JOURNAL OF BACTERIOLOGY, Dec. 1994, p. 7646–7652
0021-9193/94/$04.00+0

Vol. 176, No. 24

Wild-Type Gas Vesicle Formation Requires at Least Ten Genes in the *gvp* Gene Cluster of *Halobacterium halobium* Plasmid pNRC100

SHILADITYA DASSARMA,* PRIYA ARORA, FENG LIN, ELIZABETH MOLINARI, AND LUCY RU-SIU YIN

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received 15 August 1994/Accepted 18 October 1994

To study the functions of the 13 *gvp* genes, *gvpMLKJIHGFEDACN*, on plasmid pNRC100 of *Halobacterium halobium* in gas vesicle formation, we carried out linker scanning mutagenesis of the gene cluster. We constructed a 24.5-kb *Escherichia coli-H. halobium* shuttle plasmid, pFL2, containing the *gvp* gene cluster and introduced a kanamycin resistance (κ) cassette into each gene (except for *gvpA*). Transformation of *H. halobium* SD109, which had the entire *gvp* gene cluster deleted, with pFL2 and mutated pFL2 derivatives showed that while the unmutated gene cluster successfully programmed gas vesicle formation, derivatives with insertion of the κ cassette in any of the *gvp* genes, except *gvpM*, did not lead to production of normal gas vesicles. Insertions in *gvpL*, *-K*, *-J*, *-I*, and *-F* resulted in a complete block in gas vesicle synthesis, while insertions in *gvpH*, *-G*, *-E*, *-D*, *-C*, and *-N* resulted in greatly reduced gas vesicle synthesis. In most cases, the block in gas vesicle synthesis did not result from polar effects, since similar results were obtained for derivatives of the insertion mutants in which most of the internal portion of the κ cassette was deleted and only small (15 to 54-bp) insertions remained. The only exceptions were for *gvpH* and *gvpD*, where deletion of the internal portion of the κ insertions resulted in phenotypic reversion. Electron microscopic analysis of the κ mutants revealed that interruptions of *gvpC* and *gvpN* result in the formation of smaller gas vesicles than in the wild type, while interruptions of *gvpF*, *-G*, *-H*, *-I*, *-J*, *-K*, and *-L* produce no discernible vesicle intermediates. These results indicate that *gvpA*, *-C*, and *-N*, which have the rightward transcriptional orientation, encode structural proteins, with *gvpC* and *gvpN* necessary for late stages of vesicle formation, and *gvpL*, *-K*, *-J*, *-I*, *-H*, *-G*, and *-F*, which have the leftward transcriptional orientation, encode proteins involved in early steps in the assembly of gas vesicles.

Gas vesicles are produced by over 100 species of prokaryotic microorganisms, most of which inhabit aquatic environments (23). These microorganisms include photosynthetic and heterotrophic eubacteria as well as methanogenic and halophilic archaea. Gas vesicles provide buoyancy to cells and promote flotation, which improves the availability of oxygen for respiration and light for photosynthesis. The membrane surrounding the vesicle is proteinaceous, lipid-free, and rigid and forms a barrier to liquid water but is freely permeable to dissolved gases. Studies on the composition of gas vesicles using biochemical approaches have been greatly limited by the extreme stability of membrane proteins to solubilization (14). As a result of this difficulty, only a single protein, GvpA, was originally found in gas vesicles; a second relatively minor protein, GvpC, has recently been found in cyanobacterial and halophilic archaeal vesicles (8, 9, 24).

For the halophilic archaeum *Halobacterium halobium* NRC-1, genetic analysis of spontaneous mutants lacking gas vesicles (Vac^-) suggested that a cluster of genes on a 200-kb plasmid pNRC100 is involved in gas vesicle synthesis (4–6). Insertions of IS elements were localized in pNRC100 within the *gvpA* promoter and in two genes, *gvpD* and *gvpE*, located upstream of and transcribed divergently to *gvpA* (see Fig. 1) (12). DNA sequencing beyond the 3′ end of *gvpE* showed the presence of eight additional open reading frames, *gvpF*, *-G*, *-H*, *-I*, *-J*, *-K*, *-L*, and *-M* organized into an operon-like structure and suggested that the insertions of IS elements may have resulted in polar effects on downstream genes (13). The predicted products of *gvpJ* and *gvpM* were found to be similar to the major gas vesicle protein, GvpA, suggesting the existence of a small family of gas vesicle proteins. DNA sequencing and transcriptional analysis identified two open reading frames, *gvpC* and *gvpN*, downstream of and transcriptionally linked to *gvpA* (9). The entire cluster of 13 genes, *gvpMLKJIHGFED ACN*, was found to be flanked by two copies of an IS element, suggesting that the whole cluster may be mobile, similar to a composite transposon (18). Homologous *gvp* gene clusters were reported, including a silent copy in a second large plasmid of *H. halobium* NRC-1 (12), two copies in another strain of *H. halobium*, and one in a distantly related halophile, *Haloferax mediterraneai* (7). In the latter cases, a 14th gene, *gvpO*, was identified 3′ to *gvpN*. Taken together, these findings suggested that several and perhaps all of the *gvp* genes were necessary for gas vesicle synthesis, although direct experimental evidence for the involvement of most of the individual genes in gas vesicle formation was lacking.

