# Adaptive Evolution That Requires Multiple Spontaneous Mutations. I. Mutations Involving an Insertion Sequence

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ABSTRACT

*Escherichia coli* K12 strain χ342LD requires two mutations in the *bgl* (β-glucosidase) operon, $bglR^0 \rightarrow bglR^+$ and excision of IS*103* from within *bglF*, in order to utilize salicin. In growing cells the two mutations occur at rates of $4 \times 10^{-8}$ per cell division and $<2 \times 10^{-12}$ per cell division, respectively. In 2–3-week-old colonies on MacConkey salicin plates the double mutants occur at frequencies of $10^{-8}$ per cell, yet the rate of an unselected mutation, resistance to valine, is unaffected. The two mutations occur sequentially. Colonies that are 8–12 days old contain from 1% to about 10% IS*103* excision mutants, from which the $Sal^+$ secondary $bglR^0 \rightarrow bglR^+$ mutants arise. It is shown that the excision mutants are not advantageous within colonies; thus, they must result from a burst of independent excisions late in the life of the colony. Excision of IS*103* occurs only on medium containing salicin, despite the fact that the excision itself confers no detectable selective advantage and serves only to create the potential for a secondary selectively advantageous mutation.

ADAPTIVE evolution is an active, dynamic process that is presumed to be subject to biological constraints, and it is usually assumed that evolutionary processes can ultimately be understood in terms of those constraints. The initial event in evolutionary processes is the generation of genetic variation by mutation; thus, an understanding of evolutionary processes depends upon an accurate understanding of the nature of spontaneous mutations and spontaneous mutation rates.

Base pair substitutions have been studied very thoroughly, but there is still relatively little precise information on the rates at which these events occur. Studies on the molecular basis of substitutions have focused on errors that occur during DNA synthesis or during repair of damaged DNA. That focus, in part, has led to an infrequently stated general assumption that replication errors are the primary source of spontaneous mutations and virtually all studies of spontaneous mutation rates in bacteria have been conducted on exponentially growing cultures under conditions that are almost never found in nature.

Although we usually think of mutations in terms of base pair substitutions, mutations include any changes in the structure of the genome. It is becoming increasingly clear that mobile genetic elements play important roles in creating genetic variation in a wide variety of organisms (PFEIFER *et al.* 1983; FEDEROFF 1983; MCCLINTOCK 1965; CIAMPI, SCHMID and ROTH 1982; SCORDILIS, REE and LESSIE 1987; PRENTKI *et al.* 1986; BARSOMIAN and LESSIE 1986; SAEDLER *et al.* 1980; MILLER *et al.* 1984; MILLER, DYKHUIZEN and HARTL 1988; BREGLIANO and KIDWELL 1983; HARTL *et al.* 1986). This study focuses on adaptive mutations that result from the movement of mobile genetic elements.

Molecular evolutionists and molecular systematists make certain assumptions about the ultimate source of genetic variation. First, it is assumed that spontaneous mutations arise randomly. They may be fixed into populations by selection or by chance events, but their *occurrence* is presumed to be random and to be relatively unaffected by normal environmental conditions.

Because mutations are rare events, it is usually assumed that multiple mutations are the result of temporally separate, and therefore independent, events. This assumption underlies most methods for constructing phylogenies on the basis of molecular data. Therefore, if some phenotype requires multiple mutations in order to be exhibited, then each of these mutations is expected to be sequentially fixed into the population either by drift or by selection. This study provides evidence suggesting that both of these assumptions need to be critically reexamined.

Microorganisms have proven to be useful model systems for studying evolutionary processes (reviewed in several articles in MORTLOCK 1984; HALL 1983; HARTL 1986), and one of the most powerful approaches has been that of "directed" or "experimental" evolution (HALL 1982a, 1983; CLARKE 1984). In a variety of studies I have selected spontaneous *E. coli* mutants that have evolved the ability to utilize novel carbon and energy sources (HALL 1982b, 1983; KRICKER and HALL 1984; PARKER and HALL 1988). In most of those studies *Escherichia coli* cells were streaked onto the surface of MacConkey agar indica-

tor plates that contained a novel sugar which the cells were incapable of utilizing. On MacConkey plates the cells form colonies by utilizing small peptides in the medium, and the colonies reach a maximum size of about $10^9$ cells in two to three days. (Colony size is presumably limited by local exhaustion of the primary growth resource, peptides, from the medium.) These colonies are white because they are unable to ferment the novel sugar, but after a period of seven to 30 days (depending upon the specific new function being selected), outgrowths or papillae appear on the surface of many colonies. The papillae are red, indicating sugar fermentation, and it is easy to streak out their cells on identical medium to obtain single red colonies, and thus to isolate pure cultures of cells that have evolved the ability to utilize a novel sugar.

This approach is particularly relevant to the question of the nature of spontaneous mutations because it has permitted the isolation of mutants that cannot be obtained by direct selection on minimal medium. One of the most thoroughly studied experimental evolution systems is the evolved β-galactosidase (EBG) system in which *E. coli* strains bearing a deletion for the major portion of the *lacZ* gene were forced to evolve a new system, the *ebg* operon, for catabolism of lactose and other β-galactoside sugars (reviewed in HALL 1982a, 1983). Several years ago we showed that all of the *ebg* mutants, isolated as single-step papillae on MacConkey lactose medium, had to carry two spontaneous mutations, one in the structural gene *ebgA* and one in the regulatory gene *ebgR*, in order to grow on lactose (HALL and CLARKE 1977). I later pointed out that these spontaneous double mutants occurred some $10^8$-fold more frequently than expected on the basis of independent mutations (HALL 1982a).

Some recent observations with another system have again suggested that double mutants may occur several orders of magnitude more frequently than expected.

