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Behavioral Analysis of *Vibrio parahaemolyticus* Variants in High- and Low-Viscosity Microenvironments by Use of Digital Image Processing

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Digital image analysis and light microscopy were used to study and quantify the growth and behavior of two variants and selected flagellar mutants of *Vibrio parahaemolyticus* in glass flow cells under high- and low-viscosity conditions. The observations showed a series of surface-associated behaviors, including attachment, microcolony formation, migration, chemotactic movements, and aggregation, indicating a substantial degree of adaptive flexibility and multicellular behavior during growth of *V. parahaemolyticus* at interfaces.

Vibrio parahaemolyticus is known to be a significant member of marine fouling communities (9) and has been isolated from a variety of marine and estuarine habitats (1, 18). During colonization and growth on surfaces, *V. parahaemolyticus* is capable of expressing two phenotypes (3, 35). These phenotypic variations include cell elongation and the development of mixed flagellation (lateral and polar). In general, polar flagella are responsible for locomotion in aqueous environments, whereas lateral flagella have been shown to be active in swarming on agar surfaces or attachment to inanimate surfaces (2, 10, 14, 35, 36). The genetic control of *V. parahaemolyticus* lateral flagellum production has been extensively investigated (3, 29, 35). Belas et al. (3) used *lux* gene fusions to demonstrate that the regulation of gene expression controlling these phenotypes was physical, i.e., increasing the viscosity of the medium was sufficient to induce phenotypic change of cells to the laterally flagellated (Laf) phenotype. Additional studies have elaborated on the control of this Laf system, including the role of iron in gene induction (28, 29, 32). In addition, there can occur a spontaneous phenotypic change in which *V. parahaemolyticus* cells synthesize little lateral flagellin and do not develop into elongate morphotypes. Phenotypic switching has been described for a range of microorganisms, including xanthomonads (18) and mycoplasmas (31), and is a mechanism for generating intraspecies diversity. Genetic control of phenotypic switching is not understood in general and has not been extensively investigated in the case of *V. parahaemolyticus*. Furthermore, studies have not been directed at observing and understanding the occurrence and role of this phenomenon in the life cycle of *V. parahaemolyticus* growing at solid-liquid interfaces.

The use of digital image processing and microscopy provides a quantitative analytical approach to the study of bacterial behavior (5–8, 26). These methods have been used to quantify and describe the growth and behavior of *Pseudomonas fluorescens* and other bacteria (7, 11, 22, 24–26). In this study, such an approach was used to examine the behavior of *V. parahaemolyticus* strains in low- and high-viscosity environments during growth at a solid-liquid interface. The objectives were to define the surface-associated behavior of two variants and to evaluate the functions of polar and lateral flagella during growth of selected flagellar mutants on surfaces in viscous and nonviscous environments.

V. parahaemolyticus BB22 (translucent variant), LM2 (opaque variant), and five other mutant strains (Table 1) were obtained from M. Silverman and L. McCarter (Agouron Institute, La Jolla, Calif.). The two variants spontaneously interconverted; the opaque variant was characterized as small, polarly flagellated cells that synthesized little lateral flagellin under any conditions, whereas the translucent variant was the common wild type (27a). All strains were routinely grown in 2216 marine medium (Difco) at 75% of the recommended concentration (28 g liter^{-1}). Broth cultures were incubated at 25 ± 3°C in a rotary water bath (New Brunswick Scientific Co., Edison, N.J.). Eighteen-hour cultures were diluted 1:50 in fresh broth and incubated as described above for 1 h to provide a standard inoculum for continuous-flow slide culture experiments. Polyvinylpyrrolidone with a molecular weight of 360,000 (PVP-360; Sigma Chemical Co., St. Louis, Mo.) was used to increase the viscosity of aqueous solutions. High-viscosity medium was prepared by aseptically combining sterile 2× 2216 broth with sterile 20% (wt/vol) PVP-360 to obtain a final solution with a viscosity of 165-200 cP. The medium viscosity was measured with a Haake Rotovisco RV-2 viscometer (Gebrüder Haake, Berlin, Germany).

Continuous-flow slide culture chambers were prepared and aseptically inoculated as described previously (7, 22, 23). During high-viscosity experiments, the flow was turned off for ~5 min to facilitate initial attachment of polarly flagellated cells. A high-pressure syringe pump (model 301; ISCO, Lincoln, Nebr.) was used to provide pulse-free laminar flow.