In order to characterize the *gvp* gene cluster further and rigorously test for the possible role of each of the individual genes in gas vesicle formation, we established a genetic system for mutagenic analysis. We constructed an *H. halobium-Escherichia coli* shuttle plasmid containing the entire *gvp* gene cluster of *H. halobium* NRC-1. When this plasmid, pJHGV3, was introduced into Vac^- mutants of *H. halobium* with the entire *gvp* gene cluster deleted, the Vac^- mutants were complemented and the wild-type Vac^+ phenotype was restored (9, 10). Using a similar plasmid, pFL2, and genetic complementation and electron microscopic assays, we have now analyzed the effects of linker scanning mutations in 12 *gvp* genes of

* Corresponding author. Phone: (413) 545-2581 or (413) 545-1936. Fax: (413) 545-1578. Electronic mail address: (Internet): sds@rna.micro.umass.edu.

unknown function. The results show that at least 10 *gvp* genes are necessary for wild-type gas vesicle synthesis, indicating that gas vesicle formation is a complex process.

MATERIALS AND METHODS

Construction of pFL2 and κ insertion mutants. A precursor to pFL2, pFL1, was constructed by cloning the 13-kb *gvp* gene-containing *Eco*RI fragment from a pJHGV3 derivative into plasmid pNG11Δ12 (17). Next, the single *Pst*I site in pFL1 was deleted by digesting with *Pst*I, producing blunt ends with T4 DNA polymerase, and recircularizing with T4 DNA ligase (19). Plasmid pFL2 showed improved replication properties over pJHGV3 in *H. halobium* as a result of the presence of a complete replication region, including the A+T-rich region 5′ to the *repH* gene (17).

Linker scanning mutations (11) in pFL2 were generated by inserting the kanamycin resistance (κ) cassette, which had been constructed by ligation of a synthetic oligonucleotide adapter containing *Sse*I, *Xba*I, and *Pst*I sites to the ends of a kanamycin resistance gene-containing restriction fragment of a pUC4K (Pharmacia) derivative, as previously described (9). Linear fragments of pFL2 were generated by partial digestion with *Taq*I and purified by electrophoresis in contour-clamped homogeneous electric field agarose gels (1). The 5′ overhanging ends were filled in with T4 DNA polymerase, and the population of linearized plasmids were ligated to the κ cassette liberated from pJHKAN1 (9) by *Dra*I digestion with T4 DNA ligase. The ligation mixture was transformed into *E. coli* DH5α and transformants were selected on Luria-Bertani agar plates containing ampicillin and kanamycin (19). The κ cassette was introduced into *gvpL*, *-J*, *-G*, *-E*, *-D*, *-C*, and *-N* by this method. In place of *Taq*I, *Fsp*I was used to generate insertions in *gvpM* and *gvpF*, *Sst*I was used to generate an insertion in *gvpK*, and *Sca*I was used to generate insertions in *gvpI* and *gvpC*.

Plasmids were prepared from *E. coli* by an alkaline-sodium dodecyl sulfate (SDS) procedure (19) and characterized by restriction analysis and limited DNA sequencing analysis across the ligation junctions. DNA sequencing analysis was carried out by the Sanger chain termination procedure (20) with α-^{32}P-labelled deoxyribonucleoside triphosphates and T7 DNA polymerase on a double-stranded template, using oligodeoxyribonucleotide primers hybridizing within the κ cassette.

For excision of the internal portion of the κ cassette, either *Pac*I or *Pst*I was used followed by generation of blunt ends with T4 DNA polymerase, circularization, and transformation into *E. coli* DH5α.

Transformation of *H. halobium* SD109 and phenotypic analysis. *H. halobium* SD109, a class III (deletion) Vac$^-$ mutant of the NRC-1 wild-type strain (16), was transformed by the EDTA-polyethylene glycol procedure of Cline and Doolittle (2). Transformants were selected by plating on agar plates containing 40 μM mevinolin (15) (generously provided by Merck, Sharp, and Dohme Research Laboratories, Rahway, N.J.). *H. halobium* colonies were visually scored for Vac$^+$, Vac^{s-}, or Vac$^-$ phenotype and photographed in sunlight with a medium blue background paper, using a Nikon FE camera, a Micro-Nikkor lens with a lens focal length of 55 mm, and Kodakcolor 400 film.

H. halobium transformants were also examined by electron microscopy. For sectioned samples, cells were fixed in 3% glutaraldehyde–20% NaCl postfixed in 2% OsO_4–20% NaCl for 4 h, rinsed with 20% NaCl, stained en bloc with 5% uranyl acetate in 20% NaCl–20% acetone for 1 h, and then dehydrated by immersion in a series of isotonic acetone solutions. Samples were then embedded in Spurr medium which was polymerized at 70°C for 8 h (22). Thin sections of 600 Å (60 nm) were examined on copper grids stained with lead (21). For purified gas vesicles, samples were stained with 1% uranyl acetate. Samples were examined in a Philips CM10 transmission electron microscope at 80 kV.

RESULTS

Linker scanning mutagenesis of the *gvp* gene region. First, we constructed pFL2, a 24.5-kb plasmid by inserting a 13-kb fragment generated by partial *Eco*RI digestion and containing the entire *gvp* gene cluster into pNG11Δ12, an 11.5-kb *E. coli-H. halobium* shuttle plasmid. Transformation of *H. halobium* Vac$^-$ mutants containing large deletions of the gas vesicle gene region, e.g., SD109, with pFL2 resulted in Vac$^+$ (opaque) colonies (see Fig. 2, panel A1). This result indicated that the *gvp* gene cluster on pFL2 complements the deletion mutation, as previously reported for a similar plasmid, pJHGV3 (10). Plasmid pNG168, which confers resistance to mevinolin (Mev^r) but does not contain the *gvp* gene cluster, was used as a negative control (panel B1) and yielded Vac$^-$ (translucent) colonies (17).

