AN ABSTRACT OF THE THESIS OF

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Barry F. Sherr

A series of experiments were performed to test the hypothesis that bacterivorous protists selectively feed on specific genotypes of marine bacterioplankton, affecting bacterioplankton community structure. A study comparing the bacterioplankton community diversity estimated by a SSU rDNA gene (SSU rDNA) clone library and a collection of cultivated bacteria from the same water sample suggests that bacterioplankton is dominated by organisms that do not grow easily in enrichment cultures and are underrepresented in culture collections. Therefore, a new method, length heterogeneity analysis by PCR (LH-PCR) was developed to assess the community structure of *in situ* bacterioplankton communities. In LH-PCR, a region of the SSU rDNA which exhibits length variations among different phylogenetic groups is amplified from environmental DNA by PCR. Fragments originating from different organisms are discriminated by their length and quantified by the fluorescence emission of a labeled primer. Since the method is based on PCR, a study was performed to evaluate the introduction of bias by the reaction. Using pairwise mixtures of rDNAs I described reannealing bias, a new source of PCR bias by PCR. This bias is

caused by self-inhibition of PCR amplicons that attain elevated concentrations after several reaction cycles; thus templates with higher concentration in original gene mixtures tend to be underrepresented in products. This bias can be minimized by limiting final PCR product concentrations. I applied LH-PCR to assess changes in bacterioplankton communities in four protist exclusion experiments. Changes in the genotypic composition of the bacterial community of water samples with protists removed by filtration was followed and compared to the changes in community structure of control water samples. There were differences between filtered water samples and controls incubated for 24 to 48 hours. In the absence of bacterivores some SSU rDNAs that were insignificant in the original water samples dominated the bacterioplankton SSU rDNA pool after 48 hours. Protists appeared to be capable of controlling bacterioplankton taxonomic diversity under manipulated conditions. This supports the hypothesis that aquatic bacterioplankton communities are dominated by relatively inactive cells that are less susceptible to grazing by bacterivorous protists.

The Effect of Protistan Bacterivory on Bacterioplankton Community Structure

by

Marcelino T. Suzuki

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Redacted for privacy

Co-Major Professor, representing Oceanography

Redacted for privacy

Co-Major Professor, representing Oceanography

Redacted for privacy

Dean of the College of Oceanic and Atmospheric Sciences

Redacted for privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Marcelino T. Suzuki, Author

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CONTRIBUTION OF AUTHORS

Dr. Stephen Giovannoni was involved in the design, analysis and writing of the first three manuscripts. Dr. Michael Rappé was involved in the construction of the clone libraries, DNA sequence analysis and writing of two manuscripts. Zara W. Haimberger helped in the screening and mantainance of the culture collection, and in the DNA sequencing. Harry Winfield, Nanci Adair, Jürgen Ströbel helped in the isolation and screening of the culture collection.

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DEDICATION

This thesis is dedicated to the memory of Susanne Szentrandrasi for her courage, perseverance, and her attitude towards life.

The Effect of Protistan Bacterivory on Bacterioplankton Community Structure.

Introduction

Bacterioplankton Community Structure.

Bacterioplankton are major components of aquatic carbon cycles, representing a large fraction of the living particulate organic carbon, as well as utilizing a large fraction of the phytoplankton primary production (Cole et al. 1988). A recent study comparing global estimates of bacterioplankton respiration and phytoplankton production reports that bacterioplankton respiration may exceed phytoplankton organic carbon production, and suggests that such systems may represent a net source of carbon dioxide (del Giorgio et al. 1997). Understanding the dynamics of aquatic bacterioplankton communities is therefore an important step in the analysis of the roles of the World ocean in the atmospheric carbon dioxide balance, especially with repect to the predicted global warming scenario.

Ducklow & Carlson (1992) summarized some of the possible quantitative parameters defining community structure: "the absolute number of species in a community, the relative abundance of individuals in different species or functional groups, the ratios of biomass of producers to consumers, or the distribution of elemental fluxes among different trophic connections". Until recently, the taxonomic diversity of microbial communities was relatively unknown. Therefore, in the majority of the

surveys, bacterioplankton has been characterized in terms of the biomass, production and mortality of mixed assemblages of bacteria. This has been called the "microbial black box".

Bacterioplankton biomass has usually been estimated from the numeric abundance of cells stained with fluorochromes targeting nucleic acids, and identified as bacteria from their size and morphology using epifluorescence microscopy (Hobbie et al. 1977) or flow cytometry (Li et al. 1995, Marie et al. 1996). Cell numbers are converted to biomass from cell numbers and average cell volumes using "community-wide" conversion factors (Bratbak & Dundas 1984, Bratbak 1985, Bjørnsen & Riemann 1988, Borsheim et al. 1990). There has been general acceptance of these methods for estimating bacterioplankton biomass and such measurements have been made for decades worldwide (Berninger et al. 1991, Sanders et al. 1992, Kepner & Pratt 1994). In general, except for hypereutrophic freshwater systems, the results of such measurements show that aquatic bacterioplankton range between 10^5 and 10^7 cells x ml^{-1}. However, a number of recent studies have shown that even a basic measurement like bacterioplankton biomass may be inaccurate. The facts that different fluorochromes yield different values of abundance and biomass (Suzuki et al. 1993), and that non-specific binding of fluorochromes may overestimate bacterioplankton cell counts revived the discussion about the significance of bacterial cell counts (Zweifel & Hagström 1995, Choi et al. 1996). Furthermore, indicators of metabolic activity, such as tetrazolium or the tetrazolium derivative CTC, as well as autoradiographic measurements have shown that a large proportion of natural bacterioplankton is neither actively respiring nor utilizing substrates (Dufour et al. 1990, Dufour &

Colon 1992, del Giorgio & Scarborough 1995, Gasol et al. 1995, Karner & Fuhrman 1997). Whether the inactive fraction of the community is cells presenting low activity or remnants of once living cells is still controversial (Zweifel & Hagström 1995, Choi et al. 1996).

The measurement of bacterial secondary production has also been a subject of controversy (Riemann & Bell 1990, Ducklow & Carlson 1992). Initially, the standard methods for the measurement of bacterioplankton production measured the uptake of radiolabeled thymidine (Fuhrman & Azam 1982) as an indicator of DNA synthesis, or adenine (Karl et al. 1981, Karl et al. 1981, Bell & Riemann 1989) as an indicator of nucleic acid synthesis. In the original methods, the radioactivity was measured into total macromolecules that precipitated in cold trichloroacetic acid. The observation that the thymidine may be used as substrates by bacteria living in oligotrophic conditions (Brittain & Karl 1990) emphasizes the importance of measuring radioactivity specifically in DNA. However the vast majority of the studies in the literature use the incorporation of tritiated thymidine into total macromolecules to measure secondary production. Uptake of radiolabeled leucine to measure protein synthesis (Chin-Leo & Kirchman 1988) is a alternative method to estimate bacterioplankton secondary production. However, the requirement of balanced growth by bacterioplankton cells to estimate growth rates from rates of protein synthesis complicates the comparison of the methods (Chin-Leo & Kirchman 1990). Despite the methodological problems associated with the methods, compilations of bacterial secondary production have been published (Cole et al. 1988, Ducklow & Carlson 1992). It has been generally accepted that bacterioplankton have turnover times ranging from several

hours to weeks, and that bacterioplankton secondary production represents a significant fraction of primary production.

Since the discovery that a large fraction of eukaryotic nanoplankton are heterotrophic and prey on bacteria (Haas & Webb 1979), most studies on bacterial mortality have focused on the measurement of bacterivory rates by heterotrophic protists (Fenchel 1982, Fenchel 1982, Sherr et al. 1987, McManus & Fuhrman 1988, Simek et al. 1990). The estimation of bacterivory rates from the uptake of fluorescently labeled bacteria (Sherr et al. 1987) or *E. coli* minicells (Wikner et al. 1986, Pace et al. 1990, Vaqué et al. 1994) are now standard methods. However, the fact that bacterivorous protist show selective feeding (Gonzalez et al. 1990, Landry et al. 1991, Monger & Landry 1991, Monger & Landry 1992, Gonzalez et al. 1993) suggests that the use of bacterioplankton analogs may under- or overestimate bacterial mortality depending on the in situ bacterioplankton assemblage (Landry et al. 1991).

Lysis by bacteriophages is an alternative source of bacterioplankton mortality. The presence of viruses in the sea had long been reported (Baross et al. 1978, Torrella & Morita 1979, Moebus 1980), but not until recently has the estimation of bacteriophages numbers and effects on bacterioplankton biomass been attempted (Bergh et al. 1989, Borsheim et al. 1990, Proctor & Fuhrman 1990, Middelboe et al. 1996). Although the direct measurement of bacterioplankton lysis by bacteriophages is dependent on several assumptions including the extrapolation of burst size, and infectivity from cultivated bacteria, it has recently been suggested that bacterial mortality by viral lysis could be in the same order of magnitude as that by protistan bacterivory (Fuhrman & Noble 1995).

Summarizing, the community structure of bacterioplankton has been mostly estimated from community-wide measurements of biomass, production and mortality. Although such studies have been conducted in a multitude of environments (Cole et al. 1988, Ducklow & Carlson 1992, Sanders et al. 1992), even bacterioplankton biomass estimates are subject of controversy. We now know that bacterioplankton abundance estimated from direct counts range over several orders of magnitude (10^5-10^7cells·ml^{-1}), and that a large fraction of such cells show low or no metabolic activity. Bacterial production may represent a large fraction of the primary production, and turnover times of bacteria are on the order of days to weeks. Bacterivorous protist grazing and bacteriophage induced lysis are accepted to be the main causes of bacterioplankton mortality, although their relative importance is controversial.

Controls of Bacterioplankton Biomass.

Two decades after the publication of the landmark paper by L. Pomeroy, on the new paradigm of pelagic food webs (Pomeroy 1974), hundreds of studies have been conducted to measure biomass, production and mortality of bacterioplankton. One ultimate goal of such studies is to understand the mechanisms controlling bacterioplankton community distribution and abundance. Most of the discussion in the literature focuses on the question of whether bacterioplankton communities are controlled by the availability of nutrients and/or substrates ("bottom-up" control) or by predation ("top-down" control). Ducklow and Carlson reviewed some of the general ecological literature, and cited McQueen and colleagues. (1986) who suggested that bottom up controls are more important at the base of food

webs, whereas top down controls are more important at the top of metazoan trophic webs (Ducklow & Carlson 1992). However, microbial food webs are fundamentally different from metazoan trophic webs. These differences include mixotrophic organisms (flagellated and ciliated protists) (Boraas et al. 1988, Laval-Peuto & Rassoulzadegan 1988, Stoecker et al. 1988, Stoecker et al. 1989, Stoecker et al. 1989, Sanders 1991), predators and prey growing at similar rates (Fenchel 1982) and nutrient feedback from phagotrophs to phytoplankton and bacteria (e.g. Johannes 1965, Goldman & Caron 1985, Goldman et al. 1985, Sherr et al. 1988, Tupas & Koike 1991, Suzuki et al. 1996). Also, the theoretical view of microbial food webs as a whole being responsible for the repackaging of organic matter in a size available for metazoan food webs (Sherr & Sherr 1988), shows that the extrapolation of McQueen and colleagues' analysis for microbial food webs may be inadequate.

Studies correlating the abundance and production rates of the different compartments of microbial food webs have led to different conclusions. Regressions between bacterioplankton abundance and chlorophyll-a suggest bottom-up control of bacterioplankton biomass (Bird & Kalff 1984, Ducklow 1984). Regressions between bacterioplankton biomass and bacterial production from different systems, with the rationale that bacterial production should be a indicator of substrate availability (Ducklow et al. 1988, Billen et al. 1990, Ducklow & Carlson 1992, Dufour & Torréton 1996) suggest bottom-up control when the data analyzed represents global scales, but such a relationship is less significant within data sets (Ducklow et al. 1988, Dufour & Torréton 1996). Top-down control of bacterioplankton abundance has been indicated by the relatively steady bacterial numbers and

ratios between bacteria and bacterivores across ecosystems, suggesting that bacterivores may graze bacteria to the level of the bacterial abundance representing the threshold for random encounter between protists and bacteria (Fenchel 1982, Berninger et al. 1991, Sanders et al. 1992). Also, the measurement of bacterivorous protists clearance rates *in situ* indicates that bacterivores have the potential to ingest large fractions of the bacterial production (Fenchel 1982, McManus & Fuhrman 1988, Bloem et al. 1989, Sherr et al. 1989), and therefore could control bacterioplankton biomass.

Temperature should affect bacterioplankton biomass only if bacterioplankton production and mortality were differentially affected by temperature. There are several studies evaluating the effects of temperature on bacterioplankton secondary production (see discussion below), but less effort was taken to study the relative effects of temperature on bacterivorous protists, and so the influence of temperature on bacterioplankton biomass is relatively unknown. The effect of bacteriophages on bacterioplankton biomass is also relatively unknown since there are no direct ways to measure mortality rates by viral lysis. The results of one study estimating the impact of viruses in the bacterioplankton community mortality presented evidence that mortality by viral lysis was comparable to that by bacterivorous protist grazing (Fuhrman & Noble 1995).

In summary, the mechanisms controlling bacterioplankton abundance and biomass in aquatic systems are still the subjects of debate. It appears that when data of a large range of bacterioplankton biomass and production are compared, bacterioplankton correlates with the availability of substrates, but that measurements on smaller time and space scales suggest that protistan

bacterivory and virus lysis may control bacterioplankton abundance and biomass. The reasons for such discrepancy are unknown.

Controls of Bacterioplankton Production.

As in the case of bacterioplankton biomass, the mechanisms controlling bacterioplankton production are not well established. Temperature effects on bacterioplankton production have been subject of several studies (Pomeroy & Deibel 1986, Pomeroy et al. 1991, White et al. 1991, Autio 1992, Andersson et al. 1994), and it has been hypothesized that the fact that low temperatures affect bacterial production to a larger extent than they affect primary production may create an excess of biomass that is not utilized by the microbial food web and explain the relatively larger transfers of carbon from phytoplankton to metazoans in polar regions (Pomeroy & Deibel 1986).

The evaluation of bottom-up control of bacterioplankton production has been addressed by regression analysis between bacterioplankton production and phytoplankton production of biomass, with the rationale that phytoplankton should be ultimately the source of organic substrates for bacterioplankton. Global scale regression analyses shows good agreement between bacterioplankton production measured by different methods and both chlorophyll-a and primary production (Cole et al. 1988), suggesting that over a large spatial scale bacterioplankton production is limited by resource availability. However, the fact that bacterioplankton may be limited by some of the same inorganic nutrients as the phytoplankton (Wheeler & Kirchman 1986, Goldman et al. 1987), as well as the fact that most such nutrients are regenerated by phagotrophic protists (Johannes 1965, Goldman & Caron

1985, Goldman et al. 1985, Sherr et al. 1988, Tupas & Koike 1990, Suzuki et al. 1996), challenges the interpretation of the regressions between bacterioplankton production and phytoplankton production or biomass.

The evidence for top-down control of bacterioplankton production was mainly obtained in incubation experiments showing that protists preferentially remove larger (Chrzanowski & Simek 1990, Gonzalez et al. 1990, Monger & Landry 1990, Monger & Landry 1991, Monger & Landry 1992, Gonzalez et al. 1993, Gonzalez 1996) and active bacteria (Landry et al. 1991, Sherr et al. 1992, del Giorgio et al. 1996). Sherr and colleagues (1992) have shown that bacterivorous protists selectively feed on dividing cells, suggesting that protists may be cropping the cells responsible for production. The fact that bacterivores selectively graze cells capable of reducing the formazan derivative CTC (del Giorgio et al. 1996) confirms this hypothesis, which is also supported by the report that bacterioplankton production is better correlated with abundance of active cells than with direct total counts of bacteria (del Giorgio et al. 1997). Also, the recent observation that the size distribution of natural lake bacterioplankton numbers and productivity throughout a season is correlated with bacterivorous protist abundance (Pernthaler et al. 1996) supports top-down control of bacterioplankton production. However, the fact that bacterivorous protists are responsible for the regeneration of inorganic nutrients which may be limiting for the bacterioplankton (Johannes 1965, Goldman & Caron 1985, Goldman et al. 1985, Sherr et al. 1988, Tupas & Koike 1990, Suzuki et al. 1996) complicates the interpretation of this study.

Bacterioplankton Diversity.

As mentioned above, most studies on bacterioplankton assemblages focused on community-level parameters: bacterioplankton biomass, production and mortality. The study of bacterioplankton community structure in terms of species diversity has been precluded mainly by difficulties associated with the identification of prokaryotes. In general prokaryotes have very few morphological traits useful for identification. Also, use of biochemical traits for identification has not been extensively applied to the study of bacterioplankton since it has been long hypothesized that only a fraction of the bacterioplankton species may be amenable to cultivation on enrichment conditions (Yanagita et al. 1978, Kuznetsov et al. 1979).

The concept of copiotrophic and oligotrophic bacteria was introduced in the early 1970's as an extension of the hypothesis of the existence of "zymogenous" and "autochthonous" bacteria in soil (Winogradsky 1949) and is the subject of a recent review (Schut et al. 1997). The first evidence that bacterioplankton may be composed of species which do not grow in enrichment conditions was the discovery that direct cell counts were several orders of magnitude higher than the number of bacteria estimated from the numbers of colony forming units on enriched agar plates (Collins & Kippling 1957, Jannasch & Jones 1959, and references therein). However in these earlier studies the difference was often attributed to dead or inactive bacteria. The introduction of epifluorescence microscopy using stains targeting nucleic acids (Francisco & Rabin 1973, Hobbie et al. 1977) combined with studies on oligotrophic bacteria (Ishida & Kadota 1974, Akagi et al. 1977,

Yanagita et al. 1978, reviewed by Kuznetsov and colleagues 1979) led to two non-exclusive hypothesis: 1) bacterial communities are composed of known species that are capable of forming colonies on agar plates, but do so with low efficiency, or 2) bacterial communities are composed of unknown species which cannot easily be grown on typical microbiological media.

The introduction of molecular biology techniques (Section 2), such as cloning and sequencing of small subunit (16S) ribosomal RNA genes (SSU rDNAs) in marine systems, supported hypothesis 2, since the majority of the ribosomal genes recovered in clone libraries do not correspond and in most cases are very distantly related to those of previously cultivated bacteria (Giovannoni et al. 1990, Britschgi & Giovannoni 1991, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993, Rappé et al. Submitted). While the SSU rDNA sequences or the majority of cultivated species correspond to the gamma subdvision of the Proteobacteria, the low and high G+C gram positive phyla, and the *Flexibacter-Bacteroides-Cytophaga* phylum (Appendix 1), the SSU rDNAs cloned from environmental samples belong, in addition to the former groups, to the alpha, beta and delta subdvision of the Proteobacteria and several other phyla (Britschgi & Giovannoni 1991, DeLong et al. 1993, Fuhrman et al. 1993, Mullins et al. 1995, Giovannoni et al. 1996, Gordon & Giovannoni 1996, Field et al. 1997, Wright et al. 1997, Rappé et al. in press). Clone libraries also revealed the presence of members of the domain Archaea, none of which has been cultivated from seawater plankton samples.

However, two studies employing molecular techniques support hypothesis 1 (Rehnstam et al. 1993, Pinhassi et al. 1997). The first study used oligonucleotide hybridization to small subunit ribosomal RNA (SSU rRNA)

to study the diversity of bacterioplankton from the California coast and showed that a large fraction of the SSU rRNAs were those of strains isolated from the same environment (Rehnstam et al. 1993). Furthermore a recent study (Pinhassi et al. 1997) in which whole genomic DNA from strains isolated from the Northern Baltic Sea was hybridized to community DNA suggests that colony forming bacteria dominates the marine bacterioplankton. Sequence analysis of the SSU rDNA of the isolates suggests that a solution to the discrepancy between the sequences of SSU DNA of cultivated strains or those directly cloned from the environment could be an incomplete representation of cultivable marine bacterioplankton in culture collections.

The majority of studies on bacterioplankton diversity estimated from ribosomal gene sequences in freshwater systems has emphasized the use of in situ oligonucleotide hybridization using subdvision and phylum level probes (Amann et al. 1995, Alfreider et al. 1996, Glöckner et al. 1996, Weiss et al. 1996). A general conclusion of such studies is the relative dominance of bacteria belonging to the beta subdvision of the Proteobacteria phylum (Alfreider et al. 1996, Weiss et al. 1996). To my knowledge there are only two studies on the diversity of freshwater bacterioplankton SSU rDNA diversity employing clone libraries (Bahr et al. 1996, Hiorns et al. 1997). Both studies show that most sequenced SSU rDNAs belong to the beta-Proteobacteria. The study of bacterioplankton SSU rDNAs from seven lakes in the Adirondack mountains showed that few sequences were closely related to previous characterized species (Hiorns et al. 1997), while a study of the sequences of 16S rRNAs of both cultivated species or environmental clones from an Arctic lake, showed that all sequenced SSU rDNAs were closely

related to previously sequenced genes, most of which were from organisms on culture collections (Bahr et al. 1996).

In chapter 1 of this thesis I discuss the results of a study comparing the taxonomic diversity estimated from the diversity of SSU rDNAs of 127 strains of marine bacterioplankton and 56 bacterioplankton SSU rDNAs directly cloned from environmental DNA from the same water sample (Suzuki et al. 1997). The SSU rDNAs of cultivated species were different from those directly cloned from the environment, but were related to the rDNAs of previously cultivated organisms, supporting hypothesis 2, and emphasizing the use of methods other than cultivation for the study of marine bacterioplankton diversity.

Molecular Methods for the Study of Bacterioplankton Taxonomic Community Structure.

Environmental Clone Libraries of Small Subunit Ribosomal RNA genes.

Environmental clone libraries of small subunit rRNA genes (SSU rDNAs) are collections of SSU rDNAs representing the genes of *in situ* prokaryotic cells populations produced by recombinant DNA technology. Clone libraries of SSU rDNAs revolutionized the field of aquatic marine microbial ecology, as they first allowed the study of the *in situ* bacterioplankton species richness (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993). However the use of clone libraries for the study of bacterioplankton diversity has been limited by the fact that the construction and especially the screening of clone libraries is

very labor intensive and time consuming, leading in general to a low coverage (e.g. Suzuki et al. 1997, Chapter 1 of this thesis). Also, since most clone libraries are constructed using SSU rDNAs amplified by the polymerase chain reaction (PCR) they may be subject to its biases. Several alternative methods have been proposed to estimate the quantitative diversity of bacterioplankton SSU rDNAs, but the application of such methods to the study of aquatic bacterioplankton diversity is relatively recent.

Oligonucleotide Hybridization.

In oligonucleotide hybridization, bacterial SSU rRNA are the target for labeled oligonucleotide probes. In FISH (fluorescence *in situ* hybridization) the oligonucleotide is labeled with a fluorescent molecule and hybridized to SSU RNA in fixed bacterial cells. Labeled cells are counted via epifluorescence microscopy (DeLong et al. 1989, Amann et al. 1990, Amann et al. 1995) or flow cytometry (Amann et al. 1990, Amann et al. 1995, Wallner et al. 1995, Schonhuber et al. 1997). The low growth rates of *in situ* bacterioplankton communities is the main problem associated with the application of FISH to natural systems, as the numbers of ribosomes are proportional to growth rate of bacteria (Kemp et al. 1993, Lee et al. 1993). This appears to be especially true for marine systems, as the numbers of cells detected with FISH using oligonucleotides targeting all bacterial rRNAs is only a fraction of total counts (Karner & Fuhrman 1997). Furthermore. a recent study using image enhancement produced FISH counts similar to the number of active cells estimated by autoradiography (Karner & Fuhrman 1997).

An alternative to FISH is the hybridization of oligonucleotides to SSU rRNAs or SSU rDNAs amplified by the polymerase chain reaction (PCR) from environmental DNA. In this technique radiolabeled oligonucleotides are hybridized to nucleic acids immobilized in nylon membranes and the abundance of different SSU rRNAs or SSU rDNAs estimated from the amount of radioactivity on the membranes (Giovannoni et al. 1988, Stahl et al. 1988, Britschgi & Giovannoni 1991, Gordon & Giovannoni 1996). Such methods have been used to profile the vertical distribution of different organisms in natural systems (Giovannoni et al. 1995, Gordon & Giovannoni 1996), but a more widespread use of the technique has been limited by some methodological problems: 1) The collection of SSU rRNA for hybridization requires the collection of very large water samples, which precludes the use of the methods for bottle incubation experiments. 2) The hybridization to SSU rDNA has the problems associated with the use of the PCR and its possible biases. 3) Most importantly, the efficiency of radiolabeling of different oligonucleotides is variable, and complicates the quantitative analysis (i.e. frequently the sum of estimated percentages of different SSU rRNA types in a SSU rRNA sample exceeds 100%).

PCR-Based Methods.

The estimation of the diversity of bacterioplankton SSU rDNAs amplified by the polymerase chain reaction (PCR) has several advantages for the study of bacterioplankton *in situ* diversity: 1) Since copies of SSU rDNAs are produced *in vitro*, the size of the original DNA sample can be greatly reduced and therefore allows the estimation of diversity in samples from bottle experiments. 2) The number of copies of SSU rDNA per genome is relatively constant for individual species and so SSU rDNA proportions

correlate better to the proportions of cells of different species than do SSU rRNA proportions.

The main criticism of the measurement of the diversity of PCR-amplified rDNAs is the possible introduction of biases by the reaction. PCR biases that introduce error in interpretation of the bacterioplankton rDNA diversity are those that lead to different amplification yields for different templates. Several such biases have been hypothesized, including the different binding energies resulting from primer degeneracy (i.e. a mixture of primers with nucleotide sequences corresponding to the observed variation in the priming sites among homologs), and the influence of template folding. A documented source of bias affecting SSU rDNA diversity estimates is the mol% guanine + cytosine (G+C) content of template DNA (Liesack & Stackebrandt 1989). In this type of bias, templates with higher G+C contents are less susceptible to denaturation and therefore are less likely to be amplified.

In Chapter 2 of this thesis I discuss the results of a study of bias by PCR in the amplification of mixed templates in model systems. I report and describe template reannealling bias, a newly reported type of bias by PCR (Suzuki & Giovannoni 1996). Reannealing bias occurs when, after a certain number of replication cycles, homologues with higher concentrations in the original mixtures start to reach critical concentrations, above which template reannealling precludes primer hybridization. Templates with lower concentrations continue to amplify, until all templates reach similar concentrations, independently of the original ratio between the templates in the initial rDNA mixture. The occurrence of such bias during the amplification of rDNAs from coastal DNA samples and its effect on the

estimation of the bacterioplankton rDNA are presented and discussed in Chapter 3. The main conclusions of these studies is that PCR does not radically bias the frequencies of mixed rRNAs provided that the reactions are stopped before critical concentrations are achieved, and that PCR based methods can be used quantitatively for estimating the rDNA diversity of bacterioplankton communities.

Denaturing gradient gel electrophoresis (DGGE) is a PCR-based method applied to the measurement of the genotypic structure of bacterial communities directly from environmental DNAs (Muyzer et al. 1993, Muyzer et al. 1995, Ferris et al. 1996, Murray et al. 1996, Teske et al. 1996). In DGGE, PCR-amplified rDNA fragments are run on a low to high denaturant gradient acrylamide gel. Initially the fragments move according to their molecular weight, but as they progress into higher denaturing conditions, different rDNAs reach a point where the rDNA begins to denature, according to its sequence composition. The partial denaturation retards the progress of the molecule in the gel, and a mobility shift is observed. It is the mobility shift which can differ for slightly different sequences (Collins & Myers 1987, Myers et al. 1987). The relative abundance of different fragments can be measured by oligonucleotide probing and densitometry analysis (Teske et al. 1996). The method is relatively simple and allows a general comparison between the community structure of different samples. The main problems associated with DGGE for the analysis of bacterioplankton diversity are the need for a relatively large PCR amplicon sample (which may be subject to reannealing bias) and the difficulty of the identification of specific bands on the denaturing gel, as the bands have to be probed with oligonucleotides or cut and their rDNA sequenced, slowing the analysis.

However, the fact that SSU rDNAs can be recovered from DGGE gels is also a strength of the method, allowing species level identification of the rDNAs contributing to the bands.

Length heterogeneity analysis by PCR (LH-PCR), a new PCR-based method for the determination of the diversity of bacterioplankton rDNAs is described in Chapter 3. In LH-PCR, a region of the SSU rDNA which exhibits length variations between different phylogenetic groups is amplified from environmental DNA by PCR. Fragments originating from different organisms are discriminated by their length and quantified by the fluorescence emission of a fluorescent phosphoramidite-labeled primer. An analysis of 367 rDNA sequences of bacterial strains isolated from seawater and genes cloned directly from seawater samples indicated that LH-PCR allows a general description of the bacterioplankton genotypic community structure. However it cannot discriminate closely related organisms, and in many cases organisms belonging to different subdivisions or phyla overlap, to varying degrees in fragment size. Nonetheless, the strengths of the method are its relatively simplicity, and its capability to provide a quantitative description of bacterioplankton genotypic diversity. The method is particularly suited to manipulation experiments since it requires only small samples, and allows the analysis of a large number of samples.

Controls of Bacterioplankton Diversity.

Despite the relatively large number of studies on the diversity of bacterial SSU rDNA sequences of aquatic systems mentioned above, the mechanisms controlling bacterioplankton genotypic community structure are relatively unknown. The difference between bacterioplankton SSU

rDNAs from coastal and oceanic samples (Britschgi & Giovannoni 1991, Mullins et al. 1995, Rappé et al. in press) suggests that resources might play a role in determining bacterioplankton community structure. In addition, two recent studies reach suggest that bottom-up also may play a role (Pernthaler et al. 1997, Simek et al. 1997). These studies analyzed the effect of the bacterivorous protist *Bodo saltans* on the genotypic community structure of a mixed freshwater bacterioplankton assemblage grown under enriched continuous culture. Community structure was evaluated by *in situ* hybridization of subdivision level oligonucleotide probes. These results show that bacterivorous protists could affect bacterioplankton community structure, as large grazing-resistant members of the beta subdivision of the Proteobacteria dominated the bacterioplankton biomass under grazing pressure. Also in the latter study, alpha Proteobacteria apparently increased their growth rate and compensated for the grazing mortality.

In the last chapter of this thesis (Chapter 4) I apply the information gathered in the studies described in Chapters 1-3 to study the influence of bacterivory on the genotypic community composition of coastal bacterioplankton. In this study I followed changes in SSU rDNA diversity estimated by LH-PCR in 4 different seawater samples from which the bacterivorous protists were removed by filtration. The changes in SSU rDNA diversity with time were then compared to those of control, unmanipulated samples. In two of the experiments the incubation devices allowed the transfer of substrates and nutrients decreasing the confounding effect of disrupted nutrients and substrate feedbacks in filtered samples. The results suggested that the bacterioplankton genotypic community structure shifts in the absence of protists, as relatively insignificant gene types in the

SSU rDNA pool of original water samples dominate the SSU rRNA gene pool after 48 h of incubation in the absence of bacterivorous protists. I discuss the significance of my results to the debate on the controls of bacterioplankton community structure in Chapter 4 and in the Conclusions section of this thesis.

Chapter 1

Bacterial Diversity Among SSU rDNA Gene Clones and Cellular Isolates from the Same Seawater Sample.

Marcelino T. Suzuki, Michael S. Rappé, Zara W. Haimberger, Harry Winfield, Nanci Adair, Jürgen Ströbel and Stephen J. Giovannoni

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Abstract

Numerous investigations applying the cloning and sequencing of rRNA genes to the study of marine bacterioplankton diversity have shown that the sequences of genes cloned directly from environmental DNA do not correspond to the genes of cultured marine taxa. These results have been interpreted as support for the hypothesis that the most abundant heterotrophic marine bacterioplankton species are not readily culturable by commonly used methods. However, an alternative explanation is that marine bacterioplankton can be easily cultured, but are not well represented in sequence databases. To further examine this question, we compared the small subunit rRNA genes (SSU rDNAs) of 127 cellular clones isolated from a water sample collected off the Oregon coast to 58 bacterial SSU rDNAs cloned from environmental DNA from the same water sample. The results revealed little overlap between partial SSU rDNA gene sequences from the cellular clones and the environmental clone library. An exception was the SSU rRNA gene sequence recovered from a cellular clone belonging to the *Pseudomonas* subgroup of the gamma subclass of the Proteobacteria, which was related to a single gene cloned directly from the same water sample (OCS181, similarity = 94.6%). In addition, partial SSU rRNA gene sequences from three of the cultured strains matched a "novel" rRNA gene clone related to the gamma subclass of the Proteobacteria found previously in an environmental clone library from marine aggregates (AGG53, similarity = 94.3-99.6%). Our results support the hypothesis that many of the most abundant bacterioplankton species are not readily culturable by standard methods, but also show that heterotrophic bacterioplankton that are

culturable on media with high organic content include many strains for which SSU rRNA gene sequences are not available in sequence databases.

Introduction

The cloning of rRNA genes from natural ecosystems precipitated a fundamental shift in microbial ecology away from the study of cultured strains and towards molecular approaches that emphasized the importance of *in situ* diversity. This transition began earlier with the discovery that, in many ecosystems, bacterial numbers estimated by epifluorescence direct counts are orders of magnitude higher than colony forming units (Hobbie et al. 1977, Kogure et al. 1979). This discrepancy has been referred to as the "great plate count anomaly" (Staley & Konopka 1985).

Marine microbiologists were quick to recognize the significance of the anomalous low plate counts for heterotrophic bacterioplankton. Faced with uncertainty about the relevance of cultured species, at an early stage they moved away from cultivation-based techniques and towards a model that treated marine bacterioplankton as a functional unit with indistinguishable components, sometimes known as the "microbial black box". Later, this view was reinforced by experience with autotrophic bacterioplankton (cyanobacteria and prochlorophytes), which showed that very abundant species could be difficult to detect and cultivate without the development of specialized techniques (Johnson & Sieburth 1979, Waterbury et al. 1979, Olson et al. 1990). Subsequently, the discovery that the abundance of culturable species estimated by plate counts is increased by manipulation and confinement of seawater samples (Ferguson et al. 1984) led to the formulation of two non-exclusive hypotheses that could be applied

generally to many ecosystems: 1) bacterial communities are composed of known species that are capable of forming colonies on agar plates, but do so with low efficiency, or 2) bacterial communities are composed of unknown species that cannot easily be grown on common microbiological media (Giovannoni et al. 1995). The actual explanations for the failure of cells to form colonies may be as diverse as the species in consideration, but these two alternative hypotheses capture the essence of the debate.

