

Different *gvpC* length variants are transcribed within single filaments of the cyanobacterium *Planktothrix rubescens*

Sven Becker, Paul K. Hayes and Anthony E. Walsby

School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK

Correspondence

Sven Becker
SvenBecker@gmx.ch

Received 14 June 2004
Revised 14 September 2004
Accepted 14 October 2004

Transcripts of the gas vesicle genes *gvpA* and *gvpC* were detected in single filaments of the cyanobacterium *Planktothrix rubescens* using reverse transcription and quantitative real-time PCR. Primers were designed to amplify short sequences within *gvpA* and three length variants of *gvpC*. With genomic template DNA, and using Sybr Green to monitor product accumulation, similar amplification efficiencies were observed for each of these genes. The relative copy numbers of *gvpC* length variants in genomic DNA from five *Planktothrix* gas vesicle genotypes determined by real-time PCR were similar to those indicated by sequencing the gas vesicle gene clusters. The precipitation of *gvp* cDNA reverse-transcribed from cellular RNA from single filaments was required before amplification of the gene fragments; without this step it was not possible to detect the accumulation of the expected amplicons by dissociation analysis. Precipitation was also necessary to ensure the generation of product curves that allowed linear regression in an early stage of PCR, a prerequisite for the quantification of low-input cDNA amounts without the need for standard curves. This report shows that different *gvpC* length variants are transcribed within single *Planktothrix* filaments, both from laboratory cultures and from natural samples taken from Lake Zürich. This has implications for the efficiency of buoyancy provision by the possible production of gas vesicles of different strengths within individual cyanobacterial filaments. The hypothesis that post-transcriptional regulation may influence the type of protein (GvpC) present in gas vesicles is presented.

INTRODUCTION

In planktonic cyanobacteria, gas vesicles, which may account for about 10 % of total cell protein, provide buoyancy and enable these organisms to control their depth on vertically decreasing light gradients in lakes. Gas vesicles are an important factor in cyanobacteria outcompeting other phytoplankton organisms (Oliver, 1994). Measurements of the rates at which cyanobacteria change their buoyant density when placed at different irradiances form the basis of understanding the vertical distribution of cyanobacteria in lakes and reservoirs (e.g. Kromkamp & Walsby, 1990); however, the observations that have been made on buoyancy change are several levels of understanding away from the molecular mechanisms that control buoyancy.

The buoyant density of a cell is determined by several classes of components which make the cell either more or less dense than water; of these, only the gas vesicle provides significant buoyancy. Gas vesicles are made entirely of proteins, which form a cylindrical shell enclosing a hollow space. There are two principal protein components, GvpA,

which forms the ribs of the hollow cylindrical structure, and GvpC, which binds to the outer surface of the ribs, strengthening them against collapse by external pressure (for review see Walsby, 1994). Proteins encoded by other gas vesicle genes have been detected in gas vesicles of halobacteria (Shukla & DasSarma, 2004). GvpD and GvpE have been found to function as a repressor and activator, respectively, of the promoter for *gvpA* in *Halobacterium salinarum* (Pfeifer *et al.*, 2002).

In cyanobacteria, changes in gas vesicle production have been studied in species of *Anabaena* and *Planktothrix* (*Oscillatoria*) (Oliver & Walsby, 1984; Utkilen *et al.*, 1985), but what determines these changes at the molecular level is not known. However, it is assumed that a change in gas vesicle production must involve changes in the expression of relevant genes. The genes involved in gas vesicle production have been identified and sequenced in several cyanobacteria; the principal protein-encoding structural genes, *gvpA* and *gvpC*, occur in various arrangements in gene clusters containing additional ORFs. *Planktothrix* spp. possess *gvpA* genes that are nearly identical to those in *Anabaena* and *Calothrix* sp.; the *gvpC* genes, however, show only limited similarity to those in these two organisms

Abbreviations: C_T, threshold cycle; RT, reverse transcription.

(Griffiths *et al.*, 1992; Albouy *et al.*, 2001). In *Anabaena* there are multiple *gvpA* cistrons in tandem repeat followed by a single copy of *gvpC* (Hayes & Powell, 1995); in *Planktothrix* there are two or more copies of both *gvpA* and *gvpC* in an alternating arrangement (Beard *et al.*, 1999).

Specific promoter regions of gas vesicle gene operons have not been investigated in cyanobacteria; however, in *Calothrix* it has been demonstrated by Northern hybridization that *gvpA* and *gvpC* are co-transcribed (Damerval *et al.*, 1987), while *Anabaena* produces single and multicistronic *gvpA* transcripts and also transcripts containing both *gvpA* and *gvpC* (Hayes & Powell, 1995). The *gvp* transcripts from *Planktothrix* sp. strain CYA18 are considerably longer than predicted for the single ORFs (J. Kromkamp & P. K. Hayes, unpublished) and may include several of the alternating *gvpA* and *gvpC* genes now known to be present in many *Planktothrix* strains. Some strains of *Planktothrix* have only one length variant of *gvpC*, while others have two different variants, *gvpC*¹⁶ and *gvpC*²⁰ (e.g. strain Pla 9736), or *gvpC*²⁰ and *gvpC*²⁸ (e.g. strain PCC 7821). The occurrence of these three length variants of *gvpC* in *Planktothrix* strains is correlated with the production of gas vesicles of different diameters and, consequently, of different strengths (Beard *et al.*, 1999, 2000). This allows a selection of different gas vesicle genotypes in lakes of different depths (Walsby & Bleything, 1988; Bright & Walsby, 1999).

For cyanobacterial genotypes with two *gvpC* length variants it is not known whether the genes are expressed differentially in response to environmental conditions. In order to perform culture-independent *in situ* studies on single filaments, a sensitive and specific methodology is needed to detect very low amounts of *gvpC* transcripts. This is possible with quantitative real-time PCR. The real-time monitoring of amplicon accumulation in PCR allows calibration by the threshold-cycle method (Heid *et al.*, 1996), i.e. quantification is based on the number of cycles required to reach a certain concentration of amplicons rather than on the concentration reached after a fixed number of cycles in end-point analysis. The threshold cycle (C_T) is defined as the number of PCR cycles at which a fluorescence signal, developed by a dye–template complex or by TaqMan chemistry (Becker *et al.*, 2000), exceeds a pre-set value. The C_T value is reached in few PCR cycles if a large number of templates is initially present, but requires many cycles if the reaction starts with few templates.

