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Neomycin Resistance as a Selectable Marker in *Methanococcus maripaludis*

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We cloned the aminoglycoside phosphotransferase genes APH3′I and APH3′II between the *Methanococcus voltae* methyl reductase promoter and terminator in a plasmid containing a fragment of *Methanococcus maripaludis* chromosomal DNA. The resulting plasmids encoding neomycin resistance transformed *M. maripaludis* at frequencies similar to those observed for pKAS102 encoding puromycin resistance. The antibiotic geneticin was not inhibitory to *M. maripaludis*.

Genetic studies in methanogens and most other archaea are currently limited by a shortage of tools, including antibiotic resistance markers, plasmid vectors, transposons, reporter genes, and high-efficiency transformation protocols. In the methanococci, progress has been made in improving transformation frequencies (13, 18), developing a shuttle vector (19), and implementing insertional (4) or ex vivo transposition (5) mutagenesis. However, at present, studies are restricted to the use of a single antibiotic resistance marker for puromycin (7, 16). Additional antibiotic resistance markers will allow more sophisticated genetic analyses, including making multiple mutations, inserting reporter genes followed by a second mutation to measure the effect of that mutation, and knocking out genes which have more than one copy, such as flagellin genes (10). It has been difficult to develop markers for methanogens because archaea are generally resistant to antibiotics because they have a different ribosome structure than bacteria and lack peptidoglycan (6, 15). Some antibiotics which do inhibit methanogens do so by the toxic effect of side groups (chloramphenicol [2]) or have a detergent effect on the cell wall (tetracycline [6]) and so are not rendered noninhibitory by the action of resistance genes (2). One antibiotic which inhibits the growth of *Methanococcus maripaludis* in vivo and also inhibits protein synthesis in vitro is neomycin (6, 20). Neomycin is inexpensive and has been widely used in the bacteria. One of the resistance mechanisms, the aminoglycoside 3′-phosphotransferase (APH3′), is also used frequently for kanamycin resistance and, when coupled with a eukaryotic promoter, for G418 (geneticin) resistance (14). We report here the use of neomycin resistance as a second selectable marker in the archaeon *M. maripaludis*.

Bacterial strains and plasmids used in this study are shown in Table 1. Growth of *Escherichia coli* DH5αF′ and *M. maripaludis* (18) and the plating methods used have been described (12, 17, 18). When used as a selective agent in solid and liquid media, neomycin was included at concentrations of 250 and 1,000 μg/ml, respectively. Plasmid DNA used for cloning and restriction analysis was prepared from *E. coli* by the Easyprep method (3). DNA used for transformation of *M. maripaludis* was prepared by using QIAGEN-tip 500 columns (QIAGEN, Inc., Chatsworth, Calif.). *M. maripaludis* chromosomal DNA was prepared as follows. The cells from a 5-ml culture of *M. maripaludis* were pelleted aerobically in 1.5-ml microcentrifuge tubes by discarding the supernatant fluid between sequential centrifugations. The cell pellet was lysed by resuspension in 0.5 ml of TE (10 mM Tris, 1 mM EDTA; pH 8.0), addition of 10 μl of 20% sodium dodecyl sulfate, and incubation for 30 min at 50°C with occasional mixing. The suspension was cooled to 37°C, 10 μl of RNase A (10 mg/ml; Sigma) was added, and the mixture was incubated at 37°C for 30 min. Then 20 μl of proteinase K (10 mg/ml; Sigma) was added, and the suspension was incubated at 50°C for 2 h with occasional mixing or until it was clear. The solution was mixed once with an equal volume of phenol (buffered to pH 8.0; Intermountain Scientific) and extracted twice with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding 50 μl of sodium acetate (3 M, pH 5.2) and filling the microcentrifuge tube with 100% ethanol. The tubes were mixed gently and stored at −20°C for 2 h; this was followed by centrifugation. The DNA pellet was washed once with 70% ethanol, vacuum dried, and resuspended in 100 μl of TE overnight at 4°C. Other molecular techniques followed standard protocols (1).

