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Inter- and Intraspecies Identification of *Bartonella* (*Rochalimaea*) Species

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Species of the genus *Rochalimaea*, recently renamed *Bartonella*, are of a growing medical interest. *Bartonella quintana* was reported as the cause of trench fever, endocarditis, and bacillary angiomatosis. *B. henselae* has been implicated in symptoms and infections of human immunodeficiency virus-infected patients, such as fever, endocarditis, and bacillary angiomatosis, and is involved in the etiology of cat scratch disease. Such a wide spectrum of infections makes it necessary to obtain an intraspecies identification tool in order to perform epidemiological studies. *B. vinsonii*, *B. elizabethae*, seven isolates of *B. quintana*, and four isolates of *B. henselae* were studied by pulsed-field gel electrophoresis (PFGE) after restriction with the infrequently cutting endonucleases *Not*I, *Eag*I, and *Sma*I. Specific profiles were obtained for each of the four *Bartonella* species. Comparison of genomic fingerprints of isolates of the same species showed polymorphism in DNA restriction patterns, and a specific profile was obtained for each isolate. A phylogenetic analysis of the *B. quintana* isolates was obtained by using the Dice coefficient, UPGMA (unweighted pair-group method of arithmetic averages), and Package Philip programming. Amplification by PCR and subsequent sequencing using an automated laser fluorescent DNA sequencer (Pharmacia) was performed on the intergenic spacer region (ITS) between the 16 and 23S rRNA genes. It was found that each *B. henselae* isolate had a specific sequence, while the *B. quintana* isolates fell into only two groups. When endonuclease restriction analysis of the ITS PCR product was done, three enzymes, *Taq*I, *Hin*dIII, and *Hae*III, allowed species identification of *Bartonella* spp. Restriction fragment length polymorphism after PCR amplification of the 16S-23S rRNA gene ITS may be useful for rapid species identification, and PFGE could be an efficient method for isolate identification.

Rochalimaea spp. are gram-negative, short-rod bacteria 0.3 to 0.5 by 1.0 to 1.7 μm in size. They were included in the family *Rickettsiaceae* and the tribe *Rickettsieae*, which was subdivided into three genera: *Rickettsia*, *Coxiella*, and *Rochalimaea* (41). Recently, as a result of 16S rRNA sequencing, the genera *Coxiella* and *Rochalimaea* have been removed from the *Rickettsiaceae* family and the species of the genus *Rochalimaea* have been renamed *Bartonella* spp. (4, 39). *Bartonella* spp. grow on the surface of their eucaryotic host cells but can be grown axenically on blood agar. In the seventh edition of Bergey's manual (41), two species were described: *Rochalimaea quintana* and *R. vinsonii. R. quintana* is the etiological agent of trench fever and is transmitted by the human body louse, *Pediculus humanus*. The disease was first recognized in 1915 in Europe during World War I and afflicted many soldiers. Subsequently, this febrile syndrome occurred in Europe during World War II, as well as in Mexico and in North Africa (36, 37). *R. vinsonii* has been isolated once from a vole, *Microtus pennsylvanicus*, trapped on Grosse Isle in the St. Lawrence River in 1943 but has never been isolated from humans. In the 1990s, two new species were identified by 16S rRNA sequencing: *Bartonella henselae*, a bacterium isolated from the blood of a febrile, human immunodeficiency virus antibody-positive patient (23), and *B. elizabethae*, isolated from the blood of a patient with endocarditis (6). Over the past few years, new cases of *B. quintana* infection with pathologies different from that of trench fever have been described, but recently, a case of trench fever in Marseille, France, was described (33). Although many *B. henselae* isolates have been found in the United States, the organism has been demonstrated only once in Europe (38), by PCR followed by specific DNA hybridization, in biopsy specimens from lesions. Both *B. quintana* and *B. henselae* have been found to be associated with bacillary angiomatosis (13, 42) or with less specific clinical syndromes such as bacteremia (15, 30, 32), endocarditis (9), meningitis (15), and neurologic disorders (11). *B. henselae* is also implicated in cat scratch disease, mainly on serological evidence. Anti-*Bartonella* antibodies have been detected in the serum specimens of 84 to 88% of patients with clinical cat scratch disease (24, 43), and *B. henselae* has been isolated from the lymph nodes of two patients with cat scratch disease (8). Improvement in the detection and isolation of *Bartonella* species by prolonging incubation of blood cultures (14) and the use of cell cultures may explain, at least partly, the increase in detection of infections due to *Bartonella* species. For example, differences in the success of isolation attempts between methods using cell culture and those using direct plating onto solid agar have been described (9, 13). Also, *Bartonella* infections have been detected in people without significant antibody titers (22). Little is known about *Bartonella* genetics. The genome sizes have been estimated to be 100×10^7 Da (1.5×10^6 bp) for *B. quintana* and 133×10^7 Da (2×10^6 bp) for *B. vinsonii* (19, 20), and the G+C% is estimated at 39 to 40% for these two species (35, 40). To our knowledge, only three gene sequences have been published for these bacteria: those encoding the citrate synthase, 16S rRNA (6, 23, 39), and a 60-kDa antigen in *B. henselae* (2). The first gene sequence allows rapid identification of *B. quintana*, *B. henselae*, and *B. vinsonii* after PCR-restriction fragment length polymorphism (PCR-RFLP) (23, 25); the second is the basis for the differentiation of the four identified species; and the third allows differentiation of *B. quintana* and

