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ORIGINAL ARTICLE

Transcriptional response of *Prochlorococcus* to co-culture with a marine *Alteromonas*: differences between strains and the involvement of putative infochemicals

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Interactions between marine microorganisms may determine the dynamics of microbial communities. Here, we show that two strains of the globally abundant marine cyanobacterium *Prochlorococcus*, MED4 and MIT9313, which belong to two different ecotypes, differ markedly in their response to co-culture with a marine heterotrophic bacterium, *Alteromonas macleodii* strain HOT1A3. HOT1A3 enhanced the growth of MIT9313 at low cell densities, yet inhibited it at a higher concentration, whereas it had no effect on MED4 growth. The early transcriptomic responses of *Prochlorococcus* cells after 20 h in co-culture showed no evidence of nutrient starvation, whereas the expression of genes involved in photosynthesis, protein synthesis and stress responses typically decreased in MED4 and increased in MIT313. Differential expression of genes involved in outer membrane modification, efflux transporters and, in MIT9313, lanthipeptides (prochlorosins) suggests that *Prochlorococcus* mount a specific response to the presence of the heterotroph in the cultures. Intriguingly, many of the differentially-expressed genes encoded short proteins, including two new families of co-culture responsive genes: CCRG-1, which is found across the *Prochlorococcus* lineage and CCRG-2, which contains a sequence motif involved in the export of prochlorosins and other bacteriocin-like peptides, and are indeed released from the cells into the media.
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Introduction

Interactions such as symbiosis, competition and allelopathy are a central feature of microbial communities (Bassler and Losick, 2006; Hibbing *et al*., 2009). Even in dilute oceanic environments, microbial interactions abound: antagonistic interactions can promote biodiversity (Czaran *et al*., 2002; Pernthaler, 2005), and synergistic interactions can provide sources of sustenance in complex communities (Boetius *et al*., 2000; Croft *et al*., 2005; Azam and Malfatti, 2007; Amin *et al*., 2009, 2015). Although marine microbial interactions often occur on scales of nanometers or microns (Blackburn *et al*., 1998; Stocker *et al*., 2008; Malfatti and Azam, 2009; Seymour *et al*., 2010), they ultimately affect entire ecosystems and global biogeochemical cycles (Azam and Malfatti, 2007). Understanding these interactions requires studying them at different scales: identifying transcriptional changes that occur when organisms interact (for example, in laboratory co-cultures) being the most fundamental, as this is where the cell-to-cell 'recognition' is first expressed.

Prochlorococcus belongs to a diverse clade of cyanobacteria and is numerically dominant in large swaths of the oligotrophic ocean; they are key players in the global carbon cycle (Coleman and Chisholm, 2007; Partensky and Garczarek, 2010). On average, toward the ocean surface *Prochlorococcus* cells divide once a day (Vaulot *et al*., 1995), yet loss processes, for example by predation by grazers or infection by phage keep the total number of cells more-or-less stable (DuRand *et al*., 2001; Worden and Binder, 2003; Malmstrom *et al*., 2010; Ribalet *et al*., 2015). Carbon released through such interactions, as well as by excretion from living cells, likely provides a sizable fraction of the reduced carbon used for growth by co-occurring heterotrophic bacteria (Bertilsson *et al*., 2005; Ottesen *et al*., 2014; Biller *et al*., 2014b). Thus, like other phytoplankton, the life and death of *Prochlorococcus* are intimately coupled with that of co-occurring microbes. Although significant advances have been made in understanding 'top down' control of *Prochlorococcus* populations by phage and grazers (for example, Worden and Binder, 2003; Avrani *et al*., 2011; Pasulka *et al*., 2015), as well as 'bottom up' control

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by nutrients, temperature and light (for example, Bouman *et al*., 2006; Johnson *et al*., 2006; Saito *et al*., 2014), studying how co-occurring heterotrophic microbes affect *Prochlorococcus* is still in its infancy. Several recent studies have shown that co-occurring heterotrophic bacteria can both enhance and inhibit the growth of *Prochlorococcus* in laboratory co-cultures (Morris *et al*., 2008; Sher *et al*., 2011). Furthermore, such interactions may have significant effects on the viability of *Prochlorococcus* in the oceans, for example through scavenging by heterotrophic bacteria of reactive oxygen species (ROS) produced by *Prochlorococcus* (Morris *et al*., 2011, 2012). Finally, both *Prochlorococcus* and related *Synechococcus* strains have been shown to produce a wide diversity of ribosomally-synthesized peptides, which are then post-translationally modified to form lanthipeptides and microcins (Li *et al*., 2010; Paz-Yepes *et al*., 2013). Why these strains produce such a variety of secondary metabolites, which may have roles in signaling or allelopathy and how these putative infochemicals function in a highly diffuse marine environment remain an enigma. Thus, microbial interactions, both synergistic and antagonistic, are likely a critical determinant of *Prochlorococcus* dynamics in the oceans.

Recently, we have shown, using high-throughput laboratory co-cultures, that different microbial groups affect the growth of *Prochlorococcus* strains in distinct and phylogenetically-coherent ways (Sher *et al*., 2011). We have also shown that two closely-related *Prochlorococcus* strains, MED4 and MIT9313, belonging to the high-light I and low-light IV ecotypes, differ markedly in their response to co-culture (Sher *et al*., 2011). To get a better insight into the mechanisms behind these interactions, we selected one heterotrophic strain belonging to the *Alteromonas* clade for in-depth analysis. *Alteromonas* are typically not very common in the oceans (Eilers *et al*., 2000) yet are often identified in rRNA-based analyses of microbial communities in open water, and thus naturally co-occur with *Prochlorococcus* (García-Martínez *et al*., 2002; Ivars-Martinez *et al*., 2008; Lopez-Perez *et al*., 2012). They are metabolically-versatile copiotrophs (Pedler *et al*., 2014), rapidly responding to increases in dissolved organic matter and often dominating mesocosm experiments (McCarren *et al*., 2010). Several *Alteromonas* strains have been shown to inhibit eukaryotic phytoplankton such as diatoms, dinoflagellates and raphidophytes (reviewed by Mayali and Azam, 2004). The specific *Alteromonas* strain we used, HOT1A3, originally isolated near Hawaii (Sher *et al*., 2011), was selected for further study (including genome sequencing; Fadeev *et al*., 2016) because it inhibited the growth of MIT9313 but not of MED4 in co-cultures, and thus could potentially illuminate underlying mechanisms. Using laboratory co-cultures, we asked: (i) to what extent does the density of HOT1A3 affect the growth of the two *Prochlorococcus* strains in co-cultures? (ii) is the interaction mediated by competition for nutrients or are other mechanisms involved? (iii) what are the changes in gene expression occurring during the initial stages of co-culture, and how do these underlie the marked difference between the two *Prochlorococcus* strains in their response to HOT1A3? Here, we focus on the changes in expression of protein-coding genes of the two *Prochlorococcus* strains in response to co-culture. The changes in gene expression observed in *Alteromonas* HOT1A3 will be presented elsewhere.

Materials and methods

Strains, culture conditions and co-culture experiments
Axenic *Prochlorococcus* strains MED4 and MIT9313 were maintained in Pro99 media, whereas *Alteromonas* strain HOT1A3 was maintained in ProMM, both under constant cold while light (27 $\mu Em^{-2} s^{-1}$) at 20 °C as in Moore *et al*. (2007) and Sher *et al*. (2011). Before each experiment, the axenicity of the *Prochlorococcus* cultures was tested by inoculating 10 µl into 2 ml ProMM and marine purity test broth (Moore *et al*., 2007). At the start of each co-culture experiment, *Alteromonas* cells from stationary-stage cultures (24–72 h old) were centrifuged (15 min, room temperature, 15 000 *g*), the growth media decanted and the cells re-suspended in Pro99. The *Prochlorococcus* cultures (growing exponentially) and the re-suspended *Alteromonas* cells were then counted using an Influx fluorescence activated cell sorter (BD, San Jose, CA, USA) and the cells mixed at the ratios described in the text. The culture vessels used for each experiment and the details of sample collection and preservation are detailed in the Supplementary information.

