suggests that the accumulation of revertants should mirror the kinetics of growth. That is, one should be able to predict the number of late revertants by using the measured nonselective reversion rate of each mutant and the number of cell divisions occurring on the plate (Table 4). We calculated this expected late revertant number for each mutant and compared the expected to the observed value.

Since Lac$^+$ revertants form a visible colony in 2 days, we assumed that each late revertant colony (days 3–7) resulted from a mutation that occurred 2 days earlier (days 1–5). Total late revertants appearing from day 3 to day 7 were thus generated during growth that occurred between plating and day 5. We measured the parental cell population on day 5, subtracted the number of plated cells, and thereby determined the number of cell divisions that occurred on the plates. The product of this and the previously measured nonselective reversion rate (from Table 2) gives a predicted number of total late revertants. If reversion rates during nonselective growth apply to cells growing on selective medium, then this expected number should equal the observed number of total late revertants.

These calculations assume that late revertant colonies appear 2 days after the occurrence of late mutations. This is a conservative assumption; it maximizes the potential to account for late revertants. Assuming a longer time interval between a reversion event and the appearance of a Lac$^+$ colony would reduce expected numbers of late revertants. The expected numbers of revertants debuting on any given day would be proportional to the number of nonrevertant cells three days previous, not the higher number of nonrevertant cells two days previous. The seeding experiments with marked late revertant cells (Table 3) validate the 2-day assumption and illustrate its conservative tendency; most of the late revertant cells formed colonies in 2 days, but some required 3 days.

The ratio of the observed late revertants (from Table 2) to the calculated expectation is a coefficient of coincidence, S (Table 4). Most of the S values of Table 4

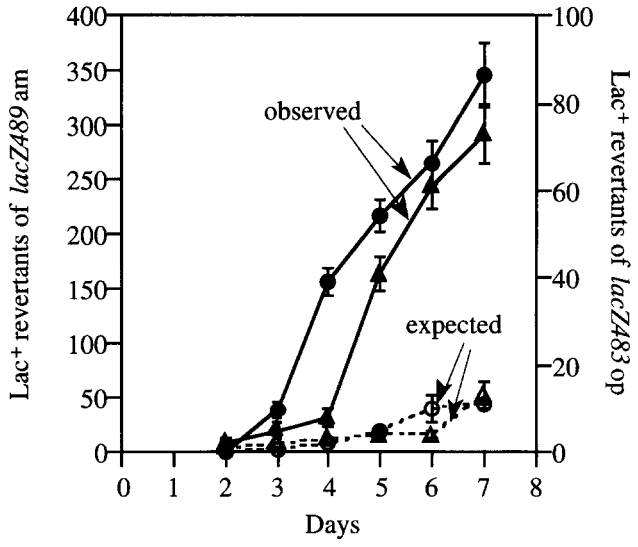

FIGURE 4.—Observed and expected Lac$^+$ revertants of strain TT18530 (*lacZ489*am; circles, left ordinate) and strain TT18524 (*lacZ483*op; triangles, right ordinate). The observed numbers of revertants (filled symbols, solid lines) are from experiments like those described in Figure 2. The expected curves (open symbols, dashed lines) are derived from the growth data of Figure 3 and the nonselective reversion rates of Table 2 (see RESULTS).

are greater than one. If we exclude the three strains producing <10 late revertants (*lacZ495*, *lacZ4632*f+1, and *lacZ502*::Tn*10*dTc), five of the remaining six show an excess of late revertants over the expected number. Predicted and observed daily revertant totals of two of these strains [TT18530 (*lacZ489*am) and TT18524 (*lacZ483*op)] are plotted in Figure 4. At all points in these plots (after 2 days) the number of observed revertants exceeds the predicted number. It should be noted that the kinetics of revertant appearance do not match the shape of the growth curve for these strains (compare Figures 3 and 4). That is, many revertants appeared early, before much growth had occurred. Also, they continued to appear after growth of the bulk parental population had stopped.

