Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaeabacterium

(plasmid/nucleotide sequence/copy number/curing/PEG-mediated spheroplast transformation)

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**ABSTRACT** We determined the complete nucleotide sequence of the 6354-base-pair plasmid pHV2 of the archaeabacterium *Halobacterium volcanii*. This plasmid is present in approximately six copies per chromosome. We have generated a strain, *H. volcanii* WFD11, cured of pHV2 by treatment of liquid cultures with ethidium bromide. We describe PEG-mediated transformation of *H. volcanii* WFD11 with intact pHV2 and with a form of pHV2 marked by a 93-base-pair deletion generated in vitro.

Since the recognition that the archaeabacteria are a form of life distinct from both eubacteria and eukaryotes (1, 2), a considerable amount of research has focused on elucidating the biological and biochemical characteristics of this ancient lineage (for reviews see refs. 3 and 4). Although there has been some progress in understanding the molecular genetics of archaeabacteria, this work has been hampered until recently by the inability to introduce exogenous DNA into cells. We have demonstrated transformation of halobacteria (5) with naked DNA from *E. coli*, a natural bacteriophage of *Halobacterium halobium* (6). In addition, Bertani and Baresi (7) have observed low-frequency transformation of *Methanococcus voltae* histidine and purine auxotrophs to prototrophy, using total wild-type DNA. However, there remained a need to obtain transformation with a plasmid as a first step toward the construction of vectors to shuttle between an archaeabacterium and *Escherichia coli*.

The halobacteria have, for purposes of research in molecular biology, an advantage over other groups of archaeabacteria in that they can be maintained and manipulated in the laboratory easily, by methods similar to those for *E. coli*. Mevarech and Werczberger (8) have discovered a natural system for genetic transfer in *Halobacterium volcanii* that invites further development of this species as a model host for halobacterial molecular biology. In the present paper, we describe a native plasmid of *H. volcanii* DS2, demonstrate its introduction by transformation into a strain of *H. volcanii* cured of this plasmid, and present its complete nucleotide sequence.

**MATERIALS AND METHODS**

**Materials.** Restriction endonucleases and the Klengow fragment of *E. coli* DNA polymerase I were obtained from Boehringer Mannheim Canada (BMC), New England Biolabs (NEB), Bethesda Research Laboratories (BRL), and Pharmacia. T4 DNA ligase was from BRL, NEB, and BMC. Exonuclease III and nuclease BAL-31 were from BMC and BRL. Nuclease S1 was from BRL and P-L Biochemicals. RNase-free DNase I was from Promega Biotec (Madison, WI). RNase A was from Sigma. *E. coli* DNA polymerase I and M13 sequencing primer were from NEB. Nucleotides were from P-L Biochemicals. 32P-labeled nucleotides were prepared by J. D. Hofman according to the method of Walseth and Johnson (9).

**Growth of Bacterial Strains.** *H. volcanii* strains DS2 (ref. 10; from C. R. Woese, University of Illinois) and WFD11 (see below) were grown as described (11). For regeneration of spheroplasts in transformation experiments, the medium for plates was modified by addition of sucrose to 15% (wt/vol). For plating *H. volcanii* PH transfected together with *H. halobium* R1 indicator lawn, we used a regeneration medium containing, per liter, 206 g of NaCl, 37 g of MgSO4·7H2O, 3.7 g of KCl, 170 µl of 10 µM MnCl2, 25 ml of 2 M Tris·HCl (pH 7.2), 5 ml of 10% (wt/vol) CaCl2·2H2O, 250 ml of 60% (wt/vol) sucrose, 3 g of Bacto yeast extract (Difco), 5 g of Bacto tryptone (Difco), and 25 g of Bacto agar (Difco) (8 g for top agar). Tryptone and yeast extract, sucrose, CaCl2, agar, and basal salt stock solutions were prepared separately and combined after autoclaving. Other growth conditions and growth of *H. halobium* R1 (ref. 12; from W. Zillig, Max-Planck-Institut für Biochemie, F.R.G.) were as described (5). *E. coli*, strain JM101, was grown and maintained according to standard procedures (13).

