Pattern and Control in Bacterial Colony Development

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Summary

As we learn more about bacterial life in the laboratory and in nature, we increasingly appreciate what highly sensitive and sophisticated organisms they are. One of the principle new insights has been the appreciation that bacteria are interactive and form organized, differentiated multicellular communities. Colonies produced on laboratory media by the standard research bacterium, *Escherichia coli*, are excellent examples. The organization of these colonies can be visualized in the microscope, by macrophotography, and by the use of special dyes and genetic engineering techniques to reveal patterns of differential gene expression. Observation of the dynamics of colony growth and the response of colonies to experimental disruptions of normal development indicate that control systems work to produce the regular patterns observed. The effects of obstacles and of other colonies on gene expression patterns indicate that non-linear responses to chemical gradients in the substrate play an important coordinating role in colony development.

1. Changing Paradigms in Prokaryotic Biology

Over the past several years, we have come to realize the limitations of standard concepts of bacteria as primitive single-celled organisms. Studies of genetic exchange, lysogeny and antibiotic resistance have shown bacteria to be masters at engineering and disseminating sophisticated DNA elements capable of contributing to survival in difficult environments[5, 30]. Analysis of transcriptional regulatory mechanisms and chemotaxis have revealed an unexpectedly rich capacity for sensing environmental parameters and for appropriately adjusting cellular behavior to optimize growth. Research on symbiosis and pathogenesis have uncovered numerous systems that bacteria use to invade and take control of eukaryotic cells in order to create suitable environments for proliferation at the expense of higher organisms[23,43]. Observations on these and other topics have made it clear that bacteria are formidable and sophisticated actors on the stage of life.

One area where the new insights have been most surprising has been the field of intercellular communication and multicellular organization. The concept of bacteria as "single-celled" organisms has had a powerful hold on biological thinking, perhaps dating back to the establishment of Koch's postulates and the demonstration that pathogenic cultures could reproducibly be grown from isolated bacterial cells. In keeping with this attitude, well-documented examples of bacterial multicellularity, such as mobile swarm colonies[38] and Myxobacterial fruiting bodies[39], have been considered exceptional specializations. Today, however, we know that the ability of bacterial cells to communicate with each other and to organize themselves into structured, differentiated multicellular communities is widespread, perhaps ubiquitous.

There has long been ample evidence of the multicellular vocation of bacteria. Whenever they were examined in natural habitats, ranging from soil[42], to the shells of marine invertebrates[40], to the lining of the cockroach intestine[3], to infected medical implants[9], bacteria were found to exist in adherent colonies, biofilms, chains and mats.

In many cases we know the advantages conferred by organized multicellular communities. Examples include the sharing of nutrients in cyanobacterial filaments composed of nitrogen-fixing and photosynthetic bacteria, the ability to trap food organisms in aqueous suspensions by predatory *Myxococcus xanthus*, and the increased resistance of biolfims and colonies to antibacterial agents like antibiotics and hydrogen peroxide[9, 15, 31]. Despite these and myriad similar observations, the concept of bacteria as multicellular organisms still seems exotic to many biologists. One reason may be the scientific tendency to focus on a few well-studied species, such as *Escherichia coli*, and consider them to be archetypes for the whole prokaryotic realm. But, as we shall see in what follows, even the laboratory workhorse, *E. coli* K-12, displays an elaborate capacity for multicellular coordination when it grows on the surface of agar medium in the laboratory.

2. E. coli Colony Organization: Alignment, Sectors, Rings and Strata.

One of the most direct ways to see that *E. coli* cells are capable of organizing themselves is to examine the structures of colonies on normal laboratory media, using either a microscope or an ordinary 35 mm camera[25]. When very young microcolonies of several hundred cells were examined microscopically, a high degree of order was observed[37]. The cells were generally aligned, and palisaded groups of cells could frequently be seen extending between two microcolonies in the process of merging (Figure 1). This kind of order was not due simply to physical packing of rod-shaped cells because it was not seen in dense cell populations immediately after they were deposited on agar. Instead, cell alignment and order appeared with growth and were evident 80 minutes after inoculation[37].

Older colonies also displayed considerable structural organization when visualized by reflected light photography to highlight details of colony surface contours (Figure 2). Many colonies displayed wedge-shaped sectors with distinct phenotypes.

Bacteriologists are accustomed to seeing sectors, and each sector is assumed to represent the descendants of a single cell that has undergone some kind of hereditary change. In other words, sectors represent **clonal** populations united by common ancestry. In addition to sectors, however, colonies also displayed patterns of concentric ridges and terraces. The bacteria in these circular ring structure were not united by common ancestry. Along any radius of the colony, the cells in one ring were descended from cells in the previous ring and were the ancestors of cells in the succeeding ring. In other words, the cells in any one concentric ring comprised a phenotypically united **non-clonal** population. Since these cells did not have a common ancestry which distinguished them from cells in other rings, some kind of non-genetic coordinating system must have been been responsible for their shared phenotype. In other words, the existence of non-clonal patterns in colonies indicated the existence of systems for multicellular coordination.

Following the organization of colonies at different stages of growth in the scanning electron microscope (SEM) revealed a wide variety of cell morphologies visible on the colony surface and spatial localization of these distinguishable cell types into zones corresponding to higher order structural features[29]. In some cases, the transitions from a region characterized by one cell morphology to a region composed of different cells were quite abrupt, suggesting that the colony periodically shifted from one growth phase to another. Such shifts were probably the source of the structural transitions seen as concentric ridges around the colonies. A microbiological parallel is the formation of concentric terraces in *Proteus mirabilis* swarm colonies which are known to arise from cyclical differentiation of cellular behavior[38]. SEM analysis of both *E. coli* and *Pseudomonas putida* colonies also demonstrated the presence of extensive coverings of extracellular polymeric material[27,29]. This material formed a viscous gel over (and perhaps through) the colony and gave structural cohesion to the multicellular aggregates.

A more high-tech method for visualizing colony organization was to place the enzyme ß -galactosidase under various control regimes by creating hybrid *lacZ* coding regions using genetic fusion technology. ß -galactosidase is handy for histochemical visualization of biochemical activity and gene expression patterns because there are substrates which can be incorporated into the growth medium that produce an insoluble dye when hydrolyzed. The most commonly used one is called XGal. When engineered strains of *E. coli* were grown on XGal indicator media, they produced variegated colonies displaying both sectors and concentric rings of differential *lacZ* expression. This laboratory has used two *lacZ* fusion systems based on the transposable genetic element bacteriophage Mu: Mu*dlacII1681*[6] and λ*placMu15*[2]. The molecular basis of patterned ß -galactosidase expression was different in each case. The Mu*dlacII1681* element was capable of replication and transposition, and the zones of ß -galactosidase activity in colonies actually represented regions where Mu*dlacII1681* replication had been derepressed in some of the cells, leading to the creation of active *lacZ* fusions[36]. The λ*placMu15* element was defective for transposition and so created genetically stable fusions[2]. ß -galactosidase expression from each λ*placMu15* insertion was assumed to represent differential transcription and/or translation from signals upstream of the inserted *lacZ* sequence. All of the λ*placMu15* colonies shown here carried the *dev1891::lacZ* insertion creating a hybrid ß -galactosidase coding sequence at 18.2 minutes on the bacterial chromosome (R. Alonso, D. Newman and J. Shapiro, unpublished).

ß -galactosidase staining permitted a more detailed view of colony patterns than surface contour photography. Figures 3 and 4 show several kinds of sectors arising on Mu*dlac* and λ*placMu15* colonies. Many other examples of sectorial phenotypes have been published previously[24,28, 30-32,34-36]. The hereditary changes at the origins of some sectors frequently altered both growth control and concentric ring patterns, indicating the creation of a whole new developmental sequence. Other sectorial

changes, however, only affected the spreading of the bacteria over the agar but left the sequence of ß -galactosidase rings largely intact. It was interesting to note in these cases how the rings were stretched according to the differential growth of the sectorial population and how the rings lined up with the parental colony at the edges of the sectors. Some sectorial changes affected only a subset of the concentric rings but left others intact[32]. The phenotypes of these sectors suggested that there could be different genetic controls over the expression of a single *lacZ* fusion in different rings, perhaps by multifactorial regulatory systems similar to those in metazoa and plants.

Among the most fascinating sector/ring interactions were the periodic changes sometimes seen in relative expansion rates of sectors and the parental colony. These changes were marked by sharp bends in the borders of a sector[30,32]. Since a straight sector border indicated that the sectorial and parental populations were expanding over the substrate at the same rate, these bends showed where growth regimes of the two populations responded differently to changing conditions. In other words, the same kinds of periodic colony-wide changes that affected *lacZ* fusion expression and gave rise to concentric ring patterns also affected the control over proliferation of genetically distinct multicellular populations. Often, a sectorial population simply "took over" at a particular stage of development and expanded transversely across one whole side of the colony. Such sectors were actually aggressive clonal neoplasms and may be thought of as "tumors" arising on the colonies.

