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Association of Lysogenic Bacteriophage MAV1 with Virulence of *Mycoplasma arthritidis*

LEROY L. VOELKER,† KEITH E. WEAVER, LACEY J. EHLE, AND LEIGH RICE WASHBURN*

Department of Microbiology, University of South Dakota School of Medicine, Vermillion, South Dakota 57069-2390

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Mycoplasma arthritidis **causes a severe polyarthritis under natural conditions in rats and under experimental conditions in both rats and mice. Although the disease itself has been extensively studied, *M. arthritidis* virulence factors remain uncharacterized. Comparison of relative arthritogenicity of 20 strains of *M. arthritidis* revealed that the strains tended to fall into two groups, a highly arthritogenic group, inducing maximum arthritis scores of ≥11 in rats, and a low-virulence group, inducing maximum scores of <6. Chromosomal DNA from the more highly arthritogenic strains possessed sequences that hybridized by Southern analysis with a probe prepared from lysogenic *M. arthritidis* bacteriophage MAV1, while DNA from low-virulence strains did not. One of the low-virulence strains, 158, was experimentally lysogenized with MAV1. Lysogenized 158 showed a significant increase in arthritogenicity over nonlysogenized 158. These data suggest that MAV1 carries a factor that is important in pathogenesis of *M. arthritidis*-induced arthritis of rats.**

Mycoplasma arthritidis is a natural pathogen of rats, although disease can also be induced experimentally in rats and mice by intravenous or intraperitoneal injection of large numbers of mycoplasmas. Experimental disease is an acute, self-limiting systemic infection involving a number of organs. However, the most noticeable characteristic is arthritis in the peripheral joints. Occasionally, infection also involves the spine, causing temporary hind-limb paralysis (11).

Several investigators have reported differences in virulence among *M. arthritidis* strains (6, 9, 18, 21, 25, 40). In attempting to account for these differences, most investigators in the past have focused on characterization of immunologic aspects of disease and antigenic analysis of *M. arthritidis*. This has led to several proposals for mechanisms of virulence, and roles have been suggested for toxins, immunomodulating factors, including the superantigen MAM, and cytadhesins (5, 11, 39). However, conclusive proof for such roles in the rat disease is not yet available.

Bacterial virulence factors are often encoded by extrachromosomal (EC) elements (plasmids, transposons, and bacteriophages). However, few such elements have been found in members of the genus *Mycoplasma*. Plasmids have been found only in *Mycoplasma mycoides* (2, 14). Insertion sequences or insertion sequence-like elements have been identified in *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis* (17), *Mycoplasma fermentans* (23), and *Mycoplasma pulmonis* (3), and bacteriophages have been isolated from *M. pulmonis* (15), *M. hyorhinis* (19), and *Mycoplasma bovirhinis* (22). None of these elements is known to be associated with virulence.

We recently isolated from *M. arthritidis* a lysogenic bacteriophage, designated MAV1, that contains linear, double-stranded DNA (32a, 34). Sequences hybridizing with MAV1 DNA were found in the chromosomes of 10 of 20 *M. arthritidis* strains examined (35). Comparison of these data with earlier studies in our laboratory on *M. arthritidis* virulence (40) suggested a possible association between the presence of MAV1 DNA-hybridizing sequences and enhanced arthritogenicity. We undertook the present study to confirm the existence of such an association.

(Parts of this study were presented at the 94th General Meeting of the American Society for Microbiology, Las Vegas, Nev., 1994 [34], and at the 10th International Congress of the International Organization for Mycoplasmology, Bordeaux, France, 1994 [35].)

MATERIALS AND METHODS

M. arthritidis **strains and cultivation.** A total of 20 *M. arthritidis* strains or isolates were used in this study. In some cases, the same strains were obtained from different sources and/or maintained in different laboratories; in this event, they were given individual designations and treated as separate strains. Strain designations and source references are summarized as follows: 158 (derived from Campo) (7), 158p10 (18), 158p10p9 (10), 14124* (Jasmin) (26), 14124 (7), 14124p10 (18), 14152* (16, 30), 14152 (7), 14152p13 (18), 19611 (PG6) (31), PG6 (31), H606 (29), 13988 (H606) (29), 23192 (Campo) (16, 30), 07 (28), H39 (28), JR3 (32), ISR1 (27), Jasmin (25), and Tru (12). Strains 158, 158p10, 158p10p9, 14124, 14124p10, 14152, 14152p13, PG6, and H606 were obtained in 1985 from Barry Cole, University of Utah. The "p" designations in strains 158p10, 158p10p9, 14124p10, and 14152p13 indicate numbers of animal passages; for example, 158p10p9 was derived from strain 158 by 10 passages through rats and another nine through mice. Since these animal-passaged derivatives are in several cases now quite different in virulence and in antigenic and restriction profiles from the original parent strains (41), they are maintained and considered as separate strains. Strain 07 was donated by Paul Smith, University of South Dakota, and strains H39, JR3, ISR1, Jasmin, and Tru were contributed by Gail Cassell, University of Alabama at Birmingham. 14124*, 14152*, 19611, 13988, and 23192 were purchased from the American Type Culture Collection and for convenience are designated by their catalog numbers. Their actual strain designations are Jasmin, Campo (or PG27), PG6, H606, and Campo, respectively.

Dilute stock cultures of each strain were prepared by inoculating 100 ml of modified Edward broth medium (EB) supplemented with 7.5% (vol/vol) horse serum and 5% (vol/vol) fresh yeast extract with 100 μl from a freshly thawed dilute stock culture and incubating the cultures at 37°C with agitation for 36 to 48 h. These cultures were stored frozen in 1-ml aliquots at −70°C (36) and usually contained 6×10^8 to 8×10^8 CFU/ml. Concentrated stocks were prepared by inoculating 100 ml of EB with 100 μl from a dilute stock culture, expanding the culture to 1 liter after 48 h of incubation at 37°C with agitation, incubating the culture for an additional 18 to 24 h under similar conditions, centrifuging the culture at 12,000 × *g* for 20 min at 4°C, and resuspending the mycoplasmas from 1 liter in 20 ml of EB prepared with 15% (wt/vol) sucrose in

* Corresponding author. Mailing address: Department of Microbiology, University of South Dakota School of Medicine, 414 East Clark St., Vermillion, SD 57069-2390. Phone: (605) 677-5170. Fax: (605) 677-5124. Electronic mail address: LWASHBUR@SUNBIRD.USD.EDU.

