Structural characteristics of halobacterial gas vesicles

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Gas vesicle formation in halophilic archaea is encoded by a DNA region (the vac region) containing 14 different genes: *gvpACNO* and *gvpDEFGHIJKLM*. In *Halobacterium salinarum* PHH1 (which expresses the p-vac region from plasmid pHH1), gas vesicles are spindle shaped, whereas predominantly cylindrical gas vesicles are synthesized by the chromosomal c-vac region of *H. salinarum* PHH4 and the single chromosomal mc-vac region of *Haloferax mediterranei*. Homologous complementation of *gvp* gene clusters derived from the chromosomal c-vac region led to cylindrical gas vesicles in transformants and proved that the activity of the c-*gvpA* promoter depended on a gene product from the c-*gvpE-M* DNA region. Heterologous complementation experiments with transcription units of different vac regions demonstrated that the formation of chimeric gas vesicles was possible. Comparison of micrographs of wild-type and chimeric gas vesicles indicated that the shape was not exclusively determined by GvpA, the major structural protein of the gas vesicle wall. More likely, a dynamic equilibrium of several *gvp* gene products was responsible for determination of the shape. Transmission electron microscopy of frozen hydrated, wild-type gas vesicles showed moiré patterns due to the superposition of the front and back parts of the ribbed gas vesicle envelope. Comparison of projections of model helices with the moiré pattern seen on the cylindrical part of the gas vesicles provided evidence that the ribs formed a helix of low pitch and not a stack of hoops.

Keywords: halophilic archaea, gas vesicle structure

INTRODUCTION

The synthesis of gas vesicles allows micro-organisms to float at a favourable depth in their watery environment. Most of these gas-filled floating devices have cylindrical middle sections with conical caps, and their length varies from 100 to 1500 nm depending on the micro-organism they derive from (for a review see Walsby, 1994). The gas vesicle envelope consists exclusively of protein and is watertight, but freely permeable to dissolved ambient gases. The complete separation and identification of protein components of the gas vesicle

envelope fails because the gas vesicle wall is resistant to conventional biochemical treatments (Krantz & Ballou, 1973; Simon, 1981). Amino acid sequencing of gas vesicle preparations, immunological analyses and electron microscopy revealed that the hydrophobic 7–8 kDa GvpA protein is the major component of the ribbed gas vesicle envelope (Walker *et al.*, 1984; Surek *et al.*, 1988; Englert *et al.*, 1990), and that the hydrophilic GvpC (20–42 kDa, depending on the species) is a minor constituent of the gas vesicle wall (Walsby & Hayes, 1988; Hayes *et al.*, 1992; Englert & Pfeifer, 1993; Halladay *et al.*, 1993). The cyanobacterial GvpC is located at the outer surface of the gas vesicle and strengthens the entire structure (Hayes *et al.*, 1992; Kinsman *et al.*, 1995). The GvpC protein is also responsible for the constant diameter of the cylindrical middle part of the halobacterial gas vesicle (Offner *et al.*, 1996). If there are other proteins involved in the structure of the gas vesicle, they have yet to be determined. High-

Abbreviations: c-vac, chromosomally located gas vesicle region of *Halobacterium salinarum*; mc-vac, gas vesicle region located at a single chromosomal position in *Haloferax mediterranei*; p-vac, gas vesicle region located on plasmid pHH1 of *Halobacterium salinarum* PHH1; Vac, gas vesicle phenotype.

resolution electron microscopy and atomic-force microscopy of the cyanobacterial gas vesicle envelope indicate that the GvpA protein, forming the 4.6 nm wide ribs, is arranged as β -chains and crosses the rib at an angle of 55° to the rib axis (Blaurock & Walsby, 1976; McMaster *et al.*, 1996). However, whether the ribs of the gas vesicle are arranged as a helix or as a stack of hoops is still a riddle.

Studies on gas vesicle formation and regulation of the gene products involved have been carried out with a few species of cyanobacteria (*Anabaena flos-aquae*, *Calothrix* PCC 7601) and halophilic archaea (*Halobacterium salinarum*, *Haloferax mediterranei*, *Natronobacterium vacuolatum*) (Hayes & Powell, 1995; Kinsman & Hayes, 1997; Damerval *et al.*, 1987; Jones *et al.*, 1991; Englert *et al.*, 1992a; Mayr & Pfeifer, 1997). In the case of the halophilic archaea, transformation experiments confirmed that the 14 different genes clustered in a region (the vac region) of approximately 9 kb are sufficient for the formation of gas vesicles (Vac⁺ phenotype) in the Vac⁻ negative recipient, *Haloferax volcanii* (Blaseio & Pfeifer, 1990; Englert *et al.*, 1992a, b). *Hf. mediterranei* contains one chromosomal DNA region (termed mc-vac) and *Hb. salinarum* PHH1 harbours two related vac regions: one named p-vac, located on the plasmid pHH1 and the other (c-vac) found in the chromosomal DNA fraction (Horne *et al.*, 1991; Englert *et al.*, 1992a). The genes of all three vac regions are identically arranged: *gvpACNO* and upstream of *gvpA* but oriented in the opposite direction, *gvpD-M*. Expression of the mc-vac or c-vac region leads to cylindrical gas vesicles in the stationary growth phase only (Englert *et al.*, 1990), whereas the p-vac region encodes predominantly short, spindle-shaped gas vesicles produced during all stages of growth (Horne & Pfeifer, 1989). The length of the spindle-shaped gas vesicles is fairly constant, around 250 nm, whereas the length of the cylindrical gas vesicles varies between 400 and 1500 nm. The diameter of both specimens varies between 100 and 200 nm (Stoeckenius & Kunau, 1968; Simon, 1981; Englert *et al.*, 1990). A peculiarity of the c-vac region is that the c-*gvpA* promoter is active only in *Hb. salinarum* strains lacking the plasmid-borne p-vac region (such as *Hb. salinarum* PHH4), implying a repressor function for one or more of the p-*gvp* gene products with regard to c-vac expression (Horne *et al.*, 1988).

Analysis of the amino acid sequences of the 14 proteins derived from *gvp* gene sequences indicates that, besides the two structural proteins GvpA and GvpC, other proteins might be minor components of the gas vesicle envelope: GvpJ (10–12 kDa) and GvpM (8–9 kDa) share sequence and structural similarity with the GvpA protein (Englert *et al.*, 1992a). As these proteins are encoded by the early-transcribed *gvpF-M* genes, they might participate in an early stage of gas vesicle assembly. The small GvpG (10 kDa) and GvpK (12.7 kDa) proteins have a hydrophobic N-terminal part and a hydrophilic C-terminus (Englert *et al.*, 1992a), which could function either as structural components of the gas vesicle or as a kind of chaperone, aiding

solubilization of the hydrophobic GvpA, GvpJ and GvpM monomers in the cytoplasm.

