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Effect of Laminar Flow Velocity on the Kinetics of Surface Recolonization by Mot$^+$ and Mot$^-$ *Pseudomonas fluorescens*

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Abstract. Computer-enhanced microscopy (CEM) was used to monitor bacteria colonizing the inner surfaces of a 1 × 3 mm glass flow cell. Image analysis provided a rapid and reliable means of measuring microcolony count, microcolony area, and cell motility. The kinetics of motile and nonmotile *Pseudomonas fluorescens* surface colonization were compared at flow velocities above (120 μm sec^{-1}) and below (8 μm sec^{-1}) the strain's maximum motility rate (85 μm sec^{-1}). A direct attachment assay confirmed that flagellated cells undergo initial attachment more rapidly than nonflagellated cells at high and low flow. During continuous-flow slide culture, neither the rate of growth nor the timing of recolonization (cell redistribution within surface microenvironments) were influenced by flow rate or motility. However, the amount of reattachment of recolonizing cells was both flow and motility dependent. At 8 μm sec^{-1} flow, motility increased reattachment sixfold, whereas at 120 μm sec^{-1} flow, motility increased reattachment fourfold. The spatial distribution of recolonizing cells was also influenced by motility. Motile cells dispersed over surfaces more uniformly (mean distance to the nearest neighbor was 47.0 μm) than nonmotile cells (mean distance was 14.2 μm) allowing uniform biofilm development through more effective redistribution of cells over the surface during recolonization. In addition, motile cell backgrowth (where cells colonize against laminar flow) occurred four times more rapidly than nonmotile cell backgrowth at low flow (where rate of motility exceeded flow), and twice as rapidly at high flow (where flow exceeded the rate of motility). The observed backgrowth of Mot$^+$ cells against high flow could only have occurred as the result of motile attachment behavior. These results confirm the importance of motility as a behavioral mechanism in colonization and provides an explanation for enhanced colonization by motile cells in environments lacking concentration gradients necessary for chemotactic behavior.

Introduction

During the colonization of surface microenvironments by *Pseudomonas fluorescens,* cells attach and grow to form discrete microcolonies [6, 12, 15]. Each

colony consists of 2^n cells, where n is the number of generations that occur following initial attachment. After the fourth generation, some cells detach and subsequently reattach at new locations where they form microcolonies. This behavior has been termed "recolonization" [15], and differs from colonization (where cells attach once and grow) in that recolonizing cells continuously redistribute over vacant surface sites when the recolonization phase begins. Thus, recolonization is a key phase during the development of a confluent *P. fluorescens* biofilm.

Previous studies demonstrated the importance of motility for successful in vitro colonization of a number of different surface types [1, 5, 7, 8, 9, 11, 14, 15]. It has also been shown that flagella may adhere to surfaces during reversible attachment [2, 15, 16, 18]. The tethered cells then rotate until either emigration or irreversible attachment occurs. During surface colonization by *P. fluorescens,* cells move upstream or cross-stream against flow rates exceeding their maximum rate of motility. These maneuvers may only be accomplished if the cells remain attached while moving across the surface, a form of behavior referred to as "motile attachment" [15]. Motile attachment behavior should enhance cell redistribution (speeding biofilm formation) and make recolonizing cells more resistant to shear stress caused by laminar flow. This study used Mot^+ and Mot^- strains to determine whether the flagellum enhanced the colonization, recolonization, and backgrowth (colonization against laminar flow) of *P. fluorescens.* The importance of motile attachment behavior in these processes was determined by comparing colonization kinetics at low flow rates with those at high flow rates. Those aspects of colonization that involve motile attachment should be flow resistant and those that do not should be flow dependent.

Materials and Methods

Organism and Culture Conditions

Slide culture experiments were conducted using *Pseudomonas fluorescens* strain CC-840406-E. This strain was isolated using a dual-dilution continuous culture enrichment [5] which selected for successful surface colonizing bacteria. Colonizing behavior was maintained in batch culture by growing cells on the surface of 50 1-mm glass beads in shake flasks (25°C and 100 rpm). Cells were subcultured by transferring a single colonized bead to fresh 10% (w/v) tryptic soy broth (TSB). Each bead was washed twice in sterile 0.1% (w/v) peptone before transfer, ensuring that only attached cells were subcultured. Cultures were stored at −80°C in sterile 5-ml cryogenic vials (Corning Glass Works, Corning, NY) containing 50 3-mm borosilicate glass beads [13]. Frozen cultures were checked for viability and purity at 90 day intervals.

Mutant Isolation

Mutant #18 (Fla^- Mot^-) *P. fluorescens* was selected following transposon 5 mutagenesis with the suicide vector pSUP1011 (containing resistance factors for chloramphenicol and kanamycin) [21] using the following procedure. *P. fluorescens* CC-860512 rif^r (a spontaneous rifampicin-resistant mutant derived from the wild-type, CC-840406-E) and *E. coli* SM10 containing pSUP1011 were grown to late log phase in the presence of rifampicin (100 $\mu g\ ml^{-1}$) and chloramphenicol (30 $\mu g\ ml^{-1}$), respectively. One milliliter of each culture was mixed and filtered onto millipore filters which

were placed on *Pseudomonas* Agar F (PAF) for 24 hours at 25°C. The bacteria were resuspended and plated on PAF (containing 100 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin): Rif-, Kan-resistant colonies were recovered at a frequency of 4×10^{-6} per recipient. Random transposition and suicide of the vector was confirmed by hybridization to EcoRI digested DNA from random colonies using Tn5 and vector sequences as probes. Approximately 9,000 Tn5 mutants were pooled and point inoculated onto the center of 10 soft agar plates (0.4% PAF). Following 10 transfers from the center of each plate, nonmotile colonies were isolated and purified.

Verification of Mutant Nonmotility

Nonmotile candidates were assayed for motility by plating the motility-negative candidate on motility agar (0.33% agar, 0.5 g liter^{-1} glucose and M salts [5]) and measuring radiant growth from the central inoculation point. Suspended log phase cells were also observed for movement using high-power phase and low-power darkfield microscopy. Last, cells and flagella were stained for phase [17] and transmission electron microscopy (TEM) analysis [23]. Mutant m#18, which had identical growth characteristics to CC-840406-E, was found to lack a flagellum. The nonmotile mutant was cultured as described for the wild-type. There was no significant difference between the growth rate of the Mot$^+$ parent and the Mot$^-$ mutant in batch culture or slide cultures.