In this study we describe a protocol for the reverse-transcription of mRNA from a single cyanobacterial filament to cDNA, which is then used as a template in real-time PCR for the detection of different gas vesicle gene (*gvp*) transcripts. We demonstrate the potential of this methodology for the measurement of transcript abundance of individual genes in single filaments grown in laboratory culture or collected from their natural environment. Such studies will extend the knowledge of buoyancy regulation in cyanobacteria and their vertical movements in lakes.

METHODS

Cyanobacterial cultures. *Planktothrix rubescens* strains Pla 9303, Pla 9316 (Walsby *et al.*, 1998) and Pla 9736 (Beard *et al.*, 1999) from Lake Zürich and PCC 7821 from Lake Gjesjøen in Norway (Skulberg & Skulberg, 1985) were grown at 20 °C in 100 ml mineral medium (Bright & Walsby, 2000) in an incident photon irradiance of 3–10 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Sampling of *Planktothrix* filaments from Lake Zürich. On August 12 and 13 2002, water samples from 8, 10.5 and 15 m depth were taken from Lake Zürich above the deepest point of the lake, immediately concentrated by filtration as described by Walsby *et al.* (1998) and stored on ice. Each single filament of *Planktothrix rubescens* was picked under a binocular microscope with a sterile syringe and washed successively in three drops of a sterile mineral medium (Bright & Walsby, 2000) containing all ingredients except FeNaEDTA and NaHCO₃. Finally, a single filament was washed in one drop of sterile nuclease-free water (Sigma), transferred to 30 μl nuclease-free water and kept on ice before it was frozen at –20 °C. The samples were shipped to Bristol on dry ice; through delay they arrived at room temperature, but were refrozen and then kept at –70 °C until further processing.

Nucleic acid extraction and synthesis of first strand cDNA from a single filament. Genomic DNA from *Planktothrix* batch cultures was extracted as described by Beard *et al.* (1999). The concentration of DNA was calculated from the A_{260} measured with a diode-array S2000 UV/Vis spectrophotometer (WPA). For RNA extraction, a single filament was picked from a *Planktothrix* laboratory culture as described above and transferred to 20 μl nuclease-free water (Sigma) with 20 units Rnasin ribonuclease inhibitor (Promega) and 2.2 mM Dithiothreitol (Sigma) before two cycles of freezing/thawing (–20 °C/on ice) were applied in order to fracture the cells, followed by freezing of the lysate at –20 °C, storing at –70 °C and thawing on ice prior to further processing.

Before DNase treatment, the thawed lysate was centrifuged for 5 min at 5000 r.p.m. at 4 °C to pellet unlysed cells and cell debris. A 15 μl quantity of the supernatant was transferred to a fresh reaction tube and mixed with 2 units RQ1 RNase-free DNase (Promega), 2 μl RQ1 DNase 10 \times reaction buffer (Promega) and 40 units Rnasin ribonuclease inhibitor (Promega). After incubation at 37 °C (45 min), 3 μl stop solution (20 mM EGTA, Promega) was added and the reaction mixtures were incubated for another 10 min at 65 °C to inactivate the DNase.

Before thawing the Lake Zürich samples from –70 °C, 40 units Rnasin ribonuclease inhibitor (Promega), 0.85 μl dithiothreitol (final concentration 2.3 mM; Sigma) and 5.25 μl nuclease-free water (Sigma) were added to the 30 μl of water that contained a single filament. The samples then underwent two cycles of freezing/thawing (see above). For DNase treatment, a larger quantity (31.4 μl) of the lysate (supernatant after centrifugation, see above) was taken and the amounts of all other components of the reaction mixtures (see above) were adjusted accordingly.

For synthesis of first strand cDNA, 7.5 μl of DNase-treated lysate from a single filament was mixed with 7.5 μl 10 μM reverse primer (Table 1), incubated at 70 °C (4 min) and 60 °C (1 min), and then kept on ice prior to the addition of 10 μl of a master mix containing the following: 200 units M-MLV Reverse Transcriptase (Promega), 5 μl 5 \times M-MLV Reverse Transcriptase reaction buffer (Promega), 1.25 mM dNTPs, 24 units Rnasin ribonuclease inhibitor (Promega) and 1.25 mM dithiothreitol (Sigma). The reaction mixtures were incubated at 42 °C for 1 h. Control reaction mixtures without reverse transcriptase were treated as above and contained the mixture

Table 1. Oligonucleotide primers for gas vesicle genes in *Planktothrix rubescens*

Primer*	Sequence (5'→3')	Target	Position (GenBank accession no.)†
GVPF	CCTCCAGTCTGGCCGAAG	<i>gvpA</i>	929–946 (AJ132357)
GVPAR	ACCGAAGGAACAGCCGC	<i>gvpA</i>	1118–1102 (AJ132357)
GVP16F	CAGAAACAGCACCAGCAAATG	<i>gvpC</i> ¹⁶	179–199 (AJ132354)
GVP16R	GGTTTAATAGGATGTCGGTGGG	<i>gvpC</i> ¹⁶	363–342 (AJ132354)
GVP20F	AAATCAGGCTTCTGAATTCGG	<i>gvpC</i> ²⁰	364–385 (AJ132357)
GVP20R	CCATTTGCTGGTGCAGAATATC	<i>gvpC</i> ²⁰	548–527 (AJ132357)
GVP28F	GTCACGGGTGTACAACAACAAC	<i>gvpC</i> ²⁸	166–188 (AJ253131)
GVP28R	TTGCTGATGCAGTTGGGC	<i>gvpC</i> ²⁸	351–334 (AJ253131)

*The melting temperature according to software Primer Express (version 2.0.0, Applied Biosystems) is 58 °C for all primers. F, forward primer; R, reverse primer.

†Position in the corresponding genes in *Planktothrix rubescens* from Lake Zürich. Accession numbers (in parentheses) according to GenBank/EMBL nucleotide sequence database.

of lysate and reverse primer, 5× M-MLV Reverse Transcriptase reaction buffer (Promega), dNTPs and water.

