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Activity of an Attached and Free-Living *Vibrio* sp. as Measured by Thymidine Incorporation, *p*-Iodonitrotetrazolium Reduction, and ATP/DNA Ratios

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Three independent techniques, [^{3}H]thymidine incorporation, the reduction rate of *p*-iodonitrotetrazolium violet (INT) to INT formazan normalized to DNA, and the ratio of ATP to DNA, were adapted to measure the activity of attached and unattached estuarine bacteria. In experiments employing the estuarine isolate *Vibrio proteolytica*, nutrient concentrations were manipulated by varying the concentration of peptone-yeast extract. In the presence of exogenous nutrients, the activity of free-living cells was greater than that of attached cells as measured by [^{3}H]thymidine incorporation and ATP/DNA ratios. In the absence of peptone-yeast extract, however, the activity of attached cells surpassed that of free-living cells as determined by [^{3}H]thymidine incorporation and INT formazan normalized to DNA. Of the three techniques, [^{3}H]thymidine incorporation was deemed most sensitive for detecting changes in activity resulting from slight differences in nutrient concentration. By this technique, attached cells were much less sensitive to changing nutrient concentrations than were free-living cells. Below a threshold concentration, attached cell activity remained constant, while the activity of unattached cells decreased as a function of decreasing nutrient concentration. The results suggest that loss of cell surface area available for substrate uptake due to attachment may be an important factor in determining the relative activities of attached and free-living cells.

With the development of new techniques for measuring bacterial activity, the theory that inert surfaces stimulate bacterial activity (39) has come into question. Investigations into this question fall into two categories; those in which nonnutritive inorganic substrata are used and those in which naturally occurring particulates are examined. In this paper we deal only with the former. Recent attempts to determine whether attachment stimulates or decreases bacterial activity have produced conflicting results depending on the organism studied, the substratum employed, and the activity parameter measured. Several investigators have reported unattached cells to be more active (9, 11, 12), while others have reported greater activity by attached cells (1, 2, 7, 14; M. Fletcher, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I73, p. 158).

We have adapted three techniques to measure the activity of bacteria both attached to polystyrene and free-living in the bulk liquid phase. The first technique is incorporation of [^{3}H]thymidine into DNA, defined as the cold-trichloroacetic acid (TCA)-precipitable fraction. This technique is commonly used as a measure of bacterial heterotrophic activity in the water column (8, 32) and for cells attached to particulate matter (19, 25) but has yet to be used to measure the activity of cells attached to an artificial substratum.

The use of *p*-iodonitrotetrazolium violet (INT) for measuring dehydrogenase or electron transport activity has recently gained acceptance (36–38). In this procedure, INT is reduced to INT formazan, which forms as dark red, water insoluble, intracellular deposits. Previous studies with marine bacteria have employed direct microscopic examination of the deposits, which allows only a percentage of active bacteria to be determined (1, 38). We adopted the methanol extraction procedure of Trevors et al. (37) for an in vivo assay of attached and free-living bacteria. We measured this per weight of DNA (INT-F/DNA), which allows quantification of the amount of formazan produced per unit of biomass over time.

The third technique measures the ratio of ATP to DNA within cells. For some organisms, ATP per cell has been found to change as growth conditions change (3, 10, 24), becoming highest during log-phase growth. DNA content, however, remains relatively constant under varying conditions (4, 33), and its value as a biomass indicator has been previously shown (27, 30, 31; J. H. Paul, W. H. Jeffrey, and M. DeFlaun, Mar. Biol., in press). Therefore, the higher the ATP/DNA ratio, the greater the activity of the cell.

The purpose of this study was to measure the activity of attached and free-living cells by three independent techniques using an estuarine isolate, *Vibrio proteolytica*, and to determine what effects nutrients may have on this activity.

MATERIALS AND METHODS

Materials. TCA was obtained from Mallinckrodt Inc., St. Louis, Mo. Thymidine, INT (grade 1), INT formazan, ATP (disodium salt from equine muscle), luciferin-luciferase firefly lantern extract (FLE-50), bovine serum albumin, Triton X-100, and arsenate buffer (FF-AS-100) were all obtained from Sigma Chemical Co., St. Louis, Mo. Hoechst 33342 and 33258 were obtained from Calbiochem-Behring, La Jolla, Calif. [*methyl*-^{3}H]thymidine (72 to 80 Ci/mmol) was obtained from ICN Pharmaceuticals Inc., Irvine, Calif. Sterile polystyrene petri dishes (60 by 15 mm) were obtained from Becton Dickinson Labware, Oxnard, Calif.

