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Molecular Epidemiology of *Acinetobacter baumannii* Bloodstream Isolates Obtained in the United States from 1995 to 2004 Using rep-PCR and Multilocus Sequence Typing

Paul G. Higgins, Katharina Janßen, Maximilian M. Fresen, Hilmar Wisplinghoff, and Harald Seifert

Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany

Using a repetitive-sequence-based (rep)-PCR (DiversiLab), we have molecularly typed *Acinetobacter* nosocomial bloodstream isolates (*Acinetobacter baumannii* [*n* = 187], *Acinetobacter pittii* [*n* = 23], and *Acinetobacter nosocomialis* [*n* = 61]) obtained from patients hospitalized in U.S. hospitals over a 10-year period (1995-2004) during a nationwide surveillance study (Surveillance and Control of Pathogens of Epidemiological Importance [SCOPE]). Patterns of *A. baumannii* rep-PCR were compared to those of previously identified international clonal lineages (ICs) and were further investigated by multilocus sequence typing (MLST) to compare the two typing methods. Forty-seven of the *A. baumannii* isolates clustered with the previously defined IC 2. ICs 1, 3, 6, and 7 were also detected. The remaining 81 isolates were unrelated to the described ICs. In contrast, *A. pittii* and *A. nosocomialis* isolates were more heterogeneous, as determined by rep-PCR. Our MLST results were in good correlation with the rep-PCR clusters. Our study confirms previous data indicating the predominance of a few major clonal *A. baumannii* lineages in the United States, particularly IC 2. The presence in the United States of *A. baumannii* ICs 1, 2, and 3 from as early as 1995 suggests that global dissemination of these lineages was an early event.

The *Acinetobacter baumannii* group comprises three species, *Acinetobacter baumannii*, *Acinetobacter pittii* (formerly *Acinetobacter* genomic species 3), and *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species [gen. sp.] strain 13TU) (20, 21). These three species are found primarily as nosocomial pathogens, but it is *A. baumannii* that is of most concern and is associated with multidrug resistance and outbreaks (11, 21).

The impact of hospital-acquired infections is costly both in human and monetary terms (6). For those tasked with tracking these infections, there now exist a plethora of techniques with which to investigate the sources, spread, and eradication of the infectious agents, with pulsed-field gel electrophoresis (PFGE) still considered the gold standard for typing outbreak-related isolates of *A. baumannii* (25). A less discriminatory method is multilocus sequence typing (MLST), but this method is primarily useful for population genetics studies (2, 9) while the semiautomated repetitive-sequence-based (rep)-PCR-based DiversiLab system has been shown to be almost as discriminatory as PFGE (24). rep-PCR has the advantage of being less labor-intensive than PFGE and MLST while allowing for the investigation of large numbers of isolates (13, 14, 17).

Portability of typing results is important, and despite attempts to standardize PFGE it is still difficult to compare results generated at different sites (25). The reproducibility of rep-PCR fingerprints between laboratories was recently tested, and clustering was found to be conserved (15). However, the most portable method is MLST, as it relies on DNA sequences. The MLST scheme as proposed by Bartual et al. has been used to identify several clonal complexes (CCs) that correlate with the previously identified European clones (1, 2, 19). An alternative MLST scheme gave similar results (9).

The few studies carried out to investigate the epidemiology of *A. pittii* and *A. nosocomialis* have shown these populations to be more heterogeneous than those of *A. baumannii*, although the typing methods used may have had an impact on the interpretation of the typing results (26, 28, 30). For example, MLST of *A. nosocomialis* isolated from different hospitals revealed that there was no relatedness between isolates from different outbreaks, i.e., outbreaks were caused by local strains that had not spread to other locations, whereas randomly amplified polymorphic DNA (RAPD) performed by Spence et al. revealed a cluster of *A. nosocomialis* isolates obtained from patients from 15 different hospitals (28, 34). These results contrast with findings for *A. baumannii*, for which Dijkshoorn and coworkers identified three clonal lineages that were later termed European clones I, II, and III (10, 32). More recently, with the use of the DiversiLab system, eight worldwide or international carbapenem-resistant clonal lineages (ICs 1 through 8) were identified, three of which corresponded to European clones I through III (9, 14).

Comparisons between rep-PCR and MLST have been performed with several bacterial species, such as *Campylobacter* spp., *Staphylococcus aureus*, and *Salmonella enterica*. These comparisons have shown these methods to be in fairly good agreement, although it was noted that the DNA extraction method affected the rep-PCR results (3, 4, 29). A recent study of 30 *A. baumannii* isolates from a hospital in west China showed good correlation between the DiversiLab system and MLST (12).

