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Diversity in surface colonization behavior in marine bacteria

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Using laminar flow chambers and time-lapse video imaging, colonization of surfaces by four marine bacteria revealed a diverse range of morphological characteristics and cell–cell interactions. The strain SW5 formed a compact, multilayered single- and double-cell biofilm on hydrophobic surfaces but developed long multicellular chains on hydrophilic surfaces. The morphologically similar SW8 showed unusual proximal vertical packing of cells on both substrata. *Vibrio* sp strain S14 exhibited cyclical colonization-detachment events on both substrata. *Pseudomonas* sp strain S9 initially displayed reversible and then irreversible adhesion apparently triggered by a cell density phenomenon that led to the development of regular microcolonies on both substrata with individual cells translocating between the colonies. The length of time bacteria were exposed to and their density at a surface influenced behavioral traits, with diverse and distinctive species-specific behavioral events.

Keywords: colonization; biofilm; diversity; proximal vertical packing; cell–cell interaction

Introduction

Although much attention has been directed towards establishing the important parameters that dictate successful adhesion to various substrata by different bacteria [12], relatively few studies have been reported on the subsequent colonization behavior of the bacteria at the surface. A range of behavioral patterns in different surface-colonizing bacteria have been studied by direct observation [26], by employing image analysis of progressively recorded sequences in single [10] or mixed [24] cultures and in natural lake water populations [9], and by employing time-lapse video recording of the colonization processes [2,8,19]. Patterns observed include: a mother-daughter [8] or shedding [9,16] strategy, whereby a cell adheres to a surface and regularly produces daughter cells; a packing [2,9,16] strategy, with dividing cells aligning adjacent to each other leading to the formation of microcolonies and to a monolayer of contiguous cells; a slow migratory [19] or spreading [9] strategy, whereby cells, after division, migrate slowly (0.15 μm min^{-1}) at random over the surface until dividing and migrating again; a reversibly adhering [19] or rolling [9,16] strategy, with the cells not firmly attached to the surface; and a filament- [15] or chain-forming [2] strategy, with biofilms developing in a filamentous manner.

The aims of the present study were to extend our knowledge on the diversity evident in surface colonization behavior in marine bacteria, a process not to be overlooked in microbial biofilm development, and to further demonstrate the cost-effective, user-friendly, and informative nature of the time-lapse video technique for this purpose. What was revealed was distinctive species-specific colonization behavioral patterns which were dependent on the stage of biofilm development over time.

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Material and methods

Bacterial strains and culture conditions

The motile bacteria *Vibrio* sp strains S14 and *Pseudomonas* sp strain S9 were isolated from the surface waters at Botany Bay, Sydney [5], whereas the non-motile bacteria SW5 and SW8 were isolated from surfboard wax after exposure to seawater off Wanda Beach, Sydney [1,17,22]. Cell surface hydrophobicity testing has shown that S9, SW8 and SW5 are relatively hydrophobic [1,5,17] and S14 is relatively hydrophilic [5]. Bacteria were grown in a minimal artificial seawater (MMM) [18] supplemented with 20 mM glutamic acid (MMMglt), except for S14, which was supplemented with 11 mM glucose (MMMglc). Glass flow chambers were employed as previously described [2]. Substrata consisted of acid-washed (hydrophilic) and silanized (hydrophobic) glass coverslips. Colonization of the continuous flow chambers was initiated by inoculating log-phase cells grown in MMMglt or MMMglc at an A_{600} of 0.1 as previously described by Dalton *et al* [2]. After 1 h an observation field was selected, flow was resumed and continuous nutrient flow maintained at a bulk flow rate of 2×10^4 μm s^{-1} throughout the period of observation. Biofilms were monitored for up to 4 days.

