

The Use of Mudlac Transposons as Tools for Vital Staining to Visualize Clonal and Non-clonal Patterns of Organization in Bacterial Growth on Agar Surfaces

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When a histochemical stain for β -galactosidase activity is applied to growth of Gram-negative bacteria on agar medium, the pigmentation is non-uniform and capable of revealing internal colony organization into different cell types. Use of an *Escherichia coli* strain with a thermo-sensitive *lac* repressor indicates that colonies expand by addition of new cells at the periphery and that older cells which have synthesized β -galactosidase early in development remain in the centre. Mixed inocula of different strains show clonal exclusiveness as they proliferate outwards. Mudlac transposons can create genetic fusions that place β -galactosidase expression under a variety of regulatory systems. Stained surface cultures of *E. coli* and *Pseudomonas putida* strains carrying Mudlac insertions in plasmids reveal a variety of flower-like staining patterns. These patterns display both clonal (i.e. sectorial) and non-clonal (circular and radial) features which are heritable within a given strain. The non-clonal aspects of the patterns reflect phenotypic differentiation without genetic change. These observations indicate that bacterial growth on agar surfaces is a highly regulated process similar, in many respects, to the development of specific multicellular tissues and organisms.

INTRODUCTION

The developmental specificity of bacterial growth on agar surfaces is frequently taken for granted. Microbiologists can usually recognize contaminant colonies on Petri dishes, and clinical bacteriologists frequently make a reliable identification of cultures on morphological grounds (see Olds, 1974). It is obvious, therefore, that bacterial colonies are specific structures whose contours are subject to hereditary determination. Observation of bacterial growth on agar for several weeks reveals reproducible patterns of morphogenesis (Legroux & Magrou, 1920; Cooper *et al.*, 1968). Thus, we can legitimately enquire about the development of bacterial colonies and pose questions about the mechanisms which organize bacterial cells and control expression of hereditary information as they proliferate on agar surfaces. In some cases, such studies of prokaryotic development are facilitated by visible cellular differentiations (*Myxococcus*, *Proteus*, *Streptomyces*), but for many bacteria there are no obvious ways to identify different cell types within the mass of bacterial growth.

In this paper I report the utility of a histochemical stain for β -galactosidase activity in revealing organizational details in intact, living colonies of Gram-negative bacteria. The use of β -galactosidase for bacterial histochemistry is greatly enhanced because expression of this enzyme and of β -galactoside permease can be placed under a wide variety of control systems by the use of Mudlac transposable elements developed by Casadaban and his colleagues (Casadaban & Cohen, 1979; Casadaban & Chou, 1984; B. de Castilho, F. Richaud, P. Olsen &

Abbreviations: XGal, 5-bromo-3-chloro-3-indolyl- β -D-galactoside; Km, kanamycin; Tp, trimethoprim; Cm, chloramphenicol; Gm, gentamicin.

M. Casadaban, personal communication). These elements are derivatives of phage Mu that have *lac* operon sequences and antibiotic resistance markers substituted for internal Mu sequences; they are therefore defective for lytic growth but retain the ability to transpose and insert at many genomic locations. The *lac* sequences are oriented so that insertion downstream of the appropriate expression signals will create a genetic fusion and permit β -galactosidase and permease expression under the control of those signals. Elements named MudI lack a promoter for an intact *lacZ* cistron and create transcription fusions; MudII elements also lack an initiating codon for *lacZ* and create translation fusions so that a hybrid β -galactosidase protein is produced.

The results presented below illustrate some applications of β -galactosidase staining for visualizing the history of colony development and certain aspects of internal colony organization. These applications became apparent when attempts to utilize a Mudlac element to place β -galactosidase expression under the control of *Pseudomonas* plasmid hydrocarbon oxidation sequences produced *Pseudomonas putida* strains displaying elaborate staining patterns (Shapiro, 1984). These patterns were heritable and had both clonal (i.e. sectorial) and non-clonal (i.e. concentric) features. Here I report experiments to clarify some aspects of the development of bacterial growth on agar and present further examples of specific patterns in *Escherichia coli* and *P. putida*, with emphasis on sectoring.

METHODS

Bacteria and genetic elements. The *E. coli* MGB series and the *P. putida* line have been described previously (MacHattie & Shapiro, 1978; Grund *et al.*, 1975). The other *E. coli* strains came from the collections of J. Beckwith and M. Casadaban. Details of the bacterial strains are given in Table 1. R751 and pPH1 were IncP/IncP-1 plasmids encoding, respectively, resistances to trimethoprim (Tp) and gentamicin (Gm). R388 was an IncW plasmid encoding resistance to Tp. Tn9 encoded resistance to chloramphenicol (Cm). CAM-OCT was an IncP-2 plasmid encoding utilization of camphor and *n*-alkanes. The *lac*^{p-155} allele blocked synthesis of a non-inducible *lac* operon repressor at 40 °C but not at 32 °C. The Mudlac elements—MudI1734, MudII1734 and MudIII1681—encoded resistance to kanamycin (Km). MudI1734 and MudII1734 were deleted for Mu *A* and *B* cistrons and so must be complemented for transposition. MudIII1681 was *A*⁺*B*⁺ and had a thermosensitive Mu *cts* 62 repressor.

Media and growth conditions. Basic growth media have been described previously (Nieder & Shapiro, 1975). Supplements were added at the following concentrations ($\mu\text{g ml}^{-1}$) XGal (Bachem, Torrance, Calif., USA), 30; Km, 20; Tp, 1000; Cm, 50; amino acids, 100; glucose, 4000; arabinose, 2000; citrate, 2500; Casamino acids (Difco, technical), 2000; glycerol, 5000. All incubations were carried out at 32 °C unless otherwise specified. To prevent agar plates from drying during prolonged incubation, they were maintained in closed metal tins that limited gas exchange with the incubator but allowed sufficient oxygen exchange for normal aerobic growth.

Plasmid transfer and Mudlac transposition experiments. Plasmid transfers were routinely carried out by one of two techniques: (a) Growth overnight of mixed spots on non-selective agar, resuspension, dilution and replating on selective agar; (b) replica-mating by patching a master plate of donor strains onto a lawn of recipients on non-selective medium, overnight incubation and further replicating to selective medium. The replica-mating technique could be carried through several transfer cycles. For example, R388::MudIII1681 plasmids could be selected by replica-plating XJS2243 to a lawn of MGB20 (selecting for Km^RCm^R clones) and the resulting plasmids could be introduced into *P. putida* by replica-plating the Km^RCm^R selection plates to a lawn of PPS587 (selecting for citrate-positive Km^R clones).

