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Comparison of Outbreak and Nonoutbreak *Acinetobacter baumannii* Strains by Genotypic and Phenotypic Methods

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Thirty-one *Acinetobacter baumannii* strains, comprising 14 strains from 14 outbreaks in different northwestern European cities and 17 sporadic strains, were compared by investigating various properties of the strains including biotype, antibiogram, cell envelope protein electrophoretic profile, ribotype pattern, and the band pattern generated by a novel genomic fingerprinting method, named AFLP, which is based on the selective amplification of restriction fragments. Results showed that 12 strains from unrelated outbreaks were linked together in two clusters according to their similarities by these typing methods, whereas sporadic strains were more heterogeneous. Outbreak strains appeared to be markedly more resistant to antibiotics than nonoutbreak strains. The uniformity of typing characters in two sets of outbreak strains suggests that strains in each cluster have a common clonal origin.

Acinetobacters are widespread in nature, and during recent decades they have emerged as nosocomial pathogens. Numerous outbreaks of nosocomial spread have been reported, with many reports commenting on the association of antibiotic resistance with outbreak strains (2–4, 9, 10, 16, 21, 22, 31).

Since 1986, the taxonomy of the genus *Acinetobacter* has been undergoing great changes. Today, the single species *A. calcoaceticus* (26) has been abandoned, and at least 20 DNA hybridization groups (genomic species; DNA groups) have been recognized (6, 8, 18, 33). Seven of these groups have species rank. It has been shown that *A. calcoaceticus* (DNA group 1), *A. baumannii* (DNA group 2), the unnamed DNA groups 3 and 13, and two recently described groups are closely related and are phenotypically very similar (5, 19, 25, 33).

Our laboratories in our respective countries are responsible for the type identification of *Acinetobacter* isolates from outbreaks in hospitals, and we found independently that strains from apparently unrelated outbreaks showed similar basic characteristics. We performed the study described here to establish whether this homogeneity in epidemiological type characters in outbreak strains was a property shared by strains from nonoutbreak situations. For this purpose, a comparison was made between *Acinetobacter* strains from outbreaks in hospitals in different cities in northwestern Europe and strains apparently not involved in outbreaks. The study was restricted to *A. baumannii*, since this species has been incriminated most in hospital outbreaks (20). Strains were collected over a period of several years, and a wide range of properties was investigated, including antibiotic susceptibility, biotype, cell envelope protein electrophoretic profile, ribotype, and the pattern obtained by AFLP, a novel high-resolution genomic fingerprinting method.

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MATERIALS AND METHODS

Strains. Fourteen *A. baumannii* strains from nosocomial outbreaks and 17 strains apparently not associated with outbreaks were investigated (Table 1). The strains originated from different cities and countries in northwestern Europe and were, with three exceptions (strains 27, 29, and 31), from hospitalized patients. Each of the 14 outbreak strains was selected from collections of isolates obtained during episodes of epidemic spread. In the present study, an outbreak is defined as a period of marked increase in the number of isolations of *Acinetobacter* spp. in one or more hospital wards with common staff and patients. The presumption of an outbreak was based on epidemiological data, supported by primary typing by using the antibiogram and/or the biotype, and was further confirmed in either of the two reference laboratories in The Netherlands and the United Kingdom by cell envelope or whole-cell protein electrophoresis. One isolate from each outbreak was selected on the basis of the fact that it shared a common protein profile with multiple isolates from the same episode. Details of the outbreaks are given in Table 1.

Identification of strains. The strains were identified as being of the genus *Acinetobacter* on the basis of the following properties: aerobic, gram-negative, nonmotile diplococci or short rods with a positive catalase reaction and a negative oxidase reaction (1). Strains were identified as being *A. baumannii* by a quantitative dot filter method (32). Briefly, bacterial preparations were hybridized on a filter with ^{125}I-labeled DNA from the type strain (ATCC 19606), and the stability of the duplexes was determined by thermal denaturation. The parameter used was $\Delta T_{m(e)}$, being the difference in the thermal denaturation midpoint between homologous and heterologous duplexes. The identified strains had $\Delta T_{m(e)}$ values of 0.0 to 3.0°C versus the type strain; other *Acinetobacter* strains had values of 5°C or higher.

AFLP. The principle of AFLP has previously been described in detail (35, 38). (The term AFLP is not an acronym but a name chosen by the inventors [35] because the method bears some resemblance to RFLP.) Total cellular DNA (1 μg), purified as described by Pitcher et al. (29), was digested with the restriction enzymes *Hin*dIII and *Taq*I, and restriction half-site-specific adaptors were ligated to all restriction fragments. Selective amplification was performed with PCR primers H01 (5′-GACTGCGTACCAGCTTa-3′) and T05 (GATGAGTCCTG ACCGAaa-3′) (selective bases are in lowercase) on 20 ng of DNA template (restriction fragments with ligated adaptors). The reactions were done on a Perkin-Elmer 9600 thermal cycler by using the stringency conditions described previously (35), except that the 12 last cycles were omitted because of the relatively low level of complexity of bacterial genomes compared with that of eukaryotic genomes (24). PCR products were separated by electrophoresis on 5% polyacrylamide–8.3 M urea sequencing gels. Since the H01 primer was

TABLE 1. Origins of the *Acinetobacter* strains used in the study and characteristics of outbreaks from which outbreak strains were selected[a]

