APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2762–2763
0099-2240/91/092762-02$02.00/0

Vol. 57, No. 9

Recovery of an Integration Shuttle Vector from Tandem Repeats in *Methanococcus maripaludis*

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Received 21 March 1991/Accepted 11 June 1991

Transformation of *Methanococcus maripaludis* by using an integration vector, pKAS102, is described. Selection and subsequent growth at high concentrations of puromycin caused pKAS102 to develop tandem repeats within the genome. As a result, total DNA isolated from the transformant could be used to recover the intact vector by direct transformation of competent *Escherichia coli*.

The understanding of the molecular biology and genetics of the methanogenic archaebacteria, while not as extensive as that of eubacteria, is advancing. Successful transformation of methanogens has been reported for only two methanogenic species. Bertani and Baresi (2) were able to transform auxotrophic mutants of *Methanococcus voltae* by using wild-type chromosomal DNA, and Worrell et al. (9) transferred 5-fluorouracil resistance between strains of *Methanobacterium thermoautotrophicum*. Gernhardt et al. (3) developed a selectable marker for puromycin resistance and made an important advance toward a shuttle vector with Mip1. This plasmid could be propagated in *Escherichia coli* and introduced into *M. voltae*, where it integrated into the genome by homologous recombination with *M. voltae hisA* sequences present on the vector. After digestion of transformant DNA with *Hin*dIII and subsequent religation, transformation of *E. coli* yielded Mip1 or portions thereof. Here we report transformation of a third methanogenic species, *Methanococcus maripaludis*, with a derivative of Mip1. In addition, we describe a facile method of recovering the vector that does not require the use of restriction enzymes which would limit the recoverability of intact clones.

M. maripaludis was grown in a defined medium previously described as WM medium (2). Procedures for growth and plating were modifications of those of Whitman et al. (7). Solid medium contained 1.5% noble agar (Difco) and was supplemented with dithiothreitol to a concentration of 3 mM after sterilization. Plates were preincubated for 24 h under a sulfide-containing atmosphere before use. Under these conditions, a spread-plating efficiency of 100% was routinely obtained. *M. maripaludis* was transformed according to the procedure of Gernhardt et al. (3), except for the plate preincubation procedure described above. Total *M. maripaludis* DNA was prepared either by the method of Silhavy et al. (6), except that the lysozyme treatment was omitted, or by a mini-prep procedure (1). For transformation with *M. maripaludis* DNA, *E. coli* DH5α cells were made competent by the RbCl procedure (4). For *E. coli*, plasmids were selected by using 50 μg of ampicillin per ml. DNA cloning, Southern hybridization (100% stringency), and probe construction were done by standard procedures (1). DNA concentrations were determined with a Milton Roy Spectronic (model 1001 plus) spectrophotometer using 260- and 280-nm-UV analysis. Plasmid Mip1 was kindly provided by A. Klein.

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By cloning wild-type *Hin*dIII-digested DNA from *M. maripaludis* into the unique *Hin*dIII site of Mip1, we constructed the plasmids pKAS100 (insert, about 3 kb) and pKAS102 (insert, 4.7 kb). These plasmids thus contain, in addition to regions of homology with the *M. maripaludis* genome, a puromycin resistance determinant for selection in *M. maripaludis* and an ampicillin resistance determinant for selection in *E. coli*. Both of these plasmids were consistently able to transform *M. maripaludis*, yielding puromycin-resistant colonies. For example, following treatment with 1 pmol of pKAS102 DNA, a plating of 100 μl (representing 2% of the transformation mixture) gave 478 puromycin-resistant colonies selected at 2 μg of puromycin per ml. This represents a transformation efficiency of 8.9×10^{-7} transformants per recipient or 3×10^3 transformants per μg of DNA. Selection at 10 μg of puromycin per ml gave about 10-fold fewer transformants. Control experiments with no DNA or with Mip1 (which lacks homology with the *M. maripaludis* genome) yielded no colonies.

pKAS100 or pKAS102 DNA was used to probe *Stu*I-digested genomic DNA from several different *M. maripalu-*

