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In Situ Identification of Bacterial Species in Marine Microfouling Films by Using an Immunofluorescence Technique

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An immunofluorescence technique was developed for the in situ identification of specific bacteria in marine microfouling films. Microorganisms adherent to glass plates after 30 days of immersion in a synthetic seawater system were cultured and classified by biochemical tests, flagellar arrangement, and the API 20E system. All isolates were gram-negative aerobic or facultative motile rods, predominantly *Pseudomonas* spp. Rabbit antisera to the five dominant organisms including *Achromobacter* spp., *Comamonas terrigena*, *P. putrefaciens*, a yellow-pigmented *Pseudomonas* sp., and *Vibrio alginolyticus* were prepared. These antisera were shown to be species specific in indirect immunofluorescence assays against a battery of 26 marine isolates from 14 bacterial species, with the exception of antisera to the *Pseudomonas* spp. which cross-reacted with each other but not with test bacteria of other genera. These immunofluorescent reagents enabled the in situ identification of all five bacterial species in microfouling films. Low-surface-energy test plates had smaller numbers of adherent bacteria in microfouling films than medium-surface-energy test plates, suggesting that the degree of microfouling may be influenced by the surface energy. In addition, the reagents could identify up to 39% of the attached bacteria in microfouling films spontaneously formed on steel plates in flow cells deployed in different areas of the Atlantic Ocean. The microbial composition of the ocean-formed films varied with the geographical area of their formation. The present results indicate that immunofluorescence techniques may provide a rapid and reliable means to identify, in situ, specific bacteria in marine microfouling films.

Marine biofouling is the process in which procaryotic and eucaryotic organisms adhere to solid surfaces immersed in seawater. Biofouling represents a major economic problem in the maritime industries, since the bacterial slime films and the large numbers of barnacles, mussels, and tunicates which accumulate on ships (11, 24) increase drag forces and surface corrosion, thereby causing additional fuel and maintenance costs. The first events in biofouling involve the deposition of a humic-like multilayer of organic matter (2) followed by colonization with specific bacterial species. The primary microbial film (10, 45) may prepare the surface for subsequent colonization by sessile organisms (10) but is itself an important problem. Microfouling can cloud optical oceanographic instruments such as periscopes (25) and can impair the transfer of thermal energy in heat exchangers (14, 16) and cooling towers (38).

The marine microflora can be studied by a number of techniques, including culture, histological staining, biochemical assays, and immunological techniques. Each of these approaches offers certain advantages and disadvantages to studies of microfouling. Microbiological culture for the identification of attached seawater microorganisms requires the removal of the bacterial cells from the attaching surface, viability of the cells, and knowledge of the appropriate in vitro growth conditions. In general, only a small proportion of the bacterial cells present in the sample will actually be recovered by culture (28, 46). Histological staining with Gram stain (29), acridine orange (13, 44), erythrosin (40), phenolic Rose Bengal, brilliant green, or Ziehls carbol fuchsin have been used for the direct enumeration of marine microorganisms (8). However, these methods do not permit bacterial speciation and require a cell density greater than 10^6 cells/ml for one bacterial cell to be detected, and stained detritus of size and shape similar to bacterial cells may be mistakenly counted (20). Biochemical assays, including limulus amebocyte lysate assay (33, 43), adenosine triphosphate determinations (27), and nucleic acid (30, 35) and muramic acid measurements (34), are useful for determining total bacterial counts but cannot distinguish between bacterial species or between procaryotic and eucaryotic organisms.

Immunofluorescence techniques overcome many of these problems. These techniques, which are based on the specificity inherent in antigen-antibody reactions, are rapid and quantitative, abrogate the need for viable microorganisms and culture, and can be used to detect a single bacterial species in a complex microbiota such as dental plaque (Y. Bonta, J. J. Zambon, M. Neiders, and R. J. Genco, Abstr. Annu. Meet. Int. Assoc. Dent. Res. 1984, J. Dent. Res. **63**:306, abstr. no. 1218). Immunofluorescence techniques have been used to assay aquatic microorganisms accumulated on membrane filters (7, 12, 26, 39, 41, 42) but not in investigations of microfouling.

