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Genetics

Shuttle vectors for the archaebacterium *Halobacterium volcanii*

(plasmid/selectable marker/transformation/mevinolin)

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ABSTRACT **Progress in archaebacterial molecular biology requires tools for genetic analysis. We describe vectors that can be selected and maintained in either *Halobacterium volcanii* or *Escherichia coli*. A genetic determinant for resistance to the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor mevinolin was isolated by "shotgun cloning" into a derivative of the endogenous *H. volcanii* plasmid pHV2, to form pWL2, which transforms sensitive *H. volcanii* to mevinolin resistance at high frequency. The resistance determinant, portions of pHV2, and an ampicillin- and tetracycline-resistance-conferring pBR322 derivative, pAT153, were ligated together to form the shuttle vectors pWL101 and pWL102. We describe conditions for the use of these vectors and provide preliminary definition of regions essential for drug resistance and for plasmid replication and maintenance.**

Methanogens, sulfur-dependent thermophiles, and extreme halophiles constitute a unique assemblage of prokaryotes that Woese and Fox call the archaebacteria (1). Archaebacteria differ from the rest of the prokaryotes (eubacteria), and from eukaryotes, in very many molecular, biochemical, and physiological features (2–5). However, our knowledge is so far based largely on comparisons of the sequences of archaebacterial genes (cloned in *Escherichia coli*) to their eubacterial and eukaryotic homologues. There is urgent need for (*i*) techniques permitting "classical" genetic analyses and (*ii*) methods that will allow the reintroduction into archaebacteria of archaebacterial genes, including genes that have been altered *in vitro*.

Mevarech and Werczberger (6) have demonstrated genetic exchange between whole cells of *Halobacterium volcanii*, and low-frequency transformation with exogenous DNA has been reported in certain methanogens (7, 8), although these methods have yet to find extensive use. In this laboratory, we showed that purified DNA of the bacteriophage ΦH can be taken up by and expressed in spheroplasted cells of the phage's host, *H. halobium*, at high frequency (up to 10^7 plaques per μg of DNA). Phage ΦH DNA can also transfect (at reduced frequency, presumably because of restriction) *H. volcanii*, which is not a natural host for the phage (9, 10). We also recently demonstrated that pHV2, a naturally occurring 6354-base-pair (bp) *H. volcanii* plasmid that we have sequenced, efficiently transforms a strain of *H. volcanii* previously cured of pHV2 (≈0.2% of regenerated spheroplasts stably transformed, at 0.1 μg of plasmid DNA; ref. 10). We indicated that, if suitable selectable marker genes could be found, pHV2 might form the starting material for construction of shuttle vectors maintainable in either halobacteria or *E. coli*. Here we describe the development of such vectors.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase were from Boehringer Mannheim and New England Biolabs. DNase I was from Promega, and RNase A was from Sigma. Nucleotides, *E. coli* DNA polymerase, and T4 DNA polymerase were from Pharmacia. [α-^{32}P]dATP and [α-^{32}P]dGTP were made by J. D. Hofman (this laboratory), according to the method of Walseth and Johnson (11). *E. coli* plasmid pAT153 (12) was obtained from K. L. Roy (University of Alberta). Mevinolin (13) was generously provided by A. Alberts (Merck Sharp & Dohme International). Polyethylene glycol 600 (PEG 600) (from Sigma) was further purified by a method described by Klebe *et al.* (14), except that the PEG was repeatedly extracted as a liquid at room temperature, with equal volumes of anhydrous ether in a separatory funnel. Residual ether was evaporated at 60°C in a waterbath.

Cell Strains and Media. *H. volcanii* DS2 was obtained from C. R. Woese (University of Illinois). It contains two plasmids, the 90-kilobase-pair (kbp) pHV1 and pHV2 (15). WFD11, a strain cured of pHV2, was derived from DS2 after exposure to ethidium bromide (10). Cells were grown in minimal or complete (rich) medium (see ref. 6), with Tris·HCl (pH 7.2) added to a final concentration of 50 mM. Solid media (including top agar) used for spheroplast regeneration were supplemented with 15% sucrose. Sucrose and agar were combined but autoclaved separately from the rest of the components. Mevinolin-resistant *H. volcanii* strains were maintained on minimal medium containing 10 μM mevinolin or on rich medium with 40 μM inhibitor. Growth was at 37°C for liquid media and 42°C for plates.

