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A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2

D. Wendoloski,[1] C. Ferrer[2] and M. L. Dyall-Smith[1]

Author for correspondence: D. Wendoloski. Tel: +61 3 8344 5693. Fax: +61 3 8347 1540.
e-mail: d.wendoloski@pgrad.unimelb.edu.au

[1] Department of Microbiology and Immunology, University of Melbourne, Melbourne, VIC 3010, Australia

[2] Departamento de Genetica Medica y Biologia Molecular, Centro Clinico San Carlos, Alicante, Spain

The mevinolin-resistance determinant, *hmg*, encodes the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and is a commonly used selectable marker in halobacterial genetics. Plasmids bearing this marker suffer from instability in *Haloferax volcanii* because the resistance gene was derived from the genome of this species and is almost identical in sequence to the chromosomal copy. In order to reduce the level of homologous recombination between introduced plasmid vectors and the chromosome of *Haloferax*, a homologue of the *hmg* determinant was obtained from the distantly related organism, *Haloarcula hispanica*. The nucleotide sequences of the wild-type genes (*hmgA*) of these two species are only 78% identical, and the predicted protein sequences show 71% identity. In comparison to the wild-type *hmgA* gene, the resistance gene from a mutant resistant to simvastatin (an analogue of mevinolin) showed a single base substitution in the putative promoter. Plasmids constructed using the new resistance determinant were stably maintained under selection in *Hfx. volcanii* and possessed very low recombination rates with the chromosome of this species. In addition, an improved strain of *Hfx. volcanii* was developed to overcome the plasmid instability and growth reduction observed in the commonly used WFD11 strain.

Keywords: HMG-CoA reductase, selectable marker gene, mevinolin, halobacteria, Archaea

INTRODUCTION

Archaea are generally resistant to most antibiotics that are active against Bacteria (Hilpert *et al*., 1981), and few drug resistance markers have been developed for use in genetic manipulations. In halobacteria, the three commonly used resistance determinants are novobiocin resistance (*gyrB*; Holmes & Dyall-Smith, 1990; Holmes *et al*., 1994), trimethoprim resistance (Zusman *et al*., 1989) and mevinolin resistance (*hmg*; Lam & Doolittle, 1989). These have been utilized in plasmid vectors, gene-knockouts, transposons, gene-expression studies, etc.

Mevinolin (Lovastatin, Merck) and its relatives, fluvastatin, pravastatin, and simvastatin, competitively inhibit HMG-CoA reductase, an enzyme found in Eucarya, Archaea and some Bacteria, and used to synthesize mevalonic acid from acetyl-CoA (see Cabrera *et al*., 1986; Lam & Doolittle, 1989, and references therein). In humans, these drugs help lower cholesterol, but in Archaea they can completely halt growth as they block production of isoprenoid lipids (Cabrera *et al*., 1986), the major lipid in the cell membrane. Resistance in *Haloferax volcanii* arises from overproduction of the enzyme, and an up-promoter mutation in *hmgA* has been described and the gene used to construct the first halobacterial shuttle vectors (e.g. pWL102; Lam & Doolittle, 1989, 1992).

The mevinolin-resistance determinant, *hmg*, as well as the other two resistance determinants, were originally isolated from the chromosome of resistant mutants of *Haloferax* spp., and since this host is recombination proficient, homologous recombination events are possible when vectors containing these genes are introduced into *Hfx. volcanii* (e.g. Lam & Doolittle, 1989; Dyall-Smith & Doolittle, 1994). This can severely compromise genetic strategies that rely on selection for drug resistance. *Hfx. volcanii* is a preferred host for the genetic

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; wt, wild type.

The GenBank accession number for the sequence reported in this paper is AF123438.

study of halobacteria, and while a recombination-deficient (*radA*) mutant of this host has been isolated, it is slow growing and is unable to maintain the replication of certain plasmids, e.g. pWL102 (Woods & Dyall-Smith, 1997). In order to lower the recombination rate between the *Haloferax* chromosome and introduced vector plasmids, we sought a selectable marker that showed less sequence similarity to the *Hfx. volcanii* genome. We report here the isolation, cloning, sequence and use in *Hfx. volcanii* of a mevinolin-resistance marker from *Haloarcula hispanica*.

