Genome size and genetic map of *Cowdria ruminantium*

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***Cowdria ruminantium* is the cause of a serious tick-borne disease of domestic ruminants, known as heartwater or cowdriosis. The organism belongs to the tribe Ehrlichieae, which contains obligate intracellular pathogens, causing several important animal and human diseases. Although a few *C. ruminantium* genes have been cloned and sequenced, very little is known about the size, gross structure and organization of the genome. This paper presents a complete physical map and a preliminary genetic map for *C. ruminantium*. Chromosomal *C. ruminantium* DNA was examined by PFGE and Southern hybridization. PFGE analysis revealed that *C. ruminantium* has a circular chromosome approximately 1576 kb in size. A physical map was derived by combining the results of PFGE analysis of DNA fragments resulting from digestion of the whole genome with *KspI*, *RsrII* and *SmaI* and Southern hybridization analysis with a series of gene probes and isolated macrorestriction fragments. A genetic map for *C. ruminantium* with a mean resolution of 290 kb was established, the first for a member of the Ehrlichieae. A total of nine genes or cloned *C. ruminantium* DNA fragments were mapped to specific *KspI*, *RsrII* and *SmaI* fragments, including the major antigenic protein gene, *map-1*.**

Keywords: *Cowdria ruminantium*, Rickettsiales, genome size, genetic map

INTRODUCTION

Cowdria ruminantium is an obligate intracellular rickettsial pathogen, a member of the group III Ehrlichieae (Allsopp *et al.*, 1996). *C. ruminantium* is transmitted by ticks of the genus *Amblyomma* and causes heartwater in domestic ruminants throughout sub-Saharan Africa (Uilenberg, 1983; Jongejan & Bekker, 1999), where it is a serious constraint to animal production. The disease is also present in the Caribbean region, where it poses the threat of spreading to the American mainland (Perreau *et al.*, 1980; Barré *et al.*, 1987). The existing vaccine is difficult to manufacture, distribute and administer and cannot be used in non-endemic areas, because it contains viable organisms (Oberem & Bezuidenhout, 1987). A new, cost-effective vaccine, preferably a recombinant one, is needed for the control of heartwater, and to

achieve this goal a better understanding of the molecular genetics of the organism is required.

All Ehrlichia species are parasites of eukaryotic cells and many species cause important diseases of domestic animals and of humans (Rikihisa, 1991). Molecular genetic analysis of the Ehrlichieae, with the exception of *Anaplasma* spp., has been severely impeded because of their obligate intracellular habitat. The organisms are therefore difficult to separate from host-cell components, especially the nuclei, which makes genomic library construction and large-fragment DNA separation difficult.

Although a few genes from *C. ruminantium* have been identified and cloned (Mahan *et al.*, 1994; Van Vliet *et al.*, 1994; Lally *et al.*, 1995; Allsopp *et al.*, 1997; Brayton *et al.*, 1997), nothing was previously known about the structure and organization of the genome. Recent advances in the purification of *C. ruminantium* (De Villiers *et al.*, 1998), using a combination of Percoll density-gradient centrifugation (Tamura *et al.*, 1982) and high-gradient magnetic cell separation (MACS) (Miltenyi *et al.*, 1990), have allowed us to study some of

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Abbreviation: MACS, magnetic cell separation.

the basic molecular genetics of this organism. The objectives of this study were to determine the genome size of the Welgevonden isolate of *C. ruminantium* by PFGE analysis and restriction endonuclease digestion, and to construct a combined physical and genetic map by integrating PFGE and Southern blot analytical data.