We tested the effect of scavenger cells on the accountability of late revertants for one strain, TT18521 (*lacZ480*op). As seen in Table 4, in the absence of scavengers this mutant produced a number of late revertants that matched the expected number. The addition of scavengers increased the S value of this strain threefold by decreasing the yield of Lac$^-$ cells sixfold while decreasing the number of late revertants twofold.

Tests of dependence on RecA functions: The role of RecA function (needed for homologous recombination and SOS induction) was tested by comparing *rec*$^+$ and *recA1* strain pairs for their ability to form late revertants. The *S. typhimurium recA1* allele causes recombination deficiency, UV sensitivity (WING *et al.* 1968), and an inability to induce the SOS response (T. GALITSKI, unpublished results). The *recA1* null allele was introduced to all strains that produced an average of six or more late revertants. In experiments performed as described for the *rec*$^+$ parents, the average total number of late revertants was determined for each RecA$^-$ derivative. In contrast to the Rec dependence of adaptive reversion of an F-plasmid-borne *lac* frameshift mutation (CAIRNS and FOSTER 1991; HARRIS *et al.* 1994), all of these chromosomally situated *lacZ* mutations show no dependence on RecA functions for reversion (data not shown). This has been noted previously for other chromosomal mutations (reviewed in FOSTER 1993).

DISCUSSION

Experimental strategy: Previous systems for study of adaptive mutation have typically involved reversion of a single mutant allele. Since the best studied examples depend absolutely on the specialized activities of experimentally introduced selfish genetic elements, we sought evidence of a more general phenomenon. We have employed a chromosome-based system to observe the reversion of 30 revertible *lacZ* mutants under selective conditions (Figure 1). This system was designed to study reversion events occurring in cells under selection while avoiding the effects of a complex genetic element or the peculiarities of any particular mutant allele. The immediate goal was to identify characteristics of mutants that revert under selection *vs.* those that do not. The mutants included a wide range of types including missense, nonsense, frameshift, and insertion alleles. The insertion element used, Tn*10*dTc, and the MudF element bearing the *lac* operon in our *S. typhimurium* strains are both unable to transpose. Observations made with this system should, in principle, apply to many types of mutations at any chromosomal locus. Ultimately, this approach may allow the elucidation of universally relevant mechanisms of adaptive mutability.

The occurrence of late revertants: The number of late revertants showed a weak correlation with residual β-galactosidase level, but not with nonselective reversion rate. Nonselective reversion rates fail to predict or account for the production of late revertants. The number of late revertants is not simply proportional to residual activity, but the two values are likely to be related. The production of late revertants requires residual enzyme activity. Other factors may limit this ability as well. This is indicated by the existence of mutants with high residual enzyme activity but low numbers of late revertants. Furthermore, we suggest that the correlation between enzyme activity and late reversion might be greater, but not perfect of course, if one could assess the actual useful activity in living cells. *In vitro* assays measure the activity in an oxidizing environment at particular conditions of salt and pH with high concentrations of an unnatural chromogenic substrate. These activities may not always reflect the activity of mutant enzymes within living cells using natural substrates. A second factor that might disturb a simple relationship

between assayed activity and *in vivo* lactose utilization is the fact that some but not all *lacZ* mutations have a polar effect on expression of the distal gene, *lacY*, encoding lactose permease. However, the contribution of lactose permease activity to capacity for lactose utilization is probably not limiting in our experiments. The most prolific late-reverting mutants all showed polarity; most of the strains with nonpolar *lacZ* mutations produced few late revertants.

