Mycoplasma Restriction: Identification of a New Type of Restriction Specificity for DNA Containing 5-Methylcytosine

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Mycoplasma bacteriophage L51 single-stranded DNA and L2 double-stranded DNA are host cell modified and restricted when they transfect *Acholeplasma laidlawii* JA1 and K2 cells. The L51 genome has a single restriction endonuclease *MboI* site (recognition sequence GATC), which contains 5-methylcytosine when the DNA is isolated from L51 phage grown in K2 cells but is unmethylated when the DNA is from phage grown in JA1 cells. This GATC sequence is nonessential, since an L51 mutant in which the *MboI* site was deleted was still viable. DNA from this deletion mutant phage was not restricted during transfection of either strain K2 or JA1. Therefore, strain K2 restricts DNA containing the sequence GATC, and strain JA1 restricts DNA containing the sequence GAT 5-methylcytosine. We conclude that K2 cells have a restriction system specific for DNA containing the sequence GATC and protect their DNA by methylating cytosine in this sequence. In contrast, JA1 cells (which contain no methylated DNA bases) have a newly discovered type of restriction-modification system. From results of studies of the restriction of specifically methylated DNAs, we conclude that JA1 cells restrict DNA containing 5-methylcytosine, regardless of the nucleotide sequence containing 5-methylcytosine. This is the first report of a DNA restriction activity specific for a single (methylated) base. Modification in this system is the absence of cytosine methylating activity. A restriction-deficient variant of strain JA1, which retains the JA1 modification phenotype, was isolated, indicating that JA1 cells have a gene product with restriction specificity for DNA containing 5-methylcytosine.

Mycoplasmas are procaryotes that have no cell walls: each cell is bounded only by a lipid-protein cell membrane (12). They are the smallest known free-living cells and have genomes 20 to 40% the size of those of typical eubacteria (12, 16). These organisms arose by degenerative evolution as a branch of the gram-positive eubacteria (16, 30).

Some strains of the mycoplasma *Acholeplasma laidlawii* are hosts for four different bacteriophages (4, 18). Mycoplasma phage L51 is a bullet-shaped virion containing circular, single-stranded (SS) DNA of 4.5 kilobases (kb). Mycoplasma phage L3 is a T7-like virion containing linear, double-stranded (DS) DNA of 39 kb. Mycoplasma phage L2 is an enveloped virion containing circular, superhelical, DS DNA of 11.8 kb. Mycoplasma phage L172 is an enveloped virion containing circular, SS DNA of 14 kb.

Bacteria have restriction-modification systems, which may have evolved to protect cells against infection by foreign DNA (for a review, see reference 27). Restriction involves endonucleases that recognize specific nucleotide sequences (≥ 4 bases in length [11, 24]) and cleave the DNA molecule at or near the sequence. A cell protects its DNA from endonuclease cleavage with a methylase that recognizes the same DNA sequence as the endonuclease and methylates a specific nucleotide within that sequence (2, 10). Specifically modified (methylated) cellular DNA is resistant to cleavage by the corresponding cellular restriction endonuclease, but in general, incoming foreign DNA is not appropriately modified and is cleaved.

A. laidlawii K2 and JA1 phenotypically restrict and modify the enveloped mycoplasma phages L2 (17) and L172 (4), but not mycoplasma phage L51 (17) or L3 (this study). A phage X propagated on *A. laidlawii* JA1 or K2 is designated

X · JA1 or X · K2, respectively. Hence, L2 · JA1 and L172 · JA1 have reduced plating efficiency on K2 cells relative to that on JA1 cells, and L2 · K2 and L172 · K2 have reduced plating efficiency on JA1 cells relative to that on K2 cells. For both L2 and L172 phages, restriction and modification are mediated at the DNA level, rather than at some other level, such as cell-virus membrane interactions, since similar restriction frequencies are observed for transfection with phage DNA and for infection with virions (4, 25).

K2 cell and L2 · K2 and L172 · K2 phage DNAs contain N⁶-methyladenine (m⁶Ade) and 5-methylcytosine (m⁵Cyt) (4, 5). L2 · K2 and L172 · K2 viral DNAs are susceptible to cleavage by *MboI* (a restriction endonuclease which cleaves GATC and GATm⁵C, but not Gm⁶ATC [11]) but not to cleavage by *Sau3A* (which cleaves GATC and Gm⁶ATC, but not GATm⁵C [11]), and L2 · JA1 and L172 · JA1 are susceptible to cleavage by both *MboI* and *Sau3A* (5; T. L. Sladek, unpublished data). Hence, when these phages are grown on K2 cells, progeny DNA must contain m⁵Cyt, but not m⁶Ade, in each GATC sequence. The basis for protection of DNA against restriction in K2 cells could be either (or both) modification of cytosine in the sequence GATC or modification of adenine in some unknown sequence. In contrast, JA1 cell and L2 · JA1 phage DNA contain no methylated bases (5), and the mechanism used to protect DNA against restriction in JA1 cells is unknown.

