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

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Summary

Determining *in situ* growth rates for specific bacterioplankton is of critical importance to understanding their contributions to energy and matter flow in the Ocean. Quantifying expression of genes central to cell division is a plausible approach for obtaining these measurements. In order to test this approach's assumptions, a quantitative PCR assay targeting the cell division gene *ftsZ* in the ubiquitous NAC11-7 group of the Rhodobacterales order of marine bacteria was developed. *ftsZ* genes and their corresponding mRNAs were measured in diel *in situ* samples and in parallel on-deck incubations. Strong correlations between *ftsZ* expression and gene abundance (R-squared=0.62) were observed *in situ*. Rapid changes in NAC11-7 *ftsZ* gene copies suggested that different populations from different water types were sampled with a significant positive correlation and between *ftsZ* expression and water temperature (R-squared=0.68, $p<0.001$). An outlier to this trend occurred at a single time point (9:00), which was remarkably consistent with a concomitant peak in *ftsZ* expression in on-deck incubations, suggesting the possibility of synchronous population growth.

Introduction

Marine bacterioplankton play pivotal roles in oceanic food webs and the cycling of organic matter, which in turn influence global carbon pools (Azam and Malfatti, 2007; Wohlers et al., 2009). In recent years, the application of molecular, genomic, and transcriptomic techniques has provided us with vast datasets, greatly expanding our knowledge of bacterioplankton phylogenetic diversity, their environmental distributions, and metabolic capabilities (for reviews see Rappé and Giovannoni, 2003; Delong, 2009). Despite this knowledge, challenges still exist in understanding the ecological functions of **specific** bacterioplankton. Among these challenges is a lack of measurements of *in situ* growth rates of bacterioplankton at high phylogenetic resolution. This information is essential for elucidating the contributions of these ecotypes to biogeochemical cycles critical to the ecosystem.

The most commonly used approaches for determining growth rates of heterotrophic marine bacterioplankton are indirect and rely on measurements of bacterial production. Growth rates are often calculated from bacterioplankton production, which are usually estimated from the incorporation of ^{3}H-thymidine or ^{3}H-leucine (Fuhrman and Azam, 1982; Kirchman et al., 1985; Simon and Azam, 1989), or non-radioactive bromodeoxyuridine (Steward and Azam, 1999). These methods regard the entire bacterial community as a "black box". They provide community-level activity measurements, and therefore do not provide information on the production and growth rates of specific bacterioplankton. While the combination of microautoradiography with fluorescence *in situ* hybridization (i.e. Micro-FISH) allows the assessment of specific bacterioplankton activities at relatively narrow phylogenetic levels (i.e. Cottrell and Kirchman, 2003; Alonso and Pernthaler, 2005; Margolin, 2005), these experiments suffer from issues inherent to confinement and tracer-based studies (i.e. bottle effects).

A plausible approach to measure *in situ* activity, and perhaps growth rates of specific bacterioplankton, involves quantifying the expression (mRNAs) of genes encoding proteins involved in cell division. The idea behind such approach would be to determine direct relationships between *in situ* expression of protein-encoding genes 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 could be made at specific phylogenetic levels, and earlier in the cell cycle (i.e. before a clear septal ring is formed), thus decreasing problems associated with preferential protistan grazing of dividing cells (Sherr et al., 1992). Requirements for successfully using a gene transcript tracking approach for **instantaneous** (i.e. at any time of the day) measurements of growth would be that populations of targeted organisms do not divide synchronously, and that the regulation of cell division protein synthesis occurs at the transcriptional level. In cases where the population divides synchronously, growth rates estimates are possible, but would not be instantaneous and would require measurements throughout a diel cycle (i.e. Carpenter and Chang, 1988; Chang and Carpenter, 1988; Holtzendorff et al., 2002).

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 populations of *Caulobacter crescentus,* a model organism for the study of bacterial cell division, the transcription of *ftsZ* is controlled by two regulators: DnaA promotes *ftsZ* transcription, while CtrA its repressor, ensures that cell division is coordinated with DNA replication (Hottes et al., 2005; McAdams and Shapiro, 2009). Levels of expression of *ftsZ* genes were also found to vary during the cell cycle in synchronized *Prochlorococcus* populations in the Gulf of Aqaba, with

the maxima of expression at the replication phase (S phase; Holtzendorff et al., 2001; Holtzendorff et al., 2002). These authors suggested that transcriptional regulation of *ftsZ* could be a major factor triggering the observed synchronized cell division of *Prochlorococcus* populations. Finally, the sequence of the FtsZ protein is highly conserved in most bacteria and its phylogeny has good congruence to 16S rRNA phylogeny (Vaughan et al., 2004), allowing putative identification of genes uncovered directly from the environment.

In this study, we investigated *in situ* expression of *ftsZ* genes putatively belonging to the NAC11-7 subclade of the *Roseobacter* group of marine bacteria. Members of the NAC11-7 clade have been implicated in DMSP degradation (Zubkov et al., 2001; Buchan et al., 2005) and the genome sequence of a NAC11-7 representative (HTCC 2255) suggests members of this subclade might be photoheterotrophic because it contains a proteorhodopsin coding gene (Newton et al 2010). We quantified *ftsZ* expression of this group over a diel cycle to determine whether evidence exists for synchronicity in cell division. Gene expression was measured as the ratio of *ftsZ* mRNA to *ftsZ* gene copies using quantitative real time PCR (qPCR) and reverse transcription qPCR (RT-qPCR), respectively, in samples collected from a station off the Chesapeake Bay and in a parallel on-board incubation.

Results

Rhodobacterales ftsZ genes from coastal waters off the Chesapeake Bay.

Degenerate primers targeting *ftsZ* from the order Rhodobacterales were designed based on conserved regions identified in alignments of representative *ftsZ* sequences obtained from GenBank. A clone library was generated from fragments amplified from coastal Atlantic Ocean surface waters and 74 *ftsZ* clones were analyzed. Phylogenetic reconstruction of these sequenced clones as well as representative reference sequences revealed three interesting groups (Figure 1):

a prevalent, yet unidentified group (designated Group I) containing 38 sequences (51% of the clones); a second unidentified group (Group II) with 31 sequences (42 % of the clones); and a group represented by 5 sequences that were most closely related to the FtsZ sequence from strain HTCC 2255 (Figure 1). 16S rRNA gene phylogeny places strain HTCC 2255 in the NAC11-7 group of the roseobacters (Buchan et al., 2005). Given the previously reported global distributions and abundances of NAC11-7 representatives and their putative photoheterotrophic metabolism, this group was selected for further investigation.

In situ diel expression of NAC11-7 ftsZ genes.

