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Adaptive Mutation and Slow-Growing Revertants of an *Escherichia coli lacZ* Amber Mutant

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ABSTRACT

We have studied revertants, selected on lactose minimal agar medium, of the *Escherichia coli* $lacZ_{am}$ strain that was first used by Cairns and his colleagues to demonstrate the phenomenon of "adaptive mutation." We have found, by performing appropriate reconstruction studies, that most of the late-arising Lac$^+$ revertants of this *lac* amber strain (appearing as colonies in 3–5 days) are slow-growing ochre suppressor mutants that probably existed in the culture prior to plating and cannot, therefore, be classified as "adaptive." The appearance of a small number of fast-growing, late-arising Lac$^+$ revertants may result from residual cell growth and turnover or from phenomena related to the fact that the $lacZ_{am}$ mutation in strain SM195 is carried on an F′ plasmid. Thus, the appearance of late-arising revertants in this $lacZ_{am}$ system does not provide convincing evidence that selective conditions specifically increase the rate of occurrence of favorable mutations.

IN 1988, Cairns and his colleagues challenged one of the central tenets of genetics by suggesting that selective conditions specifically facilitate the occurrence of mutations that permit cell growth, a phenomenon they called "directed mutation" (CAIRNS *et al.* 1988). CAIRNS' proposition was based, in part, on the observation that colonies of Lac$^+$ revertants of an *Escherichia coli* strain carrying a *lacZ* amber mutation on an F′ plasmid continue to arise on lactose minimal agar medium for several days after plating.

The *lacZ* amber mutant used by CAIRNS *et al.* (1988) was *E. coli* strain SM195 (Δ*lac-pro*/F′ $lacZ_{am}$, *pro*$^+$) (FOSTER and CAIRNS 1992). This strain contains a chain-terminating, amber (UAG codon) mutation in the *lacZ* gene, which codes for β-galactosidase. CAIRNS *et al.* (1988) reported that Lac$^+$ colonies continued to appear for several days after SM195 cells were plated on minimal agar medium containing lactose as the sole source of carbon and energy while, at the same time, valine-resistant mutants were not recovered during prolonged exposure to the lactose medium. The authors concluded that this experiment was evidence for "directed mutation" in which the presence of lactose somehow resulted in Lac$^+$ mutations but not other mutations that would be of no benefit to the cells on the lactose medium.

We have investigated the nature of the Lac$^+$ revertants of strain SM195 that appear as colonies at various times after plating. By characterizing the revertants genetically and performing reconstruction studies to evaluate their growth rates, we have been able to determine that most of the late-arising revertants in this system are simply slow-growing cells containing ochre suppressors, which can suppress amber mutations. Since these revertants could have been present in the culture prior to plating on the selective medium, they do not provide any evidence for the existence of "directed mutation" or what is now called "adaptive mutation" (FOSTER 1993). The possibility that the small number of fast-growing, late-arising revertants may result from cell growth or turnover or from F′-related phenomena is discussed.

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MATERIALS AND METHODS

Bacterial media and strains: *E. coli* strain SM195 [*ara* Δ(*lac-proB*)$_{XIII}$ Δ(*uvrB-bio*) *thiA* Rif^r/F′ *lacI*$^+$ $lacZ_{am}$ *lacY*$^+$ *pro*$^+$] was used for all the *Lac* reversion studies. *E. coli* strain XA100 [*met*$^-$ *arg*$^-$ Δ(*lac-proB*)$_{XIII}$ *thiA* Sup$^-$ Nal^r Rif^r F$^-$] was used in mating experiments to determine whether Lac$^+$ revertants of strain SM195 were intragenic. Both of these bacterial strains have been described by FOSTER and CAIRNS (1992) and were obtained from Dr. P. L. FOSTER.

