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Differential Activity of a Transposable Element in *Escherichia coli* Colonies

JAMES A. SHAPIRO[1]* AND N. PATRICK HIGGINS[2]

Department of Biochemistry and Molecular Biology, University of Chicago, 920 East 58th Street, Chicago, Illinois 60637,[1] and Department of Biochemistry, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35294[2]

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In *Escherichia coli* colonies, patterns of differential gene expression can be visualized by the use of Mu d(*lac*) fusion elements. Here we report that patterned β-galactosidase expression in colonies of strain MS1534 resulted from a novel mechanism, spatially localized replication of the Mu dII1681 element causing *lacZ* transposition to active expression sites. Mu dII1681 replication did not occur constitutively with a fixed probability but was dependent on the growth history of the bacterial population. The bacteria in which Mu dII1681 replication and *lacZ* transposition had occurred could no longer form colonies. These results lead to several interesting conclusions about cellular differentiation during colony development and the influence of bacterial growth history on gene expression and genetic change.

Escherichia coli colonies, like those of most other bacteria, are organized structures characterized by specific zones of differential biochemical activity, cell morphology, and multicellular aggregation (22, 26). Colony morphogenesis on laboratory media is but one of many cases in which bacteria form highly structured multicellular communities (27). How natural the multicellular mode of existence on a surface is for *E. coli* can be appreciated by considering the attachment of enteropathogenic strains to intestinal epithelia (18). Thus, understanding how colonies develop is relevant to a full appreciation of *E. coli* biology and, in particular, to the control of gene expression in situations such as symbiosis and pathogenesis.

A convenient way to visualize patterns of differential gene expression in *E. coli* colonies has been to grow strains with Mu d(*lac*) fusion elements (5) on XGal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) indicator agar (21, 22). We recently described results on pattern heredity in *E. coli* K-12 cultures descended from strain MS1534 that expressed β-galactosidase activity due to the presence of a transposable Mu dII1681 element in the chromosome. In this lineage, a variety of heredity changes, including Mu d(*lac*) transpositions, could alter β-galactosidase expression patterns (29). Control of *lacZ* expression in colonies of the MS1534 lineage was puzzling because it appeared to be sensitive to regulation of Mu functions. In particular, we found that the presence of a Mu c^+pAp1 prophage on a plasmid blocked β-galactosidase synthesis in MS1534 colonies. This contrasted with the behavior of Mu d(*lac*) elements in other systems, where Mu repression showed no effect (30). The experiments reported below clarified this situation by demonstrating a novel mechanism of *lacZ* expression in colonies of the MS1534 lineage. This mechanism depended on transposition of *lacZ* sequences to create active fusions during replication of the Mu d(*lac*) element. Mu d(*lac*) replication destroyed the colony-forming ability of the cells in which it occurred. Thus, colony zones with a $LacZ^+$ phenotype were composed of viable and nonviable cells. Analysis of bacteria from sectors with enhanced levels of β-galactosidase expression showed that Mu dII1681 replication was not constitutive but occurred only under certain growth conditions.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All the bacterial strains studied were derived from strain M7124 F^- *thi* Δ(*argF-lac*)*U169*. The history of MS1534 and its subclones has been described before (29). λ507 has no *lac* sequences, antibiotic resistance markers, Mu termini, or λ integration functions but expresses Mu A and B activities (3). Mini-Tn*10* insertions into the genomes of MS1534 subclones were isolated by infecting with λ1098 and selecting for Tc^r colonies. λ1098 lacks λ insertion functions, has a P amber mutation that blocks replication in M7124 descendants, and carries a mini-Tn*10* transposition system composed of a deleted transposon Tn*10* element unable to encode its own transposition functions but capable of being complemented in *cis* for insertion by an adjacent truncated IS*10* element with the "transposase" sequence under P_{tac} control (36). The following mutations were introduced into MS1534 subclones by P1 transduction after selection for the Tc^r marker of a linked Tn*10* element: *recA56*, *lexA3*, *hip-115*, *hip-157*, *himA42*, *hflA1*, and *hflB*. The *dam13*::Tn*9* mutation was introduced by P1 transduction and selection for the Cm^r marker of Tn*9*. The introduced markers were verified by testing Tc^r or Cm^r transductants for UV sensitivity (*recA*, *lexA*), growth of tester λ phages (*himA*, *hip*), or digestion of DNA by methylation-sensitive enzymes (*dam*). Because MS1534 cultures cultures can accumulate additional Mu dII1681 inserts which alter *lacZ* expression (29), all these transductions were performed with fresh subclonal cultures prepared from large single colonies on TYE (1% tryptone, 0.5% yeast extract, 0.5% NaCl) agar to ensure that parental-phenotype cultures with a single Mu d(*lac*) element were being infected. Plasmid pLP103-6-3 expresses Mu A and B products under the control of a weak kanamycin resistance promoter (33). It was introduced into MS1534::mini-Tn*10* derivatives by transformation, and β-galactosidase activity was screened on LB agar plates containing XGal (20 μg/ml).

Microbiologial methods. The methods used have been described in previous publications (22, 25). Incubations were carried out at 32°C. A special comment is required on the methods used to initiate colony development. Colonies can develop either from individual CFU or from multicellular inocula. We frequently used a spot inoculation procedure (placing a 1-μl drop containing about 10^5 bacteria on the agar surface) because it was a convenient way to obtain large colonies from different cultures at defined positions on a petri dish. For all the phenomena discussed in this paper, spot and single-CFU colonies showed equivalent physiological responses to genetic manipulations, such as the introduction of a particular mutation. Several environmental (and photographic) parameters affected the apparent intensity of XGal staining on photographs. Consequently, quantitative comparisons of β-galactosidase expression between colonies could only be made when these factors had been normalized by growing them side by side on the same petri dish.

DNA preparation and Southern hybridization. Bacterial growths from agar plates were sampled with toothpicks into Eppendorf tubes, stored frozen prior to extraction, and extracted by suspension in 0.025 M Tris (pH 8)–0.3 M sucrose–0.025 M EDTA buffer, followed by successive additions of lysozyme (to 2 mg/ml, 20 min on ice), sodium dodecyl sulfate (SDS) (to 0.1%), and proteinase K (to 7.5 μg/ml). Following prolonged proteinase digestion at 65°C, samples were deproteinized by phenol and phenol-chloroform extraction, ethanol precipitated, and suspended in 0.01 M Tris (pH 8)–0.001 M EDTA before digestion. Samples were digested with *Taq*I (New England BioLabs) in the recommended buffer at 55°C for several hours and electrophoresed in 1.5% agarose–TBE gels (30). The gels were acid-treated for 15 min in 0.25 N HCl, soaked in 0.5 N NaOH–1.5 M NaCl for 60 min, and capillary blotted onto Zeta-probe membranes in 0.4 N NaOH overnight. The following oligonucleotides were used for hybridization probes as previously described (29): IS1-20, a sequencing primer (catalog no. 1225), purchased from New England BioLabs; MuC-21, nucleotides 464 to 484 of the noncoding strand of the Mu repressor cistron; MuR-1a, the last 90 nucleotides of the right terminus of Mu (5′ at −90 from the end); MuA-22, nucleotides 2590 to 2568 of the noncoding strand of the Mu *A* cistron; and HU-4, 60 nucleotides from the coding sequence for amino acids 41 to 60 of *E. coli* Hu-1 protein (9). The positions of the Mu-specific probes are indicated on Fig. 8.

