

Characterization of aquatic bacterial communities by plate count methods and Real-time quantitative PCR of 16S rRNA genes

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In order to characterize and compare complex aquatic microbial communities their position on an *r/K*-gradient was determined. The traditional method based on growth rates on agar plates to determine the distribution of *r*- or *K*-strategists in microbial populations has been compared with a molecular approach based on variable rRNA operon copy numbers in bacterial species with different life strategies. It is assumed that multiple copies of rRNA genes enable bacteria to achieve faster doubling times than those with just one or two. The relative abundance of rRNA encoding genes in the metagenome of a specific environment might reflect the stability and resource availability in that environment. The amount of 16S rRNA genes present in diverse aquatic environments was quantitated by real-time PCR and compared with the proportion of DNA encoding for the ubiquitous single copy gene RNase P.

Introduction

Microorganisms can be found in the most diverse environments including soil, air, in and on the human body and extreme environments like hot springs. In this study the application of a culture-independent molecular approach to characterize microbial communities within highly divergent environments is described. Environments can be characterized by their tendency to select for organisms that grow fast under uncrowded conditions where resource availability is not limiting (*r*-environments) or organisms that are adapted to crowded, resource limiting conditions (*K*-environments). *K*-environments are in general more stable than *r*-environments (Andrews and Harris, 1986). Hence, extreme *r*-strategist microbes are adapted to uncrowded, nutrient rich environments and in general they have a less efficient cell metabolism. They are also thought to be more sensitive for toxins in the environment (De leij et al., 1993). *K*-strategists are more adapted to crowded environments that have reached their carrying capacity and their cell metabolism is more efficient.

In ecological studies it may be interesting to determine the distribution of *r*- or *K*-strategists. In order to characterize a complex microbial community one could start with defining the environment since an active microbial community will develop according to its environmental pressure (Caldwell et al., 1997).

The plate count method as described by De Leij et al. (1993) has been demonstrated to provide an ecological fingerprint of bacterial communities from soil, rhizosphere and

seawater environments (Verschuere et al., 1997). This method is based on the time distribution of colony emergence after plating on solid medium (Salvesen & Vadstein, 2000). However, this method requires growth and can hence be limited by the nonculturability of many bacteria. Especially *r*-strategists are more likely to be non-culturable because they are in general highly adapted to a very specific environment.

Gene redundancy is not common in bacterial genomes, yet they often contain multiple copies of the Ribosomal RNA operon. Sequence analysis of the cloned 16S rRNA genes of *Clostridium paradoxum* revealed the presence of 15 rRNA operons (Rainey et al., 1996). Operon numbers up to 7 are commonly found, but ~40% of the organisms analyzed have either one or two operons.

It is assumed that multiple copies of rRNA genes enable bacteria to achieve faster doubling times than those with just one or two. Condon et al. (1995) suggested that the significance of the seven rRNA operons in *E. coli* is not so much to support maximal growth rates but rather to allow a rapid respond to fluctuating growth conditions. Klappenbach et al. (2000) observed that the number of rRNA operon copies is related to the capability of bacteria to respond to resource availability. Multiple rRNA operons could provide an advantage under fluctuating conditions (*r*-environments), while the constitutive expression of multiple rRNA genes would confer a metabolic burden on slower growing cells (*K*-strategists) due to the overproduction of ribosomes (Fegatella et al. 1998, Klappenbach et al. 2000).

If rRNA operon copy numbers reflect the ecological strategy of bacteria in response to resource availability, the relative abundance of rRNA encoding genes in the metagenome (Venter et al., 2004) of a specific environment might reflect the stability and resource availability in that environment. Comparison of the total rDNA amounts with the total gDNA amounts or a ubiquitous single copy gene allows the positioning of a microbial community on an *r/K*-gradient.

In this study the relative abundance of 16S rRNA encoding DNA among the total amount of genomic DNA from diverse aquatic environments was assessed by real-time PCR.

The total gDNA was extracted from a water sample from a waste water treatment facility (WWTF), a surface water sample from a stratified lake and, a marine water column sample.

The relative amount of 16S rDNA was compared with the amount of DNA encoding for the single copy gene ribonuclease P (RNase P) (Brown et al. 1996, Hsieh et al. 2004). The results obtained with this molecular approach were compared with plate count observations. The amounts of 16S rRNA and RNase P genes present in the mixed gDNA extracted from three bacterial species with relatively high rDNA operon copy numbers was compared by real-time PCR with a mix of gDNA extracted from three low copy number species.

