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Proposal of *Afipia* gen. nov., with *Afipia felis* sp. nov. (Formerly the Cat Scratch Disease Bacillus), *Afipia clevelandensis* sp. nov. (Formerly the Cleveland Clinic Foundation Strain), *Afipia broomeae* sp. nov., and Three Unnamed Genospecies

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On the basis of phenotypic characterization and DNA relatedness determinations, the genus *Afipia* gen. nov., which contains six species, is described. The type species is *Afipia felis* sp. nov. (the cat scratch disease bacillus). *Afipia clevelandensis* sp. nov., *Afipia broomeae* sp. nov., and three unnamed *Afipia* genospecies are apparently not associated with cat-borne disease. All but one strain (*Afipia* genospecies 3) were isolated from human wound and respiratory sources. All *Afipia* species are gram-negative, oxidase-positive, nonfermentative rods in the α-2 subgroup of the class *Proteobacteria*. They are motile by means of a single flagellum. They grow on buffered charcoal-yeast extract agar and nutrient broth, but rarely on MacConkey agar, at 25 and 30°C. They are urease positive; but they are negative in reactions for hemolysis, indole production, H_2S production (triple sugar iron agar), gelatin hydrolysis, esculin hydrolysis, and peptonization of litmus milk. They do not produce acid oxidatively from D-glucose, lactose, maltose, or sucrose. The major cell wall fatty acids are 11-methyloctadec-12-enoic ($C_{Br19:1}$), *cis*-octadec-11-enoic ($C_{18:1omega7c}$), and generally, 9,10-methylenehexadecanoate and 11,12-methyleneoctadecanoate; and there are only trace amounts of hydroxy acids. The guanine-plus-cytosine content is 61.5 to 69 mol%. *A. felis* is positive for nitrate reduction and is delayed positive for acid production from D-xylose, but it is catalase negative. *A. clevelandensis* is negative in all of these tests. *A. broomeae* is weakly positive for catalase production and acid production from D-xylose, but it is negative for nitrate reduction.

In 1986, it was estimated that 6,000 cases of cat scratch disease (CSD) occurred annually in the United States, some 2,100 of whom were hospitalized (5.83 of 100,000 hospital admissions). Encephalitis was estimated to occur in 41 to 60 of the cases, and 4 to 6 cases were fatal (4).

The classic clinical presentation of CSD is a self-limited regional lymphadenopathy (9). However, even in this benign form, the inability to culture the etiologic agent and the lack of diagnostic tests frequently necessitate invasive diagnostic procedures (i.e., lymph node biopsy) to rule out other diseases such as lymphoma. In a minority of cases, cat scratch encephalopathy occurs, and some patients experience severe systemic disease (7, 16, 18). It has been postulated that epithelioid angiomatosis in patients with AIDS may be a manifestation of CSD, and that the same etiologic agent may be responsible for both diseases (14, 15, 29).

Until recently, only English and colleagues (8, 9), at the Armed Forces Institute of Pathology (AFIP), were successful in isolating the CSD bacillus (CSDB). However, investigators at the Centers for Disease Control, using a cell culture method, have recently isolated CSDB from 10 clinical specimens (27). A second organism, isolated from a pretibial biopsy specimen by Hall et al. (11) at the Cleveland Clinic Foundation (CCF), was shown to have the same cellular fatty acid composition as CSDB (23). CSDB and the CCF strain had very similar rRNA sequences, indicative of two species in the same genus within the α-2 subgroup of the class *Proteobacteria* (25).

We have identified, in our Special Bacteriology Reference Laboratory culture collection, six additional strains from human sources that have cellular fatty acid patterns similar to those of CSDB and the CCF strain. In this report, we show that the CSDB, the CCF strain, and the six cat scratch-like strains represent six separate species in the new genus *Afipia*. We propose the names *Afipia felis* for CSDB, *Afipia clevelandensis* for the CCF strain, and *Afipia*

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TABLE 1. Strains used in this study

Strain	Source, year of isolation	Obtained from:
A. felis B-91-007352 = AFIP strain BV = F6400 = G1492 = ATCC 53690	Lymph node, patient with CSD, 1987	D. J. Wear, AFIP
A. felis B-91-007147 = ATCC 49714[a]	Lymph node aspirate, girl, age 4 yr, fever, 1990	Tripler Army Medical Center
A. felis B-90-007209 = ATCC 49715	Lymph node aspirate, boy, age 6 yr, fever, 1990[b]	Tripler Army Medical Center
A. felis B-90-007260 = ATCC 49716	Lymph node aspirate, boy, age 6 yr, different sample, 1990[b]	Tripler Army Medical Center
A. clevelandensis B-91-007353 = F6703 = G1849 = 411m = ATCC 49720	Tibial biopsy, male, age 69 yr, 1988, died	CCF
A. broomeae B-91-007286 = G0382 = F186 = ATCC 49717	Sputum, 1981	New Zealand
Afipia sp. strain B-91-007287 = G0383 = F872 = ATCC 49721	Pleural fluid, pulmonary obstruction, fatal, 1981	Oklahoma State Department of Health
A. broomeae B-91-007288 = G0384 = F7661 = ATCC 49718	Bone marrow, woman, age 81 yr, 1985	Massachusetts Department of Public Health
A. broomeae B-91-007289 = G0385 = F8133 = ATCC 49719	Synovium, wrist abscess, male, age 60 yr, diabetic, arteriosclerosis	Kentucky Department for Health Services
Afipia sp. strain B-91-007290 = G4438 = ATCC 49722	Bronchial washing, woman, age 80 yr, 1989	Indiana State Board of Health
Afipia sp. strain B-91-007291 = G5357 = ATCC 49723	Water, 1990	Indiana State Board of Health

[a] ATCC, American Type Culture Collection.
[b] Same patient.

broomeae for three of the cat scratch-like strains and recognize three unnamed genospecies in the genus *Afipia*.

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. Strains were maintained by freezing them in defibrinated rabbit blood and storing them in a vapor-phase liquid nitrogen freezer. They were cultivated on buffered charcoal-yeast extract (BCYE) agar at 30°C prior to inoculation for biochemical and all other tests. The first CSDB strain, AFIP strain BV (B-91-007352), was isolated at AFIP (8, 9). It was from a human lymph node and was isolated directly on bacteriologic media. The three other CSDB strains were isolated at the Centers for Disease Control by a tissue culture method. This method will be described in detail elsewhere (27). Briefly, HeLa culture cell monolayers were propagated in six-well trays (Costar Corp., Cambridge, Mass.) at 3×10^5 cells per well. Prior to infection, the tissue culture medium, minimal essential medium (GIBCO, Gaithersburg, Md.) containing 10% fetal bovine serum (GIBCO), was removed, the monolayers were washed in phosphate-buffered saline (PBS), and minimal essential medium without serum was added back to the wells. Both fluid and tissue specimens were diluted in PBS and sonicated prior to inoculation. The HeLa cell monolayers were then overlaid with the sonic extracts, and infection was allowed to proceed for up to 18 h. The medium containing the inoculum was then removed, the monolayers were washed in PBS, and minimal essential medium with fetal bovine serum was added back to the wells. After several days of incubation, the medium was removed, the wells were washed with PBS, and the monolayers were collected. Portions of the monolayers were analyzed microscopically, and other portions were reinoculated onto secondary cell culture monolayers and onto BCYE agar plates.

