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Ribotyping of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex

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The *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex consists of four genotypically distinct but phenotypically very similar bacterial species or DNA groups: *A. calcoaceticus* (DNA group 1), *A. baumannii* (DNA group 2), unnamed DNA group 3 (P. J. M. Bouvet and P. A. D. Grimont, Int. J. Syst. Bacteriol. 36:228–240, 1986), and unnamed DNA group 13 (I. Tjernberg and J. Ursing, APMIS 97:595–605, 1989). Because strains in this complex cause nosocomial outbreaks, it is important to be able to identify them as completely as possible. Ribotyping could provide such identification. Therefore, ribotyping was done on 70 strains in the *A. calcoaceticus-A. baumannii* complex with known DNA group affiliations by use of restriction enzymes *Eco*RI, *Cla*I, and *Sal*I. A nonradioactive digoxigenin-11-dUTP-labeled *Escherichia coli* rRNA-derived probe was used. With any of the three restriction enzymes, banding patterns that were specific for each DNA group were seen. All 70 strains showed banding patterns that could identify them to the correct DNA group by use of any two of the three enzymes. In addition, banding patterns that could separate strains within any one DNA group were present. The discriminatory index of P. Hunter and M. Gaston (J. Clin. Microbiol. 26:2465–2466, 1988), applied to all strains with the combined results obtained with all three enzymes, revealed a value of 0.99. For strains in each DNA group, the value varied from 0.93 to 0.98. These results indicate the high discriminatory power of the system when used for epidemiological typing.

In recent years, *Acinetobacter* strains have been incriminated to an increasing extent in epidemics of hospital infections (1, 6, 7, 10, 12). Patients in intensive-care units are particularly at risk of acquiring an *Acinetobacter* infection, most often a respiratory tract infection or septicemia (2, 9, 11, 17, 23). The isolated strains have been identified phenotypically as *Acinetobacter baumannii*, on the basis of the newest terminology (2, 19, 28). However, it has been shown that strains in four different species or DNA groups ("genospecies"), *Acinetobacter calcoaceticus* sensu Bouvet and Grimont (3) (DNA group 1), *A. baumannii* (DNA group 2), unnamed DNA group 3 (3), and unnamed DNA group 13 (26), are so similar that it may not be possible to differentiate them phenotypically (13). The strains in these four DNA groups show a DNA homology of approximately 70% (3, 26) which, on the basis of a genotypic definition, may justify splitting them into different species or subspecies (27). Only tests for growth at 37, 41, and 44°C and biotyping assimilation reactions (4) (assimilation of L-phenylalanine, levulinate, citraconate, 4-hydroxybenzoate, and L-tartrate) may sometimes place these strains correctly in the appropriate DNA group. Strains of DNA group 1 grow at 37°C but not at higher temperatures. On the basis of the published taxonomic schemes, strains of DNA group 3 have a maximum temperature for growth of either 37 or 41°C (3, 13). In blinded studies in which the DNA group affiliation of the studied strains has been unknown to the investigator, some strains of DNA group 3 have been observed to grow at 44°C (11a). Strains of DNA groups 2 and 13 all grow at 41°C, and nearly all grow at 44°C. Strains of DNA group 13 nearly all belong to biotype 9; i.e., they assimilate L-phenylalanine and 4-hydroxybenzoate but not levulinate, citraconate, and L-tartrate. Strains of DNA group 3 rarely, if ever, assimilate levulinate, but strains of DNA groups 1 and 2 may assimilate all the carbohydrates used for biotyping. Thus, only strains growing at 44°C and assimilating levulinate can safely be identified as *A. baumannii*. Strains with any other phenotypic profile cannot with certainty be identified to any single DNA group. These unidentifiable strains make up the majority of *Acinetobacter* strains in hospital materials (4, 5, 8, 13, 14, 19). Although DNA group 1 (*A. calcoaceticus*) usually is considered an environmental species (16), increasing evidence indicates that strains in DNA groups 3 and 13, along with strains in DNA group 2, are important nosocomial pathogens (5, 8, 13).

