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A Plasmid Vector with a Selectable Marker for Halophilic Archaebacteria

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A mutant resistant to the gyrase inhibitor novobiocin was selected from a halophilic archaebacterium belonging to the genus *Haloferax*. Chromosomal DNA from this mutant was able to transform wild-type cells to novobiocin resistance, and these transformants formed visible colonies in 3 to 4 days on selective plates. The resistance gene was isolated on a 6.7-kilobase DNA *Kpn*I fragment, which was inserted into a cryptic multicopy plasmid (pHK2) derived from the same host strain. The recombinant plasmid transformed wild-type cells at a high efficiency (>10^6/μg), was stably maintained, and could readily be reisolated from transformants. It could also transform *Halobacterium volcanii* and appears to be a useful system for genetic analysis in halophilic archaebacteria.

Archaebacteria are phylogenetically distinct from eubacteria and eucaryotes and can be broadly divided into three groups: methanogens, sulfur-dependent thermoacidophiles, and extreme halophiles (for a review, see reference 22). Numerous halobacteria have been isolated from hypersaline environments around the world, but it is only recently that their taxonomy has been analyzed in a rigorous and comprehensive fashion. The studies of Tindall et al. (20) and Torreblanca et al. (21) identified six genera: *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Natronobacterium*, and *Natronococcus*.

Genetic analysis of halobacteria has been complicated in strains such as *Halobacterium halobium* by the high rate of spontaneous mutation due to insertion sequences and because *H. halobium* strains have complex nutrient requirements (15). However, there are halobacteria with low mutation rates that grow in a mineral salts medium containing a single carbon source (16), and these are obvious choices for studying gene structure and function if only suitable gene transfer systems, such as transformation or transduction, were available. Fortunately, an efficient DNA transformation method was recently described by Cline and Doolittle (4), using both *H. halobium* and *Halobacterium volcanii* (3), which opens the way to methods of contemporary genetic analysis. Plasmid transformation was possible, but with no selectable marker, the detection of transformants was extremely laborious (3). To date, the transformation of other archaebacteria is either extremely inefficient, such as in methanogens (1), or not currently possible, as is the case with the sulphur-dependent thermoacidophiles; so, of the three main branches of archaebacteria, the halobacteria show the most promise for genetic research.

Members of the genus *Haloferax* are aerobic chemoorganotrophs, only moderately halophilic, requiring 2.0 to 2.5 M NaCl for optimum growth, and are capable of growth on single carbon sources. We chose to use an isolate (Aa 2.2) belonging to phenon K (21). It grew rapidly in liquid and on solid media, had a low frequency of spontaneous mutation, and contained a small multicopy plasmid. These were considered to be useful properties for constructing a model genetic system in the archaebacteria, but a remaining obstacle was the lack of selectable traits, such as antibiotic resistance.

The aim of this study was to find a selectable marker for use in constructing plasmid vectors suitable for isolating and studying genes in halobacteria. We report the successful construction of such a vector by using a gene conferring resistance to novobiocin and a cryptic plasmid from *Haloferax* isolate Aa 2.2.

MATERIALS AND METHODS

Bacterial strains. The following halobacterial strains were used in this study: *Haloferax* phenon K isolate Aa 2.2 from M. Torreblanca, University of Alicante, Alicante, Spain; *Halobacterium* (now *Haloferax*) *volcanii* NCMB2012 and *Halobacterium halobium* NCMB777 from the National Collection of Marine Bacteria, Aberdeen, Scotland; and *Halobacterium halobium* DSM617 and strain RI from R. Schinzel, Würzburg, Federal Republic of Germany.

Escherichia coli XL1-Blue, used in all *E. coli* transformation experiments, was obtained from Stratagene, La Jolla, California.

Media and growth conditions. A 25% (wt/vol) solution of artificial salt water (SW) was prepared by using a slightly modified formulation of that described by Rodriguez-Valera et al. (16) and contained per liter: 197 g of NaCl, 17 g of $MgCl_2 \cdot 6H_2O$, 26 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, 5 g of KCl, and 5 g of NH_4Cl. All *Haloferax* media were prepared by using this stock solution. Growth medium (GM) contained SW (18%, wt/vol), yeast extract (0.5%, wt/vol; Oxoid Ltd., London, England) and 25 mM Tris hydrochloride buffer (pH 7.5). Solid media were prepared by adding Bacto-Agar (15 g/liter; Difco Laboratories, Detroit, Mich.). Cultures were incubated at 37°C in an orbital shaker (liquid media) or in plastic containers (solid media).

