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Identification of Variable-Number Tandem-Repeat (VNTR) Sequences in *Acinetobacter baumannii* and Interlaboratory Validation of an Optimized Multiple-Locus VNTR Analysis Typing Scheme▽†

Christine Pourcel,[1] Fabrizia Minandri,[2] Yolande Hauck,[1] Silvia D'Arezzo,[3] Francesco Imperi,[2] Gilles Vergnaud,[1,4] and Paolo Visca[2]*

Université Paris-Sud, Institut de Génétique et Microbiologie, CNRS, Orsay F-91405, France[1]; Dipartimento di Biologia, Università Roma Tre,[2] and Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani I.R.C.C.S.,[3] Rome, Italy; and DGA/MRIS, Mission pour la Recherche et l'Innovation Scientifique, 92221 Bagneux, France[4]

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***Acinetobacter baumannii* is an important opportunistic pathogen responsible for nosocomial outbreaks, mostly occurring in intensive care units. Due to the multiplicity of infection sources, reliable molecular fingerprinting techniques are needed to establish epidemiological correlations among *A. baumannii* isolates. Multiple-locus variable-number tandem-repeat analysis (MLVA) has proven to be a fast, reliable, and cost-effective typing method for several bacterial species. In this study, an MLVA assay compatible with simple PCR- and agarose gel-based electrophoresis steps as well as with high-throughput automated methods was developed for *A. baumannii* typing. Preliminarily, 10 potential polymorphic variable-number tandem repeats (VNTRs) were identified upon bioinformatic screening of six annotated genome sequences of *A. baumannii*. A collection of 7 reference strains plus 18 well-characterized isolates, including unique types and representatives of the three international *A. baumannii* lineages, was then evaluated in a two-center study aimed at validating the MLVA assay and comparing it with other genotyping assays, namely, macrorestriction analysis with pulsed-field gel electrophoresis (PFGE) and PCR-based sequence group (SG) profiling. The results showed that MLVA can discriminate between isolates with identical PFGE types and SG profiles. A panel of eight VNTR markers was selected, all showing the ability to be amplified and good amounts of polymorphism in the majority of strains. Independently generated MLVA profiles, composed of an ordered string of allele numbers corresponding to the number of repeats at each VNTR locus, were concordant between centers. Typeability, reproducibility, stability, discriminatory power, and epidemiological concordance were excellent. A database containing information and MLVA profiles for several *A. baumannii* strains is available from http://mlva.u-psud.fr/.**

The Gram-negative bacterium *Acinetobacter baumannii* has emerged worldwide as a major nosocomial pathogen and a serious threat to patients in intensive care units (ICUs) (18, 27). Hallmarks of *A. baumannii* infection are resistance to a broad range of antimicrobial agents, the tendency for epidemic spread, and long-term persistence in the hospital setting (4, 8, 27).

Most *A. baumannii* clinical strains from multiple hospital outbreaks throughout the world have been referred to a few epidemic lineages. Two of these, called international clonal lineages I and II, were first identified in northwestern Europe in the early 1980s and then worldwide (8, 17), while a third clone, called international clonal lineage III, was later detected in France, Netherlands, Italy, and Spain, probably persisting in European hospitals since the 1990s (17, 34).

Several typing methods have been developed to trace *A. baumannii* epidemiology from the local to the global scale. Among them, macrorestriction analysis with pulsed-field gel electrophoresis (PFGE) (23) and multilocus sequence typing (MLST) (2, 7) are currently considered the methods of choice for epidemiological typing and population genetic studies of *A. baumannii*. Although PFGE typing has been used extensively to characterize multihospital or nationwide outbreaks (18, 23), this technique is laborious and time-consuming, and results are difficult to compare between laboratories (18). MLST is a truly portable method that allows the generation of coded types and easily accessible databases (2, 7), but it is relatively expensive and better suited for population genetic studies than for typing (7). Recently, a PCR-based method was devised to identify the most frequent *A. baumannii* genotypes through their assignment to a few predefined sequence groups (SGs) (30). However, the diversity inferred from PCR-generated SG patterns appears too low for typing purposes (5, 28). Hence, additional typing methods combining good interlaboratory reproducibility with a high level of discrimination, meaningful epidemiological inference, and low cost are still needed.

Variable-number tandem repeats (VNTRs), consisting of consecutive occurrences of various numbers of DNA motifs, have gained attention for their practical applications in epidemiological typing (32, 36, 37). Multiple-locus VNTR analysis (MLVA), which is based on the polymorphism of such tandemly repeated DNA sequences (32), has become the reference typing method for several bacterial species (reviewed in

* Corresponding author. Mailing address: Dipartimento di Biologia, Università Roma Tre, Viale Guglielmo Marconi 446, 00146 Rome, Italy. Phone: 39 06 57 33 63 47. Fax: 39 06 57 33 63 21. E-mail: visca@uniroma3.it.

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references 36 and 37). In a typical MLVA assay, a predefined set of VNTRs is analyzed, and a code corresponding to the number of repeats at each locus can be determined for individual strains. Therefore, MLVA is a cost-effective typing method which provides portable results (MLVA codes) that can easily be stored in a database and exchanged between laboratories (9, 20, 36). A previous study identified 13 putative VNTRs in *A. baumannii* ATCC 17978, but only 2 provided sufficient repeat polymorphism for fine typing of clinical isolates showing identical PFGE profiles (29).

The aim of this study was to establish an MLVA scheme for *A. baumannii*. For this purpose, we searched for the presence of shared VNTR markers in the complete genome sequences of six *A. baumannii* strains and set up a standard MLVA protocol based on simple PCR and agarose gel electrophoresis. The overall performance of the MLVA method was assessed in an interlaboratory study of a selected collection of clinical *A. baumannii* isolates and compared with those of reference typing methods, including PFGE and SG profiling.

MATERIALS AND METHODS

Study design and participants. Participants from two institutes in two European countries took part in the study: the Institute of Genetics and Microbiology, University Paris-Sud 11, Orsay, France (center I), and the Department of Biology, University Roma Tre, Rome, Italy (center II). Both centers have already participated in multicenter studies on MLVA typing (20). Center I provided expertise in search for VNTRs and development of the MLVA method, while center II selected the collection of *A. baumannii* strains and performed SG profiling and PFGE typing. The study was coordinated by center II.

Bioinformatic detection of putative VNTRs in the genome sequences of six *A. baumannii* strains was performed using web-based tools (6, 9), and a standard laboratory protocol for MLVA was agreed upon by the two centers.