Determination of Maximum Rate of Motility for Strain CC-840406-E

The maximum rate of motility was determined for wild-type strain CC-840406-E. Cells were grown in batch culture (as described above) until an optical density (O.D.) of 0.1 (A_{660}) was reached. A 1-ml aliquot was then diluted tenfold, and injected into a flow cell containing static 10% TSB. Difference imagery (described below) was then used to quantitate the velocity of several hundred individual cells. The value reported here is the maximum observed cell velocity in micrometers per second.

Continuous-Flow Slide Culture Apparatus

The slide culture system consisted of a slide culture chamber (flow cell) mounted on the stage of a Zeiss Photomicroscope III (Oberkocken, West Germany) and observed using a Bosch S.I.T. television camera (silicone intensification target tube, Bosch, TVC9A). Growth medium was continuously pumped through the chamber and microbial colonization was quantitated using image analysis. Low (8 μm sec^{-1}) and high (120 μm sec^{-1}) media flow rates were supplied by an ISCO 301 syringe pump (ISCO, Lincoln, NE) and a Watson Marlow 501Z peristaltic pump (Watson Marlow, Cornwall, England), respectively.

Slide culture chambers were constructed as described by Caldwell and Lawrence [5, 14]. Residual organic matter present on the chamber's inner walls was oxidized by soaking the chamber overnight in a 7% hypochlorite solution. After flushing away excess hypochlorite with deionized-distilled water, the chambers were baked overnight at 170°C. The chamber was then clamped to the microscope stage and aseptically connected to the medium supply reservoir using silicone tubing (5 mm internal diameter, Cole Parmer, Chicago, IL). A 25 mm sterile filter (0.22 μm pore size, Millipore, Bedford, MA) installed at the chamber inlet sterilized the medium, removed suspended particles, and trapped air bubbles. Slide culture medium was filter sterilized (0.22 μm) before aseptically filling the supply reservoir, preventing filter clogging and microbial growth.

Inoculation and Incubation of Slide Culture

Log phase cells grown in 10% TSB were positioned on the lower surface of a flow cell by injecting a pulse of culture fluid through the slide chamber inlet tube. Injecting the inoculum directly into

the flow cell interior prevented excessive colonization of tubing walls. The density of attached cells was controlled by adjusting medium flow during inoculation or by injecting additional inoculum. Initial field densities were set at 20–50 cells per field. Inoculated flow cells were then incubated at 23 ± 3°C for a period of 8 hours (at high or low flow) using 10% TSB as irrigation medium.

Downstream Inoculation of Slide Culture for Backgrowth Study

Inoculum (prepared as described above) was injected from the downstream side of the slide culture chamber using a 38 mm, 26 G, sterile hypodermic needle. The needle was maneuvered within the slide chamber interior (observed microscopically), allowing the precise positioning of cells on the lower inner surface of the slide chamber while constant flow was maintained. A distinct "front" of attached cells on the flow cell surface was thus achieved. The inoculated flow cell was then incubated at 23 ± 3°C for approximately 35 hours as described above.

Attachment Assay

A 1-ml aliquot of Mot^+ or Mot^- culture (prepared as described above and standardized to an O.D. of 0.1 (A_{660})) was injected through the flow cell inlet tube. Flow was maintained at either 120 μm sec^{-1} or 8 μm sec^{-1} flow for 5 min. Ten random fields were chosen and results were expressed as the average number of cells $field^{-1}$ of 10 measured fields. Darkfield microscopy and CEM techniques were used as described below.

Determination of Laminar Flow Velocities

The bulk flow rate (ml $hour^{-1}$) was set by measuring the effluent volume per hour. Macroenvironment laminar flow velocities (cm sec^{-1}) were determined by dividing the bulk flow rate by the slide chamber's cross-sectional area. Microenvironment flow rates (within the hydrodynamic boundary layer) were determined as described previously [15]. Surface microenvironment flow rates of 8, 30, and 120 μm sec^{-1} corresponded linearly with macroenvironment flow rates of 10, 100, and 500 ml $hour^{-1}$.

Image Processing Hardware

A light sensitive Bosch S.I.T. television camera was mounted on Zeiss Photomicroscope III, and interfaced with an IBAS-2000 image processing computer (Kontron Inc., Eching, West Germany). This system was programmed to digitize images of growing microcolonies at 30 min intervals. The Zeiss microscope was equipped with a 100-W Halogen lamp, a 10× darkfield objective lens (Zeiss, 46 04 00, NA = 0.22), and a 2× optivar. A green interference filter (Zeiss VG-9, 46-78-05, 546 nm ± 10 nm) was used to improve microscopic resolution. Image brightness was initially adjusted using an internal diaphragm and grey neutral density filters. The S.I.T. television tube permitted the use of very low light levels during analysis, minimizing light irradiation effects on the developing biofilm. Photomicrographs were taken using Kodak Plus-X 125 ASA film (Rochester, NY).

Image Processing Procedure

Computer measurements were calibrated using the IBAS one-step scaling function and a microscope stage micrometer. A linear scale factor was then automatically converted to an area scale factor

(μm^2 $pixel^{-1}$) used in subsequent cell area measurements. Our particular lens combination (10× objective, 2× optivar) assigned a length of 0.7916 μm $pixel^{-1}$. Multiple analog images (television images) of a representative field could then be digitized and averaged. Image averaging eliminated electronic noise and limited analysis to attached, stationary bacterial cells and microcolonies. Each digital image consisted of approximately 400,000 pixels (a 768 × 512 matrix). The original image was stored as a reference image, whereas the working image was multiplied, increasing microscopic resolution and enhancing cell contrast. Discrimination (elimination of background) of the digital image produced white objects on a black background which could then be measured.

Cells moving at the slide chamber surface were also identified and counted automatically to provide a measure of cell motility. Two field images were digitized in rapid succession (0.24 sec) and subtracted, producing a "difference" image [4]. The difference image highlighted only those objects that moved, ignoring attached cells. Fifty difference images were collected every 30 min, providing a large sample of motility events.

Operating as described above, the IBAS-2000 measured microcolony count, area, size distribution, and in a linked program, the surface motility of the developing biofilm. Any distinct object identified following discrimination was measured as a single microcolony. Microcolonies consisting of many cells were still counted as a single object, therefore data were expressed in terms of microcolony count and microcolony area. Increases in microcolony area were then used to determine specific growth rates, mean colony sizes, and size distributions for the developing biofilm. Motility events were expressed as motile events $field^{-1}$ min^{-1}.