The flow cell is a recently developed apparatus for investigations of microfouling (31). It is a small, inexpensive, portable, plastic cylinder containing two test plates of known composition and surface properties. These plates become rapidly coated with microfouling materials as seawater is pumped through the flow cell. The physical composition and thickness of the microfouling layer on test plates has been determined by infrared spectroscopy, ellipsometry, and energy-dispersive X-ray techniques (5, 14), but the number and identities of specific attaching microorganisms have yet to be determined.

In the present study, we developed immunofluorescent techniques for in situ identification of the bacteria in microfouling films produced in flow cells. (Portions of this

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work were previously presented: P. S. Huber, A. E. Meyer, J. Slots, M. S. Fornalik, J. J. Zambon, and R. E. Baier, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, N44, p. 185).

MATERIALS AND METHODS

Experimental microfouling system. A microfouling model system consisting of a pair of seawater aquaria connected to a series of flow cells was used to study marine microorganisms involved in microfouling. Each aquarium contained 50 gal (187 liters) of synthetic seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio) with a complete biofouling population including barnacles, tubeworms, bryozoa, and hydroids maintained at predetermined water temperature, specific gravity, and salinity. Tank lighting was designed to simulate the solar illumination of the Atlantic coastal region at ca. 30°N. Synthetic seawater was pumped from these aquaria through flow cells for microfouling studies. The flow cells were composed of Plexiglas-stiffened silicone rubber cylinder halves containing two glass or metal test plates (5 by 1.9 by 0.08 to 0.16 cm) separated by plastic spacers to form a rectangular capillary (31). After exposure to seawater, the plates were removed and the adherent microorganisms were cultured and characterized. These bacteria were also identified in situ on additional test plates by immunofluorescence techniques with specific antisera. In addition, the effect of surface energy (3) (controlled by applying thin coatings to the test plates) on the number and types of attaching bacteria in microfouling layers was examined.

Bacterial culture and identification. Initial experiments were directed toward the isolation and speciation of microorganisms attaching to glass plates immersed in the synthetic seawater aquaria for 30 days. Material accumulated on these plates was removed with a sterile scalpel blade and transferred to 5.0 ml of sterile Ringer solution. Bacterial cells were dispersed by vortexing at the maximal setting for 5 s, and then 10-μl and 100-μl portions were plated onto Marine agar (Difco Laboratories, Detroit, Mich.) or tryptic soy agar (Difco) containing 5% sheep blood. Test plates were also placed into petri dishes containing 15 ml of Marine agar, and an additional 15 ml of molten Marine agar was poured over the surface and allowed to solidify. Bacterial colonies were enumerated on all plates after 48 h of incubation at room atmosphere and temperature.

By using established procedures (6, 32), 26 bacterial colonies were randomly selected and identified by a number of criteria, including Gram stain characteristics, motility, growth on MacConkey agar, UV fluorescence, pigment production on Marine agar, growth in the presence or absence of oxygen, oxidative-fermentative use of glucose in tubes containing 2% NaCl, and flagellar arrangement and location (21). Bacterial isolates were further characterized by the API 20E system (Analytab Products, Plainview, N.Y.) modified by the addition of 2% NaCl (M. T. MacDonell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N100, p. 194).

