Cowdria ruminantium DNA is unstable in a SuperCos1 library

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ABSTRACT

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A *Cowdria ruminantium* genomic library was constructed in a cosmid vector to serve as a source of easily accessible and pure *C. ruminantium* DNA for molecular genetic studies. The cosmid library contained 846 clones which were arrayed into microtitre plates. Restriction enzyme digestion patterns indicated that these clones had an average insert size of 35 kb. Probing of the arrays did not detect any bovine clones and only one of the known *C. ruminantium* genes, *pCS20,* was detected. Due to the high AT content and the fact that *C. ruminantium* genes are active in the *Escherichia coli* host, the *C. ruminantium* clones were unstable in the SuperCos1 vector and most clones did not grow reproducibly. The library was contaminated with *E. coli* clones and these clones were maintained with greater fidelity than the *C. ruminantium* clones, resulting in a skewed representation over time. We have isolated seven *C. ruminantium* clones which we were able to serially culture reproducibly; two of these clones overlap. These clones constitute the first large regions of *C. ruminantium* DNA to be cloned and represent almost 10 % of the *C. ruminantium* genome.

Keywords: cosmid, *Cowdria ruminantium*, library

INTRODUCTION

Cowdria ruminantium, a member of the order Rickettsiales, is an obligate intracellular parasite that causes heartwater in domestic ruminants throughout sub-Saharan Africa. Heartwater is the most important rickettsial disease of animals in Africa (Provost & Bezuidenhout 1987). In southern Africa the disease is controlled by an infection and treatment procedure which was first introduced over 50 years ago and which suffers from serious inadequacies (Oberem & Bezuidenhout 1987). The ultimate aim of our research is to develop a recombinant vaccine—a goal which will require detailed information about the molecular genetics of the organism which are almost completely lacking at present.

Although *C. ruminantium* was first successfully cultured *in vitro* in 1985 (Bezuidenhout, Patterson & Barnard 1985), molecular genetic studies have been impeded by the difficulty of obtaining *C. ruminantium* DNA uncontaminated by a large excess of host DNA. For this reason, there are very few *C. ruminantium* libraries in existence and, consequently, relatively few genes have been cloned from this organism. The gene coding for the immunodominant surface protein MAP1 was amplified from partially purified *C. ruminantium* DNA (Van Vliet, Jongejan, Van Kleef & Van der Zeijst 1994) and cloned without the benefit of a library. The 16S rRNA coding region was also

amplified by PCR and cloned from several different isolates of *C. ruminantium*, and has been used to elucidate the phylogenetic relationship of *C. ruminantium* to other *Rickettsiales* (Dame, Mahan & Yowell 1992; Van Vliet, Jongejan & Van der Zeijst 1992; Allsopp, Allsopp, Du Plessis & Visser 1996).

The first genes to be cloned from a *C. ruminantium* library were genes of unknown function, designated pCS20 and pCR9, which were obtained from a pUC19 library (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burridge & McGuire 1991) and pCS20 is in use for diagnostic purposes (Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992; Peter, Deem, Barbet, Norval, Simbi, Kelly & Mahan 1995). A second immunodominant protein gene, *map2*, was obtained from a pUC13 library (Mahan, McGuire, Semu, Bowie, Jongejan, Rurangirwa & Barbet 1994). The *C. ruminantium groE* operon was obtained from a *C. ruminantium* (Welgevonden) λZAPII library (Lally, Nicoll, Paxton, Cary & Sumption 1995). In addition, we have reported the construction of a representative *C. ruminantium* (Welgevonden) λZAPII library, from which we have isolated four *C. ruminantium* genes: *map1*, *rpoC* β subunit, a tRNA synthetase and an HflK homolog (Brayton, Fehrsen, de Villiers, Van Kleef & Allsopp 1997). While none of these genes is likely to be useful as a recombinant vaccine by itself, one or more may be useful in a pool of recombinant genes that would make up the recombinant vaccine.

A method was devised to separate intact *C. ruminantium* from host cell material by immunoaffinity chromatography (Brayton *et al.* 1997) which provided very pure DNA, contaminated with only 3 % bovine DNA. This DNA was used with great success to construct a λZAPII phage library; the library was large (> 10⁴ pfus), stable and appeared to be representative (Brayton *et al.* 1997). However, with this type of library the insert size is small, ~3 kb. We sought to construct an additional library from this material that would serve as an alternative source of easily accessible and pure *C. ruminantium* DNA for further molecular studies. A library with large inserts was desired so as to encompass the genome in as few clones as possible. Cosmids, having the largest insert size of the easily manipulated cloning vectors, would be able to span the *C. ruminantium* genome with 50–60 clones, based on an estimated genome size of 2000 kb (De Villiers, Brayton, Zweygarth & Allsopp 1998).

