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Bacterial Species Dominance within a Binary Culture Biofilm

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Studies with two species of bacteria, *Pseudomonas putida* and *Hyphomicrobium* sp. strain ZV620, were carried out to evaluate the overall net rate of accumulation of biofilm, the biofilm species composition, and individual species shear-related removal rates. Bacterial cells of either or both species were deposited onto glass or biofilm surfaces to initiate multispecies biofilms. Subsequent biofilm development was carried out under known conditions of nutrient concentration and laminar flow. Establishment of a depositing organism in a biofilm composed of another species was found to be a function of the relative growth rates of the bacterial species. In the case of simultaneous species deposition and subsequent binary culture development, the faster-growing organisms rapidly became the dominant biofilm species, but the slower-growing organisms remained established within the biofilm and continued to increase in numbers over time. The results also indicated that the rate of cell removal by fluid shear for a species was a function of biofilm cell number only if the species concentration was uniform with depth; in essence, only the upper layers of the biofilm were sheared off.

A biofilm is a biologically active matrix of cells and extracellular products attached to a solid surface. A biofilm community begins initially when a clean surface is exposed to an aqueous environment and becomes conditioned by the chemical constituents present. Inevitably, microorganisms become associated with the surface, adhere, and then attach. Once these cells are firmly bound, the activity of the community is dependent on the metabolism and growth of each member species under local surface conditions. Such metabolic activities can include substrate consumption, cellular growth and replication, and synthesis of exopolymers. Eventually, the biofilm reaches a steady state when processes producing more biofilm are counterbalanced by processes reducing or removing biofilm. Continued conversion of dissolved substrates produces biomass and soluble products that are removed by prevailing shear stresses and entrained into the surrounding fluid.

The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces, and the dominant microbial inhabitants in the biofilm. Succession or dominance of one species over another during biofilm formation has been mathematically modeled as a function of multiple soluble limiting substrates and species initial surface concentrations (8). The influence of suspended microbial species invading the biofilm from the bulk fluid has not been studied experimentally.

Here we present research on the rate of development of several binary bacterial species biofilms. The overall development of biofilm, the individual species concentrations, and the rates of removal due to shear for each species are quantified during biofilm formation. Two types of binary biofilm development are evaluated: (i) suspended cells of one bacterial species are exposed under laminar fluid flow conditions to an existing biofilm of a second species and (ii) cells of both bacterial species are exposed at time zero to a clean glass substratum. Shifts in species composition and relative rates of removal from the resultant biofilm are evaluated over time.

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MATERIALS AND METHODS

Microorganism cultivation. *Pseudomonas putida* ATCC 11172 and *Hyphomicrobium* sp. strain ZV620 were the two bacterial species used in this study. *P. putida* cells were cultivated from a freeze-dried sample by inoculating the sample into 200 ml of 001 nutrient broth (Difco Co., St. Louis, Mo.), incubating the broth for 8 h, and transferring 10 ml of the broth to glucose medium (pH 7.0) containing the following: glucose, 3 g; $MgSO_4$, 0.5 g; Na_2HPO_4, 0.32 g; KH_2PO_4, 0.37 g; $(NH_4)_2SO_4$, 0.25 g; $CaCl_2$, 0.1 g; $MnSO_4 \cdot 7H_2O$, 0.093 g; citric acid, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $CuSO_4 \cdot 5H_2O$, 0.005 g; $ZnSO_4$, 0.003 g; and distilled water, 1,000 ml. Agar slants were made from the same glucose medium, inoculated with the suspension culture, and subsequently stored at 4°C.

Hyphomicrobium sp. slants (donated by G. Hamer, Institut für Biotechnologie, Zürich, Switzerland) were grown in a suspension culture by inoculating an organism sample into 200 ml of nutrient medium (pH 7.0) (4) containing the following: methanol, 1 g; KH_2PO_4, 0.02 M; Na_2HPO_4, 0.02 M; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; $FeCl_2 \cdot 7H_2O$, 0.009 g; H_3BO_3, 0.0015 g; $ZnCl_2$, 0.0002 g; $MnCl_2 \cdot 4H_2O$, 0.001 g; $CuSO_4 \cdot 5H_2O$, 0.00016 g; $CoCl_2 \cdot 6H_2O$, 0.0002 g; $(NH_4)_6Mo_7O_{24}$, 0.0001 g; and distilled water, 1,000 ml. Agar slants were prepared with the same medium, inoculated with the suspension culture, and stored at 4°C.

