Rickettsia aeschlimannii sp. nov., a New Spotted Fever Group *Rickettsia* Associated with *Hyalomma marginatum* Ticks

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We formally propose the name *Rickettsia aeschlimannii* sp. nov. for a new spotted fever group (SFG) rickettsia, strain MC16^T, isolated from *Hyalomma marginatum marginatum* ticks collected in Morocco. This organism shows a typical rickettsial morphology when analyzed by electron microscopy. After characterization by serotyping, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western immunoblotting, PCR-restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis, and 16S rDNA sequencing, this organism was found to be different from all of the recognized SFG rickettsiae. Identical PCR-RFLP profiles have, however, been found in *H. marginatum marginatum* from Portugal and *H. marginatum rufipes* from Zimbabwe, which suggests that the distribution of this rickettsia reaches from the Mediterranean to southern Africa.

Although the first descriptions of Mediterranean spotted fever and its ecology were made in northern Africa (13) and one of the reference strains of *Rickettsia conorii* is called the Moroccan strain, there have been few recent reports about the ecology and epidemiology of rickettsioses in this area (31). In early studies, all three African spotted fever group (SFG) rickettsioses, Mediterranean spotted fever, South African tick bite fever, and Kenyan tick typhus (25), were attributed to infection with *R. conorii*. The recognized vectors were dog ticks: *Rhipicephalus sanguineus* in the Mediterranean area (11) and *Rhipicephalus simus* or *Haemaphysalis leachi* in the eastern and southern regions of Africa (22, 27). When other tick species were found to be infected with rickettsia-like organisms (RLO), they were considered to be "secondary" vectors of *R. conorii* (21). More recently, however, it has been shown that other SFG rickettsiae occur throughout Africa and that several tick species carry rickettsiae different from *R. conorii* (7, 41).

The presence of RLO in *Hyalomma* ticks of Morocco and Sudan was first reported in the 1950s (21). RLO have been detected by PCR and direct immunofluorescence in *H. impeltatum*, *H. dromedari*, and *H. anatolicum* in Egypt; however, they have not been identified (30). During a large-scale field survey in Zimbabwe, hemolymph tests showed that 11% of *Hyalomma marginatum rufipes* ticks were infected with RLO (7). Although isolation attempts were unsuccessful, PCR-restriction fragment length polymorphism (RFLP) analysis showed that these ticks contained SFG rickettsiae genotypically similar to a strain (PoTiR8) isolated from *H. marginatum marginatum* ticks from Portugal (2). Strain PoTiR8 has, however, not been further characterized.

In Morocco, *H. marginatum marginatum* is one of the most widely distributed tick species, possibly representing up to 42% of the tick burden of cattle (3, 32). Although in its juvenile stages it usually bites birds, under particular circumstances it may also infest humans (23). We present here the first description of an SFG rickettsial strain (MC16^T) isolated from Moroccan *H. marginatum marginatum*. This isolate was compared to the recognized SFG rickettsiae by serotyping, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, electron microscopy, PCR-RFLP,

pulsed-field gel electrophoresis (PFGE), and sequencing of the 16S rDNA and the citrate synthase genes (37, 38). All of these methods indicate that the MC16^T isolate is a new SFG rickettsia. We therefore propose the name *Rickettsia aeschlimannii*, in honor of André Aeschlimann, a Swiss zoologist and parasitologist who has made a wide range of scientific contributions in the field of tick-related symbionts and pathogens.

MATERIALS AND METHODS

Ticks. In March 1992, 40 adult *H. marginatum marginatum* ticks were removed from cattle in the region of Fez, Morocco, and identified according to the usual taxonomic keys (23).

