Phosphate uptake proteins as markers for the nutrient status of freshwater cyanobacteria
Dignum, M.

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Chapter 3

The *pho*-regulon in the cyanobacterium *Synechocystis* sp. strain PCC 6803: Conclusions from cell envelope protein biotinylation and DNA array.

Dignum\(^1\), M., J. W. Back\(^2\), N. Yeremenko\(^1\), V. Krasikov\(^1\), D. J. Scanlan\(^3\), and H. C. P. Matthijs\(^1\)

\(^1\) Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics/University of Amsterdam, Nieuwe Achtergracht 127, NL-1018WS Amsterdam, the Netherlands
\(^2\) Dept. of Mass Spectrometry/Swammerdam Institute of Life Sciences/University of Amsterdam, Nieuwe Achtergracht 166, NL-1018WS, Amsterdam, the Netherlands
\(^3\) Dept. of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.
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Abstract

Intact cells of the cyanobacterium *Synechocystis* sp. PCC 6803 from phosphate surplus (‘control’) and phosphate depleted cultures were treated with a non-penetrating biotinylation agent to identify differential expression of proteins at the cell periphery. Use of isolated cell wall fractions and decoration of Western blots with a streptavidin linked luminescent reporter highlighted a subgroup of proteins with covalently bound biotin amongst the multiple resolved polypeptides. Biotinylated proteins were mostly present in the P$_i$-depleted cells. Identification of selected proteins by N-terminal sequencing and MALDI-TOF mass spectrometry revealed that several gene products from in particular one of the two *pho*-regulon units in the *Synechocystis* genome, comprising genes slr1247-1250 were retrieved. The product of gene slr1841, an outer membrane phosphate transport porin of 65 kDa, was up-regulated, whereas slr1908, a 60 kDa porin, was down-regulated in P$_i$-depleted cells. The expression studies at the protein level have been accompanied by a gene array approach to estimate mRNA abundance.

*Key words:* biotinylation, cyanobacteria, gene array, MALDI-TOF mass-spectrometry, outer membrane, phosphate-binding protein, porins
Introduction

Cyanobacteria are capable of assimilating light energy to fix carbon dioxide in an oxygencic mode. In quite a few cases cyanobacteria fix molecular nitrogen as well. Freshwater environments often feature sub-optimal availability of one or more mineral factors that are essential for growth. Hence, the growth rate of cyanobacteria is naturally limited by the availability of nutrients, especially inorganic phosphate (P_i; Schindler 1977; Hecky and Kilham 1988; Correll 1999). Cyanobacteria have various pre-adaptation strategies to changing environmental conditions. These pre-adaptations are permanent, quantifiable features of the organism, which can be invoked experimentally (Reynolds 1998), and are probably genome-encoded. The nucleotide sequence of the genome of the model cyanobacterium *Synechocystis* sp. strain PCC 6803 (Kaneko et al. 1996, ‘Cyanobase’ (http://www.kazusa.or.jp/cyano/)), hereafter referred to as *Synechocystis*, gives a comprehensive overview of all the potential genes in this organism (e.g. Mizuno et al. 1996; Paulsen et al. 1998). However, this information leaves open the conditions under which these genes are actually expressed. DNA array studies (Bhaya et al. 2000; Hihara et al 2001; Kanesaki et al. 2002) and proteomics (Choi et al. 2000; Fulda et al. 2000) have been used to elucidate differential expression under changing light conditions and salt stress.

Responses to nutrient deficiency include specific strategies that help bacterial cells to increase the availability of the growth-limiting factor. In addition to specific adaptations, nutrient stress responses also provoke changes in key metabolic rates. The bacterial cell wall, the interface between the cell interior and the outside world, plays an important role in the adaptive response to changes in the environment. The cell wall is essential to maintain cell integrity; it combines an osmotic barrier function with selective molecular sieving properties and a mechanical support function (Hoiczyk and Hansel 2000). The prominent adaptation is to increase the efficiency of P_i-uptake from environments with a low P_i-concentration by inducing specific channels through the cell wall and membranes. Outer membrane pores control the diffusion of ions from the environment into the periplasm (e.g. Nikaido and Vaara 1985; Benz and Bauer 1988). Anion selectivity of pores is an effective first step in the uptake cascade. A high affinity P_i-binding protein in the periplasmic space, and an active transport system across the cytoplasmic membrane complete the P_i-uptake system. Together with this uptake strategy the cells often display a scavenging strategy; they excrete enzymes that convert otherwise unusable chemical forms of the nutrient.

Many organisms induce a multi-component high-affinity uptake system, when faced with a lack of phosphate (P_i-deficiency). In *Escherichia coli* the specific response to P_i-deficiency is very well studied, including both structural and regula-
tory genes of the so-called *pho*-regulon (e.g. Rao and Torriani 1990; Wanner 1996; Hoffer and Tommassen 2001). A high affinity phosphate uptake system, called phosphate specific transport (*pst*) operon, is part of this regulon (Rosenberg et al. 1977; Haldiman et al. 1998). The *Pst*-system consists of a high affinity $P_i$-binding protein in the periplasmic space (PhoS or PstS; Magota et al. 1984; Surin et al. 1984), and an ATP-binding cassette (ABC) dependent transport system through the cytoplasmic membrane (PstCAB; Surin et al. 1985). Homologous *Pst*-systems have been found in *Bacillus subtilis* (Qi et al. 1997) and *Pseudomonas aeruginosa*, although the *pst* operon of the latter species lacks the *pstS* gene (Nikata et al. 1996). The outer membrane porin PhoE (Overbeeke and Lugtenberg 1980; Overbeeke et al. 1983; Van der Ley et al. 1987), which exhibits strong anion selectivity (Schirmer and Phale 1999), considerably enhances diffusion of $P_i$ across the outer membrane of *E. coli* and other Enterobacteria. The enzyme alkaline phosphatase (AP) that liberates $P_i$ from organic and inorganic phospho-esters (Horiuchi et al. 1959; O'Brien and Herschlag 2002) is co-regulated with the *pho*-regulon (Willsky and Malamy 1976; Yagil et al. 1976; Tomassen and Lugtenberg 1980). A two-component regulatory system PhoBR supplies the main control for the *pho*-regulon, but cross-regulation with other regulatory systems also takes place (Wanner 1992; Kim et al. 1996). For the cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter referred to as *Synechococcus*) an analogous system has partly been characterized. It contains the $P_i$-binding protein SphX (Scanlan et al. 1993; Mann and Scanlan 1994), two AP enzymes PhoA (Ray et al. 1991) and PhoV (Wagner et al. 1995), and the sensor/regulator pair SphS/SphR (Aiba et al. 1993). The genome of *Synechocystis* contains two *pst*-like operons, sll0679-0684 and slr1247-1250 (Kaneko et al. 1996). Recently, the AP encoding gene (*phoA*) has been pinpointed (sll0654), and properties of the regulatory genes ($P_i$-sensing histidine kinase *phoR*, sll0337 and response of regulator *phoB*, slr0081) have been described (Hirani et al. 2001). A cyanobacterial equivalent to the phosphate selective PhoE porin of *E. coli* has not yet been found. Nonetheless, Hansel et al. (1998) showed that six deduced open reading frames in the *Synechocystis* genome show strong homology with outer membrane porins characterized in *Synechococcus* sp. PCC 6301. These genes are therefore assigned as encoding porins (Engelhardt and Peters 1998).