Most of the evidence supporting both hypotheses was gathered after the introduction of molecular biology techniques to aquatic microbial ecology. Hypothesis two was proposed after the cloning and sequencing of rRNA genes from environmental DNA samples showed that the most common genes recovered belong to undescribed species and, in some cases, novel groups of bacteria (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993). Since most of the marine bacteria that have been described in the systematic literature are represented in rRNA databases (Giovannoni et al. 1996), these comparisons lead to the conclusion that many marine bacterioplankton species were unknown because they are difficult to culture (Giovannoni et al. 1995). However, the same results could be attributed not to a failure to culture bacteria because of physiological differences, but instead to the incompleteness of systematic descriptions and SSU rDNA sequence databases for cultured marine bacteria. Rehnstam et al. (1993), studied bacteria cultured on Zobell's marine medium from seawater collected from Scripps pier. After partially sequencing 16S rRNAs from these isolates, they developed strain-specific oligonucleotide probes and hybridized them to genomic DNA isolated from water samples collected

from the same area. They concluded that the cultured strains were indeed significant members of the bacterioplankton community.

Here we report the results of a study in which *Hae*III restriction fragment length polymorphism (RFLP) patterns and partial gene sequences were used to compare SSU rDNAs from bacterial strains isolated on an organically "rich" medium from Oregon coast seawater to SSU rDNAs cloned from community DNA extracted from the same water sample. The results show that there is little overlap between SSU rDNA gene clones and cultured strains from the same water sample. In addition, we found that many of the cultured strains could not be identified by SSU rDNA sequence comparisons.

Material and Methods

Water samples.

Subsurface (10 m) water samples were collected in Niskin bottles at a station located 8 km west of the mouth of Yaquina Bay, OR (44°39.1' N, 124°10.6' W). The water was pre-screened through 10 µm Nitex mesh and transported in autoclaved polyethylene carboys to the laboratory for filtration and plating.

Isolation of cellular clones.

100 µl subsamples were spread onto 20 marine R2A agar plates and incubated at 15 °C in the dark in a constant temperature incubator. Marine R2A is a complex medium with the same composition as R2A medium (Reasoner & Geldreich 1985) except that inorganic salts are not added and

75% seawater is used instead of distilled water. The carbon content of this media is lower than that of marine agar, but still orders of magnitude higher than the carbon content of natural seawater (Table 1.1). All colonies appearing on 10 plates over a period of 3 weeks were characterized by several criteria related to colony morphology and streaked for isolation onto R2A plates. Subsequently, all isolated cellular clones were grown in R2A broth, and a 1 ml aliquot was frozen for storage in 10% DMSO at -80°C. After the first three weeks, only colonies that had a previously unseen morphology were picked from the 20 plates, up to a period of 3 months. After 12 months storage at -80°C, all cellular clones were inoculated into R2A broth for further experiments. Cellular clones were assigned the prefix "R2A".

Table 1.1 Composition of Marine R2A agar medium

Compound	g/liter of 75% seawater
Yeast extract	0.5
Proteose peptone (Difco)	0.5
Casamino Acids	0.5
Dextrose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
Agar	15

Cell counts.

Direct counts were performed by epifluorescence microscopy using DAPI to stain cells according to the protocol described in (Turley 1993), except that the samples were fixed with 1% formaldehyde. Most probable numbers were estimated from the number of colonies forming units appearing in 10 R2A agar plates in a period of 3 months.

SSU rRNA gene clone library.

Picoplankton from a 16 l subsample were collected by filtration onto 0.2 µm polysulfone filters (Supor-200, Gelman Inc., Ann Arbor, MI). Total cellular nucleic acids were isolated from the picoplankton sample by lysis with proteinase K and SDS, followed by phenol/chloroform extraction as previously described (Giovannoni et al. 1990). SSU rDNAs were amplified from total genomic DNA by the PCR using two general bacterial SSU rDNA primers (27F, 5'-AGA GTT TGA TCM TGG CTC AG-3'; 1522R, 5'-AAG GAG GTG ATC CAN CCR CA-3') (Giovannoni 1991) in a PTC100 thermal cycler (MJ Research, Watertown, MA). In a final volume of 100 µl, the reaction contained 0.2 µM of each amplification primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 5.0% acetamide, 10 ng of template and 2.5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA), which was added after a pre-cycling in which the reaction mixture was heated to 95°C for 5 min. and held at 80°C. *Pfu* DNA polymerase was used for DNA amplification because it exhibits proofreading activity and therefore can replicate DNA with higher fidelity that *Taq* DNA polymerase. The amplification conditions were 95°C for 1

min., 55°C for 1 min., and 72°C for 1 min. extended 5 sec per cycle for 35 cycles. Following the final cycle, the reaction was extended at 72°C for 10 min. The amplification products from six reactions were visualized by electrophoresis through a 1.0% Seakem (FMC, Rockland, ME) agarose gel in 1X TAE (40 mM Tris-acetate, 1 mM EDTA) containing 0.50 $\mu g \cdot ml^{-1}$ ethidium bromide. For each reaction, the band corresponding to the correct sized product (1.5 Kb) was cut out of the gel and purified (Qian & Wilkinson 1991). The resulting products were pooled, quantified with a UV160U spectrophotometer (Shimazu Co., Kyoto, Japan) and visualized as above. The amplified rDNA was inserted into the *Sma*I restriction site of the phagemid vector pBluescript KSII- (Stratagene) by blunt-end ligation as previously described (Giovannoni et al. 1990) with the following modifications: the ligation reaction mixture, consisting of 260 ng of insert, 50 ng of vector, and 1 unit of T4 DNA ligase in ligation buffer (10 mM Tris·HCl pH 8.3, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP), was incubated at 16°C overnight. The ligation product was diluted 2:3 with TE buffer (10 mM Tris·HCl, 1 mM EDTA pH 8.0) and used to transform competent *E. coli* XL1-blue cells (Stratagene). Transformants were subsequently plated onto LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bacto-agar) containing the antibiotic ampicillin (200 $\mu g \cdot ml^{-1}$), spread evenly with 40 µl of X-Gal (40 $mg \cdot ml^{-1}$) and 100 µl of IPTG (100 mM), and grown at 37°C overnight. Positive (white colony morphotype) transformants were streaked for isolation. For long-term storage, stab cultures of all were made in LB top agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% bacto-agar) and kept at 4°C. Clones were also stored in 7.0% DMSO at -80°C. Gene clones were assigned the prefix "OCS".

RFLP patterns: cellular clones.

Based on their colony morphology, 60 cellular clones were selected for characterization by *Hae*III RFLP analysis of their SSU rDNAs. Briefly, the cellular clones were grown in R2A broth, harvested by centrifugation (5 min. at 3000 RPM), and their genomic DNA was extracted by the CTAB protocol (Ausubel et al. 1988). The SSU rDNA of each of the clones was amplified from 10-100 ng of genomic DNA by the PCR. The reactions contained 0.5 μM of the bacterial primers 27F and 1522R, 0.2 mM of pre-mixed dNTPs, 1.5 mM MgCl and 2.5 units of *Taq* DNA polymerase (Promega, Madison WI) in a final volume of 100 μl. All PCR reactions except for the one used to amplify environmental DNA for the construction of the clone library were performed using *Taq* DNA polymerase since all templates originated from a single cellular clone and so, we had no concern about preferential amplification biases. The DNA polymerase was added after a pre-cycling step in which the reaction mixture was heated to 95°C for 1 minute and held at 80°C. The amplification conditions were: 1 min. at 94 °C, 1 min. at 55 °C, and 3 min. at 72 °C for 35 cycles. The PCR products were purified on Qiaquick-spin columns (Qiagen, Chatsworth, CA), and their concentration measured spectrophotometrically as above. For the determination of RFLPs, 700 ng of purified PCR product from each cellular clone was digested with 5 units of the restriction endonuclease *Hae*III (Promega) for 5 h. The reactions were stopped by the addition of EDTA to 50 mM. The restriction fragments were resolved by gel electrophoresis in 3% NuSieve low melting point agarose (FMC) in TAE and stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$). Photographs of the gels were digitized with a HP ScanJetIIcx scanner (Hewlett Packard Co., San Diego, CA). The images

were processed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

RFLP patterns: gene clones.

All gene clones were characterized by *Hae*III RFLPs. Plasmids bearing complete inserts, isolated by alkaline lysis, were used as templates in the amplification of environmental clone SSU rDNAs by the PCR. PCR conditions were the same as those employed above for the cellular clones, with the following exceptions. For the pre-cycling step, the gene clones were held at 95°C for 5 min. instead of 1 min. Also, each reaction contained approximately 20 ng of template plasmid DNA. Since all of the PCR reactions yielded similar amounts of product, 7 µl of non-purified PCR products were digested with 3 units of the restriction endonuclease *Hae*III for 2h. The restriction fragments were resolved by gel electrophoresis as above.

Sequencing: cellular clones.

Sequences were determined for all combinations of RFLP pattern and colony morphology that were unique. An exception was the most common colony morphotype (colony type I), which presented slight variations in colony morphology, not all of which were sequenced. rDNA amplicons from 32 separate cellular clones were sequenced bi-directionally using the primers 27F and 338R by the fluorescent dideoxy termination reaction (Applied Biosystems Inc. (ABI), Foster City, CA). The fluorescently labeled fragments were resolved by polyacrylamide gel electrophoresis in a model 373A automated DNA sequencer (ABI).

Sequencing: gene clones.

All gene clones that had unique RFLP patterns were chosen for sequencing. Briefly, template plasmid DNAs were prepared from overnight cultures by alkaline lysis using a Prep-A-Gene plasmid purification kit (Bio-Rad, Richmond, CA) or Qiaprep spin plasmid kit (Qiagen) and quantified spectrophotometrically. Plasmid DNAs were visualized by electrophoresis through a 1.0% Seakem (FMC) agarose gel as above. Plasmid DNAs were sequenced bi-directionally with conserved primers as described above, with at least 500 bases of sequence data on the 5′ end of each SSU rDNA molecule being obtained.

Phylogenetic analyses.

Unaligned sequences were submitted to the SIMILARITY_RANK (Simrank) program of the Ribosomal Database Project (RDP) to obtain a preliminary list of closest phylogenetic neighbors (Maidak et al. 1994). Sequences were then sorted according to phylum and subphylum affiliation, and manually aligned with the DNA sequence editor GDE v2.1 (supplied by Steve Smith). The consensus sequences were re-submitted to Simrank employing the MY_DATABASE option. This allowed us to append the SSU rDNA sequences available through the RDP database with all cellular and gene clone sequences obtained from this study. The results were used to sort the sequences into sets of related phylogeny from which similarity values between the cellular and gene clones and their closest relatives were calculated.

Phylogenetic trees were calculated with the neighbor-joining algorithm (Saitou & Nei 1987) using the program NEIGHBOR of the PHYLIP v3.5

software (Felsenstein 1989). Evolutionary distances were calculated using the program DNADIST and the Kimura 2-parameter model for nucleotide change, with a transition/transversion ratio of 2.0 (Kimura 1980). To check the consistency of the resulting tree, random re-sampling of the sequences (bootstrapping) was performed, and a tree representing a consensus of 100 trees was obtained (Felsenstein 1985). Similarities were calculated from partial sequences by considering all available overlapping regions, with the exclusion of ambiguous nucleotides.

Accession numbers.

Nucleotide sequences were filed in Genbank under the accession numbers: R2A5, U78932; R2A9, U78920; R2A10, U78933; R2A15, U78921; R2A28, U78936; R2A30, U78922; R2A37, U78923; R2A44, U78924; R2A54, U78939; R2A57, U78909; R2A62, U78910; R2A63, U78911; R2A81, U78925; R2A84, U78912; R2A85, U78926; R2A86, U78927; R2A88, U78928; R2A103, U78924; R2A113, U78929; R2A114, U78913; R2A117, U78914; R2A130, U78915; R2A132, U78935; R2A135, U78916; R2A148, U78930; R2A153, U78917; R2A160, U78940; R2A161, U78938; R2A163, U78918; R2A166, U78919; R2A170, U78941; R2A173, U78931; R2A180, U78937; OCS19, U78942; OCS84, U78943; OCS116, U78944; OCS122, U78945; OCS181, U78946; S34, U87407.

Results

Cell counts estimated from colony forming units on marine R2A medium were orders of magnitude lower than DAPI direct counts. The number of bacteria estimated from the number of colony forming units was 72 ± 17 ml^{-1}, and the number of bacteria estimated by DAPI direct counts was

$2.07 \cdot 10^6$ ml^{-1}. Similar results, not reported here, were obtained with other media, including Zobell's marine agar.

Colony morphology was used to sort the cellular clones into categories because a majority of the isolates could be described by a few recognizable colony morphotypes. Of 127 cellular clones examined, 27 colony morphotypes were identified. *Hae*III RFLPs of SSU rDNAs were determined for 60 cellular clones, with resampling of colony morphologies that were either very abundant or else nondescript in appearance. Our reasoning was that variety was more likely to be overlooked among the nondescript colonies. In general, each colony morphotype was associated with a unique RFLP pattern, though variation in RFLP patterns was found within 4 of the 27 colony morphotypes (colony types V, VI, IX and XVIII; Table 1.2). In three cases (RFLP types 1, 20 and 26; Table 1.2), cellular clones with different colony morphologies were found to have identical RFLP patterns.

A comparison of the *Hae*III RFLP patterns of the SSU rDNAs from the cellular and gene clones showed little overlap. The gene clone library consisted of 116 full length SSU rDNA clones, of which 54 were of plastid origin, two were chimeras, and two were not identified. The *Hae*III RFLP patterns of the remaining 58 bacterial gene clones fell into 25 RFLP types, 13 of which were unique to single clones. Three RFLP patterns were common to both cellular and gene clones (Figure 1.1, Table 1.3). Sequence comparisons showed that the cellular and gene clones with similar RFLP patterns were phylogenetically related in two of these cases (Table 1.3).

Table 1.2. Distribution of R2A cellular clones classified by colony morphotype

Colony type	No. of isolates	RFLP type	Representative isolate[a]	RDP nearest phylogenetic neighbor	Similarity (%)
I	33	1	R2A30[b]	*Azospirillum* sp. str. DSM1727	100.0
II	5	13	R2A5	*Flexibacter maritimus*	87.8
III	11	1	R2A30	*Azospirillum* sp. str. DSM1727	100.0
IV	8	10	R2A10	*Flexibacter maritimus*	86.7
V	6	1	R2A30	*Azospirillum* sp. str. DSM1727	100.0
V		2	R2A9	*Vibrio fischerii*	91.1
V		20	R2A37	environmental clone AGG53	94.3
V		21	R2A62	environmental clones OCS19 and OM42	95.6
V		29	R2A84	Sargasso Sea isolate S34	95.6
VI	4	3	R2A15	*Aeromonas salmonicida* subsp. *masoucida* str. 1-1-1	93.8
VI		4	R2A85	environmental clone AGG53	96.0
VI		5	R2A88	Unknown, unnamed purple bacterium[c]	94.4
VI		20	R2A81	environmental clone AGG53	99.6
VII	2	5	R2A113	Unknown, unnamed purple bacterium[c]	99.6
VIII	1	18	R2A44	*O. multiglobiferum*	93.5
IX	20	6	R2A54	*Arthrobacter globiformis*	89.4
IX		26	R2A135	*Blastobacter* sp. str. BF14	96.0
IX		28	R2A170	Citrus disease-associate bacteria str. UW103/A31	90.9
X	15	7	R2A153	*Sphingomonas* sp. str. SYK6	96.6
XI	2	8	R2A63	*Blastobacter* sp. str. BF14	95.1
XII	5	9	R2A103	*Capnocytophaga gingivalis*	83.7
XIII	1	26	R2A166	*Blastobacter* sp. str. BF14	96.6
XIV	4	11	R2A148	*Oceanospirillum multiglobiferum*	94.8
XV	1	19	R2A86	*Alteromonas haloplanktis*	95.2
XVI	1	12	R2A180	*Staphylococcus equorum*	98.0
XVII	1	22	R2A173	*Oceanospirillum beijerinckii*	87.4
XVIII	3	14	R2A57	environmental clones OCS19 and OM42	94.4
XVIII		24	R2A117	environmental clone OM64	95.1
XVIII		16	R2A114	environmental clone SBR2045[d]	95.2
XIX	1	15		N.D.[e]	
XX	1	18		N.D.[e]	
XXI	1	1	R2A30	*Azospirillum* sp. str. DSM1727	100.0
XXII	1	23	R2A130	*Hyphomicrobium*-like organism strain US-353	87.3
XXIII	1	17	R2A161	*Bacillus firmus*	97.4
XXIV	1	31	R2A163	environmental clone SBR2045[d]	93.6
XXV	1	30	R2A28	*Bacillus* sp. DSM8716	89.1
XXVI	1	25	R2A132	*Flavobacterium gondwanense*	87.4
XXVII	1	27	R2A160	*Micrococcus luteus*	94.1

[a]only includes those clones for which a Genbank accession number was obtained.
[b]R2A30 is the representative cellular clone for all colony types with RFLP type 1.
[c]Genbank accession number Z25522.
[d]Genbank accession number X84583
[e]N.D., no rDNA sequence data available.

Table 1.3. Summary of Oregon coast clones with overlapping *Hae*III RFLP patterns or rDNA sequence.

Cellular clone collection		Gene clone library			
RFLP type	Phylogenetic affiliation	RFLP type	Phylogenetic affiliation	same RFLP type[a]	same RDP group
1	*Pseudomonas* subgroup	35	*Pseudomonas* subgroup	+	+
29	*Roseobacter* group	14	*Roseobacter* group	+	+
7	*Sphingomonas* subgroup	37	*Rhodopseudomonas marina* subgroup	+	–
14, 16, 21, 24, 29, 31, 42	*Roseobacter* group	21,12,14	*Roseobacter* group	–	+

[a]Clones were determined to belong (+) or not to belong (-) to the same RFLP type or RDP group.

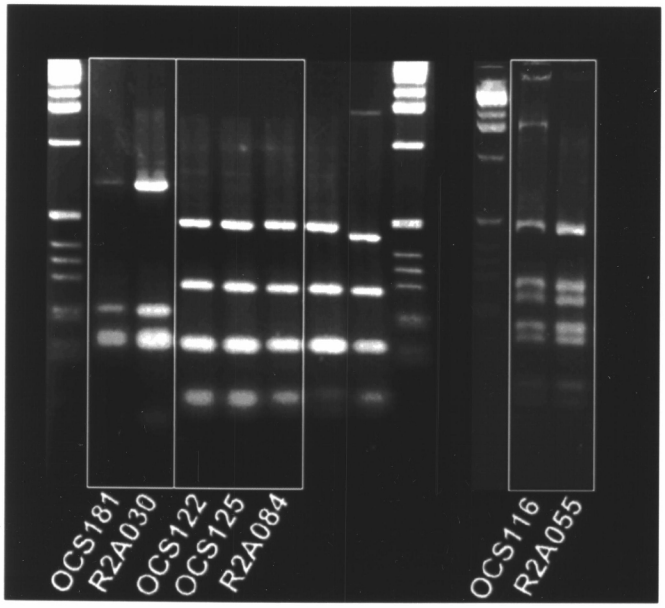

Figure 1.1 *Hae*III RFLP patterns common to cellular isolates and environmental gene clone SSU rDNAs. Cellular clones: R2A030 (RFLP type 1), R2A 084 (RFLP type 29), and R2A055 (RFLP type 7). Gene (environmental) clones: OCS181 (RFLP type 35), OCS 122 (RFLP type 14), OCS125 (RFLP type 14) and OCS 116 (RFLP type 37). The composite picture was generated by using Adobe Photoshop 3.0 (Adobe Systems Inc.)

Sequence comparisons supported the same conclusion drawn from comparisons of the *Hae*III RFLP patterns - that there was little overlap in the species present in the two sets of SSU rRNA genes. Although RFLP comparisons were determined for a larger number of cellular clones (n = 60), comparisons of 5' terminal gene sequences from 32 cellular clones provided greater resolution of genetic differences as well as phylogenetic identification. Sequencing showed that the cellular clone collection was composed, in order of abundance, of members of the gamma and alpha subdivisions of Proteobacteria, members of the *Flexibacter-Bacteroides-Cytophaga* line of descent, and members of the high and low G + C Gram-positive phyla.

The most common cellular clones, RFLP type 1, accounted for 80% of the gamma Proteobacteria cellular clones (Figure 1.2a). The SSU rDNAs of these isolates were identical to the published gene sequence of *Azospirillum* sp. str. DSM1727, a member of the *Pseudomonas* subgroup of the gamma Proteobacteria. As previously mentioned, cellular clone RFLP type 1 was one of the three cellular clone types with a *Hae*III RFLP pattern matching that of a gene clone. The second most abundant group of gamma Proteobacteria cellular clones (RFLP types 4 and 20) were related to the *Colwellia* assemblage and the *Alteromonas* group, and were most similar (94.3-99.6%) to the environmental rDNA clone AGG53, isolated by DeLong et al. from marine phytodetrital aggregates (Figure 1.3, Table 1.2) (DeLong et al. 1993).

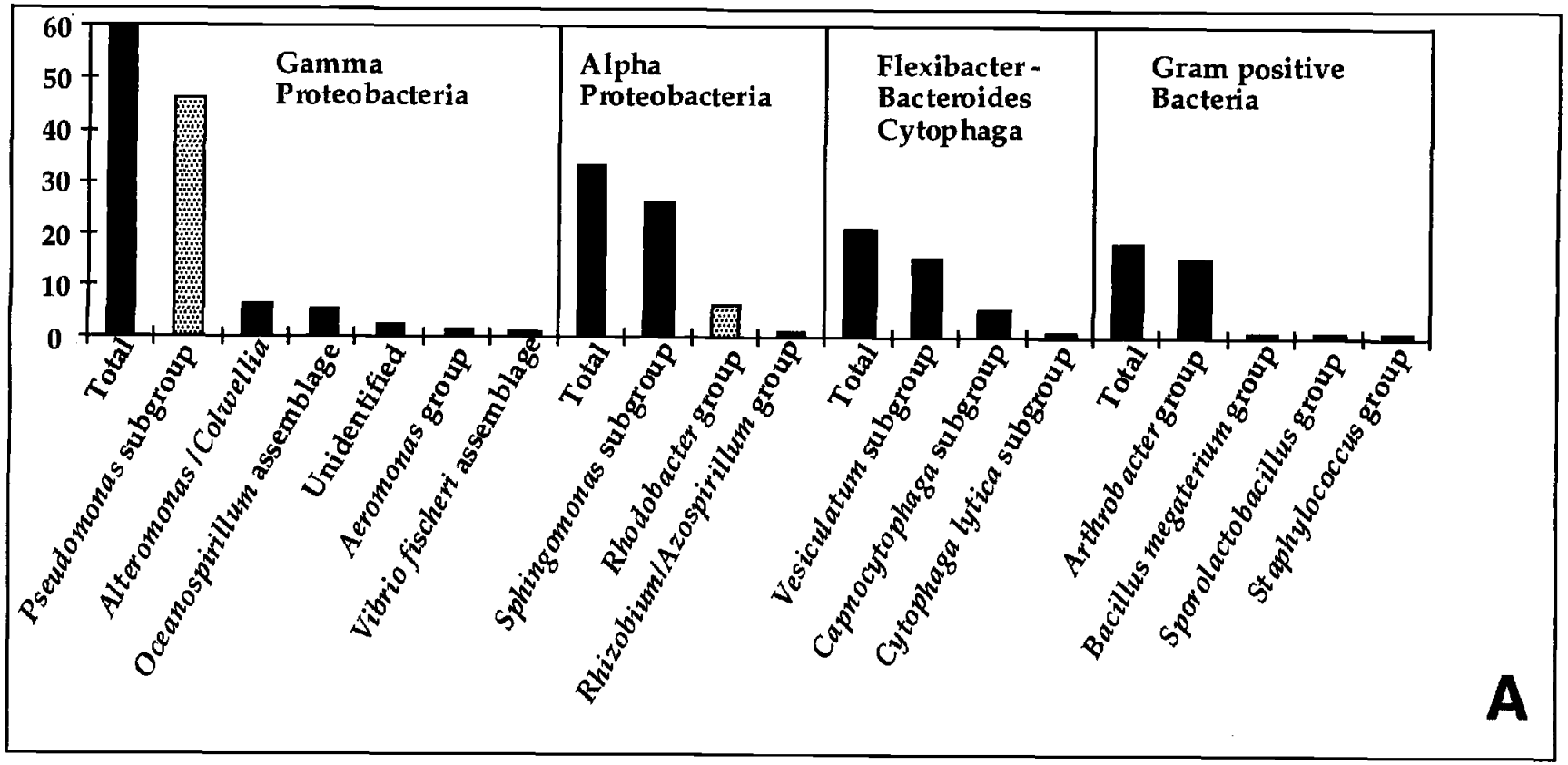

Figure 1.2. Taxonomic grouping of Oregon coast cellular clones (A) and gene clones (B), obtained by a comparison of partial (5'-end) SSU rDNA sequences to sequences available through the RDP by using the program SIMILARITY_RANK. Cellular clones and environmental clone groups with identical HaeIII RFLP patterns are indicated by shaded bars; otherwise, the clones are represented by solid bars. Values on the y axis are the number of clones

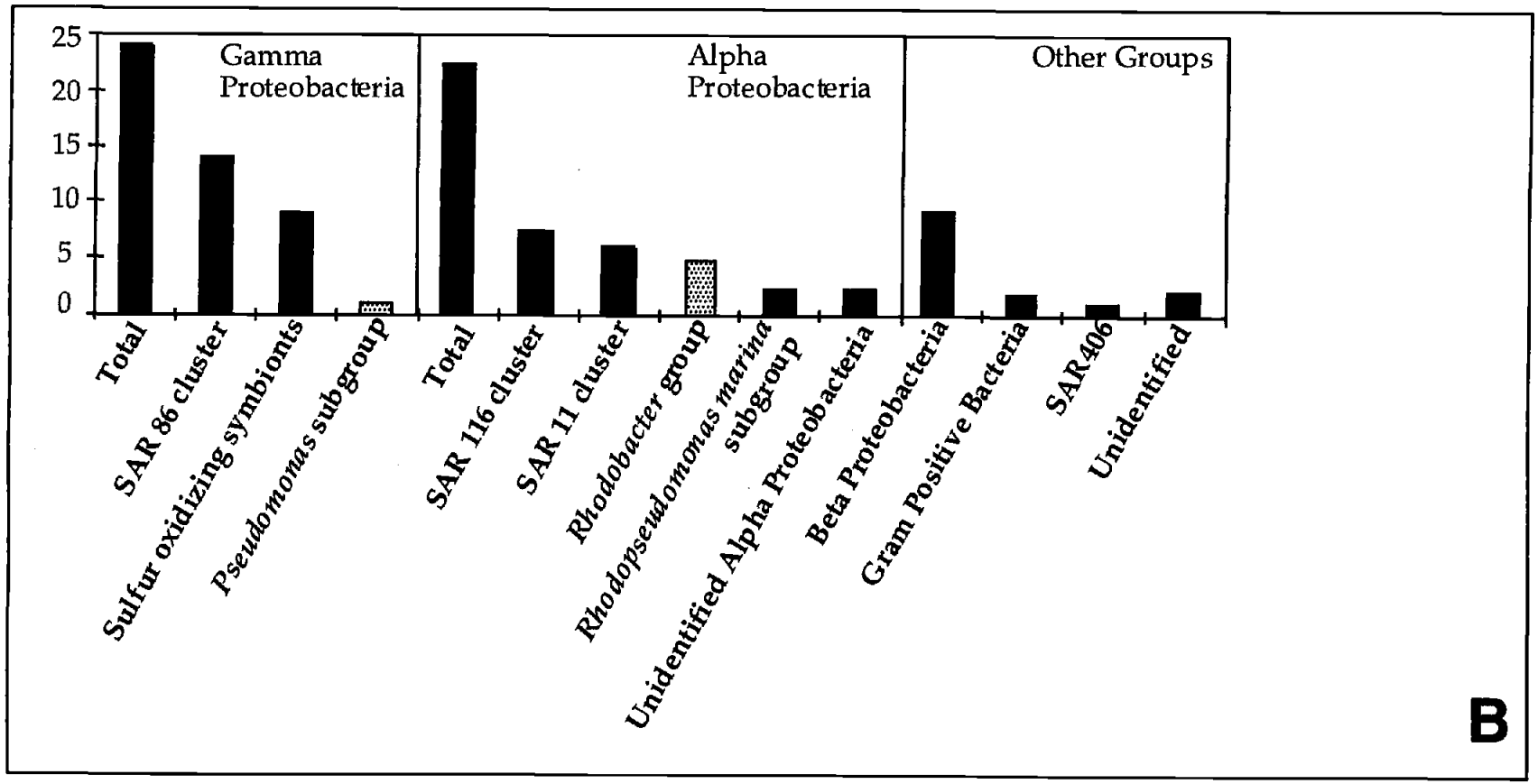

Figure 1.2. Continued

The most abundant alpha Proteobacteria cellular clones were members of the *Sphingomonas* and *Roseobacter* groups (Figure 1.2a), while the majority of the clones affiliated with the *Flexibacter-Bacteroides-Cytophaga* line of descent were members of the *Vesiculatum* and the *Cytophaga lytica* subgroups. Finally, the cellular clones belonging to the Gram-positive phyla were members of the *Arthrobacter* (high G + C) and *Bacillus* (low G + C) groups.

The gene clones were, in order of abundance, members of the gamma, alpha, and beta subdivisions of the class Proteobacteria and members of the high G + C Gram-positive phylum. All but one of the gamma Proteobacteria clones in the clone library were members of the SAR86 cluster (Mullins et al. 1995), or closely related to sulfur oxidizing symbionts (Figure 1.2b, Figure 1.3). The remaining gamma Proteobacteria gene clone (OCS181) was a member of the *Pseudomonas* subgroup. OCS181 had a *Hae*III RFLP pattern identical to that of the cellular clone RFLP type 1 (Figure 1.1), and was 94.6% similar in rRNA gene sequence to the cellular clone R2A30 (Table 1.2). Proteobacteria gene clones of the alpha subdivision were members of the SAR11 cluster (Giovannoni et al. 1990), SAR116 cluster (Mullins et al. 1995), or related to the SAR83 cluster within the *Rhodobacter* group (Figure 1.2b) (Britschgi & Giovannoni 1991). The high G + C Gram positive gene clones were members of the BDA1-5 cluster (Fuhrman et al. 1993, Rappé et al. in press).

Simrank comparisons were used to identify nearest phylogenetic neighbors. For the majority of cellular clones, the nearest phylogenetic neighbors formed monophyletic groups related by high S_{ab} values, thus permitting unambiguous identification. However, in some cases, the

Simrank results were ambiguous because they were either i) a polyphyletic list of organisms with low S_{ab} values to the submitted sequence (i.e. R2A55, cellular clone RFLP type 7), or ii) a list of uncultured environmental clones with high S_{ab} values to the submitted sequence (i.e. R2A57, cellular clone RFLP type 14). Both cases suggest that the cellular clones may represent new species.

In general, phylogenetic analyses of partial rDNA sequences supported the results obtained by Simrank by showing that, although most of the SSU rDNA sequences from cellular clones were phylogenetically related to clades of cultured species, some were instead closely related to environmental SSU rRNA gene clones. In particular, many of the cellular clones belonging to the *Rhodobacter* group were related to environmental gene clones recovered from seawater (Figure 1.4). Also, cellular clones belonging to the *Colwellia* assemblage and *Alteromonas* group were closely related to the environmental clone AGG53 (Figure 1.3) (DeLong et al. 1993).

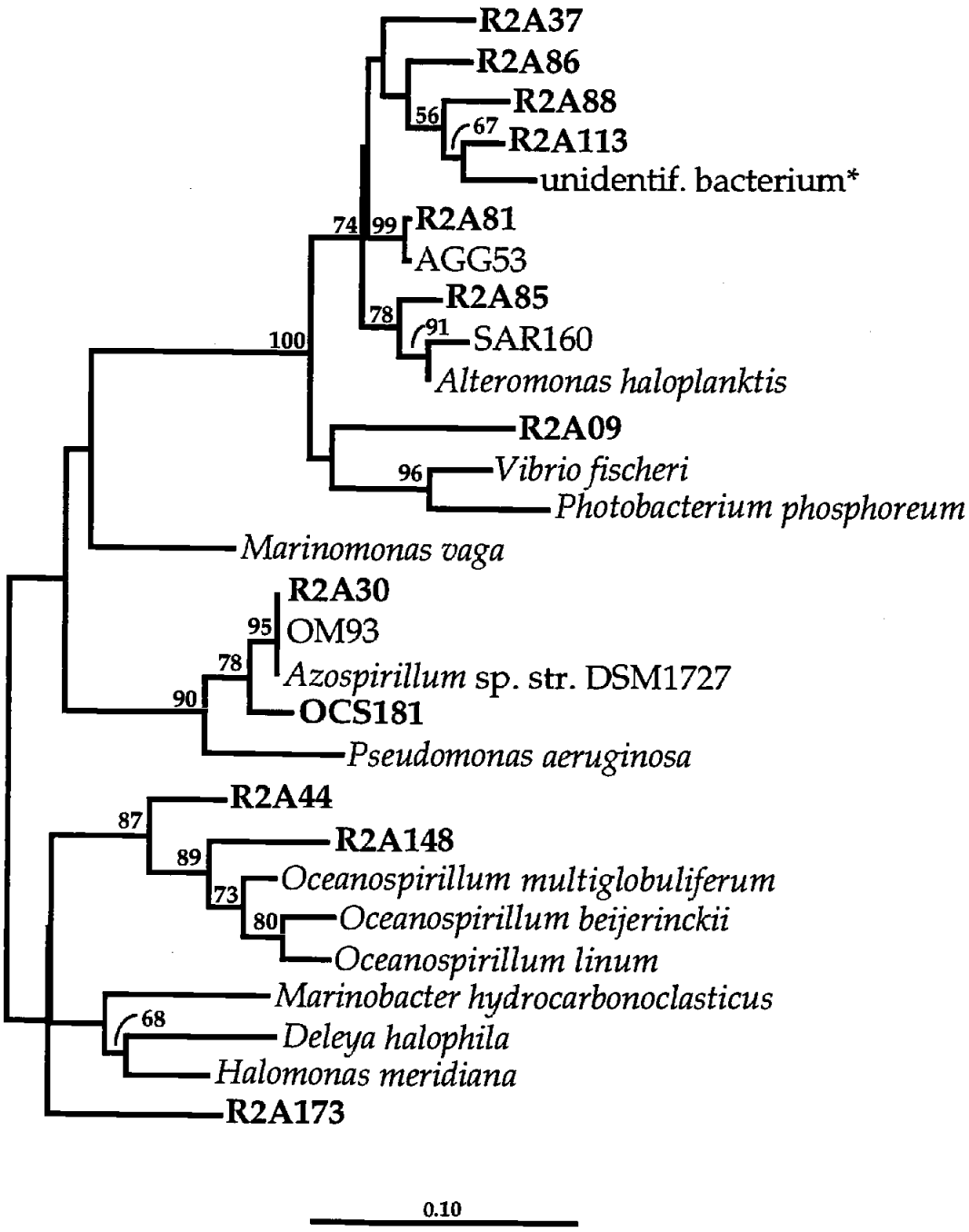

Figure 1.3. Phylogenetic tree generated by the neighbor-joining method from a mask of ca. 200 nucleotide positions, showing the relationships between Oregon coast cellular clones (R2A) and gene clones (OCS) related to the gamma Proteobacteria. Bootstrap values (n=100 replicates) are indicated for each of the branches. OM, environmental rDNA clone from the eastern continental shelf of the United States (Rappé et al. in press). *Genbank accession number Z25522.