In the present study, we used the Diversilab system to investigate the molecular epidemiology of *A. baumannii*, *A. pittii*, and *A. nosocomialis* bloodstream isolates that were collected in the United States between 1995 and 2004. The presences of the ac-

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Address correspondence to Harald Seifert, harald.seifert@uni-koeln.de.
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TABLE 1 Correlation between DiversiLab and MLST clustering and year of isolation[a]

No. of isolates	DiversiLab cluster	MLST[b]		Allele							Year of isolation
		ST	CC	*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	
4	IC 1	109	109	10	12	4	11	4	9	5	1996, 1998, 2002
1	IC 1	95	109	10	12	4	11	1	9	5	1998
9	IC 2	98	92	1	12	3	2	2	3	3	1995, 1997–99, 2001–2002
29	IC 2	223	92	1	17	3	2	2	56	3	1995–2004
1	IC 2	270	92	1	12	3	2	46	3	3	2001
3	IC 2	281	92	1	17	3	2	2	99	3	1996–1997, 2001
3	IC 2	282	92	1	17	3	2	2	116	3	1996–1997
1	IC 2	290	92	1	38	3	2	2	56	3	1997
1	IC 2	298	92	1	72	3	2	2	56	3	1997
2	IC 3	266	187	1	1	12	32	1	9	6	1997–1998
3	IC 6	196	n.a.	2	21	12	32	26	63	5	1995–1996, 1998
1	IC 7	276	276	1	15	2	28	1	104	32	1995
1	IC 7	110	276	1	15	2	28	1	52	32	1995
1	A	267	n.a.	1	1	66	12	33	117	41	2002
1	A	268	n.a.	1	1	66	12	33	129	41	2003
2	B	273	272	1	12	65	45	4	122	26	1996, 2002
1	B	272	272	1	12	65	45	4	4	26	1997
2	B	285	272	1	17	65	45	4	55	26	2002
4	C	277	s	1	15	3	31	4	1	4	2000–2101
2	D	280	s	1	17	2	12	1	138	69	1996
2	E	283	n.a.	1	17	6	1	4	56	6	1995–1996
1	E	284	n.a.	1	17	6	1	47	56	6	1995
3	E	299	n.a.	1	74	6	1	4	125	6	2002–2003
2	F	291	n.a.	1	46	96	54	4	18	59	1997, 2001
3	G	292	s	1	46	100	12	36	71	6	2001
2	H	294	s	1	52	94	11	1	117	26	1997, 2001
3	I	302	n.a.	1	81	11	48	18	24	43	1999
1	J	316	s	15	17	2	12	4	64	6	1997
2	J	317	s	15	72	2	12	4	64	6	1997
2	K	305	s	2	1	12	1	4	67	6	2002
2	L	323	s	24	58	107	2	4	54	6	2001
1	M	332	n.a.	28	38	45	1	16	40	2	1996
1	M	330	n.a.	28	17	45	1	16	4	2	1997
2	M	331	n.a.	28	38	45	1	16	4	2	1996, 2002
2	N	335	n.a.	33	17	12	49	1	54	4	2002–2003
4	O	339	s	44	73	4	11	44	121	4	1999, 2001
2	P	340	n.a.	45	77	59	2	4	56	64	2000
2	Q	341	s	46	12	110	1	16	133	50	2002–2003
2	R	307	s	3	77	42	11	16	9	5	2000, 2003
1	S	279	n.a.	1	15	62	31	4	72	45	1996
1	S	295	n.a.	1	54	62	31	4	50	45	1996
1	Unc	271	271	1	12	40	26	22	117	5	2000
1	Unc	288	271	1	34	40	26	22	1	5	1996
1	Unc	293	s	1	52	67	46	1	18	30	2001
1	Unc	318	s	18	12	80	12	1	123	3	2002
1	Unc	20	20	1	15	13	12	4	12	2	1997
1	Unc	269	s	1	1	116	60	1	132	50	1995
1	Unc	274	s	1	12	103	6	29	126	32	2002
1	Unc	17	275	1	12	12	11	4	10	3	1996
1	Unc	278	119	1	15	12	6	28	59	63	1999
1	Unc	287	s	1	17	117	11	28	11	32	1995
1	Unc	289	s	1	35	64	37	4	132	30	2001
1	Unc	81	s	1	54	59	11	4	69	45	2003
1	Unc	297	s	1	64	109	1	23	12	26	1999
1	Unc	300	s	1	76	106	46	1	131	5	2000
1	Unc	301	s	1	78	59	43	1	71	6	2001
1	Unc	303	s	1	82	112	1	1	135	66	1996
1	Unc	304	s	1	83	114	1	1	136	67	1995
1	Unc	306	s	2	79	73	59	1	9	6	2001
1	Unc	319	s	21	49	104	11	1	128	30	2003

