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Eight of Fourteen *gvp* Genes Are Sufficient for Formation of Gas Vesicles in Halophilic Archaea

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The minimal number of genes required for the formation of gas vesicles in halophilic archaea has been determined. Single genes of the 14 *gvp* genes present in the p-vac region on plasmid pHH1 of *Halobacterium salinarum* (p-*gvpACNO* and p-*gvpDEFGHIJKLM*) were deleted, and the remaining genes were tested for the formation of gas vesicles in *Haloferax volcanii* transformants. The deletion of six *gvp* genes (p-*gvpCN*, p-*gvpDE*, and p-*gvpHI*) still enabled the production of gas vesicles in *H. volcanii*. The gas vesicles formed in some of these *gvp* gene deletion transformants were altered in shape (ΔI, ΔC) or strength (ΔH) but still functioned as flotation devices. A minimal p-vac region (minvac) containing the eight remaining genes (*gvpFGJKLM-gvpAO*) was constructed and tested for gas vesicle formation in *H. volcanii*. The minvac transformants did not form gas vesicles; however, minvac/gvpJKLM double transformants contained gas vesicles seen as light refractile bodies by phase-contrast microscopy. Transcript analyses demonstrated that minvac transformants synthesized regular amounts of *gvpA* mRNA, but the transcripts derived from *gvpFGJKLM* were mainly short and encompassed only *gvpFG*(*J*), suggesting that the *gvpJKLM* genes were not sufficiently expressed. Since *gvpAO* and *gvpFGJKLM* are the only *gvp* genes present in minvac/JKLM transformants containing gas vesicles, these *gvp* genes represent the minimal set required for gas vesicle formation in halophilic archaea. Homologs of six of these *gvp* genes are found in *Anabaena flos-aquae*, and homologs of all eight minimal halobacterial *gvp* genes are present in *Bacillus megaterium* and in the genome of *Streptomyces coelicolor*.

Gas vesicles are formed by halophilic archaea, cyanobacteria, and some heterotrophic bacteria and allow these microorganisms to float at a favorable depth in their watery environment. These proteinaceous structures vary in length from 0.2 to 1.5 μm (for a review, see reference 39). The ribbed gas vesicle envelope exclusively consists of protein and appears to be watertight but is freely permeable to dissolved ambient gases. Electron micrographs indicate 4.6-nm-wide ribs arranged perpendicular to the long axis that are formed by a helix of low pitch and not by a stack of hoops (3, 30). The major constituent of the gas vesicle envelope is the hydrophobic 7- to 8-kDa protein GvpA (9, 38). Immunological studies revealed GvpC (20 to 42 kDa) as a second but minor protein constituent (8, 12, 15). In cyanobacteria, GvpC is located at the outer surface of the gas vesicle envelope and strengthens the entire structure (15, 22). In halophilic archaea, GvpC also appears to be responsible for a constant diameter of the cylindrical part of the gas vesicle (29).

Genes encoding proteins involved in gas vesicle formation have been identified in a few species of cyanobacteria (*Anabaena flos-aquae*, *Calothrix* strain PCC 7601) and in halophilic archaea (*Halobacterium salinarum*, *Haloferax mediterranei*, and the haloalkaliphilic archaeon *Natronobacterium vacuolatum*) (6, 10, 14, 20, 21, 27), as well as in the soil bacterium *Bacillus megaterium* (26). In the case of halophilic archaea, 14 *gvp* genes cluster in an approximately 9-kb DNA region termed the vac region (10, 11, 19, 20). *H. salinarum* PHH1 harbors the two related vac regions p-vac (located on plasmid pHH1) and c-vac (located in the chromosome) (10, 18, 19). In both vac regions, the 14 *gvp* genes are identically arranged: *gvpACNO* form one cluster, and *gvpDEFGHIJKLM* are located upstream of *gvpA* and oriented in the opposite direction. Two promoters located in front of c-*gvpA* and c-*gvpD* drive the expression of these genes in the c-vac region, whereas four promoters could be identified in the p-vac region, resulting in p-*gvpO* and p-*gvpFGHI JKLM* transcripts in addition to the p-*gvpA*, p-*gvpACNO*, and p-*gvpDE* mRNAs (29). The expression of the p-vac region leads to predominantly spindle-shaped gas vesicles throughout growth, whereas the c-vac region is only expressed in p-vac deletion mutants, and then in the stationary growth phase only (9, 17). Fourteen *gvp* genes designated *gvpAPQBRNFGLSK JTU* are found in the gram-positive soil bacterium *B. megaterium*; the highly similar *gvpA* and *gvpB* genes of this gene cluster could encode the major gas vesicle structural protein GvpA (26). So far, the transcription of these *gvp* genes and gas vesicle formation have not been investigated; however, *Escherichia coli* transformants containing the *gvpBRNFGLSKJTU* genes of *B. megaterium* have been reported to form tiny gas vesicles (26).

The function of some of the halobacterial Gvp proteins has been determined by transformation experiments using halobacterial shuttle vectors conferring resistance to mevinolin (2, 25) or novobiocin (pMDS20) (16) and the gas vesicle-negative (Vac^-) species *Haloferax volcanii* as the recipient strain. Also, an expression vector (pJAS35) is available which enables high-level expression of halobacterial reading frames under the control of the halobacterial ferredoxin (*fdx*) gene promoter (34). Such transformation experiments showed that (i) the entire p-vac region (construct M-O) leads to gas vesicle formation in *H. volcanii* (10), and (ii) the p-*gvpACNO* (A-O) and p-*gvpDEF GHIJKLM* (D-M) gene clusters present on different vector constructs (or A-O/F-M = ΔDE) allow the formation of spindle-shaped gas vesicles in *H. volcanii* transformants (28). The

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TABLE 1. Constructs used in this study

Construct	Restriction site(s)	Position(s) in p-vac sequence[a]	Size(s) (bp)	Second oligonucleotide used for PCR[b] or reference
D-O	*Bgl*II-*Bgl*II	4633–9751	5,119	
E-O	*Sty*I-*Ssp*I	4150–9909	5,760	
F-O	*Nsp*I-*Ssp*I	3494–9909	6,416	
G-O	*Bst*EII-*Ssp*I	3122–9909	6,788	
H-O	*Ssp*I	2622–9909	7,288	CCGGGCTTTCTAGAGGGTGATCTGCG
I-O	*Ssp*I	2226–9909	7,684	CCAGCATCTAGACGAGGTCGCCC
J-O	*Ssp*I	1909–9909	8,001	CGTCGTCTAGAGCTAGTTCCATC
K-O	*Ssp*I	1505–9909	8,405	CCACGCAGTCTAGATAGCGGCCG
L-O	*Ssp*I	721–9909	9,189	TCGCGTGTGTCTAGACTTTTGTTGGC
$L_{1/2}$-O	*Xmn*I-*Ssp*I	1061–9909	8,849	
M-O	*Mro*I-*Ssp*I	1–9909	9,909	28
D-M	*Eco*RI-*Xcm*I	292–6515	6,224	28
E-M		364–4752	4,389	ATCAGCCATGGAGAGTCGATCCCC
F-M		364–4158	3,795	GTCAATCACCATGGCAAGGAAGAG
G-M		364–3529	3,166	TTCGTGGACATTCCCATGGGCGCGGAACAG
H-M		364–3259	2,896	CAGATGCGTGACCCCATGGAGG
I-M		364–2717	2,354	ACATCGGCCATGGATGGAGG
J-M		364–2289	1,926	GCGTCCGGGAGTCCCATGGGGACGAGGTGA
K-M		364–1940	1,577	CCACGTCAGCCCATGGGACCAAATGAG
L-M		364–1623	1,260	GACACCGCCATGGCACGGCTCGCC
M-M		364–775	412	GCCGTGGCCATGGTACACGTTCGC
ΔA	*Mro*I-*Ssp*I, Δ*Xcm*I-*Stu*I	1–9909, Δ6516–6744	9,909, Δ229	28
ΔC	*Mro*I-*Ssp*I, Δ*Sca*I-*Kpn*I	1–9909, Δ6872–7716	9,909, Δ845	29