Hemolymph test and isolation attempts. After a 10-min disinfection in iodinated alcohol, the living ticks were rinsed in distilled water and dried on sterile filter paper. One droplet of hemolymph, obtained by cutting a foreleg of the tick (12), was placed on a slide and stained by the method of Giménez (20). A further droplet of hemolymph was mixed with 500 µl of brain heart infusion and inoculated into two shell vials containing monolayers of L929 cells. The vials were centrifuged at 700 × g for 1 h, and the supernatant was discarded and replaced with 1 ml of Earle's minimum essential medium (MEM) containing 4% fetal calf serum and 2 mM L-glutamine (28, 33). After 6 days of incubation in a CO₂ incubator at 32°C, the cells were gently scraped from the bottom of the shell vial and stained to detect rickettsiae (20). Cells in infected shell vials were detached with trypsin and transferred to empty 25-cm² flasks, where they were subcultured with MEM.

Rickettsiae and antigen production and purification. The following African and European rickettsial strains were grown in order to compare them with our new isolates: *R. conorii* Moroccan strain ATCC VR-141, *Rickettsia helvetica* ATCC VR-1375, *Rickettsia massiliae* ATCC VR-1376, *R. massiliae* GS (1), *Rickettsia sibirica* ATCC 232, *Rickettsia africae* ESF-5 (25), and strain Bar29 (10). *Rickettsia slovaca* 13B, Israeli spotted fever (ISF) rickettsia strain ISTTDC1, and *R. rhipicephali* 376 were kindly provided by G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). For each reference strain and the new isolates, heavily infected L929 cells from 10 flasks (150 cm²) were harvested, sonicated, and centrifuged at 150 × g for 15 min. The supernatants were layered onto equal volumes of 25% sucrose in PBS (phosphate-buffered saline, pH 7.4) and centrifuged at 7,000 × g for 30 min. The pellets were pooled and purified on a Renografin density gradient (45). Thereafter, the purified organisms were washed in PBS (three times for 10 min each time at 17,500 × g) and resuspended in distilled water at a concentration of 1 mg/ml (Total Protein Test; Bio-Rad Laboratories, Richmond, Calif.) for SDS-PAGE and Western blotting. Purification of rickettsiae for PFGE was carried out by following the same procedure, but K36 buffer (16.5 mM KH₂PO₄, 33.5 mM K₂HPO₄, 100 mM KCl, 15.5 mM NaCl) was used instead of PBS and distilled water (36). The material collected in a further flask was frozen at -80°C until required for mouse immunization, serologic typing, PCR-RFLP analysis, or nucleotide sequence determination.

Transmission electron microscopy. L929 cells were grown to a confluent monolayer in a 5-cm-diameter petri dish with MEM at 37°C in a CO₂ incubator. The monolayer was then infected with approximately 10³ MC16^T organisms and kept at 32°C for 4 days. The infected cells were fixed in a 4% glutaraldehyde solution (in PBS) for 1 h at 4°C, washed with PBS, dehydrated (70, 90, and 100% ethanol), and embedded in Epon 812. Thin transverse sections were cut on an LKB Ultratome III microtome and stained with a saturated solution of methanol-uranyl acetate and lead citrate in water. The grids were examined on a JEOL JEM 1200 EX electron microscope.

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FIG. 1. Electron micrograph of MC16^T infecting an L929 cell. R, rickettsiae; N, nucleus; C, trilaminar cell wall; S, translucent zone compatible with the presence of a slime layer. Bar, 0.2 μ m.