The *bgl* operon is cryptic in *E. coli* and cannot be expressed unless a mutation in a site or short region designated *bglR* activates the operon (PRASAD and SCHAEFLER 1974). When it is activated, the operon is inducible and permits utilization of the β-glucoside sugars salicin and arbutin. The *bglF* gene specifies a β-glucoside transport protein that both transports and phosphorylates its substrates, and the *bglB* gene specifies a phospho-β-glucosidase (PRASAD and SCHAEFLER 1974). The wild-type allele of *bglR* is designated $bglR^0$ and can be activated to the $bglR^+$ state either by point mutations (REYNOLDS *et al.* 1985; L. L. PARKER and B. G. HALL, unpublished results) or by insertion of the mobile DNA elements IS*1* or IS*5* into the *bglR* site (REYNOLDS, FELTON and WRIGHT 1981; REYNOLDS *et al.* 1985; SCHNETZ, TOLOCZYKI and RAK 1987). We recently reported that the χ342 line of *E. coli* carries a 1.4-kb foreign DNA fragment inserted into the *bglF* gene (PARKER, BETTS and HALL 1988) (Figure 1). Recent results (B. G. HALL, unpublished data) show that this foreign DNA is a newly discovered insertion sequence now designated IS*103*. Since strain χ342 carries the wild-type $bglR^0$ allele (PARKER, BETTS and HALL 1988), two events (excision of IS*103* from within *bglF* and activation of *bglR*) are required to permit the strain to grow on salicin. Since we had measured the spontaneous rate of $bglR^0 \rightarrow bglR^+$ as $2\text{–}6 \times 10^{-8}$ per cell division, and had shown that the rate of excision of IS*103* from within *bglF* was $<2 \times 10^{-10}$ per cell division (PARKER, BETTS and HALL 1988), it seemed unlikely that strain χ342 colonies consisting of about $10^9$ cells would generate salicin-utilizing ($Sal^+$) papillae.

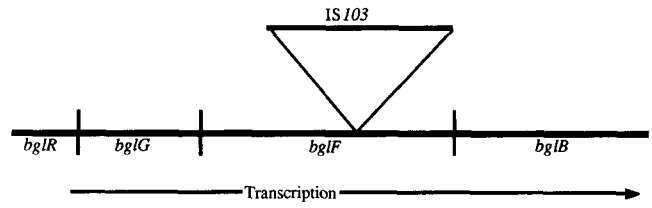


FIGURE 1.—Structure of the *bgl* operon in strain χ342. IS*103* is inserted into the *bglF* gene.

In this paper I show that after less than 2 weeks of incubation, 60% of the χ342 colonies on MacConkey salicin medium generate $Sal^+$ papillae. The source of these double mutants is determined, and the evolutionary implications of the results are discussed.

## MATERIALS AND METHODS

**Genetic nomenclature for the *bgl* operon:** Until recently "*bglC*" was used to designate the gene specifying the β-glucoside transport system and "*bglS*" to designate the gene specifying a positive regulatory locus (PRASAD and SCHAEFLER 1974). Two recent papers (MAHADEVAN, REYNOLDS and WRIGHT 1987; SCHNETZ, TOLOZYKI and RAK 1987) reversed those definitions. In order to avoid further confusion, BARBARA BACHMANN, curator of the *E. coli* Genetic Stock Center, has renamed these loci (B. BACHMANN, personal communication). In this paper we use the new designations as they will appear in the next edition of the linkage map of *E. coli*. *bglF* now designates the β-glucoside-specific enzyme II of the phosphotransferase system, *i.e.*, the transport protein. *bglG* now designates the gene encoding the positive regulator of expression of the *bgl* operon. *bglB*, whose definition is unchanged, specifies phospho-β-glucosidase B.

***E. coli* K12 strains:** Strain HfrC (HfrC *metB1 relA spoT*) and strain χ342 (HfrC *proC metB1 relA spoT* $\lambda^-$ *bglF*::IS*103*) were obtained from the *E. coli* Genetic Stock Center. Strain 1011A (HfrC *lacZ*Δ4680 $proC^+$ $metB^+$ $bglR^+$ *bglF*::IS*103* is a $bglR^+$ mutant that was ultimately derived from χ342 (PARKER, BETTS and HALL 1988). Strain χ342LD was constructed by bacteriophage P1-mediated transduction of *lacZ*Δ4680 into strain χ342 by cotransduction with $proC^+$. The $proC^+$ allele was introduced because *E. coli* can utilize proline as a carbon source, and the presence of a proline

requirement would have compromised salicin selection experiments. The *lacZ*Δ4680 allele was introduced at the same time for the purposes of another study. As a result of this manipulation, strain χ342LD is HfrC *lacZ*Δ4680 *metB1 relA spoT* λ⁻ *bglF*::IS*103*. Strain XSW1 is *bglR*⁰ *bglF*⁺ and is a spontaneous Sal⁻ IS*103* excision mutant of χ342LD (see Figure 3) whose selection is described in the text. Strain χ342LDC is a derivative of strain χ342LD that was made resistant to chloramphenicol by the introduction of Tn*9*. Strains XSW1S and XSW1N are, respectively, streptomycin-resistant and nalidixic acid-resistant mutants of strain XSW1 selected by plating strain XSW1 onto antibiotic media. The streptomycin and nalidixic acid resistance alleles were transduced by phage P1 into strain χ342LD to produce strains χ342LDS and χ342LDN, respectively. Strain CSH62T is *HfrH thi* Δ*bgl-pho201 tna*::Tn*10* (KRICKER and HALL 1984).

**Culture media and conditions:** Liquid cultures were grown at 37° with aeration either in L-broth (MILLER 1972) or in 0.01–0.05% (w/v) glucose minimal medium. Minimal medium consisted of a phosphate-buffered mineral salts solution (HALL and BETTS 1987). Solid media included 1.5% agar. Salicin was used at a concentration of 0.1% (w/v) and methionine at 100 μg/ml. MacConkey salicin indicator plates contained 1% (w/v) salicin and were prepared from MacConkey agar base according to the manufacturer (Difco). MacConkey base plates contained no added sugar.

**Molecular techniques:** Methods for the preparation of genomic and plasmid DNAs, isolation and labeling of probe DNA, and DNA/DNA hybridizations were previously described (HALL and BETTS 1987).

**Physical manipulation of bacterial colonies:** Colonies to be manipulated were inoculated from 0.5-μl drops containing 10–20 cells unless otherwise specified. In some experiments colonies were inoculated onto the surface of agar medium in 48-well microtiter plates so that there was a single colony in each well. Those colonies were resuspended by pipetting sterile buffer up and down on the surface of the medium using a Pipetman automatic micropipette. In other experiments colonies were inoculated onto the surface of sterile Millipore HAWG 0.45-μ filters resting on the surface of medium in 60-mm Petri dishes. Such filters could subsequently be transferred intact to other media. Colonies were resuspended by slicing the filter into sections containing single colonies and vortexing the slice in 1 ml of sterile medium.

