Phylogeny of the family *Moraxellaceae* by 16S rDNA sequence analysis, with special emphasis on differentiation of *Moraxella* species

Bertil Pettersson,¹ Angeli Kodjo,² Mostafa Ronaghi,¹ Mathias Uhlén¹ and Tone Tønjum^{3†}

Author for correspondence: Tone Tønjum. Tel: +47 22 86 95 10. Fax: +47 22 86 94 90.
e-mail: tone.tonjum@rh.uio.no

¹ Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-10044 Stockholm, Sweden

² Ecole Nationale Vétérinaire de Lyon, Lyon, France

³ Institute of Medical Microbiology, Department of Molecular Biology, University of Oslo, National Hospital, N-0027 Oslo, Norway

Thirty-three strains previously classified into 11 species in the bacterial family *Moraxellaceae* were subjected to phylogenetic analysis based on 16S rRNA sequences. The family *Moraxellaceae* formed a distinct clade consisting of four phylogenetic groups as judged from branch lengths, bootstrap values and signature nucleotides. Group I contained the classical moraxellae and strains of the coccal moraxellae, previously known as *Branhamella*, with 16S rRNA similarity of $\geq 95\%$. A further division of group I into five tentative clusters is discussed. Group II consisted of two strains representing *Moraxella atlantae* and *Moraxella osloensis*. These strains were only distantly related to each other (93.4%) and also to the other members of the *Moraxellaceae* ($\leq 93\%$). Therefore, reasons for reclassification of these species into separate and new genera are discussed. Group III harboured strains of the genus *Psychrobacter* and strain 752/52 of [*Moraxella*] *phenylpyruvica*. This strain of [*M.*] *phenylpyruvica* formed an early branch from the group III line of descent. Interestingly, a distant relationship was found between *Psychrobacter phenylpyruvicus* strain ATCC 23333^T (formerly classified as [*M.*] *phenylpyruvica*) and [*M.*] *phenylpyruvica* strain 752/52, exhibiting less than 96% nucleotide similarity between their 16S rRNA sequences. The establishment of a new genus for [*M.*] *phenylpyruvica* strain 752/52 is therefore suggested. Group IV contained only two strains of the genus *Acinetobacter*. Strategies for the development of diagnostic probes and distinctive sequences for 16S rRNA-based species-specific assays within group I are suggested. Although these findings add to the classificatory placements within the *Moraxellaceae*, analysis of a more comprehensive selection of strains is still needed to obtain a complete classification system within this family.

Keywords: *Moraxella*, 16S rDNA sequences, phylogeny, classification

INTRODUCTION

Several fastidious Gram-negative bacterial species, other than established human pathogens such as *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Haemophilus influenzae*, are emerging as being important

as causes of opportunistic infections, including species of the bacterial family *Moraxellaceae*. For example, all members of the classical moraxellae, that is *Moraxella lacunata*, *Moraxella nonliquefaciens* and *Moraxella bovis*, may cause eye infections (7, 9, 25, 45). *M. nonliquefaciens*, which may be part of the normal airway flora, has been found as a cause of septicaemia in leukaemic patients (3, 53). *Moraxella* (*Branhamella*) *catarrhalis* may cause eye, ear and sinus disease in children, a population displaying a high rate of colonization with this species (14, 15). This agent can also cause exacerbations of chronic bronchitis.

[†]Present address: Department of Microbiology and Immunology, University of Michigan Medical School, 5641 Medical Science Building II, Ann Arbor, MI 48109-0620, USA. Tel: +1 313 647 6765. Fax: +1 313 764 3562.
The GenBank accession numbers for the sequences reported in this paper are AF005160–AF005192.

Table 1. Bacterial strains included in the study

Species/strain	Origin	Accession no.	Reference
<i>M. lacunata</i>			
TT25: ATCC 17967 ^T	Eye infection	AF005160	Henriksen (23)
TT26: ATCC 17956	Eye infection	AF005161	Juni (34)
TT27: E 7992	Eye infection	AF005162	Juni (34)
TT28: 50141	Conjunctivitis	AF005163	Ringvold <i>et al.</i> (45)
TT29: 50142	Conjunctivitis	AF005164	Ringvold <i>et al.</i> (45)
TT41: E2371	Conjunctivitis	AF005165	Juni <i>et al.</i> (34)
TT42: CDC9833	Conjunctivitis	AF005166	Tønjum (5, 52)
TT53: E3808	Conjunctivitis	AF005167	Juni <i>et al.</i> (34)
TT54: NCTC 7911	Conjunctivitis	AF005169	Bøvre (5)
TT55: A947	Conjunctivitis	AF005168	Bøvre (5)
TT56: ATCC 11748	Conjunctivitis	AF005170	Bøvre (5)
TT57: 28419/84	Conjunctivitis	AF005171	Bøvre (7)
<i>M. nonliquefaciens</i>			
TT38: NCTC 7784	Nasopharynx	AF005172	Bøvre (5)
TT23: B2000	Septicaemia	AF005173	Tønjum <i>et al.</i> (53)
TT24: 3179/66	Nasopharynx	AF005174	Bøvre (5)
TT33: 13385/62	Nasopharynx	AF005175	Henriksen (23)
TT34: P2581/88	Septicaemia	AF005176	Tønjum <i>et al.</i> (53)
TT36: 2916/66	Nasopharynx	AF005177	Bøvre (5)
TT37: B6270/90	Nasopharynx	AF005178	Tønjum <i>et al.</i> (53)
TT39: 826/61	Nasopharynx	AF005179	Henriksen & Bøvre (24)
TT43: 270/60	Nasopharynx	AF005180	Henriksen & Bøvre (24)
TT44: 672/58	Nasopharynx	AF005181	Henriksen & Bøvre (24)
<i>M. bovis</i>			
TT21: ATCC 10900 ^T	Bovine conjunctivitis	AF005182	Hughes <i>et al.</i> (25)
TT22: 5	Bovine conjunctivitis	AF005183	Bøvre (5)
<i>M. equi</i>			
TT31: 372/72 = ATCC 25576 ^T	Equine conjunctivitis	AF005184	Hughes <i>et al.</i> (25)
<i>M. catarrhalis</i>			
TT9: Ne11 = ATCC 25238 ^T	Bronchitis	AF005185	Catlin (13)
<i>M. ovis</i>			
TT30: 199/55 = ATCC 33078 ^T	Ovine respiratory tract	AF005186	Bøvre (6)
<i>M. caviae</i>			
TT47: CCUG 355 ^T	Pharynx, guinea pig	AF005187	Enevold Falsen
<i>M. cuniculi</i>			
TT49: CCUG 2154 ^T	Oral mucosa, rabbit	AF005188	Berger <i>et al.</i> (56)
TT50: CCUG 27179	Pharynx, rabbit	AF005189	Enevold Falsen
<i>M. osloensis</i>			
TT11: 5873	Respiratory tract	AF005190	Bøvre & Henriksen (10)
<i>M. atlantae</i>			
TT12: CDC A1922	Blood	AF005191	Bøvre <i>et al.</i> (8)
[<i>M.</i>] <i>phenylpyruvica</i>			
TT10: 752/52	Spinal fluid	AF005192	Bøvre & Henriksen (10)

The classification systems for species of the *Moraxellaceae* are still incomprehensive and evolving. The genera currently belonging to the *Moraxellaceae* are *Moraxella* including the previous *Branhamella*, *Acinetobacter* and *Psychrobacter* as suggested by Rossau and co-workers (46). Genetic relationships between these species have previously been extensively studied by genetic transformation (5, 7–9, 29–33, 35). This method has added most valuable information to their genetic affinities when key conventional phenotypic

characteristics could not distinguish between these different entities. The dependence of this method on natural competence for transformation and growth caused a need for other nucleic acid techniques. Some species of genus *Moraxella*, such as *Moraxella atlantae*, *Moraxella osloensis* and [*M.*] *phenylpyruvica* are so distantly related that they can be separated and thereby identified by DNA–DNA hybridization using total genomic probes (52). One exception to this finding is the group containing the classical mor-

axellae. The species designated as *M. lacunata* unifies the previous *M. liquefaciens* and *M. lacunata*, based on observations of particularly close genetic affinities (5, 23, 34, 38).

