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Isolation and Characterization of an Archaebacterial Viruslike Particle from *Methanococcus voltae* A3

ALVIN G. WOOD,[1] WILLIAM B. WHITMAN,[2] AND JORDAN KONISKY[1]*

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

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Small amounts of a 23-kilobase covalently closed circular DNA molecule were isolated from unwashed cells of *Methanococcus voltae* A3. Further investigation indicated the presence of greater quantities of the circular DNA in the culture supernatant, complexed with protein in a manner rendering the DNA resistant to DNase. Electron-microscopic examination of supernatant material revealed the presence of particles which morphologically resemble virus. Phenol extraction of viruslike particle preparations resulted in the recovery of DNase-sensitive open-circular DNA molecules. As many as 30 viruslike particles per cell were recovered from some cultures. Hybridization data clearly indicated the presence of a chromosomally integrated copy of the viruslike particle DNA. Although *M. voltae* PS was not observed to produce viruslike particles, DNA homologous to the viruslike particle DNA was detected in its chromosome. A mutant of *M. voltae* A3 was isolated which produced no particles; its DNA was deleted for 80% of the integrated viruslike particle DNA. Despite any similarities to lysogenic bacteriophages of eubacteria, neither infectivity nor inducibility of the viruslike particles could be demonstrated.

Identification and characterization of extrachromosomal elements in archaebacteria are important for two reasons. First, plasmids and bacteriophages have historically provided the foundation for the development of genetic transfer systems in eubacteria. One anticipates that this will also prove to be the case for archaebacteria, despite their status as a third kingdom of life (23, 24) and their molecular-biological differences from eubacteria (3, 4). Regardless of any inherent ability to mediate genetic transfer, plasmids or phage genomes might be developed into cloning vehicles for use in natural or induced competence transformation systems.

The second reason for studying extrachromosomal elements in archaebacteria is less utilitarian but of equal scientific importance. Knowledge of these elements will contribute to our understanding of the evolution of plasmids, viruses, and the genomes of the organisms which harbor them. Archaebacteria represent a third line of evolutionary descent entirely distinct from eubacteria and eucaryotes (23, 24). The very existence of plasmids and viruses in all three kingdoms raises the question of whether such elements evolved in parallel within each kingdom or were transferred laterally from one kingdom to one or both of the others. If the slow evolution in archaebacteria reflects their proximity to the hypothetical progenote (23), one wonders whether archaebacterial plasmids and viruses are evolutionarily closer both to the first extrachromosomal element and to each other than are the plasmids and viruses of the other kingdoms. This closer relationship might manifest itself either in a blurring of the phenotypic differences between the two types of elements or in the occurrence of transitional forms.

The recently described viruslike particle (VLP) SSV1 of the archaebacterium *Sulfolobus solfataricus* (14, 27) may represent such a transitional form. SSV1 is a lemon-shaped particle comprising a plasmidlike DNA molecule complexed with several low-molecular-weight proteins and some lipid. It resembles a lysogenic bacteriophage in that a copy of its genome is integrated into the host chromosome in a site-specific manner and that particle production is induced by UV light. However, there is no evidence that SSV1 is infectious. We now report the isolation and preliminary characterization of a VLP (A3) from a newly described strain of *Methanococcus voltae* (22).

* Corresponding author.

MATERIALS AND METHODS

Strains and media. The isolation and characterization of the type species *M. voltae* PS (J. M. Ward, M.S. thesis, University of Florida, Gainesville, 1970) and *M. vannielii* (6), as well as 22 new *Methanococcus* strains (22), have been described. The defined medium of Whitman et al. (21) was used for 5- and 20-ml liquid cultures pressurized to 40 lb/in^2 with H_2-CO_2 (80:20) and grown at 30 or 37°C. For growth of larger cultures in modified Wheaton bottles (1) pressurized to 20 lb/in^2, this medium was supplemented with anaerobic, filter-sterilized yeast extract to 0.01%. Because of the lower initial gas pressure in the larger cultures, it was necessary to repressurize them at least once during growth to prevent hydrogen limitation. Complex medium 3 of Balch et al. (1) was supplemented with 1.5% Noble agar for plating, which was performed by the method of Jones et al. (7).