*N*-hydroxysuccinimide (NHS) biotin preferentially labels periplasmic and cell membrane proteins, but not cytoplasmic proteins in *E. coli* (Bradburne et al. 1993). In vivo biotinylation has earlier found application in the identification of phytoplankton cell surface proteins: nitrogen-regulated Nrp1 in a marine alga (Palenik and Koke 1995), and alkaline phosphatase in a marine dinoflagellate (Dyrhman and Palenik 1997). For *Synechocystis* biotinylation was used to identify thylakoid surface-exposed domains of photo system I (Xu et al., 1994).
In this study we investigate which of the genes encoding for potential components of the pho-operon are actually expressed in *Synechocystis* under P$_i$-depleting growth conditions. Our survey relies on two complementary techniques, protein analysis and comparison of specific mRNA abundance. Both methods bear in common selective extraction of fractions from the complex pools of cellular macromolecules through biotinylation. Its use for mRNA extraction from the pool of total RNA will be described in detail elsewhere (Yeremenko et al., in preparation). To investigate changes in protein expression, we used NHS-biotin to probe cell-surface accessible proteins in *Synechocystis* PCC6803. Because the current resolution in separation does not allow a full investigation of both soluble and membrane proteins that are under control of the pho-regulon, we have focused on the periphery of the cells. A selection of the labelled proteins was purified via biochemical approaches. SDS-PAGE protein separation gives distinct polypeptide patterns of P$_i$-depleted cells and of control cells grown with excess nutrients, especially clear with the biotin flag as a marker. The full genome sequence of *Synechocystis* provided the requirements for identification of proteins by peptide mass fingerprinting in MALDI-TOF mass spectrometry. We present a qualitative comparison between protein and mRNA based surveys of P$_i$-deficiency related expression to support novel findings on P$_i$-uptake in the model cyanobacterium *Synechocystis*.

**Experimental procedures**

**Culture Conditions**

Axenic batch cultures of *Synechocystis* sp. PCC 6803 were grown in BG-11 medium (Rippka et al. 1979) under continuous light (50 μM photons.m$^{-2}$.s$^{-1}$). Cultures were inoculated with 1/10 volume of exponentially growing pre-cultures from normal BG-11 medium (175 μM P$_i$) in 9/10 volume of either normal BG11 for control cells and in BG-11 without phosphate for P$_i$-depletion. The latter medium was supplemented with KCl to retain the original K-ion concentration. Measurement of alkaline phosphatase (AP) activity defined control for the onset of P$_i$-deficiency, in our culture conditions at day 5 after inoculation (see results). Cells were harvested by centrifugation (5500 rpm, 10 min at room temp).

**Alkaline phosphatase activity**

Alkaline phosphatase activity in the cultures was measured with the classical para-Nitrophenyl phosphate (p-NPP) assay (Bessey et al.1946), adapted for use in a microplate reader with tuneable measuring wavelength setting (VERSAmax, Molecular Devices, USA). A solution of 18 mM p-NPP (Sigma 104 phosphatase substrate), in 1 M Tris-HCl buffer with 10 mM MgCl$_2$, pH 8.0 was used in assays. Alkaline Phosphatase activity has been expressed as p-Nitrophenolate formation in femtomole per million cells (CAS Y 1 Cell Counter, Schärfe System) per minute.
Standard errors and the maximal number of degrees of freedom in the end result were statistically evaluated by numerical differentiation of all the partial errors. All experiments were done in triplicate with independent cultures.

**Biotinylation of peripheral proteins**

Biotin-XX-SSE (6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt, Molecular probes B-6353) was dissolved in fresh 0.2 M sodium bicarbonate buffer (pH 8.3) at 20 mg/ml directly before use. Freshly harvested cells were washed by resuspension in 10 mM HEPES-buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), at pH 7.2, and were subsequently centrifuged for 5 minutes at 8000 rpm. After two washing steps, the cell pellets were resuspended in 0.2 M NaHCO₃ to a cell count of about 2×10⁸ cells per ml. The reaction was started by adding 10-50 μl of biotin-XX-SSE solution to 1 ml of cell-suspension (equals 0.2 to 1.0 mg biotin-reagent). Incubation proceeded on ice for a range of different time spans from 1 up to 30 minutes, while stirring in dim light. At room temperature the specificity was slightly less (data not shown). The reaction was quenched by addition of 1/100 volume of 0.1 M L-lysine in 1 M Tris-buffer at pH 7.4. The cells were pelleted, washed twice with 10 mM HEPES-buffer, and the pellet of the last centrifugation was resuspended in 10 mM HEPES for subsequent cell breakage with Zirconium/glass beads (0.1 mm) in a Mini-bead-beater (Biospec products).