The transformation system for halobacteria permits functional analysis of the *gvp* genes in gas vesicle synthesis. Shuttle vectors are available that harbour genes conferring resistance to mevinolin (Lam & Doolittle, 1989; Blaseio & Pfeifer, 1990) or novobiocin (Holmes *et al.*, 1991), and compatible origins of replication. The vectors used for complementation studies with the Vac⁻ recipient *Hf. volcanii* also include an expression vector which enables high-level expression of halobacterial genes under the control of the strong promoter of the halobacterial ferredoxin (*fdx*) gene (Pfeifer *et al.*, 1994). Previous transformation experiments showed that (i) the p-vac as well as the mc-vac region present on a single vector leads to gas vesicle formation in *Hf. volcanii* (Blaseio & Pfeifer, 1990; Englert *et al.*, 1992a) and (ii) complementation of the p-*gvpACNO* and p-*gvpD-M* transcription units is possible in transformants, allowing the synthesis of mainly spindle-shaped gas vesicles (Offner & Pfeifer, 1995).

Here we report transformation experiments designed to uncover the *gvp* gene products involved in the determination of the different gas vesicle shapes (spindle shaped or cylindrical). Transformants were analysed by Western blots to observe the synthesis of GvpA, and the shape of isolated gas vesicles was inspected by electron microscopy. In addition, transmission electron microscopy of frozen hydrated samples of intact gas vesicles was carried out to gain insights into the microstructure of the gas vesicle wall.

METHODS

Strains and growth conditions. *E. coli* strains DH5 α (Hanahan, 1983) and GM1674 (*dam*) (Palmer & Marinus, 1994) were grown aerobically at 37 °C in LB broth (Sambrook *et al.*, 1989) with ampicillin at a concentration of 100 μ g ml⁻¹. *Hb. salinarum* PHH1 [renamed by Ventosa & Oren (1996); formerly *Hb. halobium* or *Hb. salinarum*] was grown in rich medium containing (l⁻¹): 245.4 g NaCl; 20 g MgSO₄·7H₂O; 2 g KCl; 50 ml 1 M Tris/HCl pH 7.2; 2 ml 10% CaCl₂·2H₂O and 15 g Oxoid peptone. *Hf. volcanii* WFD11, lacking the endogenous plasmid pHV2 (Cline *et al.*, 1989), was grown in rich medium containing (l⁻¹): 175 g NaCl; 37 g MgSO₄·7H₂O; 3.7 g KCl; 5 g Bacto tryptone; 3 g Bacto yeast extract; 25 ml 1 M Tris/HCl pH 7.2; 5 ml 10% CaCl₂·2H₂O and 17 μ l 100 mM MnCl₂. Transformants were selected on agar plates containing 0.2 μ g novobiocin ml⁻¹ and 6 μ g mevinolin ml⁻¹. Lovastatin (mevinolin) was a gift from Merck, Sharpe & Dohme (München).

Constructs used for transformation of *Hf. volcanii* and transformation procedure. Subfragments of the p-vac region used to construct plasmids are listed in Table 1 and Fig. 1. In most cases, the DNA inserts (cE-M, cA-OI, mcD-M, mcA-O, mcA) were blunt-end ligated to the halobacterial vectors pMDS20 (blunt-ended ligation into the filled-in *Hind*III site), pWL102 (blunt-ended ligation into the filled-in *Bam*HI site), or the *Sma*I site of pUBP2 (Holmes *et al.*, 1991; Lam & Doolittle, 1989; Blaseio & Pfeifer, 1990).

The construction of the pD-M, pA-O and pAA fragments are

Table 1. DNA subfragments of the p-vac, the c-vac and the mc-vac region used for transformation

The accession numbers of the p-vac sequence in the EMBL database are: X55684 for p-*gvpD-M*, and X64729 for p-*gvpACNO*. The EMBL accession number for the c-vac region is X94688.

Fragment name*	Restriction site	Position†	Size (bp)
pD-M	<i>EcoRI-XcmI</i>	294-6518	6224
pA-O	<i>HindIII-BglII</i>	6399-9771	3373
pΔA	<i>MroI-SspI</i> (Δ <i>XcmI-StuI</i>)	1-9921 (Δ6518-6761)	9920 (Δ243)
cE-M	<i>NruI-NruI</i>	approx. 250-4679	approx. 4930
cA-OI	<i>SspI-BamHI</i>	6032-8929	2897
cA-OII	-	6206-8878	2672
cAII	-	6206-6450	244
mcD-M	<i>XcmI-ScaI</i>	4104-11247	7143
mcA-O	<i>XhoI-ScaI</i>	4660-419	4241
mcA	<i>MluI-EcoRI</i>	4356-3728	628

* The first letter of the fragment name ('p', 'c', or 'mc') indicates the vac region (p-vac, c-vac, or mc-vac) it derives from. The Roman numerals (I, II) in the name of the cA-O and the cA fragment differentiate the two constructs, which contain the same *gvp* genes, but have different fragment lengths.

† The position of the restriction sites of the p-vac region is given starting from the *MroI* site (position 1) located 491 nt downstream of the p-*gvpM* gene through to the ISH2 element located downstream of p-*gvpO* in pHH1 (see Fig. 1). The start of the ISH2 element is at position 9842. The internal deletion in the ΔA construct is given in parentheses. Position 1 of the c-vac region is represented by the *PstI* site located 435 bp downstream of the stop codon of the c-*gvpM* gene. Position 1 of the mc-vac region corresponds to the *PstI* site lying 1157 bp downstream of the mc-*gvpO* gene.

Fig. 1. Subfragments of the three vac regions used for transformation experiments. The physical map of each vac region indicates the *gvpD-M* and *gvpACNO* genes as boxes. Arrows mark the start and direction of the transcripts. The subfragments used for the transformation experiments (see Table 1) are indicated by lines below the boxes. The designation of subfragments is according to the *gvp* genes present near the boundary, except for pΔA, which corresponds to the whole p-vac region, but lacking the p-*gvpA* gene. Analysis of the pD-M/pA-O transformant has already been published (Offner & Pfeifer, 1995).

described by Offner & Pfeifer (1995) and the cAII construct is published by Pfeifer *et al.* (1994).