(Continued on following page)

TABLE 1 (Continued)

No. of isolates	DiversiLab cluster	MLST[b]		Allele							Year of isolation
		ST	CC	*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	
1	Unc	320	s	21	84	64	11	4	9	5	1995
1	Unc	321	s	21	85	12	12	26	56	68	1996
1	Unc	322	n.a.	23	35	3	27	23	55	7	2000
1	Unc	333	n.a.	29	41	48	11	1	137	6	1995
1	Unc	334	s	33	12	59	11	32	11	5	2000
1	Unc	336	s	35	63	113	1	1	71	6	1995
1	Unc	337	s	37	53	67	6	42	64	7	1997
1	Unc	342	s	48	48	58	42	36	54	41	1996

[a] Only those isolates that were typed using both methods are shown.
[b] ST, sequence type; CC, clonal complex; s, singleton; n.a., clonal complex not assigned, but these STs are DLVs of other published STs.

quired bla_{OXA} carbapenem resistance determinants and the IS*Aba1* insertion sequences adjacent to bla_{OXA-51} were also investigated. Additionally, with the *A. baumannii* isolates, we compared the clustering generated by rep-PCR with that generated by MLST.

(Part of this work was presented at the 8th International Symposium on the Biology of *Acinetobacter*, Rome, Italy, 1 to 3 September 2010.)

MATERIALS AND METHODS

Bacterial isolates. *Acinetobacter* isolates (n = 271) were prospectively collected between 1995 and 2004 from patients with nosocomial bloodstream infections from 52 hospitals in the United States participating in the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) project (33). Identification to the species level (*A. baumannii* [n = 187], *A. pittii* [n = 23], and *A. nosocomialis* [n = 61]) was performed by amplified rRNA gene restriction analysis (ARDRA) and by *gyrB* multiplex PCR as described previously (35).

Imipenem susceptibility. Imipenem MICs were investigated by Etest (bioMérieux, Nürtingen, Germany). Isolates with imipenem MICs of ≤4 mg/liter and ≥16 mg/liter were interpreted as susceptible and resistant, respectively (5). MICs of other antimicrobials have been reported previously (35).

Detection of carbapenemases. Isolates with an imipenem MIC of ≥2 mg/liter were investigated for the presence of OXA-type carbapenemases as described previously (16, 36). This threshold was chosen because the highest imipenem MIC of the wild-type *A. baumannii* population is 1 mg/liter (see http://www.eucast.org/mic_distributions/). The presence of the insertion element IS*Aba1* upstream of $bla_{OXA-51-like}$ was investigated by PCR (31).

Molecular typing. Epidemiological typing of all isolates was performed by rep-PCR (DiversiLab System, bioMérieux, Nürtingen, Germany) as described previously (15). Results were analyzed with the DiversiLab software using the modified Kullback-Leibler statistical method. Isolates that clustered at >95% similarity were considered related and defined rep-PCR clusters (14, 17). Representative isolates belonging to previously identified *A. baumannii* worldwide clonal clusters 1 through 8 were included as controls (14). *A. baumannii* isolates (n = 140) were further investigated by MLST. The remaining 47 *A. baumannii* isolates were part of a large clonal outbreak at a single hospital, as shown by rep-PCR, and therefore were deemed unnecessary to be included in the MLST analysis. MLST was performed as described previously by Bartual et al. (2) with minor modifications. The following modified primers were used for amplification and sequencing: 5′-ACCCGTGAAGGTGAAATCAG-3′ (*rpoD*_F), 5′-TTCAGCTGGAGCTTTAGCAAT-3′ (*rpoD*_R), 5′-TGAAGGCGGCTTATCTGAGT-3′ (*gyrB*_F), 5′-GCTGGGTCTTTTTCCTGACA-3′ (*gyrB*_R), 5′-GAAATTTCCGGAGCTCACAA-3′ (*gpi*_F), and 5′-TCAGGAGCAATACCCCACTC-3′ (*gpi*_R). MLST sequences were uploaded to the *A. baumannii* MLST sequence type database (http://pubmlst.org/abaumannii/) to determine the alleles and sequence types (ST). Clonal complexes (CCs) were assigned using eBURST V3 (http://eburst.mlst.net/v3/) and were defined as single-locus variants (SLVs) and double-locus variants (DLVs).

