Phylogenetic Relationship of *Cowdria ruminantium*, Agent of Heartwater, to *Anaplasma marginale* and Other Members of the Order *Rickettsiales* Determined on the Basis of 16S rRNA Sequence†

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The phylogenetic relationship between *Cowdria ruminantium* and representative members of the orders *Rickettsiales* and *Chlamydiales* has been examined on the basis of the sequence of the 16S rRNA. Phylogeny reconstruction by using both parsimony and distance methods supports the conclusion that *C. ruminantium* is closely related to the *Rickettsiales* and in particular to the family *Anaplasmataceae*. A signature of nine base substitutions delineated the linkage of *Anaplasma marginale* with *C. ruminantium* and differentiated these two species from the 45 other members of the alpha group of *Proteobacteria* examined, and five of these base substitutions were unique among all members of the class *Proteobacteria* examined to date.

Heartwater, or cowdriosis, is a tick-borne disease of domestic and wild ruminants caused by the intracellular, rickettsiallike pathogen, *Cowdria ruminantium* Cowdry 1925 (15). The disease is important in tropical Africa, where it is a major barrier to improved livestock production (13), and its presence on islands in the Caribbean Sea makes it a threat to livestock on the American mainland (1). *C. ruminantium* is the only species defined in the genus *Cowdria* Moshkovski 1947, and is currently placed in the order *Rickettsiales*, family *Rickettsiaceae*, tribe *Ehrlichieae* (15). Other genera included in this tribe are *Ehrlichia* and *Neorickettsia*. Our data indicate that *C. ruminantium* is a member of the order *Rickettsiales* rather than the *Chlamydiales*, as has been previously suggested (19), but it is monophyletic with a species of the type genus of the family *Anaplasmataceae* rather than the *Ehrlichieae* (15).

The phylogenetic relationship of *C. ruminantium* to other members of the order *Rickettsiales* has not previously been determined by analysis of nucleic acid sequences, since it has not been possible to cultivate and obtain large numbers of organisms in the past. We have circumvented this difficulty by preparing DNA from *C. ruminantium* cells grown in culture with bovine endothelial cells from which we have specifically amplified, cloned, and sequenced the *C. ruminantium* 16S rRNA coding region by using the polymerase chain reaction (PCR) (9, 22, 25).

MATERIALS AND METHODS

Preparation of genomic DNA and amplified 16S rRNA gene.

Genomic DNA was prepared (17) from the Crystal Springs isolate cultured in bovine endothelial cells derived from the aorta (28). *C. ruminantium* elementary bodies were collected from culture supernatants for extraction of DNA by centrifugation for 10 min at $2,000 \times g$ to remove the remnants of bovine cells lysed by the infection followed by centrifugation at 4°C for 30 min at $30,000 \times g$ to pellet the elementary bodies. DNA prepared from *C. ruminantium* purified in this fashion contains some bovine DNA, but 90 ng of DNA from

this preparation was sufficient to amplify the 16S rRNA gene(s) in vitro via PCR (9) by using a primer set described by Wilson et al. (25). PCR was performed in a volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM deoxynucleoside triphosphates, 1 μM primer PC5, 1 μM primer PO_{MOD}, and 2.5 U of *Taq* polymerase. Thermal cycling was performed in a Coy Model 60 cyclotherm in which the target DNA was denatured by incubation at 92°C for 10 min followed by 35 cycles of denaturation (92°C for 1 min), primer annealing (55°C for 3 min), and primer extension (72°C for 3 min). At the end of cycling, the reaction mixture was held at 72°C for 10 min and cooled to 4°C . A fragment of 1,463 bp, which ran as a single ethidium bromide-staining band on a 0.8% agarose gel, was amplified. The extreme 5' and 3' ends of the 16S rRNA gene and adjacent regions were amplified by inverse PCR (10) after the genomic DNA was cut with *Xba*I and then ligated. The PCR primers at positions 64 to 50 and 1337 to 1351 (sequences shown in Table 1) were used under the conditions described above, and amplification produced a 1.8-kb fragment.

