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Evidence for Two Different Gas Vesicle Proteins and Genes in *Halobacterium halobium*

B. SUREK,[1] B. PILLAY,[1] U. RDEST,[1] K. BEYREUTHER,[2]† AND W. GOEBEL[1]*

Institut für Genetik und Mikrobiologie, Universität Würzburg, 8700 Würzburg,[1] and Institut für Genetik, Universität Köln, 5000 Cologne 41,[2] Federal Republic of Germany

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Most halobacteria produce gas vesicles (GV). The well-characterized species *Halobacterium halobium* and some GV$^+$ revertants of GV$^-$ mutants of *H. halobium* produce large amounts of GV which have a spindlelike shape. Most other GV$^+$ revertants of *H. halobium* GV$^-$ mutants and other recently characterized halobacterial wild-type strains possess GV with a cylindrical form. The number of intact particles in the latter isolates is only 10 to 30% of that of *H. halobium*. Analysis of GV envelope proteins (GVPs) by electrophoresis on phenol-acetic acid-urea gels showed that the GVP of the highly efficient GV-producing strains migrated faster than the GVP of the low-GV-producing strains. The relative molecular mass of the GVP was estimated to be 19 kilodaltons (kDa) for high-producing strains (GVP-A) and 20 kDa for low-producing strains (GVP-B). Amino acid sequence analysis of the first 40 amino acids of the N-terminal parts of GVP-A and GVP-B indicated that the two proteins differed in two defined positions. GVP-B, in relation to GVP-A, had Gly-7 and Val-28 always replaced by Ser-7 and Ile-28, respectively. These data suggest that at least two different *gvp* genes exist in *H. halobium* NRL. This was directly demonstrated by hybridization experiments with *gvp*-specific DNA probes. A fragment of plasmid pHH1 and a chromosomal fragment of *H. halobium* hybridized to the probes. Only a chromosomal fragment hybridized to the same *gvp* probes when both chromosomal and plasmid DNAs from the low-GV-producing halobacterial wild-type strains SB3 and GN101 were examined. These findings support the assumption that GVP-A is expressed by a pHH1-associated *gvp* gene and GVP-B by a chromosomal *gvp* gene.

Gas vesicles (GV) are small, hollow, gas-filled protein structures that are found in several procaryotic planktonic microorganisms. They allow the positioning of the bacteria, most of which are photosynthetic, at the favorable depth for growth (12). The best-studied GV are those from cyanobacteria. As has been shown by the amino acid sequence derived from the sequence of the GV envelope protein (GVP) structural gene (10), GV consist of a protein (GVP) which has a relative molecular mass of 7,375 daltons (Da) (*Calothrix* sp. strain PCC 7601). Purified GVP of *Anabaena flos-aquae*, another cyanobacterium, migrates in sodium dodecyl sulfate (SDS)-polyacrylamide gels as a single band at 21 kDa, although the complete amino acid sequence has demonstrated that the GVP is only 7,388 Da in size (4). Among the archaebacteria, most halobacterial strains and *Methanosarcina* spp. produce GV (1, 8, 9, 11).

By analogy with vacuolate cyanobacteria, Simon (9) demonstrated that two morphologically different GV envelopes exist in *Halobacterium salinarium* 5 and that each of these consists of a single type of protein, whereas Falkenberg (P. Falkenberg, Ph.D. thesis, Universitetet Trondheim, Trondheim, Norway, 1974) described two proteins, A and B, in a ratio of 1:4 in purified GV from this halobacterial species. A single GVP has also been isolated from *H. halobium* and shown to be identical in size to that of *H. salinarium* 5 (9).

GVP is a highly conserved protein among eubacteria and archaebacteria, and this has been demonstrated by comparing the amino acid compositions and N-terminal amino acid sequences of halobacterial and cyanobacterial GVPs (10, 11).

We had previously reported that formation of GV in *H. halobium* is highly unstable genetically (6, 13). GV-negative (GV$^-$) mutants were spontaneously obtained at frequencies of 10^{-2} to 10^{-3}. All GV$^-$ mutants examined contained insertions in a ca. 2-kilobase (kb) region of the plasmid pHH1. Revertants from such spontaneous GV$^-$ mutants could be isolated which restored wild-type GV formation (type I revertants, designated high-efficiency GV-producing strains [HGV]) or synthesized considerably fewer GV (type II revertants, designated low-efficiency GV-producing strains [LGV]).

