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Can we estimate bacterial growth rates
from ribosomal RNA content?

Paul F. Kemp
Oceanographic and Atmospheric Sciences
Brookhaven National Laboratory
Upton, NY 11973-5000
USA

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ABSTRACT

Several studies have demonstrated a strong relationship between the quantity of RNA in bacterial cells and their growth rate under laboratory conditions. It may be possible to use this relationship to provide information on the activity of natural bacterial communities, and in particular on growth rate. However, if this approach is to provide reliably interpretable information, the relationship between RNA content and growth rate must be well-understood. In particular, a requisite of such applications is that the relationship must be universal among bacteria, or alternately that the relationship can be determined and measured for specific bacterial taxa.

The RNA-growth rate relationship has not been used to evaluate bacterial growth in field studies, although rRNA content has been measured in single cells and in bulk extracts of field samples taken from coastal environments. These measurements have been treated as probable indicators of bacterial activity, but have not yet been interpreted as estimators of growth rate. The primary obstacle to such interpretations is a lack of information on biological and environmental factors that affect the RNA-growth rate relationship.

In this paper, the available data on the RNA-growth rate relationship in bacteria will be reviewed, including hypotheses regarding the regulation of RNA synthesis and degradation as a function of growth rate and environmental factors; i.e. the basic mechanisms for maintaining RNA content in proportion to growth rate. An assessment of the published laboratory and field data, the current status of this research area, and some of the remaining questions will be presented.

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Introduction

Effective methods for measuring bacterial growth in environmental samples were introduced in the early 1980's (Fuhrman and Azam, 1980, 1982; Moriarty and Pollard, 1981, 1982), and allowed more accurate evaluation of the role of bacteria as both producers and consumers of particulate organic carbon. Growth rates, or related measures such as cell or biomass production, remain a common quantitative tool used to evaluate the bacterial role in aquatic systems.

Two primary approaches are used to measure bacterial growth in natural samples. The more frequently used approach is based on the incorporation of radiolabeled precursors into newly formed macromolecules. It is axiomatic that cell production is normally accompanied by DNA replication, protein synthesis and synthesis of other macromolecules. In principle, macromolecular synthesis rates are suitable indirect measures of bacterial growth (especially during balanced growth), and they can be estimated by measuring the incorporation of appropriate radiolabelled precursors. The procedures used typically require short incubation times, and in water samples require relatively little sample disturbance.

Radiotracer methodologies are known to have numerous problems, some of which are specific to particular procedures, and considerable effort has gone toward refining and testing these methods. A brief catalog of some of the problems is: the requirement for conversion factors to estimate growth rates from radioisotope incorporation, and the known uncertainties and variability of those factors (Coveney and Wetzel, 1988); the unknown effects of community composition on conversion factors; the laborious nature of experiments required to obtain conversion factors; apparent non-specific labelling due to artifacts of the DNA extraction and purification process (Burnison and Nuttley, 1990; Servais et al., 1987); real non-specific labelling due to the use of precursors as energy sources rather than as macromolecule precursors under conditions of nutrient limitation (Hollibaugh, 1988; Iriberri et al., 1990; Carman et al., 1988a; Servais et al., 1987; Fallon and Newell, 1986);

poor detection of anaerobic and possibly of oligotrophic bacterial growth (Moriarty, 1986); high background counts and variability of uptake in heterogenous sediment matrices (Kemp, 1990); and the requirement for an incubation period after sampling, with concomitant sample manipulations which may result in disturbance artifacts (LaRock et al., 1988), especially in sediment (Findlay et al., 1985; Carman et al., 1988a,b).

Currently, the principle alternative to radioisotopic methods is incubation of bacteria after either removal of grazers, or suppression of grazing activity with metabolic poisons. The use of selective metabolic inhibitors to suppress grazing, with a few exceptions (e.g. Newell et al., 1983) has not been particularly successful (Kemp, 1990; Tremaine and Mills, 1987; Sherr et al., 1986), because inhibitors are commonly found to be partially non-specific or only partially effective when used in natural samples. Long incubations are required to detect an increase in bacterial abundance in the absence of grazing, whether grazing is reduced by selective filtration, dilution with filtered seawater (e.g. Kirchman et al., 1982; Wright and Coffin, 1984) or by metabolic inhibitors. Although "bottle effects" may be minor during incubations of bacteria in sufficiently large containers (Lee and Fuhrman, 1991), long incubations severely limit the rate at which data can be acquired, and certainly are difficult to interpret as representing instantaneous, in-situ growth rates.

Despite these problems, extensive reviews of bacterial production data, largely based on radioisotope procedures (e.g. Ducklow and Carlson, 1992), clearly show that these methods yield data that are interpretable and appear to make ecological sense, the benchmark test of any method. One can conclude that the methods are either not as prone to error as a catalog of problems might suggest, or that the magnitude of error introduced by these problems does not overshadow the real patterns in data obtained by conventional methods. What can estimates of growth rate based on RNA content contribute to the existing information on bacterial growth in aquatic environments?

