A proteomic approach to iron and copper homeostasis in cyanobacteria

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Abstract

Cyanobacteria, which are considered to be the chloroplast precursors, are significant contributors to global photosynthetic productivity. The ample variety of membrane and soluble proteins containing different metals (mainly, iron and copper) has made these organisms develop a complex homeostasis with different mechanisms and tight regulation processes to fulfil their metal requirements in a changing environment. Cell metabolism is so adapted as to synthesize alternative proteins depending on the relative metal availabilities. In particular, plastocyanin, a copper protein, and cytochrome c₆, a haem protein, can replace each other to play the same physiological role as electron carriers in photosynthesis and respiration, with the synthesis of one protein or another being regulated by copper concentration in the medium. The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 has been widely used as a model system because of completion of its genome sequence and the ease of its genetic manipulation, with a lot of proteomic work being done. In this review article, we focus on the functional characterization of knockout *Synechocystis* mutants for plastocyanin and cytochrome c₆, and discuss the ongoing proteomic analyses performed at varying copper concentrations to investigate the cyanobacterial metal homeostasis and cell response to changing environmental conditions.

Keywords: proteomic; cyanobacteria; copper homeostasis; iron homeostasis; *Synechocystis*

INTRODUCTION

Trace metals have an important role in cell metabolism by acting as redox co-factors in enzymes involved in multiple metabolic pathways [1]. In particular, iron and copper participate in essential metabolic routes, namely photosynthesis and respiration. However, at high concentrations both metals promote the generation of reactive oxygen species (ROS) and, subsequently, oxidative damage and impaired cell function and death, which are processes involved in several pathologies and neurodegenerative diseases [2, 3]. Micronutrient (especially iron) acquisition and sequestration can be crucial for competing organisms in invading particular niches or in infecting cells in a host–pathogen interaction. Thus, the importance of metal homeostasis in maintaining intracellular bioavailability of essential metal ions within a range compatible with cell viability becomes evident. Homeostatic mechanisms comprise metal sensing, chelation and transport [4, 5].

The functioning of oxygenic photosynthesis requires several membrane complexes and soluble proteins containing either iron (including iron–sulphur clusters and haem groups) or copper as co-factors [6]. Actually, the requirement for iron and copper of photosynthetic organisms exceeds that of non-photosynthetic ones. This results in higher...
metal demand and in increased sensitivity to metal limitation, along with the production of ROS during photosynthetic activity [7].

Cyanobacteria, prokaryotic organisms performing oxygenic photosynthesis, are significant contributors to the global photosynthetic productivity [8]. They grow in a wide variety of marine and terrestrial environments, in which metal deficiencies can frequently occur, thereby leading to different adaptive responses to overcome metal limitations [7, 8].

In particular, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereinafter referred to as *Synechocystis*) is a widely used model system to study photosynthesis and other metabolic processes. It is the first photosynthetic organism for which the complete genome sequence was determined [9]; it is suitable to be genetically manipulated, so becoming transformable by exogenous DNA upon homologous recombination and integration; and its metabolic versatility allows it not only to survive in a wide variety of environments—including those with different metal limitations—but also to live under non-photosynthetic conditions if a suitable carbon source, such as glucose, is available. All these features make *Synechocystis* the subject for research in many studies. In fact, several deletion mutants lacking specific genes have been analysed [10, 11], and a number of proteomic studies under different environmental conditions and stresses have been carried out [12].

In this article, we focus on the functional characterization of *Synechocystis* knockout mutants for plastocyanin (Pc) and cytochrome *c*₅ (Cyt), the two alternative soluble proteins involved in electron transport both in photosynthesis and respiration. We also discuss the comparative proteomic analysis of the native strain and the deletion mutants grown with or without copper, thus yielding relevant information on the metal homeostasis in cyanobacteria and the response of these organisms to changing environmental conditions.

**IRON–COPPER HOMEOSTASIS IN CYANOBACTERIA**

Life on our planet started up in a reductive environment, but after the development of oxygenic photosynthesis, oxygen enrichment modified the composition of the atmosphere. This event changed the bio-availability of trace mineral elements and made iron a limiting nutrient because soluble ferrous ions were oxidized to the insoluble ferric species as the prevalent form of iron in the biosphere. Simultaneously, copper changed from highly insoluble sulphides to soluble cupric salts [13]. Iron biochemistry persistence was due to the further development of Fe(III) chelators, which rendered iron once again available, and prevented potential iron toxicity by its storage in ferritin. Whereas enzymes involved in anaerobic metabolism function at low redox potential, the biochemistry of copper can take advantage of the oxidizing power of oxygen to perform reactions at higher redox potentials [13]. Furthermore, there are a number of similar reactions catalysed by analogue enzymes containing either copper or iron (Table 1).

