

# In situ activity of NAC11-7 roseobacters in coastal waters off the Chesapeake Bay based on ftsZ expression.

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| 3        | In situ Activity of NAC11-7 Roseobacters in Coastal Waters off the  |
| 4        | Chesapeake Bay based on <i>ftsZ</i> Expression  |
|          |   |
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15

#### **Summary**

16 Determining in situ growth rates for specific bacterioplankton is of critical importance to 17 understanding their contributions to energy and matter flow in the Ocean. Quantifying 18 expression of genes central to cell division is a plausible approach for obtaining these 19 measurements. In order to test this approach's assumptions, a quantitative PCR assay targeting 20 the cell division gene ftsZ in the ubiquitous, but not yet cultivated, NAC11-7 group of the 21 Rhodobacterales order of marine bacteria was developed. ftsZ genes and their corresponding 22 mRNAs were measured in diel in situ samples and in parallel on-deck incubations. Strong 23 correlations between *ftsZ* expression and gene abundance (R-squared=0.62), and between *ftsZ* 24 expression and water temperature (R-squared=0.73) were observed in situ. Rapid and cyclical 25 changes of NAC11-7 ftsZ gene copies suggested that populations from two different end-member 26 water types were sampled. This was supported by distinct ftsZ gene expression levels in 27 populations associated with these water types. An outlier to this trend occurred at a single time 28 point (9:00), and was remarkably consistent with a concomitant peak in *ftsZ* expression in on-29 deck incubations, suggesting synchronous population growth. To our knowledge this is the first 30 evidence for synchronous populations for a heterotrophic bacterioplankton group in the ocean. 31

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#### Introduction

32 Marine bacterioplankton play pivotal roles in oceanic food webs and the cycling of 33 organic matter, which in turn influence global carbon pools (Azam and Malfatti, 2007; Wohlers 34 et al., 2009). In recent years, the application of molecular, genomic, and transcriptomic 35 techniques has provided us with vast datasets, greatly expanding our knowledge of 36 bacterioplankton phylogenetic diversity, their environmental distributions, and metabolic 37 capabilities (for reviews see Rappe and Giovannoni, 2003; Delong, 2009). Despite this 38 knowledge, challenges still exist in understanding the ecological functions of **specific** 39 bacterioplankton. Among these challenges is a lack of measurements of in situ growth rates of 40 bacterioplankton at high phylogenetic resolution. This information is essential for elucidating 41 the contributions of these ecotypes to biogeochemical cycles critical to the ecosystem. 42 The most commonly used approaches for determining growth rates of heterotrophic 43 marine bacterioplankton are indirect and rely on measurements of bacterial production. Growth 44 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., 45 46 1985; Simon and Azam, 1989), or non-radioactive bromodeoxyuridine (Steward and Azam, 47 1999). These methods regard the entire bacterial community as a "black box", provide 48 community-level activity measurements, and therefore do not provide information on the 49 production and growth rates of specific bacterioplankton. While the combination of 50 microautoradiography with fluorescence in situ hybridization (i.e. Micro-FISH) allows the 51 assessment of specific bacterioplankton activities at relatively narrow phylogenetic levels (i.e. 52 Cottrell and Kirchman, 2003; Alonso and Pernthaler, 2005; Margolin, 2005), these experiments 53 suffer from issues inherent to confinement and tracer-based studies (i.e. bottle effects).

54 A plausible approach to measure *in situ* activity, and perhaps growth rates of specific 55 bacterioplankton, involves quantifying the expression (mRNAs) of genes encoding proteins 56 involved in cell division. The idea behind such approach would be to determine direct 57 relationships between *in situ* expression of protein-encoding genes and growth rates of specific 58 bacterioplankton, without additions or incubations. The rationale is analogous to that of the 59 measurement of the frequency of dividing cells (Hagstrom et al., 1979), except that the 60 measurement could be made at specific phylogenetic levels, and earlier in the cell cycle (i.e. 61 before a clear septal ring is formed), thus decreasing problems associated with preferential 62 protistan grazing of dividing cells (Sherr et al., 1992). Requirements for successfully using a 63 gene transcript tracking approach for instantaneous (i.e. at any time of the day) measurements 64 of growth would be that populations of targeted organisms do not divide synchronously, and that 65 the regulation of cell division protein synthesis occurs at the transcriptional level. In cases where 66 the population divides synchronously, growth rates estimates are possible, but would not be 67 instantaneous and would require measurements throughout a diel cycle (i.e. Carpenter and Chang, 68 1988; Chang and Carpenter, 1988; Holtzendorff et al., 2002). 69 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

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.

