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Is there a cost of virus resistance in marine cyanobacteria?

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Owing to their abundance and diversity, it is generally perceived that viruses are important for structuring microbial communities and regulating biogeochemical cycles. The ecological impact of viruses on microbial food webs, however, may be influenced by evolutionary processes, including the ability of bacteria to evolve resistance to viruses and the theoretical prediction that this resistance should be accompanied by a fitness cost. We conducted experiments using phylogenetically distinct strains of marine *Synechococcus* (Cyanobacteria) to test for a cost of resistance (COR) to viral isolates collected from Mount Hope Bay, Rhode Island. In addition, we examined whether fitness costs (1) increased proportionally with 'total resistance', the number of viruses for which a strain had evolved resistance, or (2) were determined more by 'compositional resistance', the identity of the viruses to which it evolved resistance. A COR was only found in half of our experiments, which may be attributed to compensatory mutations or the inability to detect a small COR. When detected, the COR resulted in a ∼20% reduction in relative fitness compared to ancestral strains. The COR was unaffected by total resistance, suggesting a pleiotropic fitness response. Under competitive conditions, however, the COR was dependent on compositional resistance, suggesting that fitness costs were associated with the identity of a few particular viruses. Our study provides the first evidence for a COR in marine bacteria, and suggests that *Synechococcus* production may be influenced by the composition of co-occurring viruses.

Introduction

Viruses are abundant in marine environments, often exceeding the densities of their bacterial host populations (Proctor and Fuhrman, 1990; Weinbauer and Rassoulzadegan, 2004). Since viruses are obligately parasitic, it is assumed that they are important agents of mortality for microorganisms. Indeed, virus-induced mortality of bacteria can equal the losses imposed by protist and zooplankton grazing (Fuhrman and Noble, 1995). Such findings have led to the prevailing view that viruses are important for structuring microbial communities and regulating biogeochemical cycles (Fuhrman, 1999; Wilhelm and Suttle, 1999; Weinbauer, 2004;

Bouvier and del Giorgio, 2007). In many studies, however, virus-induced mortality can be low (Fuhrman, 1999), possibly due to bacterial virus resistance.

The evolution of virus resistance has been well-documented for clinically important strains of *Escherichia*, *Salmonella* and *Pseudomonas* (Chao et al., 1977; Lenski and Levin, 1985; Buckling and Rainey, 2002; Harcombe and Bull, 2005). In these systems, populations of virus-resistant bacteria rapidly evolve, invade the microbial community, and persist through time (Bohannan and Lenski, 2000). In contrast, few studies have documented the ease by which marine bacteria evolve virus resistance or its extent in marine systems. While some reports infer that resistance to marine viruses is rare (Fuhrman, 1999), others suggest that it may be widespread (Waterbury and Valois, 1993; Middelboe et al., 2001; Jiang et al., 2003; Sullivan et al., 2003).

The potential prevalence of virus resistance sets up a 'paradox' (Weinbauer, 2004): if virus resistance readily evolves, what prevents resistant bacteria from outcompeting sensitive bacteria and driving viruses extinct? Theory suggests that the coexistence

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of sensitive and resistant bacteria may be due to a trade-off between competitive ability and reduced mortality (Lenski, 1988b). Experimental evidence indicates that virus-resistant bacteria are usually less efficient competitors for growth-limiting resources than sensitive bacteria in the absence of viruses (Buckling and Rainey, 2002); in other words, there is typically a fitness cost associated with the evolution of virus resistance (Bohannan and Lenski, 2000). For lytic viruses, resistance is often conferred through the loss or configuration change of a receptor molecule on the bacterial cell surface, which interferes with attachment of a virus to its host (Inoue et al., 1995; Bohannan and Lenski, 2000). Such mutations often translate into a fitness cost via reduced rates of resource uptake (Bohannan et al., 1999). The magnitude of this cost of resistance (COR) reflects the host's genotype and its environment (Bohannan et al., 2002). For example, different mutations can impose varied costs (for example, T7 and T4 phage), but the COR can also be influenced by the resource environment (Bohannan et al., 1999).

The laboratory systems described above provide an excellent framework for exploring the COR in strains of environmental bacteria. Very little has been carried out, however, to explore the COR in marine bacteria, which presumably experience exposure to diverse viral assemblages. Recently, there have been major advances in our understanding of the ecology and evolution of environmental viruses through the study of marine cyanobacteria, especially Synechococcus (Waterbury and Valois, 1993; Suttle and Chan, 1994; Mann, 2003; Marston and Sallee, 2003; Paul and Sullivan, 2005). In addition to being a fairly well-characterized host-parasite system, understanding viral influence on Synechococcus populations is important due to its broad geographic distribution and functional role in marine ecosystems (Bertilsson et al., 2003).

To better understand how viruses affect marine bacteria, we addressed two main questions. First, does Synechococcus incur a fitness cost when it evolves resistance to a virus? Second, what factors influence the magnitude of a COR in Synechococcus? To address these questions, we measured the growth rates of multiple Synechococcus strains that had been selected for resistance to a suite of marine viruses (Stoddard et al., unpublished data). We then determined whether Synechococcus growth rates could be explained by breadth-of-resistance (BOR) profiles, which describe a host's sensitivity or resistance to a suite of genetically different viruses. We hypothesized that not only would virus resistance in Synechococcus incur a fitness cost, but that Synechococcus growth rates would decrease with each additional virus to which it evolved resistance. In other words, we predicted there would be a negative correlation between bacterial fitness and 'total resistance' (that is, the number of viruses in a BOR profile to which a strain is resistant). In addition, Synechococcus fitness may be influenced by the identity of the viruses to which it evolved resistance. If true, we expected that there would be a relationship between a Synechococcus strain's 'compositional resistance' (as defined by the multivariate characterization of Synechococcus BOR profiles) and its fitness.

Methods

Ancestral bacterial strains
Bacterial strains were derived from four ancestral strains of marine Synechococcus that were obtained from the Woods Hole Collection of Cyanobacteria (Woods Hole Oceanographic Institution, Woods Hole, MA, USA). Strains WH7803 and WH8012 were originally isolated from the Sargasso Sea, while WH8018 and WH8101 were isolated from coastal waters adjacent to Woods Hole, MA, USA (Waterbury et al., 1986). These Synechococcus strains vary in their pigment composition and can be distinguished based on 16S-23S rRNA internal transcribed spacer sequences (Rocap et al., 2002). WH8012 belongs to Synechococcus clade II and WH8018 belongs to Synechococcus clade VI, while WH7803 and WH8101 belong to distinct subclades within Synechococcus clade V. We used SN medium (seawater-based) for isolating Synechococcus strains and AN medium (artificial seawater) for subsequent culturing and experiments (Waterbury and Willey, 1988). These ancestral Synechococcus strains were maintained at room temperature on a 14:10 light–dark cycle at $10 \, \mu M \, m^{-2} \, s^{-1}$.