In daily clinical microbiological practice, the need for detailed identification of strains in this complex, which has been named the *A. calcoaceticus-A. baumannii* complex (13), can be questioned. In an epidemic situation, however, thorough identification may be crucial. As DNA-DNA hybridization is impracticable in most clinical microbiology laboratories, there is a need for a simpler identification system for strains in the *A. calcoaceticus-A. baumannii* complex. Ribotyping may be such a system. Its efficacy for the identification and typing of several other bacterial species has been proven (15, 22, 24, 25). Nonradioactive probing techniques now have been developed, making it possible for a well-equipped clinical microbiology laboratory to use this system.

The present study was undertaken to explore this method for the identification of strains in the *A. calcoaceticus-A. baumannii* complex. A preliminary evaluation of the discriminatory power of ribotyping for subtyping of the strains was carried out as well.

MATERIALS AND METHODS

Strains. Seventy strains identified by DNA-DNA hybridization to one of the four DNA groups in the *A. calcoaceticus-A. baumannii* complex in an earlier study (13) were examined. The strains (ribotypes; see below) were as follows: DNA group 1, ATCC 17902 (1A), ATCC 23055^T (*A.*

calcoaceticus) (1D), 42 (1B), 59 (1H), 132 (1I), 64 (1C), 66 (1E), 67 (1G), 68 (1F), and 74 (= LMD 22.17) (1D); DNA group 2, ATCC 9955 (2F), ATCC 17904 (2Bf), ATCC 17978 (2G), CCUG 19096^T (= ATCC 19606^T; *A. baumannii*) (2C), 65 (2J), 91 (2Bg), 107 (2D), 133 (2I), 144 (2I), 147 (2J), 1 (2Ad), 13 (2Ac), 18 (2L), 24 (2L), 27 (2J), 28 (2Be), 29 (2H), 60 (2K), 77 (= LMD 82.54) (2E), 78 (= NCTC 7844) (2C), Ac 1141 (2Ad), 189 (2Aa), ab2444 (2Ab), ab2445 (2Ab), and 50853-82 (2Aa); DNA group 3, ATCC 17922 (3Ai), ATCC 19004 (3Cj), 40 (3I), 41 (3Fm), 55 (3B), 62 (3Ck), 79 (3Fm), 102 (3Fm), 128 (3Ah), 143 (3H), 162 (3Fn), 176 (3K), 204 (3Cl), 212 (3G), 31 (3Ck), 36 (3H), 37 (3Fm), 59 (3E), 61 (3D), and 63 (3J); and DNA group 13, ATCC 17903 (13Br), 89 (13G), 100 (13C), 165 (13Ao), 62 (13H), 65 (13Ao), Ac 2041 (13I), Ac 2284 (13Ao), Ac 2285 (13Ao), Ac 2376 (13Bq), Ac 2624 (13D), Ac 2627 (13F), 353 (13Ap), 53893-82 (13Ap), and 53937bb (13E). The ribotypes shown in parentheses consist of a number corresponding to the DNA group of the strain, indicating the difference between the ribotypes of the different DNA groups; an uppercase letter, indicating differences within each DNA group, determined by use of *Eco*RI; and a lowercase letter, indicating further differentiation, determined by use of *Cla*I and/or *Sal*I. The strains were reference strains, culture collection strains, and clinical isolates from three countries. DNA groups 1, 2, 3, and 13 were represented by 10, 25, 20, and 15 strains, respectively. All strains were considered unrelated epidemiologically.

