Response of Prochlorococcus ecotypes to co-culture with diverse marine bacteria

RESPONSE OF *PROCHLOROCOCCUS* ECOTYPES TO CO-CULTURE WITH DIVERSE MARINE BACTERIA

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Abstract

Interactions between microorganisms shape microbial ecosystems. Systematic studies of mixed microbes in co-culture have revealed widespread potential for growth inhibition among marine heterotrophic bacteria, but similar synoptic studies have not been done with autotroph/heterotroph pairs, nor have precise descriptions of the temporal evolution of interactions been attempted in a high throughput system. Here, we describe patterns in the outcome of pair-wise co-cultures between two ecologically distinct, yet closely related, strains of the marine cyanobacterium *Prochlorococcus* and hundreds of heterotrophic marine bacteria. Co-culture with the collection of heterotrophic strains influenced the growth of *Prochlorococcus* strain MIT9313 much more than that of strain MED4, reflected both in the number of different types of interactions and in the magnitude of the effect of co-culture on various culture parameters. Enhancing interactions, where the presence of heterotrophic bacteria caused *Prochlorococcus* to grow faster and reach a higher final culture chlorophyll fluorescence , were much more common than antagonistic ones, and for a selected number of cases were shown to be mediated by diffusible compounds. In contrast, for one case at least, temporary inhibition of *Prochlorococcus* MIT9313 appeared to require close cellular proximity. Bacterial strains whose 16S gene sequences differed by 1-2% tended to have similar effects on MIT9313, suggesting that the patterns of inhibition and enhancement in co-culture observed here are due to phylogenetically-cohesive traits of these heterotrophs.

Keywords: heterotrophic bacteria/interactions/phylogeny/*Prochlorococcus*

Introduction

Interactions such as symbiosis, competition and allelopathy are a central feature of microbial communities (Azam and Malfatti, 2007; Bassler and Losick, 2006; Hibbing *et al.*). Even in dilute oceanic environments, microbial interactions abound: Antagonistic interactions can promote biodiversity (Bidle and Falkowski, 2004; Czaran *et al.*, 2002; Pernthaler, 2005), and synergistic interactions can provide sources of sustenance in complex communities (Amin *et al.*, 2009; Azam *et al.*, 1983; Azam and Malfatti, 2007; Boetius *et al.*, 2000; Croft *et al.*, 2005; Tripp *et al.*, 2010). While marine microbial interactions often occur on scales of nanometers or microns (Blackburn *et al.*, 1998; Malfatti and Azam, 2009; Seymour *et al.*, 2010; Stocker *et al.*, 2008), they ultimately affect entire ecosystems and global biogeochemical cycles (Azam and Malfatti, 2007).

Heterotrophic bacteria have been shown to both enhance and inhibit the growth of marine and freshwater algae (Grossart *et al.*, 2006; Grossart and Simon, 2007; Mayali *et al.*, 2008) and cyanobacteria (Bratbak and Thingstad, 1985; Manage *et al.*, 2000; Morris *et al.*, 2008) in liquid culture and on solid media. Through these and similar studies we have come to recognize specific mechanisms of interaction which can occur in the marine environment, such as facilitation of iron uptake (Amin *et al.*, 2009; D'Onofrio *et al.*, 2010), transfer of essential vitamins (Croft *et al.*, 2005), inter- and intra-specific communication (Bassler and Losick, 2006; Vardi *et al.*, 2006) and allelopathy (Hibbing *et al.*, 2009; Mayali *et al.*, 2008). Hypothesizing that bacterium-bacterium antagonistic interactions shape microbial community structure at the microscale, Long and Azam

(Long and Azam, 2001) analyzed interactions among 86 pairs of co-isolated marine bacteria on solid media, revealing the widespread distribution of the potential for growth inhibition among these bacterial strains (Grossart *et al.*, 2004; Long and Azam, 2001; Rypien *et al.*). More recently, several strains of heterotrophic bacteria have been shown to enhance the growth of a number of ecotypes of *Prochlorococcus* – the dominant phototroph in temperate and tropical oceans (Coleman and Chisholm, 2007; Partensky and Garczarek, 2010) - at low cell concentrations on solid and liquid media (Morris *et al.*, 2008). It was shown that the mechanism of enhancement in this case was the reduction of oxidative stress, explaining in part long-standing anecdotal observations that culturing *Prochlorococcus* is usually more robust when indigenous bacterial contaminants are present.

While *Prochlorococcus* have been extensively studied *vis-à-vis* the role of environmental factors such as light, temperature and nutrient availability in shaping their ecology (Bouman *et al.*, 2006; Coleman and Chisholm, 2007; Johnson *et al.*, 2006; Moore *et al.*, 2002; Moore *et al.*, 1998), and 'top down' processes such as predation and viral lysis have also been studied to some degree (Frias-Lopez *et al.*, 2009; Lindell *et al.*, 2007; Lindell *et al.*, 2005; Sullivan *et al.*, 2005), systematic studies of their interaction with heterotrophic bacteria are limited to that of Morris and Zinser (Morris *et al.*, 2008) described above, who focused on the growth-enhancing role of bacteria in low density cultures of *Prochlorococcus.* Inspired by this work, and by systemic analyses of Long and Azam (27), we undertook a broad- based and quantitative analysis of co-cultures of two axenic *Prochlorococcus* ecotypes (Moore *et al.*, 2005; Saito *et al.*, 2002) with

hundreds of diverse heterotrophic bacteria, examining the response of the *Prochlorococcus* cells to the presence of bacteria over the entire growth curve of the cultures.

We chose two strains of *Prochlorococcus*, one adapted to low light (MIT9313) and one adapted to high light (MED4), for these studies because they are ecologically and phylogenetically distinct. Additionally, MIT9313 is known to produce a diverse array of secondary metabolites of unknown function, whereas the genes encoding this system are absent in MED4 (Li *et al.*, 2010). We paired each strain with each of 344 strains of heterotrophic bacteria isolated from an oligotrophic marine environment. We asked: 1) How does the presence of added heterotrophic bacteria influence the growth of each *Prochlorococcus* strain over the course of its growth curve? 2) Do the two ecotypes respond differently to the presence of the same heterotroph? 3) Do different strains of heterotrophs have different effects, and are they related to the phylogeny of the heterotrophs? 4) Are the observed interactions mediated by soluble compounds or do they require close cellular proximity or contact?

While the experimental system does not mimic the natural environment in many ways (Sup Info), it reveals some fundamental differences between the responses of two *Prochlorococcus* ecotypes to co-culture with hundreds of bacteria - differences that may hold clues to factors governing their realized niches in the ocean. It further highlights a strong correlation of the outcome of co-culture with the phylogeny of the heterotrophic

bacteria, yielding hypotheses for further study on the mechanisms of these interactions and their potential role in marine microbial communities.

Results and Discussion

Differences between *Prochlorococcus* MIT9313 and MED4 in outcome of co-culture

To determine what kinds of interactions occur when *Prochlorococcus* is grown in co-culture with many different strains of bacteria, we constructed a "library" of 344 heterotrophic bacterial isolates from seawater collected in the open ocean, at the Hawaii Ocean Time Series (HOT) station ALOHA (22º45' N, 158º W) (Sup Fig. 1). The heterotrophic strains were isolated on solid media (see Sup. Info) and consist of at least 65 unique ribotypes (based on partial 16S rDNA sequences) clustering into 23, 13, 8 and 6 distinct OTUs at 1%, 3%, 5% and 7% rDNA sequence divergence, respectively (Sup Fig 1). The strains belong to the gamma-proteobacteria (primarily Alteromonas, Marinobacter and Alcanivorax) and alpha-proteobacteria (Rhodobacter) classes. Each of the 344 heterotrophic strains was inoculated into co-culture with axenic *Prochlorococcus* strains MED4 and MIT9313 in 96 well plates (under our conditions the outcome of co-culture does not depend on the initial number of heterotrophs inoculated – see Sup Info, Sup Fig 2). We measured the bulk *in-vivo* chlorophyll fluorescence (FL) of the cultures, which is widely used (Grossart, 1999; Malmstrom *et al.*, 2010; Mayali *et al.*, 2008) to follow the dynamics of phytoplankton cultures in a non-invasive manner. While FL is only proportional to cell number when the cultures are in balanced growth (Log phase,

see Sup. Info) the shape of the FL curve can reveal differences between the bulk behavior of the cultures throughout the culture period.

From the hundreds of co-cultures analyzed, only a few general types of co-culture outcomes emerged, as defined by the shape of the FL curves (Fig 1). Fifty seven percent of the MIT9313 co-cultures fell into the group described as "early" (green, Fig. 1B) as these cultures entered exponential growth earlier, and reached higher maximal fluorescence than the heterotroph-free MIT9313 cultures (Fig. 1C). A small fraction of the co-cultures (3%) displayed the same initial timing as the "early" group, but fluorescence stopped increasing at an early stage and then declined rapidly ("early arrested", purple Fig. 1B). Thirty four percent of the cultures stopped increasing in fluorescence after 2-3 days, declined to undetectable levels, and then increased again much later (the "late" group, red Fig. 1B). Finally, only 6 % of the co-cultures with MIT9313 behaved similarly to the heterotroph-free cultures ("intermediate" black Fig. 1B).

The synoptic response of MED4 to co-culture with the same library of bacterial strains was dramatically different from that of MIT9313. Ninety-eight percent of the heterotroph culture collection revealed no clear effect on the growth of MED4 – as evidenced by their "intermediate" growth patterns which are very similar to the heterotroph-free cultures. The growth of *Prochlorococcus* MED4 in the remaining 2% of the co-cultures was arrested early, displaying strong inhibition by the presence of these

heterotrophs (Fig 1B). The heterotrophic bacterial strains that inhibited MED4 were the same strains that defined the "early arrested" group in the MIT9313 cultures.

Quantifying the parameter space of the MED4 and MIT9313 co-culture outcomes

To provide a quantitative estimate of the effect microbial interactions can have on *Prochlorococcus* culture dynamics, we extracted from the FL curves shown in Fig. 1 biologically-relevant descriptive parameters (similar to those used by Warringer et al (Warringer *et al.*, 2008)): the maximum growth rate (μ), the time it took the cultures to reach half of the maximal fluorescence (T_{50}), and the maximum FL (F_{max}). As was clear in the qualitative analysis, the parameter space is not homogenously covered (Fig 2; Suppl. Figs 3,4). Rather, parts of the parameter space are densely populated, whereas others regions are empty or sparse, representing parameter combinations which are not observed in our co-culture curves (e.g. co-cultures in which the log phase growth rate was significantly reduced compared to heterotroph-free cultures).

