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Evaluating bacterial activity from cell-specific ribosomal RNA content measured with oligonucleotide probes

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I. INTRODUCTION

Few methods are available to evaluate the distribution of activity among marine bacterial cells. Two approaches are the INT-formazan method[1,2] (see Padgett, this volume) and microautoradiography (see Carman, this volume). Both methods are laborious, and consequently neither is in common use. These methods are qualitative, i.e. cells are scored as positive (= metabolically active) or negative (= no evidence of metabolic activity). Both methods require incubation of bacteria with specific substrates. Sample manipulations associated with such incubations have been shown to perturb the composition, activity and growth rate of bacteria.[3,4] There is a pressing need for a quantitative approach to measuring cell-specific activity, preferably without requiring incubations.

In this chapter, we describe a procedure for measuring the cell-specific quantity of ribosomal RNA (rRNA) and DNA in order to evaluate the frequency distribution of activity among cells. The procedure is inherently quantitative, does not require sample incubation, and potentially can be taxon-specific. Fluorescently-labelled oligonucleotide probes are hybridized to the complementary 16S rRNA sequences in preserved, intact cells[5] (see DeLong, this volume). The resulting cell fluorescence is proportional to cellular rRNA content and can be measured with a microscope-mounted photometer system, or by other instrument systems (e.g. image analysis, flow cytometry). Similarly, DNA content is measured as fluorescence of cells stained with the DNA-specific fluorochrome DAPI. These are either prepared as separate samples for purposes of enumeration and DNA measurements, or are dual-labelled cells which are also hybridized with oligonucleotide probes. In the latter case, we can measure an rRNA:DNA ratio for individual cells.

A strong correlation between RNA content, or the RNA:DNA ratio, and growth rate has been demonstrated repeatedly in studies of copiotrophic "model" bacteria such as *Escherischia coli*, *Salmonella typhimurium*, and *Aerobacter aerogenes*, grown at rates ranging from 0.2-20 h^{-1}.[6-10] This correlation exists because the specific growth rate μ of bacteria is dependent on the cellular quantities and synthesis rates of ribosomal RNA, RNA polymerase and protein.[6,7] Nutrient limitation reduces rRNA synthesis rates, leading to a reduction in cellular rRNA content and consequently in protein synthesis required for growth.

We have found that rRNA $cell^{-1}$ and the RNA:DNA ratio are also highly correlated to specific growth rate for marine bacteria.[11] Ribosomal RNA was measured as probe fluorescence in individual cells, unlike the previous studies where measurements were based on extracted nucleic acids. Marine bacteria were grown in batch or chemostat culture at much slower specific growth rates (0.01-0.23 h^{-1}) which were comparable to published *in-situ* rates. Our data suggest that the RNA:DNA ratio is a more robust predictor of growth rate than rRNA $cell^{-1}$ alone, with a greater proportional response to changes in growth rate. Correlation coefficients between the RNA:DNA ratio and specific growth rate for 4 marine bacterial isolates were r^2 = 0.88, 0.98, 0.98, and 0.99 (n = 4 or 5 growth rates, $p < 0.01$). As yet, we are not able to convert a measured cellular rRNA content (or RNA:DNA ratio) to the equivalent specific growth rate in field samples: the relationship between RNA:DNA and growth rate appears to be species-specific, and we still lack data on the effects of environmental variables such as temperature. However, it is clear that rRNA content and the RNA:DNA ratio are robust measures of activity.

Oligonucleotide probes are inherently taxon-specific at some level, which depends on the degree to which the probe sequence is evolutionarily

conserved, and on the experimental conditions used to control hybridization stringency. In principle, taxon-specific oligonucleotide probes can be used to measure rRNA content in selected taxa within a mixed microbial community (see DeLong, this volume). To date, we have used mixtures of universal-level and eubacterial probes to measure the distribution of rRNA content for cultured marine bacteria, and for marine bacteria in coastal-water samples.

II. MATERIALS AND METHODS

(see also DeLong, this volume)

A. Equipment

Microphotometer (we use an Optical Technology Devices, Inc. MSA scanning microphotometer), or other instrument capable of measuring fluorescence intensity of individual bacterial cells

Epifluorescence microscope with Hg lamp

Vacuum pump and filtration manifold (e.g. Hoefer manifold)

Incubation chamber, 37°C

High-speed benchtop centrifuge

B. Supplies

Glass slides, cover slips

Glass beakers

0.2 μm Acrodisc (Gelman)

Calibration-standard microbeads (Flow Cytometry Standards Co.)

