Discrimination of *Acinetobacter* Genomic Species by AFLP Fingerprinting

PAUL JANSSEN,¹ KEES MAQUELIN,² RENATA COOPMAN,¹ INGELA TJERNBERG,³
PHILIPPE BOUVET,⁴ KAREL KERSTERS,¹ AND LENIE DIJKSHOORN^{2*}

Laboratorium voor Microbiologie, Universiteit Gent, B-9000 Ghent, Belgium¹; Department of Medical Microbiology, Leiden University Medical Center, NL-2300 RC Leiden, The Netherlands²; Department of Medical Microbiology, Malmö University Hospital, University of Lund, S-205 02 Malmö, Sweden³; and Unité des Entérobactéries, Institut National de la Santé et de la Recherche Médicale, Institut Pasteur, F-75724 Paris Cedex 15, France⁴

AFLP is a novel genomic fingerprinting method based on the selective PCR amplification of restriction fragments. The usability of this method for the differentiation of genomic species in the genus *Acinetobacter* was investigated. A total of 151 classified strains (representing 18 genomic species, including type, reference, and field strains) and 8 unclassified strains were analyzed. By using a single set of restriction enzymes (*Hind*III and *Taq*I) and one particular set of selective PCR primers, all strains could be allocated to the correct genomic species and all groups were properly separated, with minimal intraspecific similarity levels ranging from 29 to 74%. Strains belonging to genomic species 8 (*Acinetobacter lwoffii* sensu stricto) and 9 grouped together in one cluster. The closely related DNA groups 1 (*Acinetobacter calcoaceticus*), 2 (*Acinetobacter baumannii*), 3, and 13TU (sensu Tjernberg & Ursing 1989) were clearly distinguishable, with intraspecific linkage levels above 50%. Strains of the independently described genomic species 13BJ (sensu Bouvet & Jeanjean 1989) and 14TU linked together at a relatively low level (33%). Although a previous DNA-DNA hybridization study seemed to justify the unification of these genomic species, AFLP analysis actually divides the 13BJ-14TU group into three well-separated subgroups. Finally, four unclassified strains obtained from diverse sources and origins grouped convincingly together, with a similarity linkage level of approximately 50%. These strains showed no similarities in their AFLP patterns with any of the other 155 strains studied and may represent a thus-far-undescribed *Acinetobacter* species. Based on these results, AFLP should be regarded as an important auxiliary method for the delineation of genomic species. Furthermore, because AFLP provides a detailed insight into the infraspecific structure of *Acinetobacter* taxa, the method also represents a highly effective means for the confirmation of strain identity in the epidemiology of acinetobacters.

The genus *Acinetobacter* was originally proposed more than 40 years ago by Brisou and Prévot (15) and is composed of nonmotile, gram-negative, saprophytic bacteria that are strictly aerobic, usually have a coccobacillary appearance, and show a fairly broad G+C range of 38 to 47 mol% in their genomic DNA (41, 46). The genus was placed originally within the family *Neisseriaceae* (41), but DNA-rRNA hybridization studies (58, 69) and phylogenetic analysis based on 16S rRNA sequences (74) clearly showed that acinetobacters in fact belong to the γ subclass of the class *Proteobacteria*, and subsequently, Rossau et al. (59) proposed to allocate the genus *Acinetobacter* to the family *Moraxellaceae*. This proposal has recently been supported by additional 16S rRNA sequence data (25).

Acinetobacters are widespread in nature (reviewed in reference 66). They are indigenous organisms of various types of soils and waters (1, 8, 23, 54) and are occasionally found in foodstocks (26, 28). Although they are normal inhabitants of human skin (50) and are capable of transitory colonization of the upper respiratory tract (57), *Acinetobacter* spp. are considered to be normally nonpathogenic to healthy people. However, acinetobacters have been implicated in nosocomial infections in debilitated individuals (3, 34) and patients in intensive-care units are especially at risk (2, 27, 29, 60). In addition, there is a growing concern about the persistence and epidemic

spread of these organisms in the hospital environment because the strains involved are often resistant to multiple antibiotics (4, 16, 20).

A number of extensive studies based on DNA-DNA hybridization (11, 12, 48, 65) resulted in the description of 18 DNA homology groups (DG; also called genomic species), seven of which have been named. In addition, a number of yet-un-grouped strains were described, implying the existence of additional genomic species (11, 12, 32). Although many of the recognized *Acinetobacter* species could be differentiated by phenotypic criteria obtained from biochemical tests (5, 6, 11–13, 44, 45, 64), by numerical analysis of cell envelope protein profiles (18), or by multilocus enzyme electrophoresis (14), accurate species delineation was in some cases difficult or impossible (19, 33). Elucidation of the ecology and pathology of the different genomic species of *Acinetobacter* requires methods for unambiguous identification of these groups. Unfortunately, the inadequacy of identification schemes based solely on phenotypic characteristics, including commercial identification systems, may lead to nomenclatural confusion (72).

Over the past few years, various molecular techniques have been employed for the phylogenetic analysis of the genus *Acinetobacter*, including PCR fingerprinting (73), amplified ribosomal DNA restriction analysis (ARDRA) (17, 67), analysis of amplified 16S to 23S ribosomal DNA intergenic spacer regions (22, 51), restriction analysis of amplified *recA* gene sequences (53), a multiplex PCR assay that simultaneously targets *RecA*- and 16S rRNA-encoding genes (52), tRNA fingerprinting (24), and DNA sequence analysis of genes encoding 16S rRNA (38, 56) or gyrase subunit B (75). However, most of these studies either included only strains of the first 12 genomic species

* Corresponding author. Mailing address: Department of Medical Microbiology, Leiden University Medical Center, L4-P, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands. Phone: 31-71-526 39 31. Fax: 31-71-524 81 48. E-mail: dijkshoorn@rullf2.medfac.leidenuniv.nl.

originally described in 1986 by Bouvet and Grimont (56, 75) or contained only the type and/or reference strain of each genomic species (51–53, 73). In addition, some of the techniques used in these studies failed to delineate all investigated genomic species, and strains belonging to the same species could not always be differentiated from each other.

