Physiological and molecular responses to calcium supplementation in Microcystis aeruginosa (Cyanobacteria)

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Physiological and molecular responses to calcium supplementation in *Microcystis aeruginosa* (Cyanobacteria)

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Calcium is an important element in water bodies that plays a pivotal role in numerous biological processes; however, its effects on bloom-forming cyanobacteria have been studied only rarely. In the present study, the effects of calcium on *Microcystis aeruginosa*, a major bloom-forming cyanobacterium, were investigated. Significant decreases in growth, chlorophyll *a* and maximum electron transport rate were shown when calcium was absent, while similar reductions in these parameters were also found at high concentrations of calcium (240 mg/l). The expression of *psbA*, *grpE* and *recA* was up-regulated significantly for 0 and 240 mg/l calcium treatments. The activity of catalase (CAT) also increased remarkably under these two treatments, suggesting that *Microcystis* suffered from stress under both calcium absence and under high calcium concentrations. Additionally, the polysaccharide synthesis gene, *epsL*, was up-regulated at high concentrations of calcium. These data suggest that calcium plays an important role in the growth of *Microcystis*, and that enhancement in polysaccharide synthesis in response to increases in calcium concentration might be responsible for bloom formation.

Keywords: calcium; electron transport rate (ETR); extracellular polysaccharides (EPS); gene expression; *Microcystis aeruginosa*

Introduction

Accelerated eutrophication of lakes and freshwater rivers often leads to the excessive proliferation of phytoplankton, of which cyanobacteria tend to predominate. These prokaryotes develop thick layers on the water’s surface during suitable conditions, which causes problems for users and managers of aquatic environments (Smith 2003; Quiblier et al. 2008). A major bloom-forming cyanobacterium, *Microcystis aeruginosa*, has frequently been reported to form noxious blooms in eutrophic freshwater lakes, ponds and reservoirs. Worldwide, some strains of *Microcystis* synthesise toxins called microcystins (Carmichael 1994; Sinoven & Jones 1999) that threaten human and animal health (Falconer 2001; Briand et al. 2003). *Microcystis* blooms can also cause mortality of diverse species, losses in submerged aquatic vegetation and generally decrease ecosystem stability (Fleming et al. 2002). Therefore, a substantial amount work has focused on the mechanisms of bloom formation in cyanobacteria. Certain physiological strategies may be involved in *Microcystis* bloom development and sustainment (Oliver & Ganf 2002), but environmental factors, including nutrient concentrations, may also be responsible for inducing phytoplankton blooms and determining species dominance (Burford et al. 2007).

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Calcium is an important element in water bodies and it plays a pivotal role in numerous biological processes in bacteria and eukaryotes (Norris et al. 1996; Domínguez 2004). In cyanobacteria, calcium has been shown to be important for signal transduction (Bachmann 2003), heterocyst differentiation (Torrecilla et al. 2004; Shi et al. 2006), phototactic and stress resistance responses (Pitta et al. 1997; Battah & Khalil 2009; Leganés et al. 2009; Barrán-Berdón et al. 2011). Nevertheless, there is little direct evidence of the physiological and molecular mechanisms by which Ca\(^{2+}\) exerts its effects.

Lake-water calcium concentrations are currently declining in many softwater lakes worldwide, which have been considered a novel and a widespread threat to aquatic biota in freshwater ecosystems (Clair et al. 2007; Jeziorski et al. 2008). Recently, an interesting result was elucidated by Korosi et al. (2012), who found that an anomalous algal bloom was linked to lake-water calcium decline through the effect of calcium reductions on *Daphnia*. Previous studies have shown that low ambient calcium concentrations significantly affected freshwater daphnids, gammarids and crayfish (Jeziorski et al. 2008; Cairns & Yan 2009). However, to date, no detailed study has examined the relationship between calcium concentration and *Microcystis*, especially in relation to physiological and molecular effects. We hypothesise, therefore, that tolerance to calcium concentrations in *Microcystis* populations may play an important role in the development of *Microcystis* blooms.

To test this, the effects of different calcium concentrations on *M. aeruginosa* FACHB 905 were investigated under laboratory culture conditions. Additionally, to explore whether there are any effects of calcium on photosynthetic processes, DNA, protein and carbohydrate synthesis, we selected four target genes for study. These genes are associated with the core protein for photosynthetic processes (*psbA*), damage and repair of biological macromolecules (*recA* and *grpE*), and extracellular polysaccharides syntheses (*epsL*). These results will help elucidate responses of *M. aeruginosa* under the treatment of calcium at the gene and protein levels, and eventually provide insight into eco-physiological mechanisms influencing *Microcystis* blooms.

