Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis

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Anaplasma marginale is a rickettsial parasite of bovine erythrocytes causing world-wide economic losses in livestock production. Despite its importance, little is known about this rickettsia at a molecular level because it has not been cultured *in vitro*, and there is no small-animal model. Although several genes have been cloned and sequenced, the gross genome structure of the organism has not yet been well characterized. We separated intact bovine erythrocytes from leucocytes, and determined the genome size of *A. marginale* by use of restriction endonuclease cleavage and pulsed-field gel electrophoresis (PFGE). A value of 56 mol% G + C was obtained for this genome by spectral analysis. Undigested *A. marginale* DNA failed to migrate under several different electrophoretic conditions, indicating a circular genome. Digestions of intact *A. marginale* DNA were performed using restriction endonucleases *NotI*, *SfiI* and *PacI*. Complete digestion with *SfiI* resulted in 12 distinct bands ranging in size from 14 to 170 kbp. Total size determined by addition of *SfiI*-digested fragments was approximately 1200 kbp. *PacI* cleaved the *A. marginale* genome from three different isolates into just three fragments, of 598, 557 and 97 kbp. Incomplete digestion produced a band measuring 1250 kbp. These results indicate that *A. marginale* has a circular genome between 1200 and 1260 kbp, with a G + C content of 56 mol%.

Introduction

Anaplasma marginale is an obligate, intraerythrocytic parasite of the order Rickettsiales, family *Anaplasmataceae*, which is transmitted biologically by ticks (Jain, 1986). It is the aetiological agent of anaplasmosis, a disease of cattle characterized clinically by fever, marked haemolytic anaemia, haemoglobinaemia, icterus, weight loss, abortion, and often death. The prepatent period is 3 to 6 weeks, after which time the number of infected erythrocytes increases dramatically over the next 4–9 d. At peak parasitaemia, *A. marginale* initial bodies may be detected in up to 70% of the circulating erythrocytes. During this period, some cattle die. Those surviving the initial parasitaemia recover, but remain persistently infected carriers, with a small number having recurrent clinical disease.

Anaplasmosis is a disease of world-wide economic significance to the cattle industry due to mortalities,

abortions, weight loss, and veterinary and management costs. It is one of the major cattle diseases in many countries, including the United States. Despite the significant losses encountered each year, an effective vaccine has not been developed. The vaccines currently in use have disadvantages including the necessity for preparation from infected animals, neonatal isoerythrolysis in calves nursing vaccinated dams, and the potential for spread of other pathogens such as bovine leukaemia virus (Palmer, 1989). It is likely that optimal quality control, inexpensive production, and world-wide delivery of anaplasmosis vaccines in the future will be best achieved by the development and application of recombinant DNA methods.

To date, several genes for surface proteins of *A. marginale* have been cloned, sequenced, and expressed (Allred *et al.*, 1990; Barbet & Allred, 1991; Barbet *et al.*, 1987; S. M. Oberle & A. F. Barbet, unpublished results). The data have revealed that these genes may be quite polymorphic between isolates and can exist in multi-gene families. The structures of identified promotor regions are similar to the consensus *Escherichia coli* sequences.

Despite the availability of cloned genes, the gross structure and organization of the *A. marginale* genome is not well defined. There is a discrepancy in the literature

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Abbreviations: CHEF, clamped homogeneous electric field; PCV, packed cell volume; PFGE, pulsed-field gel electrophoresis.

concerning the mol% G+C content of the organism (Bear & Philpott, 1987; Normore, 1975; Weiss & Moulder, 1984), and the previously reported genome size of *A. marginale* (Bear & Philpott, 1987) is significantly smaller than values reported for other rickettsial agents (Frutos *et al.*, 1989; Kingsbury, 1969).

The objective of this study was to determine the size and mol% G+C content of the *A. marginale* genome. In addition, an analysis of restriction enzyme polymorphism between Florida, Virginia, and South Idaho isolates was performed. This information provides a framework for future studies on the organization of the *A. marginale* genome.