The taxonomic placements of the less related moraxellae such as *M. osloensis*, *M. atlantae* and [*M.*] *phenylpyruvica* are still not detailed, as they display weaker genetic affinities to the classical moraxellae by transformation (8, 10, 11), also verified by biochemical characterization and rRNA hybridization (46, 54) as well as partial 16S rDNA sequence analysis (17).

Sequence comparison of RNA genes of the ribosomal small subunit, 16S rDNA, is a powerful tool for phylogenetic analysis of bacterial species (36, 39, 48, 58). In this work we present nearly full-length primary structures of the 16S rRNA molecule from 33 strains previously classified into the genus *Moraxella*. The sequences were obtained by semi-automated solid-phase rDNA sequencing (26, 27, 40–43, 57). The sequences were used to elucidate the relationships between species belonging to the *Moraxellaceae*. The results were compared with previous classification schemes based on conventional methods and other nucleic acid techniques. Our main goals were to determine the relationships within the classical moraxellae as well as deciphering the placements of more distantly related *Moraxella* species and other members of the family *Moraxellaceae*.

METHODS

Bacterial strains and growth conditions. The strains investigated in this study are listed in Table 1. Species assignment was based on previous studies by genetic transformation in our laboratory (5–11, 23, 24, 52, unpublished data), and results from Juni and co-workers (29–34). *M. lacunata* strains E2371 and E7992 were generously supplied by Dr E.

Juni, University of Michigan, USA. Strains *Moraxella cuniculi* CCUG 2154 and *Moraxella caviae* CCUG 355 were gratefully received from Dr E. Falsen at the CCUG strain bank in Gothenburg, Sweden. Strains were grown on 5% human blood agar plates (Difco) and chocolate agar plates at 33 °C with 5% CO₂.

In vitro amplification and DNA sequencing of the 16S rRNA gene. The PCR reactions were performed in a semi-nested fashion with one of the primers biotinylated as described previously (41). The 16S rRNA genes were amplified directly from the bacterial genome with the primers detailed in Table 2. Immobilization of the biotinylated PCR products followed by strand separation and template preparation was performed with superparamagnetic beads, Dynabeads M-280 Streptavidin (Dyna). The nucleotide sequences from both strands were determined by automated solid-phase DNA sequencing (26, 27, 57) with the ALFexpress (Pharmacia Biotech) as described previously (40–43). The sequencing primers are listed in Table 2.

Analysis of the 16S rRNA gene sequences. The sequences determined in this work were edited manually and aligned with a selection of 16S rRNA sequences from members of the beta and gamma subclasses of the *Proteobacteria*. These were retrieved from the sequence collection of the Ribosomal Database Project (37) and from GenBank. The secondary structure of the 16S rRNA molecule of the *rrnB* operon of *Escherichia coli* (4, 22) was retrieved and used for proper identification of stem-loop regions in the alignment procedure. Non-resolved positions and gaps were removed prior to the phylogenetic analysis. Distance matrices were corrected for multiple base changes at single locations by the method of Jukes & Cantor (28). Phylogenetic trees were constructed from evolutionary distance matrices by the neighbour-joining method of Saitou & Nei (47) using NEIGHBOR, contained in the Phylogenetic Inference Package, PHYLIP 3.51c (18, 19). Parsimony analysis was performed by using DNAPARS. Maximum likelihood based phylogenies were constructed with the DNAML program. The results obtained by both NEIGHBOR and DNAPARS were subjected to

Table 2. Primers used for PCR and DNA sequencing of *Moraxella* species

Primer*	Position†	Sequence‡	Application	Reference
607	11–35	5'-USP-GTTTGATCYTRGYTCAGAWTGAACG-3'	PCR	This study
612	339–360	5'-USP-CTCCTAYGGGAGGCWGCAGKGG-3'	PCR	This study
390B	939–917	5'-CTTGTGCGGGYYCCCGTCAATTC-3'	PCR	(40)
621B	1517–1494	5'-RSP-CCSSTACGGITACCTTGTTACGAC-3'	PCR	This study
USP		5'-CGTTGTAAAACGACGGCCAG-3'	Sequencing	(43)
RSP		5'-CACAGGAAACAGCTATGACC-3'	Sequencing	(43)
631C	535–519	5'-ATTACCGCGGCKGCTG-3'	Sequencing	This study
538C	800–818	5'-GTAGTCCACGCCGTAACG-3'	Sequencing	(43)
390C	939–917	5'-CTTGTGCGGGYYCCCGTCAATTC-3'	Sequencing	(43)
624C	1237–1220	5'-GTAGYAYGTGTGWAGCCC-3'	Sequencing	This study
623C	1184–1200	5'-GGGGATGATGTCAARTC-3'	Sequencing	This study

* B and C indicate that a biotin or an indodicarbocyanine (Cy5) dye, respectively, are coupled to the oligonucleotide.

† Numbers are according to the *E. coli* sequence (4).

‡ USP and RSP means that the primer has a handle at the 5'-end with the sequence of the universal sequencing primer or the reverse sequencing primer. W, R, K, S and Y denote degenerated positions according to the single-letter code proposed by the Nomenclature Committee of the International Union of Biochemistry. An 'I' indicates that the primers have been synthesized with an inosine residue in the actual position.

bootstrap analysis by using SEQBOOT in sets of 500 resamplings. The CONSENSE program was used for the subsequent computerization of majority-rule and strict consensus trees from the user trees.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes from the *Moraxella* species and strains investigated in this work have the GenBank accession numbers listed in Table 1. The GenBank accession numbers for the 16S rRNA sequences of the reference strains used for comparison in this study are as follows: *Acinetobacter calcoaceticus* ATCC 11336, M34139 (58); *Acinetobacter lwoffii* ATCC 17925, U10875 (58); *Aeromonas hydrophila* ATCC 7966^T, X60404 (38); *Alcaligenes faecalis* ATCC^T, M22508 (16); *Cardiobacterium hominis* ATCC 15826^T, M35014 (16); *Escherichia coli*, J01695 (4); *Haemophilus influenzae* ATCC 33391^T, M59433 (16); *Neisseria animalis* ATCC 19573, L06172 (17); *Oceanospirillum linum* ATCC 11336^T, M22365; *Pseudomonas aeruginosa* ATCC 25330, M34133 (58); *Psychrobacter immobilis* ATCC 43116^T, U3-9399 (2); *Psychrobacter phenylpyruvicus* ATCC 23333^T, U46144 (2).

