All1371 is a polyphosphate-dependent glucokinase in *Anabaena* sp. PCC 7120

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The polyphosphate glucokinases can phosphorylate glucose to glucose 6-phosphate using polyphosphate as the substrate. ORF **all1371** encodes a putative polyphosphate glucokinase in the filamentous heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120. Here, ORF **all1371** was heterologously expressed in *Escherichia coli*, and its purified product was characterized. Enzyme activity assays revealed that All1371 is an active polyphosphate glucokinase that can phosphorylate both glucose and mannose in the presence of divalent cations in vitro. Unlike many other polyphosphate glucokinases, for which nucleoside triphosphates (e.g. ATP or GTP) act as phosphoryl group donors, All1371 required polyphosphate to confer its enzymic activity. The enzymic reaction catalysed by All1371 followed classical Michaelis–Menten kinetics, with $k_{\text{cat}} = 48.2 \text{ s}^{-1}$ at pH 7.5 and 28 °C and $K_M = 1.76 \text{ mM}$ and 0.118 mM for polyphosphate and glucose, respectively. Its reaction mechanism was identified as a particular multi-substrate mechanism called the ‘bi-bi ping-pong mechanism’. Bioinformatic analyses revealed numerous polyphosphate-dependent glucokinases in heterocyst-forming cyanobacteria. Viability of an *Anabaena* sp. PCC 7120 mutant strain lacking **all1371** was impaired under nitrogen-fixing conditions. GFP promoter studies indicate expression of **all1371** under combined nitrogen deprivation. All1371 might play a substantial role in *Anabaena* sp. PCC 7120 under these conditions.

**INTRODUCTION**

Inorganic polyphosphate, which is a linear polymer of 10–1000 orthophosphates linked by phosphoanhydride bonds, has been found in all representative living cells, including bacteria, fungi, plants, animals and archaea (Achbergerová & Naháľka, 2011; Rao et al., 2009; Remonsellez et al., 2006; Scherer & Bochem, 1983). Polyphosphate is stored in the cytoplasm, where it can be visualized as metachromic inclusions (Meyer, 1902) or electron-dense granules (Jensen, 1968). Evolutionarily, polyphosphate stands as one of the earliest polymers produced in cells. Polyphosphate is considered to be the ancestor of ATP as an energy source (Resnick & Zehnder, 2000), given that hydrolysis of the phosphoanhydride bond between each orthophosphate yields free energy comparable to that generated by cleavage of ATP. In a bacterial cell, polyphosphate functions mainly as a dynamic storage compound for phosphate and energy (Harold, 1966; Kornberg et al., 1956). However, many other functions have been proposed for the polymer, including those in stress responses, complexation of heavy metals, biofilm formation and virulence (Kornberg, 1995; Rashid & Kornberg, 2000; Rashid et al., 2000; Tsutsumi et al., 2000). In bacteria, polyphosphate metabolism is driven by two kinds of enzymes: kinases and phosphatases. Polyphosphate is synthesized by polyphosphate kinase type 1 (Ahn & Kornberg, 1990; Kornberg et al., 1956), which catalyses the formation of the phosphoanhydride bonds between the growing polymer and the γ-phosphoryl residues of ATP or another nucleotide triphosphate. Conversely, polyphosphate is degraded mainly by exopolyphosphatases (Akiyama et al., 1993; Kornberg et al., 1999) and endopolyphosphatases (Lichko et al., 2010).

**Abbreviations:** *Anabaena*, *Anabaena* sp. PCC 7120; GST, glutathione-S-transferase; PPGK, polyphosphate glucokinase; TSS, transcription start site.

Two supplementary tables and five supplementary figures are available with the online Supplementary Material.
Polyphosphate glucokinase (PPGK; EC 2.7.1.63), a paralogue of the ATP-dependent glucokinase (Hsieh et al., 1993), catalyses the transfer of the terminal phosphoryl residue of polyphosphate to glucose in order to generate glucose 6-phosphate. The first ATP/polyphosphate-dependent glucokinase was discovered in Mycobacterium phlei (Szymona & Ostrowski, 1964). Since then, such enzymes have been found in many non-eukaryotic organisms (Liao et al., 2012; Lindner et al., 2010; Pepin & Wood, 1986; Phillips et al., 1993; Szymona & Widomska, 1974; Tanaka et al., 2003). All but one of the known PPGKs are bifunctional, in that they are able to utilize both ATP and polyphosphate as phosphoryl donors. The sole known exception to that is the PPGK of the polyphosphate-accumulating bacterium Microlunatus phosphovorus (Tanaka et al., 2003), which is a strictly polyphosphate-dependent enzyme.

The potential role of PPGKs in the complex metabolism of cyanobacteria has not yet been investigated. Cyanobacteria are a widespread group of oxygenic photosynthetic prokaryotes. During photosynthesis, energy is transiently stored in the energy-rich phosphoanhydride bonds of ATP molecules. Several genera of cyanobacteria perform both photosynthesis and N₂ fixation; however, these two physiological processes are incompatible, because the oxygen-sensitive nitrogenase complex (Hill et al., 1981) is the key enzyme in N₂ fixation. The diazotrophic cyanobacteria have developed special mechanisms to allow N₂ fixation to take place under aerobic conditions (Berman-Frank et al., 2003). Some filamentous cyanobacteria, such as Anabaena sp. PCC 7120 (also called Nostoc sp. PCC 7120; hereafter Anabaena), form highly specialized cells called ‘heterocysts’, which fix N₂ in a micro-oxic environment (Adams et al., 1981). The heterocysts are semi-regularly distributed along the filaments and rely on vegetative cells to supply them with photosynthetic products. In return, the heterocysts provide the filament with reduced nitrogen compounds (Flores & Herrero, 2010; Maldener & Muro-Pastor, 2010). In contrast, some unicellular diazotrophic cyanobacteria use a diurnal rhythm to separate N₂ fixation and photosynthesis, protecting the nitrogenase from oxygen by employing it in the dark, when photosynthesis is quiescent (Mitsui et al., 1986; Toepel et al., 2008).

The purpose of this study was to characterize All1371, the PPGK from Anabaena, in vitro and to explore its biological function in vivo.

**METHODS**

Sequence analysis. A BLASTP search (Altschul et al., 1997) was performed against all cyanobacterial sequences available from the Integrated Microbial Genomes database (Markowitz et al., 2012) and against the sequences of Section V cyanobacteria identified by Dagan et al. (2013). The amino acid sequence of the PPGK from Anabaena (all1371: 657231738, gene ID Integrated Microbial Genomes database) was used as query. Similar amino acid sequences of proteins with known 3D structures were identified using the structure database PDBsum (http://www.ebi.ac.uk/pdbsum/). Sequences were aligned using CLUSTALW2 (Larkin et al., 2007), and formatted with ESPript (Gouet et al., 1999). Sequence similarities were determined using the EMBOSS needle software (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

**Bacterial strains and culture conditions.** Anabaena was grown in fourfold diluted medium of Allen & Arnon (1955) (AA/4 medium) with or without 10 mM KNO₃. Liquid cultures of Anabaena were grown under permanent illumination with white light of 70 µmol photons m⁻² s⁻¹ at 30 °C. Cultures were grown in air lift flasks (Ø 6 cm), bubbled with air enriched with 2% (v/v) CO₂. Mutants were grown in the presence of 50 µg neomycin ml⁻¹ or 4 µg spectinomycin ml⁻¹ and 1 µg streptomycin ml⁻¹. Synechocystis sp. PCC 6803 (hereafter Synechocystis) was grown on BG11 agar plates (Rippka et al., 1979) additionally containing 20 mM HEPES. Liquid cultures were grown at 28 °C and under continuous illumination as described above. Liquid cultures of Mastigocladus laminosus SAG 4.84 and Fischerella muscicola PCC 7414 were grown in Castenholz medium D (8.24 mM NaNO₃, 0.92 mM KNO₃, medium ND (without nitrate) (Castenholz, 1988) at 42 °C and under continuous illumination of 100 µmol photons m⁻² s⁻¹.