Transpositions of MudIII1681 from pPH1::MudIII1681 to CAM-OCT were selected by mixed spot matings of PPS2298 to PPS587 and selection for Met⁺ Km^R exconjugants. Since CAM-OCT suppresses the fertility of IncP-1 plasmids (cf. Fennwald & Shapiro, 1979), over 95% of the Km^R exconjugants were Gm^S (i.e. had not received pPH1) and could retransfer both Km^R and *lac* expression together with camphor or alkane utilization.

Transpositions of MudI1734, MudII1734 and MudIII1681 into R751 and R388 were obtained by incubating a master plate with patches of XJS2236-XJS2243 at 37 °C for 6 h before replica-mating to MBG20 and selection of Km^RCm^R exconjugants. Most clones of the XJS strains produced Km^RCm^R recombinants, and about one-third of these retransferred Km^R and *lac* sequences to PPS587.

Photography. All the photographs shown here were taken with a Canon FD 50 mm f3.5 Macro lens fitted with a no. 15 orange filter utilizing dark-field electronic flash illumination on Kodak Technical Pan 2415 film. For illustrations of up to one-half of a Petri dish, a 25 mm extension tube was used to yield photographic magnifications of between 1:2 and 1:1 on the negative (Figs 2 and 4). For magnifications on the negative of greater than 1:1, a 'Macro-photo coupler' reversing ring was utilized, generally in connection with a 25 mm extension tube (Figs 1, 3, 5 and 6). The light source was a square array of four bulbs placed 10 cm opposite each other and blocked by a central 9 cm square black mask to prevent direct illumination. The Petri dish was suspended on a glass plate

Table 1. *Bacterial strains*

Strain	Relevant markers	Source
<i>E. coli</i>		
MGB20	<i>lacY</i> ::Tn9	Author's collection
MGB504	<i>lacY</i> ::Tn9 (R751. <i>xyy</i> 504::MudII1734)	XJS2236 × MGB20
MGB505	<i>lacY</i> ::Tn9 (R751. <i>xyy</i> 505::MudII1734)	XJS2236 × MGB20
MGB506	<i>lacY</i> ::Tn9 (R751. <i>xyy</i> 506::MudII1734)	XJS2236 × MGB20
MGB512	<i>lacY</i> ::Tn9 (R388. <i>xyy</i> 512::MudII1681)	XJS2243 × MGB20
MGB513	<i>lacY</i> ::Tn9 (R388. <i>xyy</i> 513::MudII1681)	XJS2243 × MGB20
MGB515	<i>lacY</i> ::Tn9 (R751. <i>xyy</i> 515::MudII1681)	XJS2239 × MGB20
MGB520	<i>lacY</i> ::Tn9 (R388. <i>xyy</i> 520::MudII1734)	XJS2241 × MGB20
MGB522	<i>lacY</i> ::Tn9 (R388. <i>xyy</i> 522::MudII1681)	XJS2243 × MGB20
MGB526	<i>lacY</i> ::Tn9 (R751. <i>xyy</i> 526::MudII1734)	XJS2236 × MGB20
MGB527	<i>lacY</i> ::Tn9 (R388. <i>xyy</i> 527::MudII1734)	XJS2240 × MGB20
X5137	Δ [<i>lac-pro</i>] <i>rpsL</i> (ϕ 80 <i>dlac</i> <i>is</i> ¹⁸⁸)	J. Beckwith
XJS2236	Δ [<i>lac-pro</i>] Δ [<i>ara-leu</i>] (Mucts62) (MudII1734) (R751)	R751 into POI1734 of M. Casadaban
XJS2237	<i>ara</i> ::Mucts62 (MudII1734) (R751)	R751 into POII1734 of M. Casadaban
XJS2239	Δ [<i>lac-pro</i>] Δ [<i>ara-leu</i>] <i>rpsL</i> (MudII1681) (R751)	R751 into M8820 (MudII1681) of M. Casadaban
XJS2240	Δ [<i>lac-pro</i>] Δ [<i>ara-leu</i>] (Mucts62) (MudII1734) (R388)	R388 into POI1734 of M. Casadaban
XJS2241	<i>ara</i> ::Mucts62 (MudII1734) (R388)	R388 into POII1734 of M. Casadaban
XJS2243	Δ [<i>lac-pro</i>] Δ [<i>ara-leu</i>] <i>rpsL</i> (MudII1681) (R388)	R388 into M8820 (MudII1681) of M. Casadaban
<i>P. putida</i> *		
PPS103	<i>ilv</i>	Author's collection
PPS587	<i>his trp</i>	Author's collection
PPS2298	<i>met</i> (CAM-OCT) (pPHI::MudIII1681)	pPHI::MudIII1681 into PPS145 <i>met</i> (CAM-OCT)
PPS2299	<i>his trp</i> (CAM-OCT::MudIII1681)	PPS2298 × PPS587
PPS2306	<i>his trp</i> (CAM-OCT::MudIII1681)	PPS2298 × PPS587
PPS2334	<i>his trp</i> (CAM-OCT::MudIII1681)	Darker sector 21 of PPS2306 clone a1
PPS2335	<i>his trp</i> (CAM-OCT::MudIII1681)	Darker sector 22 of PPS2306 clone a1
PPS2336	<i>his trp</i> (CAM-OCT::MudIII1681)	Darker sector 23 of PPS2306 clone a1
PPS2357	<i>his trp</i> (CAM-OCT::MudIII1681)	Darker sector 132 of PPS2299 clone b4
PPS2358	<i>his trp</i> (CAM-OCT::MudIII1681)	Darker sector 32 of PPS2306 clone a1
PPS2422	<i>his trp</i> (CAM-OCT::MudIII1681)	Typical subclone of PPS2357
PPS2430	<i>his trp</i> (CAM-OCT::MudIII1681)	Lighter sector of PPS2334
PPS2480	<i>his trp</i> (CAM-OCT::MudIII1681)	Minority type subclone of PPS2357 on glycerol agar (see text)
PPS2491	<i>ilv</i> (CAM-OCT::MudIII1681)	PPS2357 × PPS103
PPS2495	<i>ilv</i> (CAM-OCT::MudIII1681)	PPS2430 × PPS103

* The origin of PPS2299 and PPS2306 sectors has been illustrated in Shapiro (1984). Note that each sector was purified twice before isolated colonies with novel pigmentation patterns were obtained and that sectors 21, 22 and 23 of PPS2306 clone a1 all derived from a single original sector 2. Thus PPS2334, PPS2335 and PPS2336 all descend from the same original variant clone.

either 30 mm (<1:1 magnification) or 45 mm (>1:1 magnification) above the light source. The no. 15 filter enhanced the contrast of the blue β -galactosidase stain against the agar and unpigmented bacteria. Negatives were developed in a 1:20 dilution of HC110 direct from the Kodak concentrate for 6 min at 68 °C. Prints were routinely made on no. 2 contrast paper, but occasional prints required lower or higher contrast. No systematic attempt was made to achieve 'quantitative' estimation of staining intensity from one image to another, so that comparisons are only valid within a single print. The relative densities of the various prints can be judged from the darkness of the surrounding agar. For example, in Fig. 5, the prints of the 32 °C colonies are much heavier than the prints of the 37 °C colonies, thereby emphasizing the absence of dark sectors in the low temperature colonies.

