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Digital Image Analysis of Growth and Starvation Responses of a Surface-Colonizing *Acinetobacter* sp.

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Surface growth of an *Acinetobacter* sp. cultivated under several nutrient regimens was examined by using continuous-flow slide culture, phase-contrast microscopy, scanning confocal laser microscopy, and computer image analysis. Irrigation of attached coccoid stationary-phase *Acinetobacter* sp. cells with high-nutrient medium resulted in a transition from coccoid to bacillar morphology. Digital image analysis revealed that this transition was biphasic. During phase I, both the length and the width of cells increased. In contrast, cell width remained constant during phase II, while both cell length and cell area increased at a rate greater than in phase I. Cells were capable of growth and division without morphological transition when irrigated with a low-nutrient medium. Rod-shaped cells reverted to cocci by reduction-division when irrigated with starvation medium. This resulted in conservation of cell area (biomass) with an increase in cell number. In addition, the changes in cell morphology were accompanied by changes in the stability of cell attachment. During phase I, coccoid cells remained firmly attached. Following transition in high-nutrient medium, bacillar cells displayed detachment, transient attachment, and drifting behaviors, resulting in a spreading colonization pattern. In contrast, cells irrigated with a low-nutrient medium remained firmly attached to the surface and eventually formed tightly packed microcolonies. It is hypothesized that the coccoid and bacillar *Acinetobacter* sp. morphotypes and associated behavior represent specialized physiological adaptations for attachment and colonization in low-nutrient systems (coccoid morphotype) or dispersion under high-nutrient conditions (bacillar morphotype).

Bacteria respond to changing environmental conditions by using a number of adaptive survival mechanisms. These mechanisms include phenotypic switching, often manifested both physiologically and structurally. A common structural response is the variation in cell size with changes in nutrient levels. Under low-nutrient conditions, reduced cell size increases the solute uptake capacity of the cell (13). Reduction of cell size at low growth rates has been reported for *Escherichia coli* (12) and *Pseudomonas aeruginosa* (32). Furthermore, prolonged starvation of many bacterial species results in extremely small cell size (14, 16, 30, 31). Morphological changes in bacteria have been most extensively studied in the genus *Arthrobacter*, for which a nutrient-dependent transition to rod morphology and reversion to coccus morphology have been documented (9). This morphological variation was growth rate dependent, with transition occurring at a distinct growth rate for each *Arthrobacter* species studied (26).

In addition to influencing cell size, nutrient status also affects bacterial adhesion. Kjelleberg et al. (17) demonstrated that marine bacterial communities growing in low-nutrient medium displayed greater relative adhesion than those grown in high-nutrient medium. Similarly, adhesion of *P. aeruginosa* was shown to be inversely related to specific growth rate (29), and a range of different bacteria displayed enhanced adhesion after starvation (8, 10, 15). However, increased bacterial attachment under low-nutrient response is not a universal phenomenon. It has been shown that some pseudomonads detached under nutrient-limited conditions (11, 22). Changes in bacterial attachment characteristics under different nutrient regimens have previously been suggested to be linked with changes in cell surface hydrophobicity (35).

Surface colonization behavior is a species-specific trait of sessile bacteria that often results in characteristic surface growth patterns (20, 22, 24). Colonization behavior has been shown to be modulated by motility (18) and flagellation (24) and may also be influenced by cell surface hydrophobicity (18). Furthermore, environmental parameters such as flow rate (18), viscosity (24), and nutrient status (22, 28) have also been demonstrated to alter pathways of surface colonization for specific bacterial species.

The goal of this study was to examine the effect of nutrient status on the morphology and surface colonization behavior of an *Acinetobacter* sp. isolated from a biofilm grown in an Alberta stream. Previous examinations of bacterial morphological changes have relied on light microscopic and electron microscopic evaluations of samples from planktonic populations. This study utilized continuous-flow slide culture and digital image analysis (6, 7), allowing the examination and quantitation of morphological parameters for individual attached cells under highly controlled environmental conditions. This method was also amenable to the study of changes in attachment characteristics and surface colonization behavior occurring concurrently with morphological changes.

MATERIALS AND METHODS

Isolation and cultivation of bacteria. *Acinetobacter* sp. strain GJ12 was isolated from a biofilm grown in Fish Creek, Alberta, by using submerged slide culture (33) and subsequently identified as an *Acinetobacter* sp. by using the API 20 system. This identity was confirmed by the Service de Diagnostic-FMV, Université de Montréal, Quebec, Canada, using additional metabolic tests. Cultures were stored at −70°C in sterile 5-ml cryogenic vials containing 10 3-mm boro-