Haloferax transformation medium (TM) contained SW (18%, wt/vol), sucrose (15%, wt/vol), $MnCl_2$ (1.7 μM), Tris hydrochloride (25 mM, pH 7.5), yeast extract (0.3%, wt/vol) and tryptone (Oxoid; 0.5%, wt/vol). Novobiocin was included in GM and TM at 0.1 μg/ml when required.

All solutions and growth media for *H. halobium* and *H. volcanii* were made up as previously described (3, 4). For the *H. halobium* strains, novobiocin was included in selective media at 0.2 μg/ml.

E. coli XL1-Blue was grown at 37°C in YT medium (9)

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containing tetracycline (15 μg/ml) or on YT-TAXI plates, which contained YT medium supplemented with tetracycline (15 μg/ml), ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (0.1 mM), and Bacto-Agar (15 g/liter).

Transformation of bacteria. *Haloferax* transformations were performed by using the polyethylene glycol (PEG) method described by Charlebois et al. (3). Freshly inoculated cultures were shaken at 37°C until the cells had reached a density (A_{550}) of 1 to 1.5 (corresponding to a viable count at 1×10^9 to 2×10^9 CFU/ml) and were then centrifuged (4,000 × *g*, 10 min) and suspended in a 1/10 volume of buffered spheroplasting solution. The spheroplasting solution had reduced NaCl (1 M) but was otherwise the same as described previously (3). After PEG treatment, spheroplasts were diluted in a solution containing SW (18%, wt/vol), sucrose (15%, wt/vol), and Tris hydrochloride (20 mM, pH 7.5) and allowed to recover for 5 to 12 h at 37°C, and then 100-μl samples were spread onto TM plates containing novobiocin (0.1 μg/ml) and incubated for 3 to 5 days at 37°C in plastic containers.

For transforming *H. volcanii* and *H. halobium* strains, we followed exactly the procedures described by Cline and Doolittle (4) and Charlebois et al. (3).

Isolation of plasmid DNA. For large-scale plasmid isolation from *Haloferax* strain Aa 2.2, DNA was isolated from late-log-phase cultures by the following procedure. Cells were harvested by centrifugation at 2,800 × *g* for 15 min and were washed once with 1 M NaCl. The cells were then suspended in a buffer containing 1 M NaCl and 0.1 M EDTA and lysed by the addition of 0.007 M sodium deoxycholate. This solution was left on ice for 30 min before spinning down cellular debris and chromosomal DNA at 28,000 × *g* for 30 min. The cleared lysate was transferred to a clean tube, and DNA was precipitated by the addition of 10% (wt/vol) PEG 6000 and incubation on ice for 60 min. After being centrifuged at 2,300 × *g* for 10 min, the pellet was dissolved in a small volume of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8]), and plasmid DNA was purified by centrifugation in cesium chloride-ethidium bromide density gradients (9).

Small-scale rapid plasmid preparations were performed for screening purposes by using the alkaline lysis method (9), except that cells were initially suspended in 1 M NaCl before lysis and no lysozyme was added.

DNA manipulations. Restriction endonucleases were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used according to the enzyme instructions of the manufacturers. Partial cleavage of plasmid DNA was accomplished by including 100 μg of ethidium bromide per ml in the DNA solution along with the restriction endonuclease (12). Agarose gel electrophoresis, DNA ligations, etc., were performed as described in Maniatis et al. (9).

rRNA sequencing. Total RNA was extracted from mid-log-phase cultures of *Haloferax* strain Aa 2.2 in the presence of the RNase inhibitors 1,10-phenanthroline (2 mM) and heparin (0.2 mg/ml) (6). The 16S rRNA was then sequenced by using avian myoblastosis virus reverse transcriptase (Bio-Rad Laboratories, Richmond, Calif.) (11). The universal sequencing primers for 16S rRNA described by Lane et al. (8) were used, as well as additional oligonucleotide primers, which were synthesized as required. Not all of the 16S rRNA sequence could be determined by this method because of strong stops presumably caused by secondary structure. Several sequencing ambiguities were resolved by chasing sequencing reactions with terminal deoxynucleotidyl transferase (Boehringer Mannheim) (5).