A Zeiss Photomicroscope III aligned for either phase-contrast (100× objective [Zeiss Neofluor N.A. 1.30]) or dark-field (10× objective [Zeiss N.A. 0.22]) illumination was used for all observations and photography. Photomicrographs were taken at 15- or 30-min intervals with Plus-X film (ASA 125; Kodak, Rochester, N.Y.). The photomicroscope was also interfaced with an IBAS 2000 image processor (Kontron, Eching, Germany) by using a light-sensitive cam-

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TABLE 1. Bacterial strains examined in this study

Strain	Description[a]	Behavior[b]	Reference(s)
BB22	Translucent wild type, Fla$^+$, Laf$^+$	swim$^+$, swarm$^+$	3, 28, 29
LM2	Opaque variant, Fla$^+$, Laf$^-$	swim$^+$, swarm$^-$	27a
LM761	*lux* constitutive, Tet^r, Fla$^+$, Laf$^+$	swim$^+$, swarm$^+$	27a
RS3639	*laf-3639*::mini-Mu *lux*, Fla$^+$, Laf$^-$	swim$^+$, swarm$^-$	3
LM1017	*laf-313*::*lux*, Tet^s, Fla$^+$, Laf$^-$	swim$^+$, swarm$^-$	28, 32
LM899	*flaC1* Kan^r, Fla$^-$, Laf$^+$	swim$^+$, swarm$^+$	28
ML159	*fla-159*::*lac*, Tet^r, *laf-313*::*lux*, Fla$^-$, Laf$^-$	swim$^-$, swarm$^-$	29

[a] Tet^r or Tet^s, tetracycline resistant or sensitive; Kan^r, kanamycin resistant; *lux*, *lux* gene fusion; Fla$^+$ or Fla$^-$, with or without a polar flagellum, Laf$^+$ or Laf$^-$, with or without lateral flagella.

era with a silicon intensification target (model TV9A; Bosch). Images from either dark-field or phase-contrast microscopy were digitized and averaged. The resulting image was then discriminated (thresholded) to define the cell boundary before computer measurement of the number of microcolonies per field, microcolony area, and frequency of motile cells and nearest-neighbor analyses. Details of image acquisition, digitization, and analyses for microscope images have been reported previously (12, 21–23). Biofilm development (26) and cell growth rates (7) were calculated as the slope of a plot of the natural logarithm of the area versus time.

The presence of lateral flagella on motile cells was determined by a minimum immobilizing viscosity assay. This was based on the observation that polarly flagellated cells were immobilized at viscosities of >60 cP, whereas laterally flagellated cells were motile up to 1,000 cP. The assay consisted of introducing cells from the effluent (>4 h) of continuous-flow slide culture chambers into high-viscosity medium (300 cP) and monitoring the medium for the presence of motile cells. The result was considered negative if no motile cells were detected after 1 h of incubation in high-viscosity medium.

At low viscosity, both *V. parahaemolyticus* variants were actively motile within the quiescent boundary layers at the surface of the flow cell. The macroenvironment flow rate was 10 cm s^{-1}, whereas the microenvironment (0.2-μm lamina) flow rate was only 8 μm s^{-1} as a result of increasing viscous forces near the liquid-glass interface (22, 23). Motile attachment (22, 25), in which a bacterial cell is attached to the surface yet is freely motile at the solid-liquid interface, was not necessary to sustain movement, since the maximum rate of vibrio motility (60 μm s^{-1}) (14) exceeded the velocity near the flow cell surface. Cells in association with the surface were observed to perform looping maneuvers, moving with and against the direction of flow. Both variants exhibited the preliminary chemosensory cell rotations described previously for *P. fluorescens* and several other bacteria (25, 30).

It has been demonstrated that microbial motility is affected by the viscosity of the environment. Many bacteria swim more rapidly in environments with a viscosity of 2 cP than in water (4, 13, 15). However, the mean velocity of most polarly flagellated bacteria quickly decreases when the viscosity is greater than 5 cP (16, 33). The minimum immobilizing viscosity for *Pseudomonas aeruginosa* and *Escherichia coli* was estimated to be 60 cP (16). It was similarly demonstrated in the present study that the motility of polarly flagellated *V. parahaemolyticus* strains was inhibited by a viscosity of 60 cP, whereas elongated, laterally flagellated *V. parahaemolyticus* strains were actively motile in environments with a viscosity exceeding 1,000 cP. Thus, although polarly flagellated cells could successfully attach and recolonize in low-viscosity environments, this ability was physically inhibited in high-viscosity environments (165 to 200 cP). During the early phases of surface colonization, the number of attached cells per field declined by 25 to 50% (reversible attachment). *V. parahaemolyticus* exhibited a prolonged period of reversible attachment which lasted on average 1 h, contrasting with that previously reported for *P. fluorescens*, which attached irreversibly after a period of cell rotation lasting only 1 to 2 min (25). The changes in number of cells per field of analysis during time-course experiments at high and low viscosity are shown in Fig. 1.