Viral isolates
A total of 32 marine viruses were isolated on one of the four ancestral Synechococcus strains. Detailed methods on the isolation and characterization of these viruses can be found in Marston and Sallee (2003). Briefly, seawater was collected from Mount Hope Bay, RI near Roger Williams University. Filtered seawater ($100 \, \mu l$), dense Synechococcus culture ($100 \, \mu l$ of $\sim 10^8$ cell ml^{-1}) and sterile SN media ($1000 \, \mu l$) were combined in replicate wells of a microtiter plate. The plates were incubated under constant illumination for up to 3 week, while monitoring for lysis, which was noted by a clearing of the pigmented culture relative to control wells. Viruses were isolated from the lysates via plaque purification (Marston and Sallee, 2003). Resulting plaques were sterilely picked and reinoculated into a fresh culture of the original Synechococcus strain. The lysate produced from this last step was filtered ($0.45 \, \mu m$) and used in batch culture experiments designed for the selection of virus-resistant Synechococcus. PCR amplification of the gene encoding for the capsid assembly protein (g20) indicated that all of the viruses in this study belong to the Myoviridae (Marston and Sallee, 2003). Genetic

and phenotypic similarity analyses indicated that the viral isolates were distinct from one another (Stoddard *et al.*, unpublished data). The viruses were named 'S-RIM' (*Synechococcus*-Rhode Island Myovirus) followed by the isolate number (for example, S-RIM36).

Selection for virus-resistant bacteria

We selected for virus-resistant bacteria by exposing ancestral *Synechococcus* strains to the isolated viral isolate (Stoddard *et al.*, unpublished data). We combined 100 μl of a dense *Synechococcus* culture ($\sim 10^8$ cell ml^{-1}), 10–40 μl of plaque-purified virus and 1500 μl SN media in 24-well microtiter plates. We incubated the microtiter plates and examined the wells for cell lysis. By eye, we monitored the wells for regrowth of *Synechococcus*, which occurred in 4–10 week. From wells that regrew, single colonies were isolated via dilution culturing in SN soft agar (Brahamsha, 1996) and transferred to 50 ml Erlenmeyer flasks containing SN media. We confirmed that the selected bacterial strains were virus resistant by challenging them against the original viral isolate in replicate microtiter wells. Further, in three instances, we sequentially selected for virus resistance using multiple viral isolates. In other words, we challenged a previously selected virus-resistant *Synechococcus* strain with a novel virus, monitored for cell lysis and subsequent regrowth, and then isolated the new virus-resistant strain of *Synechococcus*.

In total, we isolated 22 virus-resistant strains of *Synechococcus*: seven from WH7803, four from WH8101, three from WH8012 and eight from WH8018. We named the resistant strains by adding 'R' (for 'resistant') and the identifier of the S-RIM viruses (that is, 1–36) to the end of the ancestral strain. For example, WH8018 that became resistant to S-RIM8 was named 'WH8018R8'. In the case of 'WH8018R3R2R6', ancestral WH8018 was first selected for resistance to virus S-RIM3, then to S-RIM2, and finally to S-RIM6. Prophage was not induced with Mitomycin C (McDaniel and Paul, 2005) for any of the ancestral or virus-resistant *Synechococcus* strains (Stoddard *et al.*, unpublished data).

Breadth of resistance

We characterized the phenotype of each *Synechococcus* strain by challenging it against each of the 32 viral isolates using the microtiter-plate approach described above. If a virus caused a *Synechococcus* strain to lyse within a 3-week period, then that bacterial strain was considered 'sensitive'. In contrast, if a *Synechococcus* strain did not lyse after being challenged with a virus, then the bacterial strain was considered 'resistant' to that particular virus. Thus, a BOR profile refers to the sensitivity-resistance pattern of a *Synechococcus* strain to the 32 viral isolates (Stoddard *et al.*, unpublished data).

Collectively, the pairwise crosses between all bacteria and all viruses resulted in a binary (sensitive = 0, resistant = 1) sensitivity-resistance matrix.

We characterized the BOR profile for each of the 25 *Synechococcus* strains in two ways. First, 'total resistance' refers to the summed number of viruses to which a *Synechococcus* strain was resistant (out of the 32 viral isolates). Second, 'compositional resistance' refers to the multivariate similarity of BOR profiles among different strains of bacteria. To quantify compositional resistance, we performed a Principal Coordinates Analysis (PCoA) on a similarity matrix generated from the sensitivity-resistance matrix. The similarity matrix used in the PCoA was a slight modification of the mean character difference (Legendre and Legendre, 1998):

$$s_{ik} = 1 - \left[\frac{1}{p} \sum_{j=1}^{p} |y_{ij} - y_{kj}| \right]$$

where s_{ik} is the similarity of BOR profiles for *Synechococcus* strains i and k, j is the first of p total viruses, and y refers to the phenotypic observations for a given viral isolate. PCoA was performed in Matlab (version 6.3).

For the WH8018 strains, we constructed BOR profiles on two occasions; once when resistant strains were first isolated and again ~ 12 months later. The two sets of BOR profiles allowed us to evaluate the stability of the bacterial phenotypes over time. In particular, we were interested in determining whether a resistant phenotype reverted to a sensitive phenotype in the absence of virus.

COR: growth curves

We used two approaches to test whether *Synechococcus* experienced a cost associated with the evolution of virus resistance. The first approach compared the maximum growth rates (μ_{max}) among 25 *Synechococcus* strains in growth curve experiments (Kassen and Rainey, 2004). We inoculated 1 ml of a log-phase culture into an Erlenmeyer flask containing 20 ml of sterile AN media. Each strain was grown in triplicate for a total of 75 experimental units. We incubated the culture flasks in a Percival growth chamber at 25 °C on a 14:10 light–dark cycle at 10 μE m^{-2} s^{-1}. As a surrogate for cell density, we monitored fluorescence (ex: 515 nm, em: 570 nm for WH7803, WH8012 and WH8018 strains; ex: 515 nm, em: 660 nm for WH8101) on 150 μl sub-samples from each culture every 24 h for 18 days using a Molecular Devices SpectraMax M5 plate reader.