In this report we show that L51 SS and DS replicative form (RF) DNA is restricted by *A. laidlawii* JA1 and K2 when the cells are transfected with phage DNA, although the data do not show reproducible restriction of L51 phage during infection of these host cell strains. A restriction endonuclease cleavage map of L51 RF DNA was constructed and found to have a single *MboI* site. From the restriction data, DNA from phage grown on K2 must have the *MboI* site methylated to GATm⁵C, and DNA from phage

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grown on JA1 must have an unmethylated *MboI* site. An L51 mutant was constructed in which the *MboI* site was deleted and, since this deletion mutant was viable, it was determined that the *MboI* site (GATC) is not essential for phage growth. The L51 deletion mutant was not restricted by either strain K2 or JA1 in transfection experiments. Therefore, strain K2 has a restriction system specific for the sequence GATC and strain JA1 restricts DNA containing the sequence GATm⁵C. Using specific DNA methylases, we show that JA1 has a newly discovered type of restriction specificity, in which DNA containing m⁵Cyt is restricted independently of the m⁵Cyt-containing sequence.

MATERIALS AND METHODS

Phages, cells, and media. Mycoplasma phages L51 (15), L2 (7), and L3 (8) and *A. laidlawii* JA1 (15) were used in these studies. Other *A. laidlawii* strains were derived as follows. (i) Strain JA1-REP⁻ was isolated as a clone of JA1 that propagates DS DNA mycoplasma phages but not SS DNA mycoplasma phages (4, 21). (ii) Strain K2 was isolated from *A. laidlawii* 1305/68 as a clone with an increased plating efficiency for mycoplasma phage L3 (9). (iii) Strain 8195 was isolated from JA1-REP⁻ as a clone which showed no restriction of L2 phage grown on K2 and is described below.

Cells were assayed as CFU, phages as PFU, and transfected cells as infectious centers (PFU) on *A. laidlawii* lawns. Tryptose broth and agar plates were used for culturing cells and viruses (14).

Buffers and transfection. Buffers and the protocol for polyethylene glycol (PEG)-dependent transfection of *A. laidlawii* were described previously (25). In general, a transfection mixture contained 50 μ l of DNA solution, 250 μ l of cell suspension, and 2 ml of PEG 8000 solution (final concentration, 36%). A saturating amount of DNA was used, and cell and DNA concentrations are given for each experiment.

Phage stocks and DNA purification. L2 and L3 phage infection of early log phase *A. laidlawii* cultures has been described previously (25, 26). Centrifugation of these cells yielded infected cell supernatants which were either filtered to obtain sterile L2 or L3 phage stocks or concentrated with PEG, phenol extracted, and ethanol precipitated to prepare L2 or L3 phage DNA (25, 26).

L51 phage SS DNA was purified from supernatants of L51-infected cells. After concentration of phage from the supernatants by PEG precipitation, virions were purified by centrifugation through CsCl step gradients (26). L51 SS DNA was obtained by phenol extraction and ethanol precipitation of phage-containing CsCl gradient fractions.

To obtain L51 DS RF DNA, L51-infected cells were lysed with sodium dodecyl sulfate, and a cleared lysate was obtained by precipitation with a solution containing a high salt concentration and centrifugation (26). DNA was isolated from cleared lysates by phenol extraction and ethanol precipitation. This procedure yielded DNA preparations containing L51 RF molecules and *A. laidlawii* cellular DNA (26). These DNA preparations, termed cleared lysate RF DNA, were used for agarose gel electrophoresis and in some transfection experiments. For other transfection experiments and for restriction endonuclease mapping, cellular DNA and RF DNA were separated by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (26).

DNA (in TE buffer) concentrations were determined spectrophotometrically (26). TE buffer was 10 mM Tris (pH 8.0) and 1 mM EDTA.

Nuclease and methylase treatment. Restriction endonucleases were obtained commercially and used as recommended by the manufacturers. S1 nuclease was purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and used as described previously (26).