Biochemical tests. Gram stain, motility, and biochemical test reactions were done as described previously (6). Incubations were at 30°C, unless indicated otherwise (Table 2).

Antimicrobial susceptibility testing. Strains were grown for

TABLE 2. Biochemical reactions of *Afipia* species

Test	Result for strain[a]:							
	1	2	3	4	5	6	7	8
Nitrate reduction	+	−	−	−	−	−	−	−
D-Xylose, acid	(+^{w})	−	(+^{w})	(+^{w})	(+^{w})	(+^{w})	(+^{w})	(+^{w})
Litmus milk	(ak)	(ak)	(ak)	(ak)	(ak)	(ak)	(ak)	(ak)
Catalase	−	−	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}
Gram reaction	−	−	−	−	−	−	−	−
Oxidase	+	+	+	+	+	+	+	+
Growth at:								
25°C	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+
35°C	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}	+	+	+^{w}
42°C	−	−	−	−	−	−	−	−
Motility	+	+	+	+	+	+	+	+
Hemolysis	−	−	−	−	−	−	−	−
Nutrient broth, growth	+	(+)	+	+	+	+	+	+
Nutrient broth plus 6% NaCl, growth	−	−	−	−	−	−	−	−
MacConkey agar, growth	−	(+^{w})	−	−	−	−	−	−
Urea, Christensen[b]	+	+	+	+	(+)	+	+	+
H_2S, triple sugar iron	−	−	−	−	−	−	−	−
H_2S, lead acetate	−	−	−	−	−	−	−	+^{w}
Gelatin hydrolysis	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−
Citrate, Simmons	−	−	−	−	−	+	−	−
Esculin hydrolysis	−	−	−	−	−	−	−	−
Nitrate, gas	−	−	−	−	−	−	−	−
D-Glucose, gas	−	−	−	−	−	−	−	−
D-Glucose, acid	−	−	−	−	−	−	−	−
Lactose, acid	−	−	−	−	−	−	−	−
Maltose, acid	−	−	−	−	−	−	−	−
D-Mannitol, acid	−	−	−	−	−	+^{w}	−	−
Sucrose, acid	−	−	−	−	−	−	−	−

[a] 1, *A. felis* (CSDB; 4 strains); 2, *A. clevelandensis* (CCF strain, B-91-007353); 3, *A. broomeae* B-91-007286; 4, *A. broomeae* B-91-007288; 5, *A. broomeae* B-91-007289; 6, *Afipia* genospecies 1 B-91-007287; 7, *Afipia* genospecies 2 B-91-007290; 8, *Afipia* genospecies 3 B-91-007291. +, positive reaction within 48 h; −, negative reaction; parentheses, positive reaction in 3 to 14 days; ^{w}, weak; ak, alkaline reaction.
[b] The slant was heavily inoculated.

48 h at 30°C on BCYE agar (Carr Scarborough, Decatur, Ga.) prior to testing. Growth from BCYE agar was used to prepare a suspension in Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) to the density of a 1.0 McFarland standard. Ten microliters of this suspension was used to inoculate 10 ml of Wilkins-Chalgren broth (Difco Laboratories, Detroit, Mich.) (33). One hundred-microliter aliquots of inoculum were dispensed into the wells of Sceptor Staphylococcus, Enteric, and Gram Negative Resistant susceptibility testing plates (Becton Dickinson Microbiology Systems). The plates were incubated at 30°C and read at 48 h. In-house broth microdilution susceptibility testing plates were prepared as recommended previously (24) by using a Quick-Spense dispenser (Sandy Springs Instrument Co., Inc., Ijamsville, Md.) and Wilkins-Chalgren broth. To inoculate these plates, bacteria were suspended in 8 ml of Mueller-Hinton broth to the density of a 1.0 McFarland standard. The suspension was added to 32 ml of sterile water to make a total volume of 40 ml. This inoculum was dispensed in 1-μl aliquots by using a MIC-2000 inoculator (Dynatech Laboratories, Inc., Alexandria, Va.) into microdilution plates containing twofold dilutions of the antimicrobial agents to be tested. The final inoculum for all broth tests was 1×10^5 to 5×10^5 CFU/ml. Plates were incubated as described above. Antimicrobial agents were obtained directly from the manufacturer.

Cellular fatty acid analysis. Cells were grown for 3 to 5 days. Cellular fatty acids were determined as described previously (23).

DNA methods. The methods used to extract and purify DNA and the hydroxyapatite method for determining DNA relatedness have been described previously (3). DNAs were labeled enzymatically in vitro with [^{32}P]dCTP by using a nick translation reagent kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as directed by the manufacturer. Guanine-plus-cytosine (G+C) contents were determined spectrophotometrically and reported to the nearest 0.5 mol% by the method of Marmur and Doty (19).

Plasmid analysis. Colonies of all strains, which were grown for approximately 3 days, were assayed for the presence of plasmids by the alkaline lysis method of Birnboim and Doly (2).

Multilocus enzyme analysis. Bacteria were grown for 5 days and were harvested into cold saline. Cell extracts were prepared, and multilocus enzyme electrophoresis was done as described by Reeves et al. (28). Enzyme activities were determined as described by Selander et al. (30) and Harris and Hopkinson (12) for the following 30 metabolic enzymes: acid phosphatase, adenylate kinase, alcohol dehydrogenase, alkaline phosphatase, aspartate carbamyltransferase, benzyl alcohol dehydrogenase, catalase, dehydrogenase of unknown specificity, esterase, fumarase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, hydroxyacylglutathione hydrolase, 3-hydroxybutyrate dehydrogenase, indophenol oxidase, isocitrate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, malic enzyme, NAD-dependent glyceraldehyde 3-phosphate dehydrogenase, NADH diaphorase, NADP-dependent glutamate dehydrogenase, NADPH diaphorase, peptidase 1 (Leu-Gly-Gly), peptidase 2 (Phe-Leu), phosphoglucomutase, phosphoglucose isomerase, proline oxidoreductase, serine dehydrogenase, and shikimate dehydrogenase. Electrophoretic variants of each enzyme were considered alleles of that enzyme and were assigned different allele numbers. Each strain was characterized by a string of allele numbers for the different enzymes, and each unique string of alleles was designated as an electrophoretic type (ET). Genetic distances between pairs of strains were calculated as the proportion of weighted mismatches of alleles (30, 31), and a dendrogram showing the relatedness of the ETs was produced as described previously (13, 28).

