

Regulation of Gas Vesicle Formation in Halophilic Archaea

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Abstract

The halophilic archaea *Halobacterium salinarum* and *Haloferax mediterranei* produce gas vesicles depending on the growth phase and on environmental factors such as light, salt, or oxygen. Fourteen different *gvp* genes (*gvpACNO* and *gvpDEFGHIJKLM*) are involved in their formation, and the regulation of *gvp* gene expression occurs at the transcriptional and translational level. *Haloferax volcanii* offers a clean genetic background for the functional analysis of gas vesicle genes by transformation experiments. Such experiments show that the promoter of the *gvpA* gene encoding the major gas vesicle structural protein is activated by the endogenous basic leucine-zipper protein GvpE. On the other hand, the GvpD protein, which contains a p-loop motif, is involved either directly or indirectly in the repression of the *gvpA* promoter activity. Eight of the fourteen p-*gvp* genes (p-*gvpAO* and p-*gvpFGJKLM*) enable gas vesicle formation in *Hf. volcanii* transformants and thus constitute the minimal p-vac region.

Gas vesicles are proteinaceous flotation devices produced by some bacteria and archaea to allow these microorganisms to float to the surface of their aqueous environment where oxygen and light are present and can be used for energy metabolism (Walsby, 1994). Among the halophilic archaea, *Halobacterium salinarum*, *Haloferax mediterranei*, and the haloalkaliphilic *Natronobacterium vacuolatum* produce gas vesicles. These are spindle- or cylinder-shaped structures ranging in size from 0.2 to 1.5 μm in length and 0.2 μm in diameter. The major structural protein GvpA forms the 4.6 nm-wide ribs that are arranged perpendicular to the long axis. These ribs are formed by a helix of low pitch rather than a stack of hoops (Offner *et al.*, 1998). The gas vesicles are filled by passive diffusion with gases dissolved in the environment (Walsby, 1994). Apart from the hydrophobic 7-8 kDa GvpA protein there is a second, but minor constituent (GvpC) located at the outer surface of the ribs and strengthening the entire

gas vesicle structure (Englert and Pfeifer, 1993; Halladay *et al.*, 1993).

Hb. salinarum PHH1 contains spindle-shaped gas vesicles during all stages of growth, whereas *Hb. salinarum* PHH4 (a derivative of *Hb. salinarum* PHH1 containing a smaller plasmid) and *Hf. mediterranei* produce cylinder-shaped gas vesicles only during the stationary growth phase (Table 1). Environmental factors such as anaerobic conditions or darkness enhance the amount of gas vesicles in *Hb. salinarum* PHH4 (unpublished observations). Also the salt content of the medium affects gas vesicle formation: the moderately halophilic *Hf. mediterranei* produces gas vesicles when grown in media with a salt content exceeding 17% (Englert *et al.*, 1990). Since all these factors influence gas vesicle formation, the genes involved offer an interesting system to study gene regulation and also signal transduction.

Fourteen different *gvp* (gas vesicle protein) genes were identified that cluster in a 9-kb DNA region termed vac region (Englert *et al.*, 1992a) or *gvp* gene cluster (Jones *et al.*, 1991; DasSarma *et al.*, 1994). These genes are arranged as *gvpACNO* and, upstream and oppositely oriented, *gvpDEFGHIJKLM* (Figure 1). We use the designations p-vac, c-vac and mc-vac (and also c-*gvp*, p-*gvp* and mc-*gvp* for the respective genes) to distinguish the different vac regions and *gvp* genes according to their halobacterial origin. The largest vac region, mc-vac (*mediterranei* chromosomal), is found in the chromosome of *Hf. mediterranei* (Figure 1; Englert *et al.*, 1992a). Two different vac regions are present in *Hb. salinarum* PHH1, namely the chromosomal c-vac region and the plasmid-born p-vac region located on the 150 kb plasmid pHH1 (Englert *et al.*, 1992a). The total genome sequence determined for the related *Halobacterium* species NRC-1 indicates similar *gvp* gene clusters, with two *gvp1* gene clusters almost identical to p-vac located on the large plasmids pNRC100 (191 kb) and pNRC200 (365 kb) (DasSarma *et al.*, 1994; Ng *et al.*, 1998; Ng *et al.*, 2000). The *gvp1* gene cluster is part of an 145 kb region shared by both plasmids. The *gvp2* gene cluster found in addition on pNRC200 is almost identical to c-vac but lacks the c-*gvpM* gene and should thus not be functional (Ng *et al.*, 2000). The various vac regions are summarized in Table 1.