METHODS

Microbial strains, media, and culture methods. Halobacteria were grown in liquid or solid (1·5 % Difco Bacto Agar) modified growth medium (MGM), as described previously (Holmes & Dyall-Smith, 1990). *Haloferax volcanii* strain WFD11 (Charlebois *et al*., 1987) was grown in 18 % MGM and *Haloarcula hispanica* (Torreblanca *et al*., 1986) in 23 % MGM. Liquid cultures were incubated at 37 °C, shaken at 180 r.p.m. *Escherichia coli* strains DH5α (Hanahan, 1983) and JM110 (Yanisch-Perron *et al*., 1985) were grown on Luria–Bertani medium (Miller, 1972) with added ampicillin (50 μg ml^{-1}) or kanamycin (30 μg ml^{-1}) where necessary. Introduction of plasmid DNA into halobacterial cells was performed using the PEG method described by Cline *et al*. (1989), with selection in the presence of 4 μg ml^{-1} (10 μM) simvastatin (Merck). *E. coli* cells were transformed using the calcium chloride method (Ausubel *et al*., 1989). Minimum inhibitory concentrations (MICs) of simvastatin were determined following the general method described by Sahm & Washington (1991) but using 18 % MGM. Simvastatin (Merck) was dissolved in ethanol to a final concentration of 20 mg ml^{-1} and stored at −20 °C.

Plasmids and DNA isolation and analysis. All the plasmids referred to (pMDS95, 99, 100 and 108) are based on pOK12 (Vieira & Messing, 1991). Plasmids were isolated from *E. coli* strains using the alkaline lysis method as described by Ausubel *et al*. (1989), and from *Hfx. volcanii* using the alkaline lysis method as described by Holmes & Dyall-Smith (1990). Restriction endonucleases (AMRAD Pharmacia Biotech or New England Biolabs) were used according to the manufacturer's instructions. Vent DNA polymerase (New England Biolabs) was used for polymerase chain reactions, and PCR products were cloned into plasmid pGEM T-Easy (Promega). DNA sequencing was performed using the 'Dye-deoxy terminator' cycle sequencing kit from Applied Biosystems, with custom oligonucleotide primers. Reactions were analysed on an ABI 373A automated sequencer (Perkin Elmer).

Isolation of a simvastatin-resistant mutant and cloning the *Har. hispanica* resistance determinant. A simvastatin-resistant mutant of *Har. hispanica* was produced by sequential passage in the presence of increasing concentrations of simvastatin. The final culture grew readily in the presence of 20 μg simvastatin ml^{-1}, whereas the MIC of the wt strain was between 0·5 and 1 μg ml^{-1}. Colonies were isolated on solid media and the DNA of one mutant showed no difference in *Mlu*I digestion profile to the wt strain, indicating that resistance was not likely to be due to gene amplification. DNA from this mutant was then used to clone the *hmgA* gene. Chromosomal DNA was cut with a number of restriction enzymes and Southern blots prepared. These were hybridized at moderate stringency to a radiolabelled DNA probe prepared from the *Hfx. volcanii hmgA* gene (carried on plasmid pWL102; see Lam & Doolittle, 1989, 1992). *Bgl*II digestion produced a single band of about 4·3 kb, i.e. large enough to contain the HMG-CoA reductase gene (approx. 1·3 kb; data not shown). *Bgl*II-digested DNA of 4–5 kb was cut out from a preparative agarose gel, ligated to *Bgl*II-cut plasmid pOK12 (Vieira & Messing, 1991), introduced into *E. coli* DH5α, and transformant colonies probed using the *Hfx. volcanii hmgA*. A strongly hybridizing colony was identified which contained a plasmid with an insert of 4·3 kb. The location of the *hmgA* gene was narrowed further by Southern blot hybridization to a 1·6 kb *Not*I–*Pst*I fragment and the gene was completely sequenced (GenBank accession no. AF123438).

