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Description of *Afipia birgiae* sp. nov. and *Afipia massiliensis* sp. nov. and recognition of *Afipia felis* genospecies A

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On the basis of phenotypic characterization and DNA relatedness, two novel species are proposed, *Afipia birgiae* sp. nov. (type strain 34632^T = CIP 106344^T = CCUG 43108^T) and *Afipia massiliensis* sp. nov. (type strain 34633^T = CIP 107022^T = CCUG 45153^T). A new genospecies is described, named *Afipia felis* genospecies A, closely related to *Afipia felis*. The complexity encountered in the taxonomy of the *Bradyrhizobiaceae* group within the α-2 subgroup of the *Proteobacteria* is discussed and the description of these novel species highlights the need for new tools for phylogenetic analysis in the group. The novel species herein described are fastidious bacteria isolated from a hospital water supply in co-culture with amoebae. It is hypothesized that this group of bacteria are a potential cause of nosocomial infections.

Keywords: *Afipia*, *Afipia massiliensis* sp. nov., *Afipia birgiae* sp. nov., *Afipia felis* genospecies A, DNA–DNA hybridization

INTRODUCTION

A previously undescribed Gram-negative bacillus was isolated from lymph nodes of patients with cat scratch disease (CSD) (English *et al*., 1988). This bacterium, which was shown to belong to the α-*Proteobacteria*, was named *Afipia felis* (Brenner *et al*., 1991). Brenner *et al*. (1991) described the genus *Afipia* and two other species (*Afipia clevelandensis* and *Afipia broomeae*) and recognized three unnamed genospecies (1, 2 and 3). Apart from the report by English *et al*. (1988), very little direct microbiological evidence linking *A. felis* and CSD has been presented and it is now clear that nearly all cases of CSD are due to a different bacterium, *Bartonella henselae* (Jerris & Regnery, 1996). Birkness *et al*. (1992) demonstrated that *A. felis* has the capacity to grow intracellularly and have proposed a tissue culture protocol to isolate this species. The mechanism of intracellular survival of *A. felis* was shown to be due to a phagosome-lysosome inhibiting factor, uptake by macrophages being induced directly into a non-endocytic compartment (Brouqui & Raoult, 1993; Lührmann *et al*., 2001). This bacterium has also been shown to have similar axenic growth requirements to *Legionella* spp. These findings led us to test whether *A. felis*, like legionellae, was able to parasitize free-living amoebae (La Scola & Raoult, 1999). These co-culture studies did indeed demonstrate that *A. felis* has the ability to grow associated with amoebae, leading us to hypothesize that, *in vivo*, this species may exist in aquatic environments. By using an amoebal co-culture procedure, 68 strains of diverse α-*Proteobacteria* were isolated from our hospital environmental supplies. By amplification and sequencing of the 16S rRNA genes of these isolates, several isolates were found that were closely related to *Afipia* species (La Scola *et al*., 2000). From the results of 16 rRNA gene sequencing of members of the *Rhizobiaceae* and DNA–DNA hybridization studies, two novel species, *Afipia birgiae* sp. nov. and *Afipia massiliensis* sp. nov., and a new genospecies, *A. felis* genospecies A, closely related to *Afipia felis*, are described.

Abbreviation: CSD, cat scratch disease.

METHODS

Strains. The strains used in this study are listed in Table 1. With the exception of *A. felis* strains, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), all reference strains were obtained from the Collection de l'Institut Pasteur (CIP, Paris, France). All

Table 1. Strains used in this study

Strain	Reference(s)
Afipia felis	
B-91-007352^T (= ATCC 53690^T)	Brenner *et al.* (1991), English *et al.* (1988)
B-91-007147 (= ATCC 49714)	Brenner *et al.* (1991)
B-90-007209 (= ATCC 49715)	Brenner *et al.* (1991)
B-90-007260 (= ATCC 49716)	Brenner *et al.* (1991)
Afipia clevelandensis	
B-91-007353^T (= ATCC 49720^T)	Brenner *et al.* (1991), Hall *et al.* (1991)
Afipia broomeae	
B-91-007286^T (= ATCC 49717^T)	Brenner *et al.* (1991)
B-91-007288 (= ATCC 49718)	Brenner *et al.* (1991)
B-91-007289 (= ATCC 49719)	Brenner *et al.* (1991)
Afipia genospecies 1	
B-91-007287 (= ATCC 49721)	Brenner *et al.* (1991)
Afipia genospecies 2	
B-91-007290 (= ATCC 49722)	Brenner *et al.* (1991)
Afipia genospecies 3	
B-91-007291 (= ATCC 49723)	Brenner *et al.* (1991)
Afipia genospecies 3-related strains	
34626 (= CIP 106343 = CCUG 43110)	La Scola *et al.* (2000)
34631	La Scola *et al.* (2000)
Afipia birgiae sp. nov.	
34632^T (= CIP 106344^T = CCUG 43108^T)	La Scola *et al.* (2000)
Afipia massiliensis sp. nov.	
34633^T (= CIP 107022^T = CCUG 45153^T)	La Scola *et al.* (2000)
Afipia felis genospecies A	
76713 (= CIP 106335 = CCUG 43109)	La Scola & Raoult (1999), La Scola *et al.* (2000)

strains were routinely subcultured on BCYE agar plates (bioMérieux) at 30 °C.