To obtain specifically methylated DNA, L2 · JA1 phage DNA (i.e., unmethylated L2 DNA) was treated with commercially available methylating enzymes that had different recognition sequences. L2 · JA1 DNA (5 μ g) was incubated with 5 U of the appropriate methylase (obtained from New England BioLabs, Inc., Beverly, Mass.) at 37°C for 1 h in a 50- μ l reaction mixture containing 50 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, 5 mM 2-mercaptoethanol, and 80 μ M S-adenosylmethionine, unless noted otherwise. For *MspI* methylase, this reaction mixture also contained 100 mM NaCl. For *TaqI* methylase, the reaction mixture was 100 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, and 80 μ M S-adenosylmethionine, and incubation was at 65°C. Each methylase treatment made the DNA resistant to cleavage by the corresponding restriction endonuclease. *Escherichia coli* *dam* methylase-treated DNA was resistant to cleavage by restriction endonuclease *MboI*, which cleaves GATC but not Gm⁶ATC. Methylases were inactivated by heating the reaction mixtures to 65°C for 20 min, and the DNA was used for transfection. DNA (5 μ g) which was not methylase treated (L2 · JA1 and L2 · K2 DNA) was kept in 50 μ l of TE buffer. The number of methylase sites in L2 DNA was taken as the number of cleavage sites for the corresponding restriction endonuclease, since each methylase rendered the DNA inactive to cleavage by the corresponding endonuclease.

Agarose gel electrophoresis. Electrophoresis was carried out as described previously (26). Unless otherwise noted, gels were 1.4% agarose. Phage lambda DNA digested with *HindIII* and ϕ X174 RF DNA digested with *HaeIII* were used as molecular weight standards.

Mycoplasma phage L51 deletion mutants. L51 cleared lysate RF DNA (from strain K2) was treated with restriction endonuclease *CfoI*, *HinfI*, or *MboI*. The DNA was phenol extracted, ethanol precipitated, and suspended in TE buffer. This DNA was treated with S1 nuclease (10 U per μ g of DNA) and again phenol extracted, ethanol precipitated, and suspended in TE buffer. Between 5 and 10 μ g of each DNA was used to transfect strain K2 cells. Individual plaques were picked and used to grow phage. For each phage, L51 cleared lysate RF DNA was prepared (as described above) and treated with the restriction endonuclease originally used to cleave the respective cleared lysate RF DNA. For material originally treated with *CfoI*, RF DNA from none of the 3 clones tested was resistant to *CfoI* digestion; for material originally treated with *HinfI*, RF DNA from 1 of 14 clones tested was resistant to *HinfI* digestion; and for material originally treated with *MboI*, RF DNA from 1 of 17 clones tested was resistant to *MboI* digestion. Phage from the single *HinfI*- and *MboI*-resistant plaques (designated L51 Δ *HinfI* and L51 Δ *MboI*, respectively) were used to infect K2 cells, from which L51 Δ *HinfI* and L51 Δ *MboI* cleared lysate RF DNAs were prepared.

RESULTS

Restriction of L51 DNA. Mycoplasma phage L51 has been reported not to be restricted during infection of *A. laidlawii* K2 and JA1 (17). However, the plating efficiency for L51 phage was highly variable. A small level of restriction was sometimes seen and at other times was not (data not shown). In contrast to the L51 virion data, L51 phage DNA was reproducibly restricted when it was introduced into *A.*

laidlawii cells by transfection. The data in Table 1 show that JA1 and K2 cells restrict weakly and modify L51 SS DNA, L51 RF DNA, and L51 cleared lysate RF DNA in transfection experiments. The degree of restriction in L51 experiments was at least 10-fold less than that for L2 and L172 (4, 5, 17, 25). However, similar weak restriction has been reported for the filamentous coliphages (1), which, like L51, are small SS DNA viruses.

Treatment of L51 RF DNA with restriction endonucleases. The m⁶Ade modification, m⁵Cyt modification, or both are presumed to be the basis for protection of DNA against restriction in strain K2. Since L51 DNA isolated from strain JA1 cells was restricted by K2 cells (Table 1), modification of L51 DNA was investigated by looking at the susceptibility of L51 RF DNA to *Mbo*I and *Sau*3A nucleases (Table 2). L51 · K2 RF DNA and L51 · JA1 RF DNA were both cleaved once by *Mbo*I. However, L51 · K2 RF DNA was not cleaved by *Sau*3A, while L51 · JA1 RF DNA was cleaved once by *Sau*3A. This indicates that the single GATC site in the L51 RF molecule contains m⁵Cyt when the virus is grown on strain K2, but not when the virus is grown on strain JA1. In addition, L51 · JA1 RF DNA was not cleaved by *Bam*HI, *Bcl*II, or *Bgl*II (Table 2), indicating that the single GATC sequence in the L51 genome is not part of the sequences GGATCC (the *Bam*HI recognition sequence), TGATCA (the *Bcl*II recognition sequence), or AGATCT (the *Bgl*II recognition sequence).