Organism. The isolation and identification of *V. proteolytica* have been described elsewhere (30).

Growth and attachment of cells. Cultures were maintained in ASWJP-PY (29) supplemented with 1.66 mg of peptone and 0.33 mg of yeast extract (Difco Laboratories, Detroit, Mich.) per ml, hereafter referred to as normal growth media. All experiments employing attached and free-living *V.*

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proteolytica were initiated with a standard adhesion assay (30). A cell solution (8 ml; ~10^8 cells per ml) in ASWJP-PY was added to each of the desired number of petri dishes. The cells were allowed to adhere statically at 15°C for 90 min, after which the supernatant was collected in a sterile flask (i.e., free-living cells). Each dish was then dipped four times in each of three washes of ASWJP-PY to remove any loose cells, and the activity assays were initiated.

Thymidine incorporation. A sterile peptone-yeast extract concentrate was added to each treatment to yield the desired final concentration ranging from 0.001 to twice the normal growth media nutrient concentration. In all treatments, [^{3}H]thymidine was added so that the final concentration was 1 μM and the radioactivity was 0.4 μCi/ml.

Free-living cells (25 ml) were added to a sterile 125-ml flask which contained the desired amount of peptone-yeast extract and thymidine mixture. [^{3}H]thymidine and peptone-yeast extract were added to dishes in sterile ASWJP-PY. All samples were incubated at room temperature on a rotating shaker platform at ~100 rpm.

The macromolecular fractions of free-living cells were precipitated by addition to an equal volume of 10% TCA at 4°C. For attached cells, two dishes were removed from the shaker, and 4.0 μl of 5% (wt/vol) Triton X-100 was added to each. The dishes were sonicated for 45 s at 70 W, and a 2.0-ml subsample from each dish was added to 2.0 ml of cold 10% TCA and placed on ice. A preliminary study found that sonication did not affect the recovery of radioactivity in the TCA-precipitable fraction. After 2 h, each sample was filtered onto either 0.2-μm (pore size) Nuclepore Corp. (Pleasanton, Calif.) or Gelman Sciences, Inc. (Ann Arbor, Mich.) GA-8 filters and washed twice with 2.5 ml of ice-cold 5% TCA. Filters were prepared for liquid scintillation counting (22), and all samples were counted on an Iscocap 300 (Nuclear-Chicago Corp., Des Plaines, Ill.) liquid scintillation counter.

Incorporation of the label into the protein fraction. The percentage of tritium from [^{3}H]thymidine incorporated into the protein fraction was determined by hot-TCA fractionation (8). Chloroform formed due to heating was removed by evaporation in a stream of air. Precipitated protein was collected by filtration as described above.

INT-F/DNA. Cells were allowed to adhere as described above. To each dish, 8 ml of either peptone (1.66 mg/ml)–yeast extract (0.33 mg/ml)-INT (0.2 mg/ml)–ASWJP-PY or INT (0.2 mg/ml)–ASWJP-PY was added. The dishes were incubated at room temperature on a gyratory shaker at ~100 rpm. Replicate samples of each treatment were taken by discarding the solution from the dish and rinsing it through three washes of ASWJP-PY. INT formazan was extracted in 4 ml of methanol for 10 min. At the final sampling time, three dishes of each treatment were analyzed for DNA content.

Unattached cells were incubated in the presence of peptone-yeast extract-INT or INT only at the same final concentrations employed for attached cells. Duplicate 8-ml samples of each treatment were filtered through a Whatman, Inc. (Clifton, N.J.) GF/F filter, and INT formazan was extracted in 4 ml of methanol. Triplicate samples were filtered at the final sampling time for DNA.

Methanol extracts were clarified by filtration through a GF/F filter, and formazan content was determined by measuring the A_{485}. All A_{485} measurements were converted to grams of formazan, and each INT-F/DNA value is expressed as weight per weight.