Recently we described new isolates of halobacteria which produce only low amounts of GV, thereby resembling type II revertants (LGV) of *H. halobium* (3). These halobacterial strains do not carry pHH1 or pHH1-related plasmids and are rather stable in their GV synthesis.

In this report we show that GV from all LGV strains examined consisted of GVP which differed characteristically from those of HGV strains in their mobility on phenol-acetic acid-urea (PAU) gels and in their N-terminal amino acid sequences. In various GV$^+$ revertants obtained from GV$^-$ mutants of *H. halobium*, we could detect either a GVP of 19 kDa (GVP-A) or another GVP of 20 kDa (GVP-B). From all the halobacterial strains of the LGV type examined, only GVP-B was isolated.

MATERIALS AND METHODS

Bacterial strains. All strains used are listed in Table 1. *H. halobium* NRL (previously referred to by us as NRC817 [3]) was obtained from D. Oesterhelt (Munich). *H. halobium* strains 670 (GV$^+$) and 671 (GV$^-$) were obtained from the Deutsche Sammlung für Mikroorganismen (Göttingen). The new isolates *Halobacterium* sp. strain A2, *Halobacterium* sp. strain GRA, *Halobacterium* sp. strain GRB, *Halobacterium* sp. strain SB3, and the 671 and NRL GV revertant strains were isolated in our laboratory (3). *Halobacterium*

* Corresponding author.
† Present address: Zentrum für Molekulare Biologie, Universität Heidelberg, 6900 Heidelberg, Federal Republic of Germany.

TABLE 1. Strains used

Strain	Phenotype	Size of GVP (kDa)
Wild-type strains		
H. halobium NRL	HGV	19
H. halobium 670	HGV	19
GV^+ revertants of *H. halobium* NRL (GV^- mutant)		
H. halobium I-22	HGV	19
H. halobium I-23	HGV	19
H. halobium III-3 vd	LGV	20
H. halobium R1 Vac^+-2	LGV	20
GV^+ revertants of *H. halobium* 671		
H. halobium 671 Vac^+-I	LGV	20
H. halobium 671 Vac^+-II	LGV	20
H. halobium 671 Vac^+-III	HGV	19
H. halobium 671 Vac^+-IV	HGV	19
New isolates		
Halobacterium sp. strain A2	LGV	20
Halobacterium sp. strain GRA	LGV	20
Halobacterium sp. strain GRB	LGV	20
Halobacterium sp. strain SB3	LGV	20
Halobacterium sp. strain GN101	LGV	20
H. salinarium Vac^+-13	LGV	20

sp. strain GN101 was provided by W. Stoeckenius (University of California, San Francisco).

Source of reagents. All chemicals were obtained from Serva (Heidelberg) or Merck (Darmstadt). Nutrients were provided by Oxoid (Wesel). Membrane filters were obtained from Sartorius (Göttingen).

Isolation of gas vesicles. Intact GV were isolated from *H. halobium*, several revertant strains of GV^- mutants of *H. halobium* NRL and the GV^- strain 671, and the new isolates *Halobacterium* sp. strain A2, *Halobacterium* sp. strain GRA, *Halobacterium* sp. strain GRB, *Halobacterium* sp. strain SB3, and *Halobacterium* sp. strain GN101 by the stepwise filtration method. The halophilic strains were grown in salt medium consisting of 4 M NaCl, 0.12 M $MgSO_4$, 0.03 M KCl, 0.01 M trisodium citrate, and 1% peptone (Oxoid) with shaking and illumination at 38°C for 5 to 6 days. To lyse the cells, 0.8 mg of DNase per 100 ml was added, and the cultures were dialyzed overnight against buffer containing 0.1 M NaCl and 1 mM $NaHCO_3$, pH 8.3. The lysate was filtered at low pressure through a membrane filter (0.45-μm pore size). The procedure was repeated two times by filtration of the lysate through membrane filters with decreasing pore sizes (0.3 to 0.20 μm) to remove cell debris. The intact GV, which could be seen under the phase-contrast microscope, were mechanically collapsed and pelleted by centrifugation for 3 to 4 h at 40,000 × *g* and 10°C. They were resuspended in 0.9 to 1.0 ml of buffer. To remove residual bacterioopsin and bacterioruberins, the suspension was layered on a 45% sucrose solution in dialysis buffer and centrifuged on a cushion of 67% sucrose for 14 to 16 h at 200,000 × *g* and 10°C. The disrupted GV fraction was collected from the top of the sucrose cushion and diluted with buffer to a final concentration of 6%. The vacuole preparation was pelleted for 2 h at 40,000 × *g* and then suspended in 0.8 to 1.0 ml of H_2O. For analysis on a PAU gel, the GV envelopes were mixed 1:1 with a PAU solution containing 48% (wt/vol) phenol, 28% (vol/vol) acetic acid, and 24% (wt/vol) urea.

