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Identification of *Acinetobacter* Genomic Species by Amplified Ribosomal DNA Restriction Analysis

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A total of 53 field and reference strains, including the type strains of the seven named species (nomenspecies) and belonging to the 18 described genomic species (DNA groups) of the genus *Acinetobacter*, were studied by amplified ribosomal DNA restriction analysis (ARDRA). Restriction analysis with the enzymes *Alu*I, *Cfo*I, *Mbo*I, *Rsa*I, and *Msp*I of the enzymatically amplified 16S rRNA genes allowed us to identify all species except the genomic species 4 (*Acinetobacter haemolyticus*) and 7 (*A. johnsonii*), 5 (*A. junii*) and 17, and 10 and 11, which clustered pairwise in three respective groups. Further analysis with the enzyme *Hae*III, *Hin*fI, *Nci*I, *Scr*FI, or *Taq*I did not allow us to differentiate the species within these three clusters. However, use of a few additional simple phenotypic tests (hemolysis, growth at 37°C, production of acid from glucose, and gelatin hydrolysis) can be used to differentiate between the species within these clusters. ARDRA proved to be a rapid and reliable method for the identification of most of the *Acinetobacter* genomic species, including the closely related DNA groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13. The results of this study suggest that ARDRA can be used for the identification of *Acinetobacter* species and as such may help to elucidate the ecology and clinical significance of the different species of this genus. Since ARDRA uses universal 16S rRNA gene primers, it is expected to be applicable to the identification of most bacterial species. Furthermore, ARDRA is less prone to contamination problems than PCR for detection, since the use of cultured organisms results in a large initial quantity of target DNA.

Bacteria belonging to the genus *Acinetobacter* can be recognized as such since they are nonmotile, strictly aerobic, oxidase-negative, gram-negative, nonfermenting cocci or coccobacilli that grow well on common complex media (15, 18). By using DNA-DNA hybridization, the genus *Acinetobacter* has recently been shown to be made up of at least 18 genomic species (2, 4, 23), of which 7 have been named. In a first study, Bouvet and Grimont (2) described a total of 12 genomic species. Tjernberg and Ursing (23) found three additional genomic species, DNA groups 13, 14, and 15. Concurrently, Bouvet and Jeanjean (4) added five more species (genomic species 13 to 17) to the scheme of Bouvet and Grimont (2). Common use of reference strains allowed the conclusion that DNA group 13 sensu Bouvet & Jeanjean (4) corresponds to genomic species 14 sensu Tjernberg & Ursing (23) and that the species *Acinetobacter radioresistens*, which had been described independently (20), corresponds to genomic species 12. The taxonomy of the genus *Acinetobacter* has recently been reviewed (9, 13).

Numerous reports have commented on *Acinetobacter*-related hospital infections and outbreaks (1, 3, 6, 10–12, 17). Strains belonging to genomic species 2 (*A. baumannii*), 3, and 13 have been found most frequently to be associated with hospital infection and epidemic outbreaks (3, 6, 12). Recently, cases of septicemia and continuous ambulatory peritoneal dialysis-related peritonitis that were caused by strains of the genomic species 2, 5, 7, 8, and 10 have been reported (11). In general, the ecology and epidemiology of *Acinetobacter* species are not well understood, mainly due to the lack of practical and rapid methods to identify the isolates according to recent taxonomy.

Phenotypic identification schemes have been worked out (3, 13, 19), but these methods require up to 7 days of incubation and have been shown to be problematic in some cases (11, 22), especially in discriminating among the genetically highly related glucose-acidifying genomic species 1, 2, 3, and 13. Cell envelope protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis in combination with biochemical data can be used for presumptive identification (8) but requires thorough standardization. The problems of identification of *Acinetobacter* species have been addressed recently (26).

It has been shown previously that restriction analysis of enzymatically amplified 16S rRNA genes (16S ribosomal DNA [rDNA]) or amplified rDNA restriction analysis (ARDRA) can be used to identify the species of different genera (5, 14, 16, 24, 25). In the present study, we explored the suitability of ARDRA to identify 53 epidemiologically unrelated strains belonging to the 18 newly described genomic species of the genus *Acinetobacter*.