† Present address: Department of Comparative Medicine, University of Alabama at Birmingham, Birmingham, AL 35294.

place of horse serum. These concentrated stocks contained approximately 3×10^{10} to 4×10^{10} CFU/ml. They were stored in 1-ml aliquots at −70°C.

When a MAV1-positive strain subcultured from a dilute stock began to yield EC bacteriophage, additional stock cultures were prepared as follows. One hundred microliters of freshly thawed dilute stock culture was inoculated into 100 ml of EB and incubated in stationary culture for 48 h at 37°C. The culture was then centrifuged at 12,000 × *g* for 30 min at 25°C; mycoplasmas were resuspended in 1.2 ml of EB-glycerol (1:1) and frozen in two 0.6-ml aliquots at −70°C. These cultures are referred to as glycerol stocks.

To avoid cross-contamination, stocks were prepared one at a time. On acquisition, each strain was reidentified as *M. arthritidis* by epi-immunofluorescence using fluorescein isothiocyanate-labeled rabbit antiserum prepared against strain 158p10p9 (13, 36); strain designations were confirmed by restriction analysis of chromosomal DNA (41).

DNA preparation and Southern analysis. For preparation of mycoplasmal DNA, 100 ml of EB was inoculated with 100 μl of dilute stock culture and incubated at 37°C for 42 to 48 h without agitation; mycoplasmas were centrifuged at 12,000 × *g* for 30 min at 25°C and resuspended in 0.5 ml of 10 mM Tris-HCl–1 mM EDTA (pH 8.0) (TE buffer). Alternatively, mycoplasmas contained in 1 ml of concentrated stock culture were pelleted by centrifugation and resuspended in 0.5 ml of TE buffer. Cells were then lysed with 1% (wt/vol, final concentration) sodium dodecyl sulfate, and RNase A (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 100 μg/ml; the sample was then incubated for 30 min at 37°C and extracted twice with Tris-buffered phenol (pH 8.0), once with phenol-chloroform (1:1), and once with chloroform. After ethanol precipitation, the DNA was resuspended in 25 μl of TE buffer, quantitated by spectrophotometry, and stored at −20°C until use. For restriction analysis or in preparation for Southern hybridization, 9-μg DNA samples were digested with the appropriate restriction endonucleases, electrophoresed on 0.4% (wt/vol) agarose gels for 12 to 14 h at 25 V, and stained with ethidium bromide according to standard procedures (1).

MAV1 DNA was purified as follows. Undigested DNA from a strain known to be undergoing productive infection with MAV1 was electrophoresed at 4°C on a 0.4% (wt/vol) low-melting-point agarose gel (GeneLine LMP Agarose; Beckman, Palo Alto, Calif.) at 25 V for 20 h. Agarose fragments containing extrachromosomal bands were excised from the gel and melted at 60°C; DNA was extracted as described above with buffered phenol, phenol-chloroform, and chloroform, precipitated with ethanol, redissolved in a total of 25 μl of TE buffer, and stored at −20°C until use.

For Southern analysis, digested or undigested mycoplasmal DNA was electrophoresed as described above and vacuum blotted to nylon membranes (Hybond-N+; Amersham Life Science, Arlington Heights, Ill.) by using a Bio-Rad (Hercules, Calif.) vacuum blotter according to the manufacturer's recommendations. *Spe*I was used for most Southern analysis experiments, because it cuts MAV1 DNA once into two fragments (10.2 and 6.3 kb) (35) but cuts frequently within the *M. arthritidis* chromosome. For most experiments, membranes were probed with biotin-labeled MAV1 DNA and hybridization patterns were visualized by using a chemiluminescence detection system (Gene Images Labeling and Detection System, U.S. Biochemical, Cleveland, Ohio) with Hyperfilm-ECL X-ray film (Amersham). Biotinylated probes for Southern analysis were prepared from gel-purified MAV1 DNA with a Random Primed Images Biotin Labeling Kit (U.S. Biochemical). For one experiment, DNA was probed with [α-^{32}P]dATP-labeled MAV1 DNA and hybridization was visualized by autoradiography.

Preparation of bacteriophage stocks and lysogenization of strain 158. Bacteriophage stocks were prepared as follows. Two loopfuls of a glycerol stock culture of strain 158p10 known to produce EC MAV1 were inoculated into 100 ml of EB and incubated for 48 h at 37°C without agitation. Cultures were then centrifuged at 12,000 × *g* for 30 min at 25°C. The supernatant was passed through a 0.2-μm-pore-size filter and stored at 4°C until use. MAV1 stocks were screened by culture and shown to be free of viable mycoplasmas.

Strain 158 was lysogenized with MAV1 by combining 10 ml of EB, 50 μl of freshly thawed 158 dilute stock, and 10 μl of MAV1 stock and incubating the mixture at 37°C for 24 h without agitation. The culture was passaged four times by transferring 50 μl to 10 ml of fresh EB and incubating the mixture as before. Cultures were then expanded, and concentrated stock cultures were prepared as described above. Two batches of lysogenized strain 158 were prepared in this manner; they were designated 158L1 and 158L3.