The long period of reversible attachment noted for *V. parahaemolyticus* may be explained in terms of the regulation of adhesin production. Belas et al. (3) demonstrated that active transcription and translation of lateral flagellar (*laf*) genes occurred 30 to 60 min after cells were exposed to a surface, consistent with the period of reversible attachment observed in this study. However, since both variants and some of the mutants (which lacked lateral flagella, i.e., RS3639 and LM1017) (Table 1) exhibited similar periods of reversible attachment, adhesive exopolymer production or other factors may also provide an explanation for this behavior. The behavior of the control strain, LM761 (Fla$^+$ Laf$^+$), and other mutant *V. parahaemolyticus* strains was similar to that of both variants under low-viscosity conditions (Table 2). The exception was strain ML159 (Fla$^-$ Laf$^-$) which failed to attach and exhibited no surface-associated behavior. This observation emphasizes the importance of flagella during the initial attachment of vibrios to surfaces. The significance of polar flagella for initial attachment success of *P. fluorescens* in continuous-flow slide culture has also been demonstrated (22, 23).

After cells irreversibly attached, microcolonies developed by one of two pathways. Cells of the translucent variant underwent cell elongation after attachment, with the periodic production of daughters at the distal ends of the original mother cells. Daughters that separated from the elongating cells remained near the mother cells. In the opaque variant, cell division occurred by equal division, resulting in small, polarly flagellated daughter cells characteristic of this variant. Opaque colonies initially developed via a typical packing maneuver (24, 25) involving cell fission and cell separation, with the resulting daughter cells lying in close association in the same plane. Similar colony-forming maneuvers have been described for *P. fluorescens* and other bacteria growing in continuous-flow slide culture (24, 25) and for *E. coli* K-12 on agar-coated slides (34). Attached microorganisms have also been shown to exhibit shedding, packing, spreading, and rolling maneuvers during growth and microcolony development on surfaces (24). After one or two generations, it was common for one or more of the daughters

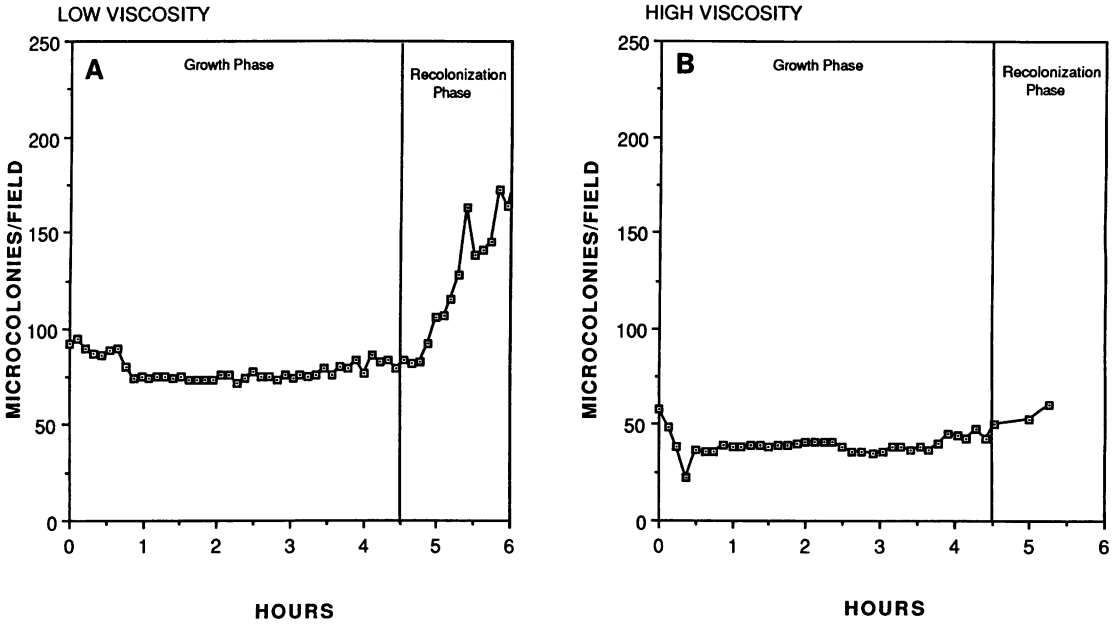

FIG. 1. Effect of viscosity on attachment, growth, and recolonization by *V. parahaemolyticus*. The number of microcolonies per field, determined by averaged image input at 6-min intervals, is shown. (A) In low-viscosity environments, the microcolony number remained stable after an initial period of instability and decline (~1 h) until 4 to 5 h, when the number of colonies increased as a consequence of cells detaching and reattaching within the field. (B) In high-viscosity environments, a decline in the number of attached cells was evident during the first hour, and the number of microcolonies per field did not increase.

