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Characterization of Mutations in the *rpoB* Gene in Naturally Rifampin-Resistant *Rickettsia* Species

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Rickettsiae are gram-negative, obligately intracellular bacteria responsible for arthropod-borne spotted fevers and typhus. Experimental studies have delineated a cluster of naturally rifampin-resistant spotted fever group species. We sequenced the 4,122- to 4,125-bp RNA polymerase β-subunit-encoding gene (*rpoB*) from typhus and spotted fever group representatives and obtained partial sequences for all naturally rifampin-resistant species. A single point mutation resulting in a phenylalanine-to-leucine change at position 973 of the *Rickettsia conorii rpoB* sequence and present in all the rifampin-resistant species was absent in all the rifampin-susceptible species. *rpoB*-based phylogenetic relationships among these rickettsial species yielded topologies which were in accordance with previously published phylogenies.

Rickettsiae are arthropod-borne, gram-negative, obligately intracellular bacteria (15). The genus *Rickettsia* has been divided into two groups, namely, the typhus group and the spotted fever group, on the basis of clinical presentation, immunological reactivity, intracellular location, and DNA G+C content (25). Phylogenetic appraisal based on a comparison of 16S rRNA genes (17, 26) has demonstrated that rickettsiae belong to the α-1 subgroup of the class *Proteobacteria*. Typhus group rickettsiae include *Rickettsia prowazekii*, the agent of epidemic typhus, and *Rickettsia typhi*, the agent of murine typhus. Phylogenetic approaches based on a comparison of sequences derived from the citrate synthase-encoding (18) and rOmpA-encoding (7) genes have resolved two principal subgroups within the spotted fever group (Table 1), the *Rickettsia conorii* subgroup and the *Rickettsia massiliae* subgroup, which also includes *Rickettsia montanensis*, *Rickettsia aeschlimannii*, *Rickettsia rhipicephali*, and the tick isolate Bar 29. Natural resistance to rifampin at MICs of 2 to 4 μg/ml is a phenotypic marker of the *R. massiliae* subgroup, whereas rickettsiae belonging to the *R. conorii* subgroup are naturally susceptible to rifampin (Rif^s) at MICs of <1 μg/ml (16). Typhus group rickettsiae are naturally susceptible to rifampin (16), but rifampin-resistant (Rif^r) strains of *R. typhi* (23) and *R. prowazekii* (14) have been selected in vitro after random mutagenesis. Alterations in the RNA polymerase β subunit resulting from mutations in *rpoB* (9, 10, 22) are the most common mechanisms for rifampin resistance (13). Indeed, amino acid substitutions in the RNA polymerase and *rpoB* point mutations have been demonstrated after in vitro selection of Rif^r *R. prowazekii* (2, 14) and *R. typhi* (23). No data are available for naturally Rif^r rickettsiae.

Therefore, we investigated the genetic basis for natural rifampin resistance in representatives of the typhus group and the two spotted fever subgroups of rickettsiae after amplification and sequencing of the *rpoB* gene. We derived phylogenetic relationships between the typhus group and spotted fever group rickettsiae and between Rif^s and Rif^r spotted fever group rickettsiae by sequence analysis of rickettsial *rpoB*. In addition, we confirmed the rifampin-based clustering of spotted fever group rickettsial species.

MATERIALS AND METHODS

Bacterial strains. The rickettsial strains used in this study are listed in Table 1. All strains were cocultivated with Vero cells in minimum essential medium