RESULTS AND DISCUSSION

In this study we investigated the imipenem susceptibility and molecular epidemiology of *Acinetobacter* bloodstream isolates from the United States. The majority of *Acinetobacter* isolates were imipenem susceptible. Forty-nine *A. baumannii* isolates had imipenem MICs of ≥2 mg/liter, of which 18 (10%) were carbapenem intermediate or carbapenem resistant. More recently, imipenem nonsusceptibility rates of 34 to 75% were reported and confirm the increasing carbapenem resistance seen in this species (1, 8, 12).

The 49 isolates were investigated by PCR for the presence of bla_{OXA} carbapenem resistance determinants. No acquired bla_{OXA} resistance determinants were detected; however, 13 carbapenem-nonsusceptible and 19 carbapenem-susceptible *A. baumannii* strains had IS*AbaI* upstream of the $bla_{OXA-51-like}$ gene. Some of these strains were isolated as early as 1996. What was later termed IS*AbaI* was first described in 2003 and was associated with overexpression of the intrinsic bla_{ampC} gene (7). Subsequent to this, IS*AbaI* was described as adjacent to bla_{OXA-58} and $bla_{OXA-51-like}$ and is often associated with carbapenem resistance (22, 31). It is interesting that over half of the isolates in our study that had IS*Aba1* associated with bla_{OXA-51} remained carbapenem susceptible but had imipenem MICs above the wild-type MIC distribution, suggesting that IS*AbaI* adjacent to bla_{OXA-51} is not in itself a good predictor of carbapenem resistance. Therefore, the acquisition of IS*Aba1* by *A. baumannii* has occurred earlier than previously described and may have represented the first step in the evolution of resistance against the carbapenems. Several recent studies investigating carbapenem-resistant *A. baumannii* from the United States have shown IS*Aba1* to be associated with bla_{OXA-51} in 46 to 65% of isolates (1, 8).

All 187 *A. baumannii* isolates were typed by rep-PCR. The comparison between rep-PCR and MLST typing of *A. baumannii* is summarized in Table 1. Using ≥95% similarity to define a cluster, we used our in-house library to detect 24 clusters, the largest of which contained 94 isolates that clustered with IC 2 (14). Evidence of clonal spread came from the IC 2 isolates that originated from 11 centers, and the IC 1 isolates, although less