Colony hybridization. Colonies grown on agar plates were lifted onto filter paper and extracted by the procedure of Miller and Barnes (14) with a few minor modifications. Whatman no. 3 filter paper proved superior to thinner grades of paper. The dry filter was placed over the colonies, pressed onto a blank area of the agar surface to initiate blotting, and left for one to several hours until completely wetted and covering the colonies. On older XGal plates, the filters could then be peeled off and generally lifted the colonies cleanly. However, it proved to be advisable on fresher plates (especially those which did not contain XGal) to press down over the colonies with a gloved finger to remove all air bubbles and ensure adherence to the filter before peeling. If done carefully, this did not perturb the colony structure. Some sectors did not peel off the agar as well as the rest of the colony. Once peeled, the filters with the immobilized colonies could be left dry at room temperature for at least a week before alkali extraction without affecting the final results. Hybridizations were carried out in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 45°C.

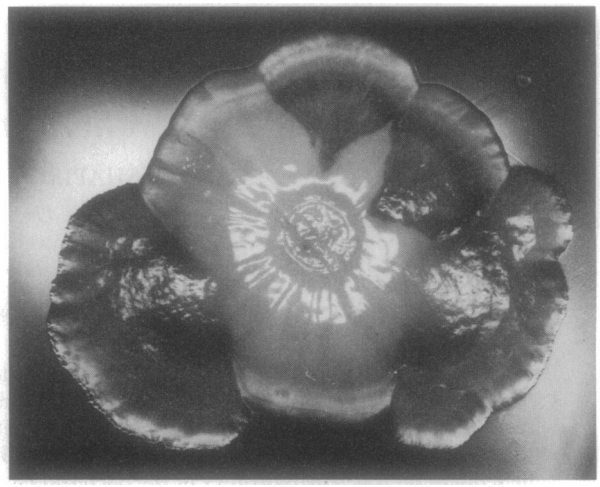


FIG. 1. Sectors on an MS1534 subclonal colony. This colony began development from a 1-μl spot inoculum containing about 10^5 CFU and was incubated on XGal indicator agar for 7 days. Note the repeat examples of two sectorial phenotypes. These phenotypes regularly appeared on colonies produced by this and related cultures. Other examples of related colonies producing dark sectors have been published (29). Magnification. ×4.1.

Four 1-h washes were performed in 4× SSC–0.1% SDS at 49°C for filters hybridized with MuA-22, MuC-21, and IS1-20 and in 0.2× SSC–0.1% SDS at 63°C for filters hybridized with MuR-1a and HU-4. Filters were stripped for rehybridization by washing in 0.2× SSC–0.1% SDS above the T_m of the particular oligonucleotide. For autoradiography, the film was placed over the colony-bearing side of the filter. The IS*1* control probes did show some variability in hybridization to different regions of the colonies, indicating that DNA extraction was not completely uniform across the colonies. Since we know that colonies are heterogeneous structures (24, 26), variations in DNA recovery on the filters were to be expected.

Photography and microscopy. Colony photography procedures with a 35-mm camera fitted with a macro lens have been described before (23).

RESULTS

Sectorial changes in growth and β-galactosidase expression. One regular feature of the spot colonies produced by MS1534 subclones harboring a single Mu dII1681 insert was the appearance, after several days of incubation, of various kinds of sectors displaying novel LacZ and growth phenotypes (Fig. 1). Among the most visually striking sector types were expansive sectors which grew beyond the colony perimeter and showed higher levels of β-galactosidase activity. We examined bacteria from the parental colonies and from the sectors in two ways: by plating them on Xgal indicator agar to determine their colony patterns and by extracting their DNA for Southern analysis of Mu dII1681 sequences.

When bacteria from the middle of expansive dark sectors were suspended, diluted, and replated on Xgal agar, colonies with two phenotypes appeared. Some resembled colonies produced by the parental culture, and some displayed a new darker-staining phenotype related to that of the sector from which they were isolated (Fig. 2). These fields of colonies were mixed, but a large number of them expressed a reproducible new phenotype, thereby showing that some

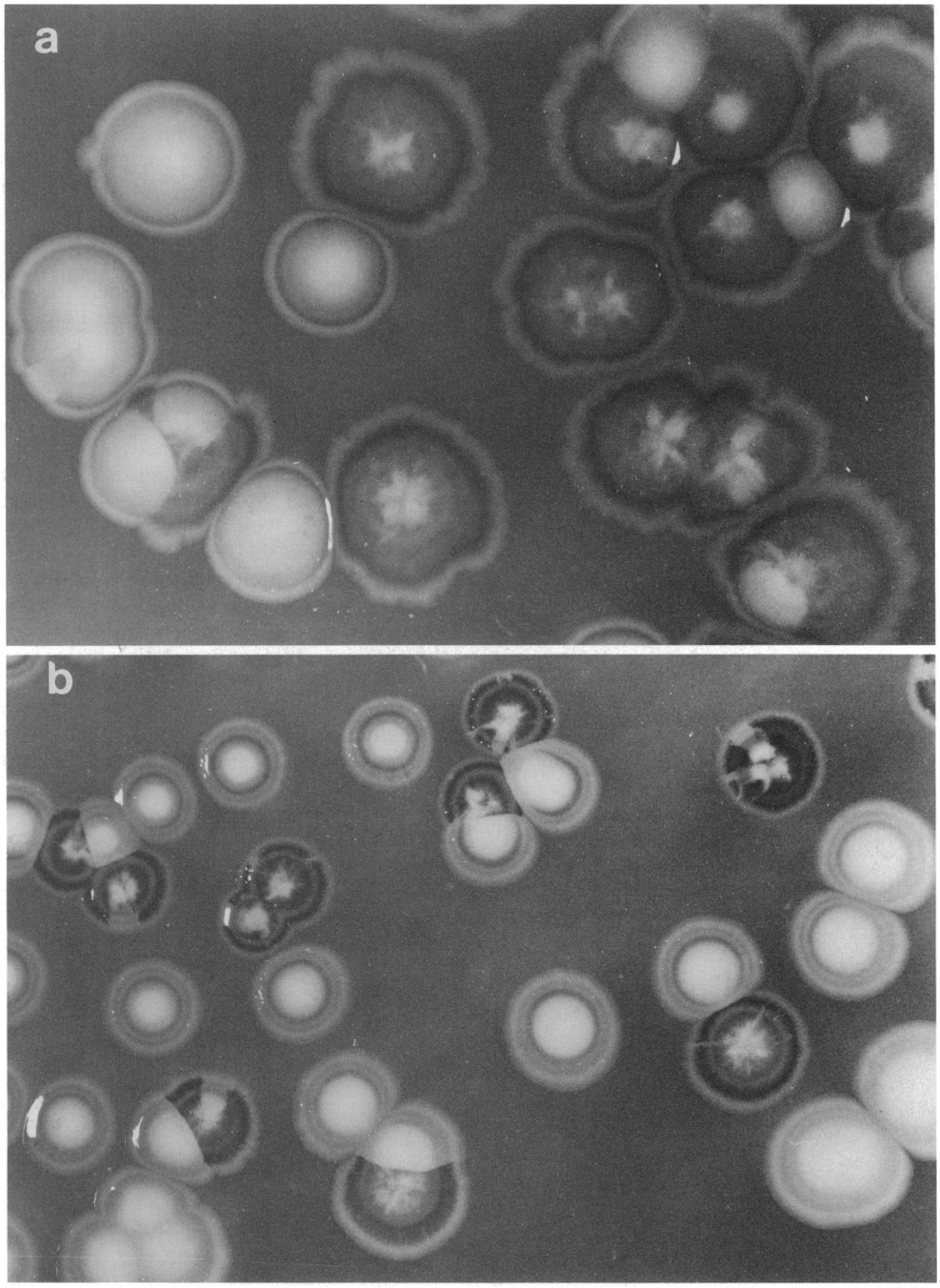


FIG. 2. Colonies produced by the individual CFU in dark expansive sectors. Sectors 5241 (a) and 5213 (b) were picked into TYE broth, vortexed, diluted 10^{-6}-fold, and immediately plated on Xgal indicator. Sector 5241 had the phenotype of the 4 and 7 o'clock sectors in Fig. 1, while sector 5213 resembled the 12 and 2 o'clock sectors on the same colony. Note that all the darker or more expansive colonies contained numerous lighter sectors. These photos were taken after 6 days of incubation. Magnification, ×4.1.

kind of hereditary change had occurred in the progenitor of each sector, leading to new growth and β-galactosidase expression patterns.

