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Stimulation of Bacterial DNA Synthesis by Algal Exudates in Attached Algal-Bacterial Consortia

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Algal-bacterial consortia attached to polystyrene surfaces were prepared in the laboratory by using the marine diatom *Amphora coffeaeformis* and the marine bacterium *Vibrio proteolytica* (the approved name of this bacterium is *Vibrio proteolyticus* [W. E. C. Moore, E. P. Cato, and L. V. H. Moore, Int. J. Syst. Bacteriol. 35:382–407, 1985]). The organisms were attached to the surfaces at cell densities of approximately 5×10^4 cells cm^{-2} (diatoms) and 5×10^6 cells cm^{-2} (bacteria). The algal-bacterial consortia consistently exhibited higher rates of [^{3}H]thymidine incorporation than did biofilms composed solely of bacteria. The rates of [^{3}H]thymidine incorporation by the algal-bacterial consortia were fourfold greater than the rates of incorporation by monobacterial biofilms 16 h after biofilm formation and were 16-fold greater 70 h after biofilm formation. Extracellular material released from the attached *Amphora* cells supported rates of bacterial activity (0.8×10^{-21} to 17.9×10^{-21} mol of [^{3}H]thymidine incorporated $cell^{-1}$ h^{-1}) and growth (doubling time, 29.5 to 1.4 days) comparable to values reported for a wide variety of marine and freshwater ecosystems. In the presence of sessile diatom populations, DNA synthesis by attached *V. proteolytica* cells was light dependent and increased with increasing algal abundance. The metabolic activity of diatoms thus appears to be the rate-limiting process in biofilm development on illuminated surfaces under conditions of low bulk-water dissolved organic carbon.

In many aquatic ecosystems, particularly when the bulk-water phase is low in nutrients, surfaces colonized by microorganisms are sites of relatively intense biological activity. Attached microbial communities occur in association with suspended particles (17, 42), as epiphytic growths on macrophytes (2), and as epilithic mats on rock surfaces (23). Stream ecosystems often contain sessile microalgal populations which are a marjor source of in situ primary production (31, 49) and attached bacterial communities which are present in larger numbers and greater biomass than are planktonic bacteria (14, 23). Consortia composed of attached microalgal and bacterial populations have essential roles in aquatic ecosystems, acting as sites of active nutrient regeneration (30, 43) and serving as a trophic resource for other organisms (8, 16, 26). Attached biofilm communities are also predominant in industrial aquatic systems in which microbial fouling is associated with corrosion and reduces the efficiency of industrial processes (9, 13).

Despite the recognized importance of attached microbial communities in nature and in industry, relatively little information is available concerning the interactions of the organisms forming these communities. Syntrophic interactions between aquatic microbial autotrophs (algae) and heterotrophs (bacteria) are known to occur within planktonic communities but have not been intensively studied in attached algal-bacterial consortia. Algal-bacterial syntrophy is mediated by the release of photosynthetically derived dissolved organic carbon (DOC) compounds from algae, which are used by bacteria as substrates for growth (7, 10, 15, 51, 52).

It has been estimated for some coastal and offshore marine ecosystems that between 10 and 50% of the carbon fixed by photosynthesis is excreted by phytoplankton as DOC and that this material is either mineralized by bacterioplankton (20) or enters the food web as bacterial biomass (3, 18, 42). Algal-bacterial interactions are likely to play an even more important role within attached microbial communities, in which the close spatial and temporal coupling between organisms would facilitate the bacterial utilization of excreted DOC. For example, the bacterium *Legionella pneumophila* (the etiological agent of Legionnaires disease) has been isolated from an algal-bacterial mat community and can utilize as its sole carbon source extracellular products released by blue-green algae (cyanobacteria) (48). The availability of algal extracellular products as a carbon source for *L. pneumophila* is at least partially responsible for the wide distribution of this organism in the environment (48).

Similarly, a close physical association is known to exist between populations of attached algae and bacteria within periphytic (2, 27, 46) and epilithic (23, 24) microbial communities. Functional relationships between attached algae and bacteria are also known to occur. The degree of bacterial colonization of stream surfaces is related to the presence of attached microalgae (45), and strong correlations between rates of epilithic algal primary production and rates of attached bacterial activity have been observed (25, 41).

