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16S rRNA Sequences of *Bartonella bacilliformis* and Cat Scratch Disease Bacillus Reveal Phylogenetic Relationships with the Alpha-2 Subgroup of the Class *Proteobacteria*

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The primary structures of 16S rRNAs of *Bartonella bacilliformis*, an isolate of the cat scratch disease (CSD) bacillus, and a strain phenotypically similar to the CSD bacillus were determined by reverse transcriptase sequencing. These microorganisms were found to be members of the alpha-2 subgroup of the class *Proteobacteria*. The sequence from *B. bacilliformis* was most closely related to the rRNA of *Rochalimaea quintana* (91.7% homology), the etiologic agent of trench fever. The sequence from the isolate of the CSD bacillus showed the greatest homology with *Brucella abortus* (89.7%) and, when compared with oligonucleotide catalog data, formed a cluster with *Rhodopseudomonas palustris*, *Pseudomonas carboxidovorans*, *Nitrobacter* species, and *Bradyrhizobium* species. The 16S rRNA sequence was also determined for the Cleveland Clinic isolate, which was previously shown to be phenotypically similar to and approximately 30% related, by DNA hybridization, to the CSD bacillus. The Cleveland Clinic isolate was isolated from a patient not diagnosed with CSD. The rRNAs from these bacteria exhibited 98.2% homology, confirming that this isolate is a second species in the same genus as the CSD bacillus. Our data suggest that neither *B. bacilliformis* nor the CSD bacillus is the etiologic agent of bacillary epithelioid angiomatosis.

Cat scratch disease (CSD), an infectious disease that occurs predominantly in children, is characterized by a self-limited lymphadenitis that is restricted to the lymph nodes that drain sites of epidermal or conjunctival inoculation. From the time that CSD was first recognized by Foshay in the 1930s, a variety of different infectious agents have been proposed as the cause of CSD, including viruses (19), chlamydia (11), mycobacteria (3), *Leptothrix* species (45), and *Rothia* species (15). In 1983, pleomorphic bacilli were observed by Wear et al. (47) in lymph node biopsy specimens from patients with CSD. These observations provided the first description of the presumptive etiologic agent of this disease. Working in the same laboratory, Margileth et al. (27) extended this observation by detecting morphologically similar bacteria in the primary skin lesions of three patients with CSD. The presence of bacteria in clinical specimens from patients with CSD was soon confirmed by others (20, 29), and in 1988 the first successful isolation and culture of the bacterium was reported by English et al. (12). Microscopic examination revealed pleomorphic, gram-negative cells that are slightly curved or rodlike with bulbous ends. Both typical cells and cell wall-defective variants were observed by transmission electron microscopy. Bacteria with complete cell walls could be converted to cell wall-less variants by incubation at 37°C. The cells were found to be motile by means of a single polar flagellum, nonhemolytic, and oxidase and urease positive; and they reduced nitrate to nitrite. They did not utilize any of 12 carbohydrates tested, either by oxidation or fermentation. They were resistant to a variety of antibiotics, including penicillin G, ampicillin, erythromycin, tetracycline, chloramphenicol, and clindomycin. While English et al. (12) made extensive morphologic and biochemical determinations for their isolate, they did not address the taxonomy of this bacterium.

The typical course of CSD begins with the development of an erythematous crusted papule at the site of a scratch or contact with a cat. Generally, the papule persists for 1 to 3 weeks (27), although some have been observed to last for as long as 3 months (25). Approximately 2 weeks after the scratch has occurred, regional lymphadenopathy develops. The lymphadenopathy usually lasts from between 2 and 4 months and then resolves spontaneously. In the majority of cases, this is the only manifestation of illness; however, in 1 to 2% of cases, patients experience prolonged morbidity with persistent high fever, suppurative lymphadenitis, malaise, fatigue, myalgia, arthralgia, skin eruptions, weight loss, or splenomegaly (26).

