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Population Morphogenesis by Cooperative Bacteria

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Abstract. In nature, most bacteria are living as population. Such bacteria are able to generate various colony patterns under experimental conditions. Studies on generation mechanisms of these population patterns highlighted unique dynamics which work dominantly in the microbial world. Spatiotemporal analyses on familiar patterns (dense-branching-morphology-like or concentric ring-like) by *Bacillus subtilis* and *Proteus mirabilis* revealed interactive and collaborative behavior of bacteria in the structured population. Bacteria seem to know well merits of being as organized population.

1. Introduction

Bacteria are known as small unicellular organisms having ability to live independently each other. For example, *Bacillus subtilis* (*B. subtilis*) shown in Fig. 1 is able to multiply from a single cell without help of neighboring cells and to swim independently in a liquid medium by rotating flagella. Translocation of each bacterium by swimming is composed of two phases, straight smooth swimming phase and tumbling phase. Tumbling is an active hovering state (flagella are rotating reversely) to change swimming directions at random. Frequency of tumbling is under the control of information generated by comparing present and past bacterial sensings of concentrations of chemicals at changing positions. When bacteria translocate by swimming from a favorite site (e.g., high concentration of galactose) to unfavorite sites, the frequency of tumbling increases (navigation sign informing the cell is going wrong directions) and chance to change directions to the favorite places increases. Thus, biased random swimming/tumbling of a restless cell is a basic mechanism of bacterial chemotaxis.

Such chemotaxis has been reported as a critical factor for generation of symmetric patterns in a semi-solid medium by *Escherichia coli* or *Salmonella typhimurium* (BUDRENE and BERG, 1991, 1995). That is, chemo-attractant (organic acids) produced by bacteria themselves are supposed to induce bacterial gathering and uneven distribution in a petri dish. Bacterial respiration in a cluster may generate anaerobic micro-milieu which is less toxic than aerobic milieu to bacteria (MA and EATON, 1992).

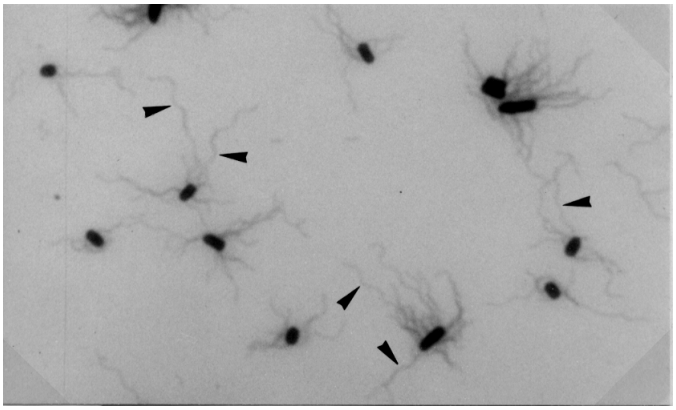

Fig. 1. Photomicrograph of flagella stained *B. subtilis*. Arrowheads indicate flagella extruding from each bacterial cell body.

In this review, two typical bacterial population patterns will be presented. However, in contrast to patterns shown by BUDRENE and BERG (1991, 1995), these two patterns are not results of chemotaxis. Most bacteria in nature are living on the surface environment (COSTERTON *et al*., 1995; MATSUYAMA, 1999) and such surface bacteria are unable to swim freely. Surface translocation of motile bacteria is designated as swarming and discriminated from swimming behavior. Bacteria on the surface are translocating by making a functional cell population. Modes of coordination making such a swarming population are different in response to biological and physicochemical factors. Analyses of characteristic population patterns produced by collective bacterial behaviors are informing us of real feature of sophisticated life of bacteria (MATSUYAMA and MATSUSHITA, 1995, 1996; SHAPIRO, 1998).

2. Unique Dynamics in the Bacterial World

2.1. Changing patterns under experimental conditions

B. subtilis is a familiar bacterial species in Japan. We can isolate *B. subtilis* from Japanese traditional food "natto" (fermented soybeans), straws, and our skin. Point inoculation of this bacterial species at the center of a sterile agar medium in a petri dish and subsequent cultivation will result in appearance of growing and spreading bacterial population (colony) with characteristic patterns (MATSUSHITA, 1997). Morphologies of these colonies have been shown to be dependent on cultivation conditions (OHGIWARI *et al*., 1992) as shown in Fig. 2. Concentration of agar and nutrients are critical factors and shown in the abscissa and the ordinate in this diagram. Agar is a solidifying agent of the medium. When agar concentration is less than some value, *B. subtilis* is able to translocate by flagella rotation. Areas C, D and E correspond to such condition permitting bacterial active translocation. In areas A and B, bacteria are unable to spread by flagella rotation, and the colony growth is achieved by volume increase of multiplying cell population. Such

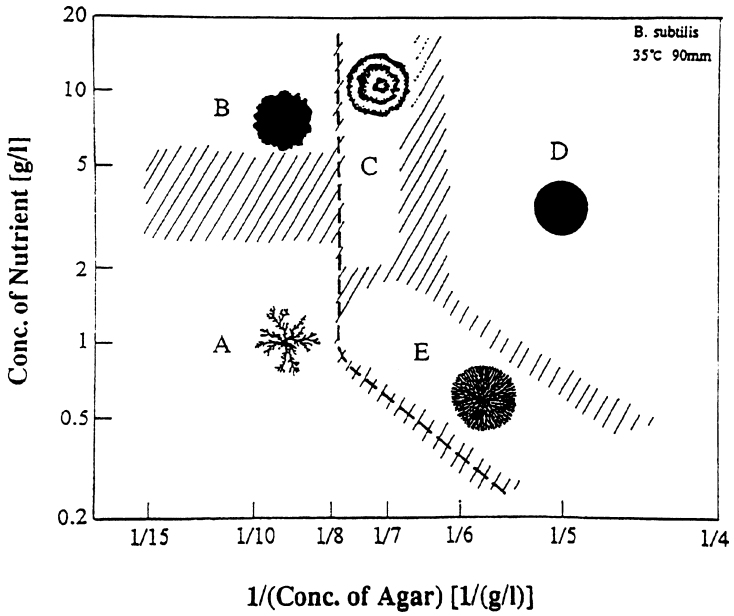

Fig. 2. Phase diagram of colonies of *B. subtilis* OG-01 (wild type). Colony patterns are classified into five types, i.e., DLA-like (area A), Eden-like (area B), concentric-ring-like (area C), homogeneously spreading disk-like (area D) and DBM-like (area E) patterns.

