The spontaneous mutation frequencies of *Prochlorococcus* strains are commensurate with those of other bacteria

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Running title: Mutation frequencies in *Prochlorococcus*

Summary

The marine cyanobacterium *Prochlorococcus*, the smallest and most abundant oxygenic phototroph, has an extremely streamlined genome and a high rate of protein evolution. High-light adapted strains of *Prochlorococcus* in particular have seemingly inadequate DNA repair systems, raising the possibility that inadequate repair may lead to high mutation rates. *Prochlorococcus* mutation rates have been difficult to determine, in part because traditional methods involving quantifying colonies on solid selective media are not straightforward for this organism. Here we used a liquid dilution method to measure the approximate number of antibiotic-resistant mutants in liquid cultures of *Prochlorococcus* strains previously unexposed to antibiotic selection. Several antibiotics for which resistance in other bacteria is known to result from a single base pair change were used. The resulting frequencies of antibiotic resistance in *Prochlorococcus* cultures allowed us to then estimate maximum spontaneous mutation rates, which were similar to those in organisms such as *E. coli* (~5.4×10^{-7} per gene per generation). Therefore, despite the lack of some DNA repair genes, it appears unlikely that the *Prochlorcoccus* genomes studied here are currently being shaped by unusually high mutation rates.

Introduction

The marine cyanobacterium *Prochlorococcus* is the smallest known oxygenic phototroph, both in terms of cell and genome size. It numerically dominates the mid-latitude oligotrophic oceans, and plays a significant role in ocean primary productivity. In addition to a small genome (1.6 – 2.4 Mb), an accelerated rate of evolution of protein-coding gene sequences has been observed for *Prochlorococcus* strains (Dufresne et al., 2005). The phenomena of accelerated protein evolution and genome size reduction have been associated with the possibility that *Prochlorococcus* may have a "mutator" phenotype, i.e., an abnormally high spontaneous mutation rate due to missing or impaired DNA repair genes (Marais et al., 2008). With regard to genome size, Marais et al. have argued that in large populations, an elevated mutation rate increases the rate of inactivation of non-essential genes (those with a lower fitness impact); such inactivated genes eventually become pseudogenes and are ultimately deleted due to deletion bias, leading to smaller genomes (Marais et al., 2008). The possibility of a mutator phenotype in our laboratory strains is supported by the observation that *Prochlorococcus* genomes, relative to other bacteria, lack a number of DNA repair enzymes (Kettler et al., 2007; Partensky and Garczarek, 2010), and that mutator strains have been found in many natural populations of bacteria (Tenaillon et al., 1999). For example, among the missing genes in high light-adapted strain MED4 are some for which mutational inactivation is strongly associated with a mutator phenotype in *E. coli* and other organisms, including *ada and ogt*, methyltransferases that remove methyl groups from O-6-methylguanine in DNA, preventing GC to AT transversions (Rebeck and Samson, 1991) , and *mutY*, an A/G-specific DNA glycosylase that removes A from 8-oxo-dG-A or A-G mispairs ((Nghiem et al., 1988)). In addition, *recQ* (encoding DNA helicase), *recJ* (encoding single-stranded DNA-specific exonuclease), *exoI*/*xseA*, and *xseB* (encoding subunits of exonuclease VII) are also missing from MED4 and are also associated with mutator phenotypes (Rebeck and Samson, 1991;Yamana et al., 2010).

We recently determined that after over 1500 generations, the number of single nucleotide substitutions (SNPs) in the genome of the high light-adapted *Prochlorococcus* strain MED4 was in the range expected for non-mutator bacteria (Osburne et al., 2010). As this finding appeared inconsistent with a mutator phenotype in MED4, we decided to investigate further by measuring the spontaneous mutation frequency (the fraction of mutant cells in a population) in MED4 and in two other *Prochlorococcus* strains, allowing us to then bound the upper limit of their spontaneous mutation rates.

