Journal of Bacteriology, June 1991, p. 3807–3813
0021-9193/91/123807-07$02.00/0
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Vol. 173, No. 12

Construction and Use of Halobacterial Shuttle Vectors and Further Studies on *Haloferax* DNA Gyrase

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Received 22 January 1991/Accepted 3 April 1991

We report here on advances made in the construction of plasmid shuttle vectors suitable for genetic manipulations in both *Escherichia coli* and halobacteria. Starting with a 20.4-kb construct, pMDS1, new vectors were engineered which were considerably smaller yet retained several alternative cloning sites. A restriction barrier observed when plasmid DNA was transferred into *Haloferax volcanii* cells was found to operate via adenine methylation, resulting in a 10^3 drop in transformation efficiency and the loss of most constructs by incorporation of the resistance marker into the chromosome. Passing shuttle vectors through *E. coli dam* mutants effectively avoided this barrier. Deletion analysis revealed that the gene(s) for autonomous replication of pHK2 (the plasmid endogenous to *Haloferax* strain Aa2.2 and used in the construction of pMDS1) was located within a 4.2-kb *SmaI-KpnI* fragment. Convenient restriction sites were identified near the termini of the novobiocin resistance determinant (*gyrB*), allowing the removal of flanking sequences (including *gyrA*). These deletions did not appear to significantly affect transformation efficiencies or the novobiocin resistance phenotype of halobacterial transformants. Northern blot hybridizations with strand- and gene-specific probes identified a single *gyrB-gyrA* transcript of 4.7 kb. This is the first demonstration in prokaryotes that the two subunits of DNA gyrase may be cotranscribed.

Plasmid cloning vectors, fundamental to the rapid advancement of archaebacterial genetics, have recently been described for use in the extreme halophiles *Haloferax* spp. and *Halobacterium* spp. Two antibiotic resistance markers (mevinolin [16] and novobiocin [12]) were developed, and several plasmid and phage replication origins have been studied in detail (1, 16). The combination of these elements has produced a number of cloning vectors which have already been applied to the study of gene expression in halobacteria (1, 12, 16). Previously, we described the cloning of a novobiocin resistance (Nov^r) determinant from a mutant of *Haloferax* strain Aa2.2 into the resident multicopy plasmid pHK2, producing pMDS2 (12). The sequence of this determinant, which has recently been published (13), was recognized as being homologous to the eubacterial *gyrB* gene.

Several laboratories have reported the existence of a restriction barrier between *Escherichia coli* and *Haloferax volcanii* strains (1, 2, 12, 16), resulting in lowered transformation efficiencies. More importantly, the majority of *H. volcanii* transformants lost their plasmid constructs, presumably because of restriction of incoming DNA followed by recombination of the drug resistance markers with the homologous genes on the chromosome. This seriously impeded genetic work with shuttle vectors, so we have investigated the effect of DNA methylation on the restriction of DNA entering *H. volcanii* cells.

Here we present the shuttle vector pMDS1 and two smaller derivatives, pMDS10 and pMDS11, which were based on pMDS2 and the *E. coli* plasmid vector pBS(+). The new vectors were shown to efficiently transform and to be stably maintained (under selection) in both hosts.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The following *Haloferax* strains were used in this study: *Haloferax* phenon K Aa2.2 obtained from M. Torreblanca (University of Alicante, Alicante, Spain); a Novr mutant of strain Aa2.2 (DS26 [12]); *H. volcanii* NCMB2012 (National Collection of Marine Bacteria, Aberdeen, Scotland); and *H. volcanii* WFD11, which lacks pHV2 (2) (kindly provided by W. F. Doolittle, Dalhousie University, Halifax, Canada). The 10.5-kb multicopy plasmid pHK2 from *Haloferax* strain Aa2.2 and a Novr determinant from the resistant mutant of this strain were used in the construction of shuttle vectors. Growth medium, transformation medium, and the polyethylene glycol transformation procedure have been described previously (12). Novobiocin was included in transformation medium plates at 0.1 and 0.2 μg/ml for *Haloferax* strain Aa2.2 and *H. volcanii*, respectively. Liquid cultures were grown at 37°C while shaking on an orbital shaker, and plates were incubated at 37°C in sealed containers.

The *E. coli* strains XL1-Blue (Stratagene, La Jolla, Calif.), JM110, JM101 (25), and JP3477 (a gift from A. J. Pittard, University of Melbourne, Victoria, Australia) were used in shuttle vector transformation experiments with the plasmid vector pBS(+) (Stratagene), providing genes for autonomous replication and ampicillin resistance in these strains. *E. coli* XL1-Blue was grown on YT-Tet medium (YT medium containing 15 μg of tetracycline per ml) (18), and transformants were grown on YT-TAXI medium (YT medium supplemented with 15 μg of tetracycline per ml, 50 μg of ampicillin per ml, 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, and 0.1 mM isopropyl-β-D-thiogalactopyranoside). The other *E. coli* strains and transformants were grown on similar media but without tetracycline.