Photomicroscopy and image processing

The glass flow chambers were mounted on the stage of an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) fitted with differential interference contrast optics (Carl Zeiss, Neofluar, 100×, NA 1.3 and 63×, NA 1.25 with oil immersion) and a green interference filter (Carl Zeiss) for on-line visualization of bacterial colonization. Video recordings were made using a Panasonic WV-BP500 CCTV camera (Matsushita Electric Industrial Co Ltd, Osaka, Japan) fitted to the microscope and connected to a Panasonic SVHS time-lapse video recorder (model AG-6720A). Images presented were obtained using Photoshop 3.0 (Adobe Systems Inc, Mountain View, CA, USA), and Video Monitor both run on a Power Macintosh 8100/80AV computer (Apple Computer, Inc, Cupertino, CA, USA).

The image processor was calibrated using a 0.01-mm slide micrometer (Olympus, Tokyo, Japan). Time measurements (to 3 s) were determined using an inbuilt timing display and frame-by-frame advance, and recordings were viewed on a 53-cm video monitor (National model WV-5490; Matsushita Electric Industrial Co Ltd). Cell movements and numbers were measured from manual tracings and photographic prints of video stills.

Results

Our initial studies on attachment and surface colonization by the marine bacterium SW5 revealed that this organism exhibited different morphological forms at hydrophobic and hydrophilic surfaces [2]. Examination of biofilms formed in laminar flow chambers using both continuous time-lapse video microscopy and scanning confocal laser microscopy revealed that isolate SW5 colonized the entire area of hydrophobic surfaces and the cells were all single or doublet forms. On a hydrophilic surface the organism formed chains of up to several hundred cells, with the terminal cell attached to the surface resulting in sparse colonization at the surface. Consequently, the structures of the biofilms on these two surfaces were entirely different [2]. Early colonization of isolate SW5 at a hydrophobic surface revealed microcolonies of cells attached longitudinally and developed by a packing strategy (Figure 1; 4, 6 and 8 h). These microcolonies developed in all directions and there appeared to be no limit to the number of cell divisions in the development of the colonies. Some cells attached to the surface, in the centre but not at the periphery of the colony, orientated into a vertical position following cell division, whereas other daughter cells were released into the flow or immediately attracted to the closest uncolonized surface. Colonization continued until the entire surface was covered with a multilayered biofilm (Figure 1; 17, 19 and 20 h). This biofilm structure was maintained throughout the observation period.

The remaining three marine bacterial species examined did not show the morphological shift seen with isolate SW5 on surfaces of different hydrophobicities nor did they respond differently to the hydrophobicity of the surface. Each organism displayed a unique colonization strategy.

The marine bacterium SW8, which resembles isolate SW5 morphologically, displayed a colonization strategy which was inceptively similar to that of isolate SW5 on a hydrophobic surface (Figure 1; 8 h and 17 h; Figure 2; 7 h and 16 h). Microcolonies, originating from single cells and doublet forms, adhered irreversibly in a longitudinal orientation at the surface, developed by a packing strategy (Figure 2; 7 h). The microcolonies developed in all directions by unlimited cell division forming a multilayered biofilm (Figure 2; 16 h) and the shed daughter cells were transported to colonize surfaces downstream. With an increase in cell numbers at the surface, the subsequent generations of cells usually orientated into a vertical position resulting in a 'honeycomb'-like structure (Figure 2; 24 h; Figure 3). Within this dense biofilm, some released daughter cells were attracted to and accommodated by the highly structured layer by the shuffling of adjacent cells so that the arrangement maximized vertical packing (Figure 2; 24 h, indicated by arrows in the enlarged views in Figures 3 and 4). Some daughter cells were shed into the flow and transported from the field of view. With time and increasing cell density, many mother cells detached resulting in low numbers attached at the surface (Figure 2; 44 and 52 h). A biofilm then developed which consisted of low numbers of single and doublet cells at the surface and multicellular aggregates and short chains (Figure 2; 65 h).