**Fluctuation tests:** Spontaneous mutation rates were measured in growing cells by the fluctuation test method (LURIA and DELBRÜCK 1943). Briefly, a large number of cultures (usually 60) were grown from small inocula (10–20 cells) to a final density that was limited by the concentration of glucose in the minimal medium. The entirety of each culture was plated onto selective medium, and the number of mutations (as contrasted with the number of *mutants*) in the culture was estimated from the proportion of cultures that contained no mutants ($P_0$). Several (usually 10) identical cultures were diluted appropriately to determine the total number of cells in each culture plated. The mutation rate (mutations per cell) was calculated as $(-\ln 2)(\ln P_0/(\text{total cells per culture})$ (LURIA and DELBRÜCK 1943). In experiments where the mutation rate was so high that there were no cultures without mutants, the method of LEA and COULSON (1949) was employed using the graphic method described by KOCH (1982).

## RESULTS AND DISCUSSION

Strains XSW1 (*bglR*⁰ *bglF*⁺, *i.e.*, wild type for the *bgl* operon), 1011A (*bglR*⁺ IS*103*::*bglF*) and χ342LD (*bglR*⁰ IS*103*::*bglF*) were plated onto MacConkey salicin medium at about 50 cells per plate and incubated at 30°. The plates were observed daily through a dissecting microscope and the proportion of colonies with Sal⁺ papillae was recorded (Figure 2). To be sure that the papillae were actually the results of mutations at the *bgl* operon, the map positions of nine independent Sal⁺ mutants isolated from papillae on the surface of χ342LD colonies were determined. Strain CSH62T carries Tn*10* in the *tna* (tryptophanase) gene adjacent to a deletion of the *bgl* operon and the tetracycline-resistance determinant of Tn*10* co-transduces about 98% of the time with the *bgl* deletion (KRICKER and HALL 1984). The nine Sal⁺ mutants were transduced with bacteriophage P1 lysates from strain CSH62T and tetracycline-resistant transductants were selected and scored for salicin fermentation. In each case, ≥98% of the Tet$^r$ transductants were Sal⁻. This indicates that the Sal⁺ mutants of χ342LD were indeed the result of mutations in the *bgl* operon, and were therefore spontaneous *bglR*⁺ *bglF*⁺ double mutants.

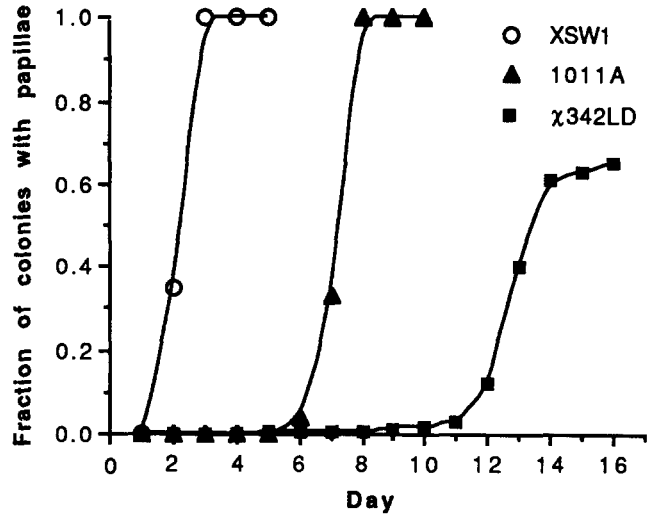


FIGURE 2.—Time course of Sal⁺ papillae formation on MacConkey salicin medium. Strain XSW1 requires only a *bglR*⁰ → *bglR*⁺ mutation to form Sal⁺ papillae, strain 1011A requires only excision of IS*103* from within *bglF* to form Sal⁺ papillae, while strain χ342LD requires both mutations.

**Basis of spontaneous double mutants:** Based upon the previously measured mutation rates (PARKER, BETTS and HALL 1988), the expected probability of a double mutation is ($6 \times 10^{-8}$) ($<2 \times 10^{-10}$) or $<1.2 \times 10^{-17}$ and the probability that a colony with $10^9$ cells will contain such a mutant is $<10^{-8}$. There are two possible explanations for the spontaneous double mutants of χ342LD: either the mutation rate (generally or individually) is greatly increased under the conditions tested, or the first mutation allows additional cell growth and thus increases the chance that the second mutation will occur in a cell that has the first mutation. However, it seems unlikely that either mu-

TABLE 1

**Mutation rates**

| Strain | Genotype | Mutation required | Permits growth on: | Mutation rate during growth[a] | Mutation rate in colonies[b] |
|---|---|---|---|---|---|
| HfrC | *bglR*$^0$ *bglF*$^+$ | *bglR*$^0$→*bglR*$^+$ | Salicin | $4 \times 10^{-8}$ | |
| 1011A | *bglR*$^+$ *bglF*::IS*103* | *bglF*::IS*103*→*bglF*$^+$ | Salicin | $<2 \times 10^{-12}$ | |
| χ342LD | *bglR*$^0$*bglF*::IS103 | BOTH | Salicin | $<1 \times 10^{-11}$ | $1 \times 10^{-8}$[c] |
| χ342LD | Wild type | Vaine resistance | High valine | $2 \times 10^{-7}$ | $3 \times 10^{-7}$[d] |

[a] Estimated from LURIA-DELBRÜCK fluctuation tests as described in MATERIALS AND METHODS.
[b] The rates estimated in colonies are not directly comparable to rates estimated during growth. Rates estimated during growth are mutations *per cell division*. Since the cells in colonies are not dividing rapidly, the rate in colonies is simply mutations *per cell*. The difference, however, is only a factor of ln2 = 0.7-fold.
[c] Estimated from the proportion of colonies that contained no Sal$^+$ mutants as described in the text.
[d] Estimated from the frequency of valine-resistant mutants in colonies by the method of LEA and COULSON (1949).

tation alone could provide the additional growth required to condition the second mutation. Excision of IS*103* from within *bglF* would simply restore a wild-type genotype in which the *bgl* operon still cannot be expressed. By itself, a mutation from *bglR*$^0$ to *bglR*$^+$ would not permit transport of β-glucosides because the *bglF* gene would still contain IS*103*. One would therefore have to postulate the unlikely case that the first mutation conditioned a special time-dependent, replication-independent mutation process for the second mutation. The first explanation, an increased mutation rate, thus seemed more likely.

**Measurement of spontaneous mutation rates:** To establish a basis of comparison, spontaneous mutation rates were measured in growing cultures by the LURIA-DELBRÜCK fluctuation test (Table 1). The mutation rate for *bglR*$^0$ → *bglR*$^+$ was very similar to that previously determined (PARKER, BETTS and HALL 1988), and the rate for excision of IS*103* from within *bglF* remained too low to detect.