The extraction of plasmid DNA from late-log-phase *Haloferax* cultures (12) and *E. coli* strains (18) was done as previously described. Plasmid DNAs were purified by cesium chloride-ethidium bromide density gradient centrifuga-

tion (18). Small-scale plasmid preparations (12, 18) were made for the rapid screening of transformants.

Northern (RNA) blot hybridization. *Haloferax* strain Aa2.2 cultures were grown to late log phase (~24 h), diluted to an optical density at 550 nm of 0.6 in prewarmed growth medium, and then grown for a further 2 h with ("induced") or without ("uninduced") 0.01 μg of novobiocin per ml. RNA was then isolated by sodium dodecyl sulfate lysis of 10-ml cultures in the presence of sodium azide (10 mM), followed by phenol and then chloroform extractions (22). It was then precipitated with ethanol and resuspended in a small volume of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8). Most solutions used during RNA isolations were pretreated with diethyl pyrocarbonate and then autoclaved.

RNA samples (2 to 10 μg) were electrophoresed on formaldehyde-agarose gels and transferred to zeta-probe nylon filters (Bio-Rad) by capillary blotting for over 4 h in the presence of 50 mM NaOH.

Four ^{32}P-labeled DNA probes were generated, a double-stranded 1.4-kb *Sal*I probe (nick translation kit; Bresatec) and three single-stranded (ss) probes, p1 (255 bases), p2 (365 bases), and p4 (245 bases), which were specific for open reading frame 1 (ORF1), ORF2, and ORF4, respectively (see Fig. 3). These ss probes, corresponding to nucleotides 773 to 519, 3913 to 3574, and 1653 to 1901 in *gyrB* (13) and *gyrA* (sequence to be published), were prepared as follows: specific primers were extended by using the Klenow fragment of DNA polymerase I (Bresatec) incorporating [^{32}P]dATP and [^{32}P]dCTP; a defined 3′ end was obtained by using a restriction endonuclease which cleaved at a desired site; and the ss labeled probes were then purified on a 6% (wt/vol) acrylamide sequencing gel (18). The nick-translated and ss probes (2×10^5 to 6×10^5 cpm) were then included in hybridizations under the high-stringency conditions recommended by the membrane manufacturer.

Plasmid vector constructions. Plasmid DNAs were digested with restriction endonucleases (Pharmacia or Boehringer Mannheim), fragments were isolated by electrophoresis onto dialysis tubing when necessary (18), and specific fragments were ligated together by using T4 DNA ligase (Pharmacia). Most plasmid manipulations were performed in *E. coli* XL1-Blue and passaged through *E. coli* JM110 prior to transformation of *Haloferax* strains. Minipreparations of plasmid from Nov^r *Haloferax* transformants were digested with restriction enzymes and analyzed by agarose gel electrophoresis to confirm the presence of the expected plasmid construct.

RESULTS

Construction of a shuttle vector. The development of a halobacterial plasmid vector (pMDS2) based on the 10.5-kb *Haloferax* strain Aa2.2 plasmid pHK2 and a 6.7-kb *Kpn*I Novr determinant from a mutant of this strain has been described elsewhere (12). This plasmid could be introduced into *Haloferax* spp. by using the polyethylene glycol-mediated transformation method (3), with the resulting transformants having a resistance phenotype very similar to that of the original mutant strain (12). We now sought to construct a shuttle vector which would allow halobacterial genes to be manipulated in *E. coli* prior to their expression in halobacteria. After partial digestion of pMDS2 with *Kpn*I, the *E. coli* plasmid pBS(+) was incorporated to provide the necessary sequences for plasmid selection and maintenance in *E. coli*. Restriction mapping revealed the location and orientation of