Calculation of Reattachment Rate

The reattachment rate of recolonizing cells was calculated using TK! Solver software (Software Arts, Wellesley, MA) written for the Macintosh™ microcomputer (Apple Computer Inc., Cupertino, CA). Third degree polynomial curves fitted to microcolony count data (from CEM analysis) were obtained using Cricket Graph (Cricket Software, Malvern, PA). Equations for curves representing Mot^+ and Mot^- growth responses at high and low flow were then entered into TK! Solver. This program was then used to determine corresponding slopes (rates of cell reattachment) at 1 hour intervals throughout the 8 hour experiment. The rate of reattachment of recolonizing cells was subsequently expressed as microcolonies $field^{-1}$ $hour^{-1}$.

Spatial Distribution of Reattached Cells

Cell distribution patterns of motile and nonmotile cells were compared following 8 hour biofilm development at 8 μm sec^{-1}. Five random cells that reattached within the field of analysis were chosen and the distance to 10 of their nearest neighbors measured using CEM. These values were averaged and expressed as the mean distance to the nearest neighboring cell, providing a measure of the randomness of cell recolonization.

Plate Counts

Slide culture effluent was collected to determine the number of viable cells washed from the slide culture chamber per milliliter as biofilm development progressed. Each sample's volume was determined by weighing. Limiting the length of downstream tubing minimized the adsorption of cells to the walls of the outlet hose. Effluent was collected at hourly intervals, diluted, and pour plated in 20 ml of 42°C molten tryptic soy agar. Plates were counted following a 2-day incubation period at 25°C.

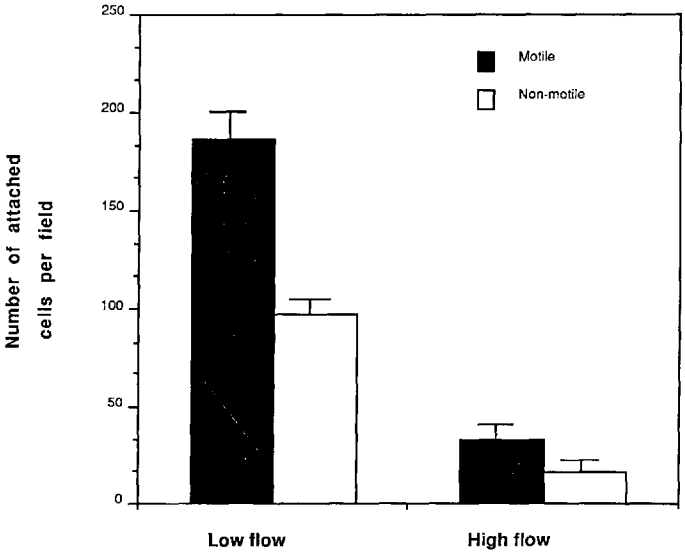

Fig. 1. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on the number of Mot$^+$ and Mot$^-$ cells that attached per field (606 × 404 μm) during a 5 min attachment period. Standardized inoculum (1 ml culture at 0.1 O.D.$_{660}$, diluted tenfold) was injected through the slide chamber inlet tube.

Results

Effect of Laminar Flow on the Initial Cell Attachment

Motile and nonmotile cells that attached to flow cell surfaces were quantitated at high and low flow rates (Fig. 1). The average number of Mot$^+$ cells (from 10 replicates) that attached over 5 min at low flow was 186.1 cells field^{-1} (SD = 20.9), whereas approximately half as many Mot$^-$ cells attached under the same conditions (97.1 cells field^{-1}, SD = 10.9). At high flow, the difference between the attachment of Mot$^+$ and Mot$^-$ strains increased (32.8 cells field^{-1}, SD = 8.3 vs 10 cells field^{-1}, SD = 2.9) indicating that high laminar flow decreased the attachment rate for both strains, but decreased the attachment of Mot$^-$ cells to a greater extent.

Effect of Laminar Flow on Recolonization of P. fluorescens

The timing and degree of recolonization by motile *P. fluorescens* was monitored in slide culture over an 8 hour period. While the timing of recolonization was flow independent, the number of reattaching cells decreased as laminar flow increased. At low flow, microcolony counts for both strains were constant until recolonization occurred at 5 hours (the 8–16 cell stage) (Fig. 2A). After the 8 hour incubation period, Mot$^+$ microcolony counts had increased to over 600% the initial cell density (47 cells field^{-1}), the result of recolonizing unicells reattaching within the field of observation. Nonmotile microcolony counts increased 130% (initial field density of 26 cells field^{-1}) (Fig. 2B) over the same period. At high flow, Mot$^+$ microcolony counts remained constant until 5 hours

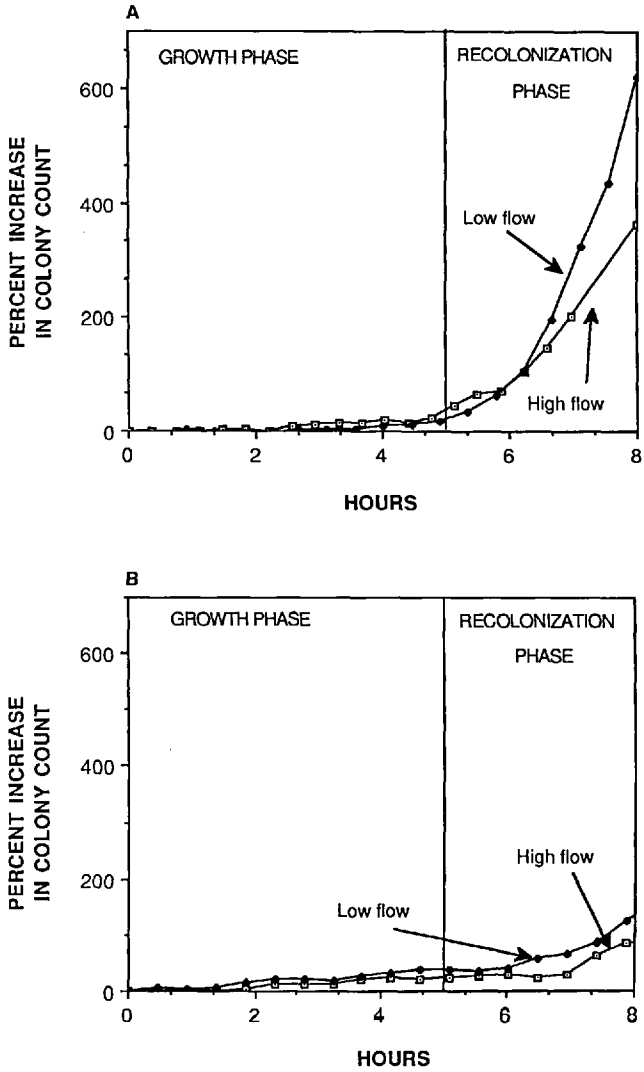

Fig. 2. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on the number of Mot$^+$ (A) and Mot$^-$ (B) microcolonies per field during slide culture. Data was reported as the percent increase in microcolony count, thereby accounting for differences in initial field densities. For Mot$^+$, the cell densities for high and low flow experiments remained constant during the growth phase (initially 47 and 40 cells field^{-1}, respectively), increasing after 5 hours. The initial field densities for Mot$^-$ cells were 26 and 24 cells field^{-1} for high and low flow, respectively.

