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Citrate Synthase Gene Comparison, a New Tool for Phylogenetic Analysis, and Its Application for the Rickettsiae†

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Using PCR and an automated laser fluorescent DNA sequencer, we amplified and sequenced a 1,234-bp fragment of the citrate synthase-encoding gene (*gltA*) of 28 bacteria belonging to the genus *Rickettsia*. Comparative sequence analysis showed that most of the spotted fever group (SFG) rickettsiae belonged to one of two subgroups. The first subgroup included *Rickettsia massiliae*, strain Bar 29, *Rickettsia rhipicephali*, "*Rickettsia aeschlimanni*," and *Rickettsia montana*, which have been isolated only from ticks. The second subgroup was larger and included the majority of the human pathogens and also rickettsiae isolated only from ticks; the members of this subgroup were strain S, *Rickettsia africae*, "*Rickettsia mongolotimonae*," *Rickettsia sibirica*, *Rickettsia parkeri*, *Rickettsia conorii*, *Rickettsia rickettsii*, the Thai tick typhus rickettsia, the Israeli tick typhus rickettsia, the Astrakhan fever rickettsia, "*Rickettsia slovacica*," and *Rickettsia japonica*. The sequence analysis also showed that the tick-borne organisms *Rickettsia helvetica* and *Rickettsia australis* and the mite-borne organism *Rickettsia akari* were associated with the SFG cluster; that *Rickettsia prowazekii* and *Rickettsia typhi*, two representatives of the typhus group, clustered together; and that *Rickettsia canada*, *Rickettsia bellii*, and the AB bacterium probably represent three new groups. We compared the phylogenetic trees inferred from citrate synthase gene sequences and from 16S ribosomal DNA (rDNA) sequences. For rickettsial phylogeny, the citrate synthase approach was more suitable, as demonstrated by significant bootstrap values for all of the nodes except those in the larger subgroup defined above. We also compared phylogenetic analysis results obtained in a comparison of the sequences of both genes for all of the representatives of the domain *Bacteria* for which the *gltA* sequence was determined. We believe that comparison of *gltA* sequences could be a complementary approach to 16S rDNA sequencing for inferring bacterial evolution, especially when unstable phylogenetic models are obtained from ribosomal sequences because of high levels of sequence similarity between the bacteria studied.

Rickettsiae belong to the order *Rickettsiales*, whose members were described as obligately intracellular gram-negative microorganisms (55). Several species cause disease in humans or in other vertebrate and invertebrate hosts and have a worldwide distribution. Recently, on the basis of 16S rRNA sequence similarity, *Rickettsia tsutsugamushi* was removed from the genus *Rickettsia* and was renamed *Orientia tsutsugamushi* (48). The genus *Rickettsia* is presently divided into two groups: the typhus group (TG), which includes three species (*Rickettsia prowazekii*, the agent of the epidemic typhus, *Rickettsia typhi*, which causes murine typhus, and *Rickettsia canada*, which has been isolated only from ticks), and the spotted fever group (SFG). The number of recognized members of the SFG increased following the development of an improved cell culture isolation technique (the shell vial technique) (26) and an increased interest in arthropod-transmitted diseases. New SFG rickettsiae have been isolated worldwide from arthropods; these organisms include *Rickettsia massiliae*, which was isolated in France (6) and in Greece (3), "*Rickettsia mongolotimonae*," which was isolated in the People's Republic of China (63), strain Bar 29, which was isolated in Spain (7), "*Rickettsia aeschlimanni*," which was isolated in Morocco (5), *Rickettsia hel-*

vetica, which was isolated in Switzerland (11), and the strain AB bacterium, which was isolated in England (57) (this strain has been characterized by genomic criteria but it remains unisolated). In addition, new SFG rickettsiae that have been isolated from humans include *Rickettsia africae* (from Zimbabwe) (24), *Rickettsia japonica* (from Japan) (49), *Rickettsia felis* (from the United States) (22, 44), the Astrakhan fever rickettsia (from Russia) (17), and "*Rickettsia mongolotimonae*" (from France) (37). These organisms were characterized by using phenotypic methods (serotyping and protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (1) and genomic methods (restriction fragment length polymorphism [RFLP] analysis of PCR amplification products [18, 38], pulsed-field gel electrophoresis [40], and 16S rRNA sequencing [41, 45]). Several studies aimed at assessing the genotypic relationships of rickettsiae have been performed by using randomly chosen DNA probes and RFLP analysis (36), PCR-RFLP profiling of the genes encoding citrate synthase (*gltA*) and protein rOmpA (a high-molecular-weight outer membrane protein) (38), or macrorestriction analysis with pulsed-field gel electrophoresis (40). These methods were useful for rickettsial identification and differentiation but were not really suitable for phylogenetic study. The most widely used approach for phylogenetic study of bacteria is based on comparisons of 16S rRNA gene sequences (60). This approach has already been used to demonstrate that the genus *Rickettsia* belongs to group 1 of the α subclass of the *Proteobacteria* and has a common origin with the genera *Ehrlichia*, *Cowdria*, and *Anaplasma* and *Wolbachia pipientis* (54). A high level of conservation in the sequence of this gene has been demonstrated

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† This work is dedicated to Natalia Balayeva, who participated in the beginning of the study and died during the time of this work.

for the members of the genus *Rickettsia* (levels of similarity between two species, more than 97.2%); thus, reliable phylogenetic analysis based on a comparison of the 16S rRNA gene sequences has been difficult, especially for the members of the SFG.

