Isolation and Restriction Endonuclease Cleavage of Anaplasma marginale DNA In Situ in Agarose

CHARLES M. KRUEGER AND GERALD M. BUENING*

Department of Veterinary Microbiology, College of Veterinary Medicine, University of Missouri-Columbia, Columbia, Missouri 65211

Received 9 November 1987/Accepted 9 February 1988

Bacterial restriction endonucleases were used to produce DNA cleavage patterns that could be useful as tools to study the relatedness among Anaplasma marginale isolates. Bovine erythrocytes infected with A. marginale were lysed, washed, and embedded in agarose. The embedded erythrocytes and bacterial pathogens were partially digested by sequential infiltration of the agarose with acetone, lysozyme, sodium dodecyl sulfate, and proteinase K. The unfragmented genomic DNA was left supported and protected in a porous matrix. The DNA was digested in situ in agarose under the following conditions: (i) brief treatment with phenol, (ii) brief washing with distilled water, and (iii) adjustment of restriction enzyme digestion mixture to compensate for the volume of the agarose. The cleaved DNA was electrophoresed horizontally to produce a DNA cleavage pattern. Of 19 restriction enzymes screened, 12 produced distinct DNA bands from the genomes of each of the five A. marginale isolates examined. The DNA cleavage pattern produced from each isolate with a given restriction enzyme was reproducible. However, the DNA cleavage patterns produced from different isolates with a given restriction enzyme were not necessarily identical. This procedure could be modified for general bacterial DNA isolation, in situ agarose digestion, and manipulations.

Anaplasma marginale is an obligately intracellular bacterial pathogen of bovine erythrocytes from the order Rickettsiales (11). A. marginale causes anaplasmosis, a significant, worldwide (20), hemolytic disease of cattle. Many isolates of A. marginale exhibit differences in morphology (9), surface antigens (18), protein structure (1), vector specificity (27), and virulence (10). Knowledge of the relatedness among the various isolates of A. marginale has importance in taxonomic, epidemiological, virulence, and vaccine development studies.

DNA cleavage patterns produced by bacterial restriction endonucleases have been used to differentiate phenotypically closely related bacteria. Such patterns have been used to study various species, strains, serovars, and isolates of Mycoplasma (19, 23, 24), Coxiella (21), Chlamydia (4, 22), Neisseria (3, 12), Streptococcus (25), Campylobacter (7), Escherichia (17), Mycobacterium (5, 6), and Leptospira (13, 28). Recently, a reclassification of leptospiral isolates belonging to several serogroups has been proposed (28) on the basis of DNA cleavage pattern analysis.

Genomic DNA cleavage patterns could be useful as additional tools for the comparison of A. marginale isolates. We have developed a procedure for the isolation and digestion of A. marginale DNA in situ in agarose. The procedure produced potentially useful DNA cleavage patterns from the genomes of five Anaplasma isolates with each of 12 restriction endonucleases.

MATERIALS AND METHODS

Parasitized bovine erythrocytes. We obtained samples of citrated or heparinized whole bovine blood which had 58 to 70% of the erythrocytes parasitized with A. marginale. The St. Croix and Virginia isolates were provided by Lucious Chieves (National Veterinary Service Laboratory, Animal Plant and Health Inspection Service, U.S. Department of Agriculture, Ames, Iowa). The Oklahoma and the Louisiana isolates were provided by Lloyd Chavez (Fort Dodge Laboratories, Fort Dodge, Iowa), and the Florida isolate was provided by Travis McGuire (Department of Veterinary Microbiology and Pathology, Washington State University, Pullman).

Isolation of Anaplasma DNA in situ in agarose. Samples of citrated or heparinized infected whole bovine blood were centrifuged at 1,800 × g for 20 min. The supernatant, buffy coat, and top layer of erythrocytes were discarded to remove the bulk of the leukocytes. The remaining erythrocytes were washed three additional times at 1,800 × g for 10 min with Vega y Martinez solution (VYMs) (29). VYMs is a modified phosphate-buffered saline (PBS)-glucose solution. After each centrifugation, the supernatant and top layer of erythrocytes were discarded to remove additional leukocytes. The erythrocytes in VYMs were filtered through cellulose to remove some of the remaining leukocytes and then washed with VYMs to remove any cellulose contamination. The remaining intact leukocytes were separated from the infected erythrocytes by centrifugation through a 70% Percoll (Pharmacia Fine Chemicals, Piscataway, N.J. [Div. Pharmacia, Inc.]) gradient at 1,800 × g for 30 min. The erythrocytes were washed with VYMs to remove Percoll and then pelleted at 1,800 × g for 10 min. The pellet of erythrocytes was frozen for a minimum of 24 h as 5-ml portions in 50-ml centrifuge tubes.