Chlorophyll a content was determined as described by de Marsac & Houmard (1988). For nitrogen starvation, exponentially grown cultures were harvested by centrifugation, washed twice with nitrate-free medium and resuspended to a final concentration of 7 µg chlorophyll ml⁻¹ for further growth. Escherichia coli strains DH5α and BL21 (DE3) (Novagen; Merck Chemicals) were grown at 37 °C as batch culture in Erlenmeyer flasks with shaking at 300 r.p.m. in Luria–Bertani (LB) medium (Bertani, 1951) supplemented with 10 µg ampicillin ml⁻¹, 150 µg neomycin ml⁻¹ or 50 µg spectinomycin ml⁻¹ when appropriate.

Construction of expression plasmid. The all1371 gene was amplified by PCR using genomic Anabaena DNA as template and oligonucleotides 5’-AGGATCCATCTCAATGGTGGAAGATAACGG-3’ and 5’-GGGCGCCGCCTTCTATAGGTGTGTTCATCTC-3’ (BanHI and NotI restriction sides highlighted in bold, stop codon underlined). The PCR product was ligated into the cloning vector pJET1.2 (Thermo Scientific) to ensure efficient restriction digests. After restriction analysis and DNA sequencing.

**Protein expression and purification.** E. coli BL21(DE3) cells were transformed with pGEX_all1371. The recombinant strain was grown in LB medium containing 100 µg ampicillin ml⁻¹ and 1% (w/v) glucose. The expression was induced with 1 mM IPTG at an OD₆₀₀ of 0.6. Cells were harvested 3 h after induction by centrifugation (15 min, 3800 g), resuspended in buffer containing 200 mM Tris/HCl (pH 8.5), 300 mM NaCl and 50 mM KCl, and disrupted by sonication. The debris was removed by centrifugation (15 min, 20000 g). The glutathione-S-transferase (GST)–PPGK fusion was purified by affinity chromatography using Glutathion-Sepharose 4B (GE Healthcare) performed in a batch technique according to the manufacturer’s instructions. To elute All1371 the GST-tagged PPGK was cleaved on the column by PreScission protease (Walker et al., 1994) (GE Healthcare) overnight at 4 °C. The purity of the enzyme was verified by SDS-PAGE.

Preparation of cell-free cyanobacterial extract, electrophoresis and protein quantification. Cyanobacterial cells were collected from liquid cultures (grown with or without nitrogen for 4 or 6 days) by centrifugation (6500 g). Sedimented cells were washed twice with 50 mM Tris/HCl buffer (pH 8.0) and stored at −20 °C. Thawed filaments of Mastigocladus laminosus and F. muscicola were pretreated...
by sonication. Cells were disrupted in a swing mill (Retsch MM 301) for 30 min at 30 Hz using glass beads (0.1 mm). Beads and crude extracts were separated by two sequential centrifugations at 10 000 g and 4 °C for 10 and 30 min. To remove small molecules the supernatants were purified using DextrASEC PRO2 columns (Applichem). The elution was performed by the original buffer. Protein concentrations were estimated according to Lowry et al. (1951) using BSA as reference. SDS-PAGE was performed on slab gels [15 % (w/v) acrylamide, 0.41 % (w/v) methylene-bisacrylamide] (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R250.

**Determination of molecular mass.** The molecular mass of native All1371 was determined by size exclusion chromatography on a Tricorn Superdex 200 10/300 GL column (GE Healthcare) calibrated with the gel filtration standards purchased from Bio-Rad (β-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; cytochrome c, 12.4 kDa). As running buffer 100 mM Tris/HCl (pH 7.5), 200 mM NaCl, 6 mM MgCl₂ (hereafter basic buffer) and 0.5 mM DTT were used at a flow rate of 0.8 ml min⁻¹. Pure All1371 (100 μg) was loaded onto the column. The elution was monitored by measuring A₂₈₀. Fractions of 0.5 ml were collected. Aliquots from these fractions were tested for PPGK activity. Precipitated fractions (Bensadoun & Weinstein, 1976) were analysed by SDS-PAGE. Two biological replicates were performed.

**Activity assays and kinetic analyses.** Glucokinase activity and kinetics of the isolated All1371 were determined in vitro by coupling glucose 6-phosphate formation to the glucose-6-phosphate dehydrogenase reaction (Hsieh et al., 1993). Glucose 6-phosphate formation was monitored indirectly by measuring NADH development spectrophotometrically at 340 nm (ε₉₆₀ = 6220 M⁻¹ cm⁻¹). Measurements were done in basic buffer with 0.6 mM NAD, 0.8 mM glucose, 0.01 mM polyP₄₅ (phosphate glass type 45; Sigma-Aldrich) and 0.02 mM polyP₃₅ (phosphate glass type 35; Sigma-Aldrich) using BSA as reference. SDS-PAGE was performed on slab gels [15 % (w/v) acrylamide, 0.41 % (w/v) methylene-bisacrylamide] (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R250.

**Deletion of all1371 in Anabaena.** An Anabaena Δall1371 mutant was generated by replacing 771 nt including all1371 [720 nt, genomic region 1625 095–1625 814 (Nakao et al., 2010)] with an antibiotic resistance cassette, not affecting other ORFs.

Upstream and downstream regions of all1371 were amplified by PCR using genomic Anabaena DNA as template. Restriction sites introduced by the primers below are highlighted in the sequence in bold type and termed in parentheses. The upstream region (position 1625 815–1625 817) was amplified using the primers 5’-ATTGA-GGCTCAAGCCGGAAAAATTACAC-3’ (Sad) and 5’-GAGTATTACCCTTTTCTTACAGCCTGG-3’ (Xhol) yielding a PCR product of 973 nt after restriction. The downstream region using the primer pair 5’-CCCGAAAATCTTAGATGTGACCTGG-GATGCGG-3’ (Xhol) and 5’-AATTGCTCGAGAAAACATCATATCCCCGTGC-3’ (Xhol). The restricted product yielded a 943 nt fragment. The fragments were successively inserted into the plasmid KSII + vector (Stratagene) resulting in pKSII+_up_down. The resistance cassette C.K3 containing the neomycin phosphotransferase II gene was released from pRL448 (Elhai & Wolk, 1988a) and inserted into XbaI site of the plasmid Xhol, yielding pKSII+_up_down via the XbaI site, yielding pKSII+_up+CK3_down. The C.K3 cassette was inserted in the same direction as all1371. The correctness of the sequence was validated by DNA sequencing. To construct pRL271_up+CK3_down used for deletion, the Sall/Xhol fragment excised from the prior plasmid was cloned into plasmid pRL271 (Black et al., 1993). This plasmid was conjugationally transferred to Anabaena by triparental mating using E. coli strain J53[RP4] and cargo strain E. coli HB101[pRL258] (Elhai & Wolk, 1988b). Neomycin-resistant double recombinants were identified by PCR and sacB selection (Cai & Wolk, 1990).

