Copper toxicity response influences mesotrophic *Synechococcus* community structure

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Summary

Picocyanobacteria from the genus *Synechococcus* are ubiquitous in ocean waters. Their phylogenetic and genomic diversity suggests ecological niche differentiation, but the selective forces influencing this are not well defined. Marine picocyanobacteria are sensitive to Cu toxicity, so adaptations to this stress could represent a selective force within, and between, “species” also known as clades. We compared Cu stress responses in cultures and natural populations of marine *Synechococcus* from two co-occurring major mesotrophic clades (I and IV). Using custom microarrays and proteomics to characterize expression responses to Cu in the lab and field, we found evidence for a general stress regulon in marine *Synechococcus*. However, the two clades also exhibited distinct responses to copper. The Clade I representative induced expression of genomic island genes in cultures and Southern California Bight populations, while the Clade IV representative downregulated Fe-limitation proteins. Copper incubation experiments suggest that Clade IV populations may harbor stress-tolerant subgroups, and thus fitness tradeoffs may govern Cu-tolerant strain distributions. This work demonstrates that *Synechococcus* has distinct adaptive strategies to deal with Cu toxicity at both the clade and subclade level, implying that metal toxicity and stress response adaptations represent an important selective force for influencing diversity within marine *Synechococcus* populations.

Introduction

Marine picocyanobacteria, including *Synechococcus* and its sister taxa *Prochlorococcus*, are a globally distributed and ecologically significant group (Scanlan *et al.*, 2009) that are surprisingly diverse (Ferris and Palenik, 1998; Rocap *et al.*, 2002; Zwirglmaier *et al.*, 2007). While marine ecologists have been interested for decades in understanding how ecologically similar microbes, such as related phytoplankton species, can coexist (Hutchinson, 1961), marine *Synechococcus* diversity, and mechanisms driving it, are not understood.

Upwards of 20 phylogenetically distinct clades of marine *Synechococcus* have been recognized using different genetic markers (Palenik, 1994; Ahlgren and Rocap, 2006; Penno *et al.*, 2006), but their distinct ecophysiologies have not been well characterized. Distinguishing adaptations have been defined for some cultured representatives, such as motility (Toledo *et al.*, 1999), but while factors such as temperature and macronutrient availability appear to drive distribution of these clades globally (Zwirglmaier *et al.*, 2008; Flombaum *et al.*, 2013; Sohm *et al.*, 2016), temporal variations in distribution are not explained by these factors alone. For example, in mesotrophic California waters, two clades of marine *Synechococcus*, clades I and IV, co-occur. Clade IV is the dominant clade most of the year at a coastal monitoring site, but trades off seasonally with Clade I, for unknown reasons (Tai and Palenik, 2009; Tai *et al.*, 2011).

Copper concentrations and chemical speciation are dynamic in the marine environment, and maintaining metal homeostasis under changing Cu conditions is a challenge for marine phytoplankton. Coastal marine environments are especially challenging since metal concentrations can fluctuate rapidly as a result of seasonal runoff and mixing (Blake *et al.*, 2004). The bioavailability and corresponding toxicity of Cu is a function of Cu speciation, with the concentration of the free, hydrated Cu^{2+} ion species considered the most bioavailable form of Cu to marine microorganisms (Sunda and Guillard, 1976). *Synechococcus* is especially prone to Cu toxicity as compared to the other major marine phytoplankton groups, exhibiting declines in growth rates at Cu^{2+} concentrations on the order of 10^{-12} M in laboratory cultures (Brand *et al.*, 1986). In natural assemblages, Cu can also influence phytoplankton distribution. For example, Cu addition experiments in coastal and open ocean sites have shown higher sensitivity of *Synechococcus* to Cu toxicity relative to other phytoplankton groups, and additions of aerosols with Cu also cause decreases in *Synechococcus* abundance (Le Jeune *et al.*, 2006; de la Broise and Palenik, 2007; Paytan *et al.*, 2009; Debelius *et al.*, 2010). Furthermore, Cu^{2+} levels (both natural and anthropogenic) have been shown to influence marine picocyanobacteria distributions (Moffett *et al.*, 1997; Mann *et al.*, 2002). Phytoplankton, including *Synechococcus*, also change metal bioavailability in surface waters by both uptake and production of strong extracellular Cu-binding ligands, which reduce Cu toxicity by decreasing the amount of Cu present as Cu^{2+} (Bruland *et al.*, 1991; Moffett, 1995; Moffett and Brand, 1996; Croot, 2003).

Within *Synechococcus,* clades or strains also have variable Cu tolerances and Cu quotas, which may influence their distribution both with geographic location and depth (Debelius *et al.*, 2009; Stuart *et al.*, 2009; Guo *et al.*, 2012). Despite this evidence that metal toxicity and stress response are important in shaping *Synechococcus* ecophysiology, how metal toxicity shapes *Synechococcus* community structure is not well understood.

Understanding the response to Cu stress and the related phenomenon of oxidative stress could help us to understand the role of environmental stress in population diversity within *Synechococcus*. Our previous work on cultures found that while mesotrophic (coastal)

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strains were more tolerant than open ocean strains, mesotrophic Clade IV-strain (*Synechococcus* sp. strain CC9902) was more Cu-sensitive than mesotrophic Clade I-member strain (*Synechococcus* sp. strain CC9311) (Stuart *et al.*, 2009). Furthermore, CC9311 has genes in horizontally transferred regions of its genome that provide Cu and oxidative stress tolerance. Abundance of these genes at a coastal monitoring site fluctuates seasonally, indicating that changing Cu levels could be an important environmental stressor to mesotrophic marine *Synechococcus* (Stuart *et al.*, 2013). We predicted that CC9902 would have a different transcriptional response to Cu than CC9311, which may provide insight into the differing ecological strategies of these two major co-existing clades.

In order to look at Cu response in cultures and natural communities, we designed a custom expression microarray with probes for all genes in two representative genomes: strains CC9902 (clade IV member) and CC9311 (clade I member). While metatranscriptomics can capture response of the abundant populations, we predicted these arrays would be advantageous for detection of strain specific low-level responses. While development of microarrays that can be used to look at key responses of many of the major marine microbial groups (Shilova *et al.*, 2014) have been applied, utilizing arrays to examine expression of specific marine populations, such as these two *Synechococcus* clades, has not been widely applied. We tested the array first on cultures of CC9902, whose response to Cu shock was previously unknown. We compared the expression results with proteomic analyses to further define Cu-shock response in CC9902. To look at the response of natural marine *Synechococcus* populations to Cu stress we conducted an expression analysis following Cu additions to seawater samples. A two-week experiment was also designed to gauge the potential for Cu tolerant subpopulations in a natural coastal marine *Synechococcus* community. Investigating the Cu stress response in this manner helps provide insight into how two phylogenetically distinct clades of *Synechococcus* can co-exist in dynamic, mesotrophic environments.

Results and Discussion

Cu gene expression analyses: CC9902 cultures

Using custom Agilent microarrays, we examined gene expression in response to a two hour copper shock treatment (10 µM CuEDTA, approximately –log[Cu^{2+}] 10.1), relative to a control. This level of Cu results in growth inhibition and a sharp decline in cell counts for CC9902 within 3 days of addition (Stuart *et al.*, 2009). Growth rate decreases relative to the control can be seen at -log [Cu^{2+}] of 11.1 and higher for CC9902 and -log [Cu^{2+}] of 10.5 and higher for CC9311 (Stuart *et al.*, 2009). This high level of copper was chosen in order to ensure activation of the stress response during the two hour incubation, and in order to compare with previous microarray results for strains CC9311 and WH8102 (Stuart *et al.*, 2009). We identified 159 genes that were significantly differentially expressed in response to 2 hours of Cu stress compared to a no-Cu control condition. Of these 159 differentially expressed genes, 125 were upregulated and 34 were downregulated (Fig. 1, Table S1).

To further verify the Cu response in CC9902, we looked at protein expression changes in response to Cu stress. Duplicate control and 8 hour Cu incubated samples were analyzed using shotgun proteomics with iTraq isobaric tags for improved relative quantitation (Zieske, 2006), and 559 proteins were identified. Of these, 62 were significantly differentially expressed in the Cu samples, with 32 upregulated and 30 downregulated (Table S1). When compared with the array results, 20 had matching regulation (Fig. 2).

A general stress regulon in marine Synechococcus

Response of the mesotrophic strain CC9902 was more similar to oligotrophic strain WH8102 than its mesotrophic cousin (Fig. 3). Unlike CC9311, CC9902 did not highly upregulate genes in genomic islands, but instead upregulated a set of core marine *Synechococcus* genes. This set of conserved genes provides evidence for a general stress regulon in marine *Synechococcus.* Tetu and colleagues (2013) predicted a set of 36 genes that were co-regulated under several stress conditions in WH8102 (of which 34 were well conserved in marine *Synechococcus*). CC9902 upregulated 13 of these genes at the transcript level (Fig. 1, red), all of which were also upregulated at the protein level (Fig. 2, red). Likewise, of the 52 genes that were differentially regulated in both CC9902 and CC9311 nine of these were part of the predicted stress regulon (Fig. 1, red). Almost all of these putative stress regulon genes were upregulated in CC9902 and WH8102 and downregulated in CC9311, which matches their respective Cu sensitivities (Stuart *et al.*, 2009). In addition, there were two genes, a high light inducible protein (Syncc9902_1000), and a chaperone (*cpn60* Syncc9902_1747), which followed the same pattern of upregulation in CC9902 and WH8102 and downregulation in CC9311, that may also be part of the general stress regulon.