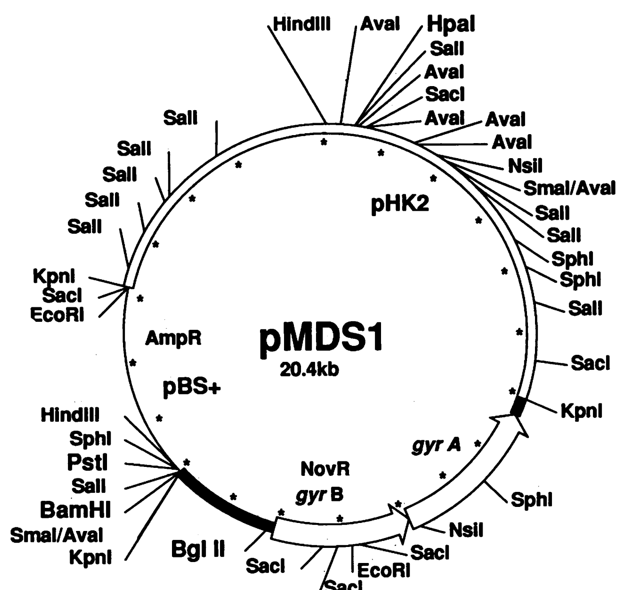

FIG. 1. Restriction map and diagram of the shuttle vector pMDS1. It includes the entire 10.5 kb of pHK2, the 6.7-kb *Kpn*I fragment containing the Novr determinant (*Sal*I sites in this fragment not shown), and the 3.2-kb *E. coli* plasmid pBS(+). This vector has unique *Hpa*I, *Bgl*II, *Bam*HI, and *Pst*I sites. Asterisks denote 1-kb divisions.

fragments within this new shuttle vector, pMDS1 (Fig. 1), which is stably maintained under selection in both hosts (results not shown). Although functional, this plasmid was rather large (20.4 kb) and contained redundant *Kpn*I, *Hin*dIII, and *Sma*I sites. To improve the vector further, we deleted regions of pHK2 and the fragment carrying the Novr determinant that were nonessential.

Locating the origin of replication in pHK2. Fragments were progressively deleted from the pHK2 sequences within pMDS1, and the resulting plasmids were introduced into *H. volcanii* cells to determine which region of pHK2 was necessary for plasmid maintenance. Removal of a 4.3-kb region, extending from the leftmost *Kpn*I site (Fig. 2) to the *Hin*dIII site in pHK2 (pMDS1 cut down to pMDS9), and then removal of a larger 6.3-kb region, extending from this *Kpn*I site to the *Sma*I-*Ava*I site (pMDS1 cut down to pMDS8; Fig. 2), did not significantly alter plasmid copy number or stability (data not shown). However, upon deletion of the region between the rightmost *Kpn*I site and the second *Sph*I site in pHK2 (pMDS1 cut with *Sph*I and religated; data not shown), the constructs could not be maintained in *H. volcanii*, indicating that there are essential sequences in this region.

The subsequent shuttle vector constructs pMDS10 and pMDS11 were based on the smallest deleted version of pHK2 identified in this way (see Fig. 6). Plasmid vectors pMDS8 and pMDS9 lacked the *E. coli* pBS(+) component but do provide an alternative cloning site (*Bgl*II) and were quite stable in *Haloferax* spp. under selection.

Transcriptional analysis. In order to determine which plasmid vector cloning sites can be utilized, it was essential that the limits of the resistance gene be defined. A considerable portion (5,112 bp) of the 6.7-kb *Kpn*I fragment has now been sequenced (Fig. 3), and the Novr determinant was identified as being homologous to the gene encoding the B