Ribotyping. Genomic DNA was isolated by the method of Wilson (34) and by the agarose plug method of McClelland et al. (20). The chromosomal DNA prepared by the agarose plug method was used for restriction with *Eco*RI, and the chromosomal DNA prepared by the method of Wilson (34) was used for double digestion with *Pst*I-*Bgl*II and *Cla*I-*Sma*I. DNA restrictions were done according to the directions of the manufacturer (New England BioLabs, Inc., Beverly, Mass.). Restriction fragments were separated by horizontal electrophoresis on 0.8% agarose (Bethesda Research Laboratories) in Tris-borate buffer at 40 V for 16 to 18 h. The separated fragments were stained with ethidium bromide, observed under 300-nm UV light, and photographed by using Polaroid 665 B/W film. The restriction fragments were denatured and transferred to Magnagraph 0.45-μm-pore-size nylon membranes (MSI Separations, Westboro, Mass.) as described previously (17). The membranes containing the DNA were probed with ^{32}P-labeled 16S and 23S *Escherichia coli* rRNA as reported previously (1). After overnight hybridization, the filter was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 37°C, and a final posthybridization wash was done with 0.15× SSC–0.1% sodium dodecyl sulfate at 37°C. The filters were autoradiographed at −70°C for 1 to 3 days by using Kodak X-OMAT X-ray film and intensifying screens. The ribosomal DNA (rDNA) fingerprint of each isolate was visually scored for the presence or absence of each unique fragment (0, absence of a fragment; 1, presence of a fragment) for each restriction enzyme or enzyme combination. Data for the three restriction enzyme or enzyme combinations were combined by concatenation. In this manner, a numerical profile was developed for each isolate. Relatedness values were calculated as the proportion of weighted mismatches of fragments, and a dendrogram showing the clustering or relatedness of the ribotypes was generated by the weighted pair group method for arithmetic averages (31) by using SAS/GRAPH software (13).

Serologic characterization. Antisera against strains B-91-007352 (AFIP strain BV) and B-91-007353 (CCF strain) were prepared in rabbits as described previously (22). The immunoglobulin G (IgG) portion of the immunoglobulin was purified by chromatography on protein A-Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) as described by Goding (10). IgG was labeled with fluorescein isothiocyanate as described previously (22). Fluorescein isothiocyanate-labeled conjugates were adjusted so that each one contained approximately 10 mg of IgG per ml. The fluorescein-to-protein ratios were approximately 25 μg/mg. Suspensions of the test strains were made in 1% Formalin in 0.01 M PBS (pH 7.2). Smears of the organisms were made and stained as described by Cherry et al. (5). The slides were examined by fluorescence microscopy as described by McKinney et al. (21). Twofold serial dilutions of the conjugate were made, and endpoint titers were expressed as the last dilution that gave 4+, or very bright, staining of the bacteria. To stringently test for the reactivities of the reagents with heterologous antigens, the dilutions were started at a 1:5 dilution (2 mg of IgG per ml). Latex reagents were prepared by using the affinity-purified IgG from the antisera against CSDB and the CCF strain. Normal rabbit globulin was used to prepare

a control latex reagent. Polystyrene amidine-modified latex beads (diameter, 0.489 μm; IDC Spheres, Portland, Oreg.) were sensitized with the globulins dissolved in 0.01 M PBS (pH 7.2). A 0.5% washed suspension of the sensitized latex was used for testing. Latex agglutination tests were done on clear glass slides with 13-mm-diameter rings. Fifty microliters of a cell suspension (approximately 3×10^8 CFU/ml) was mixed with 10 μl of latex reagent. After mixing with an applicator, the glass slide was rotated at 100 rpm for 5 min and was observed under a high-intensity lamp.

RESULTS

The biochemical reactions of the 11 strains are given in Table 2. All strains were gram negative, oxidase positive, and motile by means of a single polar, subpolar, or lateral flagellum; produced urea; gave alkaline reactions in litmus milk; grew in nutrient broth (one showed delayed growth); and grew at 25 and 30°C and (often weakly) at 35°C. All strains gave negative reactions in tests for indole; H_2S production (triple sugar iron agar); gelatin hydrolysis; esculin hydrolysis; gas production from D-glucose and nitrate; acid production by fermentation or oxidation from D-glucose, lactose, maltose, and sucrose; and growth at 42°C. Strains differed in their ability to produce acid from D-xylose or D-mannitol, reduce nitrate, grow on MacConkey agar, alkalinize Simmons citrate agar, produce H_2S (as detected by lead acetate strips), or show catalase activity. The four CSDB strains were catalase negative, citrate negative, D-mannitol negative, and positive for nitrate reduction and D-xylose (weak and delayed). The CCF strain was negative in all of these tests and gave weak, delayed growth on MacConkey agar. All other cat scratch-like strains were nitrate negative and were positive or delayed positive for catalase and acid production from D-xylose. Cat scratch-like strain B-91-007287 was citrate positive and delayed, weakly D-mannitol positive. Cat scratch-like strain B-91-007291 was weakly positive for H_2S production when lead acetate paper was used.

Strain B-91-007291 was susceptible to all antibiotics other than cefoperazone, cefoxitin, and ceftazidime. The other strains showed resistance to many, if not most, of the antimicrobial agents tested (Table 3).

The fatty acid compositions of saponified whole cells of the 11 strains are given in Table 4. Each strain was characterized by relatively large amounts of *cis*-octadec-11-enoic ($C_{18:1omega7c}$) and 11-methyloctadec-12-enoic acids ($C_{Br19:1}$), which were previously detected in both the CSDB strain (B-91-007352; AFIP BV) and the CCF strain (23). Ten of the 11 strains contained a 19-carbon acid with a cyclopropane ring ($C_{19:0cyc}$, lactobacillic acid), and 9 of these also contained a 17-carbon acid with a cyclopropane ring ($C_{17:0cyc}$). Palmitoleic ($C_{16:1omega9c}$), palmitic ($C_{16:0}$), and stearic ($C_{18:0}$) acids were also present. No branched-chain acids other than $C_{Br19:1}$ were present. Small amounts (trace to 1%) of 2- and 3-hydroxydodecanoic acids (2-OH $C_{12:0}$ and 3-OH $C_{12:0}$, respectively) and 3-hydroxyoctadecanoic acid (3-OH $C_{18:0}$) were released with acid hydrolysis, indicating that these acids are amide linked.

Because of their high G+C content (see below), 65°C was chosen as the optimal incubation temperature for DNA relatedness determinations, and 80°C was chosen as the stringent temperature. DNA relatedness results are given in Table 5. The 11 strains formed six DNA relatedness groups or genospecies. The four CSDB strains were 95% or more related at both incubation temperatures, with 0.5% divergence within related sequences. Cat scratch-like strains B-91-007286, B-91-007288, and B-91-007289 were 71 to 98% related at both incubation temperatures, with 0.0 to 2.5% divergence within related sequences. Each of the other strains was a distinct genospecies on the basis of less than species-level relatedness to the other strains (species-level relatedness is 70% or more at the optimal incubation temperature, 60% or more at the stringent incubation temperature, and 5% or less divergence within related sequences [3, 32]). On the basis of these results and phenotypic data, names are proposed in the Discussion for three of the genospecies: *A. felis* sp. nov. for the four CSDB strains, *A. clevelandensis* sp. nov. for the CCF strain, and *A. broomeae* sp. nov. for cat scratch-like strains B-91-007286, B-91-007288, and B-91-007289. The three remaining cat scratch-like strains are included in the genus *Afipia* as unnamed genospecies; B-91-007287 is *Afipia* genospecies 1, B-91-007290 is *Afipia* genospecies 2, and B-91-007291 is *Afipia* genospecies 3. The highest relatedness between *Afipia* species was 66 to 69% with 8.0 to 8.5% divergence between *Afipia* genospecies 1 and 2, 36 to 60% with 9.5 to 10.5% divergence between *A. clevelandensis* and *A. broomeae*, and 42% with 3.5% divergence between *A. felis* and *Afipia* genospecies 3.

