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6 ***In situ* Activity of NAC11-7 Roseobacters in Coastal Waters off the**  
7 **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

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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 <sup>3</sup>H-thymidine or <sup>3</sup>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).

57 A plausible approach to measure *in situ* activity, and perhaps growth rates of specific  
58 bacterioplankton, involves quantifying the expression (mRNAs) of genes encoding proteins  
59 involved in cell division. The idea behind such approach would be to determine direct  
60 relationships between *in situ* expression of protein-encoding genes and growth rates of specific  
61 bacterioplankton, without additions or incubations. The rationale is analogous to that of the  
62 measurement of the frequency of dividing cells (Hagstrom et al., 1979), except that the  
63 measurement could be made at specific phylogenetic levels, and earlier in the cell cycle (i.e.  
64 before a clear septal ring is formed), thus decreasing problems associated with preferential  
65 protistan grazing of dividing cells (Sherr et al., 1992). Requirements for successfully using a  
66 gene transcript tracking approach for **instantaneous** (i.e. at any time of the day) measurements  
67 of growth would be that populations of targeted organisms do not divide synchronously, and that  
68 the regulation of cell division protein synthesis occurs at the transcriptional level. In cases where  
69 the population divides synchronously, growth rates estimates are possible, but would not be  
70 instantaneous and would require measurements throughout a diel cycle (i.e. Carpenter and Chang,  
71 1988; Chang and Carpenter, 1988; Holtzendorff et al., 2002).

72 FtsZ initiates cell division of most prokaryotes by self-assembling into a membrane-  
73 associated Z-ring structure and by recruiting other proteins to form the cell division septum  
74 (Margolin, 2005; Dajkovic and Lutkenhaus, 2006; Osawa and Erickson, 2006). In synchronized  
75 populations of *Caulobacter crescentus*, a model organism for the study of bacterial cell division,  
76 the transcription of *ftsZ* is controlled by two regulators: DnaA promotes *ftsZ* transcription, while  
77 CtrA its repressor, ensures that cell division is coordinated with DNA replication (Hottes et al.,  
78 2005; McAdams and Shapiro, 2009). Levels of expression of *ftsZ* genes were also found to vary  
79 during the cell cycle in synchronized *Prochlorococcus* populations in the Gulf of Aqaba, with

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

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

## 96 **Results**

### 97 ***Rhodobacterales ftsZ genes from coastal waters off the Chesapeake Bay.***

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

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

#### 110 *In situ diel expression of NAC11-7 ftsZ genes.*

111 NAC11-7 *ftsZ* mRNA and DNA copies were monitored in surface waters over a 24 h  
112 period at a single station located at the mouth of the Chesapeake Bay. Reproducibility among  
113 triplicates was quite good for gene copies (Figure 2), with a single exception of 21:00 where the  
114 difference among samples was also reflected by different ARISA profiles (Supplemental Figure  
115 1). Estimated mRNA copies were more variable among triplicates, mostly likely due to RNA  
116 degradation. In 5 of 9 timepoints, a single measurement was considered as an outlier, thus  
117 averages of duplicates were taken. Gene copies, and more remarkably, gene expression, showed  
118 rapid and sometimes large (c.a. 4-fold) changes with time (i.e. from 9:00 to 12:00 and from  
119 18:00 to 21:00), suggesting distinct populations (i.e. patches) of NAC11-7 may have been  
120 sampled at different times, as a result of water advection (Figure 2). Interestingly, *ftsZ* gene  
121 expression followed closely *ftsZ* gene abundance trends, except at the last two sampling times. A  
122 regression analysis between *ftsZ* gene copies and gene expression using individual replicates  
123 showed significant correlation ( $R^2= 0.6214$ ,  $P=0.0003$ ) for the first seven sampling times,  
124 covering 18 hours from 6:00 to midnight. However, for the remaining two time points (3:00 and  
125 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).

149 ***ARISA analysis of microbial communities.***

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

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

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

169 **Discussion**

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

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

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

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

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

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

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

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

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

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

279

## 280 **Experimental Procedures**

### 281 ***Sampling***

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

287 Triplicate 590 ml water subsamples were collected and pre-filtered through GF/A filters (1.6  $\mu$ m  
288 nominal pore; Whatman, Maidstone, UK). Ninety ml of pre-filtered sample was filtered through  
289 13 mm diameter 0.2 $\mu$ m Supor200<sup>®</sup> polysulfone filters (Pall Corp., East Hills, NY) and the filters  
290 were transferred to a tube containing 130 $\mu$ l lysis buffer (2 mM NaEDTA (pH 8.0), 20 mM  
291 Tris•Cl (pH 8.0), 1.2% v/v Triton X100). The remaining 500 ml were filtered through 25 mm  
292 diameter 0.2  $\mu$ m Supor200<sup>®</sup> filters (Pall Gelman Inc.) and transferred to screw cap tubes  
293 containing 250  $\mu$ l RNALater (Ambion, Austin, TX). All samples were frozen at  $-20^{\circ}\text{C}$  aboard  
294 and within a week stored at  $-70^{\circ}\text{C}$  until nucleic acid extraction.

