The *Cowdria ruminantium* *groE* operon

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A *Cowdria ruminantium* genomic DNA library was constructed in the expression vector λ ZAPII, and an immunoreactive clone, designated λ Cr9.4, was isolated by screening with serum from a *C. ruminantium*-infected goat. Sequencing of the insert from this clone revealed two open reading frames, encoding peptides of 10462 and 58697 kDa respectively. Database searching indicated that the two genes were homologues of *groES* and *groEL*, genes encoding a group of heat shock proteins involved in protein processing, export and assembly. Western blotting experiments showed that the recombinant GroEL protein was recognized by sera raised against four isolates of *C. ruminantium* which originate from South Africa, West Africa and the Caribbean, but not by antisera to the closely related *Ehrlichia* species (*E. ovina*, *E. [Cytoecetes] ondiri*, *E. bovis*, *E. phagocytophila*) of African and European ruminants which can be expected to occur in similar geographical areas to *C. ruminantium*. This suggests that this protein may be useful in development of serodiagnostic tests for *C. ruminantium* infection which are not subject to cross-reactions with antibodies to *Ehrlichia* species. The cloning and expression of the GroE operon will also facilitate further study of the roles of the GroE proteins in the immune response to *C. ruminantium*.

Keywords: rickettsia, *Cowdria ruminantium*, heartwater, *groE* operon, heat shock protein

INTRODUCTION

Cowdria ruminantium is the causative organism of cowdriosis (heartwater), a tick-borne disease of domestic ruminants which is characterized by high fever, central nervous symptoms, hydrothorax and hydropericardium (Uilenberg, 1983). Heartwater occurs throughout much of sub-Saharan Africa, where it presents a major barrier to improved livestock production due to the extreme susceptibility of imported breeds. The disease is also present on some Caribbean islands, from where it presents a possible risk to livestock on the American continent due to the presence of *Amblyomma* ticks in both North and South America, which could act as vectors of heartwater.

One aspect of this disease which is in particular need of further research is the development of improved diagnostic tests. Currently available serological tests appear

to result in an unacceptable number of false positives, and this is thought to be due to cross-reaction with antibodies resulting from *Ehrlichia* infections (Du Plessis *et al.*, 1987). Another area in which further study is required is the investigation of immune responses to *C. ruminantium* infection. A better understanding of immunity to cowdriosis may enable the development of effective vaccines against the disease. Identification of *C. ruminantium* gene sequences may be the first step towards achieving these objectives. Cloning and expression of genes encoding antigens recognized by infected animals may be useful tools in the immunodiagnosis of heartwater. Some progress has already been made in identifying *C. ruminantium*-specific DNA sequences. Waghela *et al.* (1991) used a cloned *C. ruminantium* DNA probe to identify the organism in infected ticks. The same probe was subsequently used to identify infected sheep (Mahan *et al.*, 1992). Cloning of genes encoding *C. ruminantium* antigens will enable us to study their role in the development of immunity to cowdriosis, and may contribute towards the development of recombinant vaccines for cowdriosis.

In this study we report the sequence of an operon of *C. ruminantium* identified by immunoscreening of a genomic library constructed in the expression vector λ ZAPII. The

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Abbreviations: EB, elementary body; HSP, heat-shock protein.

The GenBank accession number for the sequence reported in this paper is U13638.

insert from this clone was partially sequenced and found to contain two open reading frames (ORFs) of 282 and 1659 bp which encoded proteins of predicted mass 10.5 kDa and 58.7 kDa, and which were homologous with the GroES and GroEL proteins, respectively, of *Escherichia coli*, and with homologous prokaryotic and eukaryotic gene products in the heat shock protein (HSP) 10 and 60 series. The 58 kDa antigen is recognized by antisera raised to several strains of *C. ruminantium* and may be of use in the development of serodiagnostic tests.

METHODS

Cowdria ruminantium isolates. The Welgevonden (Du Plessis, 1985) and Ball 3 (Haig, 1952) isolates were obtained from Dr F. Jongejan, University of Utrecht, the Netherlands. The Gardel isolate (Uilenberg *et al.*, 1985) was obtained from Dr E. Camus, CIRAD-EMVT, Point-a-Pitre, Guadeloupe. The Senegal isolate (Jongejan *et al.*, 1988) was obtained from Professor G. Uilenberg, CIRAD-EMVT, Maisons Alfort, Paris, France.

Antisera. Antisera to isolates of *C. ruminantium* were raised in British Saanen goats which had been reared in the UK, by inoculation with goat blood stabilates. Reactions were controlled by treatment with tetracycline on the second day of fever. Serum taken on days 0 (prebleed) and 28 were used for Western blotting. Serum taken on day 28 was used for screening and for preparation of recombinant-specific eluted antibodies. Bovine antiserum to experimental *C. ruminantium* (Ball 3) infection of a calf was a gift of Dr J. L. Du Plessis (Onderstepoort, South Africa).

