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# Comparison of Nucleic Acid Hybridization and Fluorometry for Measurement of the Relationship between RNA/DNA Ratio and Growth Rate in a Marine Bacterium

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**Continuous culture of *Pseudomonas stutzeri* Zobell, a marine denitrifying bacterium, was used to determine the relationship between growth rate and nucleic acid content. The trend of decreasing RNA content with decreasing growth rate, well known for enteric organisms, was found to occur in *P. stutzeri* Zobell as well, even at very long generation times such as those thought to occur in the oligotrophic ocean. When assayed by ethidium bromide fluorescence, the total RNA/DNA ratio was linear for generation times between 6 and 60 h. We also developed a 200-bp nucleic acid probe (with species-specific potential) for a portion of the 23S rRNA gene of *P. stutzeri* Zobell, which was used to quantify rRNA and rDNA by hybridization in the same continuous cultures. The rRNA/rDNA ratio also exhibited a decrease with decreasing growth rate, but the relationship, although significant, was not simply linear. The sensitivity and accuracy of the two methods are compared, and the potential for species specificity in future hybridizations is discussed.**

Microbial transformations are essential for carbon, nitrogen, and sulfur cycling in the ocean (13, 18, 30). Thus, bacteria are extremely important members of marine ecosystems. At present, the vast majority of the bacteria that exist in the ocean have been neither cultured nor characterized (2). Fundamental questions remain about how many different bacterial species are represented in a natural seawater assemblage and whether their ecology and activity are adequately described by using methods which yield average measures and thus assume homogeneity within the population. Are these bacterial cells in the ocean starved or actively growing? Are different species experiencing different growth rates under a given set of environmental conditions? Species-specific differences in growth rates are expected in the ocean, given variation in growth parameters among cultured bacteria. However, current methods for measurement of growth rates of natural bacterial populations cannot detect whether one species is rapidly dividing while another species is approaching senescence. Such differences in situ would have direct implications for biogeochemical processes which are performed by various metabolic groups of bacteria. To address the question concerning growth of individual members of the bacterial assemblage, we investigated the rRNA content of a marine bacterium as a means of developing a species-specific growth rate assay for natural samples.

The linear relationship between RNA content per cell and growth rate in eubacteria is well established (11, 20a, 23, 26, 28). A similar relationship has been demonstrated for eukaryotic algae and metazoans (6, 29). For the most part, these relationships were derived from organisms growing at near-maximal growth rates. It is not clear, however, whether this relationship between RNA and growth rate will extend to the slower growth rates more typical of nutrient-limited environments. Previous work in this area had used the commonly studied enteric strains *Escherichia coli* and *Salmonella typhimurium* with doubling times of 20 to 240 min. Typical bacterial doubling times for the bacterial assemblage as a whole in the ocean are believed to be on the order of 24 h (9).

This study was initiated to assess the relationship between the RNA content per cell and growth rate of a marine bacterium (*Pseudomonas stutzeri* Zobell) doubling at rates which we believe are representative of the marine environment. A significant positive relationship between the RNA/DNA ratio and growth rate was observed throughout the entire range of growth rates tested, using both ethidium bromide (EtBr) fluorescence and DNA hybridization methods for quantification of nucleic acids. *P. stutzeri* Zobell was chosen for these experiments because this strain was originally isolated from seawater and marine muds and is a member of the most commonly isolated denitrifying genus in soil and aquatic systems (10, 31). While denitrification is not the focus of this work, it provides a biogeochemical basis for relevance of growth rate measurements to oceanic processes and is an example of the many biogeochemical transformations for which bacterial growth rates are important determinants. Assessing this relationship between rRNA and growth rate in *P. stutzeri* Zobell establishes the feasibility of using RNA/DNA hybridization with species-specific probes to assay for growth rate of this bacterium in mixed culture and ultimately the marine environment.