While the growth rate in log phase was influenced by the presence of bacteria in most of the MIT9313 co-cultures, the median of this parameter actually increased in most of the types of co-culture outcomes compared to the heterotroph-free cultures (Sup Fig. 3) even when the overall effect was clearly one of much later onset of growth. Therefore, in agreement with other studies (Warringer *et al.*, 2008), our results suggest that a combination of different growth parameters is necessary in order to fully describe the complex effect of microbial interactions.

As described above, the most striking is the difference between the large parameter space inhabited by MIT9313 co-cultures and the much more limited space inhabited by MED4 co-cultures (Fig. 2). The suite of heterotrophic bacteria that strongly influences the growth of MIT9313, decreasing some parameters up to ten-fold or increasing them up to four-fold has minimal, if any, impact on MED4.

Heterotroph phylogeny and co-culture outcome.

We next asked whether closely related bacteria, as defined by their partial 16S rDNA sequence (ribotype), affect the growth of *Prochlorococcus* cultures similarly. As shown in Fig. 3, the heterotroph ribotypes which induced "early", "early arrested" and "late growth" phenotypes were significantly different for MIT9313 (UniFrac test with Bonferroni correction, $P \leq 0.06$ [Lozupone and Knight, 2005]), as were the groups that induced "intermediate" and "early inhibited" for MED4 ($P \leq 0.01$). For example, all but two of the heterotrophic strains which induced a "late" outcome of MIT9313 belong to two well-defined clades of Alteromonads (Fig. 3, Sup Fig. 1). Similarly, the same strains induced the "early arrested" outcome in both MED4 and MIT9313, and all of these strains belong to a well-defined clade of Rhodobacters, similar to *Marinovum algicola* and *Ruegeria sp*. In most of these cases, the differentiation between strains which inhibit *Prochlorococcus* in co-culture and strains which do not is relatively deep-rooted, within the resolution afforded by our cultured collection of heterotrophs. For example, two Alteromonad clades differing by 1-2% in their partial 16S sequence both inhibit MIT9313, whereas a third clade which differs by 4-5% from these two clades enhances MIT9313. Similarly, the clade of Rhodobacters inducing "early arrested" phenotype

differs from the most closely related strains in our collection that do not induce this phenotype by about 4% in their 16S. This level of divergence corresponds to one commonly used to delineate species or genus level differentiation (Schloss and Handelsman, 2005).

Co-culture outcome and proximity of cells

While many interactions between microorganisms are mediated by diffusible soluble compounds, some have also been observed to occur when cells live in close proximity or even necessitate direct cell-cell contact (Croft *et al.*, 2005; Mayali and Azam, 2004). To test whether close cell-cell proximity is necessary for the different co-culture outcomes observed with MIT9313, we selected five heterotroph strains representing different phylogenetic clades and co-culture outcomes, and co-cultured them with MIT9313 either separated by a membrane permeable to small molecules or mixed together as in the experiment presented above. As shown in Fig. 4, when the FL of the co-cultures increased earlier than that of the axenic cultures this happened regardless of whether or not the heterotrophic bacteria were separated from MIT9313 by a membrane. Thus, the "early" outcome of *Prochlorococcus* cultures is likely mediated in these cases by soluble, diffusible compounds, although we cannot preclude the possibility that the small number of heterotrophic bacteria than can cross the membrane during these 19-day long experiments (see Sup Info) may also directly impact the growth of MIT9313. In contrast, the late co-culture outcome occurred only when MIT9313 and Alteromonas strain HOTo1A3 were grown in close proximity and not when they were separated by a membrane.

Potential mechanisms underlying different co-culture outcomes

MIT9313 and MED4 represent two taxonomic extremes within the *Prochlorococcus* lineage, differing by ~3% in their 16SrRNA sequence. MED4 is a small cell with a highly streamlined genome, and is a member of the high-light adapted clade of *Prochlorococcus.* MIT9313, in contrast, is a slightly larger cell with a larger genome, and is better adapted for growth at the low light levels found deeper in the water column (Bouman *et al.*, 2006; Coleman and Chisholm, 2007; Johnson *et al.*, 2006; Moore *et al.*, 2002; Moore *et al.*, 1998; Rocap *et al.*, 2003). Both strains are growing in these experiments below their respective temperature and light optima (although closer to those of MIT9313, (Rocap *et al.*, 2003; Zinser *et al.*, 2007)), but have been pre-acclimated to the experimental conditions for >7 months (~120 generations) and thus the difference in co-culture outcome is likely not caused by a general stress response in one strain due to culture conditions.

The "early" culture outcome is the one most commonly observed with MIT9313, is widely distributed among the different phylogenetic groups, and in all cases tested is caused by soluble, diffusible molecules. This is consistent with a "helper" effect where the growth of *Prochlorococcus* increases as a result of basic attributes common to many lineages of heterotrophic cells, as suggested by Morris et al (Morris *et al.*, 2008). Such attributes may include scavenging of reactive oxygen species, (Morris *et al.*, 2008), increasing carbon dioxide concentration (Moore *et al.*, 2007) or cycling waste products.

MED4 as a high-light adapted strain, may be better adapted to deal with oxidative stress (often generated during photosynthesis) than MIT9313, thus the latter strain may benefit more from interacting with heterotrophs. Notably, however, MED4 can readily form colonies on solid media only with the help of heterotrophs, and thus this strain is not immune to the effect of co-occurring bacteria (Morris *et al.*, 2008).

In contrast, inhibition of MIT9313 (early arrested or late outcomes) was observed mainly in co-cultures with two-well defined groups of bacteria belonging to the Alteromonads and Rhodobacters, with the latter group being the only one to clearly affect the growth of MED4 under our conditions. Related bacteria have previously been shown to inhibit other microbes through the production of secreted allelochemicals (e.g. (Gram *et al.*; Mayali and Azam, 2004)). An intriguing observation is that inhibition of MIT9313 by an Alteromonas strain required proximity between the heterotrophic bacteria and MIT9313 – i.e. the effect could not be mimicked when the cells were kept apart by a semi-permeable membrane. Recently, close physical association (cell-cell contact) has been observed in natural seawater samples between *Synechococcus* cells, which are closely related to *Prochlorococcus*, and heterotrophic bacteria of unknown taxonomy (Malfatti and Azam, 2009; Malfatti *et al.*). These observations suggest the potential for close or contact-mediated interactions even in tiny picoplankton cells.

Conclusions

While some features of our experimental system limit extrapolation of our results to the experience of wild *Prochlorococcus* – e.g. the co-cultured strains were not co-isolated and the cell densities were higher than found in the wild (see also Sup Info) – our study has revealed some properties of these microbial interaction that likely have ecological relevance. First, the two *Prochlorococcus* ecotypes display fundamentally different responses to the presence of bacteria, both in terms of general patterns, and in terms of specific responses to specific bacterial strains. These differences could influence the connectivity of these two strains within the microbial network in the wild. If so, MIT9313 may be more susceptible to changes in the microbial community than MED4. Similar trends have been suggested for other marine bacterioplankton based on network analysis of patterns of co-occurrence in the oceans (Fuhrman and Steele, 2008).

Second, both the antagonistic and enhancing interactions in our system revealed a clear phylogenetic signature, with closely related bacteria causing similar responses in the co-cultured *Prochlorococcus*. Furthermore, only a handful of different interaction types, as measured through their effect on *Prochlorococcus* growth curves, were observed. The heterotroph culture collection we used represents only a fraction of diversity found in the oceans, and does not include many of the most common lineages. Future work with a wider diversity of bacteria may either reveal additional types of interactions or highlight unknown constrains on the types of interactions which can affect cells in the aquatic environment.

Considering the high levels of microheterogeneity in both marine microbial populations (Hunt *et al.*, 2008; Thompson *et al.*, 2005) and their environment (Azam and Malfatti, 2007; Blackburn *et al.*, 1998; Seymour *et al.*, 2010; Stocker *et al.*, 2008), the task of understanding how complex microbial populations interact in the oceans is a daunting one. Although it is encouraging, as we seek general patterns, that the co-culture outcomes we observe are not random with respect to the phylogeny of the heterortophs, the opposite has been observed in cultures of interacting heterotrophc bacteria (Long and Azam, 2001). Clearly expanded and in depth study of the network of possible interactions between microbial groups is essential, if we ever wish to incorporate microbial interactions into our understanding of marine microbial communities.

Materials and Methods

We isolated heterotrophic bacteria from the Hawaii Ocean Time Series (HOT) station ALOHA (22º45' N, 158º W), one of the most comprehensively studied sites in the ocean, with a microbial community dominated by *Prochlorococcus* and characterized in some detail (DeLong *et al.*, 2006). The heterotrophs were re-streaked for purity three times, and the final library was preserved at -80°C in 25% glycerol. *Prochlorococcus* strains MIT9313 and MED4 were isolated from the Gulf Stream and the Mediterranean Sea, respectively (Rocap *et al.*, 2003), and were maintained in the lab at 20°C and 27μE constant cold white illumination. Co-culture was initiated by adding 2μL of an overnight culture of each heterotroph from the library to 200μL of *Prochlorococcus* culture (10^6 cells/mL) in 96 well plates. The culture media was Pro99 (Moore *et al.*, 2007) with the

addition of 0.01% w/v Pyruvate, Acetate, Lactate and Glycerol as well as a vitamin mix (Morris *et al.*, 2008). The co-culture plates were maintained for 42 days at 20°C and 27μE constant cold white illumination, and the bulk chlorophyll fluorescence (ex440 em680) measured almost daily using a Bio-Tek Synergy HT plate reader. The resulting curves were filtered to retain consistent curves, defined as those in which the Euclidian Distance between normalized curves fell within the range defined by 95% of the between-plate replicates of axenic curves. The growth parameters were extracted from the growth curves using macros written in Excel VBA, which are available from the authors upon request. Hierarchical Clustering was performed in Matlab. For detailed materials and methods see Supplementary Information.