The importance of comparing results obtained for multiple molecules to test the reliability of organismal phylogeny has recently been highlighted, and it has been suggested that any alternative genes used should be genes whose products perform central cellular functions (32). At present, few genes have been sequenced in rickettsial genomes. We chose *gltA* as a possible tool for assessing more precise phylogenetic relationships among the rickettsiae. Citrate synthase is a component of nearly all living cells and is an enzyme of a central metabolic pathway, the citric acid cycle, which plays a key role in energy production and in providing important biosynthetic precursors (58). Two main types of citrate synthase have been identified, and these two types are made up of identical subunits that have M_s s of approximately 50,000; a dimeric form is found in members of the *Eukarya* and gram-positive bacteria, and a multimeric form (with four to six identical subunits) is found in members of the *Proteobacteria*. The two forms of the enzyme differ in their regulatory sensitivities; the dimer is sensitive in vitro to inhibition by ATP, whereas the multimeric form is inhibited by NADH and 2-oxoglutarate. The amino acid sequences of citrate synthase either have been determined directly from the protein or have been inferred from DNA sequences for members of the domain *Eukarya* (including pig [9], *Tetrahymena thermophila* [30], *Arabidopsis thaliana* [50], and *Saccharomyces cerevisiae* [46], for members of the domain *Bacteria* (including *Escherichia coli* [28], *Acinetobacter anitratum* [14], *R. prowazekii* [62], *Coxiella burnetii* [21], *Bartonella henselae* [29], *Pseudomonas aeruginosa* [15], *Corynebacterium glutamicum* [16], *Bacillus coagulans* [43], and *Mycobacterium smegmatis* [12]), and for a representative of the domain *Archaea*, *Thermoplasma acidophilum* (47). Multiple alignments of these protein sequences have revealed significant levels of similarity between intradomain *Eukarya* sequences (46 to 92%) and between intraphylum *Proteobacteria* sequences (56 to 75%); however, the interdomain levels of similarity are far lower (20 to 26%) (47). It appears, therefore, that the citrate synthase gene may be sufficiently informative to analyze the evolutionary relationships of closely related bacteria.

It has been determined that the rickettsiae can accumulate ATP directly from the cytoplasm of the host cell. However, these organisms are not strict energy parasites and can generate their own ATP via the tricarboxylic acid cycle and oxydative phosphorylation (59). Despite the resemblance of the morphology and cell wall chemistry of rickettsiae to the morphology and cell wall chemistry of typical gram-negative bacteria, the citrate synthase of *R. prowazekii* has been shown to resemble to the enzymes of members of the *Eukarya* and gram-positive bacteria in terms of its structure and regulation properties (33), but a sequence comparison has shown that it is most closely related to the enzymes of gram-negative bacteria. The *gltA* gene was the first rickettsial gene that was cloned and sequenced, but only one entire sequence is available, that of *R. prowazekii* (62). However, it was shown by PCR amplification that this gene was present on the chromosomes of all rickettsiae (18, 38). We amplified and sequenced the *gltA* gene of 28 rickettsiae and used alignments of the sequences as the basis for a phylogenetic analysis. The results obtained were compared with the results obtained when phylogenetic conclusions were inferred from 16S ribosomal DNA (rDNA) sequences.

MATERIALS AND METHODS

Rickettsial strains. All of the rickettsial strains included in this study are listed in Table 1.

(i) **Validly published species.** The following four strains were obtained from the American Type Culture Collection: *Rickettsia conorii* Moroccan and Seven (= Malish), *Rickettsia rickettsii* R (= Bitteroot), and *Rickettsia akari* MK (= Kaplan). *Rickettsia rhipicephali* 3-7-6 *R. africae* ESF-5, and *Rickettsia australis* Phillips were supplied by G. A. Dasch, Naval Medical Research Institute, Bethesda, Md. The following four organisms were a gift from D. H. Walker, University of Texas, Galveston: *R. japonica* YM, *Rickettsia parkeri* Maculatum 20, *Rickettsia montana* M/5-6, and *Rickettsia bellii*. Two strains, *R. massiliae* MtU1 and GS, were isolated in our laboratory from ticks found in France and in Greece, respectively. *Rickettsia sibirica* 246, *R. prowazekii* Breinl, *R. typhi* Wilmington, and *R. canadensis* 2678 were obtained from the Gamaleya Research Institute Collection. An isolate of *R. helvetica* was provided by W. Burgdorfer, Rocky Mountain Laboratory, Hamilton, Mont. *R. conorii* M1 and Indian tick typhus rickettsia were obtained from the Gamaleya Research Institute Collection.

(ii) **Species which have not been validly published.** The following three strains were a gift from G. A. Dasch: "*Rickettsia slovacica*" 13-B, Thai tick typhus rickettsia strain TT-118, and Israeli tick typhus rickettsia strain ISTT CDC1. Strain S was obtained from the Gamaleya Research Institute Collection. Ladybird beetles infected with the AB bacterium were provided by I. Zakharov, Vavilov Institute of General Genetics, Russian Academy of Science, Moscow. "*R. mongolotomanae*" HA-91 and Astrakhan fever rickettsia strain A-167 were isolated in our laboratory from ticks collected in the Alashian region of the Inner Mongolia autonomous region and in the Astrakhan region of Russia, respectively. Strain Bar 29 and "*R. aeschlimanni*" MC16 were isolated in our laboratory from ticks collected in Spain and Morocco, respectively.

Rickettsial cultivation and nucleic acid preparation. All strains were propagated on Vero cell monolayers (catalog no. CRL 1587; American Type Culture Collection). Dulbecco modified minimal essential medium (Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum (Eurobio) and 2% L-glutamine (Eurobio) was used for cultivation of *R. prowazekii*, minimal essential medium (MEM) (Eurobio) supplemented with 2% fetal calf serum and 2% L-glutamine was used for cultivation of *R. typhi*, and MEM supplemented with 4% fetal calf serum and 1% L-glutamine was used for cultivation of all of the other rickettsiae. Infected cells were maintained in a 5% CO₂ atmosphere at 35°C for *R. prowazekii* and *R. typhi* and at 32°C for all of the other rickettsiae. Infection of Vero cells was monitored by Gimenez staining, and rickettsiae were harvested at the optimal level of multiplication (after 3 to 5 days). Rickettsial cultures were centrifuged at 12,000 × g for 10 min, resuspended in Dulbecco modified minimal essential medium or MEM, and stored at -70°C.

Genomic DNA extracts were prepared as described previously (38). AB bacterium DNA was extracted from a ladybird beetle by using Qiagen columns (QIAamp tissue kit; QIAGEN, Hilden, Germany).

PCR amplification and sequencing method. PCR amplification and sequencing reactions were performed by using the oligonucleotide primers listed in Table 2. Oligonucleotides were purchased from Eurogentec S.A., Seraing, Belgium. Each PCR was carried in a Peltier model PTC-200 thermal cycler (MJ Research, Inc., Watertown, Mass.). AmpliTaq DNA polymerase and solutions of MgCl₂ and PCR buffer II were purchased from Perkin-Elmer Cetus, Norwalk, Conn., and the deoxynucleoside triphosphate solutions were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

A 380-bp fragment of the *gltA* gene was amplified by using primers Rp 877p and Rp 1258n as described by Regnery et al. (38). The 5'-terminal region and the 3'-terminal region of the citrate synthase gene were PCR amplified by using primers CS1d (or CSM18d for *R. akari* and *R. australis*) and CS890r and primers Rp877p and CS1273r, respectively.