ATP determinations. ATP was determined by the method of Karl and LaRock (17). After the attachment period, 8 ml of either normal growth media or ASWJP-PY was added to the dishes, which were incubated for 1 h at room temperature on a gyratory shaker at ~100 rpm. The supernatant was discarded, and each dish was rinsed three times in ASWJP-PY. Immediately after washing, 5 ml of ice-cold 0.06 N H_2SO_4 was added, and each dish was sonicated for 15 s at 70 W. An additional 5 ml of acid was added, and ATP was extracted for 45 min at 4°C. Four other dishes were sampled for later DNA analysis.

Unattached cells were divided into two lots. Peptone-yeast extract was added to one lot, and both lots were incubated for 1 h with attached cells. For ATP content of free-living cells, 8-ml samples were collected on GF/F filters, and each filter was extracted in 10.0 ml of 0.06 N H_2SO_4 at 2°C for 45 min.

After extraction, 2 ml each of Tris hydrochloride and 18 mg of EDTA per ml in Tris hydrochloride were added to each sample, and the pH was adjusted to 7.75 with NaOH. The extract volume was brought to 20.0 ml with Tris hydrochloride. ATP standards (0 to 8 ng/ml) were treated similarly.

Bioluminescene reaction measurements were made with a liquid scintillation counter. Two-tenths of a milliliter of each sample or standard was placed in an acid-cleaned scintillation vial, followed by 0.2 ml of luciferin-luciferase mixture. The contents of each vial were quickly mixed, placed in the liquid scintillation counter, and counted for 1 min after a 7-s delay.

Bacterial direct counts. Direct counts were performed with the DNA-specific fluorochrome Hoechst 33342 (29). All samples were counted with an Olympus model BHS-2 epifluorescence microscope.

DNA determinations. The DNA content of attached and free-living organisms was determined fluorometrically by the Hoechst 33258 method (30).

RESULTS

[^{3}H]thymidine incorporation. The efficiency of sonication for removing attached cells was examined by direct counts of cells attached to polystyrene before and after sonication. One hundred percent of the cells were removed by sonication at 70 W for 45 s.

[^{3}H]thymidine incorporation was first measured in the presence of normal growth media as well as in the absence of any exogenous nutrients. In the presence of nutrients, the thymidine incorporation rate of unattached cells averaged $(1.43 \pm 0.09) \times 10^{-19}$ mol of thymidine incorporated per cell per h or approximately two and one-half times greater than the rate for attached cells. In the absence of nutrients, however, the rate for the attached cells was $(3.23 \pm 1.26) \times 10^{-20}$ mol per cell per h or approximately twice the rate for the unattached cells.

A wider range of nutrient concentrations was then examined. In the presence of any added nutrients, the rate of thymidine incorporation of free-living cells was greater than that of attached cells (Fig. 1). Only in the absence of any exogenous nutrients were the attached cells more active than free-living cells (Fig. 1F). Rates were significantly different ($P < 0.10$) as analyzed by analysis of covariance with a statistical software package from SAS Institute Inc., Cary, N.C.

On further examination of the statistical data for each experiment in which nutrient concentrations were varied, it was observed that attached cells were much less responsive to changing nutrient levels than were free-living cells. Within

