Role of calcium in acclimation of the cyanobacterium *Synechococcus elongatus* PCC 7942 to nitrogen starvation

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A Ca2+ signal is required for the process of heterocyst differentiation in the filamentous diazotrophic cyanobacterium *Anabaena* sp. PCC 7120. This paper presents evidence that a transient increase in intracellular free Ca2+ is also involved in acclimation to nitrogen starvation in the unicellular non-diazotrophic cyanobacterium *Synechococcus elongatus* PCC 7942. The Ca2+ transient was triggered in response to nitrogen step-down or the addition of 2-oxoglutarate (2-OG), or its analogues 2,2-difluoropentanedioic acid (DFPA) and 2-methylenepentanedioic acid (2-MPA), to cells growing with combined nitrogen, suggesting that an increase in intracellular 2-OG levels precedes the Ca2+ transient. The signalling protein PII and the transcriptional regulator NtcA appear to be needed to trigger the signal. Suppression of the Ca2+ transient by the intracellular Ca2+ chelator N,N′-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl] ester (BAPTA-AM) inhibited expression of the *glnB* and *glnN* genes, which are involved in acclimation to nitrogen starvation and transcriptionally activated by NtcA. BAPTA-AM treatment partially inhibited expression of the *nblA* gene, which is involved in phycobiliprotein degradation following nutrient starvation and is regulated by NtcA and NblR; in close agreement, BAPTA-AM treatment partially inhibited bleaching following nitrogen starvation. Taken together, the results presented here strongly suggest an involvement of a defined Ca2+ transient in acclimation of *S. elongatus* to nitrogen starvation through NtcA-dependent regulation.

INTRODUCTION

Cyanobacteria are a large group of photosynthetic oxygen-evolving prokaryotes. They originated during the Precambrian era (2.8 × 109 years ago) and are known to survive a wide spectrum of environmental stresses (Tandeau de Marsac & Houmard, 1993).

The ability to cope with nutrient limitation or starvation is a requirement for survival in nature. Diazotrophic cyanobacteria circumvent nitrogen starvation by fixing nitrogen; however, in non-diazotrophic strains, nitrogen starvation triggers an acclimation process known as chlorosis or bleaching (Allen & Smith, 1969; Schwarz & Forchhammer, 2005). During chlorosis the phycobilisomes, which account for up to 50% of total cell protein, are degraded to supply the cells with amino acids for the synthesis of proteins needed for acclimation and survival (Bhya et al., 2000).

The unicellular non-diazotrophic *Synechococcus elongatus* strain PCC 7942 (hereinafter termed *S. elongatus*) is a model organism for studying molecular and physiological mechanisms during the process of chlorosis (Schwarz & Forchhammer, 2005). Several non-bleaching (*nbl*) mutants have been isolated and genes cloned (Collier & Grossman, 1994; Grossman et al., 2001; Li & Sherman, 2002). The *nbl* product, a small dimeric protein of 7 kDa helical subunits (Bienert et al., 2006), triggers the initiation of phycobilisome degradation (Collier & Grossman, 1994; Luque et al., 2001, 2003) by binding to the peripheral phycobilisome rods (Bienert et al., 2006). The expression of *nblA* is controlled by two transcriptional regulators, NblR and NtcA. NblR acts as a general transcriptional regulator that integrates different stress signals that lead to phycobilisome degradation (Schwarz & Grossman, 1998). NtcA is a transcriptional activator (seldom a repressor) of a
large number of genes mainly involved in nitrogen metabolism (reviewed by Herrero et al., 2001, 2004). NtcA belongs to the cyclic AMP receptor protein family of transcriptional regulators (Vega-Palas et al., 1992) and binds to a conserved palindromic sequence (GTA-N8TAC) (Herrero et al., 2001). 2-Oxoglutarate (2-OG), the signal of carbon/nitrogen status in cyanobacteria, directly stimulates NtcA–DNA binding as well as NtcA transcriptional activity in vitro; however, direct binding of 2-OG to NtcA has not yet been shown. Signalling protein PII, encoded by glnB, was the first 2-OG-responsive factor identified in cyanobacteria. In S. elongatus, protein PII is modified by phosphorylation on a seryl residue at position 49. The phosphorylation state of the protein is dependent on the intracellular concentration of 2-OG, reflecting the nitrogen/carbon status of the cell (Forchhammer, 2004). There is evidence of functional interaction of PII and NtcA during nitrogen deprivation in S. elongatus (Aldehni et al., 2003); recently, it has been reported that, depending on the cellular levels of 2-OG, protein PipX can form complexes with PII or NtcA providing a physical/function link between the two regulatory proteins (Espinosa et al., 2006, 2007).