Strain type and no.	Designation	Strain				Outbreak			Reference
		City (country)[b]	Yr of isolation	Specimen[c]	Ward[d]	Duration	Main specimen source[e]	No. of strains received (no. of patients)	
Outbreak strains									
1	GNU 1078	Leuven (B)	1990	Rectal mucosa	Postsurgical ICU	10 mo	Resp. tract, blood, wounds	16 (10)	
2	GNU 1079	Salford (UK)	1990	Tracheostomy site	ICU	≥1 mo	Incision sites	9 (4)	
3	GNU 1080	Salisbury (UK)	1989	CSU	Spinal unit	<12 mo	Catheter urine	11 (10)	
4	GNU 1081	Cork (IR)	1989	Tracheal aspirate	ICU	2 mo	Resp. tract	14 (9)	
5	GNU 1082	Basildon (UK)	1989	Wound	Burns unit	≥5 mo	Burns	30 (28)	
6	GNU 1083	London (UK)	1985–1988	Urine	Urological	40 mo	Urine	86 (63)	
7	GNU 1084	Sheffield (UK)	1987	Burn wound	Burns unit	1 mo	Burns	10 (9)	
8	GNU 1086	Newcastle (UK)	1989	Resp. tract	ICU	2 wk	Resp. tract	5 (5)	9, 11
9	RUH 134	Rotterdam (NL)	1982	Urine	ICUs	>12 mo	Resp. tract, urine, others	36 (36)	15
10	RUH 436	Utrecht (NL)	1984	Sputum	ICUs	12 mo	Resp. tract	13 (12)	
11	RUH 510	Nijmegen (NL)	1984	Bronchus	ICUs	>18 mo	Resp. tract	84 (60)	14
12	RUH 875	Dordrecht (NL)	1984	Urine	Surgical	9 mo	Urine	40 (40)	15
13	RUH 1752	Enschede (NL)	1986	Bronchus	ICU	3 mo	Resp. tract	23 (15)	16
14	RUH 2037	Venlo (NL)	1986	Sputum	ICU	4 mo	Resp. tract, urine, pus	36 (24)	10, 11
Nonoutbreak strains									
15	RUH 414	Leyden (NL)	1978	Ext. aud. canal					
16	RUH 1093	Rotterdam (NL)	1985	Sputum					
17	RUH 1486	Rotterdam (NL)	1985	Umbilicus					
18	RUH 1907	Rotterdam (NL)	1986	Bronchus					
19	RUH 2180	Nijmegen (NL)	1987	Sputum					
20	TU 91	Malmoe (SW)	1980	Sputum					
21	TU 144	Malmoe (SW)	1980	Wound					
22	RUH 2688	Rotterdam (NL)	1987	Pharynx					
23	TU 133	Malmoe (SW)	1980	Wound					
24	TU 147	Malmoe (SW)	1980	Wound					
25	PGS 189	Odense (DK)	1984	Crural ulcer					
26	PGS 9771	Naestved (DK)	1990	Urine					
27	PGS 10074	Vejle (DK)	1990	Urine					
28	PGS 10086	Vejle (DK)	1990	Urine					
29	MB 142	London (UK)	1982	Skin					
30	SJH 9/MB 264	London (UK)	1981	Skin					
31	SJH 26/MB 288	London (UK)	1988	Nail fold					

[a] One strain was selected from each outbreak.
[b] B, Belgium; UK, United Kingdom; IR, Ireland; NL, The Netherlands; DK, Denmark.
[c] CSU, catheter urine; ext. aud., external auditory; Resp., respiratory.
[d] Strains were of hospital origin except for isolates 27 and 31 (outpatient) and 29 (school child); ICU, intensive care unit.
[e] Resp., respiratory.

labeled with ^{32}P, selectively amplified fragments could be visualized as banding patterns by autoradiography. A recent evaluation of the AFLP method as a new tool in bacterial taxonomy included some simple tests to assess possible variation in AFLP patterns (24). In one experiment, DNA was prepared from six bacterial strains on six separate occasions following subculture at intervals of 1 to 3 days, and AFLP patterns were obtained from all 36 DNAs. For each strain, the AFLP patterns from the six separate DNA preparations were indistinguishable, and it was concluded that, under the stringent PCR conditions prescribed by Vos et al. (35), AFLP of bacterial DNAs is highly reproducible. Data acquisition was done with a RayVen RSU1 densitoscanner (X-Ray Scanner Corporation, Torrance, Calif.), and digitized signals were registered on a computer disk. Data were then further processed by GelCompar software (Applied Maths, Kortrijk, Belgium). The similarity between all possible pairs of traces was expressed by the Pearson product moment correlation coefficient (r), and cluster analysis was performed by the unweighted pair group average linkage method (UPGMA).

Ribotyping. DNA was extracted and digested with *Eco*RI as described previously (17). DNA fragments were separated by agarose electrophoresis, transferred onto nylon membranes by vacuum blotting, and hybridized with biotinylated cDNA from highly polymerized rRNA from *Escherichia coli* (Boehringer Mannheim). The profiles were compared visually, and the presence or absence of a band at each band position was scored as plus or minus. Grouping of strains according to similarity was performed by cluster analysis of the binary data by using the Advanced Statistics Facility of the SPSS software package (27). Euclidian distance was used as a measure for similarity between all possible pairs of strains, and grouping was obtained by the UPGMA clustering algorithm.

Cell envelope protein electrophoretic typing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell envelope proteins was performed as described earlier (12, 13). Briefly, cell envelope fractions were obtained by ultrasonic disruption of cells and subsequent fractional centrifugation. SDS-PAGE was performed with a stacking gel of 3% acrylamide and a running gel of 11% acrylamide. Gels were stained with fast green FCF (Sigma Chemical Co., St. Louis, Mo.). Images of photographed profiles were recorded with a Hewlett-Packard Scan Jet IIP (Hewlett-Packard Co., Vancouver, Wash.). Further processing and cluster analysis were performed by using GelCompar, with r used as the similarity measure and UPGMA used for grouping.

