

Facilitation of Robust Growth of *Prochlorococcus* Colonies and Dilute Liquid Cultures by “Helper” Heterotrophic Bacteria[∇]

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Axenic (pure) cultures of marine unicellular cyanobacteria of the *Prochlorococcus* genus grow efficiently only if the inoculation concentration is large; colonies form on semisolid medium at low efficiencies. In this work, we describe a novel method for growing *Prochlorococcus* colonies on semisolid agar that improves the level of recovery to approximately 100%. *Prochlorococcus* grows robustly at low cell concentrations, in liquid or on solid medium, when cocultured with marine heterotrophic bacteria. Once the *Prochlorococcus* cell concentration surpasses a critical threshold, the “helper” heterotrophs can be eliminated with antibiotics to produce axenic cultures. Our preliminary evidence suggests that one mechanism by which the heterotrophs help *Prochlorococcus* is the reduction of oxidative stress.

Members of the genus *Prochlorococcus* are the most abundant marine photosynthetic organisms and, as such, are major contributors to photosynthesis in the ocean (20). Over 30 strains of *Prochlorococcus* have been brought into culture, isolated from many locations within the band from 40°N to 40°S, including the North Atlantic, the North and South Pacific Oceans, the Mediterranean Sea, and the Arabian Sea (20). Despite this success, very few pure cultures of *Prochlorococcus* (e.g., those of strains PCC 9511 and MIT 9313 [18, 22]) have been obtained. The vast majority of cultures contain heterotrophic microbes as contaminants; these heterotrophs were cocultured from the marine environment during the isolation procedure, which has relied thus far exclusively on liquid cultivation. While plating for contiguous lawns of *Prochlorococcus* has proven to be productive (15), attempts at colony formation (by pour plating or surface streak plating) have thus far met with significantly less success. Recovery efficiencies of the pour plating technique of 0.1 to 10% have been reported previously for some strains (15, 24), but this technique has yet to produce pure cultures (15). The inability to readily obtain clonal, pure cultures of *Prochlorococcus* has severely limited progress in the genetic and physiological analysis of this ecologically important lineage.

The “helper” phenotype of heterotrophic bacteria. Standard dilution streaking of contaminated *Prochlorococcus* cultures onto semisolid medium failed to produce axenic colonies. Colonies formed only within a visible mass of the contaminant heterotrophic bacteria; such masses appeared typically at the sites of the earliest, heaviest dilution streaks (data not shown). One interpretation of these results was that *Prochlorococcus* was able to grow only in the presence of the contaminating bacteria, perhaps because the bacteria provide a growth factor and/or remove an inhibitory factor. Coculturing with heterotrophic bacteria is required for the growth of some bacterial

isolates (9) and is known to improve the growth of dinoflagellates (2, 7), suggesting that a similar interaction may help *Prochlorococcus*. To test this hypothesis, a heterotrophic contaminant (designated EZ55) of a culture of the *Prochlorococcus* strain MIT 9215 was isolated on ProAC medium (75% Sargasso seawater [prefiltered with a 0.2- μ m-pore-size polycarbonate filter] supplemented with 17 g of AC Difco broth liter⁻¹, 800 μ M NH₄Cl, 50 μ M NaH₂PO₄, 1 \times Va vitamin mix [27], and 15 g of granulated agar liter⁻¹ [unless otherwise noted, all chemicals were from Sigma]) and tested for its ability to help *Prochlorococcus* grow on semisolid medium. The 16S rRNA gene of EZ55 was PCR amplified (8), cloned with the TOPO TA cloning kit (Invitrogen), and sequenced, identifying the strain as a member of the *Aleromonas* genus.

