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Multiple Polymorphic Loci for Molecular Typing of Strains of *Mycobacterium leprae*

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The need for molecular tools for the differentiation of isolates of *Mycobacterium leprae*, the organism that causes leprosy, is urgent in view of the continuing high levels of new case detection, despite years of aggressive chemotherapy and the consequent reduction in the prevalence of leprosy. The slow onset of leprosy and the reliance on physical examination for detection of disease have restricted the epidemiological tracking necessary to understand and control transmission. Two genetic loci in several isolates of *M. leprae* have previously been demonstrated to contain variable-number tandem repeats (VNTRs). On the basis of these reports and the availability of the full genome sequence, multiple-locus VNTR analysis for strain typing has been undertaken. A panel of 11 short tandem repeat (STR) loci with repeat units of 1, 2, 3, 6, 12, 18, 21, and 27 bp from four clinical isolates of *M. leprae* propagated in armadillo hosts were screened by PCR. Fragment length polymorphisms were detected at 9 of the 11 loci by agarose gel electrophoresis. Sequencing of representative DNA products confirmed the presence of VNTRs between isolates. The application of nine new polymorphic STRs in conjunction with automated methods for electrophoresis and size determination allows greater discrimination between isolates of *M. leprae* and enhances the potential of this technique to track the transmission of leprosy.

The World Health Organization and its partners created the Global Alliance for Leprosy Elimination in 1999 with the aim of achieving the elimination of leprosy by the end of the year 2005, a goal originally set for the year 2000 (http://www.who.int/inf-pr-1999/en/pr99-70.html). Leprosy is effectively controlled by a multidrug therapy (MDT) regimen composed of dapsone, rifampin, and clofazimine. However, continuing large numbers of new cases are being detected in areas of the world where the disease is highly endemic, despite the application of the MDT program since 1982 (36). In order to comprehend this rising incidence of leprosy, it is necessary to identify the natural reservoir of *Mycobacterium leprae*, the route of infection, and the mode of its transmission. It is commonly believed that the human is the host and reservoir of *M. leprae* and that successful MDT will eliminate leprosy. However, other modes of transmission involving nonhuman reservoirs, such as soil (6, 15), water (20), vegetation (16), animals (including armadillos and sooty mangabeys) (22, 33), and arthropods (fleas, ticks, mosquitoes, and flies), have been suggested, as reviewed by Blake et al. (3). The route of infection is also unknown, although the entry and the exit of the bacteria via the nasal passages have been proposed (9). Methods that specifically detect *M. leprae* DNA in nasal swabs are being developed with the aim of early detection in populations at the community and village levels and for the monitoring of leprosy transmission (13, 23). Typing methods for distinguishing cases of relapse from new infections are also required. Molecular typing will make it feasible to study the global and geographical distributions of distinct clones of *M. leprae*, explore correlations between the *M. leprae* genotypes and the incidence rates of leprosy, and the type of disease manifestation (tuberculoid versus lepromatous) and provide some insight into the historical and phylogenetic evolution of the bacillus that has affected humans and stigmatized leprosy patients for centuries. The research community has been urged to develop molecular typing methods to complement the efforts of clinicians and health care workers (10, 25). These points have been emphasized in a recent review (38), which provided an elegant perspective of the general methods available for typing mycobacteria, particularly *M. tuberculosis*, and their relevance to *M. leprae*.

Approaches to uncovering polymorphisms attributed to single nucleotide mutations, insertion elements, and variable-number tandem repeats (VNTRs) in the mycobacterial interspersed repetitive unit loci in a panel of *M. leprae* strains have not identified any molecular typing markers (8, 35). However, it has been possible to recognize other potential polymorphic sites from the genome sequence of *M. leprae* (8). As is the case in several eukaryotic and prokaryotic genomes that have been sequenced, short stretches of DNA that occur in tandem repeats are found in *M. leprae* (8, 30, 32).

