Growth competition between *Halobacterium salinarium* strain PHH1 and mutants affected in gas vesicle synthesis

Steven J. Beard, Paul K. Hayes and Anthony E. Walsby

Author for correspondence: Anthony E. Walsby. Tel: +44 117 9287490. Fax: +44 117 9257374.

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

To investigate the role of the buoyancy provided by gas vesicles in the facultative anaerobe *Halobacterium salinarium* PHH1, the growth of a gas-vacuolate (Gv⁺) strain in competition with two gas-vesicle-defective (Gv^{def}) mutants was examined. The Gv⁺ strain synthesized gas vesicles throughout its growth cycle, and floated up to form a thick surface scum during the exponential growth phase in static culture. Mutant Gv^{def1} produced significantly fewer gas vesicles than the Gv⁺ strain in corresponding stages of growth, although in late stationary phase a small proportion of cells floated up to the surface of static cultures. Mutant Gv^{def2} had a much lower gas vesicle content in shaken culture and produced negligible amounts of gas vesicles in static culture. The Gv⁺ and the two Gv^{def} strains grew equally well in shaken cultures, but in static cultures, where steep vertical gradients of oxygen concentration were established, Gv^{def1} was outgrown by the Gv⁺ strain. Gv^{def2} outcompeted the Gv⁺ strain in shallow static cultures, perhaps because Gv^{def2} carried a smaller protein burden, which offset the benefits of buoyancy. This selection for Gv^{def2} was lost in deeper static cultures, although it could be restored by aerating static cultures from below. The results support the hypothesis that the role of buoyancy in halobacteria is to maintain cells at the more aerated surface of brine pools.

Keywords: buoyancy, gas vesicles, halobacteria, *Halobacterium salinarium*

INTRODUCTION

Diverse prokaryotes are made buoyant through the accumulation of gas vesicles (Walsby, 1994). The primary role of buoyancy in phototrophs is to maintain the micro-organisms either near the surface of the water or in the metalimnion of lakes, where they are exposed to favourable light intensities (Walsby, 1994). In other gas-vacuolate micro-organisms, such as the methanogen *Methanosarcina barkeri* (Archer & King, 1984) and various sea-ice bacteria (Gosink & Staley, 1995), the role of buoyancy is less obvious. Petter (1932) suggested that gas vesicles might be important to aerobic halobacteria in buoying them to the water surface, where the concentrations of oxygen are highest.

Petter's hypothesis may apply to other aerobic bacteria; it has been tested in laboratory experiments conducted

with the heterotrophic freshwater bacterium *Prosthecomicrobium pneumaticum*, an obligate aerobe that produces sufficient gas vesicles to render it positively buoyant at all stages of growth (Walsby, 1976). In static cultures, in which vertical gradients of oxygen concentration could develop, the wild-type strain outcompeted a gas-vesicle-deficient mutant. In shaken cultures (and in static growth vessels supplied with air at both the top and bottom surfaces) both strains grew at about the same rate. These competition experiments suggest that in *P. pneumaticum* the function of the gas vesicles is to buoy cells to the more aerated water surface (Walsby, 1976).

The extremely halophilic archaeon *Halobacterium salinarium* (formerly *H. halobium*) accumulates large numbers of gas vesicles and forms floating scums in static culture (Larsen *et al.*, 1967). Rodriguez-Valera *et al.* (1985) measured oxygen concentrations as low as 0.50 mg l⁻¹ in the solar salterns occupied by *H. salinarium*, whereas the concentration of oxygen in air-saturated freshwater is about 7 mg l⁻¹ at the same

Abbreviations: D_{c700}, attenuation following gas vesicle collapse; ΔT, decrease in turbidity following gas vesicle collapse; RGV, relative gas vesicle content.

temperature (40 °C). Such low oxygen concentrations are a consequence of the limited solubility of oxygen at high salt concentrations (Smith, 1928) and of respiration by micro-organisms present at high densities in these habitats (Rodriguez-Valera *et al.*, 1985). *H. salinarium* is a facultative anaerobe: it can grow under microaerobic and anaerobic conditions by using alternative terminal electron acceptors (Oren, 1991), by fermenting arginine (Hartmann *et al.*, 1980), or by using light-driven ion pumps, such as bacteriorhodopsin (Oesterhelt & Stoekenius, 1973; Rodriguez-Valera *et al.*, 1983). In environments where there are steep gradients of oxygen concentration, however, gas vesicle production may provide an advantage to the halobacteria by allowing them to float to the brine surface, where aerobic respiration can occur, as Petter (1932) suggested.