Guidelines for appropriate use and evaluation of microbial epidemiologic typing methods were followed throughout the study (33). The reproducibility (*R*) of the method was assessed at both the inter- and intralaboratory levels. Twenty-nine blind-coded isolates were analyzed independently by the two centers, and the results were collected, decoded, and compared by center II. Duplicates of four strains (ACICU, ATCC 17978, AYE, and SDF) whose complete genome sequences have been published (12, 26, 31) were included in order to assess the intralaboratory reproducibility. In addition, each center analyzed the reference strain ACICU as an uncoded internal control, for a total of 30 samples. Two sets of three epidemiologically related isolates each were used to determine the epidemiological concordance (*E*). A diversity panel of 21 epidemiologically unrelated isolates was used to determine the discriminatory power. Stability (*S*) was determined for two different subcultures of strain ACICU, the first one obtained after 10 serial passages on Mueller-Hinton (MH) agar and the second one obtained after 30 serial passages on MH agar supplemented with increasing concentrations of tigecycline (from 1 to 256 mg/liter), followed by 10 additional passages on MH agar. The *S* value of MLVA was determined by testing five different colonies for each subculture. Typeability (*T*) was estimated by analysis of 25 unique isolates.

Bacterial strains. A collection of 25 well-characterized *A. baumannii* strains was selected (Table 1), including three reference strains for the epidemic international clonal lineages I (RUH875), II (RUH134), and III (RUH5875) (17, 34) and four strains (ACICU, ATCC 17978, AYE, and SDF) whose complete genome sequences have been published (12, 26, 31). The remaining 18 strains were selected from two well-characterized *A. baumannii* collections: 10 from the Gruppo Romano *A. baumannii* (GRAB) collection (5) and eight from the Antibiotic Resistance, Prevention and Control (ARPAC) international collection (28). The criterion for strain selection was based on the concerted molecular type, as determined from a combination of SG, random amplification of polymorphic DNA (RAPD), and PFGE fingerprints (5, 28, 30).

The 10 isolates from the GRAB collection were obtained during 2004 to 2005 from clinical specimens of ICU patients (33C, 53C, 51C, 57C, 82D, 84D, 85D, 50C, and 105C) or the ICU environment (71C) (Table 1). Of these isolates, seven belonged to international clonal lineage I (82D, 85D, 84D, and 51C) or II (33C, 53C, and 71C), two were classified as variant subtypes of main PFGE and RAPD types within international clonal lineage I (57C) or II (50C), and one (105C) did not cluster with any international clonal lineage (5).

The eight *A. baumannii* clinical isolates from the ARPAC collection (A377, A374, A399, A461, A376, A389, A388, and A457) (28) were selected to further diversify the strain panel. Isolate A377, despite belonging to SG3, differed from the reference strain of international clonal lineage III (RUH5875) at the PFGE and RAPD levels; isolates A374, A399, and A461 showed SG profile 4 but were characterized by two different PFGE types and three RAPD patterns; unique PFGE and RAPD patterns were also characteristic of isolates A376 and A389, both showing SG profile 5, and A388 and A457, showing SG profiles 6 and 7, respectively (for the preliminary characterization of ARPAC strains, see reference 28). Table 1 summarizes the relationships between previously defined SG, RAPD, and PFGE types (5, 28, 30).

VNTR markers. The annotated genome sequences of *A. baumannii* ACICU (GenBank accession no. CP000863), ATCC 17978 (GenBank accession no. CP000521), AYE (GenBank accession no. CU459141), SDF (GenBank accession no. CU468230), AB0057 (GenBank accession no. CP001182) (1), and AB307-0294 (GenBank accession no. CP001172) (1) were inspected for the presence of potential VNTR loci by using the strain comparison tool available at the Microbial Tandem Repeats Database (http://minisatellites.u-psud.fr/) (6, 14, 15). VNTR loci with a repeat unit size above 9 bp were named large (L)-repeat VNTRs, whereas VNTR loci with a repeat size of up to 9 bp were named small (S)-repeat VNTRs. Each VNTR locus was designated according to the position (expressed in kilobases) of the primers designed for PCR amplification relative to the genome sequence of *A. baumannii* ATCC 17978 (i.e., Abaum_0017, Abaum_0826, Abaum_0845, Abaum_1988, Abaum_2240, Abaum_2396, Abaum_3002, Abaum_3406, Abaum_3468, and Abaum_3530), as shown in Table 2.

DNA extraction, PCR amplification, and electrophoresis. Genomic DNA was extracted by the study coordinator at center II by use of a QIAamp DNA Mini kit (Qiagen), and blind-coded samples were delivered to both centers I and II for analysis. Oligonucleotide primers targeting the 5′- and 3′-flanking regions of each VNTR locus are listed in Table 2. Primer annealing on published *A. baumannii* genome sequences was predicted *in silico* by using the PCR primer BLAST tool from the Microbial Tandem Repeats Database. PCRs were performed in a 30-μl final volume containing 1 to 5 ng of genomic DNA, 1× reaction buffer (Qbiogene), 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Qbiogene), a 200 μM concentration of each deoxynucleoside triphosphate (dNTP) (Roche), and a 0.3 μM concentration of each primer (synthesized by Eurogentec and Invitrogen for centers I and II, respectively). Amplification was performed with a PTC-200 DNA Engine (Bio-Rad) and a GeneAmp 9600 thermal cycler (PerkinElmer) at centers I and II, respectively, applying initial denaturation for 5 min at 94°C, followed by 36 cycles of denaturation for 30 s at 94°C, annealing for 30 s at the temperature reported in Table 2, and elongation at 72°C for the time reported in Table 2, with a final elongation at 72°C for 7 min.

An appropriate volume (generally 2 μl) of each PCR mix was loaded into a 2% regular agarose (Qbiogene and Gibco BRL for centers I and II, respectively) gel for L repeats or in a 3% agarose gel, composed of 1.5% regular agarose plus 1.5% Metaphor (FMC BioProducts and Cambrex for centers I and II, respectively), for S repeats. Electrophoresis was performed in 25-cm-wide gels and run at 4 V/cm in 0.5× Tris-borate-EDTA buffer (22) for about 5 h. A 100-bp or 20-bp ladder (Bio-Rad) was used as a DNA size marker for L-repeat or S-repeat VNTRs, respectively. Two microliters of the PCR product obtained from strain ACICU was loaded after every DNA size marker as an internal control. The gels were stained with 0.5 μg/ml ethidium bromide for 1 h, rinsed with water for 30 min (optional), and photographed under UV illumination.

Agarose gel image analysis. The band size was determined using BioNumerics software v. 5.1 (Applied Maths, Sint-Martens-Latem, Belgium), available at both centers. For band size optimization, the cursor was positioned on the DNA band of the internal control strain ACICU and adjusted to achieve optimum matching with the predicted amplicon size. The cursor was then positioned similarly for all other amplicons in the same gel. Minor adjustments were made to take into account the amount of DNA present in each band, as larger amounts of DNA usually result in slightly faster apparent migration.

