PURIFICATION OF DNA FOR BACTERIAL PRODUCTIVITY ESTIMATES

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MANAGEMENT PERSPECTIVE

The importance of bacteria in the aquatic environment has been known for decades. The stumbling block to detail research has been methodology. Recently, a method based on the incorporation of ^{3}H-thymidine into DNA has been developed to measure bacterial productivity. The technique has been quite controversial because a number of assumptions had to be made. The assumption that only DNA was labelled by the thymidine has been thoroughly discussed in the literature and various modifications of the procedure have attempted to isolate just the DNA fraction. We have taken a technique used in mammalian tissue research and adapted it to bacterial productivity estimates. The DNA from the lysed bacterial cells is adsorbed onto hydroxylapatite columns and the other labelled macromolecules (protein and RNA) are rinsed off the column. Subsequently, the DNA is eluted and the fraction is assayed for radioactivity. From this data bacterial production can be measured.

PERSPECTIVE-GESTION

L'importance des bactéries en milieu aquatique est reconnue depuis des dizaines d'années, mais des difficultés méthodologiques ont freiné le progrès de la recherche approfondie. Récemment, on a mis au point une méthode qui permet de mesurer la productivité bactérienne d'après l'incorporation de thymidine tritiée dans l'ADN. Cette technique a suscité la controverse car elle est fondée sur un certain nombre de suppositions. L'hypothèse selon laquelle seul l'ADN serait marqué par la thymidine a été très discutée et l'on a apporté diverses modifications à la technique pour arriver à n'isoler que la fraction d'ADN. Nous nous sommes servis d'une technique utilisée dans l'étude des tissus mammaliens que nous avons adaptée pour l'estimation de la productivité bactérienne. L'ADN de cellules bactériennes lysées est adsorbé sur des colonnes d'hydroxylapatite, tandis que les autres macromolécules (protéines et ARN) sont éliminées par rinçage. L'ADN est ensuite élué, puis on en mesure la radioactivité pour évaluer la productivité bactérienne.

RÉSUMÉ

Par chromatographie à l'hydroxylapatite, on a complètement séparé l'ADN de populations naturelles de bactéries aquatiques marqué à la (méthyl-^{3}H) thymidine de l'ARN et de la fraction protéique. On a validé cette méthode en surveillant l'augmentation du nombre d'*E. coli*, l'absorbance à 550 nm, la concentration d'ADN et l'incorporation de thymidine dans l'ADN isolé par la technique proposée. La méthode peut s'employer sur le terrain et ne nécessite ni d'hydrolyses acide-base, ni de solvants organiques volatils.

ABSTRACT

[Methyl-^{3}H] thymidine labelled DNA from natural populations of aquatic bacteria was completely separated from RNA and protein using hydroxylapatite chromatography. The procedure was validated by monitoring increases in E. coli cell count, absorbance at 550 nm, DNA concentration, and thymidine incorporation into DNA isolated by the proposed technique. The procedure can be used in the field and does not rely on the use of acid-base hydrolyses or volatile organic solvents.

INTRODUCTION

The use of [^{3}H]-methyl thymidine to measure bacterial production in aquatic habitats by Fuhrman and Azam (7) has stimulated research in microbial ecology. Their procedures have been used by others (2,19,10) and modified by subsequent workers (20,6). Although these procedures may seem to give reasonable estimates of bacterial productivity, there are three major drawbacks: (i) the imprecision of the factor which converts rates of thymidine incorporation into rates of bacterial production (9,3), (ii) the unknown percentage of the microbial population capable of incorporating extracellular thymidine (17,16), and (iii) the need to assume what fraction of the cold trichloroacetic acid (TCA) insoluble material is DNA (7,10,). Extraction and purification of DNA labelled with [^{3}H]-thymidine has been advocated by many researchers (14,20,21,22,5). Often this has been accomplished by the acid-base hydrolysis procedure originally used for aquatic bacterial production estimation (7). The procedure has been slightly modified by others (10,18) and even the nucleases, DNAse and RNAse have been used to confirm identities of the three fractions obtained (18). The acid-base hydrolysis of the TCA-insoluble fraction has recently been shown to be inadequate for quantifying labelled DNA in the presence of other labelled macromolecules (21). However, these authors do not present sufficient data to confirm that the radioactivity of the DNA fraction is influenced by the unknown nature of the possible "RNA" fraction. The need for DNA purification has been indicated and

