from mammalian cells in other ways. For example, targeting in yeast shows a more linear dependence on length of homology than in mammalian cells[15,20], and homology at the ends of the target vector is not necessary in mammalian cells[3] but seems to be crucial in yeast[21]. Collectively, these differences urge caution in extrapolating from *S. cerevisiae* and reinforce the need for systematic studies in mammalian cells. □

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New data on excisions of Mu from *E. coli* MCS2 cast doubt on directed mutation hypothesis

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ACCORDING to the directed mutation hypothesis, certain mutations in bacteria occur more frequently in environments in which the resulting phenotype is selectively favoured than in non-selective environments[1–4]. This hypothesis therefore challenges the fundamental tenet that mutations occur spontaneously, irrespective of effects on the organism's fitness[5–9]. One purported case of directed mutation is the excision of a Mu sequence from *Escherichia coli* strain MCS2 in minimal lactose–arabinose medium[1,2]. Here, we show that this case can be more simply explained by an accelerated rate of excision mutation in response to non-specific physiological stresses of starvation and by slight growth of MCS2 on minimal lactose–arabinose medium.

We used the same *E. coli* MCS2 strain as in refs 1 and 2. MCS2 is a genetically manipulated strain in which the *araC* regulatory gene is placed upstream of the *lacZY* structural genes, but is separated from them by Mu bacteriophage DNA (Mu*cts*62 prophage)[2]. If an excision of Mu produces a suitable reading frame, then the mutant cell can grow on lactose, provided that arabinose is also present as an inducer[1,2]. (J. A. Shapiro (personal communication) has shown that a Mu-encoded function is essential for most of these excisions.) Following the notation of Cairns *et al.*[1], we refer to excision mutants as Lac(Ara)$^+$, and to their MCS2 progenitor as Lac(Ara)$^-$.

Shapiro[2] observed that when Lac(Ara)$^-$ cells were spread on minimal agar plates containing lactose plus arabinose and incubated at 32 °C, no Lac(Ara)$^+$ colonies appeared on the plates for five days; after five days, Lac(Ara)$^+$ colonies began to accumulate, eventually yielding plates with more than 100 colonies. A similar pattern was observed at 37 °C, although the first colonies appeared after only 2–3 days. At both temperatures, the observed frequency of mutant colonies on old plates was orders of magnitude higher than expected from the baseline mutation rate observed in growing cultures[1,2]. Because single Lac(Ara)$^+$ cells usually produce readily scored colonies on minimal lactose–arabinose (MLA) plates within 1–2 days at either temperature[2], this delay is not a trivial consequence of arabinose induction, catabolite repression, or slow growth[1,2]. We repeated Shapiro's experiments with similar results; our data for 37 °C are shown in Fig. 1.

Cairns *et al.*[1,4] interpreted these data as evidence for directed mutation in response to the presence of lactose and arabinose, but other factors could account for the accumulation of Lac(Ara)$^+$ mutants. In particular, the rate of Mu excisions may increase as cells starve, irrespective of the sugars as selective agents[6]. To test this idea, we spread ~2.5×10^8 Lac(Ara)$^-$ cells onto minimal plates with no added sugars (M plates) and incubated them at 37 °C for 0–6 days. We estimated the number of Lac(Ara)$^+$ mutants on these plates by counting the colonies that appeared within two days of spraying them with lactose and arabinose (Fig. 2). No mutant colonies appeared within two days on plates sprayed with sugars immediately after cells were spread. However, the number of Lac(Ara)$^+$ colonies appearing within two days of spraying increased steadily as a function of the time that cells were on the plates before addition of lactose and arabinose. This indicates that an increasing number of excision mutations occur while cells are starving, even in the absence of lactose and arabinose, and it contradicts the claim that these excisions are "... genetic events that apparently occur only under conditions of selection ..."[4].

