Arch Microbiol (1985) 142:259–261

Archives of
Microbiology

A newly-isolated marine methanogen harbors a small cryptic plasmid

Alvin G. Wood [1], William B. Whitman [2], and Jordan Konisky [1]

[1] Department of Microbiology, University of Illinois, 131 Burrill Hall, 407 S. Goodwin, Urbana, IL 61801, USA
[2] Department of Microbiology, University of Georgia, Athens, GA 30602, USA

Abstract. Of 21 recently isolated strains of methanococci, one was found to harbor a small, cryptic, low copy number plasmid. Reproducible recovery was achieved by alkaline lysis of cells pretreated with proteinase K in an osmotically stabilizing buffer. The plasmid was found to contain a single *AvaI* site. No homology was detected between the plasmid and DNA from any of the other new strains or from five known species of methanococci.

Key words: Methanogenic bacteria – Plasmid isolation – Alkaline lysis – CsCl gradient – Restriction endonuclease mapping – Electron microscopy – DNA homology

Genetic analysis of methanogenic bacteria awaits the development of a means for transferring DNA from one strain to another. For this reason, there has been considerable interest in the possibility of isolating plasmids or bacteriophages from methanogens. To date, there have been two published reports of the occurrence of plasmids in methanogens. The first (Thomm et al. 1983) described a small cryptic plasmid isolated from an uncharacterized coccoid methanogen. This plasmid (pMP1) behaves somewhat atypically in that it cosediments with chromosomal DNA in cleared lysates and its isolation is not always reproducible. Meile et al. (1983) reported a plasmid (pME2001) from *Methanobacterium thermoautotrophicum* which is found in the supernatant in cleared lysates and occurs in multimeric as well as monomeric forms. We now report the isolation of a small cryptic plasmid from a recently isolated coccoid marine methanogen. This plasmid (pURB500) appears to be stably maintained since we have continued to recover it from cultures which have been transferred repeatedly in the laboratory.

Materials and methods

Samples of mud or sediment were taken from a salt marsh at Sapelo Island, Georgia, and from a brackish canal adjacent to Tampa Bay, Florida. From these samples, twenty-one new strains of methanococci were isolated and cultivated in medium 3 of Balch et al. (1979).

A quick screening for plasmids, using the method of Birnboim and Doly (1979), revealed the presence of a plasmid in one of the Georgia strains, designated C5. We then developed a procedure for quantitative isolation based on the scale-up of the Birnboim and Doly procedure described by Maniatis et al. (1982). The modifications reflect the proteinaceous nature of the cell walls of methanococci and the necessity of preventing cell lysis prior to the addition of the alkaline detergent solution.

For optimal recovery of pURB500, cells were grown anaerobically to late log or early stationary phase at 30°C in modified Wheaton bottles (Balch et al. 1979) with gentle shaking. The cultures were pressurized to 20 psi with $H_2:CO_2$ (80:20) at the time of inoculation and were regassed periodically as the pressure dropped during growth. The remainder of the procedure was performed under aerobic conditions. Cells were harvested by centrifugation at room temperature; 200 ml portions were resuspended in 3 ml of a buffer containing 50 mM Tris (pH 9), 25% sucrose, 0.4 M NaCl, 10 mM EDTA, and 50 μg/ml proteinase K (Boehringer, Mannheim, FRG). The suspension was incubated in a 50°C water bath with periodic swirling for 30 min or until most of the clumped cells dispersed. Then 6 ml of alkaline lysis buffer (0.2 M NaOH, 1% SDS) was added and the lysate was held on ice for 10 min. The pH of the lysate was neutralized by the addition of 4.5 ml of a solution which was 3 M with respect to K^+ and 5 M with respect to acetate, pH 4.8. The remainder of the procedure was exactly as described in Maniatis et al. (1982). We found it necessary to put the crude plasmid preparation through two rounds of CsCl gradient ultracentrifugation in order to eliminate all chromosomal DNA. Even so, the final dialyzed product remained heavily contaminated with protein, as evidenced by an A_{260}/A_{280} ratio of approximately 1.1. The residual protein, however, was readily eliminated by successive extractions with phenol/chloroform (1:1) and chloroform, followed by reprecipitation with ethanol. The final yield of purified plasmid (A_{260}/A_{280} = 1.7) was generally 10–15 μg per liter of cells.