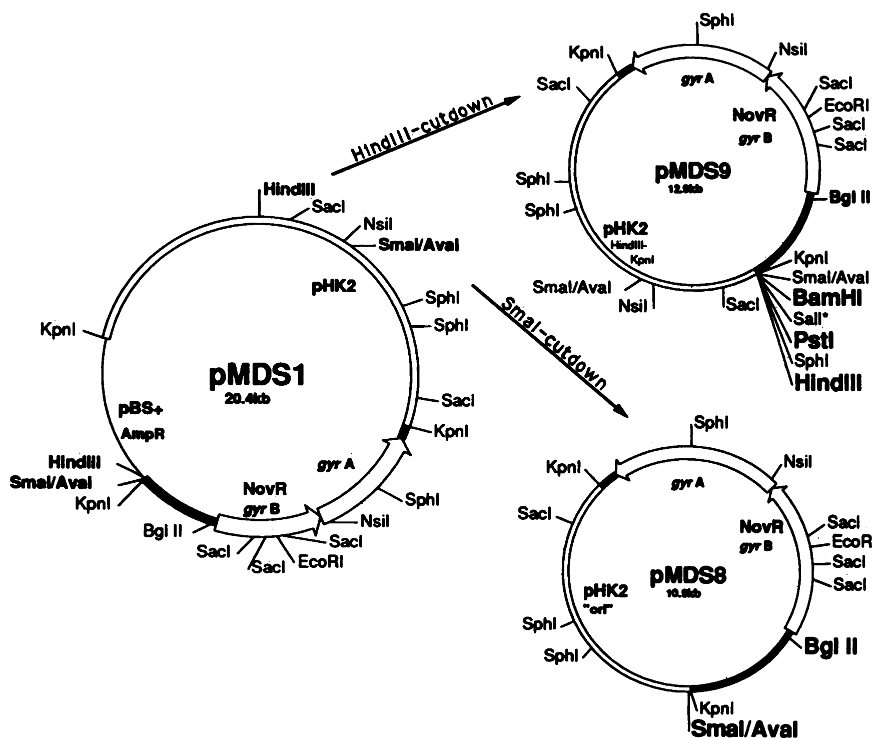

FIG. 2. Location of the region in pHK2 responsible for autonomous replication in *Haloferax* strains. The shuttle vector pMDS1 was restricted with *Hin*dIII or *Sma*I and religated, and the plasmids (pMDS9 and pMDS8, respectively) were then tested for their ability to transform *H. volcanii* WFD11 to Novr and be stably maintained in this host. The 4.2-kb *Sma*I-*Kpn*I fragment of pHK2 was the smallest fragment identified which maintained stability and copy number in *H. volcanii* WFD11.

subunit of DNA gyrase in eubacteria (13). Three other long ORFs are also present in this region (13), including the *gyrA* equivalent (ORF2) one base downstream of *gyrB* (ORF1). Preliminary Northern blot hybridization experiments, using a 1.4-kb nick-translated *Sal*I fragment from within *gyrB* to probe total RNA from *Haloferax* strain Aa2.2, identified a transcript running fractionally below the largest ribosomal RNA band (13). It was therefore estimated to be approximately 2.8 kb, somewhat larger than the predicted 2-kb *gyrB* message if ORF1 alone was transcribed but too small to

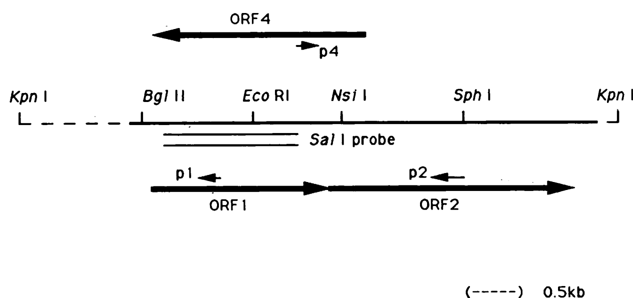

FIG. 3. Diagram of the 6.7-kb *Kpn*I fragment containing the Novr determinant. A 5.1-kb region has now been sequenced (boldface line) and contains three long ORFs (large arrows). These correspond to *gyrB* (ORF1), *gyrA* (ORF2), and an unknown gene transcribed in the opposite direction (ORF4). A fourth ORF (ORF3) (13) does not appear to be expressed and is not shown here. The relative positions of the three ss probes (p1, p2, and p4) and a double-stranded nick-translated probe (*Sal*I) used in Northern hybridizations are shown.

encode both GyrB and GyrA. This discrepancy, as well as the discovery of another ORF overlapping this probe but on the opposite strand to *gyrB*, led us to begin a more detailed analysis of gene expression in this region. Three probes were used which were both strand and gene specific (Fig. 3), and the results of the Northern blot hybridizations are shown in Fig. 4 and 5. The double-stranded *Sal*I probe and the ss-p4 probe (ORF4 specific) were both found to hybridize to the 2.8-kb mRNA previously thought to be the *gyrB* transcript (Fig. 4, lanes B and C) (13). The other two probes, p1 (*gyrB* specific) and p2 (*gyrA* specific), failed to identify any homologous transcripts (Fig. 5, lanes N u) until wild-type *Haloferax* strain Aa2.2 cultures were treated with sublethal concentrations of novobiocin prior to RNA extraction (Fig. 5, lanes N i). Both probes then hybridized to a much larger band, estimated to be 4.7 kb, indicating that *gyrB* and *gyrA* are cotranscribed and that their expression is inducible. In the same experiment, the signal from the ORF4-specific (ss-p4) probe was very weak (Fig. 5, lanes N) and a longer exposure was required (Fig. 4, lane C). Expression of ORF4 does not appear to be induced by novobiocin (Fig. 5, lanes N i; p4 signal no stronger than in N u [uninduced] lanes).