Restriction endonuclease cleavage map of L51 DS RF DNA. The susceptibility of L51 RF DNA to a number of restriction endonucleases was investigated (Table 2). Those which produced one or two cuts were used to digest L51 RF DNA and, following analysis of the DNA fragments by electrophoresis in agarose gels, a restriction endonuclease cleavage map of the 4.5-kb L51 RF genome was constructed (Fig. 1).

TABLE 1. Restriction frequencies of transfecting L51 DNA^a

Transfecting DNA	No. of infectious centers (relative no.) on strain:	
	JA1	K2
L51 · JA1 SS DNA	5.05 × 10 ³ (1.00)	9.50 × 10 ³ (0.53)
L51 · K2 SS DNA	1.40 × 10 ³ (0.28)	1.80 × 10 ⁴ (1.00)
L51 · JA1 DS RF DNA	4.00 × 10 ⁴ (1.00)	1.23 × 10 ⁵ (0.31)
L51 · K2 DS RF DNA	9.85 × 10 ³ (0.25)	3.95 × 10 ⁵ (1.00)
L51 · JA1 cleared lysate RF DNA	5.50 × 10 ³ (1.00)	1.36 × 10 ⁴ (0.44)
L51 · K2 cleared lysate RF DNA	1.90 × 10 ³ (0.35)	3.12 × 10 ⁴ (1.00)

^a Viral DNAs were isolated and transfection was performed as described in the text. For transfection with L51 SS DNA, 5 µg of L51 · JA1 DNA or 10 µg of L51 · K2 DNA was added to 1.74 × 10⁷ CFU of strain JA1 or 5.70 × 10⁷ CFU of strain K2. For transfection with L51 DS RF DNA, 10 µg of L51 · JA1 DNA or L51 · K2 DNA was added to 1.15 × 10⁸ CFU of strain JA1 or 3.43 × 10⁷ CFU of strain K2. For transfection with L51 cleared lysate RF DNA, 10 µg of L51 · JA1 DNA or 40 µg of L51 · K2 DNA was added to 7.75 × 10⁷ CFU of strain JA1 or 1.20 × 10⁸ CFU of strain K2. Since transfection efficiency varies with cell growth phase (25) and mycoplasma cultures cannot be adjusted to obtain cells in the same growth phase, transfection data from different mycoplasma cultures cannot be compared. Hence, a single mycoplasma culture must be divided, each part transfected with a saturating amount of a different DNA, and these frequencies compared. Therefore, for each experiment (i.e., infection of a strain with SS, DS RF, or cleared lysate RF DNA), the data for transfection of each strain are normalized to the number of infectious centers obtained with DNA isolated from virus grown on that strain.

TABLE 2. Restriction endonuclease cleavage sites in L51 · K2 RF DNA^a

No. of cleavage sites	Restriction endonuclease
1	<i>Cfo</i> I, <i>Hin</i> FI, <i>Hpa</i> I, <i>Mbo</i> I
2	<i>Clal</i> I, <i>Hinc</i> II
3	<i>Hae</i> III
4	<i>Hind</i> III
0 ^b	<i>Bam</i> HI, <i>Bcl</i> II, <i>Bgl</i> II, <i>Bst</i> EII, <i>Eco</i> RI, <i>Hae</i> II, <i>Hpa</i> II, <i>Msp</i> I, <i>Pst</i> I, <i>Sst</i> I, <i>Sau</i> 3A, <i>Xor</i> II

^a Some enzymes were also tested on L51 · JA1 RF DNA with the following results: no cleavage sites for *Bam*HI, *Bcl*II, and *Bgl*II; one cleavage site for *Mbo*I and *Sau*3A.

^b To ensure that these enzymes were working, a second DNA was included in the reaction mixture as a control. Phage lambda DNA usually was added as the control DNA, and in these cases, lambda DNA was cleaved but L51 RF DNA was not. When a particular restriction endonuclease did not cleave lambda DNA because of the inability of the enzyme to cleave DNA with the *E. coli* dam modification (Gm⁶ ATC), L2 · JA1 DNA was used as the control.

Isolation of mycoplasma L51 deletion mutants. Since L51 has only one *Mbo*I site, it must have only one GATC sequence. We deleted the *Mbo*I and other single restriction endonuclease cleavage sites from the L51 genome. L51 cleaved lysate RF DNA was digested with *Cfo*I, *Hin*FI, or *Mbo*I and then treated with the SS-specific nuclease S1 to remove overlapping ends generated by restriction endonuclease cleavage. These DNAs were used to transfect cells, plaques were picked, and phage was grown. RF DNA was isolated from each L51-infected culture and treated with the restriction endonuclease originally used to cleave the DNA, as described above. DNA from a clone which had originally been digested with *Hin*FI (recognition sequence GATC [11]) was found to lack the *Hin*FI site (Fig. 2, lane e). The DNA from this L51 deletion mutant, L51Δ*Hin*FI, still contained the single *Mbo*I site (Fig. 2, lane f). Likewise, a L51Δ*Mbo*I deletion mutant was found. DNA from this virus lacked the *Mbo*I site (Fig. 2, lane i), but it still contained the *Hin*FI site (Fig. 2, lane h). An internal control which showed that the restriction enzymes were working in these experiments was digestion of the cellular DNA in the cleared lysate

FIG. 1. Restriction endonuclease cleavage map of the 4.5-kilobase-pair L51 RF genome, constructed as described in the text, for endonucleases with one or two cleavage sites (Table 2).