Isolation of chromosomal DNA. DNA was extracted as described by Mekalanos (20), with minor modifications. In brief, the pellet from an overnight 5-ml broth culture of a test strain was resuspended in 500 μl of 50 mM Tris–50 mM EDTA (pH 8.0). The suspension was treated with 20 μl of lysozyme (10 mg/ml) for 30 min at 37°C. The cells were lysed by gentle mixing with 50 μl of proteinase K (10 mg/ml)–20 μl of 10% sodium dodecyl sulfate (SDS) at 56°C until the lysate cleared (approximately 30 min). Protein was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated by the addition of 1 volume of 2-propanol. After centrifugation, the DNA pellet was resuspended in 300 μl of 10 mM Tris–1 mM EDTA (pH 8.0), and DNA was reprecipitated with 100 μl of ammonium acetate (7.5 M)–800 μl of cold 96% ethanol in a refrigerator for at least 30 min. Finally, the purified DNA was pelleted by centrifugation, dried under vacuum, and resuspended in 50 to 400 μl of 10 mM Tris–1 mM EDTA (pH 8.0), depending on the amount of DNA recovered.

Restriction endonuclease digestion. The DNA was cleaved as described by the manufacturer of the restriction enzymes used (GIBCO/BRL, Life Technologies, Copenhagen, Denmark). The first restriction enzyme used was *Eco*RI; thereafter, eight additional enzymes were used to test strains not discriminated by *Eco*RI. These enzymes were *Hin*dIII, *Hae*III, *Hha*I, *Cla*I, *Sal*I, *Bam*HI, *Pst*I, and *Sma*I. All these enzymes made further discrimination for DNA group 3 possible, but only *Cla*I and *Sal*I provided further discrimination for DNA groups 2 and 13. Therefore, the latter two enzymes were chosen for further investigations.

Electrophoresis. The restriction fragments were separated in an agarose gel (Agarose Ultrapure; GIBCO/BRL), the gel concentrations being 0.7% for restriction enzymes *Eco*RI, *Hin*dIII, *Cla*I, *Sal*I, *Bam*HI, *Pst*I, and *Sma*I and 1.0% for restriction enzymes *Hae*III and *Hha*I. Electrophoresis was done with a GIBCO/BRL Horizon 20.25 electrophoresis apparatus and 90 mM Tris–90 mM borate–2 mM EDTA (pH 8.0) overnight at 50 V, except for experiments with infrequently cutting enzymes *Cla*I, *Sal*I, *Sma*I, and *Bam*HI, with which the voltage was set to 60 V. A mixture of phage lambda DNAs (Boehringer Mannheim, Ercopharm A/S, Kvistgaard, Denmark) cut with *Hin*dIII and *Sty*I or with *Hin*dIII and *Pvu*I was used as a molecular size marker.

After electrophoresis, the gel was stained in ethidium bromide (2 mg/liter) and visualized on a UV transilluminator (302 nm) (UVP Inc.) to check the intensity of the bands and to check the completeness of cutting by the enzymes.

Southern blotting. The DNA in the gel was depurinated in 0.25 M HCl for 15 min, and the fragments were transferred to a nylon membrane (ZetaProbe GT [Bio-Rad] or Hybond N+ [Amersham]) by use of a Bio-Rad vacuum blotter with 0.5 M NaOH–0.6 M NaCl as the transfer solution. A vacuum (5 in. [127 mm] Hg) was applied for 90 min. The membrane was used directly for hybridization or was air dried and kept in a plastic bag for later hybridization.

