

Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA

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Rickettsiae are classified in the order *Rickettsiales* and have been included in the α subclass of the class *Proteobacteria* on the basis of 16S rRNA gene sequence comparison. To estimate the evolutionary forces that have shaped the members of the spotted fever group (SFG) rickettsiae, the *ompA* gene (apart from the tandem repeat units), encoding an antigenic high-molecular-mass membrane protein specific for the group, was amplified and sequenced from 21 isolates. The phylogenetic relationships between SFG rickettsiae were inferred from the comparison of both the gene and derived protein sequences, using the parsimony, neighbour-joining and maximum-likelihood methods. Three strongly supported phylogenetic sub-groups were distinguished: first, the *Rickettsia conorii* complex (*R. conorii* Malish, *R. conorii* M1, *R. conorii* Moroccan, *R. conorii* Indian tick typhus, Astrakhan fever rickettsia and Israeli tick typhus rickettsia); second, a cluster including *Rickettsia africae*, strain S, *Rickettsia parkeri*, *Rickettsia sibirica* and '*Rickettsia mongolotimonae*'; and, third, a cluster including *Rickettsia aeschlimannii*, *Rickettsia rhipicephali*, *Rickettsia massiliae*, Bar 29 and *Rickettsia montanensis*. *Rickettsia rickettsii*, *Rickettsia japonica*, *Rickettsia slovacica* and Thai tick typhus rickettsia did not cluster with any other *Rickettsia* species. To test whether positive selection was responsible for sequences diversity, rates of synonymous and non-synonymous nucleotide substitutions were compared for *Rickettsia ompA* alleles and indicated that this gene is undergoing neutral evolution.

Keywords: spotted fever group rickettsiae, phylogenetic analysis, outer-membrane protein rOmpA, *Rickettsia*

INTRODUCTION

The *Rickettsia* species are small, highly fastidious Gram-negative bacteria that form a monophyletic cluster within the α subclass of the class *Proteobacteria*. Traditionally, *Rickettsia* species have been divided into two groups, namely the typhus group (TG) and spotted fever group (SFG), on the basis of the clinical presentation, immunological reactivity, DNA G+C mol% content and intracellular position (Weiss & Moulder, 1984). All species are obligatorily intracellular and maintain a life cycle in mammal cells or arthropods. In the SFG, rickettsiae are maintained by

Abbreviations: MIF, microimmunofluorescence test; SFG, spotted fever group; SPD, specificity difference; TG, typhus group.

The GenBank accession numbers for the sequences in this paper are listed in Methods.

both transovarial transmission and horizontal acquisition of organisms by means of ingestion of infested blood meals. Some SFG rickettsiae are human pathogens, causing tick-borne eruptive fevers, whereas others have only been isolated from arthropods (Raoult & Roux, 1997). Endothelial cells are specifically infected by rickettsiae, which are thus responsible for various disseminated infections. SFG rickettsiae have been isolated worldwide, but their geographical distribution varies depending of the rickettsia. The number of the SFG representatives has increased following the development of improved cell culture isolation techniques (Marrero & Raoult, 1989) and an increased interest in arthropod-transmitted diseases. Several studies aimed at assessing the genotypic relationships of these rickettsiae have been performed using DNA sequence comparison. Stothard & Fuerst (1995) and Roux & Raoult (1995) inferred

phylogenies based on the comparison of aligned 16S rRNA gene sequences.

Subsequently, Roux *et al.* (1996a) inferred phylogenetic relationships between *Rickettsia* species from the comparison of the faster-evolving citrate-synthase-encoding gene (*gltA*) sequences, showing that this tool is more sensitive than 16S rRNA comparison, but reliable phylogenetic inter-relationships could only be drawn among rickettsiae that diverged early from the common ancestor of SFG. The rOmpA-encoding gene was considered a good candidate for a phylogenetic analysis of most of the SFG representatives because it has been shown to exhibit higher degrees of interspecies difference than both the 16S rRNA gene and *gltA* by PCR-RFLP and sequencing (Eremeeva *et al.*, 1994b; Regnery *et al.*, 1991). Furthermore, as immune serum to rOmpA reacts with all the representatives of the SFG and *Rickettsia canadensis*, it appeared that the protein, and therefore the gene, were present among all SFG members (Anacker *et al.*, 1987; Vishwanath, 1991). A cloned portion of the *Rickettsia rickettsii* and *Rickettsia conorii ompA* gene has been shown to induce the expression of a truncated recombinant protein that elicits a protective immune response in mice and guinea pigs against a normally lethal inoculum of *R. rickettsii* and *R. conorii* (Sumner *et al.*, 1995; Vishwanath *et al.*, 1990). Moreover, rOmpA and rOmpB, another high-molecular-mass surface protein, are the antigenic determinants of serotyping in mice, which is the currently preferred method for identification of SFG rickettsiae species (Philip *et al.*, 1978).