Early colonization by *Vibrio* sp S14 exhibited a spreading behavior [9,19] with some cells attaching longitudinally and irreversibly. These cells showed a packing strategy giving rise to microcolony development (Figure 5; 3 and 5 h). The tightly packed groups of cells developed for 4–5 generations before daughter cells were seen to attach by one pole and oscillate. These cells then detached and were dispersed into the flow or colonized the surface downstream (Figure 5; 10 h). At this stage no packing maneuvers were seen. Cell density at the surface increased until a monolayer formed (Figure 5, 18 h) and then large numbers of cells were shed into the flow. The detachment and emigration of cells left the surface sparsely colonized (Figure 5; 45 h).

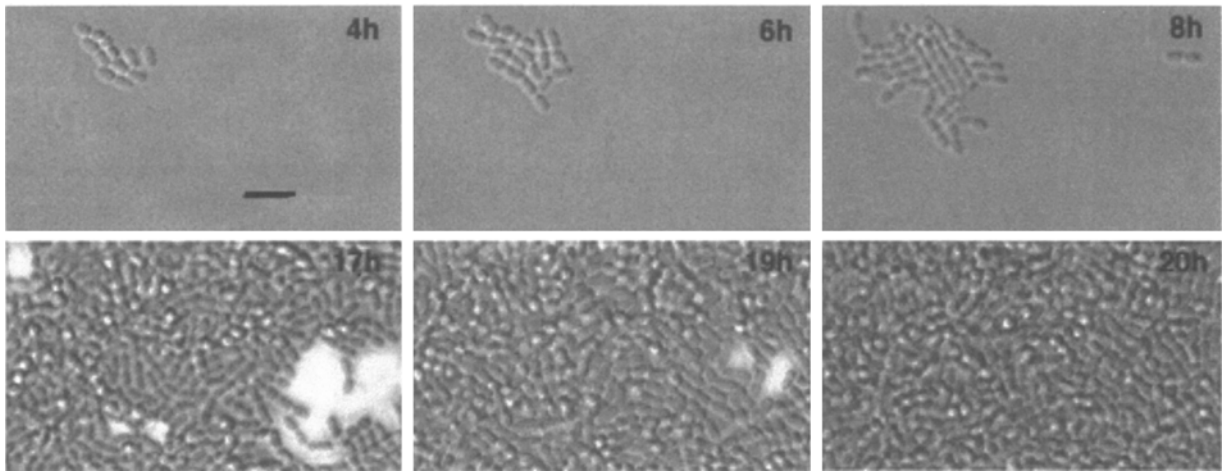

Figure 1 Images taken from on-line video recordings of the same microscopic field representing colonization features of biofilm development by isolate SW5 on a hydrophobic surface. The colonization strategy was dependent on whether the surface was hydrophobic or hydrophilic [2]. Time is given in hours after inoculation of flow chambers. Bar = 5 μm.

