JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1108–1113 Vol. 33, No. 5
0095-1137/95/$04.00+0

Identification of *Acinetobacter* Isolates in the *A. calcoaceticus-A. baumannii* Complex by Restriction Analysis of the 16S-23S rRNA Intergenic Spacer Sequences

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Received 29 August 1994/Returned for modification 29 November 1994/Accepted 26 January 1995

Members of the genus *Acinetobacter* are reported to be involved in hospital-acquired infections with an increasing frequency. However, clinical laboratories still lack simple methods that allow complete identification of some pathogenic species, i.e., those corresponding to *A. baumannii* (DNA group or genospecies 2), unnamed genospecies 3 and 13, and two new genospecies that have recently been described. In fact, a complete discrimination between these species is possible only by DNA-DNA hybridization or ribotyping. Both of these techniques are complex and time-consuming and cannot be performed in most clinical laboratories. As a consequence, isolates belonging to these genospecies are often not differentiated and included, together with the environmental genospecies 1, in the *A. calcoaceticus-A. baumannii* complex. In this report, a simple and rapid method for the identification of the genospecies belonging to the *A. calcoaceticus-A. baumannii* complex is proposed. It is based on the combined digestion by the restriction endonucleases *Alu*I and *Nde*II of the DNA fragments resulting from the amplification of the 16S-23S rRNA intergenic spacer sequences. The analysis of 36 strains characterized by DNA-DNA hybridization in previous studies showed that the restriction profiles obtained are highly reproducible and characteristic for each genospecies. Moreover, extending this study to 68 clinical strains, which were assigned to the *A. calcoaceticus-A. baumannii* complex by phenotypic tests, confirmed the existence of a panel of limited and well-conserved restriction patterns and allowed the identification of the strains tested. This study thus proposes the detection of restriction length polymorphism in the spacer sequences between the 16S and 23S rRNA genes as a method for the identification of isolates in the *A. calcoaceticus-A. baumannii* complex.

Members of the genus *Acinetobacter* are reported to be involved in hospital-acquired infections with an increasing frequency (2, 14, 17). However, the study of the pathology and epidemiology of these organisms has been hindered by the difficulties encountered in their classification and identification. The taxonomy of the genus *Acinetobacter* has undergone many changes in the last few years and is still confusing to some extent. The foundations of the present classification were laid in 1986, when Bouvet and Grimont (4) established the existence of 12 DNA groups or genospecies by DNA-DNA hybridization tests. Species names were assigned to only six of them (genospecies 1, 2, 4, 5, 7, and 8 were designated *A. calcoaceticus*, *A. baumannii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, and *A. lwoffii*, respectively), while the others were designated by group number. In the following years, other *Acinetobacter* species or DNA groups were added: *A. radioresistens*, which was described in 1988 (18) and which was later demonstrated to correspond to DNA group 12 (20); DNA groups 13 to 15, which were described by Tjernberg and Ursing (20) by using DNA-DNA hybridization; and five other proteolytic groups described by Bouvet and Jeanjean (6), with a numbering system superimposed onto that of Tjernberg and Ursing (20). Of the last two studies, only the DNA group 14 of Tjernberg and Ursing (20) corresponds to the group 13 of Bouvet and Jeanjean (6), so that, at the moment, different DNA groups have the same number (see reference 8 for a detailed account of the evolution of *Acinetobacter* classification).

Different sets of biochemical tests were proposed for the differentiation of bacterial species within the genus (5, 12, 15). However, members of some genospecies, i.e., those corresponding to DNA groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and Tjernberg and Ursing 13 are often so similar that they cannot be differentiated by phenotypic tests. For this reason, DNA groups 1, 2, 3, and 13 are referred to as the *A. calcoaceticus-A. baumannii* complex (12). Moreover, in a recent paper Gerner-Smidt and Tjernberg (11) described four isolates which genotypically clearly belonged to the *A. calcoaceticus-A. baumannii* complex, but were distinct from the four genospecies previously established. According to their results, the investigators proposed the establishment of two new genogroups, which at the moment contain two isolates each and are unnumbered. For practical reasons, they will be indicated N1 and N2 throughout this report.