Phenotypic tests. The morphological properties of the strains were studied following Gram and Gimenez staining (Gimenez, 1964). Oxidase activity was detected using a dimethyl-*p*-phenylenediamine oxalate disk (Pasteur Diagnostic). Catalase activity was detected by emulsifying a colony in 3% hydrogen peroxide and checking for the presence of microscopic bubbles. Other biochemical tests were performed by inoculation of API 20NE and API 50CH strips (bioMérieux) according to the manufacturer's instructions followed by incubation at 30 °C. These strips were respectively incubated for 7 and 15 days. The API 20NE strip tested for any reduction of nitrates, indole production, urease activity, glucose acidification, arginine dihydrolase activity, hydrolysis of gelatin and aesculin, β-galactosidase activity and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. As interpretation of arginine dihydrolase and gelatinase activities on this strip was difficult, detection of these activities was later performed, according to the manufacturer's instructions, on ADH-ODC-LDC broth (Sanofi Diagnostics) and nutrient gelatin (Oxoid), respectively, which were then incubated at 30 °C for 7 days. H_2S production was tested using sodium thiosulfate substrate (bioMérieux). The API 50CH strip tested for any acidification of glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketoglutarate and 5-ketoglutarate. Growth and haemolysis were tested at 30 °C on Columbia agar with 5% sheep blood (bioMérieux), Columbia agar (bioMérieux) enriched with 0·2% yeast extract (Merck) and 5% sheep blood, MacConkey agar (bioMérieux) and nutrient broth (Difco) alone or with 6% NaCl. Growth on BCYE agar was attempted at 25, 30, 35, 37 and 42 °C. The presence of flagella was assessed by depositing bacteria cultured on BCYE agar plates on Formvar film and staining with a 0·33% solution of uranyl acetate before examination with a JEOL JEM 1200 EX electron microscope.

Antimicrobial susceptibility testing. The strains were grown for 72–96 h on BCYE agar prior to testing. Antibiotic susceptibility testing was performed using the microbroth dilution method in nutrient broth. For the testing of cotrimoxazole, 5% lysed horse blood was added. The final inoculum for all broth tests was 1×10^5–5×10^5 c.f.u. ml^{-1}. The plates were incubated at 30 °C and read 72–96 h later. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as controls.

SDS-PAGE analysis. The strains were harvested after 5–7 days of culture and suspended in deionized water for SDS-PAGE as described previously (Laemmli, 1970). Cell suspensions

(titrated to 4 mg protein ml^{-1}) and sample buffer 0·0625 M Tris/HCl, pH 8·0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0·02% bromophenol blue) were mixed in equal volumes. The proteins were then separated by electrophoresis in a 12% resolving gel and a 5% stacking gel at a constant current (8–10 mA) at room temperature for 4 h in running buffer (25 mM Tris, 192 mM glycine, 0·1% SDS) in an electrophoretic cell (Mini Protein II; Bio-Rad).

Phylogenetic study. 16S rDNA amplification from *A. felis* genospecies A, *A. birgiae* sp. nov., *A. massiliensis* sp. nov. and *Afipia* genospecies 3-related strains has been described previously (La Scola *et al.*, 2000). The phylogenetic relationships between *Afipia* and closely related bacteria were determined using PHYLIP software. *Rickettsia prowazekii* was taken as an outgroup. A distance matrix generated by DNADIST was determined under the assumptions of Kimura (1980). A dendrogram was inferred from this matrix using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap replicates were performed to estimate the reliability of nodes of the dendrogram obtained. Bootstrap values were obtained from 1000 trees generated randomly with SEQBOOT in the PHYLIP software package.

DNA–DNA relatedness. DNA was extracted and purified as described previously (Brenner *et al.*, 1972). The procedures for labelling of DNA with tritium-labelled nucleotides and for hybridization experiments (S1 nuclease treatment, trichloroacetic acid procedure) have been detailed elsewhere (Grimont *et al.*, 1980; Khammas *et al.*, 1989).

G+C content analysis. DNA was extracted as described previously (Ausubel *et al.*, 1995). Nucleoside residue analysis was performed by HPLC using a model L6200A system pump and a UV detector L4250 (Merck). A 5 µl aliquot of sample was applied onto a Nucleosil 100-SC18 Lichrocart column (4 × 250 mm) (Merck). Elution was carried out at room temperature using a mixture of 0·2 M $NH_4H_2PO_4$ (pH 4·5) and acetonitrile (96:4, v/v). A flow rate of 1 ml min^{-1} was used and absorbance was monitored at 270 nm. The calibration curve was obtained from a mixture of four standard nucleosides (Sigma) at a concentration of 0·1 mg ml^{-1} each in distilled water. After chromatography, the relative concentration of each nucleotide was calculated on the basis of peak areas in HPLC dilution profiles and corrected by coefficients of relative molar absorption as described previously (Tamaoka & Komagata, 1984).