**Preparation of DNA.** Total DNA was isolated from halobacteria by quickly lysing resuspended cell pellets in 50 mM Tris·HCl, pH 8/50 mM EDTA/0.2% N-laurylsarcosine, followed by digestion with proteinase K at 0.1 mg/ml for several hours with gentle shaking at 37°C, before phenol extraction and ethanol precipitation. The 6.4-kilobase-pair (kbp) plasmid pHV2 (14) was prepared from *H. volcanii* DS2 using the following methods: (i) CsCl/etidium bromide buoyant density ultracentrifugation (14), (ii) alkaline extraction (method B without column purification of ref. 15), or (iii) rate zonal centrifugation on a sucrose density gradient (16). Phage *ΦH* was prepared as described (6). Plasmids and M13 RF forms from *E. coli* JM101 were prepared by alkaline extraction. The single-stranded M13 DNA was isolated and purified for sequencing as described by Messing (13).

**Cloning and Sequencing.** The 6.4-kbp plasmid pHV2 of *H. volcanii* DS2 cloned at its unique *Pst* I site into a pUC plasmid vector was kindly provided by C. J. Daniels (Ohio State University). Ordered series of deletions for each strand of three restriction fragments subcloned into various pUC or M13mp series vectors (17) were prepared using BAL-31 (18) or exonuclease III followed by nuclease S1 (19). Deleted fragments generated from pUC subclones were subsequently transferred to M13 for sequence analysis, whereas deletions generated in M13 required no further recloning. Both strands of the plasmid were completely sequenced by the dideoxy chain-termination method (13). Small gaps in the initial

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*Abbreviation: ORF, open reading frame.
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†This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J00314).
sequence data, including all restriction fragment junctions, were covered by analyzing specific gel-purified restriction fragments cloned into M13.

We have also generated clones in *E. coli* of pHV2 (cut at one or another of its unique restriction sites) inserted into pUC vectors. The *Hind*III clone, pHV2-H, was used as a nick-translated probe for hybridizations to detect pHV2 in total DNA digests (see below).

**Construction of pHV2Δ93.** A derivative of pHV2 not naturally occurring in *H. volcanii* was constructed in vitro. We excised a 93-base-pair (bp) *SnaBI*-XmnI restriction fragment (positions 4579–4672 in Fig. 1) from pHV2, and recircularized the plasmid with T4 DNA ligase to form pHV2Δ93. Approximately half of the deleted linear plasmid was recircularized, as judged by agarose gel electrophoresis. This mixture was used in a transformation of *H. volcanii* (see below) without the separation of linear from circular molecules.

**Computer Analysis.** Analysis of the sequence data was performed on a Compaq computer system, using the Microgenie software package (20) and on a Macintosh computer using DNA Inspector II (Textco, West Lebanon, NH). Shepherd's Pu-N-Py analysis (21) for protein-coding sequences was performed on a Commodore 64 computer system with programs written by R.L.C.

**Copy Number Determination.** Cell pellets from samples ranging from 10 ml to 4 ml of a *H. volcanii* DS2 culture at stages of growth from midlogarithmic phase through stationary phase were quick-frozen in liquid nitrogen, then stored at −70°C until all samples had been collected. Cell suspensions were lysed in 3 ml of 0.5 M Na2EDTA/1% N-lauroylsarcosine, pH 8, then treated with RNase A at 0.15 mg/ml for 40 min at 37°C, followed by digestion with proteinase K at 0.15 mg/ml for 18 hr at 37°C with gentle shaking. Lysates were then extracted with phenol equilibrated with TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 8), and the phenol phase was back-extracted twice with portions of TE buffer to obtain a maximum recovery of total *H. volcanii* DNA. After purification and concentration by ethanol precipitation, the DNA was digested with *Hind*III to completion. DNA samples were separated on a 1.2% agarose gel at <2 V/cm, then stained with ethidium bromide, and photographed using Polaroid film. Negative/negative gels were scanned with a microdensitometer, the tracing was enlarged, and the peaks corresponding to the plasmid and the nearby single-copy genomic bands on the gel were cut out and weighed on an analytical balance.

**Curing of pHV2.** Liquid medium (2 ml) containing ethidium bromide (20, 37.5, or 50 μM) was inoculated with 0.5–1.2 × 109 cells and incubated for 3 days at 37°C before plating. Total genomic DNA was extracted from 0.5-ml liquid cultures inoculated from resulting colonies, then digested with *Pst* I or *Hind*III, and examined by agarose gel electrophoresis. Absence of plasmid in DNA preparations was confirmed by hybridization analysis with 32P-labeled pHV2-H as probe.