DNA sequence analysis and cloning. The 1,463-bp amplified DNA fragment was sequenced directly in both strands by the dideoxy chain termination method (18) by using ^{32}P -end-labeled oligonucleotide sequencing primers labeled with T4 polynucleotide kinase (17). Suitable primers for sequencing the 16S rRNAs from members of the *Rickettsiaceae* were identified by aligning the sequences of 16S rRNAs of members of the *Rickettsiaceae* available in the GenBank data base with that of *Escherichia coli* (from the gamma group of the *Proteobacteria*) by using the multiple-sequence alignment program, CLUSTAL (Intelligenetics, Mountain View, Calif.). Regions of the 16S rRNA sequence which were sufficiently conserved between members of the *Rickettsiaceae* and *E. coli* were identified at positions 50 to 64, 297 to 311, 558 to 572, 787 to 806, 942 to 956, 1083 to 1097, and 1337 to 1351 (*E. coli* numbering). The corresponding and complementary oligonucleotide primers in Table 1 were prepared synthetically (14). In some cases, these primers were mixtures at one or two positions. The amplified DNA was also ligated into the *Sma*I site of pBluescript SK+ (Stratagene) after being prepared for cloning by polishing the ends with the Klenow fragment of DNA polymerase I (17). Regions of the sequence which were not determined with certainty by

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TABLE 1. PCR and sequencing primers

Primer sequence	Position in <i>E. coli</i> 16S rRNA
Forward	
5'AGAGTTTGATCCTGG3' ^a	8-22
5'AACACATGCAAGTCG3'	50-64
5'GAGAGGA(C/T)GATCAGC3'	297-311
5'GAAT(C/T)ACTGGGCGTA3'	558-572
5'ATTAGATACCCTGGTAGTCC3' ^b	787-806
5'TGGGAG(C/T)ATG(C/T)GGTT3'	942-956
5'TGTTGGGTTAAGTCC3'	1083-1097
5'GGAATCGCTAGTAAT3'	1337-1351
Reverse	
5'TACCTTGTACGACTT3' ^c	1507-1492
5'ATTACTAGCGATTCC3'	1351-1337
5'GGACTTAACCCAACA3'	1097-1083
5'AACC(A/G)CAT(A/G)CTCCAC3'	956-942
5'GGACTA(C/T/A)AGGGTATCTAAT3'	806-787
5'TACGCCAGT(A/G)ATTC3'	572-558
5'GCTGATC(A/G)TCCTCTC3'	311-297
5'CGACTTGCATGTGTT3'	64-50

^a PO_{MOD}.^b P3_{MOD}.^c PC5 (25).

direct sequencing of the PCR product were confirmed by sequencing the cloned fragment. The 1.8-kb amplified fragment was cloned as a blunt fragment in the *EcoRV* cloning site of pBluescript SK+, and the ends were sequenced by using T3 and T7 sequencing primers.

Phylogenetic analyses. Comparison of the *C. ruminantium* sequence with the published sequences of *Anaplasma marginale* (M60313), *Coxiella burnetii* (M21291), *Chlamydia psittaci* (M13769), *Ehrlichia risticii* (M21290), *E. coli* (J01695), *Rochalimaea quintana* (M11927), *Rickettsia prowazekii* (M21789), *Rickettsia rickettsii* (M21293), and *Wolbachia persica* (M21292), by using *Planctomyces staleyii* (M34126) as the outgroup, was performed both by parsimony

analysis with PAUP (20) and by distance analysis to calculate the Kimura two-parameter distance (6) by using the DNADIST program (4). Then, the neighbor-joining method (16) and tree construction were performed by using the NEIGHBOR and DRAWGRAM programs, respectively, in the PHYLIP program package (4). Transversions were weighted 2:1 versus transitions in parsimony analysis. All sequences were aligned by using the CLUSTAL multiple-sequence alignment program (available in the PC/Gene programs package available from IntelliGenetics) and examined by eye for regions with questionable homology.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been submitted to the EMBL data bank and assigned accession no. X61659.

