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Rochalimaea elizabethae sp. nov. Isolated from a Patient with Endocarditis

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A *Rochalimaea*-like organism (strain F9251) was isolated from a patient with endocarditis after blood drawn for culture before antimicrobial therapy was subcultured onto blood and chocolate agars and incubated for 2 weeks in 5% CO_2. The strain was phenotypically similar to known *Rochalimaea* species. The cellular fatty acid composition of strain F9251 was close to but distinct from those of the three known *Rochalimaea* species and was most similar to that of *R. vinsonii*. Labeled DNA from strain F9251 was 59 to 67% related to DNAs from type strains of the three described *Rochalimaea* species, and its 16S rRNA gene sequence was 98.9% or more homologous to their 16S rRNA gene sequences. These findings support classification of F9251 as a new *Rochalimaea* species, for which the name *Rochalimaea elizabethae* sp. nov. is proposed. The patient infected with the organism had large bacterial vegetations on his aortic valve and was cured with antibiotics and valve-replacement surgery. Recognition of the procedures required to identify this and other *Rochalimaea* species suggests that clinical laboratories should prolong the incubation times of cultures of blood and tissue from patients with suspected endocarditis, patients with fever of unknown origin, and immunocompromised patients with fever so that the full spectrum of disease caused by these organisms can be recognized.

Three *Rochalimaea* species (family *Rickettsiaceae*) have been described. *R. quintana*, the type species and causative agent of trench fever, was originally termed "*Rickettsia quintana*" by Schmincke in 1917 (35), but the organism was transferred to the genus *Rochalimaea* by Krieg (20) in 1961. The second species, originally referred to as the Canadian vole agent (2), was formally described in 1982 and was named *R. vinsonii* (50). *R. vinsonii* has been isolated only once, from voles on Grosse Isle, Quebec, Canada (2, 51). The third *Rochalimaea* species, *R. henselae* (28, 52), was first isolated by Slater, Welch, and coworkers (37, 52) and was first detected by Relman and colleagues (32) in tissue samples from patients with bacillary angiomatosis. This organism has been isolated from the blood of patients infected with the human immunodeficiency virus (HIV), transplant recipients, immunocompetent patients with bacteremia, and a patient with peliosis hepatis (52). Relman et al. (33) demonstrated that the rRNA sequences of isolates detected in the blood and organisms detected in the tissues of patients with bacillary angiomatosis represented the same species. By direct cultivation of cutaneous and osseous biopsy specimens with eukaryotic tissue culture endothelial cells, Koehler and coworkers (18) have isolated *R. henselae* from one patient and *R. quintana* from three patients with bacillary angiomatosis, suggesting an etiologic role for both species. Organisms were also recovered from the blood of these patients, but no cases of endocarditis caused by *Rochalimaea* species have been reported.

In 1986, we isolated an unidentifiable, fastidious organism from a patient with endocarditis. The organism was reexamined in 1992 after the microbiological and molecular characteristics of *R. henselae* were reported (28, 52) and was found to be a fourth species of *Rochalimaea*. We describe the patient with endocarditis from whom this organism was isolated and propose designation of the bacillus as a new species, *Rochalimaea elizabethae* sp. nov.

MATERIALS AND METHODS

Strains. *R. quintana* type strain ATCC VR-358 and *R. vinsonii* type strain ATCC VR-152, which were obtained from the American Type Culture Collection, were provided by Ted Tzianabos, Viral and Rickettsial Zoonoses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC). *R. henselae* type strain ATCC 49882 (Houston 1) was sent to CDC for identification by the Veterans Administration Hospital in Houston, Tex., and was obtained from R. L. Regnery, Viral and Rickettsial Zoonoses Branch, CDC. *R. henselae* 87-66, 88-712, 89-674, and 90-615 were obtained from David F. Welch, University of Oklahoma Health Sciences Center, Oklahoma City, and have been described previously (52). The type strain of *Bartonella bacilliformis*, ATCC 35685, has also been described (7). Strain F9251 (=B-91-002005 = ATCC 49927) was sent to CDC by the Massachusetts State Laboratory Institute, which received it from Saint Elizabeth's Hospital in Brighton, Mass.

Primary isolation. Blood obtained from the patient before the start of antimicrobial therapy was inoculated into BACTEC 460 blood culture medium (Johnson Laboratories, Towson, Md.). One bottle was vented for recovery of aerobes, and one was kept closed to allow anaerobic growth.

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The bottles were incubated at 35°C for 3 weeks and were then stored at room temperature. When the cultures had not yielded any visible growth at 1 week, subcultures (0.1 ml) from an aerobic bottle were made to tryptic soy agar (TSA) supplemented with 5% sheep blood (sheep blood-TSA; BBL Microbiology Systems, Cockeysville, Md.) and chocolate agar (BBL), as well as to sheep blood-TSA streaked with *Staphylococcus aureus*. Incubation was done at 35°C in 5% CO_2.

After 3 weeks, samples from both the aerobic and anaerobic bottles were subcultured on chocolate agar, sheep blood-TSA, Campy BAP plate (BBL), Thayer-Martin plate (BBL), and brain heart infusion agar with 5% sheep blood (BBL). The plates were incubated at 35°C in air, in 5 to 10% CO_2 (Campy pouch bags; BBL), anaerobically (GasPak pouch; BBL), and at 42°C in 5 to 10% CO_2. Once isolated, *Rochalimaea*-like strain F9251 was cultivated on chocolate agar for phenotypic tests. The organism was stored at −70°C in semisolid heart infusion agar (HIA) or in defibrinated whole rabbit blood.

Samples of blood drawn after the antimicrobial therapy was begun were inoculated into BACTEC 660-NR 6A, 7A, and 16B (resin) bottles (Becton Dickinson and Co., Paramus, N.J.) and were processed with the BACTEC 660-NR system. At the time of the patient's valve-replacement surgery, tissue from his aortic valve was ground and inoculated onto standard media, including thioglycolate and chopped meat broth. The cultures were maintained for 21 days.

