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Observations on the formation of clones containing *araB-lacZ* cistron fusions

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Summary. Casadaban (1976) developed a technique for isolating *E. coli* clones containing fusions of the amino terminal-encoding portion of any cistron with the carboxy terminal-encoding portion of *lacZ*. The technique utilizes prophage Mu homology to bring the two cistrons into proximity. I have followed the appearance over time of colonies containing *araB-lacZ* fusions from a strain where the beginning of the *araB* cistron is connected to *lacZ* by an intact Mu*cts*62 prophage. Cultures of the starting strain grown on a variety of media have fewer than 2 in 10^{10} cells capable of forming colonies within three days after plating on selective arabinose-lactose medium. At 32° C, there is a delay of between 4 and 19 days before the first colony appears. The kinetics of colony appearance over the next two to four weeks then shows a rapid increase in the number of new colonies emerging per day followed by a decline. The pattern of colonial emergence and the final numbers of fusion colonies obtained are not grossly affected by reducing the number of cells plated over five orders of magnitude. Fusion colonies sometimes show a clustered pattern when they first emerge. Innoculation of pre-existing fusion clones at specific locations on the arabinose-lactose selection plates seeded with the starting strain leads to the formation of inhibitory zones where no fusion colonies appear. Selection plates contain many microcolonies and papillae which do not proliferate into scoreable colonies but nonetheless contain cells capable of growth when replated on the same selective medium. Up to 39% of all plated cells are capable of producing fusion clones. The kinetics of fusion colony appearance can be altered by environmental and genetic manipulations. Partial derepression of the Mu*cts* prophage at 37° accelerates the appearance of colonies but also reduces the final yield. Addition of limiting concentrations of glucose to the selective medium also accelerates the appearance of colonies in a specific fashion: enrichments below the level required for maximum acceleration produce a biphasic kinetics with two waves of fusion clone emergence separated by an eight-day interval. Infection with Muc$^+$*pAp* phage produces dilysogens that have almost completely lost the ability to produce fusions. Infection with Mu*ctsAampAp* phage produces strains that are reduced in phage production and have delayed kinetics of fusion clone emergence. The implications of these observations for theories of hereditary change in bacteria are discussed.

Introduction

The formation of new protein coding sequences by the fusion of information from separate DNA regions is an important category of germinal and somatic genome reorganization. It is hard to imagine a reasonable alternative explanation for the origin of cistrons encoding hybrid proteins with coding domains derived from two or more ancestral proteins, and at least one developmental system – the formation of immunoglobulin synthesizing lymphocytes – employs coding sequence fusions as a mechanism of cellular differentiation (Tonegawa et al. 1981).

Casadaban (1976) developed an experimental system in *E. coli* for isolating bacterial clones whose DNA contains a fusion of the *lacZ* cistron to the amino terminal coding domain of any other cistron. This system involved the transposable element bacteriophage Mu and has proved extremely useful in studies of bacterial protein synthesis, transport and processing (Bassford et al. 1978). The details of the method are illustrated in Fig. 1 and will be outlined below. Here it is relevant to cite three classes of anecdotal observations which indicated that the control of the genetic events leading to the emergence of coding sequence fusion clones in this system merited further investigation: (1) Fusion clones frequently only appeared after prolonged incubation of parental cultures on selective media. Since the main application of this technique was for the study of membrane proteins, the delay was often attributed to the need to accumulate multiple mutations so that hybrid membrane proteins would not be toxic to the cell. As we shall see, the delayed appearance of fusion clones also occurs when *lacZ* is fused to a cistron encoding a soluble protein and probably represents an intrinsic feature of fusion clone formation. (2) The number of fusion clones obtained was relatively independent of the number of parental cells plated on selective medium. Hall and Silhavy (1981) noted that they obtained an equal number of *ompF-lacZ* cistron fusions after plating either 10^3 or 10^9 cells. (3) Sequencing of the hybrid proteins or the DNA regions containing novel hybrid cistrons revealed that many of them contain the last two or three dozen nucleotides of the Mu β terminus as a linker between the *lacZ* sequences and the amino-terminal sequence (Moreno et al. 1980; M. Berman and S. Benson, personal communication). Casadaban has also shown by transposition tests that at least two out of eight *ara-lac* fusion strains retained a transpositionally functional Mu β terminus (Casadaban and Chou 1984). Thus, at least some of the already characterized cistron fusions did not result from simple "in-phase" deletions directly joining the two coding sequences, and it is possible that Mu sequences played an active role in the formation of the hybrid coding sequences.

In this paper, I report quantitative observations on the appearance of clones containing *araB-lacZ* fusions. Each

experiment was carried out over a period of several weeks, generally more than one month. The results confirm and extend the anecdotal observations cited above about the kinetics and frequency with which fusion clones appear. They show that the frequency of viable cells which had undergone genetic changes enabling them to proliferate into colonies on arabinose-lactose agar was at least two orders of magnitude greater after incubation on selective medium than it was after incubation under non-selective conditions. The observed kinetics of daily colony appearance suggests that plating on selective medium triggers one or more processes involved in the formation of visible fusion colonies. This apparent "triggering" was accelerated for part of the plated population by low levels of glucose enrichment in the selective medium and was retarded by the presence in some strains of a second Mu prophage. The growth of preexisting fusion clones on selection plates inhibited the production of additional visible fusion colonies by bacteria which nonetheless proliferated when innoculated onto fresh arabinose-lactose agar. Thus, the measurement of colony numbers does not reflect the totality of genomic events forming hybrid cistrons. These results indicate that it may not be possible in many cases to infer the frequency of genomic changes in bacterial populations from simple colony counts at a fixed time.

Materials and methods

Bacterial and phage strains. Strain MCS2 was derived from MC4143 (F^- *araD*139*araB*::+Mu*cts*62 *Δ*[*lacIPOZYA,argF*]U169 *fla relA rpsL*) by homology-dependent lysogenization with λpl(209,U118) as illustrated in Fig. 1 and previously described (Casadaban 1976). λpl (209,U118) carries the W209 *trp-lacO* deletion that removes all of *lacI* and *lacP* (Mitchell et al. 1974), leaving *lacZ* and *lacY* with no functional promoter in the prophage state, and the U118 *ochre* mutation in codon 17 of *lacZ* (Zabin et al. 1978), preventing β-galactosidase synthesis even when *lacZ* is transcribed. Lysogens were selected by their immunity to λcI60. Both MC4143 and λpl(209,U118) were provided by M. Casadaban. λ*para*φlac phages were prepared as previously described (Casadaban 1976). Muc$^+$*pAp* (Leach and Symonds 1979) and Mu*ctsAam*1093pAp were obtained from strains AT3773 and AT3912, respectively, provided by Dr. H. Nakai of A.L. Taylor's laboratory. Strains MCS1236, 1238 and 1239 were prepared by transducing MCS2 to carbenicillin resistance (Cb^r) with a Muc$^+$*pAp* lysate and scoring for transductants able to grow at 42° C. These strains carried a Muc$^+$*pAp* prophage unlinked to the *ara* region because Ara^+ transductants found after P1 infection are still Cb^r and lysogenic for Muc$^+$*pAp*. (The thermosensitive transductants arising from the same Muc$^+$*pAp* infection all carried Cb^r linked to *ara*.) Strains MCS1246 and MCS1259-MCS1265 were prepared by transducing MCS2 to Cb^r with a Mu*ctsAam1093pAp* lysate. All show less than 1% survival at 42° C. MCS1259-MCS1265 carry the Cb^r marker between the *araB* and *lacZ* sequences, since *ara-lac* fusion strains and Ara^+ transductants are Cb^s. MCS1246 produces *ara-lac* fusions that are Cb^r and is a poor recipient for P1 transduction to Ara^+. MCS1260-MCS1265 produce phage as well as MCS2 (burst size >10) and these lysates contain both Mu*cts* and Mu*pAp* particles. So I presume they are dilysogens. MCS1246 and MCS1259 are impaired for lysis and phage production, although they do show evidence of limited phage production when replica-plated to a lawn of sensitive bacteria at 42° C. Induction of cultures of MCS1246 produces about 1 very weak plaque-forming unit (p.f.u.) which does not carry the beta-lactamase sequence per 10–20 induced bacteria but plating diluted populations of MCS1246 shows an efficiency of infectious center production at 42° C of 0.1 to 0.5 per cell, approaching the same level as MCS2 and MCS1260-MCS1265. MCS1259 produces fewer than 1 p.f.u. per 200 induced bacteria and shows an efficiency of infectious center production at 42° C of ≦2%. Over 90% of the phage induced from MCS1259 carry the beta-lactamase sequence. The phage produced by MCS1246 and MCS1259-1265 have not been characterized further than testing for beta-lactamase transduction. MCS2 is non-suppressing for *amber* mutations. Indicator strains for phage Mu platings were QL (Sup^+) and M7124 (Sup^0) (Muster et al. 1983).

