Phylogenetic Relationships between Some Members of the Genera *Neisseria*, *Acinetobacter*, *Moraxella*, and *Kingella* Based on Partial 16S Ribosomal DNA Sequence Analysis

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We obtained 16S ribosomal DNA (rDNA) sequence data for strains belonging to 11 species of *Proteobacteria*, including the type strains of *Kingella kingae*, *Neisseria lactamica*, *Neisseria meningitidis*, *Moraxella lacunata* subsp. *lacunata*, [*Neisseria*] *ovis*, *Moraxella catarrhalis*, *Moraxella osloensis*, [*Moraxella*] *phenylpyruvica*, and *Acinetobacter lwoffii*, as well as strains of *Neisseria subflava* and *Acinetobacter calcoaceticus*. The data in a distance matrix constructed by comparing the sequences supported the proposal that the genera *Acinetobacter* and *Moraxella* and [*N.*] *ovis* should be excluded from the family *Neisseriaceae*. Our results are consistent with hybridization data which suggest that these excluded taxa should be part of a new family, the *Moraxellaceae*. The strains that we studied can be divided into the following five groups: (i) *M. lacunata* subsp. *lacunata*, [*N.*] *ovis*, and *M. catarrhalis*; (ii) *M. osloensis*; (iii) [*M.*] *phenylpyruvica*; (iv) *A. calcoaceticus* and *A. lwoffii*; and (v) *N. meningitidis*, *N. subflava*, *N. lactamica*, and *K. kingae*. We agree with the previous proposal that [*N.*] *ovis* should be renamed *Moraxella ovis*, as this organism is closely related to *Moraxella* species and not to *Neisseria* species. The generically misnamed taxon [*M.*] *phenylpyruvica* belongs to the proposed family *Moraxellaceae*, but it is sufficiently different to warrant exclusion from the genus *Moraxella*. Further work needs to be done to investigate genetically similar species, such as *Psychrobacter immobilis*, before the true generic position of this organism can be determined. Automated 16S rDNA sequencing with the PCR allows workers to accurately determine phylogenetic relationships between groups of organisms. This method is quicker and less complex than nucleic acid hybridization, produces similar results, and has the advantage that 16S rDNA sequence information for different species can be directly compared.

The family *Neisseriaceae* was considered by Bøvre in 1984 (3) to consist of the genera *Neisseria*, *Kingella*, *Acinetobacter*, and *Moraxella*, the latter genus containing the subgenera *Moraxella* (rod-shaped bacteria) and *Branhamella* (cocci) (2). Since then it has been proposed that the genera *Psychrobacter* (10), *Mesophilobacter* (12), *Alysiella* (14), *Eikenella*, and *Simonsiella* (7) should also be included in this family.

In 1988 Stackebrandt et al. proposed the formation of a new class, the *Proteobacteria*, comprising the “purple bacteria and their relatives” (19). This class contains four subclasses, the α , β , γ , and δ subclasses, which are distinguished on the basis of 16S rRNA sequence similarities. The α , β , and γ subclasses correspond to rRNA superfamily IV, rRNA superfamily III, and rRNA superfamilies I and II of De Ley et al. (6, 13, 16); the latter was distinguished by using rRNA-DNA hybridization data. In a study in which DNA-rRNA and DNA-DNA hybridization techniques were used, Rossau et al. (14) proposed that the genera *Acinetobacter*, *Moraxella*, and *Psychrobacter* should be removed from the *Neisseriaceae* as they belong to rRNA superfamily II (γ subclass) and species belonging to these genera exhibit low levels of rRNA-DNA and DNA-DNA hybridization with members of the genera *Neisseria*, *Kingella*, *Eikenella*, *Simonsiella*, and *Alysiella*. Species belonging to the latter genera exhibit high levels of rRNA similarity (the melting temperatures of elution are between 74 and 81°C) and belong to rRNA superfamily III (β subclass of the *Proteobacteria*). Rossau et al. (15) proposed that the excluded genera should be placed in a new family, the *Moraxellaceae*, comprising two groups based on rRNA-DNA hybridization data. The *Acinetobacter* strains form one group, and the other group is

made up of the following four subgroups: (i) the authentic *Moraxella* strains, including *Moraxella catarrhalis* and the “false *neisseriae*”; (ii) *Moraxella osloensis*; (iii) *Moraxella atlantae*; and (iv) a [*Moraxella*] *phenylpyruvica*-*Psychrobacter* subgroup. However, Catlin proposed the formation of a different family, the *Branhamaceae*, to include the genera *Moraxella* and *Branhamella* (5).