This is the first report of a large insert library for *C. ruminantium* and details the difficulties we encountered trying to clone DNA from this AT-rich genome into an intracellularly maintained cloning vehicle. We have isolated seven *C. ruminantium* clones which we were able to serially culture reproducibly; two of these clones overlap. These clones constitute the first large regions of *C. ruminantium* DNA to be cloned and represent almost 10 % of the *C. ruminantium* genome.

MATERIALS AND METHODS

Bacterial strains, vectors and cultivation conditions

The Welgevonden stock of *C. ruminantium* was cultured in an endothelial cell line (E5 cell line) (Bezuidenhout *et al.* 1985). Organisms purified by immunoaffinity absorption chromatography (Brayton *et al.* 1997) were used as a source of DNA for constructing the cosmid library. SuperCos1, a 7,6 kb cosmid vector (Stratagene) was used for cloning *C. ruminantium* DNA. *Escherichia coli* XL-1 Blue MR (Stratagene) was used as a host for the cosmid clones. Recombinant clones were grown in either LB broth, LB supplemented with 5 g/l sucrose (LB/sucrose) or Terrific broth (TB) consisting of 12 g tryptone, 24 g yeast extract, 4 ml glycerol in 900 ml water to which was added 100 ml of a solution containing 0,17 g KH_2PO_4 and 0,72 g $KHPO_4$.

Library construction and manipulation

SuperCos1 vector DNA was digested with *Xba* I to linearize the cosmid, dephosphorylated and then digested with *Bam* HI to create the cloning site. Genomic *C. ruminantium* DNA was isolated by the method of Blin & Stafford (1976) from immunoaffinity purified *C. ruminantium* organisms. The bovine DNA content of the genomic DNA was estimated at 3–5 % as previously described (Brayton *et al.* 1997).

Five micrograms of *C. ruminantium* DNA was partially digested with 0,2 U *Sau* 3AI in 20 µl to yield DNA fragments of 30–50 kb. The DNA was dephosphorylated with calf intestinal alkaline phosphatase for 1 h at 37 °C. One microgram of prepared vector DNA was ligated to 2 µg *C. ruminantium* insert DNA at 4 °C overnight in a 10 µl reaction volume. The entire ligation reaction was packaged using Gigapack XL packaging extract (Stratagene). The library was transduced into XL-1 Blue MR cells and recombinants were selected by plating on LB-kanamycin (50 µg/ml) plates. The clones were arrayed by subinoculation into microtitre plate wells containing LB-kanamycin (50 µg/ml) medium and 20 % glycerol. These arrayed clones were stored at –70 °C.

Cosmid colonies were picked with sterile toothpicks and grown overnight at 37 °C. Mini preparations of DNA were prepared by the alkaline lysis method of Birnboim & Doly (1979). Large scale DNA preparations were prepared by cesium chloride density gradient centrifugation or by an ion-exchange column-based purification kit, either Nucleobond AX (Machery-Nagel) or Qiagen.

An aliquot of cosmid clone DNA (10 μg) was digested with 5 U *Not* I in 400 μℓ digestion buffer to excise the insert. Partial digestion of the subsequent fragment was performed as described in Sambrook, Fritsch & Maniatis (1989). Aliquots containing 0,5 μg of cloned DNA were pipetted into each of six wells of a microtitre plate each containing 1 μg of calf thymus DNA. *Eco* RI or *Hin*d III (4 U) was added to the first well to make a total of 60 μℓ. A 30 μℓ aliquot was transferred from the first to the second well, after mixing a 30 μℓ aliquot was transferred from the second to the third well and so on until the last well. The microtitre plate was incubated for 60 min at 37 °C and the reaction stopped by the addition of 5 μl loading dye containing 30 mM EDTA. Samples were loaded onto a 0,5 % agarose gel and electrophoresed at 10 V/cm for 3 days at 4 °C.