Biotyping. Strains were differentiated as described previously (19) on the basis of liquefaction of gelatin, hemolysis of sheep erythrocytes, production of urease, and growth at 37 and 44°C in Bacto brain heart infusion (BHI) broth (Difco). Utilization of single carbon sources was determined on *trans*-aconitate, L-histidine, malonate, histamine, citrate, levulinate, citraconate, L-phenylalanine, 4-hydroxybenzoate, and L-tartrate. Incubation was at 30°C unless otherwise indicated. The reactions for hemolysis and growth in BHI broth at different temperatures were read for up to 2 days; the remaining tests were read for up to 6 days.

Antibiotic susceptibility determination. Strains were tested by a tablet diffu-

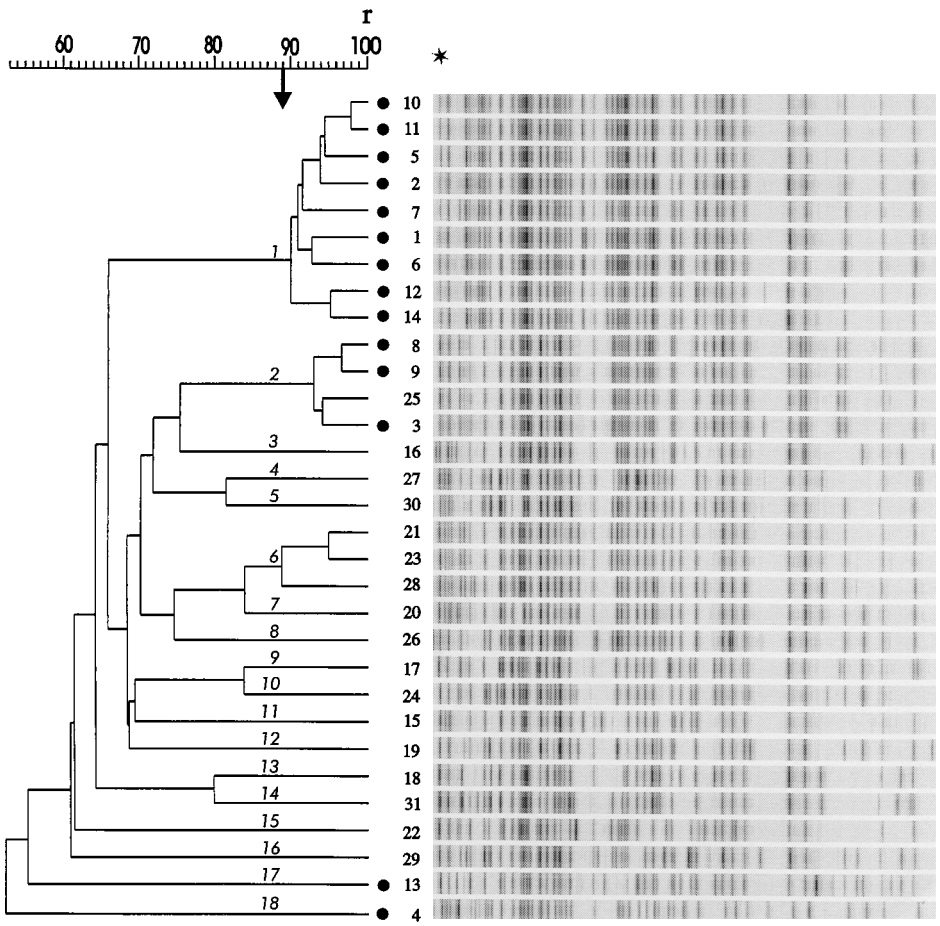

FIG. 1. Digitized AFLP patterns and dendrogram of grouping according to similarity of outbreak (●) and nonoutbreak *A. baumannii* strains. Strains are designated with the serial numbers given in Table 1. Clustering was done with GelCompar by using UPGMA linkage of correlation coefficients. The arrow denotes the cutting level for separation clusters and single strains. The asterisk indicates the top of the gel.

sion method for susceptibility to cefotaxime (30 μg), ceftazidime (30 μg), imipenem (15 μg), penicillin (5 μg), ampicillin (33 μg), piperacillin (100 μg), ticarcillin (75 μg), gentamicin (40 μg), tetracycline (80 μg), chloramphenicol (60 μg), sulfonamides (240 μg), and nalidixic acid (130 μg) (Neosensitabs, Rosco, Taastrup, Denmark). The medium was Danish Blood Agar (Statens Seruminstitut, Copenhagen, Denmark) which contained 5% defibrinated horse blood. Grouping of strains according to these susceptibilities was performed by UPGMA cluster analysis on the basis of the diameters of the inhibition zones, with Euclidian distance used as a measure of dissimilarity.

Strains were tested for susceptibility to neomycin on Iso-Sensitest agar (Unipath) containing 2% lysed blood and 32 μg of neomycin per ml. The MICs of selected antibiotics were determined by an agar incorporation method in Iso-Sensitest agar supplemented with 2% lysed horse blood. Antibiotics were incorporated with respect to their potencies, and an inoculum of approximately 10^4 CFU per spot was delivered from overnight nutrient broth cultures of the organism being tested. Control strains were *Pseudomonas aeruginosa* NCTC 10662 and NCTC 13418.

RESULTS

Epidemiology of outbreaks. The outbreaks outlined in Table 1, several of which were described elsewhere (9–11, 14–16), occurred between 1981 and 1990, most of them in intensive care units. They included brief explosive periods and prolonged episodes of endemicity involving in total at least 325 patients. In several outbreaks the organisms were mainly restricted to the respiratory or urinary tract, while in other hospitals they were recovered from different patient body sites.

