Proposals To Unify the Genera *Grahamella* and *Bartonella*, with Descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and Three New Species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov.

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Polyphasic methods were used to examine the taxonomic positions of three newly identified *Grahamella* species. A comparison of the 16S rRNA gene sequences of these organisms with the sequences available for other bacteria revealed that these three species form a tight monophyletic cluster with members of the genus *Bartonella*. This cluster is only remotely related to other members of the order *Rickettsiales*. Determinations of the levels of DNA relatedness between *Grahamella* species and *Bartonella* species (by using a modified hydroxyapatite method) revealed that all of the species belonging to these two genera are distinct but closely related. On the basis of these data and the results of guanine-plus-cytosine content and phenotypic characterization studies, we propose that the genera *Grahamella* and *Bartonella* should be unified and that the latter name should be retained. *Bartonella talpae* and *Bartonella peromysci*, new combinations for former *Grahamella* species, are created, and the following three new *Bartonella* species are described: *Bartonella grahamii*, *Bartonella taylorii*, and *Bartonella doshiae*. A taxonomic analysis of *Grahamella* species complete the study of all members of the family *Bartonellaceae*, and the results of this study support the proposal that the family should be transferred out of the order *Rickettsiales*.

Members of the family *Bartonellaceae* are gram-negative bacteria which can be grown in vitro on nonliving, blood-rich media. In vivo, these organisms are hemotrophic, parasitizing the erythrocytes of their hosts. Arthropod transmission of some members of the family has also been established (33). The family *Bartonellaceae* comprises two genera, *Bartonella* and *Grahamella* (28). The genotypic and phylogenetic relationships between these two genera are unknown, and at the present time these taxa are distinguished on the basis of the following three loose criteria: (i) *Bartonella* species, but not *Grahamella* species, infect humans; (ii) *Grahamella* species are thought to exist exclusively within the erythrocytes of their hosts, whereas *Bartonella bacilliformis* can exist on the erythrocyte cell surface; and (iii) *B. bacilliformis* has been shown to have flagella in culture, unlike *Grahamella* species (28).

For a long time the genus *Bartonella* contained only one species, *B. bacilliformis*. This bacterium is the etiological agent of Carrion's disease, a biphasic syndrome that affects the erythrocytes and skin of humans and is endemic only to the Andean region of South America (28). The number of members of the genus *Bartonella* recently increased following a study in which Brenner and his colleagues (7) confirmed the widespread belief that species of the genus *Rochalimaea* are more correctly placed in the genus *Bartonella*. This proposal was the culmination of several polyphasic studies of the taxonomic relationship between the two genera (3, 23, 27) and resulted in the unification of the two taxa, with the name *Bartonella* taking precedence (7). Thus, the genus *Bartonella* now

contains four additional species: *Bartonella quintana*, *Bartonella henselae*, *Bartonella vinsonii*, and *Bartonella elizabethae*.

The genus *Grahamella* and its type species, *Grahamella talpae*, were first proposed in 1911 in honor of G. S. Graham-Smith (8), who first observed these organisms in the erythrocytes of moles in 1905 (14). There have been many subsequent reports of observations of *Grahamella* species infecting a wide range of other animals, including fish, birds, and mammals (most commonly rodents) (18, 33). More than 40 *Grahamella* species have been proposed, although in almost all of the studies no attempts were made to culture and characterize representatives of the newly proposed species, which therefore took their names solely from the animal hosts in whose blood the organisms were observed (33). As these *Grahamella* species were so poorly characterized and no isolates existed, doubts concerning the validity of the taxa led to the name of the genus and the names of the species not being included on the Approved Lists of Bacterial Names in 1980 (30). The genus was effectively revived in *Bergey's Manual of Systematic Bacteriology* (28). The names of the genus and two species, *G. talpae* and *Grahamella peromysci*, were validated shortly thereafter (22), despite the fact that there were neither isolates nor any detailed phenotypic or genotypic information available.

For a long time the family *Bartonellaceae* has been classified in the order *Rickettsiales* (28); however, transfer of this family out of this order was recently proposed on the basis of DNA relatedness data, 16S rRNA sequence data, guanine-plus-cytosine (G+C) content data, and phenotypic characteristics (7). Members of the genus *Grahamella* were not included in the assessment which led to this proposal as isolates of the two valid species were no longer extant.

We recently isolated *Grahamella* strains from the blood of

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TABLE 1. Sources of bacterial strains

Species	Strain ^a	Source
<i>B. bacilliformis</i>	NCTC 12138 ^T (= ATCC 35685 ^T) ^b	R. Weaver, Centers for Disease Control and Prevention, Atlanta, Ga.
<i>B. quintana</i>	ATCC VR-358 ^T	American Type Culture Collection, Rockville, Md.
<i>B. vinsonii</i>	ATCC VR-152 ^T	American Type Culture Collection, Rockville, Md.
<i>B. henselae</i>	ATCC 49882 ^T	American Type Culture Collection, Rockville, Md.
<i>B. elizabethae</i>	ATCC 49927 ^T	J. Daly, Medical Center of Central Massachusetts, University of Massachusetts Medical School, Worcester
<i>Grahamella</i> species 1	NCTC 12860 ^T	Blood of small woodland mammals in the United Kingdom ^c
<i>Grahamella</i> species 2	NCTC 12861 ^T	Blood of small woodland mammals in the United Kingdom ^c
<i>Grahamella</i> species 3	NCTC 12862 ^T	Blood of small woodland mammals in the United Kingdom ^c

^a ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures.