Materials & Methods

Sample collection

Three sampling sites were selected to represent diverse aquatic environments. Two liter samples were collected with a portable peristaltic pump (Geopump™, Geotech Inc.) from the surface water of an aerated lagoon of the municipal waste water treatment facility in

Falmouth (Massachusetts, USA), Siders Pond, a small coastal salt pond in Falmouth, and Buzzards bay, an inlet of the Atlantic Ocean in southeastern Massachusetts (Figure 1). All samples were collected between June and July 2004. All water samples were processed within 6 hours after collection.

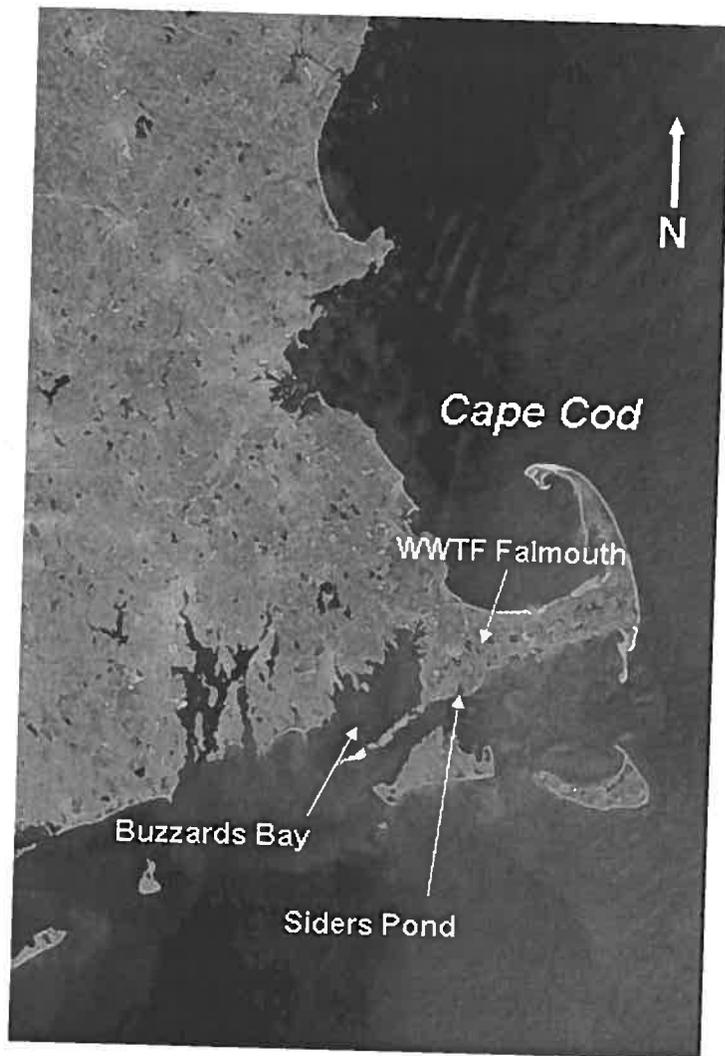


Fig.1. Cape Cod, MA, USA, with the location of the three sampling sites.

Bacterial strains

Bacterial strains with varying rRNA operon copy numbers were selected from the Ribosomal RNA Operon Copy Number Database (rrndb) (<http://rrnd.cme.msu.edu>). The rrndb is an internet accessible database that contains information on rRNA operon copy numbers among prokaryotes (Klappenbach et al., 2001). *Vibrio natriegens*, *Chromobacterium violaceum* Bergonzini and *Eschericia coli* K12 were ordered from the American Type Culture Collection (ATCC) (<http://www.atcc.org>). *Pseudomonas aeruginosa* PAO1, was kindly provided by Jean Huang (California Institute of

Technology, USA), *Lactobacillus brevis* was kindly provided by Craig Oberg (Weber State University, USA) and *Sphingomonas alaskensis* was kindly provided by Thomas M. Schmidt (Michigan State University, USA). (Table.1) All strains were grown overnight in 50 ml cultures.

