Phylogenetic Relationship of the Twenty-One DNA Groups of the Genus *Acinetobacter* as Revealed by 16S Ribosomal DNA Sequence Analysis

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The inter- and intrageneric relationships of members of the genus *Acinetobacter* were investigated by performing a comparative sequence analysis of PCR-amplified 16S ribosomal DNAs (rDNAs) from 21 strains representing all of the DNA groups that have been described. Phylogenetic treeing confirmed that *Acinetobacter* spp. form a coherent cluster within the gamma subdivision of the class *Proteobacteria* that includes strains with overall levels of 16S rDNA sequence similarity of more than 94%. The analysis of intrageneric relationships suggested that the majority of the strains cluster in five clearly distinguishable clusters, and this conclusion was supported by the results obtained with the different methods used for phylogenetic analysis (i.e., the maximum-likelihood, parsimony, and distance matrix methods). The first cluster contains the representatives of DNA groups 2 (*Acinetobacter baumannii*) and TU13, whereas the second cluster comprises representatives of DNA groups 5, “Close To TU13,” and “between 1 and 3.” The representatives of closely related *Acinetobacter* DNA groups 8 (*Acinetobacter lwofii*) and 9 belong to the third cluster, which includes the representative of DNA group 6 as well. The fourth cluster is formed by DNA groups BJ16, BJ17, and the fifth cluster comprises DNA groups 1 (*Acinetobacter calcoaceticus*), BJ14, 10, and 11. Within the fifth cluster the 16S rDNA sequences of DNA group 10 and 11 strains are nearly identical. The representatives of DNA groups 4 (*Acinetobacter haemolyticus*), 5 (*Acinetobacter junii*), 7 (*Acinetobacter johnsonii*), 12 (*Acinetobacter radioresistens*), TU14, and TU15 form individual branches that are not significantly affiliated with any of the five clusters identified. Apart from the clustering of the most closely related DNA groups, the general topology of the distance dendrogram revealed some discrepancy with previous DNA-DNA hybridization data, which may point to the inadequacy of comparative 16S rDNA sequence analysis for reflecting true evolutionary relationships of closely related bacterial taxa. Important, however, was the presence of unique sequence motifs in each of the 21 different DNA groups studied, which may be useful for rapid differentiation of DNA groups of the genus *Acinetobacter*.
meaningfulness of comparative 16S rDNA sequence analysis and to evaluate the diagnostic potential of highly variable regions for reliable differentiation of the DNA groups based on characteristic sequence motifs.

**TABLE 1. Acinetobacter DNA groups investigated and 16S rDNA accession numbers**

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</table>

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 21 bacterial isolates were analyzed (Table 1). The type strains of six of the seven validly described *Acinetobacter* species were included, as were representatives of all of the other DNA groups described by different investigators. The type strain of the seventh described species, *A. lwoffi* (DNA group 8), was not included in the study because of uncertainty about its taxonomic position (21). Instead, two other reference strains (ATCC 17925 and ATCC 9957) were investigated as representatives of DNA groups 8 and 9 described by Bouvet and Grimont (1). This was done to be sure to include strains belonging to both of these DNA groups in the study. The strains were obtained from the American Type Culture Collection and our own culture collection.

**PCR amplification and sequencing of 16S rDNA.** For amplification of the 16S rDNA, the following reagents were combined in a final volume of 50 μl: each deoxynucleoside triphosphate at a concentration of 100 mM, 10 pmol of each primer, 1X Taq buffer (Boehringer, Mannheim, Germany), and approximately 10 ng of purified RNase-treated template DNA. After 50 μl of mineral oil was added to each tube, the tubes were heated to 80°C, and 1 μl of Taq polymerase (Boehringer) was subsequently added. Amplification was carried out with a model 480 DNA thermal cycler (Perkin-Elmer, Allerod, Denmark) programmed for 30 cycles, with each cycle consisting of 1 min at 94°C (denaturation), 1 min at 52°C (annealing), and 1 min at 72°C (extension). A final extension step at 72°C was performed for 3 min. The 5′ and 3′ primers used were 5′GAGTTGATGC CGCGCTCAG3′ and 5′ACGGCTACCTTGTTACGACTT3′, targeting conserved regions 9 to 27 and 1512 to 1492 (13). The 16s rDNA products were purified by separation on 1.5% low-melting-point agarose gels. Gel fragments containing the desired product were sliced and digested with Gelase enzyme (Epitrendr Technologies, Madison, Wis.) as described elsewhere (12). Sequencing was carried out nonradioactively by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer’s instructions. The sequence primers used were those described by Lane (14). Sequence products were analyzed with a model 373A DNA sequencer (Applied Biosystems).

