Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6861–6865, September 1988
Genetics

High-frequency mutations in a plasmid-encoded gas vesicle gene in *Halobacterium halobium*

(*gvp* gene/transposable elements/insertion sequence from *Halobacterium*/bacterio-opsin gene/plasmid instability)

SHILADITYA DASSARMA*, JOHN T. HALLADAY*, JEFFREY G. JONES*, JOHN W. DONOVAN*, PAUL J. GIANNASCA*, AND NICOLE TANDEAU DE MARSAC†

*Department of Microbiology, University of Massachusetts, Amherst, MA 01003; and †Unité de Physiologie Microbienne et Centre National de la Recherche Scientifique Unité Associée 1129, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

Communicated by Walther Stoeckenius, June 10, 1988 (received for review January 29, 1988)

ABSTRACT **Gas vesicle-deficient mutants of *Halobacterium halobium* arise spontaneously at high frequency (about 1%). The mutants are readily detected, forming translucent colonies on agar plates in contrast to opaque wild-type colonies. To investigate the mechanism of this mutation, we recently cloned a plasmid-encoded gas vesicle protein gene, *gvpA*, from *H. halobium*. In the wild-type NRC-1 strain the *gvpA* gene is encoded by a multicopy plasmid of ≈150 kilobase pairs (kb). We have now characterized 18 gas vesicle-deficient mutants and 4 revertants by phenotypic and Southern hybridization analyses. Our results indicate that the mutants fall into three major classes. Class I mutants are partially gas vesicle-deficient ($Vac^{\delta-}$) and unstable, giving rise to completely gas vesicle-deficient (Vac^-) derivatives and Vac^+ revertants at frequencies of 1–5%. The restriction map of the *gvpA* gene region in class I mutants is unchanged but the gene copy number is reduced compared to the Vac^+ strains. Class II mutants can be either $Vac^{\delta-}$ or completely Vac^- but are relatively stable. They contain insertion sequences within or upstream of the *gvpA* gene. A Vac^- class II mutant, R1, contains the 1.3-kb insertion sequence, ISH3, within the *gvpA* gene, whereas four $Vac^{\delta-}$ class II mutants contain other insertion sequences upstream of the gene. Class III mutants are stable Vac^- derivatives of either the wild-type or class I mutants and have no detectable copies of the *gvpA* gene. Based on these results, we discuss the mechanisms of gas vesicle mutations in *H. halobium*.**

Extremely halophilic archaeobacteria such as *Halobacterium halobium* flourish in hypersaline brine containing 3–5 M NaCl (1, 2). Like other aquatic bacteria, many *Halobacterium* strains synthesize gas-filled vesicles, which give buoyancy and, thus, increase the availability of light and oxygen to cells (3, 4). Gas-filled vesicles diffract light, giving an opaque appearance to wild-type (Vac^+) *H. halobium* colonies on agar plates. Gas vesicle-deficient mutants (Vac^- and $Vac^{\delta-}$ mutants) of *H. halobium* arise spontaneously at frequencies of about 1% and are readily apparent as translucent colonies (5–7).

In addition to the gas vesicle phenotype, *H. halobium* also exhibits variability for several other easily detectable phenotypes, particularly for synthesis of pigments such as those in purple and red membranes (7). This variability has been ascribed to the unusual organization and plasticity of the *H. halobium* genome (8). The genome contains many repeated sequences including several well-characterized transposable elements (9–17). The transposable elements have been shown to be clustered in a physically separable, (A + T)-rich satellite component of the genome that is composed of several copies of large [≈150 kilobase pairs (kb)] covalently closed circular DNAs and several ≈70-kb segments of the chromosome (14, 18, 19). Frequent DNA rearrangements catalyzed by transposable elements are thought to result in the unusually high rate of phenotypic variation observed.

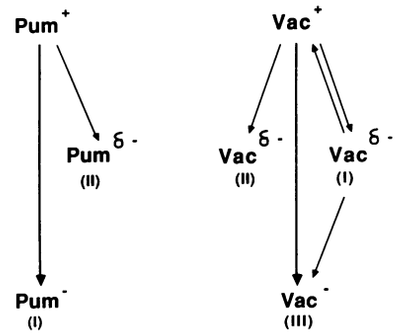

FIG. 1. Summary of phenotypic changes in *H. halobium*. Pum is the purple membrane phenotype and Vac is the gas vesicle phenotype. Superscripts are + for wild type, $\delta-$ for leaky or partial mutants, and − for mutants completely lacking gas vesicles or purple membrane. Roman numerals indicate class designations of the mutants. Arrows indicate mutations observed during our investigations (10, 11, 20).

