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The occurrence of heritable *Mu* excisions in starving cells of *Escherichia coli*

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A strain of *Escherichia coli* constructed by Shapiro has a segment of *Mu* bacteriophage DNA inserted between the *araC* and *lacZ* genes. Excision events that produce an in-frame fusion of *lacZ* to *araB* result in a cell (here designated Ara−Lac$^+$) that can grow on lactose if arabinose is present as an inducer. Whether or not these excision events occur in the absence of selection for the Ara−Lac$^+$ phenotype has figured prominently in the debate of the phenomenon known as 'directed' or 'adaptive' mutation. In an attempt to settle the issue, we have used classic fluctuation tests to show that cells capable of producing a clone of descendants that are phenotypically Ara−Lac$^+$ do, indeed, arise in stationary phase cultures kept starving in depleted minimal medium. We found that Ara−Lac$^+$ progenitors arise rapidly under these conditions, in contrast to the delayed appearance of Ara−Lac$^+$ mutants when cells are incubated on lactose−arabinose minimal plates. Similar results are reported in the accompanying paper by Maenhaut-Michel and Shapiro, who used indirect selection to isolate Ara−Lac$^+$ cells in the absence of selection. However, their sequencing data have introduced a new unexpected complication to the interpretation of all such experiments, and it is no longer clear exactly when the fusions arise.

Key words: araC/E.coli/heritable *Mu* excisions/*lacZ*/starving cells

Introduction

Shapiro (1984) constructed a strain of *Escherichia coli* in which a segment of *Mu* bacteriophage DNA containing transcription terminating signals was placed between *araC* and *lacZ*. Spontaneous excision of the *Mu*, if it results in an in-frame fusion of *lacZ* to *araB*, allows the cell to grow with lactose as an energy source provided arabinose is present to induce transcription. Such cells, previously called Lac(Ara)$^+$ (Cairns *et al.*, 1988), are here designated Ara−Lac$^+$. The excision event that produces Ara−Lac$^+$ cells appears to be under complex control because it seldom, if ever, happens during normal growth. But when Ara−Lac$^-$ cells are plated on minimal plates containing arabinose and lactose (AL minimal plates), Ara−Lac$^+$ mutants appear after a delay of several days and then continue to appear at a rapidly accelerating rate (Shapiro, 1984; Cairns *et al.*, 1988). However, when Ara−Lac$^+$ cells were incubated without an added carbon source (Shapiro, 1984) or in depleted semi-rich medium (Cairns *et al.*, 1988), and samples periodically tested on AL minimal plates, no accumulation of Ara−Lac$^+$ mutants was detected. These observations suggested that the process of excision occurs in starving cells, but only when arabinose and lactose are present, i.e. that the process is adaptive (Shapiro, 1984; Cairns *et al.*, 1988).

This conclusion has been challenged by Mittler and Lenski (1990). They showed that cultures grown to saturation in liquid minimal medium and then kept incubating with aeration for 9 days contained cells that formed colonies on AL minimal plates more quickly than did non-starved cells. Furthermore, these presumptive Ara−Lac$^+$ mutants increased in number when starved cultures were allowed a brief period of growth in fresh medium before being plated on AL minimal plates. Partly as the result of these experiments, Lenski and Mittler (1993) have argued that every example of 'directed' or 'adaptive' mutation will turn out to be illusory.

Their work resuscitates an old question. What proves that the mutants detected by some selective procedure were present in the population before it had been subjected to selection? In the present case, what would constitute proof that *Mu* can excise in such a way as to produce Ara−Lac$^+$ mutants in the absence of arabinose and lactose? Two solutions to this problem were found in the 1940s and '50s. Small samples of stationary phase cultures could be grown in a non-selective medium and these then tested on AL minimal plates. Provided the initial samples were so small that most would not be expected to contain an Ara−Lac$^+$ mutant, most of the resulting cultures should not contain any mutants. But the few cultures that started off with an Ara−Lac$^+$ mutant would produce many mutants (Cavalli-Sforza and Lederberg, 1956). If, however, Ara−Lac$^+$ mutants arise as a result of events occurring after the cells are on AL minimal plates, then the number of mutants among the cultures should have a Poisson distribution (Luria and Delbrück, 1943). There is an even more direct test. If stationary cultures contain Ara−Lac$^+$ mutants prior to selection, these rare mutants will give rise to mutant clones in non-selective medium, and these can be identified by replica plating onto AL minimal plates and then subcultured under non-selective conditions (Lederberg and Lederberg, 1952).