Primers CS1d, CSM18d, and CS1273r were chosen by referring to the *gltA* sequence of *R. prowazekii* (62), and CS890r was selected following recognition of a conserved region within the gene after alignment of the sequences of the PCR fragments amplified with primers Rp877p and Rp1258n for all of the rickettsiae included in the study. The sequencing primers used were the primers used for PCR amplification, direct primers CS113d, CS113Bd, CS409d, CS535d, and CS1048d, and reverse primers CS244r, CS428r, CS715r. These primers were complementary to *gltA* conserved regions, as determined by alignment of the sequences. The sequencing primers were labeled in the 5'-terminal region with fluorescein isothiocyanate during oligonucleotide synthesis (Eurogentec).

A 3-μl portion of rickettsial genomic DNA was amplified in a 100-μl reaction mixture containing 10 pmol of each primer, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1.25 U of AmpliTaq DNA polymerase, and 0.2 μM MgCl₂ in 1× PCR buffer II. Amplification was carried under the following conditions: an initial 2-min denaturation step at 95°C was followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 45°C for 30 s, and extension at 65°C for 55 s. Amplification was completed by incubation for 3 min at 72°C to allow complete extension of the PCR products. To verify the results of amplification, 10 μl of the PCR product was resolved at 8 V/cm in a 1% agarose gel (type LE; Sigma-Aldrich Chimie, St. Quentin Fallavier, France) by using 1× TBE migration buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA; pH 8.0). The size of the PCR product was determined by comparison

TABLE 1. Rickettsial strains studied

Taxon	Strain	Source	Geographic origin	Human disease
<i>R. conorii</i>	Moroccan	Unknown	Morocco	Mediterranean spotted fever
<i>R. conorii</i>	Seven (= Malish)	Unknown	South Africa	Mediterranean spotted fever
<i>R. conorii</i>	M1	<i>Rhipicephalus sanguineus</i>	Georgia (formerly USSR)	
<i>R. conorii</i>	Indian tick typhus rickettsia	<i>Rhipicephalus sanguineus</i>	India	
Astrakhan fever rickettsia	A-167	<i>Rhipicephalus pumilio</i>	Astrakhan region (formerly USSR)	Astrakhan fever
Israeli tick typhus rickettsia	ISTT CDC1	Human	Israel	Israeli spotted fever
<i>R. sibirica</i>	246	<i>Dermacentor nuttali</i>	Former USSR	Siberian tick typhus
" <i>R. mongolotimonae</i> "	HA-91	<i>Haemophysalis asiaticum</i>	Inner Mongolia	Unnamed spotted fever
<i>R. parkeri</i>	Maculatum 20	<i>Amblyomma maculatum</i>	Mississippi	
<i>R. africae</i>	ESF-5	<i>Amblyomma variegatum</i>	Shulu Province, Ethiopia	African tick bite fever
Strain S	S	<i>Rhipicephalus sanguineus</i>	Armenia (formerly USSR)	
" <i>R. slovaca</i> "	13-B	<i>Dermacentor marginatus</i>	Slovakia	
Thai tick typhus rickettsia	TT-118	<i>Ixodes</i> sp. or <i>Rhipicephalus</i> sp.	Thailand	
<i>R. rickettsii</i>	R (= Bitterroot)	<i>Dermacentor andersoni</i>	Montana	Rocky Mountain spotted fever
<i>R. japonica</i>	YM	Human	Japan	Oriental spotted fever
<i>R. massiliae</i>	Mtu 1	<i>Rhipicephalus turanicus</i>	Camargue, France	
<i>R. massiliae</i>	GS	<i>Rhipicephalus sanguineus</i>	Greece	
Bar 29	Bar 29	<i>Rhipicephalus sanguineus</i>	Spain	
<i>R. rhipicephali</i>	3-7-6	<i>Rhipicephalus sanguineus</i>	Mississippi	
" <i>R. aeschlimanni</i> "	MC16	<i>Hyalomma marginatum</i>	Morocco	
<i>R. montana</i>	M/5-6	<i>Microtus</i> sp.	Montana	
<i>R. australis</i>	Phillips	Human	Queensland, Australia	Queensland tick typhus
<i>R. akari</i>	MK(= Kaplan)	Human	New York, N.Y.	Rickettsial pox
<i>R. helvetica</i>	C9P9	<i>Ixodes ricinus</i>	Switzerland	
<i>R. bellii</i>	369L42-1	<i>Dermacentor variabilis</i>	Ohio	
<i>R. canada</i>	2678	<i>Haemophysalis leporispalustris</i>	Canada	
AB bacterium		<i>Adalia bipunctata</i>	England	
<i>R. prowazekii</i>	Brein L	Human	Poland	Epidemic typhus
<i>R. typhi</i>	Wilmington	Human	North Carolina	Murine typhus

with DNA molecular weight marker VI (Boehringer Mannheim). PCR products were purified by using a QIAquick Spin PCR purification Kit (QIAGEN). Sequencing reactions were carried out by using an AmpliCycle sequencing kit (Perkin-Elmer Corp., Foster City, Calif.) according to the instructions of the supplier. The reactions were performed at the optimal temperatures (T), as calculated for each primer by using the following formula: $T = [4(nG + nC) + 2(nA + nT)] - 5$, where n is the number of bases in the primer. Reactions were stopped by adding 4 μ l of stop solution (100% deionized formamide, 6 mg of dextran blue per ml, 20 mM EDTA; pH 8.3). The products of sequencing reactions were resolved on 6% denaturing polyacrylamide gels (Pharmacia, St. Quentin-Yveline, France) by using 1 \times TBE buffer, and electrophoresis was performed with a model A.L. F. DNA sequencer (Pharmacia LKB). At least two sequencing reactions were performed for each strand of DNA.