FIG. 2. Agarose gel electrophoresis of undigested and restriction endonuclease-digested cleared lysate RF DNAs from K2 cells infected with L51 wild-type or deletion mutant virus. Lane a, L51 wild-type DNA; lane b, L51 wild-type DNA + *HinfI*; lane c, L51 wild-type DNA + *MboI*; lane d, L51 Δ *HinfI* DNA; lane e, L51 Δ *HinfI* DNA + *HinfI*; lane f, L51 Δ *HinfI* DNA + *MboI*; lane g, L51 Δ *MboI* DNA; lane h, L51 Δ *MboI* DNA + *HinfI*; lane i, L51 Δ *MboI* DNA + *MboI*. The positions of cell chromosomal (CELL), and L51 open circular (OC), linear (LIN), and superhelical covalently closed circular (CCC) DNAs have been identified previously (19) and are indicated in the figure. The gel was 0.8% agarose.

DNA preparations (Fig. 2, lanes e and i). No *CfoI* deletion mutants were found.

We noted that L51 RF DNA in cleared lysates (Fig. 2, lanes a, d, and g) is largely in the form of open circular rather than superhelical molecules, probably due to nicking of superhelical molecules during preparation. Disappearance of the superhelical band in Fig. 2, lanes e and i, must reflect further conversion of superhelical to open circular molecules during incubation, since the lack of increase in linear DNA in these gel lanes rules out endonuclease cleavage in these two preparations. In contrast, the DNAs in Fig. 2, lanes f and h, have been completely converted to linear molecules, showing restriction endonuclease cleavage in these preparations.

DNA migrating slower than cellular DNA on gels was seen in the deletion mutant DNA samples. It is likely that these were oligomeric forms of the L51 DNA molecule, since digestion of deletion mutant DNA with that enzyme for which the recognition sequence had been deleted failed to change the mobility of these DNA bands. It should be noted that we have previously described dimer forms of L51 RF DNA in infected cells (26).

Restriction of deletion mutants. The finding that the single L51 GATC sequence could be deleted allowed us to test whether this sequence is required for host cell restriction by K2 or JA1 cells. If this sequence is restricted, host cell restriction of the type seen in Table 1 should not be observed in transfection experiments with L51 Δ *MboI* DNA. L51 Δ *HinfI* DNA was used as a control in these experiments, since it has a deletion but contains the *MboI* (GATC) site.

Table 3 shows the results of transfection experiments with L51 Δ *MboI* and L51 Δ *HinfI* DNAs. These data show that, whereas host cell restriction of L51 Δ *HinfI* DNA was similar to that seen in transfection experiments with wild-type DNA (cf. Table 1), restriction was not observed when L51 Δ *MboI* DNA was used. Since the restriction frequencies for L51 Δ *HinfI* DNA were similar to those for wild-type DNAs, the result with L51 Δ *MboI* DNA must be specifically due to deletion of the GATC sequence and not a general property of

L51 deletions. Also, since no restriction was seen for L51 Δ *MboI* DNA with both K2 and JA1 cells (Table 3), we conclude that the sequence GATC is restricted by K2 cells and the sequence GATm⁵C is restricted by JA1 cells.

Lack of restriction with L3 phage. We found that mycoplasma phage L3 was not host cell restricted by *A. laidlawii* K2 or JA1 (data not shown). Instead, L3 · JA1 and L3 · K2 both produce two to three times more plaques on JA1 lawns than on K2 lawns.