RESULTS AND DISCUSSION

The sequence of the *C. ruminantium* 16S rRNA sequence is shown in Fig. 1. To compare *C. ruminantium* to *A. marginale*, the 16S sequence of *A. marginale* was also amplified and sequenced as described above. During analysis of our data, the sequence of the 16S rRNA of *A. marginale* (M60313) was published (22). The sequence as reported (22) was identical to the sequence obtained in our laboratory for the Florida isolate (7) for the region comparable to bases 23 through 1491 (*E. coli* 16S rRNA numbering).

Regions comparable to bases 1 to 22, 69 to 102, 182 to 210, 454 to 478, 834 to 856, 998 to 1044, 1243 to 1294, and 1449 to 1542 of the *E. coli* sequence for all taxa were excluded from parsimony analysis. For these regions, it was not possible to confidently determine whether the bases aligned in these regions represented homologous nucleotide positions. The sequences of the regions at the ends of the gene (bases 1 to 22 and 1492 to 1542) were excluded, since the *C. ruminantium* gene sequence was available from only a single strand in these regions. The 25 bases (454 to 478) were present in *C. burnetii*, *W. persica*, *E. coli*, *C. psittaci*, and *P. staleyii* but were absent from the other taxa. A total of 1,221 character

1	AAACTTGAGA	GTTTGATCCT	GGCTCAGAAC	GAACGCTGGC	GGCAAGCTTA	ACACATGCAA	60
61	GTCGAACGGA	CAGTTATTTA	TAGTTCGGC	TATGAGTATC	TGTTAGTGGC	AGACGGGTGA	120
121	GTAATGCGTA	GGAATCTGCC	TAGTAGTATG	GAATAGCTAT	TAGAATATG	AGGTAATACT	180
181	GTATAATCCC	TGCGGGGGAA	AGATTTATCG	CTATTAGATG	AGCCTACGTT	AGATTAGCTA	240
241	GTTGGTAAGG	TAATGGCTTA	CCAAGGCATC	GATCTATAGC	TGTTCTGAGA	GGACGATCAG	300
301	CCACACTGGA	ACTGAGATAC	GGTCCAGACT	CCTACGGGAG	GCAGCAGTGG	GGAATATTGG	360
361	ACAATGGGCG	AAAGCCTGAT	CCAGCTATGC	CGCGTGAGTG	AAGAAGGCCT	TCGGGTTGTA	420
421	AAACTCTTTT	AATAGGGAAG	ATAATGACGG	TACCTATAGA	AAAAGTCCCG	GCAAACCTCCG	480
481	TGCCAGCAGC	CGCGGTAATA	CGGAGGGGGC	AAGCGTTGTT	CGGAATTATT	GGGCGTAAAG	540
541	GGCACGTAGG	TGGACTAGTA	AGTTAAAGT	GAAATACCAA	AGCTCAACTT	TGGAGCTGCT	600
601	TTTAATACTG	CTAGACTAGA	GGTCGAGAGA	GGATAGCGGA	ATTCCTAGTG	TAGAGGTGAA	660
661	ATTCGTAGAT	ATTAGGAGGA	ACACCGGTGG	CGAAGGCGGC	TATCTGGCTC	GATACTGACA	720
721	CTGAGGTGCG	AAAGCGTGGG	GAGCAAACAG	GATTAGATAC	CCTGGTAGTC	CACGCTGTAA	780
781	ACGATGAGTG	CTAAATGTGA	GGATTTTATC	TTTGTATTGT	AGCTAACGCG	TTAAGCACTC	840
841	CGCCTGGGGA	CTACGGTCGC	AAGACTAAAA	CTCAAAGGAA	TTGACGGGGA	CCCGCACAAG	900
901	CGGTGGAGCA	TGTGGTTTAA	TTCGATGCAA	CGCGAAAAAC	CTTACCACTT	TTTGACATGA	960
961	AGGTCGATC	CCTTTTAACC	GAGGGAGTCA	GTTCCGCTGG	ACCTTACATA	GGCTGTGCAT	1020
1021	GGCTGTCGTC	AGCTCGTGTC	GTGAGATGTT	GGGTAAAGTC	CCGCAACGAG	CGCAACCCCTC	1080
1081	ATCCTTAGTT	ACCAACAGGT	AATGCTGGGC	ACTCTAAGGA	AACTGCCAGT	GATAAACTGG	1140
1141	AGGAAGGTGG	GGATGATGTC	AAGTCAGCAC	GGCCCTTATA	GGGTGGGCTA	CACACGTGCT	1200
1201	ACAATGGCAA	CTACAATAGG	TTGCGAGACC	GCGAGGTTTA	GCTAATCCAA	AAAAGTTGCT	1260
1261	TCAGTTCGGA	TTGTTCTCTG	CAACTCGAGA	GCATGAAGTC	GGAATCGCTA	GTAATCGTGG	1320
1321	ATCATCATGC	CACGGTGAAT	ACGTCTCGG	GTCTTGATCA	CACTGCCCGT	CACGCCATGG	1380
1381	GAATTGGCTT	AACTCGAAGC	TGGTGTGCTA	ACCGTAAGGA	AGCAGCCATT	TAAGGTTGGG	1440
1441	TTAGTGACTA	GGGTGAAGTC	GTAACAAGGT	AGCTGTAGGT	GAACCTGCGG	CTGGATTACC	1500
1501	TCCTTT						1506