Isolation of VLPs from *Methanococcus* strain A3. Late-log- or early-stationary-phase cultures of *Methanococcus* strain A3 were centrifuged at 8,000 × *g* for 15 min. The supernatants were removed and pooled. DNase I and RNase A were each added to 1 μg/ml, and the supernatant was gently stirred for 1 h at room temperature. The concentration of NaCl was then adjusted to 1 M, and 10% (wt/vol) polyethylene glycol 8000 (PEG 8000; Union Carbide Corp., New York, N.Y.) was added with continued gentle stirring. After the PEG 8000 had dissolved, the flask containing the supernatant was immersed in ice-water for 1 h. A gray precipitate was recovered by centrifugation at 12,000 × *g* for 30 min at 4°C. For CsCl gradients, the precipitate was suspended in 7 ml of 0.1 M NaCl–10 mM $MgSO_4$–25 mM Tris (SM buffer [pH 7.5]), 3.5 g of CsCl was added, and the solution was

centrifuged for 20 h at 35,000 rpm in a 70.1 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). For nycodenz (Accurate Chemical and Scientific Corp., Westbury, N.Y.) treatment, the precipitate was suspended in a solution containing 13.5 ml of 2× SM, 3.4 ml of water, and 10.1 ml of 70% (wt/vol) nycodenz in water, and centrifugation was carried out for 36 h at 35,000 rpm in a 70.1 Ti rotor. VLP bands were collected, dialyzed against SM containing 2mM NaN_3, and stored at 4°C.

Small-scale recovery of VLPs was occasionally performed anaerobically. Cultures (5 ml) of strain A3 were centrifuged at 2,000 × *g* in a table-top centrifuge while still anaerobic. They were then taken into an anaerobic chamber (Coy Products) and unstoppered. The supernatants were removed, passed through membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and stored in sterile anaerobic tubes under N_2-CO_2 (80:20). For electron microscopy, these particles were concentrated by PEG 8000 precipitation under aerobic conditions.

DNA isolation. Total DNA from methanococci was isolated as described previously (18), except that cells were initially suspended in a buffer containing 25 mM Tris, 0.5 M NaCl, and 10 mM EDTA (pH 8). The cells were then treated with proteinase K (rather than lysozyme) at 50°C for 30 to 60 min prior to lysis with sodium dodecyl sulfate (SDS). VLP DNA was isolated in one of two ways. Small amounts of covalently closed circular VLP DNA were recovered from unwashed A3 cells as described previously (26). Large amounts of open-circular VLP DNA were recovered by phenol extraction of purified VLPs followed by precipitation with ethanol.

Gel electrophoresis. DNA was fractionated on 0.8% or 1.0% vertical agarose gels at voltage gradients of 5 V/cm or less. The running buffer was 89 mM Tris–89 mM boric acid–2.5 mM EDTA (pH 8.2) (TBE). For some experiments involving electrophoresis of intact VLPs, 0.1 M NaCl was added to TBE (TBES). DNA was visualized by staining with ethidium bromide at 0.5 μg/ml and illumination with 300-nm UV light.

Proteins were fractionated on 12% or 13% acrylamide gels with a low ratio of acrylamide to bisacrylamide (20:1) after passage through a stacking gel whose composition was described previously (12). The running buffer was glycine-Tris (12). Electrophoresis was carried out at 60 V until the tracking dye penetrated the stacking gel, and then the voltage was raised to 100 V. Gels were stained with silver as described previously (16).

Radioisotope labeling and hybridizations. VLP DNA was labeled with [α-^{32}P]dATP by nick translation with a kit from Bethesda Research Laboratories, Inc., Gaithersburg, Md. DNA from agarose gels was transferred to nitrocellulose filters as described previously (19). Filters were prehybridized and then hybridized under conditions of high stringency (65°C) in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described previously (13). Colony hybridizations were performed as described previously (5), except that 1% SDS was included in the lysis solution. After hybridization, filters were washed three times at room temperature in 0.3× SSC–0.1% SDS and once in the same buffer at 65°C. The filters were then dried and exposed to X-ray film for appropriate times.