Electron microscopy. Dilutions of the GV preparation were dialyzed against H_2O and then applied to copper grids. After drying, the grids were shadowed with platinum at 2×10^{-4} Torr (ca. 26.6 mPa) and examined by transmission electron microscopy.

Gel electrophoresis. Gel electrophoresis was carried out by using the PAU-acrylamide gel system described by Simon (8). Lysozyme (14.3 kDa) and carbonic anhydrase (30 kDa) were used as markers.

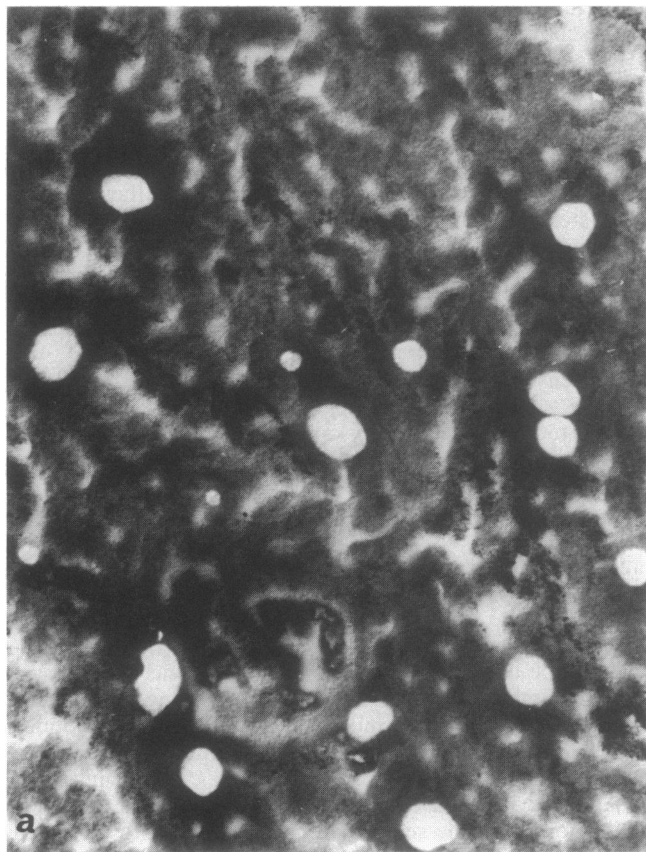
Protein sequencing. GVPs from each GV envelope preparation were sequenced at least twice with the Applied Biosystems Instruments A470 protein sequencer with on-line high-pressure liquid chromatography analysis of the phenylthiohydantoin derivatives (model 120A analyzer; Applied Biosystems, Foster City, Calif.). The separation of the phenylthiohydantoin-amino acids was carried out as specified by the manufacturer.

GV envelope suspensions or pelleted GV envelopes (40,000 × *g*) suspended in 30 μl of formic acid were transferred on glass filter disks for solid gas-phase sequencing. The filters contained 1.5 mg of Polybrene and were subjected to three cycles of Edman degradation prior to sample application. Typically, 2 μg of GVP was loaded on the filters. Identical sequencing results were obtained from envelope suspensions and pelleted envelopes.

