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Characterization of ψM1, a virulent phage of *Methanobacterium thermoautotrophicum* Marburg

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Abstract. Bacteriophage ψM1, a virulent, oxygen resistant phage of *Methanobacterium thermoautotrophicum* strain Marburg, was isolated from an anaerobic sludge digester operated at 55° C to 60° C. A reproducible plaque assay and an enrichment procedure for the preparation of high-titer lysates (2×10^{10} PFU/ml) were established. One-step growth experiments at 62° C showed that the latent period was 4 h and the burst size was 5–6 infective particles per cell. The phage infected *Methanobacterium thermoautotrophicum* Marburg but none of three other thermophilic representatives of the genus *Methanobacterium* that were tested. Electron micrographs showed that phage ψM1 has a polyhedral head of 55 nm diameter and a tail of 210 nm in length. The ψM1 genome consists of linear double-stranded DNA with a size of 30.4 ± 1.0 kb. Restriction and hybridization analysis of DNA extracted from phage particles revealed two types of linear molecules with the size of the phage genome. About 85% of the DNA molecules in such preparations were genomes of ψM1 whereas approximately 15% were multimers of the cryptic 4.5-kb plasmid pME2001 of the host. ψM1 DNA did not hybridize with chromosomal DNA of *Methanobacterium thermoautotrophicum* but it exhibited definite homology to total DNA of *Methanobacterium wolfei*.

Key words: *Methanobacterium thermoautotrophicum* – Bacteriophage – Methanogenic bacteria – Plasmid pME2001

Within the methanogen – extreme halophile branch of the archaebacteria (Woese 1987), several bacteriophages have been described for the genus *Halobacterium* (Zillig et al. 1986). The genomes of those *Halobacterium* phages that have been studied consist of linear double-stranded DNA, and with respect to their structure all these viruses are similar to eubacterial phages composed of a polyhedral head and a tail. The transcription units on the genome of halobacteriophage ΦH have been mapped (Zillig et al. 1986, 1988). This virus has been used for transfection of spheroplasts of its host, *Halobacterium halobium* (Cline and Doolittle 1987). Bacteriophage ΦN, another virus of this organism, has recently been shown to have a fully cytosine-methylated genome (Vogelsang-Wenke and Oesterhelt 1988). Information on bacteriophages from methanogens is scarce. There is a preliminary report on the isolation of a lytic bacteriophage for a *Methanobrevibacter* sp. (L. Baresi and G. Bertani, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I74, p. 133) and one which describes PMS1, a lytic phage of *Methanobrevibacter smithii* (M. R. Knox and J. E. Harris, Abstr. XIV Internat. Congr. Microbiol. 1986, P.G3–8, p. 240). This virus, with a double-stranded DNA genome of approximately 35 kilobases (kb), is reported to yield titers of 10^7 PFU/ml.

In the course of our efforts to develop gene transfer systems for *Methanobacterium thermoautotrophicum* Marburg (Rechsteiner et al. 1986) we have screened samples from mesophilic and thermophilic anaerobic habitats for lytic agents for this organism. This has led to the isolation of phage ψM1 whose properties are described in the present communication. A peculiarity of the ψM1-*Methanobacterium thermoautotrophicum* virus-host system is its efficient packaging of resident plasmid DNA from the host into phage particles.

Materials and methods

Bacterial strains and phage isolation. Methanobacterium thermoautotrophicum strain Marburg, mutants of this strain and other methanogenic bacteria used in this study are described in Table 1. Phage ψM1 was isolated from an experimental anaerobic digester operated at 55° C to 60° C. The isolation procedure involved an enrichment step, in which a mixture of 50 ml of the anaerobic sample plus 50 ml of the complete medium described below were inoculated with 2 ml of an exponentially growing culture of strain MBT1 and incubated for 48 h at 60° C under H_2/CO_2. The grown enrichment culture was centrifuged for 30 min at $20{,}000 \times g$, and the pellet was discarded. To concentrate phage particles, the supernatant was subjected to 2 h of centrifugation at $83{,}000 \times g$. The pellet was resuspended in a 1/50 volume of the supernatant by overnight incubation at 4° C on a shaker,

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Table 1. Methanogenic bacteria used in this study