[a] The p-vac sequence accession numbers in the EMBL database are X55648 (p-*gvpD-M*) and X64729 (p-*gvpACNO*). The positions refer to a combined sequence using an adenosine 486 nucleotides downstream of the p-*gvpM* stop codon (located within an *Mro*I site) as arbitrary position 1.

[b] Restriction sites are underlined (TCTAGA, *Xba*I; CCATGG, *Nco*I).

GvpD and GvpE proteins are involved in the regulation of gas vesicle formation: GvpE is a transcriptional activator required for *gvpA* promoter activity, whereas GvpD is involved in the repression of gas vesicle formation (11, 23, 24, 36).

Deletion studies have been carried out to determine the necessity of each gene found in the p-*gvpACNO* operon for gas vesicle formation (29). These experiments show that ΔA transformants (containing the entire p-vac region except for the *gvpA* gene) and ΔO transformants (p-vac region without *gvpO*) are Vac^-, whereas ΔN ($Vac^{+/-}$) and ΔC (Vac^+) transformants produce gas vesicles. ΔC transformants contain many irregularly shaped gas vesicles with various diameters throughout a single gas vesicle. The finding that gas vesicles of ΔC+C transformants regain the wild-type shape implies that GvpC is involved in shape determination (29). A different approach has been employed by S. DasSarma's group, who inserted foreign DNA into various *gvp* genes present on endogenous plasmid pNRC100 and analyzed the effect in pNRC100-negative *H. halobium* mutant strain SD109 (7). This mutant still contains the c-vac region, although the cells appear to be devoid of gas vesicles. Since the two approaches revealed different results in 6 out of 14 *gvp* cases (including the data reported here), both methods will be discussed in more detail.

In this study, we examined the necessity of genes located in the p-*gvpFGHIJKLM* cluster for gas vesicle formation. Single *gvp* genes were deleted, and the expression of the remaining *gvp* genes was analyzed in *H. volcanii* transformants. ΔH and ΔI transformants still produced gas vesicles, whereas all other deletion variants were gas vesicle free. Together with the results obtained earlier (28, 29), these experiments show that 6 of the 14 *gvp* genes can be deleted without affecting the ability of the cell to form gas vesicles. We also investigated whether the eight genes *gvpA*, *gvpO*, and *gvpFGJKLM* represent the minimal number of *gvp* genes sufficient for gas vesicle formation in *H. volcanii*.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* strains DH5α (13) and GM1674 (*dam*$^-$) (31) harboring plasmid constructs were cultured at 37°C in LB broth (37) containing ampicillin at 100 μg/ml. *H. volcanii* WFD11 (lacking endogenous plasmid pHV2) (5) was grown in rich medium containing, per liter, 175 g of NaCl, 37 g of $MgSO_4 \cdot 7H_2O$, 3.7 g of KCl, 5 g of Bacto Tryptone, 3 g of Bacto Yeast Extract, 25 ml of 1 M Tris/HCl (pH 7.2), 5 ml of 10% $CaCl_2 \cdot 2H_2O$, and 100 μl of 100 μM $MnCl_2$. Halobacterial transformants were selected on agar plates containing novobiocin at 0.2 μg/ml and/or mevinolin or lovastatin at 6 μg/ml. For inspection of the gas vesicle phenotype or isolation of total protein, transformants were grown in the medium described above, except that the NaCl concentration was raised to 206 g/liter to enhance gas vesicle formation. Lovastatin and mevinolin were a generous gift from Merck, Sharp and Dohme GmbH, Munich, Germany.

Constructs used for transformation of *H. volcanii* and transformation procedure. The subfragments of the p-vac region used for the construction of plasmids are listed in Table 1. The protruding ends of DNA fragments E-O, F-O, and G-O were blunt ended by T4 polymerase and ligated to the blunt-ended *Bam*HI site of halobacterial vector pWL102, whereas *Bgl*II fragment D-O was directly ligated to the *Bam*HI site of pWL102 (25). The $L_{1/2}$-O/pWL102 construct (Table 1) was used to yield the H-O, I-O, J-O, K-O, and L-O fragments. This construct was cut at the single *Nhe*I site located 36 bp upstream of the p-*gvpH* stop codon and at the single *Xba*I site located in the pWL102 sequence downstream of the truncated p-*gvpL* codon, resulting in a deletion of 36 bp of p-*gvpH* and all of p-*gvpIJKL*$_{1/2}$. The remaining *Nhe*I/*Xba*I fragment, $H_{9/10}$-O/pWL102 [Δ*gvp*(*H*)*IJKL*$_{1/2}$], was ligated to various fragments amplified by PCR. PCRs were performed with the p-vac region as the template and oligonucleotide CTCGAAA TTCGGCTAGCACGAACA (positions 2746 to 2723 of the p-vac region, containing the *Nhe*I site [underlined] within p-*gvpH*) as primer 1 and the respective oligonucleotide containing an *Xba*I site (as listed in Table 1) as primer 2. The PCR products started at the *Nhe*I site in p-*gvpH* and contained 36 bp derived from the 3′ end of p-*gvpH* [= p-*gvp*(*H*)], p-*gvp*(*H*)*I*, p-*gvp*(*H*)*IJ*, p-*gvp*(*H*)*IJK*, or p-*gvp*(*H*)*JKL*. These fragments were cleaved with *Nhe*I/*Xba*I and ligated to *Nhe*I/*Xba*I fragment $H_{9/10}$-O/pWL102, resulting in constructs H-O through L-O.