three alternative procedures to the acid-base hydrolysis scheme have been proposed (26,25,22). Witzel and Graf (26) ruptured [^{3}H]-labelled microorganisms by grinding with sea sand at low temperature; centrifuging the sample and then used hydroxylapatite columns to isolate the nucleic acids from protein and other radiolabelled contaminants. The RNA was eluted with 0.14 M phosphate buffer and DNA with 0.24 M phosphate buffer. Under these elution conditions the RNA and DNA overlap and are not quantitatively separated (K-P Witzel, personal communication). Marmur (11) states that cells ground in alumina or glass powder yield a lower molecular weight DNA than the DNA released by enzyme or detergent lysis. Servais et. al. (22) lysed tritiated thymidine labelled bacteria with detergent and then incubated an aliquot with DNase to selectively hydrolyse the DNA. The macromolecules from both the DNase treated and untreated solutions were precipitated with TCA and the DNA determined by the difference in radioactivity. Wicks and Robarts (25) advocate washing the TCA precipitated macromolecules (RNA has been previously removed by alkaline hydrolysis) with phenol/chloroform to remove the protein with an additional 80% ethanol wash to remove lipids and unincorporated thymidine. In this paper, we report a modification of the hydroxylapatite procedure (26) and compare it with TCA precipitation of macromolecules (7) and the phenol/chloroform wash techniques (25).

MATERIALS AND METHODS

Tritiated thymidine ([methyl-^{3}H] TdR, 3.26 TBq/mmol) in 2% ethanol was purchased from Amersham and stored at 5°C. This specific activity was used for all water samples except where noted. Water samples were collected as surface grab samples from Hamilton Harbour, Ontario. Ten ml subsamples were incubated with 10 nM [^{3}H] TdR for 20 min. The incubation was terminated by the addition of 1 mM unlabelled thymidine and by placing incubation flasks on ice (9). Three procedures were compared: i) the ice-cold trichloroacetic acid (TCA) insoluble material according to Fuhrman and Azam (7), ii) the phenol/chloroform procedure of Wicks and Robarts (25), and iii) the proposed hydroxylapatite procedure. Six replicates of water samples were processed for each procedure, and controls and DNA recoveries were run in triplicate and duplicate, respectively. For the hydroxylapatite procedure, the water samples were filtered on 0.2 μm Nuclepore polycarbonate filters and immediately placed in a liquid scintillation glass vial containing 0.5 ml lysing reagent. This reagent contained 4 mg/ml egg white lysozyme (grade 1, # L-6876, Sigma Chemical Co.) in 10 mM Tris buffer (pH 7.0), 100 mM NaCl, 10 mM EDTA, and 100 μg carrier DNA (salmon testes DNA, Type III, # D-1626, Sigma Chemical Co.). The vial was capped and frozen at least for 12 hours [field samples can be stored at this point]. At the end of this time the vial was thawed rapidly at 60°C for 3 min, then 1.5 ml 0.24 M MUP was added. MUP consisted of 0.24 M phosphate buffer, pH 6.8, containing 8 M urea. The contents of the vial were sonicated for 15 sec. (15) using the Sonic dismembrator (model 150, Artek Systems) with the