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

### 301 ***Nucleic acid extraction and quantification***

302 Total DNA was extracted from Supor200<sup>®</sup> 0.2  $\mu$ m filters as previously described, except  
303 that the DNeasy 96 Tissue Kit was used, and therefore the DNeasy 96 plates were sealed and  
304 centrifugations performed in a Sorvall<sup>®</sup> Legend<sup>™</sup> T Centrifuge with a Highplate<sup>®</sup> rotor (Kendro,  
305 Osterode, Germany) at 5250 rpm for 10 min for binding and 5250 rpm for 5 min for washes. The  
306 DNeasy 96 plate was incubated at  $70^{\circ}\text{C}$  for 15 min evaporate traces of ethanol and DNA was  
307 eluted by centrifugation at 5250 rpm, for 2 min.

308 Total RNA was extracted from Supor200<sup>®</sup> 0.2  $\mu$ m filters following an optimized protocol  
309 adapted from the Qiagen RNeasy<sup>®</sup> 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  $\mu$ l) were added into each tube (200  
312  $\mu$ m, OPS Diagnostics, Lebanon, NJ).  $\beta$ -Mercaptoethanol was mixed in a 1:100 ratio into RLT  
313 buffer and 875  $\mu$ l 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 °C. 800  $\mu$ l of the liquid phase was transferred into a new low-RNA-binding 2 ml  
316 microcentrifuge tube (Ambion, Inc.), avoiding the beads. 800  $\mu$ l of 100% ethanol was added into  
317 each tube and well mixed well. 800  $\mu$ l 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  $\mu$ l RW1 buffer and twice with 800  $\mu$ l RPE buffer at 5000 rcf for 5 min,  
320 with the last spin for 15 min. 35  $\mu$ l 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  $\mu$ l 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

333 bayesian tree containing 507 sequences (144 homologous positions) was constructed using  
334 MrBayes version 3.0 (Ronquist and Huelsenbeck, 2003) with the following parameters: 800,000  
335 generations, mixed models of amino acid substitution, and a burn in of 6000 trees. In this tree,  
336 the *Alphaproteobacteria* formed a monophyletic clade with high confidence values and several  
337 orders including *Rhizobiales* and *Rhodobacterales*, which also formed monophyletic clades (data  
338 not shown). The database was updated with public sequences belonging to the *Rhodobacterales*,  
339 and was used for the design of PCR primers and probes. Degenerate primers targeting *ftsZ* from  
340 *Rhodobacterales* (*ftsZrb2F*: 5'-AA Y GCN GTS AAY AAY AT-3', *ftsZrb2R*: 5'-YTT NCC  
341 CAT YTC RT-3') were designed and used to retrieve *ftsZ* gene sequences by PCR and cloning  
342 from a DNA sample collected at time zero.

343         1  $\mu$ l of extracted genomic DNA was used as template in a 10  $\mu$ l-volume PCR reaction,  
344 which also included 10X PCR buffer, 0.2 mM of each dNTP, 3 mM MgCl<sub>2</sub>, 500 nM forward  
345 primer *ftsZ2rbF*, 500 nM reverse primer *ftsZ2rbR* and 0.025 U/ $\mu$ l of Platinum® Taq DNA  
346 Polymerase (Invitrogen, Carlsbad, CA). Reactions were performed on a GeneAmp 9700 PCR  
347 system (Applied Biosystems) and cycling conditions were as follows: 2 min at 94 °C and 37  
348 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 2 min at 72 °C. PCR was followed by a  
349 reconditioning (Thompson et al., 2002) of 5 steps. Reconditioned PCR products were loaded on  
350 a 1% modified TAE (40 mM Tris-acetate, pH 8.0, 0.1 mM Na<sub>2</sub>EDTA) agarose gel and separated  
351 by electrophoresis. Target fragments were cut, recovered by Ultrafree®-DA (Millipore) gel  
352 extraction and used to built a *ftsZ* gene clone library using the TOPO™ TA cloning kit  
353 (Invitrogen) following manufacture's instructions. 96 clones were bidirectionally sequenced  
354 using BigDye V3.1 chemistry and capillary electrophoresis on an AB3100 genetic analyzer  
355 (Applied Biosystems Inc, Foster City, CA). These sequence data have been submitted to the

356 GenBank database under accession number HM035149-HM035222. 74 sequences identified as  
357 *ftsZ* were imported into the ARB database described above and added to the tree described above  
358 using the ADD\_BY\_PARSIMONY tool. Finally, a bayesian tree of Rhodobacterales *ftsZ* was  
359 constructed using the MPI version of Mr Bayes V3.1 (Ronquist and Huelsenbeck, 2003; Altekar  
360 et al., 2004). 188 homologous amino acid positions from 123 sequences were exported and used  
361 in this phylogenetic analysis. Two four parallel chains of 2,000,000 generations were run with  
362 mixed models of amino acid substitution; trees were sampled every 100 generations, and 11000  
363 “burnin” trees were excluded to generate the consensus tree. The average standard deviation of  
364 split frequencies was below 0.05 after 1,100,000 generations.