Hybridization. Southern hybridization with the synthetic oligonucleotide and the two other *gvp* gene probes (see text) was carried out essentially as described (3). The oligonucleotide probe used for detection of the *gvp* gene was derived from the amino acid sequence of GVP-A, using amino acids 14 to 22. First, we used a mixture of several oligonucleotides which took into consideration the codon ambiguity of the amino acids used. Later we identified by cloning and DNA sequencing a 144-base-pair (bp) *Sau*3A fragment of pHH1 which contained the sequenced amino acids 14 to 40. From these data we deduced the precise sequence for the oligonucleotide probe (GATCTGGTATAGACAAAGGTGTCGTT). The probe was labeled with [^{32}P]ATP and treated with kinase as described (3).

RESULTS

GV and GVP from *H. halobium* and related halobacteria. The two *H. halobium* strains NRL and 670 as well as some GV^+ revertants obtained from GV^- mutants of *H. halobium* NRL and the GV^- strain 671 produced large numbers of GV (HGV) which had a spindlelike shape (Fig. 1a). The new isolates, *Halobacterium* sp. strain A2, *Halobacterium* sp. strain GRA, *Halobacterium* sp. strain GRB, *Halobacterium* sp. strain SB3, Halobacterium sp. strain GN101 (3), and several other GV^+ revertants of *H. halobium* GV^- mutants contained much lower amounts of GV (LGV). The number of intact particles corresponded to 10 to 30% of that of *H. halobium* NRL and 670. In *Halobacterium* sp. strain SB3, the quantity of GV was so reduced that the cells were not able to float. The vesicles of all LGV strains were of a cylindrical form (Fig. 1b). A similar phenomenon of two different shapes of GV has already been described for *H. salinarium* 5 (9). It is interesting to note that it was not possible to isolate HGV derivatives from LGV revertants of *H. halobium* GV^- mutants or from the recently isolated LGV strains GRA, GRB, SB3, and GN101. In *H. halobium* NRL and 671, however, we could obtain, starting from GV^- mutants, spontaneous LGV revertants at a frequency of 10^{-2} to 10^{-3} and HGV revertants at a considerably lower frequency, 10^{-4} to 10^{-5}. GV envelopes were isolated from both types of GV. The purified GVPs were analyzed by PAU gel electrophoresis as described (8). As shown in Fig. 2, GVP from vacuoles of HGV halobacteria always migrated in



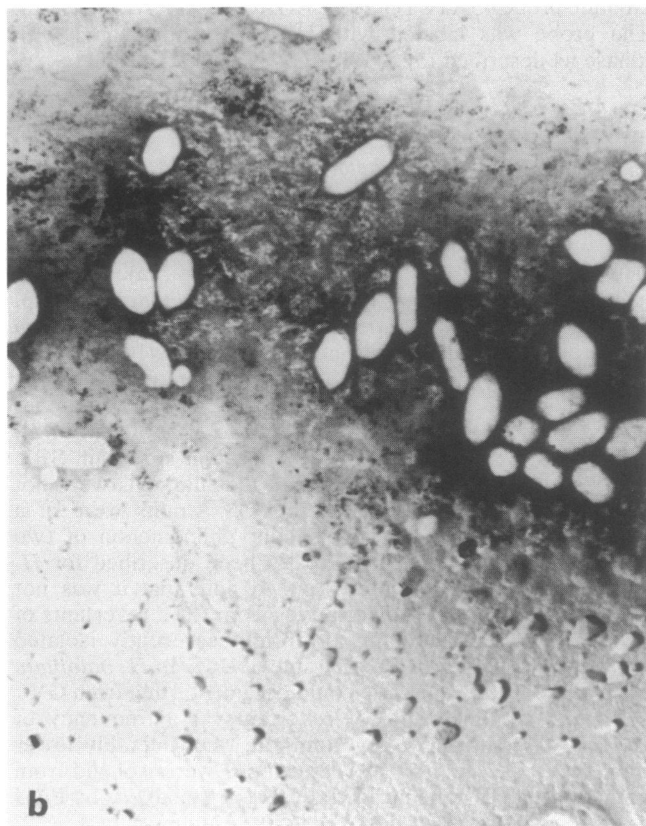


FIG. 1. Electron micrographs of purified GV (magnification, ×25,000). While the GV from *H. halobium* NRL are spindle-shaped (a), the vesicles from *Halobacterium* sp. strain GRA have a more cylindrical form (b).

