

ABSTRACT

Title of Document: IN SITU ACTIVITY OF NAC11-7
ROSEOBACTERS IN COASTAL WATERS
OFF THE CHESAPEAKE BAY BASED ON
FTSZ EXPRESSION

Daohong Yao, Master of Science, 2009

Directed By: Dr. Marcelino T. Suzuki
Marine Estuarine Environmental Science

Phylogenetic analysis of sequences of the cell division gene *ftsZ* retrieved from the Atlantic coast revealed an interesting subgroup NAC11-7, which was targeted by a specifically designed and optimized Taqman assay in diel samples collected *in situ* and in parallel on-deck incubations. Rapid changes of *ftsZ* gene copies and the patchy distribution of other phlotypes at different time points suggested that different NAC11-7 populations were sampled. Strong correlations between *ftsZ* expression and gene abundance (r-squared=0.62), and between *ftsZ* expression and water temperature (r-squared=0.73) for *in situ* samples suggested non-synchronous growth of NAC11-7 group. Contrastingly, a sharp 9:00 AM peak of *ftsZ* expression in the on deck incubation experiment suggested synchronous growth. We propose a possible mixed model in which a certain fraction of the population is synchronously dividing, while a background of asynchronously dividing NAC11-7 cells also exist, some of which are expressing *ftsZ* at any given time.

IN SITU ACTIVITY OF NAC11-7 ROSEOBACTERS IN COASTAL WATERS
OFF THE CHESAPEAKE BAY BASED ON FTSZ EXPRESSION

By

Daohong Yao

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Advisory Committee:
Assistant Professor Marcelino T. Suzuki, Chair
Assistant Professor Byron Crump
Professor Diane K. Stoecker

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Table of Contents

Acknowledgements.....	ii
Table of Contents.....	iv
List of Figures.....	vi
Title of Thesis.....	1
Abstract.....	2
Chapter 1: Introduction.....	3
Measurement of bacterioplankton growth rate.....	3
Cell division gene <i>ftsZ</i>	5
The Roseobacter group.....	5
Quantification of gene expression.....	6
Real time PCR.....	8
Chapter 2: Materials and Methods.....	11
Sampling.....	11
Nucleic acid extraction and quantification.....	13
Construction of <i>ftsZ</i> DNA PCR clone library.....	15
Real Time PCR Assays.....	18
Design.....	18
Optimization:.....	19
Primer concentration.....	19
Optimized Real Time PCR assays.....	20
Optimization of Reverse Transcription.....	21

ARISA (Automated rRNA intergenic spacer analysis)	22
Chapter 3: Results	24
Clone library and Phylogeny of Rhodobacterales <i>ftsZs</i>	24
Real time PCR and RT-PCR assays for NAC11-7 <i>ftsZ</i>	26
Diel expression of NAC11-7 <i>ftsZ</i> genes: in situ	29
ARISA results	32
Diel expression of NAC11-7 <i>ftsZ</i> gene: on board incubations.....	34
Chapter 4: Discussion	36
Chapter 5: Conclusions	43
Bibliography	46

List of Figures

Fig.1. Map showing the Southern section of the Chesapeake Bay and the study sampling site RM6.

Fig.2. Bayesian phylogenetic tree of FtsZ protein sequences from the *Rhodobacterales* group reconstructed using MrBayes v3.1. FtsZ sequences retrieved in this study are marked in boldface and labeled as “clone ftsZ05xx”, where xx represents the clone position in 96-well plate. The numbers within the parentheses indicate the FtsZ sequences retrieved from our study. The numbers on nodes represent branch confidence values.

Fig.3. NAC11-7 group: A. FtsZ amino acid sequences; B. *ftsZ* gene sequences.

Fig.4. *ftsZ* gene copy numbers (filled squares) and expression (estimated from the ratio of *ftsZ* cDNA copies to gene copies; open circles) at station RM6 on September 3 and 4, 2005, and tidal heights at Ship Shoal Inlet (37° 13'N, 75° 48'W). Error bars represent the standard deviation of triplicate samples. Pre-dawn and post-dusk sampling times are represented by the grayed areas.

Fig.5. Surface temperature (open circles) and salinity (solid squares) at station RM6, with overlaid tidal heights at Ship Shoal Inlet (37° 13'N, 75° 48'W).

Fig.6. Temperature and Salinity plot, with overlaid *ftsZ* NAC11-7 expression measurements at RM36. The *ftsZ* expression levels were in proportional to the area of the circles.

Fig.7. Percentage of different phylotypes represented by ARISA fragment sizes for *in situ* samples.

Fig.8. *ftsZ* gene copy numbers (filled squares) and expression (estimated from the ratio of *ftsZ* cDNA copies to gene copies; open circles) in samples collected at 6:00 AM on 09/03/2005 at station RM6 and incubated onboard. Error bars represent the standard deviation of triplicate samples.

In situ Activity of NAC11-7 roseobacters in Coastal Waters off the
Chesapeake Bay based on *ftsZ* Expression

Abstract

Measurements of *in situ* growth rates of specific bacterioplankton groups are of critical importance to the understanding of their contributions to the flow of energy and matter in the Ocean. Quantification of *in situ* expression of cell division gene is a possible approach towards these measurements. In order to test assumptions related to this approach regarding synchronous growth, an *ftsZ* library was constructed using primers targeting genes from *Rhodobacterales* and a phylogenetic analysis of *ftsZ* retrieved from the coast off the Chesapeake Bay revealed genes belonging to the widespread NAC11-7 subgroup. This subgroup was targeted by a specifically designed and optimized real time assay in diel samples collected *in situ* and in parallel on deck incubations. Rapid changes of *ftsZ* gene copies and the patchy distribution of co-occurring phlotypes at different time points suggested that different NAC11-7 populations were sampled *in situ*. Strong correlations between *ftsZ* expression and gene abundance ($r\text{-squared}=0.62$), and between *ftsZ* expression and water temperature ($r\text{-squared}=0.73$) for *in situ* samples suggested non-synchronous growth of NAC11-7 group. Contrastingly, a sharp 9:00 AM peak of *ftsZ* expression in the on deck incubation experiment suggested synchronous growth. We propose a possible mixed model in which a certain fraction of the population is synchronously dividing, while a background of asynchronously dividing NAC11-7 cells also exist, some of which are expressing *ftsZ* at any given time.

Chapter 1: Introduction

Measurement of bacterioplankton growth rate

Marine bacterioplankton play an important role in microbial food webs and the cycling of organic matter with global consequences (Azam, 1998; Wohlers *et al.*, 2009). In recent years the application of molecular and genomic techniques has provided us with vast genomic and metagenomic datasets, greatly expanding our knowledge of bacterioplankton phylogenetic diversity and their environmental distributions (for reviews see Rappé and Giovannoni, 2003 and Delong, 2009). Despite this knowledge, challenges still exist to the understanding of ecological functions to specific bacterioplankton. Among these challenges is the current lack of measurements of *in situ* growth rates of specific bacterioplankton ecotypes. This information is essential for the understanding of the contributions of specific organisms to biogeochemical cycles critical to ecosystem functions.

The most common approaches for determining growth rates of heterotrophic marine bacterioplankton are indirect and rely on measurements of bacterial production. Growth rates are calculated from bacterioplankton production, which are usually estimated from the incorporation of ^3H -thymidine or ^3H -leucine (Fuhrman and Azam, 1982; Kirchman *et al.*, 1985; Simon and Azam, 1989), or non-radioactive bromodeoxyuridine (Steward and Azam, 1999). These methods all regard the whole bacteria community as a “black box”

and measure the activity of the entire community. Therefore, they provide no information on the production and growth rates of specific bacteria in the community. While the combination of microautoradiography with fluorescence *in situ* hybridization (Micro-FISH) allows the assessment of specific bacterioplankton activities at narrower phylogenetic levels (i.e., Amann *et al.*, 2001; Moter and Gobel, 2000; Cottrell and Kirchman, 2003; Alonso and Pernthaler, 2005), these experiments still suffer from issues inherent to confinement and based tracer studies (i.e., bottle effects).

A possible approach to measure *in situ* activity, and perhaps growth rates of specific bacterioplankton, could involve the measurement of the expression (mRNAs) of genes coding proteins involved in cell division. The idea behind such approach would be to determine direct relationships between *in situ* production of these proteins and growth rates of specific bacterioplankton, without additions or incubations. The rationale is analogous to that of the measurement of the frequency of dividing cells (Hagstrom *et al.*, 1979), except that the measurement would be made at specific phylogenetic levels, and earlier stages in the cell cycle, thus avoiding problems associated with preferential protist grazing of dividing cells (Sherr and Sherr, 1992). Major assumptions of this type of approach would be that growth is not synchronous in the targeted organisms, and that the regulation of protein synthesis occurs at the transcriptional level.

Cell division gene *ftsZ*

Among proteins involved in bacterial cell division, FtsZ initiates cell division of most prokaryotes by self-assembling into a membrane-associated Z-ring structure and by recruiting other proteins to form the cell division septum (Margolin, 2005; Dajkovic and Lutkenhaus, 2006; Osawa and Erickson, 2006). In synchronized population of *Caulobacter crescentus* swarmer cells, the transcription of *ftsZ* gene is regulated by the cell cycle and *ftsZ* is activated concurrently with the initiation of DNA replication during the differentiation of swarmer cells into stalk cells, coordinated by global cell cycle regulators CtrA and DnaA (Kelly *et al.*, 1998; Laub *et al.*, 2000; Laub *et al.*, 2002; Hottes *et al.*, 2005). The expression of *ftsZ* genes was also found to be growth rate dependent in *Prochlorococcus* populations in the Gulf of Aqaba, with the maximum expression at the replication phase (S phase; Holtzendorff *et al.*, 2001; Holtzendorff *et al.*, 2002). These authors concluded that transcriptional regulation of *ftsZ* might be responsible for the synchronized cell division of *Prochlorococcus* populations. Finally, the sequence of FtsZ protein is highly conserved in most bacteria and *ftsZ* gene phylogeny has good congruence to 16S rRNA phylogeny (Vaughan *et al.*, 2004), allowing putative assignments of genes uncovered from the environment.

The *Roseobacter* group

The *Roseobacter* clade was chosen for our experiment due to the following reasons: first, this lineage (*Alpha-3* subdivision of *Proteobacteria*) is a ubiquitous and abundant group, broadly distributing in all the major ecological niches of the ocean and constituting more

than 20% of the total bacterial community (Suzuki *et al.*, 2001; Wagner-Dobler and Biel, 2006; Brinkoff *et al.*, 2008). Second, of all the major marine lineages, *Roseobacter* clade is one of the most easily cultured groups. Nearly one third of the known clade diversity has cultured representatives (Buchan *et al.*, 2005), making it one of the most accessible and intensively studied groups. Third, members of *Roseobacter* (roseobacters hereafter) are physiologically heterogeneous, playing a significant role in the global carbon and sulfur cycle and possibly influencing climate change. For instance, representatives of *Roseobacter* are aerobic anoxygenic phototrophs (AAnP), preserving a considerable amount of organic carbon by supplementing or substituting respiration with the light-driven generation of ATP and reductants (Kolber *et al.*, 2001; Yutin *et al.*, 2007; Jiao *et al.*, 2007). In northeastern US coastal waters, members of the roseobacter-associated clade (RAC) are the most active oxidizers of carbon monoxide - an indirect greenhouse gas -and might contribute up to 15% of the total CO oxidation (Tolli *et al.*, 2006) in coastal waters. Finally, roseobacters are actively involved in the degradation of dimethyl sulfoniopropionate (DMSP), either cleaving DMSP to the climate relevant gas-DMS or incorporating DMSP as carbon and sulfur source into food web (Kiene *et al.*, 1999; Moran *et al.*, 2003; Vila *et al.*, 2004; Malmstrom *et al.*, 2004). As the availability of a significant amount of genomic sequence information, studies of *Roseobacter* lineage are of particular importance to the understanding of ecosystem functions.

