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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, but not yet cultivated, 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), and between *ftsZ* expression and water temperature (R-squared=0.73) were observed *in situ*. Rapid and cyclical changes of NAC11-7 *ftsZ* gene copies suggested that populations from two different end-member water types were sampled. This was supported by distinct *ftsZ* gene expression levels in populations associated with these water types. An outlier to this trend occurred at a single time point (9:00), and was remarkably consistent with a concomitant peak in *ftsZ* expression in on-deck incubations, suggesting synchronous population growth. To our knowledge this is the first evidence for synchronous populations for a heterotrophic bacterioplankton group in the ocean.

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 Rappe 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", 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 is 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 was also found to vary during the cell cycle in synchronized *Prochlorococcus* populations in the Gulf of Aqaba, with

77 the maxima of expression at the replication phase (S phase; Holtzendorff et al., 2001;
78 Holtzendorff et al., 2002). These authors suggested that transcriptional regulation of *ftsZ* could
79 be a major factor triggering the observed synchronized cell division of *Prochlorococcus*
80 populations. Finally, the sequence of FtsZ protein is highly conserved in most bacteria and its
81 phylogeny has good congruence to 16S rRNA phylogeny (Vaughan et al., 2004), allowing
82 putative identification of genes uncovered directly from the environment.
83 In this study, we investigated *in situ* expression of *ftsZ* genes putatively belonging to the
84 NAC11-7 subclade of the *Roseobacter* group of marine bacteria. Members of the NAC11-7
85 clade have been implicated in DMSP degradation (Zubkov et al., 2001; Buchan et al., 2005) and
86 the genome sequence of a NAC11-7 representative (HTCC 2255) suggests members of this
87 subclade might be aerobic anoxygenic phototrophs, since the genome of HTCC 2255 contains a
88 roseobacter type *pufM* gene. We quantified *ftsZ* expression of this group over a diel cycle to
89 determine whether evidence exists for synchronicity in cell division. Gene expression was
90 measured as the ratio of *ftsZ* mRNA to *ftsZ* gene copies using quantitative real time PCR (qPCR)
91 and reverse transcription qPCR (RT-qPCR), respectively, in samples collected from a station off
92 the Chesapeake Bay and in a parallel on-board incubation.
93 ## Results
94 ***Rhodobacterales ftsZ genes from coastal waters off the Chesapeake Bay.***
95 Degenerate primers targeting *ftsZ* from the order Rhodobacterales were designed based
96 on conserved regions identified in alignments of representative FtsZ sequences obtained from
97 GenBank. A clone library was generated from fragments amplified from coastal Atlantic Ocean
98 surface waters and 74 *ftsZ* clones were analyzed. Phylogenetic reconstruction of these sequenced
99 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 *ftsZ* 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. 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* gene to gene expression inclusive of all sampling points, was not significant (R^2=0.0304， P=0.45). These results indicate that at least for some of the samples a correlation existed between gene abundance and per-cell gene expression activity.

In the first 12 hours, *ftsZ* expression and to some degree *ftsZ* gene copies appeared to be somewhat correlated with the tidal cycle, with peaks of expression at 6:00 and 18:00 and lowest

123 expression at 12:00, lagging the onshore high and low tides by about 3 hours respectively (Figure
124 2). Temperature and salinity also showed fluctuations that loosely followed the tidal cycle with
125 the same 3-hour lag (data not shown). After 18:00 a steady drop in temperature and simultaneous
126 increase in salinity was observed. Overall, these trends in salinity and temperature indicate that
127 tidal currents influenced our sampling site and that different waters with different NAC11-7
128 populations (or patches), were sampled.

129 In order to further examine the relationships between *ftsZ* gene abundance and expression
130 with water masses, we overlaid *ftsZ* gene abundances and expression to gene ratios onto
131 temperature-salinity (T-S) plots (Figure 3). These plots indicate a cyclical variation between
132 warmer, less saline waters and cooler, more saline waters. While, gene copies did not show clear
133 trends in the T-S plot (data not shown), *ftsZ* expression showed an interesting relationship. With
134 the single exception of the 9:00 sample, samples below 25.8°C showed significantly lower *ftsZ*
135 expression (two-sample t-test P=0.003) than those above 25.8°C (Figure 3). A regression
136 analysis excluding the 9:00 measurements showed a significant correlation (R^2=0.725, P=0.007)
137 between *ftsZ* expression and temperature. These data strongly indicate that populations from two
138 contrasting water types were sampled throughout the diel study. Furthermore, when the data are
139 separated between samples above and below 25.8 °C, an outlier in *ftsZ* transcript abundance is
140 evident at the 9:00 sampling time, suggestive of a peak in *ftsZ* gene expression at that time
141 (Figure 3).

142 ***ARISA analysis of microbial communities.***

143 In order to examine the dynamics of bacterioplankton communities and populations at the
144 different sampling times, these communities were examined using automated ribosomal
145 intergenic spacer analysis (ARISA). We observed that a number of ARISA peaks were present

146 at all time points analyzed, (i.e. 456, 485, 510 and 556 bp), while several peaks showed a more
147 ephemeral occurrence (i.e. 793, 799, 900 bp), suggesting that levels of patchiness differed for
148 different phylotypes (Supplemental Fig 1). At least in the case of these fluctuating phylotypes,
149 different populations (patches) appear to have been sampled at different times. Unfortunately
150 ARISA peaks sizes corresponding to the NAC11-7 group were not readily detected, as these
151 organisms were likely present in numbers below the resolution of ARISA.