METHODS

Preparation of chromosomal DNA plugs. The Welgevonden isolate of *C. ruminantium* (Du Plessis, 1985) was harvested from heavily infected bovine endothelial cell cultures (Bezuidenhout *et al.*, 1985). The organisms were tested for *Mycoplasma* contamination by three separate tests: (i) amplification by PCR using primers described by Kuppeveld *et al.* (1994), (ii) DNA fluorochrome staining (Del Giudice & Hopps, 1978) and (iii) direct culturing in MycoVito medium (International Mycoplasma). Cultures that were positive for *Mycoplasma* were decontaminated using the following methods. *Mycoplasma* contamination was removed by first of all treating the bovine endothelial cells with 10–50 µg ciprofloxacin hydrochloride ml⁻¹ (Bayer). *C. ruminantium* was cleaned by infection of mice and subsequent re-isolation into clean feeder bovine endothelial cell monolayers (Eremeeva *et al.*, 1994). The Welgevonden isolate has been in culture in our laboratory for several years and is used as our reference stock. Although the isolate has not been cloned, we have evidence, as discussed below, that it does not contain a mixture of genotypes. Endothelial cells were disrupted to release *C. ruminantium* organisms in all stages of development and host-cell debris was separated from the organisms on a discontinuous Percoll (Sigma) density gradient (Tamura *et al.*, 1982; De Villiers *et al.*, 1998). Purified organisms were stained with acridine orange and counted (Knight *et al.*, 1989), resuspended at a concentration of 1.2×10^9 cells ml⁻¹ in Dulbecco's phosphate-buffered saline (pH 7.1) and embedded in 0.5% agarose by mixing the suspension 1:1 (v/v) with FMC InCert Agarose [1% (w/v) in PBS] (FMC Bioproducts). The agarose mixture was cast into 100 µl moulds to yield plugs containing approximately 1 µg DNA per plug. Plugs were treated with lysis buffer [6 mM Tris/HCl, 1 M NaCl, 0.01 mM EDTA, 0.2% sodium deoxycholate (v/v) and 0.5% (v/v) Sarkosyl, pH 7.6] for 16 h at 37 °C. Lysis buffer was replaced with reaction buffer [10 mM Tris/HCl (pH 8.0), 0.01 mM EDTA, 1% (v/v) Sarkosyl] containing 15 units Pretaq ml⁻¹ (Roche Molecular Biochemicals) and the plugs were incubated for 2 h at 60 °C to digest all proteins. Plugs were washed twice for 20 min at room temperature with 10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA and stored at 4 °C in the same buffer. Pretaq is inactive at 37 °C and therefore does not require specific inactivation.

Restriction endonuclease digestion. Individual plugs containing intact *C. ruminantium* DNA were digested with restriction endonucleases (Roche Molecular Biochemicals). Agarose plugs were equilibrated with 100 µl suitable restriction endonuclease buffer at 4 °C for 20 min. The buffer was exchanged with 100 µl fresh buffer containing 10 units of restriction endonuclease and the mixture incubated for 4 h at the appropriate temperature. Reactions were stopped by the addition of 50 µl 0.5 M EDTA (pH 8.0).

PFGE. DNA plugs were loaded into wells of a 1% (w/v) FastLane agarose gel (FMC Bioproducts) in 0.025 M TBE (2.25 mM Tris base, 2.25 mM boric acid, 0.05 mM EDTA, pH 8.5). Electrophoresis was carried out at 13 °C in a Rotaphor Type 5 PFGE apparatus (Biomatra) using the

following separation conditions: voltage 120–125 V (logarithmic ramp); pulse rate 5–90 s (linear ramp); rotation angle 110–125° (logarithmic ramp) with a duration of 37 h. *Saccharomyces cerevisiae* chromosomal DNA markers (New England Biolabs) and Low Range PFG markers (New England Biolabs) were used as molecular size markers. Gels were stained with ethidium bromide and restriction fragments photographed and analysed with a Lumi-Imager F1 Workstation (Roche Molecular Biochemicals). Band sizes were calculated from at least three independent separations of restriction fragments.

Two-dimensional electrophoresis was carried out according to the method of Römling & Tümmeler (1991). A lane containing restriction endonuclease digested DNA was cut from a PFGE gel, incubated with 200 U of a second restriction endonuclease in 3 ml of appropriate reaction buffer and loaded across the width of a second PFGE gel. This gel was then electrophoresed at right angles to the sample strip. DNA spots were visualized with ethidium bromide staining and subsequently photographed and analysed with the Lumi-Imager F1 Workstation.

