Nitrogen starvation induces extensive changes in the redox proteome of *Prochlorococcus* sp. strain SS120

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Running title: Cys modified proteins in N-starved *Prochlorococcus*

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Summary

Very low nitrogen concentration is a critical limitation in the oligotrophic oceans inhabited by the cyanobacterium *Prochlorococccus*, one of the main primary producers on Earth. It is well known that nitrogen starvation affects redox homeostasis in cells. We have studied the effect of nitrogen starvation on the thiol redox proteome in the *Prochlorococcus* sp. SS120 strain, by using shotgun proteomic techniques to map the cysteine modified in each case and to quantify the ratio of reversibly oxidized/reduced species. We identified a number of proteins showing modified cysteines only under either control or N-starvation, including isocitrate dehydrogenase and ribulose phosphate 3-epimerase. We detected other key enzymes, such as glutamine synthetase, transporters and transaminases, showing that nitrogen-related pathways were deeply affected by nitrogen starvation. Reversibly oxidized cysteines were also detected in proteins of other important metabolic pathways, such as photosynthesis, phosphorus metabolism, ATP synthesis and nucleic acids metabolism. Our results demonstrate a wide effect of nitrogen limitation on the redox status of the *Prochlorococcus* proteome, suggesting that besides previously reported transcriptional changes, this cyanobacterium responds with post-translational redox changes to the lack of nitrogen in its environment.

Introduction

Prochlorococcus is a cyanobacterium (Chisholm et al., 1988) which has become a major model in marine ecology (Coleman and Chisholm, 2007) for different reasons. Since it is the most abundant photosynthetic organism in our planet (Partensky and Garczarek, 2010), it provides a significant contribution to primary production on Earth (Goericke and Welschmeyer, 1993; Liu et al., 1997). It has a number of features which have attracted the interest from scientists, namely the small size of its genome (Dufresne et al., 2003; Rocap et al., 2003), the existence of a large collection of cultured isolates (Moore et al., 2007) -representatives of different environments in the ocean-, and the availability of a growing number of *Prochlorococcus* genomes (Coleman et al., 2006; Kettler et al., 2007).

One of the most distinguishing features of this cyanobacterium is its outstanding ability to grow at very high concentrations in oligotrophic regions of the intertropical oceans (Liu et al., 1997; Johnson et al., 2006). In such areas permanently subjected to nutrient limitations (Howarth, 1988), *Prochlorococcus* outgrows its closer competitor, *Synechococcus*, keeping very high concentrations down to almost 200 m depth (Partensky et al., 1999b). This remarkable fact has been attributed to a number of different causes, such as their high efficiency in nutrient scavenging due to its high surface/volume ratio (Partensky et al., 1999a). Yet if we consider the tremendous amount of biomass that is comprised by *Prochlorococcus* cells in such large regions, it is obvious that this cyanobacterium had to evolve strategies to cope with nutrient limitations in order to keep its metabolism as unaffected as possible. Some examples of such adaptations have been reported in literature as the utilization of sulfolipids instead of phospholipids to reduce the phosphorus needs in phytoplankton (Van Mooy et al., 2006). The utilization of Pcb as antenna proteins in *Prochlorococcus*, evolved from stress-induced proteins (*isiA*-like), instead of the bulkier phycobilisomes (Partensky and Garczarek, 2003) and the reduction in nitrogen content in the amino acid sequences (Grzymski and Dussaq, 2011). However, the severe oligotrophy observed in the habitats where *Prochlorococcus* is more successful suggests that this organism has been subjected to nutrient limitation (and in particular, nitrogen limitation (Graziano et al., 1996) for a very prolonged

period of time, leading most possibly to a wide effect on different aspects of cell metabolism). Nitrogen is one of the key limiting nutrients in oligotrophic oceans, reaching levels in the nanomolar range (Capone, 2000). Different regions in the oceans have been described to be permanently limited in this element (Zehr and Kudela, 2011).

Light dependent disulfide exchange on cysteine residues of proteins, catalysed by the Thioredoxin (Trx) reductase, was first identified in chloroplasts and since then redox modifications and subsequent redox regulation of proteins has been linked to photosynthesis, storage, transcription, translation and protein folding (Buchanan and Balmer, 2005). The reversible modification of cysteine residues in proteins, such as oxidation, nitrosylation and formation of disulfide bonds, is a method widely utilized by cells to control protein structure and function and offers a means of reversibly regulating the activity of a significant number of important enzymes. The redox status of the thiol group is subjected to reversible reactions, allowing rapid and effective mechanisms to control enzyme activity. In previous studies, it has been shown how the redox proteome of different organisms is profoundly affected by different environmental conditions. The disulfide redox proteome of the cyanobacteria *Synechocystis sp.* PCC 6803 has been extensively studied by Lindahl and Florencio (Florencio et al., 2006; Perez-Perez et al., 2006; Mata-Cabana et al., 2007), who identified almost 80 potential Trx protein targets, with a high overlap with those identified as Trx targets in the chloroplasts of higher plants (Lindahl and Kieselbach, 2009). Our group has demonstrated that strong decreases in the N/C balance induce different changes in key enzymes, such as glutamine synthetase and isocitrate dehydrogenase, including both the expression of the corresponding genes (López-Lozano et al., 2009) and the abundance of the proteins (El Alaoui et al., 2001; El Alaoui et al., 2003; López-Lozano et al., 2009). Furthermore, different experiments suggest that both enzymes are subjected to regulation by redox oxidative modifications (Gómez-Baena et al., 2001; Gómez-Baena et al., 2006). This led us to explore the possible involvement of redox proteome changes in the response of *Prochlorococcus* to key environmental conditions, such as nitrogen starvation, which is believed to be one of the most important limitations for the growth of phytoplankton in oligotrophic oceans. In this work, we utilized novel techniques to detect changes in

cysteine-containing proteins affected by nitrogen starvation, finding variations in several proteins both directly involved in nitrogen metabolism and other pathways that may be due to secondary adaptive effects.

Effects of nitrogen starvation on protein glutathionylation in *Prochlorococcus*

The object of this work was to address the effects of nitrogen starvation on the *Prochlorococcus* redox proteome. Previous studies carried out by our group demonstrated that nitrogen starvation had little effect on a key enzyme in nitrogen metabolism, glutamine synthetase, which showed no significant changes either in the activity or concentration (El Alaoui et al., 2001; El Alaoui et al., 2003). However, in studies focused on metal-catalyzed oxidative systems, we observed that glutamine synthetase and also isocitrate dehydrogenase from *Prochlorococcus* were subjected to a process of oxidative inactivation (Gómez-Baena et al., 2001; Gómez-Baena et al., 2006). These results suggested that oxidative mechanisms might be involved in the regulation of metabolism in *Prochlorococcus*. This prompted us to study whether one of the most important limitations suffered by natural populations of *Prochlorococcus* –i.e. very low concentrations of nitrogen, given that oligotrophic oceans is the preferred habitat of this marine cyanobacterium- might produce metabolic effects through redox signaling. In order to check this hypothesis, we carried out Western blotting of control vs starved samples, to detect glutathionylated proteins. The results are shown in Figure 1, comparing control samples of *Prochlorococcus* sp. strain SS120 to samples of cultures subjected to nitrogen starvation for 24 h, mimicking the conditions of limited nitrogen availability in the intertropical oceans. A large increase in the protein glutathionylation was observed in all starved samples, suggesting that lack of nitrogen had a strong effect on the redox status of the *Prochlorococcus* sp. strain SS120 proteome. Consequently, we decided to further explore the effects of nitrogen limitation in particular, since nitrogen has been reported to be limiting in very wide regions of the oceans.