For Southern analysis and virulence assessment, several single-colony-derived clones were prepared from lysogens 158L1 and 158L3 as follows. Concentrated stock cultures were diluted 1:1,000 and passed through 0.45-μm-pore-size filters; additional 1:10,000 dilutions were made (final dilution factor, 10^{-7}), and 100 μl was plated onto Edward agar (identical to EB except for the addition of 1.4% [wt/vol] Bacto Agar [Difco Laboratories, Detroit, Mich.]). After 72 h of incubation at 37°C, agar plugs containing individual colonies were picked with Pasteur pipettes, and each plug was transferred to 2 ml of EB. The 2-ml cultures were incubated for 48 h at 37°C without agitation and expanded to 500 ml; concentrated stock cultures were then prepared as described above.

Induction and assessment of arthritis in rats. Arthritogenicity of four *M. arthritidis* strains was confirmed previously (40). In that study, male Lewis (LEW/SsNHsd) rats (Harlan Sprague-Dawley, Indianapolis, Ind.) weighing an average of 220 g were divided into groups of eight, and each rat was injected intravenously in the caudal vein with approximately 10^9 CFU of strains 158p10p9, 14152p13, PG6, and H606. To determine the relative arthritogenicity of the additional 16 strains, male Lewis rats from the same supplier, weighing an average of 178 g, were divided into groups of five to nine rats each and injected intravenously with 10^9 CFU of each strain.

Arthritogenicity of single-colony-derived filter clones from MAV1-lysogenized strain 158 (one from 158L1 and two from 158L3) was similarly assessed, using groups of seven or eight rats each (average weight, 140 g) and an inoculum size of 10^9 CFU. Control groups included rats injected with low-virulence parent strain 158 and with known arthritogenic strain 158p10 (18).

Clinical arthritis was assessed as described previously (38–40). Briefly, each peripheral joint was examined and, with the exception of the interphalangeal joints of the feet, given a score of 0 to 4, based on the degree of swelling; interphalangeal joints were assigned a score of 1 or 0, depending on the presence or absence of any degree of joint swelling. In addition, a score of 1 was assigned to each hind limb showing partial paralysis, and a score 2 represented complete paralysis. This scoring system has been used in a number of previous studies in our laboratory (38–40) and is based on those described by Cole et al. (6) and Hannan and Hughes (20). The average number of arthritic joints per rat was also calculated for each group.

Statistical analysis. For comparison of data from multiple groups, one-factorial analysis of variance was used, followed by Fisher's protected least-significant-difference test or, for comparison of multiple treatment group means with a single control, the Dunnett *t* test. Differences were considered significant only for values of $P < 0.05$.

RESULTS

Assessment of *M. arthritidis* strains for DNA sequences hybridizing with MAV1. *Spe*I-digested DNA from 20 *M. arthritidis* strains was probed under conditions of high stringency with biotin-labeled DNA from bacteriophage MAV1 as described above (Fig. 1). Ten strains possessed sequences that hybridized with the probe, and ten did not. Strains possessing these sequences were 158p10, 158p10p9, 14124, 14124p10, JR3, 14124*, 14152, 14152p13, Jasmin, and ISR1. Negative strains were 19611, PG6, 13988, H606, Tru, 158, 23192, 14152*, O7, and H39. This experiment was repeated a number of times with several different DNA preparations from each strain; hybridization patterns of chromosomal DNA did not vary.

Occasionally, derivatives of MAV1-containing strains producing EC MAV1 were identified. In such cases, MAV1 was isolated in the genomic DNA preparations. However, EC MAV1 was easily distinguished from integrated copies, because the two *Spe*I fragments comigrated with fragments produced from purified MAV1 DNA and were normally at much higher molar proportions than the chromosomal bands. None of the MAV1-positive samples shown in Fig. 1 contained EC bacteriophage DNA, because no restriction fragments that comigrated with *Spe*I fragments of gel-purified MAV1 DNA were seen (Fig. 1), and no EC bands appeared on electrophoresis of uncut DNA (not shown). This conclusion is based partly on the supposition that restriction patterns of EC MAV1 DNA isolated from different sources and at different times do not vary. In fact, no variability in restriction patterns has been observed among samples of MAV1 DNA isolated from strains 158p10, 14124, and 14152 and digested with *Spe*I, *Ssp*I, *Eco*RI, and *Nde*I (not shown).

EC bacteriophage was produced occasionally by at least three of the MAV1-positive strains, 158p10, 14124, and 14152. Two of these, 158p10 and 14124, are shown in Fig. 2. On this gel, undigested DNAs from two preparations of strain 158p10 and two of strain 14124 were compared with DNA from MAV1-negative strain 158 and gel-purified MAV1 DNA; this DNA was then blotted onto a nylon membrane and hybridized with ^{32}P-labeled MAV1 DNA (Fig. 2B). Cultures of the two MAV1-positive strains were inoculated and incubated under identical conditions; one of each expressed EC MAV1, and the other did not. However, both contained MAV1-hybridizing sequences in their chromosomes (Fig. 2B), and hybridization patterns of *Spe*I-digested chromosomal DNA were identical (not shown). Throughout this study, we continued to see ap-