AFLP. The fingerprints generated by AFLP comprised approximately 50 well-separated bands. These patterns and their groupings are depicted in the dendrogram shown in Fig. 1. At a delineation level of 89.0% similarity, a total of 18 clusters and single strains were distinguished. The majority of outbreak strains were linked in two clusters. The first cluster (cluster 1) included nine outbreak strains that linked at 89.9%, and the second cluster (cluster 2) consisted of three outbreak strains and a sporadic strain that linked at 92.8%. Strains in these clusters had patterns with differences in one or two band positions only. Overall, sporadic strains were more diverse than the outbreak strains, except for three strains in cluster 6 linked at 89.0%. The linkage levels of the other sporadic strains ranged between 61% (AFLP pattern 16) and 84% (AFLP pattern 7). Two outbreak strains (strains 4 and 13) also displayed a very low level of similarity (<60%) to each other and were clearly far less related to any of the 29 other isolates.

Ribotyping. A variety of ribotype profiles was observed, with each pattern comprising 8 to 10 bands in the 2.0- to 23.1-kb size range and with a total number of 17 different band positions. The patterns and results of cluster analysis are shown in Fig. 2. Thirteen clusters and single strains were distinguished in the dendrogram at a dissimilarity level of 14%. (The absolute distance between clusters at this level was 1.5, while the maximum distance was 10.8.) Nine strains from outbreaks in the United Kingdom, The Netherlands, Belgium, and Ireland were indistinguishable and were linked together in the dendrogram in cluster 3. Five other outbreak strains were found together

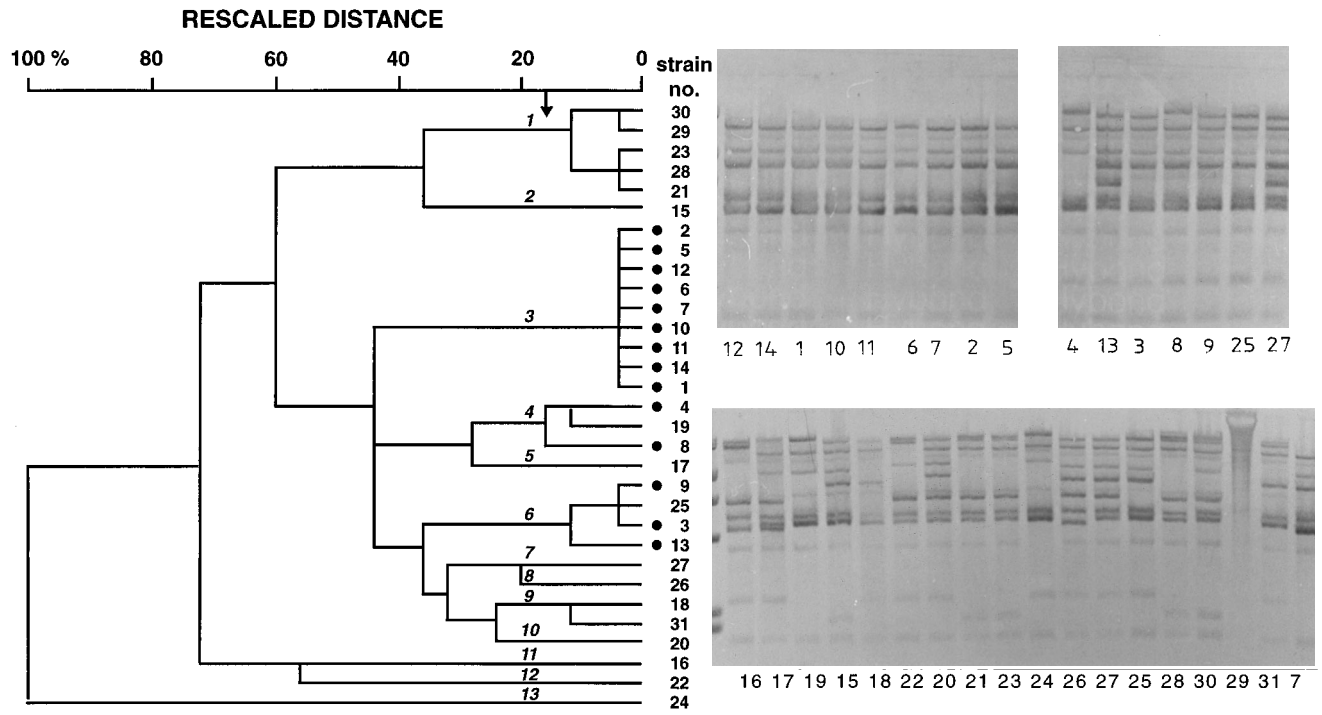

FIG. 2. Ribotypes and dendrogram of cluster analysis of visually read patterns of outbreak (●) and nonoutbreak *A. baumannii* strains. Strains are designated by the serial numbers in Table 1. Clustering was performed by using UPGMA as the clustering criterion and the Euclidian distance as the dissimilarity measure.

with sporadic isolates in clusters 4 and 6. Five nonoutbreak strains were linked in cluster 1, whereas the remaining sporadic isolates did not show a clear clustering pattern.

Cell envelope protein typing. Protein profiles were characterized by approximately 10 bands of relatively high staining intensity and 20 to 30 bands of relatively low staining intensity (Fig. 3). Cluster analysis of digitized images distinguished 10 clusters and single strains at a similarity level of 88%. Cluster 1 comprised eight outbreak and two nonoutbreak strains, while cluster 2 contained three outbreak and two nonoutbreak strains. Only one outbreak strain (strain 13) was identified in cluster 4 together with seven sporadic strains. Another outbreak strain (strain 4) was found in cluster 5, which linked to cluster 4 at 86%.

Biotyping. All strains were able to acidify glucose and grew well at 37 and 44°C, which identified them phenotypically as *A. baumannii*. Biotyping by the scheme of Bouvet and Grimont (7), based on growth on levulinate, citraconate, L-phenylalanine, 4-hydroxybenzoate, and L-tartrate, revealed 10 biotypes, i.e., biotypes 1, 2, 5, 6, 8, 9, 11, 16, 18, and 19 (Table 2). Biotype 6 was the most predominant among both outbreak and nonoutbreak strains, with eight and seven strains, respectively. Apart from biotype 9, which was observed in four strains, other biotypes occurred in only one or two strains.