Curing *Hfx. volcanii* of the smallest plasmid, pHV2. The commonly used host *Hfx. volcanii* strain WFD11 was cured of the smallest endogenous plasmid, pHV2, by ethidium bromide treatment (Lam & Doolittle, 1989). Recently, it has been found that one of the large plasmids in this strain, pHV3, is unstable and lost at a significant rate, most likely due to the use of the potential mutagen ethidium bromide in its construction. Cells without pHV3 grow more slowly, tend to filament, and lyse when spheroplasted during PEG-mediated transformation procedures (R. Charlebois, personal communication). A new derivative of the wt strain was produced which lacked pHV2 but was not treated with mutagenic agents. Firstly, plasmid pWL102 (a plasmid containing a pHV2 replicon and *hmgA*) was introduced into wt *Hfx. volcanii* NCIMB 2012 cells and simvastatin-resistant colonies selected on solid media. Several transformant colonies were subcultured into liquid medium and grown up under selection. These were passaged a further two times, after which dilutions of each culture were plated for single colonies on solid medium (with drug added). Plasmid minipreps from colonies derived from each culture were analysed by agarose gel electrophoresis and a strain that lacked pHV2, but contained pWL102, was selected. This was then grown in liquid medium without drug selection. After three passages this strain was plated for single colonies on solid media and 50 of these were patched onto plates with or without drug present. Sensitive isolates were tested for the presence of pHV2 by agarose gel electrophoresis of plasmid extracts and one was selected which showed no small plasmids (i.e. pHV2 or pWL102) present, although the larger pHV1 plasmid was retained. This was labelled strain DS70. In contrast to strain WFD11, in which about 10 % of colonies are small and slow growing, the new strain did not show any significant frequency of slow-growing colonies on solid media, and was indistinguishable in growth and transformation characteristics from the parent.

RESULTS

Cloning and sequence of *Har. hispanica hmgA*

The *Har. hispanica* simvastatin-resistance determinant was isolated from the chromosomal DNA of a drug-resistant mutant (see Methods), and a cloned fragment of 1·6 kb was completely sequenced (GenBank accession no. AF123438). The entire sequence was 1601 nt long and contained a 1218 nt ORF (nt 122–1339) encoding a 405 aa putative protein that showed high similarity to known HMG-CoA proteins. The closest sequence found in the GenBank sequence databases was that of *Hfx. volcanii hmgA*, which displayed 78 % identity at the nucleotide level, and 71 % identity in predicted amino acid sequence. The next four most similar (predicted)

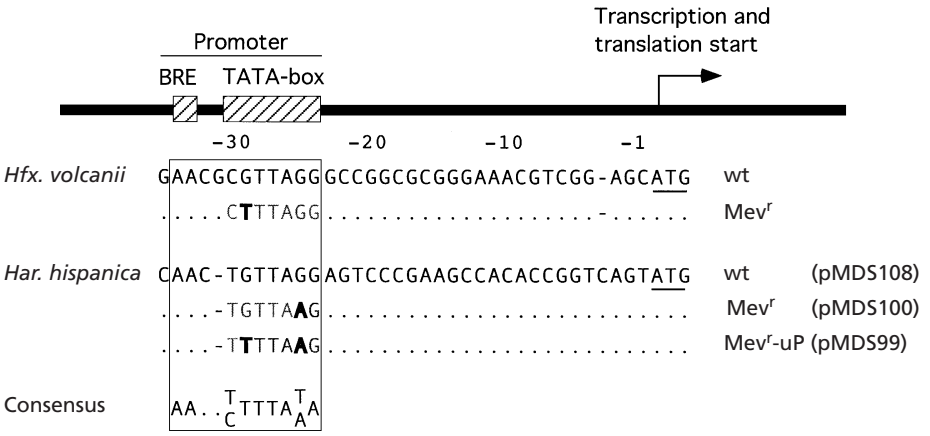

Fig. 1. Promoter regions of the *Hfx. volcanii hmgA* gene (Lam & Doolittle, 1992) aligned with the corresponding 5′ region of the *Har. hispanica* gene. Mutations giving mevinolin resistance (either spontaneous or created *in vitro*) are shown in bold letters. Dots represent unchanged nucleotides. The start codon for both genes is underlined. The consensus promoter motif is taken from Palmer & Daniels (1995) and Soppa (1999), and includes the BRE, transcription factor B responsive element, described by Soppa (1999).

proteins were all putative HMG-CoA reductases from Archaea, i.e. *Methanobacterium thermoautotrophicum*, *Sulfolobus solfataricus*, *Methanococcus jannaschii* and *Pyrococcus horikoshii*.

