

An AbrB-like protein might be involved in the regulation of cylindrospermopsin production by *Aphanizomenon ovalisporum*

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Summary

Certain filamentous cyanobacteria, including *Aphanizomenon ovalisporum*, are potentially toxic owing to the formation of the hepatotoxin cylindrospermopsin. We previously identified a gene cluster in *A. ovalisporum* likely to be involved in cylindrospermopsin biosynthesis, including amidinotransferase (*aoaA*) and polyketide-synthase (*aoaC*), transcribed on the reverse strands. Analysis of the genomic region between *aoaA* and *aoaC* identified two transcription start points for each of these genes, differentially expressed under nitrogen and light stress conditions. The transcript abundances of these genes and the cylindrospermopsin level were both affected by nitrogen availability and light intensity. Gel shift assays and DNA affinity columns isolated a protein that specifically binds to a 150 bp DNA fragment from the region between *aoaA* and *aoaC*, and MS/MS analyses identified similarity to AbrB in other cyanobacteria and in *Bacillus* sp. Comparison of the native AbrB isolated from *A. ovalisporum* with that obtained after cloning and overexpression of *abrB* in *Escherichia coli* identified specific post-translational modifications in the native cyanobacterial protein. These

modifications, which are missing in the protein expressed in *E. coli*, include N-acetylation and methylation of specific residues. We discuss the possible role of these modifications in the regulation of cylindrospermopsin production in *Aphanizomenon*.

Introduction

Certain cyanobacteria are known to produce a wide variety of secondary metabolites including non-ribosomally made peptides, polyketides and alkaloids. Some of these metabolites are toxic and their presence may constitute a serious threat to water quality in many fresh water bodies worldwide (Griffiths and Saker, 2003; van Apeldoorn *et al.*, 2007). Cylindrospermopsin (CYN) is an alkaloid toxin produced by several cyanobacteria species including *Aphanizomenon ovalisporum* (hereafter *Aphanizomenon*), *Cylindrospermopsis raciborskii*, *Anabaena Bergii*, *Umezakia nathans* and *Aphanizomenon flos-aquae* (Kaebernick and Neilan, 2001; Schembri *et al.*, 2001; Preussel *et al.*, 2006; van Apeldoorn *et al.*, 2007). Cylindrospermopsin was shown to inhibit protein and glutathione synthesis leading to cell death (Runnegar and Lu, 1994; Runnegar *et al.*, 1994; 1995; 2002; Froscio *et al.*, 2001; Metcalf *et al.*, 2004). Effects of genotoxicity and on cholesterol levels have also been reported (Humpage *et al.*, 2000; 2005; Shen *et al.*, 2002; Reisner *et al.*, 2004). Activity of cytochrome P450 enzyme system has been shown to be important for the development of CYN toxicity (Runnegar *et al.*, 1995; Norris *et al.*, 2002).

In view of its potential effect on the quality of drinking water, it is important to clarify the mechanism of CYN biosynthesis and the response of the genes involved to environmental conditions. Feeding experiments indicated that five molecules of acetate are catalytically condensed presumably by a polyketide synthase to form the carbon skeleton of CYN. Two other carbons and one of the nitrogen atoms of the guanidine moiety are donated by glycine. Guanidinoacetic acid was proposed as a source for two nitrogen atoms of the guanidino moiety used as a starter unit that initiates the biosynthetic process. The guanidinoacetic acid is probably formed by amidination of glycine by amidinotransferase, but the exact origin of the

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other N atoms in the guanidino moiety has not been resolved (Burgoyne *et al.*, 2000).

Our earlier study identified a genomic region where genes essential for CYN biosynthesis, *aoaA* encoding amidinotransferase, *aoaB* encoding non-ribosomal peptide synthetase fused to polyketide synthase (NRPS/PKS) and *aoaC* encoding a type I polyketide synthase, are clustered (Shalev-Alon *et al.*, 2002). In *Aphanizomenon*, the *aoaA* and the *aoaC* are encoded on the reverse strands (Shalev-Alon *et al.*, 2002). Southern analyses suggested a single copy of these genes in the *Aphanizomenon* genome (Shalev-Alon *et al.*, 2002). The *aoaA* is present in CYN-producing strains but apparently missing in non-toxic strains (Kellmann *et al.*, 2006). Unfortunately, despite intensive efforts we were unable to apply gene modification techniques and inactivate any of the *aoa* genes in *Aphanizomenon*. Thus, while it is most likely that the *aoa* gene cluster is involved in the formation of CYN, direct evidence is still missing.

In addition to its importance to normal growth, nutrient availability plays a role in CYN production (Bacsi *et al.*, 2006). Interestingly, replenishing S-starved cells stimulated CYN production prior to regaining of growth (Bacsi *et al.*, 2006). The high N content in CYN could potentially withdraw N resources from the cells and thus expression of *aoa* genes and CYN production might be affected by N-limitation. Studies on the response of CYN production in filamentous cyanobacteria to N availability were mostly performed under prolonged growth in N-depleted media where N₂ fixation could replace the missing inorganic N. Chemical analysis of CYN synthesis suggested that redox conditions in the cells may affect its formation. Thus, in addition to its effect on the growth rate of *Aphanizomenon*, the light intensity during growth could affect the biosynthesis of CYN via its effect on the cell's redox. Indeed, recent studies (Dyble *et al.*, 2006) demonstrated enhancement of CYN production by elevated light intensity under conditions where growth was no longer stimulated by raising the illumination. In their natural habitat the *Aphanizomenon* filaments are distributed in the photic zone (Hadas *et al.*, 1999) probably with the help of their gas vesicles.

In this study we examined the regulation of the transcript abundance of *aoaA* and *aoaC* and the CYN level shortly after exposure to N-limitation or an upshift in light intensity. We have identified a protein that binds to the genomic region between *aoaA* and the *aoaC* and is therefore most likely involved in the regulation of their transcription. Homologues of this protein in other cyanobacteria were annotated as AbrB-like proteins. AbrB (antibiotic resistance protein B) belongs to a distinct group of transcription factors (Huffman and Brennan, 2002); in *Bacillus* sp. it has been implicated in the regulation of many genes during acclimation to nutrient depletion and

in transition to stationary phase (Strauch *et al.*, 1990; Marahiel *et al.*, 1993; Fawcett *et al.*, 2000), but the specificity of binding to these genes is poorly understood. We propose that post-translational modifications of the native protein in *Aphanizomenon* might be involved in the regulation of CYN production.

Results

Expression of aoaA and aoaC and the cylindrospermopsin level

As indicated (Introduction), the structure of CYN and the procedure required for its chemical synthesis (Looper *et al.*, 2006) suggested that N-availability and light intensity may affect the rate of its biosynthesis. Therefore, we examined the effect of nitrogen starvation and of a shift to a higher light intensity on the transcript abundance of *aoaA* and *aoaC* and on the CYN level (Dyble *et al.*, 2006). Semi-continuous cultures maintained in the exponential phase of growth by frequent dilution with BG11 medium were transferred to nitrogen-depleted media for up to 72 h, followed by RNA extraction and analysis of the CYN content. As expected (Schwarz and Forchhammer, 2005), the cells responded to nitrogen starvation by decomposition of the phycobilisomes, resulting in a yellowish appearance of the cultures (Fig. 1A). Longer exposure to N-depletion led to heterocyst formation; thereafter the cells regained the blue green colour.

In Fig. 1B we present the results of one experiment, out of five independent repetitions, where the abundances of transcripts that originated from *aoaA*, *aoaC* and 16S rRNA in control and N-limited cells were determined by quantitative polymerase chain reaction (qPCR). In the control cultures, the relative abundance of 16S rRNA per total RNA was essentially constant during the experiment (72 h which is also the generation time under the control conditions) but the abundance of transcripts originated from *aoaA* and *aoaC*, normalized to the 16S rRNA level, increased threefold to fourfold (Fig. 1B). During that time the level of 16S rRNA per total RNA declined by 10–20%, in cells deprived of N but nevertheless the relative abundance of the *aoaC* transcript (per 16S rRNA) rose similarly to the control. In contrast, in all the experiments, the relative level of *aoaA* transcript increased far less (about half) than the control (Fig. 1B). These data suggested differential regulation of the transcript abundance of *aoa* genes by the N-limiting conditions; it is not known whether the formation or breakdown of the transcripts were affected.