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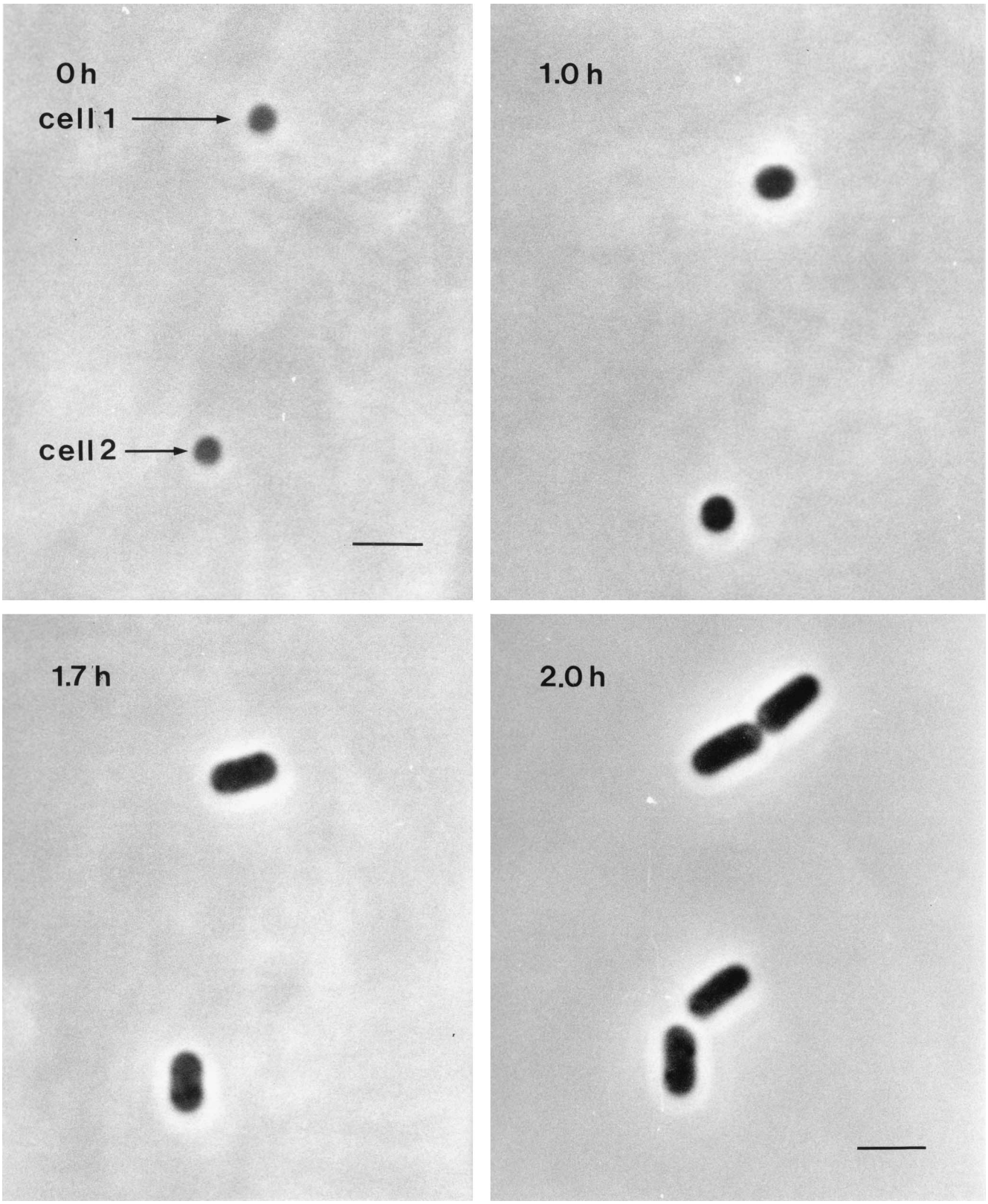

FIG. 1. Phase-contrast micrographs showing the growth response of attached stationary-phase *Acinetobacter* sp. strain GJ12 cells irrigated with CM. Coccoid cells underwent transition to bacillar morphology. Cell 1 divided at 2.55 h, and cell 2 divided at 2.47 h. Bar = 2 μm.

silicate glass beads, 30% strength tryptic soy broth, and 15% (vol/vol) glycerol. Inocula for continuous-flow slide culture experiments were grown in 150-ml shake flasks (150 rpm, 20°C) containing 50 ml of the appropriate medium. Complex medium (CM) consisted of 10% strength tryptic soy broth, while a buffered water solution (1) was used as a starvation medium (SM). Minimal medium (MM) was composed of deionized distilled water containing 31 mM $Na_2HPO_4 \cdot 7H_2O$, 20 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 7.6 mM $(NH_4)_2SO_4$, and 0.9 μM $FeSO_4 \cdot 7H_2O$, with 10 mM ethanol as the sole carbon and energy source.