MATERIALS AND METHODS

Organisms. Fifty-three *Acinetobacter* strains, including field strains (mostly of clinical origin in The Netherlands and Sweden) and type and reference strains, were studied (Table 1). A detailed list with strain designations and their origins is available upon request. Strains were chosen to represent the 18 described genomic species. All strains had been identified previously to genomic species by DNA-DNA hybridization and were selected to represent different sources and geographical sites to include intraspecific genetic variability as much as

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TABLE 1. Overview of ARDRA patterns obtained for the *Acinetobacter* genomic species and strains studied

DNA group designation[a]				Species name	Strains studied[b]	ARDRA pattern with enzyme:									
BG	TU	BJ	This study			*Cfo*I[c]	*Alu*I[c]	*Mbo*I[c]	*Rsa*I[d]	*Msp*I[c]	*Hin*fI[c]	*Scr*FI[e]	*Hae*III[e]	*Taq*I[e]	*Nci*I[f]
1	1		1	*A. calcoaceticus*	ATCC 23055^T, LMD 22.17,[g] 42[h]	2	2	1	1	3	2	2	2	2	
					67[g]	3	2	1		3	2				
2	2		2	*A. baumannii*	ATCC 19606^T	1	1	1	2	1	1	1	1	1	
					9[g], 91[h], ATCC 17904	1	1	1		3	1				
3	3		3		ATCC 19004, 31[g], 102[h]	2	1	3	1	3	2	1	1	1	
4	4		4	*A. haemolyticus*	ATCC 17906^T, 38e, 39[g], 197[h]	1	4	1	2	2	2	2	2	2	1
5	5		5	*A. junii*	ATCC 17908^T, 53[g], 124[h], 178[h]	1	2	1	2	3	1	1	1	2	
6	6		6		ATCC 17979, MGH 97923	1	1	2	2	2	1	2	1	2	
7	7		7	*A. johnsonii*	ATCC 17909^T, 68[h], 92[h], 153[h]	1	4	1	2	2	2	2	2	2	1
8	8		8TU	*A. lwoffii*	ATCC 17986^T, 43[g], 44[h], 45[g]	3	3	2	1	2	2	2	3	1	1
9	8[i]		8TU		ATCC 9957	3	3	2	1	2	2	2	3	1	1
10	10		10		ATCC 17924, 113:2[h], 198[h]	4	2	1	2	3	2	1	2	1	2
11	11		11		ATCC 11171, 51[h], 174[h], 210[h]	4	2	1	2	3	2	1	2	1	2
12	12		12	*A. radioresistens*	IAM 13186^T, LD 3517	5	3	2		3	2				
					109[h]	5	3	2	2	1	2				
	13		13		ATCC 17903	2	1	1		1	2				
					65,[g] LD 2624	2	1	1	1	3	2				
	14	13	14TU		71,[g] LD 3206	1	4	1	2	3	2				
					ATCC 17905	1	4	1		2+3	1+2				
	15		15TU		118,[h] 151a[h]	6	2	1	5	5	4				
		14	14BJ		MGH 99681, CCUG 14816	5	5	1	3	4	3				
		15	15BJ		Adam Ac 606	1	2	1	2	2	2				
		16	16		ATCC 17988	1	2	1	4	2	2				
		17	17		SEIP Ac 87.314	1	2	1	2	3	1				

[a] BG, Bouvet and Grimont (2); TU, Tjernberg and Ursing (23); BJ, Bouvet and Jeanjean (4).
[b] CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; LD, Collection of Lenie Dijkshoorn, University Hospital, Leiden, The Netherlands; LMD, Laboratory for Microbiology, Delft, The Netherlands; MGH, Collection of Malmö General Hospital, Malmö, Sweden; SEIP, Service des Entérobactéries de l'Institut Pasteur, Paris, France.
[c] All strains were tested.
[d] Only underlined and double-underlined strains were tested.
[e] Only underlined strains were tested.
[f] Only type or reference strains of genomic species 4, 7, 8TU, 10 and 11 were tested.
[g] Designations used by Dijkshoorn et al. (7, 8).
[h] Designations used by Dijkshoorn et al. (8) and Tjernberg and Ursing (23).
[i] Tjernberg and Ursing (23) omitted DNA group 9 from their nomenclature system, since they found that the reference strains of genomic species 8 and 9 according to Bouvet and Grimont (2) belonged to the same DNA homology group.

possible. In this study the genomic species are numbered according to their description by Bouvet and Grimont (2), Tjernberg and Ursing (23), and Bouvet and Jeanjean (4). However, when species numbering by these authors overlaps, we have chosen one of these numbers and added the postfix TU or BJ (Table 1).