In the diazotrophic filamentous cyanobacterium Anabaenopsis sp. PCC 7120, nitrogen starvation not only leads to increased 2-OG levels (Laurent et al., 2005), but also to increased concentrations of free Ca$^{2+}$ ions, which are found to be required for heterocyst differentiation (Torrecilla et al., 2004; Zhao et al., 2005). By using a recombinant strain expressing the Ca$^{2+}$-binding photo-protein apoaequorin (Torrecilla et al., 2000), we have found that a transient increase in intracellular free Ca$^{2+}$ (hereinafter, a Ca$^{2+}$ transient) is also triggered following nitrogen deprivation in the unicellular non-diazotrophic S. elongatus. The addition of 2-OG or its nonmetabolizable analogues 2,2-difluoropentanedioic acid (DFPA) or 2-methylenepentanedioc acid (2-MPA) (Laurent et al., 2005; Chen et al., 2006) also induced the Ca$^{2+}$ transient. We present evidence to suggest a role for Ca$^{2+}$ in the acclimation of S. elongatus to nitrogen starvation through regulation of NtcA-dependent promoters.

**METHODS**

**Cyanobacterial strains and culture conditions.** The following cyanobacterial strains were used in this study: S. elongatus PCC 7942; S. elongatus mutants MP2, lacking the PII signalling protein (Forchhammer & Tandeau de Marsac, 1995), and MntCtA, lacking the NtcA protein (Sauer et al., 1999); the S. elongatus luxAB reporter strains FAM1, carrying a full-length glnB promoter fused to luxAB, FAM2, carrying a truncated glnB promoter containing only the NtcA-dependent part (Aldehni et al., 2003), FAM-431, with a full-length glnN promoter, and FAM-84, with a truncated glnN promoter containing only the NtcA-binding site (Aldehni & Forchhammer, 2006); and S. elongatus WT-103, with the full-length nblA promoter fused to luxAB (Espinosa et al., 2007). Cells were grown in liquid BG11 medium (Rippka, 1988), except for MntCtA and derivatives expressing apoaequorin, which were grown in BG11 in which the nitrate salt was replaced by 5 mM NH$_4$Cl (BG11-NH$_4$), buffered with 5 mM HEPES, pH 7.8, on a rotary shaker at 28 °C under 65 μE m$^{-2}$ s$^{-1}$ fluorescent white light. When ammonium was used as the nitrogen source, BG11-NH$_4$ buffered with 5 mM HEPES, pH 7.8, was used. For nitrogen step-down experiments, BG11$_i$ (medium deprived of combined nitrogen) buffered with 5 mM HEPES, pH 7.8, was used. Cultures of mutants MP2 and MntCtA were maintained in the presence of 25 μg kanamycin ml$^{-1}$. Strains expressing apoaequorin were treated with 5 μg gentamicin ml$^{-1}$. Strains containing the luxAB reporter constructs were grown with 5 μg chloramphenicol ml$^{-1}$.

**Construction of apoaequorin-expressing S. elongatus strains.** A fragment of 0.78 kb containing the apoaequorin gene was PCR-amplified from template pBG2000 (Torrecilla et al., 2000) using primers aequor (5'-GCTCTAGAGGAAGCAAAATCATGACAA-GCAACAC-3') containing an XbaI restriction site, and aequorev (5'-TCCCGGGGTGCACTACACCGTA-3'), containing an Smal site. Following appropriate restriction, the fragment was cloned into the XbaI–Smal sites of pHCN2, a derivative of pCB4 (Gendel et al., 1983). Briefly, the gentamicin-resistance gene from pVZ322 (Zinchenko et al., 1999) with a modified 3' terminal part (5'-CCCCGGGTGCACTACACCGTA-3') to generate additional restriction sites (EcoRI, XbaI, SacI, SalI and Smal) was cloned into the BamHI restriction site of pCB4; into the EcoRI site of the resulting pCB-Gen plasmid, the entire promoter fragment of the PCC6803 petl gene was cloned. Ligation of the amplified apoaequorin fragment into pHCN2, such that the apoaequorin gene is transcribed from the petl promoter (Tous et al., 2001), resulted in pHCN-aeq. This plasmid was introduced into S. elongatus wild-type and mutant MP2 strains by transformation (Golden et al., 1987). pHCN-aeq was introduced into mutant strain MntCtA by conjugation (Wolk et al., 1984; Elhai & Wolk, 1988). Transformants were selected on BG11 or BG11-NH$_4$ plates containing 5 μg gentamicin ml$^{-1}$.

**In vivo aequorin reconstitution and luminescence measurements.** For aequorin luminescence measurements, in vivo reconstitution of aequorin was performed by the addition of 5 μM coelenterazine (FluoProbes) to cell suspensions of S. elongatus wild-type, MntCtA and MP2 strains expressing apoaequorin, followed by incubation for 4 h in darkness with shaking, as previously described (Torrecilla et al., 2000). Before Ca$^{2+}$ measurements were made, cells were washed twice in BG11 medium buffered with 10 mM HEPES, pH 7.5, containing 0.5 mM EGTA, to remove excess coelenterazine.

For luminescence measurements, 150 μl reconstituted cell suspension was transferred to wells of an opaque white 96-well microtitre plate (Porvair Sciences). Luminescence was recorded every 1 s for the duration of the experiment in a Centro LB 960 luminometer (Berthold Technologies).