Biochemical tests. For the initial biochemical tests we used standard methods (21). Preformed enzymes were tested by using the RapID ANA II system according to the manufacturer's directions (Innovative Diagnostic Systems, Inc., Atlanta, Ga.). Gram staining and biochemical tests were done as described previously (10). The strains were stored in defibrinated rabbit blood in a vapor-phase liquid nitrogen freezer. Inocula for biochemical evaluations were obtained by growing strains on HIA supplemented with 5% rabbit blood (rabbit blood-HIA; BBL) or on chocolate agar for 2 to 3 days at 35°C in candle extinction jars. Cells suspended in heart infusion broth were observed for motility with an oil immersion (×1,000) objective.

Antibiotic susceptibility. Antibiotic susceptibility was determined by use of the disk method of Bauer and associates (3). Colonies from a chocolate agar plate incubated for 96 h were suspended in Mueller-Hinton broth (BBL) and were used to inoculate a chocolate agar plate (BBL). Standard antibiotic-containing disks (BBL) were dropped on the plates, which were incubated for 5 days in 5% CO_2. The clear zone around each disk was measured to the nearest millimeter.

Cellular fatty acids (CFAs). Cells for fatty acid analysis were grown on rabbit blood-HIA at 35°C for 2 to 5 days. The cells were saponified, and the liberated fatty acids were methylated and analyzed by capillary gas-liquid chromatography (23). Identification of fatty acids and determination of the locations of double bond positions in monounsaturated acids were accomplished by gas-liquid chromatography and gas-liquid chromatography-mass spectrometry (23).

DNA methods. To obtain cells for DNA extraction, strains were grown on rabbit blood-HIA at 35°C for 3 to 4 days. The DNA extraction and purification procedures and the hydroxyapatite hybridization method have been described previously (6). Divergence in related sequences was estimated to be approximately 1% for each degree of decreased thermal stability in a heterologous reassociated DNA duplex compared with that in the homologous reassociated DNA duplex (6). Divergence was calculated to the nearest 0.5%. DNA was labeled enzymatically in vitro with [α-^{32}P]dCTP by use of a nick translation reagent kit (GIBCO BRL, Gaithersburg, Md.). The guanine-plus-cytosine (GC) content of DNA was determined spectrophotometrically by the method of Marmur and Doty (22). The values obtained were normalized to a *Proteus mirabilis* control strain with a GC content of 40 mol%.

PCR amplification of rDNA. Template DNA for direct sequencing was produced by enzymatic amplification of 16S rDNA by using the primers fD1 and rD1 described by Weisburg and coworkers (48). The restriction enzyme linker region present in the original oligonucleotides was omitted, resulting in a primary structure for fD1 of 5′-AGAGTTT GATCCTGGCTCAG-3′ and a primary structure for rD1 of 5′-AAGGAGGTGATCCAGCC-3′. In addition, primer rD1 was labeled at the 5′ end with biotin during oligonucleotide synthesis with biotinylated phosphoramidites (Cambridge Research Biochemicals, Wilmington, Del.). Approximately 10 ng of bacterial genomic DNA was amplified in a 100-μl reaction containing 10 pmol of each primer, 200 μmol each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), and 2.5 U of AmpliTaq DNA polymerase (U.S. Biochemical, Cleveland, Ohio) in 1× GeneAmp polymerase chain reaction (PCR) buffer (Perkin-Elmer Corp., Norwalk, Conn.). Amplification was carried out in a Perkin-Elmer DNA thermal cycler under the following conditions: an initial 3-min DNA denaturation at 94°C was followed by 25 cycles consisting of 1 min at 94°C, 1 min at 45°C, and 1.5 min at 72°C. During the last cycle, the incubation at 72°C was increased to 5 min to allow for complete extension of PCR products.

PCR products were purified by using Dynabead M-280 streptavidin-coated magnetic beads (Dynal Inc., Great Neck, N.Y.) and a modification of the method of Hultman and associates (16). The beads were washed three times in saline buffer (1 M NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA) and were resuspended in this buffer at their original concentration of 10 mg/ml. A pool of five amplification reactions was added to 200 μl of beads, and the mixture was incubated at ambient temperature, with mixing, for 15 min. A neodymium-iron-boron permanent magnet was used to sequester the beads and bound DNA, the supernatant was discarded, and the beads were washed once with 250 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The bound DNA was denatured by adding 50 μl of 0.1 M NaOH. After 15 min of incubation, the supernatant containing the eluted DNA strand was removed, neutralized with 5 μl of 1 M HCl, and buffered to pH 7.5 by adding 5 μl of 1 M Tris-HCl; the total volume was adjusted to 75 μl with H_2O. The DNA strand left bound to the beads was washed once with 250 μl of NaOH and three times with TE and was suspended in 75 μl of water.

Sequencing method and data analyses. Seven microliters of bead suspension (possessing the DNA strand complementary to the 16S rRNA) or eluted DNA strand solution was used for each sequencing reaction. Reactions were accomplished with T7 DNA polymerase (Sequenase 2.0; U.S. Biochemical), incorporating [α-^{35}S]dATP as the label. The set of 10 oligonucleotides used as sequencing primers for the eluted strand has been described previously (39). Reverse complementary oligonucleotides to the 10 oligonucleotides were used to sequence the bound strand. Sequencing reactions were resolved on 8% polyacrylamide gels containing 7 M urea (Marathon Gel-Mix 8; GIBCO BRL). After electrophoresis, the gels were transferred directly to paper and

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dried under vacuum. Autoradiographs were allowed to expose for 2 to 7 days.

The 16S rRNA sequences of 16 species of the order *Rickettsiales* and *Anacystis nidulans* were aligned by using the multisequence alignment program PILEUP, which is part of the GCG software package (12). Previously published sequences (1, 26, 28, 39, 40, 48, 49) were obtained from the GenBank data base. The sequence alignment was edited to remove regions of alignment uncertainty, leaving 1,263 positions for calculation of evolutionary distance. Phylogenetic relationships were inferred with version 3.4 of the PHYLIP software package (15). A similarity matrix was calculated by the method described by Jukes and Cantor (17). The dendrogram was constructed by using the neighbor-joining method of Saitou and Nei (34).

Nucleotide sequence accession numbers. The sequence for *R. elizabethae* F9251 has been deposited in the GenBank, EMBL, and DDBJ data bases under accession number L01260. The sequence for *R. vinsonii* was submitted to the same data bases under accession number L01259.