The only detailed investigation of a specific phenotypic variability in *H. halobium* is for purple membrane deficiency (Pum^- mutations, Fig. 1). Following isolation of the gene for bacterio-opsin (*bop*) (21), the single protein in purple membrane (22), many Pum^- mutants were isolated and characterized (9–13). These studies showed that about 90% of the Pum^- mutants resulted from insertion of the *H. halobium* insertion sequence ISH*1* or ISH*2* into the *bop* structural gene, and the mutants exhibited a stable and completely bacterio-opsin-deficient phenotype. Most of the remaining mutants were caused by insertions 5′ to the *bop* gene, one of which was shown to be a leaky mutant ($Pum^{\delta-}$) with an insertion of ISH*2* 102 base pairs 5′ to the structural gene (11, 13).

To elucidate mechanisms of the gas vesicle mutations as well as to learn more about gene structure and regulation in an archaeobacterium, we initiated an investigation of gas vesicle genes in *H. halobium*. As a first step we cloned *gvpA*, the gene encoding the major structural protein in gas vesicles from *H. halobium* by using a heterologous probe and mapped the *gvpA*-encoded mRNA (Fig. 2) (20, 23). Southern hybridization analysis showed the *gvpA* gene to be encoded by a large *H. halobium* plasmid, suggesting plasmid instability as

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Abbreviations: *gvpA*, gas vesicle protein gene; *bop*, bacterio-opsin gene; Vac, gas vesicle phenotype; Pum, purple membrane phenotype; ISH, insertion sequence from *Halobacterium* species.

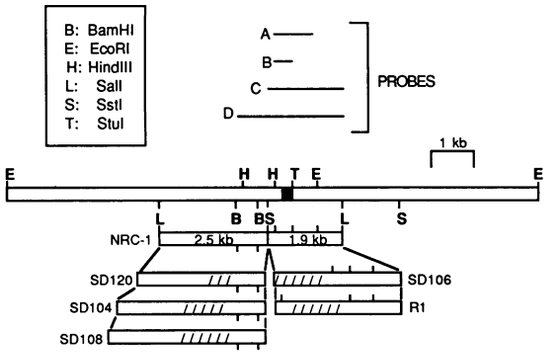

FIG. 2. Partial restriction map of the *gvpA* gene region in *H. halobium* wild-type strain NRC-1 (20) and class II mutants R1, SD104, SD106, SD108, and SD120. Restriction enzyme abbreviations, map scale, and probes used in this study are indicated above the restriction map. The coding region of *gvpA* is indicated by the black box. Approximate sites of insertions in class II mutants, R1, SD104, SD106, SD108, and SD120, into the wild-type 2.5- and 1.9-kb *Sal* I–*Sst* I restriction fragments are indicated below by the hatched regions.

a possible mechanism for the high rate of gas vesicle mutations (20). In this paper, we report on the isolation and characterization of 18 independent gas vesicle-deficient mutants with completely Vac$^-$ and partially Vac$^-$ (Vac$^{\delta-}$) phenotypes and 4 Vac$^+$ revertants. The results show the existence of three classes of gas vesicle mutants, all of which appear to have mutations within or near the *gvpA* gene.

MATERIALS AND METHODS

Materials. Enzymes were purchased from Bethesda Research Laboratories and Promega Biotec, Madison, WI. Radiolabeled nucleotides were purchased from Amersham. Nitrocellulose was purchased from Schleicher & Schuell.

