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Periodic Phenomena in *Proteus mirabilis* Swarm Colony Development

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Proteus mirabilis **colonies exhibit striking geometric regularity. Basic microbiological methods and imaging techniques were used to measure periodic macroscopic events in swarm colony morphogenesis. We distinguished three initial phases (lag phase, first swarming phase, and first consolidation phase) followed by repeating cycles of subsequent swarming plus consolidation phases. Each *Proteus* swarm colony terrace corresponds to one swarming-plus-consolidation cycle. The duration of the lag phase was dependent upon inoculation density in a way that indicated the operation of both cooperative and inhibitory multicellular effects. On our standard medium, the second and subsequent swarm phases displayed structure in the form of internal waves visible with reflected and dark-field illumination. These internal waves resulted from organization of the migrating bacteria into successively thicker cohorts of swarmer cells. Bacterial growth and motility were independently modified by altering the composition of the growth medium. By varying the glucose concentration in the substrate, it was possible to alter biomass production without greatly affecting the kinetics of colony surface area expansion. By varying the agar concentration in the substrate, initial bacterial biomass production was unaffected but colony expansion dynamics were significantly altered. Higher agar concentrations led to slower, shorter swarm phases and longer consolidation phases. Thus, colony growth was restricted by higher agar concentrations but the overall timing of the swarming-plus-consolidation cycles remained constant. None of a variety of factors which had significant effects on colony expansion altered terracing frequencies at 32°C, but the length of the swarming-plus-consolidation cycle was affected by temperature and medium enrichment. Some clinical isolates displayed significant differences in terracing frequencies at 32°C. Our results defined a number of readily quantifiable parameters in swarm colony development. The data showed no connection between nutrient (glucose) depletion and the onset of different phases in swarm colony morphogenesis. Several observations point to the operation of density-dependent thresholds in controlling the transitions between distinct phases.**

Proteus mirabilis colonies have fascinated microbiologists for over a century (13). On typical laboratory media, mature *P. mirabilis* swarm colonies display striking patterns characterized by circular symmetry and regularly spaced concentric terraces or zones (Fig. 1) (6). These terraces develop as a result of periodic events during colony growth, most notably the cyclic repetition of alternating phases: swarming (active migration) and consolidation (growth without movement of the colony perimeter) (2, 3). Colony expansion is a dynamic process involving movement over the solid substrate by multicellular rafts of specially differentiated swarmer cells (7, 18, 30, 32). The swarmer cells are elongated and hyperflagellated but have the same DNA/length ratio as the shorter oligoflagellated swimmer cells characteristic of liquid populations (14, 16).

Unsolved problems in *Proteus* swarm colony morphogenesis include the control of swarmer cell differentiation from swimmer cells, swarmer cell organization into groups capable of movement on agar surfaces, the physical basis of multicellular motility, and the origins of colony periodicities. Molecular genetics has begun to identify several molecular components needed for the execution and control of swarm colony development (1, 4, 5). As a complement to genetics, we have begun exploring swarm colony phenotypes by quantitative characterization of different macroscopic events during morphogenesis. The results reported in this paper define several different phases in swarm colony development, provide new information about the relationship between bacterial growth (biomass production) and colony expansion over the agar surface, demonstrate an unexpected robustness in the swarming plus-consolidation cycle, and describe a novel periodic feature of swarm colony development. A mathematical model to account for the robust periodicity of swarm colony morphogenesis will be presented elsewhere (10).

MATERIALS AND METHODS

Strains. Clinical isolates of *P. mirabilis* were obtained from the microbiology laboratory of the University of Chicago Hospitals (courtesy of Josephine Morello). These strains have been labelled PRM1 through PRM84. PRM1 is the parental strain of the mutants described by Belas and colleagues (4, 5). After introduction of antibiotic resistance plasmids into PRM1 and PRM2, we found that about half of the transconjugants displayed new colony phenotypes characterized by narrower terraces. These strains displayed reduced swarming motility, and the mutant phenotypes persisted even after plasmid loss. Data from six of these mutants are included in Fig. 15.

Media and culture conditions. Our basic PA minimal salts medium has been previously described (21). We routinely grew *P. mirabilis* colonies on PA agar containing 0.4% glucose, 0.2% Casamino Acids, and 0.0001% nicotinic acid at 32°C. For some of the experiments described later, different concentrations of

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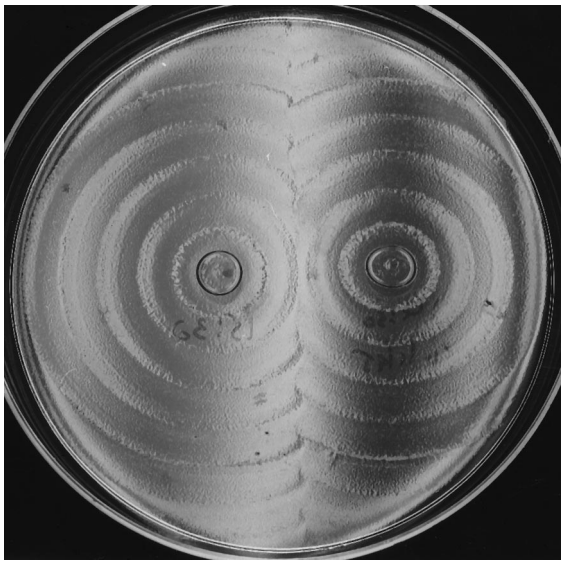

FIG. 1. Two terraced *P. mirabilis* colonies displaying spatially periodic structures. These PRM1 colonies were inoculated at a 1-h interval and then incubated for a further 42 h at 32°C on standard medium containing 2% agar. Note that the colonies displayed the same periodicity but remained out of phase with each other throughout development.

glucose or Casamino Acids were used, and we sometimes replaced Casamino Acids with yeast extract, tryptone, or peptone. Plates were routinely solidified with 2.0% agar, but in some experiments different agar concentrations were used. When the effect of agar concentration was being tested, plates were prepared by pipetting 25 ml of molten agar into a standard 9-cm-diameter petri dish and drying all plates for exactly 20 h at 37°C before inoculating. Our standard inoculation procedure was to prepare a fresh overnight liquid culture, wash the cells in PA buffer, adjust the optical density at 650 nm (OD_{650}) to 0.5 ($\sim 10^9$ CFU/ml), and spot 3 μl on the agar medium. For convenience, however, we often used inocula consisting of older suspensions of *P. mirabilis* which had been standing at room temperature for periods of up to several weeks, and sometimes we prepared inocula by resuspending cells from colonies. The effects of inoculum condition on the early phases of colony development will be described below.

Measurements of colony expansion and terracing. "Human camera" experiments involved counting terraces by visual inspection and periodic measurements of colony radius by applying a ruler to the back of the petri dish in the warm room. In some cases, colonies were inoculated off center on the plate (see Fig. 9) so that the radius could exceed half the width of the 9-cm-diameter petri dish. Periodic reflected-light photographs of the colonies were taken either with a WILD Makroskop 420 fitted with coaxial illumination or with a 35-mm camera with the ceiling light fixtures in our laboratory for illumination (22). Colony expansion and internal wave movements were monitored by time-lapse video recording using a SONY videodisk recorder. The colony perimeter, terrace boundaries, and internal waves appeared bright in dark-field illumination, and their movements could be documented in the form of time-space plots by methods previously developed for observing *Dictyostelium discoideum* (26). Briefly, time-space plots were generated as follows: the computer recorded the gray-scale levels across a thin window of each image frame, the ensemble of one-dimensional gray-scale profiles were aligned in temporal sequence, and a two-dimensional image was generated from the data (Fig. 2). Along the *y* axis, the image followed the time course of the recording. Along the *x* axis, bright lines revealed the positions of various structural features in the colony across the window. Vertical lines represented stationary features, while sloping lines represented moving features. Velocities could be measured directly from the time-space plots by reading the slope of the tangent. The intervals between internal waves at any position in the colony could be obtained by plotting gray-scale levels along a horizontal line and measuring peak-to-peak distances on the time axis (see Fig. 7).

Measurements of bacterial growth. Total biomass per colony was determined by washing the entire colony off the agar surface in 5 to 10 ml of PA buffer, reading the OD_{650} in a spectrophotometer, and multiplying by the total volume of suspension to give readings in OD-milliliter units. The biomass per unit area in a particular zone was determined by using a hollow Teflon ring to remove agar plugs of 6-mm diameter, aspirating the plug into 1 ml of PA buffer, and reading the OD_{650} in a spectrophotometer. Biomass per unit area in a whole colony was determined by measuring the total biomass in OD-milliliters and then dividing by the colony surface area in square millimeters.

