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Evidence for a Plasmid in a Methanogenic Bacterium

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Among 15 strains of methanogens, one plasmid, pMP1, was identified in the new coccoid isolate PL-12/M. It could not be detected in the cleared lysate, but it was detected in the viscous pellet. The plasmid had a molecular weight of ca. 4.6 $\times 10^6$. A restriction enzyme cleavage map of the cloned plasmid was derived.

Methanogenic bacteria are a diverse group of strict anaerobes which share the ability to produce methane. According to 16S rRNA analyses, the methanogens belong to the archaebacteria, the third kingdom of life besides eubacteria and the eucaryotic cytoplasm (1, 5, 19). Recently, the DNA-dependent RNA polymerases from representatives of different orders of archaebacteria have been isolated (20), including those from methanogenic bacteria (13), which all show striking similarities to eucaryotic RNA polymerases (20, 21). For studying archaebacterial transcription systems in more detail, especially for establishing an in vitro transcription system, homologous templates are required. In this kingdom, however, no phages or plasmids are known, the only exception being the extreme halophiles (9, 10, 12, 16, 17), which, however, seem to be less appropriate for in vitro transcription studies because of their high intracellular salt concentrations (3). We therefore screened methanogenic bacteria for the existence of plasmids. In this paper, we report on the isolation and first characterization of a cryptic plasmid from a novel methanogen.

A total of 15 strains of methanogens of the orders methanococcales and methanomicrobiales cultivates by the technique of Balch and Wolfe (2) were screened for the presence of plasmids. Included were strains obtained from culture collections and new isolates from our laboratory. Cells were lysed by a procedure described for *Escherichia coli* (4), but without the lysozyme treatment. In all of the methanogens, however, the cleared lysate, which in *E. coli* harbors the enriched plasmid DNA and fragments of chromosomal DNA (4), contained no plasmid DNA detectable by agarose gel electrophoresis (data not shown). Thus, the viscous pellet, obtained by clearing the lysate, which is known to contain the bulk of chromosomal DNA in *E. coli* (4), was analyzed for the presence of plasmids after alkaline treatment, phenol-chloroform extraction, and concentration by ethanol precipitation. In one of our new isolates, the coccoid methanogen PL-12/M, this fraction exhibited in agarose gel electrophoresis two strong additional bands besides the chromosomal DNA band (Fig. 1, lane c); the faster one probably corresponded to the covalently closed circular (ccc) and the slower one to the open circular (oc) form of a plasmid. The methanogen plasmid was further purified by cesium chloride-ethidium bromide gradient centrifugation. After ultracentrifugation, two clearly separated bands became visible, indicating the presence of ccc-DNA besides the chromosomal DNA. This result was confirmed by agarose gel electrophoresis. The heavier band consisted of the plasmid DNA (Fig. 1, lane b), and the lighter band contained the chromosomal DNA (data not shown). In control experiments, the known plasmids (9, 18) of *Halobacterium cutirubrum* (Fig. 1, lane e), *Halobacterium halobium* (data not shown), and *E. coli* DS609 (Fig. 1, lane d) could be detected by the same procedure. In contrast to the methanogen, the plasmid DNA in halobacteria and *E. coli* was found both in the viscous pellet and in the cleared lysate (data not shown).

The molecular weight of the methanogen plasmid was determined by agarose gel electrophoresis. Comparison of its electrophoretic mobility (Fig. 1, lane b) with that of marker plasmids (Fig. 1, lane a) in a calibration curve led to a molecular weight of ca. 4.6×10^6.

Analysis of the plasmid band by electron microscopy showed that it contained a single species of DNA molecule existing as a monomer in the ccc- and oc-forms (Fig. 2). The molecular weight determined by contour length measurement was $4.57 (\pm 0.11) \times 10^6$.

Repeated attempts to isolate the methanogen plasmid from transferred cultures of strain PL-12/M led to highly variable plasmid yields, indicating that the plasmid is occasionally not detectable by the isolation procedure. The reason

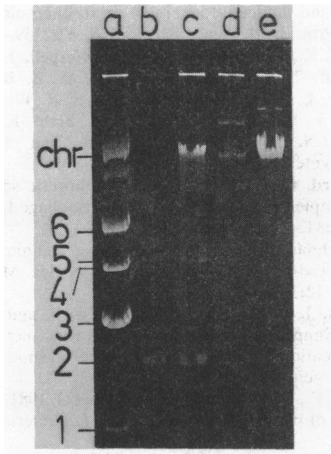

FIG. 1. Plasmids of the methanogen PL-12/M, *H. cutirubrum* and *E. coli* resolved by agarose gel electrophoresis. Electrophoresis was performed in 0.7% (wt/vol) vertical agarose gels in Tris-phosphate buffer (6) at 4 V cm^{-1} for 16 h at 5°C. (a) Marker plasmids of *E. coli*: 1, pACYC184 (2.65×10^6); 3, pJOE106 (6.05×10^6); 4, pJOE229 (9×10^6); 6, pRIT10003 (12.2×10^6). (b) Methanogen plasmid pMP1 purified by cesium chloride-ethidium bromide centrifugation: 2, ccc form; 5, oc form. (c) DNA of PL-12/M isolated without gradient centrifugation, showing chromosomal DNA (chr) and the ccc (band 2) and oc (band 5) forms of the plasmid pMP1. (d) DNA of *E. coli* DS609 and (e) *H. cutirubrum* isolated without gradient centrifugation, showing chromosomal (chr) and plasmid DNA.

for this unusual phenomenon is unknown at this point.