Neomycin sensitivity of *M. maripaludis*. The MIC of neomycin (the concentration of neomycin in liquid medium necessary to completely inhibit the growth of a 5-ml culture of *M. maripaludis* inoculated with 6×10^7 cells) was determined to be 1,000 μg/ml. Like kanamycin (20), the antibiotic geneticin (G418; Gibco BRL), which has been used extensively in eukaryotic cultures (8), did not inhibit the growth of *M. maripaludis* at levels as high as 1,000 μg/ml. Concentrations of neomycin between 500 and 1,000 μg/ml delayed growth. Cultures grown in medium containing neomycin became resistant and were no longer inhibited by 1,000 μg/ml, even when neomycin was present at levels which did not inhibit growth (300 μg/ml). The development of resistance is consistent with observations in *Methanococcus vannielii* (9), in which a change in membrane permeability resulted in resistance to neomycin. In comparison, we have not observed spontaneous resistance to puromycin.

Growth of *M. maripaludis* on solid medium containing various levels of neomycin is shown in Table 2. The number of spontaneously resistant colonies increased with the number of cells plated and decreased with increasing neomycin concentration. These results may be explained on the basis of the relative likelihood of spontaneous resistance at low antibiotic concentrations, the dilutions plated, and the tendency for a

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
E. coli DH5αF′		Gibco BRL
M. maripaludis strains		
JJ	Wild type	11
NEO 1, NEO 2, NEO 3	Colonies picked from high-dilution neomycin plates following transformation of *M. maripaludis* JJ with pJLA5 in expt 1	This work
KAN 1, KAN 2, KAN 3	Colonies picked from high-dilution neomycin plates following transformation of *M. maripaludis* JJ with pJLA6 in expt 1	This work
Plasmids		
pUC18		S. Mosely
pJLA4	pUC18 containing a 1.2-kb insertion of *M. maripaludis* DNA	This work
pUC4K	APH3′I from Tn*903*	Pharmacia
pNEO	APH3′II from Tn*5*	Pharmacia
pMEB.1	*mcr* promoter and terminator cassette from *Methanococcus voltae*	7
pMBSK	APH3′I between the *mcr* promoter and terminator	This work
pMBSN	APH3′II between the *mcr* promoter and terminator	This work
pJLA6	Neo^r integration vector, APH3′I cassette from pMBSK inserted into pJLA4	This work
pJLA5	Neo^r integration vector, APH3′II cassette from pMBSN inserted into pJLA4	This work
pKAS102	Pur^r integration vector	16

large number of cells to decrease the effective concentration of antibiotic.

Transformation of *M. maripaludis* with neomycin resistance markers. Cultures of *M. maripaludis* were transformed with pJLA5 and pJLA6 (containing neomycin resistance markers APH3′II and APH3′I, respectively) (Fig. 1), as well as with pKAS102 (encoding puromycin resistance [16]) for comparison. These plasmids were expected to integrate by homologous recombination into the genome because of the genomic fragment that they contain. Appropriate dilutions were spread onto agar plates containing the appropriate antibiotics (250 μg of neomycin per ml or 2.5 μg of puromycin per ml). A control culture subjected to the transformation procedure without adding DNA was also spread onto antibiotic plates. The total numbers of CFU were estimated by plating appropriate dilutions onto plates without antibiotics.

Since up to 3×10^8 cells were plated onto neomycin plates, some colonies arose from the control transformation (no DNA), as expected from the results of the plating experiment described above. Most of these colonies were less than 0.1 mm in diameter. In contrast, much larger colonies (diameters, 1 to 2 mm) arose from transformation with pJLA5 or pJLA6. These colonies were present along with smaller background colonies. Since the large (1- to 2-mm) colonies were easily distinguished from the small (less-than-0.1-mm) colonies, the latter were ignored in the calculations of transformation frequencies. The results showed (Table 3) that with both plasmids, neomycin resistance could be used as a selectable marker with frequencies within 1 log of those observed with puromycin.