152 ***Enclosed incubation diel expression of NAC11-7 ftsZ genes.***

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

162 ## Discussion

163 In order to test assumptions related to the measurement of *in situ* growth rates of specific
164 roseobacters based on cell division genes, a qPCR *ftsZ* assay was developed for the NAC11-7
165 clade. To the best of our knowledge these are the first quantitative data of relative expression of
166 a functional gene for a specific roseobacter subclade in natural samples. Analysis of the NAC11-
167 7 sequences reveals that although all the sequences were highly similar at the amino acid level,
168 one sequence (Clone ftsZ05F3 was different at the DNA level (Supplemental Figure 2). Based

169 on the ecotype theory (Cohan, 2001), clone ftsZ5F03 was assumed to belong to a different
170 ecotype that was not subjected to a theoretical selective sweep, as were the remaining 5
171 phylotypes. In order to maximize the chance that our measurements reflected populations most
172 coherent in their response to environmental factors, the sequence of clone ftsZ05F03 was not
173 targeted by our qPCR assay.

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

181 It is important to emphasize that our measurements of gene expression are based on a *per*
182 *cell* average for the entire population. Thus, depending on how *ftsZ* is expressed in a single cell
183 during the cell cycle, the mRNA to gene ratio may reflect: 1) the fraction of the population that is
184 actively transcribing *ftsZ* in preparation for cell division or 2) the average per cell expression
185 level [i.e. if *ftsZ* is constitutively transcribed, and FtsZ regulation occurs post-transcriptionally in
186 preparation for cell division]. If a population displays synchronous growth and *ftsZ* expression
187 varies during the cell cycle (Case 1), we expect to observe peaks in the population-level *ftsZ*
188 expression. Conversely, if *ftsZ* transcription were constitutive (Case 2), one would not expect
189 *ftsZ* expression peaks even in synchronous populations.

190 The only other study to examine *ftsZ* gene expression in natural bacterial assemblages
191 was carried out with Red Sea populations of the cyanobacterium *Prochlorococcus*. In that study,

192 *ftsZ* gene expression was found to be highly synchronous, with greatest expression occurring in
193 the twilight hours before nightfall (Holtzendorff et al., 2002). These expression profiles were
194 consistent with the known photoautotrophic physiology of the organism; these populations are
195 naturally synchronized and undergo cell division at night (Vaulot and Partensky, 1992 ; Vaulot et
196 al., 1995). *Caulobacter crescentus,* an aquatic alphaproteobacterium, which is largely used as a
197 model for cell cycle and differentiation studies (see review by McAdams and Shapiro, 2009), is
198 the closest roseobacter relative for which extensive *ftsZ* gene expression studies have been
199 undertaken. In this organism, *ftsZ* gene expression is cell cycle-dependent and is time
200 constrained (Quardokus et al., 1996; Kelly et al., 1998; Sackett et al., 1998; Martin and Brun,
201 2000; Brun, 2001). In contrast, FtsZ protein concentration per cell has been found to be constant
202 regardless of growth rate in the copiotrophic *E. coli (Gammaproteobacteria)* and *B. subtilis*
203 (*Firmicutes*) (Rueda et al., 2003; Weart and Levin, 2003; Haeusser and Levin, 2008). Several
204 arguments support time-constrained *ftsZ* expression in NAC11-7 members. As members of the
205 *Alphaproteobacteria,* these bacteria are more likely to share conserved features of central
206 physiology with *C. crescentus* than with the more distantly related *E. coli* or *B. subtilis*. In
207 addition, the genome of NAC 11-7 isolate HTCC2255 contains the genes encoding for DnaA and
208 CtrA (blastp e-value $<10^{-20}$ using the *Caulobacter crescentus* amino acid sequences), the central
209 regulators of *ftsZ* expression and cell cycle in this organism (McAdams and Shapiro, 2009).
210 Finally, it is difficult to rationalize advantages to NAC11-7, organisms adapted to somewhat
211 oligotrophic conditions, to synthesize a constant amount of FtsZ through the entire cell cycle
212 unless FtsZ has an alternative unknown function.
213 Different lines of evidence indicate that different NAC11-7 populations (or patches) with
214 different growth rates were sampled at different time points during the *in situ* experiment. First,

remarkable fluctuations of NAC11-7 *ftsZ* gene copy numbers (nearly 4-fold in 3 h) were observed *in situ* (Figure 2), implying changes in populations sizes resulting from physical processes. Second, ARISA analysis of the total bacterioplankton community structure showed evidence for patchiness, as certain phylotypes were only detected in specific time points (Supplemental Figure 1). While transient populations were likely sampled throughout the diel cycle, some trends in *ftsZ* gene and gene expression did emerge that revealed insight into *ftsZ* gene expression profiles and synchronicity of these populations. During the first seven data points (18 hrs from 6:00 to 0:00), a strong correlation (R^2= 0.6214) was observed between NAC11-7 abundance *in situ* (inferred from *ftsZ* copies/ml), and the average *per cell ftsZ* 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 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) might 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