Eleven species have been placed in the genus *Neisseria*, including the “true *neisseriae*” and four species incertae sedis (*Neisseria animalis* and the so-called false *neisseriae*, [*Neisseria*] *caviae*, [*Neisseria*] *ovis*, and [*Neisseria*] *cuniculi*) (21). Veron et al. (22) concluded from DNA-DNA hybridization and fatty acid analysis data that the false *neisseriae* form three separate genomic species which should be placed in the γ subclass of the *Proteobacteria* and thus excluded from the family *Neisseriaceae*.

TABLE 1. Strains sequenced and their accession numbers

Taxon	Strain	EMBL accession no.
<i>M. catarrhalis</i>	NCTC 11020 ^T (= ATCC 25238 ^T)	X74903
<i>M. lacunata</i> subsp. <i>lacunata</i>	NCTC 11011 ^T (= ATCC 17967 ^T)	X74895
[<i>N.</i>] <i>ovis</i>	NCTC 11227 ^T (= ATCC 11227 ^T)	X74896
<i>M. osloensis</i>	NCTC 10465 ^T (= ATCC 19976 ^T)	X74897
[<i>M.</i>] <i>phenylpyruvica</i>	NCTC 10526 ^T (= ATCC 23333 ^T)	X74898
<i>A. calcoaceticus</i>	NCTC 10292	X74899
<i>A. lwoffii</i>	NCTC 05866 ^T (= ATCC 15309 ^T)	X74894
<i>N. meningitidis</i>	NCTC 10025 ^T (= ATCC 13077 ^T)	X74900
<i>K. kingae</i>	NCTC 10529 ^T (= ATCC 23330 ^T)	X74902
<i>N. lactamica</i>	NCTC 10617 ^T (= ATCC 23970 ^T)	X74901
<i>N. subflava</i>	NCTC 04590	X74893

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TABLE 2. Primers used for 16S rDNA sequencing (5'-3')

Primer	Sequence
Po	AGAGTTTGATC
Pc3	TGGACTACCAAGGTATCTAAT
pD	CAGCAGCCGCGGTAATAC
pD ⁰	GTATTACCGCGCTGCTG
pC	CTACGGGAGGCAGCAGTGGG
pC ⁰	CCCACTGCTGCCTCCCGTAG

Catlin included these species in the genus *Branhamella* in the family *Branhamaceae* (5). In this study we used 16S ribosomal DNA (rDNA) sequencing to examine the relationships among some species belonging to the β subclass of the *Proteobacteria* (*Kingella kingae*, *Neisseria lactamica*, *Neisseria meningitidis*, *Neisseria subflava*) and to the γ subclass (*Moraxella lacunata* subsp. *lacunata*, [*N.*] *ovis*, *M. catarrhalis*, *M. osloensis*, [*M.*] *phenylpyruvica*, *Acinetobacter calcoaceticus*, and *Acinetobacter lwoffii*).

MATERIALS AND METHODS

Bacterial strains. All of the strains used (Table 1) were obtained from the National Collection of Type Cultures, Public Health Laboratory Service, London, United Kingdom.

