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Estimating the Growth Rate of Slowly Growing Marine Bacteria from RNA Content

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In past studies of enteric bacteria such as *Escherichia coli*, various measures of cellular RNA content have been shown to be strongly correlated with growth rate. We examined this correlation for four marine bacterial isolates. Isolates were grown in chemostats at four or five dilution rates, yielding growth rates that spanned the range typically determined for marine bacterial communities in nature (μ = 0.01 to 0.25 h^{-1}). All measures of RNA content (RNA $cell^{-1}$, RNA:biovolume ratio, RNA:DNA ratio, RNA:DNA:biovolume ratio) were significantly different among isolates. Normalizing RNA content to cell volume substantially reduced, but did not eliminate, these differences. On average, the correlation between μ and the RNA:DNA ratio accounted for 94% of variance when isolates were considered individually. For data pooled across isolates (analogous to an average measurement for a community), the ratio of RNA:DNA μm^{-3} (cell volume) accounted for nearly half of variance in μ (r^2 = 0.47). The maximum RNA:DNA ratio for each isolate was extrapolated from regressions. The regression of (RNA:DNA)/$(RNA:DNA)_{max}$ on μ was highly significant (r^2 = 0.76 for data pooled across four isolates) and virtually identical for three of the four isolates, perhaps reflecting an underlying common relationship between RNA content and growth rate. The dissimilar isolate was the only one derived from sediment. Cellular RNA content is likely to be a useful predictor of growth rate for slowly growing marine bacteria but in practice may be most successful when applied at the level of individual species.

For more than a decade, radioisotope incorporation methods have been the primary means of estimating the growth and production of natural communities of aquatic bacteria. These methods are based on the incorporation of radiolabelled precursors into stable macromolecules. Examples include the incorporation of thymidine into DNA, adenine into RNA and DNA, and leucine into protein. As valuable as these methods have been, they share several characteristics with other methods (e.g., dilution culturing and frequency of dividing cells) that limit their usefulness. Most methods provide estimates of growth rate averaged over the entire community, severely restricting the scope of questions which can be addressed. No information is provided on the distribution of growth rate among cells or the distribution of activity among taxa. This precludes studies of the population dynamics of specific microbes within natural marine communities. Most existing methods also rely on the incubation of samples, with concomitant sample manipulations. Associated experimental artifacts can alter community structure and compromise growth rate estimates (4, 5, 9, 16). It is difficult at best and usually impractical or impossible to ascertain whether such perturbations have occurred.

Alternative methods may circumvent these problems, and if based on new approaches, they may also provide new insights into the dynamics of marine bacteria. In this paper, we report data which suggest an alternative approach for estimating cell growth, based on measuring cellular RNA content.

Past studies of copiotrophic, model bacteria have shown that growth rate is highly correlated to various measures of RNA content. For example, in *Escherichia coli* and *Salmonella typhimurium*, stable RNA $cell^{-1}$ (which is mostly rRNA) increases by 1 order of magnitude with a four- to fivefold increase in growth rate (2, 22). DeLong et al. (7) demonstrated that in *E. coli*, both rRNA $cell^{-1}$ and the RNA:DNA ratio were highly correlated to specific growth rate over the range of μ = 0.3 to 1.6 h^{-1}. Dortch et al. (8) compiled published data showing that a single, general relationship between the RNA:DNA ratio and growth rate seemed to apply for *E. coli*, *S. typhimurium*, and *Aerobacter aerogenes*. Strong correlations between the RNA:DNA ratio and growth rate have also been demonstrated for marine phytoplankton (8), larval fish (3), and other eukaryotic organisms (see references in reference 8).

These studies suggest that RNA content measurements may be reliable indicators of bacterial growth rate. However, no study dealt with marine bacteria, which are typically slowly growing organisms often exposed to nutrient-poor environments. The previous studies are actually based on only a few bacterial taxa and therefore might be irrelevant to marine bacteria. In the present study, we examined whether a correlation exists between RNA content and growth rate for marine bacteria growing at rates characteristic of the natural environment. Furthermore, we examined whether the form of any such relationship is universal among marine bacteria or instead is unique to individual species. Our approach utilized chemostat cultures of marine bacterial isolates in which growth rate could be determined accurately and compared with various measures of RNA content.

MATERIALS AND METHODS

Bacterial isolates. Four marine bacterial strains were isolated by streaking freshly collected seawater onto agar plates (for one isolate, a very dilute suspension of sediment in filter-sterilized seawater was streaked). These have been designated SARW1 (Sargasso Sea water column no. 1), GBW1 and GBW2 (Georges Bank water columns no. 1 and 2, respectively), and GBSED1 (Georges Bank sediment no.

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1), which are mnemonics reflecting the collection locations. Phylogenetic affiliations of three of the four strains (GBW1, GBW2, and GBSED1) were determined by partial 16S rRNA sequence analysis. The reverse transcriptase procedure was used to synthesize cDNA for sequencing from RNA extracted from cultured cells during exponential growth. Sequence data (400 to 800 bases per isolate) were compared with a subset of 16S rRNA sequences selected from the National Ribosomal RNA Database. The Clustal V (12) and Phyllip (version 3.4, provided by J. Felsenstein, University of Washington) programs were used to align sequences and construct an unrooted, phylogenetic distance tree by a DNA parsimony algorithm.

