The diversity of gas vesicle genes in *Planktothrix rubescens* from Lake Zürich

S. J. Beard, B. A. Handley, P. K. Hayes and A. E. Walsby

Author for correspondence: A. E. Walsby. e-mail: A.E.Walsby@bristol.ac.uk

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

Part of the gas vesicle gene cluster was amplified by PCR from three strains of *Planktothrix rubescens* isolated from Lake Zürich, Switzerland. Each contains multiple alternating copies of *gvpA* and *gvpC*. All of the *gvpA* sequences in the different strains are identical. There are two types of *gvpC*: *gvpC*²⁰, of length 516 bp, encodes a 20 kDa protein of 172 amino acid residues (whose N-terminal amino acid sequence is homologous with the sequence of GvpC in *Planktothrix* [*Oscillatoria*] *agardhii*); *gvpC*¹⁶, of length 417 bp, encodes a 16 kDa protein of 139 amino acid residues that differs in lacking an internal 33-residue section. An untranslated 72 bp fragment from the 3' end of *gvpC*, designated Ω C, is also present in some strains. The two types of *gvpC* and presence of Ω C could be distinguished by the different lengths of PCR amplification products obtained using pairs of oligonucleotide primers homologous to internal sequences in *gvpC* and *gvpA*. Three genotype classes were found: GV1, containing only *gvpC*²⁰; GV2, containing *gvpC*²⁰ and Ω C; and GV3, containing *gvpC*¹⁶, *gvpC*²⁰ and Ω C. Subclasses of GV2 and GV3 contained either one or two copies of Ω C. The accompanying paper by D. I. Bright & A. E. Walsby (*Microbiology* 145, 2769–2775) shows that strains of the GV3 genotype produce gas vesicles with a higher critical pressure than those of GV1 and GV2. A PCR survey of 185 clonal cultures of *P. rubescens* isolated from Lake Zürich revealed that 3 isolates were of genotype GV1, 73 were of GV2 and 109 were of GV3. The PCR technique was used to distinguish the gas vesicle genotype, and thence the associated critical-pressure phenotype, of single filaments selected from lakewater samples. Sequence analysis of the 16S rDNA and of regions within the operons encoding phycoerythrin, phycocyanin and Rubisco confirmed that these strains of *Planktothrix* form a tight phylogenetic group.

Keywords: cyanobacterial genetics, gas vesicles, *Planktothrix rubescens*

INTRODUCTION

Many deep lakes in temperate latitudes contain populations of the red-coloured planktonic cyanobacterium *Planktothrix rubescens*, which forms layers in the metalimnion during the summer period of thermal stratification. After winter mixing, these cyanobacteria float back up to the metalimnion using the buoyancy provided by their gas vesicles. A gas vesicle collapses when exposed to a certain pressure termed its critical

pressure (p_c). For mechanical reasons p_c varies inversely with the gas vesicle's width (Walsby, 1982, 1991). In cyanobacteria from deep lakes there has been selection for narrower and stronger gas vesicles, which withstand the high hydrostatic pressure experienced when the organisms are mixed down to great depths (Walsby, 1994).

Differences in the p_c values of gas vesicles have been found in several strains of *Planktothrix rubescens* isolated from Lake Zürich, in Switzerland, and it has been commented that the proportions of the different strains in the population will change according to the depth of winter mixing each year (Walsby *et al.*, 1998). We describe here variations in gas vesicle genes of *Planktothrix* that are correlated with variations in the p_c

Abbreviations: 33RR, 33-residue repeat; p_c , critical pressure; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

The EMBL accession numbers for the sequences determined in this work are listed in Table 5.

of gas vesicles and can be used in studies of the natural selection of gas vesicle width.

Gas vesicles are hollow structures formed from two types of protein. GvpA is a small hydrophobic protein arranged along ribs that form the cylindrical structure and its conical end caps. The amino acid sequence of GvpA is highly conserved (Griffiths *et al.*, 1992). In *Pseudanabaena* there is only one copy of the gene that encodes GvpA (Damerval *et al.*, 1991) but in *Calothrix* sp. there are two identical *gvpA* genes (Damerval *et al.*, 1987) and in *Anabaena flos-aquae* there are at least five *gvpA*s in tandem repeat (Hayes & Powell, 1995).

GvpC is a larger hydrophilic protein; it is located on the outside of the structure (Walsby & Hayes, 1988) and is thought to tie the ribs together. It varies in size in different cyanobacteria and its amino acid sequence is much less conserved (Griffiths *et al.*, 1992). In *Calothrix* sp. the single *gvpC* gene contains four contiguous semiconserved repeats of 99 nt, which encode four 33-residue repeats (33RRs) within the 162 amino acid residue protein (Damerval *et al.*, 1987). In *Anabaena flos-aquae* the single *gvpC* encodes a 193 residue protein, with five 33RRs (Hayes *et al.*, 1988). The N-terminal sequences obtained from other cyanobacteria are too short to reveal if 33RRs are present. In the halophile *Dactylococcopsis salina* there are two GvpCs, of 17 and 35 kDa, in which, peculiarly, the first 24 residues are identical but subsequent sequences are not (Griffiths *et al.*, 1992). GvpCs in halobacteria show little sequence similarity with those in cyanobacteria, though they contain seven partially conserved repeats of 32–41 residues (Horne *et al.*, 1991; Jones *et al.*, 1991).

GvpC stabilizes the gas vesicle: the p_c decreases when GvpC is removed (Walsby & Hayes, 1988) but returns to its original value when the protein is replaced (Hayes *et al.*, 1992). Kinsman *et al.* (1995) made recombinant GvpCs with two, three, four or five of the 33RRs and found that their effectiveness in strengthening the structure increased with the number of repeats. GvpC may also determine the width of gas vesicles, which affects also their p_c . Gas vesicles grow from a biconical structure (Waaland & Branton, 1969; Lehmann & Jost, 1971), probably with the addition of new GvpA molecules at the middle of the structure where the cones contact one another (Walsby, 1994). After reaching a certain diameter the central portion extends as a cylinder. GvpC, which is distributed over the entire surface, must be attached to the ribs in the cones, as well as the cylinder, as the structure is assembled (Buchholz *et al.*, 1993). GvpC may, therefore, influence the transition from cone to cylinder, and hence control the final cylinder-diameter of the mature structure. In halobacteria mutations to *gvpC* cause changes in the shape of the gas vesicles that form (Offner *et al.*, 1996; DasSarma *et al.*, 1994).