The cDNA (25 µl) was precipitated following a protocol modified from Liss (2002): 2.5 µg glycogen (Ambion) and 625 ng polydA DNA (Amersham Pharmacia) were added to the cDNA but no carrier RNA. The dried pellet was redissolved in nuclease-free water (Sigma) by incubating for 15 min at 45 °C.

Quantitative real-time PCR. Oligonucleotide primers for gas vesicle genes *gvpA* and the three known *gvpC* length variants in *Planktothrix rubescens* (Table 1) were constructed using alignments of the *gvp* sequences published by Beard *et al.* (1999, 2000) and other sequences from the GenBank/EMBL nucleotide sequence database. The binding positions of all primers were chosen to produce amplicons of 185 to 200 bp and to achieve maximum specificity. All primer sequences were checked for specificity by a BLAST search in the GenBank/EMBL nucleotide sequence database. For quantitative PCR, 10 µl reaction mixtures contained 4 µl template (genomic DNA or cDNA), 900 nM of each primer (Table 1) and the components of the Sybr Green PCR core reagents kit (Applied Biosystems): 0.25 units AmpliTaq Gold DNA polymerase; 0.1 units Amperase UNG; 200 µM dATP, dCTP, dGTP; 400 µM dUTP; 3 mM MgCl₂ and 1 µl 10× SYBR Green buffer with ROX fluorescence dye as a passive reference for normalization. For real-time monitoring of the fluorescence increase during successive cycles in PCR, a Sequence Detection System 7000 (Applied Biosystems) was used. The thermal programme was as follows: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 20 s at 94 °C and 1 min at 68 °C.

The threshold cycle (C_T) of real-time PCR assays is defined as the number of PCR cycles at which the fluorescence signal exceeds a pre-set value (Heid *et al.*, 1996). In all assays C_T was determined at a normalized fluorescence of 0.5; log-linear standard curves were obtained by plotting the C_T of reactions with various amounts of serially diluted DNA versus the dilution factor. From the slope (s) of the curves, the amplification efficiency (E) of the PCR assays was calculated as $10^{-1/s} - 1$ (Klein *et al.*, 1999). Differences in the C_T values (ΔC_T) between separate PCR assays containing the same species and same amount of genomic template DNA, but different primer pairs, were used to calculate ratios of gas vesicle genes in *Planktothrix* genotypes. This is possible because the number of amplicons with similar lengths is the same in different assays when their fluorescence signals reach the threshold (at which the C_T value is determined). In the general case (with a PCR efficiency of 1) the ratio of the initial copy numbers between two assays is equal to $2^{\Delta C_T}$. Therefore, if $\Delta C_T = 1$,

$2^{\Delta C_T} = 2$, i.e. one assay contained twice as many initial copies as the other. In the assay with half the initial copy number, one extra cycle is required to reach the same threshold amplicon number.

The ability of primers to discriminate between the three different *gvpC* length variants (Table 1) was assessed by using the three primer pairs in separate PCR assays with a template DNA species that carries only one of the target genes. If a non-target primer pair resulted in the generation of a product curve, i.e. a C_T value could be determined, the difference between the C_T values (ΔC_T) of the target and the non-target assays was calculated. The percentage of template molecules that amplify with the non-target primers is given by the term $100/2^{\Delta C_T}$.

Dissociation analysis. The thermal programme for real-time PCR was terminated with a temperature gradient between 60 and 95 °C. Since Sybr Green fluoresces only upon binding to double-stranded DNA, the decrease of fluorescence with increasing temperature provides a measure of the melting behaviour of a certain amplicon. By determining $-dF/dT$, the negative derivative of fluorescence (F) versus temperature (T), individual melting profiles were obtained for each amplicon. The identity of amplicons derived from cDNA was checked by comparing their melting profiles with those of *gvp* fragments amplified from genomic DNA.

Gel electrophoresis. After completion of the thermal programme, 1 µl of the real-time PCR reaction mixtures was loaded on 10% polyacrylamide (acrylamide-*N,N'*-methylene-bisacrylamide, 30:0.8) gels of 0.8 mm thickness. Marker lanes contained 2 µl of a 100 bp DNA ladder (Promega). PCR products were separated in a Mini Protean II gel electrophoresis system (Bio-Rad) with 1× TBE running buffer (pH 8) at 50 V (3 h). The gels were stained for 30 min with 1× Sybr Gold nucleic acid gel stain (Molecular Probes) in 1× TAE buffer and photographed under UV illumination.

RESULTS

Evaluation of real-time PCR with genomic DNA

To evaluate the performance of the real-time PCR assays used in this study, all gas vesicle gene primer pairs were used with genomic DNA from a *Planktothrix* culture known to carry the target genes. By plotting the C_T values of the assays versus the dilution factor of DNA, log-linear standard curves were obtained over four to six orders of

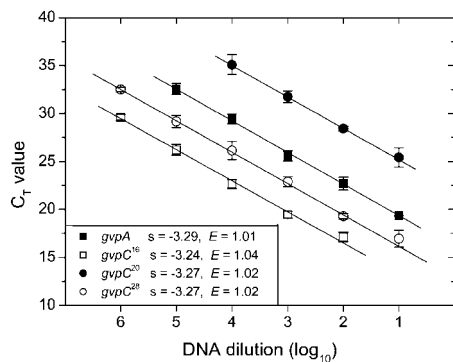


Fig. 1. Standard curves for real-time PCR amplification of gas vesicle genes in serially diluted genomic DNA from various *Planktothrix* strains. *gvpA*, strain Pla 9401 ($n=6$, $r=1.000$); *gvpC¹⁶*, strain Pla 9736 ($n=4$, $r=0.998$); *gvpC²⁰*, strain Pla 9303 ($n=8$, $r=1.000$); *gvpC²⁸*, strain CYA 137 ($n=8$, $r=1.000$). Genomic DNA purified from *Planktothrix* batch cultures was used as a template in 10 μ l real-time PCR assays with Sybr Green as a detector. DNA amount per reaction: *gvpA*, 0.39 pg to 3.9 ng; *gvpC¹⁶*, 0.82 pg to 8.2 ng; *gvpC²⁰*, 0.2 pg to 0.2 ng; *gvpC²⁸*, 0.18 pg to 18 ng. The C_T values (number of PCR cycles at which product curves exceed the fluorescence threshold) of the assays are plotted versus the \log_{10} of the dilution factor of template DNA. s , Slope of linear fit (see above for values of r); E , amplification efficiency = $10^{-1/s} - 1$ (Klein *et al.*, 1999).

