GIANT COLONY PATTERNS EXHIBITED BY A PSEUDOMONAD

GEORGE H. BORNSIDE and ROBERT L. RICHARDSON

Department of Bacteriology, State University of Iowa, Iowa City, Iowa

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A bacterial colony on the surface of a solid medium is regarded as a grouping of cells arranged as a result of physical restrictions imposed by the surface of the medium. It is limited in size by the physical nature of, and changes occurring at, this surface (Knaysi, 1951). In a biological sense the bacterial colony is a macromorphological collection of cells rarely found in nature (Pringsheim, 1955). When a Pseudomonas species displayed several unusual colony patterns it appeared that the recording of this phenomenon, together with attempts to explain the factors responsible for the colony patterns, might contribute to the knowledge of bacterial macro-morphology.

MATERIALS AND METHODS

The organism used in these studies was one of several isolated from an aqueous suspension of dental calculus which had been inoculated with woodland soil and incubated at 20 C for two weeks. Routine diagnostic media were employed for determining physiological characteristics and the type of flagellation was determined by electron microscopy.

Except where indicated, all plates were incubated in an inverted position upon a level surface at 26 to 30 C.

The principal media used were gelatin agar and blood agar. Gelatin agar contained 3 per cent gelatin (Difco), 3 per cent meat extract (Difco) and 1.5 per cent agar, and was adjusted to pH 7.6 prior to autoclaving. Blood agar was prepared using a meat infusion agar base to which was added 5 per cent defibrinated sheep blood.

Four plates (15 to 18 ml) of both media were prepared and stored at 10 C; they were rarely used if stored longer than 1 week. Prior to inoculation, plates were placed at 25 to 30 C for 1 to 2 hr. A stab inoculation into the media in the center of the plates was made with 18 to 48 hr growth of the pseudomonad from a giant colony grown on either blood or gelatin agar.

The effect on colony morphology which occurred when the organism was stabbed upon medium containing supplements to nutrient agar (basal medium) was examined. The following media were tested for their fitness to support growth of giant colonies: (1) basal, and basal containing (2) 5 per cent defibrinated sheep blood, (3) 20 per cent sterile sheep serum, (4) 40 g/L meat extract (rather than the usual 3 g/L), (5) 30 g/L gelatin (Difco), (6) 0.01 per cent yeast extract (Difco), (7) 0.1 per cent sorbitan mono-oleate. Plates containing 15 to 18 ml of these media were stored at 10 C overnight and allowed to adjust to 26 C for 2 hr prior to stab inoculation of 18 hr gelatin agar growth of the pseudomonad.

The effect of the surface moisture of the medium on giant colony formation was examined. Flasks containing 40 ml of sterile gelatin agar were prepared, cooled to 30 C, and the contents of a single flask dispensed into the lower portion of a petri dish which was subsequently covered with a petri dish lid having a disk of filter paper wedged against the inner surface. After standing at 26 C for 1 hr these lids were replaced with either a sterile earthenware (experimental) or glass (control) cover for varying periods up to 2.5 hr. Following appropriate coverage, all covers were replaced with sterile glass lids. A stab inoculation into the medium in the center of the plate was made using growth from an 18 hr giant colony of the pseudomonad on gelatin agar. Five plates were prepared per test period and inoculated consecutively without renewing the inoculum between stabs. As a biological control, one plate per test period was inoculated with growth from a 24 hr nutrient agar slant of Proteus vulgaris ATCC 9927.

RESULTS

Identification of the organism. The organism which displayed the unusual giant colonies was a gram-negative, nonsporeforming rod possessing 4 to 12 polar flagella. It produced a green fluores-
Figure 1. Growth of giant colonies following a stab inoculation on gelatin (A, C-G), blood (B) and eosin methylene blue lactose (H) agar plates. Three major patterns are shown.

Type I colonies (C and G). Narrow linear growths which occasionally branch. May be I-, Y-, X-, or L-shaped.

Type II colonies (A, B, D-F). Numerous branching filaments radiating from a small initial swarm growth.

Type III colonies (H). Swarm growth with undulate edge.

cent pigment, liquified gelatin, grew poorly at 37°C, and coagulated milk with subsequent peptonization. In arabinose and xylose broth, slight acid was produced; a pellicle or surface growth occurred in 11 other common carbohydrate media without acid being formed.