^b T = type strain.

^c See reference 4.

several small woodland mammal species in the United Kingdom and used phenotypic and genotypic methods to separate these isolates into three distinct species (4). Our results demonstrated that *Grahamella* species are not host species specific since we isolated the same *Grahamella* species from different species of mammals and different *Grahamella* species from the same species of rodent. In light of this work, the criteria used to differentiate *G. talpae* and *G. peromysci* strains into distinct species (28) clearly are no longer valid, and thus the basis of the naming of these species is now challenged.

The availability of *Grahamella* isolates allowed us to use contemporary polyphasic characterization techniques to accurately assess the taxonomic position of the three newly identified *Grahamella* species. By investigating the relationships between the members of the genus *Grahamella* and other species belonging to the family *Bartonellaceae*, we were able to identify resemblances more in keeping with members of the same genus and in doing so were able to obtain evidence which supported the proposal that the family should be transferred out of the *Rickettsiales* (7).

MATERIALS AND METHODS

Bacterial strains. The sources of the type strains used in this study are shown in Table 1. All of the strains were cultivated on Columbia blood agar containing 10% whole fresh horse blood. Plates inoculated with *B. bacilliformis* were incubated for up to 10 days at 30°C in a moist atmosphere containing 5% CO₂. Other strains were incubated under the same conditions except that the temperature was 37°C.

Biochemical tests. The phenotypes of the three newly identified *Grahamella* species were compared with each other and with the phenotypes of the *Bartonella* species by using several biochemical and physiological tests. The following assays were performed: alkaline phosphatase, arginine dihydrolase, beta-glucosidase, catalase, esculin hydrolysis, Gram reaction, hippurate hydrolysis, nitrate reductase, *o*-nitrophenyl-β-D-galactoside, oxidase, pyrazinamidase, tributyrin, urease, Voges-Proskauer, and tetrathionate reductase. In addition, the bacterial strains were tested for the ability to ferment the following 19 sugars: adonitol, L-arabinose, cellobiose, dulcitol, fructose, glucose, inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, L-rhamnose, ribose, salicin, sorbitol, sucrose, and D-xylose. The isolates were also subjected to tests for the following preformed glycosidases: β-N-acetylglucosaminidase, α-fucosidase, α-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, and β-mannosidase. Finally, all of the isolates were subjected to tests for peptidase activity toward the following amino acid-β-naphthylamide substrates: alanine, cystine, γ-glutamyl, leucine, proline, pyrrolidonyl, serine, trypsin, and valine.

All biochemical tests except the Gram stain test were performed by using methods described in the Rosco Diagnostica manual for microbiological identification (9); in all of these tests diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark) were used.

Isolation and amplification of the 16S rRNA gene. Chromosomal DNAs were extracted from the type strains of the three newly identified *Grahamella* species and *B. vinsonii* after lysis with the guanidium isothiocyanate reagent (29). The 16S rRNA gene was amplified by the PCR under conditions that have been described previously (3).

Purification of the PCR product and gene sequencing. PCR amplicons were purified by polyethylene glycol precipitation (21). PCR products were sequenced

by using the reagents in a TAQuence cycle sequencing kit (United States Biochemical Corp., Cleveland, Ohio), ³²P-end-labelled primers, and thermal cycles, as previously described (12). β-Max film (Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom) was exposed to each gel for 24 to 72 h, and base sequences were determined from the developed film by visual examination.

16S rRNA sequence analysis. The primary sequences of the test strains were generated by aligning and then combining seven partial sequences, using DNASIS (Hitachi Software Engineering America, Ltd., Brisbane, Calif.). The sequences were confirmed by examining base pairing in the predicted secondary structure. The 16S rRNA sequences of the following organisms were used in this study: *B. bacilliformis*, *B. quintana*, *B. vinsonii*, *B. henselae*, *B. elizabethae*, *Grahamella* species 1, 2, and 3, *Rickettsia prowazekii*, *Ehrlichia risticii*, *Ehrlichia chaffeensis*, *Anaplasma marginale*, *Brucella abortus*, and *Legionella jordanii*.

Unedited sequences were aligned by using version V of the CLUSTAL multisequence alignment program (17), and 100 bootstrap samples of the data were produced by using the program SEQBOOT from the PHYLIP version 3.4 suite of software (11). Each bootstrap sample was used to infer a tree by using parsimony (DNAPARS in PHYLIP version 3.4). The resulting trees were combined, and the stability of the branching of the consensus tree was determined by using CONSENSE in PHYLIP version 3.4 (11). A matrix of evolutionary distance (*K*_{nuc}) values was derived from the aligned sequences (without bootstrapping) by using DNADIST (PHYLIP version 3.4) and the assumptions of Jukes and Cantor. A tree was inferred from the matrix by using the FITCH program (PHYLIP version 3.4), in which a least-squares method is used to construct a phylogenetic tree that best fits the data. The FITCH program was also used to determine branch lengths on the consensus tree derived by a parsimony analysis of the bootstrap data. This was done by entering the consensus tree as a "user tree," to which the *K*_{nuc} values determined by DNADIST were then applied. All of the trees were produced by using the DRAWTREE utility of PHYLIP version 3.4 (11).