Relationship Between Different *Halobacterium* Species

Although the purpose of this review is to summarize our data on the regulation of gas vesicle gene expression, the relationship between the different *Halobacterium* species used to investigate gas vesicle formation

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Table 1. The *gvp* gene clusters and presence of gas vesicles during growth.

	<i>gvp</i> gene cluster(s)	Presence of gas vesicles	
		Exponential	Stationary
<i>Hb. salinarum</i> PHH1	p-vac and c-vac	++	++
<i>Hb. salinarum</i> PHH4	c-vac	—	+
<i>Hb. salinarum</i> NRC-1	2x <i>gvp1</i> ; <i>gvp2</i> *	++	++
<i>Hf. mediterranei</i> , 17–30% salt	mc-vac	—	++
<i>Hf. volcanii</i>	—	—	—
<i>Nb. vacuolatum</i> , 15–30% salt	nv-vac	+	++

**gvp1* = p-vac; *gvp2* = c-vac, but lacking the c-*gvpM* gene.

should be discussed in some detail, since there are confusions due to the frequent renaming of the halobacterial strains and species. The two *Halobacterium* strains PHH1 and NRC-1 both originate from a “*Hb. halobium*” strain kept in the strain collection of Walther Stoeckenius at UC San Francisco (USA). The American Type Culture Collection, the DSMZ in Germany (origin of “*Hb. halobium*” PHH4 = DSM 670), Ford Doolittle (origin of “*Hb. halobium*” NRC-1), Dieter Oesterhelt (origin of “*Hb. halobium*” NRC817 = “*Hb. halobium*” PHH1 strain) and also Felicitas Pfeifer obtained samples from this collection at different points in time. A later comparison of these strains based on hybridization patterns of various insertion elements and the presence of pHH1-specific plasmid sequences suggests that the further cultivation of these strains (usually accompanied by purification *via* single colonies) resulted in unexpected genome

variations which turned out to be a characteristic feature of “*Hb. halobium*” (Pfeifer *et al.*, 1981a; Pfeifer *et al.*, 1981b; Sapienza and Doolittle, 1981; Pfeifer, 1988; Pfeifer and Blaseio, 1989; Pfeifer and Ghahraman, 1993). These alterations are mainly due to the action of multiple insertion elements (ISH elements) found predominantly in the plasmid population, and in islands of (AT)-rich DNA in the chromosome of “*Hb. halobium*” (Pfeifer *et al.*, 1983; Pfeifer and Betlach, 1985; Pfeifer, 1988; Charlebois and Doolittle, 1989). The plasmid population of “*Hb. halobium*” PHH1 is highly dynamic: PHH1 usually incurred multiple insertions or deletions in phenotypic mutants such as gas vesicle minus (*Vac*[−]) mutants (Pfeifer *et al.*, 1981b; Pfeifer *et al.*, 1988; Pfeifer and Blaseio, 1989).

Also the two large plasmids of the *Halobacterium* species NRC-1 (pNRC100 and pNRC200) are related to pHH1. All three plasmids share an almost identical