Experimental design. Each isolate was grown axenically in chemostats at 25°C, at either four or five dilution rates. The medium consisted of an autoclaved, filter-sterilized (0.22-μm-pore-size filter) seawater base, supplemented with 30 ppm of yeast extract (Sigma, St. Louis, Mo.) and 30 ppm of marine broth 2216 (Difco). The yeast extract was added as a concentrated solution after filtering through a 0.2-μm-pore-size Acrodisc (Gelman) to remove autoclave-resistant yeast spores. The supplements were added to promote cell densities of ca. 10^7 cells ml^{-1}. Lower cell densities would have required excessively large samples to obtain sufficient RNA and DNA for analysis. Cell abundance was monitored by DAPI (4',6-diamidino-2-phenylindole) direct counts (19) at time intervals of ca. one generation. Photographs of each sample were taken (Ektachrome 400 film), and the mean cell size was calculated from the measured dimensions of cells in projected images. Cultures were started at dilution rates equivalent to doubling times of ca. 70 h and sampled after reaching steady-state conditions, defined as constant cell abundance for two or more sequential samples (usually after four to seven generations). After sampling, the dilution rate was increased two- to threefold.

Measurement of RNA and DNA content per cell by ethidium bromide fluorometry. RNA and DNA were measured by ethidium bromide fluorometry (20). The sample volume required to recover sufficient DNA and RNA was calculated from cell abundance (known from the monitoring samples) and a preliminary estimate of RNA $cell^{-1}$ and DNA $cell^{-1}$. After collection on 0.2-μm-pore-size Nuclepore filters, cells were resuspended in a small volume of ice-cold STE buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA [pH 7.6]). A subsample was taken to estimate the total number of cells collected. Samples were kept at <6°C during handling. The cells were pelleted (10,000 × *g*, 15 min) and stored for <2 weeks at −70°C until nucleic acid extraction. Cell pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and cells were lysed with 1% sodium dodecyl sulfate. The lysate was extracted once with an equal volume of a phenol-chloroform-isoamyl alcohol mixture (25:24:1, equilibrated with STE [pH 8.0]) and once with an equal volume of chloroform. Material at the interface between the aqueous phase and the phenol mixture was carried through to the chloroform extraction, in order to minimize the possible loss of nucleic acid. The final aqueous phase was precipitated overnight with 2 volumes of ethanol and ammonium acetate (final concentration, 2.3 M) at −20°C. The precipitate was collected by centrifugation at 10,000 × *g* for 15 min. The pellet was briefly rinsed with 70% ethanol, and a small volume of TE was added. The solution was stored at 4°C for 1 day, and the pellet was thoroughly dissolved by occasional vortexing. Immediately after the dissolution, subsamples of the nucleic acid extract were digested with DNase-free RNase (Boehringer Mannheim, Indianapolis, Ind.) for 2 h at 37°C (DNA sample). The untreated extract (containing both DNA and RNA) and the RNase-digested samples (containing DNA only) were placed at −70°C immediately after the RNase digestion and held at −70°C until fluorescence was measured.

Three to twelve microliters of the DNA or DNA-RNA samples was added to 3 ml of TE with 1 μg of ethidium bromide. Fluorescence was measured in duplicate or triplicate samples with a Perkin-Elmer spectrophotometer (excitation, 300 nm; emission, 600 nm). Standard curves for DNA or RNA were generated with known amounts of calf thymus DNA (Sigma) or *E. coli* rRNA (Sigma) over the concentration range of 0.1 to 0.8 μg of DNA (or RNA) ml^{-1}. RNase digestion had no effect on the standard curves generated with calf thymus DNA.

The standard curves were used to convert the fluorescence of samples to the equivalent weight of DNA or RNA. The amount of DNA in a sample was calculated directly from the fluorescence of the RNase-digested DNA sample. The amount of RNA was calculated by determining the difference by 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 was then calculated by dividing the total amount of DNA or RNA by the total number of cells in the extract.

Extraction efficiency was checked by determining the total recovery of known quantities of DNA and RNA standards. These were processed identically to the samples described above, at concentrations equivalent to typical nucleic acid concentrations in samples. Recoveries were 65% for DNA and 75% for RNA (n = three determinations). These values may be low: in subsequent work with natural bacterial communities, recoveries have consistently averaged 85% with the same procedures. No corrections for extraction efficiency have been applied to our data because we cannot be sure that RNA and DNA in cells will be extracted with the same efficiency as added nucleic acids.

We believe that variable recovery occurs at the extraction stage rather than during separation of RNA from DNA, because RNase digestion was invariably complete when checked on agarose gels and RNase had no effect on DNA standards (i.e., no DNase was present in the RNase). For a given sample, variations in recovery efficiency will tend to change the calculated RNA $cell^{-1}$ and DNA $cell^{-1}$ in equal proportion. This experimental artifact was expected to result in a correlation between RNA $cell^{-1}$ and DNA $cell^{-1}$. Any such artifactual correlation could obscure a real correlation between the RNA and DNA content of cells. However, the ratio of RNA to DNA eliminates this artifact, and for that reason our analyses will focus on the RNA:DNA ratio.

Statistical analyses. We are primarily interested in determining whether cellular RNA content can be used to predict growth rate, and therefore, an unreplicated, regression-based experimental design was used. The analytical strategy was as follows. We wished to evaluate whether RNA $cell^{-1}$ or any derived or related variable (e.g., RNA:volume or RNA:DNA ratio) could be used as a universal predictor of specific growth rate. This would be indicated unequivocally by finding (i) no significant differences in regression intercepts and slopes among isolates, combined with (ii) a high proportion of the variance in data accounted for by the RNA measurement and any covariates. If differences among isolates were both significant and substantial, it would be necessary to examine data for each isolate separately.