Matsuoka et al. (21) first reported that a 6-bp sequence was found as two alleles in the *rpoT* gene of *M. leprae*. This was followed by the recognition of VNTRs of the TTC triplet in an intergenic region of the genome (26). Both sets of VNTRs have been used in the first applications of genotyping to *M. leprae* isolates. In the study described in this paper, we have identified nine additional polymorphic loci in a small set of

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TABLE 1. Primers used in the PCR amplification of STR loci

Locus	Primer sequence (forward)	T_m (°C) for forward primer	Primer sequence (reverse)	T_m (°C) for reverse primer	Product length (bp)[a]
(C)20	5′-TCGGCGACTGCGGTAACATT-3′	62	5′-CGAGTGAGCGGAACGGAGTA-3′	60	197
(TA)18	5′-CGTGCGTCGTGTGTAGGCA-3′	62	5′-GACGTGGCAACATCGAAGTT-3′	60	230
(AT)17	5′-TCTCCAACATGCTGCGACA-3′	60	5′-GTACAGCGGCCTGATCGAA-3′	60	181
(CG)6	5′-CAATTCTCCGCGCCGATGA-3′	60	5′-CGGACTTAGTGCGTGCGAT-3′	60	211
(AC)9	5′-AGCGCCCGTTGTCGATAGA-3′	60	5′-TGCTGGTGACCCGCGATA-3′	58	236
(GTA)9	5′-AGCCTTAGTCGCGCAGATG-3′	60	5′-TCCGCTGTCCGTCCGCTGA-3′	64	307
(AGA)20	5′-GGACCTAAACCATCCCGTTT-3′	62	5′-CTACAGGGGGCACTTAGCTC-3′	64	201
6-7	5′-GCCATCGTTGTCGGTTCATC-3′	62	5′-CGGAGGAGGTGGGTACGGT-3′	64	268
12-5	5′-CTGGTCCACTTGCGGTACG-3′	62	5′-GGAGAAGGAGGCCGAATACA-3′	62	289
18-8	5′-GCCCGTCTATCCGCATCAA-3′	60	5′-GCAAAGATCAGCACGCCAAT-3′	60	348
21-3	5′-GAATCTGACCTTTCGGAAATG-3′	60	5′-CGATGCAGCTTCCTACGG-3′	58	312
27-5	5′-ATTGAGCAGATGGCCGGTC-3′	60	5′-AGCAGTCGGCACGCCCTT-3′	60	327

[a] As for the *M. leprae* TN strain (14).

four armadillo-derived *M. leprae* isolates and demonstrate their applicability to distinguish between isolates of *M. leprae* by the use of multiple-locus VNTR analysis (MLVA).

MATERIALS AND METHODS

***M. leprae* DNA isolation.** Clinical isolates of *M. leprae* passaged in an armadillo were obtained from Richard W. Truman, National Hansen's Disease Program, Louisiana State University, Baton Rouge, in the form of infected livers and spleens. The isolates used in this study are designated 4089, 4264, 4923, and Thai-53. About 20 g of tissue was processed as described by Lloyd and Draper (19) to obtain approximately 100 mg of purified *M. leprae* cells (containing, on average, 2.9×10^9 bacilli/mg). DNA was extracted from the purified *M. leprae* cells by methods developed for mycobacteria in general (1).

Biopsy samples collected from previous studies and stored at −70°C were processed for DNA as described in those studies (26, 37). Briefly, thin sections 5 μm thick were cut with a cryostat microtome and distributed into several cryovials, each of which contained 10 sections. DNA for PCR amplification of *M. leprae* sequences was obtained by breakage with 0.1-mm-diameter zirconium beads in the presence of Tris-EDTA buffer (TE) and a mixture of phenol, chloroform, and isoamyl alcohol. The extract was centrifuged, and the aqueous phase was boiled for 10 min before the DNA was precipitated with ethanol. The final DNA (10 μl in TE) was used as the template for one PCR.

Primer selection. Primer sets for the amplification of regions of DNA containing the short tandem repeat (STR) sites were identified by using Oligo 6 Primer Analysis software (Molecular Biology Insights, Inc., Cascade, Colo.). A tract of 400 to 500 bp that included the STRs was screened for primers (18 to 21 bases long) with melting temperatures (T_ms) between 55 and 65°C and a G+C content near 50% to produce PCR products with lengths in the range of 150 to 400 bp (Table 1). Primer pairs that could form dimers and hairpin interactions were avoided.

