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Identification of *Acinetobacter* Species and Genotyping of *Acinetobacter baumannii* by Multilocus PCR and Mass Spectrometry

Joseph A. Ecker,[1] Christian Massire,[1] Thomas A. Hall,[1] Raymond Ranken,[1] Thuy-Trang D. Pennella,[1] Cristina Agasino Ivy,[1] Lawrence B. Blyn,[1] Steven A. Hofstadler,[1] Timothy P. Endy,[2] Paul T. Scott,[2] Luther Lindler,[2] Tacita Hamilton,[2] Charla Gaddy,[2] Kerry Snow,[3] Marie Pe,[3] Joel Fishbain,[3] David Craft,[3] Gregory Deye,[4] Scott Riddell,[4] Eric Milstrey,[5] Bruno Petruccelli,[5] Sylvain Brisse,[6] Vanessa Harpin,[1] Amy Schink,[1] David J. Ecker,[1] Rangarajan Sampath,[1] and Mark W. Eshoo[1]*

Ibis Biosciences, Isis Pharmaceuticals, Inc., 1891 Rutherford Road, Carlsbad, California 92008[1]; Walter Reed Army Institute for Research, Silver Spring, Maryland 20910[2]; Walter Reed Army Medical Center, Washington, D.C. 20307[3]; Landstuhl Regional Medical Center, Landstuhl, Germany[4]; Aberdeen Proving Ground, Aberdeen, Maryland[5]; and Unité Biodiversité des Bactéries Pathogènes Emergentes (U389 INSERM), Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris Cedex 15, France[6]

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Members of the genus *Acinetobacter* are ubiquitous in soil and water and are an important cause of nosocomial infections. A rapid method is needed to genotype *Acinetobacter* isolates to determine epidemiology and clonality during infectious outbreaks. Multilocus PCR followed by electrospray ionization mass spectrometry (PCR/ESI-MS) is a method that uses the amplicon base compositions to genotype bacterial species. In order to identify regions of the *Acinetobacter* genome useful for this method, we sequenced regions of six housekeeping genes (*trpE*, *adk*, *efp*, *mutY*, *fumC*, and *ppa*) from 267 isolates of *Acinetobacter*. Isolates were collected from infected and colonized soldiers and civilians involved in an outbreak in the military health care system associated with the conflict in Iraq, from previously characterized outbreaks in European hospitals, and from culture collections. Most of the isolates from the Iraqi conflict were *Acinetobacter baumannii* (189 of 216 isolates). Among these, 111 isolates had genotypes identical or very similar to those associated with well-characterized *A. baumannii* isolates from European hospitals. Twenty-seven isolates from the conflict were found to have genotypes representing different *Acinetobacter* species, including 8 representatives of *Acinetobacter* genomospecies 13TU and 13 representatives of *Acinetobacter* genomospecies 3. Analysis by the PCR/ESI-MS method using nine primer pairs targeting the most information-rich regions of the *trpE*, *adk*, *mutY*, *fumC*, and *ppa* genes distinguished 47 of the 48 *A. baumannii* genotypes identified by sequencing and identified at the species level at least 18 *Acinetobacter* species. Results obtained with our genotyping method were essentially in agreement with those obtained by pulse-field gel electrophoresis analysis. The PCR/ESI-MS genotyping method required 4 h of analysis time to first answer with additional samples subsequently analyzed every 10 min. This rapid analysis allows tracking of transmission for the implementation of appropriate infection control measures on a time scale previously not achievable.

Acinetobacter spp. are aerobic gram-negative organisms widely distributed in the soil and water of natural environments (4) and are also important nosocomial pathogens. *Acinetobacter* outbreaks involving multidrug-resistant strains have occurred worldwide (5, 6, 13, 14, 28, 36, 39, 46, 47). In hospitalized patients, *Acinetobacter baumannii* frequently colonizes the skin and upper respiratory tract and has been isolated from human sputum, blood, urine, and feces (1, 2, 30). *A. baumannii* also persists for long periods on hospital environmental surfaces and has been isolated from various locations, including air, tap water faucets, bedside urinals, ventilators, gloves, and angiography catheters (9, 25, 29). *Acinetobacter* infections have been historically associated with military and civilian personnel injured in combat, possibly due to direct environmental contamination of wounds. For example, during the Vietnam War, *A. baumannii* was the most common gram-negative bacterium recovered from traumatic injuries to extremities (37).

A recent report described an increasing number of *A. baumannii* bloodstream infections in patients at military medical facilities that were treating service members injured in the Iraq/Kuwait region during Operation Iraqi Freedom (OIF) and in Afghanistan during Operation Enduring Freedom (32). Over a 2-year period from 2002 to 2004, military health officials identified 102 patients with blood cultures that grew *A. baumannii*. Most of the infections were reported from the Landstuhl Regional Medical Center (LRMC) in Germany and the Walter Reed Army Medical Center (WRAMC) in the United States. In both facilities, the number of patients with *A. baumannii* bloodstream infections in 2003 and 2004 significantly exceeded those reported in previous years, suggesting nosocomial transmission. This has the potential to become a serious problem in military and veterans' hospitals, where soldiers returning from active duty worldwide are treated in the same environment as other patients.

* Corresponding author. Mailing address: Ibis Biosciences, Isis Pharmaceuticals, Inc., 1891 Rutherford Road, Carlsbad, CA 92008. Phone: (760) 603-3892. Fax: (760) 603-4653. E-mail: meshoo@isisph.com.

TABLE 1. Primers used for sequencing and strain typing of *Acinetobacter* isolates

Primer	Target	Primer sequence	
		Forward	Reverse
First-generation sequencing primer pairs			
952	*adk*	GTAAAACGACGGCCAGCTGCAAGCKAARAGYGTSATGGA	CAGGAAACAGCTATGACAGTTCYTCRCCTGTSACATCATC
953	*efp*	GTAAAACGACGGCCAGGTKTKGCMGGCTTRCCGCC	CAGGAAACAGCTATGACGTTAARCCWGGYAAGGGTCARGC
954	*fumC*	GTAAAACGACGGCCAGTTGGCGGCRTTTTCRTARCCRAT	CAGGAAACAGCTATGACAAGCTTTRGCYGGACGHGATGC
957	*mutY*	GTAAAACGACGGCCAGTGGGCAGGSCTYGGSTATTAT	CAGGAAACAGCTATGACAGAGAGTAGCRCCCARRTCCAT
955	*ppa*	GTAAAACGACGGCCAGTGTTTTACCCDGCMAACTAYGG	CAGGAAACAGCTATGACGGAACAGCGATSAMTTTVGCATC
950	*trpE*	GTAAAACGACGGCCAGTCGACCTTTGGCAGGAACT	CAGGAAACAGCTATGACGAGTCAGCAACTAGCCCTG
Second-generation sequencing primer pairs			
1035	*adk*	GTAAAACGACGGCCAGGGAATCTGGCGGTTTAGTTTCAG	CAGGAAACAGCTATGACCCTGTGACATCATCTTTACCTTCC
1037	*efp*	GTAAAACGACGGCCAGCGTTCAACCGTGTAAAATTACGTAA	CAGGAAACAGCTATGACACCACCAGAAGTATCACCACG
1039	*fumC*	GTAAAACGACGGCCAGGATGCTGCTGTTTTTGCATCAG	CAGGAAACAGCTATGACCCGGATTTAATGCCGTAACTAACAT
1043	*mutY*	GTAAAACGACGGCCAGTCGTGCCCGYAATTTGCA	CAGGAAACAGCTATGACTCGCTTGAGTGTAGTCATGATT
1041	*ppa*	GTAAAACGACGGCCAGCCCGGTTATGTACCAAATACTTTG	CAGGAAACAGCTATGACGGCATCGATACCACCGTC
1033	*trpE*	GTAAAACGACGGCCAGCGCGCGGTAAAACTAAAGAAGAAG	CAGGAAACAGCTATGACCCCTGCACCAGCCTGTACATA
PCR/ESI-MS primer pairs			
1151	*trpE*	TGAGATTGCTGAACATTTAATGCTGATTGA	TTGTACATTTGAAACAATATGCATGACATGTGAAT
1156	*Adk*	TCAACCTGACTGCGTGAATGGTTGT	TACGTTCTACGATTTCTTCATCAGGTACATC
1158	*mutY*	TCGTGCCCGCAATTTGCATAAAGC	TAATGCCGGGTAGTGCAATCCATTCTTCTAG
1160	*mutY*	TTGTAGCACAGCAAGGCAAATTTCCTGAAAC	TGCCATCCATAATCACGCCATACTGACG
1165	*fumC*	TACTAGCGGTAAGCTTAAACAAGATTGC	TGAGTCGGGTTCACTTTACCTGGCA
1167	*fumC*	TCGGCGAAATCCGTATTCCTGAAAATGA	TACCGGAAGCACCAGCGACATTAATAG
1170	*fumC*	TTATAACTTACTGCAATCTATTCAGTTGCTTGGTG	TGCCGTAACTAACATAAGAGAATTATGCAAGAA
1171	*ppa*	TGGTTATGTACCAAATACTTTGTCTGAAGATGG	TGACGGCATCGATACCACCGTC