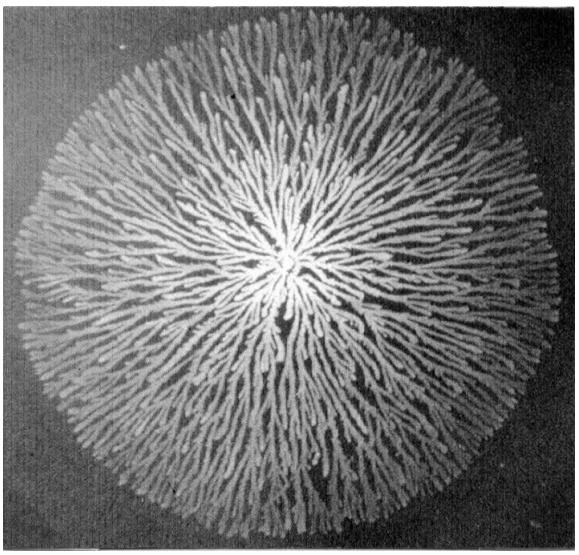

Fig. 3. DBM-like colony of *B. subtilis* in area E.

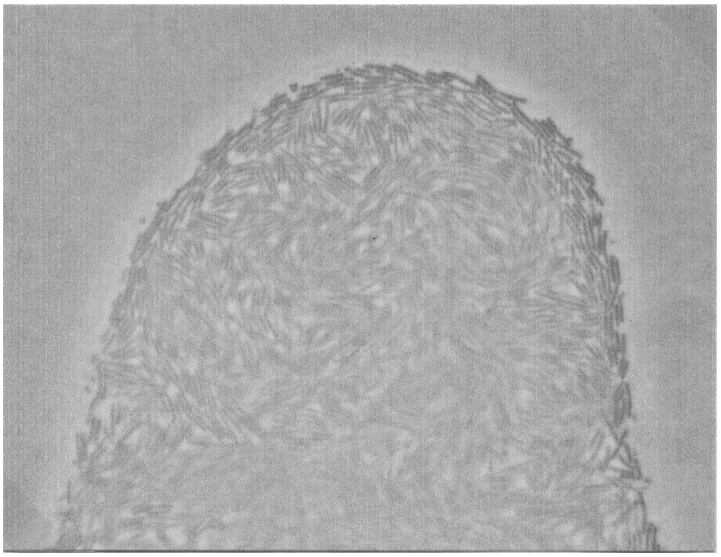

Fig. 4. Photomicrograph of a growing branch tip of DBM-like colony of *B. subtilis*. Random swirlings of inside cells (blurred images) are remarkable in contrast to non-moving cells along the branch wall.

spreading proceeds slowly (2–4 weeks are necessary) and must be distinguished strictly from morphogenesis by bacterial active migration which proceeds in 1–3 days (MATSUYAMA and MATSUSHITA, 1999). Simple phenomenological studies will miss this critical point. So, precise specific studies with immotile mutants are necessary to avoid confusion (MATSUYAMA *et al*., 1989; OHGIWARI *et al*., 1992). Mechanisms of morphogenic growth of bacterial colony in the areas A and B have been revealed by experimental investigations referring the theoretical models (MATSUSHITA and FUJIKAWA, 1990; MATSUYAMA and MATSUSHITA, 1993, 1995; WAKITA *et al*., 1997; MATSUSHITA, 1997).

Other bacterial species also demonstrate various population patterns in response to experimental conditions (COOPER *et al*., 1968; MATSUYAMA *et al*., 1989; MATSUYAMA and MATSUSHITA, 1992; HARSHEY, 1994; BEN-JACOB *et al*., 1994; SHIMADA *et al*., 1995; NAKAHARA *et al*., 1996; RUDNER *et al*., 1999).

2.2. Structured cell population for surface spreading

The colony pattern in the area E (Fig. 3) seems as a biological example of the dense-branching morphology (DBM) which is familiar in various fields (BEN-JACOB and GARIK, 1990; VICSEK, 1992; MATSUSHITA, 1997). Why does multiplying bacterial population spread radially by forming many thin branches? Microscopic examination of the branch revealed remarkably structured population of bacteria at the extending end (MATSUYAMA *et al*., 1993; WAKITA *et al*., 1998). As shown in Fig. 4, extending branch end is composed of two types of bacterial cells, not-moving cells which are forming branch walls (including the branch tip wall), and moving cells which are making a swirling cell cluster just beneath

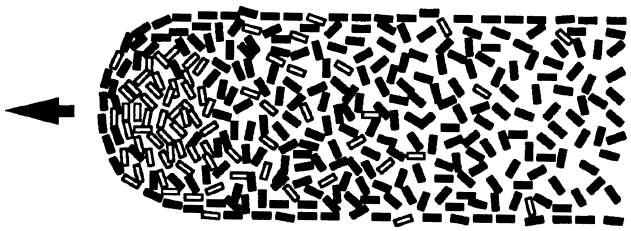

Fig. 5. Schematic illustration of an extending branch. Open box indicates an vigorously swirling bacterial cell. Closed box indicates an inactive cell. Branch extending direction is indicated by an arrow.

the tip wall. The outermost tip wall cells do not move by themselves, instead, the inner swirling cells are pushing out little by little these tip wall cells. Thus, in contrast to the moving behavior of individual cells in swirling cluster, the extension of the branch is quite slow and unidirectional as shown in Fig. 5. Since bacteria have been thought to behave independently of each other, special arrangement of differently working cells in the branch population of *B. subtilis* (division of labor in the bacterial world) was unexpected finding (MATSUYAMA *et al*., 1993; MATSUYAMA and MATSUSHITA, 1995). It is interesting that similar differential cell arrangement is present in the tissue at the growing ends of the multicellular organism "plant".

2.3. Physical factors inducing collective behavior of bacteria

It is curious that bacteria moving on the surface are always getting together. A single bacterium separated from others on the surface seemed to be immobile. Even in area D where bacterial population seemed to be spreading homogeneously, translocating bacteria in the spreading front were making raft-like clusters (WAKITA *et al*., 1994). In the world of bacteria which are so small, effective working forces are quite different from those effective on organisms of the human size. Instead of gravitation, inter-molecular forces working on the surfaces of microorganisms are critical. With the size reduction of organisms, ratio of surface area to volume of the organism will be greater and surface forces working on the organism will be more effective. In addition, water which is indispensable for life has outstandingly strong surface tension. Thus, water around microbes will restrain the movement of small unicellular organisms on the surface environments (MATSUYAMA *et al*., 1992; MATSUYAMA and NAKAGAWA, 1996). To overcome such restricting situation in the small scale surface environment, bacteria seem to evolve the translocation mechanisms by arranging collective cell groups. So, in the area E where nutrients are not enough for energy supply to every bacterial cell, active cells are gathering at the special structural part in the population. Developing this functional and differentiated population, bacteria as a whole (including immobile cells) seem to be spreading efficiently on the nutrient-poor medium surface. In spite of ubiquitous presence of DBM-like pattern in nature, its generation mechanisms have not been investigated by experimental micro-scale analyses. Morphogenic units of DBM-like pattern shown here are living bacteria which are clearly visible under an optical microscope. As shown herein, the microscopic video tracing of the development processes revealed the precise figures leading to the characteristic pattern,