This paper describes the first quantitative measurements of gas vesicle production and growth by *Halobacterium salinarium* strain PHH1. We also describe competition experiments using *H. salinarium* PHH1 (wild-type for gas vesicle synthesis; Gv⁺) and mutants affected in gas vesicle production, to investigate the role of the buoyancy provided by gas vesicles. *H. salinarium* strain PHH1 contains two gene clusters capable of directing gas vesicle synthesis, *p-vac* (located on an endogenous plasmid) and *c-vac* (located on the chromosome) (Englert *et al.*, 1992). In the Gv⁺ strain, expression of the *p-vac* cluster, which leads to the constitutive synthesis of spindle-shaped gas vesicles, represses expression of the *c-vac* cluster (Surek *et al.*, 1988; Horne & Pfeifer, 1989). The *c-vac* region is expressed only in gas-vesicle-defective (Gv^{def}) mutants, which carry rearrangements within the *p-vac* region, and this leads to the accumulation of cylindrical gas vesicles in the stationary phase of growth (Surek *et al.*, 1988; Horne & Pfeifer, 1989). A second class of gas vesicle mutants consists of those that produce no gas vesicles (Gv⁻), presumably due to mutations within both *p-vac* and *c-vac*. Mutants affected in gas vesicle synthesis occur with a high frequency, reportedly up to 1% (Pfeifer *et al.*, 1981), due mostly to insertions or deletions within *p-vac* (Pfeifer *et al.*, 1988).

METHODS

Bacterial strains and growth conditions. *Halobacterium salinarium* strain PHH1 (Gv⁺) was provided by Dr Felicitas Pfeifer (Technische Hochschule Darmstadt, Germany). Cells were grown in darkness at 30 °C, in a basal salts medium (4.0 M NaCl, 61 mM MgSO₄, 20 mM KCl and 20 mM Tris/HCl, pH 7.5) supplemented with peptone (1.5%, w/v; Oxoid) and solidified with 1.5% (w/v) agar. Spread plates were sealed in plastic bags (to minimize desiccation) and incubated at 30 °C for 2–3 weeks. The plates were then stored at room temperature until it was possible to distinguish between colonies of the Gv⁺ strain and gas-vesicle-deficient mutants: the Gv⁺ strain produces creamy pink colonies, Gv⁻ mutants produce transparent colonies, and Gv^{def} mutants produce colonies that are initially transparent, but become opaque as the cells age (Simon, 1981). The biomass in liquid cultures was estimated from the attenuation at 700 nm (path length, 1 cm) using an LKB Ultraspec spectrophotometer

(model 4050, Biochrom). Samples were pressurized (to 0.80 MPa) to collapse all gas vesicles and then diluted with growth medium to give an attenuation that was within the linear region of the instrument.

Measurement of relative gas vesicle content. The relative gas vesicle content (RGV) of cell suspensions was estimated as the ratio $\Delta T/D_{c,700}$, where ΔT is the reduction in turbidity (measured in nephelometer units) observed on collapse of the gas vesicles, and $D_{c,700}$ is the attenuation (at 700 nm) of the cell suspension following gas vesicle collapse. Turbidity measurements were made with a pressure nephelometer (Walsby, 1973). Changes in RGV were determined for shaken cultures by taking samples at intervals; changes in RGV in static cultures were determined by setting up 33 cultures in 1.4 cm-diameter glass tubes and, at each of 11 sampling times, taking samples from three replicate tubes that had been mixed (Figs 2 and 3). Inocula for these cultures were taken from starter cultures in the exponential growth phase.

Measurement of the oxygen concentration in static cultures.