Strains belonging to the *A. calcoaceticus-A. baumannii* complex can be assigned with certainty to one of the DNA groups only by DNA-DNA hybridization or, as a more recent alternative, by ribotyping (10, 12, 15). Both of these methods are complex and time-consuming; thus, simpler identification methods are very much needed. This would be of great help, for example, in tracing contamination sources in hospital-acquired infections. In fact, while *A. calcoaceticus* is considered an environmental species, *Acinetobacter* genospecies 2 (*A. baumannii*), 3, and 13 and the two new genospecies described by

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Gerner-Smidt and Tjernberg (11) are involved in hospital-acquired infections (5, 7, 11, 20).

A promising molecular approach to bacterial identification exploits the polymorphism within rRNA intergenic spacer sequences (1, 13). rRNA genes are generally present in the same order (i.e, 16S, 23S, and 5S) in their transcriptional units, and these units are present in most bacterial species at more than one copy per chromosome (19). In contrast to rRNA genes, which are remarkably well conserved throughout most bacterial species, both length and sequence variations characterize the spacer regions. Depending on the degree of these variations, the polymorphism can be used to differentiate bacteria at the genus, species (1, 13), or strain (9, 16) levels. The aim of the work described here was to explore the possibility of differentiating *Acinetobacter* isolates on the basis of a presumptive polymorphism in the 16S-23S rRNA intergenic spacer sequences.

MATERIALS AND METHODS

Bacterial strains. In the first part of the study, a group of reference strains identified by DNA-DNA hybridization (4, 11, 12) was used, including the type strains of 12 *Acinetobacter* genospecies, as well as other strains from the collections of P. J. M. Bouvet, Institut Pasteur, Paris, France, and P. Gerner-Smidt, Statens Seruminstitut, Copenhagen, Denmark (Table 1). A second group of strains consisted of clinical isolates, which were collected from patients in Padua Hospital during the period from 1990 to 1992. These strains were assigned to the *A. calcoaceticus-A. baumannii* complex by phenotypic analysis (5). No evident relationship could be established among the isolates.

All strains were grown on Luria-Bertani (LB) medium. Long-term preservation was obtained by freezing the bacteria in LB broth with 10% dimethyl sulfoxide.

DNA extraction. An overnight culture of each isolate was centrifuged and resuspended in 10 mM Tris HCl buffer (pH 8.0) containing 5 mM EDTA, washed once, resuspended in the same buffer containing lysozyme (1 mg/ml), and incubated at 37°C for 30 min. Sodium dodecyl sulfate (0.5%) and proteinase K (100 μg/ml) were then added, and the samples were incubated at 55°C until the solution became clear. DNA was then purified by one or two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and was collected by ethanol precipitation. DNA was dissolved in 10 mM Tris HCl buffer (pH 8.0) containing 1 mM EDTA and was stored at 4°C until use.

Amplification of the 16S-23S rRNA intergenic spacer sequences. Oligonucleotide primers complementary to conserved regions in the 16S and 23S rRNA genes were synthesized on a Millipore Cyclone Plus apparatus by phosphoramidite chemistry. The sequences of primer 1 (5′-TTGTACACACCGCCCGTCA-3′) and primer 2 (5′-GGTACTTAGATGTTTCAGTTC-3′) were described previously (16) and were demonstrated to be suitable for the amplification of target sequences of various bacterial species (9, 16).

Amplification reactions were performed in a volume of 100 μl and contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 μM (each) deoxynucleotide, 0.5 μM (each) primer, 3 mM $MgCl_2$, 2.5 U of *Taq* polymerase, and 10 to 100 ng of target DNA. Amplification mixtures were submitted to 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Each amplification experiment included a blank, which contained all reagents with the exception of target DNA. Amplification products were analyzed by gel electrophoresis in 2% agarose gels.