FIG. 1. Sequence of the structural gene encoding the *C. ruminantium* 16S rRNA (413 A's, 298 C's, 434 G's, and 361 T's).

FIG. 2. (A) Most parsimonious tree relating *C. ruminantium* to 10 other bacterial taxa; (B) 95% consensus tree from parsimony analysis of 100 bootstrap replications from an 11-taxon data set used for panel A.

positions were examined, and 318 were phylogenetically informative by parsimony analysis.

The most parsimonious tree was determined for the 11-taxon data set by using the branch and bound algorithm and by weighting transversions 2:1 versus transitions. This tree with a length of 1,619 steps and a consistency index of 0.493 is shown in Fig. 2A. Forty-four trees were within 31 additional steps of the most parsimonious tree. In all 44 trees, *C. ruminantium* was grouped with *A. marginale* and *C. psittaci* branching was placed at the deepest position on the tree at a large phylogenetic distance from *C. ruminantium* and the members of the *Rickettsiales*. Differences in the positioning of the other *Proteobacteria* relative to each other occurred among these longer trees. For example, the next most parsimonious tree, which was three steps longer, differed only by placement of the nodes separating *E. coli*, *W. persica*, and *C. burnetii*. The exact phylogenetic relationships of *E. risticii* and *R. quintana* to the other species in the alpha group of *Proteobacteria* and *E. coli*, *C. burnetii*, and *W. persica* among the gamma group of *Proteobacteria* are less certain (see below). The robustness of the essential features of the most parsimonious tree was examined by data resampling (bootstrap) analysis of 100 repetitions (20). This

method defined the 95% consensus tree shown in Fig. 2B. The clustering of *C. ruminantium* with *A. marginale* and of *R. rickettsii* with *R. prowazekii*, together with the deep branching of *C. psittaci*, was a fully supported feature of the tree. Thus, by parsimony analysis, *C. ruminantium* and *A. marginale* are monophyletic within the alpha group of *Proteobacteria* (*R. rickettsii*, *R. prowazekii*, *R. quintana*, *E. risticii*, *A. marginale*, and *C. ruminantium*), and this group forms a clade separate from the other three major bacterial groups, the gamma group of *Proteobacteria* (*E. coli*, *C. burnetii*, and *W. persica*), the *Chlamydiales* (*C. psittaci*), and the outgroup (*P. staley*), represented in the analysis.