RESULTS

Case report. A 31-year-old man presented with a 2-month history of fatigue, malaise, anorexia, and shortness of breath accompanied by a weight loss of 9 kg. He had sustained lacerations on the third and fourth fingers of his left hand 2 weeks before the onset of these symptoms. There was no history of intravenous drug use, exposure to pets or other animals, recent dental work, or significant medical conditions. In the patient's medical record, there was no mention of the circumstances leading to the lacerations. He was born in Puerto Rico.

On initial evaluation, the patient had an oral temperature of 39°C, a blood pressure of 105/40 mm Hg, multiple infected teeth, a 4/6 diastolic murmur of aortic regurgitation, an S3 cardiac gallop, and a splinter hemorrhage in the left index finger. A two-dimensional echocardiogram revealed large vegetations on the aortic valve and severe aortic regurgitation. A serum test for HIV antibody yielded negative results.

The patient became afebrile after 48 h of therapy with intravenous nafcillin and gentamicin, and he remained without fever throughout his hospitalization. A stool examination showed larvae of *Strongyloides stercoralis*, and the patient was treated with thiabendazole for 2 days. He developed increasing congestive heart failure, and a decision was made to replace his aortic valve surgically.

On the 16th hospital day, seven infected teeth were removed. On the 17th hospital day, because of concern about a prolonged bleeding time of greater than 15 min and the patient's imminent surgery, the antibiotic regimen was changed to vancomycin and imipenem. On the next day, the patient underwent aortic valve replacement. During the operation, he was found to have a tricuspid aortic valve with vegetations on all three cuspids (Fig. 1). Gram staining of the aortic tissue vegetation appeared to show abundant minute coccobacillary organisms.

The patient recovered from surgery and was discharged on the 24th postoperative day, after 6 weeks of intravenous antibiotic therapy. He was treated as an outpatient with oral trimethoprim-sulfamethoxazole for 5 additional weeks. There was no evidence of relapse or recurrence of endocarditis for the subsequent 2.5 years, after which he was lost to follow-up.

Primary isolation. Cultures of blood samples drawn before the patient received antimicrobial therapy showed no visible growth after 3 weeks of incubation. Subculture of a sample from the aerobic bottle done approximately 1 week after inoculation grew small, dry colonies that were adherent to the agar and that were noticed after 14 days of incubation on blood and chocolate agars at 35°C in 5 to 10% CO_2. Repeat subculture from both the aerobic and anaerobic bottles yielded a faint growth of dry, rough colonies after 10 days, confirming the original isolation of strain F9251, which was subsequently recognized as a *Rochalimaea*-like organism.

Cultures of blood obtained 2 days after the start of therapy with nafcillin and gentamicin were negative. Cultures of the tissue from the aortic valve grew catalase-positive anaerobic diphtheroids in broth medium after 2 weeks of incubation. These organisms were thought to be contaminants and were not further identified.

Strain F9251 grew best on chocolate or sheep blood-TSA at 35°C in 5 to 10% CO_2. It did not grow aerobically at 42°C, anaerobically, in stationary broth media, on Campy BAP plates, or on Thayer-Martin plates containing antibiotics. After multiple passages, the growth rate increased and colonies that were less adherent to the agar than the initial isolate could be seen after 48 to 72 h of incubation.

Phenotypic characteristics. Gram staining revealed thin, short, straight and slightly curved gram-negative bacilli (Fig. 2). Catalase, oxidase, and spot indole tests were negative. On initial isolation, the organism could not be identified as a known species. It was subsequently sent to the Massachusetts State Laboratory Institute and CDC. Neither of those laboratories could identify the organism.

However, after a *Rochalimaea* species was isolated in a patient with bacillary angiomatosis and bacteremia and the phenotypic characteristics of *R. henselae* were described (28, 37, 52), strain F9251 was recognized as being *Rochalimaea*-like and was reexamined. In the RapID ANA II system, *Rochalimaea*-like strain F9251 cleaved leucylglycine, glycine, proline, phenylalanine, arginine, and serine, yielding the code number 000671, which is identical to that obtained for *R. henselae* (28).

The phenotypic characteristics of strain F9251 were compared with those of known *Rochalimaea* species (Table 1). There were few positive characteristics other than the hydrolysis of the same six substrates. The oxidase reaction, when tested by the Kovacs procedure, was weakly positive at 9 s for strains of *R. quintana* and *R. vinsonii* but was negative for three strains of *R. henselae* and for strain F9251. Strain F9251, the type strains of *R. quintana* and *R. vinsonii*, and one of three *R. henselae* strains grew within 72 h on HIA around an X (hemin) strip. All the *Rochalimaea* strains grew on HIA supplemented with rabbit blood. F9251 produced a zone of incomplete clearing (not greening) on rabbit blood-HIA after incubation for 3 to 4 days.

Antibiotic susceptibility. Strain F9251 was susceptible to all antibiotics tested (erythromycin, ciprofloxacin, cefoxitin, gentamicin, vancomycin, and oxacillin).

Cell wall fatty acids. The CFA compositions of each *Rochalimaea* species and *Rochalimaea*-like strain F9251 were characterized by using large amounts of *cis*-11-octadecanoate ($C_{18:1\omega7c}$), hexadecanoate ($C_{16:0}$), and octadecanoate ($C_{18:0}$), which accounted for 64 to 97% of total acids (Table 2). In addition, all contained $C_{17:0}$ (1 to 21%) and trace to small amounts of $C_{16:1\omega7c}$, $C_{17:1\omega6c}$, $C_{18:2}$, and $C_{18:1\omega9c}$. No hydroxy or cyclopropane acids were present.