A lawn of 4.0×10^6 cells of strain EZ55 (prewashed in unsupplemented Sargasso seawater) was spread evenly onto each 60-mm-diameter Pro99 medium-based agar plate (containing Pro99 nutrients [15] plus 1 mM sodium sulfite [all filter sterilized separately], 0.42% Difco Noble agar [Becton Dickinson] in 18 M Ω water [autoclaved separately], and autoclaved Sargasso seawater [75% final concentration, vol/vol]) by using acid-washed, autoclaved 2.5-mm glass beads (Bio Spec). As the Pro99 plates contained no organic carbon supplement, any growth of the EZ55 cells on the plates was undetectable, and no visible lawn formed. A dilution series of a late-log-phase *Prochlorococcus* strain MIT 9215 culture was then applied to the plates, and the plates were incubated in transparent Ziploc bags under the standard incubation conditions for this study: 22°C with continuous light at 30 μ mol of quanta m⁻² s⁻¹, provided by cool white fluorescent bulbs. The plating efficiency was determined by comparing numbers of CFU to counts determined by flow cytometry, which quantifies *Prochlorococcus* particles based on size and chlorophyll-based fluorescence (4, 5). The flow cytometry count for the culture was 3.3×10^8 cells ml⁻¹, while counts of viable CFU (Fig. 1A) averaged $2.7 \times 10^8 \pm 1.6 \times 10^8$ CFU ml⁻¹. Thus, in the presence of EZ55, essentially every cell of MIT 9215 planted onto the agar formed a colony. No colonies formed on control plates lacking EZ55 (data not shown),

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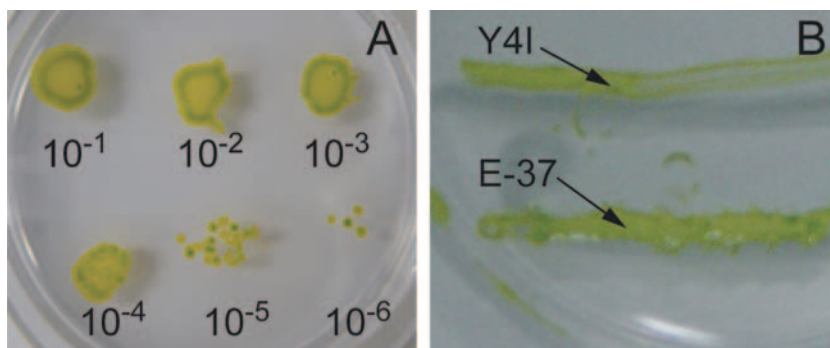


FIG. 1. (A) Dilution series of *Prochlorococcus* strain MIT 9215 on a dilute lawn of approximately 1,000,000 cells of EZ55 on Pro99 agar medium lacking organic carbon. Numbers above the dilution spots indicate the dilution (n -fold) of the source culture of 3.5×10^8 cells of MIT 9215 ml^{-1} ; a 10- μl volume was spotted for each dilution. (B) Approximately 10^6 *Prochlorococcus* strain MIT 9215 cells were spread evenly onto agar. Cells of heterotrophic roseobacters *Phaeobacter* sp. strain Y41 and *Sagittula stellata* E-37 were then applied over the MIT 9215 cells as horizontal streaks (indicated by arrows). MIT 9215 cells (green) grew only where the heterotrophs were streaked.

confirming that heterotrophs were necessary for the development of MIT 9215 colonies.

Elimination of the helpers to obtain axenic *Prochlorococcus* cultures. To utilize this method to obtain axenic cultures of MIT 9215, a genetic selection procedure was developed. We first obtained a streptomycin-resistant (Sm^r) mutant of MIT 9215 by inoculating approximately 10^{10} cells into 1 liter of Pro99 medium containing 100 μg of streptomycin ml^{-1} . The Sm^r culture that grew under these selection conditions was not pure, as Sm^r heterotrophs could be isolated if the culture was grown on ProAC rich-medium plates (data not shown). An axenic Sm^r MIT 9215 culture was then obtained by spread plating a dilution of the culture (approximately 100 Sm^r MIT 9215 cells per 60-mm agar plate) with $\sim 5 \times 10^5$ cells of wild-type, streptomycin-sensitive (Sm^s) EZ55. The wild-type EZ55 facilitated the growth of MIT 9215, which was spatially separated from the Sm^r contaminants on the plates. Green colonies were transferred with a sterile wooden toothpick or plastic pipette tip into 5 ml of liquid Pro99 medium. At the first visible sign of growth, the liquid cultures were diluted 64-fold into Pro99 medium containing 100 μg of streptomycin ml^{-1} to eliminate the EZ55 cells and, thus, establish a pure culture of MIT 9215. Due to the (low rate of) spontaneous mutation of EZ55 into an Sm^r strain, it was important to transfer as small a volume of cells as possible from Pro99 medium into Pro99 medium containing 100 μg of streptomycin ml^{-1} to ensure that all contaminants were killed upon exposure to streptomycin. Purity was confirmed by subculturing stationary-phase cultures in two types of purity test broth: a rich medium (1/10 ProAC [ProAC medium with only 1.7 g of AC Difco broth liter $^{-1}$]) and a minimal medium (PLAG [75% Sargasso seawater supplemented with 0.05% {each; wt/vol or vol/vol} sodium pyruvate, sodium lactate, sodium acetate, and glycerol; 800 μM NH_4Cl ; 50 μM NaH_2PO_4 ; 1 \times Pro99 trace metal mix; and 1 \times Va vitamin mix]). Cultures with no growth in the 1/10 ProAC or PLAG test medium after 4 weeks of incubation were considered to be axenic.