Restriction enzyme digestion of amplification products. Digestions were performed as recommended by the manufacturers (Boehringer, Mannheim, Germany). Analysis of restriction fragments was performed by electrophoresis in 4% agarose gels (NuSieve 3:1; FMC BioProducts).

Calculation of the copy number of the 16S-23S rRNA spacer sequences per genome. To assess the number of target sequences per genome, 10 μg of genomic DNA was digested with *Eco*RI, electrophoretically separated, and transferred to a nylon membrane. DNA was then hybridized with a probe obtained by amplification of the 16S-23S spacer sequences of strain ATCC 19004 in the presence of digoxigenin-11-dUTP (Boehringer). Hybridization was carried out for 12 h at 68°C, and hybrid bands were detected by binding of antidigoxigenin antibodies conjugated with alkaline phosphatase and then reaction with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate. The conditions used for both hybridization and detection were those recommended by the manufacturers (3). Because no *Eco*RI sites are present (see below) in the amplification products, template sequences in the genome should also not be split by this endonuclease. Therefore, the number of hybrid bands should correspond to the minimum number of target sequences in the genome.

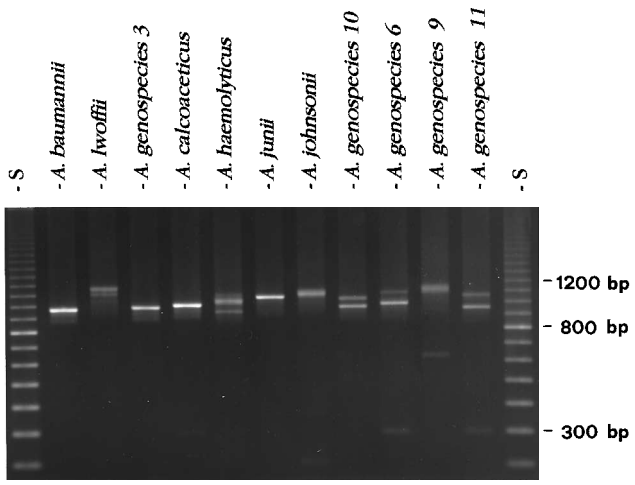

FIG. 1. DNA fragments obtained by amplification of the 16S-23S rRNA spacer sequences of 11 *Acinetobacter* type strains. A total of 5 μl of the postamplification reaction mixture was run in a 2% agarose gel. Lane S, 100-bp ladder molecular weight marker (Pharmacia, Uppsala, Sweden).

RESULTS

Amplification of *Acinetobacter* 16S-23S rRNA intergenic spacer sequences. To assess the degree of polymorphism in the target region among members of the genus *Acinetobacter*, DNAs extracted from the type strains of 11 genospecies were initially amplified. Electrophoretic analysis of the amplification products (Fig. 1) showed that each strain gave a characteristic profile consisting of one to three bands with lengths of between 300 and 1,140 bp. Moreover, fragment lengths among strains of the same genospecies were found to be highly constant (Table 1).

Almost all of the strains of the genospecies in the *A. calcoaceticus-A. baumannii* complex gave a single amplification product of very constant length, as reported in Table 1. Only three strains, belonging to genospecies 13 and N1, gave two bands of similar lengths.

An important feature is the close similarity in length among amplification products obtained from the genospecies in the *A. calcoaceticus-A. baumannii* complex. This result on the one hand confirms the close similarity between the strains belonging to the complex, but on the other hand, it renders their differentiation on the basis of this simple electrophoretic analysis more difficult.

Restriction enzyme analysis. For better discrimination, restriction polymorphism analysis of the amplification products derived from the strains belonging to the *A. calcoaceticus-A. baumannii* complex was performed. No restriction sites for *Eco*RI, *Hin*dIII, or *Bam*HI were present in any amplification product. On the other hand, *Dde*I, *Msp*I, *Cfo*I, and *Rsa*I, while cutting, did not give discriminating restriction patterns (data not shown). On the contrary, restriction endonucleases *Alu*I and *Nde*II not only were able to digest the amplification products but also gave restriction patterns that were characteristic for the different genospecies.

