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Improved Microfouling Assay Employing a DNA-Specific Fluorochrome and Polystyrene as Substratum

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With a direct count assay, 10 fouling bacterial isolates have been characterized for their ability to adhere to glass cover slips and polystyrene dishes. Although most adhered in greater numbers to polystyrene, the preference was statistically significant for only seven isolates at the 95% confidence level, due in part to the greater variability in cell attachment to glass (coefficient of variation, 32.3% for glass compared with 10.0% for polystyrene). Employing polystyrene dishes, a novel microfouling assay was developed, based on the extraction and fluorometric determination of DNA. The assay was rapid, enabled the detection of as little as 0.15 μg of DNA per dish (~5,000 cells per mm^2), and showed good agreement with the direct count assay. The DNA method resulted in less variability among three replicates (average coefficient of variation, 7.06%) and allowed for estimation of bacterial density over a larger surface area per sample (1.89×10^3 mm^2) than was feasible with epifluorescence microscopy (0.06 to 0.1 mm^2).

The study of bacterial attachment to surfaces has implication in many diverse fields, including microbial ecology (16, 21), oral hygiene and dental pathology (29), enteropathology (23), urology (14), energy conversion engineering (1, 24), hydrodynamics (26), and materials deterioration research (15). Methods to enumerate bacteria attached to surfaces have been equally diverse. Counting by use of phase-contrast microscopy (19), epifluorescence microscopy (15, 31), or scanning electron microscopy (5, 8) yields additional information (distribution and morphology) of the attached population. However, these techniques are usually tedious, and scanning electron microscopy has been criticized additionally for artifacts induced during desiccation. Enumeration by plate counts (1, 7) is hampered by the selectivity of the medium employed and the subsequent underestimation of the microbial population. Optical density measurements of nonspecifically stained, attached microbial populations (10, 33) require a transparent subtratum and measure attached nonbacterial particulates (silts and clay) and stained extracellular material as well as attached bacteria.

Light section microscopy (25) and ultrasonic interferometry (27) have been employed to determine the thickness and acoustic attenuation, respectively, of mixed microbial slimes. Directed at measuring the physical properties of microfouling communities, these techniques do not yield information on the composition or total numbers of microorganisms present.

Radioisotopically labeled bacteria have been employed to study bacterial absorption to hydrophobic chromatographic supports (6) and bacterial adherence to rat bladders (14). These techniques have been found to be quite sensitive.

A variety of cellular constituents have been measured to determine the biomass of natural bacterial populations attached to surfaces, including lipids and lipid phosphate (30), ATP, protein, carbohydrate, total organic carbon and nitrogen (1), and lipopolysaccharide (9). The lipopolysaccharide technique is preferred, varying less than the others in sensitivity, specificity, and proportionality to cell number or biomass. The measurement of lipids and fatty acids can yield additional information on the composition and structure of the microfouling community (30).

Our research efforts called for the development of a rapid, sensitive, and inexpensive technique for the enumeration of bacterial isolates adhered to surfaces. In this paper we describe a microfouling assay based on the extraction and fluorometric determination of DNA. We also describe the preferences for attachment to glass (a hydrophilic surface) or polystyrene (a hydrophobic surface) of several estuarine and marine bacterial isolates.

MATERIALS AND METHODS

Isolation of fouling bacteria. Fouling bacteria were collected by a 2-h exposure of sterile 24- by 24-mm glass cover slips, polyvinyl chloride cover slips (Fisher

Scientific Co., Pittsburgh, Pa.), or Teflon squares at a depth of 1 m off a dock in the Chesapeake Bay at Chesapeake Beach, Md. These substrata were rinsed with sterile marine medium 2216 (Difco Laboratories, Detroit, Mich.) diluted 1:3 with distilled water (termed 2216/3) and either incubated in 2216/3 broth or placed on the surface of 2216/3 agar and incubated for 48 h at 15°C.

