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Effect of Motility on Surface Colonization and Reproductive Success of *Pseudomonas fluorescens* in Dual-Dilution Continuous Culture and Batch Culture Systems

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The colonization of glass surfaces by motile and nonmotile strains of *Pseudomonas fluorescens* was evaluated by using dual-dilution continuous culture (DDCC), competitive and noncompetitive attachment assays, and continuous-flow slide culture. Both strains possessed identical growth rates whether in the attached or planktonic state. Results of attachment assays using radiolabeled bacteria indicated that both strains obeyed first-order (monolayer) adsorption kinetics in pure culture. However, the motile strain attached about four times more rapidly and achieved higher final cell densities on surfaces than did the nonmotile strain (2.03×10^8 versus 5.57×10^7 cells $vial^{-1}$) whether evaluated alone or in cocultures containing motile and nonmotile *P. fluorescens*. These kinetics were attributed to the increased transport of motile cells from the bulk aqueous phase to the hydrodynamic boundary layer where bacterial attachment, growth, and recolonization could occur. First-order attachment kinetics were also observed for both strains by using continuous-flow slide culture assays analyzed by image analysis. The DDCC system contained both aqueous and particulate phases which could be diluted independently. DDCC results indicated that when cocultures containing motile and nonmotile *P. fluorescens* colonized solid particles, the motile strain replaced the nonmotile strain in the system over time. Increasing the aqueous-phase rates of dilution decreased the time required for extinction of the nonmotile strain while concurrently decreasing the overall carrying capacity of the DDCC system for both strains. These results confirmed that bacterial motility conveyed a selective advantage during surface colonization even in aqueous-phase systems not dominated by laminar flow.

Microbial fouling begins with the sorption of a molecular film, followed closely by the attachment of a mixture of different bacterial species and their eventual growth and development into a multispecies biofilm. Competition for surface sites by a mixture of bacteria involves the mass transport of the cells from the aqueous bulk phase to the hydrodynamic boundary layer where attachment and surface colonization may occur (7, 21, 38, 39). Long-term surface colonization may then be influenced by a number of physical, biochemical, and behavioral factors (7, 20, 23). Recently, the formation of mixed-species biofilms has been investigated in an effort to understand those parameters which dictate in situ bacterial colonization processes. Banks and Bryers (1), using radiolabeled *Pseudomonas* and *Hyphomicrobium* spp., measured net biofilm accumulation and species composition as well as growth and shear-related effects on the nature of the developing biofilm. However, the unique colonization behaviors utilized by these distinct organisms were not considered during this study (14, 22). Cowan et al. (8) evaluated bacterial attachment and biofilm formation during continuous recirculation of bacterial cocultures, focusing on the adhesive nature of the contributing organisms. Microbial colonization of the oral ecosystem has also provided numerous examples of coaggregating bacteria involved in the formation of dental plaque, many of which undergo highly specific cell-cell adhesion events (18). To date, the majority of microbial competition studies have focused on the growth or substrate uptake of planktonic populations of bacteria (11, 32, 35, 40). Numerous studies exist in which the adhesion of various microbial species and strains has been evaluated in vitro (2, 10, 27). Microbial behavior has been demonstrated to play a significant role during adhesive events (20, 21, 22, 24, 28–30); however, the literature generally explains such phenomena in terms of cell-substratum physicochemistry (3, 37, 38, 42, 43).

Within flow cells dominated by laminar-flow conditions, adaptations such as the possession of a functional flagellum have previously been shown to play important roles during microbial colonization events by affecting initial attachment success, chemotaxis, and positioning behavior and by directly influencing cell redistribution, recolonization, and overall biofilm morphology (4, 17, 19, 20, 24). Here, we investigated the significance of flagellar motility during competitive and noncompetitive surface colonization assays in systems not dominated by laminar flow and during colonization of surfaces where dilution of both the aqueous and particulate phases was controlled and quantified as stress factors. Studies of this nature will assist in providing an understanding of the behavioral mechanisms which govern the formation and persistence of native biofilms in a variety of environments.

MATERIALS AND METHODS

Organism and culture conditions. The bacterial strains used in this study were *Pseudomonas fluorescens* CC-840406-E and M18. Strain CC-840406-E was isolated by using a dual-dilution continuous culture (DDCC) enrichment procedure which selected for competitive surface-colonizing bacteria (5). *P. fluo-*

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rescens M18, a Fla$^-$ Km^r Tn*5* mutant, was derived from strain CC-840406-E and previously determined to be nonmotile (20). Both *P. fluorescens* strains possessed identical growth rates (μ = 0.49) when attached or grown planktonically (20). Adherent behavior was maintained by growing cells in 0.06% (wt/vol) Trypticase soy broth (TSB; Difco, Detroit, Mich.) containing sterile 3-mm-diameter glass beads. Cells were subcultured by transferring a single colonized bead to fresh TSB-containing flasks (20). *P. fluorescens* CC-840406-E and M18 (hereafter referred to as the Mot$^+$ and Fla$^-$ strains, respectively) were cultivated by using 0.06% TSB and isolated, handled, and stored as described previously (5, 20).

Radiolabeling of bacteria. Mot$^+$ or Fla$^-$ *P. fluorescens* was grown in 250-ml Erlenmeyer flasks containing 50 ml of sterile 0.06% TSB until the late log phase was reached. Flasks were shaken at 100 rpm on an orbital flask shaker (New Brunswick Scientific Co., Edison, N.J.) maintained at 23 ± 3°C. A 5-ml aliquot of an overnight culture was then added to 30 ml of sterile 0.06% TSB containing either 1 μCi of L-[3,4,5-^{3}H(N)]-leucine (specific activity, 143.5 Ci mmol^{-1}; Dupont, Boston, Mass.) or 1 μCi of L-[U-^{14}C]glutamate (specific activity, 225 to 275 mCi mmol^{-1}) per ml of culture liquid and incubated for an additional 4 h. Radiolabeled cells were then harvested by centrifugation (2,000 × *g* for 10 min) and resuspended in sterile 0.006% TSB. This washing procedure was repeated two additional times. Cell suspensions were then standardized to an optical density at 600 nm of 0.10 and compared with a cell concentration standard curve determined by using a Petroff-Hausser counting chamber. One-milliliter aliquots (containing ~5.3 × 10^8 bacteria) of this cell suspension were then filtered by using 0.45-μm-pore-size, 25-mm Millex nitrocellulose filters (Millipore Corp., Bedford, Mass.) and a 30-filter manifold filtration unit, and the cell-associated radioactivity was measured with a scintillation counter (model LS 7500; Beckman Instruments, Inc., Irvine, Calif.). The specific activity of Mot$^+$ or Fla$^-$ bacteria labeled with [^{3}H]leucine was determined to be approximately 17,000 cpm/10^8 cells, whereas the specific activity of [^{14}C]glutamate-labeled cells was approximately 12,000 cpm/10^8 cells.