Results and discussion

Figure 1 shows electron micrographs of pURB500 and monomeric pBR322 (used as a size standard). The plasmids were prepared for electron microscopy by the Kleinschmidt technique (1968) and rotary shadowed with platinum and palladium. Contour length measurements of six pURB500 molecules indicated a size of 8.70 ± 0.17 kb, and this value agreed with that obtained by restriction fragment analysis (Figs. 2 and 3). Some apparent dimeric forms were observed. The plasmid DNA was denatured and renatured to look for

Offprint requests to: J. Konisky

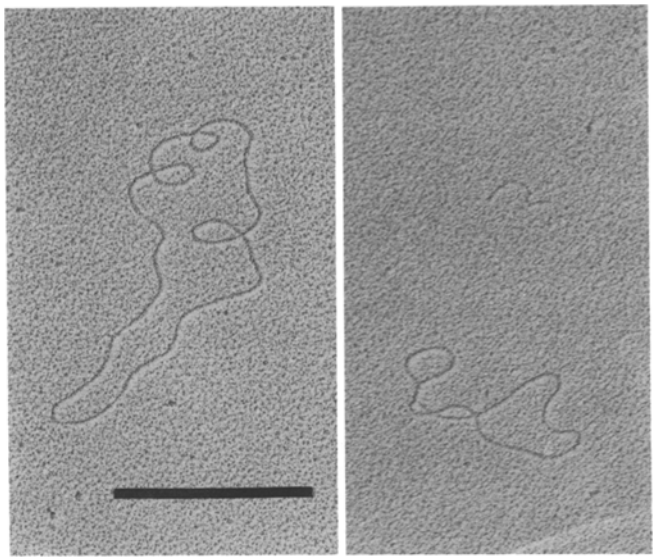

Fig. 1. Electron micrographs of open circular (**A**) pURB500 and (**B**) pBR322. Bar equals 0.2 μm

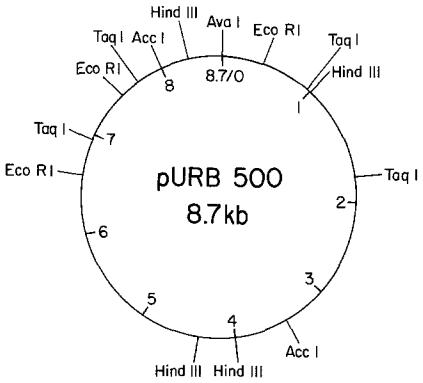

Fig. 2. Restriction map of pURB500. The single *AvaI* site was arbitrarily assigned position zero. Mapping was performed by carrying out single and double digests of pURB500 and analyzing fragments on a variety of agarose gels (ranging from 0.8% to 2%) with appropriate size standards

