Consequences of impaired microcystin production for light-dependent growth and pigmentation of Microcystis aeruginosa PCC 7806

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Abstract

Microcystis aeruginosa PCC 7806 produces potent inhibitors of eukaryotic protein phosphatases called microcystins, whose function to the organism is presently unknown. Mutants with impaired microcystin biosynthesis should provide a useful tool for investigations of microcystin function. This study has focussed on the comparison of growth and pigment content of strain PCC 7806 and its mcyB3 mutant deficient in microcystin biosynthesis, under semicontinuous culture conditions. Both wild-type and mutant are characterised by a very low light demand and low-maximum specific growth rates in comparison to other Microcystis strains studied. While growth of wild-type and mutant were similar under different light conditions, the mutant cells showed significantly higher specific absorbances in the range of photosynthetic active radiances 420–700 nm, under light-limiting conditions. The mutant cells possess lower contents of chlorophyll a, β-carotene, zeaxanthin and echinenone under light limitation and of myxoxanthophyll under saturated light conditions. Though microcystins are clearly not essential for growth, the observed effects of the mutation are a first indication of their involvement in intracellular processes. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Microcystin-deficient mutant; Microcystin function; Chlorophyll; Carotenoid; Microcystis aeruginosa

1. Introduction

Cyanobacteria are known to produce a variety of bioactive substances including antibiotics, cytotoxins, neurotoxins and hepatotoxins as well as substances with antiviral and anti-algal effects [1,2]. Scientific interests have focussed especially on Microcystis aeruginosa, due to its frequent occurrence in toxic blooms world-wide and the related health hazard for humans and livestock [3]. Most of the risk can be attributed to a family of small cyclic heptapeptides, called microcystins. Microcystins are specific inhibitors of eukaryotic protein phosphatases 1 and 2A and are primarily responsible for damage of blood-vessels in the liver of vertebrates leading to an acute haemorrhetic shock, i.e. they act as hepatotoxins [2,4]. Microcystins are produced by a number of bloom-forming species of cyanobacteria, but their function for the producing organisms remains obscure. An important role of microcystins in cellular processes of cyanobacteria has been discussed previously in relation to the compound’s ability to chelate metal ions and thus function as siderophores, possibly regulating the amount of free Fe2+ in the cell [5]. A role for microcystin as a feeding deterrent against zooplankton or other grazers has also been suggested [6,7]. Overall, the role of microcystin is still highly disputed. Even its common classification as a secondary metabolite has come under scrutiny, due to a linear correlation calculated between microcystin production and cell division rates [8].

Recently a gene cluster (mcy genes) was identified encoding non-ribosomal peptide synthetases, polyketide synthases and other enzymes involved in microcystin biosynthesis in M. aeruginosa [9,10]. Mutants unable to produce microcystins were generated by insertional mutagenesis of some of the mcy genes. These knock-out mutants (mcyB3-, MT), still producing cyanopeptolines (the second group of small peptides detected in the wild-type (WT) strain [11]) offer the possibility to find out if the loss of microcystin has phenotypic consequences for the cyanobacterial cells.
and may thus provide a clue to the function of microcystins for the cyanobacterial cell. Transcription of mcy genes is positively regulated by light in *M. aeruginosa* [12,13] suggesting a function of microcystins in light-dependent processes. Therefore, in a first attempt to elucidate a possible role of microcystins, we compared growth and pigment composition of the WT strain *M. aeruginosa* PCC 7806, a microcystin producer, and the MT mutant, lacking microcystins [11], under different light conditions.

2. Materials and methods

2.1. Cyanobacterial strains and cultivation

The microcystin-producing *M. aeruginosa* strain PCC7806 was kindly provided by R. Rippka (Institute Pasteur, Paris, France). The mutant used in this study, unable to produce microcystins, was previously generated by insertion of a chloramphenicol resistance gene cassette into mcyB [11]. MT and WT were maintained on BG11 agar medium, the MT being usually kept in medium containing 5 μg ml⁻¹ chloramphenicol. For all comparative experiments during this study, chloramphenicol was omitted from the medium to exclude an influence of the antibiotic on physiological processes and morphological characteristics. The persistence of the mutation, as indicated by the lack of toxin, was checked during and after culturing by HPLC [14,15].

