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Full Length Research Paper

Nitrogen availability for nitrogen fixing cyanobacteria upon growth on dinitrogen

Röbbe Wünschiers

University of Cologne, Institute for Genetics, Zülpicher Str. 47, D-50674 Cologne, Germany.

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The filamentous cyanobacterium *Nostoc* PCC 7120 is able to convert dinitrogen to ammonia in the absence of combined nitrogen. The expression of 20% of coding sequences from all major metabolic categories was examined in nitrogen fixing and non-nitrogen fixing growth conditions. The expression data were correlated with the nitrogen content of gene products. When growing on dinitrogen, *Nostoc* PCC 7120 incorporates more nitrogen into its proteome than in growth on ammonia. Thus, paradoxically, limitation of combined nitrogen in the culture medium leads to excessive nitrogen supply from the air. Biotechnological implications are discussed.

Key words: Cyanobacteria, gene expression, nitrogen availability, nitrogen fixation.

INTRODUCTION

Cyanobacteria are intensively used in biotechnology (Singh et al., 2005). We are interested in growing cyanobacteria in photobioreactors with the goal of photohydrogen production (Wünschiers and Lindblad, 2003; Dutta et al., 2005). One important issue is sufficient nutrient and light supply. Nitrogen sources most commonly used by cyanobacteria are either combined nitrogen (nitrate, nitrite, ammonium, urea) or atmospheric nitrogen (dinitrogen). The assimilation of these compounds provides intracellular ammonium, which itself is the preferred nitrogen source (Herrero et al., 2001). Usually, large scale bioreactors are necessary for biotechnological utilization of cyanobacteria. Thus, nutrient supply becomes an important cost factor.

Hydrogen gas produced by cyanobacteria is a byproduct of nitrogenase activity. The nitrogenase catalyzes the fixation of dinitrogen into ammonia and is only active under deprivation of combined nitrogen. Thus, in order to obtain hydrogen gas, cyanobacteria are grown in combined nitrogen deprived condition. One question to ask is, whether this has any impact on cellular constitution and consequently on biomass yield and quality. Here we focus on the nitrogen content of proteins expressed either under nitrogen fixing or non-nitrogen fixing conditions.

A powerful tool to study gene expression and its regulation is the DNA-microarray technique. Until now, only few approaches of DNA-microarray based gene-expression analyses with heterocystous cyanobacteria have been reported. With respect to nitrogen metabolism, only one experiment has been described (Ehira et al., 2003; Sato et al., 2004). The authors used a segment-based DNA-microarray, where each segment covers up to 8 predicted genes (Ehira et al., 2003). This experimental setup does not allow expression analysis of individual genes. We employed a novel, recently developed microarray technique where probe synthesis, hybridization, and signal detection take place in one device at strongly controlled physical conditions (Baum et al., 2003; Güimil et al., 2003). The expression data were correlated with the calculated nitrogen content of proteins.

MATERIAL AND METHODS

The cyanobacterium *Nostoc* sp. strain PCC 7120 (formerly *Anabaena* sp. strain PCC 7120) was grown on either dinitrogen (nitrogen fixing) or combined nitrogen (non-nitrogen fixing) in batch cultures. Non-nitrogen fixing conditions were obtained by growing cells in $BG11_0$ (Stanier et al., 1971) supplemented with 5 mM NH_4Cl and 10 mM HEPES. Nitrogen-fixing conditions were obtained by growing cells in $BG11_0$. All cultures were grown, harvested, and subjected to total RNA isolation as previously described (Oxelfelt

*Corresponding authors E-mail: robbe.wunschiers@uni-koeln.de.