Antibiogram typing. By tablet diffusion, strains were uniform in their resistance to penicillin and, with one exception, to chloramphenicol. All strains were susceptible to imipenem, and susceptibility to other antibiotics varied. Grouping according to inhibition zone diameter determined by tablet diffusion is shown in the dendrogram in Fig. 4. Eight clusters and single strains were distinguished at a relative distance between clusters of 19% (absolute distance, 345). These groups and strains were grouped together in three main clusters (clusters A, B, and C). Cluster A comprised 13 strains, 11 of which were from outbreaks; cluster B comprised 3 strains, including 2 from outbreaks; and cluster C, with 17 strains, included 1 outbreak strain. A striking feature of cluster A strains was their multiresistance according to both the number of resistance traits and the level of resistance to each antibiotic. However, all strains were susceptible to imipenem and most strains were also susceptible to nalidixic acid. Cluster C contained the most susceptible strains showing no resistance traits except different degrees of low-level resistance to the broad-spectrum β-lactam antibiotics. Cluster B contained strains with susceptibilities between those of the strains in clusters A and C. All strains in cluster B were susceptible to tetracycline but were resistant to sulfonamides. MIC determinations showed that for all but three of the outbreak strains, gentamicin MICs were ≥32 mg/liter. In contrast, the mean gentamicin MIC for the sporadic isolates was ≤1 mg/liter. Similarly, the MIC of carbenicillin for the outbreak strains was generally ≥256 mg/liter, compared with 16 to 32 mg/liter for the sporadic isolates. A further noteworthy observation was that all sporadic isolates exhibited susceptibility or intermediate susceptibility to neomycin, but only 5 of the 14 epidemic isolates were susceptible to this antibiotic.

Combination of typing results. The combined typing results (Table 2) showed that four groups of strains (groups I to IV) were distinguished; the strains within each group were more similar to each other than to the other strains. The most striking was group I, which contained nine outbreak strains. Strains in this group had AFLP pattern 1, ribotype 3, protein profiles 1 (eight strains) and 3 (one strain), biotype 6 (seven strains), biotypes 19 and 11 (one strain each), and antibiogram types 1 and 2 (six and three strains, respectively). Group II comprised four strains, including three from outbreaks, that were characterized by AFLP pattern 2, ribotype 4 or 6, and protein profile 2. Biotypes and antibiograms were not unique for this group.

Group III, comprising three nonoutbreak strains, was dis-

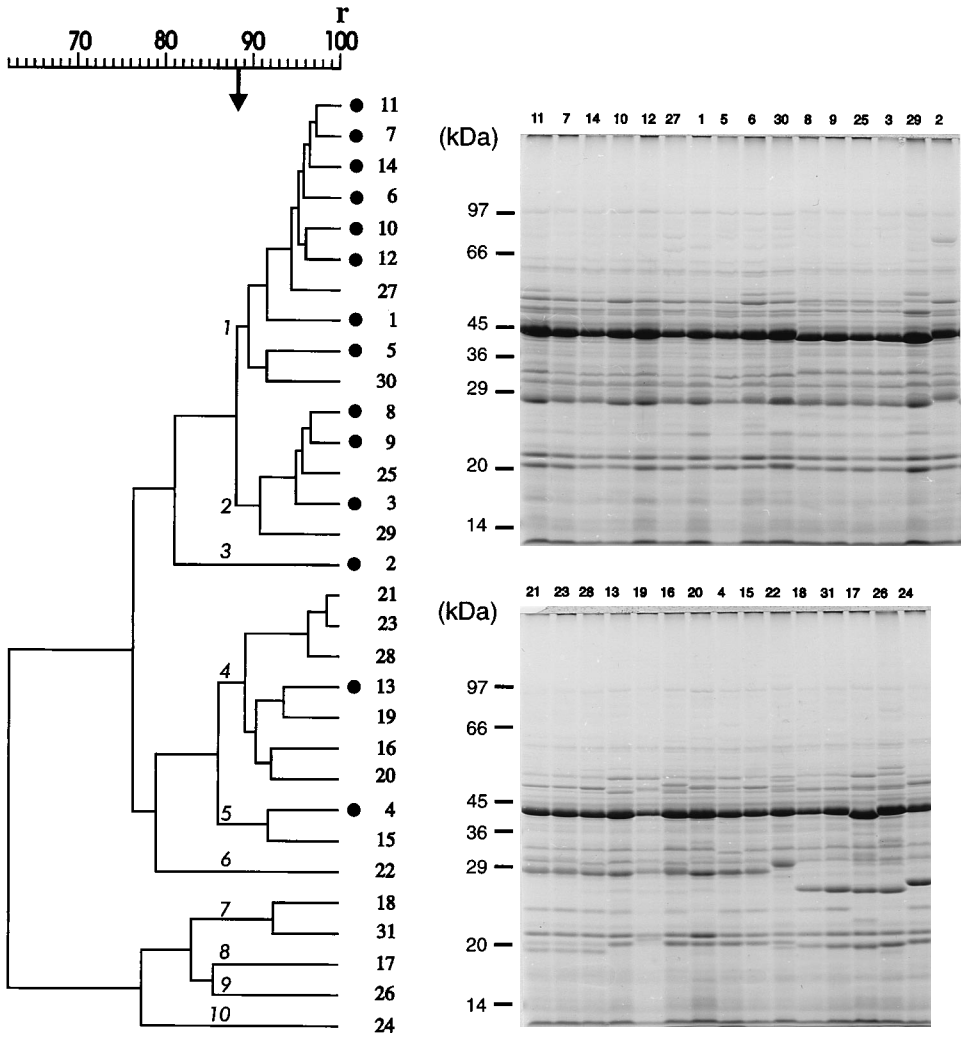

FIG. 3. Protein profiles and dendrogram of grouping according to similarity of outbreak (●) and nonoutbreak *A. baumannii* strains. Clustering of digitized patterns was performed by using GelCompar, with UPGMA as the clustering criterion and the correlation coefficient as the distance measure.

tinguished by AFLP pattern 6, ribotype 1, protein profile 4, biotype 5 or 6, and antibiogram type 6. Group IV included two nonoutbreak strains of common clusters or types, except for AFLP patterns; i.e., they had AFLP pattern 13 and 14 linked at 80% (Fig. 1). Only 2 outbreak strains failed to cluster, whereas 11 nonoutbreak strains failed to cluster.