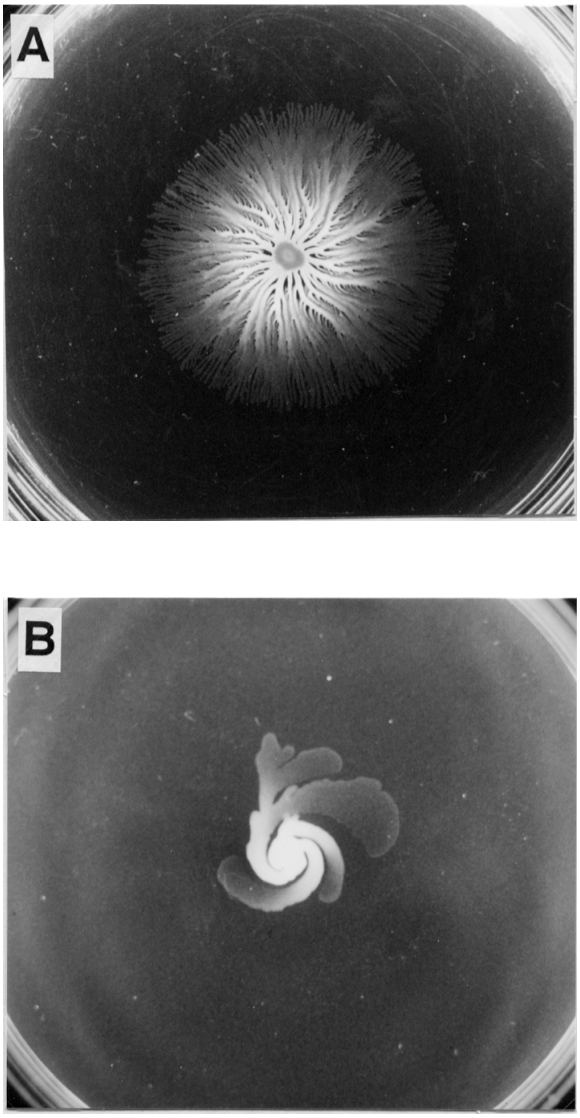

Fig. 6. A swarming colony of *S. marcescens* NS 38 with radiating many thin branches (A). A swarming colony of *S. marcescens* NS 38-09, a mutant defective in the production of biosurfactant "serrawettin W1" (B).

and the ingenious strategy of bacteria under the adverse conditions in the small scale world (MATSUYAMA and MATSUSHITA, 1995).

2.4. Role of biosurfactants in surface occupation

The second strategy of bacteria to overcome containment by surface tension of water

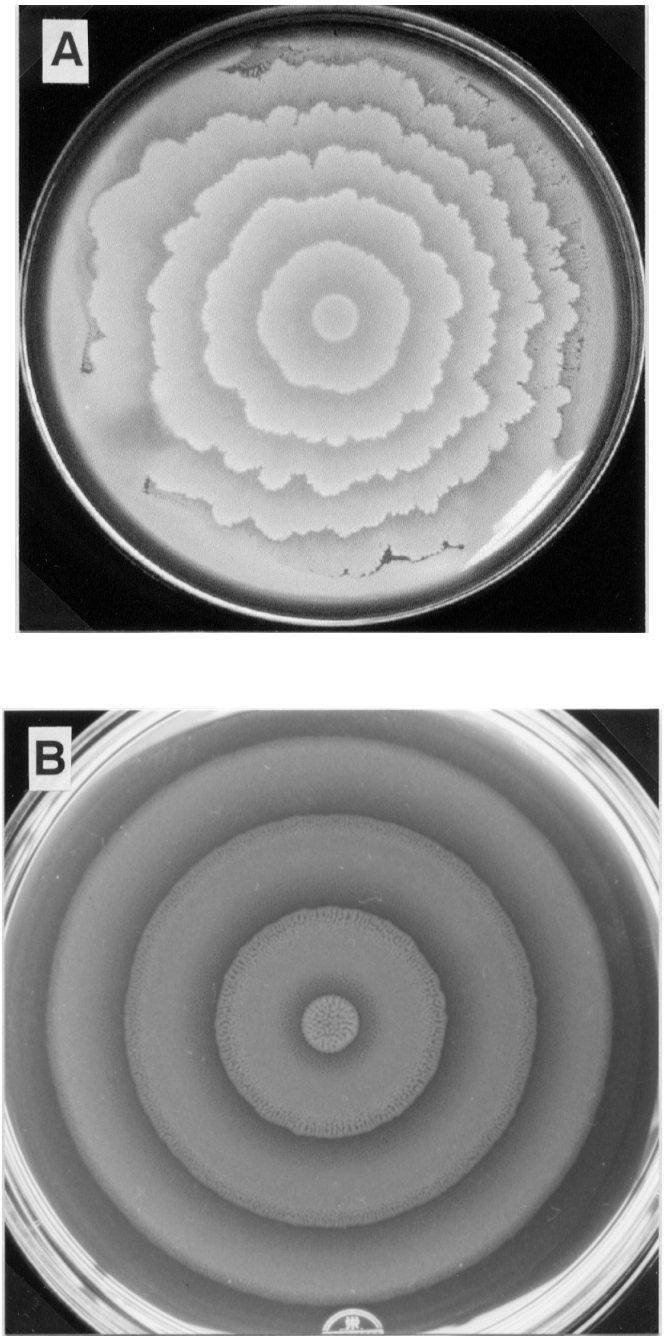

Fig. 7. Concentric ring-like colony of *B. subtilis* (A) in area C, and *P. mirabilis* (B) on hard agar medium.