Plasmid map determination. The 10.5-kilobase (kb) cryptic plasmid of *Haloferax* strain Aa 2.2, pHK2, was isolated from an agarose gel by electrophoresis onto a dialysis membrane (9), linearized with *Hin*dIII, and cloned into the *E. coli* plasmid pUC18 (23) by using T4 DNA ligase (Bresatec, Ltd., Adelaide, South Australia). A physical map was determined by restricting 0.5 to 1 μg of DNA with various restriction endonucleases (both single and double digests) and resolving the fragments on agarose or polyacrylamide gels. Sizes of fragments were then estimated by using *Hin*dIII-cut lambda DNA or 1-kilobase-pair-ladder DNA standards (Boehringer Mannheim).

As some of the *Sal*I and *Ava*I fragments were rather small and difficult to place, the nuclease digestion method (9) was also used. *Hin*dIII-cut pUC18-pHK2 (10 μg) was incubated with Bal31 nuclease (New England BioLabs, Inc., Beverly, Mass.; 0.75 U) prior to restriction with *Sal*I or *Ava*I. Samples were removed at various time intervals and analyzed on 10% polyacrylamide gels. The progressive disappearance of DNA bands then helped to position the restriction fragments within pHK2.

Determination of plasmid copy number. The copy number of pHK2 (number of plasmids per cell) was estimated by carefully extracting total DNA from mid-log-phase cultures of *Haloferax* cells grown in GM, running serial dilutions of the DNA preparation on an agarose gel (0.5%, wt/vol), and comparing intensities of DNA bands stained with ethidium bromide to those of DNA standards of known concentrations. The number of viable cells used in the DNA preparation was determined by plating dilutions onto GM plates, incubating for 2 to 3 days at 37°C, and counting colonies.

Selection of novobiocin-resistant *Haloferax* mutants. The MIC of novobiocin was first determined and found to be approximately 0.005 μg/ml. Using this value as a guide, twofold dilutions of novobiocin in GM broth were set up, inoculated with *Haloferax* strain Aa 2.2, and incubated for several days at 37°C. The culture with the highest concentration of novobiocin that showed visible growth was subcultured into fresh novobiocin broths, and the process continued until rapid growth occurred in cultures containing 0.2 μg of novobiocin per ml. After plating out onto GM agar, a single colony was chosen for further study.

RESULTS

Taxonomic status of *Haloferax* phenon K. Based upon cultural and biochemical data obtained by Torreblanca et al. (21), the organism used in this study, *Haloferax* phenon K strain Aa 2.2, was believed to be closely related to *H. volcanii*. To confirm this, total cellular RNA was purified and the 16S rRNA was sequenced directly by using synthetic DNA primers and reverse transcriptase. A total of 1,186 bases of *Haloferax* strain Aa 2.2 sequence was determined (Fig. 1), and when compared with the published rRNA sequence of *H. volcanii*, there were only three nucleotide differences (0.25%), demonstrating that the two strains were indeed closely related but not identical.

Growth characteristics of *Haloferax* phenon K. *Haloferax* strain Aa 2.2 had a generation time of 3 to 4 h when grown in liquid (GM) culture at 37°C in an orbital shaker (data not shown). Stationary phase was reached after approximately 26 h, by which time cell densities had reached 3×10^9 to 4×10^9 viable cells per ml of culture. Growth on solid media was also quite rapid, and colonies could be counted after 2 to 3