NAC11-7 *ftsZ* mRNA and DNA copies were monitored in surface waters over a 24 h period at a single station located at the mouth of the Chesapeake Bay. Reproducibility among triplicates was quite good for gene copies (Figure 2), with a single exception of 21:00 where the difference among samples was also reflected by different ARISA profiles (Supplemental Figure 1). Estimated mRNA copies were more variable among triplicates, mostly likely due to RNA degradation. In 5 of 9 timepoints, a single measurement was considered as an outlier, thus averages of duplicates were taken. Gene copies, and more remarkably, gene expression, showed rapid and sometimes large (c.a. 4-fold) changes with time (i.e. from 9:00 to 12:00 and from 18:00 to 21:00), suggesting distinct populations (i.e. patches) of NAC11-7 may have been sampled at different times, as a result of water advection (Figure 2). Interestingly, *ftsZ* gene expression followed closely *ftsZ* gene abundance trends, except at the last two sampling times. A regression analysis between *ftsZ* gene copies and gene expression using individual replicates showed significant correlation (R^2= 0.6214, P=0.0003) for the first seven sampling times, covering 18 hours from 6:00 to midnight. However, for the remaining two time points (3:00 and 6:00 on Sept 4, 2005) this trend no longer held. As a consequence, a correlation analysis of *ftsZ*

126 gene to gene expression inclusive of all sampling points, was not significant (R^2=0.0304,
127 P=0.45). These results indicate that at least for some of the samples a correlation existed between
128 gene abundance and per-cell gene expression activity.

129 In the first 12 hours, *ftsZ* expression and to some degree *ftsZ* gene copies appeared to be
130 somewhat correlated with the tidal cycle, with peaks of expression at 6:00 and 18:00 and lowest
131 expression at 12:00, lagging the onshore high and low tides by about 3 hours respectively (Figure
132 2). Temperature and salinity also showed fluctuations that loosely followed the tidal cycle with
133 the same 3-hour lag (data not shown). After 18:00 a steady drop in temperature and simultaneous
134 increase in salinity was observed. Overall, these trends in salinity and temperature indicate that
135 tidal currents influenced our sampling site and that different waters with different NAC11-7
136 populations (or patches) were sampled.

137 In order to further examine the relationships between *ftsZ* gene abundance and expression
138 with water masses, we overlaid *ftsZ* gene abundances and expression to gene ratios onto
139 temperature-salinity (T-S) plots (Figure 3). These plots indicate a cyclical variation between
140 warmer, less saline waters and cooler, more saline waters. While, gene copies did not show clear
141 trends in the T-S plot (Supplemental Figure 2), *ftsZ* expression showed an interesting relationship.
142 With the single exception of the 9:00 sample, samples below 25.8°C showed significantly lower
143 *ftsZ* expression (two-sample t-test P=0.003) than those above 25.8°C (Figure 3). A regression
144 analysis excluding the 9:00 measurements showed a significant correlation (R^2=0.68,
145 P=0.000025) between *ftsZ* expression and temperature (Figure 3). These data strongly indicate
146 that populations from different water masses were sampled throughout the diel study.
147 Furthermore, an outlier in gene-normalized *ftsZ* transcript abundance is evident at the 9:00
148 sampling time, suggestive of a peak in *ftsZ* gene expression at that time (Figure 3).

ARISA analysis of microbial communities.

In order to examine the dynamics of bacterioplankton communities and populations at the different sampling times, these communities were examined using automated ribosomal intergenic spacer analysis (ARISA). We observed that a number of ARISA peaks were present at all time points analyzed, (i.e. 456, 485, 510 and 556 bp), while several peaks showed a more ephemeral occurrence (i.e. 793, 799, 900 bp), suggesting that levels of patchiness differed for different phylotypes (Supplemental Figure 1). At least in the case of these fluctuating phylotypes, different populations (patches) appear to have been sampled at different times. Unfortunately, ARISA peaks with sizes corresponding to the NAC11-7 group were not readily detected, as these organisms were likely present in numbers below the resolution of ARISA.

Enclosed incubation diel expression of NAC11-7 ftsZ genes.

A parallel on-deck incubation experiment in which *ftsZ* gene and gene expression were quantified in incubated samples was conducted for comparative purposes. In these samples, *ftsZ* gene abundance dropped from 6:00 to 15:00 and stabilized at ca.1 x 10^4 copies/ml (a 2.5-fold decrease). Contrastingly, *ftsZ* expression showed a very remarkable 4-fold increase in expression at 9:00 relative to the 6:00 time point. However, by 12:00 expression was 5-fold less (Figure 4). Considering that the measurements were made in three separate, incubated bottles and that the C_Ts of the 9:00 cDNA samples were nearly two units lower than those of the remaining samples, we are very confident that this observation was not due to an experimental artifact.

Discussion

In order to test assumptions related to the measurement of *in situ* growth rates of specific roseobacters based on cell division genes, a qPCR *ftsZ* assay was developed for the NAC11-7

clade. Analysis of the NAC11-7 sequences reveals that although all the sequences were highly similar at the amino acid level, one sequence, clone ftsZ05F3, was different at the DNA level (Supplemental Figure 3). Based on the ecotype theory (Cohan, 2001), clone ftsZ5F03 was assumed to belong to a different ecotype that was not subjected to a theoretical selective sweep, as were the remaining 5 phylotypes. In order to maximize the chance that our measurements reflected populations most coherent in their response to environmental factors, the sequence of clone ftsZ05F03 was not targeted by our qPCR assay. This assay is highly specific to the NAC11-7 ecotype represented by the 5 phylotypes described above, since specificity tests did not show amplification for ftsZ05F03 nor any of 14 clones representing the unidentified clades shown in Figure 1 (data not shown).

We observed that levels of gene-normalized transcripts were low. This was not completely unexpected as mRNAs exist in a complex RNA pool composed primarily of rRNA and have relatively short half-lives (Belasco, 1993). Thus, the efficiency of mRNA recovery is expected to be considerably lower than that for genomic DNA. While we did not measure reverse transcription efficiencies here, we have previously shown RT efficiencies to be constant between different samples for proteorhodopsin mRNAs using an analogous method with the same reverse transcriptase and a specific primer (Shi, 2005).

It is important to emphasize that our measurements of gene expression are based on a *per cell* average for the entire population. Thus, depending on how *ftsZ* is expressed in a single cell during the cell cycle, the mRNA to gene ratio may reflect: 1) the fraction of the population that is actively transcribing *ftsZ* in preparation for cell division or 2) the average per cell expression level [i.e. if *ftsZ* is constitutively transcribed, and FtsZ regulation occurs post-transcriptionally in preparation for cell division]. If a population displays synchronous growth and *ftsZ* expression

varies during the cell cycle (Case 1), we expect to observe peaks in the population-level *ftsZ* expression. Conversely, if *ftsZ* transcription were constitutive (Case 2), one would not expect *ftsZ* expression peaks even in synchronous populations.