Attachment assays. A 1-ml aliquot (containing ~5.3 × 10^8 cells) of [^{3}H]leucine-labeled Mot$^+$ or Fla$^-$ cells was pipetted into 20-ml scintillation vials. Glass vials were cleaned prior to use with a 7% solution of sodium hypochlorite; repeated rinses in deionized-distilled water followed. The surface area exposed to cell suspensions per vial was calculated to be approximately 2.75 × 10^9 μm^2. After the addition of cell suspensions, the vials were placed on a flask shaker operating at 25 rpm and incubated for periods of 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min. The numbers of attached and unattached cells per vial were determined by removing planktonic cells at each interval from separate vials by aspiration and then rinsing twice with 1 ml of sterile diluent each time. The supernatant and diluent from each vial were then filtered through a 0.45-μm-pore-size filter. After the vials were rinsed 4 ml of ACS scintillation cocktail (Amersham Corp., Arlington Heights, Ill.) was added to each vial, and the counts per minute per vial was determined for the attached cell populations. Filters containing the planktonic cell populations were similarly dissolved in 4 ml of ACS scintillation cocktail and counted. The filtrate was also counted to determine the loss of isotope from bacteria due to leakage. Less than 1% of the filter-associated radioactivity was detected in the filtrate after completion of the attachment time course. All experiments were performed in triplicate, and scintillation counting was performed for 10 min for each sample by using internal and external standards to determine quenching.

Dual-radiolabel attachment assays were performed by incubating mixtures of Mot$^+$ and Fla$^-$ *P. fluorescens* labeled with [^{14}C]glutamate or [^{3}H]leucine, respectively. Equivalent volumes of each cell preparation were combined, added to scintillation vials, and incubated for 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min. The determination of attached and unattached cell populations at each time interval was also performed as described above.

Adsorption experiments were conducted to evaluate the equilibrium kinetics of each strain in pure culture over a range of cell densities. Radiolabeled cell suspensions of either strain, with densities ranging from ~7.5 × 10^6 to ~2.0 × 10^9 cells ml^{-1}, were added in triplicate to scintillation vials and incubated until no further cell adsorption was observed (time to equilibrium, ~180 min; data not shown). The attached and planktonic cell populations were then enumerated, as described above, by using scintillation counting.

Equilibrium adsorption isotherm parameters (k', R/k, and $1/k'$) were derived from a linearized Langmuir-type adsorption isotherm equation (10) described below:

$$[x]_s = \frac{[x]_s[x]_{ads}}{k'} + \frac{R}{k}$$

where $[x]_s$ represents the planktonic bacterial concentration, $[x]_{ads}$ is the number of attached bacteria, R/k is an indication of bacterial adsorption intensity, and k' is a constant relating the number of bacteria required for surface saturation to the unit area of the attachment surface. Cell adsorption data which had a correlation coefficient (r^2) of ≥0.85 with the adsorption isotherm equation were considered to conform with monolayer adsorption kinetics.

Continuous-flow slide culture attachment assay. The attachment of bacteria to the surfaces of glass flow cells was quantitated by using computer-enhanced microscopy (CEM) and continuous-flow slide culture (20, 21). Preparation of flow cells, assembly of apparatus, and standardization of bacterial suspensions have been reported previously (21). Assays were conducted at 23 ± 3°C for 60 min at a microenvironment flow velocity of 40 μm s^{-1} (0.37 cm s^{-1} within the macroenvironment). At this flow velocity, the Reynold's number was 36.8 and the Péclet number was 1.04; thus, the flow was laminar. The calculation of hydrodynamic parameters and descriptions of image analysis hardware and operating procedures have been presented previously (21).

Attachment of heat-treated Mot$^+$ and Fla$^-$ bacteria to the lower surfaces of a glass flow cell was measured to evaluate whether physicochemical differences between the Mot$^+$ and Fla$^-$ strains might be responsible for the attachment kinetics observed during these studies. Test tubes containing cell suspensions were placed in a 50°C shaking water bath for 2 min and then rapidly cooled to room temperature. The heat treatment inactivated cell motility. Heating was performed on Mot$^+$ or Fla$^-$ cells at standardized concentrations for immediate use in continuous-flow slide culture and CEM studies. Flagellar staining (31) was performed to determine whether heat treatment resulted in loss of the flagellum.

DDCC. The purpose of the DDCC system was to impose independently both particulate- and aqueous-phase dilution stress factors on surface-colonizing bacteria. Within the DDCC system, only bacteria which attached to surfaces could avoid being washed from the planktonic phase, and only bacteria which left surfaces and recolonized new surfaces could avoid particulate-phase washout. The DDCC apparatus consisted of presterilized, 50-ml round-bottom flasks containing 10 3-mm-diameter glass beads (Fig. 1). The beads were cleaned before

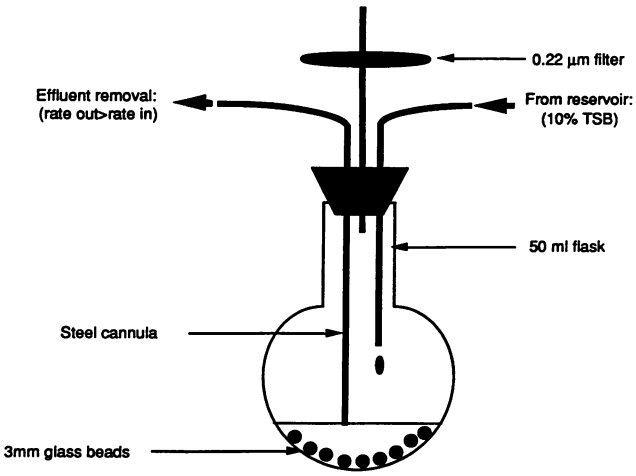


FIG. 1. Schematic illustration of the DDCC assay system.