Direct studies of the interactions within attached algal-bacterial consortia have been hampered by the need to remove organisms from surfaces before making physiological measurements, and it is not known to what extent this physical disruption of the community alters the functional interdependence of the microorganisms. We report here results obtained with a model system developed for studying algal-bacterial interactions on surfaces. The results demonstrate that sessile algal populations can act as a sole carbon source for attached bacteria and support rates of per-cell bacterial activity of the same order of magnitude as rates reported for a wide variety of planktonic ecosystems.

MATERIALS AND METHODS

Growth and attachment of cells. The diatom *Amphora coffeaeformis* was grown axenically at room temperature

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under continuous light (97 microeinsteins m^{-2} s^{-1}) in a defined artificial marine medium (modified ASP-2 [12]). Cells were grown in suspension to a density of between 1.5×10^5 and 3×10^5 cells ml^{-1}. The *Amphora* cells (7 ml; approximately 1×10^6 to 2×10^6 total cells) were added to sterile polystyrene petri dishes (15 by 60 mm; Becton Dickinson Labware, Oxnard, Calif.) containing either 3 ml of ASP-2 medium or 3 ml of bacterial suspension. The calcium concentration of the medium was increased to 2.5 mM (11), and the cells were allowed to attach for 4 h. After the attachment period, unattached cells were decanted from the petri dish and attached cells were washed twice with 10 ml of ASP-2 medium supplemented with 2.5 mM calcium. The resultant biofilm was covered with 10 ml of ASP-2 medium containing 2.5 mM calcium and incubated at room temperature under constant illumination for the specified time intervals.

The fouling bacterium *Vibrio proteolytica* (the approved name of this bacterium is *Vibrio proteolyticus* [31a]) was isolated from the Chesapeake Bay (40) and was the generous gift of J. H. Paul. Stock cultures were maintained at room temperature on artificial seawater agar containing 0.17% peptone and 0.033% yeast extract (37). For attachment, 100-ml batch cultures of cells were grown at room temperature for 16 h (optical density at 660 nm of 0.2; 2-cm path length) in ASP-2 medium enriched with 0.17% peptone and 0.033% yeast extract. The cells were harvested by centrifugation at $10,000 \times g$, and the exogenous carbon source was removed by washing twice with ASP-2 medium lacking peptone and yeast extract. Cell pellets were suspended in 100 ml of ASP-2 medium, and 3-ml portions of the cell suspension were added to polystyrene petri dishes containing either 7 ml of ASP-2 medium or 7 ml of algal culture. The calcium concentration of the incubation mixture was increased to 2.5 mM, and the cells were attached and washed as described above for *A. coffeaeformis*.

Thymidine incorporation. The incorporation of [^{3}H]thymidine into cold trichloroacetic acid (TCA)-insoluble material (primarily DNA; 21, 22, 28) was used as an index of attached bacterial activity. Live ($n = 6$) or Formalin-killed ($n = 2$ to 4) biofilms were incubated with 20 nM [*methyl*-^{3}H]thymidine (40 to 60 Ci $mmol^{-1}$; New England Nuclear Corp., Boston, Mass., or ICN Pharmaceuticals Inc., Irvine, Calif.) for 1 h. Incubations of live cells were terminated by the addition of Formalin to a final concentration of 5%. Biofilms were removed from petri dishes by scraping the dishes with a rubber policeman, and cells were extracted in ice-cold 5% TCA. After extraction, samples were passed through filters (diameter, 25 mm; pore size, 0.2 μm; Gelman Sciences, Inc., Ann Arbor, Mich.) and washed twice with 5% ice-cold TCA. The filters were transferred to glass scintillation vials, covered with 0.5 ml of Protosol (New England Nuclear), and incubated overnight at room temperature. The Protosol was then neutralized with 25 μl of glacial acetic acid, and 15 ml of ScintiVerse II (Fisher Scientific Co., Pittsburgh, Pa.) counting medium was added to the vials. The samples were assayed for radioactivity in a Tri-Carb 460 CD liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). Quench corrections were made by using the sample channels ratio method.

Enumeration of attached algae and bacteria. Attached algae and bacteria were stained with the DNA-specific fluorochrome Hoechst 33258 (Calbiochem-Behring, La Jolla, Calif.), as described by Paul (37), and counted with a Nikon Optiphot epifluorescence microscope. Algal cells were counted under low power (40× glycerol objective) by using a combination of phase and epifluorescent illumination. Bacterial cells were counted under high power (100× glycerol objective) with epifluorescent illumination. At least 10 randomly selected fields were counted on each dish.