Media. Complete TYE and minimal PA salts media have been described (Nieder and Shapiro 1975). The latter was supplemented by 0.001% thiamine and (as appropriate) 0.4% glucose, 0.5% glycerol, 0.2% lactose and 0.2% L-arabinose. Solid media were prepared by the addition of 1.5% Difco agar. Carbenicillin was added at 500 micrograms per milliliter.

Microbiological methods. MCS2 clones were routinely grown at 32° C overnight on the surface of agar plates prepared by streaking a small area (~1 cm^2) with a sterile toothpick stabbed previously into an isolated colony. In this way, a dozen clones could be prepared simultaneously on a single plate. The patch of growth contained well over 10^{10} cells, and samples of the various clones were prepared for plating by scraping with the tip of a sterile pipette and resuspension in 1 ml of PA buffer, with vigorous agitation on a vortex mixer to ensure full dispersal of the cells. Dilutions were prepared in PA buffer (for plating on selective PA-thiamine-arabinose-lactose agar) or in TYE for colony-forming-unit (c.f.u.) determinations.

Plating was performed directly from the innoculating pipette so as to avoid puddles where higher concentrations of cells might settle onto the agar surface before spreading. I have found that disposable 0.2 ml glass pipettes are very handy for this purpose. The method is to place a small aliquot (~20 μl) of cell suspension on a portion of the surface to be innoculated, spread out the liquid into a thin film, then cover other portions of the surface until it is completely covered with a liquid film; the remaining innoculum is then added and dispersed over the entire surface before any one region has started to dry in. If the plates were very dry, the innoculum was diluted with sterile PA buffer so that it could be fully plated while the innoculated surface was still wet. The use of a pipette tip rather than a spreader also allows more precise control over the shape of the innoculated surface, and it is possible to introduce small discontinuities into the bacterial lawn as shown in the bottom two plates in Fig. 4.

Plates were incubated for extended periods in metal tins to prevent dessication. I have found that the tins formerly used for autoclaving glass petri dishes permit plates to be incubated for up to three months at 37° C without visible shrinking of the agar. These tins are not air tight and permit even obligate aerobes to proliferate as fast as they do unenclosed.

Scoring fusion colonies. Plates were examined periodically after plating to score emerging colonies. As soon as an opaque area of growth became visible to the naked eye through the bottom of the petri dish against a dark lab bench, it was recorded and marked on the bottom of the dish with a magic marker. The markings made it possible to identify the newly emerging colonies at each scoring and verify previous scorings of very small colonies by their further proliferation. Use of different colors and symbols (dots, circles, crosses, etc.) made it possible to keep a record of where and when colonies emerged in the course of incubation. After about three to five weeks of incubation, reliable objective scoring became impossible because colony proliferation was inhibited and many translucent microcolonies and papillae appeared on the bacterial lawns (see text). In later experiments, plates were also recorded photographically, but photography was not as sensitive as close visual inspection for picking up freshly emerging colonies. It was not always possible to score the plates every day, and the importance of doing this was not apparent in the earliest experiments. Thus, some of the data in Figure 2 show the average daily increase in colony numbers averaged over two- or three-day periods, and the data in Figure 4 often show the earliest possible daily interval when a first fusion colony could have emerged rather than the interval when it actually did emerge.

Growth tests. To test the requirement of fusion colonies for arabinose to grow on PA agar containing lactose, I found it necessary to stab them into PA-glucose agar, grow them overnight at 32° C, and then replica-plate them to PA-agar containing either arabinose, lactose, or both. If they were stabbed into the selective medium before replica-plating or tested directly by stabbing from the selection plates, they contained enough Lac activities to continue growth in the absence of arabinose inducer.

Photography. Periodic scorings were accompanied by photographic records of the petri dishes illuminated from the sides against a dark background on Panatomic-X film with either a Pentax or Canon 35 mm camera. Contrast of the bacterial lawn and colonies against light scattered from the background agar could be increased with a number 15 deep yellow filter. The photographs shown in Fig. 7 were taken with a Canon FD 50 mm "macro" lens and a 25 mm extension tube using dark-field illumination from four electronic flash units arrayed in a square (Bowen Illumination) and a number 15 filter.

Results

The selection system

The basis of the original Casadaban (1976) technique was to use Mu prophage homology to insert *lacZ* and *lacY* cistrons downstream of the cistron to which they were to be fused. A *λplac* phage containing no *lac* promoter and having a fragment of Mu 5′ to *lacZ* can recombine with a properly oriented Mu prophage previously inserted in virtually any cistron to generate the appropriate structure (Fig. 1). If the *lacZ* cistron also has a chain termination mutation in the first two dozen triplets, only a translational fusion to an upstream cistron will permit the expression of β-galactosidase activity (necessary for growth on lactose as sole carbon source) when the upstream cistron is transcribed. The validity of these assumptions and the natures of the resulting cistron fusions have been amply documented (Silhavy et al. 1976; Moreno et al. 1980; Shuman et al. 1980).

In order to study cistron fusion without the complications of membrane proteins, I constructed strain MCS2 as described in Material and methods. MCS2 carries *lacZ* downstream of *araB*, the cistron encoding L-ribulokinase, a soluble enzyme (Lee and Bendet 1967). Separate lysogenizations gave rise to lines MCS2a, b and c. Subclones were designated MCS2a1, a2, b1, etc. and further subcloning was indicated by two-digit and three-digit designations (e.g. MCS2a211 is the result of two successive subclonings of MCS2a2). Although certain differences were noted in the behavior of the three lines on selective medium (e.g. degree of papillation of the background lawn), there was no systematic variation between them or their subclones in the parameters discussed here. From all three MCS2 lines, variants containing *araB-lacZ* fusions were isolated after plating on minimal arabinose-lactose agar. Two criteria confirmed that colonies on this medium contained *ara-lac* fusions and not Ara^+ or Lac^+ derivatives: (1) Omission of arabinose from selection plates virtually eliminated colony formation under the conditions used in most of these experiments (incubation at 32° C for 3–5 weeks), and (2) over 1100 colonies have been picked from selection plates and shown to grow only on arabinose-lactose agar but not on either arabinose or lactose agar. Several dozen putative fusion clones were repurified on non-selective medium and then tested for retention of the fusion character as well as for loss of the Mu *cts62* prophage. Subclones grew only on arabinose-lactose agar and contained no functional Mu prophage. Thus, the three MCS2 lines were assumed to contain the DNA structure depicted in Fig. 1.

Control experiments

Because a key question in quantitative studies of mutation concerns the temporal relationship of DNA reorganization to colony appearance, it was necessary to do two kinds of controls. The first was a reconstruction experiment to find out how quickly fusion colonies grew up under these plating conditions. The second was a transduction experiment where it was possible to specify the time when DNA changes began because they were initiated by phage infection.

When independent fusion colonies were subcloned onto minimal arabinose-lactose agar, they always formed distinct colonies within two days of incubation at 32° C. To verify that the emergence of scoreable fusion colonies would occur equally rapidly when plated in the presence of non-growing MCS2 cells, I did four reconstruction experiments by mixing about 100 fusion cells with over 10^8 MCS2 cells and plating on selective agar. Although the colonies were visibly retarded by the background lawn, over 95% of the colonies that would appear in the first week of incubation at 32° C had reached scoreable size by the second day after plating, and over 99% had reached scoreable size by the third day.