RNA isolation, stranded library construction and sequencing
Total RNA was extracted from two biological replicates using the mirVana miRNA kit (Ambion, Austin, TX, USA) as described in Tolonen *et al*. (2006) and Lindell *et al*. (2007). DNA was removed using Turbo DNase (Ambion). Depletion of ribosomal RNA before cDNA synthesis was performed with the Ribo-Zero kit for meta-bacteria (Epicentre Biotechnologies, Madison, WI, USA). The mRNA was purified with RNAClean XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and immediately subjected to the mRNA fragmentation step using the Fragment, Prime, Finish Mix from the TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA, USA). All the following steps were performed according to the manufacturer's protocol. Twelve mRNA libraries from one timepoint (20 h) were constructed, evaluated using a Tape Station (Agilent, Santa Clara, CA, USA), and sequenced using 50-bp single-end reads on an Illumina HiSeq2500 sequencer at the Life Sciences and Engineering Infrastructure Unit, Technion, Haifa.

RNA-Seq data analysis

Raw reads were uploaded onto the Galaxy platform (Goecks *et al.*, 2010). The first 11 base pair (bp) were trimmed and the Illumina adapter sequences were clipped. To eliminate all ribosomal RNA sequences, reads mapping to the rRNA of *Prochlorococcus* and *Alteromonas* were removed. Reads shorter than 20 bp were also removed, and the remaining reads were stringently mapped with Bowtie (Langmead *et al.*, 2009) to the *Prochlorococcus* (MED4 or MIT9313) and *Alteromonas* genomes, with no mismatches allowed and a seed length of 28. Only reads that unequivocally mapped to one organism in the co-culture and not the other were retained for further analysis. Differential expression (DE) analysis was performed using the Rockhopper program (McClure *et al.*, 2013). Similar to previous studies (Waldbauer *et al.*, 2012; Voigt *et al.*, 2014), we observed antisense expression in most of the genes (766/1716 genes in MED4 and 2060/2274 genes in MIT9313). Little is currently known about the function of these antisense RNAs, and thus we focused our analysis on protein-coding genes. Genes were considered to exhibit DE where the fold change in expression of co-culture compared with the axenic culture was >2, the q-value was <0.05 and the expression level in either axenic culture or co-culture was >10. Additional information and statistics of the analysis pipeline are presented in the Supplementary information.

Results and discussion

A high density of Alteromonas HOT1A3 inhibits the growth of Prochlorococcus MIT9313 but not MED4

Alteromonas HOT1A3 inhibits *Prochlorococcus* MIT9313 in co-culture, but has no clear effect on MED4 (Sher *et al.*, 2011). Because of the high-throughput nature of those experiments, the cultures were maintained in 96-well plates—that is, they were very small volume, and there was a significant carryover of organic carbon with the inoculated heterotrophic bacteria. To determine whether the effects of HOT1A3 on the two *Prochlorococcus* strains depend on the density of the HOT1A3 in the co-cultures, we performed similar experiments in test tubes, keeping the initial cell density of *Prochlorococcus* at 10^6 cells per ml and varying the inoculated density of *Alteromonas* HOT1A3 over three orders of magnitude (Figure 1). Little or no effect of HOT1A3 was observed on the initial growth and maximal culture fluorescence of MED4 at all cell densities (Figure 1a), but the heterotroph decreased the rate of culture fluorescence decline. In contrast at higher cell densities (10^7 cells per ml), *Alteromonas* HOT1A3 initially inhibited the growth of MIT9313

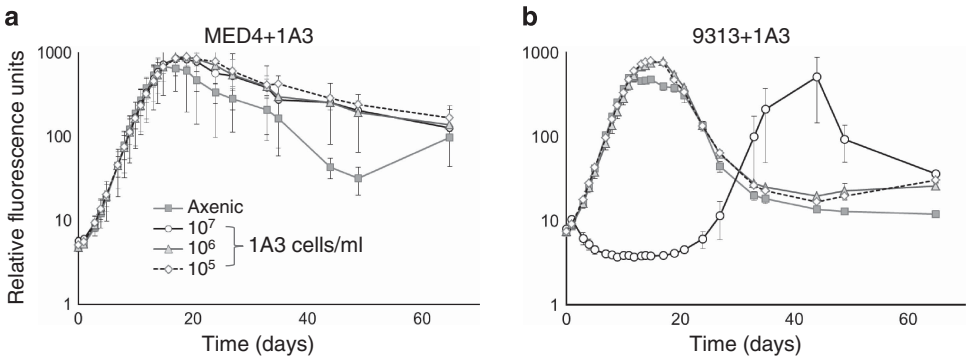
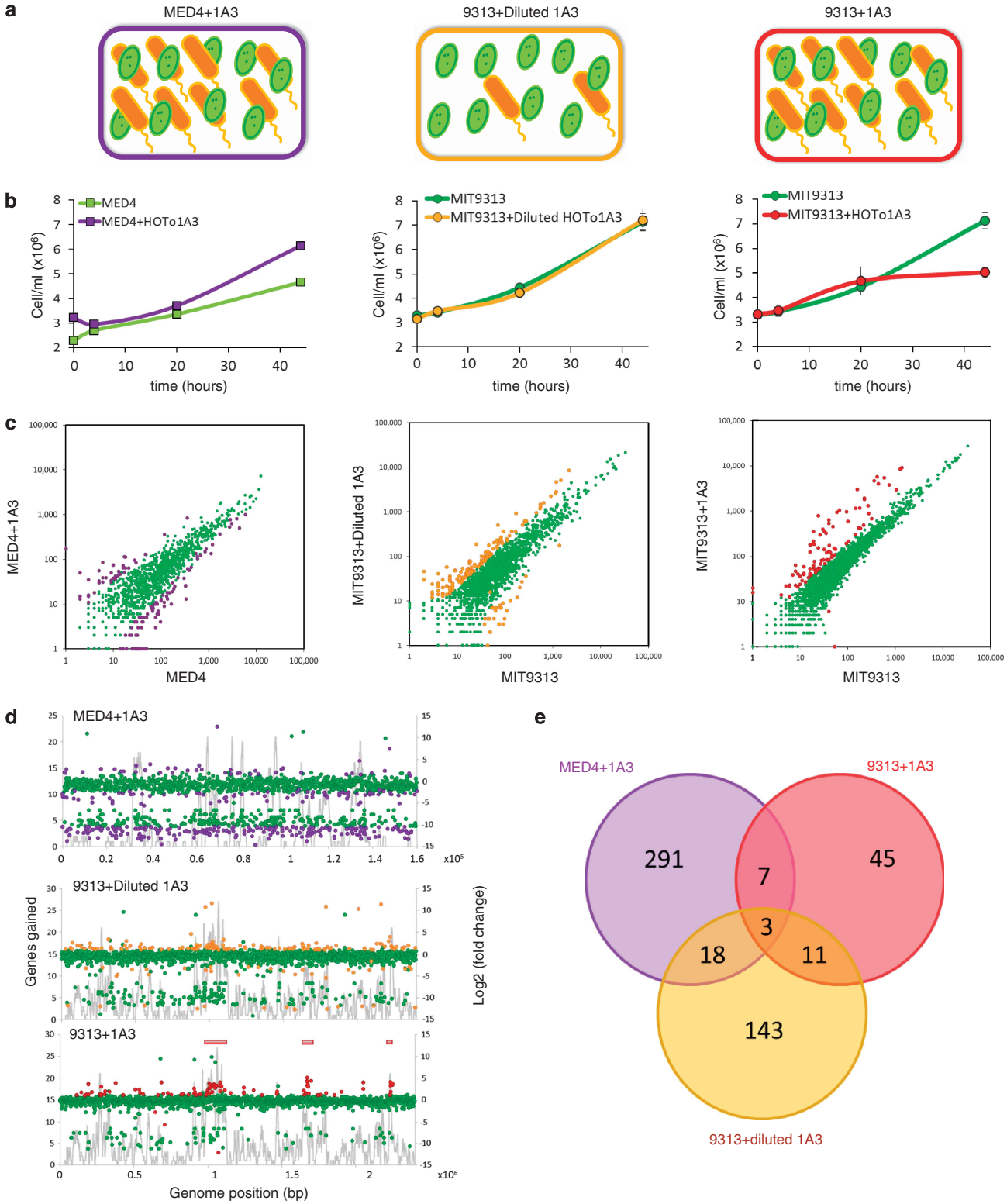


Figure 1 A high density of Alteromonas HOT1A3 inhibits the growth of Prochlorococcus MIT9313 but not MED4. Bulk culture fluorescence curves of *Prochlorococcus* MED4 (**a**) and MIT9313 (**b**) in co-culture with different densities of *Alteromonas* HOT1A3 in 25 ml tubes are shown. Data points and error bars are means and ranges of biological duplicates.

