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Development of a Multilocus Sequence Typing Scheme for Characterization of Clinical Isolates of *Acinetobacter baumannii*

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In this study a multilocus sequence typing (MLST) scheme for *Acinetobacter baumannii* was developed and evaluated by using 40 clinical *A. baumannii* isolates recovered from outbreaks in Spanish and German hospitals during the years 1990 to 2001, as well as isolates from other European hospitals and two DSMZ reference strains of *A. baumannii*. For comparison, two isolates of *Acinetobacter* species 13 (sensu Tjernberg and Ursing), two clinical isolates, and three DSMZ strains of *A. calcoaceticus* (both belonging to the *A. calcoaceticus-A. baumannii* complex) were also investigated. Primers were designed for conserved regions of housekeeping genes, and 305- to 513-bp internal fragments of seven such genes—*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*—were sequenced for all strains. The number of alleles at individual loci ranged from 6 to 12, and a total of 20 allelic profiles or sequence types were distinguished among the investigated *A. baumannii* strains. The MLST data were in high concordance with the epidemiologic typing results generated by pulsed-field gel electrophoresis and amplified fragment length polymorphism fingerprinting. The MLST scheme provides a high level of resolution and an excellent tool for studying the population structure and long-term epidemiology of *A. baumannii*.

Bacteria of the genus *Acinetobacter* are ubiquitously distributed in nature and often involved in the colonization and infection of hospitalized patients, particularly those in intensive care units (4, 10). The taxonomy of the genus *Acinetobacter* has a long and complicated history. In 1986, Bouvet and Grimont divided the genus into 12 genomic species based on DNA-DNA hybridization and proposed a variety of biochemical tests for phenotypic species identification (5). Three years later, Bouvet and Jeanjean reported new proteolytic *Acinetobacter* strains designated genomic species 13, 14, 15, and 17 (6). At the same time, three additional genomic species were proposed by Tjernberg and Ursing and designated *Acinetobacter* genomic species 13TU, 14TU, and 15TU (37). Currently, at least 32 genomic species are recognized among the genus *Acinetobacter*. This classification opened the possibility to analyze the epidemiology of this diverse genus. However, conventional or commercially available biochemical test systems are not able to identify unambiguously most genomic species (4, 23). In particular, isolates belonging to genomic species 1 (*Acinetobacter calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU are almost indistinguishable by biochemical tests (17). In 1992, the designation *A. calcoaceticus-A. baumannii* complex was therefore suggested for these genotypically distinct but phenotypically very similar bacterial species (16).

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Numerous studies have supported the observation that *A. baumannii* is the species most commonly involved in nosocomial infections such as pneumonia, wound infection, and bloodstream infection (4, 7, 15). The implication of *A. baumannii* in hospital outbreaks of nosocomial infection (8, 10, 29) has been attributed to their increasing antimicrobial resistance (21, 39), and their ability to survive on inanimate and dry surfaces (2, 24), both contributing to an increased survival time in the hospital environment.

To better understand the epidemiology and in particular the mode of spread of *A. baumannii*, a number of molecular typing systems have been developed, including PCR-based methods such as random(ly) amplified polymorphic DNA analysis (20), integrase gene PCR (25), infrequent-restriction-site PCR (42), ribotyping (16, 35), amplified fragment length polymorphism (AFLP) analysis (9), and pulsed-field gel electrophoresis (PFGE) (18, 35). PFGE restriction analysis of chromosomal bacterial DNA has been used with excellent results in epidemiologic studies of numerous *A. baumannii* outbreaks and is currently regarded as the gold standard for epidemiologic typing (8, 18).

Some of the genomic methods, in particular ribotyping and AFLP, are able to resolve bacteria of the *A. calcoaceticus-A. baumannii* complex at the species level with a high degree of discrimination (9, 16). All of these methods rely on the generation of a distinct pattern or DNA "fingerprint" that is usually visualized by ethidium bromide staining or nucleic acid hybridization. So-called comparative typing systems, i.e., methods that depend on comparisons of DNA fragment patterns on gels, such as PFGE and random(ly) amplified polymorphic

DNA analysis, are well suited for local outbreak investigation. For a global epidemiologic analysis, however, comparison of the results obtained at different laboratories would be required, but poor interlaboratory reproducibility remains a critical and unresolved issue.

Multilocus sequence typing (MLST) is a highly discriminative method of typing microorganisms (28) and has been applied successfully for the epidemiologic characterization of a variety of clinically important bacterial pathogens including *Neisseria meningitidis* (28), *Streptococcus pneumoniae* (14), *Streptococcus pyogenes* (13), *Staphylococcus aureus* (12, 19), *Campylobacter jejuni* (11), *Enterococcus faecium* (22), *Vibrio cholerae* (26), and *Haemophilus influenzae* (30).

MLST is based on the same principles as multilocus enzyme electrophoresis but relies directly on DNA sequence comparison of internal fragments of protein encoding housekeeping genes. These housekeeping genes are chosen for analysis because their products play vital function, being present in all isolates of a given species, and mutations within them are assumed to be neutral. For each gene fragment, the different sequences are assigned as distinct alleles, and each isolate is defined by the alleles at each of the housekeeping loci (the allelic profile or sequence type [ST]). MLST offers the possibility to transfer typing data from laboratory to laboratory or compare results via the internet (http://mlst.zoo.ox.ac.uk), thus providing a powerful tool for global epidemiologic studies, as well as for studies of the population biology of bacterial species. We describe here an MLST scheme for *A. baumannii* based on the nucleotide sequences of seven housekeeping loci.