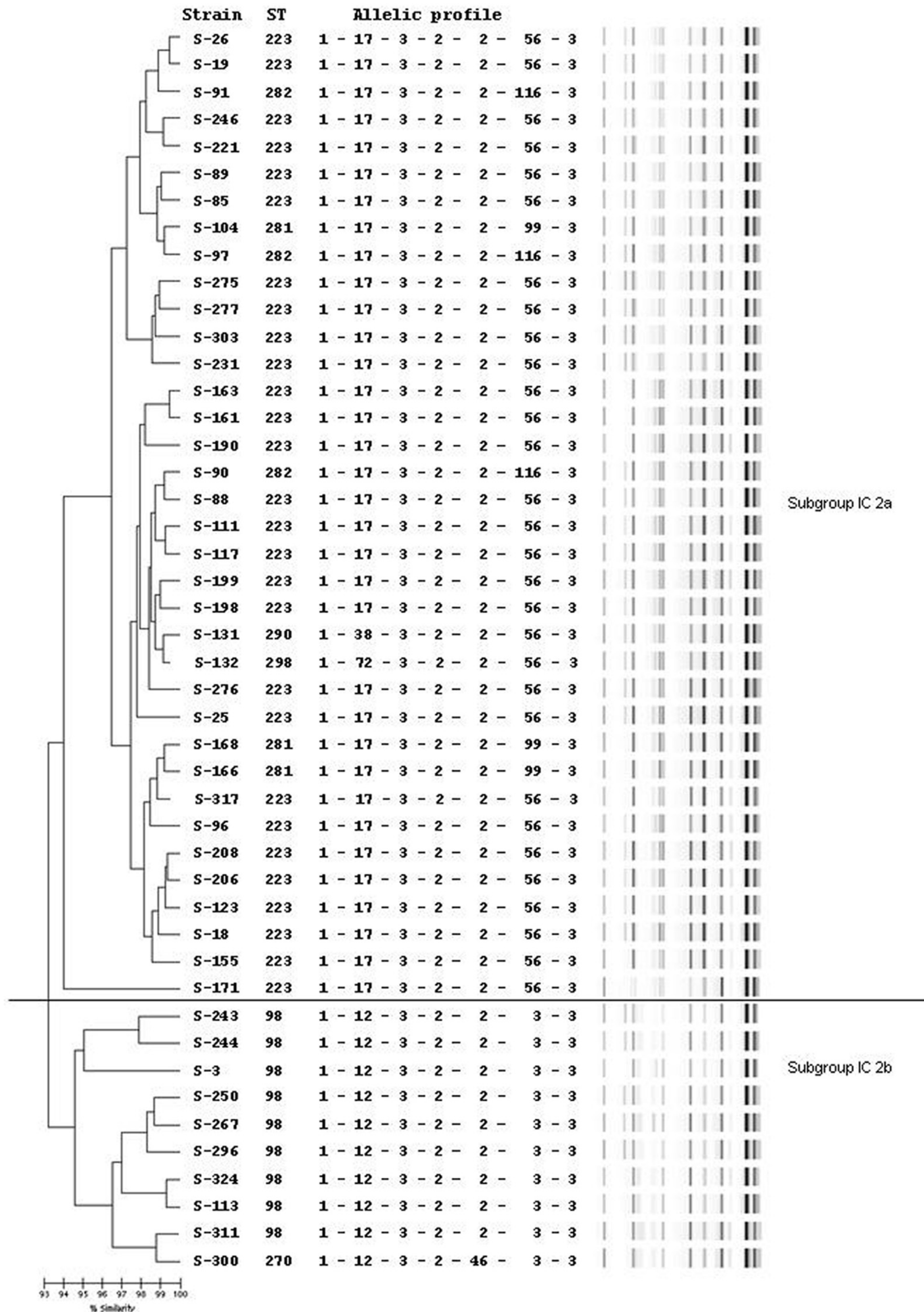

FIG 1 rep-PCR analysis. Dendrogram and computer-generated images of rep-PCR-banding patterns, including MLST sequence types (STs) and allelic profiles of *A. baumannii* IC 2 isolates. Subgroup IC 2a consists of STs 223, 281, 282, 290, and 298. Subgroup IC 2b consists of STs 98 and 270.

numerous, were recovered from 4 different centers. Overall, half the isolates were either considered singletons (i.e., not related to another ST) or were small clusters containing 2 to 6 identical isolates from the same center, suggesting small local outbreaks. These singletons and small clusters A through S did not cluster with isolates from ICs 1 through 8 (Table 1).

A possible explanation for the successful spread of IC 2 isolates in the hospital may be that they have a selective advantage over other unrelated strains. Antimicrobial resistance is one such advantage, and we found that 34 of 37 *A. baumannii* isolates with an imipenem MIC of ≥4 mg/liter were IC 2 strains originating from 7 centers (4 mg/liter [17 isolates]; 8 mg/liter [9 isolates]; 16 mg/