Demonstration of marker integration. To further demonstrate that the large colonies observed on neomycin plates spread with cultures transformed with pJLA5 and pJLA6 were in fact due to transformation and integration of the plasmid, three colonies each were picked from plates transformed with pJLA5 and pJLA6. The chromosomal DNAs from these six isolates were digested and analyzed by Southern blotting. The results from the pJLA5-transformed cultures are presented in Fig. 2. This blot was probed with pNEO (Fig. 1), which hybridizes to the pUC sequence and to APH3′II, but not to the *mcr* promoter and terminator or to the chromosomal fragment from *M. maripaludis*. When the chromosomal DNA was digested with *Eco*RI, a 1.75 kb band corresponding to the resistance cassette was seen, whereas the 3.88 kb band corresponding to pUC plus the fragment of chromosomal DNA seen in digests of the plasmid was shifted to ~9 kb because of integration of the plasmid into the chromosome. Insertion of a single copy (rather than a tandem repeat) of pJLA5 in all three isolates was demonstrated by the single band seen in the *Hin*dIII digests. Only one *Hin*dIII site exists in pJLA5, the site from the pUC18 polylinker (Fig. 1). Two restriction sites for *Sph*I exist in pJLA5, one in the pUC18 polylinker and one in APH3′II. When chromosomal DNA from transformed cultures was digested with *Sph*I, two bands were seen. The strongly hybridizing band at 3.28 kb, also seen in pJLA5, cor-

TABLE 2. Growth of wild-type *M. maripaludis* on solid medium containing various levels of neomycin

Neomycin concn (μg/ml)	No. of colonies when the following no. of cells were plated[a]:								
	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	48	7
0	L[b]	L	L	L	L	L	~500	48	7
150	L	L	~500	~200	1	0	0	0	0
250	~500	47	3	0	0	0	0	0	0
350	~200	6	0	0	0	0	0	0	0
450	8	0	0	0	0	0	0	0	0
550	0	0	0	0	0	0	0	0	0

[a] For the number of cells plated, values at low dilutions were estimated from colony counts at high dilutions.
[b] L, a lawn of growth resulted.