The lack of host cell restriction of L3 phage could mean that restriction systems are inactivated in L3-infected JA1 and K2 cells or that the L3 genome contains no GATC sequences. Therefore, L3 · JA1 and L3 · K2 DNAs were treated with restriction endonucleases *MboI* and *Sau3A* and analyzed by agarose gel electrophoresis. Neither enzyme cleaved L3 · JA1 or L3 · K2 DNA, although both cleaved L2 · JA1 DNA which was added to the L3 DNA preparations as a control to confirm that both enzymes were active (data not shown). Identical results with L3 · JA1 and L3 · K2 DNAs have been observed by Just and co-workers (Diplomarbeit, Universität Ulm, Federal Republic of Germany, 1983; W. Just, E. Schultz, K. Haberer, and G. Klotz, submitted for publication). These experiments would have detected GATC, Gm⁶ATC, or GATm⁵C sequences in L3 DNA. Therefore, we conclude that, unless L3 DNA possesses base modifications other than m⁶Ade or m⁵Cyt within the GATC sequence which block *MboI* and *Sau3A* cleavage, L3 DNA contains no GATC sequences.

Isolation of a restriction-deficient JA1 strain. We isolated a spontaneous derivative of the JA1 strain that does not restrict L2 · K2 phage or the L2 · K2 DS DNA (Table 4). This strain, designated 8195, was isolated as a clone of the JA1-REP⁻ strain (4, 21) and thus also has the phenotype of the inability to propagate SS DNA mycoplasma viruses. Table 4 shows that, although strain 8195 did not restrict L2 · K2 phage, phage grown on strain 8195 was restricted by strain K2, but not by strains JA1 and JA1-REP⁻. Therefore, 8195 cells lost the JA1 restriction phenotype, but retained the JA1 modification phenotype.

Restriction of methylated DNAs. Results of the studies (described above) with L51 deletion mutants show that JA1 cells restrict DNA containing the sequence GATC only if it

TABLE 3. Restriction frequencies of L51 deletion mutant DNAs in transfection^a

Transfecting DNA	Relative no. of infectious centers on strain ^b	
	JA1	K2
L51 Δ <i>HinfI</i> · JA1	1.00	0.22 $\pm$ 0.09
L51 Δ <i>HinfI</i> · K2	0.50 $\pm$ 0.13	1.00
L51 Δ <i>MboI</i> · JA1	1.00	0.70 $\pm$ 0.24
L51 Δ <i>MboI</i> · K2	1.12 $\pm$ 0.40	1.00

^a JA1 and K2 cells were infected with L51 Δ *HinfI* and L51 Δ *MboI* virus. Four cleared lysate RF DNAs were prepared: L51 Δ *HinfI* · JA1, L51 Δ *MboI* · JA1, L51 Δ *HinfI* · K2, L51 Δ *MboI* · K2. Each DNA was used to transfect JA1 and K2 cells. Transfection was performed as described in the text with 10 μ g of the appropriate cleared lysate RF DNA. For each transfection experiment, a JA1 or K2 culture was divided into two parts; one was transfected with deletion mutant RF DNA from a JA1 cleared lysate, and the other was transfected with the same deletion mutant RF DNA from a K2 cleared lysate. As discussed in footnote a of Table 1, data for transfection of each strain were normalized to the number of infectious centers obtained with the DNA isolated from virus grown on that strain.

^b Each value is the mean ($\pm$ standard deviation) of the data from four experiments.

contains m⁵C, the converse of the typical bacterial system in which unmethylated DNA is restricted and methylated DNA is not. From these data, it can be seen that strain JA1 restriction might be specific for the sequence GATm⁵C, for m⁵Cyt residues in a number of different sequences, or for methylated bases (m⁵Cyt and m⁶Ade) in general.

To examine these possibilities, *A. laidlawii* K2, JA1, and 8195 were transfected with mycoplasma L2 phage DNA that had been treated with specific DNA methylases, and the efficiency of plating was assayed and compared with that of unmethylated DNA. The restriction specificity for nucleotide sequences containing m⁵Cyt was investigated by transfecting with L2 · K2 DNA (which contains GATm⁵C) and L2 · JA1 DNA containing m⁵Cyt at different sites after treatment with *AluI*, *HpaII*, *MspI*, and *HhaI* methylases. Table 5 shows that unmethylated L2 · JA1 DNA is not restricted by JA1 or 8195 cells, but is restricted by K2 cells because the DNA does not have m⁵Cyt in the sequence GATC. All L2 DNAs containing m⁵Cyt were restricted by JA1 cells, but not by 8195 cells. Hence, unmethylated L2 · JA1 DNA was not restricted by JA1 cells, but L2 · JA1 DNA containing m⁵Cyt residues in at least four different sequences was restricted. The level of restriction appeared to be a function of the number of m⁵Cyt residues. The isolation of strain 8195, a spontaneous restriction-deficient variant of JA1 (described above), means that it is improbable that JA1 cells have a family of restriction activities, each of which is specific for a different m⁵Cyt-containing nucleotide sequence; instead, strain JA1 restriction is probably controlled by a single gene product. In contrast to strain JA1, K2 cells restrict all of the DNAs containing m⁵Cyt except L2 · K2 DNA, because only L2 · K2 DNA has m⁵Cyt in the sequence GATC.

