Comparison of Partial Citrate Synthase Gene (*gltA*) Sequences for Phylogenetic Analysis of *Bartonella* Species

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Nucleotide base sequence data were obtained for a 940-bp fragment of the citrate synthase-encoding gene (*gltA*) of representatives of the eight validly described *Bartonella* species and seven uncharacterized *Bartonella* strains obtained from small mammals. Complete 16S rRNA gene sequences were also determined for the uncharacterized strains, and these sequences revealed that each strain had a unique sequence which was very similar to the sequences of the previously recognized *Bartonella* species. A comparison of the *gltA* sequences of the different *Bartonella* species revealed that the levels of similarity between sequences were 83.8 to 93.5%, whereas comparisons of sequences obtained from different strains of the same species revealed that the levels of similarity were more than 99.8%. One of the uncharacterized strains had a *gltA* sequence that matched the sequence of *Bartonella elizabethae*, three uncharacterized strains had sequences which were more than 99.6% similar to each other (but less than 93.5% similar to any other sequence), and the remaining three uncharacterized strains each exhibited less than 93.5% sequence similarity to other *Bartonella* species or isolates. Phylogenetic trees were inferred from multiple alignments of both *gltA* and 16S ribosomal DNA (rDNA) sequences. Whereas the proposed intra-*Bartonella* architecture of trees inferred from 16S rDNA sequence data by using both distance matrix and parsimony methods had virtually no statistical support, the trees inferred from the *gltA* sequence data contained four well-supported lineages in the genus. The *gltA*-derived phylogeny appears to be more useful than the phylogeny derived from 16S rDNA sequence data for investigating the evolutionary relationships of *Bartonella* species, and the validity of the lineages identified by the *gltA* analysis is discussed in this paper.

The family *Bartonellaceae* has recently been subjected to considerable taxonomic reassessment, which has resulted in (i) the transfer of members of the genus *Rochalimaea* (formerly placed in the *Rickettsiaceae*) to the genus *Bartonella* (7), (ii) the unification of the two established genera in the family, the genera *Bartonella* and *Grahamella*, with the name of the former taking precedence (5), (iii) the proposal of several new species and subspecies (5, 6), and (iv) the removal of the family from the order *Rickettsiales* (7). The restructured family now includes the single genus *Bartonella*, which contains eight species. A polyphasic approach was used to develop all of these proposals, and phylogenetic data derived from a comparison of 16S rRNA gene sequences provided important supporting evidence. These studies also revealed that the *Bartonella* species are very closely related and that they exhibit very high levels of 16S rRNA gene sequence similarity (>97.8%) and significant levels of DNA-DNA homology (16 to 67%) (5, 7, 8). The paucity of 16S rRNA gene sequence differences between species limits the usefulness of this tool for inferring intragenus phylogeny and, as in other closely related taxa, does not allow measurement of small phylogenetic distances with sufficient precision to define species (14, 29).

Although 16S rRNA gene sequence analysis remains the most widely used tool in bacterial phylogeny and in most instances the data concur with the results of phenotypic and genotypic analyses, workers have expressed concern that if a single gene is used as the sole basis of phylogeny, it is possible that a recent transfer of the gene between dissimilar species could give rise to an organism that appears to be specifically

related to the donor of the gene rather than to its true relatives (27). One way of revealing such events is to compare phylogenetic trees inferred from genetic sequences independent of one another, genes which have not been recently acquired will reveal the same phylogeny (17). In a recent study, phylogenetic trees inferred from a comparison of partial methyl-coenzyme M reductase subunit A gene (*mcrI*) sequences from closely related species belonging to the family *Methanosarcinaceae* corresponded closely to trees inferred from 16S rRNA gene sequence data (28). In this study it was also determined that *mcrI* sequences evolved about three times more rapidly than 16S rRNA gene sequences; thus, closely related species exhibited markedly lower levels of sequence similarity. An increase in the number of discernible differences between such species should allow more confident estimates of their phylogenetic relationships.

Citrate synthase is the first enzyme of the tricarboxylic acid cycle and thus is a key regulator of intracellular ATP production in both prokaryotic and eucaryotic cells (32). Although this enzyme exists in two multimeric forms (a dimer and a sexmer), monomers of the two forms are similar in size and exhibit sequence homology (32). The nucleic acid sequence of the *gltA* gene, which codes for the enzyme, has been determined for a number of bacteria, including *Bartonella henselae* (9, 19–21, 33). The use of *gltA* comparisons to estimate divergence among closely related species was first proposed by Regnery and his colleagues, who demonstrated genotypic relationships among *Rickettsia* species by comparing multiple restriction endonuclease digestion patterns of partial *gltA* PCR amplicons (24), and identification schemes developed from this work were subsequently used to differentiate *Bartonella* species (23). Recently, 345-bp nucleotide sequences of these *gltA* amplicons have been determined for five *Bartonella* species (16), and a comparison of these sequences revealed levels of similarity between approximately 80 and 90%,

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TABLE 1. Sources of *Bartonella* isolates