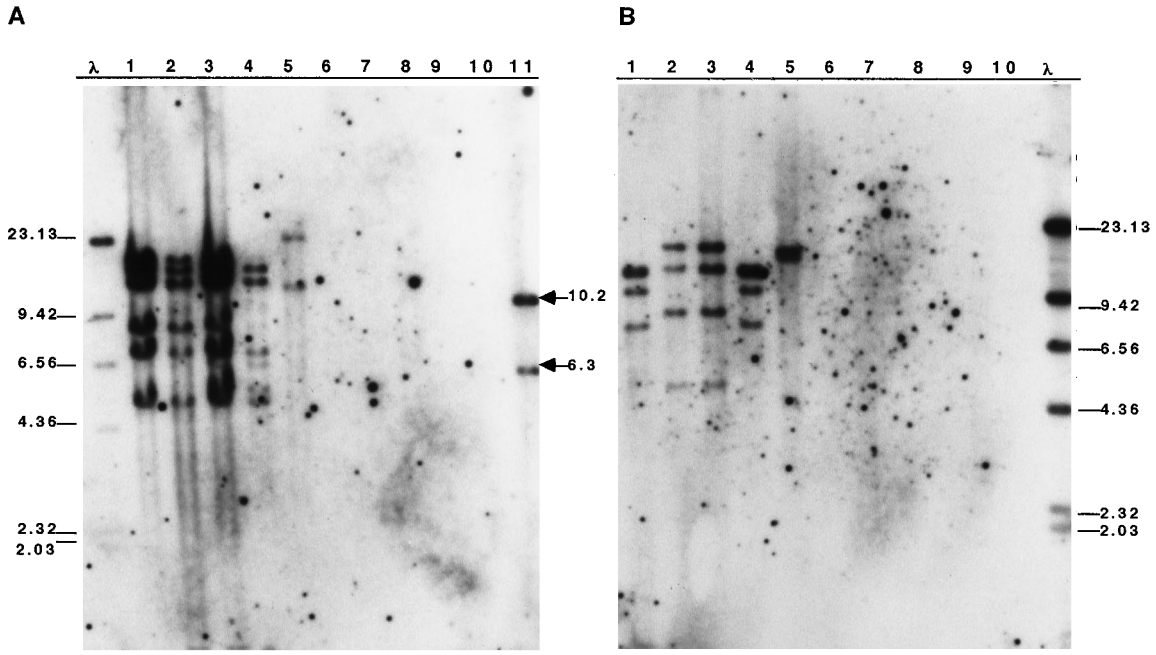

FIG. 1. DNA hybridization analysis of 20 *M. arthritidis* strains. Mycoplasmal DNA digested with *Spe*I was transferred to nylon membranes and probed with biotin-labeled MAV1 DNA. MAV1 DNA-hybridizing sequences were visualized by chemiluminescence. (A) Lanes: λ, biotinylated *Hin*dIII-digested lambda DNA; 1 to 10, DNA from strains 158p10, 158p10p9, 14124, 14124p10, JR3, 19611, PG6, 13988, H606, and Tru, respectively; 11, gel-purified MAV1 DNA. Size markers are indicated to the left in kilobases, and positions of the *Spe*I fragments of gel-purified MAV1 DNA are shown to the right. (B) Lanes: λ, biotinylated *Hin*dIII-digested lambda DNA; 1 to 10, DNA from strains 14124*, 14152, 14152p13, Jasmin, ISR1, 158, 23192, 14152*, O7, and H39, respectively. Size markers are indicated to the right in kilobases.

parently random changes in expression of EC MAV1 in strains 158p10, 14124, and 14152, but we never saw EC MAV1 DNA in the absence of integrated sequences. Similar expression of EC bacteriophage has not been seen in the other MAV1-positive strains, although these strains have not yet been thoroughly examined.

Since *Spe*I cuts the linear MAV1 genome once, into fragments migrating at 10.2 and 6.3 kb (Fig. 1A, lane 11) (34), the presence of two to six hybridizing fragments in chromosomal DNA from the positive strains (Fig. 1) suggests the integration of one to three complete or partial copies of the MAV1 genome into each chromosome. However, it is also consistent with the existence of single insertions located at different sites in chromosomes of individual members of a mixed population. To test this, single-colony-derived filter clones were prepared from MAV1-positive strains 158p10 and 158p10p9. DNA from three clones from each strain was digested with *Spe*I, blotted, and probed with MAV1 DNA. DNA from each clone showed the same six hybridizing fragments as the parent strains (not shown), indicating that individual members of these populations contained the same number of copies of the MAV1 genome integrated into the same sites.

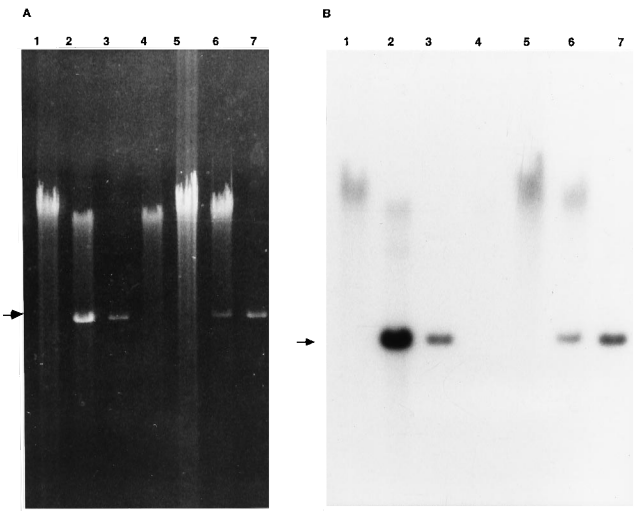

FIG. 2. Demonstration of EC MAV1 DNA. DNA prepared from two different samples of strain 158p10 (lanes 1 and 2), two samples of strain 14124 (lanes 5 and 6), one sample of strain 158 (lane 4), and EC MAV1 (lanes 3 and 7) was electrophoresed undigested, stained with ethidium bromide (A), and blotted to nylon membrane and hybridized with ^{32}P-labeled MAV1 DNA (B). Arrows indicate the position of the 16.5-kb EC MAV1 DNA genome.

Induction of arthritis by 20 *M. arthritidis* strains. Work in several laboratories, spanning several decades, has indicated that strains of *M. arthritidis* vary in pathogenicity (11). Strains used in this study that were previously reported to be capable of inducing arthritis in rats included 158p10, 14124p10, 14152p13 (18), 158p10p9 (8), Jasmin (also designated 14124) (25), ISR1 (27), and JR3 (32). According to Fig. 1, these strains all possessed MAV1 DNA-hybridizing sequences. Those showing low arthritogenic potential in earlier studies included 158, 14152* (also designated 23192), PG6 (also designated 19611), and H606 (also designated 13988) (18, 40), all of which were MAV1 negative. This finding suggested a possible correlation between the presence of MAV1 and virulence. However, it is difficult to make a statistically valid assessment of virulence differences among strains from these studies because of inconsistencies in methodology (dosages, rat strains, etc.) among laboratories. Therefore, these and additional strains in our collection that had not been previously tested were examined