TABLE 1. Rickettsial strains studied

Species	Strain	Source	Geographical origin	Human disease
R. conorii	Moroccan, ATCC VR-141^T	Unknown	Morocco	Mediterranean spotted fever
R. conorii	Seven (Malish) ATCC VR-613^T	Unknown	South Africa	Mediterranean spotted fever
R. sibirica	246, ATCC VR-151^T	*Dermacentor nuttali*	Siberia	Siberian tick typhus
R. rickettsii	R(Bitteroot), ATCC VR-891^T	*Dermacentor andersoni*	Montana	Rocky Mountain spotted fever
R. japonica	YM	Human	Japan	Oriental spotted fever
R. massiliae	Mtu1	*Rhipicephalus turanicus*	Camargue, France	Not reported
Bar 29	Bar 29	*Rhipicephalus sanguineus*	Spain	Not reported
R. rhipicephali	3-7-6	*Rhipicephalus sanguineus*	Mississippi	Not reported
R. montanensis	M/5-6	Microtus sp.	Ohio	Not reported
R. aeschlimanii	MC16	*Hyalomma*	Morocco	Not reported
R. prowazekii	Brein L	Human	Poland	Epidemic typhus
R. typhi	Wilmington	Human	North Carolina	Murine typhus

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

Primer	Nucleotide sequence (5′-3′)	Positions relative to the open reading frame
RC 1600D	GCCAATTATCGCAGTTTATGG	1,600–1,620
RC 2030R	ACGATTTGCATCATCATTTTC	2,030–2,010
Bap 3850R	GCCCAACTTCCATTTCACC	3,850–3,832
Rick 1900D	GTAATGGTAGAACCGTATGAGG	1,900–1,921
Bap 355D	GAACAAGAAGTATATATGGG	355–374
Rc7 1700R	CGTAATGAGTCGGATGTACG	1,700–1,681
Bap rpIL 350D	TAGAAGATGCTGGAGC	350–365
Rc7 450D	ACACCAGGTGAGCGATGC	450–467
Bap rpoC 300R	AGTTCTATATGACCCAT	300–284
Rc7 3400D	GATGGAACTGTGGTAGATATCG	3,400–3,421

supplemented with 4% fetal calf serum and 2 mM glutamine at 32°C for 5 days. Cultures were checked for the absence of contamination by *Mycoplasma* species with a *Mycoplasma* Detection Kit (Boehringer Mannheim, Meylan, France). DNA was extracted by a standard phenol-chloroform procedure (19).

PCR amplification of *rpoB*. Consensus PCR primers RC 1600D and RC 2030R were designed to hybridize to conserved regions of the gene identified following multiple alignments of the *rplL*, *rpoB*, and *rpoC* sequences available for the class *Proteobacteria*. Additional oligonucleotides were selected on the basis of data obtained from ongoing base sequence determinations (Table 2). All PCRs incorporated final concentrations of 2.5×10^{-2} U of *Taq* polymerase per μl, 1× *Taq* buffer, and 1.8 mM $MgCl_2$ (Gibco BRL, Life Technologies, Cergy Pontoise, France); 120 μM each dATP and dTTP and 280 μM each dGTP and dCTP (Boehringer GmbH, Hilden, Germany); and 0.2 μM each primer (Eurogentec, Seraing, Belgium). PCR mixtures were subjected to the following thermal program: 35 cycles consisting of denaturation at 94°C for 10 s, primer annealing at 52°C for 20 s, and extension at 72°C for 50 s. Every program included predenaturation at 94°C for 90 s and a final elongation step at 72°C for 5 min. Sterile distilled water and noninfected Vero cell DNA were used as negative controls in each set of reactions. The success of amplification was assessed by UV illumination of the resolution of products by electrophoresis through an ethidium bromide-stained 0.8% agarose gel.

Sequencing of *rpoB*. Amplicons were purified for sequencing by use of a QIAquik Spin PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) in accordance with the protocol of the supplier. Initial sequencing of PCR products was done with the same primers (Eurogentec) as those used for PCR. Subsequently, primers were chosen by comparison to the newly obtained sequences. Each base position was established at least three times in both the forward and the reverse directions. Sequencing reactions were carried out with the reagents in the ABI Prism dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, Calif.) in accordance with the manufacturer's instructions and with the following program: 25 cycles of denaturation at 95°C for 20 s, primer annealing at 50°C for 10 s, and extension at 60°C for 4 min. Products of sequencing reactions were resolved by electrophoresis in a 0.2-mm 6% polyacrylamide denaturing gel and recorded with an ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems) in accordance with the standard protocol of the supplier. The results obtained were processed into sequence data by use of sequence analysis software (Applied Biosystems), and partial sequences were combined into a single consensus sequence.