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TABLE 1. Oligonucleotide primers

Primer	*Bartonella* species	Nucleotide sequence (5′→3′)	ITS positions (species)
16SF[a,b]	All	AGAGGCAGGCAACCACGGTA	
23S1[a,b]	All	GCCAAGGCATCCACC	
QHVE1[b]	All	TTCAGATGATGATCCCAA	274–292 (*B. quintana*) 240–257 (*B. henselae*) 260–277 (*B. vinsonii*) 331–348 (*B. elizabethae*)
QHVE2[b,c]	All	TTGGGATCATCATCTGAA	
QHVE3[b]	All	GATATATTCAGACATGTT	898–915 (*B. quintana*) 946–963 (*B. henselae*) 904–921 (*B. vinsonii*) 1109–1127 (*B. elizabethae*)
QHVE4[b,c]	All	AACATGTCTGAATATATC	
BE1[b]	*B. elizabethae*	ATCTCATTACGCTTATCAAA	733–752
BE2[b,c]	*B. elizabethae*	TTTGATAAGCGTAATGAGAT	
BQ[b]	*B. quintana*	TTAGACAATGGAAGTAAA	625–641
BQ2[b]	*B. quintana*	TAAGCACTTAAAAGTGATGT	496–477
BH[b]	*B. henselae*	CTCATAGACGTCAATGCC	677–694
BH2[b]	*B. henselae*	GCACTTATGAAAGTAATGGA	476–461
BV[b]	*B. vinsonii*	AATGTCCATGATATATTT	637–654

[a] 16SF and 23S1 were used for PCR. The 16SF primer was chosen from a conserved region of the 3′ end of the gene encoding the 16S rRNA of *Bartonella* species, and the 23S1 primer on the antisense strand of the gene coding for 23S rRNA was determined after alignment of most of the 23S rRNA gene sequences present in the GenBank and definition of a consensus sequence.

[b] Primer was fluorescein labeled and used to sequence the PCR amplification product.

[c] Primer located on the complementary strand of DNA at the same position as the preceding primer.

B. henselae by PCR-hybridization (3). Matar et al. showed by RFLP of the PCR amplification product of the intergenic spacer region (ITS) between the 16 and 23S rRNA genes that it was possible to obtain intraspecies identification for *B. henselae* (16). We recently sequenced the ITSs of the four reference species of *Bartonella*, and our results were discrepant with the published data of Matar et al. for DNA fragment size and restriction patterns (28). The purpose of this work was the study of several *B. quintana* and *B. henselae* isolates by pulsed-field gel electrophoresis (PFGE) and sequencing of the 16S-23S rRNA gene ITS, as was done previously for other bacteria (5, 10, 12, 21, 34), in order to obtain intraspecies characterization.

MATERIALS AND METHODS

Bacteria and culture conditions. Five isolates were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga.: *B. henselae* Houston 1 (23), *B. henselae* San Ant-2 (SA2) (8), *B. henselae* CAL-1, *B. quintana* Oklahoma, and *B. quintana* SH-PERM. One isolate, *B. henselae* 90-615 (42), was obtained from D. F. Welch (University of Oklahoma, Oklahoma City). Isolates *B. quintana* Fuller (ATCC VR-358) and *B. vinsonii* ATCC VR-152 were a gift from G. A. Dasch (Naval Medical Institute, Bethesda, Md.). Four isolates of *B. quintana* were obtained from specimens cultured in our laboratory (Toulouse [17], Marseille [22], and Paris and Grenoble [9]). *B. elizabethae* F 9251 (ATCC 49927) and *B. bacilliformis* ATCC 35685 were obtained from the American Type Culture Collection. *B. bacilliformis* was cultivated by cell culture at 32°C. The other *Bartonella* isolates were cultivated on blood agar (BioMérieux, Marcy l'Etoile, France) at 37°C in a 5% carbon dioxide incubator. Bacteria were harvested and suspended in sterile water after 3 to 5 days of culture.

Nucleic acid preparation for PCR. A 450-μl sample of each bacterial suspension was mixed with 150 μl of a 20% Chelex suspension (Bio-Rad, Ivry sur Seine, France) in an Eppendorf tube, and the mixture was boiled for 30 min and centrifuged (12,000 × *g* for 10 min). The supernatant was transferred to a new Eppendorf tube and kept at 4°C until amplification.

Sequencing primers. The primers used for PCR were 16SF and 23S1. The following fluorescein-labeled primers were used to sequence the ITS PCR amplification product: 16SF, 23S1, QHVE1, QHVE2, QHVE3, QHVE4, BE1, BE2, BQ, BQ2, BH, BH2, and BV. All of the primers are described in Table 1.