During their evolution, rickettsiae may have been exposed to selective forces imposed by factors affecting their arthropod or mammalian hosts. Among those species that infect mammals, one could therefore expect this selective pressure to be directed against their immunoexposed proteins. Such a selection exerted by the human immune system has been demonstrated to occur against immunogenic surface proteins of other pathogenic organisms, including *Plasmodium falciparum* (Hughes, 1991) and *Neisseria gonorrhoeae* (Smith *et al.*, 1995). Thus, rOmpA could be a good candidate for estimating the effects of positive selection: the protein has been characterized only in SFG rickettsiae, is surface exposed (Walker *et al.*, 1992) and elicits a strong immune reaction during infection.

The *ompA* gene has previously been completely sequenced in two species of *Rickettsia*, *R. rickettsii* and *R. conorii* (Anderson *et al.*, 1990; Croquet-Valdes *et al.*, 1994). In this work, we report the DNA sequence of the entire *ompA* gene, excluding the central region containing tandem repeat units, of 21 SFG rickettsiae isolates. From the alignment of these sequences, we inferred the phylogenetic relationships within the SFG. Furthermore, to examine whether the evolution of the protein could be related to a positive selection, we have analysed the substitution rates between pairs of sequences.

METHODS

Rickettsial strains. The strains used in this study are listed in Table 1.

Rickettsial cultivation and nucleic acid preparation. The rickettsiae were propagated on Vero cell (ATCC CRL-1587) monolayers in Eagle's minimal essential medium (MEM; Seromed) supplemented with 4% fetal calf serum (Seromed) and 2 mM glutamine, at 32 °C. Harvesting of the cells was done when the degree of infection as estimated by Gimenez staining was high (3–5 d). Rickettsial cultures were centrifuged (12000 g for 10 min), resuspended in MEM and stored at –70 °C until required.

Cells were lysed by overnight incubation in a mixture of 1% SDS and 0.2 mg proteinase K ml⁻¹, in an Eppendorf tube at 37 °C. A phenol extraction and then a phenol/chloroform extraction were carried out after recovery of the aqueous phase, and DNA was precipitated by addition of two volumes of chilled ethanol held overnight at –20 °C. DNA was pelleted by centrifugation (12000 g for 10 min), then washed with cold 70% ethanol. After centrifugation, the pellet was then dried and resuspended in sterile distilled water.

PCR amplification and sequencing method. The primer Rr 190–70p has been described by Regnery *et al.* (1991). Other potentially useful primers were designed following identification of suitable hybridization sites with conserved regions of the gene after alignment of the sequences of *R. rickettsii* (Anderson *et al.*, 1990) and *R. conorii* (strain Malish) (Croquet-Valdes *et al.*, 1994). These primers are described in Table 2. For PCR amplification, one primer was labelled at the 5' end with biotin during oligonucleotide synthesis (Eurogentec) whereas the other was unmodified. Two microlitres of the DNA preparation was amplified in a 100 µl reaction mixture containing 10 pmol each primer; 200 µM (each) dATP, dCTP, dGTP and dTTP (Boehringer Mannheim); 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus); 6 µl of a 25 mM solution of MgCl₂ (Perkin-Elmer) in 1 × *Taq* buffer. Amplifications were carried in a DNA thermal cycler (PTC 200; MJ Research) under the following conditions: an initial 3 min of denaturation at 95 °C was followed by 35 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 46 °C and extension for 1 min at 65 °C. The amplification was completed by holding for 7 min at 72 °C to allow complete extension of the PCR products. Purification of PCR products and sequencing reactions were performed as previously described (Roux *et al.*, 1996a). Sequencing reactions were resolved on 6% polyacrylamide gels (Ready Mix Gels, ALF grade; Pharmacia Biotech) and electrophoresis was performed in the ALF DNA sequencer (Pharmacia Biotech) in 1 × TBE buffer, pH 8 (44.5 mM Tris/HCl, 44.5 mM boric acid, 1 mM EDTA), according to the manufacturer's instructions.

Data analysis

(i) Determination of phylogenetic relationships. The *ompA* sequences were translated into protein sequences using PC/GENE software. The *ompA* sequences and rOmpA amino acid sequences were aligned using the multisequence alignment program CLUSTAL within the BISANCE environment (Dessen *et al.*, 1990). The percentages of similarity were determined using the DNASIS software package (Hitachi Software Engineering). The phylogenetic relationships between the studied representatives of the genus *Rickettsia* were determined using version 34 of the PHYLIP software (Felsenstein, 1989). Distance matrices generated by DNADIST