The p-vac subfragments E-M, F-M, G-M, H-M, I-M, J-M, K-M, and M-M (Table 1) were produced by PCR using oligonucleotide GTCTGGACGCTAC CATGGCCTCTCGTTCCC (located downstream of p-*gvpM* at positions 351 to 380; the *Nco*I site is underlined) as primer 1 and the respective oligonucleotide complementary to different p-vac sequences as primer 2 (Table 1). Both primers contain an *Nco*I site, and the PCR products were ligated to pJAS35 cut with *Nco*I. The orientation of the DNA insert relative to the *fdx* promoter was determined by *Kpn*I cleavage. The distance between the *Nco*I site and the start codon of the first *gvp* reading frame varied between 14 and 43 nucleotides, leading to short mRNA leader sequences. The expression of functional *gvp* gene

products (especially in the case of the first *gvp* gene) was proven by the formation of gas vesicles in the respective control transformants.

The minimal p-vac region (minvac) was constructed in the following way. *Sma*I/*Asp*718-digested pUC18 was ligated with a *Psh*AI/*Asp*718 DNA fragment (fragment 1 [see Fig. 4]) containing p-*gvpFG* (= FG × pUC). This construct was linearized by *Asp*718, blunt ended, recut with *Eco*RI, and ligated to an *Sca*I/*Eco*RI fragment (fragment 2 [see Fig. 4]) harboring p-*gvpJKLM*, resulting in construct FG-JKLM × pUC. A second construct containing the *Hin*dIII/*Bgl*II p-*gvpACNO* fragment (fragment 3 [see Fig. 4]) cloned in the *Hin*dIII/*Bam*HI sites of pUC18 was cleaved with *Eco*RV to delete major portions of the p-*gvpCN* genes (= AO × pUC). Linearized AO × pUC was religated and linearized again at the *Hin*dIII site located upstream of p-*gvpA* (see Fig. 4). The *Hin*dIII site was blunt ended and used to insert the blunt-ended *Hin*dIII/*Eco*RI fragment containing p-*gvpFGJKLM* of FGJKLM × pUC. The resulting plasmid (= minvac × pUC) was analyzed by restriction digestion for the orientation of the p-*gvp* genes, which was determined as *gvpFGJKLM-gvpAO*. The minvac × pUC construct was cleaved with *Pvu*II to yield the insert, which was then cloned in the *Bam*HI-cleaved, blunt-ended pWL102 vector. The construction of the M-O, D-M, A-O, and ΔA fragments was described by Offner and Pfeifer (28), and the construction of ΔC, ACO, and ACN was described by Offner et al. (29). Prior to the transformation of *H. volcanii*, each construct was passaged through *E. coli dam* mutant strain GM1674 to avoid a halobacterial restriction barrier (16). Transformation was performed as described earlier (33). The presence of the desired plasmids in *H. volcanii* transformants was determined by Southern analyses of total DNA using a p-vac-region-specific, digoxigenin (DIG)-labeled DNA probe (DIG labeling kit of Boehringer Mannheim).

RNA isolation and Northern analyses. Total RNA was isolated as described by Chomczynski and Sacchi (4). For Northern analyses, 10 μg of each RNA was electrophoretically separated on denaturing, formaldehyde-containing 1.2% (wt/vol) agarose gels (1). Strand-specific RNA probes were synthesized using the following fragments cloned in pBluescript as the template: the 515-bp *Xho*I-*Asp*718 fragment containing the 3′ part of p-*gvpF* and p-*gvpG* (probe FG; Fig. 1), the 1,258-bp *Nco*I p-*gvpLM* fragment (probe LM), and the 476-bp *Hin*dIII-*Sca*I fragment containing p-*gvpA* (probe A). The RNA probes were synthesized using the DIG RNA labeling kit obtained from Boehringer Mannheim.

Isolation of gas vesicles and electron microscopy. *H. volcanii* transformants were grown on agar plates for 1 to 2 weeks. Cells were scraped from the agar and lysed in 10 mM Tris/HCl (pH 7.2) with 1 to 2 μl of DNase I (1 mg/ml). Gas vesicles were collected in small, narrow tubes (diameter, 4 mm) by centrifugation in an Eppendorf centrifuge for 20 min at 1,000 to 2,000 rpm to improve flotation and washed three times with 10 mM Tris/HCl (pH 7.2). For negative staining, a drop of the gas vesicle preparation was placed onto a carbon-coated copper grid and removed after 2 min with a pipette, and the grid was 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.

RESULTS

Deletion of single *gvp* genes and effect on gas vesicle formation in *H. volcanii* transformants. The deletion of a specific *gvp* gene in the p-*gvpDEFGHIJKLM* cluster was achieved by the complementation of two subfragments present on different vector plasmids, except for the deletion of *gvpM*. The ΔM transformant harbored a single p-vac construct (L-O) without the *gvpM* gene together with the "empty" pJAS35 expression vector. Each construct produced was designated according to the *gvp* gene located near the boundary of the subfragment (Table 1; Fig. 1A). Four of these fragments (D-O, E-O, F-O, and G-O) were obtained by restriction endonuclease digestions, whereas all other subfragments were, at least in part, amplified by PCR. Subfragments D-O through L-O were inserted into pWL102, and the various *gvp* genes were expressed under the control of the endogenous promoters. In contrast, constructs E-M through M-M contained *gvp* reading frames inserted into pJAS35, where they are expressed under ferredoxin (*fdx*) promoter control (Table 1).

Various combinations of two of these constructs were used to transform *H. volcanii*. Figure 1A shows the series of transformants as colony streaks according to the p-vac subfragment(s) present in the cells. For completeness, all transformants with *gvp* gene deletions are included; the deletion of genes found within the p-*gvpACNO* cluster has already been described (29). Gas vesicle-producing (Vac^+) colonies appear pink-white and turbid, whereas Vac^- colonies are red and translucent. Gas vesicles seen by phase-contrast microscopy appear as light refractile bodies inside the cells. M-O transformants contain the entire p-vac region and showed the expected pink-white and turbid Vac^+ phenotype (28). In these transformants, gas vesicle formation became visible 2 days after colony formation. The ΔC (29) and ΔH transformants were also Vac^+; the latter one started to produce gas vesicles significantly earlier than ΔC and M-O. The ΔI, ΔHI, ΔE, ΔD, and ΔDE transformants formed turbid colonies, but the amount of gas vesicles (also seen as light refractile bodies in the cells) was lower compared to the M-O wild type (Fig. 1A and data not shown). Minor amounts of gas vesicles were seen in ΔN transformants when the cells were inspected by phase-contrast microscopy (29). The lack of any of the *gvpM*, *gvpL*, *gvpK*, *gvpJ*, *gvpG*, *gvpF*, *gvpA*, or *gvpO* genes yielded red translucent colonies (Fig. 1A), and no light refractile bodies were detected in any of the cells inspected by phase-contrast microscopy. The results implied that these eight genes are essential for gas vesicle formation whereas the six genes *gvpDE*, *gvpHI*, and *gvpCN* are not required.

