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Identification and analysis of the gas vesicle gene cluster on an unstable plasmid of *Halobacterium halobium*

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Abstract. In our efforts to elucidate the mechanism of high-frequency mutation of *Halobacterium halobium* to a gas vesicle deficient state, we discovered insertions, deletions, inversions, and complex DNA rearrangements associated with a large endogenous plasmid, pNRC100. The rearrangements are mostly IS element-mediated, and when they occur in a region of pNCRC100 containing a cluster of thirteen genes, gas vesicle mutants result. We have characterized the structure and expression of this gas vesicle protein (*gvp*) gene cluster and demonstrated its requirement for gas vesicle synthesis and cell flotation by genetic transformation.
Key words. Halobacteria; plasmid; insertion sequence; gas vesicle; biotechnology.

Many halophilic archaebacterial strains produce buoyant, intracellular, gas-filled organelles called gas vesicles or vacuoles[2,21]. Mutants of halobacteria lacking gas vesicles, which appear translucent as opposed to opaque wild-type colonies, were reported as early as 1932 by Petter[25]. Since then, investigators studying *Halobacterium halobium* and related strains have been intrigued by this remarkable high-frequency phenotypic variability[21]. Studies during the 1970's showed the presence of large plasmids in halobacteria and Simon, Goebel and co-workers attempted to correlate plasmid loss or rearrangement to the gas vesicle mutation[22,26,27,37]. Although these early studies did not establish a strict correlation, they did provide strong evidence for plasmid rearrangement and loss in gas vesicle mutants. As detailed in this review, definitive studies establishing the genomic location of the gas vesicle genes of *H. halobium* and characterization of the mechanism of the gas vesicle mutation came about in the 1980's after partial sequencing of a major gas vesicle protein by Walsby and co-workers[32] and cloning of the corresponding gene by Tandeau de Marsac et al.[7,30].

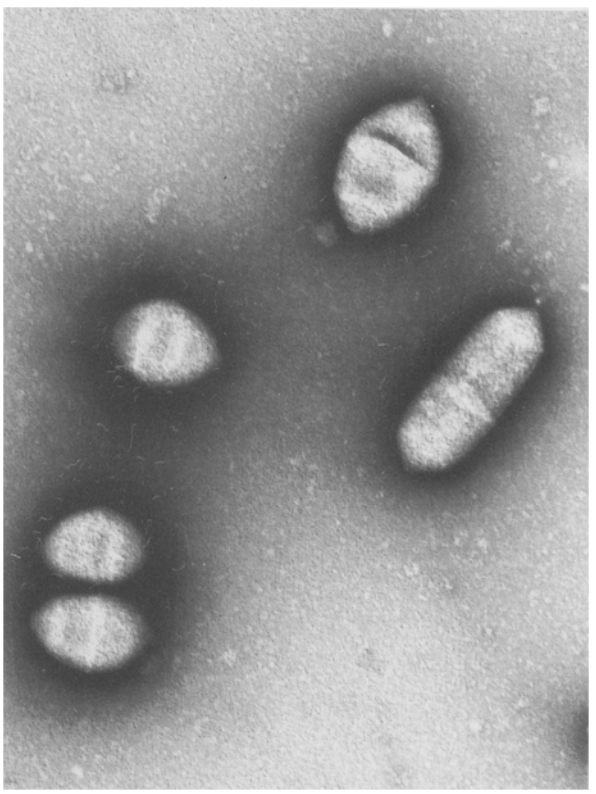

Figure 1. Gas vesicles of *H. halobium*. Two types of gas vesicles, lemon and cylinder-shaped, are apparent in the electron microscope by negative staining.

Gas vesicles

Gas vesicles are buoyant organelles synthesized by many aquatic bacteria[2,34]. Production of high levels of gas vesicles allows cells to float at the surface of the culture, increasing the availability of both oxygen and light. The vesicles also refract light, a property which may have a protective function for bacteria growing under damaging UV light intensities.