Understanding the fundamental mechanisms underlying *Acinetobacter* infections, including the original sources of the infecting organisms, their clonality, and geographical spread, is an important requirement for the development of appropriate infection control measures. Genotyping allows investigation of clonal spread and can be used to identify the source of the original infection. Traditional *Acinetobacter* strain typing methods include serotyping (38), multilocus enzyme electrophoresis (34), and DNA-based methods, including repetitive extragenic palindromic sequence-based PCR (8, 19), amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (43), and ribotyping (21, 23, 26). However, these methods are not optimal: despite strenuous efforts at standardization, it is difficult to compare results obtained in different laboratories, and the methods are labor intensive and time consuming. Multilocus sequence typing (MLST) is a high-resolution molecular tool for discriminating between closely related bacterial species (40). MLST data are reproducible and portable, facilitating comparison among laboratories worldwide. MLST approaches to strain typing of *Acinetobacter baumannii* are currently based on sequencing regions of housekeeping genes (3) or the drug efflux gene *adeB* (20). However, these MLST approaches cannot provide a real-time surveillance capability. Furthermore, *Acinetobacter* infections can be the result of one of several pathogenic *Acinetobacter* species, including *A. baumannii*, *Acinetobacter* genomospecies 13TU, or *Acinetobacter* genomospecies 3 (17, 35, 42, 45).

Here, we describe a very rapid (4 h of analysis time to first answer with additional samples analyzed every 10 min) multilocus method for the species-level identification of *Acinetobacter* spp. and the genotyping of *A. baumannii*. PCR ampli-

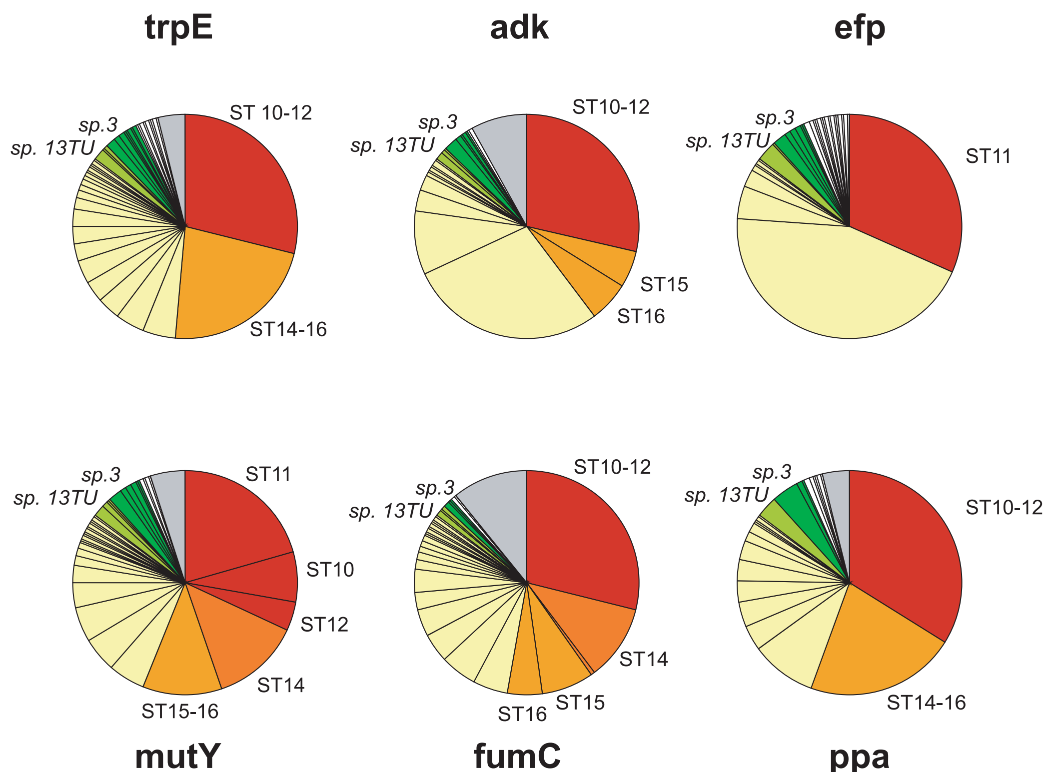


FIG. 1. Distribution of the 267 *Acinetobacter* sequences into alleles for the six individual genes. Pie slices correspond to unique alleles. The area of the slice is determined by the number of isolates containing each allele. The color coding relates to sequence type groupings (Fig. 2), as follows: red, sequences belonging to *A. baumannii* group 11 (ST1, ST10, ST11, and ST12); dark orange, *A. baumannii* group 14 (ST8 and ST14); orange, *A. baumannii* group 15 (ST15, ST45, ST46, and ST16); yellow, all other *A. baumannii* types; medium green, *Acinetobacter* genomospecies 13TU; dark green, *Acinetobacter* genomospecies 3; white, other *Acinetobacter* species; gray, no sequence data available due to failed priming.

cons from housekeeping genes are analyzed by electrospray ionization mass spectrometry (PCR/ESI-MS), and the base compositions (the numbers of adenines, guanines, cytosines, and thymines) of the amplicons are determined (15, 16, 31). The present study was performed with a collection of 216 *Acinetobacter* field isolates and 51 diverse reference strains. Results from the PCR/ESI-MS genotyping assay and PFGE analysis were compared.

MATERIALS AND METHODS

Isolates tested. A total of 267 isolates were analyzed by PCR/ESI-MS and sequencing of the six MLST loci. These included a collection of 23 isolates from European hospitals that were previously characterized by ribotyping, PFGE, and AFLP and that are representative of the major clones, I, II, and III (41). We also obtained 27 isolates from the American Type Culture Collection (ATCC) representing several *Acinetobacter* species. These were previously characterized by ARDRA, RAPD, and AFLP (23). In addition, 216 strains were isolated as part of an ongoing investigation of *A. baumannii* infections among patients at military medical facilities where U.S. service personnel were treated between 2002 and 2004 (32). One reference strain analyzed in this study, *Acinetobacter baylyi* ATCC 33405, was represented by its genomic sequence; this strain was formerly known as *Acinetobacter* sp. strain ADP1 (44). Partial sequences from strain LUH8731 were also used to characterize *Acinetobacter* genomospecies 13TU isolates (sequence data kindly provided by Lenie Dijkshoorn).

PCR/ESI-MS gene targets and selection of genotyping primers. The *Acinetobacter* genes used for PCR/ESI-MS analysis were the same as those used in the *Moraxella* MLST project (http://web.mpiib-berlin.mpg.de/mlst/dbs/Mcatarrhalis). The genes analyzed were those encoding anthranilate synthase component I (*trpE*), adenylate kinase (*adk*), translation elongation factor P (*efp*), A/G-specific DNA glycosylase (*mutY*), fumarase (*fumC*), and inorganic pyrophosphatase (*ppa*). We created an alignment of all of the sequences for each of these genes from the 267 isolates and concatenated them (total of 1,679 positions, excluding primer pair sequences). These combined sequences were grouped into 85 sequence types, wherein a single nucleotide difference in 1 of the total of 1,679 positions resulted in a new sequence type. Representatives of each sequence type were then analyzed for the design of PCR/ESI-MS genotyping primers, based on criteria previously described (15, 16).

Genome preparation, sequencing, and PCR. Genomic material from cultured samples was prepared with the DNeasy Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. All PCRs were assembled in 40-μl reaction mixtures in the 96-well microtiter plate format by using a Packard MPII liquid handling robotic platform and MJ Dyad thermocyclers (MJ Research, Waltham, MA). Sequencing of PCR-generated amplicons was performed by QIAGEN.

Sequencing of the *Acinetobacter trpE*, *adk*, *efp*, *mutY*, *fumC*, and *ppa* loci was performed with degenerate PCR sequencing primers (Table 1, first-generation sequencing primer pairs) selected for complementarity to the previously sequenced *Acinetobacter baylyi* strain ATCC 33305. These primers were synthesized with the M13 sequencing primer targets at their 5′ ends to facilitate direct sequencing of the PCR amplicons. The sequencing primers were used to generate amplicons from a diverse subset of *Acinetobacter* strains: ATCC strains 27227, 17976, 17908, 17904, 15309, and 9955 and OIF strains 197, 148, 147, 146, and 140. Due to the genetic diversity of these strains it was necessary to employ a range of PCR conditions to generate amplicons from all strains. The PCR mix consisted of 4 units of AmpliTaq Gold, 1× buffer II (Applied Biosystems, Foster City, CA), 2 to 3 mM $MgCl_2$, 0.4 M betaine, 800 μM deoxynucleoside triphosphate (dNTP) mix, and 250 nM (each) primer. The following PCR conditions were used to generate the amplicons for sequencing: 96°C for 10 min, followed by eight cycles of 96°C for 15 seconds, 52 to 58°C for 20 to 90 seconds, and 72°C for 20 seconds. The PCR was then continued for 37 additional cycles of 96°C for 15 seconds, 63°C for 10 seconds, and 72°C for 20 seconds.