### 365 ***Real Time PCR Assays***

366 PCR primers and a Taqman probe were designed to target selected members of the  
367 NAC11-7 group. Results of *ftsZ* cloning and sequencing indicated that based on amino acid  
368 sequences, five clone sequences were affiliated with strain HTCC 2255. However, the DNA  
369 sequence of one of these clones (*ftsZ*5F03) contained obviously higher variation to the remainder  
370 clones and HTCC 2255 (Supplemental Figure 3). Based on ecotype theory (Cohan, 2001), it  
371 appeared that the distinct clone could belong to a different ecotype, which we assumed was not  
372 subject to a periodic selection event as the remaining sequences. Thus we designed a primer and  
373 probe to exclusively target the remaining four sequences and HTCC 2255. The primer and the  
374 probe were manually designed with aid of probe match functions in the arb\_edit module of the  
375 ARB package.  $T_M$ , secondary structure and possible dimers were checked using Primer Express  
376 (Applied Biosystems) and the Oligo Analyzer online tool ([www.idtdna.com](http://www.idtdna.com)).

377 To test specificity of primers to the target sequences, 3 target clones and 15 non-target  
378 clones were purified using QuickLyse Miniprep kit (Qiagen), and diluted to  $10^7$  *ftsZ* copies/ $\mu$ l

379 with nuclease-free TE buffer (Ambion). Ten  $\mu\text{l}$  PCR reactions contained 5  $\mu\text{l}$  TaqMan®  
380 Universal PCR Master Mix (Applied Biosystems), 0.5  $\mu\text{M}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\text{MgCl}_2$ ,  
390 200 nM probe NAC11-7 (AAC CAACAGTAGGAGCATTAGCCGCT), 1.2  $\mu\text{M}$  SuperROX™  
391 (Biosearch Technologies, Novato, CA), 0.01 U/ $\mu\text{l}$  AmpErase® Uracil N-glycosylase (UNG)  
392 (Applied Biosystems), 0.025 U/ $\mu\text{l}$  of Platinum® Taq DNA Polymerase (Invitrogen), 2.5  $\mu\text{l}$   
393 NAC11-7 standard ( $10^4$  copies/ $\mu\text{l}$ ) and a matrix of forward and reverse primer concentrations of  
394 0.1  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1.0  $\mu\text{M}$ , 1.5  $\mu\text{M}$ . 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  $\mu\text{M}$  ftsZrbA03-2qF; 0.5  $\mu\text{M}$   
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

402 of *ftsZ* gene and mRNA. 2.5  $\mu$ l of DNA extracts were used in 25  $\mu$ l reactions and standards  
403 ranged from  $10^2$  to  $10^7$  copies/ $\mu$ l. Reactions were run in duplicate along with non-template  
404 controls using the same cycling parameters described for the primer matrix. *ftsZ* cDNA was  
405 quantified in the same manner as *ftsZ* gene except that 5  $\mu$ l of Reverse transcription products  
406 were used as template. All real time PCR measurements were calculated as copy numbers per  
407 volume of seawater, assuming that nucleic acid extraction efficiencies were constant as shown by  
408 Shi (Shi, 2005). mRNAs copies were assumed equal to cDNA copies. Since DNA and mRNA in  
409 the *in situ* experiment were measured in triplicate biological samples, *ftsZ* genes and mRNA  
410 copy numbers that were  $>2X$  or  $<X/2$  ( $X$  is the average of the remainder two replicates) were  
411 treated as outliers and removed from the analyses. Incubated samples were not subjected to  
412 outlier analysis, but the DNA samples at 3:00 were lost and not included in the analysis.

### 413 **Optimization of Reverse Transcription**

414 Since preliminary results showed relatively high  $C_T$  values from *in situ* sample cDNAs,  
415 we attempted to increase *ftsZ* cDNA copy numbers by: (1). Increasing reverse transcription  
416 efficiency, by varying template amount, RT temperature and RT duration and (2). Adding  
417 increased amounts of cDNA products in real-time PCR reactions. In optimized conditions 4  $\mu$ l  
418 purified RNA (ca. 10 ng) was reverse transcribed to cDNA using ThermoScript™ Reverse  
419 Transcriptase. Template and 10 pmol of the NAC11-7 specific primer *ftsZ*rbA03-2qR were  
420 heated to 65°C for 5 min and chilled on ice immediately, followed by the addition of 5X cDNA  
421 synthesis buffer, 5mM DTT, 2 U/ $\mu$ l RNaseOUT™ Inhibitor (Invitrogen), 0.75 U/ $\mu$  of reverse  
422 transcriptase and DEPC-treated water to a final volume of 10  $\mu$ l. Reactions were incubated at  
423 55°C for 60 min to synthesize cDNA and at 85°C for 5 min to inactivate the transcriptase. No-

424 RT controls were performed for one of the triplicate samples using the same method except  
425 substituting reverse transcriptase with DEPC-treated water.