To demonstrate that each of the constructs was expressed and produced functional Gvp proteins, the respective control transformants were prepared. Each control transformant contained the *gvp* gene missing in the gene deletion transformant as the first reading frame in the subfragment cloned in pJAS35. Figure 1B shows the colony phenotype of the gene deletion transformants in comparison to that of the respective control transformants. One would expect each control transformant to express a Vac^+ phenotype similar to that of the wild type; however, the phenotype indicated some variations. A wild-type Vac^+ phenotype (pink-white colonies and many light refractile bodies inside the cells) was observed with the cΔC, cΔG, cΔH, cΔI, cΔL, and cΔM transformants, while cΔJ indicated an overproducer phenotype. The cΔA, cΔD, cΔE, cΔN, and cΔO transformants contained somewhat fewer gas vesicles (Fig. 1B, bottom line), and only minor amounts were observed in cΔF and cΔK, as verified by phase-contrast microscopy. The ΔI and cΔJ transformants contained extremely long gas vesicles (see below). These results demonstrated that the *gvp* genes present in these control transformants were expressed and that the gene products were functional in gas vesicle formation.

Northern analyses to investigate transcription from the *gvpF-M* cluster. To analyze the *gvp* mRNA levels in the transformants, total RNA was isolated and used for Northern analyses. *H. volcanii* transformants with the wild-type p-vac region produce the 4-kb p-*gvpF-M* mRNA during exponential growth (29). Since parts of the p-*gvpFGHIJKLM* gene cluster were present on different vector constructs in the other transformants, the mRNAs derived from this region were investigated in more detail.

The FG probe (Fig. 1) was used to monitor mRNAs starting at the pF promoter present in the pWL102 constructs, whereas the LM probe was used to determine transcripts derived from the respective pJAS35 constructs in each transformant. Using the FG probe for Northern analyses, mRNAs of the expected length were detected in all gene deletion and control transformants (Fig. 2). The LM probe detected transcripts starting at the *fdx* promoter in the pJAS35 construct. This promoter usually leads to a large amount of mRNA during the exponential growth phase and a smaller amount during the stationary growth phase (34). Using the LM probe, such a transcript pattern was seen in transformants harboring the H-M, I-M, K-M, and L-M constructs whereas the amount of mRNA was smaller in transformants containing the J-M construct (ΔI and cΔJ) (Fig. 2). This was surprising, since the cΔJ transformant

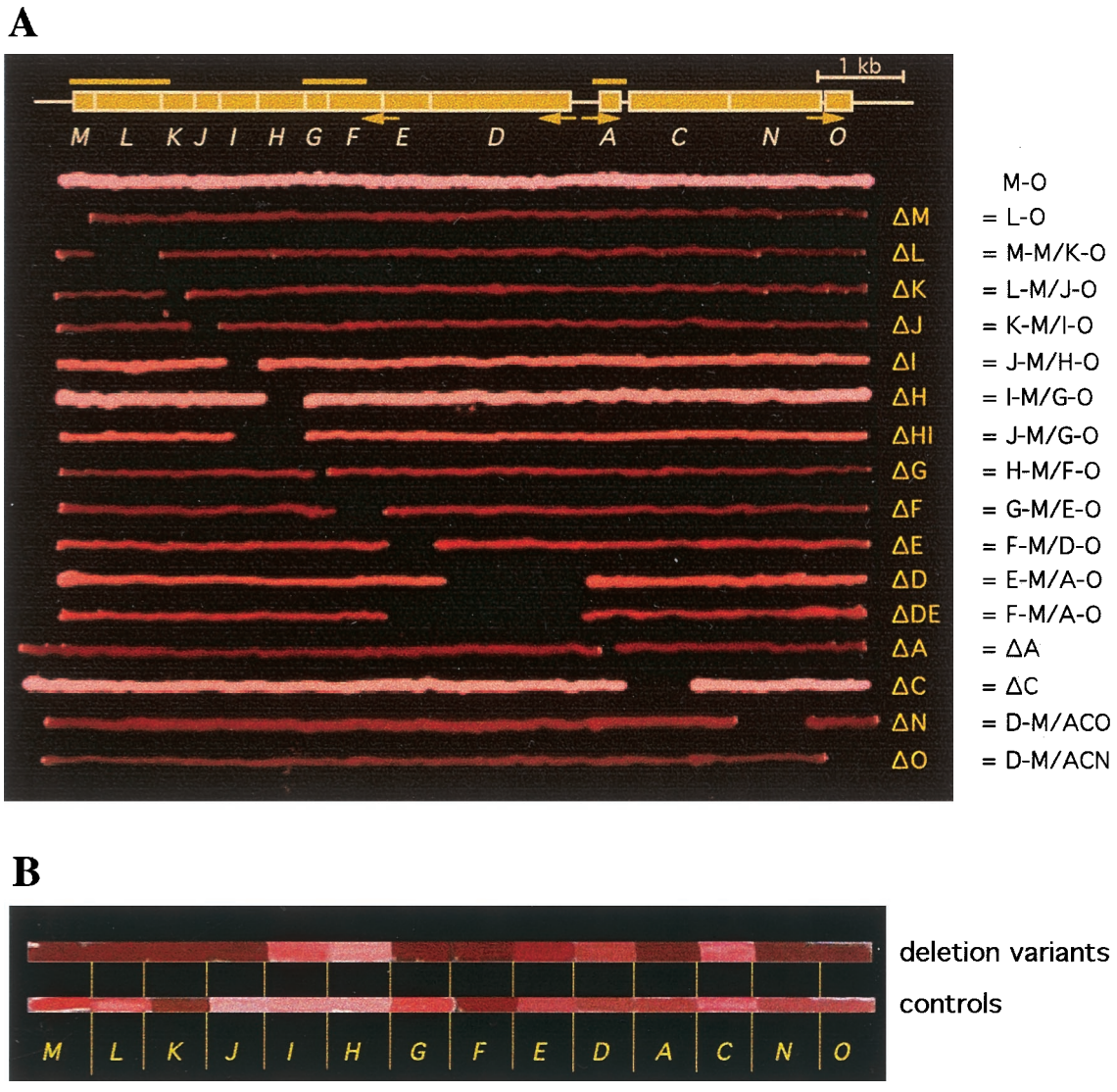

FIG. 1. The p-vac region and phenotypes of various *H. volcanii* transformants. (A) The 14 *gvp* genes constituting the p-vac region are shown as boxes (A and C through O). The four endogenous p-vac promoters are indicated by arrows underneath. The bars above represent the A, FG, and LM probes used for Northern analyses. The lines underneath represent colonies grown according to the *gvp* fragment(s) present in the cells. The M-O (entire p-vac region), L-O (ΔM), ΔA, and ΔC transformants harbor the p-vac region on a single construct (plus pJAS35 as a second construct); all other transformants contain the p-vac region on two constructs. Vac^+ colonies appear turbid and white; orange, turbid colonies possess fewer gas vesicles, and dark red translucent colonies are Vac^-. The Δ designations to the right of the plate indicate the deleted *gvp* genes. The constructs used for transformation are shown further to the right. The construction of transformants M-O, ΔA, ΔC, ΔN, and ΔO has been described previously (28, 29). (B) Various gene deletion transformants (top) in comparison to the respective control transformants (bottom). The colonies are arranged according to the respective gene deletion given underneath (i.e., M = ΔM transformant on top and cΔM control transformant underneath).

showed overproduction of gas vesicles. The M-M construct was expressed predominantly during the stationary growth phase in both cases (ΔL and cΔM) (Fig. 2). Transformants containing the M-M construct by itself showed the same pattern of *gvpM* mRNA production throughout growth (data not shown). In summary, these analyses demonstrated that each fragment was transcribed and that the observed variations in transcription did not reflect the different gas vesicle phenotypes observed.