Antisera production. A strain of each of five predominant bacterial species, *Achromobacter* spp., *Comamonas terrigena*, *Pseudomonas putrefaciens*, an orange-pigmented *Pseudomonas* sp., and *Vibrio alginolyticus*, were selected for antibody production in rabbits. Each strain was grown in air to late logarithmic or early stationary phase in 3 liters of Marine broth (Difco) at room temperature. After 48 h of incubation, bacterial cells were harvested by centrifugation at 15,000 × *g* for 20 min and washed three times in phosphate-buffered saline (PBS; pH 7.0) containing 0.3% formaldehyde. The cell pellet was resuspended in PBS to a concentration equal to a McFarland no. 5 standard, and 1 ml of bacterial suspension was combined with an equal volume of Freund incomplete adjuvant (Difco).

Eight-week-old female New Zealand white rabbits, each weighing 4 kg, initially received 1.0-ml subcutaneous and intradermal injections of the bacterial suspension in incomplete adjuvant, followed 4 weeks later by 1.0-ml intravenous injection of the bacterial suspension alone into the marginal ear vein. Trial bleedings were obtained after 2 weeks, and the serum antibody titer was determined by tube agglutination assays (17). Rabbits with an antibody titer greater than 1:512 were exsanguinated by cardiac puncture, whereas rabbits with lower antibody titers received 1.0-ml intravenous injections of bacterial suspension alone until an antibody titer greater than 1:512 was achieved. The rabbit antiserum was heated to 56°C for 30 min to destroy complement proteins and was then stored in small aliquots at −20°C until used.

Indirect immunofluorescence assays. Indirect immunofluorescence assays were carried out essentially as described by Mouton et al. (36). Each seawater isolate was grown on Marine agar for 48 h at room temperature. The bacterial cells were harvested with a platinum loop and suspended in 2% glutaraldehyde in 3.5% Instant Ocean to a density equal to a MacFarland no. 1 standard. A 10-μl portion of each bacterial suspension was placed on a glass slide, air dried, and gently heat fixed. The slides were rinsed in distilled water for 5 min to remove excess salts, air dried, and then stored at room temperature.

The working titer for each antiserum was determined by checkerboard titration. A 10-μl portion of twofold serial dilutions of rabbit antisera in PBS containing 0.05% Tween 20 was placed on the homologous bacterial smear for 20 min, rinsed with PBS-Tween, washed in PBS, and rinsed in distilled water. The slides were then incubated with 25 μl of goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (isomer I; BBL Microbiology Systems, Cockeysville, Md.) serially diluted in PBS-Tween, rinsed and washed as before, and then mounted with glycerol in PBS (2:1 [vol/vol], pH 9.0).

A Leitz Orthoplan microscope equipped for phase contrast illumination and for incident-light (epi-illumination) fluorescence was used to examine the stained bacterial smears. The light source was an Osram HBO-200 mercury lamp with a BG 12 filter and a dichroic 495-nm interference filter in its exciting pathway and a K 510 suppression filter. Fluorescence was graded from 0 to 4+ according to the following criteria; 4+, brilliant fluorescence with good cell envelope definition, center of cell is dark; 3+, moderate fluorescence with good cell envelope definition, enter of cell is dark; 2+, faint fluorescence, single cells visible but no definition of cell envelope; 1+, faint fluorescence, single cells not distinguishable; 0, no staining of bacteria visible. All reactions were recorded, but only grades 3+ and 4+ were considered serologically positive.

The penultimate twofold serial dilution of each antisera with 4+ fluorescence was selected as the working titer for use in subsequent assays. Antiserum specificity at working titer was evaluated on the basis of fluorescence reaction with heat-fixed smears of heterologous seawater microorganisms.

Field sampling. The specific antisera were used to identify bacteria in microfouling films on stainless steel plates (5 by 2 by 0.1 cm) exposed to flowing waters of the Atlantic Ocean during the 10 March to 18 April, 1983 cruise of the U.S.N.S. Lynch. Medium-surface-energy plates prepared by the radio-frequency glow discharge technique and stored in air (3)

TABLE 1. Bacteria recovered from glass surfaces exposed to a synthetic seawater aquarium system

Species or strain	No. isolated
Achromobacter spp.[a]	3
Alcaligenes pacificus	1
Alcaligenes sp. strain I	1
Comamonas terrigena[a]	2
Cytophaga sp.	1
Flavobacterium sp.	1
Halobacterium sp.	1
Moraxella sp.	1
Pseudomonas nautica	2
Pseudomonas putrefaciens[a]	3
Pseudomonas sp. strain I[a]	3
Pseudomonas sp. strain II	3
Vibrio alginolyticus[a]	3
Vibrio anguillarum	1

[a] Bacteria used to produce rabbit antisera.