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Titles and legends to figures:

Figure 1: Features of *Prochlorococcus* MED4 and 9313 growth patterns in response to co-culture with 250 different strains of heterotrophic bacteria. A) Heat maps of the normalized FL of all 338 growth curves (250 co-cultures and 88 controls) as clustered using Hierarchical Clustering (HC). A clearly different pattern can be seen between four major clusters in MIT9313 but only two in MED4. **B and C) FL curves of the 250 co-cultures (B) and 88 axenic *Prochlorococcus* cultures (C).** The curves are colored as shown in the legend based on the clustering results in panel A. Four different types of curves which differ in their growth timing and maximal FL, can be observed for MIT9313, whereas only two clusters are observed for MED4. Note the similarity in the shape of the "early arrested" outcome between MIT9313 and MED4.

Figure 2: The quantitative three-dimensional parameter spaces defining the effect co-culture on *Prochlorococcus* MIT9313 and MED4. A three-dimensional parameter space is shown, with the axes being the maximum growth rate (μ), the time it took the cultures to reach half of the maximal fluorescence (T_{50}), and the maximum FL (F_{max}). The parameter spaces shown includes both the co-cultures and the control axenic cultures, and are normalized to axenic wells on the same plates (i.e. values larger than one represent an increase in the relevant parameter compared to axenic culture, smaller than one represent a decrease). The data points are colored based on the clustering shown in Fig 1. Large circles represent the median coordinates of each co-culture outcome.

Figure 3: The relationship between patterns observed in co-cultures and the ribotype of the heterotroph. A maximum likelihood (ML) tree (partial 16S rDNA sequences) is shown, with the co-culture outcome (as defined in Fig. 1) shown for MIT9313 (middle ring) and MED4 (outer ring). Spheres on the tree branches denote >80% aLRT confidence (Anisimova and Gascuel, 2006). Different shading of the branches of the tree denotes Operational Taxonomic Units (OTUs) at 0.01 resolution (see also Sup Fig. 1 and Sup Table 1). Asterisks denote the phylogenetic position of strains used for the experiments shown in Fig. 4.

Figure 4: **Comparison of outcomes of co-culture experiments between *Prochlorococcus* MIT9313 and 5 strains of bacteria grown together in mixed culture or separated by a 0.4 µm permeable membrane.** Results shown are averages and standard deviations (n=4 for axenic MIT9313, n=2 for co-cultures). The phylogenetic positions of the strains used in this experiment are shown in Fig. 3 as asterisks.

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Supplementary Information

Detailed Materials and Methods

General considerations and caveats: The experimental system described here was designed in order to enable the quantitative analysis of hundreds of cultures in parallel. It does not mimic the natural oceanic environment in many ways: the concentrations of nutrients and cell densities were significantly higher than in the oligotrophic ocean (see below for media composition); the heterotrophs were supplemented with organic compounds in the media, and not dependent on the phototrophs for sustenance; the light intensity was constant, and the diffusion of gasses was likely limited in the 96 well plates. The heterotrophic strains used are those which are relatively easily isolated on solid medium, and do not represent the majority of bacteria in the oceans (although Alteromonads and Rhodobacters are an important part – up to 10% - of the total marine microbial population, as measured using metagenomic methods (DeLong *et al.*, 2006; Ivars-Martinez *et al.*, 2008)). Finally, batch cultures were used by necessity, whereas the ocean environment is more like a chemostat. It is important to recognize these caveats (and others, described below) when interpreting the results of the described experiments.

We isolated heterotrophic bacteria from the Hawaii Ocean Time Series (HOT) station ALOHA (22º45' N, 158º W), one of the most comprehensively studied sites in the ocean, with a microbial community dominated by *Prochlorococcus* and characterized in some detail (DeLong *et al.*, 2006). The bacteria were isolated on a relatively nutrient-poor seawater-based media, ProMM, which is similar to PLAG (Morris *et al.*, 2008) but using 100% rather than 75% seawater. This media is based on the Pro99 media used to culture *Prochlorococcus* (Moore *et al.*, 2007), with the addition of a set of defined organic compounds (0.05% w/v each of Lactate, Pyruvate, Acetate and Glycerol) and vitamins (Waterbury and Willey, 1988)). We chose to use this media because growth of *Prochlorococcus* from low cell numbers in 96 well plates is much more robust with the addition of low concentrations (1:20 v/v) of ProMM in Pro99 (manuscript in preparation). Thus, we could perform the co-culture in diluted ProMM, providing for robust growth of both *Prochlorococcus* and the co-cultured heterotroph.

Several methods are used traditionally to measure growth rates of phytoplankton cultures, including bulk fluorescence (Grossart, 1999; Malmstrom *et al.*, 2010; Mayali *et al.*, 2008; Osburne *et al.*, 2010), optical density at 600nm (OD600, (Bruckner *et al.*, 2008)), chlorophyll concentration (Bruckner *et al.*, 2008) and cell number (Grossart and Simon, 2007). We chose to measure bulk chlorophyll fluorescence (ex440 em680) (FL) since it is widely used and the easiest to measure in a non-invasive, high throughput manner. During steady-state, balanced growth, bulk fluorescence is linearly correlated with *Prochlorococcus* cell numbers (R^2 = 0.82 for MIT9313 and 0.97 for MED4, see also (Malmstrom *et al.*, 2010; Osburne *et al.*, 2010)). During lag, stationary and decline phases, however, FL is no longer correlated with cell number and thus can only be used to compare the state of one culture with respect to another. We extracted three biologically-intuitive parameters of the fluorescence curve: growth rate, growth time and

maximal fluorescence. Briefly, the growth rate, μ, was defined as the maximal slope of a linear regression, performed on Log_{10}-transformed fluorescence data, in a sliding window of four consecutive data points within the exponential growth phase. The growth time, T_{50}, was defined as the time it took the bulk culture fluorescence to reach 50% of the maximal fluorescence, F_{max}, value for that curve.

In order to simplify the experimental setup, the same volume of heterotrophic bacteria was added into each co-culture well. Thus, the number of heterotrophs initially inoculated into co-culture differs between different heterotroph strains. However, the outcome of co-culture in our experimental system does not depend on the initial density of heterotrophic bacteria added, even when this density varied over six orders of magnitude (sup Fig. 2). This was observed for 8 out of 9 strains tested in co-culture with MIT9313 and for all 8 strains tested in co-culture with MED4. The lack of dependence on initial heterotroph density is likely due to the significantly higher growth rates of the heterotrophs in this media, which reach stationary growth ("quasi steady state") typically after 1-2 days compared to 7-10 days for *Prochlorococcus* (Sup Fig. 2B, D, F).

While heterotrophs were inoculated into each well of the co-culture plates, occasionally wells were observed in which the inoculated heterotrophs did not visibly grow. If indeed no heterotrophs grew in these wells this could lead to the *Prochlorococcus* revealing a similar pattern as axenic cultures (e.g. "intermediate" response, Fig. 1). To bound how important this issue was, we monitored in parallel to the bulk culture fluorescence the turbidity (OD_{600}) for all co-cultures. While these turbidity measurements cannot allow quantitative estimation of the heterotroph numbers, they do enable us to roughly determine whether heterotroph growth occurred in a specific well, based on a sharp increase in turbidity after on a different timescale from that caused by *Prochlorococcus*. In 94% of the co-cultures with MIT9313 and 91% of the co-cultures with MED4, clear heterotroph growth was observed after 1.5 days, defined as an OD600 value of >0.086, and these included co-cultures revealing all of the different effects on *Prochlorococcus* (Early, Early arrested, Intermediate and Late growth). Of the remaining cultures, in the case of MIT9313 more than 2/3 revealed an "Early" phenotype. Thus, while we cannot rule out that some of "indifferent" co-cultures were due to a failed heterotroph inoculation, this possibility affects at most 2% of the MIT9313 and 9% of the MED4 co-cultures, and does not affect the overall conclusions of this study.

Isolation of heterotrophic bacteria from ALOHA: Seawater was collected during the C-MORE BLOOMER cruise on August 18, 2007, transported back to the lab and maintained for one month in clear polycarbonate bottles at 21ºC under 18 μE constant cold white illumination. 1-100μL of seawater were spread on ProMM agar plates, and colonies picked as they appeared over a period of 16 days into liquid ProMM. Colonies were further re-streaked three times for purity on ProMM plates, and the final library was maintained at -80°C in 25% glycerol.

PCR amplification and sequence analysis of the 16S gene: PCR amplification was performed from 2µl of the bacteria in ProMM using the universal bacterial primers 8F and 1492R and sequenced using Sanger sequencing. Several samples which did not amplify in several attempts using these primers were amplified using the ReaX kit (Q Chip, Cardiff, UK, utilizing 27F and 905R primers). Sequence traces were analyzed in CodonCode Aligner (CodonCode Corporation, Dedham, MA, http://www.codoncode.com/aligner/download.htm), with the poor quality segments of the sequences removed using clip parameters set to "maximize region with error rate below 0.01". Reads with fewer than 200 consecutive high quality base pairs were also removed from the dataset. 257 sequences (Sup Fig. 1) which passed the quality threshold were analyzed using GreenGenes ((DeSantis *et al.*, 2006a; DeSantis *et al.*, 2006b), http://greengenes.lbl.gov). The sequences were aligned to the Core Set of aligned sequences using NAST (minimum length set to 250) to produce a set of aligned 7682-character sequences (DeSantis *et al.*, 2006b), and were checked for chimeric sequences using Bellerophon 3.0 (600bp Core set threshold and 200bp window size) (DeSantis *et al.*, 2006a). The nearest neighbors and nearest isolates were determined using Simrank, and sequence divergence from near-neighbors calculated using the DNAML option of DNADIST (PHYLIP package, all performed within GreenGenes) with Lane Mask restricting calculation to 1,287 conserved columns, the A, C, G, T 16S rRNA base frequency being 0.2537, 0.2317, 0.3167, 0.1979, respectively, and the Transition:Transversion Ratio assumed to be 2.0. The phylogenetic classification is shown using the Hugenholz Genus classifier. Operational Taxonomic Unites (OTUs) were estimated using DOTUR (Schloss and Handelsman, 2005) using the furthest neighbor algorithm and a precision value of 1000. The phylogenetic trees in Fig 3 and Sup Fig. 1 were created using PhyML (model=HKY, estimated t, v and a, (Guindon and Gascuel, 2003)) with aLRT test for branches (Anisimova and Gascuel, 2006) and visualized using iTOL ((Letunic and Bork, 2007), http://itol.embl.de/). Percent divergence in the 16S gene sequences reported throughout this manuscript were based on BLAST2seq comparisons performed at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the default parameters. The sequences have been submitted to the GenBank under accession numbers HQ537134-HQ537390.

