Small ribosomal RNA content in marine Proteobacteria during non-steady-state growth

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Abstract

Nine strains of marine Proteobacteria were assayed for nucleic acid content during non-steady-state growth to assess whether a species-specific growth rate based on rRNA content is feasible for environmental samples. The large and small ribosomal subunits and genomic DNA were quantified using image analysis. It was found that the maximal intracellular concentration of 16S rRNA during batch growth for the bacteria averaged 155 fg ± 60 (S.D.) per cell for eight of the nine marine bacteria in the exponential phase (with the exception of one strain, Pac 218). The dilution/decay of 16S rRNA/cell was rapid with a return to pre-shift up values within 6–12 h for all strains except Vibrio fisheri. An overall relationship between the RNA:DNA ratio and the specific growth rate for non-steady-state growth for all bacterial strains was not observed as previously described for other Proteobacteria during steady-state growth. However, a predictable relationship between rRNA content and growth rate for many isolates during batch growth was observed. Furthermore, the rapid kinetics of intracellular rRNA levels indicates it will be feasible to assess whether specific bacteria are in steady state or non-steady state in the marine environment. If the condition of steady state is met for a specific Proteobacterial group in an environmental sample, it will be possible to estimate species-specific growth rates by measuring rRNA content. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Marine bacterium; Species-specific growth rate assay; Non-steady-state growth; Ribosomal RNA content; RNA:DNA ratio

1. Introduction

Determining the in situ growth rate of microorganisms is of fundamental importance in marine ecology. Methods which depend on measuring the concentrations of intracellular components [1] or incorporation of labelled precursors into cellular con-

stituents [1–4] have produced major advances in our understanding of the role of bacteria in marine ecosystems and are widely applied for addressing the growth rates of natural populations of marine bacteria [5,6]. These whole population approaches estimate the average growth rate of the entire population of micro-organisms and are useful for computing carbon fluxes and secondary production. However, they cannot assess the role of different species within the assemblage, or determine the relative contribution of various bacterial species to bio-
geochemical cycling under different conditions. Furthermore, we do not know whether it is reasonable to expect that the entire population of marine bacteria is all growing at a uniform rate. To better understand the ecology of a particular bacterial species in the marine environment, we need a means to measure the growth rate of a single species of bacteria in a natural sample independent of the other bacteria and eukaryotes present in that sample.

One approach to develop a species-specific growth rate assay for natural populations of marine bacteria is based on measurement of ribosomal RNA (rRNA), a cellular marker providing information on both the genetic and the metabolic condition of the cell. The relationship between rRNA content and growth rate has become well established for enteric bacteria, including *Escherichia coli*, *Salmonella typhimurium* and *Aerobacter aerogenes* cells under different growth conditions [7–9]. These and other studies demonstrated that the rRNA content per cell varies with growth rate in the bacteria examined, regardless of the type of nutrients available [7,8,10]. Much of the initial research focused on growth rates typical of these enteric strains, i.e. doubling times in the order of minutes (20–400 min). Later work extended the range of growth rates exhibiting a relationship between rRNA content to very slow doubling times typical of non-enteric bacteria in the environment, in the order of hours (6–60 h) [11–13]. Evidence has also been presented for a global relationship (at least among Proteobacteria) between rRNA content and growth rate under steady-state conditions throughout this entire range of doubling times from 0.3–60 h [12,14].

The advent of in situ hybridization techniques [13–15] for quantifying the rRNA content of specific cells ushered in a new era for growth rate estimation in complex samples. Fluorescent labels and CCD cameras have allowed researchers to detect individual cells [16] and estimate the ribosome content for approximating species-specific growth rates in the environment. However, interpretation of the degree of fluorescent signal when screening a natural sample by in situ hybridization remains problematic. For example, in the marine environment, many studies have shown Proteobacteria to be dominant members of the oceanic community [17–23]. Using in situ techniques, one can measure the amount of rRNA from a Proteobacterium in an oceanic sample and calculate an rRNA/DNA ratio. From these data, it is only possible to estimate the growth rate if we assume:

1. the global relationship between rRNA content and growth rate applies to the Proteobacterium and
2. the bacteria being monitored are in a steady state of growth.

However, it could be that many bacteria in the marine environment are not in steady state or may be in starvation/survival mode [24]. Thus, application of steady-state relationships to estimate growth rate under these conditions may be inaccurate. This study was initiated to elucidate the variability in rRNA content in marine bacteria under non-steady-state (batch growth) conditions. One question that is addressed is whether the global relationship between rRNA content and growth rate observed in Proteobacteria in steady state [12,14] is also observed during non-steady-state growth.

