Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA–DNA hybridization

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The phylogenetic relationships of 49 *Acinetobacter* strains, 46 of which have previously been classified into 18 genomic species by DNA–DNA hybridization studies, were investigated using the nucleotide sequence of *gyrB*, the structural gene for the DNA gyrase B subunit. The phylogenetic tree showed linkages between genomic species 1 (*Acinetobacter calcoaceticus*), 2 (*Acinetobacter baumannii*), 3 and TU13; genomic species 6, BJ15, BJ16 and BJ17; genomic species 5, BJ13 (synonym of TU14) and BJ14; genomic species 7 (*Acinetobacter johnsonii*), 10 and 11; and genomic species 8 and 9. The phylogenetic grouping of *Acinetobacter* strains based on *gyrB* genes was almost congruent with that based on DNA–DNA hybridization studies. Consequently, *gyrB* sequence comparison can be used to resolve the taxonomic positions of bacterial strains at the level of genomic species. However, minor discrepancies existed in the grouping of strains of genomic species 8, 9 and BJ17. The phylogenetic tree for these strains was reconstructed from the sequence of *rpoD*, the structural gene for the RNA polymerase σ^{70} factor. The latter tree was 100% congruent with the grouping based on DNA–DNA hybridization. The reliability of DNA–DNA hybridization may be superior to that of sequence comparison of a single protein-encoding gene in resolving closely related organisms since the former method measures the homologies between the nucleotide sequences of total genomic DNAs. Three strains that have not been characterized previously by DNA–DNA hybridization seem to belong to two new genomic species, one including strain ATCC 33308 and the other including strains ATCC 31012 and MBIC 1332.

Keywords: *gyrB*, *rpoD*, *Acinetobacter*, genomic species, DNA–DNA hybridization

INTRODUCTION

The genomic species is one of the major concepts of a bacterial species and is based on quantitative similarities between chromosomal DNAs of bacteria (DNA reassociation values) as determined by DNA–DNA hybridization (Grimont, 1988; Stackebrandt &

Liesack, 1993). When denatured DNAs from two organisms are incubated together under appropriate conditions, a hybrid DNA duplex is formed and the degree of nucleotide sequence similarity can be quantified by the thermal stability of the this duplex. DNA–DNA hybridization is one of the recommended standards for delineating bacterial species and a genomic species is defined as a group of bacterial strains that have DNA–DNA reassociation values of approximately 70% or more with a difference in thermal stability (ΔT_m) of 5 °C or less (Johnson, 1973; Steigerwalt *et al.*, 1976). A genomic species can be defined as a species only when it has phenotypic traits that distinguish it from its nearest neighbour genomic

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Abbreviation: AFLP, amplified fragment length polymorphism.

The GSDB, DDBJ, EMBL and NCBI accession numbers for the sequences reported in this paper are listed in Table 1.

