Phylogenetic Analysis of *Acinetobacter* Strains Based on the Nucleotide Sequences of *gyrB* Genes and on the Amino Acid Sequences of Their Products

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Partial nucleotide sequences of the *gyrB* genes (DNA gyrase B subunit genes) of 15 *Acinetobacter* strains, including the type and reference strains of genomic species 1 to 12 (*A. calcoaceticus* [genomic species 1], *A. baumannii* [genomic species 2], *Acinetobacter* genomic species 3, *A. haemolyticus* [genomic species 4], *A. junii* [genomic species 5], *Acinetobacter* genomic species 6, *A. johnsonii* [genomic species 7], *A. lwoffii* [genomic species 8], *Acinetobacter* genomic species 9, *Acinetobacter* genomic species 10, *Acinetobacter* genomic species 11, and *A. radioresistens* [genomic species 12]), were determined by sequencing the PCR-amplified fragments of *gyrB*. The *gyrB* sequence homology among these *Acinetobacter* strains ranged from 69.6 to 99.7%. A phylogenetic analysis, using the *gyrB* sequences, indicates that genomic species 1, 2, and 3 formed one cluster (87.3 to 90.3% identity), while genomic species 8 and 9 formed another cluster (99.7% identity). These results are consistent with those of DNA-DNA hybridization and of biochemical systematics. On the other hand, the topology of the published phylogenetic tree based on the 16S rRNA sequences of the *Acinetobacter* strains was quite different from that of the *gyrB*-based tree. The numbers of substitution in the 16S rRNA gene sequences were not high enough to construct a reliable phylogenetic tree. The *gyrB*-based analysis indicates that the genus *Acinetobacter* is highly diverse and that a reclassification of this genus would be required.

The nucleotide sequences of small-subunit rRNA (16S rRNA) have been used most frequently to deduce the phylogenetic relationships between bacteria (15, 24, 32, 48). However, the results of 16S rRNA sequence analysis have often disagreed with the results using DNA reassociation, which is considered to be an absolute measure of relatedness (16, 34, 44). The major reason for this discrepancy may be the low rate of base substitution in 16S rRNA genes: the numbers of substituted bases between rRNA genes from closely related bacteria are small, and a comparison of almost identical sequences is accompanied by a large statistical error. The use of protein-coding genes that are known to evolve much faster than rRNAs seems to be more appropriate for the phylogenetic analysis of closely related bacteria. We have developed universal PCR primers with which the amplification and sequencing of *gyrB* genes (the DNA gyrase B subunit genes) from a variety of bacteria were possible (49). We were thus interested in using these *gyrB* sequences in molecular systematics.

Acinetobacter strains are ubiquitous in the natural environment and are implicated in the biodegradation of a variety of hydrocarbons and in some human diseases (1, 3, 35, 40, 42, 47). The establishment of effective typing systems for this genus is especially important because of outbreaks of *Acinetobacter* infections in hospitals (8, 11). Several methods, including ribotyping, cellular fatty acid compositional analysis, cell envelope protein profiling, plasmid DNA fingerprinting, and phage typing, have been used to classify this genus (6, 8, 17, 21, 22, 26, 28, 33, 41, 46). The definition of the genus by DNA-DNA hybridization comprises at least 19 genomic species (DNA groups) (5, 7, 18, 45, 46). In this study, we carried out a phylogenetic analysis of 15 *Acinetobacter* strains by using their

gyrB sequences, and we compare the results with those from conventional taxonomic studies.