Recently, a novel PCR-based DNA fingerprinting technique, AFLP (70), was evaluated as a tool in bacterial taxonomy (39) and various conditions for a wide range of microorganisms, including *Acinetobacter*, were assessed. In two other studies, this method was shown to be very useful for the epidemiological typing of *Acinetobacter* strains (21, 40). In the present study, in which the phylogenetic structure of the genus *Acinetobacter* was analyzed by using 159 reference and type strains that were previously characterized by DNA-DNA hybridization experiments, two main issues were addressed: (i) to what extent are AFLP fingerprinting data in agreement with the previously obtained DNA-DNA hybridization data and (ii) are all genomic species currently recognized in the genus *Acinetobacter* distinguishable by a single method, AFLP?

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains studied are listed in Table 1. The collection comprised type and reference strains originally obtained from public culture collections and strains collected from clinical and environmental sources. Some strains were kind gifts from P. Kämpfer, Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, Giessen, Germany, and P. Gerner-Smidt, Statens Seruminstitut, Copenhagen, Denmark. All strains have been identified to the genomic species level by DNA-DNA hybridization analysis and were (with few exceptions) used in previous studies (10–12, 18, 32, 48, 65). Five strains, CIP 64.2 (from DG 13BJ [sensu Bouvet & Jeanjean 1989]), K. Irino 105/85 (from DG 14BJ), CCUG 14816 (from DG 14BJ), CIP 70.18 (from DG 16), and SEIP Ac 87.314 (from DG 17) (listed in Table 1 as LUH 1717, LUH 1726, LUH 1727, LUH 1731, and LUH 1736, respectively), were received from two different sources and were included in duplicate (received as ATCC 17905, Malmoe 99681, Malmoe 99871, and Malmoe 13693 from the collection of one of the authors [J.T.] and as SEIP Ac 87.314, obtained from P. Kämpfer, respectively; duplicate strains are not listed in Table 1). For DNA preparation, bacterial cultures were grown aerobically on nutrient agar containing nutrient broth (Gibco BRL) supplemented with 1.5% (wt/vol) Bacto Agar (Difco) at 28°C. Strains were stored at –70°C as grown cultures, to which 10% glycerol was added prior to freezing.

Isolation of chromosomal DNA. *Acinetobacter* DNAs were prepared by the method of Pitcher et al. (55) with minor modifications. The entire procedure for the extraction and purification of *Acinetobacter* DNAs has been reported elsewhere (39). Prepared DNAs were run on a 1% agarose gel in the presence of ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) to check for integrity and were stored at –20°C.

Preparation of template DNA for PCR amplification. Digestion of genomic DNAs with the restriction endonucleases *Hind*III and *Taq*I and subsequent ligation of restriction half-site-specific adaptors to all restriction fragments was done as outlined previously (39). Adaptor design has been described in detail by Zabeau and Vos (76) and Vos et al. (70). The *Hind*III and *Taq*I half-site-specific adaptors consisted of equimolar mixtures of the partly complementary oligonucleotides 5'-CTCGTAGACTGCGTACC-3' and 5'-AGCTGGTACGCGATC-3', and 5'-GACGATGAGTCCGAC-3' and 5'-CGGTCAGGACTCAT-3', respectively. All DNA templates were stored at –20°C.

AFLP reactions. Selective amplification of restriction fragments was achieved by the use of primers H01 (5'-GACTGCGTACCAGCTTa-3') and T05 (5'-GATGAGTCCTGACCGAaa-3'), which have one or two adenines as 3' extensions (indicated in lowercase letters), respectively. The notation of the primers is according to Janssen et al. (39). Amplification reactions, as well as the 5' end labeling of the H01 primer with ^{32}P , were done as described by Zabeau and Vos (76) with some modifications (39).

Data acquisition. All procedures and equipment have been reported elsewhere (39). Briefly, samples of the amplification reaction mixtures were loaded on a 5% denaturing polyacrylamide gel and PCR-amplified products were separated electrophoretically. Following electrophoresis, gels were dried and exposed to a 35-by-43-cm Hyperfilm-MP film (Amersham). Autoradiograms were scanned by a high-resolution densitoscanner, and images were maintained as TIFF files. Raw data were further processed with GelCompar version 3.1 software (Applied Maths b.v.b.a., Kortrijk, Belgium).

Numerical analysis of banding patterns. Digital images were normalized as outlined by Janssen et al. (39). The *Acinetobacter calcoaceticus* type strain, ATCC 23055 (Table 1), was used as the reference strain because AFLP analysis of this strain (under the conditions given above) generated a banding pattern displaying evenly distributed and well-separated bands over the entire length of the gel.

From each reference track, the same 26 bands were used for alignment. All results presented in this study were obtained by combining a total of eight polyacrylamide gels. The similarity between each pair of AFLP patterns was calculated by using the Pearson product moment correlation coefficient (r). Strains were clustered by using the unweighted-pair group method with arithmetic averages (UPGMA) (62). Using GelCompar, we calculated a standard deviation for every branch of the dendrogram. These standard deviations are indications of the significance and stability of the formed clusters and are given as $\pm$ values in Table 2 or when linkage levels are discussed in the text.