For mutagenesis, plasmid pFL2 was linearized by partial digestion with *Taq*I, the ends were converted to blunt ends with T4 DNA polymerase, and the fragment population was ligated to a *Dra*I fragment (κ cassette) containing a kanamycin resistance gene. *E. coli* transformants were selected by double selection on plates containing ampicillin and kanamycin. Plasmids were prepared from *E. coli* and characterized by restriction mapping with *Hin*dIII, which cleaves once near the middle of the κ cassette and three times in pFL2. pFL2 derivatives with insertions of κ in seven genes, *gvpL*::κ*6*, *gvpJ*::κ*1*, *gvpG*::κ*1*, *gvpE*::κ*1*, *gvpD*::κ*1*, *gvpC*::κ*1*, and *gvpN*::κ*8*, were isolated by this method (Fig. 1 and Table 1).

To obtain κ insertion mutations in the other *gvp* genes, restriction enzymes known to cleave preferentially within specific genes of interest were used in place of *Taq*I to linearize pFL2 , the ends were converted to blunt ends, and then the fragments were ligated to the κ fragment. *Fsp*I was used to generate mutations in *gvpM* (pFL2*gvpM*::κ*1*) and *gvpF* (pFL2*gvpF*::κ*5*), SstI was used to generate a mutation in *gvpK* (pFL2*gvpK*::κ*1*), and *Sca*I was used to generate mutations in *gvpI* (pFL2*gvpI*::κ*1*) and *gvpC* (pFL2*gvpC*::κ*3*). Using the above approach, we obtained at least one insertion in every *gvp* gene (except *gvpA* [Fig. 1 and Table 1]). The precise sites of insertion of κ were determined by DNA sequencing analysis across the ligation junctions.

Colony phenotypes of *H. halobium* strains harboring *gvp* gene mutations. *H. halobium* SD109 was transformed with pFL2 derivatives containing κ insertions in each of the *gvp* genes (except *gvpA*), pFL2*gvpM*::κ*1*, pFL2*gvpL*::κ*6*, pFL2 *gvpK*::κ*1*, pFL2*gvpJ*::κ*1*, pFL2*gvpI*::κ*1*, pFL2*gvpH*::κ*1*, pFL2 *gvpG*::κ*1*, pFL2*gvpF*::κ*5*, pFL2*gvpE*::κ*1*, pFL2*gvpD*::κ*1*, pFL2 *gvpC*::κ*3*, pFL2*gvpC*::κ*1*, and pFL2*gvpN*::κ*8*. Transformants were selected on agar plates containing mevinolin. The results in Fig. 2 show that only plasmids pFL2 (panel A1) and pFL2*gvpM*::κ*1* (panel C1) conferred the Vac$^+$ phenotype to transformed cells. Plasmids pFL2*gvpL*::κ*6* (panel A2), pFL2 *gvpK*::κ*1* (panel B2), pFL2*gvpJ*::κ*1* (panel C2), pFL2*gvpI*::κ*1* (panel A3), pFL2*gvpH*::κ*1* (panel B3), pFL2*gvpG*::κ*1* (panel C3), pFL2*gvpF*::κ*5* (panel A4), pFL2*gvpC*::κ*3* (panel A5), κ*1* (panel B5), and pFL2*gvpN*::κ*8* (panel C5) conferred an apparently Vac$^-$ gas vesicle-deficient phenotype (although several of the transformants did produce very low levels of gas vesicles [see below]). Plasmids pFL2*gvpD*::κ*1* (panel C4) and pFL2

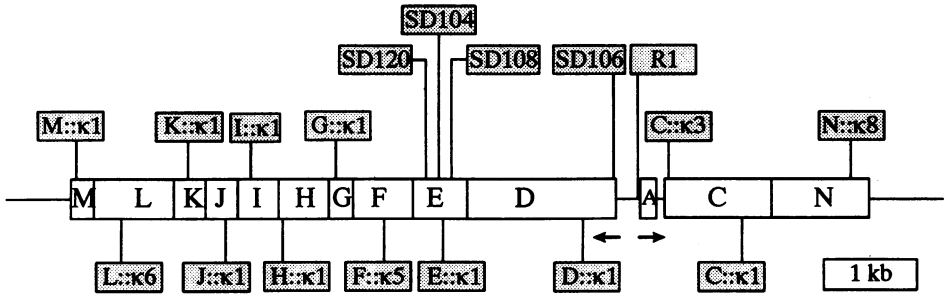

FIG. 1. Mutations in the *gvp* gene cluster. The relative locations and approximate sizes of the 13 *gvp* genes, labelled A to M, are shown by unshaded boxes. The rightward transcriptional orientation of *gvpA*, *-C*, and *-N* and the leftward transcriptional orientation of *gvpD*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, *-L*, and *-M* are indicated by the two arrows. The sites of IS element insertions in natural Vac$^-$ mutants, SD120, SD104, SD108, SD106, and R1 (12), are shown above the *gvp* genes, and the sites of κ cassette insertions, labelled *n*::κ*n*, are shown both above and below the *gvp* genes in shaded boxes. The shaded boxes do not indicate insertion sizes.

gvpE::κ*1* (panel B4) conferred a partial Vac$^{\delta-}$ phenotype, similar to those of natural mutants with insertions of IS elements in these genes (6).

Electron microscopic analysis of wild-type and *gvp* mutant strains and purified gas vesicles. Thin sections of cells of *H. halobium* SD109 transformed with pFL2 and mutated pFL2 derivatives were examined by electron microscopy (Fig. 3). Large numbers of gas vesicles were observed in transformants containing pFL2 (Fig. 3A) and pFL2*gvpM*::κ*1* (Fig. 3C). In contrast, very few vesicles were observed in transformants containing pFL2*gvpH*::κ*1* (Fig. 3H), pFL2*gvpG*::κ*1* (Fig. 3I), pFL2*gvpE*::κ*1* (Fig. 3K), and pFL2*gvpD*::κ*1* (Fig. 3L), and no gas vesicles were observed in transformants containing pNG 168 (Fig. 3B) (negative control), pFL2*gvpL*::κ*6* (Fig. 3D), pFL2*gvpK*::κ*1* (Fig. 3E), pFL2*gvpJ*::κ*1* (Fig. 3F), pFL2*gvpI*::κ*1* (Fig. 3G), and pFL2*gvpF*::κ*5* (Fig. 3J) or in the previously isolated *gvpA*::ISH*3* mutant strain R1 (Fig. 3M) (12).