Based on the *Acinetobacter* sequences generated with the first generation

Species	ST	#	isolates (clone type)	1151-trpE	1156-adk	1158-mutY	1160-mutY	1165-fumC	1167-fumC	1170-fumC	1171-ppa	2922-efp
Acinetobacter baumannii	2	1	OIF107	A45G34C21T42	A44G32C25T39	A27G21C26T21	A32G35C28T34	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C32T45	A44G35C25T43
	3	6	OIF011, 042, 106, 143, 153, 156	A44G35C22T41	A44G32C26T38	A27G20C27T21	A32G35C28T34	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C31T46	A44G35C24T44
	4	5	OIF101, 115, 116, 162, 303	A44G35C22T41	A44G32C27T37	A27G20C27T21	A32G35C28T34	A39G33C30T37	A40G35C32T40	A38G27C21T50	A35G37C30T47	A44G35C25T43
	5	1	OIF005	A44G35C22T41	A44G32C27T37	A27G21C26T21	A32G35C28T34	A39G33C30T37	A40G35C32T40	A38G27C21T50	A35G37C30T47	A44G35C25T43
	6	1	OIF035	A44G35C22T41	A44G32C27T37	A27G21C25T22	A32G35C28T34	A40G33C30T36	A40G35C35T37	A38G27C21T50	A35G37C33T44	A44G35C25T43
	7	7	OIF300, 307, 314, 315, 318, 320, 322	A44G35C22T41	A44G32C25T39	A27G21C25T22	A32G35C29T33	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C33T44	A44G35C25T43
	9	8	**ATCC9955;** OIF072, 098, 102, 113, 114, 142, 193	A44G35C21T42	A44G32C26T38	A27G21C25T22	A32G35C28T34	A39G33C30T37	A40G35C32T40	A38G27C20T51	A36G35C31T47	A44G35C25T43
	1	1	OIF157	A44G35C21T42	A44G32C26T38	A27G21C25T22	A32G35C29T33	A40G33C30T36	A41G34C34T38	A38G27C21T50	A35G37C33T44	A45G34C25T43
	10	12	OIF002, 003, 024, 081, 082, 085, 126, 148, 149, 186, 187, 189	A44G35C21T42	A44G32C26T38	A27G21C26T21	A32G35C28T34	A40G33C30T36	A41G34C34T38	A38G27C21T50	A35G37C33T44	A45G34C25T43
	11	51	OIF006, 007, 008, 015, 016, 025, 026, 027, 028, 031, 033, 034, 040, 043, 051, 052, 076, 077, 086, 088, 103, 105, 108, 112, 118, 125, 135, 141, 144, 158, 161, 163, 164, 165, 167, 168, 169, 171, 172, 173, 174, 176, 177, 178, 179, 180, 181, 182, 183, 195, 198	A44G35C21T42	A44G32C26T38	A27G21C25T22	A32G34C28T35	A40G33C30T36	A41G34C34T38	A38G27C21T50	A35G37C33T44	A45G34C25T43
	12	10	**LUH6011, 6025, 6038, 6045, 6051, 5868, 6021, 6023, 6024, 6034 (II)**	A44G35C21T42	A44G32C26T38	A27G21C26T21	A32G34C29T34	A40G33C30T36	A41G34C34T38	A38G27C21T50	A35G37C33T44	A45G34C25T43
	47	1	OIF001	A44G35C21T42	A44G32C26T38	A27G21C26T21	A32G34C29T34	A40G33C30T36	A41G34C34T38	A38G27C21T50	A35G37C33T44	A44G35C25T43
	13	8	OIF049, 095, 109, 117, 120, 121, 192, 196	A44G35C22T41	A44G32C26T38	A27G21C25T22	A32G35C28T34	A40G33C29T37	A40G35C33T39	A38G27C21T50	A35G37C33T44	A44G35C25T43
	14	28	**LUH6020, 6030, 6036, 6048, 6009, 5874 (III);** OIF017, 022, 023, 037, 038, 039, 054, 056, 063, 075, 090, 094, 110, 122, 124, 137, 138, 139, 155, 159, 188, 332	A44G35C22T41	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C29T37	A40G35C34T38	A38G27C21T50	A35G37C30T47	A44G35C25T43
	8	1	**LUH6035 (III)**	A44G35C22T41	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C29T37	A40G35C34T38	A38G27C20T51	A35G37C30T47	A44G35C25T43
	15	12	**LUH6014, 6050, 6013, 6022, 5881 (I);** OIF012, 050, 066, 067, 079, 190, 194	A44G35C22T41	A44G32C26T38	A29G19C26T21	A31G35C29T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C30T47	A44G35C25T43
	45	2	OIF166, 175	A44G35C22T41	A44G32C26T38	A29G19C26T21	A31G35C29T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C32T45	A44G35C25T43
	46	2	OIF092, 154	A44G35C22T41	A44G32C27T37	A29G19C26T21	A31G35C29T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C30T47	A44G35C25T43
	16	13	OIF009, 018, 062, 071, 074, 104, 127, 128, 130, 132, 133, 170, 197	A44G35C22T41	A44G32C27T37	A29G19C26T21	A31G35C29T34	A40G34C29T36	A41G35C32T39	A37G28C20T51	A35G37C30T47	A44G35C25T43
	17	1	OIF146	A44G35C20T43	A44G32C27T37	A27G21C25T22	A32G35C28T34	A40G33C29T37	A41G34C35T37	A38G27C21T50	A35G36C32T46	A44G35C25T43
	18	1	OIF013	A43G36C21T42	A44G32C27T37	A27G21C26T21	A31G36C28T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C31T46	A44G35C25T43
	19	1	OIF111	A43G36C21T42	A44G32C26T38	A27G21C25T22	A32G35C28T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C31T46	A44G35C25T43
	20	1	OIF316	A43G36C20T43	A44G32C27T37	A28G21C24T22	A32G35C29T33	A40G33C29T37	A40G35C33T39	A37G28C20T51	A35G37C30T47	A45G34C25T43
	21	2	OIF004, 100	A43G36C20T43	A44G32C27T37	A28G21C24T22	A32G35C29T33	A39G33C30T37	A40G35C32T40	A38G27C20T51	A35G37C33T44	A44G35C25T43
	22	1	OIF299	A43G36C20T43	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C33T44	A44G35C25T43
	23	1	OIF047	A43G36C20T43	A44G32C27T37	A27G21C26T21	A32G34C29T34	A39G33C30T37	A40G35C32T40	A38G27C21T50	A35G37C31T46	A44G35C25T43
	24	7	OIF087, 091, 184, 310, 311, 312, 327	A43G36C20T43	A44G32C27T37	A27G21C26T21	A32G35C28T34	A40G33C30T36	A40G35C34T38	A39G26C22T49	A35G37C33T44	A44G35C25T43
	25	5	OIF030, 032, 099, 147, 152	A43G36C20T43	A44G32C27T37	A27G21C25T22	A32G35C29T33	A40G33C30T36	A40G35C34T38	A39G26C22T49	A35G37C33T44	A44G35C25T43
	26	3	OIF140, 191, 333	A43G36C20T43	A44G32C26T38	A27G21C26T21	A32G35C28T34	A40G33C29T37	A40G35C34T38	A38G27C21T50	A35G37C33T44	A44G35C25T43
	27	1	OIF044	A44G35C19T44	A44G32C27T37	A27G21C25T22	A32G35C28T34	A39G33C30T37	A40G35C33T39	A38G27C20T51	A35G37C33T44	A44G35C25T43
	28	1	OIF313	A44G35C22T41	A44G32C27T37	A27G21C25T22	A32G34C28T35	A40G33C30T36	A42G34C33T38	A38G27C20T51	A35G37C31T46	A43G35C25T44
	29	3	**LUH6049 (I);** OIF020, 065	A44G35C21T42	A44G32C27T37	A27G21C25T22	A32G34C28T35	A39G33C30T37	A40G35C32T40	A38G27C20T51	A35G36C29T49	A44G35C25T43
	30	6	OIF068, 083, 084, 093, 160, 060	A44G35C19T44	A44G32C27T37	A27G21C25T22	A32G35C28T34	A39G33C30T37	A40G35C32T40	A38G27C21T50	A35G36C29T49	A44G35C25T43