Table 1. *Acinetobacter* strains used in this study

Strain designation in this study	Origin of strain*	Source	Accession no.	
			<i>gyrB</i>	<i>rpoD</i>
Genomic species 1 (<i>Acinetobacter calcoaceticus</i>)				
1 ^T	CIP 81.08 ^T , from ATCC 23055 ^T (= B46 ^T)	Soil	AB008685	
2	CIP 66.32, from H. Girard (= ATCC 17902 = B90)	Soil	AB008702	
3	CIP 66.33, from L. Rey HO-1 (= ATCC 14987 = B61)	Soil	AB008701	
Genomic species 2 (<i>Acinetobacter baumannii</i>)				
4 ^T	CIP 70.34 ^T , from ATCC 19606 ^T	Urine	AB008684	
5	CIP 70.32, from ATCC 17961 (= B55)	Blood	AB008699	
9	CIP 70.22, from ATCC 17945		AB008700	
Genomic species 3				
25	CIP 70.29, from ATCC 19004	Cerebrospinal fluid	AB008686	
26	CIP 70.15, from ATCC 17922		AB008703	
28	M. M. Adam 202 OKI 55732	Hospital environment	AB008704	
Genomic species 4 (<i>Acinetobacter haemolyticus</i>)				
32 ^T	CIP 64.3 ^T , from W. Mannheim 2446/60 ^T (= ATCC 17906 ^T = B40 ^T)	Sputum	AB008691	
38	CIP 64.4, from W. Mannheim 2181/60 (= ATCC 17907 = B44)	Pus	AB008724	
39	CIP 70.27, from ATCC 19194	Nose	AB008725	
Genomic species 5 (<i>Acinetobacter junii</i>)				
42 ^T	CIP 64.5 ^T , from W. Mannheim 2723/59 ^T (= ATCC 17908 ^T = B10 ^T)	Urine	AB008693	
43	SEIP 8.83	Blood	AB008726	
45	SEIP 14.81	Blood	AB008727	
Genomic species 6				
46	CIP A165 (= ATCC 17979)	Throat	AB008687	
47	M. M. Adam Ac 222 OKI 24697	Pus	AB008709	
Genomic species 7 (<i>Acinetobacter johnsonii</i>)				
48 ^T	CIP 64.6 ^T , from W. Mannheim 3865/60 ^T (= ATCC 17909 ^T = B8 ^T)	Duodenum	AB008692	
50	SEIP 4.81	Vaginal swab	AB008710	
54	SEIP 14.79	Mouse skin	AB008711	
Genomic species 8 (<i>Acinetobacter lwoffii</i>)				
60 ^T	CIP 64.10 ^T , from NCTC 5866 ^T (= ATCC 15309 ^T)		AB008694	AB008733
Genomic species 9				
62	CIP 70.31, from ATCC 9957 (= B25)	Gangrenous lesion	AB008688	AB008734
65	CIP A162 (= ATCC 17968)	Conjunctiva	AB008714	AB008735
66	CIP 70.19, from ATCC 17984		AB008715	AB008736
Genomic species 10				
69	CIP 70.12, from ATCC 17924		AB008689	
71	SEIP 17.81	Mouth	AB008717	
72	SEIP Ac 84.118	Urine	AB008718	
Genomic species 11				
73	CIP 63.46, from NCIB 8250 (= ATCC 11171 = B94)	Sewage	AB008690	
74	SEIP 12.79	Cow udder	AB008728	
75	SEIP 25.81	Human specimen	AB008729	
Genomic species 12 (<i>Acinetobacter radioresistens</i>)				
FO-1 ^T	(= ATCC 43998 ^T)	Cotton tampon	AB008732	
76	SEIP 12.81	Urine	AB008698	
77	M. M. Adam Ac 176 PmK 49	Hospital environment	AB008730	

Table 1 (cont.)

Strain designation in this study	Origin of strain*	Source	Accession no.	
			<i>gyrB</i>	<i>rpoD</i>
Genomic species BJ13				
82	CIP 64.2, from W. Mannheim P544/60 (= ATCC 17905 = B30). Type strain of ' <i>Achromobacter conjunctivae</i> '	Conjunctiva	AB008723	
552	SEIP Ac86.158	Skin	AB008712	
Genomic species BJ14				
382	K. Irino 105/85	Conjunctiva	AB008707	
743	R. E. Weaver CDC E655		AB008719	
Genomic species BJ15				
79	M. M. Adam Ac 606 180:40 va.	Skin	AB008721	AB008738
81	SEIP 23.78	Urine	AB008722	AB008739
Genomic species BJ16				
78	CIP 70.18, from ATCC 17988 (= B34)	Urine	AB008720	AB008737
673	SEIP Ac87.123	Urine	AB008716	AB008741
Genomic species BJ17				
942	SEIP Ac87.314	Leg ulceration	AB008731	AB008742
641	SEIP 2/87	Wound	AB008713	AB008740
Genomic species TU13				
37	CIP 70.11, from ATCC 17903		AB008706	
36	PB 377	Cerebrospinal fluid	AB008705	
45	PB 559	Sputum	AB008708	
Ungrouped strains				
	ATCC 31012	Tar on beach	AB008696	
	ATCC 33308	Soil	AB008695	
	MBIC 1332 (= T4)	Sea water	AB008697	