When we place this new Cu expression data in the context of other published transcriptional stress response in marine *Synechococcus*, many of the genes in this putative stress regulon from WH8102 appear to be commonly regulated in response to various stressors (Fig. 4). For example in WH7803, of the 36 predicted stress regulon genes, 14 were significantly differentially expressed in response to high-light hydrogen peroxide stress, and 12 in response to high-light methyl viologen stress (Fig. 4).

Included in this stress regulon is a two-component response regulator with a LuxR domain (NarL/crr1/ycf29), Syncc9902_2107, which appears to be universally conserved in cyanobacteria (Ashby and Houmard, 2006), and may be involved in regulation of some genes in this group. Marine *Synechococcus* genomes have from 10-18 two-component response regulators, far less than other members of the genus (Palenik *et al.*, 2003; Scanlan *et al.*, 2009), so the dedication of one of these master regulators to general stress response indicates the importance of this pathway for marine *Synechococcus*.

Specific Responses: Fe-related stress

The other trend in the protein-validated data was a significant down-regulation of genes associated with Fe-limitation response. This downregulation was most notable in the i*diA* (also called *futA*) genomic region, which was almost completely downregulated in CC9902 at both the transcript and protein level (Fig. 5). IdiA is a well-conserved Fe-binding periplasmic protein that is involved in Fe^{3+} transport, is induced by Fe-limitation in two *Prochlorococcus* strains, and has been used as an Fe stress transcript and protein biomarker (Webb *et al.*, 2001; Thompson *et al.*, 2011; Saito *et al.*, 2014). Along with the idiA region, CC9902 down-regulated other Fe-stress related genes including Syncc9902_1005, which is a chlorophyll-binding protein that is iron-limitation induced in some cyanobacteria (Boekema *et al.*, 2001), and an FeS-containing aconitase (Syncc9902_2296). FeS cluster assembly genes were also significantly upregulated in CC9902 and CC9311 (Fig. 1, blue). Excess Cu is known to destabilize FeS clusters in dehydratases and FeS cluster formation in *E.coli* (Macomber and Imlay, 2009; Chillappagari *et al.*, 2010), so this may be the case in *Synechococcus* as well. This was the one response shared by two mesotrophic strains only, which is not surprising considering the very different metal chemical speciation environment experienced by the mesotrophic strains as compared to the open ocean strain.

The quick shutting down of so many Fe-limitation related genes and proteins by CC9902 indicates that the high-affinity Fe-transporter may be a particular liability under high Cu levels, perhaps by allowing Cu to enter the cell. A similar response was suggested for two *Prochlorococcus* strains, which have variable Cu tolerances. *Prochlorococcus* strain MIT9313 is more sensitive to high Cu, but better able to cope with low Fe, perhaps like CC9902. *Prochlorococcus* strain MED4 is more Cu tolerant, like CC9311, but has higher Fe quotas (Mann *et al.*, 2002; Thompson *et al.*, 2011). From what is known of metal transport in phytoplankton, metal specificity is not absolute: Cu can inhibit high-affinity Zn uptake, for example (Sunda and Huntsman, 1998). Furthermore, from genome comparisons of the two mesotrophic strains it is clear that CC9311 has a higher potential for both metal transport and storage (Palenik *et al.*, 2006; Scanlan *et al.*, 2009). In particular, CC9902 lacks a gene upregulated in CC9311, DpsA, an oxidative stress induced DNA-binding protein that is involved in Cu homeostasis in *E.coli* and internal Fe transport in cyanobacteria (Shcolnick *et al.*, 2007; Thieme and Grass, 2010). This may be relevant in field populations as well, since incubation experiments with additions of Fe and Cu in the subarctic Pacific indicate that Cu additions impacted the smaller size classes of phytoplankton that were Fe-limited (Semeniuk *et al.*, 2016).

Cu gene expression analyses: Field populations

To determine whether the genes identified in strains CC9311 and CC9902 are also important in the Cu stress response of a field population of marine *Synechococcus*, samples from a station in the Southern California Bight (Fig. S2A) were incubated with and without Cu additions and RNA was isolated, amplified and hybridized to the arrays. Concentrations of Cu^{2+} went from log [Cu^{2+}] of -14.8 to -13.4 (Table 1) in the Cu-spiked incubations. This

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indicates our 8 nM total Cu addition was sufficient to bind the organic Cu-binding ligands (of unknown composition) in the seawater samples and increase free Cu^{2+} concentrations. Notably, the increase was smaller than in our culture experiment in which 10 μM CuEDTA was added, resulting in a log [Cu^{2+}] of -10.1.

Comparing array signal intensities between the culture and field samples, specificity was significantly correlated to known clade abundance and gene expression in culture. Previous DNA analysis of the *Synechococcus* community at this sampling station had shown that Clade IV was at least 5-fold more abundant than Clade I (which includes CC9311) in the surface waters (Tai *et al.*, 2011), and the signal intensities roughly confirm this pattern. The median signal intensity for the CC9902 genes from the array was 172.6 while it was only 69.9 for CC9311 genes. Genes with high signal intensities (top 100 genes) included most of the photosynthetic apparatus, phycobilisome genes, the ribosomal genes and ribulose-bisphosphate carboxylase and this was similar to CC9902 gene expression in culture (Fig. S3). This suggests the results are biologically comparable to culture studies for many genes.

In general, the significant genes from the CC9311-based gene probes had a much higher overlap with CC9311 culture-based gene induction than CC9902. As is to be expected from RNA samples from a mixed community, there was a high amount of variability between the replicates and much lower average signal intensity (leading to more signal error) than for the culture-based studies. Consequently, a false discovery rate (FDR) cutoff of 5%, as was used for the culture-based experiments, yielded only a few significant genes (14 CC9902-like genes and 23 CC9311-like genes). In order to get a broader range of genes we raised the FDR cutoff to 15%, which yielded 81 genes from the CC9902 genome and 93 genes from the CC9311 genome. Given this high FDR, we report the FDR associated with each gene as an additional measure of the significance of detected expression changes (Table S1). Of the 93 significant genes upregulated in culture-based experiments (Stuart *et al.*, 2009), we detected an increase of 32 in the natural population (Fig. 6). The CC9902-based gene set had only 4 genes that overlapped with the culture studies. Oxidative stress and Fe homeostasis response was detected in the field response. FeS assembly response was detectable in both strains – SufE (Syncc9902_0705, 5.03%) and SufS (sync_2480, 12.19%) were induced in the community samples (Table S1). Additionally, genes such as rubredoxin (sync_0241, 7.57%), glutathione-S transferase (Syncc9902_1396, 10.35%) and several genes involved in DNA damage (Syncc9902_2205, 9.60% Syncc9902_0723, 0%, and Syncc9902_1301, 4.09%) were induced.

Significantly, in field populations there was induction of the genomic island genes from CC9311-based probes (Fig. 6) that were Cu responsive in the culture-based experiments, and have been found to confer Cu tolerance in culture (Stuart *et al.*, 2013). Given the potentially low abundance of these genes in field populations (Palenik *et al.*, 2009; Stuart *et al.*, 2013), we did not expect to have the sensitivity to detect this response in the field community. Detection of induction of these low-abundance transcripts demonstrates the utility of custom arrays for this type of application. An ECF sigma factor, sync_1494 (7.33%), was the most highly induced gene in both the field and culture, and is immediately upstream of sync_1495, which has been shown to confer tolerance to Cu and oxidative stress in CC9311 (Stuart *et al.*, 2013). Metatranscriptomic analyses of marine samples have shown that *Prochlorococcus* genomic island genes are highly expressed relative to their metagenomic abundance as compared to other genes (Frias-Lopez *et al.*, 2008), and based on our results, this appears to be the case for these marine *Synechococcus* genes as well.

The low overlap between the copper response of CC9902 cultures and field populations suggests that CC9902 may not be representative of field populations at our site with respect to Cu response. Tai *et al.* found a decrease in abundance of some clade IV subgroups with depth (including the CCC9902 subgroup) while others increased (Tai *et al.*, 2011). Fe and Cu levels generally increase with depth and it could be that some subclades of Clade IV are better suited to deal with these higher metal levels. Although many of these subclades do not have sequenced representatives we hypothesize that their genomes may contain stress-related adaptations that allow them to thrive in higher metal conditions compared to reference strain CC9902.