Methods

Erythrocyte preparation. Whole blood was initially collected from a splenectomized calf experimentally infected with a Florida isolate of *A. marginale*. Similarly, blood was later collected from calves infected with South Idaho and Virginia isolates. The blood was obtained during a period when approximately 50% of the circulating erythrocytes were infected. Sodium heparin was used as an anticoagulant at a concentration of 10 units per ml of blood. Erythrocytes were separated from bovine leucocytes by passing washed blood over an α -cellulose/microcrystalline cellulose column (Sigmacell type 50, Sigma) as described by Beutler (1984), except that 1% (w/v) bovine serum albumin (BSA) was included in the wash buffer. Washed cells were resuspended to a concentration of 1.0×10^7 cells μl^{-1} , or approximately 75% packed cell volume (PCV). Blood films were prepared from isolated erythrocytes for microscopic detection of bovine leucocyte contamination and level of parasitaemia.

Intact erythrocytes were embedded in 0.7% agarose by mixing 1 part erythrocyte suspension with 2 parts of 1% (w/v) FMC InCert Agarose (FMC Bioproducts) in PBS (0.14 M-NaCl, 2.7 mM-KCl, 1.8 mM-KH₂PO₄, 10.0 mM-Na₂HPO₄, pH 7.4), 0.125 M-EDTA. In initial experiments, blood was used undiluted (75% PCV), or diluted 1/2 (37.5% PCV) or 1/3 (25% PCV) in PBS prior to embedding in agarose. The 1/2 dilution proved optimal for manipulation of plugs and visualization of ethidium bromide stained bands on clamped homogeneous electric field (CHEF) gels. This concentration was used in all further experiments. Mixtures of blood and agarose were kept at 37 °C while pipetting into moulds. Moulds containing plugs were placed on ice to set. Plugs containing PBS-washed, infected blood, without cellulose column purification were also embedded in agarose plugs to be used as a positive control for probing with bovine DNA. Plugs were incubated in 0.5 M-EDTA (pH 9.5), 1% (w/v) *N*-lauroyl-sarcosine, and 2 mg Proteinase K ml^{-1} for 48 h at 37 °C, then stored at 4 °C in fresh Proteinase K solution.

Restriction endonuclease digestion. Plugs containing intact *A. marginale* DNA from the cellulose-isolated erythrocytes were digested with restriction endonucleases *Sfi*I (Promega), *Not*I (Promega), *Sse*I (TaKaRa Biochemical), *Sgr*AI (Boehringer Mannheim) and *Pac*I (New England Biolabs). *Sfi*I, *Not*I and *Pac*I consistently produced band patterns yielding the most information. Plugs were first washed in 5 times their volume of TE (10 mM-Tris, pH 8.0, 0.1 mM-EDTA)/1.0 mM-phenylmethylsulphonyl fluoride (PMSF) for 2 h at 37 °C, and then incubated in fresh TE/PMSF at 37 °C overnight. After overnight incubation, plugs were washed twice in 50 mM-EDTA, pH 8.0, for ≥ 8 h, twice for 2 h each in TE buffer, and finally equilibrated on ice in 2 vols $1 \times$ restriction enzyme buffer plus 0.1 mg BSA ml^{-1} . A solution of 2 μl of diluted restriction enzyme (in $1 \times$ restriction enzyme buffer)

was added and DNA digestions performed according to the manufacturer's instructions. Reactions were stopped by the addition of 0.25 total reaction volume of 0.5 M EDTA/0.5% *N*-lauroylsarcosine/5 mg proteinase K ml^{-1} .

CHEF gel electrophoresis. This was done on the CHEF-DRII system (Bio-Rad). Plugs of digested or undigested DNA were electrophoresed in 1% agarose in 0.5 $\times$ TBE (0.045 M Tris/0.045 M Borate/1 mM-EDTA) buffer at 14 °C. Voltage, pulse rate and run times were varied for optimal separation of fragments. *Saccharomyces cerevisiae* (Bio-Rad), Lambda Ladder (Bio-Rad), Delta 39 Promega Markers (Promega) and Lambda DNA/*Hind*III Fragments (GIBCO BRL) were used as size standards. *Staphylococcus aureus* DNA (Promega) and *Moraxella bovis* DNA (Promega) were used as controls for restriction endonuclease activity.

Laser densitometry. Gels were stained with ethidium bromide and photographed as described elsewhere (Carle & Olson, 1984). The size of each of the band fragments of *A. marginale* produced by restriction endonuclease digestion was determined by comparison with known size standards. Photographic negatives of gels containing *Sfi*I-digested DNA were scanned with a laser densitometer (Computing Densitometer, model 300A, Molecular Dynamics) to determine if more than one fragment of *A. marginale* DNA could be migrating within a single band. Unless co-migration of two or more different fragments occurred, the densitometry reading of bands should be directly proportional to the fragment length. Results were calculated by comparing the percentage of total surface area under each peak (band density) with that which would be expected solely from difference in fragment length of the bands.