Preparation of DNA. *H. volcanii* cell pellets were resuspended and lysed in one-half growth volumes of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. Lysates were gently extracted twice with equal volumes of phenol. Total DNA was spooled onto a glass rod in ethanol and dried in ether.

Plasmids were isolated from *H. volcanii* and *E. coli* DH5α by alkaline extraction (16). Native plasmids pHV2 and pHV51, obtained from strains DS2 and WFD51, were further purified by rate zonal centrifugation on 10–40% sucrose gradients (made in 20 mM Tris·HCl, pH 8.0/5 mM EDTA/1 M NaCl) in an SW40 rotor at 35,000 rpm at 20°C for 14 hr. Recombinant plasmids isolated from mevinolin-resistant transformants were, when necessary, further purified on low-melting agarose gels.

Transformation of *H. volcanii*. *H. volcanii* spheroplasts were prepared as described (10), but the transformation procedure was modified, as follows. Input DNAs were prepared in 125 mM EDTA, typically by adding 5 μl of 0.5 M EDTA (pH 8.0) to 15-μl DNA samples (including ligation mixtures). Carrier RNA in the DNA sample does not interfere with transformation. Five minutes after the addition of DNA (20 μl) to the spheroplasts (220 μl), an equal volume (240 μl) of PEG solution [60% purified PEG 600 (vol/vol)/0.4 M NaCl/10 mM KCl/6% sucrose (wt/vol)] was blended into the spheroplast/DNA mixture by repeated gentle inversions. After a further 20-min incubation at room temperature, 1 ml of regeneration salt solution [3.5 M NaCl/150 mM $MgSO_4$/50 mM KCl/7 mM $CaCl_2$/50 mM Tris·HCl, pH 7.2/15% sucrose (wt/vol)] was added to the 480-μl transformation mixture.

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Cells were pelleted by centrifugation at 6500 rpm for 7 min at room temperature in an MSE microcentrifuge. Pellets were resuspended in 1 ml of rich medium containing 15% sucrose and incubated for 6–12 hr at 42°C. Cells were harvested and resuspended in 1 ml of regeneration salts. One-hundred-microliter samples of the appropriate dilutions were mixed with 3 ml of top agar [minimal medium containing 0.7% agar and 15% sucrose (wt/vol), kept at 60°C] and plated on minimal regeneration agar medium. Mevinolin, when necessary, was added to the regeneration agar (including top agar) to a final concentration of 10 μM.

Isolation of Mevinolin-Resistance Marker. *Eco*RI- or *Mlu* I-digested DNA (pooled from mevinolin-resistant strains) was ligated to pHV51 (linearized by these enzymes) under conditions recommended by the manufacturer. EDTA was added to ligation mixture before transformation, selecting for mevinolin resistance, as described above. Resistant colonies appeared after 10 days and were picked onto minimal agar with mevinolin. After 5 days of incubation, they were probed for the presence of pHV2 sequences by colony hybridization using ^{32}P-labeled pHV2 (as in ref. 10). Total DNAs prepared from hybridization-positive colonies were analyzed on agarose gels for plasmids larger than pHV51. These plasmids were purified and used to retransform WFD11 to show plasmid-mediated mevinolin resistance.

Hybridization. DNAs immobilized to GeneScreen*Plus* hybridization transfer membrane or Colony/Plaque Screen (NEN/DuPont) were hybridized with ^{32}P-labeled probes generated by nick-translation (16).

Shuttle Vector Constructions. Plasmid pWL2 was extracted (as described above) from *H. volcanii* in a mixture with pHV1. Unique restriction sites in the pHV2-derived portion of pWL2 were identified (see Fig. 2). Plasmid DNA was separately digested with *Eco*RI, *Hin*dIII, and *Pst* I and ligated to alkaline phosphatase-treated pAT153 digested with these enzymes. *E. coli* DH5α (Bethesda Research Laboratories) transformed with these ligation mixtures was screened for ampicillin or tetracycline resistance. Resistant colonies were picked and further analyzed by colony hybridization using ^{32}P-labeled pHV2. Plasmids from hybridization-positive colonies were identified by their restriction patterns and confirmed by transforming *H. volcanii* WFD11 to mevinolin resistance.

