The relationship between critical pressure and width of gas vesicles in isolates of *Planktothrix rubescens* from Lake Zürich

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The mean critical collapse pressure (p_c) of gas vesicles in 81 strains of the cyanobacterium *Planktothrix rubescens* from Lake Zürich, Switzerland, was bimodally distributed between a minimum of 0.86 MPa and a maximum of 1.17 MPa. Measurements were made of the cylinder diameter (d) of gas vesicles isolated from seven of the strains. The mean diameter, which varied from 48 to 61 nm, was inversely related to p_c in keeping with the theory of strength of thin-walled rigid cylinders. These measurements extended the range of p_c -width relationship of gas vesicles, which can be described by the expression $p_c = 461(d/\text{nm})^{-1.53}$ MPa. p_c was correlated with gas vesicle genotype (see the accompanying paper by S. J. Beard, B. A. Handley, P. K. Hayes & A. E. Walsby, *Microbiology* 145, 2757–2768): of the 81 strains investigated, all those with the gas vesicle genotype GV2 produced gas vesicles with a mean p_c of less than 1.0 MPa, whereas those of GV3 had a mean p_c of greater than 1.0 MPa. It is suggested that gas vesicles of the GV3 strains, which are narrower and stronger than any previously recorded in freshwater cyanobacteria, have evolved to withstand the high hydrostatic pressures during deep winter mixing in Lake Zürich.

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

INTRODUCTION

Gas vesicles, which provide buoyancy in planktonic cyanobacteria, are hollow cylindrical structures (Oliver, 1994; Walsby, 1994). They are rigid but collapse at a certain threshold pressure, referred to as the critical pressure, p_c . Gas vesicles in different genera of cyanobacteria vary in width and it has been demonstrated that their mean p_c values vary inversely with width (Hayes & Walsby, 1986; Walsby & Bleything, 1988). This inverse relationship is understood in terms of the mechanics of a thin-walled rigid cylinder (Walsby, 1991), which buckles and collapses through instability (Allen & Bulson, 1980).

Differences in the mean p_c of gas vesicles occur not only between different genera but also between different species of the same genus, e.g. between different species of *Anabaena*, *Oscillatoria* and *Trichodesmium*; it has

been suggested that these differences evolved through natural selection to withstand pressures generated by vertical mixing in natural waters (Walsby & Bleything, 1988; Walsby, 1994). Substantial differences have been found in the mean p_c of gas vesicles in different populations of *Nodularia* spp. from the Baltic Sea, which may be adapted to regions of different depth (Walsby *et al.*, 1995). It has been demonstrated that there are differences in the widths of gas vesicles in these *Nodularia* populations (Komárek *et al.*, 1993; Albertano *et al.*, 1996) but the relationship between strength and width in these populations has not been directly investigated.

Variations in gas vesicle p_c have also been demonstrated in populations of the planktonic species of *Oscillatoria*, which are now reclassified in the genus *Planktothrix* (Anagnostides & Komárek, 1988). In a green-coloured *Planktothrix* (*Oscillatoria*) *agardhii* var. *isothrix* from Deming Lake, Minnesota, the mean p_c was 0.75 MPa (Walsby & Klemer, 1974) whereas in a red-coloured *P. agardhii* from Lake Gjersjøen, Norway, the mean p_c was over 0.9 MPa (Walsby *et al.*, 1983; Walsby & Bleything,

Abbreviation: p_c , critical pressure.

1988). In *P. rubescens* from Lake Nantua, France, the mean p_c may have been even higher: the apparent critical pressure (p_a) in turgid cells was 0.82 MPa (Feuillade, 1994); allowing for turgor pressure of at least 0.2 MPa, p_c would have exceeded 1 MPa. Utkilen *et al.* (1985) reported mean p_c values ranging from 0.60 to 1.05 MPa in six strains of *Planktothrix* isolated from Norwegian lakes but, again, there was no direct investigation of the relationship between p_c and gas vesicle width.