The *Hfx. volcanii hmgA* gene, like many other halobacterial genes, produces a leaderless mRNA, i.e. with no 5′ leader sequence upstream of the start codon. Transcription begins at the A of the start codon (Lam & Doolittle, 1992), and about 25 nt upstream in the gene is a small AT-rich sequence, typical of strong archaeal promoters (Danner & Soppa, 1996; Palmer & Daniels, 1995; Reiter *et al*., 1990; reviewed by Soppa, 1999). Upstream of the start codon of the *Har. hispanica hmgA* there was a similar AT-rich sequence at a very similar distance to the promoter of *Hfx. volcanii hmgA* (see Fig. 1). Consistent with this being the promoter, the sequence of the drug-resistant *Har. hispanica* gene displayed a single base substitution (compared to the wt gene) in this AT-rich region, forming a sequence closer to the consensus for strong archaeal promoters. This change is similar in position and type to the up-promoter mutation observed in the *hmgA* determinant of *Hfx. volcanii* (Fig. 1; Lam & Doolittle, 1992). To avoid confusion with the previous literature, the marker based on the *Har. hispanica hmgA* gene will be referred to here as the Mev^r locus, even though the drug used for selection was simvastatin. Mevinolin and simvastatin are very similar chemically, and functionally equivalent, but mevinolin is not sold commercially in Australia.

Construction of *Har. hispanica hmgA*-based shuttle vectors

Wild-type and drug-resistant versions of *Har. hispanica hmgA* were PCR amplified from chromosomal DNA preparations, then cloned first into pGEM-T Easy, and finally into a plasmid (pMDS17) that contained the halobacterial replicon from pWL102, oriHV2 (Lam & Doolittle, 1989), and an *E. coli* plasmid vector (pOK12; Wang *et al*., 1990). In addition, a potential up-promoter mutation was introduced by site-directed mutagenesis into the putative promoter region of the Mev^r gene (see Fig. 1), and the modified gene was also introduced into pMDS17. The final plasmids (pMDS108, pMDS100 and pMDS99, respectively) are depicted in Fig. 2. After introduction into *Hfx. volcanii* cells all three plasmids produced simvastatin-resistant transformants at a frequency well above that expected for recombination events alone ($>10^4$ transformants per μg plasmid). Plasmids of the correct size and restriction pattern were recovered from cells transformed by each of the three plasmid constructs, and these could be reintroduced into *E. coli*. The levels of resistance to simvastatin were determined and are given in Table 1. The MIC for the untransformed host was very low (0·3 μg ml^{-1}), and was the same in both the WFD11 and DS70 strains. The transformants bearing plasmids with cloned *hmgA* genes showed much higher resistance. The lowest resistance (MIC 2 μg ml^{-1}) was shown by the cloned wt gene, and the highest resistance (MIC 19 μg ml^{-1}) was shown by the transformant carrying the simvastatin-resistance gene with two up-promoter mutations (denoted Mev^m). The actual resistance of the latter transformant may be higher as the solubility of simvastatin became limiting above 22 μg ml^{-1}. We have successfully used plasmid pMDS99 for cloning halobacterial DNA in *Hfx. volcanii* strains WFD11 or DS70 and *Har. hispanica* (data not shown).

Plasmid stability

The stability of replicating plasmids carrying the *Har. hispanica* simvastatin-resistance gene was tested in *Hfx. volcanii*. Plasmid pMDS99 (Fig. 2) was introduced into *Hfx. volcanii* DS70 cells and drug-resistant colonies grown up on plates containing 4 μg simvastatin ml^{-1}.

