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Diagnostics of *Neisseriaceae* and *Moraxellaceae* by Ribosomal DNA Sequencing: Ribosomal Differentiation of Medical Microorganisms

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Fast and reliable identification of microbial isolates is a fundamental goal of clinical microbiology. However, in the case of some fastidious gram-negative bacterial species, classical phenotype identification based on either metabolic, enzymatic, or serological methods is difficult, time-consuming, and/or inadequate. 16S or 23S ribosomal DNA (rDNA) bacterial sequencing will most often result in accurate speciation of isolates. Therefore, the objective of this study was to find a hypervariable rDNA stretch, flanked by strongly conserved regions, which is suitable for molecular species identification of members of the *Neisseriaceae* and *Moraxellaceae*. The inter- and intrageneric relationships were investigated using comparative sequence analysis of PCR-amplified partial 16S and 23S rDNAs from a total of 94 strains. When compared to the type species of the genera *Acinetobacter, Moraxella*, and *Neisseria*, an average of 30 polymorphic positions was observed within the partial 16S rDNA investigated (corresponding to *Escherichia coli* positions 54 to 510) for each species and an average of 11 polymorphic positions was observed within the 202 nucleotides of the 23S rDNA gene (positions 1400 to 1600). *Neisseria macacae* and *Neisseria mucosa* subsp. *mucosa* (ATCC 19696) had identical 16S and 23S rDNA sequences. Species clusters were heterogeneous in both genes in the case of *Acinetobacter lwoffii, Moraxella lacunata*, and *N. mucosa*. *Neisseria meningitidis* isolates failed to cluster only in the 23S rDNA subset. Our data showed that the 16S rDNA region is more suitable than the partial 23S rDNA for the molecular diagnosis of *Neisseriaceae* and *Moraxellaceae* and that a reference database should include more than one strain of each species. All sequence chromatograms and taxonomic and disease-related information are available as part of our ribosomal differentiation of medical microorganisms (RIDOM) web-based service (http://www.ridom.hygiene.uni-wuerzburg.de/). Users can submit a sequence and conduct a similarity search against the RIDOM reference database for microbial identification purposes.

Classification and diagnostic systems for microorganisms have historically been based on the existence of observable characteristics. However, because of limitations in the discriminatory power of these characteristics, problems have arisen in identification and diagnosis (29). A more recent approach for classification and identification of microorganisms involves the comparison of genetic characteristics. These molecular methods are becoming increasingly important in microbiological diagnostics (23). They are an expansion of or an alternative to phenotyping techniques if one or more of the following conditions are met: (i) microorganisms cannot be cultivated or are difficult to cultivate, (ii) organisms grow only slowly and are poorly differentiated, (iii) growth of organisms represents a hazard to laboratory staff, (iv) a suitable test method for phenotyping is not available, and (v) the extent of infection is to be quantitated (e.g., the virus load). Guidelines similar to the phenotypic methods of Koch have already been established for molecular techniques used in the identification of microorganisms involved in particular diseases (9). In most cases, one of the following genomic structures is chosen as target for a molecular diagnosis test: (i) DNA sequences bearing the code for toxic or pathogenic factors, (ii) DNA sequences of specific antigens, (iii) specific DNA plasmid sequences, (iv) DNA sequences bearing rRNA codes, and (v) small sequences, mostly species specific, which are noncoding. The rRNA genes (rDNA) are particularly suitable for identification purposes since they are ubiquitous to all living organisms. They occur as multicopy genes, making their detection relatively easy, and are composed of conserved, variable, and highly variable regions so that probes may be designed to meet a desired level of specificity. Furthermore, they are essential for survival and may be used as a molecular clock for phylogenetic studies (11, 20, 25, 40, 41).

Existing sequence databases and analytical tools (e.g., the National Center for Biotechnology Information (NCBI) GenBank or Ribosomal Database Project) are not optimal for accurate identification of clinically relevant microorganisms (17). The contents of these databases suffer many drawbacks including the presence of ragged sequence ends, faulty sequence entries (due to error-prone sequencing techniques used earlier), absence of quality control of sequence entries,

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noncharacterized entries, outdated nomenclature, and lack of type strains pertaining to many clinically important microorganisms. Furthermore, the results are not presented in a user-friendly manner. Our ribosomal differentiation of medical microorganisms (RIDOM) project is a new initiative and attempts to overcome these problems (12).