Probe preparation. The probe was made by incorporating digoxigenin-11-dUTP into first-string cDNA containing a mixture of 16 and 23S rRNAs by random priming with reverse transcriptase. The reaction mixture contained 1.5 μg of 16 and 23S rRNAs from *Escherichia coli* (Boehringer Mannheim); 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, and 0.064 mM dTTP (all from GIBCO/BRL); 0.036 mM digoxigenin-11-dUTP (Boehringer Mannheim); 0.5 mM random hexanucleotides (Boehringer Mannheim); 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL); and a buffer supplied with the reverse transcriptase 10 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM Tris-HCl [pH 8.3]) in a total volume of 20 μl. The reaction mixture was incubated overnight at 37°C, and the reaction was stopped by the addition of 2 μl of EDTA (0.2 M, pH 8.0). The labelled cDNA was purified in accordance with the instructions in the DIG DNA Labelling and Detection Nonradioactive Applications Manual (Boehringer Mannheim). The purified probe was used directly in the hybridization reaction without denaturation. The probe used for the detection of the molecular size marker was made by random priming of phage lambda DNA with digoxigenin-11-dUTP as described in the above-mentioned manual.

Hybridization. Prehybridization, hybridization, and detection were carried out as specified in the above-mentioned manual, with the exception that SDS was omitted from the washing solutions used immediately after hybridization. Hybridization was performed with sealed plastic bags at 60°C overnight. The hybrids were detected by a color reaction of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (toluidinium salt) with alkaline phosphatase-labelled antidigoxigenin antibodies. The color reaction usually took place for 2 to 3 h and was stopped by washing of the membrane in tap water. The positions of the bands were scored visually relative to those of the phage lambda DNA markers and the bands of reference strains from each DNA group run repeatedly on each gel.

Reproducibility. For comparisons and testing of reproducibility, four strains representing all DNA groups were retested on each gel. In addition, strains deviating from each other by two or fewer bands were retested for reproducibility.

Numerical analysis. Strains were analyzed by UPGMA clustering (unweighted pair-group method with arithmetic averages) of the banding patterns by use of the simple matching coefficient on an IBM-PC with the program TAXAN (D. Swartz, University of Maryland).

Discriminatory power. The discriminatory power of ribotyping as a typing method was evaluated with the discriminatory index of Hunter and Gaston (18). This index repre-

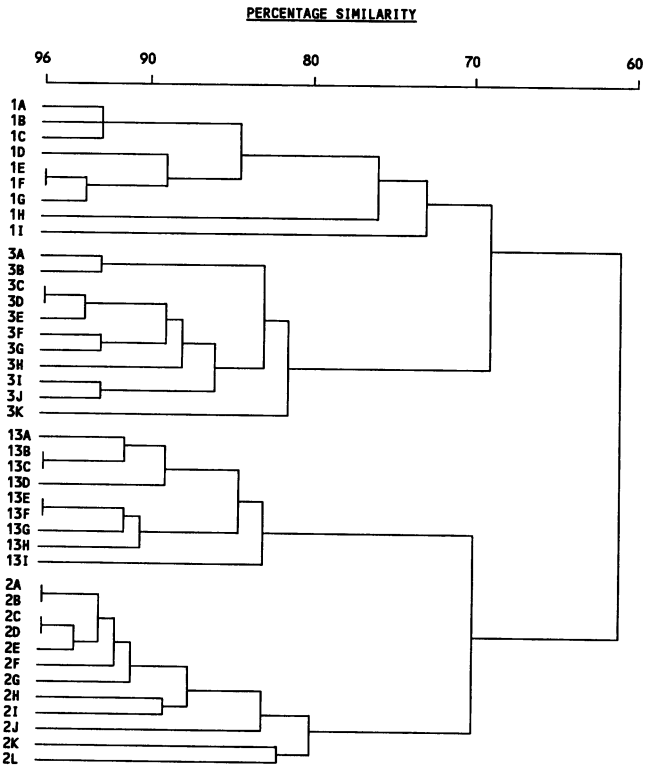

FIG. 1. UPGMA clustering dendogram of the *Eco*RI ribotypes of strains in the *A. calcoaceticus-A. baumannii* complex. The simple matching coefficient was used. The designations on the left refer to the *Eco*RI ribotype designations listed in Materials and Methods.

sents the probability that two unrelated strains will be typed differently by a given typing system. The formula reads as follows:

$$D = 1 - [1/n(n-1)] \sum_{j=1}^{s} n_j (n_j - 1)$$

where D is the discriminatory index, n is the number of strains in the study population, s is the number of different types, and n_j is the number of strains belonging to the j^{th} type.