in microcolonies of the opaque *V. parahaemolyticus* variant to undergo morphogenesis (i.e., cell elongation characteristic of the translucent variant, indicating interconversion between the two variants). Translucent cells produced daughters periodically, whereas other daughters in the same microcolony continued to undergo growth, cell fission, and separation typical of the opaque variant. The two pathways are illustrated in Fig. 2 and 3. Figure 2 shows the development of four microcolonies of *V. parahaemolyticus*, as observed with dark-field microscopy. Three colonies exhibited the morphology characteristic of the opaque variant with revertant cells, and one expressed the translucent phenotype (Fig. 2). Figure 3 illustrates the development of an opaque colony in which visible cell elongation was apparent in some daughters only after 3.5 h of colony growth. In contrast, the translucent colony (Fig. 3) initiated cell elongation without completing the first division. The development of attached microcolonies followed these pathways regardless of the viscosity of the medium. Other vibrio isolates growing under similar (low-viscosity) conditions in Duxbury chambers have exhibited patterns of attachment and growth that did not result in microcolony development (27). For example, *Vibrio* strain MH3 attached, grew, and then detached prior to cell division, which occurred when cells were suspended in the aqueous phase (27). In the case of *Vibrio* strain DW1, mother cells attached in a perpendicular orientation, growing and releasing daughter cells into the aqueous phase (20).

The viscosity of the growth medium did not affect the growth rates of any of the strains studied. The specific growth rate determined by phase image analysis was 1.2 h^{-1}, resulting in a doubling time of 0.58 h for both variants. As indicated in Fig. 4, the biofilm development rates estimated by dark-field image analysis were also similar in high- and low-viscosity environments (~0.7 h^{-1}). One previous study (17) had indicated an increased growth rate for swarmer cells

TABLE 2. Behavior of *V. parahaemolyticus* strains in continuous-flow slide culture at low and high viscosities

Strain[a]	Behavior at:							
	Low viscosity				High viscosity			
	Attachment	Microcolony formation	Emigration	Recolonization	Attachment	Microcolony formation	Emigration	Recolonization
BB22	+	+	+	+	+	+	+	−
LM2	+	+	+	+	+	+	−	−
LM761	+	+	+	+	+	+	+	−
RS3639	+	+	+	+	+	+	−	−
LM1017	+	+	+	+	+	+	−	−
LM899	+	+	+	+	−	−	+	−
ML159	−	−	−	−	−	−	−	−

[a] For description of strains, see Table 1.