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

427 One  $\mu$ l environmental genomic DNA was used to perform a 10  $\mu$ l ARISA reactions,  
428 containing 1X PCR buffer, 1.2 mM MgCl<sub>2</sub>, 0.08 mM dNTPs (Promega Corp., Madison, WI), 0.5  
429  $\mu$ M primer 1406F-FAM (Fisher and Triplett, 1999), 1.5  $\mu$ M primer 23S-Y (Dyda et al., 2009),  
430 and 0.01 U/ $\mu$ l of Platinum® Taq DNA Polymerase (Invitrogen). Reactions were run on a  
431 GeneAmp 9700 (Applied Biosystems) under the following conditions: Initial denaturation and  
432 enzyme activation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec  
433 and 65 °C for 2min. 1  $\mu$ l of each PCR reaction was mixed with 9  $\mu$ l of 1:0.06 Formamide and  
434 GS2500 size standard (Applied Biosystems), denatured at 94 °C for 2 min and separated by  
435 capillary electrophoresis using an Applied Biosystems 3100 genetic analyzer. Sizes of the  
436 fragments were analyzed by the Peak Scanner™ Software v1.0 (Applied Biosystems).

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441

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556

### 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  $2.5 \cdot 10^{-4}$  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.

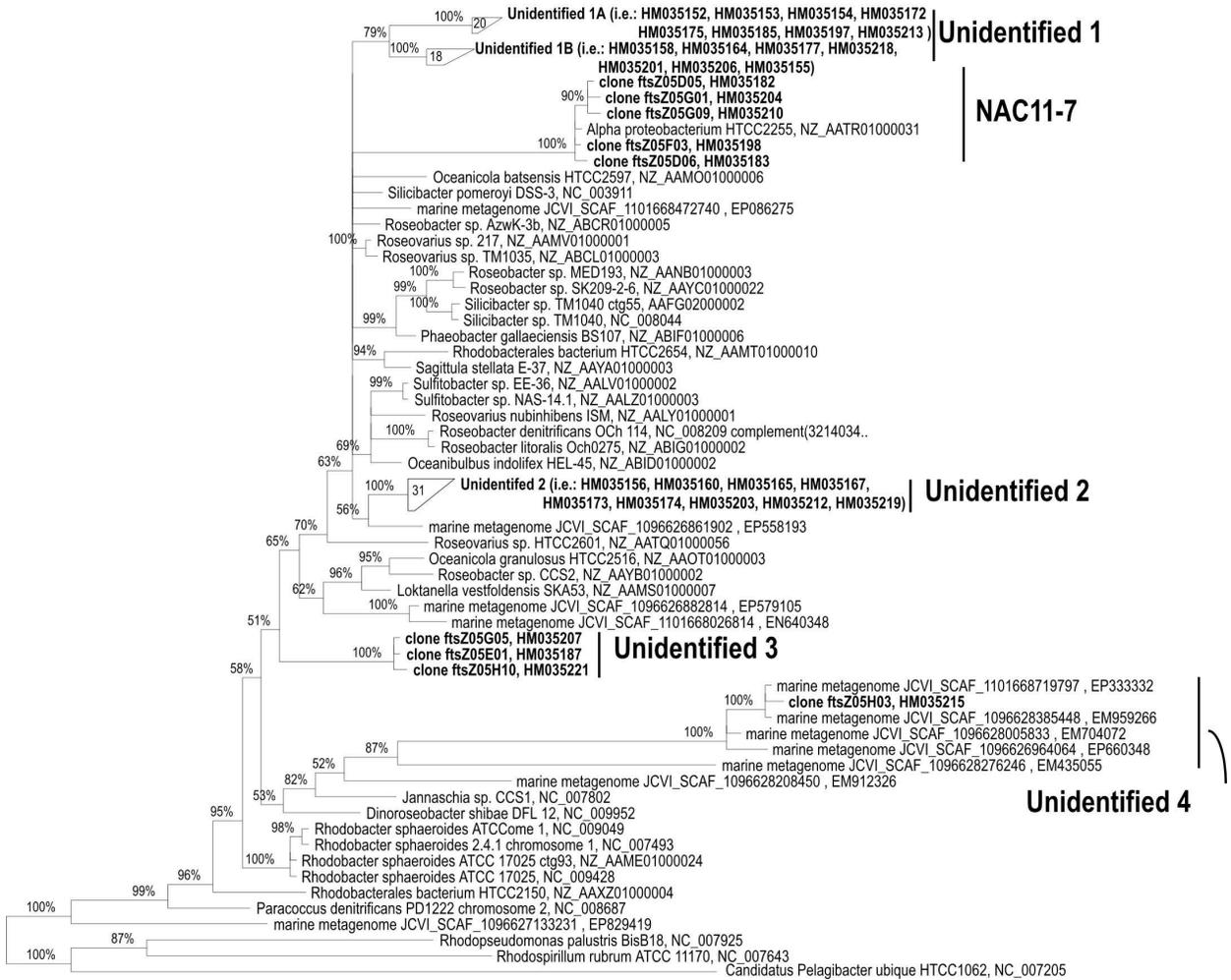
580 Supplemental Figure 1. Percentage of different phlotypes identified by ARISA fragment sizes  
581 in the *in situ* samples. The results shown in here indicate that the replicate subsamples were  
582 largely similar in composition, indicative of relatively low within-sample variation. However, in  
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  $1.0 \times 10^4$  gene copies per ml.