Figure 1.4. Phylogenetic tree generated by the neighbor-joining method from a mask of ca. 200 nucleotide positions, showing the phylogenetic relationships among Oregon coast cellular and gene clones within the alpha subdivision of the Proteobacteria. Bootstrap values (n=100 replicates) are indicated for each of the branches. OM, environmental rDNA clones from the eastern continental shelf of the United States (Rappé et al. in press).

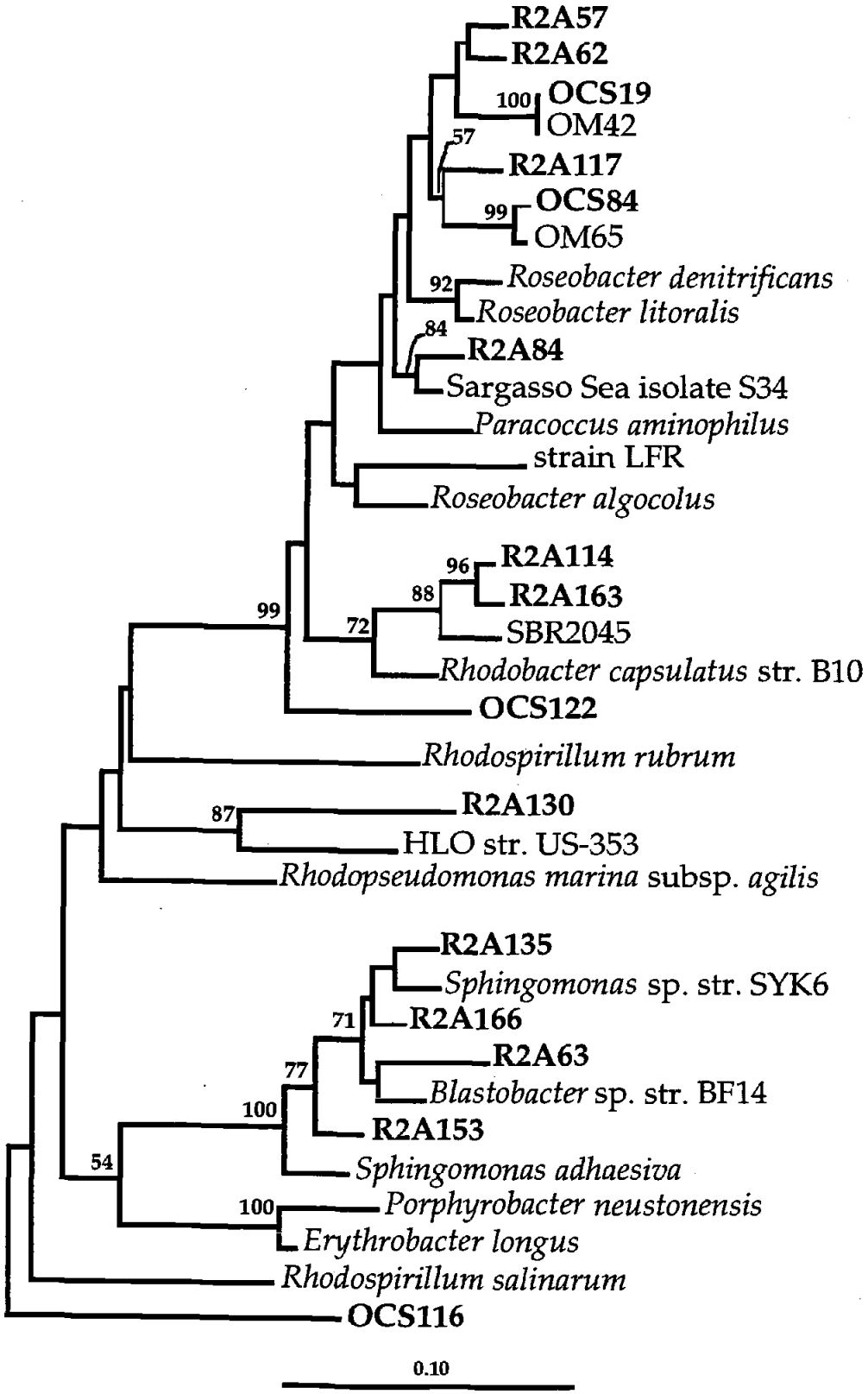
R2A57
R2A62
100
OCS19
OM42
57
R2A117
99
OCS84
OM65
92
Roseobacter denitrificans
Roseobacter litoralis
84
R2A84
Sargasso Sea isolate S34
Paracoccus aminophilus
strain LFR
Roseobacter algocolus
96
R2A114
88
R2A163
99
72
SBR2045
Rhodobacter capsulatus str. B10
OCS122
Rhodospirillum rubrum
87
R2A130
HLO str. US-353
Rhodopseudomonas marina subsp. agilis
R2A135
Sphingomonas sp. str. SYK6
71
R2A166
77
R2A63
Blastobacter sp. str. BF14
100
R2A153
54
Sphingomonas adhaesiva
100
Porphyrobacter neustonensis
Erythrobacter longus
Rhodospirillum salinarum
OCS116
0.10

Figure 1.4

Discussion

The single most important observation reported here is that the genes recovered by cloning techniques and the genes recovered from bacteria isolated into culture from the same water sample were different. Explanations that attribute this difference to biases in molecular techniques seem unlikely since investigations of bacterioplankton diversity by different gene cloning methods have led to the identification of many of the same phylogenetic lineages, regardless of the water mass investigated or the method used for gene cloning (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993, Rappé et al. in press).

Although the main conclusion of this study is that many abundant bacterial species in seawater are not easily cultured, this fact tells only a small part of the story concerning the relationship between cultured bacteria and the distributions of bacterioplankton species in nature. The observation that many of the cellular clones could not be identified by comparisons of their SSU rDNAs to sequence databases indicates that a significant fraction of the bacterioplankton that can be grown on organically "rich" media are not represented in the databases. This result suggests that microbial cultivation has not yet been employed exhaustively for determining the taxonomic identities and distributions of marine bacteria. However, an alternative explanation is that the unidentified isolates are among the ca. 26% of systematically described marine bacterial species that are not yet represented in rDNA sequence databases (Giovannoni et al. 1996). In either case, the results clearly point to a need for further investigations of the systematics of the marine heterotrophic bacteria which easily can be cultivated on common microbiological media that have high organic carbon contents.

The application of innovative culturing methods, such as seawater culture, that more accurately recreate the physical and chemical conditions found in the ocean, are likely to lead to the cultivation of microbial strains that cannot be grown on organically rich media such as the marine R2A medium we employed in this study (Button et al. 1993, Schut et al. 1993). Nature harbors microbial species with diverse growth requirements; no single cultivation medium could be expected to culture a majority of these species. Seawater culture has retrieved isolates of heterotrophic bacteria - "oligotrophs" - that cannot be grown on organically rich marine media (Ishida et al. 1986, Button et al. 1993, Schut et al. 1993). However, so far, there have been no reports of seawater culture methods being used successfully to culture microbial species corresponding to the 16S rRNA genes recovered most frequently from seawater by molecular techniques. In light of these observations it seems appropriate to regard bacteria as occupying a physiological continuum which may render them more or less amenable to cultivation. Thus, the terms "culturable" and "unculturable", although convenient, have meaning relevant only to particular, defined experimental conditions.

A third point to emerge from the comparisons we present is that the gene sequences of culturable microbes recovered from seawater in some cases are very similar but not identical to the rDNA gene sequences of closely related species. The significance of this variation is difficult to determine at this time. There is no exact relationship between SSU rDNA sequence similarity and taxonomic divisions. Furthermore, there is little comprehensive information available on rDNA variability within taxonomic groups, such as among strains of a species defined by phenotypic

criteria (Cohan 1996). Gene clusters are observed commonly among 16S rDNA clones retrieved from environmental nucleic acids; the issue of physiological variability among the closely related strains represented by these sequence clusters may be of considerable significance to microbial ecologists interested in investigating microbial distribution with gene probes (Cohan 1996, Field et al. 1997).

Our investigation uncovered some cultured isolates that are closely related to microbial species that previously had been described only from environmental clone libraries (e.g., isolate R2A81 and clone AGG53, similarity=99.6%). Interestingly, these isolates, which correspond to 16S rRNA genes recovered from marine aggregates, flocculate in suspension. These strains, which remain unidentified at a systematic level, may be of use as laboratory models for investigating the mechanisms of marine microaggregate formation.

Despite the challenges of culturing some recalcitrant species, the isolation and identification of representative marine bacterioplankton strains remains an indispensable source of physiological and genetic information about marine microbes. Phylogenetic trees can be highly informative; nonetheless, many metabolic pathways in bacteria are polyphyletically distributed, thereby limiting inferences about the biogeochemical impact of bacterioplankton based on rRNA sequence comparisons alone. Partial genomic DNA sequences retrieved from nature offer one means of obtaining more information about uncultured species. In a recent study, this approach was used to study Group I marine crenarchaeotes (Stein et al. 1996).

It was not the aim of this study to develop innovative culturing techniques. Instead, it was based on the application of those methods used most routinely in marine microbiology to recover bacterial species from seawater. Nonetheless, among the bacterial isolates recovered were many that appeared to constitute new microbial species by the criterion of SSU rRNA comparisons. In this light, we might regard genetic investigations of microbial diversity as successful not just because they employ novel methods, but also because they have invited renewed scrutiny of a fertile problem.

Acknowledgments

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Chapter 2

Bias Caused by Template Annealing in the Amplification of Mixtures of 16S rRNA Genes by PCR

Marcelino T. Suzuki and Stephen J. Giovannoni

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Abstract

The polymerase chain reaction (PCR) is used widely for the study of ribosomal RNA genes amplified from mixed microbial populations. These studies resemble quantitative applications of PCR in that the templates are mixtures of homologs and the relative abundance of amplicons is thought to provide some measure of the gene ratios in the starting mixture. Although such studies have established the presence of novel ribosomal RNA genes in many natural ecosystems, inferences about gene abundance have been limited by uncertainties about the relative efficiency of gene amplification in the polymerase chain reaction. To address this question, three ribosomal RNA gene standards were prepared by PCR, mixed in known proportions, and amplified a second time using primer pairs in which one primer was labeled with a fluorescent nucleotide derivative. The PCR products were digested with restriction endonucleases and the frequencies of genes in the products were determined by electrophoresis on an ABI 373A automated DNA sequencer in Genescan™ mode. Mixtures of two templates amplified with the 519F/1406R primer pair yielded products in the predicted proportions. A second primer pair (27F/338R) resulted in strong bias towards 1:1 mixtures of genes in final products, regardless of the initial proportions of the templates. This bias was strongly dependent on the number of cycles of replication. The results fit a kinetic model in which the re-annealing of genes progressively inhibits the formation of template-primer hybrids.

Introduction

In many applications of the polymerase chain reaction the template is a mixture of homologous genes. Three examples are: 1) the amplification of multi-gene families from the DNA of a single species; 2) the amplification of ribosomal RNA genes from genomic DNA extracted from natural communities of microbes; and 3) many quantitative PCR assays employing internal standards (e.g. Becker-Andre & Hahlbrock 1989, Leser et al. 1995, Wang et al. 1989). In each of these cases the product amplicons are derived from template DNA by a process involving complex chemical kinetics, and the relative abundance of homologs among the final reaction products is often a parameter of interest. Variability in the efficiency of PCR reactions is a common observation, but it would not be expected to influence product ratios in mixed-template reactions unless amplification efficiencies differed among homologs. Thus, information about the relative efficiency of gene amplification in mixed template PCR reactions is crucial to the interpretation of such experiments, and, in particular, the lack of such information has impeded progress in microbial ecology (Giovannoni et al. 1995, Wagner et al. 1994).

Although the mechanisms of gene amplification from complex mixtures are not well understood, PCR is nonetheless widely used for quantitative studies in molecular biology, including quantitative reverse transcriptase PCR (RT-PCR; Becker-Andre & Hahlbrock 1989, Diviacco et al. 1992, Gilliland et al. 1990, Siebert & Larrick 1992, Wang et al. 1989). In such studies, the internal standard is added at a known concentration to a reaction containing the gene of interest at an unknown concentration.

Generally, the internal standard is presumed to have priming sites identical to those of the gene under study, but it differs from the target molecules either in size or restriction endonuclease sites to allow easy detection. The concentration of the gene of interest is estimated from the ratio of the concentrations of the product amplicons from the gene of interest and the internal standard. The inclusion of internal gene standards is vital to such studies because it minimizes errors introduced by tube-to-tube variation in amplification efficiency. However, in such cases it is assumed that the amplification efficiencies of the gene of interest and the internal standards are the same. This assumption requires several further assumptions: a) the gene of interest and the gene standard are equally accessible to primer hybridization following denaturation; b) primer-template hybrids form with equal efficiency for both templates; c) both templates are extended by the polymerase with the same efficiency; d) limitations caused by substrate exhaustion equivalently affect the extensions of both templates. Two recent papers (Morrison & Gannon 1994, Raeymaekers 1995), discuss the problems associated with quantitative PCR. Raeymaekers (Raeymaekers 1995) mentions that in several published studies, violations of assumption d) may have occured, whereas Morrison and Gannon (Morrison & Gannon 1994) examine a system in which the assumptions apparently hold.

Several factors might bias the relative frequencies of genes in PCR products from mixed-template reactions. One such factor in particular, the mol% guanine + cytosine (G+C) content of template DNA, has been reported to influence gene amplification by PCR (Dutton et al. 1993, Reysenbach et al. 1992). In addition different binding energies resulting from primer degeneracy (that is, a mixture of primers with nucleotides

sequences corresponding to observed variation among homologs), and the influence of template folding are other plausible, but undocumented, sources of bias in PCR. In no case yet has the quantitative extent of template bias been well documented.

The amplification of ribosomal RNA genes from mixed genomic DNAs derived from natural microbial populations is analogous to quantitative RT-PCR, except that the gene mixtures are frequently more complex and the assumptions above must also hold for homologs with unknown sequences. In addition, universal primers employed for the amplification of ribosomal RNA genes, often contain degeneracies which may influence the formation of primer/template hybrids. Also, the mol %G+C composition of genomes in microbial populations may vary widely. Nonetheless, PCR-amplified DNA has been used in numerous studies addressing the rDNA composition of mixed populations by applying analytical techniques to detect homologous genes, including gene cloning and sequencing (Britschgi & Giovannoni 1991, DeLong et al. 1993, Fuhrman et al. 1993), denaturing gradient gel electrophoresis (DGGE; Muyzer et al. 1993), and chromatographic techniques such as capillary electrophoresis (Avaniss-Aghajani et al. 1994). Such techniques have potentially important applications to studies of spatio-temporal variations in microbial communities, where the ability to detect relative changes in gene concentrations is a foremost objective.

The study described here was undertaken to examine the potential introduction of biases by PCR in the amplification of ribosomal RNA genes from known mixtures. Our approach was to mix small-subunit ribosomal RNA genes (SSU rDNAs) from three different phylogenetic groups of

marine bacteria. Pairwise mixtures in different proportions were used as templates for amplification by PCR. To measure bias produced by PCR we compared the proportions of genes in the products with their proportions in the template mixture. Our goal was to understand mechanisms which might introduce PCR biases. Although the results described apply to a small subset of genes amplified under controlled conditions, they reveal information about mechanisms of PCR bias which can be used to identify conditions in which such biases are minimized.

Material and methods

Templates.

Since the accurate measurement of gene concentrations in the templates was critical to the assay, we chose to use either linearized plasmids containing cloned SSU rDNA inserts, or the 27F/1492R fragment of SSU rDNA, which had been synthesized by PCR. The sources of SSU rDNAs were clone libraries of bacterial SSU rDNAs (Britschgi & Giovannoni 1991, Gordon & Giovannoni, unpublished , Rappé & Giovannoni, unpublished). Briefly, 16S ribosomal RNA genes were amplified from DNA samples from the Bermuda Atlantic Time Series station in the Sargasso Sea (31°50'N, 64°10'W) via PCR using the bacterial primers 27F and 1522R (Giovannoni 1991; Table 2.1). Libraries of SSU rDNAs were obtained by cloning the PCR products into the vector pCRII (Invitrogen, San Diego, CA), as previously described (Britschgi & Giovannoni 1991). Template DNA concentrations were measured spectrophotometrically using a Shimadzu UV160U (Shimadzu Co., Kyoto, Japan) spectrophotometer.

Table 2.1. Sequences and specificity of the primers utilized.

Primer	Sequence	Specificity
EubB (27F)	AGAGTTTGATCMTGGCTCAG	Bacteria
519F	CAGCMGCCGCGGTAATWC	Universal
338R	GCTGCCTCCCGTAGGAGT	Universal
1406R	ACGGGCGGTGTGTRC	Universal
1492R	GGTTACCTTGTTACGACTT	Bacteria
EubA (1522R)	AAGGAGGTGATCCANCCRCA	Bacteria

Three clones for which the complete nucleotide sequences were known were chosen for this study: SAR432, SAR464 and SAR202. Clones SAR432 and SAR202 are affiliated with the Gram positive bacteria and *Chloroflexus / Herpetosiphon* bacterial phyla, respectively (Gordon & Giovannoni , unpublished, Rappé & Giovannoni unpublished). SAR464 is a member of the SAR11 cluster of the alpha Proteobacteria (Gordon & Giovannoni, unpublished). To avoid contamination of the cloned SSU rDNAs with genomic DNA from *E. coli*, the plasmids were purified by alkaline lysis, followed by CsCl-ethidium bromide density gradient centrifugation (Sambrook et al. 1989).

Two different sets of templates were used to evaluate the introduction of biases by PCR. The first consisted of a mixture of purified plasmids containing SSU rDNA from SAR202 and SAR464 linearized by digestion with the restriction endonuclease *Not*I (Promega, Madison, WI). The second set of templates consisted of mixtures of the 27F/1492R fragments of SAR202, SAR432 and SAR464, each of which had been amplified separately by PCR from linearized plasmids. Except when noted, the proportions were

0:1; 1:4, 2:3, 3:2; 4:1, 1:0 for each pair of genes. All template mixtures were added to 0.1 ng/µl final concentration.

PCR reaction conditions.

The primers and templates used and the number of amplification cycles varied; all other conditions were constant in all reactions. In a final volume of 100 µl, reactions contained 0.2 mM of pre-mixed dNTPs (Stratagene, La Jolla, CA), 1.5 mM MgCl, 5% acetamide and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). All reactions used the Ampliwax™ (Perkin Elmer Cetus, Norwalk, CT) hotstart protocol and a PLT100 thermal cycler (MJ Research Inc., Watertown, MA) programmed to 35 cycles (except for the one evaluating the effect of number of cycles) of 96°C denaturation, 1 min., 55°C annealing, 1 min., and 72°C extension, 3 min.

PCR primers.

Quantitative experiments used either the 27F-FAM/338R or 519F/1406R-HEX primer pairs. 27F-FAM, which was graciously supplied by Applied Biosystems Inc. (ABI, Foster City, CA), was 5' end-labeled with the phosphoramidite fluorochrome FAM (5-carboxy-fluoresceine). 1406R-HEX, purchased from Genset (San Diego, CA) was 5' end-labeled with the phosphoramidite fluorochrome HEX (6-carboxy-2'4'7',7-hexachloro-fluoresceine). All primers were added to 0.5 µM final concentration.

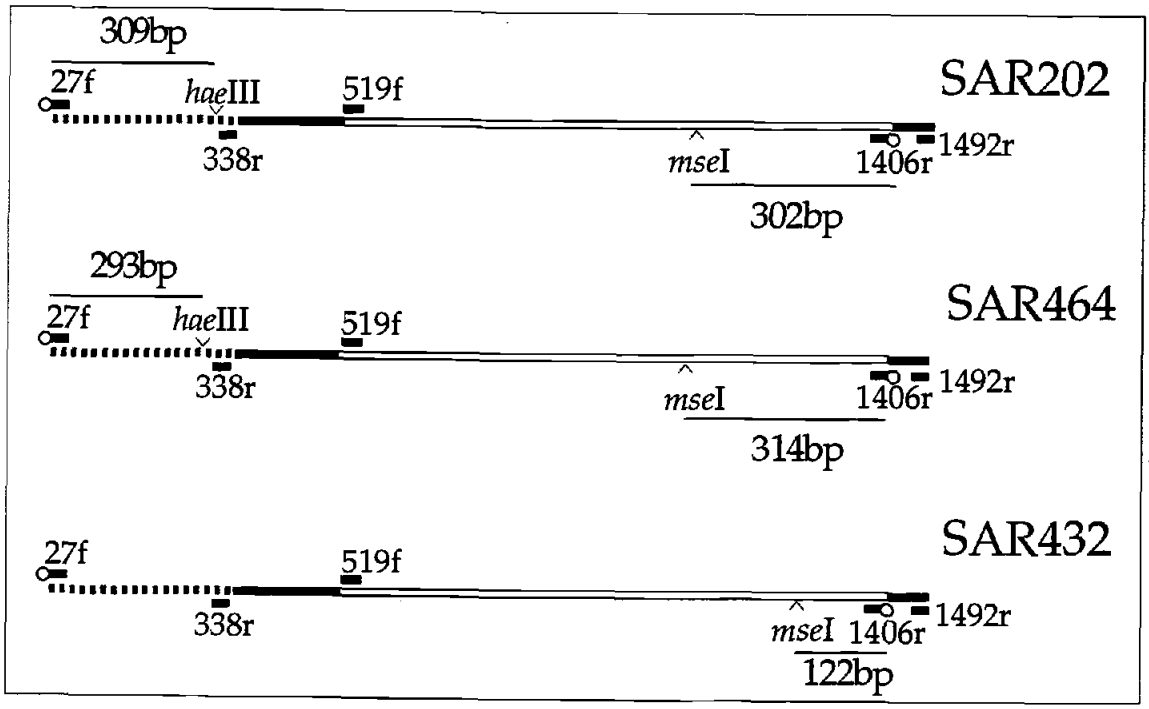

Figure 2.1. Locations of PCR primers and cleavage sites of the restriction endonucleases used in this study.

Detection of PCR products.

Labeled PCR products were digested with restriction endonucleases producing fragments of different sizes (Figure 2.1). FAM-labeled PCR products were digested with *Hae*III (Promega, Madison, WI; 5 U enzyme/μg PCR product, 2h, 37°C) and HEX-labeled PCR products were digested with *Mse*I (New England Biolabs, Beverly, MA, 5 U enzyme/μg PCR product, 2h, 37°C). Labeled fragments (50 fmoles) were chromatographically separated by PAGE in an ABI 373A automated sequencer in Genescan™ mode, which estimated both the size of fragments and the integrated fluorescence emission of individual bands. Calibration curves with FAM and HEX-labeled fragments showed linearity up to 50 fmoles. Thus, the ratios of

different PCR products were accurately represented by the ratios of peak areas.

Kinetic models.

In order to better interpret the outcome of the quantitative PCR assays, a series of kinetic numerical models were created using the modeling software Stella© (High Performance Systems Inc., Hanover, NH).

Effect of the number of cycles.

The kinetic models developed to interpret PCR biases (see results) predicted that the bias should increase with the number of cycles. To test this prediction, SAR202:SAR464 (4:1) template mixes were amplified as described above. The reactions were stopped by freezing after 10, 15, 25 or 35 cycles and the ratio of the fragments measured as described above.

Results

We used two different primer pairs to amplify mixed templates that consisted of pairwise combinations of 16S rDNAs from three different bacteria. We observed biases which were strongly dependent on choice of primers, and to a lesser extent on the templates. For one primer pair, we observed either little or no bias, and a generally low yield of product. In contrast, for the second primer pair, we observed both a strong bias, and a much higher molar yield of product. The following discussion will focus on the results obtained using PCR products as templates. The results of assays using linearized plasmids as templates were similar.

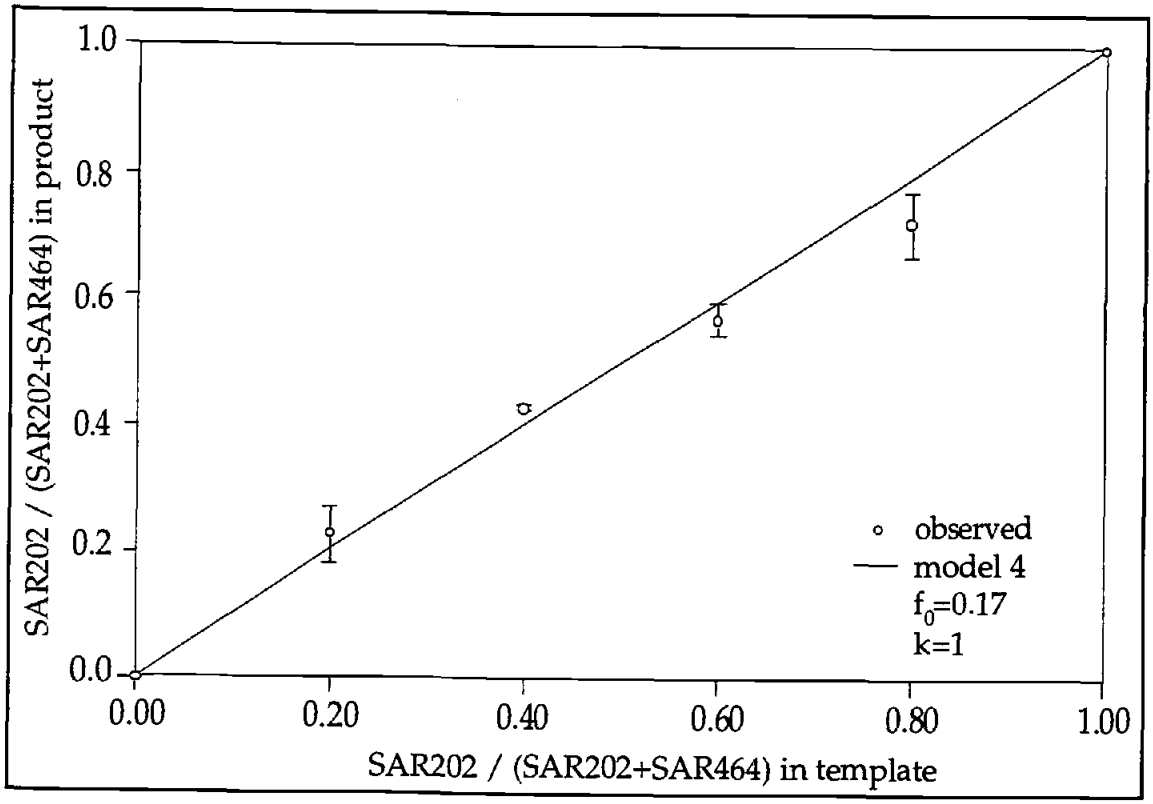

Figure 2.2 Amplification of genes with 519F/1406R-HEX from mixtures of clones SAR202 (*Chloroflexus-Herpetosiphon*)/SAR464 (SAR11 cluster of alpha Proteobacteria) and prediction of model 4, assuming k=1, f_0=0.17 cycle^{-1}.

519F/1406R-HEX primer pair.

When the template was mixed SAR202 and SAR464, the ratios of 519F/1406R PCR products did not differ from the ratios of the templates (Figure 2.2). Similar results were obtained with template mixtures of SAR432 and SAR464 (results not shown). When mixtures of SAR202 and SAR432 were used as templates, a slight bias occurred; the proportion of SAR202 in the products was higher than its proportion in the templates for all the template ratios (Figure 2.3). Deviations from linearity seen with this

template pair are consistent with predictions for templates differing in primer preference, secondary structure, or G+C content. All reactions using the primer pair 519F/1406R-HEX had lower yields than reactions using the 27F/FAM-338R primer pair, producing 16 to 40 nM of the 888 bp HEX-labeled fragment after 35 cycles.

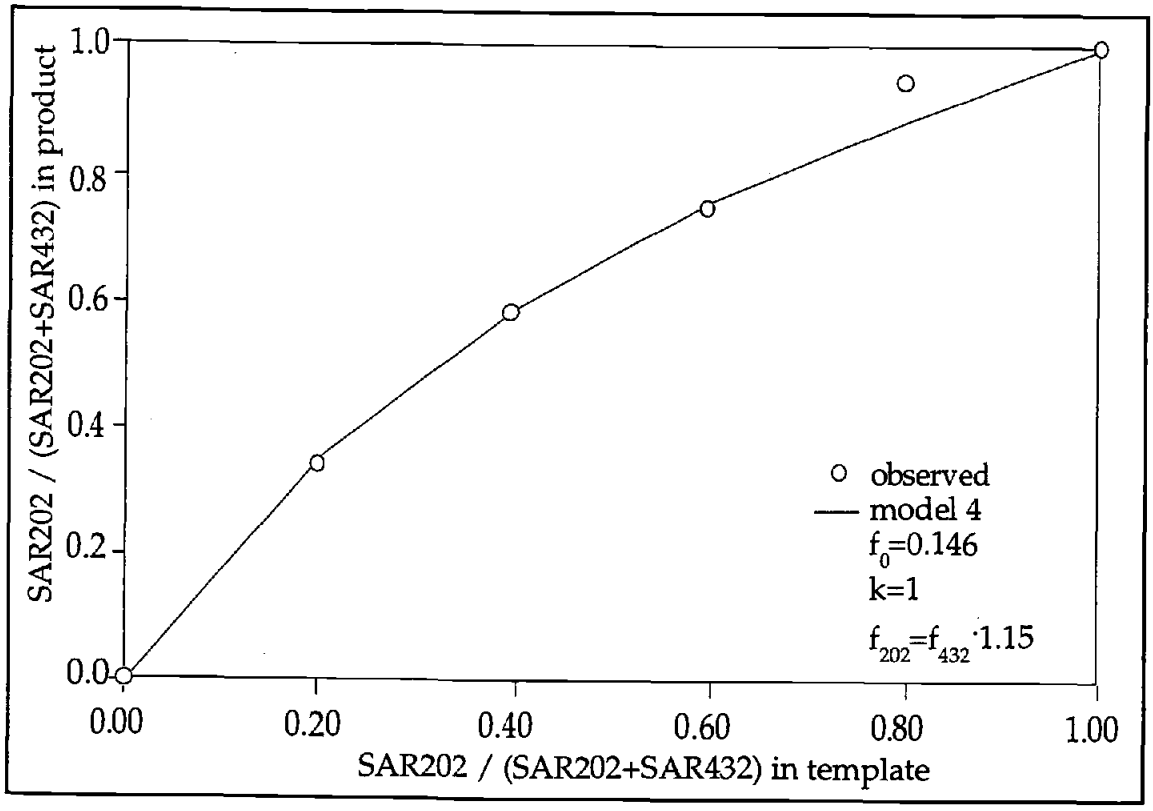

Figure 2.3. Amplification of genes with 519F/1406R-HEX from mixtures of clones SAR202 (*Chloroflexus-Herpetosiphon*)/SAR432 (marine gram positive) and prediction of model 4, assuming k=1, f_0=0.146 f_{202}=1.15·f_{432} .

27F-FAM/338R primer pair.

With the 27F-FAM/338R primer pair and mixtures of SAR202 and SAR464 as templates, we obtained the surprising result that the PCR

products were biased towards a final ratio of 1:1 (Figure 2.4) regardless of the initial ratio between the templates. This contrasted with the results obtained using the primer pair 519F/1406R-HEX, and led directly to the formulation of the kinetic model presented below. In addition, the reactions using the primer pair 27F-FAM/338R had much higher yields than the reactions using the 519F/1406R-HEX primer pair, producing about 140 nM of the 312 bp FAM-labeled amplicons after 35 cycles.

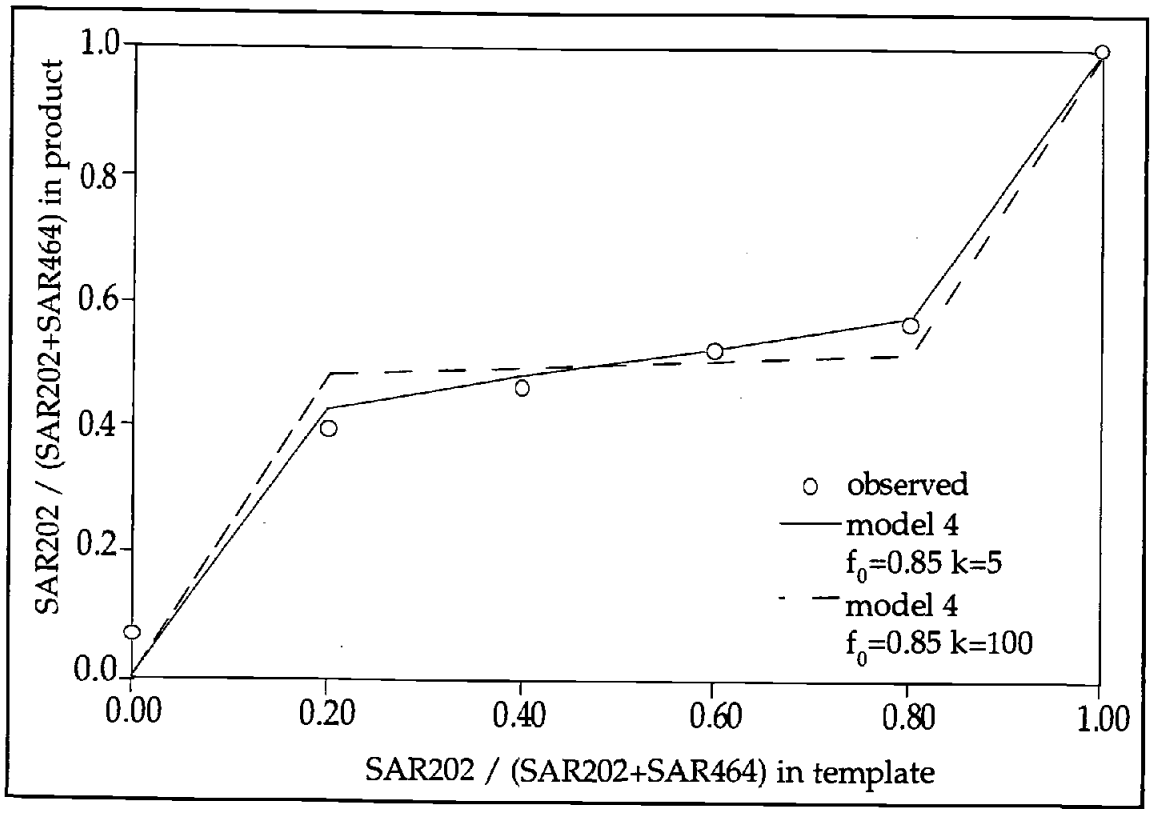

Figure 2.4. Amplification of genes with 27F-FAM/338R from mixtures of clones SAR202/SAR464 and predicted results for model 4 in which re-annealing of templates inhibits the formation of primer template hybrids. (dashed lines k=100 f_0=0.85 cycle^{-1}, solid lines k=5 f_0=0.85 cycle^{-1}).