The levels of homology between pairs of 16S rRNA gene sequences were determined by comparing the primary sequence data for the sequences, using the DNA homology search function of DNASIS.

Estimation of DNA base composition. The mean DNA base compositions of the three newly proposed species were estimated by using the thermal denaturation method (24). The results were expressed as the G+C content relative to the total base content and were compared with the previously determined value of 41 mol% for *B. vinsonii* (10).

Purification of DNA for hybridization studies. Chromosomal DNAs were extracted from strains as described above and then were purified further by using a modification of the phenol-chloroform reagent method (25). The purified DNA was resuspended overnight in 0.28 M phosphate buffer (equimolar mixture of NaH₂PO₄ and Na₂HPO₄) (pH 6.8) (PB) and was stored at 4°C. The purity and concentrations of the DNAs were determined spectrophotometrically.

DNA hybridization studies. Levels of DNA relatedness were determined by using a technique based on the batch hydroxyapatite (HA) method (5), as described below. DNA (0.1 µg) was labelled with [³²P]dCTP by using a nick translation kit (Boehringer-Mannheim, Lewes, East Sussex, United Kingdom). The labelled DNA was separated from unincorporated reagents by using a NICK column (Pharmacia Biotech, Ltd., St. Albans, Hertfordshire, United Kingdom) equilibrated with 0.28 M PB. Aliquots (100 µl) were collected from the NICK column and were assayed for radioactivity by scintillation counting. The aliquots exhibiting the highest levels of activity were then pooled, and radioactivity in the pool was counted (the typical volume was 200 µl, and the total counts were 6 × 10⁷ cpm). Prior to hybridization the labelled DNA was denatured by heating it at 100°C for 10 min.

Unlabelled DNA (60 µg) that had been dissolved in 200 µl of 0.28 M PB was placed in a 2-ml Eppendorf tube and sheared by using two 1-min bursts of sonication (amplitude, 5 µm); 0.28 M PB was then added to the tube containing

TABLE 2. Peptidase activities of *Grahamella* and *Bartonella* species

Species	Activity on amino acid- β -naphthylamine substrates								
	Alanine	Cystine	γ -Glutamyl	Leucine	Proline	Pyrrolidine	Serine	Trypsin	Valine
<i>Grahamella</i> species 1	+	—	—	+	+	—	+	—	+
<i>Grahamella</i> species 2	+	—	—	+	+	—	+	—	+
<i>Grahamella</i> species 3	+	—	—	+	—	—	+	+	+
<i>B. bacilliformis</i>	+	—	—	+	—	—	+	—	+
<i>B. elizabethae</i>	+	—	—	+	+	—	+	+	+
<i>B. henselae</i>	+	—	—	+	+	—	+	+	+
<i>B. quintana</i>	+	—	—	+	+	—	+	—	+
<i>B. vinsonii</i>	+	—	—	+	+	—	+	—	+

sheared DNA to a final volume of (typically) 380 μ l. The DNA solution was then denatured as described above.

Then 0.1 volume of the denatured labelled DNA solution (approximately 10 ng of DNA) was added to the tube containing unlabelled DNA. The final volume of this reassociation mixture was 400 μ l. The mixture was overlaid with mineral oil, and then the tube was placed in a thermal heating block and kept at 53°C. The temperature for optimum reassociation (68°C) was derived from the G+C content of the labelled DNA (13). Hybridization experiments were carried out under relaxed conditions for reassociation, at 15°C below the temperature for optimum reassociation. The incubation time required for hybridization was determined from the initial concentrations of labelled and unlabelled DNAs by using standard equations (6).

Portions (100 μ l) of HA (DNA grade Bio-Gel HTP; Bio-Rad, Richmond, Calif.) equilibrated in 0.14 M PB-0.4% sodium dodecyl sulfate (SDS) were placed in 2-ml Eppendorf tubes and allowed to settle. The Eppendorf tubes were placed in a thermal block and kept at either the temperature used for relaxed washing (53°C) or the temperature used for stringent washing (68°C).

After incubation, the reassociation reaction mixtures were diluted so that the concentration of PB was 0.14 M in a volume of 500 μ l. Aliquots of the diluted mixtures (typically 50 μ l) were then added to the relevant HA suspensions, and the preparations were mixed thoroughly. After repeated mixing, the HA was allowed to settle, and the supernatants, which contained unbound single-stranded DNA, were removed. The HA was then washed six times with 500- μ l portions of 0.14 M PB-0.4% SDS that had been preheated to the required temperature. All of the supernatants were placed directly into scintillation vials and assayed by scintillation counting. Double-stranded DNA was eluted from the HA by washing the preparation three times with 500- μ l portions of 0.4 M PB. The three supernatants were pooled and assayed for radioactivity as described above. Levels of DNA relatedness were expressed as relative binding ratios, which were calculated as previously described (6).

