Isolation and characterization of plasmids from different strains of *Methanobacterium thermoformicicum*

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Seven strains of the formate-utilizing thermophilic methanogen *Methanobacterium thermoformicicum* were screened for the presence of extrachromosomal DNA. Covalently closed circular plasmid DNA was detected in three strains, Z-245, FTF and THF. No plasmids were found in strains CB12 and SF-4, nor in two new isolates, FF1 and FF3. The plasmid from strain Z-245, designated pFZ1, and that from strain FTF, designated pFZ2, were approximately 10.5 kb in size and contained homologous and similarly sized restriction fragments. A physical map of plasmid pFZ1 was constructed and three KpnI-fragments comprising the entire plasmid were cloned in *Escherichia coli* using pUC19. Plasmid pFV1 isolated from strain THF was approximately 14 kb in size and contained regions with strong homology to pFZ1 DNA. Using the *Methanobacterium formicicum* *fdhA* gene as a hybridization probe the strains of the species *M. thermoformicicum* could be classified into two major groups.

Introduction

The concept of the Archae forming one of the three domains of living organisms (Woese et al., 1990) has generated considerable interest in identifying and characterizing archaeal extrachromosomal elements in order to study gene transfer in and develop transformation systems for these unique organisms. Furthermore, detailed knowledge of these extrachromosomal elements contributes to understanding their evolution and their possible interaction with the chromosome of their hosts.

The presence of viruses and virus-like particles has been described in various representatives of the archaeal domain, including sulphur-dependent, halophilic and methanogenic species (for reviews, see Brown et al., 1983; Wood et al., 1989). Several plasmids up to 150 kb in size have been isolated from halophilic bacteria (Brown et al., 1989). Some of these appear to be involved in genome instability (Pfeifer et al., 1981) and carry genes with known functions (DasSarma et al., 1987). In contrast, all plasmids isolated so far from methanogens are cryptic. These include four plasmids derived from *Methanococcus* (Wood et al., 1985; Zhao et al., 1988), one from *Methanosarcina acetivorans* (Sowers & Gunsalus, 1988), one from *Methanolobus vulcani* (Thomm et al., 1983), which also may be a phage (Wilharm et al., 1986), and a plasmid from *Methanobacterium thermoautotrophicum* strain Marburg (Meile et al., 1983). In this report we describe the isolation of plasmid DNA from three strains of *M. thermoformicicum* and the characterization of one of these plasmids, the cryptic plasmid pFZ1 from strain Z-245. Furthermore, we identify plasmid-free *M. thermoformicicum* strains which may be used as recipient strains in a transformation system.

Methods

Bacterial strains and vectors. Table 1 lists the strains of *M. thermoformicicum* used in this study. *M. formicicum* strain MS1 (DSM 3636) was kindly provided by G. D. Vogels (Department of Microbiology, University of Nijmegen, The Netherlands). *M. wofei* (DSM 2970) and *M. thermoautotrophicum* strains Marburg (DSM 2133) and ΔH (DSM 1053) were from the German Collection of Microorganisms, Braunschweig, FRG. *Escherichia coli* strain DH5α and plasmid pUC19 (Yanisch-Perron et al., 1985) were obtained from Life Technologies.

Growth conditions. *M. formicicum*, *M. thermoformicicum* and *M. thermoautotrophicum* were grown on minimal medium containing, per litre (J. P. Touzel, personal communication): KH₂PO₄, 0.3 g; NH₄Cl, 1.0 g; NaCl, 0.6 g; MgCl₂ · 6H₂O, 0.1 g; CaCl₂, 2H₂O, 0.06 g; NaHCO₃, 4.0 g; trace element solution, 10 ml (prepared according to Touzel & Albagnac, 1983, except that 0.05 g AlCl₃ · 6H₂O l⁻¹ was added). For cultivation in serum bottles 0.5 g cysteine hydrochloride l⁻¹ was added. The pH of the medium was adjusted to 7.2. For growth of *M. wofei*, the medium was supplemented with 2.6 mg Na₂WO₄ · 2H₂O l⁻¹ (Winter et al., 1984). After autoclaving under N₂/CO₂ (4:1, v/v) the headspace was flushed with 300 kPa H₂/CO₂ (4:1) and 0.5 g Na₂S · 9H₂O l⁻¹ was added. Except for *M. formicicum*, all methano-
Table 1. *M. thermoformicicum* strains screened for the presence of plasmid DNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid DNA (size in kb)</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-245 (DSM 3720)</td>
<td>pFZ1 (10.5)</td>
<td>DSM*</td>
</tr>
<tr>
<td>FTF (DSM 3012)</td>
<td>pFZ2 (10.5)</td>
<td>DSM</td>
</tr>
<tr>
<td>THF (DSM 3848)</td>
<td>pFV1 (14)</td>
<td>DSM</td>
</tr>
<tr>
<td>CB12 (DSM 3664)</td>
<td>-</td>
<td>DSM</td>
</tr>
<tr>
<td>SF-4</td>
<td>-</td>
<td>K. Yamamoto, Osaka City University, Japan, This work</td>
</tr>
<tr>
<td>FF1</td>
<td>-</td>
<td>This work</td>
</tr>
<tr>
<td>FF3</td>
<td>-</td>
<td>This work</td>
</tr>
</tbody>
</table>

