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Phylogenetic Diversity of the Rickettsiae

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Small subunit rRNA sequences have been determined for representative strains of six species of the family *Rickettsiaceae*: *Rickettsia rickettsii*, *Rickettsia prowazekii*, *Rickettsia typhi*, *Coxiella burnetii*, *Ehrlichia risticii*, and *Wolbachia persica*. The relationships among these sequences and those of other eubacteria show that all members of the family *Rickettsiaceae* belong to the so-called purple bacterial phylum. The three representatives of the genus *Rickettsia* form a tight monophyletic cluster within the α subdivision of the purple bacteria. *E. risticii* also belongs to the α subdivision and shows a distant yet specific relationship to the genus *Rickettsia*. However, the family as a whole is not monophyletic, in that *C. burnetii* and *W. persica* are members of the γ subdivision. The former appears to show a specific, but rather distant, relationship to the genus *Legionella*.

The order *Rickettsiales* (32) comprises a collection of procaryotes that share the property of intimate association with eucaryotic cells. The relationship in most cases is obligate intracellular parasitism, although a few of these bacteria have been grown in complex host-cell-free culture media. Although this order contains notorious pathogens of humans and animals, some of these bacteria cause no obvious damage to their host, and the relationship can be regarded as commensal. The *Rickettsiales* are clearly separate from the *Chlamydiales* (17), a narrowly defined group of energy-parasitizing, obligately intracellular bacteria. Certain fastidious parasites of plant vascular tissues and arthropods, sometimes referred to as rickettsialike (5), are not included in either category. Also, the highly heterogeneous group of bacteria that have established an endosymbiotic relationship with their hosts and for the most part have not been cultivated, including the hydrothermal-vent-associated symbionts (3, 4, 21), are viewed as distinct from the *Rickettsiales* (32) in the latest edition of *Bergey's Manual of Systematic Bacteriology*.

The order *Rickettsiales* contains three families: *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*. Our present study is confined to the family *Rickettsiaceae*, which is divided into three tribes: *Rickettsieae*, *Ehrlichieae*, and *Wolbachieae* (32). In the family *Rickettsiaceae*, rigorous criteria of classical taxonomy have been applied to the definition of most species and, in some but not in all cases, the genera. For example, good evidence was obtained by phenotypic analysis, DNA base ratio determinations, and DNA-DNA hybridization studies that in the genus *Rickettsia*, members of the typhus and spotted fever groups are related (32). However, the degree of relatedness of these microorganisms to the scrub typhus rickettsia, *Rickettsia tsutsugamushi*, remains unknown. Similarly, phenotypic analysis links the monocytic erhlichiae (*Ehrlichia canis*, *Ehrlichia sennetsu*, and *Ehrlichia risticii*) to each other (10, 22, 23), but their relationships to the granulocytic ehrlicheae (*Ehrlichia phagocytophila* and *Ehrlichia equi*) and to the endothelial pathogen *Cowdria ruminantium* remain unclear.

It is well recognized that there are great differences among some of the genera in the family *Rickettsiaceae* (32). Maintaining such diverse genera as *Rickettsia* and *Coxiella* in the tribe *Rickettsieae* and *Ehrlichia* in the family *Rickettsiaceae* (23) provides a useful guide to bench scientists, who must use comparable techniques for the isolation, identification, and cultivation of these organisms, but has no proven phylogenetic justification. Similarly, the inclusion of genera such as *Wolbachia* in the family *Rickettsiaceae* (30) has the merit of linking pathogenic and commensal microorganisms that share an ecologic niche in the arthropod but, again, has no known phylogenetic justification.

We have previously determined the 16S rRNA sequence of one member of the tribe *Rickettsieae*, namely, *Rochalimaea quintana*, the etiologic agent of trench fever, which is capable of growth in an axenic medium (28). One reason for the selection of this microorganism for our initial study was its apparent relatedness to the typhus group rickettsiae, DNA-DNA hybridization in the range of 25 to 33% having been reported previously (19). Phylogenetic analysis of its sequence showed that this organism belongs to the α subdivision of the purple bacteria and within this unit is specifically related to the plant-associated genera *Agrobacterium* and *Rhizobium* (28). As expected, it is not related to *Chlamydia psittaci* or *Chlamydia trachomatis* (27), the rickettsialike Pierce's disease bacterium of grapevines and other plants (*Xylella fastidiosa*) (5, 33), or the hydrothermal-vent-associated symbionts (7, 25).