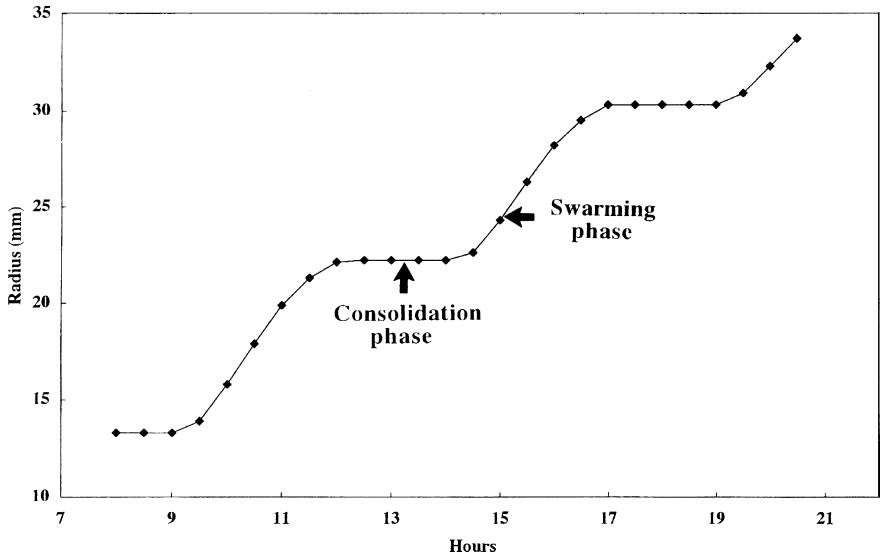

Fig. 8. Growth curve of a concentric colony of *P. mirabilis*. Lag phase is a period from time 0 to 9 hour. Radius of a colony was measured at intervals.

is the production of surface active agents. The surfactant effects are remarkable to motile and even to immotile bacteria (MATSUYAMA *et al.*, 1989, 1990, 1992, 1995). Fractal colony growth of immotile *Serratia marcescens* (*S. marcescens*) population was promoted by the bacterial surfactant "serrawettin" (MATSUYAMA *et al.*, 1989). Spreading patterns made by motile bacteria is different between population of an *S. marcescens* wild type (a producer of serrawettin) and that of the serrawettin-less mutant (Fig. 6). In the absence of surfactant, the bacterial population develops thick slowly extending branches with strong tendency to turn right. Purified biosurfactants which were externally supplied to surface population of surfactant-less bacterial strains were also effective (MATSUYAMA *et al.*, 1992; MATSUYAMA and MATSUSHITA, 1993; MATSUYAMA and NAKAGAWA, 1996). In the presence of surfactant, extension of thin branches were speedy and the tip splitting for generation of new branches was frequent.

3. Spatiotemporal and Cellular Characterization of a Concentric Colony

3.1. Time course of a concentric pattern generation

The colony pattern formed by *B. subtilis* in the area C in Fig. 2 is also intriguing (Fig. 7A). The *B. subtilis* colony with concentric rings is similar to a well known giant colony pattern (Fig. 7B) of *Proteus mirabilis* (*P. mirabilis*). In this review, well-studied ring pattern of *P. mirabilis* will be described in depth. Details of *B. subtilis* periodic colony in the area C have been published elsewhere (WAKITA *et al.*, 2001). The concentric ring colony of *P. mirabilis* has been calling attention of many scientists in a century long history of bacteriology (WILLIAMS and SCHWARZHOFF, 1978). Because, the growing process of the

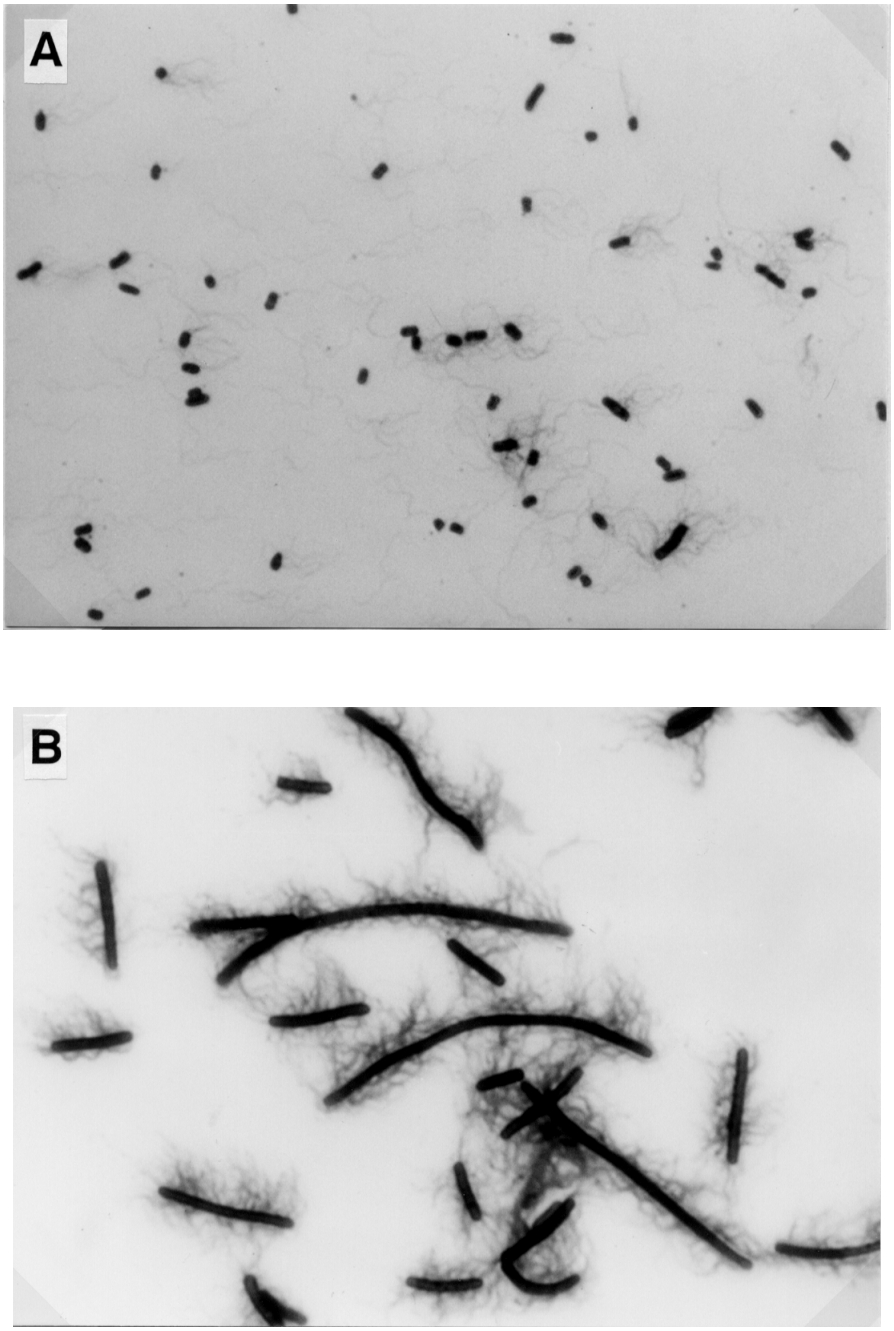

Fig. 9. Photomicrographs of flagella stained *P. mirabilis*. A, vegetative cells from the center of a concentric colony. B, swarmer cells from the swarming zone.