* DSM, German Collection of Microorganisms, Braunschweig, FRG.

genic strains were cultivated according to Schönhelt *et al.* (1980) in 0.81 and 101 fermenters containing 0.5 l and 8 l of medium, respectively. For growth in fermenters Na₂S was replaced by 0.62 g thiosulphate l⁻¹ and 0.5 g cysteine l⁻¹ (J. Korteland, personal communication). In addition 10 µg streptomycin ml⁻¹ was added. Prior to entering the vessel, the gas mixture was led through the gas phase of a closed bottle filled with 100 ml Na₂S (0.1 g l⁻¹) serving as a reducing agent. The cultivation temperature was 37 °C for *M. formicicum*, 55 °C for *M. thermoformicicum* strains Z-245, FTF, CB12, SF-4, FF1 and FF3, and 65 °C for *M. thermoformicicum* strain THF, *M. thermoautotrophicum* strains Marburg and AH and *M. wolfei*. *E. coli* strain DH5α was grown in L-broth and handled as described by Maniatis *et al.* (1982).

**Isolation of DNA.** Pelleted cells were resuspended in TE buffer (10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA) at a concentration of 1 g wet weight in 5 ml buffer. After addition of SDS to a final concentration of 0.5%, proteinase K (0.1 mg ml⁻¹; Boehringer Mannheim) and about a one-third volume equivalent of glass beads (0.3 mm), the suspension was incubated for 1 h at 56 °C. After cooling on ice the suspension was vigorously shaken for 15 s on a Vortex mixer and then kept on ice for 15 s. This shaking/cooling procedure was repeated 5–10 times until the cell suspension became viscous. Subsequently, the suspension was thoroughly deproteinized by several phenol/chloroform extractions followed by an extraction with chloroform. Finally, the nucleic acids were precipitated with ethanol and dissolved in TE buffer. In some cases, plasmid DNA was isolated by CsCl/ethidium bromide density-gradient centrifugation (Maniatis *et al.*, 1982).

For copy-number determination of plasmid pFZ1, total DNA was isolated from lysed protoplasts of *M. thermoformicicum* strain Z-245 to avoid possible nicking. For this purpose late-exponential phase cells, grown in serum bottles as described above, were harvested and incubated anaerobically with an autolysate of *M. wolfei* until protoplasts were formed, essentially as described by Mountfort *et al.* (1986). These protoplasts were washed with phosphate/sucrose buffer (Mountfort *et al.*, 1986) and resuspended in ice-cold TE buffer to lyse the protoplasts. The DNA was further purified as described above.

**Isolation of formate-utilizing, thermophilic methanogens.** Samples were taken from a thermophilic (55 °C) upflow anaerobic sludge-bed reactor (Department of Environmental Engineering, University of Wageningen, The Netherlands) and inoculated into serum bottles containing 20 ml of a selective medium for formate-utilizing methanogens. This consisted of minimal medium as described above with the addition of 50 mM-sodium formate, 50 mM-Tris/HCl, pH 7.3, 0.01 g yeast extract l⁻¹ and 10 ml vitamin solution l⁻¹ [prepared according to Balch *et al.* (1979), except that lipoic acid was omitted]. The cultures were incubated anaerobically at 55 °C under an N₂/CO₂ atmosphere (200 kPa overpressure). When the cultures became turbid, samples were plated on selective medium supplemented with 0.62 g thiosulphate l⁻¹ and 0.5 g cysteine l⁻¹ instead of Na₂S and solidified with 0.8% Gelrite (Roth), followed by incubation at 55 °C under an N₂/CO₂ atmosphere. Colonies became visible after about 1 week and were transferred anaerobically into serum bottles containing selective medium followed by incubation at 55 °C. After growth had developed the cultures were inspected microscopically and methane production was determined with a Becker gas-chromatograph model 417 with a TCD detector. The plating and inoculation procedures were repeated twice for methane-producing cultures.