MATERIALS AND METHODS

Bacterial strains. The following type strains and reference strains of genomic species 1 to 12 (5, 31, 45) were presented by P. J. M. Bouvet of Institut Pasteur in Paris, France: *Acinetobacter calcoaceticus* CIP 81.08^T (= ATCC 23055^T; genomic species 1), *A. baumannii* CIP 70.34^T (= ATCC 19606^T; genomic species 2), *Acinetobacter* sp. strain CIP 70.29 (= ATCC 19004; genomic species 3), *A. haemolyticus* CIP 64.3^T (= ATCC 17906^T; genomic species 4), *A. junii* CIP 64.5^T (= ATCC 17908^T; genomic species 5), *Acinetobacter* sp. strain CIP A165 (= ATCC 17979; genomic species 6), *A. johnsonii* CIP 64.6^T (= ATCC 17909^T; genomic species 7), *A. lwoffii* CIP 64.10^T (= NCTC 5866^T; genomic species 8), *Acinetobacter* sp. strain CIP 70.31 (= ATCC 9957; genomic species 9), *Acinetobacter* sp. strain CIP 70.12 (= ATCC 17924; genomic species 10), *Acinetobacter* sp. strain CIP 63.46 (= ATCC 11171; genomic species 11), and *A. radioresistens* SEIP 12.81 (genomic species 12). Two strains, *A. calcoaceticus* ATCC 31012 (= RAG-1) (19, 36) and *A. calcoaceticus* ATCC 33308 (= BD 413 ErpE27) (27, 39), were purchased from American Type Culture Collection. *Acinetobacter* sp. strain T4 has been isolated from the Pacific Ocean as a bacterium degrading *n*-tetradecan. Chemical taxonomy has classified this strain as a member of the genus *Acinetobacter* (unpublished data).

Preparation of chromosomal DNA. Bacteria were grown aerobically in nutrient broth at 30°C, cells from overnight cultures being washed and resuspended in a TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 7.5). Chromosomal DNA was prepared by the method described by Johnson (25).

PCR amplification and direct sequencing of *gyrB*. The *gyrB* fragments of the *Acinetobacter* strains were amplified and sequenced as described previously (49). PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Co. Norwalk, Conn.) with a PCR buffer (Perkin-Elmer Co.) containing each of the deoxynucleoside triphosphates at a concentration of 200 µM, each of the primers at a concentration of 1 µM, 1 µg of target DNA, and 2.5 U of *Taq* DNA polymerase in a total volume of 100 µl. A total of 30 amplification cycles was performed with template DNA denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 min. The amplified products were purified by preparative gel electrophoresis on 1% low-melting-temperature agarose (NuSieve GTG; FMC Bioproducts, Rockland, Maine) according to the method described by Sambrook et al. (38) before each N-terminal and C-terminal 300-bp stretch of the amplified fragments was sequenced. The sequencing reaction was conducted by using a *Taq* DyeDeoxy Terminator cycle sequencing kit according to the manufacturer's instructions, and the products were analyzed with a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.).

Phylogenetic data analysis. The nucleotide sequences of *gyrB* or their translated amino acid (GyrB) sequences were aligned by using the CLUSTAL V

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A: N-terminal region

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genomic sp. 1 1:SYKVGGLHRVGVSVNALSSKLHLMISRAGQVHEQEYQHGDPOQYPLRVIGETDKSGTTV
genomic sp. 2 1:.....G.....T.Y...I....H.....NT...
genomic sp. 3 1:.....G.....I.N....H.....S.....
genomic sp. 4 1:.....Q.T.H...E.Q...H.....RT..I.
genomic sp. 5 1:.....K...T.Q...I....C.....K.V...T...
genomic sp. 6 1:.....G.....K...I.H...T....C...S...K.....VI
genomic sp. 7 1:.....G.....R..E.T.H...HI.Q...K...V...T.V.D..TT...
genomic sp. 8 1:.....G.....E..E.T.H...KI....R...S...K.V.D.NRT..R.
genomic sp. 9 1:.....G.....E..E.T.H...KI....R...S...K.V.D.NRT..R.
genomic sp.10 1:.....G.....E.T.H...HI.Q...K...V...K...DIETT...
genomic sp.11 1:.....G.....E.T.H...HI.Q...K...V...KIV.DVETT..I.
genomic sp.12 1:..H.....G.....E.TVQ...I.....V.....V.Q.ERT..K.
ATCC 31012 1:.....G.....K...T.H...I....A.....K.V...T...
ATCC 33308 1:..H.....G.....Q.T.H...I....A.....K.V...STT...
T4 1:.....L.....K...T.H...I....A.....K.V...T...