![Structure of Biotin-XX-Sulfo-Succinimidyl Ester](image)

**Figure 1.** Structure of Biotin-XX-Sulfo-Succinimidyl Ester (Haugland 1998)

**Cell fractionation: Sucrose density gradient ultra-centrifugation**

Sucrose gradient ultra-centrifugation (Schnaitman 1970) allows separation of the three different membrane systems of cyanobacteria (Omata and Murata 1984): thylakoid, plasma, and cell wall fractions (Schrader et al. 1981; Resch and Gibson 1983; Jürgens et al. 1983). Cell wall fractions with outer membrane (Jürgens and Weckesser 1985) were purified by Triton-X-100 extraction (Schnaitman et al. 1971; Weckesser and Jürgens 1988). Recently the sucrose gradient ultra-centrifugation method was combined with two-phase separation to get pure plasma and thylakoid (Norling et al. 1998) and outer membranes (F. Huang, personal communication). The latter method was not available to us at the time this work was car-
ried out. A discontinuous gradient of sucrose solutions was made in 10 mM HEPES, 6 mM EDTA, 12 mM NaCl, pH=7.2 (from bottom to top 1 ml of 90%, 1 ml 80%, and 2 ml 55% (w/v) sucrose, in 17 ml clear polythene disposable ultracentrifuge tubes. Next, the cell extracts were loaded in 48% (w/v) sucrose, followed by 2 ml 45%, 1.5 ml 30%, and 1.5 ml of 10% (w/v) sucrose). The discontinuous sucrose density gradients were centrifuged (Kontron Instruments, rotor type swing out TST 41.14) at 18,000 rpm for 18 hours at 4°C. The bottom of the centrifuge tubes was punctuated with a needle, connected to narrow gauge tubing mounted in a peristaltic pump. Coloured fractions were collected in microfuge tubes. To the cell wall fraction (in approximately 0.8 ml, 80% sucrose), 12 ml of 20 mM HEPES, 2% Triton X-100, 10 mM MgCl₂ (pH=8.0) was added. Samples were incubated for 30 minutes at 23°C with stirring. Triton X-100 insoluble cell wall fractions were collected by ultra-centrifugation for 1 hour at 40,000 rpm (Kontron Instruments, fixed angle rotor TFT 65.13; 24,000 rpm, g max = 100 k). Samples were suspended in 20 mM HEPES, 10 mM MgCl₂, pH=8.0 and dialysed twice against this buffer to remove sucrose. Renewed centrifugation for 1 hour at 40,000 rpm (Kontron Instruments, fixed angle rotor TFT 65.13; 24,000 rpm) allowed removal of Triton-X-100. The cell wall pellets were resuspended in 50 µl of the same buffer and stored at -80°C.

Protein separation and visualisation

Whole cell extracts and cell wall fractions were loaded onto a Tris-Tricine SDS-polyacrylamide gel (Schägger and von Jagow, 1987), and separated by electrophoresis in a BioRad mini-gel set-up. The amount of protein per cell varied in the different growth conditions. Therefore we normalised the amount of sample for equal cell numbers, and applied the equivalent of about one million cells on SDS-PAGE. For Western blotting (Towbin et al. 1979; Burnette 1981), proteins were transferred onto PVDF-filter (Amersham Hybond-P, pre-wet in methanol) in Towbin buffer supplemented with 0.1% SDS, for 30 minutes at 10V in a semi-dry transfer cell (Trans-Blot, BioRad). Following transfer the membranes were equilibrated in phosphate buffered saline (PBS; 0.2 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.8 mM NaCl). Membranes were blocked for 45 minutes in PBS (PBS-T) and 5%, and washed for two times 5 minutes in PBS-T. For detection of biotinylated proteins, membranes blocked with 5% (w/v) skim milk were incubated for 1 hour at room temperature with 20 ng/ml streptavidin-horse radish peroxidase (HRP) polymer in PBS containing 0.05% (v/v) Tween20 and 1% skim milk. For immunological detection, the blocked membranes were incubated for 1 hour at room temperature with polyclonal antibody raised against PstS of the marine cyanobacterium *Synechococcus* sp. WH7803. To improve cross-reactivity, the polyclonal antibody was affinity purified. The antibody was bound with the *Synechocystis* PstS on excised pieces of Western blot and subsequently extracted according to Scanlan et al. (1997). A dilution of 1:10,000 was used for primary antibody incubation (1 hour at room temperature). The secondary antibody, HRP-conjugated goat anti rab-
bit antibody (BioRad), was added as a 1:50,000 dilution in PBS-T and incubated for 1 hour at room temperature. The membranes were developed at room temperature by incubation for 5 minutes with Western blotting detection system ECL+ (Amersham Life Science RPN 2132), or the more sensitive SuperSignal West Femto maximum sensitivity substrate (Pierce). Exposure of the Western blots to autoradiography films (Kodak Biomax Light) typically took from 15 seconds up to 30 minutes. Signal density was analysed on a gel-documentation system (Gel doc 2000, BioRad) with Quantity One software.

**Protein identification**
The amino-terminal sequence of PstS was determined via Edman-degradation (Edman 1970). Proteins were cut out from Coomassie-stained PVDF membranes after electro-blotting in CAPS-buffer (Matsudaira 1987), and analysed on a Precise model 494 sequencer (Applied Biosystems). The other proteins were identified by in-gel proteolytic digestion, mass spectrometry, and database searching. In-gel digestion of selected proteins was carried out by a protocol adapted from Shevchenko et al. (1996). Briefly, bands from Coomassie or silver stained gels were excised, proteins were reduced with dithiothreitol (DTT) and S-alkylated with iodoacetamide, and subsequently digested with trypsin (Roche). Peptides were eluted with 20 mM NH$_4$HCO$_3$, loaded onto ZipTips (Millipore), washed with 1% HCOOH, and eluted with 60% acetonitrile/1% HCOOH. 0.5μl of eluate was mixed with 0.5 μl of α-cyanohydroxysucciniminic acid (10mg/ml in 50% acetonitrile/ 50% ethanol) on a MALDI target plate. Reflectron MALDI-TOF spectra were acquired on a TOFSPEC 2EC mass spectrometer (Micromass, Whytenshawe, U.K.). Proteins were identified from the translated Cyanobase sequence database (http://www.kazusa.or.jp/cyano/) release May 2002 using the ProteinProbe software algorithm (Micromass). Molecular weights of the proteins were calculated according to Bjellqvist et al. (1993). Homology searches were conducted with the BLAST method (Altschul et al. 1990).