All variables were examined for homogeneity of variance.

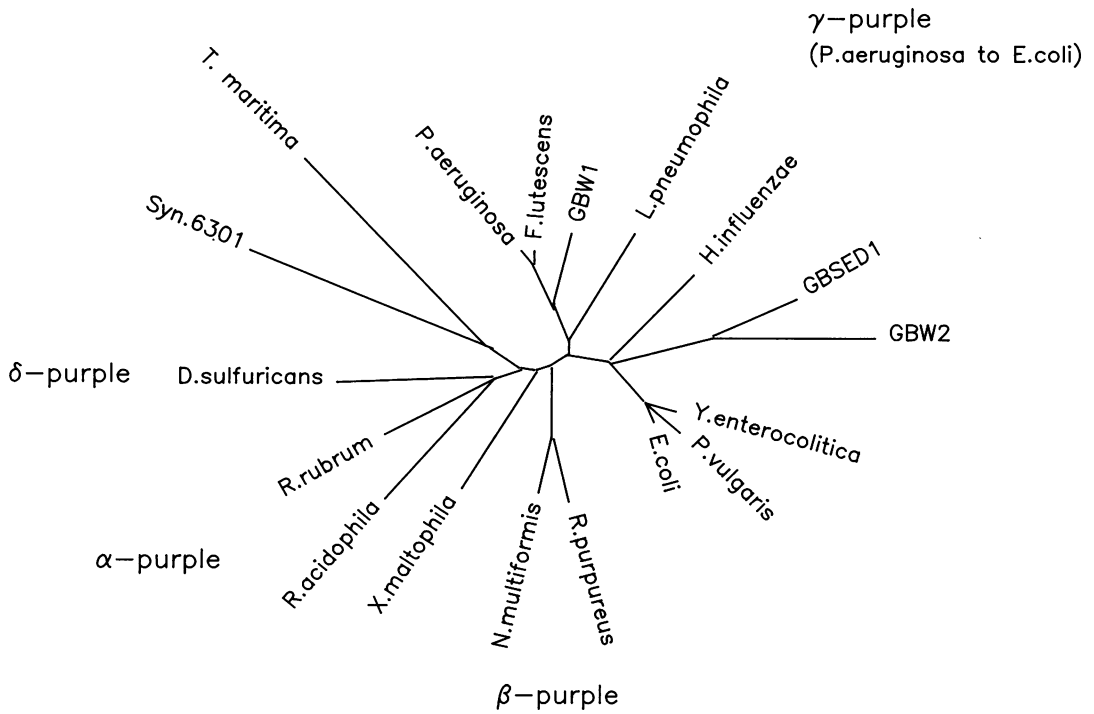

FIG. 1. Unrooted phylogenetic tree based on partial 16S rRNA sequence data. Sequences used include two non-purple bacterial sequences (*Thermotoga maritima* and *Synechococcus* sp. strain 6301) and a selection of α-, β-, γ-, and δ-purple bacterial sequences (on the basis of the classification scheme in reference 23).

Only cell volume was nonhomogeneously distributed, and accordingly, it was logarithmically (log) transformed. We found that the relationship between RNA content and growth rate consistently was linearized by log transformation of specific growth rate. For convenience, analyses are therefore based on log-transformed specific growth rate. Log transformation of RNA measurements had very little impact.

Analysis of covariance (ANCOVA) was used to test whether there were significant differences among isolates in the mean value for RNA variables. The four RNA variables used were RNA cell^{-1} (covariables: specific growth rate, DNA cell^{-1}, and cell volume), RNA μm^{-3} (covariables: specific growth rate and DNA cell^{-1}), the RNA:DNA ratio (covariables: specific growth rate and cell volume), and the RNA:DNA:volume ratio (covariable: specific growth rate). Post hoc comparisons among isolates were conducted by using the Sheffé procedure for all possible pairwise comparisons. Regression lines were examined for parallelism by testing for interactions of the covariable(s) with the main effect (isolates). All statistical calculations were completed by using the CSS:Statistica program package (StatSoft).

RESULTS

Phylogenic affiliation. The isolates GBW1, GBW2, and GBSED1 were closely aligned with the γ subgroup of purple bacteria (24, 25), on the basis of our analysis of 16S rRNA partial sequences (Fig. 1). Two of the isolates (GBW2 and GBSED1) appeared to be more closely related to each other than to any other species included in the analysis; these were related to *Vibrio* species. A third isolate was related to *Pseudomonas* species. These relationships were also found in a more extensive analysis that included the three isolates and 35 purple bacterial sequences (not shown). We do not have sufficient sequence data to assess the phylogenetic affiliation of the fourth isolate (SARW1).