Taxonomic identification of the isolates has not been performed, with the exception of isolates 8 and 9, which have been tentatively identified as *Vibrio proteolytica* and *Alteromonas citrea* by the American Type Culture Collection. The *V. proteolytica* resembles *Aeromonas hydrophila* susp. *proteolytica*, but differs in the ability to utilize histidine and arginine as carbon sources and lacks lysine decarboxylase activity (4). Organism S was isolated from surfaces incubated in an aquarium containing Instant Ocean (Aquarium Systems Inc., Wycliffe, Ohio) and bryozoan larvae, *Bugula neretina*. The marine *Pseudomonas* sp. (National Collection of Marine Bacteria [NCMB] 2021) was a gift of Madilyn Fletcher, Environmental Sciences Department, University of Warwick, Coventry, United Kingdom.

Media and maintenance of cultures. All isolates were maintained on ASWJP (salinity [‰] ~ 10 to 11 ppt) agar medium as previously described (31), except organism S and the *Pseudomonas* sp., which were maintained on triple-strength ASWJP (salinity [‰] ~ 30.5 ppt).

Attachment assays. Growth of cells for attachment assays and the techniques for attachment assays employing epifluorescence microscopy were performed at 15°C as described previously (31). Glass cover slips for attachment assays were first detergent washed with Alconox, rinsed with tap water, and then soaked in hot nitric acid for 2 h. Cover slips were then rinsed exhaustively with tap water followed by distilled water. Since detergent washing of polystyrene dishes did not improve the results of attachment assays, dishes were used directly as supplied by manufacturer.

For attachment assays employing the fluorometric DNA technique, cells were grown overnight at 15°C on a gyratory shaker (set at 141 rpm) to an optical density corresponding to 280 to 310 on a Klett-Summerson colorimeter. Cells were harvested and washed twice with the appropriate mineral salts medium (either ASWJP for isolates 1 through 9 or triple-strength ASWJP for organism S and the *Pseudomonas* sp; all wash media lacked peptone and yeast extract) and suspended to the original growth volume in mineral salt medium. Cell concentration was determined by direct counts (31). Samples of this culture were added to sterile 60- to 15-mm hydrophobic polystyrene dishes (Falcon Plastics, Oxnard, Calif.) that contained the appropriate volumes of the mineral salt medium to obtain cell concentrations of 1.35×10^5 to 7.4×10^8 in a final volume of 8 ml. Dishes were covered and incubated statically at 15°C for 10 min to 4 h. At the termination of the incubation (attachment) period, dishes were washed by dipping four times in three baths of the mineral salts medium to remove cells. The vertical side walls of the dishes were wiped with a cotton-tipped probe to remove bacteria attached to the side walls. Four microliters of 5% (vol/vol) Triton X-100 was added to each dish, and the dish was placed on a bed of crushed ice. Four milliliters of ice-cold SSC (0.154 M NaCl, 0.015 M trisodium citrate, pH 7.0) was added to each dish, and the attached cells were broken by sonication for 45 s at 100 W with a Biosonik III sonicator (Bronwill Scientific Inc., Rochester, N.Y.). It was important to rotate the dish during the sonication period so that all cells were fully exposed to the sonic energy. We have previously shown that sonication for this length of time and at this energy is sufficient to break the organisms employed in this study and natural populations of aquatic bacteria (32). However, longer periods of sonication may be necessary to break more resistant organisms (i.e., gram-positive organisms and spores).

Two milliliters of the extract liquid was added to 1 ml of either 1.5×10^{-6} M (for 0.5 to 10 μg of DNA) or 1.5×10^{-7} M (for 50 to 1,500 ng of DNA) Hoechst 33258 (Calbiochem-Behring Corp., La Jolla, Calif.) in SSC. DNA was determined fluorometrically with calf thymus DNA standards as previously described (32).