**Viability tests.** Viability tests of Anabaena and the Δall1371 mutant were carried out as a spot assay on AA-plates (Allen & Arnon, 1955) with or without 10 mM KNO₃ as a nitrogen source. A 10 μl volume of liquid cultures was applied per spot. These agar plates were exposed to continuous light of 60–70 μmol photons m⁻² s⁻¹ for 6 days. Three biological replicates were tested separately.

**Generation of a GFP promoter fusion strain.** The gfp gene was amplified by PCR using the primer pair 5’-GATGCGGCTTCTTAGA-ATGAGTAAAGGAGAAG-3’ and 5’-CTCTTAGATTAATGTTTG-ATAGTGC-3’ (Xhol in bold type) and 5’-ATGAGTAAAGGAGAAG-3’ (Xhol in bold type). The upstream region (position 1625 815–1625 817) was amplified using the primers 5’-ATTGA-GGCTCAAGCGAAAAATTACAC-3’ (Sad) and 5’-GAGTATTACCCTTTTCTTACAGCCTGG-3’ (Xhol). The restriction product yielded a 943 nt fragment. The fragments were successively inserted into the plasmid KSII + vector (Stratagene) resulting in pKSII+_up_down. The resistance cassette C.K3 containing the neomycin phosphotransferase II gene was released from pRL448 (Elhai & Wolk, 1988a) and inserted into XbaI site of the plasmid Xhol, yielding pKSII+_up_down via the XbaI site, yielding pKSII+_up+CK3_down. The C.K3 cassette was inserted in the same direction as all1371. The correctness of the sequence was validated by DNA sequencing. To construct pRL271_up+CK3_down used for deletion, the Sall/Xhol fragment excised from the prior plasmid was cloned into plasmid pRL271 (Black et al., 1993). This plasmid was conjugationally transferred to Anabaena by triparental mating using E. coli strain J53[RP4] and cargo strain E. coli HB101[pRL258] (Elhai & Wolk, 1988b). Neomycin-resistant double recombinants were identified by PCR and sacB selection (Cai & Wolk, 1990).

**Confocal microscopy.** For confocal microscopy Anabaena mutant strains (Δall1371, promoter fusion) were grown as liquid cultures with and without nitrate for 4 days. Fluorescence in cells of the Anabaena Δall1371 promoter fusion strain was visualized with a laser-scanning confocal microscope (Olympus FV-1000MPF). GFP
was excited by an argon ion laser (488 nm irradiation). Fluorescence emission was recorded at 500–545 nm (for GFP) and 570–670 nm (for chlorophyll fluorescence) using a 60× water-immersion objective (Olympus IX-81 60×/1.2 Water UPlanSApo, DIC, fourfold zoom). All confocal images for each experiment were acquired using identical adjustments. The GFP fluorescence was quantified using Olympus Fluoview version 3.1. The fluorescence of a heterocyst was compared with that of the two adjacent vegetative cells. The Δall1371 mutant strain without GFP was used as control. Background fluorescence was subtracted.

RESULTS

**All1371 as putative PPGK**

The ORF *all1371* from *Anabaena* was assumed to encode a putative PPGK (EC 2.7.1.63), as its amino acid sequence has sequence similarity to several well-characterized bacterial PPGKs, including the polyphosphate/ATP-glucosmannokinase from *Arthrobacter* sp. strain KM (Mukai *et al.*, 2003, 2004) (53.1 % similarity), the polyphosphate-dependent PPGK from *Microlunatus phosphovorus* (Tanaka *et al.*, 2003) (46.0 % similarity) and the polyphosphate/ATP-dependent glucokinase from *Mycobacterium tuberculosis* (Hsieh *et al.*, 1993, 1996a, b) (47.4 % similarity). Comparison of these sequences, including secondary structures extrapolated from the crystal structure of the polyphosphate/ATP-glucosmannokinase of *Arthrobacter* sp. strain KM (1WOQ) (Mukai *et al.*, 2003, 2004), revealed the presence of some highly conserved common motifs (Fig. 1). Seven structural and functional motifs (Fig. 1, boxed) were found in all of the sequences including All1371.

**Purification of All1371 and molecular mass determination**

The N-terminal GST-fusion protein of All1371 was expressed for 3 h in *E. coli* BL21(DE3) carrying pGEX_all1371. After on-column cleavage with the PreScission protease, the 246 aa

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**Fig. 1.** Primary structural alignments of different PPGKs. Aligned primary structures from *Arthrobacter* sp. strain KM, *Microlunatus phosphovorus* (Tanaka *et al.*, 2003), *Mycobacterium tuberculosis* (Hsieh *et al.*, 1993; Phillips *et al.*, 1999) and *Anabaena* sp. PCC 7120. Strictly conserved residues are shaded in black; similar residues are framed in black. Putative structural and functional domains are enclosed in boxes. Secondary structural elements [e.g. α helices, β sheets, turn-turns (TT)] of the polyP/ATP-glucosmannokinase from *Arthrobacter* sp. strain KM are depicted above the alignment. Residues associated with β-D-glucose binding are marked with filled triangles. The heptapeptide is underlined. The catalytic aspartate (D) is highlighted with a star. Residues involved in the binding of both phosphate molecules used as ligands (open, phosphate A; filled, phosphate B) are marked with ovals (Mukai *et al.*, 2004).
enzyme was eluted and analysed by SDS-PAGE (Fig. 2a). The enzyme appeared as a single band of 26 kDa (Fig. 2a, lane 3; apparent molecular mass). To investigate the oligomeric state of All1371, the recombinant PPGK was analysed by size-exclusion chromatography. A single symmetrical peak (Fig. 2b, inset) of approximately 39.0 kDa was obtained. Fractions corresponding to the protein elution peak showed a single protein band of 26 kDa on SDS-PAGE (Fig. 2b, inset), and exhibited polyphosphate-dependent activity in vitro (data not shown). The biochemical and kinetic properties of All1371 are summarized in Table 1.

Table 1. Biochemical and kinetic properties of All1371

Measurements were performed at 28 °C, pH 7.5; n ≥ 3; 100 % = 107.1 U mg⁻¹.

| Property                                      | Value (mean ± sd)                        |
|-----------------------------------------------|-----------------------------------------|
| Molecular mass (kDa)                          |                                        |
| Native complex*                               | 39                                      |
| Monomer, apparent†                            | 26.0                                    |
| Monomer, calculated‡                          | 26.6                                    |
| Monomer or homodimer                          |                                        |
| Oligomeric structure                          |                                        |
| \(K_M\) (mM)                                  |                                        |
| Glucose                                       | 0.118 ± 0.01                           |
| Polyphosphate                                 | 1.76 ± 0.26                            |
| \(K_M\) (mM) Glucose, polyphosphate          | 107.1 ± 15.3                           |
| \(K_M\) (mM) Glucose, polyphosphate          | 48.2 ± 6.9                             |
| \(K_M\) (mM) Mannose                         | 24.3 ± 2.36                            |
| \(K_M\) (mM) Mannose                         | 0.43 ± 0.04                            |
| \(K_M\) (mM) Mannose                         | 0.21 ± 0.017                           |
| \(v_{max}\) (U mg⁻¹)                         |                                        |
| Glucose, polyphosphate                        |                                        |
| \(k_{cat}\) (s⁻¹)                             |                                        |
| Glucose, polyphosphate                        |                                        |
| Phosphoryl donor specificity (% \(v_{max}\)) |                                        |
| ATP, GTP, UTP, CTP, ADP, AMP                  | 0                                      |
| Pyrophosphate                                 | 0                                      |
| Without polyphosphate                         | 0                                      |

