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Molecular Characterization and Evolution of Arthropod-Pathogenic *Rickettsiella* Bacteria▽

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We determined the 16S rRNA gene sequences of three crustacean "*Rickettsiella armadillidii*" strains. *Rickettsiella* bacteria overall appear to form a monophyletic group that diverged from *Coxiella* bacteria ~350 million years ago. Therefore, the genus *Rickettsiella* as a whole (not just *Rickettsiella grylli*) should be classified among the *Gammaproteobacteria* instead of the *Alphaproteobacteria*.

Members of the genus *Rickettsiella* are intracellular bacterial pathogens of arthropods (13). They are found in a wide range of hosts including insects, crustaceans, and arachnids, and they exhibit a worldwide geographic distribution (11–13). In naturally infected hosts, the *Rickettsiella*-mediated disease affects both larvae and adults and develops very slowly (13). In its crustacean hosts, "*Rickettsiella armadillidii*" induces death, preceded by loss of weight and a white coloration of intersegmentary membranes, and the host general cavity is filled with an iridescent white liquid (12). *Rickettsiella* bacteria can potentially be very contagious, since they are capable of surviving in soil for years before contaminating new hosts (13).

On the basis of ultrastructural observations, *Rickettsiella* bacteria have been classified among the *Alphaproteobacteria*, within the order *Rickettsiales*, the family *Rickettsiaceae*, and the tribe *Wolbachieae* (13). However, the 16S rRNA gene sequence of *R. grylli* isolated from the cricket suggested that this strain is a *Gammaproteobacterium* related to the genus *Coxiella* (10). Therefore, if the genus *Rickettsiella* is monophyletic (i.e., all *Rickettsiella* species share an exclusive, common ancestor), then the taxonomic position of the genus *Rickettsiella* as a whole needs to be reassessed. Otherwise, if only *R. grylli* has been misclassified among the *Gammaproteobacteria*, the genus *Rickettsiella* is polyphyletic (i.e., different *Rickettsiella* species have different evolutionary origins). To obtain new insight into the evolution of *Rickettsiella* bacteria, we characterized *Rickettsiella* molecular genetic variation by analyzing the 16S rRNA gene sequences of three strains of the crustacean pathogen "*R. armadillidii*" (12) along with a data set of *Rickettsiella*-like 16S rRNA gene sequences encompassing their entire known host spectrum, gathered through database searches.

Wild-caught individuals belonging to the three isopod crustacean species *Armadillidium vulgare* (from Camarade, Ariège, France), *Helleria brevicornis* (from Pietracorbara, Corsica, France), and *Philoscia muscorum* (from Santiago de Compostela, Galicia, Spain) were studied. The animals displayed the characteristic external symptoms of an infection by "*R. armadillidii*," as described above (12). The diagnosis was further confirmed during the dissection of the samples and by electron microscopy. We determined the nucleotide sequences of the 16S rRNA genes of the three "*R. armadillidii*" strains from the aforementioned isopods by using standard methods of DNA extraction, PCR amplification, and sequencing (2, 9). We performed PCR amplification with primers 27f and 973r and sequencing with primers 27f, 530f, 685r, and 973r (6, 9).

Taxonomic considerations. The three sequences exhibited only 0.3% divergence, based on observed nucleotide substitutions. This is more than 10 times lower than the 3.5% average divergence estimated among the three "*R. armadillidii*" strains and *R. grylli*. Therefore, on the basis of currently available data, *R. grylli* falls outside of the range of variation of "*R. armadillidii*." This result backs up the previous suggestion that "*R. armadillidii*" and *R. grylli* may represent two distinct species (5), although they are considered synonymous species in the currently accepted nomenclature (13).

To test the monophyletic or polyphyletic status of the genus *Rickettsiella*, we performed a phylogenetic analysis including both "*R. armadillidii*" and *R. grylli* strains, together with 25 *Alphaproteobacteria* and *Gammaproteobacteria* representatives (Fig. 1). The two important results that emerged from this analysis were that (i) the four *Rickettsiella* strains formed a monophyletic group supported by a bootstrap score of 100% and (ii) all *Rickettsiella* strains were most closely related to *Coxiella* bacteria (bootstrap score of 95%), within the *Gammaproteobacteria*. Therefore, our results suggest that members of the *Rickettsiella* genus are monophyletic and thus that the entire genus *Rickettsiella* (not just *R. grylli* [10]) should be classified among the *Gammaproteobacteria* rather than among the *Alphaproteobacteria*.

