JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1420–1424
0021-9193/82/091420-05$02.00/0
Copyright © 1982, American Society for Microbiology
Vol. 151, No. 3

Cytosine Methylation of the Sequence GATC in a Mycoplasma

KEVIN DYBVIG,[1] DAVID SWINTON,[2] JACK MANILOFF,[3]* AND STANLEY HATTMAN[2]

Departments of Radiation Biology and Biophysics,[1] Biology,[2] and Microbiology,[3] University of Rochester Medical Center, Rochester, New York 14642

Received 15 January 1982/Accepted 20 May 1982

Mycoplasma virus L2 is subject to host-specific restriction and modification in *Acholeplasma laidlawii* strains JA1 and K2. We have examined the DNAs from both host cells and viruses propagated on these strains with respect to susceptibility to cleavage by restriction endonucleases and for DNA base modifications. We show that, in strain K2 and L2 virus grown on K2 cells, cytosine in the sequence GATC is methylated to 5-methylcytosine and, although strain K2 and L2 viruses grown on K2 contain N^6-methyladenine in their DNA, adenine in the sequence GATC is not methylated. In contrast to K2, strain JA1 and L2 virus grown on JA1 cells contain no detectable methylated bases. It is not known which of the methylated bases in K2 is the basis for the K2 restriction-modification system operative on L2 virus.

Mycoplasmas are procaryotes that lack cell walls and have the smallest reported genome sizes (15). They appear to have arisen by degenerative evolution as a branch of the gram-positive eubacteria (20).

Some strains of the mycoplasma *Acholeplasma laidlawii* (genome size 1.0×10^9 daltons) are hosts for three morphologically distinct mycoplasma viruses: L1 are bullet-shaped virions containing circular single-stranded DNA of 1.5×10^6 daltons (12); L2 are enveloped virions containing circular superhelical double-stranded DNA of 7.8×10^6 daltons or 11.8 kilobase pairs (kbp) (11); and L3 are T7-like virions containing linear double-stranded DNA of about 26×10^6 daltons (4). From plating efficiency data, Maniloff and Das (9) concluded that *A. laidlawii* strains JA1 and K2 can modify and restrict L2 but not L1. Similar studies have shown that L3, like L1, is not modified or restricted (K. Dybvig, data not shown).

We report here studies on DNA modifications in *A. laidlawii* strains K2 and JA1. These show that strain K2 methylates the C nucleotide in the sequence GATC. No methylation was detectable in strain JA1 DNA.

MATERIALS AND METHODS

Cells and virus. *A. laidlawii* strain JA1 has been described previously (8). *A. laidlawii* strain K2 is a clone derived from *A. laidlawii* M1305/68 (4). L2 is the original group 2 mycoplasma virus isolate (3) and was obtained from R. N. Gourlay. Viruses were assayed as PFU in soft agar overlays of *A. laidlawii* on tryptose agar plates as described previously (11).

Media and buffers. Tryptose broth containing 1% glucose and 1% PPLO serum fraction was used for cultivating cells as described previously (7). Tris-EDTA-NaCl (TES) buffer (0.01 M Tris-hydrochloride [pH 7.8]–0.001 M EDTA–0.1 M NaCl) was used for purification of L2 virus and both cellular and viral DNA.

Purification of L2 virus. Overnight (late exponential phase) cultures (200 ml) of *A. laidlawii* were infected with L2 (multiplicity of infection 1 to 10) and diluted to 1 liter with fresh tryptose broth. After overnight incubation at 37°C, cells were removed by centrifugation (7 min at 9,000 rpm at 4°C in a Beckman JA-14 rotor), and the virus was pelleted (30 min at 25,000 rpm at 10°C in a Beckman SW27 rotor). The virus was suspended in 5 ml of TES buffer and purified by sedimentation (3 h at 25,000 rpm at 10°C in a Beckman SW27 rotor) in a linear 15 to 30% sucrose gradient. Fractions containing L2 were pooled and concentrated by centrifugation. After suspension in 0.5 ml of TES buffer, the virus titer was 10^{13} to 10^{14} PFU/ml.

