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Molecular Characterization and Proposal of a Neotype Strain for *Bartonella bacilliformis*

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***Bartonella bacilliformis*, the etiologic agent of bartonellosis, was characterized biochemically and by DNA hybridization, guanine-plus-cytosine content, genome size, and 16S rRNA sequencing. DNAs from the two strains in our collection exhibited 97% relatedness in hydroxyapatite reactions done at 55°C (optimal reassociation criterion) and 100% relatedness in reactions done at 70°C (stringent reassociation criterion). There was no evidence of divergence within the related sequences. *B. bacilliformis* DNA showed no relatedness to the cat scratch disease bacillus or to a strain of a second species in the same genus as the cat scratch disease bacillus in hybridization reactions done at 65°C. The guanine-plus-cytosine contents of DNAs from the two *B. bacilliformis* strains were 39 and 40 mol%. Time course reassociation, done by determining spectrophotometrically the time required for one-half of the denatured DNA to form duplexes, indicated that *B. bacilliformis* has a genome size of approximately 4×10^8. The 16S rRNA sequence analysis indicated that *B. bacilliformis* is in the α-2 subgroup of the purple bacteria, class *Proteobacteria*, and that its closest relatives are *Rochalimaea quintana* and *Brucella abortus*. Strain KC583 (= Herrer 020/F12,63 = ATCC 35685) is proposed as the type strain of *B. bacilliformis*.**

Bartonella bacilliformis is the etiologic agent of a sand fly-borne (*Phlebotomus* species and *Lutzomyia verrucarum*) disease termed bartonellosis or Carrion's disease. Bartonellosis has been documented only in Andes Mountain valleys at elevations of 500 to 3,000 m in Bolivia, Chile, Columbia, Ecuador, Guatemala, and Peru (5, 11, 13).

The disease manifests either as Oroya fever or as verruga peruana (Peruvian wart) (6, 9, 11, 20, 24, 25). Oroya fever is characterized by fever and severe intravascular hemolytic anemia (6, 8, 9, 11, 20, 24, 25). Death occurs in 40 to 90% of untreated cases (4, 5, 8). This high mortality rate is commonly the result of concomitant bacteremic infection, most frequently with *Salmonella* species (4).

Verruga peruana is a less severe form of bartonellosis, characterized by an erythematous nodular eruption. *B. bacilliformis* grows in the vascular epithelium of the skin eruption. It occurs within 2 months of the hematic phase in patients with Oroya fever. It frequently occurs in patients without Oroya fever, especially in areas endemic for the disease, indicating partial immunity against *B. bacilliformis* (8, 11). It is usually not fatal. An estimated 5 to 10% of persons in areas endemic for the disease are thought to be carriers (11).

Carrion's disease was named for Daniel Carrion, a medical student in Lima, Peru, who lost his life in 1885 proving that Oroya fever and verruga peruana are different manifestations of the same disease. He injected himself with blood from a verruga nodule and died from Oroya fever 42 days later (24). The names *Bartonella* and bartonellosis are for Alberto Barton who, between 1902 and 1905, determined that Carrion's disease is caused by a hemotropic bacterium (20, 24).

B. bacilliformis was described by Strong et al. in 1913 (24). It can and has been classified as an ungrouped gram-negative, aerobic, nonfermentative bacterium and is classified as a rickettsia in the family *Bartonellaceae*, order *Rickettsiales* (11, 17, 19). If it is a rickettsia, it is the only motile member of this order, and, other than *Rochalimaea quintana* and grahamellae, is the only rickettsia that grows in bacteriologic media.

LeBoit et al. (12) noted histologic similarities between the lesions of bartonellosis and those found in patients with epithelioid angiomatosis (described as epithelioid hemangioma-like vascular proliferations [3, 23] in patients with AIDS). On the basis of morphologic similarities among organisms seen in silver stains of lesions, several investigators have postulated that epithelioid angiomatosis may be a manifestation of cat scratch disease (10, 12, 18). On the basis of evaluation of a portion of 16S rRNA from tissue samples from patients with epithelioid angiomatosis, Relman et al. (18) stated that "the cause of epithelioid angiomatosis is a rickettsia-like organism, most closely related to *Rochalimaea quintana*" and that "this bacillus may also cause cat scratch disease." It is therefore of immediate interest to determine whether *B. bacilliformis* is related to the cat scratch disease bacillus, to the etiologic agent of epithelioid angiomatosis, or both.