Walsby *et al.* (1998) reported on the variation of p_c of gas vesicles in 10 of 12 clonal isolates of *Planktothrix rubescens* from Lake Zürich. Beard *et al.* (1999) demonstrated three distinct classes of gas vesicle genotype (GV1, GV2 and GV3) in these 12 isolates, which were distinguished by differences in the sequence, size, copy-number and arrangement of the *gvpC* genes that encode the outer-surface protein (GvpC) of the gas vesicle. In another 175 clonal isolates from the lake they found the same three classes but also distinguished subclasses (GV2a and b; GV3a and b) that differed in the number of copies of ΩC , a non-coding fragment of the *gvpC* gene.

We report here correlations between p_c and diameter of gas vesicles in seven of these *Planktothrix* strains, which extend the range and refine the description of the p_c -width relationship. We also report correlations between the GV genotype and p_c in 81 of these strains that suggest that variations in the *gvpC* gene affect the size and strength of gas vesicles. The gas vesicles in many of these Lake Zürich strains are stronger than previously encountered in freshwater lakes; the occurrence of strains that produce such strong gas vesicles may be restricted to lakes that are of comparable depth.

METHODS

Cultures. Measurements were performed on 81 strains of *Planktothrix rubescens* cultured in 250 ml Erlenmeyer flasks under an incident photon irradiance of 15–20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and at a temperature of 21 °C. Detailed measurements were made on seven of the strains, *Pla* 9303, 9316, 9401 and 9402 isolated from Lake Zürich in 1993–4 (Walsby *et al.*, 1998), and strains *Pla* 9707, 9725 and 9736 isolated in 1997 (Beard *et al.*, 1999). Investigations of gas vesicles in these strains were made using triplicate cultures grown on a light bench illuminated with a photon irradiance of $15 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ from below; cultures were repositioned daily in a Latin Square arrangement using randomly assigned coordinates.

Gas vesicle p_c values. Gas vesicle p_c distributions were determined with a pressure nephelometer (Walsby, 1973). Measurements were made with 2 ml samples of cultures mixed with 2 ml 1.0 M sucrose solution to remove cell turgor pressure. The mean and standard deviation of the p_c distribution were calculated from replicate measurements by the procedure of Walsby & Bleything (1988).

Electron microscopy of gas vesicles. Pt-C replicas of flattened isolated gas vesicle were prepared for electron microscopy (Walsby & Bleything, 1988). Measurements were made of the width (w) and length (l) of 158–240 flattened gas vesicles from each strain; measurements were also made of the cone side (s)

of 10–20 gas vesicles. The cylinder diameter was calculated as $d = 2w/\pi$. These measurements were made by converting the image of each individual gas vesicle on electron micrograph film into a digitized image in a computer bitmap using a high-resolution Cohu video camera mounted on a Leica microscope in conjunction with a Synoptics Synapse Grabber. The computer image was analysed with PC_Image and Analysis software (Synoptics). At the magnifications used, one pixel in the computer image was equivalent to 0.80 nm on the gas vesicle, a 2.5-fold improvement in measurements previously made by microscope eyepiece graticule. Comparisons of electron micrographs previously measured by Walsby & Bleything (1988) gave the same mean values but smaller standard deviations of gas vesicle widths using image analysis.

The magnification of each electron micrograph was determined by measurements of the centre line of squares in the paired image of a 463 nm diffraction grating. The lengths and heights of the 100 squares on each of three electron micrographs of the diffraction grating were measured for pin-cushion distortion: they showed that the magnification at the corner of the electron micrograph was 3.7% greater than at the centre. The mean magnification of measurements made in each of the squares, at angles from 0° to 90°, was only 0.19% greater than the magnification determined from measurement of the centre line of squares used in the calibration.