**Materials and methods**

**Strain and culture conditions**

Unicellular *M. aeruginosa* FACHB 905 was obtained from the Culture Collection of Freshwater Algae of the Institute of Hydrobiology (FACHB-Collection, Wuhan, China). CaCl\(_2\) was used as the source of calcium in the BG11 culture medium (Rippka et al. 1979). The cyanobacterium was grown in a lightbox providing white light at an intensity of 30 \(\mu\)mol/m\(^2\)/s photons and operating a 12:12 light:dark cycle at 25 ± 1 °C.

**Experimental design**

Algal cells in an exponential phase of growth were collected by centrifugation, washed three times with sterile calcium-free BG11 medium and then starved in calcium-free BG11 medium for 2–3 days. After starvation, the algal cells were inoculated into 500-ml Erlenmeyer flasks containing 250 ml of calcium-free BG11 medium. Different concentrations of calcium (0, 30, 60, 120 or 240 mg/l) were obtained using a stock CaCl\(_2\) solution. To ensure homogeneous conditions and equal illumination, the culture was aerated with filtered air and the positions of the flasks were exchanged by hand once a day. The culture pH was maintained at pH 8.0 ± 0.2 in all treatments with a buffer solution of 10 M 3-(N-morpholino) propanesulphonic acid (MOPS) (MacKenzie et al. 2004). The cultures were sampled every day to determine the concentrations of chlorophyll \(a\) (Chl \(a\)), EPS and proteins.

**Growth rate, Chl \(a\), EPS, proteins, catalase (CAT) and chlorophyll fluorescence measurements**

Chl \(a\) was extracted using 90% acetone and measured as described by Nusch (1980).
The specific growth rate ($\mu$) was calculated according to: $\mu = (\ln N_t - \ln N_0) \times t^{-1}$, where $N_t$ is the Chl $a$ content after $t$ days and $N_0$ is the initial Chl $a$ content. EPS concentrations were quantified spectrophotometrically using the phenol–sulphuric acid method with glucose as the standard (Dubois et al. 1956). Protein concentration was measured using the protein-dye binding method with bovine serum albumin as the standard (Bradford 1976). The assay used for antioxidant enzyme-catalase (CAT) activity is described in Wu et al. (2007).

Rapid light curves (RLCs) in exponential phase cultures were measured with a Phyto-PAM fluorescence monitoring system (Heinz Walz GmbH, Effeltrich, Germany), and the electron transport rate (ETR) of photosystem II (PSII) was calculated according to Wu & Song (2008).

**RNA isolation and reverse transcription**
Samples from cultures at 0, 4 and 8 days were harvested by centrifugation at 7000 rpm for 5 min. Pellets were resuspended in Trizol reagent (Invitrogen, USA) and homogenised with a mini-beadbeater. Total RNA was extracted following the manufacturer’s instructions (Invitrogen, USA). Total RNA samples were digested with RQ1 RNase-free DNase (Promega, USA). The RNA was reverse transcribed to cDNA using random primers $p$(dN)$_9$ and a reverse transcriptase kit (Generay, China).

**Quantitative real-time polymerase chain reaction (PCR)**
The primers for real-time polymerase chain reaction (PCR) are shown in Table 1. Real-time PCR was performed in a final reaction volume of 20 µl, which consisted of 10 µl Master Mix (SYBR Green, Toyobo, Japan), 0.2 µl (10 pmol/µl) each of forward and reverse primers, 1 µl cDNA, and 8.6 µl water. The amplification reactions were performed using an iCycler IQ (Bio-Rad, Hercules, CA) operating under the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Gene expression data were evaluated using the calculated $C_t$ values (Livak & Schmittgen 2001), with the 16S rRNA gene used as the housekeeping gene for normalising the expression levels of target genes. Relative transcription was calculated using the $2^{-\Delta\Delta C_t}$ method, where $\Delta\Delta C_t = (C_t$$\text{target gene} - C_t$$16S rRNA)(+Ca) - (C_t$$\text{target gene} - C_t$$16S rRNA)(-Ca)$. For each replicate, triplicate reactions were run. Reactions were considered acceptable if the amplification efficiency was 90–110% and the $\Delta C_t$ of the two slopes was less than 0.1.

