Adaptive Mutation in *Escherichia coli*: A Role for Conjugation

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When subjected to selective conditions that impose starvation, a bacterial population can accumulate mutations, called adaptive, that allow colony formation. Here, the reversion of a *lac* allele under selective conditions, in a model system using *Escherichia coli* with the *lac* mutation on an F′ plasmid, was shown to require the conjugational capacity of the plasmid. Reversion associated with transfer was shown, and when the same *lac* allele was chromosomal, reversion to Lac$^+$ was 25 to 50 times less frequent. Postplating reversion was 25 times less when mating was inhibited by the presence of detergent. Mutability associated with conjugation provides new ways of thinking about the origin of adaptive mutations.

The notion that mutations are adaptive was dispelled more than 40 years ago by the observations that: (i) Bacteria resistant to a lytic phage were present in cultures before the addition of the phage used to select those resistant mutants (*1*). (ii) Bacteria resistant to streptomycin were present before the addition of the drug used to select the mutants (*2*). The enthusiasm for rejection of the possibility of adaptive or directed mutations (those occurring because they provide a selective advantage) may have been premature, because the selections used were lethal and the mutations selected were recessive (*3*–*5*). Reversion of microbial loss-of-function mutants has provided a convenient tool for the investigation of mutagenesis under less severe conditions (*3*, *4*, *6*). Because cultures of such mutants can be deposited on a semisolid selective medium, rare mutational events that result in recovery of that function can be readily detected as those that allow colony formation. Mutant Lac$^+$ colonies, arising from a population of *lac*$^-$ *Escherichia coli* deposited on minimal medium with lactose as the sole carbon source (*4*), continued to appear during prolonged selection and did not accumulate when the bacteria were starved in the absence of the selective carbon source. This increase occurred in the absence of the accumulation of other unselected mutational events. These observations provoked the hypothesis that the reversion mutations were adaptive.

Although the early experiments suffer from many uncertainties (*7*), much of the recent work on this problem, confirming and extending the primary observations (*8*–*11*), has used an *E. coli* strain (FC40) that contains a chromosomal *lac* deletion and an F′ Lac episome with a revertible *lac* allele (*8*). This *lacI-lacZ* fusion allele, *lacI33*, is out of frame for the *lacZ* portion of the sequence and constitutes the indicator of mutation to *lac*$^+$. Because of potential nonsense triplets, only frameshift events within about 100 nucleotides in the *lacI* portion of the construct can give rise to *lacZ*$^+$ revertants. FC40 yields new Lac$^+$ colonies for many days after being plated under the selective conditions (*8*). After a few days, 90 to 95% of the revertants present on the plates seem to be the result of mutations that occur on the plate (*8*). The revertants that appear during prolonged selection have a different spectrum of sequence changes than do the mutations that arise during unselected exponential growth (*12*). These latter observations, together with some genetic evidence (*10*), suggest that they are the product of DNA replication errors in the absence of net growth of the parental cell population. The origin of this replication process has been the subject of several models involving recombination, residual cell growth, hypermutable states, and partial genome amplification [reviewed in (*5*)]. We show here that a signal for F′ conjugal transfer, or transfer itself, could provide the localized DNA replication in either the donor or the recipient cells or both, that appears to be responsible for the appearance of adaptive mutants.

In these experiments, the cultures of strains carrying the *lacI33* allele were plated on minimal lactose plates with an excess of scavenger cells (*8*). A scavenger is used to deplete the plates of residual metabolites that could permit some growth of the revertible indicator strain on the selective medium (*8*). The scavenger strain (FC29) differs from FC40 because it is rifampicin-sensitive and carries in its F′ plasmid a nonrevertible deletion of the *lac* operon that cannot complement or recombine with the revertible episome to give *lac*$^+$.