Kinetic models.

For the 27F-FAM/338R primer pair, the shapes of the curves displaying the observed biases as a function of initial ratios of template molecules were quite unlike the curves predicted by simple models based on G+C bias or primer preference; hence, we developed a kinetic model which took into account the possibility that templates might re-anneal and thereby exclude primers (Ruano et al. 1991, Sardelli 1993). This model matched the observed bias, and also provided the testable hypothesis that the bias should be a function of the number of cycles, which was verified by further experiments.

The simplest model for a PCR reaction assumes that in each cycle of replication all copies of the genes are replicated. In this case, after n replication cycles, the final molarity of the gene, M, will be equal to:

$$M = M_0 \cdot 2^n \qquad (1)$$

where M_0 is the initial concentration of template genes.

However, this model predicts an unrealistic final product concentration. A more realistic model assumes that only a fraction of the template is amplified in each replication cycle. This fraction is defined by the parameter f, the efficiency of replication per cycle:

$$M = M_0 \cdot e^{f \cdot n} \qquad (2)$$

A modification of equation 2 can also be used to predict the outcome of PCR reactions which favor one of the templates, as in the case of G+C bias, or primer preference. In such cases, a unique efficiency can be assigned to

each template, leading to bias increasing the proportion of the product with the higher amplification efficiency. This bias is described as:

$$\frac{M_1}{M_2} = \frac{M_{1_0} \cdot e^{f_1 \cdot n}}{M_{2_0} \cdot e^{f_2 \cdot n}} = \frac{M_{1_0}}{M_{2_0}} \cdot e^{(f_2 - f_1) \cdot n} \qquad (3)$$

where M_1 is the molarity of the first template, M_2 is the molarity of the second template, f_1 is the amplification efficiency of the first template and f_2 is the amplification efficiency of the second template.

The models described by equations 2 and 3 assume that the amplification efficiency, f, remains constant as the cycle number changes. Replication efficiency may actually decrease over successive cycles due to decreasing concentrations of primers and dNTPs or decreasing enzyme activity (Ruano et al. 1991, Sardelli 1993). Decreases in the concentrations of dNTPs or enzyme activity should affect the amplification efficiencies of different templates equally. Thus, equations 2 and 3 probably provide good estimations with respect to these variables.

However, for decreases in primer concentration the approximations provided by equations 2 and 3 may not hold, since as the concentration of products increases, the single strands formed at each denaturation step may re-anneal to their homologous complements during the annealing step, and so inhibit the formation of primer-template complexes (Ruano et al. 1991, Sardelli 1993). From the second order kinetics of such competing reactions, we derived the following equation for the decrease in the amplification efficiency during each replication cycle caused by the re-annealing inhibition effect:

$$f(n) = f_0 \cdot \left(\frac{P(n)}{k \cdot M(n) + P(n)}\right) \quad (4)$$

where $P(n)$ and $M(n)$ are the molarities of primer and templates, respectively, at the start of each replication cycle, f_0 is the theoretical maximum amplification efficiency, and k is the ratio between the rate constants of the re-annealing and priming reactions. In this model we also assume that in a PCR reaction containing mixed templates, each template re-anneals only to its homologous complement and so does not inhibit the priming reaction of the other template.

Calculating the values of f and k.

We performed sensitivity analyses to estimate the values of f_0 and k in equation 4 which resulted in the best predictions of observed final product concentrations and ratios among different PCR products. In these analyses the value of one of the parameters was fixed and a search was made for a best fit value of the second parameter. In the case of experiments with the 27F-FAM/338R primer set, data from experiments in which the number of cycles were varied was also considered.

The calculated values for f, using equation 2, were 0.17 cycle^{-1} for the SAR202/SAR464 template mixture and 0.15 cycle^{-1} for the SAR202/SAR432 template mixture.

The sensitivity analyses, which assumed k=1 for the reactions using SAR202/SAR464 and SAR202/SAR432 template mixtures, resulted in the same values of f_0 (Table 2.2) as calculated by equation 2. A value of k=1 assumes that the hybridization of a primer to a single stranded homolog will

occur at the same rate as the re-annealing of the homologs when primers and single-stranded homologs have the same molarities. Thus, a value of k=1 counteracts the decrease in efficiency, as well as the bias caused by template re-annealing described by equation 4. The low value of f_0 estimated by the sensitivity analysis suggests that the reactions which uses primers 519F/1406R-HEX have an inherently low initial efficiency.

Since the reaction using the SAR202/SAR432 template mixture produced a slight bias with SAR202 as the favored template, to evaluate the extent of such bias a sensitivity analysis was performed assuming k=1 and f_0 =0.15. Under such conditions, the best fit (Figure 2.3) was attained when the efficiency of amplification of SAR202 (f_{202}) was 15% higher than the efficiency of amplification of SAR432 (f_{432}).

The integrated value of f calculated using equation 2 for the SAR202/SAR464 reaction was 0.20 cycle^{-1}. Equation 4 predicted the unusual shape of the bias curve, and also predicted that a decrease in the number of cycles would reduce bias which resulted from template re-annealing, by preventing amplicon concentrations from rising to a critical level. The influence of cycle number on bias is shown in Table 2.3 and Figure 2.5. The integrated amplification efficiency was calculated by Equation 2 from the molarity of products after c cycles. The integrated amplification efficiency during the initial 10 cycles was higher (0.62 cycle^{-1}) than the integrated efficiency for 35 cycles (0.21 cycle^{-1}) (Table 2.3), indicating that the efficiency decreased with time. In fact, the integrated efficiency for the 30th to the 35th cycle was only 0.02 cycle^{-1}. Also, as predicted by model 4, the bias was not constant but increased towards a 1:1 product ratio as the number of cycles increased (Figure 2.5).

Table 2.2. Concentration of combined PCR products measured after 35 amplification cycles or calculated by the models 2 and 4 using the listed parameters as described in the text.

Templates	Primers	Method of determination	Fixed parameter(s)	Calculated parameter	Molarity of combined products
SAR202-SAR464	519F-1406R	observed			$3.79 \cdot 10^{-8}$
		model 2		f=0.17	$3.79 \cdot 10^{-8}$
		model 4	k=1	$f0$=0.17	$3.74 \cdot 10^{-8}$
SAR202-SAR432	519F-1406R	observed			$1.69 \cdot 10^{-8}$
		model 2		f=0.146	$1.69 \cdot 10^{-8}$
		model 4	k=1 $f0$=0.146	$f202$=1.15·$f464$	$2.58 \cdot 10^{-8}$
SAR202-SAR464	27F-338R	observed			$1.42 \cdot 10^{-7}$
		model 2		f=0.20	$1.42 \cdot 10^{-7}$
		model 4	$f0$=0.85	k=5	$4.98 \cdot 10^{-7}$
		model 4	$f0$=0.85	k=100	$1.53 \cdot 10^{-7}$

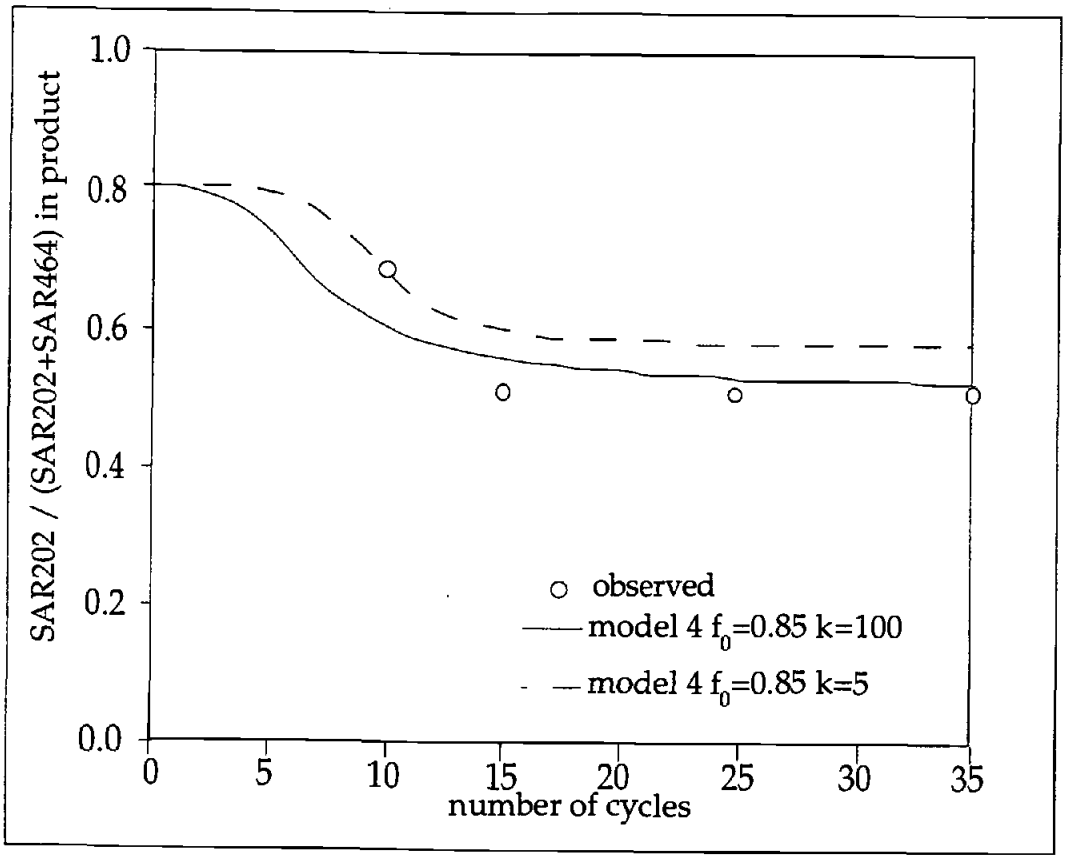

Figure 2.5. The ratio of SAR202 genes to the total PCR products (SAR202+SAR464) as a function of the number of cycles for the 27F-FAM/338R primer pair and prediction by model 4. This bias was a strong function of the number of cycles and converged at a value of 0.5 of each gene.

To find the best fit of model 4 to the observed data we took into consideration the high initial efficiencies; therefore we sought the optimum value of k for a fixed value of f_0, 0.85 cycle^{-1} (Sardelli 1993). This is a tractable procedure which avoids the complexity of fitting both the total product concentration (which decreases as k increases) and the bias (which increases as k decreases). For f_0=0.85 cycle^{-1}, the value of k for which model 4 best predicted total PCR product concentration was 100, which created somewhat more bias than observed (Figure 2.4). If, however, k was set equal to 5, the

model 4 bias prediction was more accurate, but the predicted final product concentration was about two times the observed values.

Table 2.3. Effect of the number of amplification cycles on the concentration of products, amplification efficiency, and ratio among templates.

No. of cycles	Molarity of products	Integrated f (cycle^{-1})[a]	Ratio of SAR 202 templates to SAR 202 + Sar 464 templates
0	$1.03 \cdot 10^{-10}$		0.80
10	$5.75 \cdot 10^{-8}$	0.632	0.68
15	$1.08 \cdot 10^{-7}$	0.125	0.50
25	$1.48 \cdot 10^{-7}$	0.032	0.50
35	$1.90 \cdot 10^{-7}$	0.024	0.50

[a] The integrated amplification efficiency was calculated by equation 2.

The value of k can also be approximated directly from the relative lengths of DNA molecules by the method of Wetzmur and Davidson (Wetzmur & DAvidson 1968). By this approach, we independently calculated a value of 17 for k, which agrees well with experimental observations, and falls between the values of 5, the estimated value for k which most accurately predicted the bias, and 100, the estimated value for k which most accurately predicted the yield of PCR product.

Discusssion

Several hypothetical mechanisms have been described which might contribute to the selective amplification of some templates from mixtures of more than one template, and the resulting misrepresentation of gene abundance in final PCR products. One example is the selective

amplification of low G+C templates. Because high G+C genes dissociate into single-stranded molecules with lower efficiency than low G+C templates, low G+C templates may be over-represented in the population of single-stranded molecules available for hybridization to primers, resulting in a bias in their favor. The introduction of acetamide into polymerase chain reactions has been suggested as one method of reducing the melting point of template hybrids, therefore making it possible for these genes to compete more effectively (Reysenbach et al. 1992).

Figure 2.4 provides an example of a type of bias not predicted by the above models. In these experiments we observed that the final concentrations of genes tended towards a 1:1 mixture independent of the concentrations of genes in the original reaction. It was not possible to explain these results by invoking models in which G+C content of genes or primers created biases in the representation of genes. A kinetic model was developed which explained the results. The central feature of this model is that as the concentrations of product molecules increase, the rate of the bimolecular reaction in which homologous single-stranded template molecules hybridize to each other will increase as a function of the product concentrations. Since single-stranded molecules must react with free primer to initiate extension reactions, the rate of formation of primer-template hybrids will be influenced by the proportion of template molecules in a single-stranded state. This mechanism has been previously mentioned (Ruano et al. 1991, Sardelli 1993), but not in the context of mixed templates. In reactions with mixed templates and high amplification efficiency, the template with the higher initial concentration in the mixture will reach inhibitory concentrations while the second template continues to amplify

efficiently, and thereby the original difference in concentrations decreases until a 1:1 ratio is achieved. The results of quantitative PCR assays using the primer pair 27F-FAM/338R fit the predictions of this model. The reactions had high initial amplification efficiencies, which resulted in a high concentration of products in early cycles of replication. The product concentrations were biased toward a 1:1 ratio. As predicted by the model, and demonstrated by subsequent experiments, this bias was strongly dependent on the number of cycles. As predicted by Model 4 for reactions with low amplification efficiencies, the 519F/1406R- HEX reaction did not show the bias; the molarity of the products of the 519F/1406-HEX amplification after 35 cycles was five times lower than that of the 27F-FAM/338R products.

Two factors might cause lower amplification efficiencies for the 519F/1406R-HEX primer pair. First, the 519F/1406R-HEX fragment is about three times longer than the 27F-FAM/338R fragment. *Taq* DNA polymerase may not amplify a long fragment as efficiently as a short fragment. Second, the 1406R-HEX primer is a 15-mer, which should anneal at 55°C with a lower efficiency than the 20-mer 27F-FAM or the 18-mer 338R.

The estimated best fit values of k for model 4 were of the same order as the values predicted independently by an equation which considered the influence of DNA length on hybridization kinetics. The observation that the reaction of re-annealing of PCR products is more efficient than the priming reaction is consistent with hybridization theory. The rate-limiting step in DNA annealing reactions is the recognition of a short homologous region from which the remaining strains quickly anneal (Wetzmur & Davidson 1968); a longer homologous strand has a higher probability of such

initial hybridization, explaining the higher efficiency of the re-annealing reaction than the priming reaction.

The model described by equation 4 is, to our knowledge, the first model to quantitatively estimate the effect of template re-annealing. Previous studies have attempted to model quantitative PCR (Nedelman et al. 1992, Raeymaekers 1993, Raeymaekers 1995) for the measurement of gene concentration based on the measurement of internal standards. Raeymaekers' model (Raeymaekers 1993, Raeymaekers 1995) is very similar to the model described by equation 4, except that it assigns two different constants (one for the gene of interest and one for the standard), to factor differences in the drop in efficiency for the different templates; also the model does not take in consideration the template re-annealing effect. The models described by Nedelman and colleagues (Nedelman et al. 1992) assume that the amplification efficiencies for the standard and gene of interest are the same, and also do not take into account the template re-annealing effect.

In his discussion on quantitative PCR, Raeymaekers (Raeymaekers 1995) proposes that differences in amplification efficiencies between standards and templates, as well as variations in such differences among different dilutions of the standard, may explain the deviations from linearity observed in some studies applying quantitative PCR. He suggests that differences in the amplification efficiency of sequences containing the same priming sites may be caused by differences in the kinetics of product accumulation in the non exponential phase of PCR (Nedelman et al. 1992). Our model agrees with his prediction, and explains how product accumulation causes amplification efficiencies to differ between standards

and genes of interest containing the same priming sites in the non-exponential phase of PCR. The model also explains why such difference may not be the same for all dilutions of the standard.

Morrison and Gannon (Morrison & Gannon 1994) examined the outcome of a competitive PCR assay in which they investigated the effect of different concentrations of target, holding the concentrations of one template constant, while performing a 10-fold serial dilution of the other template. They thus asked a different question than we did, as the largest initial template ratio in our experiments was 4:1. In their experiments, when one of the templates was several orders of magnitude less concentrated than the other, it did not amplify at all. Their method of assaying the outcome of the competitive PCRs, ethidium bromide staining on agarose gels, is well suited for the detection of presence and absence of product, but not sufficiently quantitative to detect smaller differences in yield. Their conclusion that the ratio of targets will be preserved in the ratio of products, since factors inhibiting PCR act equally on the amplification of both targets, is contradicted by our results using the 27F/338R primer pair as well as by the predictions of equation 4.

The only case in which we observed preferential amplification of one of the templates was the experiment using the pair SAR202/SAR432, amplified with 519F/1406R-HEX (Figure 2.3), with the calculated initial amplification efficiency for the fragment from SAR202 being 15% higher than for SAR432. The mole percent G+C content of SAR202 was higher (58%) than for SAR432 (52%), and the priming sites for both templates present the same bases at the degenerate positions of the primers 519F and 1406R. Thus, neither of these models seemed to explain the data. An alternative explanation for the

preferential amplification of the SAR202 template is differences in secondary structure, affecting either the availability of the priming sites, or the polymerization reaction.

The results presented here have implications for studies using PCR to amplify complete 16S rDNAs from DNA samples from natural ecosystems. If the environmental DNA sample contains highly diverse templates, it seems likely that the PCR produced bias described by model 4 will be small, since it is unlikely that the amplification of any particular 16S rDNA will produce products at a concentration which is high enough to produce the re-annealing inhibition effect. In addition, since the amount of this bias is dependent of the number of cycles, it can be reduced by keeping the number of cycles low. However, the complex chemistry of the polymerase chain reaction, and phenomena which might result in the differential amplification of homologs from complex DNA mixtures will require further study before final conclusions can be reached about the quantitative potential of this method.

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Chapter 3

Estimating Coastal Picoplankton Community Structure by LH-PCR

Marcelino Suzuki, Michael S. Rappé, and Stephen J. Giovannoni

Abstract

Coastal marine bacterioplankton diversity was examined by quantifying natural length heterogeneity of the 5' domain of SSU rRNA genes amplified by the polymerase chain reaction. This new technique, length heterogeneity analysis by PCR (LH-PCR), determines the relative proportions of amplicons originating from different organisms by measuring the fluorescence emission of a labeled primer used for the amplification reaction. Relationships between domain sizes and gene phylogeny were predicted by an analysis of 367 16S rDNAs sequences that included sequences from databases and from cultivated organisms and genes cloned from a water sample from the Oregon coast. We used LH-PCR to estimate the community structure of bacterioplankton in the coastal water sample and compared the results to a 16S rRNA gene clone library prepared from the same sample. LH-PCR was also used to examine the distribution of genes in the PCR products from which the clone library was prepared. The analysis revealed that the relative frequencies of genes in bacterioplankton DNA samples estimated by LH-PCR are highly reproducible, but that bias by PCR caused by the kinetics of re-annealing of product molecules can lead the frequencies of SSU RNA amplicons to misrepresent the gene frequencies of complex mixtures of SSU rRNA genes.

Introduction

Libraries of 16S rRNA gene clones prepared using the polymerase chain reaction are widely used to assess in situ microbial diversity in many ecosystems (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993, Mullins et al. 1995, Bahr et al. 1996, Hiorns et al. 1997). These studies have revealed dramatic evidence that microbial ecosystems are dominated by previously unknown microorganisms. However, quantitative comparisons using clone libraries as proxies for community structure have been limited by several factors, including 1) the undersampling of diversity often measured by coverage estimates or rarefaction curves, and 2) uncertainty about sources of bias in the cloning process, particularly the polymerase chain reaction, for there is no *a priori* knowledge of the frequency of different organisms or SSU rRNA genes *in situ*, since all alternative methods for the quantitative measurement of different types of rDNAs *in situ* are also subject to methodological problems that complicate the analysis of complex communities. We describe here, LH-PCR, a new method to quantify the frequency of SSU rRNA genes in environmental DNA sample which quickly provides an accurate profile of amplicon diversity in complex mixtures of PCR products.

LH-PCR provides information on the relative frequency of rDNA genes. In LH-PCR, a region of the SSU rDNA which exhibits length variations between different phylogenetic groups is amplified from environmental DNA by PCR. Fragments originating from different organisms are discriminated by their length and quantified by the fluorescence emission of a fluorescent phosphoramidite-labeled primer.

In a previous study we investigated biases introduced during the amplification by PCR of ribosomal RNA genes from known gene mixtures (Suzuki & Giovannoni 1996). The templates consisted of pairwise mixtures of small-subunit ribosomal RNA genes (SSU rDNAs) from three different phylogenetic groups of marine bacteria. To measure biases we compared the proportions of genes in the products with their proportions in the starting template mixture. We found that above threshold product concentrations, competition between primers and product strands could dramatically bias the frequency distribution among gene homologs relative to the starting mixture. A kinetic model was developed and accurately predicted the experimental results. The results indicated that some systematic biases in the PCR might lead to increases in the diversity among amplicons relative to genes in the native DNA mixture, and that artifacts stemming from this phenomenon could be controlled by limiting the number of replication cycles to maintain product levels below the threshold values. However, it was uncertain whether this phenomenon might influence the composition of PCR products amplified from environmental DNA samples, since in a sufficiently complicated mixture of genes it might be expected that no single gene could reach the threshold concentrations at which template reannealing begins to have a pronounced effect.

Here we present the results of a study in which LH-PCR was applied to study the community composition of bacterioplankton from a water sample collected at the Oregon coast. In order to trace the origin of the domains amplified using LH-PCR in our study we performed an analysis of the length variation of 367 SSU rDNA genes of bacterial strains cultivated or directly cloned from the same seawater sample, as well as sequences

retrieved from gene sequence databases. We found that the relative gene frequencies obtained by LH-PCR from were highly reproducible and that at high final product concentrations the kinetic bias caused by template re-annealing significantly skewed gene frequencies. Overall the results suggest that LH-PCR is a an effective tool for assessing microbial community structure, and that clone libraries may often over-represent diversity since the relative frequency of dominant genes decreases at the total product concentrations generally used in standard environmental rDNA cloning protocols.

Material and Methods

Water samples.

On April 28, 1993 a subsurface (10 m) water sample was collected by Niskin bottles at a station located 8 km off the mouth of Yaquina Bay, OR (44°39.1' N, 124°10.6' W). The water was pre-screened trough a 10 µm Nitex mesh and transported in autoclaved polyethylene carboys to the laboratory, for the remaining analysis.

LH-PCR.

Picoplankton from a 4 l (subsample 1) and a 16 l (subsample 2) subsamples were collected by filtration onto 0.2 µm polysulfone filters (Supor-200, Gelman Inc., Ann Arbor, Mich.). Total cellular nucleic acids were isolated from the picoplankton sample by lysis with proteinase K and SDS, followed by phenol/chloroform extraction as previously described (Giovannoni et al. 1990). 10 ng of purified genomic DNA from were used as

template for LH-PCR. In a final volume of 100 µl, reactions contained 0.2 mM of pre-mixed dNTPs (Stratagene, La Jolla, Calif.), 1.5 mM $MgCl_2$, 5% acetamide and 2.5 units of *Taq* DNA polymerase (Promega, Madison, Wis.). The forward primer used 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (Giovannoni 1991) was 5'-end-labeled with the phosphoramidite dye 6-FAM and graciously supplied by Applied Biosystems Inc. (Foster City, Calif.), or purchased from Genset (San Diego, Calif.). The reverse primers used were 355R (5'GCT GCC TCC CGT AGG AGT-3') (Amann et al. 1990) for Domain-A, and 536R (5'-GWA TTA CCG CGG CKG CTG-3')(Giovannoni et al. 1988) for Domain-B, synthesized at the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. All reactions used the Ampliwax™ hotstart protocol (Perkin Elmer-Cetus, Norwalk, Conn.) in a PLT100 thermal cycler (MJ Research Inc., Watertown, Mass.) programmed to 16 cycles for primer 355R (except for the one evaluating PCR bias), or 21 cycles for primer 536R of 96°C denaturation, 1 min., 55°C annealing, 1 min., and 72°C extension, 3 min.

The concentration of labeled PCR products was either measured in a Shimadzu UV160U spectrophotometer (Shimadzu Co., Kyoto, Japan) or estimated after electrophoresis in an agarose minigel stained with ethidium bromide (50 µg/ml) and comparison with mass standards. The PCR products were purified using Qiaquick-spin columns (Qiagen, Chatsworth, Calif.). Approximately 10 fmoles of the LH-PCR products were discriminated by Long Ranger (FMC, Rockland, Maine) polyacrilamide gel electrophoresis in an Applied Biosystems Inc. 377 automated DNA sequencer (Applied Biosystems Inc.) using Genescan 2.1® (Applied Biosystems Inc.), a software that estimates sizes of the bands in the gel and

their integrated fluorescence emission. The output of the software are electropherograms in which the bands are represented by peaks and the integrated fluorescence of each band is the area under the peaks. The integrated fluorescence increased linearly with concentration up to 50 fmol of PCR products, indicating that the relative proportion of the integrated fluorescence of each peak corresponded to the proportion of each amplicon in the PCR products, since (data not shown). The relative abundance of each amplicon was estimated as the ratio between the integrated fluorescence of each of the peaks and the total integrated fluorescence of all peaks.

In order to optimize the number of cycles for LH-PCR and avoid the bias described by Suzuki and Giovannoni (Suzuki & Giovannoni 1996) we performed a time course in which LH-PCR reactions of Domain-A, (using DNA purified from subsample 1 and 2 as templates) were stopped by freezing at 10, 12, 14, 16, 18, and 20, and 25 cycles. Concentrations of LH-PCR products from subsample 1 were measured spectrophotometrically using a UV160U spectrophotometer (Shimazu Co. Kyoto, Japan). Concentrations of LH-PCR products from subsample 2 were estimated from the agarose minigel as described, except for the products of the reactions stopped after 10 cycles, which was calculated assuming an amplification efficiency of 85% per cycle (Sardelli 1993).

Clone library and Culture Collection.

The SSU rRNA gene clone library and the isolation and characterization of cellular clones are described elsewhere (Suzuki et al. 1997).

Length heterogeneity analysis of published sequences.

In LH-PCR, amplicons originated from different templates are identified by the length heterogeneity of hypervariable regions of the SSU rDNA. Three of such regions occur in the 5'end of the gene, around positions homologous to *E. coli* positions 90, 190, and 450 (Figure 3.1). In order to verify the phylogenetic coherence of such variable regions, we compared the length heterogeneity of domains homologous to the domain between *E. coli* positions 8 and 355 (Domain-A) and 8 and 536 (Domain-B). The analysis included the sequences of gene clones and cellular isolates sequenced in this study as well as previously published 16S rDNA sequences of bacterial species isolated from seawater or directly cloned from DNA extracted from seawater. The sequences were retrieved from the Genbank, the Ribosomal Database Project (Maidak et al. 1994) and the ARB databases.

Bias by PCR.

To evaluate the introduction of reannealing bias by PCR in the amplification of rDNAs from mixed populations of bacteria, we estimated bacterioplankton community structure by LH-PCR of Domain-A, using genomic DNA (subsample 2) as template and compared to the community structure estimated by LH-PCR of the full length 16S rRNA amplicons used for the clone library. Triplicate LH-PCRs were performed, as above using 10 ng of genomic DNA or 60 pg of full length PCR amplicons, produced as previously described (Suzuki et al. 1997). The concentration PCR amplicons used as templates for LH-PCR was calculated in order to contain approximately the approximately the same number of copies of 16S rDNAs in 10 ng of genomic DNA. In the calculation we assumed that 50% of the

DNA was of bacterial origin, an average chromosome size of 2 Mbp and an average of 2 copies of the Ribosomal operon per chromosome.

Results

Predicted Length Heterogeneity of the 5' regions of 16S rRNAs.

Figure 3.1 is a diagram of the 5' region of bacterial 16S rRNA genes. It shows the positions of variable regions V1, V2 and V3. Insertions and deletions (indels) in these variable regions cause natural variability in the nucleotide lengths of molecules amplified with the 27F/355R primer pair (Domain A, ca. 312 to 363 bp), and the 27F/536R primer pair (Domain B, ca. 472 to 574 bp).

The lengths of domains A and B of bacteria isolated or directly cloned from seawater DNA are shown in Table 3.1. The lengths of domains A and B generally are coherent with phylogenetic relationships. Many discrete fragment lengths are monophyletic, but are shared by multiple species (e.g. 316 bp). Alpha Proteobacteria and Cyanobacteria have the shortest lengths for both domains. Beta, gamma and delta Proteobacteria and *Flexibacter-Bacteroides-Cytophaga* have intermediate lengths, and the longest domains are those from genes of low and high G+C gram positive bacteria and members of the *Vibrio fischeri* subgroup of the gamma Proteobacteria. For most phylogenetic groups there is a unique combination of lengths of domains A and B. (i.e. alpha Proteobacteria with a Domain A length of 315 bp, have Domain B lengths between 470 and 472). The lengths of domains A and B of genes with plastid origins were not included in this study, and are described elsewhere (Rappé et al. Submitted).