The mutation frequency in a prokaryote population is estimated by counting the number of pre-existing mutant cells in a population (Foster, 2006), often using resistance to antibiotics (those for which resistance arises from a single base pair change in a particular gene) as a convenient marker. Cells are grown in the absence of selective pressure, plated on antibiotic selection plates, and the number of antibiotic-resistant colonies is counted. Thus the mutation frequency for that gene (arising from a single base-pair change) is the number of antibiotic-resistant colonies divided by the total number of cells plated. This method requires that the efficiency of colony formation approaches 100% (Pope et al., 2008). Although recent improvements in efficiency have been achieved for some *Prochlorococcus* strains by co-plating them with heterotrophic bacteria (Morris et al., 2008), we were not able to duplicate those results for the *Prochlorococcus* strains used here. We therefore devised a method to estimate the mutation frequency to antibiotic resistance in liquid cultures of *Prochlorococcus.*

Results and discussion

Mutation frequency determination

The mutation rate for a base pair or a gene is generally defined as the number of mutation events per cell division (the number of cell divisions being nearly the same as the number of cells for large populations (Foster, 2005)). The mutation frequency can differ from the mutation rate, since a single mutation may be amplified (and thus over-counted) in a population,

depending upon how early or late it arose during growth. Therefore the mutation frequency can be considered to be equal to or greater than the true spontaneous mutation rate (assuming, as we do here, that the growth rates of mutant and wild type cells under nonselective conditions are approximately equal).

To measure the mutation frequency, cells were grown in culture under non-selective conditions (without antibiotics), then varying numbers of cells were diluted into tubes containing inhibitory concentrations of antibiotics. Only those culture tubes containing pre-existing antibiotic-resistant mutant(s) should be able to grow in the presence of antibiotics; thus the smallest inoculum that resulted in cell growth in the presence of antibiotic revealed the approximate number of pre-existing mutants in the culture.

Several antibiotics were chosen for this study, based on their known ability to give rise to resistant mutants resulting from a single base pair change in specific genes in other organisms. A range of drug concentrations was first tested against *Prochlorococcus* strains MED4, MIT9312, and NATL2A grown in liquid culture. The concentrations then used to determine mutation frequency are shown in Table 1.

Cells were first grown in liquid medium (described in Fig. 1), then diluted (to a target density of ~1 cell/ml to help ensure sufficient doublings to allow mutants to arise) and grown to mid log phase (approximately 25 doublings). Cells were counted by flow cytometry, concentrated by centrifugation, and then 10^6, 10^7, or 10^8 total cells were added to duplicate culture tubes containing 25 ml medium amended with antibiotics at the concentrations indicated in Table 1. Cell growth was monitored by bulk culture fluorescence for 100 days, more than ample time for a single antibiotic-resistant cell to grow to a population that would be detectable as bulk culture fluorescence. For MED4 (Fig. 1A), an initial inoculum of 10^8 cells was required for cultures to grow in the presence of any of the three antibiotics; inocula of 10^7 or fewer cells did not grow. We therefore conclude that 1 – 10 antibiotic-resistant cells were initially present in

the original inoculum of 10^8 cells, yielding a mutation frequency of $10^{-7} – 10^{-8}$/gene. Thus we estimate that the maximum spontaneous mutation rate of MED4 to antibiotic-resistance was $10^{-7} – 10^{-8}$/gene/generation. This number is consistent with rates observed in other bacteria for point mutations leading to antibiotic resistance ($5.4x10^{-7}$ per gene per generation, (Miller et al., 2002)), and for other prokaryotic genes in general (Drake et al., 1998; Whitman et al., 1998). In contrast, mutator strains of *E. coli* characterized by a mutation in *mutY* were shown to have a 290-fold increase the spontaneous mutation frequency to rifampicin-resistance (Michaels et al., 1992), and potentially higher when combined with mutations in *ogt*/*ada*, *xseA*, *recQ* and *recJ,* (Rebeck and Samson, 1991; Horst et al., 1999; Yamana et al., 2010), all of which are absent in MED4 .