Figure 2A and Table 2 show, respectively, the restriction profiles and the estimated fragment lengths that were obtained with *Alu*I. Digestion with this endonuclease gave rise to fragments of constant length for each genospecies; i.e., all of the strains which belonged to genospecies 1 had profile A, all genospecies 2 strains had profile B, and all genospecies 3 and N2 strains had profile C. In the last case, two DNA fragments,

TABLE 1. Bacterial strains and length of DNA fragments obtained by amplification of the 16S-23S rRNA intergenic spacer sequences

Genospecies	Strains[a]	Reference	Length (bp)
1	ATCC 23055, ATCC 17902, ATCC 14987	4	1,005
1	42, 59, 64, 66, 132	12	1,005
2	ATCC 19606, ATCC17961, ATCC 15149, ATCC 17978	4	975
2	9971, 10073, 10508, 10074, 10086	11	975
3	ATCC19004, ATCC 17922, ADAM 53 1984, ADAM 202 OKI 55732	4	990
3	9907, 12174a, 12398, 10790, 10078	11	990
4	ATCC 17906, ATCC 17977, SEIP 23.77	4	1,020, 935
5	ATCC 17908, SEIP 883	4	1,035
6	ATCC 17979	4	1,060, 965, 300
7	ATCC 17909	4	1,060
8	ATCC15309	4	1,140, 1,085
9	ATCC 9957	4	1,085, 625
10	ATCC17924	4	1,025, 945
11	ATCC 11171	4	1,035, 935, 300
13	ATCC 17903	20	975
13	4419, 9836, 9894,	11	975
13	10716, 12112	11	975, 1,025
N1[b]	5804	11	975
N1[b]	10090	11	975, 1,025
N2[b]	10095, 10169	11	990

[a] The type strain of a genospecies is underlined.
[b] N1 and N2 are not numbered genospecies in the *A. calcoaceticus-A. baumannii* complex (11).

each of about 175 bp, comigrated at the same position, which was established by densitometric analysis (data not shown).

Strains belonging to genospecies 13 and N1 had profiles identical or very similar to profile B. In particular, the digestion of the amplification product of 975 bp of genospecies 13 strains gave a pattern corresponding to profile B. When the additional amplification product of 1,025 bp was present, only the length of the largest restriction fragment was modified and a band corresponding to 375 bp became visible.

Although genospecies 1 was already well differentiated after *Alu*I digestion, the other genospecies showed common restriction patterns, so they were submitted to restriction analysis with *Nde*II in order to obtain a better discrimination capability. The patterns obtained are shown in Fig. 2B, and the relative fragment lengths are given in Table 2. As a general consideration, the patterns obtained with *Nde*II were more differentiated from those obtained with *Alu*I; genospecies 3 gave each of the profiles in lanes E, F, G of Fig. 2B. Profile F clearly indicates that at least some amplification products are heterogeneous, being composed of DNA fragments of the same length but of different sequences. In fact, two classes of amplification products are recognizable. In the first one, the fragment of 750 bp has a site for *Nde*II and is split into two fragments of 330 and 420 bp (as in the case of profile E); in the second one, the site for *Nde*II is not present, as in profile G. The two strains in the N2 group also showed profile E. Genospecies 13 showed a profile very similar to that in lane G of Fig. 2B, with the difference of a few base pairs in the length of the largest fragment, or profile I. Only genospecies 2 always gave the same restriction profile, which is represented in Fig. 2B, lane D.