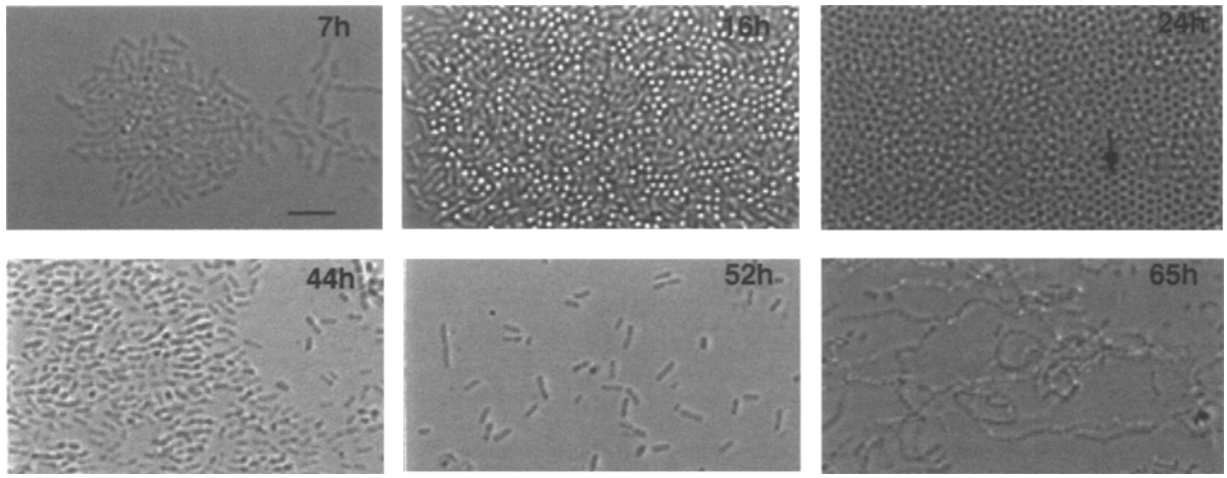

Figure 2 Images taken from on-line video recordings of the same microscopic field representing colonization features of biofilm development by isolate SW8. This colonization strategy was independent of whether the surface was hydrophobic or hydrophilic. Time is given in hours after inoculation of flow chambers. The arrow indicates an area of maximised vertical packing (see Figure 3). Bar = 10 μm.

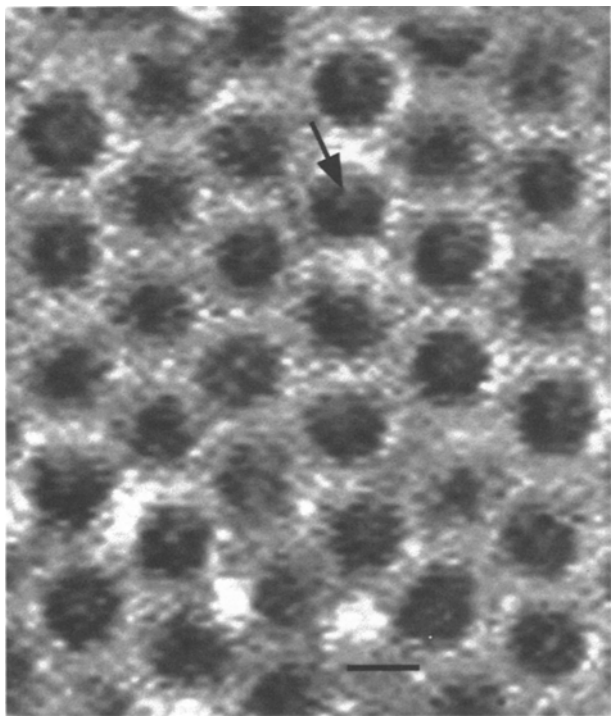

Figure 3 Enlarged view of the maximized vertical packing shown by isolate SW8 as indicated by the arrow in Figure 2. Arrow indicates a bacterium in a vertical orientation. Bar = 1 μm.

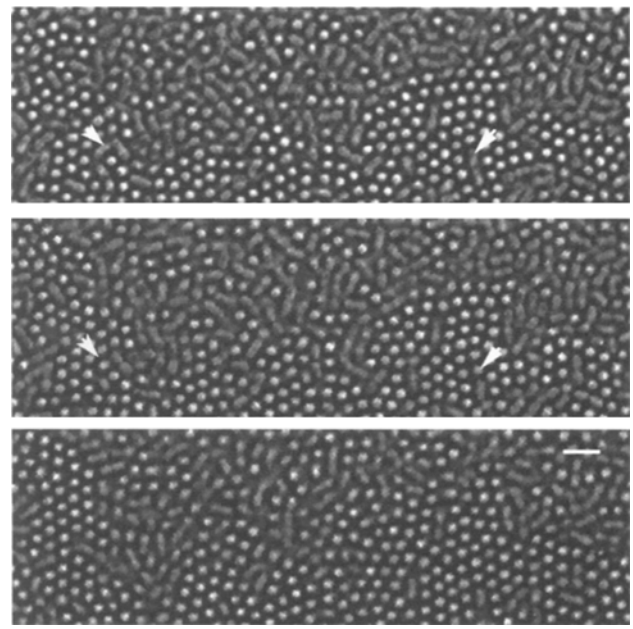

Figure 4 Enlarged images taken from on-line video recordings of the same microscopic field showing the 'honey-comb'-like structure of a biofilm formed by isolate SW8. The arrow indicates an example of the repositioning of cells from a longitudinal (arrow in a) to a vertical (arrow in b) orientation. a = 26, b = 26.5 and c = 30.5 hours after inoculation of flow chambers. Bar = 5 μm.