Effect of nitrogen starvation on the *Prochlorococcus* redox proteome

Prochlorococcus SS120 cultures growing on standard, ammonium-containing PCR-S11 medium were transferred to medium without added nitrogen, as explained previously (El Alaoui et al., 2003). Cells were harvested after 24 h and processed to detect reversible cysteine modifications by the shotgun redox proteomics method (McDonagh et al., 2009). Three independent biological replicates

were used for both control and N-starved cells. This method not only identifies the proteins involved but also the precise cysteine within each protein that is subject to a reversible modification. This has a number of advantages over traditional shotgun proteomic techniques which, can be prone to under sampling and can sometimes be overwhelmed by peptides that ionize and fragment well in MS/MS experiments, for this reason there can be little overlap between MS runs (Malmstrom et al., 2007; Nilsson et al., 2010). As we select for only those peptides containing reversibly oxidized cysteines we are greatly reducing the number of peptides and increasing confidence that they are in fact redox sensitive. However, some caution should be used in the interpretation of results, if the peptide is not detected the cysteine residue might be either reduced or overoxidised to irreversible sulfinic or sulfonic forms or alternatively the gene might be not be expressed. Also, the cys containing tryptic peptide must be amenable to analysis by MS.

Using the stringent identification criteria utilized in this study, 80 proteins were identified between control and nitrogen starved groups (Supplementary table 1). Of these, 53 proteins were consistently identified in all biological replicates from both conditions. Four proteins were identified from peptides not containing cys residues and would be considered as false positives due to misidentification by the search engine or because the peptide is attached to the biotin-HPDP (N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide). Grouping those 80 proteins into general clusters reveal that, 20 were involved in nucleotide or amino acid biosynthesis, 17 in energy or metabolic processes, 12 in transcription and translation, 10 forming part of photosynthetic system, 2 in chaperone or stress response and the rest in signaling or unknown processes.

There were 7 proteins (Table 1) that were only identified as containing peptides with reversibly modified cysteines in either the control or nitrogen starved group. Proteins only identified in the control group were ABC transporter, substrate binding protein (cys67), formyltetrahydrofolate deformylase (cys13), pyruvate dehydrogenase E1 component, alpha subunit (cys277), ribose-phosphate pyrophosphokinase (cys247) and UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase (cys98). Two proteins were identified with reversibly oxidized cysteines only in nitrogen starved cells; isocitrate dehydrogenase (cys463) and ribulose phosphate 3-epimerase

(cys68). This points to a direct response of the cell to nitrogen limitation and indeed our group has previously identified 3 of these yeast protein orthologues as being redox sensitive (Idh2p, Rpe1p and Prs3p) (McDonagh et al., 2011a). The two latter proteins have been described as thioredoxin targets in *Synechocystis* PCC 6803 (Lindahl and Kieselbach, 2009).

As a further analysis we used the Progenesis LC-MS programe to relatively quantify the abundance of detected peptides between test groups. Using an ANOVA analysis, 10 of the proteins detected in all samples (Supplementary table 1) showed a significant increase in detection of the reversibly oxidized cysteine containing peptide in nitrogen starved cells (Table 2).

Interestingly we found only two proteins directly related to nitrogen metabolism that showed significant differences between groups according to Progenesis LC-MS: the substrate binding protein of the urea ABC transporter (binding protein) and L-asparaginase II. It is worth noting that when looking at the whole list of detected proteins, some proteins in Table 2 did not meet the criteria for inclusion in Supplementary table 1, which may be due to processing of raw data in run alignments, filtering of data and file conversions between the different programes. The urea ABC transporter binding protein had a 5.3-fold increase in the N starved samples and the peptide detected contained cys145. This corresponds to the only conserved cysteine residue found in this protein in cyanobacteria (not shown).

Enzymes comprise the majority of the proteins detected by this method, given that most of the proteins possessing cysteines subjected to reversible oxidation belong to that category. When looking at the complete list of proteins (Supplementary table 1) detected by this method, we found two of the key enzymes in the nitrogen metabolism, glutamine synthetase (GS) and isocitrate dehydrogenase (IDH). These enzymes represent respectively the main pathway for nitrogen assimilation (GS/GOGAT) and the link between nitrogen and carbon metabolisms (IDH). We also detected the peptide containing cys279 of the ferroredoxin NADP oxidoreductase that can provide the reducing power for Trx and subsequently target proteins.

There were enzymes involved in the transfer of nitrogen groups -as amine or amide- and also peptidases. These include: glutamate-1-semialdehyde aminotransferase, glutamine amidotransferase

class-I: CTP synthase, aspartyl-glutamyl-tRNA amidotransferase subunits A and B, porphobilinogen deaminase, glutamate-1-semialdehyhyde amidotransferase; leucyl aminopeptidase.

Effects of nitrogen starvation on nitrogen-related enzymes

Figure 2 shows a Western blotting of glutamine synthetase from the same samples utilized in the redox proteome experiments; nitrogen starvation induced a clear increase (ca. 3-fold) in the concentration of this enzyme. Previous reports by Tolonen and coworkers (Tolonen et al., 2006) showed also a significant increase in the expression of *glnA*, encoding glutamine synthetase in cyanobacteria. However, this response seems to depend on the strain, since our team has previously shown that glutamine synthetase concentration was clearly increased after 120 h of starvation in *Prochlorococcus* strain SS120, in good agreement with the results shown here, but not in the strain PCC 9511 (El Alaoui et al., 2003).

Figure 3 shows the effect of nitrogen starvation on the isocitrate dehydrogenase concentration of the *Prochlorococcus* sp. SS120 strain. Despite the variability observed in controls, and in contrast to the results observed for glutamine synthetase, we observed no clear change under nitrogen limiting conditions, with respect to the control culture on standard culture medium. This result fits nicely with the fairly constant expression of *icd* in the *Prochlorococcus* sp. SS120 strain, the gene encoding for isocitrate dehydrogenase, under nitrogen starvation reported previously (López-Lozano et al., 2009) and could point to an effective post-translational redox control mechanism for the protein.

Discussion

The main goal of this study ~~is~~ was to utilize an unbiased, shotgun-based proteomic technique to find out whether nitrogen starvation, a wide limitation in intertropical oceans, provokes changes in the redox status of the *Prochlorococcus* proteins possessing cysteines sensitive to reversible oxidation.

Our results demonstrate this is indeed the case, therefore suggesting that redox modifications could mediate the physiological response of this marine cyanobacterium to the lack of an important element as nitrogen. To our knowledge, this is the first proteomic study on the effects of nitrogen limitation in *Prochlorococcus*.

Table 2 shows changes in the peptides detected and quantified which are statistically significant and the fold change ranged from 1.7 to 2.0. The fact these peptides were identified in all samples would potentially point to a redox modification in many constitutively expressed proteins that could alter the metabolic flow through the respective pathways, offering a means for the cell to reversibly control the activity of certain key enzymes potentially affecting key signaling pathways. This suggests that *Prochlorococcus* adapts to the lack of nitrogen by triggering proteomic changes.

A number of proteins were only detected under either control (i.e. Mn ABC transporter or formyltetrahydrofolate deformylase) or N-starved conditions (i.e., isocitrate dehydrogenase or ribulose-phosphate 3-epimerase; Table 1). In addition, two of the cysteine-modified proteins were specifically oxidized under nitrogen starvation. This feature reinforces the importance of the observed adaptations in the proteome, which does not rely solely on increases or decreases of a same subset of modified proteins.