The best conceivable application of conventional methods nonetheless has

considerable limitations. Foremost among these is the inability to consider the growth and activity of individual components (e.g. species) of the total microbial community, especially for bacteria. The existing methods measure total community turnover rates only. Estimated growth rates are dominated by growth of the most active cells, but in most studies no information is obtained which would allow some assessment of the proportion of cells which are active. Using these community-level methods, it is effectively impossible to examine such fundamental ecological problems as species diversity and succession, or the relative contributions of various specific taxa to carbon remineralization. Finally, one may reasonably argue that when methods are known to have potentially serious errors, it is worthwhile to complement them with independently derived data.

The strong correlation between bacterial RNA content and growth rate was first observed more than 40 years ago, and has been demonstrated repeatedly to occur in various bacterial species used as laboratory model systems (particularly *Escherichia coli* and *Salmonella typhimurium*). A partial compilation of the published data on the RNA-growth rate relationship is shown in Figure 1. Considering the variety of methods used to determine RNA content, the number of bacterial isolates represented, the diversity in culture conditions, and the broad range of growth rates observed, it is remarkable that the correlation of growth rate with RNA content is so strong (overall $r^2 = 0.85$).

The strength of this correlation suggests that the underlying causal relationship may be relatively universal, at least among bacterial taxa similar to those represented (Kerkhof and Ward, 1993). If so, then it may be possible to use measurements of RNA content in mixed aquatic bacterial communities in a relatively simple and straightforward manner. [Note that because most of the RNA in cells is ribosomal RNA (rRNA), total cellular RNA content is practically synonymous with rRNA content. In this paper, the term rRNA will be used only when the more specific term is justified.]

The following discussion will consider: the molecular and physiological

mechanisms involved in regulating RNA synthesis and content, and the environmental factors that may influence this regulation in nature; whether the RNA-growth rate relationship is universal, or at least whether there is a universal tendency for a strong correlation between RNA and growth rate; in broad terms, the methods that have been used to measure RNA content, and their advantages and limitations; and field applications of RNA measurements as indicators of bacterial activity.

Regulation of rRNA synthesis and content

Considerable effort has gone into understanding the physiological, biochemical and molecular responses of model bacteria to growth-limiting conditions, including those responses that determine the rate of synthesis and the concentration of cellular rRNA. This body of work has repeatedly demonstrated that rRNA synthesis and degradation rates are adjusted by complex, independent feedback mechanisms, such that the resulting concentration of ribosomes is neither in excess nor insufficient to support the current requirements for protein synthesis in the cell (Nomura et al., 1984; Lindahl and Zengel, 1986). Teleologically speaking, cells are designed to contain approximately as many ribosomes as are needed to maintain the growth rate that the environmental conditions will allow. The following is a brief synopsis of the feedback mechanisms that are hypothesized to maintain ribosomal concentration in proportion to growth rate.

Nutrient limitation of several bacterial species (e.g. *E. coli*, *Paracoccus denitrificans*, *Bacillus polymixa*, *B. licheniformis*, *Clostridium beijerinckii*) results in characteristic patterns of growth which reflect the degree of nutrient stress. The effects of nutrient availability can be divided into non-limited, chronic starvation, and starvation conditions, with a surprising consistency among these model bacteria in the growth rates which are typically associated with transitions between nutrient limitation states (Chesbro, 1988; Chesbro et al., 1990).

These transitions can be characterized by changes in the synthesis rate

and abundance of regulatory nucleotides, especially guanosine 5'-diphosphate 3'-diphosphate (ppGpp, or guanosine tetraphosphate). In the Bacteria, ppGpp influences, among other processes, translational error correction during protein synthesis (Gallant and Foley, 1979), the initiation of rRNA synthesis (Glass et al., 1986) and the protein chain elongation rate (Rojas et al., 1984). The combined effect is a reduction in cellular ribosome concentration and in protein synthesis as growth rate declines.

The cellular concentration of ppGpp is strongly and negatively correlated with growth rate and positively correlated with the proportion of RNA synthesis devoted to rRNA (Ryals et al., 1982a). In non-limiting conditions, ppGpp is at a basal level and growth is exponential. During the transition to chronic starvation condition (typically at doubling times near 15-20 h, Chesbro et al., 1979), ppGpp concentration increases, rRNA synthesis decreases and growth slows. With increasingly severe chronic starvation, the need to increase translational error correction, in particular, occupies an increasingly large fraction of cellular energy costs. As a consequence, there is a dramatic reduction in biomass synthesis in favor of redirecting energy flow to increasing maintenance requirements. At doubling times near 50-60 h, ppGpp levels increase substantially, rRNA synthesis is strongly reduced and growth rate decreases correspondingly (Van Verseveldt et al., 1984; Arbige and Chesbro, 1982). Finally, under starvation conditions ppGpp levels remain somewhat elevated, and growth is near-zero. Similar changes in ppGpp concentration are observed when cells are shifted into starvation by metabolic inhibitors (Ryals et al., 1982a), rather than entering starvation due to long-term exhaustion of resources.

The onset of severely reduced growth resulting from the shift of energy resources to maintenance functions has been termed the stringent response, originally for the specific case of the response to amino acid deficiency (Fiil et al., 1977). During and following the stringent response, specific proteins are induced which appear to increase the long-term survival of starved cells (Nystrom et al., 1990; Albertson et al., 1990). However, the

correlation between ppGpp levels and growth rate occurs not only during the onset of starvation, but as a general phenomenon over a very broad range of growth rates (Ryals et al., 1982a Fig. 2).