* Determined by size exclusion chromatography.
† Determined by SDS-PAGE.
‡ Calculated according to the primary structure, including the linker peptide of the GST tag.
Biochemical properties of All1371

Our enzyme activity assays indicated that purified All1371 uses polyphosphate to phosphorylate glucose and mannose, with a higher preference for glucose (Table 1). All1371 activity was strictly dependent on the presence of Mg$^{2+}$ or Mn$^{2+}$ (data not shown). All1371 had high substrate specificity and acted as a strict polyphosphate-dependent enzyme. No other phosphoryl group donor was accepted. Kinetic analysis indicated that the reactions of All1371 with polyphosphate and glucose followed Michaelis–Menten kinetics. The $K_M$ values for polyphosphate and glucose were 1.76 μM and 0.118 mM, respectively (at 28 °C and pH 7.5). The maximum rate of All1371-mediated catalysis was 107 U mg$^{-1}$, yielding a $k_{cat}$ of 48.2 s$^{-1}$ (Table 1). Furthermore, our kinetic analysis revealed that All1371 had a $k_{cat}$ of 0.19 s$^{-1}$ and a $K_{0.5}$ of 24.3 mM for mannose (Table 1).

To characterize the enzymic mechanism of All1371, additional kinetic analyses with glucose and polyphosphate were performed. The initial rate of All1371 activity was determined with varying concentrations of glucose and fixed concentrations of polyphosphate. We obtained a linear double reciprocal plot with parallel lines (Fig. 3a). When polyphosphate was varied, we obtained a similar graph with parallel lines (data not shown).

Distribution of putative PPGKs in cyanobacteria and PPGK activity in cell-free cyanobacterial extracts

To examine the distribution of PPGKs among cyanobacteria, we performed a BLASTP search (Altschul et al., 1997) against all sequenced cyanobacterial genomes (September 2013; 141 genomes) using the amino acid sequence of All1371 as the query. Our analysis revealed that in 34% of all sequenced cyanobacteria a putative PPGK is present. PPGKs were found in all five sections of cyanobacteria (Table 2) with the highest frequency in the heterocyst-forming species of Section IV (85%) and Section V (54.5%), followed by the non-heterocystous species of Section III (52.9%), Section II (50%), and Section I (5.7%). More complete data are presented in Table S1.

![Figure 3](image-url)

**Fig. 3.** Kinetics of the All1371 reaction. (a) Activity of All1371. Primary double-reciprocal plot of initial velocity with glucose as the variable substrate and different concentrations of polyP$_{45}$ (PP) as the fixed substrate (pH 7.5; $n=3$). (b) Schematic of the bi-bi ping-pong mechanism. Polyphosphate (PP) acts as the first substrate (A) by covalently binding to PPGK (E). The first product (P), polyphosphate reduced at one phosphate (PP$_{-1}$), is released, and binding of the second substrate, glucose (B), occurs on the phosphorylated enzyme (E-P). Finally, the second product, glucose 6-phosphate (Q), is released and the enzyme is restored (E).
Table 2. Distribution of PPGKs among cyanobacteria

| Section |
|---------|
| I       | II      | III     | IV      | V       |
| No. of PPGKs | 4 | 3 | 18 | 17 | 6 |
| No. of cyanobacteria | 70 | 6 | 34 | 20 | 11 |
| Percentage | 5.7 | 50.0 | 52.9 | 85.0 | 54.5 |

(available in the online Supplementary Material). Cell-free extracts of some of these cyanobacteria were tested for specific PPGK activity under different nitrogen conditions, including Anabaena (Section V), Mastigocladus laminosus (Section VI) (Nürnberg et al., 2014), F. muscicola (Section V) and Synechocystis (Section I), the last lacking a predicted PPGK (negative control). As expected, cell-free extracts of Synechocystis did not show any PPGK activity, whereas those of Anabaena, Mastigocladus laminosus and F. muscicola exhibited detectable PPGK activity (Table 3). In Anabaena, PPGK activity was increased slightly under nitrogen depletion. PPGK activity in cell-free extracts from the two diazotrophic, branched filamentous cyanobacterial strains of Section V was three- to fourfold higher than in non-diazotrophic, branched filamentous cyanobacterial strains of Section I (Nu¨rnberg et al., 2014). They identified numerous transcription start sites (TSSs) in Anabaena, including a TSS of all1371 at position 1625,874. The organization of the predicted all1371 promoter region is depicted in Fig. 5(a). We identified a putative palindromic motif that is likely to be a (DIF) + motif (Mitschke et al., 2011). This putative (DIF) + motif displays an inverse orientation and one mismatch [Fig. 5a; (DIF) + in bold type, mismatch in red, AGCCCT].

Expression analysis using a GFP promoter fusion

The transcriptional changes experienced by Anabaena during nitrogen-depletion-induced cell differentiation were recently analysed by Mitschke et al. (2011). They identified numerous transcription start sites (TSSs) in Anabaena, including a TSS of all1371 at position 1625,874. The organization of the predicted all1371 promoter region is depicted in Fig. 5(a). We identified a putative palindromic motif that is likely to be a (DIF) + motif (Mitschke et al., 2011). This putative (DIF) + motif displays an inverse orientation and one mismatch [Fig. 5a; (DIF) + in bold type, mismatch in red, AGCCCT].

To investigate the expression of all1371 along the filaments, the gfp gene was transcriptionally fused to the all1371 promoter and transformed into the Δall1371 mutant strain. In the absence of combined nitrogen, we noted distinct GFP fluorescence in the mature heterocysts of 4-day-old filaments of the promoter fusion strain (Fig. 5b). An overview of fluorescence (GFP, autofluorescence) of the promoter fusion strain is given in Fig. S4. No distinct GFP fluorescence was observed in the filaments of the promoter fusion strain when combined nitrogen was supplied (Fig. 5b) compared with Δall1371 (Fig. S5). GFP fluorescence in heterocysts was first detected 24 h after nitrogen step down, and persisted until the filaments were harvested 4 days later (data not shown). Quantification of GFP fluorescence of numerous cells in the promoter–gfp fusion strain in comparison with cells of the parent strain Δall1371 confirmed our microscopic observations: the morphologically mature heterocysts. Analysis of Δall1371 mutant and WT cells under different light and nitrogen conditions revealed that viability of the mutant was distinctly decreased under combined nitrogen-limiting conditions (Fig. 4b, Fig. S2). This effect was increased under light–dark cycle conditions, but in this case we also noted a reduced viability of the mutant in the presence of nitrate (Fig. S3b).