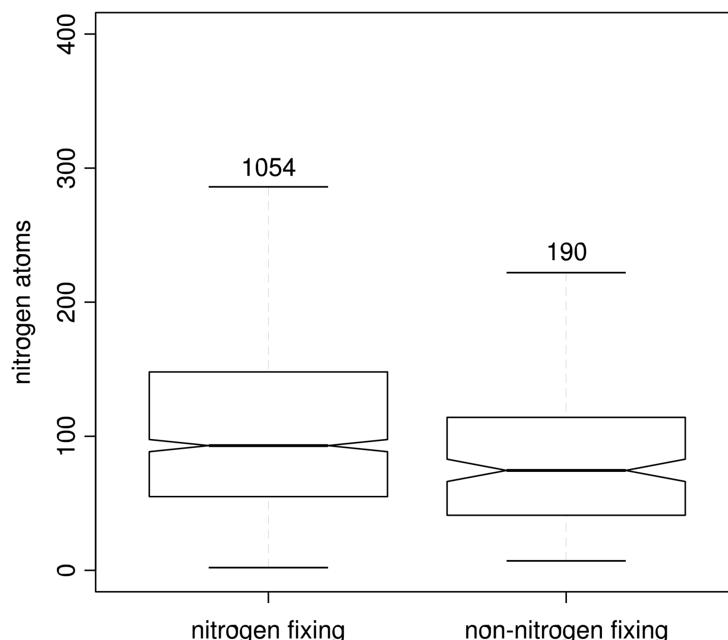

Figure 1. Median number of nitrogen atoms in genes more than 2-fold stronger expressed in *Nostoc* PCC 7120 cultures grown on either dinitrogen or combined nitrogen as sole nitrogen source. The notches around the median indicate the 95% confidence interval that the median from one box differs from the median of another box, i.e., if the notches do not overlap the corresponding medians are significantly different. The upper and lower box indicate the third and second quartile, respectively. The plot whiskers extending out from the box to the extreme values. The number above the upper whisker states the number of observations for the corresponding box.

et al., 1995; Tamagnini et al., 1997; Axelsson and Lindblad, 2002).

DNA-microarray based gene-expression analysis was carried out with a geniom one microarray system (Febit Biotec GmbH, Heidelberg/Germany) as described in Baum et al. (2003). A unique *Nostoc* PCC 7120 25-mer probe set was calculated based on the full genome sequence retrieved from CyanoBase: http://www.kazusa.or.jp/cyanobase/Anabaena/index.html. Selection criteria were as described in Lockhart et al. (1996) with modifications for the longer probes (25-mers instead of 20-mers). If available, 10 unique probes per ORF were used in the experiments. Processing of raw data, including background correction, array to array normalization and determination of gene-expression levels were carried out using the PROP-algorithm of the geniom application software which is based on the MOID-algorithm described by Zhou and Abagyan (Zhou and Abagyan, 2002). Background correction is based on probes with no corresponding mRNA-target and the average of the lowest 5% expressed genes. Data normalization is based on iteratively correcting the raw data on non-regulated genes.

All gene-expression data are saved in the Hydrogenase Database (HyDaBa, http://hydaba.uni-koeln.de). This relational database allows cross-linking of the expression data with the annotated genome data from NCBI (http://www.ncbi.nlm.nih.gov) and Cyanobase (http://www.kazusa.or.jp/cyano/Anabaena/cgi-bin/category_ana.cgi) and pathway maps available from KEGG (http://www.genome.jp/kegg). The latter is achieved in real-time via a SOAP-interface. HyDaBa is based on an Apache Webserver (http://www.apache.org), MySQL database (http://www.mysql.com) and a front-end programmed in PHP (http://www.php.net). All data are publicly accessible via this web interface that can be accessed using *guest* and *hydaba06* as user name and password, respectively.

RESULTS AND DISCUSSION

In the present study we analyzed the expression of 1249 selected genes from all 16 metabolic categories (ca. 20% of the complete genome) of *Nostoc* PCC 7120 cultures in nitrogen fixing and non-nitrogen fixing conditions. Therefore we applied a DNA-microarray based approach. All gene-expression data obtained in our study are freely available at http://hydaba.uni-koeln.de using *guest* and *hydaba06* as user name and password, respectively. The database holds both raw and processed data. Besides data management the database allows cross-connectivity of expression data with annotations from NCBI database, Cyanobase and KEGG. A most convenient feature of HyDaBa constitutes the mapping of gene-expression data onto metabolic charts from the KEGG database.

It has been shown previously for yeast and *E. coli* that enzymes involved in the assimilation of sulfur, nitrogen, or carbon are depleted in that respective atom (Baudouin-Cornu et al., 2001). Furthermore, upon sulfur depletion, yeast spares sulfur rich proteins by either reducing their expression level or by expressing isoforms that are poor in sulfur (Fauchon et al., 2002). We are interested in the global responds of *Nostoc* PCC 7120 to the depletion of both inorganic and organic combined nitrogen in the culture medium. To address this question we tested if cells grown on dinitrogen as sole nitrogen