A variety of models have been developed for estimating growth parameters from batch culture experiments (Baty and Delignette-Muller, 2004). We elected to use the modified Gompertz equation, because it is relatively simple, easy to interpret and has been shown to be effective in fitting empirical

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growth curve data (for example, Zwietering et al., 1990):

$$y = A \exp\left\{-\exp\left[\frac{\mu_{max}e}{A}(\lambda - t) + 1\right]\right\}$$

where y is cell density (or in this case fluorescence), A is the carrying capacity, μ_{max} is maximum growth rate, and λ is the lag time, which defines the duration of time between inoculation and the initiation of exponential growth (Zwietering et al., 1990). We fit $\log_{10}$ transformed fluorescence as a function of time using nonlinear regression (PROC NLIN, SAS version 8.01) with non-derivative parameter estimation (DUD).

We tested multiple hypotheses regarding the growth rates of the Synechococcus strains. First, we used one-way analysis of variance (ANOVA) to test whether there were differences in μ_{max} among the four ancestral Synechococcus strains. Second, we tested the null hypothesis that all Synechococcus strains (ancestral and resistant) had similar μ_{max} using one-way ANOVA with Tukey's post hoc comparisons of means (PROC GLM, SAS version 8.01). Third, we used one-way ANOVA with linear contrasts to test the a priori hypothesis that resistant strains of Synechococcus would have lower μ_{max} than the ancestral Synechococcus strains. Linear contrasts were used because they allow for flexibility in hypothesis testing and tend to be statistically more powerful than post hoc comparisons (Gotelli and Ellison, 2004). Fourth, we used linear regression (PROC GLM, SAS version 8.01) to test whether μ_{max} decreased with the total resistance of a strain's BOR profile. Lastly, we tested whether μ_{max} was affected by the compositional resistance of a strain's BOR profile using information generated from the PCoA. Specifically, we used the eigenvectors corresponding to the first three principal coordinate axes as independent predictors of Synechococcus μ_{max}; if there was a non-zero slope between the eigenvectors and bacterial growth rate, then we concluded that compositional resistance affected Synechococcus fitness. In addition, we calculated correlation coefficients for the principal coordinate scores generated from each PCoA axis against the resistance-sensitivity profile of each viral isolate. In this case, a strong positive or negative correlation suggests that a viral isolate was influential in a PCoA.

COR: competition assays
A second strategy for measuring fitness trade-offs is to directly compete sensitive and resistant strains of bacteria. Although logistically more difficult, this approach is often preferred because it integrates abiotic and biotic factors that influence a population's ability to persist through time (Kassen and Rainey, 2004). One obstacle with the competition approach is the challenge of distinguishing between morphologically similar strains in co-culture. We addressed this issue by conducting assays where ancestral and virus-resistant Synechococcus were competed separately against a reference strain (de Roode et al., 2005).

Although ecologically similar, certain clades of Synechococcus can be distinguished from one another based on their phycobiliprotein composition (Stomp et al., 2004). For example, all strains of WH7803, WH8018 and WH8012 contain the accessory pigment phycoerythrin. In contrast, WH8101 lacks phycoerythrin, but instead contains phycocyanin. Therefore, we assessed the competitive ability of our target strains (ancestral and virus-resistant phycoerythrin-containing strains) by competing them against a reference strain (ancestral phycocyanin-containing WH8101).

We initiated competition assays by inoculating replicate Erlenmeyer flasks containing 25 ml of sterile AN media with equal densities of the target and reference strains. Initial densities of each population were $\sim 10^6$ cell ml^{-1}. Cultures were incubated in a Percival growth chamber at 25 °C on a 14:10 light–dark cycle at 10 μE m^{-2} s^{-1} for 7–10 days. Samples from each flask were taken at the beginning (t_0) and end (t_f) of the experiment by filtering samples onto 0.2 μm black polycarbonate filters (Osmonics). Filter images were captured using a Zeiss Axioplan II microscope and Axiocam MRm camera. From these images, we estimated total Synechococcus densities (phycoerythrin- + phycocyanin-containing strains) using a CY5 filter set (ex: 640 nm; em: 680 nm). We quantified phycoerythrin-containing Synechococcus (that is, target population) using a fluorescein isothiocyanate (FITC) filter set (ex: 480 nm; em: 535 nm). We then estimated cell densities of the reference strain as the difference between CY5 and FITC counts. We calculated Synechococcus growth rates as [ln(Nt_f)/ln(Nt_0)]/t, where N is Synechococcus density (cell ml^{-1}). Relative fitness was expressed as the ratio of resistant to ancestral growth rates.

We performed two competition assays to test different COR hypotheses. The first was designed to determine whether the COR was dependent on the concentration of a growth-limiting resource. Before initiating the assay, we grew up batch cultures of an ancestral population (WH7803), a virus-resistant population (WH7803R8) and the reference population (WH8101) in either low (0.9 μM) or high (90 μM) phosphate concentrations through two serial transfers. Nitrogen, supplied as KNO$_3$, was held at a constant N:P ratio of 100. We analyzed the growth rate data using two-way ANOVA (factor 1 = phosphorus concentration, factor 2 = strain phenotype, PROC GLM, SAS version 8.01). Each treatment combination was replicated in triplicate.

The second assay was designed to test whether the COR of a strain was related to its BOR profile. We used WH8018 for this assay because, compared to the other Synechococcus groups, it contained the

largest number of strains in our collection and it exhibited the most phenotypic variation in its sensitivity-resistance matrix. Each competition trial (one of the nine WH8018 strains vs the reference strain) was replicated five times. We used the same set of hypotheses and statistical analyses for the competition data that we used for the growth curve experiments.

COR: assessing detection limits
We performed power analyses (JMP software, version 5.1) for the growth curves experiments and competitive assays to evaluate our ability to detect relative fitness costs ranging from 2.5 to 50%. This range in COR represents the 'effect size' in the power analyses. We used within-treatment standard deviations as an estimate of our measurement error. The probability of committing a Type I error (rejecting the null hypothesis when it is true) is expressed as α, while the probability of committing a Type II error (accepting the null hypothesis when it is false) is expressed as β. We assumed a typical α value of 0.05 and then estimated power $(1-\beta)$ for a range of experimental replication ($n = 3, 5$ and 10).

Results

Breadth of resistance
The ancestral and virus-resistant *Synechococcus* strains investigated in this study displayed substantial phenotypic diversity in the form of their BOR profiles. A detailed treatment of the BOR data can be found in Stoddard *et al.* (unpublished data). Briefly, total resistance, which we define as the number of viral isolates to which a bacterial strain is resistant, ranged from 10 to 15 for WH8101 strains, 13 to 21 for WH8012 strains, 2 to 13 for WH7803 strains and 3 to 30 for WH8018 strains (Table 1 and Figure 1). The compositional profiles of virus resistance varied among strains as well. For example, despite having the same total resistance, the BOR profiles for WH8101R22 and WH8101R3 were only 75% similar (Table 1). The bacterial strains were distinct from one another based on variation in their BOR profiles, as indicated by the amount of variance explained in the PCoA (Table 2 and below).