RESULTS

Characteristics of colony staining for β -galactosidase activity

The stain was the widely-used chromogenic substrate 5-bromo-3-chloro-3-indolyl- β -D-galactoside (XGal) which is colourless but releases a blue pigment when hydrolysed by β -galactosidase. The pigment was insoluble and adhered to Gram-negative bacteria. Detergent

lysis of stained cells showed that the pigment complexed with some viscous material, presumably DNA or (more likely) lipopolysaccharide. Only when there was cell lysis and liberation of β -galactosidase into the medium was pigment observed outside bacterial growth on XGal agar. The intensity of the staining reaction was proportional to the level of β -galactosidase synthesized and the reaction was more rapid in cells that possessed the *lacY*-encoded β -galactoside permease than it was in cells with no specific β -galactoside uptake system (Miller, 1972). Once a colony had acquired a specific staining pattern, some regions may have darkened, but there was no loss of detail nor 'bleeding' over periods as long as three months at room temperature. All these observations indicated that XGal staining occurred intracellularly and that the pigment stably marked the position of the cells in which hydrolysis occurred. Deeply stained areas of bacterial growth contained viable colony-forming units.

Colony expansion by the addition of cells at the periphery

In order to determine the spatial fate of older and younger cells, it was necessary to control the synthesis of β -galactosidase and to mark the bacteria at different times in colony development. This could be accomplished by the use of a strain with a thermosensitive *lac* operon repressor. Fig. 1 shows colonies of *E. coli* strain X5137 formed after incubation for varying periods at 40 °C (derepressed) and 32 °C (repressed). Very little stain was apparent in colonies incubated for 3 d at 32 °C, but initial incubation overnight or longer at 40 °C produced a clear darkly stained colony centre that remained well-defined after more than 2 d incubation at 32 °C. The size of the central zone increased with longer incubation at 40 °C, and the size of the band of non-pigmented cells increased with incubation at 32 °C. The complementary temperature-shift experiment was more difficult to accomplish because previously unstained regions of a colony would turn blue when β -galactosidase synthesis was induced. Nonetheless, the colonies incubated for almost 2 d at 32 °C and then 1 d at 40 °C generally showed lighter centres and darker edges. Thus, it appeared that new cells were added at the colony periphery. This conclusion agreed with earlier studies of colony growth where it was not possible to distinguish old and young cells (Pirt, 1967; Wimpenny, 1979).

Clonal exclusiveness

One feature of bacterial growth on agar observed repeatedly was the presence of sharp boundaries between different clonal populations in contact with each other. An example was provided by the mixed fields of growth resulting from purification of *P. putida* (CAM-OCT::MudIII1681) sectors with more intense pigmentation. These streakings often contained cells producing both parental (lighter) and variant (darker) colonies (Fig. 2). The distinctions between light and dark areas in the zones of confluent growth were sharp and were maintained down to the limits of visual resolution. There was no evidence for mixtures of cell types at the boundaries to generate zones of intermediate pigmentation.

Clonal expansion at the periphery of a mixed cell population

One very basic question about colony expansion concerned the identities of the cells at the periphery which produced daughters and gave rise to subclones extending outwards. If cell multiplication were strictly geometrical so that all cells at the periphery produced daughters and if all cells divided with the same mean frequency, then subclones would be wedge-shaped and occupy a diminishing proportion of the colonial surface as growth proceeded and the numbers of dividing cells at the periphery increased. The *Mudlac*-XGal staining system permits the visualization of subclones within colonies after genetic variation has occurred as sectors with relative differences in pigmentation intensities. Examples of different sectoring patterns have been presented in this paper and elsewhere (Shapiro, 1984). Frequently the shapes of the sectors did not follow the simple model just described. In particular, some of these sectors suggested that only a minority of cells at the colony periphery served as progenitors for outwardly expanding subclones. In order to test whether all (or most) of the cells at the edge of a bacterial population on agar gave rise to progeny clones, I performed the following experiment. I prepared a mixed suspension of cells from two strains of *P. putida*, one producing dark colonies (PPS2491) and one