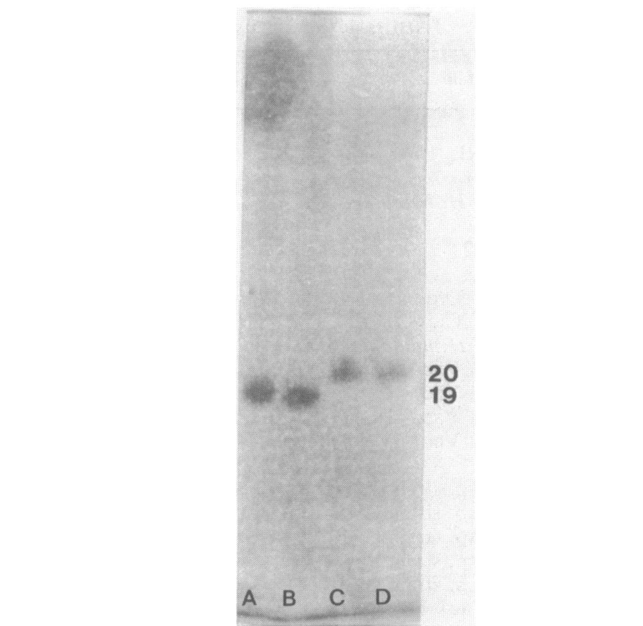


FIG. 2. Electrophoresis of gas vesicle proteins on a 10% acrylamide gel containing PAU. GVPs: *H. halobium* NRL (A), HGV revertant (Vac$^+$-IV) derived from the GV$^-$ strain *H. halobium* 671 (B), LGV revertant DSM671 (Vac$^-$-II) of *H. halobium* 671 (C), and wild-type strain *Halobacterium* sp. strain GRA (LGV phenotype) (D). The positions of GVP-B (20 kDa) and GVP-A (19 kDa) are marked.

the gel as a distinct band with a relative molecular mass of 19 kDa (GVP-A). In contrast, GVP from GV of LGV strains were slightly larger, 20 kDa (GVP-B) in this gel system. Table 1 summarizes the results for all strains tested.

It should be noted that in all LGV revertants from GV$^-$ mutants of *H. halobium* NRL or *H. halobium* 671, the 20-kDa GVP was detected, whereas the revertants with the HGV phenotype had a GVP of 19 kDa. In the new isolates, *Halobacterium* sp. strain A2, *Halobacterium* sp. strain GRA, *Halobacterium* sp. strain GRB, *Halobacterium* sp. strain SB3, and *Halobacterium* sp. strain GN101, only one type of GVP was detected, and this corresponded in size to the GVP of the LGV revertants from *H. halobium* NRL. The difference in the electrophoretic mobility in PAU gels between the two types of halobacterial GVPs disappeared after electrophoresis in SDS-PAU (5) gels containing 4 M urea, and the molecular mass for both proteins seemed to be 20 kDa (data not shown). It was recently reported (5) that the actual molecular mass of the GVP from *Anabaena flos-aquae*, a cyanobacterium, is 7,388 Da. Its relative molecular mass obtained on SDS-PAGE was also about 20 kDa, which demonstrates the tendency of the cyanobacterial GVP to form aggregates in this gel system.

Amino acid sequence of the N-terminal ends of the two types of GVPs. The amino acid sequence analyses were performed with purified GVPs from *H. halobium* NRL (HGV), one HGV revertant of an *H. halobium* NRL GV$^-$ mutant, three revertants of the LGV type, one LGV revertant (*vac*$^+$ [12])