Species	ST	#	isolates	1151-trpE	1156-adk	1158-mutY	1160-mutY	1165-fumC	1167-fumC	1170-fumC	1171-ppa	2922-efp
A. baumannii	31	3	**ATCC15308, 15473, 17961**	A44G35C22T41	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C29T37	A41G35C32T39	A37G28C20T51	A35G37C31T46	A45G34C25T43
	32	1	**ATCC15150**	A43G36C20T43	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C29T37	A41G35C33T38	A38G27C20T51	A35G37C33T44	A44G35C25T43
	33	1	**ATCC27227**	A43G36C20T43	A44G32C27T37	A27G21C25T22	A31G36C28T34	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C31T46	A44G35C25T43
	34	1	**ATCC15151**	A44G35C22T41	A44G32C26T38	A27G21C25T22	A31G36C28T34	A39G33C30T37	A40G35C32T40	A39G26C20T51	A35G37C31T46	A45G34C25T43
	35	1	**ATCC15149**	A44G35C22T41	A44G32C27T37	A27G21C25T22	A32G35C28T34	A39G33C30T37	A40G35C33T39	A38G27C20T51	A35G37C31T46	A44G35C24T44
	36	1	**ATCC17904**	A44G35C22T41	A44G32C27T37	A27G20C27T21	A32G35C28T34	A40G33C30T36	A41G34C35T37	A38G27C20T51	A35G37C30T47	A44G35C25T43
	37	1	OIF064	A44G35C21T42	A44G32C26T38	A27G20C27T21	A32G35C28T34	A40G33C29T37	A41G35C33T38	A38G27C20T51	A35G37C32T45	A44G35C25T43
	38	1	OIF319	A44G35C22T41	A44G32C27T37	A27G21C25T22	A32G34C28T35	A40G33C30T36	A42G34C33T38	A38G27C20T51	A35G36C29T49	A43G35C25T44
	39	1	OIF069	A44G35C22T41	A44G32C26T38	A27G21C26T21	A32G35C28T34	A39G33C30T37	A40G35C33T39	A38G27C20T51	A35G37C30T47	A45G34C25T43
	40	1	OIF317	A44G35C22T41	A44G32C26T38	A28G20C25T22	A32G35C28T34	A39G33C30T37	A40G35C32T40	A38G27C20T51	A35G37C33T44	A44G35C25T43
	41	1	OIF321	A44G35C21T42	A44G32C27T37	A28G20C25T22	A32G34C28T35	A40G33C30T36	A40G35C34T38	A38G27C21T50	A35G37C31T46	A45G34C25T43
	42	1	OIF070	A44G35C22T41	A44G32C26T38	A27G21C25T22	A31G36C28T34	A40G33C30T36	A41G35C33T38	A38G27C20T51	A35G37C29T48	A44G35C25T43
	43	1	OIF326	A45G34C20T43	A44G32C27T37	A28G20C25T22	A31G36C29T33	A40G33C30T36	A40G35C34T38	A39G26C22T49	A35G37C31T46	A44G35C25T43
	44	2	OIF057, 058	A44G35C21T42	A44G32C27T37	A27G21C26T21	A32G35C28T34	A39G33C30T37	A40G35C33T39	A38G27C20T51	A35G37C32T45	A45G34C25T43
A. sp. 13TU	A1	5	OIF021, 045, 046, 059, 151	A46G32C20T44	A44G33C27T36	A27G21C25T22	A30G36C26T37	A41G34C28T36	A41G39C31T36	A37G26C24T49	A34G38C31T46	A47G33C24T43
	A2	1	OIF328	A45G33C20T44	A44G33C28T35	A29G20C25T21	A31G35C29T34	A41G34C28T36	A40G40C31T36	A38G25C24T49	A34G38C31T46	A47G33C24T43
	A3	1	OIF145	A46G32C20T44	A44G33C27T36	A27G22C25T21	A30G36C27T36	A41G34C28T36	A40G40C31T36	A39G24C24T49	A34G38C31T46	A47G33C24T43
	A4	1	OIF123	A46G31C20T45	A44G33C28T35	A27G22C25T21	A30G36C27T36	A41G34C28T36	A42G38C31T36	A37G26C24T49	A34G38C31T46	A47G33C25T42
	A5	1	**ATCC17903**	A45G34C20T43	A44G33C26T37	A28G20C26T21	A29G37C27T36	A41G34C28T36	A40G40C31T36	A38G25C24T49	A34G38C31T46	A47G33C24T43
Acinetobacter sp. 3	B1	2	OIF323, 324	A42G34C21T45	A42G34C25T39	A27G22C23T23	A32G34C28T35	A38G35C28T38	A43G38C29T37	ND	A35G37C31T46	A46G35C24T42
	B2	3	OIF131, 329, 330	A41G34C22T45	A43G33C24T40	A26G23C23T23	A31G35C28T35	A39G37C26T37	A43G37C30T37	A36G27C24T49	A34G37C31T47	A46G35C24T42
	B3	2	OIF061, 331	A46G33C21T42	A43G33C24T40	A26G23C23T23	A31G35C28T35	A39G37C26T37	A44G35C30T38	ND	A34G37C31T47	A46G35C25T41
	B4	1	OIF308	A45G34C21T42	A43G33C24T40	A26G23C23T23	A30G36C27T36	A40G36C26T37	A43G37C30T37	A35G28C23T50	A34G37C31T47	A46G35C24T42
	B5	1	OIF309	A43G35C19T45	A43G33C25T39	A26G23C23T23	A30G36C27T36	A40G36C26T37	A43G37C30T37	A35G28C23T50	A34G37C31T47	A46G35C24T42
	B6	1	OIF185	A41G34C22T45	A43G33C24T40	A26G23C23T23	A30G36C27T36	A39G37C26T37	A43G37C30T37	A36G27C24T49	A34G37C31T47	A46G35C24T42
	B7	1	OIF055	A42G34C20T46	A43G33C24T40	A26G23C23T23	A30G36C27T36	A39G37C26T37	A43G37C30T37	A36G27C24T49	A34G37C31T47	A46G35C24T42
	B8	1	OIF302	A45G34C20T43	A43G33C24T40	A26G23C23T23	A31G36C26T36	A39G37C26T37	A43G37C30T37	A36G27C24T49	A34G37C31T47	A46G35C25T41
	B9	1	OIF325	A44G33C21T44	A43G33C25T39	A26G23C23T23	A30G36C27T36	A39G36C26T38	A43G37C30T37	A35G28C24T49	A35G37C32T45	A46G35C24T42
	B10	1	**ATCC19004**	A46G33C21T42	A43G33C24T40	A26G23C23T23	A31G35C28T35	A39G37C26T37	A43G37C30T37	A36G27C24T49	A34G37C31T47	A46G35C24T42
A. sp. ADP1		1	**ADP1**	A41G37C21T43	A41G34C25T40	A33G19C21T22	A35G33C29T32	A42G35C28T34	A44G38C30T35	A40G26C19T51	A35G39C27T48	A44G38C20T45
A. schindleri		1	**BAA-618**	A38G36C20T48	A42G32C27T39	A27G22C25T21	A31G37C29T32	ND	A40G37C34T36	A39G25C21T51	A35G37C33T44	A42G38C26T41
A. ursingii		1	**BAA-617**	A40G34C21T47	A39G34C26T41	A30G19C26T20	A31G37C28T33	ND	A39G38C32T38	ND	ND	A49G35C22T41
		1	OIF129	A40G34C21T47	A39G34C26T41	A28G24C22T21	A33G35C28T33	ND	A39G38C32T38	A38G27C21T50	A33G42C27T47	A49G35C22T41
A. calcoaceticus		1	**ATCC23055**	A44G35C22T41	ND	ND	A30G36C28T35	A40G35C28T36	A43G36C30T38	ND	A36G37C30T46	A45G36C21T45
A. sp. 2		1	**ATCC17976**	A41G34C20T47	A42G31C27T40	A27G24C24T20	A31G36C30T32	ND	A37G43C33T34	A38G27C20T51	A38G40C30T41	A42G37C25T43
A. haemolyticus		1	**ATCC17906**	A40G34C21T47	A39G34C24T43	A25G22C23T25	A35G35C27T32	ND	ND	A38G27C19T52	ND	A44G35C24T44
		1	OIF136	A40G34C21T47	A39G34C24T43	A25G20C26T24	A34G35C27T33	A37G34C30T38	A43G37C30T37	ND	A33G39C30T47	A45G34C24T44
A. junii		1	**ATCC17908**	A39G35C19T49	A42G32C25T41	A27G20C27T21	A32G34C30T33	ND	A40G36C29T42	A42G23C19T52	A34G41C31T43	A45G35C22T45
		1	**ATCC19001**	A39G35C19T49	A42G32C25T41	A27G21C25T22	A32G36C29T32	A37G35C29T38	A40G36C29T42	A41G24C19T52	A34G41C29T45	A44G34C23T46
A. sp. 6		1	**ATCC17979**	A41G35C20T46	A41G33C24T42	A27G21C25T22	A33G33C29T34	ND	A40G37C31T39	A36G28C23T49	A33G41C27T48	A44G33C27T43
A. johnsonii		1	**ATCC17909**	A42G34C22T44	A41G31C28T40	A25G22C23T25	A30G38C24T37	A39G36C29T35	A41G39C32T35	A40G25C21T50	A37G39C29T44	A46G35C23T43
A. lwoffii		1	**ATCC15309**	A44G35C20T43	A42G33C25T40	A26G20C30T19	A27G38C31T33	A37G36C30T36	A38G42C34T33	A38G27C20T51	A35G40C29T45	A43G35C28T41
A. sp. 9		1	**ATCC9957**	A45G34C20T43	A42G33C25T40	A25G21C28T21	A33G35C28T33	A37G36C30T36	A38G42C34T33	ND	A35G40C29T45	A43G35C28T41
A. sp. 10		1	**ATCC17924**	A43G34C20T45	A41G34C27T38	ND	A32G35C28T34	ND	ND	ND	A37G39C27T46	A43G39C21T44
		1	OIF306	A42G35C20T45	A42G34C27T37	A24G24C25T22	A31G37C29T32	A35G37C28T39	ND	ND	A38G38C27T46	A43G39C21T44
A. sp. 11		1	**ATCC11171**	A42G34C21T45	A41G34C25T40	A29G19C26T21	A32G36C29T32	ND	ND	A38G27C20T51	A36G36C32T45	A47G34C23T43
A. radioresistens		1	**ATCC43998**	A40G35C24T43	ND	A25G22C23T25	ND	A40G37C26T36	A40G38C31T38	A42G24C19T51	A36G38C29T46	A47G33C23T44
A. sp. 14TU		1	**ATCC17905**	A40G33C24T45	A42G33C26T39	A28G21C25T21	A32G38C27T32	ND	A40G37C34T36	A39G25C21T51	A34G40C30T45	A42G36C24T45
A. sp. 16		1	**ATCC17988**	A40G36C20T46	A42G33C27T38	A31G22C23T19	A30G35C33T31	ND	A41G35C32T39	A41G25C21T49	A36G37C29T47	A47G34C22T44
A. sp. OIF199		1	OIF199	A40G37C21T44	A38G33C25T44	A27G21C25T22	A34G33C30T32	A40G33C30T36	A42G35C33T37	ND	A35G38C27T49	A43G36C27T41
A. sp. OIF301		1	OIF301	A45G31C23T43	A43G33C24T40	A24G25C24T22	A28G38C28T35	A40G35C25T39	A42G37C33T35	ND	A37G39C29T44	A48G35C22T42
A. sp. OIF304		1	OIF304	A44G31C24T43	A43G33C24T40	A27G23C22T23	A30G38C30T31	ND	A39G39C33T36	ND	A37G37C32T43	A48G35C23T41

