Phylogenetic Position of *Cowdria ruminantium* (Rickettsiales) Determined by Analysis of Amplified 16S Ribosomal DNA Sequences

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The 16S ribosomal DNA sequence of *Cowdria ruminantium*, the causative agent of heartwater disease in ruminants, was determined. An analysis of this sequence showed that *C. ruminantium* forms a tight phylogenetic cluster with the canine pathogen *Ehrlichia canis* and the human pathogen *Ehrlichia chaffeensis*. Although a close relationship between the genus *Cowdria* and several members of the tribe *Ehrlichieae* has been suspected previously, the tight phylogenetic cluster with *E. canis* and *E. chaffeensis* is surprising in view of known differences in host preference and target cells.

The rickettsia *Cowdria ruminantium* is the causative agent of heartwater, a tick-borne disease of wild and domestic ruminants. This disease is endemic in sub-Saharan Africa (28). Recently, *Cowdria ruminantium* has also been detected in the Caribbean region (28). In view of the continuing spread of the African tick *Amblyomma variegatum* in the Caribbean (29), heartwater may become a serious threat to livestock on the American mainland (2).

The genus *Cowdria* is currently classified in the tribe *Ehrlichieae* in the order *Rickettsiales*, together with the genera *Ehrlichia* and *Neorickettsia* (33). The members of the genus *Cowdria* share antigenic determinants with several members of the genus *Ehrlichia* (5, 13, 17) and also with members of the genus *Chlamydia* (11). A recent electron microscopic study of the multiplication of *Cowdria* cells in cultured bovine umbilical endothelial cells showed that the genus *Cowdria* has developmental stages that resemble those of chlamydial species (14). It has been assumed that the developmental cycle consists of an extracellular stage (extracellular bodies) and an intracellular stage (reticulate bodies). A phylogenetic study of the 16S ribosomal DNA (rDNA) of *Cowdria ruminantium* was initiated to determine the taxonomic relationship of the genus *Cowdria* with *Ehrlichia* and *Chlamydia* species more precisely.

The Senegal stock (12) of *Cowdria ruminantium* was cultured in bovine umbilical endothelial cells as described previously (10). *Escherichia coli* K-12 strain PC2495, an *hsdS recA* derivative of strain JM101 (36), was used for the propagation of pBS(-) phagemid and derived clones (Stratagene, La Jolla, Calif.) and was grown overnight at 37°C in Terrific Broth (27) supplemented with 100 µg of ampicillin per ml.

Genomic DNA for polymerase chain reaction amplification was extracted from the extracellular body-containing supernatant of an infected 162-cm² culture (infection score, 2+) (10). The supernatant was centrifuged at 15,000 × *g* for 15 min, the extracellular bodies were suspended in 1 ml of TEG buffer (25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 50 mM glucose) supplemented with 4 mg of lysozyme per ml, and the preparation was incubated for 15 min at room temperature. Sodium dodecyl sulfate and proteinase K were

subsequently added to final concentrations of 0.5% and 400 µg/ml, respectively, and incubation was continued for 1 h at 55°C. The DNA was precipitated with isopropanol and then suspended in H₂O.

The 16S rDNA of *Cowdria* cells was amplified in a total volume of 100 µl by using the terminal primers fD1 and rD1 (30) and *Taq* polymerase (Promega, Madison, Wis.) under conditions described elsewhere (30). The amplification products were characterized by agarose gel electrophoresis. The 1.5-kb 16S rDNA fragment was purified by using a Gene-clean kit (Bio 101, La Jolla, Calif.) and was made blunt ended by incubating it for 15 min at 37°C with *E. coli* DNA polymerase I without deoxynucleoside triphosphates and then at 37°C for 15 min with deoxynucleoside triphosphates at concentrations of 1.67 mM (34). The DNA was phenol extracted, ethanol precipitated, and ligated into the *EcoRV* restriction site of pBS by using T4 DNA ligase. Plasmid DNAs of clones were purified by using the alkaline lysis method followed by cesium chloride-ethidium bromide density gradient centrifugation (23).

The nucleotide sequences of both strands of two cloned amplification products (clones pCRS2 and pCRS5) were determined by using the dideoxy chain termination method (24) and [α -³²P]dATP. Sequencing reactions were carried out by using a T7 sequencing kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The primers used for the sequencing of the 16S rDNA, both forward and reverse, have been described previously (30). A discrepancy between the sequences of clones pCRS2 and pCRS5 was detected at position 1447 (G and A, respectively). This part of the sequence was also determined for two other clones (pCRS7 and pCRS8), which were derived from a different amplification reaction. Both sequences had G at this position. Therefore, we decided to use G at position 1447 for the phylogenetic analyses. The sequence that was determined in this way is shown in Fig. 1.