Strain	Relevant properties	Reference
Methanobacterium thermoautotrophicum Marburg (DSM2133), MBT1	wildtype; contains plasmid pME2001	Fuchs et al. 1978; Meile et al. 1983
Methanobacterium thermoautotrophicum, MBT10	mutant of MBT1; Leu$^-$; resistant to pseudomonic acid; cured of plasmid pME2001	Kiener et al. 1986; this paper
Methanobacterium thermoautotrophicum, MBT21	mutant of MBT1; insensitive to phage ψM1	this paper
Methanobacterium wolfei (DSM 2970)	produces a lytic pseudomurein endopeptidase	Kiener et al. 1987; König et al. 1985; Winter et al. 1984
Methanobacterium thermoautotrophicum ΔH (DSM 1053)		Zeikus and Wolfe 1972
Methanobacterium thermoautotrophicum HV		Binder et al. 1981

and the concentrated solution was tested for plaque formation on a lawn of indicator bacteria.

Growth conditions. Methanogens were grown in the minimal medium described by Schönheit et al. (1980). Minimal medium was used for phage propagation as well as minimal medium supplemented with 2 g/l each of sodium acetate, sodium formate and yeast extract. The media were reduced with 0.5 g of $Na_2S \cdot 9\ H_2O$ and 0.5 g of L-cysteine-HCl per liter. Media for plates contained in addition 1.5% (w/v) agar and 1.0 mM titanium(III) citrate. Cells were cultivated at 62° C in stoppered serum flasks filled with medium to 10% their volume, and gassed to 2 bars with 80% H_2 – 20% CO_2. Anaerobic facilities, plating techniques and the conditions for growing cells on agar-medium were described by Kiener and Leisinger (1983). Growth and autolysis of *Methanobacterium wolfei* as well as the purification of pseudomurein endopeptidase from this organism have been described (Kiener et al. 1987).

Propagation and titration of bacteriophage. A low titer lysate ($1-3 \times 10^7$ PFU/ml) was obtained by addition of a single plaque of phage ψM1 to 10 ml of an exponentially growing culture (5×10^6 cells/ml) of strain MBT1 and incubation of this culture for 20 to 30 h under standard growth conditions. Large quantities of high titer lysates (2×10^{10} PFU/ml) were produced by infection of exponentially growing cells with a multiplicity of 2 to 4. After incubation under growth conditions for 3 h, the suspension of infected cells was concentrated to 0.1 volume by centrifugation at $5{,}000 \times g$ for 15 min under anaerobic conditions. Lysis occurred after incubation for 1.0 to 1.5 h at 62° C. The lysate was purified by CsCl-equilibrium centrifugation as described below and used for a subsequent cycle of phage propagation.

Bacteriophage titers were determined by the soft agar overlay method. A bacteriophage sample (0.05 ml) in minimal or complete medium was added to 5×10^6 exponential phase cells of strain MBT1 in 0.05 ml minimal or complete medium. This mixture was incubated at 25° C for 20 min to allow for phage adsorption. Soft agar (2.2 ml of 0.6% agar) was added and the mixture was poured onto fresh agar plates (Kiener and Leisinger 1983). A sheet of 3 MM filter paper was mounted in the cover of each petri dish for absorption of condensed water forming during incubation of the agar plates at 62° C for 3 to 4 days.

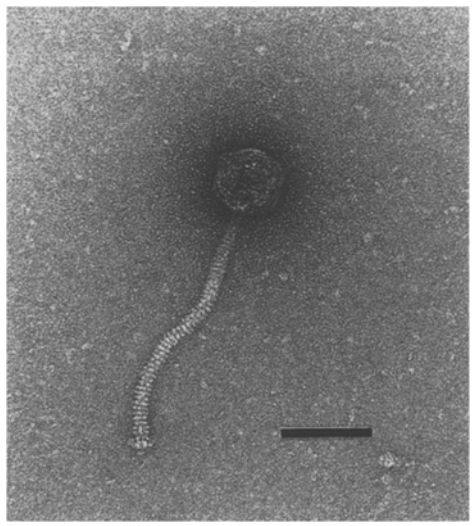

Fig. 1. Electron micrograph of phage ψM1 negatively stained with uranyl acetate. The bar represents 70 nm

Purification of bacteriophage. Phage lysates were treated with DNase I (0.025 g/l) and pancreatic RNase A (0.005 g/l) for 30 min at 37° C. If necessary, the volume was reduced to approximately 10 ml by ultrafiltration (PM30-membrane, Amicon) before CsCl-equilibrium centrifugation for 40 h at 40,000 rpm in a Ti50-rotor (Beckman). The initial density was set at 1.40. Samples with densities of 1.40 to 1.46 were collected and dialyzed against 25 mM Tris-HCl, pH 8.0 containing 5 mM $MgCl_2$.