The amount of toxin in the control cultures rose significantly during the experiment but not in the N-deprived cells. Apparently, the stress conditions resulted in a stron-

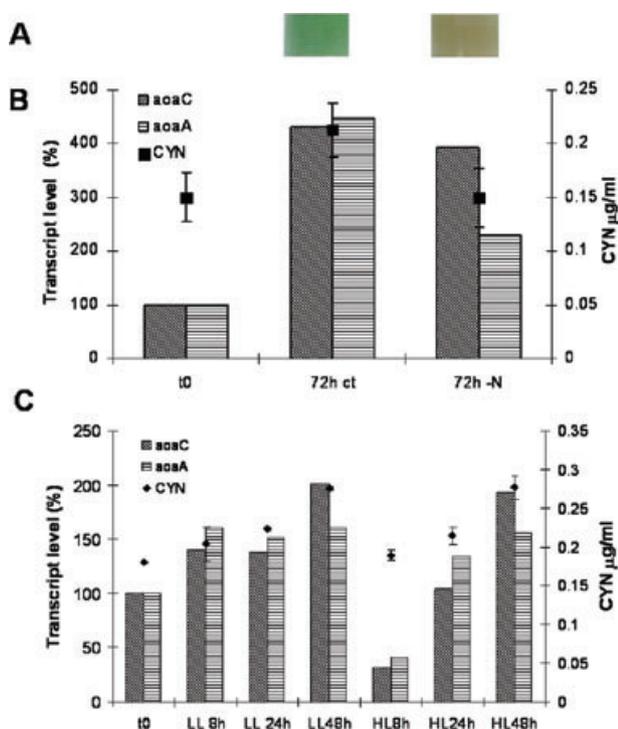


Fig. 1. Expression of *aoaA* and *aoaC* and cylindrospermopsin (CYN) levels under different environmental conditions. A. The appearance of the control and of cells transferred to N-depleted media for 72 h is shown. Decomposition of the phycobilisomes in the N-starved cells resulted in a yellowish appearance. B and C. The CYN level ($\mu\text{g ml}^{-1}$) and the transcript abundance of *aoaA* and *aoaC* (normalized to the level of the 16S rRNA) in the beginning of the experiment (t0 = 100%) and (B) after 72 h in standard (72 h-ct) or N-depleted media (72 h-N) or (C) in cultures transferred from growth at $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low light, LL) to $85 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (high light, HL) for 8, 24 and 48 h. The qPCR results presented in (B) and (C) are from one experiment each out of five independent repetitions performed on different cultures. In each of these experiments, the time-courses showed exactly the same pattern presented here. The variability in each experiment (three RNA samples isolated at each time point) did not exceed 10% of the average shown in the figure. For unknown reasons the variability between experiments was larger, possibly related to the exact conditions of the particular culture at the time of harvest, but the range of the data obtained did not exceed 20% of the average presented here. The CYN measurements shown here are the averages and SD ($n = 3$) from the same representative culture for which the qPCRs are shown.

ger impact on the level of CYN than on the transcript abundances, suggesting that CYN formation is regulated by additional factors and not solely by the transcript abundance. In contrast, studies on *C. raciborskii* (Hawkins *et al.*, 2001; Saker and Neilan, 2001) showed higher CYN level in N-deprived cells. The reason for this discrepancy is not known but one possible explanation is the presence of heterocysts in the long-term experiments performed with *C. raciborskii*. We deliberately performed short-term experiments where heterocyst formation and

complete acclimation to the N-limited conditions were avoided.

To study changes in gene expression in response to a rise in light intensity, early exponential cultures that were grown under low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were exposed to $85 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and RNA was isolated after 0, 8, 24 and 48 h. This treatment hardly affected the level of 16S rRNA per total RNA (not shown). In the low light cultures, the transcript abundance of both *aoaA* and *aoaC* (per 16S rRNA) increased during the course of the experiment (Fig. 1C). In contrast, raising the light intensity resulted in a fast decline in the levels of both *aoaA* and *aoaC* transcripts which reached a minimum within about 8 h. Bleaching of the pigments was also visualized after 8 h of the high-light treatment. Longer exposures to high light resulted in a recovery of the transcript levels of *aoaA* and *aoaC* to those depicted by the control cultures (Fig. 1C). For some reason, extraction of RNA from cultures that were maintained under the higher light intensity for longer duration was not successful; the RNA yields obtained was very low. In many of these experiments (six independent repetitions) the isolated RNA was degraded and thus we do not present data for time points longer than 48 h. The level of the toxin did not rise during the first 8 h under high light but recovered thereafter. As the toxin is relatively stable, these data may imply that toxin formation was inhibited during this period despite the fact that the transcripts originated from *aoaA* and *aoaC* were present.

Transcription start points

Differential effects of the ambient conditions on the transcript abundance of *aoaA* and *aoaC* were observed (Fig. 1). In addition, as these genes are transcribed on the reverse strands, the genomic region between them is likely to be susceptible to differential regulation. The transcription start points (TSPs) of *aoaA* and *aoaC* were determined (by 5' RACE) using RNA extracted from cells grown under the control conditions and those exposed to higher light or N-deprivation for 8 h or 72 h, respectively, as in Fig. 1. In RNA extracted from control cells, we have identified two TSPs (positions -51 and -30) upstream of the translation start point of *aoaA* and two TSPs at positions -65 and -86 upstream of the ATG of *aoaC* (Fig. 2). On the other hand, in RNA isolated from N-limited or higher light-treated cells, we noted only one TSP for each gene, at -30 and -86 upstream to the 5' ATG of *aoaA* and *aoaC* respectively (Fig. 2). Addition of 0.2 mM NH_4Cl to N-depleted cells for 24 h led to a transcription pattern similar to that observed in the control (not shown). The 5' RACE analyses suggested that in both genes one of the promoters is constitutive whereas the other is functional only under the standard growth conditions used here.

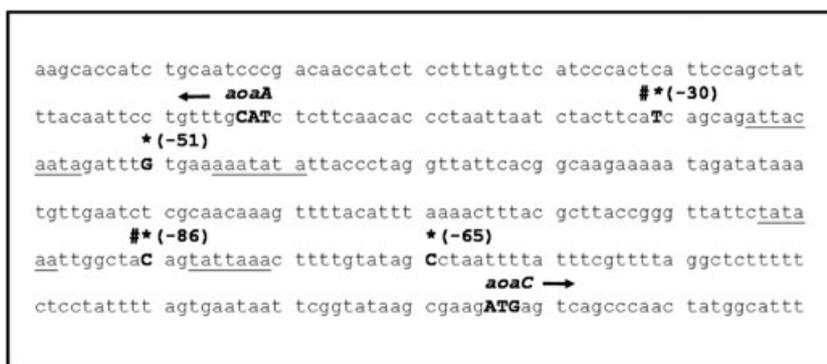


Fig. 2. Transcription start points of *aoaA* and *aoaC*. The sequence of the region between *aoaA* and *aoaC*, transcribed on the reverse strands. The translation start codons of these genes are indicated by a capital font. Transcription start points (TSPs), determined by 5' RACE, are marked by an asterisk and the nucleotide in bold. The 5' RACE analyses were performed on RNA extracted from cells grown under normal growth conditions and those exposed to higher light or N-deprivation stress for 8 or 72 h respectively. In the control cells, two TSPs (positions -51 and -30) upstream of the translation start point of *aoaA* and two TSPs at positions -65 and -86 upstream of the ATG of *aoaC* were observed. In contrast, only one TSP for each gene was detected under the stress conditions, at -30 and -86 (marked by the symbol #) upstream to the 5'-ATG of *aoaA* and *aoaC* respectively. Putative promoter sequences at -10 are underlined.