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        90        100        110        120        130        140        150        160        170        180
GAAAAGCTCA GTAACACGTG GCCAAACTAC CCTACAGAGA ACGATAACCT CGGGAAACTG AGGCTAATAG TTCATACGGG AGTCATGCTG GAATGCCGAC
       190        200        210        220        230        240        250        260        270        280
TCCCCGAAAC GCTCAGGCGC TGTAGGATGT GGCTGCGGCC GATTAGGTAG ACGGTGGGGT AACGGCCCAC CGTGCCGATA ATCGGTACGG GTTGTGAGAG
       290        300        310        320        330        340        350        360        370        380
CAAGAGCCCG GAGACGGAAT CTGAGACAAG ATTCCGGGCC CTACGGGGGC AGCAGGCGCG AAACCTTTAC ACTGCACGCA AGTGCGATAA GGGGACCCCA
       390        400        410        420        430        440        450        460        470
AGTGCGAGGG CATATAGTCC TCGCTTTTCA CGACTGTAAG GCGGTCGTGG AATAAGAGCT GGGCAAGACC GGTGCCAGCC GCCGCGGTAA TAC....
       620        630        640        650        660        670        680        690        700        710
..TACGTCCG GGGTAGGAGT GAAATCCCGT AATCCTGGAC GGACCACCGA TGGCGAAAGC ACCTCGAGAA GACGGATCCG ACGGTGAGGG ACGAAAGCTA
       720        730        740        750        760        770        780        790        800        810
GGGTCTCGAA CCGGATTAGA TACCCGGGTA GTCCTAGCTG TAAACGATGC TCGCTAGGTG TGACACAGGC TACGAGCCTG TGTTGTGCCG TAGGGAAGCC
       820        830        840        850        860
GAGAAGCGAG CCGCCTGGGA AGTACGTCCG CAAGGATGAA ACTTAAAGGA ATTGGC......
       910        920        930        940        950        960        970        980        990       1000
......CTC AACGCCGGAC ATCTCACCAG CTCCGACTAC AGTGATGACG ATCAGGTTGA TGACCTTATC ACGACGCTGT AGAGAGGAGG TGCATGGCCG
      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
CCGTCAGCTC GTACCGTGAG GCGTCCTGTT AAGTCAGGCA ACGAGCGAGA CCCGCACTTC TAATTGCCAG CAGCAGT-TT CGACTGGCTG GGTACATTAG
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
AAGGACTGCC GCTGCTAAAG CGGAGGAAGG AACGGGCAAC GGTAGGTCAG TATGCCCCGA ATGAGCTGGG CTACACGCGG GCTACAATGG TCGAGACAAT
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
GGGTTGCTAT CTCGAAAGAG AACGCTAATC TCCTAAACTC GATCGTAGTT CGGATTGAGG GCTGAAACTC GCCCTCATGA AGCTGGATTC GGTAGTAATC
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
GCATTTCAAT AGAGTGCGGT GAATACGTCC CTGCTCCTTG CACACACCGC CCGTCAAAGC ACCCGAGTGA GGTCCGGATG AGGCCACCAC ACGGTGGTCG
      1410       1420       1430       1440
AATCTGGCTT CGCAAGGGGG CTTAAGTCGT AACAAGGTAG CCGTAGG
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FIG. 1. *Haloferax* strain Aa 2.2 16S rRNA nucleotide sequence. Six primers (complementary to the regions underlined) were used to sequence directly off the RNA. Sequence numbering is the same as that used by Hui and Dennis (7) for *H. cutirubrum* and *H. volcanii* 16S rRNA. Regions where the sequence was not able to be determined (due to secondary structure) are indicated by dotted lines. Only three base changes (in boldface) were observed between this sequence and that of *H. volcanii*: positions 410 (T→A), 415 (C→T), and 428 (A→T).

days of incubation by using a hand magnifier (magnification, ×10) or left for a total of 4 to 5 days, after which they were easily countable by eye (1 to 2 mm in diameter).

Characterization of a cryptic plasmid in *Haloferax* strain Aa 2.2. Two cryptic plasmids were identified in *Haloferax* strain Aa 2.2 cells after DNA extraction and agarose gel electrophoresis: a large plasmid with a low copy number and a prominent smaller plasmid of about 10 kb (Fig. 2). Designated pHK2, the smaller plasmid was estimated to be present at approximately seven to eight copies per cell (see above; data not shown). After pHK2 was linearized with *Kpn*I, it was cloned into the *Kpn*I site of *E. coli* pUC18 (23). The recombinant plasmid was grown up in *E. coli* XL1-Blue, purified on CsCl gradients, and then digested with various restriction enzymes to determine a physical map (Fig. 3). Four unique restriction sites were found in pHK2, i.e., *Hin*dIII, *Hpa*I, *Kpn*I, and *Sma*I, but sites for *Eco*RI, *Pst*I, and *Bam*HI were not detected.