Unmodified pWL2, reconstructed from *E. coli*-grown pH455, was used as a positive transformation control. To reconstruct pWL2, 10 μg of pH455 DNA was digested to completion with *Hin*dIII. The 15.7-kbp fragment (pWL2 equivalent) was cleanly separated from the 3.7-kbp linear pAT 153 on a sucrose gradient (as described above). Fractions containing the 15.7-kbp fragment were pooled, precipitated, and self-circularized at 1 μg/400 μl. The ligation product was analyzed by agarose gel electrophoresis.

Deletion Analysis. Specific sections of the shuttle vectors were deleted. Plasmids pH455, pE3+, and pWL101 were digested to completion with various combinations of enzymes. After removing the 3′ overhangs using T4 DNA polymerase and filling in the 3′ recessed ends using the Klenow fragment of DNA polymerase (16), these DNA samples were separately self-circularized. Plasmids retrieved from transformed *E. coli* DH5α were analyzed for altered restriction patterns. Various deleted forms (summarized in Fig. 4) were tested for their ability to transform *H. volcanii* WFD11 to mevinolin resistance.

RESULTS AND DISCUSSION

Mevinolin-Resistant Mutants. Determinants available for direct selection of transformants are few; archaebacteria are insensitive to most of the antibiotics used in eubacterial vector-host systems. In 1986, Cabrera and coworkers (17) reported that mevinolin (1,2,6,7,8,8a-hexahydro-β,δ-dihydroxy-2,6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphthaleneheptanoic acid δ-lactone), an inhibitor of eukaryotic 3-hydroxy-3-methylglutaryl coenzyme A reductases, also strongly inhibits this enzyme in halobacterial extracts and prevents cell growth in liquid media. We find that growth of our wild-type *H. volcanii* strain, WFD11 is completely inhibited at mevinolin concentrations of 1–2 μM and 20–40 μM on plates of minimal and rich agar (*Materials and Methods*), respectively. Mutant colonies resistant to the drug appear spontaneously on such plates at a frequency of about 1 in 10^9 cells plated and grow well at mevinolin concentrations up to 100 μM on minimal plates.

Transformation Experiments with Uncloned and Cloned DNAs from Mevinolin-Resistant Mutants. DNAs from four spontaneous mevinolin-resistant mutants (isolated independently from minimal plates with 40 μM mevinolin) were pooled and used in transformation experiments with spheroplasts of *H. volcanii* WFD11, as described in *Materials and Methods*. After 6 hr in rich medium to allow gene expression, cells were plated on minimal regeneration medium containing 10 μM mevinolin. After 10 days on plates, resistant transformants were found at frequencies of $\approx 4 \times 10^3$ per μg of DNA—more than 2000 colonies among $1–2 \times 10^9$ regenerated spheroplasts, when 0.5 μg of total high molecular weight DNA from resistant cells was used (for instance, see Tables 1 and 2). Control transformations (wild-type or no DNA) yielded resistant colonies at a frequency of 1–5 among 10^9 regenerated spheroplasts, the same as the mutation frequency observed with intact cells.

A rare variant of pHV2, pHV51, was used for vector construction. pHV51 was fortuitously detected during plasmid screening of different colonies of *H. volcanii* strain DS2 (R. L. Charlebois and W.L.L., unpublished data). It resulted from the insertion of a single copy of the transposable element ISH*51* (18) at about position 5500 of the pHV2 sequence (see Fig 2; in ref. 10, position 1 is defined as the first nucleotide of the single *Hin*dIII recognition site). The inserted ISH*51* contains convenient sites for cloning with *Mlu* I and *Eco*RI, and further insertions into the ISH*51* moiety of pHV51 seem unlikely to disrupt functions essential for plasmid maintenance.

pHV51 DNA was isolated from *H. volcanii* cultures, digested with *Mlu* I, and ligated with partially *Mlu* I-digested DNA from mevinolin-resistant cells. Spheroplasts of *H. volcanii* WFD11 were transformed with such ligated DNA preparations (the "*Mlu* I library") and plated on minimal agar containing mevinolin. Transformants were readily obtained, the DNA preparation ligated with pHV51 showing about three times as many transformants as a DNA preparation partially digested with *Mlu* I but not ligated to *Mlu* I-digested pHV51. Transformants were screened by colony hybridization with labeled pHV2 DNA; most had reacquired pHV2-related sequences simultaneously with resistance.