A 5-ml portion was then thawed on ice after the addition of 45 ml of ice-cold PBS that contained 0.1 mM EDTA (PBS-EDTA). The solution was centrifuged at 1,300 × g for 15 min to pellet any remaining leukocyte fragments while leaving in suspension the initial bodies (individual A. marginale bacteria) with pieces of attached erythrocyte membranes. The top 40 ml of the supernatant was carefully transferred to another 50-ml centrifuge tube. The recovered supernatant was increased to its original volume by the addition of PBS-EDTA and thoroughly mixed. This centrifugation step was repeated three more times.

The top 40 ml of the supernatant was then carefully
transferred to a 40-ml superspeed centrifuge tube. The recovered supernatant was centrifuged at 20,200 \times g for 10 min to pellet the initial bodies and erythrocyte membranes. To remove hemoglobin and any free leukocyte DNA, the pellet was washed four times by resuspension in 40 ml of PBS-EDTA followed by centrifugation at 20,200 \times g for 10 min.

The pellet was thoroughly mixed by trituration with a pipette. Then, 750 \mu l of the suspension was transferred to a 1.5-ml microcentrifuge tube, and the tube was placed in a 45°C water bath. After 10 min, an equal volume of 0.8% agarose in TBE (0.089 M Tris, 0.089 M borate, 0.002 M EDTA) that had been melted and cooled to 45°C was added to the cell suspension. The solution was thoroughly mixed and immediately pipetted onto the surface of a piece of laboratory labeling tape (13 mm by 10 cm; American Scientific Products, McGraw Park, Ill.) and allowed to cool.

After the agarose had hardened into a rectangular slab approximately 1 mm thick, the thin tapered edges were removed with a scalpel and discarded. The central portion of the slab with relatively uniform thickness was then cut into plugs (1 by 5 by 5 mm). The agarose plugs were transferred to a standard 20-ml glass scintillation vial (Fisher Scientific Co., Pittsburgh, Pa.) containing 9 ml of ice-cold acetone and placed on ice for 10 min. The agarose plugs were then washed five times with 9-ml portions of TBE to remove any acetone and left in a final 9-ml volume of TBE. Lysozyme (9 mg) was added to TBE, and the agarose plugs were incubated for 60 min at 37°C. Then, 1 ml of 10% sodium dodecyl sulfate was added to the contents of the vial, and the plugs were again incubated for 60 min at 37°C. TBE (9 ml) was added to the vial to increase the volume to 19 ml. Then, 1 mg of proteinase K was added to the contents of the vial, followed by a 30-min incubation at room temperature. The solution was removed from the plugs, and the plugs were washed 10 times with 2-ml portions of TBE. The TBE was removed from the plugs and replaced with 2 ml of phenol saturated with 0.1 M TE (10 mM Tris hydrochloride, 1 mM EDTA; pH 8.0). The plugs sat in phenol without agitation at room temperature for 30 min. The plugs were then washed repeatedly with 2-ml portions of TBE until all discoloration of the agarose plugs by the phenol was gone. Enough TBE was added to cover the plugs, and they were stored at 4°C.

**Determination of DNA fragmentation.** Six agarose plugs were inserted into the central wells of an eight-well minigel (H33 "Minnie" Horizontal Submarine Agarose Gel Unit, Hoeffer Scientific Instruments, San Francisco, Calif.). The minigel was then lowered into TBE, and HindIII-cleaved lambda DNA was pipetted into the two end wells. The DNA was electrophoresed (150 V, 60 min) from the plugs into the minigel agarose.

**Determination of leukocyte DNA contamination.** Eight agarose plugs were inserted into the wells of a minigel, and the DNA was electrophoresed (150 V, 60 min) into the minigel agarose. The DNA was viewed with UV light. A single agarose strip, containing eight discrete bands of DNA (one from each plug), was cut out. The agarose strip containing the DNA bands was placed in a dialysis bag filled with TBE. The DNA was electroeluted from the agarose strip into TBE (14). The DNA solution recovered from the dialysis bag was reduced in volume with microconcentrators (Centricon-30; Amicon Corp., Lexington, Mass.). The DNA was also washed in the microconcentrators according to manufacturer suggestions. A total of 40 \mu l of Anaplasma DNA (0.625 \mu g/\mu l) was recovered.