Whole-cell fatty acid composition. Whole-cell fatty acids were analysed for *A. felis* genospecies A, *A. birgiae* sp. nov., *A. massiliensis* sp. nov. and *Afipia* genospecies 3-related strains by GC as described previously (Miller & Berger, 1985) using 3- to 5-day-old cultures of the isolates grown on BCYE agar.

RESULTS AND DISCUSSION

The biochemical reactions that allow differentiation of the strains studied are given in Table 2. No strains gave visible colonies before 72 h at 30 °C when grown on BCYE agar. All strains were Gram-negative, Gimenez-positive, oxidase-positive and weakly catalase-positive, had urease activity and grew on nutrient broth and Columbia agar with 5% sheep blood and 0·2% yeast extract, but were not haemolytic. All strains assimilated gluconate and grew at 25 and 30 °C, but gave negative reactions in tests for arginine dihydrolase activity, aesculin and gelatin hydrolysis, β-galactosidase activity, H_2S production and acid production by fermentation or oxidation of substrates tested in API 50CH. Strains failed to grow at 42 °C or on nutrient broth containing 6% NaCl. With the exception of *A. birgiae* sp. nov., all strains were motile by means of a single polar or subpolar flagellum. The strains differed in their abilities to grow on Columbia agar with 5% sheep blood, on MacConkey agar and at 35 and 37 °C, to reduce nitrate and to assimilate glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, adipate, malate, citrate and phenylacetate. *A. clevelandensis* was the only strain that grew on MacConkey agar. *Afipia* genospecies 3-related strains, *A. massiliensis* sp. nov. and *A. birgiae* sp. nov. did not grow at 35 or 37 °C; *A. clevelandensis* grew weakly at 35, but not at 37 °C. *A. felis*, *A. felis* genospecies A, *Afipia* genospecies 3-related strains, *A. birgiae* and *A. massiliensis* were able to reduce nitrate. SDS-PAGE analysis revealed that each species yielded a specific protein profile (Fig. 1). However, different strains of the same species (*A. felis*, *A. broomeae*, *Afipia* genospecies 3-related strains) yielded the same profile. *A. felis* genospecies A was distinguished from *A. felis* by the slightly lower molecular mass for a major protein band at about 50 kDa and by the lack of a major protein of 17 kDa. *A. birgiae* and *A. massiliensis* could be differentiated by comparison of their low-molecular-mass proteins. The whole-cell fatty acid compositions of the 17 strains are given in Table 3. These profiles showed that all strains shared relatively large amounts of hexadecanoic acid ($C_{16:0}$), *cis*-octadec-9-enoic acid ($C_{18:1\omega7c}$) and octadecanoic acid ($C_{18:0}$). With the exception of *A. birgiae*, all strains contained *cis*-hexadec-9-enoic acid ($C_{16:1\omega7c}$). *Afipia* genospecies 1 and *Afipia* genospecies 2 were the only species that contained *cis*-hexadec-7-enoic acid ($C_{16:1\omega5c}$). *Afipia* genospecies 3 and *Afipia* genospecies 3-related strains had no detectable *cis*-cyclo-10,11-methylene octadecanoic acid ($C_{19:0cyclo\omega8c}$) and *Afipia* genospecies 3-related strains and *A. birgiae* had no detectable 11-methyloctadec-12-enoic acids ($C_{Br19:1}$). *A. birgiae* and *Afipia* genospecies 3-related strains were the only strains that possessed 11-methyl *cis*-octadec-9-enoic acid ($C_{18:1\omega8c}$ 11-methyl). *A. felis* genospecies A differed from *A. felis* by the presence of a large amount of *cis*-octadec-9-enoic acid 11-methyl ($C_{18:1\omega7c}$ 11-methyl). Antimicrobial susceptibility testing, summarized in Table 4, showed that *A. felis* genospecies A may be differentiated phenotypically from *A. felis* by its high susceptibility to amoxicillin, cefalotin, ceftriaxon and cefepim. It is interesting to note that, if they are implicated in human infections, the only antibiotics that have reliably good efficacy on the *Afipia* group are rifampin and gentamicin, as reported for the strains studied previously (Brenner *et al.*, 1991; Maurin *et al.*, 1993).

The G+C contents of *Afipia* species ranged from 59·3 to 69·0 mol%. DNA–DNA hybridization demonstrated that *A. felis* genospecies A, *A. massiliensis* and

Table 2. Biochemical reactions and G+C contents of *Afipia* species

Species: 1, *A. felis* (4 strains); 2, *Afipia felis* genospecies A; 3, *A. clevelandensis*; 4, *Afipia* genospecies 1; 5, *Afipia* genospecies 2; 6, *Afipia* genospecies 3; 7, *Afipia* genospecies 3-related strains (2 strains); 8, *A. broomeae* (3 strains); 9, *A. birgiae* sp. nov.; 10, *A. massiliensis* sp. nov. +, Positive reaction; $+^{w}$, weak; −, negative reaction. G+C content data for strains other than *A. felis* genospecies A, *A. birgiae*, *A. massiliensis* and *Afipia* genospecies 3-related strains are from Brenner *et al.* (1991).