Table 1. Rickettsial strains studied

Species	Strain	Source organism	Geographical origin	Human disease	Reference	Supplier/source
<i>R. conorii</i>	Moroccan, ATCC VR-141T	Unknown	Morocco	Mediterranean spotted fever	Belli & Stoetner (1960)	ATCC
<i>R. conorii</i>	Seven (Malish), ATCC VR-613T	Unknown	South Africa	Mediterranean spotted fever	None	ATCC
<i>R. conorii</i>	M1	<i>Rhipicephalus sanguineus</i>	Georgia, former USSR		Golnevitch (1960)	Gamaleya Institute, Moscow
<i>R. conorii</i>	Indian tick typhus rickettsia, ATCC VR-597	<i>Rhipicephalus sanguineus</i>	India		Golnevitch (1960)	Gamaleya Institute, Moscow
Astrakhan fever rickettsia	A-167	<i>Rhipicephalus pumilio</i>	Astrakhan, former USSR	Astrakhan fever	Eremeeva <i>et al.</i> (1994a)	Isolated in our laboratory
Israeli tick typhus rickettsia	ISTT CDC1	Human	Israel	Israeli spotted fever	Goldwasser <i>et al.</i> (1974)	G. Dasch (Naval Medical Research Institute, Bethesda, MD, USA)
<i>R. sibirica</i>	246, ATCC VR151T	<i>Dermacentor nitaii</i>	Siberia, former USSR	Siberian tick typhus	Bell <i>et al.</i> (1960)	Gamaleya Institute, Moscow
' <i>R. mongolotimonae</i> '	HA-91	<i>Haemophysalis asiaticum</i>	Inner Mongolia	Unnamed disease	Yu <i>et al.</i> (1993)	Isolated in our laboratory
<i>R. parkeri</i>	Maculatum 20	<i>Amblyomma maculatum</i>	Mississippi, USA		Bell <i>et al.</i> (1963)	D.H. Walker (University of Texas, Galveston)
<i>R. africae</i>	ESF-5	<i>Amblyomma variegatum</i>	Shulu province, Ethiopia	Tick bite fever	Kelly <i>et al.</i> (1994)	G. Dasch
Strain S	S	<i>Rhipicephalus sanguineus</i>	Armenia, former USSR		Eremeeva <i>et al.</i> (1995)	Gamaleya Institute, Moscow
' <i>R. slovacca</i> '	13-B	<i>Dermacentor marginalis</i>	Slovakia	Unnamed disease	Rehacek (1984)	G. Dasch
Thai tick typhus rickettsia	TT-118, ATCC VR-599T	<i>Ixodes</i> and <i>Rhipicephalus</i> sp.	Thailand		Robertson & Wisseman (1972)	G. Dasch
<i>R. rickettsii</i>	R(Bitterroot), ATCC VR-891T	<i>Dermacentor andersoni</i>	Montana, USA	Rocky Mountain spotted fever	Bell <i>et al.</i> (1963)	ATCC
<i>R. japonica</i>	YM	Human	Japan	Oriental spotted fever	Uchida <i>et al.</i> (1992)	D. H. Walker
<i>R. massillae</i>	Miu'1	<i>Rhipicephalus turanicus</i>	Camargue, France		Beati & Raouf (1993)	Isolated in our laboratory
<i>R. massillae</i>	GS	<i>Rhipicephalus sanguineus</i>	Greece		Babalis <i>et al.</i> (1994)	Isolated in our laboratory
Bar 29	Bar 29	<i>Rhipicephalus sanguineus</i>	Spain		Beati <i>et al.</i> (1996)	Isolated in our laboratory
<i>R. rhipicephali</i>	3<->7<->6	<i>Rhipicephalus sanguineus</i>	Mississippi, USA		Burgdorfer <i>et al.</i> (1978)	G. Dasch
<i>R. montanensis</i>	M/5<->6	<i>Microtus</i> sp.	Ohio, USA		Bell <i>et al.</i> (1963)	D. H. Walker
<i>R. aeschlimannii</i>	MC16	<i>Hyalomma marginatum</i>	Morocco		Beati <i>et al.</i> (1997)	Isolated in our laboratory
<i>R. australis</i>	Phillips	Human	Queensland, Australia	Queensland tick typhus	Andrew <i>et al.</i> (1946)	G. Dasch
<i>R. akari</i>	MK (Kaplan), ATCC VR-148T	Human	New York, USA		Huebner & Armstrong (1946)	ATCC
<i>R. helvetica</i>	C9P9	<i>Ixodes ricinus</i>	Switzerland	Rickettsialpox	Burgdorfer <i>et al.</i> (1979)	W. Burgdorfer (Rocky Mountain Laboratories, Hamilton, MT, USA)
<i>R. bellii</i>	389L42<->1	<i>Dermacentor variabilis</i>	Ohio, USA		Philip <i>et al.</i> (1983)	D. H. Walker

Table 2. Oligonucleotide primers

Fragment	Primer	Nucleotide sequence	<i>ompA</i> position*
I	190-70†‡	ATGGCGAATATTTCTCCAAAA	70–90
	190-264‡	ATGGCGAATATTTCTCCAAAA	264–245
	190-485‡	GCAAAAGCTTAACTTTAAA	485–503
	190-701†‡	GTTCCGTTAATGGCAGCATCT	701–681
II	190-3588†‡	AACAGTGAATGTAGGAGCAG	3588–3607
	190-3817‡	CTGCAACTGTTCTATAG	3817–3800
	190-3968‡	TAGCAGCTGATTTAGTAGCT	3968–3986
	190-4084‡	CATCACCGATATTTCTAGC	4084–4065
	190-4406†‡	ACTATACCCTCATCGTCATT	4406–4387
III	190-4338†‡	TTCAGGAAACGACCGTACG	4338–4356
	190-4710‡	AACATTTACATTGTTATTTA	4710–4690
	190-4859‡	GCGAAATCCAAGGTACAGG	4859–4877
	190-5044‡	AACTGTAGCACCTGCCGT	5044–5028
	190-5238†‡	ACTATTAAGGCTAGGCTATT	5238–5218
IV	190-5125†‡	GCGGTTACTTTAGCCAAAGG	5125–5144
	190-5498‡	CAGCAGGTGGTTTAAAGCCT	5498–5517
	190-5561‡	CGGTTGTCATTATTAATAG	5561–5543
	190-5768‡	CACCGCTACAGGAAGCAGAT	5768–5787
	190-5831‡	GTGTCGCTAGTTTTACAAC	5831–5812
	190-6013†‡	TCTTCTGCGTTGCATTACCG	6013–5994
V	190-5917†‡	TCAGGGAATAAAGGTCCTG	5917–5935
	190-6092‡	AGATCATCGCTAACGAAGCC	6092–6073
	190-6228‡	CGTTGAAGCTATAGCATC	6228–6245
	190-6427‡	ATCTAAGCCCAGCTAGCGGT	6427–6408
	190-6585‡	CGCAATGGTTCGATTATGC	6585–6602
	190-6808†‡	CACGAACTTTCACACTACC	6808–6790