Growth of *H. halobium* and Phenotypic Analysis of Mutants. *H. halobium* strains were grown in 1% (wt/vol) peptone (Oxoid, Basingstoke, England) medium containing 4.5 M NaCl, 0.1 M $MgSO_4$, 0.025 M KCl, and 0.01 M sodium citrate (pH 7.2) at 37°C with illumination. Colonies on 2.0% (wt/vol) agar plates were examined after 7–10 days of incubation and scored as Vac$^+$, Vac$^{\delta-}$, or Vac$^-$ based initially on the qualitative level of "opacity." The phenotype was corroborated by quantitative analysis of gas vesicle preparations for the following strains: NRC-1, SD102A, SD102B, SD102C, SD112A, SD112B, SD112C, SD117A, SD117B, SD117C, SD118A, SD118B, SD118C, SD104, SD106, SD108, SD120, SD109, SD110, SD111. Vac$^+$ revertants contained 107–116% the level of gas vesicles compared to NRC-1, whereas Vac$^-$ contained <1% and Vac$^{\delta-}$ contained 1–12%. Other mutants, with only slightly lower opacity than NRC-1, were also observed but were not included in our analysis. To determine the stability of mutant strains, bacteria from single colonies were plated on agar plates and visually screened for phenotypic variants. The strains used in this investigation are listed in Table 1.

Gas vesicles were prepared by floatation (overnight centrifugation at 600 × *g*) after lysing confluent cultures on agar plates by flooding with 10 mM Tris·HCl (pH 8.0) and 0.5 μg of DNase I per ml essentially as described (24). The relative level of gas vesicles in each strain was measured spectrophotometrically by the difference in A_{600} between inflated and collapsed vesicles in the partially purified preparations. Vesicles were collapsed by high-speed centrifugation (14,000 × *g* for 2 min), which results in complete loss of absorbance.

DNA Isolation and Southern Hybridization Analysis. Genomic DNA was isolated by a previously described procedure (10) and plasmid DNA was isolated by the procedure of Casse *et al.* (25). For blotting analysis, supercoiled plasmid DNA (0.5 μg per lane) was electrophoresed on 0.7% agarose gels and restriction enzyme-digested DNA (1.0 μg of *H. halobium* plasmid per lane and 2.5 μg of genomic DNA per lane) was electrophoresed on 1% agarose gels. Following denaturation and neutralization, DNA was transferred to nitrocellulose membranes by using the procedure of Southern (26). Prehybridization and hybridization were each carried out overnight at 68°C in a solution containing 0.45 M NaCl, 0.045 M sodium citrate, 0.2% (wt/vol) each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin, and 100 μg of denatured and sheared salmon sperm DNA per ml. The *gvpA* probes used (Fig. 2) included a 926-base-pair *Hin*dIII–*Eco*RI fragment (probe A), a 268-nucleotide-long transcript extending between the *Hin*dIII and *Stu* I sites (probe B), a 1.6-kb *Hin*dIII–*Sau*3AI fragment (probe C), or a 2.2-kb *Bam*HI–*Sau*3AI fragment (probe D). Probes A, C, and D were ^{32}P-labeled by nick-translation (27) and probe B was ^{32}P-labeled by using T7 RNA polymerase (28). After hybridization, filters were washed at 68°C in a solution containing 0.45 M NaCl and 0.045 M sodium citrate for a total of 1 hr with three changes followed by a second wash in 0.045 M NaCl and 0.0045 M sodium citrate and 0.1% (wt/vol) sodium dodecyl sulfate for 30 min before autoradiography.

Table 1. *H. halobium* strains

Strain designation	Parent strain	Phenotype	Mutant class*	Gas vesicle content,† %
NRC-1‡	Wild-type	Vac$^+$	W	100
SD102B	NRC-1	Vac$^{\delta-}$	I	12
SD102A	SD102B	Vac$^-$	III	<1
SD102C	SD102B	Vac$^+$	R	107
SD112B	NRC-1	Vac$^{\delta-}$	I	5.4
SD112A	SD112B	Vac$^-$	III	<1
SD112C	SD112B	Vac$^+$	R	107
SD117B	NRC-1	Vac$^{\delta-}$	I	1.2
SD117A	SD117B	Vac$^-$	III	<1
SD117C	SD117B	Vac$^+$	R	116
SD118B	NRC-1	Vac$^{\delta-}$	I	3.1
SD118A	SD118B	Vac$^-$	III	<1
SD118C	SD118B	Vac$^+$	R	110
SD104	NRC-1	Vac$^{\delta-}$	II	2.5
SD106	NRC-1	Vac$^{\delta-}$	II	1.0
SD108	NRC-1	Vac$^{\delta-}$	II	1.8
SD120	NRC-1	Vac$^{\delta-}$	II	8.2
R1§	NRC-1	Vac$^-$	II	—
SD109	NRC-1	Vac$^-$	III	<1
SD110	NRC-1	Vac$^-$	III	<1
SD111	NRC-1	Vac$^-$	III	<1
SD113	NRC-1	Vac$^-$	III	—
SD116	NRC-1	Vac$^-$	III	—