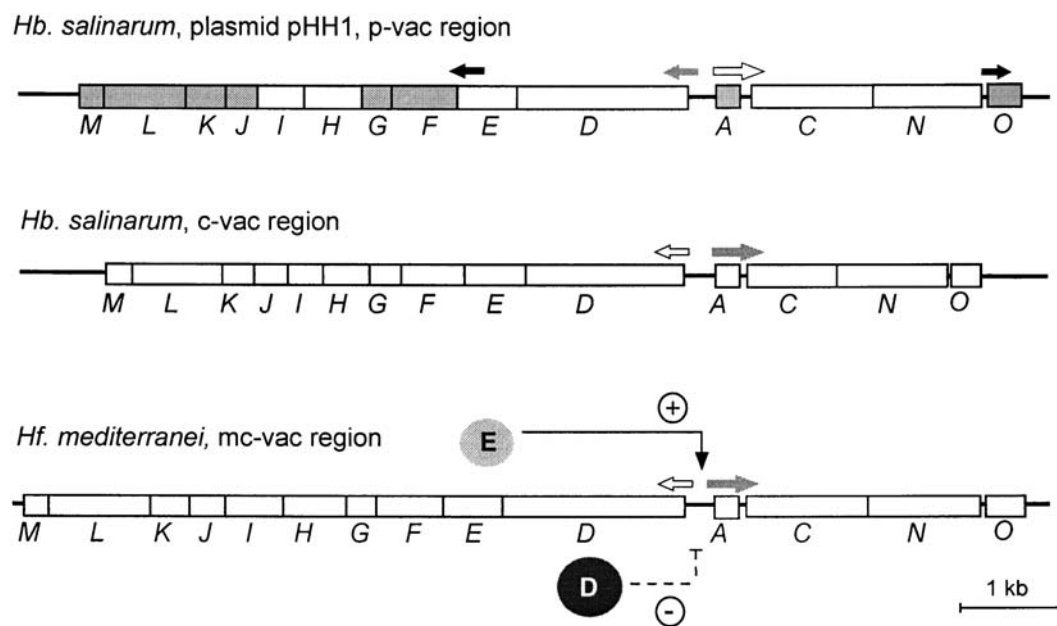


Figure 1. Genetic maps of the p-vac and the c-vac region of *Hb. salinarum* PHH1, and the mc-vac region of *Hf. mediterranei*. The fourteen *gvp* genes are depicted by boxes labeled A and C through O. The eight *gvp* genes constituting the minimal p-vac region (Offner *et al.*, 2000) are shaded in grey. Arrows above the maps indicate start sites and direction of transcription. Black arrows depict transcription during the exponential growth phase, open arrows transcription during all phases of growth, and arrows in grey indicate transcription that occurs only during the stationary growth phase. The two regulator proteins GvpE (activator) and GvpD (repressor) are shown with the mc-vac region; GvpE acts directly at the mcA and mcD promoter, whereas the action of GvpD could also be indirect.

replicon region, the p-vac region (= *gvp1* gene cluster) and adjacent sequence including various ISH elements (with ISH3 = ISH27, ISH8 = ISH26, and ISH9 = ISH28) (Ng *et al.*, 1991; Pfeifer and Ghahraman, 1993). Das-Sarma's group introduced new designations for ISH elements already published for "*Hb. halobium*" PHH1 (Ebert *et al.*, 1987; Pfeifer and Blaseio, 1990). The plasmid pNRC100 also contains an inverted repeat of 33 kb not found in pHH1, and both plasmids might in addition harbour unrelated sequences (Ng *et al.*, 1991; Pfeifer and Ghahraman, 1993). All these "*Hb. halobium*" strains and also the "*Hb. salinarium*" and "*Hb. cutirubrum*" species contain sequences homologous to pHH1 (Pfeifer *et al.*, 1981a; Pfeifer and Ghahraman, 1993). All these strains and species (including "*Hb. halobium*" NRC-1) were renamed "*Hb. salinarium*" (Tindall, 1992). However, this designation was changed again to *Hb. salinarum* due to the grammatically incorrect form "*salinarium*" (Ventosa and Oren, 1996). Since 1996 we use the designation *Hb. salinarum* for all *Halobacterium* strains that share a common ancestor (including "*Hb. halobium*" NRC817 and "*Hb. halobium*" NRC-1), and distinguish them on the basis of their major plasmids (pHH1, pHH4 and pNRC).

Eight of the Fourteen *gvp* Genes Constitute the Minimal p-vac Region

The minimal number of genes required for gas vesicle formation has been determined by transformation experiments using the gas vesicle negative species *Haloferax volcanii* as recipient (Offner and Pfeifer, 1995; Offner *et al.*, 1996; Offner *et al.*, 2000). *Hf. volcanii* is easy to transform, and this species offers a clean genetic background with respect to the *gvp* genes in contrast to *Hb. salinarum*. Single p-*gvp* genes were deleted and the remaining p-*gvp* genes analyzed for their ability to drive gas vesicle formation in the transformants. A deletion of either one of the six genes p-*gvpC*, p-*gvpD*, p-*gvpE*, p-*gvpH*, p-*gvpI*, or p-*gvpN* still leads to the formation of gas vesicles in such *Hf. volcanii* transformants (see Figure 1). Minor amounts of gas vesicles are also detectable in transformants containing a construct with the eight remaining p-*gvpFGJKLMAO* genes, indicating that these genes constitute the minimal p-vac region required for gas vesicle formation (Offner *et al.*, 2000).