Glycerol minimal medium consisted of M9 salts (MILLER 1972) supplemented with 0.1% filter-sterilized glycerol, 20 μg/ml thiamine, 1.2 μg/ml biotin and 0.001% gelatin (FOSTER and CAIRNS 1992). Lactose minimal agar contained M9 salts supplemented with 0.1% filter-sterilized lactose, 20 μg/ml thiamine, 1.2 μg/ml biotin, and 1.5% agar (FOSTER and CAIRNS 1992). For mating experiments with strain XA100, lactose minimal agar was supplemented with 40 μg/ml of methionine, arginine, proline, and nalidixic acid.

Timed isolation of revertants: Lac$^+$ revertants of *E. coli* strain SM195 were isolated in each of two separate experiments on lactose minimal agar over a period of several days, without scavenger cells, as described by FOSTER and CAIRNS (1992). For each experiment, 10 independent cultures were obtained by growing an overnight culture of strain SM195 in glycerol minimal medium supplemented with 5% LB medium and inoculating approximately 2×10^3 cells from this culture

into each of 10 flasks containing 50 ml glycerol minimal medium. After shaking at 37° until they were saturated (~40 h), the cultures were chilled and the cells were washed twice in phosphate-buffered saline (PBS) by centrifugation. Then each culture was spread at 10^9 cells per plate on petri dishes containing 25 ml lactose minimal agar. The plates were incubated at 37° under high humidity. Five newly arising colonies from each of the 10 cultures were picked after 2, 3, 4, and 5 days, and single colony purifications were performed on lactose minimal agar medium. The purified Lac$^+$ revertants were subjected to DNA colony hybridization to determine whether they contained known amber or ochre suppressor mutations. Matings were performed with strain XA100 to determine whether those revertants that did not contain known suppressor mutations resulted from intragenic mutations.

DNA colony hybridization: Each revertant was analyzed for the presence of known amber and ochre mutations by the method of KUPCHELLA and CEBULA (1991), as previously described (PRIVAL and CEBULA 1992). The DNA probes used and the temperatures for hybridization and washing of filters were as follows: *supB* (ochre), 5′-TTTAATACCGGCATTCCC-3′ (52°); *supC* or *supM* (ochre), 5′-ACTTTAAATCTGCCGTCA-3′ (50°); *supG* or *supN* (ochre), 5′-GTTGACTTTAAATCAATT-3′ (44°); *supD* (amber), 5′-AACGGACCGGTCTCTAAA-3′ (54°); *supE* (amber), 5′-TCTAATTCCGGCATTCCG-3′ (54°); *supF* (amber), 5′-ACTCTAAATCTGCCGTCA-3′ (52°).

Mating experiments: Revertants that did not hybridize with any of the ochre or amber suppressor probes were mated in microtiter wells with *E. coli* strain XA100 and replica-plated onto lactose minimal agar medium containing nalidixic acid to detect intragenic reversion mutations carried on the F′. Lac$^+$ revertants that were negative in both the DNA-colony hybridization and mating experiments were classified as unknown suppressor revertants.

Reconstruction studies: To determine how long it takes for various Lac$^+$ revertants to grow into readily visible colonies on lactose minimal medium in the presence of a lawn of SM195 cells, Lac$^+$ revertants from each of the two experiments for timed isolation of revertants were diluted in PBS and 50–300 of these cells were plated along with 10^9 cells of strain SM195. Colonies were counted each day, beginning on day 2.

In general, reconstruction studies were performed by growing purified revertants overnight in glycerol minimal medium supplemented with 5% LB and then diluting and seeding each revertant into 10^9 SM195 cells and plating the cell mixture. In both of the timed isolation experiments, reconstruction analyses were performed on all purified Lac$^+$ revertants classified either as intragenic or as unknown suppressors as well as a sampling of revertants carrying known suppressors. In addition, each colony picked after 2 or 4 days of selection in the first timed isolation experiment was immediately diluted in PBS and subjected to reconstruction analysis. The reason for plating cells picked from newly arisen colonies that were still growing was to ensure that the results of the reconstruction study would not be affected by the use of stationary phase cells or by secondary mutations arising during purification of revertants.