Calibration of changes in the free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) requires knowledge of the total available amount of reconstituted aequorin in cell suspensions (L$_{max}$) at any one point in time during the experiment as well as the running luminescence (L$_o$). For estimation of total aequorin luminescence, the remaining aequorin was discharged at the end of the experiment by the addition of 150 μl 1 M CaCl$_2$ and 5 % (v/v) Triton X-100. Rate constants of luminescence (L$_o$/L$_{max}$) were determined for each point during the experiment, and [Ca$^{2+}$]$_i$ was calculated as described by Torrecilla et al. (2000).

**Nitrogen and sulphur step-down experiments.** Nitrogen or sulphur step-down was initiated by washing cells, grown with either ammonium or nitrate until they reached the mid-exponential phase of growth (OD$_{750}$=0.5), three times with BG11$_i$ medium or BG11 deprived of a sulphur source, buffered with 5 mM HEPES (pH 7.8), and final resuspension in the same combined nitrogen- or sulphur-free medium. For sulphur deprivation experiments, MgSO$_4$, CaSO$_4$
and ZnSO₄ of medium BG11 were replaced by MgCl₂, CuCl₂ and ZnCl₂, respectively.

To facilitate cell loading of the intracellular Ca²⁺ chelator N,N’-[1, 2-ethanediylbis(oxy-2,1-phenylene)]bis[N-2-[(acetyloxy)methoxy]-2-oxoethyl]bis[(acetyloxy)methyl] ester (BAPTA-AM; FluoroProbes), cells were preincubated with the chelator at a final concentration of 300 μM for 2 h in darkness with shaking prior to nitrogen or sulphur step-down; immediately after nitrogen or sulphur step-down, the chelator at the same final concentration was again added. Experiments with BAPTA-AM were made with cells resuspended in Ca²⁺-free BG11, BG11-NH₄ or BG11₀ medium.

Analytical methods. For chlorophyll determinations, samples were extracted in methanol at 4 °C for 24 h in darkness. The chlorophyll content of the extract was estimated according to the spectrophotometric method of Marker (1972).

Phycobiliproteins were extracted after osmotic shock (Wyman & Fay, 1986); 1 ml aliquots were centrifuged and the pellets homogenized in 60–150 μl glycerol and incubated in the dark at 4 °C for 24 h. Water was then added to osmotically lyse the cells. The samples were centrifuged and the phycocyanin content was estimated according to Bennet & Bogorad (1973).

Determination of luciferase activity. To determine luciferase activity from the various luxAB reporter strains, 0.5 ml cell culture was supplemented with 0.5 ml 0.1% (v/v) emulsion of n-decanol in 0.1% (v/v) Triton X-100. Luminescence was measured using a digital luminometer (Bio-Orbit 150 luminometer). The luminometer was calibrated by setting the background counts to zero and the built-in standard photon source (a sealed ampoule of ¹⁴C with an activity of 0.26 μCi (9.62 kBq)) to 10 mV. Calculations made according to the method described by Hastings & Weber (1963) indicated that 1 U (1 mV) corresponded to a light emission of 6.7 x 10⁴ quanta s⁻¹ from the vial.

RESULTS

[Ca²⁺]i changes in S. elongatus wild-type and mutant strains MNtcA and MP2 in response to nitrogen starvation

Cell suspensions of S. elongatus wild-type, expressing apoaequorin, grown with a source of combined nitrogen, either nitrate or ammonium, were deprived of combined nitrogen as described in Methods, and any change in [Ca²⁺]i was monitored for at least 20 h. In the wild-type strain, a specific and reproducible Ca²⁺ transient could be detected in response to nitrogen starvation (Fig. 1); the transient began around 30 min to 1 h after nitrogen step-down, gradually increased in magnitude, reaching a maximum [Ca²⁺]i value of 0.376 ± 0.12 μM (n=5) after 6 h; a slow decrease followed afterwards until the [Ca²⁺]i approached the basal level (around 150–200 nM in this strain). The total length of the Ca²⁺ transient was nearly 15 h. As a control, cell suspensions were washed three times with BG11 medium containing the standard nitrate/ammonium concentration and finally resuspended in the combined nitrogen medium; these cell suspensions did not show any significant change in [Ca²⁺]i (Fig. 1), which indicated that the observed Ca²⁺ transient was not due to the manipulation of the sample or the experimental conditions of measurement; the above data strongly suggest that the observed Ca²⁺ transient is triggered by the withdrawal of the source of combined nitrogen from the external medium. Furthermore, the intracellular Ca²⁺ chelator BAPTA-AM, at a final concentration of 300 μM, was added to cells 2 h prior to reconstitution of apoaequorin with coelenterazine and again immediately after nitrogen deprivation. Experiments were repeated at least five times; the traces shown have been chosen to best represent the average results; the maximum coefficient of variation of the different experiments was 8%. Solid line, shift of cells from BG11-NH₄ to BG11₀; short dashes, shift of wild-type cells from BG11-NH₄ to BG11₀; dotted line, shift of MNtcA cells from BG11-NH₄ to BG11₀; dots and dashes, shift of MP2 cells from BG11-NH₄ to BG11₀; long dashes, treatment of wild-type cells with BAPTA-AM.