To verify that resistant phenotypes were due to genetic changes rather than to inactivation of the antibiotic over time, resistant strains were diluted into fresh medium containing selective concentrations of the antibiotic. All putative resistant strains grew rapidly and with a minimal lag period (Fig. S1), consistent with a genetic change in the culture (contrast the lag periods of the resistant and WT strains in medium containing rifampicin). Further, the relevant genes encoding rifampicin-resistance (the *rpoB* gene, encoding the β subunit of RNA polymerase) and ciprofloxacin-resistance (the *gyrA* and *topoisomerase IV subunitA* genes (Khodursky et al., 1995; Strahilevitz and Hooper, 2005)) were sequenced for two of the mutant cultures, using the primer sets shown in Table S1. Rifampicin-resistant MED4 (MED4 Rif^R) carried a substitution of methionine for isoleucine at residue 437, resulting from an A to G transition in the *rpoB* gene. An alignment of the *rpoB* genes of MED4 and *E. coli* (Fig. S2) shows that MED4 residue 437 corresponds to *E. coli* K12 residue 572, which lies in an RNA binding pocket of the β subunit of RNA polymerase and is the site of known rifampicin-resistance mutations (Ederth et al., 2006). The sequence of the ciprofloxacin-resistant

mutant.revealed that as a result of a G to T transversion, tyrosine was substituted for aspartate at residue 93 of the *gyrA* gene, discussed further below.

We also tested the spontaneous mutation frequencies of two additional *Prochlorococcus* strains, high light-adapted MIT9312 (Fig. 1B), and low light-adapted NATL2A (Fig 1C). As MED4 showed similar mutation frequencies for all three antibiotics, we used only ciprofloxacin in our analysis of these additional strains. MIT9312 and NATL2A both yielded maximum spontaneous mutation rates of 10^{-6} – 10^{-7}/gene/ generation, again commensurate with those of other bacteria. Note that the genome content of DNA repair genes in MIT9312 is similar to that of MED4, whereas several of the DNA repair genes missing from those strains (*mutY*, *xseAB* and *recJ*) are present in NATL2A. Nevertheless, the mutation frequencies of MIT9312 and NATL2A were similar, indicating that the presence or absence of those genes does not appear to have a large effect on mutation frequency.

Intrinsic resistance of diverse cyanobacterial strains to nalidixic acid

Over the course of these studies we learned that MED4 and a number of other strains of *Prochlorococcus* and *Synechococcus* are intrinsically resistant to nalidixic acid (Nal^R, Fig. S3), and encode a threonine in place of serine at position 94 of *gyrA*, corresponding to *gyrA* residue 83 in *E. coli* (Fig. S4), a mutational hotspot at which leucine or tyrosine is often substituted for serine in Nal^R mutants of *E. coli* and other bacterial (Phung and Ryo, 2002; Sáenz et al., 2003). Nal^R mutants of other bacteria are not intrinsically resistant to ciprofloxacin, but may become ciprofloxacin-resistant by acquiring an additional mutation in either *gyrA* or in the gene encoding topoisomerase IV subunit A (Sáenz et al., 2003). The *gyrA* gene sequence of a MED4 $Cipro^R$ strain revealed the threonine residue present in the WT at position 94, and a mutation resulting in the substitution of tyrosine for aspartate at residue 93 (corresponding to residue 82 in *E. coli*). This substitution lies in the same region as second-step mutations in the *gyrA* gene that lead to

ciprofloxacin-resistance in Nal^R *E. coli* strains (Vila et al., 1994; Truong et al., 1997). No mutations were found in the gene encoding topoisomerase IV subunit A.

The intrinsically Nal^R cyanobacteria studied here (Fig. S3) all contain threonine instead of serine at the hotspot position corresponding to *gyrA* residue 83 in *E. coli* , possibly accounting for the resistance phenotype. However, the dissimilarity of the C-terminal portion of the cyanobacterial *gyrA* genes relative to that of *E. coli* (Fig. S4) may also be responsible for the Nal^R phenotype.