Because the mutational target is present on an F′ plasmid, we explored the possibility of an involvement of episome transfer on the appearance of the *lac*$^+$ mutants. After reaching stationary phase, cells with an F′ can temporarily lose the surface exclusion phenotype and behave as F′ recipients without the loss of their sex factor (*13*). To determine whether *lac* reversion could be associated with such transfer during the starvation on the selective plates, we used the fact that streptomycin-sensitive cells, pretreated with the antibiotic, can act as episome donors to streptomycin-resistant cells (*14*) even though their viability is lost within a few minutes of addition of the drug. A streptomycin resistant (Sm^r) derivative of the scavenger strain was isolated (PR287) and an excess of this scavenger (10^{10}) was mixed in streptomycin containing soft agar with 10^9 streptomycin-sensitive (Sm^s) FC40 cells and layered on minimal lactose plates containing streptomycin (100 μg/ml). The plates, incubated at 37°C, were examined daily and showed that Lac$^+$ Sm^r colonies accumulated with time (*15*) (Fig. 1). Thus, the indicator allele in FC40 could be transferred to the Sm^r scavenger cells and experience mutation to *lac*$^+$. When we provided scavenger cells in a separate agar layer by first depositing them on plates in soft agar and then adding the indicator strain in another layer of soft agar, virtually no Lac$^+$ Sm^r colonies appeared (Fig. 1). Thus, physical proximity between cells from the two strains is necessary in order to obtain Lac$^+$ Sm^r revertants.

The evidence for mutation-accompanied episome transfer under these experimental conditions provoked the question of whether or not such transfer, or the capacity for such transfer, could be essential for the events that give rise to the Lac$^+$ revertants on the selective plates. If conjugation was involved, the rate at which postplating Lac$^+$ revertants appear would be expected to be enhanced with increasing concentrations of potential conjugation partners; that is, scavenger cells. To examine this possibility, about 10^8 indicator (FC40) cells were mixed with increasing numbers of scavenger cells and deposited in soft agar on minimal lactose plates previously layered with 10^{10} scavenger cells. The increase in the concentration of scavenger cells mixed with the indicator cells was accompanied by a modest increase in the rates of accumulation of the revertant Lac$^+$ colonies (Fig. 2).

Among late-appearing colonies, an increase in the concentration of scavenger cells would be expected to result in an increased likelihood of the presence of *lac*$^+$ mutant episomes in scavenger bacteria. The drug resistance genotypes of several hundred Lac$^+$ colonies that appeared on day 6 were analyzed in order to distinguish between the indicator (FC40) and the scav-

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enger (PR287) backgrounds (*16*). When indicator cells were deposited without an admixture of scavenger cells, all of the revertants bore the drug resistance genotype of the FC40 indicator strain. When the FC40 cells were mixed with 2×10^{10} PR287 scavenger cells, 40% of the colonies displayed the drug resistance genotype of the scavenger, and an additional 10% showed the presence of cells with the scavenger genotype as well as cells with the indicator genotype. These mixed colonies are likely to be products of conjugational transfer occurring after a clone of *lac*$^+$ mutants had been established. The remaining colonies were of the indicator genotype only. In a similar test, when only 10^9 scavenger cells were present, 20% displayed the drug resistance genotype of the scavenger cells and 3% were mixed.

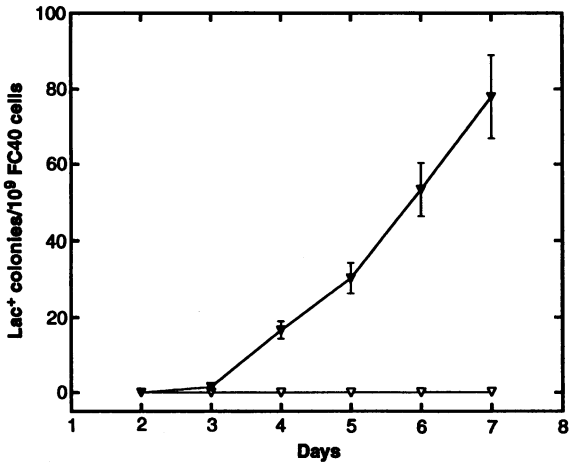

Fig. 1. F′ plasmid transfer and reversion of a *lac* frameshift mutation. About 10^9 Sm^r cells from an FC40 (*lacI33*) culture (*7*) were used for each plate (*29*). These cells and a 10-fold excess of Δ*lac* Sm^r scavenger cells (*7*) were mixed in soft agar containing streptomycin and plated on minimal lactose plates with streptomycin (100 μg/ml). The *lac*$^+$ Sm^r colonies (filled triangles) accumulate with time. These colonies must be reverted products of the transfer of the indicator episome to an Sm^r scavenger cell. No significant accumulation of *lac*$^+$ Sm^r colonies was observed when the two strains were separated by depositing the FC40 cells over minimal-lactose streptomycin plates previously overlaid with the excess of scavenger cells (open triangles). Each point represents the mean from 10 independent FC40 cultures with its standard error. Pairwise samples from the same cultures were plated mixed and separated from the scavenger.