BOR profiles for the WH8018 strains were relatively stable over time; 95% of the potential 288 interactions (nine bacterial strains × 32 viral isolates) remained fixed over a 12-month period. In addition, there was no apparent directionality for the 15 observed changes; 53% ($n = 8$) switched from resistant to sensitive (reversion), and 47% ($n = 7$) switched from sensitive to resistant. Interestingly, six of the eight reversions were associated with only two of the 32 viruses (S-RIM28 and S-RIM32, Table 1).

COR: growth curves
Before analyzing growth curve data for the COR hypotheses, we noted that the maximum growth rates (μ_{max}) of ancestral *Synechococcus* strains were not equal among the four phylogenetic groups ($P < 0.0001$, Figure 1b). In particular, the μ_{max} of WH8101 (0.59 ± 0.023) was significantly higher than that of the three other ancestral strains (0.33 ± 0.061). Therefore, subsequent tests of the COR hypotheses were performed for each group separately.

Synechococcus μ_{max} was only affected by virus resistance in the WH8101 group, which happened to have the highest maximum growth rates. A one-way ANOVA followed by Tukey's *post hoc* comparison of means indicated that the μ_{max} of ancestral WH8101 was significantly greater than that of WH8101R27 ($P_{null} = 0.014$, Figure 1). Furthermore, linear contrasts testing for differences in μ_{max} between the ancestral and all virus-resistant strains revealed that there was a significant COR ($P_{contrast} = 0.0019$, Figure 2) equating to an average 18% reduction in relative fitness.

We did not detect a COR in the remaining three groups of *Synechococcus* based on our growth curve data (Figure 1). A one-way ANOVA revealed that μ_{max} were not equal among all WH8018 strains ($P_{null} = 0.029$, Figure 1), but *post hoc* comparisons of means indicated that this was due to one virus-resistant strain (WH8018R34) having a higher μ_{max} than another virus-resistant strain (WH8018R22). A subsequent linear contrast within this phylogenetic group revealed that the ancestral and virus-resistant WH8018 strains had similar μ_{max} values ($P_{contrast} = 0.352$, Figure 1). Likewise, there were no significant differences in μ_{max} for the WH7803 or WH8012 strains (P_{null} and $P_{contrast} \geqslant 0.118$, Figure 1).

In general, BOR characterizations were poor predictors of μ_{max}. Contrary, to our prediction, μ_{max} did not decline with increasing total resistance for any of the strains belonging to the four *Synechococcus* groups (linear regression, Table 1, $P \geqslant 0.453$). Similarly, we found no relationship between μ_{max} and compositional resistance (Table 1). Although a large fraction of the BOR variation was explained via the multivariate (PCoA) analyses (Table 1), *Synechococcus* μ_{max} could not be explained by eigenvectors corresponding to its first three principal coordinates axes (Table 1, $P \geqslant 0.146$). This result means there was no relationship between a strain's fitness (as defined by μ_{max}) and the combination of viruses to which it was resistant (that is, compositional resistance).

COR: competition assays
Results from the first competition assay indicated that a virus-resistant strain of *Synechococcus* (WH7803R8) was competitively inferior compared to ancestral *Synechococcus* (WH7803) ($P = 0.036$, resistance main effect, two-way ANOVA, Figure 2). In addition, *Synechococcus* growth rates were

Table 1 Sensitivity-resistance matrix depicting the phenotypes of the nine *Synechococcus* strains from the WH8018 clade

	Bacterial strains (WH8018)									
	A	R34	R20	R16	R3	R8	R22	R26	R19†	R3R2R6
Viral isolates										
S-RIM1										
S-RIM2										
S-RIM3										
S-RIM4										
S-RIM5										
S-RIM6										
S-RIM7										
S-RIM8	Δ									Δ
S-RIM9										
S-RIM10					Δ					
S-RIM11										
S-RIM12										
S-RIM13										
S-RIM15										
S-RIM16										
S-RIM17										
S-RIM18										Δ
S-RIM19			Δ	Δ						
S-RIM20	Δ					Δ				
S-RIM21										
S-RIM23										Δ
S-RIM24										
S-RIM26										
S-RIM27										
S-RIM28			Δ				Δ	Δ		
S-RIM29										
S-RIM30										
S-RIM31										
S-RIM32					Δ	Δ		Δ		
S-RIM34										
S-RIM35										
S-RIM36										
Total resistance (t_0)	3	9	11	12	13	13	13	14	14	30
Total resistance (t_f)	5	9	12	12	13	13	12	12	N/A	29

For the bacterial strains, 'A' refers to the ancestral strain. 'R' identifies a virus-resistant strain; the number following R identifies the virus for which a bacterial strain evolved resistance. White boxes indicate sensitive phenotypes; gray boxes indicate resistant phenotypes. The sensitivity-resistance matrix was determined on two occasions; once at the beginning of the experiment (t_0) and 12 months later (t_f). Δ Indicates that a bacterial strain's phenotype changed between the time that the two sensitivity-resistance matrices were constructed († = strain died during 12-month period; second profile not constructed). The bottom two rows represent total resistance, which was calculated at t_0 and t_f, by summing the number of resistant phenotypes (gray boxes) in each column.

reduced when cultured under low phosphorus concentrations ($P = 0.014$, nutrient main effect, two-way ANOVA, Figure 2). There was, however, no resistance × nutrient interaction ($P = 0.342$, two-way ANOVA, Figure 2). Therefore, *Synechococcus* experienced a COR resulting in an average 20% reduction in relative fitness, irrespective of phosphorus availability.

The second competition experiment revealed there were significant differences among sensitive and virus-resistant strains of WH8018. One-way ANOVA followed by *post hoc* comparison of means indicated that growth rates among the WH8018 populations were marginally different ($P_{null} = 0.0717$). When linear contrasts were performed, resistant strains of *Synechococcus* had significantly lower growth rates than the ancestral population ($P_{contrast} = 0.0499$, Figure 3), equating to an 18% reduction in relative fitness.