Table 3. PPGK activities in cell-free extracts of cyanobacteria

Measurements were performed at 28–30 °C and pH 7.5; n ≥ 3; ND, not detectable (≤ 1 nmol min⁻¹ mg⁻¹).

| Strain          | Section | Growth conditions | PPGK activity (nmol min⁻¹ mg⁻¹) (mean ± SD) |
|-----------------|---------|-------------------|---------------------------------------------|
| Anabaena        | IV      | +N                | 4.3 ± 1.3                                   |
|                 |         | −N                | 5.8 ± 1.3                                   |
| Anabaena Δall1371 | IV    | +N                | ND                                          |
|                 |         | −N                | ND                                          |
| F. muscicola    | V       | +N                | 15.3 ± 2.3                                  |
|                 |         | −N                | 13.1 ± 2.1                                  |
| Mastigocladus laminosus | V | +N                | 23.6 ± 0.7                                 |
|                 |         | −N                | 13.9 ± 3.2                                 |
| Synechocystis   | I       | +N                | ND                                          |
|                 |         | −N                | ND                                          |

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fluorescence intensity in heterocysts \(n=42\) of the promoter fusion strain was \(\sim 2.1\)-fold higher than in vegetative cells \(n=71\). The ratio of fluorescence intensity from heterocysts \(n=18\) to vegetative cells \(n=34\) in filaments of the parent strain \(\Delta all1371\) was 1 : 0.9 (Table S2).

**DISCUSSION**

**Identification of the putative PPGK, All1371**

The structural and functional motifs found in all the aligned amino acid sequences (Fig. 1, boxed) are predicted to interact with the substrates glucose, mannose, ATP and polyphosphate (Liao et al., 2012; Mukai et al., 2003). Five of the identified domains have been proposed to interact with the ATP molecule: phosphate-1 and phosphate-2 are involved in binding \(\beta\)-phosphates and \(\gamma\)-phosphates, while connect-1, connect-2 and the adenosine motif interact with the adenine ring of ATP (Mukai et al., 2003). The conserved aspartate residue in the connect-1 region (Fig. 1, star) is believed to be essential for catalytic activity (Arora et al., 1991; Mukai et al., 2004). The glucose motif has been suggested to participate in glucose binding. The heptapeptide PEAPAAG (Fig. 1, underlined) was proposed to be responsible for mannose phosphorylation in the sequence.

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**Fig. 4.** The \(\Delta all1371\) mutant. (a) Schematic of the chromosomal region surrounding \(all1371\) and the gene inactivation strategy, in which \(all1371\) was replaced with the C.K3 cassette. (b) Viability analyses of *Anabaena* wild-type (WT) and the \(all1371\) knockout mutant strain \((\Delta all1371)\) on AA-agar plates containing 10 mM KNO\(_3\) \(+N\) or lacking combined nitrogen \(–N\). In total, 4.8 ng chlorophyll \(a\) per spot was plated. The plates were incubated under continuous light for 6 days.

**Fig. 5.** All1371 promoter activity in heterocysts. (a) The promoter region of \(all1371\), including the TSS and the \(-10\) region (boxed) (Mitschke et al., 2011), was integrated into a self-replicating plasmid. A promoter-less *gfp* gene was integrated via the underlined Xbal site (altered bases are depicted in lower case); the presumed DIF+ domain (AGCCCT) is shown in bold. (b) Fluorescence in the *Anabaena* \(\Delta all1371\) promoter fusion strain grown under N\(_2\)-fixing conditions \(–N\) and grown with combined nitrogen \(+N\). A heterocyst is indicated with an arrow. Bars, 5 \(\mu\)m. AF, auto-fluorescence (red); GFP, GFP fluorescence (green).
of *Arthrobacter* sp. strain KM (Mukai et al., 2003). The phosphate-3 motif was predicted to be a binding region for polyphosphate (Liao et al., 2012). Residues Trp193 and Trp198 (Fig. 1, grey shading) were proposed to be essential for catalytic activity in *Mycobacterium tuberculosis* (Phillips et al., 1999). A residue equivalent to Trp198 is present in the amino acid sequence of *Anabaena* (Fig. 1, underlined W). Furthermore, the phosphate-1 domain is likely to contain a putative polyphosphate-binding site, as both the anionic phosphates used as ligands in a crystallographic study (Mukai et al., 2004) bound at highly conserved amino acid residues similar to Lys25. It was proposed that there may be shared ATP- and polyphosphate-binding sites in the phosphate-1 and phosphate-2 regions (Mukai et al., 2004). Thus, the present amino acid sequence analysis of All1371 (Fig. 1) and the previous findings in similar proteins collectively suggest that All1371 functions as a PPGK.

**Purification of All1371, and molecular mass determination**

The purified All1371 appeared as a single protein band of 26 kDa in SDS-PAGE analysis (Fig. 2a, lane 3). This result is consistent with the expected molecular mass of 26.6 kDa calculated with the ProtParam tool (http://web.expasy.org/protparam/) (Wilkins et al., 1999) for one monomer of the recombinant All1371.

The protein peak of 39 kDa obtained in size-exclusion chromatography (Fig. 2b) indicates that the native enzyme may exist as either a monomer or a homodimer. PPGK homodimers have also been reported in *Mycobacterium tuberculosis*, *Propionibacterium shermanii* and *Propionibacterium arabinosum* (Phillips et al., 1999), whereas the polyphosphate/ATP-dependent glucomannokinase of *Arthrobacter* sp. strain KM was determined to exist as a monomer (Mukai et al., 2003).

**Biochemical properties of All1371**

All1371 uses polyphosphate exclusively to phosphorylate glucose and mannose (Table 1) and is strictly dependent on the presence of divalent cations. This requirement for divalent cations is shared with the PPGKs of *Microlunatus phosphovorus* (Tanaka et al., 2003), *Arthrobacter* sp. (Mukai et al., 2003), *Mycobacterium tuberculosis* and *Mycobacterium phlei* (Szymona & Ostrowski, 1964; Szymona & Widomski, 1974). Recently, Mg$^{2+}$ was found to be an indispensable cofactor for the PPGK of *Thermobifida fusca* (Liao et al., 2012). Here, we report that All1371 showed high substrate specificity and acted as a strict polyphosphate-dependent enzyme. This result is a notable feature, as most of the previously described glucokinases utilized either ATP alone, or ATP and polyphosphate. The previous *in vitro* studies on PPGKs revealed that these enzymes were often bi-functional and not restricted to polyphosphate. For example, the PPGKs from *Mycobacterium tuberculosis* (Hsieh et al., 1996a), *Propionibacterium shermanii* (Phillips et al., 1993) and *Corynebacterium glutamicum* (Lindner et al., 2010) were also able to use ATP or GTP. The present work showed that, along with the PPGK of *Microlunatus phosphovorus* (Tanaka et al., 2003), All1371 is one of only two known PPGKs that uses only polyphosphate as its phosphate donor.

Kinetic analyses of All1371 (Table 1) revealed a relatively low $K_M$ value obtained for polyphosphate (1.76 μM), suggesting that All1371 has a high affinity for its sole substrate. In comparison with the $K_M$ values for polyphosphate and glucose (Table 1), the polyphosphate- and ATP-dependent PPGK from *Corynebacterium glutamicum* yielded $K_M$ values of 0.2 mM for polyP$_{45}$ and 1 mM for glucose (Lindner et al., 2010); the PPGK of *Propionibacterium shermanii* yielded a $K_M$ value of 1.2 μM for polyP$_{35}$ (Phillips et al., 1993); the PPGK of *Mycobacterium tuberculosis* yielded a $K_M$ value of 4.6 μM for polyP$_{35}$ (Phillips et al., 1999); and the PPGK of *Microlunatus phosphovorus* yielded a $K_M$ of 3.8 mM for polyP$_{30}$ (Tanaka et al., 2003). The turnover number of All1371 of 48.2 s$^{-1}$ (Table 1) is comparable with the $k_{cat}$ value of 57.0 s$^{-1}$ determined for the PPGK from *Propionibacterium shermanii* against polyP$_{35}$ (Phillips et al., 1999).