Subcloning analysis had already shown that the novel sectorial phenotype was inherited in an unstable manner (29), and this insability could also be seen in colonies on Xgal agar expressing a dark expansive-growth phenotype; they all contained multiple internal lighter-staining sectors which were composed of bacteria expressing the parental phenotype (Fig. 2). This hereditary instability appeared to be the result of a large sequence duplication that reverted at high frequency by homologous recombination, as observed with *Salmonella typhimurium* clones selected for growth on low concentrations of particular carbon sources (31a). Mu-free *E. coli* K-12 strains growing on glucose-minimal agar regularly produced sectors which had a duplication that covered at least the 42- to 55-min interval of the chromosome, and these sectors contained bacteria expressing an unstable

TABLE 1. Genealogy of cultures subjected to DNA analysis[a]

Parental colony	Dark expansive sector	Single-colony subclone from sector	
		Parental	Sectorial
521	5211	5211L1 and -L2	5211S1 and -S2
524	5241	5241L1 and -L2	5241S1 and -S2
622	6221	6221L1 and -L2	6221S1 and -S2

[a] Each row gives a pedigree of derivatives from a different parental culture. Colonies 521, 524, and 622 resembled the colony in Fig. 1. Samples for DNA analysis were picked from central regions uncontaminated by sectors. Sectors 5211, 5241, and 6221 appeared on these colonies and were similar to the sectors at 4 o'clock and 7 o'clock in Fig. 1. Samples for DNA analysis were picked from the middle of each sector. These same samples were also suspended in TYE broth, diluted, and plated on TYE agar to give isolated colonies, which were picked to establish the single-colony subclones. The labeling of subclones as either L (limited parental phenotype) or S (sectorial expansive phenotype) is explained in the text, in the legend to Fig. 3, and in reference 29. These two phenotypes on Xgal agar can be seen in Fig. 2a and Fig. 3.

expansive growth phenotype similar to that of bacteria from MS1534 sectors (R. V. Sonti, personal communication). In addition, formation of the putative MS1534 duplication appeared to require homologous recombination, as observed for similar *Salmonella* duplications (31a; R. V. Sonti, personal communication). When the *recA56* mutation was transduced into MS1534 subclones expressing the parental phenotype, no expansive dark sectors appeared on the colonies after prolonged incubation (but such sectors reappeared when *recA*$^+$ was reintroduced into the strain).

In order to understand better the molecular basis of the different LacZ phenotypes, DNA analysis was applied to cell populations from the original sectoring colonies as well as to cultures derived from individual bacteria in the sectors. The basic idea was to examine any changes that might have affected the Mu dII1681 elements when DNA from parental bacteria was compared with DNA from bacteria in expansive dark sectors and from bacteria descended from individual cells in the sectors. Thus, it was necessary to keep track of each culture's ancestry prior to DNA extraction. This was done by adding a digit or letter plus digit to the culture name each time a new clone was selected (29). The nomenclature of the samples subjected to Southern analysis of Mu dII1681 sequences is summarized in Table 1 and described below.

(i) Cultures 521, 524, and 622 represented parental phenotype colonies which sported expansive dark sectors. Samples for DNA extraction were taken from colony centers after 10 days of incubation. Bacteria were carefully picked to avoid contamination with bacteria from the sectors.

(ii) Samples 5211, 5241, and 6221 were each picked from the middle of an expansive dark sector on colonies 521, 524, and 622, respectively.

(iii) Cultures 5211L1, 5211S1, 5241L1, etc., were subclonal cultures, each derived from a single colony obtained by diluting and plating a suspension of dark expansive sector 5211, 5241, or 6221. As we have described previously (29), the colonies obtained by plating the sector populations on TYE agar fell into two classes, L and S. The L colonies gave rise to parental, limited (L) colonies when picked into XGal indicator agar, and the S colonies gave rise to sectorial (S) expansive colonies (Fig. 3). As will be shown below, both

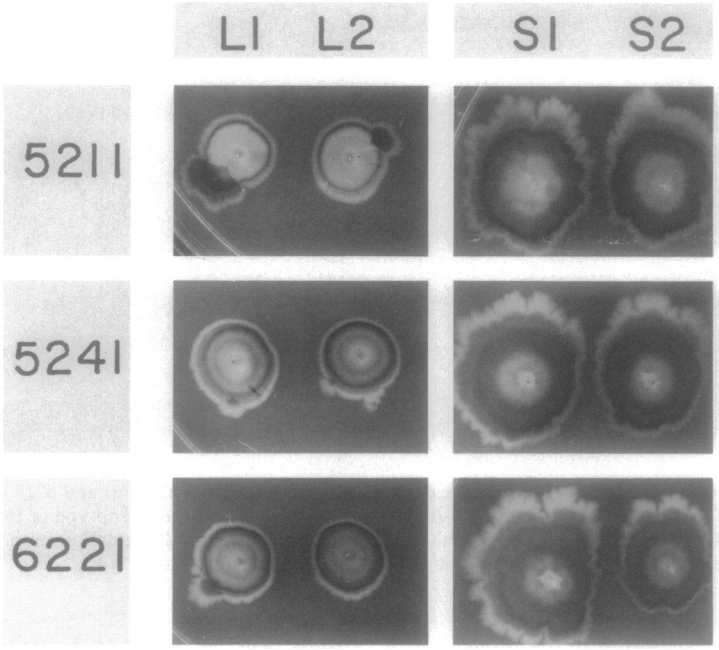


FIG. 3. Colonies produced by sectorial subclones from toothpick inoculations into XGal indicator agar. The same bacteria from sectors 5211, 5241, and 6221 used for DNA extractions (2nd, 4th, and 6th lanes in Fig. 4) were diluted and plated on TYE agar. After overnight incubation, duplicate large (L1 and L2) and small (S1 and S2) colonies were picked with sterile toothpicks and each was stabbed twice, into TYE agar and into XGal indicator agar. It is important at this point to bear in mind that the L and S phenotypes were developmental in nature and manifested themselves differently depending on the growth conditions (29); large (L) overnight TYE colonies produced limited (L) colonies when replated on XGal indicator agar, and small (S) overnight TYE colonies produced expansive sectorial (S) colonies when replated on XGal indicator agar. The two types of colonies were indistinguishable after 40 h of growth on TYE agar (29). The TYE agar stab colonies were harvested after overnight growth for DNA extraction (last six lanes in Fig. 4). The XGal agar stab colonies were incubated for 12 days, photographed as shown here, and then harvested for DNA extraction (Fig. 5). Magnification, ×1.