RESULTS AND DISCUSSION

Reproducibility of AFLP with *Acinetobacter* genomic DNAs.

Small variations in background intensities and slightly deviating electrophoretic conditions may introduce minimal differences in the correlation values between reference tracks (obtained from strain ATCC 23055^T; see Materials and Methods). However, following normalization and subtraction of background values, the similarity levels of banding patterns obtained from multiple specimens of strain ATCC 23055^T, included in several lanes of each gel to allow inter- and intragel comparison, were at least 90 and 95%, respectively (results not shown). The same similarity level (>90%) was obtained between duplicate strains analyzed on different gels (not shown). In this report as well as in previous studies (36, 39) these intra- and intergel-specific correlation values between reference samples or between duplicate strains were considered threshold values for the reproducible discrimination of genomic species by cluster analysis of AFLP patterns.

Correlation of AFLP results with existing taxonomic data.

(i) Identification of *Acinetobacter* genomic species by AFLP. Strains that belong to the same genomic species (or DG) always grouped together in one particular AFLP cluster (Fig. 1). Each AFLP cluster could be outlined as a set of strains that showed an average linkage level ranging from 29 to 74%, with most of the actual values around 45% (Fig. 1 and Table 2). Genomic species DG 11, DG 14BJ, and DG 17BJ showed a relatively low linkage level of about (38.1 ± 4.4), (40.6 ± 6.3), and (42.4 ± 0.0)%, respectively. The exceptionally low linkage level of *A. johnsonii* (DG 7) strains ($[29.4 \pm 5.5]\%$) was mainly due to the inclusion of a strain isolated from activated sludge (10) (see below). Exclusion of this strain would result in a linkage level for this group of (44.7 ± 5.5)% (Table 2). On the whole, all *Acinetobacter* genomic species that are currently recognized as distinct taxa (excluding DG 8-9 and DG 13BJ-14TU) were easily distinguished by AFLP (Fig. 1).

(ii) Interspecific phylogenetic relationships in the genus *Acinetobacter*. The results obtained from three independent DNA-DNA hybridization studies (11, 12, 65), including either reciprocal measurements between reference strains or measurements between a particular reference strain and heterologous strains from other genomic species, suggested close relationships among some of the genomic species. In particular, this appeared to be the case for (i) genomic species 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU (sensu Tjernberg & Ursing 1989); (ii) genomic species 4 (*A. haemolyticus*) and 6; (iii) genomic species 10 and 11; (iv) genomic species 8-9 (*A. lwoffii*) and 15TU; and (v) genomic species 15BJ, 16BJ, and 17BJ. The linkages among these genomic species could be fully corroborated by extensive phenotypical studies (5, 44) and, to a lesser extent, by cell envelope protein profiling (18), chemotaxonomic analysis (43), restriction fragment length polymorphism analysis of rRNA-encoding genes by using ARDRA (67), and DNA sequence analysis of 16S rRNA- and GyrB-encoding genes (38, 75). In contrast, although AFLP fingerprinting groups the highly related genomic species 1, 2, 3, and 13TU together in one cluster, it does so only at a very low linkage level (22%), and this cluster also contains *A. junii* (DG

TABLE 1. Strains used^a

Strain	Designation as received and/or other designations	Source
Genomic species 1 (<i>A. calcoaceticus</i>)		
RUH 2201 ^{Tb}	ATCC 23055 ^{T,c} , 1 ^d	Soil
RUH 54 ^b	LMD 22.17, 2 ^d	Soil
RUH 584 ^b	4 ^d	Soil
RUH 944 ^b	5 ^d	Intravenous catheter
RUH 582 ^b	6 ^d	Soil
RUH 2202 ^b	42 ^c , 7 ^d	Wound
RUH 2203	132 ^c , 8 ^d	Wound
Genomic species 2 (<i>A. baumannii</i>)		
RUH 2207 ^b	91 ^c , 9 ^d	Sputum
RUH 112	10 ^d	Wound
RUH 88	11 ^d	Wound
RUH 1147	12 ^d	Toe web
RUH 510	13 ^d	Bronchus
RUH 1052	LMD 82.54, 14 ^d	Not known
RUH 1063	NCTC 7844, 15 ^d	Not known
RUH 557	16 ^d	Urine
RUH 48	17 ^d	Wound
RUH 96	18 ^d	Urine
RUH 413	19 ^d	Pus
RUH 336	20 ^d	Sputum
RUH 2208	144 ^c , 21 ^d	Wound
RUH 871	22 ^d	Urine
RUH 230	23 ^d	Wound
RUH 872	24 ^d	Urine
RUH 2209 ^b	ATCC 17904 ^c , 25 ^d	Urine
RUH 1019	26 ^d	Wound
RUH 213	27 ^d	Sputum
RUH 882	28 ^d	Urine
RUH 436	29 ^d	Sputum
RUH 523 ^b	30 ^d	Urine
RUH 134	31 ^d	Urine
RUH 215	32 ^d	Blood
RUH 875 ^b	33 ^d	Urine
RUH 150	34 ^d	Urine
RUH 3023 ^{Tb}	CCUG 19096 ^{T,c} , ATCC 19606 ^T , 35 ^d	Urine
RUH 1093	36 ^d	Sputum
RUH 332	37 ^d	Blood
RUH 210	38 ^d	Sputum
RUH 128	39 ^d	Sputum
RUH 97	40 ^d	Sputum
RUH 447	41 ^d	Catheter tip
RUH 414 ^b	42 ^d	Ear
Genomic species 3		
RUH 2204 ^b	102 ^c , 43 ^d	Wound
RUH 502	44 ^d	Drain
RUH 1163 ^b	45 ^d	Toe web
RUH 2205	162 ^c , 46 ^d	Wound
RUH 1020 ^b	47 ^d	Bronchus
RUH 509 ^b	49 ^d	Bronchus
RUH 37	50 ^d	Blood
RUH 408	51 ^d	Not known
RUH 468 ^b	52 ^d	Urine
RUH 56	LMD 79.41, 53 ^d	Not known
RUH 532	54 ^d	Urine
RUH 2206 ^b	ATCC 19004 ^c , 55 ^d	Cerebrospinal fluid
Genomic species 4 (<i>A. haemolyticus</i>)		
RUH 2213	197 ^c , 56 ^d	Wound
RUH 415	57 ^d	Pus
RUH 55	LMD 70.9, 58 ^d	Not known
RUH 406	Gilardi 2890, 59 ^d	Not known
RUH 2215 ^T	ATCC 17906 ^{T,c} , 60 ^d	Sputum
RUH 44	61 ^d	Air
RUH 2214	61 ^c , 62 ^d	Not known