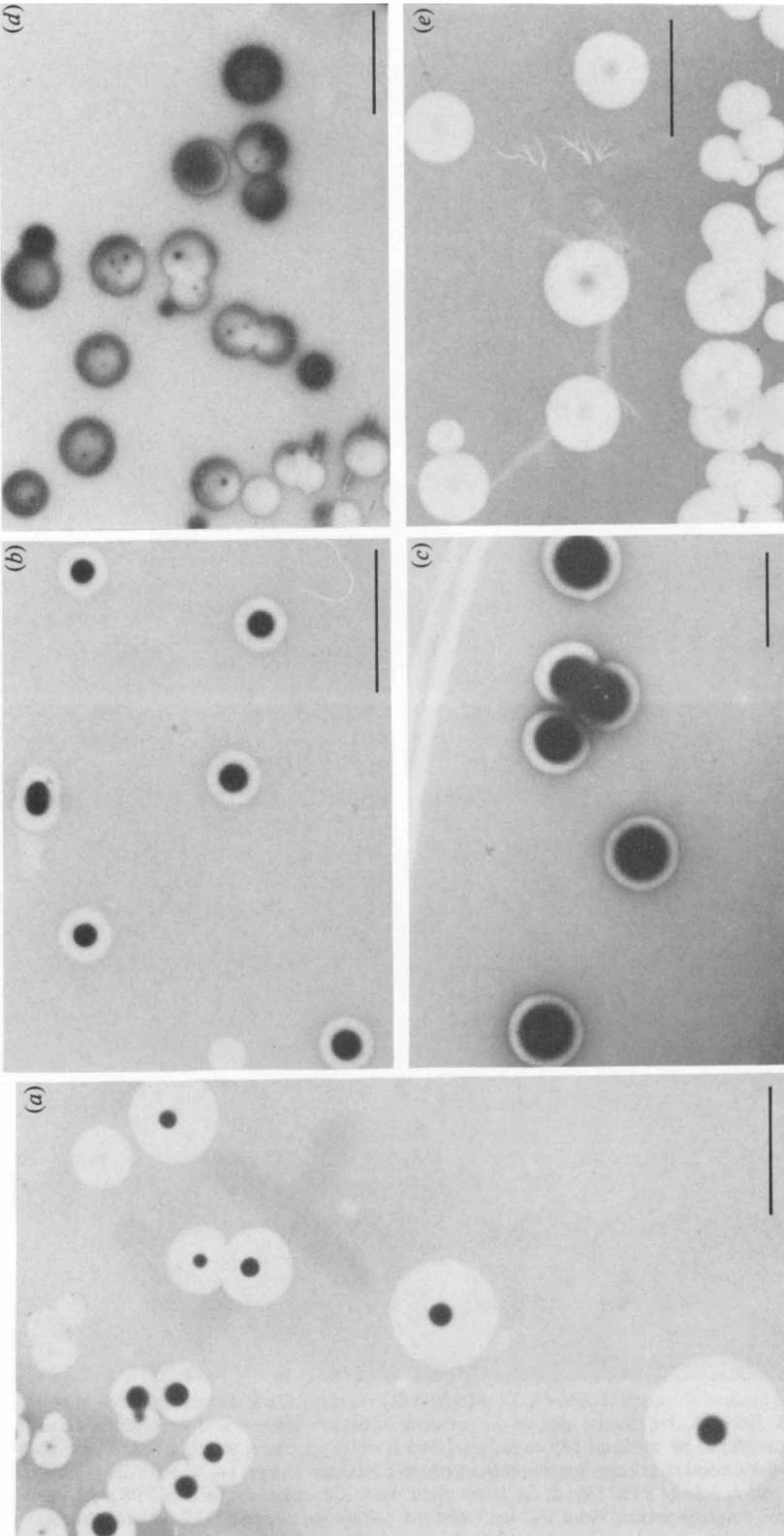


Fig. 1. *Escherichia coli* with a temperature-sensitive *lac* repressor grown under different temperature regimes. Strain X5137 was streaked on XGal/Casamino acids agar, and the plates were incubated for various times at 32 °C and 40 °C. (a) 15 h, 10 min at 40 °C, then 56 h, 30 min at 32 °C. Note that the final diameter of the colony is more sensitive to crowding effects than the size of the dark central region. (b) 22 h, 45 min at 40 °C, then 47 h, 30 min at 32 °C. (c) 41 h, 15 min at 40 °C, then 48 h, 15 min at 32 °C. (d) 41 h, 15 min at 32 °C, then 29 h at 40 °C. (e) 71 h, 30 min at 32 °C. The bars indicate 2 mm.

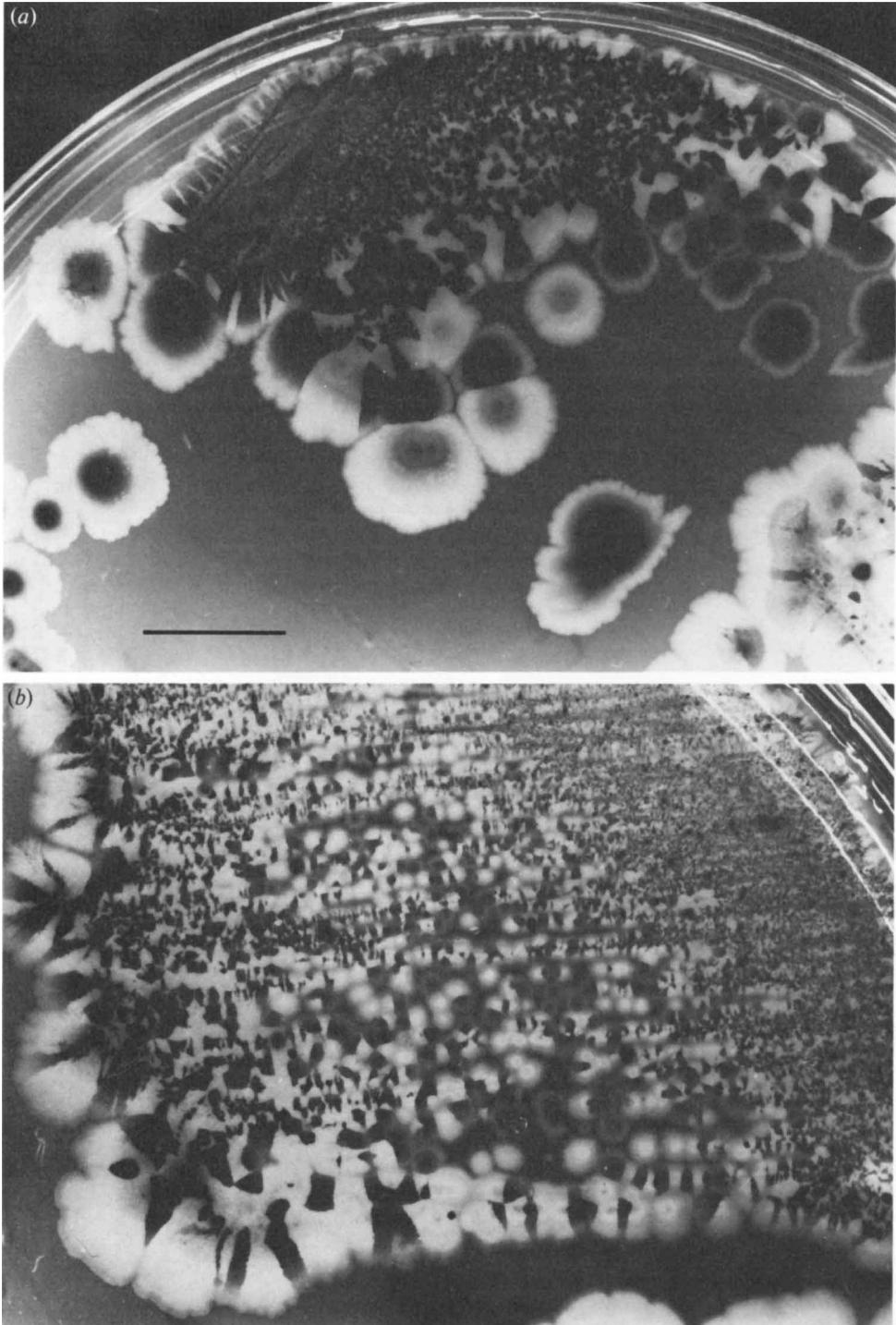


Fig. 2. Mixed fields of *P. putida* (CAM-OCT::MudIII1681) variants. Dark sectors appeared in platings of strain PPS2306 on XGal/glucose agar in the presence of octane vapours and were subjected to two restreakings on the same medium before isolated darker colony types were obtained. These photographs show the second streakings for two sectors which contained at least two clearly distinguishable colony types. (a) A field of PPS2336; at the lower right there is an adjacent field of PPS2335, derived from the same original sector. Note the half-sectored colony in the PPS2335 field. (b) A field of PPS2358. These plates were incubated for 7 d. The bar indicates 4 mm.