The organization of *E. coli* colonies into distinct zones is three-dimensional. Sections through colonies revealed a stratification of different cell types. These could be seen by standard microscopic reagents (Figure 5) or by examining ß -galactosidase expression patterns of *lacZ* fusions strains (Figure 6). In both cases, there was considerable variation in cell phenotypes organized into clearly identifiable zones. Of particular interest were the relatively poorly staining cells in the bottom third of the colony. Cell-sorter experiments showed that by 16 hours of growth *E. coli* colonies

accumulated many cells which had lost the ability to form colonies when replated (B. Hauer, H. Eippel, and J. Shapiro, unpublished). Perhaps the empty cells seen in the toluidine blue-stained sections corresponded to the non-viable bacteria detected in the cell-sorter experiments. It is also interesting to speculate on the biological utility of such a zone of dead cells. In some ways, these bacteria resembled the lysed cells in the stalks under myxobacterial fruiting bodies[39]. They may have provided some protective value to the viable bacteria in the colony. For example, when a growing *E. coli* colony encountered highly virulent bacteriophage T7, the phage infection decimated the population on the top of the colony but did not lyse the bottom layer. Eventually, bacteria in this protected layer proliferated to produce papillae of phage-resistant mutants (Figure 7). A zone of non-viable cells could also serve as a diffusion barrier protecting cells at the top of the colony against antibacterial agents in the growth substrate, in the same way as proposed for the glycocalyx of extracellular material covering antibiotic-resistant biofilms[9].

Analysis of the genetic basis of sectorial changes has just begun. This subject was approached initially by isolating bacteria from the sectors and replating them. The phenotypes of the resulting colonies provided clues to the nature of the underlying DNA changes. Some flamboyant expansive sectors affecting Mu*dlacII1681* ring patterns contained bacteria which were genetically unstable: they produced colonies with both the novel growth phenotype and the parental phenotype[35,36]. These colonies turned out to contain large chromosomal duplications of the type known to permit growth on poor carbon sources[41]. Phenotypic instability occurred because the duplications were easily eliminated by reciprocal recombination to restore the parental genetic structure. The bacteria in other expansive sectors displayed stable new colony phenotypes, and the mutations underlying the new phenotypes could be mapped and studied. One of these mutations, *dev2188*, altered the control of cell division at all growth stages and mapped near 67 minutes on the *E. coli* chromosome. Another mutation, *dev2187*, was

associated with a restriction site polymorphism near the *oriC* and *rbs* loci at 85 minutes (P. Johnson, T. Reyes and J. Shapiro, unpublished). The *dev2187* mutation did not change the pattern of cell division during early microcolony development but altered the growth response of the bacteria to changing physiological conditions after 12 -24 hours of growth to produce larger colonies of the type shown in the bottom row of Figure 2. In addition to these genetically amenable sectors, however, there were also some exceptional sectors which defied normal genetic analysis. When the bacteria from these sectors were isolated and replated, only parental-type colonies were produced. In these cases, it may be that the underlying hereditary change was some kind of DNA modification, or "imprinting," that was lost upon replating, but other explanations are also possible.

A common observation in colonies of *E. coli* and of other bacteria was the appearance of similar sectors at several, sometimes many, positions around the colony[1,24,28,30,34,36]. In many cases, the starting points of these sectors appeared to be situated in a particular concentric ring, suggesting that the underlying genetic events occurred at similar times during colony development. These observations suggested a connection between the physiological transitions that mark concentric rings and the particular DNA rearrangements that lead to a novel growth phenotype. This relationship was quite evident in Mu*dlac* colonies where the activation of Mu*dlac* replication and consequent DNA rearrangements were limited to specific concentric zones in the colony[36]. The relationship between changing growth conditions on agar and genetic variation was also well-documented in classical bacteriology, even though the underlying molecular events were completely unknown at the time[13]. The connection between a cell's physiological state and its propensity to undergo a particular kind of mutation makes sense if we think of DNA change as a biochemical process subject to regulation like other aspects of cellular biochemistry[26,30]. The apparent specificity in mutations occuring at particular times can then be understood as the activation of

particular biochemical complexes leading to a certain type of DNA change (*e.g.* recombination between defined repeat sequences).

3. Kinetics and sequence of colony development

The extensive cellular differentiation, multicellular order and macroscopic pattern observed in *E. coli* colonies of all ages tell us that colony development is a highly regulated dynamic interactive process. Direct and indirect interactions between cells and the operation of regulatory systems maintaining colony structure can be documented by following development on agar surfaces under a variety of circumstances.

Several descriptions of the very earliest stages of *E. coli* colony development from single bacteria have already been published[37]. As soon as the starting cell has divided, the two daughters interact and influence each other's growth patterns to produce a regular four-celled structure by the end of the second division cycle. That the growth pattern involves cell-cell interaction rather than internal cellular controls could be documented by examining the development of cells quite close to each other, for the daughter bacteria would sometimes deviate from the normal growth pattern to interact with cells descended from a different parent. It was intriguing to find that the natural tendency of *E. coli* cells growing on agar is to maximize cell-to-cell contact rather than maximize the exposure of each cell to substrate. Examples of the early division pattern and later microcolony interactions are shown in Figure 8. The bacteria illustrated here had a chromosomal deletion inactivating motility and chemotaxis functions, indicating that the responses of microcolonies did not use the chemotactic sensory apparatus. Despite the tendency of *E. coli* cells to line up next to each other, they divided just as rapidly on agar as in well-aerated liquid medium[32]. Thus, it appears that *E. coli* has evolved to grow in dense populations in such a way that the cells do not interfere with each other.

It has often proved useful to initiate colony development at a known position on the agar substrate by depositing a small drop of culture containing about 10^5 bacteria, and these multicellular inocula gave rise to a reproducible developmental sequence[29]. Time-lapse videos of colony development from a spot inoculum illustrated cooperative aspects of growth and expansion over the substrate. After the spot dried, bacterial divisions first filled in the interior of the inoculated zone and thickenned the ridge of bacteria at the edge before the colony began to expand over the agar. In higher magnification videos of spot inocula on microscope slides, the bacteria at the periphery were aligned radially and did not display obvious division (Figure 9). The mathematics of colony expansion have been characterized by a linear increase in diameter over time, a result geometrically consistent with a disc growing only in a limited zone at its edge[8, 19, 21]. In *E. coli* spot colonies, this zone may well have had a particular multicellular organization that needed to be constructed before expansion could begin, and the zone probably corresponded with a well-demarcated mound-like structure that was observed in scanning electron micrographs just inside the colony perimeter. This structure was bounded on the inside by a deep invagination which was observed to follow the perimeter outwards as the colonies expanded[29].

The kind of observations summarized here pointed to a defined sequence of events in *E. coli* colony development. As the bacteria proliferated, conditions changed, and the bacteria responded by forming different kinds of aggregates. Modelling bacterial surface proliferation on the assumption of autonomous cell division patterns characterized by a rate constant would not be consistent with the facts. The *E. coli* colony growth pattern actually shows parallels with the regulated development of the highly structured colonies produced by other bacterial species, such as *Proteus mirabilis*[29, 38]. It may therefore be most useful to think of the concentric structures observed on colony surfaces, like the ones illustrated in Figure 2, as reflecting periodic changes in cellular control regimes responding to the dynamic physiological and

environmental situation.

4. Regulatory phenomena in colony development

A classical approach to defining the role of biological systems in morphogenesis is to disrupt the normal developmental process and observe the effect on pattern. This kind of approach could be applied to bacterial colonies in several ways.

Certain genetic defects upset the normal sequence of bacterial proliferation on agar. For example, a *polA* mutation removed some of *E. coli*'s capacity to repair DNA damage, leading to SOS induction and filamentation in the first few hours of growth. The grossly abnormal microcolonies which resulted from the inhibition of cell division nonetheless developed into mature colonies that were indistinguishable from Pol^+ colonies in morphology at both the cellular and macroscopic levels[33]. Thus, the developing *polA* colonies changed conditions in such a way as to compensate for the repair defect, and the normal morphogenetic sequence was ultimately expressed despite the aberrant starting structure.

Another kind of morphogenetic regulation could be observed when the edge of a spot inoculum was irradiated with a beam of near-UV light using the fluorescence light source on a microscope (Figure 10). In this case, the bacteria in the illuminated area did not proliferate, and a notch appeared in the advancing colony perimeter. Within a few hours, however, expansion accelerated in the empty zone, and the colony edge resumed a relatively smooth contour. While this kind of self-adjusting behavior is rather easy to describe mathematically, it remains to be worked out what biological mechanisms generated the differential growth.

An interesting example of colony regulation has been observed during growth on scratched surfaces. A common bacteriological procedure is to streak a mass inoculum across an agar plate to separate out individual single cells or microscopic aggregates to initiate isolated colony development. When disposable glass pipettes were used for

streaking, deep creases were placed in the agar surface. These creases contained a thin layer of water, and individual bacteria released from the edge of a colony could be observed moving back in forth in this liquid, but they did not swim away from the colony. Transmitted light photography of colonies growing over scratched agar showed that the internal structure of the colony was affected by the distortions of the substrate; lines of granules formed over the scratches[32]. Nonetheless, the edges of colonies growing on scratched agar remained smooth for several days of incubation (Figure 11). When conditions changed later, regions at the colony perimeters frequently (but not always) grew out into the grooves (Figure 12). What held the colony edge intact initially and what caused it to expand into the streaks at later times are intriguing questions of changing colony behavior with age.