We report here the occurrence in *Planktothrix rubescens*, isolated from Lake Zürich (Walsby *et al.*, 1998), of two or more repeats of an alternating

gvpA–*gvpC* gene cluster that shows variation in the size of the *gvpC* gene. Strains of a variant that contain a shortened form of *gvpC*, lacking a 99 nt section, produce gas vesicles that have a higher p_c (Bright & Walsby, 1999). We demonstrate that single filaments of these variants can be identified by PCR amplification using *gvpA*- and *gvpC*-specific primers. This provides a means of determining the proportions of these variants in natural populations of the cyanobacterium, which can be used in making a quantitative study of the natural selection of gas vesicle strength.

METHODS

Clonal cultures of *Planktothrix rubescens*. The clonal cultures Pla 9303–9316 and 9401–9402 have been described previously (Walsby *et al.*, 1998). Strain CYA 1 (Staub, 1961; Utkilen *et al.*, 1985) was obtained from the Culture Collection of the Norwegian Institute for Water Research (NIVA), Oslo. Further cultures were established from single filaments isolated from samples taken from Lake Zürich in March, June, September and October 1997. Using a sterilized Terumo syringe under a dissecting microscope, single filaments were transferred through four drops of sterile growth medium and then to 5 ml medium in a sterile tube. Cultures were incubated at 14–16 °C in illuminated growth cabinets. For the first month the tubes were pressurized (1.4 MPa) at weekly intervals to collapse gas vesicles and prevent loss of filaments floating into the drying meniscus. Subcultures were made from floating filaments to select against mutants lacking gas vesicles.

Isolation of genomic DNA from *Planktothrix rubescens*. After pressurizing (1.4 MPa) to collapse gas vesicles, filaments from 1–5 ml of culture were centrifuged at 12000 g for 10 min, washed in 100 mM Tris, 20 mM EDTA (pH 8.0) and re-suspended in 200 µl of the same buffer. Lysozyme was added to 1 mg ml⁻¹ and the suspension was incubated at 37 °C for 2 h with occasional mixing. Proteinase K (200 µg ml⁻¹) was added and the suspension was incubated at 55 °C for a further 2 h. SDS was then added to 0.1% (w/v) and the cell lysate was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and then with chloroform/isoamyl alcohol (24:1, v/v). A 0.1 × volume of 3 M sodium acetate (pH 7.0) and an equal volume of 2-propanol were added to the final aqueous phase and nucleic acids were left to precipitate at room temperature for 12 h. The precipitated DNA was collected by centrifugation, washed with 70% ethanol, vacuum-dried, and redissolved in 50 µl 10 mM Tris, 1 mM EDTA (pH 8.0).

PCR amplification, cloning and sequencing. Genomic DNA from the *Planktothrix* cultures was used as a template in PCR amplifications with the primers specific to various genes (Table 1). PCR amplification reactions (50 µl) contained 0.25–0.50 µg genomic DNA, 1 × SuperTaq buffer (10 mM Tris/HCl pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.01% gelatin; H. T. Biotechnology), 1 µM of each oligonucleotide primer, 200 µM of deoxynucleotide triphosphates (Promega) and 1 unit SuperTaq DNA polymerase (H. T. Biotechnology). The reactions were performed in an OmniGene thermal cycler (Hybaid). The reaction conditions for the principal PCR amplifications of the gene loci investigated are shown in Table 2. PCR products were analysed by agarose gel electrophoresis in 1 × TAE running buffer (Sambrook *et al.*, 1989). PCR products were isolated from amplification reactions using the High Pure PCR Product

Table 1. Oligonucleotide primers used for PCR and sequence determination

Underlined regions indicate restriction sites introduced to facilitate cloning.

Primer	Sequence* (5' to 3')	Direction†	Target site	
			Gene or spacer	Position‡
GVPA-5'	GCTCTAGAYMGIATYYTIGAYAARGG	F	<i>gvpA</i>	954–971 (AJ132357)
GVPA-3'	CGGAATTCIGCRTAYTTIARRTAIGT	R	<i>gvpA</i>	1077–1060 (AJ132357)
GVPA1	CGGGATCCTATCCATAGAAGCAAGAATCGTGATCG	F	<i>gvpA</i>	1014–1048 (AJ132357)
GVPA2	CGGGATCCGAAGCTCGATTCCAACGAGGGAAAC	R	<i>gvpA</i>	1021–997 (AJ132357)
GVPA3	GAGTTCTTTAGGCTAATCAT	R	<i>gvpC</i> –A spacer	856–837 (AJ132357)
GVPA7	ATGCACAACCCACTGACACGA	F	<i>gvpA</i> –C spacer	217–237 (AJ132357)
GVPC1	TCGGAATTCCTCCCAAACTGTCTGT	R	<i>gvpC</i>	693–670 (AJ132357)
GVPC4	CTTCGAATTCCTCGATATAGGGTT	R	<i>gvpC</i> ²⁰	509–487 (AJ132357)
GVPC5	CAGGCTTCTGAATTCGCGGAAG	F	<i>gvpC</i>	368–389 (AJ132357)
GVPC6	CGGAATTCCTCCACCGACATYCTA	F	<i>gvpC</i>	684–704 (AJ132357)
GVPO2	AGCGGATCCCAAGCCATTCCC	F	<i>gvpA</i> – Ω C spacer	1019–1039 (AJ132358)
GVPO3	GGAATGGCTTGGGATCCGCT	R	<i>gvpA</i> – Ω C spacer	1039–1019 (AJ132358)
PE β F	TAYCCTAACCGTCGYWTKGCTGC	F	<i>cpeB</i>	612–634 (X04592)
PE α R	GCRCGTTGRATRGAACTTGTAC	R	<i>cpeA</i>	1133–1111 (X04592)
PC β F	GGCTGCTTGTTCACGCGACA	F	<i>cpeB</i>	720–739 (X05239)
PC α R	CCAGTACCACCAGCAACTAA	R	<i>cpcA</i>	1405–1386 (X05239)
PC α R3	GTGACTTGACGGAGGTAGTA	R	<i>cpcA</i>	648–629 (AJ131820)
CYARUB-5'	CGAMCTGTGGAARGAATTCAGTT	F	<i>rbcl</i>	2100–2123 (J01540)
CYARUB-3'	TGGGYTTGTRAACGATGAAGCTTA	R	<i>rbcl</i>	3018–2995 (J01540)
CYANO-5'	CAGCTGCATGCAAGCTTAGAGTTTGATCCTGGCTCAG	F	16S rDNA	1439–1458 (X03538)
CYANO-3'	CAGCTGTCGACGGATCCAAAGGAGGTGATCCAGCCGCA	R	16S rDNA	2918–2898 (X03538)
PLANKTO-5'	GGAAGCTTCTTGATTGTCAAC	F	16S rDNA	354–375 (AJ132250)
PLANKTO-3'	CTGTCCCTCGAATTCCTAGTAC	R	16S rDNA	1171–1151 (AJ132250)