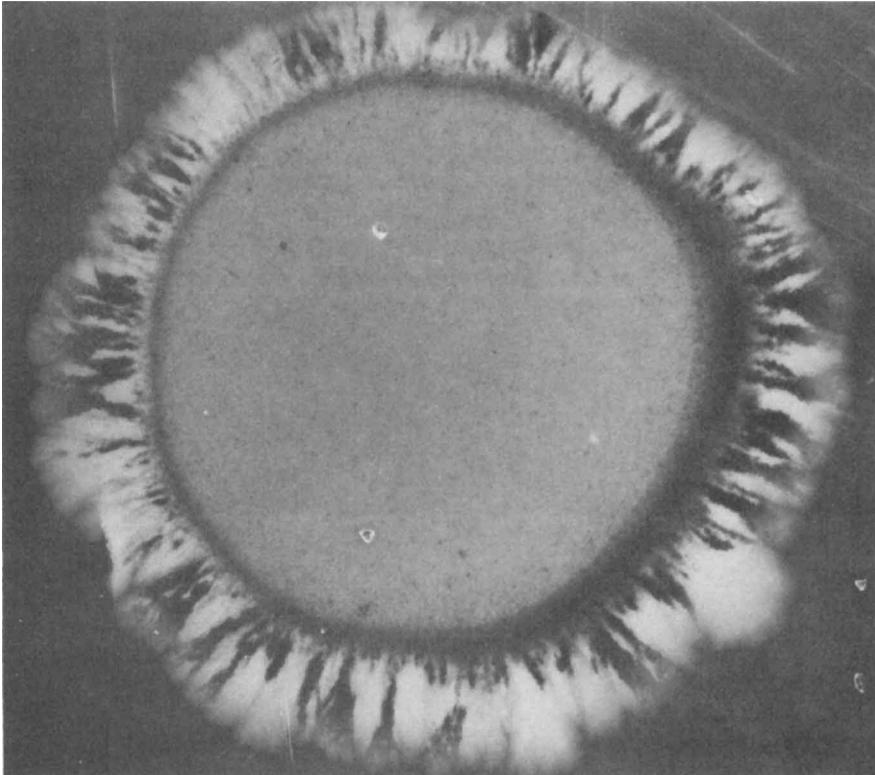


Fig. 3. Sectoring at the periphery of a random mixture of *P. putida* (CAM-OCT::MudII1681) cell types. Isolated colonies of PPS2491 and PPS2495 were resuspended in buffer at a density of about $4 \times 10^9 \text{ ml}^{-1}$, mixed in equal volumes, vortexed, and then spotted (0.05 ml) on XGal/citrate agar. The patch was incubated for 7 d before this photograph was taken. The original spot measured 1 cm across and is marked by the dark rim at the base of the sectorized zone. Note that PPS2491 and PPS2495 were produced by transfer of the CAM-OCT::MudII1681 plasmids from PPS2357 and PPS2430 which show a similar staining difference on XGal/citrate agar.

producing light colonies (PPS2495), inoculated the mixture on XGal agar, and followed the pigmentation at the edges as growth proceeded outwards from the seeded area. Abundant peripheral sectoring occurred even though the original mixture contained about 10^8 cells of each strain (Fig. 3). Thus, it appeared that only a few cells at the edge of this growing mixed bacterial population were able to produce progeny clones.

Concentric and sectorial patterns in E. coli strains

Derivatives of strain MGB20 harbouring R388::MudIac and R751::MudIac plasmids were prepared as described in Methods. It is important to note that the MudIac elements were introduced on plasmids into strains to be examined. In the plasmids which harboured MudI1734 inserts, the Mu A and B functions required for further transposition events were missing. The exconjugants which had received the various MudIac plasmids were streaked on either arabinose or glucose/Cm selective agar containing XGal, and examined after 16 or 17 d incubation at 32 °C (Fig. 4). All of the patterns displayed concentric features, and abundant sectoring was apparent in colonies of strains MGB515 and MGB522. The kind of sectoring observed with each strain was characteristic in virtually all the colonies, light broad sectors for MGB515 and thin dark jagged sectors for MGB522.

The role of Mu transposition functions in the origin of sectors could be deduced from two kinds of observations. First, sectors were abundant only in colonies of the two strains carrying the transposition-competent MudII1681 element and not in the strains carrying the $A^- B^-$