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

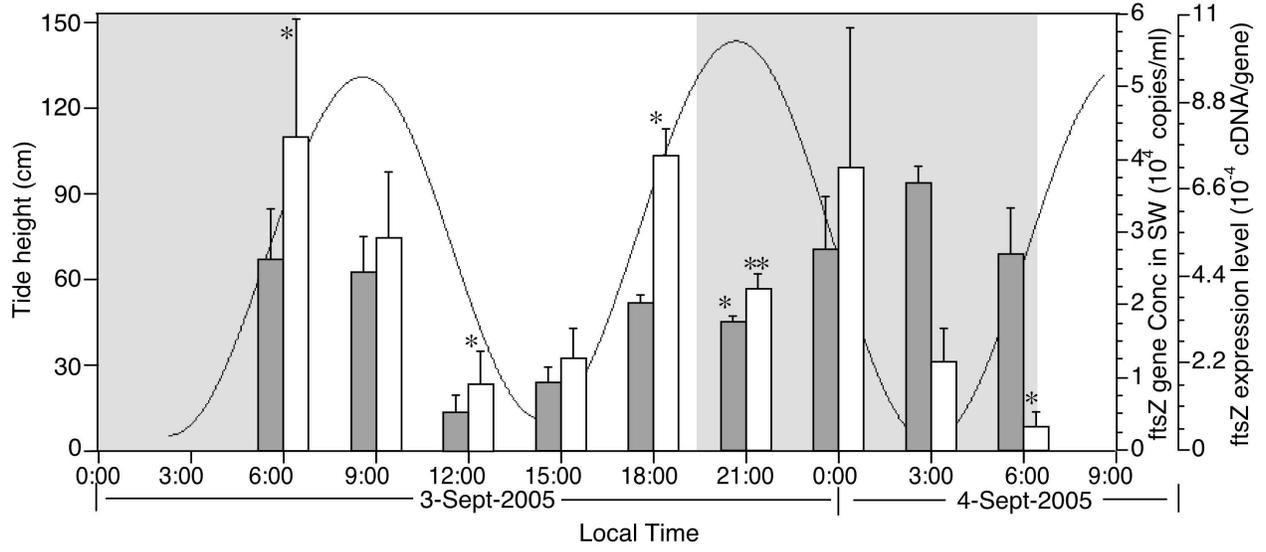
591

591 **Figure 1.**



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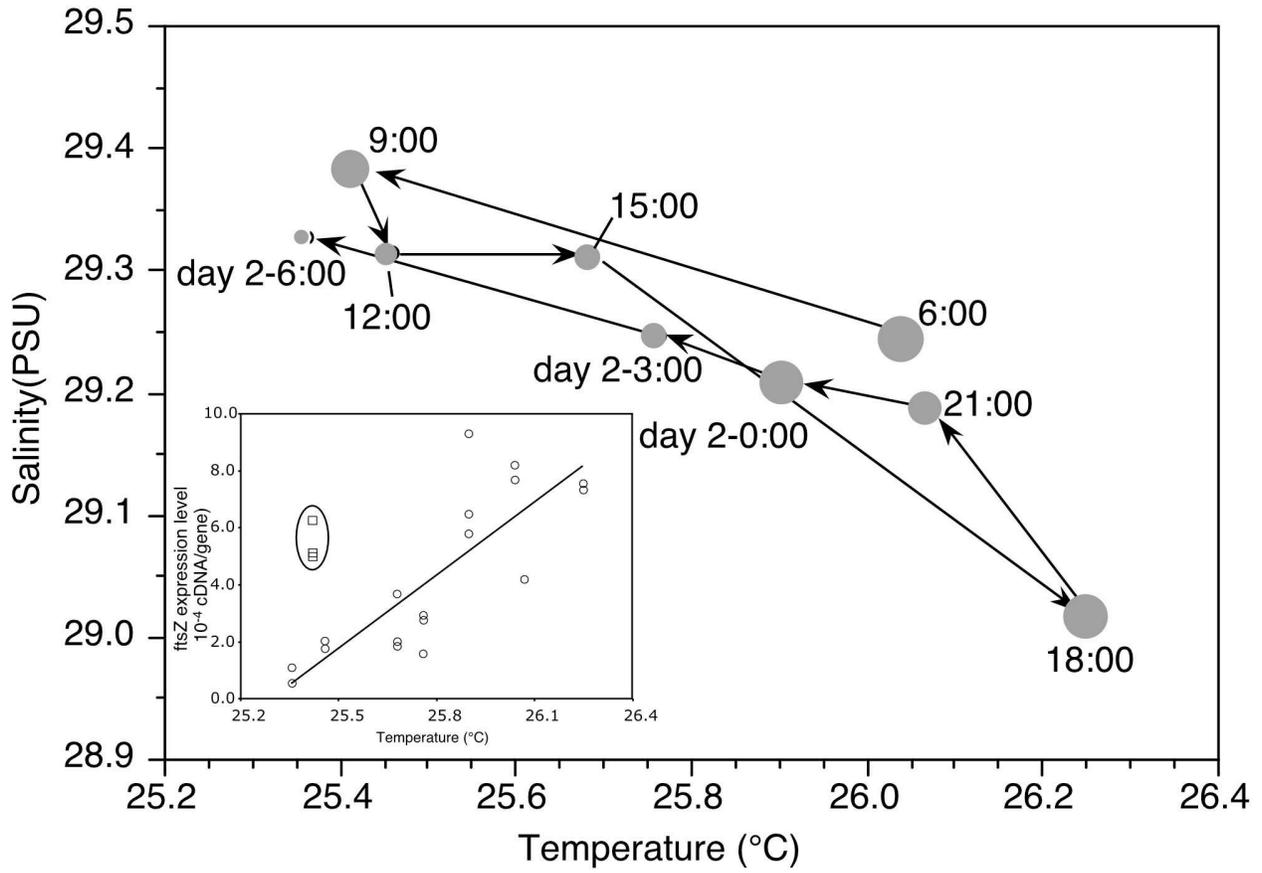
593 **Figure 2.**