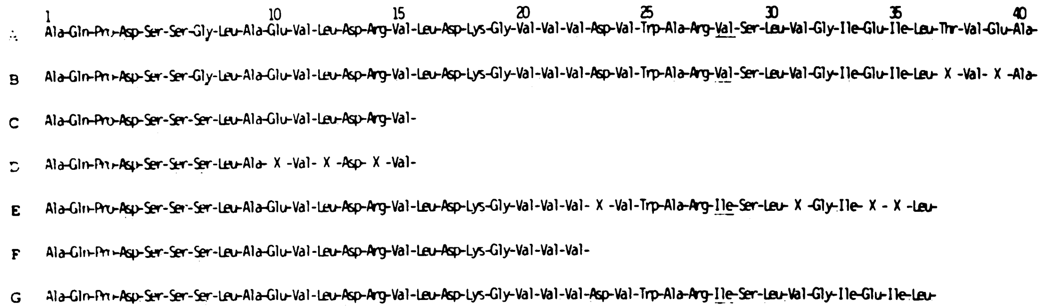


FIG. 3. Amino acid sequences of the N-terminal ends of the two types of GVP. A and B show the sequences of the N-terminal 40 amino acids of the GVPs from *H. halobium* NRL and 670. C to G represent the sequences of GVPs from the LGV revertant (III-3 vd), the LGV revertant Vac$^+$-13 of the GV$^-$ *H. salinarium* strain, the LGV revertants 671 Vac$^+$-I and R1 Vac$^+$-2, and *Halobacterium* sp. strain GRA, respectively. Amino acids which could not be determined are marked by X.

of *H. salinarium* (a GV$^-$ isolate which is closely related to *H. halobium* NRL [6]), and the LGV wild-type strain *Halobacterium* sp. strain GRA.

The sequence of 40 amino acids determined from the N-terminal part of the GVP from *H. halobium* NRL (Fig. 3A) was identical to that published previously (11). GVP isolated from an HGV revertant obtained from a GV$^-$ mutant of *H. halobium* NRL showed the same amino acid sequence as the wild-type strain NRL itself in the first 40 amino acids of the N-terminal end (Fig. 3B). However, *Halobacterium* sp. strain GRA, an LGV wild-type strain, had two changes in the first 36 amino acids of its GVP (Fig. 3G) compared with the sequence of *H. halobium* NRL (Fig. 3A). In the GVP of *Halobacterium* sp. strain GRA, Ser was found instead of Gly at position 7, and Val was replaced by Ile at position 28.

The same changes were observed in the amino acid sequence of the GVP from an LGV revertant obtained from a GV$^-$ mutant of *H. halobium* NRL (Fig. 3E). In two other purified GVPs from LGV revertants (Fig. 3C and F) of GV$^-$ mutants of *H. halobium* NRL and the GVP on an LGV revertant of the GV$^-$ strain *H. salinarium* (Fig. 3D), we could at least verify the first change in position 7 (Gly to Ser). Position 28 could not be determined in these GVPs.

The data clearly demonstrate that halobacterial isolates with a reduced number of GV compared with the *H. halobium* wild-type strain NRL show the same changes in the amino acid sequence of the N-terminal parts of their GVPs (Fig. 3C to G).

Evidence for two *gvp* genes. Determination of the N-terminal amino acid sequence of GVP-A made possible the synthesis of an oligonucleotide probe which was used to detect, by Southern hybridization, the *gvp* genes in *H. halobium* NRL and in two LGV *Halobacterium* strains, SB3 and GN101. Total DNA from these strains was fractionated by cesium chloride-ethidium bromide gradient centrifugation into covalently closed circular plasmid and chromosomal DNA (still containing open circular plasmid DNA). Both DNA fractions were cleaved with *Pst*I and hybridized with the ^{32}P-labeled oligonucleotide probe. The covalently closed circular plasmid DNA fraction of *H. halobium* NRL gave a strong signal with this probe in *Pst*I fragment 1 (Fig. 4B) and *Eco*RI fragment 6 (Fig. 5B). The chromosomal DNA fraction of *H. halobium* NRL (cut with *Pst*I) showed a weaker hybridization signal in a fragment of 7.5 kb and a stronger signal which corresponded in size (34 kb) to *Pst*I fragment 1 of pHH1. The latter hybridizing band was most likely the result of contaminating open circular pHH1 DNA in the chromosomal fraction.

