Reassessment of the Taxonomic Position of *Rickettsiella grylli*

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We determined the 16S rRNA gene sequence of *Rickettsiella grylli*, an intracellular parasite of *Gryllus bimaculatus* and related species of crickets. Phylogenetic inferences made from alignment of this sequence with the sequences of other bacteria demonstrated that *R. grylli* is most closely related to *Coxiella burnetii* and *Legionella* species in the γ subclass of the phylum *Proteobacteria*. *R. grylli* was previously thought to be related to members of the order *Rickettsiales*, but the representatives of this order have been shown to be members of the $\alpha 1$ subclass of the *Proteobacteria*. Our results indicate that *R. grylli* should be removed from the order *Rickettsiales*.

Members of the genus *Rickettsiella* are intracellular procaryotic pathogens of invertebrates, including insects, crustaceans, and arachnids. Bacterial replication takes place in cell vacuoles in the fat body, the hepatopancreas, and other organs. *Rickettsiella* species were classified in the order *Rickettsiales*, the family *Rickettsiaceae*, and the tribe *Wolbachieae*; however, this position was determined solely on the basis of the close association of these organisms with arthropods and their intravacuolar location. Three *Rickettsiella* species are currently recognized (25). The type species *Rickettsiella popilliae*, was first described in 1951 as *Coxiella popilliae* and is the bacterium responsible for blue disease, a fatal infection of the larvae of the Japanese beetle, *Popillia japonica* (6). *Rickettsiella grylli* and *Rickettsiella chironomii* were described subsequently by Vago and Martoja and by Weiser, respectively (19, 24). As *Rickettsiella* spp. replicate by using a cycle that mimics that of *Chlamydia* species, some investigators have suggested that these organisms more closely resemble members of the genus *Chlamydia* than members of the genus *Rickettsia* and should be transferred to the order *Chlamydiales*. In order to clarify the taxonomic status and the phylogenetic position of *Rickettsiella grylli*, we sequenced its 16S rRNA-encoding gene.

The *Rickettsiella grylli* strain was provided by Max Bergoin. The organisms included in the phylogenetic analysis were *Ehrlichia risticii* (HRC-IL^T (= ATCC VR-986^T) (21), *Ehrlichia chaffeensis* Arkansas (1), *Ehrlichia phagocytophila* Old Sourhope (1), *Wolbachia pipientis* (12), *Rickettsia conorii* Moroccan^T (= ATCC VR-141^T) (16), *Rickettsia prowazekii* Brienl^T (= ATCC VR-142^T) (21), *Orientia tsutsugamushi* Gilliam^T (= ATCC VR-312^T) (16), *Bartonella quintana* Fuller^T (= ATCC VR-358^T) (13), *Bartonella bacilliformis* ATCC 35685^T (11), *Brucella abortus* 11–19 (5), *Coxiella burnetii* Q117 (21), *Legionella israelensis* Bercovier-4^T (= ATCC 43119^T) (9), *Legionella anisa* ATCC 35292^T (15), *Legionella pneumophila* ATCC 33215^T (15), *Acinetobacter Iwoffii* ATCC 17925 (2), *Pseudomonas aeruginosa* DSM50071 (18), *Escherichia coli* (7), *Francisella tularensis* Utah 112 (= ATCC 15482) (26), *Wolbachia persica* ATCC VR-331^T (21), *Mycoplasma pneumoniae* FH (= ATCC 15531) (23), and *Chlamydia psittaci* 6BC^T (22).

DNA was extracted from purified elementary bodies by us-

ing Qiagen columns (QIAamp tissue kit; QIAGEN, Hilden, Germany). Amplification of the 16S rRNA gene from this extract was performed by using a PCR incorporating primers fd1 and rp2, as previously described (20). The PCR products were purified by using Qiagen columns (QIAquick Spin PCR purification kit), and sequencing reactions were carried out by using an AmpliCycle sequencing kit (Perkin-Elmer Corp., Foster City, Calif.) according to the manufacturer's instructions. The products of sequencing reactions were resolved and translated into sequence data by using an A.L.F. DNA sequencer (Pharmacia LKB) and associated software. The sequence of *Rickettsiella grylli* was aligned with other bacterial 16S rRNA gene sequences by using the multisequence alignment program CLUSTAL. Phylogenetic relationships were inferred by using the PHYLIP software package (8). An evolutionary distance matrix, generated by DNADIST, was determined by the method of Kimura (10). This matrix was used to infer a dendrogram by using the neighbor-joining method (17). The data were also examined by performing a parsimony analysis (DNAPARS). The stabilities of the predicted branching orders were assessed by generating a consensus tree from 100 trees derived from bootstrap samples of the original alignment by using SEQBOOT and CONSENSE in the same package.