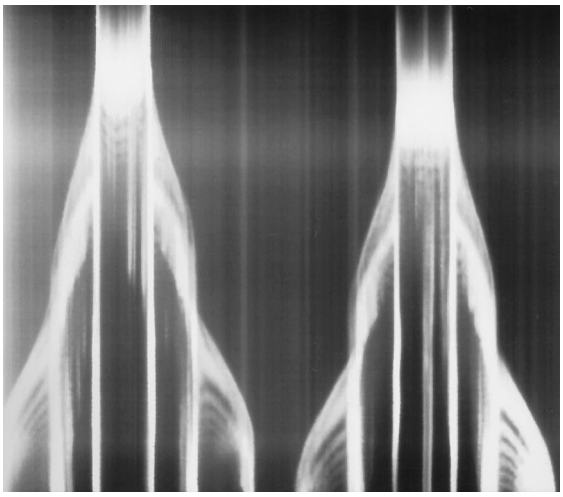

FIG. 2. A time-space plot of two PRM1 colonies developing on standard medium containing 2.1% agar at 26°C. Four hundred ninety-seven consecutive images of a slice through the two colonies at 2-min intervals were arrayed along a time axis from top to bottom (16.6-h total duration). The dark-field illumination produced images in which features such as the colony perimeter and the boundaries of terraces and internal waves were bright and uniform bacterial masses were dark, independently of how much biomass they contained. Each inoculation spot developed structure and then gave rise to the first swarming phase (expansion of the colony perimeter on each side of the spot). This process left the fixed vertical lines which marked the edges of the original inoculation zone. The first consolidation phase was then followed by the second swarm phase, which was characterized by the appearance of internal waves, visualized as the appearance of successive "ribs" in the expanding terrace, and the formation of new fixed vertical lines marking the boundaries of the first terrace. The instantaneous velocity of movement of any bright structure could be measured by drawing the tangent and measuring its slope (vertical = 0; horizontal = ∞). Since successive internal waves move faster and faster, the line representing each one lies closer to horizontal than the lines representing the leading edge and the preceding waves.

Graphical representation of the data. Graphs were prepared with CricketGraph software for the Macintosh. In all graphs except Fig. 7, the lines drawn through the datum points were fitted with the "simple curve fit" routine.

RESULTS

The different phases of swarm colony development. Our present quantitative studies of *P. mirabilis* colony development extend previous reports in the literature and supplement our own time-lapse video observations (reference 25 and unpublished data). We routinely initiated colony development by inoculating a drop of liquid culture on minimal salts-glucose-nicotinic acid-Casamino Acids agar medium and incubating it at 32°C. In some experiments, we replaced Casamino Acids with yeast extract as a source of amino acids needed for swarming (17), and one series of time-lapse videotapes was made at 26°C. Except for quantitative differences, these experimental variables did not alter the basic nature of our results. On 1.5 to 3.0% agar plates at 32°C, five distinct phases could be discerned in colony morphogenesis.

(i) Lag phase. The lag phase is characterized by the growth of the initial inoculum, the differentiation of the first swarmer cell cohorts, and the organization of swarmer cells at the colony perimeter in preparation for swarming. Time-lapse and real-time videos show that this preparation includes migration of swarmer cells from the interior of the inoculation zone to the periphery, increasingly rapid back-and-forth tangential movements of individual swarmer cells, and synchronization of flagellar movements as groups of swarmer cells align them-

selves to form the migrating rafts which have been described as the basic units of swarm motility (28, 29, 32). The lag phase ends when the first swarmer rafts emerge from the colony perimeter. The minimum length of the lag phase varies by over 40% among different clinical isolates of *P. mirabilis*, even when liquid growth kinetics and swarmer cell migrations are quantitatively similar.

(ii) First swarming phase. The first swarming phase is characterized by relatively independent movement of swarmer cell rafts which tend to coalesce into radially oriented tubes (Fig. 3b and c). As reported previously, the leading edge of migrating cells is often quite ragged in appearance (25), and measurements of edge movement over the agar surface frequently display pauses or stutters (Fig. 4a). The degree of coherence displayed by the bacteria in the first swarming phase is largely a function of the age of the inoculum. Older inocula produce more-ragged, stuttering first swarm phases with relatively low average expansion velocities (Fig. 4a), while fresh inocula produce first swarm phases that advance more smoothly (Fig. 4b). The first swarming phase ends when migration has ceased, and the leading edge remains stationary.

(iii) First consolidation phase. As the first swarming phase is coming to an end, a wave of cell multiplication and thickening of the newly colonized areas begins adjacent to the perimeter of the inoculation spot (Fig. 3e). This wave spreads outwards (Fig. 3f) until it approaches the boundary of the colonized areas, and the second swarming phase begins (25). Cell multiplication involves growth and division of undifferentiated swimmer cells as well as dedifferentiation of some of the stationary swarmer cells by septation into swimmer cells capable of further growth (14). The wave of multiplication continues to expand outwards for a short time after the second swarming phase has begun and produces the final boundary of the first terrace, which covers slightly more than the area colonized during the first swarming phase. The slight expansion of the first terrace at the onset of the second swarming phase can be seen in time-space plots (Fig. 2).

(iv) Second and following swarming phases. The second and following swarming phases are more coherent than the first swarming phase. The leading edge moves without stutters and at a velocity greater than that of the first swarming phase (Fig. 4). In time-space plots obtained at 26°C with older inocula, the first terrace expanded at 10.5 ± 1.2 μm/min ($n = 12$) while the second terrace was invariably faster, at 17.0 ± 2.0 μm/min ($n = 12$). The swarming population is also organized into larger groups which coalesce into a common front shortly after swarming begins (25). According to Bissett and Douglas (7) and to our own videotapes, some of the immobile swarmer cells from the first swarming phase are reactivated to motility during the second swarmer phase. Thus, the decision to migrate is not cell autonomous. This second swarm phase ends when the swarming front ceases to advance rather synchronously along its entire perimeter.

(v) Second and following consolidation phases. Unlike the first consolidation phase, no spreading wave of multiplication is apparent in the newly colonized area during the later consolidation phases. Instead, there is a uniform thickening of the newly formed terrace. Shortly before the next swarming phase begins, accelerating surface displacements can be observed inside the colony perimeter.

Internal waves during the second and subsequent swarming phases. On our mineral salts-glucose-nicotinic acid-Casamino Acids media, a novel feature of the second and subsequent swarming phases can be observed. This is the formation of internal waves within the expanding swarmer population. These waves are observable as surface roughenings by reflected light (Fig. 5a) or as bright bands by dark-field illumination (Fig. 5b). Although substitution of tryptone or yeast extract for Casamino Acids in PA-nicotinic-glucose agar supports the formation of internal waves, we have not observed internal waves in the first swarming phase on our standard media or in any swarming phase on 1% tryptone–0.5% yeast extract agar. The absence of visible internal waves on unbuffered rich medium probably explains why this phenomenon has not been described previously. Transmitted light microscopy of the internal waves at low magnification reveals a succession of increasingly dense layers within the swarming population as it extends from the perimeter towards the previous terrace (Fig. 6). High-magnification microscopy shows that the leading edge of the swarm population consists of several cell layers piled up like ridges in a contour map, while the layer just inside the edge is a single cell thick. We assume that each increasingly dense layer contains one cell monolayer more than its outer neighbor. At the boundaries between layers, there are islands of cell populations at the density of the inner layer. We assume that the edges of these islands, like the edges of the cells piled up at the leading edge, produce the roughness visible in reflected light and the light scatter that produces bright bands in dark-field images.

Time-space plots show that the internal waves move with variable velocities, generally faster than the leading edge (Fig. 2). Swarmer cells emerge from the previous terrace into the newly forming terrace as successively thicker populations. This increase in the density of the swarmer population occurs because the previous terrace is not inert but continues to grow and produce more and more swarmer cells. Visible waves form, apparently, because the swarmer cells are organized into discrete layers one cell thick. (Microscopy of swarming populations shows rafts in one layer moving independently of rafts in an adjacent layer.) As a result of stratification in monolayers, the increasing density of the swarm population does not appear continuously but is quantized into steps of one, two, three, and more layers, as seen in Fig. 6. More-detailed analysis of the time-space plots reveals that the intervals between successive pairs of internal bands become shorter and shorter, following a power law decay consistent with an exponentially increasing production of swarmer cells from the previous terrace (Fig. 7).

Relation of the swarming-plus-consolidation cycle to terrace formation. The macroscopic swarming and consolidation phases overlap to a small degree and are not absolutely discrete in time. The first consolidation phase begins while some swarm groups are still migrating, and bacterial multiplication (consolidation) continues after the next swarming phase has begun. Cell movements can be observed before the colony perimeter is broken, and the final boundary of each terrace is actually determined by a small expansion just after the next swarming phase has begun, as can be seen best in the time-space plots (Fig. 2). Note how the vertical line marking the terrace boundary moves slightly outwards at the start of the next cycle. Nonetheless, we follow earlier workers in denoting a clear cycle composed of one swarming phase plus the following consolidation phase and define the limits of this swarming-plus-consolidation cycle as the interval between the onsets of migration in successive swarm phases (3). Each terrace is almost entirely formed during one swarming-plus-consolidation cycle, and there is a one-to-one correspondence between terraces and cycles. Thus, we can use the equation 1 terrace = 1 swarming-plus-consolidation cycle to study periodicity over extended periods of colony development.