One of the advantages of the Casadaban (1976) technique is that once fusions have been isolated they can readily be cloned onto a λ transducing phage. Prophage induction from strains with the fusion structures illustrated in Fig. 1 will yield some phage that have excised by recombi-

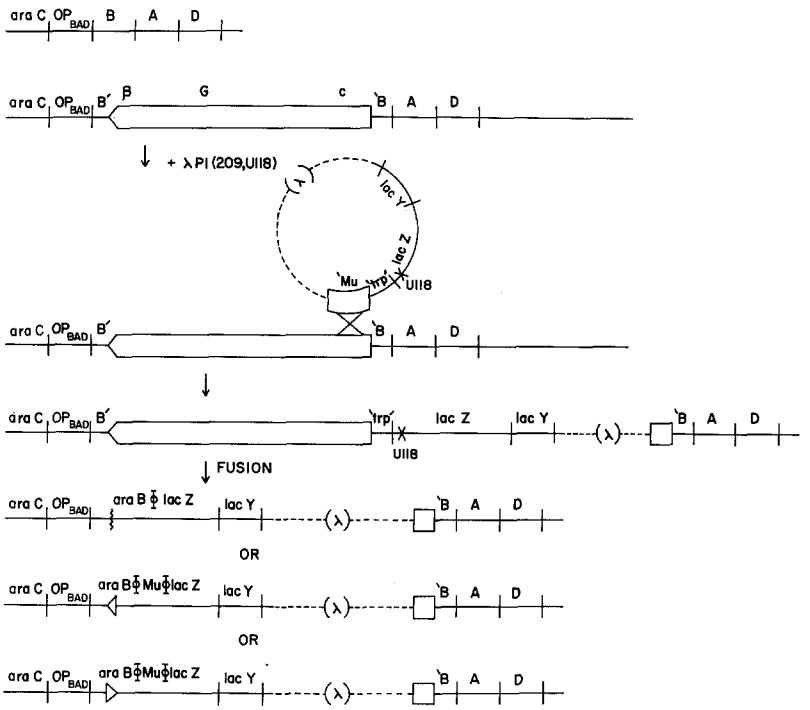

Fig. 1. Schematic representation of the DNA structures involved in the formation of *araB-lacZ* cistron fusions. *E. coli* chromosomal sequences are represented by solid lines, λ sequences by dashed lines, and Mu sequences by an open box. Apostrophes indicate interrupted or deleted sequences. The 'trp' region upstream of *lacZ* indicates *trp* operon material connected to a small segment of *lacO* by the W209 *trpA-lacO* deletion (Mitchell et al. 1974). The orientation of the Mu prophage is indicated by the pointed and blunt ends of the box and this is oriented with respect to the Mu genetic map by indicating the relative position of *c* (repressor cistron), invertible G region, and *β* region (Toussaint and Resibois 1983). The presumptive steps in fusion formation are: (i) insertion of a Mu*cts*62 prophage into *araB*; (ii) lysogenization of the *araB*::Mu*cts*62 strain with λpl(209,U118) by reciprocal homologous exchange between the Mu prophage and the Mu *c* terminus fragment of λpl (Casadaban 1976); (iii) removal of transcriptional and translational stop signals between *araB* sequences and *lacZ* sequences 3′ to the U118 *ochre* triplet to generate a functional *araB*-ϕ-*lacZ* hybrid cistron. Three possible structures are shown resulting from step (iii) – a simple in-phase deletion between *araB* and *lacZ*, a deletion between a site close to the *β* terminus of Mu and *lacZ*, and a more complex rearrangement leaving in some nucleotides from the *β* terminus of Mu but inverted with respect to their original orientation. Although no *araB*-ϕ-*lacZ* fusion have yet been sequenced, sequences of other cistron fusions generated by this method have revealed analogous structures (Moreno et al. 1980; M. Berman and S. Benson, personal communication)

nation to the left of *araC* and to the right of all essential λ sequences. Such phage will produce blue plaques on lawns of a *lac* deletion strain on medium containing L-arabinose and the chromogenic substrate XGal (Shapiro et al. 1969; Casadaban 1976). I isolated λ*paraφlac* phages from three fusion clones, confirmed that they required arabinose induction to synthesize *β*-galactosidase, and used them to measure the delay between a DNA change (i.e. introduction of the cistron fusion into the cell on a repressed phage genome) and colony formation on selective medium. When three different MCS2 clones were infected at multiplicities less than 10^{-4} with three different λ*paraφlac* phages, in each transduction between 1 and 2% of the infected cells produced scoreable colonies within two days on selective medium. These colonies represented over 90% of all transductants obtained from these infections. It thus appears that the presence of a suitable DNA structure permits MCS2 cells to proliferate rapidly (i.e. within two days) into visible clones on fresh selection plates in the presence of a large excess of non-proliferating cells.

Kinetics of fusion colony appearance

Figure 2 summarizes the numerical results of a typical series of selections for fusion derivatives from four MCS2 cultures plated at two temperatures over a 10^5-fold range of cell concentrations. Several features of these data deserve comment:

(1) At both temperatures, the final yield of "scoreable" colonies is relatively independent of the number of cells initially plated. "Scoreable" is a subjective term which here means a colony distinguishable from the background lawn. At early times, this distinction is very clear, but it becomes increasingly difficult to make after about three to five weeks incubation when numerous papillae form on the background lawn. (Picking and testing of these papillae showed that some contained cells capable of producing full growth on minimal arabinose-lactose agar and some did not.) Scoring ceased when this judgment became impossible to apply with confidence. The validity of scoring up to this time was confirmed by picking and testing putative fusion colonies for their ability to produce full growth on the selective medium. Although undiluted cultures often produced more fusion colonies than those diluted 10^4- or 10^5-fold, the differences never exceeded a factor of 4.

(2) At 32° C, there was a lag of several days before the first scoreable fusion colony appeared (at least until between the fifth and sixth days after plating for the cultures documented in Fig. 2 and never earlier than between the third and fourth days). After this initial event, there was