ITS PCR amplification and purification of the PCR product. For ITS PCR amplification, one primer was labeled at the 5′ end with biotin during oligonucleotide synthesis (Eurogentec, Seraing, Belgium) and the 5′ end of the other primer was not modified. Ten microliters of the DNA preparation was amplified in a 100-μl reaction mixture containing 10 pmol of each primer, 200 μM each dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 1.25 U of Ampli *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 6 μl of a 25 mM solution of $MgCl_2$ (Perkin-Elmer Cetus) in 1× *Taq* buffer (Perkin-Elmer Cetus). Amplification was carried out in a DNA thermal cycler (PREM III; Lep Scientific, Flobio, Courbevoie, France) under the following conditions. An initial 5-min denaturation step at 95°C was followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 52°C, and extension for 2 min at 72°C. The amplification was finished by 10 min at 72°C to allow complete extension of the PCR products. The positivity of the amplification was confirmed by electrophoresis in a 1% agarose gel. The sizes of the ITS PCR amplification products were determined by comparison with the molecular weight standard marker VI (Boehringer).

PCR products were purified by using Dynabead M-280 streptavidin-coated magnetic beads (Dynal Inc., Great Neck, N.Y.). Twenty microliters of beads was used for 40 μl of amplified DNA product. The beads were washed three times in 1× BW buffer (5 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 1 M NaCl) and resuspended in 40 μl of 2× BW buffer. Then the beads and the DNA were mixed in a 1.5-ml Eppendorf tube and incubated for 30 min with mixing at ambient temperature. The beads and bound DNA were sequestered with a Dynal MPC magnet, the supernatant was discarded, and the beads were washed twice with 1× BW buffer. The bound DNA was denatured by adding 8 μl of 0.1 N NaOH. After 5 min of incubation at 37°C, the supernatant containing the complementary DNA strand was removed and neutralized with 4 μl of 0.2 N HCl and 1 μl of 1 M Tris-HCl, pH 7.4. The DNA strand left bound to the beads was washed once with 50 μl of NaOH (0.1 N), once with 40 μl of 1× BW buffer, and once with 50 μl of water and suspended in 10 μl of distilled water.

Sequencing method. The Autoread sequencing kit (Pharmacia, Saint-Quentin Yveline, France) was used to carry out the sequencing reaction. Ten microliters of the bead suspension or eluted DNA strand solution was used for each sequencing reaction, and the fluorescent primers were labeled at the 5′ end with fluorescein isothiocyanate (Eurogentec). In a 1.5-ml Eppendorf tube, the following reagents were mixed: 10 μl of template DNA, 2 μl of the selected fluorescent primer (3 pmol/μl), and 2 μl of annealing buffer. The tube was incubated at 68°C for 10 min and then left at ambient temperature for 10 min. One microliter of extension buffer, 3 μl of dimethyl sulfoxide, and 2 μl of T7 DNA polymerase (2.5 U/μl) were added to the tube, which had been held at 37°C, and immediately 4.5 μl of this mixture was pipetted into each of four prewarmed sequencing mixes. Incubation was continued for 5 min, and 5 μl of stop solution was added to each reaction mixture.

Sequencing reactions were resolved on 6% polyacrylamide gels (Ready Mix Gel, ALF grade; Pharmacia), and electrophoresis was performed with an ALF DNA Sequencer (Pharmacia) and 1× TBE buffer, pH 8 (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA).

PCR-RFLP of the *Bartonella* species ITS. Unmodified primers 16SF and 23S1 were used to amplify DNA. Digestion was performed according to the manufacturer's instructions for 2 h by mixing 23.5 μl of PCR amplification product, 2.8 μl of 10× enzyme buffer, and 2 μl of endonuclease *Taq*I or *Hin*dIII (Boehringer). Electrophoresis was performed with the Mupid-2 minigel electrophoresis system