238 NAC11-7 group respond positively to enrichment conditions (i.e. this group is not known as
239 copiotrophic), all argue against this notion. The slow decline in *ftsZ* gene abundance in the
240 bottles might be explained by a lack of growth, and continuous mortality by viral lysis and/or
241 protistan bacterivory. The peak of *ftsZ* expression at 9:00 might be explained by a previous
242 "commitment" of the circadian rhythm. As different NAC11-7 populations (patches) were
243 sampled over the course of the *in situ* diel it is difficult to directly compare *in situ ftsZ* gene
244 expression levels with the parallel on-deck experiment. However, the fact that the *in situ* sample
245 from 9:00 was higher than expected based on the correlation between gene expression and
246 temperature (Figure 3) also agrees with the incubation results.
247 An intriguing interpretation of these seemingly contradictory results is that a certain
248 fraction of the population is synchronously dividing, with a peak in *ftsZ* expression at 9:00, while
249 a background of asynchronously dividing cells also exist, some of which are expressing *ftsZ* at
250 any given time. This "background" expression might be in fact, better correlated to temperature
251 or other parameters influencing growth. The fact that a certain level of *ftsZ* expression was
252 measured throughout the diel cycle in synchronously dividing *Prochlorococcus* populations
253 (Holzendorf et al 2001) seems to support this idea. However, in the case of *Prochlorococcus*,
254 *ftsZ* expression is at the end of the light period and cell division occurs at the dark (Holtzendorff
255 et al., 2001). Assuming that the NAC11-7 ecotype is an anaerobic anoxygenic phototroph (AAP)
256 as HTCC 2255 appears to be, one possible explanation for a peak of expression in the morning
257 with subsequent cell division might be related to the fact that in the Rhodobacterales AAP
258 studied to date (i.e. Biebl and Wagner-Dobler, 2006; Koblizek et al., 2010) bacteriochlorophyll
259 synthesis occurs at night. Division in the early morning could assure a maximum amount of this
260 pigment is available to daughter cells.

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 to the study of bacterioplankton ecology. For instance, bacterioplankton production is in most cases measured for the bulk community at short-time incubations. Thus, understanding whether specific populations are synchronous will be paramount to the full interpretation of these bulk 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 is also important. Also, future *in situ* studies 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 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 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 a 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 on the ice and 320μl low protein binding zirconium oxide beads (200 μm, OPS Diagnostics, Lebanon, NJ) were added into each tube. ß-Mercaptoethanol was mixed in a 1:100 ratio into RLT buffer and 875 μl of the mixture added to screw cap tubes. The tubes were beaten in a MM301 mixer mill (Retsch GmbH Inc., Haan, Germany) at 30.0 HZ for 2 min and incubated for 5 min at 70 ºC. 800 μl of the liquid phase was transferred into a new low-RNA-binding 2 ml microcentrifuge tube (Ambion, Inc.), avoiding the beads. 800μl of 100% ethanol was added into

each tube and well mixed well. 800 μl of the mixture was loaded into wells of RNeasy 96 plate, sealed and centrifuged at 5000 rcf for 5 min, and the procedure repeated. The columns were washed once with 800 μl RW1 buffer and twice with 800 μl RPE buffer at 5000 rcf for 5 min, with the last spin for 15 min. 35 μl Diethylpyrocarbonate (DEPC) treated water (Ambion) were added into each column, incubation for1 min at room temperature and 5 min centrifugation at 5000 rcf. This procedure was repeated with another 35 μl DEPC treated water. RNA was treated the DNA-free™ kit (Ambion) to remove co-extracted DNA.

DNA and RNA concentrations were quantified fluorometrically by PicoGreen® and RiboGreen® staining respectively (Molecular Probes, Invitrogen Corp., Carlsbad, CA) on a Spectra MAX Gemini microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA).

Construction of ftsZ DNA PCR clone library

An initial *ftsZ* and FtsZ database was developed using *arb* (Ludwig *et al*., 2004) by importing gene sequences listed by Vaughan and coworkers (Vaughan et al., 2004). 163 representative FtsZ sequences were exported and used to retrieve additional aminoacid, and coding gene sequences using blastp searches against the March 2005 NCBI *nt, env_nt* and *wgs* 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* 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.

1μl of extracted genomic DNA was used as template in a 10 μl-volume PCR reaction, which also included 10X PCR buffer, 0.2mM 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

353 "burnin" trees were excluded to generate the consensus tree. The average standard deviation of
354 split frequencies was below 0.05 after 1,100,000 generations.

355 ***Real Time PCR Assays***

356 PCR primers and a Taqman probe were designed to target selected members of the
357 NAC11-7 group. Results of *ftsZ* cloning and sequencing indicated that based on amino acid
358 sequences five clone sequences were affiliated with strain HTCC 2255. However, the DNA
359 sequence of one of these clones (F3) contained obviously higher variation to the remainder
360 clones and HTCC 2255 (Supplemental Figure 1). Based on ecotype theory (Cohan, 2001), it
361 appeared that the distinct clone could belong to a different ecotype, which we assumed was not
362 subject to a periodic selection event as the remaining sequences. Thus we designed a primer and
363 probe to exclusively target the remaining four sequences and HTCC 2255. The primer and the
364 probe were manually designed with aid of probe match functions in the arb_edit module of the
365 ARB package. T_M, secondary structure and possible dimers were checked using Primer Express
366 (Applied Biosystems) and the Oligo Analyzer online tool (www.idtdna.com).