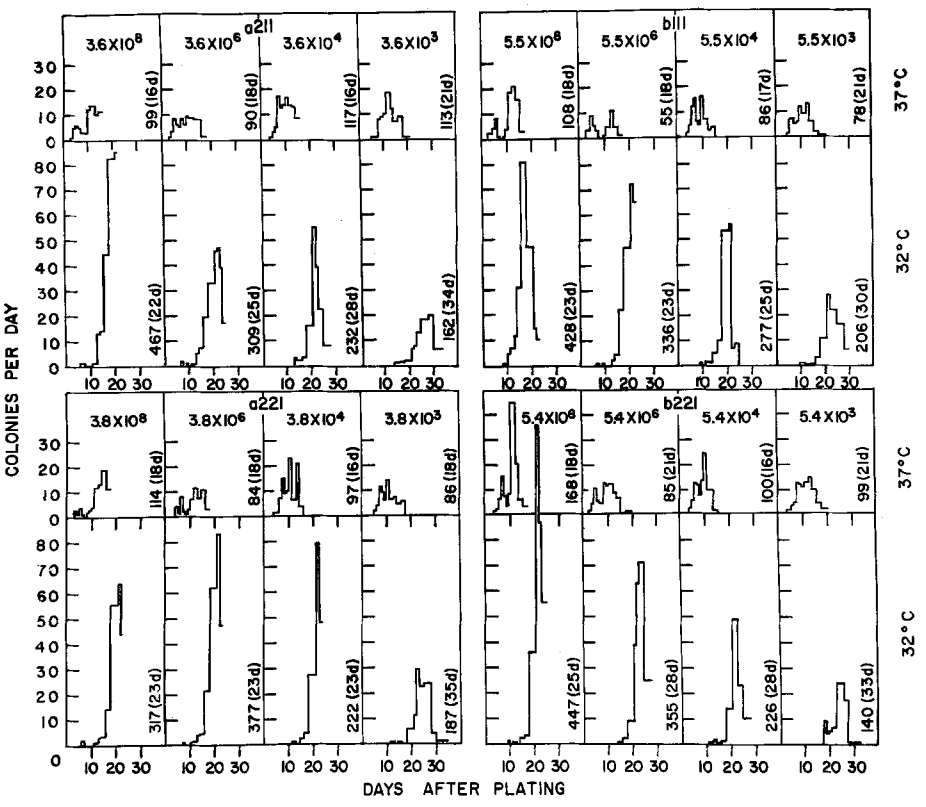

Fig. 2. Kinetics of fusion colony emergence. Four MCS2 clones were resuspended from PA-glucose agar, diluted 10^2-, 10^4-, and 10^5-fold, and each was then plated at four different concentrations on PA-lactose-arabinose agar in triplicate. One set of plates was incubated at 32° C, one at 37° C, and one overnight at 42° C followed by incubation at 37° C. (Data are not shown for the last plating.) Plates were examined, scored and photographed periodically. The abscissa indicates the days after plating, and the ordinate indicates the per diem increase in colony numbers on each plate. (If plates were scored after an interval of two or three days, the per diem increase was taken as the ratio of total new colonies to days since the last scoring.) The numbers near the top of each panel indicate the number of c.f.u. plated, and the numbers at the right of each histogram indicate the fusion colony total obtained when quantitative scoring was stopped, with the time of the last scoring given in parentheses

a period of accelerating increase in daily colony appearance, which reached a peak between 16 and 24 days after plating, followed by a decline until scoring was no longer feasible. The visible colony population changed very little after this period (see the right-hand column in Fig. 3). At the peak, the rate of colony appearance was remarkably high, often exceeding 70 or 80 per day. The rapid rise to these peak levels suggests that a synchronous process was occurring in the plated population. Lower cell concentrations generally increased the lag time and reduced the peak values achieved, but again the effects were not at all commensurate with the dilution factors. In some cases, such as clone MCS2a221, the diluted platings showed higher peaks than the undiluted plating.

(3) At 37° C, the first colonies invariably appeared between three to five days after plating, the peak rates of colony appearance were less pronounced than at 32° C, and the final total accumulations of fusion colonies were significantly lower than at 32° C. Thus, it is clear that the conditions of incubation after plating can dramatically alter the kinetics of fusion colony appearance. Plating overnight at 42° C and subsequent incubation at 37° C, the original Casadaban (1976) protocol, even more dramatically reduced the final yield of fusion colonies, presumably because lethal derepression of the Mu*cts*62 prophage killed cells which would eventually have produced colonies. According to the Mu literature, incubation of Mu*cts*62 lysogens at 37° C is not lethal and does not induce phage production but does result in expression of Mu transposition functions (Toussaint and Resibois 1983).

(4) The final densities of fusion colonies on the selection plates was much greater than one would expect from published values for the frequencies of comparable deletion events, typically on the order of $\leq 10^{-9}$ per plated cell in cultures at comparable cell titers. Based on the undiluted platings, the observed frequencies of fusion ranged from 8 to 13 per 10^7 plated cells. Of course, similar frequencies calculated for the diluted platings would be orders of magnitude higher.

Geometry of fusion colony appearance

One curious feature of how fusion colonies emerged on the selection plates was their tendency to form foci in distinct regions of the plate early (i.e. within the first two or three weeks of incubation) and later to colonize the less densely populated areas on the agar surface. While this was not always obvious and is extremely difficult to establish statistically, the point can be appreciated from serial photographs of a few plates taken at various times in the course of incubation (Fig. 3). The final colony distributions were reasonably uniform over the entire innoculated surface whereas the initial distributions showed denser localized groupings that persisted for a few days in the period when the rate of new colony appearance was accelerating. Two kinds of control experiment suggest that this early grouping was not due to significant variations in cell density over the innoculated surface as a spreading artefact: (i) the distributions of colonies on viable cell determinations plated in the same way but at lower cell concentrations were often even more uniform than the final fusion colony patterns, and (ii) the emergence of Gal$^+$ revertant colonies from a lawn of *galOP128*::IS1 bacteria over a period of more than a week did not reveal similar foci.

Effect of preplating culture conditions

In order to determine whether there was any effect of the culture conditions prior to plating on the kinetics of appear-

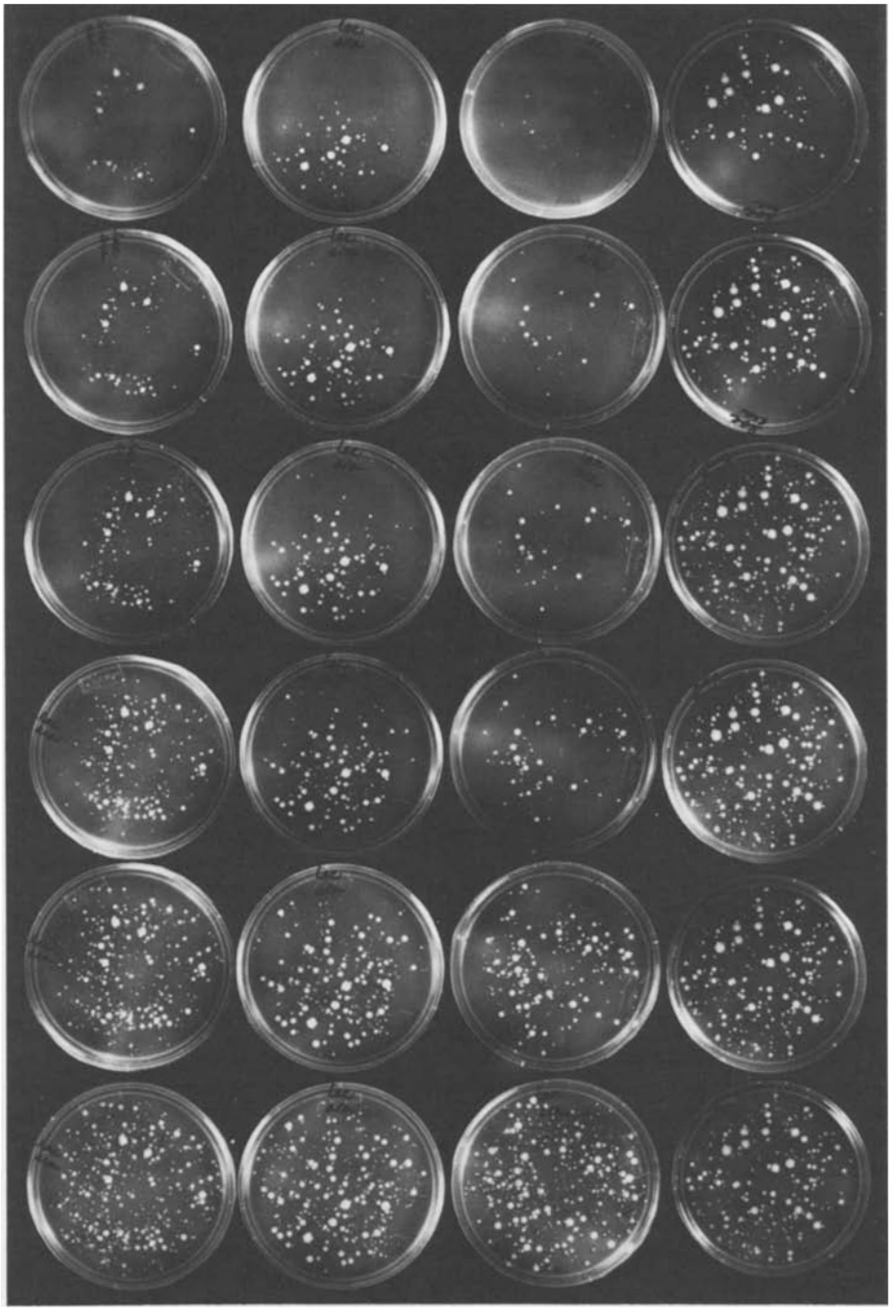

Fig. 3. Emergence of fusion colonies on PA-lactose-arabinose plates at 32° C. Each column contains successive photographs of a petri dish innoculated with samples of a glucose-grown MCS2 clone. From left to right, these are: Column 1, clone a21, 8×10^6 cells plated at 17, 18, 19, 20, 21 and 23 days after innoculation; column 2, clone b11, 8.7×10^7 cells plated, at 16, 17, 18, 19, 21, 23 days after innoculation; column 3, clone b12, 3.6×10^8 cells plated, at 15, 17, 18, 19, 21, and 25 days after innoculation; column 4, clone b11, 8.7×10^6 cells plated, photographed at 17, 20, 23, 25, 27, and 30 days after innoculation