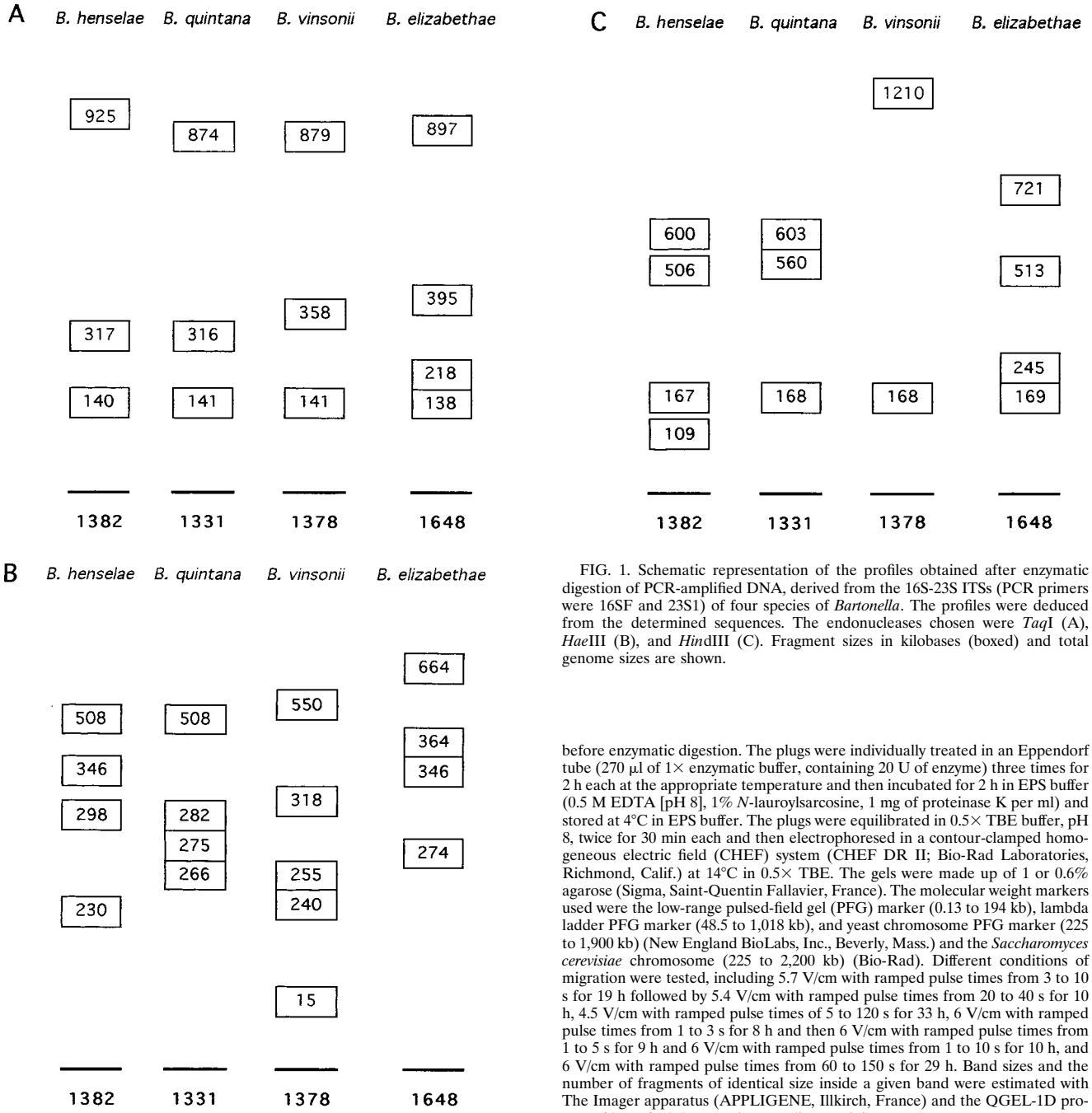

FIG. 1. Schematic representation of the profiles obtained after enzymatic digestion of PCR-amplified DNA, derived from the 16S-23S ITSs (PCR primers were 16SF and 23S1) of four species of *Bartonella*. The profiles were deduced from the determined sequences. The endonucleases chosen were *Taq*I (A), *Hae*III (B), and *Hin*dIII (C). Fragment sizes in kilobases (boxed) and total genome sizes are shown.

(Eurogentec) using a 1.5% agarose gel with 0.5× TBE buffer for 30 min at 100 V.

Determination of the endonuclease sites on the sequences. The BISANCE software package (7) was used to determine endonuclease sites.

PFGE. *Bartonella* cells were harvested and suspended in TNE buffer (10 mM Tris [pH 8], 150 mM NaCl, 2 mM EDTA). To prevent shearing of high-molecular-weight DNA, the bacterial suspension was added to an equal volume of 1% Incert agarose (FMC Bioproducts, Rockland, Maine), and plugs were made in an appropriate mold apparatus (Bio-Rad). After solidification at 4°C, the blocks were transferred to a lysis buffer (TNE buffer supplemented with 1% sodium dodecyl sulfate, proteinase K [1 mg/ml], and 0.25% Triton X-100) and incubated for 24 h twice at 50°C. Proteinase K was inactivated by two incubations (1 h each) in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.6) supplemented with 0.04 mg of phenylmethylsulfonyl fluoride per ml at 50°C. The plugs were then washed in TE buffer and stored in 0.2 M EDTA at 4°C or equilibrated in TE buffer before enzymatic digestion. The plugs were individually treated in an Eppendorf tube (270 μl of 1× enzymatic buffer, containing 20 U of enzyme) three times for 2 h each at the appropriate temperature and then incubated for 2 h in EPS buffer (0.5 M EDTA [pH 8], 1% *N*-lauroylsarcosine, 1 mg of proteinase K per ml) and stored at 4°C in EPS buffer. The plugs were equilibrated in 0.5× TBE buffer, pH 8, twice for 30 min each and then electrophoresed in a contour-clamped homogeneous electric field (CHEF) system (CHEF DR II; Bio-Rad Laboratories, Richmond, Calif.) at 14°C in 0.5× TBE. The gels were made up of 1 or 0.6% agarose (Sigma, Saint-Quentin Fallavier, France). The molecular weight markers used were the low-range pulsed-field gel (PFG) marker (0.13 to 194 kb), lambda ladder PFG marker (48.5 to 1,018 kb), and yeast chromosome PFG marker (225 to 1,900 kb) (New England BioLabs, Inc., Beverly, Mass.) and the *Saccharomyces cerevisiae* chromosome (225 to 2,200 kb) (Bio-Rad). Different conditions of migration were tested, including 5.7 V/cm with ramped pulse times from 3 to 10 s for 19 h followed by 5.4 V/cm with ramped pulse times from 20 to 40 s for 10 h, 4.5 V/cm with ramped pulse times of 5 to 120 s for 33 h, 6 V/cm with ramped pulse times from 1 to 3 s for 8 h and then 6 V/cm with ramped pulse times from 1 to 5 s for 9 h and 6 V/cm with ramped pulse times from 1 to 10 s for 10 h, and 6 V/cm with ramped pulse times from 60 to 150 s for 29 h. Band sizes and the number of fragments of identical size inside a given band were estimated with The Imager apparatus (APPLIGENE, Illkirch, France) and the QGEL-1D program (Quantigel Corporation, Madison, Wis.).