FIG. 2. Base compositions of nine selected amplicons from *Acinetobacter* housekeeping genes. Within each column, base compositions that are common to multiple types are similarly colored. Base compositions printed on a white background are unique to a particular type. #, number of isolates; ND, base composition not determined.

sequencing primers, a set of second-generation sequencing primers (Table 1) was designed and used to sequence the PCR/ESI-MS target loci of all isolates. For these reactions, the PCR mix consisted of 4 units of AmpliTaq Gold, 1× buffer II, 2 mM $MgCl_2$, 0.4 M betaine, 800 μM dNTP mix, and 250 nM (each) primer. The following PCR conditions were used to generate the amplicons for sequencing: 96°C for 10 min, followed by eight cycles of 95°C for 15 seconds, 52°C for 90 seconds, and 72°C for 40 seconds, with the 52°C annealing temperature increasing by 0.6°C each cycle. The PCR was then continued for 37 additional cycles of 96°C for 15 seconds, 58°C for 20 seconds, and 72°C for 40 seconds.

The primers used for PCR/ESI-MS analysis are shown in Table 1. The PCR mix consisted of 4 units of AmpliTaq Gold, 1× buffer II, 1.5 mM $MgCl_2$, 0.4 M betaine, 800 μM dNTP mix, and 250 nM (each) primer. The following PCR conditions were used to amplify the sequences used for PCR/ESI-MS analysis: 95°C for 10 min followed by eight cycles of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds, with the 48°C annealing temperature increasing by 0.9°C each cycle. The PCR was then continued for 37 additional cycles of 95°C for 15 seconds, 56°C for 20 seconds, and 72°C for 20 seconds.

Phylogenetic analysis. Neighbor-joining trees were constructed with the *dnadist* and *neighbor* programs of the Phylip package (http://evolution.genetics.washington.edu/phylip.html). The *efp* tree was assembled with sequence from the *efp* gene from all *Acinetobacter* species. For the *Acinetobacter* species with multiple isolates in our collection, sequences were selected to represent the allele diversity present in the *efp* gene (*A. baumannii* OIF007, OIF064, OIF139, OIF143, OIF319, OIF321, OIF327, and ATCC 17904; *Acinetobacter* genomospecies 13TU OIF145 and ATCC 17903; and *Acinetobacter* genomospecies 3 OIF061, OIF131, OIF308, OIF323, OIF325, and ATCC 19004).

Representatives of the 48 *A. baumannii* sequence types were used for the *A. baumannii* tree. Both trees were bootstrapped with the *seqboot* and *consense* programs of the Phylip package and were analyzed with *treeview* (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Final editions were created with Adobe Illustrator.

MS and base composition analysis. Following amplification, 15-μl aliquots of each PCR mix were desalted and with a weak anion-exchange protocol as described elsewhere (22). Accurate mass (±1 ppm) and high-resolution (M/dM > 100,000 full-width half-maximum resolution) mass spectra were acquired for each sample by the PCR/ESI-MS protocols described previously (16). For each sample, approximately 1.5 μl of analyte solution was consumed during the 74-second spectral acquisition. Raw mass spectra were postcalibrated with an internal mass standard and deconvolved to monoisotopic molecular masses. Unambiguous base compositions were derived from the exact mass measurements of the complementary single-stranded oligonucleotides (27). Quantitative results were obtained by comparing the peak heights with an internal PCR calibration standard present in every PCR well at 100 molecules (16).

PFGE. The PFGE protocol, described at http://www.cdc.gov/pulsenet/protocols.htm, was used with modifications for *Acinetobacter*. Bacterial DNA was prepared with a *Salmonella* standard and ApaI and XbaI restriction enzymes for *Acinetobacter* and *Salmonella*, respectively. Restriction fragments were separated with a contour-clamped homogenous electric field electrophoresis instrument (CHEF Mapper; Bio-Rad Laboratories). PFGE conditions were 6.0 V/cm gradient, 120° angle, 14°C, and 7- to 20-second pulse times for 18.5 h. Gel images were entered into the Bionumerics software (Applied Maths, Kortrijk, Belgium) for band analysis with the Dice coefficient. The unweighted pair group method with averages, with a position tolerance for comparison of 1.5%, was used to produce the dendrogram. Strain sameness was defined as greater than or equal to 90% relatedness.