In order to determine whether any changes in mutation rates at the *bgl* operon were specific to that operon, or whether they were accompanied by changed mutation rates at unrelated loci, the rate of mutation to valine resistance was also determined. *E. coli* K12 is sensitive to inhibition by high concentrations (40 μg/ml) of valine which acts as a false feedback inhibitor of isoleucine biosynthesis. Mutations at several loci eliminate this sensitivity, and the mutation rate to valine resistance reflects all types of mutations. Measuring the frequency of valine-resistance mutations has an advantage over measuring mutations that confer resistance to antibiotics because newly arisen mutants that are plated onto valine medium before they have had a chance to divide are not killed by the inhibitor and eventually form colonies. In contrast, newly arisen antibiotic resistance mutants must undergo several cell divisions before they become phenotypically resistant (phenotypic lag).

To compare the mutation rates in growing χ342LD cells with rates in χ342LD colonies on MacConkey salicin medium, individual 20-day colonies were suspended in 1 ml of sterile buffer and plated onto salicin minimal medium. A 10-μl aliquot of each suspension was diluted and plated onto rich medium to determine the number of cells per colony. Seven of the 27 colonies tested contained no Sal$^+$ mutants and the mean number of viable cells in these colonies was $6.7 \times 10^7$, while the mean number of viable cells in colonies containing Sal$^+$ cells was $2.5 \times 10^8$. On the assumption that the larger value represents Sal$^+$ mutants that grew at the expense of salicin, the lower value was used to estimate the mutation rates within colonies shown in Table 1 according to the equation $\mu = -\ln P_0/(\text{cells per colony})$. The results show that conditions present in aged colonies on MacConkey salicin plates do not increase the *general* mutation rate as estimated from mutations to valine resistance. On the other hand, the frequency of the Sal$^+$ double mutants is about 12 orders of magnitude higher than expected on the basis of the mutation rates measured during growth.

**Do the two mutations in the *bgl* operon occur simultaneously or sequentially?** Despite our current understanding of the *bgl* operon, we know very little about the conditions that obtain within a colony on a plate containing a mixture of resources. It is certainly possible that, although neither mutation alone permits growth on salicin minimal medium, one or both of the mutations might allow sufficient growth to permit the Sal$^+$ phenotype to arise via sequential mutations. If this were the case, then within those colonies that have Sal$^+$ papillae, a significant portion of the Sal$^-$ cells should have one of the two required mutations.

To test this notion, two colonies bearing Sal$^+$ papillae were resuspended and plated, and both Sal$^+$ and Sal$^-$ colonies were isolated and purified. Four Sal$^-$ isolates from colony A and five from colony B were tested genetically for the presence of a *bglR*$^+$ allele as described in PARKER, BETTS and HALL (1988). All proved to be *bglR*$^0$ (data not shown). To determine whether IS*103* had excised from within *bglF* in any of these cells, DNA from the nine Sal$^-$ isolates, plus two Sal$^+$ isolates derived from colonies A and B, was