Culture collection strains of *Neisseriaceae* and *Moraxellaceae* were studied since these families not only contain established human pathogens such as *N. meningitidis* and *Neisseria gonorrhoeae* but also contain other species which are important as emerging causes of opportunistic infections (19). Molecular identification of these particular isolates should be a good challenge for molecular diagnostic systems in general because these organisms belong to a group of bacteria that is naturally competent and frequently exchanges chromosomal genes. This exchange process could considerably complicate molecular diagnosis and identification. According to Bøvre, the family *Neisseriaceae* previously consisted of the genera *Neisseria, Kingella, Acinetobacter*, and *Moraxella*, the latter genus containing the subgenera *Moraxella* (rod-shaped bacteria) and *Branhamella* (cocci) (2, 3). On the basis of DNA hybridization and phylogenetic rDNA sequence analysis results, it is now suggested that *Neisseria, Kingella*, and *Eikenella* species are grouped in the family *Neisseriaceae* in the β subclass of the *Proteobacteria*. The genera *Acinetobacter, Moraxella*, and *Psychrobacter* are removed from the *Neisseriaceae* and included in the family *Moraxellaceae* in the γ subclass of the *Proteobacteria* (26, 27, 32). This classification system is still evolving and therefore not complete.

In practice, a defined and limited sequence run must suffice for the identification process in most cases. To decide the target that best meets the requirements for identification, coherent variable regions of the rRNA operon were studied in a total of 94 *Neisseriaceae* and *Moraxellaceae* strains. The sequence traces and further taxonomic and disease-related information on these strains have also been deposited on our RIDOM web server for prototypic demonstration purposes. The same partial 16S and 23S rDNA sequences were also used to examine the phylogenetic relationships among species of the *Neisseriaceae* and *Moraxellaceae* families.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains investigated in this study are listed in Table 1. Culture collection isolates, including the type strains, were used in this analysis when available. Strains were grown on 5% human blood and chocolate agar plates at 22, 28, or 37°C with 5% CO_2. All isolates were identified by conventional biochemical methods (19).

In vitro amplification and DNA sequencing of the 16S and 23S ribosomal RNA genes. A loopful of bacterial cells for extraction of DNA was washed with distilled water and incubated in 200 μl Tris-EDTA buffer for 10 min at 100°C. The suspension was vortexed and centrifuged at 8000 × *g* for 1 min. Two microliters of the supernatant were used for PCR amplification. PCR was performed in a total volume of 50 μl containing 200 μM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 5 pmol of each primer, 5 μl of 10-fold concentrated polymerase synthesis buffer, and 1 U of AmpliTaq DNA polymerase (PE Biosystems, Weiterstadt, Germany). Thermal cycling reactions consisted of an initial denaturation (80°C, 5 min) followed by 28 cycles of denaturation (94°C, 0.45 min), annealing (53°C or 60°C for 16S or 23S rDNA PCR, respectively, 1 min), and extension (72°C, 1.5 min), with a single final extension (72°C, 10 min). Reactions took place in a dedicated automated DNA thermal cycler (GeneAmp 2400, PE Biosystems). Negative controls containing water in place of template DNA were run in parallel in each run. The amplicons were sequenced with the PCR primers using the Taq-cycle (Big)-DyeDeoxy Terminator kit and the protocol recommended by the manufactor (PE Biosystems). Centri-Sep columns (Princeton Separations, Adelphia, N.J.) were used for purifying the sequencing products. Sequences were determined by electrophoresis with the ABI Prism 377 or 310 semiautomated DNA sequencers (PE Biosystems). The nucleotide sequences from both DNA strands were determined in this manner. The broad-range primers SSU-bact-27f (5′- AGA GTT TGA TCM TGG CTC AG -3′) and SSU-bact-519r (5′-GWA TTA CCG CGG CKG CTG -3′) reported by Lane (15) were applied for 16S ribosomal DNA (rDNA) PCR and sequencing, whereas the universal primers LSU-bact-1399f (5′- GAT GGG AAR CWG GTT AAT ATT CC-3′) and LSU-bact-1602r (5′- CAC CTG TGT CGG TTT SGG TA -3′) were used for amplification and sequencing of 23S rDNA. Identical or near-identical 23S rDNA primer binding sites have already been described by Van Camp et al. (36) and by Ludwig et al. (16). Ambiguities were resequenced and at least 98% percent of the complete double-stranded sequences of the 16S and 23S rDNA targets were obtained.