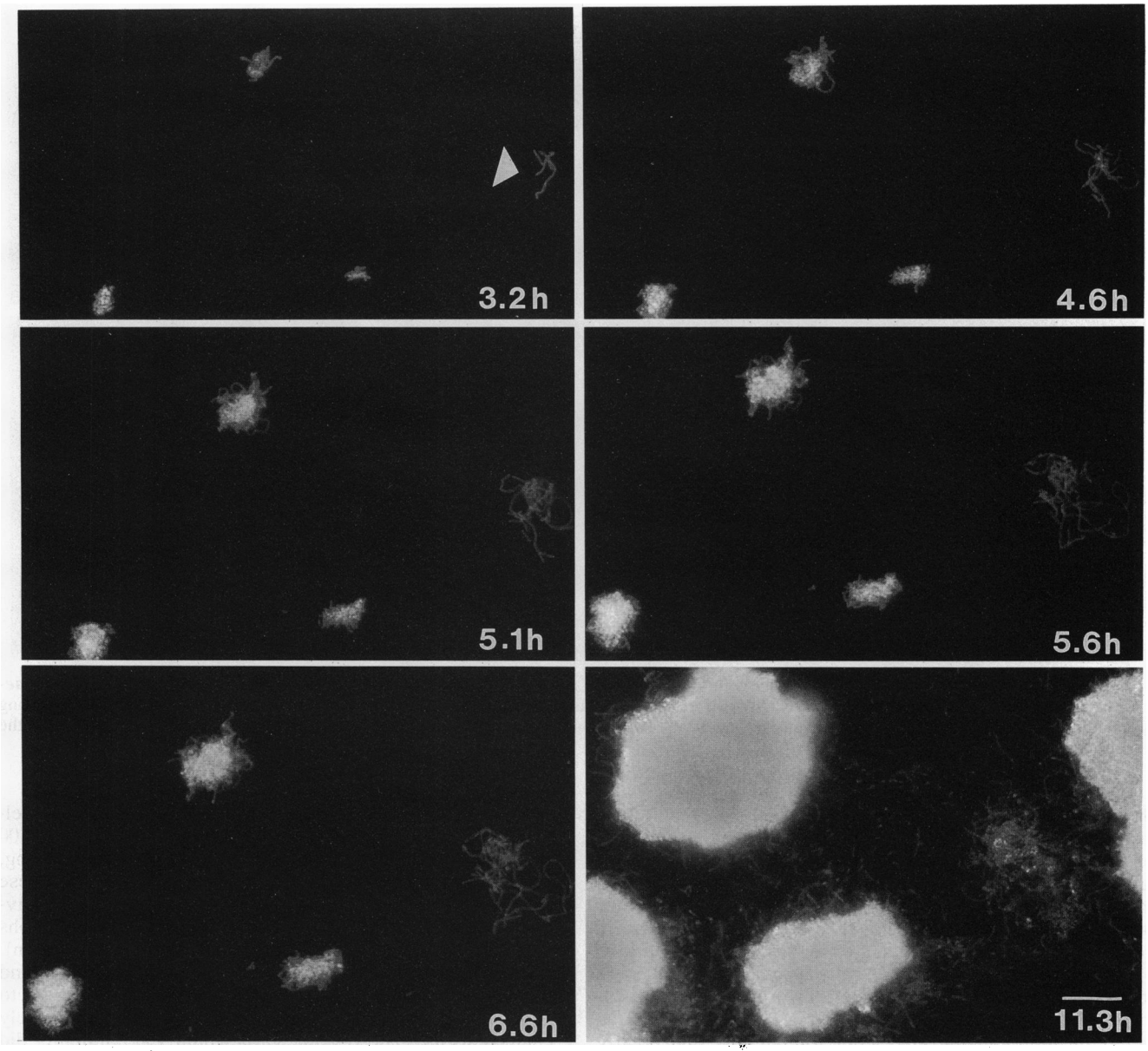

FIG. 2. Dark-field photomicrographs showing cell elongation and aggregation during the formation of microcolonies by *V. parahaemolyticus*. Colony development was monitored from the initial single cell to three-dimensional microcolonies or aggregations over a period of 11.3 h. The phenomena of cell elongation and aggregation are clearly evident in these photomicrographs; in one colony, cell elongation is evident from the first generation (the translucent variant indicated by the arrowhead), whereas in the other colonies, cell elongation was not apparent until later in microcolony development (the opaque variant). Bar = 50 μm.

of *Proteus mirabilis* PM23 grown in differentiation-supporting broth medium.

Both vibrio variants exhibited a recolonization-emigration phase during growth at low viscosity which occurred 4 to 5 h after inoculation (approximately five generations) (Fig. 1 and 5). In low-viscosity environments, only small, polarly flagellated cells (cell length, 2 μm) were observed to migrate and recolonize, whereas the elongated, apparently laterally flagellated *V. parahaemolyticus* cells remained attached to the surface and did not emigrate. Figure 1A illustrates the increase in microcolony density in low-viscosity microenvironments, where a 2.3-fold increase in microcolony number was observed after 6.0 h. Similarly, the number of motility events detected by difference imagery (Fig. 5A) increased at the same time.

Recolonization of *P. fluorescens* was shown to occur at the 8- to 16-cell stage of microcolony development (5 h) (25). Recolonizing cells of *P. fluorescens* reattached to the surface at new locations, decreasing the average colony size and increasing the random distribution of cells (22). Although recolonization by *V. parahaemolyticus* resulted in a >100% increase in the number of microcolonies per field over the analysis period, the average microcolony size continued to increase. Nearest-neighbor analyses also indicated that recolonizing cells attached primarily at or near the sites of initial microcolony growth. Indeed, as shown in Fig. 2,