The only other study to examine *ftsZ* gene expression in natural bacterial assemblages was carried out with Red Sea populations of the cyanobacterium *Prochlorococcus*. In that study, *ftsZ* gene expression was found to be highly synchronous, with a peak in expression (c.a. 10 fold increase) occurring in the twilight hours before nightfall and lasting for over 2 hours (Holtzendorff et al., 2002). These expression profiles were consistent with the known photoautotrophic physiology of the organism; these populations are naturally synchronized and undergo cell division at night (Vaulot and Partensky, 1992 ; Vaulot et al., 1995). *Caulobacter crescentus,* an aquatic alphaproteobacterium, which is largely used as a model for cell cycle and differentiation studies (see review by McAdams and Shapiro, 2009), is the closest roseobacter relative for which extensive *ftsZ* gene expression studies have been undertaken. In this organism, *ftsZ* gene expression is cell cycle-dependent and is time constrained (Quardokus et al., 1996; Kelly et al., 1998; Sackett et al., 1998; Martin and Brun, 2000; Brun, 2001), with increases in transcriptional activity greater than and 10-fold lasting for about 20% of the cell cycle (ca 30 min; Kelly et al. 1998). Given the magnitude of the transcriptional response evident from these model organisms and the expectation that natural bacterioplankton populations would have cell cycles with lengths more similar to natural *Prochlorococcus* populations than *Caulobacter* laboratory cultures, it is anticipated that a signature of elevated *ftsZ* gene expression would be evident in natural NAC11-7 populations. Particularly, if these populations demonstrated some level of synchronicity and the signal would not be masked by a portion of the population with basal level expression. In contrast, FtsZ protein concentration per cell has been found to be constant

218 regardless of growth rate in the copiotrophic *E. coli (Gammaproteobacteria)* and *B. subtilis*
219 (*Firmicutes*) (Rueda et al., 2003; Weart and Levin, 2003; Haeusser and Levin, 2008). Several
220 arguments support time-constrained *ftsZ* expression in NAC11-7 members. As members of the
221 *Alphaproteobacteria,* these bacteria are more likely to share conserved features of central
222 physiology with *C. crescentus* than with the more distantly related *E. coli* or *B. subtilis*. In
223 addition, the genome of NAC 11-7 isolate HTCC2255 contains the genes encoding for DnaA and
224 CtrA (blastp e-value $<10^{-20}$ using the *Caulobacter crescentus* amino acid sequences), the central
225 regulators of *ftsZ* expression and cell cycle in *C. crescentus* (McAdams and Shapiro, 2009).
226 Finally, it is difficult to rationalize advantages to NAC11-7, organisms adapted to somewhat
227 oligotrophic conditions, to synthesize a constant amount of FtsZ through the entire cell cycle
228 unless FtsZ has an alternative, unknown function.
229 Different lines of evidence indicate that different NAC11-7 populations (or patches) with
230 different growth rates were sampled at different time points during the *in situ* experiment. First,
231 remarkable fluctuations of NAC11-7 *ftsZ* gene copy numbers (nearly 4-fold in 3 h) were
232 observed *in situ* (Figure 2), implying changes in populations sizes resulting from physical
233 processes. Second, ARISA analysis of the total bacterioplankton community structure showed
234 evidence for patchiness, as certain phylotypes were only detected in specific time points
235 (Supplemental Figure 1). While transient populations were likely sampled throughout the diel
236 cycle, some trends in *ftsZ* gene and gene expression did emerge that revealed insight into *ftsZ*
237 gene expression profiles and synchronicity of these populations. During the first seven data
238 points (18 hrs from 6:00 to 0:00), a strong correlation (R^2= 0.6214) was observed between
239 NAC11-7 abundance *in situ* (inferred from *ftsZ* copies/ml), and the gene-normalized *ftsZ*
240 transcript abundance, indicating that levels of expression are not constant in this ecotype. This

was suggestive that more active populations yielded, or were associated, with higher abundances of these organisms. Collectively, these results support the case for non-synchronous cell division since *ftsZ* expression was not constrained to specific times (i.e. *ftsZ* was expressed at all measured time points). Finally, the plot of gene-normalized expression over a temperature-salinity diagram, and a regression analysis showed that, with the exception of the 9:00 replicates, there is a high correlation between water temperature and *ftsZ* expression (Figure 3).

We found a remarkable peak of gene-normalized *ftsZ* transcript abundance at 9:00 in the on-deck incubation experiment, which could be interpreted in several ways. First, and in contrast to conclusions drawn from the *in situ* samples, *ftsZ* expression (and by extension cell division) could be synchronous in the putatively photoheterotrophic NAC11-7 clade. An alternative explanation would be that the 9:00 peak of expression resulted from stimulation caused by confinement. The observations that 1) the response was fast and time-constrained; 2) gene abundances steadily decreased with time, and 3) there is no evidence that members of the NAC11-7 group respond positively to enrichment conditions (i.e. this group is not known as copiotrophic), all argue against this notion. The slow decline in *ftsZ* gene abundance in the bottles might be explained by a lack of growth, and continuous mortality by viral lysis and/or protistan bacterivory. The peak of *ftsZ* expression at 9:00 could be explained by a previous "commitment" of the circadian rhythm. As different NAC11-7 populations (patches) were sampled over the course of the *in situ* diel it is difficult to directly compare *in situ ftsZ* gene expression levels with the parallel on-deck experiment. However, the fact that the *in situ* sample from 9:00 was higher than expected based on the correlation between gene expression and temperature (Figure 3) corroborates the incubation results, although we only sampled a single diel cycle and there is a possibility that this was a fortuitous coincidence.

An intriguing interpretation of these seemingly contradictory results is that a certain fraction of the population could be synchronously dividing, with a peak in *ftsZ* expression at 9:00, while a background of asynchronously dividing cells could also exist, some of which would be expressing *ftsZ* at any given time. This "background" expression would be in fact, better correlated to temperature or other parameters influencing growth. The fact that a certain level of *ftsZ* expression was measured throughout the diel cycle in synchronously dividing *Prochlorococcus* populations (Holzendorf et al 2001) supports this model. However, in the case of *Prochlorococcus*, *ftsZ* expression is at the end of the light period and cell division occurs at the dark (Holtzendorff et al., 2001).

To our knowledge, this is the first study measuring the expression of cell division genes by specific heterotrophic bacterioplankton and to show that *ftsZ* expression varies between different populations of a heterotrophic ecotype. The possibility of synchronous growth while intriguing will need further exploration using combined incubation and *in situ* measurements. However the latter should include Lagrangian sampling to minimize advective effects, and allow measurement in coherent populations.

Experimental Procedures

Sampling

Surface water samples were collected every 3 hours from 6:00 local time on September 3 to 6:00 on September 4, 2005 at station RM6 (37°05.61N, 75°42.35 W) aboard the RV Cape Henlopen. Temperature and salinity were measured using a SBE9 CTD (Seabird, Bellevue, WA), and tidal effects were estimated from the height of the tide at Ship Shoal Inlet calculated using the data at the NOAA tides and current prediction page http://tidesandcurrents.noaa.gov.

Triplicate 590 ml water subsamples were collected and pre-filtered through GF/A filters (1.6 μm nominal pore; Whatman, Maidstone, UK). Ninety ml of pre-filtered sample was filtered through 13 mm diameter 0.2μm Supor200® polysulfone filters (Pall Corp., East Hills, NY) and the filters were transferred to a tube containing 130µl 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 were filtered through 25 mm diameter 0.2 μm Supor200® filters (Pall Gelman Inc.) and transferred to screw cap tubes containing 250 µl RNALater (Ambion, Austin, TX). All samples were frozen at –20ºC aboard and within a week stored at –70ºC until nucleic acid extraction.

On-deck incubation experiments were conducted using water collected at the first sampling time (6:00, 03 September, 2005). Fifteen l of surface water was used to fill 24 x 500 ml (600 ml total volume) polycarbonate bottles (Nalge Nunc International Corp., Rochester, NY), and incubated at *in situ* temperatures in an on-deck incubator. At every *in situ* sampling point, three of the incubation bottles were taken and sampled for nucleic acids as described for cast samples.