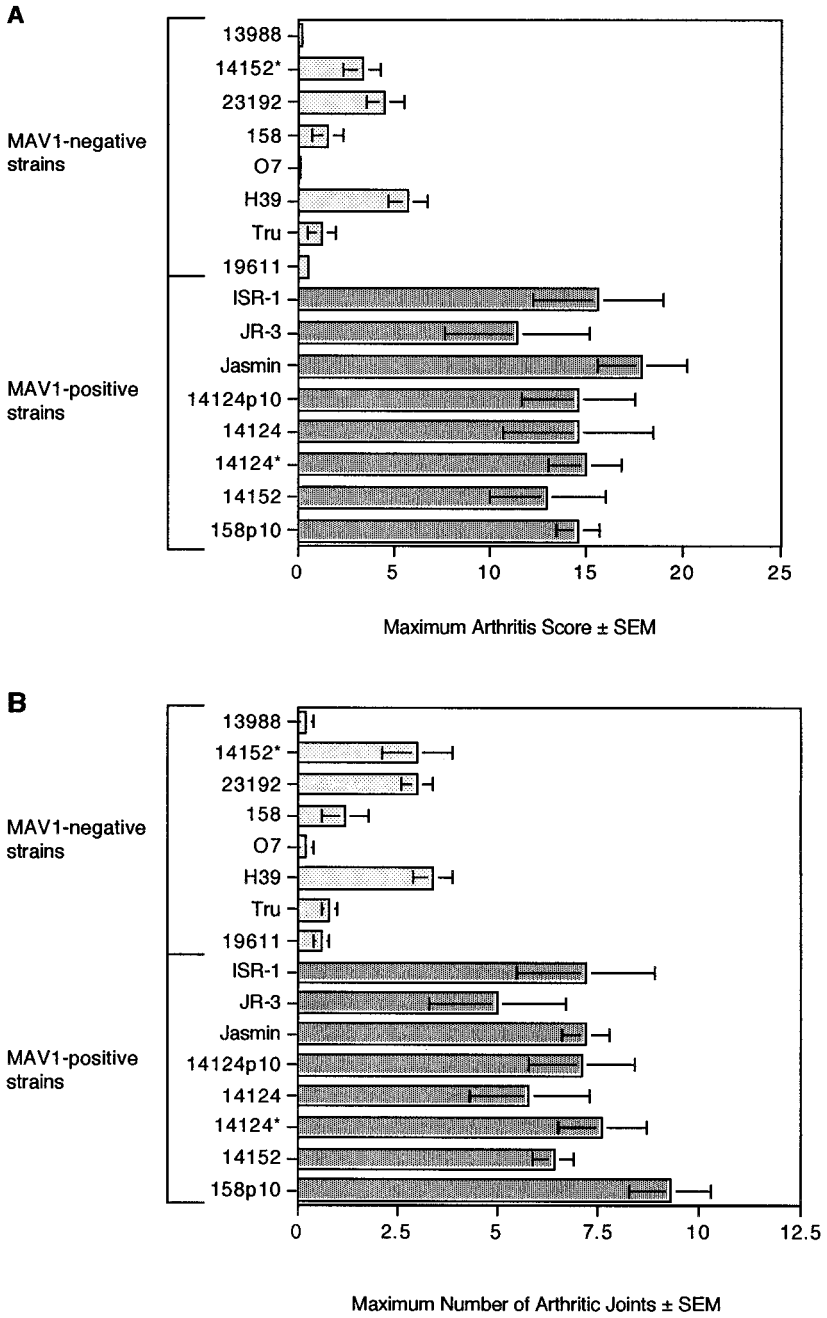

FIG. 3. Induction of arthritis in rats by 16 *M. arthritidis* strains. Maximum arthritis scores (A) and maximum numbers of affected joints per rat (B) were calculated for each group. Each point represents the mean of measurements taken from five to nine animals. Error bars represent standard errors of the means (SEM). Presence or absence of chromosomal sequences hybridizing with lysogenic bacteriophage MAV1 DNA is indicated on each panel.

under standardized conditions to provide data suitable for statistical analysis. In addition, it was important to ensure that assessments of virulence and DNA hybridization were performed on comparable samples of all of these strains, because complete in vitro passage histories from the time of the original studies were not always available, and changes occurring during the intervening decades, such as attenuation and/or loss (or acquisition) of MAV1, could not be ruled out.

Results from our laboratory with strains 158p10p9, 14152 p13, PG6, and H606 were published previously (40). In that study, arthritis scores induced by strains 158p10p9 and 14152 p13, both of which contained MAV1 DNA-hybridizing sequences (Fig. 1A, lane 2, and 1B, lane 3, respectively), were shown to be significantly greater than those induced by strains PG6 and H606, which did not (Fig. 1A, lanes 7 and 9, respectively) ($P < 0.05$). In addition, maximum numbers of affected joints per rat were also significantly higher for the MAV1-positive than the MAV1-negative strains (unpublished data; $P < 0.05$).

The other 16 strains in our collection were compared as part of the present study. Rats were observed over a 3-week period, and maximum arthritis scores and maximum numbers of ar-