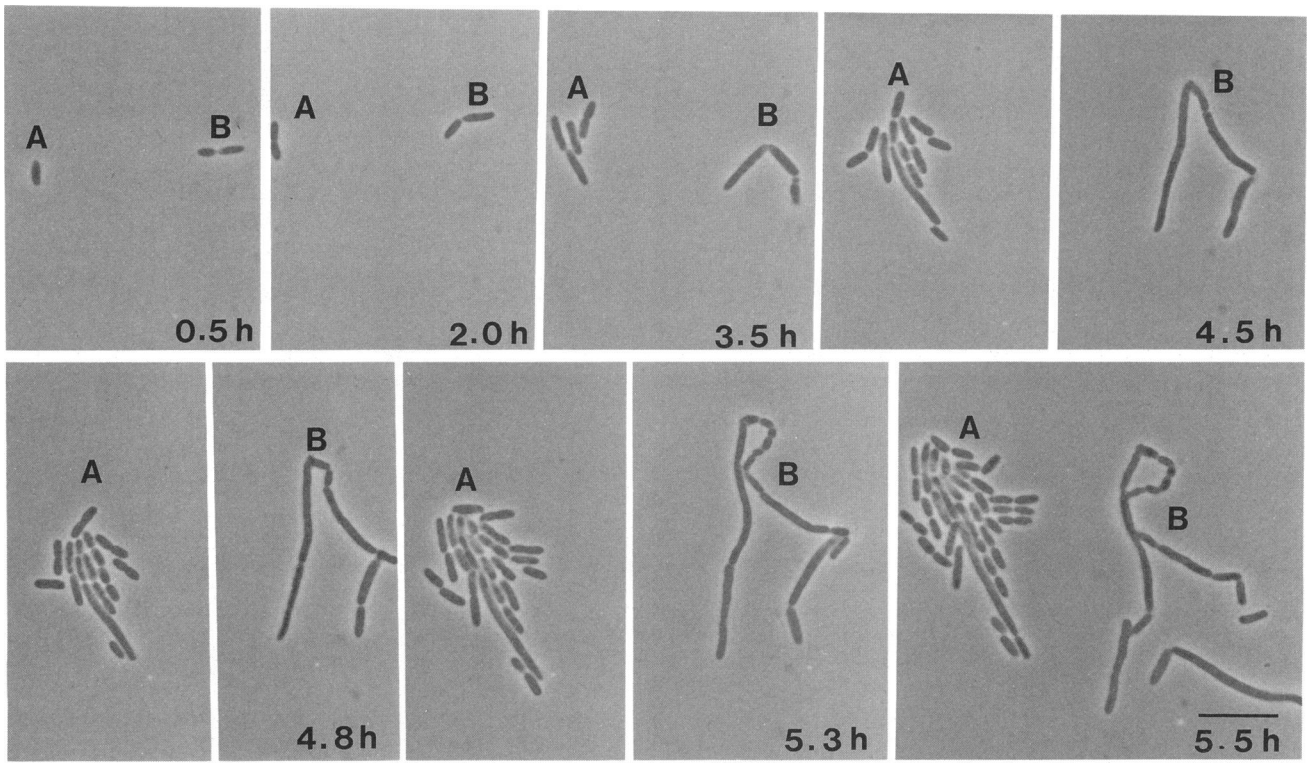

FIG. 3. Phase-contrast photomicrographs showing cell elongation and microcolony development during attached growth by *V. parahaemolyticus*. In opaque microcolonies (colony A), cell elongation was not evident until 3.5 to 4.5 h of development. In contrast, during translucent microcolony development (colony B), cell division was not completed, and the cell elongation proceeded continuously over the development period. Bar = 10 μm.

daughter cells aggregated at the original colonies, resulting in large three-dimensional colonies apparent even after 11.3 h of development. Thus, in the low-viscosity environment, laterally flagellated progeny had enhanced adhesion through multiple points of contact and acted as attachment foci for polarly flagellated recolonizing cells but were not active in cell dispersion over the surface or in the medium.

In contrast, elongated, laterally flagellated cells were found to be the only active cells in high-viscosity microenvironments (Fig. 5B and 6). This was consistent with the minimum immobilizing viscosities for laterally and polarly flagellated cells, which were >1,000 and ~60 cP, respectively. However, since the laterally flagellated cells did not reattach to the surface after emigration occurred, this was a migration phase (Table 2). Migration from existing microcolonies by cells of the translucent variant was evident in visual observations and also by the increased numbers of motile vibrios detected within the field at 4 h (Fig. 5B). Figure 5B also shows that fewer cells were observed to actively emigrate at high viscosity, reducing the magnitude of this event relative to that observed at low viscosity. It should be noted that the elongated cells were occasionally motionless and therefore not discriminated from microcolonies during the analysis, resulting in a small increase in the baseline number of microcolonies detected after 4.5 h (Fig. 1B). Observations of LM899 (Fla^- Laf^+) behavior at high viscosity supported these findings; these cells did not attach at high viscosity and remained actively motile in the surface microenvironment throughout the assay (Table 2). Thus, laterally flagellated cells (induced or constitutive) showed no tendency to attach and form microcolonies in high-viscosity environments.

During locomotion at high viscosity, the laterally flagellated cells exhibited a combination of long linear runs (>100 s), stopping, and reciprocating motions. The reciprocating, or back-and-forth, motions were also observed when these cells interacted with attached microcolonies. These behaviors are shown in dark-field and phase-contrast micrographs (Fig. 6A to C, interactive behavior; Fig. 6D, a linear run). It is suggested that this combination of linear runs and reciprocating movements constitutes a behavior parallel to "tumble-and-run" behavior at low viscosity and is likely chemosensory. The opaque variant and Laf^- mutants did not exhibit an emigration phase (Table 2). A mutant that was constitutive for production of lateral flagella (LM899 [Fla^- Laf^+]) exhibited behavior similar to that of the wild type under low-viscosity conditions, indicating that lateral flagella can function for motility and recolonization in low-viscosity environments. The behavior of the mutants also indicated that behavioral patterns such as attachment, migration, and aggregation were coupled to the induction of lateral flagella in a high-viscosity environment.

Thus, the behavior of *V. parahaemolyticus* at interfaces included a prolonged period of reversible attachment, microcolony formation by packing, a recolonization-emigration phase, and aggregation. Specific aspects of these behaviors were further modified by the viscosity of the surrounding medium. It was also shown that during development, *V. parahaemolyticus* microcolonies could form as exclusively translucent or opaque morphotypes or, most commonly, as mixed populations consisting of both opaque and translucent morphotypes. Thus, laterally flagellated progeny adapted for attachment to the surface in low-viscosity environments or

