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Thymidine Incorporation by the Microbial Community of Standing Dead *Spartina alterniflora*†

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Thymidine incorporation by the microbial community on standing dead leaves of *Spartina alterniflora* did not obey many of the assumptions inherent in the use of the technique in planktonic systems. Incorporation rates of [*methyl*-^{3}H]thymidine were nonsaturable over a wide concentration range (10^1 to 10^5 nM). Owing to metabolism by both fungi and bacteria, a major fraction of the radiolabel (mean, 48%) appeared in protein. Extraction of the radiolabeled macromolecules was inefficient, averaging 8.8%. Based on an empirically derived conversion factor, 4×10^{18} cells · mol of thymidine^{-1}, doubling times ranged from 4 to 69 h for the epiphytic bacterial assemblage.

The thymidine incorporation technique has become widely used as a potential method for measuring bacterial growth rates in nature (21, 29). However, it has received limited use with epiphytic bacterial assemblages (9, 10, 18). Bacteria play an important role in detritus formation from smooth cordgrass, *Spartina alterniflora*, in coastal marshes (2, 23), but their growth rates on dead plant material are unknown. We have examined the suitability of the thymidine incorporation technique as a means of measuring bacterial growth rates on standing dead leaves of *S. alterniflora* in Sapelo Island, Ga., marshes.

Sterile creek water (salinity, 21 to 28 g · liter^{-1}) was used as the base for all incubations. Standing dead leaves collected from the marsh were gently washed in five changes of creek water prior to use. Both [*methyl*-^{3}H]thymidine and [4,5-^{3}H]leucine (ICN Pharmaceuticals Inc.) contained 50 Ci · mmol^{-1}. Thymidine (T9250-Sigma Chemical Co.) and L-leucine (L-8000; Sigma) were used to adjust precursor concentrations. Autoclaved or 2% formaldehyde (final concentration)-killed controls were used to correct for abiotic adsorption. Controls were generally <30% of the live treatments. Total radiolabel incorporation by the live treatments was always <10% of the total added label. Protein and DNA were recovered by the method of Fuhrman and Azam (11) with the addition of 0.36% (final concentration) sodium lauryl sulfate to aid in dissolving macromolecules from the leaves (10). Material on the filters was digested (6 N HCl in vacuo, 110°C, 18 h) prior to scintillation counting. Quench correction was done with internal standard additions. [^{3}H]DNA (New England Nuclear Corp.) was used to test DNA recovery efficiency. To test the efficiency of total macromolecular radiolabel recovery, we halved replicate leaves following incubation; activity recovered by the standard fractionation protocol (11) from one set of halves was compared with that recovered from the other set of halves digested in 6 N HCl (in vacuo, 110°C, 18 h). To ensure that primarily macromolecular activity was being monitored, we gave both halves of these split leaves a postincubation creek water wash (to <1% of original soluble counts), followed by a 2-h chase incubation (no further added label). This allowed the completion of precursor incorporation into macromolecules.

In addition to the natural material, model systems were also used to test various treatments under more defined conditions. Mixed bacterial inocula obtained by filtration (filter pore diameter, 0.8 μm; vacuum, 40 cm Hg [ca. 533.32 cPa]) (2) and axenic fungal cultures obtained by micromanipulation were grown on autoclaved dead leaf extract or autoclaved dead leaves of *S. alterniflora* (26). The effects of nitrogen availability were tested by using ground, soluble nitrogen-extracted (2% sodium lauryl sulfate–0.1% EDTA, 50°C, 4 h), standing dead leaves. Nitrogen-sufficient systems received $NaNO_3$ additions (plant C/added N ratio, 3.3:1), and nitrogen-deficient systems received no N additions. Bacterial cells were counted by the acridine orange direct count method (15, 25). Fungal volume was measured by epifluorescence microscopy of water-soluble, aniline blue-calcofluor white-stained material (25).

Thymidine incorporation kinetics could not be saturated for either total alkaline-stable macromolecules (i.e., DNA and protein) (Fig. 1) or DNA (data not shown) over 10^1 to 10^5 nM added thymidine. Saturation in planktonic systems is usually observed at less than 100 nM added thymidine (1, 12, 16, 21, 24). The explanation for these observations is uncertain. Unusually high thymidine concentrations were not reponsible, since incorporation was saturated at 50 nM added thymidine in a mixed bacterial inoculum growing in dead leaf extract (data not shown). Rather, some feature of the microbial community or its physical environment may have been responsible. Multiphasic enzyme systems can cause nonsaturable kinetics (13), and diffusion resistance in attached communities may also contribute (7). The marine bacterial strain LNB-155 shows multiphasic kinetics for glucose uptake over a wide concentration range (10^{-9} to 10^{-3} M) (27). In the diverse bacterial community attached to dead leaves, multiphasic enzyme systems are one likely explanation for the kinetics we observed.