Autoradiography. Biofilms were attached to plastic cover slips (22 by 22 mm; Fisher Scientific) which were glued (Silastic adhesive; Dow Corning Corp., Midland, Mich.) to glass microscope slides. The slides and cover slips were sterilized by exposure to a 0.5% solution of sodium hypochlorite for 1 h and washed in10 rinses of sterile distilled water. The sterile slides with attached cover slips were placed in sterile polystyrene petri dishes (100 by 15 mm), and either 21 ml of *Amphora* culture (2×10^5 cells ml^{-1}) or 9 ml of *V. proteolytica* culture (optical density at 660 nm of 0.2; 2-cm path length) or both were added to each dish. When necessary, the total volume of medium in each dish was increased to 30 ml by the addition of ASP-2 medium. Cells were attached as described above, and unattached cells were removed by dipping the slides in two washes of sterile ASP-2 medium. Biofilms were incubated under constant illumination (97 microeinsteins m^{-2} s^{-1}) for 20 h, after which [^{3}H]thymidine was added to each dish (final concentration, 20 nM) and the dishes were incubated for an additional 1 h. The incubations were terminated by the addition of Formalin (final concentration, 5%), and the cells were stained with Hoechst 33258 for 1 h. The slides were then washed in 10 rinses of distilled water to remove unincorporated [^{3}H]thymidine and inorganic salts present in the medium.

Autoradiography was performed by the general procedure of Brock and Brock (6). Slides were dipped in warm (45°C) NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.) which was diluted 1:2.5 with distilled water. The coated slides were dried in a vertical position, transferred to black slide boxes, and kept at room temperature in total darkness for 23 days. The autoradiograms were developed (Kodak D-19 developer), fixed (Kodak fixer), rinsed for 0.5 h in tap water, and air dried. The developed autoradiograms were viewed with a Nikon epifluorescence microscope by using a combination of phase and epifluorescent illumination.

RESULTS AND DISCUSSION

Algal-bacterial consortia. Biofilm communities composed of autotrophic microalgae and heterotrophic bacteria can be easily prepared in the laboratory. Algal and bacterial cells grown separately in axenic suspension cultures were attached to polystyrene surfaces to form monoalgal, monobacterial, or mixed (algal and bacterial) biofilm communities. In this manner, living, physiologically functioning biofilm communities of known age in which algal exudates serve as the sole carbon source for bacterial growth can be prepared in the laboratory. Diatoms and bacteria can be combined in known proportions and form functional algal-bacterial consortia within a few hours of attachment.

Our model system was developed by using marine organisms which were isolated from geographically separate areas (South Florida and the Chesapeake Bay); we do not know if they occur together in nature. The organisms were chosen based on availability and previous work regarding their physiology and mechanisms of attachment (11, 12, 38–40). However, the technique which we developed should be applicable to other marine and freshwater algal and bacterial populations.

Algal-bacterial syntrophy. The relationship between algal growth and bacterial DNA synthesis in attached consortia was investigated by measuring algal abundance and rates of [^{3}H]thymidine incorporation into bacterial DNA. The incor-