The remaining attached cells continued to divide and spread to give a spatially heterogeneous biofilm consisting of multicellular aggregates and single cells (Figure 5; 52 h). The biofilm then underwent successive cycles of emigration and recolonization (Figure 6). Increasingly, cells at the surface became more filamentous, yet these cells produced normal sized daughter cells which were shed into the flow. Flagellar motility was evident in cells initially contacting a surface and for cells detaching from a surface or after cell division.

Cells of *Pseudomonas* sp S9 initially demonstrated a slow drift across the surface, as though restrained in some way, but did not adhere (Figure 7; 1 and 38 h). Although the bulk flow rate was 2×10^4 μm s^{-1} the rate of movement of the cells at the surface was 0.1 μm s^{-1}. This phenomenon might have been the result of a reversible attraction [14] of the cells to the surface. The eventual transition from this slow-moving state (reversible adhesion) to irreversible adhesion to the surface appeared to be a density-dependent function as it occurred in the range of 81 to 112 cells per μm^2 irrespective of the initial inoculum loading. These

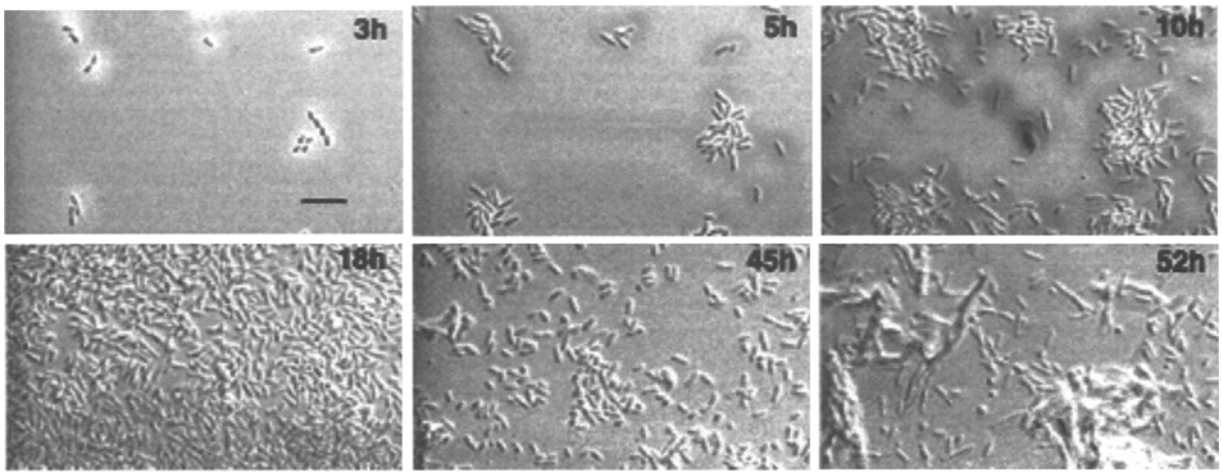

Figure 5 Images taken from on-line video recordings of the same microscopic field representing colonization features of *Vibrio* sp strain S14 biofilm development. This colonization strategy was independent of whether the surface was hydrophobic or hydrophilic. Time is given in hours after inoculation of flow chambers. Bar = 10 μm.