Electron microscopy. Intact phages were prepared for electron microscopy by negative staining with 2% uranyl acetate. Length measurements of phage DNA were carried out as described (Kleinschmidt 1968) using plasmid pKT240 (Bagdasarian et al. 1983) as an internal standard.

One-step growth of phage ψM1. 8×10^7 exponentially growing cells of MBT1 were mixed with 3×10^8 infective particles in 10 ml minimal medium and incubated at 62° C under standard growth conditions. At intervals, aliquots of 0.5 ml of the growing culture were exposed to air for at least 2 h at room temperature. Exposure to air killed the host cells but

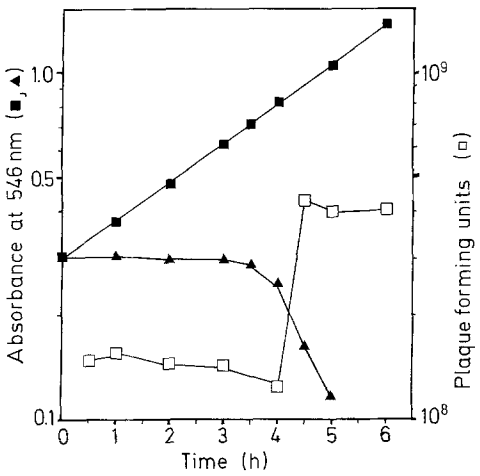

Fig. 2. One-step growth curve of phage ψM1 on cells of *M. thermoautotrophicum* MBT1. The cell density of an infected (▲) and a non-infected (■) culture was monitored at 546 nm and the total titer of free infective phage particles (□) in the medium of the infected culture was determined

did not lead to their lysis. The killed cells were removed from the oxygen-treated aliquots by centrifugation at $6{,}000 \times g$ for 5 min, and the supernatants were transferred into the anaerobic chamber. They were diluted in fresh minimal medium and used for determining the titer of infective particles.

Isolation and manipulation of DNA. Phage DNA was extracted with phenol followed by two chloroform extractions and ethanol precipitation. DNA from *Methanobacterium* sp. was isolated by the pseudomurein endopeptidase procedure developed by Kiener et al. (1987). The conditions for DNA restrictions and electrophoresis were described previously (Meile et al. 1983). DNA transfer from horizontal agarose gels to hybond-N nylon membranes (Amersham) was done according to a described method (Smith and Summers 1980). DNA hybridizations with [^{32}P]-labeled, nick-translated DNA (Rigby et al. 1977) and subsequent washing in $2 \times$ SSC containing 0.1% SDS (4 times for 1 h) were carried out at 65° C according to Southern (1975).

Results

Morphology of phage ψM1 and its plaques

The phage ψM1 was independently isolated several times from filtered samples of an experimental sludge digester operated at 55° C to 60° C. Electron micrographs of negatively stained phage ψM1 (Fig. 1) showed a polyhedral head of approximately 55 nm in diameter and a tail 210 nm long and 10 nm wide. The tail consisted of about 50 segments and exhibited a slightly enlarged terminal segment. Shortened tails suggesting contractibility were not detected. All phage particles examined showed the same structure.

Phage ψM1 formed clear plaques on lawns of MBT1. The plaque size depended on the concentration of indicator bacteria and on the type of medium used in the plaque assay. Plaques of 1 to 3 mm in diameter were formed by plating less than 10^2 phages with 1 to 5×10^6 indicator cells on plates containing minimal medium. Since smaller plaques (< 1 mm in diameter) were formed on complete medium, minimal medium was used routinely for the plaque assay. Exposure