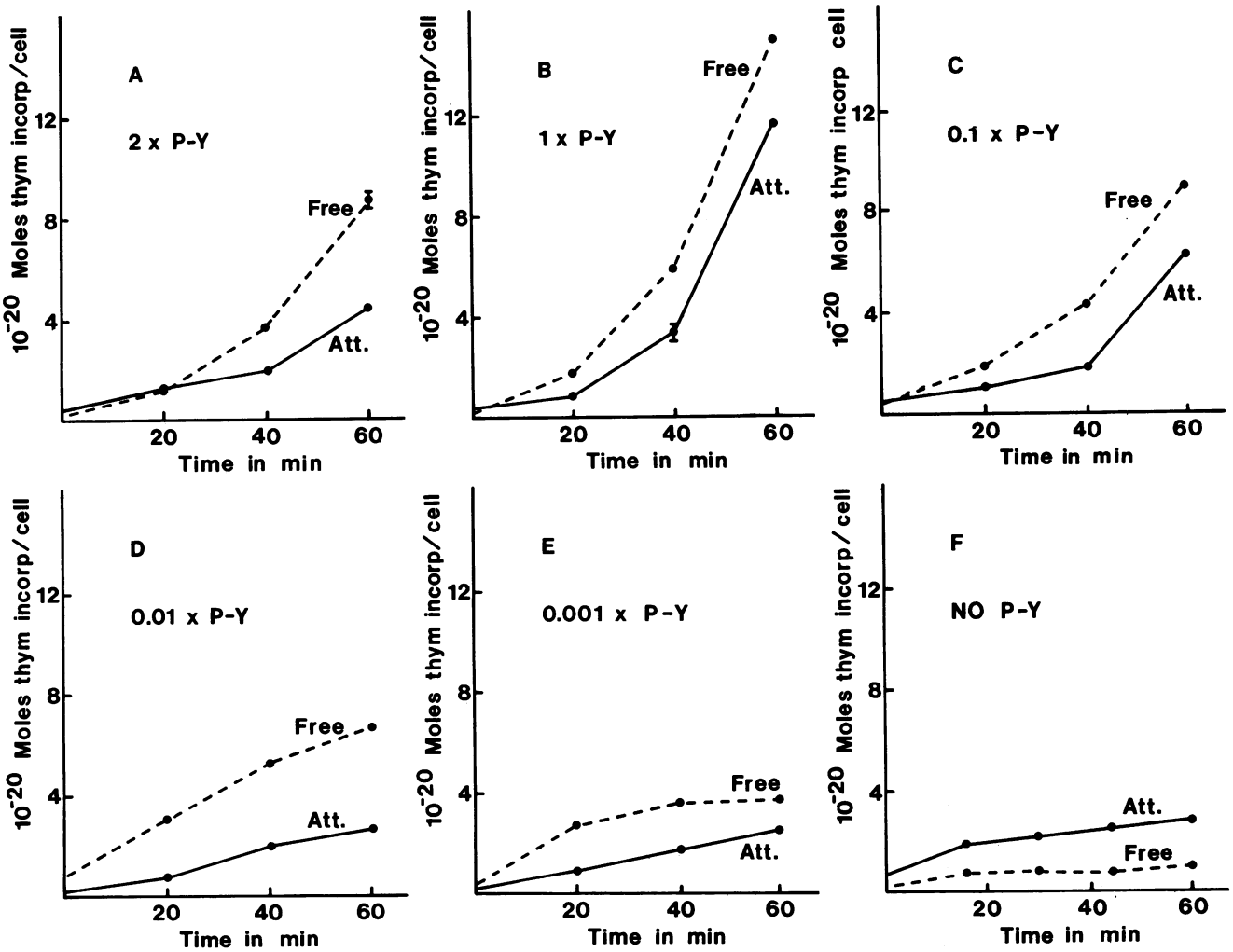

FIG. 1. [^{3}H]thymidine incorporation in the presence of a variety of nutrient concentrations. (A) Twice the normal growth media concentration; (B) Normal growth media (1.66 mg of peptone and 0.33 mg of yeast extract per ml); (C) 1/10 the normal concentration; (D) 1/100 the normal concentration; (E) 1/1,000 the normal concentration; (F) no added nutrients. Att., attached.

each experiment, activity rates for attached cells were relatively unchanged under the different nutrient conditions. The change in the difference of the relative activities of attached and free-living cells was due to a change in the activity of free-living cells. For nutrient concentrations below 1/100 the normal concentration (Fig. 1), the activity of attached cells stayed constant, but that of free-living cells decreased to below that of attached cells without nutrients. Experiments with Formalin (0.74%)-killed controls yielded no incorporation of [^{3}H]thymidine for both attached and free-living cells.

The specificity of [^{3}H]thymidine labeling was examined because other investigators have reported that the percentage of tritium incorporated into the protein fraction changes under various growth and nutrient conditions (32; R. D. Fallon and S. Y. Newell, personal communication). With free-living cells, the protein fraction was examined for tritium incorporation (Fig. 2). At the thymidine concentration used for all experiments (1 μM), no tritium was incorporated into the protein fraction whether in the presence or absence of nutrients (Fig. 2A and B). The possibility that the high thymidine concentration shut down any other enzymatic pathways preventing radiolabel incorporation into protein was also examined (Fig. 2C). At 5 nM thymidine, no label was incorporated into the protein fraction.