For further analysis of the plasmid, it was cloned into the vector plasmid pBR322. As the methanogen plasmid pMP1 was cleaved once by the restriction endonuclease *Pst*I (data not shown), *Pst*I-cleaved linear pMP1 DNA was ligated into the *Pst*I site of pBR322 (14) by a standard technique (15) and amplified in *E. coli* K12 strain HB101 (data not shown). Analysis of three recombinant plasmids by restriction enzyme cleavage revealed that the pMP1 plasmid was inserted in both orientations (data not shown). One of them, pPF1260-3, was used for detailed mapping of the restriction sites. The plasmid was digested by using various restriction enzymes either individually or in combination (see legend to Fig. 3). The positions of restriction sites within the plasmid pBR322 are taken from Sutcliffe (14). The restriction enzyme cleavage map shown in Fig. 3 is based on the sizes of the restriction fragments obtained from single and double digests.

None of the methanogens from culture collections investigated in the present study did possess a detectable plasmid. Therefore, we assumed that possible plasmids in methanogens coding for additional functions during life in nature had been lost during the serial transfers in the laboratory on artificial media. As a consequence, we isolated strains from natural habitats and screened them immediately for the presence of plasmids. One isolate from a submarine fumarole close to Vulcano Island, Italy, named PL-12/M, was found to bear a plasmid which could be isolated. To our knowledge, it is the first methanogen plasmid described.

During its enrichment, the plasmid could not be detected in the supernatant of the centrifuged cell lysate where plasmids are usually found, but it was detected almost quantitatively in the pellet, together with the chromosomal DNA. This uncommon behavior could be explained by a very close association of the plasmid with the chromosomal DNA or with the membrane.

Due to its small size, the methanogen plasmid may be well suited for studies of gene expression in methanogens, e.g., transcription studies and promoter analyses. Experiments to determine

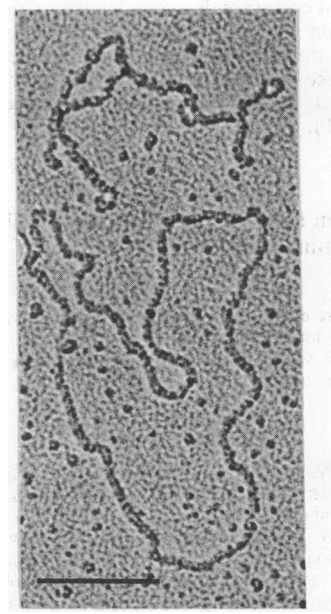

FIG. 2. Electron micrograph of ccc (top) and oc DNA (below) molecules of the methanogen plasmid. DNA molecules were prepared for electron microscopy by the cytochrome *c* spreading technique of Kleinschmidt (7). DNA was stained with 50 μg of uranyl acetate in 90% ethanol and rotary shadowed with platinum-iridium at an angle of 5°. Microscope magnifications were calibrated by using RSF2124 DNA (molecular weight, 7.35×10^6) as an internal standard. From the contour length of the pMP1 plasmid molecule (10 independent molecules measured), a molecular weight of $4.57 (\pm 0.11) \times 10^6$ was calculated. Bar equals 0.2 μm.

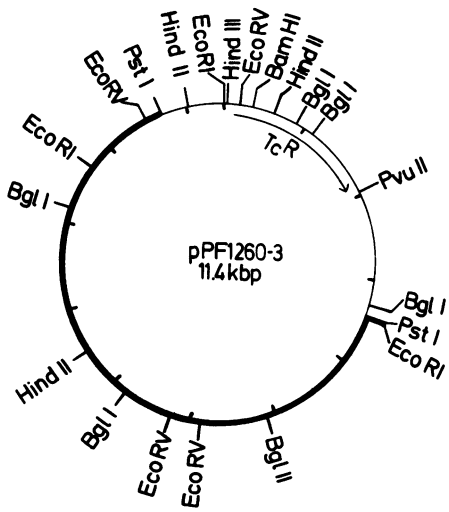

FIG. 3. Restriction endonuclease cleavage map of the recombinant plasmid pPF1260-3. The heavy line marks the extent of the methanogen plasmid, pMP1, and the thin line represents the vector pBR322 with the location and polarity of the tetracycline resistance determinant (Tc^r) indicated by an arrow (11). One scaling unit corresponds to one kilobase pair. The map is based on the sizes of restriction fragments from single and double digests obtained after electrophoresis of these fragments in 0.7% (wt/vol) horizontal agarose gels in Tris-borate (8). Electrophoresis was performed for 15 h at 4 V cm^{-1}.

the reason for the varying plasmid yield and its possible functions are in progress.

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