RESULTS AND DISCUSSION

Nucleotide sequence determination of the 16S rRNA gene

Direct solid-phase DNA sequencing resulted in an unambiguous determination of almost complete (>95%) sequences from the 16S rRNA genes of 34

strains from 11 recognized species of *Moraxellaceae* listed in Table 1. Both strands were sequenced to obtain accurate data. The obtained sequences were 1444–1451 nucleotides long, depending on the strain investigated. Polymorphic positions due to nucleotide differences between the rRNA genes at certain positions were not observed as was recently reported for certain strains of mycoplasmas and bacilli (40–43). This may indicate that either only one 16S rRNA gene is present in *Moraxella* species, that intrastrain variability of the nucleotide composition between the 16S rRNA genes does not exist or that the potentially multiple 16S rRNA genes are identical.

Phylogenetic position of the family *Moraxellaceae*

Sequence information of the 16S rRNA genes from members of the family *Moraxellaceae* and selected relatives of the *Proteobacteria* (37, 49) was used to place the accommodated genera of *Moraxellaceae* (46) within the *Proteobacteria*. For this purpose, a distance matrix was calculated for 10 species representing the *Moraxellaceae* and eight relatives in the beta and gamma lineages of the *Proteobacteria*. The matrix was calculated from a sequence alignment consisting of the 1355 positions for which data were present. An evolutionary distance tree derived from the distance data by using the neighbour-joining method (47) is

Fig. 1. Phylogenetic tree based on distance matrix analysis of 1355 positions in the 16S rRNA genes from selected members of *Moraxellaceae*, of which some were sequenced in this work, and relatives of the *Proteobacteria*. *Alcaligenes faecalis* was used as the out-group. Bootstrap percentage values obtained from 500 resamplings of the data set are given at the nodes. The tree reveals four distinct clades within the family *Moraxellaceae*, denoted groups I–IV.

Table 3. Signature nucleotide positions of the *Moraxellaceae*

The analysis included 16S rRNA sequences of the *Moraxellaceae* family generated in this study and deposited in GenBank.

Position of base or pair*	<i>Moraxellaceae</i> family	Bacteria†	Exceptions (phylum, genus or other phylogenetic group)‡
124·237	U·A	G·C	U·A: <i>Colwellia</i> assemblage, <i>Alteromonas</i> group, HP U·U: <i>Telluria</i>
423	U	G§ G : <i>Psych</i> (2) A : <i>M. atl</i>	U: <i>Mycoplasma pneumoniae</i> group (some) A: STB subdivision
440	A	U, C	A: <i>Geotoga</i> , <i>Petrogala</i> , <i>Thermus</i> , <i>Deinococcus</i> , <i>Serpulina</i> , <i>Leptospira</i> subdivision, <i>Pseudomonas</i> sub-group, <i>Francisella</i> sub-group, <i>Clostridium thermocellum</i> sub-group, <i>Acetogenium</i> sub-group, thermophilic genera U·A: <i>Chloroflexus</i> subdivision (2), <i>Leptospirillum</i> group, green sulfur bacteria, <i>Holospira</i> , <i>Oceanospirillum</i> assemblage (2), <i>Pseudomonas</i> sub-group (most), <i>Myxobacteria</i> , <i>Peptococcus</i> , mycoplasmas and relatives (most) U·G: <i>Verrucomicrobium</i> , <i>Ehrlichia</i> (few)
514·537	U·A	C·G	U·A: <i>Chloroflexus</i> subdivision (2), <i>Leptospirillum</i> group, green sulfur bacteria, <i>Holospira</i> , <i>Oceanospirillum</i> assemblage (2), <i>Pseudomonas</i> sub-group (most), <i>Myxobacteria</i> , <i>Peptococcus</i> , mycoplasmas and relatives (most) U·G: <i>Verrucomicrobium</i> , <i>Ehrlichia</i> (few)
1156	U	G	U: <i>Prevotella</i> (some), <i>Flexistipes</i> , <i>Caulobacter</i> (some), δ - and ϵ -purple (7), <i>Fusobacterium</i> (most), <i>Peptostreptococcus</i> (some), <i>Mycoplasma</i> (some)
1275	U	A	U: <i>Herpetosiphon</i> , α - and δ -purple (11), <i>Thiothrix nivea</i> sub-group (most), Gram-positive phylum (10)

* Nucleotide positions are according to the *E. coli* numbering (4).

† Dominant base or pair in the corresponding position(s) of (eu)bacteria.

‡ The number of sequences in which the actual residue is found is in parentheses. Groups and sub-groups are according to the nomenclature used by the Ribosomal Database Project (RDP) (37). Abbreviations: STB, *Spirochaeta-Treponema-Borrelia* subdivision; HP, *Haemophilus-Pasteurella* group; α , δ and ϵ , alpha, delta and epsilon subdivisions, respectively, of the purple bacteria (*Proteobacteria*).

§ Many eubacteria truncate this position.

|| A guanine is present in the actual position in 2 of the 16S rRNA sequences of *Psychrobacter* and an adenine is present in the sequence of *M. atlantae*.

presented in Fig. 1. Bootstrap percentage values as obtained from 500 resamplings of the data set are given at the nodes of the tree. *Alcaligenes faecalis* served as an outgroup. The phylogenetic tree showed that the members of the family *Moraxellaceae* constituted a monophyletic taxon forming a distinct line of descent within the gamma subclass of the *Proteobacteria* (Fig. 1). Bacterial families closely related to the *Moraxellaceae* are the *Aeromonadaceae* and the *Pseudomonas* sub-group. Bootstrap analysis and the obtained branch lengths indicated that the *Moraxellaceae* branch was phylogenetically stable. Furthermore, the branch of the *Moraxellaceae* was supported by signature nucleotide positions (Table 3). Signature nucleotides in this context are nucleotide residues explicitly found in positions within the sequence where the base present differs from that found in the majority of the other (eu)bacteria. Moreover, the signatures may be useful for the development of diagnostic probes for rapid detection of members of the family *Moraxellaceae*. One suitable motif is the presence of the

nucleotides U and A in positions 514 and 537, which is covered by the highly conserved eubacterial oligonucleotide UGUGCCAGCAGCCGCGGUAAUACA, of which over 3000 examples are known. As noted in Table 3, this target coincides only with members of mycoplasmas and relatives, *Leptospirillum* group, green sulfur bacteria, *Pseudomonas* sub-group and *Myxobacteria*.