Electron microscopy. Formvar-coated 200-mesh grids were floated on a drop of VLP preparation for 30 s and blotted dry with filter paper. The grid was then floated on a drop of 1% uranyl acetate for 5 s and blotted dry. The grid was examined under an electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) at 60 kV under standard conditions with the LN_2 contaminator in place.

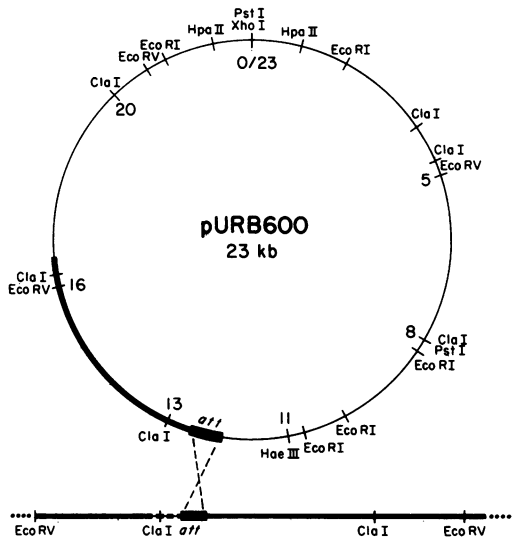

FIG. 1. Restriction map of plasmid and integrated form of A3 VLP DNA (pURB600). Symbols: ■, approximate locations of putative episomal and chromosomal attachment sites; ——, DNA not deleted from mutant strain 136; ▬▬, ambiguity in location of the leftward terminus of the deletion.

RESULTS

Isolation of VLPs from *Methanococcus* strain A3. The isolation and physiological characterization of 22 new *Methanococcus* strains have been described previously (22). Several of them are similar to *M. voltae* PS with regard to nutritional requirements such as acetate dependence. They are also similar to *M. voltae* PS at the DNA level. Thus, the cloned *M. voltae* PS *hisA* gene (25) hybridized to their DNA but not to DNA from the autotrophic isolates, and the pattern of restriction fragments generated from their DNA by the infrequently cutting enzymes *Bam*HI, *Sma*I, and *Sal*I was similar to that observed in *M. voltae* PS (A. G. Wood and J. Konisky, unpublished results). The VLP-producing strain A3 belongs to this *M. voltae* PS-like group.

Using a modified alkaline lysis plasmid isolation technique (26), we recovered variable quantities of a 23-kilobase (kb) covalently closed circular DNA (pURB600) in CsCl gradients from unwashed cells of strain A3. These covalently closed circular DNA preparations exhibited A_{260}/A_{280} ratios of 1.3, indicating protein contamination. Phenol extraction removed the protein (A_{260}/A_{280} = 1.7) and converted the DNA to the open circular form. A circular restriction map for this presumed plasmid was constructed (Fig. 1). Washing the cells led to greatly diminished recovery of pURB600, suggesting that it might reside extracellularly. This hypothesis was confirmed when agarose gel electrophoresis of phenol-extracted, PEG 8000-precipitated material from the culture supernatant revealed the presence of circular DNA with a restriction digest pattern identical to that of pURB600. Whereas the yield of pURB600 from unwashed cells was about 20 μg/liter, the yield from the supernatant was approximately 10-fold higher, corresponding to 10^{10} copies per ml.

When subjected to electrophoresis in agarose gels, uncut DNA isolated by either method migrated as two bands.