Antiserum to *Ehrlichia* (*Cytoecetes*) *phagocytophila* (feral goat strain), the agent of tick-borne fever, was raised by infection of a Blackface sheep which had been reared in tick-free conditions in Scotland. Antiserum to *Ehrlichia ovina* (Turkish strain), the agent of ovine ehrlichiosis, was a gift of Dr F. Jongejan (Utrecht), and was raised by infection of a sheep (Jongejan *et al.*, 1993). Antiserum to *Ehrlichia bovis* was from a convalescent clinical case of bovine ehrlichiosis which occurred in an endemic focus in Brazil (Dr L. Passos, unpublished). Antiserum to *Ehrlichia* (*Cytoecetes*) *ondiri* (the agent of ondiri disease) was raised by experimental infection of a cow at Kabete, Kenya (Snodgrass, 1975).

Preparation of *C. ruminantium* elementary bodies. *C. ruminantium* (Welgevonden) was cultured in bovine aorta endothelial cells as described by Pow *et al.* (1993). When the cultures were highly infected, showing elementary bodies (EBs) present in every field of a cytospin preparation when observed through a 100 $\times$ objective, the supernate was removed and centrifuged at 1000 *g* to sediment cells and debris. The resultant supernate was removed and centrifuged at 16000 *g* for 20 min to pellet the EBs, which were then washed twice in phosphate-buffered saline (PBS), and resuspended in TNE buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA).

Construction and screening of *C. ruminantium* expression library. DNA was prepared from *C. ruminantium* (Welgevonden) EBs by incubating in TNE containing 2 mg lysozyme ml⁻¹ for 30 min at 37 °C, followed by the addition of SDS and proteinase K to final concentrations of 1% (w/v) and 500 μ g ml⁻¹ respectively and incubation for a further 30 min at 37 °C. The mixture was then extracted with phenol, followed by phenol/chloroform (1:1, v/v), and the DNA was precipitated with ethanol and redissolved in TE (10 mM Tris pH 8.0, 1 mM EDTA). Separate aliquots of *C. ruminantium* DNA were digested

with *Spe*I, *Xba*I and *Nhe*I, pooled and then ligated with *Xba*I-digested, dephosphorylated λ ZAPII arms. Following *in vitro* packaging, recombinant phage were screened with goat anti-*C. ruminantium* (Ball 3) antiserum using standard procedures (Huynh *et al.*, 1985). *E. coli* JM109 was used for growth and expression of Bluescript II SK plasmid clones, XL1-blue (Stratagene) was used for screening and titration of λ ZAP recombinants, and SOLR (Stratagene) was used for *in vivo* excision.

Bluescript II SK subclones. These were prepared from λ clones by *in vivo* excision. Briefly, 200 μ l of XLI-Blue plating cells, 20 μ l of the phage stock of the clone to be excised, and 1 μ l (> 10⁸ plaque-forming units) of ExAssist helper phage (Stratagene) were mixed and incubated at 37 °C for 15 min. To this was added 3 ml L-broth followed by incubation at 37 °C for 3.5 h, then heating to 70 °C for 20 min. Aliquots of the resulting phagemid stock were mixed with SOLR plating cells, incubated at 37 °C for 15 min, then spread on L-agar plates containing 50 μ g ampicillin ml⁻¹.

Preparation of subclones in an expression vector. A 0.7 kb *Hind*III fragment (Fig. 2) which contained part of the GroEL gene (Fig. 5) was purified from an agarose gel following *Hind*III digestion of the pBSCr9.4 plasmid DNA, and cloned into pTrcHis expression plasmid (Invitrogen) by standard techniques (Sambrook *et al.*, 1989), to put the encoded sequence under the control of the *trc* promoter and in-frame with the translational start site provided by the vector; expression was induced in late-exponential-phase cultures of NM522 cells by addition of IPTG to a final concentration of 1 mM.

DNA sequencing and sequence analysis. Dideoxy chain termination DNA sequencing (Sanger *et al.*, 1977) was performed using a T7 polymerase DNA sequencing kit (Pharmacia) and double-stranded plasmid DNA from Bluescript II SK subclones as template. Synthetic oligonucleotide primers were obtained from Oswel DNA service, University of Edinburgh. DNA and protein sequence analyses were carried out using software and databases available on the SEQNET molecular biology computer facility based at the SERC Daresbury Laboratory, Warrington, UK. Programs FASTA, PILEUP, GAP, CODONFREQUENCY and PUBLISH from the GCG package (Devereux *et al.*, 1984) were used for database searching, alignment of amino acid sequences, and manipulation and presentation of data.

Preparation of antigen for Western blotting. For Western blotting of *C. ruminantium* (Welgevonden) antigens, EBs were suspended in TNE containing 0.5% Nonidet P40 and 0.5% deoxycholate and placed on ice for 30 min. EBs were then disrupted by passage five times through a 19 gauge needle, incubated at 37 °C for 15 min, then passed through the needle a further five times. This preparation was then mixed with an equal volume of 2 $\times$ SDS-PAGE sample buffer (Laemmli, 1970) and heated to 100 °C for 5 min. *E. coli* lysates for Western blotting were prepared by centrifuging 1.5 ml of a bacterial culture, which had been incubated overnight at 37 °C, at 1000 *g* for 1 min, resuspending in 100 μ l distilled water, adding 100 μ l 2 $\times$ SDS-PAGE sample buffer then heating to 100 °C for 5 min. Samples were stored at -20 °C before electrophoresis.