Similar to the results from the growth curve data, competition experiments revealed that *Synechococcus* growth rates were not affected by total resistance ($P = 0.58$, Figure 4, Table 1). In contrast, *Synechococcus* growth rates were affected by compositional resistance ($P = 0.023$, Figure 4, Table 1). Specifically, eigenvectors corresponding with PCoA axis 1, which explained 39.5% of the compositional variation in BOR for WH8018 strains, were strong predictors of *Synechococcus* growth rate ($r^2 = 0.54$, $P = 0.023$, Table 1, Figure 4). This pattern appears to have been driven in part by the sensitivity and resistance of bacteria to a few viral isolates. For example, S-RIM4 and S-RIM11 had strong negative correlations with PCoA axis 1 ($r = -0.94$ and -0.80, respectively), while S-RIM7 and S-RIM20 both had a strong positive correlation with PCoA axis 1 ($r = 0.91$). In other words, the relationship between compositional resistance and *Synechococcus*
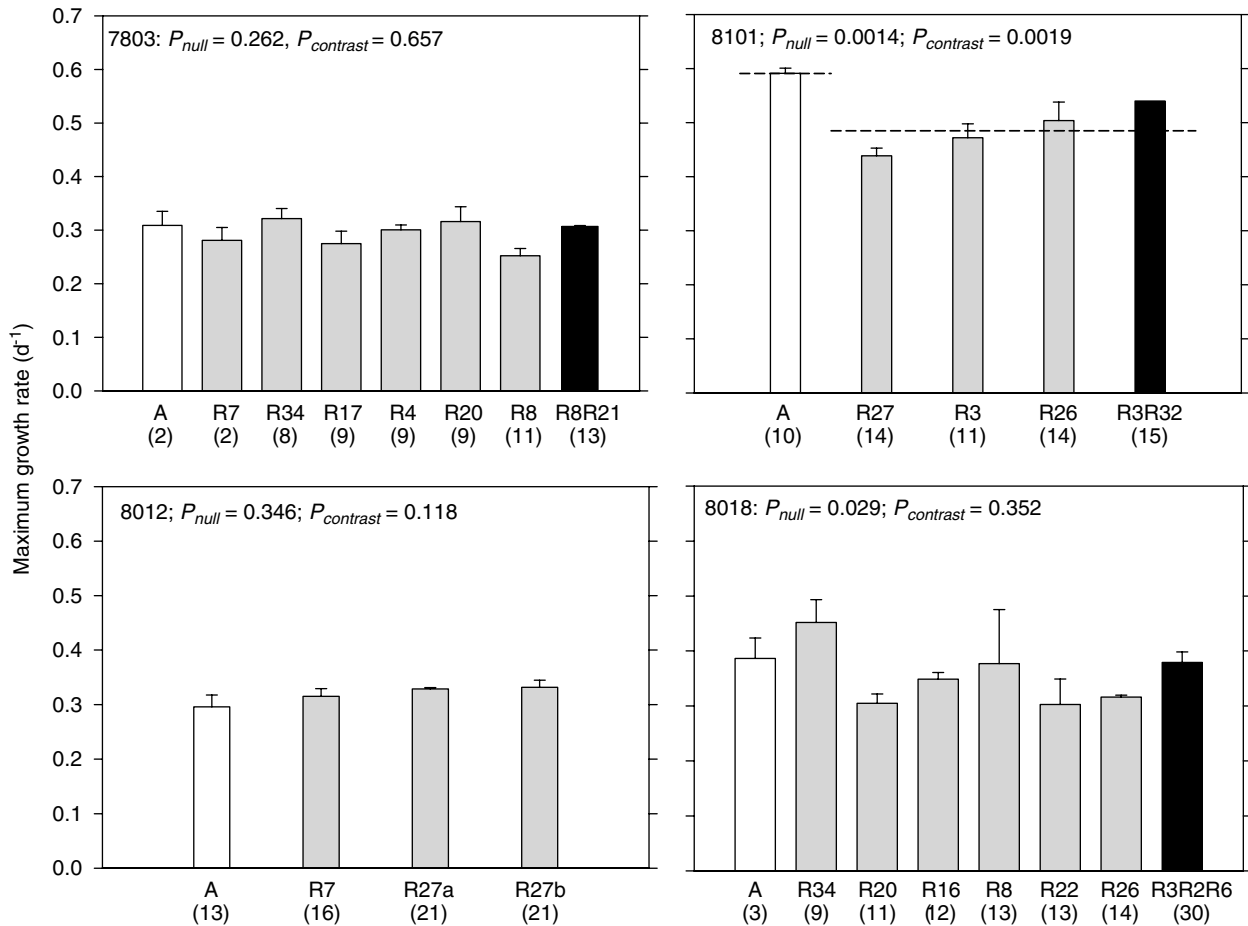
Figure 1 Maximum growth rates (μ_{max}) for ancestral (white bars) and virus-resistant strains (gray and black bars) of marine *Synechococcus*. Strains that went through one viral selection are colored gray; strains that went through multiple ($\geqslant 2$) viral selections are colored black. The four panels display results for the different phylogenetic groups of *Synechococcus* (WH7803, WH8101, WH8012 and WH8018). P_{null} refers to statistical probability that μ_{max} of all strains within a phylogenetic group are equal. $P_{contrast}$ refers to the statistical probability that the μ_{max} values of virus-resistant strains are less than that of the sensitive strain. Vertical bar data represent mean $\pm$ s.e.m. Dashed horizontal lines in the WH8101 panel represent average μ_{max} values generated from the ANOVA model with linear contrasts (that is, ancestral vs resistant). Letters below the vertical bars identify whether a strain is ancestral (A) or if is resistant (R); if resistant, the letter is followed by the number of the viral isolate for which a bacterial strain evolved resistance. Numbers in parentheses indicates the total resistance for a bacterial strain (that is, number of viruses in a BOR profile to which a strain is resistant). R27a and R27b represent resistant *Synechococcus* strains isolated after exposure to same virus (S-RIM27).

growth rate was driven by the relatively low fitness of strains that were sensitive to S-RIM4 and S-RIM11 and relatively high fitness of strains that were resistant to S-RIM7 and S-RIM20 (Table 2, Figure 4).

COR: assessing detection limits
As expected, the power to detect a COR increased with sample size and the magnitude of the COR (expressed as the percent reduction in relative fitness). Compared to the competition assays, power increased more rapidly with COR in the growth curve experiments (Figures 5a and b). This reflects an underlying difference in measurement error. For example, the average coefficient of variation (CV = standard deviation/mean) in the competition assays was 0.21, while the average CV in the growth curve experiments was only 0.10. Increasing the number of replicates from 3 to 5 would have

increased our ability to detect a 10% reduction in relative fitness by a factor of two, but we had sufficiently high power (0.79) to detect a 20% reduction in relative fitness with our experimental design (Figure 5a). In competition assays, increasing the number of replicates from 5 to 10 would have increased our power to detect a 10% reduction in relative fitness from 0.17 to 0.36 (Figure 5b). Irrespective of the sample size ($n = 3$–10), however, power was relatively low ($\leqslant 0.13$) when we simulated small reductions in relative fitness ($\leqslant 5\%$, Figure 5b).