Serological examination. Hyperimmune rabbit antiserum was raised against the type strain of each species by using previously described methods (16). The serological reactivities of presumptive strains were determined by the indirect immunofluorescence test (16).

Nucleotide sequence accession numbers. The EMBL/GenBank accession numbers of the 16S rRNA sequences used in the phylogenetic analyses were as follows: *B. bacilliformis*, X60042; *B. quintana*, M73228; *B. vinsonii*, Z31352; *B. henselae*, M73229; *B. elizabethae*, L01260; *Grahamella* species 1, Z31349; *Grahamella* species 2, Z31350; *Grahamella* species 3, Z31351; *R. prowazekii*, M21789; *E. risticii*, M21290; *E. chaffeensis*, M73222; *A. marginale*, M60313; *Brucella abortus*, X13695; and *L. jordanis*, Z32667. The 16S rRNA gene nucleotide sequences of *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshaiae* sp. nov. have been deposited in the EMBL database under accession numbers Z31349, Z31350, and Z31351, respectively.

RESULTS

Phenotypic characteristics. Microscopic examination of Gram-stained bacterial films revealed that the strains of all three *Grahamella* species were thin, short, pleomorphic, weakly staining, gram-negative bacilli or coccobacilli which were morphologically indistinguishable from all of the *Bartonella* species examined. The *Grahamella* and *Bartonella* strains were inert in almost all of the biochemical and physiological tests performed. However, unlike the five *Bartonella* species, the *Grahamella* species were Voges-Proskauer positive, with the type strain of species 3 producing a strong reaction. *Grahamella* species 3 was also the only member of either genus that hydrolyzed pyrazinamide. *Grahamella* species 1 and 3 hydrolyzed tributyrin, although only weak positive

signals were produced. Utilization of carbohydrates was not detected for any of the strains tested in either conventional fermentation tests or assays for preformed glycosidases. Our analysis of the ability of *Grahamella* and *Bartonella* strains to utilize the amino acid- β -naphthylamine substrates did, however, yield positive results (Table 2). The species of the two genera could be differentiated into four groups by comparing their aminopeptidase activities against proline and trypsin. The type strains of *B. elizabethae* and *B. henselae* hydrolyzed both of these substrates, whereas *Grahamella* species 1 and 2, *B. quintana* and *B. vinsonii* hydrolyzed proline but not trypsin, *Grahamella* species 3 hydrolyzed trypsin but not proline, and *B. bacilliformis* hydrolyzed neither compound.

16S rRNA sequence analysis. The primary 16S rRNA gene sequence generated for each of the four species studied consisted of approximately 1,400 base pairs, which was equivalent to about 93% of the complete gene. For each of the four species studied, an examination of the predicted secondary structure of the rRNA molecule derived from the sequenced genes showed that the overall structure of the molecule was maintained and that virtually all of the base differences detected occurred in regions of the molecule known to be highly variable.

We also performed a multiple-alignment analysis of the 16S rRNA gene sequences obtained for the four species examined, other *Bartonella* species, and representative species belonging to the *Rickettsiales*. Pairs of the aligned sequences were compared, and evolutionary distance (K_{nuc}) values were obtained (Table 3). The 16S rRNA gene sequence of *L. jordanis*, a member of the gamma subclass of the *Proteobacteria* and therefore an organism that is phylogenetically only distantly related to members of the *Rickettsiales*, was included in this analysis as an outgroup.

The topology of the phylogenetic tree inferred was influenced by the results of two different analyses of the aligned sequences. The FITCH program was used to impose the K_{nuc} values (derived by using the DNADIST program) onto the consensus tree which reflected the topology most often reproduced in parsimony analyses of bootstrap samples of the aligned sequence data. The resulting tree (Fig. 1) therefore reflected the most accurate phylogenetic distances within the constraints of the most reliable topology.

The three *Grahamella* species clustered among the *Bartonella* species. Inclusion of the three new species within this cluster did not alter its monophyletic nature, and the topology of the tree was consistent with the topologies described in previous phylogenetic studies (7, 10, 27).

The phylogenetic tree in Fig. 2 was constructed by using the same methods that were used to construct the tree in Fig. 1. This tree shows the internal relationships of the family *Bartonellaceae*. *Brucella abortus* was included as an outgroup. The

TABLE 3. Homology values and K_{nuc} values for partial sequences ($>92\%$ of the total sequences) of the 16S rRNA genes of members of the *Rickettsiales* and the family *Bartonellaceae* and *Brucella abortus*