The specific detection of cysteine-modified isocitrate dehydrogenase under nitrogen starvation is particularly noteworthy, given that this enzyme is located at the crossroad between the nitrogen and carbon metabolisms in cyanobacteria. Isocitrate dehydrogenase provides a carbon backbone (2-oxoglutarate) for the reaction catalyzed by glutamate synthase, allowing the net production of two glutamate molecules from glutamine and 2-oxoglutarate. The regulation of this enzyme by cysteine modification could confer a control system at a pivotal place in the cyanobacterial metabolism. It is

worth noting that our team has previously described the inactivation of both isocitrate dehydrogenase and glutamine synthetase by oxidative mechanisms (Gómez-Baena et al., 2001; Gómez-Baena et al., 2006).

Besides the list of proteins specifically detected oxidized under control or N-starved conditions, we observed a number of reversibly oxidized peptides whose levels were either increased or reduced upon N limitation. These are presented in Table 2 and will be discussed in the following paragraphs.

1. Nitrogen metabolism

The oxidized form of the urea-binding subunit of the putative urea ABC transporter (encoded by the gene *urtD*) in *Prochlorococcus* was found to increase more than five-fold with respect to the control. The urea transporter operon has been identified and characterized in *Synechocystis* PCC 6803 and *Anabaena* PCC 7120 by Valladares et al (Valladares et al., 2002). This protein was also identified as a thioredoxin target in *Synechocystis* sp. PCC 6803 by Lindahl and coworkers (Mata-Cabana et al., 2007). Interestingly, two of the proteins that comprise the urea transporter in *Prochlorococcus*, *urtA* and *urtB*, were found to be among the most upregulated genes in *Prochlorococcus* MED4 and MIT9313 cultures subjected to nitrogen starvation, in studies on global gene expression (Tolonen et al., 2006); but *urtD* was not found to show significant changes. This suggests that changes in regulation induced by nitrogen starvation include both the transcriptional stage (controlled by the master nitrogen regulator, NtcA (Luque and Forchhammer, 2008) and the post-translational stage, here exemplified by reversible modifications of cysteine residues. Redox modulation of constitutively expressed UrtD to rapidly control its activity (and metabolic flow derived from urea uptake), would complement other regulatory mechanisms of the same process based on transcriptional control of *urtA*/*urtB* expression.

L-asparaginase activity has been shown to be subjected to nonessential activation by thiolic compounds in *Erwinia carotovora* (Warangkar and Khobragade, 2010). This enzyme catalyzes the hydrolysis of asparagine to aspartic acid, removing an amine group. Since our method detects specifically the proteins with reversibly oxidized cysteins, and taking into account that the concentration of the oxidized form was three-fold lower in the N-starved cells, it might suggest that

the enzyme has become inactive due to N limitation. L-asparaginase was the only protein that had a significantly higher level of the reversibly oxidized peptide in controls respect to N-starved cells.

Interestingly, among the detected proteins, there were two enzymes involved in homoserine metabolism (homoserine dehydrogenase and O-acetyl homoserine sulfhydrylase). This pathway is involved in the metabolism of threonine and isoleucine and homoserine dehydrogenase has been shown to be inactivated in the presence of cysteine (Hama et al., 1991).

Bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase was also detected and is involved in riboflavin biosynthesis from ribulose 5-phosphate. Its yeast orthologue (Rib3p) also has an unrelated and unknown function in mitochondrial respiration. We have recently identified Rib3p in yeast as being a substrate for glutaredoxin 2 and subject to various redox interconversions including glutathionylation (McDonagh et al., 2011b).

2. Photosynthetic metabolism

Given the importance of photosynthesis in an organism which is universally recognized as one of the main primary producers on Earth, it was somehow expectable to find that several of the proteins affected by nitrogen starvation are directly related to photosynthesis.

Protein D1 is one of the most studied proteins in photosystem II, including cyanobacteria. Cross-linking studies have shown that cysteine residues in elongating D1 nascent chains are located close to the cysteine residues in the D2 protein, suggesting that the formation of a disulfide bond might be important for the stability of D1/D2 early assembled intermediates (Zhang et al., 2000). If this hypothesis holds true for *Prochlorococcus*, it might be possible that the intermediates are blocked in such an early stage due to metabolic limitations preventing the biosynthesis of the mature forms of both proteins. However, the lack of D2 proteins in the list of detected proteins under our experimental conditions might suggest that the cysteine residues from D1 might be also involved in the linking to other proteins or the corresponding cysteine-containing peptide in D2 may not be amenable to mass spectrometry analysis.

Flavodoxin is a photosystem I-electron aceptor, produced in many cyanobacteria as a replacement for ferrodoxin under conditions of iron stress (Bottin and Lagoutte, 1992). The flavodoxin sequence

in *Synechocystis* PCC 6803 contains four conserved cysteine residues belonging to the iron-sulfur cluster, and two additional cysteine residues which are conserved. The latter two were the residues detected in this work as reversibly modified, indicating that they might be involved in the regulation of this protein under stress conditions. It is worth noting that we detected this protein oxidized both in the presence and in the absence of nitrogen, although this was not under iron limiting conditions.

Coproporphyrinogen II oxidase converts coproporphyrinogen III to protoporphyrinogen IX, the sixth step in the porphyrin biosynthetic route. *Synechocystis* PCC 6803 has two different forms of this enzyme (Sll1876 and Sll1917) with a common, conserved cysteine motif (Goto et al., 2010), which had been previously described in the crystal structure of this protein in *E. coli* (Layer et al., 2003). The conserved cysteine motif invariably covers a central 4Fe/4S cluster and S-adenosyl-methionine-binding site in a loop, shielding them while providing a route of electron transfer from an electron donor docked to the exterior of the molecule.

The PsaC protein (subunit VII of photosystem I) has two cysteine residues (positions 14 and 51) ligating the Fa and Fb iron-sulfur clusters to photosystem I. By replacing these residues, it has been demonstrated that the presence of [4S-4Fe] clusters in PsaC is required for the assembly of photosystem I cores in vivo (Jung et al., 1997). Under conditions of nitrogen limitation, a general response in cyanobacteria is the degradation of the phycobilisomes, which contain a significant amount of nitrogen. *Prochlorococcus* lacks phycobilisomes, but it might be possible that other components of photosystems, as PsaC, be degraded in order to provide nitrogen for more essential functions under conditions of strong nitrogen limitation, leading to the oxidations of the cysteines.

3. Glycolytic enzymes

Triose phosphate isomerase, as previously reported, is a dimer containing two cysteine residues/subunit, Cys16 and Cys127, which could form a disulfide upon conformational changes given their close proximity in the molecule and are sensitive to oxidation (McDonagh et al., 2009).

Fructose 1,6-bisphosphatase from *Synechocystis* PCC 6803 is regulated by thioredoxin A and B, hence subjected to redox regulation by reversible modification of cysteine. The reversibly oxidized

peptides of both enzymes had double concentration in N starved cells with respect to control samples.

4. Phosphorus metabolism

Inorganic pyrophosphatase has been characterized by heterologous expression of two paralogous genes (*ppa1* and *ppa2*) from *Prochlorococcus* MED4 in *Synechocystis* PCC 6803 (Gómez-Garcia and Serrano, 2002). This study showed that the typical cyanobacterial gene (*ppa1*) encodes an inactive enzyme, while the gene *ppa2* (presumably from proteobacterial origin) encodes an active form of pyrophosphatase. Interestingly, all the cyanobacterial *ppa2* genes show three conserved cysteine residues (not shown); two of these cysteines (cys71 and 163) were detected as reversibly modified in this study. This fact had not been noticed thus far, and strongly suggest that these cysteines are important for the function of pyrophosphatase, even if they are not directly related to the catalytic center, according to reports (Gómez-Garcia and Serrano, 2002).