Gas vesicles are generally cylindrical in shape with conical caps (fig. 1). In halobacteria, most vesicles appear lemon-shaped in the electron microscope. A minor form that has a more elongated cylindrical shape has also been observed[2,28]. Vesicle synthesis is thought to be initiated at the cones followed by elongation of the central cylindrical region[18,31]. Water is reported to be excluded from the interior of the vesicle by hydrophobic forces and gases taken up as a result of diffusion across the vesicle membrane[33].

Gas vesicles have been purified from cyanobacteria and halobacteria and shown to be composed of only protein(s)[19,36]. Studies on the number and sizes of proteins present in gas vesicles have been controversial

because of the resistance of vesicle proteins to solubilization using detergents and chaotropic agents. Electrophoretic analysis using highly denaturing phenol-acetic acid-urea polyacrylamide gels showed the presence of a single major gas vesicle protein for several bacteria[36]. However, a second minor protein was reported for halobacteria[28,29]. N-terminal sequences for the major gas vesicle proteins of several cyanobacteria and halobacteria were determined and shown to be homologous[32].

Gas vesicle mutants

Mutants lacking gas vesicles are readily apparent by visual inspection of halobacterial colonies (fig. 2). Wild-type vacuolated (Vac^+) strains produce pink opaque colonies whereas non-vacuolated (Vac^-) mutants produce orange translucent colonies. For *H. halobium* strain NRC-1, the mutation rate is extraordinarily high, about 1%, resulting in the finding of mutants in every culture and Vac^- sectors in every Vac^+ colony. Three different types of Vac mutant colonies were apparent[4,7]. Mutants of the first type (class I) were partially Vac^- ($Vac^{\delta-}$) and showed alternating opaque and translucent sectors. Plating of class I mutants resulted in the finding of both Vac^+ and Vac^- colonies at very high frequencies, as well as $Vac^{\delta-}$ colonies. Class II mutants were also $Vac^{\delta-}$ but were much more stable than class I mutants. Class III mutants were completely Vac^- and could be obtained from cultures of wild-type and class I mutants.

In order to study these Vac mutants, a gas vesicle protein (*gvp*) gene probe was required. An oligonucleotide probe, designed based on the N-terminal sequence of the major gas vesicle protein[30], was synthesized and used to isolate the corresponding gene, *gvpA*, from the cyanobacterium *Calothrix* PCC 7601. The *H. halobium gvpA* gene was subsequently identified by Southern hybridization, cloned, and localized to a 200 kb plasmid, pNRC100[6] (fig. 3). This finding confirmed the suggestions of earlier studies that a plasmid is involved in gas vesicle synthesis. The *H. halobium gvpA* gene was used to analyze class I, II, and III mutants of strain NRC-1 and a stable Vac^- strain R1[7,16]. Analysis of R1 and four class II mutants showed that they resulted from insertions of IS elements in the *gvpA* gene region (fig. 3A). The insertion in R1