Partial *rpoB* amplification and sequencing in rickettsiae. Since *rpoB* sequence analysis disclosed five nonsynonymous nucleotide positions in Rif^s *R. conorii* and in Rif^r *R. massiliae* and isolate Bar 29, we further investigated these codon positions by partial *rpoB* sequencing of Rif^s and Rif^r species. Fragments of *rpoB* were amplified and sequenced with internal primers by the same methods as those described above.

Sequence data analysis. 16S rRNA sequences and *rpoB* sequences of the nonrickettsial species studied were obtained from the GenBank database, as was the *R. prowazekii* (GenBank accession no. AF034531) *rpoB* sequence. Pairwise sequence comparisons for nucleic acid or peptide sequence homology were made with PC Gene software (Intelligenetics, Campbell, Calif.). The *rpoB* sequences were aligned by use of the multisequence alignment program CLUSTAL (8). Phylogenetic relationships were inferred from this alignment by use of programs within version 3.4 of the PHYLIP software package (6). A distance matrix was generated by use of DNADIST under the assumptions of Jukes and Cantor (11) and Kimura (12). Phylogenetic trees were derived from this matrix by neighbor joining. Maximum-likelihood analysis was done with DNAMLK and a molecular clock, and parsimony analysis was done with DNAPARS.

For alignment of multiple amino acid sequences, the parsimony method was done with PROTPARS; the distance matrix was calculated by use of PROTDIST Kimura or Dayhoff algorithms. This step was followed by the neighbor-joining method. Evaluation of individual node strength was done with the SEQBOOT bootstrapping method and 100 runs.

Levels of similarity between *rpoB* sequences and RNA polymerase β-subunit amino acid sequences were determined by use of the homology search function of DNASIS (Hitachi Software Engineering America, Ltd., Brisbane, Calif.).

Nucleotide sequence accession numbers. The *rpoB* sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the following accession numbers: *R. conorii* Moroccan. AF076435; *R. conorii* Seven, AF076434; *R. massiliae*, AF076433; isolate Bar 29, AF076436; *R. prowazekii* Brein L, AF076437; and *R. typhi* Wilmington AF083622.

RESULTS AND DISCUSSION

***rpoB* variability and genetic support of natural rifampin resistance in spotted fever group rickettsiae.** Complete *rpoB* sequences were determined for *R. conorii* Seven, *R. conorii* Moroccan, *R. massiliae*, isolate Bar 29, *R. prowazekii*, and *R. typhi*. Rickettsial *rpoB* consisted of a 4,122-bp open reading frame in the spotted fever group and a 4,125-bp open reading frame in the typhus group, with a G+C content of 35%, a low

TABLE 3. Nonsilent *rpoB* sequence base positions divergent between rifampin-susceptible and rifampin-resistant spotted fever group *Rickettsia* species

Strain	Amino acid/nucleotide at position[a]:					Rifampin susceptibility
	221	524	640	973	1031	
R. conorii	Thr/ACT	Ser/AGT	Ile/ATT	Phe/TTC	Ile/ATC	S
R. rickettsii	ND	Ser/AGT	Ile/ATT	Phe/TTC	Thr/ACC	S
R. sibirica	ND	Ser/AGT	Ile/ATT	Phe/TTC	Ile/ATC	S
R. japonica	ND	Ser/AGT	Val/GTT	Phe/TTT	Thr/ACC	S
R. massiliae	Ile/ATT	Asn/AAT	Val/GTT	Leu/TTA	Thr/ACC	R
R. rhipicephali	Ile/ATT	Asn/AAT	Val/GTT	Leu/TTA	Thr/ACC	R
Bar 29	Ile/ATT	Asn/AAT	Val/GTT	Leu/TAA	Thr/ACC	R
R. montanensis	Ala/GCT	Ser/AGT	Val/GTT	Leu/TAA	Thr/ACC	R
R. aeschlimanii	Thr/ACT	Ser/AGT	Val/GTT	Leu/TAA	Thr/ACC	R