RESULTS

All organisms isolated from surfaces exposed for 2 h to Chesapeake Bay water were short, flagellated rods (organisms 1 through 9), whereas organism S was a small, coccoid form. Table 1 shows the attachment of these isolates to glass or polystyrene. Of the isolates examined, 9 of 10 adhered in numerically greater amounts to polystyrene, although the preference was significant for only 7 ($\alpha = 0.05$; 36). There was a greater variability between replicates among glass samples, resulting in a significantly greater average coefficient of variation (32.3%) compared with polystyrene (coefficient of variation, 10%; $0.001 \leq P \leq 0.002$; $n = 20$). There was also greater variability in the number of cells attached from field to field when observed under epifluorescence microscopy for glass compared with polystyrene.

Figures 1 and 2 show the results of attachment assays on polystyrene performed by direct counts or by the fluorometric determination of DNA. In these experiments three replicates per treatment (either cell concentration or sampling time) were processed for direct counts and DNA content. For direct counts, 10 to 20 fields were counted (minimum 400 cells) representing a total area of 0.06 to 0.1 mm^2 per dish. For DNA determinations, the DNA content was determined for bacteria covering the whole dish, an area of 1.89×10^3 mm^2.

Figure 1A shows bacterial attachment as a function of supernatant bacterial concentration at high bacterial concentrations. There was a linear increase in numbers of cells attached with increasing cell concentration until the surface was saturated (confluent layer). Cells were in contact with each other, and many were attached by the bacterial pole at the highest cell concentrations. The relationship between cell concentration and the number of cells attached was linear over at least 2 orders of magnitude, as

TABLE 1. Attachment of bacterial strains to glass or polystyrene[a]

Organism	Substratum[b]	Cell density[c] (10^5 cells per mm^2 ± SD)	Coefficient of variation (%)	Significance
1	G	2.46 ± 0.32	13.0	
	P	2.91 ± 0.18	6.2	+
3	G	0.025 ± 0.006	24.0	
	P	2.5 ± 0.2	8.0	+
4	G	0.028 ± 0.005	17.9	
	P	0.045 ± 0.005	11.1	+
5	G	1.52 ± 0.58	38.2	
	P	3.05 ± 0.078	2.6	+
6	G	0.82 ± 0.27	33.0	
	P	0.69 ± 0.25	36.2	
7	G	0.037 ± 0.024	64.9	
	P	0.081 ± 0.015	18.5	
8 (*V. proteolytica*)	G	2.14 ± 0.55	25.7	
	P	3.82 ± 0.086	2.3	+
9 (*A. citrea*)	G	1.80 ± 0.52	28.9	
	P	2.52 ± 0.265	10.5	+
Organism S	G	1.35 ± 0.49	36.3	
	P	2.14 ± 0.078	36.4	
Pseudomonas sp. strain NCMB 2021	G	0.033 ± 0.014	42.4	
	P	0.178 ± 0.002	1.12	+
	PLP	0.026 ± 0.008	30.8	

[a] Cells were grown to an initial cell density corresponding to an optical density of 260 on a Klett-Summerson colorimeter. Cells were washed twice and resuspended in the same volume used for growth. Four milliliters of culture medium was added to 4 ml of mineral salt medium in a polystyrene dish with or without a 24- by 40-mm cover glass and incubated for 4 h at 15°C.

[b] Substrata were glass coverslips (G), polystyrene dishes (P), or plasma-cleaned polystyrene dishes (PLP).

[c] Means of three replicate surfaces.

seen at low bacterial concentrations in Fig. 1B. The sensitivity of this technique was determined to be 150 ng of DNA per dish, which corresponds to less than 5,000 cells per mm^2.

The attachment of *Vibrio proteolytica* as a function of time appears in Fig. 2. Again there was relatively good agreement between direct counts and DNA. Curiously, neither curve could be extrapolated through the origin. Direct counts of the early samples (10 to 15 min) were obtained with difficulty due to the patchiness of cells in these samples.