2. Methods

2.1. Micro-organisms and culture conditions

Nine heterotrophic bacterial strains (collected from the Black Sea, the Atlantic, and the Pacific oceans) were tested for rRNA and DNA content along a standard growth curve. All bacteria are members of the Proteobacterial phyla based on 16S rRNA sequence data [25] and the nearest GenBank match for each isolate is presented in Table 1. For the growth rate studies, 10-day-old cultures were re-inoculated into fresh CP medium [26] diluted (1:20) with aged seawater. Twenty time points were taken along a 10-day growth curve (10 hourly time points in lag/exponential phase; and 10 time points in stationary/death phase). All samples were stored frozen at −80°C until extraction. Optical density (600 nm) was measured and samples preserved in formalin for DAPI counts [27]. A global correlation between OD and direct counts for all strains sampled throughout the different growth curves was observed (bacterial numbers = −0.74+0.12×log(OD) r² = 0.81; n = 45). Cell numbers were calculated at each time point
from OD data using the above relationship for all strains. Specific growth rates were determined from changes in the derived cell numbers at each time point during the exponential phase. Thus, the growth rate was calculated on an hourly basis for virtually every data point. Only 2 or 3 data points out of 90 reflect sampling times greater than 1 h.

2.2. Extraction of nucleic acids

Duplicate cell pellets (10⁶–10⁸ cells) were collected for nucleic acid extraction for all bacteria tested with the exception of strain DOE-8. A modified, phenol-chloroform extraction protocol [12] was used to purify both RNA and DNA. This method has been shown to quantitatively recover RNA over the range of cell concentrations used in this study [12]. Slight modifications include: 75 μl of 500 mM EDTA vs. 100 in the original method and total nucleic acids being precipitated in ethanol with glycogen (40 μg) as a carrier. All nucleic acid pellets were re-suspended in DEPC-treated water in volumes of 10–1000 μl. rRNA was separated from DNA by agarose gel electrophoresis before quantification. One percent agarose mini gels were run in 1×TAE buffer at 150 V for 1 h to achieve separation of all nucleic acid fractions.

2.3. Mass determination of ribosomal RNA and DNA

Nucleic acid masses were measured using ethidium bromide fluorescence and image analysis. This technique allows for simultaneous separation and quantification of 23S and 16S rRNA and genomic DNA [28]. (Additionally, calculation of 23S rRNA/16S rRNA or RNA:DNA ratios can be used to detect variations in extraction during the course of the experiment.) The gels were stained in zip-lock bags with ethidium bromide (4 μg ml⁻¹) for 15 min with gentle agitation. This procedure was found to minimize staining variability across the gel with coefficients of variation < 5% for DNA bands of comparable mass (data not shown). A computer image of the stained gel was captured and analyzed using the Fotoanalyst system (Fotodyne, Hartland, WI, USA). Fluorescence of specific bands was determined by creating a mapping image (copy) of each gel using the sharpen (laplacian) transformation with an 8-pixel edge detection aperture. The area and pixel intensity of each band on the original image was integrated using the mapping image as a guide.

Standards of λ HindIII DNA (Boehringer Mannheim, Indianapolis, IN, USA) and rRNA were run on all gels. In order to achieve good separation of the DNA standard, the λ sample was loaded and pre-run for 15 min prior to all other samples. rRNA standards were created and calibrated using total rRNA from six marine bacteria in early exponential phase growth. The combined 23S and 16S rRNA species were gel purified using the RNAid kit (Bio 101, Vista, CA, USA) and quantified by UV absorbance on a model DU-600 spectrophotometer (Perkin Elmer, Foster City, CA, USA) assuming a conversion of 40 μg RNA ml⁻¹ = 1 OD unit. (The OD₂₆₀/OD₂₈₀ ratios of the purified rRNAs were between 1.9 and 2.0.) The rRNA mass standard was prepared by calibrating a total RNA sample of Pseudomonas stutzeri Zobell using serial dilutions of the purified 23S and 16S rRNA mass standards from the six bacterial strains.

3. Results

Nucleic acids were extracted from samples collected throughout the entire experiment (10-day incubation). An example of a gel containing these extracts is shown in Fig. 1. Intact rRNA (low molecular mass) and DNA (high molecular mass) bands were observed for all samples throughout the growth experiment. No signs of RNA or DNA degradation were apparent, indicating uniform extraction of all nucleic acids. The mass standards of DNA and RNA included on each gel were found to exhibit linear relationships between nucleic acid mass and ethidium fluorescence with \( r^2 \approx 0.91 \) (92% of the standards had \( r^2 \approx 0.95 \)) verifying the quantitative nature and low variability of the image analysis methodology.