Quantification of gene expression

The quantification of taxonomic or functional genes and their expressions have increasingly been used to study microorganisms in natural environments (see review by

Sharkey *et al.*, 2004), after the realization that 1) more than 99% of bacteria seen under microscope are not cultivable using routine culture techniques (Kogure *et al.*, 1979; Kogure *et al.*, 1980), that 2) some abundant microorganisms in biogeochemical processes are only distantly related to or have no cultured representatives (Pace 1997; Rappé and Giovannoni 2003), and that 3) the knowledge obtained from laboratory experiments does not necessarily reflect microbial activities *in situ*. Gene expression measurements are based on the assumption that once a functional gene is expressed, its transcripts will be translated into a function protein. Therefore, understanding patterns of expression for genes involved in specific ecological roles and functions is necessary to assure that these genes are transcriptionally regulated and to understand the contribution of particular organisms within different biogeochemical cycles.

mRNA levels have been broadly used as surrogate for gene expression levels (Fey *et al.*, 2004; Bird *et al.*, 2005; John *et al.*, 2007). However, due to the labile nature of RNA, proper normalization is necessary to compensate for RNA losses during the process of cell lysis, RNA isolation, DNA removal, and reverse transcription steps (Johnson *et al.*, 2005). Moreover, changes in total mRNA levels do not always reflect transcriptional activity, as it might be caused by changes in cell numbers and consequently gene abundance (Pichard and Paul, 1993; Wyman, 1999), which emphasizes the importance of data normalization. In my study, I processed all the samples in parallel and assumed that the loss rate in all the processes were proportionally the same (Shi 2004). Furthermore, mRNA was normalized to the corresponding *ftsZ* gene concentration, accounting for possible changes in mRNA levels due to variations in cell abundance.

Various methods have been used to quantify mRNA, including Fluorescence *in situ* hybridization (FISH) (Wagner *et al.*, 1998; Pernthaler and Amann, 2004), DNA microarrays (Roth, 2002; Dennis *et al.*, 2003, Sharkey *et al.*, 2004), and reverse transcription quantitative PCR (RT-Q-PCR) (i.e., Church *et al.*, 2005; Bustin *et al.*, 2005; Smith and Osborn, 2009). Among these methods, RT-Q-PCR is one of the most extensively used methods in environmental microbial ecology research due to its sensitivity, specificity and simplicity. Q-PCR is especially suitable for environmental samples that are in low abundance and therefore was used in this study. Compared to traditional endpoint PCR, Q-PCR allows the quantification of gene (or transcript) numbers at the early exponential phase when they are proportional to the starting template amount, avoiding the inherent biases associated with the endpoint PCR due to PCR plateau effects (Livak *et al.*, 1995; Suzuki and Giovannoni, 1996).

Real time PCR

In Real time PCR (Q-PCR), the increase of amplicon numbers is recorded by the detection of a fluorescent reporter that indicates accumulation of amplicons in every cycle. Two common reporter systems are SYBR green and Taqman probes. SYBR green binds to all double-stranded DNA and emits fluorescent signal. This method is easy to operate and eliminates the complicated design of probes. However, the sensitivity and specificity is sometimes affected by non-specific binding, such as to primer-dimers. Therefore, extensive optimization is required before quantification and melting curve

analysis is necessary in every reaction to ensure the specificity (Sharkey *et al.*, 2004; Smith and Osborn, 2009). In contrast, Taqman assays utilize a sequence specific and fluorescent-labeled probe to quantify only DNA or cDNA sequences that are complimentary to the probe, which enhances specificity. The probe has a fluorescent reporter at the 5' end and a quencher molecule at the 3' end. In an intact probe, the reporter is prevented from fluorescing by the proximate quencher molecule through fluorescent resonance energy transfer (Livak *et al.*, 1995). During the annealing step in every Q-PCR cycle, primers and intact probe bind to their target sequences. As the *Taq* DNA polymerase elongates the primer strand along the template and approaches the probe, its 5' exonuclease activity will cleave the probe, which separates reporter dye and quencher dye, resulting in increased fluorescence signal (Holland *et al.*, 1991; Bustin, 2000; Zhang and Fang, 2006).

In this article, I studied *in situ* expression of *ftsZ* genes of the NAC11-7 subclade of the *Roseobacter* group. The NAC11-7 has putative roles in DMSP degradation (Zubkov *et al.*, 2001; Buchan *et al.*, 2005) and the genome sequence of NAC11-7 suggests this organism might in fact be an aerobic anoxygenic phototroph (i.e., it contains a *pufM* gene). I followed the diel variation in *ftsZ* expression to determine whether evidence exists for synchronicity in cell division. Gene expression was measured as the ratio of *ftsZ* mRNA and *ftsZ* gene copies using quantitative real time PCR (Q-PCR) and reverse transcription Q-PCR (RT-Q-PCR) in samples collected from a station in the Atlantic

coast of the USA near the mouth of Chesapeake Bay, as well as samples in parallel incubation experiments.

Chapter 2: Materials and Methods

Sampling

Surface water samples were collected every 3 hours from 6:00 AM local time on September 3 to 6:00 AM on September 4, 2005 at station RM6 (37°05.61N, 75°42.35 W) aboard the RVs Cape Henlopen using a rosette of Niskin bottles. Temperature and salinity profiles were taken simultaneously using a SBE 9CTD (Seabird, Bellevue, WA), and tidal effects were estimated from the height of the tide at Ship Shoal Inlet calculated using the data at <http://tidesandcurrents.noaa.gov/>. Triplicate 600 ml water subsamples for RNA, DNA and cell counts were collected in polycarbonate bottles (Nalge Nunc International Corp., Rochester, NY) at each sampling time. For each triplicate, 10 ml water was fixed in a 15 ml tube containing 500 µl formalin. The remaining 590 ml water was pre-filtered through GF/A filters (1.6 µm nominal pore; Whatman, Maidstone, UK). 90 ml of prefiltered water was filtered through 13 mm diameter 0.2 µm Supor200[®] polysulfone filters (Pall Corp., East Hills, NY) and transferred to a tube containing 130 µl lysis buffer. (2 mM NaEDTA (pH 8.0), 20 mM Tris•Cl (pH 8.0), 1.2% v/v Triton X100) the remaining 500 ml of prefiltered water was filtered through 25 mm diameter 0.2 µm Supor200[®] filters (Pall Gelman Inc.) and transferred to a screw cap tube containing 250 µl RNAlater (Ambion, Austin, TX). All samples were frozen at -20°C aboard and stored within a week at -70°C until nucleic acid extraction.

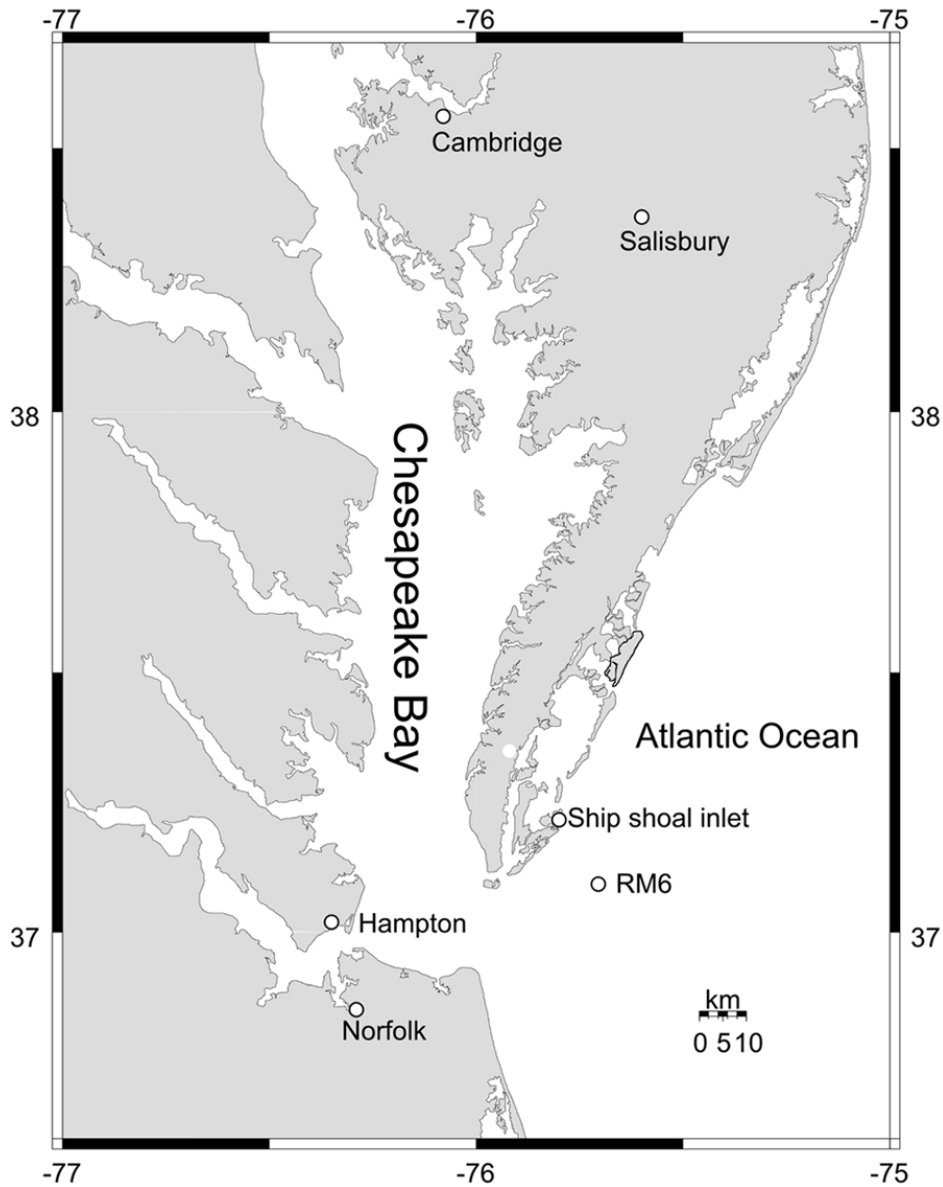


Fig.1. Map showing the Southern section of the Chesapeake Bay and the study sampling site RM6

Meanwhile, on-deck incubation experiments were conducted to control possible effects of sampling different populations *in situ*. At the first sampling time (time zero, hereafter:

6:00 AM, 03 September, 2005), 15 liter of surface water was used to fill 27 x 500 ml (600 ml total volume) clear polycarbonate bottles (Nalge Nunc), which were incubated in an on-deck incubator. At the same times that *in situ* samples were taken, three of the incubation bottles were taken and sampled for nucleic acids and cell counts as described for cast samples.

Nucleic acid extraction and quantification

Total DNA was extracted from Supor200[®] 0.2 µm filters using the DNeasy 96 Tissue Kit (Qiagen Inc., Valencia, CA) with modifications. Filters in tubes were thawed at room temperature and 50 µl lysozyme (72 mg/ml) was added into each tube, which was incubated for 1 hr at 37 °C. Five µl of Rnase A (6 U/µl, Sigma) was added into each tube, and incubated for 5 min at room temperature to digest RNA. 20 µl proteinase K (Qiagen) were added into each tube, and the mixture incubated for 30 min at 70 °C before the entire contents were transferred into a 1.5 ml tube and 410 µl of this mixture of buffer ALE was added into each of the 1.5 ml tubes, which were vortexed for 15 sec and the entire volume was transferred into wells of a DNeasy 96 plate. The DNeasy 96 plate was sealed with tape to prevent cross contamination and centrifuged in a Sorvall[®] Legend™ T Centrifuge with a Highplate[®] rotor (Kendro lab, Osterode, Germany) at 5250 rpm for 10 min at room temperature. The columns were washed with AW1 and AW2 buffer (Qiagen) at 5240 rpm for 5 min at room temperature following the DNeasy 96 kit protocol. The DNeasy 96 plate was incubated at 70 °C in an ISOTemp Incubator (Fisher Scientific Inc., Pittsburgh, PA), for 15 min evaporate traces of ethanol. DNA was finally

eluted from each column with 200 μ l TE buffer (pH 8.0, Ambion Inc., Austin, TX) by centrifugation at 5250 rpm, for 2 min.