SDS-PAGE and Western blotting. SDS-PAGE was performed according to the method of Laemmli (1970) using 7.5% or 10% (w/v) acrylamide separating gels, and 4% stacking gels. Electrophoretic transfer of proteins to nitrocellulose membranes was carried out using a semi-dry blotting apparatus at 0.8 mA cm⁻² for 30 min. Nitrocellulose membranes were blocked in 5% (w/v) dried skimmed milk diluted in PBST (PBS containing 0.05% Tween 20) for 30 min. Primary antisera were

preincubated with *E. coli* (JM109) antigens immobilized on nitrocellulose strips, at 1/50 dilution in PBST containing 5% (w/v) dried skim milk powder for 2 h at room temperature, before transfer to blots containing recombinant antigens and incubation for a further 1 h. Anti-species immunoglobulin horseradish peroxidase conjugates (rabbit anti-goat IgG or rabbit anti-bovine IgG, Sigma) were diluted to 1/500 in PBST containing 0.5% BSA and incubated with blots for 1 h. Washes between incubations consisted of two 10 min washes with PBST. Detection of bound antibody was achieved using 0.05% 4-chloro-1-naphthol in Tris-buffered saline containing 17% (v/v) methanol and 0.015% hydrogen peroxide.

Preparation of antibodies specific for the recombinant antigen. Two preparative polyacrylamide gels were run, gel 1 containing an *E. coli* lysate prepared from a JM109 clone carrying Bluescript II SK, and gel 2 containing a lysate prepared from the JM109/pBSCr9.4 clone. Proteins were transferred electrophoretically to nitrocellulose membranes, strips cut from either side, and these strips processed as for Western blotting in order to locate the position of the recombinant protein. These reference strips were then lined up with the appropriate blot, allowing a strip containing the recombinant 58 kDa protein to be cut from blot 2, and a strip from the corresponding region cut from blot 1 as a control. Strips were then blocked in 2% (w/v) dried skimmed milk diluted in PBST for 30 min, given two 10 min washes in PBST, then incubated in goat anti-*C. ruminantium* (Ball 3) antiserum diluted 1:50 for 3.5 h. This was followed by washing (3 × 10 min) in PBST. Bound antibodies were eluted from the strips by adding 1 ml 0.1 M glycine, 0.15 M NaCl, pH 2.5, and gently agitating for 5 min. This solution was neutralized by removing into 1 ml 1 M Tris/HCl pH 8.0. The strip was then washed with 3 ml PBST containing 0.5% BSA and this was added to the eluted antibody.

RESULTS

Identification and analysis of clone λ Cr9.4

Recombinant clone λ Cr9.4 was identified by screening a *C. ruminantium* genomic DNA library with goat anti-*C. ruminantium* (Ball 3) antiserum. Plaque purification of this clone followed by *in vivo* excision produced the Bluescript II SK plasmid subclone pBSCr9.4, which was found to contain an insert of approximately 7.4 kb. Western blotting of *E. coli* lysates prepared from this plasmid subclone showed that it expressed a peptide of approximately 58 kDa which was recognized by the anti-*C. ruminantium* antiserum. This peptide was not expressed by an *E. coli* clone carrying the Bluescript II SK plasmid (Fig. 1). By using *Hind*III and *Pst*I sites situated within the insert, two subclones were constructed, each containing a deletion at the 3' end of the insert (Fig. 2). Western blotting of *E. coli* lysates prepared from these clones (designated pP14.7 and pHI12.7) indicated that neither of them expressed the 58 kDa immunoreactive peptide expressed by pBSCr9.4. These results suggested that the expression of the gene encoding the 58 kDa protein, or at least the region encoding the epitopes recognized by the anti-*C. ruminantium* serum, requires the presence of sequence located downstream from the *Pst*I site at 4.7 kb (Fig. 2).

Subcloning of a 0.7 kb *Hind*III fragment (Fig. 2) into pTrcHis expression vector (to produce pTrcHisHI10.7) resulted in expression of a 35 kDa fusion protein that was

Fig. 1. A 58 kDa antigen is expressed in pBSCr9.4. Western blot analysis of *E. coli* lysates prepared from a JM109/pBSCr9.4 clone (lanes 1 and 4), a JM109/pBluescriptII clone (lanes 2 and 5), and *C. ruminantium* EB antigens (lane 3 and 6). Lanes 1, 2 and 3 were probed with anti-*C. ruminantium* (Welgevonden) antiserum, lanes 4, 5 and 6 with pre-infection serum. A peptide of approximately 58 kDa was recognized by immune serum in lanes containing pBSCr9.4 and *C. ruminantium* protein (arrowhead).

strongly recognized by antibodies in goat post-infection serum (Fig. 3). As the vector adds approximately 6 kDa to the fusion protein, the size of the fusion protein suggests that this fragment contains an ORF of approximately the same length as the fragment. Antibodies eluted from the 35 kDa antigen recognized only the 58 kDa antigen in pBSCr9.4 and in *C. ruminantium*, but not in the bacteria containing the pTrcHis vector (Fig. 3). This indicates that the 0.7 kb *Hind*III fragment expresses an antigen with common epitopes to the 58 kDa antigen. Sequence analysis indicated that the *Hind*III sites are located within the GroEL gene (see Fig. 5), which encodes a 58 kDa antigen; therefore the reaction of antibodies eluted from the 35 kDa fusion protein expressed by part of the GroEL

Fig. 2. (a) Restriction map of the pBSCr9.4 insert showing the approximate positions of *Hind*III and *Pst*I sites. (b) Inserts contained in pBluescript subclones pP14.7 and pHIII2.7, neither of which expressed the 58 kDa protein that was expressed by pBSCr9.4, and in pTrcHisHIII0.7, which expressed a 35 kDa protein. (c) Position of the *C. ruminantium* GroES and GroEL genes in the 7.4 kb insert.

gene, to the 58 kDa antigen indicates that the latter is the size of the GroEL gene product in both recombinant and native forms.