To determine whether JA1 cells also restrict DNA containing the modified base m⁶Ade, transfection was done with L2 · JA1 DNA containing m⁶Ade at different sites after treatment with *dam*, *PstI*, and *TaqI* methylases. Table 5 shows that none of the L2 · JA1 DNAs containing m⁶Ade is restricted by either JA1 or 8195 cells. Hence, L2 · JA1 DNA containing m⁶Ade in at least three different sequences is not restricted by JA1 cells. However, K2 cells restrict all of the

TABLE 4. Restriction of L2 virions and DNAs by *A. laidlawii* strains^a

Infection with:	Relative no. of infectious centers on strain:			
	JA1	JA1-REP ⁻	8195	K2
Phage				
L2 · JA1	1.00	0.84	0.92	0.03
L2 · JA1-REP ⁻	1.00	1.00	0.87	0.02
L2 · 8195	0.83	0.87	1.00	0.03
L2 · K2	0.07	0.03	0.73	1.00
DNA				
L2 · JA1	1.00		1.00	
L2 · K2	0.06		0.66	

^a For infection with L2 phage, phage was plated on lawns of all four hosts, and the data were normalized to the number of plaques on the strain on which the virus was originally grown. In these experiments, K2 cells were *A. laidlawii* strain 1305/68, the strain from which K2 was cloned. For infection with L2 DNA, transfection was performed as described in the text with L2 DNA (5 µg) added to 9.75×10^7 CFU of strain JA1 or 1.96×10^8 CFU of strain 8195. As discussed in footnote a of Table 1, in these experiments (i.e., with L2 · JA1 or L2 · K2 DNA) the data for transfection of each strain were normalized to the number of infectious centers obtained with DNA isolated from virus grown on that strain.

TABLE 5. Transfection of *A. laidlawii* strains with L2 · JA1 DNA treated with DNA methylases^a

DNA	Methylase treatment	DNA modification	No. of sites in L2 · JA1 DNA	Relative no. of infectious centers on strain:		
				K2	JA1	8195
L2 · JA1	None			0.07	1.00	1.00
L2 · K2	None	GATm ⁵ C	≥8	1.00	0.10	0.68
L2 · JA1	<i>AluI</i>	AGm ⁵ CT	≥8	0.03	0.10	1.17
L2 · JA1	<i>HpaII</i>	Cm ⁵ CGG	≥10	0.03	0.08	0.93
L2 · JA1	<i>MspI</i>	m ⁵ CCGG	≥10	0.08	0.08	0.80
L2 · JA1	<i>HhaI</i>	Gm ⁵ CGC	1	0.02	0.14	0.86
L2 · JA1	<i>dam</i>	Gm ⁶ ATC	≥8	0.02	0.74	0.77
L2 · JA1	<i>PstI</i>	CTGcm ⁶ AG	5	0.06	0.61	1.17
L2 · JA1	<i>TaqI</i>	TCGm ⁶ A	≥10	0.05	0.61	1.00

^a Each cell preparation was transfected with all of the DNAs listed. Transfection data for strain K2 are normalized to the number of infectious centers obtained from transfection of the K2 cell preparation with L2 · K2 DNA (4×10^4 PFU per transfection mixture). Transfection data for strains JA1 and 8195 were normalized to the number of infectious centers obtained from transfection with L2 · JA1 DNA; 1×10^4 PFU per transfection mixture containing JA1 cells and 2×10^4 PFU per transfection mixture containing 8195 cells.

DNAs containing m⁶Ade, because none of these has m⁵Cyt in the sequence GATC.

The specificity of *PstI* methylase had not been reported when we carried out these studies. We determined the *PstI* methylase specificity as follows. L2 · JA1 DNA, which has five *PstI* cleavage sites and contains no methylated bases, was treated with *PstI* methylase such that the DNA was no longer susceptible to *PstI* restriction endonuclease cleavage. The base composition of this DNA was then determined by high-pressure liquid chromatography (5) by David Swinton (Department of Biology, University of Rochester). The base composition (in moles percent) of the *PstI* methylase-treated L2 · JA1 DNA was 33.9% thymine, 16.1% guanine, 16.9% cytosine, <0.004% m⁵Cyt (i.e., none detectable), 33.1% adenine, and 0.05% m⁶Ade. Hence, *PstI* only methylates adenine, not cytosine, and the 11.8-kilobase-pair L2 genome contains approximately 12 m⁶Ade residues. Since the *PstI* restriction endonuclease recognition sequence is CTGCAG (24), full methylation of the five *PstI* sites in L2 DNA should yield 10 m⁶Ade residues per genome, which is experimentally indistinguishable from the measured value. Therefore, *PstI* methylates adenine in the sequence CTGCAG to CTGcm⁶AG. This conclusion is in agreement with recent reports on *PstI* methylase specificity (20, 28). The references for all other methylase specificities used in this study have been cited in reference 20.