Table 3.1. Length of the domains of the 16S rRNA lying between positions homologous to the *E coli* 16S rRNA positions 8 and 355 (Domain A) or 8 and 536 (Domain B). The lengths were calculated for the 16S rRNA of bacteria and plastids isolated from seawater, as well as for 16S rDNAs cloned from community DNA. Published sequences were obtained from the RDP and Genbank and ARB databases. We also included unpublished sequences of genes cloned from environmental DNA from the Oregon Coast (Suzuki et al. 1997; boldface) and the continental shelf off Cape Hatteras (Rappé et al. in press). The letters in brackets correspond to the peaks described in Figure 3.2. Where: alpha, beta, and gamma - alpha, beta, gamma and delta and subdivisions of the Proteobacteria; f - *Flexibacter, Bacteroides* and *Cytophaga* phylum; l - low G+C gram positive phylum; h - high G+C gram positive phylum; x - *Chloroflexus* and *Herpethosiphon* phylum.

accession number	Cellular Strains or Environmental Gene Clones		Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
	env.OCS28	**[A]**	**α**	**312**	**468**
U70681	env.OM55		α	312	468
U75264	env.SAR490		α	312	n.d.
X52170	env SAR12		c	313	n.d.
U75260	env.SAR418		α	313	n.d.
U75262	env.SAR440		α	313	n.d.
U75263	env.SAR466		α	313	n.d.
X52169	env.SAR6		c	313	470
	Synechococcus sp WH8101		c	313	471
	Synechococcus sp. str.WH8103		c	313	472
U78945	**env.OCS122**	**[B]**	**α**	**314**	**470**
U75259	env.SAR414		α	314	n.d.
	env.SAR420		α	314	472
X52171	env.SAR7		c	314	471
U64002	Rhizobium sp. BAL25		α	314	470
U63957	Zoogloea sp. BAL43		α	314	470
L10934	env. FL1		α	315	470
L10935	env.FL11		α	315	471
	env.OCS24	**[C]**	**α**	**315**	**471**
U70678	env.OM25		α	315	471
U75258	env.SAR241		α	315	471
U75254	env.SAR464		α	315	471
X78315	Roseobacter algicola		α	315	471
L15345	strain.LFR		α	315	472
U63935	Caulobacter sp. BAL3		α	316	472
U75252	**env.OCS12**	**[D]**	**α**	**316**	**472**
	env.OCS126	**[D]**	**α**	**316**	**472**
U78942	**env.OCS19**	**[D]**	**α**	**316**	**n.d.**
U78943	**env.OCS84**	**[D]**	**α**	**316**	**n.d.**
U70684	env.OM136		α	316	472

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones		Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
U70585	env.OM143		α	316	471
U70686	env.OM155		α	316	472
U70692	env.OM299		α	316	n.d.
U70679	env.OM38		α	316	472
U70680	env.OM42		α	316	472
U70682	env.OM65		α	316	473
X52280	env.SAR1		α	316	473
X52172	env.Sar11		α	316	473
U75255	env.SAR203		α	316	472
U75256	env.SAR211		α	316	472
U75257	env.SAR220		α	316	472
	env.SAR402		α	316	472
U64003	Erythrobacter sp. BAL26		α	316	472
U64005	Erythrobacter sp. BAL28		α	316	472
U64011	Erythrobacter sp. SCB34		α	316	472
U64025	Erythrobacter sp. SCB48		α	316	472
U63952	Erythromicrobium sp. BAL34		α	316	472
U63958	Flavobacterium sp. BAL44		α	316	n.d.
U63939	Rhizomonas sp. BAL11		α	316	472
U63934	Rhodobacter sp. BAL2		α	316	472
U63949	Rhodobacter sp. BAL27		α	316	472
U63956	Sphingomonas sp. BAL40		α	316	472
U63959	Sphingomonas sp. BAL45		α	316	472
U63960	Sphingomonas sp. BAL46		α	316	472
U63962	Sphingomonas sp. BAL48		α	316	485
U63937	Sphingomonas sp. BAL5		α	316	470
U63998	Sphingomonas sp. SCB21		α	316	472
U78913	**strain.R2A114**	[D]	α	**316**	**n.d.**
U78918	**strain.R2A163**	[D]	α	**316**	**n.d.**
U78919	**strain.R2A166**	[D]	α	**316**	**n.d.**
U78910	**strain.R2A62**	[D]	α	**316**	**n.d.**
U78912	**strain.R2A84**	[D]	α	**316**	**n.d.**
	env.OCS138	[E]	α	**317**	473
	env.OCS154	[E]	α	**317**	473
	env.OCS180	[E]	α	**317**	473
	env.OCS53	[E]	α	**317**	473
U70687	env.OM188		α	317	473
U70689	env.OM242		α	317	n.d.
U75649	env.SAR193		α	317	473
	env.SAR222		α	317	n.d.
	env.SAR239		α	317	n.d
	env.SAR258		α	317	n.d.
U75253	env.SAR407		α	317	473
U14583	strain.307		α	318	474
U64009	strain.BAL32		α	318	474

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones		Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
U78909	**strain.R2A57**	**[F]**	α	**318**	**n.d.**
U64019	strain.SCB42		α	318	472
	env.OCS14	**[J]**	α	**324**	**480**
	env.OCS27	**[J]**	α	**324**	**483**
U70683	env.OM75		α	326	482
U78944	**env.OCS116**	**[M]**	α	**328**	**484**
U70714	env.OM110		γ	328	n.d.
U78917	**strain.R2A153**	**[M]**	α	**328**	**n.d.**
U65915	env.SAR276		δ	329	n.d.
	env.OCS124	**[N]**	α	**330**	**486**
M58793	Microscilla marina		f	330	505
U20797	env.SAR202		x	331	488
U20798	env.SAR307		x	331	488
	env.OCS2		γ	**334**	**n.d.**
U63945	Aeromonas sp. BAL19		γ	336	n.d.
U05570	Methylobacterium pelagicum		γ	338	518
U14585	strain.34-p		α	338	520
U70702	env.OM241		γ	339	520
U70696	env.OM60		γ	339	520
L35470	env.SAR160		γ	339	520
U85887	Flavobacterium sp. A103		f	339	515
U85888	Flavobacterium sp. A265		f	339	515
D32219	strain.K189C		γ	339	n.d.
U64010	Aeromonas sp. SCB33		γ	340	n.d.
X82144	Alteromonas luteoviolacea		γ	340	522
X82147	Alteromonas rubra		γ	340	522
M93352	Deleya aquamarina		γ	340	521
	env.OCS111	**[Q]**	β	**340**	**521**
	env.OCS7	**[Q]**	β	**340**	**521**
L35469	env.SAR156		γ	340	521
U85873	Halomonas variabilis		γ	340	521
U85872	Halomonas variabilis		γ	340	521
U85871	Halomonas variabilis		γ	340	521
L42618	Halomonas venusta		γ	340	521
L35540	Methylobacterium pelagicum		γ	340	521
X72775	Methylomicrobium pelagicum		γ	340	521
RDP	Oceanospirillum kriegii		γ	340	521
U63961	Rhodoferax sp. BAL47		β	340	461
U85854	strain.IC079		γ	340	521
D32220	strain.K189B		γ	340	n.d.
D32221	strain.unid gamma proteobacterium		γ	340	n.d.
U78920	**strain.R2A9**	**[Q]**	γ	**340**	**n.d.**
X82143	Alteromonas espejiana		γ	341	522
X67024	Alteromonas haloplanktis		γ	341	522
L10938	Alteromonas macleodii		γ	341	522

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones		Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
X82140	Alteromonas undina		γ	341	523
	env.OCS43	**[R]**	β	**341**	**n.d.**
L35471	env.SAR166		γ	341	519
	env.SAR470		γ	341	n.d.
	env.SAR471		γ	341	523
U63946	Flavobacterium sp. BAL22		f	341	n.d.
U63938	Flavobacterium sp. BAL9		f	341	n.d.
X87339	Methylophaga thalassica		γ	341	521
X98336	Pseudoalteromonas antarctica		γ	341	522
U85857	Pseudoalteromonas sp. MB6-03		γ	341	522
U85858	Pseudoalteromonas sp. MB8-02		γ	341	522
U64012	Aeromonas sp. SCB35		γ	342	n.d.
U64020	Aeromonas sp. SCB43		γ	342	523
U63953	Alcaligenes sp. BAL37		β	342	n.d.
X82141	Alteromonas piscicida		γ	342	524
U63943	Cytophaga sp. BAL17		f	342	n.d.
U78946	**env.OCS181**		γ	**342**	**523**
	env.OCS44		γ	**342**	**523**
	env.OCS5		γ	**342**	**523**
	env.OCS66		β	**342**	**523**
	env.OCS98		β	**342**	**521**
U70718	env.OM111		γ	342	n.d.
U70698	env.OM133		γ	342	n.d.
U70694	env.OM23		γ	342	523
U70697	env.OM93		γ	342	523
U63954	Flavobacterium sp. BAL38		f	342	n.d.
X82134	Pseudoalteromonas atlantica		γ	342	523
X82136	Pseudoalteromonas carrageenovora		γ	342	523
U85856	Pseudoalteromonas sp. IC006		γ	342	523
U85859	Pseudoalteromonas sp. IC013		γ	342	523
U85860	Pseudoalteromonas sp. MB6-05		γ	342	523
U85861	Pseudoalteromonas sp. SW08		γ	342	523
U85862	Pseudoalteromonas sp. SW29		γ	342	523
U85870	Pseudomonas sp. A177		γ	342	523
U85868	Pseudomonas sp. ACAM213		γ	342	523
U63942	Pseudomonas sp. BAL16		γ	342	n.d.
U63944	Pseudomonas sp. BAL18		γ	342	n.d.
U63947	Pseudomonas sp. BAL23		γ	342	n.d.
U64001	Pseudomonas sp. BAL24		γ	342	29
U85869	Pseudomonas sp. IC038		γ	342	523
U65012	Pseudomonas stutzeri		γ	342	523
U26420	Pseudomonas stutzeri str. ZoBell		γ	342	523
U78922	**strain.R2A30**		γ	**342**	**n.d.**
U63941	Zoogloea sp. BAL15		β	342	n.d.
U85897	Arthrobacter sp. MB6-07		h	343	504
U85896	Arthrobacter sp. MB8-13		h	343	516

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones	Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
U85893	Arthrobacter sp. MB90	h	343	503
U63940	Cytophaga sp. BAL13	f	343	n.d.
U70693	env.OM10	γ	343	523
U65912	env.SAR248	δ	343	499
U65908	env.SAR324	δ	343	499
U85890	Flavobacterium sp. ACAM123	f	343	519
U85889	Flavobacterium sp. IC001	f	343	519
U85891	marine psychrophile IC025	f	343	519
X95640	Methylophaga thalassica	γ	343	524
U78924	**strain.R2A103** [S]	**f**	**343**	**n.d.**
U64027	Alteromonas sp. SCB50	γ	344	525
X82061	Corynebacterium glutamicum	h	344	507
U70706	env.OM156	β	344	525
U70707	env.OM180	β	344	525
	env.SAR267	x	344	502
M63811	env.SAR92	γ	344	525
L35461	env.SAR86	γ	344	525
U85879	Psychrobacter glacincola	γ	344	525
U85878	Psychrobacter glacincola	γ	344	525
U85877	Psychrobacter glacincola	γ	344	525
U85876	Psychrobacter glacincola	γ	344	525
U85875	Psychrobacter sp. IC008	γ	344	525
U85874	Psychrobacter sp. MB6-21	γ	344	525
U78941	**strain.R2A170**	**h**	**344**	**n.d.**
U64026	strain.SCB49	f	344	n.d.
M58794	"Microscilla sericea"	f	345	520
U64021	Antarcticum sp. SCB44	f	345	521
X80629	Corynebacterium glutamicum	h	345	508
X84257	Corynebacterium glutamicum	h	345	508
Z46753	Corynebacterium glutamicum	h	345	508
M62796	Cytophaga lytica	f	345	521
L10948	env.AGG13	f	345	521
L10945	env.AGG41	f	345	520
L10946	env.AGG58	f	345	519
	env.SAR242	x	345	n.d.
M58775	Flectobacilus glomeratus	f	345	521
X67022	Marinobacter hydrocarbonoclasticus	γ	345	527
U63999	Marinobacter sp. SCB22	γ	345	49
X67025	Marinomonas vaga 2	γ	345	526
U14586	strain.301	f	345	521
U85883	strain.IC054	f	345	n.d.
U85885	strain.IC063	f	345	521
U85884	strain.IC066	f	345	n.d.
U78933	**strain.R2A10**	**f**	**345**	**n.d.**
U78935	**strain.R2A132**	**f**	**345**	**n.d.**

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones	Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
U78940	**strain.R2A160**	**h**	**345**	**n.d.**
U78939	**strain.R2A54**	**f**	**345**	**n.d.**
U64015	strain.SCB38	f	345	462
M61002	Vesiculatum antarcticum	f	345	521
X82135	Alteromonas aurantia	γ	346	527
X82137	Alteromonas citrea	γ	346	527
X82137	Alteromonas denitrificans	γ	346	528
U85895	Arthrobacter sp. IC044	h	346	507
U64000	Chromohalobacter sp. SCB23	γ	346	n.d.
U85844	Colwellia sp. IC068	γ	346	527
U85845	Colwellia sp. ICP11	γ	346	527
L42615	Deleya cupida	γ	346	527
M93354	Deleya marina	γ	346	527
L42616	Deleya pacifica	γ	346	527
	env.OCS178	β	**346**	**527**
U70699	env.OM182	γ	346	527
U70704	env.OM43	β	346	527
U70705	env.OM58	β	346	
U70695	env.OM59	γ	346	527
	env.SAR226	x	346	504
	env.SAR250	x	346	503
	env.SAR259	x	346	501
	env.SAR269	x	346	504
U85863	Marinobacter sp. IC022	γ	346	527
U85864	Marinobacter sp. IC032	γ	346	527
RDP	Marinomonas communis	γ	346	527
RDP	Marinomonas vaga	γ	346	527
RDP	Oceanospirillum beijerincki	γ	346	527
U85880	Psychrobacter immobilis	γ	346	527
U78930	**strain R2A148**	γ	**346**	**n.d.**
U85881	strain.IC051	f	346	522
U78931	**strain.R2A173**	γ	**346**	**n.d.**
U78924	**strain.R2A44**	γ	**346**	**n.d.**
U78927	**strain.R2A86**	γ	**346**	**n.d.**
U78928	**strain.R2A88**	γ	**346**	**n.d.**
U64017	strain.SCB40	f	346	489
U64022	strain.SCB45	γ	346	472
M62788	Cyclobacterium marinus	f	347	522
L10944	env.AGG32	f	347	522
U70703	env.OM252	γ	347	528
	env.SAR251	x	347	504
	env.SAR432	h	347	503
U63955	Flavobacterium sp. BAL39	f	347	n.d.
U64023	Flexibacter sp. SCB46	f	347	523
X87755	Kytococcus sedentarius	h	347	508

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones	Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
DEW	Serratia rubidacea	γ	347	527
U85906	Shewanella frigidimarina	γ	347	528
U85882	strain.IC076	f	347	523
Z25522	strain.purple	γ	347	524
U78929	**strain.R2A113**	γ	**347**	**n.d.**
U78932	**strain.R2A5**	**f**	**347**	**n.d.**
U85900	Arthrobacter sp. MB6-20	h	348	509
U63951	Azospirillum sp. BAL31	γ	348	n.d.
U85841	Colwellia sp. IC062	γ	348	529
U85842	Colwellia sp. IC064	γ	348	529
L10950	env.AGG53	γ	348	528
X54745	env.WHB461	γ	348	529
X54744	env.WHB462	γ	348	529
M58784	Flexibacter litorallis	f	348	525
RDP	Oceanospirillum jannaschii	γ	348	529
M22365	Oceanospirillum linum	γ	348	529
U85855	Pseudoalteromonas sp. MB8-11	γ	348	529
U85905	Shewanella frigidimarina	γ	348	529
U85904	Shewanella frigidimarina	γ	348	529
U85903	Shewanella frigidimarina	γ	348	529
U85902	Shewanella frigidimarina	γ	348	529
U85907	Shewanella gelidimarina	γ	348	529
X81623	Shewanella putrefaciens	γ	348	529
U85886	strain.ACAM210	f	348	524
X76334	Vibrio vulnificus	γ	348	529
X74727	Vibrio vulnificus	γ	348	529
X74726	Vibrio vulnificus	γ	348	529
X76333	Vibrio vulnificus	γ	348	529
Z22992	Vibrio vulnificus	γ	348	529
X56582	Vibrio vulnificus	γ	348	529
U64004	Xanthomonas sp. BAL27	γ	348	n.d.
U85846	Colwellia sp. ACAM179	γ	349	530
U85843	Colwellia sp. IC072	γ	349	530
M58770	Cytophaga marinoflava	f	349	525
U70708	env.OM271	f	349	525
U70709	env.OM273	f	349	525
	env.SAR196	n	349	520
L35504	Nitrospina Gracilis	δ	349	508
RDP	Oceanospirillum maris	γ	349	530
U64014	strain.SCB37	f	349	480
X74685	Photobacterium angustum	γ	350	531
D25310	Photobacterium phosphoreum	γ	350	532
X74687	Photobacterium phosphoreum	γ	350	531
Z19107	Photobacterium phosphoreum	γ	350	531
U85908	Shewanella hanedai	γ	350	531

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones		Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
X82133	Shewanella putrefaciens		γ	350	532
U63948	Shewanella sp. BAL25		γ	350	n.d.
U14582	strain.90-P(gv)1		γ	350	531
U85849	strain.IC004		γ	350	531
U85852	strain.IC085		γ	350	531
U78923	**strain.R2A37**	**[U]**	γ	**350**	**n.d.**
U14581	strain.S51-W(gv)1		γ	350	531
RDP	Vibrio marinus		γ	350	531
X82134	Vibrio marinus 4		γ	350	533
U85847	Colwellia sp. IC035		γ	351	532
U85853	strain.IC067		γ	351	532
U64018	strain.SCB41		f	351	n.d.
L35468	env.SAR145		γ	352	532
M96493	Nitrococcus mobilis		β	352	533
X82132	Shewanella hanedai		γ	352	533
	env.OCS155		**h**	**353**	**509**
U41450	env.OCS307		fb	353	535
U70710	env.OM1		h	353	509
U70711	env.OM231		h	353	n.d.
U41450	env.OCS307		fb	353	535
U85851	strain.IC059		γ	353	534
X56756	Vibrio alginolyticus		γ	353	535
U34043	env.SAR406		fb	354	536
D55729	Planococcus okeanokoites		l	354	534
U85899	Planococcus sp. IC024		l	354	534
X56578	Vibrio harveyi		γ	354	536
X56581	Vibrio natriegens		γ	354	536
D25308	Photobacterium histaminum		γ	355	537
D25309	Photobacterium leiognathi		γ	355	537
X62172	Planococcus citreus		l	355	535
U85898	Planococcus sp. MB6-16		l	355	535
D83367	Staphylococcus halodurans		l	355	534
Z26896	Staphylococcus halodurans		l	355	536
X66100	Staphylococcus halodurans		l	355	536
L37600	Staphylococcus halodurans		l	355	538
U78937	**strain.R2A180**	**[V]**	**l**	**355**	**n.d.**
X56575	Vibrio campbellii		g	355	537
	env.SAR272		x	356	514
U14584	Flectobacillus sp. S38-W(gv)1		f	356	537
U78938	**strain.R2A161**		**l**	**356**	**n.d.**
	env.SAR256		x	357	514
X70642	Listonella pelagia		γ	357	n.d.
U85867	Marinobacter sp. IC065		γ	357	n.d.
X74722	Listonella pelagia		γ	358	539
X74686	Photobacterium leiognathi		γ	358	539

Table 3.1 Continued

accession number	Cellular Strains or Environmental Gene Clones	Taxonomic affiliation	size of Domain A (bp)	size of Domain B (bp)
X74691	Vibrio alginolyticus	γ	358	539
X74690	Vibrio alginolyticus	γ	358	539
X74692	Vibrio campbellii	γ	358	539
X74702	Vibrio fischeri	γ	358	539
X70640	Vibrio fischeri	γ	358	538
X74706	Vibrio harveyi	γ	358	539
X74710	Vibrio mediterranei	γ	358	539
X74714	Vibrio natriegens	γ	358	539
X74716	Vibrio nereis	γ	358	539
X74717	Vibrio nigripulchritudo	γ	358	539
X74719	Vibrio orientalis	γ	358	539
U64016	Vibrio sp. SCB39	γ	358	524
U64024	Vibrio sp. SCB47	γ	358	523
Z31657	Vibrio splendidus	γ	358	538
X74724	Vibrio splendidus	γ	358	539
U64013	Flexibacter sp. SCB36	f	360	527
U64006	Vibrio sp. BAL29	γ	360	n.d.
U64007	Vibrio sp. BAL30	γ	360	526
X62171	Marinococcus halophilus	1	363	545
X90835	Marinococcus halophilus	1	374	556
U85901	Halobacillus sp. MB6-08	1	392	574

Figure 3.1. Secondary structure of the 5'-end of the *E coli* 16S rRNA, showing the primer hybridization sites and regions in which large insertions and deletions are common (boxed).

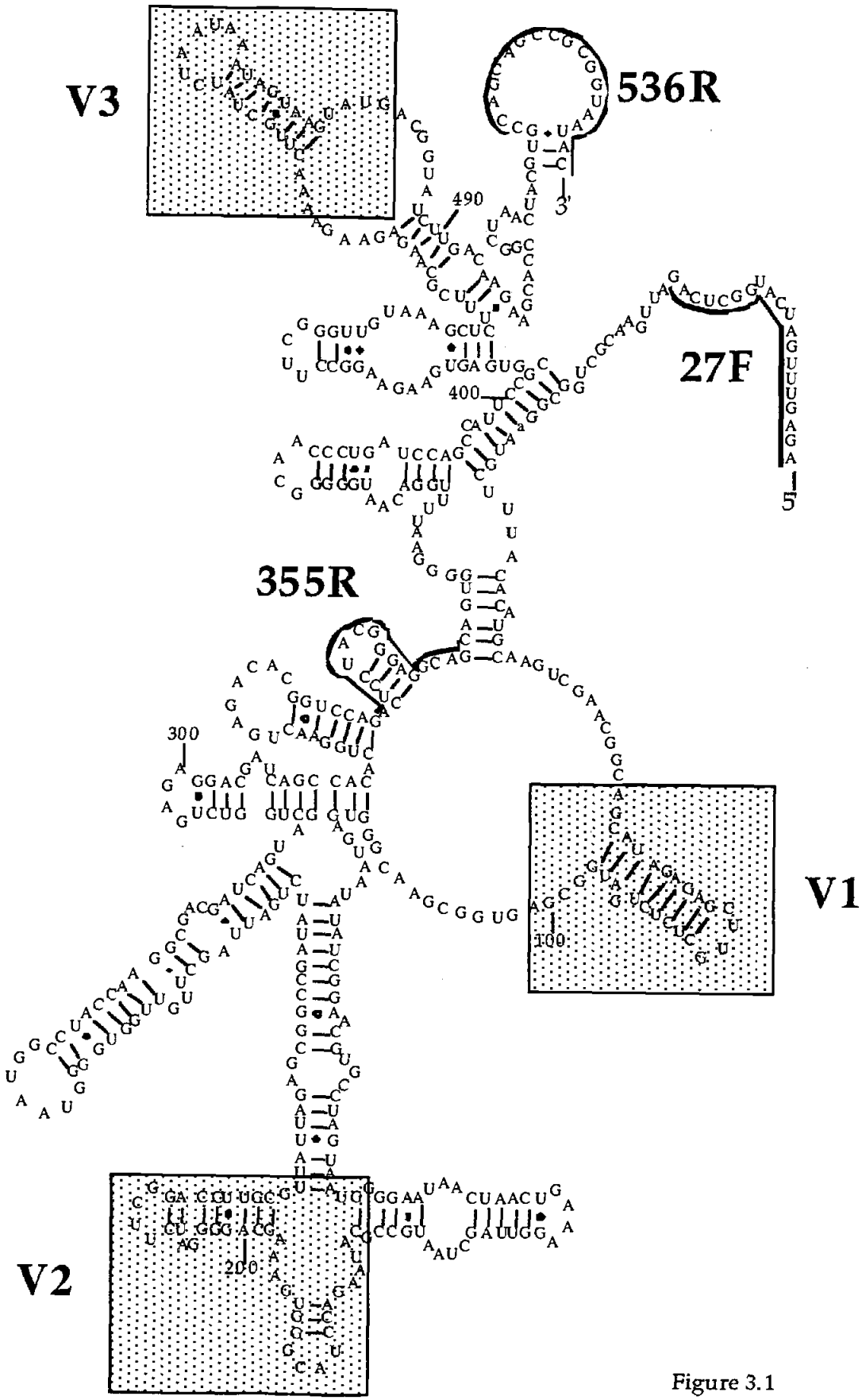

Figure 3.1

Analyses of Coastal Bacterioplankton Diversity.

An example of an electropherogram is shown in Figure 3.2. It shows length heterogeneity for Domain A for PCR products obtained directly from DNA extracted from the seawater sample. The 23 peaks are labeled A through W. The putative identities of these peaks, as determined by reference to a clone library prepared from the same seawater sample, are indicated in Table 1.

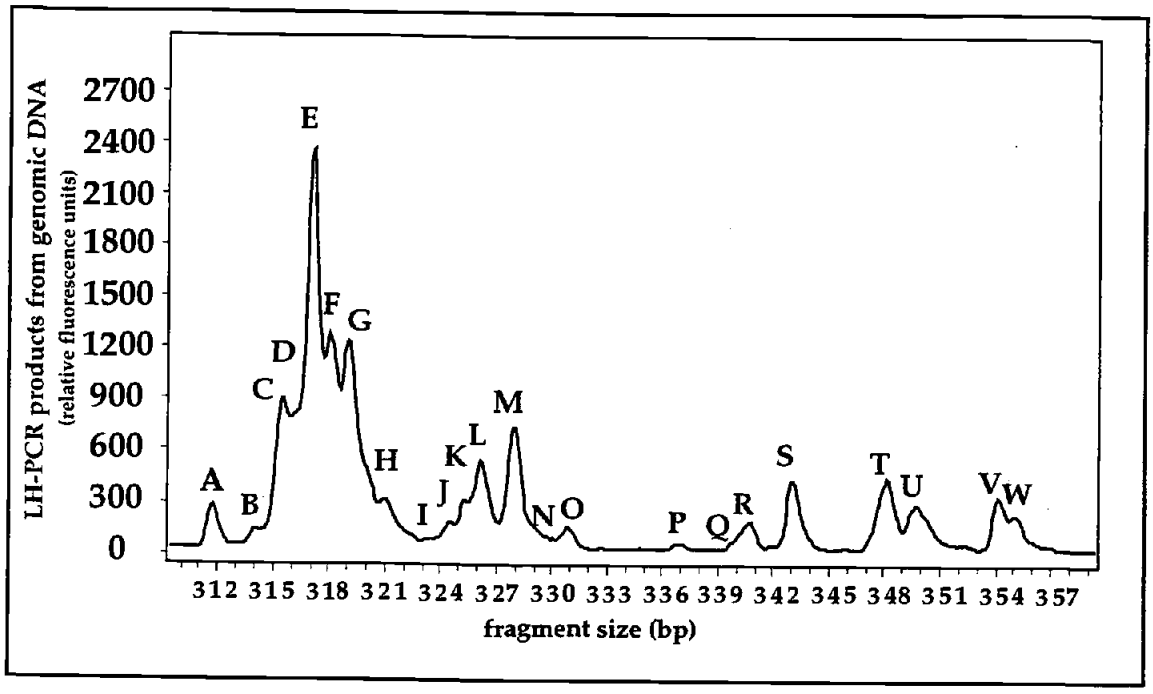

Figure 3.2 Electropherograms of Domain-A amplified by PCR from genomic DNA from subsample 2. The letters A-W correspond to the peaks detected by the Genescan 2.1.® software in at least one of triplicate reactions. The *x*-axis represents the size of domains in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). The *y*--axis represents relative fluorescence units.

We were able to assign an OCS gene clone to most peaks (Table 1). The Domain A peaks of sizes 317 bp (E), 318 bp (F) 319 bp (G) and 321 bp (H) correspond to the sizes of SSU rDNAs of plastid origin (Rappé et al. submitted). The Domain-A peak of 317 bp corresponded to sizes of both alpha Proteobacteria and plastids. There is strong evidence that the 317 bp Domain-A peak corresponds mainly to alpha Proteobacteria since the ratio between integrated fluorescence of peak E and all *bacterial* peaks is approximately the same for samples whether or not they were filtered through 0.8 μm polycarbonate membranes (Rappé et al. submitted)

Figure 3.3 presents electropherogram data in a histogram format. The data from Figure 3.2 are shown in panel A, with error bars shown to represent the standard deviation for triplicate PCR reactions from the same DNA sample. Here and in other measurements, we found the method to be highly reproducible. For this analysis the accumulation of PCR products was limited to less than 1.5 nM by controlling the number of replication cycles.

Panel B of Figure 3.3 shows the results of a Domain-A LH-PCR analysis of the full-length PCR products that were used to prepare the clone library. The template for these LH-PCR reactions was the amplicons prepared with the 27F/1542R primers from the DNA used for Panel A. There is a significant difference between the profiles in panels A and B. The comparison between panels A and B shows that the relative contributions of some peaks (A, F, G, K-L, N, P and Q) increased in the PCR amplicons, while other peaks (M, S, T and U-V) decreased. We noted that the relative proportion of peak F (319 bp) is higher than peaks D-E (317-318 bp) in the PCR amplicons, although both are major peaks that contribute significantly to the total population of molecules.

It was evident that replicated amplifications provided reproducible results; therefore, the difference between panel A and panel B can be attributed to the PCR reaction used to prepare amplicons for clone library construction. For the LH-PCR measurements, products never exceeded 1.5 nM; however, for the polymerase chain reaction used to prepare amplicons for cloning, 35 cycles of amplification were used and final product concentrations were above 10 nM. Therefore, we reason that some of the differences between panels A and B might be attributed to bias caused by the kinetic (template reannealing) effect.

Panel C of Figure 3.3 shows the distribution of clones from the gene clone library, displayed by length within the Domain-A region for comparisons to panels A and B. It is evident that the profiles in panels B and C are very different. The cause of this difference is uncertain, but probably results from one or more of three sources of error. Random error resulting from the sample size (number) of clones retrieved and identified in the library imposed limitations on the expected correspondence between LH-PCR results and clone library gene frequencies. Secondly, systematic biases in either the LH-PCR, or a step of the process of cloning genes, could explain the observations.

Figure 3.3. Comparison betwen LH-PCR and the clone library. Percentage of integrated fluorescence of each individual Domain-A produced by PCR from (A) genomic DNA (subsample 2) or (B) amplicons used to construct the clone library. The X-axis represents the size of domains in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). (C) Relative abundance of clones recovered in the OCS clone library classified according to the length of Domain-A.

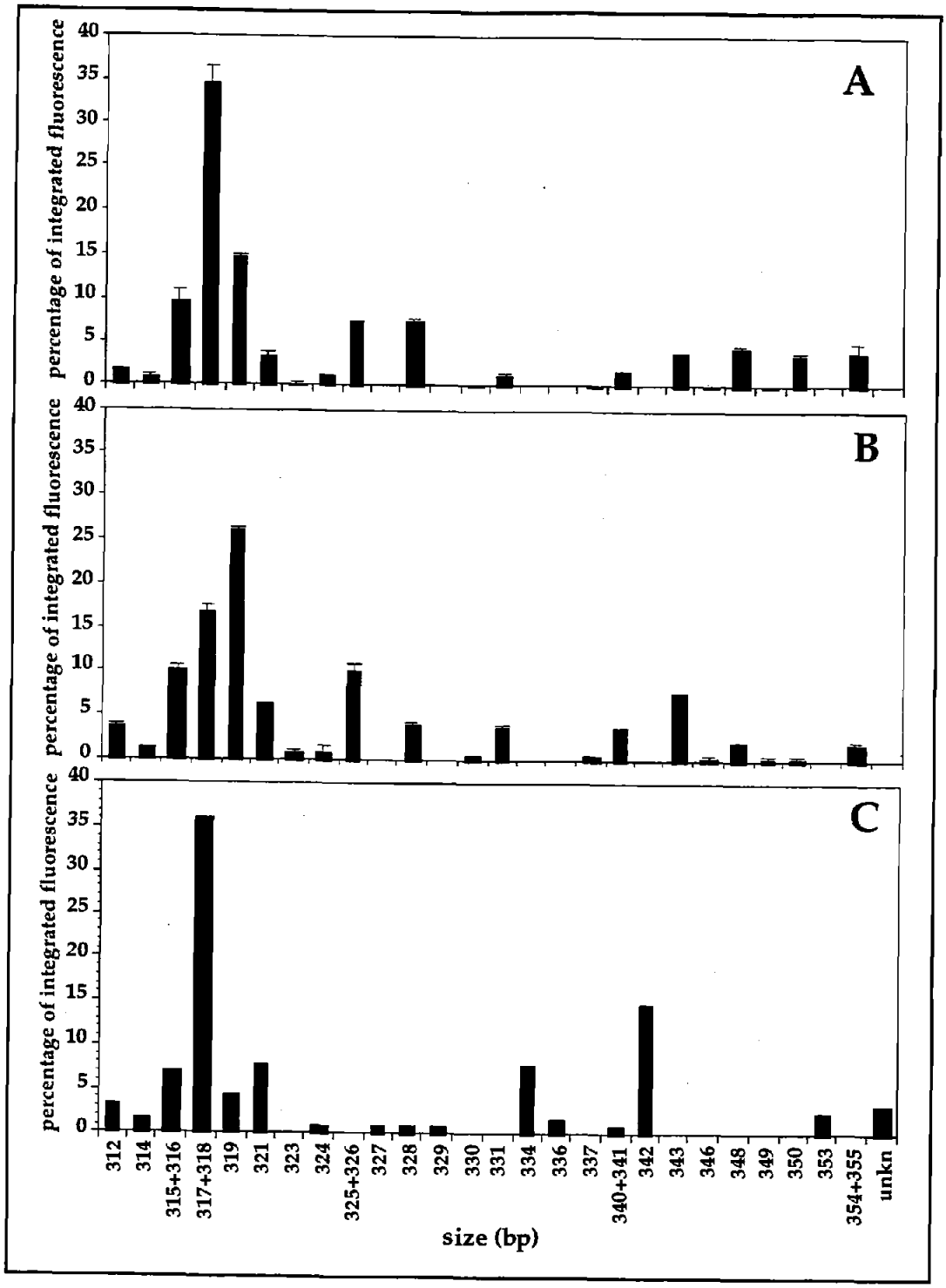
A
B
C
percentage of integrated fluorescence
0
5
10
15
20
25
30
35
40
312
314
315+316
317+318
319
321
323
324
325+326
327
328
329
330
331
334
336
337
340+341
342
343
346
348
349
350
353
354+355
unkn
size (bp)

Figure 3.3

Kinetic bias in gene frequencies.

The possibility of a kinetic bias caused by template reannealing was investigated by examining the relationship between the final concentration of products obtained and the relative frequency of dominant genes in the population. An example is provided in Figure 3.4, which shows the relative frequency of the 317 bp Domain-A (alpha Proteobacteria and Prymnesiophyte plastids) as a function of the total product molarity. The prediction for the kinetic bias effect is that the percentage of integrated fluorescence of the dominant peak should decrease, assuming that the dominant peaks are composed primarily of genes of one or a few types. The final concentrations of products for the reactions used for Figure 3.4. varied for two samples of DNA isolated independently from the same water sample (subsamples 1 and 2), and according to the number of cycles used for the amplification (12 to 25 cycles for subsample 1; 12 to 18 cycles for subsample 2). The ratio of the 317 bp fragment decreased with increasing product molarity. The results agreed with predictions for the kinetic bias effect.

LH-PCR.

In general there was good agreement between the community structure estimated by LH-PCR for domains A and B (data not shown). The main difference between LH-PCR for domains A and B was the resolution of different peaks by the Genescan 2.1 ® software. Genescan resolved more peaks in the analysis of Domain-A, and tended to merge Domain B peaks,

especially peaks for larger fragments larger than 520 bp. Some adjacent peaks of Domain-A were also merged in some of the electropherograms (peaks C and D, E and F, J and K, and S and T).

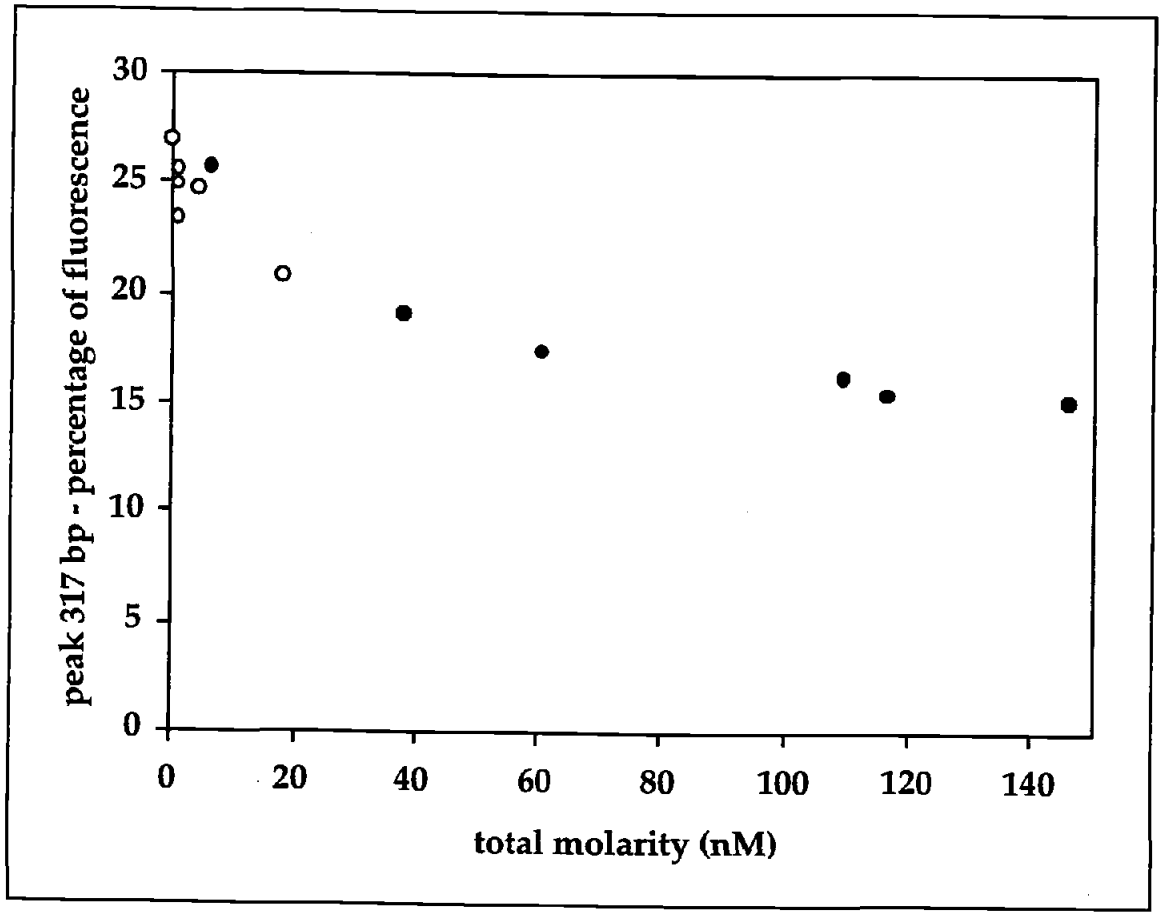

Figure 3.4. An example of PCR bias that fits the kinetic model. PCR amplicons were obtained from natural community DNA. The molar ratio of the dominant fragment (317 bp) to total products is shown plotted as a function of the final product concentration obtained. Primer set A was used for the amplification from plankton DNA subsamples 1 and 2.