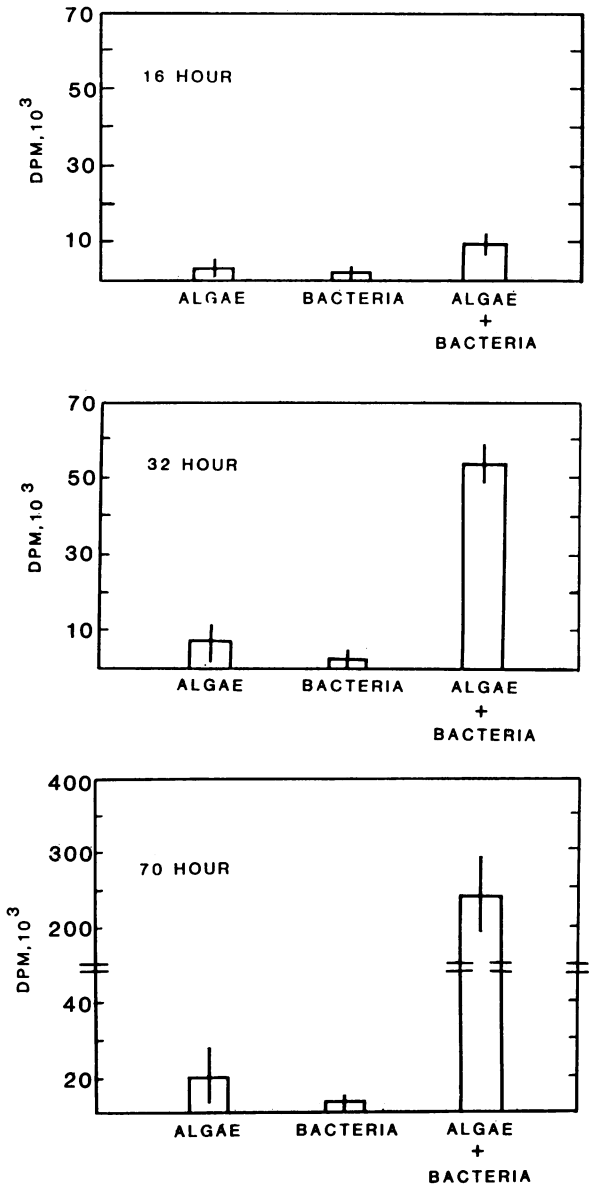

FIG. 1. Incorporation of [^{3}H]thymidine by monoalgal, monobacterial, and mixed (algal and bacterial) biofilm communities 16, 32, and 70 h after biofilm formation. Bars represent ±1 standard deviation.

poration of [^{3}H]thymidine into cold TCA-insoluble material is a good index of the rate of DNA synthesis in *V. proteolytica* (28). [^{3}H]thymidine is a specific label for DNA, and the degree of labeling does not vary with the growth state or nutrient condition of the population. None of the tritium label is incorporated into hot TCA-insoluble material (protein) by *V. proteolytica* at [^{3}H]thymidine concentrations between 5 nM and 1 μM, either in the presence or absence of nutrient enrichment (28).

We assayed the potential influence of isotope dilution on thymidine incorporation by determining the concentration of radiolabeled thymidine required to maximally label cold TCA-insoluble material. This approach has been widely used in studies of bacterial secondary production to estimate the concentration of [^{3}H]thymidine required to saturate extra- and intracellular thymidine pools (5, 21, 35, 44). For attached *V. proteolytica*, the addition of 20 nM [^{3}H]thymidine was sufficient to produce maximal labeling of cold TCA-insoluble material and thus overcome any potential isotope dilution.

The rates of [^{3}H]thymidine incorporation into cold TCA-insoluble material by mixed biofilm communities were always greater than the rate of incorporation by monobacterial biofilms (Fig. 1). Biofilms composed solely of *A. cofeaeformis* or *V. proteolytica* (which were not provided with any external carbon source) incorporated [^{3}H]thymidine poorly, if at all. The mean rates of [^{3}H]thymidine incorporation shown in Fig. 1 for monoalgal biofilms are not statistically different from the mean value for the Formalin-killed controls ($P > 0.05$; t test for grouped data).