(critical surface tension, ca. 30 dynes/cm) and low-surface-energy plates obtained when plates prepared by this technique were coated with dimethyldichlorosilane (critical surface tension of the plates, ca. 22 dynes/cm) were placed in flow cells through which water samples were pumped by a portable biofouling test unit at a rate of 350 ml/min for 7 days. Each set of plates was exposed to water from different areas of the Atlantic Ocean, from Charleston, S.C., through the Sargasso Sea to Las Palmas in the Canary Islands and, finally, to Scotland. In addition, one set of plates of each surface energy was exposed to water during the entire voyage. After exposure, the test plates were removed from the flow cells, fixed in 2% glutaraldehyde, rinsed in triple-distilled water, air dried, and sequentially stained with each of the five antisera in indirect immunofluorescent assays. The total numbers of microorganisms in the microfouling film were determined by acridine orange staining and epi-illumination microscopy (37).

RESULTS

Twenty-six strains of seawater microorganisms were cultured from glass plates immersed in the marine aquarium (Table 1). All microorganisms were gram-negative, motile rods. *Pseudomonas* spp., including *P. putrefaciens*, *P. nautica*, and two unspeciated *Pseudomonas* spp. predominated, making up 42% of the total isolates. *Achromobacter* spp., *V. alginolyticus*, and *C. terrigena* constituted 12, 12, and 8% of the isolates, respectively, and single isolates of *Alcaligenes pacificus*, *Alcaligenes* sp. strain I, *Cytophaga* sp., *Flavobacterium* sp., *Halobacterium* sp., *Moraxella* sp., and *V. anguillarum* were recovered.

The *Pseudomonas* spp. were all oxidase-positive organisms possessing large monopolar flagella (Table 2). *P. putrefaciens* liquefied gelatin and decarboxylated ornithine. *P. nautica* produced white cellular pigment, elaborated hydrogen sulfide, and utilized citrate. The two groups of unspeciated *Pseudomonas* spp. were differentiated by growth on MacConkey agar, citrate utilization, and nitrate reduction. The *Alcaligenes* spp. possessed peritrichous flagella. *A. pacificus* differed from an unspeciated group of *Alcaligenes* isolates by its inability to grow on MacConkey agar or to utilize citrate and by its inability to reduce nitrate.

The specificity of our rabbit antisera to the five predominant microbial species was determined in immunofluorescence assays with glutaraldehyde-fixed cells (Table 3). In initial studies, numerous preservation techniques were evaluated, including heat fixation of bacterial cells resuspended in PBS, 4% Instant Ocean, or 20% NaCl as well as fixation in acetone, methanol, and 95% ethanol. These fixation techniques all resulted in cell lysis of the seawater microorganisms. However, successful bacterial preparations were obtained when cells were suspended in 2% glutaraldehyde in 3.5% Instant Ocean to a density equal to a MacFarland no. 1 standard and air dried. With this fixation technique, working titer concentrations of antisera were between 1:32 and 1:64. The immunological reagents were species specific except for cross-reactions noted between *P. putrefaciens* and *Pseudomonas* sp. strain I, suggesting a close serological relationship between these organisms. In contrast, distinct patterns of immunofluorescent reactions were seen for *A. pacificus* and *Alcaligenes* sp. strain I and for *Pseudomonas* spp. strains I and II, confirming the validity of their separation based on biochemical tests. Three groups, *Alcaligenes* sp. strain I, *Flavobacterium* sp., and *Halobacterium* sp., showed no immunofluorescence reactivity with any of the reagents tested.