Southern blots and hybridization. Gels were depurinated for 15 min in 0.25 M-HCl, washed for 2×15 min in 0.4 M-NaOH/0.6 M-NaCl, and for 2×15 min in 1.5 M-NaCl/0.5 M-Tris-HCl (pH 7.5), then transferred to GeneScreen Plus membrane (DuPont) in $10 \times$ SSC (1.5 M-NaCl, 0.15 M-sodium citrate). After transfer, membranes were rinsed in 0.4 M-NaOH for 60 s, then in 0.2 M-Tris-HCl (pH 7.5)/ $2 \times$ SSC, and exposed to UV light to bind the DNA to the membrane.

Nick-translated probes were prepared from [α -³²P]dCTP (6000 Ci mmol^{-1} , 20 mCi ml^{-1} [1 Ci = 37 GBq], DuPont) labelled calf thymus or genomic *A. marginale* DNA. The genomic *A. marginale* DNA was prepared from a Florida isolate and purified by methods previously described (Barbet *et al.*, 1987). Blots were prehybridized for several hours and hybridized in 0.18 M-Na₂HPO₄ and 0.07 M-NaH₂PO₄ (pH 7.2), 0.25 M-NaCl, 1.0 mM-EDTA, 7.0% (w/v) SDS, 50% (v/v) deionized formamide/dextran sulphate solution (composed of 20% dextran sulphate in deionized formamide), and 0.1 mg herring sperm DNA ml^{-1} at 37 °C overnight. Probed blots were washed at room temperature for 2×15 min in $2 \times$ SSC/0.1% SDS, for 1×15 min in $0.5 \times$ SSC/0.1% SDS, for 1×15 min in $0.1 \times$ SSC/0.1% SDS, and for 3×15 min at 60 °C in $0.1 \times$ SSC/1.0% SDS, then rinsed in $0.1 \times$ SSC. Areas of hybridization were visualized by autoradiography.

G+C content. DNA from a Florida isolate of *A. marginale* was purified as previously described (Barbet *et al.*, 1987) from cellulose-isolated erythrocytes. The mol% of guanine plus cytosine was determined by spectral analysis (Beckman DU-64) of native DNA according to the method described by Hirschman & Felsenfeld (1966). *E. coli* strain B DNA ('ultrapure', Sigma) was included as a control.

Results and Discussion

Blood films prepared from PBS-washed, unpurified erythrocyte preparations contained low numbers of bovine leucocytes and platelets. The cellulose-isolated

Fig. 1. Undigested DNA from a Florida isolate of *A. marginale* separated by CHEF gel electrophoresis. P1, P1/2, P1/3, positive control of unpurified erythrocytes embedded into plugs at full concentration (100% PCV), 1/2 and 1/3 dilution. C1, C1/2, C1/3, erythrocytes isolated on a cellulose column and embedded into plugs at full concentration (75% PCV), 1/2 and 1/3 dilutions. (a) Ethidium bromide stained gel, containing size standards *S. cerevisiae* (SC) and Lambda Ladder (LL). Electrophoretic conditions of 200 V, 50–90 s switch rate, and a 24 h run time were used. (b) Autoradiograph of a Southern blot of this gel probed with ^{32}P -labelled calf thymus DNA. (c) The same blot, stripped and then probed with ^{32}P -labelled *A. marginale* DNA.

Fig. 2. Digested DNA from a Florida isolate of *A. marginale* separated by CHEF gel electrophoresis. Lanes 1 and 2 contain size standards of Lambda DNA/*Hind*III Fragments and Delta 39 Promega Markers, respectively. Lanes 3 and 4 contain *Sfi*I-digested and *Not*I-digested *A. marginale* DNA, respectively. Lane 5 contains *Not*I-digested *M. bovis* DNA as a control for digestion with *Not*I using the protocol described in Methods. Electrophoretic conditions of 180 V, 10 s switch rate, and a 16 h run time were used.