Co-culture setup and parameter extraction: *Prochlorococcus* strains MIT9313 and MED4 were maintained at 20°C and 27µE constant cold white illumination. Under these conditions the two strains grow at comparable rates (µ=0.32±0.04 for MIT9313, 0.43±0.01 for MED4, growth rate µ is described using base e). Duplicate mid-exponential cultures were counted by using an Influx flow cytometer (BD biosciences), diluted to a final cell density of 10^6 cells/ml in 5% ProMM in Pro99 and distributed into the wells of 96 microtitre plates. Duplicates of the heterotroph library were inoculated into 5% ProMM in Pro99 24 hours prior to the initiation of the experiment, and 2µL of the heterotroph plates were used to inoculate the co-culture plates. The outer wells of the 96 well plates were not used for the experiments as growth in these wells is less robust, possibly due to a higher evaporation rate. Each of the 96 well plates used in this experiment had at least 12 control wells (columns 2 and 11), in which *Prochlorococcus* was grown axenically, and 48 co-culture wells. The co-culture plates were maintained for 42 days at 20°C and 27µE constant cold white illumination, and the fluorescence

(ex440 em680) read almost daily using a Bio-Tek Synergy HT plate reader. The culture parameters described above were extracted from the FL curves using several macros written in Excel VBA, which are available from the authors upon request.

Selecting consistent curves and hierarchical clustering: The 96 well plates used for the experiment contained 344 heterotroph strains, of which 257 had their 16S sequences determined (Sup Fig 1B). We next removed from the study co-cultures in which the two biological duplicates were not consistent. We defined a consistent pair of fluorescence-based growth curves as one in which the Euclidian distance between the normalized curves is within the range defined by 95% of the between-plate pairs of axenic curves (Sup Fig 5). Using this definition, 94.4% of the MED4 and 77.9% of the MIT 9313 duplicate curves were consistent, and we from the 344 original strains retained for further analysis 250 strains which were consistent in co-culture with both *Prochlorococcus* strains. These include 180 strains for which partial 16S sequences was determines, and from which the phylogenetic tree in Fig. 3 was constructed. Duplicate curves deemed to be consistent and the parameters extracted from these curves were averaged. For Hierarchical Clustering, each averaged fluorescence curve was first normalized to a vector of length one. We then applied standard Hierarchical Clustering using Matlab (UPGMA linkage (Sokal and Michener, 1958)) using the default Euclidian distances and a threshold for determining the clusters of 1 for the large experiment and 0.7 for the smaller one described below. Similar results were observed in both the clustering of fluorescence curves and in the phylogenetic analysis of the outcomes both with and without the filtered "inconsistent" curves.

Testing the robustness of the observed patterns across different experiments: To test the consistency of the general pattern of interactions observed, we randomly selected a subset of 96 strains from the heterotroph library and performed a second co-culture experiment under similar conditions several months later. Similar patterns emerged in the second experiment, including the main different co-culture outcomes for MIT9313, the striking lack of effect of co-culture on MED4, and the dependence of the effect on the phylogeny of the heterotrophs (sup Fig. 6). These results show that the pattern of interactions observed across the collection of bacteria sampled, as well as the specific effect of each heterotroph strain, is highly robust.

Co-cultures separated by membranes: To test whether diffusible compounds mediated the interactions we observed, or whether close cellular proximity was needed, we performed co-culture experiments in 6-well plates containing "ThinCert" tissue culture inserts containing a transparent 0.4μm PET membrane (Greiner Bio-one). These inserts allow each well to be partitioned into two compartments separated by a porous membrane, which enables the rapid exchange of small molecules between the compartments (~95% exchange of the dye phenol red within 27 hours) but blocks almost completely the exchange of bacteria (<0.1% exchange of either *Prochlorococcus* or Alteromonas HOTo1A3 (Fig 4) in 1:20 ProMM after 24 hours, as counted by flow cytometry). The 6-well plates and tissue-culture inserts were acid washed for 15 minutes, washed extensively with sterile mili-Q water and sterilized by UV irradiation (5 minutes

using a Stratagene UV cross-linker). Each compartment contained 3.5mL of 5% ProMM in Pro99. *Prochlorococcus* MIT9313 was inoculated at 2×10^6 cells/mL into the interior compartment, and 35μL of the appropriate heterotroph strain (from an overnight culture) were inoculated either together with MIT9313 in the interior compartment or in the exterior compartment, separated by the membrane from MIT9313. The 6-well plates were maintained for 19 days at 20°C and 27μE constant cold white illumination. The fluorescence (ex440 em680) was read almost every two days a Bio-Tek Synergy HT plate reader using a 9×9 matrix per well, the middle 32 cells were averaged, and the reading from a cell not containing any *Prochlorococcus* (blank cell) subtracted to produce the final fluorescence value.

Titles and legends to supplementary figures:

Sup Fig 1: Composition and diversity of the heterotroph library used for co-culture experiments with *Prochlorococcus*. A) Phylogeny of the heterotroph library and the outcome of co-culture with *Prochlorococcus*. A maximum likelihood (ML) tree is shown built from the partial 16S rDNA sequences of 257 library strains and 13 of cultured reference strains. Spheres on the tree branches denote >80% aLRT confidence (Anisimova and Gascuel, 2006). The leaves are colored according to the genus level identity, and with black boxes showing Operational Taxonomic Units at 0.01 resolution calculated using DOTUR (Schloss and Handelsman, 2005). The results of the co-culture experiments are shown as in Figure 3. Strains which did not produce consistent curves in replicate experiments are shown in white. For the Alteromonad reference strains, the location where they were isolated is shown in parentheses to stress the global distribution of these strains. The insert shows the bacterial classes represented in the library (highlighted in red) in the context of a global phylogenetic tree ("Tree of life" downloaded from iTOL (Letunic and Bork, 2007), http://itol.embl.de/)). Cyanobacteria (the phylum including *Prochlorococcus*) are highlighted in green. **B) Order level composition of the heterotroph library**, determined using the Hugenholz Genus classifier in GreenGenes (DeSantis *et al.*, 2006a; DeSantis *et al.*, 2006b). Unknown means that either no PCR amplicon, or good quality sequence, was obtained in several reactions using the16S primers described in the materials and methods. **C) Number of Operational Taxonomic Units (OTUs) as a function of 16S rDNA gene sequence divergence.** There are 257 sequenced heterotroph strains in the library, corresponding to 65 distinct partial 16S sequences. These cluster into 23, 13, 8 and 6 distinct OTUs at 1%, 3%, 5% and 7% rDNA sequence divergence, respectively.

Sup Fig 2: The outcome of a co-culture as a function of initial density of heterotrophs. Ten-fold serial dilutions of three selected heterotroph strains (each representing one of the co-culture outcomes shown in Fig. 1), spanning 5-6 orders of magnitude, were co-inoculated with duplicate *Prochlorococcus* MIT9313 cultures. The left panels (A, C, E) show fluorescence curves, the right panels (B, D, F) show OD_{600} curves. The purpose of the OD_{600} curves is to assess the growth of the heterotrophs: during the first few days of co-culture the increase in OD_{600} is caused only by heterotroph growth, as *Prochlorococcus* cells numbers remain low and no such increase in OD_{600} is seen in axenic *Prochlorococcus* cultures. A second increase in OD_{600} mirrors the increase in culture fluorescence and is likely due to the increase in *Prochlorococcus* cell numbers. Green curves are axenic controls, colored curves show co-cultures at different serial dilutions (dark-more heterotrophic cells). Results show mean and range of duplicates. No clear difference could be seen between the outcomes of the co-cultures at different initial heterotroph densities. The growth rates of the heterotrophic strains, as calculated from the OD_{600} curves during the initial four days of co-culture, were 1.44±0.1 for Alteromonas HOTo3B7, 1.19±0.1 for Alteromonas HOTo1A3 and 1.01±0.1 for Roseobacter HOTo5H2, significantly higher than the growth rate of MIT9313 as determined using culture fluorescence (0.28-0.40 calculated during the MIT9313 exponential stage and depending on the heterotroph strain). These data support our interpretation that upon

initiation of co-culture the heterotrophic bacteria grow much faster than *Prochlorococcus* and reach a quasi-steady-state which does not depend on the initial number of cells. **A, B) Early Growth** (*Alteromonas* HOTo3B7,dilution range 2-2x10^5 cells/mL), **C, D) Late Growth** (Alteromonas HOTo1A3, dilution range 5-5x10^5 cells/mL), **E, F) Intermediate Growth** (Roseobacter HOTo5H2, dilution range 0.24-2.4x10^4 cells/mL).

Sup Fig. 3: Details of the co-culture parameter space for MIT9313. For each culture (shown by a single curve in Fig. 1) three parameters were measured: max growth rate μ, growth time T_{50} and maximal fluorescence F_{max}. Histograms are shown of these three parameters in all of the cultures belonging to a specific co-culture outcome (Early growth, intermediate growth, late growth, as defined in Fig. 1) as well as in the MIT9313-only cultures. X-axis = relative parameter value, Y-axis = number of cultures. Within each histogram the median +/- one standard deviation are written.

Sup Fig. 4: Details of the co-culture parameter space for MED4. For each culture (shown by a single curve in Fig. 1) three parameters were measured: max growth rate μ, growth time T_{50} and maximal fluorescence F_{max}. Histograms are shown of these three parameters in all of the cultures belonging to a specific co-culture outcome (Early growth, intermediate growth, late growth, as defined in Fig. 1) as well as in the MED4-only cultures. X-axis = relative parameter value, Y-axis = number of cultures. Within each histogram the median +/- one standard deviation are written.

Sup Fig. 5: Estimating the consistency of duplicate co-cultures. Histograms are shown of the Euclidian distance between pairs of axenic cultures (upper panels) and co-cultures (lower panels). Pairs of control cultures were defined as those controls found in corresponding wells from duplicate plates. The dashed line represents the 95% cutoff used to determine which co-cultures were consistent. 94.4% of the MED4 cultures and 77.9% of the MIT9313 co-cultures from the large experiment were deemed to be consistent using these criteria.