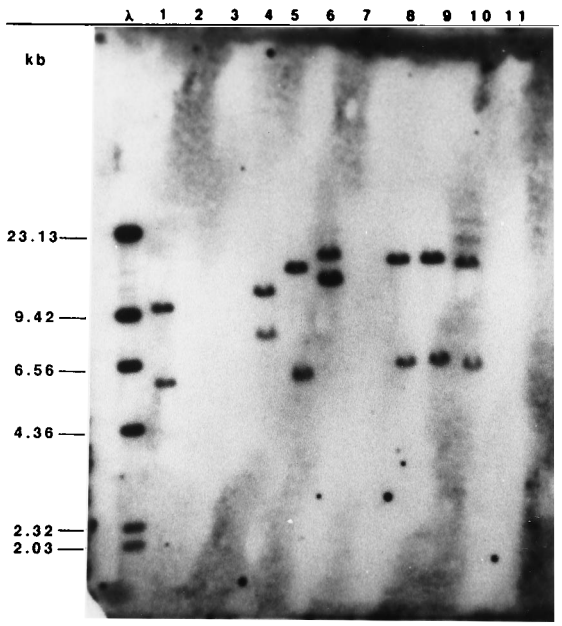

FIG. 4. DNA hybridization analysis of single-colony-derived cultures prepared by filter cloning from two preparations of 158 lysogenized with MAV1. Mycoplasmal DNA digested with *Spe*I was transferred to nylon membranes and probed with biotin-labeled MAV1 DNA. MAV1 DNA-hybridizing sequences were visualized by chemiluminescence. Lanes contain biotinylated lambda DNA digested with *Hin*dIII (lane λ), *Spe*I-digested gel-purified MAV1 DNA (lane 1), and *Spe*I-digested DNA from clones of 158L1 (lanes 2 to 5) and 158L3 (lanes 6 to 11). Lysogenized clones shown in lanes 4, 6, and 8, designated 158L1-3a, 158L3-1a, and 158L3-2a, respectively, were chosen for further study.

thritic joints per rat were calculated for each group (Fig. 3). With one exception (strain JR3 versus H39), maximum scores induced by MAV1-positive strains were significantly greater than those from MAV1-negative strains ($P < 0.05$). With two exceptions (strains JR3 and 14124 versus strains 14152*, 23192, and H39), the MAV1-positive strains also induced arthritis in a significantly greater number of joints than did the MAV1-negative strains. (While strains JR3 and 14124 induced arthritis in a greater number of joints than did strains 14152*, 23192, and H39, the differences were not statistically significant.) When data from all 20 groups of rats were examined, a statistically significant positive correlation was observed between the presence of MAV1 DNA-hybridizing sequences in chromosomal DNA and both maximum arthritis scores and maximum numbers of affected joints ($r = 0.707$, $P < 0.0001$).

Construction and analysis of MAV1 lysogens from strain 158. To confirm a possible association between the presence of MAV1 and a high-virulence phenotype, MAV1 lysogens were constructed from the low-virulence strain 158 in two separate experiments as described in Materials and Methods. Lysogenized cultures were designated 158L1 and 158L3. Several single-colony-derived cultures were prepared from 158L1 and 158L3 by filter cloning, and DNA from each clone was tested for the presence of MAV1 DNA-hybridizing sequences by Southern analysis (Fig. 4). Not all mycoplasmas in the original cultures were lysogenized, while those that were lysogenized displayed at least five different hybridization patterns. No extrachromosomal bands were seen on electrophoresis of undigested DNA from any of the MAV1-positive clones (not shown), nor did any DNA sample contain *Spe*I fragments that comigrated with those of gel-purified MAV1 DNA (Fig. 4), indicating that none of the cloned lysogens possessed EC

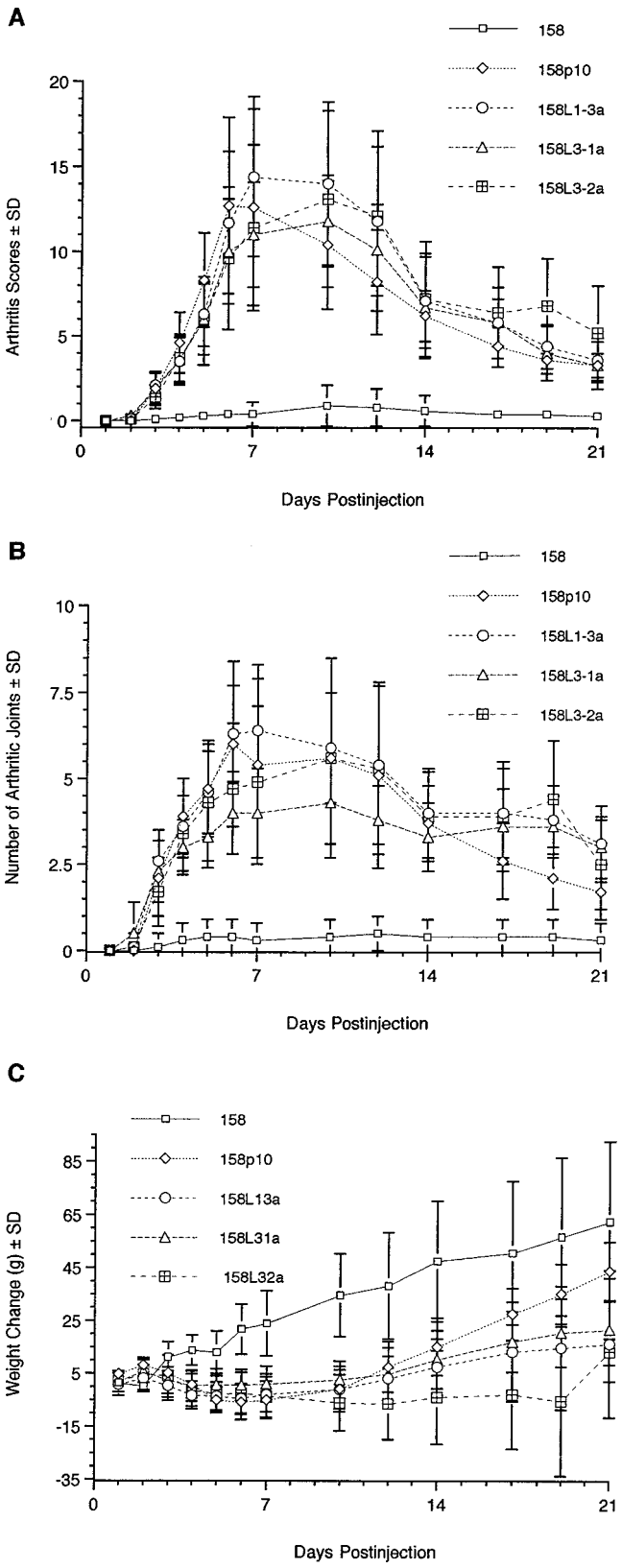

FIG. 5. Development of arthritis in rats injected with *M. arthritidis* strains 158 and 158p10 and with cloned MAV1 lysogens 158L1-3a, 158L3-1a, and 158L3-2a. Arthritis scores (A), numbers of arthritic joints per rat (B), and weight change (C) are shown over a 3-week period. Each point represents the mean of measurements taken from seven or eight rats ± standard deviation (SD).