Tailoring of the Novr determinant. Instability of plasmid shuttle vectors in *Haloferax* spp. may be due in part to homology between sequences in the constructs and genes within the chromosome. It is therefore desirable to remove any sequences from constructs which are not absolutely necessary for selection so as to decrease the chance of recombination. Despite numerous early attempts to reduce the size of the fragment containing the Novr determinant, we had not been able to subclone any fragments significantly smaller than the 6.7-kb *Kpn*I fragment which maintained the

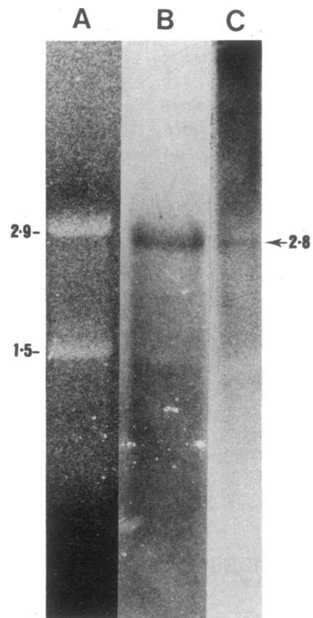

FIG. 4. Detection of a 2.8-kb transcript from ORF4 by Northern blot hybridization. Total RNA was extracted from a *Haloferax* strain Aa2.2 culture grown in the absence of novobiocin. This was then electrophoresed through formaldehyde-agarose gels and transferred to nylon membranes by capillary blotting. Ribosomal RNA bands, seen clearly after staining the agarose gels with ethidium bromide (lane A), were used as kilobase weight markers. Both ^{32}P-labeled DNA probes, p4 (specific for ORF4, lane C) and the double-stranded *Sal*I probe (lane B, complementary to regions within ORF4 and *gyrB*), hybridized to a message running fractionally below the 2.91-kb ribosomal RNA band.

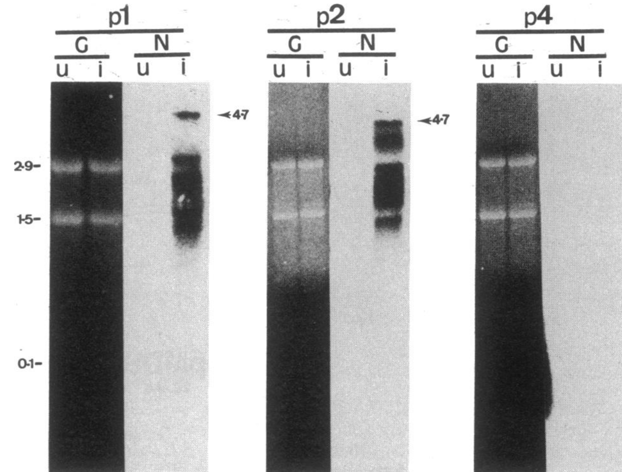

FIG. 5. Northern blot hybridization analysis of ORF1, ORF2, and ORF4 transcripts. RNAs were extracted from *Haloferax* strain Aa2.2 cultures that had been grown for 2 h in the presence (induced; i lanes) or absence (uninduced; u lanes) of 0.01 μg of novobiocin per ml. These were electrophoresed through formaldehyde-agarose gels and stained with ethidium bromide (G lanes) and then blotted onto zeta-probe nylon membranes (N lanes) and probed with ^{32}P-labeled DNA probes specific for *gyrB* (ORF1, p1), *gyrA* (ORF2, p2) or ORF4 (p4). Size markers are in kilobases.

full novobiocin resistance characteristics of the mutant of *Haloferax* strain Aa2.2. We had been frustrated by the lack of suitable restriction sites, as many endonucleases failed to cut the 6.7-kb *Kpn*I fragment (e.g., *Hin*dIII, *Pst*I, *Bam*HI, and *Sma*I), while some shredded it (e.g., *Sal*I) and others (*Eco*RI and *Sac*I) cleaved at sites later found to be within the coding region of the Novr marker. However, computer analysis of the region sequenced revealed the presence of two unique restriction sites (*Bgl*II and *Nsi*I) flanking the Nov^r *gyrB* ORF. Deletion of sequences upstream of *Bgl*II (pMDS10, Fig. 6) and downstream of *Nsi*I (pMDS11, Fig. 6) did not alter resistance to novobiocin or stability of plasmids in *H. volcanii* transformants (data not shown).

Restriction barriers. Genetic manipulations in *E. coli* followed by transformation into *H. volcanii* revealed a restriction barrier lowering the transformation frequency by several orders of magnitude. From experiments unrelated to this, we had found that unmethylated plasmid DNA (from *E. coli* JM110) was not restricted in *H. volcanii*, and we then explored this observation in more detail. The shuttle vector pMDS1 was grown up in three different *E. coli* strains (Table 1), and the state of plasmid methylation was confirmed by using methylation-sensitive restriction endonucleases (*Mbo*I and *Scr*FI; data not shown). The various pMDS1 plasmid preparations were then used to transform *H. volcanii*. Plasmid DNA with methylated A and C residues (i.e., grown up in *E. coli* JM101) showed a 10^3 drop in transformation efficiency, whereas unmethylated DNAs (i.e., from *E. coli* JM110) transformed at frequencies similar to that of plasmid prepared from *H. volcanii* (Table 1). It appears that a restriction system in *H. volcanii* is operating via adenine methylation, as pMDS1 preparations from a *dam dcm$^+$ E. coli* strain (JP3477) also transformed at high rates. It should be noted, however, that *dam* mutants tend to be unstable (19), and constructions should therefore use *dam$^+$* strains followed by passage through JM110 just prior to *H. volcanii* transformation.