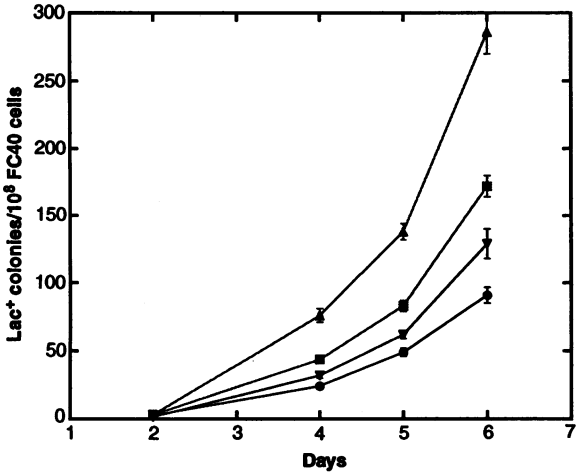

Fig. 2. Effect of cell density on the yield of mutants after plating. Minimal M9 lactose plates (*26*) were overlaid with 0.2% lactose soft agar containing 10^{10} scavenger cells. Samples with 1.5×10^8 indicator (FC40) cells from one culture were then mixed with increasing numbers of scavenger cells. 0 (circles), 1×10^9 (downward-pointing triangles), 5×10^9 (squares), and 2×10^{10} (upward-pointing triangles). The mixtures, in 0.2% lactose soft agar, constituted the top layer. Each point represents the mean of the daily counts of six plates. Error bars represent the standard errors.

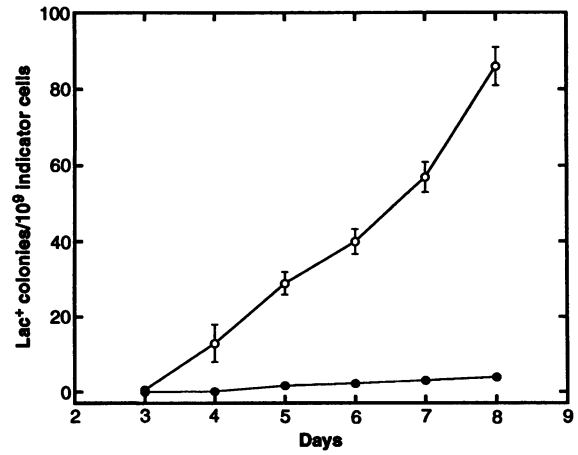

Fig. 3. The accumulation of *lac*$^+$ revertants for a chromosomal allele of *lacI33*. 10^9 cells from strains carrying the *lacI33* allele either on an episome (PR321) (open circles) or on the chromosome (PR269) (filled circles) (*17*) were layered on minimal-lactose plates together with a 10-fold excess of M182 scavenger cells. Points represent the daily means of the *lac*$^+$ colonies from 100 independent cultures. Error bars represent standard errors.

These observations are consistent with the proposal that conjugation plays a role in these reversion events. However, most of the Lac$^+$ colonies were nevertheless of the indicator genotype. If conjugation is indeed required for reversion, conjugation-specific replication of the F′ in the donor, provoked by initiation of the conjugation process, could provide the substrate for reversion without requiring successful episome transfer to another cell. Alternatively, the low plate concentration of the indicator bacteria raises the prospect that such transfer could occur within the same bacterium.