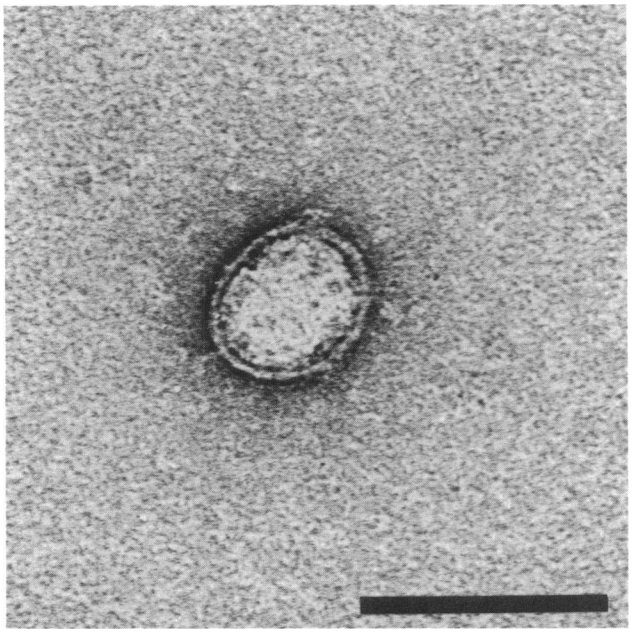

FIG. 2. Electron micrograph of a negatively stained A3-VLP isolated from a nycodenz gradient. Bar, 100 nm.

Experiments were conducted to determine the relationship between the two DNA forms (data not shown). The bands were excised from low-melting-point agarose and, following either UV irradiation or digestion with *Cla*I, run on a second gel. A UV dose sufficient to convert 50% of a control covalently closed circular plasmid to the open-circular form had no effect on the mobility of either of the DNA bands, and both molecules displayed the pURB600 *Cla*I digestion pattern. Furthermore, incubation with proteinase K at 50°C with or without SDS had no effect on mobility. These experiments suggested that pURB600 may exist in both monomeric and dimeric form. Interestingly, two of three previously characterized methogen plasmids (15, 20, 26) form dimers or multimers. However, we cannot rule out the possibility that the more slowly migrating form resulted from binding to pURB600 of a protein which is not removable by phenol, SDS, or proteinase K.

A procedure similar to that used for the recovery of bacteriophage lambda (see Materials and Methods) was used to prepare material for examination by electron microscopy. Although the use of CsCl centrifugation led a preparation consisting of a heterogeneous assortment of lemon-shaped VLPs (not shown), use of nycodenz or metrizamide gradients led to higher yields and a homogeneous preparation of VLPs (Fig. 2). Examination of several views of the particle suggests that the VLP is oblate, not spherical, and has dimensions of approximately 52 by 70 nm. The particle consists of a core and an envelope, and some images suggest that the envelope may be a capsid of subunit structure.

VLP-associated proteins. Proteins derived from the VLPs were identified by subjecting a CsCl gradient-purified VLP preparation to polyacrylamide gel electrophoresis (Fig. 3). Silver staining revealed one major species with an apparent molecular mass of 13,000 daltons in addition to three lower-molecular-mass species. Two faint bands corresponding to proteins in the 30,000- to 35,000-dalton range might represent flagellar proteins (8). In contrast, analysis of material from the 1.28-g/cm^3 CsCl band showed a major species of approximately 70,000 daltons (data not shown), presumably

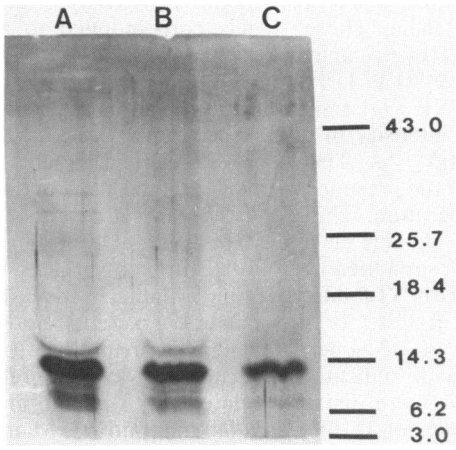

FIG. 3. Silver-stained SDS-polyacrylamide gel showing proteins from 10 μl (lane A), 5 μl (lane B), and 2 μl (lane C) of a VLP preparation (A_{280} = 16.4). Sizes of standards are indicated in kilodaltons.

representing the major S-layer protein of methanococcal isolates (9, 11).

Nuclease resistance of VLP-associated circular DNA. Incubation of a 40-μl VLP preparation at 37°C for 30 min in the presence of 5 U of DNase I affected neither the amount nor the form of the DNA liberated from the particles by phenol extraction (data not shown). However, phenol extraction prior to DNase I treatment resulted in complete digestion of the VLP DNA.