367 To test specificity of primers to the target sequences, 3 target clones and 15 non-target
368 clones were purified using QuickLyse Miniprep kit (Qiagen), and diluted to 10^7 *ftsZ* copies/μl
369 with nuclease-free TE buffer (Ambion). Ten μl PCR reaction contained 5 μl TaqMan®
370 Universal PCR Master Mix (Applied Biosystems), 0.5μM Forward primer ftsZrbA03-2qF (GTG
371 AAA AAG CTA CTG AGG GTC T) and Reverse primer ftsZrbA03-2qR (GCT TCC TGC CAG
372 ATG ATC), and 1 μl plasmid template. The cycling parameters were as follows: 2 min at 50 °C,
373 10 min at 95 °C and 30 cycles of 15 sec at 95 °C and 1min at 57 °C. All PCR products were
374 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).

In order to get the highest amplification efficiency in real time PCR, a primer concentration matrix was performed. In each 25 μl reaction, the following reagents were kept constant: 1X PCR buffer, 0.2 mM of dATP, dGTP and dCTP, 0.4 mM of dUTP, 5 mM $MgCl_2$, 200 nM probe NAC11-7 (AAC CAACAGTAGGAGCATTAGCCGCT), 1.2 μM SuperROX™ (Biosearch Technologies, Novato, CA), 0.01 U/μl AmpErase® Uracil N-glycosylase (UNG) (Applied Biosystems), 0.025 U/μl of Platinum® Taq DNA Polymerase (Invitrogen), 2.5 μl NAC11-7 standard (10^4 copies/μl) and a matrix of forward and reverse primer concentrations of 0.1 μM, 0.5 μM, 1.0 μM, 1.5 μM. Reactions were set in a MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems), which was sealed with an optical adhesive cover (Applied Biosystems) reactions 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 (1.5 μM ftsZrbA03-2qF; 0.5 μM ftsZrbA03-2qR) was used in all subsequent measurements.

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 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

398 Shi (Shi, 2005). mRNAs copies were assumed equal to cDNA copies. Since DNA and mRNA
399 were measured in triplicate biological samples, *ftsZ* genes and mRNA copy numbers that were
400 >2X or <X/2 (X is the average of the remainder two replicates) were treated as outliers and
401 removed from the analyses

402 **Optimization of Reverse Transcription**

403 Since preliminary results showed relatively high C_T values from *in situ* sample cDNAs,
404 we attempted to increase *ftsZ* cDNA copy numbers by: (1). Increasing reverse transcription
405 efficiency, by varying template amount, RT temperature and RT duration and (2). Adding
406 increased amounts of cDNA products in real-time PCR reactions. In optimized conditions 4 μl
407 purified RNA (ca. 10 ng) was reverse transcribed to cDNA using ThermoScript™ Reverse
408 Transcriptase. Template and 10 pmol of the NAC11-7 specific primer ftsZrbA03-2qR were
409 heated to 65°C for 5 min and chilled on ice immediately, followed by the addition of 5X cDNA
410 synthesis buffer, 5mM DTT, 2 U/μl RNaseOUT™ Inhibitor (Invitrogen), 0.75 U/μ of reverse
411 transcriptase and DEPC-treated water to a final volume of 10 μl. Reactions were incubated at
412 55°C for 60 min to synthesize cDNA and at 85°C for 5 min to inactivate the transcriptase. No-
413 RT controls were performed for one of the triplicate samples using the same method except
414 substituting reverse transcriptase with DEPC-treated water.

415 ***ARISA (Automated rRNA intergenic spacer analysis)***

416 One μl environmental genomic DNA was used to perform a 10 μl ARISA reactions,
417 containing 1X PCR buffer, 1.2 mM $MgCl_2$, 0.08 mM dNTPs (Promega Corp., Madison, WI), 0.5
418 μM primer 1406F-FAM (Fisher and Triplett, 1999), 1.5 μM primer 23S-Y (Dyda et al., 2009),
419 and 0.01 U/μl of Platinum® Taq DNA Polymerase (Invitrogen). Reactions were run on a
420 GeneAmp 9700 (Applied Biosystems) under the following conditions: Initial denaturation and

421 enzyme activation at 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec
422 and 65 ºC for 2min. 1 μl of each PCR reaction was mixed with 9 μl of 1:0.06 Formamide and
423 GS2500 size standard (Applied Biosystems), denatured at 94 ºC for 2 min and separated by
424 capillary electrophoresis using an Applied Biosystems 3100 genetic analyzer. Sizes of the
425 fragments were analyzed by the Peak Scanner™ Software v1.0 (Applied Biosystems).

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430

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Figure legends

Figure 1. Consensus 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. The numbers on nodes represent branch confidence values. The bar represents the mean number of substitutions per residue in the trees used to build the consensus tree.

Figure 2. *FtsZ* gene copy numbers (grey bars) and expression (estimated from the ratio of *ftsZ* cDNA copies to gene copies; white bars) 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 from a single CTD cast. Pre-dawn and post-dusk sampling times are represented by the grayed areas. The tidal height is shown with the line lacking symbols.

Figure 3. Temperature and Salinity plot, with overlaying *in situ* NAC11-7 *ftsZ* expression. Gene normalized *ftsZ* expression levels are proportional to the area of the circles. The area of the circle representing the 3:00 PM sample corresponds to 2.5 10^{-4} mRNA copies per gene copies. Inset: normalized *in situ ftsZ* expression in samples with temperatures below and above 25.8 °C.