C. Solutions and reagents

Formalin

DAPI fluorochrome, 1 mg ml^{-1} in distilled water

Glycerol

100% and 95% ethanol

Gelatin (Bio-Rad, Richmond, CA)

Sodium dodecyl sulfate (SDS), 1%

Phenol

Chloroform

Isoamyl alcohol

$CrK(SO_4)_2$ (Aldrich Chemical, Milwaukee, Wis)

PBS: 1x PBS = 145 mM NaCl, 100 mM sodium phosphate, pH 7.5

Nonidet P-40 in distilled water (Sigma), 1% and 0.1%

TE buffer: 10 mM Tris, 1 mM EDTA, pH 8.0

SET: 5x and 0.2x concentrations

1x SET = 0.15 M NaCl, 20 mM Tris, 1 mM EDTA, pH 7.8

STE buffer: 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6

Hybridization solution: contains the following in 5x SET:

probe(s) (50 ng each/30 μL solution)

dextran sulfate (10%)

bovine serum albumin (0.2%)

polyadenylic acid (0.01%)

SDS (0.1%)

DNase-free RNase (Boehringer Mannheim, Indianapolis, In)

Calf thymus DNA standard (Sigma)

Escherichia coli ribosomal RNA standard (Sigma)

III. PROCEDURES

Procedures described here are modified from DeLong et al.[5] and Giovannoni et al.[12] Modifications introduced to the original methods include cell permeabilization, composition of the hybridization solution,

and dual-labeling of cells with DAPI in order to measure DNA as well as rRNA per cell.

A. Sample preparation

1. Cells are collected on a 0.2 μm Nuclepore filter and preserved immediately by dipping the filter in an ice-cold mixture of PBS and formalin (1:9 volumes of formalin:PBS, filtered through GS filter [Millipore, Bedford, Mass]). Samples are then stored at 4°C until processed.

2. Add 1 volume of 1% Nonidet P-40 to 9 volumes of the cell suspension. Incubate overnight at 4°C, in order to increase cell permeability.

3. Slide preparation:

a. Dissolve gelatin (final conc. 0.1%) in a boiling water bath.

b. After the gelatin cools to room temperature, add $CrK(SO_4)_2$ (final conc. 0.01%).

c. Filter the gelatin solution through a 0.2 μm Acrodisc.

d. Prepare clean glass microscope slides by dipping in 95% ethanol for ca. 2 min. Wipe and dry the slides.

e. Three to 4 drops of the gelatin solution are squirted onto a slide, spread with a clean applicator (e.g. cotton-tipped swab), and dried vertically in a rack overnight.

4. Add DAPI (final concentration 10 μg ml^{-1}) to the Nonidet P-40-treated

cell suspension. Allow to stain for 10 min. Collect cells by centrifugation (10,000 x g, 15 min), and resuspend in 0.1% Nonidet P-40. For convenience, the cell suspension should be adjusted at this time to about 5 x 10^7 cells ml^{-1}.

5. Smear about 10-15 μl of the cell suspension on the gelatin coated slide (ca. 1 cm diameter circle). Dry the smear at room temperature (normally > 1 hr).

6. Dehydrate the smear by sequential ethanol baths for 3 min each in 50, 80, and 100% ethanol, and dry again at room temperature.

B. Hybridization

1. We have oligonucleotides custom-synthesized with a 5' aminolink modifier (our oligonucleotides are synthesized by Oligos Etc., Inc., Wilsonville, OR). Oligonucleotides are conjugated with the fluorochrome Texas Red sulfonyl chloride as described by DeLong (this volume). Briefly, one volume of Texas Red sulfonyl chloride (Molecular Probes, Eugene, OR) dissolved in dimethyl formamide (10 $\mu g\ \mu l^{-1}$) is mixed with 5 volumes of the oligonucleotide dissolved in 50 mM borate buffer (pH 9.2), and incubated overnight at 22°C. Fluorochrome-conjugated oligonucleotides (fluorescent probes) are purified from unconjugated fluorochromes and oligonucleotides without conjugated fluorochrome. Sephadex G-25 (Pharmacia, Uppsala, Sweden) column chromatography and polyacrylamide gel electrophoresis are used for the purification of the fluorescent probes (see DeLong, this volume).

2. Thirty μl of the hybridization solution is gently pipetted onto the cell smear, and a siliconized cover slip is placed on the droplet.

3. Slides are incubated overnight (15-18 hr) at 37°C on a raised bed with a small volume of 5x SET at the bottom in an air-tight container.

4. After the incubation, the cover slips are gently removed by dipping in 5x SET, and the slides are washed 3 times in 0.2x SET bath at 37°C for 10 min each time.

5. After the wash, the slides are dried at room temperature. Care is taken to handle the fluorochrome under subdued light at all times.