* Abbreviations: I, inosine; K, either G or T; M, either A or C; R, either A or G; W, either A or T; Y, either C or T.

† Direction indicates the primer orientation: forward (F) or reverse (R).

‡ Position of the target site is given with respect to a relevant sequence deposited in the EMBL or GenBank databases.

Purification Kit (Boehringer Mannheim). To eliminate problems associated with base misincorporation during PCR amplification, at least five separate PCR reactions were pooled prior to purification. For reactions generating more than one product the purification procedure was modified to allow fragments of the desired size to be purified from low-melting-point agarose (Gibco-BRL). Binding buffer was added to the excised agarose (300 μ l per 100 mg agarose) and the mixture was incubated at 56 °C, with occasional mixing, until the agarose had dissolved completely. 2-Propanol was added (150 μ l per 100 mg agarose), and the mixture was applied to the filter tube using the manufacturer's instructions. Purified PCR products were sequenced using an ABI Prism model 377 automated sequencer.

Some amplification products were cloned prior to sequencing. Following purification and restriction enzyme digestion, the PCR product was ligated to the plasmid vector pUC18, which was used to transform *Escherichia coli* strain DH5 α (Hanahan, 1983) to ampicillin resistance. Recombinant clones containing the desired insert were identified by single-colony PCR.

Plasmid DNA was prepared separately from at least five clones using the Wizard Miniprep DNA Purification Kit (Promega), and pooled prior to sequencing. All sequences were determined on both strands. Sequence analysis was performed using the Wisconsin Package version 9.1 (Genetics Computer Group).

Diagnostic PCR using DNA extracted from single filaments.

Planktothrix filaments in 1 l lakewater were collected on nitrocellulose filters of 50 mm diameter and 8 μ m pore size (Schleicher & Schuell) and resuspended in 5 ml filtered lakewater. Single filaments were transferred by Terumo microsyringe through three drops of sterile culture medium and finally one drop of sterile water to 25 μ l 2 $\times$ SuperTaq buffer in a 0.5 ml Eppendorf tube; one drop of mineral oil was added. The sample was heated to 94 °C for 5 min and stored at –20 °C. The PCR was set up by addition of the other reagents (dNTPs, oligonucleotide primers, SuperTaq polymerase and water) to a total volume of 50 μ l. Thermal cycling conditions were identical to those described in Table 2, except that an initial denaturing step of 10 min was used.

Table 2. Thermal cycling conditions for the principal PCR amplifications

All reactions were subjected to an initial denaturation step of 94 °C for 4 min and a final extension step of 72 °C for 5 min.

Locus	Primers	Reaction conditions
<i>gvpA</i>	GVPA-5' and GVPA-3'	4 × (94 °C, 45 s; 43 °C, 1 min; 72 °C, 1 min [ramp 2 s per °C]), 25 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min)
<i>gvpA/C</i>	GVPA1 and GVPA2	4 × (94 °C, 45 s; 65 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 70 °C, 1 min; 72 °C, 1 min)
<i>gvpC</i>	GVPC1 and GVPC5	4 × (94 °C, 45 s; 59 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 63 °C, 1 min; 72 °C, 1 min)
<i>cpeBA</i>	PEβF and PEzR	4 × (94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min)
<i>cpcBA</i>	PCβF and PCzR	4 × (94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min)
<i>cpcBA</i>	PCβF and PCzR3	4 × (94 °C, 45 s; 47 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min)
<i>rbcLXS</i>	CYARUB-5' and CYARUB-3'	4 × (94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min), 25 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 1 min)
16S rDNA	CYANO-5' and CYANO-3'	25 × (94 °C, 45 s; 50 °C, 1 min; 72 °C, 2 min)
16S rDNA	CYANO-5' and PLANKTO-3'	4 × (94 °C, 45 s; 51 °C, 1 min; 72 °C, 2 min), 25 × (94 °C, 45 s; 57 °C, 1 min; 72 °C, 2 min)
16S rDNA	PLANKTO-5' and CYANO-3'	4 × (94 °C, 45 s; 55 °C, 1 min; 72 °C, 2 min), 25 × (94 °C, 45 s; 59 °C, 1 min; 72 °C, 2 min)

RESULTS

Gas vesicle genes, *gvpA* and *gvpC*

Initially, universal *gvpA* primers GVPA-5' and GVPA-3' (Table 1), whose sequences were based on conserved amino acid sequences in cyanobacterial GvpA, were used to amplify a 128 bp segment within the *gvpA* of *Pla* 9303. Sequencing showed that the region between the primers encoded an amino acid sequence identical to residues 23–51 in GvpA of both the red (CYA 18) and green (CYA 29) strains of *Planktothrix* (*Oscillatoria*) *agardhii* (Griffiths *et al.*, 1992).

Primers GVPA1 and GVPA2 (Table 1), homologous to sequences within *gvpA* and originally designed for inverse PCR, generated a 1064 bp fragment when used in PCR with DNA from strain *Pla* 9303. Sequencing revealed a 516 bp ORF between two adjacent copies of *gvpA*. The 5' end of this ORF encodes an amino acid sequence showing homology to the N-terminal amino acid sequence of GvpC from *Planktothrix* (*Oscillatoria*) *agardhii* CYA 29 (10 of the first 12 residues identical). This ORF was designated *gvpC*²⁰, the suffix denoting the 20 kDa size of its inferred translation product GvpC²⁰ (Fig. 1). This distinguishes it from a smaller homologous ORF, *gvpC*¹⁶, found subsequently in other strains of *Planktothrix*. Primers GVPA1 and GVPA2 could be used in diagnostic PCR to distinguish the following three gas vesicle genotypes, which differed in the number of *gvpA* and *gvpC* genes, and in the sizes of the latter (Fig. 2a, Table 3).