use by soaking them overnight in 7% sodium hypochlorite, rinsing them with deionized-distilled water, and then autoclaving them. The flasks were filled with 15 ml of 0.3% (wt/vol) TSB by pumping medium (multichannel Watson Marlow 201Z) through a 6.5-cm stainless-steel cannula (1-mm inside diameter [i.d.]). The flask volume (15 ml) was maintained by continuously removing medium at a rate greater than it was added through a 13.0-cm stainless-steel cannula (1-mm i.d.). The inflow and outflow cannula, which were mounted in a no. 6 rubber stopper and fitted in the mouth of the round-bottom flask, were connected to medium reservoir and effluent containers via silicone tubing (5-mm-i.d. tubing for the outflow tube and 3-mm-tubing for the inflow tube). A 0.22-μm-pore-size presterilized Millex filter mounted on the end of a 16-gauge presterilized hypodermic needle was also inserted through the flask rubber stopper to facilitate ventilation.

Two beads colonized by the Mot^+ and Fla^- *P. fluorescens* strains were removed from log-phase cultures and added to a sterile dual-dilution culture flask placed on a shaker operating at 25 rpm. Beads used during the inoculum procedure were marked to avoid using either inoculum bead during the first subculture event. The shaker was operated at low speed to prevent agitation of the beads (which could shear cells from bead surfaces) but ensure adequate mixing of the aqueous phase. The aqueous-phase dilution rate (D of aqueous phase) was then set at either 1.2, 2.8, 3.8, or 5.0 volumes per h. At 24-h intervals, DDCC flasks were aseptically exchanged and a second sterile flask was inoculated with a bead obtained from the first DDCC flask (the particulate dilution rate was 0.09 bead day^{-1}). During the subculture procedure, the metal cannulas were flame sterilized to prevent cell carryover. Beads were rinsed in sterile diluent to confine the transfer of bacteria to those associated with the particulate phase. The number of bacteria present in the planktonic phase was then quantified by using plate count techniques; however, the number of cells present on bead surfaces required that attached cells be resuspended before enumeration. The reproducibility of the cell resuspension technique was ensured as follows. The beads to be used for enumeration of attached bacteria were rinsed in sterile diluent before each bead was transferred to a sterile dilution tube containing 10 ml of diluent. The dilution tube and bead were then vortexed at high speed for 30 s prior to removal and plating of 1 ml of the diluent. The efficiency of Mot^+ and Fla^- *P. fluorescens* resuspension was determined by incubating radiolabeled bacteria grown in batch culture on 3-mm-diameter glass beads and monitoring the release of bound radioactivity following the rinsing and resuspension procedure. The efficiencies of cell resuspension (~85%) for both *P. fluorescens* strains were not significantly different ($P \leq 0.1$). The total number of CFU per milliliter obtained from each glass bead was subsequently corrected to account for the 10-fold dilution.

The numbers of Mot^+ and Fla^- *P. fluorescens* CFU resuspended from the particulate and planktonic phases were determined by using differential plating: one set of agar plates contained 0.3% (wt/vol) TSB agar supplemented with 50 μg of kanamycin ml^{-1}, whereas the second set consisted of unsupplemented 0.3% (wt/vol) TSB agar. Subtracting the number of kanamycin-resistant CFU (Fla^- *P. fluorescens*) from the total CFU present on the TSB plates (Mot^+ and Fla^- *P. fluorescens*) provided an approximation of the number of Mot^+ and Fla^- cells present in both the particulate and planktonic phases at each sampling time. The number of CFU resuspended per bead was not corrected for resuspension efficiency and thus provided a relative measure of the number of adherent bacteria for both strains. Duplicate sampling was performed at 24-h intervals immediately prior to the subculture of the DDCC vessel.

RESULTS

Initial attachment behavior of Mot^+ and Fla^- *P. fluorescens*. Pure culture studies indicated that the Mot^+ strain attached to glass surfaces more rapidly over the 2-h assay than did the Fla^- strain, ultimately resulting in higher final cell densities (Fig. 2). However, both strains attached more rapidly to surface sites during the first hour, with rates of attachment declining over time. The attachment rate for the Mot^+ strain decreased 93% during the second hour of the assay, whereas the attachment rate of the Fla^- strain declined by 64%. The final densities of attached cells were dependent upon cell motility (2.03×10^8 cells $vial^{-1}$ or ~38% attachment of total added bacteria for the Mot^+ strain compared with 5.57×10^7 cells $vial^{-1}$ or ~11% attachment of total added bacteria for the Fla^- strain).

Initial attachment behavior of Mot^+ and Fla^- *P. fluorescens* cocultures. During 2-h competitive attachment assays between Mot^+ and Fla^- *P. fluorescens*, the Mot^+ strain attached ~2.8 times more rapidly than the Fla^- strain (Fig. 3). The final density of attached cells for the Mot^+ strain was 1.75×10^8 cells $vial^{-1}$, whereas the final cell density for the Fla^- strain was 6.26×10^7 cells $vial^{-1}$. The total number of attached cells resulting from the accumulation of both strains ($\sim 2.38 \times 10^8$ cells $vial^{-1}$) was ~17% greater than that achieved by the Mot^+ strain in pure culture ($\sim 2.03 \times 10^8$ cells $vial^{-1}$). During the competitive attachment study, the final cell density for the Mot^+ strain was reduced from the values observed in pure culture, whereas the number of Fla^- cells on the surface increased slightly.