RESULTS

Growth rates and genotypes of revertants: In two separate experiments for timed isolation of revertants, Lac$^+$ revertants of strain SM195 continued to appear after 2 days, as shown in Figure 1 and previously reported by CAIRNS *et al.* (1988). Each of the 50 revertant colonies picked on days 2 and 4 in the first experiment

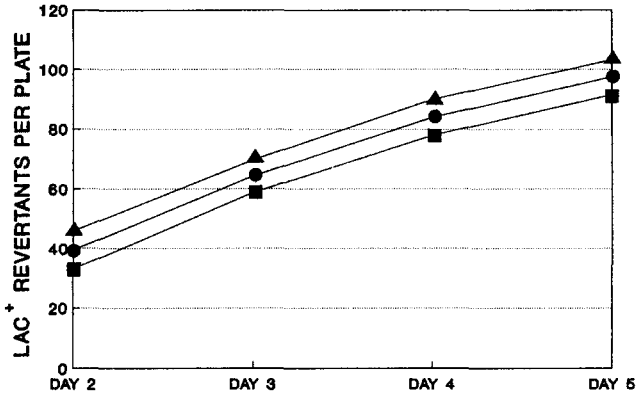

FIGURE 1.—Cumulative median numbers of Lac$^+$ revertants colonies appearing each day. Data are shown for the appearance of revertant colonies on lactose minimal agar in timed isolation of revertants: experiment 1, ▲; experiment 2, ■; and the two experiments combined, ●.

was tested in a reconstruction study in which the colony was immediately diluted in PBS and 50–250 cells from the colony plated on a lactose minimal agar plate along with 10^9 cells of strain SM195. The amount of time it took for the revertants to appear from among the SM195 cells was monitored by counting the colonies on each plate beginning 2 days after plating. Subsequently, each revertant was purified and characterized genetically.

The results displayed in Figure 2 show that all of the revertants appearing on day 2 in the original selection experiment took only 2 days to appear as colonies when plated along with 10^9 SM195 cells in the reconstruction study. However, most of the revertants that had originally appeared on day 4 took longer than 2 days to grow into visible colonies in the reconstruction study. Almost all of these slow-growing, late-arising revertants were ochre suppressor mutants. All of the revertants that gave colonies in 2 days in the reconstruction studies, whether they originally arose on day 2 or day 4, were either intragenic, amber suppressor, or unknown suppressor mutants.

All of the revertants picked during both experiments for timed isolation of revertants were purified and characterized by genetic analysis. All of those classified as intragenic or unknown suppressor revertants and representative revertants of each class of amber and ochre suppressor were subjected to reconstruction analysis. It was found that all of the amber suppressor mutants evaluated and most intragenic mutants were fast growers, giving rise to readily visible colonies in 2 days when plated with 10^9 cells of strain SM195. In contrast, all ochre suppressor mutants examined were slow growers, taking more than 2 days to appear as colonies in reconstruction studies. Purified revertants plated from overnight liquid cultures and those of the same type plated directly from newly arisen revertant colonies (Figure 2) gave similar results in reconstruction studies.

The spectra of revertants obtained on each day in

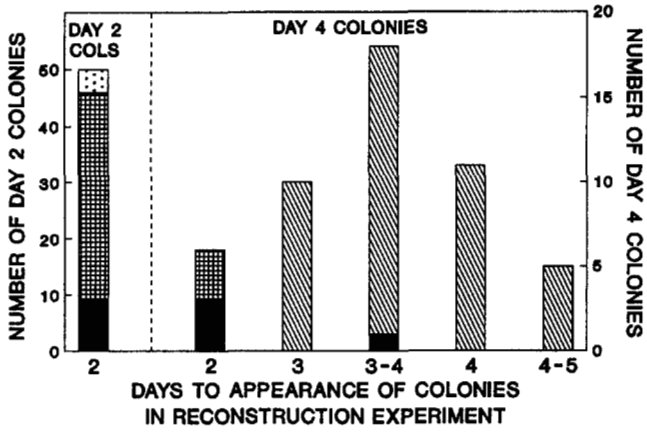

FIGURE 2.—Results of reconstruction studies on revertants picked on days 2 and 4 in experiment 1 for timed isolation of revertants. The graph shows the distribution of rates at which the revertants grew into colonies on lactose minimal agar plates in the presence of 10^9 SM195 cells as well as the nature of the mutations leading to the Lac$^+$ phenotype: solid black, intragenic; checkered pattern, amber suppressors; diagonal pattern, ochre suppressors; stippled pattern, unknown suppressors. Revertants that gave rise in 3 days to very small colonies that grew into more readily visible colonies by day 4 were classified as growing in 3–4 days, and similarly for 4–5 days.

