

## NOTES

# [<sup>3</sup>H]Thymidine Incorporation To Estimate Growth Rates of Anaerobic Bacterial Strains

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**The incorporation of [<sup>3</sup>H]thymidine by axenic cultures of anaerobic bacteria was investigated as a means to measure growth. The three fermentative strains and one of the methanogenic strains tested incorporated [<sup>3</sup>H]thymidine, whereas the sulfate-reducing bacterium and two of the methanogenic bacteria were unable to incorporate [<sup>3</sup>H]thymidine during growth. It is concluded that the [<sup>3</sup>H]thymidine incorporation method underestimates bacterial growth in anaerobic environments.**

Measurements of in situ growth rates and activity of bacteria on the basis of incorporation of radiolabelled nucleosides have been applied to environmental samples for several years. Because the ability to incorporate thymidine is widespread among aerobic bacteria, the incorporation of tritiated thymidine is a widely used method, even though bacterial strains unable to incorporate [<sup>3</sup>H]thymidine are known (6). Studies of anaerobic communities incorporating [<sup>3</sup>H]thymidine are scarce. Gilmour et al. (1) studied the incorporation of [<sup>3</sup>H]thymidine into 10 strains of sulfate-reducing bacteria and found very low rates of incorporation per cell produced (average,  $3.3 \times 10^{23}$  cells per mol of [<sup>3</sup>H]thymidine). Incorporation of [<sup>3</sup>H]thymidine in anaerobic environments has been shown (5, 7); the fermentative bacteria have been suggested as being the most important group of incorporating organisms (6). However, studies of incorporation of [<sup>3</sup>H]thymidine into fermentative and methanogenic strains are needed before this method can be used in anaerobic environmental samples. The purpose of this study was to examine the incorporation of [<sup>3</sup>H]thymidine in axenic cultures of fermentative, sulfate-reducing, and methanogenic bacteria, i.e., to evaluate the application of the method for quantification of growth rates in anaerobic environments.

**Strains.** The following fermentative strains were used: *Clostridium cellobioparum* ATCC 15832, *Clostridium formicoaceticum* ATCC 27076, and *Acetobacterium woodii* ATCC 29683. The sulfate-reducing strain was *Desulfovibrio vulgaris* G11. The methane-producing strains used were *Methanococcus vannielii*, *Methanospirillum hungatei*, and *Methanobacterium formicicum*. *C. cellobioparum* DSM 1351, *C. formicoaceticum* DSM 92, *A. woodii* DSM 1030, and *M. vannielii* DSM 1224 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSM); the other strains had been kept at the laboratory.

**Media.** *D. vulgaris* G11 and the methanogenic strains kept in the laboratory were grown in a reduced mineral salts medium (10) with 2% yeast extract and buffered with

NaHCO<sub>3</sub> under an atmosphere of N<sub>2</sub> and CO<sub>2</sub> (8:2). To *D. vulgaris* G11, 20 mM sulfate was added. The *M. hungatei*, *M. formicicum*, and *D. vulgaris* G11 cultures were pressurized with H<sub>2</sub>. The strains from DSM were grown in the media described by DSM, with the following exceptions. The trace element solution was that of Worakit et al. (10); to the medium for *C. formicoaceticum*, CaCl<sub>2</sub> (1 mg/liter) was added; to the medium for *C. cellobioparum*, clarified rumen fluid and cellobiose were added; and to the medium for *A. woodii*, another vitamin solution (3) was added. In order to evaluate any adsorption of thymidine and isotope dilution in nutrient-rich media, *M. vannielii* was grown in the reduced mineral salts media described above and in the rich media described by DSM, except that no  $\alpha$ -methylbutyric acid was added and 5% (vol/vol) clarified rumen fluid was used instead of sewage sludge. All strains were shaken and grown at 37°C except for *A. woodii*, which was grown at 30°C.

**Bacterial counts.** The total number of bacteria was determined by the use of DAPI (4',6-diamidino-2-phenylindole) as described by Porter and Feig (8). A minimum of 400 bacteria were counted in each slide.

**Methane production.** The headspace concentration of methane was measured by gas chromatography (4). The production of methane per volume of cell suspension during the incubation was calculated, assuming all methane to be in the headspace.