Chromosomal integration of the VLP DNA. Figure 4 presents DNA-DNA hybridization evidence for site-specific integration of the VLP DNA into the chromosome of strain A3. VLP DNA, DNA extracted from washed A3 cells, or DNA extracted from mutant strain 136 (whose isolation is described below) was digested with *Cla*I, subjected to electrophoresis on a 1% agarose gel, Southern blotted, and probed with nick-translated VLP DNA. Sizes of the five largest VLP-derived *Cla*I fragments (Fig. 4, lane C) are indicated. *Cla*I-digested A3 DNA (lane A) exhibited two

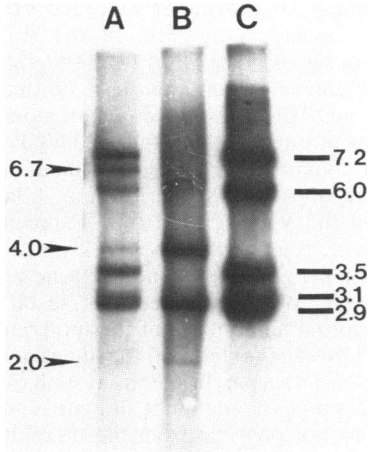

FIG. 4. Autoradiogram of *Cla*I-digested, Southern-blotted DNA from *M. voltae* A3 (4 μg) (lane A), *M. voltae* A3 mutant strain 136 (10 μg) (lane B), and pURB600 (40 ng) probed with labeled pURB600 (lane C). Sizes of pURB600 *Cla*I fragments are indicated. Arrows point to fragments containing VLP-chromosomal junctions.

hybridization signals (indicated by arrows) not observed in the VLP lane. These signals corresponded to restriction fragments of 6.7 and 4.0 kb carrying the two junctions of integrated VLP DNA and A3 chromosomal DNA. The 6.0-kb VLP *Cla*I fragment hybridization signal was diminished in intensity in lane A because it contained the VLP attachment site. That it was observed at all in lane A indicated the presence of some circular DNA in the washed-cell preparation. This could have resulted either from the presence of some plasmid form DNA (pURB6000) inside the cells or from adherence of VLPs to cell surfaces.

Absence of VLP infectivity and isolation of strain 136. VLPs purified in CsCl or metrizamide gradients or by simple filtration of A3 supernatants failed to produce plaques in assays with indicator methanococci closely related (*M. voltae*) or more distantly related (*M. vannielii* and several new isolates of *M. maripaludis*) to strain A3. Many of these strains exhibited apparent autoplaquing which was independent of VLP addition. To test the possibility that the VLPs were nonlytic bacteriophages, we devised an assay to detect VLP DNA replication in liquid cultures of potential indicator strains. Purified VLPs or VLP DNA was added to minimal liquid media containing 100 μCi of $^{32}P_i$. The media were then inoculated with a small number of cells (10^7) of the potential indicator strains, and the cultures were grown to saturation. Following removal of the cells by centrifugation, the supernates were precipitated with PEG 8000, and the precipitated material was analyzed by agarose gel electrophoresis. This assay was 10- to 100-fold more sensitive than ethidium bromide staining in the detection of VLP DNA production in strain A3 (data not shown). Although no VLP production in the indicator cultures was demonstrated, we calculated that approximately 10^7 VLPs per ml would have to have been generated for detection by this method. Thus, we cannot rule out the possibility that low-level production occurred.