Numerical analysis. A dendrogram of the genetic relationships between the different isolates of *B. quintana* was established from the restriction profiles obtained after the three enzymatic DNA digestions and PFGE. We determined the comigrating bands for each pair of *B. quintana* isolates. DNA divergence between the genomes of *B. quintana* isolates was estimated by using the Dice coefficient, and the dendrogram was made by using UPGMA (unweighted pair-group method of arithmetic averages) and Package Philip commercial computer programming.

Nucleotide sequence accession number. The ITS sequences of the Fuller and Houston isolates have been submitted to GenBank under accession no. L35100 and L35102, respectively.

RESULTS

Comparison of the ITS sequences of several isolates of the same species. The sizes of the ITS PCR products were 1.382,

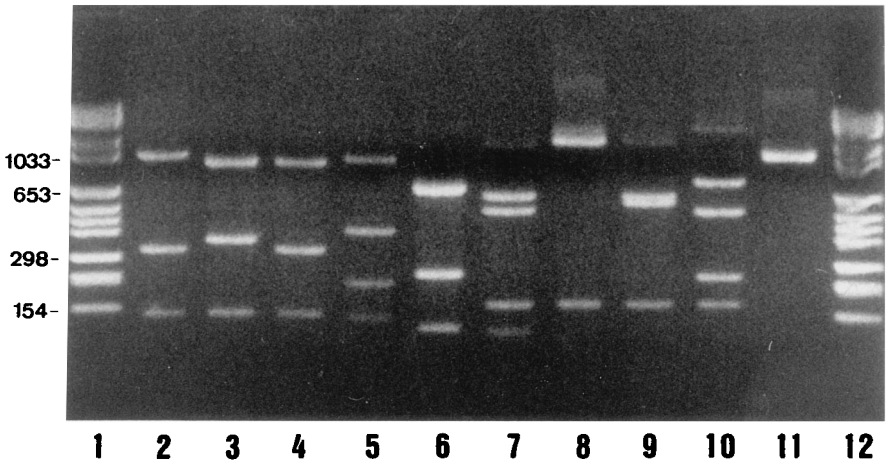

FIG. 2. Ethidium bromide-stained agarose gel of PCR-RFLP patterns of *Bartonella* species. DNAs were amplified by using 16SF and 23S1 primers and digested by *Taq*I (lane 2, *B. henselae* Houston; lane 3, *B. vinsonii*; lane 4, *B. quintana* Fuller; lane 5, *B. elizabethae*; lane 6, *B. bacilliformis*) and *Hin*dIII (lane 7, *B. henselae* Houston; lane 8, *B. vinsonii*; lane 9, *B. quintana* Fuller; lane 10, *B. elizabethae*; lane 11, *B. bacilliformis*). Lanes 1 and 12 contain DNA molecular size marker VI (sizes shown in base pairs). Migration was performed in 0.5× TBE at 100 V for 30 min.

1.331, 1.378, and 1.648 kb for *B. henselae*, *B. quintana*, *B. vinsonii*, and *B. elizabethae*, respectively. Amplified ITS PCR products for each isolate of the same species were estimated to be of the same size after migration in a 1% agarose gel. After sequencing, we compared seven isolates of the species *B. quintana* and found two subgroups: one including the Fuller and Toulouse isolates and the other including the Oklahoma, SH-PERM, Paris, Marseille, and Grenoble isolates. The differences between the two subgroups were an insertion of thymine between positions 176 and 177 and mutations at positions 860, 940, 959, and 1004 (C to G [C/G], C/G, A/G, and G/A, respectively) for isolates belonging to the Oklahoma subgroup. Four isolates of *B. henselae* were available in our laboratory, and each of them had a different sequence. In comparison with the Houston isolate, the 90-615 isolate had mutations at positions 28, 39, 348, and 910 (G/A, G/A, C/G, and A/G, respectively); for the SA2 isolate, we found the same mutations but also the insertion of a 30-bp fragment of DNA between positions 517 and 518, and for the CAL-1 isolate we found mutations at positions 348, 778, 989, and 1233 (C/G, C/T, A/G, G/A, and G/A, respectively).

Determination of endonucleases which gave suitable profiles in PCR-RFLP of ITSs. We found three enzymes which gave a characteristic pattern for each of the *Bartonella* species studied: *Hin*dIII, *Hae*III, and *Taq*I (Fig. 1). These patterns were found for each of the isolates of *B. quintana* and *B. henselae* except for *B. henselae* SA2, for which the results were a little different because of the insertion of 30 bp between bases 517 and 518 of the ITS. We digested DNAs of the five *Bartonella* species with *Taq*I and *Hin*dIII and showed that it was possible to obtain the expected profiles (Fig. 2).