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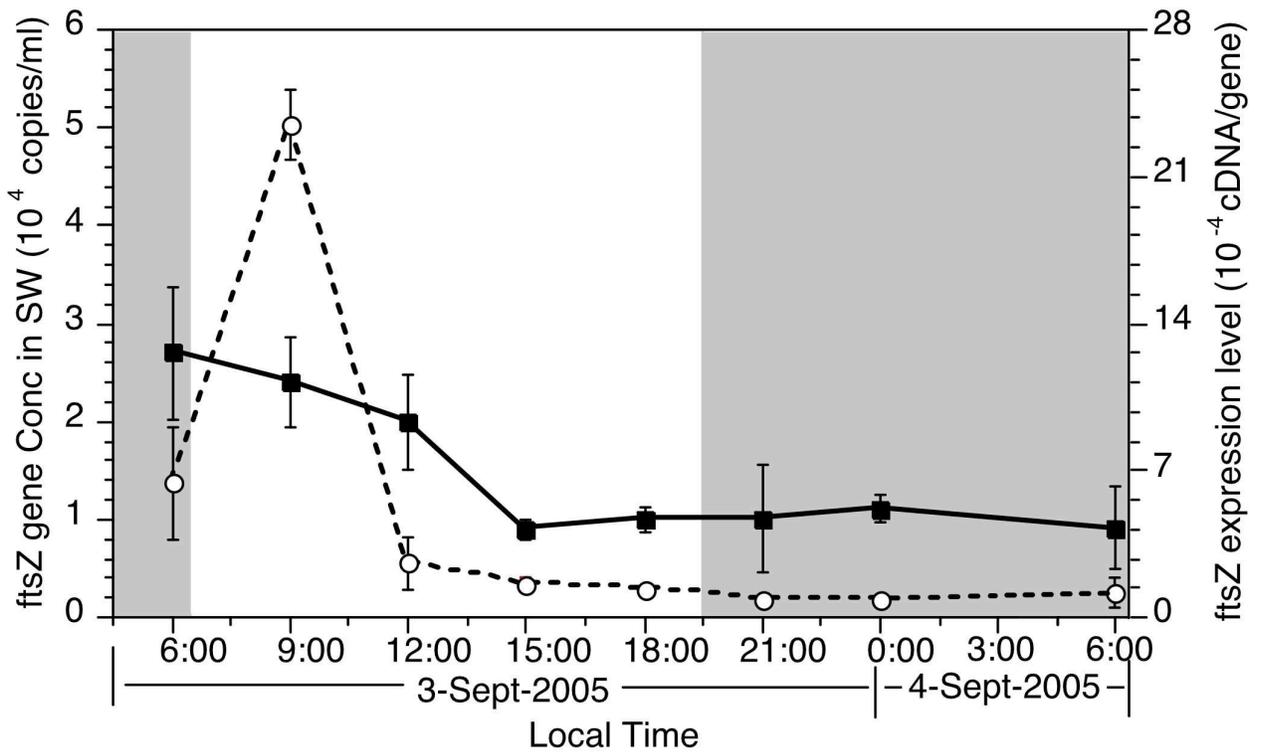
595 **Figure 3.**