Equilibrium adsorption kinetics of Mot^+ and Fla^- *P. fluorescens*. A range of radiolabeled Mot^+ and Fla^- *P. fluorescens* cell concentrations (from 7.5×10^6 to 2.0×10^9 cells ml^{-1}) was used to determine the equilibrium adsorption profiles of each strain in pure culture. The time required for equilibration between the aqueous and planktonic phases was 3 h. Langmuir-type adsorption correlation coefficients were determined for each strain: the Mot^+ strain had a correlation coefficient (r^2) of 0.86, and the Fla^- strain had a correlation coefficient of 0.94. However, the kinetics of adsorption differed with cell flagellation (Fig. 4). Calculation of Langmuir-type constants indicated that the adsorption capacity of the substra-

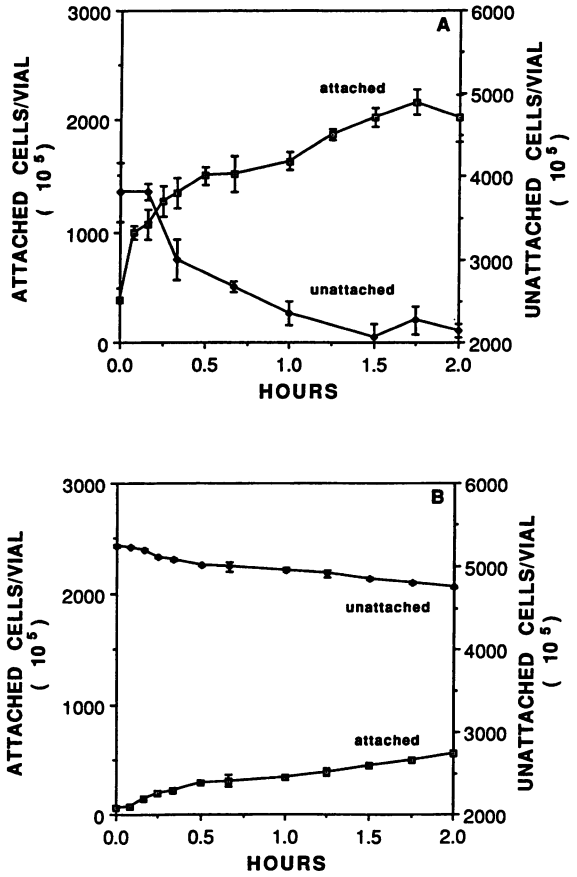


FIG. 2. Attachment of [^{3}H]leucine-labeled Mot$^+$ (A) and Fla$^-$ (B) *P. fluorescens* to glass surfaces over a 2-h time period.

tum ($1/k'$) was greater for the Mot$^+$ strain, which adhered to ~3.6 times more attachment sites than the Fla$^-$ strain even though both strains obeyed first-order adsorption profiles. Surfaces colonized by the Fla$^-$ strain became saturated at lower cell densities (2.82×10^8 cells vial^{-1}) than surfaces

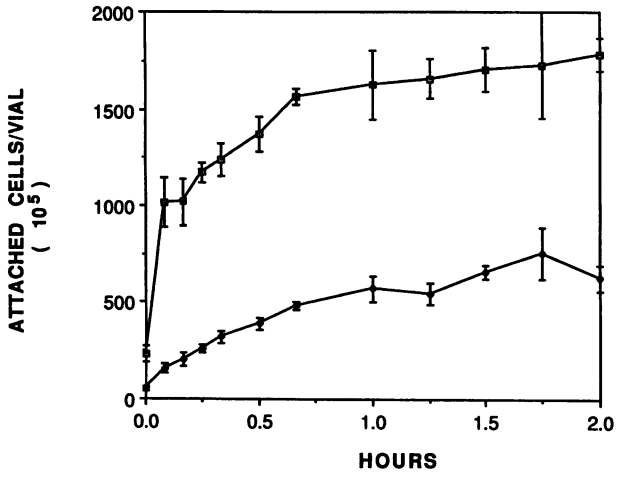


FIG. 3. Batch coculture attachment kinetics of Mot$^+$ (■) and Fla$^-$ (◆) *P. fluorescens* to glass surfaces over a 2-h time period.

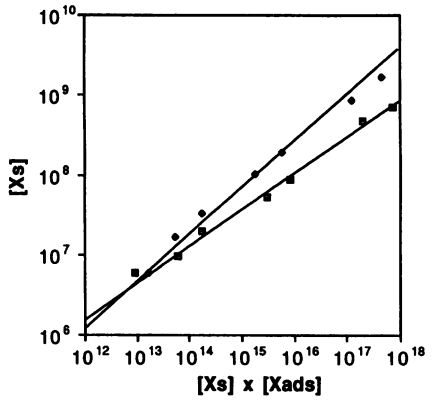


FIG. 4. Adsorption profiles comparing the equilibrium adsorption of [^{3}H]leucine-labeled Mot$^+$ and Fla$^-$ *P. fluorescens* to glass surfaces. [xs] represents the bacterial concentration at equilibrium, and [xads] represents the number of bacteria adsorbed to the surface. A range of cell concentrations was tested (~7.5×10^6 to ~2.0×10^9 cells ml^{-1}) to evaluate the relationship between planktonic cell concentrations and equilibrium cell adsorption for the two strains. Regression analysis was performed on untransformed data. Symbols: ■, motile *P. fluorescens* [$y = (6.4650e + 7) + (9.6314e - 10x)$; $r^2 = 0.868$]; ◆, nonmotile *P. fluorescens* [$y = (1.1484e + 8) + (3.4684e - 9x)$; $r^2 = 0.935$].

colonized by the Mot$^+$ strain (1.03×10^9 cells vial^{-1}). Furthermore, the intensity of adsorption (R/k) was greater for the nonmotile than the motile strain ($R/k = 1.14 \times 10^8$ and 6.46×10^7 for the Fla$^-$ and Mot$^+$ strains, respectively). At the highest cell densities used during equilibrium adsorption studies, incomplete surface coverage (on an areal basis) resulted for both strains (i.e., ~7.78×10^8 μm^2 cell area or 28.3% coverage for the Mot$^+$ strain and ~2.17×10^8 μm^2 cell area or 7.9% coverage for the Fla$^-$ strain).