RESULTS AND DISCUSSION

The range of p_c

p_c distributions were determined for 81 of the 185 clonal strains of *Planktothrix rubescens* in culture and the mean and standard deviation of p_c were calculated for each strain. The mean p_c ranged from 0.86 to 1.17 MPa (Table 1). The overall mean p_c was 1.04 MPa and the mean standard deviation was 0.093 MPa. The frequency distribution of p_c , in classes differing by 0.02 MPa, is shown in Fig. 1. The distribution contains two main peaks, which have modes at 0.94 MPa and 1.14 MPa.

Seven of the strains, including examples of the three GV genotypes, were selected for more detailed study; the selection also covered the range of p_c values found in the initial survey. These strains were grown in triplicate cultures under standardized conditions for measurement of p_c distribution (Fig. 2) and dimensions of gas vesicles (see below). Although the mean p_c values of the different strains varied by up to 0.3 MPa, the standard deviation of the p_c distributions (see Walsby & Bleything, 1988) was similar in most cases; in *Pla* 9725, however, it was considerably wider (Fig. 2).

The initial selection also contained an eighth strain, *Pla* 9319, which was originally reported to possess strong gas vesicles (Walsby *et al.*, 1998); this culture was evidently non-clonal because it contained two different genotypes.

The range of cylinder diameter

The mean dimensions of gas vesicles isolated from seven strains of *Planktothrix rubescens* are shown in Table 2. The mean cylinder diameters varied from 47.7 nm, in strain *Pla* 9736, to 61.3 nm, in strain *Pla* 9316. The

Table 1. Mean p_c values of gas vesicles in the 81 strains of *Planktothrix rubescens* from Lake Zürich, arranged by gas vesicle genotype

Genotype	Strain (Pla)	p_c /MPa	Strain (Pla)	p_c /MPa	Strain (Pla)	p_c /MPa	Strain (Pla)	p_c /MPa
GV1	9303	0.96	9307	1.00	9722	0.98		
GV2a	9304	0.93	9305	0.86	9309	0.92	9316	0.88
	9711	0.92	9713	0.91	9728	0.87	9740	0.93
	9744	0.97	9758	0.95	9760	0.89	9762	0.97
	9764	0.89	9766	0.91	9781	0.97	9783	0.99
	9790	0.94	9798	0.94	97103	0.94	97120	0.92
	97162	0.91	97127	0.90	97169	0.93	97173	0.92
	97175	0.93						
GV2b	9731	0.90						
GV3a	9401	1.10						
GV3b	9402	1.09	9701	1.14	9703	1.13	9704	1.13
	9705	1.14	9706	1.13	9707	1.14	9708	1.10
	9710	1.13	9712	1.14	9714	1.04	9715	1.09
	9716	1.05	9717	1.11	9718	1.06	9719	1.11
	9720	1.15	9721	1.02	9723	1.05	9725	1.09
	9726	1.00	9727	1.16	9729	1.14	9732	1.09
	9735	1.09	9736	1.17	9738	1.11	9741	1.02
	9743	1.12	9747	1.15	9751	1.10	9753	1.13
	9755	1.09	9757	1.15	9759	1.16	9761	1.12
	9763	1.08	9768	1.11	9773	1.12	9786	1.06
	97131	1.14	97150	1.02	97153	1.09	97157	1.06
	97160	1.12	97161	1.16	97164	1.09	97166	1.01
	97167	1.14	97170	1.14	97176	1.12

Fig. 1. Histogram of the frequency distribution of mean p_c values of gas vesicles in 81 strains of *Planktothrix rubescens*. **Fig. 2.** p_c distributions of gas vesicles in seven strains of *Planktothrix rubescens*. Symbols identify different strains listed in Table 2.

narrowest gas vesicles previously reported in freshwater cyanobacteria were 62 nm, in *Planktothrix rubescens* (*Oscillatoria agardhii*) strain CYA 18 (Walsby & Bleything, 1988). The current series of measurements therefore extends the range of width previously found in freshwater cyanobacteria.