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

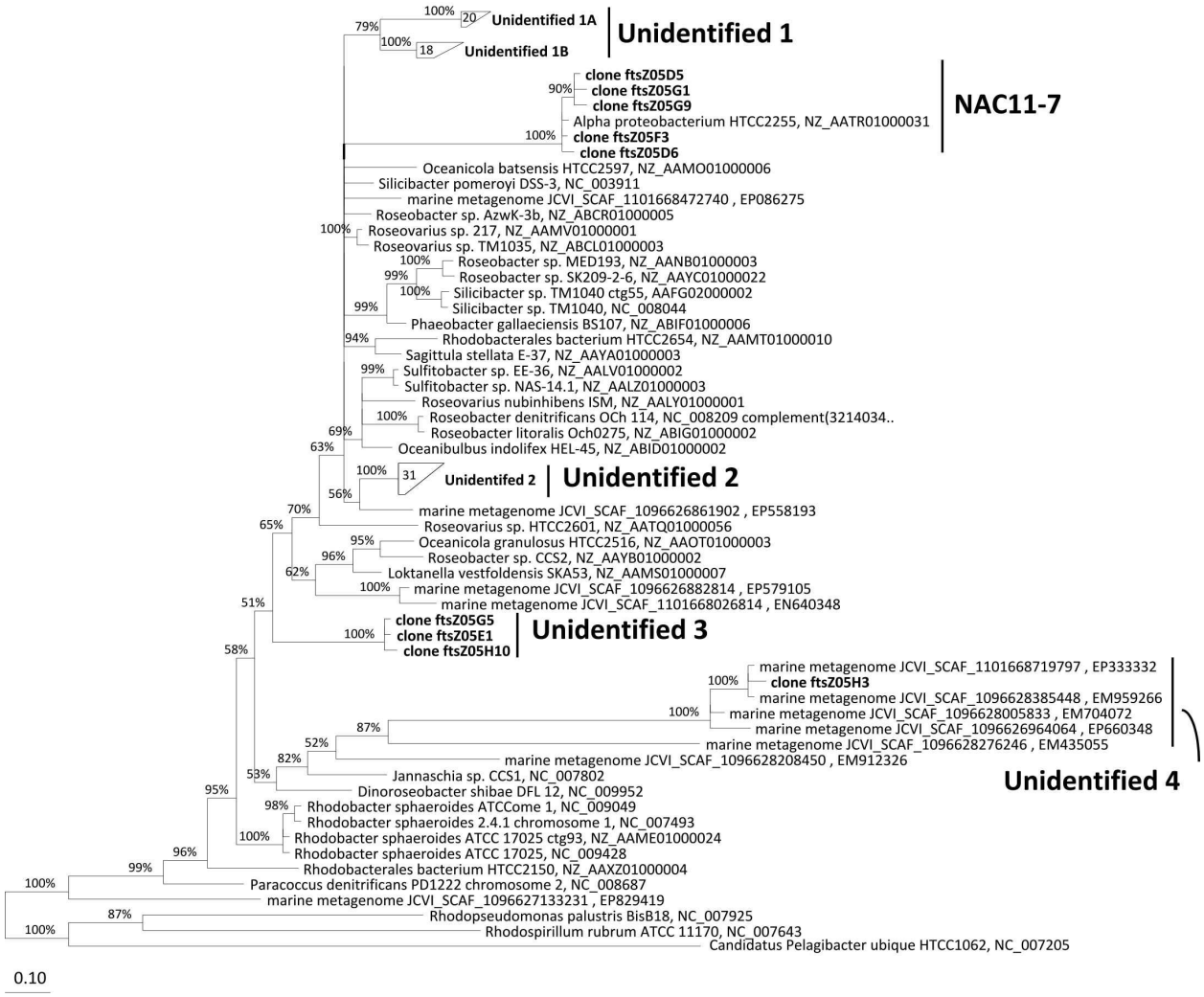
566 Supplemental Fig 1. Percentage of different phylotypes identified by ARISA fragment sizes in
567 the *in situ* samples. The results shown in here indicate that the replicate subsamples were largely
568 similar in composition, indicative of relatively low within-sample variation. However, in a few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