Culture conditions and DNA extraction. Organisms were inoculated onto Columbia agar plates containing 5% horse blood (Oxoid) and incubated overnight at 37°C in the presence of 5% CO₂. DNA was extracted by using a variation of the method of Bjorvatn et al. (1). Fresh overnight growth was harvested and resuspended in 300 μ l of 10 mM Tris (pH 8.0)–2 mM EDTA. To this preparation we added 100 μ l of lysozyme (50 mg ml⁻¹), 10 μ l of RNase A (1 mg ml⁻¹), 10 μ l of proteinase K (5 mg ml⁻¹), and 300 μ l of a solution containing 10 mM EDTA, 2% Triton X-100, and 50 mM Tris (pH 8.0), and the samples were mixed by inversion and then incubated for 30 min at 37°C. Samples were extracted once with phenol and once with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with ethanol at –20°C and collected by centrifugation. The ethanol was removed, and the pellet was resuspended in 10 mM Tris (pH 8.0)–2 mM EDTA to a final DNA concentration of 20 ng μ l⁻¹.

PCR. The 16S rDNA template was amplified by using the method of Saiki et al. (17). The universal primers used for

amplification of the 5' ends of eubacterial 16S rDNA genes, Po and Pc3 (23), were synthesized with an Applied Biosystems model 391 DNA synthesizer and were purified by using OPC columns (ABI, Warrington, United Kingdom). Amplification reaction mixtures (50 μ l) containing genomic DNA (2 ng μ l⁻¹), both primers (0.25 μ M each), four deoxyribonucleotides (0.2 mM each), *Taq* polymerase (0.025 U μ l⁻¹; Cambio, Cambridge, United Kingdom), and buffer containing *Taq* polymerase were heated at 94°C for 4 min. This was followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 1.5 min. The PCR products (5 μ l) were characterized by electrophoresis on 1% agarose gels and were visualized under UV light after they were stained with a solution containing 10 μ g of ethidium bromide per μ l.

DNA sequencing. Amplification products from the PCR were sequenced directly by using an Applied Biosystems model 373A automated DNA sequencer. PCR samples which produced a single band on agarose gels were purified for sequencing by using Centricon C-100 columns (Amicon, Gloucester, United Kingdom). Approximately 150 ng of template was sequenced by using a *Taq* dye deoxy terminator sequencing kit (ABI) and the protocol recommended by the manufacturer. The primers used for sequencing are shown in Table 2 (8, 23).

RESULTS

Amplification of genomic DNA with universal 16S rRNA primers Po and Pc3 resulted in a single 750-bp band on agarose gels. DNAs from 11 members of the genera *Moraxella*, *Neisseria*, *Acinetobacter*, and *Kingella* were amplified, and the fragments were sequenced by using the six primers shown in Table 2. At each position the nucleotides on both strands were determined to ensure correct base assignment. The sequences have been deposited in the GenBank and EMBL sequence data bases under the accession numbers shown in Table 1. The sequences were aligned and compared by using the Clustal V suite of programs (9). The sequences which we obtained were between 712 and 743 bp long, and the 5' end was located at position 44 in the *Escherichia coli* numbering system (4).

To quantify the relationships between species, the aligned DNA sequences were used to produce a distance matrix for pairs of sequences (Table 3) by using the neighbor-joining method of Saitou and Nei (18). A tree was produced from

TABLE 3. Distance matrix showing levels of homology between species, based on partial 16S rDNA sequences

Strain	% Homology with:									
	<i>M. catarrhalis</i> NCTC 11020 ^T	<i>M. lacunata</i> NCTC 11011 ^T	[<i>N.</i>] <i>ovis</i> NCTC 11227 ^T	<i>M. osloensis</i> NCTC 10465 ^T	[<i>M.</i>] <i>phenylpyruvica</i> NCTC 10526 ^T	<i>A. calcoaceticus</i> NCTC 10292	<i>A. lwoffii</i> NCTC 05866 ^T	<i>N. meningitidis</i> NCTC 10025 ^T	<i>K. kingae</i> NCTC 10529 ^T	<i>N. lactamica</i> NCTC 10617 ^T
<i>M. lacunata</i> NCTC 11011 ^T	94.6									
[<i>N.</i>] <i>ovis</i> NCTC 11227 ^T	94.9	94.8								
<i>M. osloensis</i> NCTC 10465 ^T	90.1	90.1	90.1							
[<i>M.</i>] <i>phenylpyruvica</i> NCTC 10526 ^T	89.7	89.3	88.2	89.8						
<i>A. calcoaceticus</i> NCTC 10292	87.6	87.2	87.2	85.3	86.5					
<i>A. lwoffii</i> NCTC 05866 ^T	87.7	87.7	87.0	85.4	86.2	95.6				
<i>N. meningitidis</i> NCTC 10025 ^T	81.2	82.1	81.2	79.7	80.6	81.8	80.4			
<i>K. kingae</i> NCTC 10529 ^T	81.3	80.5	80.8	78.4	79.2	80.4	79.0	92.3		
<i>N. lactamica</i> NCTC 10617 ^T	80.8	80.3	80.1	79.2	79.0	80.7	81.0	93.5	91.0	
<i>N. subflava</i> NCTC 04590	81.8	81.1	81.3	80.3	80.7	80.8	80.3	95.5	91.2	94.2