Identification of a DNA-binding protein

To identify transcription factor(s) potentially involved in the regulation of *aoaA* and *aoaC*, we fractionated crude cell-free extracts from *Aphanizomenon* by ammonium sulfate precipitation. We then applied gel shift assays using proteins from these fractions and several DNA fragments containing the relevant genomic region (Fig. 3A). These analyses revealed potential DNA-binding proteins, particularly in the cell-free fraction obtained after precipitation with 50% (w/v) ammonium sulfate (Fig. 3B). DNA affinity columns containing biotin labelled fragment 2 (see Fig. 3A for the location of the fragments used here), was used to isolate specific proteins that showed affinity to this fragment. Elution of the bound protein from the columns using 300 mM NaCl isolated a single protein of approximately 19 kDa as estimated by SDS-PAGE (Fig. 4). Mass spectrometry of the trypsin cleaved protein and analysis of the sequence of the identified peptides (in bold, Fig. S1) revealed that it is similar to various proteins recognized in other filamentous cyanobacteria including *Nodularia spumigena* CCY9414, *Lyngbya* sp. PCC 8106, *Anabaena variabilis* ATCC 29413 and *Anabaena* sp. strain PCC7120 (Fig. S1). These proteins, annotated AbrB-like proteins due to some similarity to the global transcription regulatory factor, 'transition state regulator', isolated from various bacteria including *Bacillus* sp. (Marahiel *et al.*, 1993; Huffman and Brennan, 2002; Koehler, 2002; Yao and Strauch, 2005).

In vitro DNA binding activity

To examine binding of the AbrB-like protein from *Aphanizomenon* to the relevant genomic region, degenerate primers (Table 3) were designed according to the

sequences of the *abrB*-like genes in various filamentous cyanobacteria. These primers were used to clone an *abrB*-like gene from the genomic DNA of *Aphanizomenon*. The expected gene product of the amplified *abrB*-like gene from *Aphanizomenon* (Accession No. DQ486518) is 95–98% identical to the amino acid sequences in other

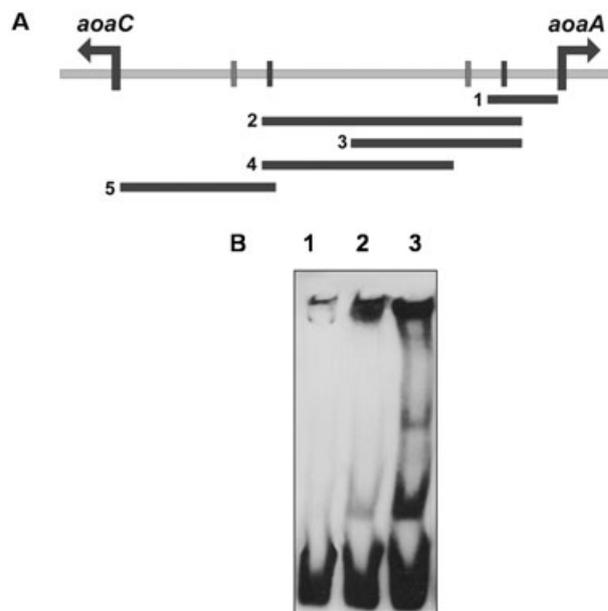


Fig. 3. Gel shift analyses. These experiments were performed using protein fractions from cell-free extracts, precipitated in various concentrations of ammonium sulfate, and several DNA fragments. A. Schematic presentation of the interregion between *aoaA* and *aoaC* showing the orientations of the two genes and the positions of the TSPs (Fig. 2). The 1–5 represents the different DNA fragments used as the templates for the gel shift analyses. B. A representative gel shift assay using fragment 2 and protein fractions obtained after precipitation with 20%, 40% and 50% (w/v) ammonium sulfate in lanes 1–3 respectively.

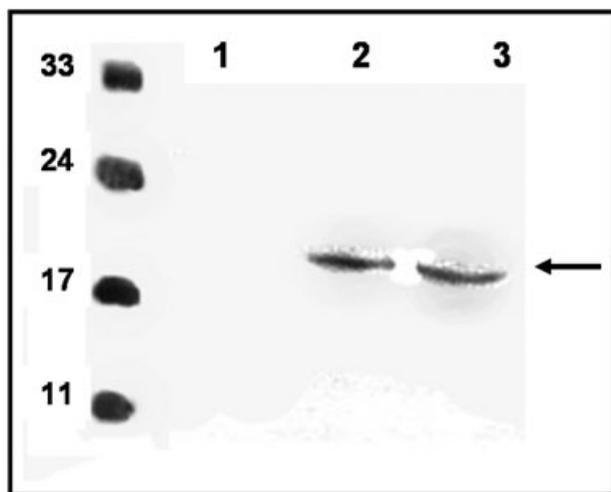


Fig. 4. Purification of the DNA-binding protein by DNA affinity columns containing biotin labelled fragment 2. Lane 1, no DNA was bound to the streptavidin coated magnetic beads; lane 2, DNA fragment 2 labelled with biotin was bound to the streptavidin-coated magnetic beads. The protein was eluted using 300 mM NaCl. MW markers, in kD, are shown on the left.

filamentous cyanobacteria (Fig. S1). The gene product of the cloned *abrB* is identical to the sequence of the protein fragments recognized by the MS/MS analyses, performed on the native protein after trypsin digestion (Fig. S1).

The *abrB*-like gene was cloned into the expression vector pHis Parallel2 (see *Experimental procedures*), bearing a His-tag on its C-terminus. This construct was selected as the N-terminus of AbrB from *Bacillus* sp. is crucial for DNA binding activity (Bobay *et al.*, 2005). The construct obtained was expressed in *Escherichia coli*

strain BL21(DE3)-pLysS (Fig. S2A, see *Experimental procedures*) and the AbrB-like protein was purified (Fig. S2B) and used in gel shift assays (Fig. S3).

Post-translational modifications in the native AbrB-like protein

Detailed MS/MS analyses of the *native* AbrB-like protein from *Aphanizomenon* identified several modifications in the amino acid sequence compared with that expected from the genomic sequence (Accession No. DQ486518). Similar MS/MS analyses performed on the recombinant protein, obtained by expressing the *abrB* gene from *Aphanizomenon* in *E. coli*, did not reveal these modifications. In Fig. 5A and B we show the MS/MS data that led us to conclude that the native protein is modified compared with that observed in the expressed protein. In Fig. 5C we marked the sites where the native protein is modified compared with that expected from the DNA sequence, including:

- The T(2) in the N-terminus is most likely acetylated. This is based on an excess mass of +42U that was observed by the MS/MS analysis at this site (Fig. 5B). Such a mass change is exactly what was expected following acetylation.
- Apparently, there is a trypsin cleavage site (R/K) upstream of K(17) unaccounted for in the DNA sequence.
- A methylation (+14 U) is likely to be present in the sequence range KVK (positions 17–19).
- The C terminus of the native AbrB-like protein appears to be LED (143), i.e. lacking the two lysines

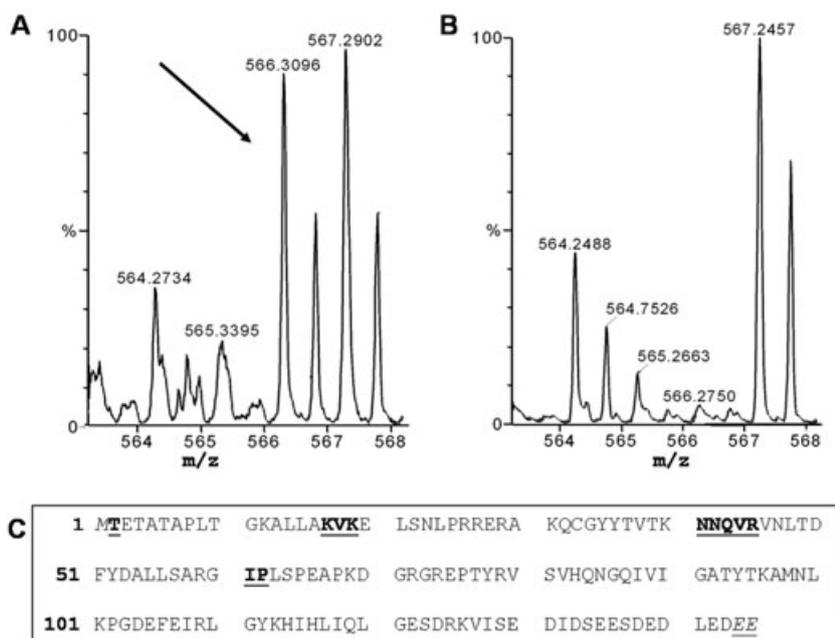


Fig. 5. Post-translational modifications in the AbrB-like protein.