Glass flow cells were constructed as previously described (7), cleaned by soaking overnight in 10% HCl, and rinsed with sterile deionized-distilled water. Medium flow was provided by a peristaltic pump (model 202U; Watson Marlow) at a flow rate of 37 ml h^{-1}, resulting in a laminar flow velocity of 25 μm s^{-1} within the 4-μm laminar layer (7). Flow cells were inoculated by stopping flow and injecting 1.0 ml of an overnight (17-h) culture. Bacteria were allowed 30 min to attach before flow was resumed, thereby washing away any unattached cells. During starvation experiments, cells were irrigated in continuous-flow slide culture with CM for 5 h, after which the medium composition was changed from CM to SM.

Microscopy. Bacteria colonizing the surfaces of flow cells were observed with a Zeiss Photomicroscope III, a 100× phase objective (numerical aperture, 1.3; Zeiss Neofluar), a green filter (Zeiss VG-9 46-78-05, 546 nm) to improve resolution, and an intensified silicon intensification target television camera (ISIT-66, MTI DAGE, Michigan City, Ind.). A neutral density filter was used to prevent inhibition of growth by high light intensities (23). Images were recorded on videotape by using a JVC BR9000U videocassette recorder (JVC, Japan) providing a real-time record of growth and behavior for cells cultivated under each nutrient regimen. Photomicrographs were obtained with either Plus-X 125 ASA or Tmax 100 ASA film (Kodak, Rochester, N.Y.).

Confocal laser micrographs were obtained with an MRC-600 Lasersharp fluorescence scanning confocal laser system (Biorad Microscience, Mississauga, Ontario, Canada) coupled with a Nikon FXA microscope equipped with a 60×, 1.4-numerical aperture objective. Biofilms were imaged by perfusing flow cell chambers with medium containing 0.01% fluorescein (5). The excitation source was an argon ion laser operated at 50 mW and 1% beam transmission. A 515-nm long-pass filter was used to detect fluorescent emission.

Computer image analysis. Phase-contrast photomicrographs were analyzed with an IBAS 2000 image processor (Kontron, Eching, Germany). An analog video signal was produced from 35-mm negatives by using a film video processor (Fotovix 87RU; Tamron, Japan) and digitized to create a 768 × 512 pixel matrix. Each digital image was the average of 10 analog images, thereby reducing electronic noise. The averaged digital image was then converted to a binary image before cells were interactively discriminated to eliminate background (21). The image processor was calibrated by using a stage micrometer. Cell area (square micrometers), length (maximum diameter in micrometers), and width (minimum diameter in micrometers) were then measured for cells in replicate fields of analysis. Cell areas were determined from the number of pixels per cell and the area per pixel in the scaled digital images. Rates of increase (instantaneous fractional increase) in cell area, cell length, and cell width for each cell were determined by plotting the natural logarithm of the cell parameter against time and calculating the slope (6, 25). Field cell area (total area of cells per field in square micrometers) and number of cells per field were measured during starvation experiments to determine changes in population size and biofilm biomass.

RESULTS

Morphological transition and reversion. Growth of attached stationary-phase cells irrigated with CM resulted in the transition from coccoid to bacillar morphology (Fig. 1). Following inoculation, the average dimensions of attached stationary-phase cells were 1.3 ± 0.2 by 1.0 ± 0.1 μm. However, after 2 h of growth, cell dimensions increased to 2.6 ± 0.6 by 1.2 ± 0.2 μm. Digital image analysis of cell dimensions during growth revealed that this transition occurred in two distinct phases. Initially, both cell width and cell length increased (phase I), resulting in enlarged coccoid cells and a constant cell length-to-width ratio of 1.3 ± 0.1 (Fig. 2). This phase lasted for approximately 1 h, after which cell width became constant while cell length continued to increase (phase II). This resulted in bacillar cells and an increasing cell length-to-width ratio (Fig. 2). The rates of increase in cell length and width were equal during phase I (0.2 ± 0.1 h^{-1}). During phase II, the rate of increase in cell length was 0.7 ± 0.1 h^{-1} and cell width remained constant. The rate of increase in cell area during phase I was 0.42 ± 0.08 h^{-1}, whereas in phase II the rate of increase in cell area was 0.81 ± 0.15 h^{-1}. Cells were capable of growth and division without morphological transition when irrigated with MM. The rate of increase in cell area during growth in MM was 0.22 ± 0.05 h^{-1}.

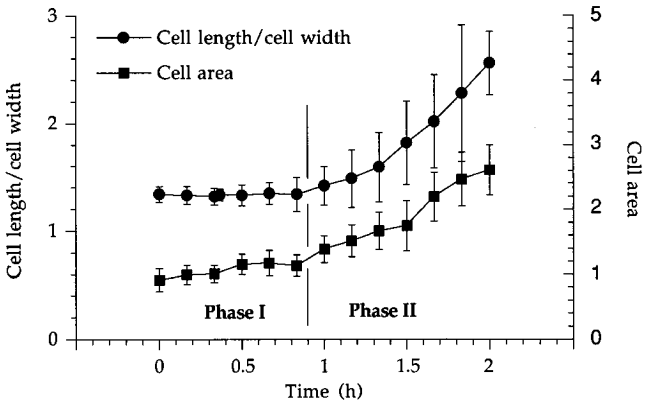

FIG. 2. Digital image analysis demonstrating the two-phase growth response of stationary-phase *Acinetobacter* sp. cells irrigated with complex medium. Phase I was characterized by an increase in both cell length and width, resulting in a constant length-to-width ratio and enlarged coccoid cells. During phase II, cell width remained constant whereas cell length continued to increase. This resulted in an increasing cell length-to-width ratio and transition to bacillar morphology. Note the greater rate of increase in cell area (i.e., steeper slope) during phase II.