Fig. 1. Effect of combined-nitrogen deprivation on the [Ca²⁺]i of S. elongatus wild-type, MNtcA and MP2 strains expressing apoaequorin. Cells were grown in BG11-NH₄ medium until they reached the mid-exponential phase of growth (OD₅₅₀=0.5). After loading the cells with coelenterazine, they were washed three times with BG11₀ medium and luminescence was continuously recorded in a luminometer over 24 h. As a control, cell suspensions were washed three times with BG11-NH₄ medium and resuspended in the same medium. The intracellular Ca²⁺ chelator BAPTA-AM, at a final concentration of 300 μM, was added to cells 2 h prior to reconstitution of apoaequorin with coelenterazine and again immediately after nitrogen deprivation. Experiments were repeated at least five times; the traces shown have been chosen to best represent the average results; the maximum coefficient of variation of the different experiments was 8%. Solid line, shift of cells from BG11-NH₄ to BG11₀; short dashes, shift of wild-type cells from BG11-NH₄ to BG11₀; dotted line, shift of MNtcA cells from BG11-NH₄ to BG11₀; dots and dashes, shift of MP2 cells from BG11-NH₄ to BG11₀; long dashes, treatment of wild-type cells with BAPTA-AM.
It has been shown that 2-OG is an intracellular signal for nitrogen status in cyanobacteria. Artificially increased levels of 2-OG mimic, to some extent, nitrogen starvation in both *S. elongatus* and the heterocystous *Anabaena* sp. PCC 7120 (Li *et al.*, 2003; Vázquez-Bermúdez *et al.*, 2003). The non-metabolizable 2-OG analogues DFPA and 2-MPA triggered heterocyst differentiation in the presence of ammonium in the *Anabaena* strain (Laurent *et al.*, 2005; Chen *et al.*, 2006). In *Anabaena*, nitrogen starvation leads not only to increased 2-OG levels but also to an increased concentration of free Ca$^{2+}$ that is necessary for heterocyst differentiation (Torrecilla *et al.*, 2004; Zhao *et al.*, 2005). The fact that in *Anabaena* free Ca$^{2+}$ ions and 2-OG are early signals following nitrogen starvation raises many questions about their role in the process of acclimation to this stress. As described, we have also found that a defined Ca$^{2+}$ transient is triggered following nitrogen step-down in *S. elongatus*; in order to shed light on a possible relationship between the two signals, 2-OG and Ca$^{2+}$, we investigated whether exogenous addition of 2-OG or either of its two analogues, 2-MPA or DFPA, triggered Ca$^{2+}$ transients in the presence of a source of combined nitrogen (nitrate/ammonium) in *S. elongatus*. In the wild-type strain, 25 mM 2-OG elicited a wide Ca$^{2+}$ transient that started around 1 h after the addition of 2-OG; the recorded Ca$^{2+}$ transient had a shoulder at 5 h 30 min reaching a [Ca$^{2+}$]$_i$ value of 0.36 ± 0.01 μM ($n = 5$), and peaked at around 15 h reaching a [Ca$^{2+}$]$_i$ maximum of 0.47 ± 0.04 μM ($n = 5$); the transient lasted more than 20 h (Fig. 2a). Strain MP2 showed a slight increase in its [Ca$^{2+}$]$_i$ basal levels; strain MNtcA also showed some fluctuations of its [Ca$^{2+}$]$_i$ basal levels but no significant Ca$^{2+}$ transient was recorded in any of the two mutant strains in any of the experiments that were carried out (Fig. 2a). As expected, treatment with BAPTA-AM suppressed the observed Ca$^{2+}$ transient in the wild-type strain. The 2-
OG analogues DFPA and MPA at a final concentration of 2 mM triggered bell-shaped Ca\(^{2+}\) transients with some differences in their temporal pattern (Fig. 2b, c, respectively). The Ca\(^{2+}\) transient induced by DFPA started shortly (30–45 min) after the addition of the analogue, peaked at around 7 h with a maximum \([\text{Ca}^{2+}]_i\) value of 0.48 \pm 0.01 \(\mu\text{M} (n=5)\), and decreased until it reached the basal level of 200 nM. The transient lasted around 13 h (Fig. 2b). The Ca\(^{2+}\) transient induced by 2-MPA started later, around 1.5–2 h after the addition of the analogue, peaked at around 12 h with a maximum \([\text{Ca}^{2+}]_i\) value of 0.58 \pm 0.05 \(\mu\text{M} (n=5)\), and decreased to basal levels; the transient lasted almost 20 h (Fig. 2c). As with 2-OG, no significant Ca\(^{2+}\) transient was recorded in mutant strains MNtcA and MP2 after the addition of the analogues, although slight fluctuations of \([\text{Ca}^{2+}]_i\) basal levels were also observed (Fig. 2b, c). Treatment with BAPTA-AM suppressed the observed Ca\(^{2+}\) transients triggered by the analogues in the wild-type strain (Fig. 2). Thus, artificial conditions mimicking nitrogen starvation (addition of 2-OG or its analogues 2-MPA and DFPA to \(S.\) elongatus cells growing with a source of combined nitrogen) triggered Ca\(^{2+}\) transients similar to that obtained following nitrogen step-down, suggesting that an increase in intracellular 2-OG is needed to induce the Ca\(^{2+}\) transients. As the MNtcA and MP2 strains do not show the Ca\(^{2+}\) transients, both proteins might also be needed to trigger the observed Ca\(^{2+}\) transients.