To show that these hybridization-positive transformants bore independently replicating plasmids with chromosomal DNA inserted into pHV51, we made plasmid DNA preparations from them. In some cases these bore more than a single inserted *Mlu* I fragment, but all of 24 tested transformants did carry plasmids with a common 7.9-kbp *Mlu* I fragment (Fig. 1). The 7.9-kbp *Mlu* I fragment from the plasmid borne by one such transformant, designated M9 in Fig. 1, was used to probe the total DNA of two independent transformants, M3 and M10, and itself. Strong hybridization signals confirmed that the cloned fragments were identical (Fig. 1 *C* and *E*).

Plasmids were also detected in mevinolin-resistant transformants obtained when WFD11 was transformed with a library prepared with *Kpn* I-digested DNA from mevinolin-resistant cells and *Kpn* I-digested pHV2. These contained

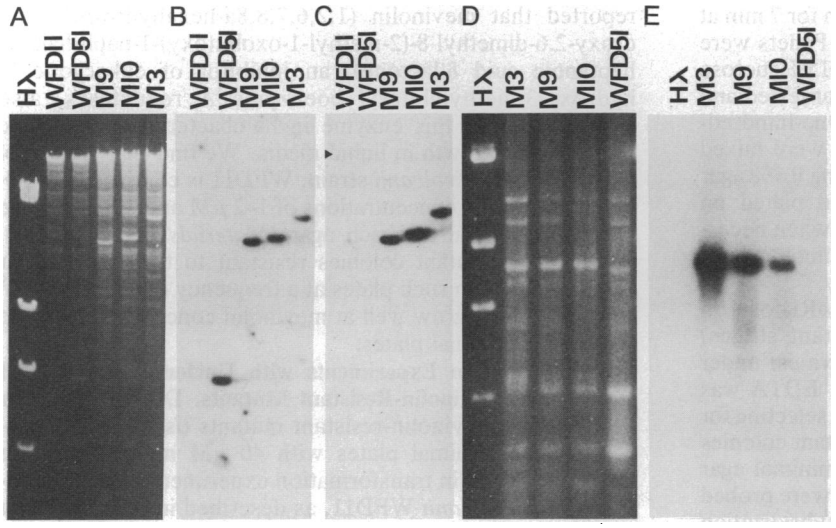

FIG. 1. Characterization of mevinolin-resistant transformants obtained with *Mlu* I library. (*A*) Total DNAs from transformants M3, M9, and M10 (see Table 2), WFD51 (which contains pHV51), and WFD11 (which has been cured of pHV2) were digested with *Hin*dIII and resolved on a 0.5% agarose gel. Hλ, *Hin*dIII-digested λ DNA as size markers. (*B*) The gel shown in *A*, blotted and probed with ^{32}P-labeled pHV2 DNA. (*C*) A duplicate blot probed with a 7.9-kbp *Mlu* I fragment of chromosomal DNA excised from the mevinolin-resistance-conferring plasmid extracted from strain M9. The dark arrowhead indicates the position of the faintly hybridizing band (visible on darker exposures) we assume to be the homologous chromosomal region of the recipient. (*D*) Plasmid DNAs isolated from mevinolin-resistant transformants, digested with *Mlu* I plus *Pst* I, and resolved on a 0.7% agarose gel. (*E*) The gel shown in *D*, blotted and probed with the same 7.9-kbp *Mlu* I fragment described in *C* above.

large (>20 kbp) insertions and have not been characterized further.

When plasmids from mevinolin-resistant transformants such as those designated M3, M9, and M10 in Fig. 1 were isolated and used once again to transform WFD11, mevinolin-resistant colonies were obtained at frequencies between 5 and 10 × 10^7 per μg of DNA, 10,000-fold higher than the frequency obtained with uncloned total DNA from resistant cells (Table 1). We presume that this increase reflects primarily the >1000-fold enrichment provided by cloning, although the fact that plasmid-borne markers need not be integrated into the chromosome to be expressed and that circular DNAs may be more easily taken up (and/or recombined into the chromosome) may well also partly explain this. When plasmids from M3, M9, or M10 were linearized by digestion with *Pst* I or *Hin*dIII (which cut once within pHV51 and not within the inserted mevinolin-resistance fragments; Table 1), transformation frequencies dropped about 100-fold.