## MATERIALS AND METHODS

**Strains and culture conditions.** *P. stutzeri* Zobell (ATCC 14405) was grown in continuous culture under carbon limitation on rich medium (5 g of Bacto Peptone, 100 mg of yeast extract [Difco Laboratories] per liter of seawater). Carbon concentration was controlled by adjustment of the fresh medium inflow rate to the culture vessel. Culture density was estimated at intervals (once or twice per day) by

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measuring optical density at 600 nm. In addition, samples from the continuous cultures were counted directly by using epifluorescence microscopy and acridine orange (17). Optical density was found to be directly correlated to cell number ($r^2$ = 0.96). Culture purity was assessed by fluorescence microscopy and plating on rich medium. Each culture was maintained at steady state for 2 to 10 generations before the cells were harvested for nucleic acid determinations. The growth rate at steady state was calculated by using standard chemostat equations (15). A 1.5-ml sample of steady-state culture ($6 \times 10^8$ to $5 \times 10^9$ cells) was pelleted in microcentrifuge tubes at 16,000 × *g* for 3 min (Brinkmann model 5415 microcentrifuge). The pelleted cells were immediately placed at −70°C without further treatment until analysis.

**Extraction of RNA and DNA.** All reagents were treated with 0.1 to 1.0% diethyl pyrocarbonate to inhibit RNases. The extraction procedure was a modification of the lysis protocol for plasmid purification in *E. coli* (22). Cell pellets were resuspended in 200 μl of cold 50 mM glucose–10 mM EDTA–25 mM Tris (pH 8.0). The same solution (100 μl) containing 10 mg of lysozyme per ml was added along with 100 μl of 500 mM EDTA (pH 8.2). The tubes were incubated at room temperature for 10 min. The cells were lysed with 50 μl of 10% sodium dodecyl sulfate (SDS). The tubes were vigorously shaken by hand, and the cell lysate was immediately extracted twice with 800 μl of phenol-chloroform-isoamyl alcohol (25:24:1) stabilized with 0.1% 8-hydroxyquinoline. The tubes were mixed by vortexing to form an emulsion. The organic phase was separated by centrifugation at 16,000 × *g* for 3 min. Fifty microliters of 3.0 M sodium acetate was added to the aqueous phase, and the nucleic acids were precipitated by addition of 1,000 μl of 100% ethanol. Nucleic acids were pelleted at 16,000 × *g* for 15 min at room temperature. The pellet was dried under vacuum and resuspended in diethyl pyrocarbonate-treated $H_2O$. Extracted nucleic acids were visualized by electrophoresis in 1% agarose minigels in 1× TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA [pH 7.8]). Gels were run at 125 to 200 V for 45 to 90 min.

**Quantification of RNA and DNA by fluormetry.** The nucleic acids from four replicates corresponding to seven different growth rates were purified by phenol extraction and ethanol precipitation as described above. When purified RNA was required, DNA was removed from the nucleic acid mixture by digestion with RNase-free DNase (Boehringer Mannheim, Indianapolis, Ind. [BM]). Purified DNA was obtained by digestion with DNase-free RNase (BM). Digestion with DNase was in 100 mM sodium acetate (pH 5.0)–5 mM $MgSO_4$ at 37°C for 3 h. Digestion with RNase was in $H_2O$ at 37°C for 3 h. A 10-μl aliquot of each sample was visualized on an agarose gel to assess whether total degradation of RNA or DNA was obtained without degradation of DNA or RNA, respectively. Seven microliters of a 10-mg/ml EtBr solution was added to 500 μl of both the RNA and DNA fractions (~1 μg). The nucleic acids were quantified by fluorometry (Farand model 3) with excitation at 260 nm and emission at 590 nm. Since we lacked suitable nucleic acid standards, RNA/DNA ratios were calculated on the basis of fluorescence intensity. Because the relative fluorescence intensity of RNA is lower than that of DNA, it was necessary to estimate a specific fluorescence coefficient for RNA before generating RNA/DNA ratios. Le Pecq and Paoletti (21) measured fluorescence at 590 nm on 0.01 to 10 μg of yeast and rat liver RNA and calf thymus DNA, using EtBr. A ratio of the slopes from Le Pecq and Paoletti's data adjusts for the relative fluorescence intensity difference of RNA and DNA (slope DNA/slope RNA = 2.82). Therefore, all RNA fluorescence intensity values were multiplied by 2.82. The RNA/DNA ratios reported are based on this corrected fluorescence intensity, and the absolute amounts of RNA or DNA were not determined.