Aligned 16S rRNA sequences of *E. coli*, *Chlamydia psittaci*, and the alpha bacteria were obtained from the small-ribosomal-subunit RNA data base (R. De Wager, University of Antwerp, Antwerp, Belgium) (20). Other 16S rRNA sequences were obtained from the EMBL and GenBank data bases. The 16S rRNA sequences of *Cowdria ruminantium*, *Anaplasma marginale* (30), and the *Ehrlichia* species (1) were aligned with the small-ribosomal-subunit RNA align-

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1- AACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCAACGGACAGTTATTTATAGCCTTGGCTATAAGTATCTGTTAGTGGCAGACGGGTGAGTAATGC - 100
 101- GTAGGAATCTGCCTAGTAGTATGGAATAGCTATTAGAAATGATAGGTAATACTGTATAATCCTCGCGGGGAAAGATTATCGCTATTAGATGAGCCTACG - 200
 201- TTAGATTAGCTAGTGTGGTAAGGTAATGGCTTACCAAGGCAATGATCTATAGCTGGTCTGAGAGGACGATCAGCCACACTGGAAGTGAATACGGTCCAGA - 300
 301- CTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGCGGAAAGCCTGATCCAGCTATGCCGCGTGAGTGAAGAAGGCCCTTCGGGTGTAAAACTCTT - 400
 401- TTAATAGGGAAGATAATGACGGTACCTATAGAAAAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGTAATACGGAGGGGGCAAGCGTTGTTCCGAATTA - 500
 501- TTGGCGGTAAAGGGCACGTAGGTGGACTAGTAAGTTAAAAGTGAAATACCAAAGCTCAACTTTGGAGCTGCTTTTAATACTGCTAGACTAGAGGTGAGAGA - 600
 601- GAGGATAGCGGAATTCCTAGTGTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCGGTGGCGAAGCGGCTATCTGGCTCGATACTGACACTGAGGTG - 700
 701- CGAAAGCGTGGGAGCAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGTAAATGTGAGGATTTTATCTTTGTATTGTAGCTAACG - 800
 801- CGTTAAGCACTCCGCTGGGGACTACGGTCGCAAGACTAAAACTCAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC - 900
 901- AACGCGAAAAACCTTACCACCTTTTGTACATGAAGTCTGTATCCCTTTTAAACCGAGGGAGTCAGTTCGGCTGGACCTTACACAGGTGCTGCATGGCTGTGC - 1000
 1001- TCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTCATCCTTAGTTACCAACAGGTAATGCTGGGCACTCTAAGGAAAAGTCCCA - 1100
 1101- GTGATAAACTGGAGGAAGGTGGGGATGATGTCAAGTCAGCACGGCCCTTATAGGGTGGGTACACACGTGCTACAATGGCAACTACAATAGGTTGCGAGA - 1200
 1201- CCGCGAGGTTTACGTAATCCAAAAAGTTGTCTCAGTTCGGATTGTTCTCTGCAACTCGAGAGCATGAAGTCGGAATCGCTAGTAATCGTGGATCATCAT - 1300
 1301- GCCACGGTGAATACGTTCTCGGGTCTGTACACACTGCCCGTCACGCCATGGGAATTGGCTTAACTCGAAGCTGGTGTCTAACGTAAGGAAGCAGCCA - 1400
 1401- TTTAAGGTTGGGTTAGTGACTAGGGTGAAGTCGTAAAGGTAGCTGTAGGTGAACC - 1457

FIG. 1. Nucleotide sequence of the 16S rRNA gene of *Cowdria ruminantium*. The nucleotide sequences of the polymerase chain reaction primers that were used to amplify the sequenced fragment are not shown.

ment (20). Because the eubacterial sequences in this alignment were aligned with eucaryotic, plastidial, mitochondrial, and archaeobacterial small-ribosomal-subunit sequences, the alignment was processed to eliminate positions where all of the 16S rRNA sequences which we used had a gap. This alignment was used for a sequence signature analysis (35) and phylogenetic analyses. Additional sequence masking that eliminated all positions where sequences had gaps, which allowed us to use only positions that were represented in all sequences, affected only the length of the branches in the evolutionary distance trees, not the branching order (data not shown).

The phylogenetic analyses which we performed were distance matrix and parsimony analyses, as described in the Felsenstein-Phylip package, version 3.3 (8). The treeing algorithms that assumed no evolutionary clock which we used with the distance matrix were the Fitch (9), NJTree (22), and UPGMA (26) algorithms. A treeing algorithm that assumed an evolutionary clock which we used with the distance matrix was the Kitsch program (9). The evolutionary distance trees that were generated by these programs and the trees that were generated by the parsimony analysis program DNAPars (7) were not significantly aberrant from the tree shown in Fig. 2. This evolutionary distance tree was generated by using the distance matrix from the analyses performed without a sequence mask (Table 1), using the Fitch program.