**Transformation of *H. volcanii* WFD11.** *H. volcanii* WFD11 was transformed with pHV2 or pHV2Δ93 using the transfection protocol we described for *H. halobium* (5), except that the spheroplasting solution used for *H. volcanii* contained 1.0 M NaCl instead of 2.0 M NaCl. Briefly, cells were grown to a density of 1.1–1.5 A660 units (≈1.1–1.5 × 108 viable cells per ml) in rich medium, then a 2-ml sample was pelleted (3300 × g) at 37°C, and resuspended in 1/10 vol of spheroplasting solution [1.0 M NaCl, 27 mM KCl, 50 mM Tris-HCl (pH 8.75), 15% (wt/vol) sucrose]. Spheroplasts were formed by adding 20 μl of 0.5 M EDTA (pH 8) with gentle agitation, before adding plasmid DNA, typically in a volume of 10 μl of spheroplasting solution. After a 5-min incubation at room temperature, an equal volume of a solution containing 6 parts of purified PEG-600 to 4 parts unbuffered spheroplasting solution was added with gentle mixing, and the mixture was allowed to stand at room temperature for 30 min. Finally, cells were pelleted in medium salts plus 15% (wt/vol) sucrose, and 100–μl samples were plated in 3 ml of regeneration top agar.

Plates bearing potentially transformed *H. volcanii* were sealed in bags and placed at 37°C. The resulting regenerated colonies were large enough to pick after 2 weeks of growth. Colonies were individually transferred to fresh plates (≈200 colonies per plate), allowed to grow for several days, then lifted onto Colony/Plaque Screen hybridization transfer membranes (New England Nuclear). Filters were hybridized with 32P-labeled pHV2-H according to the manufacturer's directions. Colonies giving a positive hybridization signal were regrown in liquid medium. Total genomic DNA extracted from the cultures was examined by agarose gel electrophoresis and Southern hybridization (16) to verify the presence of pHV2 or pHV2Δ93.

Control transfections of *H. volcanii* with ϕH DNA were performed to monitor the uptake of DNA by each batch of cells. Transfected *H. volcanii* spheroplasts were diluted to 10 ml with medium salts containing 15% (wt/vol) sucrose and pelleted (20 min, 3300 × g) to wash out PEG. Spheroplasts were gently resuspended in 0.2 ml of medium salts plus 15% (wt/vol) sucrose and plated with 200 μl of *H. halobium* R1 cells (at a culture density of about 1 × 108/unit) in 3 ml of regeneration top agar. The *H. halobium* provides an indicator lawn, since *H. volcanii* is not within the range of the PHF.

**RESULTS**

**Sequence Analysis.** Plasmid pHV2 was cloned into a pUC plasmid vector, and subclones from pUC or M13 derivatives were sequenced by the dideoxy chain-termination method. The 6354-bp, 56.0 mol% G+C sequence is shown in Fig. 1. This sequence contains a number of ORFs, four of which (Fig. 2) putatively encode polypeptides of 808, 189, 228, and 200 amino acids, or of 90, 21, 25, and 23 kDa, respectively. Products of ORF1, ORF3, and ORF4 would be acidic, with molecular weights typical of halobacteria (22), whereas the product of ORF2 would be near neutral in charge at pH 7. All four ORFs have similar codon usages that are biased suggesting expression, but biased in some way differently from known *H. halobium* protein coding genes (ref. 23 and data not shown). A high frequency of *Aat* II, *Sal* I, *Tth*1111, and *Xho* I sites (18, 20, 19, and 10 sites, respectively; data not shown) found in this plasmid may be explained by the frequent use of CUC, GAC, GAG, and GUC, four of the six codons most used. The four ORFs show conservation of the RNY codon motif (where R is a purine, N is any nucleotide, and Y is a pyrimidine) of genuine protein coding sequences (21) and also pass the minimum codon information test of Tramontano and Macchiato (24). No homology with existing protein coding sequences was found. There are in addition a number of smaller ORFs for which statistical verification becomes increasingly difficult. Five ORFs have potential coding capacity for 8- to 15-kDa polypeptides, although several of these overlap the larger ORFs.