Test	1	2	3	4	5	6	7	8	9	10
Motility	+	+	+	+	+	+	+	+	−	+
Growth on:										
Columbia agar/5% sheep blood	$+^{w}$	$+^{w}$	+	−	−	$+^{w}$	$+^{w}$	−	−	−
MacConkey agar	−	−	$+^{w}$	−	−	−	−	−	−	−
Growth on BCYE agar at:										
35 °C	+	+	$+^{w}$	+	+	+	−	+	−	−
37 °C	+	+	−	+	+	+	−	+	−	−
Nitrate reduction	+	+	−	−	−	−	+	−	+	+
Assimilation of:										
Glucose	−	−	−	+	+	−	−	−	−	−
Arabinose	−	−	−	+	+	−	−	−	−	−
Mannose	−	−	−	+	+	−	−	−	−	−
Mannitol	−	−	−	+	+	−	−	−	−	−
N-Acetylglucosamine	−	−	−	+	+	−	−	−	−	−
Gluconate	−	−	+	+	+	+	−	+	−	−
Adipate	−	−	+	+	+	+	+	+	+	+
Malate	+	+	+	+	−	+	+	+	−	−
Citrate	−	−	−	+	−	−	−	−	−	−
Phenylacetate	−	−	−	+	−	+	−	+	−	−
G+C content (mol%)	62·5	63·1	64·0	69·0	67·0	65·5	61·9	61·5	59·3	60·2

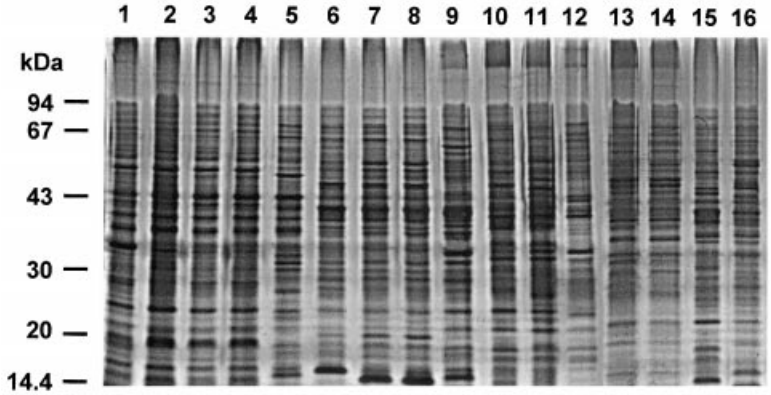

Fig. 1. Silver-stained SDS-PAGE gel of whole-cell protein preparation of *Afipia* species. Lanes: 1, *A. felis* B-91-007352^{T}; 2, *A. felis* B-91-007147; 3, *A. felis* B-90-007209; 4, *A. felis* B-90-007260; 5, *A. felis* genospecies A; 6, *A. broomeae* B-91-007289; 7, *A. broomeae* B-91-007286^{T}; 8, *A. broomeae* B-91-007288; 9, *A. clevelandensis*; 10, *Afipia* genospecies 1; 11, *Afipia* genospecies 2; 12, *Afipia* genospecies 3; 13, *Afipia* genospecies 3-related strain 34626; 14, *Afipia* genospecies 3-related strain 34631; 15, *A. birgiae*; 16, *A. massiliensis*.

A. birgiae differed from previously described *Afipia* species (Table 5). Analysis of the 16S rRNA gene sequences of our hospital isolates and previously described *Afipia* species demonstrated that they belong to the *Bradyrhizobium* group, within the *Rhizobiaceae*, within the α-*Proteobacteria* (Fig. 2). Nevertheless, the position of these bacteria within the *Bradyrhizobium* group is unclear. Within the *Bradyrhizobium* group, two major clusters can be distinguished: one that includes *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992) and *Afipia* genospecies 1 and 2 (Brenner *et al.*, 1991) and the other including *Bradyrhizobium japonicum* and *Bradyrhizobium liaoningense* (Jordan, 1982; Xu *et al.*, 1995), all other *Afipia* species and members of the genera *Nitrobacter* (Grundmann *et al.*, 2000; Navarro *et al.*, 1992; Orso *et al.*, 1994; Sorokin *et al.*, 1998), *Agromonas* (Ohta & Hattori, 1983), *Blastobacter* (Hirsch & Müller, 1985) and *Rhodopseudomonas* (Hougardy *et al.*, 2000). Moreover, several 16S rRNA gene sequences of *Afipia* genospecies available in GenBank (*Afipia* genospecies 4–14), for which no description or strain is available, are distributed among very distant groups. It is clear that most of these '*Afipia*' should not be considered as members of the genus *Afipia* or other genera of this group until strain descriptions are available. The inconsistencies in the