Measurements of oxygen concentration were made with a microelectrode which had a 700 µm diameter tip (model 768-22, Diamond General). The electrode was calibrated with *Halobacterium* growth medium (23.3%, w/v, NaCl) containing sodium sulphite (2%, w/v) to set the zero O₂ concentration, and with medium which had been bubbled with air for 24 h to set the value corresponding to 100% saturation. Oxygen concentrations were measured as the tip of the electrode was moved downward in steps of 1 mm through a culture of the Gv⁺ strain. The culture was grown in a glass tube (2.3 cm diameter) at 30 °C, but measurements were made at room temperature (21 °C). The concentration of oxygen in air-saturated 23.3% (w/v) NaCl is calculated to be 67.4 µmol l⁻¹ using the data of Smith (1928).

Measurement of critical collapse pressure of gas vesicles.

Critical collapse-pressure distributions for gas vesicles were determined by pressure nephelometry (Walsby, 1973) using cells suspended in the basal salts medium. Mean critical pressures were calculated as described by Walsby & Bleything (1988).

Electron microscopy of cell lysates. Small quantities of cell material were scraped from agar plates and applied to the edge of pioloform-coated copper grids. The grids were floated face down on drops of water and then drained with filter paper and air-dried. Specimens were examined in a JEOL transmission electron microscope.

Growth competition in batch culture. *H. salinarium* PHH1 (Gv⁺) was grown in competition with each of the gas-vesicle-defective mutants. All incubations were performed in darkness to avoid the complication of phototrophic growth. Cells were initially grown in 100 ml batch cultures in 250 ml Erlenmeyer flasks either shaken at 100 r.p.m. or left static. Subsequently, cells were cultured in 2.4 cm diameter glass tubes in which the lower end was either closed, or sealed by dialysis membrane, which was clamped in position by a glass chamber containing gas inlet and outlet tubes (Walsby, 1976). The gas inlet tube was connected to a supply of sterile moistened air. Both types of culture vessel were clamped in an upright position and contained 9 cm layers of liquid medium. Inocula for the competition experiments were taken from separate starter cultures in the exponential growth phase to give cell suspensions containing approximately equal numbers of Gv⁺ and mutant cells; cell concentrations were determined using a Weber counting chamber. At various stages in the growth cycle, samples (0.05 ml) were removed from the cultures, serially diluted in growth medium, and plated for subsequent

counting. The static cultures were mixed before samples were removed. The numbers of Gv^+ , Gv^{def} and Gv^- colonies were counted after they had grown sufficiently to allow the differences in their turbidity to be distinguished.

RESULTS

Isolation of mutants affected in gas vesicle synthesis

On agar plates, gas vesicle-defective (Gv^{def}) mutants of *H. salinarium* PHH1 appeared as reddish-pink colonies amongst the creamy-pink colonies of the Gv^+ strain. Inspection under the phase-contrast microscope showed that cells from a purified reddish-pink colony contained smaller amounts of gas vesicles, which were identified as pressure-sensitive, light-refractile bodies. This mutant was designated Gv^{def1} . Examination in the electron microscope of lysed stationary-phase cells revealed that the Gv^+ strain contained predominantly (> 99%) spindle-shaped gas vesicles, whereas the Gv^{def1} mutant contained mainly (about 55%) cylindrical gas vesicles. In static liquid cultures, Gv^+ cells floated up to the surface during the exponential and stationary growth phases to form a thick film. Under identical growth conditions cells of Gv^{def1} were suspended throughout the cultures, although some cells floated up to form a surface scum in late stationary phase. A second gas-vesicle-defective mutant (Gv^{def2}) was isolated from a culture of Gv^{def1} . Initially Gv^{def2} appeared to exhibit a Gv^- phenotype since it produced orange transparent colonies in which no gas vesicles were observed. However, the pressure-sensitive turbidity detected in shaken cultures of Gv^{def2} indicated the presence of small amounts of gas vesicles. In static cultures, cells of Gv^{def2} sank to the bottom of the growth vessel and remained there throughout the growth cycle, although in some old cultures a diffuse scum appeared in late stationary phase. Cells that were removed from these scums lacked gas vesicles and may have accumulated at the surface by swimming, since *H. salinarium* possesses flagella and has been shown to be positively aerotactic (Stoeckenius *et al.*, 1988).