Mouse immunization and microimmunofluorescence serotyping (MIF). According to previously described methods (34), 10^3 to 10^4 organisms each of MC16^T, *R. conorii*, *R. rhipicephali*, *R. massiliae*, Bar29, *R. helvetica*, *R. slovacica*, *R. sibirica*, Israeli SFG rickettsiae, and *R. africae* were inoculated intravenously into five Swiss mice on days 0 and 7. The mice were anesthetized and exsanguinated by cardiac puncture on day 10, and the sera of each group of five mice were pooled and stored at -20°C . For MIF, twofold dilutions (PBS) of the mouse polyclonal sera were reacted with both homologous and heterologous antigens. Fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulins G and M (Immunotec, Marseille, France) diluted at 1/100 in PBS were used to detect antibodies, and the highest serum dilutions giving positive reactions were recorded as endpoint titers. Specificity differences (SPDs) were calculated by following previously described methods (34): $\text{SPD} = (\text{Aa} + \text{Bb}) - (\text{Ab} + \text{Ba})$, where Aa (or Bb) is the $-\log_2$ of the endpoint titer between serum A (or B) and the homologous antigen, a (or b), and Ab (or Ba) is the $-\log_2$ of the endpoint titer of serum A

(or B) against its heterologous antigen, b (or a). If the SPD is <3 , the two strains are assumed to belong to a single serotype; if the SPD is ≥ 3 , the serotypes are different.

SDS-PAGE and Western blot immunoassay. For each strain, a 5- μ l volume of purified organisms was dissolved in 5 μ l of Laemmli (29) solution (4% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue, 0.125 M Tris hydrochloride [pH 6.8], 25% glycerol) at room temperature and SDS-PAGE was carried out with a 7.5% separating gel and a 3.9% stacking gel. The gel was run in a Mini-Protein II cell (Bio-Rad) at 10 mA in an ice bath, and protein bands were visualized by Coomassie blue staining. A high-range molecular weight standard (Bio-Rad) was used to estimate the molecular weights of the electrophoretic bands. Another, identical gel was transferred (42) to nitrocellulose paper in a Trans-blot apparatus (Bio-Rad) at 50 V for 1 h in an ice bath. Nonspecific binding sites were blocked overnight with 5% nonfat dry milk-TBS (10 mM Tris hydrochloride [pH 7.5], 250 mM NaCl, 0.01% merthiolate). After three 10-min washes in TBS, the nitrocellulose paper was overlaid with the polyclonal serum raised against MC16^T, diluted 1/100 in 3% nonfat dry milk-TBS, and rocked for 2 h. Reactive antibodies were detected with a 1/200 goat anti-mouse globulin-peroxidase conjugate (Immunotec) in 3% nonfat dry milk-TBS. Following three further 10-min washes in TBS, the bound peroxidase was detected by a solution containing 0.015% 4-chloro-1-naphthol, 0.015% hydrogen peroxide, and 16% methanol in TBS. As soon as the bands became visible, the reactions were stopped with repeated washes in distilled water.

PCR-RFLP. DNA was extracted from cells heavily infected with MC16^T, *R. massiliae*, *R. conorii*, *R. rhipicephali*, or Bar29 by using a QiAmp Tissue kit (Qiagen, Hilden, Germany). For each strain, cells harvested from a 25-cm² flask were lysed by following the producer's instructions and the DNA was eluted into 200 μ l of distilled water. A sample containing 10 μ l of the extracted DNA, 59.5 μ l of distilled water, 10 μ l of Taq buffer (Perkin Elmer Cetus, Norwalk, Conn.), 10 μ l of deoxynucleoside triphosphates (2% dATP, 2% dCTP, 2% dTTP, and 2% dGTP in distilled water; Boehringer-Mannheim, Meylan, France), 5 μ l each of citrate synthase primers Rp. CS.877 p-1258n (2 pmol/ μ l; Eurogentec, Seraing, Belgium), or 5 μ l each of 190-kDa protein gene primers Rr. 190.70p-602n (2 pmol/ μ l; Eurogentec), and 1.25 U of Taq polymerase (Perkin-Elmer Cetus, Branchburg, N.J.) was subjected to 35 cycles of denaturation (95°C for 20 s), annealing (50°C for 30 s), and sequence extension (72°C for 2 min) in a thermal cycler (MJ Research DNA Engine; MJ Research, Watertown, Mass.). A further 5-min extension at 72°C completed the enzymatic polymerization. The results of amplification were visualized on a 1% agarose gel after electrophoretic migration (100 V for 30 min) of 10 μ l of the amplified material. The agarose gels were stained with ethidium bromide and examined by UV transillumination. Following previously described protocols, 23.3- μ l volumes of the products amplified with the citrate synthase primers (or the 190-kDa protein gene primers) were then digested with 1 μ l (10 U) of restriction endonuclease *AluI* (or *RsaI* and *PstI*) (Boehringer-Mannheim). The digested products were separated on 8% polyacrylamide gels at 80 V for 4 to 5 h (Bio-Rad), stained with ethidium bromide, and examined by UV transillumination. DNA molecular weight marker V (Boehringer-Mannheim) and computer-aided densitometry were used to estimate the sizes of the observed DNA fragments (QGELEDTM program [Quantig Corporation, Madison, Wis.] connected with "The Imager" system [Appligene, Illkirch, France]). With this system, profiles could be compared with those described in other reports (4-6, 8, 9, 15, 17, 26, 35) and with the computerized data collected in the data bank of the Unité des Rickettsies in Marseille.