Nomenclature and description of MLVA profiles. The expected amplicon size, repeat length, number of repetitions, and deduced size of flanking regions were determined for reference genomes of strains ACICU, ATCC 17978, AYE, and SDF available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/genome), using the PCR primer BLAST tool from the Microbial Tandem Repeats Database. The number of repeats in VNTR alleles for isolates with an unknown genome was estimated by subtracting the flanking region size from the amplicon size and then dividing by the repeat unit length (Table 2). The number of repeats was calculated according to an excess

TABLE 1. Characteristics of *A. baumannii* strains used in this study

Study code	Strain designation	Country of origin	Source	Yr of isolation	Relevant characteristics and molecular type[a]	Reference
1	ACICU[b]	Italy	Cerebrospinal fluid	2005	Complete genome sequenced, related to international clonal lineage II[c]	12
2	ACICU[b]	Italy	Cerebrospinal fluid	2005	Duplicate of above	12
3	ATCC 17978[b]	France	Respiratory secretions	1951	Complete genome sequenced	26
4	ATCC 17978[b]	France	Respiratory secretions	1951	Duplicate of above	26
5	AYE[b]	France	Urine	2001	Complete genome sequenced, related to international clonal lineage I[d]	31
6	AYE[b]	France	Urine	2001	Duplicate of above	31
7	SDF[b]	France	Human body louse	2001	Complete genome sequenced	31
8	SDF[b]	France	Human body louse	2001	Duplicate of above	31
9	RUH134	Netherlands	Urine	1982	Prototypic strain for international clonal lineage II	17
10	33C[e]	Italy	Wound swab	2004	SG1, related to international clonal lineage II[c]	5
11	53C[e]	Italy	Urine	2004	SG1, related to international clonal lineage II[c]	5
12	71C[e]	Italy	Environmental	2004	SG1, related to international clonal lineage II[c]	5
13	RUH875	Netherlands	Urine	1984	Prototypic strain for international clonal lineage I	17
14	51C	Italy	Respiratory secretions	2004	SG2, related to international clonal lineage I[c]	5
15	57C	Italy	Respiratory secretions	2004	SG2, variant subtype within international clonal lineage I[c]	5
16	82D[e]	Italy	Wound swab	2004	SG2, related to international clonal lineage I[c]	5
17	84D[e]	Italy	Urine	2004	SG2, related to international clonal lineage I[c]	5
18	85D[e]	Italy	Respiratory secretions	2004	SG2, related to international clonal lineage I[c]	5
19	RUH5875	Netherlands	Unknown	1997	Prototypic strain for international clonal lineage III	34
20	A377	Germany	Unknown	2001–2004	SG3, RAPD type 21, PFGE type XI,[f] unrelated to any international clonal lineage	28
21	A374	Netherlands	Unknown	2001–2004	SG profile 4, RAPD type 19, PFGE type X,[f] related to international clonal lineage III	28
22	A399	Turkey	Unknown	2001–2004	SG profile 4, RAPD type 20, PFGE type X,[f] related to international clonal lineage III	28
23	A461	Portugal	Unknown	2001–2004	SG profile 4, RAPD type 22, PFGE type XIV,[f] unrelated to any international clonal lineage	28
24	A376	Austria	Unknown	2001–2004	SG profile 5, RAPD type 17, PFGE type XIII,[f] unrelated to any international clonal lineage	28
25	A389	Denmark	Unknown	2001–2004	SG profile 5, RAPD type 12, PFGE type XV,[f] unrelated to any international clonal lineage	28
26	A388	Greece	Unknown	2001–2004	SG profile 6, RAPD type 7, PFGE type IV,[f] unrelated to any international clonal lineage	28
27	A457	Estonia	Unknown	2001–2004	SG profile 7, RAPD type 24, PFGE type XII,[f] unrelated to any international clonal lineage	28
28	50C	Italy	Respiratory secretions	2004	SG profile 4, variant subtype within international clonal lineage II[c]	5
29	105C	Italy	Cerebrospinal fluid	2005	New SG profile,[c] unrelated to any international clonal lineage	5

[a] SG or SG profile, RAPD fingerprint, and PFGE type.
[b] Reproducibility panel. Duplicates of four reference strains were used to assess intralaboratory reproducibility.
[c] Previously determined by D'Arezzo et al. (5).
[d] Previously determined by Turton et al. (30).
[e] Epidemiological concordance panel. Strains 33C and 53C were isolated from two patients during the same outbreak, and 71C was isolated from the associated environment. Strains 82D, 84D, and 85D were isolated from different patients during the same outbreak.
[f] PFGE type as previously determined by Towner et al. (28).

approximation in order to also consider noninteger numbers of repeats in allele counts (as explained in reference 37). Intermediate-sized alleles, which may result from intermediate-sized repeat units, were reported as half-sized. The allelic profile was defined as the number of repeats at each VNTR locus included in the MLVA scheme. Unless otherwise stated, the "failed" designation was given when no amplification or ambiguous amplicon patterns were observed repeatedly at a given locus, and the corresponding allele was indicated with a "−" symbol.

DNA sequence analysis. Selected PCR products were subjected to DNA sequencing on both strands by center II. Sequencing reactions were performed using BigDye Terminator technology according to the manufacturer's recommendations (Applied Biosystems), and products were analyzed in an ABI 3130 capillary electrophoresis system equipped with the POP 7 matrix (Applied Biosystems).

SG profiling. SG profiles were determined with two multiplex PCRs, designed to selectively amplify group 1 or group 2 alleles of the *ompA*, *csuE*, and $bla_{OXA\text{-}51}$-like genes, according to the procedure described by Turton et al. (30). SG patterns other than those defined in reference 30 were referred to as SG profiles, since they do not define the major international clonal lineages I and II.

PFGE typing. PFGE analysis of ApaI-digested genomic DNA was performed for the whole collection of 25 unique isolates as previously described (23). The ApaI-digested genomic DNA from strain ACICU was included in every sixth lane as an internal standard for gel normalization. A dendrogram was created with BioNumerics software (Applied Maths) by using the Dice coefficient and the unweighted-pair group method using average linkages (UPGMA), with a 1.5% tolerance limit and 1.5% optimization (23). A similarity threshold of ≥85% was used to define isolates belonging to the same PFGE type (28).

Criteria for comparative evaluation of *A. baumannii* typing methods. The polymorphism index for individual or combined VNTR loci was calculated for strains included in the diversity panel and expressed as the Hunter-Gaston diversity index (HGDI), an application of Simpson's index of diversity (10, 24). The HGDI was also determined for PFGE typing and SG profiling.