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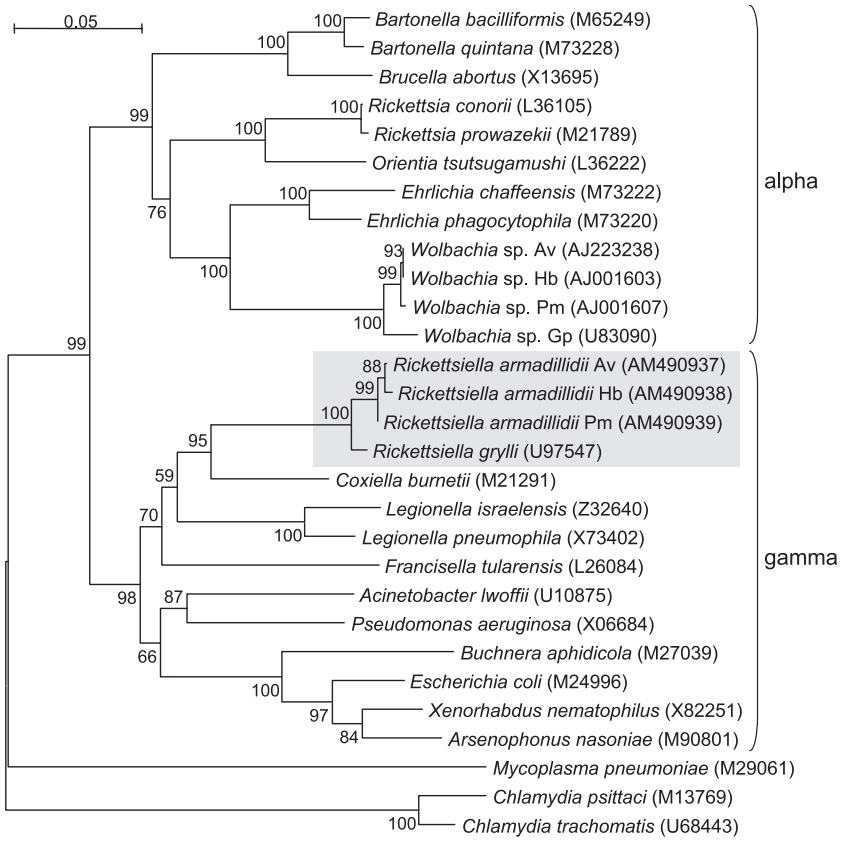

FIG. 1. Neighbor-joining tree of bacterial strains based on 16S rRNA gene sequences. The tree was built by using the software MEGA version 3.1, based on the Kimura two-parameter substitution model. Bootstrap values (based on 1,000 replicates) are shown on branches (percentages). *Rickettsiella* strains are in a gray box, demonstrating their closer relationship to the *Gammaproteobacteria* (gamma cluster) than to the *Alphaproteobacteria* (alpha cluster). GenBank accession numbers for all sequences are indicated after species or strain names. *Wolbachia* and *Rickettsiella* strains were isolated from *A. vulgare* (Av), *H. brevicornis* (Hb), *P. muscorum* (Pm), and *Gryllus pennsylvanicus* (Gp).

Genetic diversity and evolution. We queried GenBank with the 16S rRNA gene sequence of "*R. armadillidii*" from *A. vulgare* through BLASTN searches and selected the 10 best hits for further analyses. A phylogenetic analysis revealed that hits 8 to 10 are not derived from *Rickettsiella* bacteria, since they branch off in the tree at a position that is more basal than the *Coxiella-Rickettsiella* split (Fig. 2). By contrast, hits 1 to 7, together with "*R. armadillidii*" strains, form a monophyletic