RESULTS

Definitive scoring of the bands in the strains cut with *Eco*RI was only possible for bands smaller than approximately 10 kb. Above this size, the bands were too close or too dense to score unless the strains were run close together on the same gel, i.e., under comparative conditions. Twenty-nine bands could be scored, and the strains each contained 7 to 10 bands that could be scored. A comparison of the strains by use of these banding patterns revealed 9 different ribotypes in the 10 strains of DNA group 1, 12 in the 25 strains of DNA group 2, 11 in the 20 strains of DNA group 3, and 9 in the 15 strains of DNA group 13. Each ribotype was represented by one to seven strains. Twenty-two strains accumulated in four ribotypes; up to 3 strains were seen in the remaining ribotypes.

Figure 1 shows the results of UPGMA clustering of the *Eco*RI ribotypes. At a similarity level of 0.73, four clusters,

TABLE 1. Discriminative ribotype bands for the *A. calcoaceticus-A. baumannii* complex, determined with restriction enzyme *Eco*RI

DNA group	No. of strains showing a restriction band of the indicated size (kb):													
	0.8	1.8	1.85	2.0	2.4	3.6	3.9	4.7	4.9	5.0	5.2	5.4	5.8	7.1	7.8
1	10	0	0	0	10	0	4	0	9	0	1	10	10	3	8
2	25	0	25	3	18	0	22	22	0	0	25	0	0	22	13
3	18	2	0	0	18	18	1	20	0	19	1	18	14	0	20
13	0	15	12	15	14	0	15	10	0	8	0	7	0	15	3

each containing strains from only one DNA group in the *A. calcoaceticus-A. baumannii* complex, were seen. Ribotypes of DNA groups 1 and 3 clustered closer to each other (similarity level, 0.69) than to the other two DNA groups (similarity level, 0.61), as did ribotypes of DNA groups 2 and 13 (similarity level, 0.70).

This clustering reflected some *Eco*RI banding patterns that were common to most strains in each DNA group (Table 1). The presence of bands at 0.8 and 4.9 kb and the absence of bands at 1.8, 1.85, and 4.7 kb identified strains to DNA group 1 (*A. calcoaceticus*). Similarly, strains of DNA group 2 (*A. baumannii*) were characterized by the presence of bands at 0.8 and 1.85 kb, strains of DNA group 3 were characterized by the presence of bands at 4.7 and 7.8 kb and the absence of bands at 1.85 and 2.0 kb, and strains of DNA group 13 were characterized by the presence of bands at 1.8, 2.0, 3.9, and 7.1 kb and the absence of a band at 0.8 kb. *Eco*RI ribotypes of some representative strains are shown in Fig. 2. In any one DNA group, every strain showed at least two banding characteristics that could separate it from all strains in the other three DNA groups.

To increase the likelihood of strain discrimination, we cleaved DNA from 16 strains not differentiated by *Eco*RI with eight other restriction enzymes. Of these, only enzymes *Cla*I and *Sal*I provided additional discrimination for DNA groups 2, 3, and 13. All strains were therefore typed with these enzymes. The strains contained 4 to 7 bands that could be scored with *Cla*I and 6 to 12 bands that could be scored with *Sal*I. By combining the results obtained with all three enzymes, the DNA group 1 strains were split into 9 ribotypes, the DNA group 2 strains were split into 17 ribotypes, the DNA group 3 strains were split into 15 ribotypes, and the DNA group 13 strains were split into 11 ribotypes. No ribotype contained more than four strains. In addition, patterns that were specific for each DNA group were seen with both *Sal*I and *Cla*I (Tables 2 and 3). With *Cla*I, one strain in DNA group 1 repeatedly showed only one ribotype band of approximately 5.0 kb that was not shared by any other strain.