572 Supplemental Figure 2. A. Alignment of NAC11-7 FtsZ sequences. B alignment of NAC11-7
573 *ftsZ* sequences

574

575

Figure 1.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Unidentified 1B
Unidentified 1
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Alpha proteobacterium HTCC2255, NZ_AATR01000031
clone ftsZ05F3
clone ftsZ05D6
NAC11-7
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Silicibacter pomeroyi DSS-3, NC_003911
marine metagenome JCVI_SCAF_1101668472740 , EP086275
Roseobacter sp. AzwK-3b, NZ_ABCR01000005
Roseovarius sp. 217, NZ_AAMV01000001
Roseovarius sp. TM1035, NZ_ABCL01000003
Roseobacter sp. MED193, NZ_AANB01000003
Roseobacter sp. SK209-2-6, NZ_AAYC01000022
Silicibacter sp. TM1040 ctg55, AAFG02000002
Silicibacter sp. TM1040, NC_008044
Phaeobacter gallaeciensis BS107, NZ_ABIF01000006
Rhodobacterales bacterium HTCC2654, NZ_AAMT01000010
Sagittula stellata E-37, NZ_AAYA01000003
Sulfitobacter sp. EE-36, NZ_AALV01000002
Sulfitobacter sp. NAS-14.1, NZ_AALZ01000003
Roseovarius nubinhibens ISM, NZ_AALY01000001
Roseobacter denitrificans OCh 114, NC_008209 complement(3214034..
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Unidentified 2
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Loktanella vestfoldensis SKA53, NZ_AAMS01000007
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clone ftsZ05E1
clone ftsZ05H10
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clone ftsZ05H3
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marine metagenome JCVI_SCAF_1096628005833 , EM704072
marine metagenome JCVI_SCAF_1096626964064 , EP660348
marine metagenome JCVI_SCAF_1096628276246 , EM435055
marine metagenome JCVI_SCAF_1096628208450 , EM912326
Unidentified 4
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Dinoroseobacter shibae DFL 12, NC_009952
Rhodobacter sphaeroides ATCCome 1, NC_009049
Rhodobacter sphaeroides 2.4.1 chromosome 1, NC_007493
Rhodobacter sphaeroides ATCC 17025 ctg93, NZ_AAME01000024
Rhodobacter sphaeroides ATCC 17025, NC_009428
Rhodobacterales bacterium HTCC2150, NZ_AAXZ01000004
Paracoccus denitrificans PD1222 chromosome 2, NC_008687
marine metagenome JCVI_SCAF_1096627133231 , EP829419
Rhodopseudomonas palustris BisB18, NC_007925
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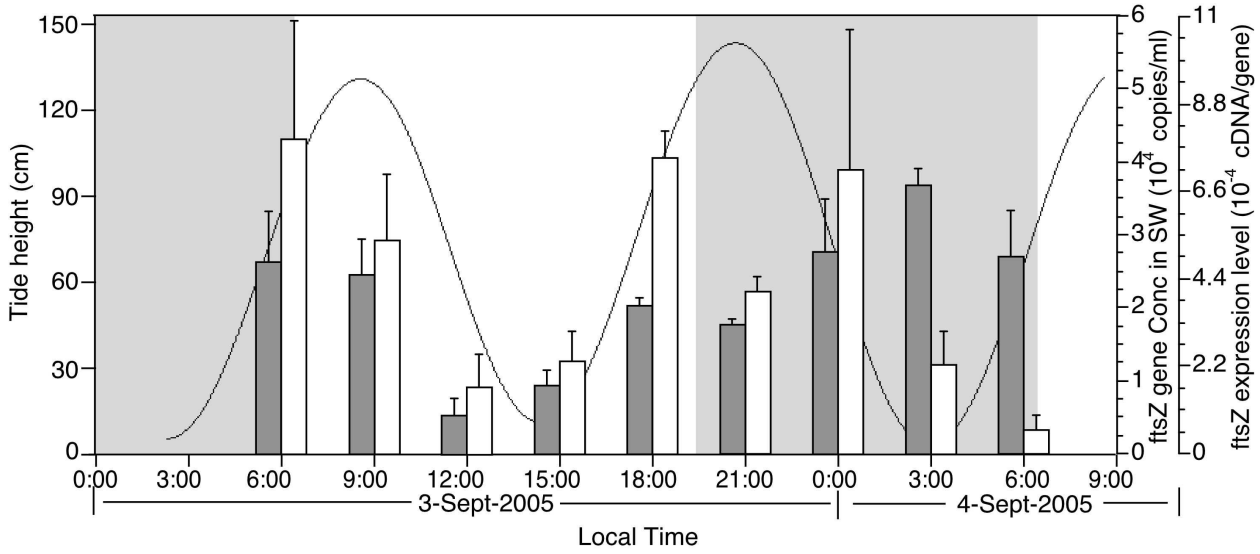
577 **Figure 2.**