Genotype GV1. In *Pla* 9303 evidence for the presence of a

second *gvpC*²⁰ downstream of the second *gvpA* was obtained by amplification of a 1059 bp segment by using the primers GVPC1 and GVPC6 (Table 1), homologous with sequences within *gvpC*²⁰. Sequencing showed that the product comprised a complete *gvpA* located between the 3' end of one *gvpC*²⁰ and the 5' end of a second *gvpC*²⁰. The sequences of the corresponding ORFs and spacers were identical to those in the amplification product obtained with the GVPA1 and GVPA2 primers, and indicated the gene arrangement shown in Fig. 3(a).

Genotype GV2. In *Pla* 9316 amplification with primers GVPA1 and GVPA2 generated products of 1064 and 595 bp (Fig. 2a). The 1064 bp product was very similar to that in *Pla* 9303 and indicated the same *gvpA*–*gvpC*²⁰–*gvpA* arrangement: subsequent studies revealed that this product is generated by two regions of the *gvp* gene cluster (Fig. 3b), which are probably identical.

The sequence of the 595 bp product showed that between two copies of *gvpA* there was a 72 bp untranslated region (designated ΩC) identical to the 3' end of the *gvpC*²⁰ (Fig. 3b). The sequenced portions of the two *gvpAs* and the ΩC–*gvpA* spacer were identical to the corresponding regions in *Pla* 9303; in the *gvpA*–ΩC spacer, however, while 137 of the first 140 bp were identical to the *gvpA*–*gvpC*²⁰ spacer, the last 17 bp showed little similarity. Complementary primers GVPΩ2 and GVPΩ3 specific to this unique sequence were used in PCR amplifications with GVPA1, GVPA7 and GVPC4 (Table 1, Fig. 3) to determine the gene arrangement shown in Fig. 3(b). The sequenced region revealed the gene order *gvpC*²⁰, *gvpA*, ΩC, *gvpA*,

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1           10           20           30
  M A L K D K W Q Q D R I G R Q Q G V Q E R Q Q Q V Q T T
L S L W Q Q E R Q N Q A S E F R E D L E Y R V T D L L A N Y Q K Q
R L E A R E T L L E D L A I F R Q T L Y R E V E E Y L G E L D I L
H Q Q M A A Q L Q Q Q L Q Q S R T E R K D A V Q K L F E D L G V F
R A E L Q D Y H L K L Q Q T V W G S S H R H S I K P Q P T V N P G
V P Q P A T L D Q P Q G *

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Fig. 1. Amino acid sequence of GvpC²⁰ inferred from the nucleotide sequence obtained from *Planktothrix rubescens* strain *Pla* 9401. The sequence is arranged in rows of 33 amino acid residues in register with the central row (bold type) that is absent from GvpC¹⁶.

Fig. 2. Diagnostic PCR used to distinguish the different gas vesicle genotypes of *Planktothrix rubescens*. The products obtained by amplification with the primer pairs (a) GVPA1/GVPA2 and (b) GVPC1/GVPC5 were analysed by electrophoresis through agarose gels (1.5% and 2%, w/v, respectively). Lanes: 1, 100 bp ladder (Life Technologies); 2, *Pla* 9303 (GV1); 3, *Pla* 9316 (GV2); 4, *Pla* 9401 (GV3); 5, no-template control.

Table 3. Summary of the arrangement of *gvp* genes in type strains of the different gas vesicle genotypes of *Planktothrix rubescens*, and the products of diagnostic PCRs used to distinguish them

+, Product present.

Genotype	Type strain	<i>gvp</i> gene order	GVPA1/GVPA2 PCR product(s)/ bp			GVPC1/ GVPC5 PCR product(s)/ bp	
			595	965	1064	227	326
GV1	<i>Pla</i> 9303	A-C ²⁰ -A-C ²⁰			+		+
GV2a	<i>Pla</i> 9316	A-C ²⁰ -A-ΩC-A-C ²⁰ -A-C ²⁰	+		+		+
GV2b	<i>Pla</i> 9731	A-C ²⁰ -A-ΩC-A-ΩC-A-C ²⁰ -A-C ²⁰	+		+		+
GV3a	<i>Pla</i> 9401	A-C ¹⁶ -A-ΩC-A-C ¹⁶ -A-C ¹⁶ -A-C ²⁰	+	+		+	+
GV3b	<i>Pla</i> 9736	A-C ¹⁶ -A-ΩC-A-ΩC-A-C ¹⁶ -A-C ¹⁶ -A-C ²⁰	+	+		+	+

Fig. 3. Diagram representing the arrangement of gas vesicle genes *gvpA* and *gvpC* in *Planktothrix rubescens* strains (a) *Pla* 9303, (b) *Pla* 9316 and (c) *Pla* 9401. The boxed regions indicate open reading frames; the boxed regions composed of broken lines indicate the position of the untranslated 3' end of *gvpC* (Ω C). The hatched area represents the 99 bp segment present in *gvpC*²⁰, but absent in *gvpC*¹⁶ (at the position indicated by ▼). The thicker lines represent regions that have been sequenced, and the thinner lines represent sections whose gene arrangement was inferred from the sizes of PCR products estimated from agarose gels. Lines underneath represent the products obtained by PCR using the primers indicated at each end (with the prefix GVP omitted). Broken lines indicate PCR products whose sequence has not been determined; the lengths given are those predicted from the lengths of the corresponding sequenced regions. The lengths of ORFs, intergenic regions and PCR products are given in bp.

*gvpC*²⁰. The only differences from the corresponding regions in *Pla* 9303 were two changes in the sequence of *gvpC*²⁰ (G → C at nt 155, encoding Arg → Thr; T → C at nt 448, encoding Ser → Pro) and two substitutions in the *gvpA*–*gvpC*²⁰ spacer.