*W, wild-type; R, revertant.
†Expressed in % compared to NRC-1. The change in A_{600} for strain NRC-1 was 2.34. Gas vesicle content was not measured for strains R1, SD113, and SD116.
‡Provided by W. F. Doolittle, Dalhousie University, Halifax, NS, Canada.
§Provided by W. Stoeckenius, Univ. of California at San Francisco.

RESULTS

The *gvpA* Gene in *H. halobium* Strains NRC-1 and R1 Is Plasmid-Encoded. To determine if the 150-kb plasmid previously reported in *H. halobium* strain NRC-1 (8) encodes *gvpA*, supercoiled plasmid from NRC-1 was electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled *gvpA* gene probe (probe A). The probe hybridized to a plasmid, designated pNRC100, that is ≈150

kb in size (Fig. 3*A*). The preparation of pNRC100 cleaved with restriction endonucleases was also analyzed by Southern hybridization using probe A. The results in Fig. 3*B* indicate that pNRC100 contains the same *Hin*dIII (lane 3), *Hin*dIII–*Eco*RI (lane 4), and *Eco*RI (lane 5) fragments in NRC-1 genomic DNA that have been shown to contain *gvpA* (20).

In the Vac$^-$ mutant strain R1 isolated several years ago (8), the *gvpA* gene is also plasmid-encoded (Fig. 3*B*, lanes 6 and 7). The size of the restriction fragments containing the gene, however, is 1.3 kb larger than the corresponding fragments in NRC-1 (compare lanes 4 and 7 and lanes 5 and 6), consistent with the possibility of insertional inactivation of the *gvpA* gene as the mechanism of this Vac$^-$ mutation. To determine the generality of this observation, we isolated and characterized a number of independent Vac$^-$ mutants (see Table 1).

Three Classes of Gas Vesicle-Deficient Mutants. The phenotype of colonies of Vac$^-$ mutants of *H. halobium* NRC-1 differs, depending on the mutant, from very slightly translucent to nearly transparent. Based on visual criteria, several mutants and derivatives were classified as either partially gas vesicle-deficient (Vac$^{\delta-}$), completely gas vesicle-deficient (Vac$^-$), or essentially wild type (Vac$^+$). The validity of these assignments was substantiated by spectrophotometric measurement of purified gas vesicle preparations (see *Materials and Methods*). Compared to NRC-1, Vac$^+$ revertants contain 107–116% the level of gas vesicles, Vac$^{\delta-}$ mutants contain 1–12%, and Vac$^-$ mutants contain <1% (Table 1). Next, Vac$^{\delta-}$ and Vac$^-$ mutants and Vac$^+$ revertants were plated to determine the stability of the phenotype. The Vac$^{\delta-}$ mutants were divided into two groups: those that are extremely unstable, giving rise to Vac$^+$ and Vac$^-$ derivatives at 1–5% frequencies, designated class I, and those that are relatively stable (mutation rate, <1%), designated class II (Fig. 1). The completely Vac$^-$ mutants, designated class III, were isolated either directly from NRC-1 or from class I mutants (Table 1, Fig. 1). Class III mutants are stable (reversion rate, <0.1%), whereas the Vac$^+$ revertants of class I mutants display wild-type mutation rates. We isolated four class I mutants and one Vac$^-$ (class III) and one Vac$^+$ derivative of each class I mutant as well as four class II mutants and five additional class III mutants directly from NRC-1 and analyzed them together with Vac$^-$ strain R1 (designated class II below) by Southern hybridization.