PFGE profiles. Endonucleases possessing 8-base recognition sequences (*Not*I) or 6-base sequences that may be rare in bacterial genomes having GC contents of 39 to 40% (*Sma*I, *Bss*HII, *Sal*I, *Mlu*I, and *Eag*I) were tested. *Bss*HII, *Sal*I, and *Mlu*I generated large numbers of small fragments (50 kb or less) that could not be resolved, while *Not*I, *Sma*I, and *Eag*I were found to be suitable for the analysis of *Bartonella* DNA. We used the DNA preparation method as modified by Schwartz and Cantor (29), which preserved the integrity of DNA with a view to subsequent investigations. However, as usual, a small part of this big molecule broke in one point and migrated like a linear fragment under the appropriate conditions. The conditions we used were a 0.6% agarose gel, pulse times of 60 to 150 s, and 6 V/cm for 29 h. We estimated the average sizes of the four *Bartonella* species to be 2.1 megabases for *B. vinsonii* and *B. elizabethae*, 2 Mb for *B. henselae*, and 1.7 megabases for *B. quintana*. By addition of the sizes of all the fragments generated after digestion by *Eag*I, *Sma*I, and *Not*I, we confirmed the mean sizes of the four *Bartonella* species to be 2.082, 2.174, 2.005, and 1.700 Mb for *B. vinsonii*, *B. elizabethae*, *B. henselae*, and *B. quintana*, respectively. *Not*I digestion resulted in three to five fragments, so it was easy to estimate their sizes and total them (Fig. 3).

After digestion with *Eag*I, 28 to 38 fragments were obtained, with molecular sizes between 208 and 4 kb, depending on the isolate (Fig. 4). After digestion with *Sma*I, 12 to 34 fragments were obtained, with molecular sizes ranging from 1,265 to 5.5 kb (Fig. 5). No digestion of the genome of *B. vinsonii* with *Sma*I could be demonstrated. With *B. quintana*, we obtained a

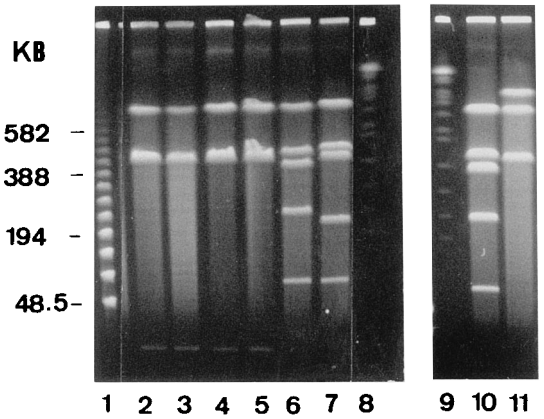

FIG. 3. PFGE of *Not*I restriction fragments of *Bartonella* DNAs. Lane 1, lambda ladder PFG marker; lane 2, Fuller strain; lane 3, Toulouse; lane 4, Oklahoma; lane 5, SH-PERM; lane 6, Houston; lane 7, SA2; lanes 8 and 9, yeast chromosome PFG marker; lane 10, Houston strain; lane 11, *B. vinsonii*. Migration was performed in 0.5× TBE at 14°C at 4.5 V/cm for 33 h with ramped pulse times from 5 to 120 s.

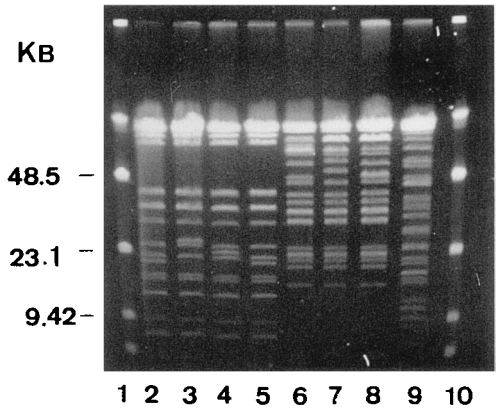

FIG. 4. PFGE of *Eag*I restriction fragments of *Bartonella* DNAs. Lanes 1 and 10, low-range PFG marker; lane 2, Fuller strain; lane 3, Toulouse isolate; lane 4, Oklahoma; lane 5, SH-PERM; lane 6, Houston; lane 7, 90-615; lane 8, SA2; lane 9, *B. vinsonii*. Migration was performed in 0.5× TBE at 14°C at 6 V/cm for 8 h with ramped pulse times from 1 to 3 s and then at 6 V/cm for 9 h with ramped pulse times from 1 to 5 s.

common PFGE profile for the seven isolates after digestion with *Not*I, but with *B. henselae* two different profiles were found, SA2 and 90-615 having the same pattern. After digestion of DNA with *Eag*I, we found a specific pattern for each isolate of both *B. quintana* and *B. henselae*. After digestion with *Sma*I, the Oklahoma and SH-PERM isolates exhibited the same profile and the Marseille and Paris patterns were similar. The other isolates had unique profiles. For *B. vinsonii* and *B. elizabethae*, it was impossible to demonstrate genome polymorphism because only one isolate was available. We were not able to digest the DNA of *B. henselae* CAL-1 in PFGE plugs.