Species	Strain	EMBL and GenBank 16S rRNA sequence accession no.	Source (reference) ^a
<i>B. bacilliformis</i>	LA6.3	Z70003	Blood of bartonellosis patient, Yumpe, Huailacayán Valley, Ancash, Peru
<i>B. doshiae</i>	R18 ^T	Z31351	NCTC 12862
<i>B. elizabethae</i>	F9251 ^T	L01260	ATCC 49927
<i>B. grahamii</i>	V2 ^T	Z31349	NCTC 12860
<i>B. henselae</i>	Houston-1 ^T	M73229	ATCC 49882
<i>B. henselae</i>	WC1		Blood of domestic cat, London, United Kingdom (10)
<i>B. henselae</i>	URLLY 8		Cat scratch disease patient (10)
<i>B. henselae</i>	URLIE 9		Endocarditis patient (10)
<i>B. quintana</i>	Fuller ^T	M73228	ATCC VR-358
<i>B. quintana</i>	Oklahoma		Gift from D. F. Welch, University of Oklahoma, Oklahoma City
<i>B. quintana</i>	Paris-1		Endocarditis patient (11)
<i>B. quintana</i>	Marseille-1		Chronic adenopathy patient (22)
<i>B. taylorii</i>	M6 ^T	Z31350	NCTC 12861
<i>B. vinsonii</i>	Baker ^T	Z31352	ATCC VR-152
<i>Bartonella</i> sp.	C1-phy	Z70006	Blood of <i>Phyllotis</i> sp., Huailacayán Valley, Ancash, Peru
<i>Bartonella</i> sp.	C4-phy	Z70007	Blood of <i>Phyllotis</i> sp., Huailacayán Valley, Ancash, Peru
<i>Bartonella</i> sp.	C5-rat	Z70008	Blood of <i>Rattus</i> sp., Huailacayán Valley, Ancash, Peru
<i>Bartonella</i> sp.	C7-rat	Z70004	Blood of <i>Rattus</i> sp., Huailacayán Valley, Ancash, Peru
<i>Bartonella</i> sp.	R-phy1	Z70005	Blood of <i>Phyllotis</i> sp., Rimac Valley, Lima, Peru
<i>Bartonella</i> sp.	R-phy2	Z70001	Blood of <i>Phyllotis</i> sp., Rimac Valley, Lima, Peru
<i>Bartonella</i> sp.	N40	Z70002	Blood of <i>Apodemus sylvaticus</i> , Staffordshire, United Kingdom

^a NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.

values that were markedly lower than the levels of similarity between 16S rRNA genes. In this study, 946-bp sequences, representing more than 75% of the complete gene, were determined for representatives of all eight *Bartonella* species and for an additional seven *Bartonella* strains which could be distinguished from these species on the basis of 16S rRNA sequence data. The selection of sequencing primers was simplified by referring to the *gltA* sequence of *B. henselae* (20). The *gltA* sequences obtained were analyzed and were used to create phylogenetic trees, which were compared with 16S rRNA trees.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The sources of the *Bartonella* strains used in this study are shown in Table 1. The strains isolated from the blood of small mammals in Peru were obtained as part of a concurrent study through collaboration with Humberto Guerra, Alejandro Llanos-Cuentas, and Kike Perez and their staff at the Instituto de Medicina Tropical Alexander Von Humboldt, University Cayatana Herrer, Lima, Peru. Strain N40 was obtained during a previous study (1). The previously uncharacterized strains were preliminarily identified as bartonellae on the basis of their phenotypic properties, including their growth requirements (the strains could not be cultivated on media lacking blood), colonial morphology, microscopic appearance (weakly staining gram-negative rods), and lack of catalase and oxidase activities, and the results of a restriction endonuclease analysis of their amplified 16S rRNA genes (2).

All strains were grown on Columbia blood agar containing 5% whole sheep blood. Most plates were incubated at 37°C in a CO₂-rich, moist atmosphere for 10 days; the exceptions were the plates inoculated with *Bartonella bacilliformis*, which were incubated at 30°C. Crude DNA extracts that were suitable for use as templates in PCR were prepared from isolates by using a Chelex 100 reagent (30).

16S rRNA gene amplification, purification, and sequence determination. The 16S rRNA genes were amplified by PCR under conditions which have been described previously (3). PCR amplicons were purified by using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions for use as templates in cycle sequencing reactions. For sequencing reactions we used the reagents of an Amplicycle kit (Pharmacia Biotech, St-Quentin Yveline, France), which included fluorescein-labelled primers (Eurogentec, Seraing, Belgium), according to the manufacturer's instructions. The primers used were universal eubacterial 16S rRNA sequencing primers that have been described previously (12). The sequencing products were resolved on 0.35-mm-thick READIMIX acrylamide gels (Pharmacia), and fluorescein-labelled products were detected and translated into sequence data by using a model ALF automated sequencer (Pharmacia) and associated software.

Amplification of *gltA*. PCR amplification of a 1,038-bp fragment of *gltA* was carried out by using an MJ Research model PTC-200 thermal cycler. Each reaction mixture contained 2 µl of crude DNA extract, 40 pmol of each primer, 1 µl of a deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, and dTTP, each at a concentration of 10 mM), 10 µl of 10× reaction buffer, 8 µl of a 25 mM MgCl₂ solution, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); the volume of the reaction mixture was adjusted to 100 µl with sterile distilled water. The primers used were primer CS140f (5'-TTACTTATG ATCKGGGYTTTA, where K is equimolar amounts of G and T and Y is equimolar amounts of C and T) and primer BhCS.1137n (20). The thermal cycle for amplification consisted of 35 cycles, with each cycle consisting of denaturation at 95°C for 10 s, primer annealing at 50°C for 20 s, and primer extension at 72°C for 50 s. Amplification products were resolved by 1% agarose gel electrophoresis and were visualized under UV light following ethidium bromide staining.