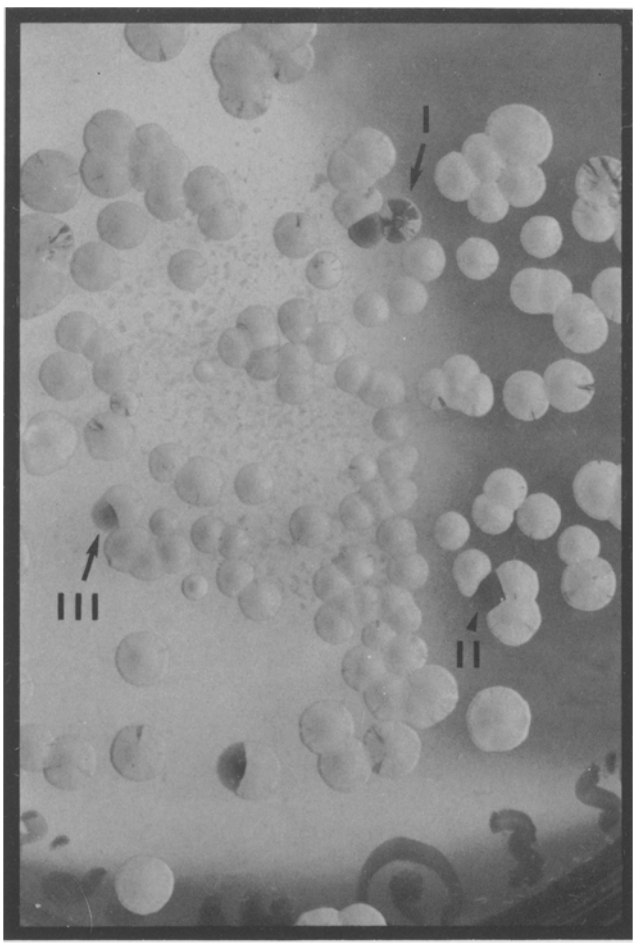

Figure 2. Gas vesicle mutants of *H. halobium*. All three classes of mutants are visible on this plate of wild-type strain NRC-1 colonies.

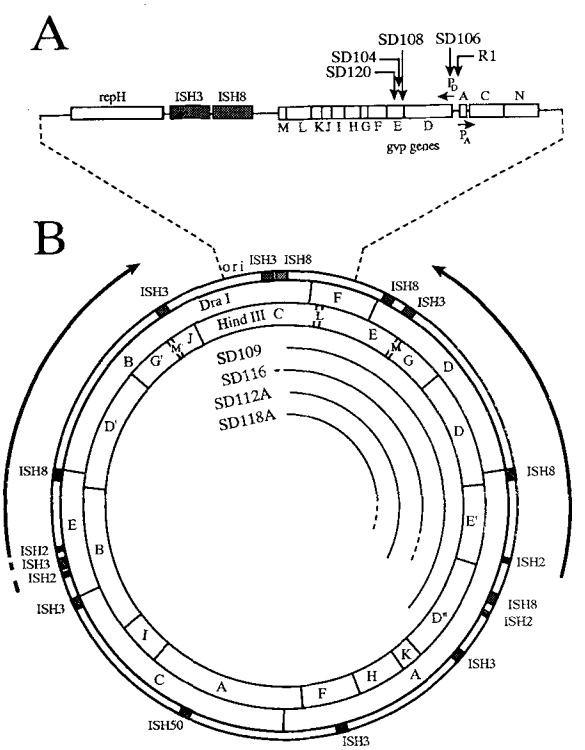

Figure 3. Map of plasmid pNRC100. The *gvp* gene region is shown enlarged in *A* with insertion sites in $Vac^{\delta-}$ mutants (R1, SD106, SD108, SD104, SD120) and the divergent promoters (P_D and P_A) indicated. A gene required for replication of mini-pNRC100 derivatives is marked *repH*. *B* shows the restriction map of pNRC100, with large inverted repeats marked by heavy arrows. The region of pNRC100 deleted in four Vac^- mutants (SD109, SD112A, SD118A and SD116) is indicated by inner arcs. IS elements are shown by shaded boxes.

(IS*H3*) occurred 14 bp 5′ to the *gvpA* transcription start site while insertions in the class II mutants, SD120 (IS*H2*), SD106 (IS*H3*), SD108 (IS*H8*), and SD104 (IS*H50*), mapped 0.2 to 2 kb further upstream. The finding of insertions upstream of *gvpA* suggested the involvement of additional genes in gas vesicle synthesis[7, 14, 16].

Four class I mutants, and single Vac$^+$ and class III Vac$^-$ derivatives of each mutant were analyzed by Southern hybridization[7]. The results indicated that class I mutants contain a heterogeneous population of plasmids, including both the wild-type pNRC100 plasmid as well as at least one plasmid derivative with deletion of the *gvpA* gene region, while the Vac$^+$ derivatives were indistinguishable from the wild-type, and the class III Vac$^-$ derivatives contained deleted pNRC100 derivatives. Thus, the sectored appearance of class I mutants can be explained by rearrangement(s) in a fraction of the pNRC100 copies in the founder cell followed by segregation of the heterogeneous plasmid population into progeny cells during growth of the colony.

In order to carry out an analysis of plasmid deletions in class III mutants, the restriction map of the wild-type plasmid pNRC100 was first established[23] (fig. 3B). Mapping by pulsed-field gel electrophoresis showed pNRC100 to be 200 kb in size, including a substantial region, about 35 kb, repeated in inverted orientation. The *gvpA* gene was localized to the small single copy region between the large inverted repeats. The presence of two inversion isomers of pNRC100 ($\alpha\delta$ and $\beta\gamma$) with different relative orientations of the single copy regions was demonstrated using restriction enzymes cleaving within both the large and small single copy regions but not within the large inverted repeats. Also, the positions of seventeen copies of IS elements were mapped in pNRC100.