The purpose of the present study was a more comprehensive phylogenetic analysis of the *Rickettsiaceae*. Six species were selected, four in the tribe *Rickettsieae* and one each in the tribes *Erlichieae* and *Wolbachieae*. Three species, *Rickettsia prowazekii* and *Rickettsia typhi* (the agents of epidemic and endemic typhus, respectively) and *Rickettsia rickettsii* (the agent of Rocky Mountain spotted fever), could be expected to be closely related to each other. The remaining three species, *Coxiella burnetii* (the agent of Q fever), *Wolbachia persica* (26) (not associated with a known disease of humans or animals), and *E. risticii* (10, 11) (the recently isolated agent of equine monocytic ehrlichiosis—Potomac

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TABLE 1. Sequence similarities and evolutionary distances among rickettsial and other eubacterial 16S rRNAs[a]

Species	% Sequence similarity (top triangle) and evolutionary distance (bottom triangle)											
	Rickettsia prowazekii	*Rochalimaea quintana*	*Ehrlichia risticii*	*Agrobacterium tumefaciens*	*Rhodospirillum rubrum*	*Escherichia coli*	*Legionella pneumophila*	*Coxiella burnetii*	*Wolbachia persica*	*Pseudomonas aeruginosa*	*Chromatium vinosum*	*Pseudomonas testosteroni*
Rickettsia prowazekii		86.6	85.7	86.5	85.7	80.9	84.1	84.5	82.3	83.0	83.3	81.8
Rochalimaea quintana	14.8		84.5	94.5	89.2	82.5	84.0	84.8	83.4	84.8	85.7	81.1
Ehrlichia risticii	15.9	17.4		85.1	83.6	80.0	81.4	82.0	79.8	81.2	81.2	79.0
Agrobacterium tumefaciens	14.9	5.7	16.6		89.3	82.3	84.2	84.8	84.2	84.4	85.7	81.1
Rhodospirillum rubrum	15.9	11.7	18.6	11.5		83.7	84.9	84.4	83.0	85.7	86.7	83.0
Escherichia coli	22.0	20.0	23.3	20.2	18.4		88.1	88.2	86.0	88.9	89.1	83.7
Legionella pneumophila	17.9	18.1	21.4	17.8	16.9	13.0		91.3	88.8	90.3	90.2	84.8
Coxiella burnetii	17.4	17.0	20.6	17.0	17.0	12.8	9.3		87.8	90.4	90.8	85.3
Wolbachia persica	20.3	18.8	23.5	17.8	19.3	15.5	12.1	13.3		88.8	87.2	83.2
Pseudomonas aeruginosa	19.3	17.0	21.6	17.6	15.9	12.1	10.4	10.3	13.1		90.6	86.6
Chromatium vinosum	18.9	15.9	21.6	15.9	14.7	11.8	10.5	9.8	14.0	10.1		86.6
Pseudomonas testosteroni	20.9	21.8	24.7	21.8	19.3	18.4	17.0	16.4	19.1	14.8	14.8	

[a] The 16S rRNA alignment was constructed by a standard method (35). Evolutionary distances were calculated as described in Materials and Methods. The sources for sequences used in the alignment (in addition to those determined in connection with the present study) are as follows: *Agrobacterium tumefaciens* and *Pseudomonas testosteroni*, reference 37; *Pseudomonas aeruginosa*, *Chromatium vinosum*, *Legionella pneumophila*, and *Rhodospirillum rubrum*, C. R. Woese et al., unpublished data; *Rochalimaea quintana*, reference 28; and *Escherichia coli*, reference 2.

horse fever), could be expected to differ widely from the genus *Rickettsia* and from each other. Among the surprising results obtained are the following: (i) a lack of close relationship between *Rickettsia* and *Rochalimaea* spp., despite the appreciable DNA-DNA homology reported (19); (ii) evidence of a common origin of the genera *Coxiella* and *Legionella*; and, most surprising, (iii) evidence of a common origin of the genus *Rickettsia* and *E. risticii*.

MATERIALS AND METHODS

Rickettsial strains. *R. prowazekii* Breinl and *R. typhi* Wilmington were cultivated in the yolk sacs of chicken embryos and separated from host constituents by procedures that included mechanical disruption of infected cells, selective absorption of host components, differential centrifugation, and separation by Renografin density gradient centrifugation as described elsewhere (29, 36).

W. persica (26) (ATCC VR 331) was grown in the yolk sacs of chicken embryos. Before purification, the wolbachiae, heavily concentrated in the lipoprotein fraction, were released by trypsin digestion and purified as described above for typhus rickettsiae.