[a] Position numbering is that of the *R. conorii* Seven sequence. S, rifampin susceptible; R, rifampin resistant; ND, not determined.

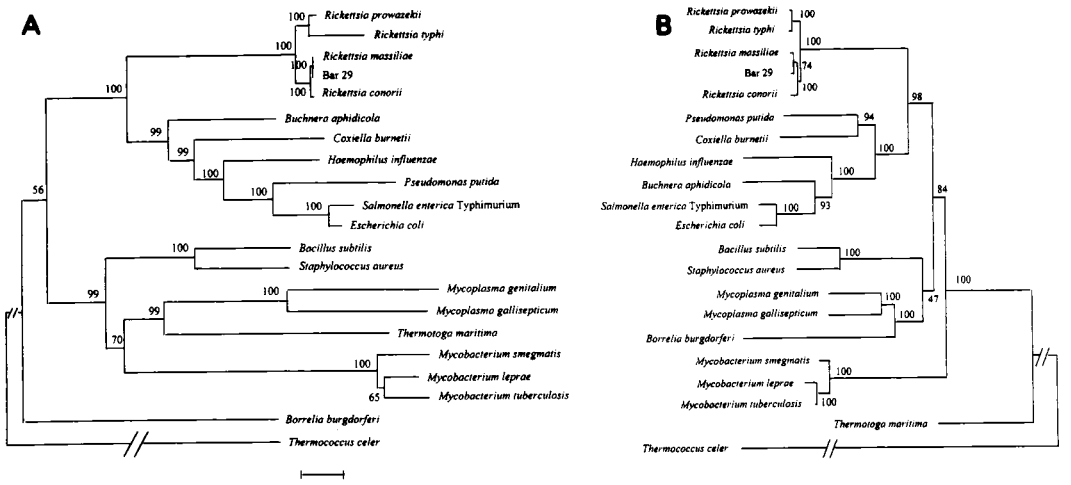

FIG. 1. Distance matrix trees derived from *rpoB* data (A) and from data for the 16S rRNA-encoding gene (B). The evolutionary distances were determined by the method of Kimura (12). These values were used to construct a dendrogram by the neighbor-joining method. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The scale bar shows 5% differences in nucleotide sequences. The GenBank accession numbers for the 16S rRNA gene sequences included are as follows: *Escherichia coli*, M24996; *Salmonella enterica* serovar Typhimurium, X80681; *Buchnera aphidicola*, Z19056; *Haemophilus influenzae*, M35019; *Pseudomonas putida*, X93997; *Coxiella burnetii*, M21291; *Rickettsia massiliae*, L36214; isolate Bar 29, L36102; *Rickettsia conorii* Moroccan, L36105; *Rickettsia typhi*, L36221; *Rickettsia prowazekii*, M21789; *Mycoplasma genitalium*, X77334; *Thermotoga maritima*, M21774; *Borrelia burgdorferi*, X85204; *Staphylococcus aureus*, X68417; *Bacillus subtilis*, AF058766; *Mycobacterium smegmatis*, M12872; and *Mycobacterium tuberculosis*, X52917. Accession numbers for the nonrickettsial *rpoB* sequences included are as follows: *Escherichia coli*, V00339; *Salmonella enterica* serovar Typhimurium, X13854; *Buchnera aphidicola*, Z11913; *Haemophilus influenzae*, U68759; *Pseudomonas putida*, X15849; *Coxiella burnetii*, U86688; *Mycoplasma genitalium*, U39717; *Thermotoga maritima*, X61562; *Borrelia burgdorferi*, X71024; *Staphylococcus aureus*, X64172; *Bacillus subtilis*, D83789; *Mycobacterium smegmatis*, U24494; and *Mycobacterium tuberculosis*, L27989. The GenBank accession numbers for the rickettsial *rpoB* sequences are given in Materials and Methods.