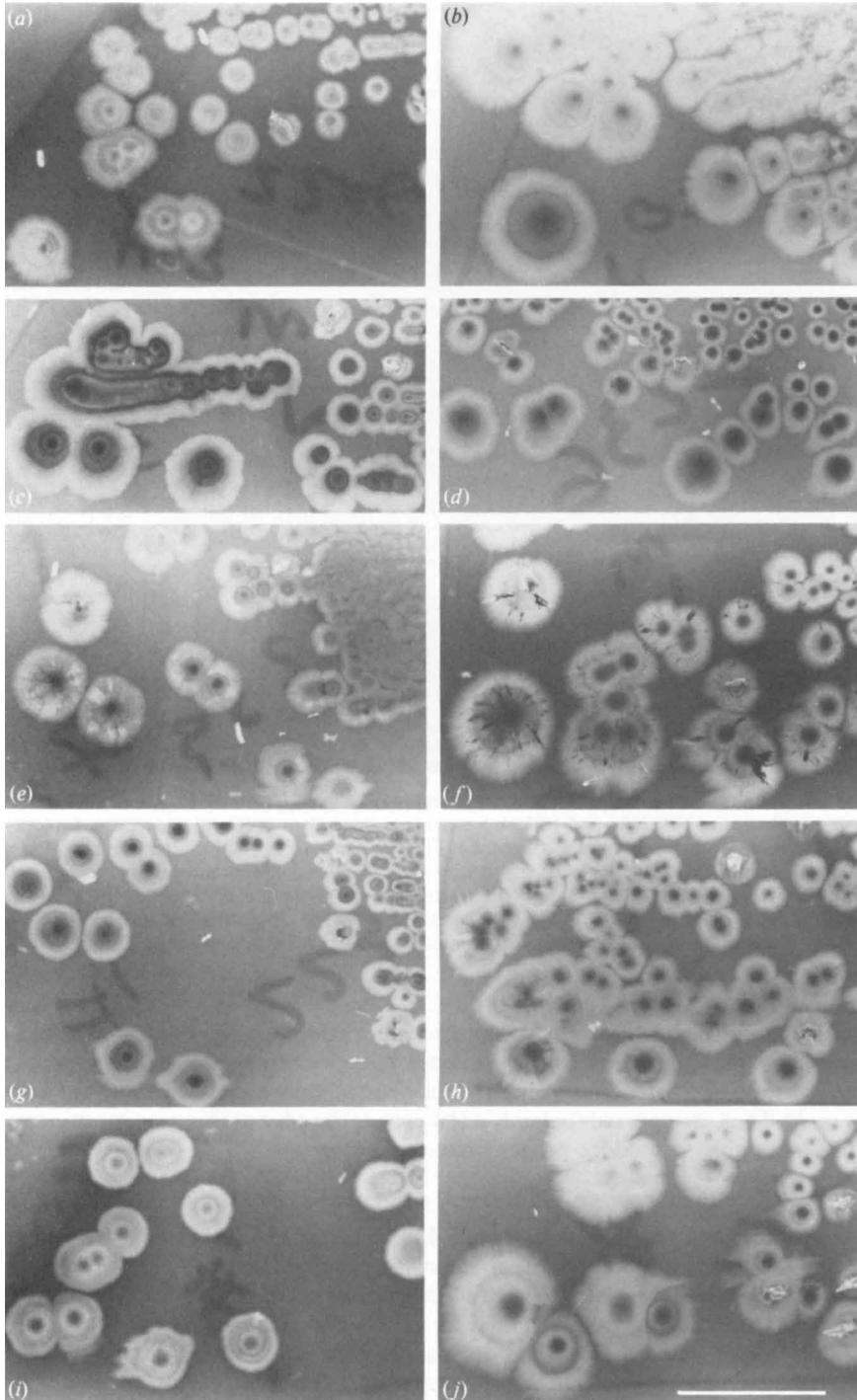


Fig. 4. *Escherichia coli* strains carrying R751::Mudlac and R388::Mudlac plasmids. Strains carrying plasmids with Mudlac insertions were isolated by replica-plate mating of donor strains to MGB20 and selection of Km^r Cm^r exconjugants as described in Methods. These exconjugants were then restreaked on XGal/glucose/Cm or XGal/arabinose agar and incubated for 16 or 17 d at 32 °C before photography. (a) Unnamed exconjugant of XJS2240 clone 3 × MGB20 on arabinose; (b) the same exconjugant on glucose/Cm. Note the effect of carbon source on the patterns of this strain. (c) MGB526 on arabinose. (d) MGB504 on arabinose. (e) MGB515 on arabinose. (f) MGB522 on glucose/Cm. (g) Unnamed exconjugant of XJS2237 × MGB20 on arabinose. (h) MGB506 on glucose/Cm. (i) MGB527 on arabinose. (j) MGB520 on glucose/Cm. The bar indicates 4 mm.

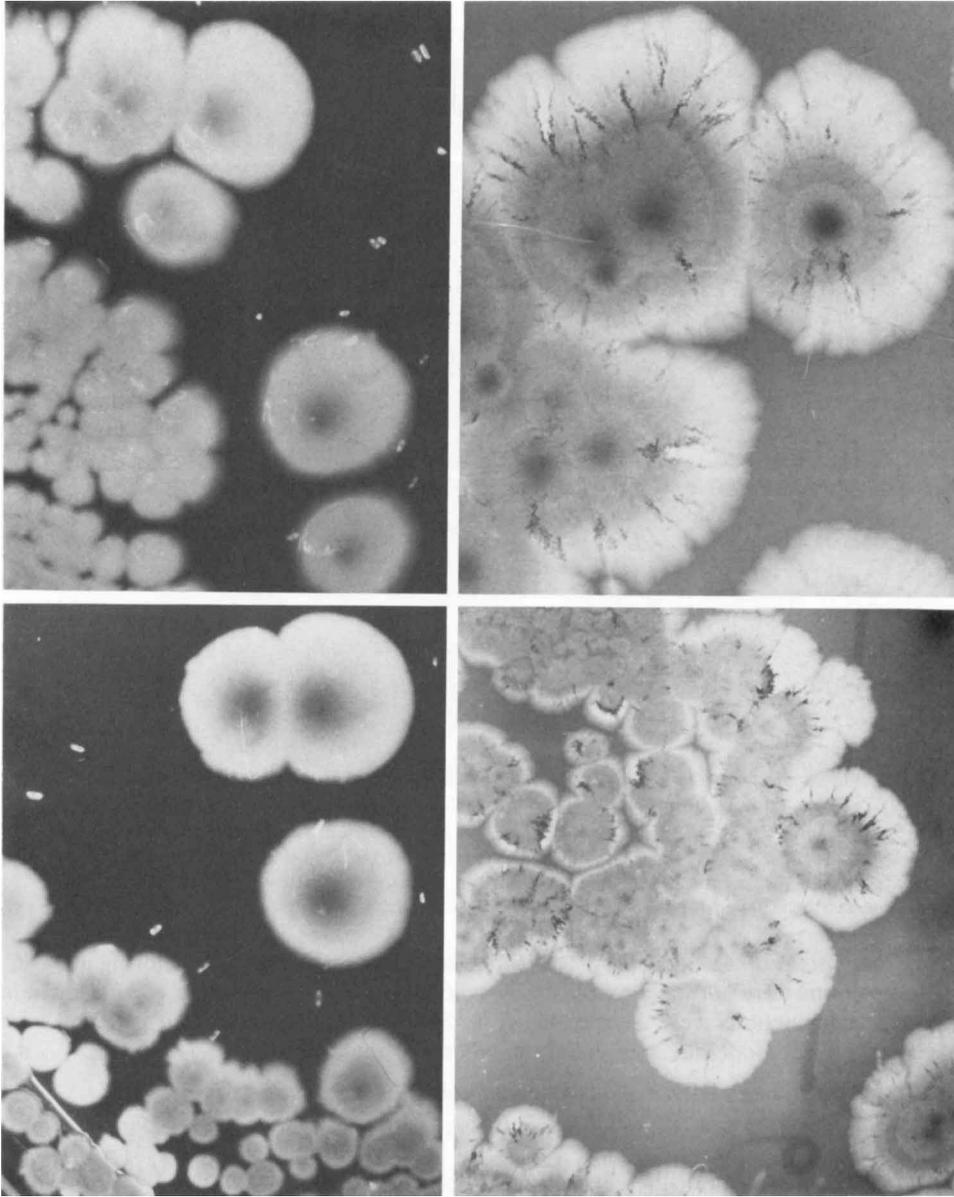


Fig. 5. Effect of temperature on *E. coli* strains carrying R388::MudIII1681 plasmids. Strains MGB512 (left) and MGB513 (right) were streaked on XGal/glucose/Casamino acids agar and incubated for 5 d at 32 °C (top) or 3 d at 37 °C (bottom).