Several reports have documented the occurrence of CSD in human immunodeficiency virus-seropositive persons (21, 24, 35, 39). In an immunocompromised host, CSD typically manifests as a systemic infection with multiple organ involvement. In addition, it has been speculated that the CSD bacillus may be associated with bacillary epithelioid angiomatosis (BEA) (7, 23). BEA is characterized by neoplastic angiomatous lesions, which typically develop in epithelioid tissue (8, 42, 46), although involvement of other tissues has been noted (28, 42). These angiomas are similar to the lesions of Kaposi's sarcoma in clinical appearance and course. Histologic examination of angioma sections stained by the Warthin-Starry method has revealed bacteria that are morphologically similar to the CSD bacillus (7, 23). The staining of these bacteria with peroxidase-labeled polyclonal antiserum raised against an isolate of the CSD bacillus (23)

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has provided further evidence that the etiologic agent of CSD is responsible for BEA.

An alternative hypothesis is that the bacteria observed in these angiomas are not the CSD bacillus but *Bartonella bacilliformis*. Some similarity has been observed between the angiomas of BEA and the dermal lesions seen in a form of bartonellosis known as verruga peruana (23, 24, 43). It has also been noted that *B. bacilliformis* reacts to the Warthin-Starry stain in a manner similar to that of the CSD bacillus (24).

B. bacilliformis has been classified in the order *Rickettsiales* as a rickettsia in the family *Bartonellaceae* (34, 37). The etiologic agent of CSD has yet to be classified. Sequencing of 16S rRNA has developed into a powerful technique for determining the phylogenetic relationships between microorganisms. We report here the complete 16S rRNA sequences for the isolate of CSD bacillus obtained by English et al. (12), the Cleveland Clinic isolate (CCI), a similar strain isolated from a patient not diagnosed with CSD (31), and a strain of *B. bacilliformis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The type strain of *B. bacilliformis* (ATCC 35685) (5) was grown on Trypticase soy agar with 5% defibrinated sheep blood. Plates were incubated at 37°C in a moist chamber for 5 to 7 days. An isolate of the CSD bacillus strain BV was provided by D. J. Wear of the Armed Forces Institute of Pathology, Washington, D.C. This strain, designated B91-007352 (ATCC 53690), and CCI strain B91-007353, which was found to be morphologically and biochemically similar to B91-007352 (31), were grown on buffered charcoal-yeast extract agar plates at 30°C for 5 days.

Isolation of rRNA. Bacterial growth was harvested from plates, and total cellular RNA was prepared as described by Stackebrandt and Charfreitag (41), with the following modifications. After washing in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), cells from 50 plates were suspended in 5 ml of 1× SSC containing 0.2% sodium dodecyl sulfate. The bacteria were disrupted by passage through a French press, and crude preparations of RNA were isolated by the procedure cited above (41).

Sequencing of 16S rRNA, gel electrophoresis, and data analyses. Reverse transcriptase sequencing reactions were carried out as described previously (41). Following synthesis of cDNA, a terminal deoxynucleotide transferase reaction was carried out to reduce ambiguities resulting from premature termination of extension by reverse transcriptase (9). *B. bacilliformis* sequencing reactions were resolved on 8% polyacrylamide gels with 7 M urea. Electrophoresis was carried out at 1,750 V for either 3 or 6 h. Following electrophoresis, gels were soaked in a 5% acetic acid solution containing 5% methanol for 15 min, to remove the urea, and were then dried under vacuum. Autoradiography was carried out for 2 to 4 days. Conditions for gel electrophoresis of sequencing reactions for the CSD bacillus and CCI have been described previously (41).

Sequences were aligned by the algorithm of Wilbur and Lipman (51) as part of the DNASTAR software package (DNASTAR, Inc., Madison, Wis.). Regions of base ambiguity were removed from all sequences prior to alignment. Other sequences used in the phylogenetic analyses were obtained from the GenBank data bank (2). Homology values were converted to evolutionary distance values via the transformation of Hori and Osawa (18). Phylogenetic trees were calculated by the method of Fitch and Margoliash (14), as executed by version 3.2 of the PHYLIP software package (13).

Nucleotide sequence accession number. These sequences have been deposited in the GenBank/European Molecular Biology Laboratory data banks under the accession numbers M65249 (*B. bacilliformis*), M65248 (CSD bacillus), and M69186 (CCI).