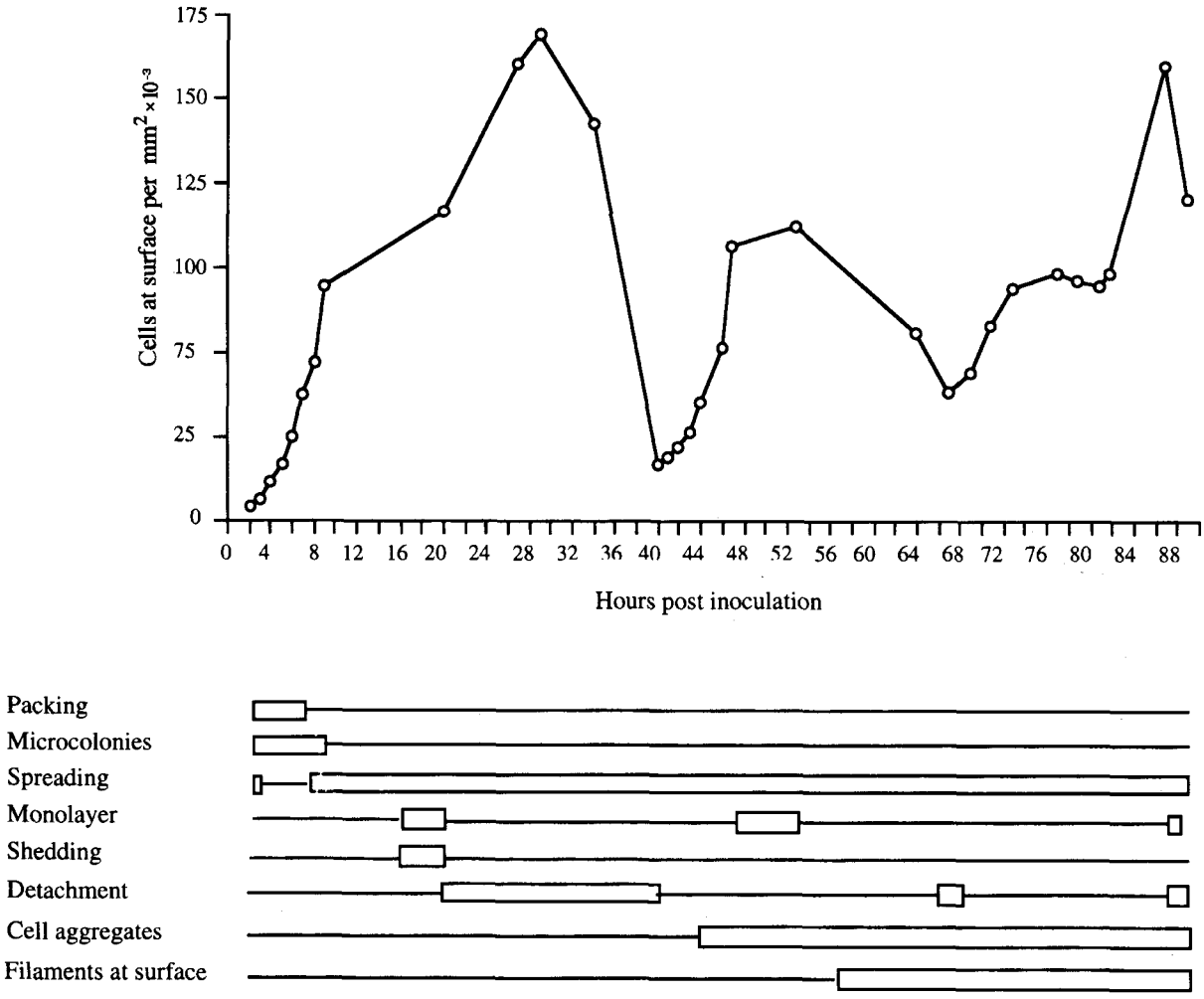

Figure 6 Cyclical colonization by *Vibrio* sp strain S14. Cell numbers at the surface of the same microscopic field are compared to behavioral characteristics of the bacteria forming a biofilm over time.