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579 **Figure 3.**

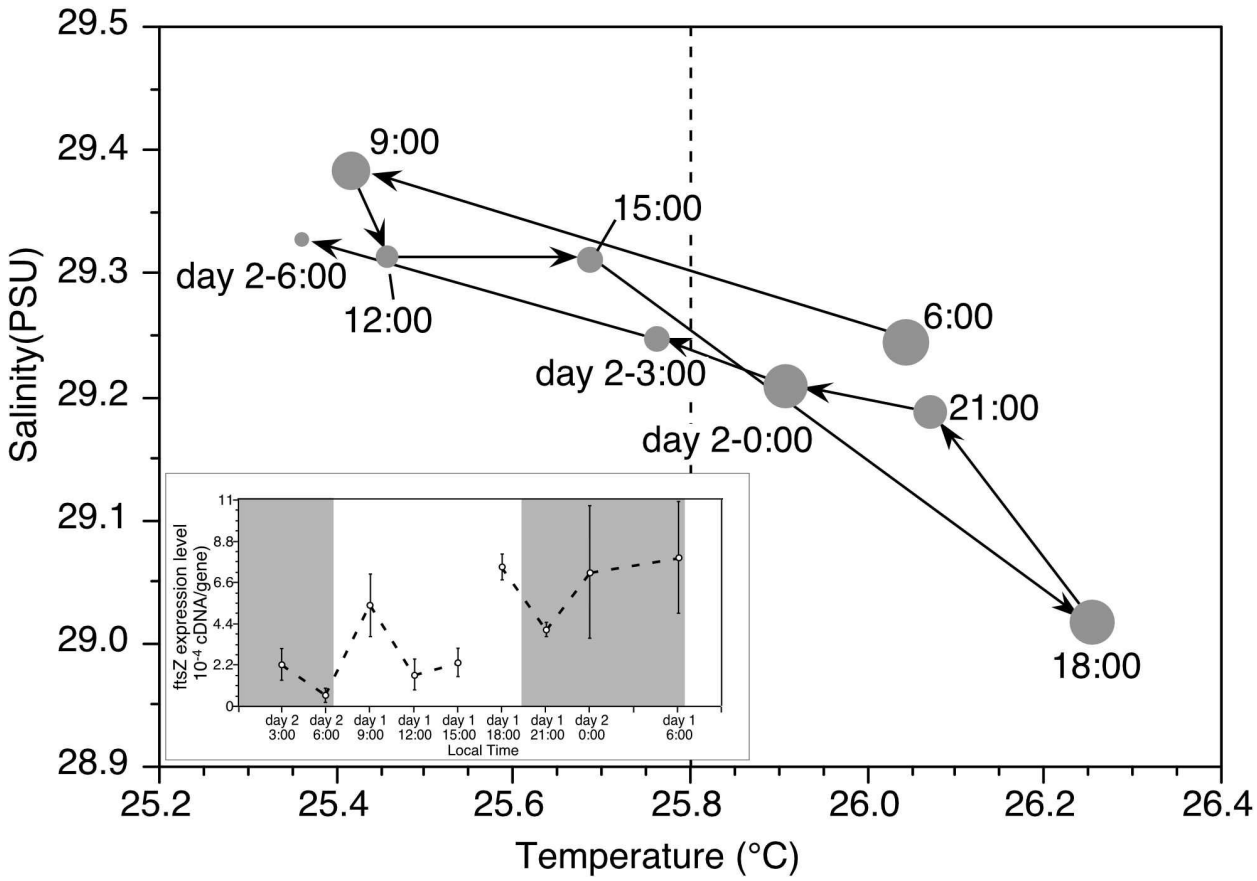

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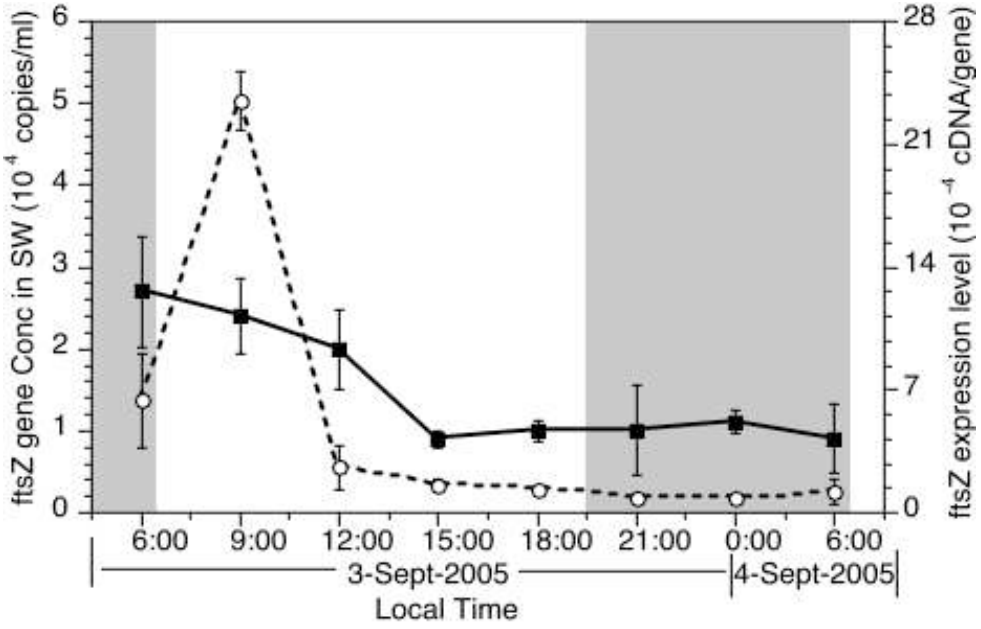
Figure 4.