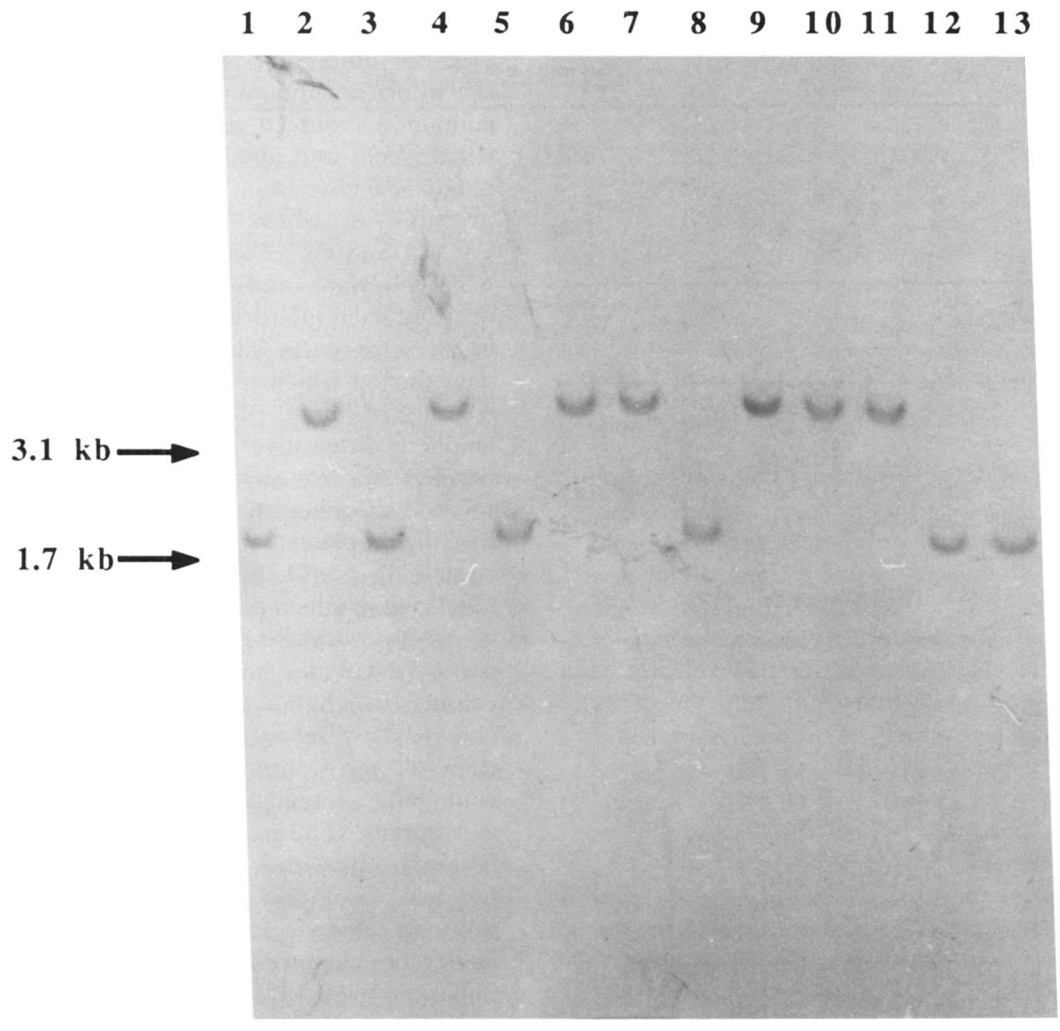


FIGURE 3.—Excision of IS*103* from within *bglF* in some Sal⁻ derivatives of χ342LD. Genomic DNA was digested with *Nci*I, electrophoresed, transferred to blotting membrane, and hybridized to a *Pvu*II-*Hpa*I fragment of the *bgl* operon. Lane 1: HfrC (wild-type *bgl* operon). Lane 2: χ342 (IS*103*::*bglF*). Lanes 3–6: XSW1, XSW2, XSW4, XSW5 (Sal⁻ isolates obtained from colony A that had formed papillae on MacConkey salicin medium). Lanes 7–11: XSW6, XSW7, XSW8, XSW9, XSW10 (Sal⁻ isolates obtained from colony B that had formed papillae on MacConkey salicin medium). Lane 12: XSR1 (a Sal⁺ isolate from colony A). Lane 13: XSR2 (a Sal⁺ isolate from colony B). Strains XSW1, XSW4 and XSW7 all have experienced excision of IS*103* and are genetically wild type at the *bgl* operon.

digested, subjected to agarose gel electrophoresis, blotted, and probed with a fragment of *bgl* operon DNA (Figure 3). IS*103* had excised from within the *bgl* operon in two of the four Sal⁻ isolates from colony A and in one Sal⁻ isolate from colony B. As expected, IS*103* had excised from within the *bgl* operon in both of the Sal⁺ isolates.

These results suggest that the two mutations arise sequentially rather than simultaneously. To obtain a clearer picture of the dynamics of this process, individual χ342LD colonies growing on MacConkey salicin medium were isolated on a daily basis for 13 days following inoculation, 5% of the colony suspension was plated onto salicin minimal medium in order to estimate the number of Sal⁺ cells in each colony, and another 1% was diluted and plated in order to estimate the total number of viable cells in the colony (Figure 4). The remainder of each colony was stored frozen at −70°.

Precise excision of IS*103* from within *bglF* restores a wild-type genotype (*bglR*$^0$*bglF*$^+$). The results of earlier experiments (Figure 2) suggested that wild-type Sal⁻ colonies on MacConkey salicin medium could be distinguished from χ342LD colonies simply on the basis of the time required for papillae to form. Dilutions of the stored colony suspensions were plated on MacConkey salicin medium so that about 100 colonies were examined from each suspension. Five independent Sal⁻ mutants of χ342LD that had lost IS*103*, as shown by probing, formed papillae on 100% of their colonies within three days, while none of five Sal⁻ isolates that retained IS*103* formed papillae on any colonies in that period. It was therefore possible to detect IS*103* excision mutants when they constituted as few as 1% of the cells in a colony. Table 2 shows the results of this experiment. The first excision mutants were detected eight days after plating and the

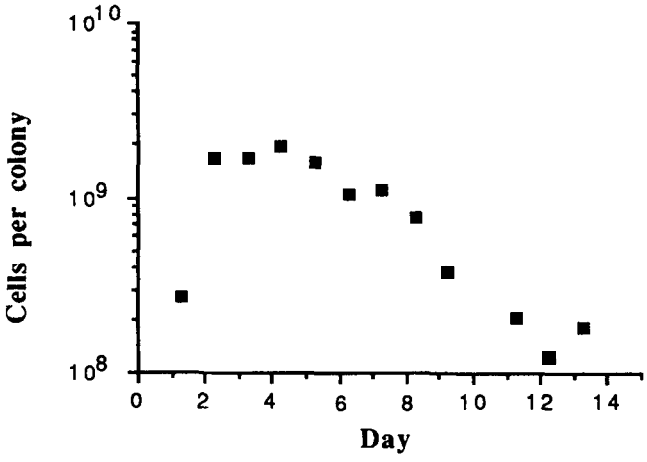


FIGURE 4.—Viable cells in χ342LD colonies growing on MacConkey salicin medium. The abscissa shows the age of the colonies in days.

TABLE 2

**Kinetics of appearance of IS*103* excision mutants**

| Days after plating | Colonies with excisions[a] | Cells in colony | Sal⁻ excision cells in colony[b] | Sal⁺ excision cells in colony[c] |
|---|---|---|---|---|
| 8 | 1 | $6.5 \times 10^8$ | $5.2 \times 10^6$ | ND |
| 9 | 1 | $3.4 \times 10^8$ | $4.9 \times 10^6$ | ND |
| 11 | 1 | $1.8 \times 10^8$ | $2.3 \times 10^6$ | ND |
| 12 | 3 | $3.8 \times 10^8$ | $3.4 \times 10^8$ | $9.9 \times 10^6$ |
| | | $1.8 \times 10^8$ | $3.1 \times 10^7$ | ND |
| | | $2.0 \times 10^8$ | $6.5 \times 10^7$ | ND |
| 13 | 6 | $8.6 \times 10^8$ | $4.3 \times 10^8$ | $5.5 \times 10^7$ |
| | | $3.5 \times 10^8$ | $1.6 \times 10^7$ | $1.9 \times 10^8$ |
| | | $2.1 \times 10^8$ | $7.9 \times 10^7$ | $1.5 \times 10^5$ |
| | | $1.3 \times 10^8$ | $2.1 \times 10^6$ | ND |
| | | $1.7 \times 10^8$ | $3.1 \times 10^7$ | ND |
| | | $1.9 \times 10^8$ | $1.2 \times 10^7$ | ND |

[a] Six colonies were assayed on each day.

[b] No excisions or Sal⁺ mutants were detected in samples from days 1 through 7. The lower limit of detection was $2 \times 10^6$ cells with excisions in a colony.

[c] ND, none detected. The lower limit of detection was 20 Sal⁺ cells in a colony.

first Sal⁺ mutant was detected on day 12.