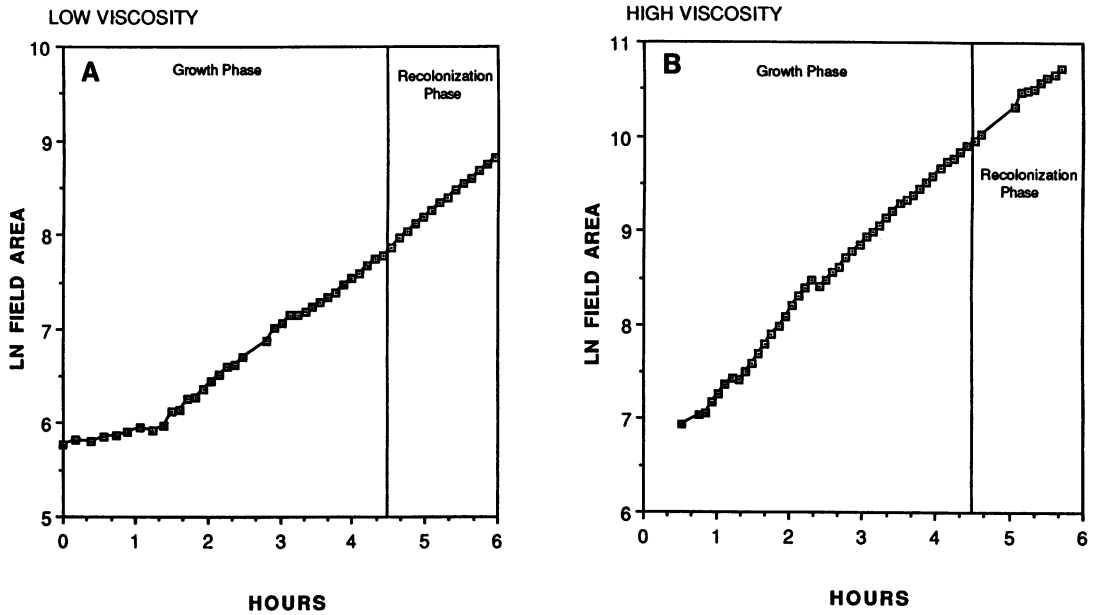

FIG. 4. *V. parahaemolyticus* biofilm development rate in high- and low-viscosity media determined by dark-field image analysis. *V. parahaemolyticus* exhibited a specific biofilm development rate of 0.69 h^{-1} at low viscosity (A) and 0.75 h^{-1} at high viscosity (B).

migration in high-viscosity environments or polarly flagellated progeny adapted for detachment and recolonization in low-viscosity environments were produced. The production of the opaque variant also provided a mechanism to create a population adapted to a planktonic existence. These studies also show the production of several functional types, indicating a degree of multicellular behavior during the formation of *V. parahaemolyticus* biofilms.

This range of adaptive flexibility is consistent with the habitat variation reported for *V. parahaemolyticus*, which is an autochthonous estuarine organism isolated from water, sediment, marine organisms, and inanimate surfaces and is

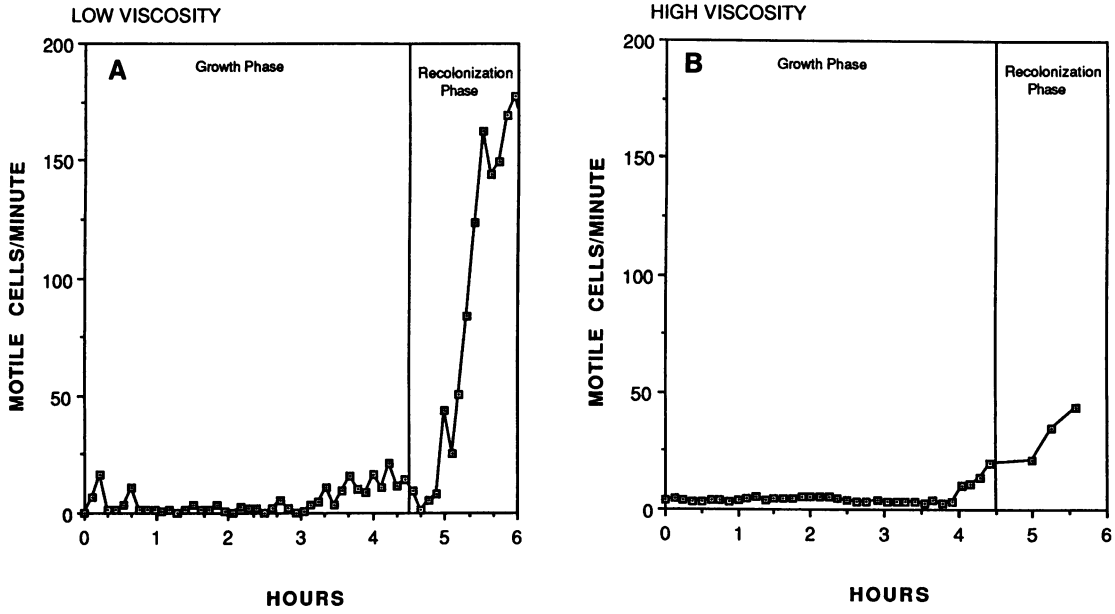

FIG. 5. Onset of recolonization in *V. parahaemolyticus* biofilms at 4 to 5 h, as indicated by increased motility detected by difference imagery. Motility was detected at a uniform background level under both high- and low-viscosity conditions until 4 to 5 h of development, at which time motility increased. The magnitude of the recolonization event was greatest under low-viscosity conditions (A) (100-fold increase in motility), whereas at high viscosity fewer cells became motile (B). However, actively motile cells at low viscosity were polarly flagellated, and those at high viscosity were elongated (laterally flagellated) cells (as shown in Fig. 6).