Species	Rickettsiales lineage ^a	Homology value or K_{true} value ^b													
		<i>B. bacilliformis</i>	<i>B. elizabethae</i>	<i>B. henselae</i>	<i>B. quintana</i>	<i>B. vinsonii</i>	<i>Grahamella</i> species 1	<i>Grahamella</i> species 2	<i>Grahamella</i> species 3	<i>A. marginale</i>	<i>R. prowazekii</i>	<i>E. risticii</i>	<i>E. chaffeensis</i>	<i>L. jordanis</i>	<i>Bruella abortus</i>
<i>B. bacilliformis</i>		97.8	0.0205	0.0169	0.0183	0.0154	0.0145	0.0130	0.0139	0.1821	0.1624	0.2081	0.1918	0.2164	0.0514
<i>B. elizabethae</i>				0.0121	0.0172	0.0129	0.0115	0.0131	0.0116	0.1958	0.1645	0.2163	0.2023	0.2252	0.0526
<i>B. henselae</i>		98.2	98.7		0.0114	0.0071	0.0072	0.0087	0.0058	0.1900	0.1662	0.2199	0.1968	0.2211	0.0543
<i>B. quintana</i>		98.0	98.2	98.8		0.0107	0.0123	0.0117	0.0109	0.1908	0.1669	0.2208	0.1975	0.2248	0.0551
<i>B. vinsonii</i>		98.3	98.6	99.3	98.9		0.0043	0.0058	0.0029	0.1909	0.1635	0.2189	0.1968	0.2250	0.0489
<i>Grahamella</i> species 1		98.4	98.6	99.1	98.6	99.4		0.0058	0.0029	0.1914	0.1619	0.2155	0.1973	0.2224	0.0484
<i>Grahamella</i> species 2		98.5	98.3	98.8	98.6	99.1	99.1		0.0029	0.1864	0.1633	0.2110	0.1944	0.2190	0.0469
<i>Grahamella</i> species 3		98.0	98.6	99.3	98.7	99.6	99.5	99.4		0.1902	0.1623	0.2187	0.1954	0.2247	0.0481
<i>A. marginale</i>	III	82.5	80.9	81.3	81.3	81.2	81.7	81.4	81.4		0.1796	0.1665	0.0828	0.2411	
<i>R. prowazekii</i>	IV	84.2	84.2	83.8	84.0	84.0	84.1	84.1	84.1	83.2		0.1956	0.2001	0.2322	
<i>E. risticii</i>	I	81.0	80.2	80.3	80.1	80.4	80.3	80.4	80.4	84.4	82.4		0.1855	0.2655	
<i>E. chaffeensis</i>	II	82.7	81.8	81.9	82.0	81.9	81.8	82.3	82.0	91.5	82.4	83.4			
<i>L. jordanis</i>		70.1	70.4	70.8	70.4	70.5	70.4	70.1	70.7	67.8	69.6	68.0	67.4		
<i>Bruella abortus</i>		94.6	94.4	94.2	94.1	94.7	94.7	94.7	94.7	80.6	83.0	80.1	81.5	70.1	

^a As defined by Brenner et al. (7).

^b The values on the lower left are homology values, and the values on the upper right are K_{nuc} values. As defined by Bremer et al. (7).

FIG. 1. Phylogenetic tree for members of the order *Rickettsiales* and the family *Bartonellaceae*, with *L. jordanis* as an outgroup. The four *Rickettsiales* species that are not members of the *Bartonellaceae* are representatives of the four other intra-*Rickettsiales* lineages, as described by Brenner et al. (7).

topology of the tree confirmed the monophyletic nature of the *Bartonellaceae* and indicated that there were no coherent subclusters within the group.

The 16S rRNA sequence homology values are shown in Table 3. The levels of similarity between the sequences of the *Grahamella* species were very high (99.1 to 99.5%), but this was also the case with other members of the *Bartonellaceae* (levels of similarity, 98.3 to 99.6%); these levels of similarity were far higher than the levels of similarity between any of the *Grahamella* sequences and the sequences of all of the other bacteria used in this study, including representatives of the other phylogenetic clusters within the *Rickettsiales*. All *Grahamella* species also exhibited markedly higher levels of homology with *Brucella abortus* than with the other non-*Bartonellaceae* members of the *Rickettsiales*. Within the *Bartonellaceae* the levels of similarity between the three *Grahamella* species and *B. bacilliformis* (mean level of homology, 98.5%) were slightly lower than the levels of similarity between the *Grahamella* species and the other *Bartonella* (formerly *Rochalimaea*) species (mean level of homology, 98.9%). *Grahamella* species 1 and 3 both exhibited the highest level of homology not with another *Grahamella* species, but with *B. vinsonii*.

The nucleotide sequences obtained for *B. vinsonii* and the three newly proposed species were deposited in the EMBL data library (see above).

Estimation of base composition. The estimated G+C contents of the *Grahamella* species were 40 mol% for *Grahamella* species 1 and 41 mol% for *Grahamella* species 2 and 3.

DNA hybridization studies. The levels of DNA relatedness between the three *Grahamella* species and members of the genus *Bartonella* are shown in Table 4. Our results indicate that each of the *Grahamella* species is distinct from the five *Bartonella* species. There was no significant difference between the levels of relatedness between the three *Grahamella* species and the levels of relatedness between *Grahamella* and *Bartonella* species.

FIG. 2. Phylogenetic tree for members of the family Bartonellaceae, with *Brucella abortus* as an outgroup.

Serological examination. Hyperimmune rabbit antiserum raised against each of the *Grahamella* or *Bartonella* species gave good homologous reactions but did not react with representatives of heterologous species (Table 5).