Of the recognized Pseudomonas species possessing similar characteristics, this organism most closely resembled Pseudomonas myxogenes (Breed et al., 1948). The major differences between our isolate and P. myxogenes (Fuhrmann, 1907) were that the latter exhibited wider fermentative capacities and produced indole in a suitable medium, whereas our organism did not. Despite these differences the organism was tentatively identified as P. myxogenes. Judging from the frequency of reports on various pseudomonads one must conclude that P. myxogenes is one of the less frequently encountered members of this genus.

Morphology of giant colonies. Giant colonies, larger than 2 cm and frequently 7 to 8 cm in diameter, were obtained following stab inoculation of the organism on gelatin agar or blood agar. Surface growth or any portion of a giant colony provided a dependable inoculum. Three
unusual types of giant colonies appeared during 96 daily transfers on blood agar and gelatin agar (figure 1).

Type I giant colonies (figure 1C and G) usually possessed an outline similar to the capital letters I, X, L, or Y. They varied in length from 4 to 9 cm, and were essentially a linear colony displaying branches. These colonies were opaque and mucoid on blood or gelatin agar and attained a maximal size in 24 hr. Type I patterns were most frequently seen during the first 30 transfers, and were demonstrated best on gelatin agar although they occurred on blood agar.

Type II giant colonies (figure 1A, B, D-F) consisted of a central circular growth (mother colony) of 1 to 5 cm in diameter, from which radiated numerous branching filaments which were 3 to 4 mm wide and 1 to 5 cm long. Located at the tip of each filament was a discrete, raised accumulation of bacterial growth (daughter colony). The morphology of these terminal daughter colonies and their manner of formation is seen in figure 2. After 20 hr incubation the filaments can be observed to be pointed and bordered along their length by a slightly raised and more concentrated growth. After 46 hr incubation the pointed filaments were rounded and a terminal daughter colony capped the end of each filament. Occasionally, the central mother colony exhibited alternate swarming and resting zones of growth (figure 3), similar to those described for Proteus by Kvittingen (1949). The essential difference between type I and type II colonies was that the former developed linearly.
from stab inoculation, whereas the latter exhibited a circular spread of 1 to 3 cm prior to the development of numerous, radial, filamentous extensions. Type II colonies were most evident during the first 50 transfers and occurred on gelatin or blood agar.

Type III giant colonies (figure 1H) were flat, circular, spreading growths 2 to 7 cm in diameter. Upon reaching maximal size these colonies developed a white, opaque, mucoid border with undulated or lobated edge. The border was similar in texture to the type II terminal daughter colonies. After 60 daily transfers this type of colony formation predominated when the organism was grown on gelatin or blood agar. Attempts to revert the type III colony to a type I or II colony were unsuccessful.

Nutritional aspects of giant colony formation. In early studies it became apparent that nutritional qualities of the medium influence, to some extent, the colony pattern of this organism. Attempts to elucidate these nutritional demands were disappointing. The basal medium failed to provide a satisfactory medium for development of giant patterns. Only a small (0.5 to 1.0 cm) circular colony developed on this medium. A similar, but larger (2.0 cm), colony formed on the basal medium supplemented with sorbitan monooleate. Addition of yeast extract to the basal medium failed to encourage giant colony formation. However, if the basal medium contained 40 g/L meat extract, growth similar in some respects to a type II colony resulted. Such colonies differed from the conventional type II colony in that the initial central swarm area was larger, and broad, arrow shaped projections replaced the usual thin, branching filaments. A larger but similar colony was seen when gelatin was used to supplement the basal medium. Sheep serum or blood added to the basal medium supported growth of typical type I or II colonies. However, after 60 transfers the serum and blood

Figure 5. A 46 hr growth (type II) on blood agar. The swarm growth (mother colony) exhibits alternate swarming and resting zones. Magnification 2X.
supplements did not support type I or II colony formation but rather, favored development of the then dominant type III colony.

The surface moisture of the medium. As indicated in table 1, the diameters of the colonies of the Pseudomonas species and P. vulgaris were reduced by drying the medium. Placement of an earthenware cover over the medium for 2.5 hr prior to inoculation decreased the diameter of the pseudomonad colony from 6.5 cm to 0.4 cm. The diameter of the P. vulgaris colony was similarly affected, but not as drastically, being reduced from 7.5 to 3.5 cm by the 2.5 hr earthenware drying period. A similar time of coverage of the medium with a glass lid decreased the pseudomonad colony 1.8 cm.