The objectives of this study are to characterize *B. bacilliformis* phenotypically and genotypically, to designate a type strain for this species, and to determine its relatedness to the cat scratch disease bacillus and to the etiologic agent of epithelioid angiomatosis.

MATERIALS AND METHODS

Strains. *B. bacilliformis* KC583 (= Herrer 020/F12,63 = ATCC 35685) and KC584 (= Herrer 157/F4,63 = ATCC 35686) were acquired in 1963 by the Special Bacteriology Reference Laboratory at the Centers for Disease Control from Aristides Herrer, Institute National de Salud Publica, Lima, Peru. Cat scratch disease bacillus strain BV (B91-

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007352 = F6400 = G1492) was provided by Douglas J. Wear, Department of Infectious and Parasitic Diseases Pathology, Armed Forces Institute of Pathology. Strain B91-007353 (= G1849 = F6703 = 411m) of an unnamed species vernacularly called the Cleveland disease isolate was isolated at the Cleveland Clinic Foundation by Geraldine S. Hall and was subsequently sent to the Special Bacteriology Reference Laboratory through the Ohio State Health Department (15). This organism is significantly related to the cat scratch disease bacillus, but it has not been implicated with cat scratch disease.

Biochemical tests. Gram stain, motility, and biochemical test reactions were done as described previously (2).

DNA methods. To obtain cells for DNA extraction, *B. bacilliformis* strains were grown on heart infusion agar containing 5% rabbit blood at 25°C. The cat scratch disease bacillus and the Cleveland disease isolate were grown on buffered charcoal yeast extract agar at 30°C. The methods used to extract and purify DNA and to determine DNA relatedness have been described previously (1b). DNA from *B. bacilliformis* KC583 was labeled enzymatically in vitro with [^{32}P]dCTP, using a nick-translation reagent kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as directed by the manufacturer. The guanine-plus-cytosine content of *Bartonella* DNA was determined spectrophotometrically by the method of Marmur and Doty (14). The genome size of *B. bacilliformis* was approximated by denaturing 100 μg of DNA contained in 0.28 M phosphate buffer (1b), allowing it to reassociate in a spectrophotometer held at 60°C for 16 h, and determining the time at which one-half of the DNA had reassociated compared with the time at which one-half of *Escherichia coli* DNA of known genome size 2.5 × 10^9 had reassociated.

16S rRNA sequencing. Sequence analysis of 16S rRNA from *B. bacilliformis* KC583 was done by the method of Stackebrandt and Charfreitag (21) as modified by O'Connor et al. (16). Percent similarity between 16S rRNA sequences was calculated by using the algorithm of Wilbur and Lipman (27). The 16S rRNA sequence of cat scratch disease bacillus strain B91-007352 was taken from O'Connor et al. (16). All other rRNA sequences were obtained from the GenBank data base (1).

RESULTS

Biochemical characteristics. *B. bacilliformis* KC583 and KC584 had identical phenotypic characteristics. They were small, gram-negative, oxidase-negative, catalase-positive, motile, aerobic rods. Motility was achieved by means of 1 to 10 polar flagella, although a small number of cells appeared to have subpolar or lateral flagella. They grew slowly (3 to 5 days), without hemolysis, on heart infusion agar with 5% rabbit blood. No growth was obtained on nutrient or MacConkey agars. Growth was best at 25°C, weak at 35°C, and absent at 42°C. They were nonfermentative, producing neither acid nor gas from D-glucose, lactose, maltose, D-mannitol, sucrose, or D-xylose in the rapid sugar test. They were negative in tests for growth on citrate (Simmons), indole production, litmus milk, and urease and did not grow on media used for esculin hydrolysis, nitrate reduction, H_2S production, and gelatin hydrolysis.

Guanine-plus-cytosine content. The guanine-plus-cytosine content, as determined spectrophotometrically, was 39 mol% for *B. bacilliformis* KC583 and 40 mol% for strain KC584.

Genome size. Fifty percent of denatured *E. coli* DNA with a known genome size of approximately 2.5 × 10^9 reassociated with a C_0t of 5.7. Similarly treated DNAs from *B. bacilliformis* KC583 and KC584 showed 50% reassociation at an average C_0t of 0.9. Therefore, the genome size of *B. bacilliformis* was 16% that of *E. coli*, or 4 × 10^8.