We reasoned that if the VLPs were lysogenic bacteriophages, the most likely candidate for reinfection would be a strain isogenic with A3 but lacking the integrated VLP DNA. We attempted to identify such a strain by hybridizing nick-translated pURB600 to A3 colonies lysed on nitrocellulose filters. Isolated colonies from a plating of a diluted A3 culture were transferred by toothpick to fresh gridded plates before being transferred to the nitrocellulose filters. We observed that the picked colonies were of two distinct size classes. Whereas all the larger colonies exhibited strong hybridization signals, about half of the smaller colonies exhibited weak signals. The DNA from several of the weakly hybridizing colonies, when Southern blotted and probed with labeled pURB600, showed a common *Cla*I pattern indicating loss of most of the integrated VLP DNA. One of these mutant colonies was picked, purified, and designated strain 136. The DNA from strain 136 (Fig. 4, lane B) showed the loss of all the VLP-derived *Cla*I fragments except the 2.9-kb fragment, retention of the 4.0-kb VLP-chromosome junction fragment, and appearance of a new junction fragment of 2.0 kb. We interpreted this result to represent deletion of approximately 80% of the integrated VLP DNA from strain 136. The extent of the deletion (Fig. 1) was determined by Southern hybridizations with *Eco*RI, *Eco*RV, *Pst*I, *Hae*III, and *Hpa*II (data not shown) in addition to *Cla*I (Fig. 4). It was not possible from these results to establish whether the deletion terminated precisely at the VLP-chromosome junction or extended into the chromosome on the left-hand side.

This isolate was grown in liquid culture for many generations and never produced detectable VLPs. Attempts to infect strain 136 with purified VLPs by using either the plaque assay or the replication assay were unsuccessful. Since the possibility existed that the 20% of the integrated VLP DNA retained by strain 136 encoded a function conferring immunity to reinfection, we tried to isolate a derivative of 136 completely cured of VLP DNA. In this case, the 2.9-kb *Cla*I fragment retained by strain 136 was cloned into pBR322 and used as the hybridization probe. Screening of several hundred 136 colonies, however, produced no completely cured strains.

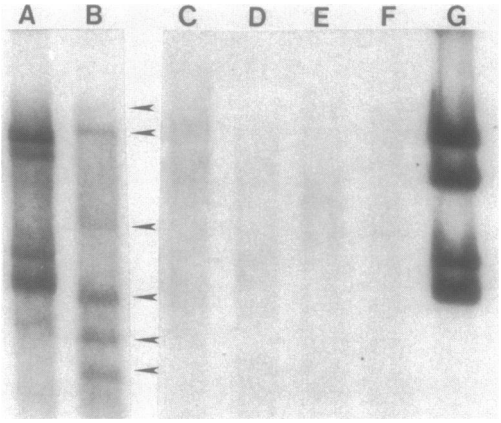

FIG. 5. Autoradiogram of *Cla*I-digested, Southern-blotted DNA from *M. voltae* A3 (4 μg) (lane A), *M. voltae* PS (4 μg) (lane B), *M. vannielii* (4 μg) (lane C), *M. maripaludis* (4 μg) (lane D), *M. deltae* (4 μg) (lane E), *M. thermolithotrophicus* (4 μg) (lane F), and pURB600 (40 ng) (lane G) probed with labeled pURB600. Arrows point to hybridizing *M. voltae* PS fragments.

***M. voltae* homology with VLP DNA.** In view of the close relatedness of strain A3 to *M. voltae*, we wondered whether the chromosome of *M. voltae* contained any sequences homologous to the A3 VLP DNA. To answer this question, DNA from several *Methanococcus* species was digested with *Cla*I, subjected to electrophoresis through a 1% agarose gel, Southern blotted, and probed with nick-translated A3 VLP DNA (Fig. 5). The VLP DNA did indeed hybridize to *M. voltae* DNA as well as A3 DNA, although the sizes of the restriction fragments probed differed between the two strains. The VLP DNA did not hybridize to DNA from *M. vannielii*, *M. maripaludis*, *M. deltae*, or *M. thermolithotrophicus*. We saw no evidence for VLP production by *M. voltae*, although we must emphasize that low-level production (less than 10^7 particles per ml) would have gone undetected. One of three explanations for these observations appears likely. First, *M. voltae* may have originally produced VLPs but lost the ability through repeated laboratory transfers. Second, *M. voltae* may produce VLPs at levels too low for us to detect; in this case, some induction event might lead to higher levels of production (see below). Finally, it may simply be that a gene or genes which are VLP encoded in A3 are chromosomally encoded in *M. voltae*.