Another method to mutate specific *gvp* genes has been employed by inserting foreign DNA into the various genes of the *gvp1* gene cluster and studying the effect of each mutation in pNRC100-negative *Hb. salinarum* NRC-1 transformants (DasSarma *et al.*, 1994). However, the presence of the pNRC200 plasmid containing the second *gvp1* and also the *gvp2* gene cluster are not mentioned in this publication. Thus, the results are very difficult to interpret. Compared to our deletion analyses the insertional mutations revealed different results for 6 out of 14 *gvp* genes (Pfeifer *et al.*, 1997). Difficulties with the insertional mutations also arise from possible polar mutations, and the authors never proved the absence of the

mutated Gvp proteins, especially in those cases where the insert occurs close to the 3' or 5' end of a gene (discussed in detail by Pfeifer *et al.*, 1997). However, a deletion of a *gvp* gene clearly destroys its function, and if the control transformant harbouring the *gvp* gene under investigation still produces gas vesicles, the phenotype of the Δ *gvp* transformant provides a good indication of the effect of the mutation.

Among the six *gvp* genes not essential for gas vesicle formation are the two genes *gvpD* and *gvpE* encoding gas vesicle regulatory proteins (Offner and Pfeifer, 1995). The Δ DE transformants produce reduced numbers of spindle-shaped gas vesicles compared to the wild-type. Also Δ N transformants contain only minor amounts of gas vesicles. Δ C transformants harbouring a p-vac region with a deletion of p-*gvpC* form large amounts of irregularly shaped gas vesicles (Offner *et al.*, 1996). Complementation of the Δ C transformants with the p-*gvpC* gene again results in spindle-shaped gas vesicles, indicating that GvpC not only stabilizes the gas vesicle structure, but is also important for the shape determination. Δ I transformants contain very long (up to 2.7 μ m) cylindrical gas vesicles, suggesting that GvpI is directly or indirectly involved in the determination of the gas vesicle length. Gas vesicles synthesized without GvpH are altered in strength, since they disaggregate into ribs when prepared for electron microscopy (Offner *et al.*, 2000).

The Mode of Transcription of the Various Vac Regions is Similar, but not Identical

Transcription in halophilic archaea depends on a single DNA-dependent RNA polymerase comprising 12 subunits that are homologous to the multiple subunits of the eukaryotic RNA polymerase II. The archaeal promoter consists of a TATA box centered around position -28 upstream of the transcription start site, and the initiation of transcription requires the TATA box binding protein TBP and the transcription factor TFB that is homologous to the eukaryotic transcription factor TFIIB (Hausner *et al.*, 1996; Qureshi *et al.*, 1995; Thomm, 1996). Many archaeal promoters mapped to date contain the TFB recognition element BRE adjacent to the TATA box (Bell *et al.*, 1999). Multiple divergent genes encoding the TFB and TBP proteins have been identified in *Haloferax volcanii* (Thompson *et al.*, 1999), and seven *tfb* and five *tbp* genes are also found in the genome sequence of *Hb. salinarum* NRC-1 (Ng *et al.*, 2000).

Despite the identical arrangement of the 14 *gvp* genes in the three vac regions, the transcription of these genes shows variations (see Figure 1). The p-vac region of plasmid pHH1 is expressed from four promoters located in front of p-*gvpA*, p-*gvpD*, p-*gvpF*, and p-*gvpO*. Three of these promoters are predominantly active during the exponential growth phase, whereas the p-*gvpDE* mRNA occurs only during the stationary growth phase (see Figure 1) (Offner and Pfeifer, 1995; Offner *et al.*, 1996). Large amounts of p-*gvpA* mRNA encoding the major gas vesicle structural protein are synthesized throughout growth, together with minor amounts of the p-*gvp ACNO*

cotranscript. Two promoters located in front of the chromosomal *c-gvpA* and *c-gvpD* genes drive the expression of the *c-vac* region (Englert *et al.*, 1992a). However, in *Hb. salinarum* PHH1 wild-type containing the *p-vac* and the *c-vac* region, only minor amounts of the relatively unstable *c-gvpDEFGHIJKLM* transcript are observed throughout growth, whereas the *c-gvpACNO* genes are not transcribed. This is the reason why the spindle-shaped gas vesicles of *Hb. salinarum* PHH1 are exclusively formed by pGvpA, and cGvpA is not involved. The cGvpD protein appears during exponential growth, whereas the cGvpE protein required for the transcription of the *c-gvpACNO* operon (see below) is not detectable, indicating that the first two reading frames of the *c-gvpDEFGHIJKLM* transcript are translated at different points in time (Krüger and Pfeifer, 1996).