The pNRC100 deletion derivatives in four class III mutants were characterized by restriction mapping and nucleotide sequence analysis. Each plasmid had lost a substantial portion of the small single copy region and most or all of one copy of the large inverted repeat of pNRC100, and several had suffered additional rearrangements[10, 24] (fig. 3B). In two plasmids, pSD109 and pSD118A, deletions extended from the end of an IS*H8* element located to the left of *gvpA* while in another, pSD116, the deletion extended approximately from the end of the IS*H3* located to the left of *gvpA*, to sites substantially beyond the right end of *gvpA*. In the fourth, pSD112A, the deletion extended from the end of an IS*H2* element in the large single copy region to a point near the same IS*H8* element at the left end of the gene cluster. The mechanism for formation of class III deletions appears to be by intramolecular transposition of IS elements, although the possibility of an intermolecular transposition followed by recombination between two IS elements cannot be ruled out.

Gas vesicle genes and gene products

A 10 kb region around *gvpA* was subjected to DNA sequence and transcript analyses for *H. halobium* NRC-1 and related strains[11, 14, 16, 17]. Thirteen open reading frames organized into two divergent operons were identified. The leftward operon contains ten genes, *gvpD*, *E*, *F*, *G*, *H*, *I*, *J*, *K*, *L*, and *M*. The rightward operon contains three genes, *gvpA*, *C*, and *N*. A fourteenth gene, *gvpO*, downstream of *gvpN*, has also been reported[9], but its involvement in gas vesicle synthesis is unclear[11].

The *gvpA-gvpD* intergenic region of strain NRC-1 is 200 bp long and contains divergent promoters with transcription start points separated by 110 bp[6, 16]. The promoter region contains a 7 bp inverted repeat suggestive of a regulatory sequence. The abundance of transcripts starting from P_A, the rightward promoter, is about five-fold greater than that from P_D, the leftward promoter. Rightward transcription is inducible during growth from early-logarithmic to mid-logarithmic phase at which point the transcript abundance plateaus to the stationary phase[38]. Induction of P_A is lowered by aeration of the culture suggesting that gene expression is responsive to the oxygen availability in the medium[38].

Currently, studies are focused on the functions of the many *gvp* gene products in gas vesicle synthesis (table 1). Two of the *gvp* gene products have so far been shown to encode mature gas vesicle proteins[11]. Antibodies raised against LacZ-GvpA and LacZ-GvpC proteins synthesized in *E. coli* were used for Western blotting analysis of gas vesicles and cell lysates of Vac$^+$ and Vac$^-$*H. halobium* strains. The GvpA protein was identified after electrophoresis on highly denaturing phenol-acetic acid-urea polyacrylamide gels whereas the GvpC protein was identified by conventional SDS-polyacrylamide gel electrophoresis. The GvpC protein contains eight imperfect copies of a repeated motif and a highly acidic region near the C-terminus[17]. In cyanobacteria, a distantly related GvpC protein has been shown to confer resistance of gas vesicles to collapse[3, 12, 35]. Two other putative Gvp proteins, GvpJ and GvpM, are small acidic proteins similar to GvpA. These may constitute a small family of proteins which could be used in different ratios for construction of gas vesicle membranes with varying geometries[17]. GvpD and GvpN contain nucleotide triphosphate binding motifs, suggestive of an energy requiring function for these putative proteins[11, 17]. Interestingly, the GvpI putative protein is highly basic, in contrast to all of the other *gvp* gene products, which like most other halophilic proteins are acidic[17].