Figure 2 Gene expression changes during the early stage of co-culture between Prochlorococcus strains and different Alteromonas densities. (**a**) Schematic illustration of the experimental setup. We inoculated triplicate cultures of *Prochlorococcus* MIT9313 and MED4 ($\sim 3 \times 10^6$ cells per ml) with either high or low *Alteromonas* HOT1A3 cell densities (5×10^6 and 0.5×10^6 cells per ml, respectively). Each of these co-cultures was compared with a control of the relevant *Prochlorococcus* strain growing axenically. (**b**) Growth curves of *Prochlorococcus* in co-culture. In all, 5×10^6 *Alteromonas* cells inhibited MIT9313 but not MED4, whereas lower *Alteromonas* doses had no discernable effect on MIT9313. Error bars represent standard deviations from three biological replicates, and may be smaller than the marker. The samples for $t = 20$ h were selected for transcriptomic analysis. (**c**) Synopsis of gene expression results at $t = 20$, comparing each co-culture with the axenic control culture and revealing different patterns of differential gene regulation. Each dot represents a gene, with colored dots (red, orange and purple for 9313+1A3, 9313+diluted 1A3 and MED4+1A3, respectively) significantly more or less abundantly expressed than the controls (see Materials and methods). Green dots reveal no significant changes in expression. (**d**) Genome position and fold change in expression levels of *Prochlorococcus* genes. Differentially expressed genes are colored as in (**c**). The gray lines show the number of genes gained along the genome, with peaks denoting hypervariable regions (Kettler *et al.*, 2007; Thompson *et al.*, 2011). Note the clustering of differentially-expressed genes in 9313+1A3 in such regions. (**e**) Venn diagram of differentially-expressed gene families (CyGOGs, Biller *et al.*, 2014a) revealing the different responses of the *Prochlorococcus* strains to each co-culture.

(Figure 1b), resulting in the same 'late-growing' phenotype that we had observed in MIT9313-HOT1A3 co-cultures in 96-well plates (Sher *et al.*, 2011). At the two lower cell densities of the heterotroph, however, this effect was not seen. On the contrary, the presence of the heterotroph resulted in a slight increase in culture fluorescence relative to the control over most of the growth curve.



The early transcriptional responses of the two Prochlorococcus strains to co-culture are different

We next focused on the initial stages of co-culture, namely the first ~48 h, with the aim of identifying transcriptional changes in the two *Prochlorococcus* strains that might explain their different responses to the co-cultured heterotroph (Figure 2). The experimental setup and *Prochlorococcus* growth curves are shown in Figures 2a and b. Similar to the results shown in Figure 1, the high *Alteromonas* concentrations (5×10^6 cells per ml) slightly enhanced the growth of MED4 (Figure 2b, purple line) but inhibited MIT9313 (Figure 2b, red line). Lower *Alteromonas* concentrations (0.5×10^6 cells/ml) had no effect on MIT9313 (Figure 2b, yellow line). We refer to these co-cultures throughout the discussion below as 'MED4+1A3', '9313+1A3' and '9313+ diluted 1A3', respectively.

On the basis of these results, we selected the time point after 20 h of co-culture for transcriptome analysis, because at this stage all co-cultures have comparable cell concentrations (Figure 2b). As shown in Figures 2c and d, clear differences were observed in the general pattern of transcriptional response to co-culture. In MED4+1A3 and 9313+ diluted 1A3, genes were observed to both increase and decrease in expression, whereas in 9313+1A3 many more genes increased in expression level. The most highly differentially-expressed genes in each co-culture are presented in Table 1, and the full data set, organized by differential expression levels, can be found in Supplementary Table S2.

Given that all co-cultures involved the same heterotrophic organism, and that after 20 h similar cell densities were observed in all co-cultures, we expected a similar set of genes to be differentially-expressed. To our surprise, only three similar genes (defined as belonging to the same cluster of orthologous genes using the V4 CyCOGs data set; Biller *et al.*, 2014a) were differentially expressed across all three conditions, encoding a putative high-light induced protein (HLIP) and two hypothetical proteins. Very few genes were even shared among any two co-culture conditions (Figure 2e) with a slightly larger percent overlap between the responses of MIT9313 to high and low 1A3 densities.

We next asked whether there were any pathways or molecular functions enriched in the subset of genes differentially expressed between co-cultures and axenic cultures, using functional categories from obtained from CyanoBase (Nakao *et al.*, 2010). The only enrichment observed in this analysis was that of 'Hypothetical' genes (*P*-value 0.013, 3.9×10^{-7} and 1.5×10^{-3} for the MED4+1A3, 9313+1A3 and 9313+diluted 1A3 co-cultures, respectively, Fisher's Exact Test with Benjamini–Hochberg FDR correction, implemented in STAMP; Parks *et al.*, 2014). These results suggest that the transcriptional response of both *Prochlorococcus* strains to co-culture consists mainly of novel, previously unstudied, genes.

Prochlorococcus cells in co-culture are not nutrient-starved

Could the different outcomes of co-culture be due to different levels of competition between the *Prochlorococcus* strains and *Alteromonas* for major macronutrients such as nitrogen (N), phosphorus (P) or iron (Fe)? Two lines of evidence suggest this is not the case. First, the transcriptomic responses of the two *Prochlorococcus* strains to co-culture were clearly different from those previously shown to occur under conditions of N, P or Fe starvation (Supplementary information and Supplementary Figure S2). Second, *Prochlorococcus* MIT9313 grows in spent media from co-cultures in which the same strain has been inhibited by *Alteromonas* HOT1A3, even when no nutrients are added to the media (Supplementary Figure S2D). Taken together, these results rule out nutrient or carbon starvation as major mechanisms underlying the inhibition of MIT9313 strain by high cell densities of *Alteromonas* HOT1A3. They also suggest that the inhibitory effect of large cell concentrations of HOT1A3 on MIT9313 is not due to interference with nutrient uptake or sensing (Bar-Yosef *et al.*, 2010).

Common themes in the responses to co-culture: cell stress and photosynthesis

The transcriptomes of all three co-cultures suggest that the *Prochlorococcus* cells are exposed to varying degrees of stress in the presence of the heterotroph (Table 2, Figure 3). In the MED4+1A3 co-culture, the only potential sign of stress was an increase in expression of two genes encoding putative antioxidants (thioredoxin-like and peroxiredoxin-like, see Table 2 and Supplementary Table S2 for detailed gene descriptions and PMT/PMM numbers not mentioned in the text). In 9313 + diluted 1A3, six genes involved in the repair of DNA mismatch or single-strand breaks were more abundantly expressed, suggesting potentially a higher level of cellular stress. In 9313+1A3, a clearer signal of cellular stress was observed: in addition to three genes involved in DNA repair, the stress-related protein chaperones Hsp90 and Hsp100 (ClpB) were more abundantly expressed, as were two proteases including one (FtsH3) predicted to be involved in repair of photosystem 1 proteins (Sacharz *et al.*, 2015). Notably, many of the genes involved in DNA damage repair were actually less abundantly expressed in MED4+1A3 (for example, recR, recO and recC), as was ftsH4 (an ortholog of ftsH3 mentioned above), lending further supports the hypothesis that MED4 is sensing significantly lower cell stress compared with MIT9313, when growing with the same density of heterotrophs. A similar reduction in the expression of genes related to DNA damage was also observed in *a Synechococcus-Vibrio* co-culture (Tai *et al.*, 2009).