The cellular abundance of ppGpp is controlled at both the production and degradation levels. ppGpp synthesis involves at least two routes, one of which is controlled by a ribosome-bound pyrophosphate transferase that requires mRNA and uncharged tRNA (i.e. tRNA not carrying an amino acid) (Goldman and Jakubowski, 1990). ppGpp degradation also is dependent on a ribosome-bound enzyme (guanosine 3',5'-bis[diphosphate] 3'-pyrophosphohydrolase, or ppGppase) that is inhibited by uncharged tRNA (Fiil et al., 1977; Richter, 1980) and by glucose exhaustion (DeBoer et al., 1975; Tetu et al., 1980). Note that it is the relative proportions of uncharged tRNA and ribosomes, rather than the absolute concentrations, that trigger the synthesis or degradation of ppGpp. This dependency on uncharged tRNA couples both synthesis and degradation of ppGpp to cellular amino acid abundance, and therefore to the supply of energy, carbon, nitrogen and sulfur (Arbige and Chesbro, 1982). Because the pyrophosphate transferase and hydrolase are both ribosome-bound, the synthesis and degradation of ppGpp are mediated in part by the abundance of ribosomes.

ppGpp appears to control rRNA synthesis at the transcriptional level by influencing the binding of RNA polymerase to RNA gene promoters. Ryals et al. (1982a) presented a model which explains the differential effects of ppGpp on stable RNA synthesis and mRNA synthesis. In their model, ppGpp-controlled conformational changes in RNA polymerase preferentially shift its binding from stable RNA (tRNA and rRNA) promoters to mRNA promoters. This model is supported by similar changes in RNA synthesis which occur in mutations that affect the sensitivity of RNA polymerase to ppGpp (Little et al., 1983), or that produce conformational changes in RNA polymerase.

Jensen and Pedersen (1990) present an alternative model for the differential effects of ppGpp on stringently controlled and other genes. They hypothesize that promoters of stringently controlled genes have lower affinity

for RNA polymerase than promoters of non-stringently controlled genes. As growth rate slows and the concentration of RNA polymerase decreases, stringently controlled gene promoters lose RNA polymerase molecules to competing promoters with higher affinity for RNA polymerase. rRNA synthesis decreases as a consequence, and the concentration of ribosomes declines until the pool of charged tRNA is sufficient to engage most ribosomes in synthesis. This triggers decreased ppGpp synthesis and increased ppGpp degradation. Steady state growth in their model is a compromise between the abundance of ribosomes, and the availability of substrate per ribosome at a given growth rate.

Severe reductions in stable RNA content and growth also have been observed for starved bacteria in the absence of ppGpp accumulation, implying that there are independent mechanisms which operate in response to starvation (Acosta and Lueking, 1987; Gaal and Gourse, 1990). In non-bacterial organisms such as the yeast *Saccharomyces cerevisiae*, ribosomal concentration is correlated with growth rate and there are strong responses to starvation that parallel the stringent response, but these occur in the absence of ppGpp (Warner, 1989). The basic requirement for maintaining a balance between ribosomal concentration and growth rate is met, but not necessarily through the ppGpp mechanism.

Ribosomal protein synthesis is also regulated by feedback mechanisms. Ribosomal protein synthesis is repressed when ribosomal proteins are present in excess relative to rRNA (Jinks-Robertson and Nomura, 1987), because free ribosomal proteins (not assembled into ribosomes) bind to the promoters of their own genes and thereby competitively inhibit transcription. Ribosomal proteins also appear to act to protect rRNA against degradation, once assembled into ribosomes. For example, when rRNA is overproduced in a conditional gene-expression system (Gourse et al, 1985), the turnover of newly synthesized rRNA is much higher than normal. Thus, mechanisms for ribosomal proteins and ribosomal RNA interact to prevent either component of intact ribosomes from being synthesized in excess.

An additional feedback regulatory mechanism involves the FIS (factor for inversion stimulation, Nilsson et al., 1992) protein, the product of the *fis* gene. The FIS protein activates the transcription of both rRNA and tRNA operons when cells are exposed to improved nutritrional conditions and shift into higher growth rates. It appears to be more important at higher growth rates (Nilsson et al., 1992), although as yet its overall importance is not well understood.

Growth rate regulation in marine bacteria

These growth-regulating mechanisms have been examined in several model bacteria and may also be general to many aquatic bacteria. However, few studies of the causes and mechanisms of bacterial growth regulation have been conducted for aquatic systems. Studies of the biochemical consequences of substrate limitation in aquatic bacteria have focused on the "starvation-survival" phenomenon in marine bacteria (Morita, 1982, 1988; Amy et al., 1983; Baker et al., 1983; Marden et al., 1985). In response to extremely low nutrient availability, marine bacteria enter a condition in which growth rates are effectively zero and biochemical adaptations occur that maximize survival rather than growth. This phenomenon is equivalent to starvation as described above for cultured model bacteria. The starvation-survival phenomenon is known in marine systems primarily from deep-ocean and extremely oligotrophic environments.