But can this control experiment account for all the excision mutants observed when sugars are present? Comparison of Figs 1 and 2 suggests that it does not: there are many more Lac(Ara)$^+$ colonies on MLA plates than on M plates incubated for the same time (including the two days after addition of lactose and arabinose). After 6 days, for example, there were on average ~200 Lac(Ara)$^+$ colonies on MLA plates, compared with ~10 on M plates. However,we have assumed in this comparison of colony numbers that the densities of Lac(Ara)$^-$ cells at risk for mutation are the same on M and MLA plates.

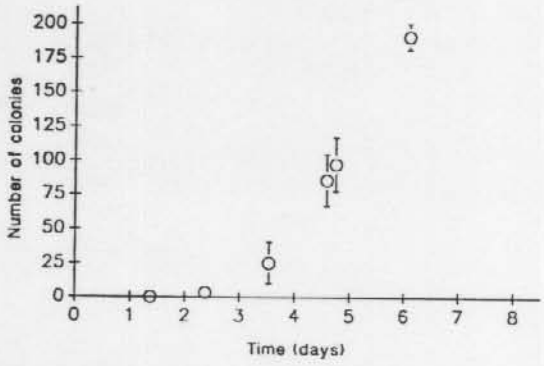

FIG. 1 The cumulative number of Lac(Ara)$^+$ colonies appearing on minimal lactose–arabinose (MLA) plates incubated at 37 °C, as a function of time. About 2.5×10^8 Lac(Ara)$^-$ cells, grown in Davis minimal liquid[10] (with 1 mg ml^{-1} glucose) for 24 h, were spread onto each of three plates. MLA plates consist of Davis minimal agar[10] with 2 mg ml^{-1} each of arabinose and lactose instead of glucose. After 6 d, the number of Lac(Ara)$^+$ colonies could no longer be scored unambiguously because large numbers of tiny colonies appeared in the vicinity of older Lac(Ara)$^+$ colonies. Error bars represent 95% confidence intervals (t-distribution, $n=3$). The first point has no error bar because the standard deviation was zero; the error bar for the second point is not visible because the standard deviation was very small.

TABLE 1 Stable persistence of Lac(Ara)$^+$ colony-forming cells during re-growth in fresh medium after a period of starvation

Treatment*	Log_{10} total viable cell density (ml^{-1})†	Log_{10} frequency of Lac(Ara)$^+$ cells‡	Proportion Lac(Ara)$^+$ visible after one day§
A. Growth in fresh medium for 1d	9.41 (0.02)	−9.70‖	—
B. As A, then 9d starvation	8.20 (0.02)	−5.66 (0.03)	0.12 (0.02)
C. As B, then re-growth for 1d in fresh medium¶	9.45 (0.02)	−5.63 (0.10)	0.89 (0.04)

* Populations were grown, starved, and re-grown at 32 °C in Davis minimal liquid[10] (with 1 mg ml^{-1} glucose). Means (and s.e.m.) were calculated from 10 independent replicates.

† Total viable cell density was estimated from colony counts on non-selective plates.

‡ Frequency of Lac(Ara)$^+$ cells was estimated from the ratio of colony counts on MLA plates after 2d incubation at 37 °C to colony counts on non-selective plates.

§ Fraction of colonies appearing on MLA plates after 48 h (2d) incubation that were already visible after only 26.5 h (1d) incubation. Most of the Lac(Ara)$^+$ colony-forming cells in the starved cultures took 2d to form visible colonies, whereas most of those that had been re-grown in fresh medium formed visible colonies after only 1d.

‖ No Lac(Ara)$^+$ colonies were obtained from any of 10 independent replicates. The upper limit for the frequency of Lac(Ara)$^+$ cells was computed as $(ndv)^{-1}$, where n is the number of replicate populations (10), d is the mean total viable cell density (2.6×10^9 ml^{-1}), and v is the sample volume tested on MLA plates (0.2 ml).

¶ One day of re-growth corresponds to ~10 generations of binary fission, based on a 50-fold dilution factor and a 17.6-fold difference in mean total viable cell density between treatments B and C: $\log_2 (50 \times 17.6) = 9.8$.