TABLE 2. Oligonucleotide primers, PCR conditions, and characteristics of VNTRs analyzed in this study

VNTR marker	Position[a]	Expected amplicon size (bp [no. of repeats]) for strain[d]:						Repeat size (bp)	Size of flanking regions (bp)[c]	Primer designation	Primer sequence (5′–3′)	PCR conditions		Source or reference
		ACICU	ATCC 17978	AYE	SDF	AB0057[b]	AB307-0294[b]					Annealing temp (°C)	Elongation time (s)	
Abaum_3406	3406043–3406564	ND	522 (10)	669 (15)	ND	549 (11)	ND	30	231	Abaum_3406_L	CACTATATTGAAGTGCTTTTA	50	45	This study
										Abaum_3406_R	GTGGTTTTTCTATTGGTACATTAC	50	45	This study
Abaum_3530	3529446–3529907	522 (7)[e]	462 (6)	462 (6)	ND	462 (6)	462 (6)	60	121	Abaum_3530_L	TGCAACCGGTATTCTAGGAAC	55	45	This study
										Abaum_3530_R	CCTTGAACAACATCGATTAC TGGA	55	45	This study
Abaum_3002	3001898–3002379	482 (7)	539 (8)[e]	482 (7)	428 (6)	483 (7)	482 (7)	57	121	Abaum_3002_L	GACTGAAGCAAGACTAAAACGT	55	45	This study
										Abaum_3002_R	TCTGGGCAGCTTCTTCTTGAGC	55	45	This study
Abaum_2240	2240480–2239857	826 (4)	624 (2)	723 (3)	625 (2)	723 (3)	722 (3)	99	494	Abaum_2240_L	CCCGCAGTACATCATGGTTC	55	60	This study
										Abaum_2240_R	AGAACATGTATACGCAACTG	55	60	This study
Abaum_1988	1988034–1988228	291 (9)	195 (5)	213 (5.5)	136 (3)	213 (5.5)	213 (5.5)	26	77	Abaum_1988_L	GGCAAGGCATGCTCAAGGGCC	55	45	This study
										Abaum_1988_R	CAGTAGACTGCTGGTTAATGAG	55	45	This study
Abaum_0826	826475–826223	322 (13)[e]	379 (19)[e]	358 (17)	349 (16)	277 (8)	283 (8)	9	208	Abaum_0826_L	TGACTACTGAAACAGTTTTTG	50	45	This study
										Abaum_0826_R	ATGATTGTACCGAGTAAAAGA	50	45	This study
Abaum_0017	17531–17706	202 (14)	239 (18)[e]	175 (11)	185 (12)	274 (22)	274 (22)	9	80	Abaum_0017_L[f]	GTGAGGGTAGAGTATTTGCTC	55	45	Alias VNTR-1
										Abaum_0017_R[f]	GAGTTAGGGAGTCTTTTATATGG	55	45	Alias VNTR-1
Abaum_0845	845900–846018	175 (10)[e]	119 (2)	238 (19)	217 (16)	189 (12)	308 (29)	7	105	Abaum_0845_L	AATTTTAATTCCAAATTGCTCC	50	45	This study
										Abaum_0845_R	ACTTAAAATCGCATTTTTATCA	50	45	This study
Abaum_2396	2396729–2396935	225 (20)[e]	219 (19)[e]	231 (21)	147 (7)	159 (9)	201 (16)	6	105	Abaum_2396_L[f]	CAAGTCCAATCAACTCATGATG	55	45	Alias VNTR-10
										Abaum_2396_R[f]	CTCCTGTAAGTGCTGTTCAGCC	55	45	Alias VNTR-10
Abaum_3468	3468315–3468539	225 (13)	225 (13)	231 (14)	189 (7)	231 (14)	225 (13)	6	147	Abaum_3468_L	CAGAAGTCACTGCATCTGCAAC	55	45	This study
										Abaum_3468_R	CGGTTGAAATTTTTTATAAT GAAG	55	45	This study

[a] First to last nucleotide, encompassing forward and reverse primers, relative to the genome sequence of *A. baumannii* ATCC 17978.
[b] Strains for which the genome sequence was considered only for *in silico* prediction of VNTR markers.
[c] Values referred to the genome of strain ATCC 17978 and considered for allele assignment.
[d] ND, not detected.
[e] Amplicon size and number of repeats were determined upon resequencing of the genomic region.
[f] Primers differ from those used by Turton et al. (29).

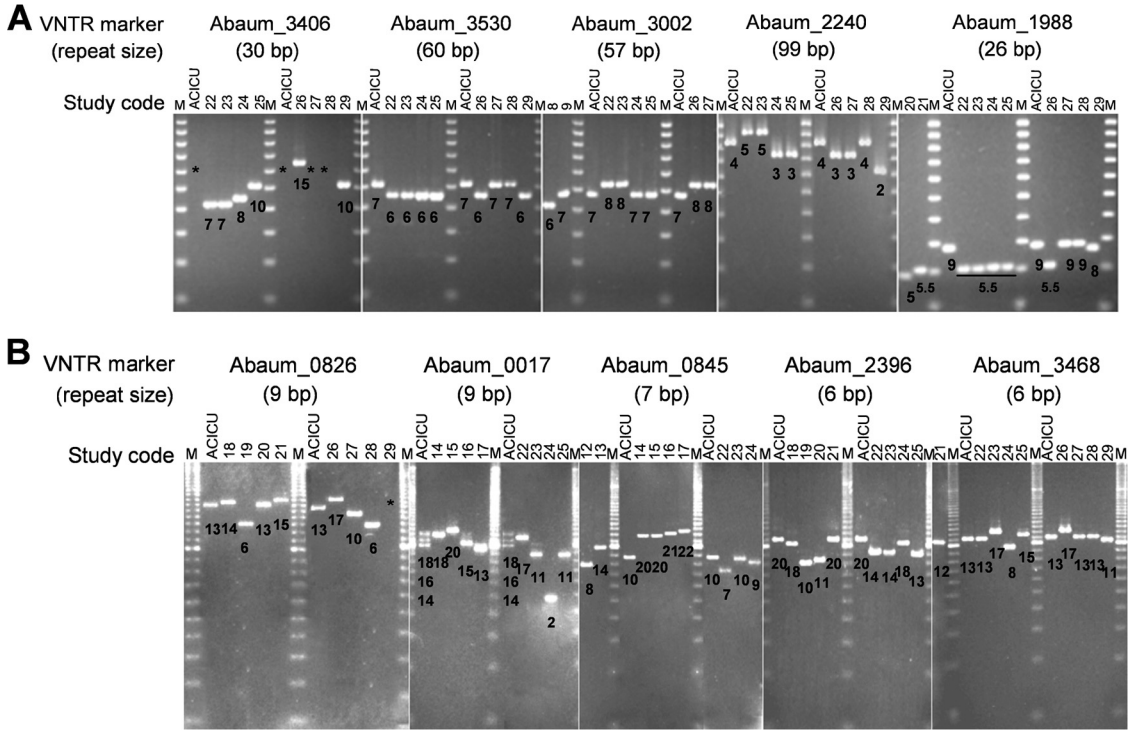


FIG. 1. Allele polymorphisms at L-repeat (A) and S-repeat (B) VNTR loci. The study code of each strain is shown above the lanes, and the size of the repeat unit is reported for each VNTR marker. The amplicon from the reference strain ACICU was loaded after each DNA size marker and used as an internal control for band size optimization. The asterisks denote amplification failures. The number of repeats is indicated below each DNA band. The PCR products were analyzed in 2% agarose gels (A; lanes M show the 100-bp ladder) or in 3% agarose gels (B; lanes M show the 20-bp ladder). Electropherograms shown in the figure were obtained in center II.