**DNA array**
Clonal arrays with shearing fragments of the complete *Synechocystis* PCC6803 genome on nylon blots were, kindly made available by Dr. S. Tabata (Kazusa DNA Research, Japan). All details on the preparation of RNA, the cleaning of cDNA to select for mRNA encoded product only, blot hybridisation, phospho-image and calculus will be presented elsewhere (Yeremenko et al., manuscript in prep.).
Results

_Alkaline Phosphatase activity as a guide for pho regulon operation_

AP activity was taken as a guide for pho-regulon related control of differential gene expression and protein synthesis. From empirical studies (not shown), it proved optimal to collect representative P_j-deficient cells 5 days (±120 h) after the inoculation of a pre-culture in P_j-free BG11 (cf. Experimental Procedures). AP activity in P_j-depleted _Synechocystis_ cultures was 167 ± 8 femtomoles _p_-Nitrophenylphosphate.10^6 cells^{-1}.minute^{-1} (8 degrees of freedom (df), 95% confidence interval), and 15.5 ± 1.1 femtomoles.10^6 cells^{-1}.minute^{-1} (9 df) in the culture supernatant after centrifugation. _p_-Nitrophenylphosphate conversion in control cultures grown with excess phosphate after a similar time of culture was 0.9 ± 1.3 (2 df), and 0.63 ± 0.10 femtomoles.10^6 cells^{-1}.minute^{-1} (7 df) in the culture supernatant.

_Biotinylation of peripheral proteins_

To be in contact with the cell environment, proteins involved in P_j-uptake must be located in the cell periphery. From that notion we pursued the search for inducible proteins by chemical labelling of exposed lysine residues with NHS-biotin (Fig. 1). The reagent was selected for minimal penetration into the cytoplasm (Bradburne et al. 1993), in order to limit the number of biotinylated proteins only to the easily accessible ones. The reaction time was optimised to negotiate between labelling specificity and intensity, by quenching of the biotinylation reaction (biotin reagent concentration 1.0 mg · ml^{-1}) at chosen intervals. P_j-depleted and control cells were lysed and processed for protein resolution in SDS-PAGE, followed by Western blot transfer, decoration with avidin-peroxidase conjugate and luminescent detection (cf. Experimental procedures; see Figure 2).

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Figure 2. Biotinylation time series (time in minutes) and proteolysis with proteinase K (P) of intact _Synechocystis_ cells, grown in nutrient replete (A) and P_j-depleted conditions (B).
In nutrient replete control cells one polypeptide with an apparent molecular weight ($M_w$) of 60 kDa was intensely labelled, this was a lot less pronounced in P-deficient cells. A polypeptide of 35 kDa showed intense labelling in $P_j$-limited cells, it lacked completely in control cells. Other changes in expression at the protein level were only marginal between $P_j$-limited and control cells, most prominently at about 45 kDa, and at about 150 kDa. Additional changes occurred in the 65-90 kDa region. Without the help of biotinylation only the difference in expression of the 35 kDa polypeptide would have been pronounced enough to be seen against a high background of other polypeptides in Silver- or Sypro orange-stained gels of whole cell extracts (results not shown). Separation of proteins from cells grown in nutrient replete controls and $P_j$-depleting conditions allows comparison of expression patterns. The nature of the labelled proteins differed between both types of cells. A limited number of proteins were labelled in the biotinylation reaction, more of them in the P-deficient than in the control cells. About 5 major and 20 minor cell-surface exposed proteins were visualised. Cells without biotin-reagent (Figure 2, $t = 0$) proved absence of endogenous biotinylated polypeptides.

**Figure 3.** Progress of the biotinylation reaction measured from chemiluminescent signal densities on the Western blots shown Figure 2. The panels show the average overall density in the lanes (A), the most intensely labelled polypeptides in the $P_j$-depleted cells, which have apparent sizes of 35 kDa (B), and 45 kDa (B), and the most intensely labelled polypeptide in the control cells with an apparent size of a 60 kDa (D). Closed triangles represent the labelling intensity in $P_j$-depleted cells, and open circles represent the labelling intensity in control cells.
The typical biotinylation rate and specificity of labelling from analysed gels led us to conclude that the reaction proceeded best on ice. On average the intensity for the selected proteins reached an optimum in 20 to 30 min. Even after 30 minutes the number of different proteins that were linked to the reagent remained fairly constant, although non-specific background stain gradually increased (Figure 2). A time course of biotinylation shows the gradual increase of total biotinylation of all bands (Figure 3A) and of three selected bands, two up-regulated ones of 35 and 45 kDa (Figure 3B,C) and a repressed one of 60 kDa (Figure 3D). Following a fast first biotin label attachment progress was gradual for all bands observed. We concluded to use a standard time of 30 min.

Penetration ‘depth’ of the reagent
A relation between the rate of label attachment and accessibility of the protein might exist. We monitored the extent of penetration of the biotinylation reagent in P₁-depleted cells by applying a low biotin reagent concentration (0.2 mg/ml) and studied subsequently the digestion of exposed proteins on intact cells by trypsin and proteinase K. The lower biotin reagent concentration only revealed the major exposed proteins. The 35 kDa protein was the first to be labelled, followed by the 45 kDa and a 90 kDa protein. These proteins were accessible from the outside as seen from (partial) degradation by protease (Figure 4). Although the 35kDa band is the most intensely biotinylated polypeptide, it was not readily accessible for tryptic digestion. In vivo digestion of intact biotinylated cells with proteinase K demonstrated high accessibility of the 150 kDa polypeptide (Table 1).

<table>
<thead>
<tr>
<th>Biotinylation</th>
<th>Proteolyis</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 5 10 20</td>
<td>0 1 2 5 10 20 30 60 O/N</td>
<td>-126 -89 -50 -33 -20</td>
</tr>
</tbody>
</table>

Figure 4. Biotinylation time series and proteolysis with trypsin (time in minutes, last lane overnight) of intact Synechocystis cells, grown P₁-depleted conditions.