genomic sp. 1 61:RFWPSELFTQTIFNVEILARRLRELSFLNAGVRIVLRDERINLGHVFDYEGGLSE
genomic sp. 2 61:.....AE..S.....E.....
genomic sp. 3 61:.....S.....E..Y.....
genomic sp. 4 61:.....AE..S.....D.....V..EN.Y.....
genomic sp. 5 61:.....S.....S.D.....V..EN.Y.....
genomic sp. 6 61:.....S.....S.....V..E..Y.....
genomic sp. 7 61:.....AE..S.....D.....W....HAE.....
genomic sp. 8 61:.....AE..S.....D.....AE.....
genomic sp. 9 61:.....AE..S.....D.....L.....AE.....
genomic sp.10 61:.....AE..S.....D.....VA.E.I..L.V...
genomic sp.11 61:.....TE..S.....D.....VA.E.....
genomic sp.12 61:.....AE..S.....D.....C.....E.....
ATCC 31012 61:.....S.....S.D.....V..E.IY..V...
ATCC 33308 61:.....GD..S.....D.....K.....V.FE.IYA...
T4 61:.....W..S.....S.D.....V..E.IY..V...

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B: C-terminal region

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genomic sp. 1 1:KSALDIAGLPGLADCQEKDPALSELVYLVEGDSAGGSAKQGRNRKMQAAILPLKGKILNVE
genomic sp. 2 1:.....
genomic sp. 3 1:.....
genomic sp. 4 1:.....
genomic sp. 5 1:.....G.....
genomic sp. 6 1:.....
genomic sp. 7 1:.....
genomic sp. 8 1:.....
genomic sp. 9 1:.....
genomic sp.10 1:.....
genomic sp.11 1:.....
genomic sp.12 1:.....F.....
ATCC 31012 1:.....
ATCC 33308 1:.....P.....
T4 1:.....

genomic sp. 1 61:RARFDKMISSQEVGTLITALGCGIGREEYNPDKLRVHKII
genomic sp. 2 61:.....
genomic sp. 3 61:.....
genomic sp. 4 61:.....
genomic sp. 5 61:.....
genomic sp. 6 61:.....
genomic sp. 7 61:.....
genomic sp. 8 61:.....R...A.....
genomic sp. 9 61:.....R...A.....
genomic sp.10 61:.....
genomic sp.11 61:.....
genomic sp.12 61:.....R...A.....V
ATCC 31012 61:.....
ATCC 33308 61:.....
T4 61:.....

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FIG. 1. Amino acid sequence alignment of the *gvrB* products of 15 *Acinetobacter* strains. Amino acids identical to those in the genomic species 1 (*A. calcoaceticus* CIP 81.08^T) sequence are indicated with dots. (A) Sequence alignment for the N-terminal region of the amplified fragment corresponds to positions 107 to 223 of the *E. coli* K-12 sequence (accession number P06982). (B) Sequence alignment for the C-terminal region corresponds to positions 394 to 493 of the *E. coli* K-12 sequence.

TABLE 1. Nucleotide sequence homology of *gyrB* between strains of the genus *Acinetobacter*

Strain no. and name	Genomic species	Nucleotide sequence homology (%) with strain:													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>A. calcoaceticus</i> CIP 81.08 ^T	1														
2 <i>A. baumannii</i> CIP 70.34 ^T	2	88.0													
3 <i>Acinetobacter</i> sp. strain CIP 70.29	3	90.3	87.3												
4 <i>A. haemolyticus</i> CIP 64.3 ^T	4	81.7	81.0	83.4											
5 <i>A. junii</i> CIP 64.5 ^T	5	82.0	81.7	81.7	82.8										
6 <i>Acinetobacter</i> sp. strain CIP A165	6	81.0	79.4	82.0	81.3	80.6									
7 <i>A. johnsonii</i> CIP 64.6 ^T	7	76.7	77.6	77.7	81.1	79.3	77.0								
8 <i>A. lwoffii</i> CIP 64.10 ^T	8	77.0	77.9	77.1	78.2	80.2	77.7	80.5							
9 <i>Acinetobacter</i> sp. strain CIP 70.31	9	77.0	77.6	76.8	77.9	80.2	77.7	80.2	99.7						
10 <i>Acinetobacter</i> sp. strain CIP 70.12	10	77.1	78.5	77.6	79.0	77.0	77.1	79.0	77.6	77.6					
11 <i>Acinetobacter</i> sp. strain CIP 63.46	11	79.6	79.7	79.4	79.6	78.3	77.6	81.1	79.1	78.8	85.6				
12 <i>Acinetobacter</i> sp. strain SEIP 12.81	12	75.4	76.8	74.2	76.5	75.0	75.4	77.3	79.6	75.3	75.3				
13 <i>Acinetobacter</i> sp. strain T4		80.0	81.4	81.1	82.2	85.9	82.8	76.7	78.3	78.0	78.6	79.1	73.6		
14 ATCC 31012		80.6	81.7	81.9	82.2	86.3	83.1	77.0	79.3	79.0	79.1	79.7	74.7	98.8	
15 ATCC 33308		70.2	73.6	70.8	71.7	73.0	69.6	73.0	73.9	73.6	70.5	72.4	75.4	70.2	71.1