It was noted that cells of *E. coli* transformed with this recombinant plasmid and grown on selective plates produced colonies that displayed surface papillae. When subcultured, these outgrowths were found to be ampicillin sensitive and did not contain plasmid DNA (data not shown). This instability was more readily demonstrated when *E. coli* transformants were grown in the absence of ampicillin selection, whereupon the plasmid was rapidly lost.

Resistance to novobiocin. Novobiocin has been shown to be a potent inhibitor of halobacteria (17, 18), and the MIC for *Haloferax* strain Aa 2.2 was found to be 0.005 μg/ml. By serially passaging this strain in liquid GM with progressively increasing levels of novobiocin, a mutant resistant to the antibiotic was selected. The MIC for the mutant was 7 μg/ml, i.e., approximately 1,000 times higher than that for the parent, yet its growth characteristics were unchanged (Fig. 4).

Cloning the novobiocin resistance gene. Transformation of *Haloferax* strain Aa 2.2 with DNA extracted from the resistant mutant produced colonies of resistant transfor-

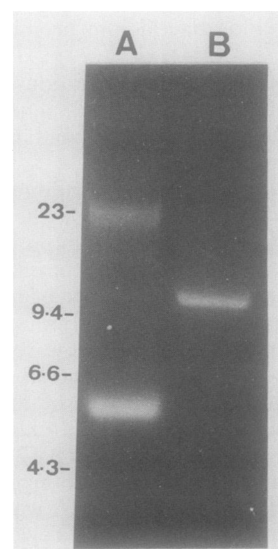

FIG. 2. Agarose gel electrophoresis of plasmid DNA isolated from *Haloferax* strain Aa 2.2. Lane A, Plasmid DNA isolated from *Haloferax* strain Aa 2.2 cells (the fainter upper band is contaminating chromosomal DNA, and the lower band is the plasmid pHK2); lane B, *Hin*dIII digest of the same DNA preparation. At the left, the positions of *Hin*dIII-cut lambda phage DNA size standards are indicated along with their approximate lengths in kilobases.

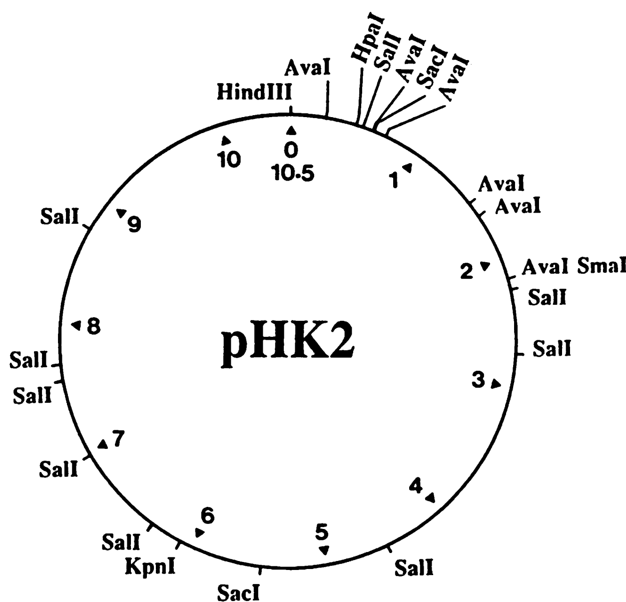

FIG. 3. Restriction map of *Haloferax* strain Aa 2.2 plasmid pHK2. The total size was estimated from gel analyses of fragments to be 10.5 kb. Enzymes which did not cut pHK2 were *Eco*RI, *Pst*I, and *Bam*HI.

mants (efficiency of transformation = 2×10^2 transformants per μg), presumably by a recombination event within the cells. Treatment of the cells with EDTA and PEG during the transformation procedure did not lead to any significant loss in cell viability, although some aggregation of the cells occurred during the PEG step. Resistant colonies were not observed in the absence of added DNA (spontaneous mutation frequency, $<10^{-10}$) or with DNA from the wild-type strain, and background growth of untransformed cells on plates was minimal or absent.