The cylinder-shaped gas vesicles formed by cGvpA of the *c-vac* region are only observed in *Hb. salinarum* PHH4 lacking the *p-vac* region (Pfeifer and Blaseio, 1989). The *c-gvpDEFGHIJKLM* mRNA (and smaller products) are detectable throughout growth, whereas the *c-gvpA* promoter is completely inactive during the exponential growth phase (Krüger and Pfeifer, 1996). Also in this case, the *c-gvpD* and *c-gvpE* genes are differentially expressed: the cGvpD protein occurs during the early exponential growth phase, whereas the cGvpE protein is detectable during the stationary growth phase. At the same time, a large amount of *c-gvpA* mRNA and minor amounts of the *c-gvpACNO* transcript occur and gas vesicles are formed (see Figure 1). The expression of the related *gvp2* gene cluster on pNRC200 of *Hb. salinarum* NRC-1 (Ng *et al.*, 2000) has not been investigated so far. Since this cluster lacks the essential *c-gvpM* gene, *gvp1*-negative mutants should be gas vesicle negative. However, this *gvp2* cluster could still be expressed.

Also, the *mc-vac* region is transcribed from two promoters, leading to minor amounts of the *mc-gvpDEFGHIJKLM* and *mc-gvpA* mRNA during exponential growth (Röder and Pfeifer, 1996). Large amounts of *mc-gvpA* mRNA only occur in the sta-

tionary growth phase, together with minor amounts of the *mc-gvpACNO* transcript (Figure 1). In contrast to the *c-vac* region, the promoter of the *mc-gvpD* gene is activated during the stationary growth phase. The resulting strong transcription leads to shorter transcripts such as the *mc-gvpDEF* and *mc-gvpD* mRNAs, and large amounts of transcripts smaller than the *mc-gvpD* gene (Röder and Pfeifer, 1996). The early termination (or processing) of the *gvpDEFGHIJKLM* transcript ensures that the products encoded by the *gvp* genes located downstream of *mc-gvpF* are only translated for a short time during exponential and early stationary growth.

Transcription of *gvp* Genes in *Hf. volcanii* Transformants and Promoter Analyses

Hf. volcanii transformants containing the entire *p-vac* or *mc-vac* region produce very similar *gvp* transcription patterns during the growth phases as determined for the respective wild-type strains. Thus, *Hf. volcanii* appears to be a useful host for comparative analyses of the different *gvp* promoters. *Hf. volcanii* transformants containing the *p-gvpA* gene by itself express this gene constitutively, demonstrating the high basal activity of the *pA* promoter. In contrast, transformants with the *mc-gvpA* gene produce very minor amounts of *mc-gvpA* mRNA (Röder and Pfeifer, 1996), whereas the *c-gvpA* gene is not expressed at all (Krüger and Pfeifer, 1996). The latter results demonstrate that the promoters of the *c-gvpA* and *mc-gvpA* genes need activation to yield the high amount of *gvpA* mRNA found in the transformants harbouring the entire *vac* region. The TATA box sequence of both promoters (*mcA* and *cA*) indicate less conservation compared to the archaeal consensus TATA box sequence, especially with respect to the central TA nucleotides, whereas the TATA box of the *pA* promoter is highly conserved (Figure 2). The factor required for the activation of the *gvpA* promoters is the product of the *gvpE* gene located in the *gvpDEFGHIJKLM* gene cluster (Röder and Pfeifer, 1996; Krüger *et al.*, 1998).