The discriminatory power of this ribotyping system, as judged by the discriminatory index, was high. The single most discriminating enzyme was *Eco*RI, with a discriminatory index of 0.97 for all 70 strains tested. Within each DNA group, this index varied from 0.85 to 0.98 (Table 4). When all three enzymes and the entire population of strains were used, the index increased to 0.99 (Table 4).

Twenty-eight strains cut with *Eco*RI, 20 strains cut with *Cla*I, and 20 strains cut with *Sal*I were run on two or more gels, with a reproducibility of 100%.

DISCUSSION

The present study has established some ribotyping patterns that are common to strains in each DNA group in the

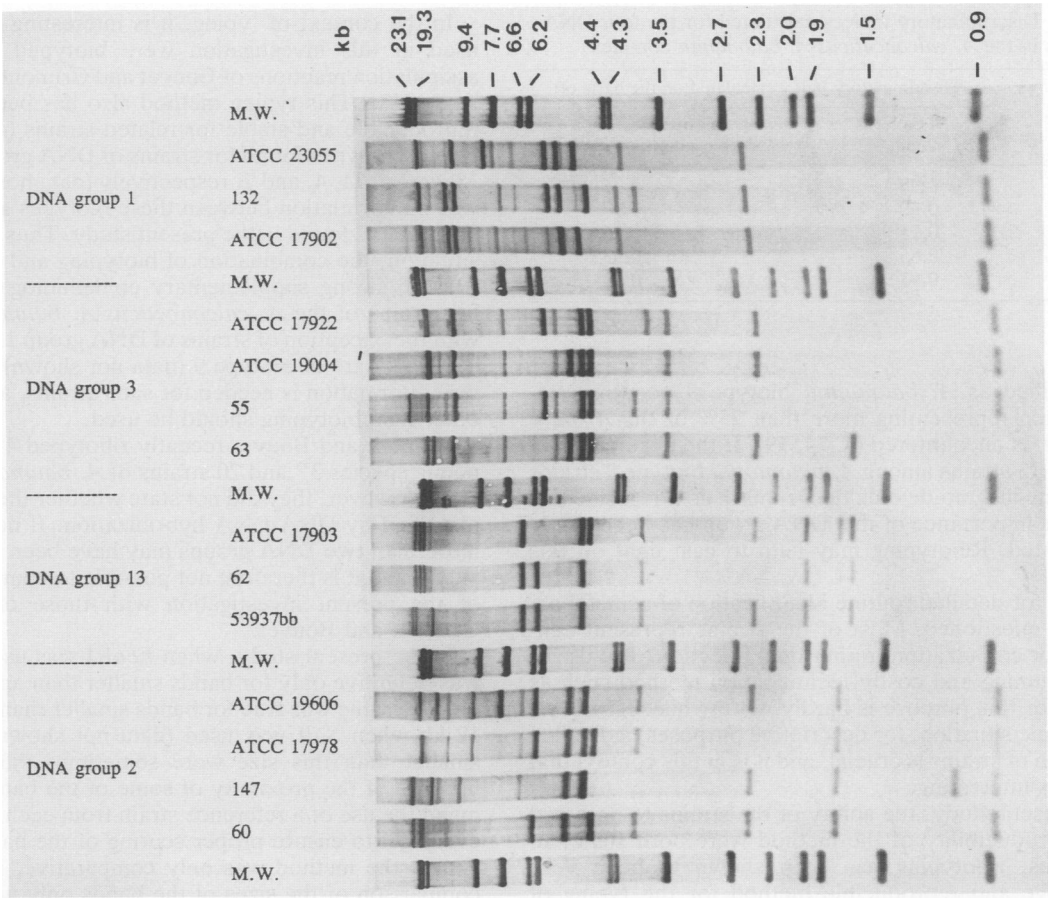

FIG. 2. *Eco*RI ribotypes of some representative strains in the *A. calcoaceticus-A. baumannii* complex. The molecular weight marker (M.W.) is a mixture of phage lambda DNAs cut with *Hin*dIII and *Sty*I.