PCR amplification of STRs. The PCR mixture consisted of Platinum PCR SuperMix (Invitrogen Corporation, Carlsbad, Calif.), which contains Platinum *Taq* polymerase, deoxynucleoside triphosphates, and buffer; 200 nM (each) primer; and 500 ng of *M. leprae* genomic DNA. Following an activation step at 94°C for 2 min, 10 cycles of touchdown PCR were performed with a model 9600 Thermocycler (Perkin-Elmer Co., Norwalk, Conn.), in which the annealing temperature was reduced from 65 to 55°C at a rate of 1°C per cycle. After the touchdown phase, 25 additional cycles were run at an annealing temperature of 55°C. The PCR was terminated with a final extension at 72°C for 5 min. An aliquot of the reaction mixture was run on a 3% agarose gel. For direct sequencing of the PCR products, the reaction products were purified on a Qiaquick Spin Column (Qiagen, Valencia, Calif.).

Microsatellite MLVA with the ABI PRISM 3100 Genetic Analyzer and GeneMapper. The forward primers (Table 1) for the (AT)17, (AGA)20, and 21-3 loci [where the locus designations indicate (repeat unit sequence)number of repeats, followed by a letter, if needed, or the length of the repeat-number of repeat units, followed by a letter, if needed] were labeled with PET, NED, and 6-FAM fluorescent dyes at the 5′ termini, while the primers for the 6-7 and 12-5 loci were labeled with VIC (Applied Biosystems, Foster City, Calif.). Following separate PCRs with the forward fluorescent labeled primer and the unlabeled reverse primer, as described above, 1 μl [8 μl for (AT)17] of each product was combined in a volume of 100 μl. A 1-μl aliquot of this sample was mixed with 12 μl of formamide and 0.5 μl of GeneScan 500LIZ (Applied Biosystems) sizing standards. The sample was denatured at 94°C for 5 min and cooled prior to electrophoresis on an ABI PRISM 3100 Genetic Analyzer under denaturing conditions on a 36-cm capillary column with performance-optimized polymer 4 polymer (Applied Biosystems) at 60°C. The instrument was previously calibrated with DS-33 matrix standards (Applied Biosystems). The electrophoresis data were analyzed with GeneMapper software (Applied Biosystems). These analyses were carried out at the Macromolecular Resources Laboratory, Colorado State University.

RESULTS

The goal of this study was to launch a systematic approach to the identification of polymorphic genetic loci applicable for the tracking of leprosy. Two elements were required for this study: target loci and a panel of distinct isolates. *M. leprae* strains are not cultivable in the laboratory, as extreme genome downsizing has resulted in an obligate intracellular niche (7). For research applications, *M. leprae* is obtained by propagating isolates in a susceptible armadillo host (28) or nude mice (31), principally at the National Hansen's Disease Program. The long doubling time of 12 to 14 days also affects the availability of *M. leprae* (18). Although small amounts of *M. leprae* for PCR applications can be obtained from biopsy specimens, slit skin smears, and the nasal mucus of leprosy patients, for these studies we found it more feasible and practical to study several loci in a limited set of available armadillo-derived isolates. Our goal was to uncover and validate polymorphic loci and also to standardize methods for MLVA before their application to clinical samples. The number of repeat units at different loci in the sequenced *M. leprae* TN strain was used as a reference with which to compare the number of repeats in the four test isolates. All STR loci that had different numbers of tandem repeats in at least one isolate compared to the sequence of the TN strain were considered polymorphic and potentially useful for typing.

Selection of potential VNTR loci. The *M. leprae* genome sequence is now in the public domain, and all potential polymorphic sites can be selected by *in silico* analysis. Using the Search Pattern Program within the Leproma website (14), the Tandem repeats finder (2), and MICdb 1.0 database (27), we interrogated the *M. leprae* TN genome for simple nucleotide repeats. There were more than 100 STRs with at least two repeat units. To streamline the screening process, we used a cutoff of eight repeats for single nucleotides, six repeats for

TABLE 2. Microsatellites in *M. leprae* TN genome targeted for MLVA[a]