The sequences, when examined by distance methods, imply the same groupings as those obtained by parsimony analysis. In the group of 11 taxa examined, 1,221 base positions were aligned as above, and a distance matrix was prepared by using the Kimura two-parameter (6) model of nucleotide substitution (Table 2) with the DNADIST program (4). When the sequences were plotted with *P. staley* as the outgroup via the neighbor-joining method (NEIGHBOR in PHYLIP [4]) (Fig. 3), *C. ruminantium* was clustered with *A. marginale* among the alpha group of *Proteobacteria* (*R.*

TABLE 2. Evolutionary distances adjusted by using Kimura two-parameter correction

Organism	Evolutionary distance										
	<i>A. marginale</i>	<i>C. burnetii</i>	<i>C. psittaci</i>	<i>C. ruminantium</i>	<i>E. coli</i>	<i>E. risticii</i>	<i>P. staley</i>	<i>R. prowazekii</i>	<i>R. rickettsii</i>	<i>R. quintana</i>	<i>W. persica</i>
<i>A. marginale</i>	0.0000										
<i>C. burnetii</i>	0.1760	0.0000									
<i>C. psittaci</i>	0.2737	0.2553	0.0000								
<i>C. ruminantium</i>	0.0517	0.1875	0.2896	0.0000							
<i>E. coli</i>	0.1910	0.1349	0.2567	0.2060	0.0000						
<i>E. risticii</i>	0.1323	0.1840	0.3080	0.1450	0.2150	0.0000					
<i>P. staley</i>	0.2786	0.2520	0.2998	0.2868	0.2882	0.2987	0.0000				
<i>R. prowazekii</i>	0.1363	0.1737	0.2813	0.1373	0.2082	0.1488	0.2930	0.0000			
<i>R. rickettsii</i>	0.1342	0.1696	0.2838	0.1352	0.2098	0.1500	0.2963	0.0041	0.0000		
<i>R. quintana</i>	0.1433	0.1612	0.2706	0.1535	0.1860	0.1745	0.2799	0.1384	0.1373	0.0000	
<i>W. persica</i>	0.1914	0.1409	0.2597	0.1904	0.1524	0.2140	0.2724	0.1957	0.1959	0.1705	0.0000

FIG. 3. Phylogenetic tree constructed from the evolutionary distances shown in Table 2 (adjusted by using the Kimura two-parameter correction). The tree is drawn to scale in arbitrary units of evolutionary distance for the taxa listed.

rickettsii, *R. prowazekii*, *R. quintana*, and *E. risticii*) (23), distinct from the gamma group of *Proteobacteria* (*E. coli*, *C. burnetii*, and *W. persica*) (23) and both *C. psittaci* and *P. staleyii*. The distance between *C. ruminantium* and *A. marginale* (0.0517) appears to be ~12 times that between the two species in the same genus, *R. rickettsii* and *R. prowazekii* (0.0041). *C. psittaci* was at a distance of >0.25 from all taxa examined. The only difference in conclusions between the parsimony and distance analyses was relative to the branching of *E. coli*, *C. burnetii*, and *W. persica*. The conclusions relative to these relationships were determined to be less reliable by the bootstrap analysis and, further, are unrelated to the present study.

A signature of nine base substitutions delineated the linkage of *A. marginale* with *C. ruminantium* and differentiated these two species from the 45 others in the alpha group of *Proteobacteria* examined in the RNA Database Project (12) (Table 3). Five of these substitutions (at positions 611, 988, 1205, 1427, and 1510) differed from those for all 105 of the other *Proteobacteria* (alpha, beta, and gamma groups) examined. A sixth base of the signature, a transversion (U→A) at position 508, was seen for only two (*Ectothiorhodospira halochloris* A and *E. halophila* SL1) of the 105 other *Proteobacteria* examined (12). A previously defined sequence signature linking the *Ehrlichia* and *Rickettsia* subgroup (23) was examined in light of the new data. Of the 15 signature sequences, 6 were shared by the *Cowdria* and *Anaplasma* spp. and by *Ehrlichia* and *Rickettsia* spp., whereas in all positions, except position 1533, the sequences of *C. ruminantium* and *A. marginale* were identical. (At this position, *C. ruminantium* has a T, and *A. marginale* has a C.) This finding further supports the linkage between *C. ruminantium* and the *Anaplasmataceae* and supports removal of *C. ruminantium* from the *Ehrlichieae*.