Nucleic acid extraction and quantification

Total DNA was extracted from Supor200® 0.2 μm filters as previously described, except that the DNeasy 96 Tissue Kit was used, and therefore the DNeasy 96 plates were sealed and centrifugations performed in a Sorvall® Legend™ T Centrifuge with a Highplate® rotor (Kendro, Osterode, Germany) at 5250 rpm for 10 min for binding and 5250 rpm for 5 min for washes. The DNeasy 96 plate was incubated at 70ºC for 15 min evaporate traces of ethanol and DNA was eluted by centrifugation at 5250 rpm, for 2 min.

Total RNA was extracted from Supor200® 0.2 μm filters following an optimized protocol adapted from the Qiagen RNeasy® 96 manual (Shi 2005). Filters in screw cap tubes were thawed

310 on the ice, along with the RNAlater, which was not removed prior to RNA extraction. Low
311 protein binding zirconium oxide beads (equivalent to ca 320 μl) were added into each tube (200
312 μm, OPS Diagnostics, Lebanon, NJ). ß-Mercaptoethanol was mixed in a 1:100 ratio into RLT
313 buffer and 875 μl of the mixture added to screw cap tubes. The tubes were beaten in a MM301
314 mixer mill (Retsch GmbH Inc., Haan, Germany) at 30.0 HZ for 2 min and incubated for 5 min at
315 70 ºC. 800 μl of the liquid phase was transferred into a new low-RNA-binding 2 ml
316 microcentrifuge tube (Ambion, Inc.), avoiding the beads. 800 μl of 100% ethanol was added into
317 each tube and well mixed well. 800 μl of the mixture was loaded into wells of RNeasy 96 plate,
318 sealed and centrifuged at 5000 rcf for 5 min, and the procedure repeated. The columns were
319 washed once with 800 μl RW1 buffer and twice with 800 μl RPE buffer at 5000 rcf for 5 min,
320 with the last spin for 15 min. 35 μl Diethylpyrocarbonate (DEPC) treated water (Ambion) were
321 added into each column, incubation for 1 min at room temperature and 5 min centrifugation at
322 5000 rcf. This procedure was repeated with another 35 μl DEPC treated water. RNA was treated
323 the DNA-free™ kit (Ambion) to remove co-extracted DNA.
324 DNA and RNA concentrations were quantified fluorometrically by PicoGreen® and
325 RiboGreen® staining respectively (Molecular Probes, Invitrogen Corp., Carlsbad, CA) on a
326 Spectra MAX Gemini microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA).
327 ***Construction of ftsZ DNA PCR clone library***
328 An initial *ftsZ* and FtsZ database was developed using *arb* (Ludwig *et al*., 2004) by
329 importing gene sequences listed by Vaughan and coworkers (Vaughan et al., 2004). 163
330 representative FtsZ sequences were exported and used to retrieve additional aminoacid, and
331 coding gene sequences using blastp searches against the March 2005 NCBI *nt, env_nt* and *wgs*
332 databases. All aminoacid sequences were aligned using ClustalW (Thompson et al., 1994) and an

bayesian tree containing 507 sequences (144 homologous positions) 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 burn in of 6000 trees. In this tree, the *Alphaproteobacteria* formed a monophyletic clade with high confidence values and several orders including *Rhizobiales* and *Rhodobacterales*, which also formed monophyletic clades (data not shown). The database was updated with public sequences belonging to the *Rhodobacterales,* and was used for the design of PCR primers and probes. Degenerate primers targeting *ftsZ* from *Rhodobacterales* (ftsZrb2F: 5'-AAY GCN GTS AAY AAY AT-3', ftsZrb2R: 5'-YTT NCC CAT YTC RT-3') were designed and used to retrieve *ftsZ* gene sequences by PCR and cloning from a DNA sample collected at time zero.

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 $MgCl_2$, 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. PCR was followed by a reconditioning (Thompson et al., 2002) of 5 steps. Reconditioned PCR products were loaded on a 1% modified TAE (40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA) agarose gel and separated by electrophoresis. Target fragments were cut, recovered by Ultrafree®-DA (Millipore) gel extraction and used to built a *ftsZ* gene clone library using the TOPO™ TA cloning kit (Invitrogen) following manufacture'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). These sequence data have been submitted to the

GenBank database under accession number HM035149-HM035222. 74 sequences identified as *ftsZ* were imported into the ARB database described above and added to the tree described above using the ADD_BY_PARSIMONY tool. Finally, a bayesian tree of Rhodobacterales *ftsZ* was constructed using the MPI version of Mr Bayes V3.1 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004). 188 homologous amino acid positions from 123 sequences were exported and used in this phylogenetic analysis. Two four parallel 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

PCR 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 strain HTCC 2255. However, the DNA sequence of one of these clones (ftsZ5F03) contained obviously higher variation to the remainder clones and HTCC 2255 (Supplemental Figure 3). Based on ecotype theory (Cohan, 2001), it appeared that the distinct clone could belong to a different ecotype, which we assumed was not subject to a periodic selection event as the remaining sequences. Thus we designed a primer and probe 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).

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/μl

379 with nuclease-free TE buffer (Ambion). Ten μl PCR reactions contained 5 μl TaqMan®
380 Universal PCR Master Mix (Applied Biosystems), 0.5μM Forward primer ftsZrbA03-2qF (GTG
381 AAA AAG CTA CTG AGG GTC T) and Reverse primer ftsZrbA03-2qR (GCT TCC TGC CAG
382 ATG ATC), and 1 μl plasmid template. The cycling parameters were as follows: 2 min at 50 °C,
383 10 min at 95 °C and 30 cycles of 15 sec at 95 °C and 1min at 57 °C. All PCR products were
384 loaded in a 2% NuSieve® (3:1) Agarose Gel (Cambrex, Rockland, ME), electrophoresed, and
385 post-stained with 1:10,000 SYBR® Gold (Invitrogen) for 30 min. The gel was visualized with a
386 FluoroChem 8900 (Alpha Innotech, San Leandro, CA).

387 In order to get the highest amplification efficiency in real time PCR, a primer
388 concentration matrix was performed. In each 25 μl reaction, the following reagents were kept
389 constant: 1X PCR buffer, 0.2 mM of dATP, dGTP and dCTP, 0.4 mM of dUTP, 5 mM $MgCl_2$,
390 200 nM probe NAC11-7 (AAC CAACAGTAGGAGCATTAGCCGCT), 1.2 μM SuperROX™
391 (Biosearch Technologies, Novato, CA), 0.01 U/μl AmpErase® Uracil N-glycosylase (UNG)
392 (Applied Biosystems), 0.025 U/μl of Platinum® Taq DNA Polymerase (Invitrogen), 2.5 μl
393 NAC11-7 standard (10^4 copies/μl) and a matrix of forward and reverse primer concentrations of
394 0.1 μM, 0.5 μM, 1.0 μM, 1.5 μM. Reactions were set in a MicroAmp® Optical 96-Well
395 Reaction Plate (Applied Biosystems), which was sealed with an optical adhesive cover (Applied
396 Biosystems) reactions and ran in an ABI Prism 7000 Sequence Detection system, following the
397 cycling parameters: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min
398 at 57 °C. The primer combination yielding the lowest C_T (1.5 μM ftsZrbA03-2qF; 0.5 μM
399 ftsZrbA03-2qR) was used in all subsequent measurements.
400 A plasmid containing cloned DNA that was purified and linearized as previously
401 described (Suzuki et al., 2000) was used to prepare standards for real time PCR for quantification