ARDRA. The ARDRA method used has been described previously (24). Briefly, strains were grown on Mueller-Hinton agar II (BBL Microbiology Systems, Cockeysville, Md.). A 1-μl loopful of colony growth was suspended in 300 μl of distilled water, boiled for 10 min, agitated thoroughly, and centrifugated briefly in a microcentrifuge. With filter-protected tips, 5 μl of the supernatant was added to 45-μl aliquots of PCR mix containing 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Emeryville, Calif.), 100 μM (each) deoxynucleoside triphosphates (Pharmacia Biotech, Uppsala, Sweden), and 0.2 μM (each) primer in reaction buffer (1.5 mM $MgCl_2$ and 50 mM KCl in 10 mM Tris-HCl, pH 8.3), overlaid with 40 μl of mineral oil. After initial denaturation at 95°C for 5 min, the reaction mixture was run through 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min. Finally, a 7-min extension period at 72°C was carried out. Oligonucleotide primers were derived from conserved regions present at the edges of the 16S rDNA (21). The sequences of the primers were 5′TGGCTCAGATTGAA CGCTGGCGGC (5′ end of 16S rRNA gene) and 5′TACCTTGTTACGACTT CACCCCA (3′ end of 16S rRNA gene). The presence and yield of specific PCR products were controlled by agarose (1%, wt/vol)-ethidium bromide (50 ng/ml) gel electrophoresis for 30 min at 7 V/cm. The length of the amplicon was about 1,500 bp. The amplified DNA was used as such for restriction.

Restriction was carried out for 1 h at 37°C (except *Taq*I, which was incubated at 65°C) in 20-μl volumes of commercially supplied incubation buffer containing 5 U of restriction enzyme *Alu*I (AGCT), *Cfo*I (GCGC), *Hae*III (GGCC), *Hin*fI (GANTC), *Mbo*I (GATC), *Msp*I (CCGG), *Nci*I [CC(GC)GG], *Rsa*I (GTAC), *Scr*FI (CCNGG), or *Taq*I (TCGA) and 3 to 10 μl of PCR product. The volume of amplicon used in the restriction mixture was adjusted arbitrarily on the basis of visually observed fluorescence intensity of the amplified rDNA fragment in the control gel. Restriction was stopped by the addition of 5 μl of 5× sample buffer (glycerol, 25% [wt/vol], sodium dodecyl sulfate, 0.5% [wt/vol], EDTA, 50 mM; bromophenol blue, 0.05%). Restriction fragment patterns were analyzed by gel electrophoresis of 10 μl of each restriction mixture at 7 V/cm for 3 h in 3% (wt/vol) Metaphor agarose (FMC BioProducts, Rockland, Maine) in Tris (89 mM)-boric acid (89 mM)-EDTA (2 mM) electrophoresis buffer, pH 8.0. Gels contained 50 ng of ethidium bromide per ml.

Gels were photographed and patterns were compared visually. The gels were scanned with a document scanner, and calculation of molecular sizes of the fragments was done with GelCompar software (Applied Maths, Kortrijk, Belgium).

RESULTS

The 16S rRNA gene (16S rDNA) was enzymatically amplified for a total of 53 strains belonging to the 18 described genomic species of the genus *Acinetobacter*, and the amplicon

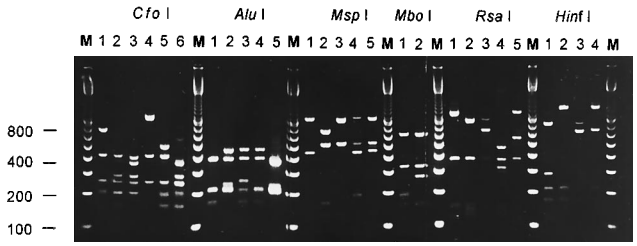

FIG. 1. Overview of the patterns obtained after restriction of the amplified 16S rRNA gene with six different enzymes. Lanes M, molecular size marker (100-bp ladder; Pharmacia Biotech). Molecular sizes are indicated on the left in base pairs. Numbers above each lane correspond to arbitrarily assigned ARDRA pattern numbers for each enzyme.