PFGE. Each of the bacterial suspensions of MC16^T, *R. massiliae* (type strain Mtu1^T and strain GS), *R. rhipicephali*, and Bar29 was added to 1 volume of Inert Agarose (FMC Bioproducts, Rockland, Maine) to form agarose blocks and lysed as previously reported (36). The plugs were digested with the endonucleases *SmaI* (Boehringer-Mannheim), *EagI* (New England BioLabs, Beverly, Mass.), and *BssHII* (New England BioLabs). The migrations were carried out in a contour-clamped homogeneous electric field system (CHEF DR11; Bio-Rad Laboratories) at 14°C in $0.5\times$ TBE (Tris-borate-EDTA buffer, pH 8). After

TABLE 1. MIF antibody titers and SPDs obtained from reciprocal cross-reactions of mouse antisera with SFG rickettsiae

Antiserum	MIF antibody titer (SPD)									
	Strain MC16 ^T	<i>R. massiliae</i>	Strain Bar29	<i>R. helvetica</i>	<i>R. conorii</i>	<i>R. sibirica</i>	<i>R. slovacica</i>	ISF rickettsiae	<i>R. africae</i>	<i>R. rhipicephali</i>
Anti-MC16 ^T	4,096 (0)	1,024 (3)	256 (6)	128 (11)	128 (12)	64 (7)	16 (10)	16 (11)	16 (12)	512 (6)
Anti- <i>R. massiliae</i>	8,192 (3)	16,384 (0)	4,096 (5)	128 (13)	128 (12)	128 (14)	256 (7)	64 (10)	64 (10)	2,048 (4)
Anti-Bar29	262,144 (6)	131,072 (5)	1,048,576 (0)	4,096 (15)	4,096 (14)	16,384 (12)	8,192 (11)	16 (20)	16 (21)	131,072 (5)
Anti- <i>R. helvetica</i>	64 (11)	64 (13)	32 (15)	4,096 (0)	32 (13)	32 (13)	32 (9)	32 (10)	32 (11)	32 (8)
Anti- <i>R. conorii</i>	64 (12)	256 (12)	128 (14)	128 (13)	8,192 (0)	256 (10)	512 (6)	1,024 (6)	256 (9)	256 (10)
Anti- <i>R. sibirica</i>	16,384 (7)	256 (14)	512 (12)	512 (13)	1,024 (10)	32,768 (0)	4,096 (4)	8,192 (4)	256 (10)	256 (12)
Anti- <i>R. slovacica</i>	256 (10)	512 (7)	64 (11)	256 (9)	256 (6)	512 (4)	1,024 (0)	128 (6)	64 (11)	64 (10)
Anti-ISF rickettsiae	512 (11)	1,024 (10)	256 (20)	512 (10)	512 (6)	1,024 (4)	512 (6)	4,096 (0)	16 (14)	64 (11)
Anti- <i>R. africae</i>	256 (12)	1,024 (10)	128 (21)	256 (11)	256 (9)	512 (10)	32 (11)	64 (14)	4,096 (0)	32 (13)
Anti- <i>R. rhipicephali</i>	256 (6)	1,024 (4)	512 (5)	1,024 (8)	64 (10)	64 (12)	32 (10)	64 (11)	32 (13)	2,048 (0)