Figure 3 shows the relationship between cells per unit area and DNA per unit area for 44 determinations (each is the mean of three replicates) from 10 experiments (different lots of cells). The correlation coefficient of the linear regression, r (36), is 0.99, and the slope is 13.74×10^{-15} g of DNA per cell, with a coefficient of variation, Sb/b (36), of 12.6%. The scatter within the plot is typical for cellular DNA determinations for different lots of cells (22).

The average coefficient of variation for all data sets (44 determinations, three replicates each) for the direct count technique on polystyrene was significantly greater ($0.01 \leq P \leq 0.02$, t test [36]) than for DNA determinations (10.2% compared with 7.06%).

DISCUSSION

A simplified microfouling assay has been developed that is based on the fluorometric determination of DNA in attached bacterial cells. Results obtained with this technique agree well with those obtained by direct count employing epifluorescence microscopy. The advantages of the DNA technique include ease in handling large numbers of samples (30 samples can be conveniently assayed in less than 2 h), better replication (smaller coefficients of variation), and less investigator bias (for example, one technician consistently counted 10 to 20% more cells than another in any one microscopic field). The specificity and quantitative nature of the interaction of Hoechst 33258 with DNA has been described elsewhere (32).

It should be emphasized that direct counting techniques based on epifluorescence microscopy are estimates of bacterial number and not biomass. DNA content of bacterial populations had been shown to correlate well with bacterial cell number, whereas the relationship between DNA content and bacterial biomass is less well defined. Torsvik and Gokskoyr (35) measured DNA by two fluorometric techniques in soil fractionated to remove organisms other than

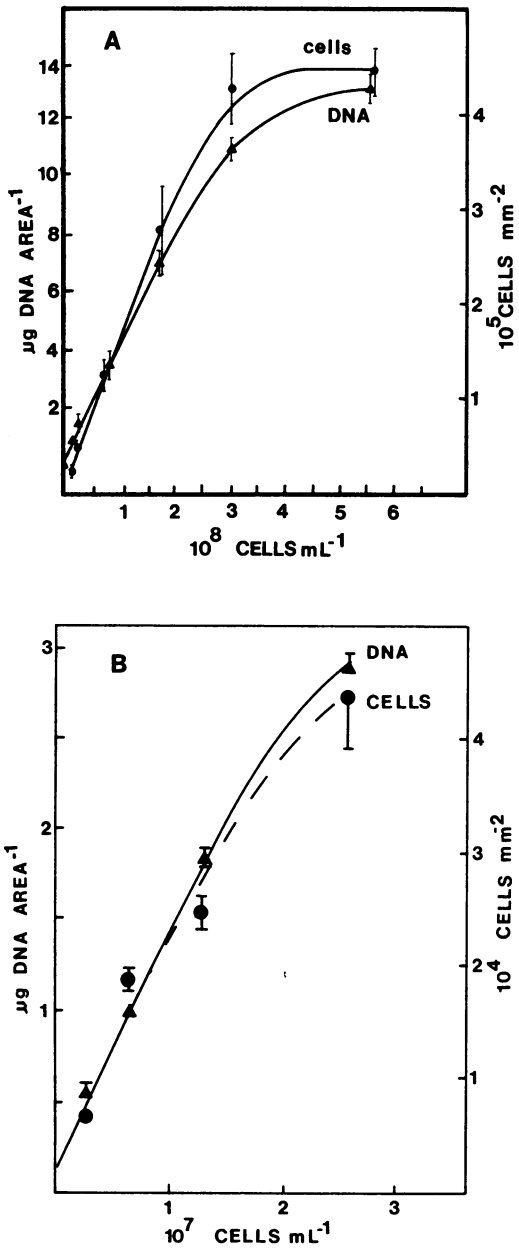

FIG. 1. Attachment of *V. proteolytica* to polystyrene at high cell concentrations (A) or low cell concentrations (B). Attached cell density was determined by direct counts (●) or estimated by total DNA content (▲) of cells attached to the dish surface ($1.89 \times 10^3/mm^2$) in Hoechst 33258 at a final concentration of 5×10^{-7} M (A) or 5×10^{-8} M (B).

bacteria and found a good correlation between DNA content and bacterial cell number. We have previously found good correlations between DNA content and cell number for bacterial cultures and natural microbial populations (32; J. H. Paul and D. J. Carlson, submitted for publication). This may be due to the relatively constant size of bacterial genomes (2, 20).