**Effect of suppression of the Ca\(^{2+}\) transient on the expression of NtcA-regulated genes involved in acclimation to nitrogen starvation in \(S.\) elongatus**

Does the observed Ca\(^{2+}\) transient have a role in acclimation to nitrogen starvation in \(S.\) elongatus? To try to answer this question, we first checked the effect of suppression of the Ca\(^{2+}\) transient on the expression of genes involved in acclimation to nitrogen starvation.

We first studied the \(glnB\) gene, whose expression is controlled by NtcA under nitrogen-deprivation conditions (Aldehni \textit{et al.}, 2003), and studied the effect of the intracellular Ca\(^{2+}\) chelator BAPTA-AM, which completely suppressed the Ca\(^{2+}\) transient, on its expression under conditions of actual or mimicked nitrogen starvation (induced by either nitrogen step-down or the addition of 2-OG or its analogues in the presence of combined

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**Fig. 3.** Time-course of \(glnB::luxAB\) induction following nitrogen step-down (a), the addition of 25 mM 2-OG (b), or the addition of 2 mM 2-MPA (c) in reporter strains FAM1 and FAM2 of \(S.\) elongatus wild-type. After shifting the cells from BG11 to BG11\(_0\) (a), from BG11 to BG11 plus 25 mM 2-OG (b), or from BG11 to BG11 plus 2 mM 2-MPA (c), bioluminescence from the reporter strains was monitored over 24 h; the intracellular Ca\(^{2+}\) was added 2 h prior to nitrogen step-down (a), the addition of 2-OG (b), or 2-MPA (c), and immediately afterwards. Three independent experiments with duplicate samples yielded similar results; for each case, results from a representative time-course experiment are shown. (●) FAM1 reporter strain, (▼) FAM2 reporter strain, (○) FAM1 reporter strain treated with BAPTA-AM, (▼) FAM2 reporter strain treated with BAPTA-AM.
nitrogen) (Fig. 3). Two reporter strains were used to study glnB expression, FAM1 and FAM2 (Aldehni et al., 2003); FAM1 contains the entire upstream region of glnB with two promoters, constitutive promoter P1 and the NtcA-regulated promoter P2, fused to luxAB; FAM 2 contains only the NtcA-dependent promoter P2. As shown in Fig. 3(a), nitrogen depletion initiated from nitrate-supplemented cultures triggered a rapid increase in luminescence in both strains: peak values 8 h after nitrogen step-down corresponded to an approximately 10- or 150-fold increase in induction for FAM1 [luminescence value of 3.23 x 10^8 ± 0.5 quanta (mg Chl)^(-1) min^(-1) at time 0, where Chl stands for chlorophyll] or FAM2 [luminescence value of 0.32 x 10^8 ± 0.06 quanta (mg Chl)^(-1) min^(-1) at time 0], respectively, compared with that for nitrate-grown cells; after 24 h, peak values corresponded to an 18- or 300-fold increase in luminescence for FAM1 and FAM2, respectively, indicating, as pointed out by Aldehni et al. (2003), that transcription from the NtcA-dependent promoter P2 is sufficient to yield the high expression of glnB under conditions of combined-nitrogen starvation. As also shown in the figure, treatment with BAPTA-AM significantly inhibited the induction of luminescence, although after 24–30 h, a 10-fold increase in luminescence in both strains treated with the chelator was observed (Fig. 3a). Similar results were obtained when cells were shifted from ammonium to combined nitrogen-free medium (not shown). The addition of 25 mM 2-OG to cell suspensions grown with nitrate induced the expression of the glnB gene in both strains, although luminescence induction was not as strong as that observed after nitrogen step-down; for strain FAM1, with a luminescence value of 25 x 10^8 ± 1.17 quanta (mg Chl)^(-1) min^(-1) at time 0, a twofold increase in induction was seen 8 h after addition of 2-OG, while at the same time point, a 10-fold increase in induction was observed for FAM2 [luminescence value 3.3 x 10^8 ± 0.30 quanta (mg Chl)^(-1) min^(-1) at time 0] (Fig. 3b). Treatment with BAPTA-AM triggered slightly different induction patterns in the two strains: while it completely inhibited induction of gene expression in FAM2, it did not inhibit induction of FAM1 at the earliest times assayed, although 5 h after addition of 2-OG there was a significant decrease of luminescence in FAM1 (Fig. 3b). The addition of 2 mM of the 2-OG analogue 2-MPA triggered a rapid induction of the glnB gene: after 7 h, peak values corresponded to a 320-fold increase in induction for FAM1 [luminescence value 0.17 x 10^8 ± 0.06 quanta (mg Chl)^(-1) min^(-1) at time 0] and a 380-fold increase in induction for FAM2 [luminescence value 0.08 x 10^8 ± 0.01 quanta (mg Chl)^(-1) min^(-1) at time 0]. BAPTA-AM completely inhibited the observed induction of glnB in both strains (Fig. 3c).