Different levels of stress between the three co-cultures may also have manifest themselves in the

Table 1 DE Genes with the highest fold change in co-cultures compared with axenic cultures

PMM	*Product*	*Log 2 fold change*
MED4+1A3		
0735	***Hypothetical protein***	12.45
1538	***50 S ribosomal protein L36***	7.44
1392	***Heat-labile enterotoxin subunit alpha***	4.64
0417	***Hypothetical protein***	3.84
0647	***Hypothetical protein***	3.54
0345	Bacterioferritin comigratory protein	3.48
0501	***Hypothetical protein***	2.91
0537	Phosphoribosylanthranilate isomerase	2.90
1053	Hypothetical protein	2.90
0181	***Hypothetical protein***	2.74
1402	Hypothetical protein	−13.18
0974	ATP-binding subunit of urea ABC transport system	−13.20
1633	Hypothetical protein	−13.27
0540	***Photosystem I reaction center subunit XII***	−13.36
0373	Cyanate hydratase	−13.43
0020	Hypothetical protein	−13.74
1151	***Translation initiation factor IF-1***	−13.74
0471	***High light inducible protein***	−14.44
0910	***Hypothetical protein***	−14.59
1110	***BolA-like protein***	−15.39
PMT	*Product*	*Log 2 fold change*
9313+diluted 1A3		
0934	***Hypothetical protein***	11.55
2051	Secreted calcium-binding protein	11.34
1684	4Fe-4 S ferredoxin	10.81
0892	***Hypothetical protein***	10.73
1906	***Hypothetical protein***	10.35
0800	***Hypothetical protein***	4.52
2112	***Alanine racemase***	3.90
0279	Hypothetical protein	3.35
1021	***Small cytokines (intecrine/chemokine)***	3.27
1437	AEC family transporter	3.05
1774	***methanol dehydrogenase beta subunit***	−12.00
0365	Hypothetical protein	−12.07
2081	Hypothetical protein	−12.10
1436	Hypothetical protein	−12.14
2262	***NADH-ubiquinone/plastoquinone***	−12.20
2266	***Hypothetical protein***	−12.23
1283	***Pterin-4-alpha-carbinolamine dehydratase***	−12.37
0845	***Histidine kinase-, DNA gyrase B-, phy***	−12.55
1693	***Hypothetical protein***	−12.60
1494	***Hypothetical protein***	−12.67
9313+ 1A3		
1571	***Hypothetical protein***	5.05
1599	***Magnesium chelatase family protein***	4.31
1570	Hypothetical protein	4.26
1572	Hypothetical protein	4.21
2117	D12 class N6 adenine-specific DNA met	4.00
1940	C9 family peptidase	3.99
1005	***Gamma-thionins family protein***	3.99
0631	***Hypothetical protein***	3.95
0328	Bromo domain-containing protein	3.78
2138	Hypothetical protein	3.75
0585	Glycosyl transferase family protein	−2.73
0651	Hypothetical protein	−5.71
0992	***High light inducible protein***	−12.17

Upregulated genes are marked with a gray background. When fewer than 10 genes are shown the list includes all of the differentially-expressed genes under this condition. Genes encoding short protein products (less than 100 amino acids) are in bold and italicized.

response of genes involved in photosynthesis. In MED4+1A3, a decrease in expression was observed in several photosynthesis-related genes, particularly those related to photosystem II (including psbD). In contrast, in 9313+diluted 1A3, higher expression levels were observed compared with axenic cultures of genes involved in the assembly or stabilization of photosystems I and II (Psb27, Liu *et al.*, 2011 and PsbI, Dobáková *et al.*, 2007; pigment biosynthesis, for example, hemF, see also Supplementary Information) and encoding electron carrier proteins (for example, menE and Ferredoxin) (Table 2, Figure 3). As the cultures were previously acclimated to the experimental illumination levels and low heterotroph cell densities were added to the culture, it is unlikely that the cells were responding to a decrease in illumination within the culture vessels. A similar increase in expression of these pathways was also observed in *Synechococcus-Vibrio* co-cultures (Tai *et al.*, 2009). In the 9313+1A3 co-culture, no changes could be observed in the key photosynthetic machinery. Instead, many genes encoding HLIPs were more abundantly expressed in this co-culture, whereas these genes were primarily less abundantly expressed in MED4+1A3 and 9313+diluted 1A3, the two co-cultures where *Prochlorococcus* was not inhibited (Table 2). HLIPs are short peptides previously suggested to be involved in photoacclimation (Steglich *et al.*, 2006; Berg *et al.*, 2011), but which may respond also to various stresses (Tolonen *et al.*, 2006) (Supplementary information). We interpret these results to suggest that the photosystem in the 9313+1A3 co-culture is exposed to significantly more stress than the other two co-cultures, and that this stress is different from that caused by a shift in light intensity or by nutrient stress.

Co-culture-specific responses: amino-acid uptake, metabolism and translation

Unlike the cell machinery involved in stress response and photosynthesis, which seems to be affected under all co-culture conditions (albeit differently), some cell processes were affected in only one or two co-culture conditions. Generally speaking, genes involved in many metabolic pathways were less abundantly expressed in MED4+1A3 compared with MED4 growing alone. These include genes involved in the biosynthesis of amino acids, purines, pyrimidines, cobalamin (Vitamin B12), fatty acids and phospholipids. Additionally, genes involved in DNA replication and cell cycle were also less abundantly expressed. Given that the growth rate of the cells was actually higher in co-culture compared with axenic culture (Figure 2b), it is not likely that these changes in expression reflect a general 'slowing down' of the cellular metabolism. Rather, we suggest that they are consistent with an increase in the availability of organic molecules such as amino acids and nucleotides due to the presence

Table 2 Selected differentially-regulated genes for which annotations are available in *Prochlorococcus* MED4 and MIT9313 in co-culture with *Alteromonas macleodii* HOT1A3