Construction of Shuttle Vectors. Plasmids from transformants M3, M9, and M10 can be used as selectable vectors for cloning into *H. volcanii*. For further development of a shuttle vector, pWL2, the mevinolin-resistance-conferring plasmid borne by strain M9, was selected as starting material. A restriction map of pWL2 is shown in Fig. 2; the chromosomal insertion determining resistance is 7.9 kbp, and pWL2 has unique sites for *Nhe* I, *Pst* I, *Spe* I, *Dra* III, and *Hin*dIII. *Eco*RI and *Sna*BI insertions can also be made easily (10).

Hybrid vectors for transformation into *E. coli* were prepared by ligation of pWL2 with the pBR322 derivative pAT153 (12), which lacks 622 bp of pBR322 but retains its ampicillin- and tetracycline-resistance determinants. These hybrid constructs are also shown in Fig. 2. For pH455, *Hin*dIII-digested pWL2 was ligated with *Hin*dIII-digested pAT153 (disrupting its tetracycline-resistance determinant). In p74, the ampicillin-resistance gene is disrupted by ligation of *Pst* I-digested plasmids. Plasmids pE3− and pE3+, obtained by ligation of *Eco*RI-digested plasmids, retain both

Table 1. Transformation of WFD11 with mevinolin-resistance-conferring plasmids from an *Mlu* I library

Source of DNA	DNA, μg	Mevinolin-resistant transformants	
		No.	No. per μg
None	—	$<10^1$	—
Mevinolin res.*	0.5	2.2 × 10^3	4 × 10^3
M3	0.02	2.3 × 10^6	1 × 10^8
M3 (*Pst* I)†	0.02	1.3 × 10^4	6 × 10^6
M9 ("pWL2")‡	0.015	1.5 × 10^6	1 × 10^8
M9 (*Pst* I)†	0.015	1.9 × 10^4	1 × 10^6
M9 (*Hin*dIII)†	0.015	1.4 × 10^4	9 × 10^5
M10	0.005	2.8 × 10^5	6 × 10^7
M10 (*Hin*dIII)†	0.005	2.6 × 10^3	5 × 10^5

Spheroplasts of strain WFD11 (cured of pHV2) were incubated with total cellular ("Mevinolin res.") or plasmid DNA preparations and PEG and plated on minimal agar in the presence of 10 μM mevinolin, in serial 10-fold dilutions. At least 100 colonies at the appropriate dilution were counted for each transformation (except "no DNA" control).

*DNA from four spontaneous mevinolin-resistant strains, mixed in equal proportions. Source of DNA for preparation of *Mlu* I library.

†DNA digested to completion with indicated restriction endonuclease, to linearize plasmid, before transformation.

‡The plasmid borne by strain M9 was designated pWL2.

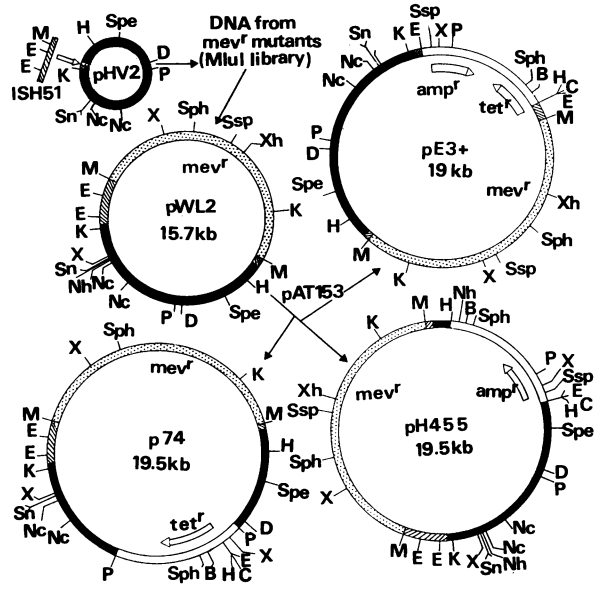

FIG. 2. Restriction maps of pWL2 and hybrids constructed with pWL2 and pAT153. pWL2 is the mevinolin-resistance-conferring plasmid isolated from transformant M9. Solid shading indicates the pHV2 sequence, hatched shading indicates the ISH*51* sequence, and the dotted region is the mevinolin-resistance-conferring *Mlu* I fragment of chromosomal DNA. The unshaded region in pH455, p74, and pE3+/pE3− is the pBR322 derivative pAT153, linearized with *Hin*dIII, *Pst* I, or *Eco*RI, respectively, and ligated into the corresponding sites on pWL2. Restriction endonuclease designations, in this and subsequent figures, are as follows: B, *Bam*HI: C, *Cla* I; D, *Dra* III; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; M, *Mlu* I; Nc, *Nco* I; Nh, *Nhe* I; P, *Pst* I; Spe, *Spe* I; Sph, *Sph* I; Sn, *Sna*BI; Ssp, *Ssp* I; X, *Xmn* I; Xh, *Xho* I.

resistance determinants and differ in the orientation of their pAT153 moieties.