* Numbering refers to the rOmpA sequence of *R. rickettsii* reported by Regnery *et al.* (1991).

† Oligonucleotide primer used for PCR amplification.

‡ 5'-Isothiocyanate-labelled primer used for sequencing.

were determined under the assumptions of Jukes & Cantor (1969) and Kimura (1980). These matrices were used to elaborate dendrograms using the neighbour-joining method (Saitou & Nei, 1987). Two other dendrograms were constructed using data processing with the maximum-likelihood and the parsimony program DNAPARS. Bootstrap replicates were performed to estimate the node reliability of the phylogenetic trees obtained by the parsimony, maximum-likelihood and neighbour-joining methods (Brown, 1994). The bootstrap values were obtained from 100 trees generated randomly with SEQBOOT and CONSENSE in the PHYLIP software package.

The phylogenetic relationships inferred from protein were determined using the neighbour-joining, parsimony and maximum-likelihood methods with PROTDIST, PROTPARS and DNAML, respectively, in the PHYLIP software.

(ii) Calculation of synonymous and non-synonymous substitution rates per site. These values were determined from the entire sequence of *ompA* by the method of Nei & Gojbori (1986) within the MEGA suite (Kumar *et al.*, 1993). The significance of the d_s/d_n value was estimated using Student's *t*-test. An equal frequency of synonymous and non-synonymous substitutions (with a d_s/d_n ratio close to 1) indicates that the

entire gene is undergoing neutral evolution. However, a ratio of > 1 indicates that purifying selection is occurring, whereas a value < 1 suggests that there is positive selection for amino acid sequence variation.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequence data reported in this paper are as follows: U83442 for *Rickettsia japonica*, U83445 for *Rickettsia massiliae* strain Mtu1, U83444 for *R. massiliae* strain GS, U83447 for *Rickettsia montanensis*, U83450 for *Rickettsia rhipicephali*, U83438 for Bar 29, U83446 for *Rickettsia aeschlimannii*, U83451 for *R. rickettsii*, U83454 for '*Rickettsia slovacica*', U83456 for Thai tick typhus rickettsia, U83455 for *Rickettsia sibirica*, U83439 for '*Rickettsia mongolotimonae*', U83449 for *Rickettsia parkeri*, U83436 for *Rickettsia africana*, U83452 for strain S, U83441 for Israeli tick typhus rickettsia, U83437 for Astrakhan fever rickettsia, U83448 for *R. conorii* strain Moroccan, U83440 for *R. conorii* strain Indian tick typhus, U83453 for *R. conorii* strain Malish and U83443 for *R. conorii* strain M1. The GenBank accession numbers for the first part of the *ompA* sequences included in Fig. 1 are as follows: U43795 for *R. japonica*, U43799 for *R. massiliae* strain Mtu1, U43793 for *R. massiliae* strain GS, U43801 for *R. montanensis*, U43803 for *R. rhipicephali*, U43792 for Bar 29, U43800 for *R. aeschlimannii*, U43804 for *R. rickettsii*,

U43808 for '*R. slovacae*', U43809 for Thai tick typhus rickettsia, U43807 for *R. sibirica*, U43796 for '*R. mongolotimonae*', U43802 for *R. parkeri*, U43790 for *R. africae*, U43805 for strain S, U43797 for Israeli tick typhus rickettsia, U43791 for Astrakhan fever rickettsia, U45244 for *R. conorii* strain Moroccan, U43794 for *R. conorii* strain Indian tick typhus, U43806 for *R. conorii* strain Malish and U43798 for *R. conorii* strain M1.