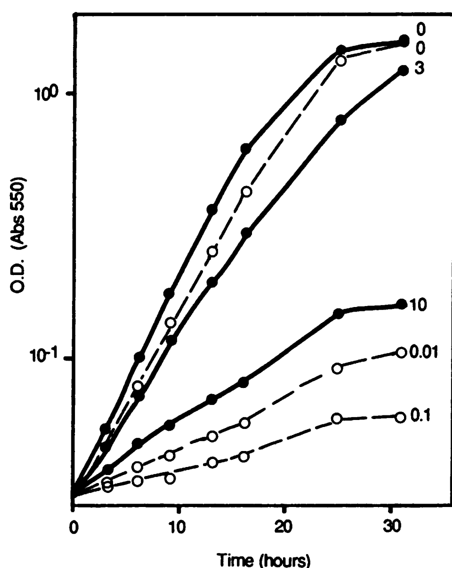

FIG. 4. Effect of novobiocin on the growth of the wild-type (---) and the novobiocin-resistant mutant (—) strains of *Haloferax* strain Aa 2.2. Mid-log-phase cultures of each strain were used to inoculate GM broths containing various concentrations of novobiocin (0 to 10 μg/ml). The concentrations are indicated at the end of each curve. Cultures were shaken at 37°C, and growth was followed for 30 h by monitoring the A_{550}. O.D., Optical density.

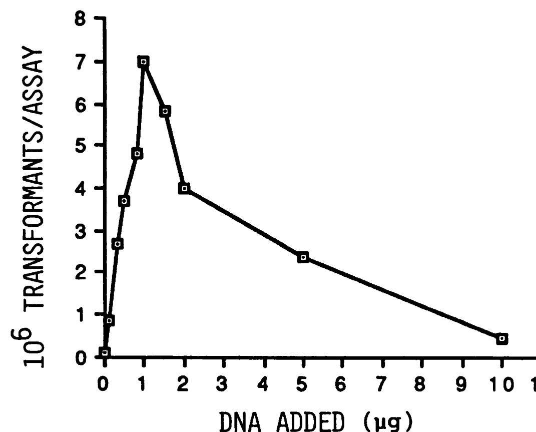

FIG. 5. Effect of plasmid concentration on the efficiency of transformation of *Haloferax* strain Aa 2.2. Cells were transformed (according to the protocol described above) with various quantities of the pHK2 novobiocin resistance plasmid (i.e., 0 to 10 μg of DNA added per 200 μl of EDTA-treated cells), and the total number of transformants was calculated from the number of colonies arising on selective plates.

We proceeded to shotgun clone the novobiocin resistance gene into the *Kpn*I site of pHK2. Chromosomal DNA was purified from the resistant mutant, digested with *Kpn*I, and ligated to *Kpn*I linearized pHK2. This mixture was then transformed into *Haloferax* strain Aa 2.2, and the cells were spread onto TM agar plates containing 0.1 μg of novobiocin per ml. Several colonies grew up, and one of these was found to contain a plasmid larger than pHK2. After purification on cesium chloride-ethidium bromide gradients, it was digested with *Kpn*I and *Sal*I to check its identity. *Kpn*I digestion produced two fragments: one equal in size to *Kpn*I-cut pHK2 and the other of about 6.7 kb (see Fig. 7, lane C). *Sal*I digestion gave an identical pattern to that of *Sal*I-cut pHK2, except that the *Sal*I fragment containing the *Kpn*I site (used for cloning) was absent and several extra bands were present. The results indicated that pHK2 had remained unchanged, except for containing a 6.7-kb *Kpn*I insert derived from chromosomal DNA of the mutant.

The recombinant plasmid transformed *Haloferax* strain Aa 2.2 cells at a very high efficiency, i.e., 10^6 to 10^7 transformants per μg of plasmid DNA, with the number of transformants being proportional to the amount of input DNA up to 1 μg/200-μl assay mixture (Fig. 5). No resistant colonies were observed if plasmid DNA was not added. At concentrations of plasmid DNA above 1 μg/200 μl of cells, the efficiency of transformation decreased rapidly because of precipitation of DNA under the high-salt conditions (4).