Inspection of the gas vesicles formed by transformants ΔH and ΔI and the respective control transformants. The cells of Vac^+ transformants ΔH and ΔI were inspected by phase-contrast microscopy. ΔH transformants contained light refractile bodies throughout growth. ΔI transformants indicated large, cylinder-shaped gas vesicles spanning the entire length of the cell and sometimes even altering the cell shape by pushing out lobes. A similar phenotype was observed with the cΔJ transformants.

Gas vesicles were isolated from ΔH and ΔI (and ΔHI) transformants and also from the respective control transformants and investigated by electron microscopy (Fig. 3). Gas vesicles of the ΔI transformant were extremely long and cylinder shaped, with an average length of more than 0.6 μm (Fig. 3). Some even measured 2.7 μm in length and were thus longer than the average *H. volcanii* cell. The appearance of these extremely long gas vesicles explained the shape alterations of ΔI transformant cells observed in the phase-contrast microscope. The control transformant cΔI, however, contained spindle-shaped gas vesicles, as found in wild-type *H. salinarum* (Fig. 3). Many gas vesicles were obtained by flotation from the ΔH transformant; however, intact gas vesicles were rarely found after stain-

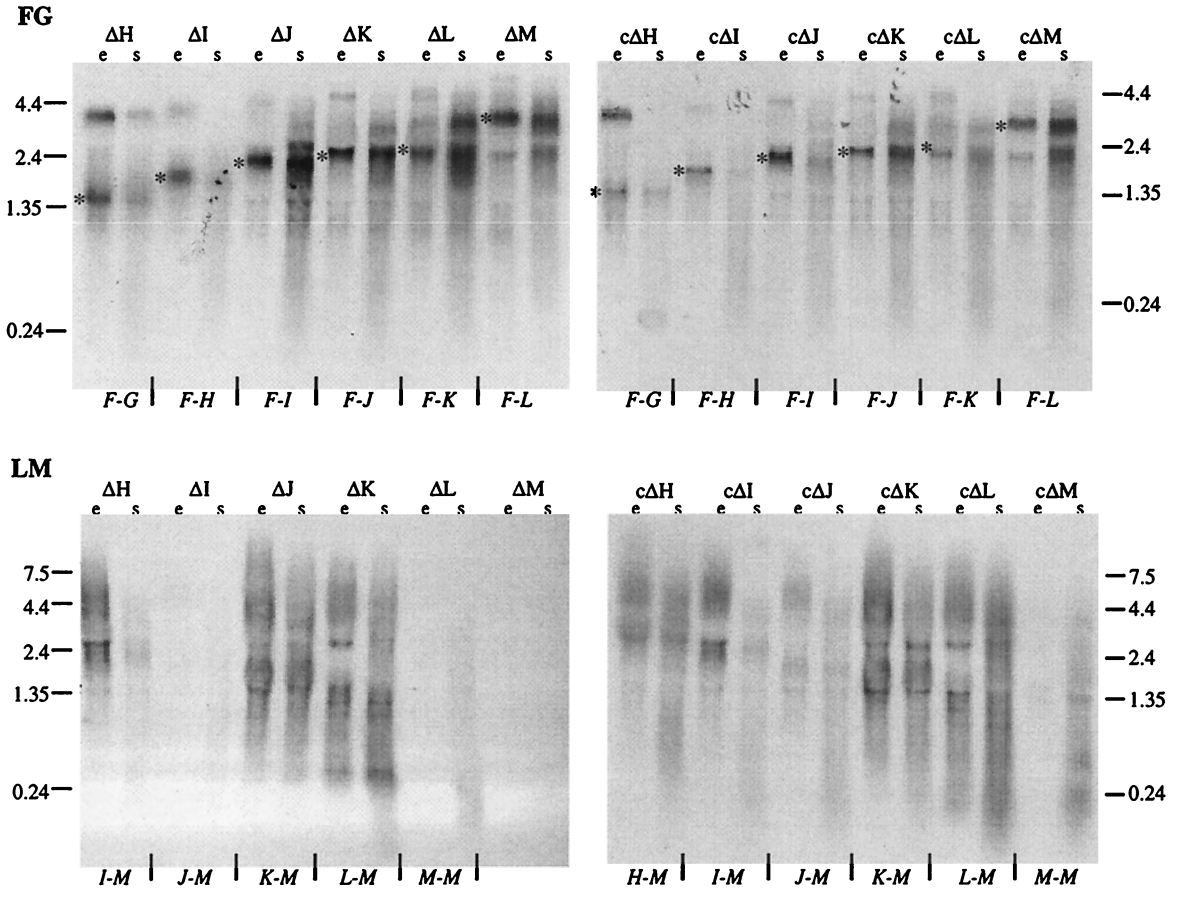

FIG. 2. Northern analyses of gene deletion and control transformants to investigate the various p-*gvpF-M* transcripts. RNA samples were derived from the exponential (e) and stationary (s) growth phases. A 10-μg sample of total RNA was applied to each slot. Transcripts starting at the pF promoter were visualized using the FG probe (top), whereas the LM probe was used to detect transcripts starting at the *fdx* promoter in pJSA35 (bottom). The construct responsible for mRNA hybridization is indicated at the bottom of each gel. For the FG probe, the expected mRNA is indicated by an asterisk. The values on the side of each gel are the sizes (in kilobases) of the RNA markers.

ing for electron microscopy (Fig. 3). They disintegrated into single ribs, demonstrating that the gas vesicles synthesized without GvpH were unstable during the staining procedure. Gas vesicles isolated from the control transformant cΔH were stable but appeared cylinder and not spindle shaped (Fig. 3). Gas vesicles isolated from the double deletion transformant ΔHI were stable and cylinder shaped.