(Fig. 2A), attaining 360% the initial cell density (40 cells field^{-1}) after 8 hours. Under similar conditions, Mot$^-$ microcolony counts increased by only 88% (initial cell density of 24 cells field^{-1}) (Fig. 2B). The rate of reattachment (at 8 hours) for the Mot$^+$ strain at low flow was 200 microcolonies field^{-1} hour^{-1} and at high flow, 50 microcolonies field^{-1} hour^{-1} (Fig. 3A) compared with 48

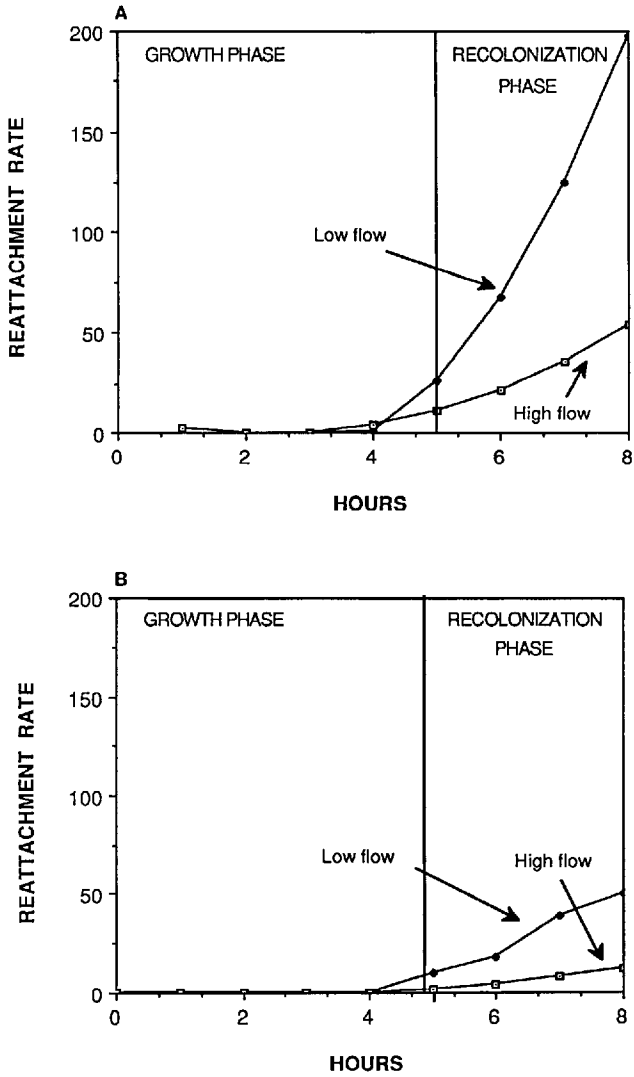

Fig. 3. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on the rate of Mot$^+$ **(A)** and Mot$^-$ **(B)** cell reattachment during the recolonization phase. Third degree polynomial curves of microcolony count data were obtained using Cricket Graph (Cricket Software, Malvern, PA). Equations for Mot$^+$ and Mot$^-$ growth curves were then entered into TK! Solver which was used to determine the hourly rate of cell reattachment. This rate was subsequently expressed as the number of microcolonies field^{-1} hour^{-1} which attached to the flow cell surface.

microcolonies field^{-1} hour^{-1} (low flow) and 13 microcolonies field^{-1} hour^{-1} (high flow) for the Mot$^-$ strain (Fig. 3B). A number of replicate experiments demonstrated that initial cell density did not influence the outcome of recolonization of either the Mot$^+$ or Mot$^-$ strain. Generally, the Mot$^+$ strain recolonized 4–5 times more rapidly at low and high flow than the Mot$^-$ strain.

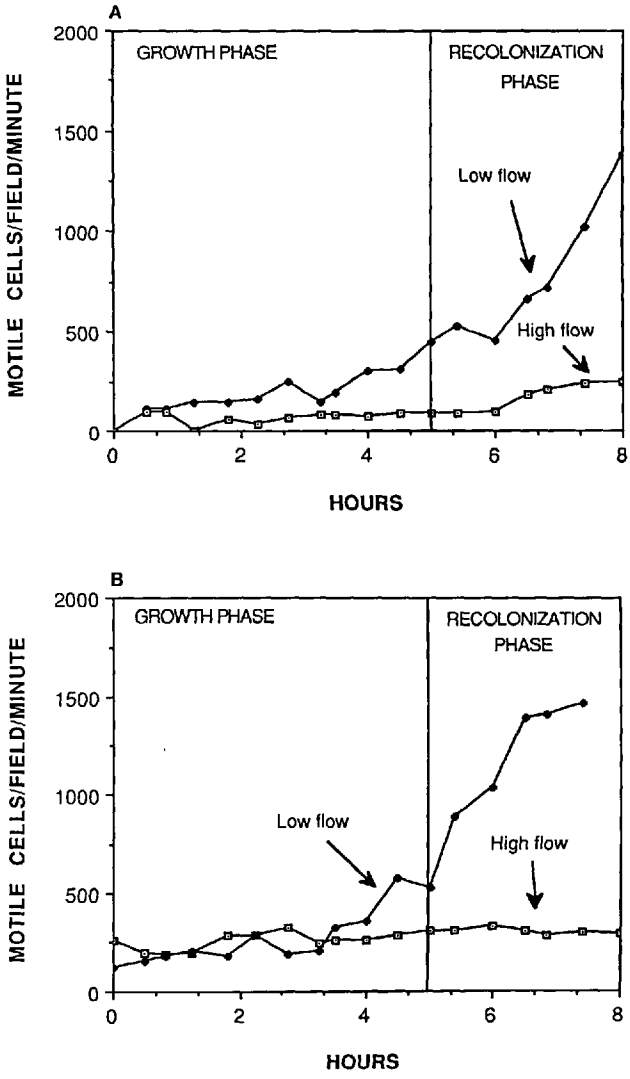

Fig. 4. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on the number of moving Mot^+ (**A**) and Mot^- (**B**) cells (motile cells $field^{-1}$ min^{-1}). Difference imagery was used to quantitate the number of cells passing near the flow cell surface.