Discussion

It is generally assumed that virus-resistant bacteria have a competitive advantage over sensitive bacteria in the presence of an infectious virus (Bohannan

Table 2 Relationships between BOR and COR for different phylogenetic groups of marine *Synechococcus*

Group	COR experiment	n	r^2	P-value	
WH7803	Growth curves	8	0.008	0.829	
WH8012	Growth curves	4	0.299	0.453	
WH8101	Growth curves	5	0.131	0.549	
WH8018	Growth curves	9	0.006	0.857	
	Competition	9	0.044	0.620	

Group	Response	PCoA Axis 1	PCoA Axis 2	PCoA Axis 3	Cumulative
WH7803	% Variance explained	44.8	22.2	15.4	82.5
	P-value (growth curves)	0.837	0.230	0.760	
	r^2	0.008	0.230	0.017	
WH8012	% Variance explained	42.4	38.1	19.5	100.0
	P-value (growth curves)	0.599	0.735	0.123	
	r^2	0.161	0.407	0.248	
WH8101	% Variance explained	39.0	35.3	15.2	89.5
	P-value (growth curves)	0.873	0.247	0.393	
	r^2	0.010	0.407	0.248	
WH8018	% Variance explained	39.5	30.0	15.1	84.6
	P-value (growth curves)	0.146	0.2311	0.834	
	r^2	0.318	0.228	0.008	
	P-value (competitions)	0.023	0.426	0.694	
	r^2	0.548	0.092	0.023	

Abbreviations: COR, cost of resistance; BOR, breadth of resistance; PCoA, Principal Coordinates Analysis.
Top: results from linear regression testing for an effect of total resistance on bacterial growth rate. COR experiment indicates whether growth rate data was generated from growth curves (μ_{max}) or competition experiments. Bottom: results from PCoA and linear regression testing for an effect of compositional resistance on bacterial growth rate. The first three axes from a PCoA were used to explain variation ('Cumulative' = sum of first three axes) in the sensitivity-resistance matrices of the different *Synechococcus* groups. In addition, eigenvectors from the first three axes of a PCoA analysis were used as predictors of *Synechococcus* growth rate in linear regression analyses. n = sample size, r^2 = coefficient of determination from regression analysis, P-value = probability that slope from regression analysis is different from zero.
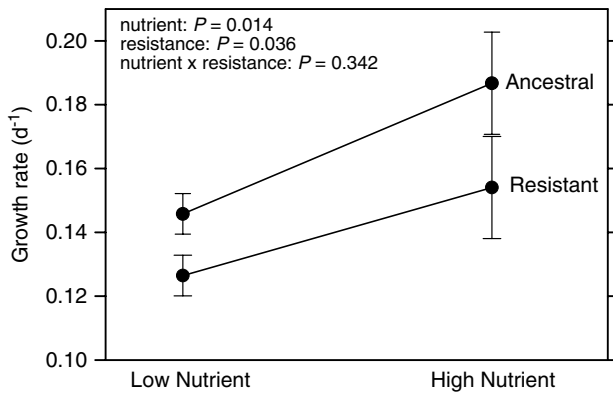

Figure 2 Growth rates for ancestral (WH7803) and virus-resistant (WH7803R8) strains of *Synechococcus* from a competition experiment designed to test whether COR was dependent on resource concentrations. Low nutrient = 0.9 μM PO$_4$; high nutrient = 90 μM PO$_4$. P-values were generated from a two-way ANOVA model.
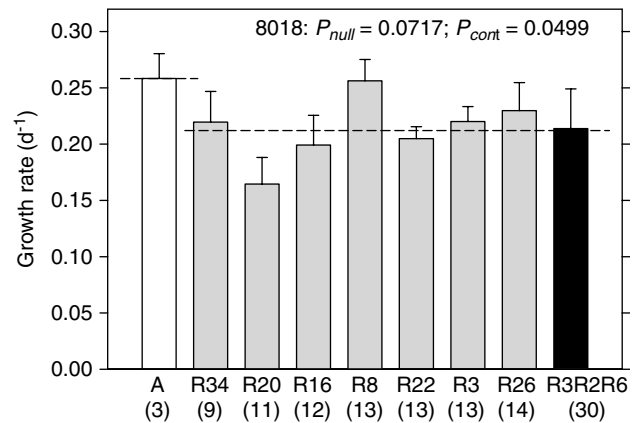

Figure 3 Growth rates from a competition experiment to test for fitness differences among ancestral (white bars) and virus-resistant strains (gray and black bars) of *Synechococcus* WH8018. Vertical bar data represent mean ± s.e.m. The dashed horizontal line represents the average growth rate generated from the ANOVA model with linear contrasts (that is, ancestral vs resistant). Letters below the vertical bars identify whether a strain is ancestral (A) or if is resistant (R); if resistant, the letter is followed by the number of the viral isolate for which a bacterial strain evolved resistance. Numbers in parentheses indicates the total resistance for a bacterial strain (that is, number of viruses in a BOR profile to which a strain is resistant).

and Lenski, 2000). Similarly, it is assumed that resistant and sensitive bacteria are able to coexist because the evolution of virus resistance comes at a fitness cost (Waterbury and Valois, 1993; Bohannan and Lenski, 2000). This trade-off may maintain genetic and phenotypic diversity in bacterial populations and thus dampen the impacts of viruses on microbial food webs (Middelboe *et al.*, 2001). Our study provides the first evidence for a cost of virus