Phylogenetic groups within the *Moraxellaceae*

The *Moraxellaceae* branched into four major lineages designated groups I, II, III and IV (Fig. 1). A tree was also constructed by including all currently known species belonging to *Moraxellaceae* of which 16S rRNA sequence data could be retrieved (not shown). The additional species and strains belonging to the *Moraxellaceae* could be assigned to any of the four clades, without significant destabilization of the nodes of groups I–IV. Therefore, the reduced tree presented in Fig. 1 representatively describes the phylogeny of

Fig. 2. Phylogenetic tree based on distance matrix analysis of 1440 positions in the 16S rRNA genes of the moraxellae sequenced in this work belonging to group I and selected members of the family *Moraxellaceae*. *Acinetobacter calcoaceticus* was used as the out-group. Bootstrap percentage values obtained from 500 resamplings of the data set are given at the nodes. The tree shows that the group I constitutes five clusters: the *Moraxella* sp. strain 27179 branch, the *M. nonliquefaciens* cluster, the *M. lacunata* cluster, the *M. bovis* cluster, and the *M. catarrhalis* cluster (see text for details).

the family *Moraxellaceae*. The four groups were considered to constitute reliable established phylogenetic units as judged from the percentage values obtained by bootstrap analysis (Fig. 1) and the signature and unique nucleotide positions presented in Table 4. The characterization of unique nucleotide features was restricted to be valid for the members of the respective group within the *Moraxellaceae*. A nucleotide residue at a certain position was said to be unique when it held for all strains within one of the four groups with no exception among the strains of any other group. A weak node positioned at the branching point between groups II and III was observed due to a bootstrap percentage of only 55% and a short common ancestral branch for these groups. The instability of this point of bifurcation was also registered by maximum likelihood analysis, where group I swapped its position with that of group III. Therefore, we do not consider the branching order among groups I, II and III to be established with certainty. However, the base pair in position 317-336 showed the composition C·G which was in common for the group II lineage and the early branches of the group III lineage. The later branches of some of the *Psychrobacter* strains showed C·C in the actual base

pair of the group III line of descent. The C·G base pair is only found in *Aquifex* (one example), *Marinobacter* (1 example), the *Actinobacillus* sub-group (few), the *Halomonas* sub-group (some), *Spiroplasma* (few) and in the *Cardiobacterium* group (two examples). Therefore, this base pair was regarded synapomorphic for the common branch of groups II and III. The tree in Fig. 1 is likely to reflect the most likely phylogenetic relationship between these two clades. Groups I and II consisted of species traditionally classified in the genus *Moraxella*, group I contained the classical moraxellae and the coccal moraxellae previously named *Branhamella* (13), while group II consisted of the two species *M. atlantae* and *M. osloensis*. Group III harboured strains of the genus *Psychrobacter* and a single strain of [*M.*] *phenylpyruvica*. Group IV contained two strains of the genus *Acinetobacter*, representing the species *Acinetobacter calcoaceticus*. The four phylogenetic groups were in agreement with those found by using DNA-rRNA hybridization to study the taxonomic relationships of the *Moraxella* and allied bacteria (46). The overall topology of the tree and positioning within the *Moraxellaceae* was also consistent with previous reports based on analysis of partial 16S rDNA sequences from a minor data set

(17), as well as genetic transformation and hybridization (5–11, 46, 52, 53).

Group I, including the genus *Moraxella sensu stricto*: the classical moraxellae and the coccal moraxellae

The phylogeny of group I was further detailed by comparison of the 16S rRNA sequences of 31 strains from eight of the recognized species included in this group. Therefore, 16S rRNA nucleotide information from close relatives of *M. lacunata* and selected members of the family *Moraxellaceae* were aligned. Gaps were removed and the final alignment consisted of 1440 nucleotide positions. A distance matrix was calculated with *A. calcoaceticus* serving as an outgroup (not shown). The members of group I showed $\geq 95\%$ similarity between their 16S rRNA sequences. *M. nonliquefaciens* was represented by only two strains because of a high nucleotide similarity within this species. A phylogenetic tree derived from distance matrix data by using the neighbour-joining method (47) is shown in Fig. 2. Group I contained species of the type genus *Moraxella*, harbouring the classical moraxellae and the coccal moraxellae. Interestingly, this group contains both rod-shaped and coccal species tightly affined within one (sub) genus. The phylogenetic analysis (Fig. 2) revealed five clusters within group I. However, bootstrap analysis by resampling the data set 500 times showed a marked instability for some of the nodes due to low percentage values. This was also indicated by a slightly different branching order obtained with the maximum likelihood method (data not shown) but without altering the species composition of the clusters. A compilation of informative 16S rRNA nucleotide positions was performed to verify the clusters of group I (Table 5). Informative nucleotide positions were restricted to the clusters of group I and defined as sites in which all members of a cluster share a nucleotide residue at a certain position in the 16S rRNA molecule which is not shared with any other member of the group I. The *Moraxella* sp. strain CCUG 27179 was excluded in the unique nucleotide analysis since this strain formed a single strain branch. The five clusters, denoted as the *M. lacunata* cluster, the *M. nonliquefaciens* cluster, the *M. bovis* cluster, the *M. catarrhalis* cluster, and the *Moraxella* sp. strain 27179 branch, could in most cases be characterized by informative 16S rRNA residues. Group I is concordant with the '*M. lacunata* homology group' as described by Rosseau *et al.* (46).

The *M. lacunata* cluster

The *M. lacunata* cluster, encompassing 13 strains of which eight strains showed unique 16S rRNA sequences, was in part relatively heterogeneous with inter-strain similarity values ranging from 98.4 to 99.9% between their 16S rRNA nucleotide sequences (data not shown). Nevertheless, all derived phylogenies resulted in one cluster with an early branch of the type strain ATCC 17967^T of *M. lacunata* and with strain ATCC 17956 forming an intermediate position with a

branch length more than twice of that of the sister strains (Fig. 2).

Interestingly, all *M. lacunata* strains belonging to the small colony variant of this species, previously designated 'group II' (34, 53) or *M. lacunata* subsp. *lacunata* (5, 23, 34), clustered tightly together with a $\geq 99.8\%$ nucleotide similarity between their 16S rRNA sequences. The high similarity was observed despite the fact that these strains originated from different geographic regions (53). In contrast, the large colony variant of *M. lacunata*, previously denoted 'group I' or *M. lacunata* subsp. *liquefaciens* (5, 34, 53), was more heterogeneous and these strains fell more peripherally within the *M. lacunata* cluster. Thus, the 16S rRNA is far more conserved among strains corresponding to the previous *M. lacunata* subsp. *lacunata* as compared with *M. lacunata* subsp. *liquefaciens* strains. Except for strain E7992 which grew with large colonies, a correlation between 16S rRNA sequences and the two distinct groups of *M. lacunata* as determined by colony morphology studies, nutritional requirements, multi-locus enzyme electrophoresis (MLEE), cell wall fatty acid patterns and auxotrophic donor transformation activities toward *Moraxella bovis* (34, 38, 53) was found (Fig. 2).

Surprisingly, *Moraxella equi* strain 327/72^T was contained within the *M. lacunata* cluster with a 16S rRNA sequence similarity of 99.9% to *M. lacunata* strain E2371 (Fig. 2). Moreover, *M. equi* shared all the informative nucleotides with the other members of this cluster (Table 5). Thus, based on 16S rRNA sequence comparison *M. equi* should be included in the *M. lacunata* group II cluster. The relationship between *M. equi* and other classical moraxellae was analysed by phenotypic characterization, genetic transformation and DNA–DNA reassociation experiments. The results suggested that *M. equi* should be taxonomically placed as a close relative of *M. bovis*, DNA–DNA relatedness being 92% (5, 53). A reassignment of *M. equi* as a non-haemolytic variant of *M. bovis* was also supported by data obtained from MLEE and genetic transformation experiments (5, 53). The 16S rRNA sequences of *M. equi* and the two *M. bovis* strains included in this study showed only 96.8 and 97.0% nucleotide similarity (data not shown). The overall genomic DNA–DNA similarity between *M. equi* and *M. lacunata* subsp. *lacunata* (group II) was found to be 57–62% in a previous study (53). Although this value was not as low as expected for non-related strains, the genome of *M. equi* was significantly more homologous to that of *M. bovis*, which is most closely related to *M. lacunata* subsp. *liquefaciens* (group I). Thus, the placement of *M. equi* based on 16S rRNA comparison is contradictory to previous reports and the classification of *M. equi* remains unresolved. This might be one example of limited concordance between total genomic and ribosomal nucleic acid sequences within certain species (20, 50).