Nystrom et al. (1990) examined the response of a marine *Vibrio* strain to complete starvation for single and multiple nutrients. Metabolic changes consistent with a stringent response mechanism (i.e. increased ppGpp levels and concomitant decreased RNA synthesis) occurred under amino acid starvation. This provided the first direct evidence that the stringent response occurs in marine bacteria, and that ppGpp is implicated in the response. Nystrom et al. found that a total of 66 proteins were starvation-inducible, and that each starvation condition tested (i.e. glucose, amino acids, ammonium, or phosphate deficiency) resulted in expression of an almost completely unique subset of

these proteins. Synthesis of additional proteins were induced by multiple-nutrient starvation. If the expression of starvation-survival proteins was inhibited, continued survival was greatly decreased. Similar responses to starvation have been observed for *E. coli* and *S. typhimurium* (Schultz and Matin, 1991; Groat and Matin, 1986; Foster and Spector, 1986; Groat et al., 1986). A few of these starvation-induced proteins have been isolated and identified or sequenced (Groat and Matin, 1986; Jenkins et al., 1988).

In summary, the regulatory mechanisms controlling bacterial growth under nutrient limitation are reasonably well understood for model bacteria, and comparable mechanisms appear to operate in at least some marine bacteria. While this is encouraging, it is important to recognize that we have no detailed information on the average physiological status of bacteria in natural aquatic systems, let alone their exact metabolic responses to different growth conditions. One cannot assume that stringent or starvation-survival responses are necessarily common, or even occur in bacteria in aquatic systems. However, as noted in the following section, there is considerable indirect evidence to suggest that these or alternative regulatory mechanisms do operate in most aquatic bacteria, and indeed in organisms other than bacteria, and regulate rRNA concentration in direct proportion to growth rate.

How universal is the RNA-growth rate relationship?

The data in Figure 1 are a subset of all published data, where the data were or could be expressed in common units (specifically, as an RNA:DNA ratio). Nine members of the Bacteria are represented; three are enteric species, one is a sulfate-reducing anaerobe, and five are isolates from marine environments. Only one is known to be a member of the alpha-Proteobacteria; the others are known or assumed to be gamma-Proteobacteria. In other words, these data, while voluminous, are severely limited in terms of the phylogenetic diversity represented. What information is available to suggest that a similar relationship between RNA and growth rate will exist for

bacteria other than the small subset that have been examined?

RNA:DNA ratios have been used as a measure of growth rate for a number of other organisms. Sutcliffe proposed as early as 1965 that ribosomal concentration could be used to predict the growth rate of marine invertebrates (Sutcliffe, 1965). Dortch et al. (1983) cited a number of examples of the use of RNA:DNA ratios to measure growth in several large invertebrates (see references cited), and found that the RNA:DNA ratio was linearly related to growth rate for marine phytoplankton. Buckley (1984) demonstrated that the RNA:DNA ratio was an excellent predictor of growth in larval fish. DiRuggiero et al. (1993) found that rRNA content increased with increasing growth rate of a hyperthermophilic Archaeon. rRNA content is also proportional to growth rate in the yeast *Saccharomyces cerevisiae* (Waldron and Lacroute, 1975; Kief and Warner, 1983; Waldron, 1977; Warner, 1989). In short, there are abundant data indicating that an RNA-growth rate relationship is a common, perhaps universal property of living organisms.

The form of that relationship may vary when taxa are compared in detail. Kemp et al. (1993) found that there were statistically significant differences among the RNA-growth rate equations determined for four marine bacterial isolates in chemostat culture. These isolates are included in the data shown in Figure 1, and suggest that at least some of the variance in Figure 1 is due to real differences among bacterial taxa. However, Kemp et al. attributed at least some of the differences among isolates to differences in their characteristic size; when cell size was taken into account, the statistical differences among isolates were greatly reduced. RNA content varies with cell size for cells growing at the same rates. Larger cells on the average contain a greater biomass and require more ribosomes to reproduce that larger biomass than do smaller cells growing at the same rate (Kemp et al., 1993). Thus, measurement methods that compensate for cell size (Poulsen et al., 1993) reduce the apparent variability of the RNA-growth rate relationship.

Environmental factors

Based on the data in Figure 1, it appears that a single, mathematically simple equation (a simple linear model fits the data in Fig. 1 nearly as well) might be used to describe the relationship that cells maintain between rRNA concentration and growth rate. Clearly, there is substantial variation also evident in Figure 1. Some of that variation may be due to variations in characteristic cell size, as discussed above. At least some of that variation is due to environmental factors. For field applications, these factors might be accounted for or eliminated. What environmental variables are likely to occur and may influence the RNA-growth rate relationship? Obvious candidates are variations in food resources, and in physical conditions such as temperature, salinity, pH, and other water properties.

Bacterial growth in aquatic environments is usually assumed to be limited by the abundance of labile dissolved organic matter (DOM), which provides the link between primary and bacterial production (Azam et al., 1983; and many others). This assumption is supported by tight coupling between the release and bacterial uptake of amino acids (Fuhrman, 1987), and various correlative and inferential data. For example, low concentrations of free amino acids are often attributed to high bacterial uptake and utilization (e.g. Jorgensen et al., 1983; Mopper and Lindroth, 1982). Diel and longer cycles in bacterial activity are often attributed to concurrent cycles in phytoplankton production and release of dissolved organic matter (Painting et al., 1989; Fuhrman et al., 1985; Turley and Lochte, 1986; Lancelot and Billen, 1984; Herndl and Malacic, 1987). For the most part, the DOM used by bacteria is uncharacterized, and little is known regarding what specific DOM components are in limiting supply.