Southern hybridization. Gels were depurinated for 15 min in 0.25 M HCl, washed twice for 30 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and for 1 h in neutralizing solution (1.5 M NaCl, 0.5 M Tris/HCl, 1 mM EDTA, pH 7.5) and then transferred to MagnaCharge nylon transfer membrane (MSI) with 20 × SSC [1 × SSC is 150 mM NaCl plus 75 mM sodium citrate (pH 7.0)] (Sambrook *et al.*, 1989). After transfer, DNA was fixed to the membrane by baking at 80 °C for 2 h. DNA probes were labelled using a Megaprime labelling kit (Amersham) and [α -³²P]dATP (3.7×10^8 Bq ml⁻¹, Amersham) according to the instructions of the manufacturer. Partially purified organisms were further purified by high-gradient MACS (Miltenyi *et al.*, 1990) as previously described (De Villiers *et al.*, 1998) to obtain genomic DNA that contained <10% bovine DNA for use as probes. Cloned genes (see Table 4) and gel-purified restriction endonuclease fragments were also used as probes. Filters were prehybridized in 0.5 M sodium phosphate (pH 7.4), 7% SDS, for at least 1 h and hybridized overnight at 64 °C in the same buffer following addition of probe. Excess probe was removed by washing at room temperature (1 × SSC, 0.5% SDS; 2 × 10 min) followed by washing in the same buffer (1 × 10 min at 64 °C). Blots were stripped with boiling 0.5% SDS and stored at 4 °C until re-use. Hybridization results were visualized by autoradiography and analysed with the Lumi-Imager F1 Workstation.

RESULTS

The genome of *C. ruminantium* consists of a circular chromosome

PFGE electrophoresis of intact, undigested *C. ruminantium* genomic DNA, under conditions designed to separate linear fragments in the 3–2000 kb size range, revealed that most of the sample DNA remained in the wells (Fig. 1a, lane 3), with a small amount of DNA migrating at a size of 1600 kb. This fragment was apparently linearized chromosomal DNA that was sheared or nicked during the isolation of the organisms or the preparation of genomic DNA. The intensity of this fragment varied from DNA preparation to DNA preparation, which supports this conclusion (data not shown). Genomic DNA from uninfected bovine endothelial cells only hybridized to lane 2 (Fig. 1b)

Fig. 1. PFGE and Southern hybridization analysis of undigested and digested *C. ruminantium* genomic DNA. (a) Ethidium bromide stained gel. Corresponding Southern hybridization with, (b) bovine (endothelial cell) genomic DNA, (c) purified *C. ruminantium* DNA and (d) *map-1* gene DNA as probe. Lanes: 1, lambda ladder and *S. cerevisiae* molecular mass markers; 2, undigested genomic bovine DNA; 3, undigested genomic *C. ruminantium* (Welgevonden isolate) DNA; 4, *RsrII*-digested, 5, *KspI*-digested and 6, *SmaI*-digested *C. ruminantium* DNA. PFGE running conditions were for 3–2000 kb fragment separation. Sizes are indicated on the left. Images were captured on the Lumi-Imager F1 Workstation and quantified and analysed with the Lumi Analyst Version 3.0 software.

containing bovine DNA. DNA in the wells, the compression front at 1900 kb, and the individual *C. ruminantium* restriction fragments gave a strong hybridization signal with genomic DNA from purified *C. ruminantium* organisms (Fig. 1c). This probe also hybridized with bovine DNA in lane 2 (Fig. 1c), because *C. ruminantium* chromosomal DNA purified on Percoll density gradients still contains 5–10% bovine DNA (De Villiers *et al.*, 1998). Southern hybridization of the same blot with a specific *C. ruminantium* gene probe, *map-1*, gave a strong signal with specific restriction fragments on which *map-1* is located, as well as undigested *C. ruminantium* DNA (Fig. 1d). The data showed that the majority of *C. ruminantium* chromosomal DNA remained in the wells during PFGE, which is indicative of a circular genome. Plasmids were not detected either by PFGE or alkaline lysis extraction.

Genome size determination

Eight restriction endonucleases that recognize GC-rich cleavage sites (*ApaI*, *KspI*, *MluI*, *NaeI*, *NotI*, *RsrII*, *SfiI* and *SmaI*) were tested for their suitability for mapping the genome. We expected that these enzymes would recognize only a few sites in the *C. ruminantium* genome due to its low G + C content (26–33 mol%) (Brayton *et al.*, 1997). Three restriction endonucleases, *KspI*, *RsrII* and *SmaI*, were found to digest the genome into a small number of DNA fragments (2–6) which could easily be

Table 1. DNA fragments from *C. ruminantium* (Welgevonden isolate) produced by digestion with three restriction endonucleases