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#### 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.

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

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108 NAC11-7 ftsZ mRNA and DNA copies were monitored in surface waters over a 24 h 109 period at a single station located at the mouth of the Chesapeake Bay. Gene copies, and more 110 remarkably, gene expression, showed rapid and sometimes large (c.a. 4-fold) changes with time 111 (i.e. from 9:00 to 12:00 and from 18:00 to 21:00), suggesting distinct populations (i.e. patches) of 112 NAC11-7 may have been sampled at different times, as a result of water advection (Figure 2). 113 Interestingly, ftsZ gene expression followed closely ftsZ gene abundance trends, except at the last 114 two sampling times. A regression analysis between *ftsZ* gene copies and gene expression using 115 individual replicates showed significant correlation ( $R^2 = 0.6214$ , P = 0.0003) for the first seven 116 sampling times, covering 18 hours from 6:00 to midnight. However, for the remaining two time 117 points (3:00 and 6:00 on Sept 4, 2005) this trend no longer held. As a consequence, a correlation 118 analysis of *ftsZ* gene to gene expression inclusive of all sampling points, was not significant 119  $(R^2=0.0304, P=0.45)$ . These results indicate that at least for some of the samples a correlation 120 existed between gene abundance and per-cell gene expression activity. 121 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

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

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 Fig 1). At least in the case of these fluctuating phylotypes,
different populations (patches) appear to have been sampled at different times. Unfortunately
ARISA peaks sizes corresponding to the NAC11-7 group were not readily detected, as these
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 gene abundance dropped from 6:00 to 15:00 and stabilized at ca.1 x  $10^4$  copies/ml (a 2.5-fold 155 156 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<sub>T</sub>s 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

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. To the best of our knowledge these are the first quantitative data of relative expression of a functional gene for a specific roseobacter subclade in natural samples. 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 2). 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.

We observed that levels of normalized transcript-to-gene ratios 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).

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 ftsZ188 expression. Conversely, if *ftsZ* transcription were constitutive (Case 2), one would not expect 189 *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,

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,

215 remarkable fluctuations of NAC11-7 ftsZ gene copy numbers (nearly 4-fold in 3 h) were 216 observed in situ (Figure 2), implying changes in populations sizes resulting from physical 217 processes. Second, ARISA analysis of the total bacterioplankton community structure showed 218 evidence for patchiness, as certain phylotypes were only detected in specific time points 219 (Supplemental Figure 1). While transient populations were likely sampled throughout the diel 220 cycle, some trends in *ftsZ* gene and gene expression did emerge that revealed insight into *ftsZ* 221 gene expression profiles and synchronicity of these populations. During the first seven data 222 points (18 hrs from 6:00 to 0:00), a strong correlation ( $R^2 = 0.6214$ ) was observed between 223 NAC11-7 abundance in situ (inferred from ftsZ copies/ml), and the average per cell ftsZ 224 transcript abundance, indicating that levels of expression are not constant in this ecotype. This 225 was suggestive that more active populations yielded, or were associated, with higher abundances 226 of these organisms. Collectively, these results support the case for non-synchronous cell division 227 since ftsZ expression was not constrained to specific times (i.e. ftsZ was expressed at all 228 measured time points). Finally, the plot of gene expression over a temperature-salinity diagram, 229 and a regression analysis showed that, with the exception of the 9:00 replicates, there is a high 230 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.