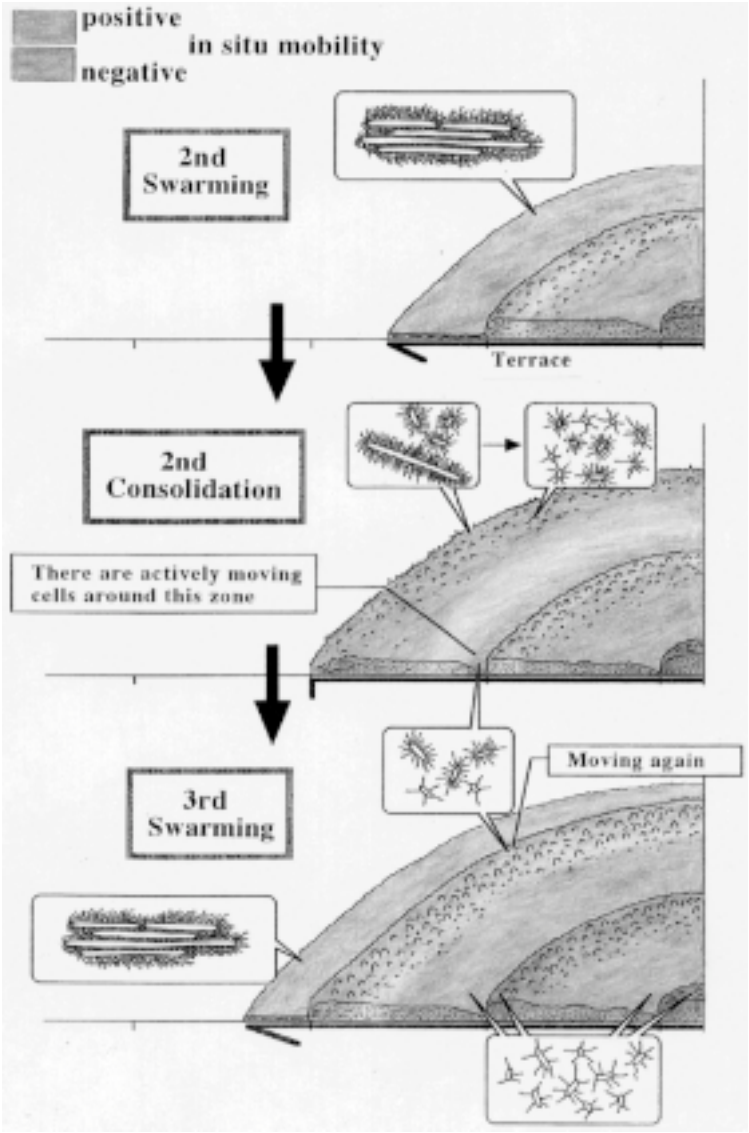

Fig. 10. Dynamic distribution of mobile cells in a swarming colony of *P. mirabilis*. Vertical arrows indicate time lapse. Horizontal arrows indicate swarming direction of the colony.

P. mirabilis colony has two peculiar characteristics, i.e., spatiotemporal periodicity and cellular differentiation (ALLISON and HUGHES, 1991). That is, the spreading of bacterial population dose not proceed continuously. As shown in Fig. 8, the colony grows intermittently. Three growth phases (lag, swarming, and consolidation phases) are present

after point inoculation of bacteria on the agar surface. In the lag phase, bacterial multiplication proceeds restricted at the inoculated site, and when the cell density reaches at some threshold value, the first swarming phase starts by expanding a circular colony margin synchronously (RAUPRICH *et al*., 1996). It is noteworthy that morphology of differentiated swarming cells (swarmer cells) are quite different from dedifferentiated multiplying cells (vegetative cells) at the inoculated site (Fig. 9). Swarmer cells are elongated hyperflagellated cells and dedifferentiate to vegetative cells when the first consolidation phase starts. In the consolidation phase, the margin of the circular colony does not expand and vegetative cells devote themselves to multiplication in the outer half of the ring. After some time interval, the second swarming phase starts by abrupt and synchronous appearance of many group of swarmer cells from the circular margin of thick cell population (the terrace) made during the foregoing consolidation phase. Thus, the concentric ring morphology of a *P. mirabilis* colony is formed by this periodic swarming/consolidation cycle (Fig. 10).

3.2. Periodical changes in developing concentric rings

P. mirabilis is famous for prompt spreading on the surface of a medium which contains high concentration (1.0–3.0%) of agar. Other bacterial species (e.g., *Escherichia coli*) have been reported to swarm on lower agar media (HARSHEY and MATSUYAMA, 1994). For efficient spreading and substantial occupation of the medium surface, *P. mirabilis* (nonproducer of biosurfactant) seems to adopt chronological separation of two indispensable processes (i.e., cell multiplication and swarming). Consequently, changes from vegetative cells to swarmer cells, and vice versa may be occurring at the special timing and site. To clarify these points, the growing concentric colony was examined for morphology and moving activity of composing cells. As shown in Fig. 10, a growing colony has a dynamic structure with cells differing in morphology and moving activity. It is noteworthy that there are actively moving cells at the inside area in an outermost consolidation ring. From the midst of the second swarming to the third swarming, cell modes of moving/nonmoving at the marginal part of a colony seem to transit gradually to the inner of the ring. When the third swarming starts, the terrace of the second consolidation ring which was once full of non-moving cells become full of moving cells. Presumably the latest (second) consolidation terrace may become the source of new active swarmer cells (ITOH *et al*., 1999). Actually, percentage of elongated cells (longer than 5 μm) in total cells in the terrace at the swarming phase was higher in comparison with that at the middle stage of the foregoing consolidation phase (MATSUYAMA *et al*., 2000).