**Data analysis.** The 21 16S rDNA sequences of the *Acinetobacter* spp. studied were manually aligned and compared with reference sequences of members of the gamma subdivision of the class Proteobacteria by focusing on members of the most closely related genera. The reference sequences were obtained from public databases (EMBL [5] and Ribosomal Database Project [15]). The intragenic relationships of the *Acinetobacter* spp. shown in Fig. 1 were deduced by comparing only the positions of a continuous stretch of 1,389 nucleotides corresponding to positions 28 through 1438 of the 16s rRNA of *E. coli* (International Union for Biochemistry nomenclature) from which unambiguous sequence information could be obtained. Three different methods of phylogenetic analysis supplied by the PHYLIP package, version 3.5c (8), were used to delineate intragenic relationships. These methods were the maximum-likelihood method (dlam) program, the parsimony method (dnapars program), and the neighbor-joining method with a distance matrix calculated with the Jukes-Cantor correction (13).

FIG. 1. Unrooted phylogenetic dendrogram constructed for 21 strains representing all known DNA groups described for the genus *Acinetobacter*. The tree was constructed by performing a maximum-likelihood analysis. The percentages of 100 bootstrap resamplings that supported the presence of five distinct clusters in the parsimony analysis (above the lines) and in the neighbor-joining analysis (below the lines) are indicated. The thresholds for defining these clusters were bootstrap values of at least 50% in the parsimony analysis and, for the same nodes simultaneously, at least 90% in the neighbor-joining analysis. Scale bar = 0.02 estimated base change per nucleotide.
The PCR primer pair used in amplification of almost complete 16S rRNA genes from the 21 strains studied. Phylogenetic treeing performed with a restricted number of nucleotide positions confirmed previous reports that members of the genus *Acinetobacter* form a phylogenetically coherent group of organisms within the gamma subdivision of the class *Proteobacteria* and that species of the genus *Moraxella* represent the most closely related lineage (data not shown) (6, 18).

In order to elucidate the intrageneric relationships of the known DNA hybridization groups of the genus *Acinetobacter*, almost complete 16S rRNA genes were comparatively analyzed. The overall levels of 16S rDNA sequence similarity for the strains were greater than 94%. The analysis of intrageneric relationships in which the maximum-likelihood method was used revealed that the majority of the strains can be assigned to five different lineages (Fig. 1). Almost identical tree topologies were obtained when the parsimony method or the neighbor-joining method based on a Jukes-Cantor distance matrix was used to construct the tree. Bootstrap analyses in which the support to some degree the phylogenetic placement of species of the genus *Moraxella* repre- sent the most closely related lineage (data not shown) (6, 18).  

The nucleotide sequences used in this study have been deposited in the EMBL Nucleotide Sequence Database under accession no. 293434 through 293454 (Table 1).

**RESULTS AND DISCUSSION**

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The bootstrap method (7) (PHYLIP package) was used to check the topology perturbations in the neighbor-joining and parsimony trees.

**Nucleotide sequence accession numbers.** The nucleotide sequences used in this study have been deposited in the EMBL Nucleotide Sequence Database under accession no. 293434 through 293454 (Table 1).

To test for statistical significance differed. When threshold values of more than 50% were used for nodes in the parsimony tree and perturbations in the neighbor-joining and parsimony trees.

The representatives of DNA groups 4, 5, 7, 12, TU14, and TU15 form individual branches that are not significantly affiliated with any of the five clusters identified (based on the bootstrap threshold values defined above). The outcome of the treeing analysis was the same whether an outgroup sequence (the 16S rDNA sequence of *Pseudomonas aeruginosa* [accession no. M34133]) was used to root the tree or not. Our data are consistent with the results of a previous study in which the authors used 16S rDNA sequences of representatives of the seven named *Acinetobacter* species and DNA group 11 (18).

A search for nucleotide positions at which the nucleotides are the same for all of the strains belonging to one of the five defined clusters but are not found in any other strain or are found in only a few other strains resulted in a set of signatures which support to some degree the phylogenetic placement of the majority of the *Acinetobacter* spp. into the five different subgroups (Fig. 1 and Table 2). The representatives of cluster I (DNA groups 2 and TU13) have a guanosine at position 648 (JUB nomenclature for *E. coli*), which is unique for these two

<table>
<thead>
<tr>
<th>DNA group</th>
<th>Sequence</th>
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**FIG. 2.** Characteristic sequence motifs for the 21 *Acinetobacter* DNA groups at the helix 6 variable region (22, 23), corresponding to *E. coli* 16S rRNA positions 70 to 101 (IUB nomenclature for *E. coli*). The stretch shown has a deletion of six nucleotides compared to the *E. coli* sequence.