Numerical analysis. A graphic representation of the numerical analysis of the *B. quintana* isolates is presented in Fig. 6. The Toulouse and Fuller isolates are grouped together, as are the Oklahoma and SH-PERM isolates and the Paris and Marseille isolates.

DISCUSSION

B. quintana has long been known as the agent of trench fever. The human body louse was the vector for *B. quintana* infection, and trench fever was rarely recognized in nonepidemic situations. In 1986, the first North American isolate of *B. quintana* was taken from the blood of a septicemic human immunodeficiency virus-infected man living in Oklahoma (42). Subsequently, this bacterium was associated with various clinical syndromes, including bacillary angiomatosis, hepatitis peliosis, endocarditis, meningitis, chronic adenopathy, and neurological disorders in immunocompetent and/or immunosuppressed individuals (9, 13, 17, 22, 42). In 1990, a novel *Bartonella* species (*B. henselae*) was isolated from the blood of a febrile human immunodeficiency virus-positive patient and on the basis of genotypic criteria was found to be related to but differentiated from members of the genus *Bartonella* (23). Since then, many isolates of *B. henselae* have been found in North America, implicated in the same clinical syndromes as described above for *B. quintana* and in cat scratch disease.

Cats have been established as reservoirs and probable vectors of *B. henselae*, but the reservoirs of contemporary *B. quintana* are not known. In endocarditis in homeless patients, ectoparasites such as lice could be implicated (9). In some cases, cats have been implicated because patients were bitten or scratched or because the animals had abundant fleas (31).

Differences in geographical distribution, clinical pathologies, isolations, and serological responses which might be correlated with phenotypic or genotypic characteristics of the organisms have been reported. A previous study of several isolates of *B. quintana* and *B. henselae* showed differences in cellular fatty acid composition (42), and subtyping of *B. henselae* isolates by PCR-RFLP of a fragment of the ribosomal operon has been described (16). The latter study has, however, recently been refuted (28). Matar et al. studied 10 isolates of *B. henselae* by PCR-RFLP of ITSs and found six and four different restriction patterns after digestion with *Alu*I and *Hae*III, respectively. However, we found differences in restriction sites, and in fact, by using PCR-RFLP it was only possible to obtain specific profiles for all the species. We determined the endonuclease sites on the ITSs sequenced by Minnick et al. (18) and by ourselves and found that three enzymes gave a convenient number of DNA fragments to establish identification of *Bartonella* species: *Taq*I and particularly *Hin*dIII and *Hae*III. This identification method is very convenient because it takes only 30 min in an agarose gel instead of the 4 h required for a polyacrylamide gel. After sequencing different isolates of the

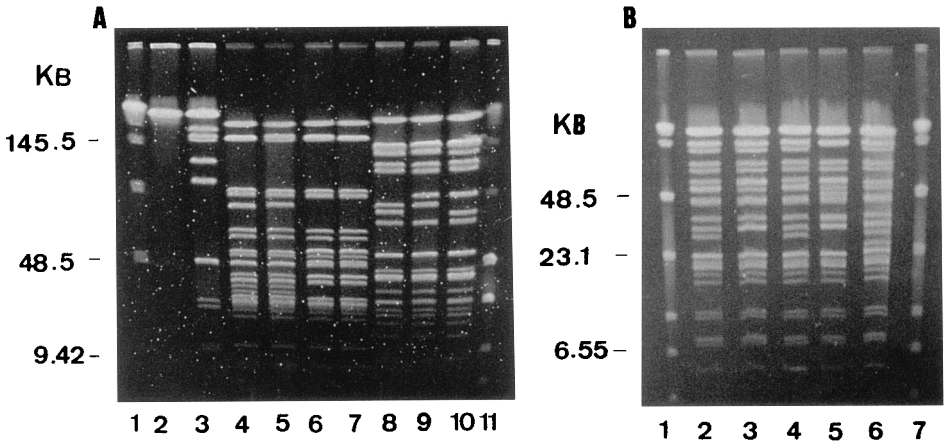

FIG. 5. PFGE of *Sma*I restriction fragments of *Bartonella* DNAs. (A) Lane 1, lambda ladder PFG marker; lane 2, *B. vinsonii*; lane 3, *B. elizabethae*; lane 4, Fuller strain; lane 5, Toulouse; lane 6, Oklahoma; lane 7, SH-PERM; lane 8, Houston; lane 9, SA2; lane 10, 90-615; lane 11, low-range PFG marker. Migration was performed in 0.5× TBE at 14°C at 5.7 V/cm for 19 h from 3 to 10 s. (B) Lanes 1 and 7, low-range PFG marker; lane 2, Marseille isolate; lane 3, Grenoble isolate; lane 4, Paris isolate; lane 5, Oklahoma isolate; lane 6, Fuller strain. Migration was performed in 0.5× TBE at 14°C at 6 V/cm for 8 h with ramped pulse times from 1 to 3 s and then at 6 V/cm for 9 h with ramped pulse times from 1 to 5 s.