TABLE 3. Sequence signature linking *C. ruminantium* and *A. marginale*

Position ^a	Base common to <i>C. ruminantium</i> and <i>A. marginale</i>	Base at position indicated for other <i>Proteobacteria</i>		
		Alpha	Beta	Gamma
45	A	G or U	U	A, G, or U
396	U	A, C, or G	A or C	A, C, G, or U
508	A	U	U	A or U
611	A	C, G, or U	C	C or U
988	U	A, C, or G	C or G	C or G
1192	U	C	C	C or U
1205	C	U	U	U
1427	A	C, G, or U	C or U	C or U
1510	U	C	C	C

^a Position (*E. coli* numbering) at which *C. ruminantium* and *A. marginale* have a common sequence which differs from those of all other members of the alpha group of *Proteobacteria* currently in the RNA Database Project (45 species). Sequence differences from the beta and gamma groups of *Proteobacteria* reflect examination of 24 and 36 species, respectively.

Parsimony and distance analyses confirm the current taxonomic placement of *C. ruminantium* among the *Rickettsiales* (15), rather than among the *Chlamydiales* as had been suggested previously (8, 19, 21). Previously, phylogenetic placement of *C. ruminantium* was based on qualitative phenotypic characters such as transmission by tick vectors, development in intracytoplasmic vacuoles, and similar host cell-type preference, etc. Conflicting conclusions have been drawn from analysis of different sets of these characters (19), since such characters are not necessarily good phylogenetic characters because of the potential for convergent evolution. Sequence data of the 16S rRNA gene refute the suggestion that *C. ruminantium* may be related phylogenetically to the *Chlamydiales* and fully support the close phylogenetic relationship between *C. ruminantium* and the *Rickettsiales*. Further, our parsimony and distance analyses of the 16S rRNA data confirm the conclusion of Weisburg et al. (24) that the *Chlamydia* form a eubacterial branch distinct from the *Rickettsiales*.

Our data strongly indicate a closer phylogenetic relationship of the genus *Cowdria* to the *Anaplasmataceae* rather than to the *Ehrlichieae* (family *Rickettsiaceae*) (15) within the rickettsial sublineage in the *Proteobacteria* (11, 23, 26, 27). The phylogenetic positions of other species sharing the property of intimate association with eukaryotic cells, namely, *Brucella abortus*, *Bartonella bacilliformis*, and cat scratch disease bacillus, were recently evaluated from 16S rRNA sequence data (2, 3, 11). These organisms grouped among the *Proteobacteria* but within the alpha-2 subgroup (2, 3, 11), represented by *R. quintana* in our studies (Fig. 2 and 3), which is distinct from the rickettsial sublineage (26a). Proper placement of *C. ruminantium* among the *Anaplasmataceae*, or determining whether it should form a tribe of its own, will require measuring the relatedness of *Anaplasma* to other genera in the *Anaplasmataceae*, including *Aegyptianella*, *Eperythrozoon*, and *Haemobartonella*. Sequences of the 16S rRNAs from species representative of these genera are not currently available, however.

The obligate, exclusive parasitism of erythrocytes among the *Anaplasmataceae* is currently a primary character linking this family. *C. ruminantium* does not share this characteristic, since it does not infect erythrocytes. Rather, it infects vascular endothelial cells, macrophages, and neutrophils. A study of the molecular phylogeny of the genera

presently placed in the *Anaplasmataceae* would address whether the adaptation to obligate parasitism of the erythrocytes and/or plasma of vertebrates is a primitive character or whether it is a convergent life-style which has arisen independently multiple times among this group of organisms. The more distant relationship of *B. bacilliformis* (alpha-2 subgroup) (2, 11) to the other members of the *Anaplasmataceae* (ricketsial sublineage) suggests that the latter is the case. On the other hand, *C. ruminantium*, like all species of *Anaplasma*, is parasitic in ruminants exclusively. This raises the possibility that *C. ruminantium* and the four *Anaplasma* species described have arisen as descendants of a common ancestral parasite of ruminants.

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