5. Other proteins

The SecA gene product likely mediates protein translocation across both the cytoplasmic and thylakoid membranes in cyanobacterial cells: this gene has been described in the cyanobacterium *Synechococcus* PCC 7942 (Nakai et al., 1994). It appears mainly as a soluble homodimer in the cytosol, but a significant amount of SecA was also detected in the membrane after fractionation of cell extracts. In good agreement, SecA was also detected in proteomic studies on membrane proteins interacting with thioredoxin (Mata-Cabana et al., 2007), indicating that the reversibly modified cysteine residues could be involved in this interaction. As suggested by Lindahl and coworkers, and reinforced by the present study, the presence in the membrane of several transporters (such as the urea-binding subunit of the ABC transporter for urea, or SecA) with sensitive cysteine residues might suggest that their functions are regulated (presumably, in the case of N starvation, in order to activate them) by the redox conditions of the cell. Global studies of gene expression showed that N limitation in *Prochlorococcus* provokes the increased expression of a number of genes encoding transporters, such as the ammonium and urea transporters (in strains MED4 and MIT9313), the cyanate transporter in MED4, and the nitrite permease in MIT9313 (Tolonen et al., 2006).

The chaperonine GroEL has been previously reported as a thioredoxin target (Lindahl and Florencio, 2003; Mata-Cabana et al., 2007), and consequently the changes in the redox status of the cysteines interacting with thioredoxin, derived from N limitation, suggest that thioredoxin is mediating the physiological response to N stress in *Prochlorococcus* SS120.

The beta subunit of ATP synthase is encoded by the *atpD* gene. It has been recently demonstrated that chloroplast F(1)-ATPase is subject to redox regulation (Kim et al., 2011), whereby ATP hydrolysis activity is regulated by formation and reduction of the disulfide bond located on the gamma subunit. A similar capability of regulation, by means of reversible modification of cysteine 227, might happen in the beta subunit of ATP synthase, detected in the present study.

The RNA-binding protein RNP-1 contains a RNA-recognizing motif highly conserved in cyanobacteria (Maruyama et al, 1999). Interestingly, an alignment of cyanobacterial RNP-1 proteins shows a single cysteine residue specifically conserved in most marine strains (highlighted with a box in Figure 4), while other amino acid residues appear at this position in other large group of strains. This might point out to a regulatory feature specific of the majority of the marine cluster, although this hypothesis should be experimentally tested.

Comparison to previous proteomic studies on cyanobacteria subjected to N starvation

The only proteome study which has described the effect of N starvation in cyanobacteria to our knowledge was carried out by Wegener and coworkers (Wegener et al., 2010), and included a quantitation of the model cyanobacterium *Synechocystis* PCC 6803 proteome under a wide series of environmental perturbations, including nitrogen depletion, nitrate repletion and ammonium repletion.–It is worth noting that the study on *Synechocystis* was focused on total quantitation of proteins, while in the present study we have specifically detected proteins subjected to reversible cysteine modification. In this regard the redox sensitive proteins we have detected show a high overlap with those identified as thioredoxin targets in *Synechocystis sp*. (Lindahl and Kieselbach, 2009). Among the proteins which showed significant changes, discussed in this paper, we found that some of them were not detected by Wegener et al: these included subunit II of photosystem I, the

RNA recoginition motif-containing protein, O-acetyl homoserine sulfhydrilase, triosephosphate isomerase, and hydroxymyristoyl-ACP dehydratase. In other cases, such as fructose 1,6-bisphosphatase II, flavodoxin, phosphoribosylformylglycinamidine synthase, coproporphyrinogen III oxidase, homoserine dehydrogenase, ATP synthase subunit beta, L-asparaginase, reaction center D1 of photosystem II and protein translocase SecA, the proteins were not detected under either N depletion or ammonium repletion. However, many of these have been identified as potential disulfide proteome Trx targets in *Synechocystsis sp.* (Lindahl and Kieselbach, 2009). Taking into account that *Prochlorococcus* SS120 (as all other cultured strains thus far) is incapable of nitrate utilization (López-Lozano et al., 2002; García-Fernández et al., 2004), we focused our comparison to the N depletion vs ammonium repletion conditions described by Wegener et al. Hereafter we will summarize the comparisons for the three cases for which data were provided (expressed as log2 ratios in the Supplemental table 6 of the manuscript by Wegener and coworkers) under boh N depletion and ammonium repletion:

GroEl from *Prochlorococcus* SS120 corresponds to GroEl1 from *Synechocystis* PCC 6803. This protein was much less abundant under N starvation than on ammonium (-0.845 vs 0.692). The difference was less marked for the urea transporter subunit UrtA (1 vs 1.273). Finally, for the RNA polymerase subunit beta (RpoB), again there was a clear decrease under N starvation (- 1.17) vs ammonium repletion (1.946).

Since reversible cysteine modification is often involved in the regulation of the protein function, it is possible that some of the proteins detected in our study will show no significant change in their concentration under N limitation such as glutamine synthetase (Fig. 2). The redox modification can produce the required effects without need to modify their concentrations, either by new protein synthesis or by degradation.

On the other hand in the three proteins which allowed direct comparison (GroEl, UrtA and RpoB), it seems that the general effect of nitrogen starvation was to decrease their concentration. However, the actual effect of nitrogen on the regulation of the metabolism might be more complex; for instance, microarray studies on *Prochlorococcus* showed that the expression of *urtA* was upregulated

(41.36-fold in the strain MED4 and 9.45-fold in the strain MIT9313), in contrast to the lower concentration of UrtA in *Synechocystis* PCC 6803 under N starvation. This apparent contradiction might be explained by a very strong turnover of UrtA under N limitation or by a very different physiological response in *Prochlorococcus* vs *Synechocystis*. Our results demonstrate that besides possible changes of *urtA* expression / UrtA concentration in *Prochlorococcus* SS120, this subunit of the transporter seems to be subjected to regulation by cysteine modification, pointing out to an important role of this ABC transporter under nitrogen limitation. This is in good agreement to the general response to stress, proposed by Wegener et al, involving the activation of urea utilization as a source of nitrogen (Wegener et al., 2010).

Concluding remarks

Nitrogen starvation had a wide range of effects on the redox proteome of *Prochlorococcus*. Key enzymes from the nitrogen metabolism were modified, but at the same time we observed changes in transporters and transaminases, suggesting that *Prochlorococcus* was adapting by activation of the uptake of nitrogen and by transfering nitrogen to essential proteins.

On the other hand, we could detect a strong effect on photosynthetic structural proteins and also enzymes involved in chlorophyll biosynthesis or in electron transfer.

In addition we observed that nitrogen limitation affected phosphorus metabolism, the status of ATP synthase, and enzymes involved in the nucleic acids metabolism. This suggests that the lack of nitrogen affects almost all metabolic pathways, far beyond the proteins directly involved in nitrogen metabolism. This is in good agreement to the observation by Tolonen and coworkers (Tolonen et al., 2006), who found that many of the genes whose expresion was affected by N starvation did not belong to the large group of genes controlled by the master N regulator, NtcA.

In conclusion our results demonstrate that beyond the transcriptional changes elicited by nitrogen starvation, *Prochlorococcus* also responds with extensive postranslational redox modifications, a reflection of the importance of nitrogen in cyanobacterial metabolism.