R. rickettsii R (ATCC VR 891) was grown in the Vero African green monkey kidney cell line, derived from ATCC CCL 81. The cell line was provided by Charles I. Pretzman, Ohio Department of Health, Columbus, Ohio, as approximately the 300th total subculture passage. The cells were grown in 175-ml flasks in a 1:1 mixture of Eagle minimal essential medium and medium 199 with Earle salts (no. 12-104-5; Quality Biologicals) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 1% minimal essential medium Eagle 100 X nonessential amino acids (no. 16-104-1; Quality Biologicals). Heavily infected cells and their culture fluids were harvested with glass beads, mechanically disrupted in a motor-driven Potter-Elvehjem (Teflon-glass) homogenizer, and purified by Renografin density centrifugation.

E. risticii Illinois (ATCC VR 986), obtained from M. Ristic of the University of Illinois, was propagated in the mouse macrophage cell line $P388D_1$ and separated from host constituents as described above and in detail in a recent publication (31).

C. burnetii Q177, an isolate obtained from a goat abortion (24), was also grown to a high titer in yolk sacs, and rickettsial cells were purified by sucrose gradient centrifugation as previously described (9).

Nucleic acid extraction. The purified microorganisms were suspended in 10 ml of TGE buffer (50 mM Tris buffer [pH 8], 25 mM glucose, 10 mM EDTA) to which 1 mg of freshly prepared lysozyme per ml was added, and then they were incubated for 30 min at room temperature. Sodium dodecyl sulfate from a 20% stock was added to a final concentration of 0.5%, as well as proteinase K (Boehringer Mannheim Biochemicals) to a final concentration of 100 μg/ml, and the samples were incubated overnight at 45°C in sealed tubes. Residual protein was removed by sequential extraction with equal volumes of phenol saturated with TE (10 mM Tris buffer [pH 8], 1 mM EDTA) and phenol-chloroform (1:1) and extraction twice with chloroform. Total nucleic acids were precipitated by the addition of ammonium acetate (final concentration, 0.3 M) and 2 volumes of ethanol followed by overnight incubation at −20°C. The pellets were washed with 10 ml of 70% ethanol and dried in vacuo.

Cloning and sequencing. The 16S rRNAs of two of the six species were sequenced as cloned genes, while those of the remaining species were sequenced directly as RNA. *R. prowazekii* DNA was partially digested with the restriction endonuclease *Sau*3A and ligated into the *Bam*HI site of bacteriophage λL47 (15) to create a gene library. Fragments from a positively identified clone were subcloned into M13 phages mp18 and mp19 for sequencing (16).

E. risticii DNA was digested with the endonuclease *Eco*RI, and a library of clones was similarly created in

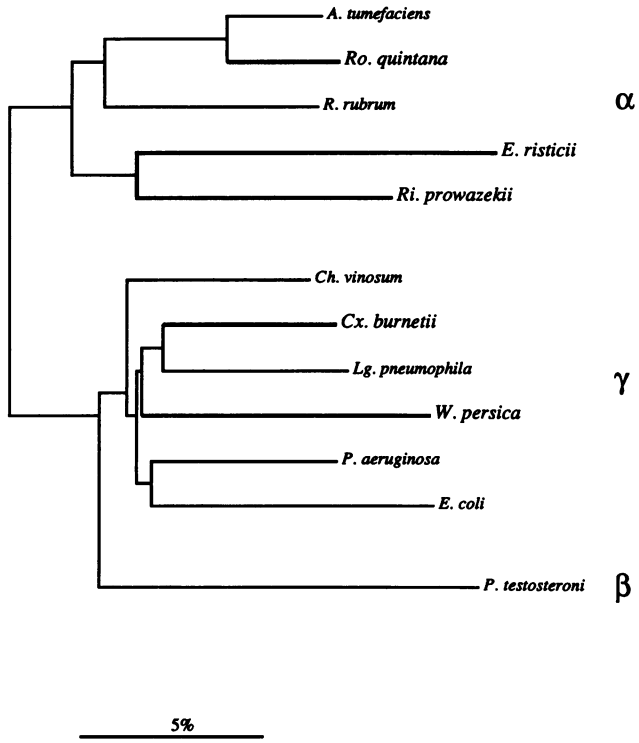

FIG. 1. Evolutionary distance tree showing relationships among the organisms in this study and representative members of the α, β, and γ subdivisions of the purple bacteria. The tree was constructed from the evolutionary distances in Table 1 as described in Materials and Methods. Scale bar corresponds to 5 nucleotide substitutions per 100 sequence positions.