In conclusion, the combined digestion with *Alu*I and *Nde*II allowed the classification of the strains in the *A. calcoaceticus-A. baumannii* complex into four groups. In fact, digestion with *Alu*I could differentiate three groups, the first corresponding to genospecies 1, the second corresponding to genospecies 3 plus strains 10169 and 10095, and the third including genospecies 2 and 13 and strains 5804 and 10090. Genospecies 2 could then be further differentiated by a very characteristic restriction profile after digestion with *Nde*II. Therefore, digestion of the amplification products by the two endonucleases allowed the correct classification of all of the strains tested, with the exception of the four strains belonging to the two new genospecies recently described by Gerner-Smidt and Tjernberg (11) and indicated here as groups N1 and N2. In fact, N1 strains grouped with genospecies 13 and N2 strains grouped with genospecies 3. These results agree with the observations of Gerner-Smidt and Tjernberg (11), who described strains 10095 and 10169 as being closely related to DNA groups 1 and 3 and strains 5804 and 10090 as being closely related to DNA group 13 (11).

Copy number of the 16S-23S rRNA spacer sequences per genome. Amplification of the 16S-23S rRNA intergenic spacer sequences of bacteria belonging to the *A. calcoaceticus-A. baumannii* complex almost always produced only one DNA fragment. This is quite unexpected, because it has been reported that in most bacterial species these sequences are present in

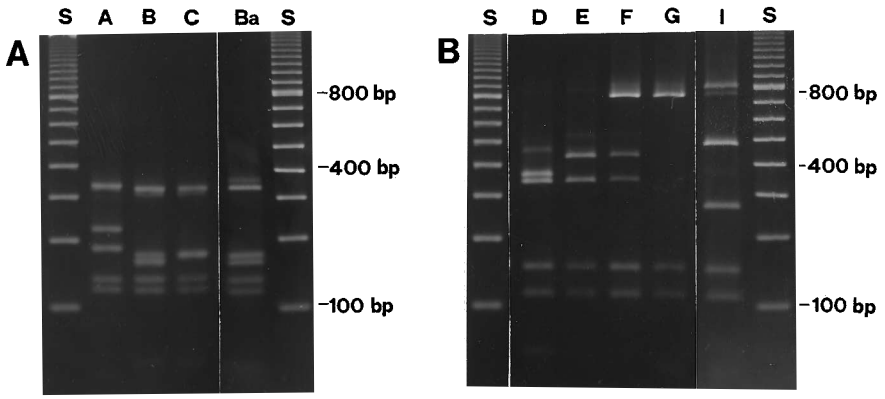

FIG. 2. Restriction patterns of the amplification products obtained from *Acinetobacter* strains in the *A. calcoaceticus-A. baumannii* complex. A total of 8 μl of the postamplification reaction mixture was digested with *Alu*I (A) or *Nde*II (B) and run in a 4% agarose gel. Lane designations indicate restriction patterns. Lane S, 100-bpair ladder molecular weight marker (Pharmacia, Uppsala, Sweden).

TABLE 2. Fragment lengths of amplification products digested with *Alu*I and *Nde*II

Restriction endonuclease and pattern[a]	Fragment length	Genospecies	Strains[b]
*Alu*I			
A	125, 135, 185, 220, 340	1	ATCC 23055, ATCC 17902, ATCC 14987, 42, 59, 64, 66, 132
B	50, 125, 135, 165, 175, 330	2	ATCC 19606, ATCC 17961, ATCC 15149, ATCC 17978, 9971, 10073, 10074, 10086, 10508
B		13	ATCC 17903, 9836, 9894, 4419
B		N1	5804, 10090
Ba	50, 125, 135, 165, 175, 330, 375	13	10716, 12112
C	50, 125, 135, 175, 330	3	ATCC 19004, ATCC 17922, ADAM 53 1984, ADAM 202, 9907, 10078, 10790, 12174a, 12398
		N2	10169, 10095
*Nde*II			
D	50, 110, 145, 330, 360	2	ATCC 19606, ATCC 17961, ATCC 15149, ATCC 17968, 9971, 10073, 10074, 10086, 10508
E	110, 145, 330, 420	3	ATCC 19004
E		N2	10169, 10095
F	110, 145, 330, 420, 750	3	9907, 12174a, ADAM 53 1984
G	110, 145, 750	3	12398, 10790, 10078, ATCC 17922, ADAM 202
H[c]	110, 145, 740	13	9836, 9894, 4419
H[c]		N1	5804, 10090
I	110, 145, 280, 475, 740, 800	13	ATCC 17903, 10716, 12112