Data analysis. The *gltA* sequences were aligned by using the multisequence alignment program CLUSTAL in the BISCANCE software package (13). Phylogenetic relationships were inferred by using version 3.4 of the PHYLIP software package (19). Evolutionary distance matrices, generated by DNADIST, were constructed by the method of Jukes and Cantor (23) and Kimura (25). The matrices were used to infer dendrograms by using the neighbor-joining method (42). The data were also examined by using parsimony analysis (DNAPARS in PHYLIP). A bootstrap analysis was performed to investigate the stability of the trees obtained. Bootstrap values were obtained for a consensus tree based on 100 randomly generated trees by using SEQBOOT and CONSENSE in the same package.

gltA and 16S rRNA sequences of *E. coli*, *Bartonella henselae*, *Bacillus coagulans*, *M. smegmatis*, *P. aeruginosa*, *Corynebacterium glutamicum*, and *Thermo-*

TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing of rickettsial species

Primer	Nucleotide sequence (5'-3')	Citrate synthase gene positions relative to the open reading frame	Organisms used in
CS1d ^a	ATGACTAATGGCAATAATAA	1-20	All except <i>R. australis</i> and <i>R. akari</i>
CSM18d ^a	GAATGAGGTATATACATTATG	-18-3	<i>R. australis</i> , <i>R. akari</i>
CS890r ^{a,b}	GCTTTAGCTACATATTTAGG	890-871	All
Rp877p ^{a,b}	GGGGACCTGCTCACGGCGG	797-815	All
Rp1258n ^{a,b}	ATTGCAAAAAGTACAGTGAACA	1178-1157	All
CS1273r ^{a,b}	CATAACCAGTGTAAGCTG	1273-1255	All
CS113d ^b	GTAGGGTATCTGCGGAAGC	113-131	All except <i>R. bellii</i>
CS113Bd ^b	GTAAAGTATATTCTGAAGCG	113-132	<i>R. bellii</i>
CS409d ^b	CCTATGGCTATTATGCTTGC	409-428	All
CS535d ^b	GCAATGTCTTATAAAATATTC	535-554	All
CS1048d ^b	CTTGAAGCTCTCCGCTCTTAA	1048-1067	All
CS244r ^b	CTTTAATATCATATCCTCGAT	244-224	All except <i>R. bellii</i>
CS428r ^b	GCAAGCATAATAGCCATAGG	428-409	All
CS715r ^b	CATTTTGCTCATGATCAGC	715-695	<i>R. bellii</i>

^a Oligonucleotide primer used for PCR amplification.

^b 5'-Isotocyanate-labeled primer used for sequencing.

plasma acidophilum and 16S rRNA sequences of the rickettsiae studied were obtained from the GenBank database. The same programs and methods as those described above were used to obtain phylogenetic inferences based on these sequences.

Levels of similarity between *gltA* sequences and between citrate synthase amino acid sequences were determined by using the homology search functions of DNASIS (Hitachi Software Engineering America, Ltd., Brisbane, Calif.). The *gltA* sequences were translated into protein sequences by using PCgene software.

Nucleotide sequence accession numbers. The *gltA* sequences of the rickettsiae studied have been deposited in the GenBank database under the following accession numbers: *R. parkeri*, U59732; *R. sibirica*, U59734; "*R. mongolotimonae*," U59731; strain S, U59735; *R. africana*, U59733; *R. conorii*, U59730; *R. rickettsii*, U59729; Astrakhan fever rickettsia, U59728; Israeli tick typhus rickettsia, U59727; Thai tick typhus rickettsia, U59726; "*R. slovaca*," U59725; *R. japonica*, U59724; *R. montana*, U74756; *R. massiliae*, U59719; strain Bar 29, U59720; *R. rhipicephali*, U59721; "*R. aeschlimanni*," U59722; *R. helvetica*, U59723; *R. australis*, U59718; *R. akari*, U59717; *R. typhi*, U59714; AB bacterium, U59712; *R. canada*, U59713; and *R. bellii*, U59716.

RESULTS

PCR amplification of rickettsial DNA and determination of the rickettsial *gltA* sequence. PCR amplification of the 5'-terminal region of the citrate synthase gene was obtained with primers CS1d and CS890r for all of the strains studied except the *R. akari* and *R. australis* strains. For these two strains, amplification of the 5'-terminal region of the gene was achieved by using an alternative primer for the direct strand of DNA, primer CSM18d, which localized 18 nucleotides upstream from the first base of the open reading frame. Subsequent sequencing demonstrated five and nine mutations in the region complementary to primer CS1d in *R. akari* and *R. australis*, respectively, which explained the lack of PCR amplification with primer CS1d for these two strains. The middle region of *gltA* was amplified for all of the strains studied with primers Rp877n and Rp1258p as described by Regnery et al. (38), and the 3'-terminal region of the gene was amplified for all of the strains with primers Rp877n and CS1273r. In all, the *gltA* sequences of three TG isolates and 25 SFG isolates were obtained. The nucleotide sequences determined were between the 21st nucleotide of the designated open reading frame and the 54th nucleotide upstream from the stop codon according to the sequence data for the *R. prowazekii* citrate synthase gene (62). The sequences were 1,234 nucleotides long for all of the samples studied except the *R. rickettsii* and *R. bellii* samples. *R. rickettsii* had a deletion of three bases at position 1006 (nucleotide positions are relative to the previously published sequence for the entire gene of *R. prowazekii*), and *R. bellii* had an insertion of three bases at position 455. The sequence data determined included data for about 89% of the open reading frame.

The sequences of four isolates of *R. conorii* (M1, Indian tick typhus rickettsia, Moroccan, and Seven) were identical. There was also no difference in the sequences of two isolates of *R. massiliae* (GS and Mtu1).

The levels of DNA sequence similarity between pairs of strains are shown in Table 3. Most of the strains exhibited between 98.5 and 99.9% similarity; this group included *R. conorii*, the Astrakhan fever rickettsia, the Israeli tick typhus rickettsia, *R. sibirica*, "*R. mongolotimonae*," *R. parkeri*, strain S, *R. africana*, *R. rickettsii*, "*R. slovaca*," the Thai tick typhus rickettsia, *R. japonica*, *R. massiliae*, strain Bar 29, *R. rhipicephali*, "*R. aeschlimanni*," and *R. montana*. However, the levels of similarity between representatives of this group and three other members of the SFG rickettsiae (*R. helvetica*, *R. akari*, and *R. australis*) were lower, ranging from 93.7 to 96.9%.