The resistance and growth characteristics of a plasmid transformant and the resistant mutant were directly compared and found to be very similar (Fig. 6), indicating that the novobiocin resistance shown by the mutant was mainly, if not entirely, due to a gene(s) carried on the 6.7-kb *Kpn*I fragment.

Stability of recombinant pHK2 plasmid. A *Haloferax* strain Aa 2.2 transformant containing the pHK2 novobiocin resistance plasmid was grown in liquid culture for 10 generations in the absence of novobiocin. Cells were plated onto GM plates without antibiotic, and 100 of the resulting colonies were replica plated onto GM plates containing 0.1 μg of

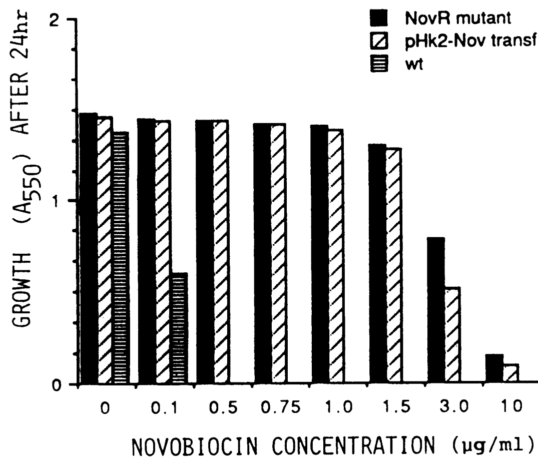

FIG. 6. Novobiocin resistance of *Haloferax* cells transformed with the pHK2 novobiocin resistance plasmid (pHk2-Nov transf) compared with the wild-type (wt) and novobiocin-resistant mutant (NovR) strains. Experimental conditions were as described in the legend to Fig. 5, except that growth was only measured after 24 h of incubation.

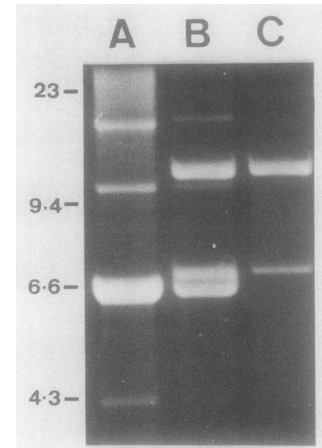

FIG. 7. Agarose gel electrophoresis of *Kpn*I-digested plasmid DNA isolated from *H. volcanii* transformed with the novobiocin resistance plasmid. Plasmids were extracted and purified on CsCl gradients (as described above). Approximately 0.5 μg of each preparation was digested with *Kpn*I and electrophoresed on a 1% agarose gel. The positions of DNA size standards are shown at the left (lambda-*Hin*dIII fragments). Lane A, Plasmid DNA from *H. volcanii*; lane B, plasmid DNA from *H. volcanii* transformed with the recombinant pHK2 plasmid; lane C, recombinant pHK2 plasmid grown in *Haloferax* strain Aa 2.2.

novobiocin per ml. All grew normally, indicating that no loss of plasmid had occurred. To confirm this, DNA was extracted from several novobiocin-resistant colonies and analyzed by gel electrophoresis. In all cases, the recombinant plasmid was present and could be used for further transformations.

Transformation of other halobacteria. The recombinant pHK2 plasmid carrying the novobiocin resistance marker was introduced into other strains of halobacteria to test if it would function in heterologous hosts. Transformation of *H. halobium* strains was performed according to the protocol of Cline and Doolittle (4), whereas *Haloferax* transformations (as discussed above) were based on the method described by Charlebois et al. (3). *H. volcanii* was found to be transformed at the same efficiency (i.e., between 10^6 and 10^7 transformants per μg) as *Haloferax* strain Aa 2.2, whereas no transformants were observed in three *H. halobium* strains tested (RI, NCMB777, and DSM617), even though two of the latter strains (DSM617 and RI) were chosen because they were believed to lack restriction systems (13; R. Schinzel, personal communication). To study *H. volcanii* transformants in more detail, plasmid DNA was isolated from one transformant, digested with *Kpn*I, and analyzed by agarose gel electrophoresis (Fig. 7, lane B). Both pHV2, the major plasmid of *H. volcanii* (3), and the recombinant pHK2 plasmid were present, as shown by the three main bands, which correspond to linearized pHV2 (6.4 kb) and the expected *Kpn*I fragments of the recombinant pHK2 (6.7 and 10.5 kb). Separate *Kpn*I digests of each plasmid were run on either side of lane B to clearly identify corresponding fragments. Fainter bands in lanes A and B are due to the larger *H. volcanii* plasmid pHV1 (3). That the transformant contained both pHV1 and pHK2 indicates that these plasmids carry compatible replicons.