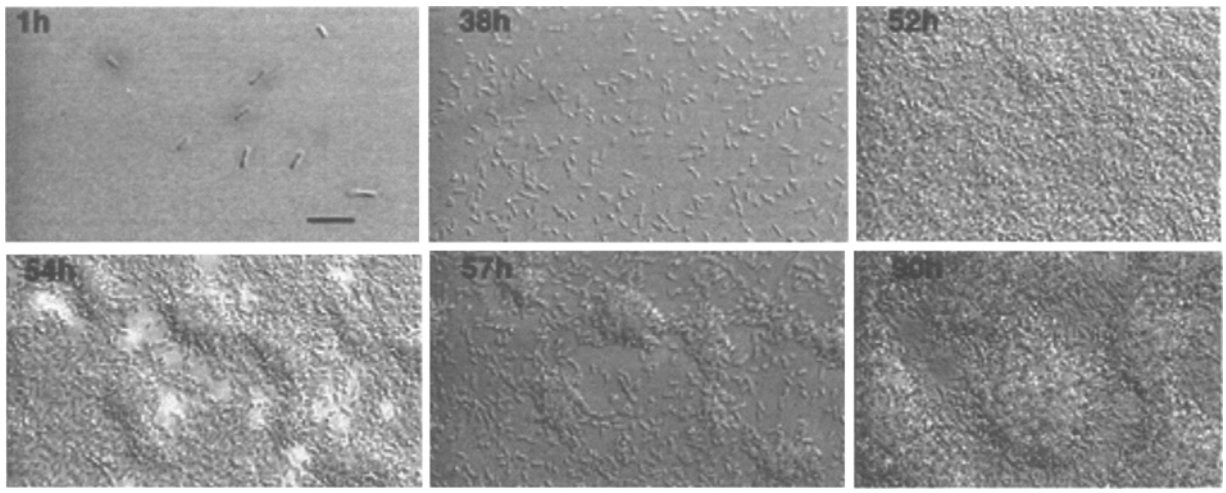

Figure 7 Images taken from on-line video recordings of the same microscopic field representing colonization features of *Pseudomonas* sp strain S9 biofilm development. This colonization strategy was independent of whether the surface was hydrophobic or hydrophilic. See text for details of the patterns observed. Time is given in hours after inoculation of flow chambers. Bar = 10 μm.

attached cells continued to multiply resulting in a confluent biofilm (Figure 7; 52 h) and eventually microcolonies (Figure 7; 54 h) were seen to develop. These microcolonies were formed by cells amassing at particular loci either by cell division or by the attraction of cells towards the loci by means of a slow (c 0.4 μm s^{-1}) translational motion across the surface resulting in large uncolonized areas between the colonies. Accordingly there was a shift from relatively firm attachment to that where cells were capable of limited movement across a surface. This cell movement was in a longitudinal direction, it was independent of the direction of flow, and the cells appeared to be tethered to the surface but not firmly attached, reminiscent of the phenomenon of temporary adhesion described in gliding bacteria [6]. The microcolonies continued to build up and bridges of translocating cells formed between colonies. A critical biofilm density was attained when an active migratory and flow-assisted dispersal of the accumulated biofilm occurred. Microcolonies then reformed at the initial sites (Figure 7; 57 h) and, within the time that biofilm observations were made, continued to increase in cell density (Figure 7; 90 h). Flagellar motility was observed, mainly in daughter cells just prior to detachment from the mother cells, only until the biofilm cells began to segregate into microcolonies.

Discussion

Diversity in bacterial behavior is epitomized by the range of strategies employed by different species when colonizing solid surfaces. The use of laminar flow chambers and time-lapse video imaging revealed diverse morphological characteristics and cell–cell interactions in four marine bacteria colonizing hydrophobic and hydrophilic surfaces. The length of time bacteria were exposed to and their density at a surface influenced behavioral traits. Distinctive bacterial species-specific colonization behaviors, with shifts in colonization strategies and subsequent biofilm development contingent on the environment at the surface, were observed for each of the organisms examined. In general terms, the unidentified strain isolate SW5 formed a compact, multilayered single and doublet cell biofilm on hydrophobic surfaces but developed long, multicellular chains on hydrophilic surfaces, the morphologically similar isolate SW8 showed unusual proximal vertical packing of cells on both substrata, *Vibrio* sp S14 exhibited cyclical colonization-detachment events on both substrata, and *Pseudomonas* sp S9 developed regular microcolonies on both substrata with individual cells slowly migrating between the colonies.