TABLE 1—Continued

Strain	Designation as received and/or other designations	Source
Genomic species 5 (<i>A. junii</i>)		
RUH 2228 ^T	ATCC 17908 ^{T,c} , 63 ^d	Urine
RUH 204	64 ^d	Blood
RUH 383	65 ^d	Not known
RUH 2229	124 ^c , 66 ^d	Surgical gown
RUH 2230	178 ^c , 67 ^d	Water
Genomic species 6		
RUH 2867	ATCC 17979 ^c , 68 ^d	Throat
LUH 286	MGH 97923, 39 ^c	Urine
Genomic species 7 (<i>A. johnsonii</i>)		
RUH 2231 ^T	ATCC 17909 ^{T,c} , 69 ^d	Gut
RUH 2233	92 ^c , 70 ^d	Urine
RUH 2855	68 ^c , 71 ^d	Urine
RUH 2856	97 ^c , 72 ^d	Urine
RUH 2857	112 ^c , 73 ^d	Urine
RUH 2858	134 ^c , 74 ^d	Urine
RUH 2232	153 ^c , 75 ^d	Feces
RUH 2859	137 ^c , 76 ^d	Urine
LUH 540	LMD 90.19, 210A ^f	Activated sludge
Genomic species 8-9 (<i>A. lwoffii</i>)		
RUH 2219 ^{Tb}	NCTC 5866 ^{T,c} , 77 ^d	Not known
RUH 551	78 ^d	Not known
RUH 45	79 ^d	Blood
RUH 2221 ^b	44 ^c , 80 ^d	Prostate secretion
RUH 548	81 ^d	Eye
RUH 74 ^b	82 ^d	Not known
RUH 302	83 ^d	Skin
RUH 1104 ^b	84 ^d	Skin
RUH 1115 ^b	85 ^d	Skin
RUH 549	86 ^d	Urine
RUH 303	87 ^d	Door
RUH 709	88 ^d	Wound
RUH 2220	202 ^c , 89 ^d	Urine
LUH 1710 ^b	62 ^c , CIP 70.31, ATCC 9957 ^c	Gangrenous lesion
LUH 1711 ^b	63 ^c , SEIP Ac84.203	Chicken
LUH 1712 ^b	64 ^c , SEIP 14.84	Sperm culture
LUH 1713 ^b	65 ^c , CIP A162, ATCC 17968 ^c	Conjunctivitis
LUH 1714 ^b	66 ^c , CIP 70.19, ATCC 17984	Not known
LUH 1715	67 ^c , SEIP 4.83	Pus
Genomic species 10		
RUH 2222	198 ^c , 90 ^d	Urine
RUH 2223	113:2 ^c , 91 ^d	Wound
RUH 2224	ATCC 17924 ^c , 92 ^d	Not known
Genomic species 11		
RUH 2234	174 ^c , 93 ^d	Contact lens
RUH 2861	CIP 63.46 ^c , ATCC 11171, 94 ^d	Not known
RUH 1050	LMD 81.109, 95 ^d	Not known
RUH 2236	51 ^c , 96 ^d	Urine
RUH 2235	210 ^c , 97 ^d	Wound
RUH 2860	58b ^c , 98 ^d	Wound
LUH 287	MGH 97931, 225 ^e	Sputum
Genomic species 12 (<i>A. radioresistens</i>)		
RUH 2225	109 ^c , 99 ^d	Urine
RUH 2864	SEIP 12.81 ^c , 100 ^d	Urine
RUH 2226	152 ^c , 101 ^d	Wound
RUH 2862	26 ^c , 102 ^d	Sputum
RUH 2865 ^T	FO-1 ^{T,c} , IAM 13186 ^T , 103 ^d	Cotton
RUH 2863	73 ^c , 104 ^d	Wound
RUH 2227	50 ^c , 105 ^d	Urine
RUH 3517		Feather pillow