A particularly useful perturbation of normal colony development has been to place fibers of glass wool on the agar surface near the advancing edge. These fibers accomplished two objectives: they forced the bacteria at the colony boundary into novel physiological situations, and they transported bacteria into a new region of the agar surface. Thus, the effects on morphogenesis of these two disruptions could easily be tested. When a colony contacted a fiber, numerous motile cells at the edge swam into the liquid around the fiber and circumnavigated it. After a time, many cells accumulated all around the fiber, settled down, and initiated the development of a satellite population. Because this satellite population required some time to achieve the organized structure necessary for expansion over the agar, relatively short fibers were engulfed by the advancing colony perimeters before they could nucleate outwardly expanding populations. After engulfment of shorter fibers, the colonies regularized their perimeters (Figure 13). On longer fibers, however, the newly established populations had time to begin outward expansion before engulfment by the colony edge.

5. Long-range effects governing gene expression patterns; non-linear responses

to gradients in the substrate

One of the more intriguing observations with patterned *E. coli lacZ* fusion colonies was the alignment of concentric ring patterns when colonies of a particular fusion strain grew together (Figure 14). This result was not particularly surprising when the colonies were growing synchronously and expressing the same pattern, but the possibility of more active coordination could not be excluded. A test for such coordination was suggested by Marc Lavenant, then a graduate student. The idea was to inoculate colonies near to each other but at different times and observe how the ring patterns interacted. If they aligned, then the colonies must somehow have communicated with each other, and this is the result that was always obtained (Figure 15). By comparing the initial pattern of *lacZ* fusion expression in the centers of younger colonies inoculated close to and far away from established colonies, it was possible to conclude that the coordination of gene expression patterns occured before the colonies came into direct contact, presumably by means of a field of diffusible chemicals in the agar substrate[32].

Testing the effects of different length glass fibers on colony ß -galactosidase patterns confirmed the conclusion from observing colony mergers. With fibers that were long enough to nucleate new growth before they were completely engulfed, a very significant result was regularly obtained[32,38]. The bacteria growing out from the fiber expressed the same concentric ring sequence as the bacteria that remained at the colony edge, and the sequence off the fiber began with the very same ring that first contacted the glass (Figure 16). In other words, despite their different physiological histories, the bacteria along the fiber and the bacteria remaining at the colony perimeter executed the same developmental sequence. Experiments with even longer fibers indicated that this coordination of the two populations involved some long-range signal that attenuated with distance (Figure 17).

The experiments just described indicated that chemical fields played a major role in bacterial colony development, just as they have long been thought to do in embryonic

development of metazoa. In other words, the bacteria were able to assess the concentrations of different substances in the agar and responded in a way consistent with the observed patterns of differential *lacZ* fusion expression. The patterns along glass fibers suggested that the bacterial response to gradients must have been non-linear, because the bacteria at different distances from the colony showed the same initial phase of gene expression, and this phase changed abruptly only at considerable distance (Figure 17).

Non-linear responses to metabolic gradients could be visualized directly by looking at ß -galactosidase ring patterns arising from localized Mu*dlac* replication in colonies. It was known that there was an element of nutritional control over this replication because it was inhibited in *cya* and *crp* mutants that lacked the components of the cAMP-catabolite activation system (Figure 18). Thus, it was not surprising to observe that ß -galactosidase expression was more intense in the crowded interior regions of an agar plate, where glucose and other nutrients had been most depleted, than at the edge of the petri dish, where there was no competition between colonies (Figure 19). However, even though nutritional depletion was necessary for Mu*dlac* replication, it was not sufficient, because only the cells in well-defined zones displayed ß -galactosidase activity. Although all of the bacteria on the starved side of these colonies were subjected to the same level of substrate depletion, only cells in particular concentric zones were capable of undergoing Mu*dlac* derepression and consequent ß -galactosidase expression. In addition to the system sensing the gradient in the agar substrate, other controls over cellular differentiation must have been active.

One convenient way to think about chemical gradients and non-linear colony patterns was suggested by the pioneering work of Legroux & Magrou[14]. Based on their observations of highly organized and sharply defined histochemical staining in colony sections, they proposed thinking of the colony as composed of physiologically distinct tissues. This concept would fit very well with our findings about the *E. coli* colony

organized into discrete zones characterized by differential cell morphologies, differential multicellular aggregation patterns, and differential gene expression. Thus, patterns of concentric rings and horizontal strata displaying ß -galactosidase activity would arise from differential gene expression in successive zones composed of fundamentally distinct cell types. In other words, the regulatory architecture of each cell type in a discrete stratum or concentric zone would be reflected in the expression of a characteristic set of tissue-specific functions.

6. Why are bacterial colony patterns important?

When biologists and other scientists first hear about the kinds of colony patterns described above, they frequently want to know how the conditions of growth on agar medium in laboratory petri dishes relate to the natural environments of *E. coli* and other bacteria. Perhaps, these questions often imply, patterning is a peculiar circumstance of growth under highly artifical circumstances. There are two ways to answer these questions. The first way is to point out that surface growth in the laboratory is a relevant model for surface growth in the environment. We have only to consider the essential roles of adhesion factors in symbiosis, pathogenesis and biofilm formation to appreciate the importance of surface attachment for bacterial life.

But there is a deeper way to answer questions about the relationship between laboratory observations and life in natural conditions. Let us grant that an agar surface is only a laboratory convenience. Nonetheless, it permits the bacteria to display the repertoire of biological control systems at their disposal. If, in a petri dish, bacteria are capable of forming complex, differentiated, organized multicellular aggregates, then they must also be capable of doing so in natural situations. Realism demands that our general theories of bacterial metabolism, genetics, cell biology and behavior take into account the potential for intercellular communication and multicellular coordination we observe under laboratory conditions. Examples of interbacterial signalling are

accumulating rapidly, as we learn more about the role of extracellular molecules in phenomena such as bioluminescence[17], sporulation[12] and genetic transfer[5, 10, 20].

The colonies described in this paper were grown under common laboratory conditions -- that is, on solid medium with an excess of nutrients. These overfed colonies tended to be compact and circular. A number of scientists have begun to investigated colony morphologies under less favorable conditions. When motile chemotactic *E. coli* swarm colonies in semi-solid medium were challenged with hydrogen peroxide, they responded by forming compact aggregation centers distributed in striking geometrical patterns[4]. This autoaggregation response system provided protection for the bacteria because dense cell populations are much more resistant to oxidative stress than dilute cells in suspension[15]. On harder agar, nutrient-starved populations of *E. coli* and *Bacillus subtilis* produced highly branched colonies with intricate geometries reminiscent of inorganic systems[1,11,16, 18]. Some of the most regular morphologies involved bacterial motility (M. Matsushita, personal communication; E. Ben-Jacob, personal communication). Thus, these patterns reflected the operation of biological control systems as much as they revealed constraints imposed by physical factors, such as diffusion of nutrients and waste products. Indeed, in some cases it appears that the simplest colony geometries, such as compact circles, actually required the highest degree of biological activity, because crippled mutants displayed more complex colony outlines[31,38] (M. Matsushita, personal communication).

Bacterial colonies are likely to become one of the main biological systems for studying general problems of self-organization and pattern formation. One reason is that bacterial systems can mimic many of the most fascinating geometries seen in inorganic systems. A second reason is that experiments with bacteria are rapid and inexpensive. Thus, models can be tested quickly in periods of stringent research budgets. A third, and very important, reason is our unmatched ability to manipulate the genomes of bacterial species. Thus, we can exploit to the fullest a major advantage of biological

systems over physical systems for the study of complexity and non-linearity: genetics. While biological systems are inherently complex beyond current scientific description, existing genetic technology allows us to modify living cells one molecule at a time and leave them otherwise intact, thereby permitting the detailed dissection of pattern-forming systems.

Science is presently undergoing its greatest change in outlook since the days of Descartes and Gallileo. This change is marked by emphasis on complexity instead of simplicity, on the behavior of systems rather than the behavior of isolated objects, on analyzing connections between components and not searching for explanations in the inherent properties of the smallest possible units. Living systems in general will play a key role in this new approach to science because thinking about all complex systems is strongly influenced by well-established biological concepts such as homeostatic feedback regulation, sensory information processing, behavioral responsiveness, and hierarchical integration. Since the realization is growing that bacteria share cellular and multicellular control capabilities with their eukaryotic relatives, it is possible that these small but highly sophisticated cells will once again be the organisms of choice for studying fundamental problems at the leading edge of science.

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Figure legends

Figure 1. *E. coli* microcolonies. A fairly dense suspension of bacteria was plated on rich agar medium to give closely spaced microcolonies and incubated 6 hours, 15 minutes incubation at 37° C. This transmitted light micrograph was photographed directly off the petri dish culture using an epi-illumination lens without coverslip correction and a defocussed condenser. Note the high degree of alignment in regions where the bacteria were individually visible and in the group of cells connecting two microcolonies in the process of merging. 600X magnification.