λgtWES.λB (14). Subcloning for sequencing was done as described above for *R. prowazekii*.

In the cases of *R. typhi*, *R. rickettsii*, *C. burnetii*, and *W. persica*, RNAs were further purified (from total nucleic acid) by DNase digestion and cesium trifluoroacetate isopycnic centrifugation.

DNA sequencing followed the standard dideoxy-chain termination method (1), with either the Klenow fragment or Sequenase (U.S. Biochemical Corp.) used as polymerase. Direct RNA sequencing was done by a similar procedure with reverse transcriptase (13). In each case, primers specific for eubacterial 16S rRNA sequences were used (13, 37).

Data analysis. The sequences were introduced into our sequence editor and aligned against our collection of 16S rRNA sequences (about 100) from various purple bacteria (34). Given the degree of similarity among all sequences, the alignment procedure is a straightforward one: it utilizes previously aligned near relatives of the new sequences, the established secondary structural constraints, and sequence conservation patterns as guides (35). The only positions in the sequence alignment that were used for analysis (Table 1 and Fig. 1) were those that met two criteria: (i) a known nucleotide is present in all sequences considered at that position, and (ii) one nucleotide accounts for at least 50% of the composition of each such position. (This last constraint removes from consideration some of the more frequently changing [phylogenetically less informative] positions; it removes from consideration none of the positions that have changed composition only once over the evolutionary span common to the considered sequences.) These constraints reduce the number of positions analyzed to just under 1,300.

Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities by the correction of Jukes and Cantor (12), as modified by G. J. Olsen (personal communication) to accommodate the actual base ratios. (This modification amounts to replacing the random background term 0.25 in the Jukes-Cantor formulation, i.e., their assumption that all four bases are present in equal amounts, by c, where $c = f_{A1}f_{A2} + f_{C1}f_{C2} + f_{G1}f_{G2} + f_{U1}f_{U2}$, the f terms being the base ratios in sequences 1 and 2.) Dendrograms were constructed from evolutionary distance matrices by the method of De Soete (6).

RESULTS AND DISCUSSION

The six rickettsial sequences generated for this study appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers M21789 (*R. prowazekii*), M20499 (*R. typhi*), M21293 (*R. rickettsii*), M21290 (*E. risticii*), M21291 (*C. burnetii*), and M21292 (*W. persica*). (They are purposely not printed herein in order to prevent their being manually transcribed, a procedure that tends to introduce error into resulting copies.) The six sequences were aligned with other 16S rRNA sequences. Table 1 displays a matrix of sequence similarities and corresponding evolutionary distances derived from the alignment (sources are described in the footnote).

The rRNAs of the *Rickettsia* species characterized, *R. rickettsii*, *R. prowazekii*, and *R. typhi*, are all extremely close (and for this reason, values for only one of them, *R. prowazekii*, are shown in Table 1). The *R. prowazekii* and *R. typhi* sequences are 99.5% similar, while these in turn are 98.2% to 98.5% similar to that of *R. ricketsii*. These values maintain the integrity of the typhus group (19, 32) as distinct from the spotted fever rickettsiae. (For comparison, rRNAs of closely related genera such as *Escherichia* and *Salmonella* are about 97% similar [34].)

Beyond the level of the genus *Rickettsia*, however, evolutionary distances increase appreciably and specific relationships tend to disappear. The tribe *Rickettsieae*, which includes the genera *Rochalimaea* and *Coxiella* in addition to *Rickettsia*, shows no phylogenetic coherence (Fig. 1) despite the reported relationship between *Rickettsia* spp. and *Rochalimaea quintana* based on DNA-DNA hybridization studies (19). Although *Rickettsia* spp. and *Rochalimaea quintana* are obviously both members of the α subdivision of the purple bacteria (Table 1 and Fig. 1), they are not specifically related to one another therein. The latter is a member of the *Agrobacterium-Rhizobium* cluster (28), while the former has no known specific relatives within the α division, with the important exception discussed below.

C. burnettii bears no relationship whatever to the other members of its tribe; it is a member of the γ purple bacteria (Fig. 1).

The polyphyletic nature of the tribe *Rickettsieae* should come as no surprise, given the paucity of common phenotypic and physiological characteristics. Although the genera *Rickettsia* and *Coxiella*, for example, are intracellular in general mode of reproduction, the specifics of their infectious cycles are quite different, e.g., their intracellular locations and their strategies for parasitism (18).