In some cases, metabolic adaptation allows the organisms to use one catalyst over the other depending on metal availability [14]. In particular, many algae and cyanobacteria synthesize the soluble electron carrier Pc or Cyt depending on copper availability, thus suggesting that these organisms can experience copper-deficiency in nature [15, 16]. Similarly, iron limitation induces the expression of new proteins, namely chlorophyll-binding proteins, and the replacement of the iron-containing protein ferredoxin by the flavin-containing protein flavodoxin to transfer electrons from photosystem I (PSI) to ferredoxin-NADP⁺ reductase [6]. Regulation of these switches involves different mechanisms (apoprotein degradation, transcriptional regulation) depending on the organism. For instance, the amount of mRNA for the Pc-encoding *petE* and Cyt-encoding *petJ* genes seem to be regulated by copper at the level of the initiation of transcription in *Synechocystis* and other cyanobacteria [17, 18].

**Table 1:** Equivalent physiological roles played by copper and iron proteins

<table>
<thead>
<tr>
<th>Function</th>
<th>Iron protein</th>
<th>Copper protein</th>
</tr>
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<tbody>
<tr>
<td>Oxygen transport</td>
<td>Haemoglobin</td>
<td>Haemocyanin</td>
</tr>
<tr>
<td></td>
<td>Haemerythrin</td>
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<tr>
<td>Hydroxylation</td>
<td>Di-iron MMO</td>
<td>Particulate MMO</td>
</tr>
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<td></td>
<td>Cytochrome P₄₅₀</td>
<td>Mononuclear tyrosinase</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Catechol dioxygenase</td>
<td>Dinuclear catechol oxidase</td>
</tr>
<tr>
<td>Electrons transfer</td>
<td>Cytochrome c₅</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>Terminal oxidase</td>
<td>Di-iron alternative oxidase</td>
<td></td>
</tr>
<tr>
<td>Anti-oxidant</td>
<td>Fe-SOD</td>
<td>Cu₉Cu₂−N₂O reductase</td>
</tr>
<tr>
<td>Nitrite reduction</td>
<td>Haem nitrite reductase</td>
<td>Cu nitrite reductase</td>
</tr>
</tbody>
</table>

SOD, superoxide dismutase; MMO, methane mono-oxygenase.

Adapted from [13].
Actually, Pc-mRNA is detected only in the presence of copper and Cyt-mRNA is detected only in the absence of copper [18].

Cyanobacteria contain internal compartments called thylakoids, which are distinct from the periplasm. Thylakoids are the site for both photosynthetic and respiratory electron transports, involving the copper proteins Pc and cytochrome c oxidase, the latter containing a binuclear copper centre oriented towards the thylakoidal lumen. Pc is imported inside the thylakoid via the typical prokaryotic Sec (for secretion) pathway for unfolded proteins [5]. Cyanobacterial thylakoids are currently the only location for copper enzymes known to require this metal entering into the bacterial cytoplasm. Consequently, copper is transported across the plasma and thylakoidal membranes by the consecutive action of two different P-type ATPases, the so-called CtaA and PacS complexes (Figure 1) [7]. Inside the cell, copper is chaperoned by Atx1, a small soluble protein that contains the typical copper-binding motif CXXC [7]. Atx1 is presumed to acquire copper from CtaA and donate it to PacS (Figure 1), although only the latter process has been demonstrated to occur [5]. Atx1 can also function, to a certain extent, in the absence of CtaA, indicating that it may acquire copper from another unknown importer [5]. Basically, a similar copper transport mechanism has been conserved in higher plants [7].

In *Synechocystis*, the iron ATP-binding cassette (ABC) transporter FutABC has been identified as a major contributor to ferric iron transport across the plasma membrane (Figure 1) [19]. This transporter is composed of four polypeptides: FutA1 and FutA2, localized in the periplasm, and FutB and FutC, probably forming an inner-membrane bound unit [19]. On the other hand, ferrous iron uptake depends on the FeoB iron transporter, although it is not essential for iron acquisition unless the ferric pathway is disabled [19]. In plants, it is not yet known whether a similar ABC transporter is involved in iron uptake [7]. Once inside the cyanobacterial cell, up to 50% of the stored Fe(II) is associated to bacterioferritin, where it is oxidized to the insoluble Fe(III) form before being transferred to the different protein substrates [7].