**Extraction of labelled macromolecules.** [Methyl-<sup>3</sup>H]thymidine (specific activity, 3.29 TBq/mmol; purity, 98.3%) (Amersham) diluted with unlabelled thymidine to a specific activity of  $1.6 \times 10^5$  Bq/nmol was added to cell cultures to a final concentration of 100 nM. Following centrifugation (8,000  $\times g$ , 5 min, 0°C) of 1.5 ml of cell culture, the cell material was washed five times with 1 ml of 0°C 5% trichloroacetic acid with centrifugations (12,000  $\times g$ , 10 min, 0°C) in between to pellet the macromolecules. Finally, the cell material was resuspended in 1 ml of 5% trichloroacetic acid and kept at 90°C for approximately 5 min to dissolve the macromolecules. One milliliter was transferred to a 6-ml vial, 5 ml of scintillation fluid (HiSafe II; LKB Wallac) was added, and radioactivity was monitored by using a scintillation counter (1209 Rackbeta; LKB Wallac). The counting efficiency was estimated by the internal standard addition

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TABLE 1. Bacterial counts, incorporation of [<sup>3</sup>H]thymidine, and methane production for selected anaerobic strains

Organism	Time (h)	AODC <sup>a</sup> (10 <sup>7</sup> /ml)	<sup>3</sup> H-TdR incorporation <sup>b</sup>		Cell production (10 <sup>18</sup> /mol of <sup>3</sup> H-TdR)	CH <sub>4</sub> production (nmol/ml)
			dpm (SD)	pmol/(liter · h)		
<b>Fermentative bacteria</b>						
<i>Clostridium formicoaceticum</i>	0	0.8	0 <sup>c</sup>	0 <sup>c</sup>		
	3	5.8	7,474 (449)	172	97	
	24	12.3	165,563 (3,022)	477	10	
<i>Clostridium cellobioparum</i>	0	0.9	0 <sup>c</sup>	0 <sup>c</sup>		
	3	1.4	8,196 (3,254)	189	9	
	24	28.9	93,117 (4,660)	268	44	
<i>Acetobacterium woodii</i>	0	0.9	0 <sup>c</sup>	0 <sup>c</sup>		
	3	2.2	bd	bd	ND <sup>d</sup>	
	24	30.8	1,183 (52)	3	3,906	
<b>Sulfate-reducing bacterium</b>						
<i>(Desulfovibrio vulgaris G11)</i>	0	10.5	0 <sup>c</sup>	0 <sup>c</sup>		
	3	10.2	bd	bd	ND	
	24	14.6	bd	bd	ND	
<b>Methanogenic bacteria</b>						
<i>Methanococcus vannielii</i> (nutrient-rich medium)	0	0.3	0 <sup>c</sup>	0 <sup>c</sup>		0
	3	1.7	bd	bd	ND	ND
	24	5.9	bd	bd	ND	17.68
<i>M. vannielii</i> (mineral salts medium)	0	0.2	0 <sup>c</sup>	0 <sup>c</sup>		0
	3	0.1	bd	bd	ND	0.06
	24	43.3	bd	bd	ND	0.38
<i>Methanospirillum hungatei</i>	0	0.8	0 <sup>c</sup>	0 <sup>c</sup>		0
	3	0.8	bd	bd	ND	0.44
	24	2.9	bd	bd	ND	2.05
<i>Methanobacterium formicum</i>	0	18.1	0 <sup>c</sup>	0 <sup>c</sup>		0
	3	28.7	14,067 (345)	324	327	0.06
	24	21.1	8,143 (413)	23	1,304	0.25

<sup>a</sup> AODC, acridine orange direct count.

<sup>b</sup> <sup>3</sup>H-TdR, [<sup>3</sup>H]thymidine. SD, standard deviation (*n* - 1). bd, below detection limit of 100 dpm, corresponding to 2 pmol/(liter · h) for 3 h of incubation and 0.3 pmol/(liter · h) for 24 h of incubation.

<sup>c</sup> By definition.

<sup>d</sup> ND, not determined.

method (Internal Standard Kit [6,6'-(n)-<sup>3</sup>H]sucrose; Pharmacia).

**Experiment.** One-hundred-milliliter serum bottles, each containing 50 ml of medium, were inoculated from growing cultures of each strain. The serum bottles were closed with butyl rubber stoppers. *C. cellobioparum*, *C. formicoaceticum*, and *A. woodii* were grown overnight, whereas the remaining strains were grown for 3 days, before the start of the experiment. The experiment was initiated with the addition of [<sup>3</sup>H]thymidine. From each bottle, three 1.5-ml samples were collected immediately for bacterial counts. In order to evaluate nonspecific adsorption of <sup>3</sup>H during extraction, *C. formicoaceticum* was sampled immediately for <sup>3</sup>H incorporation. Samples (six 1.5-ml samples) were taken after 3 and 24 h, fixed with formaldehyde (final concentration, 1%), and stored at 2°C for direct counting and triplicate extraction of macromolecules. In the methane-producing cultures, methane was measured at time 0, before and after sampling at 3 h, and before sampling at 24 h.

All of the bacterial isolates showed numerical growth during one or both of the incubation periods (Table 1). The lack of increase in cell number in one of the periods is attributed to either lag or stationary phases in the culture. All of the methanogens produced methane, which indicates metabolic activity.

A small amount of radioactivity (41 dpm/1.5 ml) was extracted from *C. formicoaceticum* at time 0. This <sup>3</sup>H is considered to have originated from nonspecific adsorption

and was subsequently subtracted from all of the incorporation data.