Table 1. DNA fragments from *A. marginale* (Florida isolate) produced by digestion with *Sfi*I

Band no.	Size (kbp)	Percentage of total 1206.5 kbp	Area under peak (band density)	Percentage of total area (total density of 15.9)	Copies of band fragments
1	170	14.1	2.2	13.8	1
2*	137.5	11.4	3.6	22.6	2
3	127.5	10.6	1.7	10.7	1
4	118	9.8	1.5	9.4	1
5*	91	7.5	2.3	14.5	2
6	78	6.5	0.8	5.0	1
7†	68.5	5.7	1.2	7.5	1
8	58.5	4.8	0.8	5.0	1
9	52	4.3	0.7	4.4	1
10*	21.5	1.8	0.7	4.4	2
11	20	1.7	0.3	1.9	1
12	14	1.2	0.1	0.6	1

* The bands produced by *Sfi*I digestion which contained two linear fragments of DNA of similar size. Copy number was determined by dividing percentage of total density by percentage of total genome size for each band.

† An artifact on the film at the location of this band caused a mild false elevation in densitometry reading.

erythrocytes appeared pure microscopically, and the level of parasitaemia was similar to that seen in the whole blood prior to red cell isolation, approximately 50%.

Undigested *A. marginale* DNA remained primarily within the wells, even under CHEF gel conditions normally used for separation of linear fragments in the 1.0–6.0 Mb size range (Fig. 1a). These results suggest that *A. marginale* has a circular genome, consistent with most prokaryotic organisms. In Southern blots of these gels, only unpurified, infected blood preparations hybridized with ^{32}P labelled calf thymus DNA (Fig. 1b),

Fig. 3. Comparison of digested genomic DNA from different geographical isolates of *A. marginale*. (a) Lanes 1 and 2 contain size standards of Lambda DNA/*Hind*III Fragments and Delta 39 Promega Markers, respectively. Florida (AmF), South Idaho (AmI), and Virginia (AmV) isolates of *A. marginale* DNA were digested with *Sfi*I and separated by CHEF gel electrophoresis (lanes 3, 4 and 5). Electrophoretic conditions of 180 V, 10 s switch rate, and a 16 h run time were used. (b) Florida (AmF), South Idaho (AmI), and Virginia (AmV) isolates of *A. marginale* DNA were digested with *Pac*I and separated by CHEF gel electrophoresis (lanes 1–3). *S. cerevisiae* size standards are in lane 4. Electrophoretic conditions of 180 V, 50–165 s ramped switch rate, and a 23.5 h run time were used.

whereas all samples hybridized with the genomic *A. marginale* DNA probe (Fig. 1c). The lack of hybridization of cellulose-isolated erythrocytes with the calf thymus DNA probe confirmed that these samples were essentially free of bovine DNA contamination.

Intact *A. marginale* DNA was cut into linear fragments using restriction endonucleases *Sse*I, *Sgr*AI, *Sfi*I, *Not*I and *Pac*I. Enzymes *Sgr*AI and *Sse*I cut the DNA into many small, poorly separated fragments. Using a Florida isolate of *A. marginale*, *Sfi*I and *Not*I produced 12 and 15 bands, respectively, with band fragments ranging from 7.5 to 170 kbp in length (Fig. 2). *Sfi*I digested the genome into fewer, larger fragments that were more distinctly separated. The size of each *Sfi*I fragment shown in Table 1 represents an average value obtained from three separate gels. Laser densitometry indicated that the 137.5, 91 and 21.5 kbp bands contained two linear fragments of similar size (Table 1). Summing the sizes of the 15 *Sfi*I fragments (12 bands) yields an estimated genome size of approximately 1200 kbp.

South Idaho and Virginia isolates of *A. marginale* were also digested with *Sfi*I. The restriction pattern and the band fragments produced by the three isolates were similar, but not identical (Fig. 3a). This clearly demonstrates restriction enzyme polymorphism between isolates of *A. marginale*, visible at the level of the complete genome, and may be useful in isolate typing. Isolate typing could be an important tool in disease surveillance for monitoring genetic changes in isolates from a particular geographical area, as well as the potential movement of isolates between geographical areas. The presence of polymorphism is also a significant consideration in the development of a subunit vaccine that will protect against all isolates of *A. marginale*. Proteins produced by genes which are conserved between various geographical isolates could be targeted as potential antigens for vaccines.