Table 3. Fatty acid composition of *Afipia* strains

Values are percentages of total fatty acids. Strains: 1, *A. felis* (a, B-91-007352^T; b, B-91-007147; c, B-90-007209; d, B-90-007260); 2, *A. felis* genospecies A; 3, *A. clevelandensis*; 4, *Afipia* genospecies 1; 5, *Afipia* genospecies 2; 6, *Afipia* genospecies 3; 7, *Afipia* genospecies 3-related strains (a, 34626; b, 34631); 8, *A. broomeae* (a, B-91-007289; b, B-91-007286^T; c, B-91-007288); 9, *A. birgiae*; 10, *A. massiliensis*. Data for strains other than *A. felis* genospecies A, *A. birgiae*, *A. massiliensis* and *Afipia* genospecies 3-related strains are from Brenner *et al.* (1991). tr, Less than 1%; –, not detected. $C_{16:0}$ 3-OH and $C_{18:1\omega9c}$ were not found in any of the strains shown.

Fatty acid	1a	1b	1c	1d	2	3	4	5	6	7a	7b	8a	8b	8c	9	10
$C_{15:0}$	–	–	–	–	–	–	2	tr	–	–	–	–	tr	–	3	–
$C_{16:1\omega9c}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–
$C_{16:1\omega7c}$	6	5	5	9	17	7	2	1	3	12	9	5	9	5	–	5
$C_{16:1\omega5c}$	–	–	–	–	–	–	4	3	–	–	–	–	–	–	–	–
$C_{16:0}$	4	3	3	4	3	3	6	6	9	23	18	4	5	4	3	5
$C_{17:1\omega9c}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	12	–
$C_{17:1\omega8c}$	–	–	–	–	–	–	tr	–	1	–	–	tr	1	–	–	–
$C_{17:1\omega6c}$	–	–	–	–	–	–	6	–	tr	–	–	–	–	–	–	–
$C_{17:0cyclo}$	7	9	13	11	13	16	–	3	–	–	–	22	15	22	25	22
$C_{17:0}$	–	–	–	–	–	–	3	–	–	–	–	–	3	–	18	1
$C_{17:0}$ iso 2-OH	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11	–
$C_{18:1(c11/t9/t6;t9/t6/c11;t6/t9/c11)}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–
$C_{18:1\omega7c}$	43	28	26	40	40	27	40	34	65	44	50	24	24	23	–	15
$C_{18:1\omega7c}$ 11-methyl	–	–	–	–	15	–	–	–	–	15	17	–	–	–	–	25
$C_{18:0}$	12	11	9	9	7	9	2	5	6	5	6	10	12	9	6	12
$C_{18:3\omega6c}$	–	–	–	–	–	–	–	–	–	tr	–	–	–	–	–	–
$C_{Br19:1}$	14	24	23	13	–	30	24	30	15	–	–	25	23	25	–	–
$C_{19:0cyclo\omega8c}$	12	28	21	14	5	5	11	15	–	–	–	9	5	9		14
$C_{19:0cyclo c11–12}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–
$C_{19:0}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–
$C_{19:0}$ iso	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	tr
$C_{20:1\omega7c}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
$C_{20:1\omega9c}$	–	–	–	–	–	1	–	–	–	–	–	1	1	1	–	–

classification of bacteria of the *Bradyrhizobium* group were highlighted soon after the official description of the genus *Afipia* (Willems & Collins, 1992) and later by others working on the phylogeny of this group of bacteria (Anzai *et al.*, 2000; Teske *et al.*, 1994; Lafay & Burdon, 1998; Wong *et al.*, 1994; Willems *et al.*, 2001; Hougardy *et al.*, 2000). The key question raised by several authors is the validity of including these species in a single genus on the basis of 16S rDNA sequences (as members of this group exhibit levels of sequence similarity of 97% or greater). It should be noted that genomic data are increasingly available for these species (up to 40 16S rDNA sequences for the *Bradyrhizobium* species) (Willems *et al.*, 2001), but phenotypic data that could help in the polyphasic taxonomy of these bacteria are still lacking. As observed among *Bradyrhizobium* species and for the species described in this study or as described previously for *Bacillus* species (Ash *et al.*, 1991; Fox *et al.*, 1992), over-reliance on 16S rDNA sequence comparisons may be foolhardy and should therefore be associated with DNA–DNA hybridization data (Stackebrandt *et al.*, 1994). In this study, *A. felis* and *A. felis* genospecies A represent two distinct genospecies on the basis of DNA–DNA hybridization and phenotypic data such as susceptibility to antibiotics, SDS-PAGE profile and whole-cell fatty acid composition (Wayne *et al.*, 1987; Grimont *et al.*, 1998), whereas they exhibit levels of 16S rDNA sequence similarity of 99·9%. An approach comparing sequences from more divergent parts of the genome such as the *rrs–rrl* intergenic spacer has been described for *Nitrobacter* species (Grundmann *et al.*, 2000) and *Bradyrhizobium* species (van Berkum *et al.*, 2000) and seems to be more powerful for differentiation. It therefore appears that even slight divergence in 16S rDNA sequences of bacteria of this group should be considered as significant. Moreover, it seems difficult to group in the same genus, human pathogens such as *Afipia*, anaerobic photosynthetic bacteria such as *Rhodobacter palustris*, a budding chemoheterotroph such as *Blastobacter denitrificans* or nitrite-oxidizers such as members of the genus *Nitrobacter*. Improved classification of the *Bradyrhizobium* group could be obtained by delineating new genera that would better reflect the heterogeneity of this group. In the tree obtained in this study, several clusters may be delineated (Fig. 2), such as a cluster with *Nitrobacter* species, which is in accordance with the denomination of members of this genus and with a previous study using more divergent