The cA-OII DNA fragment was amplified by PCR using the oligonucleotides 5'-AGATCAACCATGGCGCAACCAGACTC-3' and 5'-CCGACCATGGTGACCTGCATCACTGTGCG-3' (*NcoI* sites underlined). The PCR product was cut by *NcoI* and ligated to the *NcoI*-cleaved expression vector pJAS35 (Pfeifer *et al.*, 1994).

Prior to transformation of *Hf. volcanii*, each construct was passed through the *E. coli dam* strain GM1674 to avoid a halobacterial restriction barrier (Holmes *et al.*, 1991). Transformation was as described by Pfeifer & Ghahraman (1993). Southern analyses of total DNA of the transformants using a vac-region-specific DNA probe (digoxigenin-labelled; kit from Boehringer Mannheim) determined the presence of the desired plasmids.

Immunological detection of the GvpA protein. Proteins of halobacteria were isolated from a 5 ml culture in early stationary phase. Pelleted cells were resuspended in 0.4 ml TE buffer containing 10 µg DNase I ml⁻¹. The suspension was dialysed against 10 mM Tris/HCl, pH 7.2 overnight and centrifuged for 20 min at 12000 *g* for membrane removal. Proteins (20 µg each) were separated on 12% Tricine-SDS-polyacrylamide gels, according to Schagger & von Jagow (1987). Western analyses were performed as previously described (Englert *et al.*, 1992b) using an antiserum raised against gas vesicles of *Hf. mediterranei*; this exclusively detects the GvpA protein.

Isolation of gas vesicles and examination by electron microscopy. *Hf. volcanii* transformants were grown on agar

plates for 1–2 weeks. Stationary-phase cells were scraped from the agar and lysed in 10 mM Tris/HCl, pH 7.2, with 1–2 µl DNase I (1 mg ml⁻¹). Gas vesicles were collected by accelerated flotation in small narrow tubes (diameter 4 mm) by centrifugation in an Eppendorf centrifuge for 20 min at 1000–2000 r.p.m. and were washed three times with 10 mM Tris/HCl, pH 7.2. For negative staining, a drop of the gas vesicle preparation was placed on a carbon-coated copper grid, removed after 2 min with a pipette and air dried. Gas vesicles were treated for 1 min with a solution of 1% uranyl acetate and 0.01% glucose in water, briefly rinsed with a drop of water and then air dried. The specimens were examined with a Zeiss EM 912 transmission electron microscope operated with the OMEGA energy filter in the zero loss mode.

Electron microscopy and image processing of frozen hydrated gas vesicles. Frozen hydrated samples of gas vesicles of *Hb. salinarum* PHH1 were prepared for cryo-electron microscopy by fast-freezing thin films of sample solution on grids covered with a holey carbon film in liquid ethane with a gravity-driven plunger. Specimens were observed using a Gatan cryoholder in a Philips CM12 electron microscope equipped with a Gatan imaging filter. Images were recorded using a slow-scan CCD camera attached to the microscope. The microscope was operated at 80 kV accelerating voltage and 56900× total magnification on the CCD camera. The calibrated CCD pixel size referring to the specimen level was 0.42 nm.

Image processing, including image enhancement and helical simulations, was performed on a Silicon Graphics Indigo² workstation, using the EM program package (Hegerl, 1996). For image enhancement, areas of the original images were window-filtered using the strongest reflections of their power spectra. For better visibility, the image contrast was reversed; thus protein appears lighter than the surrounding ice. For simulations, three-dimensional maps of helices or rings, with different ratios of the diameter of the helix of the ring to the distance of helical turns or rings, were created and projected at different angles.

RESULTS

Formation of chimeric gas vesicles using heterologous *gvp* gene clusters

Homologous complementation with subfragments of the c-vac region. Initial transformation experiments were performed to test whether the *gvp* gene products of the c-vac region were sufficient to synthesize gas vesicles in transformants. For this purpose, *Hf. volcanii* was transformed with the cE-M and the cA-OI constructs (Table 1, Fig. 1). The cE-M construct lacked the *c-gvpD* gene and the native promoter for the *c-gvpD*-M transcription unit. However, *c-gvpE*-M were transcribed from a fortuitous promoter sequence of the vector, as demonstrated by primer extension (Krüger, 1996).

The cE-M/cA-OI double transformant synthesized gas vesicles (Table 2), demonstrating that (i) complementation of the *c-gvpE*-M and the *c-gvpACNO* genes led to gas vesicle formation in transformants, and (ii) as in the p-vac region (Offner & Pfeifer, 1995), the *c-gvpD* gene was not essential for the synthesis of gas vesicles. The cE-M/cA-OI double transformant was investigated by Western analyses using an antiserum speci-

Table 2. Constructs used for transformation experiments, presence of GvpA and gas vesicle phenotype

Construct*		GvpA†	Vac‡
1	2		
pA-O†	pD-M§	+	+++
cA-OI†	cE-M§	+	+++
cA-OI†	–	–	–
cA-OII‡	–	+(c-GvpA _{mut})	–
cA-OII‡	cE-M§	+(c-GvpA _{mut})	–
pA-O†	cE-M§	+	+
cA-OII‡	pD-M§	+(c-GvpA _{mut})	–
cAII‡	pΔA	+(c-GvpA _{mut})	+++
cA-OI‡	pD-M§	–	–
mcA†	–	+	–
mcA-O†	mcD-M§	+	+
mcA-O†	pD-M§	+	+++
pA-O†	mcD-M§	+	+++
mcA†	pΔA§	+	+

* The name of the construct used for transformation is given in capital letters and indicates the respective *gvp* gene cluster according to Fig. 1 (also see Table 1). The vectors used are: †, pMDS20; ‡, pJAS35; §, pUBP2; ||, pWL102.

† The GvpA protein was assigned by Western analyses.

‡ The presence of gas vesicles in the cells was determined by phase-contrast microscopy.

+++ Vac⁺ transformants from which gas vesicles could be isolated.

cally detecting the GvpA protein of the three vac regions (Englert *et al.*, 1992b), and it exhibited a faint c-GvpA-specific signal (Fig. 2a). Surprisingly, a transformant containing the cA-OI construct by itself lacked the c-GvpA protein (Fig. 2a), indicating an inactive *c-gvpA* promoter in this transformant. As the cE-M/cA-OI transformant synthesized c-GvpA, a gene product of the *c-gvpE*-M genes was postulated to serve as an activator that directly or indirectly (by repressing the action of a repressor) activates the *c-gvpA* promoter. Subsequently, the c-GvpE protein was identified as the transcriptional activator of the *c-gvpA* promoter (Krüger & Pfeifer, 1996).