For some reason, Mittler and Lenski (1990) did not choose to apply either of these classic tests, but seemed to think that the question could be answered by estimating mutation rates under different conditions. Although we would not wish to take issue with their conclusions, we felt that the matter should not be put to rest until the experiments had been performed properly. In the experiments reported here, we have applied the first of

the classic tests, fluctuation analysis, to demonstrate that cells that are able to produce a clone of Ara−Lac$^+$ cells can indeed arise in the absence of selection for the Ara−Lac$^+$ phenotype. In an accompanying paper, Maenhaut-Michel and Shapiro (1994) have taken this experiment further and used indirect selection to isolate and characterize Ara−Lac$^+$ mutants. They have shown that the mutants obtained by direct selection have a different sequence from those obtained by indirect selection. This result introduces an unexpected complication in the interpretation of the role played by selection in the production of Ara−Lac$^+$ fusions, which is considered in the Discussion.

Results

The accumulation of Ara−Lac$^+$ progenitors during incubation in liquid medium

Mittler and Lenski (1990) found that when a saturated culture of MCS2 was incubated for 9 days in liquid minimal medium, cells that could quickly form colonies on AL minimal plates appeared at a frequency of ~2×10^{-6} (or ~350 cells/ml). During this time the total viable count declined by an order of magnitude. The question left unanswered by this experiment is whether starving cultures contain actual Ara−Lac$^+$ mutants or cells in some intermediate state which raises their excision rate when they are subjected to selection on AL minimal plates. To avoid prejudging the issue, we shall refer to these quick colony formers as Ara−Lac$^+$ progenitors.

To determine the real genetic status of these cells, we began by repeating Mittler and Lenski's (1990) experiment except that we used glycerol instead of glucose as a carbon source to avoid any complication from catabolite repression. Saturated cultures (containing ~10^9 cells/ml) of MCS1260 were kept shaking at 32°C and every day aliquots were removed, the viable cell number determined, and the cells plated on AL minimal plates. The results are shown in Figure 1. Cells that were removed from the liquid cultures shortly after saturation (days 0 and 1), and thus spent most of the experiment on AL minimal plates, did not form Ara−Lac$^+$ colonies until 8 or 9 days after plating. In contrast, in the liquid cultures Ara−Lac$^+$ progenitors made their first appearance 2 days after saturation, and reached a maximum of ~400 cells/ml by day 5. However, this rapid accumulation of Ara−Lac$^+$ progenitors ceased when the cultures were plated. For example, between days 2 and 3 the number of Ara−Lac$^+$ progenitors in the liquid cultures (detected as colonies on AL minimal plates on days 5 and 6) increased 8-fold. However, during the same period no new Ara−Lac$^+$ colonies appeared on the AL minimal plates that received the samples from day 2 (Figure 1). We also found that the viable counts in the starving cultures declined to ~20% of the initial level by day 6, which may account for the fact that the accumulation of Ara−Lac$^+$ progenitors appeared to cease after day 5.

Ara−Lac$^+$ cells do not proliferate during starvation in liquid glycerol minimal medium

Because Ara−Lac$^+$ cells appear to have a survival advantage when starved in liquid minimal medium (Mittler and Lenski, 1990), it seemed possible that they might be able

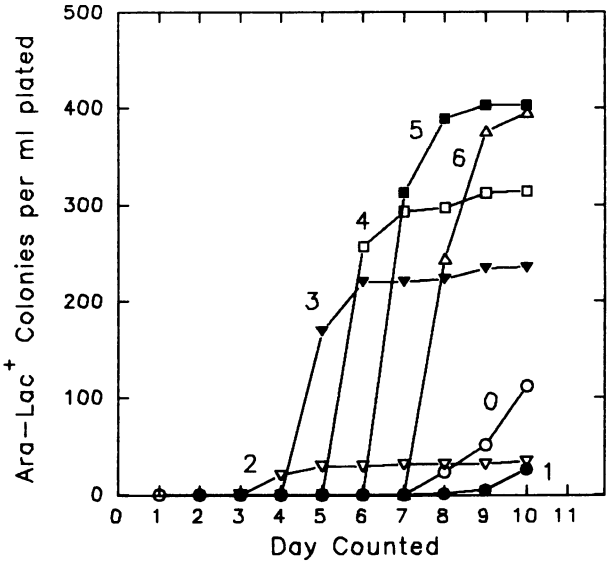

Fig. 1. The accumulation of Ara−Lac$^+$ progenitors during starvation in glycerol minimal medium. Three cultures of MCS1260 in glycerol minimal medium were kept shaking at 32°C for 6 days after reaching saturation. Every day samples were plated on AL minimal plates. The colonies that appeared were counted each day until day 10. The figure gives the means of the three cultures; the day that the sample was plated is indicated next to each curve.