Table 4. Antimicrobial susceptibility profiles of *Afipia* species

Values are MIC (mg l^{-1}). Strains: 1–10, see legend to Table 3; 11, *Escherichia coli*; 12, *Enterococcus faecalis*. AMC, Amoxicillin/clavulanic acid. For cotrimoxazole, the MIC is given for trimethoprim.

Agent	1a	1b	1c	1d	2	3	4	5	6	7a	7b	8a	8b	8c	9	10	11	12
Penicillin	32	16	16	16	1	32	256	32	32	0·5	32	16	64	32	> 256	16	64	2
Amoxicillin	8	8	8	4	⩽ 0·25	64	256	16	8	⩽ 0·25	64	16	64	64	64	32	4	1
AMC	4	4	4	4	⩽ 0·25	64	64	8	8	⩽ 0·25	2	16	64	64	0·5	32	4	1
Ticarcillin	256	64	128	128	8	128	> 256	64	64	4	128	128	> 256	256	256	128	8	64
Piperacillin	256	256	266	256	4	256	> 256	256	128	4	256	256	> 256	256	> 256	128	4	4
Cefoxitin	128	16	16	16	2	16	64	32	4	1	4	16	32	16	0·5	32	2	256
Cefalotin	8	8	8	8	0·25	0·5	128	64	⩽ 0·25	⩽ 0·25	8	0·5	2	2	4	2	8	8
Ceftriaxone	8	1	2	2	⩽ 0·25	1	> 256	128	2	1	⩽ 0·25	2	2	1	⩽ 0·25	1	⩽ 0·25	256
Ceftazidim	64	8	16	16	2	16	> 256	256	32	8	4	32	64	64	4	32	⩽ 0·25	> 256
Cefepim	16	4	8	8	⩽ 0·25	2	> 256	128	32	1	4	8	16	16	8	4	⩽ 0·25	32
Imipenem	1	⩽ 0·25	0·5	⩽ 0·25	⩽ 0·25	⩽ 0·25	32	4	1	⩽ 0·25	1	⩽ 0·25	0·5	⩽ 0·25	0·5	0·5	0·5	0·5
Amikacin	4	⩽ 0·25	⩽ 0·25	⩽ 0·25	⩽ 0·25	4	> 256	64	64	4	1	8	32	64	2	2	4	256
Netilmicin	1	⩽ 0·25	⩽ 0·25	⩽ 0·25	⩽ 0·25	2	> 256	32	8	4	⩽ 0·25	16	64	128	⩽ 0·25	32	1	8
Tobramycin	0·5	⩽ 0·25	⩽ 0·25	⩽ 0·25	⩽ 0·25	0·5	128	8	8	0·5	⩽ 0·25	1	2	4	⩽ 0·25	2	1	16
Gentamicin	1	⩽ 0·25	⩽ 0·25	⩽ 0·25	⩽ 0·25	1	32	16	4	0·5	0·5	4	16	16	⩽ 0·25	2	1	16
Ciprofloxacin	32	8	8	16	2	4	128	64	16	4	16	4	16	16	2	2	⩽ 0·25	1
Doxycycline	32	32	32	32	16	> 256	32	32	16	16	4	> 256	> 256	> 256	4	16	0·5	4
Rifampin	2	2	2	2	0·5	0·5	32	8	4	0·5	1	1	2	1	1	1	4	0·5
Cotrimoxazole	2	1	1	1	1	⩽ 0·25	> 256	> 256	> 256	> 256	> 256	8	> 256	128	> 256	16	0·5	⩽ 0·25
Erythromycin	16	8	4	4	16	2	128	64	16	64	8	16	32	32	64	16	128	2
Colimycin	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	16	> 256
Vancomycin	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	2

Table 5. DNA relatedness among *Afipia* species

Source of unlabelled DNA	Relative reassociation (%) with labelled DNA from strain:			
	34626	**34632^T**	**34633^T**	**76713**
A. felis B-91-007352^T	6	6	7	45
A. felis genospecies A strain 76713	ND	ND	6	100
A. clevelandensis B-91-007353^T	8	25	25	9
Afipia genospecies 1 strain B-91-007287^T	9	9	8	11
Afipia genospecies 2 strain B-91-007290^T	10	13	7	11
Afipia genospecies 3 strain B-91-007291^T	10	6	7	9
Afipia genospecies 3-related strain 34626	100	7	1	4
Afipia genospecies 3-related strain 34631	97 (0·9)*	5	1	3
A. broomeae B-91-007286^T	8	29	29	13
A. birgiae sp. nov. 34632^T	6	100	40	8
A. massiliensis sp. nov. 34633^T	5	41	100	9

* ΔT_m value (in °C) given in parentheses.