A. calcoaceticus-A. baumannii complex. All strains used in this study, except for one DNA group 1 strain, showed banding patterns that were specific to only one DNA group when restriction enzyme *Eco*RI, *Sal*I, or *Cla*I was used. The exceptional strain (strain 68) showed DNA group 1-specific restriction patterns with both *Eco*RI and *Sal*I but not with *Cla*I. Thus, ribotyping can be used to identify acinetobacters to the DNA group level. The identification can be done with restriction enzyme *Eco*RI alone and may be confirmed with either *Cla*I or *Sal*I. This separation of the strains is also indicated in the numerical analysis of the banding patterns by UPGMA clustering, which revealed four clusters, each containing strains from only one DNA group. Strains of DNA groups 1 and 3 clustered closer to each other than to strains in the other two DNA groups. Similarily, strains of DNA groups 2 and 13 were closer to each other. These results reflect the genetic relationship between strains, as the same patterns were seen by Tjernberg and Ursing in their DNA-DNA hybridization study (26).

This method was recently used in a Danish national survey for the identification of 28 strains collected over a 7-month period (unpublished data). In this investigation, eight of nine biotype 9 strains and one biotype 10 strain from hospitals throughout Denmark proved to be of DNA group 13. These strains had been implicated in various clinical conditions, such as septicemia, ventilator-associated pneumonia, peritonitis associated with continuous ambulatory peritoneal dialysis, and urinary tract infections. In other surveys on the nosocomial occurrence of acinetobacters, strains phenotyp-

TABLE 2. Discriminative ribotype bands for the *A. calcoaceticus-A. baumannii* complex, determined with *Cla*I

DNA group	No. of strains showing a restriction band of the indicated size (kb):												
	5.1	5.4	6.2	6.4	6.5	6.8	7.9	8.4	8.5	9.2	10.4	12.5	18.0
1	9	8	8	9	0	0	0	6	4	0	0	0	0
2	0	0	25	0	22	0	24	1	21	22	0	4	0
3	0	0	0	0	0	20	20	20	0	0	0	18	17
13	0	15	0	0	0	1	15	0	15	12	13	0	0

TABLE 3. Discriminative ribotype bands for the *A. calcoaceticus-A. baumannii* complex, determined with *Sal*I

DNA group	No. of strains showing a restriction band of the indicated size (kb):								
	3.0	3.3	3.8	4.7	4.85	6.0	6.7	8.4	11.4
1	0	0	0	3	6	0	8	0	0
2	25	0	14	23	0	20	7	0	0
3	0	19	20	0	0	0	11	0	0
13	0	0	0	15	0	0	0	13	13

TABLE 4. Discriminatory indices calculated for the four DNA groups in the *A. calcoaceticus-A. baumannii* complex

DNA group	Discriminatory index calculated on the basis of:	
	*Eco*RI patterns alone	*Eco*RI, *Sal*I, and *Cla*I patterns combined
1	0.98	0.98
2	0.90	0.97
3	0.91	0.96
13	0.85	0.93
All	0.97	0.99

ically identified as *A. baumannii* biotype 9 occurred frequently, often representing more than 25% of the *A. baumannii* isolates encountered (4, 14, 19). If the occurrence of DNA group 13 strains among *A. baumannii* biotype 9 strains is of the same magnitude outside Denmark as it is inside this country, the importance of this DNA group has been vastly underestimated. Ribotyping may help to cast light on this question.

The need for detailed routine identification of acinetobacters can be questioned. Most of the strains represent contamination or colonization, rather than infection. The use of a time-consuming and costly identification method such as ribotyping for this purpose is hardly worthwhile. However, in an epidemic situation, for descriptive purposes, a detailed identification of strains is crucial, and it is in this context that ribotyping is interesting.