Biotinylated proteins in cell wall preparations of control and P-limited cells
We fractionated broken cells by sucrose density ultra-centrifugation to reduce the overall background of proteins on gels in order to enhance secure identification. A
diffuse yellow/orange band in the 80% sucrose layer contained the cell wall fraction. In the P₁-depleted cultures this fraction was intensely coloured (brown) and extended into the 90% sucrose layer. The 35 kDa polypeptide emerged pronouncedly on silver-stained SDS-Polyacrylamide gel with cell wall fractions of P₁-depleted *Synechocystis* cells, and not in control cells (not shown). The biotinylation in sucrose gradient purified cell wall samples as judged from analysis of the banding pattern of proteins by SDS-PAGE and subsequent decoration of Western blots with peroxidase linked streptavidin showed that bands visualised in the crude preparations were mostly retained after the purification (Fig 5A and 5B respectively). A 45 kDa polypeptide repressed in P₁-limitation did not co-purify with the cell wall. According to expectations, as carried on from whole cell extract results, the banding pattern of biotinylated proteins from P₁-depleted and control cell wall fractions showed marked differences. Initial sequence information for the 35 kDa P₁-depletion de-repressed protein was derived from N-terminal sequencing by Edman-degradation. The amino-terminal end of this protein was comprised of the amino acid sequence GTLNGAG, sufficient to identify the protein as PstS (slr1247). The N-terminus of the de-repressed 65 kDa protein was blocked.

Identification of the 65 kDa-, and various other differentially expressed proteins, as well as further confirmation for the identification of PstS was done by in-gel proteolytic digestion followed by peptide mass fingerprinting and screening the resultant mass spectra against the translated Cyanobase. Table 1 lists the major differentially expressed proteins: PstS (slr1247), PhoA (slr0654), and an outer membrane porin homologue of 65 kDa (slr1841) that were derepressed. A 60 kDa outer membrane porin homologue (slr1908) was repressed by P₁-depletion.

**Table 1.** Identified cell wall bound proteins involved in high affinity phosphate uptake.

<table>
<thead>
<tr>
<th>Apparent Mw (kDa)</th>
<th>Calculated Mw (kDa)</th>
<th>Cyanobase gene nr.</th>
<th>Protein function</th>
<th>Protein name</th>
<th>Expression (P₁-depleted)</th>
<th>In situ digestion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>149.4</td>
<td>slr0654</td>
<td>Alkaline phosphatase</td>
<td>PhoA</td>
<td>+</td>
<td>99.4%</td>
</tr>
<tr>
<td>65</td>
<td>67.6</td>
<td>slr1841</td>
<td>Outer membrane porin</td>
<td>SomA</td>
<td>+</td>
<td>39.4%</td>
</tr>
<tr>
<td>60</td>
<td>64.5</td>
<td>slr1908</td>
<td>Outer membrane porin</td>
<td>SomB</td>
<td>-</td>
<td>21.9%</td>
</tr>
<tr>
<td>35</td>
<td>35.3</td>
<td>slr1247</td>
<td>P₁-binding protein</td>
<td>PstS</td>
<td>++</td>
<td>18.2%</td>
</tr>
</tbody>
</table>

* Surface accessibility for digestion by proteinase K. Intact biotinylated cells were incubated with proteinase K for 30 minutes. Digestion of the biotinylated proteins was analysed on Western blot with horseradish peroxidase conjugated streptavidin.

**Pho regulon controlled proteins PstS in replete and P-deficient Synechocystis: Results from Western analysis approaches**

Immuno-decoration with an affinity-purified polyclonal antibody raised against PstS of the marine cyanobacterium *Synechococcus* sp. WH7803 (Scanlan et al. 1997) rendered independent proof for PstS presence (Figure 5C and 5D). Cross-
reactivity occurred in four distinct bands in the cell homogenate from the control and P-replete cells (Fig 5C). In addition to the mature protein in the cell wall of P₇-depleted cells, a polypeptide with slightly higher mass (protein plus leader sequence) and two polypeptides with lower apparent molecular mass (breakdown products from redundant gene product) were present in control cells. The absence of the protein with leader-sequence and the breakdown products indicates functional use of PstS protein in the P₇-depleted cells. No incorporation of PstS protein into the cell wall was observed in control samples.

Figure 5. Western blot analysis of cell homogenates (A and C) and cell walls (B and D). Detection of biotinylated proteins with horseradish peroxidase conjugated streptavidin (A and B), and unlabelled proteins by immunological staining with an affinity-purified polyclonal antibody raised against PstS of the marine cyanobacterium *Synechococcus* sp. WH7803 (Scanlan et al. 1997) (C and D).

**Consequences of P₇-deficiency: de-repression of gene expression, analysis of relative mRNA abundance by gene array analysis**

Expression levels of potential *pho* regulon components at the mRNA level are presented in Table 2. The *pst*-operon1 (srl1247-50) was specifically de-repressed in the P₇-depleted conditions, in agreement with the results from the protein analysis in this study. The specificity was confirmed; additional experiments showed that the 35 kDa biotinylated protein in the P₇-depleted *Synechocystis* cells was absent in nitrate-depleted cells (results not shown). Expression levels of the *pst*-operon2 (sll0679-83) were also increased in P₇-depletion, but this was not unique for these conditions. The *pst*-operon2 was also expressed in nitrate-depletion and salt-stress (Yeremenko et al., manuscript in preparation). An additional open reading frame with homology to *pstS*, sll0540, showed increased expression levels in the P₇-depleted conditions. De-repression of *phoA* was not apparently differentiated between control and P₇-depleted cells from the DNA arrays, neither seemed the expression of the outer membrane porin gene srl1841 to be affected. Repression of the outer membrane porin srl1908, however, was clearly shown by the DNA array.
Strong de-repression was also observed for the gene slr1622, encoding a cytoplasmic soluble inorganic pyrophosphatase. Changes seemed to take place in the expression levels of regulatory proteins for the \textit{pho} regulon. However, the data supporting these changes did not reach acceptable significance levels (Yeremenko et al., manuscript in preparation).