A and B. Comparison of the MS/MS signals from native (A) and recombinant (B) proteins showing (pointed by the arrow) a modification, most likely acetylation, on the T(2). The native AbrB-like protein from *Aphanizomenon* was isolated using the DNA affinity column; see Fig. 4. The recombinant protein was isolated after cloning and expression of the *Aphanizomenon* gene in *E. coli*; see Fig. S2. C. Modifications in the amino acid of the AbrB-like protein from *Aphanizomenon* as revealed by the MS/MS analysis, compared with that predicted by the sequence of *abrB* (DQ486518). Bold letters designate sequence sections substantiated by MS/MS data; underlined sequences designate locations of identified modifications and irregularities (see text). The two glutamines (EE) at the C terminus, shown in *italics*, are missing in the native protein from *Aphanizomenon* but this could be due to the primers used for the cloning (see text).

in positions 144–145. These lysines are present in many of the available AbrB-like sequences from filamentous cyanobacteria (Fig. S1). Notably, these EE were present in the protein expressed in *E. coli* but in this case it could be due to the primers used to clone the gene from *Aphanizomenon*.

We examined the binding of the expressed AbrB-like protein with various DNA fragments from the genomic region between *aoaA* and *aoaC* (Fig. 3A) using the same procedure applied with the native protein extract. The central region of the promoter (fragments 2–4), between the TSPs of *aoaA* and *aoaC*, binds most efficiently with the expressed protein (Fig. S3). The regions between the TSPs and the putative translation start sites (fragments 1 and 5) showed considerably weaker binding affinity to the recombinant protein (Fig. S3).

Discussion

Because of its potential toxicity in the aquatic environment, it is important to clarify the regulation of CYN biosynthesis. Analysis of the proteins that bind to the genomic region between *aoaA* and *aoaC* identified an AbrB-like protein and revealed modifications in the native *Aphanizomenon*'s protein compared with that expected from its sequence (Fig. 5). These modifications are specific to *Aphanizomenon*; they were not observed in the recombinant protein isolated from *E. coli*. The mechanisms leading to these modifications, the enzymes and genes involved are not known but it is likely that such modifications could contribute to the regulation of CYN production and to the response of *Aphanizomenon* to the changing environmental conditions (Fig. 1). Protein acetylation as observed here is a widespread phenomenon among eukaryotes (Polevoda and Sherman, 2000) but far less abundant in prokaryotes. An exception is the well-established effect of CheY acetylation on the swimming direction of *E. coli* (Barak *et al.*, 2004). To the best of our knowledge the acetylation of AbrB observed here is the first report of protein acetylation in cyanobacteria.

As proposed, the redox conditions in the cells, which may be affected by light intensity, are likely to influence the biosynthesis of CYN (Fig. 1). Indeed, raising the light intensity experienced by *Cylindrospermopsis* sp. cultures resulted in a larger accumulation of CYN in the cells and appearance of a higher level of the toxin in the medium. Furthermore, the optimal light intensity for growth was lower than that required to reach maximal CYN production (Dyble *et al.*, 2006). Here, we examined the effects of N-depletion and upshift in light intensity on the transcript abundance of *aoaA* and *aoaC* and on the CYN level in *Aphanizomenon*. The results suggested differential responsiveness of the transcript abundances of *aoaA* and

aoaC to a short exposure to these environmental conditions (Fig. 1B and C). The results also indicated that while CYN accumulation often corresponded to the transcript abundance, this was not always the case. One example is the complete arrest of the CYN accumulation following the N-starvation while the transcript level (normalized to 16S rRNA) increased (Fig. 1B). Another example is the stable CYN level after upshift in light intensity although the transcript of *aoaA* and *aoaC* declined (Fig. 1C). These data supported the suggestion that additional parameters such as the activity of the enzymes involved in CYN biosynthesis might have been affected by the ambient conditions. The fact that CYN is relatively stable (Chiswell *et al.*, 1999) could also play an important part.

Analyses of the TSPs (Fig. 2) using RNA extracted from cells grown under standard or stress conditions revealed two TSPs for each of the *aoaA* and *aoaC* genes. Under the standard growth conditions both TSPs are functional whereas only one of the TSPs was detected under the N-depletion or the upshift in light intensity (Fig. 2A). Multiple alternating TSPs were reported in several cyanobacterial genes, including *glnA*, *psaAB* (Valladares *et al.*, 2004; Muramatsu and Hihara, 2006) and *mcyA* and *mcyD* of the *mcy* operon in *Microcystis aeruginosa* PCC 7806 (Kaebernick *et al.*, 2002). These results suggest a possible repressing mechanism of the *aoaA* and *aoaC* transcription under N-depletion or higher light, or alternatively enhancement of the transcription activity under the N-replete conditions. The TSP analyses could explain the changes in the transcript abundance of *aoaA* and *aoaC* shown under changing environmental conditions (Fig. 1).

Identification of the AbrB-like protein as a transcription factor involved in the regulation of *aoaA* and *aoaC* may shed light on the mechanism whereby CYN formation is enhanced at late growth phases (Hawkins *et al.*, 2001; Dyble *et al.*, 2006). AbrB is highly conserved in cyanobacteria (Fig. S1) but its role is poorly understood. In *Bacillus* and several other bacteria (Vaughn *et al.*, 2000; Cavanagh *et al.*, 2002; Bobay *et al.*, 2004) AbrB is involved in the transition to a stationary phase (Strauch *et al.*, 1990; Marahiel *et al.*, 1993; Fawcett *et al.*, 2000). It acts as a repressor to prevent the expression of transition stage-specific genes during lag and mid-exponential phase; it is negatively regulated by the phosphorylated form of Spo0A, thus ensuring the transition to a stationary phase and de-repression of promoters regulated by AbrB (Strauch *et al.*, 1990).

AbrB has been implicated in the regulation of more than 100 different genes during the transition state (Strauch *et al.*, 1990; Marahiel *et al.*, 1993; Hulett, 1996; Fawcett *et al.*, 2000; Stein *et al.*, 2002; Karatas *et al.*, 2003; Shafikhani and Leighton, 2004). Naturally, involvement in the regulation of large arrays of genes raises the question of specificity. Particularly interesting is the fact that the

DNA sites targeted by AbrB share no apparent consensus sequence. While AbrB has a broad DNA binding property, it does exhibit specificity, it does not bind randomly to DNA (Benson *et al.*, 2002; Strauch *et al.*, 2005). In addition, AbrB does not bind to all its cognate promoters with the same affinity, implying another level of regulation (Xu and Strauch, 1996). Studies on the DNA binding preferences of the N-terminus of AbrB revealed that AbrB–DNA interactions are strongly affected by the topology of the particular DNA region and/or its ability to adopt a suitable conformation (Bobay *et al.*, 2005). Structural studies revealed a dimeric N-terminal region consisting of a four-stranded β -sheet and a C-terminal DNA binding region forged from one α -helix and a 'looped-hinge' thus the name 'looped-hinge helix'. The conserved arginine residues in the center of this α -helix, also present in *Aphanizomenon*, have been shown to be critical for DNA recognition and also contribute to the electropositive nature of the putative DNA binding region (Huffman and Brennan, 2002). The ability of AbrB to specifically recognize various DNA sequences with no apparent homology has been attributed, in part, to a general model in which the looped-hinge regions provide a mechanism for independent reorientation of the recognition helices (Vaughn *et al.*, 2000). In this respect, binding specificity, the observed post-translational modification in the native AbrB in *Aphanizomenon*, is of particular interest. They are likely to affect the structural organization of the protein and hence its affinity to DNA.