TABLE 3. Antimicrobial susceptibility profiles of *Afipia* species

Antimicrobial agent	MIC (μg/ml) for strain[a]:					
	1	2	3	4	5	6
Amikacin	<4	16	16	>64	>64	≤4
Amoxycillin-clavulanate	2/1	32/16	4/2	16/8	4/2	2/1
Ampicillin	16	>32	8	>32	>32	4
Ampicillin-sulbactam	16/8	32/16	4/2	32/16	16/8	2/1
Cefoperazone	>64	>64	64	>64	>64	>64
Cefoxitin	64	<4–64	≤4	64	>64	64
Ceftazidime	64	16–64	8	>64	>64	>64
Ceftriaxone	8	≤4	≤4	>128	>128	≤4
Cephalothin	32	<4–8	≤4	>64	>64	≤4
Ciprofloxacin	8	2–8	4	>8	>8	2
Gentamicin	2	16	8	>16	>16	2
Imipenem	≤2	≤2	≤2	8	8	≤2
Piperacillin	>64	64	64	>64	>64	≤4
Tetracycline	>16	>16	>16	>16	>16	8
Ticarcillin	>64	64	16	>64	>64	≤4
Tobramycin	2	8	4	>16	>16	2

[a] 1, *A. felis*; 2, *A. clevelandensis*; 3, *A. broomeae*; 4, *Afipia* genospecies 1; 5, *Afipia* genospecies 2; 6, *Afipia* genospecies 3.

The G+C content was above 60 mol% for all species of *Afipia*. *A. felis* B-91-007352 had a G+C content of 62.5 mol%, that of *A. clevelandensis* was 64.0 mol%, and that of all *A. broomeae* strains was 61.5 mol%. The *Afipia* genospecies 1 strain contained 69 mol% G+C, the *Afipia* genospecies 2 strain contained 67 mol% G+C, and the *Afipia* genospecies 3 strain contained 65.5 mol% G+C.

All *A. felis* strains contained a single plasmid of approximately 29 mDa (44 kb). The plasmid bands were faint, implying a low copy number. Their concentration was too low to allow restriction endonuclease analysis. *A. clevelandensis*, *A. broomeae*, and the three *Afipia* genospecies did not contain a plasmid.

Multilocus enzyme typing divided the 11 *Afipia* strains into seven ETs that correspond to the 6 species identified by DNA hybridization (Fig. 1). The four *A. felis* strains were in a single ET, as was the single strain of *A. clevelandensis*. The three strains of *A. broomeae* were in two ETs that were

TABLE 4. Fatty acid compositions of *Afipia* strains

Strain	Composition (%) of fatty acid[a]:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
A. felis													
B-91-007352	–	6	–	4	–	–	7	–	43	12	14	12	–
B-91-007147	–	5	–	3	–	–	9	–	28	11	24	28	–
B-90-007209	–	5	–	3	–	–	13	–	26	9	23	21	–
B-90-007260	–	9	–	4	–	–	11	–	40	9	13	14	–
A. clevelandensis B-91-007353	–	7	–	3	–	–	16	–	27	9	30	5	1
A. broomeae													
B-91-007289	–	5	–	4	Tr	–	22	–	24	10	25	9	1
B-91-007286	Tr	9	–	5	1	–	15	3	24	12	23	5	1
B-91-007288	–	5	–	4	–	–	22	–	23	9	25	9	1
Afipia genospecies 1 B-91-007287	2	2	4	6	Tr	6	–	3	40	2	24	11	–
Afipia genospecies 2 B-91-007290	Tr	1	3	6	–	–	3	–	34	5	30	15	–
Afipia genospecies 3 B-91-007291	–	3	–	9	1	Tr	–	–	65	6	15	–	–

[a] 1, $C_{15:0}$; 2, $C_{16:1omega7c}$; 3, $C_{16:1omega5}$; 4, $C_{16:0}$; 5, $C_{17:1omega8}$; 6, $C_{17:1omega6}$; 7, $C_{17:0cyc}$; 8, $C_{17:0}$; 9, $C_{18:1omega7c}$; 10, $C_{18:0}$; 11, $C_{Br19:1}$; 12, $C_{19:0cyc}$; 13, $C_{20:1omega9c}$. Numbers to the left of the colon refer to the number of carbons, numbers to the right of the colon refer to the number of double bonds; omega, double bond position from the hydrocarbon end of the carbon chain; c, *cis* isomer; cyc, cyclopropane ring. Values are expressed as the percentage of total acids; Tr, less than 1%; –, not detected.

very closely related. *Afipia* genospecies 1, 2, and 3 strains were in three separate ETs.

No differences were observed among the rDNA restriction profiles of the four *A. felis* strains or between *A. broomeae* B-91-007288 and B-91-007289 with any of the three restriction enzyme combinations. rDNA restriction profiles of *A. clevelandensis* and the other cat scratch-like strains were different from the two profiles described above and from each other (Fig. 2 to 4). Although the individual patterns and the number of bands obtained with rDNA restriction by *Eco*RI (Fig. 2) or by the combination of *Pst*I and *Bgl*II (Fig. 3) were different, the strain groupings obtained were identical. Double digestion with *Sma*I-*Cla*I yielded similar results, except that the rDNA restriction profile of *A. broomeae* B-91-007286 was essentially identical to those of *A. broomeae* B-91-007288 and B-91-007289 (Fig. 4). The four *A. felis* strains are one ribotype, the three strains of *A. broomeae* comprise two ribotypes with a high relatedness index of 0.075, and *A. clevelandensis* and *Afipia* genospecies 1, 2, and 3 are separate ribotypes.