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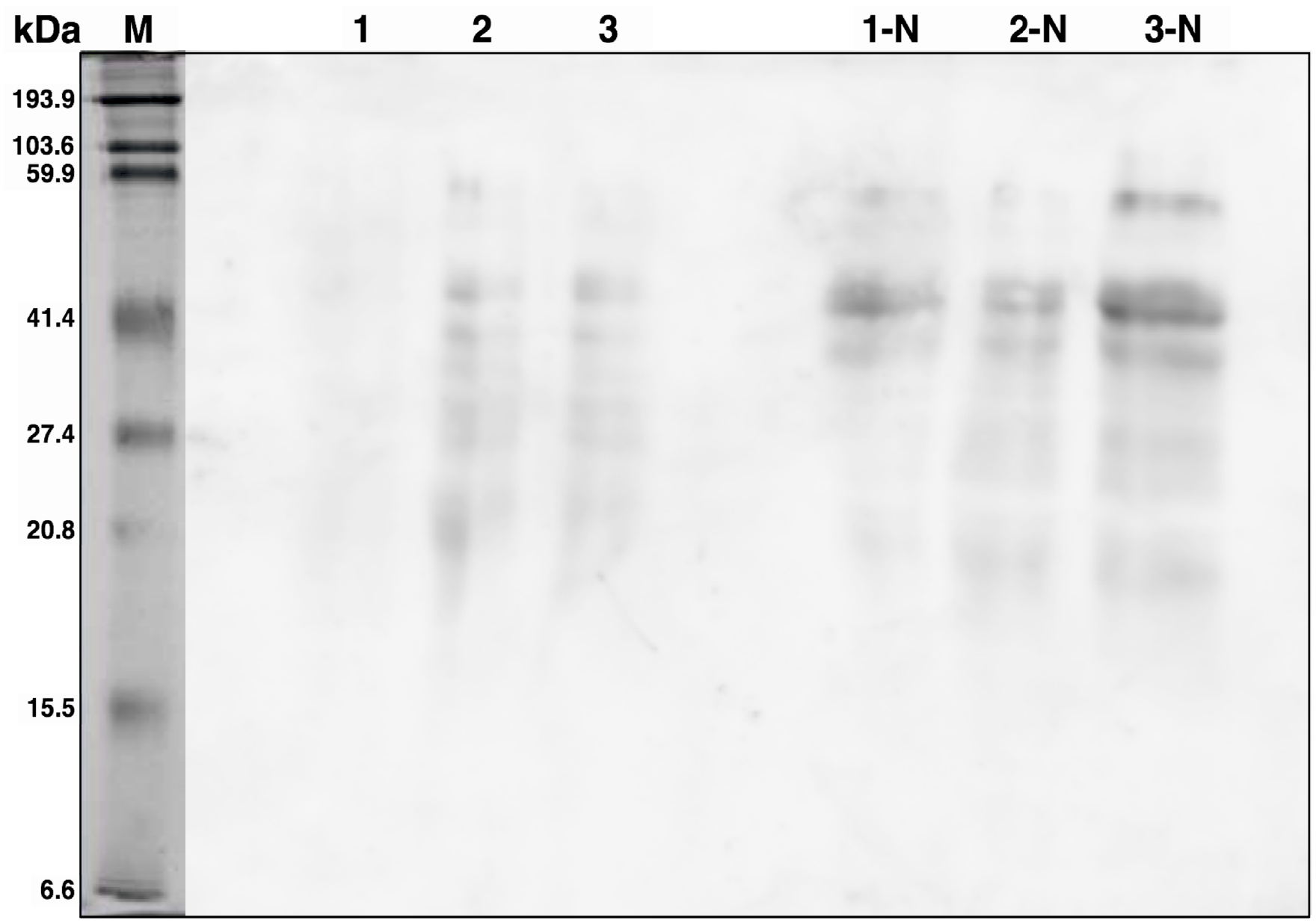
Table 1. Proteins with reversibly oxidized cysteines detected only under either control or N starvation conditions.

Protein	GI Number	Cys Peptide Detected	Condition Detected
ABC transporter, substrate binding protein, possibly Mn	157413499	67	Control
Formyltetrahydrofolate deformylase	78780180	18	Control
Pyruvate dehydrogenase E1 component, alpha subunit	254525916	271	Control
Ribose-phosphate pyrophosphokinase	33861636	247	Control
UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	91070309	98	Control
Isocitrate dehydrogenase	78780073	463	N- Starvation
Ribulose-phosphate 3-epimerase	33861323	68	N-Starvatiom

Table 2. Proteins containing reversibly oxidised cysteines with significant differences in oxidation state between N-starved vs control conditions.

Protein	GI number	Cys Detected	Fold	P Value
Bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase	72382157	Cys 43/65	1.7	0.05
Chaperonin GroEL	123969170	Cys146	2.0	0.064
Coproporphyrinogen III oxidase	123969321	Cys231	1.7	0.051
DNA-directed RNA polymerase subunit beta	124022146	Cys886/875	1.9	0.003
F0F1 ATP synthase subunit beta	159904098	Cys227	3.1	0.043
Flavodoxin FldA	126696711	Cys142	1.6	0.028
Fructose 1,6-bisphosphatase II	126696165	Cys149	2.1	0.006
Homoserine dehydrogenase	78779445	Cys344	5.8	0.045
(3R)-hydroxymyristoyl-ACP dehydratase	123969068	Cys117	1.8	0.062
*L-asparaginase II	123966129	Cys166	0.3	0.004
Phosphoribosylformylglycinamidine	33861337	Cys89	1.8	0.011
Photosystem I subunit VII	159904273	Cys21/34	1.9	0.024
Photosystem II reaction center D1	33239704	Cys19	1.7	0.033
Preprotein translocase subunit SecA	126697168	Cys577	1.5	0.086
Putative inorganic pyrophosphatase	123968086	Cys163	1.7	0.041
Putative O-Acetyl homoserine sulfhydrylase	33861199	Cys96	2.0	0.04
Putative urea ABC transporter, substrate	78779213	Cys145	5.3	0.021
RNA recognition motif-containing protein	123967549	Cys30	1.8	0.06
Triosephosphate isomerase	123968574	Cys127	1.9	0.03