Growth and late reversion: The correlation between residual β-galactosidase activity and the numbers of late revertants suggested that strains producing late revertants might be growing on minimal lactose medium. Residual activity and growth showed a high rank correlation. Furthermore, we found that mutants producing many late revertants increased their numbers ~100-fold on selective medium. This corresponded to a growth rate of about one doubling per day (assuming a negligible rate of death). Tested mutants that did not revert late did not grow on selective plates. Tested mutants that reverted late did grow on selective plates. The one exception to this showed no growth but produced a burst of revertants on the last day of the experiment. Since late reversion of other mutants often preceded growth, this strain too might show substantial growth if the experiment were continued longer than 1 week.

As was observed for residual β-galactosidase activity, there was no strict proportionality between late revertant number and growth rate or cell yield on minimal lactose medium. However, again as was observed for residual β-galactosidase activity, late reversion was associated with growth on selective plates. These results support and extend our conclusion that the residual ability to use lactose (leakiness) is a requirement of late reversion and that other unknown factors also influence rates of late reversion.

Late reversion to Lac$^+$ requiring lactose utilization: It remained possible that growth was primarily the result of crossfeeding (from revertant cells to nonrevertant cells) accelerating the accumulation of late revertants. If this were the case, crossfeeding should intensify with revertant density. This predicts that mutants showing more late revertants should show a stronger scavenger effect owing to the interception of nutrients. If the nutrient blockade has limited success, these same mutants should show a weaker scavenger effect. However, scavenger cells caused a uniform twofold reduction in late reversion for all tested mutants. Moreover, the growth of the *lacZ480*op strain was delayed more than it was slowed by scavengers. These observations are consistent with scavenging of carbon contaminants initially present in plates and available to all mutants equally. The effect of scavengers on late reversion is through restriction of the nonselective growth of the tester cell population. Thus, it is not likely that crossfeeding is a major determinant of the growth and late reversion of the *lacZ* mutants. These results indicate that the ability to metabolize lactose, not crossfed metabolites, is a requirement for reversion under selective conditions.

This association with leakiness seems to be in contrast with the observations made with an F-plasmid-borne *lac* frameshift mutation. The *Escherichia coli* K12 strain, FC40, carrying this allele produces revertants but does not grow on selective plates with an excess of scavengers (CAIRNS and FOSTER 1991; FOSTER 1994). However, when the F plasmid of this *E. coli* strain is transferred to *S. typhimurium* strains, it produces the same residual β-galactosidase activity but confers slow growth on selective medium, even in the presence of excess scavengers (GALITSKI and ROTH 1995). Furthermore, if strain FC40 were included in Table 2, it would rank sixth in terms of the number of late revertants and fourth in terms of residual β-galactosidase activity (CAIRNS and FOSTER 1991; GALITSKI and ROTH 1995). We have suggested previously that leakiness plays an important role in the adaptive reversion of strain FC40 (GALITSKI and ROTH 1995). Experiments of CAIRNS and FOSTER (1991) are consistent with this suggestion. They observed that Lac$^+$ revertants of a tryptophan-requiring derivative of strain FC40 did not accumulate in the absence of a carbon and energy source or in the absence of tryptophan. We submit that, in both situations, the failure to form revertants was due to inability to benefit from residual β-galactosidase activity; in one case this was due to absence of lactose and, in the other, to a lack of essential tryptophan. Thus, our conclusion that residual lactose metabolism is a requirement for late reversion to Lac$^+$ applies equally to strain FC40.

Accounting for late revertants: Our observations showed that chromosomal *lacZ* mutants reverted under selective conditions if they were able to grow, albeit slowly, on minimal lactose medium. These observations suggested a null hypothesis in which growing cells give rise to revertants with a nonselective mutation spectrum and rate of reversion. Thus, there would be no need to postulate the existence of adaptive mutability.

We tested this hypothesis. We attempted to account for late revertants of nine strains by measuring their growth on selective plates and applying their nonselective reversion rates (determined in fluctuation tests) to calculate an expected number of late revertants (Table 4). For three mutants that did not produce >10 late revertants, expected numbers were similar to the observed numbers. However, in spite of conservative accounting, five of six strains that produced >10 late revertants showed an excess of late revertants (Table 4 and Figure 4). For these mutations, the null hypothesis fails. We suggest that the excess of late revertants shown by these mutants arises from a general adaptive mutability.