The phylogenetic tree derived from the distance matrix analysis is presented in Fig. 1, and this tree demonstrates that *Rickettsiella grylli* belongs to the γ subclass of the phylum *Proteobacteria* and shares a common line of descent with *Coxiella burnetii* and the genus *Legionella*. The branching order in the tree derived from the parsimony analysis was the same as the branching order in Fig. 1. The order *Rickettsiales* has recently undergone substantial taxonomic reassessment on the basis of 16S ribosomal DNA (rDNA) sequence comparisons, which have revealed a high degree of evolutionary diversity. Although members of the genera *Rickettsia*, *Orientia*, *Ehrlichia*, *Cowdria*, *Anaplasma*, and *Neorickettsia* and the species *W. pipientis* group together in the $\alpha 1$ subclass of the class *Proteobacteria*, other species have been shown to fall elsewhere. *Rochalimaea*, *Grahamella*, and *Bartonella* spp., all of which are members of the genus now unified as *Bartonella*, form a distinct lineage within the $\alpha 2$ subclass of this phylum (3, 4), whereas *Coxiella burnetii* and *W. persica* are phylogenetically remote and belong to the γ subclass (21). Very recently, *Haemobartonella* and *Eperythrozoon* species have been shown to share evolutionary descent with the gram-positive members of the family *Mycoplasmaceae* (14). Our results support this phylogenetic diversity and provide a basis for removal of *Rickettsiella*

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- Phylogeny of *Legionellaceae* based on small-subunit ribosomal DNA sequences and proposal of *Legionella lytica* comb. nov. for *Legionella*-like amoebal pathogens. *Int. J. Syst. Bacteriol.* **46**:526–531.
10. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**:111–120.
 11. O'Connor, S. P., M. Dorsch, A. G. Steigerwalt, D. J. Brenner, and E. Stackebrandt. 1991. 16S rRNA sequences of *Bartonella bacilliformis* and cat scratch disease bacillus reveal phylogenetic relationships with the alpha-2 subgroup of the class *Proteobacteria*. *J. Clin. Microbiol.* **29**:2144–2150.
 12. O'Neil, S. L., R. Giordano, A. M. Colebert, T. L. Karr, and H. M. Robinson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* **89**:2699–2702.
 13. 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.
 14. Rikihisa, Y., M. Kawahara, B. Wen, G. Kociba, P. Fuerst, F. Kawamori, C. Suto, S. Shibata, and M. Futohashi. 1997. Western immunoblot analysis of *Haemobartonella muris* and comparison of 16S rRNA gene sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis*. *J. Clin. Microbiol.* **35**:823–829.
 15. Ross, T., B. Heidrich, P. N. Robinso, F. J. Fehrenbach, and A. Rolfs. Unpublished data.
 16. Roux, V., and D. Raoult. 1995. Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. *Res. Microbiol.* **146**:385–396.
 17. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
 18. Toschka, H. Y., P. Hoepfel, W. Ludwig, K. H. Schleifer, N. Ulbrich, and V. A. Erdmann. 1988. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Pseudomonas aeruginosa*. *Nucleic Acids Res.* **16**:2348.
 19. Vago, C., and R. Martoja. 1963. Une rickettsiose chez les *Gryllidae* (Orthoptera). *C.R. Acad. Sci.* **256**:1045–1047.
 20. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
 21. Weisburg, W. G., M. E. Dobson, J. E. Samuel, G. A. Dasch, L. P. Mallavia, O. Baca, L. Mandelco, J. E. Sechrest, E. Weiss, and C. R. Woese. 1989. Phylogenetic diversity of the rickettsiae. *J. Bacteriol.* **171**:4202–4206.
 22. Weisburg, W. G., T. P. Hatch, and C. R. Woese. 1986. Eubacterial origin of chlamydiae. *J. Bacteriol.* **167**:570–574.
 23. Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. L. Van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the *mycoplasmas*: basis for their classification. *J. Bacteriol.* **171**:6455–6467.
 24. Weiser, J. 1963. Diseases of insects of medical importance in Europe. *Bull. W. H. O.* **28**:121–127.
 25. Weiss, E., and J. W. Moulder. 1984. Order I. *Rickettsiales* Gieszczykiewicz 1939, 25^{AL}, p. 687–729. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams and Wilkins Co., Baltimore, Md.
 26. Wilson, K. H., H. G. Hills, and P. Zwadyk. Unpublished data.