GC content and DNA relatedness. Spectrophotometric assessments showed that the GC content of DNA from strain F9251 was 41.0 mol%; those of the type strains of *Rochalimaea* species were 40.3 mol% for *R. quintana*, 41.1 mol%

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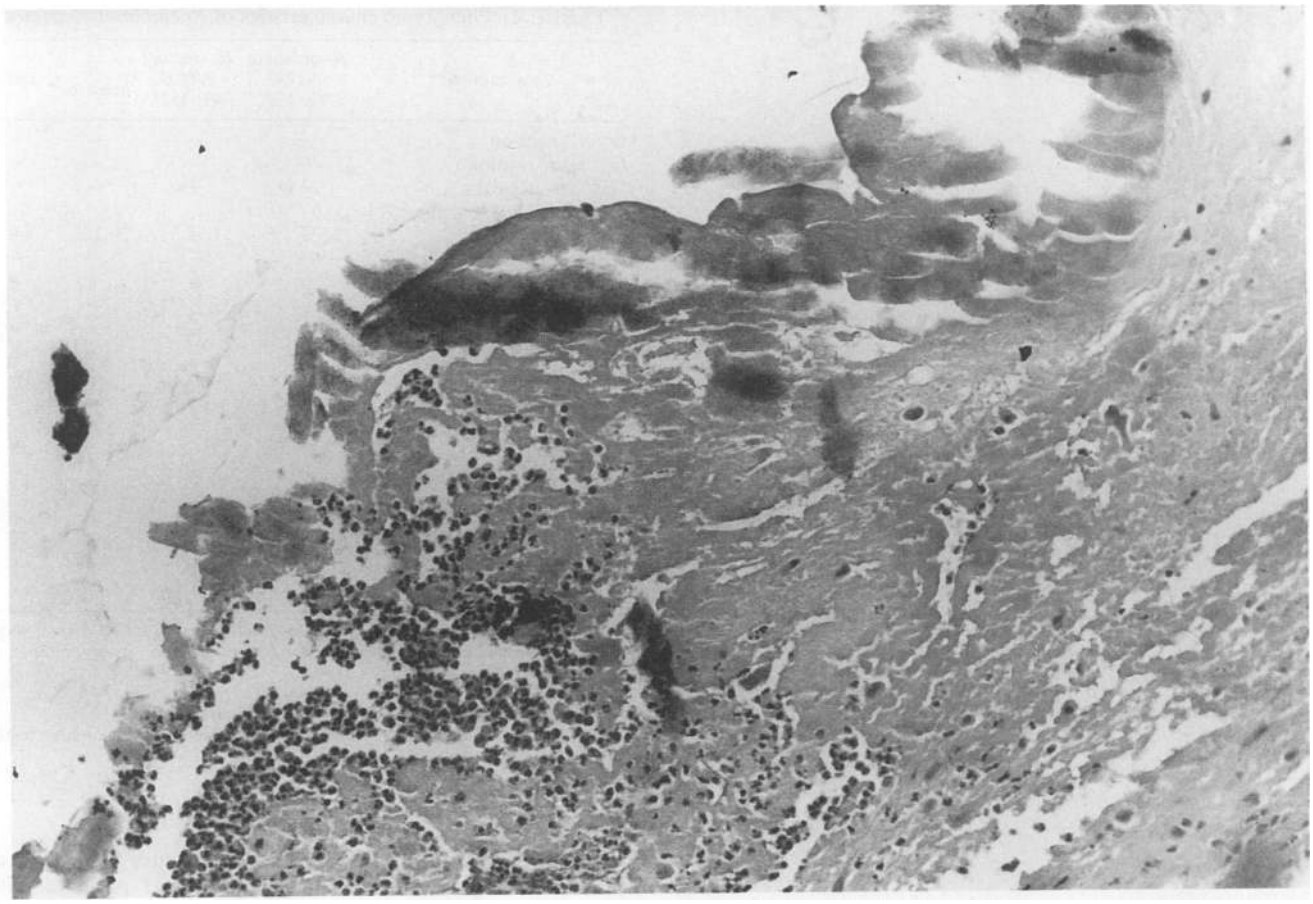

FIG. 1. Hematoxylin-eosin-stained section of aortic valve showing fibrin vegetation with acute inflammation and necrosis of underlying valve substance. Magnification, ×125.

for *R. vinsonii*, and 41.0 mol% for *R. henselae*. Labeled DNA from strain F9251 was 59 to 67% related to type strains of the three described *Rochalimaea* species at 55°C, an optimal DNA reassociation criterion (Table 3). The divergence in related sequences was 12 to 13%, and the relatedness under a stringent criterion for DNA reassociation was approximately 15%. In reciprocal reactions with labeled DNA from *R. henselae*, the relatedness was 49%, with 9.5% divergence and 16% relatedness at 75°C.

16S rRNA sequence analysis. The partial 16S rRNA gene sequence from strain F9251 is shown in Fig. 3 and is aligned with sequences from other species of *Rochalimaea* and *B. bacilliformis*. A total of 1,466 nucleotide positions, corresponding to *Escherichia coli* sequence positions 23 to 1,496 (8), were determined. This represents approximately 95% of the gene. An identical region of the 16S rRNA gene from *R. vinsonii* was also sequenced. These data show good agreement with a 16S gene sequence from the same strain of *R. vinsonii* reported by Regnery et al. (28). In an overlapping region of 1,415 nucleotides between these two sequences, only a single discrepancy was noted, the occurrence of a guanosine residue at position 875 in Fig. 3 which does not appear in the previously published sequence. The 16S rRNA sequence of *B. bacilliformis* type strain ATCC 35695 was first determined, using reverse transcriptase, by O'Connor and others (26) and was subsequently determined by Birtles and colleagues (4) and Relman and coworkers (31). Because of discrepancies between these reported sequences, a 1,471-nucleotide 16S rDNA sequence from *B. bacilliformis* ATCC 35685 was again determined. The corrected sequence shown in Fig. 3 has been submitted to GenBank, where it appears under the original accession number (M65249).

A sequence similarity matrix based on 16S sequence data from members of the family *Rickettsiaceae* is shown in Table 4. The sequence from strain F9251 was most similar (99.4%) to the *R. vinsonii* sequence, 99.2% similar to the *R. henselae* sequence, and 98.9% similar to the *R. quintana* sequence. Within the genus *Rochalimaea* an average similarity value of 99.3% was calculated. The sequence from *B. bacilliformis* exhibited a high degree of similarity to the sequences from the three *Rochalimaea* species and strain F9251, with an average value of 98.5% (this value is higher than that reported previously [26] because of corrections to the *Bartonella* sequence and masking of the sequences to remove ambiguous regions from the alignment). By grouping these five sequences, an average similarity of 99.0% is obtained. The phylogenetic ordering of these organisms is demonstrated in the dendrogram shown in Fig. 4. *Rochalimaea* species and *B. bacilliformis* form a tight cluster with a common line of descent separate from the genera *Rickettsia* and *Ehrlichia*.