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TABLE 1—Continued

Strain	Designation as received and/or other designations	Source
Genomic species 13BJ		
LUH 1717 ^b	82 ^h , CIP 64.2, ATCC 17905, B30 (also known as the type strain of <i>Achromobacter conjunctivae</i>)	Conjunctiva
LUH 1718 ^b	134 ^h , SEIP 5.84	Blood
LUH 1719 ^b	376 ^h , K. Irino 214/84	Cerebrospinal fluid
LUH 1720 ^b	496 ^h , SEIP Ac86.66	Skin
LUH 1722 ^b	943 ^h , SEIP Ac87.315	Unknown human origin
LUH 1723 ^b	1001 ^h , SEIP Ac87.348	Skin
LUH 1724 ^b	1158 ^h , SEIP Ac88.239	Skin
LUH 1725 ^b	1191 ^h , P. Osterrieth 190	Catheter
Genomic species 14TU		
RUH 2216 ^b	71 ^c , 117 ^d	Conjunctiva
RUH 2866 ^b	114 ^c , 118 ^d	Cerebrospinal fluid
RUH 2217 ^b	101 ^c , 119 ^d	Conjunctiva
RUH 2218 ^b	ATCC 17905 ^c , 120 ^d	Conjunctiva
RUH 3206 ^b	"from patient H" ⁿⁱ	Sputum
RUH 3207 ^b	"from patient I" ⁿⁱ	Sputum
RUH 3211 ^b	04/90 VI ⁱ	Respiratory flowmeter
Genomic species 13TU		
RUH 2210 ^b	ATCC 17903 ^c , 106 ^d	Not known
RUH 2624 ^b	107 ^d	Skin (forehead)
RUH 2285	108 ^d	Bronchus
RUH 2376 ^b	109 ^d	Sputum
RUH 503 ^b	110 ^d	Urine
RUH 2041	111 ^d	Post mortem
RUH 2284 ^b	112 ^d	Bronchus
RUH 2627 ^b	113 ^d	Rectum
RUH 412	114 ^d	Blood
RUH 2211	100 ^c , 115 ^d	Gastric fistula
RUH 2212	165 ^c , 116 ^d	Urine
Genomic species 14BJ		
LUH 1726	382 ^h , K. Irino 105/85	Conjunctiva
LUH 1727	513 ^h , CCUG 14816, Gilardi GLG 4638	Wound
LUH 1728	743 ^h , RE Weaver CDC E655	Not known
Genomic species 15BJ		
LUH 1729	79 ^h , M.M.Adam Ac606 180:40 va.	Skin
LUH 1730	81 ^h , SEIP 23.78	Urine
Genomic species 15TU		
LUH 1090	151a ^c , MGH 98795	Urine
LUH 1091	118 ^c , MGH 98794	Feces
Genomic species 16BJ		
LUH 1731	78 ^h , CIP 70.18, ATCC 17988	Urine
LUH 1732	673 ^h , SEIP Ac87.123	Urine
LUH 1734	1211 ^h , P. Osterrieth 312	Feces
Genomic species 17BJ		
LUH 1735	641 ^h , SEIP 2/87	Wound
LUH 1736	942 ^h , SEIP Ac87.314	Leg ulceration

Continued

TABLE 1—Continued

Strain	Designation as received and/or other designations	Source
Unclassified strains		
LUH 1737 ^b	80 ^h , SEIP 14.83	Blood
LUH 1740 ^b	930 ^h , SEIP Ac87.302	Tracheal exudate
LUH 1741 ^b	944 ^h , SEIP Ac87.316	Wound
LUH 1742 ^b	1240 ^h , RUH 65	Clinical specimen
LUH 1469 ^b	MGH 99613, 10095 ^j	Abscess
LUH 1470 ^b	MGH 99614, 10169 ^j	Sputum
LUH 1471 ^b	MGH 99896, 5804 ^j	Blood
LUH 1472 ^b	MGH 99685, 10090 ^j	Ulcer

^a LUH and RUH, collection L. Dijkshoorn, Leiden University Medical Center, Leiden, The Netherlands; ATCC, American Type Culture Collection, Rockville, Md.; LMD, LMD Culture Collection, University of Technology, Delft, The Netherlands; NCTC, National Collection of Type Cultures, London, United Kingdom; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Paris, France; SEIP, Service des Entérobactéries de l'Institut Pasteur, Paris, France; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; MGH, Collection of Malmoe General Hospital, Malmoe, Sweden.

^b AFLP patterns are given in Fig. 2 to 4 under the underlined designations.

^c Designation and strain used by Tjernberg and Ursing (65).

^d Serial number used by Dijkshoorn et al. (18).

^e Designation used by Gerner-Smidt et al. (33).

^f Designation used by Bonting et al. (10).

^g Designation used by Bouvet and Grimont (12).

^h Designation used by Bouvet and Jeanjean (11).

ⁱ Designation used by Horrevorts et al. (35).

^j Strain number used by Gerner-Smidt and Tjernberg (32).

5) (Fig. 1). Furthermore, although DGs 15BJ, 16BJ, and 17BJ can be found in close proximity, genomic species 4, 6, 10, and 11 are scattered throughout the AFLP dendrogram (Fig. 1). These findings indicate that the present AFLP methodology is of limited value in the determination of interspecific relationships in the genus *Acinetobacter*.

AFLP analysis of the Acb complex. Because interspecific DNA homology values suggested a close relationship between genomic species 1 (*A. calcoaceticus* sensu stricto), 2 (*A. baumannii*), 3, and 13TU (12, 65), and because biochemical tests were often inadequate for the proper discrimination of individual strains (5, 6, 13, 33), these species were lumped into one complex, also known as the *A. calcoaceticus*-*A. baumannii* (Acb) complex. Recently, four strains forming two new distinct groups were included in the Acb complex (32). Several authors have addressed the intricacy of this complex by using molecular typing methods, including restriction analysis of rRNA-encoding genes (22) and ribotyping (30), pulsed-field gel electrophoresis of large genomic restriction fragments (61), electrophoretic analysis of enzymes (14), repetitive extragenic palindromic sequence-based PCR (63), and tRNA spacer fingerprinting (24). In general, the use of these methods enabled the differentiation of DGs 1, 2, 3, and 13TU, although some methods failed to separate *A. calcoaceticus* (DG 1) from DG 3 or *A. baumannii* (DG 2) from DG 13TU (14, 24).