Cells were grown in 100 ml M IV/2 medium [16] in 500-ml Erlenmeyer flasks, shaken in a water bath at 20°C and illuminated in a 12-h/12-h light–dark cycle with fluorescent tubes (Lampi warmwhite LT 36 W, Germany) in a light intensity gradient between 4 and 110 μE m⁻² s⁻¹: to maintain nutrient saturated conditions cultures were grown semicontinuously (turbidostat principle), diluted every 2–3 days with MIV/2 nutrient solution.

2.2. Biovolume determination

A photometrical measurement of culture density was performed with double-frame technique [17] in order to facilitate the biovolume detection procedure for culture control. In this double-frame method priority is given to light attenuation by scattering which is caused by strain specific morphological characteristics. Optical density (light extinction), when measured using this technique is less influenced by cellular pigmentation. The obtained attenuation signals of the culture suspensions of both the WT and the MT strain at 436 nm (Photometer 1101 M, Eppendorf Gerätebau, Netheler+Hinz GmbH, Hamburg, Germany) were calibrated to their real biovolumes, which were determined microscopically both by measuring diameters of the spherical cells calculated as mean cell volume, and by cell counting. The data sets of both variants were tested for their normal distribution. Significance of differences between WT and MT was tested by comparing the slopes of the biovolume vs light extinction curves using a Student’s *t*-test and hypothesis-test (STATGRAPHICS Plus 3.0, Statistical Graphics Corp., USA).

2.3. Pigment extraction

Cells were harvested as 20-ml samples from the cultures maintained at exponential growth phase on three different days at defined biovolumes. The cells were filtered onto glass fibre filters (Whatman GF/C) in two aliquots (10 ml each). Subsequently, the filters were freeze-dried and stored at −20°C. For extraction, filters were ground in a mortar with 2 ml of cold acetone (90%), sonicated in an ice bath for 10 min and centrifuged at 8000 rpm for 10 min (4°C). The sediments were re-extracted with 1 ml acetone (100%) in the same way. Collected extracts were mixed with IPR-solution (15 mg ml⁻¹ tetrabutylammonium acetate in 77 mg ml⁻¹ ammonium acetate solution) and filtered through 0.2-μm syringe-filters into autosampler tubes. Chromatography was carried out with a binary gradient system in a Waters 600E (Waters, Milford, USA) gradient module (eluent 1: methanol/water/IPR — 80:10:10, eluent 2: methanol/acetone — 80:20). Peaks were recorded at 440 nm. A detailed description of the pigment extraction method and analysis is given in Woitke et al. [16].

Phycocyanin, which is water soluble, can not be extracted by the method described above and hence is not detected by HPLC. However it has a distinct absorption maximum at 625 nm. The intensity of the photometric signal (extent of the peak) indicates the amount of phycocyanin in the cell. Hence, cytophotometric in vivo measurements complemented the pigment analysis performed with HPLC. Prior to measurement, samples were pressurised to collapse the gas vesicles and avoid otherwise disturbing optical interferences. This was achieved by placing the cell suspension, bubble-free, in a glass syringe, sealing the needle with a rubber stopper and pressurising by 2–3 hits with a rubber mallet on the syringe pistol until light microscopy showed cells without gas vesicles. Cytophotometric absorbance spectra from 420 to 750 nm were recorded for at least 10 single cells of both a light-limited and a nearly light-saturated culture of WT and MT (Leitz DMRB with MPV-SP, Leitz, Wetzlar, Germany). Each spectrum was normalised by the actual cell diameter to obtain comparable specific absorbance values. For comparison of WT and MT data, the specific absorbances at characteristic pigment absorption maxima (435 nm — short wave maximum of chlorophyll *a* (*chl a*), 490 nm — carotenoids, 625 nm — phycocyanin, 675 nm — long-wave maximum of *chl a*) and the ratio *E*₄₂₅/*E*₅₂₅ (representing the proportion of phycocyanin in relation to *chl a*) were determined. In addition, the specific total absorbance of the cells in the range of photosynthetic active radiation (420–700 nm) was determined.
3. Results and discussion

3.1. Growth under different light conditions

In this study, microcystin producing PCC 7806 (WT) and its microcystin-deficient mutant (MT) were compared with respect to light intensity-dependent growth and pigment contents under different light conditions. We did not observe significant differences between the light and growth curves (Fig. 1) and the maximum specific growth rate (Table 1) of MT and WT. Thus, the lack of microcystins in MT had no effect on the growth under the tested light conditions. However, the low-maximum specific growth rates measured for both variants differentiate them from other strains of *M. aeruginosa* [18,19]. Similarly, light saturation intensities (\(I_{\text{sat}}\): light intensity at \(\mu=0.95\ \mu_m\)) of approximately 32 \(\mu\text{E} m^{-2} s^{-1}\) for the WT, and 38 \(\mu\text{E} m^{-2} s^{-1}\) for the MT were low compared to other *Microcystis* strains that require higher intensities (single cell strains 50 \(\mu\text{E} m^{-2} s^{-1}\), colony-forming strains 80 \(\mu\text{E} m^{-2} s^{-1}\), [18]) to reach light saturation conditions.