RESULTS

The sequenced regions of the 16S rRNAs from CSD bacillus B91-007352, CCI (B91-007353), and *B. bacilliformis* ATCC 35685 are shown in Fig. 1. Over 1,400 nucleotides of sequence information were obtained from each rRNA. Ambiguous bases resulting from sequencing artifacts represented only 0.35% (5 of 1,425 bases) of the sequence data from the CSD bacillus and related strain and 0.7% (10 of 1,431 bases) of the sequence data from *B. bacilliformis*.

Table 1 shows percent similarities and evolutionary distance values for the 16S rRNAs reported here and the rRNAs of reference organisms. *B. bacilliformis* exhibited the greatest homology with *Rochalimaea quintana* (91.7%), followed closely by *Brucella abortus* (91.3%). Both of these organisms are classified in the alpha-2 subgroup of the class *Proteobacteria* (30, 48). *Agrobacterium tumefaciens*, a plant pathogen previously shown to form a cluster with *R. quintana* and *B. abortus* (30), was 88.8% homologous. Three *Rickettsia* species and *Ehrlichia risticii*, all members of the alpha subgroup, gave values ranging from 80.9 to 78.5%. Values for bacteria of the beta and gamma branches gave values ranging from 77.8 to 76.0%. The high degree of homology of *B. bacilliformis* 16S rRNA with rRNA from organisms in the alpha-2 subgroup is evidence for its inclusion in this subgroup.

A similar finding was obtained for the CSD bacillus sequence. A homology value of 98.2% was obtained when the sequence from the phenotypically similar CCI was aligned. Such a high degree of homology can be an indication that the organisms in question represent different species within the same genus. An example can be found in Table 1, in which homology values obtained in alignments of sequences from *Rickettsia* species ranged from 97.6 to 99.3%. In descending order, the sequence from CSD bacillus was most homologous with *B. abortus*, *R. quintana*, and *A. tumefaciens*. These data indicate that the CSD bacillus and CCI are also members of the alpha-2 subgroup.

The evolutionary distance values from Table 1 were used to construct the phylogenetic tree shown in Fig. 2. The same tree was obtained irrespective of the order in which sequences were put into the algorithm. The tree shows the distinctions between the alpha, beta, and gamma subgroups, with the beta and gamma branches being more closely related to each other than to the alpha branch, as described previously (52). Within the alpha group, the three *Rickettsia* species form a tight cluster. *E. risticii* is located on the same branch, but is clearly more distantly related. The remaining alpha-2 bacteria form a cluster with the CSD bacillus and CCI, forming a branch distinct from that with the other four species. Of these four species, *B. bacilliformis* is most closely related to *R. quintana*, followed by *B. abortus* and then *A. tumefaciens*.