Nucleotide sequence accession numbers. DNA sequences for the ATCC 17978 VNTRs Abaum_0017, Abaum_0826, Abaum_2396, and Abaum_3002 and the ACICU VNTRs Abaum_0826, Abaum_0845, Abaum_2396, and Abaum_3530 have been deposited in the GenBank database under accession no. HQ656811, HQ656809, HQ656813, HQ656808, HQ656810, HQ656812, HQ656814, and HQ656807, respectively.

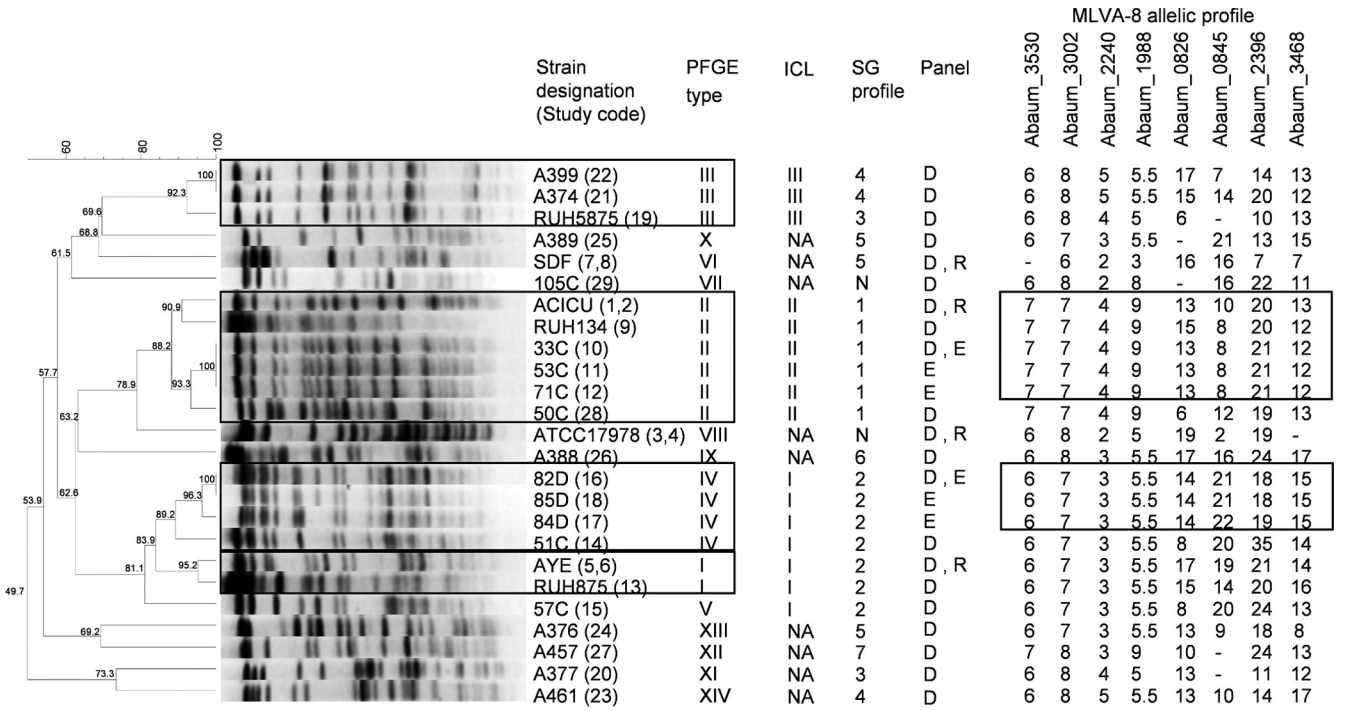
RESULTS

Detection of VNTRs in *A. baumannii* genomes. The availability of the complete genome sequences of six *A. baumannii* strains provided the opportunity to search for polymorphic tandem repeats by using the strain comparison tool available at http://minisatellites.u-psud.fr/gpms (6).

Among 38 tandem repeats detected in the genome of *A. baumannii* ATCC 17978 by use of current default parameters (i.e., total length of >50 bp and conservation of >80%), 28 were found in fewer than half of six sequenced *A. baumannii* strains (data not shown) and thus were not considered for further development of the MLVA scheme. Nine VNTRs were detected in all of the reference strains, while an additional one (Abaum_3406) was present in 50% of them (Table 2). Genome analysis predicted size polymorphisms for all of these VNTRs, and thus they were considered suitable candidates for the development of an MLVA scheme (Table 2).

Selection of VNTRs, analysis of allelic variation, and setup of the MLVA assay. The occurrence of selected VNTR loci in a diverse collection of *A. baumannii* strains was assessed. With the exception of Abaum_3406, amplification products were obtained for nearly all strains tested (see Table S1 in the supplemental material). Amplicons ranging in size between 95 and 925 bp were obtained and could be resolved clearly by agarose gel electrophoresis. Typical electrophoretic profiles for the 10 VNTR markers are shown in Fig. 1, representing 100% and 65% of the overall diversity observed for L-repeat and S-repeat VNTRs, respectively. A half-sized allele was observed only for Abaum_1988 in nearly half of the strains, also consistent with bioinformatic predictions for the reference strain AYE (Fig. 1A and Table 2). The half-sized allele could easily be distinguished from full-sized counterparts by visual inspection of the gel. This allelic variant, intermediate between 5 and 6 repeats, was arbitrarily scored 5.5 (e.g., see study codes 21 to 26 in Fig. 1A).