Purification and sequencing of *gltA* amplicons. The *gltA* amplicons were purified as described above by using QIAquick columns, and the purified products were sequenced by using the reagents of an Amplicycle kit (Pharmacia Biotech, Uppsala, Sweden) and fluorescein-labelled primers (Eurogentec) according to the manufacturer's instructions. The following primers were used for sequencing: primers CS140f, CS443f (5'-GCTATGTCTGCATTCTATCA), CSH1f (5'-GCG AATGAAGCGTGCCTAAA), and BhCS.1137n. Sequencing products were resolved and translated into sequence data as described above.

Analysis of sequence data and construction of phylogenetic trees. For each strain tested, the complete primary sequences of 16S rRNA genes and *gltA* were assembled by alignment and by combining the sequences generated by each primer, using DNASIS (Hitachi Software Engineering America, Ltd., Brisbane, Calif.). The 16S rRNA sequence data obtained were confirmed by examining base pairing in the predicted secondary structure, and *gltA* sequences were assessed by identifying codon triplets.

Complete primary 16S rRNA sequences were then aligned with each other and with 16S rRNA sequences of the recognized *Bartonella* species and other members of the *Eubacteria* by using version V of the CLUSTAL multisequence alignment program (15). The *gltA* sequences obtained in this study were aligned with each other, with the sequence of *B. henselae*, and with the sequences available for other members of the *Eubacteria* by using the same method. A total of 100 bootstrap samples for both alignments were produced by using the program SEQBOOT from the PHYLIP version 3.4 suite of software (13), and a phylogenetic tree was inferred from each bootstrap sample by using parsimony (DNAPARS in PHYLIP). The resulting trees were combined to yield a consensus tree (CONSENSE in PHYLIP). A matrix of evolutionary distances was also derived from each bootstrap alignment by using DNADIST (PHYLIP) and the assumptions of Jukes and Cantor. Trees were inferred from the matrices by using the FITCH program (PHYLIP), in which a least-squares method is used to construct a phylogenetic tree that best fits the data. The resulting trees were combined to yield a consensus tree (CONSENSE in PHYLIP).

Levels of similarity between *gltA* sequences were determined by sequentially comparing pairs by using the DNA homology search function of DNASIS.

TABLE 2. Levels of 16S rRNA gene sequence similarity and *gltA* sequence similarity

Taxon	% Similarity ^a																				
	<i>Bartonella bacilliformis</i>	<i>Bartonella doszhae</i>	<i>Bartonella elizabethae</i>	<i>Bartonella grahamii</i>	<i>Bartonella henselae</i>	<i>Bartonella quintana</i>	<i>Bartonella taylorii</i>	<i>Bartonella vinsonii</i>	Strain NP40	Strain R-phy1	Strain R-phy2	Strain C1-phy	Strain C4-phy	Strain C5-rat	Strain C7-rat	<i>Rhizobium tropici</i>	<i>Rickettsia prowazekii</i>	<i>Coxiella burnetii</i>	<i>Escherichia coli</i>	<i>Corynebacterium glutamicum</i>	<i>Acinetobacter anitratum</i>
<i>Bartonella bacilliformis</i>		97.9	97.7	97.9	97.7	97.5	98.1	98.1	98.2	97.8	97.9	97.9	97.8	97.8	97.9	92.8	83.6	71.7	69.7	75.9	70.5
<i>Bartonella doszhae</i>	83.8		98.7	99.4	99.3	98.7	99.1	99.6	99.4	99.3	99.2	99.3	99.1	99.2	98.9	93.3	84.1	71.7	70.4	76.4	70.6
<i>Bartonella elizabethae</i>	84.4	85.8		98.9	98.4	98.2	98.4	98.8	98.7	98.9	98.7	98.7	98.7	99.4	99.2	93.3	84.3	71.7	70.3	76.0	70.4
<i>Bartonella grahamii</i>	85.2	86.3	93.2		99.1	98.7	99.0	99.4	99.4	99.5	99.5	99.4	99.4	99.4	99.2	93.2	84.1	71.4	69.7	76.1	70.2
<i>Bartonella henselae</i>	86.4	87.5	87.1	88.7		98.8	98.7	99.3	99.0	99.1	98.9	99.0	98.9	99.1	98.8	92.9	83.7	71.2	70.3	76.2	70.5
<i>Bartonella quintana</i>	84.9	86.3	85.2	86.5	91.0		98.6	98.6	98.8	98.7	98.5	98.5	98.3	98.7	98.6	92.5	84.0	71.6	70.1	76.4	69.7
<i>Bartonella taylorii</i>	84.5	85.8	86.3	87.7	88.5	88.2		99.2	99.5	99.0	98.5	98.9	98.8	99.0	98.6	93.1	83.9	71.4	69.8	76.2	70.1
<i>Bartonella vinsonii</i>	85.2	86.6	88.1	88.9	88.6	87.5	90.7		99.7	99.4	99.5	99.6	99.4	99.4	99.1	93.5	84.1	71.5	70.4	76.2	70.6
Strain N40	85.3	87.0	87.8	89.6	89.6	87.3	90.2	89.4		99.4	99.4	99.4	99.2	99.3	99.0	93.4	84.1	71.3	70.1	76.4	70.4
Strain R-phy1	83.7	85.6	87.7	88.9	88.9	86.8	89.4	90.2	89.0		99.6	99.6	99.4	99.4	99.3	93.3	84.2	71.4	70.2	76.3	69.9
Strain R-phy2	84.2	87.3	88.0	88.5	88.5	86.8	89.8	90.5	89.6	93.4		99.8	99.9	99.2	99.1	93.5	84.0	71.6	69.5	76.0	70.0
Strain C1-phy	84.1	87.1	88.0	88.5	88.2	86.7	89.4	90.2	89.3	93.2	99.6		99.8	99.2	99.1	93.4	84.1	71.9	70.2	76.1	70.1
Strain C4-phy	84.0	87.0	88.0	88.3	88.0	86.6	89.3	90.1	89.4	92.7	99.9	99.7		99.1	99.0	93.4	84.0	71.6	70.0	76.0	70.3
Strain C5-rat	84.2	85.1	92.4	94.2	86.7	84.4	86.5	87.1	88.0	86.5	86.9	87.0		99.4	93.5	84.1	71.5	69.3	76.5	70.5	
Strain C7-rat	84.4	85.8	100	93.2	87.1	85.2	86.3	88.1	87.8	87.7	88.0	88.0	88.0	92.4		93.3	84.2	71.5	69.7	76.2	70.0
<i>Rhizobium tropici</i>	67.0	68.6	70.1	69.5	68.4	67.8	67.6	68.7	68.5	69.6	69.1	68.8	69.2	69.2	70.1		83.2	70.6	71.0	74.9	71.6
<i>Rickettsia prowazekii</i>	65.6	67.2	65.5	65.3	65.9	65.6	64.1	63.9	65.4	64.4	64.2	64.3	64.3	94.6	65.5	59.2		68.1	68.5	74.1	71.3
<i>Coxiella burnetii</i>	65.2	66.6	64.9	66.5	65.3	64.2	64.9	64.6	65.7	65.1	65.1	65.3	65.2	66.3	64.9	63.9	62.7		83.0	67.6	83.2
<i>Escherichia coli</i>	65.5	66.5	67.2	66.6	67.1	64.8	66.2	65.9	65.3	66.8	67.1	66.8	67.2	66.6	67.2	68.2	61.4	65.2		69.1	84.5
<i>Corynebacterium glutamicum</i>	53.9	54.4	55.6	55.0	54.2	54.4	54.4	54.4	54.0	54.2	54.6	54.2	54.4	55.5	55.6	59.8	51.7	54.5	57.9		68.9
<i>Acinetobacter anitratum</i>	67.1	64.9	66.8	67.4	66.8	67.1	64.9	65.6	65.1	65.3	65.3	65.4	65.4	66.2	66.8	64.0	62.6	65.5	67.8	56.4	