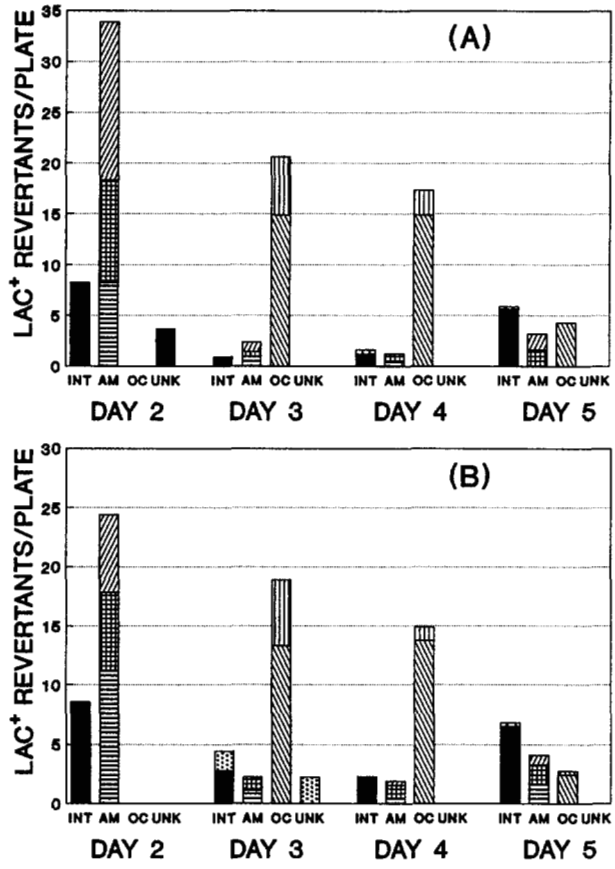

FIGURE 3.—Spectra of revertants of strain SM195 appearing each day on lactose minimal agar medium in experiments for timed isolation of revertants. (A) Experiment 1; (B) experiment 2. DAY 2, DAY 3, etc. refer to the day on which the colonies appeared on the agar medium and were picked for analysis. The number of revertant colonies of each type analyzed on each day in each experiment was normalized so that the total number of revertants represented in the figure is equal to the median number of colonies per plate for the 10 cultures on that day. INT, intragenic revertants (fast-growers, black; slow-growers, stippled pattern). AM, amber suppressor revertants, which included *supD* (horizontal pattern), *supE* (checkered pattern), and *supF* (diagonal pattern). OC, ochre suppressor revertants, which included *supC/M* (diagonal pattern) and *supG/N* (vertical pattern). UNK, unknown suppressor revertants (fast-growers, black; slow-growers, stippled pattern). Fast-growers are revertants that grew into readily visible colonies in 2 days in reconstruction studies such as that in Figure 2.

the two timed isolation experiments were similar, as shown in Figure 3. Overall, ~80% of the revertant colonies appearing after 3 or 4 days of incubation and 26% of those appearing after 5 days were ochre suppressor (*supC/M* or *supG/N*) mutants, whereas none of those appearing in 2 days contained ochre suppressors.

DISCUSSION

Slow-growing revertants: When cells of the *lac* amber mutant strain SM195 were plated on lactose minimal medium, over 70% of the Lac$^+$ revertant colonies appearing after 3 to 5 days were composed of slow-growing revertants. We conclude that these revertants could have been present in the original cultures when plated and taken more than 2 days to grow into visible colonies because of their slow growth. Most of the slow-growing revertants (94%) contained ochre suppressors; the rest resulted from intragenic mutations or contained unknown suppressors. Such slow-growers must be excluded from any analysis of the data relating to the possible occurrence of adaptive mutation.