Basic data. RNA cell^{-1} and DNA cell^{-1} were significantly correlated (r^2 = 0.76, n = 17) as expected because of the effects of variable recovery efficiency. This added source of error tended to obscure any real relationship of RNA cell^{-1} with specific growth rate (Fig. 2). The RNA:DNA ratio, which eliminates this error, was strongly correlated to specific growth rate for all four isolates (Fig. 3). Cell volume was not significantly correlated with growth rate for any isolate (data not shown). Although both cell volume and RNA content varied among isolates and therefore were signifi-

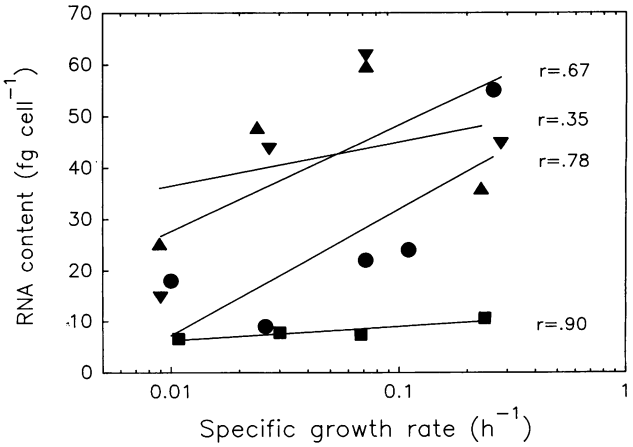

FIG. 2. Mean RNA cell^{-1} versus specific growth rate μ. Symbols: ■, SARW1; ●, GBW1; ▼, GBW2; ◆, GBSED1.

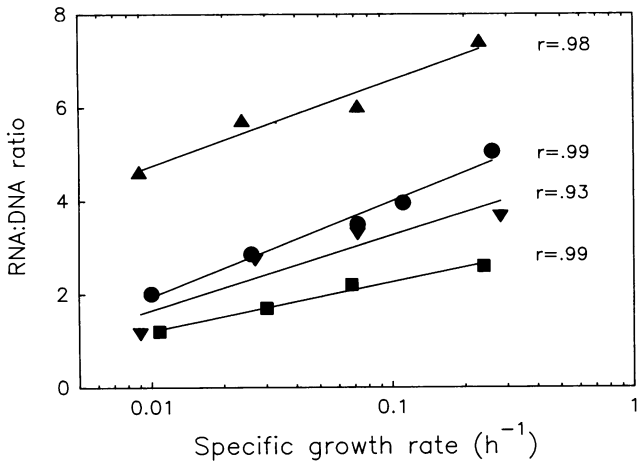

FIG. 3. Ratio of mean RNA $cell^{-1}$ to mean DNA $cell^{-1}$ versus specific growth rate μ. Symbols are described in the legend to Fig. 2.

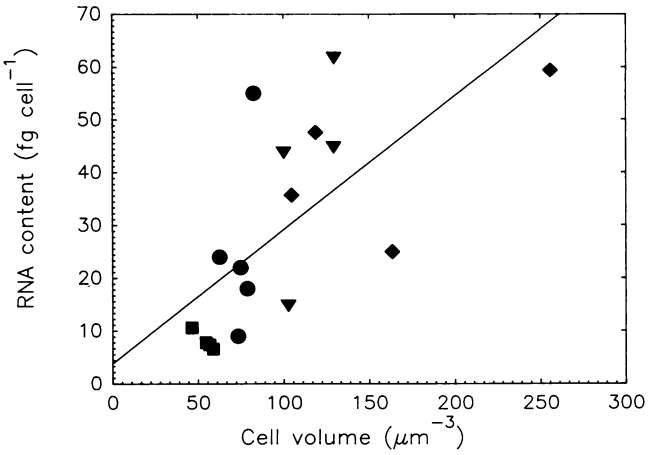

FIG. 4. Mean RNA $cell^{-1}$ versus cell volume (μm^3). Symbols are described in the legend to Fig. 2.

cantly correlated for all data combined (r^2 = 0.55) (Fig. 4), they were not correlated within isolates.

Differences among isolates. All measures of RNA content varied significantly among isolates (ANCOVA) (Table 1). Post hoc comparisons among isolates did not suggest a consistent pattern; i.e., differences and similarities among isolates depended on the RNA variable being considered (Table 1).

We examined the relationship of RNA content to growth rate, DNA content, and cell volume by multiple regressions within the analyses of covariance. This is quite different from multiple regressions for which the same variables are used but the source of the data is ignored (i.e., pooling across isolates, as shown in Table 2). Hereafter, the two will be distinguished by referring to total variance (i.e., regressions pooling data across isolates) and residual variance (i.e., regressions dealing only with residual variance after adjusting for interisolate differences in the mean RNA content; this is part of the ANCOVA).

All regressions within the ANCOVA were highly significant (r^2 = 0.63 to 0.93). There was no significant difference among isolates with regard to regression slopes (tests of parallelism, [all] $P > 0.1$). Therefore, the relationship between RNA content and growth rate is best described as a family of parallel lines with different intercepts but a common slope, as seen in Fig. 3. In the ANCOVA multiple regressions, specific growth rate and DNA content were always significant, but cell volume was never significant (Table 1). The strongest relationships were obtained for RNA $cell^{-1}$ with two significant covariables (DNA $cell^{-1}$ and specific growth rate; r^2 = 0.93) and the RNA:DNA ratio with one significant covariable (specific growth rate; r^2 = 0.91). In other words, nearly all of the residual variance in specific growth rate could be accounted for by measurements of RNA and DNA contents.

As discussed earlier, we considered the possibility that differences among isolates might be significant but perhaps small enough to be ignored for practical applications. A pooled-data regression for the RNA:DNA ratio accounted for 60% of total variance (Table 2), compared with 91% of residual variance (Table 1). We interpret this difference as reflecting a substantial component of the total variance that can only be attributed to differences among isolates, i.e., it is too large to ignore.