ND, Not done.

genes (Grundmann *et al.*, 2000), a cluster with *A. felis*, *A. clevelandensis* and *A. felis* genospecies A and a cluster with *A. broomeae*, *A. birgiae* and *A. massiliensis*. Conversely, the position of *Afipia* genospecies 3 and *Afipia* genospecies 3-related strains, which are different species but probably in the same genus, is impossible to assess. *Afipia* genospecies 1 and 2 should be placed in a separate genus with *B. elkanii*. It is thus proposed to exclude these four species from the genus *Afipia*. The development of new molecular tools, such as sequencing of the *ompA* gene for *Rickettsia* species (Fournier *et al.*, 1998), the *rpoB* gene for *Enterobacteriaceae* (Mollet *et al.*, 1997) or the *mip* gene for *Legionella* species (Ratcliff *et al.*, 1998), could help in the future to improve delineation of the different genera and species of this group.

The novel bacterial species described in this study were isolated from hospital water supplies using an amoebal co-culture procedure (La Scola *et al.*, 2000). In this study, it has been demonstrated that these bacteria were phenotypically and genotypically closely related to bacteria of the genus *Afipia*. The reservoir natural niche of members of the genus *Afipia*, which are recognized as human pathogens (Brenner *et al.*, 1991; Giladi *et al.*, 1998; Hall *et al.*, 1991; English *et al.*, 1988), remains unknown. Most *Afipia* infections, including those caused by *A. clevelandensis*, *A. broomeae* and *Afipia* genospecies 1 and 2, have been associated with either the elderly or patients with other underlying medical problems. *Afipia* genospecies 3 was isolated from water (Brenner *et al.*, 1991). An isolate of *A. broomeae* and *Afipia* genospecies 1 and 2 were isolated from pulmonary samples (Brenner *et al.*, 1991). *A. clevelandensis* was isolated from a patient after months of hospitalization (Hall *et al.*, 1991), supporting the conclusion that *A. clevelandensis* is capable of causing nosocomial infection. Furthermore, in a seroepidemiological study, several patients with elevated antibodies to this bacterium were diagnosed as having nosocomial infections including pneumonia (Drancourt *et al.*, 1997). It is speculated that, as *Afipia*-like bacteria have been isolated from hospital water supplies using amoebal co-culture, but have failed to grow on standard culture media, it is likely that, if they are causing hospital-acquired pulmonary infections, these are likely to be very difficult to diagnose.

Emended description of the genus *Afipia* (Brenner *et al.* 1992)

Afipia (A.fip′i.a. N.L. fem. n. *Afipia* derived from the acronym AFIP, for Armed Forces Institute of Pathology, where the type species was isolated).

Members of the genus are Gram-negative, but well stained by Gimenez staining, oxidase-positive, weakly catalase-positive rods in the α-2 subgroup of the *Proteobacteria*. Most are motile by means of single polar or subpolar flagella. They grow on BCYE agar and nutrient broth, but not on nutrient broth containing 6% NaCl. All grow well at 25 and 30 °C, but none grows at 42 °C. Colonies are grey-white, glistening, convex and opaque. All are urease-positive. Negative for haemolysis, arginine dihydrolase activity, aesculin and gelatin hydrolysis, β-galactosidase activity, H_2S production and acid production by fermentation or oxidation of most carbon substrates, including glucose, fructose, mannose, sucrose, mannitol and maltose. The G+C content is 59·3–64·0 mol%. The type species is *Afipia felis*.

Description of *Afipia birgiae* sp. nov.

Afipia birgiae (bir′gi.ae. N.L. fem. gen. n. *birgiae* of Birg, in honour of Marie-Laure Birg, a technician, for her many contributions to the isolation of strict intracellular bacteria, especially *Tropheryma whipplei*).