magnitude (Fig. 1, see legend for amounts of DNA per single reaction). From the slopes of the curves the amplification efficiencies in the assays were calculated to lie between 1.01 and 1.04, indicating a doubling of the short amplicons in every cycle of PCR. The contribution of any non-specific PCR products (e.g. primer dimers) to the fluorescence signal can be quantified, either by performing a dissociation analysis after the PCR run (see Methods) or by electrophoretic separation of the PCR products (see below). After completion of the thermal programme, all real-time PCR assays in this study were subjected to a dissociation analysis. With this analysis, the standard assays depicted in Fig. 1 showed single fluorescence peaks representing the desired PCR products with dissociation temperatures between 78 and 82 °C; peaks at lower dissociation temperatures that would represent primer dimers were not detected (data not shown).

The ability of the primer pairs to discriminate between the three *gvpC* length variants was assessed by the amplification of genomic DNA from two *Planktothrix* strains of contrasting genotypes. For this analysis, the same DNA was used as in the assays shown in Fig. 1; therefore, an amplification efficiency (E) of 1 was assumed for the calculation of the degree of non-target amplification. With DNA from *Planktothrix* sp. Pla 9303, a strain with two copies of *gvpA* and *gvpC²⁰* (Beard *et al.*, 2000), the lowest C_T value (28.17) was found with primers for *gvpC²⁰* (100 % signal), primers for *gvpC²⁸* produced no signal, and assays

with primers for *gvpC¹⁶* gave a C_T value of 34.91. Assuming $E=1$, the difference in the C_T values ($\Delta C_T=6.74$) shows that the signal with the primers for *gvpC¹⁶* is $100/2^{6.74}=0.94\%$ of that with primers for *gvpC²⁰*, indicating a small degree of mis-priming. With *Planktothrix* sp. CYA 137 (two copies of *gvpA* and *gvpC²⁸*; Beard *et al.*, 2000) the primer pairs for *gvpC¹⁶* and *gvpC²⁰* led to mis-priming of 0.45 % and 0.008 %, respectively. Consequently, the copy number of specific *gvpC* variants may be overestimated by a maximum of 1 %.

To test the reliability of our new real-time PCR assays, we used them to determine the relative *gvp* gene copy numbers in genomic DNA from five *Planktothrix* strains of different *gvp* genotypes that had been characterized by sequencing (Beard *et al.*, 1999, 2000). We used 10^3 -fold diluted target DNA of strains Pla 9303, Pla 9316 and CYA 137 that had been used in the assays shown in Fig. 1; thus an amplification efficiency of 1 was assumed. By the construction of log-linear standard curves, the amplification efficiency with DNA from strains Pla 97112 and PCC 7821 was checked and also found to be close to 1 (data not shown). With the C_T values of the real-time PCR assays, the ratios of the *gvp* genes were calculated and compared with those revealed by sequencing the gene clusters (Beard *et al.*, 1999, 2000). For genotypes 2a, 3b and 4a the calculated relative gene contents were similar to those found by sequence analysis, but in genotypes 1 and 6 the assays underestimated the copy number of *gvpA* relative to *gvpC²⁰* or *gvpC²⁸* copies (Table 2).

All assays from the experimental series shown in Table 2 were additionally analysed by gel electrophoresis in order to visualize the amplified fragments. As shown for genotypes 1, 2a and 3b (Fig. 2), the expected 185–190 bp amplicons were abundant in all assays (data for genotypes 4a and 6 not shown); no primer dimers were detected, but unidentified amplicons of 450 to 900 bp were present. In genotypes 2a, 3b (Fig. 2) and 4a (data not shown) an amplicon that may represent 769 bp could be explained by the amplification between two *gvpA* cistrons that occur in close proximity (Beard *et al.*, 1999). Similar long and persistent side products that may stem from genes other than those in *gvp* clusters have also been detected in gas vesicle genotyping with genomic DNA from single *Planktothrix* filaments (Beard *et al.*, 1999). We could not identify the side products by dissociation analysis (data not shown) because they may have a similar melting temperature to the desired amplicons. However, the side products contribute to the fluorescent signal and therefore cannot explain the relative underestimation of *gvpA* in genotypes 1 and 6 (Table 2).

Real-time RT-PCR with single *Planktothrix* filaments from cultures

A study by Liss (2002) on RT-PCR at the single-cell level had shown that the amplification of small amounts of cDNA can be inhibited by traces of reverse transcriptase, but this

Table 2. Ratio of *gvp* gene copy numbers in *Planktothrix* gas vesicle genotypes

Genotype (strain)	Ratio of <i>gvp</i> gene copies		
	Beard <i>et al.</i> (2000)	Real-time PCR*	(C _T values†)
1 (Pla 9303)	1 A : 1 C ²⁰	0.38 A : 1 C ²⁰	(33.31 : 31.93)
2a (Pla 9316)	4 A : 3 C ²⁰	4 A : 3.23 C ²⁰	(27.23 : 27.54)
3b (Pla 97112)	6 A : 3 C ¹⁶ : 1 C ²⁰	6 A : 3.69 C ¹⁶ : 0.84 C ²⁰	(20.58 : 21.28 : 23.11)
4a (PCC 7821)	4 A : 3 C ²⁰ : 1 C ²⁸	4 A : 2.68 C ²⁰ : 1.64 C ²⁸	(23.01 : 23.59 : 24.46)
6 (CYA 137)	1 A : 1 C ²⁸	0.41 A : 1 C ²⁸	(23.42 : 22.14)

*See Methods for calculation based on C_T values.

†Standard deviation of C_T values not shown; coefficient of variation of all assays (n=4) ≤0.04.