	MED4+1A3	*9313+ Diluted 1A3*	*9313+1A3*
Transport			
ABC transporters	Peptides (0241*), Trace metals (0125*, 0601*), Cyanate (0371*, 0373*), Phosphonate (0673*), Phosphate (0724*), Sugar (1323*)	Phosphonate (0781) amino acids (0896*, 0897), Urea* (2228)	
Other transporters		AEC family transporter (1437)	
Energy and metabolism			
Photosynthesis	HLI2*,3*,6*,8*,9*,15*,16*, 18*,20* (0064*, 1482*, 1399*, 1397*, 1396*, 1128*, 0818*, 0816*, 0471*), PSII reaction center (0251*, 0252*, 0540*, 1644, 1157*) Heme biosynthesis (0113*, 0747*, 0768*), Cytochrome b6f complex (0326*), Ferredoxin (0898*, 1449*)	HLI9* and 4* (1152*, 1594*), Heme biosynthesis (0390), PSII reaction center assembly (1260, 1840), PSI reaction center (1767), Ferredoxin (2195), phycoerythrin biosynthesis (1678, 1679, 1686), Nudix Hydrolase (1026), pentose-phosphate pathway (1453), Calvin cycle (1496*), fatty acid biosynthesis (1621, 1996), cofactor biosynthesis (2136, 2199)	HLI 7*, 9, 6, 8, 12 (0992*, 1152, 1153, 1154, 1640), Thylakoid-associated protein (1554)
General metabolism	Glycolysis (0596, 0185*), cobalamin biosynthesis (0270*, 0778*, 0863*,1160*, 1656), folate biosynthesis (0184*, 0287, 0591*), chorismate biosynthesis (0636, 0715*, 1181), ATP synthesis (1452*, 1453*), Pentose phosphate/Calvin cycles (1489*), Sugar metabolism (1208*, 1627*). Fatty acid/phospholipid biosynthesis (0136*, 0137*, 0138*, 0534*, 0798, 1085*, 1108*)		Lipoate-protein ligase (1224)
Amino-acid metabolism	Amino-acid metabolism (0166*, 0281*, 1526*, 0222*, 0387*, 0537, 0590*, 0674*, 0821*, 0887*, 0888*, 0917*, 0920*, 1051*, 1154*, 1214*, 1379*, 1565*, 1572*, 1705*)	Amino-acid biosynthesis (0426*, 1537*), Cysteine biosynthesis (0117, 0138, 0184), phenylalanine degradation (1283)	Amino-acid biosynthesis (0099, 0184)
DNA replication and cell cycle			
Purine/pyrimidine metabolism	Pur genes (0003*, 1339*), Pyr genes (0275*, 0514*, 1433*), other purine/pyrimidine biosynthesis (0467*, 0918*, 1122*)		
DNA replication and cell-cycle progression	DNA polymerase (0001*, 1647*, 1658*), DNA gyrase/helicase/topoisomerase (0005*, 1403*, 1467*), ftsZ (1309*), clpP (1313*)		
Transcription and translation			
Transcriptional regulators	PhoR (0706*), ntcA (0246*), rpoZ (1431*), bHLH transcriptional regulator (1637)	PhoB-like (0994), ntcA (1831*)	Alternative Sigma-factors (0127, 1068), two component sensor (0265)
Translation	tRNA modification (0014, 0388*, 0862*, 1165*, 1299*, 1635*, 1690*), AA-tRNA ligation (0048*, 0187*), initiation (0841, 1151*, 1494*), Ribosomes structure and maturation (0102*, 0112*1353*, 1012*, 1605*), ribosomal proteins (1519*, 1541*, 1545*, 1556*, 1538) tRNA or peptide chain release (0250*, 0349*, 1616*) signal peptide peptidase (0513*, 1180*	tRNA modification (0147*, 0356, 0750), Ribosomal structure (0549, 1420)	tRNA modification (0605

Table 2 (Continued)

	MED4+1A3	*9313+ Diluted 1A3*	*9313+1A3*
Stress			
General stress response	Stress sensing (0011), proteases (1490*, 1613*), Chaperones (0015*, 0067*, 0896*), Redox homeostasis (0242, 0345, 0955*, 1333*)		HSP90 (0696)
Proteases			Zn metalloprotease (0378), ClpB (0449), M23/M37 peptidase (0751)
DNA damage	Recombination repair (0398*, 1097*, 1105*, 1615*),	Recombination repair RecO (0211), Break/mismatch repair (0842, 1642, 2184) DNA polymerase subunits (0059, 0486)	Recombinase (0294) break/ mismatch repair (0644, 0842)
Interactions			
Cell wall modification	LPS biosynthesis (1334*, 1335*), peptidoglycan biosynthesis (0021*, 0187*, 1364*), sugar modifications (1219*, 1254*)	Peptidoglycan-associated (0190, 0543, 0915)	Peptidoglycan-associated (0915)
Drug/toxin exporters	Multidrug (0977*)	RTX exporter (0094), antimicrobial transporter (0678, 1355, 1576)	Multidrug transporter (1573)
Lantipeptides		ProcA1.7, 1.4, 3.1, 3.2, 3.4, 2.10, 2.4 (0239, 0243, 0827, 0829, 0839, 2120, 2130)	ProcA1.2, 3.1, 3.2, 3.4, 4.3, 2.8 (0245, 0827, 0829, 0836, 0926, 2122)
Other		Hemolysin-like (0929, 2051), Serine protease inhibitor Ecotin (2221)	Hemolysin-like (0929)

Abbreviation: LPS, lipopolysaccharides. The full list of differentially-expressed genes is found in Supplementary Table S2. Numbers are PMM or PMT accession numbers for MED4 and MIT9313, respectively. Genes are upregulated unless marked by an asterisk (*).

of the heterotroph. In 9313+diluted 1A3, a different pattern was observed, whereby genes involved in the uptake of amino acids and phosphonate were more abundantly expressed, as were several genes involved in biosynthesis of the amino acids, cysteine and methionine and in phenylalanine degradation. Two genes involved in the biosynthesis of many other amino acids were less abundantly expressed (Table 2). These results suggest a shift in the pool of amino acids available to the MIT9313, increasing the concentration of sulfur-containing amino acids. This could occur either as a result of metabolic exchange with the co-cultured heterotroph, or in response to some physiological requirement of the cell. Specifically, cysteine and methionine residues are especially sensitive to ROS (Arts *et al.*, 2015). Although heterotrophic bacteria, including some Alteromonas strains, may scavenge ROS and thus reduce potential oxidative stress affecting *Prochlorococcus* (Morris *et al.*, 2008; Morris *et al.*, 2011), other marine heterotrophic bacteria, again including Alteromonads, in fact produce extracellular superoxide (Diaz *et al.*, 2013). Additionally, the killing mechanism of many antibiotics ultimately involves the generation of ROS (reviewed by Dwyer *et al.*, 2009). In *E. coli*, cellular defenses against ROS include increased expression of enzymes involved in cysteine and methionine biosynthesis (Gebendorfer *et al.*, 2012), and it is tempting to speculate that the increase in the relative abundance of genes involved in cysteine and methionine biosynthesis in MIT9313 cells responding to co-culture may be related to a similar defense mechanism.

Do Prochlorococcus cells sense and specifically respond the heterotroph in co-culture?

Although many of the changes in gene expression we have observed can be rationalized as responses to changes in external or internal concentrations of nutrients or metabolites, in several cases these changes are suggestive of a specific response to the presence of other co-occurring bacteria. First, in all three co-cultures, changes were observed in the expression of genes involved in the biosynthesis or modification of peptidoglycan and lipopolysaccharides (Table 2, Figure 3). Peptidoglycan and lipopolysaccharides are components of the bacterial cell wall and outer membrane, and both have been suggested to mediate cell–cell recognition and signaling (Dworkin, 2014). Similar changes have been observed in different *Synechococcus* strains in response to co-culture (Tai *et al.*, 2009; Beliaev *et al.*, 2014). In response to nutrient starvation, the expression of these genes either did not change, or they were less abundantly expressed (Tolonen *et al.*, 2006; Martiny *et al.*, 2006; Thompson *et al.*, 2011). Second, in both MIT9313 co-cultures ABC transporters annotated as involved in the export of drugs or antimicrobials were more abundantly expressed, suggesting either that the cells were responding to the presence of toxic metabolites in the media or