Complementation of gas vesicle mutants

To determine the functions of *gvp* genes in gas vesicle synthesis, a genetic system for directed mutation of each

Table 1. Profile of predicted *gvp* gene products

Gene product	Molecular weight	Isoelectric point	Acid residues	Basic residues	Charged residues	Hydrophobic residues	Aromatic residues
GvpA	8005	4.03	18.4	7.9	26.3	31.6	3.9
GvpC	42391	3.57	30.1	7.1	37.2	14.7	9.2
GvpD	59341	4.17	16.8	9.5	26.3	25.0	7.3
GvpE	21009	4.07	19.9	10.5	30.4	27.2	4.2
GvpF	23962	4.00	22.1	9.9	31.9	24.9	7.0
GvpG	10014	4.12	24.1	12.0	36.1	28.9	8.4
GvpH	19883	3.92	25.3	11.0	36.3	23.6	1.6
GvpI	16259	10.83	15.3	24.3	39.6	9.7	1.4
GvpJ	11983	3.66	21.0	5.3	26.3	20.2	2.6
GvpK	12695	3.88	29.2	10.6	39.8	27.4	0.9
GvpL	31994	4.19	22.1	12.1	34.2	20.3	7.5
GvpM	9248	4.10	19.0	9.5	28.6	26.2	6.0
GvpN	39228	4.86	19.0	16.1	35.2	25.4	4.6

The composition of all categories of amino acid residues are expressed in per cent.

gene is required. To accomplish this goal, the *gvp* gene cluster of pNRC100 was reconstructed on shuttle plasmids capable of replication in both *E. coli* and *H. halobium* (e.g. pJHGV3)[1,10]. The shuttle plasmids contained in addition to the entire *gvp* gene cluster, the replication origin of pNRC100 with the *repH* gene[24] which is required for plasmid maintenance in *H. halobium*, a selectable marker (*mev*^{R})[20] for *H. halobium*, and an *E. coli* vector. When class III Vac$^-$ mutants with deletions of the entire *gvp* gene cluster were transformed with the reconstructed *gvp* gene cluster on shuttle plasmids, they exhibited the Vac$^+$ phenotype and were able to float at the surface of liquid cultures[10]. The first mutated derivative of plasmid pJHGV3, pJHGV33'::κ, with an insertion of a kanamycin-resistance cassette immediately 3' to *gvpN*, conferred a Vac$^+$ phenotype when transformed into class III mutants, suggesting that *gvpN* is the last gene in the rightward *gvp* operon[11].

A second gas vesicle gene cluster

Although the analysis of gas vesicle mutants implicated only *gvpA* and the surrounding cluster of genes on pNRC100 in gas vesicle synthesis, Southern hybridization showed that a second cluster of genes (*gvpB* or 'c-vac' gene cluster) is present in *H. halobium*[13,16]. In strain NRC-1, the lack of any detectable gas vesicle synthesis in class III deletion mutants suggested that the second cluster alone is not sufficient for gas vesicle synthesis[7]. However, complementation of the *gvpA* gene cluster by genes of the *gvpB* cluster is possible since the class II *gvpD* and *E* insertion mutants showed only a partial (Vac$^{\delta-}$) phenotype. In *H. halobium* strain NRC817, Pfeifer and co-workers have sequenced the *gvpB* gene cluster in its entirety and shown the presence of homologs of all thirteen or fourteen *gvp* genes[9,13,14]. In contrast to NRC-1, synthesis of gas vesicles from the *gvpB* gene cluster was reported in the stationary phase in NRC817[8,15]. The genomic location of the *gvpB* gene cluster in the two *H. halobium* strains is also different, with the cluster in NRC-1 present on a very large (~350 kb) plasmid pNRC200[5] while in NRC817 it is reportedly on the chromosome[9].

Future prospects

Analysis of gas vesicle mutants has resulted in the finding of thirteen genes involved in the synthesis of gas vesicles, suggesting that gas vesicle formation is a complex process. This finding is surprising considering that researchers had initially hypothesized the requirement of only a single self-associating gas vesicle protein. The construction of a shuttle plasmid containing the gas vesicle gene cluster which can complement Vac$^-$ mutants should soon allow mutational analysis of each gene in the cluster. Such a study should establish the roles of the various gene products in regulation, assembly, and structure of the vesicles and shed light on the exact mechanism for exclusion of water from the interior of the vesicle and for uptake of gases. Once the biosynthetic process is understood, it should be possible to genetically engineer other microorganisms to produce gas vesicles and to float, a process with significant potential for biotechnology.

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