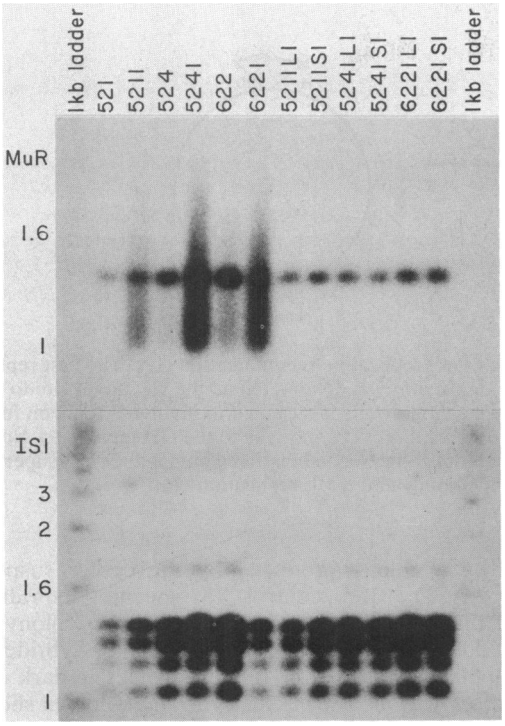


FIG. 4. Southern hybridization of DNA extracted from colony centers, sectors, and sectorial subclones. The various DNA samples were prepared and extracted as described in the text and the legend to Fig. 3. These samples were digested to completion with *Taq*I endonuclease, electrophoresed through a 1.5% agarose gel, and capillary blotted onto a Zeta-probe membrane. This membrane was then hybridized with three ^{32}P-labeled oligonucleotides: MuR-1a (Mu *R*, top panel); IS1-20 (IS*1*, bottom panel); and MuC-21 (data not shown). The 1-kb ladder (Bethesda Research Laboratories) was used as a size standard, and the positions of the 1-, 1.6-, and 2-kb fragments are indicated on the left. The filters shown here were exposed for 16 h. A 6-day exposure of the Mu *R*-hybridized filter showed smears of replicated Mu dII1681 in the first six lanes but only the single band in the last six lanes.

cell types carried the single original Mu dII1681 element, but the regulation of this element during colony development was different in the S-type cells.

Southern hybridization analysis of Mu d(*lac*) replication in MS1534 colonies and expansive sectors. In order to establish the correlations between these different growth and LacZ phenotypes and the behavior of Mu dII1681 sequences, DNA samples were extracted from bacterial masses picked directly off agar plates. The 521, 524, and 622 and 5211, 5241, and 6221 masses were picked off XGal indicator agar after 10 days of incubation. Masses of the 5211L, 5221S, 5241L, 5241S, 6221L, and 6221S cultures were picked off TYE agar after overnight incubation and off XGal indicator agar after 12 days of incubation. In this way, it was possible to determine whether different growth regimens affected the Mu dII1681 elements. The results of this analysis were somewhat surprising. As explained below, the status of Mu dII1681 was different depending on whether it came from cultures with the sectorial growth phenotype or from cultures with the parental growth phenotype. In addition, the status of Mu dII1681 DNA from the sectorial cultures depended on their growth history; evidence of replication was seen after prolonged growth on XGal indicator agar but not after overnight growth on TYE agar. The growth dependence of Mu dII1681 replication in the sectorial cultures reflected the developmental nature of Mu dII1681 regulation in colonies and is analyzed further in the Discussion.

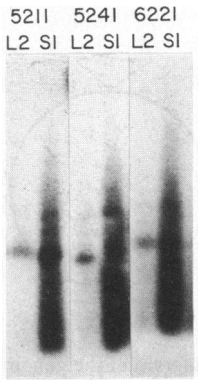


FIG. 5. Southern hybridization of DNA extracted from 12-day XGal indicator agar colonies of sectorial subclones. DNA was digested, electrophoresed, blotted, and hybridized with the MuR-1a probe as explained in the legend to Fig. 4.

All these DNA samples were digested with *Taq*I, electrophoresed, blotted, and hybridized with probes specific for Mu and IS*1* sequences (Fig. 4 and 5). With DNA from expansive dark sectors, the MuR-1a probe revealed that Mu sequences were amplified and contained many novel junction fragments, forming a continuous smear of hybridization. With DNA from the colony centers and from all the L and S subclones grown overnight on TYE agar, hybridization revealed predominantly the original junction fragment. Prolonged exposure of the autoradiograms showed that novel junction fragments were present (albeit in lower abundance) in the DNA from the 10-day-old colony centers but were not detectable in the DNA from the overnight subclones. The size range of the new junction fragments had an indefinite upper boundary but a sharp lower boundary just below the 1 kilobase (kb) size standard. This indicated that the lower size limit of the junction fragments was determined by the distance from the first *Taq*I site in *lacZ* to the Mu terminus (935 base pairs [bp]; see Fig. 8). This hybridization pattern, typified by maintenance of the original junction fragment together with the appearance of many new junction fragments producing a hybridization smear in Southern blots, is a hallmark of prophages undergoing replicative recombination (Fig. 6) (11, 13, 19). The amplification of Mu sequences in the sectorial samples was confirmed by hybridization with the MuC-21 probe, complementary to an internal Mu dII1681 *Taq*I fragment in the Mu repressor cistron (data not shown). Hybridization with the IS1-20 probe showed that rearrangement and amplification were not observed for other sequences in the genomes of bacteria from the sectors.

The results obtained with DNA from the L and S subclonal cultures were particularly interesting. No new junction fragments were detectable after growth overnight on TYE agar (Fig. 4), but samples taken after 12 days on Xgal indicator agar gave results similar to those for DNA from colony centers and from dark expansive sectors; the parental-type L cultures showed low levels of Mu dII1681 replication, and the sectorial-type S cultures displayed the abundant smears of junction fragments diagnostic for Mu dII1681 replication (Fig. 5). These results meant that the S bacteria in the sectors had not inherited additional Mu dII1681 copies but rather had acquired an enhanced propensity for Mu dII1681 replication during growth on XGal indicator agar.

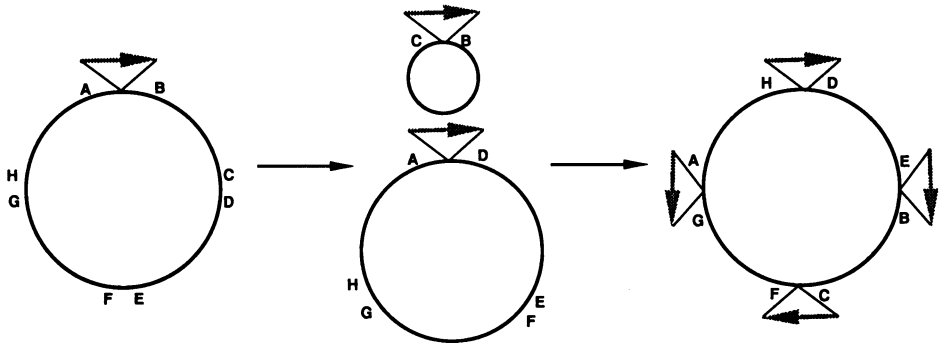


FIG. 6. Schematic diagram of the genomic consequences of two rounds of Mu d(*lac*) replicative recombination (19). The first replication event is assumed to involve recombination between the Mu d(*lac*) element at the A-Mu d(*lac*)-B donor site and the CD target site to give an adjacent deletion and excision of a Mu d(*lac*)-B-C circle. The second round of replication events is assumed to involve a replicon fusion of the Mu d(*lac*)-B-C circle into the EF target site and an adjacent inversion from the A-Mu d(*lac*)-D donor site to the GH target site. Note how the linear order of the genome has been scrambled. The knotting and catenation that replicative recombination would induce in a supertwisted genome explain the increased sedimentation of nucleoids extracted from cells undergoing active Mu replication (16).