**DNA manipulations.** Chromosomal and plasmid DNA were digested with endonucleases obtained from Life Technologies. For cloning into *E. coli* (Maniatis *et al.*, 1982), DNA fragments of both vector and insert DNA were isolated from agarose gels using a GeneClean kit as described by the supplier (Bio 101). Transfer of DNA from agarose gels to GeneScreen Plus membranes (NEN-Du Pont) was carried out using a capillary blot procedure as described by the supplier. The DNA probe was produced by labelling DNA by nick-translation (Maniatis *et al.*, 1982) with [γ³²P]ATP (Amersham). If mixed probes were used, equal amounts of DNA fragments were labelled.

**Results**

**Isolation of plasmid DNA and characterization of plasmid pFZ1.** Bacteria belonging to the species *Methanobacterium* have a rigid cell wall, with pseudomurein as the primary component, which is resistant to methods commonly used for lysis of bacterial cells. Therefore, we disrupted the cells by mechanical force using glass beads as has previously been described for the successful disruption of fungal spores (Van Etten & Freer, 1978). Both chromosomal and plasmid DNA from *M. thermoautotrophicum* strain Marburg (Meile *et al.*, 1983) could be isolated efficiently, which made the method suitable for the rapid screening of methanogens for the presence of plasmid DNA.

Using this procedure, we detected extrachromosomal DNA in *M. thermoformicicum* strain Z-245 (Fig. 1a, lane 3). To investigate the identity of this DNA, total DNA from strain Z-245 was subjected to CsCl/ethidium bromide density-gradient centrifugation and the covalently closed circular (ccc)-fraction, consisting of supercoiled plasmid, was isolated. Except for some residual chromosomal DNA, the DNA of the ccc-fraction comigrated upon agarose gel electrophoresis with the extrachromosomal DNA band found in the total DNA (Fig. 1a, lanes 3 and 4).

Comparison with supercoiled DNA markers indicated that the plasmid, which was designated pFZ1, had a size of approximately 10.5 kb. A physical map of pFZ1 containing 31 restriction sites was constructed (Fig. 2). Digestion of pFZ1 with *KpnI* resulted in the formation of three DNA fragments of 2.1, 3.7 and 4.7 kb, fragments I,
Plasmids from Methanobacterium thermoformicum 1983

Fig. 1. Agarose gel electrophoresis (a) and Southern hybridization (b) of plasmid pFZ1 DNA purified by CsCl/ethidium bromide density-gradient centrifugation from M. thermoformicum strain Z-245. Lanes 1 and 10, HindIII-digested phage λ DNA; lane 2, supercoiled size marker (with sizes from top to bottom of 16.2, 14.2, 12.1, 10.1, 8.1, 7.0, 6.0, 5.0, 4.0, 3.0 and 2.0 kb) (Life Technologies); lane 3, total undigested DNA of strain Z-245; lanes 4, 6 and 8, ccc plasmid DNA fraction, undigested, and digested by BamHI and KpnI, respectively; lanes 5, 7 and 9, oc fraction, undigested, and digested with BamHI and KpnI, respectively. The autoradiograph (b) is of the corresponding Southern blot hybridized under stringent conditions with equal amounts of KpnI fragments I, II and III of plasmid pFZ1 labelled by nick-translation. The position of linear DNA size markers (in kb) is shown on the left.

Fig. 2. Restriction map of plasmid pFZ1. The three subcloned KpnI fragments I, II and III that were cloned using pUC19 are indicated in the inner circle. Sizes are given in kb.

II and III respectively (Fig. 2). All three KpnI fragments were cloned in E. coli using pUC19 and used as probe in subsequent hybridization experiments. DNA purified from strain Z-245 by CsCl/ethidium bromide density-gradient centrifugation was digested with BamHI and KpnI, separated by agarose gel electrophoresis, blotted and hybridized to labelled pFZ1 fragments (Fig. 1b, lanes 6–9). Only the expected fragments of plasmid DNA present in the total DNA showed hybridization, indicating that no sequences homologous to plasmid pFZ1 are present in the chromosomal DNA of strain Z-245 (see also Fig. 3). Furthermore, using pFZ1 DNA as probe, no pFZ1 DNA could be detected in the supernatant of a culture of strain Z-245 with methods commonly used for the isolation of bacteriophage DNA (Maniatis et al., 1982; Wood et al., 1989).