DNA purification. Viral DNA was purified by extracting the resuspended virus twice with equal volumes of phenol saturated with TES buffer. The aqueous layer was made 0.2 M in sodium acetate, and DNA was precipitated by addition of 2 volumes of 95% ethanol. After overnight incubation at −20°C, DNA was harvested by centrifugation (1 h at 30,000 rpm at 0°C in a Beckman SW50.1 rotor) and suspended in 0.5 ml of TES buffer. DNA concentrations were typically 1 mg/ml.

Cellular DNA was prepared for agarose gel electrophoresis as described for viral DNA, except that cell DNA was precipitated with 70% ethanol and centrifuged for 15 min at 20,000 rpm. For chromatographic analysis, cellular DNA was purified by equilibrium centrifugation in a CsCl density gradient (K. Dybvig, Ph.D. thesis, University of Rochester, Rochester, NY., 1981). Fractions containing DNA were pooled,

dialyzed against TES buffer, and precipitated in ethanol.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed in a slab gel apparatus (Aquebogue Machine Shop, Aquebogue, N.Y.). The electrophoresis buffer contained 40 mM Tris (pH 7.8), 5 mM sodium acetate, and 2 mM EDTA. All gels were 1% agarose (type III; Sigma Chemical Co., St. Louis, Mo.). Samples for electrophoresis were mixed with 5 μl of marker dye solution (60% sucrose in TES buffer and 0.25% bromophenol blue) and subjected to electrophoresis for 3.5 to 4 h at 40 to 45 mA (70 to 80 V). The gels were stained with ethidium bromide (1 μg/ml), visualized by illumination with short-wavelength UV light, and photographed.

Nuclease digestion. Restriction endonucleases were obtained from Bethesda Research Laboratories (Rockville, Md.), except for *Mbo*I, which was obtained from New England Biolabs (Beverly, Mass.). All restriction endonuclease digests were performed under conditions recommended by the supplier.

High performance liquid chromatography. Up to 20 μg of DNA was precipitated with 95% ethanol and centrifuged, and the resulting pellets were washed once in cold 70% ethanol and air dried. The DNA pellets were suspended in 15 μl of 0.2 M NH_4CO_3 (pH 8.5) and 2 μl of 0.1 M $MgCl_2$. A mixture containing 50 U of DNase I, 0.5 U of snake venom phosphodiesterase, and 0.02 U of bacterial alkaline phosphatase in 5 μl was added, and the solution was incubated for at least 4 h at 50°C. The resulting nucleosides were separated as described by Singhal (17), except that a column (100 by 0.46 cm) of Aminex A-7 resin (pH 9.9 to 10.0) (Bio-Rad Laboratories, Richmond, Calif.) was used with a flow rate of 10 ml/h. The sample was introduced into the column with a high-pressure injection valve, utilizing a 20-μl sample loop.

RESULTS

Plating efficiency data. Mycoplasma virus L2 propagated on strain JA1 is designated L2 · JA1, and L2 propagated on strain K2 is designated L2 · K2. L2 · JA1 is restricted and modified by strain K2, and L2 · K2 is restricted and modified by strain JA1. The plating efficiency of L2 · JA1 on strain K2 was 0.05 relative to its plating efficiency on strain JA1, and the plating efficiency of L2 · K2 on strain JA1 was 0.002 relative to plating on strain K2. These values are similar to those reported by Maniloff and Das (9).