kDa
M
1
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3-N
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41.4
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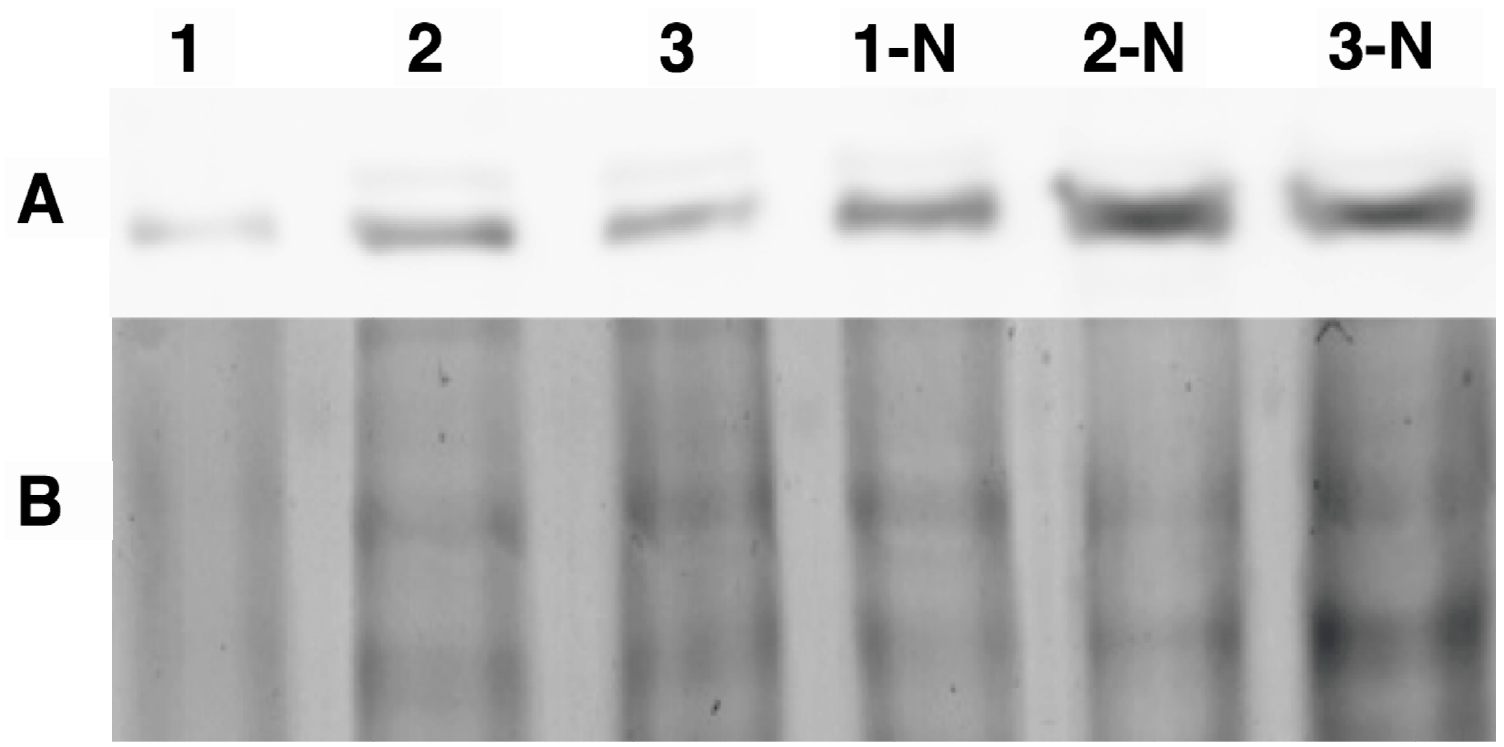
20.8
15.5
6.6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AS9601   gi|123967549   ------------------------------MSIFVGNLPFRAEREDVIQLFAPFGEVLNC
MIT9301  gi|126695350   ------------------------------MSIFVGNLPFRAEREDAIQLFAPFGEVLNC
MIT9312  gi|78778398    ------------------------------MSIFVGNLPFRAEREDVLQLFAPFGEVLNC
MIT9215  gi|157412351   ------------------------------MSIFVGNLPFRAEREDVIQLFAPFGEVVNC
MIT9211  gi|159902554   ------------------------------MSIFVGNLPFRAEQEDVIQLFAPFGEVANC
MIT9515  gi|123965247   ------------------------------MSIFVGNLPFRAEREDILELFTPYGEVMNC
NATL1A   gi|124024725   ------------------------------MSIFVGNLPFRAEQEDVMELFSPFGEVSNC
NATL2A   gi|72383177    ------------------------------MSIFVGNLPFRAEQEDVMELFSPFGEVSNC
MED4     gi|33860573    ---------MYMNYLISSFYLGYYFKVEFQVSIFVGNLPFRAEREDILELFTPYGEVMNC
MIT9313  gi|33862287    ------------------------------MSIFVGNLPFRAEQEDVIELFAPFGEVANC
MIT9303  gi|124021728   ------------------------------MSIFVGNLPFRAEQEDVIELFAPFGEVANC
RS9916   gi|116074289   ------------------------------MSIFVGNLPFRAEQEDIIELFAAHGEVTNC
CC9605   gi|78211572    ------------------------------MSIFVGNLPFRAEQEDVIELFAQFGEVTNC
RCC307   gi|148241113   ------------------------------MSIFVGNLPFRAEQEDIIELFASFGEVANC
WH8109   gi|260435624   ------------------------------MSIFVGNLPFRAEQEDVIELFAQFGEVTNC
CC9902   gi|78183598    ------------------------------MSIFVGNLPFRAEQEDVTELFAQFGEVVNC
PCC7001  gi|254431254   ------------------------------MSIFVGNLPFRAEQEDVAELFAPFGEVVSC
BL107    gi|116071793   MEHHFVSELWPDGNVPGPFGPFFRTRSTQNVSIFVGNLPFRAEQEDVTELFAQFGEVVNC
CB0101   gi|318042980   ------------------------------MSIFVGNLPFRAEQEDVAELFAPFGEVTNC
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WH7803   gi|148238350   ------------------------------MSIFVGNLPFRAEQEDVIELFAAFGEVTNC
MIT9202  gi|254526608   ------------------------------------------------------MFAPFGEVVNC
RS9917   gi|87123335    ------------------------------MSIFVGNLPFRAEQEDVIELFAAYGEVTNC
PCC7001  gi|254432360   ------------------------------MTIYVGNLSFDAEVDDVQHLFSEYGDVRKC
MIT9303  gi|124022676   ------------------------------MTIYIGNLSFQAEQEDLLDLFSQYGEVKTC
MIT9313  gi|33863352    ------------------------------MTIYIGNLSFQAEQEDLLDLFSQYGEVKTC
PCC7001  gi|254432068   ------------------------------MTIYVGNLSFQAEREDLLDLFGQYGEVRQC
WH5701   gi|87301157    ------------------------------MTIYVGNLSFDAEQEDLRDLFSQYGEVRQC
RS9916   gi|116073022   ------------------------------MTIYIGNLSFQAEQEDLLDLFSQYGEVKSA
RS9917   gi|87124729    ------------------------------MTIYIGNLSFQAEQEDLLDLFNQYGEVKSA
CC9311   gi|113953042   ------------------------------MTIYIGNLSFQAEQEDLLDLFGQYGEVKSA
WH7805   gi|88808896    ------------------------------MTIYIGNLSFQAEQEDLLDLFSQYGEVKSA
WH7803   gi|148239850   ------------------------------MTIYIGNLSFQAEQEDLLDLFSQYGEVKSA
PCC7421  gi|37520960    ------------------------------MTLFVGNLPFSATEQEIVEAFTEYGEVKSV
```

Supplementary Table 1.

Three independent cultures (N=3) for both control and N-starved *Prochlorocoocus* cells were analysed by MS/MS and an excel table containing all proteins that were identified from at least two independent cultures is presented. The Sequest score of proteins, the % coverage and number of unique peptides for each protein from each independent culture and from each strain can be examined.

Major Columns;

A, lists accession numbers

B, amino acids in protein

C, protein description

D, % coverage

E, number of unique peptides detected

Likewise the information for each unique peptide detected from each protein can be examined by opening the (+) button in column 1. The information includes;

- The sequence of each unique peptide
- The number of proteins in *Prochlorococcus* that contain the peptide sequence
- The number of protein groups
- Activation type (all CID)
- Δ score
- Sequest rank for peptide
- Peptide charge
- MH+ [Da]
- ΔM [ppm]
- First scan
- Last scan
- Retention time
- Number of ions matched
- Peptide modifications [e.g. destreak on cys residues signifies the cys was reversibly oxidised and reduced by mercaptoethanol]
- Probability
- xCorr of individual peptides.

This can be embedded again by using the (–) button in column 1.

Proteins highlighted have been identified from non-cysteine containing peptides.

					Control N=1			Control N=2		
Accession #	#AAs	Description	ΣCoverage	Σ# Peptides	Score C1	Coverage C1	eptide	Score C2	Coverage C2	eptide
gi123969282	122	50S ribosomal protein L18 [Prochlorococcus m	21,31	3	4,72	21,31	1			
gi157413499	505	ABC transporter, substrate binding protein, po	5,74	3	4,18	5,74	2	3,73	2,97	1
gi126695409	449	acetyl-CoA carboxylase biotin carboxylase sub	9,80	11	7,68	9,80	3	7,32	5,35	2
gi123969278	182	Adenylate kinase [Prochlorococcus marinus st	9,89	6						
gi123969389	586	aspartate kinase [Prochlorococcus marinus str	4,10	4	4,41	4,10	1			