Both species were maintained on slants, prepared as described above, throughout the experimental period. New slants were reinoculated every 6 weeks. For the following experiments, each species was grown in batch suspension cultures in shake flasks, agitated in an orbital water bath (150 rpm), to the early stationary phase, as determined by optical density measurements (approximately 28 h for *P. putida* and 96 h for the *Hyphomicrobium* sp.).

These two bacterial species were selected not only for their biofilm-forming abilities, but also because each can be uniquely radiolabeled through the metabolism of its individual carbon substrate. Long-term chemostat studies indicated that *P. putida* metabolized glucose exclusively, while the *Hyphomicrobium* sp. metabolized only methanol. Metabolism of the opposing carbon substrate or cometabolism of the two substrates by either species was not observed. Batch

growth studies also confirmed that the growth curve of each species was not changed by the presence of the opposing substrate (2).

Analytical methods. Glucose concentrations in the liquid phase were determined enzymatically by a glucose oxidase assay (kit no. 510; Sigma Biochemicals, St. Louis, Mo.).

Methanol concentrations in the liquid phase were determined with a GCA-9 gas chromatograph (Shimadzu Scientific Instruments, Columbia, Md.) equipped with an 80/100 Carbopack C–0.1% SP 1000 column (Supelco Inc., Bellefont, Pa.) and a flame ionization detector.

Dissolved oxygen concentrations and pH were measured periodically with a dissolved oxygen probe (Orion) and a digital pH meter (Orion).

Total bacterial counts were determined by epifluorescence microscopy as described by Hobbie et al. (5). Samples were fixed with glutaraldehyde (2% final concentration), diluted to the appropriate concentration of cells, stained with 0.01% (final concentration) acridine orange for 2 min, and filtered through 0.2-μm-pore-size black filters (Nuclepore Corp., Pleasanton, Calif.). Counts were made with the sample dilution that produced between 20 and 50 cells per field with a standard deviation of less than 15% for the 10 random fields counted.

Scintillation counts (disintegrations per minute) were measured with a Packard Tri-Carb 1900 CA liquid scintillation counter. Filter-Count (Packard, Downers Grove, Ill.) was the fluor used.

Biofilm thickness was determined with the micrometer on the focus adjustment of a Reichert-Jung Micro-Star IV (Reichert-Jung, Cambridge, Mass.) at a magnification of ×10 (1). The thickness was determined as the distance between the focus on the top of the biofilm and the focus on the top of the glass slide to which the biofilm was attached, once a correction was applied for the difference in the index of refraction between air and biofilm.

Flow cell reactor system. The overall reactor system consisted of separate, completely mixed, aerated nutrient and cell suspension containers with peristaltic pumps delivering the liquid to the biofilm flow cell reactor. During either inoculation of clean glass surfaces or the "invasion" series of experiments, both the nutrient solution and the cell suspension were delivered by separate peristaltic pumps to the biofilm reactor. During periods of biofilm accumulation, only the nutrient solution was delivered to the biofilm reactor. In all experiments, liquid was delivered to the biofilm reactor in a "once-through" mode, with effluent from the biofilm reactor being collected in a separate sterile container.

The rectangular duct flow cell is depicted in Fig. 1, and operating details are given in Table 1. This biofilm reactor, constructed of transparent Plexiglas, was designed to accept standard glass microscope slides within parallel indentations, thus forming a rectangular duct with glass as the substratum for adhesion. Prior to each experiment, the glass slides were prepared by being washed with 10% HCl and rinsed with filtered, autoclaved water. The reactor was cleaned by being soaked in 70% ethanol and rinsed with filtered, autoclaved water. A duplicate reactor was operated simultaneously for all studies.

Biofilm cultivation. Biofilms were initiated by inoculating the reactor with a flowing suspension of cells under the conditions listed in Table 1. After 6 h, the feed of bacterial cells was terminated and a sterile nutrient solution was delivered to the reactor. Biofilm samples were taken at various times during the experiment.