Sup Fig. 6: Reproducibility of general patterns observed using a smaller, randomly selected group of heterotrophs in co-cultures. A second set of co-culture experiments was performed with 96 randomly-selected strains from the heterotroph library, several months after the experiments shown in Figs 1-3. **A) Heat maps of the normalized bulk chlorophyll fluorescence of 85 co-cultures and 20 controls clustered using Hierarchical Clustering (HC). B and C) Fluorescence curves of the co-cultures (B) and axenic cultures (C).** Six main co-culture outcomes can be differentiated using HC, three of which are similar to the "Late Growth" observed in Fig. 1 but differing in the timing of the final growth. Only two co-culture outcomes can be seen for MED4. Note the similarity in the shape of the "early arrested growth" outcome between MIT9313 and MED4. **D) Similarity in the phylogenetic pattern of co-culture outcomes between the two experiments.** The maximum likelihood tree from Fig. 3 is shown, with the co-culture outcomes (see legend) of the second experiment added. Middle rings are MIT9313, outer rings are MED4. Spheres on the tree branches denote >80% aLRT confidence (Anisimova and Gascuel, 2006).

		Effect on MIT9313				Effect on MED4	
OTU number	**Order**	**Early**	**Early arrested**	**Interme-diate**	**Late**	**Early arrested**	**Interme-diate**
1	Rhodobacterales	0	4	1	0	4	1
2	Rhodobacterales	1	0	0	0	0	1
3	Rhodobacterales	7	0	0	0	0	7
4	Oceanospirillales	0	0	1	0	0	1
5	OM60	3	0	0	0	0	3
6	Alcanivoracaceae	3	0	0	0	0	3
7	Alcanivoracaceae	1	0	0	0	0	1
8	Alcanivoracaceae	1	0	0	0	0	1
9	Alcanivoracaceae	2	0	0	0	0	2
10	Marinobacter	0	0	0	1	0	1
11	Marinobacter	3	0	0	0	0	3
12	Marinobacter	19	0	2	0	0	21
13	Marinobacter	10	0	1	0	0	11
14	Marinobacter	1	0	0	0	0	1
15	Marinobacter	37	0	0	1	0	38
17	Alteromonadales	4	0	0	0	0	4
18	Alteromonadales	1	0	0	0	0	1
19	Alteromonadales	0	0	0	32	0	32
20	Alteromonadales	0	0	0	1	0	1
21	Alteromonadales	0	0	1	41	0	42

Supplementary Table 1: Operational Taxonomic Units (OTUs) at 0.01 resolution and their effect on MIT9313 and MED4 in co-culture. The OTUs are shown in the same order as in Figure 3 and Supplemental Figure 6D, ordered clockwise. The number of strains revealing each effect is shown.