of *ftsZ* gene and mRNA. 2.5 μl of DNA extracts were used in 25 μl reactions and standards ranged from 10^2 to 10^7 copies/μl. Reactions were run in duplicate along with non-template controls using the same cycling parameters described for the primer matrix. *ftsZ* cDNA was quantified in the same manner as *ftsZ* gene except that 5 μl 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 by Shi (Shi, 2005). mRNAs copies were assumed equal to cDNA copies. Since DNA and mRNA in the *in situ* experiment were measured in triplicate biological samples, *ftsZ* genes and mRNA copy numbers that were >2X or <X/2 (X is the average of the remainder two replicates) were treated as outliers and removed from the analyses. Incubated samples were not subjected to outlier analysis, but the DNA samples at 3:00 were lost and not included in the analysis.

Optimization of Reverse Transcription

Since preliminary results showed relatively high C_T values from *in situ* sample cDNAs, we attempted to increase *ftsZ* cDNA copy numbers by: (1). Increasing reverse transcription efficiency, by varying template amount, RT temperature and RT duration and (2). Adding increased amounts of cDNA products in real-time PCR reactions. In optimized conditions 4 μl purified RNA (ca. 10 ng) 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, 5mM DTT, 2 U/μl RNaseOUT™ Inhibitor (Invitrogen), 0.75 U/μ of reverse transcriptase and DEPC-treated water to a final volume of 10 μl. 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 μl environmental genomic DNA was used to perform a 10 μl ARISA reactions, containing 1X PCR buffer, 1.2 mM $MgCl_2$, 0.08 mM dNTPs (Promega Corp., Madison, WI), 0.5 μM primer 1406F-FAM (Fisher and Triplett, 1999), 1.5 μM primer 23S-Y (Dyda et al., 2009), and 0.01 U/μl 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 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec and 65 ºC 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 ºC for 2 min and separated by capillary electrophoresis using an Applied Biosystems 3100 genetic analyzer. Sizes of the fragments were analyzed by the Peak Scanner™ Software v1.0 (Applied Biosystems).

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557 Figure legends

558 Figure 1. Consensus bayesian phylogenetic tree of FtsZ protein sequences from the
559 *Rhodobacterales* group reconstructed using MrBayes v3.1. FtsZ sequences retrieved in this study
560 are marked in boldface. The numbers on nodes represent branch confidence values. The bar
561 represents the mean number of substitutions per residue in the trees used to build the consensus
562 tree. Following clone or group designations, GenBank accession numbers are provided.

563 Figure 2. *FtsZ* gene copy numbers (grey bars) and expression (estimated from the ratio of *ftsZ*
564 cDNA copies to gene copies; white bars) at station RM6 on September 3 and 4, 2005, and tidal
565 heights at Ship Shoal Inlet (37° 13′N, 75° 48′W). Error bars represent the standard deviation of
566 triplicate samples from a single CTD cast. A "*" represent samples where average of two bottles
567 were calculated; "**" represents a sample where standard deviation was estimated from
568 duplicate DNA and mRNA measurements while the ratio was calculated from a single bottle
569 where both DNA and mRNA measurements existed. Pre-dawn and post-dusk sampling times are
570 represented by the grayed areas. The tidal height is shown with the line lacking symbols.

571 Figure 3. Temperature and Salinity plot, with overlaying *in situ* NAC11-7 *ftsZ* expression. Gene
572 normalized *ftsZ* expression levels are proportional to the area of the circles. The area of the
573 circle representing the 3:00 PM sample corresponds to 2.5 10^{-4} mRNA copies per gene copies.
574 Inset: Linear regression of gene-normalized *ftsZ* transcripts and temperature excluding the 9:00

575 data points which were overlaid onto the same plot (open squares within the oval); R-
576 squared=0.68, p<0.001.

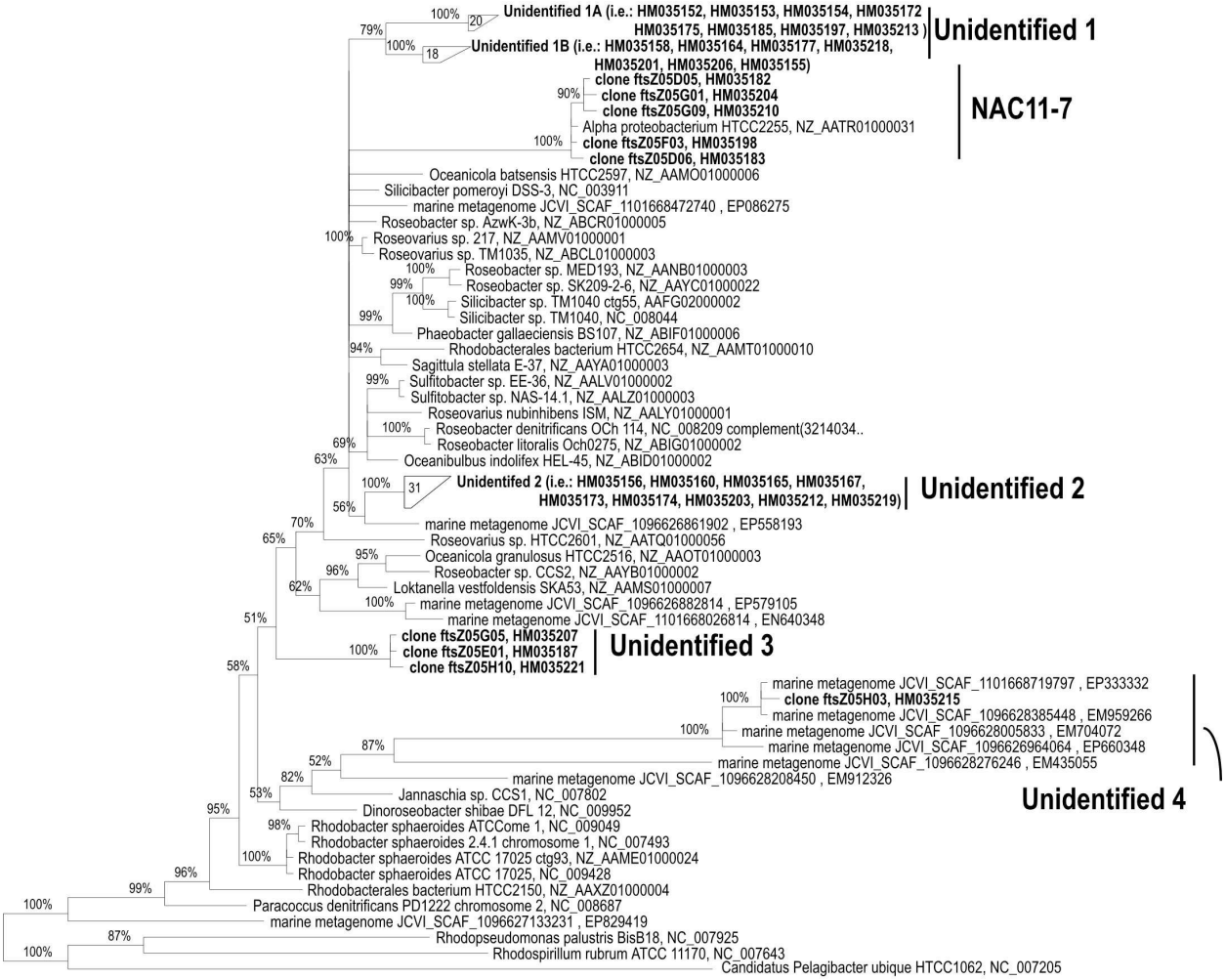
577 Figure 4. *ftsZ* gene copy numbers (filled squares) and expression (estimated from the ratio of *ftsZ*
578 cDNA copies to gene copies; open circles) in samples incubated onboard. Error bars represent
579 the standard deviation of triplicate bottles.