to proliferate under these conditions. If a few Ara−Lac$^+$ mutants arose and then were able to divide, this would explain why the accumulation of Ara−Lac$^+$ progenitors during starvation accelerates rapidly, reaches a maximum, but ceases the moment the cells are plated on AL minimal plates. To test this hypothesis, we dispensed 100 µl aliquots of newly saturated cultures of MCS1260 into microtiter wells and incubated them at 32°C. At 7 and 8 days later these were plated on AL minimal plates. If the Ara−Lac$^+$ progenitors that appear in liquid cultures were descendants of only a few Ara−Lac$^+$ mutants, then most of the microtiter wells would contain no Ara−Lac$^+$ progenitors and some would contain many. In fact, the distribution of cells capable of forming Ara−Lac$^+$ colonies was close to Poisson (Table I). Thus, at present we have no explanation for the fact that the accumulation of Ara−Lac$^+$ progenitors ceases the moment the cells are plated on AL minimal plates, except the possibility that contaminating nutrients in the agar allow the cells to avoid total starvation for a few days.

The numbers of Ara−Lac$^+$ progenitors found after regrowth

In an attempt to establish that the cells which quickly produced colonies on AL minimal plates were actually Ara−Lac$^+$ before there had been any selection, Mittler and Lenski (1990) showed that the number (but not the frequency) of these presumptive Ara−Lac$^+$ mutants increased when starved cultures were allowed a brief period of outgrowth in the absence of selection. But this experiment did not prove that the cells that gave rise to colonies on AL minimal plates after outgrowth were the progeny of pre-existing Ara−Lac$^+$ mutants. For example, because the growth medium greatly affects the subsequent rate of fusion formation (Shapiro, 1984), a brief period of growth might induce starved cells to acquire a

Table I. The number of Ara−Lac$^+$ progenitors found in small cultures starved in glycerol minimal medium

Experiment	Days incubated[a]	Increase in Ara−Lac$^+$ cells[b]	Number of cultures			Distribution of Ara−Lac$^+$ cells[c]
			Total	With no Ara−Lac$^+$	With Ara−Lac$^+$	
1	7	>370-fold	30	0	30	11 (24–33) 10 (34–43) 9 (44–53)
2	8	>500-fold	30	0	30	7 (33–42) 11 (43–52) 10 (53–62) 2 (63–82)

[a]Days after the cultures reached saturation and were dispensed into microwells.
[b]Determined from the average number of Ara−Lac$^+$ cells per microwell. When dispensed, the cultures contained less than one Ara−Lac$^+$ progenitor per ml, as detected on AL minimal plates.
[c]The numbers of Ara−Lac$^+$ cells per microwell have been grouped into ranges, and the first number is the number of microwells falling into that range. The mean and variance in the first experiment were 37 and 69, respectively; in the second experiment they were 50 and 98.

Table II. The number of Ara−Lac$^+$ progenitors found in small cultures after starvation in glycerol minimal medium and a period of growth in fresh medium

Experiment	Day diluted[a]	Dilution[b]	Increase in Ara−Lac$^+$ cells[c]	No. of cultures plated			Distribution of Ara−Lac$^+$ cells[d]	Ara−Lac$^+$ progenitors inoculated per well	
				Total	With no Ara−Lac$^+$ cells	With Ara−Lac$^+$ cells		Direct assay[e]	Indirect assay[f]
1	7	1:30	14×	25	12	13	1, 1, 2, 2, 3 4, 9, 17, 17 17, 24, 33, 119	0.90	0.73
2	9	1:100	50×	79	61	18	1, 1, 1, 1, 1, 1 1, 2, 4, 9, 11 25, 30, 62, 93 107, 253, 363	0.42	0.26
3	6	1:50	50×	59	44	15	1, 1, 1, 1, 1, 1 1, 1, 2, 4, 4, 6 9, 12, 750	0.34	0.29