Fig. 1. Critical pressure distributions for gas vesicles in the Gv^+ (○) and Gv^{def1} (●) strains of *H. salinarium* PHH1. Means and standard deviations of five (Gv^+) or three (Gv^{def1}) measurements are plotted (most of the error bars are smaller than the symbols).

Fig. 2. Changes in (a) biomass and (b) RGv of the Gv^+ strain (○) and gas-vesicle-defective mutant Gv^{def1} (●) when grown in shaken culture. Means and standard deviations of three measurements are plotted. Note the differences in the scales in (b).

Gas vesicle critical pressures

The critical pressure distributions of gas vesicles in cells from stationary-phase cultures of the Gv^+ and Gv^{def1} strains were determined (Fig. 1). As in other organisms (Walsby, 1971; Walsby & Bleything, 1988), the critical pressures of the gas vesicles in *H. salinarium* exhibited a broad normal distribution about a well-defined mean. The mean critical pressure of gas vesicles in the Gv^+ strain was 0.1138 ± 0.0013 MPa (95% confidence level, $n = 5$), with a standard deviation of the distribution of 0.0396 MPa. The mean critical pressure of gas vesicles in Gv^{def1} was 0.2012 ± 0.0004 MPa (95% confidence level, $n = 3$), with a standard deviation of the distribution of 0.0679 MPa.

Growth and gas vesicle content of the Gv^+ and Gv^{def} strains in shaken and static cultures

Measurements were made of the growth and gas vesicle content of the Gv^+ and Gv^{def1} strains, grown in 2.6 cm deep cultures that were either shaken or left static. The growth curves were broadly similar for the two strains studied (Figs 2a and 3a), with minimum mean doubling times of about 10 h in both shaken and static culture. Although the initial growth rates were similar in shaken and static culture, these rates were not sustained for so long in static culture, perhaps because the cultures became oxygen-limited.

Fig. 3. Changes in (a) biomass and (b) RGV of the Gv^+ strain (○) and gas-vesicle-defective mutant Gv^{def1} (●) when grown in static culture. Other details as in Fig. 2.

Changes in the relative gas vesicle content (RGV) demonstrated that gas vesicles were synthesized throughout the growth cycle of the Gv^+ strain in both shaken and static culture (Figs 2b and 3b). In shaken culture (Fig. 2b) the RGV of the Gv^+ strain did not change significantly during the first 44 h of the exponential phase ($P = 0.673$), which indicates that the rate of gas vesicle production matched the exponential growth rate. Thereafter the RGV increased as the growth rate decreased. A broadly similar pattern of gas vesicle production was observed when the Gv^+ strain was grown in static culture (Fig. 3b). The RGV increased at a higher rate in shaken culture (compare the slopes in Figs 2b and 3b). However, if the same measurements of RGV are plotted against culture biomass (Fig. 4a) rather than versus age of the culture, it is seen that the rise in gas vesicle formation occurs at an earlier stage of biomass development in static culture.

The amounts of gas vesicles accumulated in Gv^{def1} were much less than in the Gv^+ strain (Figs 2b and 3b). The highest mean RGV measured for Gv^{def1} ($\Delta T/D_{c,700} = 478$) was approximately sevenfold less than the highest value measured for the Gv^+ strain ($\Delta T/D_{c,700} = 3347$). In both shaken and static cultures (Figs 2b and 3b) the RGV of Gv^{def1} remained low (≤ 25) throughout the exponential growth phase. The values of RGV were, however, higher than would have been expected if no gas vesicle synthesis occurred during exponential growth and the gas vesicles present in the cells of the inoculum were simply diluted out between siblings. The RGV

Fig. 4. Changes in the RGV as a function of the culture biomass of shaken (○) and static (●) cultures of the Gv^+ strain (a) and the gas-vesicle-defective mutant Gv^{def1} (b). These measurements are the same as those presented in Figs 2 and 3. Means and standard deviations of three measurements are plotted.

increased considerably during the stationary phase in both shaken and static culture (Figs 2b and 3b). As observed for the Gv^+ strain, the RGV of Gv^{def1} began to increase at an earlier stage of biomass development in static culture than it did in shaken culture (Fig. 4b).