RESULTS

PCR amplification

ompA fragments were amplified from all the studied strains of the SFG rickettsiae except *R. australis*, *R. helvetica*, *R. akari* and *R. bellii*, for which none of the primer pairs used could amplify any fragment. For each strain, the *ompA* gene, excluding the central tandem repeat region, was amplified in five fragments. The 5' end fragment of the gene was amplified using Rr 190.70p (Regnery *et al.*, 1991) and a consensus primer 190-701 that hybridized to the *ompA* gene close to the 5' end of the tandem repeat units (Roux *et al.*, 1996b). The size of PCR product was 632 bp for all strains except *R. conorii* strain M1 (476 bp), *R. conorii* strain Moroccan (608 bp), *R. montanensis* (629 bp) and *R.*

conorii strain Indian tick typhus rickettsia (635 bp). The other four overlapping fragments were amplified using four consensus primer pairs. Primer 190-3588 hybridized to the *ompA* gene close to the 3' end of the tandem repeat units. The second amplified fragment was 819 bp long for all strains except Thai tick typhus rickettsia (813 bp), '*R. mongolotimonae*' (804 bp), *R. sibirica* (813 bp), Astrakhan fever rickettsia (822 bp) and Israeli tick typhus rickettsia (822 bp). The third fragment was 901 bp long for all strains except *R. massiliae* strains Mtul (904 bp) and GS (904 bp), Bar29 (904 bp), *R. rhipicephali* (904 bp) and *R. aeschlimannii* (904 bp). The fourth fragment was 889 bp long for all strains except *R. massiliae* strains Mtul (842 bp) and GS (842 bp), Bar29 (842 bp) and *R. rhipicephali* (842 bp). The fifth fragment was 892 bp long for all strains. The insertions and/or deletions which yielded these size variations are presented in Table 3.

Sequencing

The sequence of both DNA strands was determined twice. The same sequence was obtained for two isolates of the species *R. massiliae*, GS and Mtul. Unique

Table 3. Positions of the deletions and insertions characterized in the SFG rickettsiae studied. The positions are numbered with regard to the sequence of *R. rickettsii*

Numbering refers to positions in the nucleotide sequence of the five *ompA* fragments joined end-to-end and renumbered from 1 to 3785. Fragment I is derived from the sequenced insert between positions 70 and 701 and is renumbered from 1 to 591. Fragment II is derived from the sequenced insert between positions 3588 and 4406 and is renumbered from 592 to 1375. Fragment III is derived from the sequenced insert between positions 4338 and 5238 and is renumbered from 1347 to 2210. Fragment IV is derived from the sequenced insert between positions 5125 and 6013 and is renumbered from 2137 to 2990. Fragment V is derived from the sequenced insert between positions 5917 and 6808 and is renumbered from 2934 to 3785. ^a, Deletion; ⁱ, insertion.

Rickettsia	Fragment I	Fragment II	Fragment III	Fragment IV	Fragment V
<i>R. conorii</i> strain Malish	TTT ⁱ : 101–103 AAT ^d : 160–162				
<i>R. conorii</i> strain M1	TTT ⁱ : 101–103 AAT ^d : 160–162 156 bases ^d : 238–393				
<i>R. conorii</i> strain Moroccan	TTT ⁱ : 101–103 AAT ^d : 160–162 24 bases ^d : 360–383				
<i>R. conorii</i> strain Indian	TTT ⁱ : 101–103				
Astrakhan fever rickettsia		AAT ⁱ : 872–874			
Israeli tick typhus rickettsia		AAT ⁱ : 872–874			
Thai tick typhus rickettsia		TGA ^d : 679–681 CTT ^d : 767–769			
<i>R. aeschlimannii</i>			GGT ⁱ : 1631–1633		
<i>R. montanensis</i>	CTA ^d : 119–121	CAC ^d : 1350–1352			
Bar 29			GGT ⁱ : 1631–1633	TGC ⁱ : 2288–2290	
<i>R. massiliae</i>			GGT ⁱ : 1631–1633	TGC ⁱ : 2288–2290	
<i>R. rhipicephali</i>			GGT ⁱ : 1631–1633	TGC ⁱ : 2288–2290	
<i>R. sibirica</i>		ACGCAA ^d : 693–698			
' <i>R. mongolotimonae</i> '		CCTCTTGAT ^d : 674–682 ACGCAA ^d : 693–698			