Experimental procedures

Strain isolation and growth conditions

A toxic axenic strain of *Aphanizomenon* was originally isolated from Lake Kinneret, Israel (Pollinger *et al.*, 1998). An axenic culture was isolated using established methodology (Wolk, 1988). The cultures were grown on shakers (100 r.p.m.) in flasks containing medium BG11 (Stanier *et al.*, 1971) supplemented with 20 mM TAPS-NaOH, pH = 9 and 10 mM NaHCO₃. Light intensity was 10–15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps and the temperature was 30°C. Under these conditions the generation time was 3 days. For analysis of transcript and toxin levels, cells from mid log phase cultures were washed twice by centrifugation and re-suspended in fresh BG-11 medium at a cell density corresponding to 0.75 $\mu\text{g chlorophyll ml}^{-1}$ in 250 ml flasks under 15 or 85 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low and high light respectively). For nitrogen depletion experiments, the cells were washed twice in a BG11 media where the KNO₃ was replaced by KCl. Samples of 45 ml were withdrawn after 3 days for RNA and toxin extraction.

DNA and RNA extraction

DNA extraction was carried out as described in Shalev-Alon and colleagues (2002). For RNA extraction, cells were col-

lected by centrifugation and frozen in liquid nitrogen. Total RNA was isolated using TRI-Reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions and re-suspended in 30 μl DEPC-treated water. Then 12 μl was treated with DNase I (150 U, Roche Applied Sciences, Mannheim, Germany) for 45 min at 25°C in the presence of 0.1 M Na-Acetate, 5 mM MgSO₄, pH 5. DNase-treated samples were extracted with phenol, phenol: chloroform and the RNA was precipitated from the aqueous phase with 0.1 vol. of 3 M Na-acetate and 3 vols of cold ethanol. The final pellet was dissolved in 15–20 μl DEPC-treated water.

Quantitative PCR analysis

For the reverse transcriptase reaction, 0.75–1 μg RNA was incubated with a mixture of reverse primers for *aoaA*, *aoaC* and *16S rRNA* (Table 1) 20 pmole per 20 μl (final reaction volume) for 10 min at 70°C prior to the addition of the reverse transcriptase (ImProm-II™, Promega Madison, WI, USA) according to the manufacturer's instructions. The RT reaction was performed for 1 h at 42°C followed by 15 min at 70°C to inactivate the enzyme. The transcript levels of *16S rRNA*, *aoaA* and *aoaC* were assessed using Rotor-Gene (Corbett Research Mortlake, Australia). Absolute qPCR SYBR green mix (ABgene, Epsom, England) was used as recommended by the manufacturer. The cDNAs were diluted $\times 10$ to $\times 1000$ (according to the abundance of the relevant transcript) and the concentrations of the appropriate primers (Table 1) were 175 nM. For *aoaA* and *aoaC* genes, the amplification cycles were: one cycle of 95°C for 15 min followed by 40 cycles of 95°C for 10 s, 59°C for 20 s and 72°C for 20 s. Readings of the fluorescence were performed at 85°C and 82°C respectively. The amplification cycle for the *16S rRNA* was: one cycle of 95°C for 15 min followed by 40 cycles of 95°C for 10 s, 57°C for 20 s and 72°C for 20 s and fluorescence measurement at 81.5°C.

Determination of transcription start point

Determination of TSP was performed using the 5' RACE System Version 2.0 (Invitrogen Carlsbad, CA, USA) according to manufacturer's instructions. The cDNA was synthesized from 1 μg RNA using the primers described in Table 1. The tailed cDNA product was amplified by two sequential reactions using the reverse primers (Table 1). After denaturation at 94°C for 1 min, the amplification (35 cycles) was: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by one cycle of 72°C for 5 min and then maintained for 10°C using TaqZol (Tal-Ron, Israel). The amplified cDNAs were purified and cloned into pGEM®-T vector (Promega, Madison, WI, USA) and sequenced to recognize the relevant TSP.

Purification of *Aphanizomenon* proteins

Purification of *Aphanizomenon* proteins was performed as in Koksharova and Wolk (2002). Briefly, 3 l of cultures of *Aphanizomenon* sp. in BG11 medium was harvested by centrifugation at 4°C. Cells were washed with cold 25 mM HEPES-KOH (pH 7.5), sedimented for 15 min at 4°C and re-suspended in buffer A (50 mM HEPES-KOH pH 7.5, 1 mM disodium EDTA, 2 mM dithiothreitol (DTT), 0.5% Triton

Table 1. The primers used for the quantitative real time PCR and 5' RACE reactions.

Gene	Primer	Sequence (5'-3')	Position in AF395828
<i>aoaA</i>	amidRT (R) ^a	CACCCAGAAATCTTCACGGT	6286–6303
<i>aoaA</i>	941 (R) ^{b,c}	GACTAGCTTCGGATAGGGTA	6377–6396
<i>aoaA</i>	Gpsam (R) ^{d,c}	CTCTACCTCAAAGAATGGTG	6497–6516
<i>aoaA</i>	gps1am (R) ^c	TCATCACAGACAGGCCAAAG	6520–6536
<i>aoaA</i>	amidin (F) ^{b,d}	AAAGGAGATGGTTGTCGGGA	6755–6736
<i>aoaC</i>	gpspks (R) ^{a,c}	TCAAGTAGCAAGCGTTGCTGTG	7435–7414
<i>aoaC</i>	gps1pks(R) ^{b,c}	ATCCATGCTAACGGCTTCAC	7412–7393
<i>aoaC</i>	gps2pks(R) ^c	TTCAGAAAACCGCCGTAACG	7348–7329
<i>aoaC</i>	pksRT1 (R) ^d	CATTTTACCCGACGATGTG	7322–7304
<i>aoaC</i>	Pksin (F) ^b	TAGCCTACGATCGCAACTAG	7109–7128
<i>aoaC</i>	Pksin2 (F) ^d	GTATGAGTTGCCGTTTTCCA	7171–7190
16S rRNA	GC ^e (R) ^a	CGCGTTAGCTACGGCAGCGCTCGG	NA
16S rRNA	16srt3 (R) ^b	TTCACCGTACACCAGGAAAT	NA
16S rRNA	CC ^e (F) ^a	TGTAACGACGCGCCAGTCCAGACTCCTACGGGAGGCAGC	NA
16S rRNA	16sin3 (F) ^b	ATTGGGCGTAAAGGGTCTG	NA

a. Used in RT reaction.

b. Used in PCR.

c. Used in 5' RACE analysis.

d. Used in quantitative real-time PCR.

e. According to Rudi and colleagues (1997).

NA, not applicable; R, reverse primer, F, forward primer.

X-100, 10% glycerol and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Cells were broken using a French press. The supernatant was centrifuged at 4°C for 20 min at 16000 r.p.m. Proteins were precipitated from the soluble supernatant by the addition of (NH₄)₂SO₄ to 20, 30, 50, 70, and 90% w/v saturation. All precipitates were dissolved in a small volume of buffer A and dialysed overnight at 4°C against buffer B (50 mM HEPES-KOH pH 7.5, 0.1 mM disodium EDTA, 2 mM DTT, 0.1% Triton X-100, 10% glycerol, protease inhibitor cocktail) yielding respective protein fractions F20, F30, F50, and F70 and F90. These fractions were stored at –80°C and were then used in the gel shift assays (see below) (Koksharova and Wolk, 2002).