596

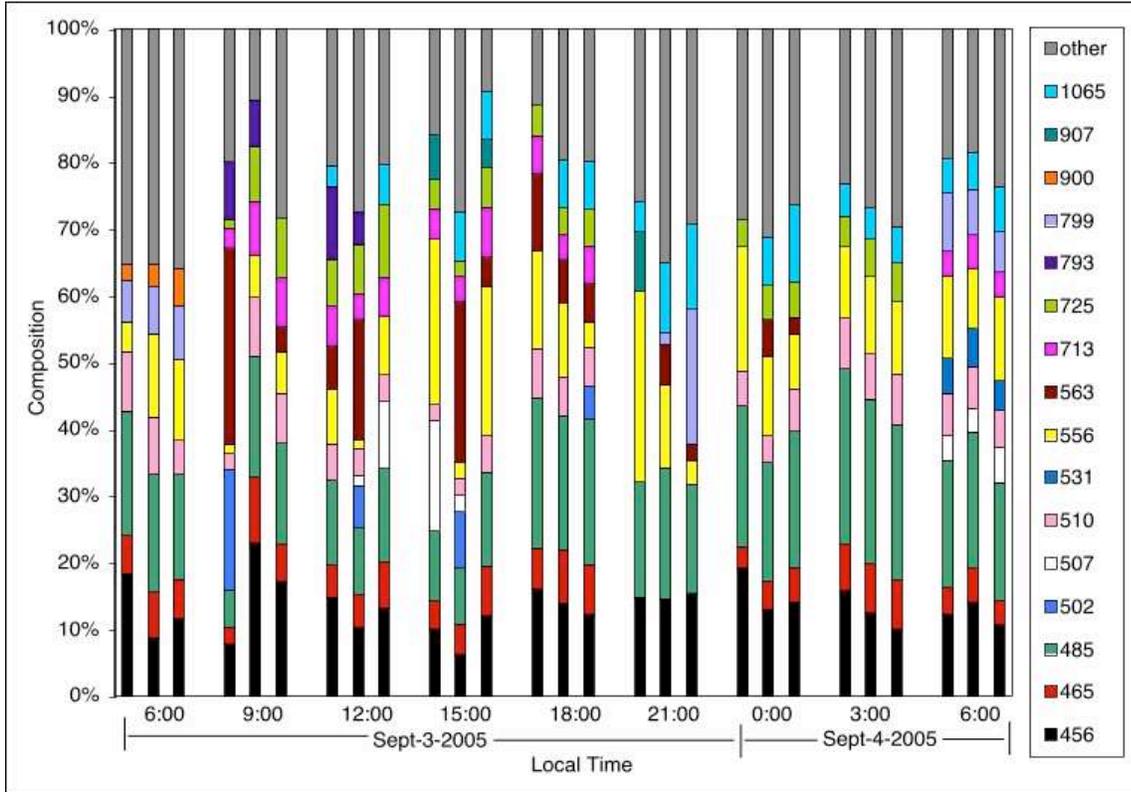
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597 **Figure 4.**



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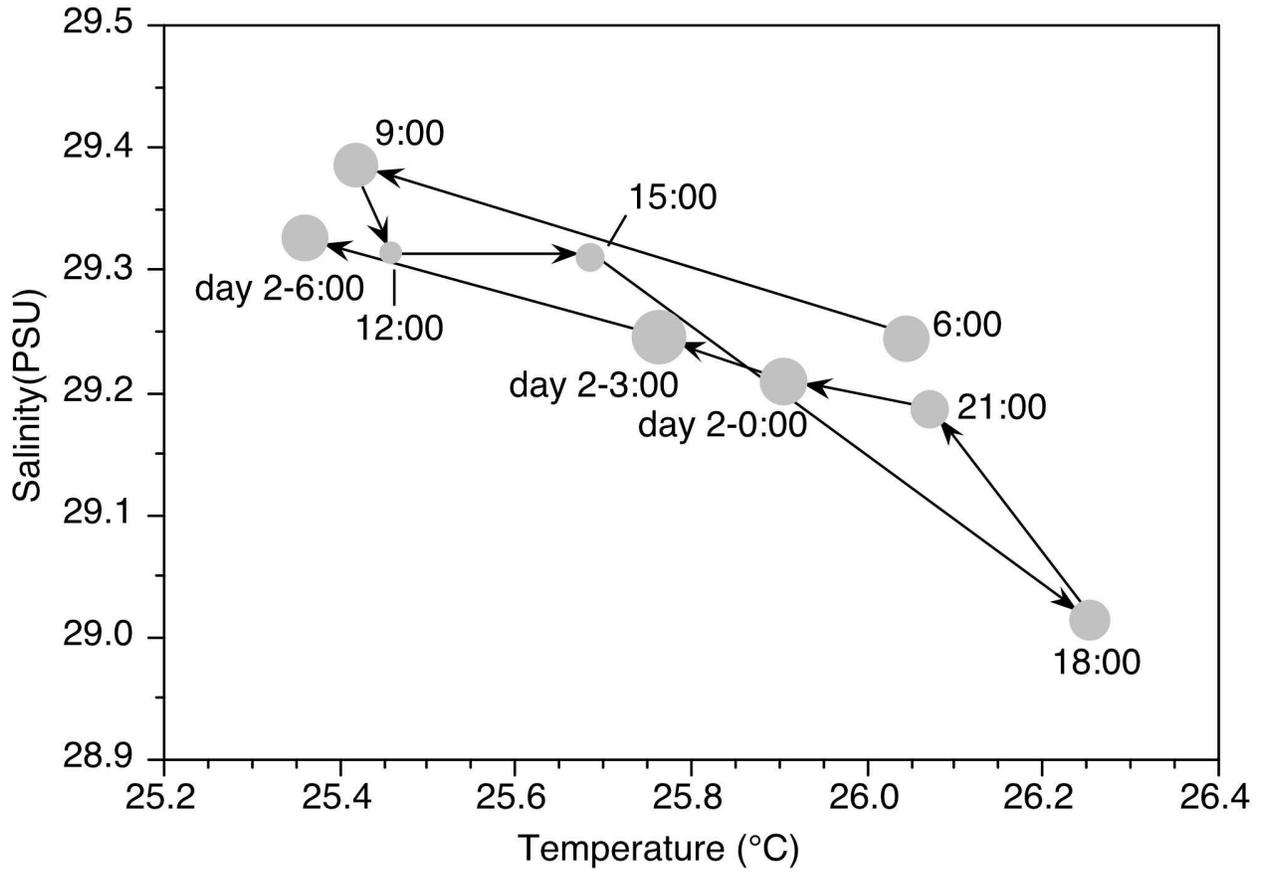
599 Supplemental Figure 1