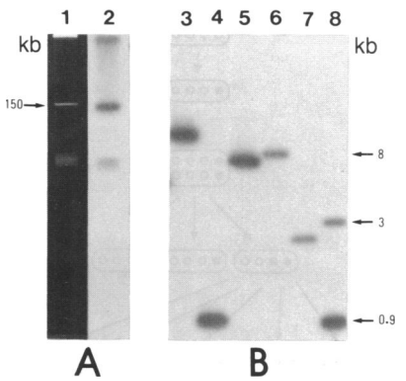

FIG. 3. Southern hybridization analysis of *H. halobium* strains NRC-1 and R1 using *gvpA* gene probe A. (*A*) Lane 1, ethidium bromide-stained lane of the supercoiled ≈150-kb plasmid pNRC100; lane 2, corresponding hybridization result. The intense intermediate band is the plasmid DNA and the fast-moving band is linear DNA. (*B*) Southern hybridization results of restriction enzyme-cleaved plasmid DNA. pNRC100: lane 3, *Hin*dIII; lane 4, *Hin*dIII + *Eco*RI; lane 5, *Eco*RI. Plasmids from strain R1: lane 6, *Eco*RI; lane 7, *Hin*dIII + *Eco*RI. Lane 8, *Eco*RI + *Hin*dIII-digested pGVH4, which contains the *gvpA* *Hin*dIII–*Eco*RI fragment cloned in pTZ18. The positions of size markers are indicated.

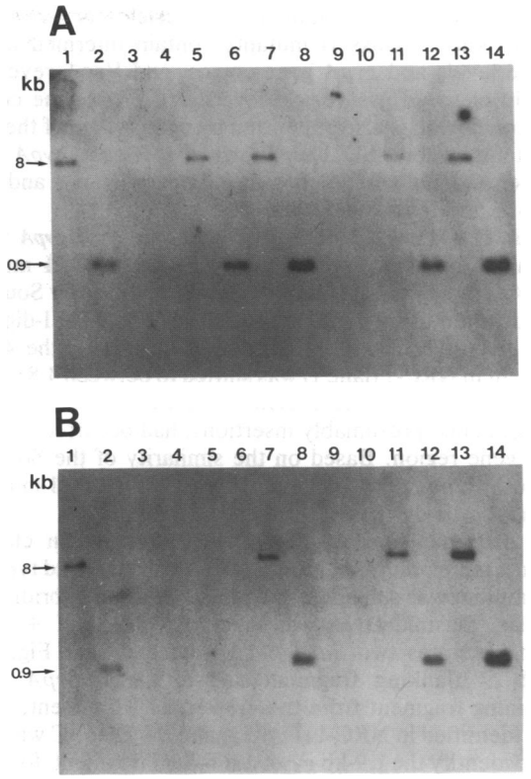

FIG. 4. Southern hybridization analysis of class I mutants and Vac$^-$ (class III) and Vac$^+$ derivatives using the 0.9-kb *Hin*dIII–*Eco*RI *gvpA* gene probe. (*A*) Analysis of mutant strains SD102A (lanes 3 and 4), SD102B (lanes 5 and 6), and SD102C (lanes 7 and 8) and SD112A (lanes 9 and 10), SD112B (lanes 11 and 12), and SD112C (lanes 13 and 14). (*B*) Analysis of mutant strains SD117A (lanes 3 and 4), SD117B (lanes 5 and 6), and SD117C (lanes 7 and 8) and SD118A (lanes 9 and 10), SD118B (lanes 11 and 12), and SD118C (lanes 13 and 14). Lanes 1 and 2 in *A* and *B* contain 8- and 0.9-kb size markers generated by digestion of DNA from a *H. halobium* NRC-1 derivative showing reduced gas vesicle content. Odd-numbered lanes contain *Eco*RI-digested DNA and even-numbered lanes contain *Hin*dIII + *Eco*RI-digested DNA.