MATERIALS AND METHODS

Bacterial isolates. A total of 49 *Acinetobacter* isolates were used in the present study: 42 *A. baumannii* isolates, including reference strains DSM 30008 and DSM 30007 (*A. baumannii* type strain ATCC 19606) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ); 2 clinical *Acinetobacter* genomic species 13TU isolates; and 5 *A. calcoaceticus* isolates, including reference strains DSM 1139, DSM 7324, and DSM 30006 (Table 1). *A. baumannii* isolates were selected to represent the major strain types involved in hospital outbreaks that were observed in different Spanish and German hospitals between 1990 and 2002; also included were three isolates from The Netherlands and from Sweden to represent other geographic locations. All isolates had been identified previously by routine phenotypic tests and assigned to the *A. calcoaceticus-A. baumannii* complex. Species identification was confirmed by 5′-end sequencing of the 16S rRNA gene of all Spanish, Dutch, and Swedish isolates (data not shown) (31) and by ribotyping of the German isolates (35).

DNA extraction. Strains were maintained at −70°C in 20% (vol/vol) glycerol in LB medium to preserve genetic variation during storage and were grown overnight on MacConkey agar at 37°C. A loopful from a colony was suspended in 500 μl of distilled water. DNA extraction was carried out by using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions. DNA was stored at −20°C until further use.

MLST. The housekeeping genes for the MLST scheme were selected on the basis of their sequence availability in GenBank and prior studies of the phylogenetic relationships for the genus *Acinetobacter* and their presence in other MLST schemes available for other bacterial species (22, 26, 28, 30). PCR primers were chosen from previous studies or were designed for amplification of the 10 selected genes: citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), RNA polymerase σ^{70} factor (*rpoD*), phospho-glucomutase (*pgm*), quinate shikimate dehydrogenase (*quiA*), and coenzyme A thiolase (*pcaf*) (Table 2).

All PCR amplifications were carried out in a MasterCycler gradient (Eppendorf, Hamburg, Germany) under the following conditions: 30 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min) preceded by a 2-min denaturation at 94°C and followed by a 2-min extension at 72°C. PCR products were directly purified from the reaction mixture with the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Sequencing of internal fragments of about 450 bp of the selected housekeeping genes was performed in an ABI Prism 377 sequencer using the ABI Prism BigDye terminator cycle sequencing ready reaction kit V2 (PE Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Specific sequencing primers and amplification lengths for each gene are listed in Table 2. Sequence data were aligned by CLUSTALX and manually corrected by using the BioEdit program version 5.09 (www.mbio.ncsu.edu/BioEdit/bioedit.html).

For each locus, distinct allele sequences were assigned an arbitrary allele number for identification; these were in-frame internal fragments of the gene which contained an exact number of codons. Each isolate was characterized by a pattern of numbers defining its allelic profile or ST. The similarities between the allelic profiles were obtained by the UPGMA (unweighted pair-group method with arithmetic averages) method available in the MEGA suite programs (version 2.1 [http://www.megasoftware.net]). This set of programs was also used to calculate the maximum percent nucleotide divergence between pairs of alleles at a given locus. The d_N/d_S ratios, d_N being the proportion of nonsynonymous substitutions and d_S being the proportion of synonymous substitutions per site, were calculated by using ST analysis and recombinational tests (START; version 1.05 http://outbreak.ceid.ox.ac.uk/software.htm). We also used this program to obtain the index of association (I_a) and the number of polymorphic nucleotide sites. The I_a is defined as the observed variance in the distribution of allelic mismatches in all pairwise comparisons of the allelic profiles divided by the expected variance in a freely recombining population, minus 1. When the alleles are in linkage disequilibrium the I_a is expected not to deviate significantly from zero.

AFLP fingerprinting. The AFLP fingerprinting was performed according to the method of Nemec et al. (32). DNA digestion was carried out with 5 U of EcoRI and 1 U of MseI, while ligation of EcoRI and MseI adaptors was performed simulteously with 1 U of T4 ligase (Invitrogen, Carlsbad, CA) in a final volume of 20 μl at 37°C overnight. Selective PCRs with fluorescently labeled primer EcoRI-C (NED) and unlabeled primer MseI-T were performed in a MasterCycler gradient with the reagents supplied with the AFLP Microbial Fingerprinting Kit (PE Applied Biosystems) according to the manufacturer's recommendations. The resulting products were denaturalized and charged on to an ABI Prism 377 sequencer for fragment separation. Normalization and fragment sizing was carried out by using GeneScan software (PE Applied Biosystems) in combination with Genographer software (version 1.6.0 [http://hordeum.oscs.montana.edu/genographer]). A similarity matrix for the presence or absence of bands was obtained by using the program Restdist in the PHYLIP 3.63 package. UPGMA was used for grouping and the Pearson product-moment coefficient (r) was used as the measure of similarity.

PFGE. Twenty-nine of the forty *A. baumannii* clinical isolates included in the present study, as well as the two *Acinetobacter* genomic species 13TU isolates, were also characterized by PFGE as described previously (18). For this analysis, two low-frequency cutting restriction enzymes, SmaI and ApaI, were used separately according to the manufacturer's specifications (New England Biolabs, Beverly, MA). DNA restriction fragments were separated in a CHEF-DR III unit (Bio-Rad Laboratories) for 20 h at 14°C and 6 V/cm, with pulse times ranging from 0.5 to 15 s when SmaI was used for restriction and from 1 to 30 s after ApaI restriction. Strain relatedness was assigned in accordance with published criteria (36).

Genome location. To locate the selected *A. baumannii* genes in the recently annotated sequence of *Acinetobacter* ADP1, a local protein database was created with the published open reading frame (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Acinetobacter_sp_ADP1). The amino acid sequences from *A. baumannii* amplified PCR products were launched onto this database using BlastP to obtain the genome position of the homologous ADP1 genes (1).