Interestingly, electron microscopic examination of transfor-

TABLE 1. *H. halobium* strains used in this study

Strain	Phenotype	Relevant characteristic(s)
NRC-1	Vac$^+$	Wild-type strain
SD109	Vac$^-$	NRC-1 mutant with a 67-kb deletion of pNRC100, including the *gvp* gene cluster (16)
SD109(pNG168)	Vac$^-$ Mev^r	pNG168 is a 8.9-kb plasmid derived from pNG11Δ12 (17) which contains the *Haloferax volcanii* Mev^r gene
SD109(pFL2)	Vac$^+$ Mev^r	pFL2 is a 24.5-kb plasmid derived from pNG11Δ12 (17) which contains the entire *gvp* gene cluster
SD109(pFL2*gvpM*::κ*1*)	Vac$^+$ Mev^r	Contains κ cassette inserted 194 bp from the 5′ end of *gvpM* in orientation A
SD109(pFL2*gvpL*::κ*6*)	Vac$^-$ Mev^r	Contains κ cassette inserted 538 bp from the 5′ end of *gvpL* in orientation A
SD109(pFL2*gvpK*::κ*1*)	Vac$^-$ Mev^r	Contains κ cassette inserted 184 bp from the 5′ end of *gvpK* in orientation B
SD109(pFL2*gvpJ*::κ*1*)	Vac$^-$ Mev^r	Contains κ cassette inserted 130 bp from the 5′ end of *gvpJ* in orientation A
SD109(pFL2*gvpI*::κ*1*)	Vac$^-$ Mev^r	Contains κ cassette inserted 295 bp from the 5′ end of *gvpI* in orientation A
SD109(pFL2*gvpH*::κ*1*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 511 bp from the 5′ end of *gvpH* in orientation A
SD109(pFL2*gvpG*::κ*1*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 184 bp from the 5′ end of *gvpG* in orientation B
SD109(pFL2*gvpF*::κ*5*)	Vac$^-$ Mev^r	Contains κ cassette inserted 304 bp from the 5′ end of *gvpK* in orientation B
SD109(pFL2*gvpE*::κ*1*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 253 bp from the 5′ end of *gvpE* in orientation A
SD109(pFL2*gvpD*::κ*1*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 352 bp from the 5′ end of *gvpD* in orientation A
R1	Vac$^-$	NRC-1 mutant with an insertion of IS*H3* in *gvpA* (5, 12)
SD109(pFL2*gvpC*::κ*3*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 33 bp from the 5′ end of *gvpC* in orientation B; contains small gas vesicles
SD109(pFL2*gvpC*::κ*1*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 824 bp from the 5′ end of *gvpC* in orientation B; contains small gas vesicles
SD109(pFL2*gvpN*::κ*8*)	Vac$^{\delta-}$ Mev^r	Contains κ cassette inserted 848 bp from the 5′ end of *gvpC* in orientation B; contains small gas vesicles
SD109(pFL2*gvpM*::κ*1*Δ)	Vac$^+$ Mev^r	Derivative of SD109(pFL2*gvpM*::κ*1*) with 48-bp insert
SD109(pFL2*gvpL*::κ*6*Δ)	Vac$^-$ Mev^r	Derivative of SD109(pFL2*gvpL*::κ*6*) with 48-bp insert
SD109(pFL2*gvpK*::κ*1*Δ)	Vac$^-$ Mev^r	Derivative of SD109(pFL2*gvpK*::κ*1*) with 15-bp insert
SD109(pFL2*gvpJ*::κ*1*Δ)	Vac$^-$ Mev^r	Derivative of SD109(pFL2*gvpJ*::κ*1*) with 18-bp insert
SD109(pFL2*gvpI*::κ*1*Δ)	Vac$^-$ Mev^r	Derivative of SD109(pFL2*gvpI*::κ*1*) with 48-bp insert
SD109(pFL2*gvpH*::κ*1*Δ)	Vac$^+$ Mev^r	Derivative of SD109(pFL2*gvpH*::κ*1*) with 54-bp insert
SD109(pFL2*gvpG*::κ*1*Δ)	Vac$^{\delta-}$ Mev^r	Derivative of SD109(pFL2*gvpG*::κ*1*) with 51-bp insert
SD109(pFL2*gvpF*::κ*5*Δ)	Vac$^-$ Mev^r	Derivative of SD109(pFL2*gvpF*::κ*5*) with 48-bp insert
SD109(pFL2*gvpE*::κ*1*Δ)	Vac$^{\delta-}$ Mev^r	Derivative of SD109(pFL2*gvpE*::κ*1*) with 51-bp insert
SD109(pFL2*gvpD*::κ*1*Δ)	Vac$^+$ Mev^r	Derivative of SD109(pFL2*gvpD*::κ*1*) with 15-bp insert
SD109(pFL2*gvpC*::κ*3*Δ)	Vac$^{\delta-}$ Mev^r	Derivative of SD109(pFL2*gvpC*::κ*3*) with 48-bp insert
SD109(pFL2*gvpN*::κ*8*Δ)	Vac$^{\delta-}$ Mev^r	Derivative of SD109(pFL2*gvpN*::κ*8*) with 48-bp insert