Class I and Class III Mutants Have Reduced *gvpA* Gene Copy Number. Genomic DNA from class I mutants SD102B, SD112B, SD117B, and SD118B as well as one Vac$^-$ (class III) derivative and one Vac$^+$ revertant of each mutant (class III: SD102A, SD112A, SD117A, and SD118A; Vac$^+$ revertants: SD102C, SD112C, SD117C, and SD118C) was purified, digested with *Eco*RI and *Eco*RI + *Hin*dIII, and analyzed by Southern hybridization using a *gvpA* gene probe (probe A). In each of the class I mutants and the Vac$^+$ revertants, the wild-type 8-kb *Eco*RI and 0.9-kb *Hin*dIII–*Eco*RI fragments containing *gvpA* are present (in Fig. 4 *A* and *B*, compare lane 1 with lanes 5, 7, 11, and 13 and lane 2 with lanes 6, 8, 12, and 14). However, the Vac$^{\delta-}$ class I mutants show a reduced level of hybridization compared to the Vac$^+$ revertants (compare lanes 6 and 8 and lanes 12 and 14 in Fig. 4 *A* and *B*). The Vac$^-$ derivatives, like other class III mutants (data not shown), are completely lacking the *gvpA* gene (lanes 3, 4, 9, and 10 in Fig. 4 *A* and *B*).‡ The phenotypic assignments, Vac$^-$, Vac$^{\delta-}$, and Vac$^+$, are consistent with the gas vesicle content measured spectrophotometrically (Table 1). Thus, there is a parallel between the intensity of hybridization and the gas vesicle phenotype such that Vac$^-$

‡When the filters in Fig. 4 were rehybridized by using a ^{32}P-labeled *bop* gene probe, a single *bop* gene band of approximately equal intensity was visible in each lane (data not shown), indicating that equivalent amounts of DNA are present in all lanes.

(class III) mutants contain no gas vesicles or *gvpA* gene copies, $Vac^{\delta-}$ (class I) mutants contain intermediate gas vesicle levels and *gvpA* gene copies, and Vac^{+} revertants contain elevated gas vesicle levels and *gvpA* gene copies. This conclusion is also supported by comparison of the class I mutants where SD117B shows the lowest *gvpA* copy number and gas vesicle content (compare lanes 5 and 11 in Fig. 4 *A* and *B* and see Table 1).

Class II Mutants Contain Insertions Near the *gvpA* Gene. Plasmid DNA from NRC-1 and R1 and class II mutants SD104, SD106, SD108, and SD120 was analyzed by Southern hybridization using *gvpA* gene probes. When *Sal* I-digested DNA was hybridized with probe B (Fig. 5*A*), the 4.3-kb fragment in NRC-1 (lane 1) was shifted to between 4.8 and 5.6 kb (lanes 2–6) for the mutants, indicating that DNA rearrangements, presumably insertions, had occurred near the *gvpA* gene region. Based on the similarity of the Southern hybridization results for strain R1 and the class II mutants, strain R1 was designated as a class II mutant.

To map the sites of DNA rearrangements in class II mutants more precisely, plasmid DNA from the wild type and each mutant was subjected to further Southern hybridization analysis. Plasmid DNA was digested with *Sal* I + *Sst* I, which generates two *Sal* I–*Sst* I fragments (see Fig. 2), a 2.5-kb 5′ flanking fragment and a 1.9-kb *gvpA* gene-containing fragment from the 4.3-kb *Sal* I fragment, previously identified in NRC-1 (Fig. 5 *A* and *B*). Probe C was used first to identify the 1.9-kb *gvpA Sst* I–*Sal* I fragment, followed by rehybridization of the same filter using probe D, which hybridizes to the 1.9-kb and 2.5-kb *Sal* I–*Sst* I fragments. The final autoradiogram is shown in Fig. 5*B*. For R1 and SD106, the mutations mapped to the 1.9-kb *gvpA* gene-containing fragment, whereas for SD104, SD108, and SD120, the mutations mapped to the 2.5-kb 5′ flanking fragment (compare lane 7 to lanes 8–12 in Fig. 5*B*). Each of the mutant restriction fragments showed an increase in size, indicating that insertions had occurred in all of these mutants (see Fig. 2).

Further Southern hybridization analysis was carried out by using *Hin*dIII, *Eco*RI, and *Bam*HI (data not shown). The conclusions are summarized in the restriction maps in Fig. 2. For R1, the insertion site was localized to the 0.3-kb *Hin*dIII–*Stu* I *gvpA* gene-containing fragment and the insertion size was found to be 1.3 kb. For SD106, the insertion site mapped to the 0.15-kb *Sst* I–*Hin*dIII fragment 5′ to the gene and the insertion size was estimated to be 1.3 kb. For SD104, SD108, and SD120, the insertion sites mapped >1 kb upstream of the gene, between the *Sal* I and the *Bam*HI sites, and the insertion sizes were estimated as 1.0, 1.7, and 0.5 kb, respectively.