12 mm probe at 90 W (60% power setting). Purification of the DNA was accomplished according to Adriaenssens et al. (1) on hydroxylapatite (HA) columns (British Drug House, product 44257). HA was suspended in 0.014 M phosphate buffer, pH 6.8, and heated for 15 min. at 85°C. A small column was made using a 23 cm Pasteur pipette. Glass wool was used to retain the hydroxylapatite which was packed to a depth of 6 cm. Being careful not to be let the column go dry, 0.24 M MUP was pumped either onto a single column using a peristaltic pump or several columns can be processed at one time using a vacuum manifold. Five to ten ml was pumped through the column before the sample was added. The sample was allowed to pass through the hydroxylapatite column by gravity flow. Twenty ml 0.24 M MUP was passed through the column which is usually sufficient to wash out all non-DNA compounds (including protein and RNA). The HA was rinsed with 10 ml 0.014 M sodium phosphate (NaP) buffer (pH 6.8) to wash out the urea. DNA was eluted from the HA column with 0.48 M NaP. The 0.48 M NaP fraction was carefully collected and the volume measured (usually 10 to 11 ml). An two ml aliquot was placed in a glass scintillation vial, 150 µL concentrated HCl added followed by heating the sample for 30 minutes at 95°C. The sample was cooled and 10 ml of ACS II (Amersham) was added, and the radioactivity was counted on a Packard Model 4430 liquid scintillation counter. The disintegrations per minute (dpm) were automatically determined using the external standard procedure. The total radioactivity of the 0.48 M NaP fraction was taken as the amount of [^{3}H]-thymidine incorporated into the DNA in the original 10 ml of lake water.

Recovery of DNA from the hydroxylapatite column was determined by adding either [thymine-methyl-^{3}H] or [thymine-methyl-^{14}C]-labelled DNA (New England Nuclear, specific activity = 632.7 MBq/mg and Amersham, specific activity = 1.44 MBq/mg, respectively). Because of the high molar specific activity of ^{14}C-DNA which increases the rate of radiation decomposition, the specific activity was lowered by adding 10 mg E. coli DNA (Type VIII, # D-2001, Sigma Chemical Co.) dissolved in 4 ml 0.05 M borate buffer (pH 8.0). Each 50 µL aliquot of labelled DNA contained approximately 10,000 DPM ^{14}C-DNA and 100 µg unlabelled DNA. Recovery of ^{14}C-DNA dissolved in 0.5 M NaOH was also tested.
The lysing reagent efficacy was tested using liquid cultures of E. coli (ATCC 11775). Five ml of culture were filtered and treated as above. The released DNA was quantified fluorometrically according to Bruck (4) using E. coli DNA as the standard. A 0.5 ml aliquot was combined with 4.5 ml of Tris buffer (100 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.0) containing 20 nanogram/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

RESULTS AND DISCUSSION

Cell disruption and DNA recovery. The lysing technique (lysozyme, freeze-thaw and sonication) we used to disrupt the microorganisms in aquatic samples quantitatively extracted DNA from E. coli (ATCC 11775). We obtained a value of $1.48 \pm 0.11 \times 10^{-14}$ g DNA per cell. Literature values for log phase cultures of E. coli strains B and B/r are 1.37×10^{-14} and 1.82×10^{-14} g DNA per

cell, respectively (23). A value of 0.90 X 10^{-14} g DNA per cell of the B/r strain has also been reported (27). Assuming E. coli is an adequate test organism, this result verifies that the lysing technique is accurate for rupturing Gram-negative cell walls. Although we did not specifically test for the lysis of Gram-positive bacteria, many are also susceptible to lysozyme action (12). Gram-negative bacteria are the predominant forms found in most freshwater ecosystems (8). Short exposure (30 minutes) to trichloroacetic acid (TCA) is assumed to cause cell lysis, but microscopic examination of the cells have not confirmed this assumption (18). Extracting the filtered cells with NaOH (26) seemed an ideal procedure to lyse cells. However, when ^{3}H-DNA is dissolved in 0.25 N NaOH, neutralized, put in MUP buffer, and run through the hydroxylapatite procedure, a recovery of only 60% was obtained.

Recovery of tritiated DNA from hydroxylapatite columns. The radioactivity in the various buffers used to adsorb and elute ^{3}H-DNA from a hydroxylapatite column is shown in Table 1. The majority of the radioactivity (93.4%) is eluted with 0.48 M sodium phosphate buffer, as expected. The initial 0.24 M MUP buffer carried 5.5% of the radioactivity through the column. The manufacturer's technical information states a 3% single stranded DNA component at the time of shipment. Single stranded DNA does not have the same affinity for hydroxylapatite as native double stranded DNA (12) and therefore is eluted with the initial buffer. Protein and RNA are also eluted in this fraction (1,12).