The members of the *M. lacunata* cluster studied in this work are characterized by the following oligonucleo-

Table 4. Signature and unique nucleotide positions of the phylogenetic groups within the *Moraxellaceae* family defined in this study

Groups are according to those defined in this study. Group I, the 'classical moraxellae' and the coccoid moraxellae; group II, *M. osloensis* and *M. atlantae*; group III, [*M.*] *phenylpyruvica* and *Psychrobacter*; group IV, *Acinetobacter*. A signature nucleotide is a nucleotide residue explicitly found in a certain position within the sequence in all members of the actual group where the base present differs from that found in the majority of other (eu)bacteria. A unique nucleotide holds for all members of a certain group within the *Moraxellaceae* but the actual residue may be found in several other (eu)bacteria. Signature nucleotides are in bold and unique nucleotides are underlined.

Position of base or pair*	Base or base pair in group:				
	I	II	III	IV	Exception(s) (phylum, genus or other phylogenetic group)†
121	U	<u>C</u>	U	U	No signature
123-238	C·G	U·A	C·G	C·G	U·A: Enterics and relatives, <i>Cardiobacterium</i> group (2), <i>Wolbachia</i> , <i>Neisseria</i> group (most), <i>Bordetella</i> sub-group, <i>Methylophilus</i> group, other purple (few)
150	U	U	U	<u>C</u>	No signature
198-219	G·C	G· <u>U</u>	G·C	G·C	No signature
257-269	G·U	G· <u>C</u>	G·U	G·U	No signature
289-311	G·C	A·U	G·C	G·C	A·U: <i>Saprosira</i> group (some), <i>Borrelia</i> group, <i>Spirillum</i> , <i>Pseudomonas</i> sub-group, <i>Plesiomonas</i> , <i>Bdellovibrio</i> , <i>Campylobacter</i> and relatives, other purple (2) G·U: <i>Nanocystis</i> , <i>Actinomyces</i> sub-group, <i>Quinella</i> , <i>Haloanaerobium</i> A·C: <i>Coprococcus</i> (1), <i>Coprococcus cuprinolyticum</i> sub-group (2)
320-333	G·C	<u>A·U</u>	U·C, G·C	G·C	No signature
443-491	<u>U·A</u>	C·G	C·G	C·G	No signature
445-489	G·C	G·C	G·C	<u>A·U</u>	No signature
446-488	G·C	G·C	<u>U·G</u>	G·C	G: <i>Serpulina</i> , <i>Spirochaeta</i> (some), <i>Treponema</i> , <i>Vibrio</i> group (some), HP (some), <i>Mycobacterium</i> (most), <i>Actinomyces</i> (most)
(475)‡	-	-	-	A, U	A, U: FCB (3)
478‡	<u>C</u>	G, U	G	G, U	No signature
479‡	<u>C</u>	G, U	<u>A</u>	U	No signature
480	U	A, U	U	G	G: <i>Francisella</i> sub-group, <i>Legionella</i> sub-group, <i>Halomonas</i> sub-group (some)
560	A	A	A	U§	U: <i>Brucella</i> assemblage (some), <i>Phyllobacterium</i> , <i>Beijerinckia</i> sub-group, <i>Bartonella</i> sub-group, <i>Bdellovibrio stolpii</i> group (2)
580-761	C·G	U·A	C·G	C·G	U·A: <i>Leptospirillum</i> , α -purple (1), β -purple (1), <i>Spirochaetes</i> and relatives (few), <i>Capnocytophaga</i> (1), <i>Alteromonas</i> group (some), <i>Vibrio</i> group, <i>Desulfuromonas</i> group, <i>Bdellovibrio bacteriovorus</i> group, <i>Myxobacteria</i> , <i>Arcobacter</i> , <i>Nocardia</i> , <i>Gardnerella</i> , <i>Arthrobacter</i> group (few), <i>Propionibacterium</i> group (few), <i>Clostridium thermobutyricum</i> sub-group, <i>Epulopiscum</i> , <i>Acidaminococcus</i> , <i>Mycoplasma hominis</i> group (some)
592-647	U·G, U·A, A·U, C·G	A·U	<u>G·U</u>	U·G, A·U,	No signature
593-646	U·G	U·G, C·G	<u>A·U</u>	U·G	No signature
616-624	G·C	G·C	G·C	<u>A·U</u>	No signatures
631	C	C	C	<u>U</u>	No signature
661-744	U·A	U·A	U·A	<u>G·C</u>	No signature
771-808	G·C	G·C	G·C	A·U	A·U: Gr- (7), <i>Arthrobacter</i> (most), <i>Corynebacterium</i> , <i>Lactobacillus</i> (most), <i>Actinomyces</i> group (most), <i>Actinopolyspora</i> , other Gr+ (15)

Table 4 (cont.)

Position of base or pair*	Base or base pair in group:				
	I	II	III	IV	Exception(s) (phylum, genus or other phylogenetic group)†
827 ·873	C·A	U·A	U·A	U·A	C: <i>Prevotella</i> , <i>Leptospira</i> , <i>Rhodobacter</i> group, <i>Bradyrhizobium</i> sub-group, methylobacteria, other (eu)bacteria (7)
1006·1023	U·A	U·A	U·A	<u>C·G</u>	No signature
1244·1293	U·A	U·A	U·A	<u>C·G</u>	No signature
1304	G	G	A	G	A: <i>Hydrogenobacter</i> (2), <i>Fervidobacterium</i> (1), <i>Chloroflexus</i> subdivision (most), <i>Deinococcus</i> , <i>Gemmata</i> , STB (few), α -purple (4), <i>Bdellovibrio</i> , <i>Myxobacteria</i> (most), Gr+ (12)
1421·1479	U·G	C¶·G	U·G	U·G	C: TOR (most), FCB (many), <i>Leptospirillum</i> group (2), <i>Ectothiorhodospira</i> assemblage, <i>Myxobacteria</i>
1423·1477	A·U	A·U	A·U, A·C	<u>G·C</u>	No signature
1425·1475	C·G	U·G	U·G, U·C	U·G	C: <i>Sporomusa</i> sub-group (most), Cyanobacteria (some), <i>Ehrlichia</i> (2), <i>Arcobacter</i> (3)
1426·1474	<u>U·G</u>	G·U	G·U	G·U	No signature
1439·1462	<u>U·A</u> , G·C: <i>M. ovis</i>	G·C	A·U, G·C: <i>M. phe</i>	C·G, G·C	A: TOR (most), <i>Simkania</i> , Cyanobacteria (some), <i>Leptospira</i> group, <i>Burkholderia</i> sub-group I, <i>Bordetella</i> sub-group, <i>Rubrivivax</i> sub-group, <i>Rhodocyclus</i> , <i>Halomonas</i> group (most), <i>Campylobacter</i> (some), <i>Mycoplasma pneumoniae</i> group (some), other Gr+ (7)

* Nucleotide positions are according to the *E. coli* numbering (4).