**SYNECHOCYSTIS PROTEOMIC STUDIES**

After genome sequencing, *Synechocystis* soon became an excellent candidate for an in-depth proteomic analysis. In the classical proteomic approach, proteins are first separated in one dimension according to their isoelectric point by isoelectric focusing, and after in the second dimension by standard SDS-PAGE. The resulting spots are stained and excised from the gel, digested by trypsin and subjected to mass spectrometry (MS) for protein identification by comparing the data obtained with the theoretical trypsin digestion products in databases [20]. The first proteomic studies [21, 22] did allow the construction of a linkage map between 2D-gels and gene expression, with the spot identification being based on micro-sequencing rather than on MS methods. Up to 234 proteins were identified in *Synechocystis* and were placed in the Cyano2Dbase as a source for future studies [9].

Subcellular fractionation can also be performed to further increase the number of proteins identified. In this way, separate data can be attained for the subproteomes of soluble and periplasmic fractions, for the plasma membrane, for the outer and thylakoidal membranes and even for the peripheral proteins associated with the latter membrane [23–28]. However, more interesting than revealing the whole catalogue of proteins that constitute the proteome of a given organism is the comparison of quantitative protein profiles when the cells are subjected to varying environmental conditions, thus allowing the identification of differentially expressed proteins. This is a much simpler way to get insight into the metabolic changes resulting from cellular adaptation to changes in the environment and from stress response.

One of the first studies aimed at identifying induced or repressed proteins in *Synechocystis* cells concerned the light-induced proteins, with changes in the expression of photosynthetic proteins and of chaperones related to general stress response being identified by micro-sequencing and MS [29]. The proteomic study of soluble proteins in cells growing heterotrophically indeed revealed a shift in central carbon metabolism and down-regulation of the photosynthetic machinery [30]. In addition, the analysis of the cytoplasmic and periplasmic proteomes of cells under acidic stress demonstrated that the proteins involved in the response to general stresses are not affected, and that the periplasmic proteome undergoes much greater changes than the cytoplasmic one [31].

In the particular case of hyperosmotic stress, it was shown that the cells face salt stress by increasing not only specific salt-responsive proteins but also many...
other general stress proteins [32]. The hyperosmotic response of the periplasmic proteome actually results in the salt-enhanced expression of proteins that alter the cell-wall composition and structure [33]. The subsequent analysis of changes in the plasma membrane proteome [34] showed that most ABC transporters are affected. Notwithstanding, it was only possible to identify the more soluble components of the membrane proteins because of the difficulty in separating the integral membrane proteins by classical protocols. Further efforts resulted in the identification of 51 membrane proteins containing transmembrane helices in a range from 1 to 12 [35], whereas the membrane protein complexes were investigated under different culture conditions by using an adapted protocol (Blue native/SDS-PAGE) that allowed the study of 20 distinct protein complexes [36].

In order to increase the throughput and access to proteins difficult to study with the traditional gel-based proteomics, shotgun proteomics has also been applied to *Synechocystis*. This technique involves sample pre-fractionation and separation, usually by liquid chromatography, before MS [12, 37]. Several sets of proteins have thus been obtained by using different protocols, indicating that the most complete proteomes are obtained when combining different fractionation approaches. Nevertheless, quantification in shotgun proteomics is a complex task as compared with classical proteomics, and no comparative studies regarding measurement of expression levels of cyanobacterial proteins have been completed [12].

Taking all this into account, the proteomic analysis of knockout mutants is an interesting approach to understand how the absence of any specific protein may affect the whole cell metabolism. In this context, a number of studies have been performed with deletion mutants of *Synechocystis* proteins, namely the FtsH protease (salt-sensitive phenotype), the LepB1 leader peptidase (essential for photoautotrophic growth) and the Hik34 histidine kinase (increased thermal tolerance) [38–40].

**petE AND petJ KNOCKOUT MUTANTS**

In this context, the effect of copper deprivation on the *Synechocystis* proteome, with particular emphasis on Pc and Cyt, was analysed because of the relevance of copper and iron homeostasis in cyanobacteria and the ample use of these organisms as model systems for higher plants [6, 41]. We thus constructed the so-called ΔpetE and ΔpetJ deletion mutants—the first lacking the Pc-coding *petE* gene, the second lacking the Cyt-coding *petJ* gene—and their behaviour when growing in the absence or presence of copper was investigated. Copper is not strictly required by *Synechocystis* under photoautotrophic conditions, as the wild-type (WT) cells do grow at similar rates with or without copper. Notwithstanding, the two deletion mutants grow at the same rate as the WT strain when cultured in media that allow them to express one of the two electron donor proteins. In fact, ΔpetJ is only able to grow at a standard rate in the absence of copper, when Cyt is being produced, whereas ΔpetE only grows when Pc is synthesized upon copper induction. However, when the ΔpetE and ΔpetJ strains are growing under conditions at which neither Pc nor Cyt is being expressed, they both show growth rates that are much lower than that of WT cells [10]. This is an interesting observation, and several proposals have been made to explain how these cultures can survive in the absence of both Pc and Cyt. Some authors have suggested the existence of a third (but relatively inefficient) electron donor to PSI [42]. Some others have proposed the formation of a supercomplex between cytochrome b$_{6}$f and PSI to allow direct electron transfer without requirement of any redox carrier [43]. And others have even discussed the existence of an alternative electron transfer pathway [44].