Heterologous complementation with subfragments of the c-vac and p-vac region. To determine whether heterologous complementations are possible and lead to chimeric gas vesicles, the *gvpACNO* and *gvpD*-M transcription units of the c-vac and p-vac regions were used for transformation experiments in both combinations (pD-M/cA-OI, cE-M/pA-O; Table 1, Fig. 1). Previous studies showed that the p-*gvpA* promoter did not need activation by another gene product and that the pD-M/pA-O transformant synthesized spindle-shaped gas vesicles (Table 2, Offner & Pfeifer, 1995).

Heterologous complementation of the cE-M and pA-O constructs allowed the synthesis of chimeric gas vesicles in double transformants as determined by phase-con-

Fig. 2. Western analyses to determine the presence of GvpA in samples of the transformants, *Hb. salinarum* PHH4, *Hf. mediterranei* and *Hf. volcanii*. The proteins (20 µg per lane) were separated on 12% tricine-SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated with the anti-gas-vesicle serum as described by Englert *et al.* (1992b). Transformants and constructs are described in Table 2 and Fig. 1. The reacting GvpA protein is indicated by an arrow. The faint signal of the GvpA protein of the pD-M/mcA-O transformant in (b) is better visible on the original autoradiogram. The gas vesicle phenotype (Vac) is indicated by plus or minus.

trast microscopy (Table 2). Unfortunately, the number of chimeric gas vesicles produced was so low that it was impossible to isolate them and determine their shape by electron microscopy. In contrast, the pD-M/cA-OI transformant did not synthesize gas vesicles (Table 2). Western analysis demonstrated that this transformant lacked *c-gvpA* expression, implying that the *p-gvpD-M* gene products failed to activate the *c-gvpA* promoter (Fig. 2a). Consequently, the absence of gas vesicle synthesis by the pD-M/cA-OI transformant could be attributed to lack of *c-gvpACNO* expression.

To bypass the lack of the c-GvpA protein in the pD-M/cA-OI transformant, the halobacterial expression vector was used to force *c-gvpACNO* expression. Briefly, the ATG start codon of the desired reading frame was inserted into the *NcoI* site downstream of the ferredoxin (*fdx*) promoter in the expression vector,

leading to a gene product with two additional amino acids (Met, Pro) at the N-terminus (Pfeifer *et al.*, 1994). The resulting construct, termed cA-OII, was used for transformation of *Hf. volcanii*. As expected, Western analysis of the cA-OII transformant indicated the presence of a large amount of c-GvpA with slightly higher molecular mass due to the two additional amino acids (c-GvpA_{mut}) (Fig. 2a). However, homologous complementation of the cA-OII construct with the cE-M construct revealed a Vac⁻ phenotype for the cE-M/cA-OII transformant (Table 2), implying that the two additional amino acids at the N-terminus of c-GvpA_{mut} prevented the formation of gas vesicles. Also, the pD-M/cA-OII transformant was Vac⁻, despite the presence of the c-GvpA_{mut} protein (Table 2; Fig. 2a). These results suggest that the c-GvpA_{mut} protein was incompatible with the *p-gvpD-M* or the remaining *c-gvp* gene products.

As the pΔA construct, containing the entire *p-vac* region except for the *p-gvpA* gene (Offner & Pfeifer, 1995), and the cAII construct, expressing the c-GvpA_{mut} protein (Pfeifer *et al.*, 1994), were available (Table 1; Fig. 1) the cAII/pΔA double transformant was tested for gas vesicle production. Indeed, the phenotype of this transformant was Vac⁺, suggesting that the c-GvpA_{mut} protein and the *p-gvp* gene products are compatible and lead to chimeric gas vesicles in transformants (Table 2). This result implied that the c-GvpA_{mut} protein was not compatible with one or more gene products of *c-gvpCNO*, because these genes constituted the only difference between the Vac⁻ cA-OII/pD-M transformant and the Vac⁺ cAII/pΔA transformant. The chimeric gas vesicles of the cAII/pΔA transformant were isolated and examined by electron microscopy (see below).

In summary, the formation of chimeric *p-vac*/*c-vac* gas vesicles by heterologous complementation experiment was hampered by regulatory or structural incompatibilities as described above. Therefore, subfragments of the *mc-vac* region were used in similar complementation experiments.

Complementation studies with subfragments of the *mc-vac* and *p-vac* region. Previous studies demonstrated that the *mc-vac* region leads to gas vesicle formation in transformants (Blaseio & Pfeifer, 1990; Englert *et al.*, 1992a). The mcD-M, the mcA-O and the mcA construct were generated and used for homologous and heterologous complementation studies (Table 1, Fig. 1). The mcD-M/mcA-O double transformant harbouring the *mc-gvpACNO* and *mc-gvpD-M* transcription units on separate vectors was Vac⁺, and Western analysis indicated the presence of the mc-GvpA protein (Table 2, Fig. 2b). Similar Western analysis of the mcA transformant showed a faint signal of the mc-GvpA protein (Fig. 2b), indicating that the *mc-gvpA* gene expression was leaky (Englert *et al.*, 1992b; Röder & Pfeifer, 1996). As minimal *mc-gvpA* expression appeared in the mcA transformant, the mcA, mcA-O and mcD-M constructs were used for heterologous complementation experi-

Fig. 3. Electron micrographs of isolated gas vesicles. Upper row: gas vesicles of *Hb. salinarum* PHH1, *Hb. salinarum* PHH4 and *Hf. mediterranei*. Lower row: chimeric gas vesicles of *Hf. volcanii* transformants containing heterologous *gvp* gene clusters.

ments with subfragments of the p-vac region. Double transformants were generated in both combinations: pD-M/mcA-O and mcD-M/pA-O (Table 2). In both cases, the transformants were Vac⁺, and Western analyses confirmed the presence of the GvpA protein (Fig. 2b). Isolated chimeric gas vesicles were used for investigation by electron microscopy (see below).

In addition, the pΔA transformant was complemented with the mcA construct (Table 2). As shown by Western analyses, a larger amount of the mc-GvpA protein was present in the pΔA/mcA transformant than in the mcA transformant (Fig. 2b), indicating activation of the mc-*gvpA* promoter by a gene product of the p-vac region. The pΔA/mcA transformant also synthesized gas vesicles, but at a very low level which was insufficient to permit isolation of these chimeric gas vesicles for electron microscopic studies.