Repeat unit (bp)	Locus ID[b]	Coordinates	Intergenic region	Intragenic region/ gene orientation
1	(T)8, (A)6	337466–337473, 337474–337479	ML0258-ML0259	
1	(T)6, (N)7, (T)8	514181–514186, 514187–514193, 514194–5141101		ML0415/−
1	(A)9	1414666–1414674	ML1196-ML1197	
1	(G)9	976857–976865		ML0823/+
1	(C)9	2658192–2658200		ML2238/−
1	(G)10a	347280–347289		ML0266/+
1	(G)10b	442993–443002		ML0350/−
1	(G)11	1309544–1309554		ML1126/−
1	(G)12	1116443–1116454		ML0946/+
1	(C)16(G)8	1987156–1987171, 1987172–1987179	ML1647-ML1648	
1	(C)20[c]	312039–312058	ML0237-ML0238	
1	(G)22	229625–229646		ML0164/+
2	(CG)6[c]	2947291–2947302		ML2472/−
2	(AC)8a	1531185–1531200		ML1285/−
2	(AC)8b	2211035–2211050	ML1824-ML1825	
2	(AC)9[c]	1452573–1452590	ML1227-ML1228	
2	(CA)6	2507097–2507108	ML2106-ML2107	
2	(TA)8	3221617–3221632	ML2676-ML2677	
2	(TA)9	2844971–2844988	ML2375-ML2376	
2	(TA)10	1744091–1744110	ML1450-ML1451	
2	(TA)13	308815–308840	ML0235-ML0236	
2	(AT)10	2951821–2951840	ML2476-ML2477	
2	(AT)15	948935–948964	ML0798-ML0799	
2	(AT)17[c]	2597735–2597768		ML2183/−
2	(TA)18[c]	984591–984626		ML0830/−
3	(ACC)5	1980049–1980063		ML1645/−
3	(GGT)5	2567251–2567265	ML2159-MI2160	
3	(AGT)5a	1237528–1237542		ML1073/−
3	(AGT)5b	1293503–1293517		ML1118/−
3	(ACT)5	2656108–2656122		ML2236/+
3	(GTA)9[c]	2583814–2583840	ML2172-ML2173	
3	(AGA)20[c]	2785435–2785494	ML2344-ML2345	
5	(CACCG)3	2562391–2562405	ML2158-ML2159	

[a] The coordinates, repeat unit sequence, number of repeat units, and the gene numbers are as for the *M. leprae* TN genome sequence (14). Pseudogenes are underlined.
[b] For easy reference, the locus identification (ID) is (repeat unit sequence)number of repeats, followed by a letter, if needed. The (AGA)20 repeat locus is the same as the TTC locus described previously (26).
[c] The loci analyzed in this report.

dinucleotide sequences, and five repeats for trinucleotide sequences to arrive at 33 promising loci for use in typing studies (Table 2). For longer repeat units (>5 bp), we selected 11 loci with at least three tandem repeats (Table 3). Our premise was that the degree of allelic diversity at a locus is likely to be higher when the number of repeat units is high. Almost all of the STRs fall within intergenic regions or within pseudogenes and are therefore unlikely to disrupt any biological pathways. A few of the STRs, particularly the minisatellites, are within genes. The primer sets used for PCR amplification (Table 1)

TABLE 3. Minisatellites in *M. leprae* TN genome targeted for MLVA[a]

Locus ID[b]	Unit size (bp)	Coordinates	(Consensus sequence of unit) number of units[c]	Intergenic region	Intragenic/gene orientation
6-3a	6	1190341–1190358	(TCGATG)3		ML1022/−
6-3b	6	2302531–2302548	(GATCAC)3		ML1918/−
6-7[d]	6	1816857–1816892	(GCACCT)7		ML1505/+
7-3	7	285076–285096	(GCGTGCC)3		ML0213/+
10-4	10	1139035–1139074	(ttATTAATAA)4		ML0970/+
12-5[d]	12	1381661–1381723	(GAGGTTGTTGAG)5		ML1182/−
15-3	15	2928131–2928175	(CCTTCTTgGCcGGaG)3	ML2454-ML2455	
18-8[d]	18	1587619–1587763	(GGCcCGCCTGGCCAGTAC)8		ML1334/+
21-3[d]	21	73077–73139	(CAAGCCAGGAATCAAGTTGAT)3		ML0058/−
23-3	23	2945487–2945555	(ATAATACTGTAGTGAACGACATC)3	ML2469-ML2470	
27-5[d]	27	687026–687160	(CCGGTGGTGcCGCCTGGTGGGTTTCcC)5		ML0568/+

[a] The coordinates, consensus sequence, number of repeat units, and the gene numbers are as for the *M. leprae* TN genome sequence (14). Pseudogenes are underlined.
[b] Each locus was given an identification (ID) for easy reference (length of repeat-number of repeats, followed by a letter, if needed). The 6-3a locus has been studied previously (21). The 21 bases upstream of the 21-3 locus are homologous to the repeat and could form a fourth repeat.
[c] A lowercase letter in a consensus sequence is a nonconserved base in tandem repeats.
[d] The loci analyzed in this report.