**Labeling of digoxygenin probes.** Digoxygenin probes of a uniform length and label incorporation were generated via the polymerase chain reaction (PCR) (27). A 200-bp *Hin*dIII fragment from the 5′ end of the *P. stutzeri* Zobell 23S rRNA gene was subcloned into pBluescript (Stratagene, La Jolla, Calif.) as described previously (19). The resulting plasmid, pPE H303, was purified by alkaline lysis miniprep (22). One-fifth of the miniprep plasmid (~1 μg) was cleaved with *Pvu*II, and the insert DNA was separated from the vector by gel electrophoresis. The insert DNA was purified from the agarose by binding to a silica matrix (Geneclean; Bio 101, La Jolla, Calif.) and resuspended in 200 μl of $H_2O$. One microliter (~300 pg) of the purified insert was combined with 0.5 U of AmpliTaq (Perkin-Elmer/Cetus, Norwalk, Conn.), 100 ng each of the M13 forward and reverse or the SK and KS pBluescript sequencing primers (Stratagene), 2 nmol of digoxygenin-dUTP (BM), 15 nmol of deoxynucleoside triphosphates, 10 μl of 10× of PCR buffer (100 mM Tris [pH 8.3], 500 mM KCl, 0.1% gelatin, 4.5 mM $MgCl_2$), 80 μl of $H_2O$, and 60 μl of mineral oil. The amplification was for 40 cycles with a 1-min denaturation at 94°C, 1.5-min reannealing at 45°C, and 2-min extension at 72°C. The last step in the amplification was a final 5-min extension at 72°C to complete elongation. The probe could be used directly without further purification.

**Slot blotting.** The treatment of samples varied depending on whether RNA or DNA was to be detected on the slot blot. For RNA samples, 5 μl of a total nucleic acid extract (~150 ng) was combined with 5 U of RNase-free DNase (BM), 1 μg of poly(A) RNA, and 1 U of Inhibit-Ace (5′→3′ Prime Inc., Boulder, Colo.) in 50 μl of 100 mM sodium acetate (pH 5.0)–5 mM $MgSO_4$. The sample was incubated at 37°C for 3 h. Twenty microliters of 37% formaldehyde and 30 μl of 24× SSPE (24× SSPE = 4.32 M NaCl, 0.24 M $NaPO_4$, and 0.24 M EDTA [pH 7.5]) was added to each sample. The RNA was denatured by incubation at 60°C for 15 min. The sample was immediately filtered via moderate vacuum (125 to 175 mm Hg). Each sample tube and tip was washed with 100 μl of 12× SSPE containing a trace amount of methylene blue, and this wash was added to the appropriate slot.

For DNA samples, 20 μl of a total nucleic acid extract (~600 ng) was combined with 10 μl of $H_2O$ containing 2 to 5 μg of RNase per ml and 10 U of *Eco*RI (BM). The sample was incubated at 37°C for 3 h. One hundred microliters of 6× SSPE–30% formamide was added to each sample. The DNA was denatured in boiling water for 15 min. The samples were plunged into ice water and then immediately filtered. Each sample tube, tip, and slot was washed as described above.

The faintly blue slots on each slot blot were demarcated by placing holes in the filter on both sides of the slot, using a 23-gauge needle. Nucleic acids were bound to the membranes by baking under vacuum at 60 to 80°C for 0.5 to 2 h. Internal standards of purified *P. stutzeri* Zobell total RNA and genomic DNA were included on each slot blot (see below for quantification).

**Hybridizations.** Blots were prehybridized for 1 h in 6× SSPE–50% formamide–5% blocking reagent (BM)–0.1% Sarkosyl–0.02% SDS. Hybridizations were performed overnight at 61°C with probe concentrations of ~100 ng/ml. The filters were washed twice for 30 min in 2× SSPE–0.1% SDS and twice for 30 min in 0.2× SSPE–0.1% SDS at 65°C. A 1.0%

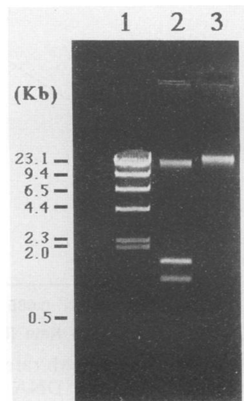


FIG. 1. Agarose gel of nucleic acid extracts from laboratory cultures of *P. stutzeri* Zobell. The molecular sizes of the lambda standard are indicated. Lanes: 1, lambda *Hin*dIII molecular weight marker (500 ng); 2, 10 μl of total nucleic acid extract; 3, 10 μl of total nucleic acid extract treated with RNase.