Initial attachment behavior of heat-treated Mot$^+$ and Fla$^-$ *P. fluorescens*. The attachment of heat-treated Mot$^+$ and Fla$^-$ *P. fluorescens* to flow cell surfaces was compared with the attachment of untreated cells by using CEM. These control experiments were performed to evaluate whether the Fla$^-$ strain had undergone physicochemical outer membrane changes resulting from mutagenesis, thereby affecting the adsorption tendencies of the strain. Heat-treated motile bacteria attached at the same rate as did the heated and untreated nonmotile bacteria during a 1-h attachment time course. Untreated Mot$^+$ *P. fluorescens* attached at approximately five times the rate of heat-treated Mot$^+$ cells, heat-treated Fla$^-$ cells, and untreated Fla$^-$ cells (~1,544 cells field^{-1} h^{-1} for the Mot$^+$ *P. fluorescens* strain compared with ~300 cells field^{-1} h^{-1} for the Fla$^-$ strain and heat-treated strains; (Fig. 5). Overall, nonmotile cells (the Fla$^-$, heated Mot$^+$, and heated Fla$^-$ cell preparations) reached approximately 19% of the final cell densities observed for the Mot$^+$ strain.

Behavior of Mot$^+$ and Fla$^-$ *P. fluorescens* in DDCC. Under the conditions employed during the DDCC study, the Mot$^+$ strain was the most effective competitor in the two-strain coculture (Fig. 6). When the aqueous-phase dilution rate was less than the maximum growth rate (μ_{max}; $D = 1.2$ volumes h^{-1}; the doubling time for *P. fluorescens* is 1.45 h), both strains of bacteria should have been able to coexist in suspension because of their identical growth rates. However, the Fla$^-$ strain eventually became extinct over a 250-h period, whereas the Mot$^+$ strain was observed to attain steady-state cell concentrations on surfaces and in suspension, with approximately 4.9×10^8 CFU ml^{-1} detected in the planktonic phase

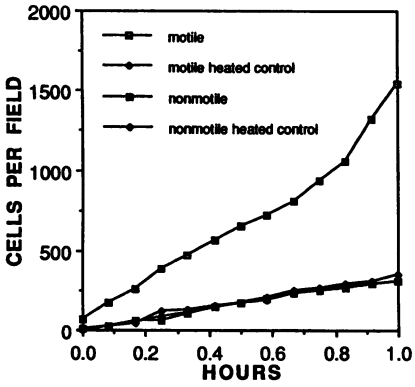


FIG. 5. Control experiment illustrating the role of motility during attachment to glass surfaces in continuous-flow slide culture by using low-power (×10) dark-field CEM. Suspensions of Mot$^+$ and Fla$^-$ cells were allowed to attach to glass flow cell surfaces, and the results were compared with the results from the attachment of heat-treated (50°C, 2 min) Mot$^+$ and Fla$^-$ cells.

and approximately 1.7×10^9 CFU ml^{-1} recovered from the particulate phase. In general, the numbers of planktonic-phase CFU detected for the Mot$^+$ and Fla$^-$ strains were approximately three times less than those resuspended from bead surfaces.

Increasing the aqueous-phase dilution rate (1.2, 2.8, 3.8, and 5.0 volumes h^{-1}) decreased the time required for elimination of the Fla$^-$ strain and concurrently decreased the number of Mot$^+$ cells able to persist in both the planktonic and particulate phases. At a D of 2.8 volumes h^{-1}, the Fla$^-$ strain became extinct after ~170 h, whereas the numbers of Mot$^+$ cells present on surfaces and in suspension at equilibrium were 5.4×10^8 and 1.5×10^8 CFU ml^{-1}, respectively. At dilution rates of 5.0 volumes h^{-1}, both strains eventually became extinct (>100 h); however, the Mot$^+$ strain remained in the system for 48 h after the Fla$^-$ strain had been eliminated.

DISCUSSION

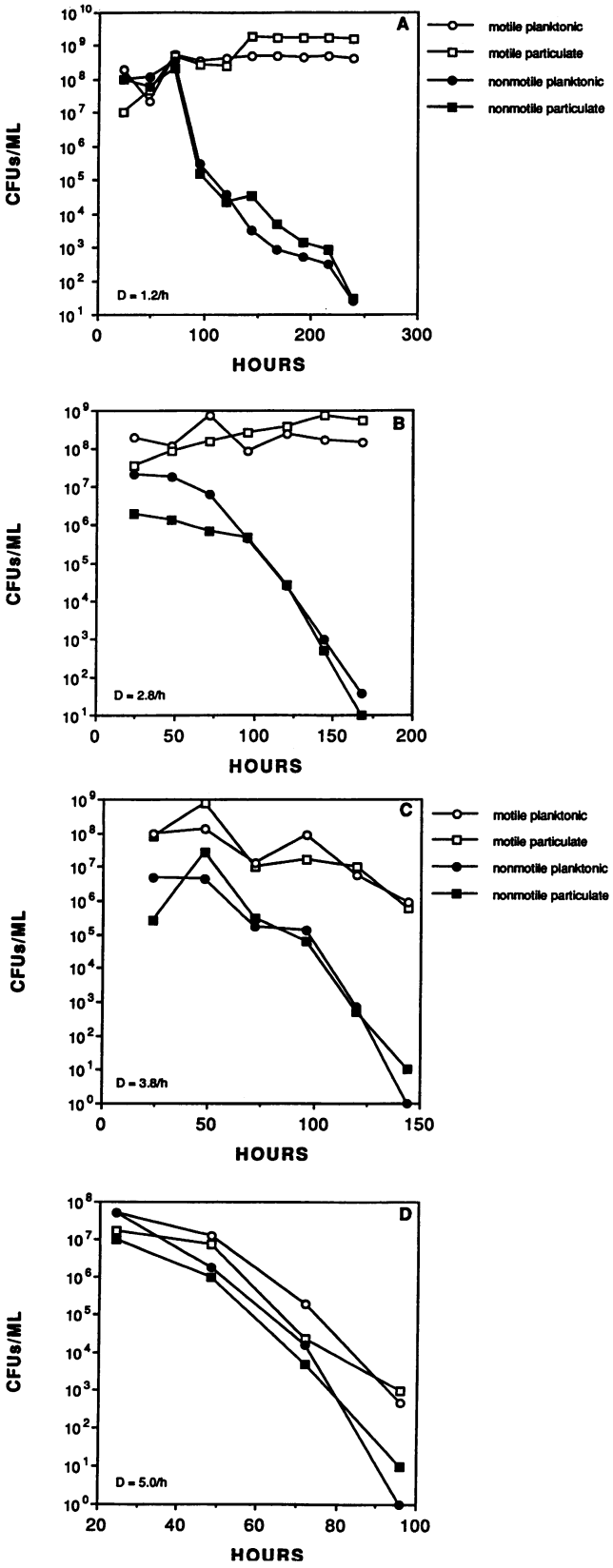
Initial attachment behavior of Mot$^+$ and Fla$^-$ *P. fluorescens*. During the initial stages of biofilm formation, motile cells colonize available surface sites by moving from the bulk aqueous phase to the surface microenvironment where attachment to vacant sites may occur. The mechanisms which facilitate bacterial transport to surface microenvironments include Brownian diffusion, gravitational cell settling, vortex currents, and motile cell behavior (7, 17, 21, 28, 29). In laminar-flow systems, it has been suggested that cellular diffusion is likely the dominant cell transport mechanism (33). However, by ignoring the effect of motility during cellular diffusion processes, it has been calculated that the number of cells which may reach any given point may be underestimated by as much as 50-fold (15). Studies of bacterial transport through porous media indicate that flagellar locomotion may also be of significance in groundwater systems. For example, Jenneman et al. (16) determined that a motile *Enterobacter aerogenes* strain responding to a sucrose gradient present within Berea sandstone moved three- to eightfold-greater distances than did a nonmotile *Klebsiella* strain.