Visual examination of the rDNA fragment patterns revealed 9 unique *Eco*RI fragments, 15 unique *Sma*I-*Cla*I fragments, and 15 unique *Pst*I-*Bgl*II fragments, for a total of 39 unique fragments. A dendrogram constructed from the ribotyping data (Fig. 5) shows the degree of relatedness among the isolates. Seven ribotypes were obtained that

TABLE 5. DNA relatedness of *Afipia* strains

Source of unlabeled DNA	% Relatedness to labeled DNA from[a]:														
	B-91-007352			B-91-007353			B-91-007289			B-91-007287			B-91-007290		
	65°C	D	80°C	65°C	D	80°C	65°C	D	80°C	65°C	D	80°C	65°C	D	80°C
A. felis															
B-91-007352	100	0.0	100	32	11.5	16	16	15.5		21	18.0		17	18.0	
B-91-007147	98	0.5	99	26	13.5	12									
B-90-007209	96	0.5	97	25	13.5	11									
B-90-007260	95	0.5	99	28	14.0	12	12	17.0							
A. clevelandensis B-91-007353	35	7.0	10	100	0.0	100	36	10.5		24	17.0		22	16.0	
A. broomeae															
B-91-007289	22	11.0		60	10.5	42	100	0.0	100	22	18.0		24	17.5	
B-91-007286	25	10.0		56	9.5	37	77	2.5	71						
B-91-007288	23	10.5		59	9.5	39	98	0.0	98						
Afipia genospecies 1 B-91-007287	19	11.5		29	14.0	15	19	14.0		100	0.0	100	66	8.0	53
Afipia genospecies 2 B-91-007290	18	11.5		34	12.5	19	32	9.5		69	8.5	56	100	0.0	100
Afipia genospecies 3 B-91-007291	42	3.5	34	35	11.5	20	25	12.0		43	10.0		32	12.5	

[a] A blank space indicates that the reaction was not done. D, divergence, calculated to the nearest 0.5%.

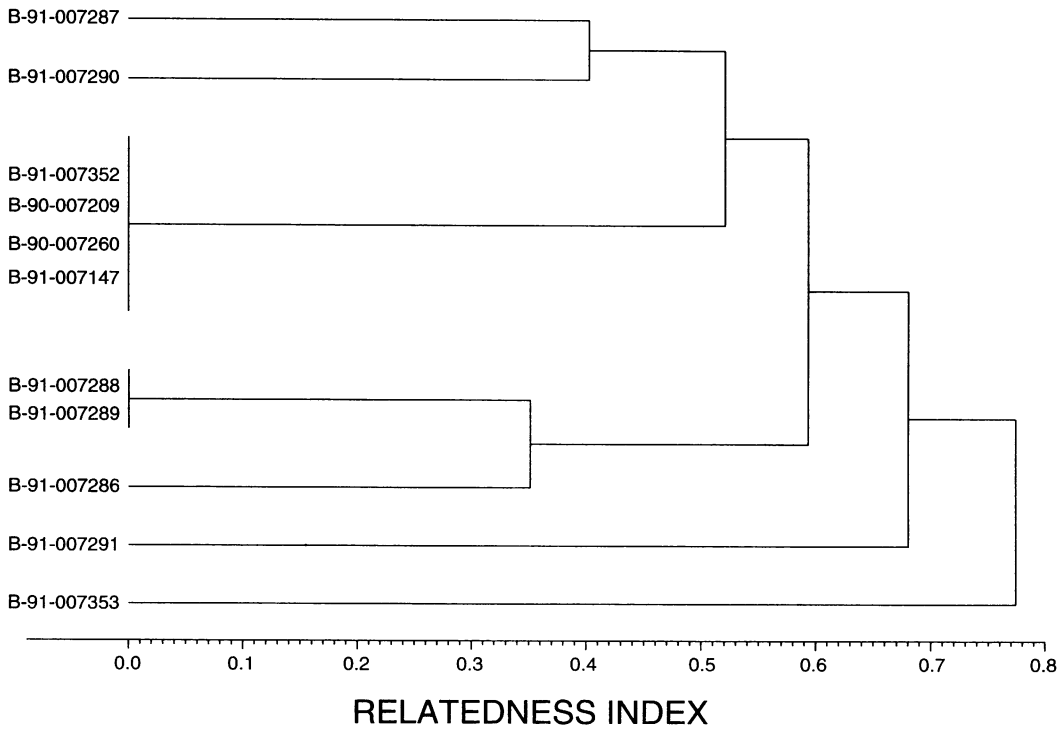

FIG. 1. Dendrogram of ETs obtained with *Afipia* strains. The vertical axis has no units, and the vertical distance between ETs has no quantitative meaning.

corresponded to the seven ETs and the six species identified by DNA hybridization.

The fluorescent-antibody conjugate made against *A. felis* B-91-007352, AFIP strain BV, reacted at a titer of 1,280 with both the homologous strain and *A. felis* B-91-007147, B-90-007209, and B-90-007260, the three CDSB strains isolated at the Centers for Disease Control, but did not react with strains of *A. clevelandensis*, *A. broomeae*, or the unnamed *Afipia* genospecies (Table 6). The fluorescent-antibody conjugate made against *A. clevelandensis* B-91-007353 showed a homologous titer of 640. The conjugate prepared against *A. clevelandensis* did not react with *A. broomeae*, any of the *Afipia* genospecies, or *A. felis* BV (B-91-007352), but it did show weak cross-reactivity with the three *A. felis* strains isolated at the Centers for Disease Control (Table 6).

The latex reagent prepared with the IgG from the anti-*A. felis* rabbit serum agglutinated all four *A. felis* strains. This reagent did not react with *A. clevelandensis*, *A. broomeae*, or *Afipia* genospecies 1, 2, and 3. The latex reagent prepared with the IgG from the anti-*A. clevelandensis* rabbit serum agglutinated only the homologous strain.

DISCUSSION

Previously reported rRNA sequence analyses showed that CSDB and the CCF strain were in the α-2 subgroup of the class *Proteobacteria* and that they were not related at the genus level to either *Bartonella bacilliformis* or *Rochalimaea quintana* or to the putative agent of bacillary epithelioid angiomatosis (on the basis of the partial rRNA sequence reported by Relman et al. [29]) (25). On the basis of their overall phenotypic similarity and the DNA relatedness data reported here, we identified four additional species that belong in the same genus. While rRNA sequence analyses

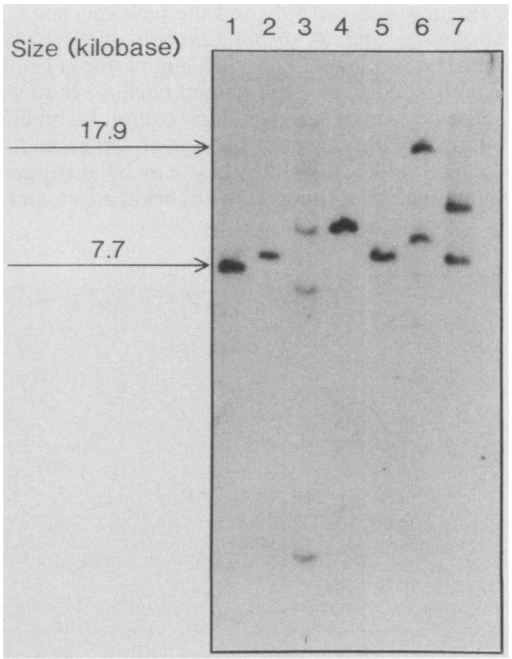

FIG. 2. rDNA restriction fragment patterns (ribotyping) of *Afipia* strains after digestion of total DNA with *Eco*RI. Lane 1, *A. felis* B91-007352, B90-007209, B90-007260, and B91-007147; lane 2, *A. clevelandensis* B91-007353; lane 3, *Afipia* genospecies 2 strain B91-007290; lane 4, *A. broomeae* B91-007288 and B91-007289; lane 5, *A. broomeae* B91-007286; lane 6, *Afipia* genospecies 3 strain B91-007291; lane 7, *Afipia* genospecies 1 strain B91-007287.