In the present study, the ability of the strains to be typed and the reproducibility of the method were both 100%. In other studies, ribotyping has been shown to be a very discriminative and reproducible method for the typing of other bacterial species (15, 22, 24, 25). Stull et al. (25) showed that the ribotypes of three strains each of *E. coli* and *Pseudomonas cepacia* did not change after 16 serial subcultures. *P. cepacia* strains exhibiting different colony morphologies exhibited identical ribotypes, too. These investigators also found that three epidemiologically related strains of *Haemophilus influenzae* shared the same ribotype, whereas among 10 randomly selected strains of the same species, nine ribotypes were seen. Such results also have been found to apply to strains in the *A. calcoaceticus-A. baumannii* complex. Fourteen related strains of DNA group 13 showed an identical ribotype, as did two related strains of DNA group 2, in a study comparing multilocus enzyme electrophoresis and ribotyping for the typing of *Acinetobacter* strains (unpublished data). In a multinational study on the typing of strains in the *A. calcoaceticus-A. baumannii* complex from five outbreaks in three countries, the ribotype patterns were found stable for each outbreak (unpublished data). The stability of the restriction patterns of single isolates was confirmed by the reproducibility experiments in the present study. The discriminatory power of ribotyping for typing acinetobacters also can be judged from this study, because none of the strains studied a priori were thought to be related. The discriminatory index, which represents an average estimate of the probability that two unrelated strains will be typed differently, was very high (Table 4). Fifty-two ribotypes were seen among the 70 strains examined when the results of all three enzyme analyses were combined. Some of these 52 ribotypes included up to four strains each. This result may indicate a clonal relationship between epidemiologically unrelated strains, a phenomenon that is well known for *H. influenzae* (21).

In the context of typing, it is interesting that the strains used in this investigation were biotyped by use of the assimilation reactions of Bouvet and Grimont (4) in an earlier study (13). This typing method also has been shown to be reproducible and stable for related strains (4, 5). The numbers of biotypes found for strains of DNA groups 1, 2, 3, and 13 were 5, 11, 4, and 2, respectively (data not shown). There was no correlation between these biotypes and the ribotyping patterns found in the present study. Thus, in an epidemic situation, the combination of biotyping and ribotyping may provide strong supplementary epidemiological information for strains of the *A. calcoaceticus-A. baumannii* complex, with the exception of strains of DNA group 13, which almost invariably are of biotype 9 (data not shown). If supplementary information is needed for such strains, a typing method other than biotyping should be used.

Grimont and Bouvet recently ribotyped 4 strains of "genomic species 3" and 20 strains of *A. baumannii* using *Cla*I (16). However, they did not state whether these strains were identified by DNA-DNA hybridization. If not, strains from more than two DNA groups may have been present in their study, and it is therefore not possible to compare the results of the present investigation with those of the study of Grimont and Bouvet.

In the present study, when *Eco*RI was used, the method was definitive only for bands smaller than approximately 10 kb. The same was true for bands smaller than approximately 12 kb when *Sal*I was used (data not shown). Even bands smaller than this size were sometimes difficult to score, because of the proximity of some of the bands. We recommend the use of a reference strain from each DNA group on every gel to ensure proper scoring of the bands. For larger bands, the method was only comparative, i.e., allowing a comparison of the sizes of the bands only when the strains involved were run on the same gel, preferably in adjacent lanes; however, the numbers of bands could always be compared, even when the strains were run on different gels.

In conclusion, it has been shown that ribotyping may be used as a method for the taxonomic identification of strains in the *A. calcoaceticus-A. baumannii* complex. The method is highly discriminatory, and differences in the position of even a single band are reproducible. Thus, ribotyping has the potential to become a reference method for the typing and identification of strains in the *A. calcoaceticus-A. baumannii* complex.

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