Examination of radiolabeled Mot$^+$ and Fla$^-$ *P. fluorescens* attachment kinetics indicated that the Mot$^+$ strain attached ~3.5 times more rapidly to surfaces than the Fla$^-$ strain over the 2-h time course, reflecting previously reported trends (20) in which the accumulation of motile *P. fluorescens* to lower-flow cell surfaces exceeded that of a nonmotile strain by >6 times. The rates of attachment for both strains decreased over time from the initial rates (93 and 64% for the Mot$^+$ and Fla$^-$ strains, respectively) as surface sites became occupied by attached cells (Fig. 2). A time-dependent reduction in the rate of cell attachment may have resulted from one or more of the following events: (i) the probability of an effective cell surface contact decreased with diminishing planktonic cell concentrations; (ii) the number of suitable attachment sites became limiting in number over time as cells attached to favorable locations (e.g., monolayer attachment); or (iii) surface conditioning by macromolecules present within the suspending medium interfered with cell attachment in a time-dependent fashion. In each case, a functional flagellum would increase the number of bacteria which would reach potential attachment sites by virtue of increased cell mass transport.

Since the relative attachment rates for both strains decreased during the second hour of the attachment assay (rate decreases for the Mot$^+$ and Fla$^-$ strains were 93 and 64%, respectively) more than the relative decrease in the remaining number of planktonic cells (decreases in cell concentration for the Mot$^+$ and Fla$^-$ strains were 63 and 12%, respectively), factors other than the numerical probability of successful cell attachment appear to have been active. Furthermore, considering the final number of attached cells at the completion of the attachment assay (for the Mot$^+$ strain, $\sim 2.03 \times 10^8$ cells vial^{-1}; for the Fla$^-$ strain, $\sim 5.57 \times 10^7$ cells vial^{-1}), the estimated surface area of the cells (~0.75 μm^2 cell^{-1}), and the total available surface area ($\sim 2.75 \times 10^9$ μm^2), cell-cell attachment or multilayer cell attachment was not likely to have occurred. Monolayer attachment kinetics would indicate that only a finite number of attachment sites exists and the probability of locating such a site would decrease over time. Nevertheless, both strains were tested under equivalent conditions; therefore, any difference in cell adsorption kinetics may be attributed to possession or lack of a functional flagellum and the associated behavior.

First-order, or monolayer, attachment kinetics may have important consequences during the colonization of surfaces by bacteria in natural systems since successful attachment by a rapidly colonizing species would leave fewer attachment sites available for competitors. A number of examples exist in which bacteria have been observed to obey monolayer attachment kinetics, with surface coverage by various bacterial species ranging from 1 to 45% (10, 25, 33). Korber et al. (21) demonstrated that attachment rates for Mot$^+$ and Fla$^-$ *P. fluorescens* strains remained relatively constant over a 45-min attachment time course, whereas rates of two *Vibrio parahaemolyticus* strains increased over time, even though multilayer attachment was not observed in either case. During this study, results from radiolabeled cell attachment assays conducted over longer time periods suggest that the rapid occupation of a limited number of surface sites by aggressively colonizing bacteria would provide an advantage during subsequent stages of surface growth since that population which occupied the greatest number of attachment sites could eventually grow to dominate the biofilm. The competitive significance of flagellar motility has been postulated as a result of a number of in situ colonization studies involving *Pseudomonas* spp. and root and leaf surfaces (9, 13).

Initial attachment behavior of Mot$^+$ and Fla$^-$ *P. fluorescens* cocultures. The significance of a functional flagellum was further tested by using dual radiolabeling ([^{3}H]leucine and [^{14}C]glutamate), allowing the simultaneous determination of the attachment success of Mot$^+$ and Fla$^-$ *P. fluorescens*



strains. Dual-radiolabel attachment studies have been used previously to demonstrate the effect of lateral flagellation during surface colonization by *V. parahaemolyticus* strains (2). During this study, equilibrium adsorption profiles of laterally flagellated (Laf^+) and polarly flagellated (Fla^+) cells to chitin surfaces were compared, demonstrating that Laf^+ *Vibrio* strains generally obeyed first-order attachment kinetics, whereas Fla^+ *Vibrio* strains adsorbed to surfaces proportional to the number of bacteria added to the system. This is in contrast to our observations with *Pseudomonas* strains, in which polarly flagellated and Fla^- *P. fluorescens* exhibited monolayer attachment. During coculture attachment assays, the attachment success of each strain reflected those results obtained during pure culture studies (i.e., the motile strain attached more than three times more rapidly than the nonmotile strain, occupying the majority of available attachment sites and consequently outnumbering the nonmotile strain); however, a slightly higher percentage of available surface sites was colonized when both strains were present (i.e., 33 versus 37%; Fig. 3). Furthermore, the final cell density for the Mot^+ strain was reduced from values observed in pure culture, whereas the number of Fla^- cells on the surface increased slightly. It is possible that the higher surface affinity constant for the Fla^- strain ($R/k = 6.4 \times 10^7$ for the Mot^+ strain and 1.14×10^8 for the Fla^- strain) resulted in slightly fewer attachment sites remaining for the Mot^+ strain or that the two strains simply attached to two different classes of adsorption sites (e.g., high and low affinity). High- and low-affinity binding sites have been identified previously by using adsorption isotherms for *Streptococcus sanguis* strains attaching to hydroxyapatite (12) but have not been documented for glass surfaces. Gibbons et al. (12) determined that cells preferentially attached to more-specific, high-affinity sites when cells were present in low concentrations but switched to low-affinity sites when higher concentrations of cells were added to the system. Multiple binding sites may similarly exist for Mot^+ and Fla^- strains of *P. fluorescens* and provide an explanation for the observed trends, with the ability of the cells to reach either class of attachment site dependent on the motile condition of the cell.