Fragment	Fragment size* (kb) obtained with:		
	<i>KspI</i>	<i>RsrII</i>	<i>SmaI</i>
1	572 ± 35	1453 ± 35	525 ± 35
2	452 ± 27	181 ± 2	295 ± 39
3	271 ± 30		259 ± 29
4	174 ± 17		257 ± 29
5	82 ± 15		189 ± 18
6			20 ± 3
Total	1551 ± 114	1633 ± 15	1545 ± 143

* Mean of at least three independent gels with standard deviations indicated. The fragments were designated S, K and R to indicate the corresponding restriction endonuclease *SmaI*, *KspI* and *RsrII* followed by the fragment number.

distinguished and sized by PFGE (Fig. 1a). Table 1 summarizes the mean sizes of the fragments obtained from at least three separate gels with the restriction endonucleases selected for this study.

Digestion of chromosomal *C. ruminantium* DNA with the restriction endonuclease *RsrII* yielded two fragments

Fig. 2. PFGE of restriction fragments obtained from (a) partially digested *C. ruminantium* DNA. Lanes: 1, *KspI*; 2, *SmaI*. (b) PFGE of restriction fragments obtained from double digestion of *C. ruminantium* DNA with *KspI* and *RsrII*. Lanes: 1, *KspI*; 2, *KspI* and *RsrII*; 3, *RsrII* only. (c) PFGE of restriction fragments obtained from double digestion of *C. ruminantium* DNA with *KspI* and *SmaI*, and *SmaI* and *RsrII*. Lanes: 1, *RsrII*; 2, *SmaI*; 3, *KspI*; 4, *KspI* and *SmaI*; 5, *SmaI* and *RsrII*. PFGE running conditions for all gels were for 3–2000 kb fragment separation. Sizes are indicated on the left of each panel. Images were captured on the Lumi-Imager F1 Workstation.

Table 2. Fragment sizes resulting from double digestion of *C. ruminantium* (Welgevonden isolate) DNA

The fragments were designated S, K and R to indicate that the corresponding *SmaI*, *KspI* and *RsrII* fragment was not cut by *SmaI*, *KspI* and *RsrII* respectively. The fragments resulting from the cleavage of a *SmaI* fragment with *KspI* are designated SK followed by a number (1 to 6). Similarly fragments were designated SR and KR followed by a number when they were produced by *SmaI* + *RsrII* and *KspI* + *RsrII* cleavages, respectively.

Fragment	<i>KspI</i> + <i>RsrII</i> size (kb)	Fragment	<i>SmaI</i> + <i>RsrII</i> size (kb)	Fragment	<i>KspI</i> + <i>SmaI</i> size (kb)
K1	572	S1	525	S1	525
K3	271	S2	295	SK1	271
KR1	253	S3	259	S5	189
R2	181	S5	189	SK2	189
K4	174	R2	181	K4	174
K5	82	SR1	70	SK3	94
KR2	18	S6	20	K5	82
		SR2	6	SK4	40
				S6	20
				SK5	11
Total	1551		1539		1572

(Fig. 1a, lane 4), fragment 1 (R1) which migrated lower than the compression front at approximately 1453 kb and another smaller fragment 2 (R2) at 181 kb. Addition of these two fragments gave a genome size estimate of 1633 ± 15 kb (Table 1). Southern hybridization with a *C. ruminantium* genomic DNA probe yielded strong signals with residual DNA in the well and with the R1 fragment and a faint signal with the R2 fragment (Fig. 1c, lane 4).

The restriction endonuclease *KspI* produced five fragments (Fig. 1a, lane 5), which added up to 1551 ± 114 kb (Table 1). *SmaI* produced six fragments (Fig. 1a, lane 6) with a total length of 1545 ± 143 kb (Table 1). Based on the data obtained by these three enzyme digestions, we estimated the *C. ruminantium* genome size at 1576 ± 91 kb.

Construction of a physical map

Both physical methods and hybridization analysis were used in the construction of a physical map for *C. ruminantium* (Welgevonden). To supplement the data obtained with restriction endonuclease digestion of whole genomic DNA with *KspI*, *RsrII* and *SmaI*, partial digestions with these endonucleases were obtained either by limiting the amount of restriction endonuclease added to a reaction or by limiting the reaction time of the digestions (Fig. 2a).