We have experienced difficulty in cutting pHV2, but not several independent *E. coli* JM101-propagated clones of pHV2 in pUC, with certain restriction enzymes, notably Spe I and Nhe I. These restriction endonucleases, together with Xho I, also cut total genomic DNA from *H. volcanii* very infrequently and share the tetranucleotide core CTAG, sug-

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Fig. 1. Nucleotide sequence of pHV2. The complete nucleotide sequence of 6354 bp was determined by the dideoxy chain-termination method. Numbering begins arbitrarily at the first nucleotide of the HindIII recognition method. The four largest open reading frames (ORFs) are indicated by boxes.

gesting DNA modification near these sites. Mae I, recognizing this core, also does not cut native plasmid.

Copy Number of pHV2 in H. volcanii. As HindIII linearizes the plasmid and cuts the H. volcanii genome rather infre-
Fig. 2. Restriction map and putative ORFs of pHV2. Restriction endonucleases with one (boldface) or two recognition sites only are indicated. Numbers refer to map positions (in kbp) and are consistent with those of Fig. 1. The predicted direction and extent of translation for each of the four longest ORFs is indicated by an arrow.

Eventually, it was straightforward to compare the relative staining intensities of the 6.4-kbp pHV2 band with well-separated single-copy genomic fragments in the 5- to 8-kbp range. The relative content of pHV2 in H. volcanii DS2 was found to be constant over all stages of growth tested, from midlogarithmic phase through stationary phase, and corresponded to 6.4 plasmids per chromosome (data not shown).

Curing H. volcanii of pHV2. Ethidium bromide treatment was used to rid H. volcanii DS2 of pHV2. One colony of 96 colonies screened was found to contain no detectable pHV2 band upon agarose gel electrophoresis, whereas DNA from the other 95 colonies showed obvious 6.4-kbp plasmid bands. Restriction endonuclease-digested genomic DNA of the cured cells was hybridized, after electrophoresis and Southern transfer, to 32P-labeled pHV2-H (an E. coli-propagated clone of pHV2), to verify the absence of pHV2 sequences (Fig. 3, lane 3). We named this cured strain WFD11. There is no visibly apparent phenotypic effect or difference in growth rate from H. volcanii DS2 associated with the loss of the plasmid, in either rich medium or the defined medium described by Mevarech and Werczberger (8). A larger H. volcanii plasmid, the 60-MDa pHV1 (14), is still present in WFD11 (L. C. Schalkwyk, personal communication).

Transformation. The procedure we described for transfecting H. halobium with bacteriophage ΦH DNA (5) was used with reduced NaCl to transform H. volcanii WFD11 with 0.1 μg of pHV2. We picked individual regenerated colonies to gridded plates and allowed these to grow before colony hybridization. Of 10,100 regenerated colonies picked, 18 gave a positive hybridization signal with 32P-labeled pHV2-H. Upon further characterization by agarose gel electrophoresis of extracted total DNA, 17 of these proved to contain pHV2. A representative example is shown in Fig. 3, lanes 7 and 11. These results extrapolate to an efficiency of 107 transformants per μg of pHV2 with 2% of the regenerated spheroplasts expected to be transformed. Spheroplast regeneration was 30% relative to the viable count of the original culture. The control hybridization with H. volcanii with 0.34 μg of ΦH DNA produced 58 plaques, a typical value.

Although cryptic contamination of WFD11 cultures by pHV2-containing cells seemed unlikely, we repeated these experiments using a "tagged" plasmid, obtained by deleting 93 bp between ORF2 and ORF3 in native pHV2. When H. volcanii WFD11 was transformed with 25 ng of circular pHV2Δ93, 2 out of 1300 colonies individually picked and screened by colony hybridization were positive. This represents a frequency of transformation consistent with the frequency seen with natural pHV2. Nru I and Alu I digests of DNA recovered from these two isolates clearly demonstrated that the transformed plasmids both carry the expected deletion in the appropriate restriction fragments (Fig. 3). Both the 2091-bp Nru I band and the 1115-bp Alu I band are smaller by ~90 bp in the two pHV2Δ93-transformed isolates (lanes 5, 6, 9, and 10) compared to both the wild-type H. volcanii DS2 control (lanes 4 and 8) and the pHV2-transformed WFD11 isolate (lanes 7 and 11).