According to our analyses, All1371 is a polyphosphate-dependent glucomannokinase. Interestingly, the heptapeptide in the sequence of *Arthrobacter* sp. strain KM (Fig. 1, underlined), which is assumed to be responsible for mannose phosphorylation (Mukai et al., 2004), is not present in the corresponding *Anabaena* sequence. The results of additional kinetic analyses (Fig. 3a) were consistent with the so-called ‘bi-bi ping-pong’ mechanism (Cleland, 1963). As illustrated in Fig. 3(b), this mechanism is a particular multi-substrate reaction that includes two substrates and two products (bi-bi) and is characterized by alternating processes of substrate binding and product release (ping-pong) for the two substrates. In a first step, polyphosphate is covalently bound to All1371, which is then phosphorylated. Approximately one orthophosphate-reduced polyphosphate is released from the enzyme as the first product. In a second step, glucose is bound to the phosphorylated enzyme, and the second substrate is phosphorylated. Glucose 6-phosphate is released as a second product, and the enzyme returns to its initial state (Fig. 3b). In contrast, the ATP/polyphosphate-dependent PPGK of *Mycobacterium tuberculosis* (Hsieh et al., 1996a) and the ATP-dependent glucokinase from *Streptomyces coelicolor* (Imrisko et al., 2005) were both found to display ordered bi-bi sequential mechanisms. The ordered bi-bi sequential mechanism differs from the bi-bi ping-pong mechanism in that both substrates (glucose and ATP or polyphosphate) bind to the enzyme first before the two products are released.

**Putative PPGKs in cyanobacteria**

Cyanobacteria may be grouped into five sections according to their morphology (Rippka et al., 1979). While species of Sections I and II are unicellular forms, those of Sections III, IV and V show filamentous forms. Cyanobacteria of
Sections IV and V are additionally able to form heterocysts. The highest level of complexity is seen among Section V strains, which form true branches within their filaments (Golubic et al., 1996). Diazotrophic growth has been observed in both unicellular and filamentous strains (reviewed by Stal, 1995). Our BLASTP search revealed that PPGKs were found very frequently in cyanobacteria of Sections IV and V, which are all diazotrophic strains forming heterocysts. All genomes of the analysed Section IV and Section V strains contained PPGK genes. In about half of the analysed genomes of Section III we found putative PPGK genes (52.9%). In 11 of these 18 PPGK gene-containing genomes (61%) we also found nifH genes encoding the key enzyme of N₂ fixation (Table S1). Some of these Section III organisms are known to fix N₂ under micro-oxic conditions, such as *Pseudanabaena* sp. ATCC 27183 (Rippka & Waterbury, 1977) (synonymous with *Pseudanabaena* sp. PCC 6802). Among cyanobacteria of Section II, 50% of sequenced unicellular strains were also predicted to contain a PPGK. All are known to fix N₂ under anaerobic conditions (Rippka et al., 1979) or to have a nitrogenase complex (Rippka & Waterbury, 1977), or a putative dinitrogenase has been annotated in the genome (Markowicz et al., 2012). Furthermore, strains belonging to *Chroococcidiopsis* are closely related to the heterocyst-forming cyanobacteria (Fewer et al., 2002). These facts may suggest a possible correlation between PPGK appearance and the ability to fix N₂ under anoxic/micro-oxic conditions provided by either heterocysts or the environment. This presumption is supported by the results obtained by analysing Section I organisms. Only four (5.7%) of the unicellular strains of Section I were found to contain a putative PPGK. *Synechococcus* sp. PCC 7335 and *Synechococcus* sp. PCC 7502 arrose through morphological transition events (Robertson et al., 2001; Shih et al., 2013). Interestingly, putative PPGKs were not found in the genomes of *Cyanothecae* strains that are able to grow diazotrophically in diurnal rhythm. Based on the present findings, we hypothesized that the presence of a PPGK in cyanobacterial genomes is strongly related to the organism’s ability to fix N₂ in heterocysts. A correlation between PPGK appearance and an organism’s ability to fix N₂ under anoxic conditions is possible but has to be analysed further, especially from a phylogenetic point of view.

To determine whether PPGK activity was present in vivo, PPGK activity in cell-free extracts of some heterocyst-forming cyanobacteria with putative PPGKs was determined. As summarized in Table 3, PPGK activity in *Anabaena* is increased after 4 days of nitrogen depletion. An increase of PPGK activity under this condition is in line with the results of Flaherty et al. (2011). Using deep sequencing analyses performed 21 h after nitrogen deprivation, they found a 4.8-fold increase in the mRNA expression level of *all1371*. Furthermore, the increased PPGK activity is in line with a previous report (Thompson et al., 1994), showing that in *Anabaena flos-aquae*, phosphate is stored as sugar phosphate under N₂-fixing conditions, but as polyphosphate in the presence of combined nitrogen. We found that PPGK activity was higher in cell-free extracts from thermophilic Section V strains of *F. muscicola* and *Mastigocladus laminosus* than in *Anabaena* (Table 3). A higher in vitro activity might be the result of a higher robustness of the PPGK due to its thermophilic origin (Beadle et al., 1999). Interestingly, we observed an increased PPGK activity in cell-free extracts obtained from these strains grown in the presence of nitrate than under diazotrophic conditions. The higher complexity of Section V strains differing in the regulation of diazotrophic growth (Nürnberg et al., 2014) might explain this observation.

### Viability of Δ*all1371*

The impaired viability of the mutant implies that All1371 plays an important role in providing glucose-6-phosphate in *Anabaena*, supporting the canonical hexokinase. This is supported by the PPGK activity measured in cell-free extracts of *Anabaena* obtained from nitrate-supplemented cultures (Table 3). Under diazotrophic conditions heterocysts are not able to fix carbon dioxide. Carbon compounds, probably in the form of sucrose (Curatti et al., 2002), are imported from vegetative cells. NAD(P)H, needed as a reducing equivalent, is generated in heterocysts (Maldener & Muro-Pastor, 2010). There, glucose 6-phosphate is used as substrate for glucose-6-phosphate dehydrogenase, a main enzyme of the oxidative pentose phosphate pathway. Because of the decreased viability of the mutant observed under diazotrophic conditions, we conclude that All1371 may represent an alternative enzyme completing the hexokinase under ATP-consuming (diazotrophic) growth conditions.

### Expression analysis using a GFP promoter fusion

In cyanobacteria, the nitrogen-regulated genes are mainly controlled by the transcriptional regulators NtcA and HetR (Kumar et al., 2010). Recently, chromatin immunoprecipitation analysis followed by high-throughput sequencing was used to identify all of the NtcA-binding sites of *Anabaena* at 3 h after a nitrogen step down (Picossi et al., 2014). Interestingly, they detected an internal NtcA-binding site in *all1371* whereas the impact of binding of NtcA remains unclear. Further HetR-controlled promoters characterized by a differentiation-related change (DIF) + motif (TCCGGGA, a palindrome at or close to position −35) were identified by comparing results found in *Anabaena* with a ΔHetR mutant 8 h after a nitrogen step down (Mitschke et al., 2011). The putative (DIF) + motif with an inverse orientation located in the promoter region of *all1371* (Fig. 5 a) additionally indicates that the promoter might be HetR-dependent. In fact, the promoter of *all1371* responded to nitrogen depletion in WT but not in the ΔhetR mutant, indicating a HetR dependency (W. Hess, personal communication). Our results obtained with a GFP promoter fusion strain show that the *all1371* promoter activity is particularly enhanced under nitrogen
starvation in mature heterocysts. \(\text{N}_2\) fixation in heterocysts is an energy-intensive process requiring 16 molecules of ATP to reduce one molecule of \(\text{N}_2\) (Hill et al., 1981; Howard & Rees, 1996). The ability of PPGKs to utilize polyphosphate instead of ATP for glucose phosphorylation might allow the heterocysts to save ATP for the essential process of \(\text{N}_2\) fixation.