DISCUSSION

Epidemic spread of multiresistant *Acinetobacter* species is well documented, but the determinants of the epidemic behavior of strains are unknown. The aim of our study was not to evaluate typing methods but to compare strains from outbreaks of infection with those from sporadic cases of infection or colonization. We used four methods to investigate different levels of genetic expression, including ribotyping, protein electrophoretic typing, biotyping, and antibiogram typing, which had previously been evaluated for the identification of *Acinetobacter* strains (11). In addition, a novel high-resolution genomic fingerprinting method, AFLP, was applied. AFLP has recently been found to be a powerful method for the delineation of strain relatedness below the species level for several genera, including the genus *Acinetobacter* (23, 24). The strains included were identified as being *A. baumannii* by DNA-DNA hybridization, because this is the standard method for separating this group from the other closely related groups of the *A. calcoaceticus-A. baumannii* complex (5, 19, 25, 33).

The most striking finding of our study was the distinction of four groups of strains that were highly similar by at least one genomic and one other typing method (Table 2), which could be explained by a common clonal origin (28). Two groups (groups I and II) mainly comprised strains from outbreaks at different locations in northwestern Europe. Whether these strains have some unknown virulence attribute related to invasiveness, transmissibility, or an enhanced ability to colonize compromised patients remains to be established. Spread of these strains may have occurred by transfer of patients between hospitals, cities, and countries over the course of time, but there is no evidence of this. Another possibility is that strains with these characteristics circulate in the community and are selected in hospitals through selective antibiotic pressure. Studies on the population structure of *A. baumannii* in and outside hospitals might provide an explanation for the high prevalence of the similarity groups in outbreaks. Strains of two other clones (groups III and IV) were, to our knowledge, not

TABLE 2. Grouping of strains according to combination of typing results[a]

Group and strain no.	City (country)[b]	AFLP type	Ribotype	Protein electrophoretic type	Biotype	Antibiogram type
Group I[c]						
7●[d]	Sheffield (UK)	1	3	1	6	1
6●	London (UK)	1	3	1	6	1
12●	Dordrecht (NL)	1	3	1	6	1
5●	Basildon (UK)	1	3	1	6	1
11●	Nijmegen (NL)	1	3	1	6	1
1●	Leuven (B)	1	3	1	6	2
14●	Venlo (NL)	1	3	1	6	2
10●	Utrecht (NL)	1	3	1	19	1
2●	Salford (UK)	1	3	3	11	2
Group II						
8●	Newcastle (UK)	2	4	2	2	5
9●	Rotterdam (NL)	2	6	2	1	1
25	Odense (DK)	2	6	2	2	3
3●	Salisbury (UK)	2	6	2	9	3
Group III						
23	Malmoe (SW)	6	1	4	5	6
28	Vejle (DK)	6	1	4	6	6
21	Malmoe (SW)	6	1	4	6	6
Group IV						
18	Rotterdam (NL)	13	9	7	6	6
31	London (UK)	14	9	7	6	6
Ungrouped strains						
16	Rotterdam (NL)	3	11	6	5	6
27	Vejle (DK)	4	7	1	19	4
30	London (UK)	5	1	1	6	1
20	Malmoe (SW)	7	10	4	6	7
26	Naestved (DK)	8	8	9	6	6
17	Rotterdam (NL)	9	5	8	16	6
24	Malmoe (SW)	10	13	10	18	6
15	Leiden (NL)	11	2	5	9	6
19	Nijmegen (NL)	12	4	4	9	6
22	Rotterdam (NL)	15	12	6	8	8
29	London (UK)	16	1	2	8	6
13●	Enschede (NL)	17	6	4	6	6
4●	Cork (IR)	18	4	5	9	4

[a] Types were determined by AFLP, ribotyping, protein electrophoresis, and antibiogram determination correspond to the clusters in Fig. 1 to 4; biotypes were determined by the scheme of Bouvet and Grimont (6).

[b] UK, United Kingdom; NL, The Netherlands; B, Belgium; DK, Denmark; SW, Sweden; IR, Ireland.

[c] Groups are based on linkage in the same cluster or are designated the same type by at least one genotypic and one phenotypic method.

[d] ●, outbreak strain.

associated with epidemic spread and were not particularly resistant to antibiotics.

The typing results showed that strains of the epidemic clones of *A. baumannii* were, with few exceptions, homogeneous in their genomic fingerprint and protein profiles, but were more variable in their biotype and antibiogram profiles. The variation in these phenotypic characteristics may be of use for further discrimination of these strains in complex situations (30), e.g., during the concurrent spread of different strains of the same clone.