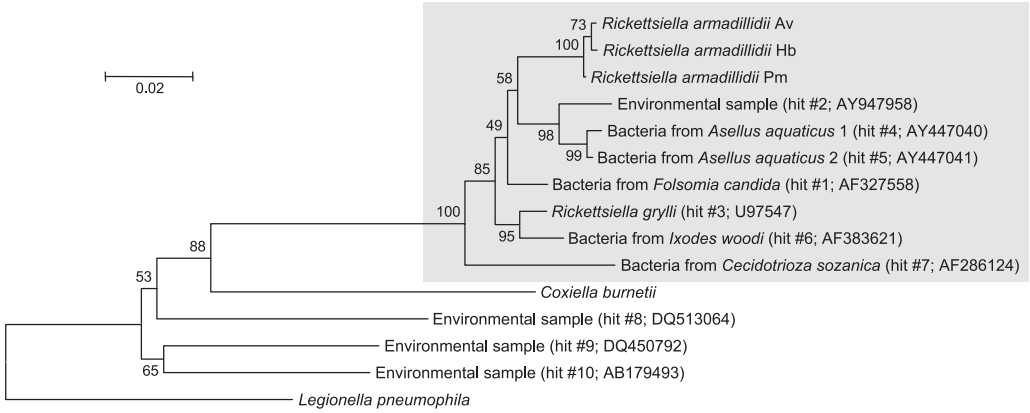

FIG. 2. Neighbor-joining tree of bacterial strains based on 16S rRNA gene sequences. The tree was built as described in the legend to Fig. 1. Bootstrap values (based on 1,000 replicates) are shown on branches (percentages). The monophyletic group of *Rickettsiella* strains is in a gray box. Strains followed by a hit number correspond to the 10 best hits resulting from a BLASTN search using the 16S rRNA gene sequence of "*R. armadillidii*" isolated from *A. vulgare* (Av) as a query against GenBank (hit accession numbers are shown). GenBank accession numbers for other sequences and *Rickettsiella* strain names are as in Fig. 1. Host systematics for the 10 best hits are as follows: *Insecta*, hits 1, 3, and 7; *Crustacea*, hits 4 and 5; *Acari*, hit 6; unknown, hits 2, 8, 9, and 10.

group, suggesting that all of these sequences are derived from *Rickettsiella* bacteria. Quantitatively, these conclusions are also supported by the fact that "*R. armadillidii*" strains and *Coxiella burnetii* exhibit an average divergence of 13.9% versus 13.5% between "*R. armadillidii*" strains and hits 8 to 10. In comparison, hits 1 to 7 show an average divergence of only 4.0% with "*R. armadillidii*" strains, similar to the 3.5% divergence observed between "*R. armadillidii*" and *R. grylli*. Interestingly, the 10 *Rickettsiella* strains for which 16S rRNA gene sequence data are available (Fig. 2) encompass the whole range of arthropod organisms known to be natural hosts of *Rickettsiella* bacteria, including insects, crustaceans, and arachnids (13). This observation provides further support for the suggestion that the *Rickettsiella* genus, as a whole, has been misclassified among the *Alphaproteobacteria*.

The average divergence between the 10 *Rickettsiella* strains and *C. burnetii* was estimated to be 14.1%. Assuming a substitution rate of the eubacterial 16S rRNA gene of 1% per 50 million years (My) (7), the origin of *Rickettsiella* bacteria is estimated to have occurred ~350 My ago. On the other hand, the divergence among the 10 *Rickettsiella* strains is 3.6% ± 1.6%, suggesting that diversification within the genus *Rickettsiella* started 90 ± 40 My ago. These 10 *Rickettsiella* strains are found in crustaceans, insects, and acari (Fig. 2), which probably diverged back in the Precambrian era (3) over 500 My ago. If this is so, the presence of ~100 My-old *Rickettsiella* bacteria in such a wide range of arthropod hosts makes the occurrence of horizontal transmission of these bacteria necessary. This is not unexpected, since *Rickettsiella* bacteria are known to be able to survive in the soil outside of their arthropod hosts for years (13).

Concluding remarks. The crustacean populations studied here are also known to harbor intracellular *Wolbachia pipientis* bacteria (2, 4, 8) (Fig. 1). This intracellular arena (1) could therefore represent a remarkable environment in which to promote evolutionary novelty by horizontal gene transfer among unrelated bacteria. Beyond this evolutionary interest, *Rickettsiella* spp. are pathogenic bacteria whose introduction into laboratory insectaries or other animal collections might be devastating and needs to be prevented (13). Therefore, our results not only offer new insight into the evolution of these bacteria but also provide the basis for a genetic test to easily recognize the presence of *Rickettsiella* in suspect samples.

Nucleotide sequence accession numbers. The three "*R. armadillidii*" sequences generated in this study have been deposited in GenBank under accession numbers AM490937 to AM490939.

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