Recognition of the 58 kDa antigen by antibodies to *C. ruminantium*

In order to identify the *C. ruminantium* protein corresponding to the 58 kDa protein expressed by pBSCr9.4, antibodies specific for the recombinant peptide were eluted from a Western blot. This antibody preparation recognized a band of approximately 58 kDa on a Western blot of *C. ruminantium* EBs (Fig. 3). This 58 kDa protein of *C. ruminantium* is therefore likely to be the protein corresponding to the recombinant 58 kDa protein expressed by pBSCr9.4. The recombinant 58 kDa protein was in all cases strongly recognized by immune sera raised in goats to the heterologous *C. ruminantium* isolates Ball 3, Gardel and Senegal, which originate from South Africa, Guadeloupe and Senegal, respectively (Fig. 4). No detectable reaction of the antigen with pre-infection sera of these animals was observed, nor did it react with antisera from a sheep pre- or post-infection with *Ehrlichia phagocytophila*, or post-infection with *E. ovina* (Fig. 4). The antigen was recognized by antiserum from a cow immunized with the Ball 3 stock, but not by post-infection sera from infections of cattle with *E. bovis* or *E. omdiri* (Fig. 4).

DNA sequencing

The insert from pBSCr9.4 was partially sequenced using plasmid DNA prepared from this clone, and from a series of additional plasmid subclones containing restriction

Fig. 3. Identification of the *C. ruminantium* protein corresponding to the 58 kDa recombinant protein and evidence that the 58 kDa protein is encoded by the GroEL gene. Antibody against *C. ruminantium* eluted from nitrocellulose strips containing the 58 kDa protein expressed by pBSCr9.4, or the 35 kDa protein expressed by pTrcHisHIII0.7, recognizes a 58 kDa antigen in *C. ruminantium*. Lanes 1 to 3, antibody eluted from the 58 kDa protein expressed by pBSCr9.4: lane 1, eluted antibody recognizes only the 58 kDa protein (arrowhead at left) in a lysate of JM109/pBSCr9.4; lane 2, lysate of JM109/pBluescript; lane 3, antibody eluted from the recombinant 58 kDa protein recognizes a 58 kDa antigen in the *C. ruminantium* EB. Lane 4, Western blot of *C. ruminantium* EB antigen probed with anti-*C. ruminantium* antiserum (immunoblot transfer from edge of gel). Lanes 5, 6, 7 and 9, antibody eluted from the 35 kDa protein expressed by a subcloned 0.7 kb fragment of the GroEL gene (pTrcHisHIII0.7) recognizes a 58 kDa antigen present in the *C. ruminantium* EB (lane 5), and in a lysate of JM109/pBSCr9.4 (lane 6) and recognizes only the 35 kDa protein expressed by JM109/pTrcHisHIII0.7 (lane 7) but not other antigens present in a lysate of JM109/pTrcHis (lane 9). Lane 8, anti-*C. ruminantium* antiserum recognizes a 35 kDa antigen in a lysate of JM109/pTrcHisHIII0.7 (arrowhead at right).

fragments of the pBSCr9.4 insert. Sequencing was first undertaken on the 0.7 and 2.0 kb *Pst*I fragments, which originate between 4.7 and 5.5 kb, and 5.4 and 7.4 kb, from the 5' end of the insert (relative to the orientation shown in Fig. 2). Sequence obtained from these *Pst*I fragments included a large ORF which encompassed the entire 0.7 kb fragment and ended 113 bp downstream from the beginning of the 2.0 kb *Pst*I fragment. Sequencing was then continued upstream from the 0.7 kb *Pst*I fragment. A total of 2440 bp were sequenced (Fig. 5) and this included two ORFs. The shorter ORF of 282 bp spanned nucleotides 91–372 and encoded a hypothetical peptide of 10462 Da; the other ORF of 1653 bp spanned nucleotides 472–2124 and encoded a peptide of 58697 Da. Purine-rich putative ribosome-binding sites were identified 6 bp upstream from the shorter ORF start codon and 7 bp from the longer ORF (Fig. 5). In addition, sequences resembling *E. coli* –35 and –10 promoter sequences were identified at positions 9–14 and 29–34 respectively. There was also a putative terminator consisting of a region of dyad symmetry spanning bp 2155–2178. The