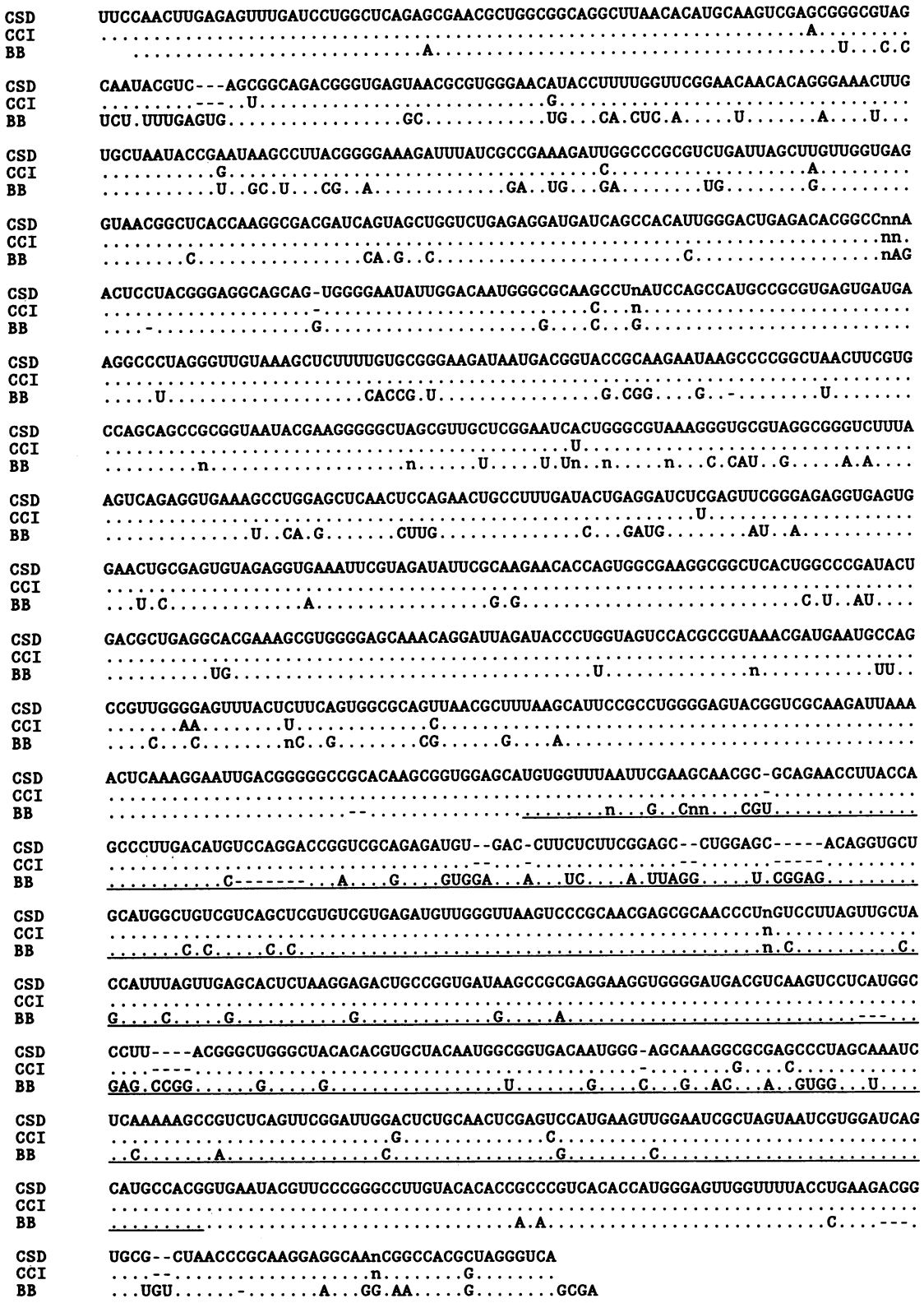

FIG. 1. Aligned 16S rRNA sequences of CSD bacillus (CSD), CCI, and *B. bacilliformis* (BB). Bases for which an unambiguous identification could not be made are indicated by *n*. Consensus with the sequence of CSD is indicated by a period; gaps inserted by the Wilbur and Lipman (51) algorithm to optimize alignment are indicated by a hyphen. The underlined region corresponds to the sequence BA-TF.

TABLE 1. Percent similarity between 16S rRNA sequences of *B. bacilliformis*, CSD bacillus, CCI, and other members of the class *Proteobacteria* and evolutionary distance between 16S rRNA sequence pairs

Organism[a]	% Similarity and evolutionary distance[b]												
	AT	BB	BA	CS	CV	ER	NG	PT	RP	RR	RT	RQ	EC
AT		0.1213	0.0757	0.1416	0.1391	0.2178	0.2367	0.2576	0.1941	0.1993	0.1876	0.0982	0.2491
BB	**88.8**		0.0925	0.1624	0.1587	0.2534	0.2632	0.2892	0.2204	0.2326	0.2204	0.0880	0.2661
BA	**92.8**	**91.3**		0.1108	0.1166	0.2019	0.2151	0.2436	0.1851	0.1915	0.1838	0.0790	0.2245
CS	**87.1**	**85.4**	**89.7**		0.0182	0.2151	0.2285	0.2450	0.1889	0.1928	0.1876	0.1332	0.2367
CV	**87.3**	**85.7**	**89.2**	**98.2**		0.2164	0.2395	0.2422	0.1851	0.1889	0.1864	0.1344	0.2367
ER	**81.1**	**78.5**	**82.3**	**81.3**	**81.2**		0.2534	0.2590	0.1851	0.1941	0.1980	0.2353	0.2408
NG	**79.7**	**77.8**	**81.3**	**80.3**	**79.5**	**78.5**		0.1711	0.2548	0.2661	0.2747	0.2258	0.2032
PT	**78.2**	**76.0**	**79.2**	**79.1**	**79.3**	**78.1**	**84.7**		0.2520	0.2576	0.2647	0.2395	0.2045
RP	**82.9**	**80.9**	**83.6**	**83.3**	**83.6**	**83.6**	**78.4**	**78.6**		0.0244	0.0070	0.1876	0.2506
RR	**82.5**	**80.0**	**83.1**	**83.0**	**83.3**	**82.9**	**77.6**	**78.2**	**97.6**		0.0244	0.1941	0.2718
RT	**83.4**	**80.9**	**83.7**	**83.4**	**83.5**	**82.6**	**77.0**	**77.7**	**99.3**	**97.6**		0.2006	0.2819
RQ	**90.8**	**91.7**	**92.5**	**87.8**	**87.7**	**79.8**	**80.5**	**79.5**	**83.4**	**82.9**	**82.4**		0.2313
EC	**78.8**	**77.6**	**80.6**	**79.7**	**79.7**	**79.4**	**82.2**	**82.1**	**78.7**	**77.2**	**76.5**	**80.1**	