Microcolony development was common to the four organisms, yet the character of the microcolonies and their development differed depending on the duration of cell–cell and cell–substratum interactions. Isolate SW5 (on hydrophobic surfaces) and isolate SW8 formed microcolonies by a packing strategy, conceivably determined by the cell surface hydrophobicity and the nature of the substratum surface, that led to multidirectional development of the microcolonies. The cell surface hydrophobicity may explain the marked steadfastness by which the cells were attracted to the surface. The microcolonies of *Vibrio* sp S14 occurred early in surface colonization and also were formed by a packing strategy, but were limited in size by the number of cells forming the colonies (4–5 generations) as reported previously in *Pseudomonas fluorescens* [10]. The cell surface of *Vibrio* sp S14 is relatively hydrophilic [5] and may be a determining factor in the development of instability in microcolonies of this organism. The relatively hydrophobic *Pseudomonas* sp S9 [5] did not form microcolonies by a packing strategy and, in fact, microcolony formation did not occur until the density of slow drifting cells reached a level whereby the cells became firmly attached to the surface as a continuous layer. The microcolonies then developed at particular foci, either by rapid division at or by attraction of slow migrating cells to these sites. It is not clear at this stage whether localization at

these foci was the result of a concentration of nutrients or an accumulation of a favorable polymer at the sites, or because of cell–cell signaling resulting from a shift in cell physiology [25]. The translocation of the *Pseudomonas* sp S9 cells did not appear to be organized, as seen with swarming organisms [23], but their behavior could indicate some alteration in gene expression to enable movement across the surface [4]. Depending on nutrient and surface modifications, *Vibrio* sp S14 and isolate SW5 established multicellular and filamentous biofilms with few cells attached to the surface. The advantages of such filamentous biofilms for the organisms might be the increased opportunities for the cells to forage for nutrients further into the flowing aqueous phase.

The dispersal of the microcolonies of *Vibrio* sp S14 and *Pseudomonas* sp S9 may have been a consequence of environmental stress rendering surface attachment undesirable for continued survival and growth. These stresses may include nutrient depletion, anaerobiosis, or the build-up of toxic metabolic byproducts. Bacteria may respond to such conditions by surface modifications, including loss of proteinaceous adhesins [7] or adhesive exopolysaccharides [20,27], or the production of hydrophilic exopolymers by normally hydrophobic bacteria [3,21]. It is conceivable that with *Pseudomonas* sp S9 environmental stress was created when microcolonies were joined by bridges of cells, occluding nutrient within that environment or creating adverse surface modifications. The fact that microcolonies reformed following dispersal implies that the former rather than the latter situation occurred.

Vertically attached cells have been reported previously [2,8,10,11]. To the best of our knowledge, however, the development of the proximal vertical packing seen with isolate SW8 has not been reported. With rod-shaped microorganisms many more cells would be accommodated by adhering in a vertical orientation than longitudinally. The strategy would ensure maximal cell growth within a biofilm in times of nutrient abundance and, during adverse conditions, dispersal from the surface would be facilitated. This space-maximizing behavior may result from direct cell–cell interactions, rather than by specific surface-imposed forces, such as the localization of cell surface hydrophobicity as described by Marshall and Cruickshank [13]. The observation of some daughter cells shuffling into and being accommodated in the highly organized layer tends to favor this explanation.

This study reinforces previous reports [8,9,11,19] where surface colonization patterns were found to be species-specific characters, but emphasizes that subsequent biofilm development is not easily predictable on the basis of the early colonization behavior of the particular organism in question. Many aspects of the adhesion and subsequent colonization behavior of different microorganisms at surfaces are almost certainly subject to modifications in the expression of various genes as a result of contact with surfaces [2,4]. With the surface behavioral traits of the four marine bacteria established, the foundations have been laid for further studies on the dynamics involved in mixed species biofilm formation and structure.

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