Restriction endonuclease studies. A restriction endonuclease cleavage map of the L2 · K2 genome was constructed (Fig. 1). The *Hin*dIII and *Hpa*I sites confirm previously published results involving L2 · JA1 DNA (11). Only one *Xba*I site was identified in the previous study, but two close *Xba*I sites were resolved in the experiments reported here. Although L2 · JA1 DNA was reported to have a single *Bgl*II site (11), L2 · K2 DNA was not cleaved by *Bgl*II. To determine whether the lack of *Bgl*II cleavage of L2 · K2 DNA was due to a host modification in the *Bgl*II recognition sequence, two types of experiments were done. First, to be certain that L2 · K2 DNA preparations did not contain an inhibitor of *Bgl*II, the enzyme was added to a mixture of L2 · K2 DNA and coliphage λ DNA. After digestion and agarose gel electrophoresis, the standard *Bgl*II fragment pattern of λ DNA was observed (data not shown). Hence, the L2 · K2 preparation does not contain an inhibitor of *Bgl*II activity. Second, to confirm the presence of a *Bgl*II recognition sequence in L2 · K2 DNA, JA1 cells were infected with L2 · K2 virus to regenerate a stock of L2 · JA1 virus. DNA from this stock was cleaved by *Bgl*II at the expected site (data not shown). We concluded that mycoplasma virus L2 contains a *Bgl*II site which must be modified when the virus is grown in strain K2.

Since the *Bgl*II recognition sequence is AGATCT (16), several specific restriction endonucleases were used to investigate the base sequence recognized by the K2 cell modification system. A typical experiment is shown in Fig. 2, and data from many experiments are summarized in Table 1. The sensitivity of virus L2 DNA to *Bgl*II was the same as that of its host cell; both strain K2 and mycoplasma virus L2 · K2 DNAs were not cleaved by *Bgl*II. Since *Bam*HI did not cleave L2 · JA1 or L2 · K2

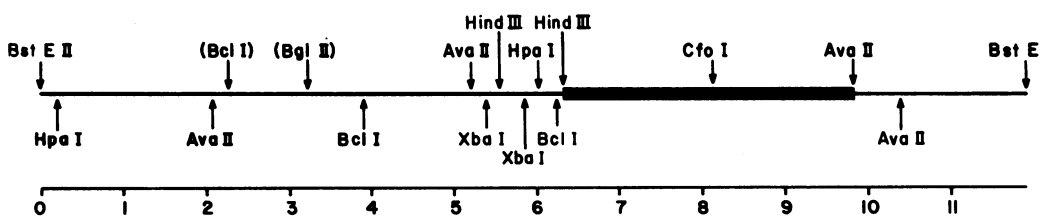

FIG. 1. Restriction endonuclease cleavage map of L2 DNA. Cleavage sites were mapped as described by Nowak and Maniloff (11). The circular L2 genome was broken at the single *Bst*EII site to allow a linear representation. The scale is in kilobase pairs (kbp). L2 DNA is 11.8 kbp. The heavy line indicates the region containing the L2 integration site (K. Dybvig and J. Maniloff, manuscript in preparation). The parentheses around the *Bgl*II site and one of the *Bcl*I sites denote that these sites are cleaved in L2 · JA1 DNA but not in L2 · K2 DNA.

TABLE 1. Susceptibility of DNAs to restriction endonuclease cleavage[a]

Restriction enzyme	Sequence recognized[b]	DNA source			
		Strain K2	L2 · K2	Strain JA1	L2 · JA1
*Bgl*II	AGATCT	−	−	+	+
*Bam*HI	GGAT$\overset{*}{C}$C	−	−	+	−
*Sau*3A	GAT$\overset{*}{C}$	−	−	+	+
*Mbo*I	G$\overset{*}{A}$TC	+	+	+	+

[a] Cleavage was determined by agarose gel electrophoresis of enzyme-treated DNA. +, DNA cleaved at one or more sites; −, DNA not cleaved.

[b] The asterisk (*) above a nucleotide denotes the base that is modified to render the site not susceptible to the nuclease (e.g., *Bam*HI cleaves GGATCC but not GGATm^{5}CC). Literature citations in text.

DNA, we concluded that the L2 genome lacks the *Bam*HI sequence GGATCC (16). Since *Bam*HI cleaves cellular DNA from strain JA1 but not from strain K2, the *Bam*HI sequence is modified in strain K2. *Sau*3A recognizes the sequence GATC (16). *Sau*3A cleaves DNA from strain JA1 but not from strain K2. L2 · JA1 DNA has more than 10 *Sau*3A cleavage sites, but L2 · K2 DNA was not cleaved by *Sau*3A (Fig. 2; Table 1). The *Bam*HI, *Bgl*II, and *Sau*3A data are consistent with the recognition sequence of the K2 modification system being GATC.