Because replication was systematically more extensive in the S colonies than in the L colonies, it was not simply the nonspecific consequence of bacterial ageing after prolonged incubation. Some aspect of hereditary control must have been involved.

These observations demonstrated a correlation between increased *lacZ* expression and Mu dII1681 replication. This connection provided a logical explanation for the inhibitory effects on *lacZ* expression of additional Mu elements in the genomes of MS1534 descendants. If the original chromosomal Mu dII1681 insert in MS1534 (linked to *thyA*) did not express *lacZ* from adjacent transcription and translation signals, then all *lacZ* expression in MS1534 descendants would actually be due to new fusions resulting from Mu dII1681 replicative recombination. A Mu c^+pAp1 element in the genome would inhibit Mu dII1681 replication by increasing stable repressor levels and thus block *lacZ* expression (29).

Colony hybridization analysis of Mu d(*lac*) amplification. In order to explore further the notion that Mu dII1681 replication caused *lacZ* expression, we examined the relative spatial abundance of Mu sequences in colonies produced by MS1534 descendants. Intact colonies were immobilized on Whatman no. 3 filter paper, lysed in situ by alkali, neutralized, and probed with oligonucleotides for Mu or IS*1* sequences. As the filter shown in Fig. 7a illustrates, colony structure was very well preserved by this procedure. There was a definite correlation between the XGal staining and the Mu probe hybridization patterns. The IS*1* hybridization patterns showed reasonably uniform levels of IS*1* sequence abundance in regions containing sectors which were clearly distinguished by both XGal and the Mu probe. Thus, these results confirmed the expectation that amplification of Mu dII1681 sequences would coincide spatially with zones of more intense *lacZ* expression.

Filter hybridization of colonies produced by plating bacteria suspended from an expansive sector and from the central region of the parent colony gave similar results (Fig. 7b). The sectorial suspension produced a mixture consisting of a majority of expansive colonies with greater β-galactosidase activity and a minority of parental-type colonies, whereas the center suspension produced only parental-type colonies with an occasional expansive dark sector. The Mu-specific hybridization clearly distinguished between the two colony types and even revealed two expansive dark sectors on a colony produced by the center suspension (arrow). Again, the control IS*1*-specific hybridization showed that the difference between the two colony types was not due to differential DNA extraction or hybridization. The amplification of Mu dII1681 sequences in dark expansive sectors was also seen clearly in the colonies shown in Fig. 7c.

The colony hybridization method made it possible to show that XGal in the agar was not involved in β-galactosidase expression and Mu d(*lac*) derepression. Colonies produced by MS1534 subclonal cultures that expressed different levels of β-galactosidase on XGal indicator agar still showed the appropriate differential hybridization results with a Mu-specific probe after growth on agar free of XGal (data not shown). Control hybridizations with a probe for HU coding sequences were more uniform.

Genetic analysis of the relationship between Mu d(*lac*) replication and β-galactosidase expression. The Southern and colony blots established a correlation between increased *lacZ* expression and Mu dII1681 replication and suggested a logical explanation for that correlation. To prove that Mu dII1681 replication was in fact necessary for *lacZ* expression, two kinds of genetic experiments were performed. The first was to transduce MS1534 subclones with transposon-linked mutations affecting functions involved in DNA metabolism and Mu regulation. Mutations in the *recA*, *lexA*, *hflB*, and *dam* loci (not required for Mu replicative recombination) did not prevent β-galactosidase expression; the *hflA1* mutation reduced β-galactosidase expression and gave pale colonies on Xgal agar; and mutations in the *himA* and *hip* loci (essential for Mu replicative recombination) completely blocked β-galactosidase expression and led to the formation of white colonies on XGal agar. The latter two classes of mutations created deficiencies in the IHF protein, which is required for Mu gene expression and replication (12, 17).

The second kind of genetic experiment was to isolate mini-Tn*10* insertions into the MS1534 genome and screen for those which affected β-galactosidase expression. Three insertions gave white colonies on XGal indicator agar and were 100% linked to the Km^r marker of Mu dII1681 in P1 transduction tests. These represented mini-Tn*10* insertions into the Mu dII1681 element. Two of the insertion derivatives, MS2095 and MS2098, showed increased survival at 42°C, and one, MS2108, remained thermosensitive. The loss

of thermosensitivity by MS2095 and MS2098 suggested that the mini-Tn*10* insertions in those strains had interrupted a sequence needed for lethal derepression of Mu dII1681 replication at 42°C and that the same block to replication accounted for the LacZ$^-$ phenotype. This conclusion was tested by providing Mu replication functions in *trans*. Complementation of MS2095 and MS2098 with either the pLP103-6-3 plasmid or the λ507 phage expressing Mu A and B functions restored β-galactosidase expression, demonstrating that these insertions affected Mu replication functions and not *lacZ* sequences. Complementation of MS2108 for Mu A and B did not restore β-galactosidase expression. The location of the mini-Tn*10* insertion in the Mu AB region of strain MS2098 was confirmed by Southern blotting, which showed an increase in the size of the *Hin*dIII fragment hybridizing with the MuA-22 probe (Fig. 8). More precise mapping of the insert was achieved by cloning the *Bam*HI fragment carrying the left end of the Mu dII1681::mini-Tn*10* element in MS2098. Digestion of the cloned fragment with *Hin*cII, *Hpa*I, and *Hin*dIII placed the mini-Tn*10* element at coordinate 2.0 in the *A* cistron on the Mu dII1681 map (Fig. 8).

DISCUSSION

The results presented above have made it possible to identify Mu dII1681 replication and consequent *lacZ* transposition as the molecular events underlying patterned β-galactosidase expression in MS1534 colonies. The observations established a correlation between β-galactosidase synthesis and Mu dII1681 replication, demonstrated that Mu transposition and replication functions were required for *lacZ* expression, and showed that elevated levels of Mu dII1681 replication in sectorial cultures was dependent on growth history. Considered together with earlier results (20, 26), these data lead to interesting corollary conclusions about the regulation of Mu dII1681 replication during growth on agar, the heterogeneous cellular composition of phenotypically different colony zones, and the effect of growth history on genome rearrangements.

Mu dII1681 replication was the molecular basis for patterned β-galactosidase expression in MS1534 colonies. Because three different genetic blocks to Mu dII1681 replication prevented the appearance of β-galactosidase activity, it is clear that Mu dII1681 replication was responsible for *lacZ* expression in MS1534 colonies. These blocks included the inhibitory effect of a Mu c^+pAp1 element (29), defects in IHF activity resulting from *himA* or *hip* mutations, and mini-Tn*10* insertion into the Mu *A* cistron (Fig. 8). These data demonstrated that *lacZ* was not connected to functional transcription and translation signals at its original location. Since replication involved the joining of each Mu dII1681 extremity to many new sequences in the MS1534 genome (Fig. 6) (13, 19), the resulting *lacZ* transpositions created a variety of new genetic fusions. MS1534 is probably not unique in showing replication-dependent *lacZ* expression from a transposable Mu d(*lac*) element. We previously reported that β-galactosidase synthesis directed by an R388::Mu dII1681 plasmid was repressed by a Mu c^+pAp1 prophage in the chromosome (28), and this result indicated that Mu dII1681 replication was likely to be the basis for *lacZ* expression in that case as well.