Influence of inoculum density on the lag phase and first swarming phase. Inoculum density affected the duration of the

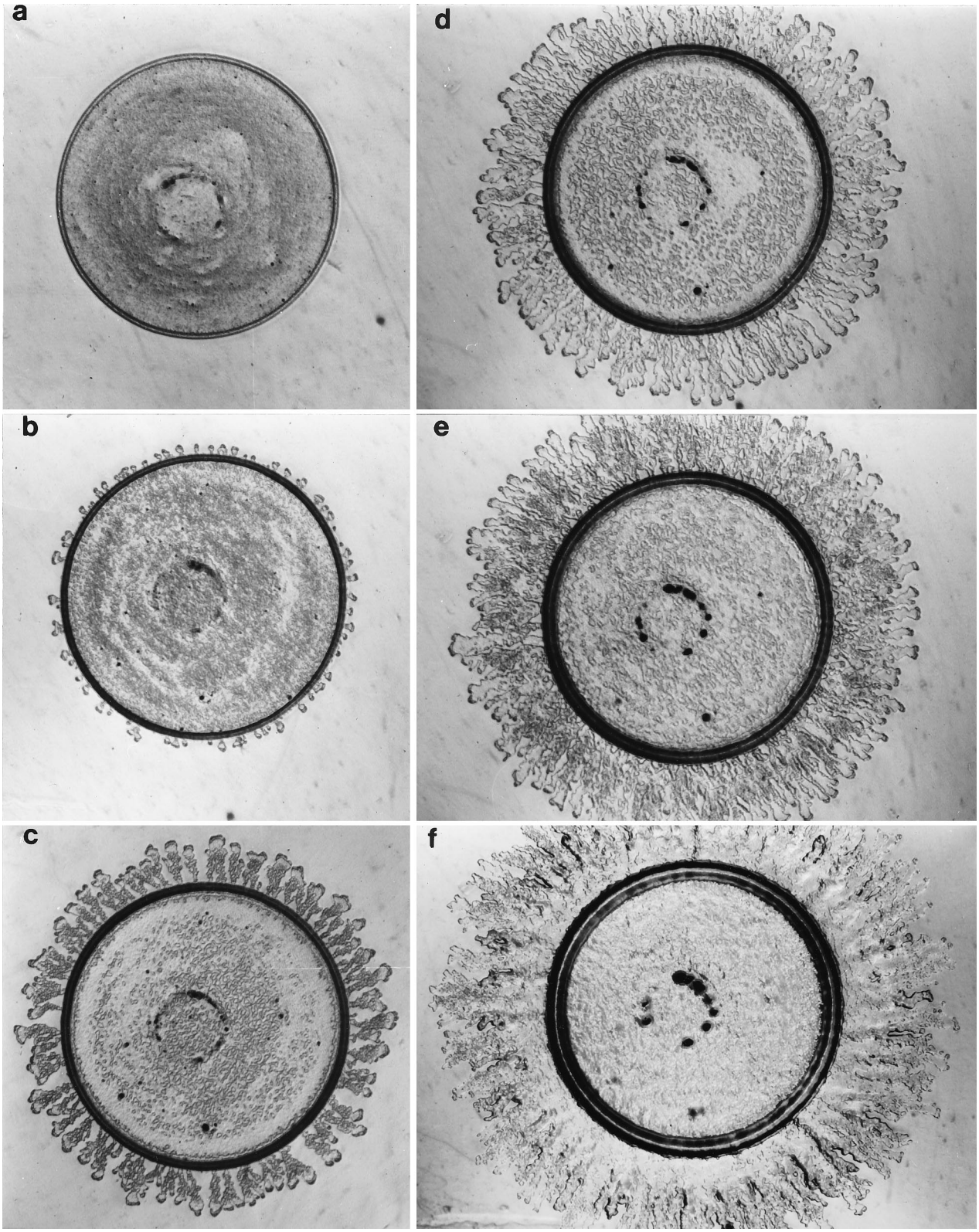

FIG. 3. First swarming phase of an older PRM1 inoculum. (a) Before emergence of first rafts (photographed 4.75 h postinoculation); (b) initial emergence of rafts at the start of the first swarm phase (7.75 h postinoculation); (c and d) expansion of the swarm population (9.25 and 10.5 h postinoculation, respectively); (e) initiation of cellular multiplication from the edge of the inoculation zone as swarmer cell spreading slows down (11.3 h postinoculation); (f) continued expansion of cellular multiplication as edge movement ceases and the first consolidation phase begins (12.5 h postinoculation). The inoculation spot measured 5 mm in diameter.

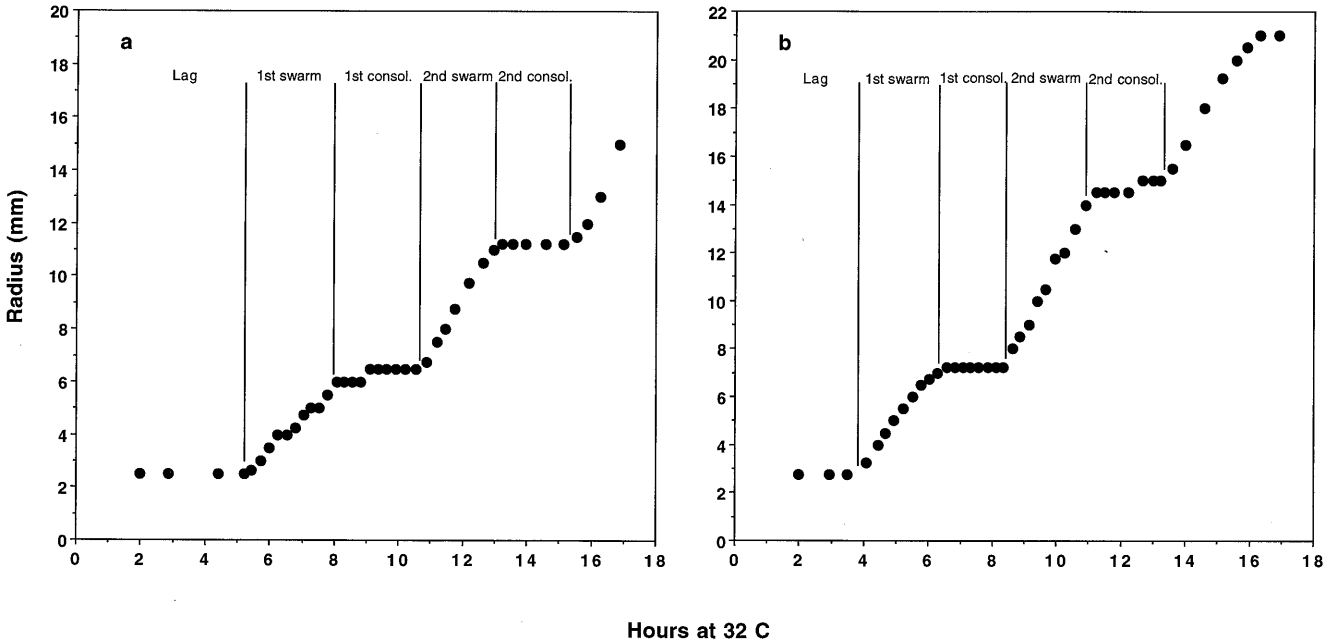

FIG. 4. Human camera experiment tracing the movement of the colony edge during the first two cycles of colony expansion. The lag, swarm, and consolidation phases are demarcated. Different PRM1 liquid cultures were used as inocula: >1 week old (a) and overnight (b). Note that in panel a that the colony front moved jerkily during the first swarming phase but smoothly during the second swarming phase.

lag phase in a strain-specific manner. We examined two clinical isolates, PRM1 and PRM2, in detail. These strains have the same doubling time in standard glucose-nicotinic acid-Casamino Acids liquid medium at 32°C (0.9 h) and the same swarming-plus-consolidation cycle time at 32°C (4.7 h).

Under standard conditions, the lag time was 4.7 h for PRM1 and 6 h for PRM2 on 2% agar medium. With each strain, different concentrations of bacteria were inoculated in standard 3-μl aliquots in order to test the idea that the lag phase duration depended simply on achieving a certain cellular density needed to trigger swarming. Concentrations were varied by diluting the cultures in buffer or concentrating them by centrifugation and resuspension in a smaller volume. The results at low inoculum densities were similar for the two strains, showing a logarithmic dependency of lag time on bacterial concentration (Fig. 8). At high cell densities, however, there was a marked difference between the two strains. For PRM1, the lag phase remained at 4.7 h over a 64-fold range of inoculum densities (1.5×10^6 to 9.6×10^7 CFU per spot). For PRM2, in contrast, the lag time actually increased reproducibly