Figure 2. The surface structures of some *E. coli* colonies after 6 days incubation at 37° C on minimal salts-glucose agar. These colonies were inoculated with a 1 μ l spot of culture containing about 10^5 bacteria. Note the concentric ridge patterns on all colonies and the wedge-shaped sectors on four of them. The colonies in the top row showed the phenotype of this laboratory's standard strain, while the colonies in the bottom row were larger because they carried the *dev2187* mutation isolated from bacteria in a spreading sector. These colonies were photographed by reflected light optics: by placing the petri

dishes at the appropriate angle to reflect the light from fluorescent light fixtures in the ceiling into the lens and focussing on the colonies, surface structure could be recorded in great detail. The colonies in the top row measured about 1 cm across.

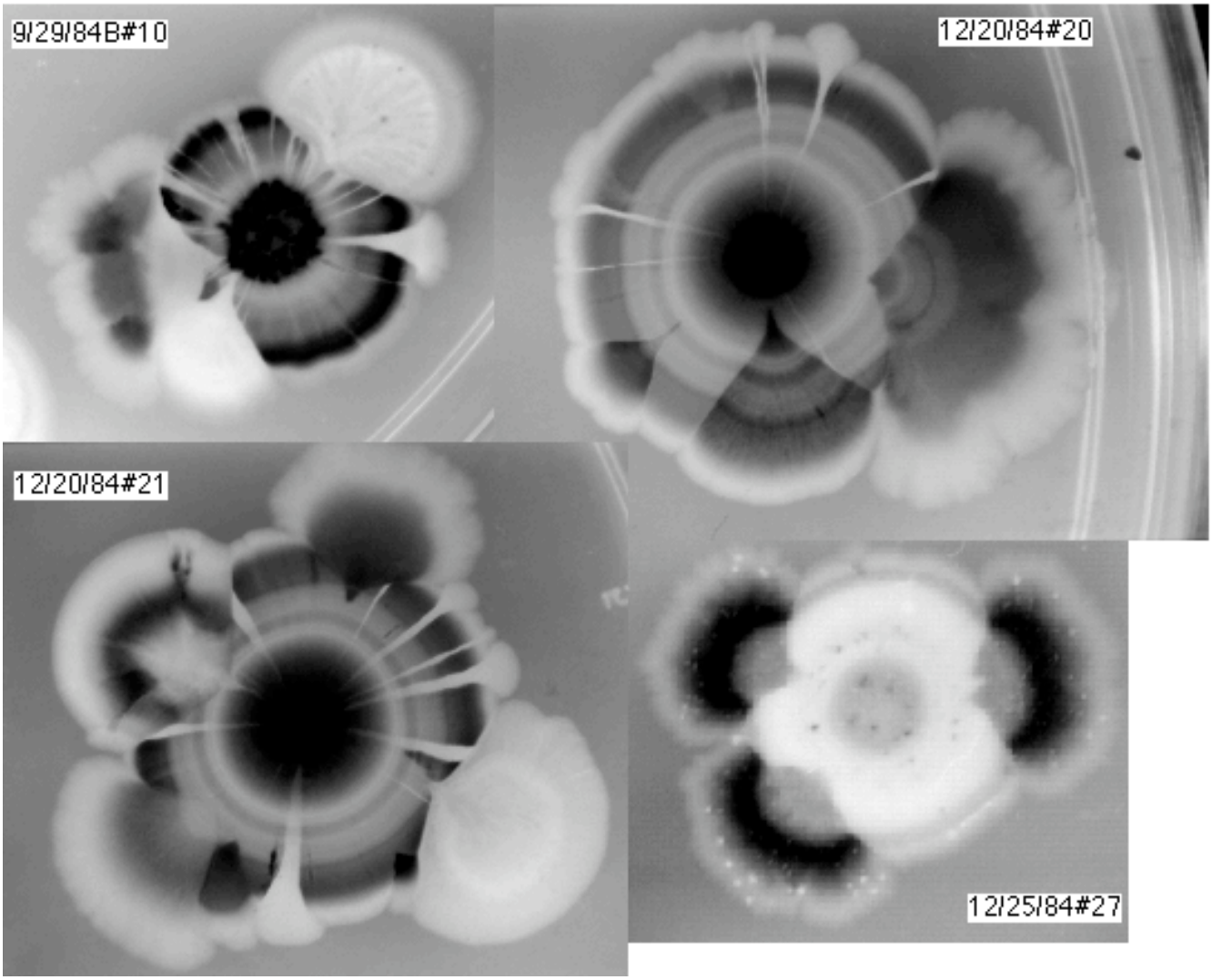


Figure 3. *E. coli* colonies displaying variegated ß -galactosidase expression due to the presence of the Mu*dlacII1681 lacZ* fusion element. These colonies were incubated for 10 days at 32° C on minimal salts-glucose agar containing the chromogenic substrate known as XGal. XGal hydrolysis by ß -galactosidase led to the deposition of an insoluble blue dye. Note the expansive neoplastic sectors and the sectors with altered staining patterns. These colonies measured about 1 cm across.

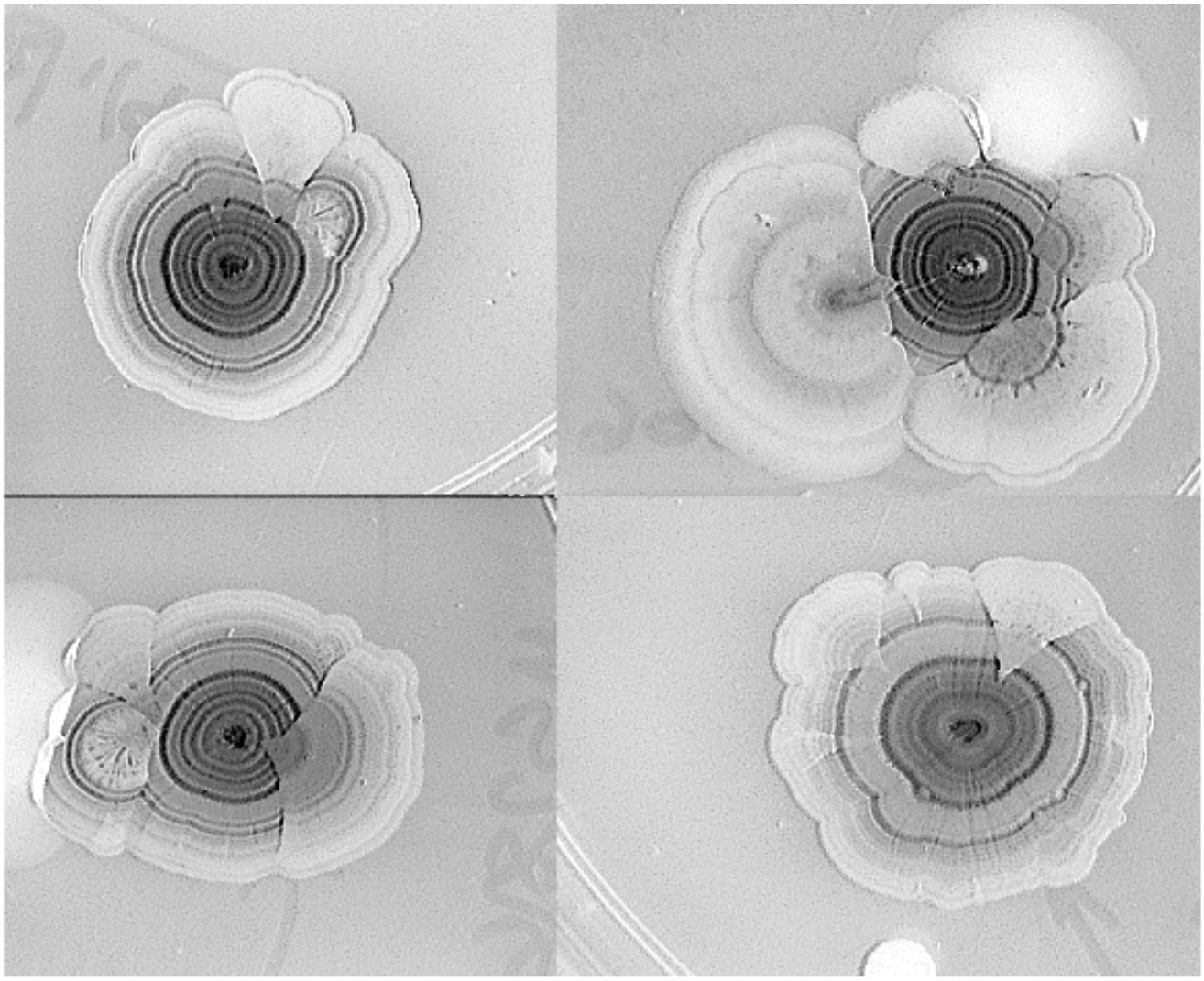


Fig. 4

Figure 4. *E. coli* colonies displaying variegated ß -galactosidase expression due to the presence of the λ*placMu15 lacZ* fusion element. In these *dev1891::lacZ* colonies, *lacZ* was stably fused to a protein-coding sequence at 18.2 minutes on the *E. coli* chromosome. These colonies were inoculated by stabbing a dense bacterial mass into the agar with a sterile toothpick and incubated for 8 days at 37° C on miniminal salts-glucose-casamino acids agar containing XGal. Note that phenotypically similar sectors arose on independent colonies. 4.8X magnification.