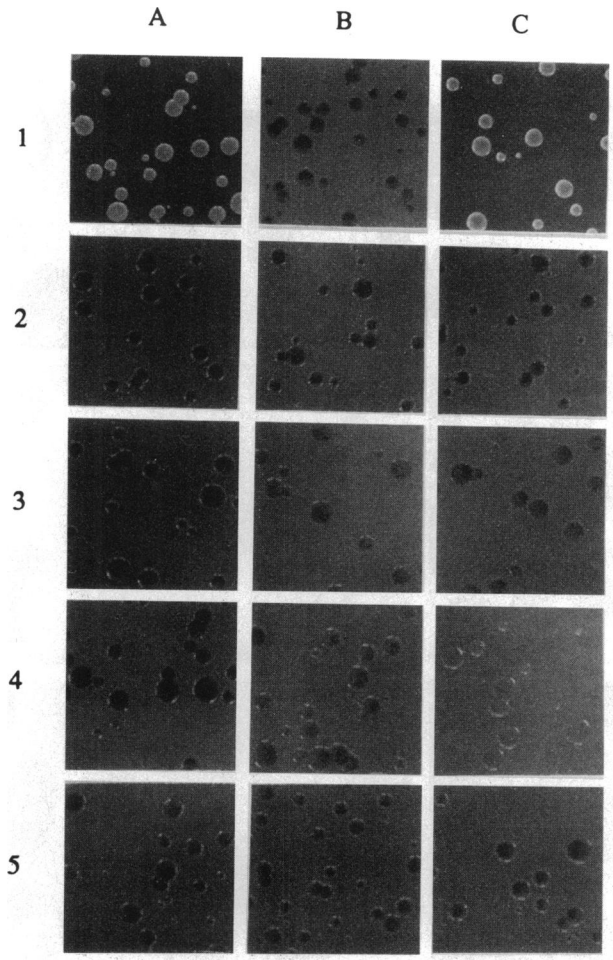

FIG. 2. Colony phenotypes of *H. halobium* SD109 derivatives containing mutated *gvp* genes. Colonies of the following strains are shown: SD109(pFL2) (A1), SD109(pNG168) (B1), SD109(pFL2*gvpM*::κ*1*) (C1), SD109(pFL2*gvpL*::κ*6*) (A2), SD109(pFL2*gvpK*::κ*1*) (B2), SD109 (pFL2*gvpJ*::κ*1*) (C2), SD109(pFL2*gvpI*::κ*1*) (A3), SD109(pFL2*gvpH*:: κ*1*) (B3), SD109(pFL2*gvpG*::κ*1*) (C3), SD109(pFL2*gvpF*::κ*5*) (A4), SD109(pFL2*gvpE*::κ*1*) (B4), SD109(pFL2*gvpD*::κ*1*) (C4), SD109 (pFL2*gvpC*::κ*3*) (A5), SD109(pFL2*gvpC*::κ*1*) (B5), and SD109(pFL2 *gvpN*::κ*8*) (C5).

mants containing pFL2*gvpC*::κ*3* (Fig. 3N) and pFL2*gvpN*::κ*8* (Fig. 3O) revealed large numbers of gas vesicles smaller than those of the wild-type. These gas vesicles were purified by centrifugally accelerated flotation and examined after negative staining by electron microscopy (Fig. 4). The sizes of gas vesicles from SD109(pFL2), which had an average length of 291 ± 65 nm and an average width of 211 ± 42 nm (± standard deviation for 53 vesicles), were substantially larger than for vesicles from SD109(pFL2*gvpC*::κ*3*) (average length of 118 ± 21 nm and average width of 86 ± 12 nm for 54 vesicles) and from SD109(pFL2*gvpN*::κ*1*) (average length of 80 ± 13 nm and average width of 61 ± 8 nm for 55 vesicles), indicating that *gvpC* and *gvpN* are necessary for the formation of gas vesicles of wild-type size.

Construction and phenotypic analysis of κ deletion mutants. In order to assay for possible polar effects of κ insertions on downstream genes, we constructed derivatives of each pFL2*gvp*::κ plasmid by deletion of the internal portion of κ. (For this purpose, κ had been constructed with pairs of rare restriction enzyme sites near its termini [9].) The deletion derivatives retained 5 to 18 codons (15 to 54 bp) from the termini of κ (Table 1). Transformation of *H. halobium* SD109 with these pFL2*gvp*::κΔ plasmids produced results analogous to the plasmids containing the original κ insertions in *gvpM*, *-L*, *-K*, *-J*, *-I*, *-G*, *-F*, *-E*, *-C*, and *-N* (data not shown). The only exceptions were for pFL2*gvpH*::κ*1*Δ and pFL2*gvpD*::κ*1*Δ which produced Vac^+ transformants in contrast to the Vac^{s-} transformants produced by the parent plasmids (data not shown). The phenotypic differences in these latter cases came about either because the mutant gene products were functional in gas vesicle synthesis or because there were no direct roles for these genes in gas vesicle formation.

DISCUSSION

We have mutagenized the *gvp* gene cluster of *H. halobium* plasmid pNRC100 and found that nearly all of the *gvp* genes are necessary for normal gas vesicle formation. First, linker scanning mutagenesis was carried out with a kanamycin resistance cassette to construct a set of κ insertion mutants. Next, most of the κ cassette was deleted by using rare restriction sites, which had been incorporated near the ends of the κ cassette, to construct a set of κ deletion mutants with small insertions. Comparison of the phenotypes of the κ mutants to those of the κ deletion mutants allowed us to distinguish between insertional inactivation of interrupted genes and polar effects on downstream genes. The strategy also permitted genetic analysis of *gvp* gene function in an isogenic background. The results of this genetic analysis clearly showed that at least 10 of the 13 genes, including *gvpL*, *-K*, *-J*, *-I*, *-G*, *-F*, *-E*, *-A*, *-C*, and *-N*, are necessary for normal gas vesicle formation. The involvement of the other three genes, *gvpM*, *-H*, and *-D* in gas vesicle formation was neither supported nor ruled out by the data. Moreover, colony phenotypes and electron microscopic analysis of mutants suggested that the genes with the rightward transcriptional orientation, *gvpA*, *-C*, and *-N*, encode proteins necessary for gas vesicle structure and/or growth, while most genes with the leftward transcriptional orientation, including *gvpF*, *-G*, *-I*, *-J*, *-K*, and *-L*, are most likely involved in early steps in assembly of gas vesicles.