DISCUSSION

The introduction of phylogenetic analysis into the taxonomic study of the order *Rickettsiales* has provided a potent tool that has allowed effective restructuring of an order recognized as a taxon in which accurate classification has been very difficult. The advent of the PCR has circumvented the need to culture large amounts of organisms known to be either extremely fastidious or obligately intracellular parasites, and suitable templates for gene sequencing and comparison are now easily obtained. However, representative isolates of several species of the *Rickettsiales* are no longer extant and are thus beyond the reach of even the PCR. Taxonomic protocol is such that these species cannot be ignored, and thus their paper existence remains an obstacle around which taxonomic restructuring must proceed. Only if they are reisolated can these "lost" genera and species be included in taxonomic reevaluations.

Grahamella was an example of a genus for which isolates no longer existed. However, the names of the genus and two species were revived in 1984 (28). The genus was classified in the order *Rickettsiales* as a member of the family *Bartonellaceae*. The genus *Bartonella* was the second and only other member of this family, and three criteria for differentiating the two genera were described (see Introduction). The subsequent

transfer of four *Rochalimaea* species to the genus *Bartonella* led to two of the three criteria becoming redundant. First, *B. vinsonii* has not been shown to infect humans; it has been isolated only from the blood of a Canadian vole (1). And second, none of the four former *Rochalimaea* species possess flagella (34). Thus, the only criterion which remains is that *Grahamella* species exist exclusively within erythrocytes, whereas *Bartonella* species can exist on the erythrocyte cell surface. However, even the validity of this criterion must be considered doubtful as extracellular grahamella-like organisms have been observed in Giemsa-stained blood films obtained from infected animals (14) and the entry of *B. bacilliformis* into erythrocytes has been carefully studied (2).

The recent unification of the genera *Rochalimaea* and *Bartonella* led to an emended description of the genus *Bartonella*. The typical characteristics which are now used for members of the genus is that they are all gram-negative, oxidase-negative, fastidious, aerobic, rod-shaped organisms which grow best on blood-enriched media in an atmosphere containing 5% CO₂. All of these characteristics are equally applicable to *Grahamella* species. Thus, on the basis of these phenotypic criteria, the only source of comparison available, it is not possible to differentiate between members of the genus *Grahamella* and members of the genus *Bartonella*.

Following the recent isolation of three newly recognized *Grahamella* species, the taxonomic position of the genus was reappraised by using modern polyphasic taxonomic methods. The results of these analyses are discussed below.

The estimated DNA base compositions of the three *Grahamella* species were very similar to those reported for the *Bartonella* species and thus were significantly higher than the DNA base compositions of *Rickettsia* species (34).

The new method used to determine levels of DNA relatedness in this study was evaluated by comparing the results obtained with this method with results obtained by using a previously described method which is used to estimate levels of relatedness between members of the same group of organisms. Daly and her colleagues determined the levels of relatedness between *Rochalimaea* (*Bartonella*) *elizabethae* and other members of the genus by using the HA column method (10). The results obtained by Daly et al. and the relevant results obtained in this study are shown in Table 4; the levels of DNA relatedness calculated in the two studies are very similar. The method which we describe above is a quick and reliable alternative to other HA methods. Furthermore, with our method the radiological hazards are minimized through the use of very small volumes of labelled reagents which can be handled entirely in disposable plasticware.

The DNA relatedness values suggest that all three *Grahamella* species are closely related to, but distinct from, the *Bartonella* species (32). There is no marked difference in the levels of relatedness obtained when *Grahamella* species are compared with each other and when *Grahamella* species are compared with *Bartonella* species. Although all three *Grahamella* species are closely related to the four former *Rochalimaea* species (levels of relatedness, 30 to 54%), DNA relatedness values indicate that there is a weaker, but still significant, relationship between the *Grahamella* species and *B. bacilliformis* (level of relatedness, 16%). The overall level of relatedness between the *Grahamella* species and *Bartonella* species (37%) indicates that the *Grahamella* species are indeed valid members of the family *Bartonellaceae* and strongly suggests that all of the species should be classified in the same genus.

The phylogenetic tree in Fig. 1 presents evidence of the close evolutionary relationships between the *Grahamella* and *Bartonella* species. Brenner and his colleagues (7) divided the

TABLE 4. Relative binding ratios for members of the *Bartonellaceae*

Source of unlabelled DNA	Wash temp	Relative binding ratios (%) with the following sources of labelled DNA:			
		<i>Grahamella</i> species 1	<i>Grahamella</i> species 2	<i>Grahamella</i> species 3	<i>B. elizabethae</i>
<i>Grahamella</i> species 1	Optimum	100	49	40	64
	Stringent	100	19	18	42
<i>Grahamella</i> species 2	Optimum	51	100	44	41
	Stringent	22	100	15	8
<i>Grahamella</i> species 3	Optimum	43	51	100	41
	Stringent	16	25	100	9
<i>B. bacilliformis</i>	Optimum	16	NT ^a	NT	NT
	Stringent	6	NT	NT	NT
<i>B. henselae</i>	Optimum	34	44	41	43 (67) ^b
	Stringent	14	23	16	9 (16)
<i>B. quintana</i>	Optimum	31	39	30	42 (59)
	Stringent	6	18	12	7 (15)
<i>B. vinsonii</i>	Optimum	38	38	37	46 (66)
	Stringent	15	19	16	9 (16)
<i>B. elizabethae</i>	Optimum	54	41	37	100
	Stringent	31	19	9	100
Salmon testis DNA	Optimum	3	2	2	NT
	Stringent	<1	<1	<1	NT

^a NT, not tested.^b The values in parentheses are the DNA relative binding ratios obtained by Daly et al. (10).