Probing for Cu stress resistant subpopulations

In order to address this hypothesis, a two-week experiment was designed to detect and isolate *Synechococcus* populations that were able to rebound following Cu toxicity. Samples were taken from the end of the SIO pier in La Jolla, CA (Fig. S2B, 32°53’N, 117°15’W), where preliminary Cu speciation indicated that an addition of 31.5 nM of $CuSO_4$ would result in a Cu^{2+} concentration of approximately -log [Cu^{2+}] 11.5. Our final Cu^{2+} concentration in the Cu-treated incubations was approximately –log [Cu^{2+}] 11.5 (Table 2). This Cu addition, which was much higher than the addition for field expression analyses (which increased from -log [Cu^{2+}] of 14.8 to 13.4), resulted in a rapid, significant decrease of the *Synechococcus* population (Fig. 7). By day 11, *Synechococcus* numbers began to rebound in the Cu-treated samples (Fig. 7). Compared with other *in situ* Cu additions, this addition was similar to 31.9 nM additions from de la Broise *et al.* (2007), which yielded similar results. This field Cu addition was lower than our culture-based Cu additions (-log [Cu^{2+}] of 10.1, 10 µM), and lower than the threshold of -log [Cu^{2+}] of 11.1 for growth declines in CC9902 in culture.

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Mann *et al.* (2002) also found some variability between *in situ* and culture based growth rate declines in response to Cu, where *Synechococcus* growth declined at (-log $[Cu^{2+}]$ of 11.04) at one site, and no growth declines in culture with up to -log $[Cu^{2+}]$ of 10.0.

Synechococcus Clade IV members persisted in both the Cu-treated and control incubations, but subclade membership may have shifted in the Cu-treated incubations. QPCR results showed that Clade IV maintained its dominance relative to Clade I in both treatments over time, with an average of 71±11% and 84±6% in the control and Cu-treated conditions, respectively (Fig. S4). *Rpoc1* clone libraries made from the final day of the experiment (Day 14) showed there were different dominant subgroups of both Clades I and IV in the Cu-treated community relative to the control (Fig. 8).

We isolated and cultured a member of one of these dominant subgroups from the day 14 Cu-treated incubations, *Synechococcus* RhCu13. From *Rpoc1* gene sequence analysis this strain was found to be from Clade IV (Fig. 8). Marine *Synechococcus* is quite sensitive to methyl viologen, which generates superoxide anions ($O_2^{\cdot -}$) (Imlay, 2008), and has some overlapping response with Cu stress (Stuart *et al.*, 2013). We tested the oxidative stress tolerance of Strain RhCu13 and found it was able to grow after a 50 nM methyl viologen addition, albeit slower than the control condition, whereas CC9902 did not grow (Fig. S5). Together, these results show that Clade IV harbors subclades that have distinct higher stress tolerances than CC9902.

Conclusions

Cu toxicity and the oxidative stress response play an important role in shaping mesotrophic marine *Synechococcus* diversity. Our incubation studies show that less abundant subclades harbor higher levels of stress tolerance, indicating there are tradeoffs involved with regards to Cu tolerance. The expression response of strain CC9902 suggests that one of these fitness tradeoffs for gaining tolerance to Cu toxicity may be alterations to iron homeostasis. Our expression analyses also demonstrate that a general stress response represents a conserved *Synechoccocus* trait.

We did not find evidence that Cu tolerance alone was advantageous to one clade over the other in field populations—the subpopulation that recovered from Cu toxicity had members from both Clades I and IV. Yet, our expression analyses highlight the distinct expression responses of these two groups to Cu toxicity. Together, this suggests that these two phylogenetically divergent clades, which co-occur globally (Sohm *et al.*, 2016), are both well adapted to their mesotrophic niche, where Cu fluctuations are common, but have evolved separate, convergent, strategies to deal with this common stressor. Therefore, while it is unlikely that Cu fluctuations alone drive the temporal shifts in clade dominance seen at a coastal monitoring site (Tai and Palenik, 2009), it is clear from our expression analyses that the two clades have distinct tradeoffs and sensitivities that may contribute to their temporal shifts.

Our results show the importance of Cu toxicity in facilitating intra-clade diversity. Cu toxicity is likely a selective pressure that enables maintenance of diversity within the clade (species) (Cohan and Perry, 2007), and horizontally acquired genomic island genes are the mechanism for enhanced Cu tolerance. Hypervariable genomic islands are a distinctive feature of marine picocyanobacteria genomes (Palenik *et al.*, 2003; Coleman *et al.*, 2006; Palenik *et al.*, 2009). Our previous work characterized CC9311 genomic island genes that increased Cu tolerance, and further documented shifts in abundance of these genes within field populations (Stuart *et al.*, 2013). Remarkably, using custom arrays we are able to detect induction of these genes in response to Cu in field populations where Clade I is not dominant, demonstrating the utility of these arrays. Recent evidence supporting the changes in abundance we observed from sister clade *Prochlorococcus* single cell genomes show that subpopulations, with distinct genomic backbones and genomic islands, fluctuate over seasonal timescales (Kashtan *et al.*, 2014). Given the dynamic nature of mesotrophic marine Cu levels, there may be high-Cu microenvironments which help these subpopulations to persist. For example, marine *Synechococcus* have been found attached to sinking particles and in sediments and there is some evidence that they may be able to survive heterotrophically (Lochte and Turley, 1988; Cottrell and Kirchman, 2009; Sohrin *et al.*, 2011). These particles could potentially have higher metal levels (Huang and Conte, 2009; Boyd *et al.*, 2010) than found in the surface water. The ability to maintain and draw upon this pool of diversity within subpopulations helps explain *Synechococcus* success in dynamic mesotrophic environments.

Experimental Procedures

Cell culture and growth assays. 1 L or 2 L batch cultures of *Synechococcus* sp. strain CC9902 were grown in chelexed SOW media with stirring as previously described in Stuart *et al*. (2009), with the exception that CC9902 is not axenic and heterotrophic bacteria were present at low levels throughout growth based on qualitative microscopy. While it is possible that these bacteria may have produced Cu-binding ligands, which would result in higher Cu tolerances for CC9902 than measured, since CC9902 is more sensitive than CC9311, this does not change our interpretation. Additionally, other studies have found that contaminating bacteria do not influence *Prochlorococcus* Cu tolerance (Mann *et al.,* 2002).

Media constituents were as follows: NaCl (4.2×10^{-1} M), Na_2SO_4 (2.88 × 10^-2 M), KCl (9.39×10^{-3} M), $NaHCO_3$ (3×10^{-3} M), KBr (8.4×10^{-4} M), H_3BO_3 (4.85×10^{-4}), NaF (7.14×10^{-5} M), $MgCl_2{\bullet}6H_2O$ (5.46×10^{-2} M), $CaCl_2{\bullet}2H_2O$ (1.05×10^{-2} M), $SrCl_2{\bullet}6H_2O$ (6.38×10^{-5} M), $NaHPO_4$ (7.7×10^{-5} M), $NaNO_3^-$ (2×10^{-3} M), Na_2CO_3 (9.4×10^{-5}), Na_2 EDTA•$2H_2O$ (1.345×10^{-5} M), $ZnSO_4{\bullet}7H_2O$ (7.72×10^{-7} M), $MnCl_2{\bullet}4H_2O$ (7.07×10^{-6} M), $CoCl_2{\bullet}6H_2O$ (8.59×10^{-8} M), $Na_2MoO_4{\bullet}2H_2O$ (1.16×10^{-6} M), $FeCl_3$ (7.4×10^{-7} M), Na_2SeO_3 (5×10^{-8}), $NiCl_2$ (5×10^{-8} M), Citric acid hydrate (3×10^{-5}), Vitamin B_{12} (1 µg L^{-1}), Thiamine HCl (200 µg L^{-1}), D-Biotin (1 µg L^{-1}), Cultures were grown in acid-washed polycarbonate bottles, following trace metal clean procedures.

RNA extraction. For microarray expression triplicate 2 L cultures of both no-Cu controls and 10 µM 2 hour CuEDTA addition were grown up to mid-exponential and harvested by centrifugation and RNA was extracted as described in Stuart *et al.* (2009). Details are described in supplemental methods.

Array expression analyses. Agilent expression microarray was designed using Agilent's microarray design website (earray.chem.agilent.com). An custom single color 8 X 15k slide (8 arrays per slide) was designed with 150004 60 bp oligonucleotide probes based on all annotated genes in both *Synechococcus* sp. strain CC9902 and *Synechococcus* sp. strain CC9311. Each gene had three unique probes optimized to span the length of the gene. A randomized design was used with 20 randomized replicate probes in addition to Agilent-designed controls. RNA processing and hybridization are described in supplemental methods.