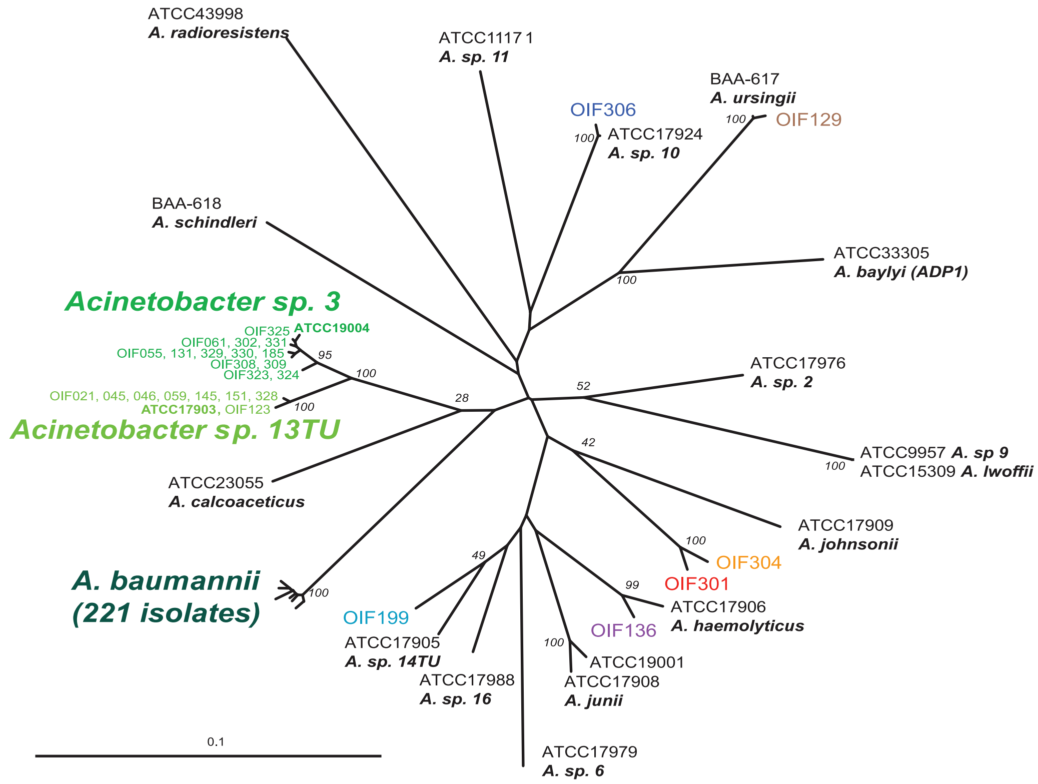
RESULTS

Diversity of PCR/ESI-MS target genes for *Acinetobacter*. To identify a set of genes suitable for creation of an PCR/ESI-MS database for *Acinetobacter*, we selected the set of housekeeping genes used in the *Moraxella catarrhalis* MLST database. *Moraxella* is the organism closest to *Acinetobacter* for which an MLST database exists (both *Acinetobacter* and *Moraxella* are members of the family *Moraxellaceae*). Using primer pairs derived from the completely sequenced *Acinetobacter baylyi* strain, homologous regions of the housekeeping genes *trpE*, *adk*, *efp*, *mutY*, *fumC*, and *ppa* were sequenced from 267 *Acinetobacter* isolates collected from outbreaks in European hospitals, from samples associated with the military conflict in Iraq, and from reference strains in the ATCC culture collection. This set of diverse isolates contains several different *Acinetobacter* species, including representatives of the major clones, I, II, and III, obtained from the European hospital collection (12, 41) Target gene sequences from these isolates showed a considerable range of allelic variation (Fig. 1), virtually all (>98%) due to silent mutations. The *efp* gene was the most conserved and showed very little variation among *A. baumannii* isolates, suggesting that the *efp* gene is under more stringent evolutionary constraints that the other genes analyzed. As a result, the *efp* gene was the least useful of the six target genes for parsing *A. baumannii* isolates into different subgroups. Only eight alleles were indicated by the *efp* gene (Fig. 1), and the sequences differed from the consensus sequence by single point mutations. However, the *efp* gene had significant allelic variation among different species of *Acinetobacter* and was the most useful among the six target genes for characterizing *Acinetobacter* isolates at the species level. The other five genes, *mutY* (25 alleles), *fumC* (25 alleles), *trpE* (21 alleles), *adk* (13 alleles), and *ppa* (14 alleles), had significant sequence diversity at the subspecies level for *A. baumannii* (Fig. 2) and were used to design nine primer pairs for PCR/ESI-MS analysis (one each for *trpE*, *adk*, *efp*, and *ppa*, two for *mutY*, and three for *fumC*).

Analysis of isolates by PCR/ESI-MS. The entire collection of 267 isolates was analyzed by PCR/ESI-MS; as expected, the base compositions determined experimentally were in agreement with the sequence-derived base compositions (summarized in Fig. 2). The relatedness of the isolates was assessed by using sequences from the *efp* gene, the gene in the set for which PCR amplicons for sequencing could be obtained for all isolates tested (including the non-*baumannii Acinetobacter* species) (Fig. 3, top). The uniqueness of the different *Acinetobacter* species is especially visible in base composition space of amplicons generated with the *efp* PCR/ESI-MS primer pair (Fig. 3, bottom). The majority of OIF samples (Fig. 2) fell into three tight clusters designated *A. baumannii* (189 isolates), *Acinetobacter* genomospecies 13TU (8 isolates), and *Acinetobacter* genomospecies strain 3 (13 isolates).

***A. baumannii* genotyping.** The 221 *A. baumannii* isolates (189 OIF strains, 23 European strains, and 9 ATCC strains) are depicted in Fig. 3 as a tight wedge and are shown on an expanded scale in Fig. 4. This group, although numerically abundant in this sample set, was tightly clustered on the *efp* phylogenetic tree. These *A. baumannii* isolates cluster into 48 different sequencing genotypes that are distinguished by 116 mutations, significantly fewer than the 531 positions that exhibit variation within all of the *Acinetobacter* isolates evaluated. Twenty-one of the 48 *A. baumannii* sequence types were represented by two or more isolates of identical genotype. Notably, of the 48 types identified by sequencing, 47 types were also distinguishable by PCR/ESI-MS using the set of nine PCR primer pairs. The one strain type identified only by sequence was a single-isolate variant of type ST12 (LUH6011). This reference strain was distinguished within the *ppa* locus, by a C-to-T and a T-to-C mutation, from the nine other ST12 reference strains; these simultaneous mutations did not change the amplicon mass and were therefore not distinguished by PCR/ESI-MS.

Clusters of related types that share four or five alleles are readily apparent. In particular, the first cluster contained the



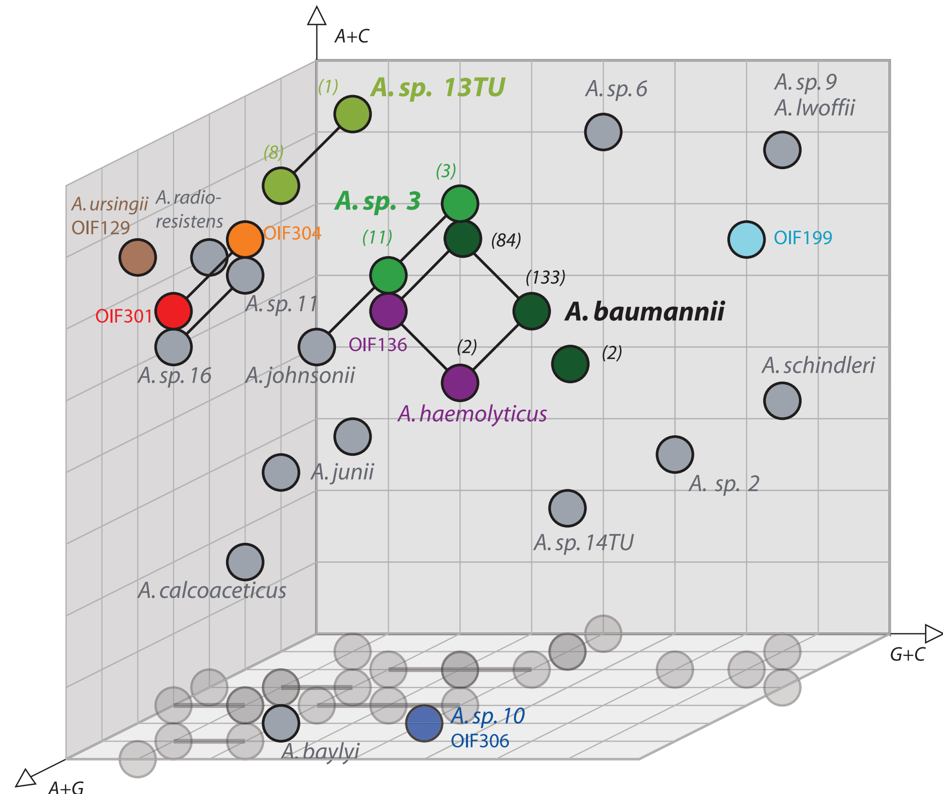


FIG. 3. (Top) Phylogenetic relationships of *Acinetobacter* species as inferred from the analysis of 298 conserved positions of the *efp* gene. (Bottom) Distribution of the base compositions obtained for a primer pair encompassing 91 positions in *efp*. Since all amplicons have the same length, base compositions can be accurately mapped in a three-dimensional (G+C, A+C, A+G) space. The numbers of isolates that map to the same position are indicated in parentheses. Solid lines connect base compositions that are a single transition apart from one another. To facilitate depth perception, shadows are cast on the horizontal plane.