Growth curves obtained for Gv^{def2} (data not shown) were similar to those obtained for the Gv^+ and Gv^{def1} strains, with minimum mean doubling times of about 10 h in shaken culture, and 9 h in static culture. Gas vesicles were detected in the late stationary phase of the shaken cultures at 0.5% of the level in the Gv^+ strain, and in these cultures the RGV varied considerably ($\Delta T/D_{c,700} = 18.3 \pm 25.1$, $n = 3$).

Investigation of the vertical gradient of oxygen concentration in a static culture of the Gv^+ strain

A steep vertical gradient of oxygen concentration was measured in a static culture of the Gv^+ strain (Fig. 5). The culture, which was in the exponential phase of growth ($D_{c,700} = 0.375$), had an RGV of 1.6×10^2 and contained a thin layer of buoyant cells at the surface. The concentration of oxygen at the surface of the culture was 66% of that in air-saturated growth medium, which corresponds to $45 \mu\text{mol l}^{-1}$. At a depth of 1 mm, however, the oxygen concentration was only 7.3% saturation (approximately $5 \mu\text{mol l}^{-1}$). The oxygen concentration then fell gradually over the next 34 mm to reach 3.1% saturation (approximately $2 \mu\text{mol l}^{-1}$).

Fig. 5. Vertical gradient of oxygen concentration (percentage saturation) in a static culture of the Gv^+ strain. The concentration of oxygen corresponding to 100% saturation in a 23.3% (w/v) NaCl solution is $67.4 \mu\text{mol l}^{-1}$ (see Methods).

Fig. 6. Ratio of $Gv^+ : Gv^{\text{def1}}$ strains in shaken (○) and static (●) cultures after different times. Means and standard deviations of three measurements are plotted.

Fig. 7. Ratio of $Gv^+ : Gv^{\text{def2}}$ strains in shaken (○) and static cultures (●) after different times. Means and standard deviations of three measurements are plotted.

Growth competition between the Gv^+ and Gv^{def} strains in shaken and static cultures

The Gv^+ strain was grown in competition with either Gv^{def1} or Gv^{def2} in 2.6 cm deep shaken and static cultures. When the Gv^+ and Gv^{def1} strains were grown

Fig. 8. Ratio of $Gv^+ : Gv^{\text{def2}}$ strains when grown together in shaken (a) and static (b) flasks, and in special culture vessels supplied with air at the top (c), or at top and bottom (d). Open bars, ratio in the inoculum; hatched bars, ratio after 10 d incubation. Means and standard deviations of three measurements are given.

together in shaken culture (Fig. 6) the proportions of the two strains did not change significantly over a 22 d period ($P = 0.962$). In static culture, however, Gv^{def1} was outgrown by the Gv^+ strain (Fig. 6).

Two sets of experiments were performed to investigate the competition between the Gv^+ and Gv^{def2} strains. In the first experiment (Fig. 7) we detected a small, but significant decrease in the ratio of $Gv^+ : Gv^{\text{def2}}$ after 9 d in shaken culture ($P = 0.003$), although after 22 d the ratio was not significantly different from that in the inoculum ($P = 0.150$). There was no significant change in the ratio over the course of the second experiment in shaken culture ($P = 0.630$) (Fig. 8a). In static flasks, however, the Gv^+ strain was outgrown by Gv^{def2} (Figs 7 and 8b). This result suggested that the burden of producing gas vesicles in the Gv^+ strain resulted in a lower growth rate. In the second set of experiments additional cultures were grown in static vessels that contained 9 cm deep layers of growth medium, and in these cultures there was no significant change in the ratio of $Gv^+ : Gv^{\text{def2}}$ ($P = 0.360$) (Fig. 8c). This suggested that growth of Gv^{def2} was limited by the low oxygen tension at the bottom of the deeper cultures and that this counteracted the intrinsically high growth rate shown in Fig. 8(b). Further 9 cm deep cultures were grown in special culture vessels in which air was supplied to both the top and bottom. In these cultures the growth advantage of Gv^{def2} was partially restored (Fig. 8d), which indicated that growth of Gv^{def2} was indeed limited by the reduced availability of oxygen in the deeper cultures (Fig. 8c). The growth medium used in this study could not support the growth of *H. salinarum* under anaerobic conditions: cultures that were sparged with oxygen-free nitrogen failed to grow.