The weaker-hybridizing band appeared to be caused by an additional chromosomal band. The latter assumption is supported by the hybridization data obtained with the DNA fractions of strains SB3 and GN101. Plasmid DNA from these strains did not hybridize with the *gvp*-specific oligonucleotide probe (Fig. 4B). This is in line with our previous data (3), which showed the absence of pHH1-related sequences in these strains. The chromosomal DNA fractions of both strains (cut with *Pst*I), however, hybridized to a band indistinguishable in size from the weaker-hybridizing *Pst*I fragment of *H. halobium*. Similar results (with a higher nonspecific background; data not shown) were obtained by using a *gvp* gene probe from the cyanobacterium *Calothrix* sp. strain pCC7601 (generously provided by N. Tandeau de Marsac, Institut Pasteur). In addition, we cloned and identified by DNA sequence analysis a *gvp* gene-specific *Sau*3A fragment of 144 bp from pHH1 (B. Pillay and U. Rdest, unpublished results). With this fragment as a hybridization

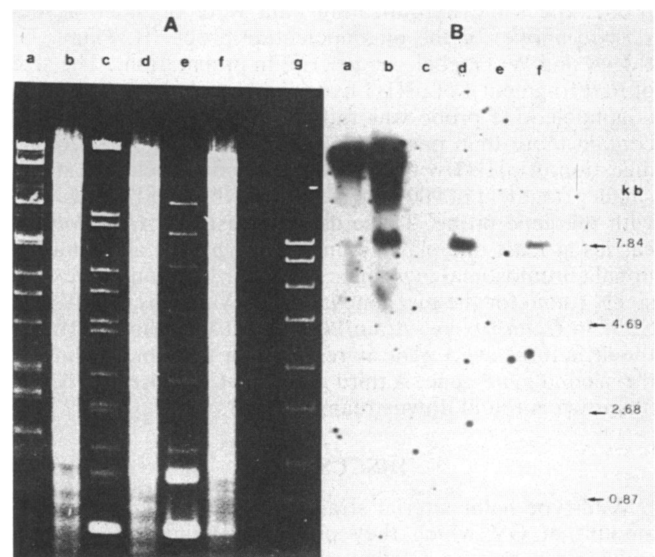


FIG. 4. (A) Agarose gel electrophoresis of *Pst*I-cleaved plasmid and chromosomal DNA and (B) autoradiography of this DNA after hybridization with a ^{32}P-labeled *gvp*-specific oligonucleotide probe. (a) Plasmid pHH1 DNA, (b) chromosomal DNA from *H. halobium* NRL; (c) plasmid and (d) chromosomal DNA from *Halobacterium* sp. strain GN101; (e) plasmid and (f) chromosomal DNA from *Halobacterium* sp. strain SB3; (g) phage SPP1 DNA cut with *Eco*RI as a size marker.

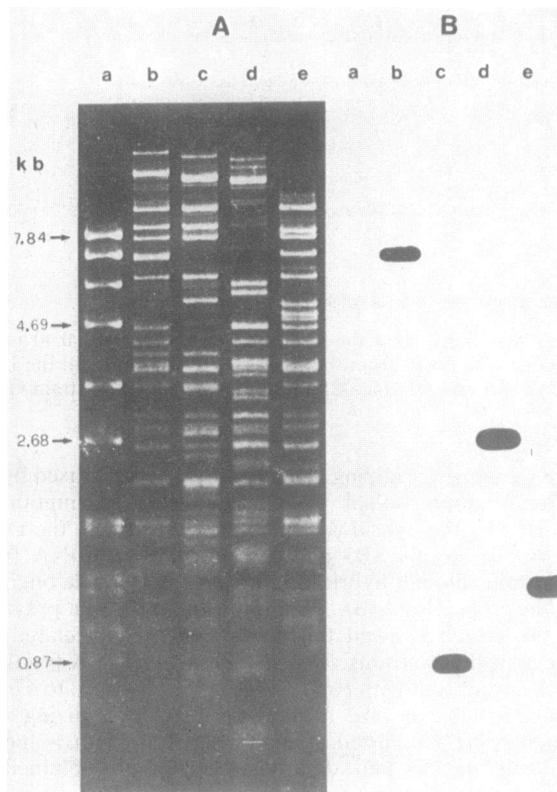


FIG. 5. (A) Agarose gel electrophoresis and (B) autoradiography after hybridization with a ^{32}P-labeled *gvp*-specific oligonucleotide probe of plasmid pHH1 of *H. halobium*. (a) Phage SPP1 DNA cut with *Eco*RI as a size marker; pHH1 after cleavage with (b) *Eco*RI, (c) EcoRI and *Hin*dIII, (d) EcoRI and *Bgl*II, or (e) *Eco*RI and *Bam*HI.