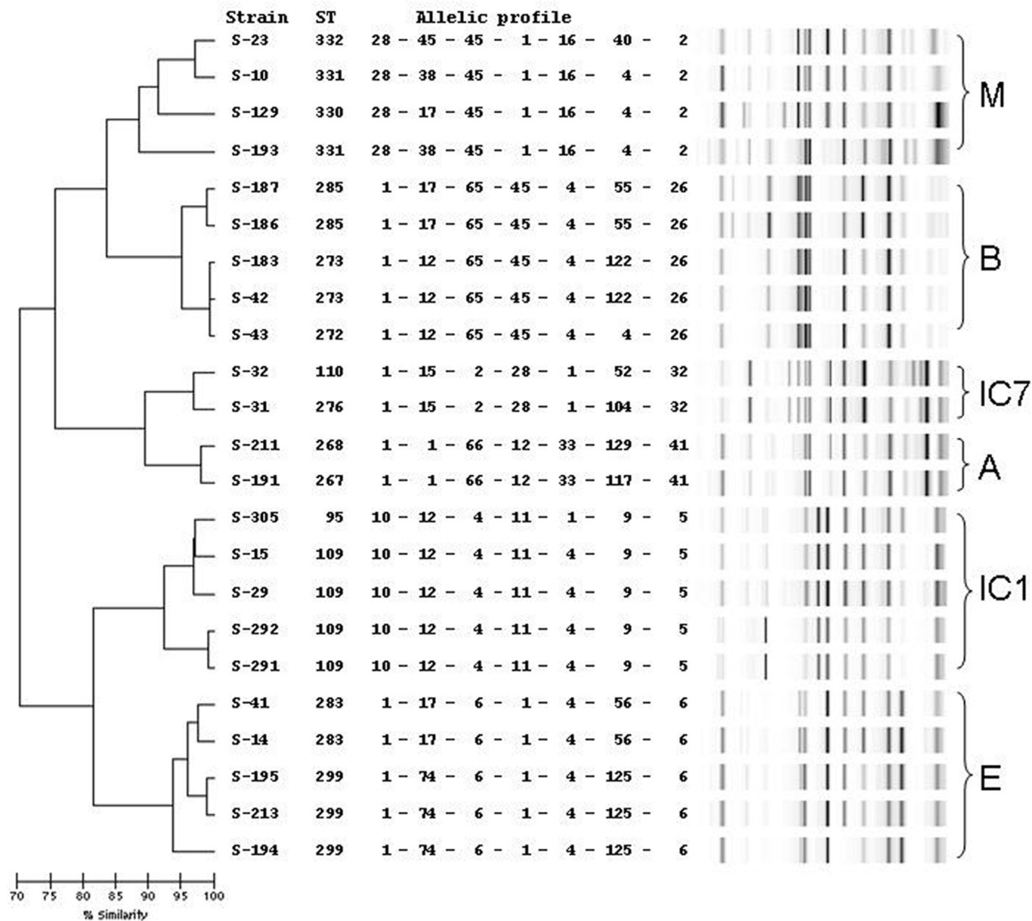

FIG 2 Dendrogram and computer-generated images of rep-PCR-banding patterns, including MLST sequence types (STs) and allelic profiles of the *A. baumannii* rep-PCR clusters M, B, IC 7, A, E, and IC 1.

liter [5 isolates]; 32 mg/liter [2 isolates]; and >32 mg/liter [2 isolates]). Similarly, a previously reported analysis of antimicrobial susceptibility (35) revealed that CC92 isolates were more resistant to levofloxacin, doxycycline, and ampicillin-sulbactam (96%, 21%, and 77% of isolates, respectively) than all other STs combined (23%, 1%, and 4% of isolates, respectively) (data not shown).

These data indicate that IC 2 isolates had spread to many U.S. centers as early as 1996. Similarly, we found IC 1 isolates from the United States as early as 1995. It is worth noting that although IC I (EUI) was described first in 1996, the earliest isolate dates from 1977, and therefore it is not inconceivable that it had spread to the United States before 1995 (10, 18). We did not detect IC 5 isolates, which was surprising because in a previous study IC 5 was the second-most-prevalent cluster and was almost entirely composed of isolates from North and South America (14). However, there were differences in strain composition between the former and latter studies; in the former study, which comprised more recent isolates, IC 5 isolates were carbapenem nonsusceptible, while in this study the majority of isolates were carbapenem susceptible.

With MLST we further typed 140 *A. baumannii* isolates (Table 1), and we detected 68 sequence types (STs). Using BURST software (http://pubmlst.org/analysis/), we found that the STs formed 24 clusters comprising SLVs/DLVs (the same number of clusters as determined by rep-PCR clustering). Clustering by MLST correlated with rep-PCR clusters, with the exception of ST271 and ST288, which are DLVs but show <90% similarity with rep-PCR. Each rep-PCR cluster had a corresponding sequence type(s) that in some instances included SLV/DLVs (Table 1). We analyzed these data with all published sequence types available on the MLST website to identify clonal complexes (Table 1, see CC column). The IC 2 cluster correlated with CC92, which has been shown to correlate with EUII isolates, while CC109 correlated with IC 1 (1, 23). Using rep-PCR, we split the IC 2 cluster into two subgroups, which also corresponded with the sequence types (Fig. 1). Subgroup IC 2a comprised isolates with ST223 or single-locus variants, while IC 2b comprised ST98 and a single ST270 isolate. ST223 and ST98 are double-locus variants (Fig. 2). A clear delineation between rep-PCR clustering and sequence types is shown in Fig. 2.