^a The values on the upper right are levels of 16S rRNA gene sequence similarity, and the values on the lower left are levels of *gltA* gene sequence similarity.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences which we determined have been deposited in the EMBL data bank under the following accession numbers: strain C1-phy, Z70006; strain C4-phy, Z70007; strain C5-rat, Z70008; strain C7-rat, Z70004; strain R-phy1, Z70005; strain R-phy2, Z70001; strain N40, Z70002; and *B. bacilliformis* LA6.3, Z70003. The 16S rRNA gene sequence accession numbers for the eight currently recognized *Bartonella* species are shown in Table 1, and the 16S rRNA gene sequence accession numbers for the organisms used for comparison are as follows: *Rickettsia prowazekii*, M21789; *Acinetobacter anitratum*, U10874; *Corynebacterium glutamicum*, X80629; *Rhizobium tropici*, U38469; *Escherichia coli*, Z46753; and *Coxiella burnetii*, M21291.

The *gltA* sequences which we determined have been deposited in the EMBL data bank under the following accession numbers: *B. bacilliformis* LA6.3, Z70021; *Bartonella doszhae*, Z70017; *Bartonella elizabethae*, Z70009; *Bartonella grahamii*, Z70016; *Bartonella quintana* Fuller^T (T = type strain), Z70014; *Bartonella taylorii*, Z70013; *Bartonella vinsonii*, Z70015; strain C1-phy, Z70022; strain C4-phy, Z70019; strain C5-rat, Z70018; strain C7-rat, Z70020; strain R-phy1, Z70010; strain R-phy2, Z70011; and strain N40, Z70012. The *gltA* sequence accession numbers for the reference organisms are as follows: *Rhizobium prowazekii*, M17149; *A. anitratum*, M33037; *Corynebacterium glutamicum*, X71489; *Rhizobium tropici*, Z34516; *E. coli*, J01619; and *Coxiella burnetii*, L33409.

RESULTS AND DISCUSSION

Sequence alignment. The primary 16S rRNA gene sequences generated for the seven uncharacterized *Bartonella* isolates studied consisted of between 1,409 and 1,411 bp; i.e., they were equivalent to almost the entire gene. The primary sequence generated for *B. bacilliformis* LA6.3 consisted of 1,380 bp. For the multiple alignment of 16S rRNA gene sequences we used the sequences of the seven uncharacterized strains (strains C1-phy, C4-phy, C5-rat, C7-rat, R-phy1, R-phy2, and N40), *B. bacilliformis* LA6.3, the eight currently recognized *Bartonella* species, *Rickettsia prowazekii*, *A. anitratum*, *Corynebacterium glutamicum*, *Rhizobium tropici*, *E. coli*, and *Coxiella burnetii* (the last six species were chosen because *gltA* data were available for them).