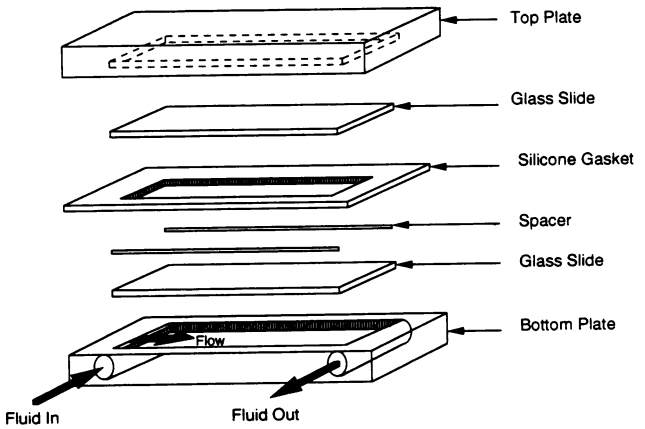

FIG. 1. Flow cell reactor.

Biofilm sampling. Biofilm samples were taken after a prescribed period by disassembling the reactor, scraping 2 cm^2 of the biofilm from the glass slides, and resuspending this sample in 10 ml of phosphate buffer (5.4 g of K_2HPO_4/liter; pH 7.0). Six samples were taken for each time period. These samples were exposed to 0.5 μCi of ^{14}C-labeled substrate per ml (glucose for *P. putida* and methanol for the *Hyphomicrobium* sp.) for 2 h. The cells were centrifuged, washed three times with phosphate buffer, and filtered through 0.22-μm-pore-size filters. Scintillation counts (disintegrations per minute) were made with the filter. The results used were averages of all the data. Calibration curves to determine the specific activity of the labeled cells were prepared to relate disintegrations per minute to cell numbers. Radioactively labeled cells of each organism were enumerated by epifluorescence cell counts with suspension culture samples. Calibration curves were made by plotting

TABLE 1. Operating details of the rectangular flow cell reactor

Internal dimensions	
Channel width (cm)	2
Channel length (cm)	7.5
Channel height (cm)	0.5
Internal surface area (cm^2)	39.5
Vol (cm^3)	7.5
Constant operating conditions	
Volumetric rate of flow to flow cell (cm^3/min)	2.0
Fluid velocity in flow cell (cm/s)	3.2×10^{-2}
Reynolds number[a]	2.67
Shear stress at substratum (N/m^2)	3.9×10^{-4}
Temp (°C)	20
Biofilm growth conditions	
Glucose concn (mg/liter)	500
Methanol concn (mg/liter)	533
Particle deposition and attachment conditions	
Suspended *P. putida* concn (cells/ml)	1×10^8 to 2×10^8
Suspended *Hyphomicrobium* sp. concn (cells/ml)	4×10^7 to 5×10^7

[a] Reynolds number = $d_h v_m \rho/\mu$, where d_h = hydraulic diameter defined for noncircular cross sections as [4(cross-sectional area/wetted perimeter)], v_m = mean fluid velocity, ρ = fluid density, and μ = fluid viscosity.

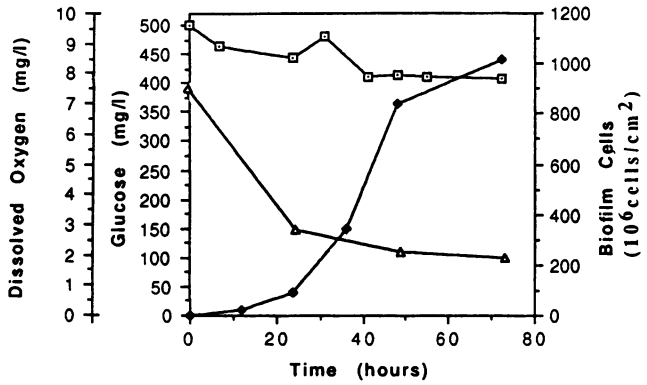

FIG. 2. Development of a pure-culture *P. putida* biofilm. Symbols: ⊡, glucose; △, dissolved oxygen; ◆, biofilm cells.