Total RNA was extracted from Supor200[®] 0.2 μ m filters following an optimized protocol adapted from the Qiagen RNeasy[®] 96 manual (Shi 2005). Filters in screw cap tubes were thawed on the ice and 320 μ l (measured by filling a thin wall 0.6 ml PCR tube) Low Protein Binding Zirconium Oxide beads (200 Micron, OPS Diagnostics, Lebanon, NJ) were added into each tube. β -Mercaptoethanol was added in a 1:100 ratio to RLT buffer (Qiagen) and 875 μ l of the mixture was added to each of the screw cap tubes. The tubes were beaten in a MM301 mixer mill (Retsch GmbH Inc., Haan, Germany) at maximum speed (30.0 HZ) for 2 min and then incubated for 5 min at 70 °C. 800 μ l of the liquid phase was transferred into a new low-RNA-binding 2 ml microcentrifuge tube (Ambion, Inc.), avoiding transferring the beads. 800 μ l 100% ethanol was added into each tube and mixed well, resulting 1.6 ml crude lysate. 800 μ l of this mixture was transferred into wells of RNeasy 96 plate, which was sealed with airpore tape and centrifuged at 5000 rcf for 5 min at room temperature. The remaining 800 μ l of the crude lysate was added into the corresponding wells of RNeasy 96 plate and the centrifugation repeated. The columns were washed once with 800 μ l RW1 buffer and twice with 800 μ l RPE buffer at 5000 rcf for 5 min at room temperature, with the last spin for 15 min. 35 μ l Diethylpyrocarbonate (DEPC) treated water (Ambion) were added into each column, followed by 1 min incubation at room temperature and 5 min centrifugation at 5000 rcf. Another 35 microliter DEPC water was used to repeat this elution, resulting in c.a. 60 μ l RNA extracts. RNA was treated with deoxyribonuclease (DNase) I using the DNA-free[™] kit

(Ambion) to remove co-extracted DNA before the downstream reverse transcription and RNA quantification.

DNA and RNA concentrations were quantified fluorometrically by PicoGreen® staining and RiboGreen® staining respectively (Molecular Probes, Invitrogen Corp., Carlsbad, CA) on a Spectra MAX Gemini microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). For DNA quantification, the DNA standard (provided with PicoGreen® kit) was diluted in duplicate with TE buffer to generate a standard curve, ranging from 1000 pg/μl to 5.25 pg/μl and with a dilution factor of 0.35. RNA was quantified in a similar way using the RiboGreen kit, except for the following: RNA standard was diluted using DEPC treated water to generate the standard curve, ranging from 1000 pg/μl to 31.25 pg/μl and with a dilution factor of 0.5. Samples were diluted 1:100 using DEPC treated water before their measurements. Standards and samples were quantified by Ribogreen fluorescent dye on the same spectrofluorometer using the same software module.

Construction of *ftsZ* DNA PCR clone library

An initial *ftsZ* database was developed using *arb* (Ludwig *et al.*, 2004) by importing DNA sequences listed by Vaughan *et al.* 2004. 163 representative FtsZ sequences were exported and used to retrieve additional *ftsZ* DNA sequences using *blastp* searches against the March 2005 NCBI *nt*, *env_nt* and *wgs* databases. These sequences were translated and the amino acid sequence aligned using *clustalW* (Thompson *et al.*, 1994). An amino acid-based (144 homologous position) bayesian tree containing 507 sequences

was constructed using MrBayes version 3.0 (Ronquist and Huelsenbeck, 2003) with the following parameters: 800,000 generations, mixed models of amino acid substitution, and a burnin of 6000 trees. In this initial tree the *Alphaproteobacteria* formed a monophyletic clade with high confidence values and several orders including *Rhizobiales* and *Rhodobacterales* also formed monophyletic clades. This database was continually updated with public sequences belonging to the *Rhodobacterales*, and was used for the design of PCR primers and probes.

Based on the sequences in this database, a preliminary clone library was constructed using degenerate primers designed using CODEHOP (Rose *et al.*, 2003) to target all *Alphaproteobacteria ftsZs*: ftsZalphaF (5'-GCW GYN AAY CAN GAY GCN CA-3') and ftsZalphaR (5'-ACR TCN GCR AAR TCN ARR TT-3'). Preliminary results showed that among 48 sequences, 21 were *ftsZ* sequences, all belonging to the SAR11 clade (i.e., closely related to the *ftsZ* from *Pelagibacter ubique*) and none of them to the *Roseobacter* group. Therefore, our database was updated and I designed two degenerate primers targeting *ftsZ* from *Rhodobacterales* more specifically. FtsZ amino acid sequences of *Rhodobacterales* were exported and used to retrieve additional *ftsZ* DNA sequences using *blastp* searches against the March 2007 NCBI *nt* database. I retrieved 111 *ftsZ* DNA sequences that were imported to the existing ARB database and translated into amino acid sequences, which were aligned using the alignment tool in ARB_EDIT, followed by manual editing. In addition, the amino acid sequence of *Rhodobacter sphaeroides* was blasted against the GOS ORF PEPTIDE DATABASE (<http://camera.calit2.net/>) with 500 maximum sequence hits. These sequences were then retrieved from the NCBI database.

Among these 500 amino acid sequences, 232 sequences not closely related to *Candidatus Pelagibacter ubique* were added into the ARB database and aligned. The 343 new FtsZ amino acid sequences were added into the original bayesian tree using ARB_Parsimony. Primers ftsZrb2F (5'-AAY GCN GTS AAY AAY AT-3') and ftsZrb2R (5'-YTT NCC CAT YTC RT-3') were successfully designed and used to retrieve *ftsZ* gene sequences by PCR and cloning from a subsample from 6:00 AM on September 6, 2005.

Approximate 1 µl of extracted genomic DNA was used as template in a 10 µl-volume PCR reaction, which also included 10X PCR buffer, 0.2 mM of each dNTP, 3 mM MgCl₂, 500 nM Forward primer ftsZ2rbF, 500 nM Reverse primer ftsZ2rbR and 0.025 U/µl of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA). Reactions were performed on a GeneAmp 9700 PCR system (Applied Biosystems) and cycling conditions were as follows: 2 min at 94 °C and 37 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 2 min at 72 °C. The initial PCR was followed by a reconditioning PCR of 5 steps as recommended by Thompson et al. (2002). All reconditioned PCR products were loaded on a 1% modified TAE (40 mM Tris-acetate, pH 8.0, 0.1 mM Na₂EDTA) agarose gel and separated by electrophoresis. Target fragments were cut, recovered by Ultrafree®-DA (Millipore) gel extraction and used to build an *ftsZ* gene clone library using the TOPO™ TA cloning kit (Invitrogen) following the manufacturer's instructions. 96 clones were bidirectionally sequenced using BigDye V3.1 chemistry and capillary electrophoresis on an AB3100 genetic analyzer (Applied Biosystems Inc, Foster City, CA). 73 sequences identified as *ftsZ* were imported into the ARB database described above and added to the tree above using the ADD_BY_PARSIMONY tool.

A bayesian tree of all *Rhodobacterales* was constructed using Mr Bayes V3.1 (Ronquist and Huelsenbeck, 2003). 188 homologous amino acid positions from 123 sequences were exported and used in the phylogenetic analysis. Two parallel four chains of 2,000,000 generations were run with mixed models of amino acid substitution; trees were sampled every 100 generations, and 11000 “burnin” trees were excluded to generate the consensus tree. The average standard deviation of split frequencies was below 0.05 after 1,100,000 generations.

Real Time PCR Assays

Design

The primers and a Taqman probe were designed to target selected members of the NAC11-7 group. Results of *ftsZ* cloning and sequencing indicated that based on amino acid sequences five clone sequences were affiliated with the strain HTCC2255 *ftsZ*. However the DNA sequence of one of these clones (F3) contained obviously higher variation than the remainder of clones and HTCC 2255 (Figure 3). In accordance to the ecotype concept (Cohan, 2001), it appeared that the distinct clone might belong to a different ecotype, and was not subject to an assumed periodic selection event. In view of this possibility, the primers and probe were designed to exclusively target the remaining four sequences and HTCC 2255. The primer and the probe were manually designed with aid of probe match functions in the arb_edit module of the ARB package. T_M , secondary

structure and possible dimers were checked using Primer Express (Applied Biosystems) and the Oligo Analyzer online tool (www.idtdna.com).

Optimization:

To test specificity of primers to the target sequences, 3 target clones and 15 non-target clones were purified using QuickLyse Miniprep kit (Qiagen), and diluted to 10^7 *ftsZ* copies/ μ l with nuclease-free TE buffer (Ambion). Ten microliter PCR reaction contained 5 μ l TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.5 μ M Forward primer *ftsZrbA03-2qF* (GTG AAA AAG CTA CTG AGG GTC T) and Reverse primer *ftsZrbA03-2qR* (GCT TCC TGC CAG ATG ATC), and 1 μ l plasmid template. The cycling parameters were as follows: 2 min at 50 °C, 10 min at 95 °C and 30 cycles of 15 sec at 95 °C and 1min at 57 °C. All PCR products were loaded in a 2% NuSieve® (3:1) Agarose Gel (Cambrex, Rockland, ME), electrophoresed, and post-stained with 1:10,000 SYBR® Gold (Invitrogen) for 30 min. The gel was visualized with a FluoroChem 8900 (Alpha Innotech, San Leandro, CA).

Primer concentration

In order to get the highest amplification efficiency in real time PCR, a primer concentration matrix was performed. In each 25 μ l reaction, the following reagent concentrations were kept constant: 1X PCR buffer, 0.2 mM of dATP, dGTP and dCTP, 0.4 mM of dUTP, 5 mM MgCl₂, 200 nM probe NAC11-7 (AAC CAACAGTAGGAGCATTAGCCGCT), 1.2 μ M SuperROX™ (Biosearch Technologies,

Novato, CA), 0.01 U/ μ l AmpErase® Uracil N-glycosylase (UNG) (Applied Biosystems), 0.025 U/ μ l of Platinum® Taq DNA Polymerase (Invitrogen), 2.5 μ l NAC11-7 standard (10^4 copies/ μ l) and a matrix of forward and reverse primer concentrations of 100 nM, 500 nM, 1000 nM, 1500 nM. Reactions were set in a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems), which was sealed with an optical adhesive cover (Applied Biosystems) and ran in an ABI Prism 7000 Sequence Detection system, following the cycling parameters: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 57 °C. The primer combination yielding the lowest C_T was used in all subsequent measurements.

Optimized Real Time PCR assays

A plasmid containing cloned DNA that was purified and linearized as previously described (Suzuki *et al.*, 2000) was used to prepare standards for real time PCR for quantification of *ftsZ* gene and mRNA. 2.5 μ l of DNA extracts were used in 25 μ l reactions, containing 1X PCR buffer, 0.2 mM of dATP, dGTP and dCTP, 0.4 mM of dUTP, 5 mM MgCl₂, 1.5 μ M Forward Primer *ftsZ*rba03-2qF and 0.5 μ M reverse primer *ftsZ*rba03-2qR, 200 nM NAC11-7 probe, 1.2 μ M SuperROX™ (Biosearch Technologies, Novato, CA), 0.01 U/ μ l AmpErase® Uracil N-glycosylase (Applied Biosystems), and 0.025 U/ μ l of Platinum® Taq DNA Polymerase (Invitrogen). Standards ranged from 10^2 to 10^7 copies/ μ l and were run in duplicate along with non template controls. All reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following cycle parameters: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 57 °C. *ftsZ* cDNA was quantified in the same manner as *ftsZ* gene except that 5 μ l of reverse transcription products were used as

template. All real time PCR measurements were calculated as copy numbers per volume of seawater, assuming that nucleic acid extraction efficiencies were constant as shown in Shi (2004). mRNAs copies were assumed to be the same as cDNA copies. Since DNA and mRNA were measured in triplicate biological samples, *ftsZ* genes and mRNA copy numbers that were $>2X$ or $<X/2$ (X is the average of the remaining two replicates) were treated as outliers and removed from the analyses.