Reversion to coccoid from bacillar morphology occurred when the nutrient regimen was switched from CM to SM (Fig. 3). This reversion required two or four divisions per cell, depending on the initial cell length. This process occurred incrementally (i.e., a long rod divided into two shorter rods, each of which divided into two cocci). Digital image analysis of microscopic fields during starvation revealed a disparity between the rate of increase in cell number and the rate of increase in total cell area per field (Fig. 4). The rates of increase in field cell area and cell number prior to starvation were 0.51 ± 0.22 h^{-1} and 0.65 ± 0.31 h^{-1}, respectively. Starvation had an immediate effect on the rate of increase in field cell area, which declined to 0.08 ± 0.06 h^{-1} over the first 3 h of starvation. In contrast, the rate of increase in cell number declined only to 0.22 ± 0.8 h^{-1} during this time period. After approximately 3 h of starvation, field cell area and cell number became constant, with this stasis lasting for the duration of the experiments (24 h).

Attachment and surface colonization. Cells did not exhibit Brownian or flow-induced motion in continuous-flow slide culture when irrigated with SM or MM. Cell growth and division in MM, coupled with firm attachment, resulted in the formation of tightly packed microcolonies. Thus, irrigation of attached *Acinetobacter* sp. strain GJ12 cells with MM led to formation of an aggregated biofilm composed of discrete microcolonies (Fig. 5A).

Irrigation with CM resulted in a change in attachment stability. After approximately 2 h of growth (following transition to bacillar morphology), cells displayed Brownian motion, detachment, and flow-induced drifting (Fig. 6) and often became polarly attached. These attachment characteristics culminated in the spread of surface-colonizing bacteria and in the formation of a dispersed biofilm (Fig. 5B). Loosely attached cells became firmly attached when the medium was changed from CM to SM.

DISCUSSION

The growth and behavior of *Acinetobacter* sp. strain GJ12 on surfaces varied, depending on the composition of the irrigation