ance of fusion colonies, several different growth media besides minimal glucose agar were tested: rich TYE agar, minimal glycerol agar, and minimal glycerol agar with arabinose to induce *araB* transcription. In addition, the plated suspensions used in two experiments were replated after either 15 or 28 days "starvation" in PA buffer at room temperature without agitation. There was a 40% difference in one experiment on the final yield of cells between TYE-grown cultures and those grown on minimal medium. But the kinetics of colony appearance showed no striking reproducible differences. In particular, there was no discernable acceleration or retardation in the rate of appearance of the initial fusion colonies on selection medium (Fig. 4).

Effect of pre-existing fusions

The initially clustered geometry of colonial emergence (Fig. 3) and the lag period followed by a rapid acceleration in the rate of fusion colony appearance at 32° (Fig. 2) both suggested that a positive feedback process might operate whereby the first fusion clones would somehow stimulate the formation of new fusion clones. In order to test this idea, I spread replicate plates with identical aliquots of the same MCS2 suspensions and then stabbed pre-existing fusion clones from a prior selection at the periphery of the bacterial lawn on one or more of the replicas. Incubation and scoring of these plates yielded the opposite result of

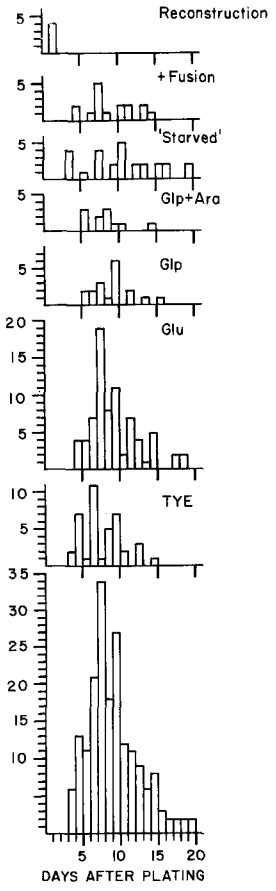

Fig. 4. Time of appearance of the first fusion colonies at 32° C. The abscissa indicates the earliest daily interval when a scoreable fusion colony could have appeared on a plate seeded with an MCS2 clone, and the ordinate indicates the number of independent platings that had colonies which could have emerged in each interval. Since many cultures were not examined every day and the first interval after a zero scoring is indicated, the data may be biased towards earlier colony emergence. The bottom panel gives all results; the top panels are labelled to indicate the growth substrate, whether the cell suspensions were "starved" in buffer at room temperature without agitation before plating, or if pre-existing fusion clones were stabbed at the periphery of the lawn after innoculation. The results of the four reconstruction experiments discussed in the text are indicated for comparison

the one predicted by the working hypothesis. The pre-existing fusions did not accelerate the appearance of initial fusion colonies (Fig. 4), nor did they augment the rate of appearance or final yield of colonies. On the contrary, the prexisting fusion clones accelerated the shut-down phase of colony appearance and thereby reduced the final yield (Fig. 5). Moreover, they had a striking effect on the geometry of colonial emergence, creating a clearly visible zone of inhibition (Fig. 6). The inhibitory effect was also seen when there was a discontinuity of two or three millimeters in the bacterial lawn (Fig. 6), showing that the inhibition resulted from depletion or excretion of a diffusible substance rather than from some effect requiring direct cell to cell contact.

It was possible to demonstrate that the inhibition acted after the genetic events necessary for an MCS2 variant to form a colony on minimal arabinose-lactose agar had been completed. Once the zone of inhibition was visible on two 22 day-old plates, I suspended a fusion colony from the central area of each in buffer, diluted them, and plated 5 µl aliquots on a blank selection plate as well as within the zones of inhibition. Within two days, distinct colonies formed on the control plate, but there was no visible colony formation in the zones of inhibition for up to two weeks after this secondary innoculation.

Examination of microcolonies from plates innoculated at low cell densities

The inhibition phenomenon observed with pre-existing fusions suggested an explanation for the shutdown phase observed in periodic colony counts (Figs. 2 and 5). This was that the shutdown resulted from inhibition of the growth of new colonies by established ones rather than from a cessation in the requisite DNA reorganizations. If this explanation was correct, then it would be possible to show that the selection plates contained cells capable of forming colonies on the same medium but which had not proliferated. This possibility could most readily be tested on plates initially innoculated with only a few thousand cells. These plates contained many barely visible microcolonies in the appropriate numbers to represent each initially plated cell which had not produced a scoreable fusion colony (Fig. 7). When removed from the plate intact on a plug of agar and resuspended after 35 days of incubation, these microcolonies proved to contain between 1.5×10^6 and 1.2×10^7 colony-forming units when plated on minimal glucose agar (Table 1). When the entire microcolony population from a single area of the selection plate was sampled (with care to avoid scored fusions, as illustrated in Fig. 7), stabbed into minimal glucose agar, and tested for the presence of

Table 1. Characterization of plates innoculated with 3000–6000 MCS2 cells

MCS2 clone plated	c.f.u. per resuspended microcolony	(a) Cells plated	(b) Scoreable fusions	(c) Microcolonies tested	(d) Microcolonies containing fusions	Frequency of fusions [(b)/(a)+(d)/(c)]
a211	1.2×10^7 3.4×10^6 4.5×10^6	3620	162	36	3	0.12
a221	3.4×10^6	3800	187			
b111	2.9×10^6 6.6×10^6 7.5×10^6 1.5×10^6	5520	206	72	25	0.39
b221	4.0×10^6 9.3×10^6	5400	140	69	10	0.18

Microcolonies were assayed for c.f.u. by collecting them intact on agar plugs with a Pasteur pipette, resuspending them thoroughly in 1 ml of PA buffer, diluting and plating on PA glucose agar. An area where a plug was removed from one of these plates can be seen in the lower left-hand corner of the top panel in Fig. 7. Microcolonies were assayed for the presence of fusion cells by picking with sterile toothpicks into PA glucose agar and replica-plating to PA-lactose, PA-arabinose and PA-lactose-arabinose agar. A typical picked area can be seen in the bottom of the lower panel of Fig. 7