Cell volume contributed significantly to pooled-data regressions (Table 2), for which the partial correlation between the RNA variable and growth rate was highest for the RNA:DNA:volume ratio (Table 2, Δr^2 = 0.47 versus 0.28 for the RNA:DNA ratio).

TABLE 1. Comparisons of RNA content among isolates by ANCOVA tests for differences in mean value of the RNA variable

RNA variable	Covariable(s)	ANCOVA		r^{2a}
		P	Comparison[b]	
RNA $cell^{-1}$	Log(μ),[c] log(vol), DNA $cell^{-1}$[c]	0.003	GBW2 = GBSED1	0.93
RNA μm^{-3}	Log(μ),[c] DNA $cell^{-1}$[c]	0.05	SARW1 = GBSED1; GBW1 = GBW2 = GBSED1	0.74
RNA:DNA	Log(μ),[c] log(vol)	<0.001	All significantly different	0.91
RNA:DNA μm^{-3}	Log(μ)[c]	0.03	SARW1 = GBW1 = GBSED1; SARW1 = GBW2 = GBSED1; (GBW1 ≠ GBW2)	0.63

[a] Correlation of covariables with the RNA variable, after adjustment for differences among isolates ($P < 0.001$, for all).
[b] For post hoc comparisons, the groupings shown are the isolates which were not significantly different ($P > 0.05$) in pairwise comparisons.
[c] Covariable which was significant in multiple regressions ($P \leq 0.05$).

TABLE 2. Contributions of variables to multiple regressions of data pooled across isolates

Dependent or independent variable(s)[a]	Δr^{2b}	P_{var}[c]	P[d]
RNA cell^{-1}			
Log(vol)	0.54	0.001	<0.001
Log(μ)	0.18	0.01	
DNA cell^{-1}	0.15	0.002	
RNA μm^{-3}			
DNA cell^{-1}	0.33	0.003	0.001
Log(μ)	0.29	0.005	
RNA:DNA			
Log(vol)	0.32	0.003	0.001
Log(μ)	0.28	0.007	
RNA:DNA μm^{-3}, log(μ)	0.47	0.007	0.002

[a] Variables are listed in the order of entry in a forward-stepwise regression procedure.
[b] Δr^2, increment in r^2 due to adding a variable (total r^2 = sum of Δr^2).
[c] P_{var}, probability level for each variable within regression.
[d] P, probability level for regression with all variables listed.

Isolate-specific regressions. Since there were significant and substantial differences among isolates, we proceeded to calculate separate multiple regressions for each isolate (RNA content, dependent variable; specific growth rate, DNA cell^{-1}, and cell volume, independent variables, as appropriate). We assumed that DNA content and cell volume are independent attributes of cells that potentially may influence RNA content but that are not themselves dependent on specific growth rate. There is, in fact, no apparent relationship between growth rate and either DNA content or cell volume in our data (data not shown).

Results for each isolate are shown in Table 3. Cell volume was never significant as an independent variable, possibly because there was little dynamic range in volume within any one isolate. In most cases, multiple correlations were very high (e.g., $r^2 \geq 0.96$ for 10 of 16 regressions). Weak correlations were found only for regressions based on RNA μm^{-3} and RNA:DNA μm^{-3}. The small sample size and lack of replication meant that even apparently strong relationships (up to $r^2 = 0.985$ for some degrees of freedom) were not necessarily significant at the traditional α level of 0.05. Thus, we have very little capability of assessing the significance of any one regression. However, the cumulative probability of so many high correlations is very low. For example, by

TABLE 3. Multiple regression equations for individual isolates[a]

Dependent or independent variable	Isolate			
	SARW1 (n = 4)	GBW1 (n = 5)	GBW2 (n = 4)	GBSED1 (n = 4)
RNA cell^{-1}				
Log(μ)	4.060 (0.81)	19.744 (0.29)	21.585 (0.49)	9.519 (0.83)
DNA cell^{-1}	0.973 (0.16)	2.988 (0.67)	4.754 (0.49)	5.892 (0.17)
Log(vol)	— (0.00)	68.475 (0.02)	— (0.00)	— (0.00)
Intercept	9.15	−98.65	−0.573	12.565
r^2	0.97	0.96	0.99	0.99
P	0.2	0.03	0.1	0.05
RNA μm^{-3}				
Log(μ)	0.123 (0.78)	0.243 (0.70)	0.129 (0.31)	0.079 (0.20)
DNA cell^{-1}	0.032 (0.19)	0.039 (0.28)	0.038 (0.54)	0.007 (0.02)
Intercept	0.174	0.369	−0.033	0.336
r^2	0.97	0.99	0.84	0.22
P	0.2	0.01	0.4	0.9
RNA:DNA				
Log(μ)	1.421 (0.96)	2.042 (0.98)	1.846 (0.86)	1.738 (0.96)
Log(vol)	4.774 (0.03)	2.057 (0.01)	−2.500 (0.01)	−1.262 (0.03)
Intercept	−4.467	2.211	10.342	10.994
r^2	0.99	0.99	0.87	0.99
P	0.08	0.01	0.4	0.07
RNA:DNA μm^{-3}				
Log(μ)	0.027 (1.00)	0.027 (0.89)	0.010 (0.62)	0.022 (0.43)
Intercept	0.072	0.081	0.037	0.072
r^2	1.00	0.89	0.62	0.43
P	0.001	0.01	0.2	0.3

[a] Probabilities and r^2 values are rounded and reported to 1 and 2 significant digits, respectively. Numbers in parentheses are Δr^2 upon entry into forward-stepwise multiple regressions (i.e., the proportion of the remaining variance accounted for by a new variable, after previously entered variables have been considered). Cell volume was excluded from some regressions for RNA (— symbol) because the F statistic to enter regression was <0.001.

random chance alone, one might expect 1.6 of the 16 regressions to have a P of ≤ 0.1; instead, P was ≤ 0.1 for 9 of the 16. If the number of false-positive regressions is Poisson distributed and all regressions were wholly independent, then the probability of attaining nine false-positive regressions is <0.0003. For the four regressions based on the RNA:DNA ratio alone, the probability of obtaining three of four regressions significant at an α value of 0.1 is <0.008.