Analysis of the rDNA sequences. The region from base positions 54 to 510 for the 16S rDNA and the region from positions 1400 to 1600 for the 23S rDNA were analyzed (corresponding to *Escherichia coli* 16S and 23S rDNA positions, respectively). Sequences from primer regions were therefore not included in this analysis. Sequences were aligned using the CLUSTAL W program (34). This program was also used to construct phylogenetic trees from distance matrices using the neighbor-joining method of Saitou and Nei (28) with the correction for multiple substitutions option turned off. The mean sequence divergence level within each taxon and between each pair of related taxa and genera was calculated as the mean of all pairwise comparisons.

EMBL accession numbers for the partial sequences of the 16S and 23S rDNA genes in the *Neisseriaceae* and *Moraxellaceae* strains investigated in this study are listed in Table 1. The EMBL accession numbers for the 16S rDNA sequences used to determine error rates in public databases are as follows: *Acinetobacter baumannii* X81660, *Acinetobacter calcoaceticus* X81661, *Acinetobacter haemolyticus* X81662, *Acinetobacter johnsonii* X81663, *Acinetobacter junii* X81664, *Acinetobacter lwoffi* X81665, *Acinetobacter radioresistens* X81666, *Chromobacterium violaceum* M22510, *Eikenella corrodens* M22512, *Iodobacter fluviatile* M22511, *Kingella kingae* M22517, *Moraxella catarrhalis* U10876, *Moraxella lacunata* D64049, *Neisseria animalis* L06172, *Neisseria canis* L06170, *Neisseria elongata* subsp. *elongata* L06171, *Neisseria macacae* L06169, *Neisseria weaveri* L10738, and *Suttonella indologenes* M35015.

RIDOM implementation. The software selected for the RIDOM project had to be freely available for academic use as well as efficient and applicable without the need of a specific platform. FASTA version 2.0 is used for similarity searches, whereas CLUSTAL W version 1.7 is utilized for constructing multiple alignments and nearest-neighbor trees (22, 34). C-source code is available in the case of both programs. Phylogenetic trees are drawn with the aid of DRAWTREE version 3.5 of the PHYLIP software package (7). A MySQL (MySQL AB, Stockholm, Sweden) database holds all taxonomic, disease-related, and species information. Depending on the user query results, dynamically generated HTML pages are published with the aid of an APACHE HTTP server. The database, FASTA, and CLUSTAL W are linked to the common gateway interface of the Web server using programmed Perl script, which also interprets similarity search results. The client's World Wide Web browser should support at least HTML version 3.2. Some HTML extensions such as JavaScript, frames, and cascading style sheets are used occasionally for greater clarity of presentation. These extensions, however, are not essential and therefore older browsers can also be used. Due to the frequent use of tables, however, text-only browsers such as Lynx are not well suited to the task of viewing the contents of RIDOM.

RESULTS

A total of 202 nucleotides of the 23S rRNA gene from 94 isolates and 457 bp of the 16S rDNA from 90 *Neisseriaceae* and *Moraxellaceae* strains were determined by direct DNA sequencing (Table 1). When compared to the type species of the genera *Acinetobacter*, *Moraxella*, and *Neisseria*, an average of 30 polymorphic positions were observed within the partial 16S rDNA for each species and an average of 11 polymorphic positions were observed within the 202 nucleotides of the 23S gene (Table 2). Some of the polymorphic positions were found to be species specific, and a subset of these were present in

TABLE 1. *Neisseriaceae* and *Moraxellaccae* strains included in the study[a]