[a] AT, *Agrobacterium tumefaciens* (54); BB, *Bartonella bacilliformis*; BA, *Brucella abortus* (10); CS, cat scratch disease bacillus; CV, Cleveland isolate; ER, *Ehrlichia risticii* (48); NG, *Neisseria gonorrhoeae* (38), PT, *Pseudomonas testosteroni* (54); RP, *Rickettsia prowazekii* (48); RR, *Rickettsia rickettsii* (48); RT, *Rickettsia typhi* (48); RQ, *Rochalimaea quintana* (49); EC, *Escherichia coli* (6).

[b] Percent similarity is indicated in boldface type, and evolutionary distance is indicated in light face type. Percent similarity was calculated with the algorithm of Wilbur and Lipman (51). Evolutionary distance values were calculated as previously described (18).

DISCUSSION

Comparison of 16S rRNA sequences has become the "gold standard" for the elucidation of phylogenetic relationships among microorganisms. As the number of sequences available for analysis continues to grow, the structure of phylogenetic trees derived from these sequences becomes both more intricate and more accurate. One branch on the eubacterial tree that has become much better defined in recent years is the alpha branch of the class *Proteobacteria*. This branch was originally divided into three subgroups (16, 53), with a fourth subgroup having since been recognized (52). Each subgroup of the alpha branch contains both photosynthetic and nonphotosynthetic species. The alpha-2 subgroup contains a variety of species that are of particular interest because they can exist in close association with eukaryotic cells. These interactions range from endosymbiotic relationships with leguminous plants (i.e., *Rhizobium* species), plant-bacteria interactions of a pathogenic nature (*A. tumefaciens*), and a number of species pathogenic for mammalian hosts which form pericellular or intracellular associations during the course of infection (*B. abortus* and *R. quintana*). Our data allow for the placement of three more