FIG. 2. SDS-PAGE of the antigens of *R. rhipicephali* (lane 1), strain MC16^T (lane 2), strain Bar29 (lane 3), *R. massiliae* (lane 4), *R. conorii* (lane 5), *R. africae* (lane 6), *R. slovaca* (lane 7), *R. sibirica* (lane 8), and *R. helvetica* (lane 9). Standard molecular mass markers, from the bottom, 49.5, 80, 116, and 205 kDa, were in lane 10.

restriction with *Bss*HII, the migration conditions were 33 h at 4.5 V/cm with ramped pulse times varying from 5 to 120 s. A low number of bands is commonly observed after digestion with *Bss*HII. Therefore, this enzyme is particularly interesting for evaluation of the overall size of rickettsial genomes. To discriminate all of the bands obtained after restriction with *Sma*I and *Eag*I, two different protocols were used for migration of the digests: 19 h at 5.7 V/cm (ramped pulse times from 3 to 10 s) and a further 10 h at 5.4 V/cm (ramped pulse times from 20 to 40 s) or 8 h at 6 V/cm (ramped pulse times from 1 to 3 s) followed by 9 h at 6 V/cm (ramped pulse times from 1 to 5 s). Low-range and lambda ladder PFGE markers (New England BioLabs) were used for estimation of the molecular sizes of the digested products. Computer-aided estimation of the molecular weights was performed as described for PCR-RFLP analysis.

16S rRNA gene sequencing and data analysis. The DNA was extracted from MC16^T as described in the PCR-RFLP protocol. The primer pairs used for the amplification and sequencing of the gene encoding the 16S rRNA have been previously described (37). The amplicons were sequenced by using an Autoread sequencing kit (Pharmacia, St. Quentin-Yvelines, France). The sequencing reactions were resolved on a Readymix acrylamide gel (Pharmacia) in an A.L.F. automated DNA sequencer (Pharmacia). The sequence of the 16S rRNA gene of MC16^T was aligned with the corresponding sequences of the other SFG rickettsiae (37) by using the Clustal (version V) alignment program of the BISANCE software package (14). Levels of similarity between pairs of sequences were determined by using DNASIS (Hitachi Software Engineering America Ltd., Brisbane, Calif.). A matrix of evolutionary distances was derived from the alignment (DNADIST, PHYLIP 3.4 software), and a tree was inferred from these distances by using the neighbor-joining method (PHYLIP) (18, 24, 39).

RESULTS AND DISCUSSION

Hemolymph test and isolation attempts. Six of 40 ticks were found to be infected with RLOs. This infection rate of 15% is consistent with the infection rates of *H. marginatum rufipes* ticks in Zimbabwe (6 to 14%) and *H. marginatum marginatum* ticks in Portugal. However, in Portugal it has been observed that the infection rate may vary from 11 to 30%, depending on the geographic area (1a). Five isolates were obtained after centrifugation of the hemolymph on shell vials; however, cell lines were found to be contaminated with *Mycoplasma* spp., and after decontamination (16), only a single isolate, MC16^T, could be recovered. The contaminated strains were, however, analyzed by PCR-RFLP and had profiles identical to that of strain MC16^T.

Electron microscopy. Strain MC16^T is an intracellular organism which occurs free in the cytoplasm of its host cell (Fig. 1). At a higher magnification, MC16^T shows typical rickettsial

morphology, with a trilaminar cell wall surrounded by a translucent area compatible with the presence of an outer slime layer (Fig. 1). The length of MC16^T cells varies from 0.7 to 1.1 μ m, and the mean diameter is 0.3 μ m.