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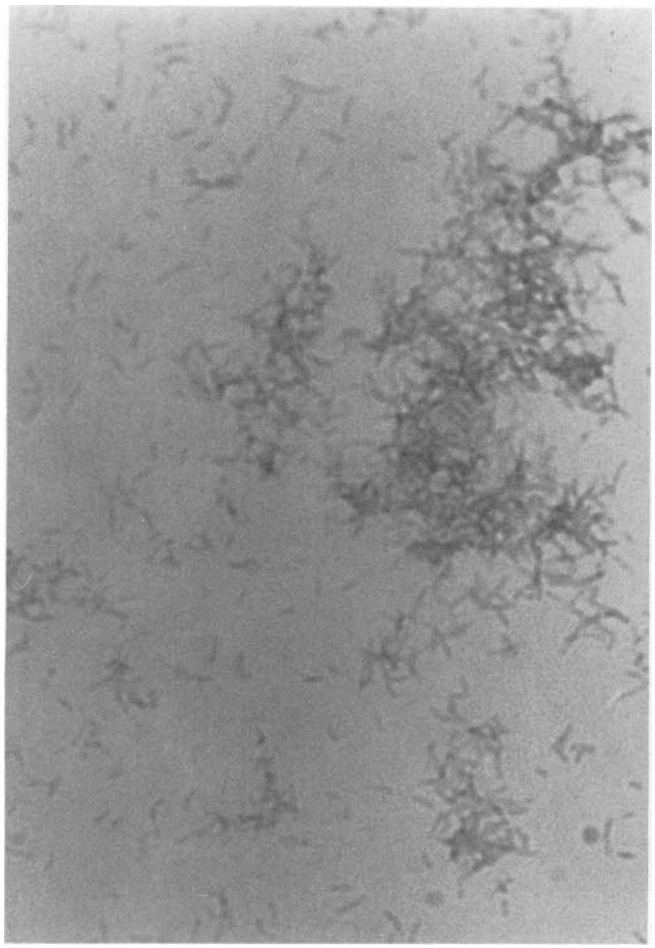

FIG. 2. Gram-stained smear of strain F9251 taken from a colony grown on a chocolate agar incubated for 96 h at 35°C in 5% CO_2. Magnification, ×1,250.

TABLE 1. Phenotypic characteristics of *Rochalimaea* species

Characteristic[a]	*R. quintana* ATCC VR-358[T]	*R. vinsonii* ATCC VR-152[T]	*R. henselae*[b]	F9251
Gram reaction	−	−	−	−
Oxidase, routine	−	−	−	−
Oxidase, Kovacs	+w	+w	−	−
Growth on HIA with:				
X factor	+	+	1/3	+
V factor	−	−	−	−
Rabbit blood	+	+	+	+
Hemolysis	−	−	−	+[c]
Motility	−	−	−	−
Indole production, spot test	−	−	−	−
Ornithine decarboxylase	−	−	−	−
Urea, Christensen	−	−	−	−
Acid from carbohydrates[d]	−	−	−	−
Hydrolysis of:				
Leucylglycine	+	+	+	+
Glycine	+	+	+	+
Proline	+	+	+	+
Phenylalanine	+	+	+	+
Arginine	+	+	+	+
Serine	+	+	+	+

[a] +, positive reaction within 48 h; −, negative reaction after 7 days; T, type strain; w, weak reaction; fraction, number of positive strains/number of strains tested.
[b] Strains ATCC 49882[T], 87-66, and 88-712.
[c] Zone of incomplete clearing at 3 to 4 days on rabbit blood.
[d] D-Glucose, lactose, maltose, D-mannitol, D-xylose, and sucrose tested with a rapid sugar test (10).

DISCUSSION

Our patient initially presented with culture-negative endocarditis, an entity that is becoming increasingly rare as laboratories develop better techniques for culturing fastidious organisms (41, 43). Because our patient's history, clinical evaluation, and echocardiographic findings provided compelling evidence of bacterial endocarditis, we used assessment methods designed to enhance the detection of fastidious organisms. Strain F9251 was isolated only when an aliquot of broth containing the patient's blood was subcultured onto chocolate agar plates and the plates were kept for 14 days in an atmosphere of 5 to 10% CO_2. Standard clinical laboratory practice would have dictated holding the subculture plates for only 48 to 72 h, an incubation period which is usually adequate for recovery of organisms known to cause endocarditis, such as of the *Haemophilus-Actinobacillus-Cardiobacterium-Eikenella-Kingella* group (46). In contrast, *Rochalimaea* species require 10 to 21 days to grow after subculture from broth medium or after culture of blood samples by the lysis-centrifugation method (37). Our experience in isolating F9251 and appreciation of the techniques required to isolate the other *Rochalimaea* species from blood or tissue specimens (28, 37, 52) indicate that changes are necessary in the methods of blood culture processing in patients with suspected endocarditis, patients with fever of unknown origin, and immunocompromised patients with fever (28), in order to allow recognition of the full spectrum of diseases associated with *Rochalimaea* and related species.

Organisms that are difficult to identify and that appear to be *Rochalimaea*-like species should be tested for preformed enzymes, and their CFA composition should be determined. Since the reactions of all the *Rochalimaea* strains we tested in the RapID ANA II system (profile number 000671) were the same as those for the type strain of *R. henselae* (28), the system may be useful for identification to the genus level, although it does not differentiate between the species of *Rochalimaea*. The only phenotypic characteristic that distinguished strain F9251 from the strains of the other species was a delayed hemolytic reaction on rabbit blood agar. At present, only DNA hybridization, 16S rRNA sequencing, and PCR-restriction endonuclease fragment length polymorphism analysis of the citrate synthase gene (18, 28) can be used to identify *Rochalimaea* isolates to the species level. Presumably, a species-specific antiserum, such as that produced from *R. henselae* by Welch and coworkers (52), could be prepared against strain F9251 for use in identifying other strains. It may be possible to develop genus-specific *Rochalimaea* antiserum to aid in identification.