Optimization of Reverse Transcription

I tested whether different starting amount of template in reverse transcription would affect estimations of *ftsZ* cDNA copies/ μ l extracted RNA. Three different amounts (1 μ l, 2 μ l and 3 μ l) of RNA extracted from a single sample were used to synthesize cDNA in duplicate 10 μ l RT reactions. Briefly, template and 10 pmol of the reverse primer *ftsZ*rba03-2qR were heated to 65°C for 5 min and immediately chilled on ice, followed by the addition of 5 X cDNA synthesis buffers, 5 mM DTT, 2 U/ μ l RNaseOUT™ Inhibitor (Invitrogen) and 0.75 U/ μ l of ThermoScript™ Reverse Transcriptase (Invitrogen). Reactions were incubated at 52 °C for 55 min to synthesize cDNA and at 85 °C for 5 min to inactivate the transcriptase. 2.5 μ l of these RT products were used in subsequent 25 μ l real time PCR reactions.

Since preliminary results indicated that low copy numbers of *ftsZ* cDNA were added to Q-PCR reactions (lower than 100 copies/ μ l), I attempted to increase *ftsZ* cDNA copy numbers using two strategies: (1). Increasing the reverse transcription efficiency, by optimizing RT temperature and duration. (2). Adding increased amounts of cDNA

products in real-time PCR reactions. A 2.5 μ l RNA sample from the *in situ* diel sampling was used as template to synthesize cDNA in 10 μ l reactions. Reactions were conducted at two temperatures 52 °C and 55 °C and were run in triplicate using the NAC11-7 specific primer ftsZrbA03-2qR and ThermoScript™ Reverse Transcriptase for 60 minutes. Different RT incubation times (60 min, 90 min, and 120 min) were also tested and longer incubation times did not produce higher yields of *ftsZ* cDNA (data not show).

Four microliter purified RNA was reverse transcribed to cDNA using ThermoScript™ Reverse Transcriptase. Template and 10 pmol of the NAC11-7 specific primer ftsZrbA03-2qR were heated to 65 °C for 5 min and chilled on ice immediately, followed by the addition of 5X cDNA synthesis buffer, 5 mM DTT, 2 U/ μ l RNaseOUT™ Inhibitor (Invitrogen), 0.75 U/ μ l of reverse transcriptase and DEPC-treated water to a final volume of 10 μ l. Reactions were incubated at 55 °C for 60 min to synthesize cDNA and at 85 °C for 5 min to inactivate the transcriptase. No-RT controls were performed for one of the triplicate samples using the same method except substituting reverse transcriptase with DEPC-treated water.

ARISA (Automated rRNA intergenic spacer analysis)

One microliter of environmental genomic DNA was used to perform a 10 μ l ARISA reactions, containing 1X PCR buffer, 1.2 mM MgCl₂, 0.08 mM dNTPs (Promega Corp., Madison, WI), 0.5 μ M primer 1406F-FAM (M. M. Fisher and E. W. Triplett, 1999), 1.5 μ M primer 23S-Y (Dyda *et al.*, 2009), and 0.01 U/ μ l of Platinum® Taq DNA Polymerase (Invitrogen). Reactions were run on a GeneAmp 9700 (Applied Biosystems) under the

following conditions: Initial denaturation and enzyme activation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 65 °C for 2min. 1 µl of each PCR reaction was mixed with 9 µl of 1:0.06 formamide and GS2500 size standard (Applied Biosystems), denatured at 94 °C for 2 min and separated by capillary electrophoresis using an Applied Biosystems Genetic 3100 analyzer. Sizes of the fragments were analyzed by the Peak Scanner™ Software v1.0 (Applied Biosystems).

Chapter 3: Results

Clone library and Phylogeny of Rhodobacterales *ftsZs*

Degenerate primers were designed based on *Rhodobacterales* FtsZ amino acid sequences and retrieved gene sequences from a surface seawater sample of coastal Atlantic Ocean. An *ftsZ* gene clone library was built using the TOPO™ TA cloning kit and clones sequenced. Preliminary placement of sequences using *blastx* against the NCBI *nr* database showed that sequences of 76 out of 93 clones were those of *ftsZs*. Among these *ftsZ* sequences, 63 were associated with *Rhodobacterales*.

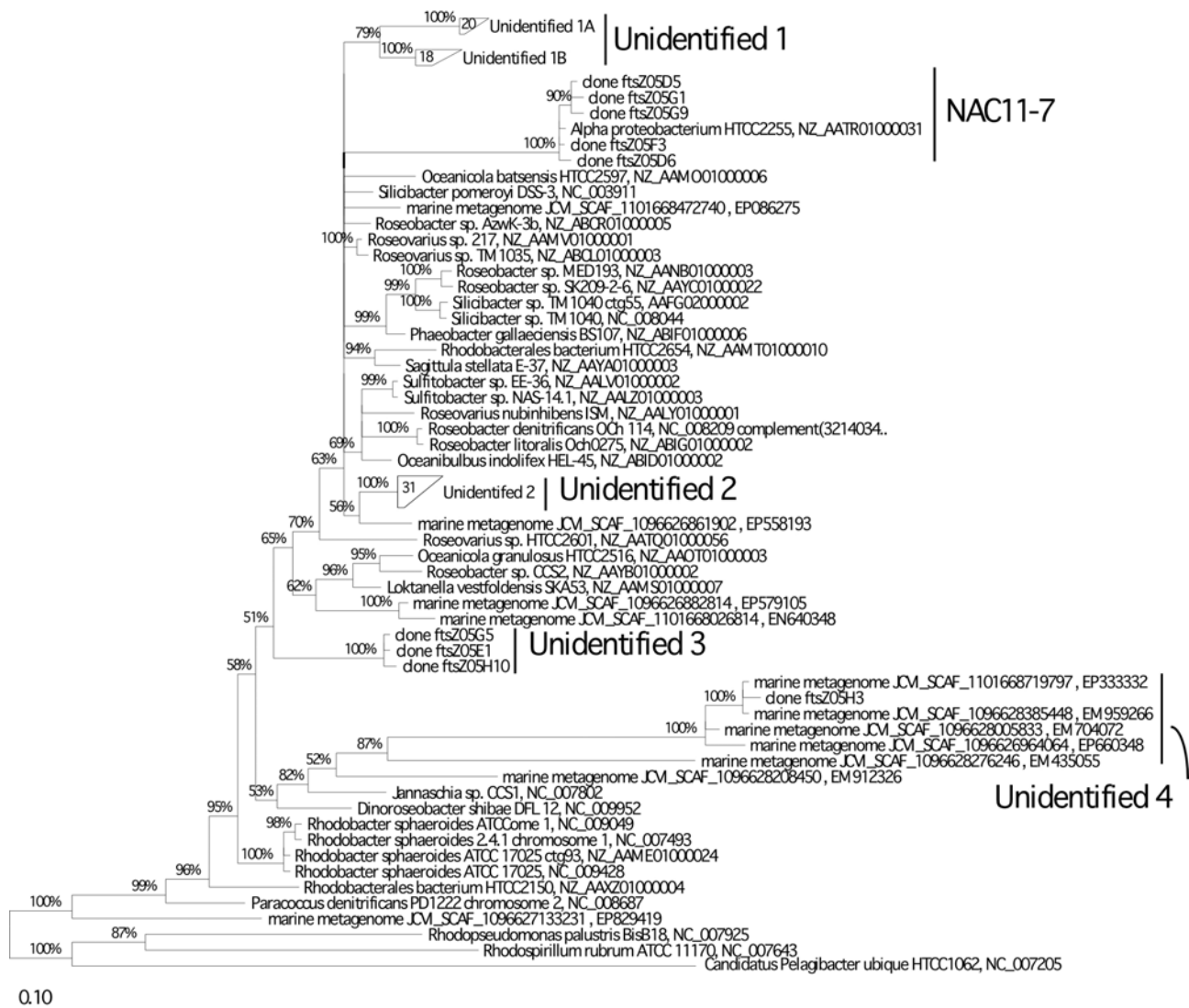


Fig.2. Bayesian phylogenetic tree of FtsZ protein sequences from the *Rhodobacterales* group reconstructed using MrBayes v3.1. FtsZ sequences retrieved in this study are marked in boldface and labeled as “clone ftsZ05xx”, where xx represents the clone position in 96-well plate. The numbers within the parentheses indicate the FtsZ sequences retrieved from our study. The numbers on nodes represent branch confidence values.

Diverse FtsZ amino acid sequences were retrieved in this study, as shown in the Bayesian FtsZ phylogenetic tree (Figure 2). FtsZ sequences retrieved from our experiment

clustered into 5 groups within the *Rhodobacterales*. One interesting group was related to strain HTCC 2255, which based on 16S rRNA phylogeny is a member of the NAC11-7 group (Buchan *et al.*, 2005). The other four groups were unidentified and clustered with sequences from the Global Ocean Survey (Rusch *et al.*, 2007). Surprisingly, a yet unidentified group (Group 2) contained 31 sequences, which represented more than 30% of the library, suggesting that this prevalent clade might be an important roseobacter of our sampling site.

Real time PCR and RT-PCR assays for NAC11-7 *ftsZ*

I developed a real-time PCR assay for *ftsZ* from a putative NAC11-7 ecotype. A primer specificity test showed that all targeted clones were amplified and produced clear single bands in an Agarose Gel (not shown), while no amplified bands were detected for 15 controls, including “clone *ftsZ*05F03”, which I assumed to belong to a different NAC11-7 ecotype (Figure 3). In addition to primer-led specificity, a Taqman fluorogenic probe was designed with no degeneracies to specifically target the selected NAC11-7 ecotype, further ensuring the specificity of our assay. The combination of 1500 nM Forward primer and 500 nM reverse primer showed the lowest C_T and the highest amplification efficiency in primer matrix experiments, and was used in all subsequent assays.

Fig.3. NAC11-7 group: A. FtsZ amino acid sequences; B. *ftsZ* gene sequences.

In order to test whether the efficiency of RT reactions was dependent of the starting amount of mRNAs, cDNA copy numbers were compared between RT reactions where increasing amounts of RNA were used as templates. A single factor ANOVA analysis showed that there was no significant difference ($P=0.172$) between *ftsZ* cDNA copies per μl extracted RNA, when 1, 2 and 3 μl of total extracted RNA were used in the RT reactions. Incubation temperature in reverse transcription reactions significantly affected the efficiency of reverse transcription and two-sample t-test (assuming equal variances) analysis showed that the number of copies *ftsZ* cDNA synthesized from RTs at 55 °C was significantly higher than that of 52 °C RTs. ($P=0.0037$). An inhibitory effect was observed when larger amounts of reverse transcription (RT) products were used as templates in real time PCR, seen as a decrease in the estimated copy numbers per microliter in RT products with increased volume of RT products as templates. No significant inhibition effect was observed when 5 μl and 2.5 μl RT products were used in the real time PCR (two-sample t-test $P=0.219$), while there was significant inhibition when 7 μl RT products were used in the real time PCR compared to 2.5 μl (two-sample t-test $P=0.0026$). Therefore, *ftsZ* cDNAs were measured using 5 μl RT products as templates.