Species	Strain	EMBL accession number (16S/23S)
A. baumanni	DSM 30007^T, LMG 994	AJ247197/AF124611; AJ247198/N.D.
A. calcoaceticus	DSM 30006^T	AJ247199/AF124612
A. haemolyticus	DSM 6962^T, LMG 1033	AJ247200/AF124613; AJ247201/N.D.
A. johnsonii	DSM 6963^T, LMG 10584	AJ247202/AF124614; AJ247203/AF242446
A. junii	DSM 6964^T, LMG 10577	AJ247204/AF124615; −/−
A. lwoffii	DSM 2403^T, LMG 1134, LMG 1138, LMG 1154, LMG 1299, LMG 1300	AJ247205/AF124616; −/−; AJ247207/AJ242449; AJ247206/AJ242448; N.D./−; AJ247208/−
A. radioresistens	DSM 6976^T, LMG 10614	AJ247209/AF124617; AJ247210/AJ242450
Chromobacterium violaceum	DSM 30191^T	AJ247211/AF124618
Eikenella corrodens	DSM 8340^T, LMG 15557, Oslo 31745/80	AJ247212/AF124619; −/−; AJ247213/−
Iodobacter fluviatilis	DSM 3764^T	AJ247214/AF124620
Kingella denitrificans	ATCC 33394^T, NCTC 10997	N.D./AJ228757; AJ247215/AJ242451
K. kingae	DSM 7536^T	AJ247216/AF124626
Kingella oralis	ATCC 51147^T	AJ247217/AF124627
M. (Branhamella) catharralis	ATCC 25238^T, LMG 1133	AJ247218/AJ228758; AJ247219/AJ242452
Moraxella (B.) caviae	ATCC 14659^T, CCUG 355	AJ247220/AF124637; −/N.D.
Moraxella (B.) cuniculi	LMG 8382^T, CCUG 27179	N.D./AJ228759; AJ247221/N.D.
Moraxella (B.) ovis	LMG 8381^T, ATCC 33078	AJ247222/AJ228760; −/−
Moraxella (Moraxella) atlantae	LMG 5133^T, CDC 5118	N.D./AF124638; N.D./AJ242453
Moraxella (M.) bovis	LMG 986^T, LMG 1006	AJ247223/AF124639; AJ247224/AJ242454
Moraxella (M.) canis	LMG 11194^T	AJ247225/AF124640
Moraxella (M.) caprae	NCTC 12877^T	N.D./AF124641
M. (M.) equi	LMG 5315^T, ATCC 25576	AJ247226/AF124642; −/N.D.
M. (M.) lacunata[b]	ATCC 11748^T, ATCC 17956, ATCC 17967, LMG 1008	AJ247228/AJ242455; −/AJ242456; AJ247227/AF124643;−/AJ242457
Moraxella (M.) lincolnii	LMG 5127^T	AJ247229/AF124644
Moraxella (M.) nonliquefaciens	DSM 6360^T, NCTC 7784, LMG 1045	AJ247230/AF124645; −/−; AJ247231/N.D.
Moraxella (M.) osloensis	LMG 5131^T, LMG 6916	AJ247232/AF124646; AJ247233/N.D.
N. animalis	NCTC 10212^T, ATCC 19573	AJ247234/AF124628; −/−
N. canis	LMG 8383^T	AJ247235/AJ228761
Neisseria cinerea	DSM 4630^T, LMG 8380	AJ247236/AJ228762; −/−
N. elongata subsp. *elongata*	LMG 5124^T	AJ247252/AJ228763
N. elongata subsp. *glycolytica*	NCTC 11050^T	AJ247253/AF124634
N. elongata subsp. *nitroreducens*	ATCC 49377^T, ATCC 49378	AJ247254/AF124635; −/−
Neisseria flava	Berger 122	AJ247237/AF124629
Neisseria flavescens	ATCC 13115^T, LMG 5297	N.D./−; N.D./AJ228764
N. gonorrhoeae	DSM 9188^T, DSM 9189 Wue 799, Wue 920	AJ247238/AJ228765; AJ247239/−; −/−; −/−
Neisseria iguanae	ATCC 51483^T	AJ247240/AF124630
Neisseria lactamica	DSM 4691^T, Berger 116	AJ247241/AJ228766; −/−
N. macacae	ATCC 33926^T	AJ247243/AF124631
N. meningitidis	DSM 10036^T, 2120, 25/634, 264/93, ATCC 13102, ATCC 35559, BZ163, H41/88, H44/76, MC58, Z2491, Z6422	AJ247244/AJ228767; N.D./−; −/−; −/−; N.D./AJ242458; AJ247245/AJ242459; −/−; N.D./−; N.D./−; −/−; −/−; N.D./−
N. mucosa subsp. *heidelbergiensis*	ATCC 25999^T, Berger 114, LMG 5136[c]	AJ247258/AJ242462; −/−; AJ247260/N.D.
N. mucosa subsp. *mucosa*	ATCC 19696^T, Berger 112, DSM 4631	AJ247257/AJ242460; −/−; AJ247255/AJ228768
Neisseria perflava	LMG 5284^T, Berger 120	AJ247246/AF124632; −/−
Neisseria polysaccharea	ATCC 43768^T	N.D./AJ228769
Neisseria sicca	LMG 5290^T	AJ247248/AJ228770
Neisseria subflava	LMG 5313^T	AJ247249/AJ228771
N. weaveri [Anderson]	ATCC 51223^T	AJ247250/AJ228772
N. weaveri [Holmes]	LMG 5135^T, ATCC 51462	AJ247251/AF124633; −/−
Oligella ureolytica	LMG 6519^T	AJ247261/AF124621
Oligella urethralis	DSM 7531^T, Oslo 1098	AJ247262/AF124622; AJ247263/N.D.
Psychrobacter immobilis	DSM 7229^T	AJ247264/AF124623
Psychrobacter phenylpyruvicus	Oslo A9911	AJ247266/AJ242462
Suttonella indologenes	DSM 8309^T	AJ247267/AF124625