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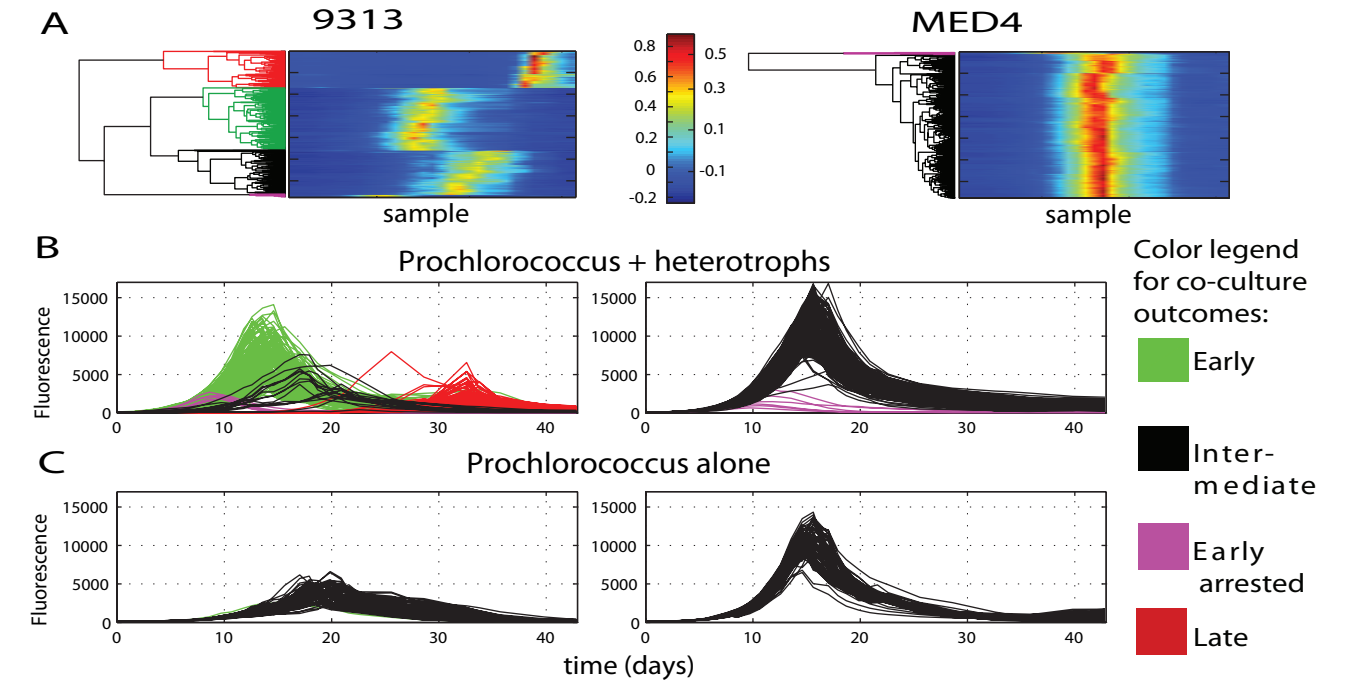
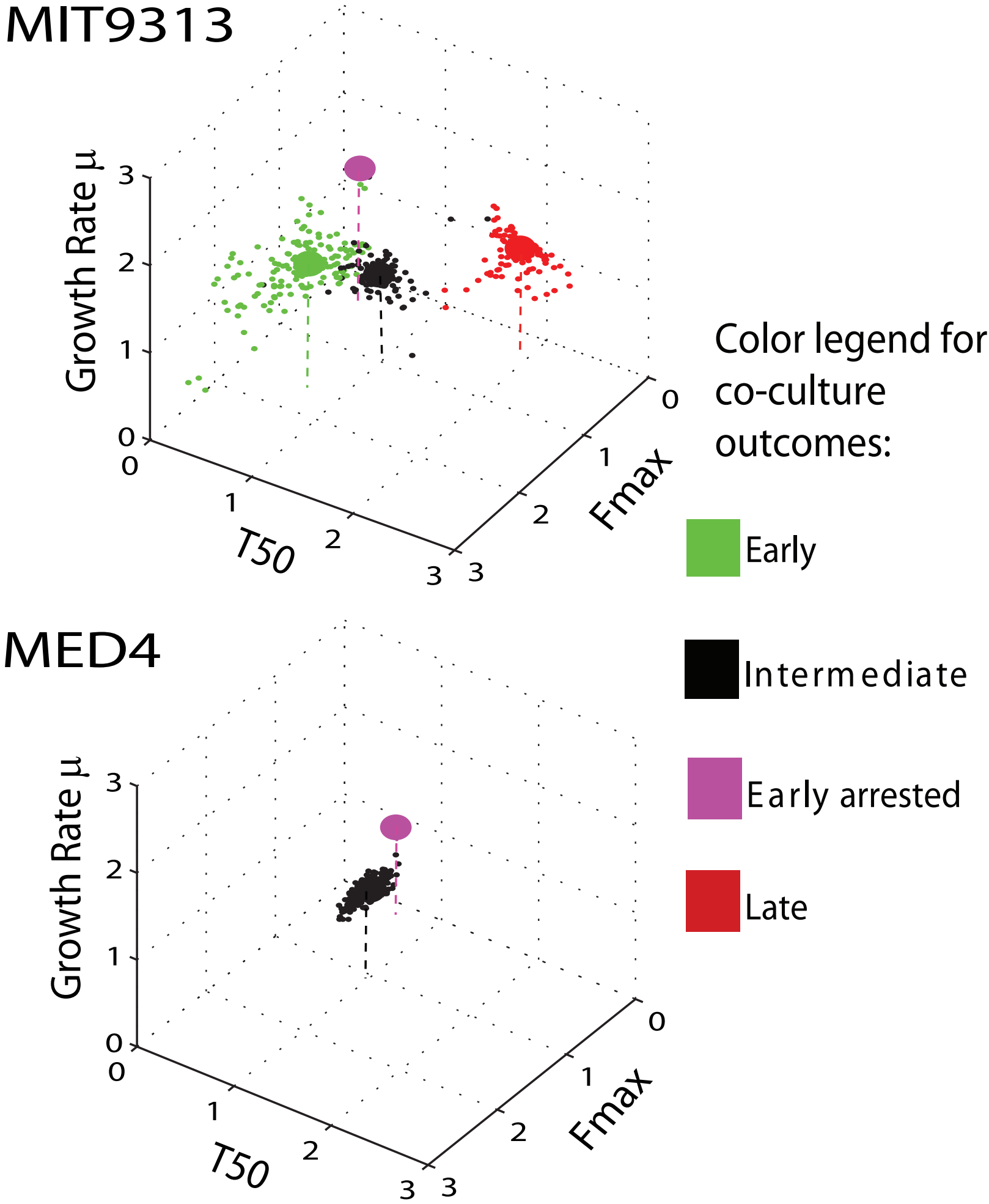
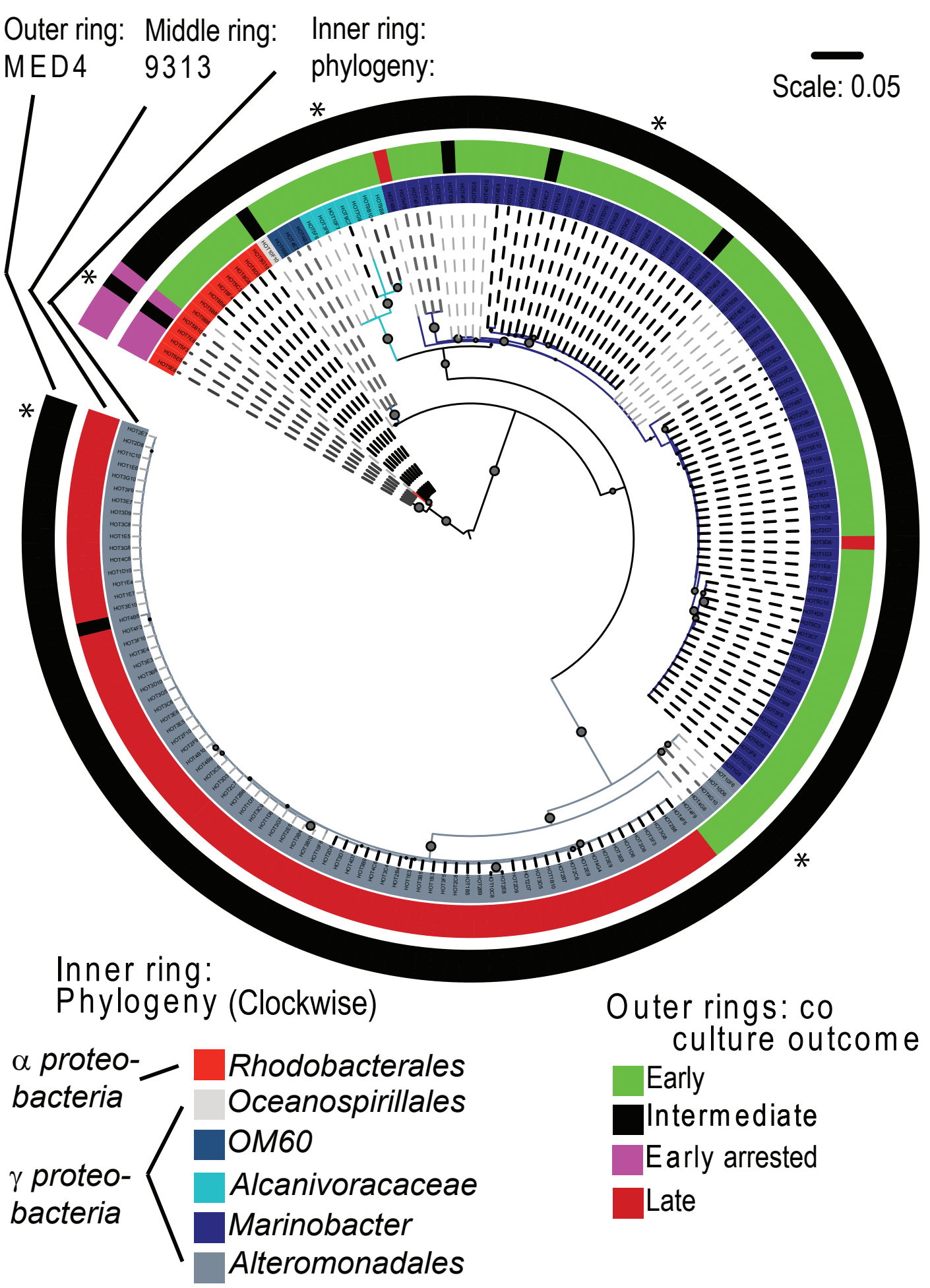
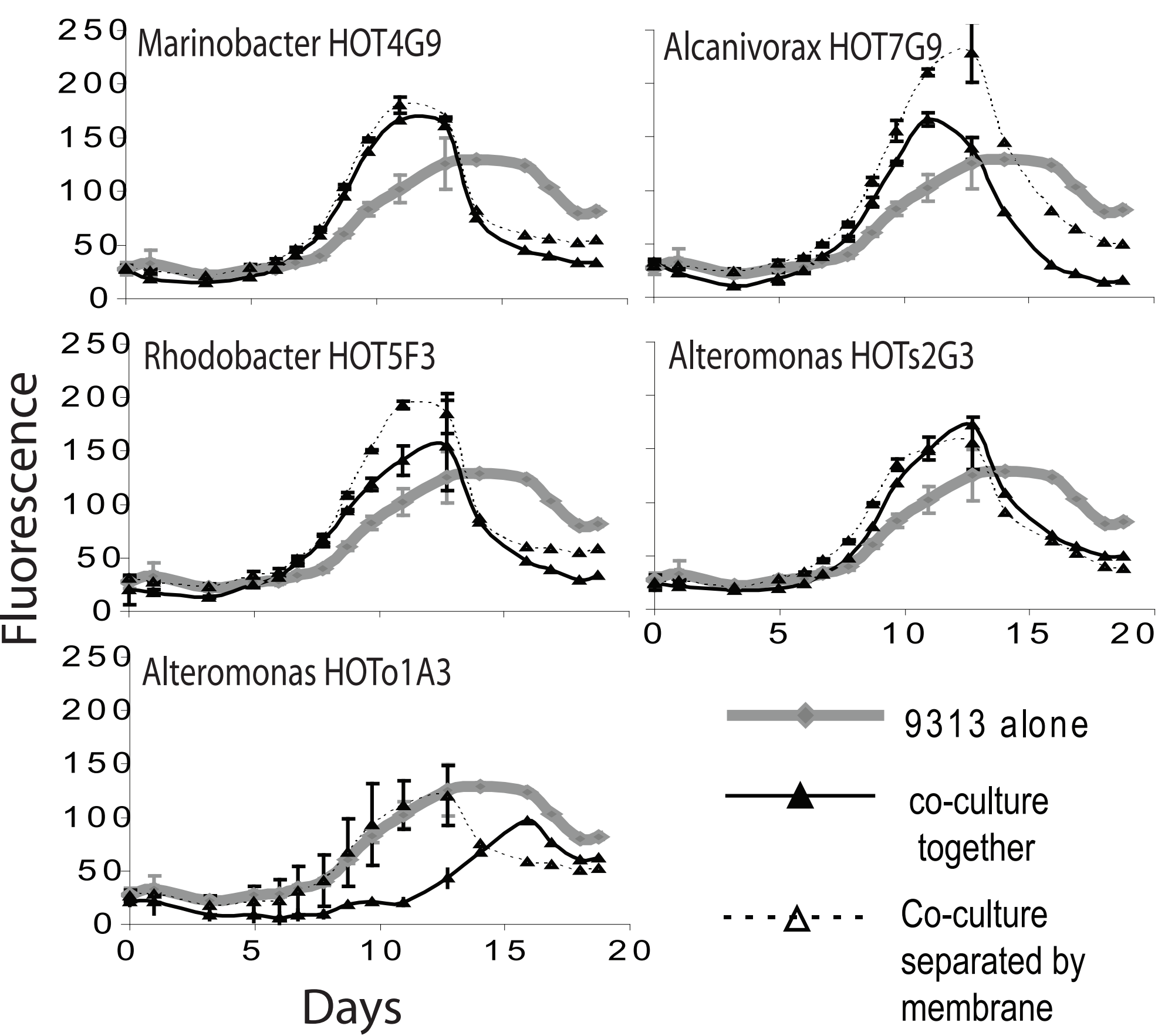
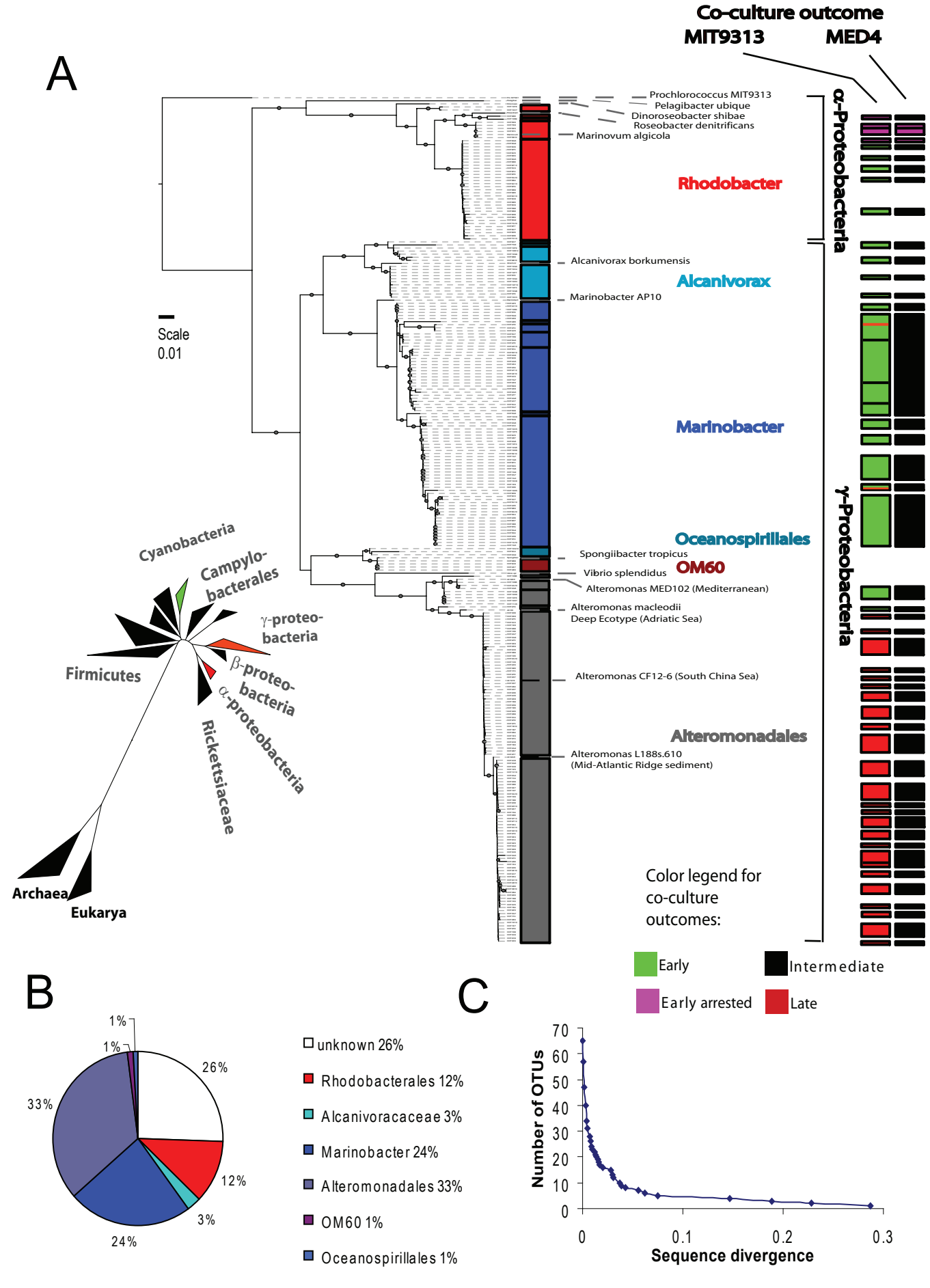
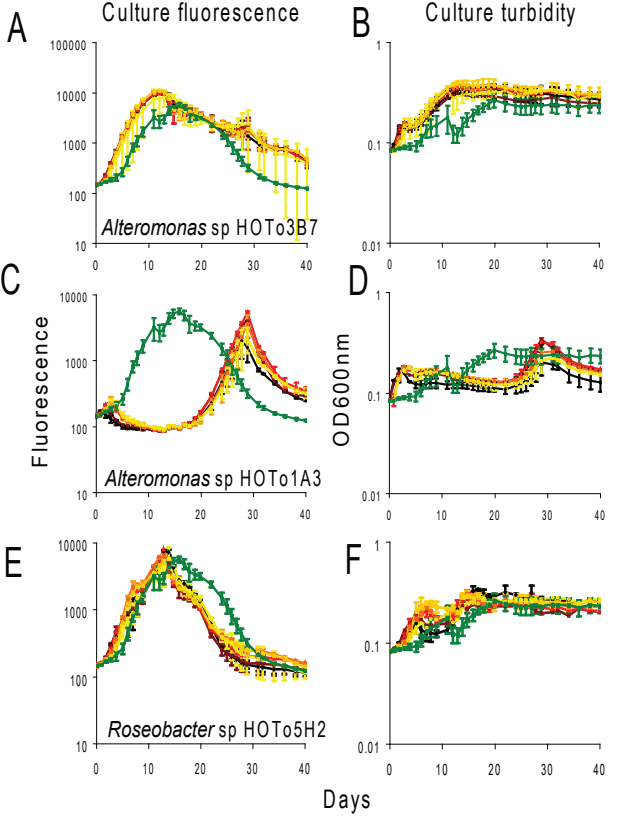