Two outbreak isolates (strains 4 and 13) were clearly different from strains of clones I and II, which shows that outbreaks are not exclusively associated with strains of clones I and II. In addition, one nonoutbreak strain (strain 25) shared features with clone II (Table 2). Nevertheless, identification of strains with characteristics of clones I or II in some hospital wards should be a cause of concern, and rapid and simple methods are needed for tracing these strains. The electrophoretic methods used in the present study are time-consuming, require experience and strict standardization, and are not likely to be performed in routine microbiology laboratories. Given the explosive evolution of molecular typing methods, it should be possible to develop a strategy for the rapid and easy recognition of the clonal types. For instance, the readily performed PCR fingerprinting methods that make use of arbitrary primers (36) or primers that correspond to conserved motifs in bacterial repetitive elements (31, 37) could be tried. A first step would be to establish whether these primers provide fingerprints specific for clones I and II. Difficulties related to intra- and interlaboratory variations in PCR fingerprinting (34) could be overcome by inclusion of strains representative for each clone in each run. (We propose that strains RUH 134 [strain 9] and RUH 875 [strain 12] be used as reference strains for clones I and II, respectively.) Thus, outbreak isolates could be screened for their similarity to clones I and II in different laboratories. Combinations of genotypic and phenotypic methods are necessary to allocate strains definitely to a given clone. It may also be worthwhile to investigate the development of clone-specific DNA probes.

In conclusion, our study showed so many identical phenotypic and genotypic traits in strains from outbreaks in different locations that a common clonal origin is a likely explanation. Early recognition and careful monitoring of these organisms and, if necessary, implementation of infection control measures may halt their spread in hospitals. Furthermore, studies investigating the global distribution of these and perhaps other clones should be considered for epidemiological reasons. Finally, a repertoire of methods for the identification of genetically distinct strains of *Acinetobacter* is now available, and fur-

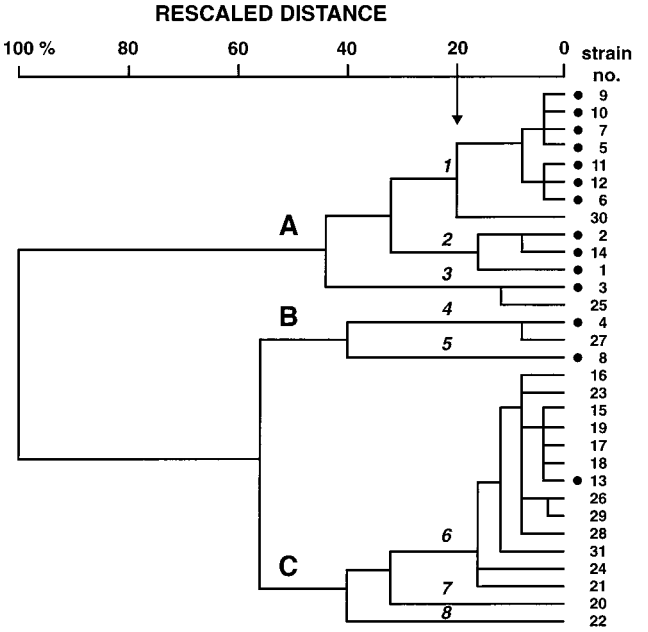

FIG. 4. Grouping of outbreak (●) and nonoutbreak *A. baumannii* strains according to antibiotic susceptibility pattern (zone sizes of tablet diffusion). Clustering was based on UPGMA and Euclidian distance.

ther investigation should address the epidemiological factors that favor the emergence of specific strains in hospital wards.