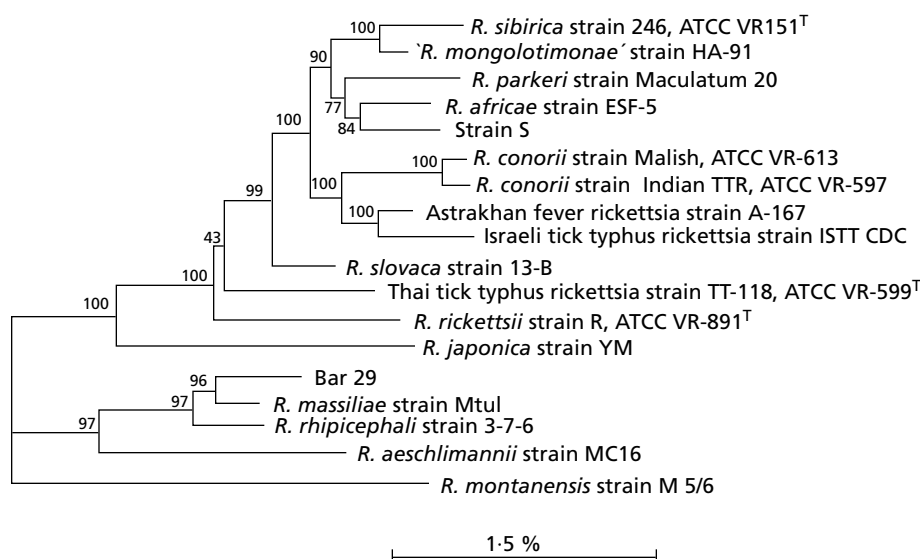


Fig. 1. Phylogenetic tree of bacteria belonging to the SFG rickettsiae inferred from comparison of the *ompA* gene sequences. The sequences were aligned using the multisequence alignment program CLUSTAL, which is a part of the BISANCE software package. Phylogenetic relationships were inferred with version 3.4 of the PHYLIP software package. The evolutionary distance values were determined by the method of Kimura (1980), and these values were used to construct a dendrogram by the neighbour-joining method. The scale bar represents 1.5% difference in nucleotide sequences. The numbers at nodes are the proportion of 100 bootstrap resamplings that support the topology shown.

sequences were found for each of four strains of the species *R. conorii*. The sequenced fragments of the *ompA* gene were analysed from base position 91 to 680 and from 3608 to 6789 with respect to the *R. rickettsii* published sequence (Anderson *et al.*, 1990). The first and second fragments were united and the complete sequence was renumbered from base 1 to base 3785.

Sequence comparison

(i) Nucleotide sequences. The nucleotide sequences obtained for *R. rickettsii* and *R. conorii* strain Malish were identical to those reported previously (Anderson *et al.*, 1990; Crocquet-Valdes *et al.*, 1994). Variations in the sizes of both the 5'-end and the 3'-end fragments of *ompA* were observed among the SFG strains. However, the most important source of genetic variation between rickettsiae was due to base substitutions, which are scattered throughout the entire sequence. Of the 489 sites at which point mutations occurred within the rOmpA-encoding gene, 173 (35.4%) occurred in the first position of a codon, 164 (33.5%) in the second position and 152 (31.1%) in the third position. Among mutations, 345 (70.5%) were transitions and 144 (29.5%) were transversions. Sequence similarities ranged from 95.2% (*R. montanensis*/*R. conorii* Malish) to 100% (*R. massiliae* GS/*R. massiliae* Mtu1).

(ii) Amino acid sequences. Of the 489 sites at which a base substitution was found, 148 (30.3%) produced non-synonymous amino acid changes. Sequence similarities ranged from 89% between *R. montanensis* and *R.*

conorii strain Moroccan to 100% between the two *R. massiliae* strains.

Phylogeny inference

The dendrograms obtained with the three different tree-building analysis methods used showed similar organization for most of the rickettsiae whether inferred from the gene or the derived protein sequences. Only the positions of *R. rickettsii* and Thai tick typhus rickettsia were unstable, as is reflected in low bootstrap value at the node from which branched *R. rickettsii* (43%). All other nodes were well-supported (> 70% bootstrap values) (Fig. 1). We identified three monophyletic groups within the tree: one made of *R. aeschlimannii*, *R. rhipicephali*, *R. massiliae*, *R. montanensis* and Bar 29, another including the *R. conorii* complex (*R. conorii* strains, Astrakhan fever rickettsia and Israeli tick typhus rickettsia) and a third including *R. africae*, strain S, *R. parkeri*, *R. sibirica* and '*R. mongolotimonae*'. *R. rickettsii*, *R. slovaca*, Thai tick typhus rickettsia and *R. japonica* did not cluster with any other *Rickettsia* species.

d_s and d_N values

Mean percentages of synonymous and non-synonymous substitutions per site were determined from the entire sequence. The d_s and d_N values were 2.85 ± 0.6 and 2.87 ± 0.3 , respectively. These values are significantly different ($P < 10^{-6}$). A mean overall d_s/d_N value

of 0.99 suggests that this gene is undergoing neutral evolution.

DISCUSSION

Phenotypic criteria (morphology and antigenic and metabolic characters) have long been the basis of bacterial taxonomy. However, since the 1960s, molecular approaches to the study of evolution have involved sequence analysis of proteins (cytochrome *c*, fibrinopeptides, ferredoxins ...) (Wilson *et al.*, 1977). In the 1980s, PCR and rapid sequencing techniques for DNA became available and comparison gene sequences became the most useful tool to make phylogenetic inferences. It was established that the most adapted molecular chronometers were universal molecules with highly conserved function that were established at early stages in evolution, functions that are not affected by changes in the organism's environment. Genes encoding rRNA were chosen universally as molecular chronometers (Woese, 1987). The bacterial phylogeny that emerged from molecular sequence comparisons had little in common with preconceptions. This is particularly true in the order *Rickettsiales*, a taxon that was originally a repository for intracellular bacteria, which, by their nature, could be characterized only on the basis of a few non-specific phenotypic criteria. Phylogeny-based taxonomic reconstructions have led to numerous genera being removed from the order [*Rochalimaea* (Weisburg *et al.*, 1991), *Coxiella* (Weisburg *et al.*, 1989), *Rickettsiella* (Roux *et al.*, 1997), *Eperythrozoon*, *Hemobartonella* (Rikihisa *et al.*, 1997)], renamed [*Rochalimaea/Bartonella* (Brenner *et al.*, 1993), *Grahamella/Bartonella* (Birtles *et al.*, 1995)] or created [*Orientia* (Tamura *et al.*, 1995)], and it was found that the taxonomy inside the cluster including *Ehrlichia*, *Wolbachia*, *Anaplasma*, *Cowdria* and *Neorickettsia* species is inadequate. The evolutionary relationships among the members of the genus *Rickettsia* were first assessed through phylogenetic studies based on analysis of sequences of gene encoding 16S rRNA (Roux & Raoult, 1995; Stothard & Fuerst, 1995). However, the close evolutionary proximity of species, reflected by very highly conserved 16S rRNA gene sequences, prevented a stable, well-supported phylogeny from being obtained. The relative 'insensibility' of 16S rRNA gene-based assessments for inferring intra-genus phylogenetic relationships is not limited to *Rickettsia* species (Birtles & Raoult, 1996; Hookey *et al.*, 1993; Martinez-Murcia *et al.*, 1992). After the period of emphasis placed on rRNA analysis, genes encoding universally distributed and conserved proteins are now receiving renewed attention in those instances where less conserved chronometers are needed and the reliability of phylogenetic pattern derived from nucleic acid analyses needs to be tested by markers with different evolutionary origins (Olsen & Woese, 1993). In this way, Roux *et al.* (1996a) have completed a comparative sequence analysis of the citrate synthase gene among *Rickettsia* species. How-