In these experiments it was observed (table 1) that the diameters of the pseudomonad colonies were smaller on the fifth than on the first plate inoculated. To test further the effect of the size of the inoculum on colony size, 30 consecutive inoculations, 3 per plate, were made on 10 blood agar plates without renewing the inoculum. Plates were prepared in triplicate. Results indicated that 6 consecutive inoculations with the same inoculum reduced the diameter of the pseudomonad colony 2.0 cm.

Whether the surface moisture of the medium altered the pattern of colony, as judged by favoring formation of type I or II colonies can not be ascertained, since these experiments were performed after the organism had been transferred 60 times and was at that time forming the type III pattern. However, in earlier experiments it was observed that media stored at 10 C and subsequently placed at 37 C for longer than 3 hr resulted in a decrease in colony size without altering the pattern (types I-III).
Influence of lines of stress, gravity, and obstacles, upon the direction of filaments. It was suspected that the narrow filamentous components of giant colonies would exhibit an elastoc-taxic response (i.e., would grow along lines of stress imposed upon a solid medium). The validity of this idea was tested using the technique of Stanier (1942). Rectangular pieces of solid medium were cut from plates of blood agar and gelatin agar, and draped across a glass rod (4.5 or 9.0 mm diameter) lying in a petri dish. This induced lines of stress which extended perpendicularly along the rectangle of agar from the glass rod to the area of contact between the sheet of agar and the bottom of the dish. The pseudomonad was inoculated at a central area of the medium where the sheet of agar rested upon (1) the bottom of the dish, (2) or on top of the glass rod, or in an area midway between (1) and (2).

These experiments were performed prior to the thirty-fifth transfer and all colonies exhibited the type I pattern. The direction of growth taken by the filaments of these colonies was unpredictable. Filaments at times followed the lines of stress. However, just as frequently they crossed over or grew diagonally to the imposed stress. On some occasions the filaments extended from one area of the rectangle up and over the incline imposed by the glass rod and down onto the other slope of the medium.

This latter observation raised the question of the effect of gravity on the direction of filament growth. To test the effect of gravity gelatin agar plates were inoculated in the center of the plate or in an area corresponding to "9 o'clock" and incubated in a vertical position for 24 hr at 28 C. Despite the over-all downward flow of the colony, some filaments grew diagonally across the medium.

Figure 6. A 12 hr growth on blood agar. (Top) Limited growth and lack of filaments on adjacent sides of parallel slice inoculations. (Bottom) Young type II colony arising from stab inoculation.
TABLE 1

Influence of surface moisture of gelatin agar on colony size

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<thead>
<tr>
<th>Type of Petri Dish Cover</th>
<th>Organisms Inoculated</th>
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<tr>
<td></td>
<td><em>Proteus vulgaris</em></td>
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<td>Plate 1</td>
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<td>hr</td>
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<tr>
<td>Glass</td>
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<tr>
<td>Glass</td>
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<td>Earthenware</td>
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<tr>
<td>Earthenware</td>
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<td>2.0</td>
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<td>Earthenware</td>
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* Colony diameters in cm following incubation at 30°C for 24 hr. Protocol: Glass or earthenware covers replaced filter paper interlined glass lids which had been in place 1 hr following pouring of plates. After appropriate coverage, a dry glass petri dish replaced the earthenware or glass covers. Five plates per test time were consecutively stabbed with a single inoculum of the pseudomonad. One plate per test period inoculated with *Proteus vulgaris* ATCC 9927. All plates contained 40 ml medium.

and some even followed a horizontal direction after first exhibiting growth in a diagonal direction.

To examine the effect which an array of obstacles would have upon the growth of filaments several sterile glass beads were scattered on the surface of gelatin agar and blood agar plates prior to a stab inoculation. The beads did not appear to attract or repel the filamentous growth and obstruction by a bead seemed to occur by chance. When a filament encountered a glass bead during growth, further migration ceased. During the entire study it was rare to see one filament merge into another (figure 4). Filaments appeared to repel each other, and there were indications that close proximity of growth would inhibit filament formation (figure 5).

DISCUSSION

The documentation of unusual growth patterns by this *Pseudomonas* species affords opportunity for consideration of giant colonies and their possible relationships to bacterial colonial morphology.