A total of 64 strains of the Acb complex with known DNA group affiliations were analyzed by AFLP fingerprinting. Strains representing *A. calcoaceticus*, *A. baumannii*, and the genomic species 3 and 13TU clearly grouped in four separate AFLP clusters at an intraspecific similarity of 50 to 60% (Fig. 1 and Table 2). Four strains (designated LUH 1469 to 1472 [Table 1]), which genotypically belong to the Acb complex but are distinct from the four other genomic species (32), grouped separately in two clusters, UG1 and UG2, of two strains each (Fig. 1 and 2). Pairwise grouping of these strains by AFLP analysis was in agreement with the observations made by

FIG. 1. Cluster analysis of 159 *Acinetobacter* strains with *Hind*III-*Taq*I-digested genomic DNAs. All amplification reactions were performed with the same primer set, H01-T05 (see Materials and Methods). AFLP patterns used for numerical analysis were derived from eight different gels. Genomic species are given as DGs. UG, ungrouped strains; *, deviating *A. johnsonii* strain (DG 7); *r*, Pearson product moment correlation coefficient. The dendrogram was constructed by using UPGMA.

Gerner-Smidt and Tjernberg (32), whose findings were based on DNA hybridization experiments. However, phylogenetic relationships that were reported between the two new groups (UG1 and UG2) and the rest of the *Acb* complex (22, 24, 32, 38) could not be demonstrated by AFLP.

AFLP analysis of *A. haemolyticus*, *A. junii*, and *A. johnsonii*. In 1986, Bouvet and Grimont (12) proposed to name genomic species 4, 5, and 7 *A. haemolyticus*, *A. junii*, and *A. johnsonii*, respectively.

(i) *A. haemolyticus*. Strains belonging to *A. haemolyticus* are occasionally isolated from patients and the hospital environment (12). Seven *A. haemolyticus* strains, including the reference strains RUH 2213 and RUH 2215^T (= ATCC 17906^T) (Table 1) from two independent DNA hybridization studies, were analyzed with AFLP and could be grouped together at a linkage level of (50.6 ± 3.2)% (Fig. 1 and Table 2).

(ii) *A. junii*. *A. junii* is predominantly found in clinical specimens (12) and has been associated with outbreaks in neonates (7, 68). Five *A. junii* strains were subjected to AFLP analysis and grouped together at a similarity level of (54.7 ± 7.5)% (Fig. 1 and Table 2). Although this group can be found as part of the *Acb* complex in the large dendrogram (Fig. 1), it is not possible to determine interspecific relationships by the current AFLP methodology and none of the previous taxonomic studies have indicated a particular relationship between *A. junii*

and members of the complex. The positioning of *A. junii* in the dendrogram (Fig. 1) should therefore be regarded as purely incidental.

(iii) *A. johnsonii*. *A. johnsonii* is occasionally isolated from the hospital environment (31), but its etiological role in human infection remains unclear. *A. johnsonii* has also been associated with biological phosphate removal in activated sludge systems (23), although the role of acinetobacters in these processes has recently been de-emphasized (9, 71). A total of nine *A. johnsonii* strains were analyzed by AFLP fingerprinting. Eight strains from clinical sources convincingly grouped together at (44.7 ± 5.5)% similarity, but the remaining strain, LUH 540 (= LMD 90.19 [Table 1]), only linked at (29.4 ± 5.5)% (Fig. 1 and Table 2). This strain was originally isolated from activated sludge as strain 210A and could be identified as *A. johnsonii* by phenotypic methods and DNA hybridization (5, 10). The AFLP banding pattern of this strain strongly deviated from those of the other *A. johnsonii* strains (Fig. 1; patterns not shown), in spite of the apparently high DNA homology with the *A. johnsonii* type strain ATCC 17909 (with a ΔT_m of only 1.5°C) (10).

AFLP analysis of *A. lwoffii*. Bouvet and Grimont (12) originally described the genomic species 8 and 9 as two distinct taxa. However, because the type strain of *A. lwoffii* fell into DG 8 and genomic species 8 and 9 could not be separated by phenotypic tests, *A. lwoffii* became synonymous with DG 8-9 (with DG 8 as *A. lwoffii* sensu stricto). The unification of DGs 8 and 9 was justified by additional DNA hybridization experiments

TABLE 2. Linkage levels of *Acinetobacter* DGs according to AFLP

DG ^a	Species name	No. of strains	Linkage level (%) ^b
1	<i>A. calcoaceticus</i>	7	52.5 ± 2.6
2	<i>A. baumannii</i>	34	57.4 ± 5.0
3		12	58.1 ± 6.2
4	<i>A. haemolyticus</i>	7	50.6 ± 3.2
5	<i>A. junii</i>	5	54.7 ± 7.5
6		2	55.6 ± 0.0
7	<i>A. johnsonii</i>	9	29.4 ± 5.5
	Excluding LUH 540	8	44.7 ± 5.5
8-9 ^c	<i>A. lwoffii</i>	13/6	51.7 ± 7.6
10		3	51.9 ± 0.9
11		7	38.1 ± 4.4
12	<i>A. radioresistens</i>	8	60.3 ± 6.6
13BJ		8	39.5 ± 6.2
14BJ		3	40.6 ± 6.3
15BJ		2	74.1 ± 0.0
16BJ		3	48.5 ± 5.9
17BJ		2	42.4 ± 0.0
13TU		11	60.5 ± 2.0
14TU		7	33.3 ± 1.9
15TU		2	50.6 ± 0.0
13BJ-14TU ^d		8/7	33.6 ± 5.8
UG1 ^e	LUH 1469, 1470	2	49.5 ± 0.0
UG2 ^e	LUH 1471, 1472	2	50.2 ± 0.0
UG3 ^f	LUH 1737, 1740-1742	4	50.8 ± 2.2

^a Genomic species (DGs) are numbered according to Bouvet and Grimont (12) (no suffix), Bouvet and Jeanjean (11) (suffix BJ), or Tjernberg and Ursing (65) (suffix TU).