The presence and relative amounts of the fatty acids in *Rochalimaea* species are unique for this genus and currently represent the most practical and rapid way to differentiate these organisms from other bacterial genera. Initially, CFA data provided a strong indication that strain F9251 was in the genus *Rochalimaea* and was possibly a new species. These data showed that the CFA content of F9251 was typical of, but not identical to, that of other *Rochalimaea* species. *R. henselae* and *R. quintana* have virtually identical CFA

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TABLE 2. CFA composition of *Rochalimaea* species

Organism (no. of strains tested)	% Fatty acids[a]									
	$C_{15:0}$	$C_{16:1\omega7c}$	$C_{16:0}$	$C_{17:1\omega8c}$	$C_{17:1\omega6c}$	$C_{17:0}$	$C_{18:2}$	$C_{18:1\omega9c}$	$C_{18:1\omega7c}$	$C_{18:0}$
R. henselae (7)	–	Tr	17	–	Tr	3	1	1	57	20
R. quintana (8)	–	Tr	18	–	Tr	1	Tr	Tr	59	21
R. vinsonii (1)	2	2	20	–	3	9	Tr	Tr	54	9
Rochalimaea-like strain F9251 (1)	2	1	13	1	8	21	1	1	43	8

[a] Numbers before the colons are the numbers of carbon atoms; numbers after the colons are the numbers of double bonds; ω, double-bond position from the hydrocarbon end of the carbon chain; c, *cis* isomer; values are the mean percentages of total fatty acids; Tr, 0.4 to 0.9%; –, not detected.

features and cannot be distinguished from each other by fatty acid analysis. *R. vinsonii* and strain F9251 had similar CFA compositions, which differed from those of *R. quintana* and *R. henselae* by the presence of $C_{15:0}$, $C_{17:1\omega6c}$, and larger amounts of $C_{17:0}$. However, strain F9251 contained more $C_{17:0}$ and less $C_{16:0}$ and $C_{18:1\omega7c}$ than did *R. vinsonii*. Although the differences in CFA composition remained consistent upon repeated regrowth and retesting of each species, they must be considered tentative until additional strains are tested. The data in Table 2 are in general agreement with those reported by Welch and associates (52), except that the $C_{13:1}$ acid observed by those investigators was not present in any *Rochalimaea* strain that we tested.

There were other differences between our results and those reported previously. Our results indicate that the GC contents of strain F9251 and named *Rochalimaea* species were between 40.3 and 41.1 mol%. These values were greater than the level of approximately 39 mol% previously observed for *R. quintana* and *R. vinsonii* (42, 50). All GC determinations made by us and others were done spectrophotometrically by means of thermal denaturation analysis. We used the equation of Marmur and Doty (22) whereas the earlier evaluations used the equation of De Ley (11) to determine the mol% GC from thermal denaturation. The resulting difference in values was probably due to either impurities in the DNA used or the calibration temperatures of the spectrophotometer.

DNA-DNA hybridization remains the method of choice for defining species (24, 47). The high level of DNA relatedness of *Rochalimaea*-like strain F9251 to the three described *Rochalimaea* species (49 to 67%; Table 3) leaves little doubt that it represents a new species in the genus *Rochalimaea*. The 16S rRNA sequence of F9251 is most homologous to the 16S rRNA sequences of the three existing *Rochalimaea* species (98.9% or greater), supporting placement of this strain in the genus *Rochalimaea*. The magnitudes of dissimilarity between the F9251 sequence and the other three *Rochalimaea* sequences are similar to those obtained by comparing the sequences from the three *Rochalimaea* species previously defined by DNA-DNA hybridization (52). These findings, as well as the similarities observed in the cultural, biochemical, and CFA characteristics, support the classification of F9251 as a new species in the genus *Rochalimaea*. We propose the name *Rochalimaea elizabethae* sp. nov. for this organism. The description of the new species is given below.

The topology of the tree in Fig. 4 reveals that the genus *Rochalimaea* is a monophyletic group which shares a common line of descent with *B. bacilliformis*, a phylogenetic relationship that has been noted previously (4, 26, 31). The 16S rRNA sequence reported by O'Connor et al. (26) was 91.7% homologous to that of *R. quintana*. This level of homology was well below that expected from the 32 to 47% DNA relatedness between *B. bacilliformis* and *Rochalimaea* species shown previously (52) and in the present study. We therefore resequenced *B. bacilliformis* 16S rRNA (Fig. 3), obtaining 98.4% homology with *R. quintana* (Table 4). In conjunction with the hybridization results, the genetic data support the reclassification of the genus *Rochalimaea* into the family *Bartonellaceae* and suggest the reordering of these organisms into a single genus.

B. bacilliformis and *Rochalimaea* species are related both genetically and by similarities in their associated clinical presentations and transmission by vectors. As Welch and colleagues (52) have observed, the histologic appearance of bacillary angiomatosis (27); the chronic form of bartonellosis, verruga peruana; cat scratch disease; and Kaposi's sarcoma show some similarities. Transmission by a vector

TABLE 3. DNA relatedness of *Rochalimaea* species

Source of unlabeled DNA	% Relatedness to labeled DNA from:					
	Rochalimaea-like strain F9251			*R. henselae* ATCC 49882^T		
	55°C	D[a]	70°C	55°C	D	70°C
Rochalimaea-like strain F9251	100	0.0	100	49	9.5	16
R. henselae ATCC 49882^T	67	12.5	16	100	0.0	100
R. henselae 89-674				100	1.0	100
R. henselae 90-615				99	0.5	98
R. henselae 87-66				100	0.0	100
R. henselae 89-712				92	1.0	99
R. quintana ATCC VR-358^T	59	13.0	15			
R. vinsonii ATCC VR-152^T	66	12.0	16			
B. bacilliformis ATCC 35685^T	32	17.0		47	16.5	

[a] D, divergence within related sequences, calculated to the nearest 0.5%.