Gel mobility shift assays for DNA–protein complexes

Mobility shift assays were performed by using the DIG gel shift kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations. DNA fragments from the promoter region (360, 220, 150 and 91 bp) were prepared by

PCR with the primers shown in Table 2. The smaller fragments (41 and 57 bp) were raised by annealing the single-stranded nucleotides to form a double-stranded DNA fragment. The two oligonucleotides (corresponding to 5 OD₂₆₀ per 100 µl) were dissolved in STE buffer (10 mM Tris buffer pH 8.0, 50 mM NaCl, 1 mM EDTA) at equal molar amounts, warmed to 94°C followed by slow cooling to room temperature.

The DNA fragments were 3' end-labelled by using DIG-11-ddUTP and terminal transferase. Alternatively, labelled fragments were raised by PCR using 5' DIG-labelled primers (see Table 2). Labelled DNA probes (2 ng µl⁻¹) were incubated with the protein extracts from *Aphanizomenon* in a binding buffer (BB, 25 mM HEPES pH 7.8, 5 mM MgCl₂, 25 mM NaCl, 1 mM DTT, 5% glycerol) (Koksharova and Wolk, 2002) containing 1 µl from 100 ng µl⁻¹ of poly (dl-dC), 200 ng µl⁻¹ of Calf Thymus DNA and 100 ng µl⁻¹ of poly-L-Lysine in a final volume of 20 µl. For gel shift competition, proteins were allowed to bind with an unlabelled competitor DNA for 25 min at room temperature before addition of the probe. Incubation

Table 2. The primers used to raise the DNA fragments for the gel mobility shift assays.

Fragment size	Primer	Sequence (5'-3')	Position in AF395828
360	Amipro(F)	CTCCTTTAGTTCATCCCACTC	6749–6769
360	Pkspro(R)	AGCTAGTTGCGATCGTAGGCT	7130–7110
150 ^a	Amiproin(F)	CTACTTCATCAGCAGATTAC	6821–6840
150 ^a	Pksproin(R)	CTGTAGCCAATTTATAGAATAACC	6948–6972
57	Amiproin1	CTAGGTTATTCACGGCAAG	6867–6885
57	Pksproin1	CCTTGTTGCGAGATTTCAAC	6902–6920
41 ^a	Ami(F)	CTCTTCAACACCCTAATTAATCTA CTTCATCAGCAGATTAC	6800–6840
41	Ami(R)	GTAATCTGCTGATGAAGTAGATTAATTAGGGTGTGAAGAG	6800–6840
91 ^a	Pks(F)	GGCTACAGTATTAACCTTTTGTAT AGCC	6965–6992
91	Pks(R)	CTTCGCTTATACCGAATTATTCAC	7032–7055

a. 5' modification of the primers by DIG or Biotin-TEG labeling.

Primers Amipro and Pksproin, Amiproin and Pksproin1 and Pksproin1 were used to raise the 220, 100 and 110 bp fragments respectively.

with the labelled probe lasted for 30 min. The mixture was then loaded on to a native gel [5% polyacrylamide gel, 37.5:1 acrylamide-bisacrylamide in 0.5× TBE buffer (Sambrook *et al.*, 1989)] that had been prerun for 1 h at 4°C in 0.5× TBE buffer at 80 V. Electrophoresis was performed at 4°C in a 0.5× TBE buffer for 2–2.5 h at 80 V. The gel was then electroblotted onto Hybond-N⁺ membrane (Amersham Biosciences, Freiburg, Germany) at 400 mA in a 0.5× TBE buffer for 30 min. The nucleic acids–protein complex was cross-linked to the membrane by UV and chemiluminescence was detected according to the instructions of the manufacturer (Roche, Mannheim, Germany). Gel retardation assay using overexpressed protein was stained by GelStar[®] Nucleic Acid Stain (Cambrex, Rockland, MA, USA).

DNA affinity chromatography

Streptavidin-coated magnetic beads (Dynabeads M-280, Dynal Biotech, Lake Success, NY, USA) were used as recommended by the manufacturer. A 150 bp DNA fragment (designated 2 in Fig. 3) from the promoter region was amplified by PCR using the oligonucleotides shown in Table 2, labelled with Biotin-TEG (Integrated DNA Technology, Coralville, IA, USA). The streptavidin-coated magnetic beads (1 mg) were incubated with labelled fragment 2 (50 pmole) in buffer A (5 mM Tris pH 8, 0.5 mM EDTA, 1 M NaCl) for 15 min with gentle rotation. The beads were washed three times with buffer A and three times with buffer BB (used in the gel-mobility shift assay). The proteins were preincubated with competitor DNA (200 ng µl⁻¹ of Calf Thymus DNA) in BB with 100 ng µl⁻¹ of poly-L-lysine for 25 min at room temperature. The preincubated proteins were added to the fragment two-beads complex to a final volume of 400 µl for 30 min with gentle agitation at room temperature. The column was washed three times with BB and the proteins were eluted with homogenization buffer (20 mM Tris pH 8, 1 mM EDTA, 1 mM DTT, 10% glycerol and protease inhibitor) and increasing concentrations of NaCl (0.3–1 M). The eluted proteins were analysed by SDS-PAGE and were stained with SeeBand Protein Staining solution (Gene Bio Application Ltd, Kefar Hanagid, Israel). The relevant protein bands were excised from the gels and used in the MS/MS analyses.

Identification of AbrB-like protein

Reduction, alkylation and trypsinization steps were carried out as described in Rosenfeld *et al.* (1992) with some modifications (Wilm *et al.*, 1996). Gel pieces with protein samples measuring 2–5 µg were treated with 0.15 µg trypsin (Promega, Medissoon, WI, USA) for 17 h at 37°C followed by wash, reduction and alkylation all in 0.05 M NH₄HCO₃. The

peptides were extracted from the gels using 60% CH₃CN and 1% CHOOH followed by evaporation to dryness, the peptide mixture was solid phase extracted with C18 resin filled tip (ZipTip, Millipore, Billerica, MA, USA) into a volume of 6 µl of 50% CH₃CN in 1% CHOOH solution.

Mass spectrometry and database searching

All experiments were performed on a Micromass Q-TOF2 mass spectrometer (Micromass, Altrincham, UK) equipped with a nanospray attachment (Wilm and Mann, 1996) purchased from Micromass. The capillary voltage used was 1200 V and the cone voltage was 35 V. The peptide mass and the tandem-MS data were analysed using the biolynx package (Micromass, England) and database searches were performed with the Mascot package (Matrix Science, England). Similarity searches of sequences determined via manual analysis were carried out using BLAST.

Overexpression and purification of AbrB-like protein from *Aphanizomenon*

In view of the homology revealed of the purified protein from the MS/MS analysis to AbrB, we used degenerate primers to amplify, clone and sequence the region encoding the AbrB-like protein from *Aphanizomenon* (Table 3). We then raised the same fragment using specific primers with NdeI and XhoI restriction sites. The PCR product was verified by sequencing and cloned in the expression vector pHis-Parallel2 bearing His-Tag at the C-terminus.

The resulting plasmid, designated pHAb4, was transformed to *E. coli* strain BL21(DE3)-pLysS (Novagen, Darmstadt, Germany). The transformed cells were grown in 2 ml of LB auto-induced media (Studier, 2005) supplemented with 0.5% glycerol, 0.05% glucose, 0.2% α-lactose, NPS (100 mM PO₄, 25 mM SO₄, 50 mM NH₄, 100 mM NaCl, 50 mM KCl and 1 mM MgCl₂) and 25 µg chloramphenicol ml⁻¹ overnight at 37°C. The cells were harvested, re-suspended in lysis buffer (50 mM Tris pH 8, 10% glycerol, 0.1% Triton X-100, protease inhibitor and 3 units of DNase) and incubated on ice for 30 min. They were then lysed by sonication six times for 10 s each followed by centrifugation for 20 min at 4°C. The overexpressed protein was examined by electrophoresis on 11.5% SDS-PAGE analysis followed by Coomassie blue staining and Western blot analysis using goat-α-His antibody.