**FIG. 3.** Characteristic sequence motifs for *Acinetobacter* DNA groups at the helix 18 variable region (22, 23) compared to the *E. coli* 16S rRNA sequence at positions 453 to 477 (IUB nomenclature for *E. coli*).

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**TABLE 2.** Sequence motifs that support the presence of the five distinct groups derived from the phylogenetic analysis

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</table>

* See Fig. 1.

+ Positions according to the IUB nomenclature for *E. coli*.

+ Clusters are defined as shown in Fig. 1. Cluster I contains DNA groups 2 and TU13; cluster II contains DNA groups 5, 6, 7, 8, 9, and 10; cluster III contains DNA groups 11 and 12; cluster IV contains DNA groups 13, 14, 15, and 16; and cluster V contains DNA groups 17, 18, 19, and 20.

+ The nucleotides which are highly indicative for clusters are in boldface type.
sequences in the 21 sequences of *Acinetobacter* strains studied. Similarly, the representatives of cluster II (DNA groups 3, CTTU13, and 1-3) are characterized by a unique guanosine at position 658 ( *E. coli* numbering). The representatives of cluster III (DNA groups 6, 8, and 9) are defined by two coordinated nucleotide substitutions. The first of these is a C-G base pair at positions 206 and 213 ( *E. coli* numbering), which is shared only by the representative DNA group 12. The second coordinated substitution is a T-A base pair at positions 586 and 755 ( *E. coli* numbering) that is shared only by the representative DNA group TU15. The members of cluster IV (DNA groups BJ15 through BJ17) are also characterized by two coordinated nucleotide substitutions. The first coordinated substitution is a G-C base pair at positions 591 and 648 ( *E. coli* numbering), which is shared by the representatives of DNA groups 4 and 7. The finding that in addition to members of cluster IV, both of these coordinated nucleotide substitutions are also present in the representative DNA group 4 suggests that the DNA group 4 strain may be a member of cluster IV, which is also consistent with the topology of the tree shown in Fig. 1. Five unique nucleotides are highly indicative for members of cluster V (DNA groups 1, 10, 11, and BJ14). These nucleotide signatures, which are not found in any of the remaining 17 strains, are located in a highly variable region characterized by helix 6 (numbering of Van de Peer et al. [22, 23]) and include the nucleotides at positions 79 (T), 81 (G), 82 (C), 83 (T), and 85 (C) ( *E. coli* numbering).

We did not include the type strain of *A. lwofii* in this study because of the uncertainty of its taxonomic affiliation. Bouvet and Grimont (1) placed the type strain of *A. lwofii* (NCTC 5866) and strain ATCC 17925 in DNA group 8 and strain ATCC 9957 in DNA group 9. In the study of Tjernberg and Ursing (21), type strain NCTC 5866 and strain ATCC 9957 clustered together, whereas strain ATCC 17925 clustered at a marginal position in their DNA group 8/9 (*A. lwofii*). In order to examine strains belonging to both DNA group 8 and DNA group 9 described by Bouvet and Grimont (1), strains ATCC 9957 and ATCC 17925 were selected for the present study. These two strains had almost identical rDNA sequences.

When our data are compared with previously published DNA-DNA hybridization data (1, 2, 11, 21), both similarities and differences are found. As determined by DNA-DNA hybridization, DNA groups 1 through 3, TU13, CTTU13, and 1-3 form a tight cluster (the *A. calcoaceticus-A. baumannii* complex) (10); proteolytic DNA groups 4 through 6, TU14, and BJ14 through BJ17 are also more closely related to each other than to other acinetobacters (1, 2, 21), as are DNA groups 7 through 9 and TU15 (1, 21); DNA groups 10 and 11 are related as determined by DNA hybridization; and DNA group 12 is a separate entity (1, 21). rDNA sequencing confirmed the close relationship between DNA groups 10 and 11 and the close relationship between DNA groups 8 and 9. The unique position of DNA group 12 within the genus determined in all previous studies was also reproduced. However, the DNA groups in the *A. calcoaceticus-A. baumannii* complex were split into three clusters (clusters II, III, and V). The very close relationship between DNA groups 2 and TU13 was confirmed, but DNA group CTTU13 clustered with DNA groups 3 and 1-3. In cluster V, DNA group 1 was placed with DNA groups 10, 11, and BJ14. Likewise, the proteolytic DNA groups were distributed in different clusters, with only the rDNA sequences of DNA groups BJ15 through BJ17 identified as a tight cluster. Also, DNA groups TU15 and 7 are separated from each other and from the strains of DNA group 8/9 by the rDNA sequence analysis data. One important reason for the discrepancies between the results of the 16S rDNA analysis and the DNA-DNA hybridization data may be the fact that the majority of the nucleotide differences between the strains that were investigated occur in highly variable regions as defined by Van de Peer et al. (22). Nucleotide positions in these areas are known to have a very fast evolutionary rate, based on fixation of random mutation events resulting in a loss of phylogenetic information. In addition, lateral gene transfer between closely related bacteria followed by gene conversion leading to homogenization of multiple rRNA gene copies in the genomes of individual lineages may eclipse fast mutational rates in the highly variable regions (4). However, the meaningfulness of the highly variable regions for phylogenetic inference is still under investigation (20) and may vary with the bacterial group being studied. Therefore, sequence motifs, especially those like coordinated substitutions between distantly located sequence positions as determined for members of cluster III (T-A base pair at positions 586 and 755) and members of cluster IV (G-C pair at positions 591 and 648), represent helpful markers for inferring true phylogenetic affiliations among closely related microorganisms, like the *Acinetobacter* spp.