Competent cells of *E. coli* strain DH5α were transformed with the hybrid constructs shown in Fig. 2. Plasmids were prepared from the ampicillin- or tetracycline-resistant *E. coli* transformants obtained and used to transform *H. volcanii* WFD11 spheroplasts, selecting for resistance to mevinolin (Table 2). As one control, we "reconstructed" pWL2 by *Hin*dIII digestion of *E. coli*-propagated pH455, removal of pAT153, and religation, as described in *Materials and Methods*. (This plasmid, designated pΔAT in Table 2; should lack modifications characteristic of DNA prepared from *H. volcanii* cells). With pΔAT, or with any of the constructs containing pAT153, mevinolin-resistant transformants were obtained at frequencies of about 10^4 per μg of DNA. When DNA was prepared from such transformants, plasmids of ≈20 kbp (the expected size) were detected. Crude alkaline preparations of pH455-bearing *H. volcanii* strains transformed *E. coli* DH5α to ampicillin resistance, and the ampicillin-resistant transformants acquired plasmids with the same restriction endonuclease digestion pattern as pH455—confirming its utility as a shuttle vector.

We attribute the relatively low transformation frequencies obtained with hybrid constructs prepared from *E. coli* (compared to around 10^8 per μg obtained with *H. volcanii*-propagated pWL2) to restriction in the archaebacterial host—a similar reduction is observed when DNA from phage ΦH grown in *H. halobium* is used to transfect *H. volcanii*, and yet burst sizes in the two hosts are the same (10). Although a mutant of *H. volcanii* lacking this restriction system(s) has not been isolated, utility of the hybrid constructs described here as shuttle vectors is, for most purposes, not seriously compromised by restriction. A microgram of vector DNA prepared in *E. coli* will produce thousands of mevinolin-resistant *H. volcanii* transformants in a typical experiment, whereas on average less than one new spontaneously resistant mutant appears on plates with comparable numbers (10^8) of regenerated spheroplasts after mock (no DNA) transformations.

Preliminary Tailoring of Shuttle Vectors. To reduce the size and complexity of subsequent generations of shuttle vectors, specific regions of pWL2 and of the hybrid constructs were experimentally deleted. With pH455, deletions of part or all of the major pHV51-derived sequence (the 6.0 kbp between *Eco*RI sites) could be made, without destroying the ability to transform strain WFD11 to mevinolin resistance. Because positive results in such transformation experiments can come not only from acquisition of a stable resistance-conferring plasmid but also from reintegration by homologous recombination between the 7.9-kbp plasmid-borne mevinolin-resistance region and the chromosome of the sensitive recipient, we wished to reduce the size of this region further. A deletion (resulting also in the loss of tetracycline resistance) between the two *Sph* I sites of the construct pE3+ (Fig. 2) did not destroy the ability to transform strain WFD11 to mevinolin resistance. Even a subsequent deletion between *Kpn* I sites, which removed more of the mevinolin-resistance region and all but a few hundred base pairs of the pHV51 moiety, allowed transformation to resistance, albeit at very low frequency (presumably reflecting recombination of the mevinolin-resistance region with the chromosome).

Table 2. Transformation of WFD11 with pWL2-pAT153 hybrid constructs (Fig. 3) prepared from *E. coli*

Source of DNA	DNA, μg	Mevinolin-resistant transformants	
		No.	No. per μg
None	—	1×10^1	—
Mevinolin res.*	0.5	2.0×10^3	4×10^3
pH455	2	2.9×10^4	1×10^4
p74	2	3.0×10^4	2×10^4
pE3−	2	2.4×10^4	1×10^4
pΔAT†	1	2.1×10^4	2×10^4

*DNA from four spontaneous mevinolin-resistant strains, mixed in equal proportions. Source of DNA for preparation of *Mlu* I library.
†Product of removal of pAT153 sequences from pH455 grown in *E. coli*. Should be identical in sequence to pWL2 (designated M9 in Table 2) but lacks *H. volcanii*-specific modifications.