To investigate whether the presence of lactose and arabinose affected the population dynamics of Lac(Ara)$^-$ cells, we sampled 'plugs' from a series of MLA and M plates incubated for different times. MLA plates had significantly more Lac(Ara)$^-$ cells than the M plates, even after one day (Fig. 3), and after several days the densities differed by at least an order of magnitude. Growth by Lac(Ara)$^-$ cells on plates supplemented with lactose and arabinose apparently offsets cell death, which produces a sharp decline on plates without sugars. We suggest that the higher density of Lac(Ara)$^-$ cells on MLA plates was initially due to slight growth on lactose and arabinose, or on trace contaminants such as glucose. The increased difference after several days reflects cross-feeding by Lac(Ara)$^-$ cells on metabolites released from growing Lac(Ara)$^+$ colonies, which appear in increasing numbers on MLA plates. Indeed, we often observed 'haloes' of background growth around Lac(Ara)$^-$ colonies on old MLA plates; and in minimal lactose-plus-arabinose liquid medium, Lac(Ara)$^-$ cells grew significantly faster when Lac(Ara)$^+$ cells were also present (data not shown).

These pronounced differences in the densities of Lac(Ara)$^-$ cells are sufficient to explain the differences in the total numbers of Lac(Ara)$^+$ colonies that accumulate on MLA and M plates (Figs 1 and 2). To verify this, we crudely estimated the rate of mutation to Lac(Ara)$^+$ per viable cell per day, as described in the legend to Fig. 4. Note the striking increase with time in the inferred mutation rate on both M and MLA plates; moreover, these rates are comparable. There is some indication that excision mutations occur slightly earlier on MLA plates than on M plates, but there were fewer Lac(Ara)$^+$ colonies on the M plates sprayed immediately after cell spreading than on MLA plates. Variations in sugar application or local concentrations might be responsible for these subtle effects. Also, our analysis makes two assumptions that could underestimate the mutation rate on M plates relative to MLA plates. First, we assume that Lac(Ara)$^+$ mutants do not die on M plates before spraying. We found an average mortality rate of ~9% per day for Lac(Ara)$^+$ cells on M plates between days 1 and 5 (data not shown), versus ~78% per day for Lac(Ara)$^-$ cells under comparable conditions (Fig. 3), but as the estimated mortality rate for Lac(Ara)$^+$ cells was not significantly different from zero, we have conservatively assumed no death of mutants on M plates. Second, we assume that all mutant cells form visible colonies within two days of lactose and arabinose application. Any Lac(Ara)$^+$ mutants present at the time of spraying and taking more than two days to form visible colonies would not be included in our estimation of the mutation rate.

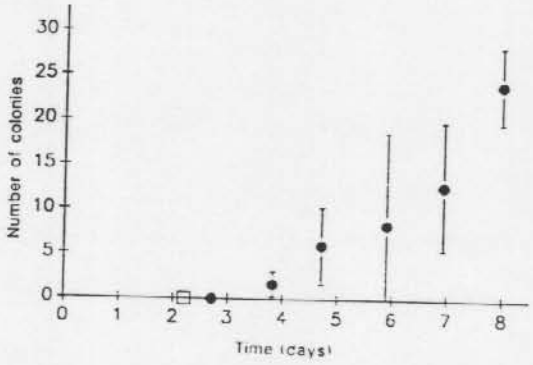

FIG. 2 The cumulative number of Lac(Ara)$^-$ mutants occurring on minimal (M) plates incubated at 37 °C, as a function of time; lactose and arabinose were added to plates 2 d before Lac(Ara)$^+$ colonies were scored. Day 6, for example, corresponds to a plate that sat for 4 d before addition of lactose and arabinose, and was then scored for Lac(Ara)$^+$ colonies after two more days. About 2.5×10^8 Lac(Ara)$^-$ cells, grown as described for Fig. 1, were spread onto each of 21 plates. Each day for 7 d (including day 0), an atomizer was used to add ~0.1 ml of a solution containing 5% lactose and 5% arabinose to 3 of these plates. The open square indicates the set of plates sprayed with lactose and arabinose immediately after cells were spread (day 0), which were counted two days later. M plates consist of Davis minimal agar without any added sugars. Error bars represent 95% confidence intervals (t-distribution, $n = 3$). The first two points have no error bars because the standard deviations were zero.