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REFERENCES

1. **Barrow, G. I., and R. K. A. Feltham (ed.).** 1993. Cowan and Steel's manual for the identification of medical bacteria, 3rd ed. Cambridge University Press, Cambridge.
2. **Beck-Sagué, C. M., W. R. Jarvis, J. H. Brook, D. H. Culver, A. Potts, E. Gay, B. W. Shotts, B. Hill, R. L. Anderson, and M. P. Weinstein.** 1990. Epidemic bacteremia due to *Acinetobacter baumannii* in five intensive care units. Am. J. Epidemiol. **132:**723–733.
3. **Bergogne-Bérézin, E.** 1995. The increasing significance of outbreaks of *Acinetobacter* spp.: the need for control and new agents. J. Hosp. Infect. **30:**441–452.
4. **Bergogne-Bérézin, E., and M. L. Joly-Guillou.** 1985. An underestimated nosocomial pathogen, *Acinetobacter calcoaceticus*. J. Antimicrob. Chemother. **16:**535–538.
5. **Bernards, A. T., L. Dijkshoorn, J. van der Toorn, B. R. Bochner, and C. P. A. van Boven.** 1995. Phenotypic characterisation of *Acinetobacter* strains of 13 DNA-DNA hybridisation groups by means of the Biolog system. J. Med. Microbiol. **42:**113–119.
6. **Bouvet, P. J. M., and P. A. D. Grimont.** 1986. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. Int. J. Syst. Bacteriol. **36:**228–240.
7. **Bouvet, P. J. M., and P. A. D. Grimont.** 1987. Identification and biotyping of clinical isolates of *Acinetobacter*. Ann. Inst. Pasteur/Microbiol. **138:**569–578.
8. **Bouvet, P. J. M., and S. Jeanjean.** 1989. Delineation of new proteolytic genomic species in the genus *Acinetobacter*. Res. Microbiol. **140:**291–299.
9. **Cefai, C., J. Richards, F. K. Gould, and P. McPeake.** 1990. An outbreak of *Acinetobacter* respiratory tract infection resulting from incomplete disinfection of ventilatory equipment. J. Hosp. Infect. **15:**177–182.
10. **Crombach, W. H. J., L. Dijkshoorn, M. van Noort-Klaassen, J. Niessen, and G. van Knippenberg-Gordebeke.** 1989. Control of an epidemic spread of a multi-resistant strain of *Acinetobacter calcoaceticus* in a hospital. Intensive Care Med. **15:**166–170.
11. **Dijkshoorn, L., H. M. Aucken, P. Gerner-Smidt, M. E. Kaufmann, J. Ursing, and T. L. Pitt.** 1993. Correlation of typing methods for *Acinetobacter* isolates from hospital outbreaks. J. Clin. Microbiol. **31:**702–705.
12. **Dijkshoorn, L., M. F. Michel, and J. E. Degener.** 1987. Cell envelope protein profiles of *Acinetobacter calcoaceticus* strains isolated in hospitals. J. Med. Microbiol. **23:**313–319.
13. **Dijkshoorn, L., I. Tjernberg, B. Pot, M. F. Michel, J. Ursing, and K. Kersters.** 1990. Numerical analysis of cell envelope protein profiles of *Acinetobacter* strains classified by DNA-DNA hybridization. Syst. Appl. Microbiol. **13:**338–344.
14. **Dijkshoorn, L., R. van Dalen, A. van Ooyen, D. Bijl, I. Tjernberg, M. F. Michel, and A. M. Horrevorts.** 1993. Endemic acinetobacter in intensive care units: epidemiology and clinical impact. J. Clin. Pathol. **46:**533–536.
15. **Dijkshoorn, L., A. van Ooyen, W. C. J. Hop, M. Theuns, and M. F. Michel.** 1990. Comparison of clinical acinetobacter strains using a carbon source growth assay. Epidemiol. Infect. **104:**443–453.
16. **Dijkshoorn, L., J. L. Wubbels, A. J. Beunders, J. E. Degener, A. L. Boks, and M. F. Michel.** 1989. Use of protein profiles to identify *Acinetobacter calcoaceticus* in a respiratory care unit. J. Clin. Pathol. **42:**853–857.
17. **Garaizar, J., M. E. Kaufmann, and T. L. Pitt.** 1991. Comparison of ribotyping with conventional methods for the type identification of *Enterobacter cloacae*. J. Clin. Microbiol. **29:**1303–1307.
18. **Gerner-Smidt, P., and I. Tjernberg.** 1993. *Acinetobacter* in Denmark. II. Molecular studies of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. APMIS **101:**826–832.
19. **Gerner-Smidt, P., I. Tjernberg, and J. Ursing.** 1991. Reliability of phenotypic tests for identification of *Acinetobacter* species. J. Clin. Microbiol. **29:**277–282.
20. **Grimont, P. A. D., and P. J. M. Bouvet.** 1990. Taxonomy of *Acinetobacter*, p. 25–36. *In* K. J. Towner, E. Bergogne-Bérézin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York.
21. **Hartstein, A. I., A. L. Rashad, J. M. Liebler, L. A. Actis, J. Freeman, J. W. Rourke, T. B. Stibolt, M. E. Tolmasky, G. R. Ellis, and J. H. Crosa.** 1988. Multiple intensive care unit outbreak of *Acinetobacter calcoaceticus* subspecies *anitratus* respiratory infection and colonization associated with contaminated, reusable ventilator circuits and resuscitation bags. Am. J. Med. **85:**624–631.
22. **Horrevorts, A., K. Bergman, L. Kollée, I. Breuker, I. Tjernberg, and L. Dijkshoorn.** 1995. Clinical and epidemiological investigations of *Acinetobacter* genomospecies 3 in a neonatal intensive care unit. J. Clin. Microbiol. **33:**1567–1572.
23. **Huys, G., R. Coopman, M. Vancanneyt, I. Kersters, W. Verstraete, K. Kersters, and P. Janssen.** 1993. High resolution differentiation of aeromonads. Med. Microbiol. Lett. **2:**248–255.
24. **Janssen, P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters.** Evaluation of the DNA fingerprinting method AFLP™. Microbiology, in press.
25. **Kämpfer, P., I. Tjernberg, and J. Ursing.** 1993. Numerical classification and identification of *Acinetobacter* genomic species. J. Appl. Bacteriol. **75:**259–268.
26. **Lautrop, H.** 1974. Genus IV. *Acinetobacter* Brisou and Prévot 1954, p. 436–438. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
27. **Norusis, M. J.** 1988. SPSS/PC+ advanced statistics, version 2.0, p. B-71–B-101. SPSS Inc., Chicago.
28. **Ørskov, F., and I. Ørskov.** 1983. Summary of a workshop on the clone concept in epidemiology, taxonomy, and evolution of the Enterobacteriaceae and other bacteria. J. Infect. Dis. **148:**346–357.
29. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
30. **Seifert, H., B. Boullion, A. Schulze, and G. Pulverer.** 1994. Plasmid DNA profiles of *Acinetobacter baumannii*: clinical application in a complex endemic setting. Infect. Control Hosp. Epidemiol. **15:**520–528.
31. **Struelens, M. J., E. Carlier, N. Maes, E. Serruys, W. G. V. Quint, and A. van Belkum.** 1993. Nosocomial colonization and infection with multiresistant *Acinetobacter baumannii*: outbreak delineation using DNA macrorestriction analysis and PCR-fingerprinting. J. Hosp. Infect. **25:**15–32.
32. **Tjernberg, I., E. Lindh, and J. Ursing.** 1989. A quantitative bacterial dot method for DNA-DNA hybridization and its correlation to the hydroxyapatite method. Curr. Microbiol. **18:**77–81.
33. **Tjernberg, I., and J. Ursing.** 1989. Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. APMIS **97:**595–605.
34. **Van Belkum, A., J. Kluytmans, W. van Leeuwen, R. Bax, W. Quint, E. Pieters, A. Fluit, C. Vandenbroucke-Grauls, A. van den Brule, H. Koeleman, W. Melchers, J. Meis, A. Elaichouni, M. Vaneechoutte, F. Moonens, N. Maes, M. Struelens, F. Tenover, and H. Verbrugh.** 1995. Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **33:**1537–1547.
35. **Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Freijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau.** AFLP™: a new concept for DNA fingerprinting. Nucleic Acids Res. **23:**4407–4414.
36. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18:**7213–7218.
37. **Woods, C. R., J. Versalovic, T. Koeuth, and J. R. Lupski.** 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. **31:**1927–1931.
38. **Zabeau, M., and P. Vos.** March 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1, bulletin 93/13.