We also detected plasmid DNA in M. thermoformicum strains FTI and THF; these plasmids were designated pFZ2 and pFV1, respectively (Table 1). Plasmid pFZ2 was similar in size to plasmid pFZ1 (10.5 kb, see below). In contrast, plasmid pFV1 was larger than plasmid pFZ1, with a size of approximately 14 kb as revealed by comparison of ccc-DNA from plasmid pFV1 isolated by CsCl centrifugation with supercoiled DNA.
Fig. 3. Southern hybridization of total DNA from *M. thermoformicicum* strains Z-245, FTF and THF with labelled pFZ1 DNA. Total DNA (about 1.5 μg each) of strains Z-245 (lanes 2 and 6), FTF (lanes 3 and 7) and THF (lanes 4 and 8) was digested with *XhoI* (panels *a*, *b* and *c*) and *HindIII* (panel *d*) and separated by agarose gel electrophoresis. Blots were hybridized under stringent conditions to equal amounts of *KpnI* fragments I (*a*), II (*b*) and III (*c*), and to all three fragments (*d*), labelled by nick-translation. Lanes 1', 1'' and 1''' contain purified *KpnI* fragments I, II and III of plasmid pFZ1, respectively, used as the probe; lane 5 contains all three *KpnI* fragments. The position of linear DNA size markers (in kb) is shown on the left.

markers separated by agarose gel electrophoresis (data not shown). No extrachromosomal DNA was detected in *M. thermoformicicum* strains CB12 and SF-4 or in the new isolates, strains FF1 and FF3.

**Determination of the copy-number of plasmid pFZ1**

To determine the copy-number of plasmid pFZ1 several dilutions of total DNA isolated from strain Z-245 were analysed by agarose gel electrophoresis. By comparing the intensity of plasmid and chromosomal DNA bands we found that the intensity of the ccc-plasmid band in the undiluted fraction was comparable with that of the chromosomal DNA band in the fraction diluted 40 times. Assuming that the size of the chromosomal DNA of *M. thermoformicicum* strain Z-245 is equivalent to that of *M. thermoautotrophicum* strain Marburg (1.2 × 10⁹ Da) (Brandis *et al*., 1981), this indicates that strain Z-245 contains at least four copies of pFZ1 per chromosome.

**Sequence homologies between plasmid pFZ1 and DNA from other methanogens**

None of the labelled *KpnI*-fragments of pFZ1 (Fig. 2) hybridized to total DNA of *M. thermoautotrophicum* strains Marburg and AH, *M. formicicum* strain MS1 or *M. thermoformicicum* strains CB12 and SF-4 and the new isolates FF1 and FF3 (data not shown). In contrast, hybridization of these pFZ1 DNA fragments was observed to total DNA isolated from strains FTF and THF. Subsequent experiments using undigested and digested DNA of both these strains showed that this was due to homology of pFZ1 DNA with the plasmid DNA of strains FTF and THF (data not shown).

To determine the degree of homology between the plasmids, total DNA isolated from *M. thermoformicicum* strains Z-245, FTF and THF was digested with *XhoI* and *HindIII*, separated by agarose gel electrophoresis, blotted and hybridized to the individually labelled *KpnI* fragments I, II and III of pFZ1 (Fig. 2). The results (Fig. 3) indicate that (i) the hybridizing fragments of DNA
from strains Z-245 and FTF are of identical size (Fig. 3, lanes 2, 3, 6 and 7), corresponding with that expected from pFZ1 (Fig. 2); (ii) a different hybridization pattern is obtained with DNA from strain THF (Fig. 3, lanes 4 and 8); and (iii) that pFZ1 has no detectable homology with the chromosomal DNA of all three strains.

To analyse the high similarity of plasmid DNA from strains M. thermoformicicum Z-245 (pFZ1) and FTF (pFZ2) in more detail, total DNA from both strains was digested with restriction enzymes known to have several recognition sites in plasmid pFZ1 and, after separation and blotting, the DNA was hybridized to labelled pFZ1 DNA. All 31 restriction fragments derived from digestion of plasmid DNA of both strains with EcoRI, PstI, ApaI, RsaI, AluI, HaeIII and MspI were found to have the same size (data not shown).