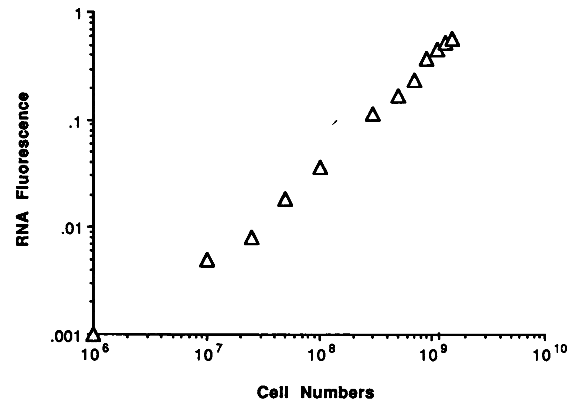


FIG. 2. RNA pool quantified by using EtBr versus total number of cells extracted. Cell numbers ranged from $1 \times 10^6$ to $1.5 \times 10^9$. The least-squares fit for the data is RNA fluorescence units = $-3.4 \times 10^{-3} + 3.9 \times 10^{-10}$ (cell numbers) ($r^2 = 0.994$, $n = 12$).

diethyl pyrocarbonate treatment was found to work best when the hybridization mix was boiled for 15 min and quickly cooled in ice water. All hybridizations were performed in plastic bottles, using a Wheaton roller culture apparatus (model 348922) placed in a small oven with variable temperature control (25 to 68°C).

**Detection of probes and quantification of hybridization.** Digoxygenin probes were detected by using either the protocol supplied in the Genius kit (BM) or the fluorescent alkaline phosphatase substrate AttoPhos (JBL Scientific, San Luis Obispo, Calif.). Quantification of hybridized probe was achieved by carefully trimming away excess filter from the slots. Each slot (3 to 4,600 pg of target nucleic acid) was placed in 1.5-ml microcentrifuge tubes with 350 μl of AttoPhos. The tubes were incubated at room temperature for 8 h in the dark. Before analysis, an additional 450 μl of methanol was added to each tube to elute the reacted AttoPhos from the filters. The fluorescence intensity of the solubilized substrate was read on a Farand MK 2 fluorometer. The linearity and sensitivity of this method were determined with digoxygenin-labeled pBR328 DNA supplied in the Genius Kit (BM) and with purified *P. stutzeri* Zobell total RNA and genomic DNA as internal standards on each slot blot. This quantification method has been shown to have a linear range >1,250 pg and detection limits of 3 pg (20). The *P. stutzeri* Zobell DNA internal standards were independently quantified by Hoechst dye 33258 fluorescence, using a TKO 100 minifluorometer (Hoefer Scientific Instruments, San Francisco, Calif.), and *P. stutzeri* Zobell total RNA was quantified by the orcinol method (14).

## RESULTS

**Extraction of nucleic acids.** Total nucleic acids were extracted from laboratory cultures of *P. stutzeri* Zobell by the extraction procedure described above. Extracts were visualized by agarose gel electrophoresis with EtBr staining. A single high-molecular-weight band and three lower-molecular-weight bands were clearly visible in the gel (Fig. 1). The bands exhibited no smearing, indicating negligible degradation. The addition of RNase confirmed that the lower-molecular-weight bands were RNA (Fig. 1, lane 3).

The effect of sample size on our nucleic acid extraction procedure was determined on the RNA pool by using serial dilutions of a *P. stutzeri* Zobell laboratory culture. The RNA pool was chosen for this study because it is more subject to cleavage than is DNA. We reasoned that extraction of the DNA pool would prove to be less variable than extraction of the RNA pool. Therefore, if extraction of RNA was reproducible, extraction of DNA would also be reproducible. RNA was quantified only in relative terms as amount of RNA-EtBr fluorescence. The amount of RNA fluorescence was linearly related to the number of cells extracted (RNA fluorescence units = $-3.4 \times 10^{-3} + 3.9 \times 10^{-10}$ [cell numbers] [$r^2 = 0.994$, $n = 12$]; Fig. 2).

**RNA content as an indicator of growth rate.** The relationship between RNA content per cell and growth rate in *P. stutzeri* Zobell was determined on cells from steady-state conditions (known growth rate). Seven separate doubling times, ranging from 6 to 60 h, were achieved by using continuous culture techniques. Cell densities at each steady state in the culture vessels ranged from $4.2 \times 10^8$ at a doubling time of 6 h to $2.1 \times 10^9$ ml$^{-1}$ at a doubling time of 60 h. The nucleic acids were extracted from four subsamples at each growth rate, containing $6 \times 10^8$ to $5 \times 10^9$ steady-state cells. The RNA and DNA were quantified fluorometrically by using EtBr as described above. After correcting for the specific fluorescence of RNA, an RNA/DNA ratio was calculated on the basis of fluorescence intensity. This RNA/DNA ratio decreased as the specific growth rate decreased, indicating a reduction of RNA content in more slowly growing cells. In addition, the RNA/DNA ratio was found to be linearly related to the specific growth rate throughout the entire range tested (Fig. 3).