Organism	Designation	rRNA c.n.	ATCC#	Medium	T
<i>Vibrio natriegens</i>	-	13	14048	marine broth, ATCC 2216	25 °C
<i>Chromobacterium violaceum</i>	Bergonzini	8	12472	Luria-Bertani broth (LB)	32 °C
<i>Escherichia coli</i>	K-12	7	10798	(LB)	32 °C
<i>Pseudomonas aeruginosa</i>	PAO1	4	baa-47	(LB)	32 °C
<i>Sphingomonas alaskensis</i>	RB2256	1	-	R2B medium (Difco)	25 °C
<i>Lactobacillus brevis</i>	-	2	8290	M.R.S. broth	25 °C

Table. 1. Bacterial strains with variable rRNA operon copy numbers (c.n.).

DNA extraction

Water samples of 500 ml were pre-filtered through a 11µm glass microfibre filters (Whatman International) and subsequently filtered through a 0.2 µm membrane filter (Millipore). DNA was extracted from the biomass accumulated on the membrane filters using the UltraClean Soil DNA Isolation Kit (MoBio) as described by Stepanauskas et al. (2003). The filters were transferred to a 50 ml sterile plastic tube together with 1.5 ml of lysis buffer (40mM EDTA, 50 mM Tris [pH 8.2]). One portion of lysis beads, 550 µl of cell lysis solution, 60 µl of S1 solution and 200 µl of IRS solution were added to the tube followed by 15 min of vortexing at maximal speed. The resulting slurry (450 µl of) was transferred to 1.5 ml sterile Eppendorf tube. DNA extraction was completed according to manufacturers protocol.

A 1 ml sample of each overnight culture was centrifuged for 1 min at maximum speed. The pellet was resuspended in 550 µl of cell lysis solution and transferred to a Bead Tube. DNA extraction was completed according to manufacturers protocol (Ultra Clean soil extraction kit, MoBio). All DNA extracts used for Real-Time PCR were diluted to a final concentration of 2.5 ng/µl.

Plate count assay

Dilution series were made from each pre-filtered water sample. 100 µl of each dilution was plated out on solid agar plates. The WWTF sample and the Siders Pond sample were plated out on R2A Agar plates (Difco) the Buzzard Bay sample was plated out on Sea Water Complete (SWC) medium plates (750 ml seawater, 250 ml distilled water, 5 g Bacto tryptone, 3 g yeast extract, 3 ml glycerol, 20 agar,; [pH 7.0]). Plates were incubated at room temperature for six days. Each day colonies that were visible were marked and

enumerated. Plates that contained between 1 and 250 colonies were selected for enumeration. When plates became too crowded, a higher dilution was used for enumeration. The number of visible colonies counted each day was expressed as a percentage of the total count after six days. The colonies that became visible within 48 hours were defined as fast growers.

Growth curves

Three strains with a diverse 16S rRNA operon copy number were subjected to a growth curve analysis. The optical density of the culture at 600 nm (OD^{600}) in 1 ml of a 100 pure culture of *Vibrio natriegen*, *Escherichia coli* and *Sphingomonas alaskensis* was determined at different time points with a Smartspec plus photospectrometer (Biorad). The OD^{600} values were plotted against time in a graph (Figure 2).

Real-time quantitative PCR

The 7300 Real Time PCR System (Applied Biosystems) was used to enumerate bacterial 16S rRNA and RNase P genes in the environmental samples and in the mixes of extracted DNA from high copy number species and low copy number species. The high copy number mix consisted of the gDNA from *Vibrio natriegens*, *Chromobacterium violaceum* and *Escherichia coli* K12. The low copy number mix consisted of the gDNA extracted from *Pseudomonas aeruginosa* PAO1, *Sphingomonas alaskensis* RB2256 and *Lactobacillus brevis*. The Bacterial 16S amplification mixture contained 11 μ l distilled H_2O , 12.5 μ l IQ SYBR Green mix (Biorad), 0.2 μ l D16S-F (50 pmol/ μ l), 0.2 μ l D16S-R (50 pmol/ μ l) (Table 2), 2.5 μ l DNA. The RNase P amplification mixture contained 11 μ l distilled H_2O , 12.5 μ l IQ SYBR Green mix (Biorad), 0.2 μ l Bpi-F (50 pmol/ μ l), 0.2 μ l Bpi-R (50 pmol/ μ l) (Table 2), 3.5 μ l DNA. The following program was performed: 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting of 94°C for 30 sec, 52°C for 30 sec and 72°C for 45 sec. The program was followed by a melting curve analysis to determine the melting point of the formed double stranded DNA products.