FIG. 1. Rooted phylogenetic tree. Horizontal distances are equivalent to genetic distances; vertical distances have no meaning.

these data, and the tree illustrates the relationships between species based on genetic distances (Fig. 1). The tree was rooted at the midpoint of its longest branch. All of the species used in this study fell into one of two large groups on the tree. The upper group included *M. lacunata* subsp. *lacunata*, [*N.*] *ovis*, *M. catarrhalis*, *M. osloensis*, [*M.*] *phenylpyruvica*, *A. calcoaceticus*, and *A. lwoffii*, and the lower group included *K. kingae*, *N. lactamica*, *N. meningitidis*, and *N. subflava*. The levels of sequence homology between members of the two groups varied from 78.4% (*M. osloensis* and *K. kingae*) to 82.1% (*M. lacunata* subsp. *lacunata* and *N. meningitidis*).

M. lacunata subsp. *lacunata*, [*N.*] *ovis*, and *M. catarrhalis* formed a tight cluster on the dendrogram (the *M. lacunata* cluster), with levels of DNA homology of at least 94.6%. *M. osloensis* and [*M.*] *phenylpyruvica* exhibited lower levels of homology with members of this cluster (90.1 and 88.2 to 89.7%, respectively) and with each other (89.8%). The two *Acinetobacter* species were more closely related to each other (95.6% homology) than to members of the *M. lacunata* cluster (87.0 to 87.7% homology), *M. osloensis* (85.3 to 85.4% homology), or [*M.*] *phenylpyruvica* (86.2 to 86.5% homology). In the *Neisseria* group the true neisseriae were more closely related to each other (93.5 to 95.5% homology) than to *K. kingae* (91.0 to 92.3% homology), and the levels of DNA homology between *N. meningitidis* and *N. subflava* were slightly higher than the levels of homology between *N. meningitidis* and *N. lactamica*.

In addition to the sequences of the 11 isolates described above, the sequences of four clinical isolates of *M. catarrhalis* obtained from Scotland, Belgium, Australia, and South Africa were also determined. These isolates exhibited very high levels of DNA homology with the type strain (99.8 to 100%) (data not shown).

DISCUSSION

rDNA sequencing is the most useful tool for studying bacterial evolution because the sequences of the genes involved are subject to different rates of change. This means that sequence comparisons can be used to measure the relationship between distant or close relatives (24). 16S rDNA is the most

widely studied of the ribosomal genes as it contains sufficient information to accurately determine most phylogenetic relationships up to about the species level (11). We used an automatic DNA sequencer and the PCR (8) to sequence approximately one-half of the 16S rDNAs from 11 species belonging to the *Proteobacteria*.

Relationship between the *Neisseria*-*Kingella* group and the *Moraxella*-*Acinetobacter* group. DNA-DNA and rRNA-DNA hybridization studies of species belonging to the *Neisseriaceae* led to the proposal that the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* should be excluded from this family (14) and should instead be placed in a new family, the *Moraxellaceae* (15). We found little genetic similarity between members of the emended family *Neisseriaceae* and members of the proposed family *Moraxellaceae*. Our sequence data indicate that on the basis of rRNA sequence signatures (19), the true *Neisseria* species and *K. kingae* belong to the β subclass of the *Proteobacteria*, whereas the genera *Moraxella* (including [*N.*] *ovis*) and *Acinetobacter* belong to the γ subclass. We support the proposal of Rossau et al. (15) that [*N.*] *ovis* should be renamed *Moraxella ovis* as it is very closely related to other *Moraxella* species and is only distantly related to members of the *Neisseriaceae*.