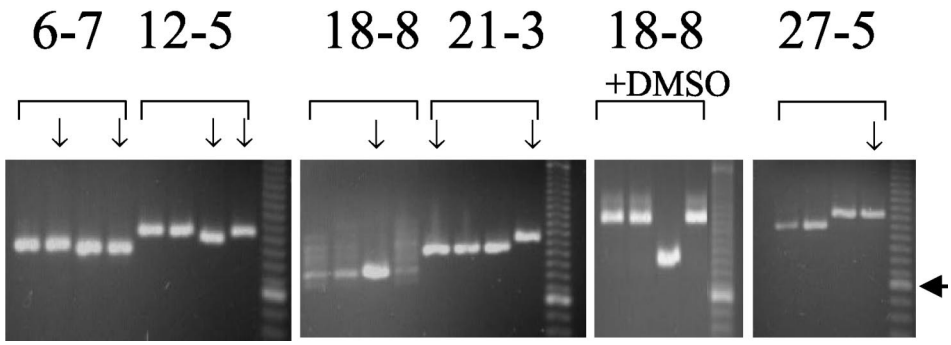

FIG. 1. VNTRs at the minisatellites 6-7, 12-5, 18-8, 21-3, and 27-5 loci in *M. leprae*. PCR products from *M. leprae* isolates 4089, 4264, 4923, and Thai-53 were resolved in that order on a 3% agarose gel. DNA size standards (20-bp ladder) were run in the last lane. The horizontal arrow indicates the position of the brightest 200-bp size marker. The vertical arrows above the gels indicate that the PCR product was also sequenced. DMSO, dimethyl sulfoxide.

were chosen as described in the Materials and Methods section.

VNTR polymorphism at five minisatellite loci. We began with five loci that have more than two repeats in the TN strain. For screening of this set of loci, it was possible to detect VNTRs between isolates by simply resolving the PCR products on a 3% agarose gel. Figure 1 demonstrates that there are at least two alleles for the 6-7, 12-5, 21-3, and 27-5 loci. To confirm that alleles at a locus varied in size due to VNTRs, some representative PCR products were sequenced. The data are summarized in Table 4. In the case of the 18-8 locus, PCR amplification yielded one distinct product for isolate 4923, but a staggered ladder was seen for the other isolates. This phenomenon was reproducible. Interestingly, *in silico* prediction of the secondary structure of the PCR product revealed a large hairpin loop containing the repeat region and a T_m of greater than 70°C. Addition of dimethyl sulfoxide to the PCR mixture to destabilize the secondary structure alleviated this problem. The PCR product amplified from isolate 4923 was considerably shorter than that expected from strain TN. In fact, sequencing confirmed that there are only three repeats in isolate 4923, while there are eight in strain TN. This locus could potentially have several alleles, a desired trait in strain typing.

VNTR polymorphism at four of five microsatellite loci. The PCR products of the (C)20, (AT)17, (TA)18, and (GTA)9 loci were resolved on an agarose gel (Fig. 2). There was variation in the fragment sizes that can be attributed to VNTRs. However, due to the small size of the repeat unit, it was necessary, as was done for the minisatellites, to sequence the products directly to verify the number of repeats. Isolates 4089, 4264, and 4923 had 11, 13 and 15 repeats, respectively, at the (AT)17 locus, correlating well with the mobilities in the agarose gel. There were only nine C residues at the (C)20 locus in isolate 4923.

We also tested the (AGA)20 locus, which corresponds to the previously discovered (TTC)21 locus (26), in three of the armadillo-derived isolates and two biopsy samples obtained from Leonard Wood Memorial Hospital, Cebu, Philippines, for VNTRs (Fig. 3). Sequencing revealed that the numbers of TTC repeats were 12 for isolate 4923, 10 for isolates 4264 and 4089, and 14 for two separate biopsy samples, also in accordance with the migration patterns in the agarose gel. Further gel migration analysis revealed that a fourth allelic variation existed within the Thai-53 isolate.

We also amplified the (CG)6 locus and saw no difference between the different isolates. Furthermore, when the DNA from strain Thai-53 was sequenced, the number of repeats was six, as was the case for the TN strain. It therefore seems reasonable that this locus can be eliminated from the list of suitable targets. We will, however, refer to this STR as a negative control and will continue to test it against new, uncharacterized isolates. From the agarose gel electrophoresis migrations, the (AC)9 STR may similarly be nonpolymorphic.