We have shown that the glnB gene is induced not only after shifting nitrate- or ammonium-grown cells to combined nitrogen-free medium (nitrogen step-down experiments) but also when nitrogen-starvation conditions are mimicked by the addition of 2-OG or its analogue 2-MPA to cells growing with a source of combined nitrogen. The expression of glnB under nitrogen-starvation conditions seemed to be regulated by [Ca^{2+}]_o as the Ca^{2+} chelator BAPTA-AM, which suppressed the observed Ca^{2+} transients (Figs 1 and 2), significantly inhibited the induction of expression of the gene. We wished to check whether other genes involved in acclimation to nitrogen starvation whose promoters were also regulated by NtcA could also be regulated by Ca^{2+}. We chose glnN and nblA for this purpose. glnN encodes a nitrogen starvation-specific glutamine synthetase in S. elongatus. The promoter of S. elongatus glnN displays an imperfect NtcA-binding site (Aldehni & Forchhammer, 2006). We used two luxAB reporter strains, strain FAM431, which contains the full-length glnN promoter fused to luxAB, and FAM84, which only retains the NtcA-binding site in its promoter fused to luxAB (Aldehni & Forchhammer, 2006). As shown in Fig. 4, nitrogen starvation induced glnN expression in both strains, although the luminescence induction was moderate in both strains as compared with the level of induction of glnB (Fig. 3). The FAM84 reporter strain displayed more activity [a 30-fold increase in induction after 24 h of starvation from an initial luminescence value of 0.8 x 10^8 ± 0.08 quanta (mg Chl)^(-1) min^(-1)] than FAM431 [a 19-fold increase in induction after 24 h of starvation from an initial luminescence value of 0.7 x 10^8 ± 0.09 quanta (mg Chl)^(-1) min^(-1)], as already observed by Aldehni & Forchhammer (2006), who have suggested that
this effect is due to a negatively acting cis-element in the upstream region of the glnN promoter. Intracellular Ca$^{2+}$ depletion by BAPTA-AM significantly inhibited glnN expression, particularly in the FAM84 reporter strain, which has a shorter promoter containing the imperfect NtcA-binding site (Fig. 4), again confirming control by Ca$^{2+}$ of an NtcA-regulated promoter. We could not detect any clear induction of the glnN gene 48 h after the addition of 2-OG or its analogue 2-MPA to cells growing with either nitrate or ammonium. Expression of the nblA gene is controlled by two regulators, NtcA and NblR; nblA dependency on NtcA is nitrogen-specific, while the response-regulator NblR acts as the general regulator of nblA transcription in response to a variety of stresses; both regulators seem to stimulate transcription from the major nblA promoter PnblA-2 (Luque et al., 2001). As shown in Fig. 5, nitrogen starvation induced nblA expression, as expected [40-fold increase in expression after 8 h of starvation from an initial luminescence value of $0.37 \times 10^6 \pm 0.01$ quanta (mg Chl)$^{-1}$ min$^{-1}$]; [Ca$^{2+}$] depletion by BAPTA-AM partially inhibited nblA expression at shorter times (i.e. after 8 h, a 40-fold increase in induction in the control vs a 25-fold induction in cultures treated with BAPTA-AM); in the longer term (24 h) there was no difference in expression levels between control and those treated with BAPTA-AM (Fig. 5). We could not detect any clear induction of the nblA gene 48 h after the addition of 2-OG or its analogue 2-MPA to cells growing with either nitrate or ammonium. If Ca$^{2+}$ is regulating NtcA-dependent promoters, the observed partial effect of Ca$^{2+}$ depletion on expression of nblA in the short term might indicate that NtcA and Ca$^{2+}$ are needed to rapidly upregulate nblA expression under nitrogen-starvation conditions. The residual response of nblA expression indicates an NtcA-independent regulatory mechanism, probably involving the NblR factor, which is also required for nblA induction following sulphur deprivation (Schwarz and Grossman, 1998). The Ca$^{2+}$ chelator itself did not have any negative effect on growth in the presence of ammonium (results not shown), suggesting that it did not cause an intracellular Ca$^{2+}$ depletion that could compromise the viability of the cells and have a negative effect on gene expression. To reinforce this, we checked the effect of BAPTA-AM on nblA activation under sulphur deprivation; nblA is activated not only by nitrogen deprivation but also by sulphur and phosphate deprivation (Collier & Grossman, 1994; Schwarz & Forchhammer, 2005); nblA dependency on NtcA has been shown to be nitrogen-specific (Luque et al., 2001), while NblR acts as the general regulator of nblA transcription in response to most stresses. We did not find any significant Ca$^{2+}$ transient following sulphur step-down (not shown); as also shown in Fig. 5, nblA expression was induced within a few hours of sulphur deprivation [fivefold increase in luminescence 6 h after sulphur stepdown from an initial luminescence value of $0.24 \times 10^6 \pm 0.05$ quanta (mg Chl)$^{-1}$ min$^{-1}$]; as shown, the chelator did not have any effect on nblA expression under sulphur-starvation conditions.