Construction of the minvac region containing eight *gvp* genes. The results of the transformation experiments implied that the genes p-*gvpFGJKLM* and p-*gvpAO* constitute the minimal number of genes required for gas vesicle formation. In order to prove that these genes are really sufficient, a plasmid containing these eight *gvp* genes was constructed: the *gvpDE* and *gvpHI* genes were deleted from the p-*gvpDEFGHIJKLM* cluster, as were the *gvpCN* genes from the p-*gvpACNO* gene cluster. The resulting minimal p-vac (minvac) region contained the remaining *gvp* genes arranged consecutively under the control of the endogenous promoters pA, pO, and pF (Fig. 4). The *H. volcanii* transformants containing the minvac plasmid did not contain light refractile bodies and were Vac^-, suggesting that the six *gvp* genes that were lacking (*gvpCN*, *gvpDE*, and *gvpHI*) could not be deleted all at once and that the remaining eight *gvp* genes were not sufficient for gas vesicle formation. To identify the missing gene(s) required for gas vesicle formation, double transformants were produced containing the minvac construct together with a second plasmid harboring an additional fragment of the p-vac region (A-O or E-M). The minvac/A-O transformants were Vac^-, whereas the minvac/E-M transformants were Vac^+, suggesting that the p-*gvpHI* genes of the p-*gvpFGHIJKLM* unit might be lacking. Double transformants containing minvac plus further deletions in E-M (H-M, I-M, J-M, or K-M) were produced, and the cells were inspected for gas vesicle formation. Light refractile bodies were found in the minvac/H-M and minvac/I-M transformants and even in the minvac/J-M transformants, although the *gvp* genes present on construct J-M were already present on the minvac plasmid (Fig. 5 and data not shown). The minvac/K-M transformants did not reveal light refractile bodies and were thus Vac^-. These results suggested that the *gvpHI* genes are not required for gas vesicle formation and that the *gvpJKLM* genes present on the minvac construct (especially *gvpJ*) were not sufficiently expressed.

Northern analyses were performed to investigate the amount of *gvp* transcripts using the A, FG, and LM probes. The A probe detected a large amount of 0.27-kb *gvpA* mRNA in each transformant (Fig. 6). The FG probe used to detect the *gvpFG JKLM* mRNA indicated a small amount of a 3.5-kb transcript that could span the entire *gvpFGJKLM* region (Fig. 4). In addition to this transcript, larger amounts of 1.8- and 1.4-kb mRNAs were also detected, suggesting early termination or degradation of the 3.5-kb transcript (Fig. 6). The LM probe indicated the small amount of the 3.5-kb mRNA in minvac

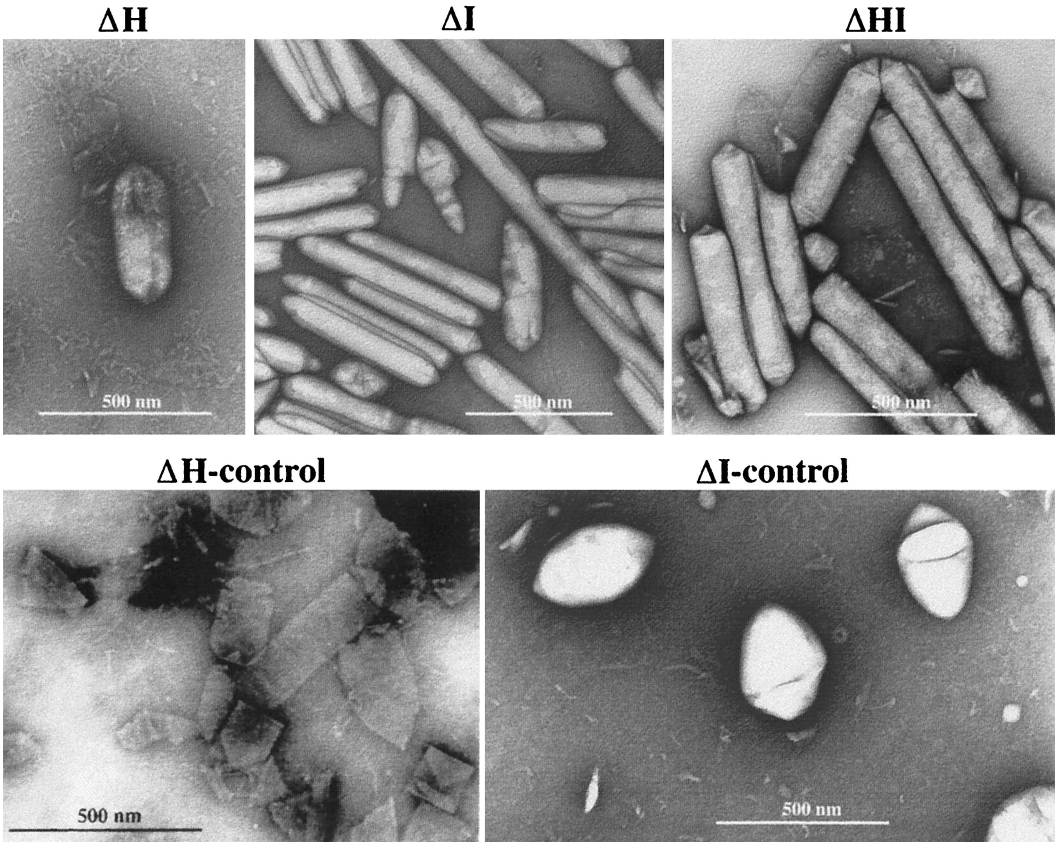

FIG. 3. Electron micrographs of isolated gas vesicles of ΔH, ΔI, ΔHI, and the respective control transformants.

transformants. The double transformant minvac/I-M or minvac/J-M contained a large amount of I-M or J-M mRNA due to the expression of these genes under *fdx* promoter control in pJAS35 (Fig. 6). Thus, the lack of gas vesicles in minvac transformants might be due to the early termination (or processing) of the *gvpFGJKLM* mRNA.

DISCUSSION

The results presented here suggest that 8 out of 14 *gvp* genes found in the vac regions of halophilic archaea are sufficient for gas vesicle formation: *gvpA* (encoding the major gas vesicle structural protein); *gvpO*, whose function is unknown; and *gvpFGJKLM*. In contrast to the deletion analysis described here, DasSarma's group reported that gas vesicle formation in *H. salinarum* (formerly *H. halobium*) requires at least 10 out of 13 *gvp* genes (*gvpO* was not recognized at that time; reference 7). That group inserted foreign DNA into various *gvp* genes derived from pNRC100 and analyzed the effect on *H. salinarum* mutant strain SD109 lacking the pNRC100-encoded vac region. One reason for the discrepancies in 6 out of 14 cases could be the use of different recipient strains for the transformation experiments. We chose *H. volcanii* as the recipient because this strain is easy to transform, grows faster than *H. salinarum*, and—most importantly—offers a clean genetic background for the functional analysis of *gvp* genes. The expression of various *gvp* genes in this recipient strain appears to be similar to that found for *H. salinarum* or *H. mediterranei*. *H. salinarum* strains harboring plasmid deletion variants lacking the p-vac region (such as *H. salinarum* PHH4 or SD109; reference 7) are deficient in the plasmid vac region but still contain the c-vac region.