These antisera were then used in indirect immunofluorescence assays to determine the bacterial composition of microfouling films produced on glass plates by aquarium seawater pumped through flow cells for 6 days at a rate of 325 ml/min. All five bacterial species could be detected in these films (Table 4); 39% of the attaching microorganisms were identified as *V. alginolyticus* on the medium-surface-energy plates (critical surface tension, ca. 31 dynes/cm),

TABLE 2. Differential characteristics of *Pseudomonas* and *Alcaligenes* spp. isolated from synthetic seawater aquarium system

Characteristic	*P. putrefaciens*	*P. nautica*	*Pseudomonas* spp.		*Alcaligenes sp.* strain I	*A. pacificus*
			I	II		
Oxidase reaction	+	+	+	+	+	+
Flagellar arrangement	Monopolar	Monopolar	Monopolar	Monopolar	Peritrichous	Peritrichous
Gelatin liquefaction	+	−	−	−	−	−
Hydrogen sulfide production	+	+	−	−	−	−
Ornithine decarboxylase	+	−	−	−	−	−
Nitrate reduction	+	−	−	+	+	−
Growth on MacConkey agar	+	−	−	+	+	−
Citrate utilization	+	+	−	+	+	−
Pigment formation	Yellow	White	Yellow	Yellow	White	White
Mannitol fermentation	ND[a]	−	−	−	−	−
Arabinose fermentation	ND	−	−	−	−	−
Growth at 4°C	ND	−	−	−	+	+

[a] ND, Not determined.

TABLE 3. Determination of antisera specificity to seawater microorganisms by immunofluorescence assays

Test strain	Specificity of antisera to:				
	Achromobacter spp.	*C. terrigena*	*P. putrefaciens*	*Pseudomonas* sp. strain I	*V. alginolyticus*
Achromobacter spp.	4+	0	1+	0	1+
Alcaligenes pacificus	0	0	2+	2+	0
Alcaligenes sp. strain I	0	0	0	0	0
Comamonas terrigena	0	4+	1+	2+	0
Cytophaga sp.	0	1+	0	1+	0
Flavobacterium sp.	0	0	0	0	1+
Halobacterium sp.	0	0	0	0	0
Moraxella sp.	0	0	0	0	0
Pseudomonas nautica	0	2+	2+	2+	1+
Pseudomonas putrefaciens	0	0	4+	4+	1+
Pseudomonas sp. strain I	0	2+	4+	4+	0
Pseudomonas sp. strain II	0	0	0	1+	0
Vibrio alginolyticus	0	2+	0	0	4+
Vibrio anguillarum	0	1+	0	0	0

whereas only very few cells from each of the other bacterial species could be found. On the dimethyldichlorosilane-coated (low-surface-energy) glass plates, in contrast, *V. alginolyticus* was not detected and only small numbers of *Achromobacter* sp., *P. putrefaciens*, and *Pseudomonas* sp. strain I were identified. The highest total number of bacteria as determined by acridine orange staining occurred in the medium-energy plates. However, medium-surface-energy plates subjected to increased shear rate of 1,400 ml/min for the previous hour gave significantly lower bacterial counts than other similar plates and which approximated the original numbers on the low-energy plates.

The utility of our immunofluorescent reagents to identify the bacteria in natural microfouling films was examined by using stainless steel test plates fouled by Atlantic Ocean waters (Table 5). Again, each bacterial species could be identified in these films with our antisera. *Achromobacter* spp. could be detected on plates from each segment of the voyage and in proportions up to 28% of the total number of adherent cells. *V. alginolyticus*, also present on each plate, made up ≤20% of the microflora. The other seawater microorganisms were seen far less often. *P. putrefaciens* was found in samples from three of the cruise legs in proportions up to 24% but was not detectable in plates exposed for the full cruise. *Pseudomonas* sp. strain I was found in two of the cruise legs, and *C. terrigena* appeared in samples from the second cruise leg and from the entire voyage. Also, similarly to our findings in the aquarium seawater system, a large number of bacterial cells were found on the medium-surface-energy plate exposed for the entire length of the cruise, whereas significantly fewer cells (ca. one-half) were found on the corresponding low-surface-energy plate.