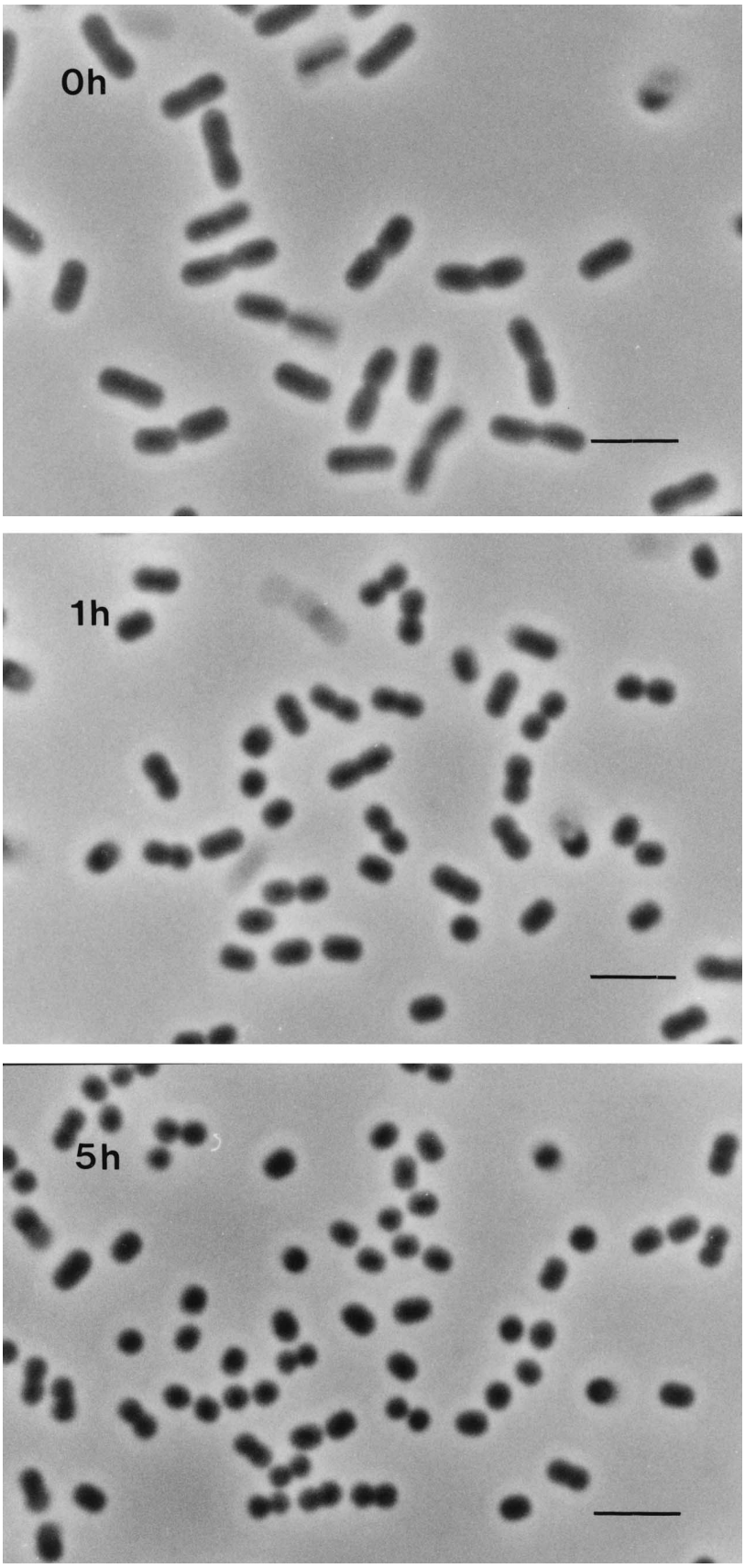

FIG. 3. Phase-contrast micrographs showing the starvation response of sessile *Acinetobacter* sp. cells following a change from irrigation with CM to SM. Cells ceased growth but continued to divide, resulting in an increase in the number of cells and a reduction in cell size. Bar = 5 μm.

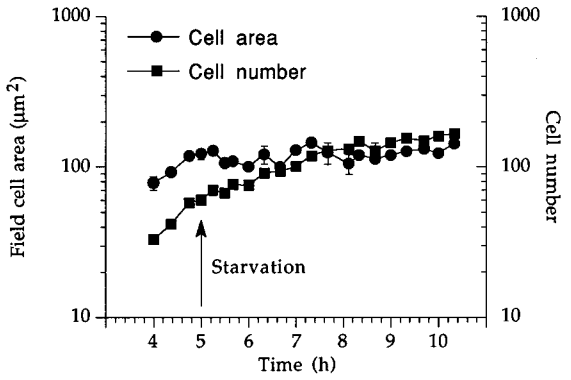

FIG. 4. Image analysis of the starvation response of *Acinetobacter* sp. strain GJ12. Prior to starvation, cells were irrigated with CM and both the number of cells and total cell area per field were increasing (surface colonization). Starvation conditions caused an immediate cessation of the increase in cell area, whereas the number of cells continued to increase for approximately 3 h of starvation.

medium. Under high-nutrient conditions (CM), transition from coccoid to bacillar morphology occurred, followed by a decrease in attachment stability. This behavior resulted in dissemination of cells across the surface and formation of a dispersed biofilm structure. This alteration in morphology and attachment characteristics was reversed when cells were subjected to starvation conditions. Morphological transition and dispersive behavior did not occur under low-nutrient conditions (MM), with cells grown under these conditions forming an aggregated biofilm composed of discrete, tightly packed microcolonies. The pathways of surface growth and starvation responses are summarized in Fig. 7.

The transition to bacillar morphology and reversion to coccoid morphology of *Acinetobacter* sp. strain GJ12 analyzed in this study were similar to the morphogenic cycle of *Arthrobacter* spp. as described by Clarke (9). In both cases, transition and reversion were nutritionally controlled. Previous studies have also indicated that the morphological transition of *Arthrobacter* spp. was related to specific growth rate, with transition in a chemostat occurring at a distinct dilution rate for each species studied (26). A similar relationship between growth rate and transition in *Acinetobacter* sp. strain GJ12 was suggested during the present study, in which growth rate was determined from the increase in cell area. Transition of *Acinetobacter* sp. strain GJ12 occurred in media providing a relatively high growth rate (e.g., CM) but not in media providing a lower growth rate (e.g., MM). The greater rate of increase in cell area during transition (phase II) than during the period of growth before transition (phase I) also suggests that this transition was related to growth rate, because the rate of increase in cell area was proportional to the growth rate (6). However, cell width increased during phase I and was constant during phase II. This finding suggests that cell thickness (*z* dimension, i.e., height of cells above the plane of the glass) also increased during phase I and was constant during phase II. Thus, the two-dimensional (*x* and *y*) rate of increase in cell area would not include the increase in cell thickness and would consequently underestimate the rate of growth. These difficulties emphasize the importance of developing methods for three-dimensional image analysis to determine the actual growth responses of sessile microorganisms.