As previously demonstrated in other studies, the *gpi* allele showed the most variation within a CC, while *gltA*, *recA*, *gdhB*, and *rpoD* were the most conserved (1, 23, 34). *gpi* encodes glucose-6-phosphate isomerase, but why this allele has so many variants is not understood. In a recent study using comparative genomics, extensive recombination was found in EUI and EUII strains, but this was linked most often with genes encoding cell-surface-asso-

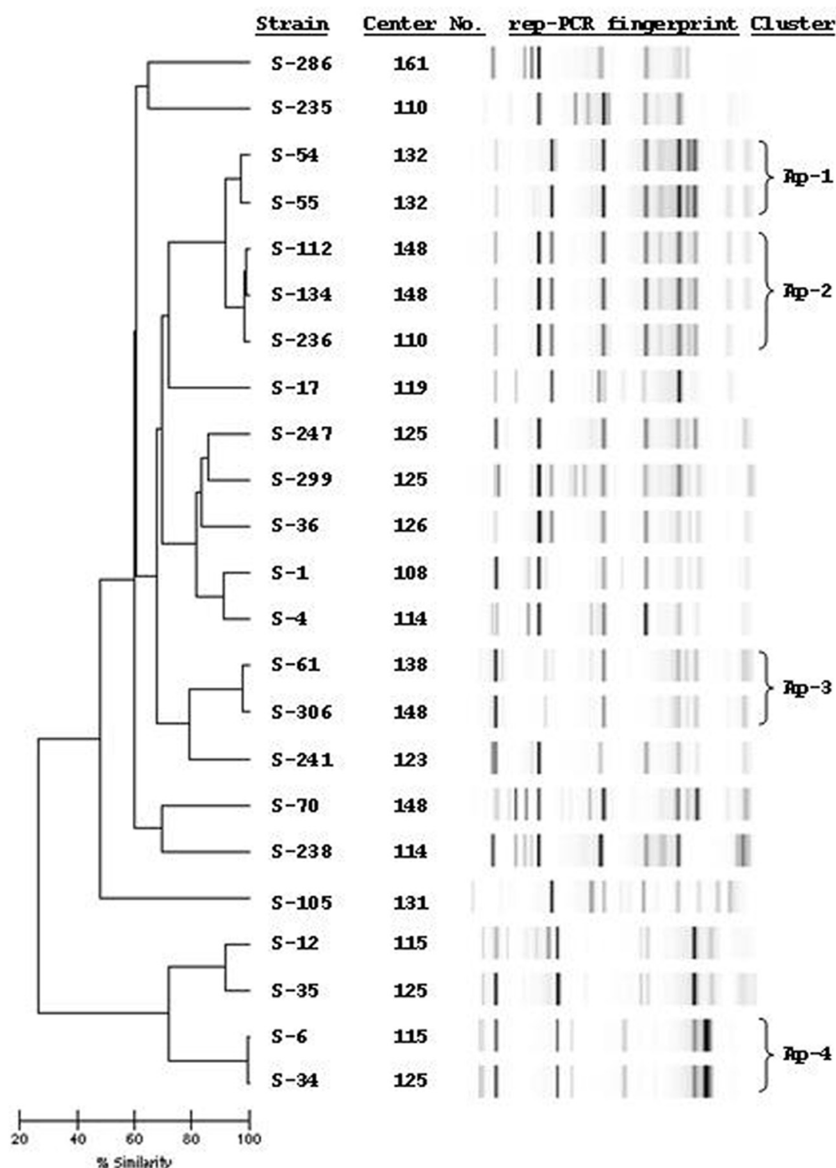

FIG 3 rep-PCR dendrogram of *Acinetobacter pittii* isolates showing clusters Ap-1 to Ap-4.

ciated proteins (27). However, we have analyzed the published *A. baumannii* genomes and found that the location of *gpi* is not adjacent to any such gene.

Thirty-one STs were considered singletons, as they did not cluster with any other published STs. Sixteen STs were not assigned clonal clusters but were SLVs/DLVs of published STs of which a "founder" has not been identified (Table 1). For example, rep-PCR cluster E was represented by three STs (283, 284, and 299) (Table 1). Cluster analysis using all published STs from the MLST website revealed that these three STs also cluster with previously assigned ST40 and ST120, but no CC has been assigned.

Typing of *A. pittii* by rep-PCR revealed that the majority of isolates were singletons. Of the 23 isolates, 14 did not cluster with any other strain. The remaining 9 isolates formed 4 clusters, with isolates showing a similarity of >97% (Fig. 3). Two of these clusters involved strains isolated from separate hospitals (clusters Ap-3 and Ap-4), suggesting horizontal spread.