It should be noted that the ochre suppressor mutants did give rise to visible colonies within 2 days when streaked alone on lactose minimal agar medium, although these colonies were smaller than those of amber suppressor mutants or most intragenic mutants. Their delayed appearance when plated in the presence of 10^9 SM195 cells underscores the necessity of determining the growth characteristics of late-arising mutants by reconstruction studies to determine whether they arose during selection or before plating. Previous studies have emphasized the importance of such reconstruction studies (HALL 1990; PRIVAL and CEBULA 1992; STEELE and JINKS-ROBERTSON 1992). They are not necessary, however, when DNA sequence analysis shows that mutants are of the same type as previously characterized revertants whose growth characteristics are known.

Fast-growing revertants: Overall, combining the data from the two experiments for timed isolation of revertants shown in Figure 3, 84% of the revertants appearing on day 3 of selection on lactose minimal agar medium, 83% of those appearing on day 4, and 28% of those appearing on day 5 were simply mutants that

grew too slowly to give rise to visible colonies in 2 days on a minimal lactose plate containing 10^9 SM195 cells. The median numbers of colonies per plate arising each day on days 2–5 were 39, 25, 20, and 14, respectively, in the two experiments. If only fast-growing revertants are considered, then the numbers of colonies per plate appearing on days 2–5 are 39, 4, 3, and 10, respectively. It is only the relatively small number of late-arising (days 3–5), fast-growing revertants that may be considered as possibly relevant to the phenomenon of adaptive mutation; such revertants were exclusively amber suppressor and intragenic mutants.

The median number of late-arising (days 3–5) amber suppressor mutants was only about 0.23 times the number appearing on day 2. Mutation rates during growth in liquid medium were estimated from day 2 colony counts by using Equation 8 of LURIA and DELBRÜCK (1943) and the amount of cell growth on plates that would be required to result in the appearance of the observed numbers of late-arising mutants was calculated as described by HAYES (1964). It was found from these calculations that even the highest increase in late-arising amber suppressor mutants (30% in *supE*) could be accounted for if cell growth and turnover had amounted to no more than 80% of the cells originally plated, assuming that the mutation rates are the same under selective conditions on the agar plates as they are in the nonselective liquid growth medium. Increased cell numbers in the range of 80% are consistent with the known leakiness of the *lac* amber mutation in strain SM195 and the fact that background growth and some cross-feeding of the cells become readily visible by day 6.

The question of whether a phenomenon of "directed" or "adaptive" mutation occurs when strain SM195 is plated on minimal lactose agar thus rests on the interpretation of the origin of the small number of late-arising, fast-growing intragenic revertants. Residual growth of strain SM195 on the minimal lactose agar selection medium would be expected to contribute to the appearance of intragenic revertants as it presumably did for the amber suppressor revertants. Late-arising intragenic revertants amounted to about 1.28 times the number appearing on day 2. Calculations using the equations of LURIA and DELBRÜCK (1943) and HAYES (1964), as described above, show that in order to attribute these late-arising mutants to cell growth, an increase in cell number to more than three times the original cell number would have to have occurred, assuming that mutation rates are the same on the plates as in the liquid medium. FOSTER and CAIRNS (1992), in an experiment similar to ours, have reported that by day 5 intragenic revertants of strain SM195 increased to approximately five times the number seen on day 2, which would require an increase to about seven times the original number of cells plated if these revertants are to be accounted for by cell growth alone. It seems unlikely that growth from 1×10^9 to 7×10^9 cells per plate would have gone unnoted by the authors. Thus, while cell growth on the agar plates probably contributed to the appearance of the fast-growing, late-arising intragenic revertants, such growth may not be sufficient to account for all such revertants.