6. Three to 4 drops of mountant (9 volumes of glycerol mixed with 1 volume of 1X PBS) are dropped on the cell smear and covered with a cover slip. Excess mountant is removed by lightly pressing the slide against absorbant paper tissue.

7. Slides are stored in the dark, either at 4°C for several days or at -70°C for long-term storage.

C. Measurement of rRNA and DNA by microphotometry

i. Instrument

We measure fluorescence of individual cells with a scanning microphotometer (Optical Technology Devices, Inc.) mounted on a Nikon Optiphot microscope. Photometer output is passed to a Metrabyte analog/digital converter and recorded on an IBM-compatible computer. The microphotometer incorporates a target-spotting aperture system which precisely defines the area over which fluorescence intensity is measured. Detector apertures of 0.09 mm to 0.35 mm are used to restrict the target area to a minimum of 1 um diameter at 1250x total magnification. The microphotometer has been modified such that its photomultiplier detector

is parfocal with the binocular eyepieces. Thus, the operator can simply center a cell or cell-free background area at the target location (for example, using an ocular grid) while viewing the field normally. Automatic scanning and recording of excitation wavelength and fluorescence intensity are triggered by appropriate single-key commands at the computer keyboard. Background fluorescence associated with fluorescence of the mounting medium and glass is measured and automatically subtracted from cell fluorescence.

ii. Instrument calibration and measurement

Calibration-standard fluorescent microbeads (e.g. from Flow Cytometry Standards Co.) are used to correct for day-to-day fluctuations in instrument readings, which result from variations in line voltage, lamp output, and gain and sensitivity settings. Daily standardization is absolutely essential, because day-to-day variation in photometer output is substantial.

1. Establish a reference calibration curve by measuring mean bead fluorescence at full excitation intensity, then at lower intensities using combinations of neutral-density filters to achieve 1/2, 1/4, and 1/8 intensity levels. Mean bead fluorescence should be linearly related to intensity with a zero-intercept. This reference curve should be repeated periodically.

2. Daily calibration can consist of a single-point measurement of the mean fluorescence of standard beads ($n \approx 100$) at full excitation intensity.

3. Fluorescence of cells in samples is measured at emission wavelengths of 455 nm (for DNA by DAPI fluorescence) or 600 nm (for RNA by Texas Red

fluorescence). About 150-180 cells are measured for each subsample.

4. Daily measurements of cell fluorescence are expressed as fractions or multiples of the mean fluorescence of standard bead on that day. Measurements expressed as "bead units" are reproducible and comparable among days and samples.

D. Calibration of probe and DAPI fluorescence

We obtain independent estimates of RNA $cell^{-1}$ using ethidium bromide (EtBr) fluorometry, and construct the regression of EtBr-measured RNA $cell^{-1}$ on probe fluorescence. This regression is used thereafter to convert fluorescence measurements to rRNA $cell^{-1}$. Similarly, the regression of EtBr-measured DNA $cell^{-1}$ on DAPI fluorescence is used to convert fluorescence to DNA $cell^{-1}$. Note that although the RNA extraction procedure described below isolates both rRNA and other forms of RNA, nearly all (ca. 90%) of cellular RNA is ribosomal. Therefore, calibration of probe fluorescence (measures rRNA) against EtBr-RNA (measures total RNA) introduces very little bias. Procedures similar to the following can be used to calibrate the user's particular instrument.

1. Cultures of marine bacterial isolates are grown at various rates, using batch or chemostat culture methods. Samples are collected on 0.2 μm Nuclepore filters.

2. The cells are resuspended in a small volume of ice-cold STE buffer, then a subsample is taken to estimate (by AO or DAPI direct counts, see chapters in this volume) the total number of cells collected. Samples are kept at < 6°C during handling.

3. The cells are pelleted (10,000 x g, 15 min), and stored for < 2 wk at

-70°C until nucleic acid extraction.

4. Cell pellets are resuspended in TE buffer and cells are lysed with 1% SDS.

5. The lysate is extracted once with an equal volume of phenol-chloroform-isoamyl alcohol mixture (25:24:1, equilibrated with STE), and once with an equal volume of chloroform. In each extraction, the aqueous phase is transferred to the next step while the phenol phase is discarded. Material at the interface between the aqueous phase and the phenol mixture is carried to the chloroform extraction, in order to minimize the possible loss of nucleic acid during the extraction.

6. The final aqueous phase is precipitated overnight with 2 volumes of ethanol and ammonium acetate (final conc. 2.3 M) at -20°C.

7. The precipitate is collected by centrifugation at 10,000 x g for 15 min. The pellet is briefly rinsed with 70% ethanol, and a small volume of TE buffer is added.