Amplification between the primers GVP Ω 2 and GVPC4 generated two products: the sequence of the smaller 881 bp product (Fig. 3b) established the *gvpA* and *gvpC*²⁰ downstream of Ω C; the larger 1.9 kbp product indicated a second *gvpA* and *gvpC*²⁰ downstream of this. Amplification between the primers GVP Ω 3 and GVPA1 also generated two products, a smaller 263 bp product and a larger 1.3 kbp product, which indicated another *gvpA* upstream (Fig. 3b).

In another strain of genotype GV2 (*Pla* 9731), amplification between primers GVP Ω 3 and GVPA1 generated an additional product of 0.85 kbp. The sequence of this product indicated two copies of Ω C in the gene arrangement *gvpA*, Ω C, *gvpA*, Ω C. The genotypes of

strains *Pla* 9316 (one copy of Ω C) and *Pla* 9731 (two copies of Ω C) are designated GV2a and GV2b, respectively (Table 3).

Genotype GV3. In *Pla* 9401 amplification with primers GVPA1 and GVPA2 generated products of 595 and 965 bp (Fig. 2a). The 595 bp product had the same sequence as that in *Pla* 9316, except for a single substitution in the *gvpA*– Ω C spacer. The 965 bp product contained a similar gene arrangement to that found in the 1064 bp product from *Pla* 9303 and *Pla* 9316 but the *gvpC* ORF lacked a 99 bp segment (corresponding to nt 184–282 in *gvpC*²⁰). This smaller *gvpC*, which encodes a 16 kDa protein of 139 amino acid residues, was designated *gvpC*¹⁶. It showed only one other difference from *gvpC*²⁰ in *Pla* 9303, a change of T → C at the position corresponding to nt 448 in *gvpC*²⁰ (encoding a change of Ser → Pro).

A similar set of amplifications to those used with *Pla* 9316 gave products that supported the gene arrangement

Table 4. Gas vesicle genotype identities of 186 strains of *Planktothrix rubescens* isolated from Lake Zürich, and held in the Bristol Collection

The full strain identities contain the prefix *Pla*, year (e.g. 93) and accession number for the year, e.g. *Pla* 9303. The year number is given for only the first of each group. CYA 1 was obtained from the NIVA Collection, Oslo.

Date of sampling	Strains of gas vesicle genotype:				
	GV1	GV2a	GV2b	GV3a	GV3b
1958 (Staub, 1961)	CYA 1				
19 February 1993	9303, 07	9304, 05, 09, 16		9301	9312
30 August 1994				9401	9402
18 March 1997	9722	9711, 13, 28, 33, 34, 37, 39, 40, 44, 50, 52	9731	9709	9701, 03–08, 10, 12, 14–21, 23–27, 29, 30, 32, 35, 36, 38, 41–43, 46–49, 51, 53–57
27 June 1997		9758, 60, 62, 64, 66, 67			9759, 61, 63, 65, 68
30 September and 2 October 1998		9772, 79–81, 83, 85, 87, 89, 90, 92, 93, 95, 98–100, 102, 103, 105–109, 111, 115, 119, 120, 122, 124, 125, 127, 128, 130, 133, 134, 136, 138, 139, 143, 149, 151, 152, 154, 158, 159, 163, 165, 168, 169, 171, 173, 175			9769, 71, 73–78, 82, 84, 86, 88, 91, 94, 96, 97, 101, 104, 110, 112–114, 116–118, 121, 123, 126, 129, 131, 132, 135, 137, 140–142, 144–148, 150, 153, 155–157, 160–162, 164, 166, 167, 170, 172, 176–179
Total	4	72	1	3	106

shown in Fig. 3(c): it was the same as that in *Pla* 9316 but with three *gvpC*¹⁶s instead of the three *gvpC*²⁰s. The two *gvpA* ORFs that were sequenced were identical to those in *Pla* 9303 and *Pla* 9316; the *gvpA*–*gvpC* and *gvpC*–*gvpA* spacers were identical to those in *Pla* 9316. The sequenced regions of the first two *gvpC*¹⁶ ORFs were identical.

In addition to *gvpC*¹⁶, a copy of *gvpC*²⁰ was revealed by amplification with GVPA7 and GVPA3 (Table 1); the sequence of this *gvpC*²⁰ differed from that of *gvpC*²⁰ in *Pla* 9303 in only one position (G → C at nt 155, encoding Arg → Thr). The location of this gene was determined by amplification between the GVPΩ2 site, which is unique to the *gvpA*–ΩC spacer, and the GVPC4 site, which is unique to the 99 bp segment in *gvpC*²⁰.

With a similar set of amplifications to those used on *Pla* 9401 we determined the nucleotide sequence of *gvpA*, *gvpC*¹⁶ and *gvpC*²⁰ in the GV3 strain *Pla* 9736, which was shown by Bright & Walsby (1999) to possess the narrowest and strongest gas vesicles. The sequences of *gvpA* and *gvpC*¹⁶ were identical to those in *Pla* 9401, and the sequence of *gvpC*²⁰ differed in only one position (T → C at nt 448, encoding Ser → Pro). Strain *Pla* 9736 was also shown to possess two copies of ΩC: amplification

between primers GVPΩ3 and GVPA1 generated an additional product of 0.85 kbp, whose sequence indicated two copies of ΩC in the gene arrangement *gvpA*, ΩC, *gvpA*, ΩC. The genotypes of strains *Pla* 9401 (one copy of ΩC) and *Pla* 9736 (two copies of ΩC) are designated GV3a and GV3b, respectively (Table 3).