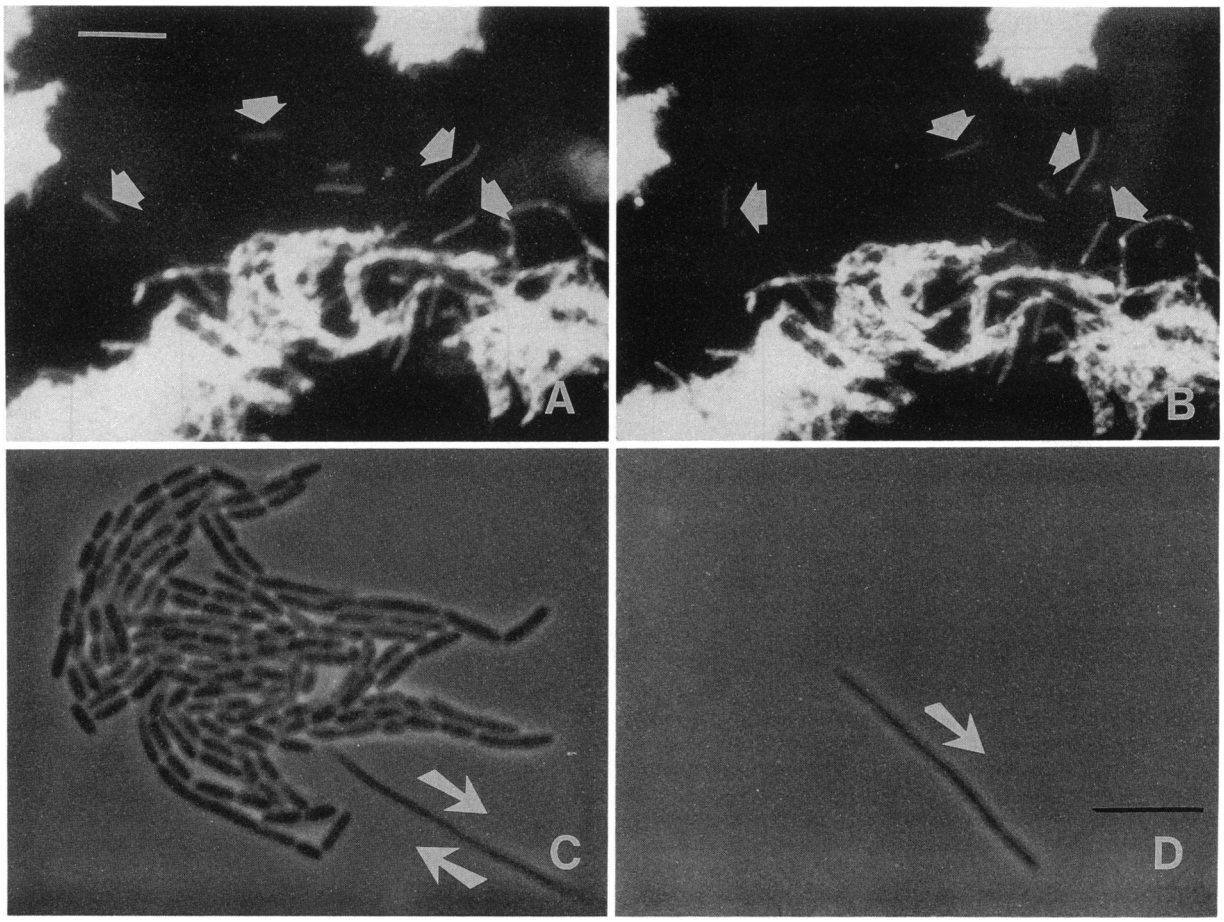

FIG. 6. Dark-field (A and B) and phase-contrast (C and D) photomicrographs showing *V. parahaemolyticus* microcolonies in a high-viscosity environment after 5 h of attached growth. (A) Microcolonies and associated elongated motile cells; (B) same field 10 s later, showing movement of the migrating cells; (C and D) laterally flagellated cell performing reciprocating motions (C) and, later, the same cell during an extended run (D). Bars: dark field, 50 μm; phase contrast, 10 μm.

also associated with gastroenteritis in humans (19). However, the regulation and selective advantages of specific microbial behaviors, such as the chemosensory nature of reciprocating movements, migration, and aggregation, require further study by behavioral and genetic analyses.

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