Nucleotide sequence accession numbers. GenBank accession number ranges for the sequences used in this study are as follows: for *cpn60*, D156565 to DQ156613; for *gdh*, from DQ156614 to DQ156661; for *gpi*, from DQ156662 to DQ156709; for *gt1A*, from DQ156710 to DQ156758; for *gyrB* from Q156759 to DQ156806; for *recA*, from DQ156807 to DQ156855; and for *rpoD*, from DQ156856 to DQ156904.

RESULTS

Sequencing of housekeeping gene fragments. Ten metabolic genes were initially selected to develop the MLST scheme. For two of the selected genes, phospho-glucomutase (*pgm*) and quinate shikimate dehydrogenase (*quiA*), the primers failed to

TABLE 1. Epidemiologic characteristics of *Acinetobacter* isolates used in the present study[a]

Strain no.	Designation	Origin or hospital[b]	City of isolation	Yr of isolation	Species	Source	PFGE type	AFLP type[c]	ST[d]	Allele no.						
										gltA	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*
1	62791	HB	Barcelona	1993	*A. baumannii*	Blood	A	a	1	1	1	1	1	1	1	6
2	52400	HB	Barcelona	2000	*A. baumannii*	Urine	A	a	1	1	1	1	1	1	1	6
3	151101	HB	Barcelona	1994	*A. baumannii*	Blood	B	b	2	2	2	2	1	1	2	7
4	288611	HB	Barcelona	1999	*A. baumannii*	Blood	B	b	2	2	2	2	1	1	2	7
5	218011	HB	Barcelona	2000	*A. baumannii*	Urine	A	a	1	1	1	1	1	1	1	6
6	228620	HB	Barcelona	2000	*A. baumannii*	Urine	A	a	3	1	1	1	1	2	1	4
7	220383	HB	Barcelona	2000	*A. baumannii*	Bronchial aspirate	B	b	2	2	2	2	1	1	2	7
8	213278	HB	Barcelona	1999	*A. baumannii*	Blood	B	b	2	2	2	2	1	1	2	7
9	60127	HB	Barcelona	1997	*A. baumannii*	Wound swab	D	c	5	1	3	3	2	2	3	1
10	113402	HB	Barcelona	1999	*A. baumannii*	Blood	D	c	5	1	3	3	2	2	3	1
11	118151	HB	Barcelona	1997	*A. baumannii*	Blood	E	c	6	1	4	3	2	2	3	3
12	224220	HB	Barcelona	2000	*A. baumannii*	Bronchial aspirate	E	c	6	1	4	3	2	2	3	3
13	135172	HB	Barcelona	2001	*A. baumannii*	Blood	D	c	5	1	3	3	2	2	3	1
14	135323	HB	Barcelona	2001	*A. baumannii*	Bronchial aspirate	D	c	5	1	3	3	2	2	3	1
15	135467	HB	Barcelona	2001	*A. baumannii*	Wound swab	D	c	5	1	3	3	2	2	3	1
16	61588	UC	Santander	2000	*A. baumannii*	Unknown		c	7	3	3	3	2	3	3	8
17	61812	UC	Santander	2000	*A. baumannii*	Unknown		a	8	4	4	4	4	4	5	9
18	62309	UC	Santander	2000	*A. baumannii*	Unknown		c	9	1	3	3	2	2	6	8
19	2002-34100	HE	Elche	2002	*A. baumannii*	Wound swab		c	10	1	4	3	2	2	7	3
20	2002-9198	HE	Elche	2002	*A. baumannii*	Wound swab		c	6	1	4	3	2	2	3	3
21	2002-26724	HE	Elche	2002	*A. calcoaceticus*	Bronchial aspirate				8	5	ND	8	5	ND	10
22	2001-30645	HE	Elche	2001	*A. baumannii*	Bronchial aspirate		c	6	1	4	3	2	2	3	3
23	2001-12583	HE	Elche	2001	*A. baumannii*	Sputum		c	6	1	4	3	2	2	3	3
24	2001-11094	HE	Elche	2001	*A. baumannii*	Bronchial aspirate		c	6	1	4	3	2	2	3	3
25	2001-16313	HE	Elche	2001	*A. calcoaceticus*	Wound swab				9	6	5	7	6	1	11
26	RUH2207	LUMC	Malmö	1980	*A. baumannii*	Sputum		c	11	4	11	6	1	7	8	6
27	RUH134	LUMC	Rotterdam	1982	*A. baumannii*	Urine		c	6	1	4	3	2	2	3	3
28	RUH875	LUMC	Dordrecht	1984	*A. baumannii*	Urine		a	12	8	4	4	4	4	5	5
29	DSM 1139	DSMZ			*A. calcoaceticus*					5	8	ND	4	8	1	ND
30	DSM 7324	DSMZ			*A. calcoaceticus*					6	9	7	5	9	1	12
31	DSM 30008	DSMZ			*A. baumannii*			c	13	1	7	8	9	1	4	14
32	DSM 30006	DSMZ			*A. calcoaceticus*					7	ND	9	3	10	4	13
33	DSM 30007	DSMZ			*A. baumannii*			c	14	1	10	10	6	1	4	14
34	W 5420	CUH	Cologne	1991	*A. baumannii*	Tracheal aspirate	F	f	15	1	12	11	10	1	9	4
35	U 1901	CUH	Cologne	1991	*A. baumannii*	Tracheal aspirate	F	f	15	1	12	11	10	1	9	4
36	U 10247	CUH	Cologne	1991	*A. baumannii*	Urine	G	g	16	10	12	4	11	11	9	5
37	U 11177	CUH	Cologne	1991	*A. baumannii*	Urine	G	g	16	10	12	4	11	11	9	5
38	St 284	CCH	Cologne	1991	*A. baumannii*	Blood	H	h	17	1	12	12	11	4	10	3
39	St 14733	CCH	Cologne	1990	*A. baumannii*	Blood	H	h	17	1	12	12	11	4	10	3
40	St 20820	CMH	Cologne	1991	*A. baumannii*	Blood	I	i	18	10	13	4	11	12	11	5
41	St 21359	CMH	Cologne	1991	*A. baumannii*	Catheter	I	i	18	10	13	4	11	12	11	5
42	St 15598	CMH	Cologne	1991	*A. baumannii*	Catheter	K	k	19	1	14	3	2	2	9	3
43	St 14970	CMH	Cologne	1991	*A. baumannii*	Catheter	K	k	19	1	14	3	2	2	9	3
44	St 17093	CMH	Cologne	1991	*A. baumannii*	Blood	L	k	20	1	15	13	12	4	12	2
45	V 7459	CMH	Cologne	1991	*A. baumannii*	Tracheal aspirate	L	k	20	1	15	13	12	4	12	2
46	St 11681	CMH	Cologne	1991	*Acinetobacter* 13TU[e]	Blood	M			11	16	14	13	13	13	15
47	St 7961	CMH	Cologne	1991	*Acinetobacter* 13TU[e]	Blood	M			11	16	14	13	13	13	15
48	St 1650	CMH	Cologne	1992	*A. baumannii*	Blood	N	k	21	1	12	15	2	2	9	3
49	St 1954	CMH	Cologne	1992	*A. baumannii*	Blood	N	k	21	1	12	15	2	2	9	3