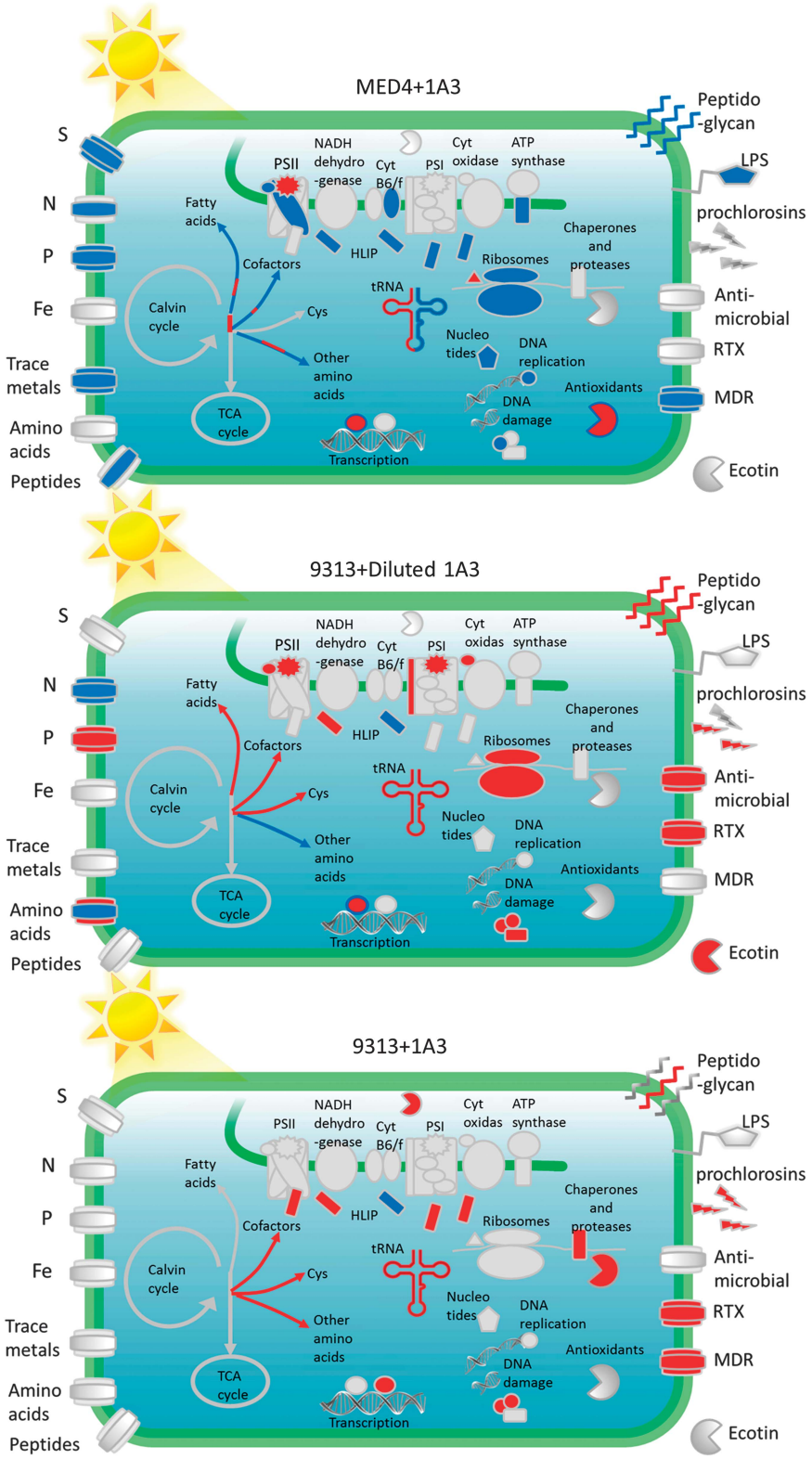


Figure 3 Schematic models of Prochlorococcus MIT9313 and MED4 showing the main cellular functions, metabolic pathways and transporters responding to co-culture based on the transcriptome changes after 20 h in co-culture. Cells take up nutrients on the left, process them in the middle of the cell and excrete substances on the right. Functions, pathways or transporters are shown as more abundantly expressed (red), less abundantly expressed (blue) or not differentially regulated (gray) relative to the axenic cultures. PSII/PSI, photosystem II and I; Cyt, cytochrome; Cys, cystein biosynthesis; RTX, hemolysin-like proteins containing RTX (repeats in toxin) domains; MDR, multi-drug resistance/macrolide transporter.

were producing such metabolites themselves (Table 2, Figure 3). Strikingly, when MIT9313 was co-cultured with both low and high densities of HOT1A3, changes in expression of many different prochlorosins were observed. Prochlorosins are peptide metabolites (lanthipeptides) produced by several strains of *Prochlorococcus* and *Synechococcus* and likely secreted from the cells (Li *et al.*, 2010). Their similarity to lanthibiotics suggests they may be involved in cell–cell signaling or allelopathy. Finally, genes with hemolysin-like or RTX (repeat in toxin) domains were also more abundantly expressed in MIT9313, as has previously been observed in proteomic analysis of secreted proteins from co-cultures between *Synechococcus* WH8102 and the α-proteobacterium *Ruegeria pomeroyi* DSS-3 (Christie-Oleza *et al.*, 2015). Although such genes are often annotated as involved in pathogenicity, their actual role is as yet unknown. Taken together, these results suggest that both *Prochlorococcus* strains, but especially MIT9313, were mounting a specific transcriptomic response to the presence of a different type of cells in the co-cultures.

Gene families encoding short peptides form a major part of the response of MIT9313 to co-culture

During the analysis of the transcriptional changes in co-cultures, we noticed two peculiarities about the response of MIT9313 as it was stressed by an inhibitive dose of *Alteromonas* HOT1A3 (9313+1A3): (a) much of the transcriptional response seemed to be clustered around three regions along the genome, which were also associated with the presence of many newly acquired genes (Figure 2d) (Kettler *et al.*, 2007; Thompson *et al.*, 2011). (b) Many of the genes that were differentially regulated had a short predicted protein product (~100 amino acids long, Figure 4a, Supplementary Figure S3). Recent studies in eukaryotes have shown that short genes are transcribed and translated rapidly compared with longer genes, perhaps providing a mechanism for fast response to changes in conditions or during development (Shah *et al.*, 2013; Heyn *et al.*, 2014). Such genes also evolve rapidly and may be important in shaping genomic novelty and evolutionary plasticity (Grishkevich and Yanai, 2014; Heyn *et al.*, 2014). Genes encoding for short proteins are common in *Prochlorococcus* (Figure 4c) yet, for the most part, their roles are still unknown (Zhaxybayeva *et al.*, 2007; Whidden *et al.*, 2014). A closer look at the differentially-expressed short genes revealed that some of them belong to at least four defined families. Two of these, encoding HLIPs and prochlorosins, have been discussed above. Two additional families were initially annotated in the genome sequences as 'kinesin motor domains' and hypothetical genes but in fact are two novel gene families encoding putative short proteins. We term these here co-culture responsive genes (CCRGs). The CCRG-1 gene family is found in most *Prochlorococcus* strains sequenced to date, as well as in one *Synechococcus* strain and

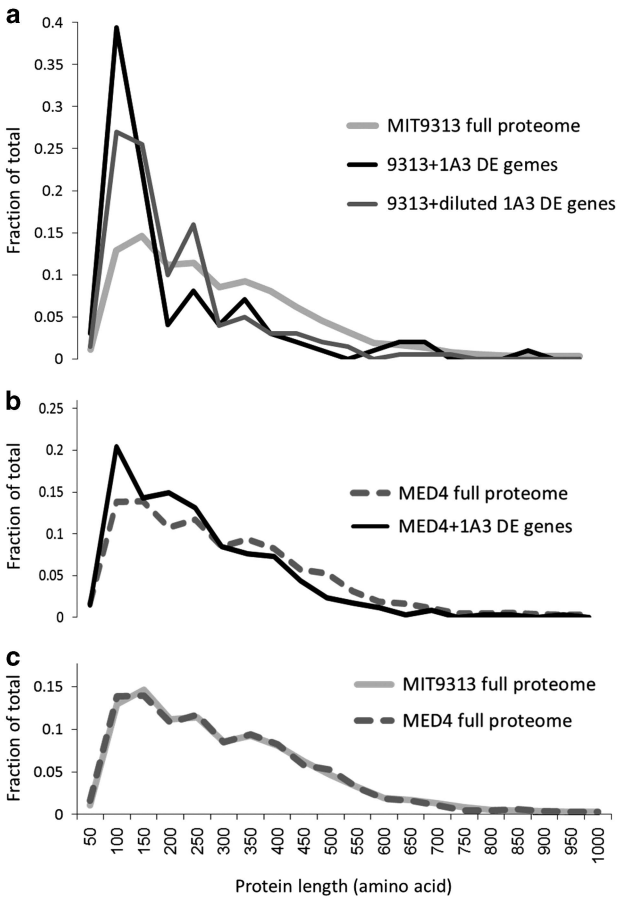


Figure 4 Genes encoding short (50–100 amino acids long) predicted proteins form a large fraction of the transcriptomic responses of Prochlorococcus MIT9313 to co-culture. (**a**, **b**) The distribution of predicted protein length of the DE genes in 9313 (**a**) and MED4 (**b**) compared with the full predicted proteomes of the two Prochlorococcus strains. Short proteins form almost half of the differentially-expressed genes in 9313+1A3, but are not overly abundant in MED4+1A3. (**c**) The distribution of predicted proteins in the full MED4 and MIT9313 proteomes is similar, suggesting that the relative abundance of differentially-expressed genes encoding short proteins observed in the MIT9313 transcriptomes is not a result of a different genome structure.