Analysis of wild-type and chimeric gas vesicles by electron microscopy. Gas vesicle preparations of halobacterial species and of Vac⁺ transformants were examined by

electron microscopy. The shape of the gas vesicles seen in electron micrographs was correlated with the genotype of the respective micro-organism in order to identify the transcription unit harbouring the genetic determinants responsible for gas vesicle shape. In general, a cylindrical gas vesicle shape depends on the presence of a cylindrical middle section with constant diameter. Absence of this in spindle-shaped gas vesicles causes a difference in length. As the diameter of gas vesicles varies regardless of their shape, only the length was used for characterizing the gas vesicle shape.

Electron micrographs of wild-type gas vesicles showed that 95 % of the gas vesicles from *Hb. salinarum* PHH1 are spindle shaped, with a mean length of 280 nm (Stoeckenius & Kunau, 1968; Englert *et al.*, 1990). In contrast, *Hb. salinarum* PHH4 and *Hf. mediterranei* synthesize predominantly cylindrical gas vesicles, with mean lengths of 347 nm and 455 nm, respectively (Fig. 3). Transformants containing *gvp* genes of the p-vac region (pD-M/pA-O) are spindle shaped (Offner &

Fig. 4. (a) Electron micrograph of a frozen hydrated gas vesicle of *Hb. salinarum* PHH1. Protein is darker than the surrounding ice due to its stronger scattering of electrons. The dashed box encloses the region magnified in (b). (b) Magnified part of (a) after image enhancement and contrast reversal; thus, the protein appears lighter than the surrounding ice. (c) Model helix, with a value of 22.6 for the ratio of the diameter of the model to the distance of the ribs, projected at an angle of 88.1° against the helical axis. In (b) and (c) the arrows indicate the position of the extinction stripes in the moiré patterns; the dashed lines correspond to the position of the projection of one full helical turn.

Pfeifer, 1995), whereas transformants containing the c-vac (cE-M/cA-OI), or mc-vac region (mcM-O) synthesized predominantly cylindrical gas vesicles (Englert *et al.*, 1992b and data not shown).

Only with the p Δ A/cAII (c-GvpA_{mut}), pD-M/mcA-O and mcD-M/pA-O transformants were chimeric gas vesicles obtained in sufficient amounts to be isolated for electron microscopy (Fig. 3). Measurement of length and determination of shape gave the following results. Spindle-shaped gas vesicles, with a length of 157 nm or 235 nm were synthesized in a significant proportion (50%) only by the p Δ A/cAII and the pD-M/mcA-O transformant, respectively. The cylindrical gas vesicles of the two transformants had a mean length of about 325 nm and 573 nm, respectively (Fig. 3). Obviously, the

addition of two amino acids in the c-GvpA_{mut} protein of the p Δ A/cAII transformant did not hinder the formation of 50% spindle-shaped gas vesicles, but these were slightly shorter and wider than wild-type gas vesicles (Fig. 3). In contrast, gas vesicles of the mcD-M/pA-O transformant were exclusively cylindrical, with a mean length of 640 nm (Fig. 3). Since the pD-M/mcA-O transformant contained a significant proportion of spindle-shaped gas vesicles, the *gvp* genes involved in the formation of the spindle shape are localized in the p-*gvpD-M* DNA region and not in the p-*gvpACNO* gene cluster. The mc-GvpA protein constituted the cylindrical and spindle-shaped gas vesicles in the pD-M/mcA-O transformant, whereas the p-GvpA protein formed the cylindrical gas vesicles of the mcD-M/pA-O transformant.

Fig. 5. (a) Electron micrograph of a second image of the same gas vesicle shown in Fig. 4, but tilted by 5° relative to it. The dashed box indicates the region magnified in (b). (b) Magnified part of (a) after image enhancement and contrast reversal. (c) Same model helix as in Fig. 4(c), projected at an angle of 87.0° .

Analysis of the microstructure of the gas vesicle wall by transmission electron microscopy and model calculations

Electron microscopy and image processing of frozen hydrated gas vesicles. A gas vesicle preparation of *Hb. salinarum* PHH1 was investigated in a frozen hydrated state, which is supposed to yield the best structural preservation for proteins. Figs 4(a) and 5(a) show two projections of the same intact, gas-filled gas vesicle, which was used in this investigation; the sample in the second image was tilted by 5° relative to the first one. The tilt-axis was almost parallel to the long axis of the gas vesicle. The gas vesicle was cylindrical, with a small change in diameter in the middle of the particle, and had conical ends. The contrast of the images originated from the density differences between the proteinaceous gas vesicle and the surrounding ice. Therefore, at the edge of the gas vesicle where the ribs run parallel to the viewing

direction, the outline of the particle appeared darker than the ice, whereas in the central region the gas-containing hollow part of the structure was lighter than the surrounding ice. The hollow structure of the gas vesicle was not only the reason for its good contrast, but also seemed to be the reason for its extreme sensitivity to irradiation by electrons; the ribs vanished after an irradiation of about 10 electrons per $\text{\AA}^2$. Nevertheless, the good contrast and conservation of this gas vesicle with a diameter of about 100 nm made it possible to identify the positions of single ribs.

As the rib structure on the upper and lower surfaces produced roughly the same contrast, it was expected that moiré patterns would be formed by superposition of the two lattices, depending on the viewing angle. These moiré patterns could be recognized in images of many investigated gas vesicles and can, in fact, be used to decide whether the ribs form a helix or a stack of

Fig. 6. Comparison of moiré patterns produced by tilting and projecting a helix and a stack of circular rings. The diameter of model/distance of ribs = 6, projection angles 90.0°, 85.2°, 81.0°, 76.2° and 71.6°. The dashed lines indicate the positions of the extinction stripes in the moiré patterns. A, light areas due to integrated density along the projection axis; B, areas where two loops or ellipses are superimposed; C, empty areas; D, areas containing only a single loop or ellipse.

hoops. Due to the background noise, these features were not clearly visible in the original images, but they could be enhanced by image processing. The areas of the images marked with a dashed line in Figs 4(a) and 5(a) were window-filtered using the strongest reflections of their power spectra. The resulting images are shown in Figs 4(b) and 5(b). To adapt the images better to the perceptivity of the human eye, the image contrast was reversed so that the protein appears lighter than the ice. Obviously, the visibility of the ribs varies within the areas: in Fig. 4(b) there is one, and in Fig. 5(b) there are three stripes parallel to the particle's long axis where the ribs become faint (indicated by the arrows). These are areas in which the rib structures of both surfaces are shifted by half a period, as expected for such a moiré pattern. Across these regions the ribbed pattern jumps by half a period. Moreover, at the edge of the gas vesicle there is a series of bright spots: those at the upper edge are in line with the ribs whereas those at the lower edge are located halfway between the ribs.