The shuttle vectors pMDS10 and pMDS11 were prepared in *E. coli* XL1-Blue and passaged through *E. coli* JM110, and they were shown to transform *H. volcanii* WFD11 at frequencies similar to that of pMDS1 (Table 2).

NA resistance. Resistance to nalidixic acid (NA) is generally associated with amino acid substitutions in the A subunit of DNA gyrase (8, 9, 15, 26), but in *E. coli* two resistant strains were found to have point mutations in *gyrB* (24). In order to check whether the three point mutations we identified in the Nov^r *Haloferax gyrB* affected the sensitivity of this strain to NA, MICs were determined for several different *Haloferax* strains (Table 3). NA is inactivated at high levels of magnesium (Mg^{2+}) (7, 23), so saltwater media with reduced Mg^{2+} were used for these determinations. The MICs for *H. volcanii* were 150 μg of NA per ml (at 25 mM Mg^{2+}) and 100 μg of NA per ml (at 10 mM Mg^{2+}). *Haloferax* strain Aa2.2 was inhibited by 150 μg of NA per ml at 25 mM Mg^{2+} and grew so poorly at 10 mM Mg^{2+} that an MIC could not be determined. The Novr mutant of this strain had exactly the same MIC as did the wild type, indicating that the three point mutations at the amino terminus of *gyrB* do not alter the sensitivity of *Haloferax* DNA gyrase to NA. We also found that the MIC of novobiocin for the Novr mutant was greatly reduced at lower Mg^{2+} levels, with 0.1 μg of novobiocin per ml being sufficient to almost completely inhibit growth at 25 mM Mg^{2+} compared with 7 μg of novobiocin per ml at 136 mM Mg^{2+} (the normal concentration in growth medium).

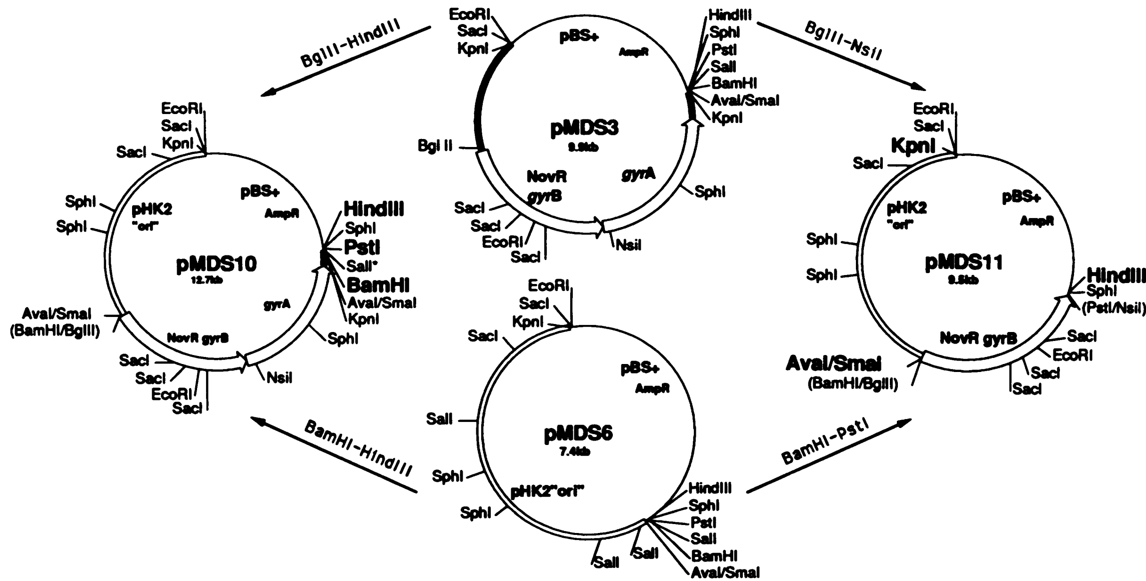

FIG. 6. Construction of shuttle vectors pMDS10 and pMDS11. Two pBS(+) clones, containing the 6.7-kb *Kpn*I Nov[r] determinant (pMDS3) and the 4.2-kb *Sma*I-*Kpn*I fragment identified as responsible for the autonomous replication of pHK2 (pMDS6), were the sources of shuttle vector fragments. The vector pMDS10 contains complete *gyrB-gyrA* genes, whereas pMDS11 contains *gyrB* and only 190 bases at the 5′ end of *gyrA*. These constructs could be shuttled between *H. volcanii* WFD and *E. coli* JM110 without significant loss in transformation efficiency or plasmid stability. Names of unique cloning sites are enlarged. *Sal*I sites are not shown in the pHK2 "ori" or the Nov[r] determinant fragment of pMDS3, pMDS10, and pMDS11.