The reversion of *Acinetobacter* sp. strain GJ12 to coccoid morphology was also similar to observations reported for species of *Arthrobacter*, for which a gradual reversion rather than a fragmentary reversion was reported (9). The reduction of cell size resulting from starvation likely reflects a survival response. Such a response results in an increase in cell number, which increases the probability of survival, while biomass is conserved. A decrease in cell size accompanied by an increase in population has also been observed during starvation of marine bacteria (2, 30). The ubiquity of bacterial cell size fluctuations following nutrient level changes underscores the importance of phenotypic flexibility (3). Furthermore, reduction of cell size may have industrial significance by improving the penetrability of bacterial inoculants for in situ bioremediation and plugging operations (19, 27).

In addition to the morphological response to nutrient status, a change in attachment characteristics of *Acinetobacter* sp. strain GJ12 occurred. During starvation or growth in low-nutrient medium, cells remained firmly attached, whereas growth under high-nutrient conditions resulted in unstable attachment. Conditions promoting unstable attachment were also conditions which permitted the highest rate of increase in cell area (i.e., growth rate). These results are in agreement with reports of enhanced adhesion of slowly growing (29) as well as starved (8, 10, 15) bacteria. Cell surface hydrophobicity has been identified as an important factor in microbial adhesion and was shown to be influenced by growth rate (35) and starvation (15). *Acinetobacter calcoaceticus* demonstrated greater adhesion to hydrocarbons (i.e., greater hydrophobicity) during stationary phase than during logarithmic phase, and it was suggested that this was due to a lack of thin pili on logarithmic-phase cells (34). However, there were no differences in piliation between logarithmic-phase and stationary-phase *Acinetobacter* sp. strain GJ12 cells (14a).

The difference in attachment characteristics of cells grown under low-nutrient and high-nutrient conditions contributed to a difference in surface colonization by *Acinetobacter* sp. strain GJ12. The stable attachment of slowly growing cells resulted in the formation of microcolonies. However, under high-nutrient conditions, cells did not become firmly attached but continuously migrated across the surface in what has been previously termed the spreading maneuver (20). A similar type of spreading behavior was observed during surface growth of a *Pseudomonas* sp. which was scavenging surface-bound substrate (28). The change in surface colonization behavior of *Acinetobacter* sp. strain GJ12 contrasted with that observed for *Pseudomonas syringae*, which detached under low-nutrient conditions and formed adherent microcolonies under high-nutrient conditions (22). Overall, these observations are in agreement with hypotheses stating that surface colonization behavior is a species-specific characteristic (4, 21) and may reflect different starvation survival strategies of these species. Since *P. syringae* was motile, detachment under adverse conditions followed by motile chemotaxis could result in the return of cells to a favorable environment. In the case of the nonmotile *Acinetobacter* sp. strain GJ12, starvation survival may entail firm adhesion and cell dormancy until the nutrient status becomes more favorable.

During this study, it was determined that *Acinetobacter* sp. strain GJ12 cells cultivated on surfaces responded to changes in nutrient state by changing both cell morphology and adhesion characteristics. These responses likely reflect growth rate-dependent surface colonization strategies for survival and reproductive success. Dispersive behavior (i.e., spreading) during growth under high-nutrient conditions allows expansion of the population over a surface. Under low-nutrient or starvation conditions, population expansion becomes secondary in importance to survival and cells display an aggregative behavior (microcolony formation). This study also demonstrates the utility of continuous-flow slide culture and computer image