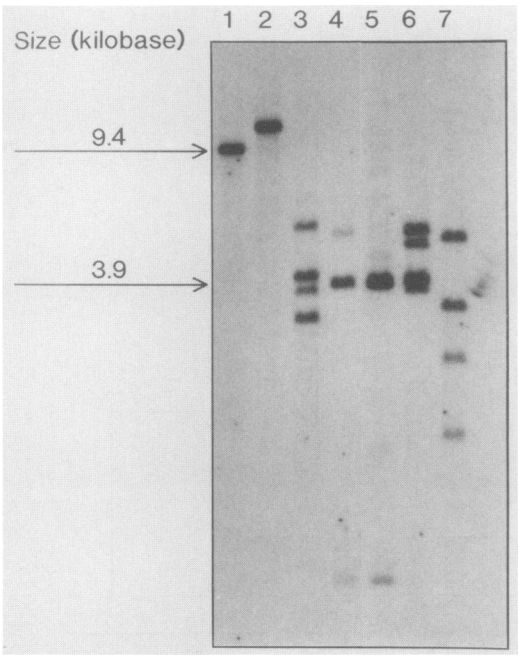

FIG. 3. Ribotyping of *Afipia* strains after digestion of total DNA with *Pst*I-*Bgl*II. Lane designations are the same as those in the legend to Fig. 2.

were not performed for the four additional species, their interspecies DNA relatedness values leave almost no doubt that all six species represent a single genus within the α-2 subgroup of the *Proteobacteria*. Formal taxonomic proposals for the new genus *Afipia* and the new species *A. felis*, *A. clevelandensis*, and *A. broomeae* are given below. Also described, but not named, are three new *Afipia* genospecies.

The genospecies were not named because they were each represented by single strains which cannot be biochemically differentiated with certainty from each other and from the three named species. While they can be distinguished by enzyme typing, ribotyping, DNA hybridization, and presumably, serotyping, we agree with Wayne et al. (32) that it is best to be conservative in naming genospecies.

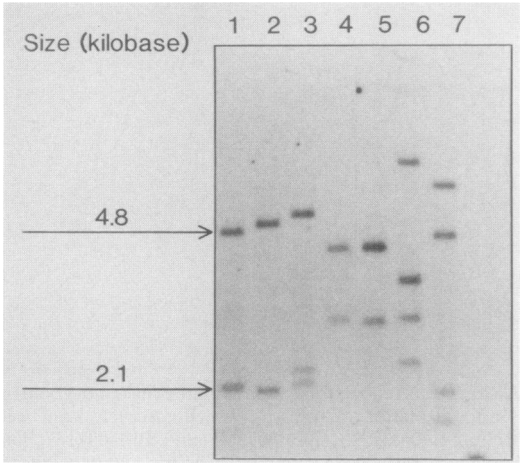

FIG. 4. Ribotyping of *Afipia* strains after digestion of total DNA with *Sma*I-*Cla*I. Lane designations are the same as those in the legend to Fig. 2.

In contrast to *A. felis*, which has not been isolated in a clinical laboratory, *A. clevelandensis*, *A. broomeae*, and *Afipia* genospecies strains were all cultured directly on bacteriologic media. While they grow somewhat slowly, there is no indication that they are difficult to isolate or maintain in culture.

Because they are slow growing and do not grow well at 37°C, the strains were incubated under conditions different from those normally used in determining MIC breakpoints. Therefore, one cannot describe resistance according to the standards developed by the National Committee for Clinical Laboratory Standards (24). The discussion below is subject to this caveat. *A. felis* was resistant to a broad array of antimicrobial agents, including many beta-lactam compounds, ciprofloxacin, and tetracycline. However, it was susceptible to the aminoglycosides, imipenem, and ceftriaxone. The three *A. broomeae* isolates varied in their susceptibility profiles to beta-lactams and ciprofloxacin, suggesting diversity within this species. The *A. clevelandensis* isolate was more susceptible than *A. felis* to beta-lactam compounds. Of the three *Afipia* genospecies, genospecies 1 and 2 were resistant to virtually all antimicrobial agents tested, while genospecies 3 was exquisitely susceptible to the agents tested, with the exception of cefoperazone, cefoxitin, and ceftazidime. Cefoperazone may have utility in a selective medium for the genus.

The fatty acid profiles of all strains were similar except for that of *Afipia* genospecies 3 strain B-91-007291, which lacked 17- and 19-carbon acids with a cyclopropane ring, and *Afipia* genospecies 1 strain B-91-007287, which contained $C_{15:0}$ and $C_{17:1omega6}$ that were absent in other strains. Strain B-91-007287 also lacked $C_{17:0cyc}$ and contained small amounts (3 to 4%) of $C_{17:0}$ and $C_{16:1omega5}$. These differences were reproducible; however, both B-91-007291 and B-91-007287 were easily recognized as members of the genus *Afipia* by the large amount of $C_{Br19:1}$. The presence of $C_{Br19:1}$ as a major fatty acid is a useful chemical marker for this group, since this acid has been detected only in some *Brucella* and *Pseudomonas* species, and always at trace to 2% levels.

On plasmid analysis, all of the *A. felis* strains contained a faint 29-mDa plasmid. Large-scale plasmid preparations are being made to allow examination of possible differences in these plasmids by restriction endonuclease analysis.

Enzyme typing and ribotyping divided the *Afipia* strains into the same six species that were obtained by DNA hybridization. However, without the DNA relatedness data and the recommended definition of a species based on DNA relatedness (3, 32), it would be difficult to correctly interpret species relatedness as judged by ET. For the genus *Afipia*, at least thus far, interspecies relatedness by enzyme typing is between a genetic distance of 0.35 and 0.40, and by ribotyping, interspecies relatedness is represented by a relatedness index of more than 0.075 to 0.18. These values will not be the same for all groups of organisms. The presence of relatively few fragments that hybridized with the rRNA probe in restriction digests indicates that members of the genus *Afipia* may have very few ribosomal operons. This may account in part for their relatively slow growth.