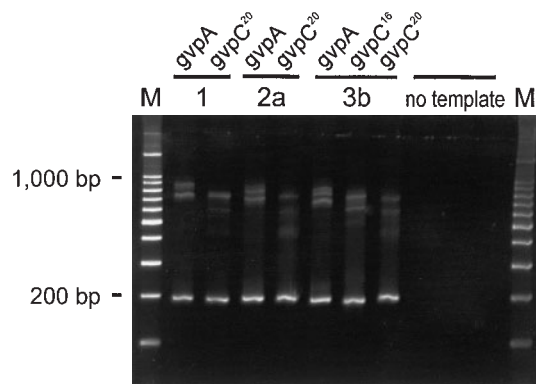


Fig. 2. Separation of real-time PCR products from assays for the amplification of gas vesicle genes *gvpA*, *gvpC*¹⁶ and *gvpC*²⁰ in *Planktothrix* gas vesicle genotypes 1, 2a and 3b (see Table 2). 10 µl PCR assays contained 4 µl 10³-fold diluted genomic DNA as a template. After completion of the thermal programme, 1 µl of the assays was loaded on a 10% acrylamide gel. Three no-template PCR assays contained primer pairs for genes *gvpA*, *gvpC*¹⁶ or *gvpC*²⁰ (assays shown in this order). M, 100 bp DNA ladder (Promega).

inhibition can be prevented if the cDNA is purified by precipitation. We found that reverse transcriptase, if added to a conventional PCR assay in the amount that is usually carried over with the cDNA template, completely inhibited the amplification of genomic DNA, and that this problem could not be circumvented by heat inactivation of the RT (data not shown).

Consequently, we decided to evaluate the effect of cDNA precipitation on real-time PCR performance with cDNA from single filaments of a *Planktothrix* Pla 9303 culture that had been grown in batch cultures for 20 to 57 days. One set of PCR primers (for *gvpA* or *gvpC*²⁰) was used on subsamples of unprecipitated and precipitated cDNA derived from the same filament. For all the 20 to 57 day old cultures, irrespective of the cDNA treatment, we obtained a real-time PCR signal (C_T value) (Table 3). No signal was observed in control assays with a portion of the cell lysates that were not subjected to reverse transcription (-RT control). Precipitation not only led to exponential product curves that had a linear relationship before the plateau phase (data not shown), but also significantly reduced the C_T value of most of the assays, indicating removal of PCR

Table 3. Effect of cDNA precipitation on *gvp* transcript detection in single filaments of *Planktothrix* strain Pla 9303

Filament	Days after inoculation	Target gene	C _T value* (amplicon†)		-RT control*
			Unprecipitated	Precipitated	
1	20	<i>gvpA</i>	35.95 (-)	25.05 (+)	ND
2	20	<i>gvpC</i> ²⁰	33.38 (+)	23.21 (+)	ND
3	27	<i>gvpA</i>	43.76 (-)	27.54 (+)	ND
4	27	<i>gvpC</i> ²⁰	31.47 (+)	24.16 (+)	ND
5	33	<i>gvpA</i>	41.79 (-)	24.92 (+)	ND
6	33	<i>gvpC</i> ²⁰	29.83 (+)	24.14 (+)	ND
7	57	<i>gvpC</i> ²⁰	27.56 (+)	27.99 (+)	ND
8	57	<i>gvpC</i> ²⁰	27.34 (+)	27.07 (+)	ND

ND, No signal detected.

*Mean of n=2.

†Confirmation (+) of the amplicon identity by dissociation analysis (see Methods).

inhibitors (most probably reverse transcriptase). In only one assay (Table 3, filament 7) did precipitation increase the C_T value slightly, possibly because of the loss of a small amount of template. The carrier DNA that is used in the protocol usually prevents this, however. Dissociation analysis confirmed that *gvpA* amplicons were present only when precipitated cDNA was used as the template (Table 3). It appears, therefore, that purification of cDNA by precipitation is essential for real-time RT-PCR detection of certain *gvp* transcripts in a single cyanobacterial filament.

In a separate experiment we amplified *gvpC*²⁰ cDNA from single filaments of two other *Planktothrix* strains (PCC 7821 and Pla 9316) and separated the PCR products by gel electrophoresis. With precipitated undiluted or 10-fold diluted cDNA from three filaments, the expected *gvpC*²⁰ fragment (185 bp) was amplified; no products accumulated in the -RT controls (Fig. 3). In contrast to results with genomic DNA (Fig. 2), the production of unidentified amplicons was significantly reduced, particularly with the 10-fold diluted cDNA. The identity of the *gvpC*²⁰ amplicon was confirmed by dissociation analysis in all assays (Fig. 4a for filament 1, other data not shown).

Detection of *gvpC* transcripts in single *Planktothrix* filaments from cultures and lake samples

With the new methodology described above (using 10-fold diluted precipitated cDNA in real-time PCR) we were able

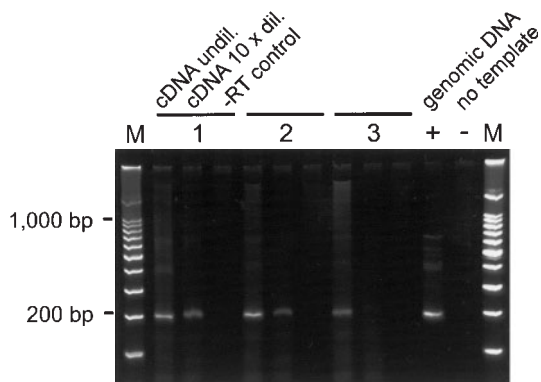


Fig. 3. Single filament RT-PCR. First strand *gvpC*²⁰ cDNA from three *Planktothrix* filaments (compare sections 1 to 3 of the gel) was amplified in 10 μ l real-time PCR assays. Filaments 1 and 3, *Planktothrix* strain PCC 7821; filament 2, strain Pla 9316. A 4 μ l quantity of undiluted or 10-fold diluted cDNA was used as a template or lysate that was not subjected to reverse transcription (-RT control). A 4 μ l quantity of 10³-fold diluted genomic DNA from strain PCC 7821 was amplified as a positive control (+). After completion of the thermal programme, 1 μ l of the assays was loaded on a 10% acrylamide gel. M, 100 bp DNA ladder (Promega).