[a]Days after the cultures reached saturation.
[b]Dilution of the original cultures into fresh glycerol minimal medium. In every case the overall increase in cell number determined by plating was approximately equal to the dilution factor.
[c]Determined by dividing the average final number of Ara−Lac$^+$ cells per microwell by the average number of Ara−Lac$^+$ progenitors inoculated, which was calculated from the indirect assay.
[d]Among the cultures that had Ara−Lac$^+$ cells.
[e]Determined by plating the cultures on AL minimal plates.
[f]Calculated from the proportion of wells that had no Ara−Lac$^+$ cells after outgrowth.

predisposition to become Ara−Lac$^+$, and this could account for the increase in the numbers of Ara−Lac$^+$ mutants.

To establish whether Ara−Lac$^+$ cells actually do appear in the absence of selection for the Ara−Lac$^+$ phenotype, we diluted starved cultures into fresh glycerol minimal medium such that 100 μl contained less than one Ara−Lac$^+$ progenitor, and distributed 100 μl aliquots into microtiter wells. As discussed above, if Ara−Lac$^+$ mutants pre-exist before the cells are exposed to arabinose and lactose, then after outgrowth cultures should contain either none or many Ara−Lac$^+$ cells. On the other hand, if the Ara−Lac$^+$ mutants only appear after plating on AL minimal plates, then their distribution should be Poisson. The results of three such experiments (Table II) show that there is great fluctuation in the number of Ara−Lac$^+$ progenitors per culture; most cultures contained no progenitors and some contained many. In fact, a few cultures contained more Ara−Lac$^+$ progenitors than would have been expected from the amount of growth that had occurred. Overall, the total increase of Ara−Lac$^+$ progenitors (i.e. the total number found in all the microwells divided by the estimated number initially present in these microwells) roughly equalled the total growth of the cultures. So it seemed likely that the excessive variation in the final number of Ara−Lac$^+$ progenitors was the result of a great variation in the time at which individual cells can start to divide when returned to an energy-rich medium after prolonged starvation. A few cells start straight away and produce large clones, but some cells have not started to grow by the time the medium is exhausted and so are recorded as singles when the culture is finally plated on AL minimum plates.

To check that this explanation was correct, we repeated the fluctuation test with a culture to which a few Tet^r cells had been added. After 6 days of starvation, the Tet^r-enriched culture was diluted into fresh glycerol minimal medium as before so that each microwell would contain less than one Tet^r cell. After outgrowth, the microwell cultures were plated on glycerol-tet minimal plates and titered on glycerol minimal plates. As shown in Table III, the behavior of the Tet^r cells was quite similar to the

Table III. The behavior of a minority population of Tet^r cells under the same conditions that give rise to Ara−Lac$^+$ mutants

Day diluted	Dilution[a]	Increase in Tet^r cells[b]	Number of cultures			Distribution of Tet^r cells[c]	Tet^r cells inoculated per well	
			Total	With no Tet^r cells	With Tet^r		Direct assay[d]	Indirect assay[e]
6	1:100	670×	68	53	15	4, 5, 22, 116 139, 253, 310 342, 369, 647 684, 843, 1596 2476, 3624	0.16	0.25

[a]Dilution of the original culture into fresh glycerol minimal medium. The overall increase in cell number determined by plating was equal to the dilution factor.
[b]Determined by dividing the average number of Tet^r cells per microwell by the average number inoculated, which was calculated from the indirect assay.
[c]Among the cultures that had Tet^r cells.
[d]Determined by plating the culture on tetracycline plates.
[e]Calculated from the proportion of wells that had no Tet^r cells after outgrowth.

Ara−Lac$^+$ progenitors—many cultures had either less or more than the number that would be expected if all the cells could start growing immediately. From this experiment we can conclude that the Ara−Lac$^+$ progenitors are behaving like added Tet^r cells and are therefore capable of producing clones of Ara−Lac$^+$ cells in the absence of selection for the Ara−Lac$^+$ phenotype.