The 3.5-kbp *Sph* I–*Kpn* I fragment, which, from these experiments, must contain a functional mevinolin-resistance determinant, was recloned into pH455ΔM [pH455 religated after removal of the 7.9-kbp *Mlu* I fragment bearing the mevinolin-resistance determinant (Fig. 3)]. The resultant plasmid, pWL101 (Fig. 3), contains complete pHV51 and pAT153 sequences. It was subjected to functional analyses by deletion into the pHV2 moiety from the unique *Cla* I and *Bam*HI sites near the boundaries of the pAT153 component. (pAT153 replication and ampicillin-resistance functions remain intact in such deletions.) Results of these analyses are presented in Fig. 4. A deletion of 762 bp of pHV2 between *Cla* I and *Spe* I sites reduced transformation frequency by >20-fold. Deletions from the opposite direction up to the *Nco* I site do not affect ability to transform WFD11 to mevinolin resistance, but a further deletion, extending to the *Dra* III site, does, again reducing transformation frequency by 20-fold. We assume that these experiments define a region(s) important to plasmid replication in *H. volcanii* and that the smaller number of transformants obtained with plasmids lacking this region(s) (or indeed lacking all pHV2-derived material) results from integration of the mevinolin-resistance determinant into the WFD11 chromosome.

When DNA preparations are made from mevinolin-resistant *H. volcanii* transformants obtained with the deleted vectors designated ΔBM, ΔK, ΔBSn, and ΔBNc in Fig. 4, covalently closed-circular DNAs were found in all of 20 cases. Digestion with *Cla* I produced linearized plasmid DNAs of the expected sizes, with no obvious insertions or deletions. These showed themselves upon probing with la-

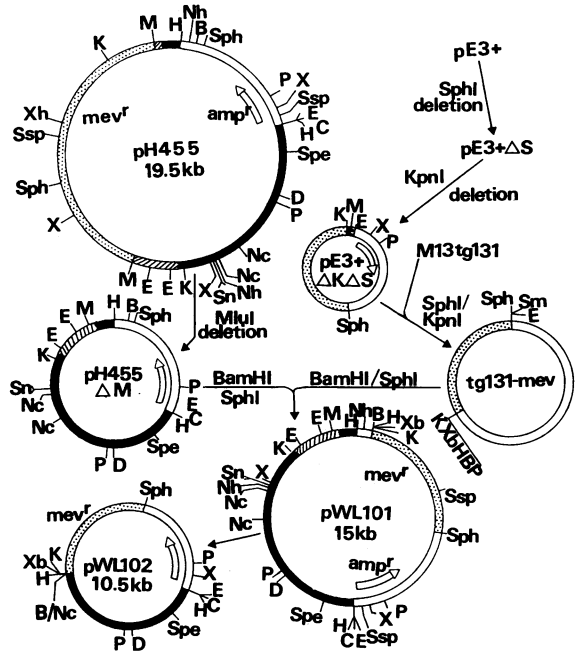

FIG. 3. Construction of pWL101. The mevinolin-resistance fragment was excised from pH455 with *Mlu* I to produce pH455ΔM. This was digested with *Bam*HI and *Sph* I and ligated with *Bam*HI–*Sph* I-digested M13-tg131-mev, which was obtained by ligation of the 3.5-kbp *Kpn* I/*Sph* I mevinolin-resistance determinant of pE3+ into M13-tg131 vector from Amersham. Xb, *Xba* I; Sm, *Sma* I.