Serologic reactions of *Afipia* species with antibodies made against *A. felis* and *A. clevelandensis* reveal significant antigenic diversity within the genus, and perhaps some diversity within *A. felis*. Investigations with polyclonal and monoclonal antibodies are now under way to further evaluate the cross-reactions of some *A. felis* strains with antise-

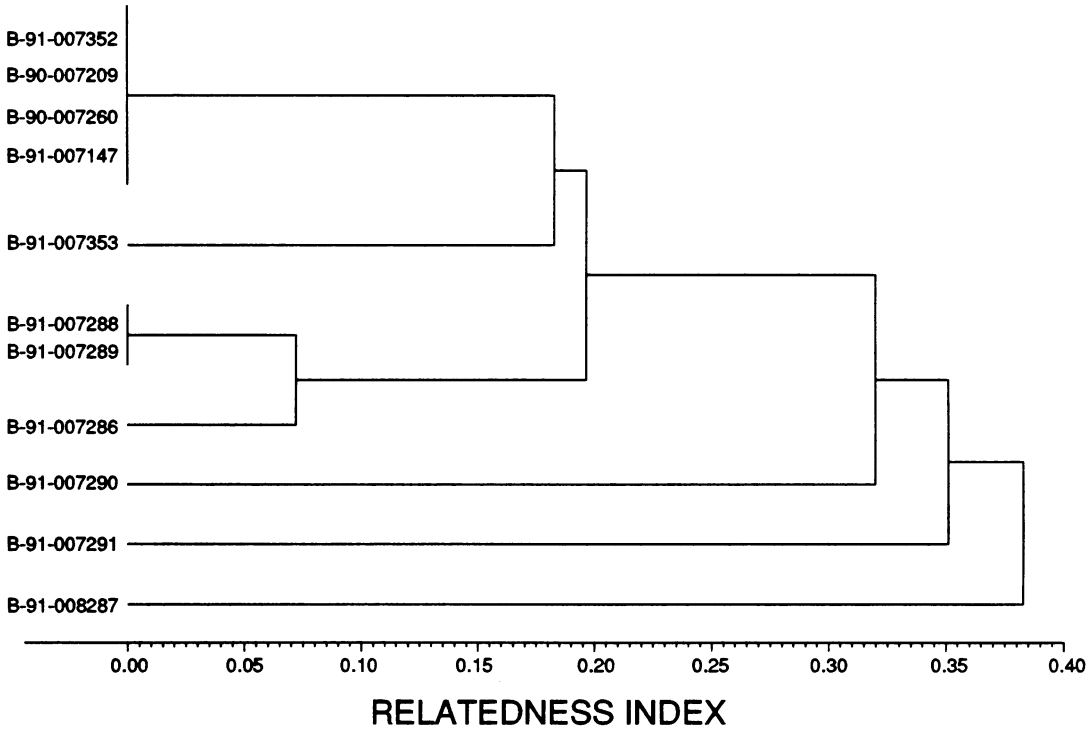

FIG. 5. Dendrogram of ribotypes obtained with *Afipia* species.

rum made to *A. clevelandensis* and to identify the antigen that is responsible. Studies are also under way to determine whether a polyclonal or monoclonal assay can be developed for the rapid diagnosis of CSD. While the latex reagent prepared with the IgG from anti-*A. felis* serum was species specific in tests with pure cultures, its utility, if any, as a diagnostic reagent or in the rapid identification of *A. felis* needs to be determined by additional tests with mixed cultures and clinical specimens.

The *A. felis* strains formed a single ET and a single ribotype, although there is some indication that the AFIP strain was separable from the other three strains by its lack of cross-reaction with antibody against *A. clevelandensis*. *A. felis* B-90-007209 and B-90-007260 were isolated from the same patient, and B-91-007147 was isolated from another patient in the same hospital, so perhaps it is not surprising that they gave identical reactions by ET, ribotyping, and serologic typing. Further subtyping analyses of these four *A. felis* strains and of many more strains are necessary before one can conclude anything about the clonal homogeneity of this species.

The role of *A. felis*, which was first isolated by English and colleagues (8, 9), as the etiologic agent of CSD has now been confirmed. Quinn and Birkness (27) have isolated 10 strains from lymph node tissues of nine patients with typical clinical presentations of CSD. Furthermore, a chromosomal probe designed by O'Connor and Swaminathan (26) against the original AFIP isolate (B-91-007352) has been used successfully to detect the presence of *A. felis* in more than 20 lymph nodes taken from persons with CSD. The three *A. felis* isolates from Hawaii were from two febrile children who presented with clinical CSD (Table 1). Neither child had underlying medical problems.

None of the other *Afipia* species have been linked with cats, and none of the persons from whom they were isolated had symptoms characteristic of CSD, although these possibilities have not been ruled out. Three of the seven strains were from wounds, three were from pleural fluid (two strains) or sputum, and one was from water. When known, the ages of infected persons were 60 to 81 years. *A. clevelandensis* was isolated from a pretibial biopsy specimen from a man who was hospitalized and who had multiple surgical procedures for severe necrotizing pancreatitis. He was in the intensive care unit for 8 months, had many episodes of sepsis and pneumonia, and died from complications of his diseases (11). *A. broomeae* was isolated from the synovial fluid of a man who had diabetes and arteriosclerosis. *Afipia* genospecies 2 was isolated from a woman who was 90% blind because of bilateral cataracts, who had Parkinson's disease, and who had a history of chronic progressive dementia. She complained of pain in the left upper chest and shoulder area. Radiology and computed tomography scans of the chest revealed a wedge-shaped infiltrate in the left upper lobe. Bronchoscopy revealed a golden yellow endobronchial exudate with underlying hemorrhage, and the organism was isolated from samples obtained during bronchoscopy.

Thus, *Afipia* species other than *A. felis* caused opportunistic respiratory and wound infections in patients who either were elderly or had other medical problems.

Description of the genus *Afipia* gen. nov. *Afipia* (A.fip'i.a. N. L. fem. n. *Afipia*, derived from the abbreviation AFIP, for Armed Forces Institute of Pathology, where the type strain of the type species was isolated). Members of the genus are gram-negative, oxidase-positive rods in the α-2 subgroup of the class *Proteobacteria*. They are motile by means of a single polar, subpolar, or lateral flagellum. They grow on BCYE agar and nutrient broth, but not nutrient broth containing 6% NaCl, and rarely on MacConkey agar at 25 and 30°C; strains grow at least weakly at 35°C, but growth does not occur at 42°. Organisms are gray-white, glistening,

TABLE 6. Fluorescent-antibody reactions

Strain	Reactivity of *Afipia* strains with antibody conjugates[a]	
	Anti-B-91-007352	Anti-B-91-007353
A. felis		
B-91-007352	4+ (1,280)[b]	–
B-91-007147	4+ (1,280)[b]	2+ (20)
B-90-007209	4+ (1,280)[b]	2+ (20)
B-90-007260	4+ (1,280)[b]	2+ (20)
A. clevelandensis B-91-007353	–	4+ (640)
A. broomeae		
B-91-007286	–	–
B-91-007288	–	–
B-91-007289	–	–
Afipia genospecies 1 B-91-007287	–	–
Afipia genospecies 2 B-91-007290	–	–
Afipia genospecies 3 B-91-007291	–	–

[a] 4+, very bright staining of cells; weak fluorescence was graded as 3+ or 2+ on a diminishing scale; –, a negative reaction. Numbers in parentheses are the reciprocal of the highest dilution of the conjugate giving the indicated fluorescence intensity.