Fig. 4. The 58 kDa recombinant antigen is recognized by antisera raised to various strains of *C. ruminantium*. Western blots of lysates of JM109/pBSCr9.4 or JM109/pBluescript (control), were reacted with antisera raised to *C. ruminantium* or various *Ehrlichia* species. Reaction to the 58 kDa antigen (arrowhead) was detected only with antisera raised to *C. ruminantium*, and only in blots containing pBSCr9.4. Odd-numbered lanes, JM109/pBluescript lysates; even-numbered lanes, JM109/pBSCr9.4 lysates. Lanes 1 and 2, caprine *C. ruminantium* (Welgevonden) infection, day 0 post-infection; lanes 3 and 4, day 28 post-infection. Lanes 5 and 6, caprine *C. ruminantium* (Senegal) infection, day 0; lanes 7 and 8, day 28. Lanes 9 and 10, caprine *C. ruminantium* (Gardel) infection, day 0; lanes 11 and 12, day 28. Lanes 13 and 14, caprine *C. ruminantium* (Ball 3) infection, day 0; lanes 15 and 16, day 28. Lanes 17 and 18, ovine *E. phagocytophila* infection, day 0; lanes 19 and 20, day 28. Lanes 21 and 22, ovine *E. ovina*, 4 weeks post-infection. Lanes 23 and 24, bovine *E. bovis* infection, convalescent sera. Lanes 25 and 26, bovine *E. ondiri* infection, convalescent serum. Lanes 27 and 28, bovine *C. ruminantium* (Ball 3) infection, 6 weeks post-infection.

58 697 Da protein encoded by the 1653 bp ORF probably corresponds to the 58 kDa peptide that was observed on Western blots of clone pBSCr9.4.

Homology of the *C. ruminantium* sequence with other bacterial sequences

The amino acid sequences encoded by the cloned *C. ruminantium* ORFs were compared with sequences in the SwissProt protein sequence database. They were found to be homologous with the GroES and GroEL proteins of *E. coli*, and with GroE homologues in other species. The *C. ruminantium* GroES and GroEL amino acid sequences are aligned with those from several other bacterial species in Fig. 6. The *C. ruminantium* sequences showed the closest similarity to those of *Ehrlichia chaffeensis*, with 83.2% and 93.5% identity for GroES and GroEL respectively. For the other bacterial sequences shown the percentage of identical amino acids was between 30 and 41% for GroES and between 46 and 52% for GroEL. The *groES*–*groEL* intergene spacer region differs in length by 5 nucleotides between *C. ruminantium* and *E. chaffeensis*, and very considerably between these species and the

corresponding region in *Rickettsia tsutsugumushi* and *Coxiella burnetii*, where it is 72 bp and 27 bp, respectively.

When the *C. ruminantium* and *E. chaffeensis* GroEL amino acid sequences are aligned over their entire lengths there are 37 amino acid differences, and an additional amino acid (asparagine) close to the C-terminus in the predicted GroEL of *C. ruminantium*. Most of the amino acid differences consist of conservative substitutions. Perhaps of interest is the fact that these mismatches include four cysteine residues which are present in the *C. ruminantium* sequence but not in the *E. chaffeensis* sequence. Another interesting feature is the presence of a repeating M-G-G motif at the C-terminus of the *C. ruminantium* protein.

DISCUSSION

We have cloned and sequenced the *groE* operon of *C. ruminantium*. Homologues of GroES and GroEL, also known as HSP10 and HSP60 respectively, have been identified in a variety of different organisms and are involved in protein folding, oligomerization and export (Zeilstra-Ryalls *et al.*, 1991). They are expressed constitutively at all temperatures but their expression is

1 gaotgatacttgaaatataatttctgtatatatttatggttttaatoagotgatgotgataaatttttttaacagaggtagtat
-35 -10 SD