Equilibrium adsorption kinetics of Mot^+ and Fla^- *P. fluorescens*. A range of radiolabeled Mot^+ and Fla^- *P. fluorescens* cell concentrations was tested to evaluate equilibrium attachment kinetics by comparing the fit of the adsorption data with that of a linearized Langmuir-type isotherm. The criteria for accepting or rejecting the data as fitting the isotherm equation (and, hence, obeying either monolayer or proportional attachment kinetics) was artificially imposed at an r^2 of ≥ 0.85. Both strains obeyed first-order attachment kinetics ($r^2 = 0.86$ for the Mot^+ strain and 0.94 for the Fla^- strain; Fig. 4). However, surfaces colonized by the Fla^- strain became saturated at a lower cell density (2.82×10^8 cells $vial^{-1}$) than surfaces colonized by the Mot^+ strain (1.03×10^9 cells $vial^{-1}$), suggesting that the possession of a functional flagellum enabled the Mot^+ strain to colonize locations inaccessible to nonmotile cells. In contrast, the intensity of adsorption (R/k) was greater for the nonmotile strain, indicating that once a surface contact was established, binding occurred rapidly and irreversibly. Calculated values for the area occupied by the Mot^+ and Fla^- *P. fluorescens* strains after application of the

FIG. 6. Colonization of DDCC by Mot^+ and Fla^- *P. fluorescens* at dilution rates of 1.2 (A), 2.8 (B), 3.8 (C), and 5.0 (D) volumes h^{-1}. Symbols: ○, motile planktonic; □, motile particulate; ●, nonmotile planktonic; ■, nonmotile particulate.

highest concentration of cells (based on a mean cell size of 0.5 by 1.5 μm) were 28 and 8%, respectively. Therefore, the saturation of surfaces by a monolayer of colonizing bacteria does not equal the total available area during attachment of *P. fluorescens*.

At the highest cell densities tested, about four times as many motile cells as nonmotile cells were observed to adhere at equilibrium. Sjollema and Busscher (36) previously investigated blocking effects during the deposition of polystyrene particles to surfaces, demonstrating that objects present on a surface in a system with laminar flow inhibit the subsequent deposition of additional particles in their immediate vicinity. Site blocking may thus account for decreased nonmotile strain attachment, as turbulent flow in the vicinity of attached cells would inhibit further adsorption of the particle-like nonmotile cells. Chemical variations present on the surface may also play a role in defining the adsorption potential of a particular surface for colonizing bacteria (7). These observations are in agreement with the results of the present study, in which the total available area did not equal the number of preferential sites of adsorption.

While physicochemical theories and formulae designed for molecular adsorption (39, 42) may not fully explain the attachment of many bacterial species, assessment from a behavioral perspective may help to explain inconsistencies. For example, interpretation of the adsorption equation parameter, R/k (an indication of the intensity of adsorption), suggested that the Fla^- strain adsorbed to surfaces more securely than the Mot^+ strain despite evidence that the Mot^+ strain was numerically more successful during attachment assays. While this finding contrasts the overall attachment tendencies of the two strains of *P. fluorescens*, it agrees with behavioral studies where Mot^+ *P. fluorescens* bacteria frequently exhibited reversible attachment and rotational movements whereas the nonmotile strain underwent rapid attachment with little observable movement following initial surface contact (21, 22). Thus, while hydrodynamic effects and site blocking may have resulted in fewer Fla^- *P. fluorescens* cells encountering accessible attachment sites, cells which reached vacant sites rapidly became irreversibly attached. Since areal calculations indicated that neither strain saturated the total area available for attachment during pure culture assays, it is likely that the presence of a functional flagellum primarily facilitated cell transport from the bulk phase to surface microenvironments and that the ability of motile cells to locate less-accessible attachment locations was of secondary importance.

Initial attachment behavior of heat-treated Mot^+ and Fla^- *P. fluorescens*. Heat treatment was applied to Mot^+ *P. fluorescens* cells, inactivating cellular motility and providing a mechanism for evaluating whether transposon mutagenesis altered the adhesive nature of the Fla^- strain. Long-term heating (30 min) of *Pseudomonas aeruginosa* cells has resulted previously in relatively small differences in attachment tendencies when compared with mechanical deflagellation of cells. In contrast, deflagellation alone reduced attachment relative to untreated cells by 90% (41). In our study, the attachment of heated Mot^+ cell suspensions to the surfaces of glass flow cells was determined by using CEM and reflected that of the untreated Fla^- strain (Fig. 5). Fla^- cell suspensions were also heated, and the deposition of the heated Fla^- cells was identical to that of the heated Mot^+ strain and the untreated Fla^- strain. Therefore, the adhesive nature of both strains utilized during this study remained constant for all practical purposes, with the only perceivable difference between the strains being the presence or absence of a functional flagellum. The possession of a nonfunctional flagellum (the heated Mot^+ strain) apparently did not influence the kinetics of attachment. Consequently, active flagellar motility, and not the flagellar structure, was the principle agent responsible for enhanced attachment of *P. fluorescens*. The similar rates of attachment for heat-treated Mot^+ and Fla^- *P. fluorescens* suggest that, in the absence of motility, the capacity and mechanism for attachment were equivalent for both strains.