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
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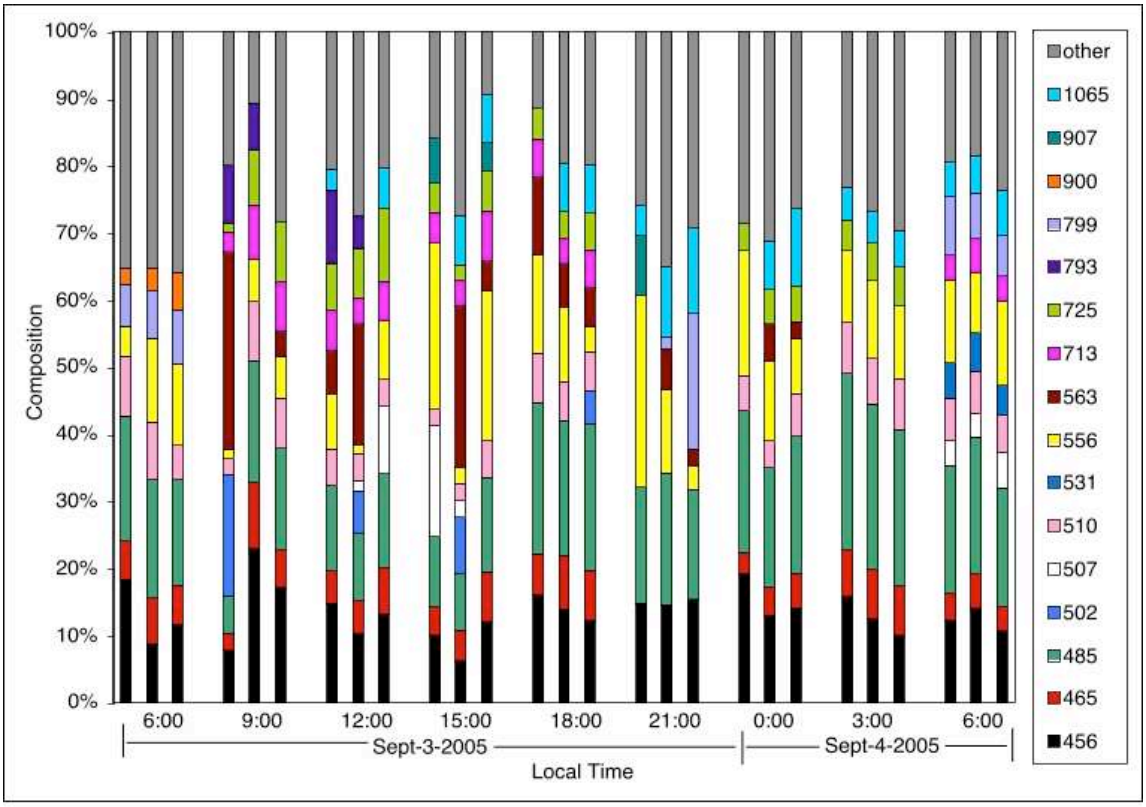
3-Sept-2005
4-Sept-2005
Local Time

583 **Supplemental Figure 1**



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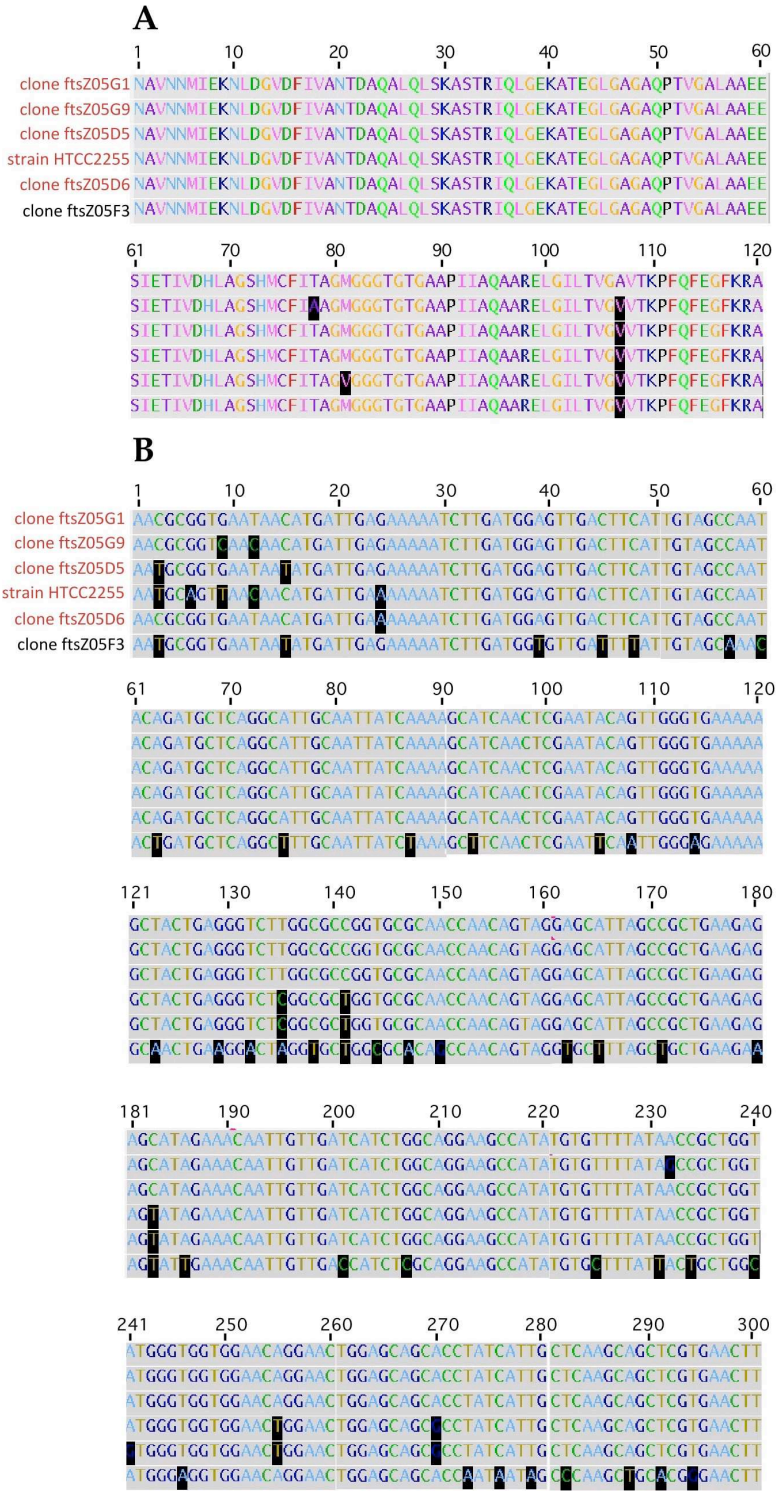
585 Supplemental Figure 2



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