to determine which *gvpC* transcripts were present in single filaments of two laboratory *Planktothrix* cultures. In the three filaments of strain Pla 9736 investigated we detected *gvpC*¹⁶ and *gvpC*²⁰ transcripts and in one filament of strain PCC 7821 the presence of *gvpC*²⁰ and *gvpC*²⁸ transcripts was observed (Table 4). Transcripts of *gvpA* were found in all filaments investigated and no signal occurred in the

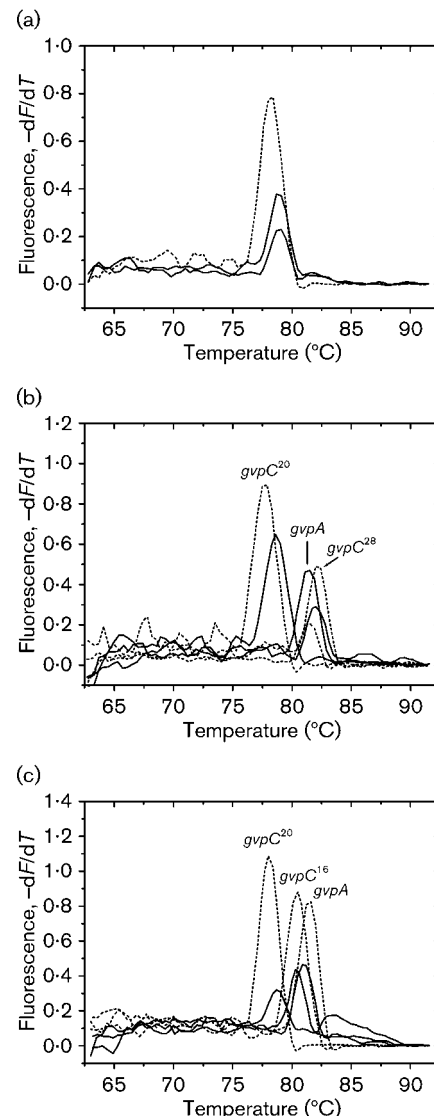


Fig. 4. Dissociation analysis of amplified *gvp* cDNAs from single *Planktothrix* filaments. The negative derivative of fluorescence versus temperature ($-dF/dT$) is plotted against temperature. Solid lines, cDNA amplicons from single filaments; dashed lines, amplicons from genomic *Planktothrix* DNA. (a) *gvpC*²⁰ in cultured strain PCC 7821 (filament 1 in Fig. 3) with undiluted (high peak) and 10-fold diluted cDNA (low peak). (b) *gvpA*, *gvpC*²⁰ and *gvpC*²⁸ in cultured strain PCC 7821 (filament 4 in Table 4). (c) *gvpA*, *gvpC*¹⁶ and *gvpC*²⁰ in natural filament 2 from Lake Zürich (Table 5).

Table 4. Transcripts of *gvp* in single filaments of two *Planktothrix* strains

Filament	Strain	C_T value*				-RT control
		<i>gvpA</i>	<i>gvpC</i> ¹⁶	<i>gvpC</i> ²⁰	<i>gvpC</i> ²⁸	
1	Pla 9736	36·14	34·5	<u>28·64</u>	–	ND
2	Pla 9736	36·46	35·78	34·86	–	ND
3	Pla 9736	37·18	34·94	36·8	–	ND
4	PCC 7821	<u>31·49</u>	–	<u>28·7</u>	35·52	ND

ND, No signal detected.

*With 10-fold diluted cDNA; results from undiluted cDNA are underlined.

–RT controls. In all assays the identity of the corresponding amplicon was confirmed by dissociation analysis (Fig. 4b for strain PCC 7821, other data not shown). However, in three assays this confirmation was possible only with undiluted cDNA; note that in Fig. 3 only undiluted cDNA from filament 3 resulted in an amplification product. In Fig. 4(b) we observed a shift between the fluorescence signals generated with the genomic template and *gvpC*²⁰ cDNA; the major area of the latter peak was, however, within the peak of the control DNA.

We also applied the new real-time PCR assays to *Planktothrix* filaments from Lake Zürich (Table 5). In the summer of 2002, single filaments were picked from water samples that had been taken from various depths of the lake. Real-time PCR was performed with cDNA from single filaments and primer pairs for gas vesicle genes that have been identified in *Planktothrix rubescens* from this habitat (Beard *et al.*, 2000). In all assays, an amplification signal (C_T value) was observed, no signal occurred in the –RT controls. The presence of the expected amplicons could only be confirmed (by dissociation analysis) for filaments 2 and 5 (Fig. 4c for filament 2, other data not shown). In both of these filaments, transcripts of *gvpA*, *gvpC*¹⁶ and *gvpC*²⁰ were present.

DISCUSSION

RT-PCR detection of *gvpC* length variant transcripts in a single cyanobacterial filament

The presence of different length variants of the gas vesicle gene *gvpC* in *Planktothrix rubescens* is associated with the occurrence of gas vesicles with different diameters and strengths (Bright & Walsby, 1999; Beard *et al.*, 2000). In genotypes with two *gvpC* length variants it is not known whether the genes are transcribed differentially in response to environmental conditions. We therefore evaluated real-time RT-PCR to analyse transcript patterns in single cyanobacterial filaments.

Real-time PCR can be calibrated by the threshold-cycle (C_T) method (Heid *et al.*, 1996), which is based on the number of amplification cycles required to reach a certain concentration of amplicons. To evaluate the overall performance and amplification efficiencies of the new PCR assays used in this study, we constructed log–linear standard curves with genomic *Planktothrix* DNA as a template (Fig. 1). The assays targeting *gvpA* and three *gvpC* length variants showed amplification efficiencies between 1·01 and 1·04, indicating a doubling of the amplicon concentration in each PCR cycle. The detection limits of the assays were similar, between 0·18 and 0·82 pg DNA. With a mean detection limit of 0·4 pg DNA per assay, a molecular mass of 650 Da of one base pair (bp) and an assumed *Planktothrix* genome size of 4·5 Mbp, the analytical sensitivity of the 10 μ l assays can be calculated as 82 genomes (Becker *et al.*, 2000).