It is fortunate, then, that the cause of nutrient limitation (e.g. carbon versus nitrogen starvation, single amino acid deficiency versus multiple deficiencies) does not appear to influence the form of the rRNA-growth rate relationship. Indeed, the most common method for manipulating bacterial growth rates in the studies represented in Figure 1 was to alter

food quality as well as quantity, for example by altering the carbon and energy source provided in a nutrient medium. One way of phrasing it is that the effects of nutrient limitation on bacterial growth rate appear to be interpreted through their effects on the activity, synthesis, and concentration of ribosomes. Although much more work needs to be done to test this hypothesis for aquatic bacteria, the data for cultured bacteria supports it. What other environmental variables influence the rRNA-growth rate relationship and are likely to be important in aquatic systems?

The specific growth rate μ of bacteria can be described by a theoretical relationship which incorporates the synthesis rates of RNA, RNA polymerase and protein (rewritten from eqns. in Bremer, 1975; Ryals et al., 1982b):

$$\mu = k_0 \sqrt{(\epsilon_r \alpha_p \phi_s c_s \beta_p k_1) / (k_2 k_3 k_4)} \quad (1)$$

where ϵ_r = rate of protein production per ribosome; α_p = proportion of synthesized protein which is RNA polymerase; ϕ_s = fraction of RNA polymerase synthesizing ribosomal RNA (rRNA); c_s = stable RNA chain elongation rate per RNA polymerase; and β_p = active fraction of RNA polymerase. The constants k_0 - k_4 are: k_0 = dimensional constant; k_1 = ratio of rRNA to total stable RNA; k_2 = ratio of length of complete stable RNA transcripts (including spacer regions) to mature stable RNA; k_3 = rRNA nucleotides per ribosome; and k_4 = amino acids per RNA polymerase molecule.

Nutrient limitation has its primary effect in reducing rRNA synthesis rates (c_s), at least partly mediated by the effects of charged and uncharged amino acid concentrations on ppGpp concentration, as discussed above. Reduced rRNA synthesis rates result in a reduction in cellular rRNA content and consequently in protein synthesis. In addition, nutrient limitation can also suppress other growth-limiting processes such as peptide chain elongation rate (ϵ_r), because ribosomes in cells starved for a specific amino acid tend to "pause" in translation when that amino acid is required in the growing peptide chain.

Temperature has a strong effect on both the rRNA and protein chain elongation rates (c_s and ϵ_r). In *E. coli*, ribosomal and RNA polymerase

activity both have typical, and rather similar, Q_{10} responses to changes in temperature. For a given rRNA concentration, higher temperatures result in higher rRNA and protein synthesis rates, and consequently higher growth rates. Ryals et al. (1982b) noted that the effect of temperature on growth rate appeared quite simple, but suggest that this apparent simplicity is actually the result of interactions among more complex effects. As a practical matter, however, temperature can be incorporated into an rRNA-growth rate equation as a simple linear variable, orthogonal to rRNA content. Buckley (1984) was able to predict larval fish growth using an empirical, predictive equation for larval fish, where growth rate was a linear function of both temperature and rRNA content. The equation explained most of the variance in weight-specific growth rates of several species in aquaculture. Similarly, Juinio et al. (1992) found that temperature effects must be taken into account to use RNA:DNA ratios as a measure of the nutritional status of postlarval lobsters; the RNA:DNA ratio was inversely correlated with temperature and positively correlated with food ration.

In relatively short-term culture conditions, rRNA content per cell is invariant with changes in temperature for *E. coli* (Ryals et al., 1982b) and *S. typhimurium* (Schaechter et al., 1958). Under field conditions, however, the average rRNA content of cells appears to decline with increasing temperature (Lee et al. 1993) over seasonal time scales, suggesting that fewer ribosomes are needed at higher temperatures to maintain the same average growth rate. Alternatively, community composition may change over seasonal time scales (Lee and Fuhrman, 1991), and the seasonal changes in average rRNA concentration observed by Lee et al. may relate more to changes in species than to changes in temperature. At present, it seems likely that it will be necessary to incorporate temperature effects into predictive rRNA-growth rate equations for natural aquatic communities; however, seasonal changes in response to temperature may prove to complicate matters.

There is now limited evidence that rRNA content varies with diel periodicity in some organisms, including bacteria. Kramer and Singleton

(1993) found strong diel periodicity in *Synechococcus* (phototrophic cyanobacterium) populations in coastal marine waters, such that ribosome concentrations were highest at noon and lowest at night. It was not known whether this diel change was in proportion to growth rate, but the existence of diel patterns points out that measurements of RNA content must be interpreted in the context of periodic changes in the environment.