In addition, the levels of DNA-DNA relatedness in the proteolytic groups vary between 33 and 68% with DNA group 5 being least similar to the other groups. Thus, the DNA-DNA clustering of these proteolytic groups may not be reliable, and it may not be surprising that they are split into different 16S rDNA clusters. Consequently, in the present situation, reliable correlation between the 16S rDNA sequence analysis and DNA-DNA hybridization is difficult to achieve.

At variance with the 16S rDNA sequence data but consistent with the DNA-DNA hybridization data is the fact that the *gyrB* gene sequence analysis data reported by Yamamoto and Harayama (24) revealed a close relationship among strains in the *A. calcoaceticus-A. baumannii* complex. However, only strains belonging to DNA groups 1 through 3 in this complex were investigated in the study of Yamamoto and Harayama. Strains belonging to the proteolytic DNA groups were also found to be more closely related to each other on the basis of *gyrB* gene data than on the basis of 16S rDNA sequence analysis data. Yet, protein-encoding genes like *gyrB* evolve much faster than rRNA genes and may thus provide precise phylogenetic information about closely related species. Moreover, disagreement between the results of 16S rRNA sequence and DNA-DNA hybridization studies has been described in detail by Stackebrandt and Goebel (19). An interesting example is the almost complete identity of the 16S rRNA genes derived from *Bacillus anthracis* and *Bacillus cereus* although the two species are well-differentiated on the basis of DNA-DNA hybridization data and phenotypic traits (9). Martinez-Murcia et al. (16) found a similar discrepancy in their study of the genus *Aeromonas*.

We tried to identify sequence stretches with diagnostic potential. The current investigation was based on direct sequence analysis of PCR-amplified 16S rDNA. This approach produces a consensus 16S rDNA sequence for each strain studied, and it may be possible to detect sequence microheterogeneities (polymorphisms) due to nucleotide differences between 16S rRNA genes which may be present in multiple copies in the bacterial genome. Our results have the potential for differentiating *Acinetobacter* spp. not only for reliable identification of an isolate as a member of the genus *Acinetobacter*, but also for placement of an isolate in the proper DNA group. This is possible because of unambiguous sequence differences in highly variable regions (22), especially in the areas characterized by helices 6 and 18 (22), corresponding to positions
around positions 70 to 100 (Fig. 2) and 450 to 480 (Fig. 3) (IUB nomenclature for *E. coli*), respectively. The representative of each of the 21 DNA groups had a stretch of unique sequence motifs either in one region or in both regions. To our knowledge, this is the first time that the 21 DNA groups have been clearly differentiated. In our hands, when one reverse primer targeting positions 536 to 519 (IUB nomenclature for *E. coli*) was used, it was possible to read the complete stretch to the 5’ end of the PCR-amplified fragments encompassing both highly variable regions. Consequently, the use of this sequencing tool, coupled with automated fluorescent sequencing, should allow identification of unknown isolates as members of the genus *Acinetobacter* through the more conserved regions of partially analyzed 16S rRNA genes and reliable and rapid DNA group differentiation based on the two highly variable areas characterized by helices 6 and 18 (22).

In conclusion, our data confirmed that the 21 *Acinetobacter* DNA groups form a coherent group of organisms. We also documented the intrageneric relationships of certain DNA groups, especially DNA groups 8 and 9. DNA groups 8 and 9 are indistinguishable phenotypically (1) and by most molecular methods, including DNA-DNA hybridization with the hydroxypatite method (21), and we propose that they should be included in one species, *A. lwofii*. However, our delineation of intrageneric relationships revealed some disagreement with previous DNA-DNA hybridization data. Our findings also highlighted the potential of using direct sequencing of PCR-amplified 16S rDNA as a rapid tool for identification and delineation of *Acinetobacter* strains. A final conclusion on this point cannot be drawn until more representatives of each DNA group are sequenced.

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