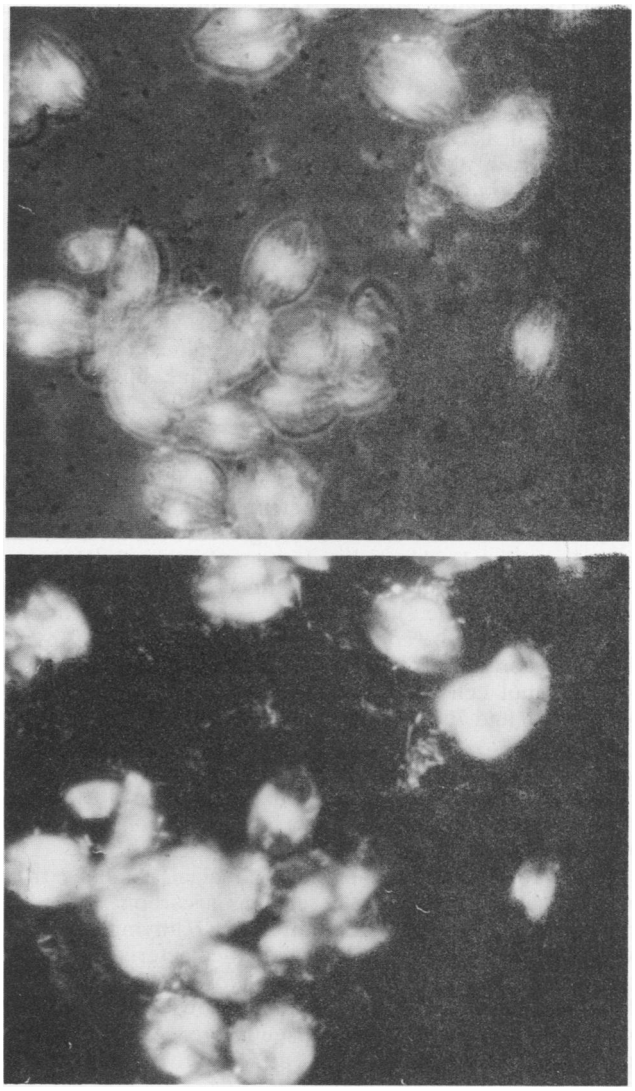

FIG. 2. Autoradiograms of attached algal-bacterial consortia photographed with a combination of phase and epifluorescent illumination. The top photo shows developed silver grains (dark shadows) in association with Hoechst 33258-stained bacteria (small white areas). Note the absence of silver grains associated with Hoechst 33258-stained algal nuclei (larger white areas inside algal cells). The bottom photo shows the same microscope field with less phase illumination to better illustrate the location of stained bacteria and algal nuclei. Magnification, ×1,000.

TABLE 1. Incubation conditions and rates of bacterial growth in attached algal-bacterial consortia

Time elapsed since formation of biofilm (h)	Incubation condition[a]	Algal abundance (10^6 cells dish^{-1})	Thymidine incorporation (10^{-21} mol cell^{-1} h^{-1})	Bacterial doubling times (days)	Bacterial production		Algal DOC release (fg of carbon released algal cell^{-1} h^{-1})[b]
					10^6 cells produced dish h^{-1}	10^{-8} g of carbon produced dish^{-1} h^{-1}	
16	Light	1.0	0.83	29.5	0.11	0.20	4.1
32	Light	2.4	4.16	5.9	0.63	1.12	9.3
32	Dark	0.7	0.31	79.1	0.05	0.08	2.4
70	Light	7.4	17.98	1.4	2.64	4.69	12.7

[a] Light intensity was 97 microeinsteins m^{-2} s^{-1}.
[b] Assuming that 50% of the DOC utilized by the bacteria was respired.

A. coffeaeformis grown alone or in conjunction with *V. proteolytica* also did not incorporate [^{3}H]thymidine in autoradiographic studies. Hoechst 33258-stained algal nuclei and bacteria were clearly visible under epifluorescent and phase illumination. Developed silver grains were not found in association with algal nuclei in either monoalgal or mixed biofilms (Fig. 2); therefore, [^{3}H]thymidine was not incorporated into algal DNA. Grain densities associated with bacteria grown in algal-bacterial consortia were greater than background grain densities in biofilms composed solely of *A. coffeaeformis* or *V. proteolytica*, which did not have access to a carbon source. Clusters of silver grains, which are indicative of [^{3}H]thymidine uptake by *V. proteolytica*, were often observed when the bacteria were grown in conjunction with *A. coffeaeformis* (Fig. 2). Therefore, we conclude that *A. coffeaeformis* did not incorporate significant amounts of [^{3}H]thymidine and that the [^{3}H]thymidine uptake which occurred in algal-bacterial consortia was due to bacterial activity only.

The rates of [^{3}H]thymidine incorporation by the algal-bacterial consortia were fourfold greater than the rates of incorporation by the monobacterial biofilms 16 h after biofilm formation and were 16-fold greater 70 h after biofilm formation (Fig. 1). Algal abundance and rates of [^{3}H]thymidine incorporation bacterial cell^{-1} increased with the age of the consortia (Table 1). When *V. proteolytica* was grown in conjunction with *A. coffeaeformis*, the rates of [^{3}H]thymidine incorporation *V. proteolytica* cell^{-1} increased from 0.83 $\times$ 10^{-21} mol cell^{-1} h^{-1} to 17.98 $\times$ 10^{-21} mol cell^{-1} h^{-1} between 16 and 70 h after biofilm formation (Table 1). DNA synthesis by *V. proteolytica* was light dependent, since consortia incubated in the dark for 32 h and then assayed for [^{3}H]thymidine incorporation exhibited rates of DNA synthesis 10 times lower than those exhibited by consortia incubated under constant illumination (Table 1).