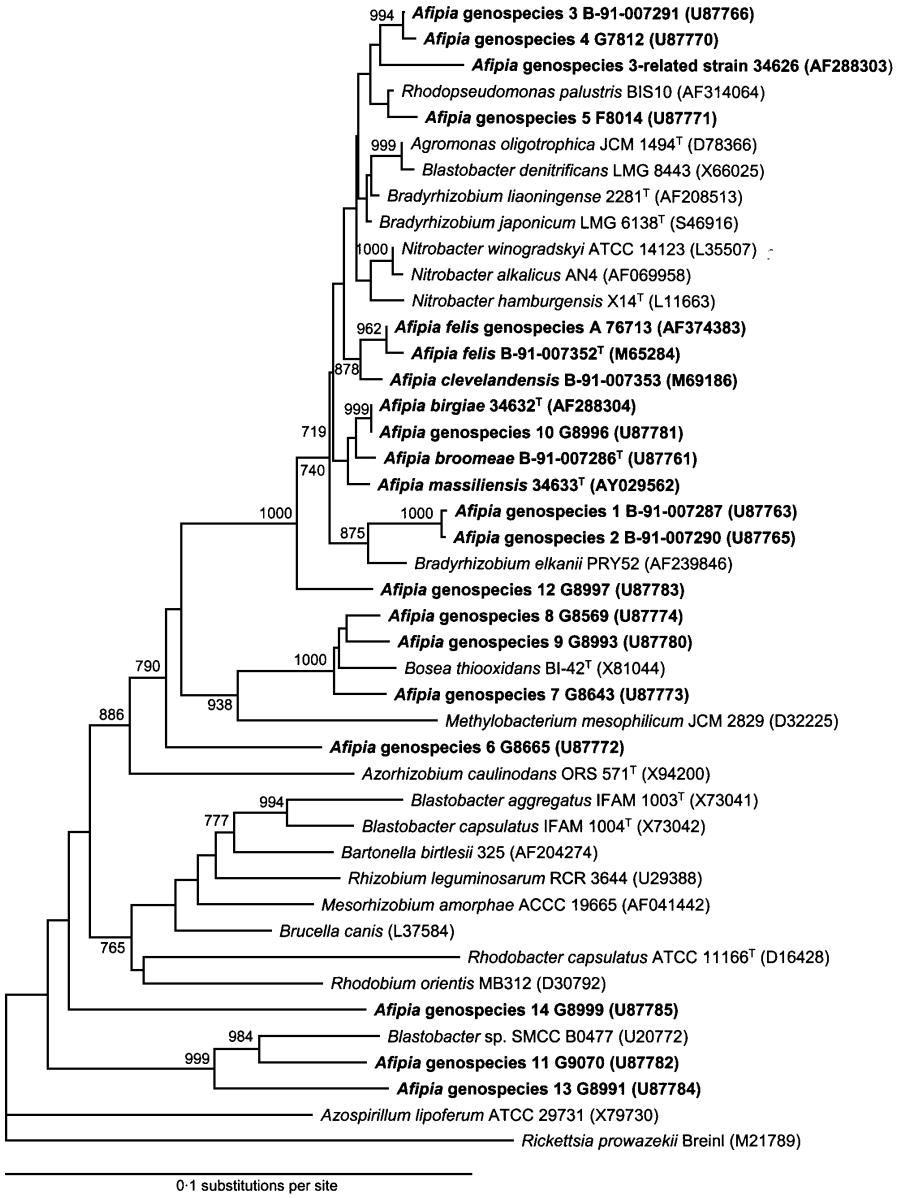

Fig. 2. Dendrogram representing phylogenetic relationships of *Afipia* species within the *Bradyrhizobiaceae* group and closely related bacteria among the α-2 subgroup of the *Proteobacteria*. The tree was derived from a 1202 bp fragment of the 16S rRNA gene. Support for each branch, as determined from 1000 bootstrap samples, is indicated by the value at the node if $\geqslant$ 700/1000.

Exhibits all of the characteristics of the genus, does not grow at 35 °C or on Columbia agar with 5% sheep blood and is not motile. Grows in co-culture with *Acanthamoeba polyphaga* in Page's amoebal saline. Reduces nitrate. Biochemical characteristics of use in differentiating this organism from other *Afipia* species are given in Table 2. The whole-cell fatty acid profile is given in Table 3. The antimicrobial susceptibility profile is given in Table 4. The G+C content is 59·3 mol%. Isolated from the water supply of the La Timone Hospital Centre (Marseilles, France). The type strain is strain 34632^T (= CIP 106344^T = CCUG 43108^T).

Description of *Afipia massiliensis* sp. nov.

Afipia massiliensis (mas.si.li.en′sis. L. fem. adj. *massiliensis* referring to Massilia, Latin name of Marseille, where the organism was isolated).

Exhibits all of the characteristics of the genus, does not grow at 35 °C or on Columbia agar with 5% sheep blood and is motile. Grows in co-culture with *Acanthamoeba polyphaga* in Page's amoebal saline. Reduces nitrate. Biochemical characteristics of use in differentiating this organism from other *Afipia* species are given in Table 2. The whole-cell fatty acid profile is given in Table 3. The antimicrobial susceptibility

profile is given in Table 4. The G+C content is 60·2 mol%. Isolated from the water supply of the La Timone Hospital Centre (Marseilles, France). The type strain is strain 34633^T (= CIP 107022^T = CCUG 45153^T).

Description of *Afipia felis* genospecies A

Exhibits all of the characteristics of the genus. Unique among members of the genus in its susceptibility to penicillins and cephalosporins. Reduces nitrate, grows at 37 °C and in co-culture with *Acanthamoeba polyphaga* in Page's amoebal saline. Does not assimilate gluconate or adipate. Biochemical characteristics of use in differentiating this organism from other *Afipia* species and members of the genus *Afipia* are given in Table 2. The whole-cell fatty acid profile is given in Table 3. The antimicrobial susceptibility profile is given in Table 4. The G+C content is 63·1 mol%. Isolated from the water supply of the La Timone Hospital Centre (Marseilles, France). The reference strain is strain 76713 (= CIP 106335 = CCUG 43109).

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