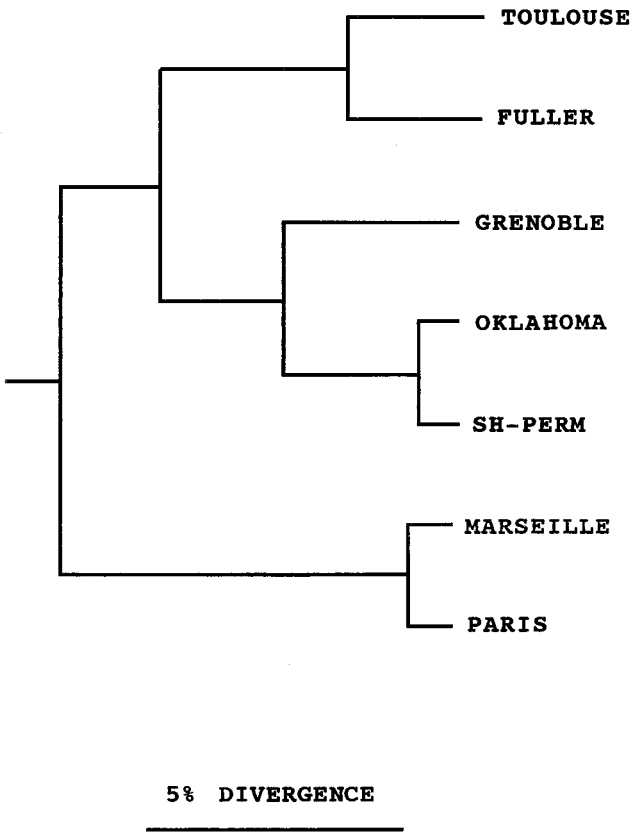

FIG. 6. Genetic relationship dendrogram of different *B. quintana* isolates established from PFGE patterns obtained after *Sma*I, *Eag*I, and *Not*I digestion by using the Dice coefficient and UPGMA and Package Philip programming.

same species, we found the percentage of mutation in this part of the DNA to be low (0.6% for *B. quintana* isolates and 0.5 to 4% for the isolates of *B. henselae*) and to usually not affect restriction sites. Consequently, PCR-RFLP of the 16S-23S ITS is probably not useful in epidemiological studies.

When intact DNAs from isolates of *Bartonella* species were subjected to PFGE after digestion with restriction endonucleases, the mean calculated genome size was between 1,700 and 2,174 kb, depending on the species. The values found for *B. quintana* and *B. vinsonii* agree with those published by Reschke et al. of 1,700 and 2,100 kb, respectively (26), calculated from the fragments obtained after digestion of the total DNA by *Sfi*I and *Not*I and PFGE and are slightly higher than the values determined by the renaturation rate method of Gillis et al. (1,500 and 2,000 kb for *B. quintana* and *B. vinsonii*, respectively) (19, 20). The genome sizes that we found for the three *B. henselae* isolates were similar, while the genome sizes of the seven *B. quintana* isolates were identical. However, comparison of the profiles of different isolates of one species obtained after enzymatic digestion and PFGE revealed many differences, showing that this method is adequate for intraspecies differentiation, as has been established for other bacteria including *Coxiella burnetii* (34), *Staphylococcus aureus* (21), *Leptospira* spp. (12), and *Listeria monocytogenes* (5). PFGE is more discriminatory than ITS sequencing for studying *Bartonella* isolates. The limitations of PFGE relate to the impossibility of obtaining a profile for one isolate of *B. henselae*, apparently because of nondigestion of the bacterial membranes or because of a precipitate which obstructs the pores of the plugs, preventing enzymatic digestion. We found an important heterogeneity in the profiles of the four *Bartonella* species, allowing characterization of each of them, but the intraspecies differences were small and the general patterns for *B. quintana* and *B. henselae* are conserved. *Not*I is not a good tool for investigating the intraspecies genetic diversity of *Bartonella* spp., because we obtained few fragments and noted no difference between *B. quintana* isolates and little difference in *B. henselae* patterns. *Eag*I and *Sma*I are very efficient in discriminating isolates; specific profiles were obtained for each isolate with *Eag*I, and with *Sma*I only the SH-PERM and Oklahoma isolates and the Paris and Marseille isolates were identical. For *B. quintana* isolates, we used the same numerical analysis previously reported for spotted fever group rickettsia species (27). It is interesting that isolates Fuller and Toulouse are the closest among the *B. quintana* species. The former was isolated 50 years ago in Yugoslavia, and the latter was isolated in 1992 in France (17). On the other hand, isolate Oklahoma was isolated in the United States and SH-PERM was isolated in Russia. Our phylogenetic study as established from PFGE profiles does not appear to correlate with geographical distribution. Similarly, there are no apparent correlations between clinical data for people infected with *B. quintana* and genotypic characterization of the organism. For example, the Fuller isolate was from a patient suffering from trench fever, and the Toulouse isolate was from a patient with bacillary angiomatosis. Genetic variability in *Bartonella* spp. is important, as it allows the differentiation of isolates found in the same year in the same country and with the same clinical picture (Toulouse and Grenoble isolates). The genetic variability of *B. henselae* isolates was more pronounced than that of *B. quintana* isolates after PFGE and sequencing of the 16S-23S rRNA ITSs. However, this could be related to the bacteriophage-like particle reported to be in *B. henselae* (1), a transposable element promoting DNA rearrangements and genetic alteration in microorganisms.

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