gi87119361	939	ATP-dependent helicase HepA [Marinomonas s	1,81	101	13,14	1,81	25	9,04	1,81	12
gi254526296	617	ATP-dependent metallopeptidase HflB subfami	5,02	6						
gi123967588	412	bifunctional ornithine acetyltransferase/N-acet	4,37	22	14,61	4,37	4	3,78	4,37	1
gi123968143	765	carboxysome shell protein CsoS2 [Prochloroco	3,40	14	10,29	3,40	3	11,75	3,40	2
gi78212390	369	cell division protein FtsZ [Synechococcus sp. C	5,42	20	8,98	5,42	2	4,14	5,42	1
gi159903848	619	cell division protein FtsH3 [Prochlorococcus m	2,60	10	4,05	2,44	1	3,83	2,44	1
gi157878583	80	Chain A, Nmr Solution Structure Of Unbound, (	36,25	20	5,58	20,00	2	3,02	20,00	1
gi33861992	544	chaperonin GroEL [Prochlorococcus marinus su	12,32	127	59,48	4,96	19	25,50	4,96	10
gi91070621	422	conserved hypothetical protein [uncultured Pro	3,55	9	6,48	3,55	2	3,37	3,55	1
gi78779352	282	dihydrodipicolinate reductase [Prochlorococcus	9,57	6	5,73	9,57	2	6,20	9,57	2
gi78779778	479	dihydrolipoamide dehydrogenase [Prochlorococ	5,85	7						
gi33861126	349	dihydroorotase [Prochlorococcus marinus subs	5,44	3						
gi78780012	311	DNA-directed RNA polymerase subunit alpha [	8,36	15	6,67	8,36	2	3,51	8,36	2
gi126697012	1366	DNA-directed RNA polymerase subunit beta' [I	1,32	13	6,94	1,32	3	10,36	1,32	3
gi78779962	1097	DNA-directed RNA polymerase subunit beta [P	6,29	12	7,94	5,20	5			
gi157414109	399	elongationfactor Tu [Prochlorococcus marinus	14,54	141	65,03	10,78	32	25,69	14,54	9
gi123967763	430	Enolase [Prochlorococcus marinus str. AS9601	9,30	24	14,67	9,30	4	9,30	9,30	2
gi123965550	260	enoyl-(acyl carrier protein) reductase [Prochlo	12,31	42	10,77	12,31	7	9,57	12,31	7
gi157414052	505	F0F1 ATP synthase subunit alpha [Prochloroco	2,76	24	4,73	2,76	5			
gi33861994	486	F0F1 ATP synthase subunit beta [Prochlorococ	6,79	6	3,68	3,09	2			
gi33860771	318	FAD-dependent pyridine nucleotide-disulphide c	5,66	29	10,18	5,66	3	5,47	5,66	1
gi126696520	321	ferredoxin-NADP oxidoreductase (FNR) [Prochl	7,79	19	7,93	7,79	4			
gi78780180	284	formyltetrahydrofolate deformylase [Prochlorc	8,80	2	4,00	8,80	1			
gi123968362	333	fructose 1,6-bisphosphatase II [Prochlorococcu	6,01	96	59,81	6,01	18	17,95	6,01	6
gi157413172	637	ATP-dependent metalloprotease FtsH [Prochlor	2,83	20	6,74	2,83	4	6,64	2,83	4

gi91070190	431	fumarate lyase:adenylosuccinate lyase [uncult	8,58	6	5,66	8,58	2			
gi78779161	431	glucose-1-phosphate adenylyltransferase [Proc	7,89	21	18,54	7,89	5			
gi33861477	473	Glutamine synthetase, glutamate--ammonia li	11,63	47	12,89	6,55	5	25,98	11,63	7
gi33861458	634	heat shock protein 90 [Prochlorococcus marin	5,05	18	7,20	5,05	3			
gi78779445	433	Homoserine dehydrogenase [Prochlorococcus r	5,31	4	4,05	5,31	1			
gi123968332	145	hypothetical membrane protein [Prochlorococc	14,48	5	5,88	14,48	2			
gi123969110	91	hypothetical protein A9601_15781 [Prochlorocc	40,66	11				7,00	19,78	2
gi78779387	106	hypothetical protein PMT9312_1003 [Prochloro	36,79	59	9,61	36,79	6			
gi126696506	387	inositol-5-monophosphate dehydrogenase [Proc	15,76	18	8,65	10,59	3	3,36	4,65	1
gi78780073	474	isocitrate dehydrogenase [Prochlorococcus ma	3,59	7						
gi221538460	329	ketol-acid reductoisomerase [Prochlorococcus	7,55	15	5,47	7,55	2	4,27	7,55	2
gi78779814	490	leucyl aminopeptidase [Prochlorococcus marin	11,43	20	12,33	8,57	4			
gi78779353	1336	magnesium chelatase [Prochlorococcus marinu	2,77	14	16,73	2,77	5	3,65	1,87	1
gi125658551	627	methanol dehydrogenase large subunit [Methyl	3,51	4	4,22	3,51	1	6,17	3,51	2
gi33860964	328	O-acetylserine (thiol)-lyase A [Prochlorococcu	6,71	14	16,99	6,71	5			
gi123969070	712	outer envelope membrane protein-like protein	3,23	6						
gi78779106	331	Periplasmic phosphate binding protein [Prochlo	12,99	35	16,60	6,95	8	10,20	12,99	4
gi123967626	545	phosphoglucomutase [Prochlorococcus marinus	7,16	6						
gi78779172	221	phosphoribosylformylglycinamidine synthase I	18,55	12	7,34	11,31	2	4,40	11,31	1
gi91070307	298	phosphoribulokinase [uncultured Prochlorococc	21,48	22				7,61	9,40	3
gi33862079	742	photosystem I P700 chlorophyll a apoprotein A	4,18	7				4,99	4,18	2
gi91070529	241	photosystem I PsaA protein [uncultured Proch	11,20	10						
gi123968061	184	Photosystem I PsaF protein (subunit III) [Proc	14,13	196	131,28	14,13	28	115,07	14,13	27
gi123969255	199	photosystem I reaction center protein subunit	27,14	33	10,18	10,55	4	38,53	27,14	10
gi123967786	279	Photosystem II manganese-stabilizing protein	17,56	281	167,16	17,56	46	192,07	17,56	52
gi33861714	460	Photosystem II PsbC protein (CP43) [Prochlor	7,39	3				5,85	7,39	2
gi126695860	316	porphobilinogen deaminase [Prochlorococcus m	13,92	23	16,46	13,92	5	3,42	5,38	1
gi123968452	120	possible cAMP phosphodiesterases class-II [Pr	41,67	32	20,09	41,67	5	17,77	41,67	5
gi126696524	119	possible Villin headpiece domain-containing pr	15,97	3	4,08	15,97	1	4,28	15,97	1
gi145353264	130	predicted protein [Ostreococcus lucimarinus C	20,00	13				4,76	20,00	3
gi123969381	943	preprotein translocase subunit SecA [Prochlorc	5,83	18	12,74	3,71	4			
gi123968086	195	putative inorganic pyrophosphatase [Prochloro	19,49	31	10,62	19,49	5	10,74	17,95	3