Although resistance to nalidixic acid is common among pathogenic bacterial strains that have been exposed to the antibiotic, it seems unlikely that these cyanobacteria have been exposed to significant levels of nalidixic acid, given their origins in oligotrophic regions of the open ocean,.Therefore other selective pressures must have driven them to carry a resistant *gyrA* gene. DNA gyrase catalyzes the ATP-dependent negative super-coiling of double-stranded closed-circular DNA (Reece and Maxwell, 1991). Nal^R DNA gyrases in other organisms have reduced supercoiling ability (yielding more relaxed DNA), which facilitates gene transcription as compared with more highly supercoiled DNA (Bagel et al., 1999). In the case of *Prochlorococcus* and *Synechococcus*, potentially reduced supercoiling ability may increase the efficiency of gene transcription, since it is possible that the increased osmolarity in ocean environments may otherwise lead to an intrinsically higher degree of DNA supercoiling (Schlick et al., 1994).

Conclusions

Our data indicate that antibiotic-resistance mutation frequencies in these *Prochlorococcus* strains do not appear to be elevated relative to those of other bacteria. For antibiotic resistance, the mutation frequency data for MED4 leads to a maximum spontaneous mutation rate of 10^{-7} – 10^{-8}/gene/generation, about the same or slightly lower than that found for other bacteria (Drake et al., 1998; Whitman et al., 1998). These results are also consistent with our previous findings

regarding the number of SNPs in one MED4 isolate after growth in culture for more than 1500 generations (Osburne et al., 2010). Although the sporatic appearance of mutator strains may have played a past role in shaping *Prochlorococcus* genomes, potentially affecting their rate of protein evolution, it is clear that despite the lack of some "mutator" DNA repair genes, these *Prochlorococcus* strains do not appear to have a mutator phenotype. With regard to genome size, it has recently been suggested (Luo et al., 2011) that factors involved in size reduction may be complex, potentially combining mutational bias toward gene deletion, the relaxation of purifying selection on nonessential gene families resulting in the loss of nonessential genes (Kuo and Ochman, 2009), and potential advantages derived from reduced cell size (e.g., increased surface-to-volume ratio facilitating nutrient uptake) in the oligotrophic environment inhabited by *Prochlorococcus* (Gregory et al., 2009). It is expected that further analyses will elucidate the roles played by these and other factors.

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345 **Table 1. Antibiotics used to inhibit *Prochlorococcus* cultures**

Antibiotic	***Cellular target***	***Gene(s)known to encode resistance mutations in other organisms***	***Concentration used in this study*** **(μg/ml)**
ciprofloxacin	DNA gyrase, A subunit, Topoisomerase IV, A subunit	*gyrA*, and *topoIVA*,	2
kanamycin	30S ribosome	*16S rRNA*, ribosomal protein genes, other genes	50
rifampicin	RNA polymerase, β subunit	*rpoB*	5

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Figure Legends

Figure 1. Mutation frequency determination in *Prochlorococcus* cultures.

Cells were first grown as described in the text in 25 mm borosilicate glass tubes with continuous light (22-25 µmol Q m^{-2} s^{-1} for MED4 and MIT9312, and at 10 µmol Q m^{-2} s^{-1} for NATL2A) using cool white fluorescent bulbs), at 22°C in filtered Sargasso Sea Water amended with Pro99 nutrients (Moore et al., 2007). Growth was monitored by fluorometric detection of chlorophyll autofluorescence using a Turner Design fluorometer 10-AU. Then 10^8: ○, 10^7: □ , or 10^6: Δ, cells were added to duplicate tubes containing PRO99SSW medium and either kanamycin (25 µg/ml), ciprofloxacin (2 µg/ml), or rifampicin (5 µg/ml). Cells were then allowed to grow in continuous light. Culture growth in the presence of an antibiotic indicates that 1-10 pre-existing mutants were present in the culture inoculum. A: MED4; B: MIT9312; C: NATL2A. All mutation frequency experiments were repeated a minimum of three times, and yielded the same result each time.