For some VNTR markers (Abaum_3530, Abaum_0826, Abaum_0845, and Abaum_2396 in strain ACICU and Abaum_3002, Abaum_0826, Abaum_0017, and Abaum_2396 in strain ATCC 17978), discrepancies were evidenced between the amplicon size predicted from the available genome sequences and that observed experimentally (Table 2). Therefore, we verified the sequences of the corresponding alleles in ACICU and ATCC 17978 by direct sequencing of PCR products and noticed that discrepancies were exactly coincident in size with one or more repeats. For instance, Abaum_3530 was predicted to be 462 bp in ACICU, while it was observed to be 522 bp, corresponding to a difference of a single 60-bp repeat unit; likewise, Abaum_3002 was predicted to be 482 bp in ATCC 17978, while it was observed to be 539 bp, corresponding to a difference of



Strain designation (Study code)	PFGE type	ICL	SG profile	Panel	MLVA-8 allelic profile: Abaum_3530	Abaum_3002	Abaum_2240	Abaum_1988	Abaum_0826	Abaum_0845	Abaum_2396	Abaum_3468
A399 (22)	III	III	4	D	6	8	5	5.5	17	7	14	13
A374 (21)	III	III	4	D	6	8	5	5.5	15	14	20	12
RUH5875 (19)	III	III	3	D	6	8	4	5	6	-	10	13
A389 (25)	X	NA	5	D	6	7	3	5.5	-	21	13	15
SDF (7,8)	VI	NA	5	D , R	-	6	2	3	16	16	7	7
105C (29)	VII	NA	N	D	6	8	2	8	-	16	22	11
ACICU (1,2)	II	II	1	D , R	7	7	4	9	13	10	20	13
RUH134 (9)	II	II	1	D	7	7	4	9	15	8	20	12
33C (10)	II	II	1	D , E	7	7	4	9	13	8	21	12
53C (11)	II	II	1	E	7	7	4	9	13	8	21	12
71C (12)	II	II	1	E	7	7	4	9	13	8	21	12
50C (28)	II	II	1	D	7	7	4	9	6	12	19	13
ATCC17978 (3,4)	VIII	NA	N	D , R	6	8	2	5	19	2	19	-
A388 (26)	IX	NA	6	D	6	8	3	5.5	17	16	24	17
82D (16)	IV	I	2	D , E	6	7	3	5.5	14	21	18	15
85D (18)	IV	I	2	E	6	7	3	5.5	14	21	18	15
84D (17)	IV	I	2	E	6	7	3	5.5	14	22	19	15
51C (14)	IV	I	2	D	6	7	3	5.5	8	20	35	14
AYE (5,6)	I	I	2	D , R	6	7	3	5.5	17	19	21	14
RUH875 (13)	I	I	2	D	6	7	3	5.5	15	14	20	16
57C (15)	V	I	2	D	6	7	3	5.5	8	20	24	13
A376 (24)	XIII	NA	5	D	6	7	3	5.5	13	9	18	8
A457 (27)	XII	NA	7	D	7	8	3	9	10	-	24	13
A377 (20)	XI	NA	3	D	6	8	4	5	13	-	11	12
A461 (23)	XIV	NA	4	D	6	8	5	5.5	13	10	14	17

FIG. 2. Typing concordance between MLVA-8 and PFGE typing or SG profiling. Cluster analysis of 25 *A. baumannii* strains analyzed by PFGE following ApaI digestion is shown on the left. The dendrogram was generated using the Dice coefficient and UPGMA clustering, with a 1.5% tolerance limit and 1.5% optimization as standard settings (23). The PFGE types were defined at a similarity threshold of ≥85% (28). SG and MLVA-8 allelic profiles are provided for direct comparison. The boxes denote strains belonging to the same PFGE type or MLVA-8 complex. Gel images were analyzed with BioNumerics version 5.1 software. PFGE type and SG profile determinations were performed in center II. Abbreviations: D, diversity; R, reproducibility; E, epidemiological concordance; ICL, international clonal lineage; NA, not assigned; N, new SG profile.

a single 57-bp repeat unit. The sizes of expected, observed, and resequenced amplicons as well as the sizes of flanking regions for the reference strains used in this study are shown in Table S2 in the supplemental material. It should be pointed out that sequence length discrepancies were always due to the absence of one or multiple repeat units and were observed only in *A. baumannii* ACICU and ATCC 17978 genome sequences obtained by the Roche 454 high-density pyrosequencing technology (12, 26), which provided reads of 100 to 200 bp.

Among the 10 putative VNTR markers analyzed in this study, 8 yielded amplification products for most isolates and allowed unambiguous assignment of the number of tandem repeats, while 2 provided unsatisfactory results. In particular, poor amplification was observed for Abaum_3406, raising the possibility that this locus is not widespread in the *A. baumannii* population (see Table S1 in the supplemental material), while Abaum_0017 yielded an ambiguous amplification pattern in center II, with three and two bands for reference strains ACICU and ATCC 17978, respectively (Fig. 1B and data not shown). Preliminary stability evaluation of Abaum_0017 provided evidence that this marker is unstable over time, as demonstrated by the presence of a 14- or 18-repeat allele in two subcultures of the reference strain ACICU (data not shown). As a result, Abaum_3406 and Abaum_0017 were judged unsuitable for inclusion in a first-line MLVA scheme, although these VNTRs could be reconsidered under circumstances in which additional informativeness is needed.

Based on the above results, an MLVA scheme (MLVA-8) was developed to include eight VNTR markers, in the order Abaum_3530, Abaum_3002, Abaum_2240, Abaum_1988, Abaum_0826, Abaum_0845, Abaum_2396, and Abaum_3468.

Typeability of the MLVA-8 assay. Analysis of the whole collection of 25 different *A. baumannii* strains showed amplifiability and full typeability ($T = 1.00$) for VNTR markers Abaum_3002, Abaum_2240, Abaum_1988, and Abaum_2396 (Fig. 2). The remaining four markers, namely, Abaum_3530, Abaum_0826, Abaum_0845, and Abaum_3468, yielded lower though still good typeability values ($0.88 \leq T \leq 0.96$). This was due mainly to failures of some PCR amplifications, even though for one marker a double-band profile was observed repeatedly in both centers. In particular, no amplification was obtained for Abaum_3530 in strain SDF, while double bands were observed for Abaum_3468 in ATCC 17978 by both centers and were scored as failures (Fig. 2; see Table S1 in the supplemental material). Therefore, a T value of 0.96 was determined for both of these markers. Moreover, no amplification was obtained by both centers for Abaum_0826 in strains A389 and 105C ($T = 0.92$) and for Abaum_0845 in strains RUH5875, A377, and A457 ($T = 0.88$) (Fig. 2; see Table S1 in the supplemental material).

Reproducibility of the MLVA-8 assay. Reproducibility was assessed at the intra- and interlaboratory levels. For intralaboratory reproducibility, the MLVA-8 profiles of the four sequenced strains were determined in two independent assays. For interlaboratory reproducibility, the whole collection of strains was tested separately by the two different centers.