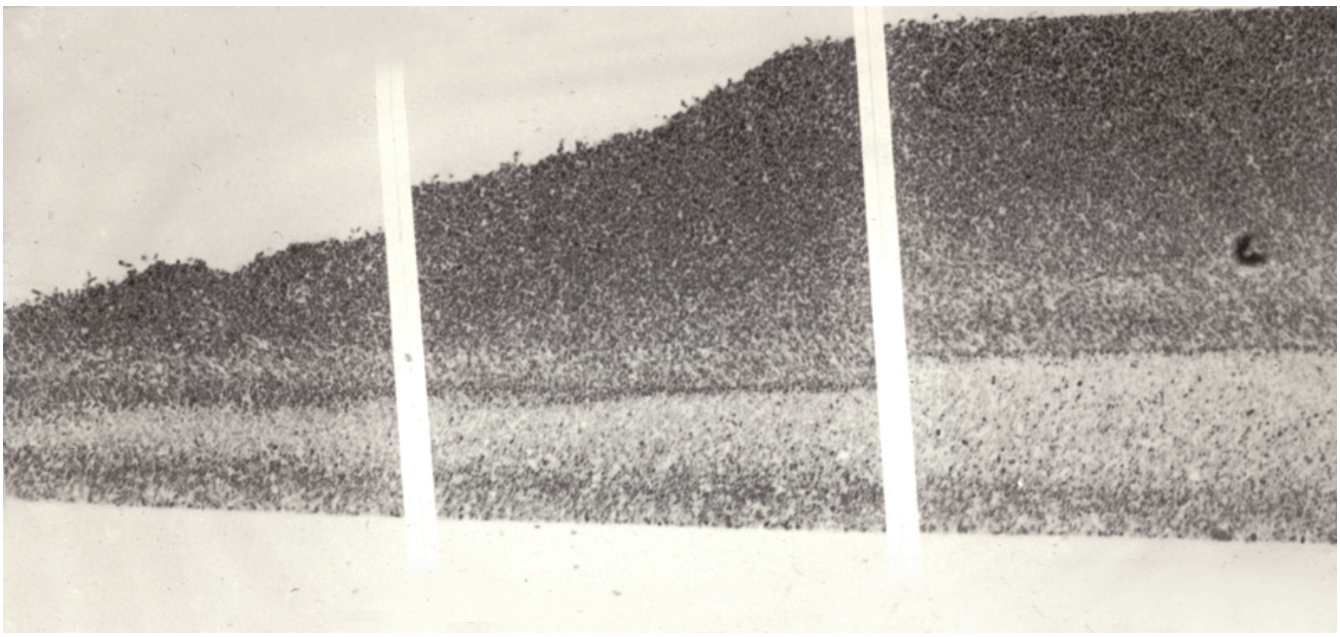


Figure 5. Cross-sections of three neighboring areas at the edge of an *E. coli* colony. This colony was encased in agar after two weeks incubation at 37 C (Puzyr and Mogilnay[22]), fixed with formaldehyde, desiccated in ethanol and embedded in Epon. 1 μ m sections were stained with toluidine blue and photographed under oil immersion through a Zeiss Axiophot microscope. 1200X manification.

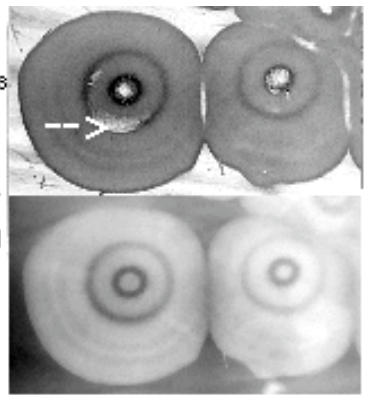


Figure 5. Photos of XGal-stained colonies of strain MS1891. The bottom panel was illuminated from the side. The top panel also had coaxial illumination. Note how the rings of XGal staining correspond to highlighted features of the colony surface (arrow).

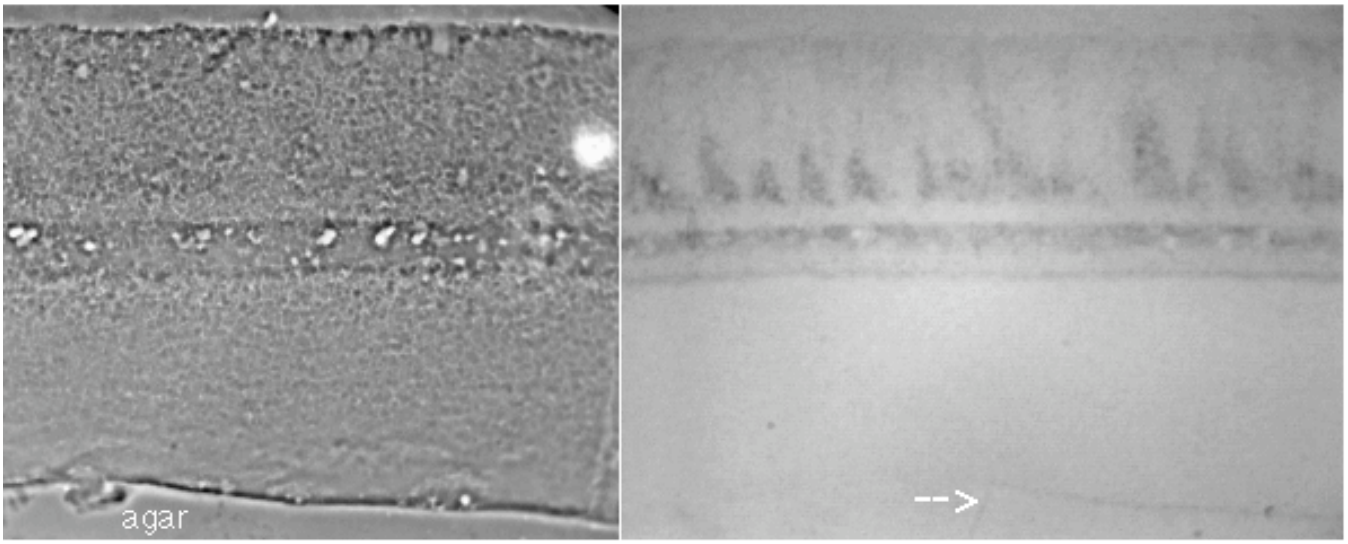


Fig. 7. 10µ paraffin section of an XGal-stained dev1891::lacZ colony. Left: Phase contrast. Right: Transmitted light, XGal staining. The colony was approximately 50µ thick. Note thin layer of XGal staining at bottom of colony on right (arrow).

Figure 6. Two views of a section through a *dev1891::lacZ* colony (labeled Fig. 7 in the digitized image). The colony was grown for two weeks on XGal indicator agar. The right panel was photographed with transmitted light optics to reveal ß -galactosidase staining, and the left panel was photographed with Nomarski optics to reveal the entire colony outline. Note the localized enzyme activity in a thin line at the very bottom of the colony, two continuous strata in the middle of the colony, and flares in the top half of the colony. This colony was prepared like the one in Figure 5 except that it was embedded in paraffin and the sections were 10 μ m thick. 1200X magnification.