To further examine the proposal that the observed mutability is characteristic of its presence on an F′ plasmid, we introduced the *lacI33* allele into its normal chromosomal location, creating strain PR269 (*17*). In parallel, we constructed in the same background a strain PR321 that carried the FC40 F′ plasmid (*18*). When the latter strain was plated on lactose minimal plates, it behaved qualitatively like FC40, as shown by the continuous appearance of Lac$^+$ revertants during several days of incubation (Fig. 3). The frequency of mutants that occurred during unselected growth in liquid medium and the rate of accumulation after day 2 on the selective plate were about 10 times less in this background than in the FC40 strain. This is consistent with previous observations showing that the background from which FC40 is derived (P90C) displays elevated episomal *lac* reversion rates when compared with another *E. coli* background (*19*). Analysis of the postplating reversion of the chromosome-borne *lacI33* allele in strain PR269 showed that the rate of accumulation of revertants was 25 to 50 times less than for the chromosomally isogenic strain with *lacI33* on the episome (Fig. 3). The residual time-dependent accumulation of *lac* revertants of the chromosomal allele has not been investigated but could reflect the uncovering of low-frequency revertants with a spectrum of reduced growth rates on lactose.

Our final evidence for the requirement of a capacity for episome transfer in the generation of postplating revertants was obtained by physical impairment of conjugation. A low concentration (0.01%) of the detergent SDS dissociates mating pili into subunits and interferes with cell aggregation and conjugational transfer (*20*). This low concentration of SDS severely reduced the conjugation capacity of FC40, although it did not affect the growth rate in liquid culture, the viability under starvation conditions on a semisolid medium (*21*), or the

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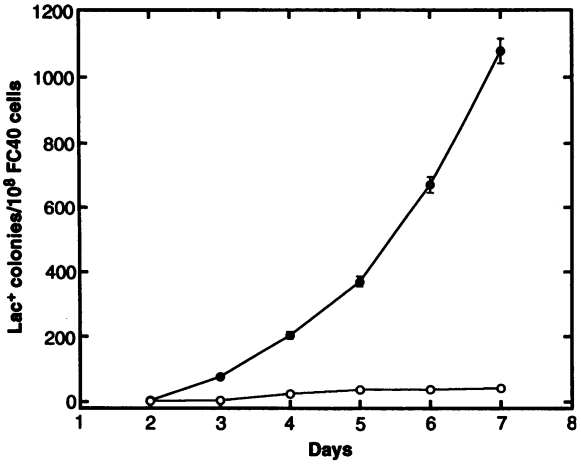

Fig. 4. Effect of detergent on the appearance of postplating revertants. Strain FC40 was grown from an inoculum of 10^3 bacteria per culture in minimal 0.1% glucose medium containing 0.01% SDS. Ten samples containing 3×10^7 cells from one culture were then mixed with 10^{10} scavenger FC29 cells each and plated on minimal lactose with (open circles) or without (filled circles) 0.01% SDS (n = 10).

plating efficiency of Lac$^+$ cells, under conditions used to detect Lac$^+$ revertants. The presence of SDS in the selective plates markedly reduced the rate of accumulation of Lac revertants during the postplating incubation, whereas it did not alter the yield of early mutant colonies that are presumably products of mutations occurring during the period of unselected growth in liquid (Fig. 4). Similar results were obtained with cells that were initially grown in the presence or absence of 0.01% SDS (*22*).

Thus, our experiments suggest a requirement for the formation of mating aggregates in order to obtain postplating Lac revertants with strain FC40. Results obtained with a *Salmonella* system (*23*) provide evidence for a similar conclusion. Vigorous F′ transfer in agar surface lawns of *E. coli* populations subject to nonlethal selection has been observed (*24*). The conjugation-provoked replication of the F′ plasmid in nongrowing cells could display a characteristic mutability or enlarge the pool of targets for new mutations as the result of a net increase in the plasmid population. The basis for the reported requirement for recombination functions (*8*, *11*) is unclear but could reflect a need for those functions, under starvation conditions, to allow conjugation to occur or to process conjugation products. The conjugation-associated replication of the episome, either in the indicator or the scavenger cells, could provide the localized DNA synthesis required for the accumulation of the adaptive mutants (*10*, *12*). If mismatch repair were slow in starving cells, as suggested by Stahl (*25*), a transient *lac*$^+$ DNA sequence could be transcribed, providing a source of energy that allows the replication and fixation of the revertant sequence.