resistance in marine bacteria. A COR was only found in a few of our experiments. When detected, however, it resulted in an 18–20% reduction in relative fitness. This cost of virus resistance is
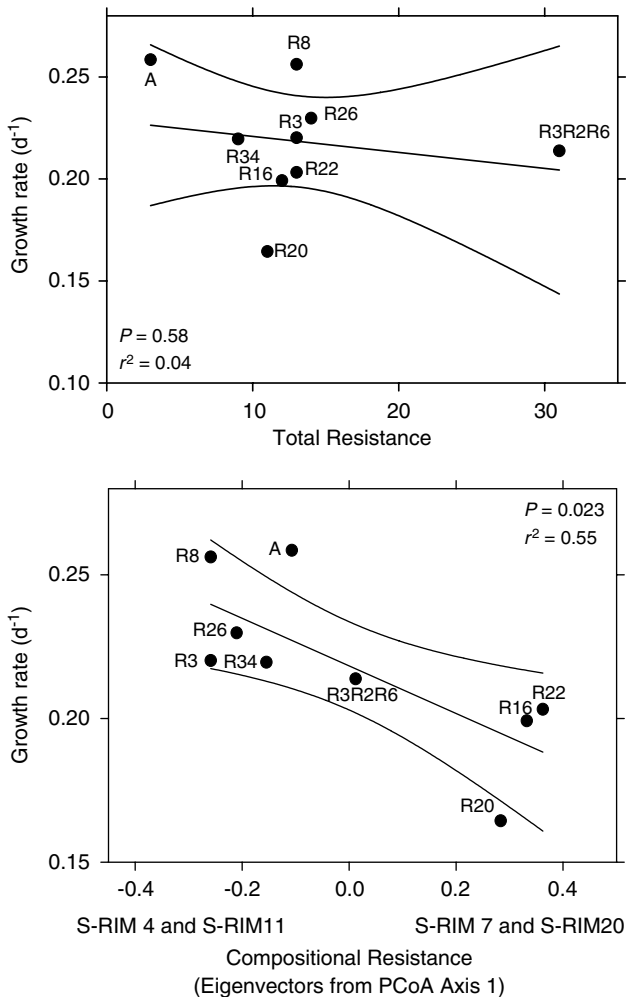
Figure 4 Growth rates from a competition experiment to test for an effect of breadth-of-resistance (BOR) on *Synechococcus* fitness. Top panel: total resistance (number of viruses to which a *Synechococcus* strain was resistant) in a BOR profile was a poor predictor of mean ($n = 5$) *Synechococcus* growth rate. Bottom panel: compositional resistance of a strain's BOR was a significant predictor of mean ($n = 5$) *Synechococcus* growth rate. Compositional resistance was calculated by generating a similarity matrix from a binary sensitivity-resistance matrix (Table 1). We then performed a Principal Coordinates Analysis (PCoA) on the similarity matrix and used the resulting eigenvectors from the first PCoA axis as predictors of *Synechococcus* growth rate. In this case, the eigenvectors from the first PcoA axis were used (see Table 1). Letters next to data points indicate whether a strain is ancestral (A) or if is resistant (R); if resistant, the letter is followed by the number of the viral isolate for which a bacterial strain evolved resistance.

in-line with the fitness reductions reported in other bacteria-phage systems (Lenski, 1988a). Moreover, we observed that a bacterial strain's growth rate was affected by the identity of the different viruses to which it evolved resistance, as described by its BOR profile. These results are important because the magnitude of a strain's COR has the potential to modify competitive outcomes and influence microbial community composition (Bohannan *et al.*, 2002).
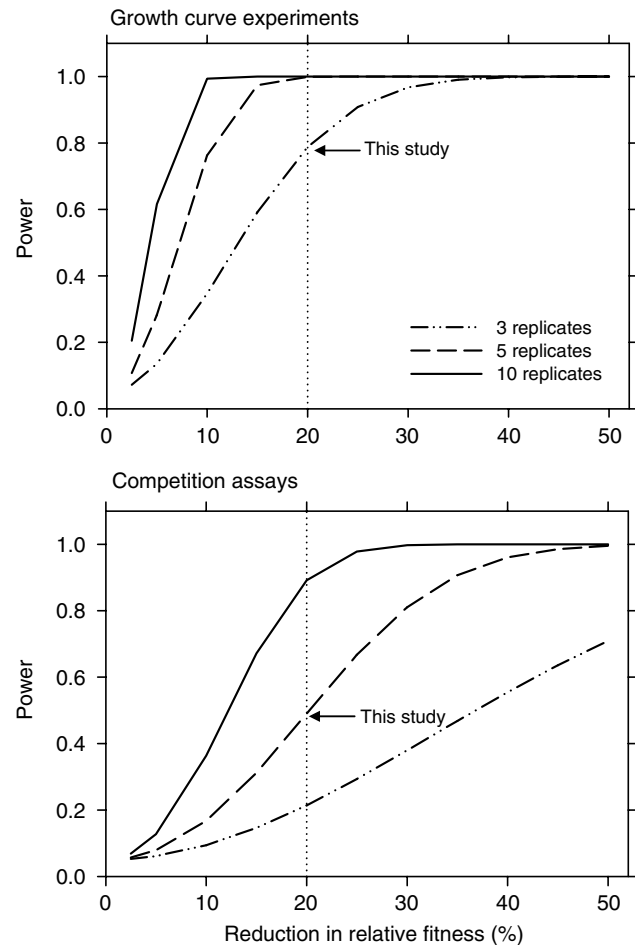

Figure 5 Power curves used to assess COR detection limits in growth curve experiments (top) and competition assays (bottom). Power is plotted as a function of the reduction in relative fitness (that is, COR) for three different levels of replication ($n = 3, 5, 10$). The dashed vertical line at 20% reduction in relative fitness represents the COR magnitude detected in some of our experiments.

Detecting a COR in Synechococcus

A COR was only detected in half of our experiments. An extensive review of the plant literature revealed that a cost of pathogen or herbivore resistance was only found half of the investigated cases (Bergelson and Purrington, 1996). Likewise, a fitness cost is not always apparent in bacteria that have evolved resistance to antibiotics (Lenski, 1998; Gagneux *et al.*, 2006) or viruses (Lythgoe and Chao, 2003; Brockhurst *et al.*, 2005). As theory predicts, the absence of a COR can result in the extinction of both the ancestral bacterial host and virus as in the case of *Escherichia coli* and T5 phage (Lenski and Levin, 1985).

There are a number of potential explanations for the lack of a COR in some of our experiments. First, a COR does not have to be large to be biologically important (Sasaki, 2000; Bohannan *et al.*, 2002). There are, however, constraints on our ability to detect a small COR. For example, our power

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analyses suggest that it is difficult to detect small fitness costs, even when measurement error is relatively low (Figures 5a and b). Increased statistical power can be achieved through increased replication, but this strategy will often be met by a reduction in the number of experimental treatments or strains that can be investigated (Cottingham et al., 2005). Thus, our results suggest that the difficulty of detecting a COR – in this study and in other systems – may be due in part to the statistical challenge of detecting small, but meaningful biological effect sizes.