Table 2. Differential expression levels of mRNA from genes encoding proteins involved in P\textsubscript{i}-incorporation in \textit{Synechocystis} sp. PCC 6803.

<table>
<thead>
<tr>
<th>Gene nr.</th>
<th>Function</th>
<th>Protein name</th>
<th>Size (aa)</th>
<th>Expression level*</th>
<th>**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pst-operon 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>slr1247</td>
<td>Periplasmic P\textsubscript{i}-binding protein</td>
<td>PstS</td>
<td>333</td>
<td>18±6</td>
<td>48±17</td>
</tr>
<tr>
<td>slr1248</td>
<td>P\textsubscript{i}, transport system permease</td>
<td>PstC</td>
<td>328</td>
<td>18±8</td>
<td>42±15</td>
</tr>
<tr>
<td>slr1249</td>
<td>P\textsubscript{i}, transport system permease</td>
<td>PstA</td>
<td>290</td>
<td>20±8</td>
<td>38±5</td>
</tr>
<tr>
<td>slr1250</td>
<td>ATP-binding protein</td>
<td>PstB</td>
<td>271</td>
<td>19±4</td>
<td>38.4±1.7</td>
</tr>
<tr>
<td><strong>pst-operon 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sll0679</td>
<td>P\textsubscript{i}-binding protein precursor</td>
<td>SphX</td>
<td>336</td>
<td>21±4</td>
<td>50±10</td>
</tr>
<tr>
<td>sll0680</td>
<td>P\textsubscript{i}, transport system permease</td>
<td>PstS</td>
<td>383</td>
<td>23±5</td>
<td>41.0±1.5</td>
</tr>
<tr>
<td>sll0681</td>
<td>P\textsubscript{i}, transport system permease</td>
<td>PstC</td>
<td>317</td>
<td>12±3</td>
<td>40±7</td>
</tr>
<tr>
<td>sll0682</td>
<td>P\textsubscript{i}, transport system permease</td>
<td>PstA</td>
<td>287</td>
<td>13.6±1.5</td>
<td>37±5</td>
</tr>
<tr>
<td>sll0683</td>
<td>ATP-binding protein</td>
<td>PstB</td>
<td>269</td>
<td>12±6</td>
<td>44.0±1.5</td>
</tr>
<tr>
<td>sll0684</td>
<td>ATP-binding protein</td>
<td>PstB</td>
<td>266</td>
<td>11±5</td>
<td>33±14</td>
</tr>
<tr>
<td><strong>Additional P\textsubscript{i}-binding protein homologue</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>sll0540</td>
<td>Putative P\textsubscript{i}-binding protein</td>
<td></td>
<td>307</td>
<td>23±13</td>
<td>41±13</td>
</tr>
<tr>
<td><strong>Putative pore-forming proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>slr1841</td>
<td>Outer membrane porin</td>
<td>SomA</td>
<td>630</td>
<td>52±14</td>
<td>72±24</td>
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<tr>
<td>slr1908</td>
<td>Outer membrane porin</td>
<td>SomB</td>
<td>591</td>
<td>47.7±1.6</td>
<td>21±5</td>
</tr>
<tr>
<td>slr0042</td>
<td>Outer membrane porin</td>
<td>SomC</td>
<td>576</td>
<td>60±17</td>
<td>51±10</td>
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<tr>
<td>sll0772</td>
<td>Outer membrane porin</td>
<td>SomD</td>
<td>546</td>
<td>28±4</td>
<td>29±13</td>
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<tr>
<td>slr1550</td>
<td>Outer membrane porin</td>
<td>SomE</td>
<td>544</td>
<td>17±5</td>
<td>12±4</td>
</tr>
<tr>
<td>slr1271</td>
<td>Outer membrane porin</td>
<td>SomF</td>
<td>572</td>
<td>46±5</td>
<td>26±9</td>
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<tr>
<td>slr1272</td>
<td>SLH*** containing protein</td>
<td></td>
<td>254</td>
<td>117±36</td>
<td>65±33</td>
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<tr>
<td><strong>Phosphatases</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>sll0654</td>
<td>Extracellular alkaline phosphatase</td>
<td>PhoA</td>
<td>1409</td>
<td>29±8</td>
<td>27±4</td>
</tr>
<tr>
<td>sll0222</td>
<td>Putative purple acid phosphatase</td>
<td></td>
<td>326</td>
<td>47±13</td>
<td>40±13</td>
</tr>
<tr>
<td>slr1622</td>
<td>Soluble inorganic pyrophosphatase</td>
<td>Ppa</td>
<td>233</td>
<td>34±11</td>
<td>72±5</td>
</tr>
<tr>
<td>sll1546</td>
<td>Exopolyphosphatase</td>
<td>Ppx</td>
<td>540</td>
<td>43±11</td>
<td>34±11</td>
</tr>
</tbody>
</table>

| **Regulatory proteins of the \textit{pho} regulon** | | | | | |
| sll0337 | Sensory kinase | SphS | 430 | 56±22 | 39±7 | - |
| slr0081 | Response regulator | SphR | 262 | 7±4 | 35±14 | ++ |
| slr0741 | Regulatory inhibitor | PhoU | 224 | 69±31 | 38±11 | - |

* Data expressed as percentage of total signal multiplied by 1000 (average ± standard deviation of three independent experiments)

** Qualitative interpretation of change in expression levels in Pi-depleted cells relative to controls with excess nutrients (0: no change, +: probable increase, ++: significant increase, -: probable decrease, --: significant decrease)

***SLH: surface layer homology domain


Discussion

\( P_1 \)-deficiency forces cells to differentiate gene expression. In an overall analysis several hundreds of the 3167 genes in *Synechocystis* showed very specific or less specific up-regulation or down-regulation (Yeremenko et al., manuscript in preparation). Many of those regulated genes have been annotated in Cyanobase as ‘hypothetical protein’. A full survey of stress induced changes in gene expression eventually requires evaluation of the protein counterpart in which the localisation of proteins in the cell may help to assign functional annotation.