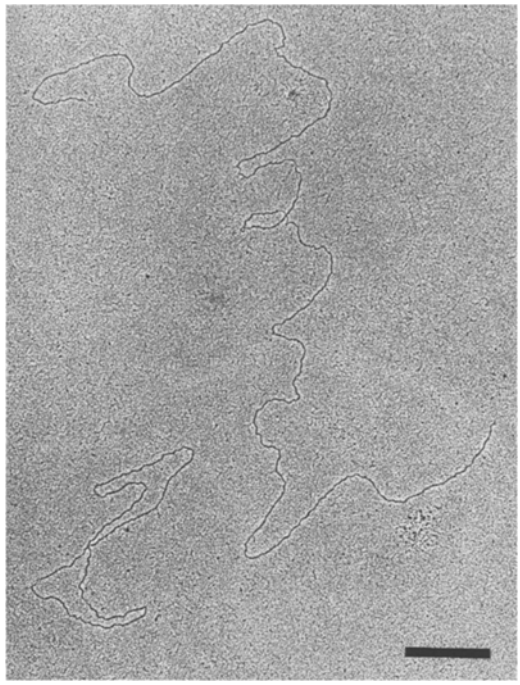

Fig. 3. Electron micrograph of ψM1 DNA (linear molecule) and of the length standard plasmid pKT240 (circular molecule). The bar represents 0.5 μm

to air did not affect the phage titer nor did storage at 4° C for at least 4 weeks.

Phage propagation

A representative one-step growth curve of phage ψM1 is illustrated in Fig. 2. Bacterial growth stopped immediately upon phage infection and the cells began to lyse after 4 h of incubation. 8×10^6 host cells per ml were exposed to 3×10^7 infective particles per ml. After adsorption the titer of PFU's decreased to 1.5×10^7 per ml, indicating a m.o.i. of about 2. According to the Poisson law (Stent 1963) 15% of the cells were not infected, and the infected cells (6.8×10^6/ml) produced 4×10^7 infective particles per ml. This corresponds to a burst size of 5.9. Burst sizes around 6 were observed in many other phage propagation experiments in minimal or complete medium. The unusually low burst size was confirmed by estimation of the amount of the DNA contained in phage lysates. For this purpose DNA extracted from lysates was subjected to agarose gel electrophoresis, stained with ethidium bromide, and the intensity of the band compared with the intensity of known amounts of marker DNA assuming a genome size of 30 kb for phage ψM1.

Because of the low burst size, it was not possible to obtain high-titer lysates in a single propagation cycle. High-titer lysates (2×10^{10} PFU/ml) were obtained by three subsequent cycles of phage propagation using a concentrated and purified lysate for each round of infection (described in Materials and methods).

Host specificity

Different thermophilic representatives of the genus *Methanobacterium* were tested as hosts for phage ψM1. Neither