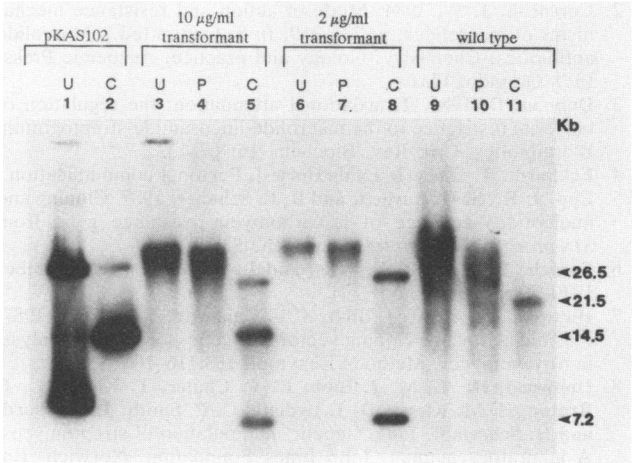

FIG. 1. Southern blot analysis of *M. maripaludis* first transformed and then probed with pKAS102. Lanes: 1 and 2, pKAS102 DNA; 3 to 5, total DNA from a transformant selected at 10 μg of puromycin per ml; 6 to 8, total DNA from a transformant selected at 2 μg of puromycin per ml; 9 to 11, total DNA from untransformed *M. maripaludis*. Lanes 1, 3, 6, and 9, uncut DNA (U); lanes 4, 7, and 10, partial *Stu*I digests (P); lanes 2, 5, 8, and 11, complete *Stu*I digests (C).

TABLE 1. Numbers of ampicillin-resistant colonies obtained after transformation of *E. coli* with whole genomic DNA from pKAS102-transformed *M. maripaludis*[a]

Amt of DNA	No. of colonies obtained with DNA from:				
	Wild type	pKAS102 transformants[b]			
		S, 10; G, 10	S, 2; G, 2	S, 10; G, 10	S, 10; G, 2	S, 2; G, 20
100 ng	0	19	1	1	0	1
1 μg	0	80	3	22	0	35
10 μg	0	50	3	16	1	101

[a] The first two, middle two, and last two columns of data each constitute a separate experiment.

[b] S, original selection of *M. maripaludis* transformant; G, growth of *M. maripaludis* transformant. Numbers are concentrations of puromycin in micrograms per milliliter.

dis transformants (*Stu*I was found to cut only once in each plasmid, in the puromycin resistance cassette [5]). Figure 1 shows a representative result with pKAS102. In all cases, the presence of two bands of the sizes appropriate to contain integration junctions showed that the plasmid had integrated by a single homologous recombination event, as was found in most cases by Gernhardt et al. (3).

Interestingly, when the transformants were selected and maintained at 10 rather than 2 μg of puromycin per ml, an additional intense band that comigrated with *Stu*I-cut pKAS102 was observed (Fig. 1). The same was observed with pKAS100. This observation most likely indicates that tandem repeats had arisen in response to selection for more copies of the puromycin resistance determinant. Gernhardt et al. (3) discussed what may be a similar amplification of MipI in some of their *M. voltae* transformants. A second formal possibility that would explain the additional band is that we had fortuitously cloned an origin of replication and that the pKAS plasmids were present as stable episomes. However, plasmid forms could not be detected by Southern hybridization with uncut total DNA (Fig. 1), by plasmid mini-preps, or by large-scale CsCl plasmid preps (8).

We obtained total DNA from *M. maripaludis* that had been transformed with pKAS102 and selected at 10 μg of puromycin per ml. When we used this DNA to transform competent *E. coli* cells, a significant number of ampicillin-resistant colonies arose (Table 1). DNA from a pKAS100 transformant of *M. maripaludis* behaved similarly (data not shown). In addition, when the *M. maripaludis* transformant was originally selected at 2 μg/ml but subsequently grown at 20 μg of puromycin per ml, similar results were obtained. In contrast, very few colonies arose when the transformant was selected and grown at 2 μg/ml or when it was selected at 10 but subsequently grown at 2 μg of puromycin per ml. Restriction analysis of plasmid mini-preps from transformed *E. coli* showed that, in all cases in which the *M. maripaludis* transformant had been grown at higher levels of puromycin, the colonies contained intact pKAS102, while in some cases in which 2 μg of puromycin per ml had been used the colonies did not contain the plasmid and had apparently arisen by spontaneous mutation to ampicillin resistance.

These results demonstrate that the use of high concentrations of puromycin enables the pKAS vectors to behave as recoverable shuttle vectors. Tandem repeats might result in more homologous looping out of the vector in *M. maripaludis* such that transformation of *E. coli* can occur from these transient "episomal" DNA species. Alternatively, transformation of *E. coli* might occur with linear pieces of DNA containing the tandem repeats which in *E. coli* are resolved into intact plasmids. Presumably, any plasmid containing the puromycin resistance cassette and a sufficiently large piece of homologous DNA might be made to transform *M. maripaludis* and be recoverable in the presence of the appropriate puromycin selection. This methodology will allow selection of cloned genes by complementation of *M. maripaludis* mutants followed by recovery of the complementing clones. This phenomenon should be applicable to the methanococci in general and may be applicable to other competent methanogenic genera sensitive to puromycin.

We thank John Jelesko, Peter Kessler, and Kelly Hughes for helpful discussions and A. Klein for MipI.

This work was supported by NSF grant DMB-8552553.

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