8. The solution is stored at 4°C for 1 day. The pellet is thoroughly dissolved by vortexing occasionally.

9. After dissolution, subsamples of the nucleic acid extract are digested with DNase-free RNase (Boehringer Mannheim, Indianapolis, In) for 2 hrs at 37°C (DNA sample). The untreated extract (containing both DNA and RNA) and the RNase-digested samples (containing DNA only) are stored at -70°C until fluorometric measurement.

10. Three to 12 μl of the DNA or DNA+RNA samples are added to 3 ml of TE with 1 μg of EtBr.

11. Fluorescence is measured in duplicate or triplicate samples using a Perkin-Elmer spectrophotometer (excitation 300 nm, emission 600 nm).

12. Standard curves for DNA or RNA are generated with known amounts of calf thymus DNA (Sigma) or *Escherichia coli* ribosomal RNA (Sigma), over the concentration range of 0.1-0.8 ug DNA (or RNA) ml^{-1}. Standard curves generated with RNase-digested calf thymus DNA showed no difference from the curves generated with untreated calf thymus DNA.

13. The standard curves are used to convert fluorescence of DNA or DNA+RNA samples to the equivalent weight of DNA or RNA. The amount of DNA in a sample is calculated directly from the fluorescence of the RNase-digested DNA sample. The amount of RNA is calculated by difference using the disappearance of fluorescence after RNase digestion: i.e. the difference between fluorescence in the undigested DNA+RNA sample and in the matching, RNase-digested DNA sample. DNA or RNA content per cell is then calculated by dividing the total amount of DNA or RNA by the total number of cells in the extract.

14. Extraction efficiency is checked by determining the total recovery of known quantities of DNA and RNA standards added at concentrations equivalent to the nucleic acid concentrations in samples, and processed identically to normal samples. We have found that extraction efficiency is relatively high (ca. 75-100%) using this procedure.

E. Example of rRNA frequency distribution data

Figure 1A shows the distributions of rRNA $cell^{-1}$ at four growth rates, from a chemostat culture of a γ-purple bacterium isolated from the Sargasso Sea water column (50 m). The mean rRNA $cell^{-1}$ for this isolate was highly correlated to specific growth rate (μ = 0.01-0.23 h^{-1}, r^2 =

0.996). Figure 1B shows the frequency distributions of DNA cell^{-1} for the same samples of this isolate.

IV. NOTES AND COMMENTS

1. The primary obstacle to measuring rRNA cell^{-1} in natural bacterial assemblages is the low rRNA content of many slow-growing cells. We have found that with a single probe, as few as 33% of bacteria in coastal water samples are visibly labeled.[13] The fluorescence of the dimmest visibly-labelled cells falls below the detection limit of the microphotometer. However, it is possible to design and utilize multiple probes with comparable levels of specificity.[13] Since each probe acts independently of the others, the fluorescence per rRNA increases in additive fashion with increasing number of probes. Using this approach, we have found that the percent of natural bacterial cells (Long Island coastal water, in spring) which contain a measurable quantity of rRNA increases asymptotically with increasing number of probes (up to 5) to ca. 75%. Even in these relatively eutrophic coastal waters, the remaining ca. 25% of cells contain extremely little rRNA, i.e. less than our current detection limit of 0.24 fg rRNA cell^{-1}. The majority of cells which remained unmeasurable were coccoidal and relatively small.

2. The procedures described here should be readily applicable to other instruments such as image analysis systems (see Verity and Sieracki, this volume) and flow cytometers (see Olson and Zettler, this volume; Button and Robertson, this volume). Although the photometry system we use is designed to have excellent sensitivity to low-fluorescence signals, alternative instruments may provide greater sensitivity or faster sample processing times.

3. Calibration of probe fluorescence: We used the regression line

obtained from culture studies to estimate RNA cell^{-1} from probe fluorescence in a natural bacterial community sample. When compared to independent measurements of RNA cell^{-1} using EtBr fluorometry, the two estimates consistently agree to within 15%.[13] We conclude that our culture-based calibration of probe fluorescence is robust and applicable to natural samples.

4. The fluorochrome 4',6-diamidino-2-phenylindole (DAPI) intercalates between adenosine and thymine base pairs in DNA. Because DAPI is AT-specific, it can be used to measure the relative DNA content per cell if the exact GC:AT ratio is unknown. If the GC:AT ratio is known, measured or assumed (e.g. to be 1:1), then the absolute DNA content per cell can be estimated. It may be possible to use AT-specific and GC-specific fluorochromes simultaneously to measure the GC:AT ratio directly in natural bacterial cells.

5. Nucleic acid samples prepared for EtBr fluorometry by the method described above were clean enough for RNase and DNase digestion. When checked by agarose gel electrophoresis, characteristics bands of DNA or RNA disappeared completely after 2 h digestion with DNase or RNase, respectively.

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