The question of whether these images are projections of a helix or a stack of rings can, in principle, be answered from the distribution of moiré extinction stripes: these must be asymmetric in the case of a helix, and symmetric for stacked rings. Because of the small ratio of the rib distance to the vesicle diameter, the moiré stripes are not easily recognizable in all cases, and those that are close to the edge of the particle are particularly difficult to see. We therefore made simulations of both cases and compared them with the processed images.

Simulations of helices and stacked hoops. Fig. 6 shows a comparison of projections of a helix and a stack of circular rings. At a projection angle of 90° to the long axis, a helix appears as a sine function (in our case much like a zigzag line), whereas a stack of rings is projected into a set of parallel lines. At decreasing angles, the projection of a full helical turn forms a loop, while that of a ring forms an ellipse. Depending on the projection angle and the ratio of the diameter of the helix/rings to the distance between the helical turns (pitch) or rings and the projection angle, the projections of different loops/ellipses overlap or cross each other (or they do not touch at all for angles close to 90°). Loops cross themselves once and can cross increasing numbers of other loops as the tilt angle is decreased. The projection of rings looks similar despite the fact that the ellipses do not cross themselves. Looking at the grey levels of the projections in Fig. 6 one may differentiate between four types of areas: (A) areas which are lightest, because of integrated density along the projection axis; (B) areas where two loops/ellipses are superimposed; (C) empty areas and (D) areas containing only one loop/ellipse.

Referring to the moiré patterns in Figs 4 and 5, (B) and (C) above correspond to areas where the rib structures are visible, while they are smeared in the area corresponding to (D). The dashed lines in Fig. 6 indicate the position of the extinction stripes in all projections. The main difference between the projection of a helix and a stack of rings is that the moiré pattern is asymmetric to

the axis in the case of a helix, whereas it is symmetric for the projection of rings.

Figs 4(c) and 5(c) show projections of a helical model with parameters similar to those of the gas vesicle, with a value of 22.6 for the ratio of the diameter of the particle to the distance between ribs. In Fig. 4(c), the model was projected at an angle of 88.1°, at which angle the different loops do not yet touch. The projection angle in Fig. 5(c) is 87.0°, which results in two overlapping neighbouring loops. The helical model simulations (Figs 4c and 5c) agree so well with the images of the gas vesicle after image enhancement (Figs 4b and 5b), that even the position of single loops could be identified. The dashed lines in Figs 4(b) and 5(b) indicate the positions of such a loop. From this result we conclude that the ribs of gas vesicles form a helix.

DISCUSSION

Localization of the genes responsible for different gas vesicle shapes

Transformation experiments and electron microscopy of isolated gas vesicles were performed to determine if chimeric gas vesicles (i.e. ones in which the gene products involved in gas vesicle assembly derive from two different vac regions) are produced, and to localize the gene(s) responsible for the different gas vesicle shapes. The complementation of the *c-gvpE-M* and *c-gvpA-CNO* genes of the *c-vac* region led to the synthesis of *c-GvpA* and cylindrical gas vesicles in transformants (*cE-M/cA-OI*), implying that the *c-gvp* gene products were functional in *Hf. volcanii* and that complementation experiments were possible. However, expression of the *c-gvpA* gene required an activating gene product from the *c-gvpE-M* region because the transformant harbouring the *cA-OI* construct by itself could not synthesize the *c-GvpA* protein. Subsequent transformation experiments assigned the *c-gvpE* gene product as the activator of the *c-gvpA* promoter (Krüger & Pfeifer, 1996; Krüger, 1996). The sequence of the *c-gvpA* promoter box A element (5'-GTTTTC-3') does not follow the archaeal consensus promoter sequence ($T^{\text{C}}/TTA^{\text{T}}/A^{\text{G}}$) (Pfeifer *et al.*, 1997; Hain *et al.*, 1992), reflecting the fact that expression of the *c-gvpA* gene needs an activating gene product. In contrast, the *p-gvpA* promoter box A element (5'-CTTATG-3') completely matches the archaeal consensus promoter sequence and is active without the help of another *gvp* gene product (Offner & Pfeifer, 1995). The heterologous complementation in the *pD-M/cA-OI* transformant did not lead to production of the *c-GvpA* protein, implying that activation of the *c-gvpA* promoter must be specifically carried out by *c-GvpE*. The homologous gene product of the *p-vac* region (if present) did not activate the *c-gvpA* promoter, indicating that in *Hb. salinarum* PHH1 wild-type activation of the *c-gvpA* promoter cannot be achieved by the *p-GvpE* protein.

As found for the *c-vac* region, complementation of the *mc-gvpD-M* and the *mc-gvpACNO* transcription units

of *Hf. mediterranei* resulted in *Vac*⁺ transformants. In contrast to the *c-vac* region, the *mcA* transformant synthesized the *mc-GvpA* protein, but only in small amounts. Thus, the *mc-gvpA* promoter is leaky, and subsequent transformation experiments indicated that strong *mc-gvpA* gene expression depended on *mc-GvpE* (Röder & Pfeifer, 1996). Also in this case, the *mc-gvpA* box A element (5'-ATTTTG-5') (Englert *et al.*, 1992a) does not follow the promoter box A consensus sequence (Hain *et al.*, 1992). As the *mc-gvpA* transcription was leaky, the *mc-vac* subfragments were useful for heterologous complementation studies. Western analyses demonstrated that the amount of *mc-GvpA* was significantly higher in the *mcM-O*, *mcD-M/mcA-O* and *pΔA/mcA* transformants than in the *mcA* transformant containing the *mcA* construct by itself (Englert *et al.*, 1992b and this report). In the case of the *pΔA/mcA* transformant, activation of *mc-gvpA* expression can apparently also be mediated by *p-gvpD-M* gene products.

Chimeric gas vesicles could be obtained with double transformants harbouring transcription units from different vac regions (*cE-M/pA-O*, *pΔA/cAII*, *mcD-M/pA-O*, *pD-M/mcA-O*, *pΔA/mcA*). Obviously, the similarity of the *gvp* gene products of the three vac regions, which is between 50% and 97% (Englert *et al.*, 1992a), is sufficient for replacing various *Gvp* proteins. This heterologous assembly of the gas vesicle envelope presupposes a certain structural flexibility of the assembly machinery. The gas vesicles of the *pΔA/cAII* and *pΔA/mcA* transformant were indeed chimeric, because the structural components *GvpA* and *GvpC* were derived from different vac regions. In addition, the mutated *c-GvpA* protein of the *pΔA/cAII* transformant (i.e. insertion of two amino acids, Met and Pro, at the N-terminus) led to gas vesicles, though with a slightly altered outline: the gas vesicles were wider than the wild-type gas vesicles, but the feature of the spindle shape was conserved.