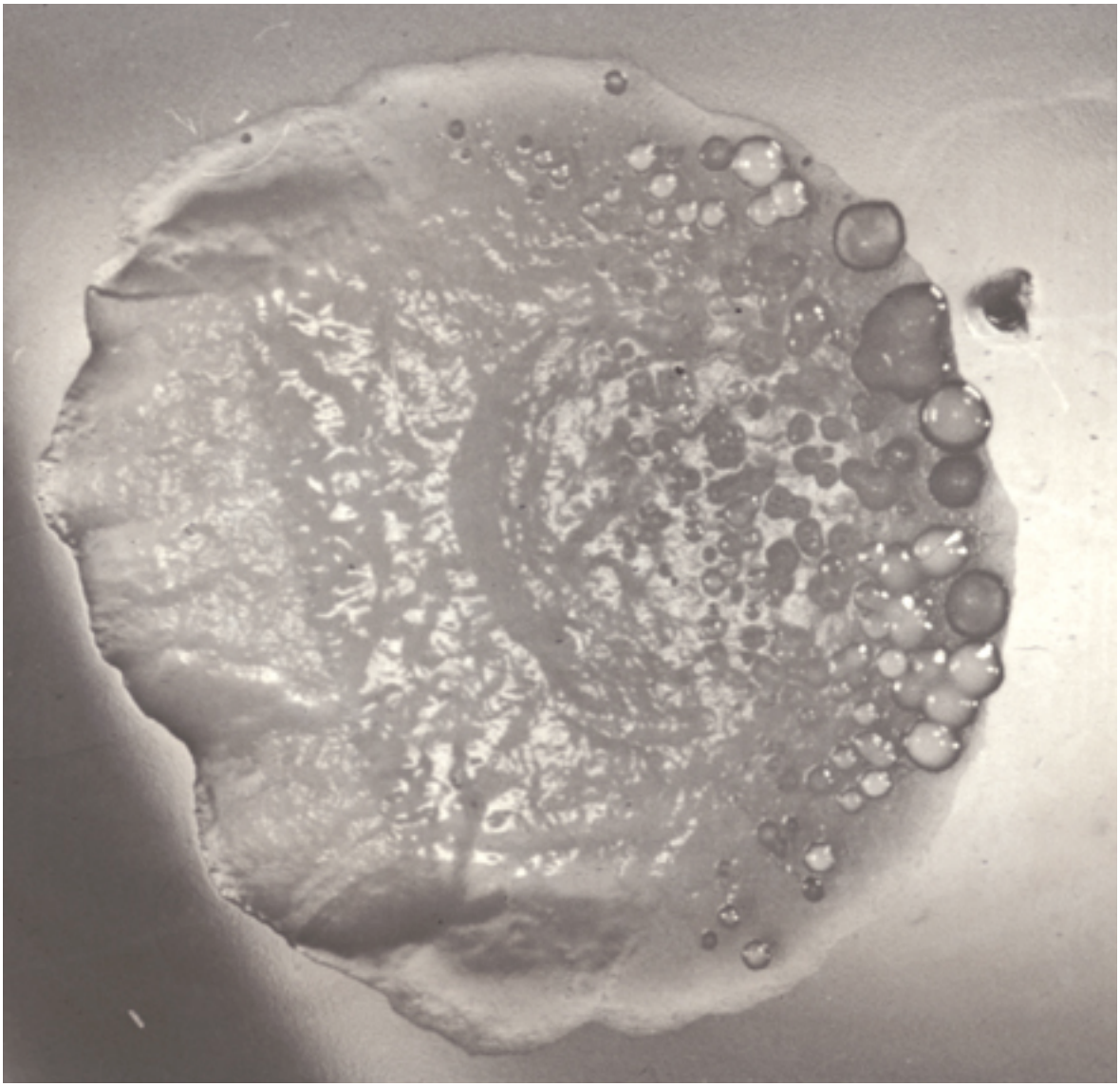


Figure 7. An *E. coli* colony several days after encountering a spot of bacteriophage T7. The colony was inoculated with a spot of culture next to a small deposit of T7 phage marked by the toothpick hole just to the right of the colony. After approximately 2 days incubation at 37° C on rich medium, the colony grew into the phage and lysis began at the left and continued to progress across the top of the colony. This photograph was taken after 6 days incubation. Note the papillae of resistant bacteria growing up in the earliest zones to be attacked on the right side of the colony. This colony measured about 1 cm across. VIDEO AVAILABLE AT

http://shapiro.bsd.uchicago.edu/index3.html?content=bacteria.html

CLICK ON “BACTERIAL VIDEOS” THEN “ECO T7.MOV”.

Figure 8. Frames from a time-lapse video sequence showing *E. coli* cells proliferating on an agar surface on a microscope slide under a coverslip at approximately 32° C. The elpased time since inoculation, date and actual time are given in the numerals at upper left. These bacteria had a deletion of the *flhD-flhA* region at 41 minutes on the chromosome which inactivated motility and chemotaxis sensory systems. Note the mergers of initially separate microcolonies. The bacteria were observed with phase-contrast optics using an oil immersion lens. Approximately 3000X magnification.
VIDEO AVAILABLE AT
http://shapiro.bsd.uchicago.edu/index3.html?content=bacteria.html.

CLICK ON "BACTERIAL VIDEOS" THEN "3cellsmicrocolony.mov" OR

"5cell_fusingmicrocolonies.mov".

Figure 9. Organization of the bacteria at the edge of an expanding colony. A spot inoculum on a microscope slide was observed under the same conditions as in Figure 8. The two frames were captured 1 hour 28 minutes apart. Note how the cells in the inoculum were initially disordered but filled in and acquired considerable alignment at the edge less than 1.5 hours later. Approximately 3000X magnification.
VIDEO AVAILABLE AT
http://shapiro.bsd.uchicago.edu/index3.html?content=bacteria.html.

CLICK ON "BACTERIAL VIDEOS" THEN "spot_phasecontrast2.mov".

Figure 10. Adjustment of the edge of an irradiated microcolony. The edge of a spot inoculum on a rich agar medium in a petri dish was placed under the fluorescence excitation beam of a microscope for 3 minutes and then videotaped during incubation at approximately 32° C. These frames show how the perimeter had become smooth about three hours after filling in of the inoculated zone. VIDEO AVAILABLE AT
http://shapiro.bsd.uchicago.edu/index3.html?content=bacteria.html
CLICK ON "BACTERIAL VIDEOS" THEN "SPOT UV REGULATION.MOV".

Fig. 3

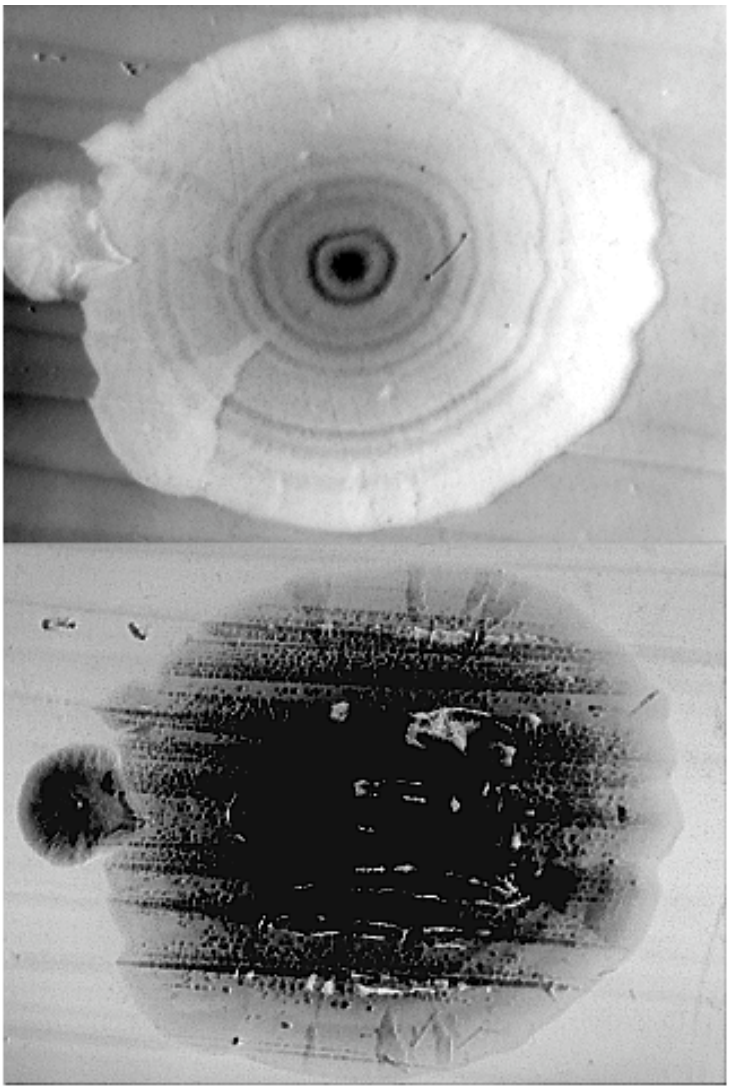


Figure 11. *E. coli* colonies growing on scratched agar. These colonies were inoculated at 12:58 on 1-16-86 on minimal salts-glucose-casamino acids agar and incubated at approximately 32° C during videotaping. THIS IMAGE IS OF A DIFFERENT COLONY GROWING ON SCRATCHED AGAR. NOTE THAT THE SCRATCHES DO NOT AFFECT THE RING PATTERN.

Figure 12. Older *E. coli* colonies on scratched agar. These Mu*dlac* colonies were incubated 10 days on rich medium containing glucose and XGal indicator at 32° C. Note the growth processes extending into some (but not all) of the scratches. The rows of

internal granules that formed over the scratches can be seen in the bottom panel. 15X magnification.