INT-F/DNA. The effect of methanol extraction of the formazan deposits on the recovery of DNA from each sample was determined. No effect was observed for free-living cells, while attached samples averaged a 14% decrease in DNA per dish after methanol extraction. Therefore, the attached DNA value was multiplied by 1.16 to correct for this loss, which may have been the result of detachment of cells by methanol.

In the absence of nutrients, the activity of attached cells surpassed that of free-living cells (Fig. 3A). In the presence of nutrients, no difference was found in the rates (Fig. 3B). Formalin controls had no activity as indicated by INT reduction (Fig. 3A).

ATP/DNA. Sonication for 15 s at 70 W slightly increased (~7%) the extraction of ATP from attached cells but had no effect on its extraction from free-living cells. Two acid concentrations were compared for extraction of ATP, 0.6 N H_2SO_4, as recommended by Karl and LaRock (17), and 0.06 N H_2SO_4. Both procedures gave similar results, but the variability between replicates was much greater for 0.6 N H_2SO_4. The higher acid concentration also gave a much

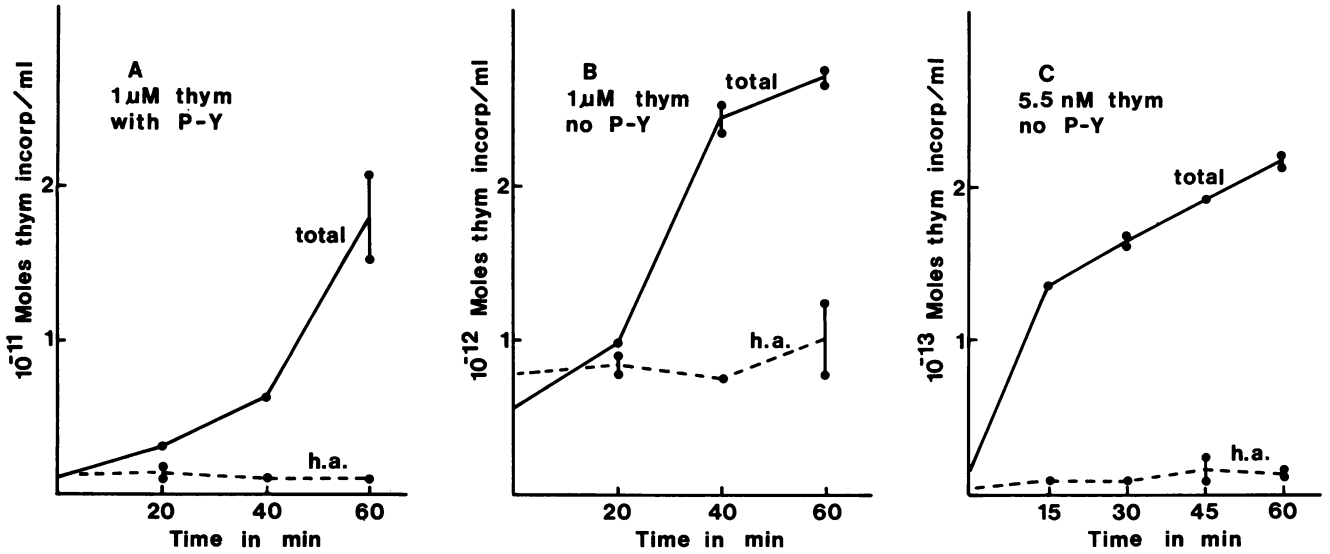

FIG. 2. [^{3}H]thymidine incorporation into the protein fraction of *V. proteolytica*. Tritium incorporation into the cold-TCA-precipitable fraction (total) and the hot-acid (h.a.) or protein fraction is shown. The slopes of incorporation into the protein fraction were 0 ($P < 0.05$).

lower bioluminescence signal, which decreased the sensitivity of the assay.

ATP/DNA ratios were measured during a growth experiment for *V. proteolytica* and found to be greatest during log-phase growth (Fig. 4). In the absence of nutrients, ATP/DNA ratios were equivalent for attached and free-living cells. In the presence of nutrients, however, the ATP/DNA ratio for free-living cells was significantly greater ($P < 0.001$) than the ratio for attached cells (Table 1).