TABLE 3. HGDI for individual or combined (MLVA-8) VNTR loci, PFGE types, and SG profiles calculated for 21 unrelated *A. baumannii* strains (diversity panel)

VNTR marker or analysis	No. of alleles or types	HGDI
L-repeat VNTR loci		
Abaum_3530	2	0.39
Abaum_3002	3	0.57
Abaum_2240	4	0.73
Abaum_1988	5	0.68
S-repeat VNTR loci		
Abaum_0826	9	0.88
Abaum_0845	12	0.95
Abaum_2396	12	0.94
Abaum_3468	9	0.87
MLVA-8	21	1.00
PFGE typing	14	0.95
SG profiling	8	0.89

The MLVA profiles were in complete agreement for the four duplicates of reference strains within individual centers, as well as for all 25 unique strains of the collection tested independently by the two centers (Fig. 2; see Table S1 in the supplemental material), yielding an *R* value of 1.00.

Epidemiological concordance of the MLVA-8 assay. For the assessment of epidemiological concordance, a panel composed of two sets of epidemiologically related *A. baumannii* isolates was used (Fig. 2). Epidemiological concordance was excellent, with an *E* value of 1.00 for all VNTR markers except for Abaum_0845 and Abaum_2396 ($E = 0.83$). While one group of epidemiologically related isolates (33C, 53C, and 71C) (Table 1) was characterized by identical MLVA types, the other (82D, 84D, and 85D) (Table 1) showed two MLVA profiles differing in only one unit at two S-repeat VNTRs (Abaum_0845 and Abaum_2396) (Fig. 2).

Stability of the MLVA-8 assay. To assess the stability of our MLVA scheme, two subcultures of the reference strain ACICU were analyzed and compared to the original strain. MLVA-8 provided indistinguishable allelic profiles between the original strain and the subcultures, corresponding to an *S* value of 1.00 for all VNTR markers.

Discriminatory power of the MLVA-8 assay and concordance with PFGE and SG profiling. Twenty-five different strains were typed comparatively by means of MLVA-8, PFGE, and SG profiling. The dendrogram generated by PFGE analysis was then matched with the results of both SG profiling and MLVA-8 for direct comparison (Fig. 2). Diversity index (HGDI) values for the three typing methods were estimated using the diversity panel of 21 unrelated isolates, which includes only one strain for each set in the epidemiologically related panel (Fig. 2). Individual HGDI values for the eight VNTR markers included in the MLVA-8 scheme ranged between 0.39 and 0.95 (Table 3). Greater diversity was generally observed for the S-repeat markers ($0.87 \leq \text{HGDI} \leq 0.95$). Although all of the VNTRs included in the MLVA-8 scheme showed excellent stability ($S = 1$), the S-repeat loci Abaum_0826, Abaum_0845, Abaum_2396, and Abaum_3468, with the highest HGDI, could represent rapidly evolving markers with less phylogenetic value (36).

Considering the diversity panel of 21 unrelated isolates, the MLVA-8 assay defined 21 different types, represented by individual allelic profiles, while 14 and 8 different types were defined by PFGE (with an 85% similarity threshold) and SG profiling, respectively (Table 3). Thus, MLVA-8 (HGDI = 1.00) was more discriminatory than PFGE typing (HGDI = 0.95) and SG profiling (HGDI = 0.89) (Table 3).

For epidemiological typing purposes, it should be pointed out that the MLVA-8 allelic profile defines the genotype, while closely related genotypes can be clustered into complexes, where a different weight is assigned to the different VNTR markers according to their HGDI. Based on epidemiological concordance results and taking into account the relatively low weight that should be assigned to the more highly variable markers (36), for operative purposes we suggest the following two criteria to assign *A. baumannii* strains to the same MLVA-8 complex: (i) identical L-repeat VNTR profiles and (ii) a maximum of three differences of no more than two repeats at S-repeat VNTRs.

To assess the effectiveness of this assumption, the concordance between MLVA-8, PFGE, and SG profiling was evaluated by side-by-side comparison of results for all 25 strains. Fifteen isolates were found to group together into four PFGE clusters, well matching their assignment to the three international clonal lineages. The remaining 10 isolates showed unique PFGE types, and all but 1 (57C) were not assigned to any international lineage (Fig. 2). MLVA-8 clustered 8 isolates into only two main complexes, while the remaining 17 isolates were characterized by unique MLVA types which could not be assigned to any complex (Fig. 2). The largest MLVA-8 complex matched five of six isolates included in PFGE type II (international clonal lineage II) and typed as SG1 (Fig. 2). The only exception was strain 50C, which differed markedly at the level of S-repeat VNTRs, consistent with a previous report which assigned this strain to a variant subtype of PFGE type II (5). The second group matched three of four isolates included in PFGE type IV, all belonging to SG2 (international clonal lineage I) (Fig. 2), while the last isolate (51C) belonged to a unique MLVA type because of differences in the S-repeat profile. Of the remaining 15 isolates with unique MLVA-8 types, 10 were assigned to unique PFGE types (A389, SDF, 105C, ATCC 17978, A388, 57C, A376, A457, A377, and A461), while 5 were clustered within PFGE types III (A399, A374, and RUH5875) and I (AYE and RUH875), referable to international clonal lineages III and I, respectively (Fig. 2).

DISCUSSION

MLVA has become the reference typing method for *Bacillus anthracis*, *Yersinia pestis*, *Mycobacterium tuberculosis*, *Escherichia coli* O157, and *Salmonella enterica* (11, 14, 15, 16, 21, 36) and is considered a promising alternative to established typing methods for other microbial species, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Legionella pneumophila* (13, 19, 20, 38), especially when highly discriminatory, portable, and fast results are needed (32). Therefore, in this work, we set the basis for the application of MLVA to the epidemiological typing of *A. baumannii* for possible use as a complement or alternative to previously established methods.

In an attempt to increase the repertoire of previously estab-

lished VNTR markers (29), we searched for new VNTR candidates in six *A. baumannii* genome sequences. With the aim of combining technical simplicity with high discriminatory power, we selected eight VNTR markers and tested their typing capability with a diverse collection of *A. baumannii* strains. The MLVA-8 scheme we propose represents an easily manageable combination of four moderately diverse L-repeat VNTR markers (repeat units of 26 to 99 bp), characterized by a small number of alleles with low variability, and four highly variable S-repeat VNTR markers (repeat units of 6 to 9 bp) showing high levels of polymorphism (Table 3). The L-repeat loci are suitable for establishing phylogenetic relationships among strains evolving over a long period (40). Conversely, S-repeat loci, particularly those with high HGDI values, are characterized by more rapid evolution and provide more subtle diversification, which is crucial to discriminate between closely related isolates (20), as in the case of disease surveillance and/or investigations of outbreaks (29). By combining four L-repeat and four S-repeat VNTR markers whose individual diversity indices (HGDI) ranged between 0.39 and 0.95, an excellent discriminatory power of the MLVA-8 assay was achieved (HGDI = 1.00) (Table 3), at least with the present diversity panel of *A. baumannii* strains.