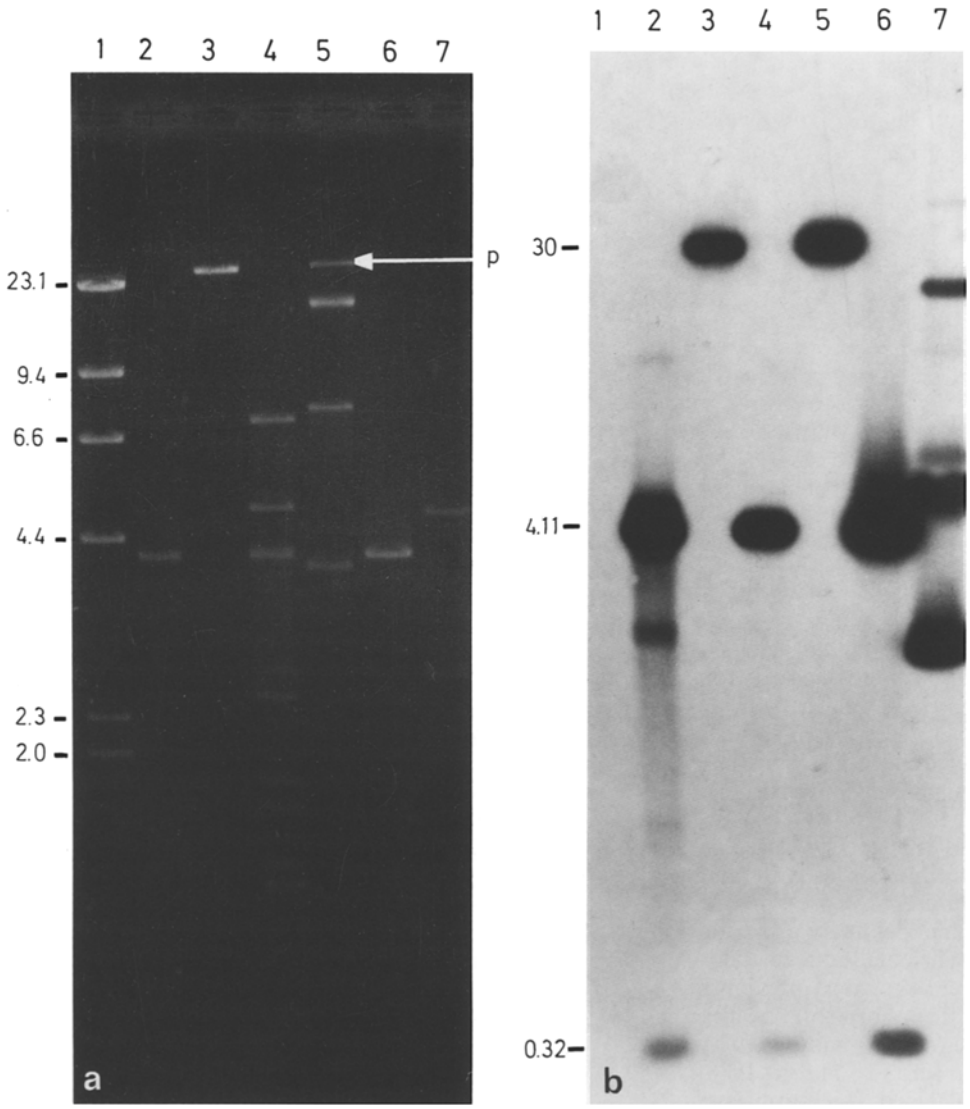

Fig. 4a, b
Restriction digestion analysis of DNA extracted from phage ψM1 particles on 0.8% agarose (**a**) and autoradiography of corresponding Southern hybridizations with [^{32}P]-labeled plasmid pME2001 DNA (**b**). *Lanes: 1, Hin*dIII-digested λ DNA; *2, Bam*HI-digested DNA corresponding to band p in *lane 5; 3,* undigested DNA from phage particles; *4, Bam*HI-digested DNA from phage particles; *5, Hin*dIII-digested DNA from phage particles; *6, Bam*HI-digested plasmid pME2001; *7,* undigested plasmid pME2001 exhibiting from top to bottom the oc dimer, the ccc dimer, the oc monomer, and the ccc monomer. Sizes of linear fragments are in kilobases

cells of *M. thermoautotrophicum* strain ΔH or HV nor *M. wolfei* were lysed within 20 h after infection with a m.o.i. of 3. However, when washed cells of all these strains were suspended in a crude ψM1 lysate obtained on strain MBT1, they were lysed within 3 h from the outside by the lytic activity contained in the lysate. Strain MBT10 (Table 1) is a leucine-auxotrophic, pseudomonic acid-resistant derivative of strain MBT1 which has undergone two mutagenesis treatments with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Kiener et al. 1986). It has recently been observed to be free of the cryptic plasmid pME2001 (Meile et al. 1983), demonstrating that this genetic element is dispensable for *M. thermoautotrophicum* Marburg (L. Meile, unpublished data). In the present survey we have found that strain MBT10 is resistant to phage ψM1 although it is susceptible to the lytic activity of lysates. Spontaneous ψM1-resistant mutants of strain MBT1 were isolated on confluently lysed agar plates. They occurred at a frequency of approximately 10^{-7}. Strain MBT21, a representative ψM1-resistant mutant containing plasmid pME2001 and sensitive to the lytic activity of ψM1-lysates is listed in Table 1.

The genome of ψM1

DNAse I and a variety of restriction endonucleases, but not pancreatic RNase A, degraded the nucleic acid of phage ψM1, indicating double-stranded DNA as its genomic material. Electron micrographs showed exclusively linear molecules of phage DNA (Fig. 3). The average size of ψM1 DNA was determined by measuring the contour length of single molecules. It was calculated to be 30.4 ± 1.0 kb by comparison with the length of plasmid pKT240 (12.5 kb). One hundred and sixteen molecules were inspected.