The relative proportions of LH-PCR-A and LH-PCR-B confirm previous observations that alpha Proteobacteria dominate clone libraries in surface samples. Peaks A-E and K-M represents about 65% of the total fluorescence,

and is represented by sequences of alpha Proteobacteria (Table 3.1), and plastids (Rappé at al., submitted). Peaks E, F, G, H, J, K, P and V have sizes corresponding to plastid sequences (Rappé at al., submitted) Most remaining peaks are not phylogenetic coherent. Peaks P and Q, which represented about 7 % of the total fluorescence, correspond to the sizes of beta and gamma and delta Proteobacteria, *Flexibacter-Bacteroides-Cytophaga*, and high G+C gram positive bacteria, many of which are cultivated strains. Peaks S represent about 5 % of the total fluorescence corresponds to the sizes of previously cultivated gamma Proteobacteria finally, Peaks T-V represents about 9 % of the total fluorescence and corresponds to the sizes of several phylogenetic groups.

Discussion

LH-PCR is a promising method for the analysis of the diversity of *in situ* microbial populations. The method avoids several of the steps needed in the analysis of bacterioplankton SSU rRNA or SSU rDNA by cloning or hybridization techniques. LH-PCR provides a quick assessment of the diversity of bacterioplankton communities, and is very reproducible. The observations that Fragment-A peaks with sizes corresponding to those of plastid rDNAs disappear after filtration of the water sample through 0.8 μm and that there are significant differences between SSU rDNAs in genomic DNA and in full length PCR products support the applicability of the technique. Furthermore, due to its relative simplicity, the method can be applied to manipulation experiments with natural bacterioplankton communities or cultivated organisms (Suzuki, this thesis, chapter 4).

The main problems to the application of LH-PCR for *in situ* bacterioplankton communities are associated to the inaccuracy of peak detection, especially when longer domains are used, although, improvements on the automated sequencers and in the Genescan 2.1® software might increase the applicability of the method for longer domains in the future. Currently the use of LH-PCR for the Domain-B is useful to confirm the results obtained with the Domain-A and to differentiate between phylogenetic groups with overlapping Domain-A sizes. Finally, biases by PCR, are associated with the method, although good agreement between replicates indicates that such biases do not occur randomly, but may be rather systematic. Reannealing bias apparently occurred when high final product concentrations were achieved, and therefore we recommend reactions to be stopped at a minimal concentration level to allow detection.

The relative proportions of the different domains, agrees with previous observations that alpha Proteobacteria are major components of environmental clone libraries constructed from DNA extracted from surface seawater (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993, Mullins et al. 1995, Rappé et al. in press). Also, the observation that most SSU rDNAs of organisms cloned or cultivated from the same water sample have sizes which correspond to the peaks in the LH-PCR electropherograms supports to the applicability of the method. However, the comparison between the relative frequency of different sizes of Domain-A estimated by LH-PCR or the SSU clone library is inconclusive. Although the frequencies of Domain-A sizes of the clones in the SSU rDNA library is similar to that of genomic DNA estimated by LH-PCR, the genes with a 317-318 bp Domain-A in the clone library are mainly those of

Prymnesiophyte plastids, while we have strong evidence that the 317-318 bp Domain-A (Peak E) in environmental DNA should correspond to those of alpha Proteobacteria since the ratio between integrated fluorescence of Peak E and all *bacterial* peaks is approximately the same for samples whether or not they were filtered through 0.8 μm polycarbonate membranes (Rappé et al. submitted). Such discrepancy can be reasoned by the relatively low coverage by the clone library or the unlike possibility that the Prymnesiophyte plastid SSU rRNAs were abundant in the subsample filtered through a 0.8 μm polycarbonate membranes. Finally, biases associated with the construction of the clone library could also contribute to this discrepancy.

The suggestion that PCR reannealing bias occurred during the amplification of rDNA from mixed populations of bacteria (Figure 3.3a and b), contradicts our previous expectation that reannealing biases should not be important in the amplification of DNA from mixed bacterial populations (Suzuki & Giovannoni 1996). Furthermore, the shift in the dominance from the peaks 317 to the peak of 319 bp also contradicts our previous expectation that reannealing bias would lead amplicons originating from different templates to reach similar concentrations. A possible explanation for this discrepancy is the fact each of the LH-PCR peaks represent SSU rDNAs of several different organisms. Cross-reannealing between the domains originating from different organisms could explain the shift in the dominance of the peaks. If the degree of similarity between the sequences with Domain-A of 317 bp were high enough for PCR amplicon cross hybridization and kinetic inhibition while the degree of similarity between the sequences with Domain-A of 319 bp were lower and not lead to cross

hybridization, one could envision that each of 319 bp amplicons would experience lower levels of kinetic inhibition. The average identity between four 317 bp Domain-A types was 0.93 (.89-1.00), while the degree of identity of the two 319 bp Domain-A types was 0.85, supporting our hypothesis. Another explanation to explain the peak shift would be the diversity index for the organisms corresponding to each of the peaks, as template reannealing inhibition should be lower for peaks with more gene types, or without a dominant gene type. We do not have a way to directly test this hypothesis based in our data.

Acknowledgments

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Chapter 4

The Effect of Protistan Bacterivory on Bacterioplankton Community Structure

Marcelino T. Suzuki

Abstract

Four protist exclusion experiments were conducted to examine the hypothesis that marine bacterivorous protists selectively feed on different bacterioplankton genotypes and may affect the taxonomic diversity of coastal marine bacterioplankton communities. In these experiments the changes in the genotypic composition of the bacterial community of water samples with protists removed by filtration was followed and compared to the changes in community structure of untreated control water samples. Bacterioplankton community structure was inferred from the relative abundance of bacterial Small Subunit rRNA genes (SSU rDNAs) by a recently developed technique (Length Heterogeneity analysis by PCR (LH-PCR); Chapter 3 of this thesis). The results of the experiments showed that the bacterioplankton community structure did not change dramatically up to a 24 hour incubation period in any of the treatments. However there were significant differences in filtered water samples and controls between 24 and 48 hours of incubation. In the absence of bacterivores some SSU rDNAs that were insignificant in the original water samples dominated the bacterioplankton SSU rDNA pool after 48 hours of incubation. Protists appeared to be capable of controlling bacterioplankton taxonomic diversity under manipulated conditions, and the results further agree with the hypothesis that aquatic bacterioplankton communities are dominated by relatively inactive cells that are less susceptible to grazing by bacterivorous protist.

Introduction

The relative significance of different mechanisms controlling bacterioplankton populations in aquatic systems is a subject of debate in microbial ecology. Several studies have attempted to estimate the relative importance of bottom-up versus top down controls of bacterioplankton biomass, but the question remains unanswered (Shiah & Ducklow 1995, Dufour & Torréton 1996). Nonetheless, in the past decade, both theoretical and empirical evidence demonstrated that bacterivorous protists can select their prey based on size (Chrzanowski & Simek 1990, Gonzalez et al. 1990, Monger & Landry 1992) or quality (Mitchell et al. 1988, Landry et al. 1991, Gonzalez et al. 1993) and could potentially control the size distribution of aquatic bacterioplankton. Sherr and co-workers have shown that bacterivorous protists selectively graze on dividing cells, and suggested that protists grazing should represent a larger impact on the bacterioplankton production than on bacterioplankton standing stock (Sherr et al. 1992). Furthermore, recent studies showed that different size classes of freshwater bacterioplankton are differentially susceptible to bacterivory (Pernthaler et al. 1996) and that protist grazing rates are higher on active than on inactive bacterioplankton cells (del Giorgio et al. 1996). The results of these studies support the hypothesis that a large fraction of aquatic bacterioplankton is composed of small cells that escape predation, which are in a state of low physiological turnover (Kjelleberg et al. 1987).

Assuming that active and inactive bacterioplankton are represented by different taxonomic groups, the same hypothesis suggests that bacterivorous protists could affect bacterioplankton genotypic community structure. Two

recent studies (Pernthaler et al. 1997, Simek et al. 1997) have examined the effect of a bacterivorous protist strain on the genotypic community composition of a mixed assemblage of freshwater bacterioplankton growing in enriched continuous culture conditions. Both studies used oligonucleotide hybridization to bacterial SSU rRNA to assess community diversity and showed that the community shifts under grazing pressure. In these experiments the bacterial biomass became dominated by elongated, grazing-resistant cells belonging to the beta subdivision of the Proteobacteria. Neither study showed evidence of dominance of the bacterioplankton by small, slow growing bacteria in communities subject to bacterivory.

Here I report evidence that bacterivorous protists preferentially feed on faster growing bacterioplankton genotypes in natural seawater samples incubated for 48 hours, and thus have the potential to affect the taxonomic diversity of natural bacterioplankton communities. To evaluate the effect of selective bacterivory by protists on marine bacterioplankton phylogenetic community structure, I followed the changes in the bacterioplankton community structure inferred using Length Heterogeneity analysis by PCR (LH-PCR), a recently developed PCR-based method (Suzuki et al., Chapter 3 of this thesis) in four protist exclusion experiments. In these experiments the changes in bacterioplankton community structure of coastal water that had protists removed by gentle filtration was compared to the changes in community structure in untreated controls which contained the natural assemblage of baterivorous protists.

Material and methods

Four protist exclusion experiment were performed using four different water samples from the Oregon coast. In these experiments the bacteria were separated from bacterivorous protists by size fractionation. The community structure estimated by LH-PCR was followed with time and compared with that of an unfiltered control. Cell counts were performed for all samples, except when otherwise noted. The experiments are referred to in the remaining of the text as PROTEX 1-4.

Water samples.

Water samples were collected at different times and locations off the Oregon coast. The water samples used for PROTEX 1 (March 28, 1993) and PROTEX 4 (July 24, 1997) were collected at subsurface (10 m) by Niskin (General Oceanics, Miami, Fla.) bottles (PROTEX 1) or from the surface using a bucket (PROTEX 4), aboard the RV Sacajawea at a station located 8 km off the mouth of Yaquina Bay, Oregon (44°39.1' N, 124°10.6' W). The water sample used for PROTEX 2 (October 3, 1993) was collected at subsurface (10 m) by Niskin bottles (General Oceanics) aboard the RV Wecoma at a station located off the mouth of the Columbia River, Oregon (47°17.06' N, 124°44.8' W). The water sample used for PROTEX 3 (March 25, 1997) was collected from the surface with a bucket at the South Jetty of the Yaquina Bay, Oregon (44°36.8' N, 124°10.6' W).

Protist Exclusion Experiment 1.

The water sample was pre screened through a 10 µm mesh. Two 4 liter subsamples were filtered through a 0.8 µm polycarbonate membrane (Poretics, Osmonics, Minnetonka, Minn.) and used to fill two 4 liter polycarbonate bottles. The controls consisted of two 4 liter polycarbonate bottles filled with unfiltered water. At time zero and 24 after the start of incubation in the dark at 15°C, the water from one of the treatment bottles and one of the control bottles were filtered through 0.2 µm polysulfone filters (Supor-200, Gelman Inc., Ann Arbor, Mich.). The filters were immersed in sucrose lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris·HCl pH 9.0) and stored at -80°C for later analysis.

Protist Exclusion Experiment 2.

Nine 4 liter subsamples were filtered through a 2.0 µm polycarbonate membrane and used to fill nine 4 liter polycarbonate bottles. Nine 4 liter polycarbonate bottles were filled with unfiltered water and served as controls. The water from three of the treatment bottles and three of the control bottles was filtered through 0.2 µm polysulfone filters (Supor-200, Gelman Inc.) at time zero and after 12 and 24 h incubation at surface temperature in a on deck Plexiglass incubator. The filters were immersed in sucrose lysis buffer and stored at -80°C.

Protist Exclusion Experiment 3 and 4.

Seven 500 ml subsamples were filtered once through GFF glass fiber filters (Whatman, Kent, England) and twice through 0.8 µm polycarbonate membranes. To avoid the effects caused by separation of bacteria from bacterivorous protists and eukaryotic phytoplankton on substrate availability and nutrient feedback to the bacterioplankton, the samples were incubated in 500 ml polysulfone filter holders with receivers (Nalgene, Rochester, NY., item 300-4050). The unfiltered controls were poured into the bottom collection flask, the filtered subsamples were gently poured in the top filtration tower closed with a lid and the subsamples were separated by a 0.2 µm polycarbonate membrane that allowed inorganic nutrients and organic substrates to exchange. at time zero and after 16, 24, 30, 37, 42 and 48 h (PROTEX 3) or 14, 24, 30, 34, 42 and 48h (PROTEX 4) of incubation in the dark at 15°C under gentle shaking (the filtration flasks were lying in their sides), the water from the treatment and control were filtered through 0.2 µm polysulfone filters (Supor-200) and the filters stored as above.

Cell Counts.

Samples of 20 ml were collected from all treatment and control subsamples, preserved, stained, and filtered onto 0.2 or 0.8 µm polycarbonate filters (Poretics, Osmonics) for enumeration of bacteria and of heterotrophic flagellates via DAPI staining (Sherr et al. 1993, Turley 1993). Bacterivorous protists were not enumerated in the controls of PROTEX 1.

LH-PCR.

Total cellular nucleic acids were extracted from the polysulfone filters by lysis with proteinase K and SDS, followed by phenol/chloroform extraction as previously described (Giovannoni et al. 1990) 10 ng of purified genomic DNA were used as the template for LH-PCR. Details of the LH-PCR protocol are described elsewhere Suzuki et al. Submitted. Briefly, in a final volume of 100 µl, reactions contained 0.2 mM of pre-mixed dNTPs (Stratagene, La Jolla, Calif.), 1.5 mM $MgCl_2$, 5% acetamide and 2.5 units of *Taq* DNA polymerase (Promega, Madison, Wis.). The forward primer used, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (Giovannoni 1991) 5'-end-labeled with the phosphoramidite dye 6-FAM was purchased from Genset (San Diego, Calif.) The reverse primer used was 338R (5'-GCT GCC TCC CGT AGG AGT-3') (Amann et al. 1990), synthesized at the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. All reactions used the Ampliwax™ hotstart protocol (Perkin Elmer-Cetus, Norwalk, Conn.) in a PLT100 thermal cycler (MJ Research Inc., Watertown, Mass.) programmed to 16 cycles of 96°C denaturation, 1 min., 55°C annealing, 1 min., and 72°C extension, 3 min.

The concentration of labeled PCR products was estimated after electrophoresis in an agarose minigel stained with ethidium bromide (50 µg/ml) and comparison with mass standards. The PCR products were purified using Qiaquick-spin columns (Qiagen, Chatsworth, Calif.). Approximately 10 fmoles of the LH-PCR products were discriminated by Long Ranger (FMC, Rockland, Maine) polyacrylamide gel electrophoresis in an Applied Biosystems Inc. 377 automated DNA sequencer (Applied

Biosystems Inc., Foster City, Calif.) using Genescan® (Applied Biosystems Inc.). The output of the software are electropherograms in which the bands are represented by peaks and the integrated fluorescence of each band is the area under the peaks (Figure 4.3). The relative abundance of each amplicon was estimated as the ratio between the integrated fluorescence of each of the peaks and the total integrated fluorescence of all peaks.

Results

Cell Counts.

In all experiments the total numbers of bacteria in the filtered water and the controls increased with time (Table 4.1, Figure 4.1), and except for PROTEX 6 (Figure 4.1b), there was no decline in the control populations. In all experiments in which the water was filtered through 0.8 μm the initial numbers of bacteria in the treatment subsamples were lower than in the controls, and had a longer lag phase. Net growth rates, calculated from the increase in total cell numbers and assuming exponential growth were, in general, lower in the controls (data not shown), suggesting that the protists were grazing on the bacterial population.

Bacterivorous protist numbers increased with time after a lag phase in the controls of PROTEX 5 and 6 (Figure 4.1) and did not grow in the 0.8 μm filtered samples. In PROTEX 6 bacterivores apparently controlled the increase in bacterial numbers since the increase in bacterivores was followed by a decrease in the total numbers of bacteria. Bacterivores did not show increasing or decreasing trends in the controls of PROTEX 2 (Table 4.1), but

were present and increased in numbers in the 2.0 μm filtered samples, with final numbers being about 40 % of the numbers in the controls (Table 4.1).

LH-PCR.

Analysis of replicated bottles for PROTEX 2 confirmed that the community structure inferred by LH-PCR was very consistent between bottles, with a coefficient of variation, in general, lower than 10% (Figure 4.2, Table 4.2). Furthermore, the consistency of the patterns between samples collected in the time series of PROTEX 3 and 4 strongly suggest that the patterns are a result of changes in the community structure inferred by LH-PCR, rather than random experimental error. As in the original description of the method, LH-PCR was not able to consistently discriminate the peaks of adjacent fragments, and therefore, the fluorescence of some adjacent fragments were combined (i.e. fragments of 348 and 349 bp). Although this represents a complicating factor in the analysis of the results, in general, peaks of fragments with similar sizes represent the same phylogenetic groups (Suzuki et al. Submitted).

Except for PROTEX 4, the initial community structure was very similar for all the samples (Table 4.4, Figures 4.2a and 4.3a). The fragments with sizes between 315 and 317 base pairs (Peak 1) were the most abundant fragments in the original samples representing up to 50% of the fluorescence of all amplified fragments. The database analysis of the size of the region between primers 27F and 338R for 366 sequences of SSU rDNAs directly cloned from DNA samples from seawater or belonging to bacteria isolated from seawater shows that these fragment sizes correspond exclusively (except for the case of Prymnesiophyte plastids) to the SSU rDNAs sequences

of the alpha subdivision of the Proteobacteria. Also, most of these sequences in the databases represent SSU rDNAs directly cloned from environmental DNA samples (Suzuki et al. Submitted.)

The observation that the relative abundance of the peaks sized between 315 and 317 bp does not significantly decrease after filtration through 0.8 μm is a good evidence that these fragments are of bacterial origin. The 348 and 349 bp fragments (Peak 3) are the second most abundant peak representing up to 20% of the fluorescence of all amplified fragments. The size analysis of SSU rDNAs showed that this fragment size is shared by different phylogenetic groups, including the gamma and delta subdivisions of the Proteobacteria, and the *Flexibacter-Bacteroides-Cytophaga* phylum. Most of the sequences correspond to those of cultivated organisms (i.e. *Flexibacter littoralis*, *Oceanospirillum* spp., *Shewanella* spp.) (Suzuki et al. Submitted).

The filtration through 0.8 or 2.0 μm does not dramatically change the community structure of the bacterioplankton community inferred by LH-PCR (Table 4.4, Figures 4.2a-4.2b and Figures 4.3a-4.3b), indicating either that most bacterioplankton cells are smaller than 0.8 μm and/or that a similar fraction of organisms with SSU rRNA contributing to different peaks is smaller than 0.8 μm.

Figure 4.1. Time series of bacteria and bacterivorous protists in experimental (filtered) and control (unfiltered) water samples. Cell numbers of PROTEX 2 are averages and standard deviations for triplicate bottles.

	PROTEX1		PROTEX2			
	Bacteria (10^6 cells x ml^{-1})		Bacteria (10^6 cells x ml^{-1})		Protists (10^2 cells x ml^{-1})	
	< 0.8 µm	unfiltered	< 2.0 µm	unfiltered	< 2.0 µm	unfiltered
t = 0 h	1.67	2.07	2.21+0.15	2.14±0.12	1.51±1.33	8.42±3.79
t = 12 h			2.86±0.07	2.29±0.11	0.85±0.58	10.4±2.87
t = 24 h	2.90	2.37	3.34±0.14	1.95±0.20	3.37±1.54	8.59±3.64

	PROTEX3			PROTEX4		
	Bacteria (10^6 cells x ml^{-1})		Protists (10^3 cells x ml^{-1})	Bacteria (10^6 cells x ml^{-1})		Protists (10^3 cells x ml^{-1})
	< 0.8 µm	unfiltered	unfiltered	< 0.8 µm	unfiltered	unfiltered
t = 0 h	0.40	0.79	n.d.	n.d.	1.18	1.33
t = 14 h	0.41	0.97	n.d.			
t = 16 h				0.09	3.30	1.33
t = 24 h	0.28	1.25	0.30	0.12	3.69	2.84
t = 30 h	0.68	2.43	0.36	0.23	3.66	3.69
t = 34 h				0.35	2.19	5.72
t = 37 h	0.82	2.39	0.42			
t = 42 h	1.24	3.10	0.73	1.53	2.56	6.42
t = 48 h	2.03	4.49	1.03	3.01	1.17	5.03

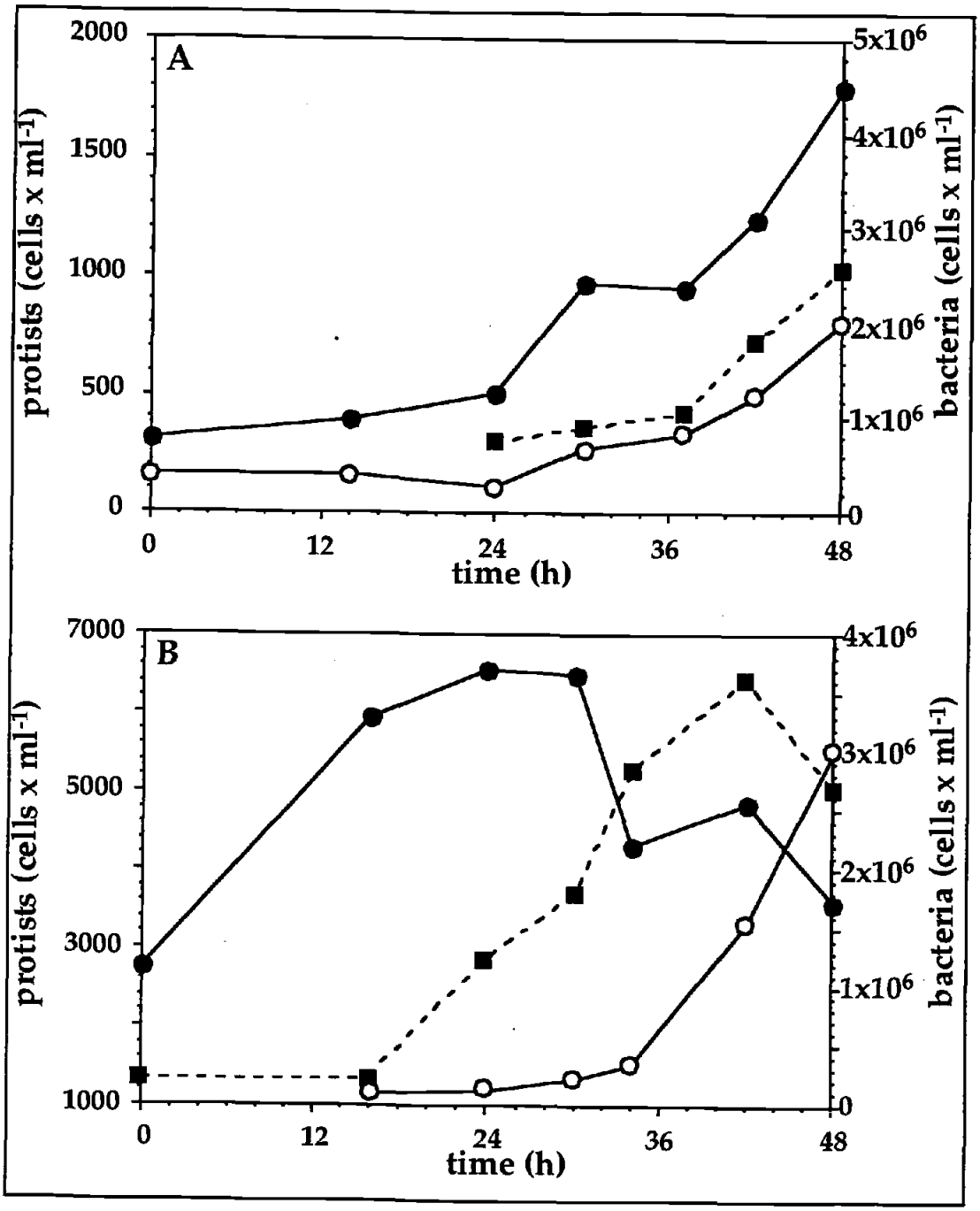

Figure 4.1. Time course of bacteria and bacterivorous protists. (A) PROTEX 3. (B) PROTEX 4. Open circles, bacteria - unfiltered water; Black circles, bacteria - 0.8 μm filtered water; Black squares; bacterivorous protists.

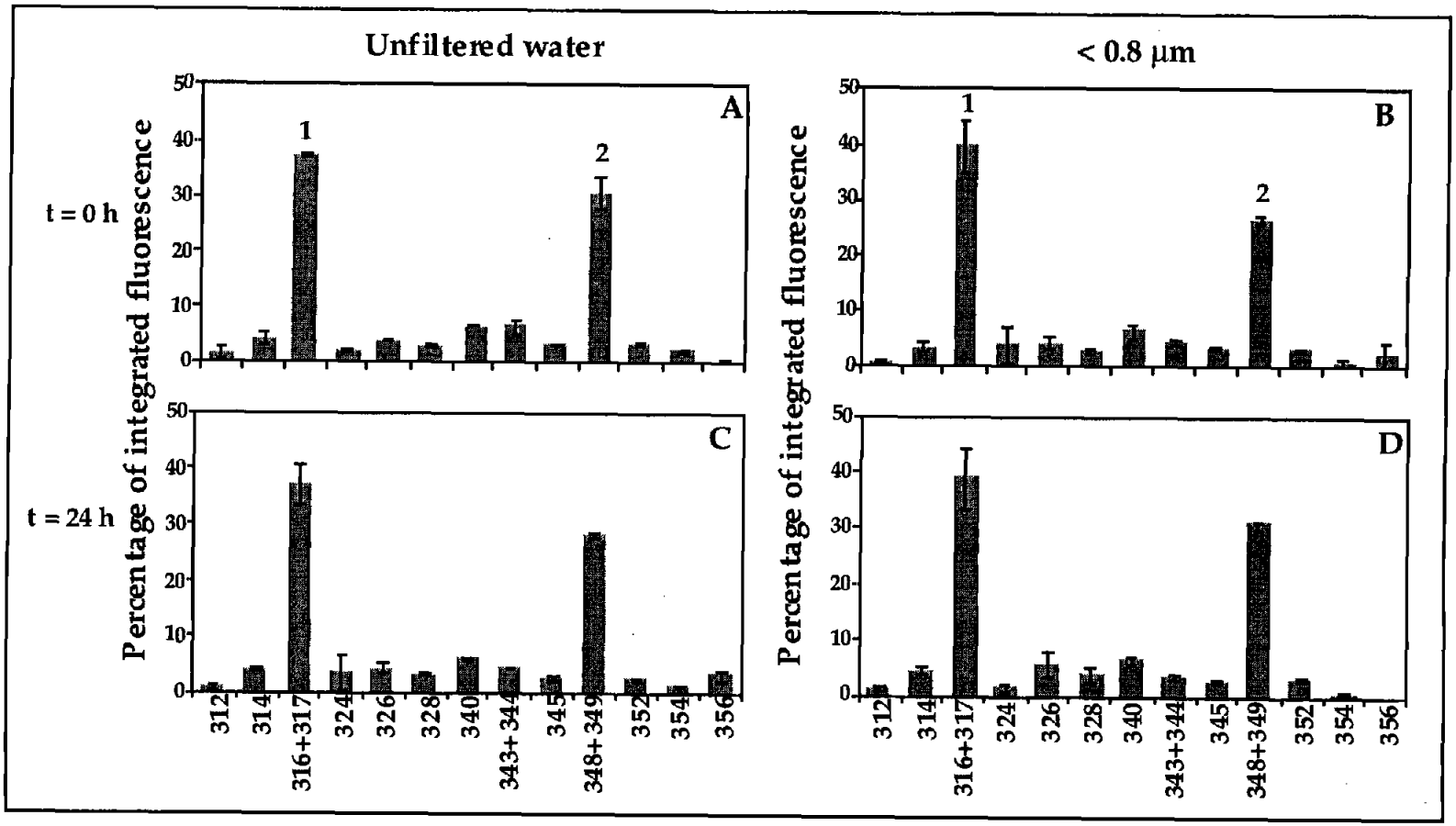

Figure 4.2. PROTEX 2: Frequency histograms of the percentage of integrated fluorescence emission by rRNA of different lengths produced by PCR using primers 27F (5' labeled with 6-FAM) and 338R. The x-axis represents the size of domains in base pairs, estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.) The error bars are one standard deviation from the average between duplicate (unfiltered water) and triplicated bottles. (A) Contol bottles, t=0 h (B) Treatment bottles t= 0h (C) Control Bottles t=24 h (D) Treatment bottles, t=24h.

Table 4.2. Time series of the ratio (in percent) of integrated fluorescence of PCR amplified SSU rDNAs of different sizes and the total integrated fluorescence of all PCR amplifed fragments assigned to bacterial SSU rDNAs. The percentages of PROTEX 2 are averages and standard deviation for duplicate (unfiltered water) and triplicate (< 0.2 μm) bottles

PROTEX 1 March 28, 1993	Fragment Size (bp)												
	312 bp	314 bp	316+317 bp	340 bp	343 bp	346 bp	348bp	349 bp	352+353 bp	354 bp	355 bp		
	Percentage of integrated fluorescence												
Unfiltered water													
t=0h	3.1	1.7	57.6	3.7	4.8	0.0	10.1	8.9	1.7	8.4	0.0		
t=24h	2.2	1.9	42.7	1.9	6.3	2.0	21.1	12.8	3.4	3.0	1.1		
Filtered (0.8 μm)													
t=0h	3.9	2.5	50.3	2.4	6.5	0.0	11.5	8.1	2.4	12.4	0.0		
t=24h	1.9	1.2	48.9	1.2	5.9	0.9	22.2	9.4	2.4	4.5	1.5		
PROTEX2 October 3, 1993	Fragment Size (bp)												
	312 bp	314 bp	316+317 bp	324 bp	326 bp	328 bp	340 bp	343+344 bp	345 bp	348+349 bp	352 bp	354 bp	356 bp
	Percentage of integrated Fluorescence												
Unfiltered water													
t=0h	1.3±1.3	3.8±1.1	36.8±0.1	1.5±0.1	3.8±0.1	2.6±0.3	6.1±0.2	6.1±1.3	3.0±0.2	30.6±2.8	2.8±0.3	1.5±0.3	0.2±0.2
t=24h	0.8±0.2	4.2±0.5	36.9±3.8	3.5±3.1	4.3±0.9	3.0±0.1	5.9±0.1	4.4±0.1	2.4±0.3	28.2±0.4	2.1±0.1	1.2±0.0	2.8±0.9
Filtered (0.8 μm)													
t=0h	0.5±0.2	3.5±0.9	39.7±4.7	3.7±3.3	4.3±1.1	2.6±0.5	6.4±1.4	4.4±0.4	2.9±0.3	26.6±0.6	3.0±0.5	0.6±0.6	1.8±2.6
t=24h	0.8±0.4	4.4±0.9	38.8±5.3	1.3±0.6	5.6±2.3	3.5±1.3	6.0±0.7	3.3±0.3	2.4±0.2	30.4±0.9	2.8±0.3	0.5±0.2	0.0±0.0

Table 4.2. Continued

PROTEX 3 March 25, 1997	Fragment Size (bp)											
	312 bp	314 bp	315-317 bp	340 bp	341 bp	343+344 bp	345 bp	348 bp	349+350 bp	352 bp	354 bp	355 bp
	Percentage of integrated fluorescence											
Unfiltered water												
t=0h	1.0	0.0	48.3	3.3	0.0	10.3	3.8	18.7	10.6	2.6	1.4	0.0
t=14h	0.5	0.0	45.4	3.7	0.0	8.6	4.6	18.3	13.7	5.2	0.0	0.0
t=24h	0.0	0.0	30.6	2.5	0.0	2.0	4.6	30.8	16.4	13.1	0.0	0.0
t=30h	0.0	0.0	19.6	4.7	0.0	3.5	6.3	31.4	19.0	15.5	0.0	0.0
t=37h	0.0	0.0	23.5	6.4	0.0	3.8	6.6	31.2	17.2	11.3	0.0	0.0
t=42h	0.0	0.0	21.8	7.2	0.0	4.4	6.8	34.8	15.2	9.8	0.0	0.0
t=48h	0.0	0.0	23.9	5.8	0.0	1.7	5.6	39.3	15.2	8.6	0.0	0.0
Filtered (0.8 µm)												
t=0h	1.0	0.8	44.1	2.5	0.0	12.1	5.2	15.0	15.0	1.6	1.6	1.0
t=14h	0.9	0.4	46.7	2.1	0.0	10.5	4.5	14.4	15.0	1.5	1.7	2.1
t=24h	0.5	0.3	29.7	0.8	2.3	7.0	9.1	26.0	16.6	6.3	1.3	0.0
t=30h	0.0	0.0	11.5	0.0	4.9	4.9	13.5	35.3	19.5	10.4	0.0	0.0
t=37h	0.0	0.0	7.4	0.0	8.0	3.5	12.8	29.4	18.6	20.3	0.0	0.0
t=42h	0.0	0.0	2.6	0.0	23.2	2.2	2.9	19.3	12.1	37.7	0.0	0.0
t=48h	0.0	0.0	2.6	0.0	18.4	2.7	2.8	14.1	10.2	49.3	0.0	0.0

PROTEX 4 July 24, 1997	Fragment Size (bp)											
	312 bp	314 bp	316+317 bp	340 bp	341 bp	343+344 bp	346 bp	348 bp	349+350 bp	352 bp	354 bp	355 bp
	Percentage of integrated fluorescence											
Unfiltered water												
t=0h	0.0	1.1	24.8	0.0	5.2	12.0	5.2	36.7	8.1	5.2	1.8	0.0
t=16h	0.0	0.6	31.0	0.0	4.4	3.9	3.5	47.8	7.1	1.7	0.0	0.0
t=24h	0.0	0.0	25.0	0.0	4.7	5.0	4.2	49.6	7.0	4.7	0.0	0.0
t=30h	0.0	0.8	24.7	0.0	6.9	6.5	4.6	45.4	7.7	3.4	0.0	0.0
t=34h	0.0	1.3	26.5	0.0	5.0	5.2	4.6	43.7	7.2	6.6	0.0	0.0
t=42h	0.0	0.9	26.0	0.0	7.6	4.6	3.3	45.8	9.6	2.2	0.0	0.0
t=48h	0.0	0.0	20.6	0.0	6.1	5.1	3.7	47.1	8.7	8.0	0.0	0.0
Filtered (0.8 µm)												
t=0h	0.8	0.0	42.0	0.0	1.7	18.4	3.3	18.2	11.3	0.0	3.0	1.2
t=16h	0.5	0.0	39.2	0.0	3.8	19.3	3.7	19.3	10.7	0.0	3.4	0.0
t=24h	0.4	0.0	38.5	0.0	7.9	12.9	3.1	21.1	9.8	2.9	3.4	0.0
t=30h	0.0	0.0	7.9	0.0	33.6	8.3	0.0	25.8	7.3	14.9	0.0	2.3
t=34h	0.0	0.0	0.4	1.4	37.9	4.8	2.0	25.9	9.8	17.7	0.0	0.0
t=42h	0.0	0.0	0.7	3.5	75.4	0.0	0.0	7.9	0.0	12.6	0.0	0.0
t=48h	0.0	0.0	0.7	1.5	62.5	0.0	0.0	12.5	0.0	22.8	0.0	0.0

The community structure inferred by LH-PCR of the water sample used in PROTEX 4 was different from previous water samples. (Figure 4.4). The 348 bp fragment represents the larger fraction of the fluorescence (~40%) followed by fragments of 315 and 316 bp (~25%). This difference might be attributed to the samples being collected during a phytoplankton bloom (personal observation) possibly caused by coastal upwelling. Furthermore, filtration through 0.8 μm changed the initial community structure, with an increase of the relative proportion of Peak 1. This observation suggests that the bacteria contributing to Peak 3 in PROTEX 4 were larger or were associated with particles.