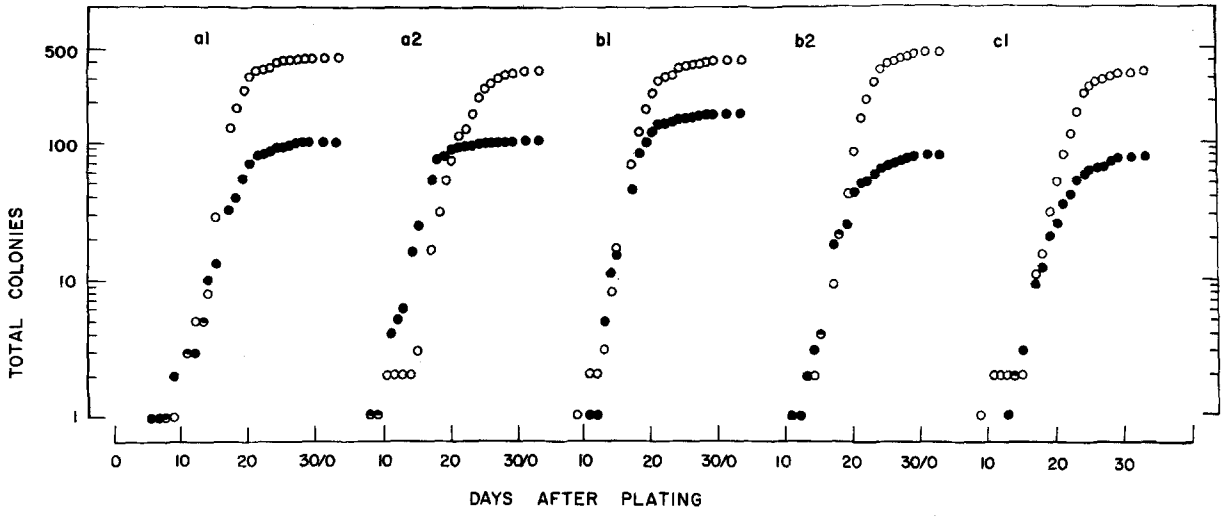

Fig. 5. Kinetics of fusion colony accumulation in the presence (●) and absence (○) of pre-existing fusions. Five independent MCS2 clones were plated in duplicate on PA-lactose-arabinose agar, and three pre-existing fusions previously isolated from the same clones were innoculated into three points 90° apart on the periphery of the seeded area on one of the duplicates for each clone (see Fig. 6, top two dishes). Plates were then scored periodically for the emergence of fusion colonies after incubation at 32° C. The abscissa indicates the days after plating when colonies were scored, and the ordinate indicates the accumulated total of colonies on a logarithmic scale

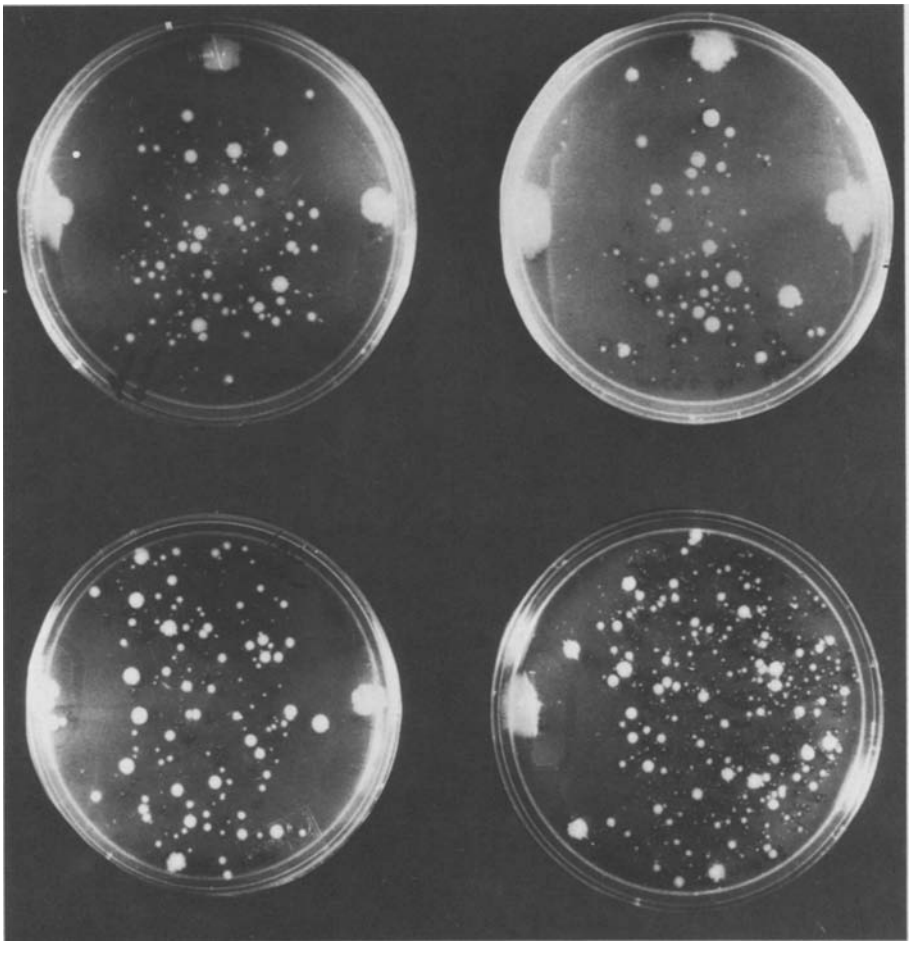

Fig. 6. Selection plates with pre-existing fusions. The plates were prepared either as described in the legend to Fig. 5 (top two plates) or by stabbing only one or two pre-existing fusions at the periphery (bottom two plates). Note that the bacteria in the bottom two plates were spread in such a way that the lawn has a small discontinuity close to a pre-existing fusion. The innocula (and times of incubation at 32° C) of these plates, clockwise from upper left, are clone b1 (34 days), clone b2 (34 days), clone a21 (61 days) and clone b12 (61 days)

fusions by replica-plating, between 8 and 35% of the micro-colonies turned out to harbor enough cells capable of growth on minimal arabinose-lactose agar to be scored as clear positives (Table 1).

Effect of glucose enrichment

When low concentrations of glucose (0.004 to 0.04%) were added to the selection medium, the growth of the back-

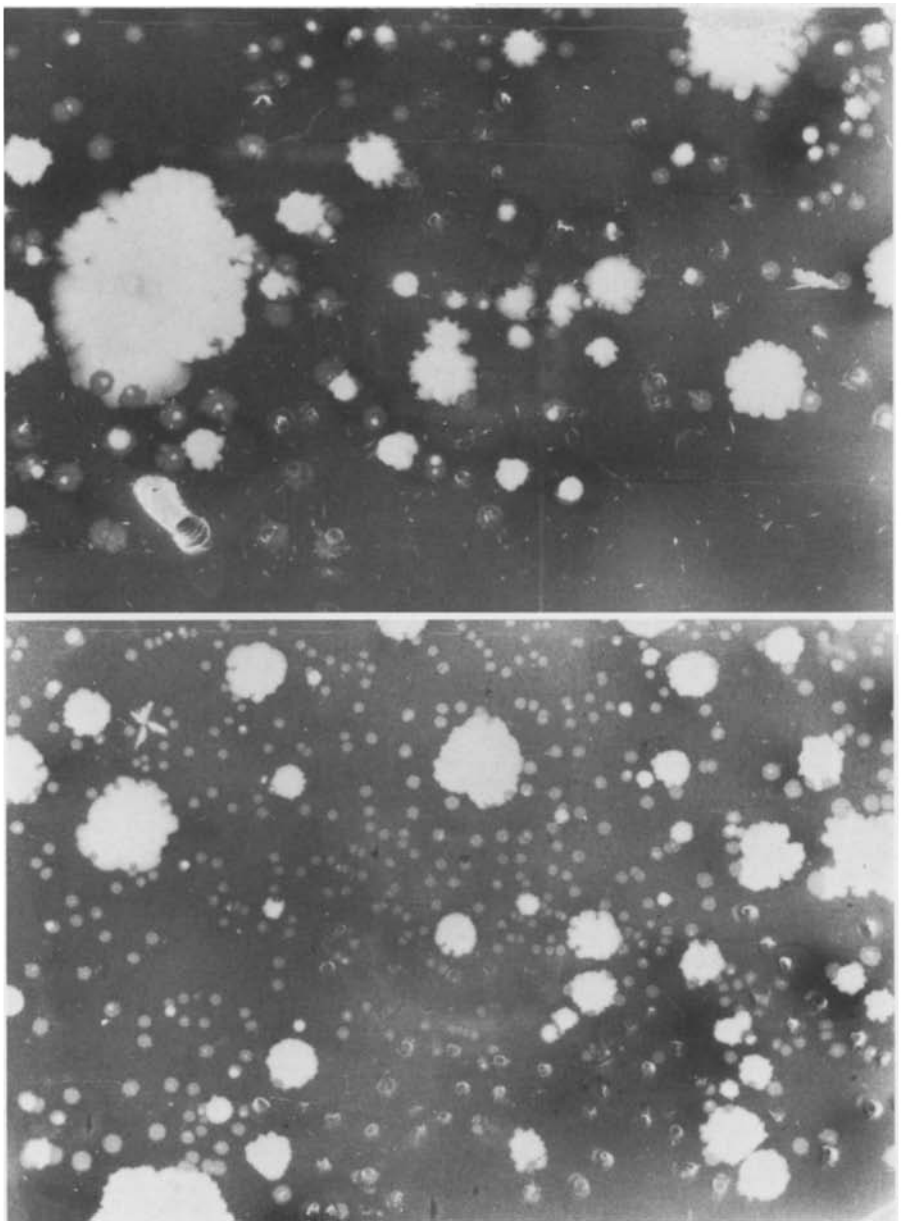

Fig. 7. Portions of the surface of plates innoculated at low density. The upper panel is clone a211, 3.8×10^3 cells plated, and the bottom panel is clone b221, 5.4×10^3 cells plated, both at 39 days at 32° C after innoculation. Note the areas where microcolonies have been picked with sterile toothpicks or (top panel) where an intact colony was removed with a Pasteur pipette