[a] Letters correspond to the lanes in Fig. 2.
[b] The strains with each particular restriction profile are listed.
[c] Not shown in Fig. 2.

more than one copy per bacterial genome (16, 19), and these sequences often differ in both sequence and length. Our results could be due to the inability of primers to anneal with some of the *Acinetobacter* target sequences. This appears to be unlikely, because these primers were able to recognize the corresponding sequences in many bacterial species (9, 16) and primer 1 perfectly matches with the only *Acinetobacter* 16S rRNA gene sequence covering the region of our interest, which is deposited in GenBank (accession number M34139). No sequences are deposited for *Acinetobacter* 23S rRNA genes.

Another possible explanation could be that in these bacteria only one target sequence is present per genome. To clarify this aspect, we hybridized *Eco*RI-digested genomic DNA with amplification products labelled with digoxigenin. Because *Eco*RI does not cut amplification products, each hybrid band should correspond to at least one copy of a sequence complementary to the probe. The results obtained with four strains are shown in Fig. 3. The minimum numbers of target sequences are five for strains ATCC 19004 (*Acinetobacter* genospecies 3) and 9836 (*Acinetobacter* genospecies 13) and six for strains ATCC 23055 (*Acinetobacter* genospecies 1) and ATCC 19606 (*Acinetobacter* genospecies 2).

Restriction analysis of amplification products obtained from clinical isolates. Sixty-eight clinical isolates, which were assigned to the *A. calcoaceticus-A. baumannii* complex by phenotypic analysis, were characterized by amplification of the 16S-23S rRNA intergenic spacer sequences and subsequent restriction analysis of the amplified products. All of them gave amplification products and restriction profiles perfectly corresponding to those already described in this report. In particular, 59 strains could be classified as *A. baumannii*, 7 could be classified as *Acinetobacter* genospecies 3 or N2, 1 could be classified as *A. calcoaceticus*, and 1 could be classified as *Acinetobacter* genospecies 13 or N1 (Table 3).

DISCUSSION

This work has demonstrated a polymorphism in the spacer sequences between the genes for the 16S and 23S rRNAs among bacteria in the genus *Acinetobacter* and has exploited it for their identification. Particular attention was paid to bacteria belonging to the *A. calcoaceticus-A. baumannii* complex, because they are phenotypically very similar and can be identified with certainty only by DNA-DNA hybridization or ribotyping.

Amplification of the target sequences of 12 *Acinetobacter* genospecies demonstrated that one to three amplification products of characteristic length could be obtained. For *Acinetobacter* species in the *A. calcoaceticus-A. baumannii* complex, amplification products were very constant, allowing at least

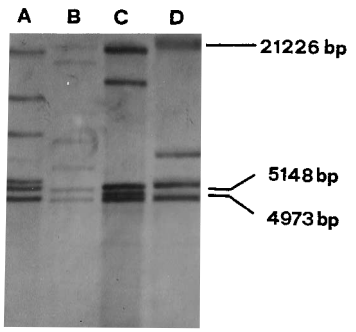

FIG. 3. Southern blot analysis of four *Acinetobacter* strains. Genomic DNAs (lane A, strain ATCC 23055; lane B, ATCC 19606; lane C, ATCC 19004; lane D, 9836; corresponding, respectively, to genospecies 1, 2, 3, and 13) were digested with *Eco*RI, transferred to a nylon membrane, and hybridized to a probe obtained by amplification of the 16S-23S rRNA intergenic spacer sequences of strain ATCC 19004 in the presence of digoxigenin. The migration positions of the molecular weight markers are indicated.