Microarray results were analyzed using Agilent GeneSpring GX software to normalize signal intensity across arrays and generate both gene-level and probe level signals. Log_2 transformed signal ratios were then calculated on each replicate Cu and no-Cu control and Statistical Analyses of Microarrays algorithms used with a 5% (or 15%) false discovery rate (FDR) cutoff and a 0.4/-0.4 $\log_2$ fold change cutoff as described in Stuart *et al.* (2009) to generate significant gene hits and probe hits. When hybridized with CC9902 culture cDNA, the median signal intensity from CC9902-designed probes was 3300 whereas the median signal for CC9311-designed probes was 14.9, demonstrating array probe specificity was high. There were 64 CC9311 genes that did have an average signal intensity above 1000 in the CC9902 culture-based study (Table S1). This included genes that were well conserved between these two strains making specific probe design difficult. In the field analyses these 64 genes were not included in comparative analyses between the groups and are considered as non-specific within marine *Synechococcus.* Real time PCR (QRTPCR) validation described in supplemental methods (Fig. S1, Table S2). Custom array design files and raw and processed experimental data have been deposited in NCBI's Gene Expression Omnibus (Edgar and

Lash, 2002) and are accessible through GEO Series accession number GSE89262 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89262).

Proteomics analyses. Two 1.5 L cultures were grown up to mid-exponential at which point they were combined and re-separated and 10 μM CuEDTA was added to one. After 8 hours both were harvested by centrifugation and this process was repeated for a duplicate set. Crude protein extract was extracted for all samples as done in Johnson *et al.* (2011). Samples were trypsin digested and labeled with isobaric tags (iTRAQ, ABSCIEX, (Ross *et al.*, 2004)), described in detail in supplemental methods.

The collected data were analyzed using and ProteinPilot software v.4.0 (Applied Biosystems, MDS-Sciex) to identify and quantify proteins with the Paragon search algorithm (Shilov *et al.*, 2007). The database used for the search included *Synechococcus* CC9902 proteins and a reverse decoy database generated by ProteinPilot, which also generated a false discovery rate (FDR) for proteins detected. Proteins with less than two unique peptides hits at 95% confidence were discarded (136 proteins, 19% of total) and duplicate ratios were averaged. Ratio cutoffs with an average $\log_2$ fold change above 0.3 or below -0.3 (+/- one standard deviation between replicates) were considered significant. Duplicates with opposite regulation (that is, one $\log_2$ fold change greater than 0.3 and one less than -0.3) were discarded (18 proteins, 22% of total). Proteomics data have been deposited in the MASSIVE repository (MSV000080326).

Cu incubations and sample collection. Samples were collected in July 2007 on a research cruise in the Southern California Bight from surface waters (5 m depth) in a mesotrophic region (Fig.S2 A, 32.513°N;-118.214°W). Triplicate bottles of Cu-spiked (8 nM $CuSO_4$) and no-Cu control samples of seawater were incubated onboard for two hours. Samples were collected in a 30 L Teflon-lined GoFlo bottle on a synthetic, non-metallic line. All samples were collected and processed using trace metal clean techniques in a clean van. Six 2.7 L polycarbonate bottles were rinsed and filled, and, to three of them, 8 nM CuSO4 was added. All bottles were incubated onboard in a flow-through incubator shaded to 1% light. After 2 hours, all bottles were prefiltered through an 11 μm filter to remove larger eukaryotic phytoplankton and then microorganisms were filtered onto a 0.2 μm Sterivex filter unit (Millipore, Billerica, MA) using a peristaltic pump for 30 min, snap frozen in liquid nitrogen and stored at -80°C until processed. 250 ml of sample from each bottle was also 0.2 μm filtered (acid-washed polycarbonate filters) and stored at -20°C for Cu speciation measurements. *Synechococcus* cell counts were between 4.5 and 5.1 x 10^4 cells/ml in all samples before and after the two-hour incubations, indicating no significant cell death occurred as a result of the 2-hour Cu shock.

Pier collection. 50 L of water from the Scripps Institution of Oceanography (SIO) pier was collected on August 8, 2011 in an acid washed carboy using a trace metal clean pumping system and aliquoted into four 2.7 L acid-washed polycarbonate bottles in a laminar flow hood. Also collected using a trace-metal clean pole sampler was 500 ml water for Cu speciation measurements. 31.5 nM $CuSO_4$ was added to two bottles (Cu-treated bottles) and sterile milliQ water was added to the other two bottles (no-Cu control bottles). Bottles were incubated in shaded rooftop incubators with constant flow-through of seawater for 14 days ("Day 0" is the day of sample collection). All subsequent sampling from bottles was done in a laminar flow hood. 1 ml samples were taken daily from all 4 bottles and fixed for 10 min in 0.25% glutaraldehyde after which they were frozen at -80°C until analyzed. On days 0, 2, 5, 11 and 14, 250 ml from each bottle was removed in the laminar flow hood and filtered onto a

0.2 µm Supor filter (Pall Corporation, Port Washington, NY) and the filter was frozen at -80°C. After removal of the 250 ml volume the bottles were left uncapped to equilibrate in the laminar flow hood for approximately 1 hour and then replaced in the incubator.

Cu speciation measurements. Cu measurements were conducted as described in Bundy *et al.* (2013), using competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV), described in detail in supplemental methods. For pier experiments, total dissolved Cu was determined the week before the experiment took place, to give a general idea of ambient Cu concentrations to be used for speciation calculations (due to the 8-hour UV oxidation time necessary for determination of Cu totals). This total dissolved Cu concentration was used for speciation calculations on the day of the experiment, in order to quickly determine the appropriate concentration of the Cu spike. To determine how much Cu should added to the seawater in order to achieve toxic Cu conditions (log[Cu^{2+}] = -11.5), a filtered seawater sample was analyzed using CLE-ACSV before setting up the experiment. The speciation results were then plotted using Cu* versus Cu^{2+} (Moffett *et al.*, 1997; Buck and Bruland, 2005), and interpolated where ambient dissolved Cu concentrations would lead to toxic Cu conditions (log[Cu^{2+}] = -11.5). The ambient total dissolved Cu concentration was then subtracted from the determined Cu* concentration to calculate the necessary Cu spike. The calculated Cu spikes are shown in Table 2.

Flow cytometry, DNA extraction and QPCR. Synechococcus and picoeukaryote abundance was assessed using flow cytometry, wherein *Synechococcus* cells are distinguished by the fluorescence of the phycoerythrin pigments. Samples were analyzed using the highest flow rate (approximately 45 µl/min) on a FACsort flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) as described in Tai and Palenik (2009). DNA was extracted from filters as

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described in Tai and Palenik (2009). QPCR was performed on a Qiagen RotorGene-Q with Promega GoTaq qPCR mastermix according to the manufacturer's specifications using Clade I and IV primers from Tai and Palenik (2009).

Enviromental clone libraries. RPOC libraries were constructed from DNA samples from day 14 of both the Cu and no-Cu control (replicate 1) bottles as described in Tai and Palenik (2009) with the following exceptions. PCR was done with GoTaq master mix (Promega, Madison, WI) and cyanobacteria-specific primers that amplify 841 bp of the *rpoC1* gene as described in Tai *et al.*, 2009 (17). The PCR products were ligated into the pCR®2.1-TOPO® vector (Life Technologies, Carlsbad CA) and cloning performed according the manufacturer's specifications. 10-20 colonies were picked from each cloning reaction and submitted for sequencing. Resulting 22 sequences (11 of each Cu and Control treatments) have been deposited in GenBank (accession numbers KY053360- KY053381).

Isolation and culturing of putative oxidative stress resistant strains. On day 14 of the Cu incubation study, aliquots of the Cu treated samples were diluted (1:10, 1:100 and 1:1000) and plated on seawater based (SN) agar plates and incubated at 23°C under constant light for 2-3 weeks. Once colonies became visible they were picked into 2ml of SN media grown up and maintained in 50 ml batch cultures. Methyl viologen growth assays were carried out in acid-washed glass tubes, with three biological replicates of each strain. Triplicate cultures of the different strains were grown in SN media to early exponential and 50 nM or 100 nM methyl viologen was added as well as control with no methyl viologen added. Sample were fixed at start of addition and after 24 hours and cell counts determined using flow cytometry.

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Tables

Table 1 Cu speciation from station A

Sample	Cu added	[Cu] measured	[Cu] ±	[L1]	L1 ±	log k1	K1 ±	[L2]	log [Cu2+]
2.5 µM SA:									
Control	0	1.13	0.08	5.6	0.2	14	0.05	3.88	-14.76
8 nM Cu	8	9.69	0.07	13.6	0.02	13.26	0.01		-13.41
10 µm SA:									
Control	0	1.13	0.08	4.4	0.2	14.62	0.07	6.2	-15.25
8 nM Cu	8	9.69	0.07	7.12	0.1	13.9	0.07		-13.77

Table 2 Cu speciation station B, all done with a 2.5 µM SA window

Sample	[Cu]	[Cu] ±	L_2	L_2 ±	logK_2	logK_2 ±	log[Cu^{2+}]	Calculated Cu spike	Resulting log [Cu2+]
Estimate	2.47	0.43	4.38	0.01	13.33	0.23	-12.90	31.54	-11.50
Initial Conditions	1.21	0.14	3.10	0.02	13.09	0.27	-13.68	31.41	-11.50

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Figure Legends

Fig. 1: Significant differentially expressed genes from strain CC9902 that were also differentially expressed in response to Cu in previous strains tested, CC9311 and WH8102 (Stuart *et al.*, 2009). 51 genes overlapped with CC9311 and 33 with WH8102. Microarray expression values are average $\log_2$ fold change of three biological replicates. Labels correspond to strain CC9902 gene locus number and a gene product name. Points with only a number label are conserved hypothetical genes. Red colored points are putative stress cluster genes from (Tetu *et al.*, 2013), blue are Fe-homeostasis related and green are commonly differentially expressed in all three strains (excluding stress cluster genes).