Further studies of the phenomenon were hampered owing to the cessation of designs other than those of type III. However, from observational and experimental studies it was apparent that the growth patterns described were largely attributable to the physical conditions of the medium. Of these, the surface moisture of the solid media and the chemical nature of the medium were most important. Undoubtedly, other conditions which were not elucidated operated in determining the colony forms. However, it is suggested that in the final analysis the degree of motility of the organism, as influenced by nutritional and physical environment, and the degree of moisture of the solid medium were the major determinants of the giant colonies. Sykes and Reed (1949) demonstrated a relationship between motility and colony form, as influenced by the composition of the medium, by incorporating boric acid in blood agar to inhibit the swarm of proteus.

Filaments are a common biological structure; their formation implies orientation or polarity of the cells composing the filament (Bonner, 1932). Impression slides of young and old giant colonies failed to demonstrate cellular orientation. Despite this, one wonders if there existed an unobserved orientation, or perhaps some degree of flagellar synchronization which contributed to the filament formation and pattern development. Evidence is available (Loedel and Meffert, 1952; Nettleton et al., 1953) that a synchronization of flagellar movement in heavy suspensions of *Pseudomonas fluorescens* and *Escherichia coli*, and also certain ciliated protozoa, can cause distinct cultural patterns.

It is possible that the filaments may have been initiated at zones of increased bacterial density which ultimately overflowed their initial area to extend upon a region of the medium which offered little resistance to flow. Despite the most delicate care during inoculation, a microscopic spatter from the stab inoculation might have initiated such zones. A model demonstrating this phenomenon is provided in the different spatter patterns obtained by drops released from varying heights above the surface. In this connection it is of interest that a miniature of the final pattern of a giant colony could frequently be faintly seen 3 hr following inoculation. However, as an argument against this spatter hypothesis for the formation of filaments, one has the occasional demonstra-
tion of filaments which occurred following a straight line, slice type of inoculation (figure 5).
One may also entertain the idea of molecular arrangement within the medium enhancing the filamentous growths. Weiss (1939) commented on the various factors determining direction of nerve fibers, and provided experimental evidence that an oriented substratum guided the developing fibers.

Two colonial manifestations of motile bacteria, swarming in *Proteus* species and motile colonies deserve comment. The type III colony was principally a swarming growth; in some instances even characterizing the alternate resting and swarming zones described for *Proteus* species by Kvittingen (1949). Swarming is a phenomenon which has intrigued bacteriologists for over 60 years and despite a respectable degree of attention still lacks an acceptable explanation as to cause. In the most commonly described swarming development, blocks or islands of cells break away from the parent colony and frequently form arms or branches of growth onto the agar (Stuart, 1956). It may be several hours before the space between the filaments is filled in by swarming growth. Thus, a pattern similar in some aspects to a type II colony may be found in a developmental stage of the *Proteus* swarm.

The discrete daughter colonies at the tips of filaments of the type II colonies are definitely not motile colonies. However, during their formation a feature similar to one occurring among motile colonies was encountered. Beginning with the first description of motile colonies (Muto, 1904) there are accounts of processes or offshoots from a colony or from streak inoculations. These offshoots generally consisted of two thin, parallel ridges of dwarf colonies which ended in a colony of the same width. Henriksen and Svendsen (1946) described these processes as resembling wheel marks of a vehicle or railway tracks. Thus the more heavily concentrated growth bordering the filament of the type II colony (figures 2 and 3) becomes increasingly interesting. Associated with the migration of a motile colony is also rotation, either clockwise or counterclockwise. A study of several species of the genus *Bacillus* (Murray and Elder, 1949) indicated that chance does not determine the direction or rotation of the curved path traced by a motile colony. Accordingly, it is of interest that the filamentous growth described for the type I and II colonies were usually linear although an occasional curve developed along the course of the filament.

The three major configurations described would appear to have a graded interrelationship. The type II colony is considered the key colony, as it combines features of the other two types. The mother moiety of the type II colony resembled the type III swarm colony; the radial branching filaments and daughter moiety suggest a higher degree of colony complexity and organization than that found in the linear, sparsely branching type I colony. It is noteworthy that the initial pattern observed (from stab inoculation) of this organism was a type II colony.

**ACKNOWLEDGMENTS**

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**SUMMARY**

An organism, tentatively identified as *Pseudomonas myxogenes*, was found to display three different but related giant colonies when grown on suitable medium following stab inoculation. The colonies were best demonstrated on gelatin agar or blood agar. The chemical nature and the surface moisture of the medium were shown to be influential in establishing these giant colonies. The terminal design after 96 daily transfers was a swarming type of growth. Attempts to revert this terminal pattern to those encountered earlier were unsuccessful.

**REFERENCES**