261 To our knowledge, this is the first indication that specific heterotrophic bacterioplankton 262 grow synchronously in the environment, and if proven true, this will have very important 263 implications to the study of bacterioplankton ecology. For instance, bacterioplankton production 264 is in most cases measured for the bulk community at short-time incubations. Thus, 265 understanding whether specific populations are synchronous will be paramount to the full 266 interpretation of these bulk measurements. Since there is putative evidence that some 267 representatives of NAC11-7 are photoheterotrophic, understanding the relationships between 268 light-driven metabolism and synchronous growth in nature is also important. Also, future in situ 269 studies should include Lagrangian sampling to minimize advective effects, and allow 270 measurement in coherent populations. 271 **Experimental Procedures** 272 Sampling 273 Surface water samples were collected every 3 hours from 6:00 local time on September 3 274 to 6:00 on September 4, 2005 at station RM6 (37°05.61N, 75°42.35 W) aboard the RV Cape 275 Henlopen. Temperature and salinity were measured using a SBE9 CTD (Seabird, Bellevue, 276 WA), and tidal effects were estimated from the height of the tide at Ship Shoal Inlet calculated 277 using the data at the NOAA tides and current prediction page http://tidesandcurrents.noaa.gov. 278 Triplicate 590 ml water subsamples were collected pre-filtered through GF/A filters (1.6  $\mu$ m 279 nominal pore; Whatman, Maidstone, UK). Ninety ml of pre-filtered sample was filtered through 280 13 mm diameter 0.2µm Supor200<sup>®</sup> polysulfone filters (Pall Corp., East Hills, NY) and the filters 281 transferred to a tube containing 130µl lysis buffer (2 mM NaEDTA (pH 8.0), 20 mM Tris•Cl (pH 282 8.0), 1.2% v/v Triton X100). The remaining 500 ml were filtered through a 25 mm diameter 0.2 283  $\mu$ m Supor200<sup>®</sup> filters (Pall Gelman Inc.) and transferred to screw cap tubes containing 250 µl

284 RNALater (Ambion, Austin, TX). All samples were frozen at -20°C aboard and within a week
285 stored at -70°C until nucleic acid extraction.

286 On-deck incubation experiments were conducted using water collected at the first

sampling time (6:00, 03 September, 2005). Fifteen 1 of surface water was used to fill 24 x 500

288 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,

290 three of the incubation bottles were taken and sampled for nucleic acids as described for cast

samples.

#### 292 Nucleic acid extraction and quantification

Total DNA was extracted from Supor200<sup>®</sup>  $0.2 \mu m$  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<sup>®</sup> Legend<sup>TM</sup> 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 70°C 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<sup>®</sup> 0.2  $\mu$ m filters following an optimized protocol 299 300 adapted from the Qiagen RNeasy<sup>®</sup> 96 manual (Shi 2005). Filters in screw cap tubes were thawed 301 on the ice and  $320\mu$ l low protein binding zirconium oxide beads (200  $\mu$ m, OPS Diagnostics, 302 Lebanon, NJ) were added into each tube. ß-Mercaptoethanol was mixed in a 1:100 ratio into 303 RLT buffer and 875  $\mu$ l of the mixture added to screw cap tubes. The tubes were beaten in a 304 MM301 mixer mill (Retsch GmbH Inc., Haan, Germany) at 30.0 HZ for 2 min and incubated for 305 5 min at 70 °C. 800 µl of the liquid phase was transferred into a new low-RNA-binding 2 ml 306 microcentrifuge tube (Ambion, Inc.), avoiding the beads. 800µl of 100% ethanol was added into

ach tube and well mixed well. 800  $\mu$ l 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  $\mu$ l RW1 buffer and twice with 800  $\mu$ l RPE buffer at 5000 rcf for 5 min, with the last spin for 15 min. 35  $\mu$ l 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  $\mu$ l DEPC treated water. RNA was treated the DNA-free<sup>TM</sup> kit (Ambion) to remove co-extracted DNA.

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

#### 317 Construction of ftsZ DNA PCR clone library

318 An initial *ftsZ* and FtsZ database was developed using *arb* (Ludwig *et al.*, 2004) by 319 importing gene sequences listed by Vaughan and coworkers (Vaughan et al., 2004). 163 320 representative FtsZ sequences were exported and used to retrieve additional aminoacid, and 321 coding gene sequences using blastp searches against the March 2005 NCBI nt, env nt and wgs 322 databases. All aminoacid sequences were aligned using ClustalW (Thompson et al., 1994) and an 323 bayesian tree containing 507 sequences (144 homologous positions) was constructed using 324 MrBayes version 3.0 (Ronquist and Huelsenbeck, 2003) with the following parameters: 800,000 325 generations, mixed models of amino acid substitution, and a burn in of 6000 trees. In this tree 326 the Alphaproteobacteria formed a monophyletic clade with high confidence values and several 327 orders including *Rhizobiales* and *Rhodobacterales* also formed monophyletic clades (data not 328 shown). The database was updated with public sequences belonging to the Rhodobacterales, and 329 was used for the design of PCR primers and probes. Degenerate primers targeting ftsZ from

330 Rhodobacterales (ftsZrb2F: 5'-AAY GCN GTS AAY AAY AT-3', ftsZrb2R: 5'-YTT NCC

331 CAT YTC RT-3') were designed and used to retrieve *ftsZ* gene sequences by PCR and cloning
332 from a DNA sample collected at time zero.