Fig. 2: Overlap of significantly differentially expressed genes and proteins in response to Cu addition. 21 genes had agreement in expression changes in both microarray expression and in protein expression. Microarray expression calculated as average $\log_2$ fold change of three biological replicates and proteins between two biological replicates. Number in each label is CC9902 locus number. "hsp" stands for heat shock protein. Red points are stress regulon genes (Dupont *et al.*, 2012; Tetu *et al.*, 2013) and green are genes that were also differentially expressed in response to Cu in WH8102 or CC9311 (excluding stress regulon genes).

Fig. 3: Conservation patterns of genes that were differentially expressed in response to Cu. All significantly differentially upregulated genes in each of three strains (CC9902, WH8102 and CC9311) were categorized based on ortholog presence in eleven marine *Synechococcus* genomes (determined by Dufresne *et al.* (2008), ortholog analysis). Orthologs were binned based on presence in either 1-3, 4-6, 7-9 or 10-11 genomes. Percentages were calculated for each category of gene conservation as a fraction of total differentially expressed genes for each strain. Gene regulation data for strains WH8102 and CC9311 from (Stuart *et al.*, 2009).

Fig. 4: Differential expression of stress regulon genes identified in strain WH8102 by Tetu and colleagues (Tetu *et al.*, 2013). Log_2 fold changes from published genome-wide microarray stress studies are compared to this work on CC9902 Cu-stress response (column 1). Orthologs of stress genes identified in WH8102 present in strains CC9902, CC9311 and WH7803 are compared. Number following gene names are CC9902 locus numbers. Columns 2 and 11 from (Stuart *et al.*, 2009) and columns 3, 13-14 from (Tetu *et al.*, 2009). Columns 4-5 & 12 from (Tetu *et al.*, 2013). Column 10 from (Dupont *et al.*, 2012), columns 6-9 from (Blot *et al.*, 2011). Treatments in columns include: ‘Cu’ for Cu additions, ‘early P’ for early growth phase phosphate limitation, ‘EB’ for ethidium bromide additions, ‘MC’ for mitomycin C additions, ‘LL’ for low light and ‘HL’ for high light, ‘H2O2’ for hydrogen peroxide additions, ‘MV’ for methyl viologen additions, ‘Ni_NH4’ for nickel limitation with ammonium as the N source, ‘late P’ for late growth phase phosphate limitation and ‘SYNW0947-(P-stress)’ for a WH8102 mutant (a putative phosphate response regulator gene knock out) under phosphate limitation. ** in labels are genes predicted from CC9902 data, not included in original predicted stress regulon.

Fig. 5: Response of IdiA gene region to Cu in strain CC9902. ‘array’ bars represent CC9902 transcriptional expression array (2h Cu shock), and ‘protein’ bars represent proteomic (8h Cu shock). All bars represent average $\log_2$ fold change between three or two biological replicates and errors bars represent one standard deviation. Numbers on x-axis represent CC9902 locus numbers. ‘chp’ indicates conserved hypothetical proteins, ‘CRP’ indicates cyclic AMP receptor protein bacterial regulators.

Fig. 6: Genes upregulated in both community and culture (CC9902 or CC9311) expression microarrays. Labels represent locus number from either CC9902 (squares) or CC9311

(circles) and gene description. Labels without a description are conserved hypothetical genes. Green labels are genomic island genes and red labels are putative stress regulon genes. Microarray expression values are average $\log_2$ fold change of three biological replicates.

Fig. 7: Flow cytometric counts from Cu additions and no-Cu control bottles incubated over 14 days. Error bars represent one standard deviation between two biological replicates. Arrow indicates day at which clone libraries and strain isolation were conducted.

Fig. 8: Comparison of control and Cu-treated *Synechococcus* populations after 14-day incubations. Phylogenetic tree of nucleotide sequence alignment of 841 bp of RPOC gene from environmental clone library. Alignment was done using MUSCLE and tree built using PhyML. Values on branches represent bootstrap values from 100 iterations. Branch tip labels with "Control" are clones from no-Cu control treatments and labels with a "Cu" are from Cu-incubated treatments.

Supplemental Material

Supplemental Methods

Table S1: Array and proteomic data (excel spreadsheet). Worksheets include 'CC9902_array_sig' with CC9902 microarray significantly differentially expressed genes, 'CC9902_prot_sig' with CC9902 proteomic significantly differentially expressed proteins, 'Community_array_sig' with field microarray significantly differentially expressed genes, 'CC9902_all' and 'DCM07_all' with microarray results for all genes in CC9902 culture and field, respectively, and 'non_specific_genes_array' with gene loci with non-specific binding.

Table S2: Primers used in QRTPCR verification of microarray expression

Supplemental Figure Legends

Fig. S1: QRTPCR validation of 10 genes chosen from microarray expression results. All points represent average fold change of three biological replicates. Line represents linear regression, with reported slope and R^2.

Fig. S2: Map of sampling locations in Southern California Bight for both Cu incubation experiments. "A" is location where the 2 hour Cu-expression sampling was conducted. "B" marks the Scripps Pier where sampling and incubation of the 2 week experiment was conducted.

Fig. S3: Average normalized signal intensities of all CC9902 genes from microarray expression experiment with both CC9902 cultures ("culture") and mixed community experiments.

Fig. S4 Relative abundance of Clade I to Clade IV *Synechococcus* in the control ("Control") and Cu-treated ("Cu") incubations over 14 days. Data based on QPCR of *rpocI* gene using primers from (Tai and Palenik, 2009). Points represent ratio of average QPCR calculated gene abundance from two biological replicates of each treatment and error bars represent one standard deviation.

Fig. S5: Higher tolerance of RhCu13 to methyl viologen than CC9902. Bars show ratio of cell counts before and after 24 hours of methyl viologen additions. Error bars represent one standard deviation between three biological replicates and * indicates mean >1 (t-test, p>0.05).

SIGNIFICANCE STATEMENT

We present results that are a rare effort to test a microbial organism's response to an environmental stress at the molecular level both in cultures and field populations. This involved difficult trace metal experiments using trace metal clean techniques and trace metal speciation measurements.

We also demonstrate the role that microarrays can play in microbial ecology that is synergistic with other 'omics data. In meeting these technical challenges we were able to define a *Synechococcus* general stress response, but also distinct copper responses that were found in different *Synechococcus* species. Our field experiments also suggest that subpopulations of less copper sensitive *Synechococcus* strains are present in the marine environment. This has implications for longer term responses of the environment to copper stress.