Most bacterial strains grew to an optical density of 0.2–0.3 within 15 h of inoculation (Fig. 2). All strains, with the exception of BS5 30A, were in stationary phase within 24 h. Using the fluorescence and cell numbers data it was possible to calculate 16S rRNA concentration per cell during growth by averaging the mass of two 16S rRNA bands and
dividing by the cell number at any particular time point. The results of the nucleic acid measurements for exponential phase growth are presented in Fig. 2A–H. All strains with the exception of Alcaligenes faecalis homari (Fig. 2H) and Vibrio fisheri (Fig. 2I) exhibited a sharp peak in 16S rRNA during exponential phase. The amount of 16S rRNA during this peak in concentration averaged 155 fg ± 60 (S.D.)/cell for eight of the nine marine strains tested. One strain exhibited a much higher peak in 16S rRNA content during early exponential phase (1040 fg/cell for strain Pac 218; Fig. 2G). Dilution/decay of 16S rRNA within the bacterial cells was initiated in mid-exponential phase for most cultures with the exception of V. fisheri (Fig. 2I). Rapid rates of dilution/decay were found in most strains with a return to pre-shift up rRNA intracellular concentrations by the onset of stationary phase (6–12 h; Fig. 2A–I).

The computer images also allowed the calculation of 23S rRNA/16S rRNA ratios to test for variability in extraction between samples during growth. These ratios were determined by dividing the mass of the rRNA species in each extraction (lane). The ratios were then averaged and standard deviations were calculated. Since the rRNAs are most likely co-transcribed [29], any deviations in the 23S/16S ratio would indicate selective extraction of either large or small rRNAs during the experiment. The results are presented in Fig. 3. Large to small rRNA ratios ranged from less than one to four with most bacterial strains having 23S/16S ratios between two and three. However, for all strains (with the exception of 2563D) nearly constant 23S/16S ratios were observed during the first 24 h of the experiment indicating peaks in 16S rRNA per cell are accompanied by peaks in 23S rRNA per cell. Small variability is seen in most samples (with the exception of 2563D) indicating the extraction procedure is robust and highly reproducible during this time period. These results suggest the observed peaks in 16S rRNA/cell (Fig. 2) represent changes in intracellular concentrations and are not due to variations in extraction of rRNA subunits during exponential phase growth. The nearly constant 23S/16S ratios also indicate little

<table>
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<th>Strain</th>
<th>Nearest GenBank match</th>
<th>Percent identity</th>
<th>Overlap</th>
<th>Reference</th>
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<tr>
<td>2593A</td>
<td>Vibrio splendidus</td>
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<td>[35]</td>
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<td>96.4</td>
<td>514/533</td>
<td>[35]</td>
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<td>488/498</td>
<td>–</td>
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<td>BS5 30A</td>
<td>Mariana Trench clone 01</td>
<td>99.1</td>
<td>535/540</td>
<td>[33]</td>
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<tr>
<td>BS5 40A</td>
<td>Pseudoalteromonas sp. ANT6</td>
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<td>430/437</td>
<td>[18]</td>
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<tr>
<td>DOE-8</td>
<td>V. splendidus</td>
<td>95.7</td>
<td>491/513</td>
<td>[35]</td>
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<tr>
<td>Pac 218</td>
<td>Pele’s Vent bacterium OTU 5</td>
<td>97.6</td>
<td>480/492</td>
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degradation is occurring during extraction, since more of the 16S rRNA would be degraded per unit time than 23S and the ratio would change. However, two strains (DOE-8 and Pac 218) exhibited 23S/16S ratios less than one indicating excess 16S rRNA in these particular bacteria or a systematic bias for the small rRNA that is constant throughout the experiment.

Using the cell number data, it was possible to determine specific growth rate and compare with 16S rRNA/cell during the incubation (Fig. 4). Three patterns were apparent in the data. One strain, BS5 30A, demonstrated a direct linear relationship between specific growth rate and 16S rRNA/cell (BS5 30A $r^2 = 0.85$). Most other bacteria in the study, represented by DOE-8, had the peak in specific growth rate before or after the peak in 16S rRNA/cell. Finally, one strain (*V. fisheri*) did not have an obvious relationship between specific growth rate and 16S rRNA content.