The helper phenotype in liquid medium. The helping phenomenon was found to occur in liquid medium as well as on semisolid medium. A dilution series of an axenic Sm^r MIT 9215 culture was inoculated into fresh Pro99 medium, and

chlorophyll-based fluorescence, quantified with a TD700 fluorometer (Turner Designs), was used to measure the relative *Prochlorococcus* cell concentrations during the incubation (28). At the highest cell inoculum tested, 3.5×10^6 cells ml^{-1} (as determined by flow cytometry), axenic Sm^r MIT 9215 colonies grew well as pure cultures (Fig. 2A), confirming that the helping phenomenon is not necessary for concentrated axenic cultures of *Prochlorococcus*. However, the growth of cultures from smaller inocula was severely limited. Cultures with an initial concentration of 3.5×10^5 cells ml^{-1} grew but exhibited variable and overall lower final yields of chlorophyll-based fluo-

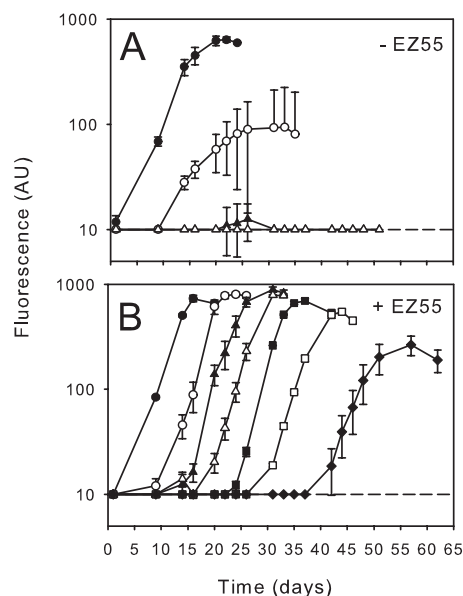


FIG. 2. Growth of MIT 9215 in liquid Pro99 medium with or without helper heterotrophs. Dilutions of MIT 9215 were grown in the absence (A) or presence (B) of $\sim 5 \times 10^5$ CFU of EZ55 ml^{-1} . Initial concentrations of MIT 9215 cells were 3.5×10^0 (\blacklozenge), 3.5×10^1 (\square), 3.5×10^2 (\blacksquare), 3.5×10^3 (\triangle), 3.5×10^4 (\blacktriangle), 3.5×10^5 (\circ), and 3.5×10^6 (\bullet) cells ml^{-1} . Error bars indicate one standard deviation of the mean for three replicate cultures. The dotted horizontal lines represent the limit of detection for chlorophyll-based fluorescence (10 arbitrary units [AU]); values below this limit were reported as 10 AU.

rescence than the cultures initiated with 10 times as many cells. Cultures from inocula of 35 to 35,000 cells ml⁻¹ failed to grow to detectable levels of fluorescence. Hence, the ability of pure cultures of Sm^r MIT 9215 to grow was strongly dependent upon the initial cell density.

In contrast, there was effectively no density dependence of the growth of Sm^r MIT 9215 when the strain was coinoculated with approximately 10⁶ cells of EZ55 ml⁻¹. From inocula of 35 to 3,500,000 cells ml⁻¹, growth was robust and highly reproducible: triplicate cultures exhibited the same growth rates and yields (Fig. 2B). The lowest initial cell dilution assayed, 3.5 cells ml⁻¹, was the one exception, producing cultures with a lower growth rate and maximum fluorescence yield. Nevertheless, all three cultures started at this very low inoculum grew to detectable levels of fluorescence by day 40 postinoculation. Thus, as on semisolid medium, the ability of initially low concentration cultures of Sm^r MIT 9215 to grow was dependent on the presence of heterotrophic helper bacteria.