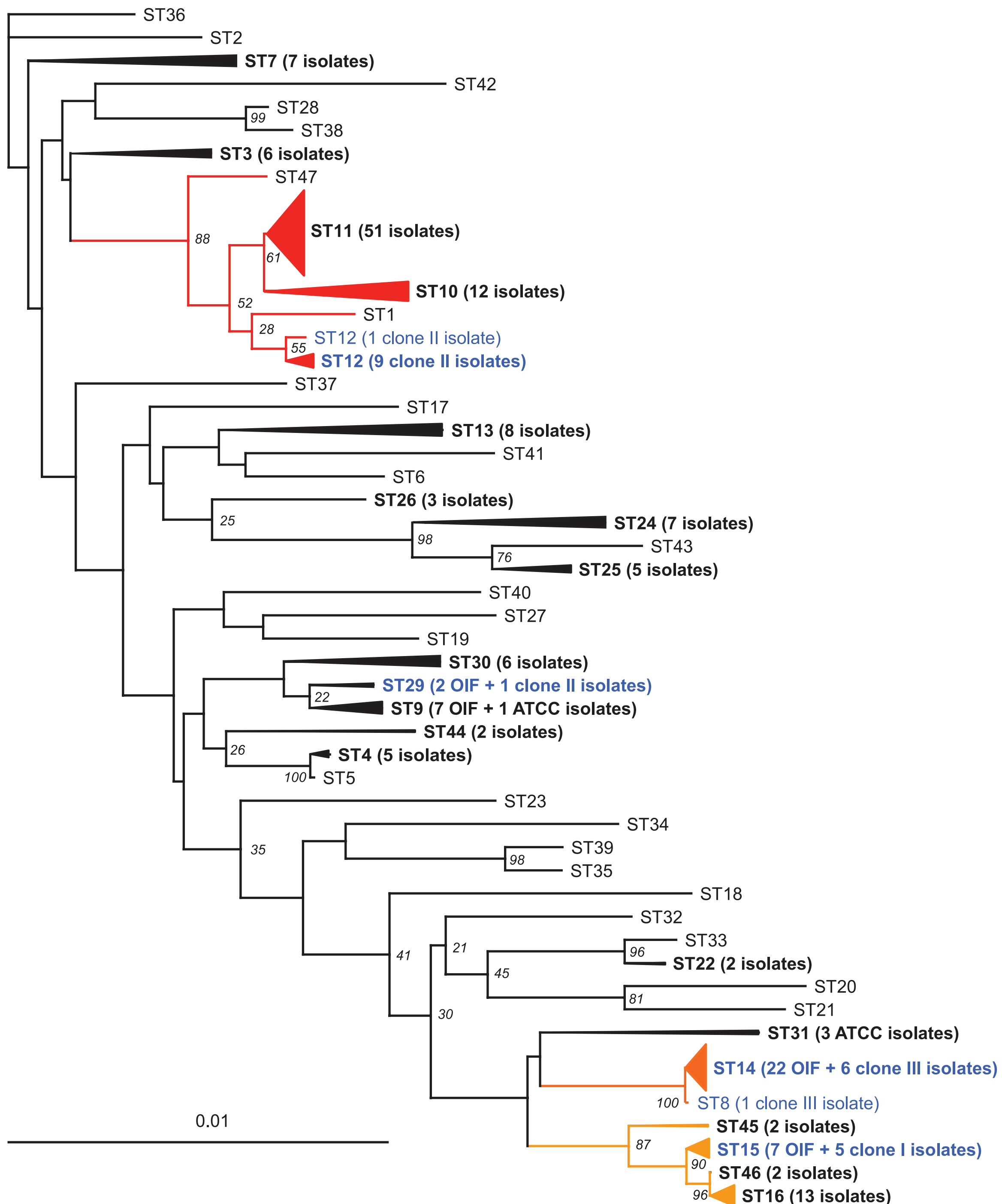


FIG. 4. Phylogenetic relationships of *Acinetobacter baumannii* sequence types as inferred from the analysis of 1,679 conserved positions from the *trpE*, *adk*, *efp*, *mutY*, *fumC*, and *ppa* genes. Established clusters of types that differ in a single gene are indicated by red-orange shading.

most-represented type (ST11, 51 isolates) and *mutY* variants in types ST1, ST10, ST12, and ST47. A second cluster consisted of types ST15, ST16, ST45, and ST46. Similarly, types ST14 and ST8 differed only by a single mutation in the *fumC* gene. These clusters are highlighted in red and orange in Fig. 1, 2, and 4.

The *A. baumannii* group included nine reference strains from the ATCC and 23 well-characterized European *A. baumannii* strains, with representatives of European hospital clones I and II and the recently identified pan-European multidrug-resistant clone III (represented in blue in Fig. 4) (12, 41). Twelve of the 23 European strains were identical at all six loci studied with one or more OIF isolates, providing a direct comparison between ribogroup and PCR/ESI-MS typing (Fig. 2). In particular, type ST15 contained seven OIF isolates and five clone I European isolates. Cluster ST14 contained 22 OIF

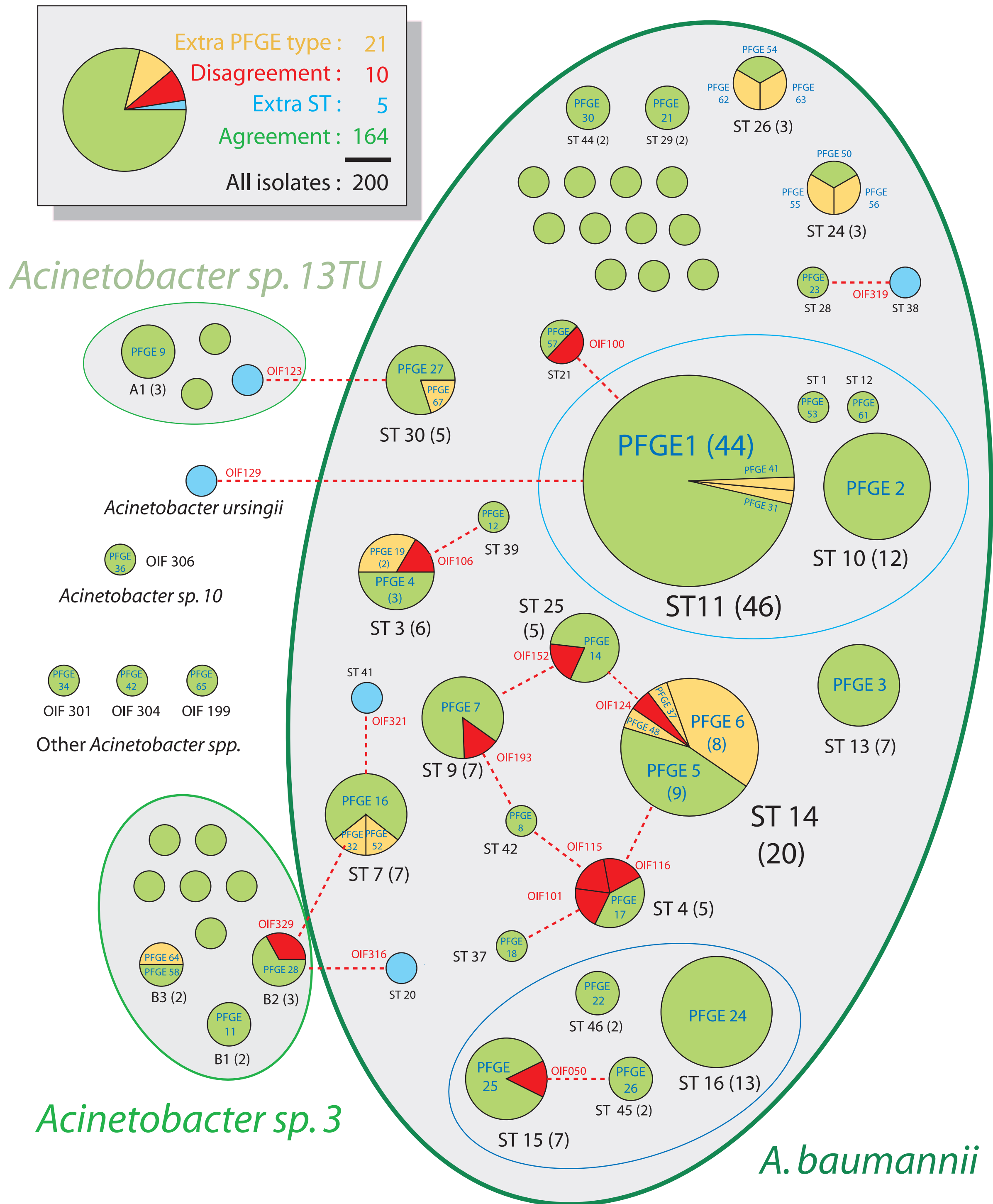


FIG. 5. Relationship between PCR/ESI-MS typing and PFGE genotyping. Both typing methods were used to analyze 200 isolates, and the isolates were distributed among pies in accordance with their sequence types (black labels). Within each pie, isolates were segregated according to PFGE types (blue labels). Green slices or pies represent agreement between typing methods. Yellow slices indicate isolates that are further discriminated by PFGE relative to the PCR/ESI-MS typing method. Blue pies represent isolates that are further discriminated by PCR/ESI-MS typing relative to PFGE. Red slices indicate disagreement between the PFGE and PCR/ESI-MS typing methods; red dashed lines connect the PCR/ESI-MS type with the PFGE type that was actually observed. Ellipses cluster together closely related types.

isolates and 6 clone III European isolates. No OIF isolate was found to be identical to clone II European isolates, found exclusively in ST12. However, 51 OIF isolates differed from clone II by only four mutations at the *mutY* locus. Overall, these data strongly suggest that the 111 OIF isolates belonging to the ST15/16/45/46, ST1/10/11/12, and ST8/14 clusters are phylogenetically associated with European hospital clones I, II, and III, respectively.