Another difference is the mutation method used for the analyses. Insertion of foreign DNA into a *gvp* gene can cause a polar mutation affecting not only the expression of the *gvp* gene investigated but also that of *gvp* genes located farther downstream, especially when they are cotranscribed. Without the determination of the expression of these *gvp* genes, the results obtained are not sufficient to define the function of a single *gvp* gene. Another problem is encountered when the integration site is close to the 5′ or 3′ terminus of the *gvp* gene and the

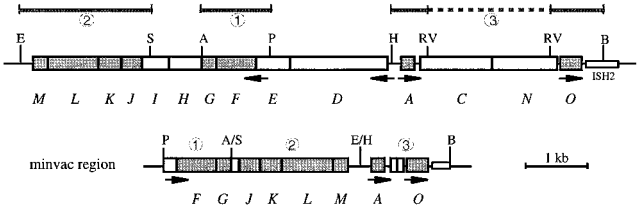

FIG. 4. Genetic map of the p-vac region and strategy for the construction of the minvac region. The 14 *gvp* genes of the p-vac region are represented by boxes labeled A and C through O. Essential *gvp* genes are represented by grey boxes, and nonessential genes are represented by white boxes. Arrows indicate the locations of the endogenous promoters. Restriction sites used to obtain p-vac subfragments 1 to 3, used for the construction of the minvac plasmid, are shown at the top as follows: A, *Asp*718; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Psh*AI; RV, *Eco*RV; S, *Sca*I. The respective subfragments are shown as bars. For further explanations, see Materials and Methods. The minvac construct at the bottom acquired deletions of *gvpDE*, *gvpHI*, and *gvpCN*. ISH2, halobacterial insertion element.

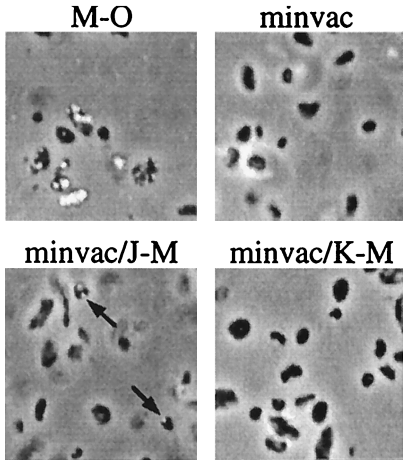

FIG. 5. Phase-contrast microscopy of various *H. volcanii* transformants. Gas vesicles are visible as white bodies inside the cells. The constructs present in the cells are shown above the images. The M-O transformant contains the entire p-vac region and produces many light refractile bodies, whereas the minvac and minvac/K-M transformants are gas vesicle free. Gas vesicles found in minvac/J-M cells are indicated by arrows. Magnification, ×1,050.

insert does not destroy the gene function at the protein level. In all such cases, the presence or absence of the respective *gvp* gene products (mRNAs or proteins) should be determined; however, this has not been done (7). In contrast, deletion of a single *gvp* gene within the vac region clearly destroys its function and if the control transformant containing the respective *gvp* gene produces gas vesicles, the phenotype of the *gvpΔX* transformants provides a good indication of the effect of the mutation. The deletion of the *gvp* gene of interest is, however, often achieved by the complementation of *gvp* genes present on two vector plasmids. In such cases, *gvp* genes are expressed either by their endogenous promoter(s) or under ferredoxin (*fdx*) promoter control (28, 29, and this report). In such double transformants, gas vesicle synthesis could be affected by an imbalance of Gvp proteins due to different plasmid copy numbers or the higher *fdx* promoter activity compared to the normally regulated *gvp* gene expression. However, *gvp* gene function can still be determined by this method, especially when the respective control transformant indicates normal gas vesicle formation. The differences obtained with mutations in the *gvpACNO* gene cluster have already been discussed (29, 35).

The analyses of the *gvpFGHIJKLM* genes presented in this report indicated that deletion of the gene *gvpF*, *gvpJ*, *gvpK*, or *gvpL* in the p-vac region resulted in Vac$^-$ transformants, whereas the respective control transformants produced gas vesicles. Similar results have been obtained by insertional mutation of these genes (7). Contradictory results were observed with the *gvpG*, *gvpH*, *gvpI*, and *gvpM* genes. Deletion of p-*gvpG* or p-*gvpM* (and also mc-*gvpM*; reference 11) resulted in Vac$^-$ transformants, whereas gas vesicle production was still observed when each of these *gvp* genes was mutated by a respective insertion (7). However, the integration site of the insertion in the *gvpM* gene is very close to the 3′ end and the absence of GvpM has not been checked in these transformants. An insert in *gvpH* results in transformants containing minor amounts of gas vesicles (7), but the ΔH transformants described in this report produced large amounts of gas vesicles that could be isolated by flotation but were unstable in electron microscopy. These results imply that the presence of GvpH is important for the formation of stable gas vesicles. Transformants carrying an insert in the *gvpI* gene are Vac$^-$ (7), whereas the ΔI transformants described here contained extremely long, cylinder-shaped gas vesicles, and the respective ΔI/I control transformant harbored large numbers of spindle-shaped gas vesicles. All of these discrepancies indicate that careful analyses are required to unequivocally determine a *gvp* gene function.

Our deletion analyses suggested that the p-*gvpC*, p-*gvpDE*, p-*gvpHI*, and p-*gvpN* genes are not essential for gas vesicle formation and that the remaining eight genes (*gvpAO* and *gvpFGJKLM*) consequently represent the minimal number of genes required for their assembly. In order to test whether these eight genes are indeed sufficient, the minvac construct containing these genes was used to transform *H. volcanii*. The minvac construct was initially inadequate for the formation

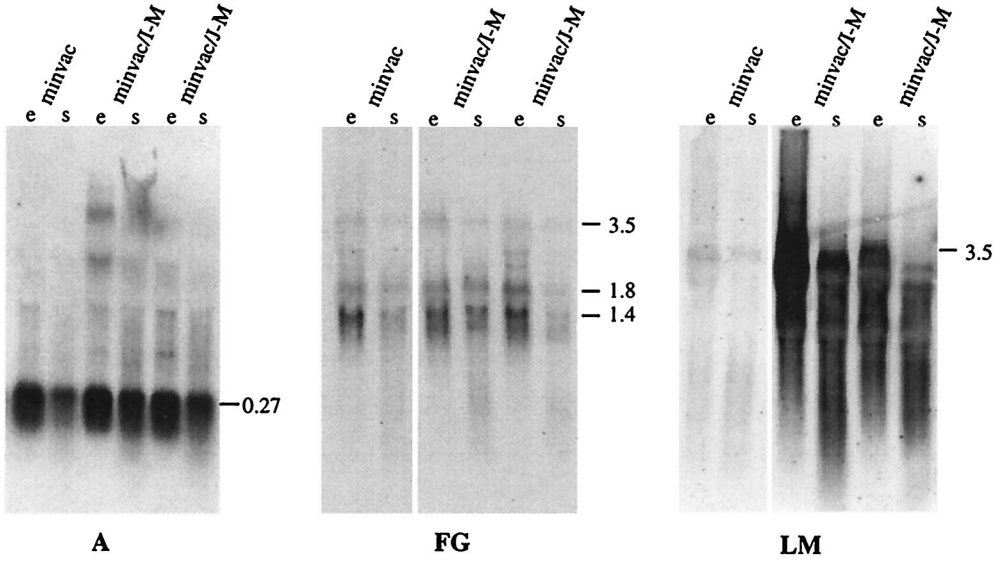

FIG. 6. Northern analyses of minvac and minvac double transformants to detect *gvpA* and *gvpF-M* mRNA. RNA samples were derived from the exponential (e) and stationary (s) growth phases. A 10-μg sample of total RNA was applied to each slot. Transcripts starting at the pA promoter were visualized using the A probe (A), and transcripts derived from the pF promoter were visualized using an FG probe (FG), whereas the LM probe also detected transcripts starting at the *fdx* promoter in pJSA35 in the cases of the I-M and J-M constructs (LM). The values on the right side of each gel are the sizes (in kilobases) of the hybridizing mRNAs.