Discussion

Our experiments show that cultures of MCS1260, held in stationary phase in liquid minimal medium depleted of a source of energy, give rise to cells that appear to be Ara−Lac$^+$ before there has been any selection for the Ara−Lac$^+$ phenotype, i.e. cells which when provided with a non-selective energy source form a clone of descendants capable of making colonies on AL minimal plates. These rare cells were identified because they produced clones when old stationary cultures were diluted into fresh medium, distributed among a large number of small cultures, allowed to regrow and then plated on AL minimal plates. The cells in these old cultures are apparently slow to resume growth when returned to favorable conditions, and so only a minority of the population are responsible for most of the population increase in fresh medium. A few of the Ara−Lac$^+$ progenitors produced many descendants, but roughly half had not started growing before the resources of the fresh medium were exhausted by the cells first off the mark. There is, however, no evidence that the Ara−Lac$^+$ progenitors behaved differently from the rest of the population, or from a minority class marked with antibiotic resistance. Thus, we concur with Mittler and Lenski (1990) that cells capable of giving rise to Ara−Lac$^+$ clones can arise during conditions of starvation in the absence of selection for the Ara−Lac$^+$ phenotype. Still unexplained is the fact that Ara−Lac$^+$ progenitors did not accumulate when cells were held in a salt solution without a carbon source (Shapiro, 1984) or starved in depleted semi-enriched medium (Cairns *et al.*, 1988; Cairns, 1990). All the experiments reported here were performed by P.L.F. In cultures grown by J.C., no Ara−Lac$^+$ progenitors appeared in the absence of arabinose and lactose (even if the cultures were plated on AL minimal plates by P.L.F.), and this is apparently due to some difference between the media prepared in the two laboratories (Cairns, 1990). The discrepancy may ultimately be explained when the physiological regulation of the fusion process is understood completely (Shapiro and Higgins, 1989; Shapiro, 1993; Maenhaut-Michel and Shapiro, 1994). It is also worth remembering that Ara−Lac$^+$ cells may have a survival advantage when starving in minimal medium (Mittler and Lenski, 1990) but not in depleted semi-enriched medium (Cairns, 1990), and thus the selective pressures are not the same in the two cases.

Results like ours are reported in an accompanying paper by Maenhaut-Michel and Shapiro (1994). However, these authors have gone on to show that the Ara−Lac$^+$ cells that arise when starved cultures are given glucose contain a wide spectrum of different fusions ('non-standard fusions') that are quite distinct from the one class of fusion ('standard fusions') found when cultures are starved on AL minimal plates or when starved cultures are plated on AL minimal plates. These unexpected results completely alter the possible interpretations we can place on our experiments. If the type of fusion found depends on whether a selective or a non-selective source of energy was used to capture the Ara−Lac$^+$ cells, the final step that produces each fusion may not be occurring until after the starved cell receives that source of energy. Both we (Table II) and they (Table I in Maenhaut-Michel and Shapiro, 1994) found that selective and non-selective energy sources capture approximately equal numbers of Ara−Lac$^+$ cells. Any interpretation must encompass that awkward fact. There seem to be three obvious possibilities.

(i) Selective and non-selective sources of energy capture roughly equal numbers of Ara−Lac$^+$ cells because in starved cultures there is just one class of Ara−Lac$^+$ precursor, and it can make or stabilize one or the other class of fusion when given a source of energy; for some reason the exact source of energy determines the type of fusion that is formed.

(ii) Cells with standard fusions and cells with non-standard fusions both pre-exist in the starving culture and are present in roughly equal numbers. For some reason the addition of arabinose and lactose kills, or prevents the growth of, starved cells with non-standard fusions, and the addition of glucose or glycerol kills, or prevents the growth of, starved cells with standard fusions.

(iii) Ara−Lac$^+$ progenitor cells contain fusions, but these fusions are unstable. Non-standard fusions may be more numerous than standard fusions, but this difference is, by coincidence, exactly counterbalanced by the length of the period during which a fusion can be captured. For example, when a starved pre-fusion cell is given glucose or glycerol it may have only a few minutes in which to stabilize whatever fusion it contains, and so Ara−Lac$^+$ cells isolated in this way may have the most common class of fusion. In contrast, a starved pre-fusion cell on an AL minimal plate may have many hours in which to stabilize its fusion, and for some reason the rarer type of fusion is captured. Note that this hypothesis applies whether an individual cell contains several different fusions or only one.

Of course, none of these explanations addresses the central question, which is how the nature of the source of energy determines the end result. One possible contributing factor is that arabinose induces transcription of the region and transcription could influence the type of fusion made. Another possibility is that the non-standard fusions may not be immediately expressed, and so do not allow the starved cell to grow on lactose. Clearly, more experiments are needed to determine the exact molecular mechanisms by which the different classes of fusion occur.