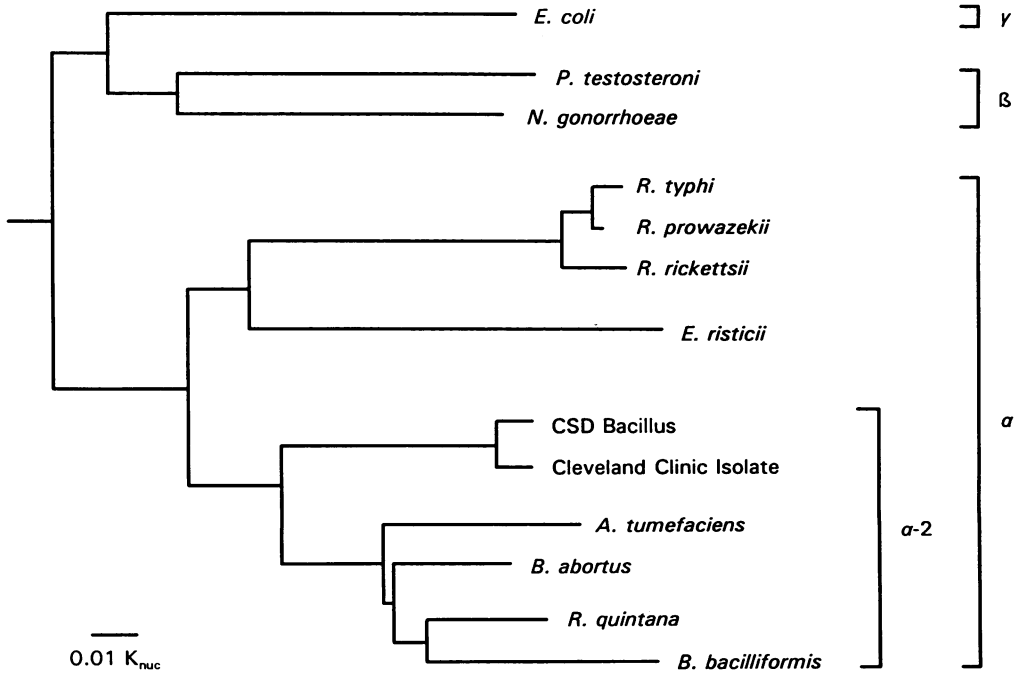

FIG. 2. Phylogenetic tree showing relationships of CSD bacillus, CCI, and *B. bacilliformis* among the *Proteobacteria*. The tree was constructed by the method of Fitch and Margoliash (14) by using the 16S rRNA sequence of *Anacystis nidulans* (44) as an outgroup. K_{nuc}, evolutionary distance.

bacterial species, two of which are known to be pathogenic for humans, into the alpha-2 subgroup.

B. bacilliformis is presently classified as a rickettsia in the family *Bartonellaceae*. Included in this family is the genus *Grahamella*, a pathogen that infects small mammals. These two genera and a third species of rickettsia, *R. quintana*, are fundamentally different from other organisms classified as rickettsiae by virtue of their ability to grow in axenic media. Additionally, *B. bacilliformis* is the only motile member of the order *Rickettsiales*. Previously, Weisburg et al. (48, 49) reported the sequence of 16S rRNAs from seven members of the family *Rickettsiaceae* representing five different genera. They determined that this family represents a polyphyletic group of organisms, with three species of *Rickettsia*, *E. risticii*, and *R. quintana* belonging to the alpha subdivision, while *Coxiella burnetii* and *Wolbachia persica* fell within the gamma subdivision. Of the five species in the alpha subdivision, the three *Rickettsia* species formed a tight cluster in the alpha-3 subgroup, with *E. risticii* being a more distantly related organism of the same lineage. *R. quintana*, while in the same subdivision, belonged to a different subgroup (alpha-2), forming a cluster with the plant-associated genera *Agrobacterium* and *Rhizobium*. Our analyses confirmed these relationships within the alpha subdivision (Fig. 2) and allowed placement of *B. bacilliformis* within the alpha-2 subgroup, specifically in a cluster with *R. quintana*, *B. abortus*, and *A. tumefaciens*. Inclusion of *B. bacilliformis* in this subgroup is supported by the occurrence of six signature oligonucleotides (AUUUAUCG, positions 185 to 192; AUAAUG, positions 423 to 428; CCUUUG, positions 580 to 585; AUAUUCG, positions 650 to 656; UCCAUUACUG, positions 689 to 698; UUUUACCCG, positions 1,372 to 1,380) identified within the sequence that are associated of the alpha-2 subgroup (1, 53).

The phylogenetic clustering of *B. bacilliformis* and *R. quintana* is consistent with a variety of characteristics associated with these species. Each species is capable of survival and multiplication within eukaryotic cells, but they also grow on axenic media. Each has been associated with invertebrate hosts, sand flies of the genus *Phlebotomus* in the case of *B. bacilliformis* (22) and the body louse in the case of *R. quintana*. The guanosine-plus-cytosine content of *B. bacilliformis* DNA is 39 mol% (5), and the value for *R. quintana* is 38.5 mol% (50), while the calculated genome size for *B. bacilliformis* of 4×10^8 (5) is similar to values determined for *Rickettsia* species (50).