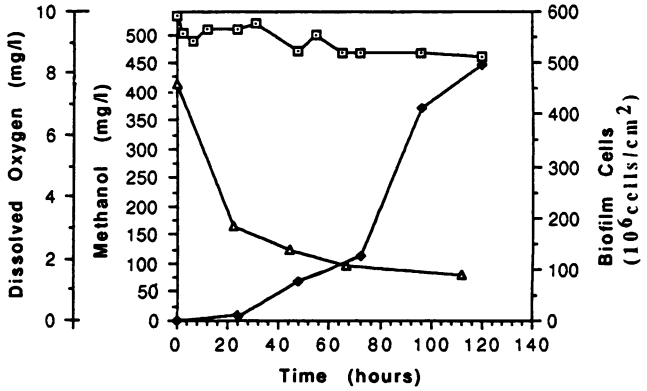

FIG. 3. Development of a pure-culture *Hyphomicrobium* sp. biofilm. Symbols: ⊡, methanol; △, dissolved oxygen; ◆, biofilm cells.

^{14}C counts against cell numbers. Calibration gradients were found to be 1,075 dpm/10^6 cells for the *Hyphomicrobium* sp. and 2,440 dpm/10^6 cells for *P. putida*.

Liquid phase sampling. Glucose and methanol, dissolved oxygen, and numbers of cells were measured in the liquid leaving the biofilm flow cell reactor. Cells found in the effluent were enumerated by the same radioactive labeling technique as that described above. Cells in the liquid phase represented those sheared off the biofilm and entrained into the liquid.

Bilayer biofilm studies. In this experimental series, pure-culture biofilms of either *P. putida* or the *Hyphomicrobium* sp. were first cultivated to a biofilm thickness of approximately 90 μm. Then, the invading bacterial species (either *P. putida* or the *Hyphomicrobium* sp.) was grown to the early stationary phase in batch cultures, centrifuged, and resuspended in biofilm nutrient medium. This cellular suspension was delivered to the biofilm reactor under the conditions listed in Table 1. After 6 h, the flow of the cellular suspension was terminated and the flow of nutrient medium containing both methanol and glucose was initiated. Effluent methanol and glucose concentrations, cell numbers, and species percentages were quantified over time. At prescribed intervals, the flow was stopped, the reactor was disassembled, a sample of 2 cm^2 of the biofilm was removed from the microscope slides, and the biofilm sample was resuspended in 20 ml of phosphate buffer. This suspension was divided in half, one subsample was exposed to ^{14}C-methanol, and the other subsample was exposed to ^{14}C-glucose. Biofilm cell numbers of each species were determined as described above.

Binary culture biofilm studies. In this experimental series, clean glass slides were placed in the reactor and a suspension of *P. putida* and *Hyphomicrobium* sp. cells was delivered under the conditions listed in Table 1. After 6 h, the flow of the cellular suspension was terminated and a nutrient medium containing both glucose and methanol was delivered to the reactor. Effluent glucose and methanol concentrations, suspended cell numbers, and species percentages were measured. At prescribed intervals, the reactor was disassembled and 2 cm^2 of the biofilm was removed and resuspended in 20 ml of phosphate buffer. The resulting cellular suspension was divided, one subsample was labeled with ^{14}C-methanol, and the other subsample was labeled with ^{14}C-glucose. Cell numbers of each species were determined as previously described.

RESULTS

Pure-culture biofilm studies. Pure culture biofilms of both the *Hyphomicrobium* sp. and *P. putida* were grown by the experimental techniques described above. For the *P. putida* biofilm, Fig. 2 shows the increase in biofilm bacterial cell concentration with time and the corresponding decreases in bulk liquid glucose concentration and dissolved oxygen concentration. In Fig. 3, the development of the *Hyphomicrobium* sp. biofilm showed the same trends over a slightly longer time. The results revealed that the biofilms of both *P. putida* and the *Hyphomicrobium* sp. were cultivated under oxygen mass transfer and stoichiometric limitations. The residual oxygen concentrations shown were the result of reaeration during sampling.

Bilayer biofilm studies. The first bilayer study consisted of exposure for 6 h of a suspension of *Hyphomicrobium* sp. cells to an existing *P. putida* biofilm. During and after the deposition period, media containing both methanol and glucose were delivered to the reactor. Figure 4 shows the cell number and the relative percentage of each bacterial species over time. Essentially, the *Hyphomicrobium* sp. did not become successfully established in significant numbers in the *P. putida* biofilm.