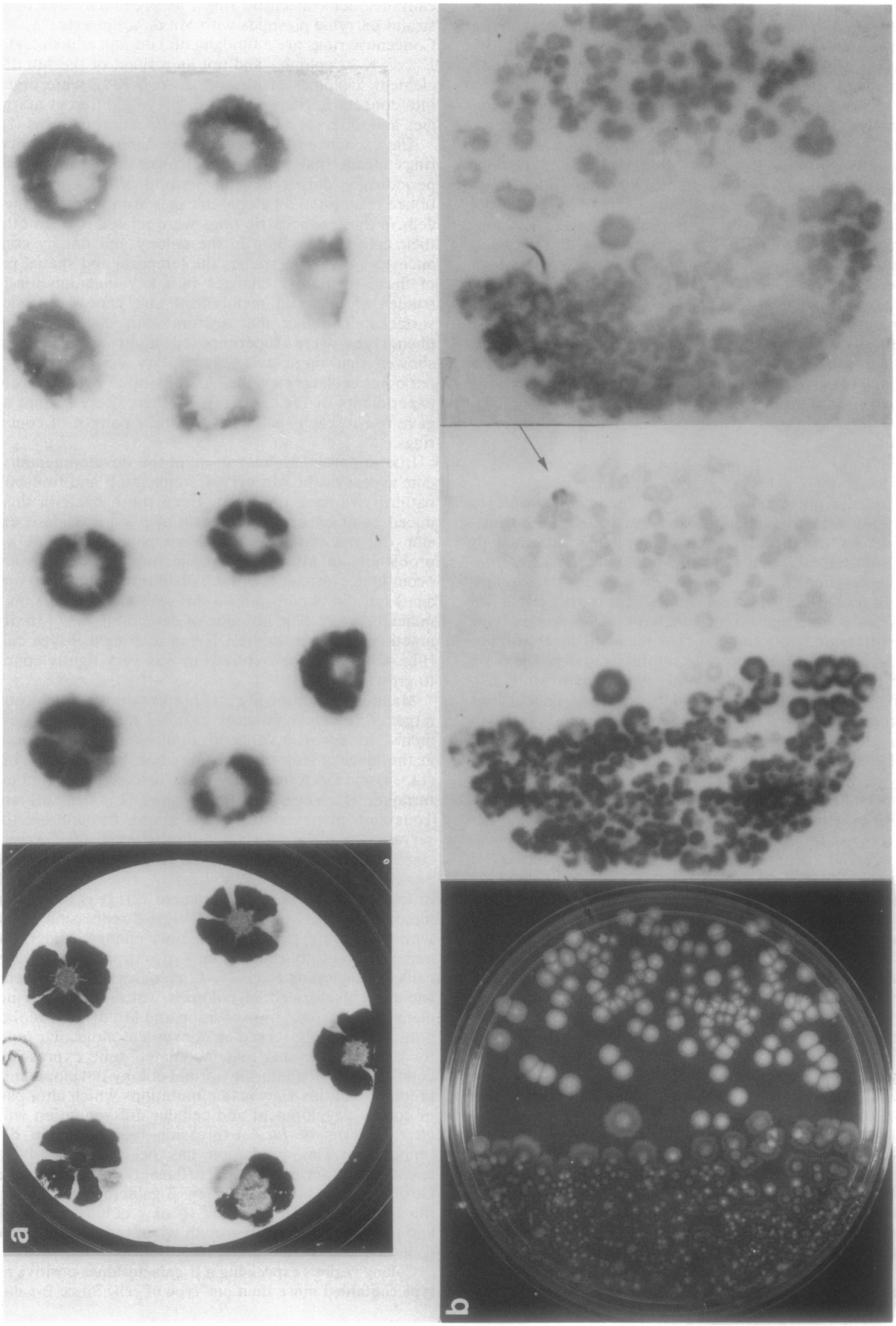
Colony patterns reflected differential activity of Mu dII1681 replication and transposition functions during colony development. The basic pattern of *lacZ* expression and Mu dII1681 replication in MS1534 colonies was a series of phenotypically distinct concentric rings, as previously described for strains carrying plasmids with Mu d(*lac*) inserts (21, 22, 28). Concentric rings are a fundamental organizational feature of *E. coli* K-12 colonies and not an artifact of the Mu dII1681 element. Colonies of Mu-free *E. coli* K-12 were organized into concentric rings of cells displaying different morphologies and different multicellular aggregation patterns (26).

The existence of phenotypically differentiated concentric rings meant that regulatory changes must have occurred periodically during development to affect similarly positioned bacteria of all clonal lineages around the colony. The cells in these concentric rings were related to each other by their common position in the colony and not by common ancestry. What determines the temporal and spatial pattern of these regulatory changes is a key question for future studies of bacterial metabolism and gene expression on surfaces. The fact that sectors with distinct concentric phenotypes were superimposed upon the ring patterns showed that there was a hereditary component to these periodic regulatory events. As illustrated by the subcloning experiments in Fig. 2 and 3, the hereditary change which gave rise to each sector led to a new pattern of concentric rings.

It is important to bear in mind the developmentally specific aspect of the Mu dII1681 replication and transposition patterns we have observed. Even those bacteria that produced colonies with high levels of β-galactosidase expression did not have a constitutive phenotype with a fixed probability of Mu dII1681 replication. Replication did not occur under one set of growth conditions (overnight on TYE agar) but did occur under a different set (12 days on XGal indicator agar). The absence of detectable new Mu dII1681 junction fragments in the DNA of overnight S-type colonies (Fig. 4) showed that replication was very tightly connected to growth history.

Maintenance of the Mu *c*ts62 repressor was the most likely target of periodic regulatory changes during colony development. Mu repressor concentration is known to be controlled at the level of transcription initiation by repressor binding (12, 34) and DNA topology (8) and also at the level of mRNA turnover (J. Vogel, N. P. Higgins, L. Desmet, and A. Toussaint, manuscript in preparation). In addition, the observation that the *hflA1* mutation lowered *lacZ* expression suggested that the product of this locus participated in proteolytic repressor destabilization, consistent with its role in the regulation of λ development (2). It is likely that the molecular events affecting Mu *c*ts62 repression coincided with some of the periodic regulatory changes underlying the appearance of concentric rings of morphologically distinct cells in developing *E. coli* K-12 colonies (26). Since concentric patterns formed in colonies which contained no Mu elements (26), *lacZ* transposition and Mu dII1681 replication patterns probably served as convenient molecular reporters for regulatory events that modulated gene expression and cellular physiology during normal colony development. One prediction of this view is that mutations which alter patterns of colony development and cellular differentiation will also alter patterns of *lacZ* expression based on Mu dII1681 replication. This prediction has been confirmed by the isolation of a series of mini-Tn*10* insertions into the MS1534 chromosome which led to new β-galactosidase patterns in the MS1534 background and to new colony morphologies and new patterns of cell growth when transduced into other genetic backgrounds (unpublished observations).