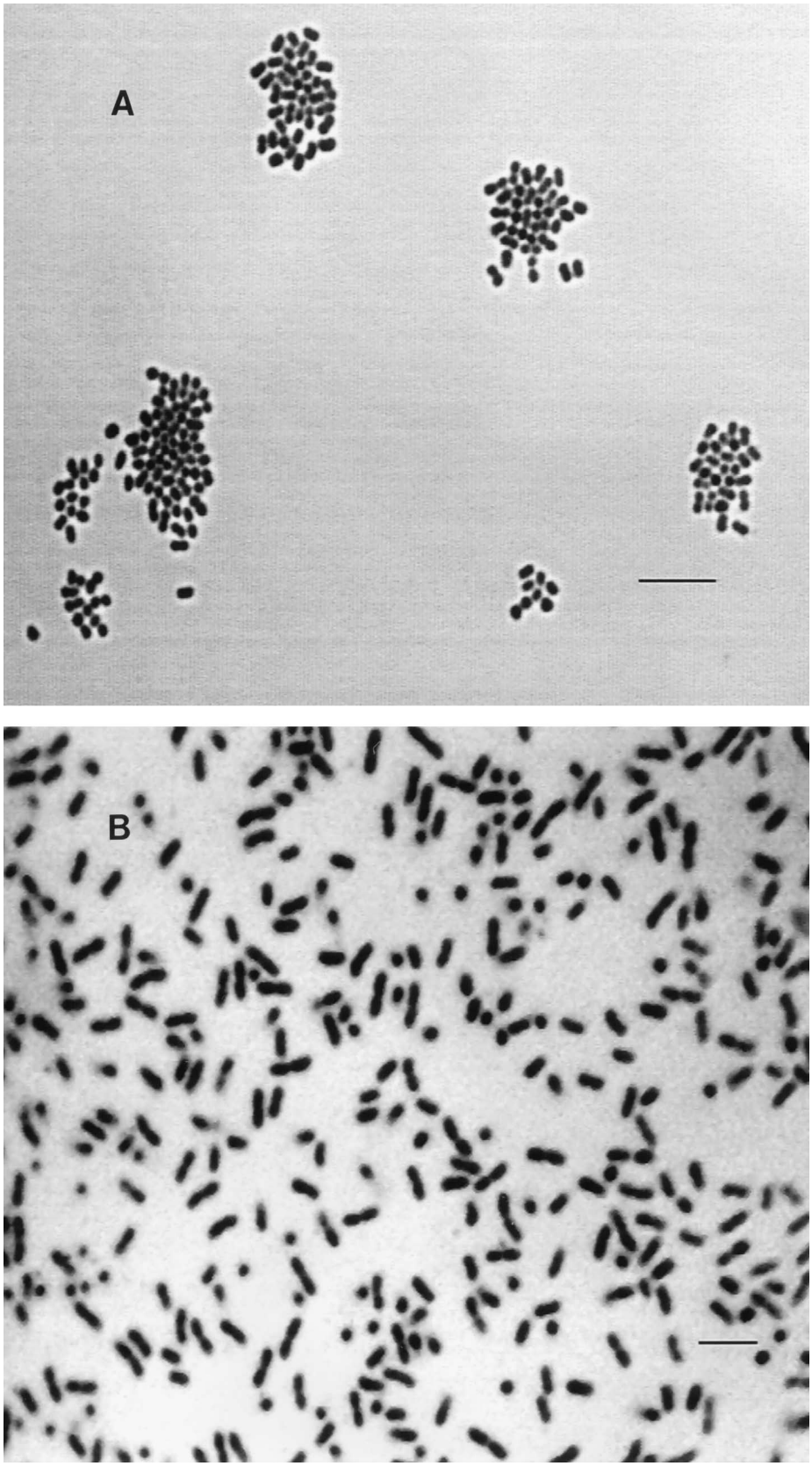

FIG. 5. Confocal laser micrographs showing surface colonization patterns of *Acinetobacter* sp. cells grown under low-nutrient (A) and high-nutrient (B) conditions for 12 h. Cells remained firmly attached under low-nutrient conditions, resulting in microcolony formation and an aggregated biofilm. High-nutrient conditions resulted in dispersive behavior (unstable attachment) and a relatively dispersed biofilm.