The level of similarity between *R. prowazekii* and *R. typhi*, two members of the TG, was 96.5%.

The levels of sequence similarity of *R. canada* and the AB

bacterium with representatives of the SFG rickettsiae and the TG rickettsiae were 90 to 93 and 89 to 90%, respectively.

R. bellii exhibited the lowest levels of sequence similarity (86.2 to 86.7%) with most other representatives of the SFG; the two exceptions were *R. akari* (level of similarity, 95.5%) and *R. australis* (95.8%). *R. bellii* also exhibited low levels of similarity with members of the TG rickettsiae (85 to 85.5%).

Similarities among the bacterial *gltA* sequences available in the GenBank database and among the corresponding amino acid sequences. Similarity data are shown in Table 4. The levels of *gltA* sequence similarity within the *Proteobacteria* and within the gram-positive bacteria were between 56.3 and 68.4% (between 56.7 and 75% for proteinic sequences [PS]) and between 46.1 and 47.1% (between 29.2 and 34.3% for PS), respectively. The levels of sequence similarity between members of the *Proteobacteria* and the gram-positive bacteria were between 44.8 and 60.5% (between 24.3 and 51.2% for PS). The levels of similarity for the member of the *Archaea*, *Thermoplasma acidophilum*, and representatives of the domain *Bacteria* were between 44.5 and 51% (between 25.7 and 31.2% for PS).

Phylogenetic analysis. Dendrograms were inferred by using neighbor-joining (Fig. 1) and parsimony methods. Analyses of the *gltA* sequence data by these two methods yielded slightly different trees (the architectures of the subgroup including strain S, *R. africana*, *R. parkeri*, *R. sibirica*, and "*R. mongolotimonae*" were different).

At this time, *R. prowazekii*, *R. typhi*, and *R. canada* are classified in the TG. All of the other rickettsiae are placed in the SFG. The phylogenetic analysis based on the *gltA* sequence comparison showed that *R. bellii*, *R. canada*, and the AB bacterium are on a branch which diverged before the split of the SFG and TG species. *R. canada* is outside the TG rickettsial cluster made up of *R. typhi* and *R. prowazekii*. *R. bellii* and the AB bacterium are outside the SFG cluster. *R. australis* and *R. akari* clustered together. *R. helvetica* was found on a distinct branch between the *R. akari*-*R. australis* cluster and the other SFG rickettsiae. All of the other SFG rickettsiae were unified into two subgroups, one consisting of five organisms ("*R. aeschlimanni*," strain Bar 29, *R. massiliae*, *R. rhipicephali*, and *R. montana*) and the other consisting of the 12 remaining organisms.

A bootstrap analysis of the data was performed to measure the reliability of the branching order proposed by each analysis. A consensus tree derived from 100 trees generated by the neighbor-joining and parsimony methods had exactly the same structure as the tree shown in Fig. 1. The values for the nodes were greater than 82% for all of the clusters except the large cluster of SFG made up of 12 strains (bootstrap values, 17 to 78%).

We compared the trees constructed on the basis of the nucleotide sequences of *gltA* and the nucleotide sequences of the 16S rRNA-encoding gene of rickettsiae. A bootstrap analysis of the 16S rDNA-derived trees revealed a lack of statistical significance for most of the proposed branching orders.

We compared the results of phylogenetic analyses obtained by comparing the sequences of both the citrate synthase and 16S rRNA-encoding genes for all representatives of the domain *Bacteria* for which citrate synthase gene data were determined. The results are summarized in Fig. 2. *Thermoplasma acidophilum*, a member of the domain *Archaea*, was used as the outgroup. Analysis of the *gltA* sequence data by neighbor-joining and parsimony methods yielded the same tree. The dendrograms inferred from 16S rDNA sequences were slightly different when the two methods were used. Phylogenetic results inferred from the comparisons of *gltA* and 16S rDNA sequences clustered together the representatives of the α sub-

TABLE 4. Levels of similarity between *gltA* sequences, between citrate synthase amino acid sequences, and between 16S rDNA sequences

Species	% Similarity ^a									
	<i>Rickettsia prowazekii</i>	<i>Bartonella henselae</i>	<i>Escherichia coli</i>	<i>Coxiella burnetii</i>	<i>Pseudomonas aeruginosa</i>	<i>Acinetobacter anitratum</i>	<i>Corynebacterium glutamicum</i>	<i>Mycobacterium smegmatis</i>	<i>Bacillus coagulans</i>	<i>Thermoplasma acidophilum</i>
<i>Rickettsia prowazekii</i>	100	65.2 (60.3)	61.4 (58.9)	61.5 (56.7)	56.3 (59.4)	62.6 (60.3)	51.2 (48.9)	44.5 (29.4)	48.7 (26.5)	48.1 (29.9)
<i>Bartonella henselae</i>	83.8	100	65.9 (69.3)	63.3 (62.1)	60.5 (65.4)	65.4 (65.6)	52.6 (48.7)	45.8 (28.1)	48.0 (29.8)	44.5 (28.6)
<i>Escherichia coli</i>	68.5	69.4	100	63.6 (61.3)	68.4 (69.0)	67.0 (67.0)	57.8 (49.1)	44.1 (24.6)	45.1 (29.5)	47.9 (25.7)
<i>Coxiella burnetii</i>	68.1	71.2	83.0	100	62.8 (66.6)	64.0 (64.6)	52.5 (46.1)	47.5 (28.3)	45.8 (31.9)	46.1 (30.7)
<i>Pseudomonas aeruginosa</i>	69.3	69.9	85.0	84.7	100	66.2 (75.0)	60.5 (51.2)	48.3 (24.3)	44.8 (31.6)	47.0 (28.3)
<i>Acinetobacter anitratum</i>	71.3	70.5	84.5	83.2	87.3	100	55.6 (50.5)	49.9 (31.0)	47.7 (32.7)	48.9 (25.7)
<i>Corynebacterium glutamicum</i>	74.1	75.3	69.1	67.6	75.3	69.0	100	47.0 (30.2)	47.1 (34.3)	49.3 (25.9)
<i>Mycobacterium smegmatis</i>	74.1	76.0	68.6	67.6	69.6	68.7	89.0	100	46.4 (29.2)	51.0 (31.2)
<i>Bacillus coagulans</i>	51.4	71.1	77.3	76.5	76.9	76.9	75.2	61.6	100	48.2 (28.4)
<i>Thermoplasma acidophilum</i>	59.6	58.9	59.2	56.0	58.4	58.5	49.5	49.4	57.9	100

^a The values on the upper right are the levels of similarity between *gltA* sequences and (in parentheses) between citrate synthase amino acid sequences, and the values on the lower left are the levels of similarity between 16S rDNA sequences.

class of the *Proteobacteria*, the representatives of the γ subclass of the *Proteobacteria*, and the representatives of the gram-positive bacteria. However, the organization of these clusters was different. The bootstrap values for all of the nodes provided high levels of confidence in the two analyses for the most part; the two exceptions were the branching of *Coxiella burnetii* (62%) on the dendrogram inferred from citrate synthase gene data and the branching of *Bacillus coagulans* (75%) on the 16S rRNA gene dendrogram.