Results obtained at a flow velocity of 30 μm sec^{-1} were not significantly different from those at 8 μm sec^{-1}.

CEM Determined Cell Motility

Motility remained negligible for both Mot^+ and Mot^- strains during the first 4 hours of slide culture incubation, regardless of flow velocity. However, motility counts (motility events $field^{-1}$ min^{-1}) increased from 0 (during the first 4 hours) to 1,440 (at 8 hours) for the Mot^+ strain during low flow studies (Fig. 4A). The Mot^- strain showed a similar increase in motility after 4 hours during low flow

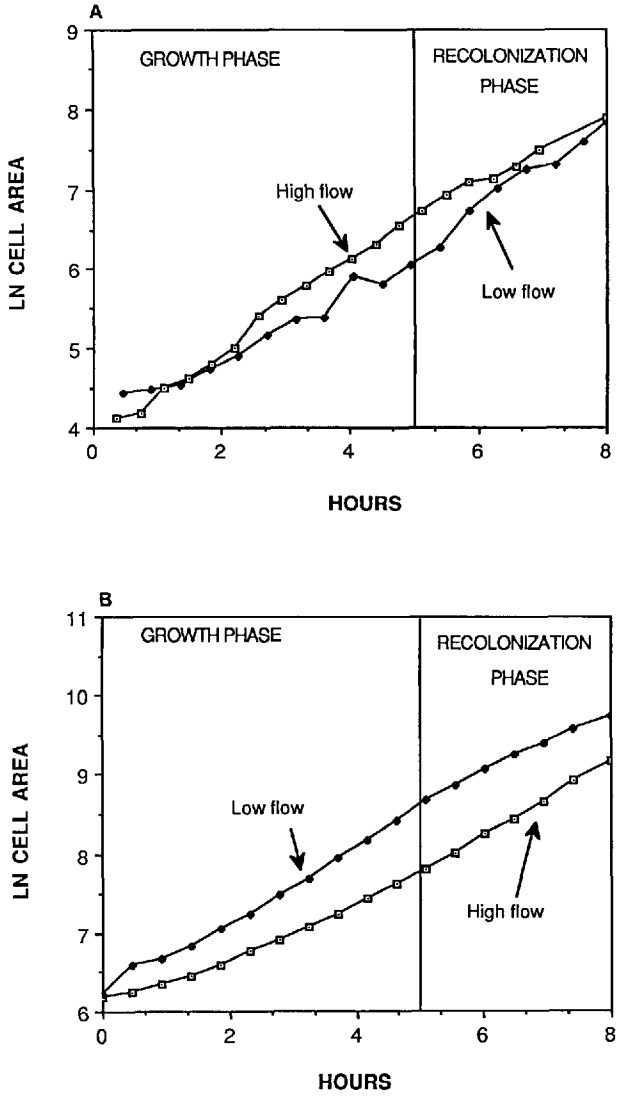

Fig. 5. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on Mot^+ (**A**) and Mot^- (**B**) microcolony cell area (μm^2).

studies (Fig. 4B), suggesting that Mot^- cells were capable of timed detachment without flagella. However, nonmotile cells showed a reduced tendency to reattach. High flow motility counts for both strains showed slight increases following 4 hours.

Effect of Laminar Flow on Specific Growth Rates

Growth of Mot^+ and Mot^- microcolonies in 10% TSB irrigation media was not flow dependent. Specific growth rates for Mot^+ *P. fluorescens* (Fig. 5A) virtually remained constant ($\mu = 0.47$, $R = 0.99$), as did the nonmotile strain ($\mu = 0.45$, $R = 0.99$) (Fig. 5B).

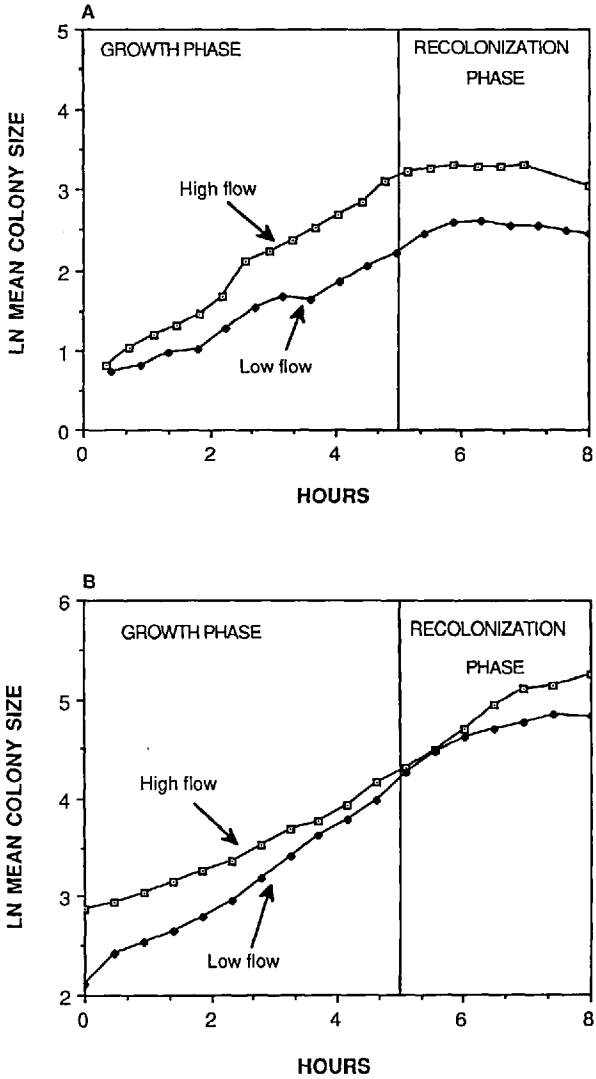

Fig. 6. Effect of high and low flow (120 and 8 μm sec^{-1}, respectively) on the average microcolony size (μm^2) of Mot$^+$ (A) and Mot$^-$ (B) cells grown in slide culture. With Mot$^+$, a decrease in mean microcolony size coincided with the initiation of the recolonization phase, thereby reducing the average size of Mot$^+$ microcolonies during high and low flow experiments. With Mot$^-$, no decrease in average microcolony size was observed during the recolonization phase, as reattaching Mot$^-$ cells were unable to redistribute themselves randomly over the surface. These results were consistent with distance-to-nearest-neighbor data shown in Figure 10.