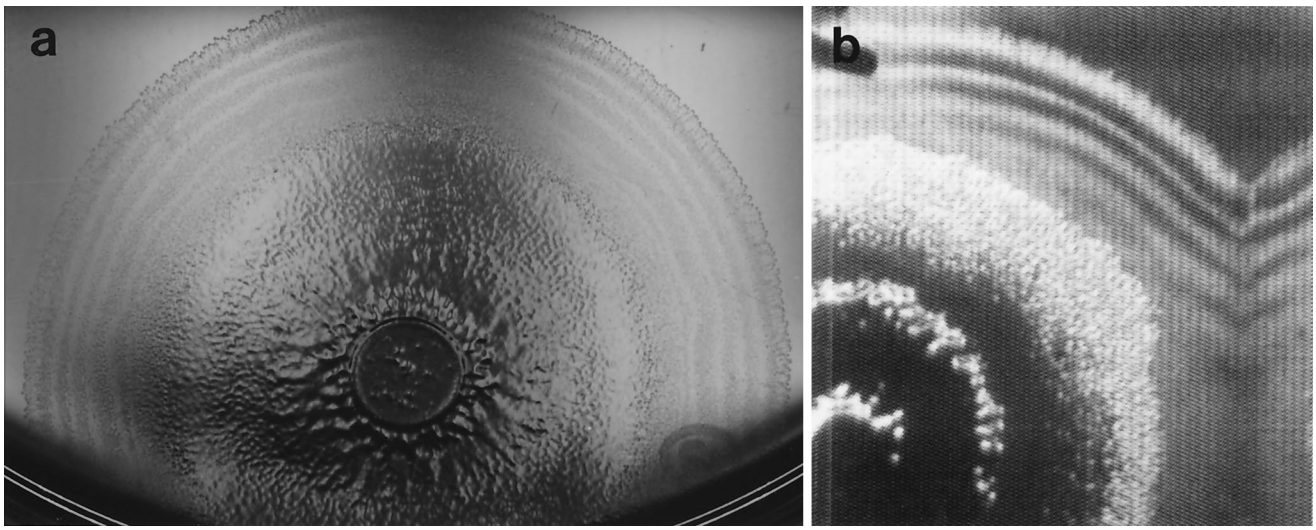

FIG. 5. Internal waves visualized by reflected-light (a) and dark-field (b) illumination. The concentric bands that appear as surface roughness on the left and as bright zones on the right correspond to regions with many small islands of two distinct thicknesses, as seen at higher magnification in Fig. 6. Roughness and brightness appear to result from light scatter at the boundaries of these islands.

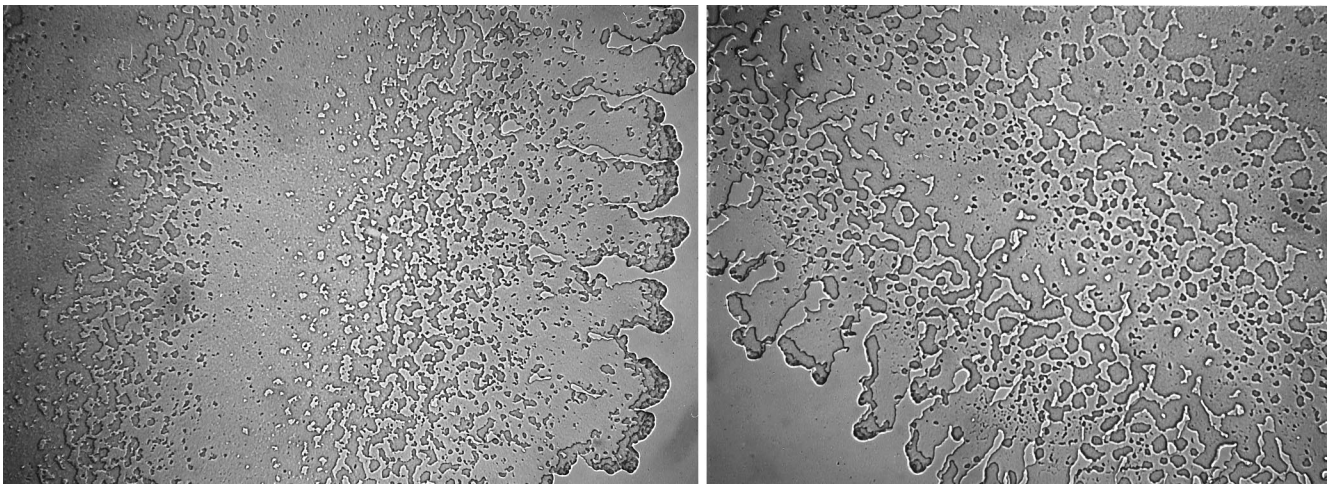

FIG. 6. Transmitted-light micrographs of internal waves in the swarm phase of two PRM1 colonies after 19.5 h incubation at 32°C. The increasing densities of the populations appear as increasingly dark layers. The populations at the swarming fronts are four to five cells thick. Just inside the front, the population is a monolayer. In the next darkest region, two independently moving cell layers can be distinguished. These micrographs were taken with a 2.5× objective and a long-working-distance condenser passing light through the agar plate with a Zeiss Axiophot microscope. The image on the left is approximately 5 mm across.

at the highest inoculum density tested, 4.8×10^7 CFU per spot (Fig. 8), indicating an inhibitory effect on the start of swarming by high concentrations of swimmer cells.

Influence of agar concentration on growth and swarming. It has long been known that higher agar concentrations inhibit swarming (e.g., see reference 29). Modifying the agar concentration allowed us to control swarming without measurably affecting growth. Initial growth rates of the bacteria within the inoculation zone were the same at 1.5, 2.0, and 2.5% agar (data not shown).

Colony expansion was dramatically affected by changing the agar concentration, and this effect on expansion influenced the total colony biomass produced after a fixed period of time. Figure 9 shows colonies inoculated on PA–glucose–nicotinic acid–0.3% yeast extract medium containing different agar concentrations (2.0 to 3.0%) after 33 h of incubation at 32°C. Note that these colonies of very different sizes all had the same number of terraces. Figure 10 gives the quantitative relationships between colony growth and agar concentration. There was an inverse relationship between colony expansion and agar concentration (Fig. 10a) but a direct monotonic relationship between colony expansion and total biomass produced over 2 days of incubation (Fig. 10b). Terracing was not affected by agar concentration (Fig. 10c). When colony density (biomass per surface area colonized) was plotted as a function of colony expansion rate, it could be seen that bacterial density increased

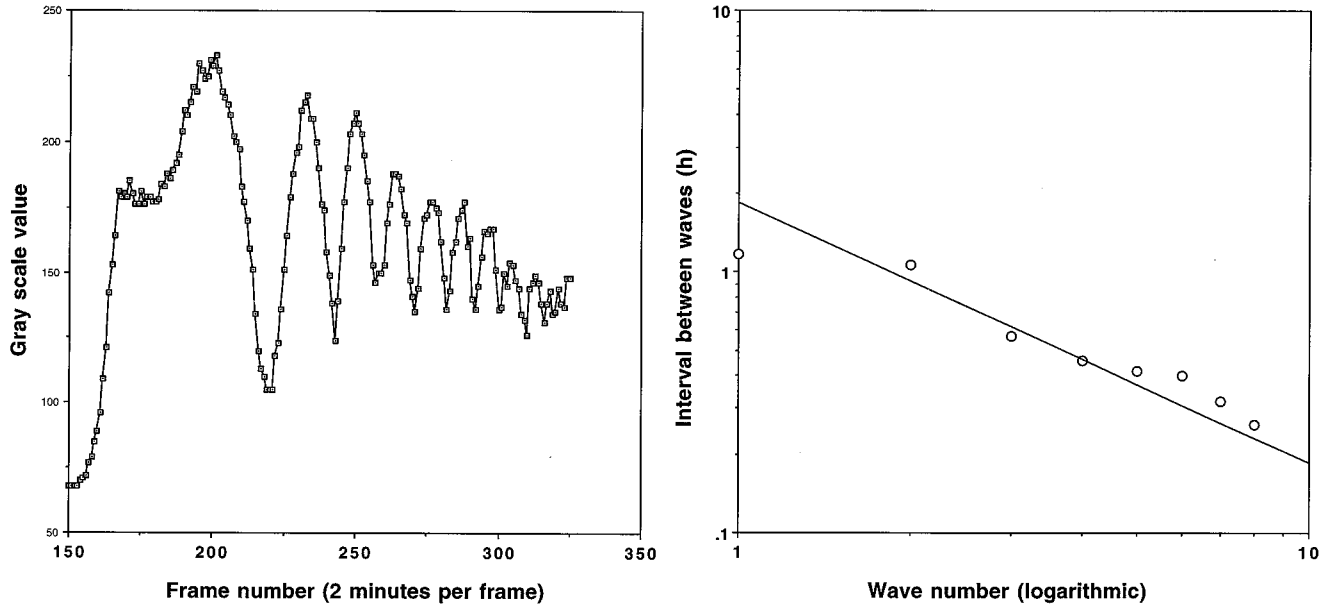

FIG. 7. Analysis of the emergence of successive internal waves. (Left) Tracing of gray-scale values across a time-space plot window to reveal the temporal distribution of the waves visualized by dark-field illumination as illustrated in Fig. 5b. (Right) Log-log plot of the time intervals between successive pairs of waves; the line is arbitrarily fitted.