Preparation of hybridization filters

The clones were transferred from the microtitre plate using a 96-well replica plater (Sigma) onto nylon filters (MSI) on an LB-kanamycin (50 μg/mℓ) plate and grown overnight at 37 °C. The cells were lysed and the DNA denatured and prepared for hybridization according to the filter manufacturer's instructions. Slot blots of cloned DNA were prepared on nylon filters (MSI) according to manufacturer's instructions. Agarose gels for Southern blots were treated according to the manufacturer's (MSI) instructions prior to transfer to nylon membranes. DNA was bound to the filters by baking at 80 °C for 2 h.

Hybridization probes and hybridization conditions

Probes specific to known DNA fragments, genes and genomic DNA were generated by a multiprime labelling system (Amersham) according to manufacturer's instructions using [α-^{32}P]dCTP. Filters were prehybridized in hybridization buffer (0,5 M sodium phosphate (pH 7,4), 7 % SDS) for at least 1 h and hybridized overnight at 65 °C with the addition of probe. Excess probe was removed by washing twice for 10 min in 0,1 x SSC, 0,1 % SDS at room temperature and twice in 0,1 x SSC, 0,1 % SDS for 15 min at 65 °C.

High specific activity RNA probes (riboprobes) were generated from either the T3 or T7 end of cosmid clones with an RNA transcription kit (Stratagene) and [α-^{32}P]dCTP according to manufacturer's instructions. Filters were hybridized and washed as above.

T3 and T7 oligonucleotides were end-labelled with [α-^{32}P]dATP employing a terminal transferase kit (Promega). Filters were prehybridized in hybridization buffer for at least 1 h and hybridized for 4 h at 42 °C with the addition of labelled probe. Filters were washed twice for 10 min in 6 x SSC at room temperature and then in 6 x SSC, 0,1 % SDS for 10 min at 40 °C. Hybridization results were visualized by autoradiography.

RESULTS AND DISCUSSION

Storage and initial characterization of the *C. ruminantium* cosmid library

C. ruminantium genomic DNA, estimated to contain 3 % bovine DNA, was partially digested by *Sau* 3AI to yield fragments in a size range of 20–60 kb. The DNA was not size selected for the desired size range of 30–50 kb. Instead, it was treated with alkaline phosphatase to prevent concatenated inserts. The DNA was ligated into the SuperCos1 cosmid vector, packaged into λ phage heads *in vitro* and transduced into *E. coli* strain XL-1 Blue MR to produce a total of 846 kanamycin resistant colonies. Given a genome size of 2000 kb (De Villiers *et al.* 1998) a random 846-clone library should be nearly 15-fold redundant.

All colonies were picked and arrayed into wells of microtitre plates and stored as glycerol stocks at –70 °C. Screening replicas of the library with ^{32}P-labelled calf thymus DNA detected no bovine clones, although the starting material was estimated to contain 3 % bovine DNA. In addition, we screened with DNA probes from two known *C. ruminantium* genes, *pCS*20 (Waghela *et al.* 1991) and *map*1 (Van Vliet *et al.* 1994; Brayton *et al.* 1997) and only the former could be detected. These findings indicated that the library was not representative of the starting material or of the genome of *C. ruminantium*. Hybridization of colony lifts with riboprobes proved to be unsatisfactory owing to large differences between the growth rates of the different cosmid clones which led to widely different intensities in the hybridization signals. This complicated our interpretation of the results to such an extent that we abandoned characterization of the library using colony lifts. Despite these problems, this was the only large-insert *C. ruminantium* library available and we proceeded to isolate and investigate as many unique clones as possible.

Individual clone characterization from the library

As an alternative approach to clone isolation we subinoculated individual clones from the stored arrays and found that only 53 % of the clones would grow. Cosmid mini-preparations were made from the 448 clones which did grow. Restriction digestion analysis with *Not* I revealed that 18 % of the clones contained concatenated vector (Fig. 1, lane 6). This may be due to the fact that we did not size select the DNA for cloning after partial digestion with *Sau* 3AI, and a second vector was incorporated into small clones such that they had the appropriate size for packaging. A further 19 % of the clones had a small or no

insert (Fig. 1, lane 3). In order for cosmids to be packaged in phage heads they must contain 35–50 kb of DNA, therefore the small clones which we observed are most probably the result of deletions during subculturing of the clones. The insert sizes of the remaining 282 clones (63 %) averaged 35 kb. Analysis of a number of these clones by restriction digestion with *Eco* RI revealed a high percentage (>98 %) of unique clones (Fig. 1, lanes 4 and 5). While all the clones had what appeared to be large inserts, not all of the insert DNA was intact. Some preparations had a non-stoichiometric minor population of DNA bands having a pattern which differed from that of the major population of DNA bands (Fig. 2, lane 2). We picked 32 clones from which to make large DNA prepara-

tions. This subset of 32 clones was hybridized with riboprobes from each of the clones and it was found that two of the clones overlapped, 1H12 and 2C11.