On the basis of our evaluation of the cumulative probability of error with many regressions, we reject the possibility that we found predominantly high correlations by chance. Instead, we conclude that in most cases, these regressions account for nearly all of the variability in the data.

DISCUSSION

Regressions and experimental data. RNA content was strongly correlated to specific growth rate for four marine isolates. When data were pooled across isolates, the composite ratio RNA:DNA:cell volume accounted for 47% of total variance in μ. Such pooled-data regressions are analogous to predictors of the community-average growth rate, such as measurements of thymidine uptake. In comparison, we consider that thymidine-based production estimates may be accurate only within a factor of 5 (14). From that standpoint, even pooled-data regressions of RNA on growth rate may be at least comparable in accuracy to the thymidine method. However, in ANCOVA which accounted for differences among isolates, RNA and DNA together accounted for over 90% of residual variance in μ. In regressions calculated for individual isolates, the RNA:DNA ratio accounted for an average of 94% of variance in μ. This suggests that taxon-specific procedures (as opposed to the non-taxon-specific procedures used here) may provide very powerful tools for estimating growth rates.

The differences among isolates were substantially due to their differences in mean cell size. Given two cell types growing at the same rate, if one is twice as large in biovolume, it should produce about twice the biomass per unit of time. To synthesize twice the protein biomass, larger cells should have more RNA than smaller cells growing at the same rate. We observed a general correlation between RNA content and cell size (Fig. 4). Furthermore, the RNA:DNA regressions on specific growth rate (Fig. 3) were ordered in approximately the same manner as the average cell volume of isolates. We had hypothesized that the RNA:volume ratio would tend to be constant among different-sized bacterial species growing at the same rate. If so, then normalizing RNA content to cell size should eliminate differences among isolates. This was generally, but not entirely, true. Instead, RNA μm^{-3} was lower for the smallest isolate, SARW1 (Table 1), while the remaining three isolates were not significantly different. Similarly, most, but not all, of the differences among isolates were eliminated when the RNA:DNA ratio was normalized to cell volume (Table 1, compare RNA:DNA to RNA:DNA:volume). We conclude that volume-normalized RNA content is more nearly universal among taxa, although some taxon-specific differences in the RNA-growth rate relationship remain.

We found that both RNA $cell^{-1}$ and the RNA:DNA ratio were linearly related to log(μ), which is inconsistent with the results of some previous studies. The form of the RNA-growth rate relationship has been reported in several studies as linear for both variables for the RNA:DNA ratio (1–3, 7, 8). For RNA $cell^{-1}$, the relationship has been reported as linear in growth rate and logarithmic in RNA content (2, 7, 22). As yet, we have no explanation for the form of the relationship between RNA content and growth rate, either in past studies or in the current study.

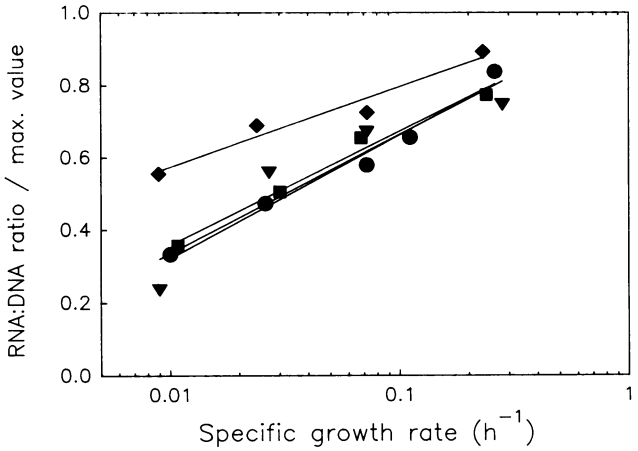

FIG. 5. The RNA:DNA ratio, divided by the predicted asymptotic maximum RNA:DNA ratio, versus specific growth rate. Symbols are described in the legend to Fig. 2.

Interpretation of RNA as growth rate. The volume-normalized RNA content appeared to be more general among isolates than RNA content alone. However, some differences among taxa remained and may be problematic when attempting to interpret RNA data collected for naturally mixed communities. It may be possible to find a truly universal expression of the RNA-growth rate relationship, one which accounts for differences among taxa. For example, RNA content can be expressed as a fraction of the maximum RNA content expected for a taxon, which presumably occurs when cells are growing at maximal rates. We fit semi-log regressions to the RNA:DNA data for each isolate: RNA:DNA ratio = a + $b$log(μ), where μ is the specific growth rate. These are the regressions shown in Fig. 3, which appear linear on a semi-log plot but on a linear-linear scale rise to an asymptotic maximum RNA:DNA value given by the constant a. We divided each measurement of the RNA:DNA ratio by the value of a for that isolate (Fig. 5). The relationship between RNA:DNA/a and μ is almost identical for three of four isolates, indicating that an underlying common relationship may indeed exist. It is intriguing that the single dissimilar isolate was GBSED1, the only one isolated from sediment. For data pooled across all four isolates, the overall correlation of (RNA:DNA)/$(\text{RNA:DNA})_{max}$ with μ was highly significant ($r^2 = 0.76$, $P < 0.001$).