| 333 | $1\mu$ l of extracted genomic DNA was used as template in a 10 $\mu$ l-volume PCR reaction,           |
|-----|---|
| 334 | which also included 10X PCR buffer, 0.2mM of each dNTP, 3 mM MgCl <sub>2</sub> , 500 nM forward       |
| 335 | primer ftsZ2rbF, 500 nM reverse primer ftsZ2rbR and 0.025 U/ $\mu$ l of Platinum® Taq DNA             |
| 336 | Polymerase (Invitrogen, Carlsbad, CA). Reactions were performed on a GeneAmp 9700 PCR                 |
| 337 | system (Applied Biosystems) and cycling conditions were as follows: 2 min at 94 °C and 37             |
| 338 | cycles of 30 sec at 94 °C, 30 sec at 55 °C and 2 min at 72 °C. PCR was followed by a                  |
| 339 | reconditioning (Thompson et al., 2002) of 5 steps. Reconditioned PCR products were loaded on          |
| 340 | a 1% modified TAE (40 mM Tris-acetate, pH 8.0, 0.1 mM Na <sub>2</sub> EDTA) agarose gel and separated |
| 341 | by electrophoresis. Target fragments were cut, recovered by Ultrafree®-DA (Millipore) gel             |
| 342 | extraction and used to built a $ftsZ$ gene clone library using the TOPO <sup>TM</sup> TA cloning kit  |
| 343 | (Invitrogen) following manufacture's instructions. 96 clones were bidirectionally sequenced           |
| 344 | using BigDye V3.1 chemistry and capillary electrophoresis on an AB3100 genetic analyzer               |
| 345 | (Applied Biosystems Inc, Foster City, CA). These sequence data have been submitted to the             |
| 346 | GenBank database under accession number HM035149-HM035222. 74 sequences identified as                 |
| 347 | ftsZ were imported into the ARB database described above and added to the tree described above        |
| 348 | using the ADD_BY_PARSIMONY tool. Finally, a bayesian tree of Rhodobacterales ftsZ was                 |
| 349 | constructed using the MPI version of Mr Bayes V3.1 (Ronquist and Huelsenbeck, 2003; Altekar           |
| 350 | et al., 2004). 188 homologous amino acid positions from 123 sequences were exported and used          |
| 351 | in this phylogenetic analysis. Two four parallel chains of 2,000,000 generations were run with        |
| 352 | 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<sub>M</sub>, 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  $\mu$ l PCR reaction contained 5  $\mu$ l 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  $\mu$ l 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 1 min at 57 °C. All PCR products were loaded in a 2% NuSieve® (3:1) Agarose Gel (Cambrex, Rockland, ME), electrophoresed, and 374

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).

| 377 | In order to get the highest amplification efficiency in real time PCR, a primer  |
|-----|--|
| 378 | concentration matrix was performed. In each 25 $\mu$ l reaction, the following reagents were kept                        |
| 379 | constant: 1X PCR buffer, 0.2 mM of dATP, dGTP and dCTP, 0.4 mM of dUTP, 5 mM $MgCl_2$ ,                                  |
| 380 | 200 nM probe NAC11-7 (AAC CAACAGTAGGAGCATTAGCCGCT), 1.2 $\mu$ M SuperROX <sup>TM</sup>                                   |
| 381 | (Biosearch Technologies, Novato, CA), 0.01 U/µl AmpErase® Uracil N-glycosylase (UNG)                                     |
| 382 | (Applied Biosystems), 0.025 U/ $\mu$ l of Platinum® Taq DNA Polymerase (Invitrogen), 2.5 $\mu$ l                         |
| 383 | NAC11-7 standard (10 <sup>4</sup> copies/ $\mu$ l) and a matrix of forward and reverse primer concentrations of          |
| 384 | $0.1 \mu\text{M}, 0.5 \mu\text{M}, 1.0 \mu\text{M}, 1.5 \mu\text{M}$ . Reactions were set in a MicroAmp® Optical 96-Well |
| 385 | Reaction Plate (Applied Biosystems), which was sealed with an optical adhesive cover (Applied                            |
| 386 | Biosystems) reactions and ran in an ABI Prism 7000 Sequence Detection system, following the                              |
| 387 | cycling parameters: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min                           |
| 388 | at 57 °C. The primer combination yielding the lowest C <sub>T</sub> (1.5 $\mu$ M ftsZrbA03-2qF; 0.5 $\mu$ M              |
| 389 | ftsZrbA03-2qR) was used in all subsequent measurements.  |
| 390 | A plasmid containing cloned DNA that was purified and linearized as previously   |
| 391 | described (Suzuki et al., 2000) was used to prepare standards for real time PCR for quantification                       |
| 392 | of <i>ftsZ</i> gene and mRNA. 2.5 $\mu$ l of DNA extracts were used in 25 $\mu$ l reactions and standards                |
| 393 | ranged from $10^2$ to $10^7$ copies/ $\mu$ 1. Reactions were run in duplicate along with non-template                    |
| 394 | controls using the same cycling parameters described for the primer matrix. ftsZ cDNA was                                |
| 395 | quantified in the same manner as $ftsZ$ gene except that 5 $\mu$ l of Reverse transcription products                     |