MIF. Calculated SPDs between strain MC16^T and the other considered SFG rickettsiae were all ≥ 3 , indicating that MC16^T is a new SFG serotype (Table 1).

SDS-PAGE and Western immunoblotting. Although the MC16^T antigenic profile shares some species-specific high-molecular-weight bands with *R. massiliae* and strain Bar29, its migration patterns are unique and easy to differentiate from those of all other SFG rickettsiae. The approximate molecular masses of the species-specific bands have been evaluated as follows: (116, 120), 140, and 145 kDa for *R. rhipicephali*; 95, (116), and 150 kDa for MC16^T; (116), 135, 140, and 150 kDa for Bar29; 110, (116), 130, (150), and 160 kDa for *R. massiliae*; 120 and (135) kDa for *R. conorii*; 120, (138), and 155 kDa for *R. africae*; 120, (125), 135, and 140 kDa for *R. slovaca*; (120), 130, and 155 kDa for *R. sibirica*; and (90, 120), and 140 kDa for *R. helvetica* (the more prominent bands are in parentheses) (Fig. 2).

By Western blotting, anti-MC16^T antibodies reacted with low-molecular-mass bands (<50 kDa) of each of the rickettsiae. Cross-reactions with high-molecular-weight species-specific antigens were found only with *R. massiliae*, *R. rhipicephali*, and strain Bar29. The migration patterns of the cross-reacting bands revealed were different, however, allowing one to distinguish these four rickettsiae (Fig. 3).

PCR-RFLP. After amplification with the citrate synthase primers and digestion with *Alu*I, the profile of MC16^T was identical to that of the other classical SFG rickettsiae (45, 95, 105, and 135 bp) (Fig. 4). The 190-kDa protein gene fragment of MC16^T showed a profile identical to that of *R. rhipicephali* when digested with *Rsa*I (95, 106, 118, and 220 bp). Following restriction with *Pst*I, however, the profile was different from

FIG. 3. Western immunoblot assay. Antibodies of anti-MC16^T sera reactive with *R. rhipicephali* (lane 1), strain MC16^T (lane 2), strain Bar29 (lane 3), *R. massiliae* (lane 4), *R. conorii* (lane 5), *R. africae* (lane 6), *R. slovaca* (lane 7), *R. sibirica* (lane 8), and *R. helvetica* (lane 9). The bars on the left correspond to molecular masses (from the bottom, 49.5, 80, 116, and 205 kDa).

FIG. 4. Ethidium bromide-stained profiles of DNA fragments of strain MC16^T (lane 1), *R. massiliae* (lane 2), strain Bar29 (lane 3), *R. rhipicephali* (lane 4), and *R. conorii* (lane 5) amplified with the citrate synthase primers and digested with *AluI*. Molecular size standards are represented by the bars on the right (from the bottom, 79, 162, 210, and 291 bp).

that of *R. rhipicephali* (260 and 280 bp) and similar to that of *R. slovaca* and *R. japonica* (125, 165, and 265 bp) (15). This combination of PCR-RFLP migration patterns (Fig. 5 and 6) has already been observed during PCR-RFLP analysis of crude extracts of Zimbabwean *H. marginatum rufipes* (7) and Portuguese *Hyalomma* isolate PoTiR8 (2).

PFGE. After restriction of the whole genome with *Bss*HII, the genome size of MC16^T could be estimated to be 1,336 kb (Fig. 7), suggesting that MC16^T, like strain Bar29, *R. massiliae*, and *R. helvetica*, is characterized by a genome size slightly greater than that of the other SFG rickettsiae (1,216 to 1,289 kb) (36). PFGE results obtained after restriction with *Bss*HII, *Sma*I, and *Eag*I show that MC16^T is clearly different from other SFG rickettsiae, including the closely related species *R. rhipicephali* and *R. massiliae* and strain Bar29 (Fig. 8).