probe, the same hybridization data were obtained as described above for the oligonucleotide probe (B. Pillay, U. Rdest, and W. Goebel, manuscript in preparation). The size of *Pst*I fragment 1 of pHH1 hybridizing with the *gvp*-specific oligonucleotide probe was rather large (34 kb), and it may contain more than one gene. As shown in Fig. 5B, double digestion of pHH1 with *Eco*RI and *Hin*dIII yielded a much smaller fragment of 900 bp which also hybridized exclusively with the gene probe. These data suggest that *H. halobium* carries at least one plasmid-encoded *gvp* gene and an additional chromosomal *gvp* gene. The latter location was exclusively found for the *gvp* gene in the LGV strains GN101 and SB3. In *Calothrix* sp. strain PCC7601 it was shown (2) that the structural *gvpA* gene is repeated in tandem, leading to the second *gvpB* gene. A third gene (*gvpC*) whose function is unknown is found downstream of *gvpB*.

DISCUSSION

Wild-type halobacterial strains differ considerably in the amount of GV which they produce. While *H. halobium* synthesizes a large number of GV, the number of these proteinaceous structures is drastically reduced in most other wild-type isolates.

Revertants obtained from spontaneously occurring GV$^-$ mutants of *H. halobium* fall into two different classes with respect to the amount of GV which they synthesize. Most revertants obtained from spontaneous GV$^-$ mutants of *H. halobium* are of the LGV phenotype, producing small amounts of GV. Only a few revertants which exhibit a level of GV similar to that of the wild-type *H. halobium* (HGV revertants) were isolated. LGV wild-type halobacteria and LGV revertants derived from GV$^-$ mutants of *H. halobium* synthesize a GVP (GVP-B) that can be distinguished from the GVP-A of HGV halobacteria by two criteria. (i) GVP-B migrates on PAU gels as a protein with a molecular mass of 20 kDa. This could represent a multimer, as has been demonstrated previously for a cyanobacterial GVP (4). GVP-A migrates under the same conditions as a slightly smaller protein (19 kDa). This difference in size is not observed on SDS-PAU gels and may be explained by only partial denaturation of these hydrophobic proteins on PAU gels. (ii) The amino acid sequence of the N-terminal halves of the two types of proteins differs in two defined positions in the first 40 amino acids. These changes were consistently found in GVP-B of *Halobacterium* sp. strain GRA, an LGV wild-type strain, and LGV revertants derived from GV$^-$ mutants of *H. halobium*. Since the total amino acid composition of GVP-A (19 kDa) and GVP-B (20 kDa) differs significantly in some amino acids (data not shown), additional amino acid changes may occur in the remaining parts of the GV proteins. The two amino acid exchanges documented here can be explained by two G→A transitions in the DNA sequence. It seems highly unlikely that (at least) two mutations are always occurring at exactly the same positions in a single *gvp* gene of *H. halobium*. From this we postulated that the synthesis of GVP-A and GVP-B is controlled by two different genes.

By using an oligonucleotide probe which was primarily deduced from the determined amino acids of GVP-A and which took into consideration the codon usage of *H. halobium* plus the fact that the plasmid pHH1 and hence its genes have a higher A+T content than the chromosome (3), we could identify a plasmid pHH1-associated and a chromosomal DNA fragment in *H. halobium* which specifically hybridized with this oligonucleotide. The two LGV halobacterial strains SB3 and GN101 which we tested showed hybridization only in the chromosomal fragment. This fragment (7.5 kb) appeared indistinguishable in size in all three halobacterial strains, suggesting that not only the chromosomal *gvp* gene itself but also its flanking regions may be conserved in *Halobacterium* spp. A *gvp* gene probe derived from *Calothrix* sp. strain PCC7601 (10) and a cloned 144-bp *Sau*3A fragment from pHH1 (which by DNA sequence analysis proved to be an internal part of the *gvp* gene [Pillay and Rdest, unpublished results]) hybridized with the same two DNA fragments of *H. halobium* and the single chromosomal fragment of *Halobacterium* sp. strains SB3 and GN101. Together, these data suggest that the two GVPs identified here are encoded by two different *gvp* genes, GVP-A by the plasmid pHH1-borne gene and GVP-B by the chromosomal *gvp* gene.

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