was restricted independently with a total of 10 different restriction enzymes. The amplified 16S rDNA of all strains was restricted with the enzymes *Alu*I, *Cfo*I, *Msp*I, *Mbo*I, and *Hin*fI, while only one or two strains of each genomic group were studied with *Rsa*I, only one strain of the genomic species 1 to 11 was studied with the enzymes *Hae*III, *Scr*FI, and *Taq*I, and only the type or reference strains of the genomic species 4, 7, 8TU, 10, and 11 were studied with *Nci*I.

The results are summarized in Table 1. Restriction patterns were numbered arbitrarily. Figure 1 gives an overview of all of the patterns (except *Mbo*I 3) observed. *Mbo*I patterns had 616-, 328-, and 160-bp fragments in common. Pattern *Mbo*I 1 was characterized by the presence of a 192-bp fragment; pattern *Mbo*I 2, by the presence of a 274-bp fragment; and pattern *Mbo*I 3, by the absence of fragments within the 328- to 160-bp region. Faint fragments on the gel (which are probably due to minor incomplete digestion) are due to overloading (which was done to ensure good visible fragments) and are not essential to differentiate between the different patterns. For the 18 genomic species studied, only a limited number of patterns were observed for each enzyme, with a maximum of six patterns for *Cfo*I (Fig. 1). Combination of the patterns obtained after separate restriction with *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, and *Msp*I enabled us to divide the 18 genomic species into 20 separate groups (designated rDNA groups), of which two were present within the species *A. calcoaceticus*, *A. baumannii*, and *A. radioresistens* and the genomic species 13 and 14TU. On the other hand, genomic species 4 and 7, 10 and 11, and 5 and 17 could not be differentiated from each other. Strains of *A. baumannii* (genomic species 2), the clinically most abundant species, could be identified by the combination of patterns *Cfo*I 1, *Alu*I 1, and *Mbo*I 1.

For one strain (ATCC 17905; genomic species 14TU), a double pattern was observed for the enzymes *Msp*I and *Hin*fI; i.e., the fragments observed for patterns *Msp*I 2 and *Msp*I 3 and *Hin*fI 1 and *Hin*fI 2, respectively, were present simultaneously (data not shown). Since this superposition of patterns also persisted after reisolation, the observation did not result from the presence of strains from two species in a single culture. Also, *A. calcoaceticus* 67 had an aberrant pattern (*Cfo*I 3 instead of *Cfo*I 2).

The reproducibility of ARDRA was high; i.e., different cultures, cell suspensions, amplifications, and restrictions of the same strain yielded identical restriction patterns (data not presented).

The intraspecific variability was low. Within each DNA group, all strains (except strains 67 and ATCC 17905), which had been selected to represent strongly varying sources (clinical and environmental) and different geographical origins, yielded identical patterns for all enzymes (except *Msp*I) tested.

Restriction with *Msp*I, which for the purpose of genomic species identification is needed only to differentiate DNA group 14TU from the genomic species 4 and 7, enabled us to divide the species *A. baumannii* and *A. radioresistens* and the genomic species 13 and 14TU into two rDNA groups each. For *A. baumannii*, this could be further substantiated by studying clinical strains from different hospitals in different countries. Using ARDRA, a total of 37 strains were identified as *A. baumannii* because of the combination of restriction patterns *Cfo*I 1, *Alu*I 1, and *Mbo*I 1, and it was found that 11 strains had *Msp*I pattern 1, while the other 26 strains had *Msp*I pattern 3 (data not presented).

DISCUSSION

Currently, most applications of PCR aim at the sensitive and specific detection of microbial pathogens directly from clinical samples. Although appealing, this approach is restricted mainly to pathogens that are difficult to cultivate, since for routine diagnostics, several PCRs would be needed for each specimen to cover the spectrum of possible pathogens. Also, susceptibility testing continues to rely on the Kirby-Bauer antibiogram, since genotypic susceptibility testing suffers from the same problems outlined for specific-detection PCR: the different resistance mechanisms are spread over the genome and have to be searched for with different primers (and PCRs). Since the Kirby-Bauer antibiogram depends on cultured organisms, culture remains necessary, even after successful detection of an organism with PCR. Nevertheless, application of PCR for the identification of cultured bacteria could offer an alternative in routine microbiology to current phenotypic identification, which has several shortcomings.