3.3. Mystery of migration cease

It has been reported that time interval between the swarming end and the next swarming end (time for the one cycle of swarming and consolidation) is constant for each *P. mirabilis* strain, and a duration ratio of swarming to consolidation is dependent on the medium composition (RAUPRICH *et al*., 1996). For *P. mirabilis* strain ATCC 29906, the combined time is *ca*. 5.1 hour. At the end stage of the swarming phase, swarmer cells at the front area (not at the rear area of the ring as shown in Fig. 10) cease to translocate before they divide into shorter vegetative cells. Why do these swarmer cells become immobile? Are these cells energetically exhausted or functionally disabled? Swarmer cells taken from the front in the transitional stage from swarming to consolidation phase were examined for

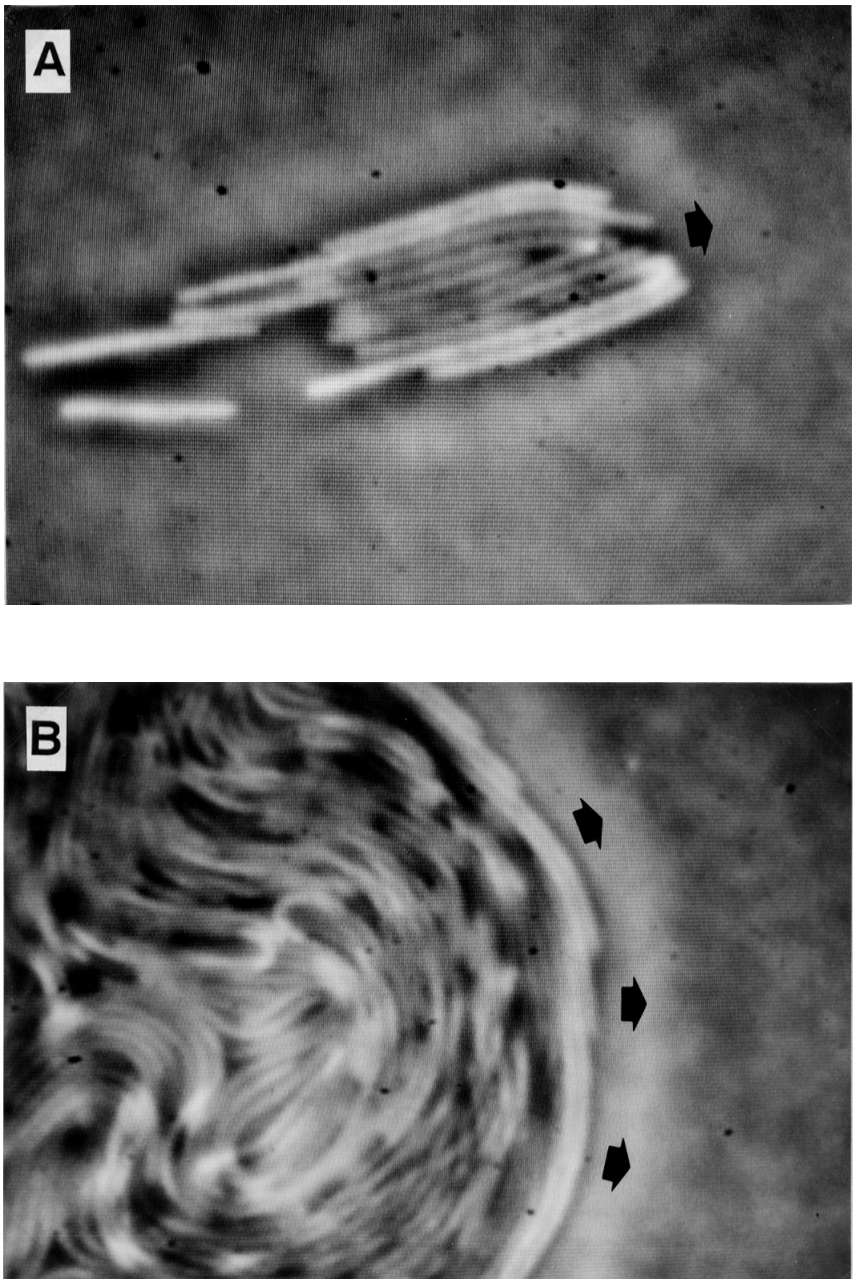

Fig. 11. Photomicrographs of translocating *P. mirabilis* cell clusters. A, a cluster on low agar medium. B, a giant cluster on hard agar medium. Arrows indicate cluster-translocating (or expanding) directions. In B, a cluster is expanding to the direction perpendicular to the sliding directions of each cell.

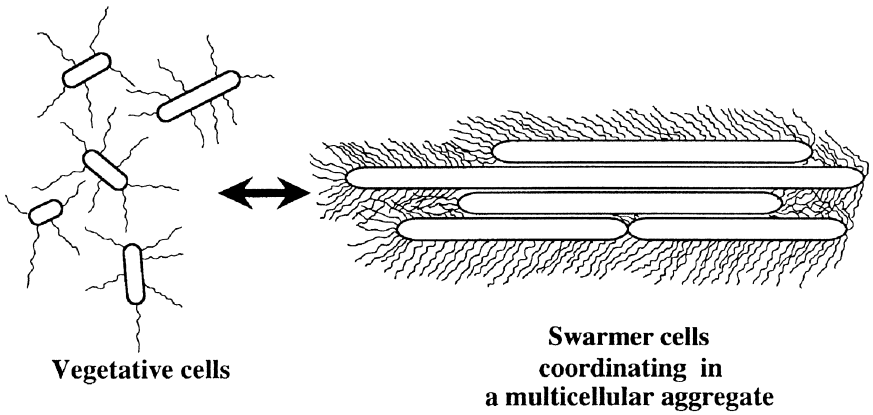

Fig. 12. Schematic illustration of vegetative and swarmer cells. Vegetative cells are immobile on the surface in spite of having flagella. Swarmer cells are easy to contact and slide each other and translocate little by little as a group.

their swimming speed in a viscous solution (bacteria swim better in a lightly viscous solution). Their swimming activity was shown to be normal (MATSUYAMA *et al.*, 2000). So, nontranslocating elongated hyperflagellated cells in the ceasing front is functionally intact. Other factors seemed to be working for translocation halting.

3.4. Collective behavior and chemotaxis

In the viscous solution, elongated hyperflagellated swarmer cells were swimming solely and independently without making multicellular clusters. On the other hand, swarmer cells on the surface of solid media never translocate solely, as mentioned previously in Subsec. 2.3. They were migrating always as multicellular clusters. The size of a cluster is dependent on the hardness of the agar media. When the concentration of agar was low, swarmer cells formed a small raft as shown in Fig. 11A. On a harder agar plate (e.g., 1.5% agar concentration), swarmer cells aligned along the colony front-line and formed disk-like arrangement (Fig. 11B). Irrespective of the size of a cluster population, swarmer cells were sliding each other, back and forth along their elongated cell bodies, consequently they migrated slowly (in comparison with a speed of a single cell sliding) as a whole on the surface. Thus they were making an interactive cell group for surface translocation (Fig. 12) which is in different type of cooperation in comparison with the "division of labor" shown in Fig. 5.