Within each strain, variation in gas vesicle width was encountered; frequency distributions of gas vesicle width are shown in Fig. 3. The distribution of gas vesicle

diameters was tested for normality using the Anderson–Darling test: in none of the seven strains did it depart significantly from a normal distribution.

Correlation of p_c with width of gas vesicles

The inverse relationship between p_c and width of gas vesicles in different genera of cyanobacteria (Hayes &

Table 2. Dimensions and p_c values of gas vesicles isolated from seven strains of *Planktothrix rubescens*

The strains are bracketed into groups that show no significant difference in diameter.

Strain (<i>Pla</i>) ... Gas vesicle genotype ...	[9316] GV2a	[9303] GV1	[9401 GV3a	9402 GV3b	9725] GV3b	[9707 GV3b	9736] GV3b
Critical pressures							
Mean p_c /MPa	0.88	0.96	1.10	1.09	1.09	1.14	1.17
Mean p_a /MPa	0.61	0.72	0.81	0.88	0.80	0.82	0.79
Turgor pressure/MPa	0.27	0.24	0.29	0.21	0.22	0.35	0.38
Gas vesicle measurements							
No. measured	199	158	185	234	168	172	240
Length/nm	523.3	352.1	263.3	257.1	300.8	323.0	282.0
Side of cone/nm	64.7	70.3	67.9	67.6	67.3	66.6	66.2
Collapsed width/nm	95.4	88.0	83.9	84.8	82.6	77.1	74.1
Gas vesicle density/kg m ⁻³	159	179	194	193	192	203	213
Diameter/nm	61.3	56.6	53.9	54.5	53.1	49.6	47.7
Group mean diameter/nm	61.3	56.6		53.8		48.6	
Group standard error/nm	0.15	0.22		0.21		0.15

Fig. 3. Histograms of gas vesicle diameters measured by electron microscopy in seven strains of *Planktothrix*, compared with the theoretical diameters (lines) calculated from the p_c distributions by using equation (3).

Walsby, 1986) has previously been described by the negative exponential expression

$$p_c = 275(r/\text{nm})^{-1.67} \text{ MPa} = 875(d/\text{nm})^{-1.67} \text{ MPa} \quad (1)$$

where r is the radius and d is the diameter of the central cylinder of the gas vesicle (Walsby & Bleything, 1988).

Fig. 4. Relationship between mean p_c and cylinder diameter of gas vesicles in seven strains of *Planktothrix rubescens* (Δ) and the species of cyanobacteria investigated by Walsby & Bleything (1988) ($\bullet$) and Kinsman (1995) ($\circ$). The solid line indicates the exponential relationship of p_c/d calculated from equation (2), using all the data; the dashed line is calculated from equation (1), excluding the new data on *Planktothrix* gas vesicles.

Our new measurements of gas vesicle p_c and width in *Planktothrix rubescens* extend the range of the relationship to stronger and narrower gas vesicles (Fig. 4). With inclusion of these data the coefficients are recalculated, by using the Walsby & Bleything (1988) procedure, as

$$p_c = 160(r/\text{nm})^{-1.53} \text{ MPa} = 461(d/\text{nm})^{-1.53} \text{ MPa} \quad (2)$$

It is seen that the new p_c values are only slightly lower than would be predicted by extrapolating the relationship defined in equation (1); inclusion of the new values gives little change in the relationship over the range of the original measurements (Fig. 4).

Comparison of measured gas vesicle width distribution and that calculated from p_c

If the variation in gas vesicle diameter were the only cause of variation in p_c then the diameters of gas vesicles that collapsed at different pressures could be calculated from the relationship described in equation (2); rearranging gives

$$d = (p_c/461 \text{ MPa})^{-(1/1.53)} \text{ nm} = 55.7(p_c/\text{MPa})^{-0.655} \text{ nm} \quad (3)$$

Using these values the frequency distributions of p_c shown for each strain in Fig. 2 were transformed into the frequency distributions of diameters shown in Fig. 3, where they are compared with the distribution of measured diameters. In most cases the means and standard deviations of both measured and calculated diameter distributions were fairly similar. This indicates that much of the variation in p_c could be explained by variation in gas vesicle width.