The helping phenomenon is common in marine heterotrophs. To address the specificity of the helping interaction, one member of each ecotype (ecologically distinct lineage) of *Prochlorococcus* (1) was assayed with (i) marine heterotrophs isolated as described above for EZ55 from seven cultures of *Prochlorococcus* representing all six ecotypes, (ii) representatives of the *Roseobacter* clade, and (iii) bioluminescent species of the genus *Vibrio*. Several roseobacters have been shown previously to improve the growth of phototrophic dinoflagellates and algae (2, 7), suggesting that they may be able to perform a similar role for *Prochlorococcus*. A rapid screening assay was developed to facilitate the high-throughput *Prochlorococcus*-heterotroph interaction survey. In contrast to the prior plating method, the *Prochlorococcus* strain, rather than the heterotroph, was spread onto the plate as the dilute lawn (approximately 10⁶ cells from exponentially growing cultures per 60-mm-diameter plate). The *Prochlorococcus* was then overlaid with heterotrophs by being streaked with a wooden toothpick carrying a patch of cells from a colony. Accumulations of green biomass indicating *Prochlorococcus* growth occurred only where helpers were streaked (Fig. 1B). While the *Prochlorococcus* cultures tested in this assay were not axenic, the contaminants did not affect the assay, since *Prochlorococcus* grew only where the much denser test heterotrophs were streaked on top. For the low-light-ecotype strains NATL2A, SS120, MIT 9211, and MIT 9313 (16, 17), plates contained only 0.29% agar, as this concentration provided more robust growth than the 0.42% agar plates (data not shown).

The majority of heterotrophic strains tested helped *Prochlorococcus* strains MIT 9215, MED4, NATL2A, and MIT 9313 grow, although the amount of time required for visible cyanobacterial growth to appear varied extensively among both heterotrophic and *Prochlorococcus* strains (Table 1). In contrast, far fewer heterotrophic strains facilitated the growth of *Prochlorococcus* strains SS120 and MIT 9211. Additionally, the amount of time before growth was evident was typically greater for these strains. Only one heterotroph, *Silicibacter lacuscaeruleus*, universally helped all six strains of *Prochlorococcus* grow, although seven other heterotrophs (including all four *Vibrio* spp. tested) helped five strains. Heterotroph isolates from the *Prochlorococcus* cultures exhibited no preferential helping of the cognate *Prochlorococcus* strains over the other

Prochlorococcus strains tested, and not all isolates from the *Prochlorococcus* cultures were able to help the cognate strains. Hence, these initial studies indicate that the helping phenomenon appears to be common, though not universal, among all marine heterotrophs. Despite the relative crudity of this assay, it is nevertheless a significant observation that all strains of *Prochlorococcus* tested in this study—representing each of the six known ecotypes—were helped by at least four of the heterotrophic species surveyed.

The helping phenomenon is linked to oxidative stress. Heterotrophs appeared to play an active role in the facilitation of *Prochlorococcus* growth. EZ55 Sm^s cells helped Sm^r MIT 9215 form colonies on standard Pro99 plates (see above) but not on plates containing 100 μg of streptomycin ml⁻¹ (data not shown). Thus, streptomycin-killed cells were unable to facilitate MIT 9215 growth. One heterotroph activity we hypothesized to be important in the helping phenomenon is the scavenging of reactive oxygen species (ROS) from the medium. There is growing evidence that standard agar media contain levels of ROS that restrict the growth of many bacteria: the overall efficiency of plating of bacteria from marine, soil, and atmospheric environments can be dramatically improved by the introduction of a hydrogen peroxide (HOOH)-scavenging agent such as catalase or pyruvate to the medium (3, 10–12, 14, 19, 23). Of note, cells of the marine heterotroph *Vibrio vulnificus* that appear to have entered a “viable but nonculturable” state during exposure to low temperatures have lost their catalase activity (9) and can in fact be cultured if the medium contains catalase (3). In light of these findings of earlier studies, we found it intriguing that the genomes of *Prochlorococcus* strains lacked homologs of all known genes for catalases and heme-containing peroxidases (21). We therefore hypothesized that the removal of ROS from the medium by ROS-scavenging heterotrophic bacteria might be an important component of the helping phenomenon.