Diel expression of NAC11-7 *ftsZ* genes: *in situ*

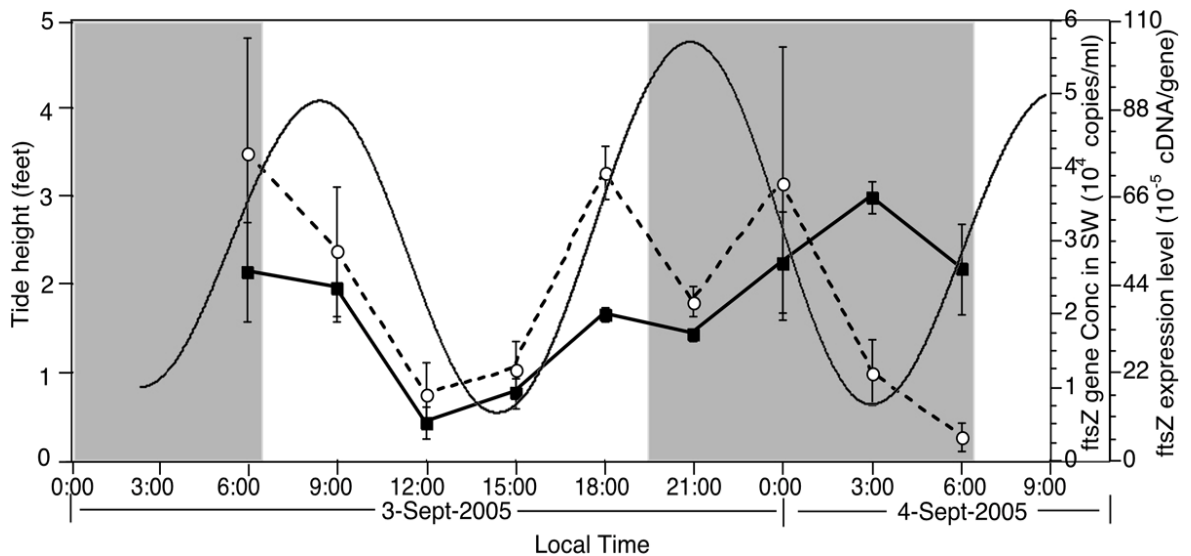


Fig.4. *ftsZ* gene copy numbers (filled squares) and expression (estimated from the ratio of *ftsZ* cDNA copies to gene copies; open circles) at station RM6 on September 3 and 4, 2005, and tidal heights at Ship Shoal Inlet (37° 13'N, 75° 48'W). Error bars represent the standard deviation of triplicate samples. Pre-dawn and post-dusk sampling times are represented by the grayed areas.

FtsZ transcriptional activity was measured as the ratio of *ftsZ* mRNA concentration in the seawater normalized to *ftsZ* gene concentration in the seawater (per gene normalization). Gene copies, and more remarkably, gene expression showed rapid and sometimes large (around four fold) changes with time (i.e., from 9:00 AM to 12:00 PM and from 6:00 PM to 9:00 PM), suggesting that distinct populations (i.e., patches) were sampled at different times as a result of water advection (Figure 4). Interestingly, except at the last two sampling times, *ftsZ* expression followed closely the *ftsZ* gene abundance trends. A regression analysis between *ftsZ* abundance and expression using individual replicates

showed significant correlation ($R^2= 0.6214$, $P=0.0003$) for the first 7 sampling times. However, after 3:00 AM on Sept 4, 2005 this trend was no longer visible and the correlation between *ftsZ* abundance and expression was not significant ($R^2=0.0304$, $P=0.45$) when all data points were included in the analysis. These results indicate that at least for some of the samples a correlation existed between abundance and per-cell activity.

In the first 12 h, *ftsZ* expression, and to some degree *ftsZ* copies appeared to be somewhat correlated with the tidal cycle, with peaks of expression at 6:00 AM and 6:00 PM and lowest expression at 12:00 PM, lagging the high tides and low tide onshore by about 3 hours (Figure 4).

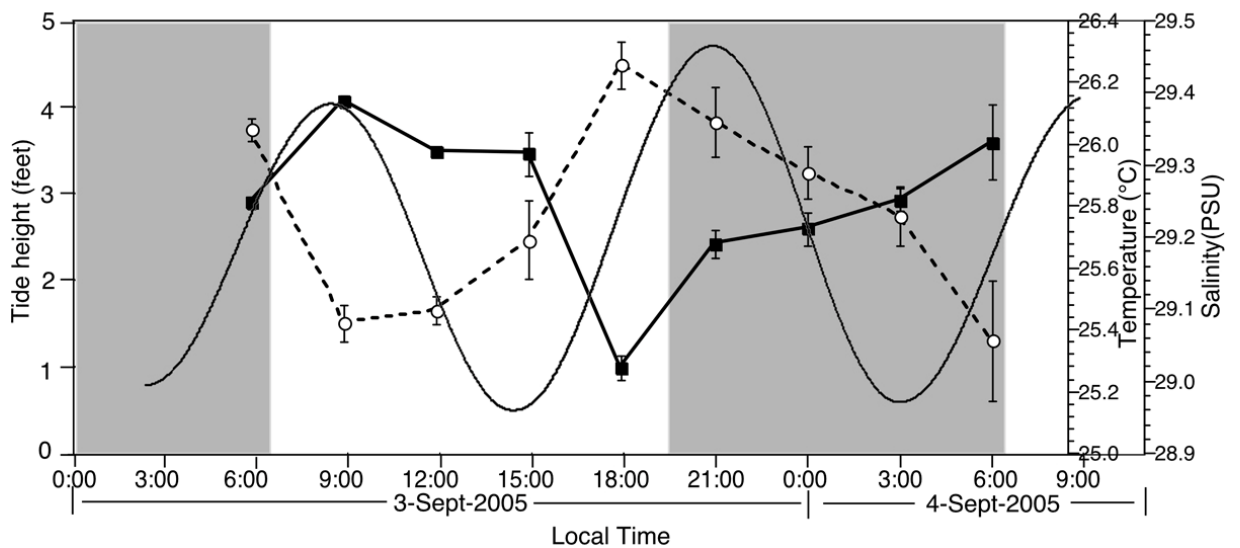


Fig.5. Surface temperature (open circles) and salinity (solid squares) at station RM6, with tidal height of Ship Shoal Inlet (37° 13'N, 75° 48'W) plotted on top.

Temperature and salinity also showed fluctuations that loosely followed the tidal cycle with a 3 h lag (Figure 5). For the first 12 h, surface temperatures were highest c.a. 3 h before the high tide on shore and lowest before the low tide. After 6:00 PM a steady drop in temperature and increase in salinity was observed. Overall the trends in salinity and temperature suggest that our sampling site was likely influenced by tidal currents and that different water masses with different communities and perhaps different NAC11-7 populations (or patches) were sampled (see ARISA results below).

In order to further examine the relationships between gene abundance and expression, and water masses, I overlaid gene abundance and expression values onto temperature-salinity (T-S) plots. These plots indicate a cyclical variation between warmer, less saline waters and cooler, more saline waters. While, gene copies did not show clear trends in the T-S plot (data not shown), *ftsZ* expression showed an interesting relationship where with the exception of the 9:00 AM sample, samples below 25.8 °C showed significant lower *ftsZ* expression (two-sample t-test $P=0.003$) than those above 25.8 °C (Figure 6). A regression analysis excluding 9:00 AM measurements showed a significant correlation ($R^2=0.725$, $P=0.007$) between *ftsZ* expression and temperature. Samples at 9:00 AM were excluded from this regression analysis after evidence for synchronous *ftsZ* expression was observed in the on-deck incubated samples.

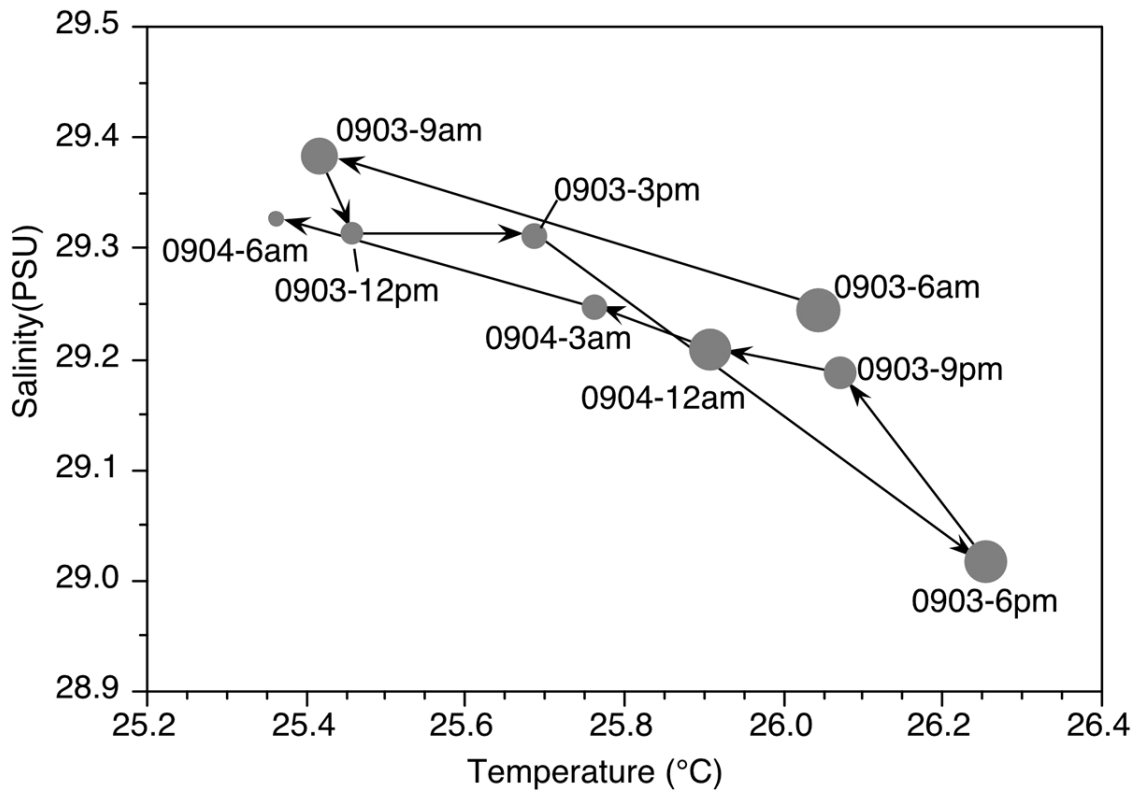


Fig.6. Temperature and Salinity plot, with *ftsZ* expression data of NAC11-7 group in 2005 Diel RM36 samples plotted on top. The *ftsZ* expression levels were in proportional to the area of the circles.

ARISA results

Finally, in order to examine the dynamics of bacterioplankton communities and populations at the different sampling times, these communities were examined using automated ribosome intergenic spacer analysis (ARISA).

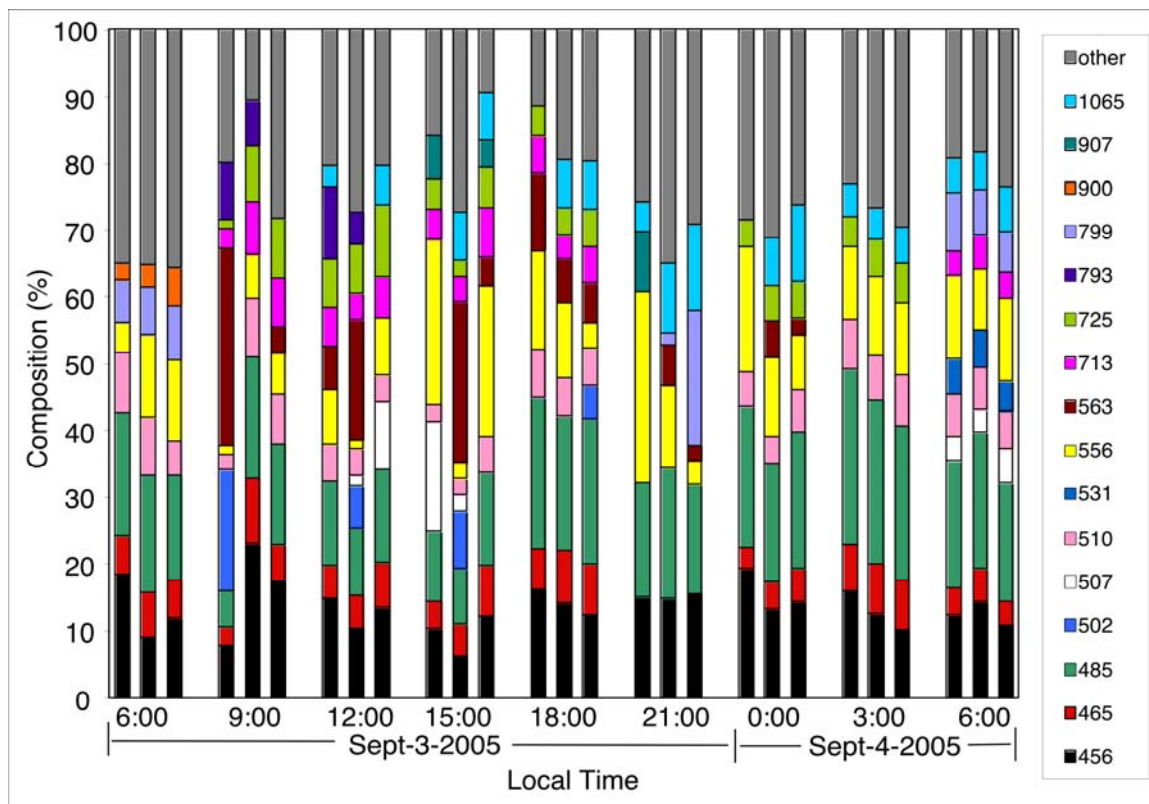


Fig.7. Percentage of different phylotypes represented by ARISA fragment sizes for 2005 diel *in situ* samples.