Colony regions expressing a β-galactosidase-positive phenotype contained more than one type of cell. Since β-galactosi-


a
b

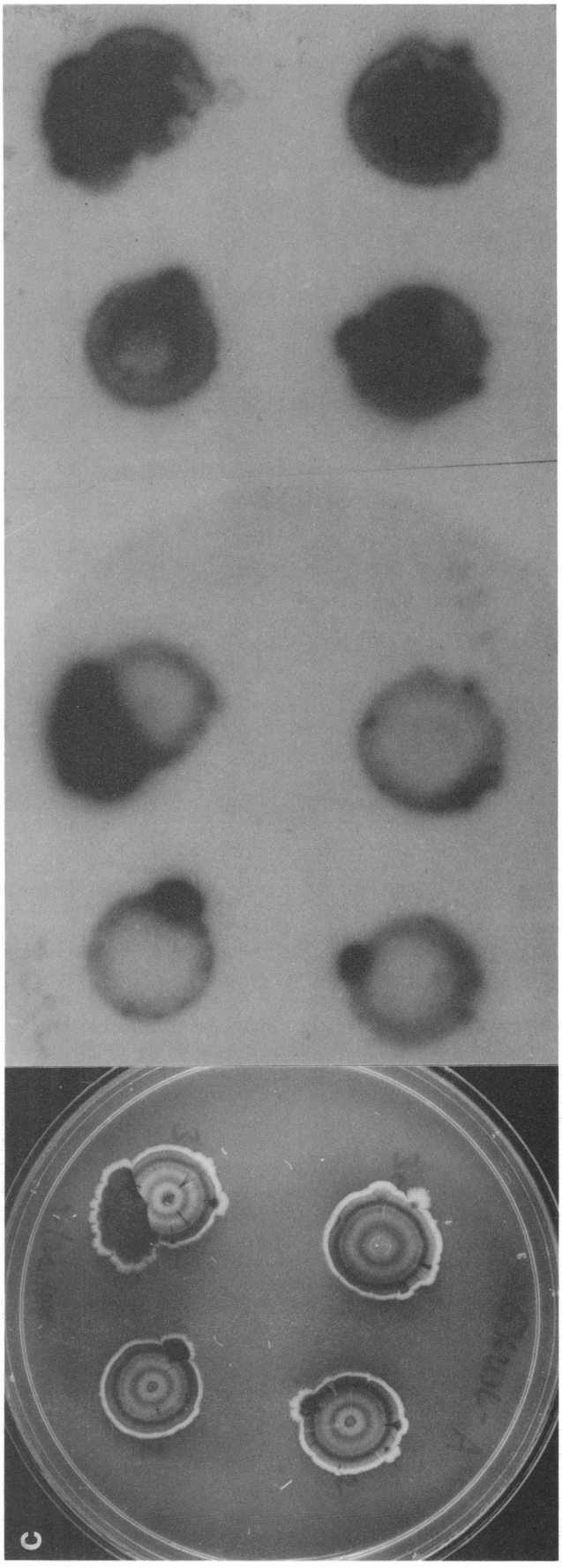


FIG. 7. Correlation between XGal staining and Mu-specific hybridization in colonies immobilized on filter paper. Colonies grown on XGal indicator agar were lifted onto filters of Whatman no. 3 paper, extracted with NaOH, neutralized, and then hybridized with probe MuC-21 (center) or IS1-20 (right). (a and b) The initial hybridization used the Mu probe, which was then stripped, and the second hybridization used the IS*1* probe. (c) The IS*1* probe was used first and then stripped to permit use of the Mu probe. (a) Colonies produced by spotting subcultures of a suspended dark expansive sector. The left photograph shows the colonies immobilized on a 7-cm filter 25 days after inoculation. Note the lighter-staining sectors. The very edges of these colonies were not stained by XGal and are not visible against the filter paper background but can be seen in the IS*1* hybridization. (b) Colonies produced by plating 10^{-6} dilutions of bacteria picked from the colony center (right) or from a dark expansive sector (left). The photo at the left shows the original petri dish after 5 days of incubation. The colonies were lifted onto filter paper when the plate was 37 days old. The arrows indicate two small dark sectors on a colony at the edge of the center field. (c) Colonies containing dark expansive sectors produced by spotting parental-type cultures. The photo on the left shows the original petri dish. Photographs and filters were prepared after 6 days of incubation.

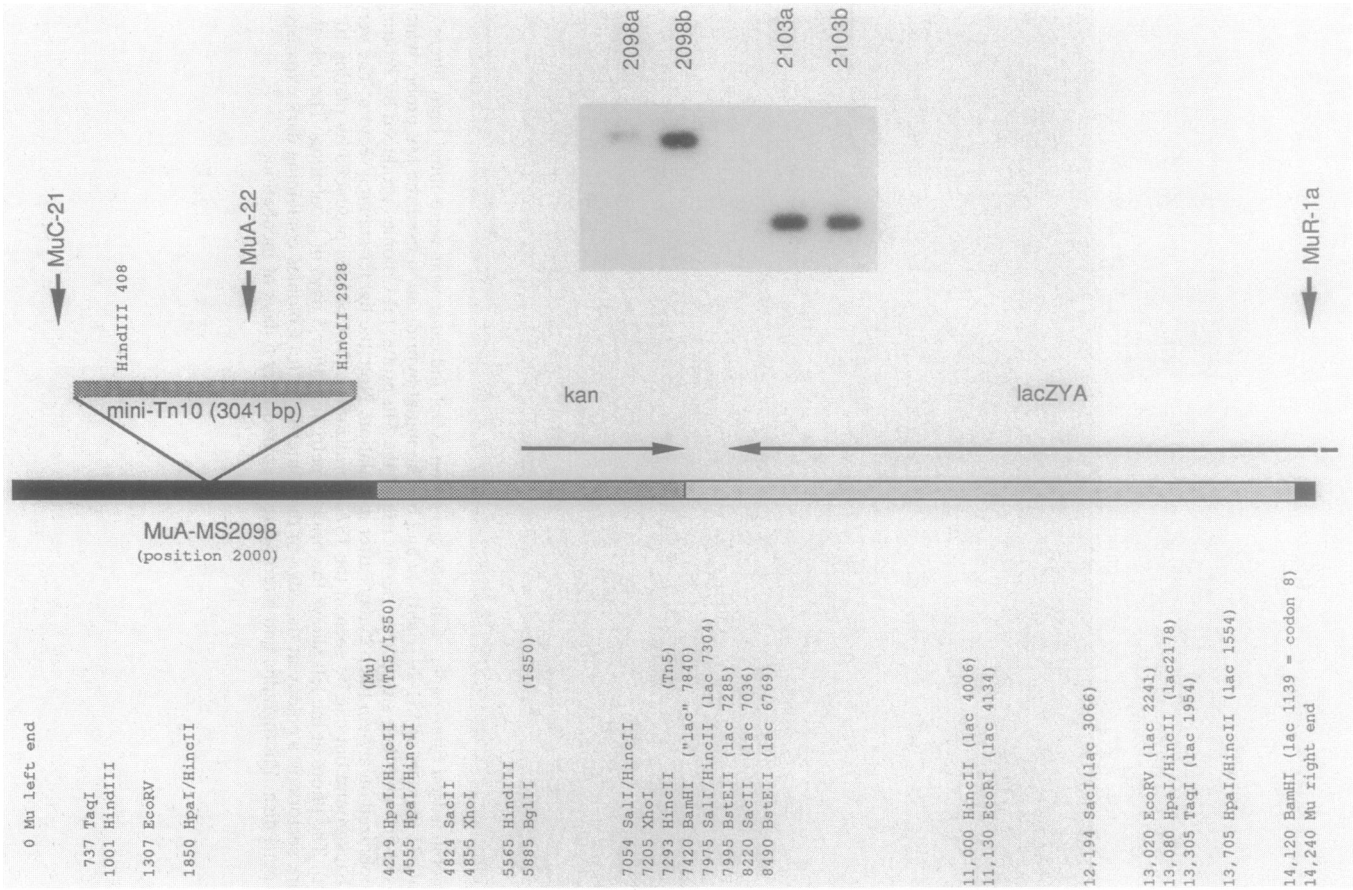


FIG. 8. Mini-Tn*10* insertion in the Mu *A* cistron of strain MS2098. The drawing shows the Mu dII1681 map from Shapiro and Higgins (29) plus the mini-Tn*10* insert detected in strain MS2098. The heavy arrows indicate the positions of the three Mu-specific oligonucleotide probes used in this study. The Southern hybridization shows a *Hin*dIII digest of total DNA from duplicate subclones of strains MS2098 and MS2103 probed with the MuA-22 oligonucleotide. Strain MS2103 carries a mini-Tn*10* insertion unlinked to the Mu dII1681 element and so produces the parental 4.6-kb *Hin*dIII fragment between coordinates 1001 and 5565 on the Mu dII1681 map. Strain MS2098 produces the hybrid 6.2-kb *Hin*dIII fragment extending from coordinate 408 of mini-Tn*10* (36) to coordinate 5565 of Mu dII1681. The position of the mini-Tn*10* insertion was confirmed from a cloned *Bam*HI fragment by noting the absence in *Hpa*I digests of the *Hpa*I-*Hin*cII fragment (1850 to 4219) of Mu dII1681 and detecting the hybrid 2.3-kb *Hin*cII fragment extending from coordinate 2928 of mini-Tn*10* to coordinate 4219 of Mu dII1681. Not all the *Hin*cII sites of the mini-Tn*10* are shown.