It also points out that ribosomal concentration can alter over relatively short time scales. We have found that rRNA of marine bacterial isolates increased to a new equilibrium level after growth rate upshifts, over times less than or equal to one generation time (Kemp et al., 1993); i.e. within hours for faster growing cells. For cells undergoing growth rate downshifts, rRNA content of marine isolates decreased to the new level equally rapidly (Kemp, unpublished data). The rapidity with which ribosomal concentration is adjusted means that rRNA concentrations at any given time should reflect the current (or at worst very recently past) growth rate.

Kramer and Singleton (1992), however, found that the rate at which two marine *Vibrio* species reduced ribosome concentration depended on the species and the cause of the nutrient downshift. *V. alginolyticus* shifted from nutrient-replete to nutrient-depleted medium lost rRNA slowly, in comparison to cells allowed to exhaust the nutrients in the original medium. In *V. furnissii*, however, cells entering starvation by either route lost rRNA rapidly. Thus, there is evidence to suggest that under certain conditions, some bacteria may retain excess rRNA (not necessarily as functional ribosomes) over extended periods. This is of some concern, because it implies that the rRNA content of these bacteria does not necessarily reflect the current growth rate, but rather may be a relict of earlier events. More work is certainly needed to test whether the phenomenon of ribosome retention is common or atypical. To date, we have not observed it during extensive work with four marine gamma-Proteobacteria isolates (Kemp, unpublished data). Work is underway to look for this phenomenon in a larger suite of isolates.

Oxygen clearly also has a strong influence on cell metabolism. Dramatic

changes in metabolic pathways occur when organisms switch between aerobic and anaerobic metabolism, and there is relatively little information available to judge whether these changes affects the regulation of rRNA synthesis and the RNA-growth rate relationship. Georgellis et al. (1993) found that there was a large increase in RNA stability when *E. coli* cells were grown anaerobically, relative to cells grown aerobically. However, there was no difference in rRNA concentration in aerobically and anaerobically grown *E. coli* at comparable growth rates, because rRNA synthesis was reduced in anaerobic cells, thus compensating for the increased half-life of the RNA. This suggests that the cells use compensatory changes in synthesis and degradation rates to maintain the same proportionality between ribosome concentration and growth rate as they switch between aerobic and anaerobic metabolism (Georgellis et al., 1993). This is particularly encouraging because at least some conventional methods for measuring bacterial growth rate are believed to be unreliable in anaerobic conditions (e.g. Moriarty, 1986). RNA-based methods may not be as problematic as conventional methods when used in anaerobic environments.

In summary, the single environmental factor known to be important under at least some conditions is temperature. Diel changes in environmental conditions, especially light, may be important to photosynthetic bacteria. Food quality does not appear to alter the form of the RNA-growth rate relationship. Cell size is important and must be taken into account. There are little or no data to judge whether other environmental variables such as oxygen, salinity and pH will present problems in field applications.

Methods used

Measurements of RNA content can be acquired by several alternate approaches. In broad terms, these approaches can be divided into biochemical measurements, and nucleic acid hybridization methods. The various specific methods differ in the information returned, their sensitivity and detection limits, and their ease of use.

Biochemical methods utilize colorimetric reactions, or alternatively,

nucleic acid-specific fluorochromes. Much of the published data on rRNA content and growth of bacteria is based on measurements of bulk RNA in nucleic acid extracts, originally through colorimetric assays, and more recently using fluorochromes such as ethidium bromide. These data are typically normalized to cell numbers, in recognition that it is the RNA content per cell mass that is proportional to growth rate. In many cases, cell counts are not available, or are difficult to relate to the quantity of RNA extracted because the efficiency of extraction is not measured or is not well known (i.e. it is not certain how many cells are actually represented in a quantity of extracted nucleic acids). The most common solution is to normalize RNA to DNA in the same nucleic acid extract. This treats DNA as a surrogate for cell abundance, assuming that the DNA content of bacterial cells is much less variable than their RNA content.

On the positive side, RNA:DNA ratios have the advantage of correcting for variations in the efficiency of nucleic acid extraction, because much of the variation in extraction efficiency appears to occur at the level of co-purification of RNA and DNA from intact cells, rather than later in the extraction when RNA and DNA are separated (Kemp et al., 1993). The disadvantage is that to the extent that DNA content also varies with growth rate, the RNA:DNA ratio approach will actually result in a loss of dynamic range of the variable used to predict growth rate. That is, the slope of a line describing the change in RNA:DNA ratio per unit change in growth rate is proportionally less steep than the slope of a line describing the change in RNA per unit change in growth rate.

More recently, hybridization methods are finding increasing use because they provide a greater capacity to target specific taxa and to measure rRNA content in individual cells. These methods are based on hybridization of nucleic acid probes to target sites on the rRNA molecules. The probes carry a radioisotopic or fluorescent label, or alternatively carry a target for a secondary detection probe.