The results shown in Figure 7 indicate that although replicate subsamples were reasonably similar indicating low within-sample variation, in some cases (i.e., 9:00 AM, 9:00 PM) variations between replicates were quite obvious. I observed that a number of ARISA peaks were present during the entire sampling period, (i.e., 456, 485, 510 and 556 bp), while several peaks showed a more ephemeral occurrence (i.e., 793, 799, 900 bp), indicating that levels of patchiness were different for different phylotypes, and that at least for these phylotypes different populations (patches) might have been sampled at

different times. Unfortunately ARISA peaks corresponding to the NAC11-7 group were not detected, as these organisms were likely present in numbers below the resolution of ARISA.

Diel expression of NAC11-7 *ftsZ* gene: on board incubations

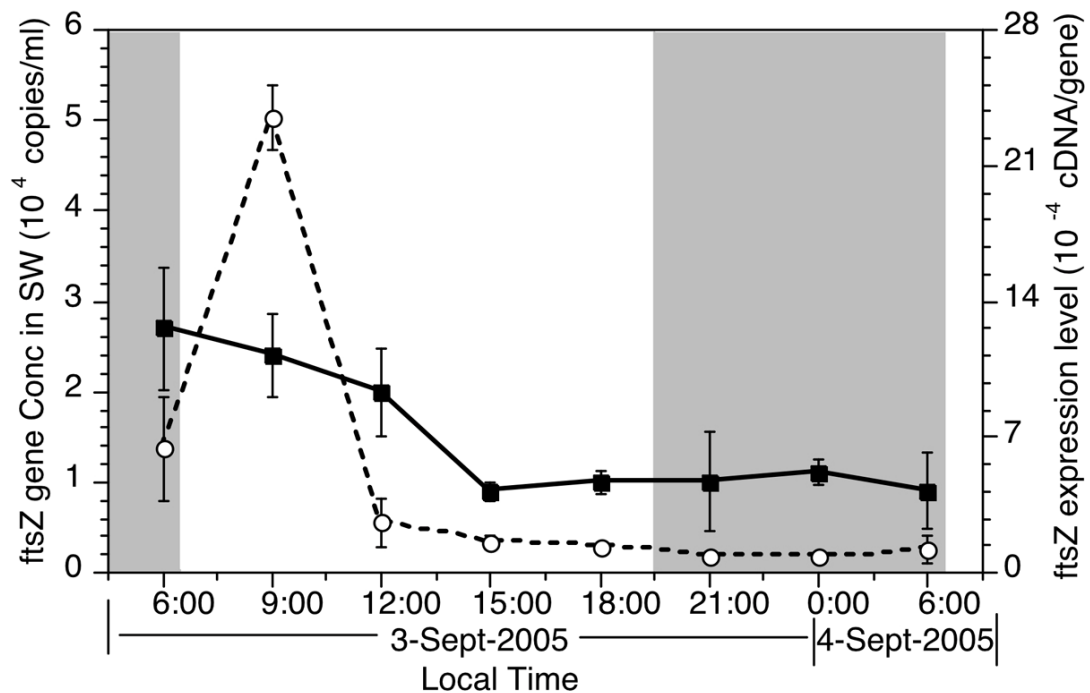


Fig.8. *ftsZ* gene copy numbers (filled squares) and expression (estimated from the ratio of *ftsZ* cDNA copies to gene copies; open circles) in samples collected at 6:00 AM on 09/03/2005 at station RM6 and incubated onboard. Error bars represent the standard deviation of triplicate samples at the same sampling time.

A parallel on-deck incubation experiment was conducted to control possible effects of advection and sampling of different bacteria populations (patches). *ftsZ* gene and transcripts in those incubated samples were quantified as for *in situ* samples and the results are shown in Figure 8. *ftsZ* gene abundance dropped from 6:00 AM to 3:00 PM

and stabilized at c.a. 10000 copies/ml (a 2.5-fold decrease). Contrastingly, *ftsZ* expression showed a very remarkable 4-fold increase in expression at 9:00 AM followed by a sharp 5-fold decrease at 12:00 PM. Considering that the measurements were made in three separately incubated bottles and that C_{TS} of the 9:00 AM cDNA samples were nearly two units lower than those of the remaining samples, I am very confident that this observation was not an experimental artifact.

Chapter 4: Discussion

In order to test assumptions related to the measurement of *in situ* growth rates of specific roseobacters based on cell division genes, a *Rhodobacterales ftsZ* gene clone library was built and phylogenetic analysis indicated sequences from two interesting subgroups were retrieved: a prevalent, yet unidentified group (c.a. 30% of the library) and the NAC11-7. Further analysis of the NAC11-7 sequences reveals that although all the sequences have similar amino acid sequences, one sequence *ftsZ5F03* was different at the DNA level (Figure 3). Based on the ecotype theory (Cohan, 2001), the “clone *ftsZ5F03*” clone was assumed to belong to a different ecotype, since it was not subjected to the theoretical selective sweep assumed to have happened in the remaining 5 phylotypes. Clone “clone *ftsZ05F03*” was therefore not targeted by our Q-PCR assay. Using this assay, *ftsZ* gene and transcripts of NAC11-7 group were quantified in diel *in situ* and on-deck incubation experiments.

It is important to emphasize that our measurements of expression are an average *per cell* for the whole population. Thus, depending on how *ftsZ* is expressed during the cell cycle in a single cell, the mRNA to gene ratio would in fact reflect: 1) the fraction of the population that is transcribing *ftsZ* [i.e., if expression only happens in a short time span during the cell cycle] 2) the average per cell expression level [i.e., *ftsZ* is constitutively transcribed] or perhaps 3) a combination of both [i.e., *ftsZ* has a basal level of expression, but transcription increases for a short span of time, related to cell division]. If

synchronous growth occurs in combination cases 1 or 3 above, one would observe peaks in the average *ftsZ* expression, as a large proportion of the population would be concurrently transcribing *ftsZ* in a short time span. In contrast, if case 2 above is true, one would not expect peaks of *ftsZ* expression even in synchronous populations. Finally even for synchronous populations, with time-constrained *ftsZ* expression, it is conceivable that one would observe "basal" *ftsZ* transcription reflecting expression by the non-synchronous fraction of the population. This last measurement would be in fact also correlated to the whole population growth rates, assuming that synchronous and non-synchronous cells are dividing at the same rates.

In the 2005 diel *in situ* experiment, several evidence indicate that different NAC11-7 populations (or patches) with different growth rates were likely sampled at different time points. First, remarkable fluctuations of NAC11-7 gene copy numbers (nearly 4 fold in 3 hours) were observed *in situ* (Figure 4), implying changes in populations resulting from advective processes. Second, ARISA analysis of the total bacterioplankton community structure showed evidence for patchiness, as certain phylotypes were only detected in specific time points (Figure 7). The fact that different subpopulations were sampled complicated our interpretation of the data, and currently it is not completely clear whether the cell division in NAC11-7 clade is or is not synchronous *in situ*.

During the first seven data points (6:00 AM, 9:00 AM, 12:00 PM, 3:00 PM, 6:00 PM, 9:00 PM, and 0:00 AM), a strong correlation ($R^2 = 0.6214$) was observed between NAC11-7 gene abundance and *in situ* expression (inferred from *ftsZ* copies/ml and *per*

cell ftsZ expression), suggesting that more active populations yielded or were associated with higher abundances of these organisms. This strong correlation also supports the case for non-synchronous cell division since *ftsZ* expression was not constrained to specific times. On the other hand, the correlation between abundance and expression was reversed at the last two data points (3:00 AM and 6:00 AM), with high gene abundance associated with low gene expression, indicating that some degree of temporal variation in gene expression also occurs. Interestingly, the plot of gene expression data over a Temperature-Salinity diagram showed that excluding 9:00 AM samples a high correlation between water temperature and *ftsZ* expression.

In addition to the good correlations between *ftsZ* gene expression and gene copies, and *ftsZ* gene expression and temperature, I found a remarkable peak of expression (not associated with a gene copy increases) at 9:00 AM in the on-deck incubation experiment, strongly suggesting that *ftsZ* expression (and by extension cell division) is synchronous in this possibly photoheterotrophic NAC11-7 ecotype. The fact that the *in situ* sample from 9:00 AM deviated from the correlation between gene expression and temperature (Figure 6) also seem to support this hypothesis. Remarkably, although there is peak *ftsZ* expression at 9:00 AM sample in the incubation experiment, expected increases of gene copy number in the subsequent samples were not observed (Figure 8). The drop in gene copy numbers from 6:00 AM sample to 3:00 PM sample suggest a large percentage of NAC11-7 group might have been grazed or lysed. A possible explanation for the disconnection between the increased *ftsZ* expression at 9:00 AM and lack of subsequent increment in copy numbers in these bottles is that cells were already preconditioned to

expressing *ftsZ* before the start of the incubation, but did not proceed to the division phase due to changes in conditions.

Prochlorococcus strains were found to grow synchronously in nature, tightly phased to the light-dark cycle, with DNA replication in the late afternoon and cell division at night (Vaulot *et al.*, 1995; Liu *et al.*, 1997, Partensky *et al.*, 1999). An explanation for this circadian growth of *Prochlorococcus* is that this division pattern might make efficient use of daylight hours for carbon fixation via photosynthesis in order to prepare for cell division during the night (Shalapyonok *et al.*, 1998). Synchronized expression of *ftsZ* gene has also been shown for *Prochlorococcus* spp. populations in the Red Sea, with maximum expression within the DNA replication phase in the afternoon, and transcriptional control of *ftsZ* was speculated to be a major factor triggering the synchronized cell division of *Prochlorococcus* (Holtzendorff *et al.*, 2001; Holtzendorff *et al.*, 2002). If NAC11 *ftsZ* is expressed at a limited time span, as in *Prochlorococcus* and *Caulobacter crescentus* (Kelly *et al.*, 1998), the single peak expression of *ftsZ* observed would indicate synchronous growth in NAC11-7. Ample evidence shows that at least in the case of the alphaproteobacterium *Caulobacter crescentus*, *ftsZ* is expressed during a limited period during the cell cycle (Quardokus *et al.*, 1996; Sackett *et al.*, 1998; Martin and Brun, 2000; Brun, 2001). On the other hand, FtsZ protein concentration per cell was also found to be constant regardless of growth rates in copiotrophic *E.coli* and *B.subtilis* (Rueda *et al.*, 2003; Weart and Levin, 2003; Haeusser and Levin, 2008).

Several arguments support constrained *ftsZ* expression in NAC11-7. As members of the *Alphaproteobacteria*, these bacteria are more closely related to *C. crescentus* than to *E.coli* or *B. subtilis*. In addition, the genome of HTCC2255 contains the gene coding for CtrA, a two-component signaling protein that coordinates *ftsZ* expression and DNA replication to ensure correct division in *C. crescentus* (Laub *et al.*, 2000; Laub *et al.*, 2002; Quardokus and Brun, 2003; Skerker and Laub, 2004). Finally, it is difficult to envision reasons NAC11-7, an organism adapted to somewhat oligotrophic conditions would synthesize a constant amount of FtsZ proteins through the whole cell cycle unless FtsZ has an alternative function in these organisms. Since members of NAC11-7 are putatively AAnPs, the cell cycling of this group might be triggered by irradiance levels, similar as in *Prochlorococcus* (Jacquet *et al.*, 2001). Although light is a possible factor regulating cell cycle in both NAC11-7 group and *Prochlorococcus* populations, there is a difference in the time of peak *ftsZ* expression between *Prochlorococcus* (afternoon) and NAC11-7 (morning). One possible reason would be differences in general metabolism between phototrophs and photoheterotrophs. AAnP do not fix significant amounts of inorganic carbon, using reduced organic carbon for anabolism. In addition, Bacteriochlorophyll (BChla) of many AAnP is mainly synthesized at night-time (Yurkov and Beatty, 1998). Therefore, it is probable that NAC11-7 synthesizes enough BChla at night prior to cell division.