[a] ^{T} designates the type strain of this species; −, sequence determined but not submitted to EMBL because another species sequence was identical and already submitted; N.D., sequence not determined; subsp., subspecies. ATCC, American Type Culture Collection; Berger, Ulrich Berger *Neisseriaceae* strain collection, Heidelberg, Germany; CCUG, Culture Collection University of Göteborg; CDC, Centers for Disease Control and Prevention; DSM, Deutsche Sammlung von Mikroorganismen; EMBL, European Molecular Biology Laboratory; LMG, Laboratorium voor Microbiologie Gent (Belgian Coordinated Collections of Microorganisms); NCTC, National Collections of Type Cultures; Oslo, Oslo strain collection; Wue, Würzburg strain collection. Multiple 16S/23S groups in column 3 correspond to strains in column 2.

[b] *Moraxella lacunata* is proposed to consist of at least two subspecies, i.e., subsp. *lacunata* (small colony variant or group II) and subsp. *liquefaciens* (large colony variant or group I) (24).

[c] No phenotypic information available to classify this strain as a *N. mucosa* subspecies.

TABLE 2. Analysis of polymorphisms in the 16S and 23S rRNA genes of *Acinetobacter*, *Neisseria*, and *Moraxella* species

Species[a]	No. of isolates	Alleles		Total no. of polymorphic positions		Species-specific polymorphisms[b]		Species-specific conserved polymorphisms[c]	
		16S	23S	16S	23S	16S	23S	16S	23A
A. baumanni	1	1	1	22	8	0	5	–	–
A. haemolyticus	1	1	1	20	7	1	0	–	–
A. johnsonli	2	2	1	26	9	2	2	1	2
A. junii	2	1	1	19	8	2	1	2	1
A. lwoffii	5	3	2	47	17	15	2	0	0
A. radioresistens	2	2	2	33	21	7	6	7	6
ACINETOBACTER (MEAN ± SD)		1.67 ± 0.82	1.33 ± 0.52	27.83 ± 10.69	11.67 ± 5.85	4.50 ± 5.68	2.67 ± 2.39	2.50 ± 3.11	2.25 ± 2.63
M. (B.) catharralis	2	2	2	37	19	2	0	1	0
M. (B.) caviae	1	1	1	40	18	2	3	–	–
M. (B.) ovis	1	1	1	29	12	3	1	–	–
M. (M.) bovis	2	1	2	25	5	0	0	0	0
M. (M.) canis	1	1	1	38	19	3	1	–	–
M. (M.) equi	1	1	1	2	0	1	0	–	–
M. (M.) lacunata	3	2	3	1	16	0	0	0	0
M. (M.) lincoinii	1	1	1	45	26	12	8	–	–
M. (M.) nonliquefaciens	2	1	1	7	10	0	0	0	0
M. (M.) osloensis	1	1	1	58	15	19	2	–	–
MORAXELLA (MEAN ± SD)		1.20 ± 0.42	1.40 ± 0.70	28.20 ± 9.35	14.00 ± 7.54	4.2 ± 6.29	1.50 ± 2.51	0.25 ± 0.50	0.00 ± 0.00
N. animalis	2	1	1	40	19	1	4	1	4
N. canis	1	1	1	43	7	10	3	–	–
N. cinerea	2	1	1	11	4	0	0	0	0
N. elongata subsp. *elongata*	4	3	2	52	7	5	1	4	0
N. elongata subsp. *glycolytica*	1	1	1	51	6				
	1	1	1	51	6				
N. elongata subsp. *nitroreducens*	2	1	1	50	6				
N. flava	1	1	1	26	5	1	1	–	–
N. gonorrhoeae	3	2	1	1	0	0	0	–	–
N. iguanae	1	1	1	30	35	6	15	–	–
N. lactamica	2	2	1	36	3	3	1	0	1
N. macacae	1	1	1	35	5	0	0	–	–
N. meningitidis	7	2	2	12	4	1	0	0	0
N. mucosa subsp. *heidelbergienis*	5	4	3	58	8	4	1	0	0
	2	2	1	51	6				
N. mucosa subsp. *mucosa*	3	2	2	36	7				
N. perflava	2	2	1	25	5	0	0	0	0
N. sicca	1	1	1	35	7	0	1	–	–
N. subflava	1	1	1	32	4	0	0	–	0
N. weaveri	3	1	1	31	17	1	0	1	–
NEISSERIA (MEAN ± SD)		1.54 ± 0.92	1.27 ± 0.59	31.13 ± 15.06	8.67 ± 8.83	2.13 ± 2.95	1.80 ± 3.84	0.75 ± 1.39	0.63 ± 1.41
ALL SPECIES (MEAN ± SD)		1.45 ± 0.77	1.32 ± 0.60	29.55 ± 15.48	10.97 ± 8.06	3.26 ± 4.75	1.87 ± 3.14	1.06 ± 1.91	0.88 ± 1.75