* ATCC, American Type Culture Collection, Manassas, VA, USA; B, University of California, Berkeley, CA, USA; CIP: Collection de l'Institut Pasteur, Paris, France; MBIC, Collection of the Marine Biotechnology Institute, Kamaishi, Japan; NCTC, National Collection of Type Cultures, London, UK; NCIMB, National Collection of Industrial Bacteria, Aberdeen, UK; SEIP, Service des Enterobactéries de l'Institut Pasteur, Paris, France; M. M. Adam, National Institute of Hygiene, Budapest, Hungary; PB: Collection of P. J. M. Bouvet, l'Institut Pasteur, Paris, France (Bouvet & Jeanjean, 1995); H. Girard, France; K. Irino, Brazil; W. Mannheim, Marburg, Germany; R. E. Weaver, CDC, Atlanta, GA, USA. Clinical specimens were isolated from humans unless indicated otherwise.

species (Wayne *et al.*, 1987). The similarity of small-subunit rRNA (16S rRNA) sequences is increasingly being used for the classification of bacteria (Fox *et al.*, 1992; Ochman & Wilson, 1987; Olsen *et al.*, 1994; Stackebrandt & Goebel, 1994; Woese, 1987). However, the resolution of 16S rRNA sequence analysis is insufficient to distinguish closely related genomic species because of the extremely slow rate of base substitution in 16S rDNAs (Yamamoto & Harayama, 1998) and cannot replace the DNA-DNA hybridization method (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). On the other hand, phylogenetic analysis based on protein-encoding genes provides a greater degree of resolution than that based on 16S rRNA genes since the former genes evolve faster than the latter (Ochman & Wilson, 1987; Yamamoto & Harayama, 1998). Various protein-encoding genes such as *recA*, *groEL*, *hsp75*, *rpoB*, *rpoD* and *gyrB* have been used for the classification of bacteria at the intragenomic level (Bustamante *et al.*, 1995; Haake *et*

al., 1997; Karlin *et al.*, 1995; Kullen *et al.*, 1997; Nowak & Kur, 1995; Pai *et al.*, 1997; Yamamoto & Harayama, 1996, 1998).

It has been reported that the grouping of *Acinetobacter* strains based on 16S rRNA sequence analysis, DNA-DNA hybridization analysis and phenotypic methods is inconsistent and therefore the establishment of reliable methods for unambiguous identification of these strains is quite urgent (Gerner-Smidt *et al.*, 1991; Ibrahim *et al.*, 1997; Rainey *et al.*, 1994). We are interested in examining the possibility that phylogenetic analysis using protein-encoding genes could be used for the identification of *Acinetobacter* strains and to provide information equivalent to that of DNA-DNA hybridization analysis. In this study, we examined this possibility using 49 *Acinetobacter* strains.

METHODS

Bacterial strains. The *Acinetobacter* strains used in this study are listed in Table 1. Currently, two different numbering

Table 2. PCR primers and sequence primers used in this study

Primer UP-1E was designed from the conserved regions of the amino acid sequences of GyrB proteins of *Escherichia coli* (SWISS-PROT code, GYRB-ECOLI), *Pseudomonas putida* (GYRB-PSEPU) and *Bacillus subtilis* (GYRB-BACSU), while primer APrU was designed from the conserved GyrB sequences among 15 *Acinetobacter* strains, including 12 genomic species (Yamamoto & Harayama, 1996). The universal sequence primers M13 reverse and M13(–21) were appended at the 5' ends of the degenerated sequences of PCR primers UP-1M and APrU, respectively. Direct sequencing of the PCR fragments was performed by using the universal sequencing primers M13 reverse or M13(–21). PCR primers and sequencing primers for *rpoD* were also designed from conserved regions of the amino acid sequences of RpoD proteins of *Buchnera aphidicola* (RP70_BUCAP), *Escherichia coli* (RP70_ECOLI), *Salmonella typhimurium* (RP70_SALTY) and *Pseudomonas aeruginosa* (RP70_PSEAE) (Yamamoto & Harayama, 1998). N, any base; R, A or G; S, C or G; Y, C or T; M, A or C.