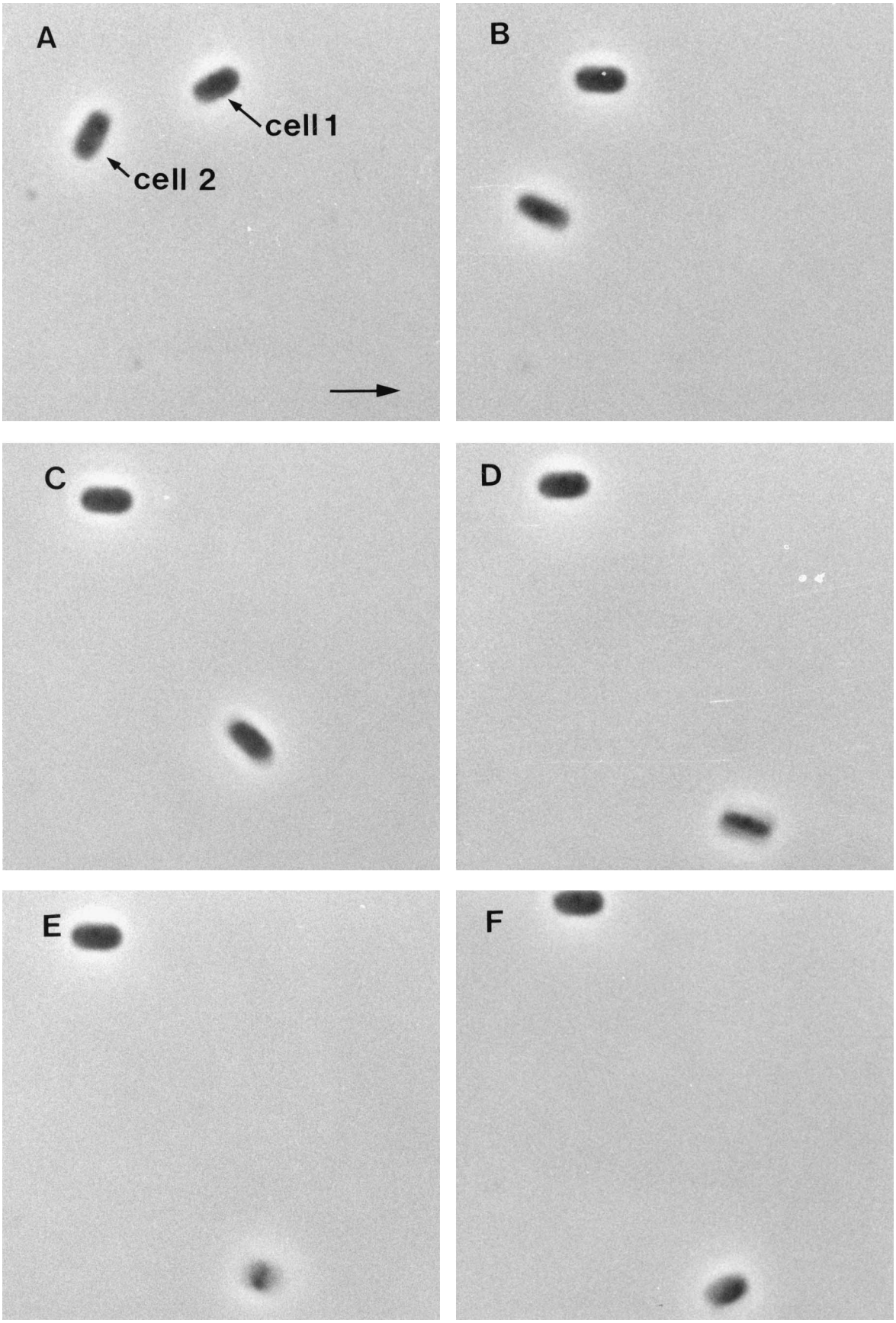

FIG. 6. Phase-contrast micrographs showing the dispersive behavior of an *Acinetobacter* sp. cell (cell 2) relative to that of a cell which remained stably attached during the same time interval (cell 1) during growth under high-nutrient conditions. Micrographs were taken at approximately 30-s intervals; the direction of medium flow is indicated by the arrow (A), which is 5 μm long. The cell displayed a drifting movement (A to D) followed by a rolling movement (E and F).

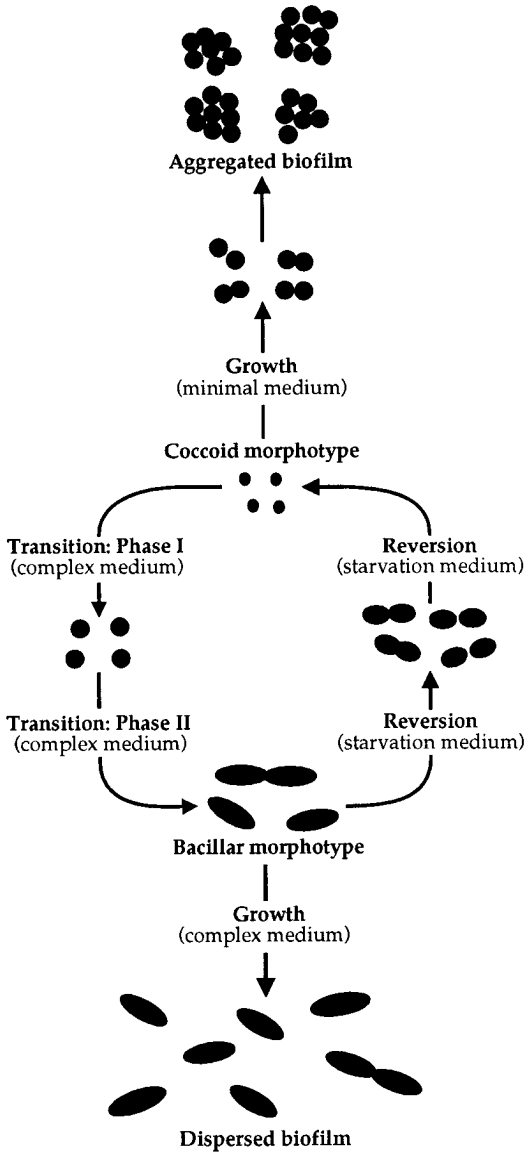

FIG. 7. Diagram of the *Acinetobacter* sp. strain GJ12 life cycle and surface colonization patterns. Starved and stationary-phase cells were small cocci but underwent a two-phase transition to bacillar form when irrigated with CM. The attachment of rod-shaped cells was unstable, resulting in a spreading surface colonization behavior and a dispersed biofilm. The bacillar morphotype reverted to coccus morphology by reduction-division when irrigated with SM. Growth and division without transition to bacillar morphology occurred when cells were irrigated with MM. Attachment of cocci was stable, resulting in a biofilm consisting of aggregated microcolonies.

analysis for the study of morphological responses of attached bacteria to defined solution chemistry.

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