computer program (23). Evolutionary trees were constructed with the PHYLIP computer program package (13), using the neighbor-joining method (37).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers (first and second numbers indicate the 5' and 3' regions of *gyrB*, respectively): genomic species 1, D73427 and D73412; genomic species 2, D73428 and D73413; genomic species 3, D73429 and D73414; genomic species 4, D73430 and D73415; genomic species 5, D73431 and D73416; genomic species 6, D73417 and D73432; genomic species 7, D73433 and D73418; genomic species 8, D73434 and D73419; genomic species 9, D73435 and D73420; genomic species 10, D73436 and D73421; genomic species 11, D73437 and D73422; genomic species 12, D73438 and D73423; ATCC 31012, D73440 and D73425; ATCC 33308, D73441 and D73426; and T4, D73439 and D73424.

RESULTS AND DISCUSSION

Nucleotide sequences of *gyrB*. The partial nucleotide sequences of *gyrB* from 15 *Acinetobacter* strains were determined as described in Materials and Methods. The alignment of the *GyrB* amino acid sequences translated from the obtained *gyrB* nucleotide sequences is shown in Fig. 1. The percentage of nucleotide substitutions in the *gyrB* genes varied from 0.3 to 30.4% (Table 1), while the percentage of substitutions in the

amino acid sequences was between 0.5 and 13.3%.

Phylogenetic structure based on *gyrB*. The phylogenetic tree based on the *gyrB* nucleotide sequences is shown in Fig. 2. *A. calcoaceticus* (genomic species 1), *A. baumannii* (genomic species 2), and an *Acinetobacter* sp. (genomic species 3) formed one cluster (87.3 to 90.3% identity), while *A. lwoffii* (genomic species 8) and an *Acinetobacter* sp. (genomic species 9) formed another cluster (99.7% identity). The topologies of the phylogenetic trees produced by the neighbor-joining method (37), maximum-parsimony method (14), maximum-likelihood method (12), and unweighted pair-group method (43) were almost identical (data not shown).

Strains ATCC 31012 and ATCC 33308 have been classified as members of *A. calcoaceticus* (genomic species 1) (19, 27, 36, 39), but they were localized in branches different from the branch of the *A. calcoaceticus* type strain. The crude oil-degrading strain ATCC 31012 (RAG-1) producing the biosurfactant emulsan (19) was closely related to the *n*-tetradecan-degrading *Acinetobacter* sp. strain T4 (98.8% identity) and, to a lesser extent, to *A. junii* CIP 64.5^T (genomic species 5; 86.3% identity). Strain ATCC 33308 has been used as the competent

FIG. 2. Phylogenetic tree for the strains of *Acinetobacter* based on the *gyrB* nucleotide sequences by the neighbor-joining method. The numbers shown next to the nodes indicate percent bootstrap values of the 500 replicates. Genetic distances were computed by using Kimura's two-parameter model (29).

FIG. 3. Phylogenetic tree for the strains of *Acinetobacter* based on the amino acid sequences of *gyrB* products by the neighbor-joining method. The numbers shown next to the nodes indicate percent bootstrap values of the 500 replicates. Distances were computed by using maximum-likelihood estimates based on the Dayhoff PAM matrix (10).

strain of Juni's transformation assay for the identification of *Acinetobacter* strains at the genus level (27), and this strain was not strongly linked to any genomic species.