396 were used as template. All real time PCR measurements were calculated as copy numbers per

397 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  $4 \mu l$ 407 purified RNA (ca. 10 ng) was reverse transcribed to cDNA using ThermoScript<sup>™</sup> Reverse Transcriptase. Template and 10 pmol of the NAC11-7 specific primer ftsZrbA03-2qR were 408 409 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<sup>™</sup> Inhibitor (Invitrogen), 0.75 U/µ of reverse 411 transcriptase and DEPC-treated water to a final volume of 10  $\mu$ l. 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)

#### 415 ARISA (Automateu TRIVA intergenic spacer un

416 One  $\mu$ l environmental genomic DNA was used to perform a 10  $\mu$ l ARISA reactions,

417 containing 1X PCR buffer, 1.2 mM MgCl<sub>2</sub>, 0.08 mM dNTPs (Promega Corp., Madison, WI), 0.5

- 418  $\mu$ M primer 1406F-FAM (Fisher and Triplett, 1999), 1.5  $\mu$ M primer 23S-Y (Dyda et al., 2009),
- 419 and 0.01 U/ $\mu$ l 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 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec  |
|--------------------------|--|
| 422                      | and 65 °C for 2min. 1 $\mu$ l of each PCR reaction was mixed with 9 $\mu$ l of 1:0.06 Formamide and  |
| 423                      | GS2500 size standard (Applied Biosystems), denatured at 94 °C 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 <sup>™</sup> Software v1.0 (Applied Biosystems).   |
|                          |  |
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| 548        |   |

## **Figure legends**

| 549 | Figure 1. Consensus bayesian phylogenetic tree of FtsZ protein sequences from the                       |
|-----|---|
| 550 | Rhodobacterales group reconstructed using MrBayes v3.1. FtsZ sequences retrieved in this study          |
| 551 | are marked in boldface. The numbers on nodes represent branch confidence values. The bar                |
| 552 | represents the mean number of substitutions per residue in the trees used to build the consensus        |
| 553 | tree.   |
| 554 | Figure 2. $FtsZ$ gene copy numbers (grey bars) and expression (estimated from the ratio of $ftsZ$       |
| 555 | cDNA copies to gene copies; white bars) at station RM6 on September 3 and 4, 2005, and tidal            |
| 556 | heights at Ship Shoal Inlet (37° 13'N, 75° 48'W). Error bars represent the standard deviation of        |
| 557 | triplicate samples from a single CTD cast. Pre-dawn and post-dusk sampling times are                    |
| 558 | represented by the grayed areas. The tidal height is shown with the line lacking symbols.               |
| 559 | Figure 3. Temperature and Salinity plot, with overlaying in situ NAC11-7 ftsZ expression. Gene          |
| 560 | normalized <i>ftsZ</i> expression levels are proportional to the area of the circles. The area of the   |
| 561 | circle representing the 3:00 PM sample corresponds to 2.5 10 <sup>-4</sup> mRNA copies per gene copies. |
| 562 | Inset: normalized <i>in situ ftsZ</i> expression in samples with temperatures below and above 25.8 °C.  |
| 563 | Figure 4. $ftsZ$ gene copy numbers (filled squares) and expression (estimated from the ratio of $ftsZ$  |
| 564 | cDNA copies to gene copies; open circles) in samples incubated onboard. Error bars represent            |
| 565 | the standard deviation of triplicate bottles.   |
|     |   |

| 566 | Supplemental Fig 1. Percentage of different phylotypes identified by ARISA fragment sizes in              |
|-----|---|
| 567 | the <i>in situ</i> 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 |   |

#### Figure 1.





**Figure 2.** 









**Figure 4.** 



## 583 Supplemental Figure 1



585 Supplemental Figure 2