Most host-specific modifications of DNA resulting in changes in restriction endonuclease susceptibility are either methylation of adenine (Ade) to N^6-methyladenine (m^{6}Ade) or methylation of cytosine (Cyt) to 5-methylcytosine (m^{5}Cyt). K2 does not modify the GATC sequence to Gm^{6}ATC (Table 1) for the following reasons. (i) *Bgl*II cleaves AGm^{6}ATCT (13), (ii) *Bam*HI cleaves GGm^{6}ATCC (5), (iii) *Sau*3A cleaves Gm^{6}ATC (19), and (iv) *Mbo*I, which cleaves DNA from L2 · K2 and strain K2, does not cleave Gm^{6}ATC (2). However, the data in Table 1 are consistent with the modification of GATC to GATm^{5}C in K2 cells for the following reasons: (i) *Bam*HI does not cleave GGATm^{5}CC (5), and (ii) *Sau*3A does not cleave GATm^{5}C (18).

Base composition studies. If the K2 modification is GATm^{5}C, then we expect that strain K2 and L2 · K2 DNAs should contain m^{5}Cyt. To test this, high performance liquid chromatography was used to analyze the base compositions of L2 · JA1, L2 · K2, strain JA1, and strain K2 DNAs (Table 2). About 1.6% of Cyt bases in L2 · K2 DNA were methylated, and about 2.2% of Cyt bases in strain K2 DNA were methylated. Thus, the presence of m^{5}Cyt in mycoplasma virus L2 · K2 and host K2 DNAs was established.

We also learned that (i) about 0.3% of Ade bases in strain K2 DNA and L2 · K2 were methylated; hence, there may be another K2 modification system based on m^{6}Ade; (ii) L2 · JA1 DNA and strain JA1 DNA had no detectable methylated bases; and (iii) L2 DNA was 32% G+C, and strain JA1 and K2 DNAs were 31 to 32% G+C (Table 2). These latter G+C compositions are within the 31 to 36% range reported for *A. laidlawii* strains (10). The 32% G+C value for L2 DNA was confirmed by isopycnic CsCl density gradient analysis of L2 · JA1 DNA (data not shown).

DISCUSSION

We studied the DNA from two *A. laidlawii* strains (JA1 and K2) having different restriction and modification specificities and the DNA from a mycoplasma virus grown on each strain. Data on the susceptibility of these DNAs to cleavage by several restriction endonucleases (Table 1) led to the following conclusions. (i) In *A. laidlawii* strain K2, the DNA sequence GATC is modified, making it resistant to cleavage by *Bgl*II, *Bam*HI, and *Sau*3A; (ii) in K2 cells, adenine in the sequence GATC is not methylat-

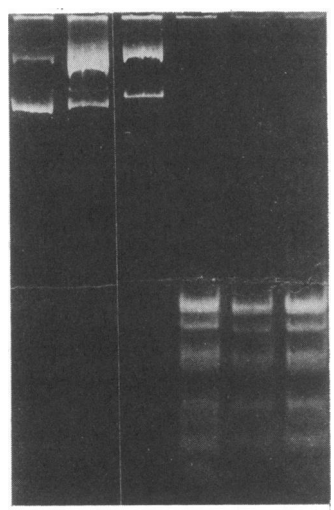

FIG. 2. Agarose gel electrophoresis of L2 DNAs after cleavage with restriction endonucleases *Sau*3A and *Mbo*I. Each lane contained the following DNA: (a) untreated L2 · K2; (b) untreated L2 · JA1; (c) L2 · K2 treated with *Sau*3A; (d) L2 · JA1 treated with *Sau*3A; (e) L2 · K2 treated with *Mbo*I; and (f) L2 · JA1 treated with *Mbo*I. In lanes a to c, the upper bright band is relaxed open circular L2 DNA, and the lower bright band is superhelical covalently closed circular L2 DNA. In lane b, the faint band between these circular DNAs is linear L2 DNA.