These results clearly suggest that algal exudates from sessile *A. coffeaeformis* can serve as a carbon source for attached *V. proteolytica*. The rates of [^{3}H]thymidine incorporation *V. proteolytica* cell^{-1} when the bacterial cells were grown in conjunction with *A. coffeaeformis* were similar to the rates of activity reported for freshwater and marine bacterioplankton (for a review, see reference 47) as well as for natural bacterial populations attached to particles (1, 19) and to artificial surfaces (29).

Rates of [^{3}H]thymidine incorporation (moles dish^{-1} hour^{-1}) were converted to rates of bacterial production (grams of carbon dish^{-1} hour^{-1}) by using conversion factors developed for bacteria in nearshore marine ecosystems. The thymidine incorporation rate was multiplied by a factor of 1.7 $\times$ 10^{18} cells produced mol^{-1} of thymidine incorporated (22). The cellular production rates were converted to carbon equivalents by multiplying them by an estimate of the average volume cell^{-1} (0.147 μm^3; 36) and by 1.21 $\times$ 10^{-13} g of carbon μm^{-3} (50). Thymidine conversion factors specific for *V. proteolytica* are being developed (J. Paul, personal communication), and when they become available we will use them to refine our carbon budget. In the absence of specific conversion factors for *V. proteolytica*, the nearshore marine conversion factors should provide a reasonable estimate of bacterial production.

Substantial rates of bacterial secondary production occurred in attached consortia composed of *A. coffeaeformis* and *V. proteolytica* (Table 1). The rates of bacterial production ranged from 0.20 $\times$ 10^{-8} g of carbon produced dish^{-1} h^{-1} 16 h after biofilm formation to 4.69 $\times$ 10^{-8} g of carbon produced dish^{-1} h^{-1} 70 h after biofilm formation and corresponded to bacterial doubling times of 29.5 to 1.4 days. The bacterial doubling times in our model system were well within the range reported for marine (18, 32) and freshwater (4, 34) planktonic systems, as well as for coral reef sediments (33). Extracellular material released from sessile *Amphora* cells was thus capable of supporting rates of activity by attached bacteria (thymidine incorporation cell^{-1} hour^{-1}) and bacterial growth (doubling time) comparable to values reported for a wide variety of aquatic ecosystems. This strongly suggests that our model system is capable of supporting levels of algal-bacterial interactions similar to those which occur in natural systems.

The amount of carbon released per *Amphora* cell was calculated from our estimates of bacterial secondary production, assuming that 50% of the DOC utilized by the bacteria was respired. This calculation was based solely on the observed rates of bacterial production and was not corrected for algal carbon which may have been released but not utilized by the bacteria. The amount of carbon released by *A. coffeaeformis* was light dependent, and when cells were incubated under constant illumination, the amount of carbon released per cell^{-1} hour^{-1} appeared to increase with the age of the consortia (Table 1). This apparent increase in the availability of carbon could have resulted from either an increase in the amount of carbon released per *Amphora* cell^{-1} hour^{-1} or from an increase in the DOC release resulting from a greater number of senescent algal cells in the older consortia. It is clear, however, that release of DOC from algal cells on the order of only a few femtograms of carbon cell^{-1} hour^{-1} can support substantial rates of bacterial secondary production.

The process of bacterial attachment results in a reduction of the amount of cell surface available for nutrient uptake. In *V. proteolytica*, between 15 and 20% of the cell surface is in direct contact with the substratum and is not available for nutrient transport (28). This decrease in surface area is believed to be responsible for the observation that under conditions of nutrient enrichment unattached *V. proteolytica*

cells are more active than are attached cells (28). Under oligotrophic conditions, however, attached cells are more active than unattached cells are, and the loss in effective surface area may be overcome by the availability of surface-absorbed trace nutrients (28).

The colonization of surfaces by algae could provide an enriched microenvironment in which the availability of algal exudates can offset the loss of bacterial cell surface area associated with attachment and support high rates of bacterial growth. Jeffrey and Paul (29) noted that marine fouling communities, composed of diatoms and bacteria, which colonized polystyrene surfaces exhibited higher rates of bacterial activity than did bulk-water planktonic populations. Our results are consistent with this observation and with previous work on stream epilithic communities in which a tight coupling between rates of algal primary production and rates of bacterial activity was noted (25, 41).

The system which we developed can be used as a model to study the role of algal-bacterial syntrophy on naturally occurring and man-made surfaces which do not act as a carbon source for bacterial growth. We suggest that when such surfaces are illuminated and exist under conditions of low bulk-water DOC, metabolic activity by sessile algae controls the rates of secondary production by attached bacteria.

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