PCR fingerprinting techniques such as ARDRA and tRNA fingerprinting (27) are genotypic culture identification approaches which can be applied to the identification of most bacterial species and which are flexible since patterns of newly described species can be added directly to a database without the need for sequence information. These PCR fingerprinting techniques require only simple (single-step) DNA extraction procedures, are technically less demanding than most other molecular biology approaches, and are less prone to contamination problems, since one starts from cultures and thus from large amounts of target DNA. Pattern interpretation is simple and can be automatized by using the sophisticated electrophoresis apparatuses known as automated sequencers. In order to develop these techniques, pattern variation should be validated by the use of well-defined collections of reference strains.

Identification of acinetobacters according to the recent taxonomy is problematic (9, 13, 22, 27). In this study, we explored the possibility of amplification of the 16S rDNA followed by restriction analysis to identify 18 species of this genus. Results showed that strains of 12 of the 18 described genomic species could be identified unambiguously and that strains of the remaining 6 species could be allocated into three separate groups.

Strains within the *A. calcoaceticus-A. baumannii* complex, i.e., DNA groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13 (13), could easily be identified as separate groups. Strains of these groups are difficult to identify by phenotypic tests (11), and DNA hybridization has shown that these four DNA groups are genotypically more closely related to each other than to other DNA groups (23).

Bouvet and Grimont (2) originally described the genomic species 8 (*A. lwoffii*) and 9 as separate entities. However, Tjernberg and Ursing (23), also using DNA-DNA hybridiza-

TABLE 2. Phenotypic differentiation of genomic species for which ARDRA patterns are identical

Genomic species[a]	% of strains positive for[b]:			
	Gelatin hydrolysis	Hemolysis	Acid from glucose	Growth at 37°C
4 (*A. haemolyticus*)	90	95	76	100
7 (*A. johnsonii*)	0	0	0	0
5 (*A. junii*)	0	38[c]	0	100
17	100	100	0	100
10	0	0	100	100
11	0	0	0	0

[a] Number of strains tested: *A. haemolyticus*, 21; *A. johnsonii*, 20; *A. junii*, 21; genomic species 17, 1; genomic species 10, 3; genomic species 11, 7.
[b] According to Gerner-Smidt et al. (13), and Kämpfer et al. (19) for genomic species 17.
[c] Results obtained using sheep blood. With human blood, 62% of 21 strains were positive.

tion, showed that the reference strains of DNA groups 8 and 9 were highly related and therefore omitted genomic group 9 from their numbering system. Using 10 different enzymes, we could not find any differences between the ARDRA patterns of the four strains belonging to genomic species 8 and the one strain of genomic species 9 (sensu Bouvet & Grimont [2]).

ARDRA could not differentiate between genomic species 4 and 7, 5 and 17, or 10 and 11, although DNA homology between species within each of these pairs is less than 45%.

Strains belonging to DNA groups 10 and 11 are very similar in phenotypic tests (19) and in protein electrophoretic patterns (8). The ability of DNA group 10 strains to acidify glucose and to grow at 37°C can be used for differentiation from strains of DNA group 11 (Table 2).

In contrast, strains of *A. haemolyticus* (DNA group 4) and *A. johnsonii* (DNA group 7) have very few phenotypic characteristics in common and can easily be distinguished from each other phenotypically. However, they seem to have highly similar 16S rDNA sequences: 10 different restriction enzymes could not establish a single restriction site difference for the strains of each species tested.

Also, the four strains of species 5 (*A. junii*) and the single strain of genomic species 17 were indistinguishable according to ARDRA patterns obtained with six different restriction enzymes, while genomic species 17 is less than 22% related to *A. junii* according to DNA hybridization data (4).

However, when four simple phenotypic tests (gelatinase production, hemolysis, production of acid from glucose, and ability to grow at 37°C) are added to the ARDRA identification scheme, strains of DNA groups 4 and 7, 5 and 17, and 10 and 11 can be identified to the genomic species level (Table 2).