[a] ND, the sequence could not be determined.
[b] HB, Hospital Bellvitge; UC, Universidad de Cantabría; HE, Hospital General de Elche; LUMC, Leids Universitair Medisch Centrum; CUH, Cologne University Hospital; CCH, Cologne Childrens Hospital; CMH, Cologne Community Hospital.
[c] AFLP type, determined for *A. baumannii* isolates only.
[d] ST, determined for *A. baumannii* isolates only.
[e] *Acinetobacter* genomic species 13TU.

amplify the fragment in some of the *A. baumannii* isolates. Examination of the sequence traces of the beta-ketoadipyl CoA thiolase gene (*pcaf*) showed that there were two overlapping peaks, suggesting two different nucleotides at a few sites. For the remaining seven genes, the results were satisfactory, i.e., all strains produced one single amplicon and the number of different alleles appeared to be sufficiently discriminative to be used for typing (Table 3). The selected gene fragments were sequenced from each of the 40 clinical *A. baumannii* isolates and the two reference strains, DSM 30007 (ATCC 19606) and DSM 30008. All sequences had the same size for each gene ranging in length from 305 bp (*gpi*) to 513 bp (*rpoD*) (Table 3). Obtained alleles were defined as distinct if they differed at a single nucleotide site and were numbered consecutively for identification. Homologous genes in the *Acinetobacter* genomic species 13TU and *A. calcoaceticus* isolates were also sequenced to be used as outgroups in the dendrogram construction. *A. calcoaceticus* strains 21, 29, and 32 were not used in the MLST scheme because sequences could not be obtained for all loci (Table 1). In *A. baumannii* isolates, the number of alleles at each locus ranged from 6 alleles for *gltA* to 12 alleles for *gpi*. The overall mean value for *A. baumannii* isolates was 10 alleles per locus, and the percent variable nucleotide sites for these housekeeping genes ranged from 2.13% (*cpn60*) to 18.03%

TABLE 2. Details of loci and oligonucletide primers used in the present study

Locus	Gene product	Primer	Sequences (5′→3′)	Amplicon size (bp)	Usage
gltA	Citrate synthase[a]	Citrato F1	AATTTACAGTGGCACATTAGGTCCC	722	Amplification/sequencing
		Citrato R12	GCAGAGATACCAGCAGAGATACACG		Amplification/sequencing
gyrB	DNA gyrase subunit B[b]	APRU F	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	909	Amplification
		M13 [−21]	TGTAAAACGACGGCCAGT		Sequencing
		UP1E R	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA		Amplification
		M13 F	CAGGAAACAGCTATGACC		Sequencing
gdhB	Glucose dehydrogenase B[a]	GDHB 1F	GCTACTTTTATGCAACAGAGCC	775	Amplification
		GDH SEC F	ACCACATGCTTTGTTATG		Sequencing
		GDHB 775R	GTTGAGTTGGCGTATGTTGTGC		Amplification
		GDH SEC R	GTTGGCGTATGTTGTGC		Sequencing
recA	Homologous recombination factor[c]	RA1	CCTGAATCTTCYGGTAAAAC	425	Amplification/sequencing
		RA2	GTTTCTGGGCTGCCAAACATTAC		Amplification/sequencing
cpn60	60-kDa chaperonin[a]	CPN 3F2	ACTGTACTTGCTCAAGC	479	Amplification/sequencing
		CPN R2	TTCAGCGATGATAAGAAGTGG		Amplification/sequencing
gpi	Glucose-6-phosphate isomerase[a]	GPI F1	AATACCGTGGTGCTACGGG	508	Amplification/sequencing
		GPI R1	AACTTGATTTTCAGGAGC		Amplification/sequencing
rpoD	RNA polymerase sigma factor *rpoD* (Sigma-70)[b]	70F RPOD	ACGACTGACCCGGTACGCATGTAYATGMGNGARATCGC NACNCT	492	Amplification
		70FS	ACGACTGACCCGGTACGCATGTA		Sequencing
		70R RPOD	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTG YTTYTT		Amplification
		70RS	ATAGAAATAACCAGACGTAAGTT		Sequencing

[a] Primers for citrate synthase (accession no. M33037), glucose dehydrogenase B (*gdhB*; accession no. X15871), 60-kDa chaperonin (*cpn60*; accession no. AY123669), and glucose-6-phosphate isomerase (*gpi*; accession no. X89900) were constructed by using available *Acinetobacter* sequences from conserved regions of each gene.
[b] Primers for DNA gyrase subunit B (*gyrB*) and RNA polymerase sigma factor rpoD (Sigma-70) (*rpoB*) were selected and used as described previously (41).
[c] Homologous recombination factor (*recA*) primers were selected and used as described previously (33).