To provide further information about the linkage of fragments, double digestion of total genomic DNA was performed with combinations of *KspI* and *RsrII*, *SmaI* and *RsrII*, and *KspI* and *SmaI* (Table 2). Fragments K1, K3, K4 and K5 were not digested by *RsrII* (Fig. 2b). K2

Fig. 3. Determination of *SmaI* restriction endonuclease sites in *KspI* fragments by two-dimensional PFGE. A lane containing *KspI* digestion fragments was excised from a PFGE gel, digested with *SmaI* and loaded lengthwise and separated on a second PFGE gel together with *KspI*- and *SmaI*-digested DNA. Lanes: 1, *SmaI*; 2, *KspI*; 3, two-dimensional separation of *KspI* fragments digested with *SmaI*. Sizes are indicated on the left. Images were captured on the Lumi-Imager F1 Workstation (part of the same gel as in Fig. 2c).

was digested at two sites by *RsrII*, yielding the R2 fragment and two additional fragments, KR1 and KR2, of 253 and 18 kb, respectively (Table 2, Fig. 2b, lane 2). Fragments S1, S2, S3, S5 and S6 were not digested by *RsrII*. S4 was digested at two sites by *RsrII*, yielding the R2 fragment and two additional fragments, SR1 and SR2, of 70 and 6 kb, respectively (Table 2, Fig. 2c, lane 5). The results with *KspI* and *SmaI* were more complex. Fragments K4 and K5 were not digested by *SmaI*, and fragments S1, S5 and S6 were not digested by *KspI*. *SmaI* digested fragment K1 into three fragments, S1, SK4 and SK5 (Table 2, Fig. 2c, lane 4).

These results were confirmed with two-dimensional restriction fragment analysis. Chromosomal DNA was digested with *KspI* and the resulting fragments (K1 through K5) were separated by PFGE. The lane containing the DNA bands was cut out, digested with *SmaI*, and separated in the second dimension. As a control, *KspI*- and *SmaI*-digested chromosomal DNA samples were also separated on the same gel (Fig. 3). The results showed that fragment K1 had two *SmaI* sites, yielding three fragments: a 525 kb fragment (Fig. 3, lane 3 fragment S1), a 40 kb fragment (Fig. 3, lane 3 fragment SK4) and a smaller fragment of 11 kb (Fig. 3, lane 3 fragment SK5). Fragment K2 also had two *SmaI* sites, yielding three fragments, a doublet consisting of S5 and SK2, and SK3 (Fig. 3, lane 3). A small amount of undigested K2 was also visible. Fragment K3 had a single *SmaI* site, yielding two fragments, SK1 and S6 (Fig. 3, lane 3). With data obtained from the partial digests, double digests and two-dimensional restriction

Table 3. Hybridization with genomic restriction fragments isolated from agarose gels

DNA probe	Hybridized to fragment(s):		
	<i>KspI</i>	<i>RsrII</i>	<i>SmaI</i>
K1	1	1	1, 2, 4
K2	2	1, 2	3, 4, 5
K3	3	1	2, 6
K4	4	1	3
K5	5	1	4
R2	2	2	4
S1	1	1	1
S2	1, 3	1	2
S3/4*	2, 4, 5	1, 2	3, 4
S5	2	1	5
S6	3	1	6

*This band contained two fragments which could not be separated from each other.

fragment analysis, a physical map was constructed which was confirmed by hybridization analysis.

Southern blots of single- and/or double-digested *C. ruminantium* genomic DNA were probed with individual restriction fragments (Table 3) and with cloned or PCR-amplified genes of *C. ruminantium* obtained from various sources (Table 4). Individually labelled restriction fragments were hybridized to PFGE-separated single- and/or double-digested chromosomal DNA fragments. For example, the K1 fragment hybridized to fragments S1, S2 and S4, indicating the linkage between these three fragments (Table 3). The *rpoC* gene probe hybridized to fragments K1 and S2 and to a 45 kb fragment resulting from the *KspI*/*SmaI* double digest, indicating that fragments K1 and S2 were linked with a 45 kb common fragment on which the *rpoC* gene was located (Table 4, Fig. 4). In a similar fashion, most of the *KspI*, *SmaI* and *RsrII* fragments were located on the *C. ruminantium* physical map thus confirming its topology (Fig. 5).