Even though the absolute number of late-arising, fast-growing intragenic revertants is not very different from the total number of late-arising amber suppressor revertants, comparison with the early arising (day 2) revertants shows that there has been a shift in the relative rates of these mutations toward the intragenics. The fact that these relative rates are different implies that the mechanisms by which these revertants arise on the plates are different from the mechanisms by which they arise during exponential growth in liquid, or at least the mechanisms operate with different efficiencies under the two conditions. The shift from extragenic suppressor to intragenic reversion on lactose selective agar is similar to the increase in intragenic reversion that we have previously reported for chromosomal *hisG* mutants of *Salmonella typhimurium* under selective conditions on agar plates (PRIVAL and CEBULA 1992). It is possible that the mechanisms responsible for this shift are the same in the *E. coli lac* amber system as they are in the *S. typhimurium hisG* strains. For example, increased transcription of the relevant genes induced by the selective conditions may increase mutation rates and cause the shift toward intragenic revertants, as originally suggested by DAVIS (1989). However, the fact that the *E. coli lac* amber mutation is carried on an F′ plasmid suggests other models for the induction of intragenic revertants in strain SM195.

Conjugal transfer of F′ plasmids to F^- cells results in *recA*-independent increases in the frequencies of transition and transversion mutations in *lacI*, possibly as a result of low fidelity of conjugation-associated replication (KUNZ and GLICKMAN 1983). While there are no F^- cells in our system, transfer of F′ to other cells containing F′ plasmids is known to occur on agar plates (PETERS and BENSON 1995). Thus replication of F′ on the agar plates may contribute to intragenic reversion of SM195 to Lac^+.

An extensive body of recent work on the frameshift mutation *lacI33* carried on an F′ plasmid has shown that the high rate of reversion of this mutation under starvation conditions is dependent upon the RecA-RecBCD system and the functioning of the system (*tra*) that permits transfer of F′ between cells (HARRIS *et al.* 1994; FOSTER and TRIMARCHI 1995a,b; GALITSKI and ROTH 1995; RADICELLA *et al.* 1995b; PETERS *et al.* 1996; ROSENBERG *et al.* 1996), though there is evidence indicating that actual conjugal transfer is not necessary for the appearance of revertants (FOSTER and TRIMARCHI 1995a,b). There are important differences between the revertants recovered after starvation of strains containing the *lacI33* mutation and those from strain

SM195, including the very high rate of reversion observed with *lacI33* strains (CAIRNS and FOSTER 1991) and the fact that the *lacI33* revertants are predominantly single base pair deletions (FOSTER and TRIMARCHI 1994; ROSENBERG *et al.* 1994). The possible uniqueness of the *lacI33* allele has been discussed by RADICELLA *et al.* (1995a). Thus, the mechanism that accounts for starvation-associated reversion in *lacI33* strains may not be completely relevant to the reversion of the *lac* amber mutant SM195. Nevertheless, the fact that the *lacZ* amber mutation is carried on an F′ in strain SM195 suggests the possibility that the small number of intragenic revertants seen in this strain under starvation conditions may be a consequence of F′ plasmid biology.

In conclusion, it is not possible, based on the available data, to exclude the possibility that cell growth and turnover alone or in combination with replication-associated functions of F′-lac or some other F′-specific process could account for the appearance of late-arising intragenic revertants in strain SM195. While such a plasmid-related phenomenon might be embraced under the broad rubric of "adaptive mutation," it is certainly not consistent with the original, revolutionary notion of "directed mutation," in which selective conditions specifically increase the probability of occurrence of mutations that permit cell growth (CAIRNS *et al.* 1988; STAHL 1988, 1990; HALL 1989, 1990). Obviously, determination of whether or not "directed" or "adaptive" mutation occurs in the absence of mediation by F′-specific processes would require focusing attention on chromosomal, rather than episomal, mutations that occur under selective conditions (RYAN 1955; HALL 1989, 1990, 1992; GIZATULLIN and LYOZIN 1992; JAYARAMAN 1992; PRIVAL and CEBULA 1992; STEELE and JINKS-ROBERTSON 1992).

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