TABLE 2. Comparison of *gvp* loci identified in archaea and bacteria

Species or region	*gvp* loci identified													
Halobacterium salinarum	A	C	D	E	F	G	H	I	J	K	L	M	N	O
minvac	A	—[d]	—	—	F	G	—	—	J	K	L	M	—	O
Anabaena flos-aquae[a]	A	C							J	K	L		N	
Bacillus megaterium[b]	A	—	—	—	F	G	—	Q	J	K	L	S	N	R
Streptomyces coelicolor[c]	A	—	—	—	F	G	—	—	J	K	L	S	—	O

[a] Published as the *gvpACNJKL* gene cluster (21).
[b] Published as the *gvpAPQ-gvpBRNFGLSKJTU* gene cluster (26).
[c] Found as the *gvpOAFGxxJLSK* gene cluster on cosmid 1E6 in the EMBL gene sequence data bank.
[d] —, a homologous *gvp* gene has not been identified in the *gvp* gene cluster investigated.

of gas vesicles; however, the addition of extra copies of the *gvpJKLM* genes resulted in transformants containing gas vesicles whereas extra copies of *gvpKLM* revealed Vac$^-$ transformants, suggesting that the lack of gas vesicles in minvac transformants is mainly caused by the lack of sufficient GvpJ protein. Northern analyses demonstrated that the J-M genes on the original minvac construct were insufficiently expressed due to early termination of transcription. Nevertheless, since only the *gvpAO* and *gvpFGJKLM* genes were present in the minvac/JKLM transformants, these eight *gvp* genes represent the minimal number of genes required for gas vesicle formation. Thus, it is possible to delete six *gvp* genes simultaneously without losing the ability to synthesize gas vesicles. Similar experiments have not been done for the system used by DasSarma et al. (7).

The essential gene products determined in this study involve all of the small Gvp proteins with molecular masses of 8 to 12.7 kDa (GvpA, GvpG, GvpJ, GvpK, and GvpM) that also exhibit hydrophobic stretches in their amino acid sequences (10, 28). Surprisingly, GvpJ and GvpM show more than 60% sequence similarity to the GvpA protein, suggesting that they are structural components of the gas vesicle (20, 32). However, the amino acid composition of isolated gas vesicles exclusively reflects that of the GvpA protein and does not indicate a recognizable proportion of other proteins (9); thus, GvpM and GvpJ may only be required in early stages of gas vesicle assembly, being replaced by GvpA later on. Neither a GvpA-specific antiserum (12) nor an anti-gas vesicle serum (8) reacts with other Gvp proteins in a gas vesicle preparation. For some of the products encoded by the *gvpFGHIJKLM* gene cluster, chaperone functions have been suggested; they might keep the highly hydrophobic GvpA protein in a conformation that is required for the assembly process or even comprise an incorporation mechanism.

Comparison of the minvac region to *gvp* gene clusters found in bacteria. With the recent finding of gas vesicle genes in *B. megaterium* (26) and also in *Streptomyces coelicolor* (cosmid 1E6; EMBL database) it is interesting to compare these eight essential *gvp* genes of halophilic archaea with gas vesicle genes identified in bacteria. In the cyanobacterium *A. flos-aquae*, six homologs to essential archaeal *gvp* genes have been identified, including multiple copies of *gvpA* and the genes *gvpC*, *gvpN*, and *gvpJKL* (22). According to our studies, homologs to *gvpFG*, *gvpM*, and *gvpO* are still lacking (Table 2). Since neither transcript analyses nor transformation experiments have been done, it is not clear whether the *gvp* gene cluster of *Anabaena* is complete.

The gram-positive bacterium *B. megaterium* contains a *gvp* gene cluster consisting of 14 genes (*gvpAPQ-gvpBRNFGLS KJTU*) (26). This *gvp* gene cluster has been used to transform *E. coli*, leading to the formation of tiny gas vesicles (average length, 40 nm). The first three genes (*gvpAPQ*) of this *gvp* cluster can be deleted without disturbing the ability of *E. coli* transformants to synthesize gas vesicles (26). The product of the *gvpB* gene presumably constitutes the major gas vesicle structural protein. The product of the second gene of this cluster, GvpR, exhibits 44% similarity to the GvpO protein assigned to *S. coelicolor* and 39% similarity to the GvpO protein of halophilic archaea. Thus, GvpR might be a more distant homolog of GvpO (Table 2). The *gvpNFGLKJ* genes are homologs to essential genes determined in this study. The *gvpS* gene product shows similarity to GvpA and GvpJ and could be a more distant homolog of the GvpM protein of halophilic archaea. A phylogenetic tree constructed with the sequences of GvpA, GvpJ, and GvpS indicates that all three sequences cluster together (data not shown). The *gvpTU* genes located at the end of this gene cluster have no archaeal homolog. Thus, among the 14 *gvp* genes found in *B. megaterium*, all eight essential *gvp* genes determined for halophilic archaea are present (Table 2).

During the genome sequencing project of the gram-positive bacterium *S. coelicolor*, eight *gvp* genes were found in cosmid 1E6; these genes are arranged as a *gvpOAFGxxJLSK* cluster (with *xx* representing two hypothetical protein genes; EMBL gene sequence data bank). The *gvpS* gene product is highly similar to the GvpS protein of *B. megaterium* and most likely a more distant homolog of GvpM. The arrangement of certain *gvp* genes (i.e., *gvpLSK*) is the same in both gram-positive bacteria but differs from the arrangement of the homologous genes (*gvpKLM*) in halophilic archaea. Strikingly, the eight *gvp* genes found in *S. coelicolor* exactly match the *gvp* genes in minvac that are required for gas vesicle formation in *H. salinarum* (Table 2). The expression of *gvp* genes in the gram-positive bacteria has not been investigated. It might be interesting to see whether, where, and when *S. coelicolor* produces gas vesicles and what functions these flotation devices might have in an organism that forms mycelia and does not usually exist in an aquatic environment. The possession of genes encoding gas vesicles is obviously more widely distributed than currently thought, and they occur in archaea as well as in gram-positive and gram-negative bacteria.

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