**Fitness of excision mutants within χ342LD colonies:** The excision mutants present within a colony could either be siblings descended from a single mutant cell (model A of Figure 5), or could represent a large number of mutants that arose independently late in the life of the colony (model B of Figure 5). The time required for strain 1011A (*bglR*⁺ IS*103*::*bglF*) to form papillae (Figure 2) suggests that excisions do not begin to occur prior to five days after inoculation, *i.e.*, after colony growth has stopped. In order for an excision mutant that arose on day 3 to reach a density of $5 \times 10^6$ cells by day 8, the excision mutant (*i.e.*, a wild-type cell) would have to achieve a doubling time of 5.4 hr within a colony. In order to measure the growth rate of an excision mutant within a colony, a reconstruction experiment was conducted. "Mixture" colonies were prepared by inoculating 0.5-μl drops onto filters on the surface of MacConkey salicin plates containing chloramphenicol. Each drop contained about $10^5$ χ342LDC (chloramphenicol-resistant) cells and about 50 streptomycin-resistant or nalidixic acid-resistant cells. Four such mixtures were prepared in which the minority population was χ342LDS (Str$^r$), χ342LDN (Nal$^r$), XSW1S (Str$^r$), or XSW1N (Nal$^r$), and filters were inoculated with 6 drops of each mixture spaced at even intervals about its circumference. The plates were incubated for 4 days during which time the χ342LDC cells formed colonies of about $10^9$ cells, but the minority chloramphenicol-sensitive population did not increase in number. After 4 days the filters were transferred to spent MacConkey salicin plates without chloramphenicol. (Spent plates were prepared by inoculating drops containing χ342LDC onto filters on the surface of MacConkey salicin plates and incubating those plates in parallel with the chloramphenicol plates.) At this point the colonies were the equivalent of 4-day old colonies in which a few excision mutations had occurred. Daily thereafter, colonies from one filter from each set were suspended and plated onto L agar containing either streptomycin or nalidixic acid as appropriate. The number of XSW1 (IS*103* excision mutant) cells within a colony was equivalent to the number of colonies on the plates containing streptomycin or nalidixic acid. Mixtures in which the minority streptomycin-resistant or nalidixic acid-resistant mutants were χ342LDS or χ342LDN served as controls. Figure 6 shows that XSW1 cells did not increase in number over the period of the experiment. Their failure to do so cannot be attributed to the drug-resistance mutations that were used to monitor their frequency, since the drug-resistant mutants of χ342LD were, if anything, slightly fitter than the XSW1 mutants. Furthermore, when two papillae that had formed on the surface of colonies containing XSW1N were tested, the Sal⁺ mutants were chloramphenicol-resistant and nalidixic acid-sensitive, showing that they had arisen from the majority χ342LDC population rather than from the preexisting XSW1N minority population in the colonies.

Since excision mutants like XSW1 do not grow within a colony consisting primarily of χ342LD cells, the excision mutants that are present in an 8–12-day colony must be the result of independent excision events as shown in model B of Figure 5.

These results suggest the following sequence of events to explain the observed double mutants. Colonies reach a maximum size of about $10^9$ cells within 3 days, at which time a slow decline in the number of viable cells begins. In the period between 8 and 12 days, 1 to 89% of the cells in a colony experience an excision of IS*103* from within *bglF*. That burst of

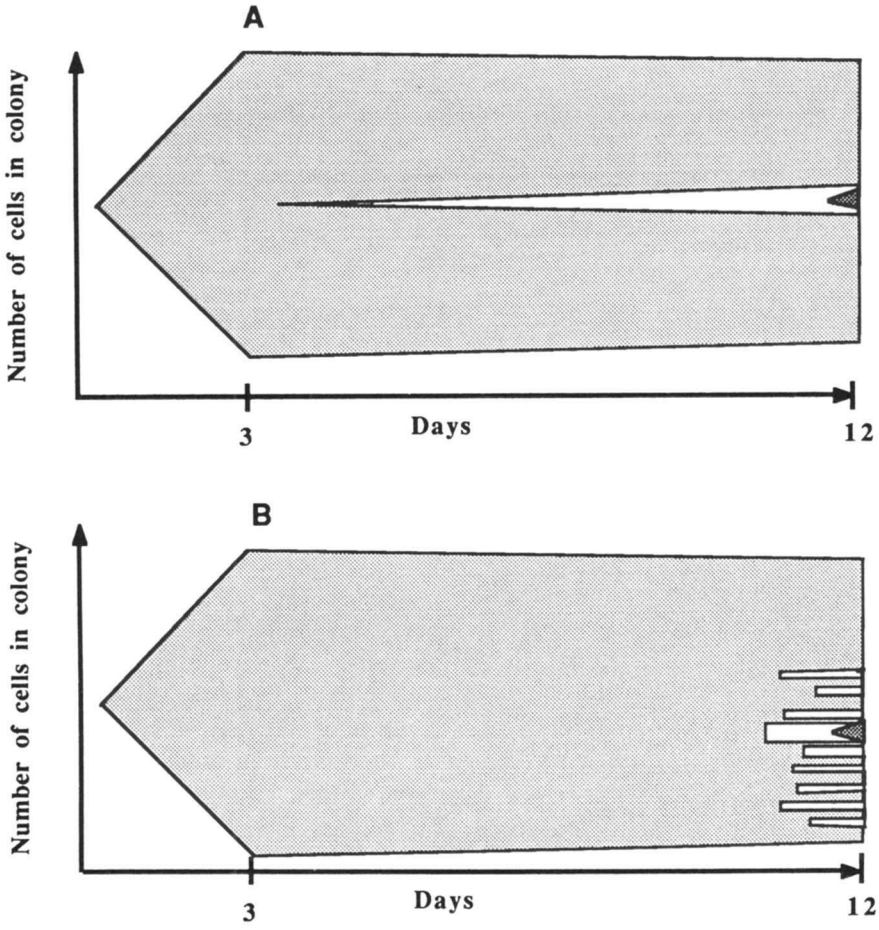


FIGURE 5.—Two models for the appearance of IS*103* excision mutants within χ342LD colonies on MacConkey salicin medium. The stippled area represents the population of χ342LD cells in the colony over time. The white area represents the population of excision mutants. The gray area represents the population of Sal$^+$ mutants that forms a papilla. In model A, an excision mutation occurs shortly after the colony has stopped growing and the excision mutant continues to grow, so that all excision mutants are descended from the original mutant. In model B, a large number of excision mutants arise independently late in the life of the colony, so that excision mutants are no more closely related to each other than they are to other cells in the colony.