Comparison of TCA, phenol/chloroform and hydroxylapatite.

Three of the commonly employed procedures were tested for thymidine incorporation into DNA, DNA recovery, radioactivity of controls and reproducibility. Table 2 shows that the cold-TCA precipitate contained the highest radioactivity in the sample as well as the control, the lowest sample coefficient of variation, and the best DNA recovery. However, many authors (21,22) have previously shown and we have also demonstrated (unpublished results) that macromolecules (protein, RNA and perhaps lipids) other than DNA are labelled in the presence of tritiated thymidine. The radioactivity retained by TCA precipitation would therefore be expected to be greater than methods which selectively recover only DNA. Riemann (18) has stated there seems no reason to differentiate the TCA insoluble material into specific macromolecular fractions, because of appreciably lower results. The phenol/chloroform procedure recovered 76.8% of the added tritiated DNA, but had very variable control values (89% C.V.) as well as variable sample values (29.1% C.V.). Other disadvantages, with our samples, were very slow filtration rates at the TCA ppt. step and often at the phenol/chloroform wash step, and the obnoxious smell of the phenol/chloroform reagent. The proposed hydroxylapatite procedure recovered 90.7% of the tritiated DNA, had very low consistent controls, and reasonable reproducibilities of sample values (11% C.V.). For stable liquid scintillation counting of the radioactivity in all procedures, we found it necessary to acid hydrolyse the DNA before addition of the fluor.

E. coli growth experiment. Figure 1 illustrates the logarithmic growth phase of an E. coli culture. Bacterial growth was monitored by increases in optical density, cell count, DNA concentration and incorporation of tritiated thymidine into DNA isolated on hydroxylapatite columns. The slopes of the lines generated from plotting the log n versus time indicate a close correlation between all variables except increases in cell number. Recently, Coveney and Wetzel (5) have shown changes in biovolume are more important than cell number changes when determining the conversion factors relating thymidine incorporation to bacterial growth.

The isolation of DNA labelled by tritiated thymidine on hydroxylapatite columns is an efficient technique which removes protein and RNA contaminants. The lysis of the microorganisms seems to be complete using lysozyme and sonication. The released native DNA is not subjected to harsh bases, acids or organic solvents, and therefore is readily adapted to field use. Controls, consisting of tritiated thymidine, are consistently low and recoveries of radioactive DNA are high and reproducible.

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TABLE 1. Recovery of ^{3}H-DNA from hydroxylapatite columns

Eluent buffer	Volume collected[a] (mL)	% Recovered
Run #1:		
0.24 M MUP	12.5	5.33[b]
0.014 M NaP	7.3	0.14
0.48 M NaP	10.5	93.13
Additional 2 ml	2.0	1.41
Run #2:		
0.24 M MUP	12.6	5.67[a]
0.014 M NaP	7.5	0.15
0.48 M NaP	10.5	93.82
Additional 2 ml	2.0	0.37

[a] Two ml from collected volume counted by liquid scintillation

[b] Probably single-stranded DNA, see Results and Discussion for details.