Name	Target gene	Sequence (5'–3')	Length (bases)
UP-1E	<i>gyrB</i>	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	37
M13R		CAGGAAACAGCTATGACC	18
APrU	<i>gyrB</i>	TGTAACACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	38
M13(–21)		TGTAACACGACGGCCAGT	18
70F	<i>rpoD</i>	ACGACTGACCCGGTACGCATGTAYATGMNGARATGGGNACNGT	44
70Fs		ACGACTGACCCGGTACGCATGTA	23
70R	<i>rpoD</i>	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT	44
70Rs		ATAGAAATAACCAGACGTAAGTT	23

systems are used for the identification of *Acinetobacter* genomic species and we have used genomic species numbers preceded by the initials of the authors who described the genomic species (i.e. BJ, Bouvet & Jeanjean; TU, Tjernberg & Ursing) following the numbering system of Ibrahim *et al.* (1997). The following 18 genomic species were examined in this study: 1–12 (Bouvet & Grimont, 1986; Nishimura *et al.*, 1988; Tjernberg & Ursing, 1989); BJ13 (synonym of TU14)–BJ17 (Bouvet & Jeanjean, 1995); and TU13 (Tjernberg & Ursing, 1989).

PCR amplification and sequencing of *gyrB* and *rpoD*.

Nucleotide sequences of *gyrB* genes were determined directly from PCR fragments amplified by using PCR primers UP-1E and APrU as described previously (Yamamoto & Harayama, 1995). The PCR primers and sequence primers used in this study are summarized in Table 2. Chromosomal DNAs from *Acinetobacter* strains used as PCR templates were prepared by the methods described by Brenner *et al.* (1982). PCR amplification of *gyrB* was performed with a DNA Thermal Cycler 480 (Perkin-Elmer) by using PCR reaction buffer (Perkin-Elmer) containing each of the deoxy-nucleoside triphosphates at a concentration of 200 µM, each of the primers at a concentration of 1 µM, 1 µg target DNA and 2.5 U *Taq* DNA polymerase (Perkin-Elmer) in a total volume of 100 µl. A total of 35 cycles of amplification was performed with template DNA denaturation at 94 °C for 1 min, primer annealing at 57 °C for 1 min and primer extension at 72 °C for 2 min. Amplified products were electrophoresed on 0.8% low-melting-temperature agarose gels (SeaPlaque GTG, FMC Bioproducts) and purified by using QIAquick (Qiagen) following the manufacturers' instructions. Sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing Kit and a 373A DNA Sequencer (Perkin-Elmer) according to the manufacturer's instructions.

The sequencing of *rpoD* (the structural gene for RNA polymerase σ^{70} factor) of 10 strains belonging to genomic species 8, 9, BJ15, BJ16 and BJ17 was also done by direct

sequencing of PCR fragments by using the PCR primers shown in Table 2 (Yamamoto & Harayama, 1998).

Data analysis. *GyrB* and *RpoD* sequences translated from *gyrB* and *rpoD* sequences, respectively, were aligned using the CLUSTAL W computer program (Thompson *et al.*, 1994). The *gyrB* and *rpoD* sequences were aligned according to the alignments of the *GyrB* and *RpoD* sequences, respectively. Phylogenetic trees were constructed with the PHYLIP computer program package (Felsenstein, 1989), using the neighbour-joining method (Saitou & Nei, 1987) with genetic distances computed by using Kimura's 2-parameter model (Kimura, 1980). Synonymous distances were obtained by applying the method of Nei & Gojobori (1986), using the MEGA computer program (Kumar *et al.*, 1993). The correction for multiple substitutions was made by the Jukes–Cantor formula (Jukes & Cantor, 1969).

RESULTS

Phylogenetic structure based on *gyrB* sequences

Use of PCR primers UP1-E and APrU generated 909 bp *gyrB* nucleotide sequences of the 49 *Acinetobacter* strains corresponding to nucleotide positions 316–1227 of the *Escherichia coli* K-12 sequence (Yamagishi *et al.*, 1986), including gaps. A phylogenetic tree constructed from these *gyrB* sequences by using the neighbour-joining method is shown in Fig. 1. The grouping of the strains examined in this tree was almost congruent with the genomic species determined by DNA–DNA hybridization studies (Bouvet & Jeanjean, 1989; Bouvet & Grimont, 1986; Tjernberg & Ursing, 1989). The genetic distances between the sequences within the same genomic species were mostly within 0.041 of the Kimura 2-parameter distances and within 0.183 of the synonymous distances (Jukes–

Fig. 1. Phylogenetic trees of 49 *Acinetobacter* strains reconstructed from the nucleotide sequences of *gyrB* genes by using the neighbour-joining method (Saitou & Nei, 1987). The genetic distances were computed by using Kimura's 2-parameter model (Kimura, 1980). The scale bar indicates a genetic distance of 0.05. The number shown next to each node indicates the percentage bootstrap value of 1000 replicates.