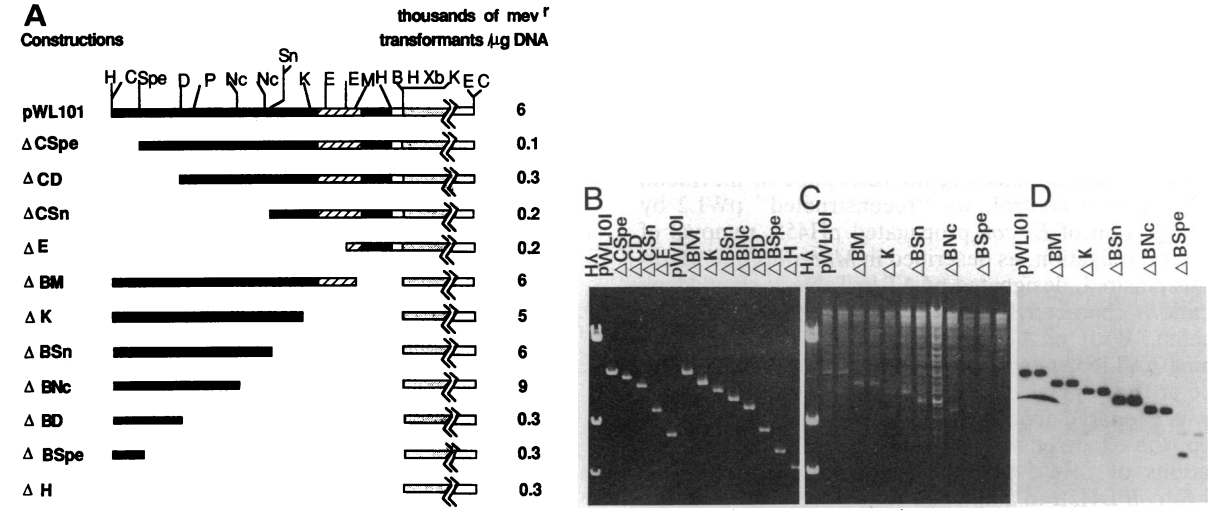

FIG. 4. Deletion analysis of pWL101. Deletions were made by digesting pWL101 with the indicated enzymes, religating and transforming *E. coli* DH5α, with selection for ampicillin resistance. (*A*) Schematic representation of DNA remaining after deletion. pWL101 is shown as linearized at its unique *Cla* I site. The mevinolin-resistance region and the ampicillin-resistance region of pAT153 (between broken lines) are not displayed, as these are retained in all deletions. Shading as in Figs. 2 and 3. Frequencies of transformation of WFD11 to mevinolin resistance obtained with each deleted plasmid are indicated. (*B*) Size analysis of deleted plasmids, prepared in *E. coli* DH5α. Plasmids were linearized and resolved on a 0.7% agarose gel. (*C*) *Cla* I digestions of total DNA obtained from mevinolin-resistant *H. volcanii* colonies from transformation with some of the deleted plasmids shown in *B*. Two independent transformants are presented for each deleted plasmid. (*D*) Southern hybridization of gel shown in Fig. 2*C*, with pAT153 DNA probe.

beled pAT153 to bear sequences from this *E. coli* plasmid (see Fig. 4 *C* and *D*). No such plasmids were seen in preparations of DNA from the few mevinolin-resistant transformants obtained with deletions designated ΔBD and ΔBSpe, but colony hybridization detected pAT153 in some of these transformants, as might be expected from chromosomal integration, by a single crossover, of nonreplicating but circular input DNA. With two such ΔBSpe transformants, Southern hybridization signals obtained when *Cla* I-digested total DNA was probed with pAT153 suggest that there are indeed chromosomal copies of this eubacterial sequence (Fig. 4*D*). It should prove easy to use recombination with nonreplicating selectable markers for insertional mutagenesis and gene tagging.

The 10.5-kbp pWL102 is an effective vector for shuttling between *E. coli* and *H. volcanii*, with selection for ampicillin and mevinolin resistance, respectively. It bears no IS*H51* sequences and contains unique sites for *Kpn* I, *Xba* I, *Sph* I, *Bam*HI, *Nco* I, *Cla* I, and *Eco*RI. Insertions can be made into any of these sites without disrupting plasmid maintenance or resistance functions. The 15.0-kbp pWL101 has additional (*Sna*BI and *Mlu* I) sites for cloning. Further tailoring of vectors and hosts could increase utility, but the system as it is is of high efficiency and fully adequate for (*i*) studies of gene structure and function using archaebacterial genes site-specifically mutagenized *in vitro*, (*ii*) studies of expression of eubacterial and eukaryotic genes in archaebacteria, (*iii*) determination of linkage and dominance, and (*iv*) strain construction.

In our view, pWL102 and pWL101 are only the first in what should become an extensive collection of cloning vehicles. Low molecular weight plasmids potentially exploitable for these purposes have for instance been described and sequenced from *H. halobium* (19, 20). Likely both *H. halobium* and *H. volcanii* will be developed as systems for experimental molecular genetics—the former because it elaborates the bacteriorhodopsin-containing purple membrane so long of interest to biophysicists and the latter because it seems the more stable genetically, grows readily on defined minimal media, and sports a natural system of genetic exchange (6).

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