^b Average linkage levels of genomic species are expressed as the percent Pearson product moment correlation coefficient at the first branching point of each cluster and are indicated with the standard deviation as calculated by GelCompar.

^c DGs 8 and 9 form one species according to Tjernberg and Ursing (65).

^d Assuming the independently described DGs 13BJ and 14TU unite in one genomic species (5, 44, 65) (see text).

^e Isolates that belong to the *Acb* complex but are distinct from any of the four genomic species described within this complex (32).

^f Ungrouped strains described by Bouvet and Jeanjean (11).

FIG. 2. Chromosomal AFLP patterns of some of the *Acinetobacter* strains representing the six currently known genomic species within the *Acb* complex. *r*, product moment correlation coefficient; *, DNA hybridization reference strain. The dendrogram was constructed by using UPGMA.

(65). A total of 19 *A. lwoffii* strains were analyzed by AFLP. These strains formed a homogeneous group at a linkage level of $(51.7 \pm 7.6)\%$ similarity (Fig. 1), with AFLP patterns of NCTC 5866^T (DG 8) (65) and CIP A162 (DG 9) (12) nearly identical ($r > 0.9$) (Fig. 3a), illustrating the identity of the two DGs.

AFLP analysis of *A. radioresistens*. In 1979, an *acinetobacter* displaying elevated resistance against gamma rays was isolated from a cotton sample (42). This strain (FO-1) and other radioresistant *Acinetobacter* strains isolated from soil were characterized by phenotypic tests and DNA-pairing experiments (47, 48), leading to the proposal in 1988 of a new species, *A. radioresistens* (49), with the FO-1 strain as the type strain. The following year, based on DNA hybridization data, strains FO-1^T (41) and SEIP 12.81 (12) were grouped together with 30 clinical isolates in a single genomic species, DG 12 (65). AFLP analysis fully corroborated this finding by grouping strains FO-1^T, SEIP 12.81, and six of the 30 clinical *A. radioresistens* strains as a very homogeneous species displaying a high linkage level of $(60.3 \pm 6.6)\%$ similarity (Fig. 1).

AFLP analysis of *Acinetobacter* species without nomenclatural rank. (i) DGs 6, 10, and 11 *sensu* Bouvet & Grimont 1986. Besides DG 3, three other genomic species from the original Bouvet and Grimont study (12) remained unnamed. Genomic species 6 is difficult to distinguish phenotypically from *A. haemolyticus* (5, 44) except for the inability of DG 6 strains to metabolize 4-aminobutyrate (5, 12). Only two strains of DG 6 were available for AFLP analysis, which linked them at $(55.6 \pm 0.0)\%$ similarity. Their AFLP patterns did not show any resemblance to the banding patterns obtained for *A. hae-*

moliticus (with an interspecific similarity below 12%; AFLP patterns not shown). The genomic species 10 and 11 share a number of genotypical (12, 67, 75) and phenotypical (5, 44) features but clearly represent two separate taxa. This could be confirmed by AFLP analysis, which grouped 3 DG 10 and 10 DG 11 strains in two separate clusters, with linkage levels of $(51.9 \pm 0.9)\%$ and $(38.1 \pm 4.4)\%$ similarity, respectively (Fig. 1), and an interspecific linkage below 15%.

(ii) DGs 13BJ to 17BJ. In 1989, Bouvet and Jeanjean (11) characterized 27 proteolytic *Acinetobacter* strains that were phenotypically different from the 12 previously described *Acinetobacter* species (12). Twenty of these strains could be allocated to five distinct DNA hybridization groups (genomic species 13BJ to 17BJ), of which 15BJ, 16BJ, and 17BJ appeared to be more closely related to each other than to any other genomic species in the genus. All 27 strains were included in this study and subjected to AFLP fingerprinting. The 20 strains that represented the five DGs 13BJ to 17BJ formed five corresponding AFLP clusters at an average linkage level of approximately 40 to 74% (Fig. 1 and Table 2). The remaining seven strains could not be assigned to any of the currently described genomic species (not all results shown; see below). The species 13BJ appeared to be heterogeneous, because its reference strain, LUH 1717 (= CIP 64.2 = ATCC 17905), together with strain LUH 1719 (related to the reference strain by an AFLP-based correlation of 70.2%), were linked with the other 13BJ strains ($r = [74.1 \pm 4.7]\%$) at only $(39.5 \pm 6.2)\%$ (these linkage levels were found by a separate numerical analysis including only 13BJ strains [i.e., excluding 14TU strains]; dendrogram not shown; see below).

FIG. 3. (a) AFLP patterns of *Acinetobacter* strains belonging to genomic species 8 (*A. lwoffii* sensu stricto) (■) and 9 (□) form one homogeneous AFLP cluster. (b) AFLP patterns of four ungrouped *Acinetobacter* strains that were previously described by Bouvet and Jeanjean (11) and that may be part of a new genomic species. *, DNA hybridization reference strain. All strains are listed in Table 1. The scale represents the Pearson correlation coefficient (r). Dendrograms were constructed by using UPGMA.