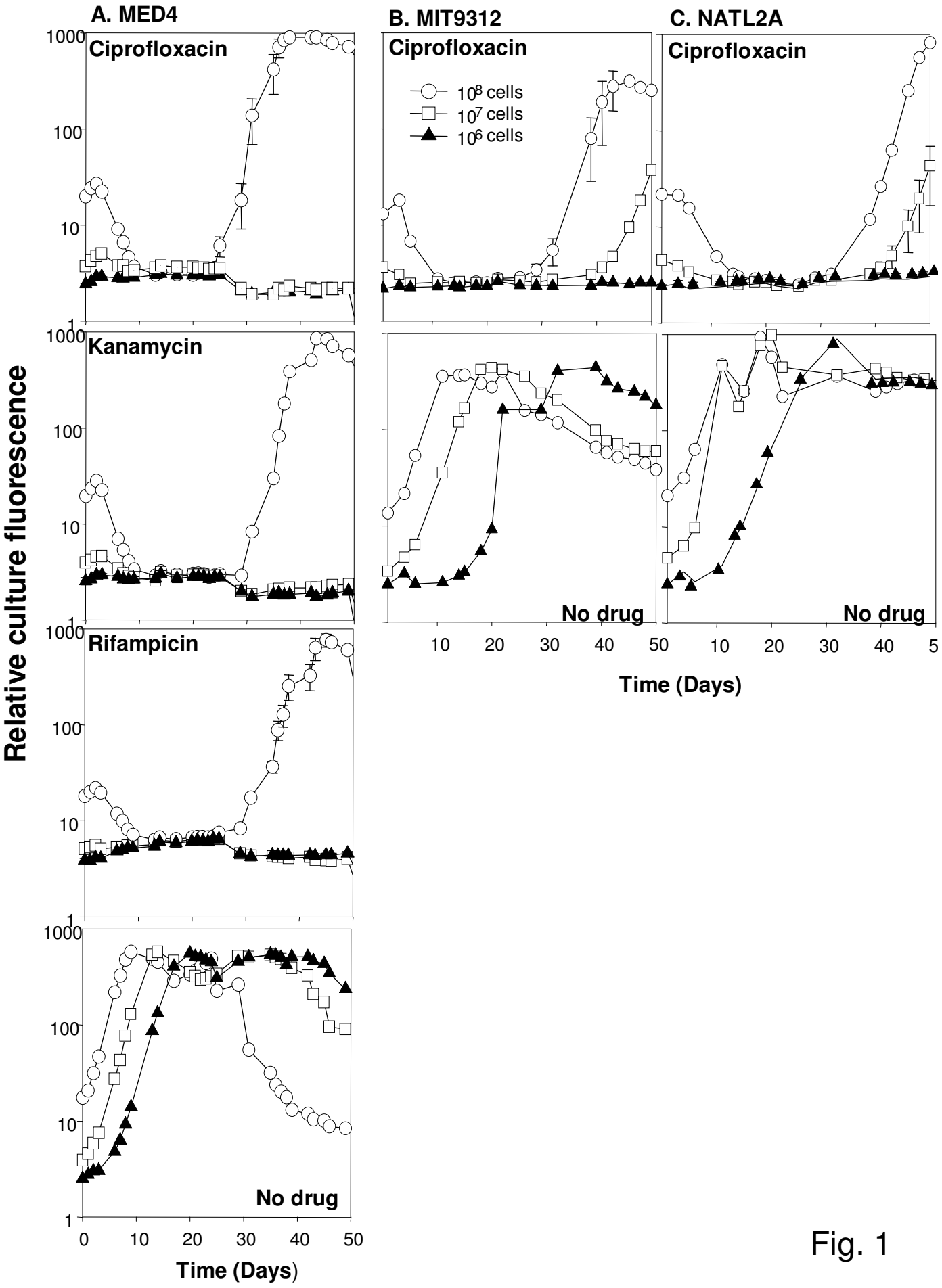
A. MED4
B. MIT9312
C. NATL2A
Ciprofloxacin
Kanamycin
Rifampicin
No drug
10^8 cells
10^7 cells
10^6 cells
Relative culture fluorescence
Time (Days)
1000
100
10
1
0
10
20
30
40
50
5