Phylogenetic structure based on the amino acid sequences of GyrB. The phylogenetic tree based on the amino acid sequences of GyrB is shown in Fig. 3. The branching order of the GyrB-based tree was very similar to that based on the *gyrB* nucleotide sequences; the only difference between these trees was the position of *A. baumannii* (genomic species 2).

Nucleotide substitution which provokes amino acid substitution is less frequent than synonymous substitution. The rate of amino acid substitution may be influenced by the percentage of amino acids essential for function, and each protein can be expected to have its own evolutionary speed. In the case of *gyrB*, the amino acid substitution rate was calculated to be one-third of the nucleotide substitution rate (Fig. 4). To examine the phylogeny of closely related bacteria, the analysis of nucleotide sequences may be superior to that of amino acid sequences, as higher numbers of substitutions are found in the former sequences. When the divergence of two compared nucleotide sequences is high and base substitutions can be expected to have occurred at most potential sites, the analysis of amino acid sequences may give a more accurate phylogenetic tree. In the case of the taxonomic position of *A. baumannii*, the nucleotide sequence analysis may provide better resolution, as the number of substituted amino acids between the sequences of *A. baumannii* and other related strains was small, being around 10.

Comparison with other phylogenetic analyses. The result of a phylogenetic analysis using *gyrB* nucleotide sequences was consistent with those using DNA-DNA hybridization and phenotype comparison. These classical analyses have demonstrated the clustering of genomic species 1, 2, and 3 and that of genomic species 8 and 9 (5, 45). The cluster analysis of 95 carbon source utilization profiles generated by the Biolog system (4) also suggested linkages between genomic species 4 and 6, between genomic species 10 and 11, and between genomic species 8 and 12 (2). All of these linkages were observed in the *gyrB*-based phylogenetic tree (Fig. 2).

On the other hand, the topology of the phylogenetic tree

based on the 16S rRNA sequences was quite different from that based on the *gyrB* sequences (34, 50). In the 16S rRNA sequence analysis done by Rainey et al., four genomic species (1, 4, 7, and 8) and two genomic species (2 and 5) were grouped (34). Neither of these clusters was detected by DNA-DNA hybridization, by phenotypic characterization, or by our *gyrB* sequence analysis. Thus, the 16S rRNA gene sequence analysis may not be appropriate to deduce phylogenetic relationships among closely related bacteria. The aberrant structure of the 16S rRNA gene-based phylogenetic tree may have been due to statistical errors during the analysis of the small numbers of substitutions. Since a large part of the primary and secondary

FIG. 4. Comparison of the nucleotide substitution frequencies of *gyrB* and of the amino acid substitution frequencies of *gyrB* products. Substitution frequencies between all the possible combinations of the *Acinetobacter* strains were calculated and plotted. The nucleotide substitution frequencies were corrected for multiple substitutions by using the Kimura and Ohta equation (30). R^2 indicates the coefficient of correlation.

structures of 16S rRNA is important for its functions, the evolution speed of 16S rRNA is extremely low (9, 20).

Conclusions. As shown in this study, the *gyrB* sequence analysis is a rapid and effective method for identifying bacterial species and for examining phylogenetic relationships. Theoretically, any protein-coding sequence can be used for a phylogenetic analysis. However, many genes, especially those for catabolism, are known to spread horizontally among different bacterial species, and they cannot be used to trace the evolutionary record of host bacteria. Criteria for selecting appropriate taxonomic marker genes may be that (i) they are essential for housekeeping activities such as DNA replication, transcription, and translation; (ii) they are a single copy on each genome; and (iii) at least two regions are conserved for PCR amplification by using primers complementary to the conserved regions. We have conducted a search for such genes and conclude that *gyrB* may be the best for such a purpose.

The genus *Acinetobacter* is a highly diverse taxonomic group (69.6 to 99.7% identity in the *gyrB* sequences), in comparison with the genetic proximity between *Pseudomonas putida* IFO 14164^T (= ATCC 12633^T) and *Escherichia coli* K-12 (74.6% identity; Fig. 2 and Table 1). It will be necessary to clarify the taxonomic positions of the *Acinetobacter* genomic species with respect to other closely related genera.

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