An analysis of the helices between positions 180 and 220 (*E. coli* numbering) (20) indicated that the genus *Cowdria* is related to the alpha subdivision of the purple bacteria (35). The sequence signature analysis showed that the 16S rRNA sequence of the genus *Cowdria* matches the signature sequences of the alpha subgroup of the purple bacteria in 58 of 66 positions (35), and similarity data and evolutionary distance data showed that the genus *Cowdria* is related to other

FIG. 2. Evolutionary distance tree showing the relationships among ehrlichial and rickettsial species. All of the species belonging to the tribe *Ehrlichieae* are shown in boldface type. The tree was constructed by using the evolutionary distances shown in Table 1.

TABLE 1. Percentages of sequence identity and evolutionary distances among ehrlichial, chlamydial, and other eubacterial rRNA and rDNA sequences

Organism	% Sequence identity and evolutionary distance (10 ⁻²) ^a										
	<i>Cowdria ruminantium</i>	<i>Ehrlichia canis</i>	<i>Ehrlichia chaffeensis</i>	<i>Ehrlichia phagocytophila</i>	<i>Ehrlichia equi</i>	<i>Ehrlichia risticii</i>	<i>Ehrlichia sennetsu</i>	<i>Anaplasma marginale</i>	<i>Rickettsia prowazekii</i>	<i>Escherichia coli</i>	<i>Chlamydia psittaci</i>
<i>Cowdria ruminantium</i>		96.8	97.0	92.2	92.1	83.0	83.3	92.1	81.4	75.8	71.7
<i>Ehrlichia canis</i>	2.4		98.1	92.3	92.3	82.4	83.1	91.9	80.6	74.7	71.3
<i>Ehrlichia chaffeensis</i>	2.0	1.4		92.7	92.7	82.6	83.3	91.8	80.7	74.9	71.9
<i>Ehrlichia phagocytophila</i>	7.2	7.5	7.0		99.9	83.4	83.9	96.2	83.4	76.5	72.3
<i>Ehrlichia equi</i>	7.2	7.6	7.1	0.1		83.3	83.8	96.2	83.3	76.5	72.3
<i>Ehrlichia risticii</i>	17.6	18.2	17.7	16.5	16.6		99.0	83.8	81.7	76.4	71.9
<i>Ehrlichia sennetsu</i>	17.3	17.9	17.4	16.5	16.5	0.5		83.4	81.1	75.7	71.2
<i>Anaplasma marginale</i>	7.1	7.3	7.0	2.6	2.7	17.2	17.1		82.6	77.8	72.9
<i>Rickettsia prowazekii</i>	19.2	20.0	19.8	18.9	19.0	20.4	20.2	19.2		77.4	74.4
<i>Escherichia coli</i>	27.9	29.3	28.6	26.3	26.4	28.1	28.1	26.1	26.4		72.9
<i>Chlamydia psittaci</i>	34.2	34.7	33.8	32.9	33.0	35.3	35.4	32.9	31.1	32.2	

^a The values on the upper right are percentages of sequence identity, and the values on the lower left are evolutionary distances.

members of the order *Rickettsiales* within the alpha subdivision of the purple bacteria. The closest relatives of the genus *Cowdria* are *Ehrlichia canis*, the causative agent of canine ehrlichiosis (21), and the recently described human pathogen *Ehrlichia chaffeensis* (1, 3) (Fig. 2). The members of this phylogenetic cluster exhibited a level of sequence identity of 97 to 98%, which was greater than the level of sequence identity between the 16S rRNAs of two species of the genus *Chlamydia* (95.5%) (32). More distantly related (92% sequence identity) are the granulocytic members of the tribe *Ehrlichiae*, *Ehrlichia phagocytophila* and *Ehrlichia equi* (which have recently been reported to be members of the same species) (1), and the erythrocyte-borne rickettsia *Anaplasma marginale*. Sequence signatures that link the genus *Cowdria* with the genus *Ehrlichia* and with *Anaplasma marginale* and distinguish the genus *Cowdria* from a set of other bacteria belonging to the alpha subdivision are shown in Table 2.

The genus *Ehrlichia* has been reported previously to be phylogenetically diverse (1). The close relationship among

the genus *Cowdria*, *Ehrlichia canis*, and *Ehrlichia chaffeensis* was not expected on the basis of host preference and target cells. Members of the genus *Cowdria* infect endothelial cells of capillaries and larger blood vessels (28) and to a lesser extent granulocytes (18), whereas *Ehrlichia canis* infects only canine monocytes (21). Although the target cells for *Ehrlichia chaffeensis* are not yet known (3), morulae that resemble ehrlichial inclusions have been reported in human monocytes (19).