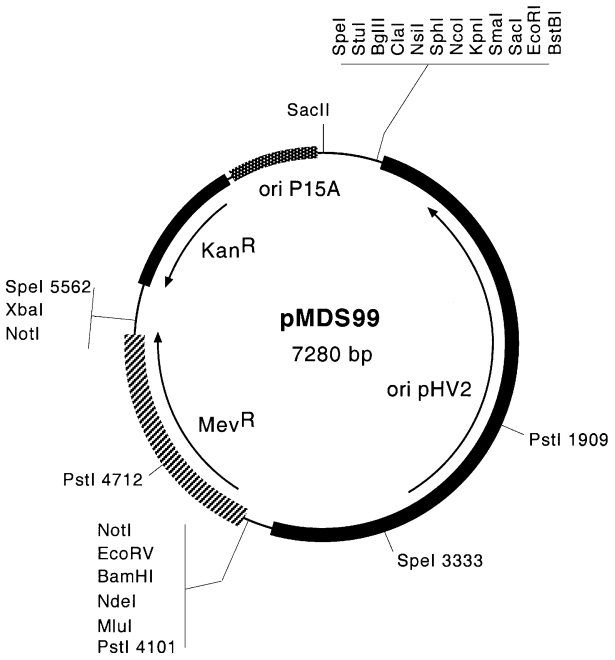

Fig. 2. Map of plasmid pMDS99, showing the sizes and orientation of the major genes, as well as the more important restriction sites. Plasmids pMDS100 and pMDS108 are the same except they contain the promoter sequences (in the *Har. hispanica* mevinolin-resistance gene) as shown in Fig. 1. For replication and selection in *E. coli* there is a medium-copy replicon (ori P15A) and a kanamycin-resistance gene (Kan^R), respectively. Replication in halobacteria is via the pHV2 replicon (ori pHV2; see Charlebois *et al.*, 1987).

One colony was subcultured into liquid medium and grown for 10 generations in the absence of simvastatin, and then plated for single colonies on solid medium (without simvastatin). Colonies were patched onto plates with or without 4 μg simvastatin ml^{-1}. Of 120 colonies examined, 104 had retained resistance to simvastatin (i.e. a mean loss rate of 1·3% per generation). Several of these resistant colonies were grown up in liquid culture (with simvastatin) and their plasmids examined by standard plasmid miniprep isolation and agarose gel electrophoresis. All contained pMDS99, as shown by the number and sizes of restriction fragments separated on agarose gels (data not shown).

Recombination with *Hfx. volcanii*

To determine whether the *Har. hispanica* simvastatin-resistance gene recombined at a high frequency with the genome of *Hfx. volcanii* (most likely at the *hmgA* locus), plasmid pMDS95, containing this gene between the *Pst*I site and the *Not*I site of pOK12, but unable to replicate in halobacteria because it lacks a halophilic origin of replication, was introduced into *Hfx. volcanii* cells and resistant colonies selected on plates with 4 μg simvastatin ml^{-1}. The results of a typical experiment are shown in Table 2. Resistant colonies were observed in very low numbers, i.e. 0–3 colonies per 100 μl of plated transformation mixture ($\leqslant 10^2$ transformants per μg DNA), and this did not change if the plasmid was linearized beforehand (by digestion with *Not*I). These values were comparable to those with control cells that had either no DNA added or a plasmid containing only the novobiocin-resistance determinant (pMDS20; Holmes *et al.*, 1990). Cell competency was checked using replicating plasmids with a mevinolin-resistance marker (i.e. pWL102, pMDS99), and these produced high counts ($> 10^4$ transformant colonies μg^{-1}).

DISCUSSION

A simvastatin (mevinolin) resistance determinant was isolated from *Har. hispanica* that allows selection in *Hfx. volcanii* cells without the disadvantage of recombination at the *hmgA* locus. The low level of recombination fits well with the results of a previous study by Cline & Doolittle (1992), who looked at the reverse situation. They showed that the *Hfx. volcanii* mevinolin-resistance gene did not recombine with the chromosome of *Har. hispanica* cells. The *Har. hispanica* Mev^r marker may be of particular use in experimental strategies where homologous recombination is unwanted but is likely to occur (e.g. transposon muta-

Table 1. Simvastatin resistance in *Hfx. volcanii* strains carrying mevinolin-resistance genes

Species	Strain*	Plasmid (gene)†	MIC (μg ml^{-1})‡
Har. hispanica		–	0·2
Hfx. volcanii	WFD11	–	0·3
Hfx. volcanii	DS70	–	0·3
Hfx. volcanii	DS70	pMDS108 (*Har. hispanica hmgA* Mev^a)	2·0
Hfx. volcanii	DS70	pMDS100 (*Har. hispanica hmgA* Mev^r)	10
Hfx. volcanii	DS70	pMDS99 (*Har. hispanica hmgA* Mev^m)	19
Hfx. volcanii	DS70	pWL102 (*Hfx. volcanii hmgA*)	19

* The WFD11 strain was originally described by Lam & Doolittle (1989); the DS70 strain is described in this study.

† The mutations in the promoter motifs of the mevinolin-resistance genes of drug-resistant mutants are given in Fig. 1.

‡ Simvastatin precipitated out at concentrations above 22·5 μg ml^{-1}.