The primary *gltA* sequences generated for the *Bartonella* strains were 946 bp long and represented the sequence be-

tween positions 136 and 1082 of the *B. henselae* *gltA* sequence, as determined previously (19). For the multiple alignment of *gltA* sequences we used the sequences of *B. bacilliformis* LA6.3, *B. doszhae*, *B. elizabethae*, *B. grahamii*, *B. quintana* Fuller^T, *B. taylorii*, *B. vinsonii*, strains C1-phy, C4-phy, C5-rat, C7-rat, R-phy1, R-phy2, and N40, *Rickettsia prowazekii*, *A. anitratum*, *Corynebacterium glutamicum*, *Rhizobium tropici*, *E. coli*, and *Coxiella burnetii*.

Analysis of uncharacterized *Bartonella* isolates by comparison of 16S rRNA gene sequences. Phylogenetic inferences were made on the basis of a 1,327-bp multiple alignment of 16S rRNA gene sequences from which regions of ambiguity (although not regions of hypervariability) had been removed. All seven uncharacterized *Bartonella* isolates had unique 16S rRNA gene sequences, although all of these organisms exhibited very high levels of sequence similarity with each other and with the type strains of the eight *Bartonella* species (Table 2). There appears to be one region of the 16S rRNA gene which is highly variable in *Bartonella* species; this region corresponds to positions 183 to 187 (*E. coli* structural model), in variable region V2. At this point, the sequences of the seven uncharacterized isolates have between one and three base insertions. An examination of 16S rRNA secondary-structure models suggests that these changes should result in a slight lengthening of helix 10 or a slight enlargement of the loop that it carries. The hypervariability of this region has been observed previously in other *Bartonella* isolates, notably a strain of *B. vinsonii* which has a 27-bp insertion at this point (6).

An analysis of the phylogenetic tree (Fig. 1) derived from the distance matrix indicated that the seven uncharacterized strains belong to a monophyletic cluster which includes the eight recognized *Bartonella* species and no other organisms. Of the non-*Bartonella* species included in this study, the two representatives of the alpha subdivision of the *Proteobacteria*, to

FIG. 1. Comparison of Fitch distance matrix trees derived from a 940-bp fragment of *gltA* (right side) and from the 16S rRNA gene (left side) for *Bartonella* strains and other representatives of the *Eubacteria*. The support for each branch, as determined from 100 bootstrap samples, is indicated by the value at the node. The lengths of vertical lines are not significant. The scale inside the dashed box is 10 times larger than the scale outside the box. Abbreviations: B., *Bartonella*; Rh., *Rhizobium*; R., *Rickettsia*; C., *Corynebacterium*; Cx., *Coxiella*; A., *Acinetobacter*; E., *Escherichia*.

which *Bartonella* species are also assigned, lie in closest evolutionary proximity. Within the *Bartonella* cluster, *B. bacilliformis* remains the most outlying species. Previously, it could have been proposed that the geographic isolation of this species (which is limited to the inter-Andean valleys of South America) influenced its divergence from the other *Bartonella* species; however, the six uncharacterized *Bartonella* strains which originated from the same region do not exhibit a closer evolutionary relationship to *B. bacilliformis* than to the other *Bartonella* species, which originated in Europe and the United States. The four isolates obtained from *Phyllotis* mice grouped together on the tree, although support for this cluster was only moderate (65 of 100 bootstrap samples). Although the isolates obtained from Peruvian rats also occur close to one another, they also exhibit high levels of homology with *B. elizabethae*, a species presently represented by a single isolate obtained from a patient with endocarditis in the United States (8). In general, the branching order of the tree has only moderate support, and the instability of the proposed structure was confirmed by the results of a parsimony analysis of the sequence data (Fig. 2). Other than the branch containing *B. bacilliformis*, no branch of the genus, on either tree, had the support of more than 90% of the bootstrap samples. Indeed, a comparison of the branching order of the *Bartonella* species on the two trees revealed

marked differences. Conversely, outside the *Bartonella* cluster, the stability of the proposed branching order was considerably higher; when a distance matrix analysis was performed, four of the six branches were retained in more than 90% of bootstrap samples, whereas when a parsimony analysis was performed, all six branches had this level of support.

Although the finding described above may suggest that there are no distinct evolutionary subgroups in the genus *Bartonella*, it more likely emphasizes the insensitivity of 16S ribosomal DNA (rDNA) sequence comparisons for inferring evolutionary relationships at this level. It is clear that inferences are difficult to make with confidence when there are so few base differences between sequences and when the base differences occur at highly variable positions where multiple base changes may have taken place.