To verify the EtBr data, a method to quantify the RNA/DNA ratio by using a chemically labeled (nonradioactive) DNA probe was developed (20). Nucleic acid extracts from six of the continuous cultures were quantified by probing with a 200-bp *Hin*dIII fragment subcloned from the 5′ end of the *P. stutzeri* Zobell 23S rRNA gene. Standards of purified *P. stutzeri* Zobell RNA and DNA were included with the continuous culture samples. Triplicate samples from each cell extract at a given growth rate were assayed by using the 200-bp DNA probe. The concentration of RNA and DNA in each sample was calculated on the basis of the fluorescence signal from the internal standards. Coefficients of variation were <7% for most nucleic acid measurements. The exceptions were DNA measurements with coefficients of variation

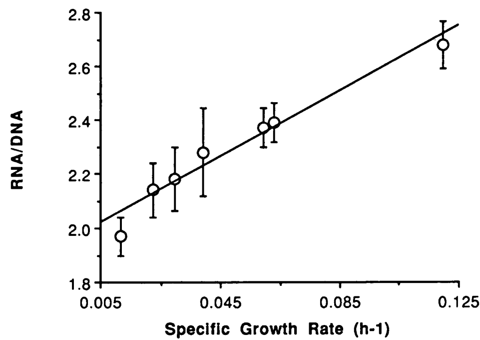


FIG. 3. RNA/DNA ratio versus specific growth rate of *P. stutzeri* Zobell cells grown in continuous culture with doubling times of 6 to 60 h. Nucleic acids were measured by using EtBr. Error bars indicate standard deviation of the ratio (24). The least-squares fit for the line is RNA/DNA ratio = 2.0 + 6.4 (specific growth rate) ($r^2$ = 0.96, $n$ = 28, i.e., four replicates at each growth rate).

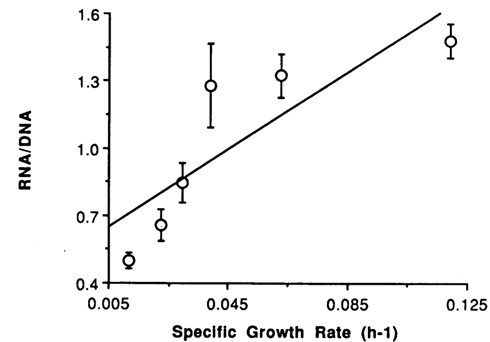


FIG. 4. RNA/DNA ratio versus growth rate of *P. stutzeri* Zobell cells. Nucleic acids were measured by DNA hybridization. Error bars indicate standard deviation of the ratio (triplicate measurements of one sample at each growth rate) (24). The least-squares fit for the line is RNA/DNA ratio = 0.60 + 8.6 (specific growth rate) ($r^2$ = 0.71, $n$ = 6).

of 10 and 13% for samples with specific growth rates of 0.022 (doubling time = 31 h) and 0.039 (doubling time = 18 h), respectively. One RNA measurement had a coefficient of variation of 10% for the samples with a specific growth rate of 0.030 (doubling time = 24 h).

An RNA/DNA ratio based on mass was calculated by using the *P. stutzeri* Zobell internal standards of total RNA and DNA (Table 1). The RNA/DNA ratio generated by this hybridization method also exhibited a decrease when the growth rate decreased (Fig. 4). The relationship between RNA/DNA and growth rate with use of the DNA probe did not appear to be a simple linear relationship with respect to growth rate. The best fit to the data is logarithmic and described by the following equation: RNA/DNA ratio = 2.6 + 1.1 × (log [specific growth rate]) ($r^2$ = 0.89). However, additional scatter in the probe data is to be expected since the hybridization method is a multistep process, whereas the EtBr method is a single-step process. We have approximated the relationship by using a least-squares linear regression, since a logarithmic model implies minimal changes in RNA/DNA ratios at both very high and very low specific growth rates.