The time course of community structure showed certain patterns common to almost all of the experiments (Table 4.2, Figures 4.2-4.4). First, except for PROTEX 2, the relative fluorescence of Peak 1 decreased with time in all filtered samples as well as in all the controls, while the relative fluorescence of Peak 3 increased with time. This observation, along with the fact that the total numbers of bacteria increase with time, suggests that the bacteria contributing to Peak 1 had lower growth rates in the bottles, than did the bacteria contributing to Peak 3. Second, the community structure did not dramatically change in the filtered water or in the controls for the first 24 hours of incubation. Finally, in the first 24 hours of incubation in PROTEX 1-3 there were no significant differences between the changes in community structure in the filtered samples and in the controls.

The time course between 24 and 48 hours of incubation in PROTEX 3 and 4 (Table 4.2, Figure 4.3e-i, Figure 4.4g-n) show that there were significant differences in genotypic community structure between filtered samples and unfiltered controls, suggesting that protists were selectively grazing bacteria

with specific genotypes. In both experiments, the community structure of the 0.8 µm filtered samples showed major shifts in the proportions of peaks between 24 ad 48 hours, whereas no such dramatic changes occurred in the controls. In PROTEX 3, the relative fluorescence of Peak 1 decreased several fold, while that of Peak 3, and several other peaks dominated the population (Figure 4.3h,j,i,n). After 42 hours of incubation, the 341 bp (peak 2) and 352 bp (Peak 4) fragments represented about 25 % and 40% of the total fluorescence, respectively. After 48 h incubation, peak 352 represented about 50% of the total fluorescence. In PROTEX 4, Peak 2 represented about 30 % of the total fluorescence after 30 h, and was the sole dominant peak (fluorescence about 65% of the total) after 48 h.

The size analysis of the SSU rDNAs indicates that a fragment of 341 bp is shared by different phylogenetic groups, including the beta and gamma subdivisions of the Proteobacteria, and the *Flexibacter-Bacteroides-Cytophaga* phylum, most of which are cultivated species (i.e. *Pseudoalteromonas* spp. , *Flavobacterium* spp.), and one of the sequences represents a gene cloned from environmental DNA from a sample from the Oregon coast (environmental clone OCS143) Suzuki et al. Submitted. The 352 bp fragment is shared by few marine strains or gene clones of the gamma (environmental clone SAR145, *Shewanella hanedai*) and beta (*Nitrococcus mobilis*) subdivisions of the Proteobacteria (Suzuki et al. Submitted).

Figure 4.3. PROTEX 3: Electropherograms of the rDNA amplified by PCR using primers 27F (5'-labeled with 6-FAM) and 338R. The *x*-axis represents the size of fragments in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). The *y*-axis represents relative fluorescence units. (A, t=0h; C , t=24h; E, t=30h; G, t=42h and I, t=48h) control bottles; (B, t=0h; D, t=24h; F, t=30h; H, t=42h and J, t=48h) treatment bottles.

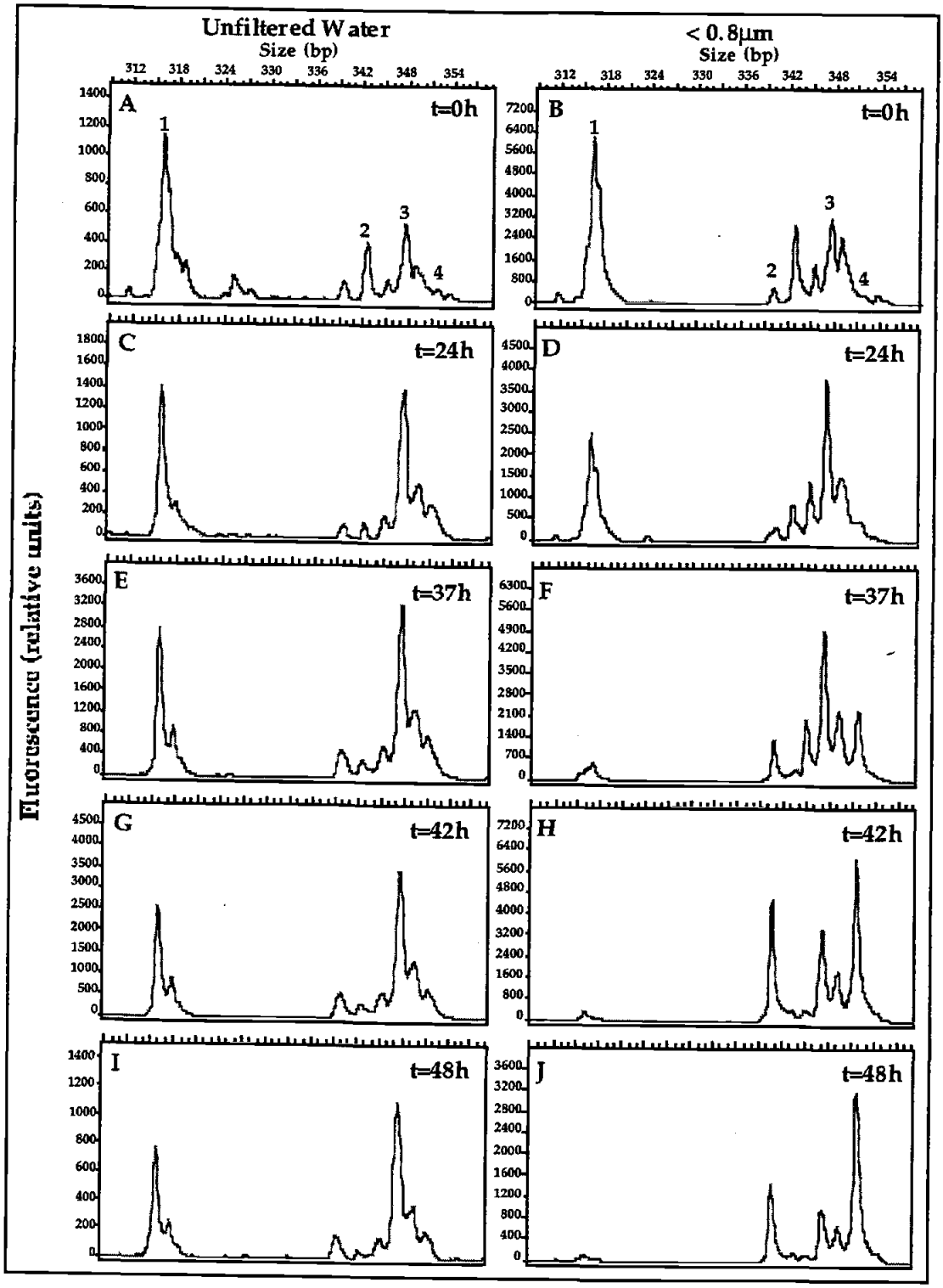
Unfiltered Water
Size (bp)
< 0.8µm
Size (bp)
312 318 324 330 336 342 348 354
Fluorescence (relative units)
A t=0h
B t=0h
C t=24h
D t=24h
E t=37h
F t=37h
G t=42h
H t=42h
I t=48h
J t=48h
1
2
3
4

Figure 4.3

Figure 4.4. PROTEX 4, Frequency histogram of the percentage of integrated fluorescence emission by different sized rDNAs amplified by PCR using primers 27F (5'-labeled with 6-FAM) and 338R. The X-axis represents the size of fragments in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). The Y-axis represents relative fluorescence units. (A, t=0h; C , t=14h; E, t=24h; G, t=30h; I, t=37h;K, t=42h and M, t=48h) control bottles; (B, t=0h; D , t=14h; F, t=24h; H, t=30h; J, t=37h; L, t=42h and N, t=48h) treatment bottles.

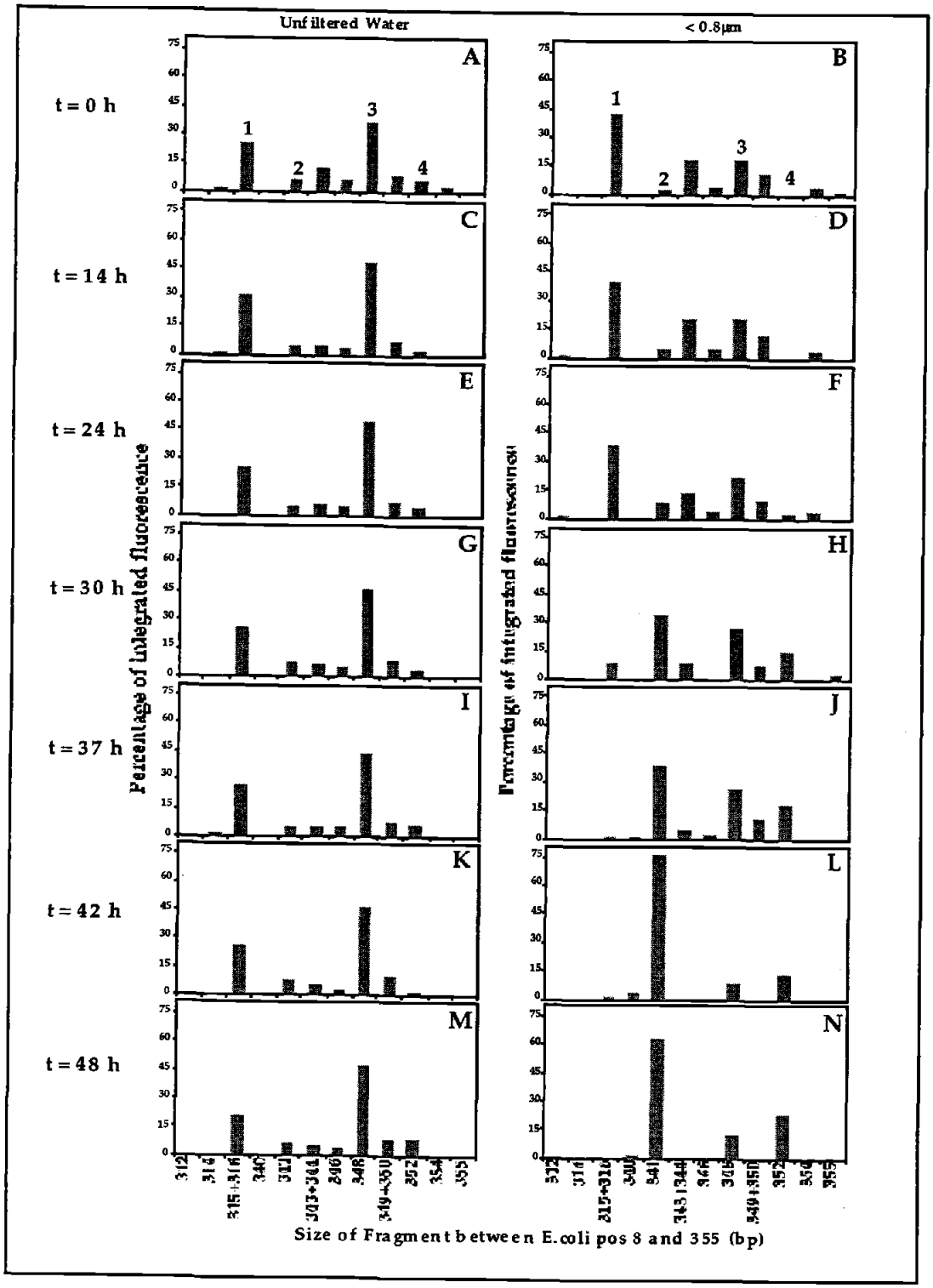
Unfiltered Water
< 0.8µm
t = 0 h
t = 14 h
t = 24 h
t = 30 h
t = 37 h
t = 42 h
t = 48 h
A
B
C
D
E
F
G
H
I
J
K
L
M
N
1
2
3
4
Percentage of integrated fluorescence
Size of Fragment between E.coli pos 8 and 355 (bp)

Figure 4.4

Discusssion

The capability of aquatic bacterivorous protists to discriminate bacterioplankton cells based on size or quality has been shown in several empirical experiments (Chrzanowski & Simek 1990, Gonzalez et al. 1990, Landry et al. 1991, Monger & Landry 1992, Sherr et al. 1992, Gonzalez et al. 1993, del Giorgio et al. 1996, Pernthaler et al. 1996) and demonstrated by physical models (Monger & Landry 1990, Monger & Landry 1991, Gonzalez 1996). In early studies bacterivorous protists were shown to selectively feed on larger bacterial analogs (Chrzanowski & Simek 1990, Gonzalez et al. 1990). Later studies employing live stained bacteria showed that the selection between live and killed bacteria was only significant for motile bacteria, and suggest that the physical forces leading to the encounter between bacterivores and their prey, rather than active selection by the bacterivores may explain the protistan food selectivity (Monger & Landry 1992, Gonzalez et al. 1993). The observations that protists selectively graze dividing cells, led to the hypothesis that protists are not only grazing bacterial standing stock, but more importantly, directly feeding on the fraction of the bacterioplankton responsible for bacterioplankton production (Sherr et al. 1992). This hypothesis was supported by the results of a time series analysis of bacterioplankton size structure in a freshwater lake showing that bacterivory effects were higher on the most active bacterial size class, and the existence of abundant small sized and relatively inactive size class of bacteria, which was weakly affected by protistan bacterivory (Pernthaler et al. 1996). Also, the recent observation that protist grazing on cells capable of reducing the tetrazolium salt CTC (del Giorgio et al. 1996) supports the hypothesis that bacterivorous protists may exert higher impact on

bacterioplankton production rather than in bacterioplankton standing stock *per se*.

The results of the PROTEX 3 and PROTEX 4 experiments also suggest that protists preferentially graze on faster growing bacteria supporting the hypothesis of selective feeding by protists on the active fraction of the bacterioplankton community. In the absence of protists, community structure showed major shifts and the bacteria representing Peaks 2, 3 and 4 dramatically increased the frequency of their SSU rDNAs on the gene pool while the bacteria contributing the highest frequency of SSU rDNAs in the gene pool at the beginning of the experiment are insignificant after 48 h. Since the total numbers of bacteria always increased in the absence of bacterivorous protists, it is very unlikely that the shifts in gene frequency were caused by mortality of the bacteria representing Peak 1. The most likely explanation for the increase in proportion of Peaks 2, 3 and 4 is that the bacteria contributing to those peaks had higher growth rates than those contributing to Peak 1, as the proportions between the peaks should not have changed had the growth rates been the same. However, it is not possible to calculate the magnitude of the differences in growth rate since the number of copies of rRNA operons and the genome size are unknown for the organisms contributing to those peaks.

Assuming that there was a sufficient exchange of nutrients and substrates between the chambers of the incubation devices, the fact that the proportion of Peaks 2, 3, and 4 increased to a lesser extent in the controls suggest that the bacteria contributing to these peaks had a higher mortality rate than the bacteria contributing to Peak 1 in the controls. There is no apparent reason why viral lysis should differ between filtered and unfiltered

samples and therefore I suggest that bacterivorous protists preferentially grazed on the faster growing cells associated with peaks 2, 3, and 4. An alternative explanation is that the availability of substrates and inorganic nutrients was lower in water filtered through 0.8 μm, and that the organisms contributing to peaks 2, 3 and 4 are better adapted to conditions of substrate limitation. However the fact that the community growth rates and the final standing stock of bacteria in filtered samples (Table 4.1) were similar to those of the controls, particularly in PROTEX 4 contradicts this explanation.

There is little evidence that the bacteria contributing to Peaks 2, 3 and 4 were larger in size than those contributing to Peak 1, since, except for PROTEX 4, the relative proportion between the peaks in 0.8 μm filtered samples at the time of experimental setup was similar to that of the unfiltered sample. Also, the longer lag periods in 0.8 μm filtered samples suggest that the bacteria contributing to peaks 2, 3 and 4 might not have been active in the original water sample and were stimulated by sample manipulation. It has been suggested in the past that the number of cultivable bacteria increase after confinement and manipulation (Ferguson et al. 1984). The fact that most of the known SSU rRNA sequences of bacteria cultivated or cloned from seawater and with size corresponding to peaks 2, 3 and 4 are indeed those of previously cultivated bacteria support this hypothesis. Therefore, although I showed that bacterivorous protists have the potential to preferentially graze on fast growing, opportunistic bacteria in manipulation experiments, the extrapolation of the results to *in situ* microbial communities should be taken with caution. A more direct way to examine this question would be to study the contents of protist food

vacuoles. Two recent studies have succeeded in hybridizing oligonucleotides to the ribosomes of the bacteria inside bacterivores food vacuoles (Gunderson & Goss 1997, Pernthaler et al. 1997). However, both experiments were performed using cultivated bacteria as prey, and the fact that natural bacterioplankton communities may be dominated by slow growing cells which may not have enough ribosomes to allow detection, poses a problem to the application of oligonucleotide hybridization to the bacteria inside food vacuoles of *in situ* bacterivorous protist communities.

Finally, the observations that: 1) all known SSU rRNA sequences of bacteria cultivated or cloned from seawater and with the sizes of Peak 1 in sequence databases represent sequences of alpha Proteobacteria, 2) that this peak is very abundant and represents organisms that grow slower in seawater culture and that are grazed to a lesser extent than other bacterial groups support the hypothesis that a large fraction of aquatic bacterioplankton is composed of small cells that escape predation, and which may be in a state of low physiological turnover (Kjelleberg et al. 1987). This hypothesis may also explain the discrepancy between the sequences of SSU rDNA of bacterioplankton directly cloned from seawater to those of marine bacterioplankton obtained by cultivation methods (Giovannoni et al. 1990, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993), as well as the recent observations that a large proportion of natural bacterioplankton communities are relatively inactive (del Giorgio & Scarborough 1995, Gasol et al. 1995, del Giorgio et al. 1997). However, since the results present in the present study can be the result of bottle effects rather than reflect the dynamics of bacterioplankton populations *in situ*, the controversy regarding the phylogenetic diversity of active and inactive

fractions of bacterioplankton populations (Rehnstam et al. 1993, Pinhassi et al. 1997, Suzuki et al. 1997) are far from being solved.

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Conclusions

Detailed discussion of the results of each of the individual studies composing this thesis is presented in the respective chapters. In this section I discuss the relevance of the results to the understanding of marine bacterioplankton community diversity and its controlling factors.

Bias by PCR

The results of the study evaluating bias by PCR suggests a new mechanism of bias by PCR in the amplification of SSU rDNA mixtures. Such bias is caused by the accumulation of products which decreases the amplification efficiency of the reaction. Bias occurs when different templates are present in different concentration at the start of the reaction. As the amplification efficiency decreases faster for templates with higher initial concentrations, amplicons of templates with lower initial concentrations tend to be over-represented in PCR products. This type of bias can be avoided if the final concentrations of reaction products are kept low by minimizing the number of amplification cycles. Another type of bias occurred in one of the reactions, with one of the templates being over-represented regardless of its initial ratio in the templates. This bias did not dramatically change the proportion between the templates. This second type of bias suggests that different templates may have inherently different amplification efficiencies but could not find a source for such difference in amplification efficiencies.

The hypothesis that reannealing bias would be lower in complex rDNA mixtures (Suzuki & Giovannoni 1996 ; Chapter 2) proved not to be true (Chapter 3). The relative abundance of the dominant amplicons produced by LH-PCR from an environmental DNA sample decreased with increasing concentrations of LH-PCR products suggesting that reannealing bias occurred. However such relationship was not clear between 0.07 and 6 ng/µl final product concentration and I could not determine whether the reactions were subjected to bias below these final product concentrations, since they are below the detection limits of the DNA sequencer.

The comparison of the relative proportions of amplicons in an environmental DNA sample or in near-complete genes produced after 35 cycles by PCR from the same DNA sample, also shows that PCR can bias the proportions of SSU rDNAs amplified from complex environmental SSU rDNA samples. Furthermore, the fact that the dominant SSU rDNAs differs in environmental DNA or PCR amplified DNA suggests that cross reannealing between closely related genes may influence the reannealing bias by PCR reactions, over-representing groups presenting genes with higher rates of substitutions.

In summary, PCR may bias the proportion of mixed SSU rDNAs. Most results could be explained by reannealing bias. Reannealling bias is proportional to total PCR product concentrations, and therefore can be minimized by keeping the such concentrations below critical levels. A second type of bias was observed but we could not determine its source. The impact of such bias to the proportion of SSU rDNAs produced by PCR from mixed templates was smaller than that of reannealing bias.

LH-PCR

The results of the analysis of the diversity of the bacterioplankton community from the Oregon coast and of the study addressing protists selective bacterivory showed the applicability of LH-PCR to studies of bacterioplankton community structure. LH-PCR is a relatively simple technique that allows a general preliminary assessment of the structure of marine bacterioplankton communities. One strength of this method is the fact that LH-PCR electropherograms can be used to measure the ratio of different SSU rDNA types in the community. Also, low coefficients of variation between triplicate LH-PCR reactions from a single DNA sample as well as from DNA samples from triplicate incubation experiments bottles show that the method is accurate. Furthermore, since the method is based on PCR amplification of SSU rDNAs, the method requires small amounts of genomic DNA, allowing the application of the method for bottle experiments and situations when only a small DNA sample is available for analysis.

The main problems with the use of LH-PCR for the analysis of marine bacterioplankton are associated with the relatively low resolution between different components of marine bacterioplankton communities. The discrimination of different taxa based on the size of hypervariable regions is far from perfect. First, the DNA sequencer and the software used in this study was not always able to discriminate between SSU rRNA of similar length. Second, in most cases, the length variation of hypervariable regions of the SSU rDNA is not phylogenetically coherent, and in many cases taxa from different bacterial phyla share common lengths. Also, since the

method is based on PCR, the method has the same potential sources of bias discussed above, although the method's ability of the to show a variation of SSU rDNA ratios in time series experiments (Figures 4.3 and 4.4) are a good indication that such bias do not lead to a random representation of the bacterioplankton community structure

Restriction endonuclease digestion of fragments is an alternative way to increase the resolution of the method in discriminating different SSU rRNAs ((Suzuki & Giovannoni 1996), Chapter 2). However, due to the general lack of *a priori* knowledge of the sequences of SSU rDNAs in different bacterioplankton community as well as difficulties associated with partial digestion by restriction endonucleases, the use of the technique for the analysis of *is situ bacterioplankton diversity* remains as a subject to be addressed by futures studies.

Bacterioplankton diversity

The results of the study comparing SSU rDNAs marine bacterioplankton cellular clones from a water sample from the Oregon coast and SSU rDNAs cloned from environmental DNAs from the same water sample (Chapter 2) support the hypothesis that bacterial communities are composed of unknown species which cannot easily be grown on typical microbiological media (Hypothesis 2). There were no perfect matches between the SSU rRNA from the cellular clones and gene clones, and although gamma and alpha Proteobacteria were numerically dominant both in the cellular clone collection and in the gene clone library, the SSU rDNA genes of cellular clones and gene clones belonged to different subgroups of the alpha and gamma Proteobacteria.

Furthermore, LH-PCR analysis of SSU rDNAs from the same water sample (Figure 3.3a) indicates that SSU rDNAs with Domain-A sized 316 to 317 or 348 to 349 bp are the most abundant SSU rDNAs *in situ*, representing about 45% and 25% of the SSU rDNAs respectively, whereas the cellular clones with Domain-A sized 316 or 317 bp (RLFP types IX, XIII, XI, XVIII, XXIV) represent about 10% the cellular clones and no cellular clones with Domain-A sized 348 or 349 bp were isolated. (Table 3.1). In contrast the dominant cellular clones (RFLP types I, and X) have Domain-A sized 328 and 342 bp respectively, which were present in concentrations which were not detected by LH-PCR (Figure 3.3a). These results suggest that the cultivation method biased the representation of the genotypic diversity of the bacterioplankton community, as species capable of forming colonies in agar plates are relatively rare species in the original population.

These results contrast to those published by (Rehnstam et al. 1993) and (Pinhassi et al. 1997), who hypothesized that species capable of forming colonies on agar plates are also the dominant genotypes *in situ*. Bias by PCR could explain such a discrepancy if the genes of species capable to form colonies on agar plates were amplified with lower efficiencies. However there is no evidence for a mechanism producing this type of bias. Reannealing bias by PCR cannot explain this discrepancy, since this type of bias should overestimate the proportion of rare peaks as explained in chapters 2 and 3, thus overestimating the proportion of rare species. The discrepancy between my results and those of Rehnstam et al. (1993) can be partially explained by the relative non-specificity of the oligonucleotide probes used in that study. In other hand, Pinhassi et al. (1997) used a whole genome hybridization, a more specific method to quantify the abundance of

different genotypes, and therefore, the best explanation for the discrepancy between my results and that of Pinhassi et al. (1997) are differences inherent to the water samples. The Baltic Sea is a brackish and shallow, and perhaps the bacterioplankton community may resemble that of some eutrophic freshwater systems, such as an eutrophic Arctic lake which is dominated by species closely related to previously cultivated species (Bahr et al. 1996).

The fact that the SSU rDNAs of rare species *in situ* also dominate the SSU rDNAs in exclusion experiments after 48h of incubation without protists, and represent most of the isolates in enrichment cultures suggests the existence of opportunistic species (r-strategists, Andrew & Harris 1986) in coastal bacterioplankton, which are rare members of bacterioplankton communities *in situ*, but are better adapted to higher substrate concentrations or relief from bacterivory produced by manipulation of samples. The early observation that the number of colony forming units increases after manipulation or confinement of samples (Ferguson et al. 1984) also supports this hypothesis. Such opportunistic species loosely correspond to the so called copiotrophs (Schut et al. 1997), but they do not necessarily require substrate levels much higher than those *in situ*.

The size analysis of the SSU rDNA of marine bacteria (Table 3.1) suggests that such opportunistic species are possibly members of the gamma Proteobacteria and the *Flexibacter-Bacteroides-Cytophaga* phylum, whereas the community *in situ* is in general dominated by the members of the alpha Proteobacteria. This fact may partially explain the discrepancy between the SSU rDNAs of cultivated species and those directly cloned from the environment (Chapter 2, Giovannoni et al. 1990; Schmidt et al. 1991; DeLong et al. 1993; Fuhrman et al. 1993).

Controls of Bacterioplankton Community Structure.

The results of the study evaluating the effects of bacterivorous protists on bacterioplankton community structure suggest that bacterivorous protists graze selectively on different bacterioplankton genotypes. The LH-PCR analysis does not allow a definitive identification of the organisms preferentially grazed by bacterivorous protists. The results did suggest that grazing by bacterivorous protists was higher on the opportunistic fraction of the population which represented a low fraction of the SSU rDNAs of *in situ* populations or in bottles with bacterivores but represented the dominant SSU rDNA population in the absence of protists.

These results are similar to those of del Giorgio et al. (1986), which showed that actively respiring cells represent a larger fraction of the bacterial biomass in communities incubated without protists compared to those incubated with protists. The results also support the hypothesis that bacterivorous protists feed on large and actively growing cells, leading the bacterioplankton community to be composed of smaller inactive cells. Whether the same cells that grow in bottle experiments are also growing *in situ* remains unknown. The results of Pernthaler et al. (1996) showed selective feeding by protists on active cells belonging to specific size fractions *in situ*. However, the authors did not attempted to identify the taxonomic composition of the active fraction of the population.

The identification of the food vacuole contents of bacterivorous protists is a different way to address the question of what are the genotypes active *in situ* being selectively grazed by bacterivores. Two recent studies report the possibility of targeting oligonucleotide probes to bacterial cells inside bacterivore food vacuoles (Gunderson & Goss 1997; Pernthaler et al. 1997).

However, the same problems associated with the application of *in situ* hybridization techniques for slow growing cells is a problem to the quantification of relative proportions of different types of bacteria inside protist food vacuoles.

I attempted to evaluate the food vacuole contents of bacterivorous protists using cultures of the flagellate *Cafeteria* sp. and samples from PROTEX 4 after feeding the protist assemblage with paramagnetic beads, fixing the samples and magnetically separating protists from bacteria. The food vacuole contents were analyzed by LH PCR using DNA extracted from isolated bacterivores. The main problems I experienced with this approach were the relative low uptake of the paramagnetic beads by non-starved protists, and the difficulty of completely separating the bacterivores from the bacteria. The results of a preliminary experiment using the PROTEX 4 sample shows that the 316 and 317 bp fragments that composed a large fraction of the SSU rDNAs in the sample incubated for 48h with protists were absent in the DNA extracted from magnetically separated protists. This result can possibly due to experimental artifacts, but nonetheless is in agreement with the results of the protist exclusion experiments that suggest that protists grazing rates on organisms contributing to peaks 316 and 317 is lower than in peaks 341 and 352.

The significance of my results to the question of what controls bacterial populations is dependent on the extrapolation of the results of bottle experiments to *in situ* bacterioplankton communities. Assuming that bacterivorous protists *in situ* present the same type of selective feeding on actively growing bacteria, bacterivorous protists may be directly removing bacteria responsible for bacterioplankton production. If that is the case,

actively growing active cells that may be responsible for most of the community metabolism (del Giorgio et al., 1997), are kept in low abundance by the bacterivores explaining the low percentages of active cells *in situ* (del Giorgio & Scarborough 1995; Gasol et al. 1995). This hypothesis is also in agreement with observations that bacterivorous protist grazing rates are similar to bacterioplankton growth rates (Fenchel 1982; McManus & Fuhrman 1988; Bloem et al. 1989; Sherr et al. 1989).

In other hand, if small cell size and low growth rates are a refuge from bacterivory (Kjelleberg et al. 1983), the standing stock of cells with such characteristics is mainly dependent of growth rates and sources of mortality other than bacterivore grazing. Assuming that growth rates of these small slow growing cells are correlated with substrate availability and that sources of mortality other than grazing are relatively constant across systems, one can envision that the total numbers of these small slow growing cells, which compose the largest fraction of bacterioplankton in terms of abundance are dependent on substrate availability. This bottom-up controls of bacterioplankton biomass is in agreement with regression analysis between bacterioplankton biomass and production (Billen et al. 1990; Ducklow & Carlson 1992; Dufour & Torréton 1996) especially since variations in bacterioplankton biomass are a better reflection of variations of bacterioplankton abundance than of variations in cell volumes (Dufour & Torréton 1996).

The existence of bacterioplankton species with different metabolic strategies has been long hypothesized (Ishida & Kadota 1974; Akagi et al. 1977; Yanagita et al. 1978). However, whether the fast growing cultivated cells that apparently also dominate the bacterioplankton community in

manipulation experiments or the uncultivated organisms represented in clones libraries of rDNAs are growing *in situ* remains unknown. The identification of the organisms active *in situ*, and their physiological characteristics, especially under the predicted global change scenario remains as a major challenge for future studies on aquatic bacterioplankton communities.

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Appendix

List of bacterial strains isolated from seawater and their SSU rDNA sequences.

This list is a compilation of bacterial species with strains isolated from seawater, with cross reference to available SSU rRNA sequences. This list was produced by search in the "Bergeys Manual for Systematic Bacteriology" and World Wide Web searches of online culture collection databases from the American Type Culture Collection (ATCC), the Deustsche Sammlung von Mikroorganismen und Zellkulturen (DSZM), the Centraalbureau voor Schimmelcultures (CBS), the Belgium Co-ordinated Collection of Microorganisms (BCCM) and the Pasteur Collection of Cyanobacteria (PCC).

The species are grouped according to the "Bergeys Manual for Systematic Bacteriology". In species described after the publication of the "Bergeys Manual for Systematic Bacteriology", were included in the sections according to Genus. Genus not described in the "Bergeys Manual for Systematic Bacteriology" are listed at the end of the table along with species with names not approved by the International Committee on Systematic Bacteriology.

The names of bacteria in boldface correspond to names of bacteria officially recognized by the International Committee on Systematic Bacteriology. The names were cross-referenced with the online database of "bacterial nomenclature up to date" provided by the DSMZ. The names in Italic correspond to other denominations of strains belonging to each of the species.

The sequence data was obtained by searching for the species on table in the online databases Genbank, from the National Center for Biotechnology

information, the Ribosomal Database Project (RDP) (Maidak, 1984), and the ARB database from the Technische Universität München. The Genbank accession numbers correspond to the strains listed in the Appendix.

WWW addresses of online databases searched

Culture collections-databases

ATCC: http://www.atcc.org

DSZM: http://www.gbf-braunschweig.de/DSMZ/dsmzhome.html

CBC: http://www.cbs.knaw.nl/www/database.html

BCCM: http://www.belspo.be/bccm/

PCC: http://www.pasteur.fr/Bio/PCC/

Gene sequence databases

Genbank: http://www.ncbi.nlm.nih.gov/Web/Search/index.html

RDP: http://rdpwww.life.uiuc.edu/

ARB: http://pop.mikro.biologie.tu-muenchen.de/

Abbreviations

AL: Name included in the Approved list of bacterial names (Skerman et al 1980).

VL: Names validated in the "Validation lists of the publications of new names previously effectively published outside the IJSB"

IJSB: International Journal of Systematic Bacteriology.