Frequency of different GV genotypes in Lake Zürich

The gas vesicle genotypes of clonal *Planktothrix* cultures, isolated from Lake Zürich water samples on various dates between 1993 and 1997, were determined from the lengths of the PCR products obtained with primers GVPA1 and GVPA2 (Fig. 2a). Of 187 cultures established over the whole period, 185 were clonal; of these, 3 (2%) were of genotype GV1, 73 (39%) were GV2 and 109 (59%) were GV3 (Table 4). Two of the original cultures, *Pla* 9302 and *Pla* 9319, contained two genotypes. Strains of genotypes GV2 and GV3 were also screened for the presence of two copies of ΩC (diagnostic of GV2b and GV3b, respectively) by amplification with primers GVPΩ3 and GVPA1. Of the 73 strains of genotype GV2 only one (*Pla* 9731) contained two ΩCs; of the 109 strains of genotype GV3, however, 106 contained two ΩCs (Table 4). In the sets of cultures

established at different sampling times the ratio of the different genotypes varied; no firm conclusions can be drawn from this because the method of collection was biased. For example, in March 1997 we selected filaments that remained buoyant in samples collected at depths exceeding 100 m in an attempt to obtain strains with stronger gas vesicles. We are currently investigating the proportions of GV genotypes by collecting much larger samples of single filaments for genotype analysis using PCR. It is worth commenting, however, on the apparent disappearance of genotype GV1: two out of the first 10 clonal cultures established in 1993 were GV1 whereas in the 1997 isolates only one out of 175 was of this genotype; moreover, a culture of *Planktothrix rubescens* established from a Lake Zürich water sample by Staub in 1958 (Staub, 1961), and maintained for 40 years as strain CYA 1 in the NIVA collection, is also of genotype GV1.

A second set of diagnostic PCR primers was designed to enable discrimination between those strains containing just *gvpC*²⁰, and those containing both *gvpC*¹⁶ and *gvpC*²⁰. Primers GVPC1 and GVPC5 were used to amplify the central portion of both *gvpC*¹⁶ and *gvpC*²⁰, generating products of 227 and 326 bp, respectively (Fig. 2b, Table 3). Amplification using these primers revealed that strains of genotypes GV1 and GV2 generated only the 227 bp product, whereas those of GV3 generated both products. These results confirm that strains GV1 and GV2 contain only *gvpC*²⁰ whereas GV3 strains contain both *gvpC*¹⁶ and *gvpC*²⁰.

Amplification with primers GVPC1 and GVPC5 generated an additional product which gave a band with a mobility indicating 0.35 kbp when performed with DNA isolated from strains of the GV3 genotype (Fig. 2b). The smaller products, of 227 and 326 bp, were cloned into separate plasmids but the '0.35 kbp' product could not be cloned. When the plasmid containing the 227 bp product was subjected to PCR amplification with the GVPC1 and GVPC5 primers, only a 227 bp product was obtained; similarly only a 326 bp product was given by the plasmid containing the 326 bp product (not shown). When both plasmids were mixed together, however, PCR gave the '0.35 kbp' product in addition to the two smaller products. It is concluded that the '0.35 kbp' product is a heteroduplex formed from the homologous sequences from the *gvpC*¹⁶ and *gvpC*²⁰ genes.

Ratio of GV genotypes in Lake Zürich water samples

An investigation was made of the gas vesicle genotypes of *Planktothrix* filaments in water samples taken from a depth of 10 m in Lake Zürich on 30 September and 2 October 1997. From 40 separate filaments picked from the water sample, 26 clonal cultures were established. Diagnostic PCR using the GVPC1 and GVPC5 primers with DNA prepared from each culture showed that 13 (50%) contained only *gvpC*²⁰ while 13 also contained *gvpC*¹⁶. One hundred and thirty-three single filaments were transferred directly into 2 × PCR buffer and used in subsequent diagnostic PCR: of the 106 single filaments

that gave an amplification product, 57 (54%) contained only *gvpC*²⁰ while 49 (46%) also contained *gvpC*¹⁶. The results obtained using the two methods were not significantly different ($\chi^2 = 0.604$, $P = 0.44$). An example of a gel used to distinguish the two genotypes in single filaments selected from a Lake Zürich lakewater sample is shown in Fig. 4.

Sequences of other gene clusters

In order to determine if the observed variation in GV genotype occurred within or between different *Planktothrix* species, the variation in DNA sequences of four other gene clusters was investigated: (a) 16S rRNA gene; (b) phycoerythrin β and α subunit genes (*cpeB* and *cpeA*); (c) phycocyanin β and α subunit genes (*cpcB* and *cpcA*); (d) Rubisco large and small subunit genes (*rbcL* and *rbcS*).

16S rRNA genes. The 16S rRNA gene was amplified in two overlapping fragments from genomic DNA isolated from non-axenic cultures, using primers CYANO-5' and CYANO-3' (complementary to the 5' and 3' ends, respectively, of the 16S rDNA of *Synechococcus* sp. PCC 6301; Table 1) in combination with two *Planktothrix*-specific primers. Primer PLANKTO-5' (Table 1) was used with CYANO-3' to amplify a 1128 bp region covering the 3' end; primer PLANKTO-3' was used in combination with CYANO-5' to amplify a 1208 bp region covering the 5' end. The sequence of PLANKTO-5' was based on that of primer CN described by Rudi *et al.* (1997). Primer PLANKTO-3' was designed on the basis of an alignment of the 16S rDNA sequence of strain *Pla* 9402 with a number of other bacterial 16S rDNA sequences. The region analysed corresponds to nt 28–1521 in the *E. coli* 16S rRNA gene sequence (EMBL accession no. J01695) of Brosius *et al.* (1978). The 16S rDNA sequences obtained from strains *Pla* 9303, 9316, 9401 and 9402 were identical, although there were ambiguities at one position in *Pla* 9303, and at at least four positions in *Pla* 9316, which may reflect sequence heterogeneity in multiple copies of the rDNA gene (von Wintzingerode *et al.*, 1997). The best match is with the 16S rDNA sequence (EMBL accession no. X84811) from *Planktothrix* (*Oscillatoria*) *agardhii* strain CYA 18 (Nelissen *et al.*, 1996): it differed in only two positions: G → C at base 1274, and an extra C after base 326.

***cpeB*–*cpeA* gene cluster.** The primers (PE β F and PE α R; Table 1) used for the amplification of part of the operon encoding phycoerythrin were designed on the basis of an alignment of *cpeB* and *cpeA* sequences from *Pseudanabaena* sp. strain PCC 7409, *Calothrix* sp. strain PCC 7601 and *Synechococcus* sp. strain PCC 6701 (EMBL accession nos X63073, X04592 and M33812, respectively).