DISCUSSION

Currently, serological typing by microimmunofluorescence with mouse polyclonal antisera remains the reference method for identification of rickettsiae, and determination of a new species is officially based on the specificity difference values calculated from microimmunofluorescence results (35). The antigenic determinants for this serotyping are two high-molecular-weight outer membrane proteins, rOmpA and rOmpB, for the SFG rickettsiae and rOmpB for the TG rickettsiae. This method, which requires culture of all recognized serotypes and production of antibodies in laboratory animals, is time-consuming, expensive, and sometimes hazardous. It was proposed almost 20 years ago because only phenotypic criteria were available at the time. Today, it is evident that a polyphasic approach, in keeping with the approaches used for other organisms, would be more suitable. Relatedness by DNA-DNA hybridization, the "gold standard" for bacterial species definition (52), has been used to study relationships between *R. rickettsii* and other species, including *R. conorii* (levels of DNA-DNA hybridization, 91 to 94%), *R. sibirica* (70 to 74%), *R. montana* (73%), *R. australis* (53%), *R. akari* (46%), *R. prowazekii* (47%), *R. typhi* (42%), and *R. canada* (47%). If "the 70% relatedness rule" were followed, *R. rickettsii*, *R. conorii*, *R. sibirica*, and *R. montana* would belong to the same species (51). However, *R. rickettsii* would be considered a species different from *R. akari* and *R. australis*, two members of the SFG, and would be considered different from *R. prowazekii*, *R. typhi*, and *R. canada*, three representatives of the TG (27, 51). On the basis of this criterion, *R. prowazekii* and *R. typhi* (level of relatedness as determined by DNA-DNA hybridization, 72%) would belong to the same species, but *R. canada* would be considered a

different species than *R. prowazekii* (45%) and *R. typhi* (42%) (27). Defining rickettsial species by using this criterion, is, however, problematic. First, culture of these bacteria is difficult and time-consuming; thus, using this method each time that a new strain is isolated is almost impossible. Second, interspecies DNA-DNA homology assessments were first made with members of the *Enterobacteriaceae*, and the critical value of 70% was proposed based on the results obtained for representatives of this family. The genome of the rickettsiae is four times smaller than the genomes of enteric bacteria (1.0 to 1.4 Mb for most of the rickettsiae and 4.8 Mb for *E. coli*). It has been hypothesized that the obligately intracellular bacteria evolved from free-living bacteria which lost genes which became unnecessary (53). Therefore, most likely, the compositions of the genomes of these bacterial species are more similar. Thus, the observations established by using members of the *Enterobacteriaceae* are not necessarily applicable to the rickettsiae.

The introduction of molecular techniques has revolutionized the study of gene and genome evolution and has allowed new approaches to phylogenetic inference. In particular, molecular sequences are useful for comparisons between distantly related species or when biochemical characteristics have proven to be inadequate. Recently, a universal phylogenetic study of a vast spectrum of organisms was performed in which 16S rDNA sequences were compared, and this study defined three domains, *Archaea*, *Eukarya*, and *Bacteria* (61). The domain *Bacteria* presently comprises more than 11 phyla, some of which (*Proteobacteria*, gram-positive bacteria) are subdivided. The rickettsiae are located in group 1 of the α subclass of the phylum *Proteobacteria*. The order *Rickettsiales* as described in *Bergey's Manual of Systematic Bacteriology* has been shown to be phylogenetically diverse. *Coxiella burnetii*, the agent of Q fever, has now been removed from the order as it has been shown to be evolutionarily distinct, and to be a member of the γ subclass of the *Proteobacteria* (54). Moreover, the genus *Rochalimaea* was united with the genus *Bartonella* in group 2 of the α subclass of the *Proteobacteria* and was also removed from the *Rickettsiales* on the basis of significant taxonomic differences, including phylogenetic diversity (10). 16S rRNA-based phylogeny has also been used to infer unexpected evolutionary relationships within the order *Rickettsiales*. Members of the genus *Ehrlichia* have been found to form two clearly distinct