(*gpi*) (Table 3). The d_N/d_S ratio was calculated for all loci and ranged from 0.0318 for *gtlA* to 0.2570 for *cpnd60* (Table 3). The index of association (I_a) was used to test for linkage disequilibrium between alleles at the seven housekeeping genes. For the entire *A. baumannii* population the I_a value was 2.592. Using only one representative strain for each ST this value was reduced to 1.393 but was still greater than 1. A concatenated loci alignment for each of the strains was used to construct the dendrogram with the UPGMA method (Fig. 1).

Mapping of the selected genes. The only available genome sequence of the genus *Acinetobacter*, *Acinetobacter* sp. strain ADP1 (3), was used to estimate the genome position of the seven selected *A. baumannii* genes (Fig. 2). Five of these genes (*gyrB*, *recA*, *gpi*, *cpn60*, and *rpoD*) were found to have one copy within the ADP1 genome. The citrate synthase gene (*gtlA*) seems to have a paralogous gene (*prpC*) with homologous function. Although both genes are present within the ADP1 genome, the nucleotide differences between the *gtlA* and *prpC* genes (sequence identity 47%) are sufficient to be differentiated by standard PCR. No homologous nucleotide or protein sequence with the *gdhB* gene was detected in the ADP1 genome. As had been expected from our sequence data the genome analysis also revealed the presence of two highly conserved copies of the beta-ketoadipyl CoA thiolase gene (98% nucleotide sequence identity) within the ADP1 genome (*pcaf* and *catF*), precluding its use for further analysis.

Evaluation of the *A. baumannii* MLST scheme. Table 1 shows the 20 different allelic profiles or STs identified among the 42 *A. baumannii* isolates. The MLST scheme was evaluated by comparing allelic profiles with restriction fragment patterns generated by AFLP and PFGE.

All *A. baumannii* isolates, including 13 isolates that were not characterized by PFGE, the two *Acinetobacter* genomic species 13TU isolates, and also the five *A. calcoaceticus* isolates, were analyzed by AFLP (Fig. 3). Using a similarity cutoff value of 50% for species delineation (9), the strains were grouped into three distinct clusters, one including the reference strain *A. baumannii* DSM 30007 and all *A. baumannii* clinical isolates, a

TABLE 3. Variation in loci used in the present *A. baumannii* MLST scheme

Locus	Fragment size (bp)	No. of alleles	No. of polymorphic nucleotide sites	% Variable sites	% Nucleotide divergence between pair of alleles		d_N/d_S[a]
					Max	Avg	
gltA	484	6	45	9.29752	0.093	0.033	0.0318 (0.0273)
gyrB	457	11	22	4.81400	0.031	0.017	0.0786 (0.0275)
gdhB	396	11	23	5.80808	0.038	0.019	0.0594 (0.0520)
recA	371	8	9	2.42588	0.016	0.009	0.0561 (0.0094)
cpn60	421	7	9	2.13777	0.083	0.025	0.2570 (0.0483)
gpi	305	12	55	18.03280	0.141	0.060	0.0415 (0.0327)
rpoD	513	10	8	1.55945	0.022	0.009	0.1232 (0.0826)

[a] The d_N/d_S values for all *Acinetobacter* gene sequences obtained in this study are given in parentheses. Max, maximum.