Fig. 3. Hybridization analysis of wild-type, cured, and transformed strains of Halobacterium using a pHV2-specific probe. (A) Restriction digests of total DNA separated on a 1.2% agarose gel and stained with ethidium bromide. DNA samples were extracted from H. halobium R1 (lane 2), H. volcanii WFD11 (lane 3), H. volcanii DS2 (lanes 4 and 8), WFD11 transformed with pHV2 (lanes 7 and 11), and two examples of WFD11 transformed with pHV2-Δ93 (lanes 3, 6, 9, and 10). Samples in lanes 2–7 were digested with Nru I. Samples in lanes 8–11 were digested with Alu I. Lanes 1 and 12 are HindIII-digested bacteriophage λ DNA used as a size marker. (B) Autoradiogram resulting from the hybridization of 32P-labeled pHV2-H (pHV2 cloned at its HindIII site into pUC) to the Southern blot of the gel shown in A.
DISCUSSION

The sequence of pHV2 alone does not suggest any function for the plasmid, and its presence is not required for growth by *H. volcanii* in either rich or defined medium. The lack of any homology between the plasmid and the genomes of either *H. halobium* or *H. volcanii* (Fig. 3, lanes 2 and 3) indicates the lack of repetitive elements, common in these bacteria (25). Earlier work by Pfeifer et al. (14) failed to find homology between pHV2 and plasmids in other species of Halobacterium.

Here, we have demonstrated efficient PEG-mediated spheroplast transformation of *H. volcanii* WFD11 (a derivative of strain DS2 cured of pHV2) with pHV2 and with pHV2Δ93, an artificial construct. Using our method, the efficiency of uptake and expression of *H. volcanii*-propagated pHV2 by *H. volcanii* (10⁷ transformants per μg of DNA) was comparable to the efficiency of transfection of *H. halobium* with *H. halobium*-propagated ΦH DNA (10⁵-10³ transfectants per μg of DNA (5)). When results are normalized to 1 μg of DNA, the frequency of transformation of *H. volcanii* with pHV2 (∼2% of spheroplasts) is also comparable to the frequency of transfection of *H. halobium* with ΦH DNA (0.5-1% of spheroplasts (5)). We have shown (5) that transfection efficiency and frequency are proportional to input DNA, and we expect that this is true for plasmid transformation as well. Phage ΦH DNA is a linear molecule, whereas pHV2 is circular; thus, our method appears to work well for either of these forms.

A method of regenerating normal, dividing cells is essential for a spheroplast transformation system to be broadly useful. As is the case with other halobacterial species (5, 26, 27), we have found that *H. volcanii* spheroplasts regenerate their cupped, disc-shaped appearance (implying regeneration of the glycoprotein cell envelope (26)) when Mg²⁺ levels in the growth medium are returned to normal. Regeneration of *H. volcanii* spheroplasts under our conditions is reasonably efficient: 30-80% of the viable count of the initial culture.

We individually picked and plated regenerated colonies on grid plates for purposes of collecting data on transformation efficiency and frequency. When accurate quantitation is not needed, screening may be more simply performed by direct lifting of colonies from soft agar regeneration plates (data not shown).

To avoid difficulties with restriction of DNA upon entering the cell, transformations described above were performed with native plasmid or with pHV2Δ93, which was constructed from plasmid directly isolated from *H. volcanii* DS2. We have observed a 10⁴-10⁵ times reduction in transfection efficiency in *H. volcanii* DS2 or WFD11 relative to *H. halobium* R1, when *H. halobium* R1-grown ΦH DNA is used. ΦH burst size in *H. volcanii* appears to be within reported ranges (6) for *H. halobium* (unpublished data); thus phage replication is presumably unimpaired. We tentatively attribute the reduction in transfection efficiency to restriction in *H. volcanii*, especially as restriction has already been convincingly demonstrated for other halobacterial species (28-30).

It is encouraging for future prospects to find that the efficiency of transformation in an archaebacterium can compare quite favorably to the most efficient eu bacterial systems. To increase the usefulness of this system, further refinements in both the host bacterium and plasmid vector will be necessary. Such improvements should include trimming the plasmid to its minimal replicating size, inserting a marker that can be selected in the halobacterial host, adding *E. coli* plasmid segments to make the plasmid a shuttle vector, and, for the host strain, overcoming any restriction barrier and perhaps compromising recombination activity.

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