Thus, for some species there was little overall agreement between ARDRA grouping, i.e., partial 16S rRNA gene sequence information, and data from DNA-DNA hybridization, which is considered to be the gold standard for species delineation.

Strain ATCC 17905 was found to have double *Msp*I and *Hin*fI patterns. We have reported similar observations previously (25). This phenomenon may be explained by the fact that the multiple copies of the 16S rRNA gene, present within each bacterial genome, have undergone sequence divergence.

One of four *A. calcoaceticus* strains (67) had an aberrant *Cfo*I pattern (*Cfo*I 2 instead of *Cfo*I 3), while patterns obtained after restriction with four other enzymes were identical. Accordingly, results obtained by restriction with *Msp*I indicate that some other species can be subdivided into at least two rDNA groups. For example, a collection of 37 strains of clinical origin, identified with ARDRA as *A. baumannii*, could be separated into two groups according to *Msp*I patterns.

The reproducibility of ARDRA can be expected to be high on theoretical grounds, since the technique makes use of genetic information in a rather direct way. Because a well-conserved region is involved, intraspecific variability is expected to be low, and this is confirmed by this and earlier studies (5, 14, 16, 24). For each enzyme, only a few patterns were observed for the complete genus, also indicating that intraspecific variability can be expected to be low. The reproducibility of the technique is also enhanced by the ease of interpretation of the simple patterns, which are composed of only a few fragments.

The application of ARDRA for the identification of *Acinetobacter* species has several advantages over phenotypic identification. A major difficulty with phenotypic methods is that the use of high identification probabilities ($\geq$0.99) for the identification matrices results in a low number of identifiable strains (60%), while lower probability levels ($\geq$0.80) yield a large number (10%) of misidentifications (13). The applicability of commercial identification kits such as API 20E and API 20NE has been questioned (11). Also, commercially available identification systems which are based on assimilation tests are of little use for the identification of *Acinetobacter* species (11). In particular, discrimination between the genomic species 1, 2, 3, and 13 is difficult with phenotypic methods; therefore, it has been suggested that these four DNA groups be considered a single complex, named the *A. calcoaceticus-A. baumannii* complex (13). Moreover, some strains, such as the type strain (ATCC 23055^T) of *A. calcoaceticus*, the type strain of *A. radioresistens*, (IAM 13186^T), and the reference strain (ATCC 17903) of DNA group 13 are phenotypically highly atypical (13), whereas these strains are identified unambiguously by ARDRA patterns obtained after restriction with *Cfo*I, *Alu*I, and *Mbo*I.

ARDRA is a universally applicable method which can be used for the identification of virtually all bacterial species. The technique could also be used as a detection method, when at least one of each primer used is chosen to complement a 16S rDNA sequence specific for *Acinetobacter* species.

In summary, amplification of the 16S rDNA followed by independent restriction of the amplicon with five different restriction enzymes allows the recognition of 20 rDNA groups within the genus *Acinetobacter*. The four genomic species within the *A. baumannii-A. calcoaceticus* complex can easily be identified, and *A. baumannii*, the species most frequently encountered in clinical situations, can be recognized by restriction with three enzymes only and can be separated into two groups by restriction with the enzyme *Msp*I. On the other hand, strains of *A. haemolyticus* and *A. johnsonii*, DNA groups 10 and 11, and DNA groups 5 and 17 cannot be recognized as separate entities, but some simple phenotypic tests can be used to differentiate between these species. Finally, it was observed that some results obtained with ARDRA seem to be in discordance with taxonomic relationships based on DNA relatedness, and this requires further study. ARDRA may be a rapid and more practical alternative procedure for the identification of *Acinetobacter* species according to recent taxonomy and thus may contribute to a better understanding of the clinical importance, epidemiology, and the relative abundance of the different DNA groups (and rDNA groups) in different clinical and environmental samples.

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ADDENDUM IN PROOF

After this manuscript had been accepted, we were informed that a manuscript describing the application of tRNA fingerprinting to the identification of *Acinetobacter* species had been accepted for publication (M. Wiedmann-Al-Ahmad, H.-V. Tichy, and G. Schön, Appl. Environ. Microbiol. **60:**4066–4071, 1994).

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