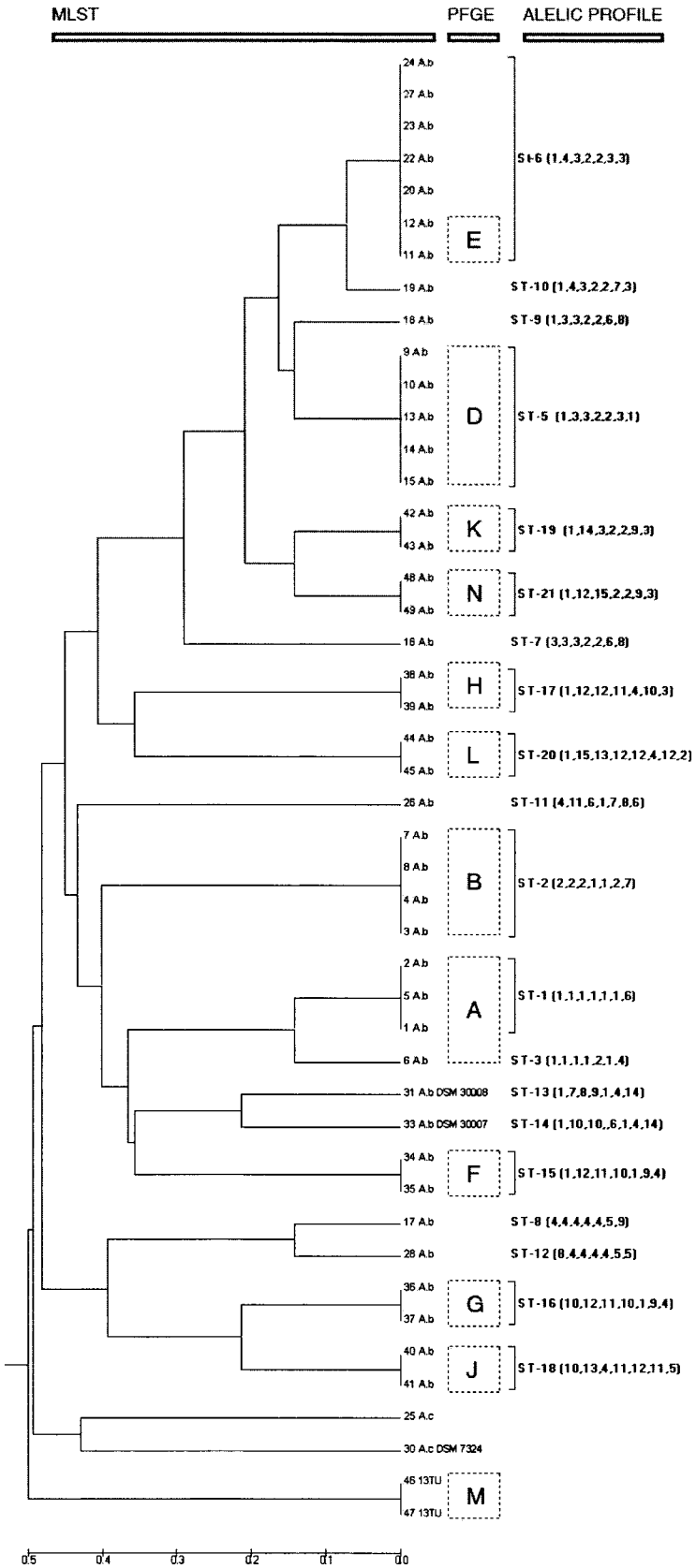

FIG. 1. Dendrogram constructed by UPGMA cluster analysis based on the nucleotide differences obtained by sequencing of *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* gene fragments. From left to right are indicated isolate number, species identification (A.b, *A. baumannii*; 13TU, *Acinetobacter* genomic species 13TU; A.c, *A. calcoaceticus*), PFGE type (boxed capital letters), ST, and allelic profile.

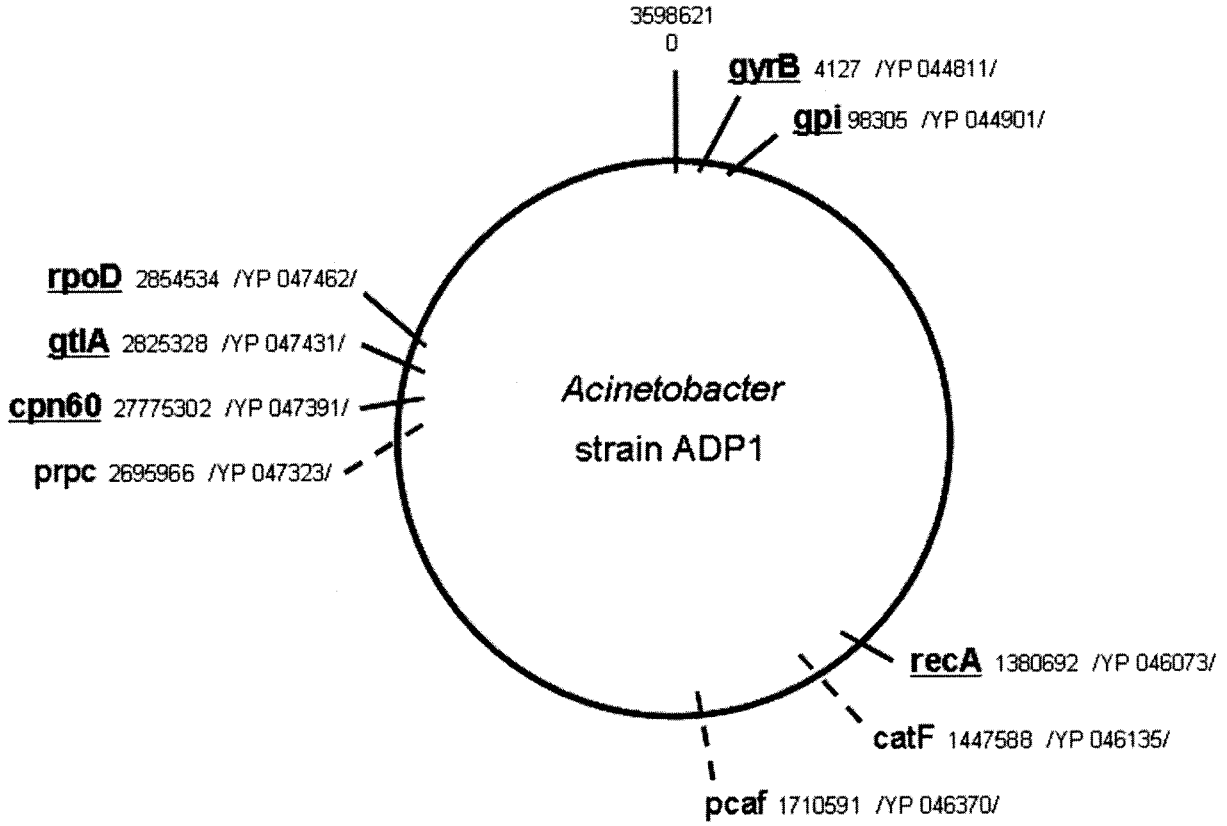

FIG. 2. Relative location of the selected housekeeping genes used in the present study on a schematic map of the genome of *Acinetobacter* strain ADP1 (NC_005966.1). The *A. baumannii cpn60* gene is homologous to chaperone Hsp60 from ADP1. Genes used in the present MLST scheme are underlined. The coordinates are given in bases, and the protein accession numbers are indicated.