In a second experiment, a *P. putida* cell suspension was

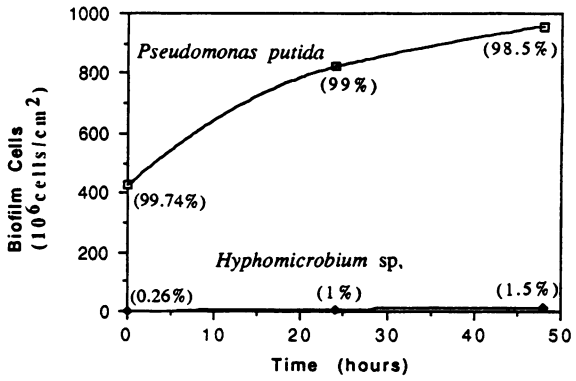

FIG. 4. Deposition and subsequent growth of *Hyphomicrobium* sp. cells in a *P. putida* biofilm. Shown are cell numbers and species percentages.

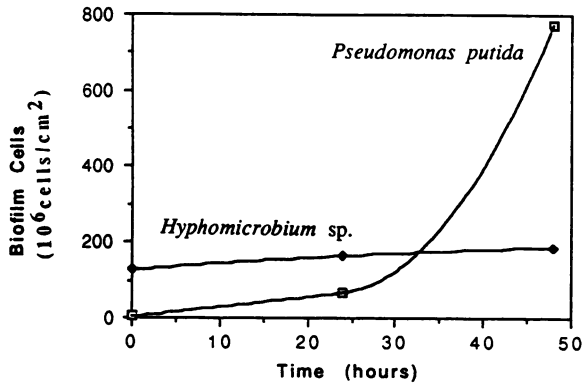

FIG. 5. Deposition and subsequent growth of *P. putida* cells in a *Hyphomicrobium* sp. biofilm. Shown are cell numbers.

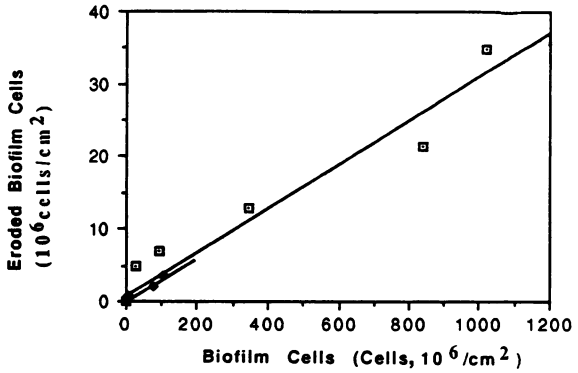

FIG. 7. Removal of *P. putida* cells from pure- and mixed-culture biofilms. Symbols: ⊡, *P. putida* pure-culture biofilm; ◆, *P. putida*-*Hyphomicrobium* sp. mixed-culture biofilm.

exposed for 6 h to a *Hyphomicrobium* sp. biofilm, during and after which nutrient media containing both methanol and glucose were delivered to the reactor. Figure 5 shows the concentration of each species at various times. *P. putida* cell numbers increased rapidly after 24 h while the original biofilm bacterium, the *Hyphomicrobium* sp., grew at a steady rate. Between 24 and 48 h, *P. putida* became the dominant species in the biofilm.

Unfortunately, this form of sampling destroys the spatial segregation that must exist in these artificially derived bilayer biofilms. Thus, Fig. 5 represents only the relative species composition within a homogenized biofilm sample.

Binary culture biofilm studies. In the binary biofilm studies, a suspension containing both *P. putida* and the *Hyphomicrobium* sp. was exposed to an initially clean glass surface for 6 h under the experimental conditions listed in Table 1. During and after the deposition period, nutrient media containing both methanol and glucose were delivered to the reactor.

Figure 6 illustrates the biofilm cell numbers of each species over time. Between 24 and 48 h, the concentration of the attached *P. putida* cells exceeded that of the *Hyphomicrobium* sp., a result which was expected, since *P. putida* grows faster ($\mu_{max} = 0.54\ h^{-1}$) on glucose than the *Hyphomicrobium* sp. does on methanol ($\mu_{max} = 0.24\ h^{-1}$) (2). The concentration of the attached *Hyphomicrobium* sp. cells increased slightly between 48 and 72 h, implying an increase