However, homologous and heterologous complementation experiments demonstrated that some combinations of the transcription units were incompatible and did not result in gas vesicle synthesis: (i) in a regulatory manner, where the gene products of the *p-gvpD-M* region failed to activate the *c-gvpA* promoter and consequently the gas vesicle formation was repressed and (ii) in a more structural manner, where the two additional amino acids at the N-terminus of the *c-GvpA_{mut}* protein hindered the gas vesicle formation. The *c-GvpA_{mut}* protein could not be used by the *c-gvpCNO* gene products for the formation of gas vesicles in the *Vac*⁻ *pD-M/cA-OII* or *cE-M/cA-OII* transformants, whereas in the *pΔA/cAII* transformant, the *c-gvpA_{mut}* gene led to gas vesicles in combination with the *p-gvpCNO* genes.

Chimeric gas vesicles of the *pD-M/mcA-O* and *mcD-M/pA-O* transformant were subjected to electron microscopy to investigate which gene product(s) determine the spindle-shaped, rather than cylindrical,

morphology of the gas vesicles. Roughly half of the gas vesicles of the pD-M/mcA-O transformant (expressing mc-GvpA) were spindle shaped, whereas the other half were cylindrical. The mc-GvpA protein usually forms the cylindrical gas vesicles of *Hf. mediterranei*. In contrast, the mcD-M/pA-O transformant (expressing the p-GvpA protein) formed mainly cylindrical gas vesicles, despite the fact that the p-GvpA protein constitutes the spindle-shaped gas vesicles of *Hb. salinarum* PHH1. Consequently, the amino acid sequence of p-GvpA, which differs from mc-GvpA by six amino acids (Englert *et al.*, 1990), does not exclusively determine the spindle shape of the gas vesicles. More likely, one or more gene product(s) of the p-*gvpD*-M gene cluster are also involved in the shape determination of the gas vesicles. However, a particular *gvp* gene cluster did not absolutely determine the shape of the respective wild-type gas vesicle, since the pD-M/mcA-O transformant synthesized cylindrical and spindle-shaped gas vesicles in roughly equal quantities. All these aspects led to the conclusion that the difference in shape and length of the gas vesicles does not depend on a single protein. More likely, a dynamic equilibrium of two or more *gvp* gene products is responsible for the determination of the shape during gas vesicle formation. In the case of the pD-M/mcA-O transformant, this equilibrium was affected by the gene product(s) of the p-*gvpD*-M genes that directed assembly of the gas vesicle envelope towards the spindle shape.

Determination of the rib structure of the gas vesicle

Transmission electron microscopy with frozen hydrated samples of intact gas vesicles was carried out to answer the old question of whether the ribs oriented perpendicular to the longitudinal axis of the gas vesicle are a stack of hoops or a helix. Walsby (1972) pointed out that this question might be solved by analysing the images of negatively stained collapsed gas vesicles. Superficially, a homogeneous appearance of the collapsed structure would then support the hoop model (Walsby, 1972). However, this question proved to be difficult to answer by analysing collapsed gas vesicles. The data presented in this study resulted from the analysis of intact gas vesicles that maintain their three-dimensional structure in a frozen, hydrated state. In this case, the moiré patterns produced by the images of the gas vesicle made it very likely that the ribs consisting of GvpA are formed by a helix of low pitch. Thus, the enlargement of the prestructures of the gas vesicles proceeds by the addition of GvpA to a helical structure, as implied by theoretical considerations discussed by Walsby (1994).

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REFERENCES

- Blaseio, U. & Pfeifer, F. (1990). Transformation of *Halobacterium halobium*: development of vectors and investigation of gas vesicle synthesis. *Proc Natl Acad Sci USA* **87**, 6772–6776.
- Blaurock, A. E. & Walsby, A. E. (1976). Crystalline structure of the gas vesicle wall from *Anabaena flos-aquae*. *J Mol Biol* **105**, 183–199.
- Cline, S. W., Schalkwyk, L. & Doolittle, W. F. (1989). Transformation of the archaeobacterium *Halobacterium volcanii* with genomic DNA. *J Bacteriol* **171**, 4987–4991.
- Damerval, T., Houmard, J., Guglielmi, G., Csiszar, K. & Tandeau de Marsac, N. (1987). A developmentally regulated *gvpABC* operon is involved in the formation of gas vesicles in the cyanobacterium *Calothrix* 7601. *Gene* **54**, 83–92.
- Englert, C. & Pfeifer, F. (1993). Analysis of gas-vesicle gene expression in *Haloferax mediterranei* reveals that GvpA and GvpC are both gas-vesicle structural proteins. *J Biol Chem* **268**, 9329–9336.
- Englert, C., Horne, M. & Pfeifer, F. (1990). Expression of the major gas vesicle protein in the halophilic archaeobacterium *Haloferax mediterranei* is modulated by salt. *Mol Gen Genet* **222**, 225–232.
- Englert, C., Krüger, K., Offner, S. & Pfeifer, F. (1992a). Three different but related gene clusters encoding gas vesicles in halophilic archaea. *J Mol Biol* **227**, 586–592.
- Englert, C., Wanner, G. & Pfeifer, F. (1992b). Functional analysis of the gas-vesicle gene cluster of the halophilic archaeon *Haloferax mediterranei* defines the vac-region boundary and suggests a regulatory role for the *gvpD* gene or its product. *Mol Microbiol* **6**, 3543–3550.
- Hain, J., Reiter, W.-D., Hüdepohl, U. & Zillig, W. (1992). Elements of an archaeal promoter defined by mutational analysis. *Nucleic Acids Res* **20**, 5423–5428.
- Halladay, J., Jones, J., Lin, F., MacDonald, B. & DasSarma, S. (1993). The rightward gas-vesicle operon in *Halobacterium* plasmid pNRC100: identification of the *gvpA* and *gvpC* gene products by use of antibody probes and genetic analysis of the region downstream of *gvpC*. *J Bacteriol* **175**, 684–692.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Hayes, P. K. & Powell, R. S. (1995). The *gvpA/C* cluster of *Anabaena flos-aquae* has multiple copies of a gene encoding GvpA. *Arch Microbiol* **164**, 50–57.
- Hayes, P. K., Buchholz, B. & Walsby, A. E. (1992). Gas vesicles are strengthened by the outer-surface protein, GvpC. *Arch Microbiol* **157**, 229–234.
- Hegerl, R. (1996). The EM program package: a platform for image processing in biological electron microscopy. *J Struct Biol* **116**, 30–34.
- Holmes, M. L., Nuttall, S. D. & Dyll-Smith, M. L. (1991). Construction and use of halobacterial shuttle vectors and further studies on *Haloferax* DNA gyrase. *J Bacteriol* **12**, 3807–3813.
- Horne, M. & Pfeifer, F. (1989). Expression of two gas vacuole protein genes in *Halobacterium halobium*. *Mol Gen Genet* **218**, 437–444.