gi91070055	376	putative nicotinamide nucleotide transhydroge	10,64	12	6,79	10,64	2	3,56	4,52	1
gi157413687	376	putative nicotinamide nucleotide transhydroge	9,84	14	13,77	9,84	4	3,56	4,52	1
gi91069943	84	putative protein [uncultured Prochlorococcus r	21,43	22	12,19	21,43	5	7,44	21,43	2
gi254525916	357	pyruvate dehydrogenase E1 component, alpha	4,20	3	4,77	4,20	2	3,30	4,20	1
gi78779253	327	pyruvate dehydrogenase E1 beta subunit [Proc	7,95	6				3,25	7,95	1
gi33861636	331	ribose-phosphate pyrophosphokinase [Prochlor	6,95	3	7,11	6,95	2			
gi33861323	251	Ribulose-phosphate 3-epimerase [Prochlorococ	9,96	6						
gi123967549	203	RNA-binding region RNP-1 (RNA recognition m	12,32	21	16,76	12,32	6	9,82	12,32	3
gi78778702	413	S-adenosylmethionine synthetase [Prochloroco	10,17	17	12,29	10,17	3	8,04	5,08	2
gi126695619	423	serine hydroxymethyltransferase [Prochlorocc	4,26	10	6,49	4,26	4			
gi33861413	194	thioredoxin peroxidase [Prochlorococcus marir	12,89	25	12,29	12,89	6	20,15	12,89	4
gi25453310	80	Transcription elongation factor 1 homolog	22,50	7	3,95	22,50	3			
gi254526762	691	translation elongation factor G [Prochlorococc	7,81	10	5,41	4,34	2	8,34	5,93	2
gi33633963	230	Triosephosphate isomerase [Prochlorococcus r	9,57	8				4,13	9,57	1
gi254526324	260	two-component response regulator [Prochloroc	12,50	10	7,31	12,50	2			
gi91070309	344	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-	3,49	4	3,66	3,49	1	3,43	3,49	1
gi123965821	234	uridylate kinase [Prochlorococcus marinus str.	20,51	18	13,78	20,51	4	8,44	9,40	2

Control N=3			N-Starvation N=1			N-Starvation N=2			N-Starvation N=3		
Score C3	Coverage C3	Peptides	Score N1	Coverage N1	Peptides N1	Score N2	Coverage N2	Peptides N2	Score N3	Coverage N3	Peptides N3
			6,26	21,31	1				3,73	21,31	1
			9,82	9,80	3				9,34	9,80	3
			5,27	9,89	2	5,38	9,89	2	4,86	9,89	2
4,28	4,10	1				4,13	4,10	1	4,36	4,10	1
10,27	1,81	13	12,26	1,81	21	7,75	1,81	16	7,75	1,81	14
5,41	2,51	2	8,87	5,02	3	6,26	2,51	2			
14,90	4,37	4	14,77	4,37	4	14,03	4,37	4	14,79	4,37	5
5,30	3,40	1	16,17	3,40	4	6,34	3,40	2	8,05	3,40	2
13,03	5,42	3	27,90	5,42	7	21,35	5,42	5	9,92	5,42	2
7,16	2,60	2	18,62	2,60	5				3,77	2,44	1
8,66	20,00	4	17,60	36,25	7	12,11	20,00	4	6,45	20,00	2
73,67	12,32	27	85,97	4,96	27	64,06	4,96	21	60,14	4,96	23
7,26	3,55	2				6,43	3,55	2	6,40	3,55	2
						5,59	9,57	2			
9,30	5,85	3				3,72	5,85	1	10,08	5,85	3
4,01	5,44	1				4,46	5,44	1	4,03	5,44	1
8,07	8,36	3	9,42	8,36	3	8,50	8,36	3	6,39	8,36	2
			10,94	1,32	4				6,96	1,32	3
			5,59	3,10	3				5,95	2,28	4
72,76	10,78	23	70,51	10,78	24	62,99	10,78	29	84,94	10,78	24
11,60	4,19	3	24,39	9,30	6	14,12	9,30	4	19,19	9,30	5
6,37	12,31	4	12,51	12,31	10	10,56	12,31	7	10,19	12,31	7
4,50	2,76	6	4,98	2,76	4	5,02	2,76	5	3,65	2,76	4
4,24	3,70	1							5,70	6,79	3
25,01	5,66	7	25,52	5,66	8	17,86	5,66	5	18,08	5,66	5
7,71	7,79	3	11,59	7,79	5	7,08	7,79	2	10,36	7,79	5
3,98	8,80	1									
50,76	6,01	16	58,17	6,01	19	48,68	6,01	17	63,92	6,01	20
6,41	2,83	4	7,26	2,83	4	7,10	2,83	4			

			5,67	8,58	2				5,46	8,58	2
14,03	7,89	4	19,02	7,89	5	14,18	7,89	4	12,64	4,18	3
19,46	9,09	5	34,48	11,63	12	18,92	6,55	8	32,17	11,63	10
12,70	5,05	5	10,46	5,05	4	7,34	5,05	3	7,18	5,05	3
3,55	5,31	1				3,69	5,31	1	3,70	5,31	1
3,41	14,48	1	6,58	14,48	2						
10,69	40,66	3	6,97	40,66	2	6,86	40,66	2	6,84	40,66	2
8,90	36,79	11	29,58	36,79	23	12,47	36,79	13	7,68	36,79	6
10,05	10,59	3	15,91	15,76	4	13,16	15,76	4	10,90	10,59	3
			6,76	3,59	3	6,30	3,59	2	6,07	3,59	2
8,62	7,55	3	8,16	7,55	4	5,26	7,55	2	5,26	7,55	2
14,49	8,57	4	13,59	8,57	4	10,59	11,43	4	11,94	8,57	4
			8,64	2,77	3	5,85	2,77	2	11,03	2,77	3
						4,40	3,51	1			
13,06	6,71	4	9,39	6,71	2	7,79	6,71	2	5,20	6,71	1
3,66	3,23	1	9,87	3,23	3	5,86	3,23	2			
14,59	12,99	5				28,55	6,95	11	19,78	6,95	7
3,51	7,16	2	8,73	4,40	2	8,34	4,40	2			
7,88	11,31	2	8,83	18,55	3	7,92	11,31	2	7,84	11,31	2
15,73	21,48	6	13,79	9,06	5	10,69	14,09	4	11,30	14,09	4
			8,73	1,75	5						
14,74	11,20	4				6,73	11,20	3	8,88	11,20	3
168,67	14,13	37	197,90	14,13	43	127,38	14,13	28	154,85	14,13	33
29,06	22,11	6	15,26	10,55	4	15,77	22,11	4	19,96	22,11	5
129,29	17,56	39	222,94	8,60	56	207,76	17,56	56	124,61	17,56	32
3,34	5,65	1									
17,00	13,92	4	18,13	13,92	5	13,05	12,97	3	18,60	13,92	5
16,07	41,67	5	25,46	41,67	7	18,46	41,67	6	14,44	41,67	4
									4,14	15,97	1
3,39	20,00	2				7,34	20,00	4	5,75	20,00	4
16,02	5,73	5	17,86	3,71	5	13,23	3,71	4			
7,92	17,95	3	16,23	17,95	5	21,53	17,95	8	19,02	17,95	7

6,96	10,64	2	13,77	10,64	3	5,78	10,64	2	7,30	10,64	2
4,06	4,52	1	16,04	9,84	5				9,70	9,84	3
8,88	21,43	3	15,24	21,43	5	8,79	21,43	3	12,68	21,43	4
6,65	7,95	2							9,38	7,95	3
3,60	6,95	1									
7,16	9,96	2	4,69	9,96	1	4,00	9,96	1	7,15	9,96	2
12,57	12,32	3	15,75	12,32	4	9,35	12,32	2	11,35	12,32	3
12,47	10,17	3	12,56	10,17	3	13,09	10,17	3	10,81	10,17	3
			6,06	4,26	3				5,49	4,26	3
7,65	12,89	5	11,29	12,89	3	14,34	12,89	4	11,06	12,89	3
						4,28	22,50	2	4,09	22,50	2
			6,65	4,34	3	8,28	5,93	2	4,04	2,46	1
9,21	9,57	2	8,52	9,57	2	9,50	9,57	2	4,24	9,57	1
8,17	12,50	2	7,52	12,50	2	7,08	12,50	2	8,01	12,50	2
3,56	3,49	1							3,93	3,49	1
12,91	20,51	3	15,39	20,51	4	13,73	20,51	3	8,87	9,40	2