DISCUSSION

These results indicate that, for *V. proteolytica*, attachment imparts a physiological advantage only under nutrient deprivation conditions. In addition, attached cells demonstrated much lower sensitivity to changing nutrient concentrations than did free-living cells.

Each of the techniques used proved to be a valuable indicator of activity, yet each has its own limitations. DNA synthesis is a complex process, which ultimately relies on the supply of energy and nutrients to the cell. The rate-limiting step for [^{3}H]thymidine incorporation into DNA is most often the DNA polymerase reaction (32). When the extracellular thymidine concentration is low and intracellular dTTP is high, the thymidine kinase reaction becomes the rate-limiting step (28). Under these conditions, intracellular isotope dilution may become a problem (32). High thymidine concentrations (as employed in this study) will reverse the inhibition of thymidine kinase by dTTP (34). Isotope dilution has been shown to be insignificant at thymidine concentra-

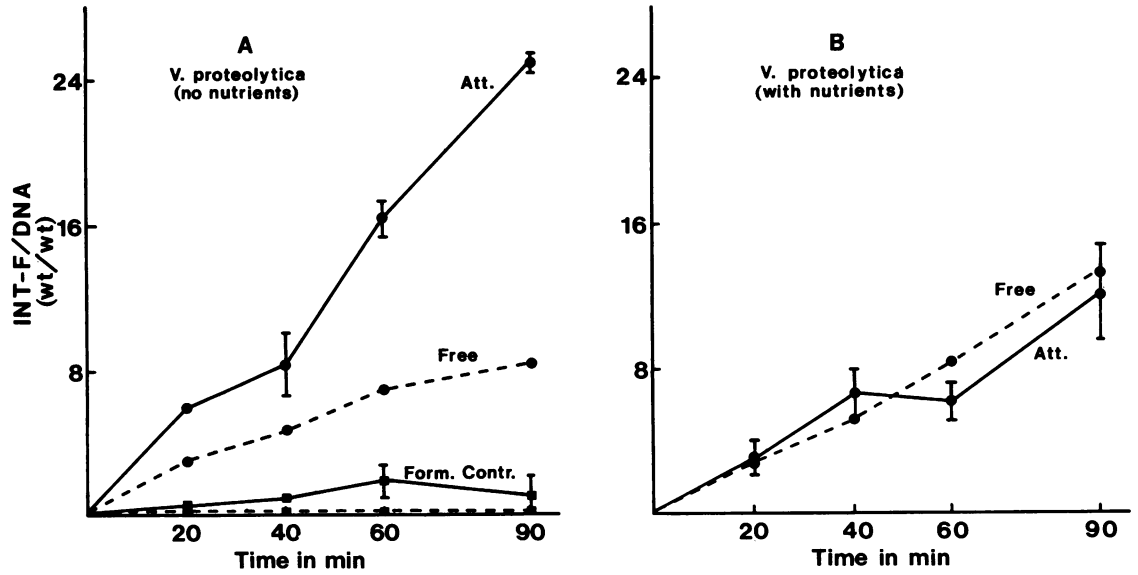

FIG. 3. INT-F/DNA for attached and free-living *V. proteolytica* cells. (A) In nutrient-free media; (B) in normal growth media. Att., Attached; Form. Contr., Formalin controls.

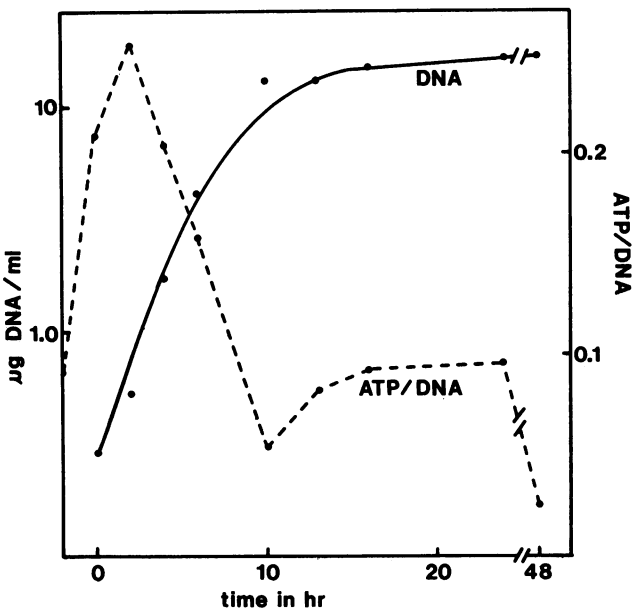

FIG. 4. ATP/DNA ratios of *V. proteolytica* during a growth curve. Stationary-phase cells were inoculated into fresh growth media at 0 h. Note the elevated values at the early exponential phase and the low values in the late stationary phase.

tions greater than 35 nM (32). Therefore, the different [^{3}H]thymidine incorporation rates are not due to thymidine transport kinetics but rather to a difference in cell growth rates.