Our preliminary evidence supports this oxidative stress reduction hypothesis. Unlike its wild-type parent (ESR1, a derivative of ES114) (Table 1), a *katA* mutant (KV433) of *V. fischeri* that lacks the periplasmic catalase (26) was unable to help MIT 9215 grow on plates (data not shown). This finding indicates that catalase activity in this strain is necessary for the helping phenomenon. The *katA* mutant has no growth defects (as indicated by the rate or yield) in rich medium; indeed, the level of *katA* expression during logarithmic growth is low, but expression is induced by HOOH additions or entry into stationary phase (26). These results thus suggest that the elimination of extracellular HOOH by the periplasmic catalase of *V. fischeri* is a necessary component of this organism's helping phenotype. Direct tests for the sufficiency of purified catalase to help *Prochlorococcus* grow were complicated by the fact that catalase is vulnerable to photoinactivation (6, 13, 25). This loss of activity is likely very significant given the long incubation periods in the light that are required for growth on solid medium (1 to 2 months for colonies). Nevertheless, purified catalase showed a significant positive effect on the growth of dilute lawns of *Prochlorococcus*. Without catalase, an even spread of 10⁶ cells of MIT 9215 was unable to form a lawn on Pro99 semisolid medium. However, on plates containing 50, 100, and 200 U of catalase ml⁻¹, the same amount of cells grew robustly

TABLE 1. *Prochlorococcus* growth on plates streaked with heterotrophic bacteria^a

Organism	Accession no.	Catalase result ^k	No. of wks required for visible growth of <i>Prochlorococcus</i> strain:					
			MIT 9215	MED4	NATL2A	MIT 9211	SS120	MIT 9313
Coisolates of <i>Prochlorococcus</i> strains								
<i>Alteromonas</i> sp. strain EZ32 ^b	AF493971	±	1	3	—	—	—	—
<i>Rhodospirillaceae</i> strain EZ35 ^b	AF493974	±	2	3	3	—	—	—
<i>Alteromonas</i> sp. strain EZ33 ^c	AF493972	+	1	2	5	—	—	—
<i>Halomonas</i> sp. strain EZ34 ^c	AF493973	++	2	3	7	—	—	—
<i>Rhizobiaceae</i> sp. strain EZ36 ^c	AF493975	++	2	3	5	—	—	—
<i>Flavobacteriaceae</i> strain EZ40 ^d	EU591706	±	1	3	5	—	—	9
<i>Sphingomonas</i> sp. strain EZ41 ^d	EU591707	+	1	3	3	—	—	9
<i>Alteromonas</i> sp. strain EZ42 ^d	EU591708	±	2	3	5	—	—	9
<i>Marinobacter</i> sp. strain EZ43 ^e	EU704111	+	2	3	5	—	—	9
<i>Marinobacter</i> sp. strain EZ44 ^e	EU591709	±	5	3	3	—	—	—
<i>Alteromonas</i> sp. strain EZ45 ^f	EU591710	±	5	3	3	—	—	—
<i>Alcanivorax</i> sp. strain EZ46 ^f	EU591711	+	—	3	3	—	—	—
<i>Pseudoalteromonas</i> sp. strain EZ48 ^g	EU704112	±	5	3	—	—	—	—
<i>Rhodospirillaceae</i> strain EZ49 ^g	EU704113	±	2	3	1	—	9	7
<i>Alteromonas</i> sp. strain EZ55 ^h	EU704114	±	2	2	1	—	—	7
<i>Rhodospirillaceae</i> strain EZ54 ^h	EU704115	±	2	2	3	—	—	7
<i>Vibrio</i> spp. ⁱ								
<i>V. fischeri</i> ES114	CP000020	+	3	2	4	10	—	9
<i>V. fischeri</i> 7744	AY341436	+	3	3	5	10	—	9
<i>V. harveyi</i> B392	DQ068936	±	2	2	3	9	—	—
<i>V. loeigi</i> 15382	AY292932	+	2	3	3	14	—	9
Roseobacters ^j								
<i>Phaeobacter</i> sp. strain Y4I	AY457919	+	3	3	3	14	—	—
<i>Phaeobacter</i> sp. strain Y3F	AF253466	+	3	3	—	—	—	7
<i>Silicibacter lacuscaerulensis</i> ITI-1157	U77644	±	3	2	5	14	9	5
<i>Silicibacter</i> sp. strain TM1040	AY332662	±	3	—	—	—	—	5
<i>Silicibacter pomeroyi</i> DSS-3	AY457918	+	2	3	3	—	—	5
<i>Sagittula stellata</i> E-37	AF253465	±	2	5	5	—	9	7
<i>Sulfitobacter</i> sp. strain NAS-14.1	AY729963	+	3	—	—	—	—	5
<i>Sulfitobacter</i> sp. strain EE36	AY457920	++	2	4	3	—	—	5
<i>Sulfitobacter pontiacus</i> ChLG10	AY457921	+	3	4	5	—	9	5
<i>Rhodobacteraceae</i> strain SE62	AY038920	±	2	5	5	—	—	5
<i>Rhodobacteraceae</i> strain PSPC-2	AY149624	++	2	—	4	—	—	5
<i>Citricella</i> sp. strain SE45	AY457916	+	1	3	3	—	—	5
<i>Roseovarius nubinihibens</i> ISM	AY457917	+	2	2	6	—	—	5