3.2. Biovolume vs absorption

We could not detect significant differences in the size of cells between WT and MT. Surprisingly, WT and MT variants exhibited significant differences in the biovolume vs light extinction curves (Fig. 2). At the same biovolume a 30% higher optical signal was measured from the WT vs light extinction curves (Fig. 2). At the same biovolume WT and MT variants exhibited significant differences in the biovolume of WT and MT cells between WT and MT.

The observed cellular pigment concentrations reflect adaptation of the pigment apparatus to light intensities between 4 and 110 \(\mu\text{E} m^{-2} s^{-1}\). Both the WT and the MT reflected mainly influenced by cellular surface structure, refraction is determined by the internal organisation of membrane systems, the nature and size of inclusions and the occurrence of gas vesicles within the cell. The double-frame technique for measuring photometric signals, used in this study, is based on light attenuation as a consequence of light refraction (scattering). Therefore, the significantly lower photometric absorbance signals observed in MT cultures in comparison to the WT, are an indication of internal structural differences in MT cells.

3.3. Pigment composition

Structural changes in the MT are also suggested by the significantly higher specific absorbance values (for individual pigments), but lower chl \(a\) and carotenoid contents (Fig. 3, Table 2). To investigate possible adaptive responses to different light intensities, the pigment contents of WT and MT cells were analysed by HPLC. Clear separation of peaks was seen for chl \(a\), the carotenoids \(\beta\)-carotene (\(\beta\)-car) and zeaxanthin (zea), the ketocarotenoid echinenone (ech), and the carotenoid-glycoside myxoxanthophyll (myx). The detected crocetindial (cro) represents an artificial degradation product of \(\beta\)-car or zea due to oxidative splitting by the carotene oxygenase enzyme complex [20]. Due to the highly variable occurrence of cro in the samples, the sum of \(\beta\)-car, zea and cro was used to compare WT and MT.

The observed cellular pigment concentrations reflect adaptation of the pigment apparatus to light intensities between 4 and 110 \(\mu\text{E} m^{-2} s^{-1}\). Both the WT and the

![Fig. 1. Light intensity-dependent growth of PCC 7806 WT and MT, means (\(n=25\)) and confidence limits; Values of filled symbols were tested with Student’s \(t\)-test (\(P=0.05\)) for significant differences at comparable light supply. Grey filled symbols – tested values without significant differences; black filled symbols – tested values with significant difference; open symbols – not tested; Light-growth curves were estimated by non-linear regression to a modified Mitscherlich model [17] \((\mu=\mu_m(1-\exp(-\ln2(I-I_0)(K_1-I_0)))\).](#)

![Fig. 2. Light extinction vs biovolume ratios of WT and MT cultures with linear regression lines.](#)

### Table 1

Model parameters of estimated light–growth curves (Fig. 2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu_m) (day(^{-1}))</td>
<td>0.211 (0.205–0.217)</td>
<td>0.208 (0.202–0.214)</td>
</tr>
<tr>
<td>(I_0) ((\mu\text{E} m^{-2} s^{-1}))</td>
<td>1.85 (0.73–2.97)</td>
<td>0.61 (–0.43–1.70)</td>
</tr>
<tr>
<td>(K_1) ((\mu\text{E} m^{-2} s^{-1}))</td>
<td>8.79 (8.16–9.42)</td>
<td>9.44 (8.60–10.28)</td>
</tr>
<tr>
<td>(I_{\text{sat}}) ((\mu\text{E} m^{-2} s^{-1}))</td>
<td>31.8</td>
<td>37.7</td>
</tr>
</tbody>
</table>

\(\mu_m\): maximum specific growth rate; \(I_0\): minimum light demand; \(K_1\): half saturation constant; \(I_{\text{sat}}\): saturating light supply, calculated at \(\mu=0.95\) \(\mu_m\)

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*Standard deviations
MT adapted to light by altering their intracellular concentrations of chl a and carotenoids. Chl a, β-car+zea+cro and ech decreased with increasing light intensity, especially in the low light range, while high irradiance resulted in higher myx concentrations. When normalised to chl a, all pigment ratios increased with increasing light supply, largely reflecting a much greater reduction in chl a relative to accessory carotenoids. The carotenoid/chl a ratios were identical for both variants.