A. nosocomialis isolates were more clonal; 28 singletons with the remaining 33 isolates formed 13 clusters with ≥95% similarity. While some of these clusters included strains from the same center, we also found evidence of horizontal spread, with clusters containing strains isolated from multiple centers with rep-PCR fingerprints showing >95 to 98.5% similarity (Fig. 4).

In summary, these data illustrate several key facts about the epidemiology of the *A. baumannii* group that causes bloodstream infections. The predominant species is *A. baumannii*. Global dissemination of the major *A. baumannii* clones seems to have been an early event, since we found isolates in the United States that clustered with the international (previously European) clones as

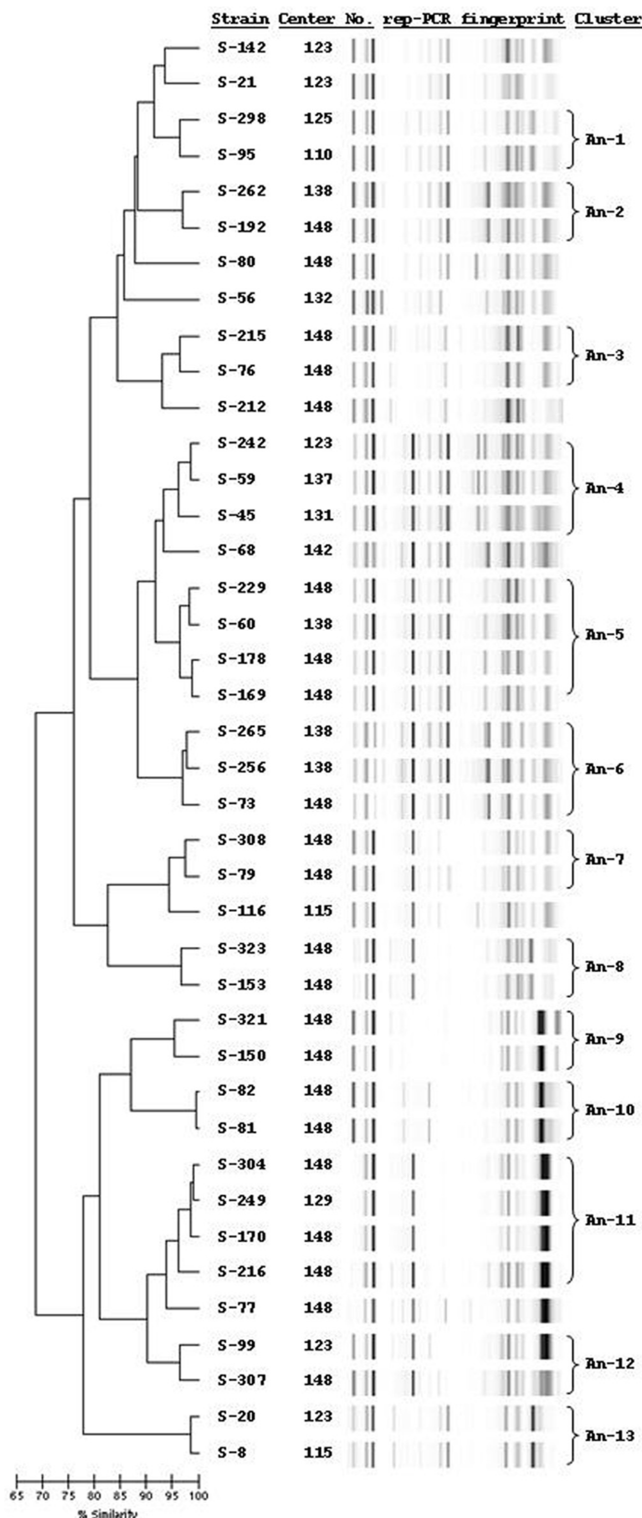

FIG 4 rep-PCR dendrogram of selected *Acinetobacter nosocomialis* isolates showing singletons and clusters An-1 to An-13.

early as 1995. We also found the IS*AbaI* element adjacent to $bla_{\text{OXA-51}}$, even when it was not associated with imipenem resistance, in isolates from 1996. MLST correlates very well with DiversiLab rep-PCR typing. *A. pittii* and *A. nosocomialis*, in contrast, do not appear to be as widespread and are less clonal in nature than *A. baumannii*.

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