Brenner et al. (5) discussed the higher-order classification of *B. bacilliformis* and *R. quintana* with respect to 16S rRNA sequence data. The data support the classification of *B. bacilliformis* in the order *Rickettsiales*, but suggest that *R. quintana* should be moved from the family *Rickettsiaceae* to the family *Bartonellaceae*. This recommendation may also hold for the second species of *Rochalimaea*, *Rochalimaea vinsonii*, but at present, sequence data for this species are lacking. Sequence data are also lacking for *Grahamella* species. It would be interesting to see whether *Grahamella* species and *R. vinsonii* are more closely related to each other than to either *B. bacilliformis* or *R. quintana*, given the fact that each organism is a pathogen for small mammals and has not been shown to cause infection in humans.

The taxonomy of the CSD bacillus isolated by English et al. (12) has been entirely unaddressed to date. The data presented here support its addition to the alpha-2 subgroup of the *Proteobacteria*. This determination is based upon quantitative data derived from homology values (Table 1) and qualitatively by the presence of five signature oligonucleotides indicative of the alpha-2 subgroup (AAACUUG, positions 147 to 153; AUUUAUCG, positions 185 to 192; AUUAAUG, positions 423 to 428; CCUUUG, positions 581 to 586; AUAUUCG, positions 651 to 657). As seen in Fig. 2, while the CSD bacillus belongs in the alpha-2 subgroup, its relationship to members of the *A. tumefaciens-R. quintana* branch is a relatively distant one. When the CSD bacillus sequence was broken down into a catalog of oligomers on the basis of ribonuclease T1 digestion and compared with catalogs of other organisms classified as alpha-2 (1, 16, 17, 40), it was found to cluster with *Rhodopseudomonas palustris*, *Pseudomonas carboxidovorans*, *Nitrobacter* species, and *Bradyrhizobium* species (data not shown). This is a second case in the alpha-2 subgroup in which pathogens and nonpathogens form a tight cluster of organisms, the first being the previously mentioned clustering of *R. quintana* with members of the family *Rhizobiaceae* noted by Weisburg et al. (49).

Because of the variety of organisms postulated as the etiologic agent of CSD and the extreme difficulty with which the CSD bacillus is cultured from clinical material, it is reasonable to question whether the lone isolate of English et al. (12) used in this study is indeed the etiologic agent of CSD. These investigators believed that this bacillus is the etiologic agent of CSD for several reasons; it was morphologically similar to bacteria seen in lesions from patients with CSD, patients exhibited rises in antibody titers or elevated titers to this organism, antibody raised against their isolate reacted with known CSD bacilli in human tissue, and the organism induced dermal lesions in the armadillo that were identical to those seen in humans. A polymerase chain reaction (PCR) assay with oligonucleotides based on the nucleotide sequence from B91-007352 has yielded positive results with clinical specimens from patients with CSD (33), indicating that this isolate is the etiologic agent of CSD.

The CSD bacillus exhibits some traits characteristic of other organisms in the alpha-2 subgroup. Intracellular parasitism is one such characteristic of pathogens in this subgroup. The CSD bacillus has been observed in macrophages and endothelial cells (12, 47), brucellosis is characterized by invasion of macrophages and histiocytes, and bartonellosis entails multiplication of bacteria within erythrocytes and endothelial cells. Entry of these organisms into the host also exhibits a common motif. The CSD bacillus typically gains entry to the host via the skin, as a result of a cat scratch or bite. Although normally ingested, *B. abortus* can gain entry via abrasions, as does *R. quintana*. *B. bacilliformis* is introduced through the skin through the feeding of its vector.

Another common theme of organisms in the alpha-2 subgroup is an association with soil. Many species live either in the soil or in endosymbiotic or pathogenic relationships with plants. *B. abortus* is capable of survival for several months in the soil. This raises interesting questions about the epidemiology of CSD. It is not known how cats acquire the bacterium. The CSD bacillus does not appear to cause feline infection, and to date, it has not been detected in cats. If the CSD bacillus can survive as a free-living organism in the soil, then it may be transiently acquired by cats when they are outdoors. No data have yet been collected regarding the incidence of CSD in households with cats that are allowed outside versus the incidence in those with cats that are kept indoors.