Taking into account that the cyanobacterial respiratory and photosynthetic electron transfer chains share a number of redox components, namely Cyt, Pc, plastoquinone and cytochrome b$_{6}$f (Figure 1) [45, 46], the ability of the WT, ΔpetE and ΔpetJ strains to grow in glucose-supplemented culture media was also investigated. Under such heterotrophic conditions, glucose is used as an organic carbon source [47] and the electrons are transported from the sugar molecule to dioxygen throughout the respiratory pathway. None of the cell strains (including the WT one) can grow in copper-free medium, as expected from the specific requirement for copper of cytochrome c oxidase [46]. In copper-supplemented medium, however, the WT and ΔpetJ strains synthesize Pc and show a normal growth level, but the ΔpetE strain produces neither Cyt nor Pc and is thus unable to grow.
because of the absence of any electron donor to cytochrome c oxidase.

The in vivo PSI reduction kinetics of the Synechocystis WT and mutant strains were indeed specifically analysed [10]. When the WT cells grow in copper-supplemented culture medium, PSI reduction is just due to Pc (as Cyt synthesis is repressed) and the PSI reduction kinetics are well fitted to the monoexponential curves typical of simple collisional mechanisms [10]. When the WT cells grow in copper-depleted medium, in which Pc synthesis is repressed, the Cyt-dependent PSI reduction follows a biphasic kinetic, which corresponds to a more evolved mechanism [10, 48]. In ΔpetE and ΔpetJ cells, growing under conditions that allow the expression of just one of the two electron donor proteins, the in vivo PSI reduction follows kinetics similar to those observed in the WT strain for the same donor protein, but no PSI reduction is detected in mutant cells in which neither Pc nor Cyt is expressed.

The WT and mutant species can be further characterized by fractionation of cells grown either in the absence or in the presence of copper. In the WT strain, in which the soluble fraction and the thylakoidal and plasma membranes can be clearly resolved upon sucrose gradient centrifugation, the absence or presence of copper does not significantly affect the gradient pattern. As regards the deletion mutants, both ΔpetE in the absence of copper and ΔpetJ in the presence of copper show a fractional pattern similar to that of WT cells. The extracts of mutant cells in which neither Pc nor Cyt is expressed do exhibit neither the band corresponding to thylakoidal membranes nor the blue colour typical of soluble phycobiliproteins, thereby suggesting that both the membrane organization and the synthesis of accessory pigments are significantly modified because of the lack of electron donors to PSI and to the terminal oxidase [49].

A comparative scheme summarizing the whole proteomic analysis of WT and mutant Synechocystis cells is shown in Figure 2. The soluble fractions of the cell extracts, upon photoautotrophic growth in the absence or presence of copper, are subjected to 2D-electrophoresis, and some of the differentially expressed proteins are selected and analysed by MS. The mutants grown under restrictive conditions show a pattern of over-expressed proteins (thioredoxins, superoxide dismutase, etc.) similar to that induced by oxidative stress. This is as expected because the lack of any redox carrier transferring electrons from cytochrome b6f to PSI blocks the photosynthetic chain, with the concomitant accumulation of photooxidized chlorophyll P700 in PSI, the over-reduction of the quinone pool, the oxidation of the final acceptors and the over-production of ROS. The response of the mutants to such an oxidative stress is the over-expression of peroxiredoxin [50] and the DnaK protein [51]. Moreover, the over-expression of enzymes involved in the main metabolic pathways has also been observed, as expected from the metabolic alteration induced by oxidative stress.

It must also be noted that the ΔpetJ and ΔpetE mutants are frequently able to revert spontaneously under restrictive metal conditions, despite they were constructed by using the technique of gene deletion by homologous recombination with an antibiotic-resistance cassette. Thus, the revertants are probably affected in the copper-dependent regulation of some metabolic processes and/or in the metal intake itself.
The in-depth characterization of such mutant strains, along with a detailed proteomic analysis of the differential expression of proteins in the WT and knockout mutants at different metal availabilities, will let us understand the complex process of copper homeostasis in cyanobacteria.

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