In this work, we have targeted lysine residues accessible from the outside of intact control and \( P_1 \)-deficient *Synechocystis* cells, by covalent bonding to a specific agent with a coupled biotin molecule. The latter served as an easy guide to recognise the labelled lysine residues in proteins after SDS-PAGE separation from broken cell preparations. The biotinylation of surface exposed proteins on intact *Synechocystis* cells and detection with a chemiluminescent method greatly enhanced the visibility of low-abundance proteins in the cell envelopes. The labelling intensity, measured as signal density from Western blots, increased over time. We also observed an increase in background staining, which could not be attributed to specific polypeptides. With a low concentration of labelling agent, additional polypeptides were labelled during the experiment. This indicates that penetration of label into additional cell compartments took place within the time-frame of the experiment. More proteins were found to be labelled before sucrose gradient centrifugation when compared to the situation after purification.

Differential label incorporation reflects the adaptability of the cell envelope in adverse conditions. Depletion of \( P_1 \) urges the cells to synthesize more proteins in the cell envelope that are in contact with the external medium. The increased resolution in the sucrose gradient fractions rendered clearer expression patterns. One protein of about 60 kDa was repressed in the \( P_1 \)-depleted conditions, and proteins with estimated masses of 35, 65, 90 and 150 kDa were derepressed. The identity of a number of proteins was deduced from the sequence information in CyanoBase. These proteins are specifically expressed under \( P \)-deficiency, and functionally contribute to \( P_1 \)-accumulation.

**Expression of \( P_7 \)-binding protein PstS**

\( P_7 \)-binding proteins are involved in facilitating the diffusion of \( P_7 \) into the periplasm at low external concentrations by tightly binding \( P_7 \) and delivering it to the cytoplasmic membrane transporter. \( P_7 \)-binding protein PstS (slr1247) had a very high accessibility for biotinylation in intact cells (Figures 1 and 3), but was not readily accessible for proteinase K (Table 1) or trypsin digestion (Figure 4). The labelling development of PstS showed some interesting characteristics (Figure
The polypeptide band widened, and shifted to a higher apparent molecular weight. This can be explained by progressive labelling of additional residual lysine sites on the protein, each biotin moiety adding an extra 0.47 kDa. PstS was at least partially co-purified with the cell wall (Figure 5). These results suggest that PstS is a periplasmic protein that is associated with the cell wall.

The marine cyanobacterium *Synechococcus* sp. WH7803 induces a cell wall associated polypeptide with apparent molecular weight of 32 kDa (Scanlan et al. 1993; Mann and Scanlan 1994) that is homologous to the *E. coli* periplasmic Pj-binding protein (Carr and Mann 1994). These authors detected no *pstS* gene homologues in fresh water cyanobacterial strains, and no immunological cross-reactivity with anti-PstS polyclonal antibodies against the marine cyanobacterium *Synechococcus* sp. WH7803. In contrast to these findings, we now show clear cross-reactivity with the anti-PstS polyclonal antibodies in *Synechocystis* sp. PCC 6803 after affinity purification. Cross-reactivity was also detected in *Synechococcus elongatus* PCC 7942 and *Prochlorothrix hollandica* PCC 9006 (unpublished results). The occurrence of four distinct bands with anti-PstS cross-reactivity in the cell homogenate from the control *Synechocystis* cells (Figure 6C) could be explained as follows. In addition to a low level of the mature protein that is seen in the cell wall of Pj-depleted cells, a PstS protein precursor with slightly higher apparent molecular mass might have been present in the control cells. Calculation of the size of the leader sequence (2.8 kDa) from the experimentally determined N-terminus agrees with this difference in apparent molecular weight. The two bands in the homogenate of control cells with lower apparent molecular mass are possibly breakdown products. Much less turnover was observed in the Pj-depleted cells, and PstS is quantitatively exported to the cell envelope (Figure 6D). No incorporation of PstS protein into the cell wall was observed in P-replete samples (Figure 6D). From these findings we conclude that the expression level of PstS is regulated at the transcriptional level (de-repression) as well as post-transcriptional level (transport and digestion).

Two complete *pst*-operons with P1-specific ABC-transporter and matching Pj-binding proteins are encoded in the *Synechocystis* genome (Kaneko et al. 1996). The srl1247-1250 operon contains the *pstS* gene encoding the cell wall associated and biotinylation accessible Pj-binding protein identified in the present work. It is interesting to note that the sll0679-sll0684 operon has two genes encoding putative Pj-binding proteins; the sll0679 gene encodes a homologue to the Synechococcus SphX-type Pj-binding protein (Scanlan et al. 1993; Mann and Scanlan 1994), and the sll0680 gene encodes a PstS homologue. Our results show that the srl1247-1250 region encodes the primary *pst*-operon under specific Pj-control. The sll0679-sll0684 operon is expressed in several other stress conditions (Yeremenko et al. manuscript in preparation). An additional putative Pj-binding protein,
sll0540, shows an increased expression level in P$_i$-depleted conditions (Table 2). None of these P$_i$-binding proteins were retrieved by our protein analysis. The presence of tandem high affinity P$_i$-uptake systems is an important difference with the situation in Enterobacteria, which possess distinct P$_i$-uptake systems (Rosenberg et al 1977; Willsky and Malamy 1980; Harris et al. 2001). The Pit-system with low affinity for P$_i$ is not found in *Synechocystis* by BLAST similarity search (Altschul et al. 1990). Instead, *Synechocystis* has two different high affinity uptake systems, of which one is specifically derepressed in P-depletion. The other system seems to be regulated by a more general response.