Effect of Laminar Flow on Mean Microcolony Size

The average microcolony size (Fig. 6A) for attached Mot$^+$ microcolonies decreased after 5 hours during both high and low flow treatments, consistent with the reattachment of single recolonizing unicells (Fig. 2A). No decrease in the

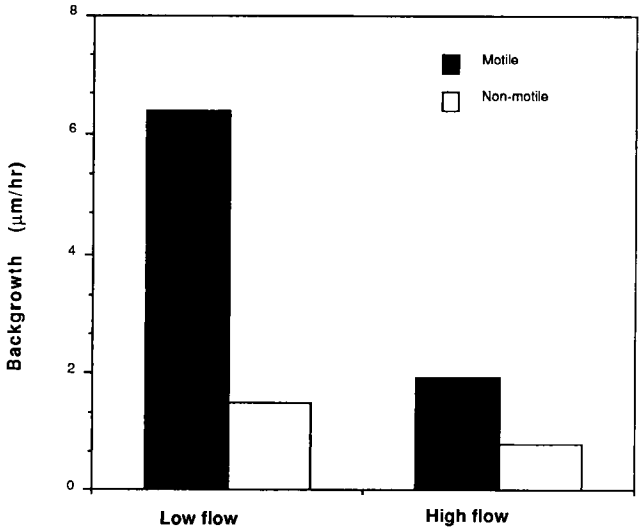

Fig. 7. The effect of high and low flow (120 and 8 μm sec^{-1}), respectively on the backgrowth of Mot$^+$ and Mot$^-$ *P. fluorescens* during extended (35 hour) continuous-flow slide culture experiments.

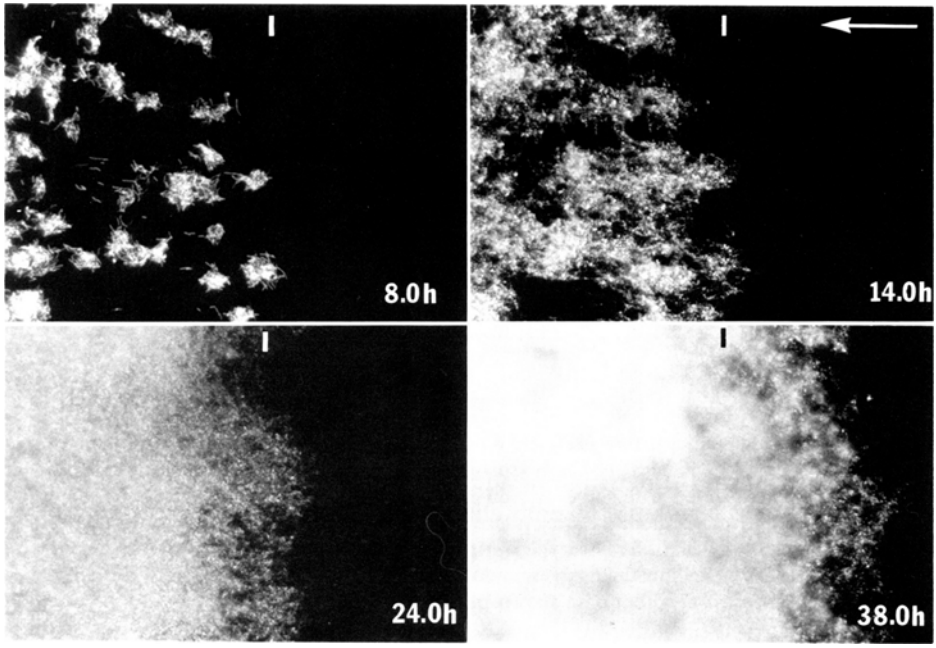

Fig. 8. Darkfield photomicrographs of Mot$^+$ biofilm backgrowth at low flow (8 μm sec^{-1}). The biofilm "front," which advanced against flow at a rate of 6.4 μm hour^{-1}, progressed 250 μm over a 38 hour period and is shown 8, 14, 24, and 38 hours after inoculation. The vertical bar positioned near the top central region of each photograph represents the initial position of the bacterial "front" for each time period, and the arrow indicates scaling (100 μm) and the direction of flow.

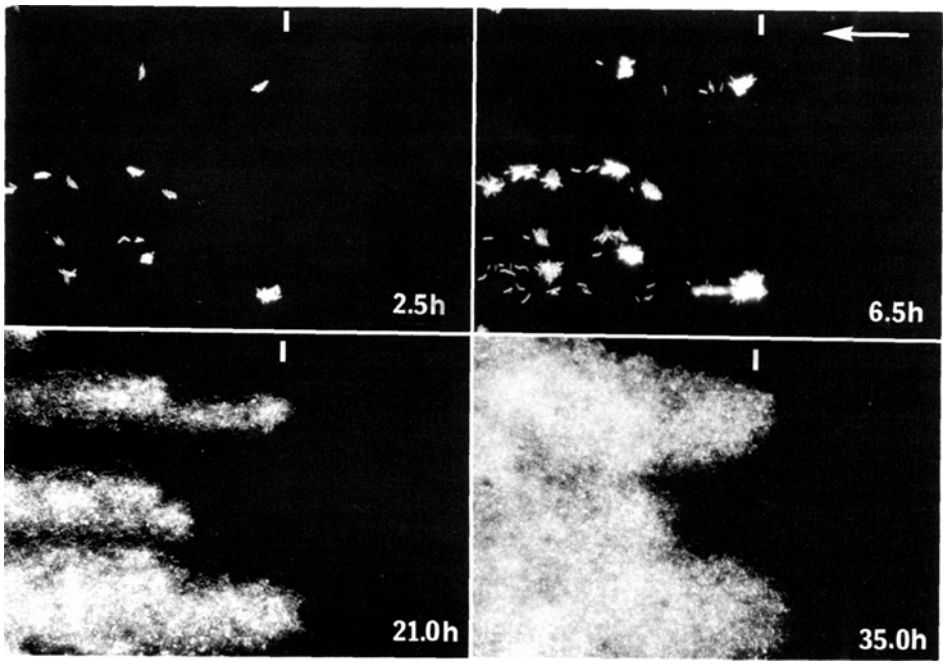

Fig. 9. Darkfield photomicrographs of Mot⁻ biofilm backgrowth at low flow (8 μm sec^{-1}) over 35 hours. The biofilm "front," shown at 2.5, 6.5, 21, and 35 hours after inoculation, advanced against flow at 1.5 μm hour^{-1}. The characteristic contours (horizontal streaks, 21 hours) of the Mot⁻ biofilm front result from the inability of nonmotile cells to redistribute themselves effectively during recolonization. The vertical bar positioned in each photograph represents the initial position of the bacterial "front" for each time period, and the arrow indicates scaling (100 μm) and the direction of flow.