Protein expressing cells were harvested by centrifugation (10 min at 4°C), re-suspended in cold PBS buffer and harvested again at 8000 r.p.m. for 10 min at 4°C. The pellet was re-dissolved in 10 ml of lysis buffer (50 mM Tris pH 8, 0.3 M NaCl, 10% glycerol and protease inhibitor) and lysed by sonication four times of 20 s each. The lysate was centrifuged

Table 3. Primers used to raise and to overexpress AbrB-like protein from *Aphanizomenon*.

Primer	Sequence (5'–3')	Technique for which was used
AbrB(F)	ATGACTGAAACTGCAACCGCG	PCR
AbrB(R)	TTAYTCTTCRTCWTCCAAATC	PCR
AbNdeI	TTCCATATGACTGAAACTGCAACCGCGCCA	Overexpression
AbXhoI	TGCTCGAGTTCTTCGTCTTCCAAATCTTCATC	Overexpression

for 15 min at 8000 r.p.m. at 4°C. The supernatant was mixed with equilibrated 500 µl Ni-NTA agarose resin (Qiagen, Hilden, Germany, pre-washed twice with 10 ml water and once with 10 ml lysis buffer) for 90 min at 4°C with gently agitation. The columns were washed twice in a 10 ml lysis buffer. AbrB-like protein was eluted in two steps; in a lysis buffer containing 100 mM and 250 mM imidazole respectively. Samples from each step were analysed by SDS-PAGE and stained by Coomassie blue. The purified protein was dialysed overnight against gel-shift binding buffer and used in gel-shift assays.

References

- van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J.A., and Bakker, G.J.I. (2007) Toxins of cyanobacteria. *Mol Nutr Food Res* **51**: 7–60.
- Bacsi, I., Vasas, G., Suranyi, G., Hamvas, M., Mathe, C., Toth, E., *et al.* (2006) Alteration of cylindrospermopsin production in sulfate- or phosphate-starved cyanobacterium *Aphanizomenon ovalisporum*. *FEMS Microbiol Lett* **259**: 303–310.
- Barak, R., Prasad, K., Shainskaya, A., Wolfe, A.J., and Eisenbach, M. (2004) Acetylation of the chemicals response regulator CheY by acetyl-CoA synthetase purified from *Escherichia coli*. *J Mol Biol* **342**: 383–401.
- Benson, L.M., Vaughn, J.L., Strauch, M.A., Bobay, B.G., Thompson, R., Naylor, S., and Cavanagh, J. (2002) Macromolecular assembly of the transition state regulator AbrB in its unbound and complexed states probed by micro-electrospray ionization mass spectrometry. *Anal Biochem* **306**: 222–227.
- Bobay, B.G., Benson, L., Naylor, S., Feeney, B., Clark, A.C., Goshe, M.B., *et al.* (2004) Evaluation of the DNA binding tendencies of the transition state regulator AbrB. *Biochemistry* **43**: 16106–16118.
- Bobay, B.G., Andreeva, A., Mueller, G.A., Cavanagh, J., and Murzin, A.G. (2005) Revised structure of the AbrB N-terminal domain unifies a diverse superfamily of putative DNA-binding proteins. *FEBS Lett* **579**: 5669–5674.
- Burgoyne, D.L., Hemscheidt, T.K., Moore, R.E., and Runnegar, M.T.C. (2000) Biosynthesis of cylindrospermopsin. *J Org Chem* **65**: 152–156.
- Cavanagh, J., Thompson, R., Bobay, B., Benson, L.M., and Naylor, S. (2002) Stoichiometries of protein – Protein/DNA binding and conformational changes for the transition-state regulator AbrB measured by pseudo cell-size exclusion chromatography-mass spectrometry. *Biochemistry* **41**: 7859–7865.
- Chiswell, R.K., Shaw, G.R., Eaglesham, G., Smith, M.J., Norris, R.L., Seawright, A.A., and Moore, M.R. (1999) Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: effect of pH, temperature, and sunlight on decomposition. *Environ Toxicol* **14**: 155–161.
- Dyble, J., Tester, P.A., and Litaker, R.W. (2006) Effects of light intensity on cylindrospermopsin production in the cyanobacterial HAB species *Cylindrospermopsis raciborskii*. *Afr J Mar Sci* **28**: 309–312.
- Fawcett, P., Eichenberger, P., Losick, R., and Youngman, P. (2000) The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **97**: 8063–8068.
- Froschio, S.M., Humpage, A.R., Burcham, P.C., and Falconer, I.R. (2001) Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin. *Environ Toxicol* **16**: 408–412.
- Griffiths, D.J., and Saker, M.L. (2003) The palm island mystery disease 20 years on: a review of research on the cyanotoxin cylindrospermopsin. *Environ Toxicol* **18**: 78–93.
- Hadas, O., Pinkas, R., Delphine, E., Vardi, A., Kaplan, A., and Sukenik, A. (1999) Limnological and ecophysiological aspects of *Aphanizomenon ovalisporum* bloom in Lake Kinneret. *J Plankton Res* **21**: 1439–1453.
- Hawkins, P.R., Putt, E., Falconer, I., and Humpage, A. (2001) Phenotypical variation in a toxic strain of the phytoplankter, *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) during batch culture. *Environ Toxicol* **16**: 460–467.
- Huffman, J.L., and Brennan, R.G. (2002) Prokaryotic transcription regulators: more than just the helix-turn-helix motif. *Curr Opin Struct Biol* **12**: 98–106.
- Hulett, F.M. (1996) The signal-transduction network for Pho regulation in *Bacillus subtilis*. *Mol Microbiol* **19**: 933–939.
- Humpage, A.R., Fenech, M., Thomas, P., and Falconer, I.R. (2000) Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. *Mut Res* **472**: 155–161.
- Humpage, A.R., Fontaine, F., Froschio, S., Burcham, P., and Falconer, I.R. (2005) Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. *J Toxicol Environ Health A Curr Issues* **68**: 739–753.
- Kaebbernick, M., and Neilan, B.A. (2001) Ecological and molecular investigation of cyanotoxin production. *FEMS Microbiol Ecol* **35**: 1–9.
- Kaebbernick, M., Dittmann, E., Börner, T., and Neilan, B.A. (2002) Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. *Appl Environ Microbiol* **68**: 449–455.
- Karatas, A.Y., Cetin, S., and Ozcengiz, G. (2003) The effects of insertional mutations in *comQ*, *comP*, *srfA*, *spo0H*, *spo0A* and *abrB* genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochim Biophys Acta* **1626**: 51–56.
- Kellmann, R., Mills, T., and Neilan, B.A. (2006) Functional modeling and phylogenetic distribution of putative cylindrospermopsin biosynthesis enzymes. *J Mol Evol* **62**: 267–280.
- Koehler, T.M. (2002) *Bacillus anthracis* genetics and virulence gene regulation. *Curr Top Microbiol Immunol* **271**: 143–164.
- Koksharova, O.A., and Wolk, C.P. (2002) Novel DNA-binding proteins in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **184**: 3931–3940.
- Looper, R.E., Runnegar, M.T.C., and Williams, R.M. (2006) Syntheses of the cylindrospermopsin alkaloids. *Tetrahedron* **62**: 4549–4562.
- Marahiel, M.A., Nakano, M.M., and Zuber, P. (1993) Regulation of peptide antibiotic production in *Bacillus*. *Mol Microbiol* **7**: 631–636.
- Metcalfe, J.S., Barakate, A., and Codd, G.A. (2004) Inhibition of plant protein synthesis by the cyanobacterial hepato-