TABLE 1. Quantitation of internal standards of *P. stutzeri* Zobell total RNA and genomic DNA from probed slot blots

| Nucleic acid | Amt (ng) | Intensity at 560 nm[a] |
|---|---|---|
| RNA | 125 | 660 |
| | 94 | 470 |
| | 63 | 279 |
| | 25 | 108 |
| | 12 | 72 |
| | 0 | 45 |
| DNA | | |
| | 270 | 960 |
| | 203 | 710 |
| | 135 | 438 |
| | 54 | 163 |
| | 27 | 103 |
| | 0 | 39 |

[a] Least-squares linear regressions for RNA ($r^2$ = 0.985) and for DNA ($r^2$ = 0.995).

## DISCUSSION

These two independent means of assessing rRNA content of *P. stutzeri* Zobell cells clearly indicate a reduction in cellular ribosomes with decreasing growth rate, as is commonly observed. The relationship that we report between RNA/DNA ratio and growth rate for *P. stutzeri* Zobell (as determined from the EtBr assay) is very similar to relationships previously reported for other bacteria (1, 20a, 23, 26). This historical data set includes three different bacteria, doubling at near-maximal growth rates. The nucleic acids in the historical studies were measured by colorimetric techniques or UV absorption (3, 4). The slopes from the *P. stutzeri* Zobell data set and the historical data are not significantly different (Student's $t$ test, $P < 0.50$), and the data have been pooled in Fig. 5. The *P. stutzeri* Zobell data that we report are a significant extension of the RNA/DNA ratio-growth rate relationship to much slower growth rates. The fluorometry data support extension of this macromolecular composition relationship to very low growth rates, such as those thought to occur in the marine environment.

The similarity between RNA/DNA ratio and growth rate for these four bacterial species implies a fundamental relationship between macromolecule composition and bacterial growth rate (1). This quantitative relationship appears to

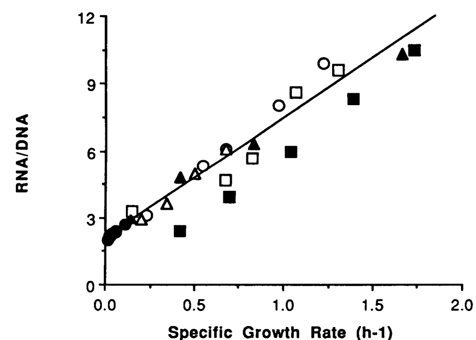


FIG. 5. RNA/DNA ratio of *P. stutzeri* Zobell and a historical data set versus specific growth rate. Symbols: △, *Aerobacter aerogenes* (23); ▲, *S. typhimurium* (20a); □, *A. aerogenes* (26); ○, *S. typhimurium* (26); ■, *E. coli* (1); ●, *P. stutzeri* Zobell (this study). The least-squares fit for the entire data set is RNA/DNA ratio = 2.0 + 5.2 (specific growth rate) ($r^2$ = 0.922).

apply to all bacteria thus far examined and to extend over a wide range of growth rates. While we do not know with any certainty the taxonomic composition of natural marine bacterial assemblages, it is widely thought that most are gram negative organisms in the phylum *Proteobacteria*. Thus, we might expect that an RNA/DNA ratio would be a useful indicator of growth rate for many marine bacteria. Laboratory studies such as the one described here could be used to produce calibration curves for different bacteria or groups of bacteria. The calibration could then be used to derive the in situ growth rate from the theoretically simple measurement of the RNA/DNA ratio by using species-specific probes in the natural population.

The study that we have described represents the first attempt to determine RNA/DNA ratios of any marine bacterium at growth rates realistic for the open ocean environment. The EtBr data that we obtained for *P. stutzeri* Zobell at our lowest measured specific growth rate ($\mu$ = 0.01; doubling time = 60 h) indicate a ratio of total RNA to DNA of approximately 2. Assuming that the measured DNA value corresponds to one cellular chromosome and that all of the RNA measured in our assay was rRNA, the EtBr data indicate that the relative rRNA mass would be nearly equivalent to the mass of two chromosomes. The size of the *P. aeruginosa* genome has been estimated at $5.9 \times 10^6$ bp (25). If *P. stutzeri* Zobell's genome is of comparable size, the RNA content at $\mu$ = 0.01 is therefore $11.8 \times 10^6$ bp. Assuming a ribonucleotide content per ribosome of 4,566 bp (sum of 5S + 16S + 23S fragments in *E. coli*), the amount of RNA is equivalent to about 2,584 ribosomes. Not only does this tell us the mass of nucleic acids to expect in slowly growing cells, but it implies that 2,584 copies of rRNA are present and sets the target for detection of rRNA in natural samples on a single-cell basis.