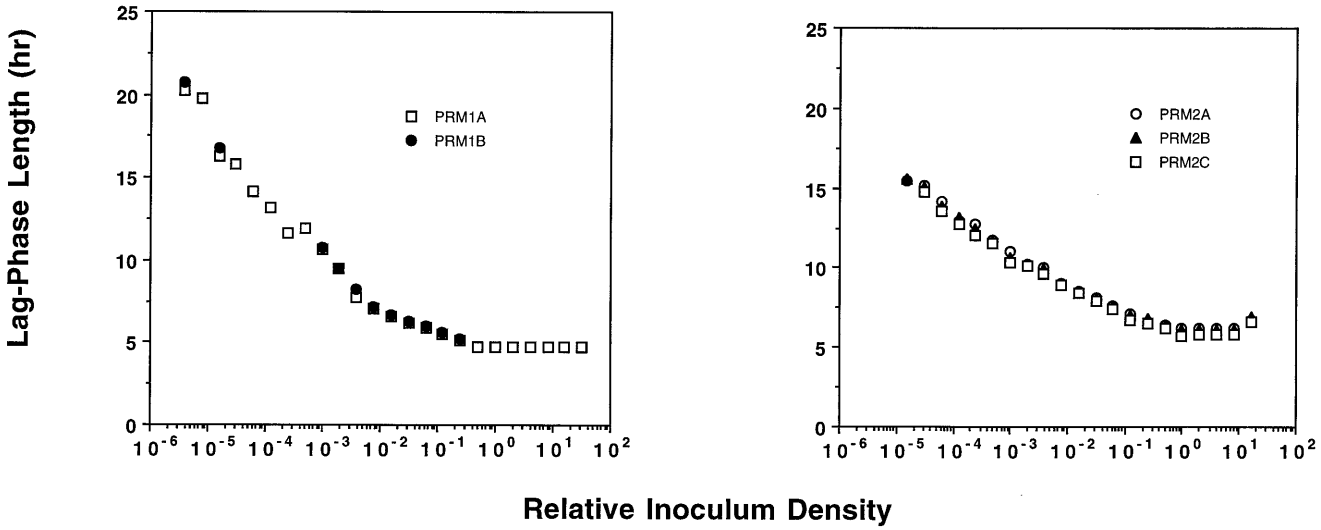

FIG. 8. Lag-phase length versus inoculum density for PRM1 (left) and PRM2 (right) at 32°C on 2.0% agar medium. Different relative concentrations of each culture were inoculated onto standard medium in 3-μl aliquots, and the interval before the emergence of the first swarm rafts was determined. The abscissa gives the population density relative to the standard inoculum (3×10^6 CFU). Dilutions of two independent PRM1 and three independent PRM2 cultures are shown.

in colonies which expanded more slowly at higher agar concentrations, even though these colonies had less total biomass than faster-spreading colonies (Fig. 10d).

These results indicated that swarming is an effective strategy for maximizing biomass production on agar. The higher density observed in smaller colonies can be understood as resulting from a competition between colony expansion and inward glucose diffusion from uncolonized areas. Apparently, the outer

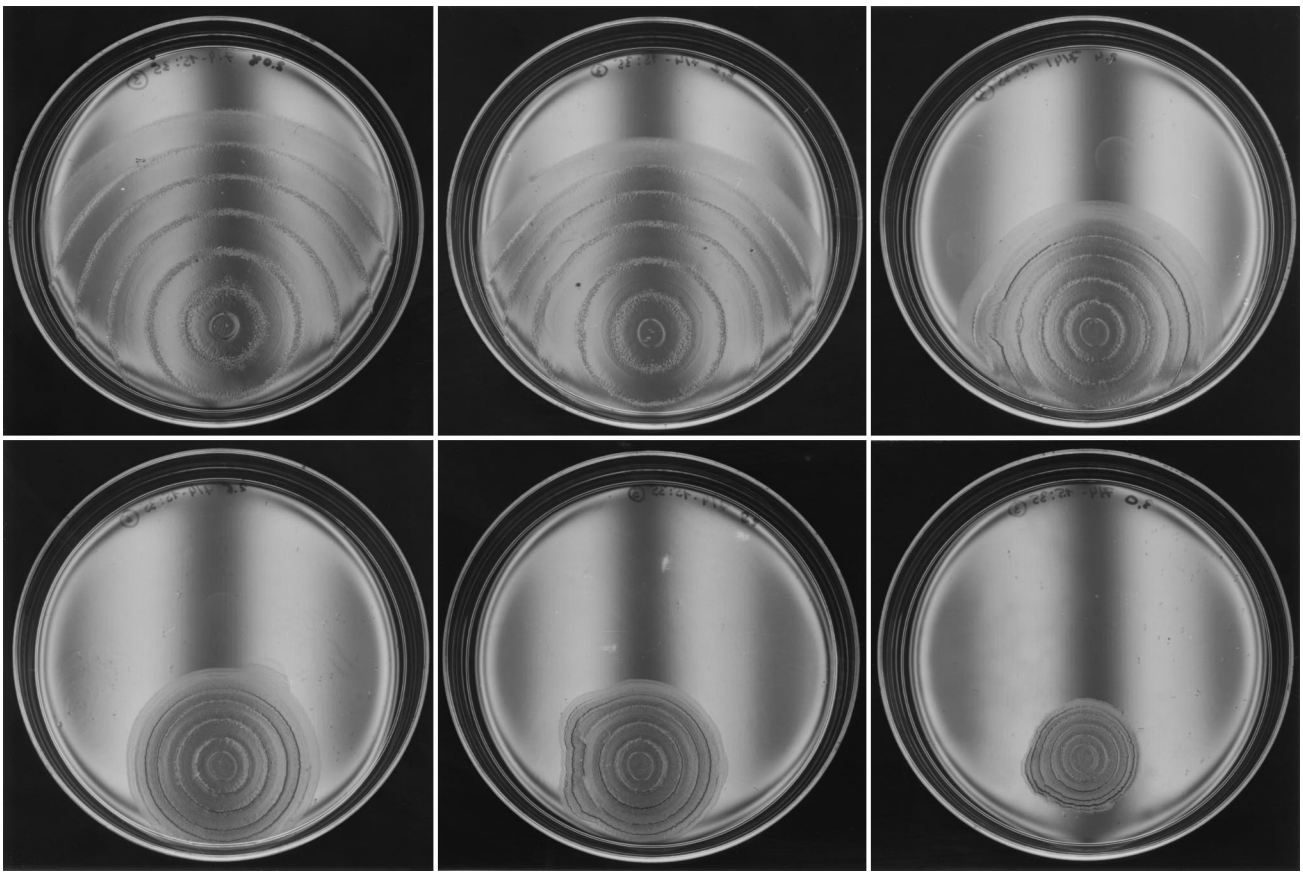

FIG. 9. Effect of agar concentration on colony expansion. PRM1 colonies were grown for 33 h at 32°C on medium containing 0.4% glucose, 0.3% yeast extract, and agar concentrations of 2.0, 2.2, and 2.4% (top, left to right) and 2.6, 2.8, and 3.0% (bottom, left to right).

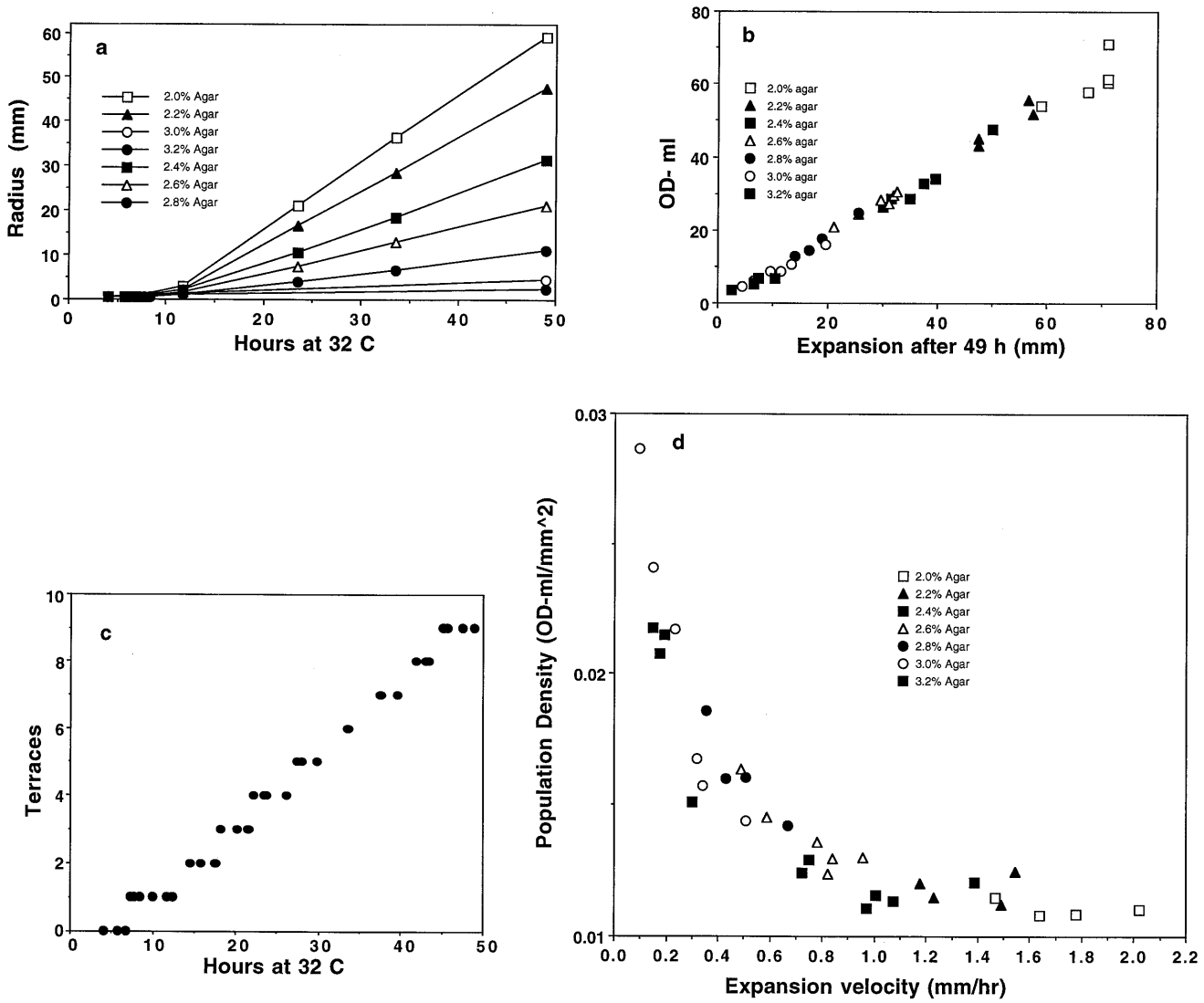

FIG. 10. Effect of agar concentration on colony expansion over time (a), biomass production (b), terracing (c), and final population density after 49 h of growth at 32°C (d) produced by strain PRM1 on standard medium. Only 1 colony per agar concentration is shown in panel a, but all 34 colonies measured (4 or 5 colonies for each agar concentration) are indicated in panels b, c, and d. Note the linear relationship between colony expansion and total biomass produced in panel b. The different agar concentrations are not distinguished in panel c.