The results of studies on the serological cross-reactivity between members of the genus *Cowdria* and members of the tribe *Ehrlichiae* support the phylogenetic relationship which we observed. For instance, cross-reactive antibodies have been reported between members of the genus *Cowdria* and *Ehrlichia canis*, *Ehrlichia equi* (17), or *Ehrlichia phagocytophila* (13), but not between members of the genus *Cowdria* and the more distantly related species *Ehrlichia risticii* and *Ehrlichia sennetsu* (17). *Cowdria* antigens have been used to detect antibodies to *Ehrlichia canis* by immunofluorescence (6). Although studies on possible serological cross-reactivity between the genus *Cowdria* and *Ehrlichia chaffeensis* have not been conducted, it is known that *Ehrlichia canis* and *Ehrlichia chaffeensis* are serologically strongly cross-reactive, both in immunofluorescence tests and in Western blotting tests (3, 19). Moreover, *Ehrlichia canis* antigen can be used to detect human ehrlichiosis, which is presumably caused by *Ehrlichia chaffeensis* (4).

Cowdria ruminantium (16), *Ehrlichia canis* (25), *Ehrlichia phagocytophila* (21), and *Anaplasma marginale* (15) all develop within target cells of several species of ixodid ticks. For the more distantly related organisms *Ehrlichia risticii* and *Ehrlichia sennetsu* no vector has been identified yet. In view of the 99.9% rRNA similarity between *Ehrlichia equi* and the tick-borne fever-causing organism *Ehrlichia phagocytophila* (1), we expect that vector transmission of *Ehrlichia equi* occurs. Although it is suggestive that one-third of the patients with human ehrlichiosis have a history of tick bites before the onset of illness, tick transmission has not been conclusively demonstrated (3). We postulate that the closely related rickettsial organisms may have evolved from common ancestors that originated in their invertebrate tick hosts.

Finally, although the developmental stages of the genus *Cowdria* resemble those of chlamydial species (14) and common antigenic determinants have been found (11), we

TABLE 2. Sequence signature positions that link the genera *Cowdria* and *Ehrlichia* with *Anaplasma marginale* and distinguish this group from other alpha subgroup species

Position in 16S rDNA ^a	Nucleotide in:	
	<i>Cowdria</i> , <i>Ehrlichia</i> , and <i>A. marginale</i>	Other alpha bacteria ^b
501	U	C
829	A	G
859	U	C
890	C	G
904	C	U
929	A	G
1163	A	G
1246	C	U
1388	U	C

^a Position (E. coli numbering) (20) at which the 16S rRNA sequences of *Cowdria*, *Ehrlichia*, and *A. marginale* have a common composition that differs from the common composition of a collection of 10 16S rDNA sequences from other bacteria belonging to the alpha subdivision of the purple bacteria.

^b The alpha bacteria which we used were *Agrobacterium tumefaciens*, *Aquaspirillum magnetotacticum*, *Brucella abortus*, *Hyphomicrobium vulgare*, *Rhodobacter capsulatus*, *Rhodocyclidium vannielii*, *Rhodospseudomonas acidophila*, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rickettsia typhi*, and *Rochalimaea quintana*.

found no significant phylogenetic relationship (Fig. 2 and Table 1).

The tight clustering of the genus *Cowdria* with *Ehrlichia canis* and *Ehrlichia chaffeensis* indicates that the present subdivision of the tribe *Ehrlichieae* needs readjustment. Before this matter can be fully addressed, the 16S rRNA sequences of other stocks of *Cowdria* strains (for instance, the mouse-pathogenic Kümme, Kwanyanga, and Nonile stocks) will have to be determined, as will the sequences of other possibly closely related ehrlichial species, such as *Ehrlichia bovis*, *Ehrlichia ondiri*, and *Ehrlichia ovis*. The fact that the erythrocytic rickettsia *Anaplasma marginale* (30) clusters with the granulocytic genus *Ehrlichia* (Fig. 2) and the previously reported phylogenetic diversity of the rickettsiae (31) indicate the need for a reorganization of the order *Rickettsiales*. Before this can be done, the 16S rRNA sequences of more rickettsial species that are currently placed in three families of the *Rickettsiales* (*Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*) will also have to be taken into account.

Cross-reactivity between members of the genus *Cowdria* and members of the genus *Ehrlichia* should be further investigated and should be taken into account in studies on the serological diagnosis of cowdriosis and ehrlichial diseases.

Nucleotide sequence accession number. The nucleotide sequence of the amplified 16S rDNA of *Cowdria ruminantium* has been assigned accession number X62432.

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ADDENDUM IN PROOF

The 16S ribosomal DNA sequence of the Crystal Springs isolate of *Cowdria ruminantium* has been reported recently by Dame et al. (J. B. Dame, S. M. Mahan, and C. A. Yowell, *Int. J. Syst. Bacteriol.* 42:270–274, 1992). The two sequences differ by only 4 nucleotides.

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