Table 2. Recombination of the cloned resistance determinant with the *Hfx. volcanii* genome

Plasmid	Halobacterial replicon	Marker (origin)	Transformation (c.f.u. μg^{-1})*	
			Linear plasmid	Uncut plasmid
pWL102	pHV2	*hmgA* (*Hfx. volcanii*)	5×10^2	$9{\cdot}6 \pm 0{\cdot}9 \times 10^4$
pMDS95	–	*hmgA* Mev^r (*Har. hispanica*)	3×10^2	$\leqslant 1 \times 10^2$
pMDS99	pHV2	*hmgA* Mev^m (*Har. hispanica*)	$\leqslant 1 \times 10^2$	$2{\cdot}3 \pm 0{\cdot}1 \times 10^4$
No DNA	–	–	–	$< 1 \times 10^2$

* Error values are standard deviations. Where no error values are shown, the plate counts were very low and included plates with 0 or 1 colony, resulting in high standard deviations.

genesis), or where a vector is desired to be shuttled between different species of halobacteria (excluding *Haloarcula* spp.), such as between *Hfx. volcanii* and *Halobacterium salinarum*. The marker provides additional functionality to the limited set of drug-resistance markers available but still shares a significant sequence similarity to the homologues in *Hfx. volcanii* and *Hb. salinarum*. Recently we have adapted a bleomycin-resistance marker (ShBle; Nuttall *et al.*, 2000) for use in halobacteria. The ShBle gene comes from fungi and shares no homologue in haloarchaea, so should not suffer from any homologous recombination events when introduced into these cells.

The *Har. hispanica* gene is similar to other archaeal *hmgA* genes and retains the critical catalytic residues in the predicted protein, i.e. amino acids 66 (Glu), 101 (Glu) and 193 (Asp) (see Wang *et al.*, 1990). The cloned wt gene conferred increased resistance upon *Hfx. volcanii* cells, showing that simply increasing the gene copy is sufficient to produce a resistant phenotype. The copy number of the pHV2 replicon has previously been estimated to be about six per cell (Charlebois *et al.*, 1991). The underlying mutation in the isolated resistance gene appears to be the same as that observed for the mevinolin-resistance gene of *Hfx. volcanii* (Lam & Doolittle, 1992), i.e. a single up-promoter mutation causing overproduction of HMG-CoA reductase. Further evidence that the mutation occurred within the promoter of the gene was obtained by introducing an additional change nearby, forming a sequence motif even closer to the consensus for strong halobacterial promoters (Danner & Soppa, 1996; Palmer & Daniels, 1995; Soppa, 1999). The additional up-promoter mutation increased the level of drug resistance about twofold.

The WFD11 strain of *Hfx. volcanii* was derived from the wt strain by ethidium bromide treatment, to cure it of the smallest cryptic plasmid pHV2 (Charlebois *et al.*, 1987). Unfortunately, this process appears to have introduced mutations into the genome, and resulted in the observation (R. Charlebois, personal communication) that a significant proportion of the cells in a population of the WFD11 strain are relatively slow growing, and lack the 442 kb plasmid pHV3. Such strains are less suitable for genetic work. The pHV3 plasmid has now been fully sequenced and contains many recognizable ORFs (R. Charlebois & J. Shaw, personal communication) and one tRNA gene. We avoided the use of mutagens, which may affect the maintenance of pHV3, and eliminated pHV2 from *H. volcanii* NCIMB 2012 using plasmid incompatibility (with pWL102) followed by screening for spontaneous plasmid loss. Strain DS70 does not show the small-colony (slow-growing) pHV3$^-$ variant seen in strain WFD11, and performs equally as well in transformations, plasmid isolations, etc.

The halobacterial replicon of pMDS99 was obtained from pWL102, and this derives from a segment of the *Hfx. volcanii* cryptic plasmid pHV2 (Charlebois *et al.*, 1987; Lam & Doolittle, 1989). The latter plasmid is extremely stable in its natural host but the cloned replicon is less so. A recent study of pWL102 stability in *Hfx. volcanii* showed a rate of loss of 49% over 28 generations, or 1·75% per generation (Ortenberg *et al.*, 1999). Our results show a similar loss of 1·3% per generation, but both this and the previous values are far higher than the rates originally reported by Lam & Doolittle (1989) in *Hfx. volcanii*, and by Cline & Doolittle (1992) in *Haloarcula* spp. (i.e. <5% loss over >30 generations, or $<0{\cdot}16$ % per generation). While this instability can be a problem when cells are not kept under selection, it has the advantage of allowing easy recovery of cured hosts.

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