Library analysis after 2 years storage at −70 °C

Optimization of growth conditions

The value of a library depends on the ability to culture the clones consistently and reproducibly and extract sufficient amounts of high quality DNA. In the quest for high DNA yields there is a trade off to be made: the richer the broth and the more the aeration, the faster the cells will grow; but when under pressure to grow faster, the clones are more likely to undergo deletion to minimize the time it takes to
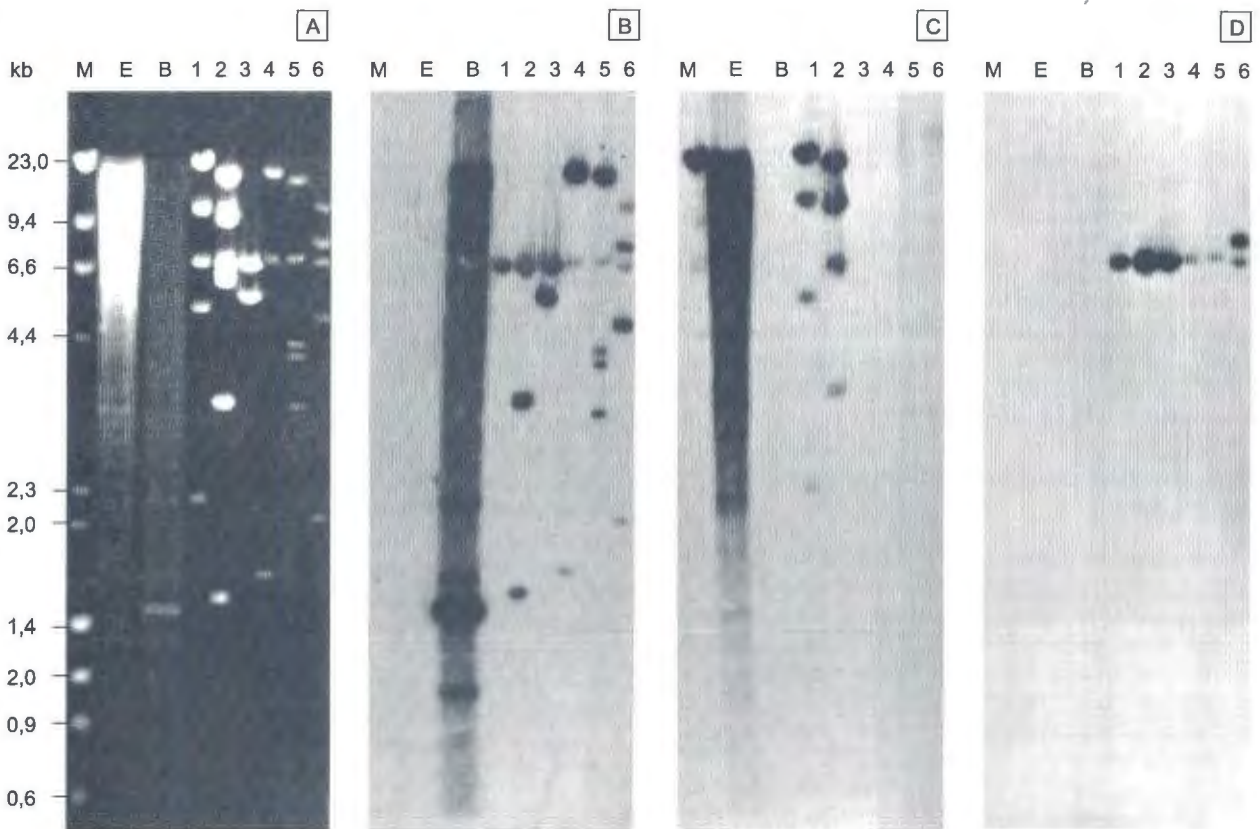