Restriction digestion of DNA extracted from phage particles with *Hin*dIII and gel electrophoresis produced three DNA fragments of a total length of 27.1 ± 1.0 kb and a fourth substoichiometric band at the position of unrestricted ψM1 DNA on the agarose gel (Fig. 4a, lanes 3 and 5, band p). Band p was suspected to represent unrestricted multimeric pME2001 DNA. The 4.5-kb plasmid pME2001 contains no *Hin*dIII cleavage site and two *Bam*HI recognition sites which yield fragments of 0.32 and 4.11 kb (Meile et al. 1983). This restriction pattern is in accordance with the mobility and the hybridization signal to labeled pME2001

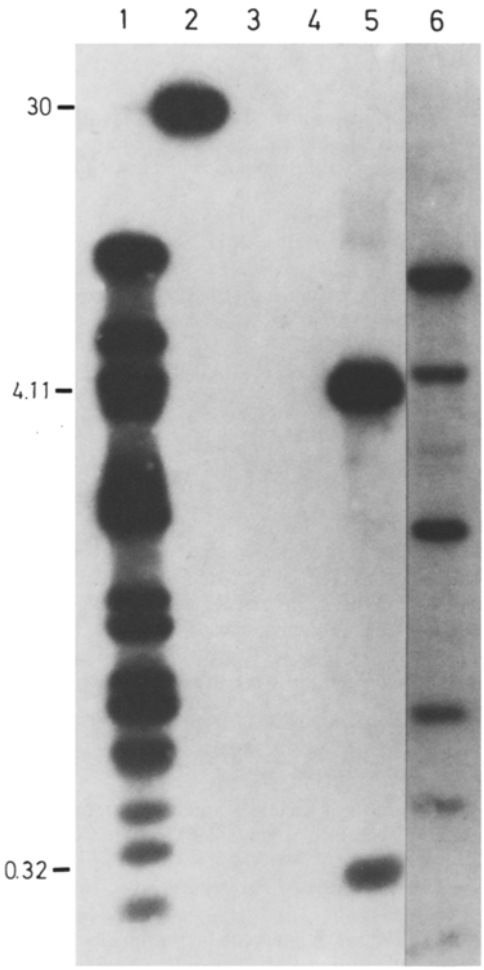

Fig. 5. Autoradiography of a Southern hybridization of *Bam*HI-restricted DNA from different *Methanobacterium* sp. with [^{32}P]-labeled DNA extracted from phage ψM1 particles containing ψM1 DNA as well as multimeric pME2001 DNA. *Lanes: 1*, *Bam*HI-digested DNA extracted from phage particles; *2*, undigested DNA from phage particles; *3*, *Bam*HI-digested DNA from *M. thermoautotrophicum* ΔH; *4*, *Bam*HI-digested DNA from strain MBT10; *5*, *Bam*HI-digested DNA from strain MBT1; *6*, *Bam*HI-digested DNA from *M. wolfei*. Sizes of linear fragments are in kilobases

DNA of band p after *Hin*dIII digestion of DNA extracted from phage particles (Fig. 4a and b, lanes 3 and 5). It is also in accordance with the banding pattern (Fig. 4a, lane 2) and the hybridization signals (Fig. 4b, lane 2) that were obtained when the DNA of band p was isolated, digested with *Bam*HI, electrophoresed and hybridized to [^{32}P]-labeled pME2001 DNA. Band p yielded two major signals corresponding to the fragments obtained upon *Bam*HI cleavage of pure plasmid pME2001 (Fig. 4a and b, lanes 6) as well as to two *Bam*HI fragments present in *Bam*HI-digested DNA extracted from phage particles (Fig. 4a and b, lanes 4). The substoichiometric hybridization signal of approximately 3.0 kb in the *Bam*HI digest of band p (Fig. 4a and b, lane 2) appeared upon overexposure of the X-ray film and may represent an endfragment arising during packaging of plasmid multimers into phage particles (see below).

In conclusion the experiment described in Fig. 4 demonstrates that the DNA extracted from ψM1 phage particles is heterogeneous. Its major part consists of ψM1 DNA. A minor fraction, 10 to 15% of the total, as estimated by the intensity of ethidium bromide-stained bands on agarose gels, is composed of plasmid pME2001 DNA. This DNA is not integrated into the phage genome but is packaged into phage particles as plasmid multimers. The two types of DNA have the same length (Fig. 4a, lane 3), probably as a consequence of a headful packaging mechanism of phage ψM1.

The sum of restriction fragments obtained by cleavage of ψM1 DNA with different restriction enzymes, excluding fragments of pME2001 multimers, added up to less than 30 kb. Restrictions with *Pvu*II (3 fragments), *Eco*RI (6 fragments), *Mlu*I (4 fragments), and *Hin*dIII (3 fragments) indicated total sizes of the phage genome of 27.1, 27.4, 27.1 and 27.2 kb, respectively. The difference between these apparent sizes and the genome size of 30.4 kb obtained from electron microscopy together with the existence of substoichiometric restriction fragments (data not shown), suggest that the ψM1 genome consists of circularly permuted, terminally redundant DNA molecules.