**Statistical analysis**
All experiments were performed three times, each time with three independent replicates. Significant differences were determined by analysis of variance (ANOVA) using the Microcal Origin software (Version 6.1, OriginLab Corporation, Northampton, MA, USA). Differences were considered significant at $P < 0.05$.

**Table 1** Primers used for real-time polymerase chain reactions.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>GAGCGGGGTGAGATACGCCCTTAA/CACATTGCGGAAAATTCGCCC</td>
<td>Shao et al. (2009)</td>
</tr>
<tr>
<td>psbA</td>
<td>GGTCAAGARGAAGAAAACCTACAGTG/TTG/AAACCCGTTAGGTTGAA</td>
<td>Shao et al. (2009)</td>
</tr>
<tr>
<td>recA</td>
<td>TAGTTGACCAGTTAGTGGTTGTTCTT/TCATTGGATTTTGCAGTAGGT</td>
<td>Shao et al. (2009)</td>
</tr>
<tr>
<td>grpE</td>
<td>CGCAAAACGCACAGCCAGGAAAGGTAATACCACAATCTGCGGTGG/CGGATTACTGGGCTTCCAG</td>
<td>Shao et al. (2009)</td>
</tr>
<tr>
<td>epsL</td>
<td>CGATGGGTGCGGTATCTTCC/GCCGATTACTGGGCTTCC</td>
<td>This study</td>
</tr>
</tbody>
</table>
Results

Growth

Concentrations of Chl a in *M. aeruginosa* varied with the concentration of calcium in the culture medium. Chl a levels were greatest at a calcium concentration of 30 mg/l, followed by cultures at 60 mg/l ($P > 0.05$, ANOVA). When cultured in 0 and 240 mg/l calcium, cyanobacterial growth was slow and the slowest increase in Chl a occurred at 0 mg/l calcium during the 8-day experiment (Fig. 1). Compared with the growth rate in the absence of calcium, higher specific growth rates were found in cultures containing calcium at 30 and 60 mg/l (Table 2, $P < 0.05$, ANOVA).

Chlorophyll fluorescence, Extracellular polysaccharides (EPS) concentrations and catalase (CAT) activity

Different RLCs were determined for the cultures containing the various concentrations of calcium (Fig. 2). Greatest maximum ETR (ETR$_{max}$) was observed in cultures containing 60 mg/l calcium, while lower ETR$_{max}$ values were found in the other treatment groups. There was a significant decrease in ETR$_{max}$ in cultures completely lacking calcium compared with cultures containing 60 mg/l calcium (Table 2, $P < 0.05$, ANOVA).

*Microcystis aeruginosa* produced more EPS in higher concentrations of calcium. After 8 days of culture, the EPS concentrations in

![Figure 1](image1.png)

Figure 1 Chl a levels in *Microcystis aeruginosa* FACHB 905 cultured in different CaCl$_2$ concentrations. Error bars are ± one standard deviation of triplicate measurements.

<table>
<thead>
<tr>
<th>Calcium concentration (mg/l)</th>
<th>Specific growth rate ($\mu$, day$^{-1}$)</th>
<th>ETR$_{max}$ (µmol/m$^2$/s electrons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.12±0.02</td>
<td>47.15±3.18</td>
</tr>
<tr>
<td>30</td>
<td>0.17±0.02*</td>
<td>56.23±1.33*</td>
</tr>
<tr>
<td>60</td>
<td>0.20±0.01*</td>
<td>66.45±1.77*</td>
</tr>
<tr>
<td>120</td>
<td>0.15±0.01</td>
<td>54.12±3.21</td>
</tr>
<tr>
<td>240</td>
<td>0.14±0.00</td>
<td>53.20±0.71</td>
</tr>
</tbody>
</table>

*Significant difference ($P < 0.05$) compared with the 0 mg/l calcium treatment group.
the cultures containing 240 mg/l calcium were 99.1% and 32.0% greater than those cultures containing 0 and 60 mg/l calcium, respectively (Fig. 3).

The CAT activity of *M. aeruginosa* under different concentrations of calcium was assayed (Fig. 4). After 8 days of culture, the activities of CAT in the 0 and 240 mg/l calcium treatments were increased by 2.26 and 3.07 times, respectively, compared with the 60-mg/l calcium treatment.