TABLE 2. Base composition of strain K2, strain JA1, L2 · K2, and L2 · JA1 DNA

DNA source	Mol%					
	Thy	Gua	Cyt	m^5Cyt	Ade	m^6Ade
Strain K2	36.2	15.0	15.9	0.36	33.2	0.10
L2 · K2	34.2	16.0	16.1	0.26	33.4	0.10
Strain JA1	34.0	15.5	16.7	<0.003	33.6	<0.003
L2 · JA1	34.6	15.8	16.6	<0.004	33.0	<0.004

ed; and (iii) in K2 cells, cytosine in the sequence GATC is methylated to m^5Cyt. The presence of m^5Cyt in strain K2 and virus L2 · K2 DNAs was confirmed by high performance liquid chromatography.

These data do not allow conclusions on whether the GATC to $GATm^5C$ modification in K2 is the biochemical basis of modification of mycoplasma viruses by these cells. The presence of m^6Ade in K2 cells indicates that a second modification system is possible. The lack of detectable methylated bases in JA1 means that modification of mycoplasma viruses in JA1 cells is significantly different than in K2 cells. In fact, it is a mystery how restriction-modification works in JA1 in the absence of detectable m^5Cyt and m^6Ade. From the base composition data, each L2 · JA1 DNA molecule must contain <0.15 m^5Cyt residue and <0.31 m^6Ade residue. The possibility that JA1 modifies the viral coat rather than viral DNA can be eliminated, since recent data (T. Sladek and J. Maniloff, unpublished data) show similar restriction frequencies for infection with L2 · K2 virions and transfection with DNA from L2 · K2 virions.

In a study of five mycoplasmas (four *Mycoplasma* species and an *A. laidlawii* oral strain), Razin and Razin (14) found m^6Ade (0.08 to 0.8 mol%) in all five, but m^5Cyt was detected (0.8 mol%) in only one *Mycoplasma* species. These results, together with those presented here, indicate significant diversity in the DNA methylation patterns among the mycoplasmas.

Although the modification which makes the *Bgl*II recognition sequence (AGATCT) resistant to cleavage has not been reported, these studies indicate two possibilities. The identical pattern of low-molecular-weight DNA fragments was seen for L2 · JA1 digested with either *Sau*3A or *Mbo*I and L2 · K2 DNA digested with *Mbo*I. Hence, the distribution of GATC sequences must be similar in L2 · JA1 and L2 · K2 DNAs, and there must be little or no m^6Ade in GATC sequences in K2 cells. (The absence of Gm^6ATC sequences is consistent with the observation that *Dpn*I [recognition sequence Gm^6ATC; 6] does not cleave either L2 · K2 or L2 · JA1 DNA [data not shown].) Since *Sau*3A cannot cleave L2 · K2 DNA, every GATC must be methylated to $GATm^5C$. Therefore, we conclude that in L2 · K2 DNA, the resistant *Bgl*II recognition sequence must contain m^5Cyt, i.e., $AGATm^5CT$. However, since L2 · K2 DNA also contains m^6Ade, we cannot rule out the possibility that the resistant *Bgl*II site is m^6AGATm^5C. Either or both methylations might be necessary to make the site resistant to *Bgl*II.

In addition to the modification of the *Bgl*II site, growth of L2 virus in K2 cells also causes one of the three *Bcl*I sites in the L2 DNA to become resistant to cleavage by *Bcl*I (data not shown). The *Bcl*I site (recognition sequence TGATCA; 1) at 2.25 kbp on the L2 map (Fig. 1) is cleaved in L2 · JA1 DNA but not in L2 · K2 DNA. Hence, not all *Bcl*I sites are identically modified in K2 cells. As discussed above, since all GATC sites are modified, in L2 · K2 DNA the *Bcl*I recognition sequence must be present as $TGATm^5CA$. Therefore, *Bcl*I can cleave $TGATm^5CA$, and the *Bcl*I site at 2.25 kbp on the L2 map must have a modification in addition to m^5Cyt. Hence, a second modification system in K2 cells must modify a sequence which overlaps the *Bcl*I sequence at 2.25 kbp on the map.