Cantor distance), as summarized in Table 3. The correlation between the *gyrB*-based genetic distances and the DNA reassociation values was good, particularly when the latter values were higher than 50%. It has been observed that DNA-DNA hybridization values are less quantitative when these values are 50% or less (Owen & Pitcher, 1985). Minor discrepancies existed between the *gyrB*-based phylogeny and that resolved by DNA-DNA hybridization studies: (i) genomic species 8 and 9 were not clearly separated by the *gyrB*-based analysis and (ii) the *gyrB*-based genetic distance between two members of genomic species BJ13 and that of genomic species BJ17 were much larger than 0.041 (Table 3). We then examined the 843 bp *rpoD* sequences of the strains belonging to genomic species 8, 9, BJ15, BJ16 and BJ17. The phylogenetic tree reconstructed from the *rpoD* sequences (Fig. 2) was congruent with the grouping

determined by DNA-DNA hybridization studies (Table 3).

Phylogenetic structure of the genus *Acinetobacter*

DNA-DNA hybridization data have indicated the linkage between genomic species 1 (*Acinetobacter calcoaceticus*), 2 (*Acinetobacter baumannii*), 3 and TU13. Using electrophoretic polymorphism of certain enzymes (Bouvet & Jeanjean, 1995) or tRNA spacer fingerprinting (Ehrenstein *et al.*, 1996), strains in this complex could not be differentiated. However, the *gyrB*-based tree correctly assigned the genomic species in this complex (Fig. 1).

The linkage of genomic species BJ15, BJ16 and BJ17 was previously suggested by Bouvet & Jeanjean (1989) from the results of DNA-DNA hybridization and also by Janssen *et al.* (1997) from the results of amplified

Table 3. Ranges of genetic distances within each genomic species

Evolutionary distances between *gyrB* and *rpoD* nucleotide sequences were obtained by applying Kimura's 2-parameter model (Kimura, 1980), using the MEGA computer program (Kumar *et al.*, 1993). The synonymous distances between *gyrB* and *rpoD* nucleotide sequences were obtained by applying the method of Nei & Gojobori (1986), using the MEGA computer program. The correction for multiple substitution was made by using the Jukes–Cantor formula (Jukes & Cantor, 1969). ND, Not determined.

Genomic species	<i>gyrB</i> distance		<i>rpoD</i> distance	
	Kimura model	Synonymous	Kimura model	Synonymous
1	0.006–0.019	0.020–0.076	ND	ND
2	0.000–0.018	0.000–0.081	ND	ND
3	0.000–0.001	0.000–0.005	ND	ND
4	0.010–0.013	0.034–0.102	ND	ND
5	0.011–0.024	0.044–0.103	ND	ND
6	0.012	0.054	ND	ND
7	0.025–0.041	0.101–0.183	ND	ND
9	0.000–0.110	0.000–0.393	0.005–0.027	0.022–0.103
10	0.002	0.005–0.010	ND	ND
11	0.002–0.006	0.005–0.019	ND	ND
12	0.001–0.018	0.000–0.072	ND	ND
BJ13	0.079	0.402	ND	ND
BJ14	0.029	0.134	ND	ND
BJ15	0.000	0.000	0.000	0.000
BJ16	0.021	0.096	0.007	0.028
BJ17	0.066	0.315	0.015	0.057
TU13	0.006–0.009	0.020–0.035	ND	ND

Fig. 2. Neighbour-joining tree of *Acinetobacter* strains that belong to genomic species 8, 9, BJ15, BJ16 and BJ17 (Table 1) reconstructed from the nucleotide sequences of *rpoD* genes. The genetic distances were computed by using Kimura's 2-parameter model (Kimura, 1980). The scale bar indicates a genetic distance of 0.1. The number shown next to each node indicates the percentage bootstrap value of 1000 replicates.

fragment length polymorphism (AFLP) fingerprinting. The clustering of these genomic species was confirmed in the *gyrB*-based tree, identifying genomic species 6 as the closest relative.