Fig. 1

Table S1. Primer sets used for sequencing the *rpoB* gene of MED4Rif^R and the *gyrA* gene of MEDCipro^R

Gene/primer set number		Primer Pairs
rpoB	1	5'-GATATTTGTTGAATTCAAGACTAAAATCTCG-3' 5'-CATCTTTGAAATAGACCCCTGGACTACG-3'
rpoB	2	5'-CCTTTAATGACTGAGAGAGGGACCTT-3' 5'-CTGTCTCGCCAACAGTCATCC-3'
rpoB	3	5'-GTTGGAGAACTTCTTCAAAACCAAG-3' 5'-CTAAGAAGGGAATGAGAGAAGTCGC-3'
rpoB	4	5'-CTTTCTCCTGTTCAAGTTATTTCAGT-3' 5'-CAGGTGGCTGATCTGATTCTCCC-3'
rpoB	5	5'-GAAATGGGAATTATCAGAACAGGTGC-3' 5'-CAACAGCTACTGGTTGATCAAAAGG-3'
rpoB	6	5'-CAAAGCAAGATGGTAAGGATTGGG-3' 5'-GTCCTTTGCCCCCAATCC-3'
rpoB	7	5'-GTTGTTACTCAAAGATGGTAGAACAGGCGA-3' 5'-CCAATCCATTATTCTTTGAGGTGAAGC-3'
rpoB	8	5'-CATGAGGATGTTCTATCGACAATTG-3' 5'-GATGAGGCGGAACTTTCTCAAAATC-3'
gyrA	1	5'-GTTGATTCTGAGAATTCTGGTTTGAG-3' 5'-GGGGGATCCTAGCTGGTAAAAC-3'
gyrA	2	5'-GTTTTACCAGCTAGGATCCCCC-3' 5'-CTTGAGGATAGGCATCTCTTTTAAGCTC-3'
gyrA	3	5'-GAGCTTAAAAGAGATGCCTATCCTCAAG-3' 5'-AGAATCTCTGTTTTTCTCGGAGATG-3'

gyrA	4	5’-CATCTCCGAGAAAAACAGAGATTCT-3’ 5’-CCAAGAGGTCTTAATTCATTAGTATCTAACC-3’
gyrA	5	5’-GGTTAGATACTAATGAATTAAGACCTCTTGG-3’ 5’-GGTAAATCAAGAATATTTAAAATGGGTTG-3’

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Supporting Figure Legends

Figure S1. Growth of MED4 and MED4 Rif^R strains in media containing rifampicin. 10^8 cells of MED4 or MED4 Rif^R were inoculated into 25 ml PRO99SSW medium amended with rifampicin (5 μg/ml) and grown under constant light as described in the legend to Fig. 1.

Figure S2. Alignment of the MED4 WT (top, GenBank accession number NP_893602.1) and *E. coli K12* (bottom, GenBank accession number NP_418414.1) RNA polymerase, β subunit protein sequences. Sequences were aligned using the Emboss alignment tool (http://www.ebi.ac.uk/Tools/emboss/align/). The green shaded box denotes the correspondence between MED4 residue 437 and *E. coli* residue 572, a known mutational hotspot for rifampicin-resistance.