Rickettsiales into five groups that represent five distinct evolutionary lineages. The genus *Bartonella* forms group V in this classification. Representatives of the other four groups, namely, *E. risticii* (group I), *E. chaffeensis* (group II), *A. marginale* (group III), and *R. prowazekii* (group IV), were therefore included in this study.

The *Bartonella* and *Grahamella* species form a single tight cluster which is only remotely related to the representative species of the other phylogenetic lineages in the *Rickettsiales*. The branching order of the five intra-*Rickettsiales* lineages indicates that the four non-*Bartonellaceae* lineages cluster together on a branch that is deeply divergent from the branch carrying the *Bartonella*-*Grahamella* cluster. The inclusion of the genus *Grahamella* in this tree confirms that all members of the family *Bartonellaceae* are phylogenetically only remotely related to other members of the *Rickettsiales* and provides additional evidence which supports the proposal that the family *Bartonellaceae* should be transferred out of the order *Rickettsiales* (7).

The results of our investigation of the evolutionary relationships within the *Bartonella*-*Grahamella* cluster are shown in the phylogenetic tree in Fig. 2. A comparison of the phylogenetic tree derived solely by a FITCH analysis of K_{nuc} values with the

phylogenetic tree derived solely from a parsimony analysis of bootstrap sample data revealed that these trees have different overall topologies (data not shown). In our tree we attempted to combine these conflicting views, however, because the actual branch lengths could not be reasonably accommodated within the topology derived by the parsimony method, many zero-length branches resulted, and the tree was therefore dominated by a central node. The levels of sequence homology between the species belonging to the cluster are extremely high. The range of homology values (98.3 to 99.6%) reflects between 8 and 24 nucleotide differences in a sequence of more than 1,400 base pairs. With so few differences from which to infer phylogenetic relationships, it is perhaps not surprising that different methods of analysis produce different results. Although this finding may illustrate the limitations of 16S rRNA gene sequence comparison as a method for inferring precise intragenus relationships between very closely related species of bacteria, the levels of phylogenetic homology observed between members of the *Bartonella*-*Grahamella* cluster are clearly consistent with the levels of homology observed between species of the same genus.

Our assessment of the phenotypic characteristics of the *Grahamella* species indicated that these organisms have much

TABLE 5. Immunofluorescence test titers for the type strains of *Bartonellaceae* species

Antiserum (immunizing species)	Titers with the following antigens ^a :							
	<i>B. bacilliformis</i>	<i>B. elizabethae</i>	<i>B. henselae</i>	<i>B. quintana</i>	<i>B. vinsonii</i>	<i>Grahamella</i> species 1	<i>Grahamella</i> species 2	<i>Grahamella</i> species 3
<i>B. bacilliformis</i>	1,600	<100	<100	<100	<100	<100	<100	<100
<i>B. elizabethae</i>	<100	1,600	<100	<100	<100	<100	<100	<100
<i>B. henselae</i>	<100	<100	800	wk100 ^b	wk100	<100	<100	<100
<i>B. quintana</i>	<100	<100	<100	800	<100	<100	<100	<100
<i>B. vinsonii</i>	<100	<100	<100	wk100	1,600	<100	<100	<100
<i>Grahamella</i> species 1	<100	wk100	wk100	wk100	wk100	1,600	wk100	wk100
<i>Grahamella</i> species 2	<100	<100	wk100	wk100	wk100	<100	800	wk100
<i>Grahamella</i> species 3	<100	<100	wk100	<100	wk100	wk100	wk100	1,600

^a A titer of <100 was considered negative.^b wk100, weak fluorescence was observed at an antibody dilution of 1:100.

in common with the *Bartonella* species. Morphologically, the species of the two genera appear to be very similar; members of both genera are inert in almost all commonly used biochemical assays and are not able to utilize carbohydrates. The *Grahamella* species can be differentiated from the *Bartonella* species by the Voges-Proskauer test, as only members of the former genus give positive results in this test. Individual *Grahamella* species can be distinguished from one another by using the tributyrin utilization test and the pyrazinamidase test. Amino acid- β -naphthylamine substrate utilization assays are useful for identifying and differentiating *Grahamella* and *Bartonella* species; the value of such tests for identification of *Bartonella* species has been described previously (35). The clearest indication of phenotypic variation between species of the two genera can be obtained by using polyclonal antiserum. For all members of both genera, an antiserum was reactive in immunofluorescence tests only with the species against which it had been raised; no reaction with isolates belonging to other *Grahamella* or *Bartonella* species was observed.