Questions about mutability in the absence of growth and questions about adaptive mutability should not be confused; there is no *a priori* reason for this association.

Wild populations of bacteria growing at low rates under adverse conditions would benefit from adaptive mutability. We should not expect this ability to require a complete cessation of growth. We suggest that mutability during limited growth is the appropriate subject of investigation even though it presents some experimental difficulty. Experiments with strain FC40 have been designed to suppress growth to preclude trivial interpretations involving standard random mutagenesis during rounds of cell division (CAIRNS and FOSTER 1991; FOSTER 1994).

In our system, efforts to suppress growth by employing scavenger cells prevented neither growth nor late reversion. However, scavengers did affect the accounting of late revertants. When tested with the one mutant that produced high but expected numbers of late revertants (*lacZ480*op), scavengers reduced growth sixfold and observed revertants twofold. Thus, the presence of scavengers, demanding utilization of lactose (not contaminating or crossfed carbon compounds), resulted in a threefold excess of revertants. This result suggests that the S values of Table 4 and the plots of Figure 4 underestimate the production of excess late revertants. The effect of scavenger cells was to weaken the null hypothesis of a random nonselective spectrum of mutations occurring during rounds of cell division under selective conditions.

The inadequacy of the null hypothesis of simple growth-dependent mutability is further illustrated by the differences in the kinetics of growth and the kinetics of reversion. If late reversion events were simply due to random mutations occurring strictly during rounds of chromosome replication and cell division, then the accumulation of revertants should reflect the growth of the population. That is, the shape of the growth curve should predict the shape of the reversion curve (with a 2- or 3-day delay to allow revertant individuals to form macroscopic colonies). This was generally not observed. This was evident both at earlier times when revertants had begun to rapidly accumulate but rapid growth had not begun and at later times when growth had ceased a few days previous but revertants continued to accumulate rapidly. The addition of scavenger cells exacerbated such discrepancies (data not shown). We conclude that the relationship between growth and reversion under selection is not a simply causal one. Growth and reversion under strong selection are parallel manifestations of a nonzero fitness (leakiness). We suggest that the ability to revert under selection is further limited by mechanistic constraints that have yet to be fully explored.

Recombination and adaptive reversion: Adaptive reversion of an F-plasmid-borne *lac* frameshift mutation has been shown to depend on homologous recombination functions, RecABC (CAIRNS and FOSTER 1991; HARRIS *et al.* 1994; reviewed in ROSENBERG *et al.* 1995). This is an important mechanistic difference between reversion under selective and nonselective conditions in this F-plasmid-based system. We tested the effect of a *recA* null allele on all mutants producing six or more late revertants. In contrast, we found a total lack of RecA dependence for late reversion of chromosomal *lacZ* mutants. The RecA dependence of adaptive reversion in the FC40 system applies only when the mutant allele is on (in *cis*) a F-prime plasmid with mutationally derepressed *tra* operon functions, not when the *lac* locus is in the chromosome (FOSTER and TRIMARCHI 1995; GALITSKI and ROTH 1995). The need for homologous recombination results from the conjunction of rather specialized circumstances. Furthermore, apparent adaptive reversion at several chromosomal loci other than *lac* does not depend on RecA function (reviewed in FOSTER 1993). Overall, dependence on homologous recombination functions for reversion under selection is unusual rather than a defining characteristic.

Mechanisms: The excess revertants produced under selection reflect a phenomenon of adaptive mutability. These events are adaptive in the following sense—natural selection is required to produce the revertant genotype. Selection appears to drive this mutability since leakiness is a requirement for the production of excess revertants. The requirement for residual ability to use lactose implies that the presence of lactose is also required. We have confirmed this for the *lacZ489*am mutant, which produced the greatest excess of late revertants; that is, it produces no excess Lac^+ revertants during slow growth on other carbon sources (T. GALITSKI, unpublished results). The models proposed below describe mechanisms of reversion in which selection promotes formation of the revertant genotype.