DISCUSSION

Haloferax strain Aa 2.2, like other archaebacteria, is resistant to a large number of commonly used antibiotics (2, 14) but is extremely sensitive to novobiocin, with concentrations as low as 0.005 μg/ml being sufficient to considerably retard growth. In eubacteria, the target of novobiocin is the DNA gyrase subunit encoded by *gyrB* (19), with inhibition leading to a shutdown of DNA synthesis. Studies on halobacteria have not been as detailed, but novobiocin has been shown to inhibit cell growth and DNA synthesis and to alter plasmid supercoiling (17, 18). These observations indicated that a cellular gyrase was affected, so the primary site of action of novobiocin was assumed to be the same in both eubacteria and archaebacteria. Halobacteria show a similar level of sensitivity to novobiocin as do eubacteria, whereas eucaryotes are much more resistant. This led Sioud et al. (18) to propose that the halobacterial gyrase is more related to the eubacterial than the eucaryotic homolog (18). Our initial sequencing data from the cloned novobiocin resistance gene has confirmed that this is the case (work in progress).

Transformation of *Haloferax* strain Aa 2.2 using chromosomal DNA indicated that an efficient recombination system was operating, consistent with the results of Mevarech and Werczberger (10), who demonstrated recombination between auxotrophic mutants of *H. volcanii*. Recombination may be a problem in retaining cloned chromosomal genes in plasmid constructs, although the recombinant pHK2 plasmid used in our study, which contained 6.7 kb of genomic DNA, was stably maintained in *Haloferax* strain Aa 2.2.

Restriction barriers are commonly found in halobacteria (13), and in at least one strain, two different restriction systems were found to be operating. We believe both *H. volcanii* and *Haloferax* strain Aa 2.2 contain at least one restriction system for the following reason. When the pHK2 recombinant plasmid containing the 6.7-kb *Kpn*I insert was cloned into the pUC18 vector (using the *Hin*dIII site of each) and grown up in *E. coli*, the unmodified plasmid DNA transformed both strains at least 1,000-fold less efficiently than the original recombinant pHK2 plasmid grown in *Haloferax* strain Aa 2.2 or *H. volcanii* (data not shown). In addition, the *E. coli* vector sequences were often deleted after transformation into *Haloferax* strain Aa 2.2, making it difficult to introduce the plasmid back into *E. coli*.

Recently, another antibiotic resistance gene, which con-

fers resistance to mevinolin (W. L. Lam, A. Cohen, and W. F. Doolittle, Abstr. 3rd Int. Conf. Mol. Biol. Archaebacteria, 1988, section 2, p. 11), was reported for halobacteria. However, this compound is expensive and difficult to obtain (no longer supplied by Sigma Chemical Co., St. Louis, Mo.). Another possible selection mechanism uses genes that complement auxotrophic mutations of the host cell (4a). As a selection system, this has several disadvantages, including slower growth rates on glucose-mineral salts media and a host range restricted to those with suitable mutations.

The general utility of the novobiocin resistance marker will need to be tested by cloning it into other halobacterial plasmids, since the pHK2 replicon did not appear to function in *H. halobium* strains. We aim to improve the vector described in this study by reducing its size and increasing the number of cloning sites. Restriction-negative mutants of *Haloferax* strain Aa 2.2, with and without active modification systems, would also be a great advantage for transfer of plasmids between different host cells. We are using the current vector to clone other genes of *Haloferax* strain Aa 2.2, such as the β-galactosidase gene, which may be a useful addition to future cloning-expression vectors.

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