Investigation of the contributions of mutation and conjugation to the results of the competition experiments

The ratio of $Gv^+ : Gv^{\text{def}}$ strains could be affected by three processes: selection, mutation and the conjugal transfer of DNA encoding the gas-vacuolate phenotype. To

investigate the possibility that changes in the mutation frequency may have influenced our results we measured the frequencies with which gas vesicle mutants appeared in clonal cultures of each of the three strains. After growing the cultures for 22 d in shaken and static flasks, cells were plated and the numbers of Gv⁺, Gv^{def} and Gv⁻ colonies were counted. For each strain, colonies exhibiting an altered gas vesicle phenotype appeared at a frequency of less than 1×10^{-2} . From this it was concluded that mutation made a negligible contribution to the changes in the ratio of Gv⁺:Gv^{def} that were observed in the competition cultures. To determine whether the *p-vac* gene cluster could be transferred from the Gv⁺ strain to Gv^{def1} by conjugation we grew the Gv⁺ strain in competition with an anisomycin-resistant derivative of Gv^{def1}, and plated cells on medium containing anisomycin ($8 \mu\text{g ml}^{-1}$). Only colonies with a Gv^{def} phenotype grew on this medium, which indicated that there was no detectable transfer of *p-vac* from the Gv⁺ strain to Gv^{def1}.

DISCUSSION

The gas-vesicle-defective mutant Gv^{def1} contained predominantly cylindrical gas vesicles, which were stronger than the spindle-shaped gas vesicles produced by the Gv⁺ strain (Fig. 1). These observations are consistent with those of Hayes & Walsby (1986), who demonstrated an inverse relationship between the width and strength of gas vesicles in cyanobacteria. Walsby & Bleything (1988) demonstrated that wider gas vesicles have a higher ratio of gas space to wall volume and are therefore more efficient in providing buoyancy. This is the basis of the hypothesis that there has been natural selection of gas vesicles with the maximum width allowed by the requirement to withstand pressure (Hayes & Walsby, 1986). Halobacterial gas vesicles are the widest (Larsen *et al.*, 1967; Stoeckenius & Kunau, 1968; Simon, 1981) and weakest (this study; Walsby, 1971) known. They are strong enough to withstand the combination of the low hydrostatic pressures and negligible cell turgor pressure in halobacteria growing in shallow brine pools (Walsby, 1994). The stronger, cylindrical gas vesicles of gas-vesicle-defective mutants may be an adaptation to provide buoyancy in deeper brine lakes, but there has been no investigation of this.

The patterns of gas vesicle production seen in the Gv⁺ and Gv^{def} strains of *H. salinarium* (Figs 2 and 3) correlate well with the patterns of *p-gvpA* and *c-gvpA* gene expression (*gvpA* encodes the major gas vesicle protein, GvpA): in the Gv⁺ strain maximal levels of *p-gvpA* mRNA were detected during the exponential growth phase, and in *p-vac* deletion mutants maximal levels of *c-gvpA* mRNA were detected in stationary phase (Horne & Pfeifer, 1989). The observations that the RGV of both the Gv⁺ and Gv^{def1} strains began to increase at an earlier stage in the growth cycle when grown in static culture (as compared to when grown in shaken culture) indicate that gas vesicle synthesis may have been induced in response to the lower concen-

trations of oxygen dissolved in the static cultures. This has also been suggested by Horne & Pfeifer (1989), who have stated that in *Halobacterium* sp. SB3 there may be greater gas vesicle production under anaerobic growth conditions. Furthermore, Yang & DasSarma (1990) demonstrated that aeration significantly reduces the transcriptional induction of *gvpA* in *H. salinarium* NRC-1. Gas vesicle synthesis in *Haloferax mediterranei* was shown to be stimulated by growth at higher salt concentrations, in which the dissolved oxygen concentration would have been lower (Englert *et al.*, 1990). These observations suggest that gas vesicle production in halobacteria is stimulated in oxygen-limited environments.