[b] Maximal staining was observed.

convex, and opaque colonies and are 1.5 mm wide with entire edges; they are formed after 72 h of incubation at 32°C on blood agar (9) or on BCYE agar, except that the size range is from 0.5 to 1.5 mm. They are urease positive and turn litmus milk alkaline. *Afipia* species are negative in reactions for hemolysis, gas production from nitrate, indole production, H_2S production (triple sugar iron method), gelatin hydrolysis, and esculin hydrolysis and are nonfermentative. Acid is not produced oxidatively from D-glucose, lactose, maltose, or sucrose.

Afipia species contain 11-methyloctadec-12-enoic ($C_{Br19:1}$), *cis*-octadec-11-enoic ($C_{18:1omega7c}$), and generally, 17- and 19-carbon acids with a cyclopropane ring as major acids, with only trace amounts of hydroxy acids. The G+C content is 61.5 to 69 mol%. The interrelatedness of species by DNA hybridization is 12 to 69%.

Organisms in the genus *Afipia* contain the etiologic agent of CSD, and other species are presumptively considered to be pathogenic for humans. The type species is *A. felis*.

Description of *A. felis* sp. nov. *A. felis* (fe'lis. L. gen. n. *felis*, of the cat, because a cat scratch or bite is the means by which this organism infects humans). *A. felis* exhibits all of the characteristics of the genus. It is 0.2 to 0.5 μm in diameter and 0.2 to 2.5 μm long (9). Cells grown in brain heart infusion broth for 72 h at 32°C and then incubated at 37°C for 3 weeks form delicate pleomorphic forms (9). *A. felis* is catalase, citrate, and D-mannitol negative. It reduces nitrates and produces acid oxidatively from D-xylose in a delayed, weak reaction. The biochemical characteristics of use in differentiating *A. felis* from other *Afipia* species are given in Table 2.

Antimicrobial susceptibility data are given in Table 3. *A. felis* has previously been reported by English and colleagues (8, 9) to be resistant to ampicillin, cefamandole nafate, cefazolin sodium, cefoperazone sodium, cephalothin sodium, chloramphenicol, clindamycin hydrochloride, erythromycin, nitrofurantoin, penicillin G, and tetracycline hydrochloride; to have intermediate or moderately susceptible to piperacillin sodium, sulfamethoxazole plus trimethoprim, ticarcillin disodium, and vancomycin hydrochloride; and to be susceptible to amikacin sulfate, cefotaxime sodium, cefoxitin sodium, gentamicin sulfate, mezlocillin sodium, netilmicin sulfate, and tobramycin sulfate by the API UniSept microvolume broth dilution breakpoint procedure (9).

The G+C content in each of the four *A. felis* strains was 62.5 mol%.

A. felis is pathogenic for humans and is the causative agent of CSD. The type strain is AFIP strain BV (= B-91-007352 = ATCC 53690), which was isolated by English and colleagues (8, 9) from a lymph node obtained from a person with CSD.

Description of *A. clevelandensis* sp. nov. *A. clevelandensis* (cleve.land.en'sis. N. L. fem. adj. *clevelandensis*, coming from Cleveland, Ohio, where the type strain was isolated). *A. clevelandensis* exhibits all of the characteristics of the genus, except that it gives delayed, weak growth on MacConkey agar. The species is catalase negative and negative in tests for nitrate reduction, citrate, and acid production from D-xylose and D-mannitol. The biochemical characteristics of use in differentiating this organism from other *Afipia* species are given in Table 2.

The antimicrobial susceptibility profile of *A. clevelandensis* is given in Table 3.

A. clevelandensis is presumptively pathogenic for humans. The type strain, and the only strain isolated to date, is B-91-007353 (= ATCC 49720). It has a G+C content of 64 mol%. It was isolated in Ohio in 1988 from a tibial biopsy specimen taken from a 69-year-old man.

Description of *A. broomeae* sp. nov. *A. broomeae* (broome'a.e. N. L. gen. n. *broomeae*, to honor Claire V. Broome for her many contributions to microbiology and epidemiology, especially with regard to legionellosis, toxic shock syndrome, and Brazilian purpuric fever, and for providing the stimulus and resources that made this study on *Afipia* possible). *A. broomeae* exhibits all of the characteristics of the genus. It is weakly catalase positive and shows delayed, weak acid production from D-xylose. The organism has negative reactions in tests for nitrate reduction, citrate, and D-mannitol. Growth at 35°C is weak. The biochemical characteristics of use in differentiating this organism from other *Afipia* species are given in Table 2.

The antimicrobial susceptibility profile of *A. broomeae* is given in Table 3.

The G+C content of each of three strains was 61.5 mol%.

The organism was isolated from human bone marrow, synovial fluid, and sputum; and it is presumptively pathogenic for humans. The type strain is B-91-007286 (= ATCC 49717), which was isolated from human sputum in New Zealand in 1981.

Description of *Afipia* genospecies 1. *Afipia* genospecies 1 exhibits all of the characteristics of the genus. It is weakly catalase positive and is negative for nitrate reduction. It is citrate positive and is weakly positive for acid production from D-xylose and D-mannitol. The biochemical characteristics of use in differentiating it from other *Afipia* species are given in Table 2. It can be differentiated from *A. clevelandensis* serologically, by DNA hybridization, enzyme typing, and ribotyping.

The antimicrobial susceptibility pattern of *Afipia* genospecies 1 is given in Table 3.

The organism is presumptively pathogenic for humans.

The type and only strain is B-91-007287 (= ATCC 49721), which was isolated in 1981 in Oklahoma from human pleural fluid in a fatal case of pulmonary obstruction. It has a G+C content of 69 mol%.

Description of *Afipia* genospecies 2. *Afipia* genospecies 2 exhibits all of the characteristics of the genus. It is weakly catalase positive and is delayed, weakly D-xylose positive. It does not reduce nitrate, grow on citrate, or produce acid from D-mannitol. The biochemical characteristics of use in differentiating it from other *Afipia* species are given in Table 2.

The antimicrobial susceptibility pattern of *Afipia* genospecies is given in Table 3.

The organism is presumptively pathogenic for humans. The type and only strain is B-91-007290 (= ATCC 49722), which was isolated in 1989 in Indiana from the bronchial wash of an 80-year-old woman with pneumonia. It has a G+C content of 67 mol%.

Description of *Afipia* genospecies 3. *Afipia* genospecies 3 exhibits all of the characteristics of the genus. It is weakly catalase positive and has delayed and weak acid production from D-xylose. It is nitrate, citrate, and D-mannitol negative. It has weak production of H_2S (lead acetate method only). The biochemical characteristics of use in differentiating it from other *Afipia* species are given in Table 2.

The antimicrobial susceptibility pattern of *Afipia* genospecies 3 is given in Table 3.

It is presumptively pathogenic for humans. The type and only strain is B-91-007291 (= ATCC 49723), which was isolated in 1990 from water in Indiana. It has a G+C content of 65.5 mol%.

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