Recent work on the genes with the rightward orientation, *gvpA*, *-C*, and *-N*, had shown that at least two genes, *gvpA* and *gvpC*, encode structural gas vesicle proteins (9). GvpA and GvpC were found in vesicles by immunoblotting analysis, and polycistronic transcripts encoding GvpA, GvpC, and GvpN were mapped. Additionally, an insertion of an IS*H3* element in the *gvpACN* promoter in strain R1 was found to produce a completely Vac^- phenotype (Fig. 3M) (6). Now we have shown that mutations of *gvpC* and *gvpN* reduce the size of gas vesicles (Fig. 4). This finding indicates that the GvpC and GvpN proteins are necessary for the late stages of growth of gas vesicles to the wild-type size.

In cyanobacteria, a GvpC protein similar to the halobacterial GvpC protein has also been detected in gas vesicles (3, 24). Treatment of cyanobacterial vesicles with denaturing agents such as SDS resulted in the release of GvpC without collapse of vesicles. The resulting vesicles were more easily collapsed by hydrostatic pressure, and they were strengthened by readdition of GvpC. These results suggested that GvpC is present on the outer surface of the vesicle and functions as "molecular glue" to strengthen the vesicle membrane. If GvpC has a similar function in *H. halobium*, the protein is likely to be bifunctional, acting in both gas vesicle assembly and stability.

Beyond the 3′ end of the *gvpACN* operon, a 14th gene, *gvpO*, has been found in a related strain of *H. halobium* and has been