**Expression of alkaline phosphatase PhoA**

Alkaline Phosphatase (AP) enzymes have an important function in the recycling of organically bound P in aquatic systems (e.g. Siuda 1984; Jansson et al. 1988). The increased expression levels of this type of enzyme in bacteria experiencing a shortage of P$_i$ have long been known (Horiuchi et al. 1959), but are not a common property of all microorganisms (Kuo and Blumenthal 1961). The usefulness of AP activity as a test for conditions of P-deficiency for cyanobacteria and other aquatic phototrophs was also recognized early (Fitzgerald and Nelson 1966). In early literature ample attention was paid to the location of AP. The cell-bound AP is found in the periplasmic space or firmly attached to the outer surface of the cell wall. The sheath may act as a secondary location for AP activity. At least a portion of the enzyme is excreted into the surroundings. In the cyanobacterium *Plectonema boryanum* AP activity is located in the periplasmic space, no activity was found in culture supernatant. *Anabaena variabilis* excretes small amounts of AP into the medium after five days in P$_i$-free medium (Doonan and Jensen 1977). *Coccolithus peniocytis* (an unicellular coccoid) shows strong constitutive AP activity of cell-bound enzyme but also excretes it into the medium. *Anabaena cilindrica* shows AP activity firmly bound to the cell that is increased sevenfold in P$_i$-depleted conditions, and *Oscillatoria woronichinii* excretes small amounts of AP in P$_i$-depleted conditions (Doonan and Jensen 1980). AP activity measurements showed that both *Synechocystis* and *Synechococcus elongatus* PCC 7942 (results not shown) excrete about 10% of AP into the medium; thus most of the enzyme is bound to the cells. Derepression of AP activity at low P$_i$-concentrations is well known for *Synechococcus* sp. (Ihlenfeldt and Gibson 1975). The enzyme responsible for the increased activity was identified to be a 145-kDa protein (Block and Grossman 1988). Although this enzyme shows little sequence similarity with the *E.coli* AP, it was called PhoA in analogy (Ray et al. 1991). The enzyme is located in the periplasm or loosely bound to the cell wall (Block and Grossman 1988). *Synechococcus* contains a second, but constitutive AP of 61.3 kDa, designated PhoV (Wagner et al. 1995). Recently the increased transcription of a *Synechocystis*
gene (sll0654) was described, encoding an AP homologue to the *Synechococcus* PhoA (Hirani et al. 2001). Transcription of the other two genes potentially encoding AP (sll0222 and slr0509) enzymes was not detected in that study by Northern analysis under the conditions used (0 or 2.24 μM K₂PO₄; Aiba et al. 1993). Expression levels of mRNA for the potential AP-encoding genes did not increase in P₁-depleted cells. However, both AP activity measurements and identification of the 150 kDa protein as PhoA (sll0654), which is absent in control conditions, strongly suggest its derepression. The lack of response in the DNA array is probably an artefact, resulting from the strong repression of the neighbouring extracellular nuclease (sll0656), represented in the same clones (Yeremenko et al. manuscript in preparation).

**Expression of outer membrane porins SomA and SomB**

Pore-forming proteins are the major polypeptides in the outer membrane of gram-negative bacteria. They facilitate diffusion of molecules with a relative molecular mass of about 600, and are essential for the molecular sieving properties of the cell wall. These proteins, referred to as porins, form stable trimeric channels with an apparent molecular mass of 100 kDa in Enterobacteria (Engel et al. 1985). In addition to the major, non-specific porins OmpF and OmpC, *E. coli* and several other Enterobacteria induce an anion-selective porin in P₁-deficient conditions (Overbeeke and Lugtenberg 1980; Bauer et al. 1985; 1988). This porin (PhoE) is part of the *pho* regulon in *E. coli* (Rao and Torriani 1990). A different type of P₁-deficiency inducible outer membrane porins, larger than enterobacterial porins (about 48 kDa), is found in the Pseudomonads (Poole and Hancock 1986; Hancock et al. 1990; Leopold et al. 1997). This class of porins is represented by the P₁-selective porin OprP of *Pseudomonas aeruginosa* (Hancock et al. 1982; Siehnel et al. 1990). Whereas PhoE is only weakly anion-selective, OprP is very strongly selective for P₁ over other anions, due to a P₁-binding site in the channel involving three lysine residues (Hancock et al. 1990). In addition, *P. aeruginosa* possesses a polyphosphate-selective porin, OprO (Siehnel et al. 1992).

Polypeptide patterns of the outer membranes of several *Synechococcus* strains are dominated by two proteins with very similar apparent molecular weights of about 52 k (Resch and Gibson 1983; Scanlan et al. 1989). The polypeptide patterns of cell wall fractions and isolated outer membranes of *Synechocystis* PCC 6714 are dominated by two proteins with apparent molecular weight of 61 k and 67 k (Jürgens et al. 1985; Jürgens and Weckesser 1985). A protein fraction (40-80 kDa) from the outer membrane of *Anabaena variabilis* showed pore-forming activity (Benz and Böhme 1985). Thus, the cyanobacterial major outer membrane proteins have a higher apparent molecular weight (50-70 k) than those of most heterotrophic bacteria (30-40 k) (Hoiczyk and Hansel 2000). The two major porins of
Synechococcus sp. PCC 6301 were characterized (Hansel et al. 1994; Hansel and Tadros 1998) and sequenced (Hansel et al. 1998). These proteins closely migrate at about 52 kDa and were called SomA and SomB, after the Synechococcus elongatus PCC 7942 homologue SomA, which was sequenced earlier (Umeda et al. 1996), but not recognized as a porin at the time. The presence of a $\text{P}_1$-regulated porin in Synechococcus elongatus PCC 7942 was suggested, because of the appearance of a 32 kDa polypeptide in $\text{P}_1$-depleted cultures (Scanlan et al. 1989). With the current knowledge, this seems rather small for a cyanobacterial porin, and it is more likely to have been a co-purified $\text{P}_1$-binding protein. The presence of multiple genes encoding outer membrane porins in Synechocystis (Hansel et al. 1998; Engelhardt and Peters 1998) invokes questions about their function and expression. Recently, Sauer et al. (2001) have reported the accumulation of the porins SomA and SomB in Synechococcus due to increased expression following nitrogen-depletion. In this contribution, we report the differential expression in $\text{P}_1$-deficient conditions of two porins in Synechocystis, slr1841 and slr1908. Specific regulation of expression of the major outer membrane porins in cyanobacteria probably optimises the permeability of the cells for anionic nutrients.

**Acknowledgements**

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