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**REFERENCES**

Achbergerová, L. & Nahálka, J. (2011). Polyphosphate – an ancient energy source and active metabolic regulator. *Microb Cell Fact* 10, 63.

Adams, D. G., Carr, N. G. & Wilcox, M. (1981). The developmental biology of heterocyst and akinete formation in cyanobacteria. *Crit Rev Microbiol* 9, 45–100.

Ahn, K. & Kornberg, A. (1990). Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate. *J Biol Chem* 265, 11734–11739.

Akiyama, M., Crooke, E. & Kornberg, A. (1993). An exopolyphosphatase of *Escherichia coli*. The enzyme and its ppx gene in a polyphosphate operon. *J Biol Chem* 268, 633–639.

Allen, M. B. & Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol* 30, 366–372.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389–3402.

Arona, K. K., Filburn, C. R. & Pedersen, P. L. (1991). Glucose phosphorylation. Site-directed mutations which impair the catalytic function of hexokinase. *J Biol Chem* 266, 5359–5362.

Baier, A. (2013). Untersuchungen zum stickstoffinduzierten Phosphobilisomenabbau - NHA, ein kleines Protein mit großer Wirkung. Doctoral thesis, Humboldt-Universität zu Berlin.

Beadle, B. M., Baase, W. A., Wilson, D. B., Gilkes, N. R. & Shoichet, B. K. (1999). Comparing the thermodynamic stabilities of a related thermophilic and mesophilic enzyme. *Biochemistry* 38, 2570–2576.

Bensadoun, A. & Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. *Anal Biochem* 70, 241–250.

Berman-Frank, I., Lundgren, P. & Falkowski, P. (2003). Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Res Microbiol* 154, 157–164.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 62, 293–300.

Black, T. A. & Wolk, C. P. (1994). Analysis of a Het- mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. *J Bacteriol* 176, 2282–2292.

Black, T. A., Cai, Y. & Wolk, C. P. (1993). Spatial expression and autoregulation of hetR, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* 9, 77–84.

Cai, Y. P. & Wolk, C. P. (1990). Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J Bacteriol* 172, 3138–3145.

Castenholz, R. W. (1988). Culturing methods for cyanobacteria. *Methods Enzymol* 167, 68–93.

Cleland, W. W. (1963). The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. *Biochim Biophys Acta* 67, 188–196.

Curatti, L., Flores, E. & Salerno, G. (2002). Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp. *FEBS Lett* 513, 175–178.

Dagan, T., Roettger, M., Stucken, K., Landan, G., Koch, R., Major, P., Gould, S. B., Goremykin, V. V., Rippka, R. & other authors (2013). Genomes of Stigonematal cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. *Genome Biol Evol* 5, 31–44.

de Marsac, N. T. & Houmard, J. (1988). Complementary chromatographic adaptation: physiological conditions and action spectra. *Methods Enzymol* 167, 318–328.

Elhai, J. & Wolk, C. P. (1988a). Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol* 167, 747–754.

Elhai, J. & Wolk, C. P. (1988b). A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene* 68, 119–138.

Fewer, D., Friedl, T. & Büdel, B. (2002). *Chroococcidiopsis* and heterocyst-differentiating cyanobacteria are each other’s closest living relatives. *Mol Phylogenet Evol* 23, 82–90.

Flaherty, B. L., Van Nieuwerburgh, F., Head, S. R. & Golden, J. W. (2011). Directional RNA deep sequencing sheds new light on the transcriptional response of *Anabaena* sp strain PCC 7120 to combined-nitrogen deprivation. *BMC Genomics* 12, 332.

Flores, E. & Herrera, A. (2010). Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol* 8, 39–50.

Golubic, S., Hernandez-Marine, M. & Hoffmann, L. (1996). Developmental aspects of branching in filamentous *Cyanophyta*. *Cyanobacteria*. *Arch Hydrobiol Suppl Algol Stud* 83, 303–329.

Gouet, P., Courcelle, E., Stuart, D. I. & Métoz, F. (1999). ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15, 305–308.

Harold, F. M. (1966). Inorganic polyphosphates in biology: structure, metabolism, and function. *Bacterial Rev* 30, 772–794.

Hill, S., Kennedy, C., Kavanagh, E., Goldberg, R. B. & Hanau, R. (1981). Nitrogen fixation gene (*nifL*) involved in oxygen regulation of nitrogenase synthesis in *K. pneumoniae*. *Nature* 290, 424–426.

Howard, J. B. & Rees, D. C. (1996). Structural basis of biological nitrogen fixation. *Chem Rev* 96, 2965–2982.

Hsieh, P. C., Shenoy, B. C., Jentoft, J. E. & Phillips, N. F. (1993). Purification of polyphosphate and ATP glucose phosphotransferase from *Mycobacterium tuberculosis* H37Ra: evidence that poly(P) and ATP glucokinase activities are catalyzed by the same enzyme. *Protein Expr Purif* 4, 76–84.

Hsieh, P. C., Kowalczyk, T. H. & Phillips, N. F. (1996a). Kinetic mechanisms of polyphosphate glucokinase from *Mycobacterium tuberculosis*. *Biochemistry* 35, 9772–9781.

Hsieh, P. C., Shenoy, B. C., Samols, D. & Phillips, N. F. (1996b). Cloning, expression, and characterization of polyphosphate glucokinase from *Mycobacterium tuberculosis*. *J Biol Chem* 271, 4909–4915.

Imriskova, I., Arreguin-Espinosa, R., Guzmán, S., Rodríguez-Sandoja, R., Langley, E. & Sanchez, S. (2005). Biochemical characterization of the glucose kinase from *Streptomyces coelicolor* compared to *Streptomyces peucetius* var. caesius. *Res Microbiol* 156, 361–366.
Jensen, T. E. (1968). Electron microscopy of polyphosphate bodies in a blue-green alga Nostoc pruniforium. Arch Mikrobiol 62, 144–152.

Kornberg, A. (1995). Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177, 491–496.

Kornberg, A., Kornberg, S. R. & Simms, E. S. (1956). Metaphosphate synthesis by an enzyme from Escherichia coli. Biochim Biophys Acta 20, 215–227.

Kornberg, A., Rao, N. N. & Ault-Riché, D. (1999). Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68, 89–125.

Kumar, K., Mella-Herrera, R. A. & Golden, J. W. (2010). Cyanobacterial heterocysts. Cold Spring Harb Perspect Biol 2, a000315.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). Clustal W and Clustal_X version 2.0. Bioinformatics 23, 2947–2948.

Liao, H., Myung, S. & Zhang, Y. H. (2012). One-step purification and immobilization of thermophilic polyphosphate glucokinase from Thermotoga maritima ZYK81: glucose-6-phosphate generation without ATP. Appl Microbiol Biotechnol 93, 1109–1117.