[a] *Acinetobacter* spp. were compared with *A. calcoaceticus* DSM 30006^T, *Moraxella* spp. were compared with *Moraxella lacunata* ATCC 17967^T, and *Neisseria* spp. were compared against *N. gonorrhoeae* DSM 9188^T. SD, standard deviation.

[b] Polymorphisms found only in corresponding species.

[c] Polymorphisms found in every isolate analyzed of that species. 0, no conserved polymorphisms; −, no conserved polymorphisms although species-specific polymorphisms were detected.

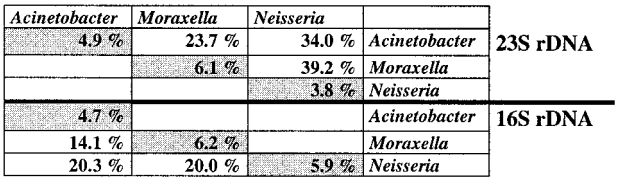

FIG. 1. Interspecies variation between (white fields) and within (grey fields) genera for 37 isolates of *Neisseria* (15 different species), 16 isolates of *Moraxella* (10 species), and 10 isolates of *Acinetobacter* (7 species). Values are the percent of variation based either on 457 bp in the case of 16S rRNA (bottom) or 202 bp in the case of the 23S rRNA gene (top).

every isolate of the species concerned (conserved polymorphism). It is interesting that differences in diversity within a species group were observed, depending on which gene was being considered. For example, in the case of *N. mucosa*, four and three alleles were observed for the 16S and 23S genes, respectively, whereas *M. lacunata* exhibited two and three alleles, respectively. On the other hand, the complexity of *N. meningitidis* and *N. weaveri* were similar, as exemplified by the number of alleles observed for the 16S and 23S genes. In this context alleles are defined as observed rDNA sequence differences of different isolates of the same species, as determined by direct sequencing of PCR products (10).

Intraspecies variation for the 16S and 23S genes was highest in *A. lwoffii* (4.35 and 3.4%), *M. lacunata* (1.66 and 2.49%), *M. catharralis* (0.55 and 2.25%) and *Neisseria mucosa* (1.64 and 1.05%), respectively. *Acinetobacter* isolates showed the highest average intraspecies variation (2.10 and 1.76%), followed by *Moraxella* (0.90 and 1.50%) and *Neisseria* (0.36 and 0.42%), respectively. Interspecies variation within a genus (Fig. 1) ranged from 6.2 to 6.1% for *Moraxella* spp. to 4.7 to 4.9% for *Acinetobacter* spp. Interspecies variation between the genera (Fig. 1) ranged from 39.2% for *Moraxella* and *Neisseria* spp. 23S rRNA gene sequences to 14.1% for *Acinetobacter* and *Moraxella* 16S rRNA gene sequences.

To demonstrate the relationships between species, the aligned DNA sequences were used to produce a distance matrix for pairs of sequences by using the neighbor-joining method of Saitou and Nei (28). All species used in this study fell into one of two large groups on the tree. One group included all isolates of the genera *Chromobacterium*, *Eikenella*, *Iodobacter*, *Kingella*, *Neisseria*, and *Oligella* and comprises the β subclass of the *Proteobacteria*. The other group contained all isolates of the genera *Acinetobacter*, *Moraxella*, *Psychrobacter*, and *Suttonella* and constitutes the γ subclass of the *Proteobacteria*. It is interesting that *Oligella* spp. clustered with the β subclass of *Proteobacteria* according to 16S rDNA sequence analysis, but to a separate group as analyzed by partial 23S rDNA sequences.