16S rRNA gene sequencing and data analysis. A 1,432-bp sequence, representing 95% of the entire 16S rRNA gene, was generated for MC16^T and has been deposited in the GenBank database under accession number U74757. Comparison of 16S rRNA gene sequences derived from SFG species revealed a high level of similarity between strain MC16^T and strain Bar29 (99.6%), *R. massiliae* (99.5%), and *R. rhipicephali* (99.6%). As already pointed out (25, 37), the levels of 16S rRNA similarity among SFG rickettsiae are all very high. Between MC16^T and the other SFG rickettsiae, similarity varies from 98% (*Rickett-*

sia akari) to 99.4% (*R. conorii*, *R. japonica*, and *R. slovaca*). Similarly, evolutionary distances between MC16^T and the other SFG rickettsiae must also be considered to be very low, varying from 0.0199×10^3 nucleotides with the more distant species *R. akari* to 0.0042×10^3 to 0.0049×10^3 nucleotides with *R. massiliae*, *R. rhipicephali*, and strain Bar29. The tree inferred from the distance matrix (Fig. 9) suggests that strain MC16^T clusters with *R. rhipicephali*, *R. massiliae*, strain Bar29, and *R. montana*. Although the topology of this tree has no statistical support (bootstrap values are <80%) and must therefore be considered with the greatest caution, the clustering of MC16^T with at least *R. rhipicephali*, *R. massiliae*, and strain Bar29 has been confirmed by a statistically supported phylogenetic analysis based on comparison of citrate synthase gene sequences (GenBank accession numbers for the citrate synthase genes: *R. rhipicephali*, U59721; *R. massiliae*, U59719; strain Bar29, U59720; strain MC16^T, U59722) (38). Comparison of the citrate synthase genes provides stronger statistical support for the topology of the deeper branches of the tree, including the "MC16^T cluster." Nodes at this branching level are characterized by bootstrap values varying from 95 to 96% (38). Interestingly, by Western blot immunoassay, strain MC16^T, *R. massiliae*, *R. rhipicephali*, and strain Bar29 are the only organisms that have antigenic epitopes in common in the high-molecular-weight protein bands.

FIG. 5. Ethidium bromide-stained profiles of DNA fragments of strain MC16^T (lane 1), *R. massiliae* (lane 2), strain Bar29 (lane 3), *R. rhipicephali* (lane 4), and *R. conorii* (lane 5) amplified with the 190-kDa protein primers and digested with *Rsa*I. Molecular size standards are represented by the bars on the left (from the bottom, 79, 162, 210, 291, and 392 bp).

FIG. 6. Ethidium bromide-stained profiles of DNA fragments of strain MC16^T (lane 1), *R. massiliae* (lane 2), strain Bar29 (lane 3), *R. rhipicephali* (lane 4), and *R. conorii* (lane 5) amplified with the 190-kDa protein primers and digested with *Pst*I. Molecular size standards are represented by the bars on the left (from the bottom, 79, 162, 210, 291, and 392 bp).

Justification for a new species. MIF has long been considered the reference method for rickettsial identification and classification (34). This method does not, however, fulfill the polyphasic criteria generally accepted as necessary for the description of new bacterial species (19, 44). Furthermore, compared with other genotypic and phenotypic analysis methods, MIF is a fastidious and sometimes hazardous technique, the use of which can no longer be justified. All other analysis, including SDS-PAGE, Western immunoblotting, PCR-RFLP, PFGE, and 16S rRNA gene sequencing, concurred with the MIF results in indicating that the MC16^T strain is a new rickettsial pheno- and genotype.