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601

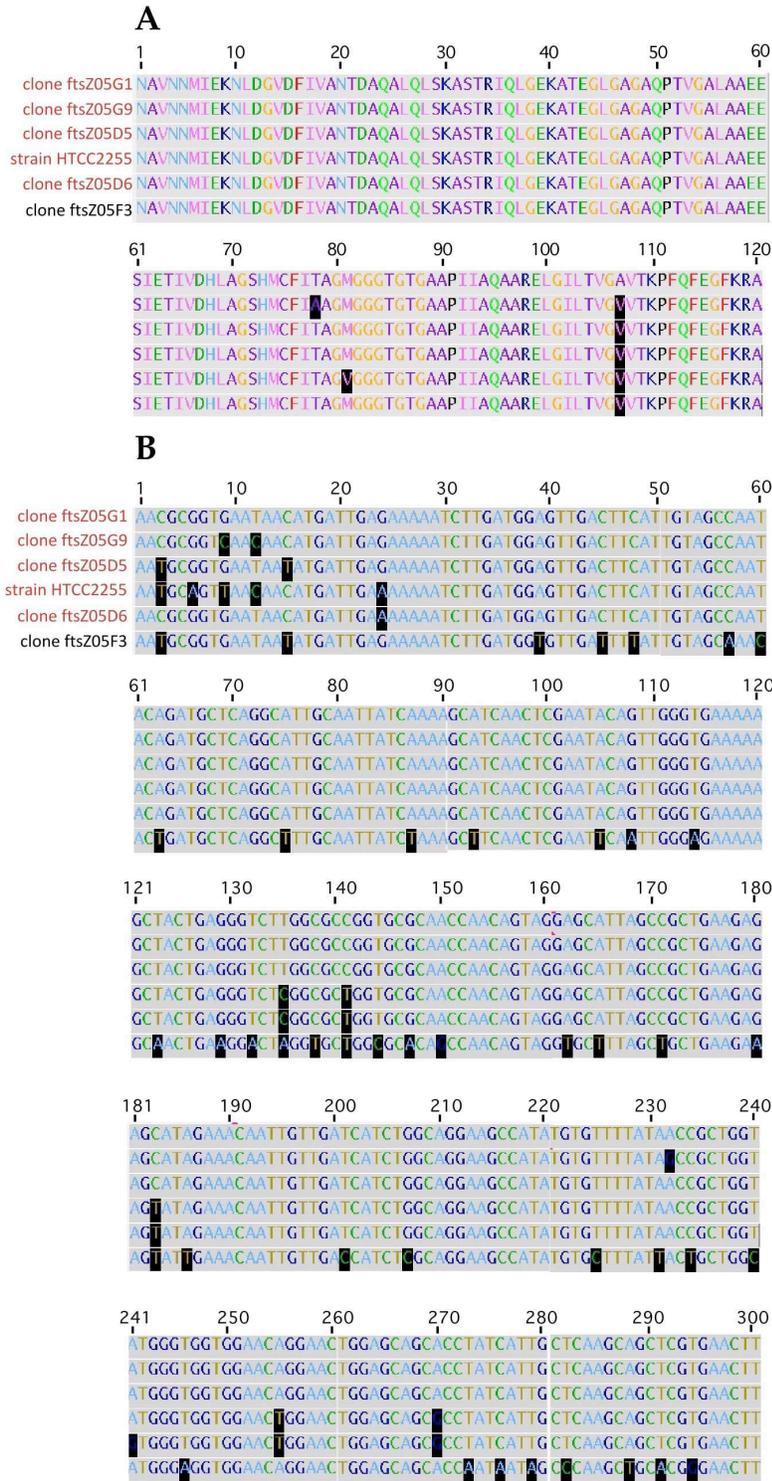
601 Supplemental Figure 2



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603 Supplemental Figure 3



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