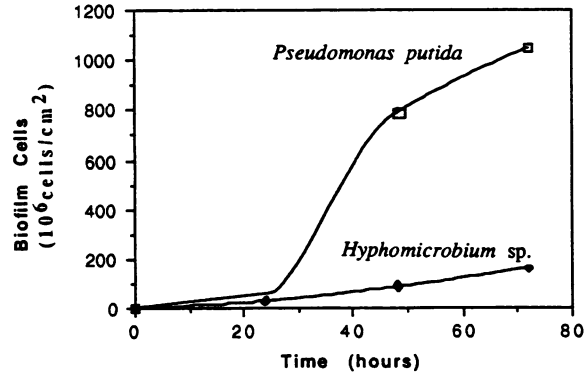

FIG. 6. Simultaneous deposition and growth of *P. putida* and *Hyphomicrobium* sp. cells. Shown are cell numbers.

in the net growth rate of these organisms relative to *P. putida*. We attributed this resurgence of the *Hyphomicrobium* sp. to incomplete oxygen penetration to the depths of the biofilm in the later stages of the experiment. As the experiment continued to proceed, the faster-growing *P. putida* dominated the upper layers of the biofilm, thus depleting both its specific electron donor and the common electron acceptor. As the *Hyphomicrobium* sp. was suppressed to the lower depths of the biofilm, it initially grew slowly on whatever substrate penetrated to its depths. Should the *Hyphomicrobium* sp. exhibit a lower saturation constant for oxygen than *P. putida*, it is possible that in the later stages of the experiment the *Hyphomicrobium* sp. would grow faster at the lower biofilm depths than *P. putida*.

Rates of removal of biofilm cells. Shear stress-related rates of removal of each species were evaluated during the accumulation of the binary culture biofilm. In general, the biofilm flow cell reactor can be treated as an ideal plug flow reactor producing suspended cells as a result of the shear removal rate, $r_r[M_x/(L^2 - t)]$, so that

$$V/F = \tau = \int_{X_{in}}^{X_e} \frac{dX}{(-r_r A/V)} \quad (1)$$

represents the residence time τ in the reactor of volume V at the influent flow rate F, where X_{in} and X_e are, respectively, the suspended-cell concentrations in the influent and effluent of the reactor (M_x/L^3) and A is the reactor surface area (L^2).

Because of the time scale of biofilm growth, one can assume that during any one sampling period, the biofilm cell concentration, B (M_x/L^2), and the resultant effluent suspended-cell concentration are constant. In addition, assuming that the effluent suspended-cell concentration is a result of a shear removal rate process that is uniformly distributed over the entire biofilm area, one can treat the system as a differential reactor; thus, equation 1 reduces to equation 2,

$$V/F = (X_{in} - X_e)/(-r_r A/V) \quad (2)$$

Assuming that the influent to the reactor is sterile, equation 2 can be rearranged to solve for the removal rate. Further, one can assume that the removal rate is a function of the biofilm-bound species cell concentration, such that

$$r_r = k_r B^n = FX_e/A \quad (3)$$

where k_r is the specific removal rate constant. Literature on biofilm removal rates as well as inspection of the data below

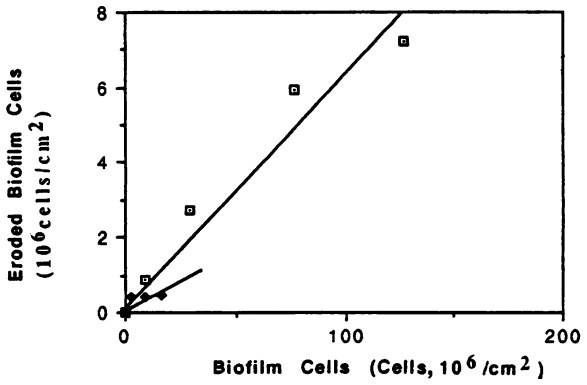

FIG. 8. Removal of *Hyphomicrobium* sp. cells from pure- and mixed-culture biofilms. Symbols: ⊡, *Hyphomicrobium* sp. pure-culture biofilm; ◆, *P. putida-Hyphomicrobium* sp. mixed-culture biofilm.

indicate that removal rate is a first-order function of B; thus $n = 1$. Reducing equation 3 to equation 4 yields

$$k_r = FX_e/BA \quad (4)$$

and the reaction rate constant, k_r, can be determined from the data shown in Fig. 7 and 8 for pure- and mixed-culture biofilms; the resultant values are given in Table 2 for each biofilm type.