91 atgaattttaaatattgttacatgacaattgtttaaatttaggottttagaagagagtottaataaatttoctatacaaaccttoctgaatcagca
M N L N M L E D N V L I E A L E S L N N S P I Q L P E S A
181 aaaaaagccaaactaaaagggaaagtagtagctgttgggtocagggtottacaataataatggtaacctoattcoctatgactcttaaagta
K K K P T K G K V V A V G P G S Y N N N G N L I P M T L K V
271 ggtgtatgtattgtttatogccagtggtgtgttaagtgaggattttotgtataagaatacatagttatgagaagagagtataaatt
G D V F V F Y R Q W A G N E V E F S D K K Y I V M K E S D I I
361 gogaagaagataataaattgtatttagtatattaataaataatagtttttttgcattgtgtgaagtataggtgttttagttaattttt
A K E V *
451 cattataaatggagtgtaaaaaacatggcacaatatggttgttaacagggggagcaactagacaaaatcaattagagaagttgtcoatttttagaa
SD M A N M V V T G E Q L D K S I R E V V R I L E
541 gaogcagtaggatgtcacagctgtgtccaaaaggattgactgtagatttagtaaaactattgtgtgaccagaggtgtgacaaaagattggatat
D A V G C T A G P K G L T V A I S K P Y G A P E V T K D G Y
631 aggtttatgaaaagcataaagccagaagattccattggctotttgtattgtctaataaattgtccaagtgtctotcaattgtaacgataaaa
K V M K S I K P E D D P L A L A I A N I A Q S A S Q C N D K
721 gtagggtgtgaaactacatgtcttctattttaactgcaaaggttattgaggaagtattotaaggttaaggtgtgtgtgcagatatttatt
V G D G T T T C S I L T A K V I E E V S K V K A A G A D I I
811 tbtgttaggggaaggtgtacttaagctaaagaggcgagttattagaagcttttaaatgcctggaagcgtggaagtgttatctgaagaagaatt
C V R E G V L K A K E A A V L E A L K C M K R E V L S E E I
901 gcccaattgtcaactatctogcaatgtgtgataaaaaattataggtactaaaattgccagctgtgttaagaagttggtaagagtggygtt
A Q V A T I S A N G D K N I G T K I A Q C V K E V G K D G V
991 attacagtgtaagaagaagatttaaaagagtttagattgttgaaaagactgtgtgtatgcaattgtgatagaggtatcttctotctatt
I T V E E S K G F K E L D V E K T D G M Q F D R G Y L S P Y
1081 ttgttactaactoaagaaaagattgttagttgagtttgaaaatoottatattttataacagaaaagaaactaaataataacaaactota
F V T N S E K M L V E F E N P Y I L L T E K K L N I I Q P L
1171 ttgccaatttttagagaataattgttagatcaggttagaccaacttttaattattgcaagaggtgtgagaaggtgaagcaatttagtacaactgtta
L P I L E N I A R S G R P L L I I A E D V E G E A L S T L V
1261 ttgaataaagttgctgtgtgtttacatgtagctgcagtttaagctocagggtttggagatagaagaagaatgatgttaggttagatttgca
L N K L R G L G L E V A A V K A P G F G D R R K D M L G D I A
1351 attttaactggagctaaacatgtataaattgatgagcttgtattataaattggaagatttgactctatgtgtattaggaactgtctaagaat
I L T G A K H V I N D E L A I K M E D L T L C D L G T A K N
1441 ataogtattacaaaagataactacaactatttggtagtgtagataaattgtgtgtotcattgttocaagtagaattgttocaatttagaattg
I R I T K D T T T I I G S V D N S C A H V Q S R I C Q I R M
1531 caaattgataaattctaatogattatgtataagaagaagctacaagagcgttttagcaaggtttcttgggtgtgtgtgtttgaaagta
Q I D N S T S D Y D K E K L Q E R L A K L S G G V A V L K V
1621 ggtggatctagtgaagttgaagtttaaggaacgttaagatogtgtagaagatgtctcttcagcaactagagctgtgtgtgaagaaggtgtt
G G S S E V E V K E R K D R V E D A L H A T R A A V E E G V
1711 gtgcccaggtgtgtgtgtgttattatcacattatcagolggtgacaacttaaaaagtagaagatgatgatgaacagttgggttttaatt
V P G G G A A L L Y T L S A L D N L K S K N D D E Q L G I N
1801 attgtaaaogtgcttttaacagctcctataaagoggattattataaaaatgctggatcagaataatgcacactgtgtttattgtctattttg
I V K R A L Q A P I K R I I K N A G S E N A P C V I A H L L
1891 aagcaaaatgataaggaaactattatcttaattgtgtatgaagaattttgtcaatgcattactcttgaggtaattgtatccgttaaaagta
K Q N D K E L I F N V D V T N F A N A F T S G V I D P L K V
1981 gtgogatagcaatttggatttctgtatcttctgtgcagttatttgacacttaattgtattgtgtgtatattocatcaagagatg
V R I A F D F A V S L A A V F M T L N A I V V D I P S K D D
2071 aatagtgctgcoggtgtgtgtgtatgggaagcagtggtgtgtatgggtgttttaagtaaaccatatccttttaattattatgtaggga
N S A A G G A G M G G M G G M G G F * <<<<<<
2161 agttgtttaactcctcgttaogttattgatgtgatttttttaagtttaacttatcotaattttatttaggtttttattattcogtga
<< >>>>>>>>
2251 tttacaagtaactgataagtggtgtgtattatggttagagtagttgtaatttttagtatattactgacaaacatgtgtatttttagtattgt
2341 tatgttccagaattaaaaaagtgtttaattgtgtgtagtattccttaagttccaaaaaagtagtgttgctaatagttactgtatgtctgtt

2431 ctattaaaga 2440

Fig. 5. Nucleotide and deduced amino acid sequences of the *C. ruminantium* *groES* and *groEL* genes. The putative promoter sequences are underlined, as are the ribosome-binding sites (SD). A putative terminator sequence is also indicated (<<<< >>>>). The *Hind*III sites which were used in cloning the 0.7 kb fragment into pTrcHisIII0.7 are indicated by a line above the *Hind*III recognition sequence (AAGCTT).

increased considerably under conditions of heat shock or other metabolic stress. In *E. coli*, and in several other species of bacteria including *Ehrlichia chaffeensis* (Sumner *et al.*, 1993), *Rickettsia tsutsugamushi* (Stover *et al.*, 1990), *Coxiella burnetii* (Vodkin & Williams, 1988), *Brucella abortus* (Lin *et al.*, 1992), *Chlamydia psittaci* (Morrison *et al.*, 1990) and *Chlamydia trachomatis* (Cerrone *et al.*, 1991), the genes encoding GroES and GroEL are adjacent to one another and are apparently co-transcribed from a single

promoter. Analysis of the *C. ruminantium* DNA sequence described above suggests that this may also be the case in this organism.