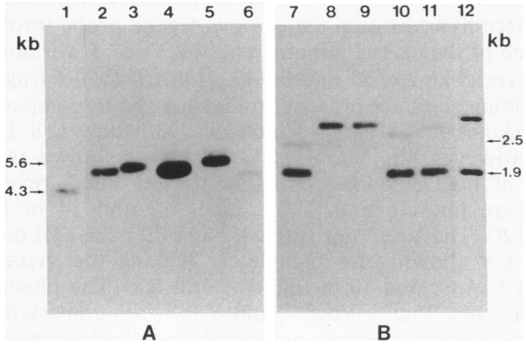

FIG. 5. Southern hybridization analysis of class II mutants using *gvpA* gene probes. Plasmid DNA from strains NRC-1 (lanes 1 and 7), R1 (lanes 5 and 8), SD104 (lanes 4 and 12), SD106 (lanes 3 and 9), SD108 (lanes 2 and 10), and SD120 (lanes 6 and 11) was digested using *Sal* I (*A*, lanes 1–6) and *Sal* I + *Sst* I (*B*, lanes 7–12). (*A*) Hybridized with probe B. (*B*) Hybridized with probes C and D. The sizes of restriction fragments are indicated.

DISCUSSION

We have shown that each of the 18 gas vesicle-deficient mutants of *H. halobium* strain NRC-1 studied has a mutation in the *gvpA* gene region of plasmid pNRC100. The mutants fall into three classes: (I) those with a reduced copy number of the *gvpA* gene, (II) those with insertion sequences in the *gvpA* gene region, and (III) those with no copies of the *gvpA* gene. These results are distinct from those obtained for purple membrane-deficient mutations (9–13), nearly all of which resulted from insertional inactivation of the *bop* gene (Fig. 1).

Class I mutants display a partially gas vesicle-deficient phenotype ($Vac^{\delta-}$) and are extremely unstable, giving rise to Vac^{+} revertants and completely Vac^{-} (class III) mutants at frequencies of 1–5%. The gas vesicle content and the apparent copy number of the *gvpA* gene are intermediate between the Vac^{+} and Vac^{-} derivatives, suggesting a gene dosage effect on gas vesicle content. A mechanism for generating such changes in *gvpA* copy number could involve selective deletion and/or amplification of a region of pNRC100. Such rearrangements occur at high frequencies for DNA flanked by directly repeated sequences such as insertion sequences (e.g., refs. 29 and 30) and have previously been reported in similar *Halobacterium* strains (7, 31). *H. halobium* plasmids are known to harbor multiple copies of ISH elements (14). An alternative potential mechanism for reduction in *gvpA* copy number is by the loss of pNRC100 copies by blocking either replication or partitioning (32). Preliminary analysis of plasmid DNA from class III mutants is consistent with the deletion model.

A model involving deletion of the *gvpA* gene followed by random segregation of the resulting heterogeneous plasmid population explains the observed interconversions between the wild-type Vac^{+} strains, $Vac^{\delta-}$ class I, and Vac^{-} class III mutants (Fig. 1). In Fig. 6, the model is schematically represented for a plasmid replicating according to a 4–8–4 cycle (newly divided cells have four plasmid copies and cells ready to divide have eight copies), consistent with the estimated plasmid copy number (6–7) in a similar *H. halobium* strain (6). The initial mutational event, a deletion, in a

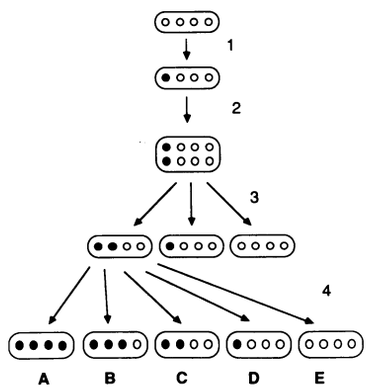

FIG. 6. Hypothetical model for interconversion between the wild-type and class I and III gas vesicle-deficient mutants of *H. halobium*. The large ovals represent *H. halobium* cells and the small circles represent plasmids. Open circles are wild-type plasmids and closed circles are mutant. Step 1, a mutational event occurs in one of four pNRC100 copies in a cell. Step 2, wild-type and mutant plasmids replicate. Step 3, random segregation of the heterogeneous plasmid population occurs. Step 4, further rounds of replication, random segregation, and cell division result in heterogeneous plasmid content (cell types A–E; see text and ref. 32 for discussion).