terraces of the more rapidly expanding colonies consumed all the glucose at the periphery and so prevented it from diffusing to the interior; consequently, these colonies all had the same final density which was determined by the initial concentration of carbon source in the substrate. In contrast, the centers of more slowly expanding colonies could be fed by additional glucose diffusing in from the exterior, resulting in higher bacterial densities. The diffusion coefficient for glucose (31) indicates a linear migration of fresh nutrients of approximately 0.6 mm/h, which is close to the colony expansion value below which we observe a marked increase in population density (Fig. 10d).

A more detailed look at the influence of increasing agar concentration on swarming revealed two separate effects in the reduction in colony expansion: (i) the velocity of the swarm front was lower, and (ii) the swarming phase was shorter (Fig. 11). Interestingly, the reduction in the length of the swarming phase was compensated by an increase in the length of the consolidation phase so that swarming-plus-consolidation cycle time was essentially the same at all agar concentrations.

Relation of growth to colony development phases. The observation that colony expansion played a role in biomass production led us to examine the relationship of bacterial growth to swarming in more detail. This was particularly important because some of the early hypotheses for periodic swarming by *P. mirabilis* were based on the idea that swarming was a direct behavioral response to depletion of nutrients (20) or accumulation of negative chemotactic factors (19).

We measured bacterial growth during development by taking samples of defined area from the colony center and the third terrace and measuring the increase in bacterial mass at these locations over time (Fig. 12). We found that exponential increase in bacterial mass continued in the colony center for about 15 to 17.5 h, well into the second swarming phase, and

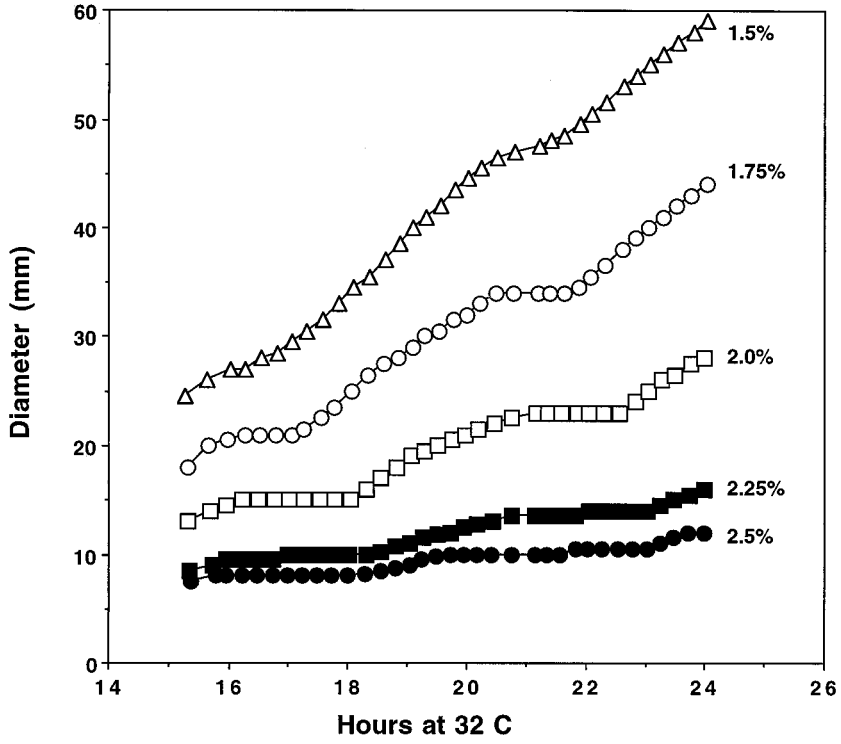

FIG. 11. Human camera experiment showing movement of the colony edge at different agar concentrations.

that growth at a lower rate continued into the fourth swarming-plus-consolidation cycle. Thus, swarming did not appear to be a response to exhaustion of growth substrate. The kinetics of biomass increase in the third terrace were more complex because bacteria entered the terrace by successive waves of swarming to build up an initial biomass in a short period of time. Nonetheless, biomass increase in the third terrace continued into the sixth swarming-plus-consolidation cycle, indicating again that nutrient exhaustion was not essential to initiate swarming.

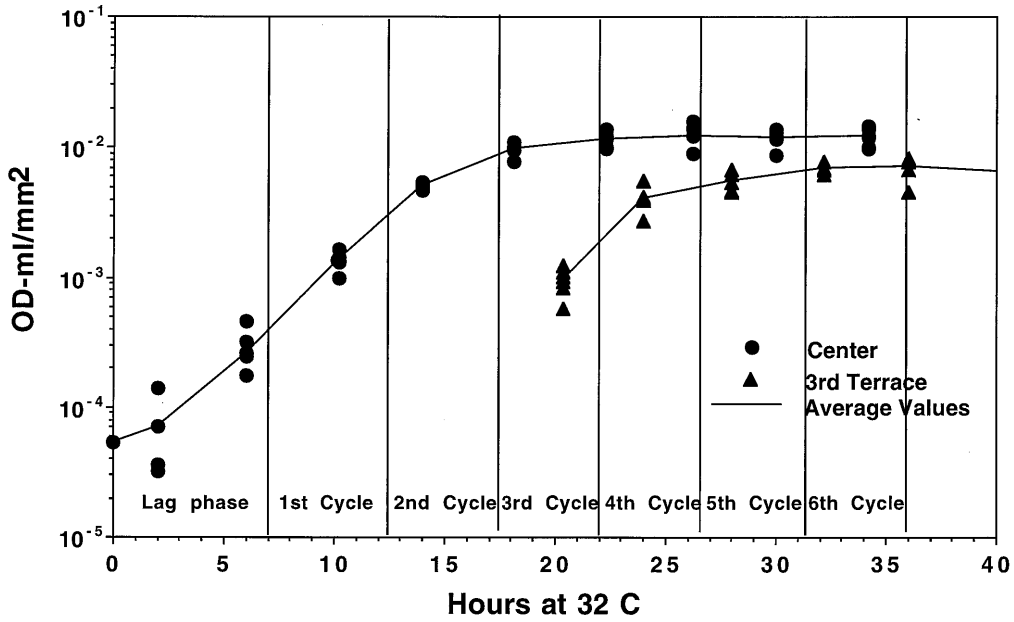

FIG. 12. Growth in the colony center and third terrace. Replicate PRM1 cultures were inoculated onto 2.0% agar medium and incubated at 32°C. At intervals, 6-mm agar plugs were removed from the colony center or from the third terrace, the bacteria were suspended in 1 ml of buffer, and their biomass in OD units per square millimeter was determined. At least four independent determinations were made at each time.

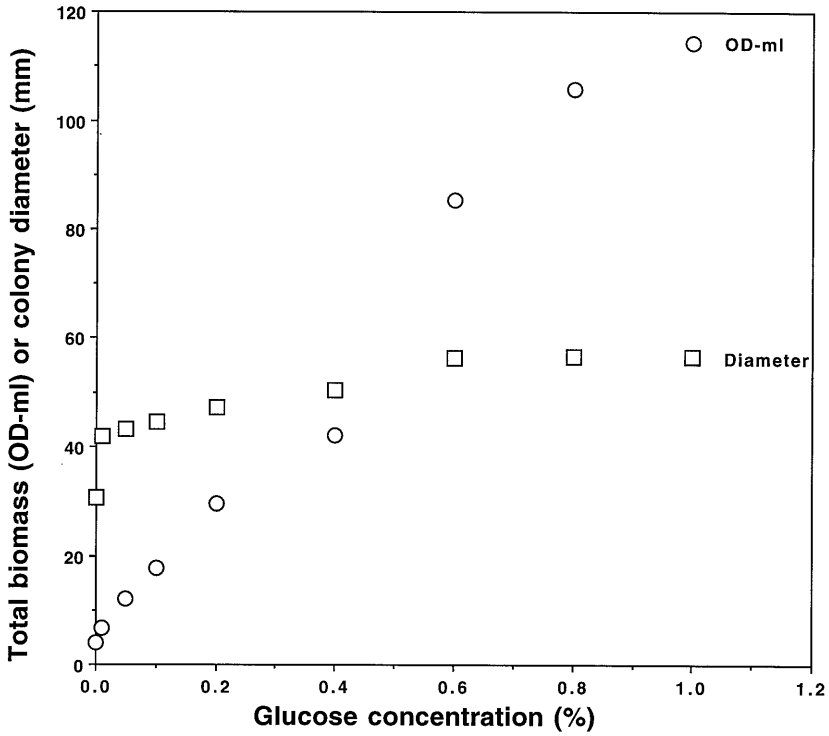

FIG. 13. Effect of glucose concentration on colony expansion and biomass production. Colonies were grown with different glucose concentrations at 32°C for 55 h, at which time the colony diameter was measured in millimeters and the bacteria were washed off the plate to be assayed for total biomass in OD-milliliter units as described in Materials and Methods. Only a single ordinate is given because the numerical values for diameter (squares) and biomass (circles) fell within the same numerical order of magnitude.