Steep gradients of oxygen concentration were shown to exist in the 2.6 cm deep static cultures of the Gv⁺ strain (Fig. 5). The selection in static culture for the Gv⁺ strain over the gas-vesicle-defective mutant Gv^{def1} (Fig. 6) supports the hypothesis of Petter (1932) that in unstirred habitats it is advantageous for halobacteria to float to the more-aerated brine surface. Similar results were obtained in competition experiments conducted with the gas-vacuolate wild-type and gas-vesicle-deficient mutants of *Prosthecomicrobium pneumaticum* (Walsby, 1976). It is likely that the buoyancy provided by gas vesicles benefits the growth of *P. pneumaticum* throughout its growth cycle, since the cells are positively buoyant at all stages of growth (Walsby, 1976). *H. salinarium*, in contrast, achieves buoyancy only in the late exponential and stationary growth phases, which suggests that gas vesicles may be more important in the survival or dispersal of this organism. In the natural environment, gas vesicles must confer a selective advantage upon *H. salinarium* since the *p-vac* gene cluster has not been lost despite the extremely high frequency of *p-vac* mutants (Pfeifer *et al.*, 1988).

An explanation is needed for the different results obtained in the competition experiments with the two Gv^{def} strains. The Gv^{def2} mutant produced negligible amounts of gas vesicles in static culture and therefore would have carried a smaller protein burden than either the Gv⁺ or Gv^{def1} strains. This appeared to offset any advantage that the Gv⁺ strain may have had in floating nearer the surface of the culture (Fig. 8b). This explanation is supported by the finding that net selection of the Gv^{def2} mutant did not occur in deeper cultures where a greater proportion of the non-buoyant mutant cells were further from the oxygenated surface (Fig. 8c). The reason why Gv^{def2} was not selected for in shaken culture (Fig. 8a) may have been that the Gv⁺ strain produced fewer gas vesicles in the early stages of growth in shaken culture (Fig. 4a). In deeper cultures the advantage enjoyed by the Gv^{def2} mutant was partially restored by providing aerobic conditions at the bottom surface (Fig. 8d). Therefore, it seems likely that gas vesicles become progressively more beneficial to the growth of *H. salinarium* as the depth of the culture increases (and the availability of oxygen decreases). There have been no comparable studies on how the growth rates of other gas-vacuolate micro-organisms

are affected by the proportions of protein that are diverted into gas vesicle production. However, it has proved to be difficult to demonstrate the effects of a protein burden on the growth rate of *Escherichia coli* (Koch, 1983).

ACKNOWLEDGEMENTS

We would like to thank Mrs Avril Avery for technical assistance and Dr Felicitas Pfeifer for helpful discussions and the kind gift of *H. salinarum* strain PHH1. We are grateful to Professor John Parkes and Mr Alistair Lawson of the Department of Geology, University of Bristol, for assisting us with the measurements of oxygen concentration. This work was supported by a grant GR3/8987 from the NERC.