How do these results affect the phenomenon that has come to be called 'directed' or 'adaptive' mutation? One of the first theories postulated to explain the phenomenon is that adaptive mutations may require two processes: (i) the generation of genetic variation among non-growing cells, and (ii) the immortalization of this variation when a cell achieves a useful mutation (Cairns *et al.*, 1988; Stahl, 1988; Hall, 1990; Cairns and Foster, 1991). Thus, it is to be expected that at the appropriate moment some mutations will be 'captured' non-selectively if the cell is allowed to grow. It is clear that an important source of genetic variation in non-growing cells is the movement of insertional elements (Arber *et al.*, 1978; Shapiro, 1984; Cairns *et al.*, 1988; Hall, 1988, 1994; Mittler and Lenski, 1990, 1992; Pfeifer and Blaseio, 1990; Tormo *et al.*, 1990; Naas *et al.*, 1994), and such movement is under complex control (Shapiro and Higgins, 1989; Shapiro and Leach, 1990; Shapiro, 1993; Maenhaut-Michel and Shapiro, 1994). Whether this source of variation can give rise to adaptive mutations remains to be seen.

Materials and methods

Bacterial strains and growth media

The Ara−Lac$^-$ strain used here, MCS1260, is the same as the parent strain, MCS2, except that the β-lactamase sequence of the *Mu pAp* bacteriophage (Leach and Symonds, 1979) is internal to the *Mu cts*62 component of the fusion to the *araB* gene. The rate and kinetics of production of Ara−Lac$^+$ fusions by MCS1260 is identical to that of MCS2 (Shapiro, 1984). The strain is also Δ(*lacIPOZYA*)U169 *fla relA rpsL* (Shapiro, 1984). A tetracycline-resistant (Tet^r) derivative was made by P1 transduction of a *mini*Tn*10* element at 24 min on the chromosome (Singer *et al.*, 1989).

Liquid minimal medium and minimal plates were M9 (Miller, 1972) with 5 μg/ml thiamine and 0.1% glycerol (Sigma; glycerol minimal medium or glycerol minimal plates) or 0.1% lactose (Difco) and 0.02% arabinose (Sigma; AL minimal plates). Sugars were filter sterilized. Gelatin (0.001%) was added to liquid minimal media. Arabinose−lactose−tetrazolium (AL−tetrazolium) plates were prepared as described previously (Miller, 1972) with 1% lactose and 0.2% arabinose. This is a nutrient-rich medium that allows all cells to produce colonies; Ara−Lac$^-$ colonies are red and Ara−Lac$^+$ colonies are white. Tetracycline was added to minimal plates at 10 μg/ml (glycerol-tet minimal plates).

Fluctuation tests

Three separate experiments were performed (Table II), each with three independent cultures of MCS1260 grown to saturation in 25 ml glycerol minimal medium at 32°C with shaking. Every day 0.5 ml samples were removed, titered on AL−tetrazolium plates and plated in top agar on AL minimal plates. Plates were incubated at 32°C. Viable counts were calculated from the number of colonies on AL−tetrazolium plates 2 days after plating, and the frequencies of Ara−Lac$^+$ progenitors were calculated from the number of colonies appearing on AL minimal plates 3 days after plating. On days 6, 7 or 9 one culture was diluted into fresh glycerol minimal medium so that 100 μl would contain less than one Ara−Lac$^+$ progenitor. 100 μl aliquots of this dilution were dispensed into the wells of a microtiter dish, the plate lids were sealed with parafilm and the plates incubated at 32°C. After 2 days the entire contents of between 25 and 80 wells were each plated in top agar on AL minimal plates, and a few pooled samples were titered on AL−tetrazolium plates. These plates were incubated at 32°C and counted 3 and 2 days later, respectively.

In one reconstruction experiment (Table III) the same protocol was followed except that ~160 Tet^r cells/ml were spiked into a culture of MCS1260. The cells were diluted into fresh medium on day 6, dispensed into microtiter wells, given 2 days of outgrowth and then the contents of 68 wells were each plated on glycerol-tet minimal plates and pooled samples titered on glycerol minimal plates. In a control experiment (Table I) 100 μl aliquots of two just-saturated cultures of MCS1260 were dispensed without dilution into microtiter wells and then incubated at 32°C for 7 and 8 days. The cells from 30 wells were then plated on AL minimal plates and pooled samples titered on glycerol minimal plates.

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