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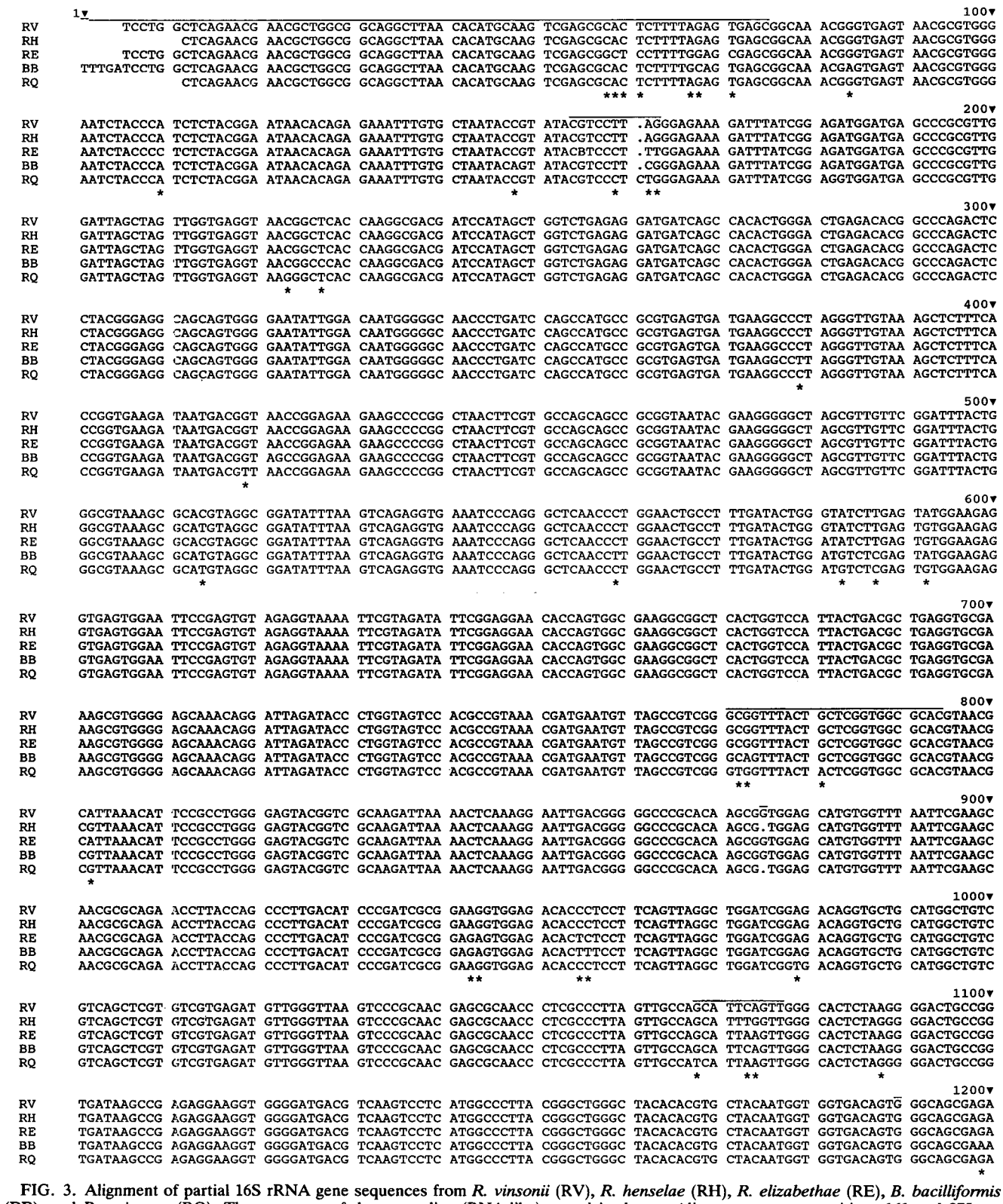

FIG. 3. Alignment of partial 16S rRNA gene sequences from *R. vinsonii* (RV), *R. henselae* (RH), *R. elizabethae* (RE), *B. bacilliformis* (BB), and *R. quintana* (RQ). The sequence of the noncoding (RNA-like) strand is shown. Alignment gaps at positions 161 and 875 are indicated by dots. The asterisks indicate the lack of consensus at positions where the nucleotide sequence was determined for all genes in the alignment. The positions of alignment uncertainty, observed in an alignment of 16 sequences from members of the order *Rickettsiales* (data not shown) that were omitted from the phylogenetic analysis, are indicated by a line above these positions.

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                                                                                                                    1300▼
RV      CCCCGAGGTC GAGCTAATCT CCAAAAGCCA TCTCAGTTCG GATTGCACTC TGCAACTCGA GTGCATGAAG TTGGAATCGC TAGTAATCGT GGATCAGCAT
RH      CCGCGAGGTC GAGCTAATCT CCAAAAGCCA TCTCAGTTCG GATTGCACTC TGCAACTCGA GTGCATGAAG TCGGAATCGC TAGTAATCGT GGATCAGCAT
RE      CCGCGAGGTC GAGCTAATCT CCAAAAGCCA TCTCAGTTCG GATTGCACTC TGCAACTCGA GTGCATGAAG TTGGAATCGC TAGTAATCGT GGATCAGCAT
BB      CCGCAAGGTC GAGCTAATCT CCAAAAGCCA TCTCAGTTCG GATTGCACTC TGCAACTCGA GTGCATGAAG TCGGAATCGC TAGTAATCGT GGATCAGCAT
RQ      CCGCGAGGTC GAGCTAATCT CCAAAAGCCA TCTCAGTTCG GATTGCACTC TGCAACTCGA GTGCATGAAG TTGGAATCGC TAGTAATCGT GGATCAGCAT
          *                                                                                   *
                                                                                                                    1400▼
RV      GCCACGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGT TTTACCCGAA GGTGCTGTGC TAACCGCAAG GAGGCAGGCA
RH      GCCACGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGT TTTACCCGAA GGTGCTGTGC TAACCGCAAG GGGGCAGGCA
RE      GCCACGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGT TTTACCCGAA GGTGCTGTGC TAACCGCAAG GAGGCAGGCA
BB      GCCACGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGT TTTACCCGAA GGTGCTGTGC TAACCGCAAG GAAGCAGGCA
RQ      GCCACGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTTGGT TTTACCCGAA GGTGCTGTGC TAACCGCAAG GAGGCAGGCA