Lichko, L. P., Kukakovskaya, T. V. & Kulaev, I. S. (2010). Properties of partially purified endopolyophosphatase of the yeast Saccharomyces cerevisiae. Biochemistry (Mosc) 75, 1404–1407.

Lindner, S. N., Knebel, S., Pallerla, S. R., Schobert, S. M. & Wendisch, V. F. (2010). Cg2091 encodes a polyphosphate/ATP-dependent glucokinase of Corynebacterium glutamicum. Appl Microbiol Biotechnol 87, 703–713.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265–275.

Maldener, I. & Muro-Pastor, A. M. (2010). Cyanobacterial heterocysts. In eLS. Chichester: Wiley. [doi:10.1002/9780470015902.a0000306.pub2]

Markowitz, V. M., Chen, I. M., PALaniappan, K., Chu, K., Szeto, E., Grechkin, Y., Ratner, A., Jacob, B., Huang, J. & other authors (2012). IMG: the Integrated Microbial Genomes database and comparative analysis system. Nucleic Acids Res 40 (Database issue), D115–D122.

Meyer, A. (1902). Orientierende Untersuchungen über Verbreitung, Morphologie, und Chemie des Volitins. Bot Zeitschr 62, 113–152.

Mitschke, J., Vioque, A., Haas, F., Hess, W. R. & Muro-Pastor, A. M. (2011). Dynamics of transcriptional start site selection during nitrogen stress-induced cell differentiation in Anabaena sp. PCC7120. Proc Natl Acad Sci U S A 108, 20130–20135.

Mitsui, A., Kumazawa, S., Takehashi, A., Ikemoto, H., Cao, S. & Ariai, T. (1986). Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. Nature 323, 720–722.

Mukai, T., Kawai, S., Matsukawa, H., Matuo, Y. & Murata, K. (2003). Characterization and molecular cloning of a novel enzyme, inorganic polyphosphate/ATP-glucomannokinase, of Arthrobacter sp. strain KM. Appl Environ Microbiol 69, 3849–3857.

Mukai, T., Kawai, S., Mori, S., Mikami, B. & Murata, K. (2004). Crystall structure of bacterial inorganic polyphosphate/ATP-glucomannokinase. Insights into kinase evolution. J Biol Chem 279, 50591–50600.

Nakao, M., Okamoto, S., Kohara, M., Fujiyoshi, T., Fujisawa, T., Satoh, S., Tabata, S., Kaneko, T. & Nakamura, Y. (2010). CyanBase: the cyanobacteria genome database update 2010. Nucleic Acids Res 38 (Database issue), D379–D381.

Nürnberg, D. J., Mariscal, V., Parker, J., Mastroianni, G., Flores, E. & Mullineaux, C. W. (2014). Branching and intercellular communication in the Section V cyanobacterium Mastigocladus laminosus, a complex multicellular prokaryote. Mol Microbiol 91, 935–949.

Pepin, C. A. & Wood, H. G. (1986). Polyphosphate glucokinase from Propionibacterium shermanii. Kinetics and demonstration that the mechanism involves both processive and nonprocessive type reactions. J Biol Chem 261, 4476–4480.

Phillips, N. F., Horn, P. J. & Wood, H. G. (1983). The polyphosphate- and ATP-dependent glucokinase from Propionibacterium shermanii: both activities are catalyzed by the same protein. Arch Biochem Biophys 300, 309–319.

Phillips, N. F., Hsieh, P. C. & Kowalczyk, T. H. (1999). Polyphosphate glucokinase. Prog Mol Biol Cell 23, 101–125.

Picosi, S., Flores, E. & Herrero, A. (2014). ChIP analysis unravels an exceptionally wide distribution of DNA binding sites for the NtcA transcription factor in a heterocyst-forming cyanobacterium. BMC Genomics 15, 22.

Rao, N. N., Gómez-Garcia, M. R. & Kornberg, A. (2009). Inorganic polyphosphate: essential for growth and survival. Annu Rev Biochem 78, 605–647.

Rashid, M. H. & Kornberg, A. (2000). Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97, 4885–4890.

Rashid, M. H., Rao, N. N. & Kornberg, A. (2000). Inorganic polyphosphate is required for motility of bacterial pathogens. J Bacteriol 182, 225–227.

Remonsellez, F., Orell, A. & Jerez, C. A. (2006). Copper tolerance of the thermoacidophilic archaean Sulfolobus metallicus: possible role of polyphosphate metabolism. Microbiology 152, 59–66.

Resnick, S. M. & Zehnder, A. J. (2000). In vitro ATP regeneration from polyphosphate and AMP by polyphosphate:AMP phosphotransferase and adenylate kinase from Acinetobacter johnsonii 210A. Appl Environ Microbiol 66, 2045–2051.

Rippka, R. & Waterbury, J. B. (1977). Synthesis of nitrogenase by non-heterocystous cyanobacteria. FEMS Microbiol Lett 2, 83–86.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111, 1–61.

Robertson, B. R., Tezuka, N. & Watanabe, M. M. (2001). Phylogenetic analyses of Synechococcus strains (cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. Int J Syst Evol Microbiol 51, 861–871.

Scherer, P. A. & Bochem, H. P. (1983). Ultrastructural investigation of 12 Methanosarcinae and related species grown on methanol for occurrence of polyphosphatelike inclusions. Can J Microbiol 29, 1190–1199.

Shih, P. M., Wu, D., Latifi, A., Axen, S. D., Fewer, D. P., Talla, E., Calteau, A., Cai, F., Tandeau de Marsac, N. & other authors (2013). Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. Proc Natl Acad Sci U S A 110, 1053–1058.

Stal, L. J. (1995). Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol 131, 1–32.

Szymona, M. & Ostrowski, W. (1964). Inorganic polyphosphate glucokinase of Mycobacterium phlei. Biochim Biophys Acta 85, 283–295.

Szymona, M. & Widomska, J. (1974). A kinetic study on inorganic polyphosphate glucokinase from Mycobacterium tuberculosis H37RA. Physiol Chem Phys 6, 393–404.

Tanaka, S., Lee, S. O., Hamaoka, K., Kato, J., Takiguchi, N., Nakamura, K., Ohlke, H. & Kuroda, A. (2003). Strically polyphosphate-dependent glucokinase in a polyphosphate-accumulating bacterium, Microlunatus phosphovorus. J Bacteriol 185, 5654–5656.
Thompson, P. A., Oh, H. M. & Rhee, G. Y. (1994). Storage of phosphorus in nitrogen-fixing Anabaena flos-aquae (Cyanophyceae). J Phycol 30, 267–273.

Toepel, J., Welsh, E., Summerfield, T. C., Pakrasi, H. B. & Sherman, L. A. (2008). Differential transcriptional analysis of the cyanobacterium Cyanothece sp. strain ATCC 51142 during light-dark and continuous-light growth. J Bacteriol 190, 3904–3913.

Tsutsumi, K., Munekata, M. & Shiba, T. (2000). Involvement of inorganic polyphosphate in expression of SOS genes. Biochim Biophys Acta 1493, 73–81.

Walker, P. A., Leong, L. E., Ng, P. W., Tan, S. H., Waller, S., Murphy, D. & Porter, A. G. (1994). Efficient and rapid affinity purification of proteins using recombinant fusion proteases. Biotechnology (N Y) 12, 601–605.

Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D. & Hochstrasser, D. F. (1999). Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 112, 531–552.