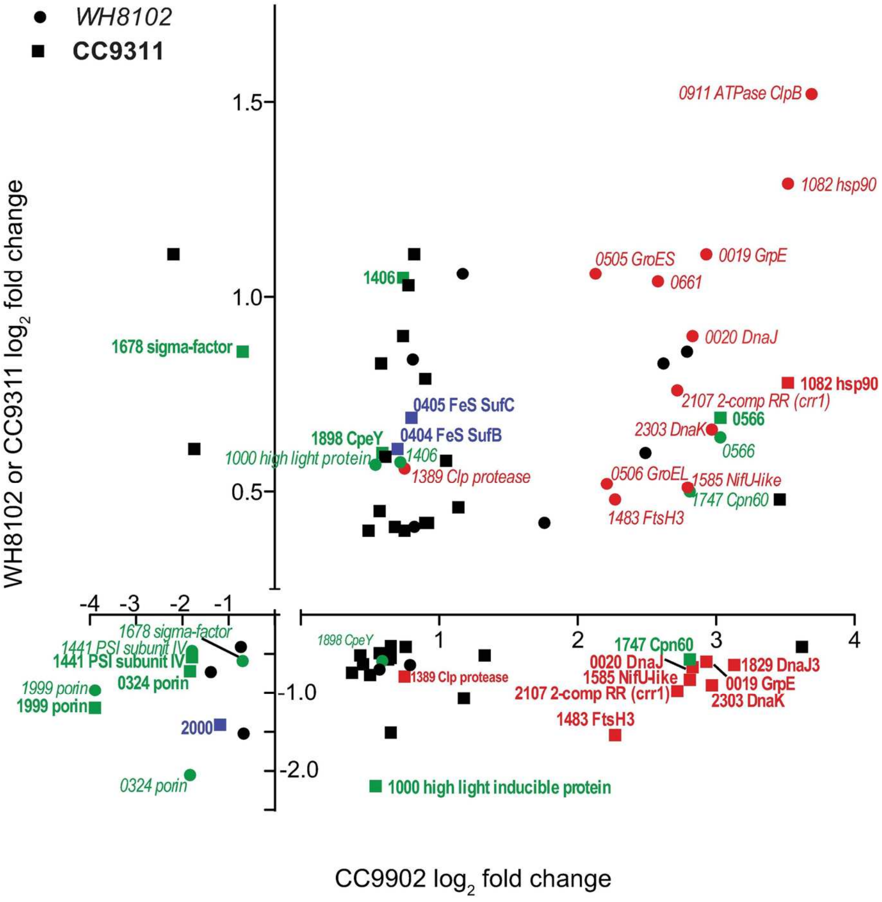
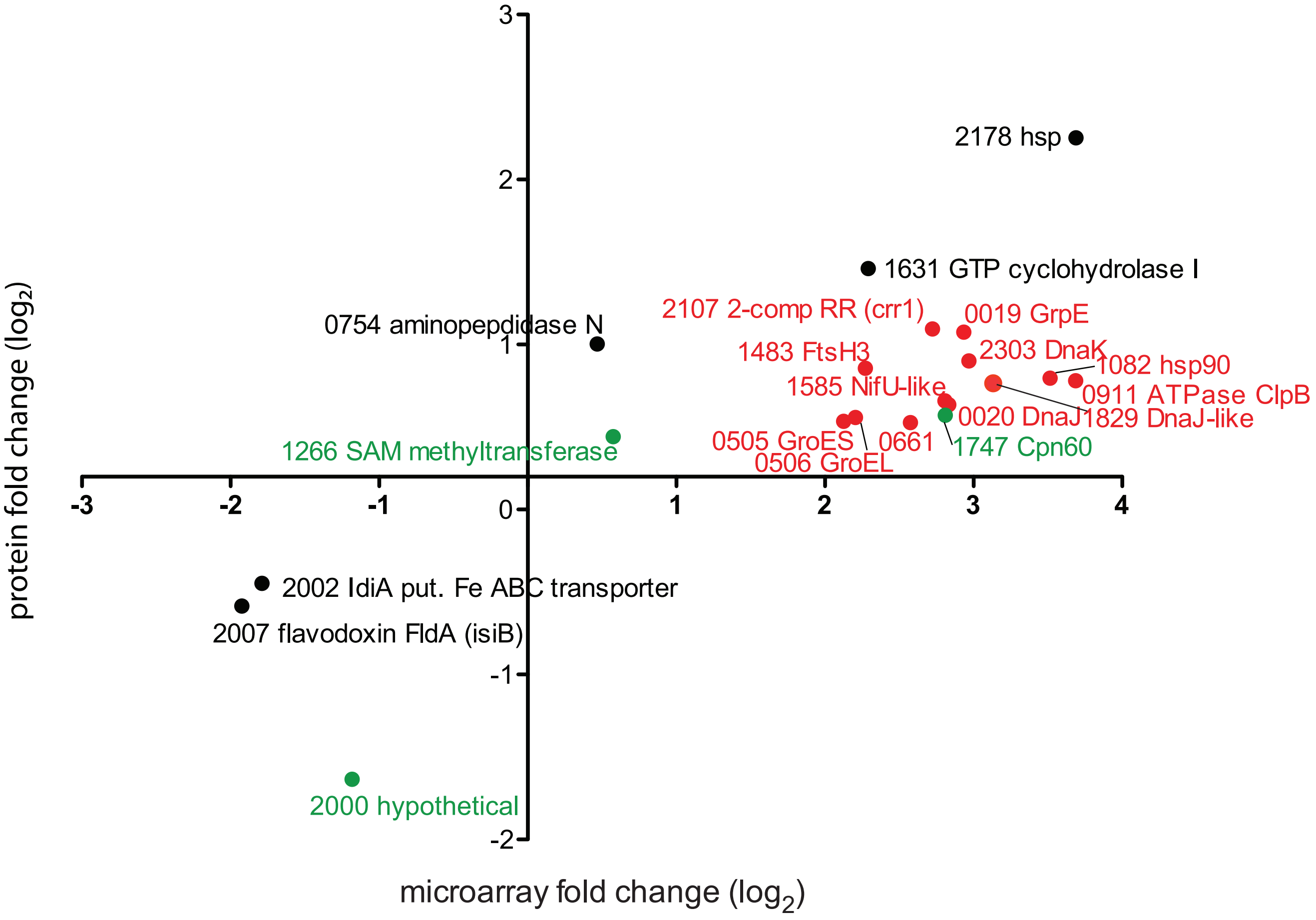
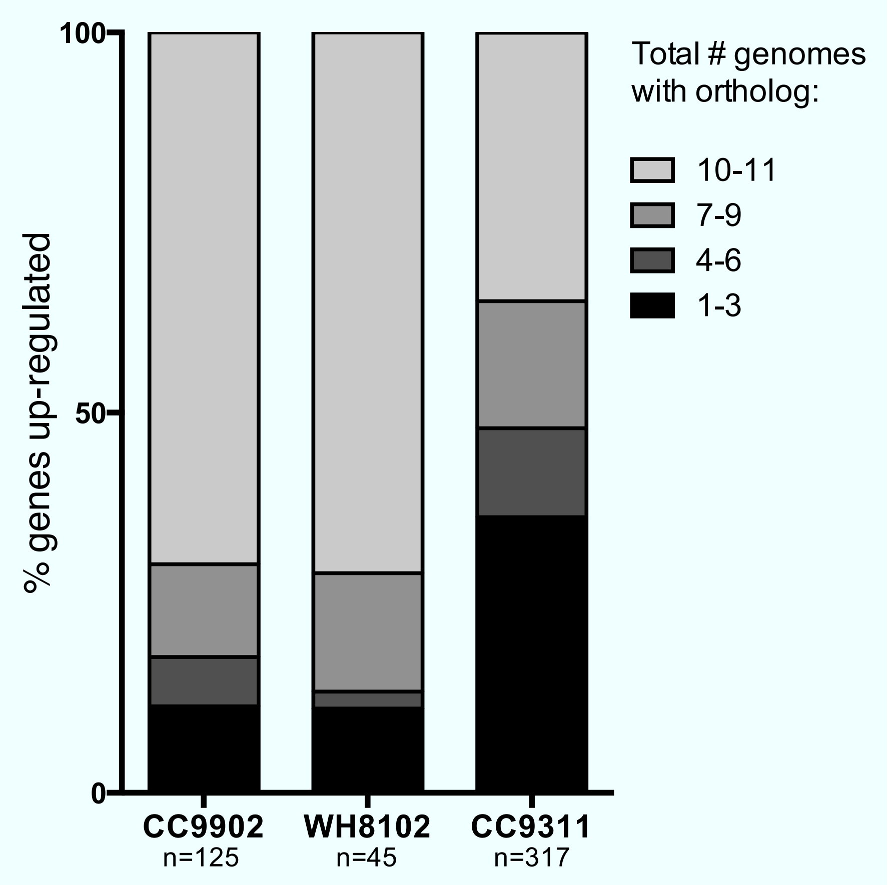
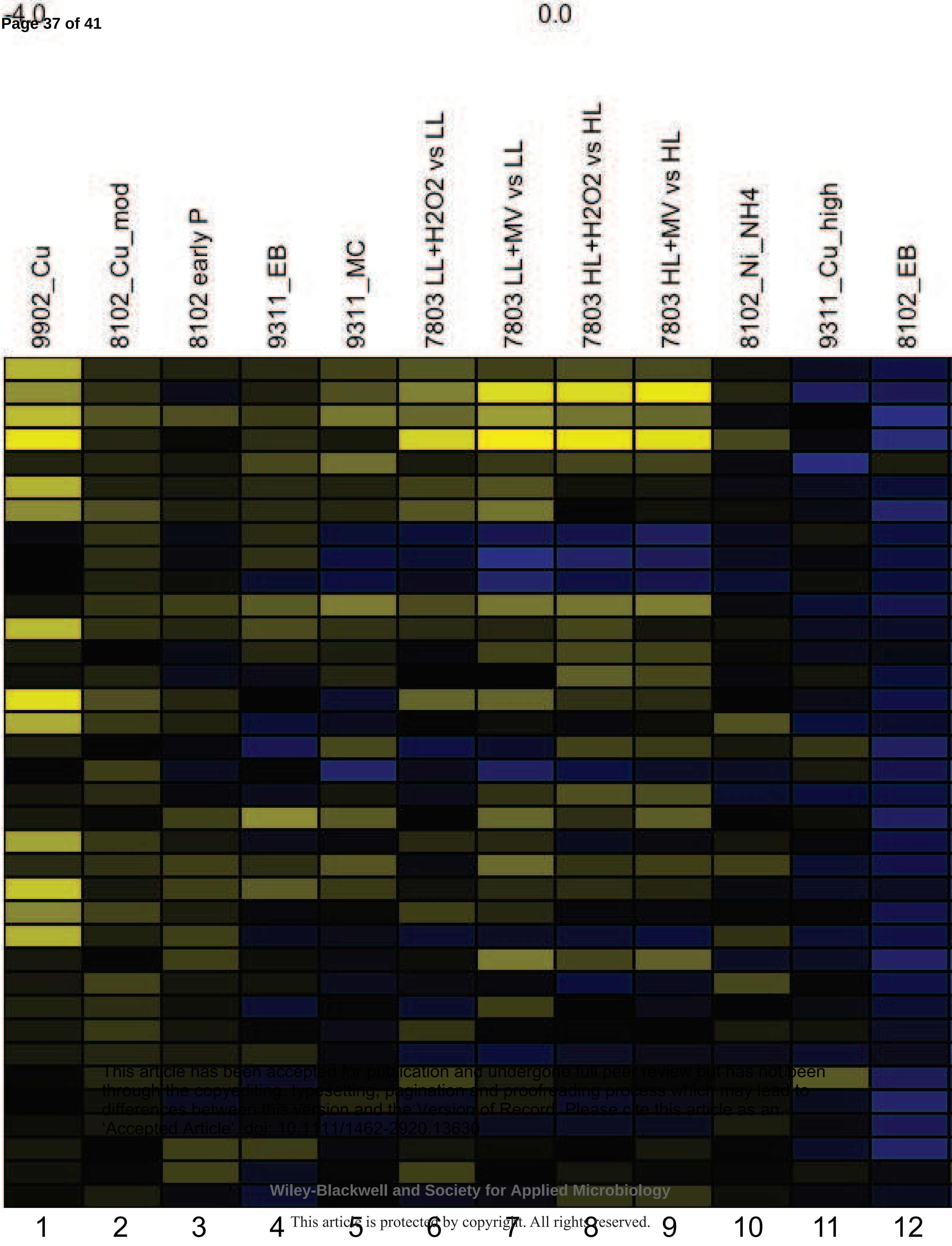