For the periodic swarming behavior of *P. mirabilis*, chemotaxis has been proposed (LOMINSKI and LENDRUM, 1947) as a mechanism. However, as described already, mechanisms of chemotaxis is dependent on restless random swimming of independent cells. Such chemotactic mechanisms seem to be improper for bacteria migrating as a

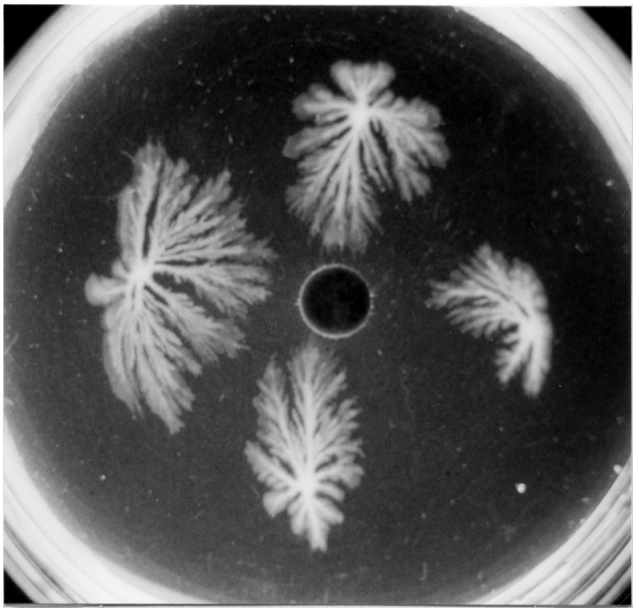

Fig. 13. Chemotaxis-like behavior of *P. mirabilis*. At the center of minimal agar medium, a paper disk containing L-alanine (2.5 μmol) was placed, and at the four points around the disk, *P. mirabilis* was point inoculated, then incubated for 41 hours.

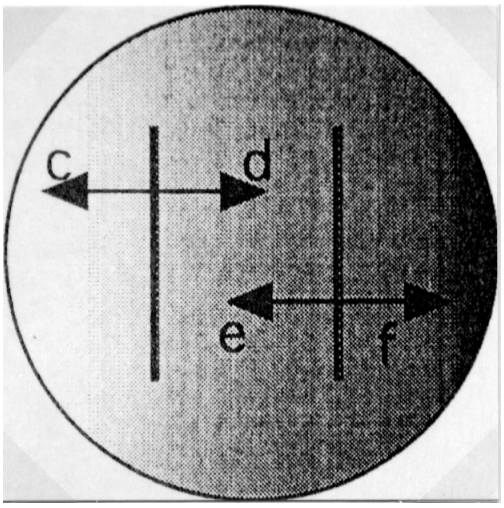

Fig. 14. A gradient plate for examination of surface chemotaxis. Concentration of alanine is higher at the right side of the plate. Vertical two lines indicate bacterial inoculation lines. Arrows from these lines indicate colony extending length. When surface chemotaxis occur, length of the d-arrow will be longer than that of the e-arrow. In repeated experiments with *P. mirabilis*, length of the d-arrow was always shorter than that of the e-arrow.

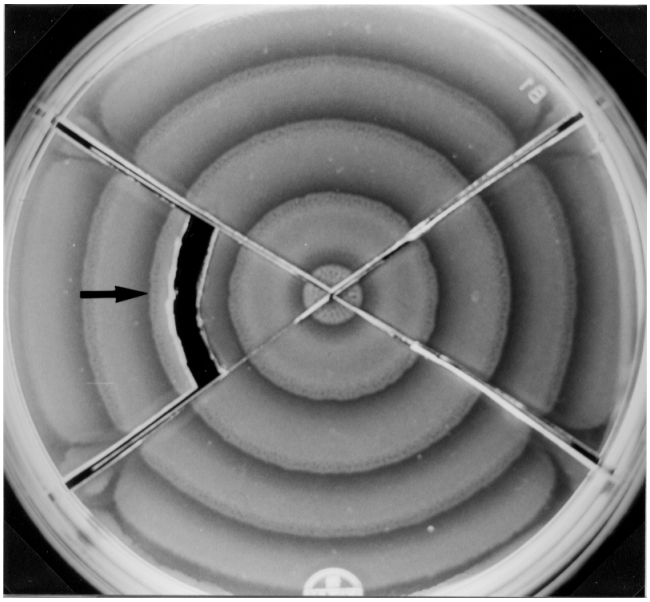

Fig. 15. Focusing of critical cell population necessary for the achievement of ongoing swarming program. When the front line of a growing colony reached the point (indicated by an arrow), the colony was cut by a knife to isolate cell population at the frontal zone from the central and surrounding area. Thereafter, cultivation was resumed. The swarming from the isolated zone progressed with identical time schedule to the neighboring area.

coordinating population on the surface. In Fig. 13, however, bacterial surface behavior suggesting chemotaxis is observable. Toward a paper disk containing amino acid (alanine), a population of *P. mirabilis* seems to be extending branches. In the true meaning, chemotaxis is a translocation behavior by sensing the gradient of concentration of chemicals. Phenomenon shown in Fig. 13 may, however, be simply explainable by the promotion effect on cell migration. To confirm this, the experiment designed in Fig. 14 was done. No positive results showing chemotaxis of *P. mirabilis* population on the surface were obtained (TAKAGI, 1998). In the additional experiment, promotion of bacterial growth was not recognized in the presence of alanine. Thus, chemotaxis-suggesting behavior of *P. mirabilis* in Fig. 13 may be due to the swarming promotion effect of alanine. Such effect (simple promotion or inhibition of swarming) must be discriminated strictly from the chemotaxis.

3.5. Preventive trials against swarming cease

Then we looked for the way to prevent the swarming cease which is expected to occur soon. Accumulation of inhibitory metabolites at the swarming front seemed to be responsible for the swarming cease. This possibility has also been shown to be negligible by the following experiments.

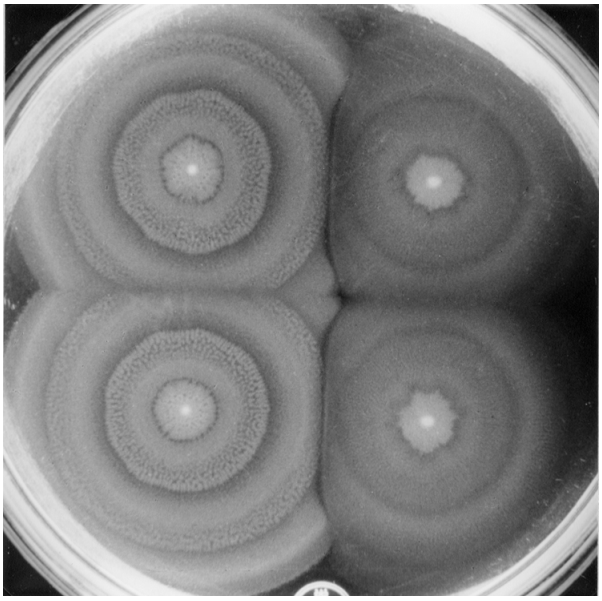

Fig. 16. Repulsion effects between different strains of *P. mirabilis*. At the left side two points, strain ATCC 29906 was point inoculated and at the right side two points strain PRM was point inoculated. A clear repulsion line was present between colonies of different strains, and absent between colonies of the identical strain.