If the amplification efficiency is known and genomic DNA is used as a template, differences in the C_T values between real-time PCR assays can be used to calculate the copy number ratio of different target genes. For three of the five *Planktothrix* gas vesicle genotypes investigated, the ratios of *gvpC* genes were similar to those determined by DNA sequencing (Table 2), although slight deviations from the expected ratios were observed in the genotypes 3b (*gvpC*¹⁶) and 4a (*gvpC*²⁸). However in genotypes 1 and 6, the relative

Table 5. Transcripts of *gvp* in natural *Planktothrix* filaments from Lake Zürich

Filament	Sampling date (dd/mm/yy)	Water depth (m)	C_T value* (amplicon identity†)			–RT control*
			<i>gvpA</i>	<i>gvpC</i> ¹⁶	<i>gvpC</i> ²⁰	
1	12/08/02	8	27·81 (+)	23·55 (–)	23·41 (–)	ND
2	13/08/02	8	27·71 (+)	25·00 (+)	23·80 (+)	ND
3	13/08/02	10·5	27·54 (–)	25·87 (–)	24·21 (–)	ND
4	13/08/02	10·5	28·04 (–)	21·47 (+)	23·82 (–)	ND
5	13/08/02	15	27·63 (+)	24·18 (+)	23·63 (+)	ND
6	13/08/02	15	29·38 (–)	34·00 (+)	24·35 (–)	ND

ND, No signal detected.

*Mean of $n=2$.

†Confirmation (+) of the amplicon identity by dissociation analysis (see Methods).

gvpA copy number was underestimated by a factor of about 2.5. We assume that the observed deviations are due to variation in the amplification efficiencies of single assays that can lead to shifts of C_T values. Additionally, calculations based on the slopes of standard curves as shown in Fig. 1 may overestimate the amplification efficiency of single reactions (Ramakers *et al.*, 2003). This can lead to amplification efficiencies slightly higher than 1 (compare Fig. 1). Therefore, for quantitative transcription analysis, and because it is impossible to construct standard curves with cDNA from single cyanobacterial filaments, it is essential to monitor the amplification efficiencies of single reactions (see below).

Real-time PCR not only provides a sensitive and specific detection method, but, when performing a dissociation analysis with Sybr Green as a detector, also allows the amplicon identity to be checked in the same assay, a prerequisite for reliable transcription studies. The dissociation analysis also circumvents the time-consuming electrophoretic separation of PCR products and makes it possible to identify the contribution of non-specific PCR products, such as primer dimers, to the fluorescence signal.

In this study we describe a protocol for the extraction of mRNA from a single cyanobacterial filament. After reverse transcription, small amounts of cDNA were detected in real-time PCR. By comparing the C_T values of these assays with the results of gel-based amplicon separation (Fig. 3) and fluorescent dissociation analysis (Fig. 4), we showed that it is possible to detect transcripts of more than one type of *gvpC* variant in a single *Planktothrix* filament. Hence, transcripts of *gvpC*¹⁶ and *gvpC*²⁰ or *gvpC*²⁰ and *gvpC*²⁸ were identified in laboratory cultures of *Planktothrix* strains Pla 9736 and PCC 7821, respectively (Table 4). These results indicate that both *gvpC* length variants are transcribed under our laboratory conditions.

In single *Planktothrix* filaments from Lake Zürich (Table 5) transcripts of both *gvpC*¹⁶ and *gvpC*²⁰ were found. These two length variants have been identified in *Planktothrix* isolates from this habitat (Beard *et al.*, 2000). The presence of different *gvpC* transcripts in a single cyanobacterial filament has not previously been described. Currently no information is available to indicate whether the presence of two different *gvpC* transcripts reflects the expression (i.e. the translation to protein) of the two genes. Immunoblotting of proteins from pure *Planktothrix* gas vesicles has shown that only one GvpC protein species was present in gas vesicles isolated from strains carrying two *gvpC* length variants (S. J. Beard, unpublished results). It remains possible, however, that gas vesicles of different diameters and strengths may co-occur within a single filament under appropriate environmental conditions; this might contribute to the efficiency of buoyancy provision in *Planktothrix*. We hypothesize that post-transcriptional regulation may influence the type of protein (GvpC) present in gas vesicles. Information on gas vesicle gene promoters in cyanobacteria as well as insights into a possible translational regulation of

gas vesicle formation and the structural assembly of the protein cylinders are necessary.

Quantification of transcripts in a single cyanobacterial filament

The level of transcription of a particular gene can be estimated by reverse transcription of mRNA to cDNA and measuring the number of cDNA copies by quantitative PCR. To achieve quantitative results, the preservation of mRNA in samples is crucial at the time of sampling. Since it might be difficult to maintain the required low temperature for working with RNA while processing samples, they could be treated directly with reagents that preserve the mRNA profile in the cells and allow storage at room temperature for a sufficient period of time. To achieve efficient reverse transcription of different mRNA species, it might be necessary, however, to purify extracted mRNA from single cyanobacterial filaments after preservation of the transcript profile with reagents such as RNAprotect Bacteria Reagent (Qiagen) or RNAlater (Ambion) (S. Becker, unpublished results).

In this study we confirmed results of Liss (2002), who had shown that it is essential to remove PCR inhibitors (e.g. carry-over of reverse transcriptase) from small amounts of cDNA that will be used as a template in real-time PCR. This can be done by the precipitation of cDNA. From the difference in the C_T values between the assays with precipitated and unprecipitated cDNA it is calculated that the input template amount would have been underestimated by a factor of up to 10^3 in the absence of the precipitation step (Table 3). It may be essential to use precipitated cDNA to confirm the presence of certain *gvp* amplicons by dissociation analysis (Table 3), a prerequisite for reliable transcription studies. Additionally, precipitation of cDNA appears to be essential for obtaining product curves that enable linear regression of the section before the plateau phase (data not shown), a prerequisite for quantitative analysis after Ramakers *et al.* (2003) (see below).