- Horne, M., Englert, C. & Pfeifer, F. (1988). Two genes encoding gas vacuole proteins in *Halobacterium halobium*. *Mol Gen Genet* 213, 459–464.
- Horne, M., Englert, C., Wimmer, C. & Pfeifer, F. (1991). A DNA region of 9 kbp contains all genes necessary for gas vesicle synthesis in halophilic archaeobacteria. *Mol Microbiol* 5, 1159–1174.
- Jones, J. G., Young, D. C. & DasSarma, S. (1991). Structure and organization of the gas-vesicle gene cluster on the *Halobacterium halobium* plasmid pNRC100. *Gene* 102, 117–122.
- Kinsman, R. & Hayes, P. K. (1997). Genes encoding proteins homologous to halobacterial Gvps N, J, K, F and L are located downstream of *gvpC* in the cyanobacterium *Anabaena flos-aquae*. *DNA Seq* 7, 97–106.
- Kinsman, R., Walsby, A. E. & Hayes, P. K. (1995). GvpCs with reduced numbers of repeating sequence elements bind to and strengthen cyanobacterial gas vesicles. *Mol Microbiol* 17, 147–154.
- Krantz, M. J. & Ballou, C. E. (1973). Analysis of *Halobacterium halobium* gas vesicles. *J Bacteriol* 3, 1058–1067.
- Krüger, K. (1996). *Untersuchungen zur Regulation der Gasvesikelsynthese in dem halophilen Archaeon Halobacterium salinarium PHH4*. Dissertation, LMU München. Munich: Shaker Verlag.
- Krüger, K. & Pfeifer, F. (1996). Transcript analysis of the c-vac region, and differential synthesis of the two regulatory gas-vesicle proteins GvpD and GvpE in *Halobacterium salinarium* PHH4. *J Bacteriol* 178, 4012–4019.
- Lam, W. L. & Doolittle, W. F. (1989). Shuttle vectors for the archaeobacterium *Halobacterium volcanii*. *Proc Natl Acad Sci USA* 86, 5478–5482.
- McMaster, T. J., Miles, M. J. & Walsby, A. E. (1996). Direct observation of protein secondary structure in gas vesicles by atomic force microscopy. *Biophys J* 70, 2432–2436.
- Mayr, A. & Pfeifer, F. (1997). The characterization of the *nv-gvpACNOFGH* gene cluster involved in gas vesicle formation in *Natronobacterium vacuolatum*. *Arch Microbiol* 168, 24–32.
- Offner, S. & Pfeifer, F. (1995). Complementation studies with the gas vesicle-encoding p-vac region of *Halobacterium salinarium* PHH1 reveal a regulatory role for the p-*gvpDE* genes. *Mol Microbiol* 16, 9–19.
- Offner, S., Wanner, G. & Pfeifer, F. (1996). Functional studies of the *gvpACNO* operon of *Halobacterium salinarium* reveal that the GvpC protein shapes gas vesicles. *J Bacteriol* 178, 2071–2078.
- Palmer, B. & Marinus, M. (1994). The *dam* and *dcm* strains of *Escherichia coli* – a review. *Gene* 143, 1–12.
- Pfeifer, F. & Ghahraman, P. (1993). Plasmid pHH1 of *Halobacterium salinarium*: characterization of the replicon region, the gas-vesicle gene cluster and insertion elements. *Mol Gen Genet* 238, 193–200.
- Pfeifer, F., Offner, S., Krüger, K., Ghahraman, P. & Englert, C. (1994). Transformation of halophilic archaea and investigation of gas-vesicle synthesis. *Syst Appl Microbiol* 16, 569–577.
- Pfeifer, F., Krüger, K., Röder, R., Mayr, A., Ziesche, S. & Offner, S. (1997). Gas vesicle formation in halophilic archaea. *Arch Microbiol* 167, 259–268.
- Röder, R. & Pfeifer, F. (1996). Influence of salt on the transcription of the gas-vesicle genes of *Haloferax mediterranei* and identification of the endogenous transcriptional activator gene. *Microbiology* 142, 1715–1723.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schägger, H. & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166, 368–379.
- Simon, R. (1981). Morphology and protein composition of gas vesicles from wild type and gas vacuole deficient strains of *Halobacterium salinarium* strain S. *J Gen Microbiol* 125, 103–111.
- Stoeckenius, W. & Kunau, W. H. (1968). Further characterization of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and isolation of gas vacuole membrane. *J Cell Biol* 38, 337–357.
- Surek, B., Pillay, B., Rdest, U., Bayreuther, K. & Goebel, W. (1988). Evidence for two different gas vesicle proteins and genes in *Halobacterium halobium*. *J Bacteriol* 170, 1746–1751.
- Ventosa, A. & Oren, A. (1996). *Halobacterium salinarum* nom. corrig., a name to replace *Halobacterium salinarium* (Elazari-Volcani) and to include *Halobacterium halobium* and *Halobacterium cutirubrum*. *Int J Syst Bacteriol* 46, 347.
- Walker, J. E., Hayes, P. K. & Walsby, A. E. (1984). Homology of gas vesicle proteins in cyanobacteria and halobacteria. *J Gen Microbiol* 130, 2709–2715.
- Walsby, A. E. (1972). Structure and function of gas vacuoles. *Bacteriol Rev* 36, 1–32.
- Walsby, A. E. (1994). Gas vesicles. *Microbiol Rev* 58, 94–144.
- Walsby, A. E. & Hayes, P. K. (1988). The minor cyanobacterial gas vesicle protein, GvpC, is attached to the outer surface of the gas vesicle. *J Gen Microbiol* 134, 2647–2657.

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