A sectorial part of a swarming ring colony was excised and immediately replica-printed onto fresh agar medium. When the mother cell population in the midst of swarming phase, the replica-printed cell population continued the ongoing swarming. Whereas, replica-printed cell population from the ending stage of swarming phase ceased the swarming as scheduled and started the swarming after the scheduled consolidation time interval (MATSUYAMA *et al.*, 2000). Since printed fresh medium has no history of bacterial multiplication, participation of medium-accumulated waste products in swarming halting is improbable. The results of these experiments also indicate that the diffusion of nutrients is irrelevant to the periodic colony growth. Experimental analyses on the concentric ring colony of *B. subtilis* (Fig. 7A) indicate the same conclusion (WAKITA *et al.*, to be published elsewhere).

Lastly we tried to disrupt the internal population structure of the swarming ring. Only by this procedure of mixing cells randomly in the ring population by a platinum loop, the scheduled ceasing of swarming was canceled. Thus, specific population structure was shown to be important for regular periodic swarming (MATSUYAMA *et al.*, 2000).

3.6. The schedule practitioner of the periodic growth

The experiments described above seem to indicate that bacterial intrinsic program determines the swarming time course. To identify the bacterial group specially working for the programmed spreading of *P. mirabilis*, sub-population of swarming colony was

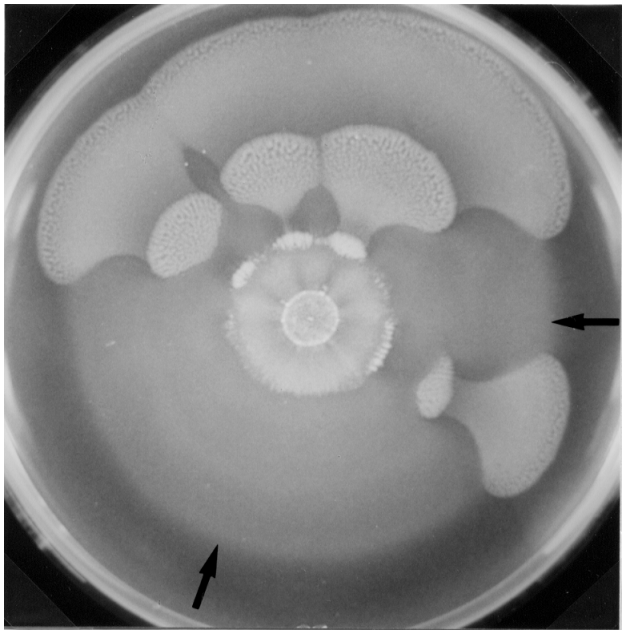

Fig. 17. A giant colony made after point inoculation of a mixture suspension of strain ATCC 29906 (labeled with GFP, so looks as a brighter cell population) and strain PRM (dark cell population, indicated by arrows). Cells of each strain seem to have excluded each other and made discrete swarming subpopulation respectively.

isolated and examined for the schedule exerting ability.

It was shown that a small cell population locating at the colony spreading front is enough for keeping the ongoing periodic colony growth (Fig. 15). Cells populating at the colony center were not responsible for scheduled ring formation. For details, please see our recent article (MATSUYAMA *et al*., 2000). So, central control of programmed spreading for concentric ring formation may be absent. In addition, the swarming is not a stereotypic expression of the schedule. The bacteria seem to start the swarming by recognizing the threshold cell density of their own (RAUPRICH *et al*., 1996; BELAS *et al*., 1998; ITOH *et al*., 1999).

4. Biological Requirements for Bacterial Coordination

4.1. Many questions

As described above, bacteria are organisms living cooperatively by forming a functional population. Many questions arise by facing those new findings. How are they recognizing their cooperative colleagues? How are they exerting productive collective behavior? Simple back and forth sliding with each other will be direction-less migration. Bacteria forming a raft (Fig. 11A) seemed to be sliding by a common intention with regard to the migration direction. How are they communicating with each other for the productive behavior? Are there extracellular communication substances? What about the direct

contact communications?

Recently, signal substances for cell-to-cell communications have been discovered from many species of bacteria. Several review articles have been published (SHAPIRO, 1998; GREENBERG, 1999).

4.2. Are they really recognizing cooperative partners?

When an identical strain of *P. mirabilis* was separately point inoculated on the same agar plate, two growing colonies fuse together. However, when different strains were point inoculated separately on the same agar plate, concentric ring colonies mostly do not fuse each other (Fig. 16). Thus, *P. mirabilis* seems to have ability to reject different strains at the population level. Does this occur at the cellular level when cells of two strains were forcibly mixed and point inoculated on agar medium?

To answer this question, we examined pattern change of a concentric ring colony formed by mixed two-strain population. Fluorescent label (green fluorescence protein, GFP) was tagged to one of the strain to examine the distribution of labeled *P. mirabilis* strains in the concentric ring colony. The result is shown in Fig. 17, the presented ring pattern shows that two strains did not work together in swarming. Different strain cells seem to make swarming population by excluding each other (MATSUYAMA *et al.*, to be published elsewhere). If they did not recognize the difference of each other and made cooperative swarming (as a colony made by a single strain, shown in Fig. 8B), mixed population might have distributed evenly without making irregular ring patterns such as a giant colony in Fig. 17.

5. Prospects

Various colony forms made by bacteria are precious experimental objects for analysis of pattern formation mechanisms. Bacteria have a suitable size for microscopic observation and a representative morphogen in a small scale world. Time and space for the development of the colony pattern are also appropriate for experiments. Patterns in nature are telling us something. Investigations with bacteria on characteristic patterns are disclosing details of physical and biological principles in nature.

Bacteria are living in nature as various types of population (e.g., biofilms on the surface). Such a life-style of microbes are known to have a intimate relationship to our human life. More than 60% of infectious diseases are mentioned to be due to microbial biofilms. Studies on the morphogenesis by the bacterial population are opening a new way for revealing the cooperative population strategy of bacteria.

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