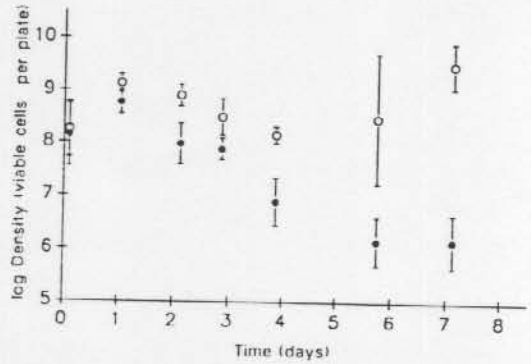

FIG. 3 Density of viable Lac(Ara)$^-$ cells on MLA plates (open circles) and M plates (filled circles) incubated at 37 °C, as a function of time. About 2.5×10^8 Lac(Ara)$^-$ cells, grown as described in the legend to Fig. 1, were spread onto each of three MLA and three M plates. Densities were estimated by vigorously vortexing 0.81 cm^2 agar plugs in saline, spreading appropriate dilutions onto lactose-arabinose tetrazolium indicator plates[10], and counting the resulting Lac(Ara)$^-$ colonies (which are red). After several days, many Lac(Ara)$^+$ colonies (which are white) also appeared in the samples from MLA plates, but these were not counted. Error bars represent 95% confidence intervals based on the log-transformed data (t-distribution, $n = 3$).

Although approximations, assumptions and sources of statistical error enter into the calculation of these mutation rates, it is clear that the rate of excision mutation per viable cell per day increases by orders of magnitude as cells sit starving for several days, irrespective of whether lactose and arabinose are present. We conclude that there is no compelling evidence for an increase in the mutation rate when the Lac(Ara)$^+$ phenotype is selectively favoured, beyond that caused by the non-specific stresses of starvation.

Of course, the possibility can never be excluded that some arbitrarily small fraction of mutants is the result of a directed process. Moreover, it is often possible to produce another hypothesis which is consistent with observations that are themselves inconsistent with the earlier hypothesis: for example, the stress of starvation might have predisposed Lac(Ara)$^-$ cells to directed gene mutation when exposed to lactose and arabinose. To test this *ad hoc* variant of the directed mutation hypothesis, we monitored the frequency of Lac(Ara)$^+$ cells in grown, starved, and re-grown liquid cultures at 32 °C (Table 1). In freshly grown cultures, the frequency of Lac(Ara)$^+$ cells is $\sim 10^{-10}$ or less[1,2]. When these populations of MCS2 are starved for 9 days in glucose-limited minimal medium, the frequency of Lac(Ara)$^+$ colony-forming cells increases to $\sim 10^{-6}$. When we transferred cells from these starved cultures into fresh minimal glucose liquid, where they grew for 10 generations, the Lac(Ara)$^+$ colony-forming cells (most of which formed visible colonies after only one day) persisted at this increased frequency. Had the Lac(Ara)$^+$ colony-forming cells been genotypically Lac(Ara)$^-$ cells that were physiologically predisposed to directed mutation, then the frequency of Lac(Ara)$^+$ colony-forming cells should have returned to the low value characteristic of freshly grown cells. We conclude that the genetic events causing the frequency of Lac(Ara)$^+$ colony-forming cells to increase by several orders of magnitude occurred before exposure to lactose and arabinose, contrary to the *ad hoc* hypothesis we have discussed.