We also studied the influence of glucose concentration on growth and swarming. Colonies were inoculated onto 2% agar medium with PA buffer, nictotinic acid, and Casamino Acids as usual but with different concentrations of glucose. The plates were then incubated for 55 h at 32°C before being measured for expansion and total biomass (Fig. 13). The results show that some glucose was necessary for rapid expansion (diameter increased 30% upon addition of 0.01% glucose) but that average colony diameters changed only about 10% over 2 orders of magnitude in glucose concentration (0.01 to 1%). Human camera experiments showed that the speed of swarmer migration and the length of the swarming phase were relatively constant from 0.01 to 1% glucose (Fig. 14). In contrast, total colony biomass production was roughly proportional to glucose concentration over this entire range. All colonies on plates containing glucose displayed the same number of terraces, but the terrace boundaries were much less distinct at lower glucose concentrations. Like amino acids (17), glucose appears to play some important concentration-independent role in swarming. At 0% glucose, with Casamino Acids as the sole carbon source, the colony perimeter did not advance smoothly (Fig. 14), and neither terraces nor cycles were reliably observed (data not shown). Increasing the Casamino Acids concentration to 0.6% produced greater colony biomass but did not restore cyclic swarming behavior.

TABLE 1. Effects of temperature on PRM1 growth in liquid medium and on timing of PRM1 swarming-plus-consolidation cycles[a]

Temp (°C)	Doubling time (h)	Cycle time (h)
22	1.8	8.5
32	0.9	4.7
37	0.7	3.5

[a] PRM1 was grown in standard PA salts–0.4% glucose–0.2% Casamino Acids–0.0001% nicotinic acid medium with aeration and measured periodically for OD_{650} to determine the culture doubling time. Cycle times were determined by plotting terrace numbers on standard solid medium over time and measuring the mean interval between successive terraces.

Robustness of the swarming-plus-consolidation cycle and factors affecting its length. All the results cited above indicated that factors which have strong influences on growth and swarmer cell migration have little effect on the length of the swarming-plus-consolidation cycle, whether measured directly (e.g., Fig. 11 and 14) or by counting terraces formed over time. In particular, there was no correlation between colony expansion rates and cycle times. The Hughes laboratory has reported a similar result with a swarming-inhibited mutant which makes restricted terraces and expresses swarming-associated pathogenicity functions with the same periodicity as the parental strain (12). To confirm and extend this conclusion, we accumulated the results from 125 plates incubated at 32°C after inoculation with different *P. mirabilis* strains (clinical isolates PRM1, PRM2, and PRM48 plus six spontaneous swarm-inhibited mutants of PRM1 and PRM2) onto media of different volumes, different agar or glucose concentrations, and different degrees of drying. The 649 datum points show a wide dispersion of values for colony expansion over time but virtually no deviation from a simple function for terrace numbers over time (Fig. 15). This result indicated a remarkable robustness to the swarming-plus-consolidation cycle time of 4.7 h at 32°C on our standard nutrients.

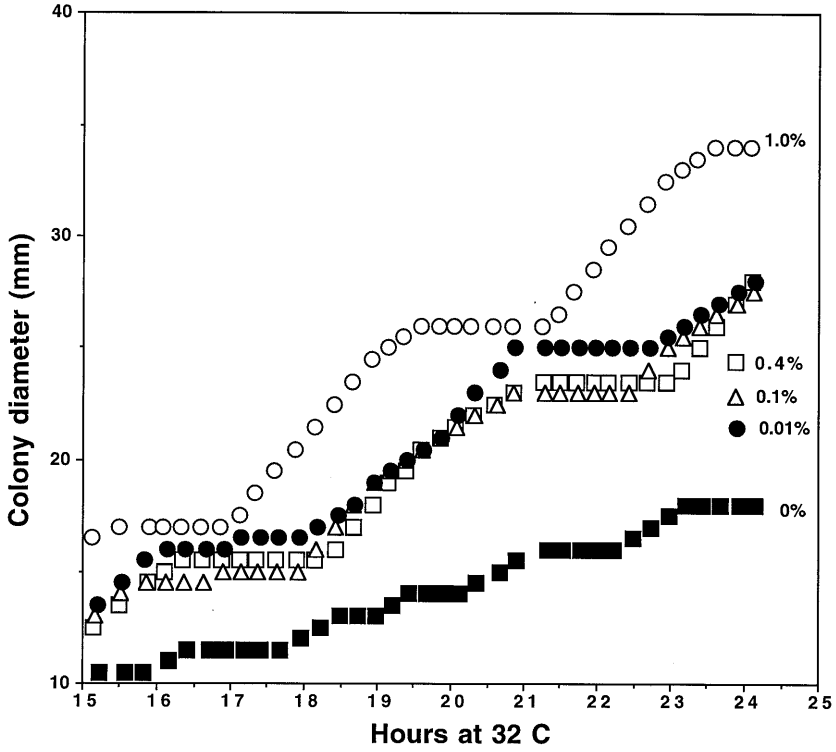

FIG. 14. Human camera experiment showing movement of the colony edge at different glucose concentrations.

Certain variables did affect cycle times, however. Temperature was the most effective. For PRM1, for example, cycle times were shorter at higher temperatures and depended on temperature in approximately the same quantitative manner as did the doubling time in liquid medium (Table 1). Nutrients could also affect the cycle time up to 20%. Addition of various concentrations of peptone, tryptone, or yeast extract in place of Casamino Acids could increase the number of terraces formed after 42 h from 8 to 10, with yeast extract showing the strongest effect (data not shown). These results indicated that the duration of the swarming-plus-consolidation cycle is sensitive to metabolic influences. Thus, the periodic behavior of *Proteus* colonies differs in two important respects from a temperature-compensated biological clock.

Although our mutants of PRM1 do not show variation of cycle times, we did find a spread of cycle lengths from 4.0 to 5.4 h at 32°C among 29 different *P. mirabilis* clinical isolates, two of which are illustrated in Fig. 16. Thus, there is no fundamental species constant for the duration of swarming-plus-consolidation cycles, and the robust value of 4.7 h at 32°C that

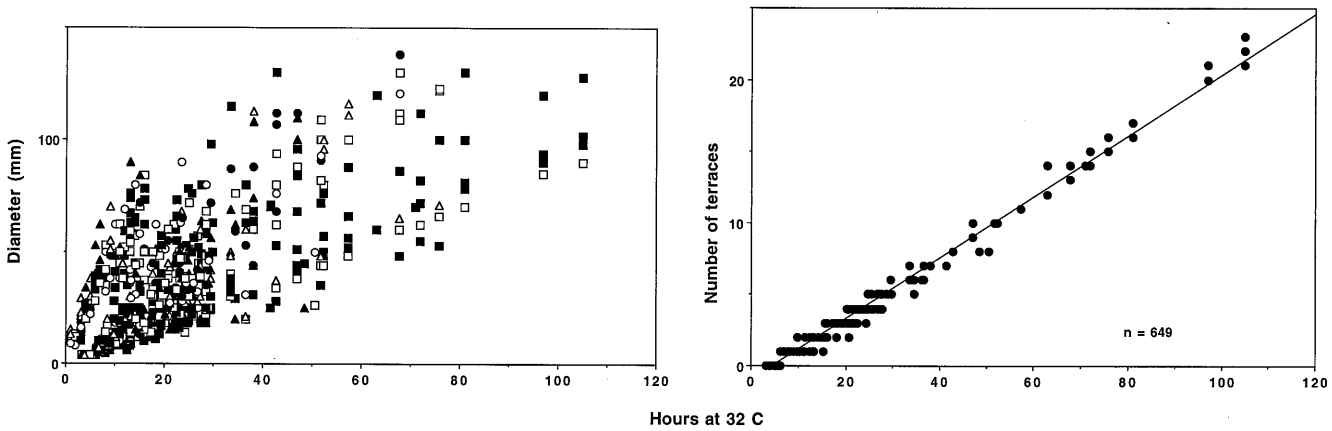

FIG. 15. Effect of multiple variables on colony expansion (a) and terrace formation (b). Nine different *P. mirabilis* strains were inoculated onto a total of 125 petri dishes under different conditions. The strains were clinical isolates PRM1, PRM2, and PRM48 plus three reduced-spreading mutants each of PRM1 and PRM2. The environmental variables were agar volume (5 to 50 ml per plate), agar concentration (2 or 2.5%), and glucose concentration (0.05 to 0.4%). Nicotinic acid concentration was also varied, but all concentrations tested were saturating. At intervals, colony diameters were measured in millimeters and colony terraces were counted. A total of 649 datum points were collected.