**Effect of suppression of the Ca$^{2+}$ transient on bleaching caused by nitrogen starvation**

*nblA* is a key gene controlling phycobilisome degradation in response to environmental stress conditions such as nutrient deprivation. As Ca$^{2+}$ seems to some extent to control nblA expression, we checked the effect of BAPTA-AM treatment on the bleaching process in the wild-type, MNTca and MP2 strains. As shown in Fig. 6, after 3 days of nitrogen starvation, BAPTA-AM partially protected the wild-type strain from bleaching (Fig. 6a); the phycocyanin: chlorophyll a ratio decreased fourfold in the control wild-type versus 1.7-fold in the wild-type treated with BAPTA-AM (Fig. 6b); BAPTA-AM-treated cells retained the red phycobiliprotein autofluorescence that the non-treated cells had almost completely lost by this time of nitrogen starvation (not shown). As mutants MNTca and MP2 did not show the Ca$^{2+}$ transient (Figs 1 and 2), treatment with BAPTA-AM did not have any effect on their bleaching pattern. MNTca, as already described (Sau er et al., 1999; Espinosa et al., 2007), showed a delay in phycocyanin degradation compared with the wild-type; after 3 days, the phycocyanin: chlorophyll a ratio decreased twofold after nitrogen step-down both in cultures treated and in those not treated with BAPTA-AM (Fig. 6a, b); the

![Fig. 5. Time-course of nblA::luxAB induction following nitrogen (●) or sulphur (▼) step-down in reporter strain *S. elongatus* WT-C103. After shifting the cells from BG11 medium to BG11$_0$, bioluminescence from the reporter strains was monitored over a time period of 24 h. The intracellular Ca$^{2+}$ chelator BAPTA-AM, at a final concentration of 300 µM, was added 2 h prior to nitrogen step-down and immediately afterwards. Three independent experiments with duplicate samples yielded similar results; for each case, results from a representative time-course experiment are shown. (●) *S. elongatus* WT-C103 reporter strain, (○) *S. elongatus* WT-C103 reporter strain treated with BAPTA-AM, (▼) *S. elongatus* WT-C103 reporter strain treated with BAPTA-AM.](http://mic.sgmjournals.org)
**Fig. 6.** Effect of BAPTA-AM on bleaching of *S. elongatus* wild-type (WT), MNtcA and MP2 mutant strains. (a) Cultures of WT, MNtcA and MP2 grown in BG11-NH₄ (+N), incubated in BG11₀ (−N) or BG11₀ plus 300 µM BAPTA-AM (−N + BAPTA) for 72 h. (b) Phycocyanin : chlorophyll a ratios of cultures of WT, MNtcA and MP2 incubated as indicated in (a). PC, phycocyanin; Chla, chlorophyll a. At least five independent experiments were performed; results from a representative experiment are shown.

MP2 mutant showed the same pattern of bleaching as the wild-type, as already described (Sauer et al., 1999), but no protection by BAPTA-AM was observed. BAPTA-AM partially protected the wild-type from bleaching for at least 9 days (results not shown).

**DISCUSSION**

In this study, we report an early Ca²⁺ transient that is triggered under conditions of nitrogen starvation in *S. elongatus* (Fig. 1). The Ca²⁺ transient is very similar to the one that we recorded in the heterocystous *Anabaena* sp. PCC 7120 in shape, time of appearance (45 min to 1 h) and magnitude (0.37 µM vs 0.39 µM in *Anabaena*), although it has a longer duration (15 h vs 4 h in *Anabaena*) (Torrecilla et al., 2004). The Ca²⁺ transient occurred not only after removing nitrate or ammonium from the growth medium (nitrogen step-down) but also after the addition of 2-OG or its analogues DFPA or 2-MPA to cells growing with nitrate or ammonium (Fig. 2). The latter conditions mimic, to some extent, nitrogen starvation in *Anabaena* sp. PCC 7120 (Li et al., 2003; Laurent et al., 2005; Chen et al., 2006). Interestingly, 2-OG and both its analogues also trigger Ca²⁺ transients in *Anabaena* similar to the ones observed in *S. elongatus* (F. Leganes and F. Fernandez-Piñas, unpublished results).