Homology of ψM1 DNA with total DNA of Methanobacterium wolfei

Total DNA from different strains of thermophilic *Methanobacterium* sp. was digested with *Bam*HI and hybridized with a nick-translated ψM1 DNA preparation representing a mixture of ψM1 DNA and multimeric pME2001 DNA. DNA from strain MBT1 exhibited no homology to ψM1 DNA (Fig. 5, lane 5). Both signals observed in lane 5 originated from plasmid pME2001 which is present in this strain. Such signals were absent in hybridizations with total DNA of the plasmid-free mutant MBT10 (Fig. 5, lane 4). DNA from *M. thermoautotrophicum* strain ΔH did not hybridize with ψM1 DNA (Fig. 5, lane 3), whereas total DNA of *M. wolfei* exhibited definite homology to ψM1 DNA (Fig. 5, lane 6). Since previous hybridization experiments have shown that *M. wolfei* does not contain plasmid pME2001 or DNA sequences related to this element, the signals in the DNA of this organism are due exclusively to hybridization with the ψM1 component of the labeled probe.

Discussion

In this report we present the first detailed description of a bacteriophage for a methanogenic bacterium. The morphology of phage ψM1 is similar to that of halobacterial phages (Schnabel et al. 1982; Torsvik and Dundas 1974; Vogelsang-Wenke and Oesterhelt 1988) and that of many eubacterial phages with a polyhedral head and a tail. It thus appears that unusual virus structures predominate in the branch of archaebacteria comprising the sulfur-dependent *Thermoproteales* and *Sulfolobales* (Zillig et al. 1986) whereas the viruses for organisms of the methanogen-extreme halophile branch, that have been described so far, exhibit – with one exception (A. G. Wood, A. M. Weege, P. Delannoy and J. Konisky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I37, p. 178) – classical bacteriophage morphology.

A special feature of the *M. thermoautotrophicum* – ψM1 system is the capacity to efficiently pack multimers of plasmid pME2001 into phage particles. Our data (Fig. 4) suggest that about 10% of the virus particles do not contain ψM1 DNA but head-to-tail hexamers of the 4.5-kb cryptic plasmid pME2001 linked to an incomplete terminal copy of

pME2001. Within the error of the determination (± 1.0 kb) the total size of these plasmid multimers is identical to the size of 30.4 kb of the ψM1 DNA molecules. This indicates that the packaging mechanism of ψM1 is precise for both ψM1 and pME2001 DNA. Hybridization experiments (Fig. 4) have indicated that there is no homology between these two elements. Multimers up to hexamers have been observed in preparations of plasmid pME2001 (Meile et al. 1983). At present it is not clear whether these resident multimers are packaged or whether multimerization is enhanced upon phage infection thereby leading to the high frequency of plasmid-containing virus particles. Encapsidation of head-to-tail concatemers of plasmid pBR322 has been observed with a cytosine-substituted mutant of bacteriophage T4 (Takahashi and Saito 1982a, b). In this system the frequency of particles with plasmid multimers amounted to 1% of the infective particles, which is at least 10 times lower than in the ψM1 system.

The efficient packaging of plasmid DNA by phage ψM1 gave rise to the question whether this virus encapsidates chromosomal DNA. Preliminary experiments (L. Meile, unpublished) have shown that this is the case. ψM1 had the capacity to transduce a number of chromosomal markers. It may therefore be developed into a useful tool for strain construction and mutant analysis in *M. thermoautotrophicum*.

ψM1 DNA hybridized to fragments with a total size of about 16 kb in a *Bam*HI digest of DNA from *M. wolfei* (Fig. 5). This organism lyses upon energy starvation (König et al. 1985), and the lytic enzyme responsible for this process has been shown to be a pseudomurein endopeptidase (Kiener et al. 1987). The homology between *M. wolfei* and ψM1 DNA rises the possibility that *M. wolfei* carries in its genome a defective phage which encodes pseudomurein endopeptidase. In this context it will be interesting to compare the lytic enzyme coded for by ψM1 with the pseudomurein endopeptidase of *M. wolfei*.

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