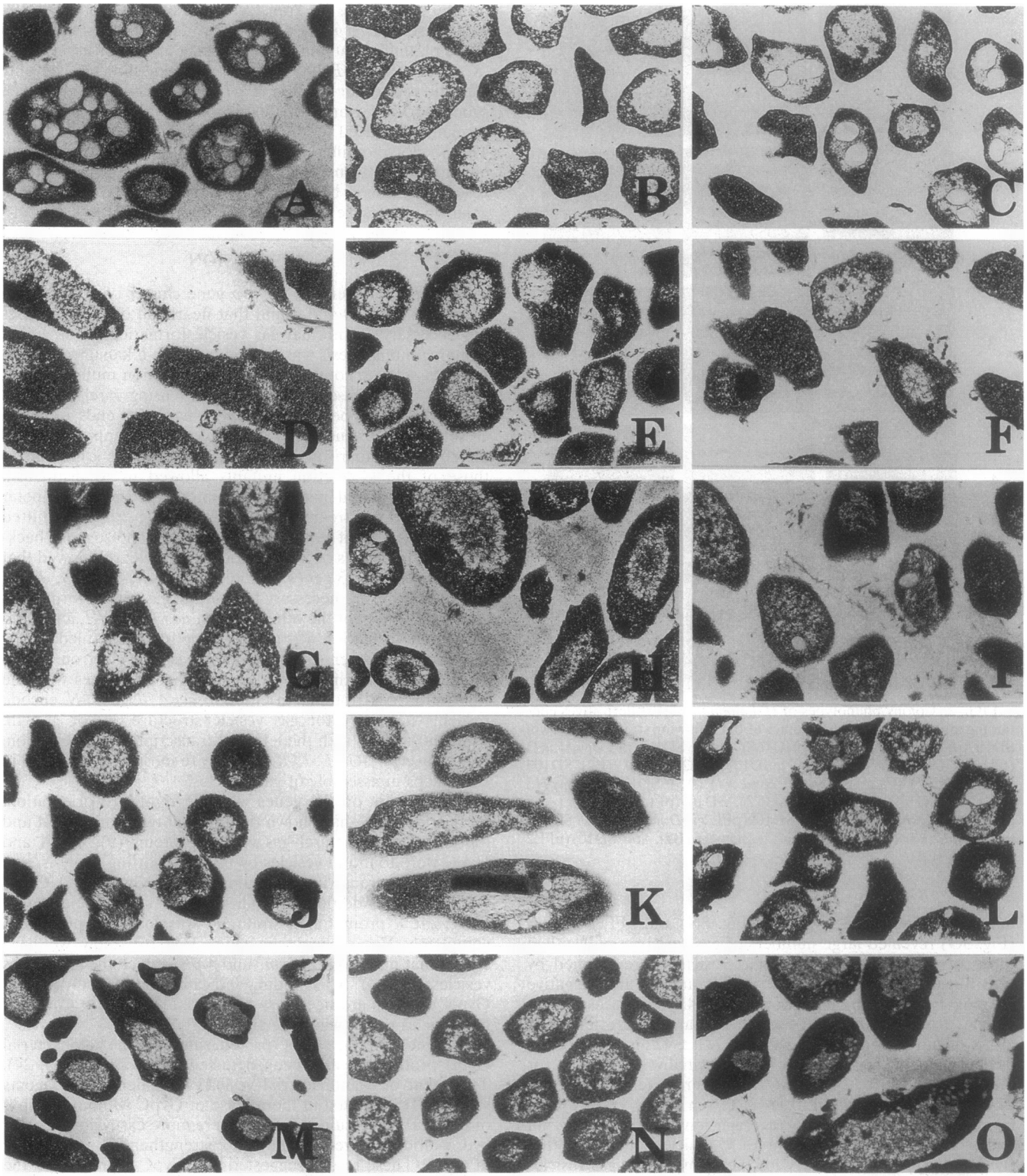

FIG. 3. Thin sections of *H. halobium* SD109 derivatives containing mutated *gvp* genes. The following strains are shown: SD109(pFL2) (A), SD109(pNG168) (B), SD109(pFL2*gvpM*::κ*1*) (C), SD109(pFL2*gvpL*::κ*6*) (D), SD109(pFL2*gvpK*::κ*1*) (E), SD109(pFL2*gvpJ*::κ*1*) (F), SD109(pFL2*gvpI*::κ*1*) (G), SD109(pFL2*gvpH*::κ*1*) (H), SD109(pFL2*gvpG*::κ*1*) (I), SD109(pFL2*gvpF*::κ*5*) (J), SD109(pFL2*gvpE*::κ*1*) (K), SD109(pFL2*gvpD*::κ*1*) (L), R1(*gvpA*::IS*H3*) (M), SD109(pFL2*gvpC*::κ*3*) (N), and SD109(pFL2*gvpN*::κ*8*) (O). Magnification, ×9,800.

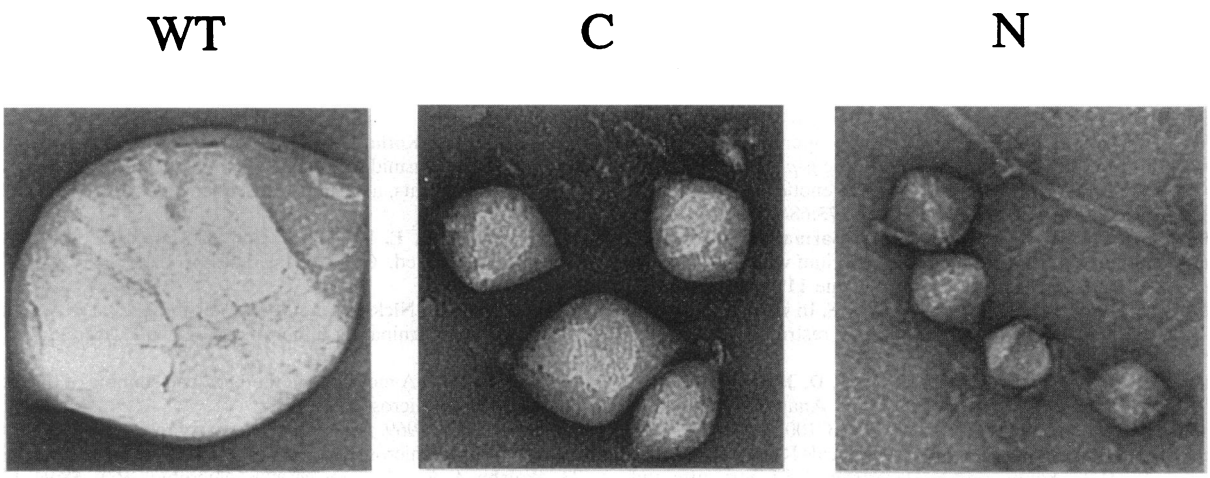

FIG. 4. Relative sizes of gas vesicles from *gvpC* and *gvpN* mutants. Gas vesicles purified from NRC-1 (wild type [WT]), SD109(pFL2*gvpC*::κ*1*) (C), and SD109(pFL2*gvpN*::κ*8*) (N) stained with uranyl acetate are shown.

suggested to play a role in gas vesicle formation (8). However, in previous analysis, *gvpO* was not found to be cotranscribed with the *gvpACN* operon, and a κ insertion between *gvpN* and *gvpO* resulted in a Vac^+ phenotype (9). Moreover, six additional linker scanning mutations located 2 to 4 kb downstream of the *gvpN* gene also produced Vac^+ transformants (data not shown). Thus, it is likely that *gvpN* is the last gene at the right end of the gene cluster necessary for gas vesicle synthesis.

The results of mutagenesis suggest that many if not most of the genes with the leftward transcriptional orientation, which include *gvpD*, *-E*, *-F*, *-G*, *-H*, *-I*, *-J*, *-K*, *-L*, and *-M*, are involved in the initiation of gas vesicle formation. Consistent with such a role, no vesicles were detectable in mutants containing insertions in *gvpF*, *-I*, *-J*, *-K*, and *-L*, while two other mutants, *gvpG* and *gvpH*, produced very low levels (less than 1% of parental levels). The products of these seven genes may be necessary for the formation of nucleation sites around which the major gas vesicle proteins assemble to form the membrane. A direct role in promoting assembly of the membrane is also possible for the gene products.

Two genes, *gvpD* and *gvpE*, are at least partially dispensable for gas vesicle formation, since both κ mutants and IS*H* element mutants (5, 12) produced low levels of gas vesicles. Moreover, for *gvpD* mutants, removal of the internal portion of the κ cassette resulted in reversion of the partial Vac^{s-} phenotype. These findings together with the observation of an "overproducer" phenotype for a mutant with an internal *gvpD* deletion (8) suggested that *gvpD* may be involved in regulation of gas vesicle synthesis. If so, the *gvpD* gene product may act as both a repressor for leftward transcription and an activator for rightward transcription. A site at which GvpD could bind and mediate both effects, switching transcription from the left to the right, has been identified in the *gvpA* to *-D* intergenic region (25; unpublished work). Further mutagenic and transcriptional analysis is necessary to substantiate the proposed role of *gvpD* in transcriptional regulation.