ever, no reliable phylogenetic analysis for all the SFG representatives could be inferred from this study as the sequences derived from different species were too similar. Not all molecular chronometers are equally useful for reconstructing phylogenetic analyses. Some are restricted to particular taxonomic levels and/or particular bacterial groups. This is true among members of the same genus, in which case an additional more rapidly evolving housekeeping molecule is required as an alternative phylogenetic tool. In this work, we obtained base sequence data for all but the tandem repeat region of the *ompA* gene from SFG rickettsiae, allowing us to infer a well-supported phylogenetic study for most of the SFG rickettsiae, thus extending the phylogenetic studies of the entire order *Rickettsiales* and the genus *Rickettsia* undertaken with the genes encoding the 16S rRNA (Roux & Raoult, 1995; Stothard & Fuerst, 1995) and citrate synthase (Roux *et al.*, 1996a).

The usefulness of *ompA* gene comparison for the differentiation of SFG rickettsiae was first recognized by Regnery *et al.* (1991), who demonstrated a clear heterogeneity in the gene sequences of different SFG species using restriction endonuclease analysis of PCR-amplified *ompA* gene fragments. It was clear from these studies that intra-SFG variability in *ompA* was markedly higher than variability in either the 16S rRNA or the *gltA* genes. By obtaining complete *ompA* sequences except for the repeat domain, we were able to confirm and quantify these earlier observations.

Within the SFG, we have identified three distinct phylogenetic groups. Inside the first one clustered Eurasian isolates, the four *R. conorii* strains (M1, Moroccan, Malish and Indian tick typhus), Astrakhan fever rickettsia and Israeli tick typhus rickettsia, forming the *R. conorii* complex. *R. africae*, strain S, *R. parkeri*, '*R. mongolotimonae*' and *R. sibirica* clustered together and formed the second group. At the present time, *R. africae* has been isolated only in Africa, *R. parkeri* in the USA and *R. sibirica*, '*R. mongolotimonae*' and strain S in Eurasia. The third group included *R. massiliae*, Bar 29 and *R. aeschlimannii*, encountered around the Mediterranean Sea, and *R. montanensis* and *R. rhipicephali* isolated from ticks in USA, and in USA and Europe, respectively.

The existence of the sub-group including *R. massiliae* is supported by dendrograms inferred from the comparison of 16S rDNA, *gltA* and *ompA* sequences, and high bootstrap values were calculated for all supporting nodes using *gltA* and *ompA* analysis. The pathogenic potential in humans of all these rickettsiae is unknown at the present time, although a possible pathogenic role of Bar 29 in humans was recently proposed (Beati *et al.*, 1996; Bella *et al.*, 1991). The close evolutionary relationship between *R. massiliae* and Bar 29 is reflected in genotypic similarities. The species were isolated from *Rhipicephalus* tick species in France and Greece, and in Spain, respectively. *R. rhipicephali* has been also isolated from *Rhipicephalus*

ticks in the USA and characterized in ticks in France (Drancourt *et al.*, 1992) and Portugal (Bacellar *et al.*, 1995), making it the only SFG rickettsia associated with ticks present in both the New and the Old Worlds. The other two representatives of this sub-group, *R. aeschlimannii* and *R. montanensis*, were isolated not from *Rhipicephalus* species but from *Hyalomma* and *Dermacentor* species, respectively. Recently, a phylogenetic study of ticks based on the comparison of mitochondrial 16S rDNA sequences suggested that *Hyalomma* species share a common ancestor with the Rhipicephalinae (Black & Piesman, 1994), suggesting that the divergence pattern of the tick hosts parallels the recent divergence of the *Rickettsia* species that they carry.