A perhaps simpler hypothesis would suggest that the lactose requirement for the appearance of new mutants reflects a low level of lactose metabolism in the FC40 indicator cells (*9*), that is inadequate for cell duplication but sufficient for pilus assembly and plasmid replication. The reported failure of *lac*$^+$ mutants to accumulate when the lactose selection is carried out under conditions lacking a required amino acid (*8*) would be consistent with this hypothesis. The predominance of revertant F′ plasmids in the indicator cells could reflect the greater stringency of the *lac* deletion allele in the scavenger cells and the consequent difficulties in replication of transferred DNA.

Whatever the mechanism that will ultimately be elaborated, a role for conjugation in the occurrence of mutations under conditions of selection stress introduces a new consideration and could have implications when mechanisms of evolutionary change are considered.

Note added in proof: Foster and Trimarchi have described observations showing that the RecA-dependent yield of postplating revertants of the *lacI33* allele "requires that the *lac* allele be on the episome and is enhanced by the expression of conjugal functions" (*30*).

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15. The rate at which *lac*$^+$ colonies appeared was about one-tenth of the rate observed when no streptomycin was present. The rate was not changed by incubation of the FC40 strain with streptomycin for 16 hours before adding Sm^r scavenger cells and plating. In reconstruction experiments using an Sm^s *lac*$^+$ revertant plasmid–containing strain, F′ transfer to give Lac$^+$ Sm^r colonies by day 2 was 1 in 200 to 1 in 1000 of the Lac$^+$ bacteria plated. Lac$^+$ Sm^r colonies continued to appear to increase about fourfold over the next 4 days.
16. For each group, 200 well-separated Lac$^+$ colonies were collected from at least eight plates representing different cultures from the experiment shown in Fig. 2. Each colony was patched on a set of minimal lactose plates containing, in order, streptomycin (100 μg/ml), rifampicin (100 μg/ml), both drugs, or no antibiotics. Patches with cells resistant to rifampicin but sensitive to streptomycin were classified as the indicator strain. Conversely, patches with cells sensitive to rifampicin but resistant to streptomycin were classified as the scavenger strain. None of the colonies displayed the capacity to grow on the plates with both antibiotics. Ninety-nine percent of the colonies tested grew on the final lactose-minimal plate.
17. For the construction of strain PR269, a P1 lysate was made on a strain containing the *zah281*::Tn10 insertion, which is linked to *lac* (*26*). The lysate was used to transduce FC40. Tetracyclin-resistant *lac*$^-$ transductants were selected and used to obtain a new P1 lysate that was used to transduce M182, which carries a deletion in the *lac* operon (Δ*X74*) (*27*). A tetracyclin-resistant product of this transduction that yielded pale blue colonies in the presence of X-Gal and was able to revert to *lac*$^+$ was used for the experiments.
18. PR 321 was constructed by transfer of the F′ plasmid from FC40 to a derivative of strain M182 (*27*) that carries an insertion mutation disrupting the *proAB* genes (*26*) to ensure plasmid stability.
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21. To test the viability of the cells under the selection conditions in the presence of the detergent, about 400 *lac*$^+$ cells from a revertant FC40 culture were mixed with 10^{10} scavenger cells and plated on minimal plates with no carbon source with or without 0.01% SDS. After 0, 2, 3, and 5 days of incubation at 37°C, duplicate plates from each group were overlaid with 3 ml of minimal soft agar containing 1.2% lactose and incubated for another 48 hours. For both sets of plates, with and without detergent, the Lac$^+$ cells maintained their full capacity to form colonies.
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29. Unless specified, all selective plates were M9 minimal with 0.2% lactose (*28*). Indicator strains were always grown for 20 hours at 37°C to glucose exhaustion in M9-minimal medium with 0.1% glucose from inocula of less than 10^4 cells. Scavenger strains were grown in Luria-Bertani broth, then washed and concentrated in M9 salts. Cells were deposited on the selective plates by mixing them with 0.2% lactose M9 soft agar and layering. All incubations were at 37°C.
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