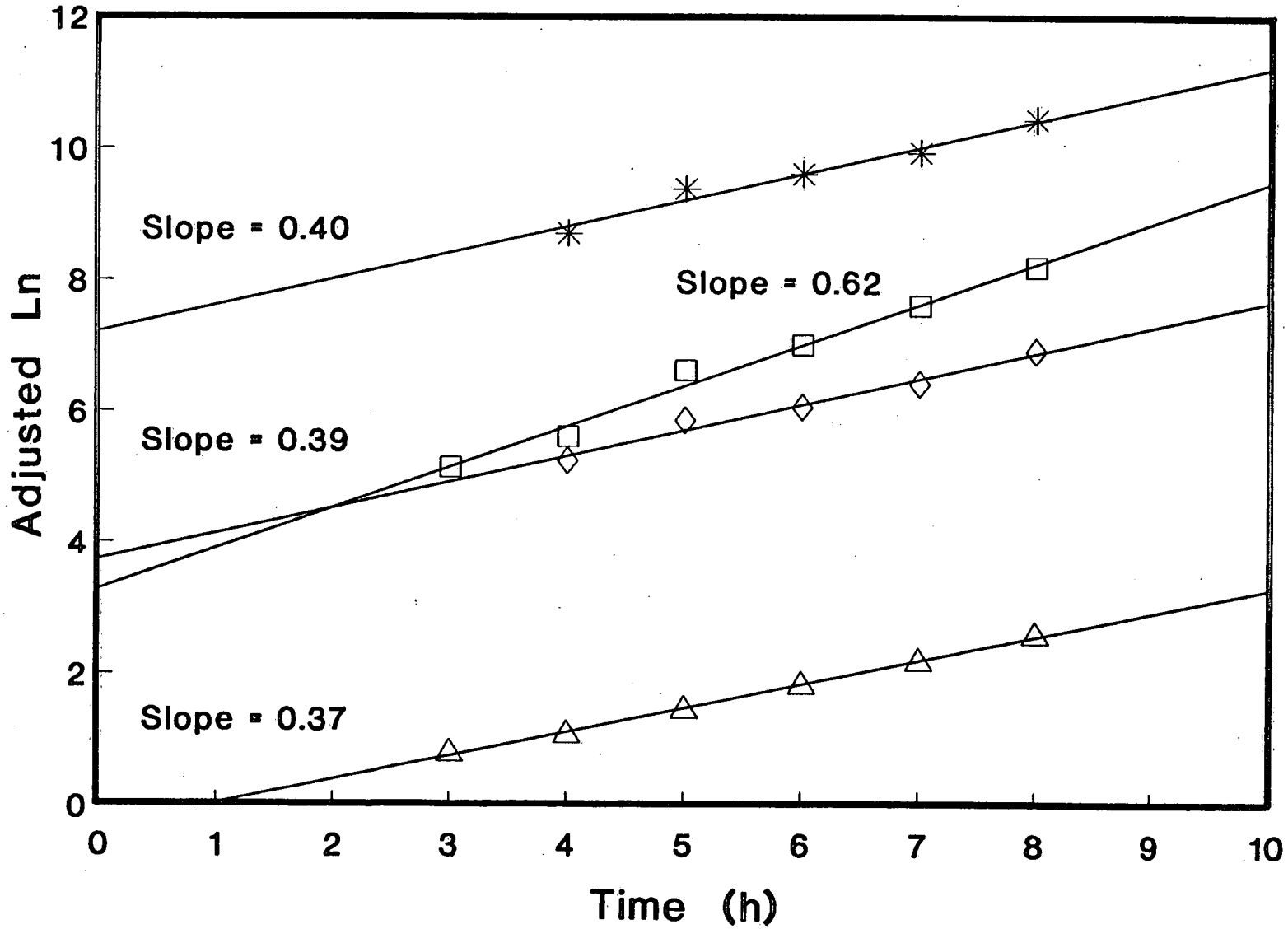
TABLE 2. Radioactivity of TCA precipitate, phenol/chloroform washed filter, and hydroxylapatite isolated DNA

	TCA ppt.	Phenol/ chloroform	Hydroxylapatite adsorption
Hamilton Harbour			
Surface water[a]	38726 (4.0)[b]	27205 (29.1)	29557 (11.0)
Control	2954 (58.2)	2123 (89.0)	500 (2.0)
% DNA recovery	91.8	76.8	90.7

[a] Samples corrected for control radioactivity and % DNA recovery.

[b] Coefficients of variation in parentheses; samples n=6, controls n=3, DNA recovery n=2.

FIG. 1. Increase in A_{550} (△), DNA concentration (◇), [^{3}H]TdR incorporation (*) and bacterial cell numbers (□) in a batch culture of E. coli with time.


Adjusted Ln
Time (h)
Slope = 0.40
Slope = 0.62
Slope = 0.39
Slope = 0.37
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9
10