Section 2 - Aerobic/microaerophilic motile helical/vibrioid Gram negative

taxa	strain	accession number	source	Reference
Marinomonas communis	ATCC27118t			(Baumann et al. 1972) Van Landschoot & de Ley 1984 [VL]
				Van Landschoot & de Ley 1983
	IAM12914	D11181 D11230 D11279		Kita-Tsukamoto et al. 1993
=*Oceanospirillum commune*	ATCC27118t	RDP		Woese, unpublished
=*Alteromonas communis*	ATCC27118t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
Marinomonas vaga	ATCC27119t			(Baumann et al. 1972) Van Landschoot & de Ley 1984 [VL]
				Van Landschoot & de Ley 1983
	ATCC27119t	RDP		Woese unpublished
	IAM12923	D11182 D11231 D11280		Kita-Tsukamoto et al. 1993
=*Oceanospirillum vagum*	ATCC27119t	X67025		Gauthier et al. 1992
=*Oceanospirillum vagum*	ATCC27119t			Bowditch et al. 1984
=*Alteromonas vaga*	ATCC27119		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
Oceanospirillum beijerincki subsp beijerinckii	ATCC12754t			(Williams & Rittenberg 1957) Hylemon et al. 1973 [AL]
	ATCC12754t	RDP		Woese unpublished
=*Spirillum beijerinckii*	NCMB52t		Intertidal Zone of Coastal USA	Williams & Rittenberg 1957
Oceanospirillum kriegii	ATCC27133t		Coastal waters off Oahu, Hawaii	Bowditch et al. 1984 [VL]
				Bowditch et al. 1984
	ATCC27133t	RDP		Woese unpublished
Oceanospirillum jannaschii	ATCC27135t		Coastal waters off Oahu, Hawaii	Bowditch et al. 1984 [VL]
	ATCC27135t	RDP		Woese unpublished
Oceanospirillum linum	ATCC11336t			(Williams & Rittenberg 1957)Hylemon et al. 1973
	ATCC11336t			Hylemon et al. 1973
	ATCC11336t	M22365		Woese unpublished
=*Spirilum linum*	ATCC11336		Intertidal Zone of Coastal USA	Williams & Rittenberg 1957
Oceanospirillum maris subsp. maris	ATCC27509t		Seawater	Hylemon et al. 1973 [AL]
	ATCC27649		Seawater	Hylemon et al. 1973
	ATCC27649	RDP		Woese unpublished
Oceanospirillum maris subsp. willliamsae	ATCC29547t		Mixed culture	Linn & Krieg 1978
=*Oceanospirillum lunatum*			Intertidal Zone of Coastal USA	Williams & Rittenberg 1957

Section 3 - Nom motile curved Gram negative

Cyclobacterium marinus	ATCC25205t			(Raj 1976)Raj & Maloy 1990
=*Flectobacillus marinus*	ATCC43824	M62788 M27801		Woese et al. 1990
=*Microcyclus marinus*	ATCC25205		Dendraster (sand dollar)	Raj 1976
=*Flectobacillus marinus*	ATCC25205			Borrall & Larkin 1978
=*Flectobacillus marinus*	ATCC43824 ATCC43825		2m from seafloor - Guaymas Basin	Bazylinski & Jannasch unpubl.
Flectobacillus glomeratus	ACAM171t		Antarctic lake with marine connection	McGuire et al. 1988 [VL]
				McGuire et al. 1987
	ATCC43844t	M58775		Woese, unpubl.

Section 4 - Gram negative aerobic rods and cocci

taxa	strain	accession number	source	Reference
Agrobacterium ferrugineum	ATCC25652t			ex Ahrens & Rheinheimer 1967 Rüger & Hofle 1992
	ATCC25652t		Seawater, Baltic Sea	Ahrens & Rheinheimer 1967
Agrobacterium gelatinovorum	ATCC25655t			ex Ahrens 1968 Rüger & Hofle 1992
	ATCC25655t		Seawater, Baltic Sea	Ahrens 1968
Alteromonas macleodii	ATCC27126t		Coastal waters off Oahu, Hawaii, surface, 5, 100, 750 and 1100m	Baumann et al. 1972 [AL]
		L10938		DeLong et al. 1993
	IAM12920t	X82145		Gauthier et al. 1995
Halomonas aquamarina	ATCC14400t			(ZoBell & Upham 1944)Dobson & Franzmann 1996
=Deleya aquamarina	ATCC14400t	M93352		Dobson et al. 1993
=Deleya aquamarina	ATCC14400t			Akagawa & Yamasato 1989
=Achromobacter aquamarinus	ATCC14400t		Seawater	ZoBell & Upham 1944
=Deleya aesta	ATCC27128			Baumann et al. 1983
=Alcaligenes aestus	ATCC27128		Coastal waters off Oahu, Hawaii 100, 150, 200 and 600 m	Baumann et al. 1972
Halomonas cupida	ATCC27124t	L42615		(Baumann et al. 1972)Dobson & Franzmann 1996
=Deleya cupida	ATCC27124t			Baumann et al. 1983
=Alcaligenes cupidus	ATCC27124t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
Halomonas marina	ATCC25374t			(Cobet et al. 1970)Dobson & Franzmann 1996
=Deleya marina	ATCC25374t	M93354		Dobson et al. 1993
=Deleya marina	IAM12928 =ATCC27129	D11177 D11226 D11275		Kita-Tsukamoto et al. 1993
=Deleya marina	ATCC25374t			Baumann et al. 1983
=Pseudomonas marina	ATCC27129		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
=Pseudomonas marina	ATCC27179		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
=Arthrobacter marinus	ATCC25374t		Littoral water sample at Woods Hole, MA	Cobet et al. 1970
Halomonas pacifica	ATCC27122t	L42616		(Baumann et al. 1972)Dobson & Franzmann 1996
=Deleya pacifica	ATCC27122t			Baumann et al. 1983
=Alcaligenes pacificus	ATCC27122t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
Halomonas venusta	ATCC27125t	L42618		(Baumann et al. 1972)Dobson & Franzmann 1996
=Deleya venusta	ATCC27122t			Baumann et al. 1983
=Alcaligenes venustus	ATCC27122t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
Methylophaga thalassica	ATCC33146t			Janvier et al. 1985
	ATCC33146t	X87339		Janvier & Grimont 1995
	DSM5690t	X95460		De Zwart et al. unpubl.
	ATCC33146t			Janvier et al. 1985
=Methylomonas thalassica	NCMB2163t		Coastal Seawater	Yamamoto et a 1980
Methylomicrobium pelagicum	ACM3505t	X72775		(Sieburth et al. 1987)Bowman et al. 1995
=Methylomonas pelagica		L35540		Mullins et al. 1995
=Methylomonas pelagica		U05570		Distel & Cavanaugh 1994
=Methylobacter pelagicus	ACM3505t			Bowman et al. 1993

taxa	strain	accession number	source	Reference

=*Methylomonas pelagica*	NCMB2265t		Sargasso Sea. Top of thermocline and half TC depth	Sieburth et al. 1987
Pseudoalteromonas atlantica	IAM12927t	X82134		(Akagawa-Matsushita et al. 1992) Gauthier et al. 1995
=*Alteromonas atlantica*	ATCC19962t			Akagawa-Matsushita et al. 1992
=*Pseudomonas atlantica*	NCMB301t		Seaweed	Yaphe 1957
=*Pseudomonas atlantica*	ATCC43667		Seawater - California	Corpe, 1970
=*Pseudomonas atlantica*	ATCC43666		Seawater - California	Corpe, 1970
Pseudoalteromonas aurantia	ATCC33046t	X82135		(Gauthier & Breittmeyer 1979) Gauthier et al. 1995
=*Alteromonas aurantia*			Seawater	Gauthier & Breittmeyer 1979
Pseudoalteromonas carrageenovora	IAM12662t	X82136		(Akagawa-Matsushita et al. 1992) Gauthier et al. 1995
=*Alteromonas carrageenovora*	IAM12662t			Akagawa-Matsushita et al. 1992
=*Pseudomonas carrageenovora*	ATCC43555t		Seawater, Nova Scotia, Canada	Yaphe and Morgan unpublished
Pseudoalteromonas citrea	NCMB1889t	X82137		(Gauthier 1977)Gauthier et al. 1995
=*Alteromonas citrea*			Surface water, Mediterranean sea near Nice, France	Gauthier 1977
Pseudoalteromonas denitrificans	ATCC43337t	X82138		(Enger et al. 1987) Gauthier et al. 1995
=*Alteromonas denitrificans*	ATCC43337t		Fjord system off Norway 90-100m	Enger et al. 1987
Pseudoalteromonas espejiana	NCIMB2127t	X82143		(Chan et al. 1978) Gauthier et al. 1995
=*Alteromonas espejiana*	ATCC29659t		Seawater off North California	Chan et al. 1978
=*Alteromonas espejiana*	IAM12640	D11171 D11220 D11269		Kita-Tsukamoto et al. 1993
Pseudoalteromonas haloplanktis	ATCC14393t	X67024		(ZoBell & Upham 1944) Gauthier et al. 1995
=*Alteromonas haloplanktis*	ATCC14393t	D11172 D11221 D11270		Kita-Tsukamoto et al. 1993
=*Vibrio haloplanktis*	ATCC14393t		Sessile associated with Phytoplankton	ZoBell & Upham 1944
=*Alteromonas marinopraesens*	ATCC27127		Coastal waters off Oahu, Hawaii	Baumann et al. 1972
=*Vibrio marinopraesens*	ATCC19648		Seawater	ZoBell & Upham 1944
Pseudoalteromonas luteoviolacea	MCIMB1893t	X82144		(Gauthier 1982) Gauthier et al. 1995
=*Alteromonas luteoviolacea*			Surface water, Mediterranean sea near Nice, France	Gauthier 1982
=*Chromobacterium marinus*			Atlantic Ocean water	Hamilton & Austin 1967
Pseudoalteromonas piscicida	ATCC15057t			(Bein 1954) Gauthier 1976
=*Pseudomonas piscicida*	C201CERBOM	X82141	Seawater near Brest, France	Gauthier et al. 1995
=*Flavobacterium piscicida*	ATCC15057		Red tide water	Bein 1954
Pseudoalteromonas rubra	ATCC29570t	X82147		(Gauthier 1976) Gauthier et al. 1995
=*Alteromonas rubra*	NCMB1890		Mediterranean waters off Nice	Gauthier 1976
Pseudoalteromonas undina	ATCC29660t	X82140		(Chan et al. 1978) Gauthier et al. 1995
=*Alteromonas undina*	ATCC35257		Seawater off North California	Chan et al. 1978
Pseudomonas doudoroffii	ATCC27173t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972 [AL]
Pseudomonas elongata	ATCC10144t		Seawater	Humm 1946 [AL]

taxa	strain	accession number	source	Reference
Pseudomonas gelidicola	IAM1127t		Seawater, Maizuru Bay, Japan	Kadota 1951 [AL]
Pseudomonas nautica	ATCC27132t		Coastal waters off Oahu, Hawaii	Baumann et al. 1972 [AL]
	IAM12929	D11189 D11238 D11287		Kita-Tsukamoto et al. 1993
Pseudomonas stanieri	ATCC27130		Coastal waters off Oahu, Hawaii	Baumann et al. 1983 [AL]
Pseudomonas stuzeri str. Zobell	ATCC14405t			Döhler et al. 1987
	ATCC14405t	U65012		Kerkhoff unpubl.
	ATCC14405t	U26420		Bennasar et al. 1996
=*Pseudomonas perfectomarinus*	ATCC14405t		Seawater and marine mud	ZoBell & Upham 1944
Roseobacter algicola	ATCC51440	X78315	Associated with cultures of Prorocentrum lima	Lafay et al. 1995
	ATCC51441	X78313	Associated with cultures of Prorocentrum lima	Lafay et al. 1995
	ATCC51442	X78314	Associated with cultures of Prorocentrum lima	Lafay et al. 1995
Shewanella putrefaciens	ATCC8071t			(Lee et al. 1977)MacDonell & Colwell 1986 [VL]
	ATCC8071t			MacDonell & Colwell 1985
	LMG26268t	X81623		Rossello-Mora et al. 1994
	ATCC8071t	X82133		Gauthier et al. 1995
	IAM12079	D11183 D11232 D11281		Kita-Tsukamoto et al. 1993
=*Alteromonas putrefaciens*	SS65 SS191		Seawater	Lee et al. 1977
=*Pseudomonas putrefaciens*	ATCC8071		Butter	Derby & Hammer 1931
Shewanella hanedai	ATCC33224t			Jensen et al. 1980MacDonell and Colwell 1986 [VL]
	ATCC33224t			MacDonell & Colwell 1985
	IC050	U85908	Antarctic Sea Ice	Bowman et al. unpubl.
	CIP103207	X82132		Gauthier et al. 1995
=*Alteromonas hanedai*	ATCC33224		Arctic sediments	Jensen et al. 1980
=*Alteromonas hanedai*	str 282-283		Seawater in the Anterctic	Jensen et al. 1980
=*Alteromonas hanedai*	str 284-291		Sannich Inlet BC, Canada 110-220m	Jensen et al. 1980

Section 5 - Facultative anaerobic Gram negative rods

Photobacterium angustum	ATCC25915t			Reichelt et al. 1979 [AL]
	ATCC25915t	X74685		Ruimy et al. 1994
	ATCC25915t	D25307		Okuzumi et al. 1994
		D11183 D11232 D11281		Kita-Tsukamoto et al. 1993
	ATCC25915		Coastal waters off Oahu, Hawaii 750 m	Baumann et al. 1971
	many strains		Coastal waters off Oahu, Hawaii 7.5, 300 800 and 1300 m	Baumann et al. 1971

taxa	strain	accession number	source	Reference
Photobacterium histaminum	JCM8978	D25308	Skin of Labracoglossid fish	Okuzumi et al. 1994
	many strains		Seawater, Tokyo and Sagami bays	Okuzumi et al. 1994
Photobacterium leiognathi	ATCC25521t		Luminous organ of fish	Boisvert et al. 1967
	ATCC25521t	X74686		Ruimy et al. 1994
	PL721	Z21730		Haygood et al. 1992
	ATCC25521t	D25309		Okuzumi et al. 1994
	ATCC25521t	D11184		Kita-Tsukamoto et al. 1993
		D11233		
		D11282		
=*Photobacterium mandapanensis*	ATCC27561		Seawater, Band island, Indonesia	Reichelt & P 1973
Photobacterium phosphoreum				(Cohn 1875) Beijerinck 1889 [AL]
	ATCC11040t			Hendrie et al. 1970
	Og61	Z19107		Haygood et al. 1992
	ATCC11040t	X74687		Ruimy et al. 1994
	ATCC11040t	D21310		Okuzumi et al. 1994
	ATCC11040t	D11186		Kita-Tsukamoto et al. 1993
		D11235		
		D11284		
	many strains		Oceanic and Coastal waters	Baumann et al. 1971
=*Bacterium phosphoreum*	ATCC11040		Not mentioned	Deposited in ATCC : F. H. Johnson
Listonella pelagia	ATCC25916t			(Baumann et al. 1971) MacDonell & Colwell 1986 [VL]
	ATCC25916t			MacDonell & Colwell 1985
	ATCC25916t	Z22991		Martinez-Picado et al. 1994
	ATCC25916t	X74722		Ruimy et al. 1994
	ATCC25916t	D11180		Kita-Tsukamoto et al. 1993
		D11229		
		D11278		
=*Vibrio pelagicus*	NCMB1900t	X70642		Wiik et al. 1995
=*Beneckea pelagia*	ATCC25916t		Coastal waters off Oahu, Hawaii	Baumann et al. 1971
Serratia rubidaea	ATCC27593t		Not mentioned	(Stapp 1940)Ewing et al. 1973 [AL]
	ATCC27593t	AB004751		Harada & Ishikawa 1996
=*Bacterium rubidaeum*	ATCC27593t			Stapp 1940
=*Serratia marinorubra*	ATCC27614		Seawater	ZoBell & Upham 1944
Vibrio alginolyticus	ATCC17749t			Sasazaki 1968 [AL]
	ATCC17749t			Baumann et al. 1980
	ATCC17749t	X56576		Dorsch et al. 1992
	ATCC17749t	X74690		Ruimy et al. 1994
	CIP7065	X74691	*Not Type Strain	Ruimy et al. 1994
	NCMB1903t	D11192		Kita-Tsukamoto et al. 1993
		D11241		
		D11290		
=*Beneckea alginolytica*	ATCC17749	many strains	Coastal waters off Oahu, Hawaii	Baumann et al. 1971
=*Vibrio alginolyticus*	ATCC17749t			Sasazaki 1968
= *Oceanomonas alginolytica*	ATCC17749t		Spoiled horse mackerel, causing food poisoning	Miyamoto et al. 1961
Vibrio campbelli	ATCC25920t			Baumann et al. 1981 [VL]
	ATCC25920t	X56575		Dorsch et al. 1992
	ATCC25920t	X74692		Ruimy et al. 1994
	ATCC25920t	D11193		Kita-Tsukamoto et al. 1993
		D11242		
		D11291		
=*Beneckea campbelli*	ATCC25920		Coastal waters off Oahu, Hawaii	Baumann et al. 1971

taxa	strain	accession number	source	Reference
Vibrio fischeri	ATCC7744t			Beijerinck 1889 Lehmann & Neumann 1896 [AL]
	ATCC7744t	X74702		Ruimy et al. 1994
	ATCC7744t	Z21729		Haygood et al. 1992
	NCMB1281t	X70640		Wiik et al. 1995
	NCMB1274	X71813	Merluccius vulgaris	Wiik et al. 1995
	ATCC7744t	D11203 D11252 D11301		Kita-Tsukamoto et al. 1993
=*Photobacterium fischeri*	ATCC7744t			Reichelt & P 1973
=*Photobacterium fischeri*	many strains		Coastal waters off Oahu, Hawaii	Baumann et al. 1971
=*Achromobacter fischeri*	ATCC7744t		Dead Squid	Johnson & Shunk 1936
Vibrio harveyi	ATCC14126t			(Johnson & Shunk 1936) Baumann et al. 1981 [VL]
	ATCC14126t			Baumann et al. 1980
	ATCC14126t	X56578		Dorsch et al. 1992
	ATCC14126t	X74706		Ruimy et al. 1994
	n.a.	M58172		Rosson and Schmidt 1991 unpubl.
	ATCC14126t	D11205 D11254 D11303		Kita-Tsukamoto et al. 1993
=*Beneckea harveyii*	many strains		Seawater	Reichelt & P 1973
=*Achromobacter harveyi*	ATCC14126		Dead amphipod (Talorchestria sp.)	Johnson & Shunk 1936
Vibrio marinus	ATCC15381t			Baumann et al. 1984 [VL]
	ATCC15381t			Baumann et al. 1984
	NCIMB1144t	X82134		Gauthier et al. 1995
	ATCC15381t	D11208 D11257 D11306		Kita-Tsukamoto et al. 1993
	ATCC15381t	RDP		Woese unpublished
	ATCC15381t		Pacific Ocean off Oregon 1200m	Colwell & Morita 1964
=*Moritella marinus*	ATCC15381t			Steven 1990
Vibrio mediterranei	CECT621t			Pujalte & Garay 1986
	CIP103203t	X74710		Ruimy et al. 1994
Vibrio natriengens	ATCC14048t			(Payne et al. 1961) Baumann et al. 1981 [VL]
	ATCC14048t			Baumann et al. 1980
	ATCC14048t	X56581		Dorsch et al. 1992
	ATCC14048t	X74714		Ruimy et al. 1994
	CCM2575t	D11211 D11260 D11309		Kita-Tsukamoto et al. 1993
=*Beneckea natriegens*	107-110,112		Coastal waters off Oahu, Hawaii	Baumann et al. 1971
=*Pseudomonas natriegens*	ATCC14048		Salt marsh mud	Payne et al. 1961
Vibrio nereis	ATCC25917t			(Harwood et al. 1980) Baumann et al. 1981 [VL]
	ATCC25917t			Baumann et al. 1980
	ATCC25917t	X74716		Ruimy et al. 1994
	ATCC25917t	D11212 D11261 D11310		Kita-Tsukamoto et al. 1993
=*Beneckea nereida*	ATCC25917t			Harwood et al. 1980
=*Beneckea nereida*	ATCC25917t		Coastal waters off Oahu, Hawaii	Baumann et al. 1971

taxa	strain	accession number	source	Reference
Vibrio nigropulchritudo	ATCC27043t			(Baumann et al. 1971) VL Baumann et al. 1981
	ATCC27043t			Baumann et al. 1980
	ATCC27043t	X74717		Ruimy et al. 1994
	ATCC27043t	D11213 D11262 D11311		Kita-Tsukamoto et al. 1993
=*Beneckea nigropulchrituda*	ATCC27043		Coastal waters off Oahu, Hawaii 50 and150 m	Baumann et al. 1971
Vibrio orientalis	ATCC33934t		Seawater, Yellow Sea China	Yang et al. 1983
	ATCC33934t	X74719		Ruimy et al. 1994
	ATCC33933	Z21731	Shrimp, Yellow Sea China	Yang et al. 1983, Haygood and Distel unpubl.
	ATCC33934t	D11215 D11264 D11313		Kita-Tsukamoto et al. 1993
Vibrio splendidus	ATCC33125t			(Beijerinck 1900)Baumann et al. 1981 VL
	ATCC33125t			Baumann et al. 1980
	ATCC33125t	X74724		Ruimy et al. 1994
	NCB8	Z31657		Rehnstam-Holm et al. unpublished
=*Beneckea splendida*	ATCC33125		Coastal Waters of Denmark and North America	Reichelt et al. 1976
Section 11 - Oxygenic Phototrophs Not in the aproved list of bacterial names.				
Synechococcus sp. Marine group A	WH8103t		Seawater	Waterbury et al. 1986
Waterbury et al. 1986	WH8005		Seawater	Waterbury et al. 1986
	WH7803		Seawater	Waterbury et al. 1986
	WH6501		Seawater	Waterbury et al. 1986
	WH7803		Seawater	Waterbury et al. 1986
	WH6501		Seawater	Waterbury et al. 1986
	WH8011		Seawater	Waterbury et al. 1986
	WH8102		Seawater	Waterbury et al. 1986
	WH8112		Seawater	Waterbury et al. 1986
	WH8113		Seawater	Waterbury et al. 1986
Synechococcus sp. Marine group B	WH5701t		Seawater	Waterbury et al. 1986
	WH8007		Seawater	Waterbury et al. 1986
	WH8101		Seawater	Waterbury et al. 1986
	PCC7001		intertidal mud	Stanier et al. 1971
Synechococcus sp.	PCC7336		Seawater tank - Berkeley, CA	Waterbury unpublished
	PCC7309		Seawater - City Island , NY	Van Baalen 1962
	PCC7922		Brakish water - Finland	Vaara et al. 1979
	PCC7923		Brakish water - Finland	Vaara et al. 1979
	PCC8806		Lagoon, Gabon	M. Magot unpublished
	PCC8806		Lagoon, Gabon	M. Magot unpublished.
	PCC8905		Brakish marshland	Thiery et al. 1991
	PCC8916		Brakish marshland	I. Thiery unpublished.
	PCC8966		Brakish marshland	L. Nicolas unpublished.
	PCC8975		Brakish marshland	L. Nicolas unpublished.
	PCC8978		Brakish marshland	I. Thiery unpublished.
	PCC9213		Brakish marshland	L. Nicolas unpublished.

Group 12 - Aerobic chemolithotrophs

taxa	strain	accession number	source	Reference
Nitrococcus mobilis	ATCC25308t		Pacific Ocean 200 m off Fernandino Island Galapagos archipelago. Surface	Watson & Waterbury 1971 [AL]
	ATCC25308t	L35510		Teske et al. 1994
	Nc2	M96403		Head et al. 1993
Nitrosococcus oceani	ATCC19707t			(Watson 1965) Watson 1971 [AL]
=*Nitrocystis oceanus*	ATCC19707		Atlantic Ocean 370 km east of Long Island. 600m	Watson 1965
	C-107=Nc10	M96395		Head et al. 1993
	C-27=Nc9	M96398		Head et al. 1993
	C-56=Nm63	M96400		Head et al. 1993
	Nm22	Z46990		Pommerening-Roeser et al. 1996
Nitrospina gracilis	Nb-211		Atlantic Ocean, 200 miles off Amazon river mouth, Surface	Watson & Waterbury 1971 [AL]
	Nb-211	L35504		Teske et al. 1994
	Nb-3	L35503		Teske et al. 1994
Nitrospira marina	ATCC43039t			Watson et al. 1986 [VL]
	ATCC43039t		Gulf of Maine 206 m	Watson et al. 1986 [VL]
	Mb-295	X82559		Ehrich et al. 1995
	Mb-295	L35501		Teske et al. 1994

Group 13 - Budding and Appendaged

Caulobacter halobacteroides	ATCC15269		Filtered Seawater	Poindexter 1964 [AL]
Caulobacter maris	ATCC15268t		Filtered Seawater	Poindexter 1964 [AL]
Hyphomonas neptunium	ATCC29201t			(Leifson 1964) Moore 1984
=*Hyphomicrobium neptunium*	ATCC29201		Seawater from Barcelona Harbour, Spain	Leifson 1964
Pirelulla marina	ATCC49069t			Schlesner & P 1987
	IFAM 1313	X62912		Liesack et al. unpubl.
	ATCC49069t	RDP		Woese unpublished
=*Pirella marina*	ATCC49069t		Brackish waters	Schlesner 1986
Pirellula staleyi	ATCC27377t			Schlesner & P 1987
	DSM6068	X81946		Stackebrandt unpubl.
=*Pirella staleyi*	many strains		Brackish waters, hypesaline lagoon	Schlesner & Hirsch 1984
=*Planctomyces staleyi*	ATCC27377t	M34126		Woese and Oyaizu unpubl.
=*Pasteuria ramosa*	ATCC27377		Lake Lansing, Michigan	Staley 1973
Planctomyces maris	ATCC29201t		Neritic waters, Puget Sound, Washington	Bauld & Staley 1980 [VL]
	ATCC29201t			Bauld & Staley 1976
	ATCC29201t	X62910 S39798		Liesack & Stackebrandt 1992
Prosthecomicrobium litoralum	ATCC35022t		Littoral marine waters, Oregon and Washington	Bauld et al. 1983

Group 15 - Non photosynthetic non fruiting, Gliding

taxa	strain	accession number	source	Reference
Cytophaga latercula	ATCC23177t			Lewin 1969[AL]
	ATCC23177t		Outflow of marine aquarium	Lewin & Lounsbery 1969
	ATCC23177t	D12665		Nakagawa & Yamasato 1993
	ATCC23177t	M58769		Woese unpublished
Cytophaga lytica	ATCC23178t			Lewin 1969 [AL]
	ATCC23178t		Mud, Limon, Costa Rica	Lewin & Lounsbery 1969
	ATCC23178t	D12666		Nakagawa & Yamasato 1993
	ATCC23178t	M62796 M28058		Woese et al. 1990
	ATCC23174		Seawater, British Columbia, Canada	Lewin & Lounsbery 1969
Cytophaga marinoflava	ATCC19326t			Reichenbach 1989 [VL]
	ATCC19326t			Colwell et al. 1966
	NCIMB397	D12668		Nakagawa & Yamasato 1993
	ATCC19326t	M58770		Woese 1991 unpublished
=*Flavobacterium sp*	ATCC19326		North Sea off Aberdeen, Scotland	Spencer 1960
Flexibacter litoralis	ATCC23117t			Lewin 1969 [AL]
	ATCC23117t		Outflow of marine aquarium	Lewin & Lounsbery 1969
	ATCC23117t	M58784		Woese 1991 unpublished
Microscilla marina	ATCC23134t			(Prigsheim 1951) Lewin 1969[AL]
	ATCC23134t		Outflow of marine aquarium	Lewin & Lounsbery 1969
	ATCC23134t	M58793		Woese 1991 unpubl.

Group 16 - Myxobacteria

Myxococcus coralloides				Thaxter 1892 [AL]
	ATCC25202t		Soil	McCurdy & Wolf 1967
=*Chondrococcus coralloides*	ATCC25202	M94278		Shimkets and Woese unpubl.
	many strains		Bay of St. John, Virgin Islands. Surface	Brookman 1973
Myxococcus fulvus				(Cohn 1875)Jahn 1911 [AL]
	ATCC25199t		Soil	McCurdy & Wolf 1967
	many strains		Bay of St. John, Virgin Islands. Surface	Brookman 1973
Myxococcus stipitatus	Windsor M78		Dung	Thaxter 1897 [AL]
	many strains		Bay of St. John, Virgin Islands. Surface	Brookman 1973
Myxococcus xanthus	ATCC25232t		Dried cow dung	Beebe 1941 [AL]
	MD207	M34114		Oyaizu & Woese 1985
	many strains		Bay of St. John, Virgin Islands. Surface	Brookman 1973

Section 17 - Gram positive cocci

taxa	strain	accession number	source	Reference
Kytococcus sedentarius	DSM20547t	X87755		(ZoBell & Upham 1944) Stackebrandt et al. 1995
=*Marinococcus sedentarius*	CCM314t		Seawater	ZoBell & Upham 1944
Marinococcus halophilus	ATCC27964t			(Novitsky & Kushner 1976) Hao et al. 1985 VL
	ATCC27964t			Hao et al. 1984
	DSM20408t	X90835		Lawson et al. 1996
	HK713		Seawater	Hao et al. 1984
	NCIMB2178t	X62171 S49893		Farrow et al. 1992
=*Planococcus halophilus*	ATCC27964t		Salted Mackerel	Novitsky & Kushner 1976
Planococcus citreus				Migula 1894 AL
	ATCC14404t			Kokur et al. 1970
	NCIMB1493t	X62172 S49897		Farrow et al. 1992
=*Micrococcus aquivivus*	ATCC14404t		Seawater	ZoBell & Upham 1944
Planococcus okeanokoites	ATCC33414t	D55729		(ZoBell & Upham 1944) Nakagawa et al. 1996
=*Flavobacterium okeanokoites*	CCM320t		Seawater	ZoBell & Upham 1944
Staphylococcus haemolyticus	DSM20263t		Human skin	Schleifer 1975 AL
	DSM20263t	Z26896		MacLean et al. unpublished
	ATCC29970t	D83367		Takahashi et al. unpublished
	ATCC29970t	X66100		Ludwig et al. unpublished
	ATCC29970t	L37600		Wilson and Hills unpublished
=*Micrococcus halodurans*	ATCC15796		Seawater	Buck 1965

Section 13 - Endospore Forming Gram-positive Rods and Cocci

Bacillus licheniformis				(Weigmann 1898) Chester 1901 AL
	DSM13t		Dairy	Gibson 1944
	DSM13t	X68416		Ludwig et al. 1992
	NCDO1772	X60623		Ash et al. 1991
	B-6-4J	D31739		Nagashima unpublished
	LGM7627-32		Seawater	Bonde, unpublished.

Group 15 - Irregular Nonsporing Gram positive Rods

Corynebacterium glutamicum	ATCC23032t			(Kinoshita et al. 1958)Abe et al. 1967 AL
	DSM20300t	X80629		Rainey et al. 1995
	NCIB10025t	X84257		Pascual et al. 1995
	IAM12435t	X82061		Ruimy et al. 1995
=*Corynebacterium sp.*	NCIMB10025t	Z46753		Chun and Goodfellow unpubl.
=*Micrococcus glutamicus*	DSM46307		Seawater	Bousfield, I.J. unpubl.
	ATCC23032t			Kinoshita et al. 1958
	534t		Seawage	Kyowa Ferm. Inc. unpubl.

Genus not mentioned in Bergey's Manual of Determinative Bacteriology

Colwellia hadaliensis	BNL1t			Deming et al. 1988 VL
	BNL1t		Puerto Rico Trench 7410m Sediment trap 10m above seafloor	Deming et al. 1988
Filomicrobium fusiforme	ATCC35158t			Schlesner 1988 VL
	ATCC35158t		Kiel Bight, Baltic Sea 2m	Schlesner 1987
Gelidibacter algens	A296	U62916	Antarctic Sea Ice	Bowman et al. 1997
	A374	U62915	Antarctic Sea Ice	Bowman et al. 1997
	C8ST5	U62914	Antarctic Sea Ice	Bowman et al. 1997
Hirschia baltica	DSM5838t		Kiel Bight, Baltic Sea	Schlesner et al. 1990
	IFAM1418	X52909		Wolters unpubl.

taxa	strain	accession number	source	Reference
Mesophilobacter marinus	IAM13185t		Otsushi bay seawater. Indian Ocean strain also mentioned	Nishimura et al. 1989
Marinobacter hydrocarbonoclasticus	ATCC49840t	X67022	Sediments - Gulf of Fos, France Seawater also mentioned	Gauthier et al. 1992
Marinobacterium georgiense	ATCC700074t		Coastal seawater containing lignin rich pulp mill water.	Gonzalez et al. 1997
Microbulbifer hydrolyticus	ATCC700072t		Coastal seawater containing lignin rich pulp mill water.	Gonzalez et al. 1997
Polaromonas vacuolata	34-Pt	U14585	Antarctic marine waters	Irgens et al. 1989
Prochlorococcus marinus	SSW5	X63140		Urbach et al. 1992
Psychroserpens burtonensis	ACAM188	U62913	Antarctic Sea Ice	Bowman et al. 1997
	ACAM181	U62912	Antarctic Sea Ice	Bowman et al. 1997
	ACAM167	U62911	Antarctic Sea Ice	Bowman et al. 1997
Sagitulla stellata	ATCC700073t		Coastal seawater	Gonzalez et al. 1997

Species not in the aproved listof bacterial names or validation lists of bacterial name published outside of the IJSB

"Agrobacterium agile"	ATCC25651t		Seawater, Baltic Sea	Ahrens 1968
"Agrobacterium luteum"	ATCC25657t		Seawater, Baltic Sea	Ahrens & Rheinheimer 1967
"Agrobacterium kieliense"	ATCC25656t		Seawater, Baltic Sea	Ahrens 1968
"Agrobacterium sanguineum"	ATCC15918		Seawater, Baltic Sea	Ahrens & Rheinheimer 1967
"Achromobacter colinophagum"	ATCC25659t		Seawater enriched with coline	Shieh 1964
"Microscilla sericea"	ATCC28182t		Outflow of marine aquarium	Lewin & Lounsbery 1969
	ATCC28182t	M58794		Woese 1991 unpubl.
"Nitrosomonas aestuarii"	Nm36		Seawater, North Sea	Koops et al. 1991
	Nm36	Z46985		Pommerening-Roeser et al. 1996
"Nitrosomonas cryotolerans"	ATCC49181t		Kasitsna Bay, Alaska. Surface	Jones et al. 1988
	Nm55t	Z46984		Pommerening-Roeser et al. 1996
"Nitrosomonas halophila"	Nm1		Seawater, North Sea	Koops et al. 1991
	Nm1	Z46987		Pommerening-Roeser et al. 1996
"Nitrosomonas marina"	Nm22		Seawater, South Pacific	Koops et al. 1991