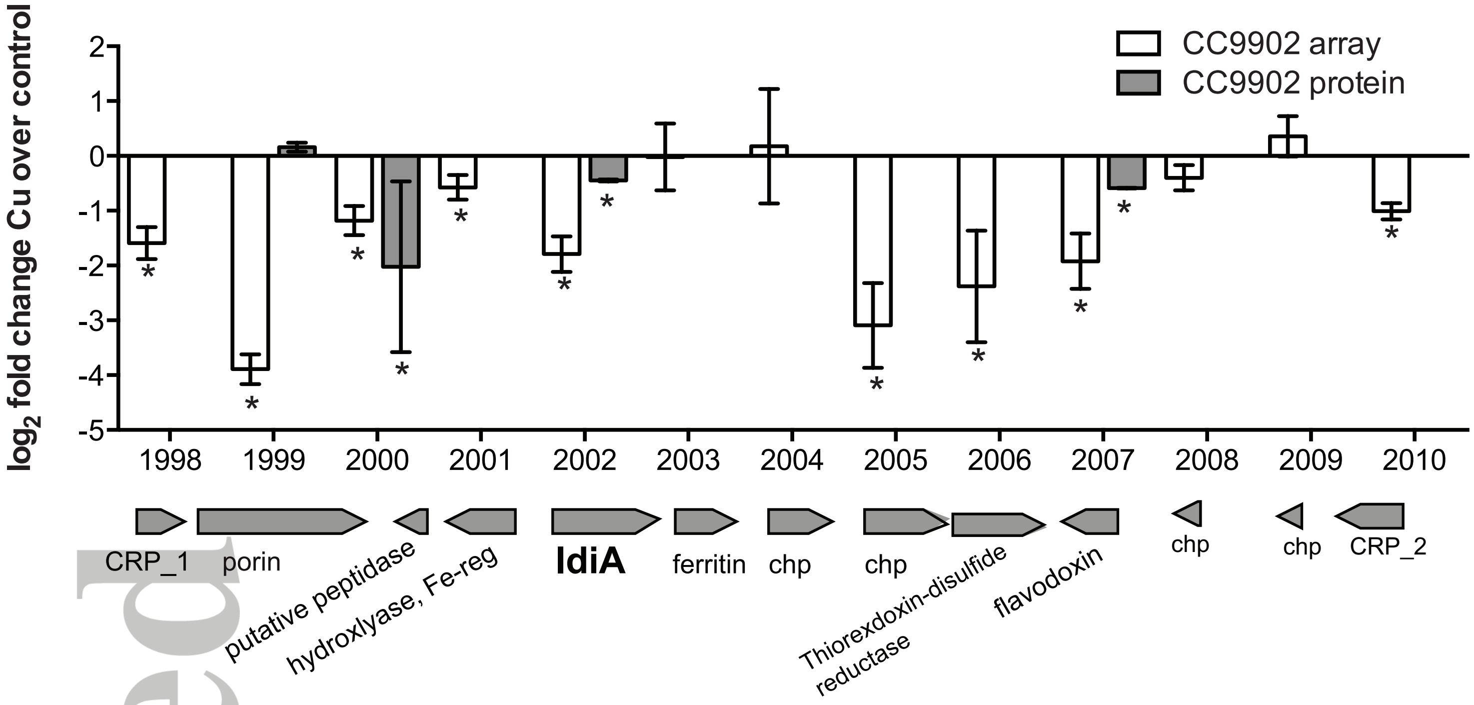
Fig. 1: Significant differentially expressed genes from strain CC9902 that were also differentially expressed in response to Cu in previous strains tested, CC9311 and WH8102 (Stuart et al., 2009). 51 genes overlapped with CC9311 and 33 with WH8102. Microarray expression values are average log2 fold change of three biological replicates. Labels correspond to strain CC9902 gene locus number and a gene product name. Points with only a number label are conserved hypothetical genes. Red colored points are putative stress cluster genes from (Tetu et al., 2013), blue are Fe-homeostasis related and green are commonly differentially expressed in all three strains (excluding stress cluster genes).

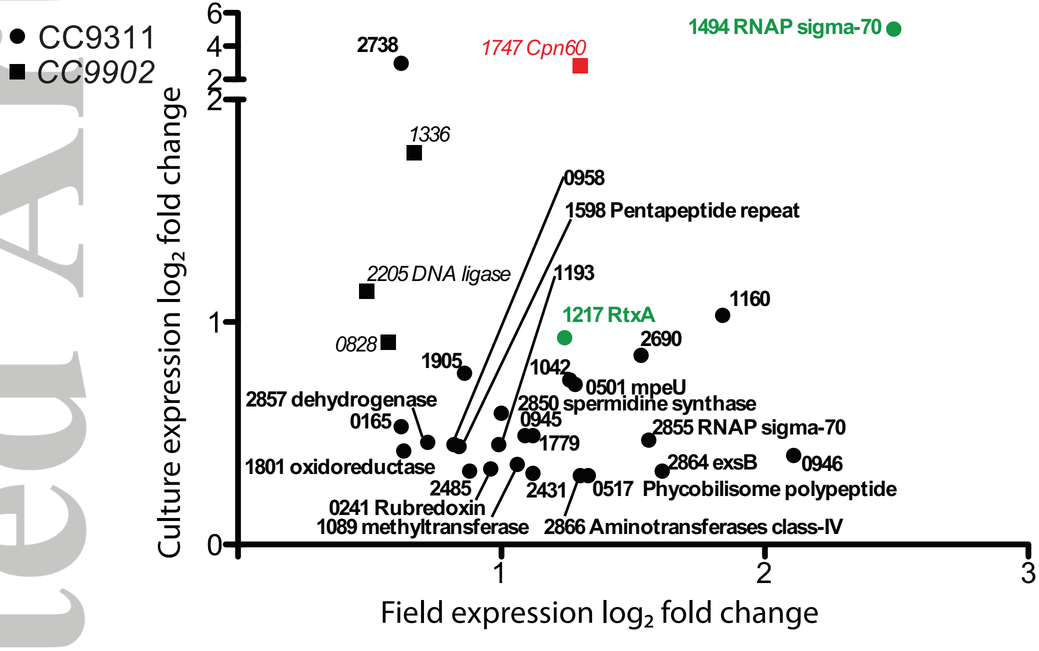
160x151mm (300 x 300 DPI)


2178 hsp
1631 GTP cyclohydrolase I
0754 aminopepdidase N
2107 2-comp RR (crr1)
0019 GrpE
1483 FtsH3
2303 DnaK
1082 hsp90
1585 NifU-like
0911 ATPase ClpB
0020 DnaJ
1829 DnaJ-like
1266 SAM methyltransferase
0505 GroES
0661
1747 Cpn60
0506 GroEL
2002 IdiA put. Fe ABC transporter
2007 flavodoxin FldA (isiB)
2000 hypothetical
protein fold change ($\log_2$)
microarray fold change ($\log_2$)
3
2
1
0
-1
-2
-3
-2
-1
1
2
3
4


100
50
0
% genes up-regulated
CC9902
n=125
WH8102
n=45
CC9311
n=317
Total # genomes with ortholog:
10-11
7-9
4-6
1-3