In all of the *Planktothrix* strains investigated, PCR amplification resulted in a single product of 517 bp. The sequence of the 471 bp between the primers indicated: (a) the 310 bp of the 3' end of *cpeB*; (b) a 83 bp non-coding region; (c) the first 78 bp of *cpeA*. Three sequence variants were identified: (1) PE1, which shows 72%

Fig. 4. Diagnostic PCR performed on single filaments of *Planktothrix rubescens* picked directly from Lake Zürich water samples. The products obtained by amplification with primers GVPC1 and GVPC5 were analysed by electrophoresis through a 2% (w/v) agarose gel (lanes 1–5 and 7–11). Lane 6 contains a 100 bp ladder (Life Technologies); lane 12 contains a no-template control.

identity with part of the *cpeBA* operon from *Pseudanabaena* sp. strain PCC 7409 in the EMBL database; (2) PE2, which differed from PE1 in only one position (C → T, encoding Thr → Ile at nt 240) in the 310 nt sequence at the 3' end of *cpeB*; (3) PE3, which showed two additional differences (G → A encoding Val → Ile at nt 167; C → G, encoding Ala → Gly at nt 276) in the same 310 nt sequence at the 3' end of *cpeB*.

***cpcB–cpcA* gene cluster.** The primers (PC β F and PC α R; Table 1) used for the amplification of part of the phycocyanin operon were those designed by Neilan *et al.* (1995). DNA polymorphisms identified previously within this operon (Neilan *et al.*, 1995; Hayes & Barker, 1997) indicated that this may be a useful genetic marker for distinguishing between cyanobacterial species.

In *Planktothrix* strain 9307, PCR amplification resulted in a 695 bp product. The sequence of the 655 bp between the primers indicated: (a) the 260 bp of the 3' end of *cpcB*; (b) a 101 bp non-coding region; (c) the first 294 bp of *cpcA*. The sequence showed 68% identity with part of the *cpcBA* operon from *Synechococcus* sp. strain PCC 7002 (accession no. K02659). A *Planktothrix*-specific reverse primer (PC α R3) was designed from the sequence obtained from strain *Pla* 9307 (complementary to nt 287–268 in *cpeA*). Amplification with primers PC β F and PC α R3 (Table 1) generated a 668 bp product from strains *Pla* 9303, 9316, 9401 and 9402. In each strain the sequence between the primers (628 bp) was identical to the corresponding sequence in *Pla* 9307.

***rbcl–rbcX–rbcS* gene cluster.** The primers used for the

amplification of genes encoding Rubisco, CYARUB-5' and CYARUB-3', were designed on the basis of an alignment of *rbcl* and *rbcS* sequences from *Synechococcus* sp. PCC 7002, *Prochlorothrix hollandica* and *Anabaena* sp. PCC 7120 (EMBL accession nos D13971, X57359 and J01540, respectively).

In all of the *Planktothrix* strains investigated, PCR amplification resulted in a single product of 970 bp. The sequence of the 922 bp between the primers indicated: (a) the 25 bp of the 3' end of *rbcl*; (b) a 131 bp non-coding region; (c) a 402 bp ORF designated *rbcX* (Rudi *et al.*, 1998); (d) a 69 bp non-coding region; (e) the first 295 bp of the 5' end of *rbcS*. Three sequence variants were found: (1) RBC1, which differed at only one position, T → G at nt 64 in the first spacer, from the *rbclX* sequence of *Planktothrix rubescens* CYA 55 (Rudi *et al.*, 1998); (2) RBC2, which was identical to the *Planktothrix* CYA 55 sequence; (3) RBC3, which showed the same difference of T → G at position 64 in the first spacer, and an additional difference, G → T at position 247 in *rbcX*. This sequence variant was obtained by Rudi *et al.* (1998) in *Planktothrix* strain CYA 1 (Staub, 1961).

DISCUSSION

Genetic diversity and species classification

Comparison of the rRNA sequences of the Lake Zürich strains of cyanobacteria investigated here with published rRNA sequences from strains of *Planktothrix* isolated

Table 5. Summary of the genotypic variation associated with the gene clusters investigated, and EMBL database accession numbers

The genotypes were identified from nucleotide sequences except for those in parentheses, which were inferred from diagnostic PCRs using primer pairs GVPA1/GVPA2 and GVPA1/GVP Ω 3. –, Nucleotide sequence not determined.

Strain	Genotype					EMBL accession no.				
	GV	PE	PC	RBC	16S rDNA	<i>gvpAC</i>	<i>cpeBA</i>	<i>cpcBA</i>	<i>rbcLXS</i>	16S rDNA
9303	1	1	1	1	1	AJ132357	AJ132245	AJ132279	AJ132248	AJ132251
9307	(1)	1	1	1	–	–	AJ132245	AJ131820	AJ132248	–
9316	2a	2	1	2	1	AJ132358	AJ132246	AJ132279	AJ132249	AJ132252
9401	3a	1	1	2	1	AJ132354	AJ132245	AJ132279	AJ132249	AJ132250
						AJ132361				
9402	(3b)	1	1	2	1	–	AJ132245	AJ132279	AJ132249	AJ132250
9736	3b	3	–	–	–	AJ238351	AJ132247	–	–	–
						AJ23852				
						AJ23853				
9743	(3b)	2	–	–	–	–	AJ132246	–	–	–

from Norwegian lakes (Walsby et al., 1983; Utkilen et al., 1985) confirms that these organisms form a tight clade, separate from other species of *Oscillatoria* (Rudi et al., 1997, 1998). We therefore support the suggestion of Anagnostides & Komárek (1988) that these planktonic forms should be separated into the genus *Planktothrix*, but we maintain the specific epithet, *P. rubescens*, used in previous description of this population in Lake Zürich (Thomas & Märki, 1949; Walsby et al., 1998).

Our investigation of the DNA sequences of the rDNA, *cpe*, *cpc* and *rbc* gene loci revealed little or no difference in the seven strains of *Planktothrix* investigated (Table 5). In each case the variation was limited to changes in three or fewer bases in the 471–1443 bp sequenced. This very low degree of variability indicates that the seven strains are members of the same species and of the same population within the lake. It is noted that the three variants of the *cpe* gene cluster (PE1, PE2 and PE3) occur in combination with one of the *gvp* genotypes (GV3), and that two of the variants each occur in combination with two of the *gvp* variants (PE1 with GV1 and GV3; PE2 with GV2 and GV3). It is therefore possible that there has been lateral transfer of genetic information between strains, as suggested by Rudi et al. (1998).