**Gene expression**

To explore whether there were changes in processes associated with photosynthesis, DNA, protein and EPS syntheses at different concentrations of calcium, *psbA*, *recA*, *grpE* and *epsL* were selected as target genes, respectively. The expression data for *psbA*, *recA*, *grpE* and *epsL* in response to different concentrations of calcium are shown in Fig. 5. Expression of *psbA* was up-regulated significantly in the 0 and 240 mg/l calcium treatments (*P* < 0.05, ANOVA), while expression remained unchanged after 8 days exposure to 60 mg/l calcium. Expression of *recA* did not change significantly after exposure to 60 mg/l calcium for 8 days, but after this period expression of *recA* at 0 and 240 mg/l calcium were 1.99 and 2.35 times greater than the 60 mg/l calcium treatment, respectively (*P* < 0.05, ANOVA). Additionally, *grpE* expression also changed significantly in the 0 and 240 mg/l calcium treatments. Compared with the 0 mg/l calcium treatment, expression of *epsL* was up-regulated significantly in the presence of calcium.

**Discussion**

Calcium is an essential element for growth and development in vascular plants, but little is known regarding the calcium requirements of prokaryotic cells (Norris et al. 1996). Debus (1992) indicated that Ca\(^{2+}\) requirements for cyanobacterial cells may also be as different from those in plants as it is among various species of cyanobacteria. In the present study, calcium deficiency for *M. aeruginosa* was associated with reduced growth, suggesting that this element is required for the growth of this organism. A similar result was also found in
Figure 3 Production of extracellular polysaccharides by *Microcystis aeruginosa* FACHB 905 cultured in media containing 0, 60 and 240 mg/l calcium. Error bars are ± one standard deviation of triplicate measurements. *Significant difference (*P* < 0.05) and **highly significant difference (*P* < 0.01) compared with the 0 mg/l calcium treatment group.

Figure 4 The catalase (CAT) activity of *Microcystis aeruginosa* FACHB 905 cultured in media containing 0, 60 and 240 mg/l calcium. Error bars are ± one standard deviation of triplicate measurements. *Significant difference (*P* < 0.05) and **highly significant difference (*P* < 0.01) compared with the 0 mg/l calcium treatment group.
the blue-green alga *Calothrix parietina* where a significant decrease in growth resulted from Ca$^{2+}$ starvation (Zaki 1999). Recent evidence is increasingly implicated in a number of bacterial functions, including heat shock, pathogenicity, chemotaxis, differentiation and the cell cycle (Norris et al. 1996). Similar results are also reported in cyanobacteria, whose photosystem II (PSII) activity, N$_2$ fixation, heterocyte differentiation, phosphate uptake and phototactic responses show a number of Ca$^{2+}$-dependent processes (Zaki 1999). In this study, moderate concentrations of calcium increased the growth of *Microcystis*, suggesting that calcium might be involved in cell differentiation or the cell cycle of *Microcystis*. Additionally, high concentrations of calcium (>120 mg/l) were also observed to reduce the growth of *M. aeruginosa*, and in fact greatest growth was found at 60 mg/l calcium (Fig. 1, Table 2). The results were corroborated by data from Carneiro et al. (2011), who showed that growth in *Cylindrospermopsis* was inhibited by a continued exposure to high Ca$^{2+}$ (8 mM). A field survey was implemented by Ye et al. (2010), who showed that Ca$^{2+}$ concentration was about 57.73 mg/l in the northern rivers of the Taihu Basin where large *Microcystis* blooms often occurred. Korosi et al. (2012) indicated that the decline of calcium concentration increased chlorophyll *a* levels in the water column of Lake George, suggesting that calcium decline might be an additional mechanism for potential increases in algal blooms.

Calcium has been suggested to play an important role in cyanobacterial photosynthesis (Piccioni & Mauzerall 1976). Debus (1992) showed that the influence of Ca$^{2+}$ ions on electron transfer and oxygen evolution might be different between cyanobacteria and higher plants. Miqyass (2008) indicated that high levels of calcium were essential for every step of electron transfer from the manganese cluster to the secondary electron donor tyrosine Z in PSII. In the present study, the ETR$_{\text{max}}$ of PSII decreased significantly in the absence of calcium (Fig. 3, Table 2), which suggests that