The range of gas vesicle length

Measurements were also made of the lengths of the gas vesicles and, when they were not distorted by collapse, of the sides of the collapsed cones (up to 20 in each strain). From these measurements, using procedures described by Walsby & Bleything (1988), the mean volumes of wall (V_w) and enclosed gas space (V_i) could be calculated for gas vesicles of each strain. The ratio of these volumes can be used to calculate the buoyant density of the gas vesicles (Table 2), which is inversely related to their efficiency in providing buoyancy. The relatively high densities, from 159 to 213 kg m⁻³, are principally a consequence of the small widths.

Increased length carries no penalty in p_c , and brings some decrease in density. It might be expected, therefore, that where narrow gas vesicles are required to withstand high pressures, they should be made as long as possible. Paradoxically, those in six of the seven *Planktothrix* strains are shorter (in the range of 257–352 nm) than those in most other cyanobacteria. The ratio of V_i/V_w in the conical end caps is lower than that in the central cylindrical part of a gas vesicle and for this reason the overall value of V_i/V_w increases with gas vesicle length but asymptotically approaches a maximum value. For gas vesicles with diameters less than 60 nm, 90 % of the maximum V_i/V_w ratio is obtained at lengths exceeding 260 nm (see Fig. 6 of Walsby & Bleything, 1988). The gas vesicles in all of the strains exceed this efficiency level.

Correlation of p_c with genotype

Beard *et al.* (1999) determined the GV genotypes of the 81 strains of *Planktothrix* investigated here. The three different classes of GV genotype are distinguished in the frequency distribution of mean p_c values in Fig. 1: the p_c ranges were 0.96–1.0 MPa for the 3 isolates of GV1, 0.86–0.99 MPa for the 26 isolates of GV2, and

1.0–1.17 MPa for the 52 isolates of genotype GV3. No distinction can be drawn between the p_c values of GV2a and GV2b or between GV3a and GV3b. The p_c distributions of genotypes GV2 and GV3 are non-overlapping and clearly different. (That of genotype GV1 appears to be mid-way between the other two, but the number of isolates of this genotype available is too small to allow firm conclusions to be drawn.)

The gas vesicle gene cluster of genotype GV2 is distinguished by having three copies of a *gvpC*²⁰ gene, whereas that of GV3 has three copies of the smaller homologous *gvpC*¹⁶ gene as well as a copy of *gvpC*²⁰ (Beard *et al.*, 1999). A possible explanation for the observed correlation between phenotype and genotype is that the smaller GvpC¹⁶ product of the *gvpC*¹⁶ gene causes narrower gas vesicles to form (perhaps by causing the growing conical cap to extend as a cylinder at a smaller diameter); the resulting narrower gas vesicles would have a higher p_c .

If this explanation is correct then the size of the GvpC molecule must have two opposing effects on gas vesicle strength. Kinsman *et al.* (1995) showed that when *Anabaena* gas vesicles were stripped of their native GvpC (containing five 33-residue repeats, 33RRs) and then allowed to bind recombinant GvpCs containing different numbers (2–5) of the 33RRs the resulting p_c increased with number of 33RRs. In those experiments, however, the width of the gas vesicles would already have been fixed, during their formation; the restrengthening that occurred on rebinding GvpC then depended on the number of ribs each molecule crossed, a function of the number of 33RRs. What we are suggesting here is that during gas vesicle assembly the number of 33RRs also influences the diameter of the gas vesicle that forms and this is a major determinant of critical collapse pressure (Walsby, 1982).