The fermentative bacteria (*C. formicoaceticum*, *C. cellobioparum*, and *A. woodii*) all incorporated [<sup>3</sup>H]thymidine. The sulfate-reducing bacterium *D. vulgaris* G11 might have been in late logarithmic phase by the time of [<sup>3</sup>H]thymidine addition, as the cell number was high and the culture showed only slow growth during incubation. This strain did not incorporate [<sup>3</sup>H]thymidine under the present growth conditions, which is in agreement with the results of Gilmour et al. (1).

Among the methanogens, only *M. formicum* incorporated thymidine. *M. vannielii* grew well on both the nutrient-rich medium and the mineral salts medium, as is evident from the increase in cell number, with the methane production being considerably higher when the methanogens were grown on the rich medium. Thus, isotope dilution and adsorption of thymidine to constituents of the rich medium can be ruled out as causes for the absence of [<sup>3</sup>H]thymidine incorporation.

The amount of [<sup>3</sup>H]thymidine taken up per cell produced was calculated (Table 1) without accounting for isotope dilution, which would lower the calculated cell production. The values varied from 9 × 10<sup>18</sup> to 3,906 × 10<sup>18</sup> cells per mol of [<sup>3</sup>H]thymidine and were as high as or higher than the empirical values reported, 0.2 × 10<sup>18</sup> to 59 × 10<sup>18</sup> cells per mol of [<sup>3</sup>H]thymidine (2, 9). This indicates some constraints on the [<sup>3</sup>H]thymidine incorporation, especially by *A. woodii*

( $3,906 \times 10^{18}$  cells per mol of thymidine) and *M. formicicum* ( $1,304 \times 10^{18}$  cells per mol of thymidine). Little or no ability to take up exogenous thymidine and/or lack of thymidine kinase to incorporate thymidine into DNA are probable reasons for the lack of [ $^3\text{H}$ ]thymidine incorporation.

In conclusion, this study confirms that fermentative bacteria are able to incorporate [ $^3\text{H}$ ]thymidine and that sulfate-reducing and methanogenic bacteria generally do not possess this ability. From this study it seems as if measuring growth rates in anaerobic environments on the basis of [ $^3\text{H}$ ]thymidine incorporation would exclude methanogenic and sulfate-reducing bacteria, and the measured growth rates would mainly represent the fermentative bacteria. Therefore, alternative methods for measuring microbial growth rates in natural anaerobic environments must be applied.

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#### REFERENCES

- Gilmour, C. C., M. E. Leavitt, and M. P. Shiaris. 1990. Evidence against incorporation of exogenous thymidine by sulfate-reducing bacteria. *Limnol. Oceanogr.* **35**:1401-1409.
- Jeffrey, W. H., and J. H. Paul. 1988. Underestimation of DNA synthesis by [ $^3\text{H}$ ]thymidine incorporation in marine bacteria. *Appl. Environ. Microbiol.* **54**:3165-3168.
- Madsen, T., and J. Aamand. 1991. Effects of sulfuroxy anions on degradation of pentachlorophenol by a methanogenic enrichment culture. *Appl. Environ. Microbiol.* **57**:2453-2458.
- Madsen, T., and J. Aamand. 1992. Anaerobic transformation and toxicity of trichlorophenols in a stable enrichment culture. *Appl. Environ. Microbiol.* **58**:557-561.
- McDonough, R. J., R. W. Sanders, K. G. Porter, and D. L. Kirchman. 1986. Depth distribution of bacterial production in a stratified lake with an anoxic hypolimnion. *Appl. Environ. Microbiol.* **52**:992-1000.
- Moriarty, D. J. W. 1986. Measurement of bacterial growth rates in aquatic systems from rates of nucleic acid synthesis. *Adv. Microb. Ecol.* **9**:245-292.
- Pollard, P. C., and D. J. W. Moriarty. 1984. Validity of the tritiated thymidine method for estimating bacterial growth rates: measurements of isotope dilution during DNA synthesis. *Appl. Environ. Microbiol.* **48**:1076-1083.
- Porter, K. G., and Y. S. Feig. 1980. The use of DAPI for identifying and counting microflora. *Limnol. Oceanogr.* **25**:943-948.
- Smits, J. D., and B. Riemann. 1988. Calculation of cell production from [ $^3\text{H}$ ]thymidine incorporation with freshwater bacteria. *Appl. Environ. Microbiol.* **54**:2213-2219.
- Worakit, S., D. R. Boone, R. A. Mah, M.-E. Abdel-Samie, and M. M. El-Halwagi. 1986. *Methanobacterium alcaliphilum* sp. nov., an  $\text{H}_2$ -utilizing methanogen that grows at high pH values. *Int. J. Syst. Bacteriol.* **36**:380-382.