The DNA from *Vibrio natriegens* in different dilutions was used to establish a standard curve for both primer pairs.

Primer	Target	Sequence	Reference
D16S-F	Bacterial 16S rDNA	5' cggtaatacgttcycgg 3'	Marcelino et al., 2000
D16S-R	Bacterial 16S rDNA	5' ggwtacctgttacgactt 3'	Marcelino et al., 2000
Bpi-F	Ribonuclease P	5' gaggaagtccliigc 3'	Brown et al., 1996
Bpi-R	Ribonuclease P	5' taagccggrttctgt 3'	Brown et al., 1996

Table 2. Primers used in this study.

Results

Plate count assay

The total number of colony forming units (cfu) per ml water sample was determined by calculating the total number of colonies after 6 days of incubation from 100 μ l diluted sample to 1000 μ l undiluted sample. The difference in numbers of cfu per ml between the three tested samples was huge (Table 3).

The numbers of visible colonies counted each day were transformed into percentages in order to compare the different environments (Figure 2).

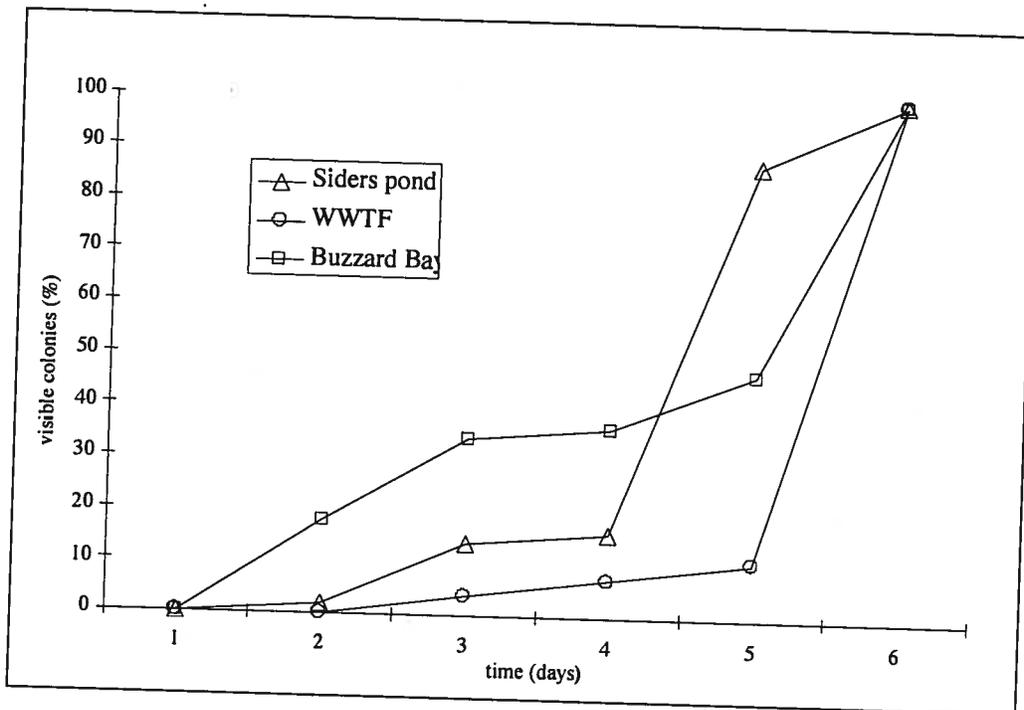


Fig. 2. Plate count assay. Proportions (%) of the total number of visible colonies appearing over a period of six days.

Sample site	cfu /ml water	medium
WWTF	1724185	R2B
Siders Pond	1110	R2B
Buzzards Bay	253	SWC

Table 3. Total counts obtained after 6 days of incubation expressed in colony forming units (cfu) per ml water sample.

Growth curves

The growth rates derived from the OD₆₀₀ measurements were very different for each strain. The growth rate of *Vibrio natriegens*, which contains 13 rRNA operon copies, was much higher than the growth rate of *Sphingomonas alaskensis*, which contains only one operon. The growth rate of *E. coli* (8 copies) was in between (Figure 3).