![Figure 5](image_url)  
**Figure 5** Relative normalised expression of *psbA*, *recA*, *grpE* and *espL* by *Microcystis aeruginosa* FACHB 905 cultured in media containing 0, 60 and 240 mg/l calcium. Error bars are ± one standard deviation of triplicate measurements. *Significant difference (P < 0.05) and **highly significant difference (P < 0.01) compared with the 0 mg/l calcium treatment group.
Calcium is involved in photosynthesis and affects growth of *M. aeruginosa*. However, high levels of calcium (240 mg/l) were also found to slightly impact on the ETR\textsubscript{max} in this study (Fig. 3, Table 2). Pandey et al. (1996) reported that the cation uptake pattern was biphasic, consisting of rapid binding of cations to the negatively charged cell surface and its metabolism based on intracellular import, suggesting that high concentrations of intracellular \(\text{Ca}^{2+}\) may influence the binding or import of cations. Smith et al. (1987) showed an increase in size of the intracellular \(\text{Ca}^{2+}\) pool with increasing \(\text{Ca}^{2+}\) concentration in the growth medium. These results implied that low binding or import of cations may be a reason for ETR decline of *Microcystis* at high \(\text{Ca}^{2+}\) concentration. In addition, the result also supported the theory that native PSII units contain a finite but small number of \(\text{Ca}^{2+}\) ions (Kashino et al. 1986; Ono & Inoue 1988). Interestingly, we found that high concentrations of calcium increased the number of small colonies seen in the culture (data not shown). These observations are consistent with a previous report by Wang et al. (2011). van Boekel (1992) indicated that \(\text{Ca}^{2+}\) was essential for colony firmness and morphology in *Phaeocystis*, suggesting that it might play a role in colony formation and firmness in *Microcystis*. Previous studies have indicated that colony morphology plays a significant role in bloom formation of *Microcystis* (Fulton & Paerl 1987; Reynolds 2007; Wu et al. 2007), suggesting that calcium might also show a significant relationship with *Microcystis* blooms.

In the present study, the expression of *psbA*, *recA* and *grpE* were used to determine the response of *M. aeruginosa* to environmental stress. PSII is very sensitive to changes in the environment, and the ability of PSII to be repaired is determined by the *de novo* synthesis of the D1 protein, which is encoded by *psbA* (Aro et al. 1993). When the photodamage rate exceeds repair by D1, photoinhibition occurs (Madhavi et al. 2007). *recA* encodes a co-protease that stimulates the cleavage of LexA and several other proteins and this leads to the initiation of SOS repair of DNA (Khil & Camerini-Otero 2002). GrpE, encoded by *grpE*, function is to prevent heat-induced protein aggregation and oxidative stress (Van Bogelen et al. 1987; Kim et al. 2007). In the present study, the syntheses of *psbA*, *grpE* and *recA* mRNA were up-regulated in both the absence of calcium and under high concentrations (240 mg/l) of calcium, suggesting that this did cause cellular stress, perhaps also explaining the observed lower growth rate. These data suggest that oxidative stress might take place at 0 and 240 mg/l calcium. In order to confirm this hypothesis further, we determined the CAT of *M. aeruginosa*. CAT can catalyse the \(\text{H}_2\text{O}_2\) into \(\text{H}_2\text{O}\) and \(\text{O}_2\) (Zamocky et al. 2008), being representative for the most important antioxidant enzymes in cells. As shown in Fig. 4, the activities of CAT were enhanced under the 0 and 240 mg/l calcium treatments, suggesting that *Microcystis* cells encountered oxidant stress when cultured at 0 and 240 mg/l calcium. This supports the finding of Zaki (1999) that protein and nitrogen fixation are inhibited significantly in cultures containing 80 mg/l \(\text{Ca}^{2+}\). Interestingly, *espL*, which encodes a polysaccharide deacetylase family protein (Kaneko et al. 2007), showed significant up-regulation at 60 and 240 mg/l calcium and this coincided with increases in EPS production, supporting the conclusion of Wang et al. (2011), who suggested that greater EPS production was not a stress response to the high calcium concentrations. Thus, photosynthesis and changes in related gene expression regulation might be responsible for the observed increases in EPS production. Jeziorski et al. (2008) predicted that the ecological effects of environmental calcium decline were likely to be both widespread and pronounced. Thus, more studies need to be carried out in order to clarify the impacts of calcium on different aspects of primary production and algal blooms.

In conclusion, the results of this study indicate that both the absence of calcium and high concentrations of calcium result in
decreases in growth rate and ETR, but increases of CAT activity and up-regulation of psbA, grpE and recA. Conversely, high concentrations of calcium resulted in increases in the production of EPS. A moderate concentration of calcium increased the growth rate and ETR\textsubscript{max}. These findings suggest that calcium is needed for the growth of \textit{M. aeruginosa} and its concentration might be responsible for bloom formation either directly or indirectly in soft water lakes.

Acknowledgements
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