By contrast, *E. coli* growing at a doubling time of 1 h has 13,400 ribosomes per cell (1). The number of ribosomes per cell in *E. coli* is exponentially related to growth rate ($r^2$ = 0.988) in the range of 0.6 to 2.5 doublings $h^{-1}$. (The RNA/DNA ratio is linear with growth rate because of the effect of changing genome number with growth rate.) If we extrapolate the exponential relationship to 0.01 $h^{-1}$, we expect to find 3,664 ribosomes per cell in *E. coli*. The data (1) from which this relationship was derived are shown in Fig. 5. These data form a good linear relationship with growth rate, but the regression line for this data set alone ($-0.312 + 4.3 \times \mu$ [$r^2 = 0.999$]) is different from that of the pooled data and implies a negative ribosome content at very low growth rates. Given the multiple sources of error in this calculation, we conclude simply that a few thousand ribosomes per cell is the likely rRNA content of *P. stutzeri* Zobell growing slowly in the marine environment.

Our data were obtained from carbon-limited cells growing in rich medium at steady state, and it is well to consider whether these conditions are applicable to growth conditions of bacteria in the sea. Marine bacteria in the environment are probably exposed to a complex array of organic substrates, each occurring at a very low concentration. Whether growth of heterotrophic bacteria in the sea is steady state or episodic cannot be determined with present methodology. Although previous researchers found no effect of medium composition on RNA/DNA ratios (26), important differences in the factors which limit growth in the sea could conceivably affect the RNA/DNA ratio independent of growth rate. Whether limitation by a nutrient other than carbon or growth under non-steady-state conditions yields a different calibration curve remains to be determined. These factors, as well as the effect of temperature, may be important in interpreting RNA/DNA ratios obtained from natural samples.

Our results from the DNA hybridization method used to measure RNA/DNA ratios have implications for the use of rDNA and rRNA probes in general. It appears from our attempts to quantify rRNA by filter hybridization that such detection is reproducible but not tremendously efficient. In principle, a hybridization method should measure all 23S rRNA molecules and 23S rDNA structural genes present in a sample. If we were to calculate an RNA/DNA ratio on the basis of this hybridization signal, the RNA/DNA ratio should be proportional to the number of rRNA molecules and the number of rDNA structural genes rather than the total mass of nucleic acids. We estimated the number of rRNA molecules at the low end of our growth curve to be about 2,500, and we have determined from previous Southern hybridization results that *P. stutzeri* Zobell has four rDNA operons (19). If the 200-bp *Hin*dIII DNA probe hybridized with each ribosome from the cell and with each of the rRNA genes in the genome, we should detect an RNA/DNA ratio close to 600 (2,500/4). Thus, it is very surprising that our hybridization assay produced RNA/DNA ratios ranging from 0.5 to 1.5.

At present, we are unable to explain this result. By converting the hybridization signal to a total nucleic acid signal via the internal standards, we should see RNA/DNA ratios identical to the EtBr data. However, the ratios that we measure are lower than the EtBr data. We suspect that these low ratios result from inefficient detection of the target rRNA sequence in the ribosomal nucleic acids. That is, the hybridization conditions used to detect rRNA, although reproducible, actually detect a very small fraction of the 23S rRNA sequences present in a sample. Previous research into ribosome structure by using hexanucleotide probes indicates highly variable hybridization throughout the 16S and 23S rRNA subunits (16). This dampening of rRNA hybridization signal in our filter-based, nonradiolabeled system is clearly seen in both the detection limit and the signal from increasing target with the internal standards (Table 1). The detection limit, i.e., the amount of RNA that yielded a signal twice the blank, for total RNA is 17 ng. Assuming that the *P. stutzeri* Zobell ribosome is the same size as the *E. coli* ribosome (4,566 bp per ribosome) and that 85% of the total RNA is ribosomal, then the amount of target RNA (calculated from the linear regression) present at the detection limit with use of the 200-bp *Hin*dIII probe is 633 pg. The detection limit for total genomic DNA is 22 ng, similar to the detection limit for total RNA. If we assume that there are four rRNA operons and that *P. stutzeri* Zobell has a genome size of $6 \times 10^6$ bp, the detection limit for rDNA with use of the 200-bp *Hin*dIII probe is 3 pg. Furthermore, for every nanogram of total RNA, the target sequence is ~1,000-fold greater that for every nanogram of total DNA. The signal generated from probing increasing amounts of rRNA does not reflect the increase in target. Clearly, the probe is less sensitive for 23S rRNA than for 23S rDNA. Nearly 200-fold fewer rRNA target sequences are detected by the probe compared with DNA target sequences. This difference in detection limits for RNA and DNA by the hybridization method could contribute to the scatter seen in RNA/DNA ratios with growth rate (Fig. 4).