While the adaptation to different light intensities was shown to be the same in MT and WT, the pigment concentrations revealed differences between the two variants independent of light intensities (Fig. 3). The cellular concentrations (means) of all pigments were approximately 20% lower in the MT compared to the WT. Significant differences were found at light limited conditions for chl a and ech, and at light saturation for myx.

The cytophotometrically determined absorbance data are displayed in Table 2. Under nearly light saturated conditions, there were no significant differences in the specific absorbances, the ratio of $E_{625}/E_{675}$ and total specific absorbances between 420 and 700 nm. However, the cytophotometric in vivo absorbance spectra revealed a significantly higher PC content ($E_{625}/E_{675}$) relative to chl a in the

![Graphs and tables]

**Table 2** Cytophotometrically determined values of specific absorbances of light limited and nearly saturated WT and MT cells

<table>
<thead>
<tr>
<th>Specific absorbance ($\mu$m$^{-1}$)</th>
<th>WT (5.3 $\mu$E m$^{-2}$ s$^{-1}$)</th>
<th>MT (4.0 $\mu$E m$^{-2}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{435}$</td>
<td>0.055$^{a}$</td>
<td>0.083$^{a}$</td>
</tr>
<tr>
<td>$E_{490}$</td>
<td>0.017$^{a}$</td>
<td>0.023$^{a}$</td>
</tr>
<tr>
<td>$E_{625}$</td>
<td>0.037$^{a}$</td>
<td>0.053$^{a}$</td>
</tr>
<tr>
<td>$E_{675}$</td>
<td>0.041$^{a}$</td>
<td>0.055$^{a}$</td>
</tr>
<tr>
<td>Total specific absorbance (420–700 nm)</td>
<td>7.1$^{a}$</td>
<td>10.1$^{a}$</td>
</tr>
<tr>
<td>$E_{625}/E_{675}$</td>
<td>0.89$^{a}$</td>
<td>0.97$^{a}$</td>
</tr>
<tr>
<td>WT (34.6 $\mu$E m$^{-2}$ s$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{435}$</td>
<td>0.042</td>
<td>0.040</td>
</tr>
<tr>
<td>$E_{490}$</td>
<td>0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>$E_{625}$</td>
<td>0.027</td>
<td>0.026</td>
</tr>
<tr>
<td>$E_{675}$</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td>Total specific absorbance (420–700 nm)</td>
<td>5.9</td>
<td>5.8</td>
</tr>
<tr>
<td>$E_{625}/E_{675}$</td>
<td>0.86</td>
<td>0.85</td>
</tr>
</tbody>
</table>

$^{a}$Values are significantly different, tested with Student’s t-test at a level of $P<0.05$.

![Graphs and tables]
MT cells under light limitation. PC is the main component of the phycobilisomes in *M. aeruginosa*, which act as light-harvesting systems and transfer collected light energy to the photosynthetic reaction centres, primarily to PS II [21].

The lower chl a content, compared to the WT, and the simultaneously increasing ratio of PC to chl a, exhibited under light limited conditions in MT cells, point toward a changed PS I/PS II ratio. In cyanobacteria light adaptation mechanisms are guided by variations in the PS I/PS II ratio [22]. Therefore, it can be speculated that microcystins could play a role in light adaptation processes. The light-regulated transcription of the microcystin synthetase gene cluster [13] and the subcellular localisation of microcystins on thylakoid membranes [23] support such assumptions. In this case, presumably, compounds with similar chemical structure (i.e. cyanopeptolines, microginins [24]) serve similar functions in non-microcystin-containing WT strains.

On the other hand our observations could be explained by structural changes in the cellular membrane system caused by the lack of membrane bound microcystin. Then the assumed change in the PS I/PS II ratio could be a consequence of the lack of space for the photosynthetic reaction centres and the expanded antennae assembly under light-deficient conditions.

It is likely that more information will be gained, when the second cyclic peptide present in PCC 7806 cyanopeptoline is absent also.

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