The only apparent exception in the degree of genetic diversity is with the sequences of the *gvpC* ORFs. It is noted first, however, that the number of single-base differences between the three GV genotypes, only one to four over the *gvpA*–*gvpC* repeat, is similar in frequency to that of the other four gene loci. Moreover, although the other differences involve larger changes, all of them can be explained by single genetic events: the duplication of *gvpA*–*gvpC* repeats; the loss of 99 nt elements from *gvpC*²⁰ to generate *gvpC*¹⁶; and the loss of the N-terminus of *gvpC* to leave the Ω C fragment. The role of Ω C is unknown, but we suggest that the other genetic rearrangements have been maintained in the population

because they produce a change in the phenotype that is subjected to positive natural selection.

The *gvp* gene arrangements

Homologies with *gvpA* and *gvpC* sequences in other cyanobacteria. The *gvp* gene clusters in the three strains of *Planktothrix* investigated resemble those of other cyanobacteria in the occurrence of multiple copies of *gvpA*, but differ in possessing multiple copies of *gvpC* and in the alternating arrangement of *gvpA* and *gvpC*.

The sequence of *gvpA* is perfectly conserved in all of the Lake Zürich strains of *Planktothrix rubescens*. The inferred amino acid sequence is identical over the corresponding regions to the N-terminal amino acid sequences of GvpAs from both the red (first 46 residues) and green (58 residues) variants of *Planktothrix* (*Oscillatoria*) *agardhii* isolated from Lake Gjørsjøen in Norway. Of GvpA sequences in other cyanobacteria it most closely resembles that in *Pseudanabaena* sp. (Damerval et al., 1991): both depart from the GvpA consensus sequence in having Val at residue 5, Ser at 40 and Ala at 64, and in having 71 rather than 70 residues; the *Planktothrix* sequence differs from the *Pseudanabaena* sequence, however, in three of the last seven residues (cf. Griffiths et al., 1992).

The degree of sequence similarity of GvpCs in cyanobacteria is much lower than that of GvpA (Griffiths et al., 1992). The homology of the inferred N-terminal amino acid sequence of the GvpC in the *Planktothrix* strains with the directly determined amino acid sequence of GvpC in *Planktothrix* (*Oscillatoria*) *agardhii* CYA 29 confirms the identities of the *gvpC* genes in the Lake Zürich isolates. We discuss below their sequence variation, the occurrence of internal sequence repeats and the occurrence of multiple copies of *gvpC* in other organisms.

Cryptic and overt 33RRs in *gvpC*. Since *gvpC*¹⁶ differs from *gvpC*²⁰ only in the absence of a 99 bp section it may have originated from *gvpC*²⁰ by gene duplication followed by deletion of the 99 bp section. There is no other instance of this known in cyanobacteria but in *Dactylococcopsis salina* gas vesicles it was demonstrated that there are two types of GvpC, whose N-terminal amino acid sequence is identical over the first 24 residues but shows little similarity over the following 30 residues (Griffiths *et al.*, 1992). This might also be explained by the deletion of a section of the encoding gene.

No repeating elements were immediately apparent in either the nucleotide sequence of the *gvpC*s from the *Planktothrix* strains or the encoded amino acid sequences of the GvpC products. Nevertheless, it is significant that *gvpC*¹⁶ and *gvpC*²⁰ differ by a 99 bp segment, which is identical to the length of the repeating elements that encodes the 33RRs in GvpCs of *Calothrix* (Damerval *et al.*, 1987) and *Anabaena* (Hayes *et al.*, 1988). When the amino acid sequence is arranged in 33-residue rows (aligned in Fig. 1 with the 33-residue sequence that is present in GvpC²⁰ but missing from the GvpC¹⁶) significant similarity is seen in vertical columns. We used pair-scoring logic to compare the number of identical residues at the five positions in each column: two identical scores 2 for 1 pair; three identical scores 6 for 3 pairs; four identical scores 12 for 6 pairs. The mean score for each column is 2.5; the scores for arrangements in rows of 31, 32, 34 and 35 residues are all between 1.4 and 1.6. A similar analysis performed on the standard simplified classification of amino acids (given by the 'Simplify' function in the Wisconsin package) gives scores of 6.5 for arrangements of 33 residues per row and between 4.1 and 4.6 for rows of 31, 32, 34 and 35 residues. These analyses suggest that there may be cryptic functional 33RRs in GvpCs of *Planktothrix*.

Correlation between GV genotypes and gas vesicle p_c phenotypes

The companion study (Bright & Walsby, 1999) describes the p_c distribution of the gas vesicles in 81 of the 185 clonal *Planktothrix* strains isolated from Lake Zürich. It demonstrates that the mean p_c of all genotype GV2 strains is less than 1.0 MPa while that of all genotype GV3 strains exceeds 1.0 MPa. (The few GV1 strains isolated have p_c values close to the 1.0 MPa borderline.) The strong correlation between *gvpC* variants and p_c suggests, but does not prove, that the observed differences in the combinations of *gvpC* genes in *Planktothrix rubescens* may be causative of the associated gas vesicle phenotypes. Whether or not they directly determine gas vesicle phenotype it is clear that they provide a valuable marker for gas vesicle strength or width.

Population studies of *gvp* genes

With the development of a system of primers capable of distinguishing the two classes of *gvpC* genotypes that are correlated with strong and weak gas vesicle pheno-

types we can now use diagnostic PCR to investigate the proportion of the two strains present at different periods of the year. This method provides several advantages over the alternative method of isolating single filaments in culture and then measuring the p_c of their gas vesicles: (1) it involves fewer laboratory resources, fewer manipulations and less time; (2) it can be performed on many more filaments per sample (with filaments stored frozen for subsequent analysis) compared with the number that can be isolated into culture; (3) with a success rate of 80–95% (our unpublished results) it is less susceptible to sampling bias than the 44% establishment rate of single filament cultures.

It has been suggested that there is natural selection for strains of *Planktothrix rubescens* with strong gas vesicles during the vernal recruitment to the metalimnion in Lake Zürich, when only the buoyant filaments float up from great depths after winter mixing (Walsby *et al.*, 1998). We are currently using diagnostic PCR to investigate changes in the proportions of the different GV genotypes in this *Planktothrix* population, in order to quantify the process of natural selection of different gas vesicle phenotypes that occurs in this lake.

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