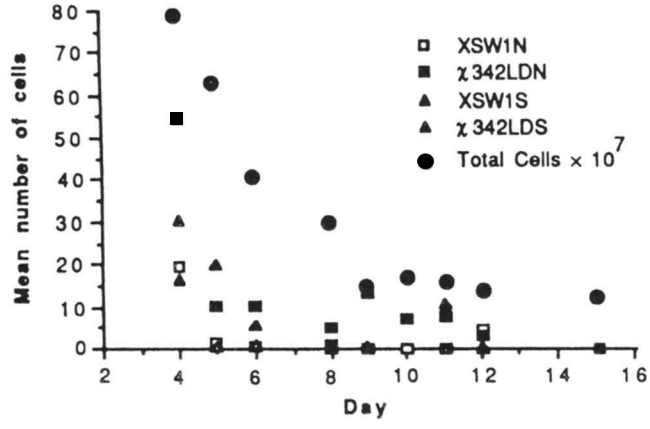


FIGURE 6.—Change in frequency of IS*103* excision mutants within χ342LDC colonies over time. XSW1N and XSW1S are nal$^r$ and str$^r$, respectively, mutants of XSW1; χ342LDN and χ342LDS are control strains bearing the same nal$^r$ and str$^r$ mutations.

excision mutants provides a population of wild-type cells sufficiently large that a replication-independent *bglR*$^0$ → *bglR*$^+$ mutation occurs in one of them and generates a Sal$^+$ cell that grows by utilizing salicin to form a papilla.

An alternative explanation is that the majority of Sal$^+$ cells arise from those IS*103* excisions that occur in the *bglR*$^+$ mutants present within the colony as a result of mutations that occurred during the growth of the colony. In a colony of about $5 \times 10^8$ cells with a mutation rate for *bglR*$^0$ → *bglR*$^+$ of $4 \times 10^{-8}$ per cell division, about 40 mutations would occur during the growth of the colony, producing a median of about 240 *bglR*$^+$ cells in the colony as calculated according to KOCH (1982). When the excision mutations occur, about 10% of these *bglR*$^+$ cells would be expected to experience an IS*103* excision and thus to produce a Sal$^+$ papilla by the next day. If this were the case, then the time of appearance of Sal$^+$ papillae from strain χ342LD should be no greater than 2 days more than the time of appearance of Sal$^+$ papillae from strain 1011A. Contrary to this expectation, the curve for appearance of papillae from χ342LD follows that for strain 1011A by about 5 days (Figure 2). The failure of significant numbers of Sal$^+$ papillae to appear prior to day 12 suggests that the average number of *bglR*$^+$ mutants within a χ342LD colony is considerably lower than anticipated from the colony size and mutation rate. It is possible either that *bglR*$^+$ mutations occur less frequently during growth in a colony than they do in liquid culture, or that *bglR*$^+$ mutants die preferentially in the period between 2 and 8 days. Although IS*103* excisions may occur occasionally in preexisting *bglR*$^+$ mutants, the long lag time before papillae appear on the surfaces of χ342LD colonies

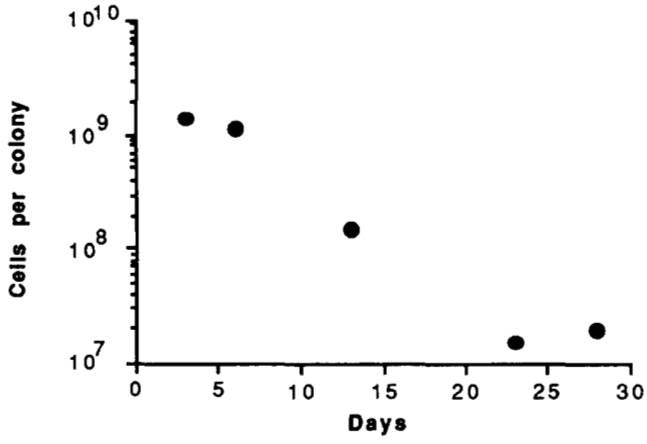


FIGURE 7.—Viable cells per colony in colonies growing on MacConkey base medium.

makes it unlikely that it is the usual sequence of events.

IS*103* is never observed to excise in growing cells, and within colonies the excision probability seems to depend on time rather than cell divisions. This is not particularly surprising, in that the activities of transposition and excision proteins of IS*103* could well respond to the physiological conditions that exist within a colony that has depleted its environment of nutrients. What is surprising is that an event that is so rare in growing cells ($<2 \times 10^{-12}$ per cell, Table 1) should occur so frequently (in 1–89% of the cells, Table 2) in aged colonies.

Salicin has been tacitly assumed to play no role in *generating* $Sal^+$ papillae, only in the *selection* that favors $Sal^+$ double mutants and leads to papillae. To test this reasonable expectation, χ342LD cells were inoculated onto MacConkey agar base medium, which is identical to MacConkey salicin except that no sugar is present. Colonies were suspended periodically to determine the number of viable cells (Figure 7) and, after 28 days of incubation, 20 colonies were suspended and the entirety of each suspension was plated onto salicin minimal medium in order to detect any $Sal^+$ mutants that might have arisen. None were detected. If, at 13 days, each colony contained on average one $Sal^+$ mutant and no additional mutants were generated, then the $Sal^+$ mutants would be subject to cell death at the same rate as the other cells in the colony and by 28 days there would be an average of about 0.13 $Sal^+$ cells per colony. In that case, from the Poisson distribution the proportion of colonies with no mutants would be $e^{-0.13}$ or 0.878 and the probability of failing to detect any $Sal^+$ mutants in 20 colonies would be $0.878^{20}$ or 7.4%.

Even if the $Sal^+$ mutants were missed by chance alone, the excision mutants should still constitute about 10% of the cells in colonies on MacConkey base. Thirty-two days after inoculation onto MacConkey agar base, 18 colonies were suspended, diluted, and plated onto MacConkey salicin medium to detect excision mutants by their ability to form papillae within 3 days. A minimum of 200 colonies were screened from each of the original 18 colonies and no excision mutants were detected. If excision mutants constituted as little as 0.002 of the population in these colonies, then there would be an average of 0.4 excision colonies ($0.002 \times 200$) per suspension plated and only 67% of the suspensions would be expected to contain no excision colonies. Thus, the probability that no excisions would have been detected is $0.67^{18}$ or 0.00074.

From these experiments it is concluded that the excision of IS*103* from within *bglF* does not occur in the absence of salicin in the medium.

## CONCLUSIONS

The results presented here warrant consideration from a variety of perspectives.

First is the observation that mutation rates measured in growing cultures need not, and probably do not, reflect mutation rates under other conditions. This is of particular importance in considering mutation rates in adaptive evolution. It is clear that bacteria spend little time in exponential growth conditions. *E. coli* are estimated to double once or twice a day in nature, rather than once or twice an hour. It seems likely that conditions within old colonies on nutritionally depleted solid surfaces are more representative of typical natural conditions than are conditions in well aerated liquid media with nutrients in gross excess and the temperature held at an optimal 37°. There have been almost no studies of mutation rates under these suboptimal conditions, and those that have been done suggest that mutation rates are different from those measured in growing cells (RYAN 1955; RYAN *et al.* 1959).