By combining the results of the genotypic and phenotypic analyses we convincingly demonstrated that the genera *Grahamella* and *Bartonella* should be combined and established that the long-recognized phenotypic similarities between members of the two genera are a reflection of a close genotypic and phylogenetic relationship. Changes in the classification of the two genera are warranted. Below, unification of the two genera is proposed, and as the names *Bartonella* and *B. bacilliformis* predate the revalidation of the names *Grahamella* and *G. talpae*, the name *Bartonella* should be retained for the unified genus. Although isolates of *G. talpae* and *G. peromysci* are no longer extant, the species names must be preserved, and thus new combinations must be created. The three recently described but previously unnamed *Grahamella* species used in this study are formally named as three new *Bartonella* species. *Grahamella* species 1 is named *Bartonella grahamii* in honor of G. S. Graham-Smith, who recognized grahamellae as a new form of intraerythrocytic parasites in 1905. *Grahamella* species 2 is named *Bartonella taylorii* in honor of Anthony Taylor, who has been instrumental in initiating and maintaining investigations of members of the *Bartonellaceae* and the diseases that they cause at the Central Public Health Laboratory. *Grahamella* species 3 is named *Bartonella doshiae* in honor of Nivedita Doshi, who has made invaluable contributions to the exacting technical work required for this and other projects at the Central Public Health Laboratory. The incorporation of these five species into the genus *Bartonella* means that the description of the genus needs to be emended. Our taxonomic proposals are given below.

Emendation of the genus *Bartonella* (Strong, Tyzzer, and Sellards 1915) Brenner, O'Connor, Winkler, and Steigerwalt 1993. The proposed unification of the genera *Bartonella* and *Rochalimaea* requires emendation of the description of the genus *Bartonella*, as follows.

Gram-negative, oxidase-negative, fastidious, aerobic rods. One species (*B. bacilliformis*) is motile by means of polar flagella. The best growth is obtained on media containing 5% or more rabbit, sheep, or horse blood in the presence of 5% CO₂. The optimal incubation temperatures range from 25°C (*B. bacilliformis*) to 37°C. Carbohydrates are not utilized. Four species have been shown to be pathogenic for humans (10, 15, 26, 31). Five species (including one pathogenic species, *B. henselae*) have been isolated from other mammals (1, 4, 19). Arthropod vectors have been identified for *B. bacilliformis* and *B. quintana* and have been implicated for all other species except *B. elizabethae* (1, 19, 20). One or more species cause bartonellosis, trench fever, bacillary angiomatosis, peliosis

hepatitis, septicemia, endocarditis, and cat scratch disease. The G+C contents of the DNAs are 38.5 to 41 mol%. The type species is *B. bacilliformis* (Strong, Tyzzer, Brues, Sellards, and Gastiaburu 1913) Strong, Tyzzer, and Sellards 1915.

Description of *Bartonella talpae* comb. nov. The description of *Bartonella talpae* is the same as the description given for *Grahamella talpae* (28). No type strain is available.

Description of *Bartonella peromysci* comb. nov. The description of *Bartonella peromysci* is the same as the description given for *Grahamella peromysci* (28). No type strain is available.

Description of *Bartonella grahamii* sp. nov. *Bartonella grahamii* (gra.ham'i.i. N. L. gen. n. *grahamii*, of Graham, named after G. S. Graham-Smith, who observed similar organisms, subsequently named *Grahamella* [8], in the blood of moles in 1905 [14]). The type strain is V2 (= NCTC 12860), which was isolated from the blood of *Clethrionomys glareolus*. Although the type strain is generally inert in conventional biochemical tests and is not able to utilize carbohydrates, it is Voges-Proskauer positive and is able to hydrolyze tributyrin, albeit weakly. The type strain is also able to hydrolyze amino acid- β -naphthylamine substrates, including proline but not trypsin. The G+C content of the type strain is 40 mol%, and its 16S rRNA gene nucleotide sequence has been deposited in the EMBL database.

Description of *Bartonella taylorii* sp. nov. *Bartonella taylorii* (tay.lor' i.i. N. L. gen. n. *taylorii*, of Taylor, named after A. G. Taylor, whose foresight led to the initiation of studies of legionellae, listeriae, and bartonellae at the Central Public Health Laboratory). The type strain is M6 (= NCTC 12861), which was isolated from the blood of *Apodemus* spp. Although the type strain is generally inert in conventional biochemical tests (including tributyrin hydrolysis tests) and is not able to utilize carbohydrates, it is Voges-Proskauer positive. The type strain is also able to hydrolyze amino acid- β -naphthylamine substrates, including proline but not trypsin. The G+C content of the type strain is 41 mol%, and its 16S rRNA gene nucleotide sequence has been deposited in the EMBL database.

Description of *Bartonella doshiae* sp. nov. *Bartonella doshiae* (do'shi.ae. N. L. gen. n. *doshiae*, of Doshi, named after Nivedita Doshi, whose technical skills have been central to the work on legionellae and bartonellae at the Central Public Health Laboratory). The type strain is R18 (= NCTC 12862), which was isolated from the blood of *Microtus agrestis*. Although the type strain is generally inert in conventional biochemical tests and is not able to utilize carbohydrates, it is Voges-Proskauer positive, is able to hydrolyze butyrin (albeit weakly), and exhibits pyrazinamidase activity. The type strain is also able to hydrolyze amino acid- β -naphthylamine substrates, including trypsin but not proline. The G+C content of the type strain is 41 mol%, and its 16S rRNA gene nucleotide sequence has been deposited in the EMBL database.

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