in one phage (Figures 5a and b). The phylogeny of the CCRG-1 gene family broadly corresponds to that of the strains themselves, suggesting that it originated before the *Prochlorococcus* diversification and potentially has a common role in the biology of these organisms (Figure 5b). Within the low-light IV *Prochlorococcus* clade, the CCRG-1 gene family has undergone at least two duplication events, including a very recent one within the MIT9313 lineage that resulted in four identical genes (Figure 5b). The predicted CCRG-1 protein products are not similar in sequence or predicted structure to any known proteins, and thus the role of this gene family is currently unknown.

CCRG-2 genes are found in the genomes of all low-light IV *Prochlorococcus* strains sequenced to date, as well as in the genomes of three *Synechococcus* strains, RS9916, WH8016 and CC9605 (Figure 5d).

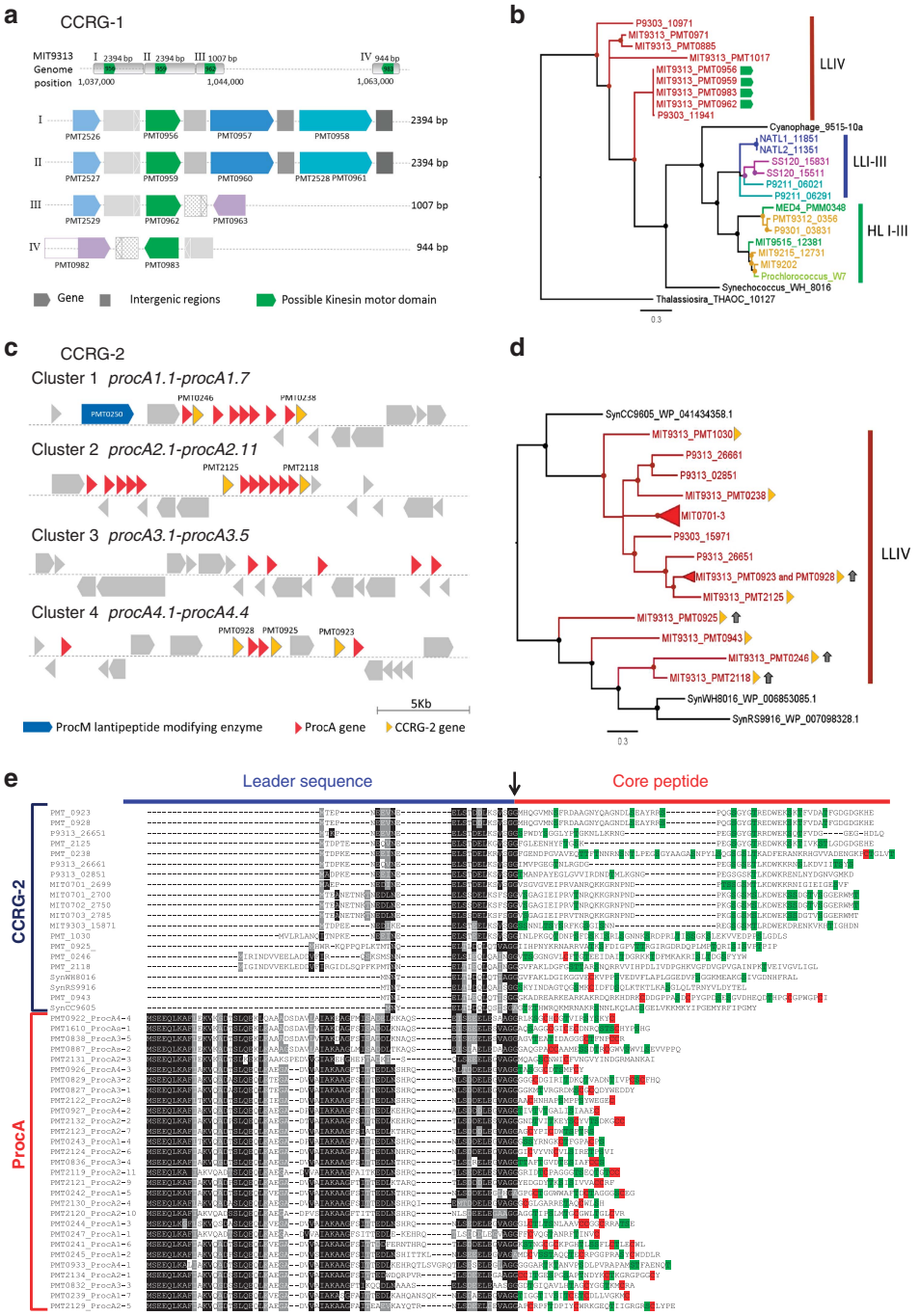


Figure 5 CCRG-1 and 2, two novel gene families encoding short, co-culture induced proteins. (**a**) CCRG-1 genes are found in a recently duplicated segment of the MIT9313 genome. Four repeats are found on the MIT9313 genome, two of which (marked I and II) are identical over 2394 bp, the two others are identical over smaller regions. (**b**) CCRG-1 genes are found in all *Prochlorococcus* clades. A Bayesian tree constructed from protein sequences is shown, with circles on the nodes denoting >0.5 probability. Clade assignment and color coding are according to Kettler *et al.* (2007). *Prochlorococcus* w7 is a sequenced genome from a single cell sorted from an environmental sample (Malmstrom *et al.*, 2012). Green arrowheads mark the genes shown in (**a**). (**c**) CCRG-2 genes (yellow triangles) are found in prochlorosin clusters at the same orientation as the prochlorosin genes. Two CCRG-2 genes (PMT0943 and PMT1030) are not found within a prochlorosin cluster, as are some of the prochlorosin genes themselves (Li *et al.*, 2010). (**d**) CCRG-2 genes are found only in *Prochlorococcus* strains belonging to the LLIV clade as well as in related *Synechococcus* strains. The phylogenetic tree was produced and colored as in (**b**). Strains MIT0701-3 are described in Biller *et al.* (2014a). Yellow arrowheads mark the genes shown in (**c**) and gray arrows mark upregulated genes in 9313+1A3 co-cultures. (**e**) CCRG-2 reveals similarity to Prochlorosins in the C'-terminus of the leader sequence. Residues identical and conserved among more than 50% of the sequences are highlighted in black and gray, respectively. Cysteine and serine/threonine residues potentially modified in lanthipeptides to form the lanthionine bridge (Li *et al.*, 2010) are marked in red and green, respectively. The arrow marks the GG/GA cleavage site.