Therefore, nitrogen starvation leads not only to increased 2-OG levels but also to increased concentrations of free Ca²⁺ ions both in *Anabaena* and *S. elongatus* intracellular 2-OG levels increase immediately in response to nitrogen limitation in both strains (Laurent et al., 2005; Schwarz & Forchhammer, 2005), while Ca²⁺ ions start to accumulate 45 min–1 h after starvation (this work; Torrecilla et al., 2004); the fact that artificially increased 2-OG or analogue levels also trigger the Ca²⁺ transients in both strains suggests that an increase in intracellular 2-OG precedes the Ca²⁺ transient.

Also, the fact that the MNtcA and MP2 mutant strains of *Synechococcus* did not show the calcium signal implies that both proteins might be needed in conjunction with high levels of intracellular 2-OG to trigger the Ca²⁺ transient; Shi et al. (2006) have already found that the regulation of [Ca²⁺]ᵢ in *Anabaena* depends on NtcA.

Our results also showed that glb expression can be induced by the addition of 2-OG or its analogue 2-MPA in the presence of combined nitrogen, while that of nbla and glnN cannot. glb has a canonical NtcA-binding site (Aldehni et al., 2003), glnN displays an imperfect NtcA-binding motif and a negatively acting cis-element in the upstream region of its promoter (Aldehni & Forchhammer, 2006), and nbla has a complex regulatory region containing multiple promoters: under nitrogen deprivation, its major promoter (pnbL-A-2) requires both NtcA and NblR for maximal activation, although the structure of this promoter does not match that of a typical NtcA-activated promoter (Luque et al., 2001). The lack of a canonical NtcA-activated promoter and the presence of other factors or regulators may hamper induction of glnN and nbla expression by 2-OG under nitrogen-replete conditions.

*Anabaena* responds to the removal of a combined nitrogen source by differentiating heterocysts, specialized cells that fix molecular nitrogen. Our group and that of Zhao (Torrecilla et al., 2004; Zhao et al., 2005) have demonstrated that the Ca²⁺ transient in *Anabaena* is necessary for heterocyst development; non-diazotrophic strains such as *S. elongatus* respond to nitrogen deprivation by an acclimation process that begins with pigment degradation (phycobiliprotein proteolysis followed by chlorophyll a loss) that leads to chlorosis before differentiating into non-pigmented cells able to endure long periods of starvation (Görül et al., 1998). A relevant question is whether the observed Ca²⁺ transient in response to nitrogen starvation in *S. elongatus* has a role in this process of acclimation; we have found that suppression of the Ca²⁺ transient by the intracellular Ca²⁺ chelator BAPTA-AM inhibits induction of glb and glnN expression under nitrogen-starvation conditions (Figs 3 and 4); both genes are involved in acclimation to nitrogen limitation in *S. elongatus* and both are transcriptionally activated by NtcA (Aldehni et al., 2005).
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2003; Aldehni & Forchhammer, 2006; Schwarz & Forchhammer, 2005). Furthermore, BAPTA-AM partially inhibited induction of expression of nblA (Fig. 5), a crucial gene involved in the process of phycobiliprotein degradation that is controlled by two transcriptional regulators, NtcA (specifically under conditions of nitrogen starvation) and NblR. The inhibitory effect of BAPTA-AM occurred only in the short term after starvation; in the longer term (24 h), BAPTA-AM did not have any effect. This partial inhibitory effect might indicate that Ca$^{2+}$ is acting through NtcA but not NblR in the induction of expression of nblA under nitrogen limitation; if that is true, it also might imply that NtcA is an early activator of transcription of nblA, while the main activator at longer times of starvation is NblR. This partial inhibitory effect of the chelator on nblA expression correlated with the observed partial inhibition of phycobiliprotein degradation (Fig. 6). In Anabaena, we have also found that suppression of the Ca$^{2+}$ transient by treatment with the chelator arrests heterocyst differentiation at an early stage, as evidenced by the absence of patterned loss of phycobiliprotein fluorescence, suggesting a role of the Ca$^{2+}$ transient in proteolysis of the phycobiliproteins (Torrecilla et al., 2004). The results presented here strongly suggest that Ca$^{2+}$ ions are involved in the induction of NtcA-regulated genes by nitrogen starvation; Ca$^{2+}$ might act at different levels: it may physically interact with NtcA and facilitate DNA binding and/or stabilize PipX–NtcA complexes that have been proposed to activate NtcA-dependent promoters (Espinosa et al., 2006, 2007). In vivo, the PipX–NtcA complexes are thought to be formed under high intracellular levels of 2-OG and, as we have reported, increased intracellular 2-OG triggers the Ca$^{2+}$ transient. A stabilizing role of Ca$^{2+}$ has already been proposed for the NAGK–PII complex in S. elongatus (Maheswaran et al., 2004), although in this case, Ca$^{2+}$ and 2-OG seem to act antagonistically. Alternatively, the Ca$^{2+}$ transient, probably acting synergistically with increased intracellular 2-OG levels, could sense C/N imbalance and transduce it, by a yet unknown signalling mechanism, to some other factor(s) (perhaps PII and/or PipX) that may facilitate NtcA transcriptional activation.

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