Automated MLVA. Microsatellite analysis of the combined PCR products of the (AT)17, (AGA)20, 6-7, 12-5, and 21-3 loci of the four isolates, based on PCR and multiplex electrophoresis of fluorescently labeled PCR products by automated electrophoresis, yielded patterns that corroborated the findings of agarose gel electrophoresis. The product sizes were in agreement with the sequence data (Table 4). The ability to distinguish the isolates with just five loci demonstrates the potential of MLVA for molecular typing (Fig. 4). The sensitivity of the method allows the PCR "stutter" products that occur at microsatellites (1- to 4-bp repeat units) to be detected and distinguished from the main allele peak (34). The (AT)17 locus in isolate 4264 appeared to contain two alleles, with the major peak having 13 repeats and another one having 14 repeats.

TABLE 4. PCR product lengths of STR loci in various isolates

Locus	PCR product length (bp)[a]				
	TN	4089	4264	4923	Thai-53
(AT)17	181	168	173	177	ND
(C)20	197	ND	ND	186	ND
(CG)6	211	211*	211*	211*	211
6-7	268	268*	268	262*	262
12-5	289	289*	289*	277	289
18-8	348	ND	ND	258	ND
21-3	312	291	291*	291*	312
27-5	327	ND	ND	327*	327
(AGA)20	201	168	168	174	183

[a] The DNA lengths of the indicated loci were obtained by direct sequencing of the PCR products. The values for the *M. leprae* TN strain are based on the published sequence (14). Values marked with an asterisk were inferred from gel comparisons (Fig. 1). In addition, for the (CG)6 locus, the entire sequence and length of product were confirmed after the PCR products were cloned into a pGEM-T vector (Promega, Madison, Wis.) and sequenced with primers that annealed to the vector. ND, not determined.

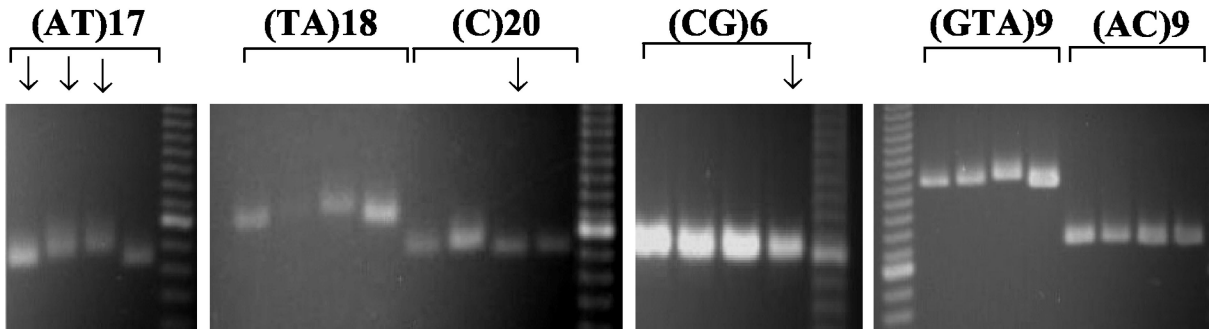

FIG. 2. VNTRs at the (AT)17, (TA)18, (C)20, and (GTA)9 loci in *M. leprae*. PCR products from *M. leprae* isolates 4089, 4264, 4923, and Thai-53 were resolved on a 3% agarose gel. DNA size standards (20-bp ladder) were run in the rightmost lane, and the brightest band is the 200-bp size marker. The vertical arrows above the gels indicate that the PCR product was also sequenced.

Such patterns may become important in assessing the stability of the marker and the purities of the isolates. For the (AGA)20 locus and minisatellites, minor products differing by 1 bp are likely due to incomplete 3′ dA additions by the polymerase, as observed in other STR analyses, and could be avoided by modifying the reverse unlabeled primer (5). As such, these products do not pose a problem in allele definition for these loci.

DISCUSSION

Molecular typing methods are frequently based on DNA sequence differences between isolates which can arise from single nucleotide changes, deletions and insertions (such as those arising from mobile elements), and repetitive stretches of sequences (tandem repetitions) at different sites of a genome. STR loci, loosely classified as microsatellites (repeat units of less than 6 bp) or minisatellites (repeat units of 6 to 100 bp), are excellent sources of polymorphism, because the number of repeat units can increase or decrease due to slippage of the DNA strands during replication by the DNA polymerase. The most recent advance has been in the molecular typing of the VNTRs by PCR amplification, multiplex electrophoresis, and automated detection and analyses (29). The digitization of the data allows the fingerprinting method to be portable and standardized.