Citrated whole blood from a healthy bovine was centrifuged at 1,800 \times g for 20 min. The supernatant was removed and discarded. The buffy coat was then recovered and washed twice with ice-cold PBS. Bovine DNA (0.416 \mu g/\mu l) was isolated (15) from the pelleted leukocytes.

Denatured samples of the recovered Anaplasma DNA (0.625, 1.250, and 2.500 \mu g) and bovine genomic leukocyte DNA (0.416, 0.832, and 1.664 \mu g) were bound to a nitrocellulose filter by using a slot blot apparatus (Hybri-Slot; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and fixed to the filter by baking for 2 h at 80°C in a vacuum oven. The nitrocellulose filter was hybridized with a 32P-labeled leukocyte genomic DNA probe (nick translation kit; Bethesda Research Laboratories) overnight at 65°C, washed under medium-stringency conditions, air dried, and autoradiographed by the methods of Maniatis et al. (16), except that formamide was not used.

Bovine leukocyte DNA was cleaved with the 12 restriction enzymes chosen for use with the five A. marginale isolates. The cleaved bovine DNA was then electrophoresed and photographed under conditions identical to those used for the isolated Anaplasma DNA.

**Cleavage of DNA in situ in agarose.** A total of 19 restriction enzymes (AatII, Aval, BamHI, BglII, BglIII, BsiEll, EcoRI, EcoRV, HincII, HindIII, NotI, PstI, PvuII, SalI, SfiI, SmaI, SpII, SrtI, and TthIII) were screened for their ability to produce useful cleavage patterns of A. marginale genomic DNA.

The plugs containing genomic A. marginale DNA were cleaved with the enzyme used, depending on the enzyme used, to 10,000 base pairs. Anaplasma DNA isolated in agarose was washed five times with 1 ml of distilled water and then digested overnight at 37°C in 200-\mu l reaction mixtures which contained 14 to 20 U of a bacterial restriction enzyme (Bethesda Research Laboratories). The reaction conditions were those recommended by the manufacturer, except that the amount of water in the reaction mixture was reduced by 25 \mu l to adjust for the volume of the agarose plug.

After digestion, the plugs were inserted into the wells of agarose gels (4 by 20 by 25 cm). The gels consisted of 0.4% agarose in TBE with 0.5 \mu g of ethidium bromide per ml. The samples were electrophoresed horizontally at 50 V (1.4 V/cm) for 18 h at room temperature in TBE buffer which contained 0.5 \mu g of ethidium bromide per ml. The gels were photographed by using 300-nm UV light and a red gelatin filter (no. 23A; Eastman Kodak Co., Rochester, N.Y.).

**RESULTS**

**Determination of DNA fragmentation.** When electrophoresed, Anaplasma DNA isolated in situ in agarose formed a discrete band that migrated slightly above the 23,000-base-pair marker of lambda DNA cleaved by HindIII (data not shown).

**Determination of leukocyte DNA contamination.** A 32P-labeled bovine leukocyte genomic DNA probe did not hybridize with Anaplasma DNA that had been isolated in situ and then electroeluted, but it did hybridize with bovine leukocyte DNA controls (data not shown).

Bovine leukocyte DNA cleavage patterns were obtained for comparison with the Anaplasma DNA cleavage patterns (data not shown). As expected, because of the large size of the bovine genome, digestion by the restriction enzymes produced a long thick smear of DNA. In some cases, a fragment population was produced that was large enough to be seen as a band within the background smear. Depending on the enzyme used, 1 to 10 bands could be seen.