REFERENCES

- Archer, D. B. & King, N. R. (1984). Isolation of gas vesicles from *Methanosarcina barkeri*. *J Gen Microbiol* **130**, 167–172.
- Englert, C., Horne, M. & Pfeifer, F. (1990). Expression of the major gas vesicle protein gene in the halophilic archaeobacterium *Haloferax mediterranei* is modulated by salt. *Mol Gen Genet* **222**, 225–232.
- Englert, C., Krüger, K., Offner, S. & Pfeifer, F. (1992). Three different but related gene clusters encoding gas vesicles in halophilic archaea. *J Mol Biol* **227**, 586–592.
- Gosink, J. J. & Staley, J. T. (1995). Biodiversity of gas vacuolate bacteria from Antarctic sea ice and water. *Appl Environ Microbiol* **61**, 3486–3489.
- Hartmann, R., Sickinger, H.-D. & Oesterhelt, D. (1980). Anaerobic growth of halobacteria. *Proc Natl Acad Sci USA* **77**, 3821–3825.
- Hayes, P. K. & Walsby, A. E. (1986). The inverse correlation between width and strength of gas vesicles in cyanobacteria. *Br Phycol J* **21**, 191–197.
- Horne, M. & Pfeifer, F. (1989). Expression of two gas vacuole protein genes in *Halobacterium halobium* and other related species. *Mol Gen Genet* **218**, 437–444.
- Koch, A. L. (1983). The protein burden of *lac* operon products. *J Mol Evol* **19**, 455–462.
- Larsen, H., Omang, S. & Steensland, H. (1967). On the gas vacuoles of the Halobacteria. *Arch Mikrobiol* **59**, 197–203.
- Oesterhelt, D. & Stoeckenius, W. (1973). Functions of a new photoreceptor membrane. *Proc Natl Acad Sci USA* **70**, 2853–2857.
- Oren, A. (1991). Anaerobic growth of halophilic archaeobacteria by reduction of fumarate. *J Gen Microbiol* **137**, 1387–1390.
- Petter, H. F. M. (1932). *Over roode en andere bakteriën gezouten visch*. Doctoral thesis, University of Utrecht, Utrecht, The Netherlands.
- Pfeifer, F., Weidinger, G. & Goebel, W. (1981). Genetic variability in *Halobacterium halobium*. *J Bacteriol* **145**, 375–381.
- Pfeifer, F., Blaseio, U. & Ghahraman, P. (1988). Dynamic plasmid populations in *Halobacterium halobium*. *J Bacteriol* **170**, 3718–3724.
- Rodriguez-Valera, F., Nieto, J. J. & Ruiz-Berraquero, F. (1983). Light as an energy source in continuous cultures of bacteriorhodopsin-containing halobacteria. *Appl Environ Microbiol* **45**, 868–871.
- Rodriguez-Valera, F., Ventosa, A., Juez, G. & Imhoff, J. F. (1985). Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern. *Microb Ecol* **11**, 107–115.
- Simon, R. D. (1981). Morphology and protein composition of gas vesicles from wild-type and gas vacuole defective strains of *Halobacterium salinarum* strain 5. *J Gen Microbiol* **125**, 103–111.
- Smith, D. F. (1928). The solubility of gases in solutions. In *International Critical Tables of Numerical Data, Physics, Chemistry and Technology*, pp. 271–283. Edited by E. W. Washburn. New York: McGraw-Hill.
- Stoeckenius, W. & Kunau, W. H. (1968). Further characterization of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and isolation of gas vacuole membranes. *J Cell Biol* **38**, 337–357.
- Stoeckenius, W., Wolff, E. K. & Hess, B. (1988). A rapid population method for action spectra applied to *Halobacterium halobium*. *J Bacteriol* **170**, 2790–2795.
- Surek, B., Pillay, B., Rdest, U., Beyreuther, K. & Goebel, W. (1988). Evidence for two different gas vesicle proteins and genes in *Halobacterium halobium*. *J Bacteriol* **70**, 1746–1751.
- Walsby, A. E. (1971). The pressure relationships of gas vacuoles. *Proc R Soc London, B* **178**, 301–326.
- Walsby, A. E. (1973). A portable apparatus for measuring relative gas vacuolation, the strength of gas vacuoles, and turgor pressure in planktonic blue-green algae and bacteria. *Limnol Oceanogr* **18**, 653–658.
- Walsby, A. E. (1976). The buoyancy-providing role of gas vacuoles in an aerobic bacterium. *Arch Microbiol* **109**, 135–142.
- Walsby, A. E. (1994). Gas vesicles. *Microbiol Rev* **58**, 94–144.
- 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.
- Yang, C.-F. & DasSarma, S. (1990). Transcriptional induction of purple membrane and gas vesicle synthesis in the archaeobacterium *Halobacterium halobium* is blocked by a DNA gyrase inhibitor. *J Bacteriol* **172**, 4118–4121.

Received 17 June 1996; revised 11 September 1996; accepted 13 September 1996.