dase synthesis in MS1534 colonies depended upon Mu dII1681 replication, the dark sectors and rings contained cells with transposed *lacZ* sequences connected to many different regions of the chromosome. Direct evidence of this was seen in the Southern blots (Fig. 4 and 5). Did all of the bacteria in these zones have new *lacZ* fusions, or was the β-galactosidase phenotype the result of expression by a distinct subpopulation of cells? Sampling and replating experiments showed that sectors and rings contained more than one cell type and indicated that β-galactosidase synthesis occurred in cells which were no longer colony-forming units. When bacteria were picked from dark rings on parental MS1534 colonies and streaked on XGal indicator agar, only parental colony phenotypes were observed (29). Similarly, when expansive dark sectors were resuspended, diluted, and plated, two colony types were observed (Fig. 2 and 3) (29). Of the CFU from the expansive dark sectors, neither type contained new Mu dII1681 junction fragments (Fig. 4). Thus, no colony formers with transposed *lacZ* sequences were isolated from dark rings or sectors. This meant either that the cells which had undergone Mu dII1681 replication, if viable, were such a small minority of the total population that they could not be detected by these plating tests or that these cells had lost their ability to form colonies. The former possibility could be excluded by quantitative analysis. Densitometry of junction fragments in bacteria from dark sectors gave a figure of 8 to 13 new junctions per genome equivalent, indicating that a minority component (<10%) of viable cells with active fusions would each carry over 80 new Mu d(*lac*) insertions. Moreover, microscopy of bacteria resuspended from a dark sector showed that at least 5% of the cells stained blue. Thus, CFU with new junctions would have been detected in our platings. Since they were not observed, Mu dII1681 replication must have been lethal. As illustrated in Fig. 6, this process drastically rearranged the MS1534 chromosome. We also know that Mu-specific replication dramatically changes the structure of the *E. coli* genome (apparently tying it in knots) because Mu induction results in increased nucleoid compaction (16).

The observation that a particular colony or regional phenotype could arise from a mixed cell population may well be general for bacteria growing on surfaces. Scanning electron microscopy examination of developing *E. coli* colonies revealed many zones containing multiple cellular morphologies (26). An increasing number of phenotypes are known to be subject to phase variations (1, 15, 31, 32, 35). It is

significant that many of these phenotypes involve envelope proteins and appendages important in bacterial aggregation and attachment to surfaces. These phase variations have functional utility in the context of bacterial proliferation on surfaces, where cell tasks have to change as development proceeds. In *E. coli* and *Neisseria gonorrhoeae* pathogenesis, for example, the initial infecting bacteria have to establish themselves on host epithelia, whereas cells that are formed later in the colonization process will primarily aggregate with other bacteria and elaborate toxins and protective extracellular slime materials. Thus, mixed populations of phenotypically differentiated cells are likely to prove the rule rather than the exception with bacteria in natural situations.

Genome rearrangements occurred in a physiologically specific manner on agar substrates. Our results showing that Mu dII1681 transpositions and rearrangements were temporally and spatially regulated did not represent an isolated case. There have been other examples of physiologically specific and functionally meaningful genetic changes occurring episodically in bacteria growing on agar surfaces. One closely parallel case was the involvement of the Mu *c*ts62 prophage in the formation of coding sequence fusions by the original Casadaban technique (20). The formation of hybrid *araB-lacZ* cistrons was studied in detail; fusions arose only in response to specific selection and with a very characteristic periodicity (20). This case has recently been cited as an example of "directed mutation" (4). The *araB-lacZ* fusion system was very similar to the Mu dII1681 situation in three important ways: (i) genetic rearrangements were necessary to express *lacZ*; (ii) an unlinked Mu c^+pAp1 prophage prevented the rearrangement; and (iii) certain growth conditions (low levels of glucose enrichment in the selective agar) led to periodic waves of DNA rearrangement (20).

Studies of *bgl* activation in *E. coli* colonies on MacConkey-salicin agar (7) demonstrated that Mu-based systems were not the only examples of growth history-dependent DNA rearrangement. These contemporary examples of physiologically specific genetic events are instances of the phenomenon known in the early part of this century as "microbic dissociation" (6). Microbic dissociation referred to pleiotropic hereditary changes that were observed to occur regularly in older bacterial colonies, giving rise to papillae and sectors in which multiple phenotypes diagnostic for the parent strain (morphology, fermentation, antigenicity, etc.) had "dissociated" into a new set of characters. It has been pointed out that regulation of genetic change by growth history has very important evolutionary consequences (4, 7). One of the main advantages to studying colonies on agar substrates, compared with working with liquid cultures, is that colonies preserve a history of all clonal relationships and make it possible to discern pattern and regularity in hereditary changes. Each colony is, in effect, an evolutionary microcosm.

Future directions in the study of bacterial morphogenesis on surfaces. On laboratory media, the basic geometry of colony organization is circular. Concentric patterns arise in bacterial colonies because of sequential cellular differentiations during growth. As a consequence, the colony is a heterogeneous but organized structure. The morphogenetic capabilities displayed on agar plates are an expression of bacterial systems for organization of growth on substrates in nature. Because bacterial growth is so highly structured, it is not realistic to understand the physiological, biochemical, or even genetic activity of a colony or any bacterial surface population in terms of an "average" cell.

Investigating how different cells and groups of cells interact to produce a proliferating multicellular community will involve a variety of approaches, including many of those applied to *Drosophila melanogaster* and other higher organisms, such as in situ hybridization and immunostaining (15). Since we have identified Mu dII1681 replication as one process sensitive to sequential cellular differentiations, we can use our knowledge of Mu regulation to provide an array of molecular probes for examining how specific regulatory elements (e.g., IHF and similar DNA-binding proteins and HflA protease) vary during colony development. It will also be necessary to have ways to score genetic differences affecting specific functions involved in orderly colony morphogenesis. The Mu d(*lac*)–XGal vital staining system provides one easy way to test known markers and identify new mutations (22). As mentioned above, we have used this system to isolate a series of mini-Tn*10* insertions into the MS1534 chromosome leading to altered colony development. The inserts were picked up because they led to new β-galactosidase staining patterns. All but three were not linked to the Mu dII1681 element by P1 transduction. A majority of the inserts altered the morphologies of mature colonies and affected the patterns of cell division and microcolony organization in the first few hours of development. Our results with filter paper immobilization of colonies will also prove useful in further studies of different phase variation systems and their regulation. The ability to visualize the spatial organization of Mu DNA replication within colonies immobilized on filter paper means that a molecular analysis of colony structure is not limited to special situations involving histochemical strains. We anticipate that this method can be applied to a variety of different macromolecular syntheses.

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