In order to interpret RNA data as measures of growth rate, one must also understand the time frame represented by the measurement. The determination of thymidine uptake, for example, ideally shows the ongoing DNA synthesis rate during the actual incubation; i.e., it should be a measure of the instantaneous growth rate. A nontrivial amount of time is required to alter the RNA concentration (2), and, therefore, RNA content should integrate the recent growth history of the cells. One useful consequence is that the effects of shorter-term fluctuations in macromolecular synthesis rates are minimized. For example, artifactual or natural changes in synthesis rates during sampling are unlikely to affect measurements of RNA content.

Our chemostat data are based primarily on measurements

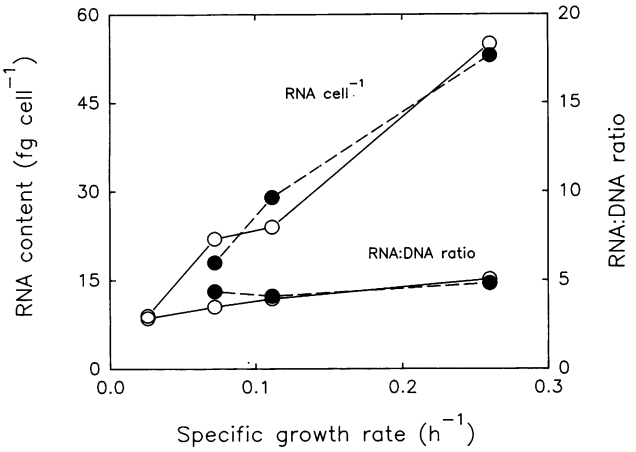

FIG. 6. For isolate GBW1, RNA cell^{-1} and the RNA:DNA ratio measured one generation time after the growth rate was increased (filled symbols with dashed lines) and again after the new equilibrium cell density was established (open symbols with solid lines).

at equilibrium. However, in one experiment (with GBW1), the RNA content of cells was measured one generation time after the growth rate was increased and again after cell abundance had stabilized. Both RNA cell^{-1} and the RNA:DNA ratio were similar to their equilibrium values after only one generation time (Fig. 6). This is consistent with previous studies showing that bacterial RNA accumulates at a rate approximately equal to the growth rate (2). As a rule of thumb, the RNA content probably reflects the recent growth history over time scales equal to the generation time. For marine bacteria, this would be roughly 1 day. RNA measurements may not be useful for evaluating changes in bacterial growth over shorter time scales.

Application to natural marine environments. The growth rates in our chemostat experiments are comparable to those determined for entire marine bacterial communities in coastal waters and sediment, and it seems reasonable to expect that correlations between RNA and growth rate will exist for marine bacteria in nature. Moriarty (18) compiled published estimates of specific growth rates at 19 sites ranging from polar to temperate and estuarine to marine. The average of all median values in his summary table is 1.08 day^{-1}, or equivalently about 0.05 h^{-1}. The lowest growth rates in our chemostat experiments were fivefold lower, and the RNA-growth rate correlation held for the lowest growth rates. It is possible that this relationship may not hold for even lower growth rates, and certainly the relationship becomes meaningless when growth is effectively zero, i.e., for cells in starvation conditions.

Prior to conducting these experiments, we had speculated that slowly growing marine bacteria might behave like slowly growing *E. coli*. *E. coli* tends to retain excess ribosomes at very low growth rates (15). The loss of ribosomes by starving *E. coli* is accompanied by loss of viability (6). Copiotrophic marine *Vibrio* species also retain a residual complement of ribosomes when growing slowly, which again seems important to viability (10). In effect, these bacteria exhibit a nonlinear relationship between RNA content and growth at very low growth rates. In our experiments, however, RNA content was strongly and linearly correlated with growth rate (log[μ]) to a μ of 0.01 h^{-1}, which is more than 10-fold slower than the lowest growth rates for *E. coli*, *A. aerogenes*, and *S. typhimurium* in studies summarized by Dortch et al. (8). Thus, there is no evidence of excess ribosomal capacity in these marine isolates, at growth rates which would be considered extremely low for a copiotroph. The control of rRNA synthesis at low growth rates may operate very differently in normally slowly growing, noncopiotrophic marine bacteria.

The RNA-growth rate relationship was highly significant for data pooled across isolates but much stronger at the isolate-specific level. For practical applications, it may be preferable to develop taxon-specific methods and predictive regressions, perhaps only for the important (however importance is defined) bacterial species present in a given environment. There are very few data available to assess the difficulty of this task, which must depend on whether marine bacterial communities typically are dominated by relatively few or many species and whether community structure is highly variable. Lee and Fuhrman (17) found that community composition can shift dramatically over relatively short spatial scales. However, at least some bacterial taxa appear to be rather cosmopolitan in distribution (23), and there is evidence that these taxa are also numerically dominant or at least abundant where they occur (11).