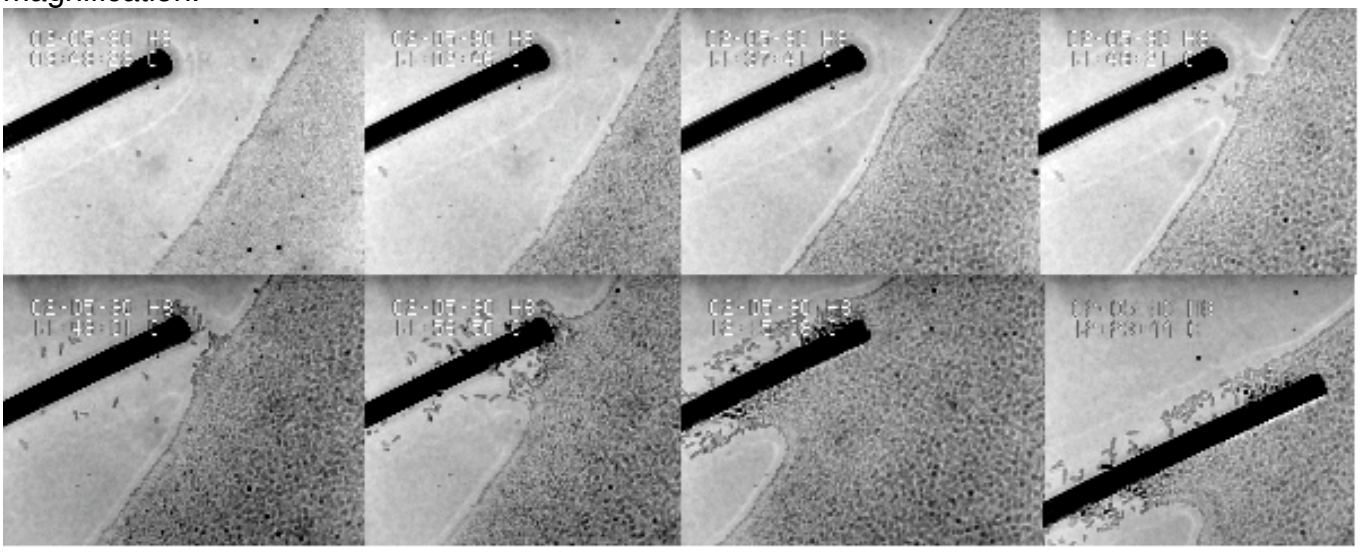
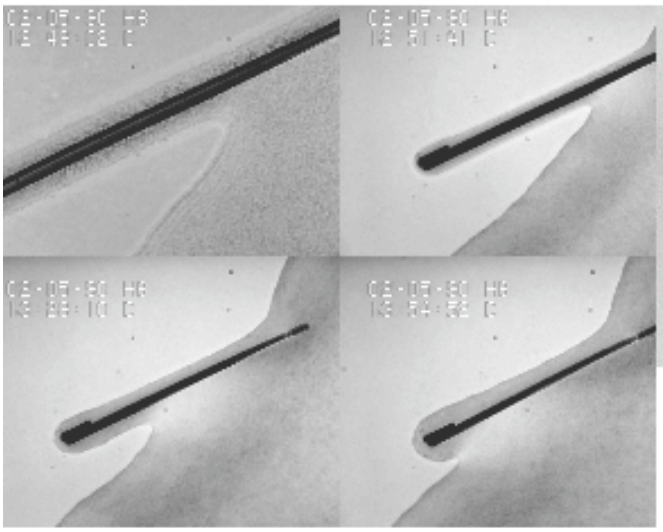


Fig. 14. Encounter of a colony with a glass fiber on an agar petri dish. Frames from a time-lapse videotape were taken at 9:48, 11:02, 11:37, 11:48 (top row), 11:49, 11:56, 12:15 and 12:23 (bottom). Note the delay before the edge began to move.



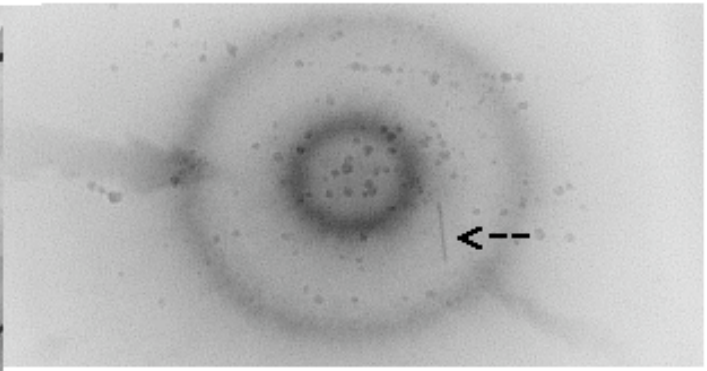


Fig. 16. The first two XGal rings in a dev1891 colony which had engulfed a short glass fiber (arrow).

Fig. 15. Lower magnification pictures of the encounter that began in Fig. 14. Frames were taken at 12:49, 12:51, 13:28, and 13:54. By 15:00 the fiber was engulfed and the edge had begun to smooth out.

Figure 13. Colony engulfing a glass fiber. Images were taken from a time-lapse video at the following hour:minute intervals from the top frame - 0:49, 1:53, 2:44, 4:34.

VIDEO AVAILABLE AT

http://shapiro.bsd.uchicago.edu/index3.html?content=bacteria.html

CLICK ON “BACTERIAL VIDEOS” THEN “fiberengulfment.mov“

Figure 14. Alignment of concentric ß -galactosidase rings in merged colonies of a *dev1891::lacZ* strain. Cultures were streaked on Xgal indicator agar and incubated at 37°

C for several days before photography. The streak inocula produced colonies arising from single bacteria or from microscopic aggregates. Top panel, 5 days incubation, 4.8X magnification; bottom panel, 3 days incubation, 19X magnification.

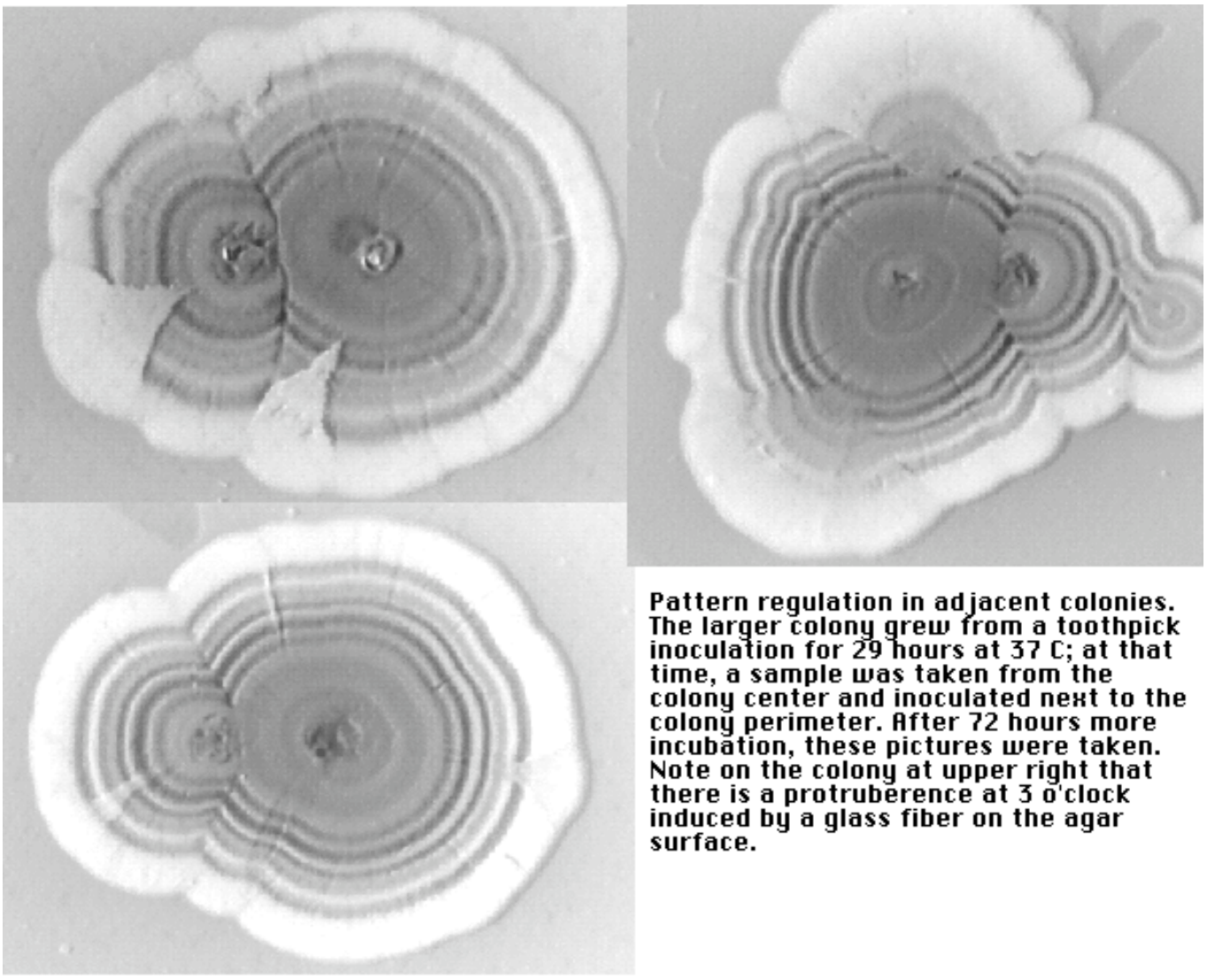


Figure 15. Alignment of concentric rings in merged colonies of different ages. A *dev1891::lacZ* colony was inoculated with a toothpick stab on Xgal indicator agar and incubated 29 hours at 37° C. A second toothpick sample was taken from the center of this colony and inoculated at two sites on the same plate, one about 2 mm and one about 4 cm from the edge of the growing colony. These photographs were taken 3 days after

the secondary colonies were inoculated. Note how the center of the distal colony displayed more intense ß -galactosidase expression than the center of the proximal colony. 4.8X magnification.