Over a wide range of experiments, the incorporation ratio (disintegrations per minute incorporated into DNA/disintegrations per minute incorporated into DNA plus protein) was 0.62 (standard deviation, ±0.21; $n = 10$), well below values often reported in the literature for the pelagic, euphotic zone (6, 11, 17). Experimental data also showed a significant negative correlation ($r = -0.51$, $P < 0.01$, $n = 40$) between the incorporation ratio and the concentration of added thymidine. Tritium from thymidine was incorporated by fungi into protein but not significantly into DNA, and parallel

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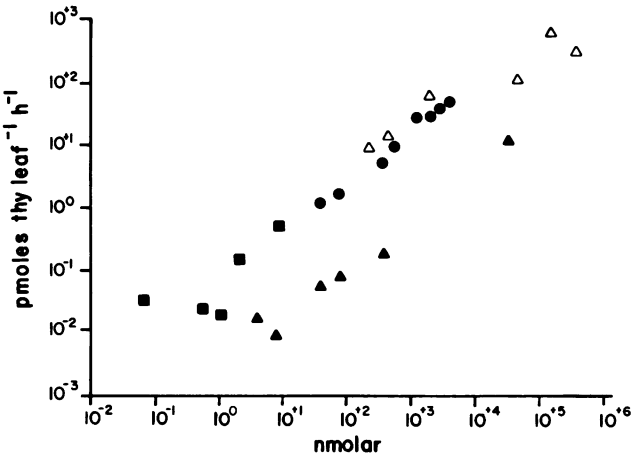

FIG. 1. Total macromolecular radiolabel incorporation rate versus added thymidine (thy) concentration (abscissa). Symbols: ▲, ■, and ●, three separate experiments with natural material; △, autoclaved standing dead leaves with a mixed bacterial assemblage.

rates of incorporation into protein were much lower for thymidine than for leucine, a direct protein precursor (Table 1). Thus, fungal metabolism may be one cause for the high relative rates of radiolabel incorporation into protein in dead leaves. However, when 50 nM thymidine was added to dead leaf extract, cell volume-specific incorporation rates for the fungi were about 100- to 1,000-fold lower than those for the mixed bacterial assemblage from *S. alterniflora*. In addition, model systems containing a mixed bacterial assemblage growing on dead leaves often showed similarly low incorporation ratios, indicating that bacterial metabolism alone could account for our observations. In pairwise comparisons between nitrogen-sufficient growth and nitrogen-deficient growth of mixed bacteria on autoclaved dead leaves, there was a significant treatment effect ($P = 0.008$, $n = 7$ [Sign test]) (28), showing that there was a strong tendency for nitrogen-deficient conditions to result in low incorporation ratios. The nitrogen treatment effect and the negative correlation between the concentration of added thymidine and the incorporation ratio observed in the bacterial model systems suggest that alternative processing pathways for thymidine (i.e., other than the "salvage" pathway [3, 21]) may have been enlisted by the bacteria under nitrogen-deficient conditions. Similarly low incorporation ratios have been seen in planktonic populations growing under "stressful" conditions (5, 14, 20). Perhaps thymidine can better serve as a C, N, or energy source rather than as a direct DNA precursor under such conditions.

TABLE 1. Radiolabel distribution in fungal species

Fungus (grown on dead leaf extract)	Radiolabel distribution[a] with indicated substrate:			
	Thymidine (nM)[b]			Leucine (410 nM)
	41	410	4,100	
Phaeosphaeria typharum	79 (0.15)	100 (1.3)	86 (11)	80 (867)
Buergenerula spartinae	61 (0.047)	ND[c]	ND	80 (867)

[a] Data represent acid-stable (protein) radiolabel incorporation as a percentage of the total macromolecular incorporation; the numbers in parentheses represent the total macromolecular incorporation rate in picomoles per cubic millimeter per hour.

[b] Mean percent radiolabel in the thymidine treatments: *P. typharum*, 88; *B. spartinae*, 61; all treatments, 75.

[c] ND, Net incorporation not detected.

Macromolecule recovery was very inefficient, with a mean value of only 8.8%. However, epifluorescence microscopy indicated that all bacterial cells were lysed. The recovery of [^{3}H]DNA standards in parallel experiments averaged 57.4%, indicating that inefficient recovery was primarily due to a failure to release macromolecules from the leaf pieces. When the [^{3}H]DNA was precipitated onto cellulose filter paper and extracted, only 13% of the radiolabel could be redissolved. Radiolabel that could be dissolved was recovered at 94% efficiency. Therefore, the poor recovery in the dead leaf experiments appeared to result primarily from the failure to dissolve macromolecular counts, perhaps due to condensation reactions between the radiolabeled macromolecules and leaf cellulose (30), and not from inefficient recovery of the dissolved material. Some previous reports suggested that DNA can be recovered at >50% efficiency (8–10, 22). However, low efficiencies (<25%) have been reported under some conditions (4, 18, 19).

[*methyl*-^{3}H]thymidine metabolism by the microbial community on standing dead leaves of *S. alterniflora* precludes the use of many of the assumptions common to the technique as used in planktonic systems. Tritium incorporation by fungi and the influence of potential N limitation on bacteria suggest that production estimates should be based on DNA incorporation, not total macromolecular incorporation, because one cannot assume a constant incorporation ratio (11). Standard values for converting thymidine incorporation rates into cell production rates are generally derived from experiments done under saturated conditions (1, 6, 11, 12, 17). The inability to saturate incorporation kinetics on dead leaves suggest that these standard values may not be applicable. Therefore, we used an empirical approach similar to that of Kirchman et al. (17) to derive a conversion factor. A mean value of 4 (range, 1.4 to 7.1; $n = 3$) $\times 10^{18}$ cells · mol of thymidine^{-1} was observed for mixed bacterial inocula growing on autoclaved dead leaves. Applying this value to field material, we estimated bacterial turnover times of 4 to 69 h under moist conditions, depending on the dead leaf history and incubation temperture. These observations show that bacterial growth rates on dead leaf material can be as rapid as those in eutrophic planktonic systems.

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