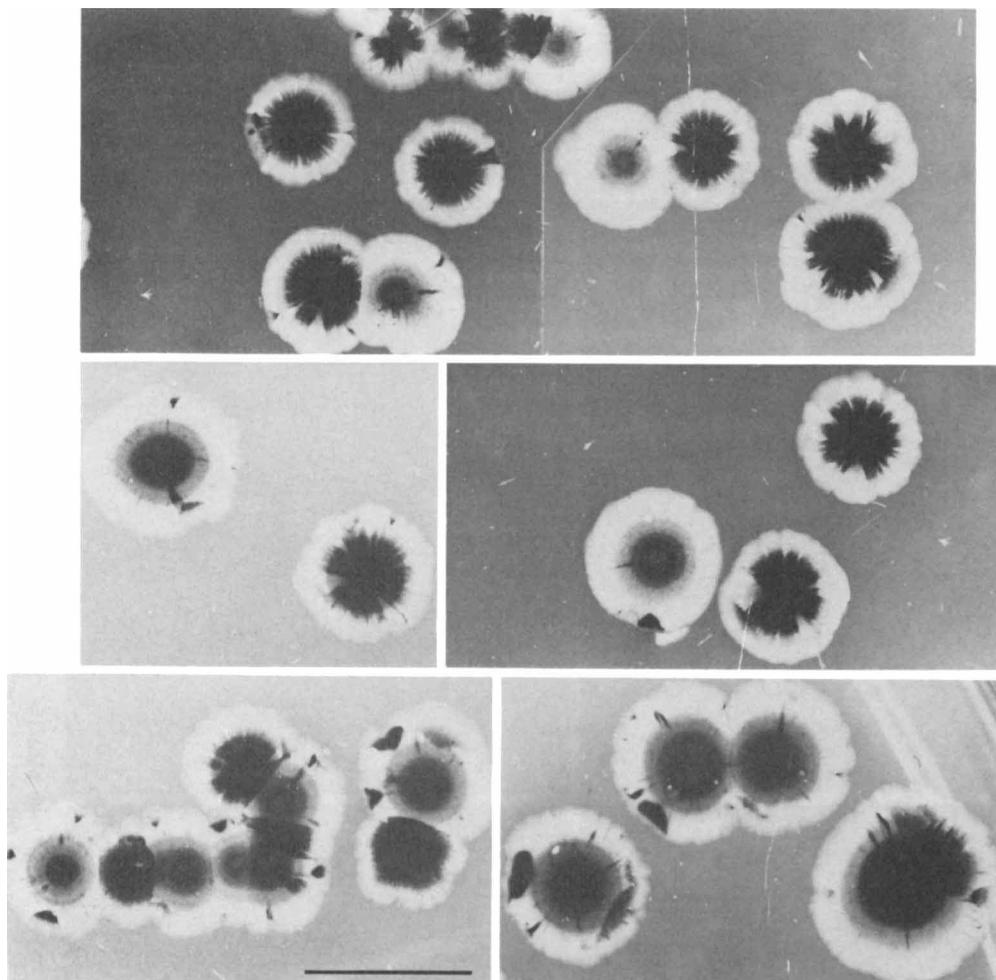


Fig. 6. Colony dimorphism of *P. putida* PPS2357 on XGal/glycerol agar. Two isolated colonies of PPS2357 on XGal/glycerol agar were restreaked on the same medium. One colony had a large dark centre, and some of its progeny are shown in the top row and the middle right panel. The other colony (designated PPS2480) had the lighter bull's-eye pattern, and some of its progeny are shown in the bottom row and the middle left panel. These colonies were incubated for 10 d. The bar indicates 4 mm.

MudII1734 elements (Fig. 4). Secondly, some MudII1681 strains showed no sectoring at 32 °C, where Mu functions were normally repressed, but did show frequent sectoring at 37 °C, where Mu transposition functions were derepressed (Fig. 5). Note that the sectoring patterns were characteristic and reproducible from colony to colony in the two strains.

Sectoring patterns and colony dimorphism in a P. putida strain

Several examples of XGal staining patterns in colonies of *P. putida* strains harbouring CAM-OCT::MudIII1681 plasmids have been presented elsewhere (Shapiro, 1984). One strain, PPS2357, showed a characteristic dark colony with multiple light peripheral sectors when streaked on XGal/glycerol agar, but there were also a few colonies with a different, bull's-eye pattern with dark sectors. The second colony type was originally considered to represent a heritable variant and was assigned a different strain number (PPS2480). However, restreaking of each colony type produced fields of progeny colonies like those illustrated in Fig. 6. About one-quarter (33/133) of the PPS2357 progeny resembled PPS2480, and about two-fifths (16/41) of the PPS2480 progeny resembled PPS2357. It thus appeared that this strain was dimorphic for colony type as revealed by XGal staining on glycerol agar. Within each colony type, there was

also a noteworthy consistency of sectoring. For example, all of the PPS2480-type colonies had one or more thin dark streaks across the middle band of intermediate pigmentation, and at least one-third displayed one or more large triangular dark sectors in the outer white band.

Non-heritable cellular differentiation in P. putida

The concentric bands of differential pigmentation in *P. putida* (CAM-OCT::MudIII1681) colonies did not result from heritable changes, such as loss of the *Mudlac* element. This was demonstrated by picking cells from the dark centre and the light edge of a single colony of either PPS2334 or PPS2422, restreaking on the same growth medium, and observing that the parental pattern with dark centres and light edges was reproduced in the colonies formed by cells from either location (data not shown).