Second is the surprising observation that, within a colony, a usually stable insertion sequence can excise from within a gene in over $10^6$ individuals in a short time. For the population, the result of these precise excisions (the only ones detected) is the generation of genetically identical but independently derived mutants. This contravenes a basic assumption in constructing phylogenies, that a set of indistinguishable mutants within a population reflects a single event that occurred earlier in the history of the population, all the mutants descending from a single ancestor. In this case the mutants are no more closely related to each other than they are to any other randomly chosen member of the population.

Third is the observation that excision of IS*103* from within *bglF* is only detected when it creates the potential for a subsequent advantageous second mutation (if one accepts the order of events that I prefer), or when it is selectively advantageous (if one prefers the alternative order of events). Excision of IS*103* from

within *bglF* has been observed only on MacConkey salicin medium, and not on MacConkey medium without sugar. Excision itself is not advantageous. Even on MacConkey salicin medium, an advantage is realized only when a second mutation ($bglR^0 \rightarrow bglR^+$) occurs. This has the appearance of anticipatory evolution, a phenomenon that would itself be strongly selected, but for which it is very difficult to imagine a molecular mechanism (but see CAIRNS, OVERBAUGH and MILLER 1988). Despite appearances, it is not necessary to invoke anticipatory evolution to explain these results. Physiological regulatory feedback loops could modulate the probabilities of mutations at specific loci when the cell is under stress, and thereby produce results similar to these. We now take for granted the notion that gene expression is regulated, *i.e.*, subject to modulation by environmental conditions, but this notion was not always in mind (KELLER 1983). We now have to examine the notion implied by these results, that mutation, like other biological processes, is subject to regulation by environmental factors.

There are two reasons to believe that these observations are not simply reflections of the unusual biology of a particular mobile DNA element, IS*103*, which have no real relevance to evolution or to mutation rates in general.

First, DNA rearrangements mediated by and including the transpositions of mobile DNA elements are a significant portion of the mutational repertoire of cells. That repertoire also includes base pair substitutions, large and small deletions, large and small additions, inversions, and (in higher organisms) translocations. All of these forms of mutation can either increase or decrease the effectiveness of a particular gene. In higher organisms, rearrangements that move genes between euchromatic and heterochromatic regions can dramatically affect levels of expression (SPOFFORD 1976; RUSHLOW and CHOVNICK 1984; RUSHLOW, BENDER and CHOVNICK 1984). Major rearrangements are often seen when comparing genetic structures of closely related species (discussed in ALEXANDER 1976 and HESS 1976) and have been implicated in speciation mechanisms (WHITE 1978). All of these evolutionarily important rearrangements can be mediated by mobile DNA elements. Insertion sequences are best known for mutating genes by disrupting coding sequences (JORDAN, SAEDLER and STARLINGER 1968; SHAPIRO 1969; SAEDLER *et al.* 1972), but many other effects have been demonstrated. Insertion sequences can provide promoters for other genes (CIAMPI, SCHMID and ROTH 1982; PRENTKI *et al.* 1986), they can activate cryptic genes by inserting into upstream regions (REYNOLDS, FELTON and WRIGHT 1981; REYNOLDS *et al.* 1985; L. L. PARKER and B. G. HALL, unpublished results), they can promote replicon fusions (BARSOMIAN and LESSIE 1986), and they can reduce the level of expression of genes by topological regulation when they are as far away as 5 kbp from the affected coding region (STOKES and HALL 1984). Insertion sequences can generate both inversions and deletions (CRAIG and KLECKNER 1987; KLECKNER 1981), both of which can lead to gene fusions and thereby potentiate novel genetic functions. In one case, excision of IS*5* generated a short deletion that has been shown to increase the expression of a nearby gene and thereby to alter fitness (MILLER, DYKHUIZEN and HARTL 1988). Although we do not yet know the extent to which the phenomena reported here can be generalized to other mobile elements and other genes, it is clear that transposition of mobile DNA elements is evolutionarily important and particularly warrants investigation when that transposition is related both to adaptation and to environmental conditions.

Second, the phenomena reported here are not unique. Several years ago SHAPIRO (1984) pointed out that in a strain in which excision of another mobile element, bacteriophage Mu, fused the arabinose and lactose operons to permit growth on lactose-arabinose medium, the excision did not occur in growing cells. In striking similarity to this study, the excision of Mu occurred only several days after the cells had been plated onto selective medium, and did not occur during the same period when the cells were plated onto medium that did not contain both lactose and arabinose. J. CAIRNS has been studying similar phenomena with several genetic systems in *E. coli*, and has concluded that there is an entire class of mutations that occur only when they are advantageous to the cell (CAIRNS, OVERBAUGH and MILLER 1988; J. CAIRNS, personal communication). Indeed, this entire study, begun as a study of apparently simultaneous multiple mutations, was stimulated by discussion with CAIRNS. The results of SHAPIRO (1984) and CAIRNS, OVERBAUGH and MILLER (1988) can no longer be classed as anomalies uniquely associated with Mu.

There are two principal lessons from these experiments. The first is that we are ignorant of the fundamental mechanisms and rates of mutations in nongrowing but metabolizing cells. We have studied an evolutionarily fundamental event (mutation) under unrealistic circumstances and in a portion of the life cycle (exponential growth) that represents only a small part of the life histories of microorganisms. We have assumed that what we have learned in those limited studies is sufficiently general that we can extrapolate from that knowledge to generate realistic evolutionary theories and expectations. As a result, we discuss mutation rates as though they were constants, generally related to cell division, that reflect only the properties that are inherent in the genome of the cell. We

have ignored the possibility that mutation rates might be highly variable and subject, like other cellular processes, to environmental modulation. These results suggest that spontaneous mutation is a more complex process than we have believed, that mutation rates are not biological constants, and that probabilities of mutation are modulated by normally encountered environmental factors.

The second lession is that evolutionary biologists need to critically examine the assumptions that underlie methods of deducing evolutionary rates and of constructing phylogenetic trees. If the same mutation can, under appropriate selective conditions, occur in a large number of independent individuals (as occurs with excision of IS*103* from within *bglF*), then individuals that share derived characters are not necessarily more closely related to each other than they are to other members of the population. If the probabilities of particular mutations are subject to environmental modulation, then the number of observed differences between two sequences may be completely unrelated to the time since they diverged.

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