-4.0
0.0
9902_Cu
8102_Cu_mod
8102 early P
9311_EB
9311_MC
7803 LL+H2O2 vs LL
7803 LL+MV vs LL
7803 HL+H2O2 vs HL
7803 HL+MV vs HL
8102_Ni_NH4
9311_Cu_high
8102_EB
1
2
3
4
5
6
7
8
9
10
11
12


log$_2$ fold change Cu over control
2
1
0
-1
-2
-3
-4
-5
CC9902 array
CC9902 protein
*
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
CRP_1
porin
putative peptidase
hydroxlyase, Fe-reg
IdiA
ferritin
chp
chp
Thiorexdoxin-disulfide reductase
flavodoxin
chp
chp
CRP_2


● CC9311
■ CC9902
Culture expression log$_2$ fold change
Field expression log$_2$ fold change
6
4
2
2
1
0
1
2
3
2738
1747 Cpn60
1494 RNAP sigma-70
1336
0958
1598 Pentapeptide repeat
2205 DNA ligase
1193
1160
1217 RtxA
2690
0828
1905
1042
0501 mpeU
2857 dehydrogenase
2850 spermidine synthase
0165
0945
2855 RNAP sigma-70
1779
1801 oxidoreductase
2864 exsB
0946
2485
2431
0517
Phycobilisome polypeptide
0241 Rubredoxin
1089 methyltransferase
2866 Aminotransferases class-IV

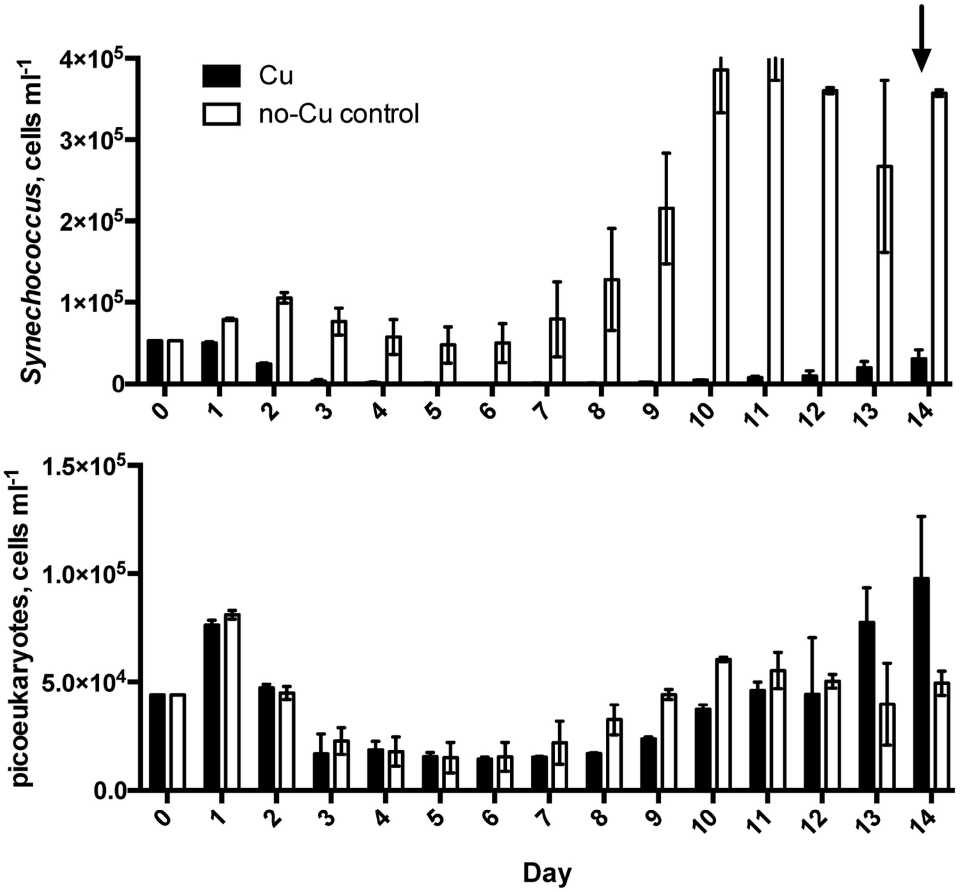


Fig. 7: Flow cytometric counts from Cu additions and no-Cu control bottles incubated over 14 days. Error bars represent one standard deviation between two biological replicates. Arrow indicates day at which clone libraries and strain isolation were conducted.

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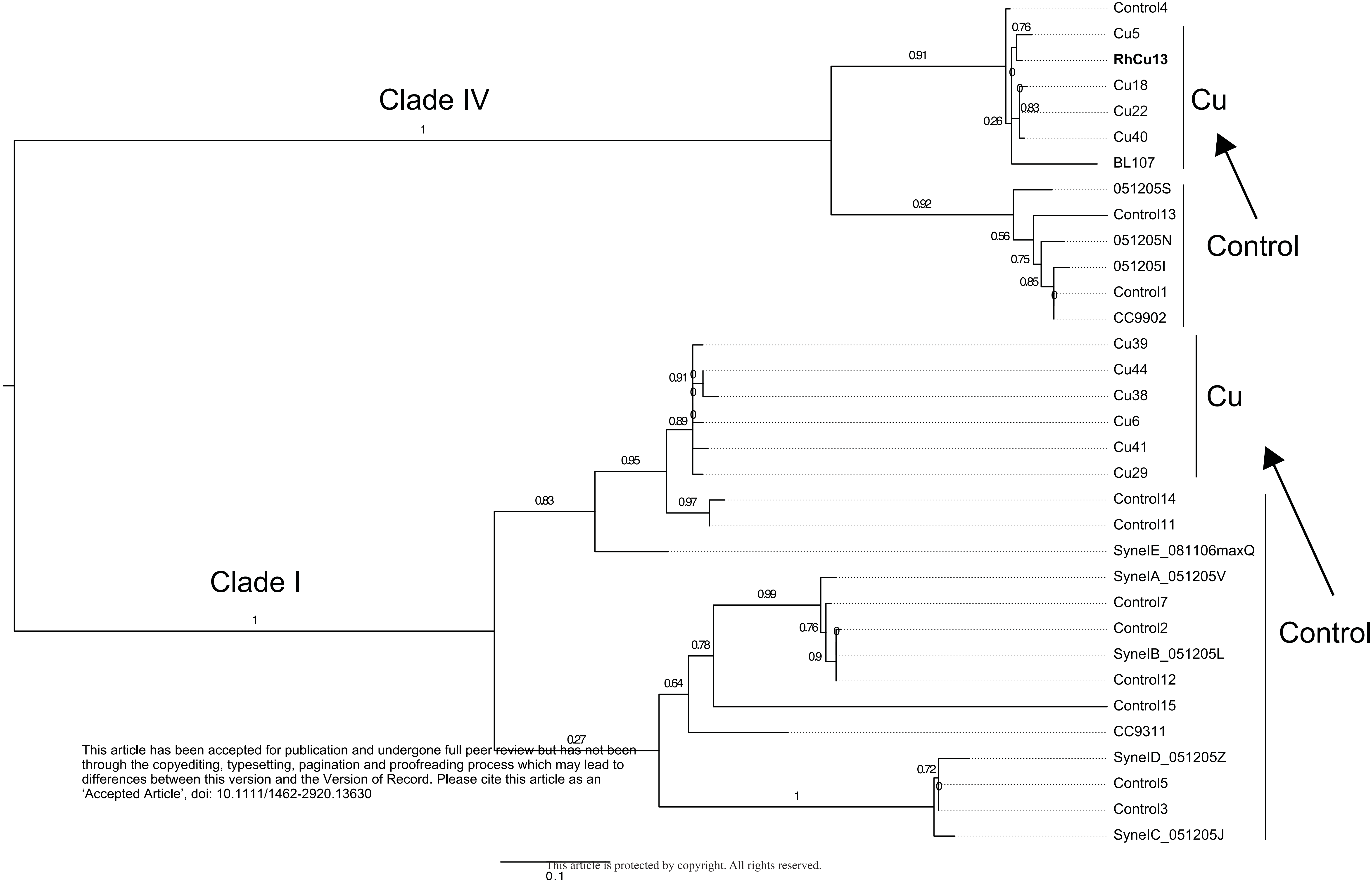
Clade IV
1
0.91
0.76
0
0.26
0
0.83
Control4
Cu5
RhCu13
Cu18
Cu22
Cu40
BL107
Cu
0.92
0.56
0.75
0.85
0
051205S
Control13
051205N
051205I
Control1
CC9902
Control
0.95
0.91
0
0
0.89
0
0.97
Cu39
Cu44
Cu38
Cu6
Cu41
Cu29
Cu
Control14
Control11
SynelE_081106maxQ
Clade I
1
0.83
0.99
0.76
0
0.9
0.78
0.64
0.27
1
0.72
0
SynelA_051205V
Control7
Control2
SynelB_051205L
Control12
Control15
CC9311
SynelD_051205Z
Control5
Control3
SynelC_051205J
Control
0.1