second cluster contained all *A. calcoaceticus* isolates, and the third cluster comprised the two *Acinetobacter* genomic species 13TU isolates. Within the *A. baumannii* cluster, using a clustering level of 90% (9), analysis of AFLP fingerprints showed nine different clusters. The largest cluster contained strain 27 (RUH134), a representative of the so-called European clone II, and 14 Spanish isolates from Barcelona, Elche, and the University Hospital of Cantabria. Among these isolates, six different STs (ST5, ST6, ST7, ST9, ST10, and ST11) were identified that differed from each other at one to four loci with one exception; strain 26 (ST 11) differed from strain 27 (RUH134) at all seven loci. Strain 28 (RUH875), the reference strain for European clone I, clustered with outbreak strains from Barcelona and the University Hospital of Cantabria. Among these strains, four different STs were identified (ST1, ST3, ST8, and ST12), with ST1 and ST3, as well as ST8 and ST12, being double-locus variants each. In contrast, most of the German outbreak isolates, the two *A. baumannii* reference strains (both environtmental isolates), and one group of outbreak-related isolates from Barcelona exhibited different STs and also clustered distinct from each other by AFLP. Three German epidemic strains exhibiting three STs (ST19, ST20, and ST21, two of them being double-locus variants) clustered together by AFLP.

PFGE analysis of 29 of the *A. baumannii* clinical isolates generated 11 different PFGE patterns (Table 1). Fifteen of these isolates were representative of the main clonal types (PFGE types A, B, D, and E) involved in a sustained outbreak in Hospital Universitari de Bellvitge (Barcelona) that took place between 1993 and 2001. Another 14 *A. baumannii* isolates (PFGE types F to L and N), as well as two *Acinetobacter* genomic species 13TU isolates (PFGE type M), were from eight major outbreaks in various hospitals in Cologne, Germany, that occurred between 1990 and 1992. Epidemiologic details of these strains have been described previously (35). The PFGE clusters were in high concordance with the dendrogram constructed by using MLST data for these strains (Fig. 1). The Barcelona isolates exhibited four different PFGE types and clustered into five different STs, while seven different PFGE types and seven STs were identified among the Cologne isolates. One of the Barcelona epidemic strains (ST6) was also responsible for the outbreak observed in Elche General Hospital in 2001 and 2002. The two *Acinetobacter* genomic species 13TU isolates, as well as the *A. calcoaceticus* isolates, clustered distantly from the *A. baumannii* isolates by MLST.

DISCUSSION

A. baumannii has emerged as an important nosocomial pathogen in various countries in Europe, Asia, the United States, and Latin America (27, 39). This organism is known for its involvement in hospital outbreaks and has sometimes caused interinstitutional spread (27). Whereas several researchers emphasized the genetic heterogeneity among epi-