DISCUSSION

Only gram-negative motile rods, predominantly *Pseudomonas* spp., were cultured from glass plates immersed in the synthetic seawater system for 30 days. Biochemically, these microorganisms closely resembled those found on naturally fouled surfaces. Short rod-shaped bacilli, especially *Pseudomonas* spp., are the first microorganisms to colonize solid surfaces exposed to natural seawater, followed by *Achromobacter* spp., *Flavobacterium* spp., and *Vibrio* spp., (9, 10, 15, 22, 23, 47). Since these same types of microorganisms are recovered from microfouling films produced in our aquarium seawater system, it appears that this system does mimic the natural ocean environment and is an appropriate model for the study of microfouling.

Several distinctions were noted among the cultivable *Pseudomonas* spp., which included *P. putrefaciens*, *P. nautica*, and two unknown *Pseudomonas* spp. The *Pseudomonas* spp. were differentiated on the basis of biochemical tests, including growth on Mac Conkey agar, citrate utilization, pigmentation, gelatin liquefaction, mannitol and arabinose fermentation, and growth at 4°C (Table 2). Serological findings by indirect immunofluorescence assays indicate that several of these groups may be taxonomically distinct. Some cross-reactions were noted between *P. putrefaciens* and *Pseudomonas* sp. strain I, but no positive immunofluorescent reactions occurred with these same antisera to the other *Pseudomonas* organisms. Accordingly,

TABLE 4. Immunofluorescence identification of microorganisms attached to substrates of different surface energy

Surface energy of test plate	% Bacterial cells (total no. of attached cells) detected by antisera to:					Mean total no. of bacterial cells/10^3 mm^2
	Achromobacter spp.	*C. terrigena*	*P. putrefaciens*	*Pseudomonas* sp. strain I	*V. alginolyticus*	
Low[a]	1 (119)	0 (100)	1 (112)	1 (101)	0 (100)	106.4
Medium[b]	1 (177)	1 (123)	1 (202)	1 (160)	39 (132)	158.8
Medium with terminal flush[c]	0 (108)	0 (114)	0 (117)	1 (106)	1 (130)	113.8

[a] Dimethyldichlorosilane coated glass.
[b] Detergent-washed glass.
[c] Detergent-washed glass exposed to a flow rate of 1,400 ml/min for 1 h.

TABLE 5. Immunofluorescent determination of microfouling organisms in the Atlantic Ocean

Cruise leg[a]	Surface energy of test plate	% of total no. of adherent cells					Total no. of adherent microorganisms/10^3 mm^2
		Achromobacter spp.	*C. terrigena*	*P. putrefaciens*	*Pseudomonas* sp. strain I	*V. alginolyticus*	
1	Low[b]	ND[c]	ND	ND	ND	ND	ND
	Medium[d]	6	0	0	0	13	274
2	Low	1	7	1	0	11	423
	Medium	0	0	15	39	6	538
3	Low	4	0	0	0	4	177
	Medium	15	0	24	0	20	283
4	Low	13	0	0	0	0	239
	Medium	16	0	0	15	4	311
5	Low	28	0	0	0	0	407
	Medium	0	0	3	0	1	493
Full cruise	Low	16	2	0	0	0	433
	Medium	5	2	0	1	1	836

[a] From the March to April 1983 voyage of the U.S.N.S. Lynch.
[b] Dimethyldichlorosilane-coated stainless steel.
[c] ND, Not detected.
[d] Radio frequency-glow-discharge treated stainless steel.

several species of *Pseudomonas* were present in our aquarium system.