N. macacae and *N. mucosa* subsp. *mucosa* ATCC 19696 had identical 16S and 23S rDNA sequences. The 23S genes of *Moraxella equi*, *M. lacunata* ATCC 17967, and of *Neisseria elongata* subsp. *glycolytica* and *N. elongata* subsp. *nitroreducens* also showed sequence identity. The species clusters were heterogeneous in both genes for *A. lwoffii*, *M. lacunata*, and *N. mucosa*. *N. meningitidis* isolates failed to cluster in one group only in the case of the 23S rDNA subset. These *N. meningitidis* isolates grouped neither according to serotypes nor to multilocus enzyme electrophoretic types. No clustering according to the *Moraxella* subgenera *Branhamella* or *Moraxella* could be observed.

The EMBL database contained 19 sequences from the same culture collection strains that covered the 16S rDNA region examined in this study. These sequences were compared against our newly generated sequence chromatograms. The EMBL sequences contained 56 ambiguities, whereas our own sequences showed only 3 ambiguities. Furthermore, we detected eight substitutions, two insertions, and one deletion that could be attributed to possible sequencing errors in these published sequences (i.e., an error rate of 0.78%).

DISCUSSION

Our objective was to find a hypervariable rDNA stretch, flanked by strongly conserved regions, which can be used for molecular species identification of species within the *Neisseriaceae* and *Moraxellaceae* families. The longest coherent variable region in the 16S rDNA fulfilling these criteria spans the region from *E. coli* positions 54 to 510 and in the 23S rDNA spans the region from positions 1400 to 1600 (16). This is well illustrated by the quantitative map of nucleotide substitution rates in bacterial rDNAs published by Van de Peer et al. (37). The inter- and intrageneric relationships of members of the *Neisseriaceae* and *Moraxellaceae* were therefore investigated by carrying out comparative sequence analysis of PCR-amplified partial 16S and 23S rDNAs of these regions in a total of 94 strains. An ideal region should show a low intraspecies and a high interspecies variability. When the DNA sequences of the 16S and 23S rRNA genes were used as a measure of the intraspecies and interspecies distances within a genus, no great differences between the two regions could be observed (Fig. 1). Only in the case of the 23S rDNA in *Neisseria* was the interspecies variation significantly lower than that of the 16S rDNA. The interspecies distances between genera for the 23S rDNA sequences on the other hand, were consistently higher than those for the 16S rDNA. A comparison of an absolute measure (i.e., polymorphic positions), however, showed that 16S rDNA always had significantly more variable positions and this was because the relevant region in the 16S rDNA was more than twice as long as that in the 23S rDNA (Table 2). We therefore concluded that the selected region of the 16S rDNA is more suitable than that of the 23S rDNA for identification purposes because of its greater length.

The intraspecies variation within *A. lwoffii* alone was nearly as high as the interspecies differences in the genus *Acinetobacter* in general, with the result that molecular identification of this heterogeneous species will be relatively difficult. This finding pinpoints a potential problem in the interpretation of rDNA sequence analysis and indicates that a reliable classification system will require a complete genetic database. The taxonomy of *Acinetobacter* is, however, still incomplete and evolving, with only seven species currently named (nomenspecies) out of a total of 18 known genomic species, which indicates that a further subdivision of *Acinetobacter* species may be needed (5, 13, 38). Another potential limitation of the 16S and 23S rDNA method is the insufficient discriminatory power for recently diverged species, as seen in this study in the case of *N. macacae* and *N. mucosa* subsp. *mucosa* ATCC 19696 (8, 21,

39). On the other hand, these two entities are even by phenotypic means not clearly distinct species. In general, a sequential (e.g., second line) sequencing of the more variable 16S/23S rDNA spacer region (1, 14), and a polyphasic approach (i.e., a combination with other pheno- or genotypic techniques) (31), should solve this problem. A further problem in using the rDNA sequencing approach for identification purposes is the possible intercistronic heterogeneity between different rRNA operons (18). Despite these limitations, DNA sequence-based microbial identification is expected to play a major role in clinical microbiology laboratories in the future because of its speed, reproducibility, and potential for automation. High-quality DNA sequence data, in combination with DNA microarray techniques, may revolutionize diagnostic laboratory procedures (35).

The lack of adequate quality control procedures for public database entries and the associated difficulties when this data is used in medical diagnostics is documented by the high error rates of sequences detected in this study. Therefore, a diagnostic library preferably should rely on culture collection strain sequences and make their primary sequence data (i.e., the sequence chromatograms) available to users for purposes of intersubjective control of their data.