In real-time PCR, two quantification methods based on C_T values are available: absolute quantification by the construction of log-linear standard curves as shown in Fig. 1 (Heid *et al.*, 1996) and relative quantification by the comparative C_T method (Livak & Schmittgen, 2001). However, standard curves with cDNA from single cyanobacterial filaments are not possible and both methods are prone to quantification errors if possible deviations in the amplification efficiencies between sample and standard assays are not taken into consideration (Ramakers *et al.*, 2003). Therefore, as suggested for TaqMan chemistry (Becker *et al.*, 2000), when using Sybr Green as a detector, the amplification efficiency in real-time PCR assays needs to be monitored in order to achieve reliable quantitative results. According to Ramakers *et al.* (2003), the input template copy number in a real-time PCR assay is reflected by the input fluorescence of the assay, which can be calculated by a regression of the linear section of an

exponential product curve in the early stage of PCR. This quantification method is independent from standard curves and can be used to monitor the amplification efficiencies between samples. The measurement of cDNA from single cyanobacterial filaments in which the mRNA has been preserved, and normalization of the PCR results to the filament biomass or number of cells (based on the filament length determined by image analysis, cf. Walsby & Avery, 1996), seem feasible for quantitative transcription studies in cultures and natural samples.

ACKNOWLEDGEMENTS

We are grateful to F. Schanz, B. A. Handley and S. J. Beard for sampling Lake Zürich. B. A. Handley kindly provided genomic DNA from *Planktothrix* cultures. This work was funded through the Natural Environment Research Council (UK) by grants to A. E. Walsby and P. K. Hayes. A research fellowship by Deutsche Forschungsgemeinschaft to S. Becker is gratefully acknowledged.

REFERENCES

- Albouy, D., Castets, A. M. & Tandeau De Marsac, N. (2001). The gas vesicle gene (*gvp*) cluster of the cyanobacterium *Pseudanabaena* sp. strain PCC 6901. *DNA Seq* **12**, 337–344.
- Beard, S. J., Handley, B. A., Hayes, P. K. & Walsby, A. E. (1999). The diversity of gas vesicle genes in *Planktothrix rubescens* from Lake Zürich. *Microbiology* **145**, 2757–2768.
- Beard, S. J., Davis, P. A., Iglesias-Rodríguez, D., Skulberg, O. M. & Walsby, A. E. (2000). Gas vesicle genes in *Planktothrix* spp. from Nordic lakes: strains with weak gas vesicles possess a longer variant of *gvpC*. *Microbiology* **146**, 2009–2018.
- Becker, S., Böger, P., Oehlmann, R. & Ernst, A. (2000). PCR bias in ecological analysis: a case study for quantitative *Taq* nuclease assays in analyses of microbial communities. *Appl Environ Microbiol* **66**, 4945–4953.
- Bright, D. I. & Walsby, A. E. (1999). The relationship between critical pressure and width of gas vesicles in isolates of *Planktothrix rubescens* from Lake Zürich. *Microbiology* **145**, 2769–2775.
- Bright, D. I. & Walsby, A. E. (2000). The daily integral of growth by *Planktothrix rubescens* calculated from growth rate in culture and irradiance in Lake Zürich. *New Phytol* **146**, 301–316.
- Damerval, T., Houmard, J., Guglielmi, G., Csiszár, K. & Tandeau de Marsac, N. (1987). A developmentally regulated *gvpABC* operon is involved in the formation of gas vesicles in the cyanobacterium *Calothrix* 7601. *Gene* **54**, 83–92.
- Griffiths, A. E., Walsby, A. E. & Hayes, P. K. (1992). The homologies of gas vesicle proteins. *J Gen Microbiol* **138**, 1243–1250.
- Hayes, P. K. & Powell, R. S. (1995). The *gvpA/C* cluster of *Anabaena flos-aquae* has multiple copies of a gene encoding GvpA. *Arch Microbiol* **164**, 50–57.
- Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. (1996). Real-time quantitative PCR. *Genome Res* **6**, 986–994.
- Klein, D., Janda, P., Steinborn, R., Müller, M., Salmons, B. & Günzburg, W. H. (1999). Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification. *Electrophoresis* **20**, 291–299.
- Kromkamp, J. & Walsby, A. E. (1990). A computer model of buoyancy and vertical migration in cyanobacteria. *J Plankton Res* **12**, 161–183.
- Liss, B. (2002). Improved quantitative real-time RT-PCR for expression profiling of individual cells. *Nucleic Acids Res* **30**, e89.
- Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.
- Oliver, R. L. (1994). Floating and sinking in gas-vacuolate cyanobacteria. *J Phycol* **30**, 161–173.
- Oliver, R. L. & Walsby, A. E. (1984). Direct evidence for the role of light-mediated gas vesicle collapse in the buoyancy regulation of *Anabaena flos-aquae* (cyanobacteria). *Limnol Oceanogr* **29**, 879–886.
- Pfeifer, F., Gregor, D., Hofacker, A., Plosser, P. & Zimmermann, P. (2002). Regulation of gas vesicle formation in halophilic archaea. *J Mol Microbiol Biotechnol* **4**, 175–181.
- Ramakers, C., Ruijter, J. M., Deprez, R. H. & Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**, 62–66.
- Shukla, H. D. & DasSarma, S. (2004). Complexity of gas vesicle biogenesis in *Halobacterium* sp. strain NRC-1: identification of five new proteins. *J Bacteriol* **186**, 3182–3186.
- Skulberg, O. M. & Skulberg, R. (1985). Planktic species of *Oscillatoria* (Cyanophyceae) from Norway. Characterization and classification. *Arch Hydrobiol Suppl* **71**, 157–174.
- Utkilen, H. C., Oliver, R. L. & Walsby, A. E. (1985). Buoyancy regulation in a red *Oscillatoria* unable to collapse gas vacuoles by turgor pressure. *Arch Hydrobiol* **102**, 319–329.
- Walsby, A. E. (1994). Gas vesicles. *Microbiol Rev* **58**, 94–144.
- Walsby, A. E. & Avery, A. (1996). Measurement of filamentous cyanobacteria by image analysis. *J Microbiol Methods* **26**, 11–20.
- Walsby, A. E. & Bleything, A. (1988). The dimensions of cyanobacterial gas vesicles in relation to their efficiency in providing buoyancy and withstanding pressure. *J Gen Microbiol* **134**, 2635–2645.
- Walsby, A. E., Avery, A. & Schanz, F. (1998). The critical pressures of gas vesicles in *Planktothrix rubescens* in relation to the depth of winter mixing in Lake Zürich, Switzerland. *J Plankton Res* **20**, 1357–1375.