Recent reviews detail the molecular basis for hybridization methods

(e.g. Ward et al., 1992; Wolcott, 1992). In brief, these methods capitalize on the evolutionarily conservative nature of ribosomal RNA, and the resulting ability to select target locations on the rRNA molecule whose nucleotide sequences are diagnostic for a specific taxon. Segments of the rRNA molecules (5S, 16S, and 23S rRNA are the three rRNA molecules incorporated into bacterial ribosomes) vary in the degree to which their nucleotide sequences are evolutionarily conserved. Nucleotide sequences in conservative segments are common to relatively broad phyla, whereas nucleotide sequences in non-conservative regions may be unique even at the species or subspecies level. Consequently, probe sequences can be selected which are characteristic of specific phylogenetic groups, ranging from universal probes, to kingdom-level (Bacteria, Archaea, or Eucarya), to extremely specific probes. DeLong et al. (1989), for example, used rRNA probes to distinguish between two closely related bacterial species. Gobel et al. (1987) developed rRNA probes capable of discriminating between congeneric bacteria. Salama et al. (1991) describe the development of rRNA probes to distinguish *Lactococcus lactis* sub. *cremoris* from its conspecifics. Specificity is manipulated both by target site selection, and by altering the conditions of the hybridization.

Hybridization methods can be applied to bulk extracts of nucleic acids, e.g. by hybridizing DNA probes against RNA extracted from natural samples. Like biochemical methods discussed earlier, these methods yield total RNA per sample, which is then normalized to either cell numbers or to a surrogate measure, such as total DNA abundance, or the abundance of rDNA genes (Kerkhof and Ward, 1993). The important difference from data that is obtained using biochemical measurements is that hybridization techniques incorporate taxonomic specificity. Ideally, these methods can be used, for example, to measure the RNA:DNA ratio of a specific bacterium in a mixed community (e.g. Kerkhof and Ward, 1993).

An alternative form of hybridization methods uses the same principle of targeting sites on the rRNA molecule with a labelled DNA probe, but this is done in intact cells rather than extracted nucleic acids. Most commonly, the

probe carries a fluorochrome and hybridization is detected using microfluorometry. These "in situ hybridizations" are now used commonly to detect and enumerate targeted bacteria in natural and laboratory samples (e.g. Amann et al., 1990; Manz et al., 1993; McSweeney et al., 1993; Kemp, 1994; Wagner et al., 1993). It is a natural extension to that work to measure the fluorescence signal obtained after hybridization, to learn to interpret that fluorescence intensity as a measure of rRNA content, and to explore the meaning of that rRNA content as an indicator of growth rate.

The advantage to this approach is that the rRNA content of individual cells is measured with the same taxonomic specificity as in bulk nucleic acid hybridization methods. Ideally, such data can be interpreted as indicators of the growth rate of individual cells, and it should be possible to determine the distribution of growth rate among cells in bacterial populations. Such cell-specific data have been difficult to acquire with conventional methods (e.g. using microautoradiography, Tabor and Neihof, 1982). Indeed, it is not yet known how useful rRNA-frequency distributions may be as measures of the ecological status and responses of aquatic bacteria.

A principle disadvantage to the in-situ hybridization approach is that the fluorescence signal is low for relatively inactive cells containing few ribosomes, and therefore the method tends to be biased in favor of active cells. Solutions for this problem are to enhance the fluorescence signal, increase the sensitivity of the detector (e.g. cooled-CCD camera systems rather than optical photometers), or simply accept that the method is biased. The bias, it should be noted, exists as well for virtually any growth rate method that pools entire populations of cells, including RNA methods and macromolecular labelling methods (e.g. thymidine and leucine labelling) based on bulk nucleic acid extracts.

Enhancement methods are problematic. Multiple independent probes act to increase fluorescence additively and have been used successfully in field samples (Lee et al., 1993; Lee and Kemp, 1994). For a given target group, it is probably feasible to design a few independent probes of similar specificity

and similar requirements for hybridization (a function of the base sequence of probes, their length, and the number of mismatches expected from the target sequence), but this produces only a limited enhancement of 2-5x. Other methods that have been explored include: labelling with multiple fluors; attachment of long polythymidine "tails" detected with polyadenine secondary probes; and the use of biotinylated probes detected with an fluorochrome-labelled avidin secondary probe. The former two methods have been found to result in variable signal strength, depending on cell permeability, and undesirable non-specific probe binding. The biotin-avidin method, however, has worked well for labelling rRNA in marine protists (Lim et al., 1993), and is a promising approach for aquatic bacteria as well.

Improving instrumentation is an effective method for increasing sensitivity and improving detection limits. Cooled-CCD cameras are much more sensitive than optical photometers, in our experience; indeed, typical cooled-CCD cameras are considerably more sensitive to low fluorescence than the human eye. Using these detectors, it is common to find that cells are seen in captured images that were not visible to the eye. With an optical microphotometer, we were able to detect as little as 0.3 femtograms of RNA per cell (Lee and Kemp, 1994). The cooled-CCD camera we now employ is substantially more sensitive, although other factors are involved in setting the absolute detection limit, including background and non-specific fluorescence, and software limitations in analyzing captured images.

Field applications: examples

There are, as yet, relatively few examples of the use of rRNA measurements to study natural aquatic bacterial communities. The rRNA-growth rate relationship has not been used to evaluate bacterial growth in field studies. Instead, RNA measurements have been treated as probable indicators of bacterial activity rather than as estimators of growth rate. This level of interpretation is appropriate. The rRNA-growth rate relationship is not yet sufficiently well understood to predict the growth rate of complex natural

communities, based solely on the rRNA-growth relationship determined for model organisms. However, a more complete interpretation waits only on additional information. The most urgently needed are data on the RNA-growth rate relationship for bacterial taxa actually present in natural populations, and comparisons with other measures of growth rate.