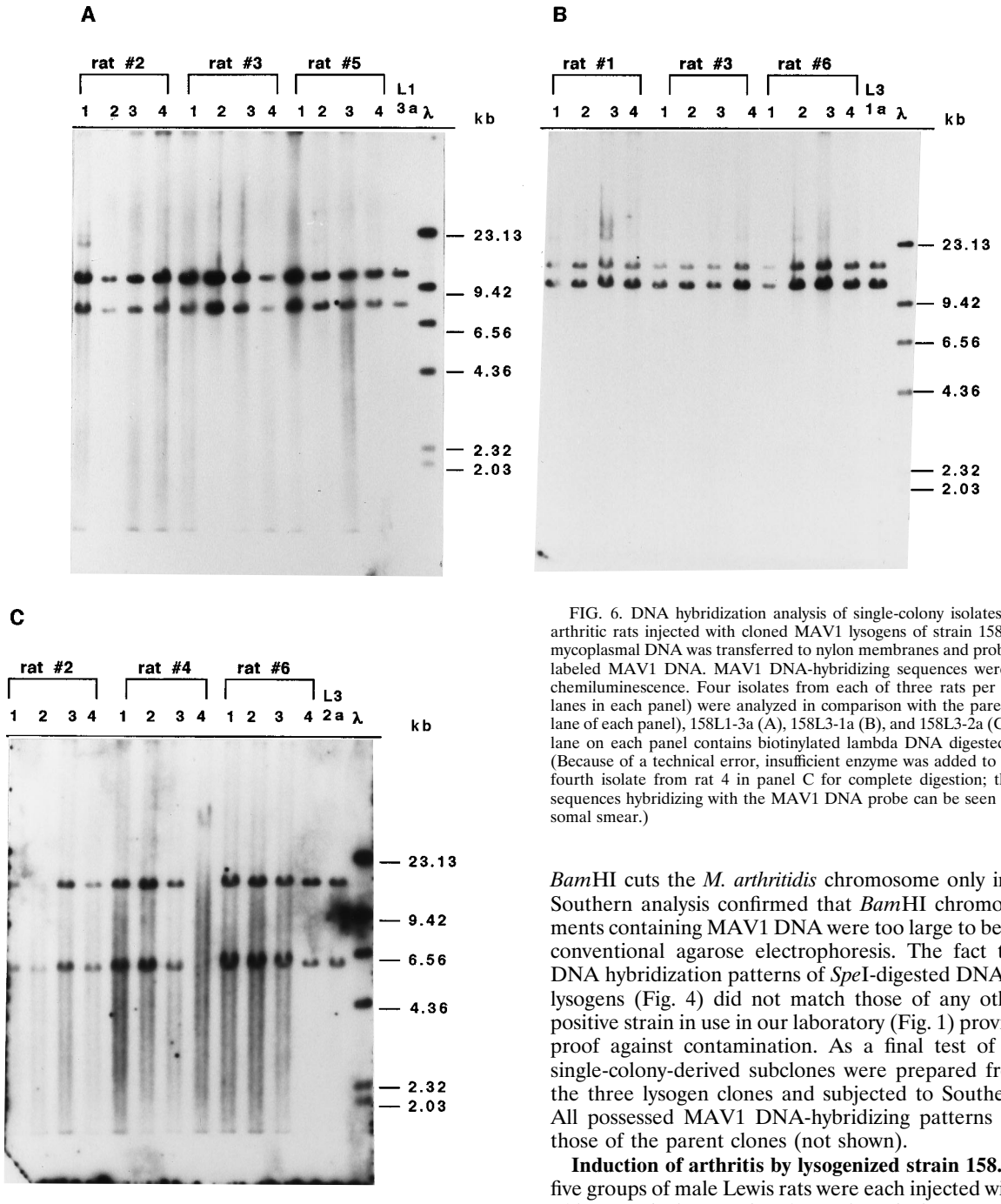

FIG. 6. DNA hybridization analysis of single-colony isolates cultured from arthritic rats injected with cloned MAV1 lysogens of strain 158. *Spe*I-digested mycoplasmal DNA was transferred to nylon membranes and probed with biotin-labeled MAV1 DNA. MAV1 DNA-hybridizing sequences were visualized by chemiluminescence. Four isolates from each of three rats per group (first 12 lanes in each panel) were analyzed in comparison with the parent clones (13th lane of each panel), 158L1-3a (A), 158L3-1a (B), and 158L3-2a (C). The far right lane on each panel contains biotinylated lambda DNA digested with *Hin*dIII. (Because of a technical error, insufficient enzyme was added to DNA from the fourth isolate from rat 4 in panel C for complete digestion; the presence of sequences hybridizing with the MAV1 DNA probe can be seen in the chromosomal smear.)

MAV1. Three clones, each showing a different MAV1 DNA hybridization pattern, were chosen for further study. These clones were designated 158L1-3a, 158L3-1a, and 158L3-2a (Fig. 4, lanes 4, 6, and 8, respectively).