A way to reconcile the somewhat contrasting results from the *in situ* and incubation experiments is a model in which a certain fraction of the population is synchronously dividing and expresses *ftsZ* at 9:00 AM, while a background of asynchronously dividing

cells also exist, some of which are expressing *ftsZ* at any given time. This "background" expression might in fact be better correlated to temperature or other parameters. The fact that a certain level of *ftsZ* expression was measured throughout the diel cycle of synchronously dividing *Prochlorococcus* populations (Holzendorf *et al.*, 2001) seems to support our model.

Although I have compelling evidence for synchronous growth, our sampling was limited to a single diel sampling, and a single geographic location, and therefore it is obvious that more studies are required to examine whether synchronized *cell division* occurs at some later time in the day. I was unable to observe such spike in cell division in the incubated bottles, since the population steadily decreased likely due to effects of confinement. Future *in situ* studies should also include Lagrangian sampling (Mariano *et al.*, 2002) to minimize advective effects, and to ensure the measurement in more coherent populations. To our knowledge this is the first indication that specific heterotrophic bacterioplankton grow synchronously in the environment, and if proven true, this will have very important implications for the study of bacterioplankton ecology, and raises interesting questions for future research. For instance, as bacterioplankton production is in most cases measured for the bulk community at short time incubations, and thus understanding whether synchronized and unsynchronized cells in single populations, or synchronous and non-synchronous species have the similar rate of cell division will be paramount to the interpretation of these measurements. Since there is putative evidence that some representatives of NAC11-7 are photoheterotrophic, understanding the relationships

between light-driven metabolism and synchronous growth in nature would also be of interest.

Chapter 5: Conclusions

In conclusion, I quantified the *in situ* expression of cell division gene *ftsZ* of NAC11-7 group in diel experiments and tested whether synchronized growth exists in the roseobacter group.

Specifically, two degenerate primers were designed successfully based on the existing *ftsZ* gene database of *Rhodobacterales* and used to construct a PCR clone library from one sample of coast Atlantic Ocean. 63 sequences were retrieved from the library and used to build a Bayesian phylogenetic protein tree, the analysis of which revealed an interesting group clustered with HTCC2255 (NAC11-7 group). A real time PCR (Taqman) assay was specifically designed and optimized to quantify *ftsZ* gene and transcripts of the NAC11-7 group in diel samples collected *in situ* and on deck parallel incubations. Since NAC11-7 was not abundant in our samples, I enhanced the efficiency of RT-Q-PCR (Reverse Transcription Quantitative Polymerase Chain Reaction) through the optimization of template amount and incubation temperatures. Those optimized procedures could be applied to other gene expression studies where the mRNAs of interest are in low abundance. In addition, we normalized *ftsZ* mRNA abundance (copies per volume seawater) to *ftsZ* gene abundance (copies per volume seawater) to account for changes of NAC11-7 abundances, which are not unexpected in the samples from the ocean.

From this study, we observed rapid changes of *ftsZ* gene copies which could not be explained solely by biological factors such as grazing or cell division. Moreover, our ARISA analysis of *in situ* samples showed patchy distribution of specific phylotypes at different time points. Both those facts above suggest different subpopulations (patches) of NAC11-7 group might have been sampled due to physical processes which complicated the interpretation of my data. I could not determine with certainty whether the changes of *ftsZ* expression was due to the change of transcription activity or the change of NAC11-7 populations. Therefore I suggest that a Lagrangian sampling scheme be used in future experiments to minimize advective effects and monitor more coherent microbial populations.

We found a strong correlation between *ftsZ* expression and gene abundance (r-squared=0.62), and between *ftsZ* expression and water temperature (r-squared=0.73) for *in situ* samples of NAC11-7 group. The fact that *ftsZ* expression was not confined to narrow time periods and was related by temperature to some degree suggests the existence of non-synchronous growth within NAC11-7 groups. However, we also found evidence for synchronous growth of this group. For instance, a sharp 9:00 AM peak expression of *ftsZ* gene was observed in the on-deck incubation diel samples and a deviation of *ftsZ* expression and water temperature was also detected at 9:00 AM time point sample in the *in situ* diel experiment. This is the first indication, to our knowledge, that heterotrophic bacterioplankton might have synchronized growth and if proven true, it

will have significant consequences to microbial ecology research. For instance, growth rates estimated from classic methods (tracer uptake) will probably be underestimates, since the incubation might be taken when certain cells are not dividing and do not synthesize DNA or protein. In order to confirm the existence of synchronized *cell division* in heterotrophic bacterioplankton, experiments including several diel cycles and at different geographic locations, and targeting specific bacterial cells should be performed. Moreover, one could design experiments to test whether the 9:00 AM peak expression of *ftsZ* in NAC11-7 incubated samples is caused by light (and not other bottle effects) by incubating parallel samples in dark conditions or by starting experimental setup from times other than 6:00 AM.

Finally, I propose a possible model, in which, a certain fraction of the NAC11-7 population is synchronously dividing, while a background of asynchronously dividing NAC11-7 cells also exist, some of which are expressing *ftsZ* at any time. The coexistence of two types of cell cycles within one closely related gene cluster or likely one ecotype might result from the complexity of seawater habitats where heterotrophic bacterial populations thrive. While a large proportion of the synchronized population might be free living, some cells might deviate from this cycle due to responses of different substrate conditions (i.e., in particles).

Bibliography

- Alonso, C. and J. Pernthaler (2005). "Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters." *Appl. Environ. Microbiol.* **71**: 1709-1716.
- Amann, R. I., W. Ludwig, and K. H. Schleifer (1995). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation." *Microbiol. Rev.* **59**:143–169.
- Amann R, Fuchs BM, Behrens S (2001). "The identification of microorganisms by fluorescence in situ hybridization." *Curr. Opin. Biotechnol.* **12**:231–236.
- Azam, F. (1998). "Microbial control of oceanic carbon flux: the plot thickens." *Science* **280**(5364): 694-696.
- Bird, C., J. Martinez Martinez, A. G. O'Donnell, and M. Wyman. (2005). "Spatial distribution and transcriptional activity of an uncultured clade of planktonic diazotrophic {gamma}-proteobacteria in the Arabian Sea." *Appl. Environ. Microbiol.* **71**:2079-2085.
- Brinkhoff, T., H. A. Giebel, and M. Simon (2008). "Diversity, ecology, and genomics of the Roseobacter clade: a short overview." *Arch. Microbiol.* **189**:531-539.
- Brun, Y. V. (2001). "Global analysis of a bacterial cell cycle: tracking down necessary functions and their regulators." *Trends Microbiol.* **9**(9): 405-407.
- Buchan, A., J. M. Gonzalez and M. A. Moran (2005). "Overview of the marine *Roseobacter* lineage." *Appl. Environ. Microbiol.* **71**(10): 5665-5677.
- Bustin SA. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." *J. Mol. Endocrinol.* **25**: 169–193.
- Bustin, S. A., V. Benes, T. Nolan, and M. W. Pfaffl. (2005). "Quantitative real-time RT-PCR—a perspective." *J. Mol. Endocrinol.* **34**:597-601.
- Campbell, L. and E. J. Carpenter (1986). "Diel patterns of cell division in marine *Synechococcus* spp. (Cyanobacteria): use of the frequency of dividing cells technique to measure growth rate." *Mar. Ecol. Prog. Ser.* **32**: 139-148.
- Church, M. J., C. M. Short, B. D. Jenkins, D. M. Karl, and J. P. Zehr.(2005). "Temporal patterns of nitrogenase (nifH) gene expression in the oligotrophic North Pacific Ocean." *Appl. Environ. Microbiol.* **71**:5362-5370.
- Cohan, F. M. (2001). "Bacterial species and speciation." *Syst. Biol.* **50**(4): 513-524.
- Cottrell, M. T. and D. L. Kirchman (2003). "Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary." *Limnol. Oceanogr.* **48**: 168-178.
- Dajkovic, A. and J. Lutkenhaus (2006). "Z ring as executor of bacterial cell division. ." *J. Mol. Microbiol. Biotechnol.* **11**: 140-151.
- Delong, E. F. (2009). "The microbial ocean from genomes to biomes." *Nature* **459**: 200-206.
- Dennis, P., E. A. Edwards, S. N. Liss, and R. Fulthorpe (2003). "Monitoring gene expression in mixed microbial communities by using DNA microarrays." *Appl. Environ. Microbiol.* **69**:769-778.

- Dyda, R.Y., Suzuki, M.T., Yoshinaga, M.Y. and Harvey, H.R. (2009). "The response of microbial communities to diverse organic matter sources in the Arctic Ocean." *Deep-Sea Research II*, this issue.
- Fey, A., S. Eichler, S. Flavier, R. Christen, M. G. Höfle, and C. A. Guzman. (2004). "Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism." *Appl. Environ. Microbiol.* **70**:3618-3623.
- Fisher, M. M. and E. W. Triplett (1999). "Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities." *Appl. Environ. Microbiol.* **65**(10): 4630-4636.
- Fuhrman, J. A. and F. Azam (1982). "Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results." *Mar. Biol.* **66**(2): 109-120.
- Haeusser, D. P. and P. A. Levin (2008). "The great divide: coordinating cell cycle events during bacterial growth and division." *Curr. Opin. Microbiol.* **11**: 94-99.
- Hagstrom, A., U. Larsson, P. Horstedt and S. Normark (1979). "Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments." *Appl. Environ. Microbiol.* **37**(5): 805-812.
- Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand (1991). "Detection of specific polymerase chain reaction product by utilizing the 59339 exonuclease activity of *Thermus aquaticus* DNA polymerase." *Proc. Natl. Acad. Sci. USA* **88**:7276-7280.
- Holtzendorff, J., D. Marie, A. F. Post, F. Partensky, A. Rivlin and W. R. Hess (2002). "Synchronized expression of *ftsZ* in natural *Prochlorococcus* populations of the Red Sea." *Environ. Microbiol.* **4**(11): 644-653.
- Holtzendorff, J., F. Partensky, S. Jacquet, F. Bruyant, D. Marie, L. Garczarek, I. Mary, D. Vaultot and W. R. Hess (2001). "Diel Expression of Cell Cycle-Related Genes in Synchronized Cultures of *Prochlorococcus* sp. Strain PCC 9511." *J. Bacteriol.* **183**(3): 915-920.
- Hottes, A. K., L. Shapiro and H. H. McAdams (2005). "DnaA coordinates replication initiation and cell cycle transcription in *Caulobacter crescentus*." *Mol. Microbiol.* **58**: 1340-1353.
- Jacquet, S., F. Partensky, D. Marie, R. Casottii, and D. Vaultot (2001). "Cell cycle regulation by light in *Prochlorococcus* strains." *Appl. Environ. Microbiol.* **67**(2): 782-790.
- Jiao, N., Y. Zhang, Y. Zeng, N. Hong, R. Liu, F. Chen, and P. Wang.(2007). Distinct distribution pattern of abundance and diversity of aerobic anoxygenic phototrophic bacteria in the global ocean. *Environ. Microbiol.* **9**:3091-3099.
- John, D. E., Wang, Z. A., Liu, X., Byrne, R. H., Corredor, J. E., L'opez, J. M., Cabrera, A., Bronk, D., Tabita, F.R., and Paul, J. H. (2007). "Phytoplankton carbon fixation gene (*RuBisCO*) transcripts and air-sea CO₂ flux in the Mississippi River Plume." *The ISME Journal.* **1**:517-531.
- Johnson, D. R., P. K. Lee, V. F. Holmes, and L. Alvarez-Cohen (2005). "An internal reference technique for accurately quantifying specific mRNAs by real-time PCR with application to the *tceA* reductive dehalogenase gene." *Appl. Environ. Microbiol.* **71**:3866-3871.