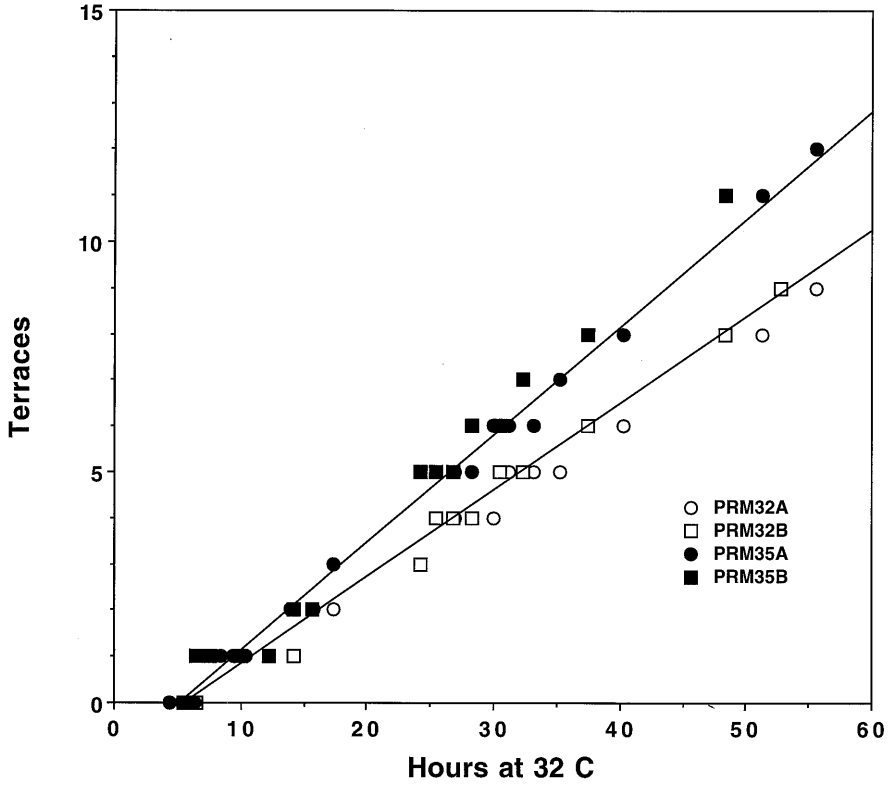

FIG. 16. Terrace formation by two different clinical isolates with different cycle times. Duplicate inoculations of strains PRM32 and PRM35 were made on 2.5% agar, and terrace numbers were counted periodically.

we observed for PRM1, PRM2, PRM48, and their mutants on glucose-Casamino Acids agar (Fig. 15) apparently must find its explanation in one or more basic metabolically determined parameters. Since strains with different cycle times (PRM32 and PRM35) (Fig. 16) have the same doubling time as PRM1 and PRM2 in liquid medium (0.9 h at 32°C), the variable(s) underlying cycle time cannot represent physiological processes fundamental to growth under all conditions.

DISCUSSION

Interactive process of swarm colony development. Previous descriptions in the literature and the observations just presented all indicate that *Proteus* swarm colony development involves a complex sequence of regulated multicellular events organized into distinct phases. The final symmetrical colony geometry is the integrated result of all these events. The morphogenetic process involves cellular differentiation and dedifferentiation (2, 3, 14, 16, 30), the formation of structured multicellular populations capable of specific behaviors (most notably swarming), and the interaction of different parts of the colony (as in the formation of the internal waves by swarmer cells produced in the previous terrace). After the first terrace is formed, the different phases of colony development are organized into a repeating cycle whose duration is robust to various treatments that alter some of the component phases.

The high degree of spatial and temporal order in *Proteus* colonies indicates that multicellular cooperativity and intercellular signalling compensate for fluctuations at the level of individual cells and rafts. The tendency of swarm colonies is to become increasingly synchronized and coherent as development proceeds (25). This tendency is particularly evident in the elimination of stutters in colony edge movement between the first and second swarm phases (Fig. 4a). The recent demonstration of a role for a homoserine lactone in swarming of *Serratia liquefaciens* is suggestive of the role that small extracellular molecules can play in information transfer (9). In addition, extracellular polymers can have multicellular integrating functions (23), which would be consistent with the role hypothesized below for capsular polysaccharides in creating a fluid environment necessary for swarming.

Relationship between growth and swarming. Continued colony growth internal to expanding swarm populations (Fig. 12) and the absence of an effect of additional glucose on cycle timing (Fig. 14) both indicate that swarming is not a response to nutrient exhaustion, as previously suggested (20). The events which trigger swarming are metabolically determined (both nutrition and temperature alter cycle timing) but apparently independent of final growth yield (Fig. 13). One general hypothesis suggested by the periodic nature of *Proteus* colony expansion is that swarming initiates when the population in the last terrace has reached a certain threshold, well before nutrient depletion occurs, even at low glucose levels.

Swarming appears to be a plausible strategy for maximizing biomass production and substrate utilization. We observed a constant bacterial density per unit of area at higher swarming velocities and increasing densities as swarming was inhibited by higher agar concentrations (Fig. 10d). These data are consistent with the hypothesis that rapidly expanding swarm colonies exhaust the nutrients under them and that nutrients cannot diffuse into the colony center because they are consumed by

the peripheral population. Swarming definitely facilitates total biomass production (Fig. 10b). However, our present knowledge of bacterial swarming is insufficient to determine whether biomass maximization is actually the goal of swarming behavior or merely a secondary consequence of a growth mode that evolved for other reasons.

The effects of agar on swarming. Among our more interesting observations was the finding that swarming velocity and the duration of the swarming phase are both exquisitely sensitive to the agar concentration (Fig. 11). We believe that the inhibitory effect of high agar concentrations is an important clue to the physics of swarming, and we suggest a specific hypothesis to explain our results. Swarming is dependent upon flagellar function (1, 4, 11, 15). This indicates that the swarming bacteria are moving in a fluid environment, and we have obtained evidence of fluid movements by tracking the behavior of small beads among swarming *Proteus* cells (8, 11a). To create this fluid environment, the bacteria must extract water from the agar gel under them. We believe that extraction is achieved by the elaboration of the acidic capsular polysaccharide observed to accompany swarming (27, 33). According to our hypothesis, the fluidity of the swarming environment would be determined by the relative osmotic activities of the agar polymers in the substrate and the acidic capsular polysaccharide around the bacteria. Lowering the capsular polysaccharide/agar osmotic activity ratio would reduce the ability of *P. mirabilis* to migrate over the substrate, and this is exactly what is found when the ratio is lowered, either by increasing the agar concentration (Fig. 11) or by a mutation which blocks polysaccharide biosynthesis (12).

Periodicity of *Proteus* colonies. For each temperature and medium, there is a clock-like aspect to *Proteus* colony development. From the start of the second swarming phase, the intervals between successive swarming phases are constant. This constant interval comprises the swarming-plus-consolidation cycle and is subject to variation by genetics, nutrition, and temperature but is unaffected by several treatments which have dramatic effects on swarming (Fig. 15). The robustness of the swarming-plus-consolidation cycle for a particular strain at a given temperature with a given source of amino acids is one of the principal aspects of our experimental results. This robustness could reflect the operation of some as yet unidentified oscillator governing the overall swarming-plus-consolidation cycle. However, mathematical modelling indicates that it is not essential to postulate an independent oscillator. A mathematical model submitted elsewhere shows how *Proteus* swarm colony periodicity can be based upon the interplay among a metabolically dependent cellular characteristic, the functional lifetime of a swarmer cell, and the interactive dynamics of population structure which govern swarmer motility (10). This model accounts for the lack of entrainment between adjacent colonies (Fig. 1) by postulating that age-weighted swarmer cell density, a nontransmissible internal colony property, is the key determinant of when swarming phases begin and end.

***Proteus* and other bacterial colonies as experimental models for self-organizing complex systems.** Control at both the cellular and multicellular levels is characteristic of virtually all biological morphogenesis. Bacterial colonies offer important advantages as experimental material for probing pattern formation and self-organization of complex systems (24): rapid and inexpensive experimentation, ready documentation of morphogenetic processes at the microscopic (cellular) and macroscopic (colonial) levels, and the potential for applying molecular genetics with nucleotide-by-nucleotide resolution. Others have begun to demonstrate the genetic potential of using *P. mirabilis* as a morphogenetic model system (1, 4, 5). We have exploited the geometric regularities of *Proteus* colonies to demonstrate how useful results can be extracted with very simple laboratory procedures. We expect that the combined application of molecular genetics, kinetic analysis of microscopic and macroscopic behaviors, and mathematical modelling to the remarkable symmetry and periodicity of *P. mirabilis* colonies will prove valuable to a wide variety of scientists interested in the dynamic evolution of complex systems.

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