Figure S3. *Prochlorococcus* strains MED4, MIT9313, MIT9215, NATL2A and *Synechococcus* stain WH8102 are resistant to naladixic acid. All cultures were grown in duplicate in PRO99SSW medium, with or without 50 μg/ml of nalidixic acid (NAL), at 21ºC in either 10 μmol Q m^{-2} s^{-1} continuous light (NATL2A, WH8102, MIT9313) or 20 μmol Q m^{-2} s^{-1} continuous light (MED4, MIT9215). Culture growth was monitored daily by fluorometric detection of bulk chlorophyll autofluorescence over the course of the growth cycle using a Turner Designs 10-AU fluorometer.

Figure S4. Alignment of the MED4 WT (top, GenBank accession number NP_893180.1) and *E. coli K12* (bottom, GenBank accession number NP_416734.1) DNA gyrase subunit A protein sequences. Sequences were aligned using the Emboss alignment tool as for Figure S2. The green shaded box denotes a mutational hotspot at position 83 of *E. coli* and the corresponding position (94) for MED4.

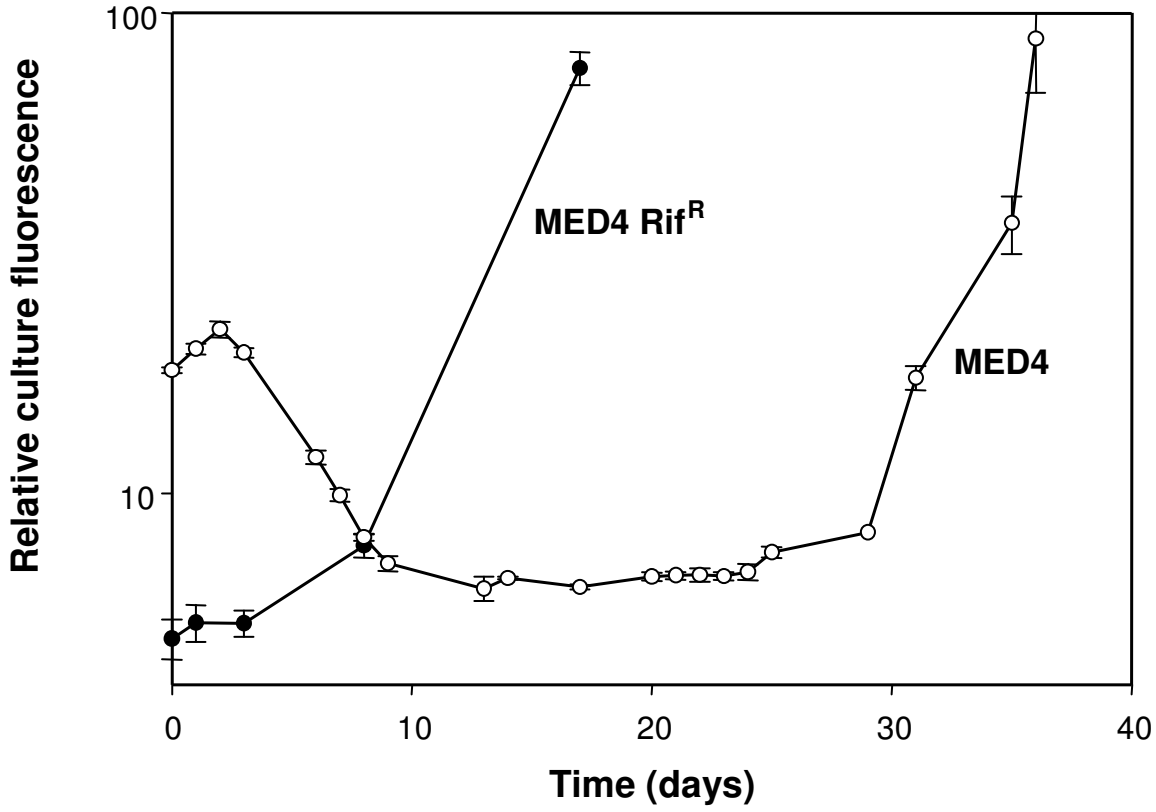
100
10
Relative culture fluorescence
MED4 Rif^R
MED4
0
10
20
30
40
Time (days)