We also determined the 16S rRNA sequence of an isolate that is phenotypically similar to the CSD bacillus but that was obtained from a patient not diagnosed with CSD. Several lines of evidence indicate that this isolate is a second

species in the genus represented by the CSD bacillus. Both strains contain 11-methyloctadec-12-enoic acid, which has not previously been detected in bacteria (31). The guanine-plus-cytosine mole percent for each strain is similar (62.5 versus 64 mol%), and the chromosomal DNAs were found to be approximately 30% related (4); a value typical of species within a genus. Finally, the high degree of homology observed for the 16S rRNAs of these isolates (98.2%) is also indicative of relationship at the genus level. Brenner et al. (4) have identified six additional strains of human origin, but not from patients with clinically diagnosed CSD, with the same fatty acid profile as that of the CSD bacillus. Collectively, these eight strains have been shown by DNA hybridization to represent six species. Whether or not the isolate of English et al. (12) is the only member of the genus that is capable of causing classic CSD has yet to be determined.

For different reasons, both the CSD bacillus and *B. bacilliformis* have been suggested as the etiologic agents of BEA. Our data do not specifically address this question, but we can comment on their putative role in light of a recent report by Relman et al. (36). Those investigators amplified a segment of 16S rDNA by a PCR by using DNA from a biopsied lesion from a patient with BEA as the template. The approximately 480 bases of sequence data (designated BA-TF) that were obtained were found to be most closely related (98.3%) to the 16S rRNA of *R. quintana*. It was concluded that "the cause of bacillary angiomatosis is a previously uncharacterized rickettsia-like organism, closely related to *R. quintana*" (36). If this conclusion is correct, then the CSD bacillus is not the etiologic agent of BEA, since its 16S rRNA shows greater homology with *B. abortus* (89.7%) than with *R. quintana* (87.8%). When the region of the sequence analyzed is restricted to that amplified by Relman et al. (36) (denoted by the underline in Fig. 1), the homology with *B. abortus* and *R. quintana* rises to 89.9%, but it is still substantially lower than the reported value for the BA-TF sequence (a portion of this difference is undoubtedly due to the different methods used in calculating homology values). The PCR assay for CSD bacillus supports this conclusion, since we have yet to detect a product when DNA extracted from BEA specimens is used as a template (32). Although we found that *B. bacilliformis* is most closely related to *R. quintana*, the same reasoning applies. Limiting the sequence analyzed to the region covered by BA-TF yields a homology value of 91.0%. Since sequences obtained from different patients with BEA varied by no more than 0.7% from the BA-TF sequence, the degree of heterogeneity between *B. bacilliformis* and BA-TF suggests that *B. bacilliformis* is also not the etiologic agent of BEA.

Relman et al. (36) identified regions in the BA-TF sequence that they reported were specific for BA-TF. Primers based on these regions did not amplify DNA extracted from tonsillar and splenic tissue from patients without BEA. We used these primers and the PCR conditions reported by Relman et al. (36) with the aim of unequivocally ruling out a role for *B. bacilliformis* in BEA. Unfortunately, these primers yielded a product of the proper size when *B. bacilliformis*, *R. quintana*, or *B. abortus* DNA was used as a template (32) (no product was detected from CSD DNA), indicating a lack of specificity which precludes the possibility of making any conclusions about *B. bacilliformis* and BEA.

An absolute answer on the role of *B. bacilliformis* in BEA will not be possible until the BA-TF sequence becomes available for comparison or until a sensitive and specific assay for the detection of *B. bacilliformis* in clinical specimens is produced. A PCR assay is being developed which uses a primer based on the 16S rRNA sequence in a region upstream from the BA-TF primers. Preliminary tests of specificity have shown that this primer does not anneal to DNA from CSD bacillus (or other species in the genus), *Brucella* species, or *Rochalimaea* species. With this assay, we expect to conclusively eliminate *B. bacilliformis* from the list of putative etiologic agents of BEA.

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ADDENDUM

The sequence BA-TF has been released into the GenBank data base. Alignment of this sequence with the analogous region of the 16S rRNAs from the following species gave the indicated homology values: *R. quintana*, 97.4%; *B. bacilliformis*, 92.4%; CSD bacillus, 89.8%; CCI, 89.1%. These values provide further evidence that the etiologic agent of BEA is most likely a member of the genus *Rochalimaea*.

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