The enzyme *Pac*I cleaved the *A. marginale* genome in just three positions, producing fragments of 598, 557 and 97 kbp from Florida, South Idaho and Virginia isolates,

Fig. 4. Hybridization of the 1250 kbp band with *A. marginale* genomic DNA. *A. marginale* Florida isolate DNA was digested for 1 h with various amounts (0.01, 0.1, 1.0 and 10 units) of *Sfi*I and probed with 32 P-labelled genomic *A. marginale* DNA. Electrophoretic conditions of 200 V, 50–90 s ramped switch rate, and a 24 h run time were used.

respectively (Fig. 3*b*). Addition of the *Pac*I fragments yields a genome size of 1252 kbp, in reasonable agreement with the *Sfi*I data. Although the South Idaho, Virginia and Florida isolates have slightly different restriction patterns with *Sfi*I, the identical patterns produced by these three isolates when digested with *Pac*I confirms that their genomes are of equal size, approximately 1252 kbp.

In many of the gels containing undigested *A. marginale* DNA, and in some gels where restriction enzyme digestion was incomplete, a band was seen consistently migrating just above the 1125 kbp standard (Fig. 1*a*). This band hybridized with 32 P-labelled *A. marginale* genomic DNA (Fig. 4) and with probes prepared from two cloned *A. marginale* genes, *msp1 α* and *msp1 β* (Allred *et al.*, 1990; Barbet & Allred, 1991) (data not shown). Lack of sufficient exposure time prevents visualization of

this band in Fig. 1(*c*). The size of this band, 1250 kbp, is similar to the genome size determined by summation of the linear fragments produced by complete digestion with *Sfi*I or *Pac*I. Our studies indicate that this is the entire linearized genome of *A. marginale*, probably sheared during some stage of blood collection or plug formation. These data further support the accuracy of the genome size determined by addition of *Sfi*I- and *Pac*I-digested fragments.

The G + C content of purified *A. marginale* DNA was determined by spectral analysis to be 56 mol %. Analysis of *E. coli* strain B DNA yielded a G + C content of 51.2 mol %, close to the 50 mol % G + C content reported by the manufacturer. The G + C content of 56 mol % is slightly higher than previously reported values of 49.9–50.7 and 51 mol % (Normore, 1975; Weiss & Moulder, 1984). However, it is significantly higher than the previously reported value of 33 mol %, determined by Bear & Philpott (1987) when calculating the genome size of *A. marginale* by renaturation kinetics. An accurate determination of the G + C content is essential in calculating the genome size of organisms by renaturation kinetics (Bove, 1984; Pyle *et al.*, 1988; Razin, 1985). This may in part account for the difference in genome size of *A. marginale* reported here, 1200–1260 kbp, and that previously reported using renaturation kinetics, 297–340 kbp (Bear & Philpott, 1987). Genome sizes determined by renaturation kinetics can also yield erroneous results if the DNA is not pure or if numerous multiple-copy genes are present in the genome (Bove, 1984; Pyle *et al.*, 1988; Razin, 1985). Bovine DNA contamination is a common problem in preparing *A. marginale* DNA from bovine blood by standard methods. We have eliminated this source of potential error from our DNA preparation by the use of cellulose columns.

Recently, restriction endonucleases and PFGE have been used by many investigators for molecular analysis of different bacterial genomes (Frutos *et al.*, 1989; Lee & Smith, 1988; McClelland *et al.*, 1987; Mehdi & Barbour, 1989; Pyle *et al.*, 1988; Taylor *et al.*, 1991). Using these techniques the genome sizes of other prokaryotic organisms have been found to be significantly larger than previously determined by renaturation kinetics (Frutos *et al.*, 1989; Pyle *et al.*, 1988). The genome size of *A. marginale* we report is quite close to the 1500–2100 kbp reported for several other rickettsial agents (Frutos *et al.*, 1989; Kingsbury, 1969), but is much larger than the previously reported 297–340 kbp genome size (Bear & Philpott, 1987).

Building on the information presented here, a restriction map for the entire *A. marginale* genome can be developed (McClelland *et al.*, 1987; Smith *et al.*, 1987; Romling & Tummler, 1991), and an ordered clone bank

produced. Given the small genome size and the availability of new, high-capacity cloning vectors (Sternberg, 1990; Sternberg *et al.*, 1990), the complete genome could be cloned in < 20 recombinants. Physical maps and ordered clones representing the complete genome of *A. marginale* would be a valuable resource for future research at a molecular level. This resource might be used to rapidly identify and obtain cloned genes and to investigate complex gene relationships involved in pathogenesis.

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