Construction of a genetic map

Nine genes or cloned DNA fragments were located on the *C. ruminantium* physical map by hybridization to restriction endonuclease digested genomic DNA separated by PFGE. The probes used in this study, and the results obtained, are listed in Table 4. For example, the *map-1* gene probe hybridized to the R1 fragment and not to the R2 fragment. It further hybridized only to the K1 and S1 fragments (Fig. 1d, Table 4), indicating that it was located on fragment 1 of *RsrII*, *KspI* and *SmaI*. In the same manner, all the genes and cloned DNA fragments were located on the physical map as indicated in Table 4 and Fig. 5, with position 0/1576 chosen at random.

Table 4. Restriction fragments resulting from *KspI*, *RsrII* and *SmaI* digestion which gave a hybridization signal with several DNA probes

DNA probe	Source	Reference	Hybridized to fragment(s):		
			<i>KspI</i>	<i>RsrII</i>	<i>SmaI</i>
16S rRNA (gene)	Oligonucleotide (18 bp)	Allsopp <i>et al.</i> (1997)	3	1	2
<i>map-1</i> (gene)	pBluescript clone (3.2 kb)	Brayton <i>et al.</i> (1997)	1	1	1
<i>pcs20</i> (ORF)	PCR amplicon (1.3 kb)	Waghela <i>et al.</i> (1991)	1	1	1
<i>groEL</i> (gene)	PCR amplicon (1.6 kb)	Lally <i>et al.</i> (1995)	2	2	4
28G (ORF)	PCR amplicon (450 bp)	Perez <i>et al.</i> (1997)	3	1	2
<i>rpoC</i> (gene)	PCR amplicon (426 bp)	Brayton <i>et al.</i> (1997)	1	1	2
1H12 (cosmid)	Cosmid clone (20.1 kb)	Brayton <i>et al.</i> (1999)	3	1	2
2C11 (cosmid)	Cosmid clone (35.6 kb)	Brayton <i>et al.</i> (1999)	3	1	2
6D4 (cosmid)	Cosmid clone (45 kb)	Brayton <i>et al.</i> (1999)	3	1	2

Fig. 4. PFGE of restriction fragments obtained from double digestion of *C. ruminantium* DNA with *KspI* and *SmaI* and probed with the *rpoC* gene. (a) Ethidium bromide stained gel. (b) Corresponding Southern hybridization with *rpoC* gene. Lanes: 1, *SmaI*; 2, *KspI*; 3, *KspI* and *SmaI*. PFGE running conditions for all gels were for 3–2000 kb fragment separation. Sizes are indicated on the left. Images were captured on the Lumi-Imager F1 Workstation (part of the same gel as in Fig. 2c). **Fig. 5.** Physical and preliminary genetic map of the *C. ruminantium* chromosome. The map was constructed from partial and double digestions obtained with *KspI*, *RsrII* and *SmaI* followed by PFGE separation and subsequent Southern hybridization of ^{32}P -labelled fragments cut out from restriction endonuclease digested genomic DNA separated by PFGE. DNA probes used for genetic mapping are shown in Table 4.

DISCUSSION

We have determined a complete physical map of the genome of the Welgevonden isolate of *C. ruminantium*. This is the first such map for a member of the tribe *Ehrlichieae*. The Welgevonden isolate has not been somatically cloned, but the polymorphic *map-1* gene of this isolate has been cloned and sequenced on different occasions by different laboratories over a period of more than 6 years (Brayton *et al.*, 1997; A. Bensaid & D. Martinez, personal communication, 1999; Sulsona *et al.*, 1999). In all but one unusual case (Sulsona *et al.*, 1999) the sequence of this gene has remained identical,

which provides good evidence that this is not a mixed isolate. More significantly, recent macrorestriction profile analysis by PFGE of the Welgevonden isolate also gave a consistent and clear pattern, further indicating that the isolate is genetically stable and does not contain a mixture of genotypes (De Villiers *et al.*, 2000).

In the exception noted above, Sulsona *et al.* (1999) found that the *map-1* sequence of the Welgevonden isolate (GenBank accession number AF125274) grown in their lab for a number of years was 86.88% identical to the earlier Welgevonden sequence (GenBank accession

number U49843). The sequence they obtained was reported to be identical to a new Zimbabwean isolate called Lemco (GenBank accession number AF125277). However, examination of the sequences reported in GenBank show these sequences to share only 99.6% identity. This is the only known report of two different pathogenic isolates of *C. ruminantium* having identical *map-1* sequences. All other laboratories that have sequenced the *map-1* gene from the Welgevonden isolate have obtained the same sequence as U49843 (Brayton *et al.*, 1997; A. Bensaid & D. Martinez, personal communication, 1999).