Phylogeny of *Bartonella* species based on partial *gltA* sequences. A pairwise comparison of *gltA* sequences revealed similarity values between 83.7 and 100% for the *Bartonella* strains (Table 2), whereas levels of similarity of less than 70% were observed between the sequences of *Bartonella* species and the sequences of organisms belonging to other genera. The sequences of the four isolates of *B. henselae* were virtually identical, differing at only one base site (Table 3); this level of similarity was also observed for the four strains of *B. quintana*

FIG. 2. Comparison of parsimony trees derived from a 940-bp fragment of *gltA* (right side) and from the 16S rRNA gene (left side) for *Bartonella* strains and other representatives of the *Eubacteria*. The support for each branch, as determined from 100 bootstrap samples, is indicated by the value at the node. This analysis provided tree topology only, and the lengths of both the vertical lines and the horizontal lines are not significant. Abbreviations: *B.*, *Bartonella*; *Rh.*, *Rhizobium*; *R.*, *Rickettsia*; *C.*, *Corynebacterium*; *Cx.*, *Coxiella*; *A.*, *Acinetobacter*; *E.*, *Escherichia*.

tested (Table 3). The highest level of similarity between sequences from different *Bartonella* species was 93.2%. These observations are consistent with the results of other genotypic analyses of *Bartonella* species; a comparison of the hypervariable spacer region sequence between the 16S and 23S rRNA genes revealed very high levels of similarity between strains of the same species (seven *B. quintana* strains differed at a maximum of 5 sites in a 1,331-bp sequence), whereas the differences between species were great enough that reasonable alignments could not be obtained (levels of similarity, less than 50%) (25). A macrorestriction analysis of *Bartonella* strains in which pulsed-field gel electrophoresis was used also revealed that strains of the same species had very similar profiles which could be clearly differentiated from the profiles obtained for other species (25).

Interestingly, the sequence of strain C7-rat was indistinguishable from that of *B. elizabethae*. As this result was unex-

pected, the identities of the isolates were confirmed in two ways. First, partial 16S rRNA gene and *gltA* sequences determined for multiple samples of both C7-rat and *B. elizabethae* matched the sequences of original preparations used in this study. Second, a restriction endonuclease analysis of PCR-amplified 16S/23S intragenic spacer regions (25) of C7-rat and *B. elizabethae* revealed profiles which were very similar but distinguishable (data not shown).

The *gltA* sequences of strains C1-phy, C4-phy, and R-phy2 exhibited levels of similarity of more than 99.6% with each other, but levels of similarity of less than 93.5% with the sequences of all other strains. The remaining uncharacterized isolates exhibited less than 93.5% similarity with all other *Bartonella* strains.

A phylogenetic tree (Fig. 1) was constructed from the distance matrix. Although the genus *Bartonella* is monophyletic and *B. bacilliformis* is the most outlying species in this group, the architecture of this tree differs markedly from the architecture of the tree derived from 16S rDNA data.

Although the alpha subdivision representative *Rickettsia prowazekii* is close to the *Bartonella* cluster, *Rhizobium tropici* is in a position markedly more distant than the position inferred from the 16S rRNA gene sequence comparison. However, the sequence data available for *Rhizobium tropici* were derived from *gltA* in a plasmid rather than genomic DNA (21) and as such cannot be considered representative of the evolution of the species. Although the relationship between the *Bartonella* species and the species not included in the alpha subdivision inferred from *gltA* sequence data is markedly different from the relationship derived from 16S rDNA analysis, there is no statistical support for its architecture. From the tree derived from the parsimony analysis (Fig. 2), the instability of the proposed extra-*Bartonella* architecture was confirmed, as a different

TABLE 3. Levels of similarity between *gltA* sequences of *B. henselae* and *B. quintana* strains

Strain	% Similarity						
	<i>B. henselae</i> Houston-1 ^T	<i>B. henselae</i> WC1	<i>B. henselae</i> URLLY8	<i>B. henselae</i> URLIE9	<i>B. quintana</i> Fuller ^T	<i>B. quintana</i> Oklahoma	<i>B. quintana</i> Paris-1
<i>B. henselae</i> WC1	99.8						
<i>B. henselae</i> URLLY8	99.9	99.9					
<i>B. henselae</i> URLIE9	99.8	100	99.9				
<i>B. quintana</i> Fuller ^T	91.0	91.0	91.0	91.0			
<i>B. quintana</i> Oklahoma	91.1	91.1	91.1	91.1	99.9		
<i>B. quintana</i> Paris-1	91.1	91.0	91.0	91.0	99.8	99.9	
<i>B. quintana</i> Marseille-1	91.1	91.0	91.0	91.0	99.8	99.9	100

branching order, again without the support of the bootstrap analysis, was produced.

However, unlike the results obtained in the 16S rDNA analysis, the intra-*Bartonella* phylogeny derived from *gltA* data has some statistical significance. The following four well-supported (>90% of bootstrap samples) clusters were identified in the genus: (i) consistent with the 16S rDNA analysis data, a unique, relatively deep-rooted divergence of *B. bacilliformis*, (ii) a cluster containing *B. grahamii*, *B. elizabethae*, and the two Peruvian rat isolates, (iii) a cluster containing the four isolates obtained from *Phyllotis* mice, and (iv) the cluster containing *B. henselae* and *B. quintana* (supported by distance matrix data but not by the results of a parsimony analysis of bootstrap samples).

Previous studies have shown that different small mammal species that inhabit the same geographical location are susceptible to infection by the same *Bartonella* species and that different *Bartonella* species can infect the same species of small mammal (4). However, there is clear evolutionary divergence not only between the strains isolated from *Phyllotis* mice and the strains isolated from rats in the Huailacayán Valley, but also between *B. grahamii* and the other United Kingdom small mammal isolates. Why such divergence between these strains occurred is not clear; however, species of small mammals are probably infected by the same *Bartonella* species by sharing the same flea fauna, which serves as a vector for the bacteria. As several different species of fleas are naturally found as ectoparasites on small mammals (26), infection by different *Bartonella* species may originate from different flea species, with each *Bartonella* species exhibiting species specificity for its vector. Such a proposal clearly needs further investigation. Interestingly, isolates obtained from *Phyllotis* mice in the geographically remote Rimac Valley shared a specific relationship with isolates obtained from the Huailacayán Valley; isolates of *B. grahamii* and *B. taylorii* have also been obtained from geographically distinct populations of small mammals in the United Kingdom (14).