Indirect immunofluorescence examination of flow cell test plates exposed to the synthetic seawater revealed each of the five predominant microorganisms originally isolated from the seawater tanks; however, the flow cell test plates showed a microbiota which quantitatively differed considerably from that of the original glass plates. For example, *V. alginolyticus* was the most prominent bacterial species on the flow cell test plates but made up only ca. 10% of the microflora on the static glass plates. The most likely reason for this difference in microbial composition is the length of exposure of each surface. Although initial bacteriological culture was performed after the microfouling film had accumulated for 30 days, test plates were examined after seawater had been pumped through the flow cells for only 6 days. Additional exposure of the test plates might have resulted in a microfouling film with a proportion of bacterial species similar to that on the glass plates, and such studies are obviously facilitated by the immunofluorescent reagents now available. Alternatively, the difference in proportion of attached bacterial species may reflect variations in the relative strength of bacterial attachment. Seawater microorganisms were cultured from glass plates which were static in the seawater tanks, whereas microfouling films on test plates were formed as seawater was pumped through the flow cells. The predominant bacteria on the static plates, with the exception of *V. alginolyticus*, may not be able to adhere strongly enough to resist the water flow. The large number of *V. alginolyticus* on the medium-surface-energy test plate suggests that it can tenaciously attach to test plates; however, even this organism was not able to resist high-shear terminal flushing. The vast majority of microorganisms on the flow cell test plates could not be identified with the present battery of immunological reagents and may represent species which were absent or present in only low numbers on the static glass plates. These organisms presumably include species with particularly high attachment strength to the test surface.

The observation that a smaller number of total microorganisms attached to low-surface-energy plates agrees with the previous findings of biofouling occurring to a lesser degree on these surfaces (1, 4, 14). The surface energy, charge, and texture dictates the quality of the initially adsorbed glycoprotein layer which in turn affects the degree and nature of the subsequent microfouling layer (14). An implication of this finding is that microfouling may be reduced by alterations in surface energy (18, 19).

Even though the antisera used in these studies, with the exception of *Pseudomonas* antisera, appeared to be species specific, bacteria may be present in microfouling films which possess cross-reacting antigens and which may give false-positive reactions. In the synthetic seawater system such microorganisms may be of little importance, since the reagents were determined species specific in immunofluorescence assays against all 26 isolates from the static glass plates and since only small numbers of fluorescing cells were detected in flow cell experiments. In natural microfouling films, however, the potentially greater complexity of the seawater microflora and the observed higher proportions of fluorescing cells point to the greater likelihood of cross-reacting species. For *Pseudomonas* spp., the cross-reactions seen between *P. putrefaciens* and *Pseudomonas* sp. strain I hampers the use of immunofluorescence to distinguish between these two species. However, since the cross-reacting antigenic determinants may play a role in bacterial adherence, the polyclonal antisera directed toward these components may be useful in studying adherence mechanisms.

The immunofluorescent reagents were also capable of detecting microorganisms in natural microfouling films formed from the waters of the Atlantic Ocean. As found on the synthetic seawater test plates, the bacterial composition of the natural microfouling films varied considerably. *Achromobacter* spp. and *V. alginolyticus* were found in microfouling films formed in all parts of the Atlantic Ocean sampled, whereas the other study bacteria were found in certain areas. For example, *P. putrefaciens* appeared in microfouling films formed only during the second and third legs of the cruise. These initial studies suggest that the geographical area of formation of microfouling films influences the type and number of attaching bacteria.

In conclusion, we have shown that immunofluorescence techniques are potentially useful for the in situ identification of bacteria in microfouling films. However, in view of the apparent complexity of these microflora, a greater number of specific immunological reagents must be developed to more completely characterize the microbiological constituents of these films.

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