It is not surprising that of the available GroE sequences the *C. ruminantium* sequence showed closest similarity to that of *E. chaffeensis*. Recent work on the 16S ribosomal RNA genes of *C. ruminantium* indicated that this organism formed a close phylogenetic cluster with *E. canis* and *E.*

Fig. 6. Amino acid sequences of *C. ruminantium* GroES and GroEL aligned with those of *E. chaffeensis*, *Rickettsia tsutsugamushi*, *Coxiella burnetii*, *Escherichia coli*, *Chlamydia psittaci*, and *Mycobacterium tuberculosis*. Identical amino acids are indicated by a hyphen (-). Gaps introduced to allow alignment are indicated by a full stop (.).

GroES

[illegible]

	81	104
C. rumi	NEVEFSDNKY	IVMKESDIIA KEV.
E. chaf	-I--HE---	---A.
R. tsut	T-IKLES-D-	V-I-----LL VKS.
C. burn	T--KLAGE-	---R-D--MG VIEK
E. coli	KSEKIDNEEV	LI-S-----IVEA
C. psit	Q-LTVDGEE-	VIVQ---EVM-VLK.

GroEL

[illegible][illegible][illegible]

	241											320
C. rumi	IARSGRPILI	IAEDVEGEAL	STLVLNKLRG	GLEHVAVKAP	GFGDRRUDML	GDIAILTGAK	EVINDELAIK	MEDL	TLCDL			
E. chaf	V-----						S-D-----					AE
R. tsut	V-HT-K-VL	-D-D-	TA-I-N-K	SIK-V-	E-----	E-----	NGE	TEQ-G	L-KVNDTSK			
C. burn	V-K-----V	-I-----	A-V-NI	VVK----	A-----	Q-V-----	G-----	SE-VGLS	L-A-AS-D			
E. coli	V-KA-K----		A-A-V-TI	IVK----	A-----	Q-----	T-GT	SE-IGME	L-K-A-E-			
C. psit	V-E-----	-I-----	A-V-R-A	FR-C----	A-----	E-----	GQ	SE-GM	L-N-T-AM			
M. tube	VIGA-K----		V-I-----	TPKSV----	A-----	Q-M-----	QQ	SE-VGLT	L-N-AD-SL			

[illegible][illegible][illegible]

Fig. 6. For legend see facing page.

chaffeensis (Van Vliet *et al.*, 1992). The degree of DNA sequence conservation between the GroE operons of *C. ruminantium* and *E. chaffeensis* (84.6% identity in DNA sequence over 2277 bp) is less than that of the 16S rRNA genes of these species (97% over 1457 bp; Van Vliet *et al.*, 1992), and is as low as 70% in the 104 nucleotides in the intervening sequence between the stop codon of GroES and the start codon of GroEL. The variation in sequence conservation between regions of the GroE operon suggests that this genetic locus may be of value for phylogenetic comparison of *Cowdria* strains and *Ehrlichia* species.

The repetitive M-G-G motif present at the C-terminus of *Cowdria* GroEL has also been reported in *R. tsutsugamushi*, *C. burnetii*, *Mycobacterium leprae*, *E. coli* and *Ehrlichia chaffeensis* sequences (Stover *et al.*, 1990; Vodkin & Williams, 1988; Gillis *et al.*, 1985; Hemmingsen *et al.*, 1988; Sumner *et al.*, 1993). It has been suggested that these repeats may serve as an anchor to the membrane or peptidoglycan fraction in the cases of *C. burnetii* and *M. leprae*, since in these species there is evidence that GroEL is membrane-associated (Vodkin & Williams, 1988; Gillis *et al.*, 1985). The cellular location of the *C. ruminantium* GroEL is not known, but the recognition of a 58 kDa antigen of the *Cowdria* EB by antibody eluted from the recombinant 58 kDa antigen (Fig. 3) suggests that the GroEL antigen is present in the extracellular stage of the organism.

The codon usage in the *Cowdria groES* and *groEL* genes indicated a bias towards the use of AT-rich codons. Codons ending in A or T are used more frequently than those ending in G or C at synonymous sites, for example there were no codons ending in G or C for histidine, tyrosine and phenylalanine in 5, 7 and 12 codons respectively. In the case of leucine and arginine the bias extends to the first and second codon positions, and only in the case of lysine (27/20 codons, ratio 1.35) was the ratio for A + T codons to G + C codons less than 3. The bias towards AT-rich codons is more pronounced in *groEL* than in *groES*. A similar bias towards the use of codons ending in T was noted for the *C. burnetii groEL* gene (Vodkin & Williams, 1988). The use of AT-rich codons by the *C. ruminantium groE* genes is probably associated with the high A + T content of the *C. ruminantium* genome, as deduced from other available *C. ruminantium* DNA sequences, which have reported G + C contents of 26.0 mol% (Mahan *et al.*, 1992), 29.3 mol%, 29.6 mol% (Waghela *et al.*, 1991), and 30.4 mol% (Van Vliet *et al.*, 1994).