Conclusions and future directions. The correlation between the pool size of a macromolecule (RNA cell^{-1} and related measures) and a rate (cell-specific growth rate) may seem counterintuitive. In general terms, the correlation occurs because ribosomal abundance influences protein synthesis rates, which in turn are essential to cell division rates. Of course, the control of bacterial growth is not so simple, and metabolic processes are not affected uniformly by environmental variables, such as nutrient availability. Is there a mechanistic relationship between nutrient availability, RNA concentration, and protein synthesis that would allow prediction of growth rate from the RNA concentration? Jensen and Pedersen (13) and others (2) have proposed that ribosomal availability is the rate-limiting factor controlling protein synthesis. This implies that the form of nutrient limitation (nitrogen versus carbon, for example) will affect growth rate but in general will not alter the relationship between the rRNA concentration and growth rate. The validity of this model for marine bacteria must be tested before measurements of RNA content can be interpreted reliably as indicators of growth rate. Furthermore, practical applications of this approach will require an understanding of the effects of temperature, which appears to alter growth rate independently of RNA content (3, 21). In addition, in these experiments we did not address the issue of unbalanced growth, during which the correlation between RNA and growth rate may alter.

Finally, we have noted that taxon-specific methods are likely to be more powerful than the ethidium bromide method we have employed here to explore the RNA-growth rate relationship. In a separate paper, we will describe a method for measuring the rRNA contents of individual cells, on the basis of fluorescent oligonucleotide probes targeted to 16S rRNA. The method is inherently taxon specific. We are working toward the capability of interpreting such data as the distribution of growth rate among cells of a targeted species. With the increasing availability of 16S rRNA sequence data, this taxon-specific approach has real promise for studying the population dynamics of marine bacteria in nature.

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REFERENCES

1. **Båmstedt, U., and H. R. Skjoldal.** 1980. RNA concentration of zooplankton: relationship with size and growth. Limnol. Oceanogr. **25**:304–316.
2. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
3. **Buckley, L. J.** 1984. RNA-DNA ratio: an index of larval fish growth in the sea. Mar. Biol. (New York) **80**:291–298.
4. **Carman, K. R., F. C. Dobbs, and J. B. Guckert.** 1988. Consequences of thymidine catabolism for estimates of bacterial production: an example from a coastal marine sediment. Limnol. Oceanogr. **33**:1595–1606.
5. **Carman, K. R., F. C. Dobbs, and J. B. Guckert.** 1988. Nonspecific labelling, catabolism, and recycling of [methyl]^{3}H- and [methyl]^{14}C-thymidine in a marine sediment. Limnol. Oceanogr. **33**:1595–1606.
6. **Davis, B. D., S. M. Luger, and P. C. Tai.** 1986. Role of ribosome degradation in the death of starved *Escherichia coli*. J. Bacteriol. **166**:439–445.
7. **DeLong, E. F., G. S. Wickman, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1363.
8. **Dortch, Q., T. L. Roberts, J. R. Clayton, Jr., and S. I. Ahmed.** 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. Mar. Ecol. Prog. Ser. **13**:61–71.
9. **Findlay, R. H., M. B. Trexler, and D. C. White.** 1990. Response of a benthic microbial community to biotic disturbance. Mar. Ecol. Prog. Ser. **62**:135–148.
10. **Flärdh, K., N. H. Albertson, L. Adler, and S. Kjelleberg.** 1991. Stability of ribosomes in the marine *Vibrio* sp. S14 during multiple nutrient starvation, abstr. I-57. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
11. **Giovannoni, S.** Personal communication.
12. **Higgins, D. G., and P. M. Sharp.** 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci. **5**:151–153.
13. **Jensen, K. F., and S. Pedersen.** 1990. Metabolic growth rate control in *Escherichia coli* may be a consequence of subsaturation of the macromolecular biosynthetic apparatus with substrates and catalytic components. Microbiol. Rev. **54**:89–100.
14. **Kemp, P. F.** 1990. The fate of benthic bacterial production. Rev. Aquat. Sci. **2**:109–124.
15. **Koch, A. L.** 1971. The adaptive responses of *E. coli* to a feast and famine existence. Adv. Microb. Physiol. **6**:147–217.
16. **LaRock, P. A., J. R. Schwartz, and K. G. Hofer.** 1988. Pulse labelling: a method for measuring microbial growth rates in the ocean. J. Microbiol. Methods **8**:281–297.
17. **Lee, S., and J. A. Fuhrman.** 1990. DNA hybridization to compare species compositions of natural bacterioplankton assemblages. Appl. Environ. Microbiol. **56**:739–746.
18. **Moriarty, D. J. W.** 1986. Measurement of microbial growth rates in aquatic systems using rates of nucleic acid synthesis. Adv. Microb. Ecol. **9**:245–292.
19. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943–948.
20. **Prasad, A. S., E. DuMouchelle, D. Knovich, and D. Oberleas.** 1972. A simple fluorometric method for the determination of RNA and DNA in tissues. J. Lab. Clin. Med. **80**:598–602.
21. **Ryals, J., R. Little, and H. Bremer.** 1982. Temperature dependence of RNA synthesis parameters in *Escherichia coli*. J. Bacteriol. **151**:879–887.
22. **Schaechter, M., O. Maaløe, and N. O. Kjeldgaard.** 1958. Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. **19**:592–606.
23. **Schmidt, T. M., E. F. DeLong, and N. R. Pace.** 1991. Analysis of a marine phytoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173**:4371–4378.
24. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
25. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eucarya*. Proc. Natl. Acad. Sci. USA **87**:4576–4579.