According to 16S rDNA sequences analysis, the genera *Chromobacterium*, *Eikenella*, *Iodobacter*, *Kingella*, *Neisseria*, and *Oligella* form one cluster, which is a part of the β subclass of the *Proteobacteria*. *Acinetobacter*, *Moraxella*, *Psychrobacter*, and *Suttonella* are separated into another cluster belonging to the γ subclass of the *Proteobacteria* (6, 13, 24). Signature sequence positions for these subclasses determined by Woese (41), most notably the *E. coli* position 485, also support this grouping. In contrast, the relationships among subgroups in our study differ slightly depending on the gene analyzed and also differ from previous results. This might be due to interspecies recombination events, since these species belong to a group of bacteria that frequently exchange chromosomal genes (30).

New RIDOM entries of bacterial genera are compared with the *American Society for Microbiology's Manual of Clinical Microbiology* to guarantee that all medically relevant pathogens are included (19). For quality control reasons, only sequences from strains held in culture collections are included in the database. In addition, the electropherograms of the sequence are deposited on our World Wide Web server thus allowing detailed comparison of the sequences generated. The classification of species entries is made in accord with the NCBI's "Guidelines and Conventions for the purpose of Biological classification." Therefore, we implemented the phylogeny tree of the Ribosomal Database Project (17) to reflect current phylogenetic knowledge on prokaryotes. In the case of nomenclatural terms, we adhered as closely as possible to the recommendations of the draft BioCode as found at the Royal Ontario Museum Website (http://www.rom.on.ca/biodiversity/biocode/biocode1997.html). To ensure updated taxonomic entries and avoid outdated nomenclature, bacterial names are checked with the aid of the Deutsche Sammlung von Mikroorganismen und Zellkulturen's Bacterial Nomenclature up-to-date database (http://www.dsmz.de/bactnom/bactname.htm), which is based on valid names published in the *International Journal of Systematic and Evolutionary Microbiology*. The hierarchic ordering and naming of different levels within the "organ-browser" is adapted from the "NLM-MeSH Tree Structures—Category C. Diseases" as found at the National Library of Medicine Website (http://www.nlm.nih.gov/mesh/). The Council for International Organizations of Medical Sciences' *International Nomenclature of Diseases* (4) and the World Health Organization's *International Classification of Diseases* (42) were used to relate standardized disease terms to specific microorganisms and to facilitate low background noise links to Internet databases.

The logic incorporated in the recently published and now commercially available MicroSeq system (PE Biosystems, Forster City, Calif.) is comparable to that in the RIDOM system (33). It also uses nonragged 16S rDNA sequences for microbial identification purposes. The database of MicroSeq currently contains over 1200 full-length, high-quality ATCC culture collection bacterial strain sequences. A feature-rich Macintosh analysis software enables the comparison of any unknown sequence with the sequences in the database. However, some fundamental differences exist between the two systems. Most notable of these is the fact that the RIDOM system, due to its open hypertext structure, allows the incorporation of other useful Internet sources. Another important difference is the inclusion of phenotypic methods and non-rDNA targets for species identification purposes (a polyphasic approach). Furthermore, the RIDOM approach is far-reaching in that it not only tries to include sequences and species names in its database but also includes additional information related to taxonomy and disease. Finally, RIDOM is specifically designed for medically important organisms for both humans and animals, whereas MicroSeq in its current form concentrates on environmental isolates for food and pharmaceutical industry quality control needs.

The RIDOM system currently offers the *Neisseriaceae* and *Moraxellaceae* dataset for general use and demonstration purposes, and a rapid and constant increase in the number of entries pertaining to other classes of bacteria and fungi is now under development. If the similarity search score is too low because the species in question is not yet incorporated in our database, the user may directly conduct a search of the NCBI GenBank using NCBI's sequence similarity search tool BLAST. Even if the user has no sequence for comparison, our database can still be searched or an Internet metasearch for species information related to nomenclature, phylogeny, or disease can be carried out. The RIDOM persistent uniform resource locator is http://purl.oclc.org/net/ridom, which is currently associated with the following URL: http://www.ridom.hygiene.uni-wuerzburg.de/. E-mail contact is possible using the address webmaster@ridom.hygiene.uni-wuerzburg.de.

In conclusion, our data show that it is possible to identify most *Neisseriaceae* and *Moraxellaceae* species by partial rDNA sequencing and that the 16S rDNA region examined in this study is more suitable for molecular diagnosis than the partial 23S rDNA. A genetic database should be exhaustive, that is, it should include more than just one representative strain of each species. A molecular diagnosis system should involve both different molecular targets and additional analytical procedures, since not all species can be differentiated by partial 16S rDNA sequencing alone.

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