(iii) **DGs 14TU to 15TU.** An independent DNA hybridization study with strain RUH 2216 as a reference (65) allocated the 13BJ reference strain, CIP 64.2 (= ATCC 17905) (see above), to DG 14TU. This finding, which could be fully corroborated by phenotypic results (5, 44), suggested that DGs 13BJ and 14TU may represent the same genomic species. We analyzed seven strains of DG 14TU by AFLP fingerprinting and found that five of the seven strains, including reference strain RUH 2216 (Table 1), linked tightly together at $(87.3 \pm 1.5)\%$ similarity (dendrogram not shown). However, the remaining two strains, RUH 2217 and RUH 2218 (= ATCC 17905), linked with the other five strains at only $(33.3 \pm 1.9)\%$ and $(35.4 \pm 1.5)\%$, respectively. It is interesting to note that these two strains displayed a relatively low degree of DNA relatedness with the 14TU reference strain, RUH 2216 (65), and that the cell envelope protein profiles of strains RUH 2216 and RUH 2217, although similar, were clearly distinguishable from each other (18). When AFLP data obtained for DGs 13BJ and 14TU are combined in one dendrogram, three subgroups, A, B, and C, linking at similarity levels of $(69.5 \pm 0.6)\%$, $(87.3 \pm 1.5)\%$, and $(70.6 \pm 4.5)\%$, respectively, appear to exist within the 13BJ-14TU union (Fig. 4). Recently, nine strains of the 13BJ-14TU group were analyzed by tRNA spacer fingerprinting (24), but only one strain of the 14TU group, ATCC 17905, was included and the typing results obtained on these strains were inconsistent with their DG affiliations. Also, these data were not in agreement with the ABC subdivision of the 13BJ-14TU group as suggested by AFLP analysis. An earlier study with ARDRA (67) included only three 14TU strains and none of the originally described 13BJ strains, but a recent continuation of this work (17) eventually led to the analysis of all 13BJ and 14TU strains listed in Table 1. Intriguingly, these

new ARDRA data also seem to point out incongruities within the 13BJ-14TU group. Clearly, additional DNA hybridization data combined with a polyphasic approach using various genotypic and phenotypic methods is needed to unravel the precise structure of the 13BJ-14TU group. Genomic species 15TU was represented by only two strains (Table 1), which were linked by AFLP analysis at 50.6% similarity (Fig. 2).

(iv) **A novel *Acinetobacter* genomic species?** Seven of the 27 proteolytic *Acinetobacter* strains described in 1989 by Bouvet and Jeanjean (11) could not be characterized by DNA hybridization. These strains showed a 28 to 60% DNA relatedness with genomic species 13BJ to 17BJ (11). Unfortunately, further taxonomic information on these strains is scarce, although very recently the same seven strains were analyzed by ARDRA (17). AFLP analysis revealed that three of the seven strains remained ungrouped and did not link to any other *Acinetobacter* strain included in the present study (results not shown). The other four strains (LUH 1737 and LUH 1740 to 1742) (Table 1) convincingly grouped in a separate AFLP cluster, UG3, at $(50.8 \pm 2.2)\%$ similarity (Fig. 1). Their AFLP patterns are given in Fig. 3b. The overall data presented here and in previous reports (36, 39) on the ability of AFLP to discriminate genomic species strongly suggest that AFLP cluster UG3 represents a new genomic species in the genus *Acinetobacter*. This concept was recently further strengthened by the fact that all four strains displayed the same unique ARDRA profile (17). Nonetheless, additional DNA-DNA hybridization experiments must be performed to confirm these results.

Conclusions. The entire taxonomic structure of the genus *Acinetobacter* could be resolved by a single method, AFLP. By

FIG. 4. AFLP analysis of *Acinetobacter* strains belonging to the genomic species 13BJ (□) and 14TU (■). Strains displaying highly similar AFLP patterns ($r \geq 69\%$) could be found in three separate subclusters, A, B, and C. The scale represents the Pearson product moment correlation coefficient (r). *, DNA hybridization reference strain. The dendrogram was constructed by using UPGMA.

using this method, all genomic species currently known in the genus as distinct taxa were delineated correctly and individual strains within a species were easily distinguished, although interspecific relatedness could not be addressed by AFLP. Identification of acinetobacters at the genomic species level is often difficult and usually requires the determination of DNA homology values by DNA-DNA hybridization. These data, however, cannot be stored as reference entities for future comparative analyses. Consequently, each new *Acinetobacter* isolate that needs unambiguous identification at the genomic species level will require additional hybridization experiments. This is not the case for AFLP fingerprints, which after storage and processing by computer, can be used to establish identification libraries (37). In this context, AFLP should be seen as an ideal preliminary screening method for large numbers of isolates, with the ultimate confirmatory role reserved for DNA hybridization analysis, in which DNA-DNA pairing experiments can be more directed.

ACKNOWLEDGMENTS

This research was carried out in the framework of contract G.O.A. 91/96-2 with the Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek (Belgium). In addition, part of this research was funded by the Stichting Microbiologie Leiden.

Peter Gerner-Smidt and Peter Kämpfer are thanked for their generous provision of strains. We are indebted to Barbara van Harselaar for her dedicated review of strain specifications and gratefully thank Dirk Dewettinck for the meticulously prepared figures. Finally, we also acknowledge C.P.A. van Boven for his invaluable advice and continuous support.

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