Although the results presented here do not support the directed mutation hypothesis, they agree with the more widely held view that physiological stresses such as starvation may increase the activity of transposable genetic elements[6]. Under what circumstances this increased activity improves the fitness of the cell, the transposable element, or both, remains an interesting question. □

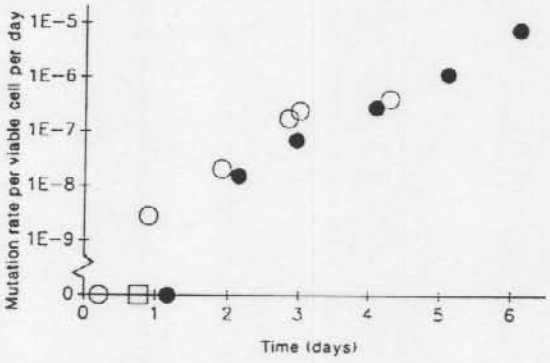

FIG. 4 Inferred rates of mutation per viable cell per day from Lac(Ara)$^-$ to Lac(Ara)$^+$ on MLA plates (open circles) and M plates (filled circles and open square) incubated at 37 °C, as a function of time. These rates were calculated as $u(t) = c(t')/d(t)$, where $u(t)$ is the time-dependent mutation rate, $d(t)$ is the number of Lac(Ara)$^-$ cells at risk at time t, and $c(t')$ is the rate of appearance of new colonies at some later time t'. The time t' is given by $t' = t + h(t)$, where $h(t)$ is the elapsed time between occurrence of a mutation and appearance of the resulting colony. Freshly grown Lac(Ara)$^+$ cells typically produce visible colonies within 1 d after plating on MLA, but starved Lac(Ara)$^+$ cells usually require 2 d to form colonies (Table 1, footnote). We therefore set $h(t) = 1 + t/(1 + t)$, such that $h(0) = 1$, $h(1) = 1.5$, $h(2) = 1.67$, and so on. The precise form of this function has little effect on the overall appearance of this figure, provided that $1 < h(t) < 2$. The rate of appearance of Lac(Ara)$^+$ colonies, $c(t')$, was estimated from the difference between adjacent means in Figs 1 and 2, divided by the time elapsed between them; the later time point for each pair was designated t'. The number of Lac(Ara)$^-$ cells at risk for mutation, $d(t)$, was obtained by interpolation between adjacent logarithmic means in Fig. 3, to provide estimated cell densities $h(t)$ days before each estimate of $c(t')$. Solving for t requires iteration of $t' = t + h(t)$. Inferred mutation rates are not available for MLA plates after 4 d because it becomes too difficult to score the number of Lac(Ara)$^+$ colonies after 6 d (Fig. 1).

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Dependence of the torsional rigidity of DNA on base composition

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THE *Escherichia coli* phage 434 repressor binds as a dimer to the operator of the DNA helix. Although the centre of the operator is not in contact with protein, the repressor binding affinity can be reduced at least 50-fold by changing the sequence there[1]: operators with A·T base pairs near their centre bind the repressor more strongly than do operators with G·C base pairs at the same positions. To explain these observations, it has been proposed that the base composition at the centre of the operator affects the affinity of the operator for repressor by altering the ease with which operator DNA can undergo the torsional deformation necessary for complex formation [1,2]. In this model, the variation in binding affinity would require the torsion constant to have specific values and to change in a sequence-dependent manner[1]. We have now measured torsion constants for DNAs with widely different base compositions. Our results indicate that the torsion constants depend only slightly on the overall composition, and firmly delimit the range of values for each. Even the upper-limit values are much too small to account for the observed changes in affinity of the 434 repressor. These results rule out simple models that rely on substantial generic differences in torsion constant between A·T-rich sequences and G·C-rich sequences, although they do not rule out the possibility of particular sequences having abnormal torsion constants.

Torsion constants are obtained from the time-resolved fluorescence polarization anisotropy (FPA) of intercalated ethidium dye. By monitoring the rotational motion of the transition dipole of the intercalated dye, it is possible to infer the torsion constant of the host DNA. Details of the theory and experiment are described elsewhere[3-10]. The estimated torsion constant depends on the value assumed for the dynamic bending rigidity, or dynamic persistence length P_d. A lower bound torsion constant α is obtained by assuming $P_d = \infty$, which assigns all depolarization to twisting motions. An upper bound torsion constant, $\alpha' = (1.9)\alpha$, is obtained by assuming $P_d = 500$ Å (ref. 11). The