value consistent with that previously reported (24). A comparison of *rpoB* sequences showed five nonsynonymous mutations in Rif^r isolate Bar 29 and *R. massiliae* versus Rif^s *R. conorii* (Table 3). When these mutations were investigated with a larger representation of the Rif^s subgroup and the more distant rickettsial species *Rickettsia bellii*, $Phe_{973} \rightarrow Leu_{973}$ was found to be the only one common to the five members of the Rif^s subgroup. This single point mutation, which appeared to be specific for the naturally rifampin-resistant subgroup, was not previously implicated in rifampin resistance in other bacteria.

In *Escherichia coli*, clusters of mutations were precisely determined (10): cluster I, encoding peptide amino acids 507 to 511 and 513 to 533; cluster II, encoding peptide amino acids 563 to 564 and 572; and cluster III, containing the single codon for Arg_{687}. Clusters I and II comprise more than 90% of the mutations in Rif^r *E. coli* (20, 21). A region homologous to clusters I and II has been reported to acquire mutations in Rif^r *Mycobacterium leprae* (5, 9), *Mycobacterium tuberculosis* (22), *Neisseria meningitidis* (4), and *Staphylococcus aureus* (1). A unique point mutation changing Arg_{546} to Lys has been reported for Rif^r *R. prowazekii* (2, 24), and three amino acid changes at residues 151, 201, and 271 have been described for Rif^r *R. typhi* (23). It is noteworthy that all mutations reported to date were derived from rifampin-resistant isolates selected after a rifampin treatment failure in patients, in vitro selection, or directed mutagenesis. This study reports for the first time mutations associated with natural rifampin resistance among closely related bacterial species.

The clinical significance of these data remains hypothetical, since none of the five rifampin-resistant species has been isolated from patients. A randomized trial of 5-day rifampin versus 1-day doxycycline for treating Mediterranean spotted fever in Barcelona, Spain, revealed delayed apyrexia in the rifampin group, and the trial was stopped (3). No strain was isolated from patients during this trial; interestingly, however, the *rpoB* mutant isolate Bar 29 has been recovered from *Rhipicephalus sanguineus* ticks in the Barcelona area. The hypothesis that isolate Bar 29 was responsible for rifampin-resistant Mediterranean spotted fever in the Barcelona area remains to be confirmed by the isolation of this rickettsial species from any patient.

***rpoB*-based phylogenies of rickettsiae.** The sequences of *rpoB* of *R. conorii* Moroccan and Seven determined in this study were identical, as were those of *R. prowazekii* Brein L and Madrid E (14). Among the spotted fever group rickettsiae, the *rpoB* coding sequences were 97% similar, whereas among the typhus group rickettsiae, they were about 90% similar. A sequence similarity of about 92.8% was found between the spotted fever and typhus group rickettsiae, but only about 54.7 to 65.5% similarity was found between spotted fever group *rpoB* and *rpoB* from nonrickettsial bacteria. Dendrograms inferred from the entire *rpoB* alignment by neighbor-joining (Fig. 1) and parsimony methods yielded similar topologies with clusters of rickettsiae. The two typhus group species clustered together, as did the three spotted fever strains with bootstrap values of >80%. *rpoB*-based phylogenies supported the divergence of Rif^r strains from other members of the spotted fever group, as previously indicated by partial *gltA* sequencing (16, 18) and sequence comparisons of rickettsial *ompA* (encoding an antigenic high-molecular-weight membrane protein) from all spotted fever group species (7).

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