All members of the genus *Bartonella* except *B. bacilliformis* exhibit very similar and significant levels of DNA-DNA homology (5, 7, 8). An analysis of the evolutionary distances derived from a *gltA* comparison of these species revealed that these distances are also very similar, with a narrow standard deviation (0.024) about a mean of 0.132 K_{nuc} (range, between 51 and 121% of the mean). In contrast, evolutionary distances derived from a 16S rRNA gene comparison exhibit a far broader standard deviation of 0.004 about a mean of 0.009 (range, between 33 and 176% of the mean). This comparison suggests that *gltA*-derived data are more compatible with DNA-DNA homology results than data derived from 16S rRNA genes are. However, such an observation is not unexpected, as the evolutionary distances between *Bartonella* species inferred from 16S rRNA comparisons are so small that minute differences appear to be significant. The evolutionary distances between *Bartonella* species inferred from *gltA* comparisons are 14.7 times greater (mean) than the evolutionary distances derived from 16S rRNA genes; thus, slight differences in distances between species are not very influential. Previous studies of other genera of bacteria have also revealed apparent disagreements between data derived from DNA-DNA homology studies and data derived from comparisons of very similar 16S rRNA gene sequences (14, 18), and such reports have culminated in a recent taxonomic proposal suggesting that meaningful taxonomic conclusions cannot be confidently inferred from comparisons of sequences that exhibit more than 97% sequence homology (29).

Furthermore, although the *gltA* sequences of different *Bar-*

tonella species have diverged at a considerably faster rate than 16S rRNA gene sequences have diverged, the apparent rates of divergence of the two gene sequences in closely related strains appear to be similar. Indeed, *B. elizabethae* and strain C7-rat, despite having identical *gltA* sequences, are separated by a branch length similar to the branch length observed between different species on the tree inferred from the 16S rRNA gene comparison. Which of these two models is the most accurate is unclear; however, this example demonstrates again that data derived from sequences which are virtually identical cannot be relied on for accurate phylogenetic reconstructions. It could also be proposed that C7-rat and *B. elizabethae* share *gltA* sequences as a result of a recent transfer of genetic material between the organisms. Such a proposal again highlights the potential problems associated with phylogenetic inferences derived from a comparison of only a single gene.

The correspondence of *B. elizabethae* and C7-rat *gltA* sequences is unexpected as the former organism has been encountered on only one occasion in the United States (8). Although additional comparisons of these organisms are clearly necessary, it is likely that C7-rat is a strain of *B. elizabethae*. This finding provides the first evidence that *B. elizabethae* infection, like *B. henselae* infection, is a zoonosis. The high levels of homology among C4-phy, R-phy2, and C1-phy (separated by less than 0.04 K_{nuc} , whereas the closest two *Bartonella* species are 0.66 K_{nuc} apart) suggest that these strains may also belong to a single species. The relationship of the remaining uncharacterized isolates, C5-rat, R-phy1, and N40, to other *Bartonella* species remains unclear; in particular, N40 exhibits no specific relationship with any of the recognized species or other uncharacterized strains.

Intra-*Bartonella* phylogeny remains confusing, and it is likely that this problem will become more complicated as additional isolates are obtained and additional characterization studies are performed. *Bartonella*-like organisms have been observed in the erythrocytes of a wide range of animals (31), and thus it is likely a large number of strains and species have yet to be identified and characterized. However, whereas 16S rDNA-based phylogenies must be considered of little use at this level, data derived from comparisons of *gltA* sequences have resulted in the identification of some distinct evolutionary divergence within the genus. Extended analysis of the *gltA* gene from a greater number of isolates should help improve our understanding of the evolutionary relationships in the genus *Bartonella*. It would also be interesting to assess the utility of this approach for determining the phylogenetic relationships between *Bartonella* species and other members of the alpha sub-division of the *Proteobacteria*.

REFERENCES

1. Ball, M. L. 1994. M.Sc. thesis. Liverpool School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom.
2. Birtles, R. J. 1995. Differentiation of *Bartonella* species by restriction endonuclease analysis of PCR-amplified 16S rRNA genes. *FEMS Microbiol. Lett.* 129:261-266.
3. Birtles, R. J., T. G. Harrison, N. K. Fry, N. A. Saunders, and A. G. Taylor. 1991. Taxonomic considerations of *Bartonella bacilliformis* based on phylogenetic and phenotypic characteristics. *FEMS Microbiol. Lett.* 83:187-192.
4. Birtles, R. J., T. G. Harrison, and D. H. Molyneux. 1994. *Grahamella* in small woodland mammals in the UK: isolation, prevalence and host specificity. *Ann. Trop. Med. Parasitol.* 88:317-327.
5. Birtles, R. J., T. G. Harrison, N. A. Saunders, and D. H. Molyneux. 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshaiae* sp. nov. *Int. J. Syst. Bacteriol.* 45:1-8.
6. Breitschwerdt, E. B., D. L. Kordick, D. E. Malarkey, B. Keene, T. L. Hadfield, and K. Wilson. 1995. Endocarditis in a dog due to infection with a novel *Bartonella* subspecies. *J. Clin. Microbiol.* 33:154-160.
7. Brenner, D. J., S. P. O'Connor, W. W. Winkler, and A. G. Steigerwalt. 1993.

- Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. *Int. J. Syst. Bacteriol.* **43**:777–786.
8. Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor. 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J. Clin. Microbiol.* **31**:872–881.
 9. Donald, L. J., and H. W. Duckworth. 1987. Expression and base sequence of the citrate synthase gene of *Acinetobacter anitratum*. *Biochem. Cell Biol.* **65**:930–938.
 10. Drancourt, M., R. J. Birtles, G. Chaumentin, F. Vandenesch, J. Etienne, and D. Raoult. 1996. New serotype of *Bartonella henselae* in endocarditis and cat-scratch disease. *Lancet* **347**:441–443.
 11. Drancourt, M., J. L. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Leher, J. Etienne, F. Goldstein, J. Acar, and D. Raoult. 1995. *Bartonella* (*Rochalimaea*) *quintana* endocarditis in homeless men. *N. Engl. J. Med.* **332**:419–423.
 12. Embley, T. M. 1991. The linear PCR: a simple and robust method for sequencing amplified rRNA genes. *Lett. Appl. Microbiol.* **13**:13–24.
 13. Felsenstein, J. 1989. PHYLIP: phylogeny inference package. *Cladistics* **5**:164–166.
 14. Fox, G. E., J. D. Wisotzkey, and P. Jurtschuk, Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**:166–170.
 15. Higgins, D., A. Bleasby, and R. Fuchs. 1992. Clustal V: improved software for multiple sequence analysis. *Comput. Appl. Biosci.* **8**:188–192.
 16. Joblet, C., V. Roux, M. Drancourt, J. Gouvenet, and D. Raoult. 1995. Identification of *Bartonella* (*Rochalimaea*) species among fastidious gram-negative bacteria on the basis of the partial sequence of the citrate synthase gene. *J. Clin. Microbiol.* **33**:1879–1883.
 17. Ludwig, W., J. Neumaier, N. Klugbauer, E. Brockmann, C. Roller, S. Jilg, K. Reetz, I. Schachtner, A. Ludvigsen, M. Bachleitner, U. Fischer, and K. H. Schleifer. 1993. Phylogenetic relationships of bacteria based on comparative sequence analysis of elongation factor Tu and ATP-synthase beta-subunit genes. *Antonie van Leeuwenhoek* **64**:285–305.
 18. Martinez-Murcia, A. J., S. Benlloch, and M. D. Collins. 1992. Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by ribosomal 16S rRNA sequencing: lack of congruence with results of DNA-DNA hybridization. *Int. J. Syst. Bacteriol.* **42**:412–421.
 19. Ner, S. S., V. Bhayana, A. W. Bell, I. G. Giles, H. W. Duckworth, and D. P. Bloxham. 1983. Complete sequence of the gltA gene encoding citrate synthase in *Escherichia coli*. *Biochemistry* **22**:5243–5249.
 20. Norman, A. F., R. Regnery, P. Jameson, C. Greene, and D. C. Krause. 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. *J. Clin. Microbiol.* **33**:1797–1803.
 21. Pardo, M. A., J. Lagunez, J. Miranda, and E. Martinez. 1994. Nodulating ability of *Rhizobium tropici* is conditioned by a plasmid-encoded citrate synthase. *Mol. Microbiol.* **11**:315–321.
 22. Raoult, D., M. Drancourt, A. Carta, and J. A. Gastaut. 1994. Letter. *Lancet* **343**:977.
 23. Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr. 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *J. Clin. Microbiol.* **30**:265–274.
 24. Regnery, R. L., C. L. Spruill, and B. D. Plikaytis. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **173**:1576–1589.
 25. Roux, V., and D. Raoult. 1995. Inter- and intraspecies identification of *Bartonella* (*Rochalimaea*) species. *J. Clin. Microbiol.* **33**:1573–1579.
 26. Smit, F. G. A. M. 1957. Handbooks for the identification of British insects, vol. 1. Royal Entomological Society of London, London.
 27. Sneath, P. H. A. 1993. Evidence from *Aeromonas* for genetic crossing-over in ribosomal sequences. *Int. J. Syst. Bacteriol.* **43**:626–629.
 28. Springer, E., M. S. Sachs, C. R. Woese, and D. R. Boone. 1995. Partial gene sequences for the A subunit of methyl-coenzyme M reductase (*mcrf*) as a phylogenetic tool for the family *Methanosarcinaceae*. *Int. J. Syst. Bacteriol.* **45**:554–559.
 29. Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846–849.
 30. Stein, A., and D. Raoult. 1992. A simple method for amplification of DNA from paraffin-embedded tissues. *Nucleic Acids Res.* **20**:5237–5238.
 31. Weinmann, D., and J. P. Kreier. 1977. *Bartonella* and *Grahamella*, p. 197–233. *In* J. P. Kreier (ed.), *Parasitic protozoa*, vol. 4. Academic Press, London.
 32. Wiegand, G., and S. J. Remington. 1986. Citrate synthase: structure, control, and mechanism. *Annu. Rev. Biophys. Biophys. Chem.* **15**:97–117.
 33. Wood, D. O., L. R. Williamson, H. H. Winkler, and D. C. Krause. 1987. Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. *J. Bacteriol.* **169**:3564–3572.