Behavior of Mot^+ and Fla^- *P. fluorescens* in DDCC. To date, continuous culture systems involving quantitative two-phase dilution schemes have not found wide application in microbiology. Maigetter and Pfister (26) utilized continuous culture containing kaolinite particles during competition between *Chromobacterium lividum* and a *Pseudomonas* sp. The addition of kaolinite particles resulted in a decrease in the number of free *C. lividum* cells; however, the number of cells associated with the particulate kaolinite was not determined, and the dilution rate of the particulate phase was not set independently from the dilution rate of the aqueous phase. Microbial adhesion and growth on surfaces has also been considered in attempts to explain results obtained in predator-prey chemostat studies. Ratnam et al. (34) silanized the walls of their chemostat vessel and demonstrated that in cases in which repeated oscillations of species densities occurred during predator-prey studies, minimum *Escherichia coli* densities were lower by several orders of magnitude than when bacteria were allowed to colonize the chemostat vessel walls. It was hypothesized that untreated chemostat walls allowed sustained growth of attached bacteria without predation pressure (the ciliate was a suspension-feeding organism), which consequently seeded the aqueous phase, resulting in elevated planktonic *E. coli* counts. Overall, while adhesion and cell aggregation likely play a significant role in natural systems (where the majority of cells exist in the attached state), the effects of surfaces or wall growth during continuous culture are commonly overlooked.

DDCC allowed independent dilution of both the aqueous and particulate phases at different rates (5) and was used during the present study to evaluate the adaptive significance of flagellar motility during long-term surface colonization experiments. In general, the results agreed with findings obtained during direct attachment assays in parallel-plate flow cells and during radiolabel attachment studies. The Mot^+ strain persisted in suspensions and on surfaces in greater numbers than the Fla^- strain, which eventually became extinct in the DDCC system at all tested dilution rates. When the aqueous-phase dilution rate was less than the maximum growth rate, both strains of bacteria coexisted in suspension because of their identical growth rates. On the basis of radiolabeled cell and continuous-flow slide culture attachment assays, the Mot^+ strain attached to vacant surface sites within DDCC more rapidly and in higher numbers than the Fla^- strain and thus occupied a greater percentage of available surface sites on bead surfaces. Once in the attached condition, both *P. fluorescens* strains grew and developed into biofilms at the same rate; however, the higher initial density of attached cells and the ability of the Mot^+ strain to colonize new surfaces more effectively resulted in a greater percentage of the Mot^+ strain persisting on bead surfaces after repeated subculture. After subculture of colonized beads to sterile DDCC flasks, only those organisms which had successfully colonized new bead surfaces were transferred to new flasks; thus, selection pressure for attached growth and effective recolonization existed even though planktonic growth of the two strains occurred at the same rate.

Increasing the rate of aqueous-phase dilution (D = 2.8, 3.8, and 5.0 volumes h^{-1}) decreased the time required for elimi-

nation of the Fla$^-$ strain (time to extinction were 170, 145, and <100 h, respectively) while concurrently decreasing the number of Mot$^+$ cells able to persist in both the planktonic and particulate phases (Fig. 6). Since only single beads were subcultured, the effective migration of either strain of *P. fluorescens* from the transferred bead to new bead surfaces imposed a stronger selection pressure as aqueous-phase dilution rates were increased. During DDCC, the wall-associated cell population did not have sufficient time to become a stable source of sloughed or recolonizing cells from which the planktonic cell pool could be seeded. This is in contrast to chemostat studies in which resuspended cells from wall populations of *E. coli* varied little even though the planktonic cell populations varied by several orders of magnitude (6). However, since the number of attached CFUs determined at each time period in DDCC was about three times greater at all dilution rates than the corresponding aqueous-phase determinations for either strain, the presence of unsuccessful recolonizing cells or sloughed biofilm materials provides an explanation for the occurrence of planktonic cell counts where the dilution rate was greater than the growth rate. At the highest dilution rate tested (i.e., $D = 5$ volumes h^{-1}), the Fla$^-$ strain was eliminated from the DDCC system in less than 100 h. Under these conditions, the Mot$^+$ strain was also unable to persist at equilibrium concentrations and became extinct at ~48 h after the Fla$^-$ strain. In this case, the capacity for motile behavior to facilitate successful recolonization was exceeded and planktonic cells could not effectively reach new beads and attach before being washed from the system.

Previous growth studies of motile and nonmotile *P. fluorescens* in continuous-flow slide culture (laminar flow) indicated that Mot$^+$ cells recolonized four to five times more effectively than Fla$^-$ strain cells (20). These values agree with the observed differences between the attachment success of the two strains during adsorption time course and equilibrium studies. Thus, successful recolonization may be due primarily to the effective reattachment of detached cells, and overall, the expected colonization success of the Fla$^-$ strain would likely be four to five times less than that of the Mot$^+$ strain per recolonization event. On the basis of this assumption, it was determined that if a stable population of 10^9 attached Fla$^-$ cells existed at inoculation and the dilution rate equaled 1.2 volumes h^{-1}, after 250 h (i.e., 10 subculture events), only 102 cells would be left in the system since the Mot$^+$ strain would gradually exclude the competitor by occupying available surface sites. This closely approximates the actual time required for loss of the Fla$^-$ strain from the DDCC system at this dilution rate. If multilayer attachment occurred, any advantage conferred by early colonization would not necessarily equate with exclusion of a competing strain. The success of the flagellated *P. fluorescens* strain during these studies therefore appears to be linked to a monolayer mode of surface colonization.

Overall, the DDCC system provided selective pressure for nearly all aspects of surface colonization by *P. fluorescens* cells, namely, (i) the initial mass transport of cells from the bulk aqueous phase to surface microenvironments, (ii) the adsorption of cells to surface sites, and (iii) the effective redistribution, or recolonization, of bacteria from established growth sites to new, uncolonized sites. Recolonization behavior encompasses all aspects of surface colonization and thus provides an advantage for growth on surfaces in flowing microenvironments. Further evidence suggests that a functional flagellum also influences the pathways of biofilm formation, possibly through active cell redistribution events, resulting in motile *P. fluorescens* biofilms containing higher cell densities than biofilms formed by nonmotile *P. fluorescens* (19). Under the well-defined conditions of DDCC, a functional flagellum was pivotal to the success of the motile *P. fluorescens* and represents an important behavioral adaptation responsible in part for the widespread success of this bacterium. The current study also confirms that, in systems not dominated by laminar flow, the flagellum still confers advantages for surface-colonizing bacteria.

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