Published studies of variations in bacterial rRNA content under field conditions are so far limited to exploratory work. Kramer and Singleton (1993) measured variations in rRNA content of heterotrophic bacterial communities and of photosynthetic *Synechococcus* in coastal marine waters. The approach used was probing bulk-extracted rRNA with either Bacteria- or Cyanobacteria-specific 16S rRNA oligonucleotide probes. In this demonstration study, they were able to detect substantial diel variations in rRNA content of *Synechococcus*. They also found that heterotrophic bacteria were stimulated, in terms of increased rRNA content, by additions of glucose but not by additions of amino acids. Their work demonstrated the potential for rRNA measurements to reveal patterns in bacterial activity, including changes in activity induced by changes in the nutrient field.

In our laboratory, we have begun an extensive series of measurements of rRNA content in field populations of marine bacteria, using in-situ hybridization and analyses of rRNA content in individual cells by microfluorometry. Lee et al. (1993) and Lee and Kemp (1994) reported on the effectiveness of multiple independent probes to detect the rRNA content of individual cells in nearshore bacterial communities. Data were presented showing that there are pronounced changes in mean rRNA content in these communities over seasonal time scales. To some extent (but not entirely), these changes are consistent with the effects expected from seasonal changes in mean water temperature.

In more recent work, Kemp (1994) presented a preliminary data set showing a section plot of bacterial rRNA content across the continental shelf. Variations in rRNA were compared to changes in temperature, salinity, nutrients, chlorophyll, and other water properties. rRNA content was also

compared to measurements of thymidine incorporation and leucine incorporation. In general, rRNA content showed very strong spatial pattern that was to a considerable extent statistically explained by variations in other measured variables. For example, there was a large increase in the mean rRNA content of bacteria in locations associated with the outflow of the Chesapeake Bay estuary, marked by high temperature and low salinity. A second site of high rRNA content coincided with an area of high chlorophyll concentration at mid-shelf near the bottom.

Thymidine and leucine incorporation showed equally strong spatial pattern, and in general terms were elevated in the same water masses. However, the three different measures of bacterial activity were weakly intercorrelated, indicating that each represented some different aspect of the relationship of bacterial activity to the environment, such that detailed point-by-point comparisons revealed differences in spatial distributions. This points out the value of having multiple independent measures of activity, each of which provides some unique glimpse into the relationship of bacteria to their environment.

We have found that, on the average, approximately 65% of bacterial cells in coastal marine communities contain sufficient rRNA to be detected and measured using an in-situ hybridization approach and multiple independent probes. The remainder contain less than ca. 0.3 fg rRNA per cell, and we assume that these represent the least active cells. Most are small and coccoidal (Lee and Kemp, 1994).

Conclusions

Clearly, there is a large body of laboratory data that supports the hypothesis that rRNA content is strongly and consistently related to growth rate in bacteria, and there is ample evidence to suggest that the existence of such a relationship is a universal property. However, there are as yet insufficient data to assume that the exact form of an equation describing the relationship is also universal among bacteria. Existing data indicate that

such an equation must explicitly or implicitly recognize at least rRNA content, temperature, and cell size. Other environmental factors may prove to be important as well. The available data suggest that a simple (e.g. linear) proportionality of growth rate to rRNA content (expressed as rRNA per cell biomass or other measure of cell size) and temperature is sufficient. With these measurements, it is now possible to use RNA content measurements to estimate growth rate, for specific cultures in laboratory conditions.

Early field work with in-situ hybridization and bulk hybridization approaches have demonstrated that measurements of rRNA content are useful indicators of bacterial responses to environmental conditions. However, the current understanding of the rRNA-growth rate relationship is still too primitive to justify more than a very cautious interpretation of rRNA content as a predictor of growth rate for field populations. The current status of the method might be described as having moved from laboratory demonstration to field validation. All data now available suggest that the approach will prove robust, and a useful complement to conventional methods for measuring bacterial growth rate.

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Figure legend

Fig. 1. Partial compilation of published data on the RNA:DNA ratio versus specific growth rate. Data for marine species in filled symbols, freshwater or enteric in open symbols. Original data sources are: *Escherichia coli*, Rossett et al. (1966), Churchward et al. (1982), Neidhardt (1987); *Salmonella typhimurium*, Rossett et al. (1966), Schaechter et al. (1958), Kjeldgaard and Kurland (1963); *Aerobacter aerogenes*, Rossett et al. (1966); strain PT2 Poulsen et al. (1993); *Pseudomonas stutzeri*, Kerkhof and Ward (1993); strains SARW1, GBW1, GBW2, and GBSED1, Kemp et al. (1993). A) shows specific growth rate on a linear scale; B) shows growth rate on a log scale.

○ <i>Salmonella typhimurium</i>	▼ <i>Pseudomonas stutzeri</i>
□ <i>Aerobacter aerogenes</i>	● SARW1
◇ <i>Escherichia coli</i>	■ GBW1
△ PT2	▲ GBW2
	◆ GBSED1