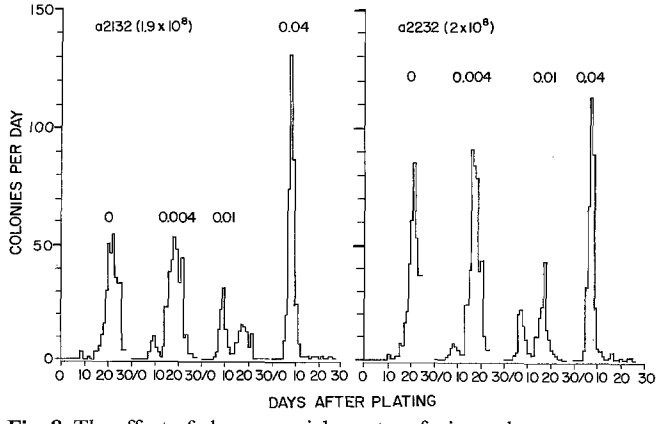

Fig. 8. The effect of glucose enrichment on fusion colony emergence at 32° C. The data are presented in the same way as in Fig. 2. The percentage glucose concentration in the selective medium is indicated over each histogram

ground lawn was visible accelerated in the first day of incubation, but the lawn remained thin enough for fusion colonies to be readily scored. Glucose enrichment also accelerated the appearance of fusion colonies, but not in the manner expected from a simple increase in cell numbers. Figure 8 shows two representative experiments. With no enrichment, these two cultures showed peaks of colony appearance that reached their high points in the 20–23 day interval. At 0.004% glucose in both cultures, there was a small peak of early colonies centered around the 8–9 day interval followed after a decline by a second larger peak centered around the 16–18 day interval. At 0.01% glucose, these two peaks were still distinct but now about equal in size. At 0.04% glucose, virtually all the colonies had entered the first peak which was much larger and centered on the 7–8 day interval. Even at the highest glucose enrichment, no fusion colonies emerged before the fourth day after plating.

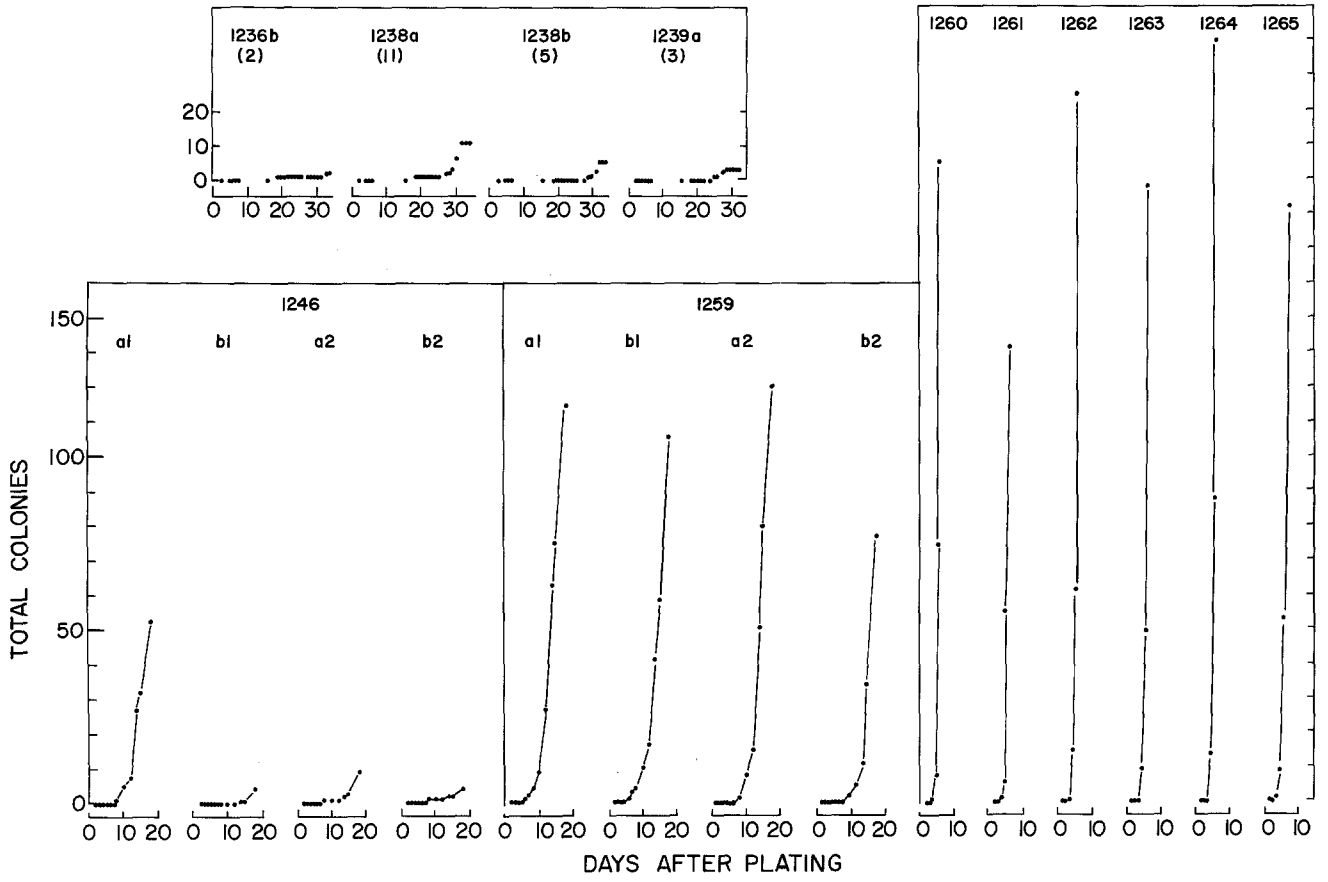

Fig. 9. The accumulation of fusion colonies on glucose-enriched (0.04%) selective medium at 32° C by Mu*pAp* transductants. The data are presented in the same way as in Fig. 5. See text for explanation

Effects of changing the Mu prophage

The presence of Mu termini in many fusions (Moreno et al. 1980; Casadaban and Chou 1983; M. Berman and S. Benson, personal communication) and the 37° C effect seen in Fig. 2 both hinted at a role for Mu functions in fusion formation. To investigate this possibility further, I attempted to convert the resident Mu*cts* prophage in MCS2 to one that had a stable repressor or to one that lacked essential Mu functions. The strategy for accomplishing this was to infect with Mu*pAp* phages at low temperature, select for the β-lactamase marker, and score for recombinants that had also incorporated other markers on the infecting Mu*pAp* genome. While these infections gave somewhat different results from those I anticipated, they produced five classes of Cb^r strains which were altered in Mu phage production and sometimes in fusion colony emergence.