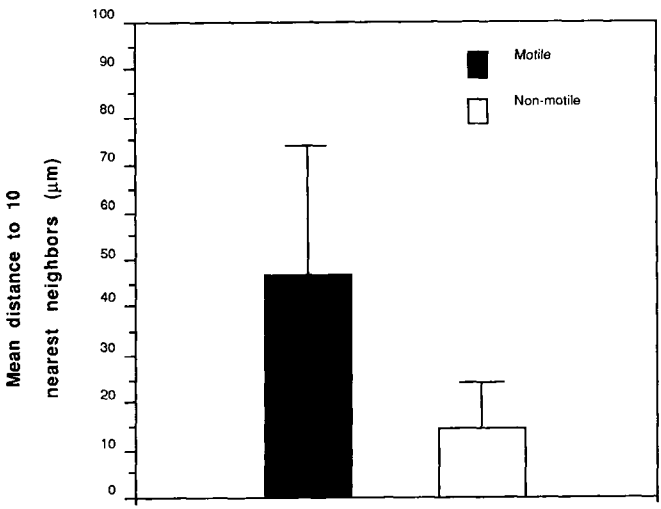

Fig. 10. The effect of low flow (8 μm sec^{-1}) on the spatial distribution of Mot⁺ and Mot⁻ cells recolonizing the flow cell surface during slide culture.

Fig. 11. Darkfield photomicrograph showing the 35 hour distribution pattern at the "front" of recolonizing Mot⁻ *P. fluorescens* during continuous-flow slide culture. Nonmotile cells reattached most frequently on the "lee" side of existing microcolonies. The arrow represents 200 μm.

Mot⁻ mean microcolony size was evident during high and low flow studies (Fig. 6B), indicating that although reattachment of recolonizing unicells occurred (confirmed by an increased microcolony count at 5 hours), their uneven distribution resulted in their being counted as fewer large colonies rather than many small colonies. This was confirmed by mean-distance-to-neighbor data obtained from Mot⁻ biofilms.

Backgrowth and Redistribution of Motile and Nonmotile Strains

Mot⁺ and Mot⁻ cells at the biofilm "front" were monitored to quantitate cell backgrowth during biofilm development. At low flow, the Mot⁺ strain grew against flow at 6.4 μm $hour^{-1}$ over 38 hours (Figs. 7, 8). During this period, many motile cells were observed migrating against the flow, but few cells reattached. The Mot⁻ strain grew against flow at 1.5 μm $hour^{-1}$ over 35 hours under similar conditions (Figs. 7, 9). During high flow, the Mot⁺ strain's rate of backgrowth decreased to 1.9 μm $hour^{-1}$, compared with a rate of 0.8 μm $hour^{-1}$ for the Mot⁻ strain. The redistribution of recolonizing Mot⁺ and Mot⁻ cells in similar fields (i.e., cell density and stage of development) was also quantitated using CEM (Fig. 10). Following 8 hour incubation at 8 μm sec^{-1} flow, the mean distance from recolonizing Mot⁺ cells to their nearest neighbor was 47.0 μm (SD = 28.5), whereas the mean distance from Mot⁻ cells to their nearest neighbor was 14.2 μm (SD = 6.2), indicating that redistribution of recolonizing Mot⁻ cells occurred less randomly. Motile cells reattached in an evenly distributed, random manner, whereas nonmotile cells formed "windrows" or "drifts" of reattached cells strung out directly behind the parent colony (Fig. 9 (21 hours) and Fig. 11).

Effect of Laminar Flow on Cell Washout

Increases in the number of viable cells washed from the flow cell per min were consistent with the timing of recolonization. Near 4–5 hours, the number of

viable cells washed from the system increased by three orders of magnitude (data not shown). Although large increases in effluent cfu's (colony forming units) occurred after 5 hours, differences between Mot^+ and Mot^- strain washout were not distinguishable using the plate count technique.

Discussion

Role of Flagella During Initial Attachment

Previous studies of motile and nonmotile bacterial surface colonization demonstrated that motility facilitates the infection, spread, and attachment of many bacterial species in a number of diverse microenvironments [1, 3, 7, 8, 9, 19]. In investigations where flagellar adherence to surfaces was considered, it was shown that flagella occasionally act as adhesive appendages [2, 14, 16, 18]. Our findings revealed that motility facilitated initial attachment in high and low flow environments. Work done by Guentzel and Berry [8] also demonstrated the importance of motility during colonization of mammalian tissue. Nonmotile vibrios (Fla^-) failed to associate with intestinal mucosa and were subsequently nonpathogenic in mice. It was thought that the nonflagellated cells were unable to access the intervillar spaces necessary for long-term colonization of the bowel. Similar results were obtained by De Weger et al. [7] using a potato-root model system. De Weger et al. found that nonmotile, siderophore-producing *P. fluorescens* failed to effectively colonize potato roots in numbers high enough to stimulate improved plant growth. This study confirms that flagellar motility provides an advantage during the initial attachment of *P. fluorescens* cells to glass surfaces. During the low flow CEM adhesion assay, Mot^+ cells attached to lower flow cell surfaces at two times the rate of Mot^- cells. Motile cells attached at three times the rate of Mot^- cells during high flow, although both strains attached less rapidly than during low flow. High laminar flow was also shown to decrease the recolonization of Mot^+ and Mot^- *P. fluorescens*; however, motile cells were significantly more resistant to these effects. These results indicated that alternate surface contact mechanisms for nonmotile cells (such as cell settling, brownian motion and vortex currents) became less effective as flow velocity increased. It also clearly demonstrated the importance of the flagellum during colonization of rapidly flowing surface microenvironments.

Role of Flagella During Cell Detachment

The importance of flagella during the emigration of cells from established microcolonies was also addressed during these studies. If the flagellum is necessary for cell detachment, then nonmotile cells should fail to emigrate from mature microcolonies. If flagella simply facilitate cell detachment, a greater number of motile cells should emigrate from microcolonies when compared with nonmotile cells.