Second, the cost of virus resistance can be affected by genotype × environment interactions (Bohannan and Lenski, 2000), such that relative fitness is reduced under particular environmental conditions. In plant systems, a COR to herbivory often involves increased allocation of nutrients to secondary compounds used to deter grazing pressure (Bergelson and Purrington, 1996; Siemens et al., 2003), suggesting that a COR may be expressed more in nutrient-limited environments. For bacteria, it is assumed that virus resistance often involves the modification of pilus structure or another surface receptor molecule (Lythgoe and Chao, 2003; Mizoguchi et al., 2003). Such mutations may decrease host fitness by reducing nutrient uptake rates, thus influencing competitive interactions. In E. coli, for example, mutations that confer resistance to virulent lambda phage usually modify the LamB protein (Szmelcman and Hofnung, 1975), which is involved in the uptake of maltose and trehalose, but not glucose (Travisano and Lenski, 1996). Thus, the COR to virulent lambda phage is dependent upon whether it is supplied with glucose or trehalose (Bohannan et al., 1999). We tested the hypothesis that the COR in Synechococcus would be greater under reduced phosphorus concentrations, since phosphorus limitation has been implicated in viral infection dynamics (Wilson et al., 1996). Synechococcus growth rates were reduced under low phosphorus concentrations, but this had no effect on the magnitude of the COR for the resistant strain we tested. This suggests that some viruses may target Synechococcus receptors that are not involved in phosphorus acquisition. Future studies should address whether other environmental variables, such as nitrogen concentrations, temperature or light levels influence the COR to viruses in marine bacteria.

There is also evidence that a COR may be expressed more under competitive conditions. For example, the COR for T4-resistant E. coli was half the magnitude in batch culture than continuous culture, where it is assumed that bacteria experience more intense resource competition (Bohannan et al., 2002). Results from our experiments with Synechococcus are consistent with this view; we were unable to detect a COR in growth curve experiments with WH8018 strains, but measured an 18% reduction in relative fitness with the same strains in competition assays.

Third, the COR can be eliminated if resistant strains are capable of reverting to a sensitive phenotype. Initially, no COR was detected in studies of virus-resistant Pseudomonas strains (Brockhurst et al., 2005). Subsequent experiments within the same study revealed that certain resistant strains were reverting to a sensitive phenotype in <24 h, thus eliminating the COR. This reversion pattern, however, was not observed for other strains of Pseudomonas that had evolved resistance to different viruses (Brockhurst et al., 2005). In this study, Synechococcus BOR profiles were generally stable over time under our culturing conditions, suggesting that reversions did not play a large role in altering a strain's COR over the course of our experiment. Of the 134 resistant phenotypes tested, only 6% reverted to a sensitive phenotype. Of those reversions, most were associated with two viruses, suggesting that some resistance mutations are less stable than others. Nevertheless, reversions may not be rare; Synechococcus infections were more common when host strains had been cultured in the absence of viruses for at least 2 years, during which time they presumably lost their resistance (Waterbury and Valois, 1993).

In addition to reversions, the COR can be diminished through time owing to compensatory mutations. Compensatory mutations refer to host mutations at secondary loci that increase fitness independent of the mutation involved in viral resistance (Wijngaarden et al., 2005). For example, the COR for T4-resistant E. coli declined by ~50% over 400 generations in the absence of T4 (Lenski, 1988b). Although potentially important, our study was not designed to test for the effect of compensatory mutations on the COR in marine Synechococcus.

Lastly, there is some theoretical evidence to suggest that a COR is not required for the coexistence of virus-sensitive and virus-resistant bacterial populations. For example, metapopulation models, which include spatial heterogeneity allow for the coexistence of host and parasite populations without assuming a COR (Thrall and Burdon, 2000). Similarly, gene-for-gene models with finite population sizes can maintain phenotypic diversity through genetic drift and periods of selective neutrality (Salathe et al., 2005). To our knowledge, these models have not yet been tested. Also, it is possible that some viral 'infections' actually increase host fitness. For example, it has been hypothesized that viruses may be a source of nutrition and novel genetic information for marine bacteria (Fuhrman, 1999).

COR affected by compositional resistance

Previous studies suggest that the number of viruses to which a bacterium has evolved resistance will influence the COR. For example, the COR in E. coli is greater when it has evolved resistance to both T4

and T7, instead of T4 alone (Lenski, 1988a). There-fore, we predicted that the COR would increase with total resistance, which we defined as the number of viruses that a *Synechococcus* strain was resistant to out of the 32 viral isolates tested in this study. Despite substantial variation in the predictor and response variables, total resistance was not posi-tively correlated with a strain's fitness. Pleiotropy, the effect of one mutation on multiple phenotypes, is one explanation for the lack of a relationship between total resistance and fitness (Lenski, 1988a). For example, *E. coli* resistance to colicins arises non-independently, such that bacterial resistance to one colicin type confers resistance to additional colicin types (Feldgarden and Riley, 1999). Similarly, on average, marine *Synechococcus* gained resistance to eight additional viruses when challenged against a single viral isolate (Stoddard *et al.*, unpublished data). Therefore, total resistance may not reflect the number of viruses to which a host has become independently resistant. This non-additive effect was especially apparent with the observation that *Synechococcus* fitness did not decline after going through multiple, independent selections for virus resistance (black bars in Figures 1 and 3).

Under competitive conditions, however, *Synecho-coccus* growth rate could be explained in part by the compositional resistance observed in BOR profiles. In other words, although fitness was unaffected by total resistance, it was affected by the identity of the specific viruses to which it evolved resistance. In particular, it appears that WH8018 resistance to S-RIM7 and S-RIM20 comes at a particularly large cost. As in *E. coli*, it is possible that *Synechococcus* resistance to these viral isolates involves mutations that result in a modified physiology that is particu-larly costly (Lenski, 1988a). Future studies should evaluate whether different mechanisms of virus resistance (for example, modifications of different receptor molecules) lead to different fitness costs in marine *Synechococcus*. Together, our results have important implications for understanding the diver-sity and function of marine microbial food webs. In theory, even if small, a COR can allow for the coexistence sensitive bacteria, resistant bacteria and a virus population (Bohannan and Lenski, 2000). Hence, by detecting a significant COR in some of our experiments, our results are consistent with the view that viruses play a role in promoting *Synecho-coccus* diversity in marine waters (Muhling *et al.*, 2005). Results from our study also provide insight into potential evolutionary controls on the virus-induced mortality of marine bacteria. Specifically, our results suggest that the composition of co-occurring viruses may influence virus-induced mortality rates via varied fitness costs. For example, if on average, the viruses present in a microbial community impose a small COR, then resistant bacteria should be common and lead to lower viral abundances and lower virus-induced mortality. In contrast, if the viral community imposes a high COR, then resistant strains of bacteria should be relatively rare and lead to higher viral abundances and higher virus-induced mortality. Thus, variation in fitness costs associated with virus resistance has the potential to structure microbial communities and regulate biogeochemical cycles in marine ecosystems.

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