580 Supplemental Figure 1. Percentage of different phylotypes identified by ARISA fragment sizes
581 in the *in situ* samples. The results shown in here indicate that the replicate subsamples were
582 largely similar in composition, indicative of relatively low within-sample variation. However, in
583 a few of the samples (i.e. 9:00, 21:00) noticeable variation between replicate subsamples was
584 evident. These findings highlight the heterogeneity in marine environments and reinforce the
585 value of replicate subsampling in natural systems.

586 Supplemental Figure 2. Temperature and Salinity plot, with overlaying *in situ* NAC11-7 *ftsZ*
587 gene copies. *ftsZ* copies are proportional to the area of the circles. The area of the circle
588 representing the 3:00 PM sample corresponds to 1.0×10^4 gene copies per ml.

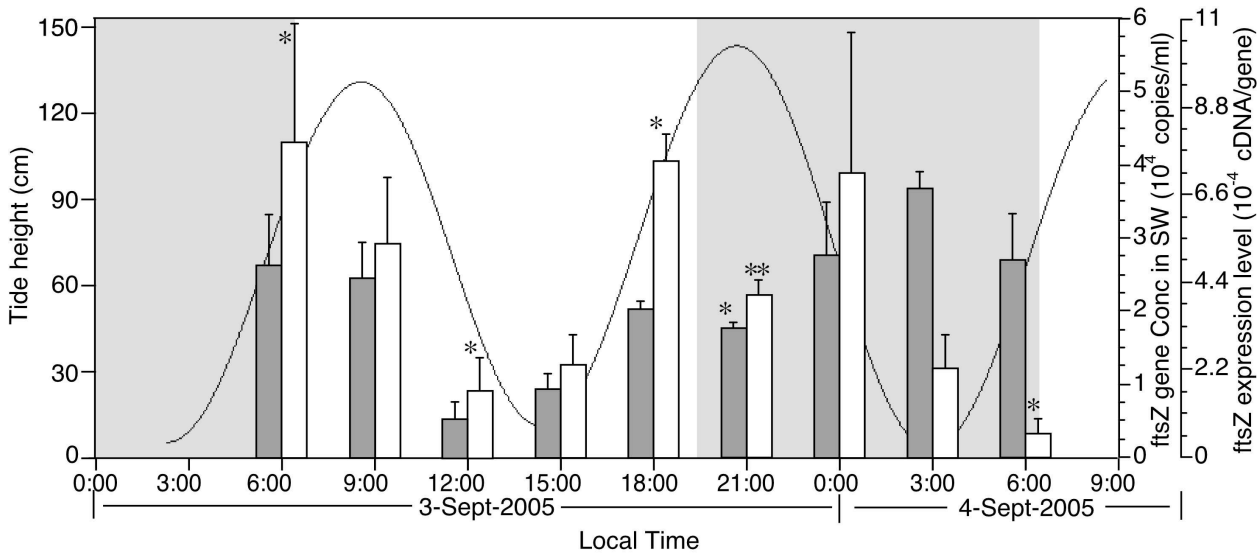
589 Supplemental Figure 3. A. Alignment of NAC11-7 FtsZ sequences. B alignment of NAC11-7
590 *ftsZ* sequences
591