Western blotting experiments showed that goats infected with four different isolates of *C. ruminantium* each developed antibodies which recognized a recombinant 58 kDa antigen, and antibody eluted from the recombinant antigen recognized a similar-sized antigen in the *Cowdria* EB. The identity of the 58 kDa antigen as a homologue to the GroEL protein of *E. coli* was shown by reaction of antibody eluted from a fusion protein expressed by a 0.7 kb fragment of the *groEL* gene to the 58 kDa antigen present in the EB of *C. ruminantium*. The 58 kDa antigen is of the size predicted from the sequence

of the *groEL* gene and must have been expressed through recognition of the *Cowdria groE* promoter sequences in *E. coli*, as reported for expression of the *groEL* gene of *Ehrlichia chaffeensis* (Sumner *et al.*, 1993). However, an immunoreactive antigen of the expected size of the GroES protein was not detected in immunoblots, as also reported for the recombinant GroES protein of *Rickettsia tsutsugamushi* (Stover *et al.*, 1990). An antibody response to the GroEL antigen following infection with *C. ruminantium* is not surprising, since temperature changes and intracellular processing of the organism by antigen-presenting cells during the febrile response of the infected animal are likely to result in high levels of expression and presentation of this peptide. Additionally, strong immune responses to GroEL have been described in infection with several pathogens including *Mycobacterium tuberculosis* (Shinnick, 1987), *Mycobacterium leprae* (Young *et al.*, 1988), *Chlamydia trachomatis* (Morrison *et al.*, 1989) and *Coxiella burnetii* (Vodkin & Williams, 1988), each of which have, as is the case with *Cowdria ruminantium*, an obligate intracellular stage in the infection of animal hosts. However, previous studies of antibody responses to *C. ruminantium* infection have not described reactivity to an antigen of approximately 58 kDa (Rossouw *et al.*, 1990; Jongejan & Thielmans, 1989). The immunodominant antigen in *C. ruminantium* infections appears to be a protein of approximately 32 kDa which is conserved among at least nine *C. ruminantium* isolates (Jongejan & Thielmans, 1989). In another study (Rossouw *et al.*, 1990), two peptides of 31 kDa (which may be the same as the 32 kDa band of Jongejan & Thielmans, 1989) and 29 kDa were identified as immunodominant, and conserved among nine different *C. ruminantium* isolates. However, close inspection of the Western blots in this publication (Rossouw *et al.*, 1990) reveals another antigen of between 50 and 60 kDa which is recognized by sera raised against at least three separate *C. ruminantium* isolates. The authors do not comment on this band, presumably because it was not so consistently recognized as the 29 and 31 kDa bands. The lack of consistency with which this 50–60 kDa antigen appears to be recognized by immune serum in Western blots may be a result of different methods of preparation of *C. ruminantium* antigen rather than a true variation in reactivity, or absence in particular isolates.

Antigenic differences between strains of *C. ruminantium* are recognized (Jongejan *et al.*, 1988), and therefore the recognition of *C. ruminantium* GroEL by serum from animals infected with four distinct strains of the organism, but apparently not by antibodies to four of the most significant ehrlichial infections of ruminants, indicates that the recombinant antigen may be useful in the development of a specific serodiagnostic test for heartwater. In view of the close similarity between the amino acid sequences of the GroEL proteins of *C. ruminantium* and *E. chaffeensis*, it is to be expected that species in these genera will have some GroEL epitopes in common, but that there may also be regions of the protein which contain *C. ruminantium*-specific determinants. Studies on GroEL from other bacterial species indicate that both conserved and species-specific epitopes are present (Gillis

et al., 1985; Plikaytis *et al.*, 1987; Stover *et al.*, 1990). Monoclonal antibodies to HSP60 of *Ehrlichia risticii* and *E. sennetsu* did not recognize the HSP60 of other bacteria tested, including *Chlamydia*, *Rochalimea*, *Rickettsia typhi* and *Wolbachia* (Dasch *et al.*, 1990). The observation that the *C. ruminantium* GroEL protein sequence has cysteine residues that are not found in the *E. chaffeensis* sequence may be of interest in terms of trying to identify regions which may contain species-specific epitopes. The cysteine residues in the *C. ruminantium* protein may participate in disulphide bonding and consequently form conformational epitopes. If these cysteine residues are not conserved in *Ehrlichia* species then such conformational epitopes are likely to be *C. ruminantium*-specific.

The observation that strong T- and B-cell immune responses to bacterial GroEL proteins occur in a variety of infections has led to the suggestion that they may play a role in protective immunity (Young *et al.*, 1988). However, GroEL is a highly conserved protein, with homologues having been identified in both prokaryotes and eukaryotes. Generation of immune responses to such highly conserved proteins has been associated with autoimmune reactions (Young, 1990), and a greater risk of immunopathology must exist in infections in which the host is persistently infected with the pathogen. Stress response proteins, such as GroEL, may be expected to be expressed in response to adverse intracellular environments, such as may result during immunological reactions to infected cells (Thole & Van der Zee, 1990). Immunological responses to GroEL have been implicated in the development of arthritis (Van Eden *et al.*, 1988), and it has been suggested that a similar mechanism may occur in certain chlamydial disease conditions (Morrison *et al.*, 1989). Infection of ruminants with *C. ruminantium* is recognized to result in a carrier state, with infectivity to ticks being retained for up to 8 months (Andrew & Norval, 1989). It is possible that immunopathological consequences may result through a persistently stimulated immune state to *Cowdria* or *Ehrlichia* antigens. The cloning and expression of the *C. ruminantium* GroE operon should therefore facilitate the development of serodiagnostic tests and enable investigation of the role of GroEL in protective immunity and pathogenesis of heartwater.

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