Fragmentary data on DNA-DNA homology among SFG rickettsiae indicate that most of these organisms belong to the same species (43), an observation supported by the high level of similarity observed among SFG rickettsial 16S rRNA gene sequences (37). It has been suggested, however, that bacterial species identification cannot be confidently made on the basis of phylogenetic analysis of 16S rDNA sequences which share a level of similarity of >97% (40). It has also been observed (25) that the species identification criterion of >70% DNA-DNA homology, which was initially proposed for the classification of enterobacteria, may not be suitable to the classification of rickettsiae. Moreover, SFG rickettsiae are strictly intracellular

FIG. 7. Schematic representation of the PFGE profiles obtained after digestion of the rickettsial genome with *Bss*HIII. TOT, total.

bacteria whose main hosts and reservoirs are hard-tick species. Although the relationship between rickettsiae and ticks is not necessarily obligately specific, some rickettsiae have never been found in more than one tick species, including strain MC16^T, which has been found to be associated only with *H. marginatum* ticks. Furthermore, there have been no reports of more than one rickettsial strain being harbored by the same tick specimen. Although the possibility of horizontal transmission of rickettsiae through infected vertebrates or cofeeding processes cannot be completely excluded, the fact that rickettsiae are maternally transmitted strongly suggests that rickettsial lineages can be considered to occupy isolated evolutionary niches within specific tick lines. Within this niche, rickettsiae would have scarce opportunity for recombination by genomic exchange with other, related bacteria. As formal criteria for the definition of rickettsial species have yet to be defined, we

FIG. 8. Ethidium bromide-stained PFGE profiles of strain GS (lanes a), *R. massiliae* (lanes b), strain MC16^T (lanes c), and strain Bar29 (lanes d) digested with *Sma*I (A) and *Eag*I (B). Molecular sizes are marked on the right (from the bottom, 9, 23, 48, 97, 145, and >200 kb).

FIG. 9. Phylogenetic tree for SFG rickettsiae derived from the 16S rRNA gene. The sequence of *Rickettsia typhi* was used as an outgroup. Evolutionary distances were determined by the method of Kimura, and the tree was constructed by the neighbor-joining method (39). knuc, 10^3 nucleotides.

believe that a polyphasic approach to SFG taxonomy, based on phenotypic, genotypic, and ecological data, is the most suitable (25). On the basis of these considerations, we propose that strain MC16^T, isolated from an *H. marginatum marginatum* tick in Morocco, be regarded a separate taxonomic species named *Rickettsia aeschlimannii*.

Description of *Rickettsia aeschlimannii* sp. nov. *Rickettsia aeschlimannii* (ae.schli.man'ni.i, L. gen. n. *aeschlimannii*, of Aeschlimann, named after André Aeschlimann, a Swiss zoologist, is an obligate intracellular bacterium of the tick *Hyalomma marginatum*, which grows in several cell lines (Vero, L929, and Hel). It can be grown in Earle's MEM supplemented with 4% fetal calf serum and 2 mM L-glutamine at 32°C. After Giménez staining, this organism is rod shaped (data not shown). As determined by electron microscopy, this rickettsia has features in common with the other SFG rickettsiae. Mouse antisera against this rickettsia show cross-reactivity against antigens of other SFG rickettsiae. MIF, SDS-PAGE, Western immunoblotting, PFGE, PCR-RFLP, and citrate synthase gene and 16S rDNA sequencing indicated that this rickettsia is clearly different from all other recognized SFG rickettsial species, the most closely related organisms being *R. massiliae*, *R. rhipicephali*, and strain Bar29. No information is available about the possible pathogenicity of this organism for vertebrate hosts. The geographic distribution of this bacterium is likely to correspond to the areas where *H. marginatum* ticks have been analyzed (Morocco, Zimbabwe, and Portugal).

Type strain. The type strain MC16^T, isolated from *H. marginatum marginatum* in Morocco, is held in the collection of the World Health Organization Collaborative Center for Rickettsial Reference in Marseille, France.

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