† The number of sequences in which the actual residue is found is shown in parentheses. Exceptions are listed for signature residues only. Groups and sub-groups are according to the nomenclature used by the Ribosomal Database Project (RDP) (37). Abbreviations: FCB, *Flexibacter*–*Cytophaga*–*Bacteroides* phylum; STB, *Spirochaeta*–*Treponema*–*Borrelia* subdivision; α and β , alpha and beta subdivision of the purple bacteria (*Proteobacteria*); TOR, thermophilic oxygen reducers; Gr– and Gr+, Gram-negative and Gram-positive (eu)bacteria.

‡ Many (eu)bacteria truncate this region. Parentheses denote that this position is missing in *E. coli* and the number indicates the adjacent position which is present in the 16S rRNA sequence of *E. coli*.

§ Comparison restricted to *Proteobacteria*.

|| This might be a non-basepairing position in the 16S rRNA molecule of the classical *Moraxella*.

¶ Comparison restricted to Gram-negative (eu)bacteria.

tide starting at position 184, UCUTACGAGA, in the vicinity of the tetra-nucleotide loop starting at position 187 of the variable region V2 (21) of the 16S rRNA molecule. This potential probe has four unique nucleotides (bold) and will detect only strains of the *M. lacunata* cluster and distinguish them from all other strains of group I except for *Moraxella* sp. strain CCUG 27179, which also shares this oligonucleotide sequence. The probe will be evaluated elsewhere.

The *M. nonliquefaciens* cluster

M. nonliquefaciens strains overall showed a high interstrain similarity ($\geq 99.8\%$) on the 16S rRNA level (Table 6). Nevertheless, only 2 out of 10 strains shared their exact 16S rRNA sequences with another strain of this species, indicating a certain heterogeneity. A phylogenetic tree was constructed including all *M. nonliquefaciens* strains sequenced in this study. No formation of subclusters was observed as determined

from bootstrap values and single nucleotide analysis (data not shown). Therefore, *M. nonliquefaciens* was only represented by the two strains, NCTC 7784 and B2000 (Fig. 2). Two nucleotide positions in the *M. nonliquefaciens* 16S rRNA gene can be utilized for diagnostic purposes as they are only shared with *M. catarrhalis*. These residues are situated at positions 669 (G) and 737 (C). Since *M. nonliquefaciens* is so closely related to *M. lacunata* and both species colonize similar habitats in humans, rapid and reliable methods for the detection and differentiation between these species are needed. Therefore, the informative residues presented for *M. lacunata* and the characteristic nucleotides of *M. nonliquefaciens* could be highly valuable in a PCR or hybridization diagnostic assay based on the 16S rRNA gene. Moreover, *M. nonliquefaciens* differs from *M. lacunata* by the oligonucleotide GGGUCUU-(C)UUA(G)AAGAC, starting at position 835. The residues present in the differing positions in the 16S rRNA molecule of *M. nonliquefaciens* are in bold and

Table 5. Informative nucleotide positions of the 16S rRNA gene correlated to the phylogenetic clusters of the moraxellae belonging to group I

Cluster	Nucleotide at position:*													
	(75)	78	79	90	91	92	97	98	184	186	191	193	1000	1040
<i>M. lacunata</i>	A	U	C	G	A	C	G	A	U	U	A	A	U, A	A, U
<i>M. nonliquefaciens</i>	A	U	C	G	A	C	G	A	A	C	G	U	U	A
<i>M. catarrhalis</i>	U	G	G	C	U	G	A	C	A	C	G	U	U, A	A, U
<i>M. bovis</i>	A	U	C	G	A	U	G	A	A	C	G	U	C	G

* Nucleotide positions according to the *E. coli* numbering (4). The strain *Moraxella* sp. 27179 is excluded (see text for details). Parentheses denote that the corresponding position to that of *Moraxella* spp. is not present in *E. coli*. The number within parentheses is the adjacent position in the *E. coli* sequence to that of the actual residue in the 16S rRNA sequence of the moraxellae.

those present in the members of the *M. lacunata* cluster are shown in parentheses.

In the maximum likelihood analysis of the members of group I, *M. nonliquefaciens* and *M. lacunata* also formed distinct clusters, but were not positioned at the same branch as in the tree derived by using the neighbour-joining method. The phylogenetic distinctions between these species are supported by genotypic and biochemical properties, such as differences in colony morphology and growth characteristics and the presence of *M. lacunata* serum liquefaction (5, 53). We believe that *M. nonliquefaciens* and *M. lacunata* should be placed on the same phylogenetic branch as determined by 16S rRNA comparisons (Fig. 2, Table 5). For example, these species share common nucleotide patterns as the G·C, G·U and U·A base pairs in positions 77·92, 96·103 and 139·224, respectively. They also truncate the U·A base pair at position 76·93 (present in all the other species of group I) of the stem capped by the loop starting in position 83. This truncation constitutes a higher order structural attribute in the 16S rRNA molecule of these species, and is probably a common feature of an apomorphic homologous character.

The *M. bovis* cluster

The two *M. bovis* strains and the single strain of *M. ovis* formed a cluster, with *M. ovis* branching off from this line of descent (Fig. 2). The 16S rRNA sequences obtained from the two *M. bovis* strains differed from each other by 0·3%. The 16S rRNA genes of *M. bovis* and *M. ovis* were 97·1–97·4% homologous. This striking similarity clearly shows that *M. ovis* belongs within the genus *Moraxella* and was previously indicated by results from hybridization (52) and transformation (34). The *M. bovis* cluster constituted an unstable clade due to a low bootstrap percentage value (Fig. 2) and the fact that its phylogenetic significance could only be supported by three informative nucleotide positions, namely 92 (U), 1000 (C) and 1040 (G) (Table 5). Additional strains from *M. bovis* and *M. ovis*

should be analysed to evaluate whether this cluster constitutes a true phylogenetic lineage.

The *M. catarrhalis* cluster

M. catarrhalis, *M. caviae* and *M. cuniculi* represented by four strains clustered together in all trees, however with low bootstrap values, indicating that this cluster might not be stable if more species of group I are subjected to phylogenetic analysis. We found that the strain CCUG 24965, previously classified as *M. cuniculi*, should be assigned as a strain of the species *M. caviae*, since it had a 16S rRNA nucleotide sequence identical to that of *M. caviae* strain 355^T. The lack of affinity of the coccal *M. ovis* to *M. catarrhalis*, *M. caviae* and *M. cuniculi* was evident.

Strikingly, *M. catarrhalis* and *M. caviae* in this cluster shared the composition of 5 bp with *M. bovis*, *M. nonliquefaciens* and the *M. lacunata* subsp. *lacunata* (including strain E7992) in the vicinity of the tetra-loop starting at position 1013. However, the removal of these nucleotides from the alignment, since they might contribute to the destabilization of the node of the *M. catarrhalis* cluster, revealed an identical tree topology to that in Fig. 2 without a substantial improvement of the bootstrap value of the actual node. This cluster can also be justified by eight informative nucleotide positions (Table 5) and by the idiosyncrasy (a truncation of one nucleotide) in a position between 75 and 76 (*E. coli* lacks this position) of the 16S rRNA molecule. Therefore, the oligonucleotide GUUAGGRAGCUUGCCUYCUGAUACYUA surrounding the tetra-loop (underlined) starting at position 83, constitutes a target of the 16S rRNA molecule for identification of the *M. catarrhalis* cluster.