ACKNOWLEDGMENTS

These studies were supported, in part, by Public Health Service research grants AI10605 (to J.M.) from the National Institute of Allergy and Infectious Diseases and GM29227 and GM26892 (to S.H.) from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Bingham, A. H. A., T. Atkinson, D. Sciaky, and R. J. Roberts.** 1978. A specific endonuclease from *Bacillus caldolyticus*. Nucleic Acids Res. 5:3457–3467.
2. **Dreiseikelmann, B., R. Eichenlaub, and W. Wackernagel.** 1979. The effect of differential methylation by *Escherichia coli* of plasmid DNA and phage T7 and λ DNA on the cleavage by restriction endonuclease *Mbo*I from *Moraxella bovis*. Biochim. Biophys. Acta 562:418–428.
3. **Gourlay, R. N.** 1971. Mycoplasmatales virus-laidlawii 2, a new virus isolated from *Acholeplasma laidlawii*. J. Gen. Virol. 12:65–67.
4. **Haberer, K., G. Klotz, J. Maniloff, and A. K. Kleinschmidt.** 1979. Structural and biological properties of mycoplasmavirus MVL3: an unusual virus-procaryote interaction. J. Virol. 32:268–275.
5. **Hattman, S., T. Keister, and A. Gottehrer.** 1978. Sequence specificity of DNA methylases from *Bacillus amyloliquefaciens* and *Bacillus brevis*. J. Mol. Biol. 124:701–711.
6. **Lacks, S., and B. Greenberg.** 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneu-*

moniae with respect to DNA methylation. J. Mol. Biol. **114**:153–168.
7. **Liss, A., and J. Maniloff.** 1971. Isolation of Mycoplasmatales viruses and characterization of MVL1, MVL52, and MVG51. Science **173**:725–727.
8. **Liss, A., and J. Maniloff.** 1973. Infection of *Acholeplasma laidlawii* by MVL51 virus. Virology **55**:118–126.
9. **Maniloff, J., and J. Das.** 1975. Replication of mycoplasmaviruses, p. 445–450. *In* M. Goulian, P. Hanawalt, and C. F. Fox (ed.), DNA synthesis and its regulation. W. A. Benjamin, Reading, Mass.
10. **Neimark, H. C.** 1970. Division of mycoplasmas into subgroups. J. Gen. Microbiol. **63**:249–263.
11. **Nowak, J. A., and J. Maniloff.** 1979. Physical characterization of the superhelical DNA genome of an enveloped mycoplasmavirus. J. Virol. **29**:374–380.
12. **Nowak, J. A., J. Maniloff, and J. Das.** 1978. Electron microscopy of single-stranded mycoplasmavirus DNA. FEMS Microbiol. Lett. **4**:59–61.
13. **Pirrotta, V.** 1976. Two restriction endonucleases from *Bacillus globiggi*. Nucleic Acids Res. **3**:1747–1760.
14. **Razin, A., and S. Razin.** 1980. Methylated bases in mycoplasmal DNA. Nucleic Acids Res **8**:1383–1390.
15. **Razin, S.** 1978. The mycoplasmas. Microbiol. Rev. **42**:414–470.
16. **Roberts, R. J.** 1981. Restriction and modification enzymes and their recognition sequences. Nucleic Acids Res. **9**:r75–r96.
17. **Singhal, R. P.** 1972. Ion-exclusion chromatography: analysis and isolation of nucleic acid components, and influence of separation parameters. Arch. Biochem. Biophys. **152**:800–810.
18. **Streeck, R. E.** 1980. Single-strand and double-strand cleavage at half-modified and fully modified recognition sites for the restriction nucleases *Sau*3A and *Taq*I. Gene (Amst) **12**:267–275.
19. **Sussenbach, J. S., C. H. Monfoort, R. Schiphof, and E. E. Stobberingh.** 1976. A restriction endonuclease from *Staphylococcus aureus*. Nucleic Acids Res. **3**:3193–3202.
20. **Woese, C. R., J. Maniloff, and L. B. Zablen.** 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. U.S.A. **77**:494–498.