Table 3 Identification by tandem mass spectrometry of CCRG-1 and CCRG-2 gene products in spent media and cells from an exponentially growing, axenic MIT9313 culture

Gene product	*Sequence*	*AUC (spent media, 1 mg)*	*AUC (cells, 2 mg)*
CCRG-1			
PMT0956	MYSLFDSVFDVPFGYSIPR**DRVVVIPDSQYNK**LRAQENERQVAKLEARKEHHSQVIER **LNEQISELQAALPAAEPDKELAATKE**	148.2 ± 147.9	7485.2
PMT0971	MDFDSQKPYTSRSGGDVYRRPALVLTTFLVRSEILMASCCRGLELNDMYSRF-DEVFNGPFGYTIPRDR**VVVIPDSAYK**AAQERQNAQRVARLEARRAEYLS**VVDQLEK QIAELQPSQQEPVPDKGLAAAKT**	13.5 ± 6.0	1336.2
PMT0885	MASCCRGLELHELYSRFDEDLNAPFGYTIPRDRVVVIPDSEYKAAQERQTAQRVAGLE-ARRAERLSVVDQLEKQIDELQPSQ	ND	ND
CCRG-2			
PMT0923	M**HQGVMNSFR**DAAGNYQAGNDLSEAYRRSPQGSGYGGTREDWEKSKTFVDATFG DGDGKHE	ND	95.6
PMT0928	MHQGVMNSFRDAAGNYQAGNDLSEAYRRSPQGSGYGGTREDWEKSKTFVDATFGD GDGKHE	ND	ND
PMT0925	**IIHPNY**KRNARRVATK**SFDIGPVTTRGIRGDRDQPLMPTQR**IS**ISPVPTPIP**	136.8 ± 124.1	ND
PMT2125	**FGLEENHYFTGSKPEGSG**YGGTREDWKKSKTIV**KSTLGDGDGEHE**	4.7 ± 1.9	ND
P9313_26651	SPWDYSGGLYPTGKNLLKRNGPEGSGYGGTRRDWEK**SQTFVDGGEGHDLQ**	12.8 ± 6.2	255.7
PMT0238	**FGENDPGVAVEQTTFTNNR**NSNTLPEGSGYAAGASNPYLSQGSGSGLTKADFERA NKRHGVVADENGKPCTGLVT	28.0 ± 29.4	69.7
P9313_26661	IMVPGEGTNLRGDGPEGSGSGETLKDWKKRNS**LKDVIITSYS**	153.2 ± 168.6	188.1
P9313_02851	TMANPAYEGLGVVIRDNDTMLKGNGPEGSGSGKTLKDWK**RENLNYDGNVGMKD**	22.9 ± 19.7	182.1
PMT0943	GKADREARKEARKAKRDQRKHDRKCDDGPPASDCPYGPDSESGVDHHEQDTHPG CGPWGPCI	ND	ND
PMT0246	NGVLC**FTGTEEIDAITDGR**KKTDFMKAKRISLTDGSFYYWR	ND	ND
PMT2118	GVFAK**LDGFGSTTAR**SNQRRVVIHPDLIVDPGHKVGFDVGPVGAIRNPKTVEIVGVLIGL	42.3 ± 15.8	ND
PMT1030	INLPKGQTDNPSFDSKISRLSGNNKSRDPRLTISSGKSLEKVVEDMPPSLGDLS	ND	ND

Abbreviation: CCRG, co-culture responsive gene. AUC represents the average area under the curve of the ion chromatograms of the three most intense peptides form each protein ($\times 10^6$), and is a semiquantitative estimate of the relative abundance of that protein in the two samples. Average and standard deviation for spent media are from two independent MS/MS runs of the same spent media collected using two different solid phase extraction columns (strata X and Strata XL, see Materials and methods). Underlined and bold amino acids represent peptides identified in one or both of the spent media analyses.

Intriguingly, with the exception of CC9605, these are the same strains of *Prochlorococcus* and *Synechococcus* that contain a gene encoding an ortholog of ProcM, and therefore likely produce prochlorosins (Li *et al.*, 2010). Additionally, in *Prochlorococcus* MIT9313 almost all of the CCRG-2 genes are found within the four prochlorosin clusters and in the same orientation of the prochlorosin structural genes themselves (ProcAs, Figure 5c). The predicted CCRG-2 gene products comprise a short leader peptide and a 41–75 amino-acid long core peptide (Figure 5e). In fact, the CCRG-2 leader peptide is very similar to the C'-terminus of the leader peptides of prochlorosins, including the 13-amino-acid sequence motif ending with an amino-acid diad (GG/GA) involved in cleavage, maturation and export of class II lantibiotics, bacteriocins and other secreted peptides (Figure 5e, Chatterjee *et al.*, 2005; Wang *et al.*, 2011). However, many of the predicted CCRG-2 core peptides do not contain cysteine residues and thus cannot be modified by the ProcM enzyme to form the lanthionine ring that is one of the hallmarks of lanthipeptides (Figure 5e). Notably, in some prochlorosin genes, transcription may start downstream of the predicted open reading frame, leading to translated proteins which lack much of the N'-terminal leader sequence (Voigt *et al.*, 2014). It is unclear whether these truncated prochlorosins can be recognized and modified by the ProcM enzyme.

To test the hypothesis that CCRG-2 genes encode secreted peptides, we collected spent media and cells from an exponentially growing, axenic MIT9313 culture, and searched for the CCRG-1 and CCRG-2 gene products using tandem mass spectrometry (MS/MS). As shown in Table 3, several of the CCRG-2 gene products were significantly more abundant in the spent media compared with the cell pellet, whereas the opposite was observed for CCRG-1. For some of the CCRG-2 products, we observed in the spent media peptides covering essentially all of the mature peptide, including the N' and C' termini (Table 3). Taken together, these results suggest that the CCRG-1 gene family encodes cellular proteins, whereas some CCRG-2 s are efficiently secreted into the media, and that at least some of these secreted peptides are unmodified. We therefore speculate that the CCRG-2 gene family, as well as the truncated prochlorosins, may encode novel peptides, which are not modified by the ProcM enzyme yet are secreted by the same pathway used by prochlorosins. Notably, CCRG-2 transcripts were identified in a meta-transcriptomic data set from the Gulf of Aqaba (Red Sea, Steglich *et al.*, 2015). The sequences from the Red Sea were similar in their

signal peptide yet encoded very different putative mature peptides, an observation reminiscent of the high diversity of putative prochlorosins identified in the Global Ocean Survey metagenomic data set (Li *et al.*, 2010) (Supplementary Figure S4).

Summary—common and unique responses to co-culture

As phytoplankton and heterotrophs interact in co-culture, they are affected by contrasting pressures to compete and cooperate. In our experimental system, the same heterotrophic bacterium elicits contrasting responses from two strains of *Prochlorococcus* that have distinct physiologies. Several interesting responses were evident: first, there is little evidence for non-specific interaction through competition for or recycling of nutrients. The *Prochlorococcus* cells in our experiments are apparently not nutrient-starved (Supplementary Figure S2), and while potentially synergistic interactions through the exchange of nutrients or organic compounds may be occurring (especially in MED4), additional studies are required in order to test this hypothesis. Second, core metabolic processes in the cell such as photosynthesis, amino-acid biosynthesis and translation are being affected by co-culture. Whether this is in response to specific metabolic exchange, to changes in the oxidative levels of the cell or to general cell stress is currently unclear. Third, both *Prochlorococcus* strains are likely responding specifically to the presence of other cells in co-culture, through modification of the cell wall and membrane (a common occurrence in co-culture; Tai *et al.*, 2009; Beliaev *et al.*, 2014) and, in the case of MIT9313, increased expression of genes potentially involved in the production of or response to antimicrobial or signaling compounds (infochemicals). These include transporters and the prochlorosin and CCRG-2 gene families. Taken together, these results suggest that these small, planktonic cells that live in the most nutrient-poor regions of the world and consequently have small and streamlined genomes, nevertheless have a 'genetic toolkit' enabling them to engage in complex chemically-mediated interactions. Finally, the most significant fraction of genes differentially expressed in co-culture compared with axenically growing cells have no known function. This highlights how much we have yet to learn about the mechanisms and molecules underlying microbial interactions.

Conflict of Interest

The authors declare no conflict of interest.

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