Our results show that *M. catarrhalis*, *M. caviae* and *M. cuniculi* group together and support these coccal species being taxonomically judged as members of the genus *Moraxella*, forming a phylogenetic cluster within group I (6, 24, 56), or alternatively as a new genus within *Moraxellaceae*.

The *Moraxella* sp. strain CCUG 27179

A fifth branch was formed by the strain CCUG 27179, which previously was classified as *M. cuniculi*. However, this could not be regarded as a strain of *M. cuniculi* since it only showed a 95.7% similarity with the 16S rRNA sequence of the type strain CCUG 2154^T of *M. cuniculi*. Strain CCUG 27179 branched early from the line of the *M. nonliquefaciens* and *M. lacunata* clusters in the dendrogram constructed by the neighbour-joining method (Fig. 2). Since strain CCUG 27179 formed the earliest branch of group I when the maximum likelihood method was used for phylogenetic inference (not shown), the phylogenetic position of this strain could not be solved in this study.

General group I considerations

Except for the case in *M. nonliquefaciens* and *M. lacunata* subsp. *lacunata*, group I was generally highly heterogeneous as seen from the bootstrap values obtained by 16S rDNA analysis (Fig. 2) and the branching order between the clusters and between the members within the clusters could not be fully established. A careful check of the variable positions within the alignment revealed that certain nucleotide positions were common to species occupying different clusters of the phylogenetic tree. Moreover, as discussed for the *M. catarrhalis* cluster, some segments appeared to have been shuffled between species which phylogenetically belong to different clusters. These segments and the extent of their occurrence deserve further investigation. Almost 80% of the variable positions (i.e. 98 out of 123 positions) were found to be positioned in highly variable locales of the molecule (21). This means that the trees are derived from nucleotide information present in regions which are prone to mutation. Therefore, it is likely that a certain 'noise' has been incorporated into the phylogenetic analysis: the tree in Fig. 2 should be regarded as tentative and Table 5 might need some revision when 16S rRNA sequence data from more strains and species of group I have been sequenced. Nevertheless, we believe that the five tentative clusters of group I and Table 5 constitute a valuable guide in the future taxonomic and diagnostic work.

In spite of the heterogeneity detected, a relatively high similarity was observed between the species within group I. Typically, the similarity values ranged from 95 to 98.8%, except for that between the *M. lacunata* strains and *M. equi* which were 99.0–99.8%. Thus, the similarity values lie in the range 95–97%, which has been suggested as a general cut-off for species definition within a genus (16). On the other hand, DNA–DNA reassociation experiments may, however, motivate a classification into new species regardless of high 16S rRNA sequence similarities (20, 50).

A taxonomic challenge is to decipher whether the entities within the classical moraxellae, namely *M. nonliquefaciens*, *M. lacunata* and *M. bovis*, should

represent different species, or whether they should be considered as sub-groups or genovars within one species, exhibiting various biochemical and growth requirements, as well as different habitats. This is one example of how problematic it still is to give strains that appear to be intermediate between known species the optimal designation. When analysed by 16S rDNA sequencing, the classical moraxellae and the coccal moraxellae appeared as five major clusters, representing *M. lacunata*, *M. nonliquefaciens*, *M. bovis*, the coccal moraxellae and one unspecified *Moraxella* sp. Previous DNA–DNA hybridization with total genomic probes gave insufficient distinction for identification purposes between the three closely related species, *M. nonliquefaciens*, *M. lacunata* and *M. bovis*, in spite of their A+T-rich DNAs (52, 53). Another challenge is to determine whether the sub-groups present within *M. lacunata* require more distinctive nomenclature. If so, should these (sub)species correspond to what was previously designated as *M. liquefaciens* and *M. lacunata*, or the *M. lacunata* group I and group II strains (34, 53)? The clustering in these groups by 16S rDNA analysis appeared to correspond well to the two sets of *M. lacunata* strains found by cell wall fatty acid patterns, as well as to hybridization analysis (53) and the two donor transformation activities towards *M. bovis* (34). The difference between the *M. lacunata* groups was so marked, that although they are mutually more closely related than to the other moraxellae, they are so distinct that the previous species distinctions of *M. lacunata* and *M. liquefaciens* (5) should be reconsidered.

In medical microbiology, the distinction between strains of *M. catarrhalis*, *M. nonliquefaciens* and *M. lacunata* is most relevant, as these species colonize similar habitats in humans. *M. catarrhalis* is easily recognized by its characteristic growth pattern and ease in agar surface sliding (12). Serum liquefaction and growth requirements (5, 31) are traits that may help distinguish *M. nonliquefaciens* and *M. lacunata*, but such tests are often not employed in ordinary routine diagnostic work and their value as specific key reactions is not fully exploited. Separation between the two species has previously been demonstrated by genetic transformation (5, 31), but the laborious quantitative assay needed for this fine distinction is time-consuming and dependent on competent strains. The target regions of the 16S rRNA molecule outlined in this paper will be of importance in the development of new PCR-based diagnostic tools for these species.

Distinction between *M. lacunata* and *M. bovis* can usually be obtained easily by the expression of haemolysis of *M. bovis* as well as by difference in habitat (9, 34). A problem in this context are non-haemolytic strains of *M. bovis*, as well as the non-haemolytic entity *M. equi*, found in horses (5, 34). This type of taxonomical problem is likely to be more pronounced when a wider environment is investigated for the presence of strains of group I, as already shown by Kodjo and co-workers (35).

Group II, including *M. osloensis* and *M. atlantae*

Group II comprised only the two species *M. osloensis* strain 5873 and *M. atlantae* strain 1922 which formed a common and distinct clade (Fig. 1, Table 4) in all derived phylogenies. A partial 16S rRNA sequence consisting of 729 nucleotides could be retrieved for the type strain ATCC 19976^T of *M. osloensis* (17) to which strain 5873 showed only a few nucleotide differences. A tree was constructed from a data set consisting of only partial 16S rRNA sequences and the two *M. osloensis* strains clustered tightly together (tree not shown). The strains of *M. osloensis* and *M. atlantae* were only distantly related to each other, displaying a 93.4% nucleotide similarity between their 16S rRNA genes and $\leq 93\%$ to the other members of the *Moraxellaceae* family including the moraxellae of group I.