A
9313
MED4
0.8
0.6
0.4
0.2
0
-0.2
0.5
0.3
0.1
-0.1
sample
sample
B
Prochlorococcus + heterotrophs
Fluorescence
15000
10000
5000
0
0
10
20
30
40
C
Prochlorococcus alone
time (days)
Color legend for co-culture outcomes:
Early
Inter-mediate
Early arrested
Late


MIT9313
Growth Rate μ
T50
Fmax
MED4
Growth Rate μ
T50
Fmax
Color legend for co-culture outcomes:
Early
Intermediate
Early arrested
Late


Outer ring:
MED4
Middle ring:
9313
Inner ring:
phylogeny:
Scale: 0.05
*
*
*
*
*
Inner ring:
Phylogeny (Clockwise)
α proteo-
bacteria
γ proteo-
bacteria
Rhodobacterales
Oceanospirillales
OM60
Alcanivoracaceae
Marinobacter
Alteromonadales
Outer rings: co
culture outcome
Early
Intermediate
Early arrested
Late

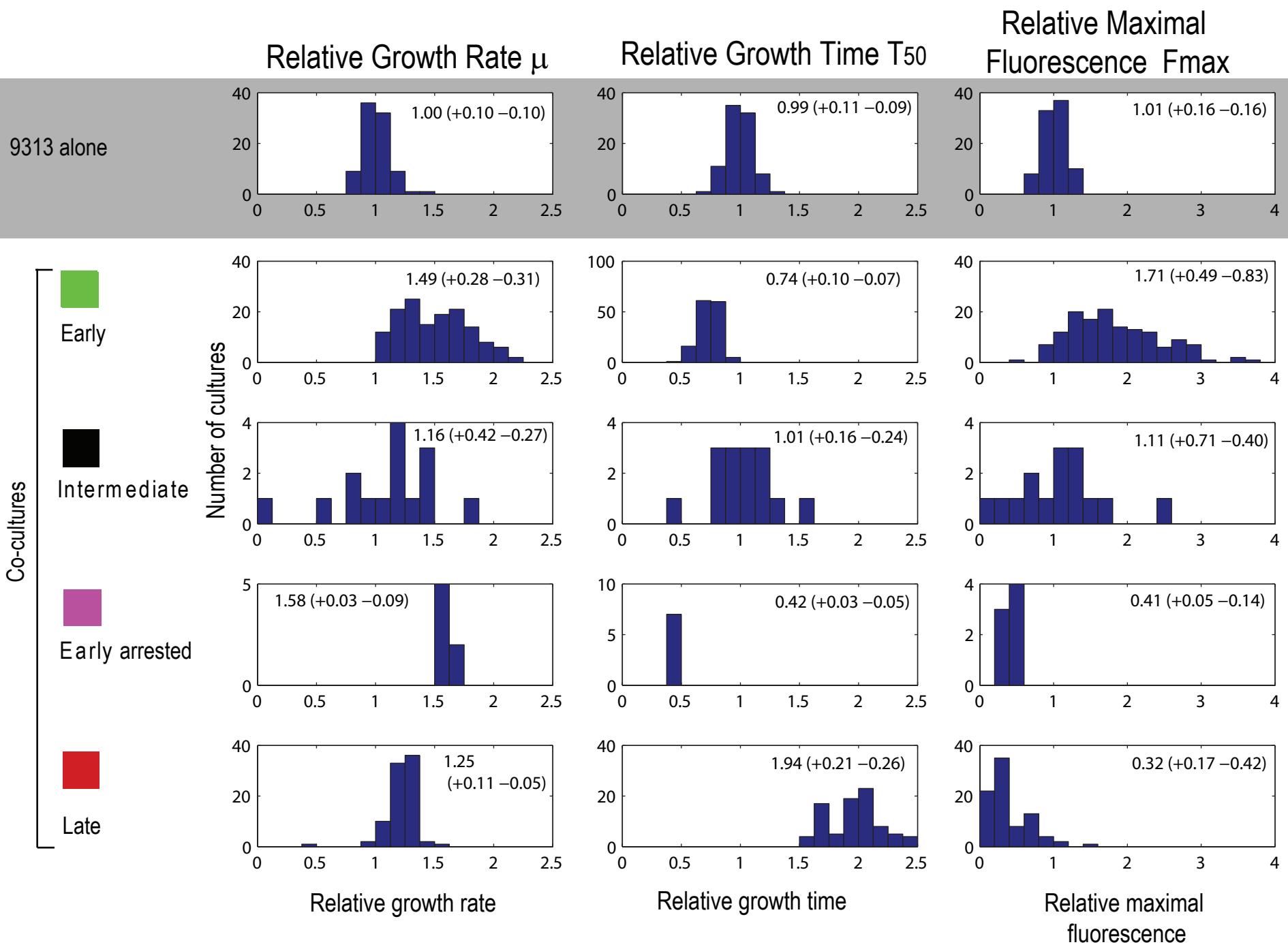

Marinobacter HOT4G9
Alcanivorax HOT7G9
Rhodobacter HOT5F3
Alteromonas HOTs2G3
Alteromonas HOTo1A3
Fluorescence
Days
250
200
150
100
50
0
0
5
10
15
20
9313 alone
co-culture together
Co-culture separated by membrane

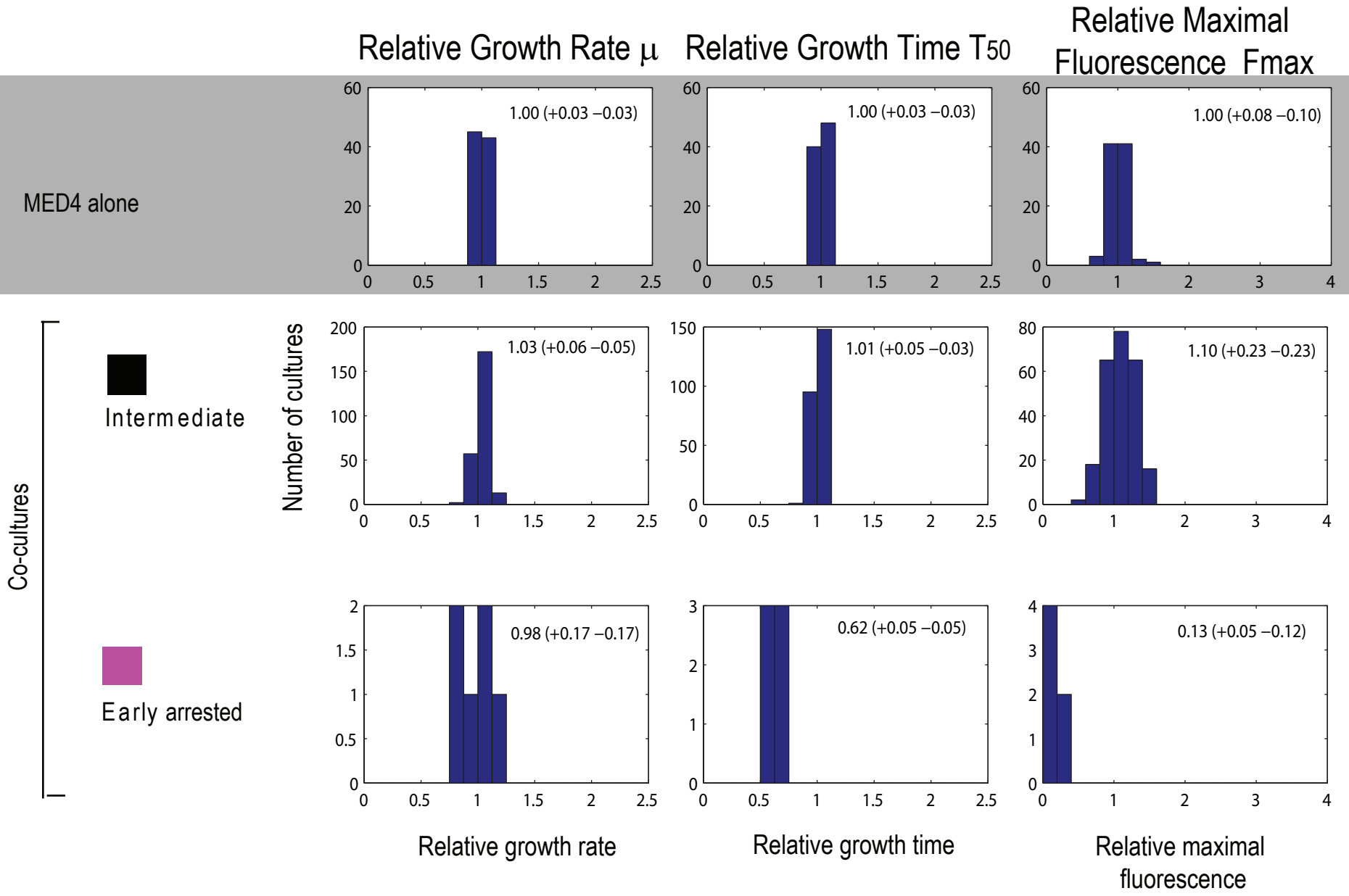

A
Co-culture outcome
MIT9313
MED4
Prochlorococcus MIT9313
Pelagibacter ubique
Dinoroseobacter shibae
Roseobacter denitrificans
Marinovum algicola
Rhodobacter
α-Proteobacteria
Alcanivorax borkumensis
Alcanivorax
Marinobacter AP10
Scale
0.01
Marinobacter
Oceanospirillales
Spongiibacter tropicus
OM60
Vibrio splendidus
Alteromonas MED102 (Mediterranean)
Alteromonas macleodii
Deep Ecotype (Adriatic Sea)
Alteromonas CF12-6 (South China Sea)
Alteromonadales
Alteromonas L188s.610
(Mid-Atlantic Ridge sediment)
γ-Proteobacteria
Cyanobacteria
Campylo-
bacterales
γ-proteo-
bacteria
β-proteo-
bacteria
α-proteobacteria
Rickettsiaceae
Firmicutes
Archaea
Eukarya
Color legend for
co-culture
outcomes:
Early
Intermediate
Early arrested
Late
B
1%
1%
26%
33%
12%
3%
24%
unknown 26%
Rhodobacterales 12%
Alcanivoracaceae 3%
Marinobacter 24%
Alteromonadales 33%
OM60 1%
Oceanospirillales 1%
C
Number of OTUs
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
Sequence divergence