	archaeal consensus:	BRE RNWAAWNYTTAWG	TATA	promoter activity in <i>vac</i> region context	
				exp	stat
pA	TTACAGGAGACATAACGACTGGTGAAACCATACACATCCTTATGTGATGCCCGAGTATAGTTAGAGAT*		<u>CCTTATGTGATGCCCGAGTATAGTTAGAGAT</u>	++	++
cA	TTACGAGCGCCGAAACGGGGTTGAACTCACAACGGCGGTTTTCCGGACACTCCCTGTAGTTGCGGGT*		<u>TTTTCCGGACACTCCCTGTAGTTGCGGGT</u>	-	++
mcA	TTACGAGAGGTGAAACGGTTGCTGAACCAACACGAATGATTTTGTACTTGCCAACACGTTTTTCAGAT*		<u>GATTTTGTACTTGCCAACACGTTTTTCAGAT</u>	(-)	++
mcD	GTTTCAGCAACCGTTTTACCTCTCGTAATGAGTCAAACATCTAAGTAGTTTGGCAGATGAATGACA*		<u>CTAAGTAGTTTGGCAGATGAATGACA</u>	(+)	++
pD	TTTCACCAGTCGTTATGTCTCCTGTAATGAGTCGTCATCTAAGTACGGAGAGTGTAAGCTTCTTAG*		<u>CTAAGTACGGAGAGTGTAAGCTTCTTAG</u>	-	+
cD	TCGTAATAGTTGCTGATTTGTACAAAATCTTGAACAAGATAAAAATGCGTTCTTATTGTTTTCAACACT*		<u>GATAAAAATGCGTTCTTATTGTTTTCAACACT</u>	+	+
pF	TTTGGGGCAGACCTGAGTCCGGGTACAGTATACCCGCATTTAAATGACCTTGACGTCGAAGGTGTACT*		<u>TTTAAATGACCTTGACGTCGAAGGTGTACT</u>	++	-
pO	AATAGAATCCGCGATCGACGACATGGAAGTCGCCCTTTCTTAAGATCCGGGGTCTCTACATAGAAGC*		<u>CTTAAGATCCGGGGTCTCTACATAGAAGC</u>	++	(+)

Figure 2. Sequences of the various *gvp* promoters. The start site of the transcription (+1) is marked by a star. The TATA box centered around -28 is underlined, and the consensus sequence of the TATA box and of the putative BRE element are given above (R = A or G, N = any base, W = A or T, Y = C or T). The plus and minus on the right describe the relative activity of the promoters during the exponential (exp) and stationary (stat) growth phase as determined by the amount of *gvpA* mRNA seen in Northern analyses using RNA derived from the respective wild-type strains. Minus = no activity; (-) = minor activity; (+) = low activity; += intermediate activity; ++ = high activity.

Transformants containing the *c-gvpA* or *mc-gvpA* gene together with the respective *gvpE* gene produce high amounts of *gvpA* mRNA and also GvpA protein, suggesting that the *gvpE* gene encodes a transcriptional activator (see below).

Quantitative Analyses of *gvpA* Promoter Activities Using a β -Galactosidase Gene as Reporter

The *bgaH* reading frame encoding the β -galactosidase of *Haloferax alicantei* (Holmes and Dyll-Smith, 2000) can be used as reporter gene to investigate the basal and induced activities of the various *gvpA* promoters at a more quantitative level. *Hf. volcanii* lacks detectable β -galactosidase activity and offers in this respect a clean genetic background. Transformants expressing the β -galactosidase turn blue when sprayed with Xgal on agar plates, and the activity of this enzyme can be quantified by a standard ONPG assay (Holmes and Dyll-Smith, 2000). The application of this system for *Hb. salinarum* has recently been reported (Patenge *et al.*, 2000).

Transformants containing fusions between the promoter regions of the various *gvpA* genes and the *bgaH* reading frame (*A-bgaH*) yield β -galactosidase activities that correlate well with the amount of the respective *gvpA* mRNA (Gregor and Pfeifer, 2001). The *cA-bgaH* fusion gene is not expressed due to the completely inactive *cA* promoter, the *mcA-bgaH* gene reveals low β -galactosidase activity, and the *pA-bgaH* fusion gene leads to a constitutive production of β -galactosidase in *Hf. volcanii* transformants. Transformants harbouring an *A-bgaH* construct together with the homologous *gvpE* reading frame expressed under the control of the ferredoxin (*fdx*) gene on the expression vector pJAS35 yield severalfold higher β -galactosidase activities, indicating that all three *gvpA* promoters are activated by GvpE (Gregor and Pfeifer, 2001). Each *A-bgaH* gene was also tested for the activation by the heterologous GvpE proteins. From these experiments it appears that cGvpE is the strongest and pGvpE the weakest transcriptional activator. Each GvpE protein is able to activate the *pA* or the *mcA* promoter, whereas the *cA* promoter in *cA-bgaH* is only activated by the homologous cGvpE protein (Gregor and Pfeifer, 2001). A chimeric *pAcA* promoter consisting mainly of the *cA* promoter sequence (including the TATA box) but containing 21 nucleotides 5' to the TATA box substituted with the respective *p-gvpA* promoter sequences, still lacks the basal promoter activity. Nevertheless, this chimeric promoter acquired the ability for activation by all three GvpE proteins. These results imply that the 21 nt sequence upstream of the TATA-box is important for the promoter activation mediated by GvpE (Gregor and Pfeifer, 2001).