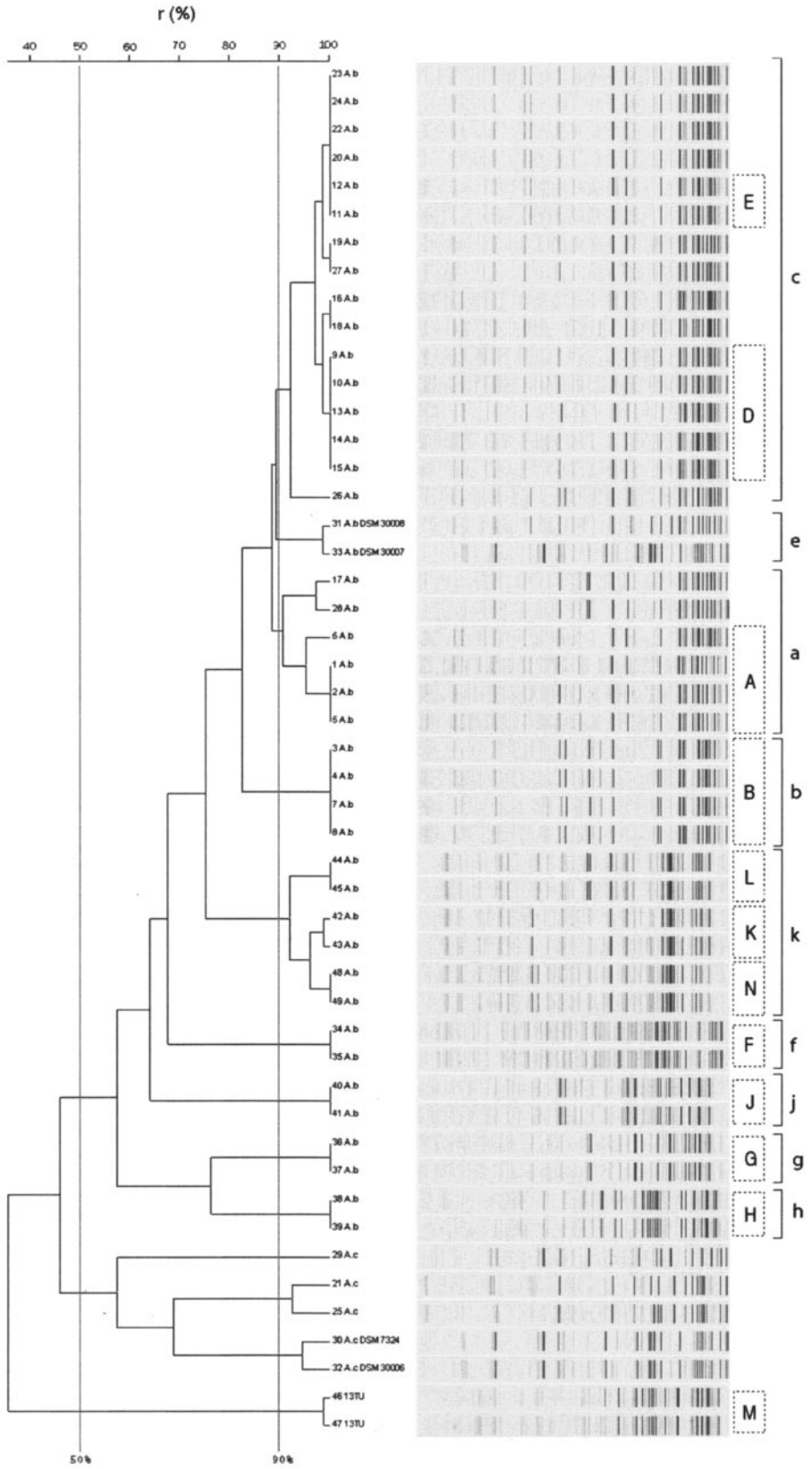

FIG. 3. Fluorescently labeled AFLP patterns and UPGMA/product-moment cluster analysis of the 49 *Acinetobacter* strains. Twenty-seven different patterns were obtained after PCR with EcoC and MseT templates among all isolates. Levels of correlation are expressed as percentages of similarity. The known PFGE types are indicated inside boxes in capital letters for comparison. AFLP types are indicated at the far right side.

demic *A. baumannii* strains (34, 35, 40), recent data indicate that a few successful epidemic *A. baumannii* strains (clones) circulate in Europe, including the so-called EU clones I and II that have been isolated in northern Europe (9, 32), and the geographically more widespread EU clone III (38). The contribution of these widespread clones to the overall burden of epidemic *A. baumannii* strains remains to be determined. In view of the worldwide expansion of multidrug and even panresistant epidemic *A. baumannii* strains, a better understanding of the global epidemiology of this the species is urgently needed. For early recognition of epidemic *A. baumannii* strains a typing system is required that is characterized by excellent discriminatory power but even more importantly by high interlaboratory reproducibility that permits the development of a central databank of strain patterns. After local analysis, typing data can then be submitted to the central databank and compared to other patterns of known epidemic strains, thus alerting the investigator if an epidemic strain or a strain with particular virulence properties has been detected.

The main objective of the present study was to develop a new typing scheme for *A. baumannii* that allows unambiguous comparison of typing data generated at different laboratories. A major advantage of MLST compared to other typing methods results from the use of nucleotide sequence data that offer the possibility of data exchange via an electronic platform. This feature makes this technique ideally suited for national or international surveillance programs involving multiple laboratories and for monitoring the spread of drug-resistant clones.

The *A. baumannii* MLST scheme described here is based on the allelic variations in seven housekeeping genes of *A. baumannii* by using a sample of 40 clinical isolates and 2 reference strains of *A. baumannii* and 7 other isolates belonging to the *A. calcoaceticus-A. baumannii* complex. Several candidate loci were eliminated, since the chosen gene fragments could not be readily amplified from all initial test isolates. The internal fragments of the seven loci that were selected for the final MLST scheme—*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*—could be amplified from all isolates that were investigated. The d_N/d_S ratio was less than 1 for all of the selected housekeeping genes, indicating a very low contribution of environmental selection to variation in these genes. The allele diversity ranged from 2.1 to 18% and was relatively large. For comparison, in *N. meningitidis* the allelic diversity ranged from 5.6% (*gdh*) to 33.9% (*aroE*) (28). Considering the smaller number of strains investigated, our data are comparable and consistent with a species of average genetic diversity. Therefore, the selected housekeeping genes are probably adequate for population-genetic studies of *A. baumannii* (11).

The clustering of *A. baumannii* isolates achieved by MLST was in good agreement with strain grouping obtained by AFLP and PFGE; both techniques have proven their discriminatory power for *A. baumannii* in previous studies. Although our strain collection comprised 20 STs, 9 AFLP types were identified. Similarly, among the 29 isolates subjected to PFGE, 11 PFGE types were discernible, corresponding to 12 STs. The MLST scheme was validated by showing that pairs of isolates with the same allelic profile produced identical or very similar fragment patterns by PFGE. The collection of isolates that we examined included the type strains for EU clone I (RUH 875; ST12) and EU clone II (RUH 134; ST6) (9). Although ST12 was not identified among the remaining isolates, ST6 was the strain type involved in hospital outbreaks in Barcelona and in Elche, confirming the widespread occurrence of this successful epidemic *A. baumannii* clone over a period as long as two decades. Thus, the *A. baumannii* MLST scheme provides a promising method for unambiguous epidemiologic strain characterization.

Our study is based on only a limited number of *A. baumannii* isolates from different hospitals distributed throughout Spain, as well as from Germany and from a few other European locations. Overall, these isolates should represent a significant fraction of the European variability within this species. To broaden our understanding of the molecular epidemiology of *A. baumannii*, the present study should be expanded to larger strain collections from diverse locations around the world. It would also be warranted to extend MLST typing of *Acinetobacter* species 13TU. Since conventional identification of acinetobacters does not permit one to discern this species from *A. baumannii*, it would considerably add to the usefulness of our MLST scheme if *Acinetobacter* species 13TU isolates could be unambiguously characterized and separated from *A. baumannii* by MLST without prior species identification. The only *Acinetobacter* species 13TU strain investigated in the present study differed from all *A. baumannii* isolates at all loci, thus holding promise that our MLST scheme might also be applicable for this species.

In summary, the *A. baumannii* MLST scheme developed in the present study provides a powerful tool for molecular epidemiologic studies of clinical strains of *A. baumannii* and offers a new way to study the population biology of this pathogen. The discriminatory power of the MLST typing system is comparable to both PFGE and AFLP. It provides a portable method that is suitable for global epidemiologic study and allows the recognition of epidemic, multiresistant, and virulent clones and monitoring of their international spread. Investigations with more strains from widespread origins will allow a better understanding of the population genetics of this important nosocomial pathogen.

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