Extracellular isotope dilution may have been caused by the presence of traces of thymidine in peptone-yeast extract. [^{3}H]thymidine incorporation rates for cells in twice the normal nutrient concentration (Fig. 1A) were lower than the rates for cells in normal growth media (Fig. 1B). These results indicate either that high nutrient concentrations had some inhibitory effect on the activity of the organism or that thymidine in peptone-yeast extract diluted the isotope. Because thymidine incorporation was used only as an activity indicator and not as a productivity measure, it was assumed that no difference in isotope dilution for attached and free-living cells would occur within the same experiment.

[^{3}H]thymidine is used for activity measurements because it is believed to be a specific DNA precursor. However, recent evidence has shown that significant amounts of tritium may be incorporated into either protein (8; R. D. Fallon and S. Y. Newell, personal communication) or RNA (16) as well as into DNA. The lack of incorporation of tritium from [^{3}H]thymidine into the protein fraction of *V. proteolytica* agrees with results found for *Serratia marinorubra* (16). In natural populations, however, approximately 15 to 20% of the tritium is incorporated into protein (8, 15, 16; R. D. Fallon and S. Y. Newell, personal communication). The difference in specificity of labeling between organisms in pure culture and environmental samples cannot be explained at this time.

The [^{3}H]thymidine incorporation rates of cells in nutrient-free media may be a result of DNA excision and repair processes rather than growth. Such processes would presumably be proportional to intracellular enzyme activity and still be an indicator of cellular activity.

The use of methanol extraction of formazan in the INT-F/DNA procedure has several advantages over the more commonly used technique of direct counts of the formazan deposits. First, it allows for quantification of the activity level within the population. Direct counts of deposits only allow a percentage of the active cells to be determined. Another advantage is time. Methanol extraction eliminates the time-consuming and often subjective task of direct microscopic examination of samples. In addition, deposits within small cells are often very hard to see. Nonspecific particulates in the water sample may interfere with the identification of formazan deposits, and immersion oil used in slide preparation extracts the formazan if the samples are stored for over 2 h (38).

INT-F/DNA measures the activity of a variety of enzymes involved in oxidation-reduction reactions and may be affected by cell density and spatial relationships between organisms, E_h oxygen tension, ion exchange, surface area, and adsorption (36). These enzymes are not as closely regulated by the growth process as those enzymes involved in DNA synthesis.

The effect of nutrients on the activity of cells as measured by INT-F/DNA was somewhat different than that indicated by [^{3}H]thymidine. The presence of nutrients had little or no effect on the activity of free-living cells. For attached cells, the presence of nutrients seemed to decrease the rate of INT reduction, perhaps due to some competition effect of the nutrients with INT. These results may have been due to normalization to DNA, since DNA content in the presence of nutrients increased while INT reduction remained relatively constant.

Previous investigations are contradictory as to whether cellular ATP content changes under various growth conditions, depending on the organism examined and whether ATP is expressed as ATP per cell, ATP per g of dry weight, or ATP per g of protein (21). There are several investigations, however, in which ATP per cell did change (3, 10, 24, 35). DNA content correlates very well with cell number (27, 31), and therefore the ATP/DNA ratio is equivalent to ATP per cell.

Contaminating transphosphorylase enzymes in the crude firefly extract (FLE-50) used for ATP determinations may have also reacted with other phosphorylated nucleotides, causing overestimation of ATP (6). However, the corroboration with the other activity measurement and the growth curve data in Fig. 4 indicate that, for *V. proteolytica*, ATP/DNA ratios are a valuable biochemical indicator of activity.