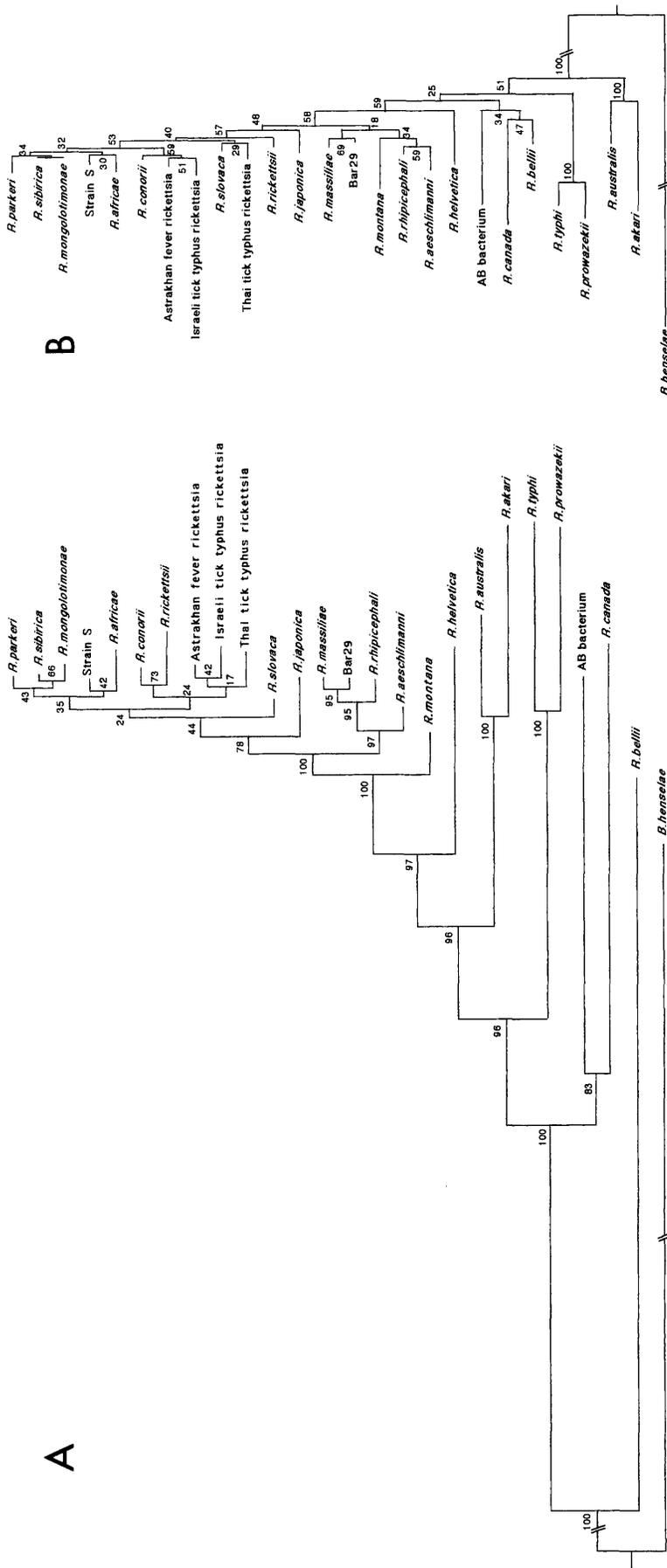


FIG. 1. Comparison of phylogenetic trees for members of the genus *Rickettsia* derived from *gltA* sequence data (A) and from 16S rRNA gene sequence data (B). The evolutionary distance values were determined by the method of Kimura, and the trees were constructed by the neighbor-joining method. Scale bar = 1% difference in nucleotide sequences for *gltA* and for 16S rDNA. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The GenBank accession numbers for the 16S rRNA gene sequences included are as follows: AB bacterium, U04163; *R. prowazekii*, M21789; *R. helvetica*, L36212; *R. japonica*, L36213; *R. massilliae*, L36214; *R. rhipicephali*, L36218; *R. rickettsii*, L36219; Thai tick typhus rickettsia, L36220; *R. typhi*, L36221; Israeli tick typhus rickettsia, L36223; “*R. slovaca*,” L36224; *R. bellii*, L36103; *R. canada*, L36104; *R. conorii* Moroccan, L36105; *R. africae*, L36098; *R. akari*, L36099; Astrakhan fever rickettsia, L36100; *R. australis*, L36101; strain Bar 29, L36102; *R. parkeri*, L36673; strain S, U25042; and “*R. aeschlimanni*,” U74757. The GenBank accession numbers for *gltA* sequences are given in Materials and Methods.

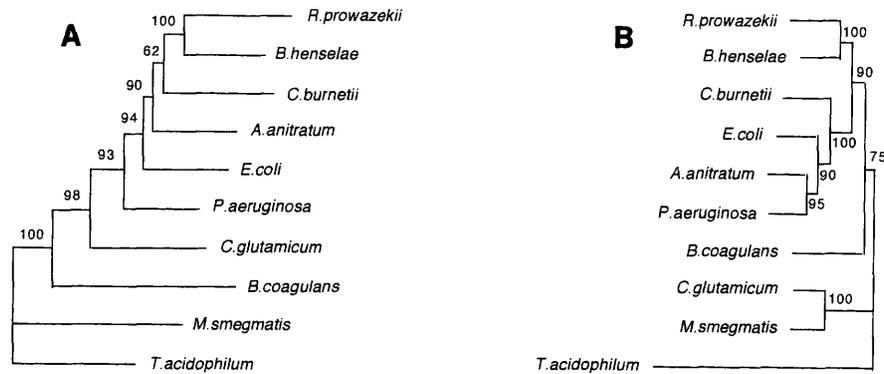


FIG. 2. Distance matrix trees derived from *gltA* data (A) and from data for the 16S rRNA-encoding gene (B). The evolutionary distances were determined by the method of Kimura. These values were used to construct a dendrogram by the neighbor-joining method. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. Scale bar = 18% difference in nucleotide sequences. The GenBank accession numbers for the *gltA* sequences included are as follows: *R. prowazekii*, M17149; *Bartonella henselae*, L38987; *Coxiella burnetii*, M36338; *Acinetobacter anitratum*, M33037; *E. coli*, J01619; *P. aeruginosa*, M29728; *Corynebacterium glutamicum*, X66112; *Bacillus coagulans*, M74818; *M. smegmatis*, X60513; and *Thermoplasma acidophilum*, X55282. The GenBank accession numbers for the 16S rRNA gene sequences of these bacteria are M21789, M73229, M21291, U10874, M24996, X06684, X80629, D16267, X52922, and M38637, respectively.

lineages (2). Furthermore, *R. tsutsugamushi* has been shown to exhibit only 90% sequence similarity with other members of this genus, which resulted in a proposal that it should be transferred to the new genus *Orientia* (48). However, the usefulness of the 16S rRNA gene as a tool for evolutionary inference for other members of the genus *Rickettsia* is less certain. Phylogenetic analyses of members of the SFG or the TG result in models whose branching orders have no statistical significance (41, 45). In addition to these problems, shortcomings in overreliance of bacterial phylogeny on comparisons of 16S rRNA gene sequences have been highlighted. We therefore investigated the usefulness of an alternative tool for phylogenetic inference in the genus *Rickettsia*. To date, only a few genes in the rickettsial chromosome have been sequenced, and we chose one of these, the *gltA* gene, as the gene product is an enzyme central to the metabolism of nearly all living cells (56).