TABLE 1. Percent homology of *B. bacilliformis* 16S rRNA to those of selected *Proteobacteria*

Species	Subgroup	% Homology
Bartonella bacilliformis	α-2	100
Rochalimaea quintana	α-2	91.7
Brucella abortus	α-2	91.3
Agrobacterium tumefaciens	α-2	88.8
Cat scratch disease bacillus	α-2	85.4
Rickettsia prowazekii	α	80.9
Rickettsia typhi	α	80.9
Rickettsia rickettsii	α	80.0
Ehrlichia risticii	α	78.5
Neisseria gonorrhoeae	β	77.8
Escherichia coli	γ	77.6
Desulfovibrio desulfuricans	δ	77.3
Pseudomonas testosteroni	β	76.0

DNA hybridization. Labeled DNA from *B. bacilliformis* KC583 was used for DNA hybridization reactions. It was 97% related to unlabeled DNA from strain KC584 in reactions done at 55°C, with 0% divergence within the related sequences. Relatedness between DNAs from these strains was 98% at 65°C and 100% at 70°C. *B. bacilliformis* was 1% related to the type strain (NCTC 11432) of *Flavobacterium gleum*. *F. gleum* was used as an unrelated control because the guanine-plus-cytosine content of its DNA (37 mol%) is close to that of *B. bacilliformis*. *B. bacilliformis* was 1 and 0% related to cat scratch disease bacillus strain B91-007352 and was 3 and 0% related to the Cleveland disease isolate strain B91-007353 in reactions done at 55°C and 65°C, respectively.

16S rRNA sequence analysis. The complete 16S rRNA sequence of *B. bacilliformis* is shown elsewhere (16). On the basis of 16S rRNA homology with previously sequenced and classified bacteria, *B. bacilliformis* was shown to be a member of the class *Proteobacteria* (22), which includes the "purple bacteria and their relatives" (28). It belongs to the α-2 subgroup of *Proteobacteria*, and its closest relatives are *R. quintana* and *Brucella abortus*. Homology values are given in Table 1.

DISCUSSION

B. bacilliformis KC583 and KC584 were characterized biochemically and by guanine-plus-cytosine content, genome size, and DNA hybridization. Both strains conformed to the biochemical description of the species (19). Their DNA genome sizes and guanine-plus-cytosine contents were very similar, and they were essentially identical in DNA relatedness reactions.

Although *B. bacilliformis* is a validly published species, the absence of a type strain casts some doubt on its standing, since its description is incomplete. More importantly, since a species is defined by its type strain, the absence of a type strain affects anyone who wants to use *B. bacilliformis*, especially in comparative studies with other bacteria. We hope to alleviate these problems by proposing strain KC583 (= ATCC 35685) as the neotype of *B. bacilliformis*.

The two strains used in this study are the only two *B.*

bacilliformis strains available at the Centers for Disease Control and the American Type Culture Collection. We presume, but do not know, that they were isolated from cases, rather than from vectors. We certainly do not know whether they were isolated from persons suffering from Oroya fever or verruga peruana disease. Carrion's tragic experiment and literature studies seem to convincingly attribute both diseases to the same organism (20, 24). However, the possibility that two related species are responsible has not been specifically ruled out. This possibility can now be easily tested with isolates from persons with both manifestations of the disease.

It has become increasingly difficult to define rickettsia. Most species that fall into this group are obligate intercellular parasites and are nonmotile, and their cells are smaller than those of other bacteria. There are now three genera that violate these general rules, *Rochalimaea*, *Bartonella*, and *Grahamella*, all of which can be cultivated on bacteriologic media. Furthermore, *B. bacilliformis* is motile.

Neither DNA relatedness nor rRNA hybridization studies have been done to determine the taxonomic relationship of *B. bacilliformis* to *Grahamella* species, the other genus in the family *Bartonellaceae*, or rickettsiae in other families. Unfortunately, a type strain has not been designated for either *Grahamella* species, and cultures are apparently not available in the American Type Culture Collection. When available, these data will not only be inherently important taxonomically but will also aid in comparing bartonellosis with other, seemingly similar, diseases of unknown etiology and in comparing bartonellosis with grahamellosis, which occurs in small mammals.