Infection of MCS2 with Muc$^+$*pAp* produced Cb^r transductants about half of which appear to have incorporated the *Ap* region into the resident prophage. They are still *cts* and have β-lactamase located between the *araB* promoter and the *lacZ* sequence since all fusions are Cb^s. These strains have fusion kinetics indistinguishable from MCS2. The other Cb^r transductants (represented by MCS1236, 1238, 1239) have a Muc$^+$*pAp* prophage unlinked to the *araB-lacZ* region because it is retained in both *araB-lacZ* fusion clones and Ara$^+$ transductants. Infection of MCS2 with Mu*ctsAampAp* produced mainly transductants with the β-lactamase incorporated near the site of the original prophage. There are two classes in this group. Strains MCS1260-1265 exhibit normal phage production and yield particles with and without the beta-lactamase marker and so appear to be polylysogens. Strains MCS1259 is severely inhibited for phage production and yields only particles with the beta-lactamase marker. A third class of Cb^r transductant resulting from Mu*ctsAampAp* infection is strain MCS1246. The beta-lactamase marker in this strain does not appear to lie between *ara* and *lac* sequences, and phage production (chiefly of particles without the beta-lactamase marker) is only moderately inhibited. The physical structures of the prophages in these Mu*ctsAampAp* transductants have not yet been investigated, but it is clear that they were formed by events other than simple homologous recombination between the infecting phage genome and the resident prophage (see Materials and Methods for more details). Figure 9 shows the kinetics of fusion colony appearance for the last four classes of Cb^r strains on selective medium enriched with 0.04% glucose at 32° C. Strains MCS1260-MCS1265 behave essentially like MCS2, producing well over 100 fusion colonies within 8 days of plating under these accelerating conditions. MCS1236, 1238 and 1239 with the Muc$^+$*pAp* prophage are severely inhibited for fusion formation. Three other cultures plated in this same experiment (MCS1236a, MCS1239b and c) gave no fusion colonies in 34 days on this medium, and the same four cultures plated without glucose enrichment produced no fusion clones in 28 days incubation. MCS1246 and MCS1259 are clearly different from their siblings

MCS1260-MCS1265 and also from each other. Except for one clone, MCS1246 cultures produced less than 10 fusion colonies in 18 days incubation, whereas MCS1259 cultures were clearly slower than MCS1260-1265 but still all produced about 100 colonies in the 18 day period. Thus, there are at least four inheritable kinetic patterns of fusion colony emergence, typified by MCS2, MCS1236, MCS1246 and MCS1259.

Discussion

The data presented above show that the events leading to the appearance of colonies containing cells with *araB-lacZ* fusions are subject to a more elaborate set of environmental, populational and genetic controls than anticipated from stochastic models of hereditary variation. These results make it possible to ask the following series of questions:

(1) Why are there no pre-existing fusions in unselected MCS2 cultures that will produce a colony within 2 days of plating (Fig. 4)? Even on the accelerated plates containing glucose, the earliest colonies appear between the third and fourth days after plating (Fig. 8 and 9). We can calculate a *minimum* estimate for the frequency of fusions in a selected population by taking the lowest proportion of cells plated at low density that produce detectable fusions (0.12, Table 1) and dividing by the maximum number of cells per microcolony on these plates (1.2×10^7, Table 1). This gives a result of 1 per 10^8 cells. In the experiments summarized in Fig. 4, 187 suspensions were plated containing 3.3×10^{10} cells. If the frequency of fusions per cell were constant, then we should have expected over 300 pre-existing fusion cells in these suspensions, and they clearly were not present.

(2) What happens in the period after plating that results in such a sharply synchronous emergence of colonies with delays that may be over two weeks (Fig. 2)? How does glucose enrichment alter this synchrony in such a manner that certain glucose levels produce two waves of fusion colony emergence (Fig. 8)? Is the synchrony also related to the geographic clustering that is often seen (Fig. 3)? Two sets of observations in other systems are worth noting in this context. (a) Reversion experiments with *gal*::IS1 and *lac*::Tn*9* mutants to produce colonies on minimal-galactose or minimal-lactose agar showed different kinetics of colony emergence from those described above. Cultures of all strains contained cells immediately capable of forming colonies on the selective medium, but some strains produced no (or very few) additional colonies after incubation for several weeks whereas others strains continued to produce additional colonies for one or two weeks until the plates were saturated at a few hundred colonies (unpublished data). (b) Examination of sectoring patterns in strains of *E. coli* and *P. putida* containing Mu*dlac* fusion elements (Casadaban and Chou 1984) showed that the occurence of clonal changes within a colony was characteristic for each strain and for the particular medium on which that strain was grown. The nature of the sectors (i.e. whether they display higher or lower levels of β-galactosidase expression), their shapes and sizes, their frequencies, and their places of origin in the colony were strain-specific and medium-specific (Shapiro 1984a, b). For example, colonies of a *P. putida* (Mu *dlac*) strain may show no sectors when growing on glucose, large sectors from the center when growing on citrate, and thin peripheral sectors when growing on casamino acids. Both these sets of data combine with the results presented above to indicate that in many (if not most) situations the formation of variant clones on agar is regulated by an interaction between the genomic configuration and growth history of a bacterial population (either a lawn or a developing colony).

(3) What is the mechanism by which fusion clones that have already grown up inhibit the emergence of other clones? This mechanism may account for the rapid decline in new colony appearance a few days after the peak (Fig. 2) as well as for the striking effect of pre-existing fusions (Fig. 6). It is possible that nutrient depletion may explain these effects, but it should be noted that these plates can support the appearance of over twice as many fusion colonies when innoculated with very high cell densities. The appearance of the plates in Figure 6 suggests the excretion of an inhibitory substance into the agar. If this is correct, then it would be interesting to find out whether this substance is an unavoidable metabolic product or rather serves to control bacterial growth. Similar inhibitory effects can easily be noted between nearby colonies on agar medium, where the interactions often lead to marked changes in colony shape and the formation of inter-colony boundary zones (Shapiro 1984a, b).

(4) How do the different Mu prophage configurations affect the kinetics of fusion formation (Fig. 9)? (Note that all strains do produce fusions, but the number and timing is altered.) The strong inhibition in MCS1236, 1238 and 1239 may indicate that Mu derepression is one element in the events leading to colony formation. According to this hypothesis, it would be much more difficult to inactivate the c^+ protein product than the already enfeebled *cts* product. But such an explanation would still leave unanswered how the *cts* repressor is inactivated and cannot account for the observed differences with MCS1246 and MCS1259. (The properties of these two strains also illustrate how little is yet known about the structure of dilysogens formed by infection of a Mu lysogen with a second Mu prophage.)

We cannot expect to answer questions like those listed above simply by defining the nature of DNA changes that have taken place. Even if large numbers of *ara-lac* fusions were characterized physically and found either to fall into clearly defined structural classes or to be unclassifiable, the same regulatory problems would remain (as, indeed, they do for mechanistically well-defined rearrangements like IS1 and Tn9 excision). Questions such as the above may seem unusual in discussions of bacterial variation only because we are trained to think about this topic in terms of the issues current in the 1940's and 1950's. At that time it was important to show that variants could arise in the absence of selection, and this was accomplished by the classic experiments of Luria and Delbrück (1943), Newcombe (1949) and the Lederbergs (1952). It should be kept in mind that these experiments involved immediately lethal selective agents (virulent phages or streptomycin) and so could only produce mutants in the absence of selection. Statistical analysis of the variation in mutant frequencies from culture to culture in such experiments is not really very informative about the regulation of the mutagenic process because any reasonably complex system will look stochastic. Mutational selections that do not involve lethal platings (such as reversion of auxotrophies or the acquisition of the ability to utilize a particular carbon source) make it possible to per-

ceive controls that were not visible in the earlier experiments. The dynamics of bacterial growth and death in populations plated under non-lethal conditions will definitely need to be considered in explaining the production of clones with new genetic capabilities (cf. Fig. 7). While this may appear to make non-lethal platings more complex than lethal platings, it must be remembered that the mutants which have survived bactericidal selections arose during a previous growth process. There is no evidence to make us believe that pre-selection population dynamics are any less complex than what happens post-selection, and in many cases the selected population is much more amenable to experimental manipulation and observation so that different factors operating in the mutational process can be disentangled. Indeed, now that we know about mobile genetic elements, inducible mutator systems and multiple biochemical activities that reorganize DNA molecules, the most pertinent questions in studies of hereditary change must be questions of control and regulation.

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