The interlaboratory reproducibility of the MLVA-8 assay was excellent (see Table S1 in the supplemental material), although in preliminary assays some discrepancies were occasionally observed. Troubleshooting involved primarily amplicon sizing and PCR specificity. The former could be ascribed to the way the cursor was placed on the band to estimate the band size when reading agarose gels. When the cursor was placed in the middle of a large band resulting from DNA overloading, the allele size was generally underestimated, as indicated by the sizing of the amplicons from reference strain ACICU. This error was suppressed by loading similarly small amounts of PCR products in the gel to get sharp bands or, when this was difficult to obtain, by placing the cursor at the trailing part of the band peak. Only in center II, double bands were initially observed for markers Abaum_0826 and Abaum_2396, in very few isolates. This problem was easily solved by using more stringent PCR conditions upon agreement between the two centers.

The epidemiological concordance was excellent when epidemiologically related isolates 33C and 53C, from two patients during the same outbreak, and isolate 71C, from the associated environment in the same hospital (Table 1), were analyzed. For these strains, we observed identical allele profiles (Fig. 2). Intriguingly, the MLVA-8 results for epidemiologically related isolates 82D, 84D, and 85D, obtained from different patients during the same outbreak in the same hospital (Table 1), showed differences in two S-repeat VNTR markers (Abaum_0845 and Abaum_2396) (Fig. 2). It is important to consider that Abaum_0845 and Abaum_2396 are the S-repeat VNTR loci which showed the highest levels of diversity among the MLVA-8 markers (Table 3). Thus, as suggested for such rapidly evolving markers (29), it is possible that alterations in the repeat number occur so rapidly that the VNTR profile changes in a relatively small number of generations, such as during the course of an outbreak. Therefore, while the evolution of the most variable VNTRs may be helpful in monitoring short-term genome variations in bacterial pathogens, their intrinsic variability should be taken into account in comparing MLVA-8 allelic profiles between epidemiologically related isolates during an outbreak. This led us to propose criteria for the assignment of strains to MLVA-8 complexes, i.e., (i) identical L-repeat VNTR profiles and (ii) a maximum of three differences of no more than two repeats at S-repeat VNTR loci. Even at the MLVA complex definition level, the discriminatory power of MLVA-8 was greater than that observed for PFGE typing and SG profiling, enabling differentiation of unrelated isolates which clustered together by PFGE type or SG (Fig. 2). Moreover, when MLVA-8 results were interpreted according to the above criteria and in spite of the limited number of strains analyzed, a remarkable congruence between MLVA-8- and PFGE-based strain clustering was observed. However, future studies with a larger *A. baumannii* population are needed to analyze the evolution of individual VNTRs and to verify the proposed criteria for MLVA-8 complex definition.

It is worth addressing the possible contributions of *A. baumannii* VNTRs to genome polymorphism and/or evolution. Expansion or contraction of the number of tandem repeats can have an impact on the expression, structure, and/or activity of cellular proteins. When a mutation event changes the number of tandem repeats at a given VNTR locus, the effect of this event depends on the location and structure of the specific VNTR. Tandem repeats located within regulatory regions can cause a modification of gene expression at the transcriptional level. Tandem repeats located within gene coding regions and endowed with repeat units that are not multiples of three base pairs can induce a reversible premature end of translation (35, 39). Conversely, VNTR loci localized within gene coding regions and characterized by repeat units that are multiples of three base pairs can promote variations of the protein sequence and possibly lead to altered protein structure and/or activity (41). The coding potential of genes and the predicted functions of the translated VNTR sequences or the noncoding regions encompassing VNTRs described here are listed in Table S3 in the supplemental material for the six sequenced *A. baumannii* strains. Notably, 8 of the 10 VNTR loci are entirely or partially located within coding regions of putative orthologous genes. Among these VNTR loci, the only two having repeat units that are not multiples of three (Abaum_1988 and Abaum_0845) marginally overlap the 3′ regions of genes and are not predicted to generate longer coding sequences. Some VNTR loci with repeat units that are multiples of three lie within coding regions (see Table S3), and a change in repeat number will result in quite different coding regions. VNTR loci in specific coding regions can potentially lead to variations in surface-exposed proteins related to pathogenicity, allowing bacteria to alter their capability to adhere to and invade the host cell (3) as well as to elude the host's immune response (25). Since VNTR loci Abaum_0826, Abaum_3002, Abaum_3406, Abaum_3468, and Abaum_3530 are internal to genes responsible for processes which occur in the cell envelope (see Table S3 in the supplemental material), it is tempting to speculate that their variability could actually promote structural and perhaps antigenic alterations of some cell envelope proteins of *A. baumannii*.

Our results with *A. baumannii* strengthen the notion that MLVA is a rapid, easy-to-perform, cost-effective typing method with excellent reproducibility and discriminatory power, enabling interlaboratory transfer and unequivocal comparison of typing

data. When large numbers of isolates need to be analyzed and S-repeat VNTR loci are used, it could be advisable to perform multiplex PCR using fluorescent primers and to separate the products by means of high-precision capillary electrophoresis equipment such as a DNA sequencer. Preliminary experiments performed in center I, using fluorescent primers and capillary electrophoresis, showed that *A. baumannii* MLVA-8 can be performed with two multiplex PCRs (see the text and Table S4 in the supplemental material). Moreover, successful MLVA-8 results were obtained in both centers by direct amplification of the VNTR markers from thermolysates of *A. baumannii* colonies, avoiding the genomic DNA extraction step and thereby reducing the time and cost of the assay (data not shown). Lastly, this study evidenced some discrepancies between observed amplicon sizes and the sizes expected on the basis of the available genome sequences of strains ACICU (for VNTR markers Abaum_3530, Abaum_0826, Abaum_0845, and Abaum_2396) and ATCC 17978 (for VNTR markers Abaum_3002, Abaum_0826, Abaum_0017, and Abaum_2396). As shown by sequencing of the PCR products, both the ACICU and ATCC 17978 published genome sequences contain deletions in the sequences of the above VNTRs, probably due to erroneous assembly of short pyrosequencing-generated reads (100 to 200 bp) (12, 26). It is therefore possible that some VNTR candidates that initially were discarded by the bioinformatic analysis because of their apparent low degree of polymorphism could be reconsidered. In the future, improvement of the number of isolates typed by MLVA-8 and the design of multicenter studies for interlaboratory comparison of MLVA-8 proficiency will contribute further to validation of the method. This study also made it possible to establish the first *A. baumannii* MLVA-8 database that can be queried via the Internet (http://mlva.u-psud.fr/).

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