**Cleavage of DNA in situ in agarose.** A total of 19 bacterial restriction endonuclease enzymes were screened for their
ability to produce useful cleavage patterns from isolated Anaplasma DNA (data not shown). The cleavage patterns produced by AarI, Smal, SpiI, and SstII (six-base recognition sites); NotI and SfiI (eight-base recognition sites); and TliIII (nine-base recognition site) were unsatisfactory because AarI, Smal, SpiI, SstII, and TliIII produced high background smears, while NotI and SfiI cleaved the Anaplasma genome into very few fragments. The rest of the restriction enzymes examined (six-base recognition sites) produced useful cleavage patterns. These enzymes were chosen to cleave the DNA of five A. marginale isolates (Florida, Louisiana, Oklahoma, St. Croix, and Virginia) (Fig. 1 to 3). Under the conditions described, these enzymes totally digested the Anaplasma genome into DNA fragment populations that could be separated into distinct bands by electrophoresis.

DISCUSSION

Recently, Smith et al. (26) have reported successes in the preparation of unbroken Escherichia coli by embedding cells in agarose and in the digestion of the DNA in situ in agarose with restriction enzymes. We isolated pure unfragmented A. marginale DNA in situ in agarose. The organism, embedded in agarose, was partially digested. This left the genomic DNA supported and protected in a porous matrix. It was not possible to use standard methods to quantify the amount of DNA isolated, since removal of the DNA from the agarose resulted in a simultaneous loss of DNA. However, our gel electrophoresis results qualitatively suggested that we recovered a larger amount of Anaplasma DNA from the same amount of starting material with our method than we did with extraction precipitation techniques (13).

The DNA supported in the porous matrix could be digested by restriction enzymes under the following conditions: (i) brief treatment with phenol, (ii) brief washing with distilled water, and (iii) adjustment of the restriction enzyme digestion mixture to compensate for the volume of the agarose plug. The DNA was digested in situ in three different lots of agarose (ultrapure; Bethesda Research Laboratories), and the digestions were successful only under the above-described conditions. It is possible that phenol destroys protease activity and that distilled water removes EDTA.

A. marginale cannot be cultured in vitro except for very short periods and cannot be grown in the usual laboratory animals (8). Since A. marginale must be isolated from its normal bovine host, it is difficult to obtain Anaplasma DNA that is not contaminated with bovine leukocyte DNA. This procedure yielded DNA that did not hybridize with bovine leukocyte DNA. Also, if the A. marginale DNA had been contaminated with bovine leukocyte DNA, then its restriction endonuclease cleavage pattern would have been superimposed on the bovine leukocyte DNA smear produced by that same restriction enzyme. This was not the case (Fig. 1 to 3), and the few bands distinguishable in the bovine leukocyte smears were absent from the A. marginale DNA cleavage patterns.

The lack of background smears in the DNA cleavage patterns demonstrated the absence of at least three undesirable conditions: (i) contamination with a high-molecular-weight DNA component that had many recognition sites for
FIG. 3. Genomic DNA cleavage patterns obtained from each of five *A. marginale* isolates after digestion with either *PstI*, *BstEII*, *PvuII*, or *SalI*. Sample treatment and the order of isolates for each enzyme are as described in the legend to Fig. 1. Lanes: A to E, *PstI*; F to J, *BstEII*; K to O, *PvuII*; P to T, *SalI*.

...the restriction enzyme used, (ii) fragmentation of the DNA during isolation and subsequent manipulations, and (iii) too many recognition sites in the subject DNA for the restriction enzyme used.

Bear and Philpott (2) examined restriction endonuclease cleavage patterns obtained from the Wyoming isolate of *A. marginale*. The *A. marginale* DNA isolated does not hybridize with calf thymus DNA, indicating a high state of purity. They found that most of the restriction enzymes they examined produced blurred, indistinct patterns after digestion and electrophoresis. Specifically, good DNA cleavage patterns are not obtained from the Wyoming isolate with *HindIII*, *BamHI*, *SalI*, *KpnI*, and *PstI* (2). These enzymes incompletely digest the DNA and produce DNA cleavage patterns with bands that are indistinct (2). Good restriction endonuclease cleavage patterns are obtained from the Wyoming isolate with *EcoRI* (2). In agreement with Bear and Philpott, we also obtained good restriction cleavage patterns from all five of the isolates we examined with *EcoRI* (Fig. 2).

We have produced potentially useful DNA cleavage patterns from five *Anaplasma* isolates with each of twelve restriction endonucleases. Preliminary examination of five *A. marginale* isolates indicated that different isolates did not have identical patterns. This procedure could be useful as an additional tool for the comparison of *A. marginale* isolates. This procedure could be modified for general bacterial DNA isolation, in situ agarose digestion, and manipulations.

**LITERATURE CITED**


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