fraction of the plasmid population would result in cells containing wild-type and mutant plasmids (step 1). In step 2, the plasmids replicate, followed by random segregation and cell division in step 3. Our calculations indicate that about 21% of the progeny cells will contain two copies of the mutant and wild-type plasmids. The colony resulting from this cell would be scored as a $Vac^{\delta-}$ (or class I) mutant due to the gene dosage effect. Multiple rounds of replication, random segregation, and cell division (step 4) would result in progeny with additional plasmid heterogeneity (e.g., types A–E in recently divided cells). Calculations published by Novick (32) indicate that cells with an equal ratio of wild-type to mutant plasmids would give rise to completely Vac^- (type A or class III) and Vac^+ (type E or wild-type) progeny at 1.4% frequency. The observed mutation rates for class I mutants to Vac^+ and Vac^- phenotypes are in the 1–5% range. An interesting and testable prediction of this model is that the fraction of A and E (Vac^+ and Vac^-) type cells increases at a constant rate (32).

Class II gas vesicle mutants contain insertion sequences within or upstream of the *gvpA* gene. This conclusion is supported by Southern hybridization analysis using six restriction enzymes (Fig. 2). Based on size, restriction map, and hybridization analysis, we have compared these gas vesicle gene-inactivating insertion sequences to previously characterized ISH elements involved in insertional inactivation of the *bop* gene (e.g., ISH*1*, ISH*2*) (9, 10). Although none of the mutant restriction fragments hybridizes to the ISH*1* probe, hybridization using the ISH*2* probe indicates that the 0.5-kb insertion sequence in SD120 is ISH*2* (data not shown). For R1, SD104, SD106, and SD108 the data suggest that each mutant contains a different element distinct from the known ISH elements. For R1, the 1.3-kb insertion sequence has been cloned, and nucleotide sequence analysis shows that it contains an ISH*51*-like element (16) 14–15 nucleotides 5′ to the *gvpA* transcription start site (20). Our designation for this element is ISH*3* (J.W.D. and S.D., unpublished).

Four of the five class II mutants contain insertions substantially (120 base pairs to at least 1.0 kb) upstream of the *gvpA* gene and they are partially gas vesicle-deficient (Figs. 2 and 5, Table 1). The *gvpA* transcription start site is only 20 nucleotides upstream of the coding region (20). The results suggest that these class II mutations have interrupted a previously uncharacterized region required for synthesis of wild-type levels of gas vesicles. This region does not contain the second gas vesicle protein gene, *gvpC*, which is found 3′ to *gvpA* (T. Damerval, N.T.deM., J.G.J., and S.D., unpublished). Whether the upstream mutations are resulting in disruption of cis-acting (e.g., enhancer) or trans-acting (e.g., regulatory or structural gene) functions is currently unknown.

Previously, investigations of gas vesicle mutants of *Halobacterium* strains were published by Simon (33, 34) and Pfeifer *et al.* (6, 7). Agarose gel electrophoretic analysis of plasmid DNA from mutant strains was carried out in the absence of cloned gas vesicle gene probes. These investigations concluded that plasmid instability is correlated with gas vesicle mutations, but although Simon found that loss of a large *Halobacterium salinarium* plasmid was responsible for the mutation, Pfeifer *et al.* concluded that DNA rearrangements were the likely cause in *H. halobium*. Our results show that in gas vesicle-deficient mutants of *H. halobium* strain NRC-1, loss of and rearrangements in the gas vesicle gene region have occurred. Further analysis of the gas vesicle mutants will help to define the precise molecular events altering the gas vesicle gene region and to determine the role, if any, of the mutations in normal regulation of gas vesicle synthesis in *H. halobium*.

We thank Drs. T. G. Lessie, S. J. Robinson, N. Hackett, and N. Kleckner and T. Damerval for gifts of plasmid DNA and strains and for helpful advice. This work was supported by National Science Foundation Grant DMB8703486.

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