We used similar plots to ascertain the time shift necessary to superimpose the peak in specific growth rate and 16S rRNA content for the remaining bacteria used in the study. This alignment of the peaks

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**Fig. 2.** Graphs of optical density (△), 16S rRNA/cell (▼) through time for the following marine strains: (A) 2593A, (B) 2563 C, (C) 2563 D, (D) BS5 30A, (E) BS540A, (F) DOE-8, (G) Pac 218, (H) *A. faecalis homari*, (I) *V. fisheri*. Error bars indicate S.D.
allows for comparison of rRNA content and specific growth rates. The data are summarized in Table 2. A linear relationship between growth and 16S rRNA/cell under non-steady-state conditions was only observed in three strains, BS5 30A, DOE-8 and Pac 218 ($r^2 = 0.80^{0.95}$). Correlation coefficients ranged from $r^2 = 0.21$ to 0.95. The global 16S rRNA/cell vs. growth rate relationship was not found to be significant ($r^2 = 0.199$) under non-steady-state conditions.

### 4. Discussion

Two important findings came out of this study. Primarily, the data presented here confirm that interpretation of rRNA signals from environmental samples using either fluorescent in situ hybridizations or probing of total RNA is not straightforward. Although the range of peak intracellular concentrations of 16S rRNA varied by a factor of 2–4-fold for most marine Proteobacteria at comparable portions of the growth curve, one strain (Pac 218) was found to contain significantly more 16S rRNA during exponential phase growth than the other marine strains. If you compare the highest and lowest 16S rRNA content per cell during early exponential growth (1040 fg/cell for Pac 218; and 70 fg/cell for *A. faecalis homari*), a 15-fold difference is observed. This data suggest that there may be a small number of strains detected in the environment displaying 15-fold differences in 16S rRNA intracellular concentration that are actually at the same numbers and growing at the same rate as most other bacteria in the sample. An important implication of this finding is that techniques that rely on estimating bacterial abundance and distribution based on probing of total RNA with universal probes will be significantly

<table>
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<tr>
<th>Strain</th>
<th>Correlation coefficient</th>
<th>Time shift (h)</th>
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<td>DOE-8</td>
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<td><em>V. fisheri</em></td>
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</tr>
<tr>
<td>Global</td>
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</table>
influenced by those bacterial strains containing high levels of 16S rRNA, such as Pac 218.

With respect to growth rate estimations from environmental samples using rRNA content, the interpretation of data will also be difficult. If specific populations of bacteria in the marine environment are in steady state, growth rate can be reasonably estimated using the Proteobacterial RNA : DNA vs. growth rate relationship previously described [12,14]. However, during non-steady-state conditions, intracellular 16S rRNA levels were not found to be linearly related to growth rate for most of the strains tested (Table 2). Hence, estimations of growth rate for these non-steady-state population will not be as accurate.

If species-specific growth rates are to be precisely determined, it will be necessary to ascertain whether the bacterial group in the sample is in steady state. Our results suggest that assessment of whether many marine microorganisms (not some *Vibrios*) are in steady state in the ocean can be done by sampling through time and measuring large increases or decay in 16S rRNA/cell. In contrast with previous reports of slower decay or retention of ribosomes during stationary phase for *Desulfobacter lactus* [30] and *Vibrio* spp. [31,32], most marine strains in this study demonstrated a return to pre-shift up levels in 16S rRNA/cell within 6–12 h. Additionally, samples were collected for 10 days after inoculation to assess whether ribosomes were retained in non-growing cells. No strain exhibited significant ribosome retention during stationary phase. Therefore, if the 16S rRNA content per cell in a specific microbial population in a natural sample was observed to remain stable over a 3–6-h time period, the bacterial cell will most likely be in steady state. If the steady-state criteria are met, then universal steady-state relationships [14] between RNA:DNA ratios and growth (i.e. RNA/DNA = 1.65 + 6.01 mu^0.73, r^2 = 0.85) can be reasonably applied to estimate species-specific growth rates.

In conclusion, intracellular concentrations of 16S rRNA were not found to be globally related to all specific growth rates in all marine bacteria tested during non-steady-state growth in contrast with previous reports of a highly consistent global relationship for Proteobacteria in steady-state growth. Although a global relationship for non-steady-state growth was not found, it is highly likely that there is a predictable relationship between RNA and growth rate for each isolate, even though it may not be revealed by a simple linear regression. Our data show consistent patterns of peak rRNA concentration associated with maximal growth rates. Furthermore, since many of the Proteobacterial strains used in this study are found in samples or 16S rRNA clonal libraries ranging from Antarctica [18] to Ha-

![Fig. 4. Graphs of 16S rRNA/cell (▼) and specific growth rate (□) through time for the strains indicated. Error bars indicate S.D.](image-url)
waii [22] and the Mariana trench [33] to surface waters, these results should be applicable to other parts of the ocean. Finally, additional research is warranted to determine whether other molecules such as pre-16S mRNA can better predict growth rate [34] on a species-specific basis in natural samples for bacteria experiencing non-steady-state growth.

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