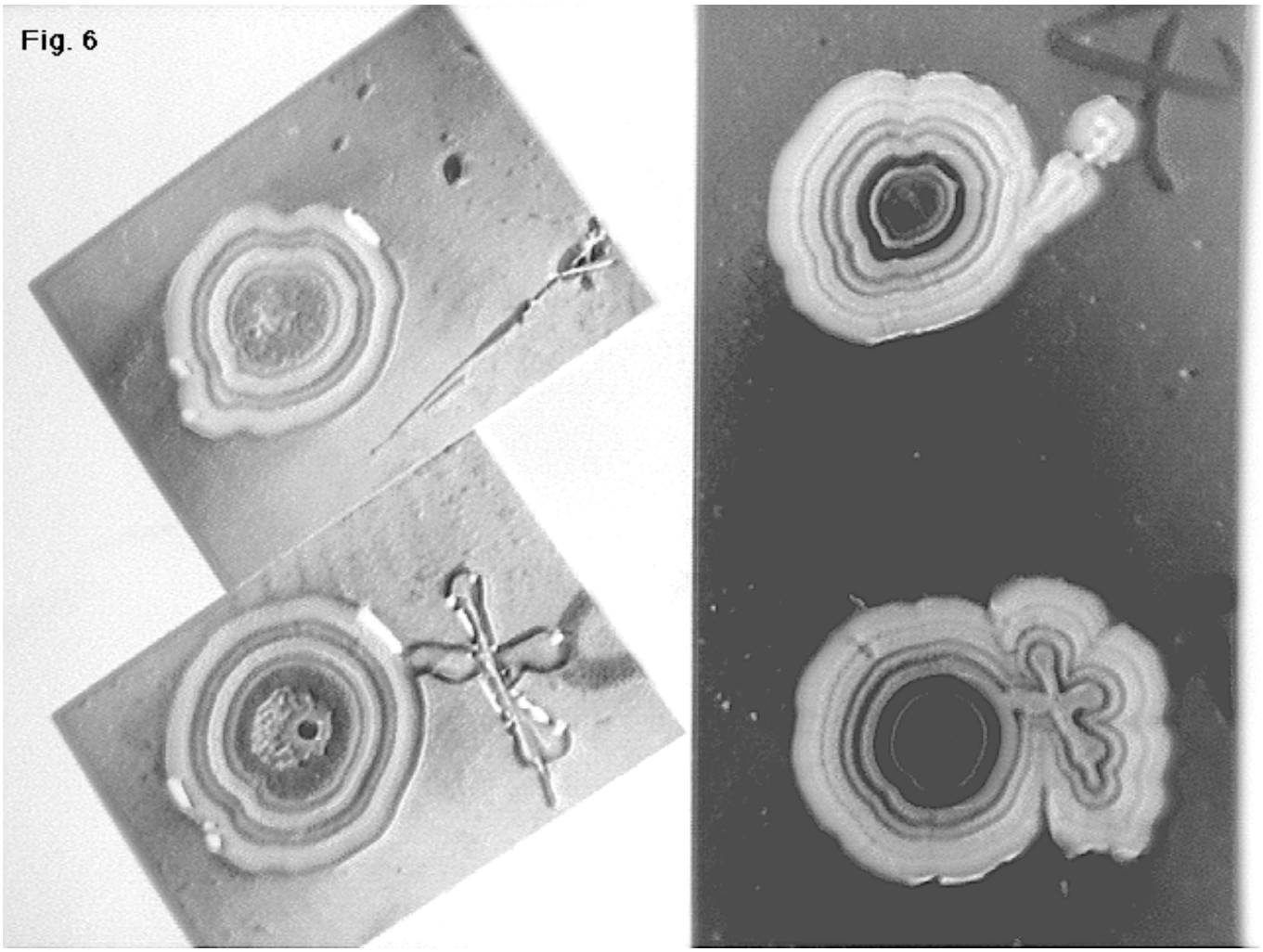


Figure 16. The effect of glass fibers on concentric ring patterns. Duplicate colonies were inoculated on XGal indicator agar with drops of a single *dev1891::lacZ* culture. After one day of incubation at 37° C, glass fibers were placed on the agar next to the colonies. The left panels were photographed on day two, when the colony on the left had already contacted and coated the fibers. The right panel was photographed on day six. Note how the rings around the fibers coincided with the rings on the parent colonies and how the ring sequences began only at the point of contact. 8X magnification.

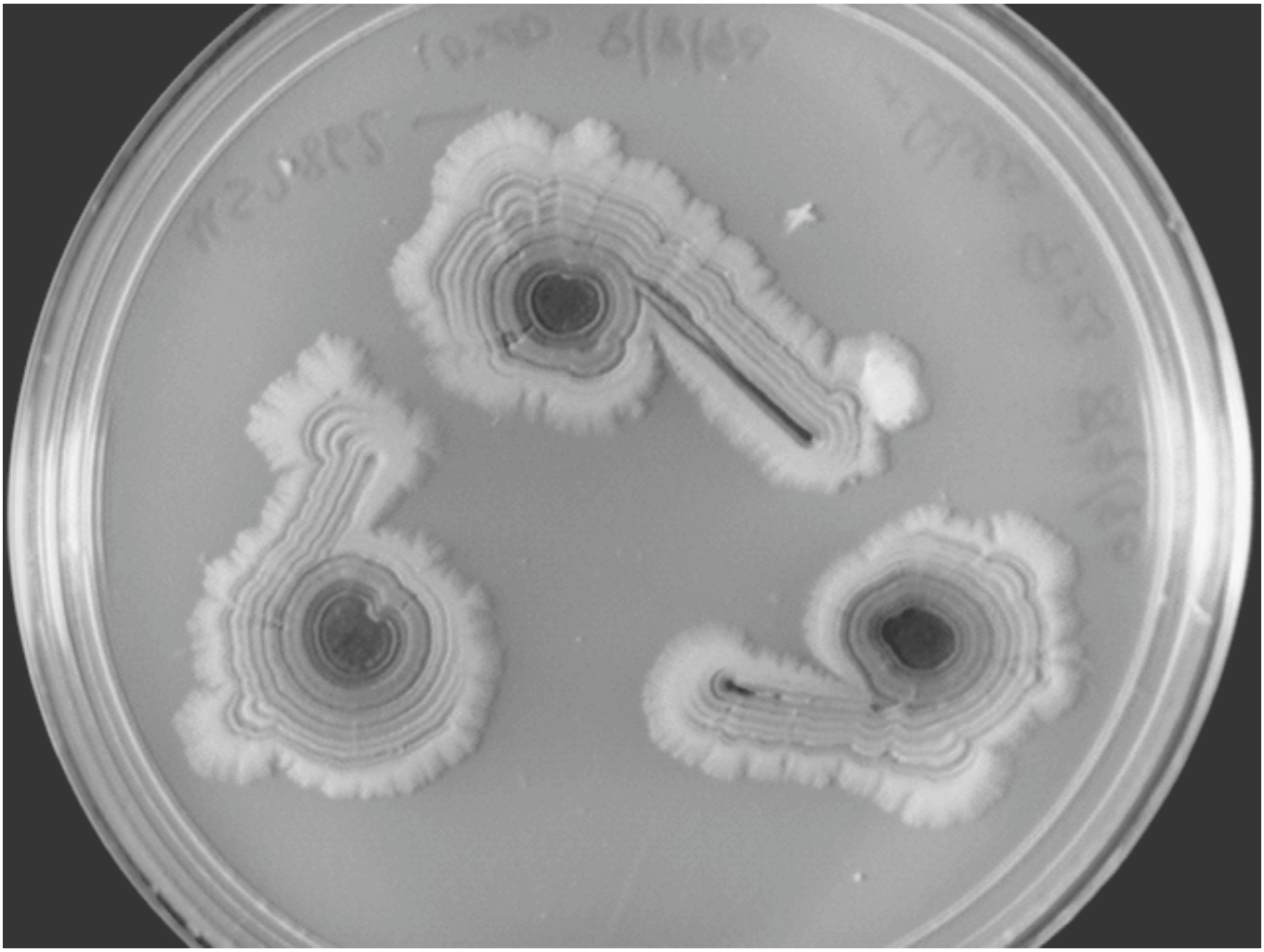


Figure 17. Attenutation on very long fibers of the signal(s) coordinating concentric ring patterns. Very long pieces of glass wool were placed next to two *dev1891::lacZ* colonies on XGal indicator agar, and incubation was continued at 37° C for 14 days. Note how the bacterial populations emerging the farthest ends of each fiber displayed a distinct, more intense level of ß -galactosidase activity. 1.6X magnification.

Figure 18. Effect of mutations altering the cAMP system on Mu*dlac* patterns. Replicate colonies of three different *E. coli* Mu*dlacII1681* strains were toothpick inoculated in rows on XGal indicator agar and incubated 6 days at 32° C. The bacteria in the top row contained a *cya* mutation inactivating the enzyme adenylate cyclase responsible for cAMP synthesis. The bacteria in the bottom row contained a *crp* mutation inactivating the cAMP Receptor Protein (CRP) which controls transcription in response to changing levels of cAMP. Note that both cAMP and CRP appear to be needed for normal Mu*dlac*

derepression and consequent ß -galactosidase expression. 1.3X magnification.

Figure 19. The effect of metabolic gradients in the agar substrate on Mu*dlac* patterns. These were 6 out of 41 evenly-spaced toothpick inoculated colonies on a XGal indicator agar plate incubated 6 days at 32° C. The edge of the plate can be seen at lower right. Note how the colonies near the edge grew larger away from the other colonies which had depleted the agar medium. A gradual decrease in ß -galactosidase expression reflecting the nutritional gradient can also be observed, but note the strict spatial localization of XGal staining in the upper regions of these colonies and throughout the more interior colonies. 4.8X magnification.