It is possible that the small size of rickettsial cells and their diminished metabolic capacity (compared with those of the cells of most other bacteria) correlate with a smaller DNA molecular weight. When measured, the genome size of rickettsia is 1.0×10^9 to 1.5×10^9 (26), which is significantly smaller than those of most bacteria. In the present study, we approximated the genome size of *B. bacilliformis* at 4×10^8, which is more like previous estimates for rickettsiae than it is for those of other bacteria. It would be informative to systematically determine genome sizes in rickettsial species to see whether this characteristic is of taxonomic and determinative value.

DNA relatedness studies indicated that *B. bacilliformis* is a species distinct from both the cat scratch disease bacillus and the Cleveland disease isolate. We have now identified by DNA hybridization four additional species in the same genus as the cat scratch disease bacillus and the Cleveland disease isolate (1a). *B. bacilliformis* has been shown to be essentially unrelated to three of these by hybridization. Since the guanine-plus-cytosine content of *B. bacilliformis* is 20 to 30% less than that of all six of these species, it is definitely a separate species.

On the basis of 16S rRNA sequence analysis, there is no doubt that *B. bacilliformis* is in the α-2 subgroup of the class *Proteobacteria*. Since all rickettsiae that have been sequenced are in the α subgroup, and since *B. bacilliformis* is most closely related to *R. quintana*, the sequence data are consistent with the classification of *B. bacilliformis* in the order *Rickettsiales*. However, since *R. quintana*, a member of the family *Rickettsiaceae*, is more closely related to *B. bacilliformis*, a member of the family *Bartonellaceae*, than it is to members of other genera within its own family, the position of *R. quintana* within the *Rickettsiaceae* should be reassessed.

B. bacilliformis 16S rRNA is 85.4% homologous to that of the cat scratch disease bacillus, less than its level of homology to *B. abortus* or *Agrobacterium tumefaciens*. These data not only support placement of *B. bacilliformis* and the cat scratch disease bacillus in different genera but also indicate that they are probably not members of the same family. This is not at all surprising, since the guanine-plus-cytosine content of the cat scratch disease bacillus is above 60% (7, 16) and its genome size is similar to that of *E. coli* (1a).

We cannot yet definitively determine the relationship between *B. bacilliformis* or the cat scratch disease bacillus and the agent of epithelioid angiomatosis; however, we can comment on some of the possibilities. We cannot totally exclude the possibility that *B. bacilliformis* is the same species or a species similar to the organism that causes epithelioid angiomatosis; however, its rRNA is only 91.7% homologous to *R. quintana*, whereas Relman et al. (18) reported 98.3% homology of a partial 16S rRNA sequence of the epithelioid angiomatosis agent and *R. quintana*. Other information that may conflict with this possibility is the inability to culture the agent of epithelioid angiomatosis.

Relman et al. (18), on the basis of partial 16S rRNA sequence analysis, concluded that the agent of epithelioid angiomatosis is "rickettsia-like. . . most closely related to *Rochalimaea quintana*" and believed that it (the agent of epithelioid angiomatosis) "may also cause cat scratch disease." According to our data presented here and elsewhere (16), no more than one of these conclusions can be correct. If the extremely close relationship of the agent of epithelioid angiomatosis to *R. quintana* (98.3%), based on analysis of approximately 30% of the total sequence, is correct, then it is markedly different from the cat scratch disease bacillus. Alternatively, if the two diseases are caused by the same organism, it is unlikely that it is rickettsia-like.

O'Connor et al. (16) showed that 16S rRNA from the cat scratch disease bacillus is only 87.8% homologous to *R. quintana*, whereas it is 89.7% homologous to *B. abortus*. Moreover, the genome size of the cat scratch disease bacillus is substantially larger than those of rickettsiae, and its guanine-plus-cytosine content is over 60 mol%, whereas it is 30 to 40 mol% in *R. quintana* and *B. bacilliformis*. Of course it is possible, although highly unlikely, that the total 16S rRNA sequence of the agent of epithelioid angiomatosis differs substantially from the partial sequence reported by Relman et al. (18) and, perhaps, is close to that of the cat scratch disease bacillus. If that occurs, then it is not rickettsia-like. These questions will be answered definitively only when there is an isolate of the agent of epithelioid angiomatosis or there is a direct comparison of their 16S rRNA sequences.

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