FIG. 1 Southern analysis of cosmid clones

Panel A shows the ethidium bromide stained gel of cosmid clones digested with *Eco* RI which was transferred to a nylon membrane for Southern analysis

Panel B has been hybridized with *C. ruminantium* DNA

Panel C has been hybridized with *E. coli* DNA

Panel D has been hybridized with the cosmid vector

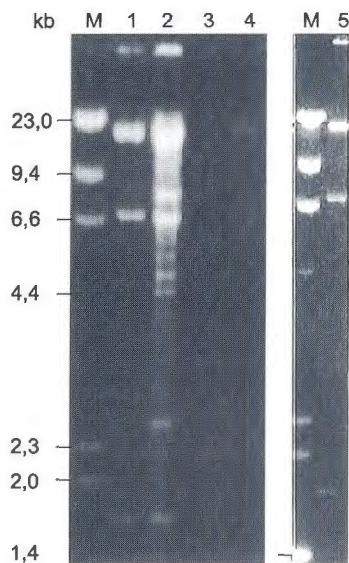|	
M	λ-*Hin*d III and φχ-*Hae* III molecular mass markers
E	*E. coli* DNA digested with *Eco* RI
B	bovine DNA digested with *Eco* RI
1	*E. coli* clone
2	mixed *E. coli/C. ruminantium* clone
3	deleted *C. ruminantium* clone
4 and 5	*C. ruminantium* clones
6	a clone containing concatenated vector and a small *C. ruminantium* insert

FIG. 2 Optimization of growth conditions

Eco RI digests of cosmid 1H12 grown as indicated:

M	λ-Hind III and φχ-Hae III molecular mass marker
Lane 1	37 °C at 400 rpm
Lane 2	RT at 400 rpm
Lane 3	RT at 300 rpm
Lane 4	30 °C at 300 rpm
Lane 5	37 °C at 300 rpm
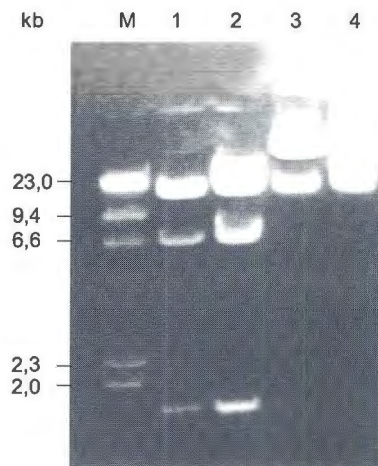
FIG. 3 Eco RI digests of cosmid 1H12 after growth in LB, LB+sucrose or TB. The digestion profiles indicate that rearrangement has occurred when the clone was grown in LB+sucrose or TB

M	λ-Hind III molecular mass marker
Lanes 1 and 2	two different DNA preparations grown in LB
Lane 3	LB+sucrose
Lane 4	TB

replicate. The recommendations of the supplier of the cloning vector were that clones should be grown for a minimal time of 8–10 h with as much aeration as possible. After the library had been in storage for 2 years we went back to the microtitre plate arrays to obtain more DNA from the clones. When the recommended approach was tried, we were not able to obtain any DNA and, in fact, the cultures had not begun to grow after 10 h. Therefore, it was necessary to investigate what conditions would provide consistent growth of our clones.

First we examined growth media: LB, LB+sucrose and TB were used in this study. Growth in the rich media, TB and LB+sucrose caused the clones to grow more quickly, leading to deletion and/or rearrangement of inserts (Fig. 3). LB was then used in all further experiments. To modify the growth rates of the clones, we tried growing them at different temperatures (room temperature, 30° C and 37° C) and aeration rates (shaking at 300 and 400 rpm). Deletions and rearrangements occurred in most of the clones (Fig. 2), leading to the conclusion that there was no single set of conditions for growth of all the cosmids which would ensure their stability. This meant that each clone would need to be considered independently of the others, an approach impractical in terms of both time and expense. Conditions were decided upon that ensured growth of the clones while not necessarily guaranteeing stability: 37 °C at an aeration rate of 300 rpm. These conditions were

used for all further work on growth and characterization of the cosmids.

By following this approach, 382 clones were grown successfully. Twenty per cent of these clones contained concatenated vectors and 86% of the clones had undergone deletions. Only 48 clones harbored large inserts, that is, only 13% of the clones grown 2 years after the library was made contained large inserts, compared to the initial study in which 63% of the clones contained large inserts. This is a large increase in the number of deleted clones, indicating that the library had not been stable over time, despite being stored at −70 °C.