***M. osloensis*.** The systematic work regarding the taxonomic position of *M. osloensis* has been extensively investigated by genotypic and phenotypic studies (10, 17, 30, 46, 54). However, the placement of this species has remained uncertain. The last taxonomic effort led to the proposal to classify *M. osloensis* in the *M. lacunata* rRNA homology group (46). We searched the 16S rRNA sequences through for unique nucleotide positions which were shared between *M. osloensis* and all members of group I of *Moraxellaceae*. Only two positions were found, namely the residues at position 453 (G) and 861 (U). Interestingly, only two positions were also found to be shared between the strain of *M. atlantae* and the *M. lacunata* rRNA homology group. These positions were 1355 (A) and 1367 (U). Moreover, an additional thymidine residue was found situated between positions 649 and 650 in the 16S rRNA gene of both *M. osloensis* strains. This structural element was not present in any other member of the *Moraxellaceae* and was rarely found among other (eu)bacteria. The resulting U constitutes a unilaterally bulged residue in the actual locale of the matured 16S rRNA molecule and is a higher-order structural idiosyncrasy for the species *M. osloensis*. Thus, our analysis showed that there is no evidence on the 16S rRNA level to include *M. osloensis* in group I. Also, there are other properties which support a definitive exclusion of *M. osloensis* as a member of the *M. lacunata* rRNA homology group (group I), such as its distinct fatty acid profile and findings by genetic transformation (9, 10, 30). This is the only bacterium with pronounced formation of fusiform cells when cultured at sublethal concentrations of penicillin.

***M. atlantae*.** The strain of *M. atlantae* had easily the longest single branch in this part of the phylogenetic tree. One can speculate whether this species has a significantly higher evolutionary rate. However, more strains of *M. atlantae* have to be sequenced to allow clarification of whether the long branch is due to phylogenetic information or to phylogenetic noise, resulting in a long-branch artifact (18). *M. atlantae* shares characteristics, such as the cellular lipid com-

position which resembles that of [*M.*] *phenylpyruvica*, but *M. atlantae* differs from [*M.*] *phenylpyruvica* by containing true waxes (8, 9). Also, *M. atlantae* grows with particularly small colonies which display highly fimbriated phenotypes by virtue of its colony morphology and pronounced twitching motility.

Group III, including [*M.*] *phenylpyruvica* 752/52 and the genus *Psychrobacter*

Group III included a single *Moraxella* strain, namely [*M.*] *phenylpyruvica* strain 752/52 (11), which was sequenced in this work, and the genus *Psychrobacter* recently investigated by Bowman *et al.* (2). The group III line of descent was regarded reliable as judged from the consistency measures (Fig. 1, Table 4). The members of the *Psychrobacter*-[*M.*] *phenylpyruvica* group were only distantly related to the species of the other three groups of the *Moraxellaceae* family, with a 16S rRNA sequence similarity $\leq 93.1\%$.

[*M.*] *phenylpyruvica*. [*M.*] *phenylpyruvica* strain 752/52 (different from ATCC 23333^T) was found to be positioned early on the same branch as the *Psychrobacter* strains in all inferred phylogenetic trees (represented by Fig. 1). Interestingly, the [*M.*] *phenylpyruvica* strain 752/52 revealed a 16S rRNA sequence similarity of only 95.9% with the recently established *Psychrobacter phenylpyruvici* type strain which indicated that 752/52 is not a strain of this species (which hosts the previous type strain ATCC 23333^T of *M. phenylpyruvica*) (2, 11). Moreover, strain 752/52 was found to be only distantly related to the other members of the *Moraxellaceae* due to a 16S rRNA sequence similarity value of $\leq 93.1\%$. In comparison, strain 752/52 showed a significantly higher 16S rRNA sequence similarity value ($\geq 94.6\%$) to the species of genus *Psychrobacter*, which is still not very high. The group containing *Psychrobacter* spp. and strain 752/52 has long since been described as both genotypically and phenotypically heterogeneous (11, 31–33, 46). The genus status of *M. phenylpyruvica* has also been questioned repeatedly (8, 11, 46).

Studies based on comparison of features other than ribosomal sequence differences also conflict with a classification of [*M.*] *phenylpyruvica* strain 752/52 into the genus *Moraxella*, as well as into the genus *Psychrobacter*. Strains of [*M.*] *phenylpyruvica* are usually phenylalanine-deaminase- and urease-positive, and true waxes are absent in the fatty acid profile (8, 11). Until now, no naturally competent culture of [*M.*] *phenylpyruvica* strain 752/52 has been found. The complete lack of transforming activity between [*M.*] *phenylpyruvica* strain 752/52 as donor DNA and *Psychrobacter* sp. strongly goes against there being a close affiliation between these entities (33). The 16S rDNA sequence analysis displays this divergence, however to a lesser extent. The branch of [*M.*] *phenylpyruvica* and *Psychrobacter* spp. is characterized by several common signature and unique 16S rRNA residues (Table 4), but one difference is noteworthy. In

position 1336, the 16S rRNA gene of [*M. phenylpyruvica*] has a cytosine residue, while *Psychrobacter* sp. displays an adenine residue, representing a signature for the latter species. The results from this study suggest that [*M.*] *phenylpyruvica* strain 752/52 is neither a species of the genus *Moraxella* nor a member of *Psychrobacter*, and that it should be placed in a new genus.

Group IV, including the genus *Acinetobacter sensu stricto*

This group we only analysed by means of pre-existing 16S rDNA sequences from the databases. Group IV consisted solely of species belonging to the genus *Acinetobacter*, represented by a strain of *A. calcoaceticus*. The phylogenetic relation among the *Acinetobacter* species has been published previously (1, 44, 55, 59) and was not focused on in this work. The genus *Acinetobacter* formed an early branch of the *Moraxellaceae* family in all constructed trees (Fig. 1). Binary similarity values between members of *Acinetobacter* and groups I–III ranged between 86 and 90%. In general, group IV showed the slightly higher 16S rRNA sequence similarity percentage value to group I (the *M. lacunata* rRNA homology group). Despite the phylogenetic proximity exhibited among different species of *Acinetobacter* on the 16S rRNA level (44), studies on the total genome level have demonstrated that species of the genus *Acinetobacter* are very heterogeneous, to such an extent that some *Acinetobacter* species show very low DNA reassociation values, indicating that they should be placed in separate genera (51).

Overall nucleotide distribution and variability between *Moraxella* species on the 16S rDNA level

Higher diversity was found on the 16S rDNA level among the *Moraxella* species than could be expected based on phenotypic similarities, on overall nucleic acid homologies by hybridization and on ratios in genetic transformation. Considering the extensive genetic exchange that goes on in nature among these bacteria, due to their naturally high competence for constitutive transformation throughout their life-cycle combined with a target that could be subject to homologous recombination with DNA from a vast amount of bacterial species, a relatively high diversity on the 16S rDNA sequence level is not unlikely. Compared to species that exhibit particularly high conservation on the 16S rDNA sequence level, such as the *Mycobacteria*, strains of species of *Moraxellaceae* contain relatively high 16S rDNA sequence divergence. This finding promotes the use of 16S rDNA sequence analysis for fine discrimination between strains and entities, but limits its use for species discrimination of this bacterial family.

Concluding remarks

We believe that the 16S rRNA gene sequences from strains of the type genus *Moraxella* determined and discussed in this work constitute an important part of the framework for taxonomic studies of the members of the family *Moraxellaceae*. Taxonomic realignment of genera and species within the *Moraxellaceae* is not proposed in this report. The analysis of additional strains within each species/entity will be necessary to obtain a more finite classification and complete phylogenetic status of this family. Areas of interest deserving the highest priority in this pursuit are the genus *Moraxella sensu stricto* and the genus *Psychrobacter* and allied bacteria. Moreover, nucleotide sequence data of the 16S rRNA genes from members of both groups II and III are needed for proper taxonomic affiliation of *M. osloensis*, *M. atlantae*, [*M.*] *phenylpyruvica* and related species within these groups.

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