ATP/DNA ratios support the results obtained with the

TABLE 1. ATP and DNA contents of attached and free-living *V. proteolytica* in the presence and absence of nutrients

Conditions	µg of ATP ± SD[a]	µg of DNA ± SD[b]	ATP/DNA ratio
No nutrients			
Free[c]	0.245 ± 0.003	1.240 ± 0.040	0.198
Attached[d]	0.569 ± 0.072	3.200 ± 0.140	0.178
With nutrients			
Free[c]	0.373 ± 0.014	1.450 ± 0.180	0.257[e]
Attached[d]	0.832 ± 0.082	4.130 ± 0.260	0.202

[a] Mean of three determinations ± one standard deviation.
[b] Mean of four determinations ± one standard deviation.
[c] Values expressed as micrograms per milliliter.
[d] Values expressed as micrograms per dish.
[e] Statistically different from attached cells (α = 0.001).

other techniques in the presence of nutrients. Equal ATP/DNA ratios in nutrient-free media may be due to inherent differences in the types of activity measured. [^{3}H]thymidine and INT-F/DNA ratios rely on incubation and uptake of a substrate, while ATP/DNA ratios are instantaneous biochemical indicators of activity. The difference in the results may reflect either a difference in substrate availability or cell surface area available for substrate uptake by attached and free-living cells (see below).

Ellwood et al. (7) proposed that surface-associated cells are more active owing to a localized higher concentration of extruded protons. These protons can be passed back into the cell, generating ATP and increasing cell activity. This theory is supported by Kjelleberg and Dahlback (20), who reported higher ATP concentrations per biovolume for starved cells attached to glass. While *V. proteolytica* had higher thymidine incorporation and INT reduction rates when attached in the absence of nutrients, the cellular ATP levels were no different from those of unattached cells. These differences may be due to differences in experimental designs or organisms employed.

By the direct viable count method of Kogure et al. (23), approximately 45% of the cells in both the attached and unattached populations were active (data not shown). In addition, no difference was observed by epifluorescence microscopy in the average cell size of attached and unattached cells. The difference in measured activities, therefore, is not due to a difference in the percentage of active cells or cell size but rather to a physiologic difference in those cells which are active.

Attachment is often viewed as an advantageous starvation response (5, 20). In a preliminary study on the effects of starvation, *V. proteolytica* attached less as starvation time increased (data not shown). Therefore, attachment is not a starvation or low-nutrient response for this organism.

Nutrients are known to adsorb to surfaces in seawater (26), and attached bacteria have been shown to have an advantage in scavenging adsorbed nutrients (18). Therefore, higher activities for attached cells in nutrient-free media may be the result of greater availability of trace nutrients adsorbed to the surface. Several investigators have found that bacterial attachment increases activity in low-nutrient environments (14, 20). Bright and Fletcher (1, 2) reported greater amino acid assimilation by surface-associated cells for nutrient ranges of 10 to 1,000 μg of C per liter. Similar results were found for *V. proteolytica* only in the absence of added nutrients, where total organic carbon may have been 300 μg of C per liter. However, the lowest concentration of added nutrients, 2,000 μg of peptone-yeast extract per liter (~830 μg of C per liter), may not have been low enough to cause the effect reported by others.

Greater activity by unattached cells in the presence of nutrients has been reported by others. Hattori and Furusaka (12, 13) reported higher succinate oxidation rates for unattached *Escherichia coli* and *Azotobacter agile*. Gordon et al. (9) tested the supposition that adsorption of organic nutrients or bacterial cells or both to inorganic surfaces increases bacterial activity. The addition of particles did not stimulate activity whether bacteria, organic nutrients, or both adhered to the surface. In all cases, free-living bacteria had higher respiratory rates than did attached cells.

When nutrients are in ample supply, the rate of growth is limited by the cell surface area available for nutrient transport. Attached cells have a decreased surface area (losing part in contact with the substratum) and thus cannot absorb or transport nutrients as rapidly as can unattached cells (9, 11). Based on scanning electron microscopic photographs, an estimated 15 to 20% of the surface area of *V. proteolytica* could be lost owing to attachment. If [^{3}H]thymidine incorporation rates are corrected for this, the rates of attached and free-living cells in the presence of the midrange nutrient concentrations examined become equivalent in approximately 50% of the experiments. Cell surface area available for nutrient uptake lost owing to attachment may be one factor which alters nutrient supply to cells when other conditions are equal.

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