DISCUSSION

The photographs presented in this paper show how staining for β -galactosidase expression can reveal a richness of structural detail in colonies derived from cells harbouring *Mudlac* transposons. These transposable elements are particularly useful in morphological studies for two reasons: (1) they are readily introduced into a wide range of Gram-negative organisms on promiscuous plasmid vectors; and (2) it is possible to isolate different *Mudlac* insertions which lead to distinct staining patterns. Very high levels of β -galactosidase expression make it hard to visualize structure in the XGal stain, but deliberate underexposure in printing generally uncovers patterns even in dark colonies. Other visualization methods can yield comparable complex colony patterns, such as penicillinase staining in *Staphylococcus aureus* (R. Novick, personal communication) or bioluminescence in marine *Vibrio* spp. (M. Simon, personal communication), and experience in examining stained colonies makes it easier to perceive sectoring and other features in unstained material. Nonetheless, the advanced development of the *Mudlac*-XGal system makes it likely to prove the most widely applicable vital staining system for studies of bacterial morphogenesis on agar surfaces. Because *Mudlac* elements can insert into almost any genetic locus, it will be possible to monitor the expression of many functions during colony development.

It is obvious from the photographs presented here and elsewhere (Shapiro, 1984) that bacterial populations on agar are neither uniform nor random mixtures of different cell types. There is clearly visible, sharply defined phenotypic heterogeneity within colonies. This means that population averages are almost certain to prove misleading representations of data about metabolic activity and regulatory systems that operate as bacteria grow on agar surfaces. For example, two strains may produce colonies with the same average β -galactosidase activity per unit mass, but in one case the enzyme may be found in rings of concentrically organized cells, whereas in the other case the enzyme may be produced only after clonal variation events and be localized in radial wedge-shaped sectors. It is useful to consider that we do not know whether a similar heterogeneity prevails in liquid cultures of bacteria. There is no clear evidence to show that suspension cultures are actually as homogeneous as is usually assumed, and examination of single cells by a variety of methods has revealed a wide range of individual differentiation (Henrici, 1928; Ellis & Delbrueck, 1939; Spudich & Koshland, 1976). Moreover, it is a rare culture which does not trace its ancestry back to an original inoculum (e.g. an isolated colony) produced on agar.

The use of XGal staining has made it possible to delineate three features of bacterial development on agar: (1) cells formed early in colonial history remain concentrated in the centre (Fig. 1); (2) clones maintain their identities in areas of mixed growth (Fig. 2); and (3) clonal proliferation at the periphery of an area of growth begins with a small subset of all cells (Fig. 3). The nature of the processes which determine the identities of progenitor cells and the locations of their progeny remain to be clarified. The sectoring patterns presented here and elsewhere (Shapiro, 1984) indicate that differential rates of cell mass increase are important, because some clones expand and some contract relative to others. The physical properties of the bacterial mass

must also play a role, especially in maintaining clonal exclusiveness, for colonies are highly viscous and covered by a canopy of slime that can be visualized in scanning electron micrographs of vapour-fixed colonies (J. A. Shapiro & H. Swift, unpublished observations). While it is possible to envisage theoretical models of colony development (Wimpenny, 1981), data generated by XGal staining indicate that many parameters regulating this process are yet to be discovered. For example, since different closely related strains show distinct shapes for sectors and colony borders (Shapiro, 1984, and Fig. 4), the geometry of clonal expansion outwards must be subject to specific influences.

Two general kinds of bacterial organization are visible in XGal-stained colonies: clonal and non-clonal. Clonal organization is reflected in sectors which generally extend radially outwards towards the colony periphery. Picking and restreaking experiments frequently confirm the presence in sectors of genetically different bacteria which give rise to new colony types (Shapiro, 1984, and unpublished observations). Non-clonal organization is reflected in concentric patterns which change with distance from the colony centre. Since the cells within a particular concentric phenotypic zone are clonally related to cells in the preceding and succeeding zones rather than to each other, the mechanisms generating these zones cannot involve heritable genetic changes, as demonstrated by restreaking experiments. The detailed structures of the non-clonal patterns are complex and can vary widely between closely related strains. There are concentric rings of different intensities, speckled discs, radial streaks and textured bands. Sometimes the borders of these zones are diffuse, but often they are sharp. In addition, sectoring patterns frequently respond to the non-clonal organization, so that (for example) sectors may reproducibly originate in specific concentric zones of a particular colony type (Fig. 6).

Reflection on these and other XGal-stained colonies raises basic questions about the mechanisms of pattern formation by bacteria. Clearly the patterns are subject to genetic control because a given strain produces many similar colonies and different strains produce distinguishable sets of colonies under specific growth conditions. The reproducibility of colony pattern can be complex, as seen with the dimorphism of PPS2357 colonies on glycerol agar (Fig. 6), and such situations may reflect the action of cellular differentiation systems analogous to the one governing flagellar phase variation in *Salmonella* (Silverman & Simon, 1983). In the case of patterns revealed by XGal staining of *Mudlac* strains, the transcriptional and translational controls of the hybrid β -galactosidase sequence are almost certain to play a key role, and these will vary for each fusion. Although the patterns shown here involve plasmid fusions and so are presumed not to affect sequences essential for colony formation by *E. coli* and *P. putida*, the presence of the *Mudlac* element can influence colony development in several ways. In particular, the hybrid β -galactosidase protein and XGal hydrolysis products may have significant physiological effects, and the mutagenic potential of Mu transposition functions appears to play a role in clonal variation (Figs 4 and 5). The explanation of any single colony pattern will have to take all these factors into account, but the general observation remains that XGal staining reveals internal colony organization. Two general classes of explanation may be invoked for how colonies become organized clonally and non-clonally. (1) There are no specific control mechanisms involved, but cellular proliferation and metabolism create physical and chemical gradients that generate concentric zones and influence clonal proliferation (Pirt, 1967; Cooper *et al.*, 1968; Wimpenny, 1979, 1981). (2) Bacterial development on agar involves the operation of morphogenetic regulatory systems that are analogous to those controlling the development of complex multicellular structures in higher organisms. Although the physico-chemical explanation prevails in the literature, my own opinion is that the patterns revealed by XGal staining are too specific and too diverse to be explicable solely by the creation of physical and chemical gradients. In particular, the reproducible strain-specific patterns of clonal variation are very difficult to explain by stochastic processes. The idea of multicellular morphogenetic mechanisms is clearly necessary to account for certain bacterial phenomena, such as fruiting body formation in *Myxococcus* (Dworkin, 1966). It certainly appears from these data that formation of a colony is also a complicated process requiring coordination of the activity of many cells, indicating that genetically controlled morphogenesis is a general, rather than an exceptional, feature of the growth of bacterial populations on agar.

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