Lack of evidence for induction of A3 VLPs. UV irradiation of *Sulfolobus solfataricus* B12 increases the production of SSV1 from barely detectable levels to more than 10^{10} particles per ml (14). With this in mind, we attempted to induce VLP production in *M. voltae* or influence levels of production in A3 by subjecting cultures to various stresses. Neither UV irradiation, temperature shift from 30°C to 37°C, hydrogen (energy) starvation, growth in mitomycin C (which activates SOS repair in *Escherichia coli*), nor growth in

acridine orange (which intercalates into DNA and can cure episomes) affected VLP production in either strain.

DISCUSSION

Plasmids and bacteriophages make up the two categories of extrachromosomal elements in procaryotes. The VLPs of *Methanococcus* strain A3, however, do not at present fall neatly into either category. Although some VLP DNA can be recovered from washed cells by a plasmid isolation procedure, most (at least 90%) of the VLP DNA clearly exists extracellularly. Methanococci autolyse under conditions of hydrogen limitation (2; our unpublished observations), and it is probable that some cells lyse even during exponential growth. Therefore, one could assert that the extracellular VLP DNA is simply plasmid complexed with protein from lysed cells. Two considerations argue against this interpretation. First, we have recently described a small cryptic plasmid in another *Methanococcus* isolate (26). Although this strain autolyses as readily as does A3, we have never recovered the plasmid quantitatively from the culture supernatant. Second, if the plasmid were becoming entangled with cell debris, one would expect little specificity in the DNA-associated proteins. On the contrary, we have shown that the association manifests substantial specificity.

We have been unable to demonstrate that the A3 VLPs are infective. Possible reasons for this failure include biological inactivation of the particles during purification and lack of an appropriate uninfected host strain. Infections were attempted by using particles prepared in three different ways to minimize the first possibility. The higher degree of morphological homogeneity of the particles recovered from nycodenz and metrizamide gradients compared with those from CsCl gradients suggested a sensitivity to high salt concentrations.

The possibility that we do not have a suitable strain with which to demonstrate infectivity seems more likely. Ideally, such a strain would be isogenic with A3 except for the absence of the integrated VLP DNA. The 20% of the VLP DNA retained by the variant strain 136 may well encode some immunity function which prevented reinfection. The cross-hybridizing DNA of *M. voltae* may encode a similar function. Less closely related strains may lack appropriate receptors for viral attachment or restrict incoming viral DNA. Eliminating the retained VLP DNA from strain 136 may prove difficult, and there is currently no method for site-specific mutagenesis in methanogens. Therefore, it may be necessary to attempt to isolate an isogenic uninfected strain from nature.

In the event that the observed lack of infectivity cannot ultimately be ascribed to technical problems, we may be forced to conclude that the A3 VLPs are simply not infectious. They might be defective bacteriophages requiring an unidentified helper phage for infectivity. A more exciting possibility is that they are a previously uncharacterized type of extrachromosomal element unique to archaebacteria, perhaps representing an evolutionarily transitional form between plasmids and bacteriophages. In this regard, their resemblance to the VLPs of *S. solfataricus* B12 may be significant. Both VLPs comprise circular DNA complexed with low-molecular-weight proteins. Both elements can be isolated in either plasmid or VLP form, and the chromosomes of both *Methanococcus* strain A3 and *S. solfataricus* harbor copies of the episomal DNA integrated at a specific site. The induction of SSV1 but not A3 VLP by UV light may reflect the different habitats of the host bacteria: *Sulfolobus* species occur in solfataric waters, whereas *Methanococcus* species are found in subsurface anaerobic sediments and rarely encounter sunlight. Although very little is known about mechanisms of DNA repair in methanogens, a methanococcus related to *M. voltae* was found incapable of repairing UV-induced damage to its DNA by photoreactivation (10).

A recent description of VLPs in *Desulfurolobus ambivalens* (28) and our unpublished observations of possible VLPs in at least one other *Methanococcus* isolate suggest that these elements may be characteristic of archaebacteria. In future studies, we intend to sequence portions of the A3 VLP genome relating to chromosomal integration and production of the putative coat protein, as well as to characterize VLP-encoded transcripts. This will permit comparisons with SSV1, which has been completely sequenced and analyzed for transcripts (17), at a more fundamental molecular level.

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