^a Results of the streak plate assay show the number of weeks required for *Prochlorococcus* growth to become visible on at least two of three replicate plates streaked with the indicated heterotrophic bacteria. — indicates that no growth occurred within the allotted time period (16 weeks).

^b Isolated from a *Prochlorococcus* strain MED4 culture (S. Bertilsson, unpublished results).

^c Isolated from a *Prochlorococcus* strain MIT 9313 culture (Bertilsson, unpublished).

^d Isolated from a *Prochlorococcus* strain MIT 9312 culture (this study).

^e Isolated from a *Prochlorococcus* strain MIT 9211 culture (this study).

^f Isolated from a *Prochlorococcus* strain NATL2A culture (this study).

^g Isolated from a *Prochlorococcus* strain SS120 culture (this study).

^h Isolated from a *Prochlorococcus* strain MIT 9215 culture (this study).

ⁱ Source, P. Fidopiastis.

^j Source, A. Buchan.

^k Relative vigor of bubble formation upon exposure to HOOH. ++, immediate, vigorous bubbling; +, immediate, slower bubbling; ±, delayed, slow bubbling (<1 bubble s⁻¹).

into lawns (data not shown), demonstrating that catalase can significantly enhance the growth of *Prochlorococcus* on plates.

Clearly, HOOH scavenging is implicated as a mechanism of helping by *V. fischeri* and may play a similar role for the other helpers. All of the heterotrophs assayed for the ability to help *Prochlorococcus* grow also demonstrated visible signs of catalase activity (as judged by the “bubbling” of colonies upon exposure to 3 or 30% HOOH at 22°C) (Table 1). However, there was no clear correlation between the intensity of catalase activity and the ability of an organism to help; indeed, *Silicibacter lacuscaerulensis*, the only organism capable of helping all strains of *Prochlorococcus* tested, also had the lowest

catalase activity. Whether or not peroxidases (which do not form gas bubbles) substitute for catalase in the low-catalase-activity helpers is currently unknown, but we suspect that ROS scavenging may not be the only mechanism of helping. The apparent specificity of some helpers for certain strains of *Prochlorococcus* (e.g., EZ46) (Table 1) and the identification of only one universal helper among 33 candidate strains tested suggests that multiple factors are responsible for the helping phenomenon.

In this work, we have presented a two-step method whereby strains of *Prochlorococcus* may be made clonal and eventually pure. By plating the strains with heterotrophic helper bacteria,

isolated clones can be obtained as colonies, with plating efficiencies approaching 100%. Subsequently, the clones can be grown in liquid medium and the cultures can be made axenic by the addition of streptomycin to eliminate the helpers once the *Prochlorococcus* cells are dense enough to grow as a pure culture. This advance in our ability to grow *Prochlorococcus* colonies should dramatically enhance our ability to isolate new, clonal strains from the oceans and to improve the genetic manipulation of this organism, which has thus far been limited by the inability to isolate individual mutants (24). In addition, our preliminary evidence indicates that a wide range of marine heterotrophs can facilitate the growth of the different ecotypes of *Prochlorococcus*, with the reduction of oxidative stress as an important component of this helping phenomenon. Clearly, much work remains to be done in order to understand the mechanism(s) of this helping phenomenon and to determine what connection, if any, this interaction between heterotroph and phototroph plays in the oceans.

Nucleotide sequence accession numbers. The sequences from heterotrophic strains EZ40, EZ41, EZ42, EZ43, EZ44, EZ45, EZ46, EZ48, EZ49, EZ55, and EZ54 obtained in this study have been deposited in GenBank under accession numbers EU591706, EU591707, EU591708, EU704111, EU591709, EU591710, EU591711, EU704112, EU704113, EU704114, and EU704115, respectively.

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