Given that the tribe *Rickettsieae* is a polyphyletic taxon, it is somewhat surprising that at the level of the family *Rickettsiaceae* (which includes the tribes *Rickettsieae*, *Ehrlichieae*, and *Wolbachieae*), the phylogenetic dispersion of species does not become more severe. Not only does the 16S

TABLE 2. Sequence signature linking the genera *Rickettsia* and *Ehrlichia* (RE group)

Position in 16S rRNA[a]	Composition in:		Ancestral composition[b]
	RE group	Other α bacteria	
38	A	G	G
43	U	C	C
399	A	G	G
671	A	G	G
735	U	C	C
931	U	C	C
965	U	A(G)	?
974	A	C	A
976	A	G	G
1051	U	C	C
1189	C	U(G)	?
1383	U	C	C
1400	U	C	C
1410	G	A	A
1533	U	C	C

[a] Position (*Escherichia coli* numbering) in which *Rickettsia* strains and *E. risticii* strains (RE group) have a common composition that differs from that common to all other α purple bacterial sequences, of which more than 20 now exist in our collection. (In two cases there is one exception among the remaining α subdivision sequences, whose composition is given in parentheses.)

[b] Composition judged to be ancestral for the purple bacteria on the basis of a phylogenetically broader selection of eubacteria.

rRNA sequence of *E. risticii* localize in the α subdivision of the purple bacteria (Table 1), but it also groups specifically (albeit somewhat distantly) with the *Rickettsia* lineage (Fig. 1), a relationship that can also be demonstrated by parsimony analysis (not shown) and by sequence signature (i.e., an extensive list of positions whose compositions are common, unique, and [mainly] derived for these two sequences within the α subdivision) (Table 2). (Note that these differences from the other members of the α subdivision exist despite the fact that the rickettsiae and ehrlichiae show a typical α subdivision sequence signature, e.g., that seen in Table 4 of reference 34.) This relationship is particularly interesting in light of the recent finding that *E. risticii* is capable of some in vitro ATP synthesis in a manner comparable to that of *R. typhi* (32a). Considering the great evolutionary distance between the genera *Ehrlichia* and *Rickettsia*, this trait and their common parasitic mode of existence must either be very ancient in origin or have arisen independently in the two lineages, given common biochemistries and thus similar evolutionary proclivities.

Another unexpected relationship suggested by this study is between the genera *Coxiella* and *Legionella*, which also grows intracellularly, though not obligately so (Fig. 1). The relationship between the two is clearly not a close one, and these parasites are different in their modes of intracellular development: *C. burnetii* grows intracellularly within a phagolysosome type of structure, while *Legionella* spp. multiply in phagosomes (18). Although not firmly established, the relationship between these two organisms is considered likely, because it is (to a first approximation) independent of the exact makeup of the alignment and of the exact positions used in the analysis. The relationship is also given by parsimony analysis of the data (not shown).

The 16S rRNA sequence of *W. persica* places this organism in the γ purple bacteria as well. (Both it and *C. burnetii* can be grouped within the γ subdivision by sequence signature, and there is no evidence of a β subdivision affiliation for either one; see Table 4 in reference 34.) However, within the γ subdivision, the exact phylogenetic position of the genus *Wolbachia* is unclear. Figure 1 shows *W. persica* as a peripheral member of the *Coxiella-Legionella* cluster, but this placement is not a consistent one. Whether or not the *W. persica* lineage appears to be a sister group of the *Coxiella-Legionella* cluster depends on which other eubacterial sequences are included in the alignment and which positions are used in the analysis. Since the *Wolbachia* lineage is relatively rapidly evolving, all analyses used will tend artificially to place it more deeply in the branching pattern than is actually the case (8, 20), and this would tend to prevent its appearing to be a sister group of the *Coxiella-Legionella* cluster. Additional sequence data will be required to resolve the relationship of *W. persica* to these other intracellular parasites.

In summary, we now have a reasonable sampling of 16S rRNA sequences from a variety of rickettsiae (28), rickettsia like organisms (33), vent-associated endosymbionts (7, 25), chlamydiae (27), mycobacteria, and other assorted intracellular forms by which to examine the nature of intracellular bacteria. They are derived from several of the major eubacterial phyla (34), but, as with the polyphyletic rickettsiae, many appear to have originated from a variety of free-living ancestors among the purple bacteria (34). The lineages of intracellular bacteria which parasitize eucaryotic hosts probably arose at about the time that the eucaryotic hosts were radiating to their current diversity.

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