Screening of unique clones for C. ruminantium sequences

Forty clones from both studies were screened against genomic DNA from bovine (*C. ruminantium* host cells), *Mycoplasma* (a potential contaminant of cultured cells), *E. coli* (a potential contaminant of cloning experiments) and *C. ruminantium* starting material. Twenty-two (55%) of the clones were found to harbor *C. ruminantium* inserts. No clones were found to hybridize with bovine or *Mycoplasma* DNA (data not shown). However, eight (16%) of the cosmid clones were found to contain full length *E. coli* inserts, i.e. the entire insert was derived from *E. coli* DNA (Fig. 1, lane 1). Four clones (10%) contained inserts which hybridized to *E. coli* and *C. ruminantium*

genomic DNA, indicating they contain mixed inserts (Fig. 1, lane 2). Six clones did not hybridize to any of the genomic DNA probes.

To find such a high proportion of *E. coli* inserts in this pool of clones was strange. The starting material did not contain *E. coli* DNA and a λZAPII library (Brayton *et al.* 1997) made from this same starting material also did not contain *E. coli* clones. The *E. coli* contamination most likely was introduced during the preparation of the cosmid vector and this is supported by the fact that the cosmid DNA has a faint background smear on hybridization to *E. coli* DNA (data not shown). This contamination is not visible on an agarose gel when analyzing 1 μg of vector. Therefore, if we estimate that there was 1 ng of *E. coli* DNA present for every 1 μg of vector in the ligation reaction used to construct this library, one would expect to obtain one *E. coli* clone for every 2000 *C. ruminantium* clones (0,05 %). Yet we have obtained at least 1 % of *E. coli* clones (8/846), 20 times more than we would expect. In our pool of viable clones, there is a representation of 25 % *E. coli* clones (12/48), 500 times more than expected. That the proportion of *E. coli* clones is so much more than expected is another indication that there is selection against *C. ruminantium* clones in this cloning system.

Summary

A number of problems was experienced when attempting to clone DNA from *C. ruminantium* in the SuperCos1 vector. We believe this has to do with a number of factors: the first being the high AT content of the *C. ruminantium* genome. *C. ruminantium* genes are ~70 % AT (Mahan *et al.* 1994; Van Vliet *et al.* 1994) and clones containing high AT inserts have been found by other investigators to be difficult to grow (Pan, Ravot, Tolle, Frank, Mosbach, Türbachova & Bujard 1999). We suspect that the lower melting temperature of AT rich clones decreases their stability during culture at 37 °C, giving rise to rearrangements. Another possibility is that the difference in AT content between the clone and the host cell serves to mark the clone as a foreign intruder and the host cell then deletes the insert to minimize this difference. A further difficulty may result from the fact that *C. ruminantium* promoters are known to be active in *E. coli* (Van Vliet *et al.* 1994; Brayton *et al.* 1997) and we believe that expression of certain *C. ruminantium* genes may lead to suppression of host cell growth. For instance, a full length *map*1 gene that is transcribed from its own promoter leads to lysed cultures when grown in the intracellularly maintained vector, pBluescript (Brayton *et al.* 1997). The SuperCos1 cosmid is maintained at high copy number, which may exacerbate these problems. It is interesting to note that these problems are not encountered in a phage library made from the same DNA starting material (Brayton *et al.* 1997). Since a phage will maintain the foreign DNA within the phage particle, the naked phage DNA will be present for a minimal time in contact with the cytoplasm of the cloning host and the foreign DNA is therefore not available to be processed. We were unable to find any reports in the literature stating that high copy number cosmid vectors are unsuitable as a cloning vehicle for bacterial genomes or genomes that have a high AT content.

Despite the problems detailed, this is the first report of a large insert library for *C. ruminantium*. Out of a total of 40 clones that were screened, seven clones (1H12, 2C11, 2E1, 6B4, 6D4, 6G1 and 9C3) were confirmed as bona fide *C. ruminantium* clones with intact inserts. These clones are the first large regions of the *C. ruminantium* genome to be cloned and represent about 170 kb of *C. ruminantium* sequence or almost 10 % of the total genome. Two of these clones overlap, to provide 55 kb of contiguous genomic sequence. Clone 1H12 is approximately 27 kb and is in the process of being sequenced.

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