The Transcriptional Activator GvpE Resembles a Basic Leucine-Zipper Protein

A closer inspection of the GvpE protein sequence implies that GvpE resembles a basic leucine-zipper

protein typically involved in the regulation of gene expression in eukaryotes. A molecular modeling study of the carboxy-terminal portion of cGvpE indicates an amphiphilic helix (AH6) suitable for the formation of the leucine-zipper structure within a cGvpE dimer. Close to the N-terminal end of the AH6 helix is a cluster of basic amino acids that could constitute the DNA binding site (DNAB, Krüger *et al.*, 1998). A model of the cGvpE dimer docked onto DNA suggests that the side-chains of the basic amino acids found in DNAB could perfectly interact with the negatively charged phosphate groups of the DNA backbone. Mutations in this DNAB motif generated cGvpE proteins that were unable to activate the *c-gvpA* promoter in *Hf. volcanii* transformants, indicating that these amino acids are required for cGvpE activity (Krüger *et al.*, 1998). Other mutations affecting conserved amino acids in the hydrophobic surface of AH6 should prevent the dimer formation of the putative leucine-zipper. Most of these cGvpE "zipper mutants" were unable to activate the *c-gvpA* promoter *in vivo*, underlining the importance of this structure for the transcriptional activator activity (P. Plöber and F. Pfeifer, unpublished). Biochemical analyses of the isolated cGvpE proteins and cGvpE mutants are in progress and will provide further insights into cGvpE dimer formation, the DNA binding activity of cGvpE, and the importance of distinct amino acids for the cGvpE activator function.

GvpD is Involved in the Repression of Gas Vesicle Formation

The involvement of *gvpD* in the repression of gas vesicle formation was first observed in *Hf. volcanii* transformants containing an *mc-vac* region with an internal deletion in *mc-gvpD* (ΔD transformants, Englert *et al.*, 1992b). These ΔD transformants are gas vesicle overproducers (Vac^{++}), whereas $\Delta D/D$ transformants, containing ΔD and in addition the *mc-gvpD* reading frame under the control of the *fdx* promoter in pJAS35, do not form gas vesicles and are Vac^{-} (Figure 3) (Pfeifer *et al.*, 1994). The function of mcGvpD in repression can also be observed at the transcriptional level when the amount of *mc-gvpA* transcripts is investigated in these transformants. The Vac^{++} ΔD transformants contain high amounts of *mc-gvpA* mRNA predominantly in the stationary growth phase, whereas the amount of this transcript is significantly reduced in the Vac^{-} $\Delta D/D$ transformants, implying that mcGvpD is able to prevent the GvpE-mediated *mc-gvpA* promoter activation (Pfeifer *et al.*, 2001).

The deduced amino acid sequences of all three GvpD proteins indicate two interesting features that are important for this repressor function: a putative nucleotide-binding site (p-loop motif) near the N-terminus, and two basic regions (Pfeifer *et al.*, 2001). Alterations of conserved amino acids in the p-loop motif of mcGvpD (36LVNGAPGTGKT44, conserved amino acids are underlined) reveal mutated proteins (D_{mut}) that have

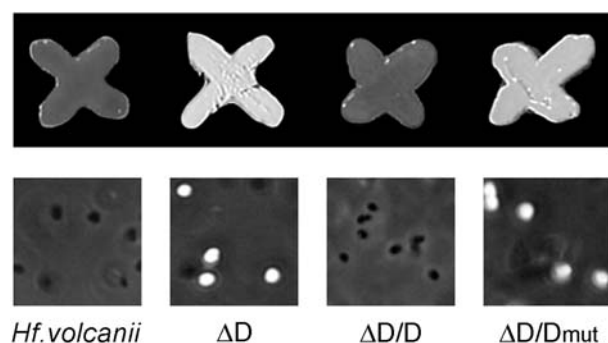


Figure 3. Phenotype of *Hf. volcanii* and various transformants. Top: colonies streaked in cross-shape on agar medium; bottom: cells (length 1 μm) observed by phase-contrast microscopy. Vac^{++} colonies and cells appear white due to the high level of light reflection of the gas vesicles. The Vac^{-} cells appear dark, and the Vac^{-} colonies show a dark red colour like the colonies of *Hf. volcanii* wild-type.