Characterization of other Acinetobacter species. Two groups of OIF isolates were distinct from the *A. baumannii* isolates and matched the reference strains for *Acinetobacter* genomospecies 13TU and *Acinetobacter* genomospecies 3 (Fig. 3). The *Acinetobacter* genomospecies 13TU group contained eight OIF isolates (seven patient samples from Walter Reed Army Medical Center and Landstuhl Regional Medical Center and one environmental sample) and reference strain ATCC 17903 and was comprised of five unique genotypes, A1 to A5. The *Acinetobacter* genomospecies 3 group contained 13 OIF isolates (nine environmental and four patient samples) and reference strain ATCC 19004 and was comprised of genotypes B1 to B10. The assignment of these isolates to different species was firmly established due to the uniqueness of the base composition signatures (in Fig. 2, note how the olive and bright green base compositions are restricted to these species).

Six OIF isolates (OIF129, OIF136, OIF199, OIF301, OIF304, and OIF306) were genotypically interspersed with the diverse non-*baumannii Acinetobacter* reference strains (Fig. 3). *A. baumannii* is the predominant species of *Acinetobacter* that causes human disease, but many other species of this genus have also been shown to infect humans (14, 24). Isolate OIF129 was assigned to the species *A. ursingii*, based on the base composition and sequence homology found in both *efp* (variation at a single position between OIF129 and the reference strain BAA-617) and *trpE* (seven variations). Isolates OIF136 and OIF306 were similarly assigned to the species *A. haemolyticus* and *Acinetobacter* genomospecies 10, respectively.

Relationship between PCR/ESI-MS and PFGE genotyping. A comparison of the classifications obtained by PCR/ESI-MS typing and PFGE is shown in Fig. 5. Dual classification was available for 200 isolates, mapped to 57 pies representing the number of sequence types; pies were then sliced to reflect the classification of these isolates into the 66 observed PFGE types. Fifty-two sequence types and PFGE types were clearly correlated and provided a consistent classification for 164 isolates (82% of the total evaluated [green in Fig. 5]). Twenty-one isolates were further discriminated by PFGE (Fig. 5, yellow). In particular, isolates belonging to ST14 were almost equally split into two PFGE types regardless of origin, suggesting that the PCR/ESI-MS genotyping method could be further improved for higher resolution. Conversely, five isolates were resolved by PCR/ESI-MS and not by PFGE (Fig. 5, blue pies) indicating that strain resolution by PFGE is also not exhaustive. For example, PFGE type 23 was composed of the closely related types ST28 and ST38. Isolates OIF123 and OIF129, characterized by PCR/ESI-MS as non-*baumannii Acinetobacter* species, shared PFGE types with *A. baumannii* isolates belonging to ST11 and ST30, respectively. PFGE and the PCR/ESI-MS genotyping methods were not in agreement for the 10 remaining isolates (Fig. 5, red).

DISCUSSION

Acinetobacter baumannii is a pathogen that has been associated with hospital outbreaks worldwide (6, 7, 9, 11, 18, 28, 33). This organism is particularly problematic due to the large number of multidrug-resistant strains, including the pan-European clone types I, II, and III, that have become endemic in hospital settings (12, 41). Recently, infections by *Acinetobacter baumannii* have resulted in critical complications for troops injured in Iraq and Afghanistan (47). The injured were treated in a variety of locations in the field and in European hospitals. As part of a public health investigation to identify the possible source(s) of this outbreak, to determine the likely mode(s) of transmission, and to assist in generating and implementing infection control countermeasures, we set out to develop a rapid *Acinetobacter baumannii* strain typing assay. The assay we developed is similar to traditional MLST strain typing, but analysis of PCR amplicons is performed with ESI-MS rather than standard DNA sequencing. The base composition of the PCR amplicons is determined from the masses of the PCR amplicons and provides enough information to type the strains. Previously we developed a similar assay for the rapid strain typing of respiratory pathogens (15, 16).

To develop the *A. baumannii* typing assay, we assembled a collection of 267 diverse isolates. Included in this collection were 216 isolates from U.S. and Iraqi patients involved in an outbreak in U.S. military hospitals associated with OIF, 23 isolates containing representatives of the endemic pan-European clones types I, II, and III, and a diverse collection of commercially available clones representing *Acinetobacter baumannii* and other *Acinetobacter* species.

Analysis of the collection of 216 *Acinetobacter* isolates from U.S. and Iraqi patients treated in U.S. military hospitals identified 31 (14.4%) *A. baumannii* isolates that were indistinguishable from the European reference strains that are plaguing European hospitals. Additionally, 80 OIF isolates (37%) differed from the endemic European reference strains by minor variations at a single locus, suggesting a relationship with the European endemic strains. The remaining 105 isolates represented more diverse strains.

Twenty-seven OIF *Acinetobacter* isolates (12.5%) were found to represent at least seven additional *Acinetobacter* species. Two of these were represented by multiple isolates and displayed intraspecies allelic variation; we were able to identify these two species as *Acinetobacter* genomospecies 13TU and *Acinetobacter* genomospecies 3 through comparison with relevant reference strains. Interestingly, these results mirror a recent study that identified the same two species amid infections by *A. baumannii* in hospital settings (10).

Three OIF isolates did not match any of our reference species and may represent novel *Acinetobacter* species. Due to sequence variation of the non-*baumannii Acinetobacter* isolates, we were not able to generate PCR/ESI-MS or sequencing PCR amplicons for these isolates from all primer pairs. Based upon the *efp* sequence and PCR/ESI-MS analyses from the other loci, we determined that these isolates were not *Acinetobacter baumannii* and did not match any of the 18 representatives of diverse *Acinetobacter* species tested. The unexpected diversity of these isolates highlights the potential of the PCR/ESI-MS approach to detect and strain type de novo infections from other *Acinetobacter* species.

Sequence analysis of the entire strain collection, including the European reference strains and the commercially available strains, identified 48 unique *A. baumannii* strain types. Interestingly, only one of the nine *A. baumannii* reference strains from the ATCC collection (ATCC 9955, ST9) was represented in the OIF isolates, suggesting that the bulk of

the OIF sequences are distinct from the strains archived at the ATCC.

Base composition, though not as information rich as sequence data, has nearly the same resolving power for *Acinetobacter baumannii* strain typing as sequencing. By selecting primer pairs targeting nine information-rich regions of six loci, we were able to resolve by PCR/ESI-MS 47 of the 48 *Acinetobacter baumannii* strain types identified by sequencing 1,679 nucleotides from the six loci. The only case in which PCR/ESI-MS analysis did not resolve the strain type involved the two mutations (C to T and T to C) that distinguish isolate LUH6011 from the other clone III isolates: these mutations are mutually compensating, resulting in no net change in the PCR amplicon mass. Additional primers specifically targeting this isolate would bring no value to the resolution of other types, as the use of these nine primer pairs had already maximized the average number of mutations between all characterized types.

Our sequence analysis showed that the *efp* gene was not useful for discrimination of *Acinetobacter baumannii* isolates (Fig. 1), the initial goal of this study. However, the *efp* gene was particularly useful for discriminating *Acinetobacter* strains at the species level. The incorporation of the *efp* primer pair expanded the discrimination power of the PCR/ESI-MS *Acinetobacter baumannii* MLST assay to include the identification and typing of other *Acinetobacter* species.

In summary, we have used an *A. baumannii* MLST-like strain typing assay based on PCR/ESI-MS of nine PCR amplicons to identify 47 *A. baumannii* strain types. Fifteen additional types represented the related *Acinetobacter* genomospecies 13TU and *Acinetobacter* genomospecies 3. Using a portion of the *efp* locus, we distinguished at least 16 additional *Acinetobacter* species. The PCR/ESI-MS strain typing method is both rapid and low cost and can be performed in an automated modality, enabling the rapid surveillance of *Acinetobacter* spp. in a variety of settings. Use of this *A. baumannii* typing assay identified a genetic relationship between the endemic European isolates and many of the isolates found in patients and in military hospitals treating casualties from OIF. Further analysis of the OIF isolates and their locations of collection should provide better understanding of the origins of these infections and will ultimately improve infection control and prevention measures.

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