591 **Figure 1.**



592

593 **Figure 2.**



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595 **Figure 3.**

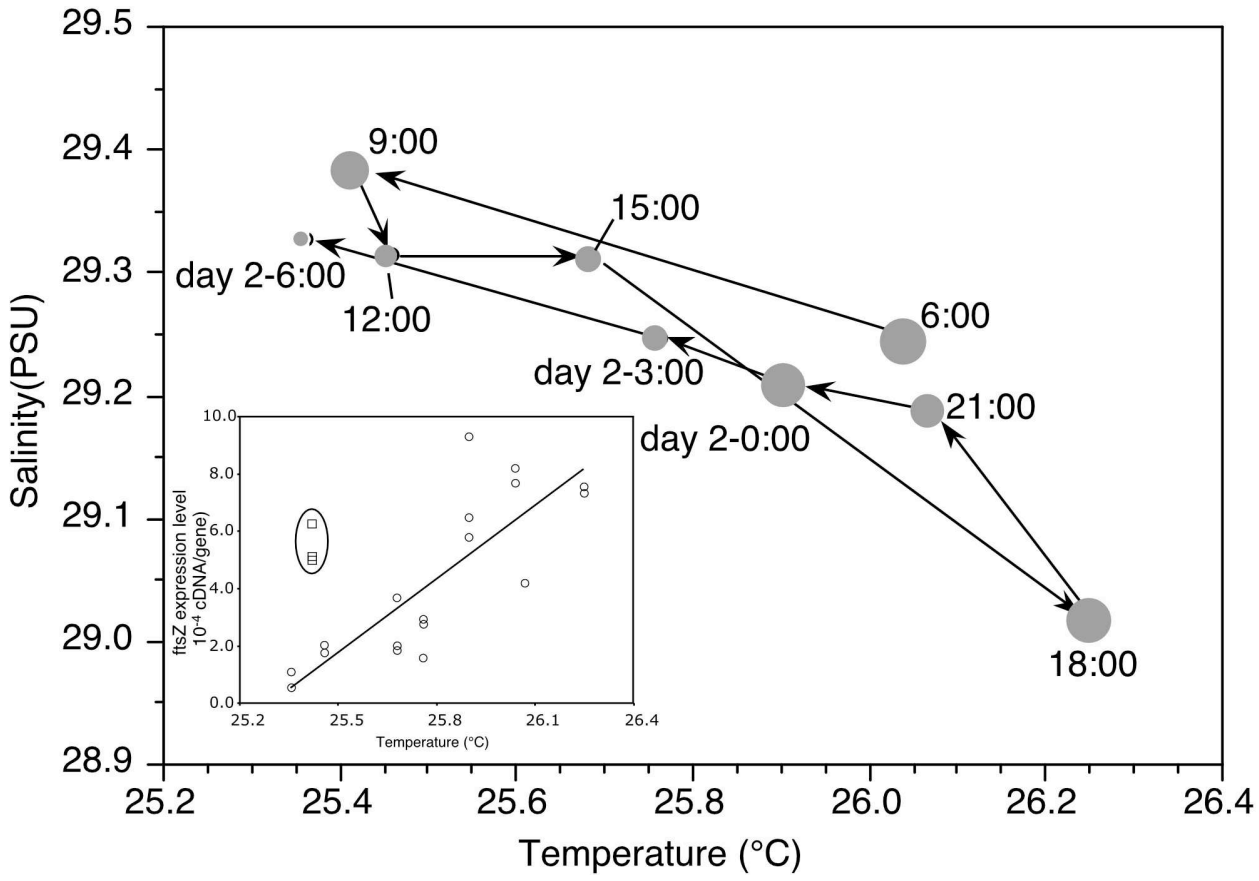

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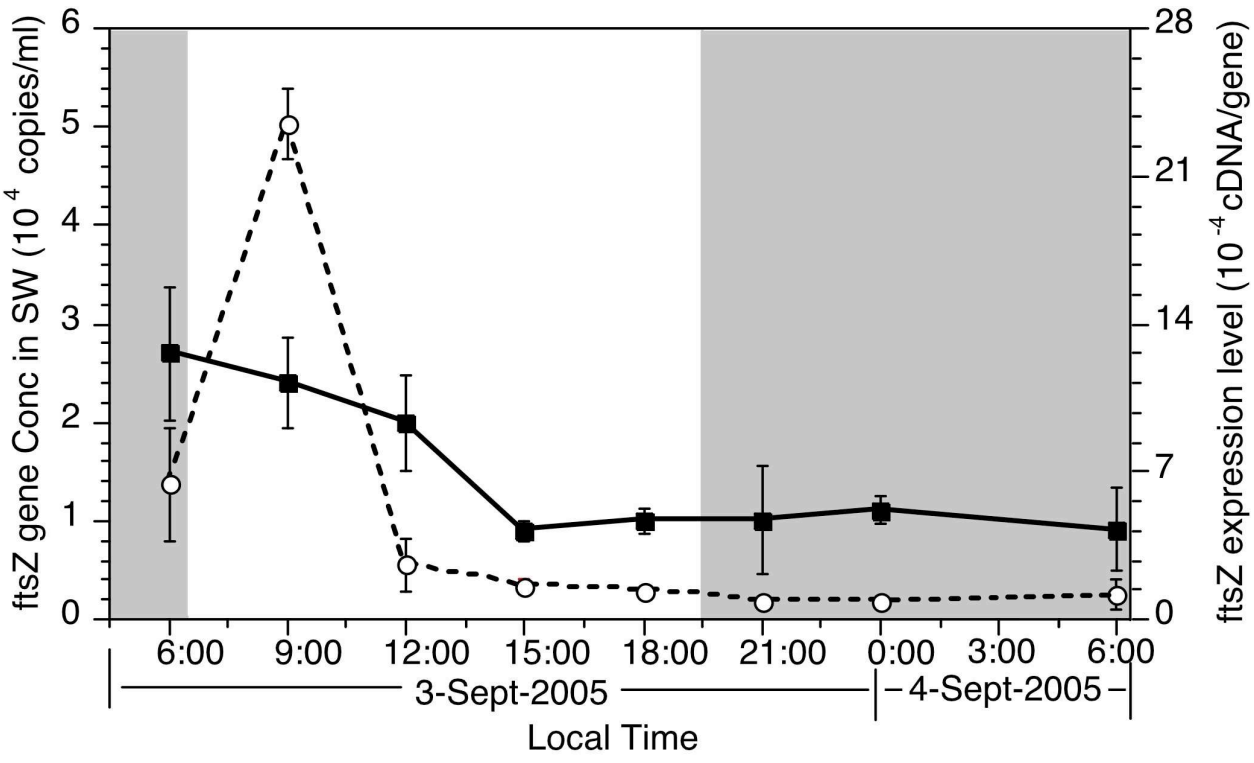
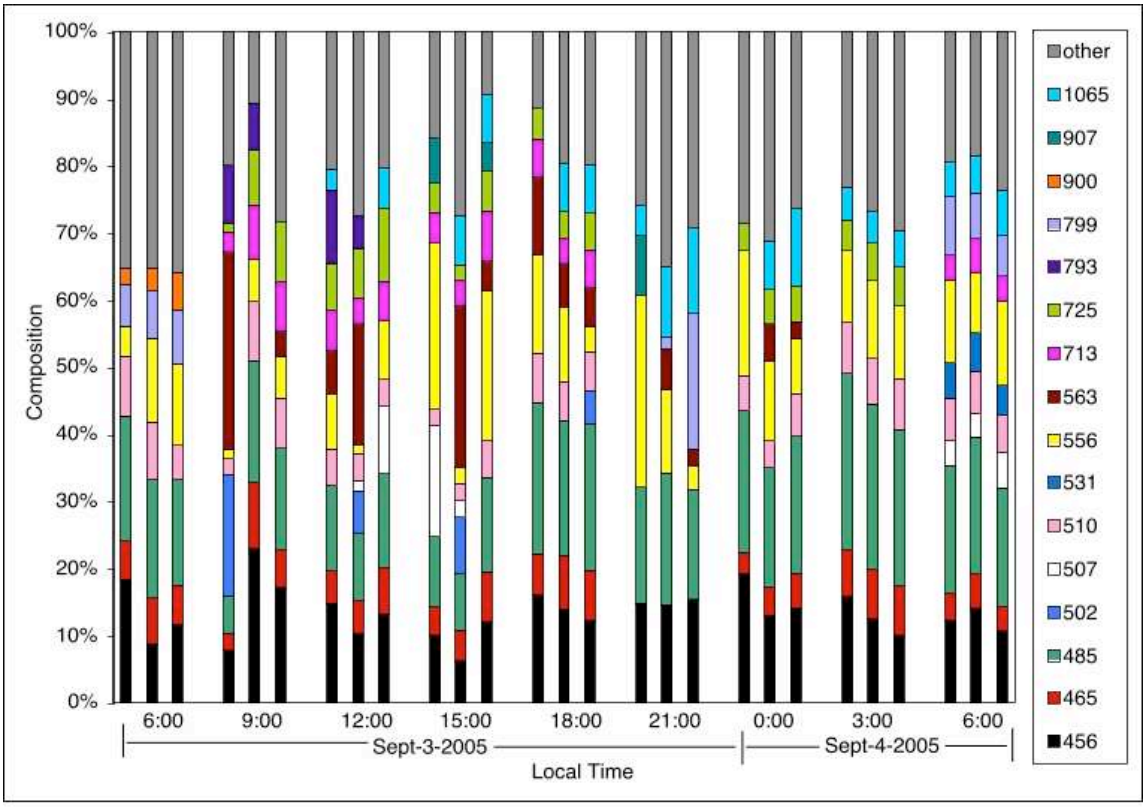
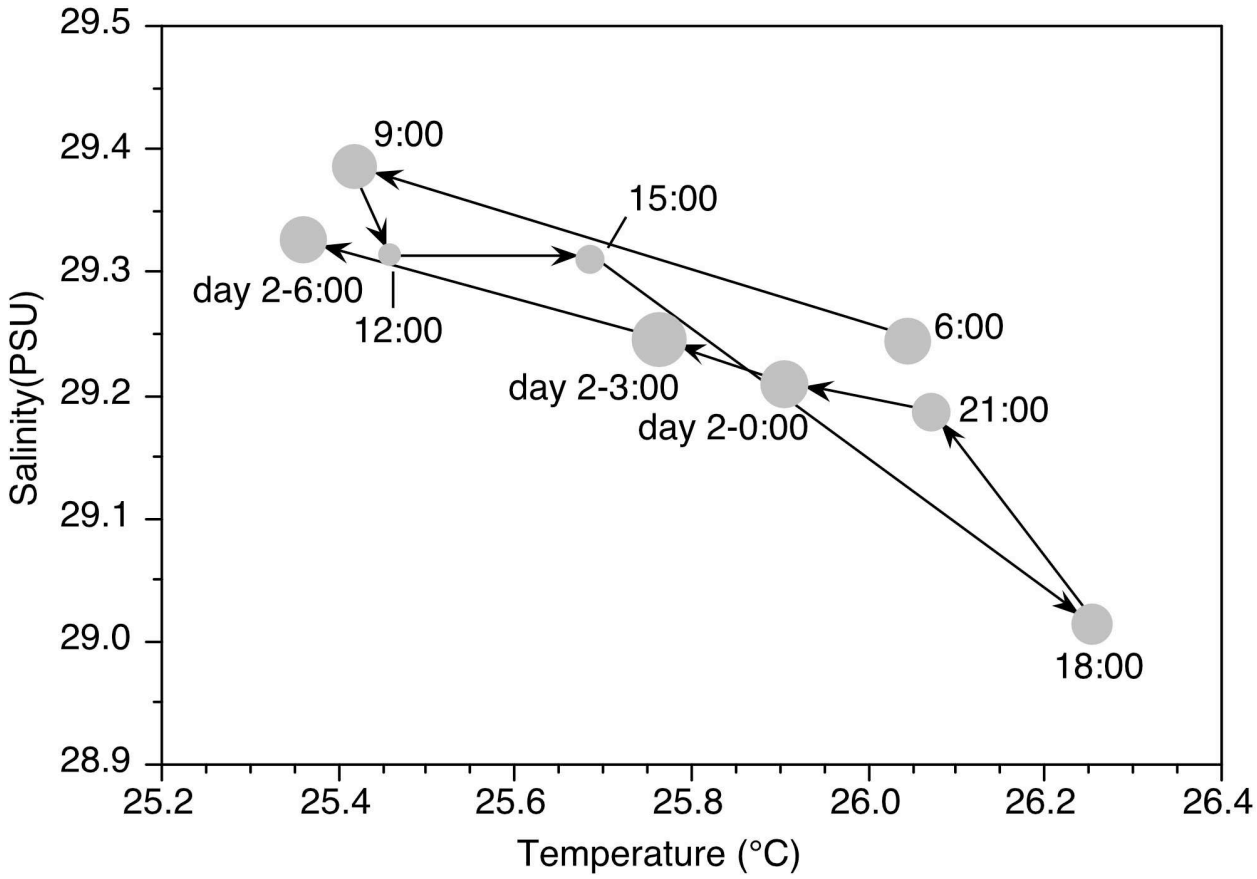
Figure 4.