There is some variation in mean p_c between different strains within each GV genotype (Fig. 1) and it is possible that they contain additional genetic differences that affect gas vesicle collapse. These might include changes in other *gvp* genes (Kinsman & Hayes, 1997) or minor differences in nucleotide sequence within *gvpA* or *gvpC* that do not change the lengths of the PCR amplification products used to characterize each GV genotype (Beard *et al.*, 1999). It is noted, however, that the sequence of GvpC²⁰ in strain *Pla* 9736, which has the narrowest and strongest gas vesicles (Table 2), differs in only one amino acid residue (proline instead of serine at residue 150) from that of *Pla* 9401. Comparisons of *Pla* 9316 and *Pla* 9303, whose *gvpC*²⁰s differ in the same way, suggest that this single amino acid change does not account for the difference in p_c .

At least part of the variation in mean p_c must be due to variations in the state or condition of the cultures. In clonal cultures of *Anabaena flos-aquae* the p_c varies with a standard deviation of 15 % of the mean value of 0.615 MPa; about half of this variation is explained by variations in the width of gas vesicles (Walsby & Bleything, 1988). Other causes of variation include the

age distribution of the gas vesicles and physiological conditions that affect p_c (Hayes & Walsby, 1984). Values of mean p_c in different investigations may vary by $\pm 6\%$. This is similar to the variation within the GV2 genotypes ($\pm 6\%$) and only slightly less than that within the GV3 genotypes ($\pm 8\%$).

Natural selection of gas vesicle p_c in Lake Zürich

The natural selection of gas vesicle p_c in populations of *Planktothrix* has been discussed in some detail by Walsby *et al.* (1998). Filaments with stronger gas vesicles maintain their buoyancy at greater depths during winter mixing of a lake and are then able to float up in the spring and contribute to the inoculum of the next season's population in the metalimnion. Filaments with gas vesicles that withstand the pressure at the deepest point of the lake would always form the greatest proportion of buoyant filaments. There is, however, a greater cost in producing stronger, narrower gas vesicles and this produces counter-selection for wider and weaker gas vesicles (Walsby, 1994). The compromise solution is determined by the balance of the cost in making stronger, narrower gas vesicles and the benefit of the additional filaments recruited to the metalimnetic population.

In Lake Zürich the population of *Planktothrix rubescens* is mixed down to great depths during the winter (Thomas & Märki, 1949) and in cold winters is circulated to the deepest point of the lake at 136 m (Walsby *et al.*, 1998). Gas vesicles with a p_c of 1.53 MPa would be required to withstand the combination of cell turgor pressure (0.2 MPa) and hydrostatic pressure at the deepest point of the lake (1.33 MPa at 136 m). Filaments that have gas vesicles with the highest mean p_c of 1.17 MPa would remain buoyant down to depths of 99 m (below which only 3% of the lake volume occurs), while those that have gas vesicles of the lowest p_c (0.86 MPa) would remain buoyant down to depths of only 67 m (below which is 17% of the lake volume).

Walsby *et al.* (1998) observed that in the mild winter of 1994–5 the mixing depth did not exceed 80 m; 80% of the population remained above this depth and 78% of the filaments there retained their buoyancy. There would have been little difference in the selection of strains with the weakest and strongest gas vesicles. In the colder winters of 1993–4 and 1996–7, however, some mixing penetrated to the lake bottom and (in the latter year) only 25% of the filaments in the top 80 m remained buoyant. A much greater proportion of strains with the weakest gas vesicles would have lost their buoyancy and there would have been positive selection for strains with the strongest gas vesicle.

The fact that *Planktothrix* strains with both stronger and weaker gas vesicles persist in the Lake Zürich population indicates the dynamic nature of the selection process from one year to the next. A quantitative description of this process could be produced by determining the ratio of the different strains in relation

to the depth of winter mixing in different years. It would be difficult to obtain unbiased quantitative samples by the isolation and culture of single filaments, but the ability to identify GV genotypes that correlate with different p_c phenotypes could provide the quantitative information required (Beard *et al.*, 1999).

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