                                                                                                          1471▼
RV      ACCACGGTAG GGTCAGCGAC TGGGGTGAAG TCGTAACAAG GTAGCCGTAG GGAACCTGTG GCTGGATCAC CT
RH      ACCACGGTAG GGTCAGCGAC TGGGGTG
RE      ACCACGGTAG GGTCAGCGAC TGGGGTGAAG TCGTAACAAG GTAGCCGTAG GGAACCTGTG GCTGGATCAC CT
BB      ACCACGGTAG GGTCAGCGAC TGGGGTGAAG TCGTAACAAG GTAGCCGTAG GGAACCTGCG GCTGGATCAC CT
RQ      ACCACGGTAG GGTCAGCGAC TGGGGTG
```

FIG. 3—*Continued*

has been suggested for *R. vinsonii*, which was isolated from the spleens of voles and then experimentally transmitted by the ear mite (*Trombicula microti*) to a hamster by Baker (2). *B. bacilliformis* and *R. quintana* are transmitted by vectors to humans (25, 45). In Oroya fever, the acute form of bartonellosis (an infection transmitted by the sandfly), organisms can be isolated from the blood of patients when blood samples are subcultured to medium containing rabbit blood and incubated at 28 to 30°C (19). *R. quintana* (transmitted by the louse) has been easily isolated for as long as 1 month after the illness from the blood of patients with trench fever and from one volunteer who was inoculated experimentally by Varela and associates (44). Koehler and coworkers (18) have reported exposure to possible vectors in two of their patients with bacillary angiomatosis; one patient whose samples grew *R. quintana* owned a pet rat and the patient whose samples grew *R. henselae* owned cats. The serological data reported by Regnery and colleagues (29) implicate *Rochalimaea* species as a possible etiologic agent of cat scratch disease (30), and *R. henselae* has been cultured from lymph nodes in two patients (53) and from the blood of a domestic cat (29). However, the etiology of cat scratch disease remains controversial. The proposed etiologic agent was first reported to be a bacterium (14) that subsequently was named *Afipia felis* (5); it has been detected by PCR in a lymph node of a patient with the disease (9) and produced a serological response in another patient (13). On balance, the present data indicate that *R. henselae* and possibly other *Rochalimaea* species may cause most cases of cat scratch disease, but a possible role for *A. felis* has not been ruled out, nor has the possibility of dual infection.

Our patient represents the first reported case of infection caused by *R. elizabethae* sp. nov. and the first case of endocarditis caused by a *Rochalimaea* species. We can only speculate why this infection developed. The patient had no known exposure to animals or insect vectors, was not apparently immunosuppressed, and was HIV antibody negative, in contrast to most patients reported to have bacillary angiomatosis or bacteremia caused by *R. henselae* (37, 38, 52). He did not have any predisposition to endocarditis, such as cardiac valvular abnormalities. A risk factor for bacteremia was his poor dentition and, possibly, intestinal strongyloidiasis, an infection known to be associated with a predisposition to bacteremia resulting from organisms in the stools of patients with overwhelming infestations (36). Until the ecological niche of *R. elizabethae* is identified, it cannot be determined whether our patient's parasitic infection caused by *S. stercoralis* played a part in the acquisition of this organism.

Rapid, direct identification of *Rochalimaea*-like organisms in blood or tissue would facilitate appropriate treatment for future cases because blood cultures are likely to remain negative for weeks. Cure of endocarditis depends on the use of appropriate bactericidal agents for a prolonged time period, and antimicrobial therapy for culture-negative endocarditis generally has consisted of a combination of ampicil-

TABLE 4. 16S rRNA similarity matrix[a]

Organism	% Similarity				
	R. vinsonii	*R. henselae*	*R. quintana*	*R. elizabethae*	*B. bacilliformis*
R. vinsonii					
R. henselae	99.6				
R. quintana	99.1	99.4			
R. elizabethae	99.4	99.2	98.9		
B. bacilliformis	98.5	98.6	98.6	98.4	

[a] Numbers represent percent similarity corrected for multiple base changes by the method of Jukes and Cantor (17).

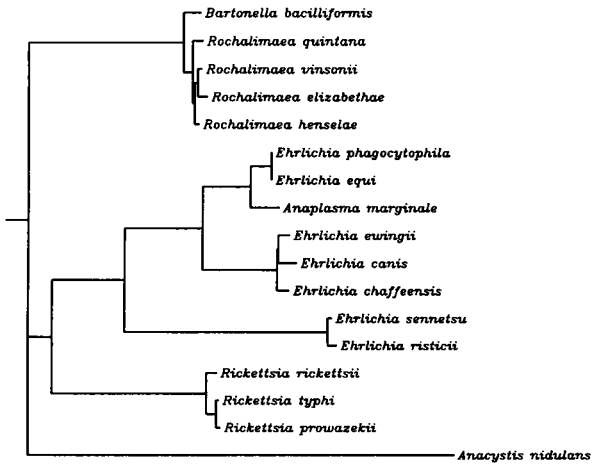

FIG. 4. Phylogenetic tree for members of the order *Rickettsiales*, with *A. nidulans* as the outgroup. The tree was inferred from 16S rRNA similarity data by the neighbor-joining method (34). The bar represents a 5% difference in nucleotide sequence.

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lin or vancomycin and gentamicin. When given intravenously for 2 to 6 weeks, this regimen produces cure rates of 92% in patients who become afebrile (43). This therapy, however, may not be ideal for patients with *Rochalimaea* infections, even though the organisms appear to be susceptible to most antibiotics. Our patient was cured with a combination of medical and surgical therapy, and *R. elizabethae* sp. nov. was not recovered from blood cultures obtained after 2 days of treatment with nafcillin and gentamicin therapy or from the cardiac valve removed after 18 days of therapy, despite the presence of organisms on pathologic examination. This suggests that the *R. elizabethae* sp. nov. was susceptible in vivo to either nafcillin or gentamicin or both of these agents, but the ideal therapy is not known.

As laboratories become aware of the growth characteristics of *Rochalimaea* species and physicians better understand the illnesses caused by these pathogens, it is likely that more cases will be recognized. With an increase in the frequency of detection, more in vitro information about antimicrobial susceptibility and in vivo data about clinical risk factors for infection will emerge to help clinicians correctly diagnose and treat patients infected with these organisms.

Description of *R. elizabethae* sp. nov. *R. elizabethae* (e.liz' a.beth.e.a. N.L. fem. adj. *elizabethae*), named after Saint Elizabeth's Hospital, Brighton, Mass., where the organism was isolated. Biochemical reactions are given in Table 1. Its closest phenotypic relative is *R. henselae*, from which it appears to be differentiated by incomplete hemolysis on rabbit blood agar. It is presumed to be pathogenic for humans.

Description of the type strain. The type strain of *R. elizabethae* is F9251 (=B91-002005 = ATCC 49927). It shares all the characteristics of the species. The GC content of DNA was 41.0 mol%. It was isolated from the blood of a man with endocarditis.

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ADDENDUM IN PROOF

DNA from the paraffin-embedded tissue block of the patient's heart valve was amplified using *Rochalimaea* genus-specific 16S RNA primers and was positive (personal communication, B. Swaminathan, Centers for Disease Control).

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