***Moraxella*-*Acinetobacter* group.** We placed the *Acinetobacter* and *Moraxella* species which we studied into the following four groups: (i) *Acinetobacter* species; (ii) the *M. lacunata* cluster, comprising *M. catarrhalis*, *M. lacunata* subsp. *lacunata*, and [*N.*] *ovis*; (iii) *M. osloensis*; and (iv) [*M.*] *phenylpyruvica*. These results are consistent with the results of other workers obtained by using rRNA-DNA and DNA-DNA hybridization methods (15, 22). Catlin (5) proposed that the genus *Acinetobacter* should be removed from the same family as the genus *Moraxella* because of phenotypic differences and a lack of phylogenetic evidence. Fatty acid analysis and rDNA-DNA hybridization results (22) suggested that there is a close relationship between the genera *Moraxella* and *Acinetobacter*, which was confirmed by our data. We therefore agree with Rossau et al. (15) and Veron et al. (22) that the genus *Acinetobacter* should be placed in the same family as the genus

Moraxella despite the small phenotypic differences discussed by Catlin (5).

M. osloensis forms a separate group within the *Moraxellaceae* according to our data and the hybridization results of Rossau et al. (15). Rossau et al. proposed that this species should be removed from the genus *Moraxella* on the basis of their results, but a recent report (20) confirmed that *M. osloensis* is a genuine member of the genus *Moraxella* as it has melting temperatures of elution (as determined by rRNA-DNA hybridization) similar to those of a new *Moraxella* species, *Moraxella lincolnii*. *M. osloensis* and *M. lincolnii* have melting temperature of elution differences of less than 5°C, which, together with the phenotypic similarities between *M. osloensis* and other members of the genus, suggests that *M. osloensis* is a genuine member of the genus *Moraxella*. [*M.*] *phenylpyruvica* is not as closely related to the *M. lacunata* group as *M. osloensis* is but is more closely related than either of the *Acinetobacter* species is. Rossau et al. (15) expressed doubts concerning whether this organism should be placed in the genus *Moraxella* as it is closely related to *Psychrobacter immobilis* genotypically but is not a member of this species on the basis of the results of transformation studies (10). We agree with Rossau et al. that this species should be placed in the *Moraxellaceae* on the basis of phylogenetic and phenotypic data, but there is little evidence to keep this species in the genus *Moraxella*. Further study of *P. immobilis* will be required to establish the taxonomic position of [*M.*] *phenylpyruvica*.

Neisseria-Kingella group. We found that the true neisseriae and *K. kingae* belong to the β subclass of the *Proteobacteria* on the basis of 16S rDNA sequence data (19). These organisms form a cluster with DNA homology values of $\geq 91.0\%$. *N. meningitidis* 16S rDNA exhibits a slightly higher level of sequence identity with *N. subflava* DNA than with *N. lactamica* DNA. These results are in contrast to the findings of Rossau et al. (14), who, on the basis of DNA-DNA hybridization results, suggested that *N. lactamica* and *N. meningitidis* are members of a subgroup distinct from *N. subflava*. Our results could be explained by the fact that we used a different strain of *N. subflava* (NCTC 04590) than Rossau et al. used (ATCC 10555).

Using the PCR and an automated DNA sequencer, we sequenced about one-half of the 16S rDNAs of 11 bacterial species. Our results are in general agreement with the results found in similar phylogenetic studies in which hybridization techniques were used, but the 16S rDNA sequencing approach has the advantage that sequences, which are deposited in the GenBank and EMBL data bases, can be compared quickly with sequences of any other bacteria. The speed and simplicity of automated 16S rDNA sequencing are comparable to the speed and simplicity of hybridization methods.

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