DISCUSSION

The absence of adenine methylation at the sequence GATC in *H. volcanii* (17) had been demonstrated previously by using restriction endonucleases which recognize the sequence only when it is methylated (*Dpn*I) or unmethylated (*Mbo*I). We have now shown that *H. volcanii* possesses a restriction system similar to the *E. coli* restriction system which recognizes and degrades DNA which is methylated at adenine residues (10). The passaging of plasmids through *dam E. coli* strains such as JM110 should improve the utility of shuttle vectors and facilitate the screening of *E. coli*-grown plasmid libraries in *Haloferax* spp.

In the process of eliminating nonessential sequences from the initial shuttle vector, pMDS1 (20.4 kb), the genes for the autonomous replication of pHK2 were located within a 4.2-kb *Sma*I-*Kpn*I fragment. Further reduction in the size of this fragment was difficult because of a lack of suitable restriction sites, since remaining *Sal*I and *Sph*I sites appear to be located within crucial sequences. The corresponding region in the *H. volcanii* plasmid pHV2 was found to be 3.7 kb and included a long ORF of 2.4 kb (2, 16).

To simplify the shuttle vector further, the actual limits of the Nov[r] determinant were defined by Northern blot hybridizations so that regions within the 6.7-kb *Kpn*I fragment adjacent to the resistance marker could be removed. The lengths of predicted and observed transcripts are in good agreement if *gyrB* and *gyrA* are cotranscribed: the *gyr*-specific probes (p1 and p2) hybridized to a message of approximately 4.7 kb, and the length of a message from the *gyrB* transcriptional start site (13) to a putative termination sequence at a string of six T's downstream of the *gyrA* ORF is 4,633 bp. Therefore, it was not surprising that the derivative pMDS10 (which includes both *gyrB* and *gyrA* genes) maintained the ability to confer novobiocin resistance to *H. volcanii*, but the deletion of *gyrA* sequences downstream of the *Nsi*I site also produced a selectable plasmid vector pMDS11. In this case, there may be a fortuitous termination sequence in the adjacent *E. coli* pBS(+) sequence.

Very few transcriptional analyses of eubacterial *gyrB*s have been published, but it would seem that our results are the first example of the DNA gyrase genes being cotranscribed. Judging from the close proximity of *gyrA* and *gyrB* in *Staphylococcus aureus* and *Mycoplasma pneumoniae*, it is conceivable that they are also cotranscribed in these organisms (4, 14), but as yet the necessary experiments have not

TABLE 1. Effect of DNA methylation on the transformation of *H. volcanii* by plasmid pMDS1

Plasmid DNA source	Methylation genotype[a]	*H. volcanii* Nov[r] transformants/μg of pMDS1
E. coli		
JM101	*dam*[+] *dcm*[+]	2.5×10^2
JP3477	*dam dcm*[+]	3×10^5
JM110	*dam dcm*	5×10^5
H. volcanii WFD11		10^6

[a] *dam*[+] strains have methylated A residues in the sequence GA^{m}TC; *dcm*[+] strains have methylated C residues within the sequence CC^{m} ($^{A}_{T}$) GG (19).

TABLE 2. Transformation efficiencies of shuttle vectors pMDS10 and pMDS11

Plasmid DNA source	Transforming plasmid	*H. volcanii* WFD11 Nov[r] transformants/μg of plasmid DNA
H. volcanii WFD11	No DNA	0
	pMDS10	8.7×10^6
	pMDS11	1.3×10^6
E. coli JM110 (*dam dcm*)	pMDS10	2.8×10^6
	pMDS11	0.4×10^6

TABLE 3. MICs of NA for *Haloferax* strains with various concentrations of Mg^{2+}

Strain	Novobiocin concn (μg/ml)	Growth at NA concn (μg/ml) of[a]: 0	50	100	150	MIC[a] (μg/ml)
H. volcanii WFD11	0	+ (+)	+ (+)	+ (−)	− (−)	150 (100)
Haloferax strain Aa 2.2						
Wild type	0	+ (S)	+ (−)	− (−)	− (−)	100 (ND)[b]
Novr mutant	0	+ (S)	+ (−)	− (−)	− (−)	100 (ND)
Novr mutant	0.1	S (−)	− (−)	− (−)	− (−)	ND (ND)