Matsuoka et al. (21) were the first to report on a 6-bp sequence present as two alleles within the *rpoT* gene of *M. leprae*. *M. leprae* isolates from patients in Japan (except Okinawa) and Korea carried three copies of the repeat unit, while those from Brazil, Haiti, and Okinawa, Japan, had four copies. The second influential milestone was the identification of VNTRs of the TTC triplet in an intergenic region of the genome (26). This locus is very valuable, because alleles with as few as 10 repeats and as many as 37 repeats were detected in clinical isolates. These two lines of experimentation inspired us to initiate a more thorough and comprehensive analysis of other potential STRs in *M. leprae*, because when several independent STR DNA loci of clinical isolates are evaluated for VNTRs, unique patterns or fingerprints emerge.

Although this report is limited to data for a small number of *M. leprae* isolates propagated in an armadillo, it is significant, because nine new polymorphic loci have been identified. The full range of allelic diversity at each locus is not available from this sample size; even so, this information can now be applied to the typing of *M. leprae* isolates from clinical samples (biopsy and nasal swab specimens) by MLVA. For epidemiological studies, it is necessary to include a set of reproducible, stable, and discriminating loci (11, 12). The ability to discriminate strains can be quantified and is called the discriminatory power. By analysis of additional isolates and loci, one should seek a discriminatory power of 0.95 or higher (12).

The MLVA method described here is similar to the method for the detection of VNTRs in mycobacterial interspersed repetitive units in *M. tuberculosis* isolates (29). The method is sensitive because PCR with suitable flanking primers is used to amplify the regions containing the STRs, and small amounts of genomic DNA are sufficient to amplify several STR loci. This method has proved to be successful with microorganisms for which genome sequences have recently become available, such as *Bacillus anthracis* (24), *Yersinia pestis* (17), and *Candida albicans* (4). On the basis of this background knowledge, we have initiated MLVA-based molecular typing for distinguishing strains of *M. leprae*.

Our long-term vision is to identify a minimal set of typing markers for a minimal amount of template DNA and the

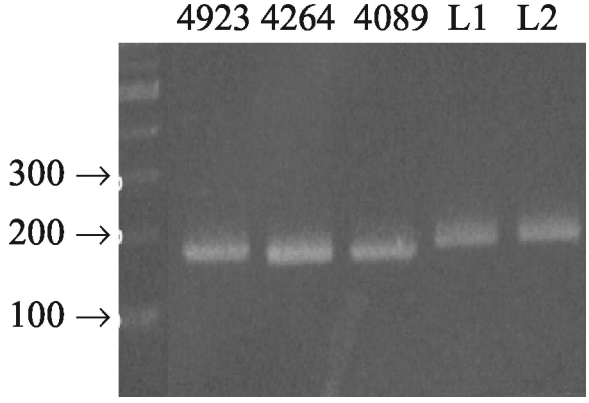

FIG. 3. VNTR at the (AGA)20 locus in *M. leprae* obtained from infected armadillo tissues and frozen sections of two biopsy specimens (L1 and L2). PCR products for the indicated isolates were resolved on a 3% agarose gel. The (AGA)20 locus was previously described as the TTC repeat (26). DNA size standards (100-bp ladder) were run in the first lane, and the numbers on the left are in base pairs.