This reduced sensitivity for the 23S rRNA subunit in comparison with sensitivity to rDNA has also been shown with fluorescently labeled oligonucleotide probes hybridized to single cells (5). The reduction in filter hybridization signal for rRNA could result from RNases in the system, interfer-

ence from ribosomal proteins copurifying with the rRNA, incomplete denaturation of target sequence, or loss of rRNA from the membrane during hybridization. Each of these possibilities has been explored by varying the treatment of total RNA prior to hybridization (19). The effects of RNase in the system should be minimal since each RNA sample has 1 μg of poly(A)$^+$ RNA carrier added. This represents over 100-fold excess carrier to target, essentially overwhelming the effects of any RNase contamination. In addition, the internal standards of purified *P. stutzeri* Zobell RNA exhibit excellent linearity. One would expect more scatter in the standards if RNase were present. Second, the possibility of ribosomal protein contamination of the RNA samples has been tested by a guanidinium isothiocyanate addition to the extraction protocol or a proteinase K treatment prior to denaturation. The resulting RNA signal was improved a meager twofold. An improvement of some 1,000-fold is necessary for detection of every RNA target molecule. However, ribosomal proteins have been detected in the nucleic acid extracts by SDS-polyacrylamide gel electrophoresis, and a modification of the extraction procedure is warranted. Third, we investigated many different denaturation techniques for both RNA and DNA. Most commonly used denaturation methods, although sufficient to allow acceptable detection limits for presence/absence-type analyses, were found to yield comparable results, an improvement of detection ranging around a factor of 2 or 3. In addition, the 200-bp *Hin*dIII DNA probe used in the hybridizations corresponds to a large loop (bases 70 to 270) in the proposed secondary structure map of the *Anacystis nidulans* 23S rRNA gene (7), indicating reduced secondary structure compared with the remainder of the 23S rRNA molecule. Finally, the possibility of RNA target loss during probe hybridization was assessed by UV cross-linking of the 23S rRNA to the Nytran membranes before baking. A fivefold increase in RNA signal was obtained. Thus, every improvement to the protocol used to obtain the data in Fig. 4 that we tried could increase the apparent RNA/DNA ratio; however, they do not completely explain the reduced sensitivity that we found in probing rRNA.

In conclusion, two independent methods for measuring the RNA content of *P. stutzeri* Zobell were compared and found to yield similar results. Less scatter, resulting in a tighter statistical relationship, was obtained with the nonspecific EtBr method. We conclude from the data reported here that while the EtBr method yielded a more reliable relationship initially, the probe data are also consistent with the general RNA/DNA-growth rate relationship. Although the described probing method requires significant improvement to detect most of the rRNA target sequences in the sample, the method is still more sensitive (target detection limits of 600 pg for RNA) than the EtBr quantification method, which has detection limits in the nanogram range for both RNA and DNA. Moreover, the hybridization method can be species specific, given the variable nature of rRNA sequences. Since species specificity is not possible with measurements based on fluorescence of bulk nucleic acids, the hybridization assay has potential for specificity and sensitivity that, at present, is not attainable any other way.

Additionally, the range of bacterial growth rates over which the relationship between RNA/DNA ratio and growth applies has been extended by more than 10-fold toward the minimum growth rate that can be measured reliably in continuous culture. Our ability to detect rRNA at low concentrations has potential applications beyond the laboratory systems. This usefulness of assaying for rRNA arises because the intracellular concentration of rRNA is linearly related to the steady-state growth rate and a particular rRNA subunit within a mixed sample can be targeted by using species-specific nucleic acid probes (5, 8, 12). We have demonstrated the potential for measurement of the variation in rRNA with growth rate by nucleic acid probing in the laboratory. The next step is developing a species-specific growth rate assay for *P. stutzeri* Zobell in its natural marine environment.

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