[a] Cells were grown in 25 mM Mg^{2+} or (in parentheses) 10 mM Mg^{2+}. +, growth; −, no growth; S, slight growth. Growth was recorded after 3 days of incubation at 37°C on an orbital shaker. Mid- to late-log-phase cultures were diluted 1/100 prior to addition of NA.
[b] ND, MIC could not be determined because of the poor growth of cells in medium without NA.

been published. In *E. coli*, *gyrB* is expressed at very low levels unless DNA supercoiling is relaxed, at which time specific relaxation-stimulated transcriptional sequences respond by enhancing expression approximately 10-fold (20, 21). A similar system may exist in *Haloferax* spp., as novobiocin concentrations similar to those required to block gyrase activity resulted in the level of *gyrB-gyrA* message being increased markedly compared with that of uninduced cells (Fig. 5). Previously, we identified direct and indirect repeated sequences upstream of the *gyrB* ORF (13), and these could be important in *gyrB* expression and in novobiocin-induced enhancement. However, sequences identical to the relaxation-stimulated transcription sequences of *E. coli* (21) and *Klebsiella pneumoniae* (5) could not be identified.

It appears that *gyrB* in *Haloferax* spp. is normally expressed at low levels and is tightly regulated. No *gyrB-gyrA* transcript (4.7 kb) was identified in Northern blots of RNAs from *Haloferax* strain Aa2.2 containing the plasmid pMDS2 (13) even though these genes were present on a multicopy plasmid (approximately seven copies per cell) (12). The observed differences in novobiocin sensitivities between wild-type *Haloferax* strains and mutant or plasmid-transformed strains harboring the Nov^r *gyrB* are due to the three known point mutations and not to overexpression of *gyrB* (13). The inducibility of *gyrB* expression upon the addition of sublethal concentrations of novobiocin suggests that the *gyrB* promoter could potentially be used to enhance the transcription of halobacterial genes cloned in downstream of it.

The single mRNA of approximately 2.8 kb identified previously in uninduced *Haloferax* cultures by using the double-stranded *Sal*I probe overlapping both ORF1 and ORF4 (13) probably corresponds to a transcript produced from ORF4, since the ORF4-specific probe (p4) hybridized to a message of this size (Fig. 4, lane C). The function of the ORF4 product is still unknown, but if it is essential to the viability of *Haloferax* cells, the constraints upon possible mutations in ORF1 resulting in Novr could be very significant. This would explain the observed low frequency of mutations giving rise to high levels of novobiocin resistance in *Haloferax* spp. (12). The three point mutations we identified in the Nov^r *gyrB* sequence (13) did not result in the premature termination of ORF4 on the opposite strand. In fact, they produced fairly conservative amino acid substitutions.

It is possible that mutations in *gyrA* may result in increased resistance to quinolones and that another drug resistance marker could be obtained in this way. The three mapped *gyrB* mutations do not alter the sensitivity of DNA gyrase to NA, as is indicated by the identical NA MICs for wild-type and Novr mutant strains of *Haloferax* spp.

Haloferax strains are relatively resistant to NA, with concentrations of 100 to 150 μg of NA per ml being required to inhibit the growth of cultures. It is possible that the so-called new quinolones (norfloxacin, enoxacin, ofloxacin, and ciprofloxacin) would inhibit *Haloferax* spp. at lower concentrations, as these drugs are much more potent inhibitors of *E. coli* (26). All of these quinolones possess chemical groups which can chelate Mg^{2+} ions, and it is these structures which are essential for antibacterial action (23). High Mg^{2+} concentrations effectively inactivate quinolones, so levels of Mg^{2+} in saltwater media were significantly reduced prior to the determination of *Haloferax* NA MICs. It is also interesting to note that high salt concentrations destroy the electrostatic forces necessary for DNA-gyrase complex formation in *E. coli* (11) and that the *Haloferax* DNA gyrase must have evolved to function in the high internal salt concentrations found in these organisms (6).

Further tailoring of our plasmid shuttle vectors is under way, with the rearrangement of *E. coli* pBS(+) sequences such that the multicloning site is left intact. This will facilitate the identification by blue/white selection of *E. coli* transformants with desired clones prior to *Haloferax* transformation. The inclusion of alternative cloning sites is desirable, but as it stands, pMDS1 and its derivatives (pMDS8, pMDS9, pMDS10, and pMDS11) collectively provide eight unique restriction sites (*Bgl*II, *Bam*HI, *Pst*I, *Hpa*I, *Sma*I, *Ava*I, *Hin*dIII and *Kpn*I) for the cloning of genes in *Haloferax* spp.

ACKNOWLEDGMENTS

This research was financed by a grant from the Australian Research Council. M.H. and S.N. were supported by Australian Postgraduate Research Awards.

The technical assistance of V. Triantafillou is gratefully acknowledged.

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