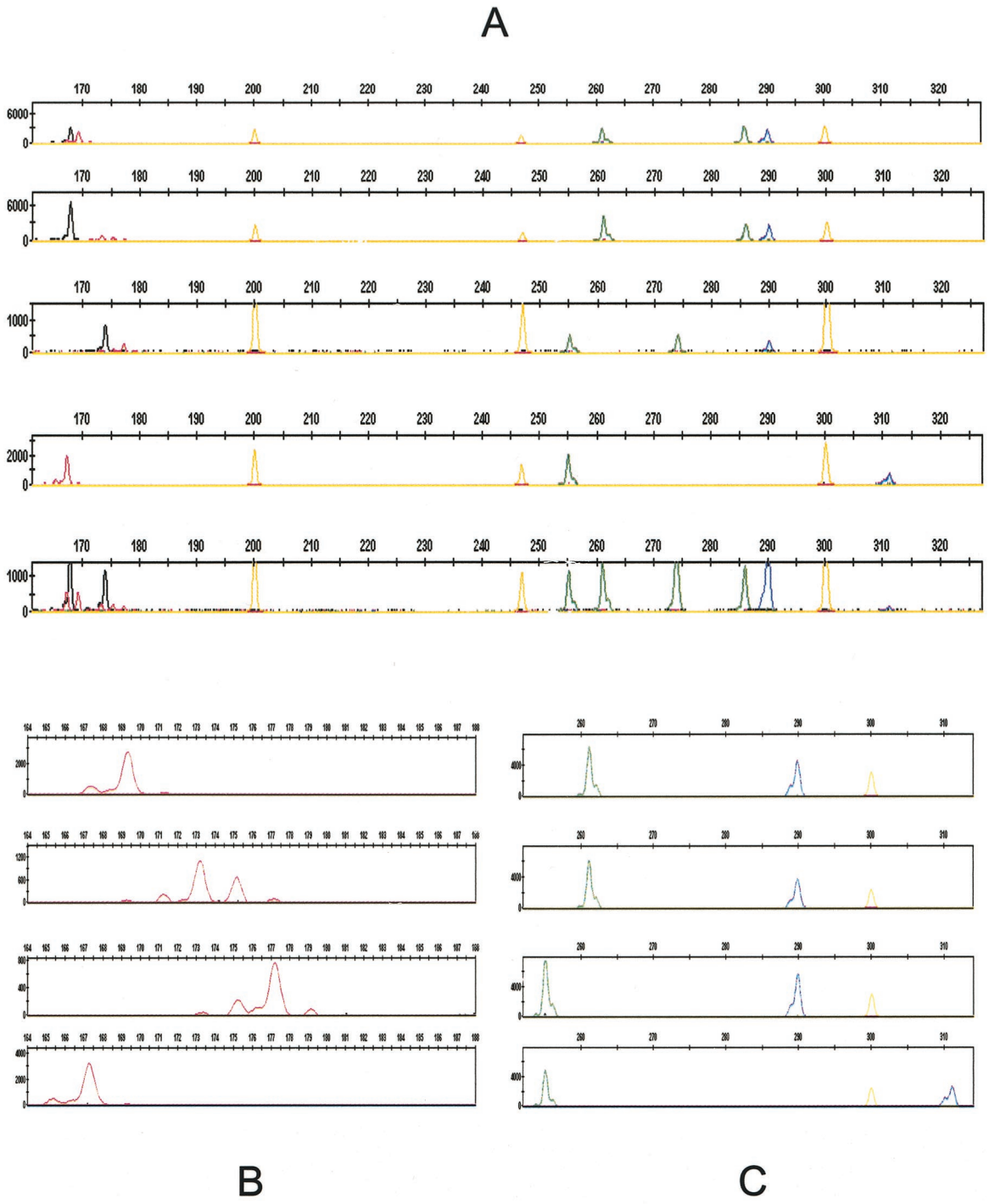

FIG. 4. MLVA analysis of (AT)17, (AGA)20, 12-5, 6-7, and 21-3 loci. (A) Each panel presents the multiplex electrophoresis results for the (AT)17 (red), (AGA)20 (black), 6-7 (first green peak), 12-5 (second green peak), and 21-3 (blue) fluorescence-labeled PCR products compared with those of the GeneScan 500LIZ (yellow) size standards obtained on the ABI Prism 3100 Genetic Analyzer. The electropherograms were obtained with GeneMapper software. The *y* axis is in fluorescence units, and the *x* axis is in base pairs. The first four panels contain products from isolates 4089, 4264, 4923, and Thai-53 (from top to bottom, respectively). Only the (AT)17, 6-7, and 21-3 loci are depicted for the Thai-53 isolate. The fifth (bottom) panel presents the results for the PCR products from all four isolates combined. (B) An enlarged view of the (AT)17 locus in the four isolates depicting stutter bands obtained by the PCR. (C) An enlarged view of the 6-7 and 12-5 loci depicting incomplete dA tailing of the PCR products in isolates 4089, 4264, 4923, and Thai-53 (from top to bottom, respectively).

optimal analytical conditions applicable to leprosy in the clinical setting. Fixed clinical samples or DNA products may then be transferred to a reference laboratory for molecular typing and matched against a database of sequences. Collaborations are in progress to transfer the methodology.

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