**Hybridization of M. thermoformicicum strains to fdhA**

As described above M. thermoformicicum strains Z-245, FTF and THF harbour plasmids that are (partially) homologous whereas other strains lack plasmid DNA. Using the M. formicicum fdhA gene as a hybridization probe, the relationship between the M. thermoformicicum strains was examined. As shown in Fig. 4 M. thermoformicicum strains fell into two groups on the basis of hybridization patterns of total DNA: one group, designated the Z-245 group, comprised strains Z-245, FTF and THF (lanes 1, 2 and 3); a second, designated the CB12 group, included strains CB12 and SF-4 (lanes 4 and 5). Within these two groups the hybridization patterns were identical except that strain THF showed a different minor hybridization species (Fig. 4, lane 3).

The fdhA gene was also hybridized to DNA of the new strains FF1 and FF3. These strains were assigned to M. thermoformicicum because of their capacity to produce methane at 55 °C using either H2/CO2 or formate as substrate, and their similar morphology to known M. thermoformicicum strains. The hybridization results (Fig. 4) show that strains FF1 and FF3 seem to be different from each other since the major hybridization species has a slightly different mobility in each case (lanes 6 and 7). Both strains, however, show very similar hybridization patterns that resemble that of strain Z-245, and therefore these new strains may be assigned to the Z-245 group (lanes 2, 6 and 7). Interestingly, both strains FF1 and FF3 lack plasmid DNA, in contrast to all other strains of the Z-245 group (Table 1).

**Discussion**

The present report describes the identification, isolation and characterization of extrachromosomal DNA from the thermophilic, formate-utilizing M. thermoformicicum strains Z-245, FTF and THF. The extrachromosomal DNA of strain Z-245 has the characteristics of plasmid DNA, being isolated in a ccc-form by CsCl/ethidium bromide density-gradient centrifugation and not detectable in the culture supernatant as described for virus-like particles of Methanococcus voltae (Wood et al., 1989). In addition, pFZ1 shows no homology with the chromosomal DNA of the host strain Z-245 nor with that from other species of the genus Methanobacterium tested in this study (Figs 1 and 3). This is, to our knowledge, the first report of the presence of plasmid DNA in formate-utilizing, thermophilic methanogens. Plasmid pFZ1 is a relatively low-copy-number plasmid with a minimal four copies per chromosome of strain Z-245 and is stably maintained in its host during several serial transfers in the laboratory. At present we have not been able to
assign a specific function to pFZ1, which therefore remains cryptic, as are all other methanogen plasmids described so far.

Hybridization experiments revealed that *M. thermoformicicum* strains Z-245 and FTF harbour the very similar, if not identical, 10.5 kb plasmids pFZ1 and pFZ2, respectively. Both plasmids are homologous to similar, if not identical, 10.5 kb plasmids pFZ1 and pFZ2, respectively. Both plasmids are homologous to.

These results indicate that the plasmids of *M. thermoformicicum* share a common ancestor. The presence of highly similar plasmid DNA has been described for *Methanosarcina acetivorans* and two other marine acetotrophic strains, all three of which were isolated from the same location (Sowers & Gunsalus, 1988). In contrast, strains Z-245, FTF and THF were isolated from geographically very different locations (Touzel et al., 1988; Zhilina et al., 1984; Zinder & Koch, 1984).

Several attempts have been described to determine the relationship among *M. thermoformicicum* strains. Yamamoto et al. (1989) suggested that the strains may be divided into two groups, based on the cross-reaction of antibodies raised against strain SF-4 with strain CB12 and Z-245 but not with strain FTF. In contrast, DNA-DNA homology studies (J. P. Touzel, personal communication), indicated that strain FTF is more related to strains Z-245 and THF than to strain CB12 (strain SF-4 was not tested). Our observation that strains Z-245, FTF and THF harbour (partially) homologous plasmid DNA and the results based on the hybridization patterns with the *fdhA* gene support the classification of strains Z-245, FTF and THF into one group and strains CB12 and SF-4 into another group. On the basis of their hybridization patterns with the *fdhA* gene the new isolates FF1 and FF3 appear to be related to the Z-245 group. Since they are plasmid-free, those strains could be good candidates as recipients for plasmid pFZ1 or derivatives in transformation studies.

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**References**