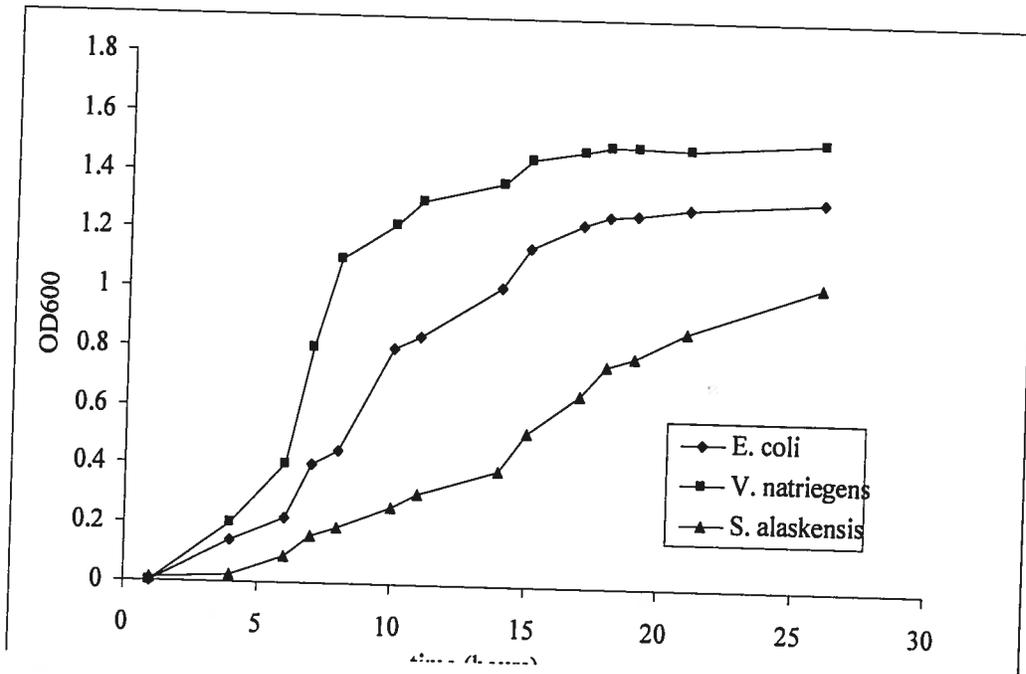


Fig. 3. Growth curves for *Vibrio natriegens*, *Escherichia coli* and *Sphingomonas alaskensis*

Real-time PCR

No results were obtained from the real-time PCR assays. The Quantitation of 16S rRNA and RNase P encoding DNA within the gDNA mixes and environmental DNA extractions and the establishment of a standard curve failed, probably due to low specificity of the primer sets and nonoptimal PCR programme conditions.

Discussion

When it is assumed that doubling times on their own are valid parameters to identify microorganisms as *r*- or *K*-strategists, then the results obtained from the plate count assay are remarkable. Since *r*-strategists are defined as fast growing organisms adapted to less stable and nutrient rich environments, it is surprising to find relatively more slow growers in the WWTF sample. The conditions in waste water are fluctuating and the resource availability is very high, hence one would expect this environment to be more *r*-selective. Since the marine water column offers a very stable environment where resources are limited one would expect this environment to be more *K*-selective.

However, it must be noted that by transforming the plate counts into percentages information about nutrient availability in the environment is partly lost. Therefore, it should be noticed that the high total cfu count on the WWTF plates represents the nutrient richness of this environment. The low counts on the Buzzard Bay plates indicate that this environment is relatively poor or that other conditions are adverse for microbial growth.

The growth curve assay seems to confirm again that there is a correlation between growth rate and rRNA operon copy numbers (Klappenbach et al., 2000).

Venter et al (2004) obtained that when methods relying on rRNA were used to assess the species diversity in seawater samples collected from the Sargasso Sea, abundance was often overestimated due to the varying number of copies of rRNA genes between taxa.

Although this study was unable to deliver the proof of principle, it still seems challenging to evaluate this method in further detail in future research.

Considering the Real-time PCR assay it's obvious that primers and PCR conditions need to be optimized in order to apply this method. Assessment of the primer specificity is highly recommendable. The total amount of extracted DNA should be quantitated and normalized for each sample, although DNA quantitation by photospectrometry might not be accurate enough. Instead the fluorometer method for dsDNA quantitation using PicoGreen (Turner BioSystems) would be a more suitable alternative.

It should be considered that the genome size of the organisms also influences the relative amount of 16S rRNA genes within the total gDNA. Therefore, normalization to a single copy gene would be favorable.

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