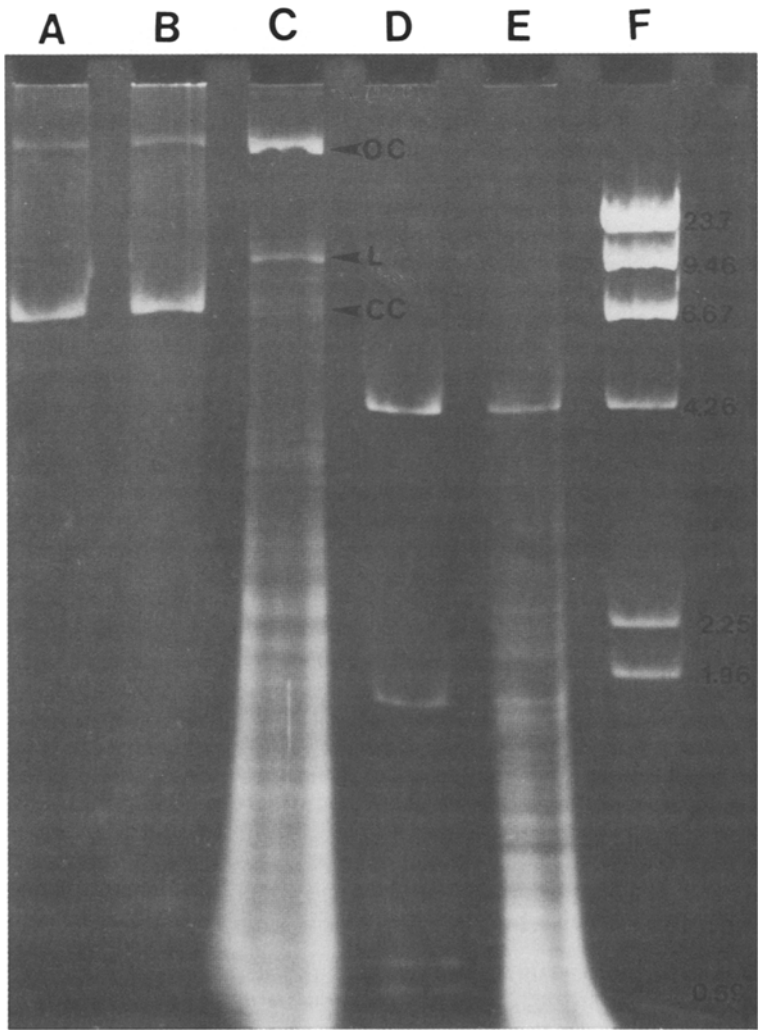

Fig. 3
Photograph of 1% agarose gel stained with ethidium bromide. *A* Undigested, CsCl gradient-purified pURB500; *B Sau3AI*-treated pURB500; *C Sau3AI*-digested C5 total DNA; *D TaqI*-digested pURB500; *E TaqI*-digested C5 total DNA; *F HindIII*-digested bacteriophage lambda DNA. Symbols: *OC* open circular; *L* linear; *CC* covalently closed circular. Assignment of plasmid forms to specific bands was made according to the method of Hintermann et al. (1981). Size standards are in kilobase pairs. The low proportion of CC plasmid in lane *C* is due to nicking during preparation or subsequent freezing and thawing of the total DNA

loop or bubble structures indicative of inverted repeats, but no such forms were found.

A restriction map for pURB500 is shown in Fig. 2. The plasmid was found to contain a single recognition site for *AvaI*, two *AccI* sites, three *EcoRI* sites, and four sites each for *HindIII* and *TaqI*. Restriction enzymes possessing five or more sites (all unmapped) included *AluI*, *HinfI*, *RsaI*, and *AhaIII*. No recognition sites were found for *BamHI*, *BglII*, *ClaI*, *HpaI*, *PstI*, *PvaI*, *SalI*, *Sau3AI*, *SmaI*, *XbaI*, or *XhoI*. Given the low G + C content of DNA from methanococci (approximately 31%, see Balch et al. 1979), we were not surprised at the failure of enzymes such as *BamHI* and *PstI* to cut pURB500. It seemed peculiar, however, that the four-base enzyme *Sau3AI* (recognition sequence GATC) did not cut the methanogen plasmid, when, as shown in Fig. 3 (lane C), *Sau3AI* cut the chromosomal DNA into predominantly small-sized fragments.

From Fig. 3 (lanes D and E) it is apparent that the largest *TaqI* fragment present in this strain is plasmid-derived. We exploited this observation to obtain an estimate of the plasmid copy number by digesting various quantities of pure plasmid and total DNA with *TaqI*, subjecting them to agarose gel electrophoresis in alternating lanes, staining with ethidium bromide, and observing equivalent fluorescence in the large *TaqI* fragment (data not shown). From the equivalence of 10 ng pURB500 with 1 μg total DNA, and assuming a genome size equal to that of *Methanococcus voltae* (Klein and Schnorr 1984), we have estimated a plasmid copy number of three per genome.

To determine whether related DNA sequences (plasmid or chromosomal) might be present in any of the other new strains, we biotin-labeled (Langer et al. 1981) pURB500 and used it to probe *HindIII*-digested, Southern-blotted (Southern 1975) total DNAs from these strains and from *Methanococcus voltae*. Even under conditions of somewhat reduced stringency, hybridization occurred only to DNA from strain C5 (data not shown). Thus, the sequences present on pURB500 appear to be unique to this strain. Taxonomic characterization of all our new isolates is underway.

Acknowledgments. This work was supported by National Science Foundation grants NSF PCM 82-14068 (to W. W.) and NSF PCM 83-13901 (to J. K.) and Department of Energy grant DOE DEFG 02-84ER13241 (to J. K.).

The authors acknowledge the assistance of Margery Sullivan of Genex Corporation in preparing the electron micrographs.

References

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Hintermann G, Fischer H-M, Crameri R, Hutter R (1981) Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid 5:371–373

Klein A, Schnorr M (1984) Genome complexity of methanogenic bacteria. J Bacteriol 158:628–631

Kleinschmidt AK (1968) Monolayer techniques in electron microscopy of nucleic acids. Methods Enzymol 12:361–377

Langer PR, Waldrop AA, Ward DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc Natl Acad Sci USA 78:6633–6637

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Meile L, Keiner A, Leisinger T (1983) A plasmid in the archaebacterium *Methanobacterium thermoautotrophicum*. Mol Gen Genet 191:480–484

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Thomm M, Altenbuchner J, Stetter KO (1983) Evidence for a plasmid in a methanogenic bacterium. J Bacteriol 153:1060–1062

Received April 12, 1985/Accepted April 17, 1985