TABLE 3. Classification of 68 clinical isolates on the basis of the restriction polymorphism of the 16S-23S intergenic spacer sequences

Genospecies	No. of isolates	No. of isolates with the following profile distribution[a]							
		*Alu*I			*Nde*II				
		A	B	C	D	E	F	G	H
1	1	1							
2	59		59		59				
3 or N2	7			7		4	1	2	
13 or N1	1		1						1

[a] All letters correspond to lanes in Fig. 2.

one of the genospecies (*A. calcoaceticus*) to be recognized on this basis. However, because the similarities of the amplified DNA fragments hindered their electrophoretic separation, a restriction analysis was performed for a clearer differentiation. Two restriction endonucleases, *Alu*I and *Nde*II, gave discriminatory restriction patterns. By digesting the amplification products with both of these enzymes, a reliable classification into four groups of 36 strains belonging to the *A. calcoaceticus-A. baumannii* complex could be obtained. The first one, corresponding to genospecies 1, and the second one, including genospecies 3 and the two strains in the N2 DNA group, had characteristic restriction patterns after digestion with *Alu*I. The third group, corresponding to genospecies 2, and the fourth, including genospecies 13 and N1, could be differentiated after further restriction analysis with *Nde*II. No discriminatory restriction pattern could be obtained for the four isolates representing the two new DNA groups in the *A. calcoaceticus-A. baumannii* complex described by Gerner-Smidt and Tjernberg (11) and indicated in this report as N1 and N2. In our system, therefore, the two isolates of the N1 group would be classified as genospecies 13 and the two isolates of the N2 group would be classified as genospecies 3. This classification, although not fully satisfactory, reflects the relatedness between genospecies 13 and N1 as well as the relatedness between genospecies 3 and N2 that were found by DNA-DNA hybridization (11). The detection of restriction polymorphism of the 16S-23S rRNA intergenic spacer sequences is therefore a less discriminatory identification method compared with DNA-DNA hybridization. However, it should be considered that restriction analysis of amplification products is a much simpler technique, and also a relatively rapid one, because the whole procedure can be completed within 2 days of less than fully dedicated working time. Moreover, it should also be noted that the reference strains tested were of different geographic origins and that no new restriction pattern was observed after extension of the analysis to a significant number of Italian clinical isolates. Therefore, even if we cannot exclude the existence of restriction patterns different from those described in this report, we can reasonably presume that the proposed identification system is capable of recognizing a very high percentage of the strains that are eventually tested.

A remarkable result of the study is the amplification of a single DNA fragment from strains in the *A. calcoaceticus-A. baumannii* complex. Because the results obtained by Southern hybridization experiments indicated the presence of five to six target sequences in genomic DNA, we should conclude that at least the length of the 16S-23S rRNA intergenic spacer sequences is very well conserved, both between copies of the rRNA transcriptional units in the same chromosome and between different isolates. This conclusion is rendered more interesting by the observation that the restriction patterns obtained with all of the endonucleases tested are often identical within the same DNA group (data not shown), thus justifying the impression that, in contrast to other bacterial genera (9, 16), these regions are highly conserved in *Acinetobacter* species. While this high degree of conservation allows reliable identification of genospecies, it prevents subtyping; therefore, for epidemiological classification, a separate approach should be used. The reasons for the high degree of conservation of the 16S-23S rRNA intergenic spacer sequences in *Acinetobacter* species will be the object of further work.

In conclusion, the present study has demonstrated that restriction polymorphism in the spacer sequences between the 16S and the 23S rRNA genes allows identification of *Acinetobacter* genospecies in the *A. calcoaceticus-A. baumannii* complex. The techniques used for its detection, i.e., DNA amplification and restriction analysis, are still not suitable for routine analysis in diagnostic laboratories. However, they are much simpler than DNA-DNA hybridization or ribotyping and can easily be implemented by those laboratories that are interested in the study of *Acinetobacter* infections.

ACKNOWLEDGMENTS

We are grateful to P. Gerner-Smidt (Statens Seruminstitut, Copenhagen, Denmark) and to P. J. M. Bouvet (Institut Pasteur, Paris, France) for providing some of the strains. Special thanks are due to P. Gerner-Smidt for critical reading of the manuscript.

This work was supported by 40% and 60% grants from Italian MURST.

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