DISCUSSION

No study to date has investigated biofilm ecology resulting from the invasion of one bacterial species into a developed biofilm of a second pure culture. Two series of experiments were done: (i) a *Hyphomicrobium* sp. being deposited and growing in a *P. putida* biofilm and (ii) *P. putida* being deposited and growing in a *Hyphomicrobium* sp. biofilm. The experimental results indicated that while *Hyphomicrobium* sp. cells were deposited and grew on a *P. putida* biofilm, they only contributed to a very small percentage of the total cell population. In the contrary experiment, after being deposited on a *Hyphomicrobium* sp. biofilm, *P. putida* outgrew the *Hyphomicrobium* sp. within 48 h and became the dominant species in the biofilm. The change in biofilm species composition due to the effect of bacterial invasion appeared to be dependent on the comparative growth rates of the biofilm species and the deposited species, with the faster-growing species having the competitive advantage. Destructive sampling and analysis of species concentrations within the biofilm were unable to depict the obvious artificially constructed bilayer nature of these biofilms.

TABLE 2. Shear-related removal rate constants

Biofilm species	k_r (h^{-1}) for the following species removed:				Reference or source
	P. putida	*Hyphomicrobium* sp.	*P. aeruginosa*	*Bacillus cereus*	
P. putida	0.1				This study
Hyphomicrobium sp.		0.2			This study
Binary culture	0.1	0.098			This study
P. aeruginosa			0.31		3
B. cereus				0.37	6

A third experimental series, the binary culture study, involved depositing both *Hyphomicrobium* sp. and *P. putida* cells simultaneously, onto a clean glass surface. Experimental results showed that the cells were initially present in comparable numbers (65% *P. putida* and 35% *Hyphomicrobium* sp.). Between 24 and 48 h, *P. putida* outgrew the *Hyphomicrobium* sp; after 48 h, the *Hyphomicrobium* sp. fraction appeared to marginally increase. This phenomenon may have been due to the slower growth rate of the *Hyphomicrobium* sp. (as shown in Fig. 2 and 3) or to the inability of the *Hyphomicrobium* sp. to adjust quickly to new environmental conditions. Also, as discussed earlier, should the *Hyphomicrobium* sp. exhibit a lower saturation constant for oxygen than *P. putida*, the *Hyphomicrobium* sp. may grow faster in the later stages of the experiment.

Biofilm removal rates were determined for three studies: (i) a pure *P. putida* biofilm-*P. putida* cells, (ii) a pure *Hyphomicrobium* sp. biofilm-*Hyphomicrobium* sp. cells, and (iii) a binary-culture biofilm-*P. putida* and *Hyphomicrobium* sp. cells. The specific removal rate constants for these three studies are shown in Table 2. The results showed that the rate constants for the removal of *P. putida* and *Hyphomicrobium* sp. biofilm cells from pure-culture biofilms were different. More importantly, depending on the type of biofilm (e.g., mixed versus pure culture), the shear-related removal rate constants for *Hyphomicrobium* sp. changed significantly. This difference may be attributed to the spatial distribution of *Hyphomicrobium* sp. cells in the two biofilms. In the mixed-culture biofilm, *P. putida* was the faster-growing biofilm species and eventually dominated the upper layers of the biofilm, while the slower-growing *Hyphomicrobium* sp. cells were relegated to the lower layers. Presuming that shear stresses continually erode biofilm material at the fluid-biofilm interface, it is understandable that the observed rate of removal of *Hyphomicrobium* sp. cells from the binary biofilm was slower than that of the *Hyphomicrobium* sp. cells from the pure-culture biofilm. The removal rate constants for *P. putida* cells eroding from binary- and pure-culture biofilms were equal, as expected.

Literature provides a limited number of studies on the removal of cells from a biofilm by shear. Trulear and Characklis (7) reported that the biofilm mass removal rate was a function of biofilm biomass for a *Pseudomonas aeruginosa* biofilm cultivated in a rotating annular reactor. The removal rate expression, r_r, calculated from their published data is

$$r_r \text{ (mg/m}^2\text{-min)} = 0.02\ B^{0.8} \quad (5)$$

In other published studies on shear-related removal rates, investigators have all assumed a first-order dependency on the attached-cell concentration, as summarized in Table 2. The results found in this study indicate that, in pure-culture biofilms, biofilm removal due to shear is definitely a function of biofilm cell concentration. In mixed-culture biofilms, the rate of removal of a species may not be correlated to the concentration of that species should the biofilm develop species spatial segregation, i.e., a nonuniform distribution of bacteria in the biofilm.

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