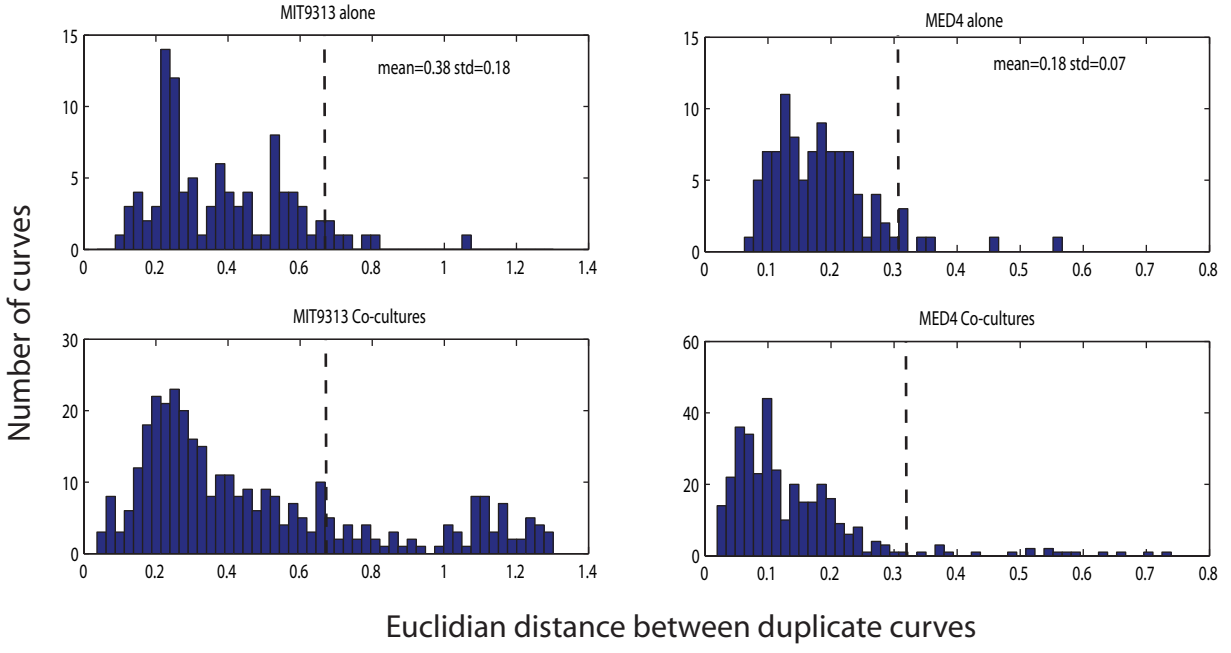


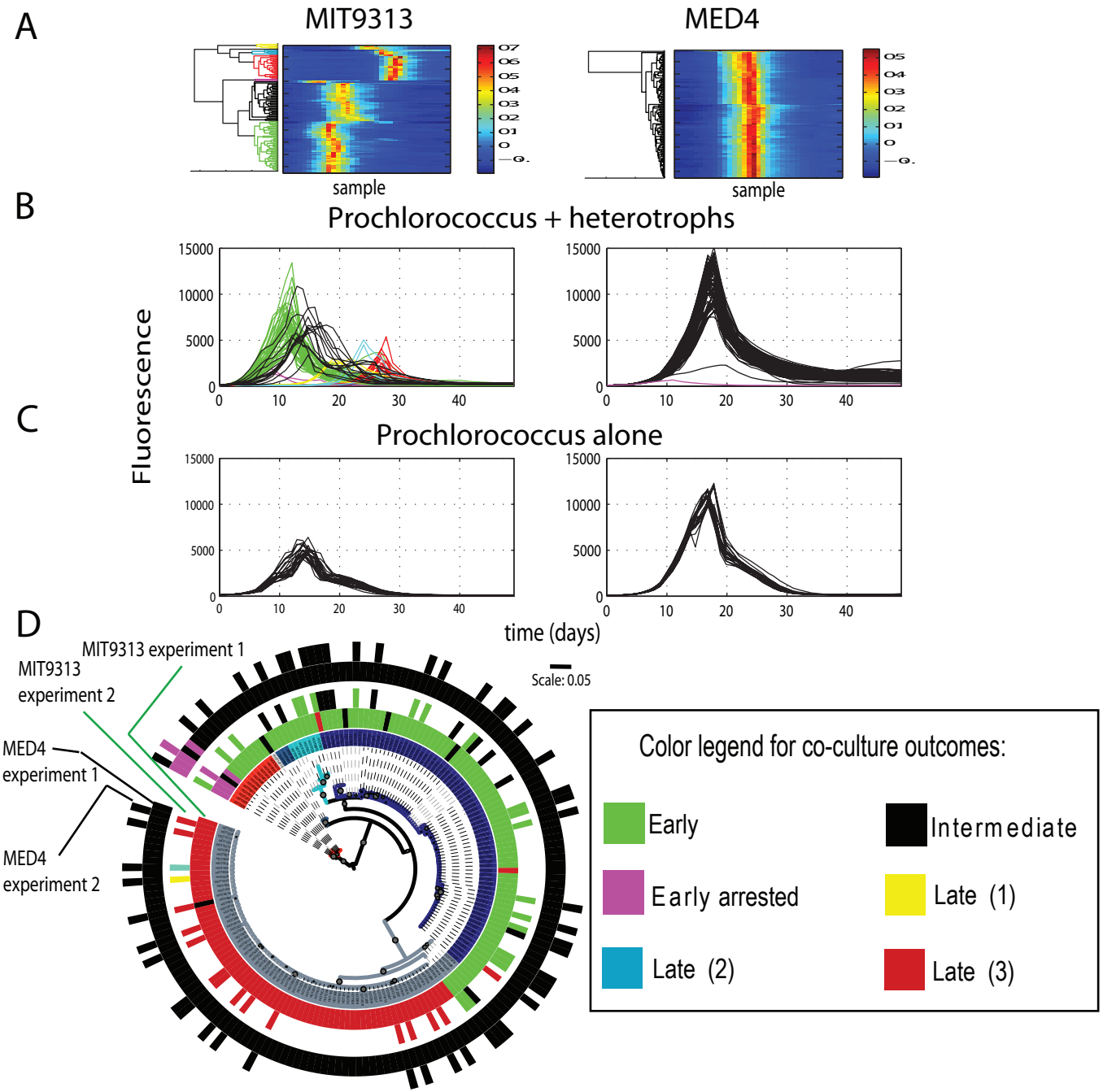
Heterotroph density (cells/mL)	Alteromonas HOTo3B7	Alteromonas HOTo1A3	Roseobacter HOTo5H2
	2x10^5	5x10^5	2.4x10^4
	2x10^4	5x10^4	2.4x10^3
	2x10^3	5x10^3	240
	200	500	24
	20	50	2.4
	2	5	0.24

Mean all controls


Relative Growth Rate μ
Relative Growth Time T50
Relative Maximal Fluorescence Fmax
9313 alone
1.00 (+0.10 −0.10)
0.99 (+0.11 −0.09)
1.01 (+0.16 −0.16)
Co-cultures
Early
Intermediate
Early arrested
Late
Number of cultures
1.49 (+0.28 −0.31)
0.74 (+0.10 −0.07)
1.71 (+0.49 −0.83)
1.16 (+0.42 −0.27)
1.01 (+0.16 −0.24)
1.11 (+0.71 −0.40)
1.58 (+0.03 −0.09)
0.42 (+0.03 −0.05)
0.41 (+0.05 −0.14)
1.25 (+0.11 −0.05)
1.94 (+0.21 −0.26)
0.32 (+0.17 −0.42)
Relative growth rate
Relative growth time
Relative maximal fluorescence


Relative Growth Rate μ
Relative Growth Time T50
Relative Maximal Fluorescence Fmax
MED4 alone
1.00 (+0.03 −0.03)
1.00 (+0.03 −0.03)
1.00 (+0.08 −0.10)
Co-cultures
Intermediate
Early arrested
Number of cultures
1.03 (+0.06 −0.05)
1.01 (+0.05 −0.03)
1.10 (+0.23 −0.23)
0.98 (+0.17 −0.17)
0.62 (+0.05 −0.05)
0.13 (+0.05 −0.12)
Relative growth rate
Relative growth time
Relative maximal fluorescence


MIT9313 alone
mean=0.38 std=0.18
MED4 alone
mean=0.18 std=0.07
MIT9313 Co-cultures
MED4 Co-cultures
Number of curves
Euclidian distance between duplicate curves


A
MIT9313
MED4
sample
sample
B
Prochlorococcus + heterotrophs
Fluorescence
C
Prochlorococcus alone
time (days)
D
MIT9313 experiment 1
MIT9313
experiment 2
MED4
experiment 1
MED4
experiment 2
Scale: 0.05
Color legend for co-culture outcomes:
Early
Intermediate
Early arrested
Late (1)
Late (2)
Late (3)