Genomic species 5, BJ13 (= TU14) and BJ14 formed a subgeneric cluster in the *gyrB*-based tree. The genetic distance between two strains in genomic species BJ13 was larger than the distances observed in other genomic species (Fig. 1). Recently, Janssen *et al.* (1997) provided some evidence that genomic species BJ13 is heterogeneous. Therefore, it may be necessary to analyse more strains in this genomic species to clarify actual genetic diversity in this taxon. Strains ATCC 31012 (Reisfeld *et al.*, 1972) and MBIC 1332 (Komukai-Nakamura *et al.*, 1996) were located in the group of genomic species 5, BJ13 and BJ14 and seem to constitute a new genomic species. Since both strains were isolated from marine environments, it may be of interest to examine whether or not this taxonomic group is constituted exclusively from marine *Acinetobacter* strains.

Strains of genomic species 4 (*Acinetobacter haemolyticus*) are isolated from clinical specimens and also from activated sludge (the ecology of genomic species 4 is not well understood). The *gyrB* sequences of three strains of genomic species 4 were not identical, but formed a cluster which was distinctly separated from any other genomic species. A similar observation was made for strains of genomic species 7 (*Acinetobacter johnsonii*).

Strain ATCC 33308 was used as the competent strain in Juni's transformation assay to determine whether or

not any tested strains belonged to the genus *Acinetobacter* (Juni, 1972). This strain was distantly related to all other strains examined. Thus, it could be a member of a new genomic species.

While the *gyrB*-based tree could not differentiate genomic species 8 and 9 (Fig. 1), the *rpoD*-based tree could (Fig. 2). Genomic species 8 and 9 are phenotypically indistinguishable, but have been proposed to be separated into two taxa by DNA–DNA hybridization (4). However, the ΔT_m values for genomic species 8 and 9 were in the range 4.5–6 °C and the unification of genomic species 8 and 9 has been proposed by several investigators (Janssen *et al.*, 1997; Tjernberg & Ursing, 1989). If the two genomic species are identical, it is not surprising that the *gyrB* analysis failed to differentiate them.

DISCUSSION

The genus *Acinetobacter* Brisou and Prévot 1954 is a group of Gram-negative, non-motile and non-fermentative bacteria commonly present in soil and water. Recent taxonomic studies have placed this genus within the γ -subclass of the *Proteobacteria* (Rainey *et al.*, 1994) and the genus has been resolved into seven valid species and at least 14 genomic species (DNA groups) by DNA–DNA hybridization studies (Bouvet & Jeanjean, 1989; Bouvet & Grimont, 1986; Gerner-Smidt & Tjernberg, 1993; Nishimura *et al.*, 1988; Tjernberg & Ursing, 1989). *Acinetobacter* is a normal inhabitant of the skin in healthy people and is an opportunistic pathogen involved in outbreaks of hospital infections (Bifulco *et al.*, 1989; Bouvet & Jeanjean, 1995; Seifert *et al.*, 1997; Towner, 1997; Towner *et al.*, 1991). The nosocomial infections of some *Acinetobacter* strains are difficult to treat because of resistance to β -lactams and aminoglycosides (Gerner-Smidt & Frederiksen, 1993; Joly *et al.*, 1990). The increasing pathogenic importance of *Acinetobacter* has stimulated the development of reliable typing methods for these strains. We have previously carried out the phylogenetic analysis of 15 *Acinetobacter* strains belonging to 12 different genomic species by using the nucleotide sequence of *gyrB* (Yamamoto & Harayama, 1996). In this study, we extended the *gyrB* analysis to 49 *Acinetobacter* strains covering 18 different genomic species.

When strain pairs exhibiting a *gyrB*-based genetic distance of less than 0.041 were grouped, the grouping mostly corresponded to genomic species determined by DNA–DNA hybridization. Therefore, it is likely that the 70% DNA reassociation value, the proposed limit of genomic species identity, corresponds to a genetic distance of 0.041 with *gyrB* sequences. The inconsistencies in the assignment of genomic species 8 and 9, and genomic species BJ13 by *gyrB* sequences could be due to the identity of genomic species 8 and 9 and to the heterogeneity of genomic species BJ13, respectively, as discussed earlier. However, since the *rpoD*-based classification could assign these genomic