Restriction analysis of *Bam*HI-digested chromosomal DNA confirmed that these three clones were identical to parent strain 158 and different from strain 158p10 (not shown), thus ruling out the possibility of contamination of the lysogenized cultures by the strain from which the phage stock was derived. Lysogenization of strain 158 might reasonably be expected to result in the appearance of novel *Bam*HI restriction fragments; however, no such fragments were seen. This is because the 16.5-kb MAV1 genome contains no *Bam*HI sites (33, 34), and *Bam*HI cuts the *M. arthritidis* chromosome only infrequently. Southern analysis confirmed that *Bam*HI chromosomal fragments containing MAV1 DNA were too large to be resolved by conventional agarose electrophoresis. The fact that MAV1 DNA hybridization patterns of *Spe*I-digested DNA from these lysogens (Fig. 4) did not match those of any other MAV1-positive strain in use in our laboratory (Fig. 1) provided further proof against contamination. As a final test of purity, five single-colony-derived subclones were prepared from each of the three lysogen clones and subjected to Southern analysis. All possessed MAV1 DNA-hybridizing patterns identical to those of the parent clones (not shown).

Induction of arthritis by lysogenized strain 158. Animals in five groups of male Lewis rats were each injected with 10^9 CFU of known virulent strain 158p10, low-virulence strain 158, and 158 lysogen clones 158L1-3a, 158L3-1a, and 158L3-2a. Rats were examined over a 3-week period. Development of arthritis and weight loss are shown in Fig. 5. Arthritis scores from the three groups injected with lysogenized 158 were equal to or greater than those from the 158p10-injected control group from days 3 through 21 (with the exception of day 5); numbers of affected joints showed a similar relationship from days 3 to 21 (with the exception of day 6), as did weight loss, from days 4 through 17. At all of these time points, values from the group injected with unlysogenized strain 158 were significantly less than those for the 158p10-injected controls ($P < 0.05$).

All rats were killed after 3 weeks of observation. Pus from arthritic joints of rats injected with the three 158 lysogens was

cultured on Edward agar. After 72 h, agar plugs containing single colonies were transferred to broth medium. DNA extracted from expanded cultures was digested with *Spe*I and probed with biotin-labeled MAV1 DNA by Southern analysis (Fig. 6). Controls consisted of DNA extracted from samples of the cultures used to inject these rats. Altogether, 12 isolates per group were examined (four isolates from each of three rats). All isolates hybridized with MAV1 DNA, and integration patterns were identical to those of the original inocula. None contained EC MAV1.

DISCUSSION

Work in our laboratory over the last decade has been directed at identifying a relationship between arthritogenicity and some structural or antigenic characteristic of the *M. arthritidis* cell. These attempts have revealed differences among *M. arthritidis* strains in antigenic specificity, surface protein patterns on one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and DNA restriction profiles (37, 40, 41). However, none of these correlated with the dramatic differences in virulence for rats that characterize the strains in our collection. Recently we identified and have begun to characterize a lysogenic bacteriophage from *M. arthritidis* designated MAV1. The MAV1 genome is approximately 16.5 kb in size and has been mapped (32a, 34). In the present investigation, we observed that MAV1 DNA hybridized under highly stringent conditions with chromosomal sequences from 10 highly arthritogenic strains but failed to hybridize with DNA from 10 additional strains capable of inducing no or only low-grade arthritis in rats. Further evidence for an association between MAV1 and virulence was obtained by demonstrating that the arthritogenic potential of the low-virulence strain 158 could be significantly enhanced by lysogenization with MAV1.

Hybridization analysis of DNA from filter-cloned progeny of strains 158p10 and 158p10p9 confirmed that MAV1 DNA could integrate into different sites in the chromosome and that multiple copies could exist in the same chromosome. Ability to integrate into different sites was further confirmed when broth cultures of strain 158 were exposed to MAV1, and single copies of the bacteriophage genome were shown to integrate into the chromosomes of different cells in at least five different sites. Numerous progeny were derived from these 158 lysogens by subcloning in vitro and by passage through rats; hybridization patterns did not vary among progeny derived from a single cell, and although only a limited number have been tested, it is interesting that none were negative. This finding suggests that once the MAV1 genome has integrated, it is not easily lost.

However, MAV1 does seem to be able to revert periodically to an EC existence, although the mechanism by which it does this and the stimuli that induce it are completely unknown. The growth conditions described in Materials and Methods only occasionally yielded EC bacteriophage. Preliminary work also suggests that differences in degree of aeration, exposure to UV light, length of incubation, temperature, and medium constituents all seem irrelevant to this process (32a). Additional studies are under way.

Data presently available give no indication as to the mechanism by which MAV1 enhances virulence. The fact that several MAV1-negative strains are not completely avirulent (Fig. 3 and reference 18) suggests that some of the necessary factors are already present in these strains. This in turn suggests that the virulence factor may not actually be present on the MAV1 genome; instead, the act of integration into certain preferred sites could have an activating or suppressive effect on a critical chromosomal element. However, this hypothesis cannot easily explain the fact that integration into each of at least three different sites in the 158 genome affected pathogenicity in exactly the same way. Other possibilities include the presence in MAV1 of genes for a *trans*-acting transcription control element or an antiphagocytic factor or toxin that increases invasive and/or survival potential. The *M. arthritidis* superantigen MAM is an interesting candidate, since several other bacterial superantigens are known to be encoded by mobile genetic elements (24). However, MAV1-negative strain PG6 has been used by Cole et al. as a source of purified MAM (4), and further analysis has shown that there is no correlation between MAM production and the presence of MAV1 DNA-hybridizing sequences in the *M. arthritidis* chromosome (3a). Cytadherence mechanisms are also classified as virulence factors for many bacterial pathogens. We have identified two surface-exposed putative adhesins (39), neither of which is expressed by all 20 strains (41). However, the presence of MAV1 DNA-hybridizing sequences is also unrelated to expression of either of these adhesins (35a). Additional molecular characterization of MAV1 may help resolve the question of its precise mode of action.

Although the mode of action is not yet known, this is the first report of a bacteriophage-associated virulence factor within the class *Mollicutes*. The other *Mycoplasma* species from which bacteriophages have been isolated are also pathogenic for their natural hosts, and two of the three are arthritogenic. The occurrence of EC elements encoding virulence factors, as with other eubacteria, may be more common within the genus *Mycoplasma* than is currently appreciated.

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