been tested *in vivo* in $\Delta\text{D}/\text{D}_{\text{mut}}$ transformants for their ability to reduce the amounts of gas vesicles in these transformants from Vac^{++} to Vac^{-} . All D_{mut} proteins with an alteration of an amino acid at a conserved position in the p-loop motif are unable to repress gas vesicle formation (see Figure 3). Thus, the nucleotide binding (and hydrolysis?) is important for the mcGvpD repressor function. Mutations in basic region 1 (position 201–221 in the mcGvpD sequence) also abolish the repressing activity of mcGvpD, whereas the alteration of basic region 2 (position 494–496) leads to ambiguous results: the alteration of RRR to AAA in this region results in a “super”-repressor protein, completely blocking the mc-*gvpA* expression in $\Delta\text{D}+\text{D}_{\text{AAA}}$ transformants (Pfeifer *et al.*, 2001). In contrast, a mcGvpD protein containing an alteration of RRR to ADA in this region is unable to repress. A positive charge in basic region 2 is obviously not important for the mcGvpD repressor function.

The question as to whether additional mc-*gvp* genes are required for the repression of the mc-*gvpA* promoter activity was addressed by constructing transformants harbouring the constructs ADE, ΔADE or $\Delta\text{ADE}+\text{D}$ (Pfeifer *et al.*, 2001). Construct ADE contains the three genes mc-*gvpA* and mc-*gvpDE* in the native arrangement and expressed from the endogenous promoter; the ΔADE construct incurred an internal deletion within the mc-*gvpD* reading frame, and this construct is complemented with mc-*gvpD* expressed under *fdx* promoter control in the $\Delta\text{ADE}+\text{D}$ transformant. The amount of mc-*gvpA* mRNA is high in ΔADE transformants, slightly lower in ADE transformants, and significantly reduced in the $\Delta\text{ADE}+\text{D}$ transformants. The slightly lower amount of mc-*gvpA* mRNA in ADE compared to ΔADE transformants presumably reflects the relatively weak expression of mc-*gvpD* under the endogenous promoter control, whereas the strong reduction of the mc-*gvpA* mRNA in the $\Delta\text{ADE}+\text{D}$ transformants is most likely due to an earlier and stronger synthesis of mc-*gvpD* mRNA (and mcGvpD) under *fdx* promoter control. Since the amount of mc-*gvpA* mRNA is not reduced to the minor

amounts observed during exponential growth with transformants containing the entire mc-*vac* region, these results suggest that other mcGvp proteins are involved in mc-*gvpA* regulation. However, the lack of these other mcGvp proteins can be complemented by higher amounts of mcGvpD protein in case of the $\Delta\text{ADE}+\text{D}$ transformants. It is not known so far whether mcGvpD acts directly or indirectly at the mc-*gvpA* promoter, since it is also possible that mcGvpD inactivates mcGvpE, or acts at the mc-*gvpE* mRNA level by reducing the amount of mcGvpE produced during exponential growth.

Conclusions

The genes involved in gas vesicle formation offer an interesting system to study gene regulation at the transcriptional (and also the translational level) in halophilic archaea. Despite the eukaryotic RNA polymerase and promoter structure, most archaeal gene regulator proteins characterized to date are of the bacterial type. Examples are a repressor protein involved in the regulation of nitrogen fixation in *Methanococcus maripaludis* (Cohen-Kupiec *et al.*, 1997), or the regulator of the arginine fermentation in *Hb. salinarum* (Ruepp and Soppa, 1996; Soppa *et al.*, 1998). So far, the only example of a regulatory protein with structural similarities to eukaryotic basic leucine-zipper regulators appears to be the GvpE protein described here. The activities of various *gvpA* (and *gvpD*) promoters depend on this protein, and the repression of these promoters involve the action of the GvpD protein. Whether and where the GvpE protein binds in the *gvpA* promoter region is currently under investigation; these experiments will give further insight into the importance of specific DNA sequences. How the environmental factors light, oxygen and salt are transduced to these regulators and influence *gvp* transcription is not known to date. Some of these factors have been investigated with respect to the amount of *gvp* gene expression. Higher amounts of mc-*gvpA* mRNA (and also of other mc-*gvp* mRNAs) are found in response to high salt concentrations with *Hf. mediterranei* (Englert *et al.*, 1990; Röder and Pfeifer, 1996), and aeration caused a 5-fold higher expression of (p)-*gvpA* in the case of *Hb. salinarum* NRC-1 (Yang and DasSarma, 1990). The investigation of the signal transduction pathways involved is in progress and should help to uncover the general regulatory networks affecting gene expression in halophilic archaea.

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