ftsZ gene Conc in SW (10^4 copies/ml)
ftsZ expression level (10^-4 cDNA/gene)
0
1
2
3
4
5
6
0
7
14
21
28
6:00
9:00
12:00
15:00
18:00
21:00
0:00
3:00
6:00
3-Sept-2005
4-Sept-2005
Local Time

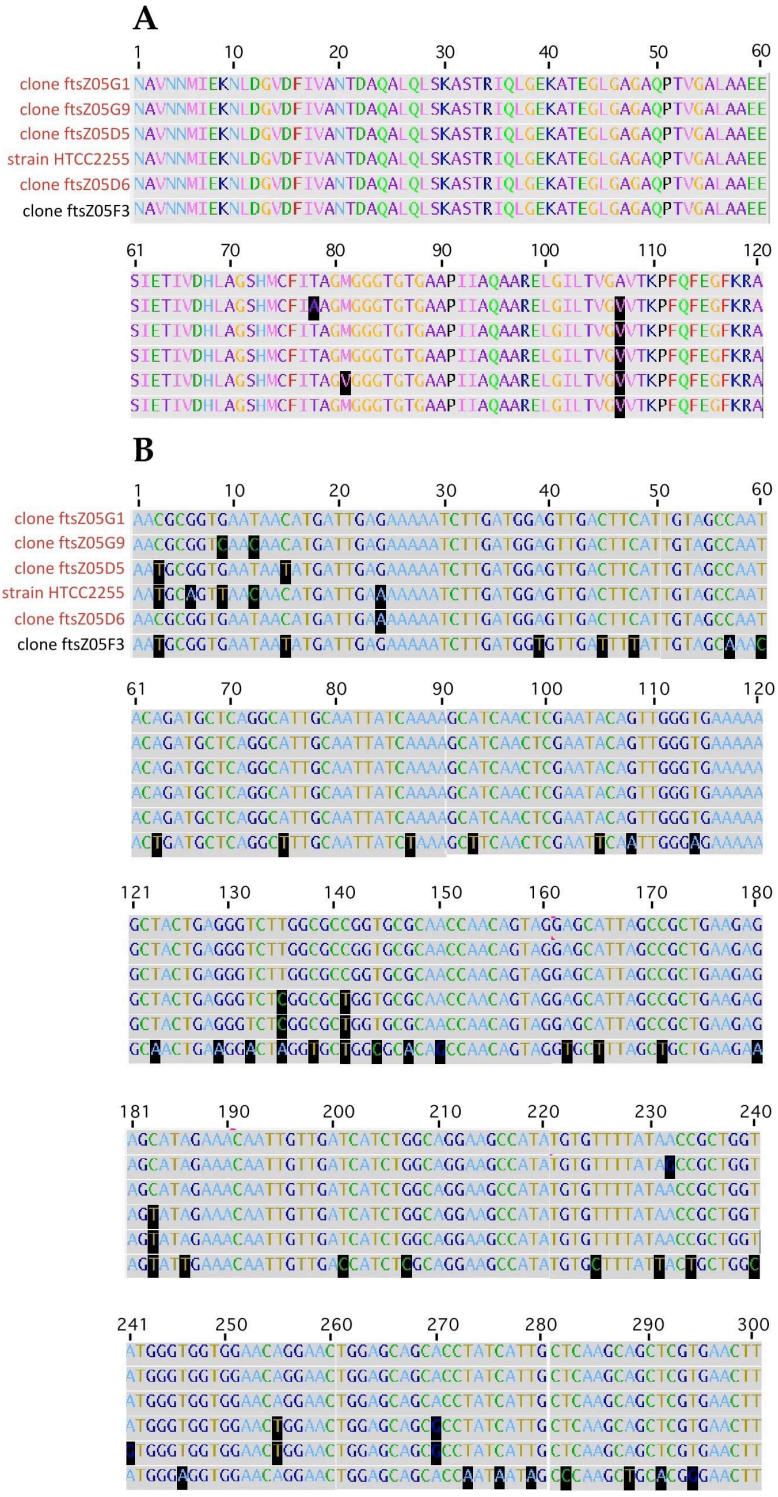
Supplemental Figure 1



Supplemental Figure 2



603 **Supplemental Figure 3**



604