Holm-Hansen (17) found a correlation between cell size and DNA content in various eukaryotic microalgae. Cellular DNA content was correlated to organic carbon content since algal DNA content varies among different-sized species by several orders of magnitude (17). Breter et al. (3) also proposed a relationship between biomass and DNA content. However, in studies of natural phytoplankton populations, Holm-Hansen (18) found DNA to be a poor estimate of biomass or particulate organic carbon.

The general preference of marine fouling bacterial isolates for hydrophobic surfaces (found in this study) has also been found by others (12, 13). Cell surface hydrophobicity has been implicated as an important factor in the interaction of bacteria with various interfaces (6, 28, 34). The greater variability observed in the numbers of cells attached to glass may be due to inhomogeneities and inconsistencies in the glass surface or the inability to truly clean glass, a reactive substance. Polystyrene is presently employed in our studies on the physiology of bacterial attachment due to the greater reproducibility.

Interestingly, this preference for hydrophobic surfaces may not occur in natural populations of marine bacteria. For example, Dexter et al. (9) found that the number of bacteria attached to glass in Woods Hole Harbor exceeded that attached to polystyrene (and other hydrophobic, low-energy surfaces) by 1 to 3 orders of magni-

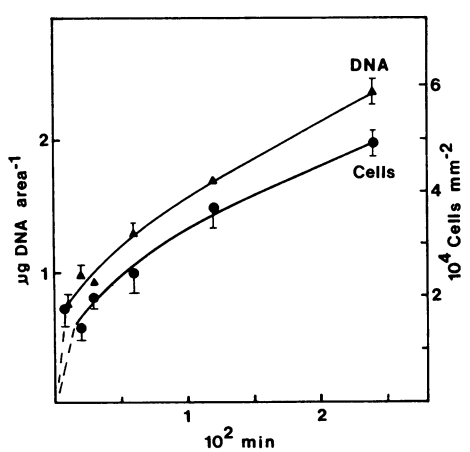

FIG. 2. Attachment of *V. proteolytica* to polystyrene as a function of time. Symbols are as in Fig. 1. The initial cell concentration was 2.43×10^7 cells per ml. The dashed line indicates extrapolation through the origin.

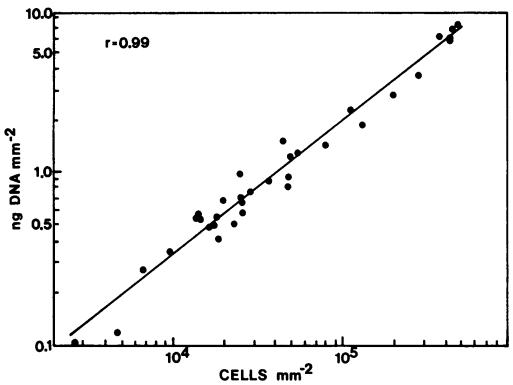

FIG. 3. Relationship between DNA content per unit area and number of attached cells for *V. proteolytica*. Each point is the mean of three determinations.

tude. The reasons for the discrepancies observed between bacterial isolates and natural populations of bacteria remain unclear.

Curves for the rate of bacterial attachment for *Vibrio protolytica* strongly resemble those obtained by Fletcher (11) for *Pseudomonas* sp. NCMB 2021 in that extrapolation of the curve does not result in intersection with the origin. This suggests that the rate of attachment is biphasic, with a very rapid rate of attachment occurring in the first 10 to 30 min, followed by a slower, more linear rate after 30 min. The meaning of these biphiphasic kinetics is not clear, but may suggest the involvement of two temporarily separate processes.

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