The *R. conorii* complex was well-supported by *ompA*-derived phylogenetic analysis. This was not the case when analysis was performed by comparison of *gltA* and 16S rDNA sequences. Human diseases are associated with these rickettsiae and, clinically, the main difference is the frequency of the eschar (0, 23 and 72% for Israeli tick typhus, Astrakhan fever and Mediterranean spotted fever, respectively). The taxonomic position of Astrakhan fever rickettsia and Israeli tick typhus rickettsia remains unclear. Using the current criteria for the definition of a *Rickettsia* species, the microimmunofluorescence test (MIF), they belong to the same species (*R. conorii*), as specificity difference (SPD) values are ≤ 3 (Eremeeva *et al.*, 1994a). However, the strains can easily be differentiated using phenotypic methods (SDS-PAGE and immunoblotting) and genotypic methods (restriction profiles of chromosomal DNA obtained by pulsed-field gel electrophoresis, 16S rDNA, *gltA* and *ompA* sequencing). From an ecological perspective, Astrakhan fever rickettsia, Israeli tick typhus rickettsia and *R. conorii* were all isolated from *Rhipicephalus* ticks in Eurasia, but the geographical location of *R. conorii* is not restricted to a specific area as it has been isolated all around the Mediterranean Sea, in India, and around the Black Sea, and was isolated in Africa, where it was also found in *Haemophysalis* ticks. Reliance solely on the MIF for species identity is no longer justified, and the introduction of genotypic, phylogenetic and ecological criteria is required. In this way, species can be defined by polyphasic means.

The monophyletic relationship among members of the *R. sibirica* sub-group is evidenced by the phylogenetic analysis inferred from 16S rDNA, *gltA* and *ompA* sequence comparison. The geographical areas of distribution of '*R. mongolotimonae*' (China and France) and *R. sibirica* (China, former USSR and Pakistan) overlap. These two rickettsiae are genotypically very close. '*R. mongolotimonae*' was isolated from *Haemophysalis asiaticum* and *R. sibirica* from *Haemophysalis concinna* and *Dermacentor* species; thus horizontal transfer of rickettsiae may have occurred after the separation of *Dermacentor* and *Haemophysalis* clades. The geographical dispersion of the other three representatives of this cluster, which are evolutionarily

close, is great, as *R. africae*, strain S and *R. parkeri* were isolated from Africa, Armenia and USA, respectively. Furthermore, their vectors (*Rhipicephalus sanguineus* for strain S and *Amblyomma* species for *R. africae* and *R. parkeri*) are phylogenetically distant (Black & Piesman, 1994).

The phylogenetic position of *R. japonica* was the same whichever analysis system was considered. It was intermediate between the *R. massiliae* sub-group and the other rickettsiae described, which presented a common ancestor.

R. rickettsii, '*R. slovacca*' and Thai tick typhus rickettsia did not cluster with any other rickettsiae but branched alone. It seems that these species lacked a reliable phylogenetic analysis among the SFG.

The study of synonymous and non-synonymous substitutions allowed us to assess the manner of rOmpA evolution. Protein evolution depends on both the probability that a substitution will be compatible with the biochemical function of the protein and the dispensability of the protein to the organism. The regions of *ompA* we sequenced were undergoing neutral evolution. For free-living bacteria, the host immune system plays a major role as a positive selection factor. The obligate intracellular nature of *Rickettsia* sets them apart from the free-living bacteria, and thus the importance of host immunity on selection is unclear. The nature of the relationship between rickettsiae and arthropods suggests that their evolution is likely to be influenced far more by arthropod factors than those of transient warm-blooded hosts because rickettsiae are mainly maintained by transovarial transmission between tick generations. However, horizontal transfer of rickettsiae between vectors certainly occurs, as proven by the multiplicity of vectors for *R. conorii* or *R. rickettsii* (Raoult & Roux, 1997). Furthermore, owing to their strict intracellular position, the mechanisms commonly implied in genetic transfer in free-living bacteria, such as plasmid-mediated conjugation, phage infection and transduction, transformation by unassociated DNA or conjugative transposition of mobile genetic elements, may have a low impact on the genetic evolution of rickettsiae. However, within tick hosts, co-infections with other bacteria such as *Ehrlichia*, *Coxiella* or *Borrelia* species may occur (P. Parola, L. Beati, M. Cambon & D. Raoult, unpublished results). It is not clear whether there is intimate contact between different co-infectors, but, if so, genetic transfer may be possible. However, evidence for this is yet to be found.

Recently, new *Rickettsia* species were characterized in insects [AB bacterium (Werren *et al.*, 1994) and pea aphid rickettsia (Chen *et al.*, 1996)], attesting to the association of these bacteria with a large clade of arthropod hosts. In future, the goal should be to obtain more isolates from different hosts and from different geographical regions. Analysis including an enlarged panel of strains should reinforce and clarify our understanding of the phylogeny of rickettsiae.

Moreover, to confirm our results and to obtain a precise and reliable phylogenetic position for all the SFG members, other bases for phylogenetic inference are required. Only very few genes have been sequenced for the SFG rickettsiae. The rOmpB protein gene, which is also strongly immunogenic and is common to the SFG and TG (Gilmore *et al.*, 1991), may prove to be useful for this goal. In fact, it seems likely that outstanding phylogenetic analysis of micro-organisms will follow from comparison of part or whole bacterial chromosomes in their organization and in their sequencing.

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