DISCUSSION

We identified two acholeplasma restriction-modification systems: *A. laidlawii* K2 with a typical bacterial-type system, which includes restriction of DNA containing a specific unmethylated sequence (GATC) and modification by methylation of a specific residue (cytosine) in this sequence; and *A. laidlawii* JA1 with a unique mechanism, which includes restriction of DNA containing m⁵Cyt independent of the sequence containing the m⁵Cyt and modification by not methylating cytosine residues. Since mycoplasmas arose by degenerative evolution from the eubacteria (16, 30), their genetic complexity is limited by their small genome size (19). Hence, there must have been some selective advantage for

mycoplasmas to maintain restriction-modification systems during the phylogenetic reduction in genome size. The ubiquity of achleoplasma habitats may give them constant exposure to foreign DNAs, providing selective pressure for restriction-modification activities. Although modification methylation has been suggested to have a role in DNA repair (for a review, see reference 23), it is probably not involved in mycoplasmas. *A. laidlawii* JA1 (which has no detectable methylated bases) is proficient in the repair of DNA damage (6), and *A. laidlawii* K2 (which has both methylated adenine and cytosine) is repair deficient (22).

The weak restriction observed for L51 DNA (Table 1), as compared with restriction of L2 and L172 phage and DNA (4, 5, 25), may reflect differences in a number of GATC sequences in the phage genomes. Since both 11.8-kb DS L2 and 14.0-kb SS L172 DNAs contain many GATC sequences (4, 5), these DNAs should be more efficiently restricted by a system specific for GATC than the 4.5-kb SS L51 DNA molecule containing only one GATC sequence. A similar argument has been given to explain the weak restriction observed for some of the small SS DNA coliphages (1).

Weak host cell restriction of L51, coupled with variability in L51 adsorption (26), might explain our inability to reproducibly demonstrate restriction of L51 virions. Slight differences in adsorption (due to culture or physiological variation [26]) could mask the small amount of restriction possible for L51.

L3 phage was not host cell restricted by *A. laidlawii* K2 and JA1, and we found no *MboI* or *Sau3A* cleavage sites (GATC sequences) in the L3 genome. This is surprising, since one would expect many of these sequences in the 39-kb L3 genome. Hence, L3 virus may have evolved a genome containing no GATC sequences to escape host cell restriction.

Several questions remain concerning host cell modification and restriction of phages by *A. laidlawii* JA1 and K2. We have shown that strain K2 restricts DNA containing the sequence GATC (e.g., L2 · JA1 and L51 · JA1 DNAs), but not DNA containing GATm⁵C (e.g., L2 · K2 and L51 · K2 DNAs) or no GATC (e.g., L51Δ*MboI* DNA). DNA of K2 cells and phages grown in K2 cells contain m⁵Cyt in their GATC sequences (since they are not susceptible to cleavage by *Sau3A*), which protects them from K2 restriction. Hence, we expect K2 cells will be found to have a restriction endonuclease that recognizes the sequence GATC and cleaves the DNA, but does not restrict when the sequence contains m⁵Cyt. K2 cells must also have a complementary methylating enzyme that methylates cytosine in the sequence GATC.

JA1 cells have a newly discovered type of restriction specificity. All previously reported restriction specificities are for sequences of at least four nucleotides, and only one other restriction-modification system has been described in which methylated DNA is restricted and unmethylated DNA is not. This is *Diplococcus pneumoniae* restriction endonuclease *DpnI* (13) (and its isoschizomers [24]), which cleaves the tetramer GATC only if it contains m⁶Ade. In contrast, the *A. laidlawii* JA1 restriction activity described here is specific for the single methylated nucleotide m⁵Cyt. A restriction endonuclease with this specificity would be the first of a new class of restriction endonucleases and would have a variety of experimental uses, particularly in studies of the role of methylated nucleotides in cellular processes.

The *HinI* and *MboI* sites that were deleted from L51 DNA without inactivating the virus are only about 150 base pairs apart, which suggests that the region spanning these

two sites may be nonessential. This region may be analogous to the intercistronic regions of icosahedral and filamentous SS DNA phages (29). Such a region in L51 might be useful for insertion of foreign DNA for cloning in mycoplasmas. In addition, since L51 phage particles longer than unit length have been observed in virion preparations (3, 15), there may not be strong constraints on the amount of DNA that L51 can package. Hence, the L51 deletion mutants described here may allow development of a mycoplasma cloning vector.

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