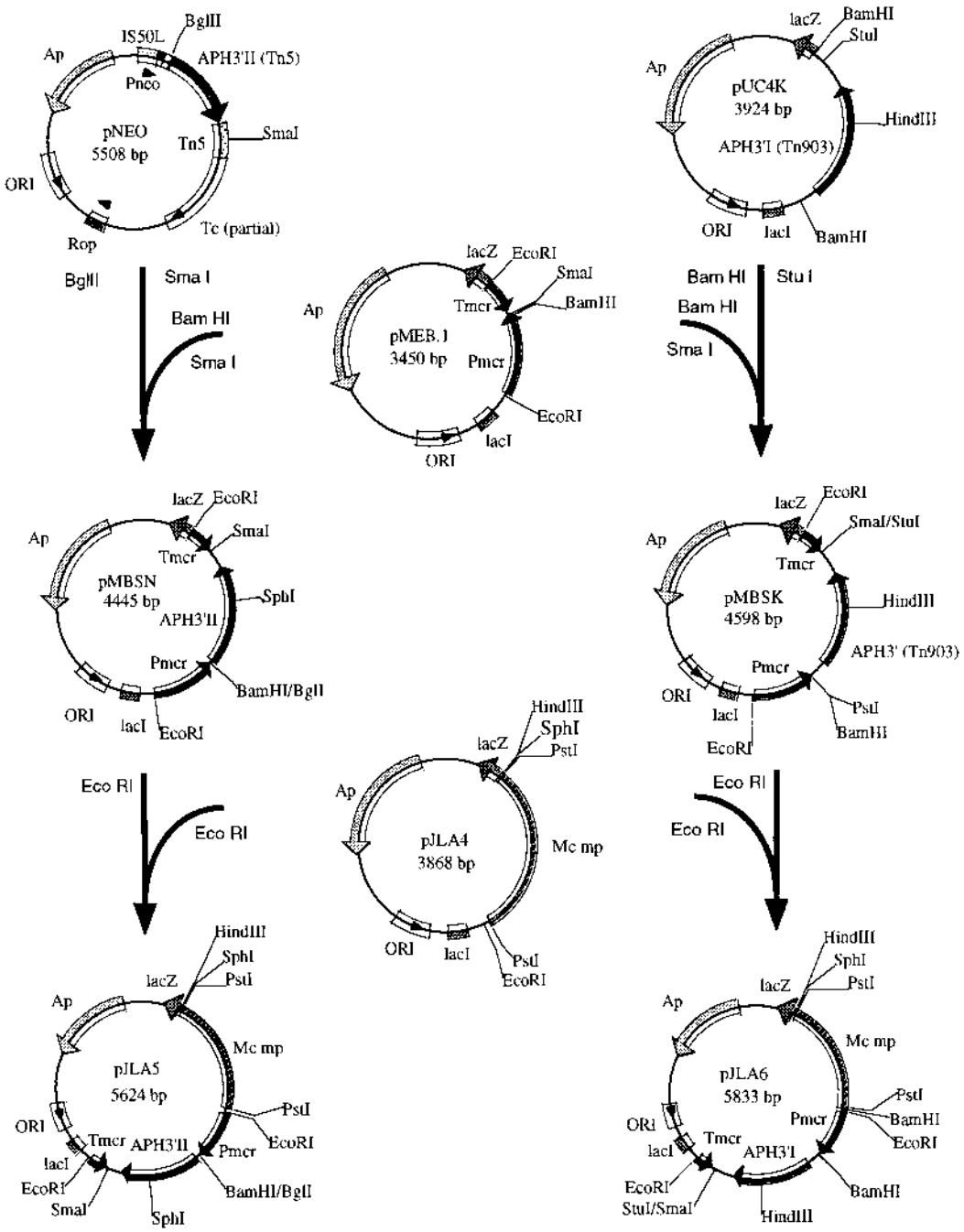

FIG. 1. Construction of pJLA5 and pJLA6. pMEB.1 contains the promoter (Pmcr) and terminator (Tmcr) of the *Methanococcus voltae* methylreductase gene (7). pJLA4 was created by cloning a 1.2-kb fragment of *M. maripaludis* DNA into the *Pst*I site in pUC18.

responded to the vector, the *mcr* promoter (no hybridizing DNA), and a portion of APH3′II, while the weakly hybridizing band containing the balance of APH3′II and the *mcr* terminator (no hybridizing DNA) was shifted to ~4 kb from 2.34 kb because of integration of the plasmid. Wild-type DNA did not bind the probe (Fig. 2).

The results of probing chromosomal digests from pJLA6-transformed cultures with pUC4K are shown in Fig. 3. The major differences in restriction patterns between pJLA6 and pJLA5 are that the numbers of restriction sites for *Sph*I and *Hin*dIII are reversed (Fig. 1) and APH3′I is 100 bp larger than APH3′II. Thus, the results shown in Fig. 2 and 3 were expected to be similar. Instead, pJLA6 was apparently present in tandem repeats in two of the three cultures (KAN 1 and KAN 3). When the chromosomal DNAs from KAN 1 and KAN 3 were digested with *Eco*RI, not only were there bands at 1.95 and ~9 kb corresponding to the APH3′I cassette and the vector, respectively, integrated into the chromosome, but also a 3.88-kb band was present, which corresponded to the vector plus only the 1.2-kb chromosomal fragment from the plasmid. Similarly, when the chromosomal DNAs from KAN 1 and KAN 3 were digested with *Sph*I, which has only one restriction site in pJLA6

TABLE 3. Comparison of transformation efficiencies with pKAS102, pJLA5, and pJLA6[a]

Plasmid	Expt	No. of transformants per μg (10^5)	No. of transformants per pmol (10^5)	No. of transformants per CFU (10^{-5})
pKAS102	1	11.7	93.5	32.0
	2	1.16	9.25	6.27
pJLA5	1	1.33	4.95	1.8
	2	4.85	18.0	10.4
pJLA6	1	1.13	4.33	1.5
	2	0.703	2.7	1.83

[a] Duplicate experiments were performed. Cultures were transformed with 0.1 pmol of DNA (0.799 μg of pKAS102, 0.371 μg of pJLA5, or 0.384 μg of pJLA6). Numbers of transformants were determined by determining the average numbers of colonies on duplicate plates. For enumeration of colonies on neomycin plates, only colonies of more than 0.1 mm in diameter were counted. Colonies less than 0.1 mm in diameter were ignored. In experiment 1, all background colonies (colonies arising from control [no DNA] transformation) were less than 0.1 mm in diameter. In experiment 2, a few background colonies (1.4% for pJLA5 and 6.9% for pJLA6) were more than 0.1 mm in diameter; the numbers of such colonies were subtracted from the numbers of colonies counted on transformant plates.