species correctly, it is possible that *gyrB* sequences in some members of these genomic species were biased from the 'expected' sequences. Such a bias may simply be due to a fortuitous drift of mutation frequency, or may be provoked by non-conventional DNA rearrangements such as horizontal transfer of *gyrB*. If certain members of genomic species 8 and 9 possess hybrid genomes caused by horizontal DNA transfer of *gyrB*, the phylogenetic relationship of these strains deduced from *gyrB* could be different from that deduced from *rpoD*. The occurrence of a mosaic gene structure has been reported in many bacteria, including *Campylobacter jejuni* (Ehrenstein *et al.*, 1996), *Streptococcus pyogenes* (Kapur *et al.*, 1995), *Rhodobacter capsulatus* (Nikolskaya *et al.*, 1995) and *Mycobacterium tuberculosis* (Philipp *et al.*, 1996).

Thus, the reliability of DNA–DNA hybridization analysis may be superior to that of the sequence comparison of a single protein-encoding gene when applied to the classification of recently diverged strains since DNA–DNA hybridization measures the similarity between total genomic sequences. In other words, a measure of risk may accompany the analysis of single gene species in the identification of strains and the analysis of more than one gene species is desirable to increase the reliability in the classification. Nevertheless, sequence analysis of essential protein-encoding genes is effective for genomic species identification and extremely useful for the determination of branching orders in evolution that is difficult to achieve by using DNA–DNA hybridization.

Although DNA–DNA hybridization is a recommended method for the identification of genomic species (Murray *et al.*, 1990), this method is unpopular among scientists due to the fact that it requires the preparation of pure chromosomal DNAs from all strains to be examined and that the hybridization has to be conducted for all combinations of strains. Fingerprinting of PCR products is much easier to perform than DNA–DNA hybridization. Janssen *et al.* (1997) have reported the usefulness of the AFLP fingerprinting method in the identification of *Acinetobacter* strains. They examined classified strains representing 18 genomic species by AFLP and all the strains could be allocated correctly to the genomic species and all groups were properly separated. As we report here, the nucleotide sequence analysis of protein-encoding genes may also become a preferred method as the technologies for nucleotide sequencing and sequence analyses have advanced rapidly in recent decades. Furthermore, sequence data deposited in databases can be used for phylogenetic analysis on demand (Larsen *et al.*, 1993) and the accumulated sequence data can be used to design specific PCR primers useful for rapid identification of particular strains.

The *gyrB*-based phylogenetic structure was incongruent with that based on published 16S rRNA sequence analyses (Ibrahim *et al.*, 1997; Rainey *et al.*, 1994). Ibrahim *et al.* (1997) have reported linkages

between genomic species 1, BJ14, 10 and 11, and between genomic species 6, 8 and 9 in the 16S-rRNA-based phylogenetic tree. However, only the linkages between genomic species 10 and 11 and between genomic species 8 and 9 were recognized in the *gyrB*-based tree. Rainey *et al.* (1994) also reported the close relationships between genomic species 1, 4, 7 and 8 using 16S rRNA sequence analysis, but these relationships were neither recognized in the *gyrB*-based tree nor in the 16S-rRNA-based tree drawn by Ibrahim *et al.* (1997). We have observed the correlation between the phylogenetic tree of *Pseudomonas* strains based on the nucleotide sequences of 16S rRNAs and that based on protein-encoding genes (*gyrB* and *rpoD*) only when the sequences of non-variable regions of 16S rRNAs were used for the analysis (Yamamoto & Harayama, 1998). Both Rainey *et al.* (1994) and Ibrahim *et al.* (1997) have used 16S rRNA sequences, including the sequences of variable regions, for their phylogenetic analyses. This may be the cause of the inconsistency between their 16S-rRNA-based trees and our *gyrB*-based tree. The resolution of phylogenetic analysis based on the non-variable regions of 16S rRNAs seems insufficient to resolve the genomic species in *Acinetobacter*, but the use of the nucleotide sequences of protein-encoding genes such as *gyrB* or *rpoD* seems to be more suitable for the identification and delineation of these bacteria.

At present, approximately 1000 *gyrB* sequences have been deposited in the ICB database (<http://www.mbio.co.jp/>) and a program has been developed to align *GyrB* sequences in relation to their tertiary structures (K. Watanabe, personal communication). Therefore, the *gyrB* sequence may be one of the best suited to the identification of bacterial strains at the level of genomic species.

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