- Kelly, A. J., M. J. Sackett, N. Din, E. Quardokus and Y. V. Brun (1998). "Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*." *Genes Dev.* **12**: 880-893.
- Kiene, R. P., L. J. Linn, J. M. González, M. A. Moran, and J. A. Bruton (1999). Dimethylsulfoniopropionate and methanethiol are important precursors of methionine and protein-sulfur in marine bacterioplankton. *Appl. Environ. Microbiol.* **65**:4549-4558.
- Kirchman, D. L., E. K'Neas and R. Hodson (1985). "Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems." *Appl. Environ. Microbiol.* **49**(3): 599-607.
- Kogure, K. U. Simidu and N. Taga (1979). "A tentative direct microscopic method for counting living marine bacteria." *Can. J. Microbiol.* **25**(3): 415-420.
- Kogure, K. U. Simidu and N. Taga (1980). "Distribution of viable marine bacteria in neritic seawater around Japan." *Can. J. Microbiol.* **26**(3): 318-323.
- Kolber, Z. S., F. G. Plumley, A. S. Lang, J. T. Beatty, R. E. Blankenship, C. L. Van Dover, C. Vetriani, M. Koblizek, C. Rathgeber, and P. G. Falkowski (2001). "Contribution of aerobic photoheterotrophic bacteria to the carbon cycle in the ocean." *Science* **292**:2492-2495.
- Laub, M. T., S. L. Chen, L. Shapiro and H. H. McAdams (2002). "Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle." *Proc. Natl. Acad. Sci.* **99**(7): 4632-4637.
- Laub, M. T., H. H. McAdams, T. F. Feldblyum, Claire M and L. Shapiro (2000). "Global analysis of the genetic network controlling a bacterial cell cycle." *Science* **290**: 2144-2148.
- Liu, H., H. A. Nolla, and L. Campbell (1997). "Prochlorococcus growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean." *Aquat. Microb. Ecol.* **12**:39-47.
- Livak, K., S. Flood, J. Marmaro, W. Giusti, and K. Deetz (1995). "Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system for detecting PCR product and nucleic acid hybridization." *PCR Methods Appl.* **4**:357-362.
- Malmstrom, R. R., R. P. Kiene, and D. L. Kirchman (2004). Identification and enumeration of bacteria assimilating dimethylsulfoniopropionate (DMSP) in the North Atlantic and Gulf of Mexico. *Limnol. Oceanogr.* **49**:597-606.
- Margolin, W. (2005). "FtsZ and the division of prokaryotic cells and organelles." *Nat. Rev. Mol. Cell Biol.* **6**: 862-871.
- Mariano, A. J., A. Griffo, T. M. Ozgokmen and E. Zambianchi (2002). "Lagrangian analysis and predictability of coastal and ocean dynamics." *J. Atmos. Oceanic Technol.* **19**: 1114-1126.
- Martin, M. E. and Y. V. Brun (2000). "Coordinating development with the cell cycle in *Caulobacter*." *Curr. Opin. Microbiol.* **3**: 589-595.
- Moran, M. A., J. M. González, and R. P. Kiene (2003). Linking a bacterial taxon to sulfur cycling in the sea: studies of the marine *Roseobacter* group. *Geomicrobiol. J.* **20**:375-388.
- Moter, A., and U. B. Gobel (2000). "Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms." *J. Microbiol. Methods* **41**:85-112.

- Newell, S. Y. and R. R. Christian (1981). "Frequency of dividing cells as an estimator of bacterial productivity. ." *Appl. Environ. Microbiol.* **42**(1): 23-31.
- Osawa, M. and H. P. Erickson (2006). "FtsZ from divergent foreign bacteria can function for cell division in *Escherichia coli*." *J. Bacteriol.* **188**: 7132-7140.
- Pace, N. R. (1997). "A molecular view of microbial diversity and the biosphere." *Science* **276**:734-740.
- Partensky, F., W. R. Hess, and D. Vaulot (1999). "Prochlorococcus, a marine photosynthetic prokaryote of global significance." *Microbiol. Mol. Biol. Rev.* **63**:106-127.
- Pernthaler, A and R. Amann (2004). " Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria." *Appl. Environ. Microbiol.* **70**(9):5426-5433.
- Pichard, S. L., and J. H. Paul. (1993). "Gene expression per gene dose, a specific measure of gene expression in aquatic micro-organisms." *Appl. Environ. Microbiol.* **59**:451-457.
- Quardokus, E. M. and Y. V. Brun (2003). "Cell cycle timing and developmental checkpoints in *Caulobacter crescentus*." *Curr. Opin. Microbiol.* **6**: 541-549.
- Quardokus, E. M., N. Din and Y. V. Brun (1996). "Cell cycle regulation and cell type-specific localization of the FtsZ division initiation protein in *Caulobacter*." *Proc. Natl. Acad. Sci.* **93**: 6314-6319.
- Rappe, M. S. and S. J. Giovannoni (2003). "The uncultured microbial majority." *Annu. Rev. Microbiol.* **57**: 369-394.
- Ronquist, F. and J. P. Huelsenbeck (2003). "MrBayes 3: Bayesian phylogenetic inference under mixed models." *Bioinformatics.* **19**: 1572-1574.
- Rose, T. M., J. G. Henikoff and S. Henikoff (2003). "Codehop (consensus-degenerate hybrid oligonucleotide primer) PCR primer design." *Nucleic Acids Res.* **31**:3763-3766.
- Roth C. M.(2002). "Quantifying gene expression." *Curr. Issues. Mol. Biol.* **4**:93–100
- Rueda, S., M. Vicente and J. Mingorance (2003). "Concentration and assembly of the division ring proteins FtsZ, FtsA, and ZipA during the *Escherichia coli* cell cycle." *J. Bacteriol.* **185**(11): 3344-3351.
- Rusch, D.B., A.L. Halpern, G. Sutton, K.B. Heidelberg, S. Williamson and S. Yooseph et al.(2007). " The Sorcerer II Global Ocean Sampling Expedition: Northwest Atlantic through Eastern Tropical Pacific." *PLoS Biol.* **5**(3):0398-0431.
- Sackett, M. J., A. J. Kelly and Y. V. Brun (1998). "Ordered expression of ftsQA and ftsZ during the *Caulobacter crescentus* cell cycle." *Mol. Microbiol.* **28**(3): 421-434.
- Shalapyonok, A., R. J. Olson, and L. S. Shalapyonok (1998). "Ultradian growth in *Prochlorococcus* spp." *Appl. Environ. Microbiol.* **64**:1066-1069.
- Sharkey FH, Banat IM, Marchant R (2004). " Detection and quantification of gene expression in environmental bacteriology".*Appl Environ Microbiol* **70**:3795–3806
- Simon, M. and F. Azam (1989). "Protein content and protein synthesis rates of planktonic marine bacteria." *Mar. Ecol. Prog.* **51**: 201-213.
- Smith, C. J. and A. M. Osborn (2009). " Advantages and limitations of quantitative PCR (Q-PCR) based approaches in microbial ecology." *FEMS. Microbiol. Ecolo.* **67**(1): 6-20.

- Skerker, J. M. and M. T. Laub (2004). "Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*." *Nat. Rev. Microbiol.* **2**: 325-337.
- Steward, G. F. and F. Azam (1999). "Bromodeoxyuridine as an alternative to H-3-thymidine for measuring bacterial productivity in aquatic samples." *Aquat. Microb. Ecol.* **19**: 57-66.
- Suzuki, M., and S. J. Giovannoni (1996). "Bias caused by template annealing in the amplification mixtures of 16S rRNA genes by PCR." *Appl. Environ. Microbiol.* **62**:625-630
- Suzuki, M. T., C. M. Preston, O. Beja, J. R. de la Torre, G. F. Steward and E. F. Delong (2004). "Phylogenetic screening of ribosomal RNA gene-containing clones in Bacterial Artificial Chromosome (BAC) libraries from different depths in Monterey Bay." *Microb. Ecol.* **48**(473-488).
- Suzuki, M. T., O. Beja, L. T. Taylor and E. F. Delong (2001). "Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton." *Environ. Microbiol.* **3**(5): 323-331.
- Suzuki, M. T., L. T. Taylor and E. F. Delong (2000). "Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays." *Appl. Environ. Microbiol.* **66**(11): 4605-4614.
- Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). "Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Res.* **22**(22): 4673-4680.
- Tolli, J. D., S. M. Sievert and C. D. Taylor (2006). Unexpected diversity of bacteria capable of carbon monoxide oxidation in a coastal marine environment, and contribution of the Roseobacter-associated clade to total CO oxidation. *Appl. Environ. Microbiol.* **72**: 1966–1973.
- Vaughan, S., B. Wickstead, K. Gull and S. G. Addinall (2004). "Molecular evolution of FtsZ protein sequences encoded within the genomes of archaea, bacteria, and eukaryota." *J. Mol. Evol.* **58**: 19-29.
- Vaulot, D., D. Marie, R. J. Olson, and S. W. Chisholm (1995). "Growth of *Prochlorococcus*, a photosynthetic prokaryote, in the Equatorial Pacific Ocean." *Science* **268**:1480-1482
- Vila, M., R. Simó, R. P. Kiene, J. Pinhassi, J. M. González, M. A. Moran, and C. Pedrós-Alió (2004). Use of microautoradiography combined with fluorescence in situ hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. *Appl. Environ. Microbiol.* **70**:4648-4657.
- Wagner-Döbler, I., and H. Biebl. 2006. "Environmental biology of the marine Roseobacter lineage." *Annu. Rev. Microbiol.* **60**:255-280.
- Wagner, M., M. Schmid, J. Stefan, K. H. Trebesius, A. Bubert, G. Werner and K. H. Schleifer. 1998. "In situ detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*." *FEMS. Microbiol. Lett.* **160**(1): 159-168.
- Weart, R. B. and P. A. Levin (2003). "Growth rate-dependent regulation of medial FtsZ ring formation." *J. Bacteriol.* **185**(9): 2826-2834.
- Werbrouck, H., N. Botteldoorn, M. Uyttendaele, L. Herman and E. Van Coillie (2007). "Quantification of gene expression of *Listeria monocytogenes* by real-time reverse

- transcription PCR: Optimization, evaluation and pitfalls." *J. Microbiol. Meth.* **69**: 306-314.
- Wohlbers, J., A. Engel, E. Zollner, P. Breithaupt, K. Jurgens, H. G. Hoppe, U. Sommer and U. Riebesell (2009). "Changes in biogenic carbon flow in response to sea surface warming." *Proc. Natl. Acad. Sci.* **106**(17): 7067-7072.
- Wyman, M. (1999). "Diel rhythms in ribulose-1,5-bisphosphate carboxylase/oxygenase and glutamine synthetase gene expression in a natural population of marine picoplanktonic cyanobacteria (*Synechococcus* spp.)." *Appl. Environ. Microbiol.* **65**:3651-3659.
- Yurkov, V. V., and J. T. Beatty (1998). "Aerobic anoxygenic phototrophic bacteria." *Microbiol. Mol. Biol. Rev.* **62**:695-724.
- Yutin, N., M. T. Suzuki, H. Teeling, M. Weber, J. C. Venter, D. B. Rusch, and O. Béjà. (2007). Assessing diversity and biogeography of aerobic anoxygenic phototrophic bacteria in surface waters of the Atlantic and Pacific Oceans using the Global Ocean Sampling expedition metagenomes. *Environ. Microbiol.* **9**(6): 1464-1475.
- Zhang, T., and H. P. Fang (2006). "Application of real-time polymerase chain reaction for quantification of microorganisms in environmental samples." *Appl. Microbiol. Biotechnol.* **70**:281-289.
- Zubkov, M. V., B. M. Fuchs, S. D. Archer, R. P. Kiene, R. Amann and P. H. Burkill (2001). "Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea." *Environ. Microbiol.* **3**(5): 304-311.