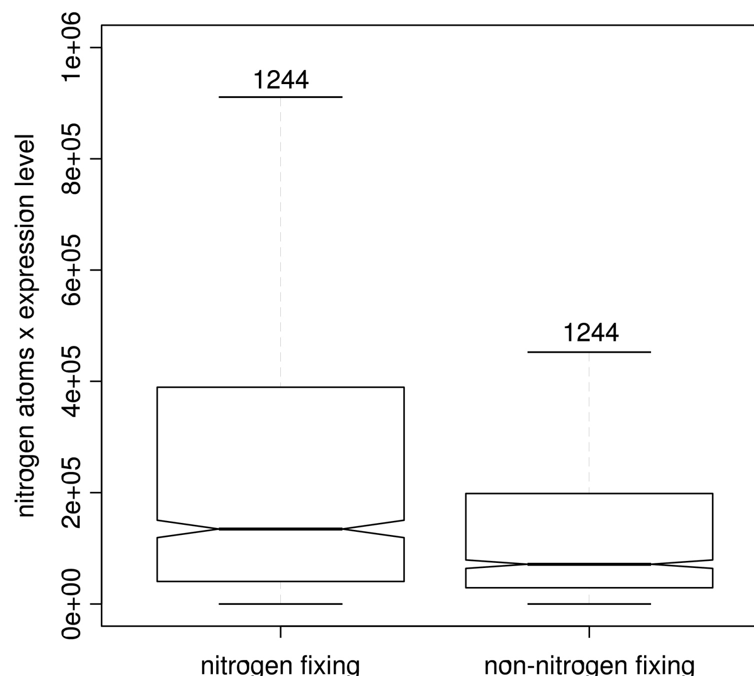

Figure 2. Totally expressed proteinous nitrogen for all analyzed genes. See Figure 1 for an explanation of the box plot.

Table 1. Expression differences for the HLIP-superfamily involved in adaptations and atypical conditions. All proteins are stronger expressed in growth on combined nitrogen. Four genes of the HLIP-superfamily were less than 2-fold stronger expressed (asl0514, asl2354, asl3726, asr5262).

Gene	Description	Expr-Diff
asl0449	CAB/ELIP/HLIP-related protein	2.3
asl0873	CAB/ELIP/HLIP-superfamily	4.4
asr3042	CAB/ELIP/HLIP-superfamily of proteins	11.3
asr3043	CAB/ELIP/HLIP-superfamily of proteins	7.9

source tend to spare nitrogen. This should be reflected in the nitrogen content of the gene products. As a rough approximation we assume that the expression level of a particular gene is proportional to the amount of protein the gene is encoding for. Then we can analyze whether the proteome composition differs between both growth conditions. Figure 1 shows the nitrogen content for all analyzed genes that are more than 2-fold stronger expressed in *Nostoc* PCC 7120 cultures grown on either dinitrogen or combined nitrogen. Genes expressed in nitrogen fixing conditions contain significantly larger numbers of nitrogen atoms than genes expressed in non-nitrogen fixing conditions (means: 118.5 versus 91.7; Welch two sample t-test: p-value < $2.2e^{-16}$). In summary, more nitrogen-rich proteins are expressed in growth on dinitrogen. Figure 2 shows, based on all analyzed proteins, the difference of expressed nitrogen atoms (number of nitrogen atoms per gene times expression value) in nitrogen fixing and non-nitrogen fixing conditions. Again, we observed a significant difference between these two conditions (means: 391634 versus 183051; Welch two sample t-test: p-value $8.8e^{-6}$). Obviously, *Nostoc* PCC 7120 does not save but spend nitrogen in nitrogen fixing conditions. We conclude that the cells are freed from any limitations in nitrogen utilization once the nitrogenase enzyme complex is expressed and active.

This finding is supported by the fact that members of high light-induced proteins (HLIP-family; Table 1) are up to 11-times stronger expressed in growth on combined nitrogen. These proteins belong to the CAB/ELIP/HLIP-superfamily and take over photo-protective functions (Heddad and Adamska, 2002). It is believed that HLIP-proteins fulfill their photoprotective role by either transient binding of free chlorophyll molecules or by participating in energy dissipation (Montané and Kloppstech, 2000). Photooxidative stress in not necessarily connected to high light fluxes but can also be caused by nutrient depri-

vation that ultimately lead to oversaturation of the photosynthetic electron transport chain.

Obviously, growth on dinitrogen is not sensed as nitrogen starvation by *Nostoc* PCC 7120. This raises the question why *Nostoc* does not permanently fix dinitrogen. This can presumably be explained by the metabolic costs the cell has to invest in order to synthesize and fuel the nitrogen fixing apparatus. Cells growing on combined nitrogen sources show higher growth rates and are ecologically competitive. In photobioreactors for photobiological hydrogen production we demand high hydrogen but little biomass yields. Furthermore, investments in nutrients should be minimized while the biomass, as a byproduct, should be of high nutritional quality. Only then it can be used, e.g., in livestock farming. In conclusion, apparent combined nitrogen deprivation has an overall positive effect on biomass quality, a fact that has to be considered when utilizing nitrogen fixing cyanobacteria in biotechnology.

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