P. fluorescens growing in continuous-flow slide culture developed at a constant rate until the 8–16 cell stage was reached (4–5 hours), regardless of flow

or motility. Caldwell and Lawrence [5] previously demonstrated flow-dependent growth responses by *P. fluorescens* during flow-on flow-off slide culture studies. The present study utilized a complex growth medium (10% TSB) rather than 100 mg liter^{-1} glucose in minimal medium, and did not include a flow-off component (as used by Caldwell and Lawrence), therefore decreases in growth rates were not observed during periods of decreased flow. It remains possible that a strain that randomly distributed recolonizing progeny over a surface might develop more rapidly than one that recolonized site specifically, as nutrient limited regions associated with high cell concentrations would be avoided.

After 5 hours of surface growth, some cells within *P. fluorescens* microcolonies became unstable and detached, initiating the recolonization phase. The timing of this detachment was confirmed using the computer parameters microcolony count (counts began to increase at 5 hours), and motility (the number of detached Mot$^+$ cells field^{-1} min^{-1} began to increase at 4 hours). Increases in the number of cells present in slide culture effluent were also consistent with the timing of recolonization. Cfu's washed from the system remained constant until 5 hours, at which time a 1,000-fold increase in cell washout occurred. Cell detachment did not appear to require a flagellum, as flagellated and nonflagellated cells both recolonized at 5 hours. Therefore, some mechanism other than the activation of motility must control cell emigration. Van Loosdrecht et al. [22] reported that the surface hydrophobicity of *P. fluorescens* CC-840406-E (Mot$^+$) first increases and then decreases during a batch culture time course. P. Delaquis (personal communication) investigated this phenomenon further, demonstrating that surface hydrophobicity decreased as Mot$^+$ *P. fluorescens* CC-840406-E depleted available substrate (glucose). Decreases in hydrophobicity correlated with large increases in the number of planktonic cells, indicating that motile cells actively emigrated from surfaces when glucose was exhausted. Similarly, cells within 8–16 cell microcolonies might also become nutrient limited during slide culture, leading to decreased hydrophobicity of those nutrient limited cells. By releasing cells from developing microcolonies, the parent colony's size becomes reduced (reducing the nutrient deficiency within the microcolony), and detached progeny may colonize new surfaces. Therefore, chemical changes affecting cell surface chemistry may explain the timed detachment of Mot$^+$ and Mot$^-$ *P. fluorescens* from surfaces.

Role of Flagella During Bacterial Recolonization

The flagellum also plays a significant role during the reattachment and relocation of bacterial cells, a behavior termed recolonization. The greatest reattachment of recolonizing cells (in slide culture) occurred with the Mot$^+$ strain during conditions of low flow. Under such conditions, Mot$^+$ microcolony counts increased to over 600% of the initial cell density (a 200 microcolony field^{-1} hour^{-1} attachment rate at 8 hours), whereas the Mot$^-$ strain increased by only 130% (50 microcolonies field^{-1} hour^{-1} at 8 hours). It was observed that high laminar flow decreased the recolonization of both strains by about 50%, however, flagellated cells still reattached in fourfold greater numbers than Mot$^-$ cells. The reduction of recolonization success by high flow suggested that most

recolonizing cells detached and entered the rapidly flowing bulk phase where they were washed from the system. This was consistent with increased cell washout observed during the recolonization phase and decreased reattachment during high flow studies. The mutant's inability to move across laminar flow boundaries near the flow cell surface [10], or their inability to respond chemotactically to nutrient gradients [3, 20] likely contributed to these observed differences. In addition, any flagellum-mediated attachment would be impossible during colonization by the Mot$^-$ (Fla$^-$) mutant strain.

Those flagellated cells that reattached despite high flow (Fig. 2A) were typically observed traveling along surfaces against flow rates exceeding *P. fluorescens* maximum rate of motility (85 μm sec^{-1}). As flow velocity exceeded *P. fluorescens* measured rate of motility, motile attachment would explain how cells "tractor" themselves upstream against extreme flow rates [14]. This behavior would explain a 4.2-fold greater rate of backgrowth for motile cells at low flow (6.4 μm hour^{-1} for motile vs 1.5 μm hour^{-1} for nonmotile) (Fig. 7). The maximum Mot$^-$ backgrowth that might have occurred over 35 hours can be determined by multiplying the number of cell divisions by the average length of the cell, assuming cells divided in an end-to-end fashion. Using a cell length of 3 μm and 23.3 cell divisions (doubling time of 1.5 hours), potentially 2 μm hour^{-1} backgrowth by nonflagellated cells may have occurred under optimal conditions (a total of 70 μm), whereas 52.5 μm backgrowth was actually measured over 35 hours. Obviously, rates of backgrowth exceeding this value must have occurred due to motility or motile attachment, the latter being most significant where flow velocity exceeded *P. fluorescens* maximum rate of movement. The flagellum was also observed to affect the distribution of motile *P. fluorescens* progeny over available surface attachment sites. The average microcolony size for Mot$^-$ microcolonies did not decrease at 5 hours as observed with motile microcolonies (Fig. 6A, B), confirming that nonmotile cells primarily reattach near preexisting microcolonies. This correlated with a CEM-determined cell distribution pattern for Mot$^+$ cells which was 3.3 times more random (Fig. 10) than for Mot$^-$ cells. As a result, Mot$^-$ microcolonies were larger and less numerous, increasing the mean microcolony size. Motile cells evenly distributed themselves during recolonization, presenting small, separate objects, thereby decreasing the mean microcolony size after 5 hours. It therefore appears that nonflagellated cells develop as a biofilm less rapidly than flagellated strains because (1) they do not recolonize or "seed" vacant surfaces as evenly as motile strains (thus slowing the formation of a confluent biofilm), and (2) microcolonies of the nonmotile strain are incapable of rapid backgrowth in either high or low flow environments. Those nonmotile cells that successfully reattached were always positioned on the "lee" side of the microcolony from which they emigrated (Fig. 9 (21 hours) and Fig. 11). This characteristic pattern of Mot$^-$ cell reattachment likely occurred as a result of vortex back eddies [10], or "quiet" zones immediately downstream of established colonies. Only within these regions is it likely that detached cells were able to recontact the surface and reattach.

The flagella did not appear to influence cell detachment in flowing microenvironments, but it certainly played an important role during the following phases of bacterial surface colonization: the initial attachment phase (flagella facilitated initial cell adsorption to surfaces), the recolonization phase (flagella

significantly increased the reattachment and random redistribution of recolonizing cells over the surface), and the motile attachment phase (flagella increased cell resistance to washout, and more significantly, facilitated cell backgrowth under conditions of high laminar flow). These observations confirm that the flagellum is a significant attachment mechanism as well as an organelle of movement.

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