1. Models invoking a starvation-induced hypermutable state have been proposed to explain cases of adaptive mutability (HALL 1990). Starvation may induce a small subpopulation of cells to undergo catastrophic hypermutation. Most will die, but some will acquire an adaptive mutation, allowing rapid growth and escape from the hypermutable state. While plausible, this model is difficult to reconcile with our data. A starvation-induced state does not predict that reversion under selection would require leakiness; one should predict that more stringent starvation would cause heavier mutagenesis. Nonetheless, the hypermutable state remains a viable explanation that is neither supported nor excluded by our observations. Moreover, it could be superimposed on a variety of other models including the ones that follow. The strongest prediction would be a high frequency of unrelated mutations among revertants. The spectrum of late revertant types could be either similar or different from the nonselective spectrum depending on the types of mutations whose rates are increased by starvation.
2. Multiple mutations contribute incremental fitness

gains ultimately resulting in a revertant phenotype (LENSKI *et al.* 1989). Mutations conferring a small fitness gain are likely to be common whereas single mutations conferring a fully revertant phenotype are rare. Frequent partially revertant subpopulations will form microscopic clones while accumulating additional mutations. These will ultimately appear as a macroscopic fully Lac^+ revertant colony. This predicts that late revertants will have multiple mutations contributing to their Lac^+ phenotype, whereas preexisting revertants will have single mutations. Leakiness (an innate fitness greater than zero) might be a predisposing condition facilitating further fitness gains.

3. Amplification of genes with residual activities under selection results in local mutagenesis (ROTH *et al.* 1996). Random duplications or amplifications of mutant genes with some residual activity are known to occur (*e.g.*, TLSTY *et al.* 1984). Further amplification, allowing limited growth, will be selected. Overreplication of the selected locus in individual chromosomes and during slow growth of amplified strains will produce more mutations of the selected locus. A base change correcting the mutation in any single copy of the amplified locus will be preserved by selection as the amplification recedes. This model predicts that adaptive mutability will be observed at loci whose amplification increases fitness, *i.e.*, intragenic revertants and dominant suppressors. Some examples of amplification (*e.g.*, TLSTY *et al.* 1984) are known to require homologous recombination functions. However, some amplification mechanisms may not require homologous recombination.
4. Sluggish repair of mismatched or damaged DNA residues under starvation allows preferential fixation of mutations that relieve the nutrient limitation and allow DNA replication and rapid growth (STAHL 1988). This type of model might involve mutation fixation through mutant transcripts templated by mismatched bases or miscoding lesions (BRIDGES 1995b). Selective conditions may further bias this process toward the selected locus through transcription patterns (DAVIS 1989; PRIVAL and CEBULA 1992; DATTA and JINKS-ROBERTSON 1995). Limited growth and DNA replication may accelerate this process metabolically by producing more damage and mismatches. This model predicts a unique mutational spectrum associated with reduced activity of one or more DNA repair systems.

We are currently testing the above models using the *lacZ489*am mutant, which produced an eightfold excess of late revertants. Preliminary results suggest that revertants arising under selection are of types consistent with the second model. That model, like the others, offers a satisfying explanation for the phenomenon of adaptive mutability. An evolutionarily relevant understanding of genetic variation requires a detailed study of mutability under selection.

We thank J. CAIRNS, P. FOSTER, M. FOX, R. HARRIS, N. P. HIGGINS, J. LAWRENCE, J. P. RADICELLA, S. ROSENBERG, and F. STAHL for especially influential discussions. This work was supported by a grant GM-27068 (J.R.R.) and a predoctoral training grant 5 T32 GM07464-18 (T.G.) from the National Institutes of Health.

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Communicating editor: P. L. FOSTER