Two of the 13 *gvp* genes, *gvpM* and *gvpH*, may be dispensable for gas vesicle formation. For *gvpH*, the κ mutant was Vac^- but the κ deletion mutant was Vac^+, while for *gvpM*, both the κ and κ deletion mutants were Vac^+. These phenotypes could result from the lack of requirement for these genes in gas vesicle formation. Alternatively, the mutated *gvpN* and *gvpM* genes giving rise to Vac^+ transformants could result from gene products functional for gas vesicle synthesis, especially since the mutations are near the 3′ ends of the genes. It is also possible that *gvpA* or *gvpJ* complements mutations in *gvpM*.

Analysis of mutations of *gvp* genes in an isogenic *H. halobium* background has shown that at least 10 genes in the *gvp* gene cluster are necessary for gas vesicle synthesis in *H. halobium*. The data collected thus far is consistent with the involvement of up to 13 or 14 genes in this process, many more than the 1 or 2 genes originally hypothesized. The genes with rightward transcriptional orientation encode the structural gas vesicle proteins, while those with leftward orientation most likely encode proteins necessary for initiation and regulation. Further genetic and structural analyses will yield greater insights into this unusual biosynthetic process.

ACKNOWLEDGMENTS

This work was supported by grant MCB-9221144 from the National Science Foundation and by Public Health Service grant GM41980 from the National Institutes of Health.

We thank Chris Woodcock for invaluable advice on electron microscopy.

REFERENCES

1. **Chu, G., D. Vollrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234**:1582–1585.
2. **Cline, S. W., and W. F. Doolittle.** 1987. Efficient transfection of the archaebacterium *Halobacterium halobium*. J. Bacteriol. **169**:1341–1344.
3. **Damerval, T., J. Houmard, G. Guglielmi, K. Csiszàr, and N. Tandeau de Marsac.** 1987. A developmentally regulated *gvpABC* operon is involved in the formation of gas vesicles in the cyanobacterium *Calothrix* 7601. Gene **54**:83–92.
4. **DasSarma, S., and P. Arora.** 1993. Gas vesicle proteins and genes. Mol. Biol. (Life Sci. Adv.) **12**:93–98.
5. **DasSarma, S., T. Damerval, J. G. Jones, and N. Tandeau de Marsac.** 1987. A plasmid-encoded gas vesicle protein gene in a halophilic archaebacterium. Mol. Microbiol. **1**:365–370.
6. **DasSarma, S., J. T. Halladay, J. G. Jones, J. W. Donovan, P. J. Giannasca, and N. Tandeau de Marsac.** 1988. High-frequency mutations in a plasmid-encoded gas vesicle gene in *Halobacterium halobium*. Proc. Natl. Acad. Sci. USA **85**:6861–6865.
7. **Englert, C., K. Krüger, S. Offner, and F. Pfeifer.** 1992. Three different but related gene clusters encoding gas vesicles in halophilic archaea. J. Mol. Biol. **227**:586–592.

8. **Englert, C., and F. Pfeifer.** 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.
9. **Halladay, J. T., J. G. Jones, F. Lin, A. B. MacDonald, and S. DasSarma.** 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.
10. **Halladay, J. T., W.-L. Ng, and S. DasSarma.** 1992. Genetic transformation of a halophilic archaebacterium with a gas vesicle gene cluster restores its ability to float. Gene **119:**131–136.
11. **Heffron, F., M. So, and B. J. McCarthy.** 1978. In vitro mutagenesis of a circular DNA molecule using synthetic restriction sites. Proc. Natl. Acad. Sci. USA **75:**6012–6016.
12. **Jones, J. G., N. R. Hackett, J. T. Halladay, D. J. Scothorn, C.-F. Yang, W.-L. Ng, and S. DasSarma.** 1989. Analysis of insertion mutants reveals two new genes in the pNRC100 gas vesicle gene cluster of *Halobacterium halobium*. Nucleic Acids Res. **17:**7785–7793.
13. **Jones, J. G., D. C. Young, and S. DasSarma.** 1991. Structure and organization of the gas vesicle gene cluster on the *Halobacterium halobium* plasmid pNRC100. Gene **102:**117–122.
14. **Krantz, M. J., and C. E. Ballou.** 1973. Analysis of *Halobacterium halobium* gas vesicles. J. Bacteriol. **114:**1058–1067.
15. **Lam, W. L., and W. F. Doolittle.** 1989. Shuttle vectors for the archaebacterium *Haloferax volcanii*. Proc. Natl. Acad. Sci. USA **86:**5478–5482.
16. **Ng, W.-L., P. Arora, and S. DasSarma.** 1994. Large deletions in class III gas vesicle-deficient mutants of *Halobacterium halobium*. Syst. Appl. Microbiol. **16:**560–568.
17. **Ng, W.-L., and S. DasSarma.** 1993. Minimal replication origin of *Halobacterium* plasmid pNRC100. J. Bacteriol. **175:**4583–4596.
18. **Ng, W.-L., S. Kothakota, and S. DasSarma.** 1991. Structure of the gas vesicle plasmid in *Halobacterium halobium*: inversion isomers, inverted repeats, and insertion sequences. J. Bacteriol. **173:**1958–1964.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Sato, T.** 1967. A modified method for lead staining of thin sections. J. Electron Microsc. **16:**133.
22. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31–43.
23. **Walsby, A. E.** 1994. Gas vesicles. Microbiol. Rev. **58:**94–144.
24. **Walsby, A. E., and P. K. Hayes.** 1988. The minor cyanobacterial gas vesicle protein, GVPc, is attached to the outer surface of the gas vesicle. J. Gen. Microbiol. **134:**2647–2657.
25. **Yang, C.-F., and S. DasSarma.** 1990. Transcriptional induction of purple membrane and gas vesicle synthesis in the archaebacterium *Halobacterium halobium* is blocked by a DNA gyrase inhibitor. J. Bacteriol. **172:**4118–4121.