PFGE separation of intact *C. ruminantium* chromosomal DNA indicated that the chromosome was circular, in common with most other prokaryotic organisms, with an estimated size of 1576 kb. A physical map was constructed by a combination of complementary techniques. Single, double and partial digests separated with normal PFGE and two-dimensional PFGE in combination with hybridization experiments with genetic markers and isolated restriction fragments. A total of 13 restriction fragment sites were positioned on the map (5 *Ksp*I, 2 *Rsr*II and 6 *Sma*I sites) with a mean resolution of 290 kb. Similar methods were used to construct the *Haemophilus influenzae* (Lee *et al.*, 1989) and *Mycoplasma mycoides* (Pyle & Finch, 1988) physical maps.

Relatively few genes of *C. ruminantium* have been identified and therefore only a limited genetic map could be constructed by Southern hybridization with nine probes (Table 4). Interestingly, the majority of these probes were located on one half of the map with the three stable cosmids from a SuperCos1 library clustered on the 271 kb *Ksp*I–*Sma*I fragment together with the 16S rRNA and the 28G ORF.

Previously, using PFGE of uncut circular DNA, we estimated the *C. ruminantium* chromosome size at 1900 kb (De Villiers *et al.*, 1998). The discrepancy is probably due to the fact that circular DNA does not migrate predictably during PFGE (Birkelund & Stephens, 1992) and the large molecular mass markers behaved erratically under the PFGE conditions used (New England Biolabs, Yeast Chromosome PFG Marker technical bulletin). In our earlier study, a 815 kb extrachromosomal element was also identified, which upon sequencing of the PCR-amplified product turned out to be an unknown *Mycoplasma* sp., most closely related to *Mycoplasma indiane*. All cultures used in this study were tested and shown to be free of mycoplasma contamination by three different methodologies as described earlier.

Several close relatives of *C. ruminantium* genomes have been determined with PFGE. The closest relative of *C. ruminantium* is the group III *Ehrlichia* sp. *Ehrlichia chaffeensis* with a genome size of 1225.8 kb (Rydina *et al.*, 1999). Very close in size to the *C. ruminantium* chromosome is the unnamed agent of human granulocytic ehrlichiosis (HGE), which has a genome size of 1494 kb (Rydina *et al.*, 1999). *Anaplasma marginale*

has a slightly smaller genome, between 1200 and 1260 kb (Alleman *et al.*, 1993). *Ehrlichia sennetsu* and *Ehrlichia risticii*, on the other hand, have substantially smaller genomes of 878 kb and 880 kb, respectively (Rydina *et al.*, 1999). Unfortunately, no physical or genetic maps are available for these organisms. *Rickettsia prowazekii*, the causative agent of epidemic typhus and only distantly related to the *Ehrlichiae*, is the only rickettsial organism to have its genome completely mapped and sequenced (Andersson *et al.*, 1998). This parasite has a genome size of 1 111 523 bp. The size of the *C. ruminantium* genome, therefore, appears to be close to that of its relatives, whose small genomes are consistent with their obligate intracellular lifestyles. Due to the limited amount of genetic data available for *C. ruminantium* and the fact that only a small number of genes can be compared with this method, it is impossible to make further comparisons between our map and that of *R. prowazekii*.

However, the *C. ruminantium* physical map has already been shown to be valuable for comparative genome analyses of *C. ruminantium* isolates. The restriction endonucleases identified for the construction of the physical map were applied in macrorestriction profile analysis by PFGE to distinguish seven isolates of *C. ruminantium* from geographically different areas (De Villiers *et al.*, 2000). Statistical analysis of the macrorestriction profiles indicated that all isolates were indeed distinct from each other. These data will contribute to a better understanding of the molecular epidemiology of this pathogen and may be further exploited for the identification of genotype-specific DNA probes. In addition, the estimated genome sizes for the seven isolates ranged from 1546 to 1675 kb, in agreement with the genome size calculated in this paper.

The physical and genetic map of the *C. ruminantium* genome constitutes a significant step forward in the study of the molecular biology of this organism, and will considerably aid the completion of the genome sequencing project which is currently under way.

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