Comparisons of similarity values showed that this gene is less conserved than the 16S rRNA gene in rickettsiae. This observation was particularly true for the deepest-branching representatives of the genus *Rickettsia*. The dendrograms inferred from 16S rDNA and *gltA* sequences had different architectures in the cluster which included *R. parkeri*, *R. sibirica*, "*R. mongolotimonae*," strain S, *R. africae*, *R. conorii*, *R. rickettsii*, the Astrakhan fever rickettsia, the Israeli tick typhus rickettsia, the Thai tick typhus rickettsia, "*R. slovacica*," and *R. japonica*. The phylogenetic positions of *R. rickettsii* and the Thai tick typhus rickettsia were different; however, in both trees there was no statistical support for their locations. The problem of the phylogenetic organization within this subgroup therefore remains. For these serotypes an additional more rapidly evolving housekeeping molecule is required as an alternative phylogenetic tool. Perhaps the nucleotide or protein sequences of proteins rOmpA and rOmpB, which are specific for the SFG rickettsiae and the members of the genus *Rickettsia*, respectively, may be suitable candidates. These molecules have been shown to exhibit higher degrees of difference between different strains with PCR-RFLP (18, 38, 39) or sequencing (39) than other molecules (citrate synthase, 17-kDa protein, 16S rRNA). Differences in the phylogenies inferred from *gltA* and 16S rDNA sequences were found in the organization of the bacteria which represented the most outlying rickettsiae (*R. bellii*, *R.*

canada, the AB bacterium, *R. prowazekii*, *R. typhi*, *R. australis*, *R. akari*) (Fig. 1). Although *R. canada* and *R. bellii* have some characteristics of the SFG (serological cross-reactions, ticks are the arthropod reservoir, transovarial transmission, and growth in the cytoplasm and nucleus of the host cell), they also have some of the characteristics associated with the TG (identical G+C contents) (27, 34). Furthermore, the similarities in the sodium dodecylsulfate-polyacrylamide gel electrophoresis patterns of the TG and SFG strains were greater than the similarities between either group and *R. bellii* (34). The AB bacterium was assigned to the genus *Rickettsia* on the basis of the results of a comparison of 16S rDNA and *gltA* sequences and the results of PCR amplification of parts of the genes encoding two specific rickettsial proteins (17-kDa protein and rOmpB) (4, 57). Characterization of this strain has not yet been completed because cell culture is not possible yet. *R. canada*, *R. bellii*, and the AB bacterium probably represent three new groups distinct from the SFG or the TG. With both methods of analysis *R. prowazekii* and *R. typhi* clustered on the same branch with high bootstrap values (100%). This finding is consistent with other phenotypic and genotypic characteristics. These two bacteria are without doubt two representatives of the TG.

R. akari and *R. australis* clustered together, and it is reasonable to consider them outlying members of SFG on the basis of the following characteristics: presence of *ompA* in the *R. akari* genome (20), serological cross-reactions, and G+C contents and genome sizes identical to those of other representatives of the SFG (40, 55). The phylogenetic position of *R. helvetica* and the existence of a subgroup that includes *R. massiliae*, strain Bar 29, *R. rhipicephali*, *R. montana*, and "*R. aeschlimanni*" were confirmed statistically by the *gltA* analysis. Recently, Birtles and Raoult (8) showed that the intra-*Bartonella* architecture of trees inferred from a *gltA* sequence comparison included four well-supported lineages within the genus, whereas the trees inferred from 16S rDNA sequences had no statistical support. The levels of similarity between *Bartonella gltA* sequences were markedly lower than the levels of similarity between 16S rDNA sequences (8). The average rate of sequence change in *gltA* is faster than the average rate of sequence change in 16S rRNA;

thus, for close relationships the *gltA* sequences can be quite valuable.

We wanted to test the *gltA* sequence comparison approach for general phylogenetic inferences. We obtained from GenBank the *gltA* sequences available for representatives of the *Bacteria* and compared the phylogenetic inferences made for these bacteria on the basis of the *gltA* and 16S rDNA data. The phylogenies inferred from the two approaches were similar. We observed that the species assigned to the α and γ proteobacterial subclasses by the rRNA analysis also clustered in *gltA* trees. The species included in the gram-positive bacterial cluster based on 16S rDNA sequences also clustered together when *gltA* sequences were compared. However, the levels of similarity between the *Corynebacterium glutamicum* *gltA* sequence and the *gltA* sequences of members of the *Proteobacteria* (60.5 to 51.2%) were higher than the levels of similarity obtained for two other members of the gram-positive bacterial cluster, *M. smegmatis* and *Bacillus coagulans* (49.9 to 44.1%). This difference is not consistent with segregation of these organisms into high- and low-G+C-content groups, as *Corynebacterium glutamicum* and *M. smegmatis* cluster with the high-G+C-content gram-positive bacteria and *Bacillus coagulans* clusters with the low-G+C-content gram-positive bacteria. On the basis of citrate synthase amino acid sequences, Sutherland et al. concluded that the *Eukarya*, *Bacteria*, and *Archaea* are three separate groups (47). However, no gram-positive bacterial sequence was included in this study. We showed that the division of the *Archaea* and *Bacteria* is not so evident on the basis of citrate synthase amino acid and *gltA* sequence comparisons. In this case, the organization of gram-positive bacteria and the low levels of similarity within this phylum might be apparent only because of the peculiar story of the citrate synthase gene. More *gltA* sequences will be necessary to study the evolution of representatives of the domain *Bacteria* (particularly other phyla, including the spirochetes, the *Planctomyces-Chlamydia* subgroup, the *Cyanobacteria*, and other subclasses of the *Proteobacteria*) and to determine the utility of the technique. We found unexpected results for the gram-positive bacterial phylum which need to be confirmed and explained.

In rickettsial phylogeny, although 16S rDNA data must be considered not useful, data derived from comparisons of *gltA* sequences are consistent with other phenotypic and genotypic characteristics of these organisms. The *gltA* sequence analysis allowed us to determine more reliable evolutionary relationships within the genus *Rickettsia*. Based on the calibration of rRNA gene evolution by Ochman and Wilson (31), the divergence of the genera *Orientia* and *Rickettsia* occurred 500×10^6 years ago, and divergence among the members of the genus *Rickettsia* occurred more recently. At present, based on results obtained for *Bartonella* species and *Rickettsia* serotypes, phylogenetic studies based on comparisons of *gltA* sequences should be considered an interesting approach which should be considered when phylogenetic relationships between taxa cannot be significantly resolved by 16S rDNA data, particularly when the phylogenetic organization of bacteria whose divergence is as recent as that of members of the genus *Rickettsia* is studied.

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