(Fig. 1), two bands were present, one of which was the correct size to be a copy of pJLA6 (5.83 kb), whereas in KAN 2 only one band was present. The results of the *Hin*dIII digestion also demonstrated the presence of both plasmid bands plus one additional band in KAN 1 and KAN 3, whereas KAN 2 contained only the 3.36-kb band corresponding to the vector, promoter, and a portion of APH3′I and a faint band at ~3.5 kb corresponding to the rest of APH3′I, the terminator, and a portion of the chromosome.

The presence of tandem repeats was also observed after transformation of *M. maripaludis* with pKAS102 during selection on high levels of puromycin (16). It is possible that the neomycin resistance marker in pJLA6 was only marginally effective in a single copy, whereas in multiple copies resistance to neomycin (250 μg/ml on plates and 1,000 μg/ml in subsequent liquid culture) was easily achieved. In general, avoiding the use of excessive concentrations of neomycin may help prevent multiplicity. The reason(s) why pJLA5 integrated as a single copy, whereas pJLA6 was present in tandem, is not

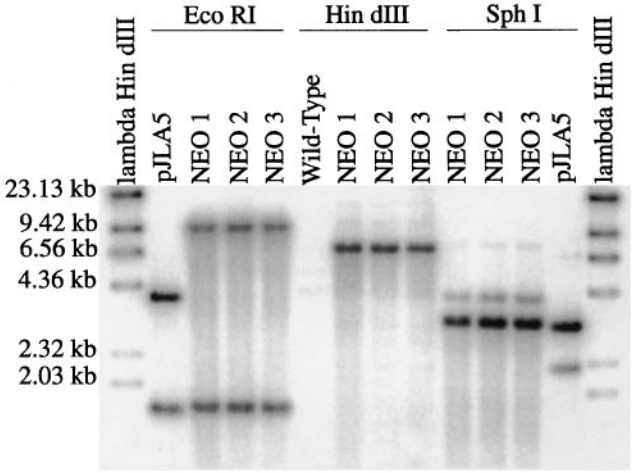

FIG. 2. Southern blot of chromosomal DNAs from three isolates picked from a neomycin plate following transformation of *M. maripaludis* with pJLA5 and grown in the presence of 1,000 μg of neomycin per ml. The blot was probed by using a mixture of labeled lambda DNA and pNEO.

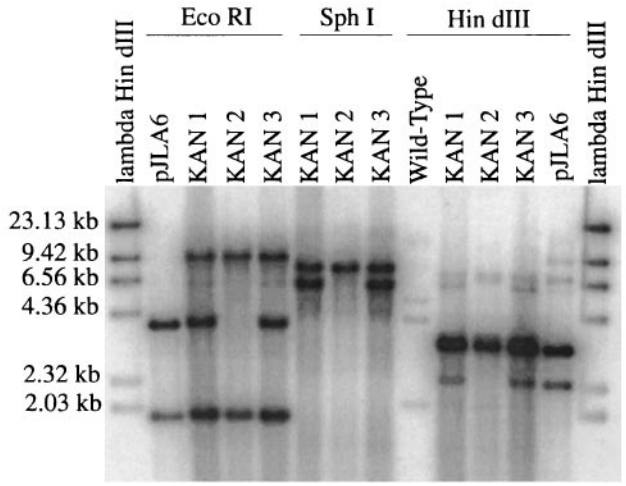

FIG. 3. Southern blot of chromosomal DNAs from three isolates picked from a neomycin plate following transformation of *M. maripaludis* with pJLA6 and grown in the presence of 1,000 μg of neomycin per ml. The blot was probed by using a mixture of labeled lambda DNA and pUC4K.

clear. APH3′I and APH3′II are related enough at the DNA level to be used as probes for each other in Southern blots (level of identity, 44%) and are 34% identical and 61% similar at the protein level. Duplication of integrated plasmid sequences also occurs in members of the *Bacteria* (21, 22).

Conclusion. We have shown that APH3′I and APH3′II can be used to provide neomycin resistance in *M. maripaludis*, making neomycin the second selectable marker shown to work in methanococci. The eukaryotic antibiotic geneticin does not appear to be inhibitory to *M. maripaludis.*

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