Fig. S1

Fig. S2

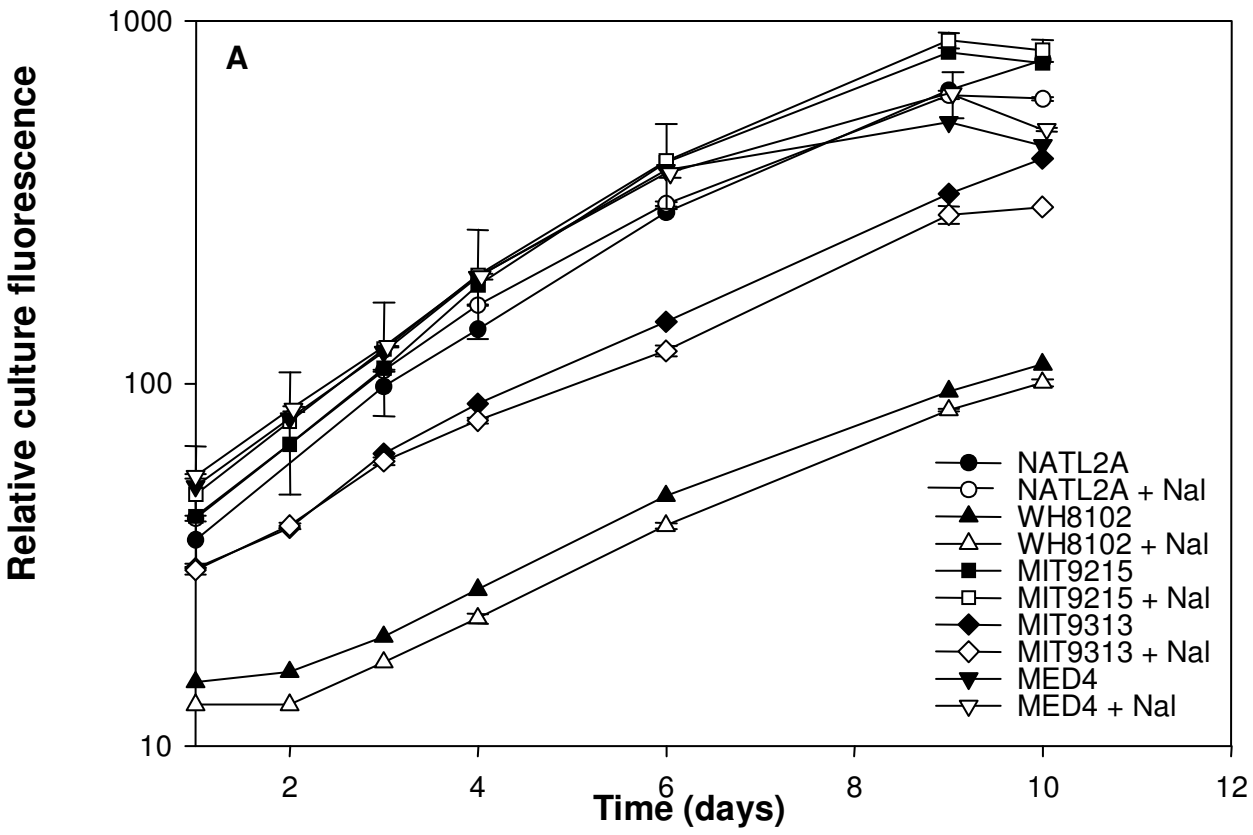
A
1000
100
10
Relative culture fluorescence
2
4
6
8
10
12
Time (days)
NATL2A
NATL2A + Nal
WH8102
WH8102 + Nal
MIT9215
MIT9215 + Nal
MIT9313
MIT9313 + Nal
MED4
MED4 + Nal

Fig. S3

Fig. S4