- toxin, cylindrospermopsin. *FEMS Microbiol Lett* **235**: 125–129.
- Muramatsu, M., and Hihara, Y. (2006) Characterization of high-light-responsive promoters of the *psaAB* genes in *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* **47**: 878–890.
- Norris, R.L.G., Seawright, A.A., Shaw, G.R., Senogles, P., Eaglesham, G.K., Smith, M.J., et al. (2002) Hepatic xenobiotic metabolism of cylindrospermopsin in vivo in the mouse. *Toxicol* **40**: 471–476.
- Polevoda, B., and Sherman, F. (2000) N alpha-terminal acetylation of eukaryotic proteins. *J Biol Chem* **275**: 36479–36482.
- Pollinger, U., Hadas, O., Yacobi, Y.Z., Zohary, T., and Berman, T. (1998) *Aphanizomenon ovalisporum* (Forti) in Lake Kinneret, Israel. *J Plankton Res* **20**: 1321–1339.
- Preussel, K., Stuken, A., Wiedner, C., Chorus, I., and Fastner, J. (2006) First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicol* **47**: 156–162.
- Reisner, M., Carmeli, S., Werman, M., and Sukenik, A. (2004) The cyanobacterial toxin cylindrospermopsin inhibits pyrimidine nucleotide synthesis and alters cholesterol distribution in mice. *Toxicol Sci* **82**: 620–627.
- Rosenfeld, J., Capdevielle, J., Guillemot, J.C., and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence-analysis after 1-dimensional or 2-dimensional gel-electrophoresis. *Anal Biochem* **203**: 173–179.
- Rudi, K., Skulberg, O.M., Larsen, F., and Jakobsen, K.S. (1997) Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. *App Environ Microbiol* **63**: 2593–2599.
- Runnegar, M., and Lu, S. (1994) The role of glutathione (Gsh) in the hepatotoxicity caused by the cyanobacterial alkaloid cylindrospermopsin (Cy). *FASEB J* **8**: A419–A419.
- Runnegar, M.T., Kong, S.M., Zhong, Y.Z., Ge, J.L., and Lu, S.C. (1994) The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem Biophys Res Commu* **201**: 235–241.
- Runnegar, M.T., Kong, S.M., Zhong, Y.Z., and Lu, S.C. (1995) Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem Pharmacol* **49**: 219–225.
- Runnegar, M.T., Xie, C.Y., Snider, B.B., Wallace, G.A., Weinreb, S.M., and Kuhlenkamp, J. (2002) *In vitro* hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. *Toxicol Sci* **67**: 81–87.
- Saker, M.L., and Neilan, B.A. (2001) Varied diazotrophies, morphologies, and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from northern Australia. *Appl Environ Microbiol* **67**: 1839–1845.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. New York, USA: Cold Spring Harbor.
- Schembri, M.A., Neilan, B.A., and Saint, C.P. (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environ Toxicol* **16**: 413–421.
- Schwarz, R., and Forchhammer, K. (2005) Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology* **151**: 2503–2514.
- Shafikhani, S.H., and Leighton, T. (2004) *AbrB* and *Spo0E* control the proper timing of sporulation in *Bacillus subtilis*. *Curr Microbiol* **48**: 262–269.
- Shalev-Alon, G., Sukenik, A., Livnah, O., Schwarz, R., and Kaplan, A. (2002) A novel gene encoding amidinotransferase in the cylindrospermopsin producing cyanobacterium *Aphanizomenon ovalisporum*. *FEMS Microbiol Lett* **209**: 87–91.
- Shen, X.Y., Lam, P.K.S., Shaw, G.R., and Wickramasinghe, W. (2002) Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. *Toxicol* **40**: 1499–1501.
- Stanier, R.Y., Kunisawa, R., Mandel, M., and Cohen Bazire, G. (1971) Purification and properties of unicellular blue-green algae (order *Chroococcales*). *Bacteriol Rev* **35**: 171–205.
- Stein, T., Borchert, S., Kiesau, P., Heinzmann, S., Kloss, S., Klein, C., et al. (2002) Dual control of subtilin biosynthesis and immunity in *Bacillus subtilis*. *Mol Microbiol* **44**: 403–416.
- Strauch, M., Webb, V., Spiegelman, G., and Hoch, J.A. (1990) The *SpoA* protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc Natl Acad Sci USA* **87**: 1801–1805.
- Strauch, M.A., Ballar, P., Rowshan, A.J., and Zoller, K.L. (2005) The DNA-binding specificity of the *Bacillus anthracis* *AbrB* protein. *Microbiol* **151**: 1751–1759.
- Studier, F.W. (2005) Protein production by auto-induction in high-density shaking cultures. *Protein Expr Purif* **41**: 207–234.
- Valladares, A., Muro-Pastor, A.M., Herrero, A., and Flores, E. (2004) The *NtcA*-dependent *P-1* promoter is utilized for *glnA* expression in N_2 -fixing heterocysts of *Anabaena* sp strain PCC 7120. *J Bacteriol* **186**: 7337–7343.
- Vaughn, J.L., Feher, V., Naylor, S., Strauch, M.A., and Cavanagh, J. (2000) Novel DNA binding domain and genetic regulation model of *Bacillus subtilis* transition state regulator *AbrB*. *Nat Struct Biol* **7**: 1139–1146.
- Wilm, M., and Mann, M. (1996) Analytical properties of the nanoelectrospray ion source. *Anal Chem* **68**: 1–8.
- Wilm, M., Shevchenko, A., Houthaev, T., Breit, S., Schweigener, L., Fotsis, T., and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nanoelectrospray mass spectrometry. *Nature* **379**: 466–469.
- Wolk, C.P. (1988) Purification and storage of nitrogen-fixing filamentous cyanobacteria. *Methods Enzymol* **167**: 93–95.
- Xu, K., and Strauch, M.A. (1996) *In vitro* selection of optimal *AbrB*-binding sites: comparison to known *in vivo* sites indicates flexibility in *AbrB* binding and recognition of three-dimensional DNA structures. *Mol Microbiol* **19**: 145–158.
- Yao, F., and Strauch, M.A. (2005) Independent and interchangeable multimerization domains of the *AbrB*, *abh*, and *SpoVT* global regulatory proteins. *J Bacteriol* **187**: 6354–6362.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Alignment of the AbrB-like sequence from various filamentous cyanobacteria. *Aphanizomenon ovalisporum* (DQ486518, *A. ovalisporum*); *Anabaena variabilis* ATCC 29413 (ABA20148, *A. variabilis*); *Anabaena* sp. strain PCC 7120 (Alr0946, *Anabaena* PCC7120); *Nodularia spumigena* CCY9414 (*N. spumigena*); *Lyngbya* sp. PCC 8106 (*Lyngbya* PCC8106). Note the very high conservation of this protein in various cyanobacteria. Bold letters represents residues identified by the MS/MS.

Fig. S2. Overexpression and purification of the recombinant AbrB-like protein.

A. Western analysis of proteins expressed in *E. coli* using goat- α -His antibody. The *abrB*-like gene from *Aphanizomenon* was cloned into the expression vector pHis Parallel2, the construct obtained was introduced into *E. coli* strain BL21(DE3)-pLysS⁺ (lanes 1–3), the protein was expressed under auto-induced media at 28°C and 37°C (lanes 2 and 3 respectively) as described in the *Experimental procedures*. Lane 1 is the un-induced control.

B. The AbrB-like protein was purified from the crude protein extract using Ni-NTA column. Lanes 1 and 2, samples from

the soluble and insoluble fractions subsequent to breakage of the bacterial cells; lanes 3 and 4, samples from the unbound and first washes of the Ni-NTA column respectively. Lanes 5–8, two elution steps using lysis buffer containing 100 mM imidazole followed by elution with lysis buffer containing 250 mM imidazole (each step performed twice).

Fig. S3. Binding of the recombinant AbrB with various DNA fragments from the genomic region between *aoaA* and *aoaC*. Gel shift analyses using the overexpressed AbrB-like protein and the various DNA fragments depicted in Fig. 3A – fragments 5, 4, 3, 2 and 1 (lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9 and 5 and 10 are the treatments with and without the protein respectively). The central region of the promoter (fragments 2, 4 and 3) between the transcription start points of *aoaA* and *aoaC* binds most efficiently with the overexpressed AbrB. The regions between the transcription start points and the putative translation start sites (fragments 5 and 1) showed considerably weaker binding affinity to the recombinant protein.

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