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Isothermal Amplification and Molecular Typing of the Obligate Intracellular Pathogen *Mycobacterium leprae* Isolated from Tissues of Unknown Origins

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Molecular diagnostic and epidemiology studies require appreciable amounts of high-quality DNA. Molecular epidemiologic methods have not been routinely applied to the obligate intracellular organism *Mycobacterium leprae* because of the difficulty of obtaining a genomic DNA template from clinical material. Accordingly, we have developed a method based on isothermic multiple-displacement amplification to allow access to a high-quality DNA template. In the study described in this report, we evaluated the usefulness of this method for error-sensitive, multiple-feature molecular analyses. Using test samples isolated from lepromatous tissue, we also evaluated amplification fidelity, genome coverage, and regional amplification bias. The fidelity of amplified genomic material was unaltered; and while regional differences in global amplification efficiency were seen by using comparative microarray analysis, a high degree of concordance of amplified genomic DNA was observed. This method was also applied directly to archived tissue specimens from leprosy patients for the purpose of molecular typing by using short tandem repeats; the success rate was increased from 25% to 92% without the introduction of errors. This is the first study to demonstrate that serial whole-genome amplification can be coupled with error-sensitive molecular typing methods with low-copy-number sequences from tissues containing an obligate intracellular pathogen.

Advances in genomic technologies have greatly facilitated molecular-based epidemiological studies, providing new insights into disease epidemiology and transmission. In addition, the increasing array of whole-genome sequences permits bioinformatic comparisons of clinical strains and laboratory isolates with the potential of revealing genetic determinants of disease. Although genomic analytical strategies have had a substantial impact on epidemiological studies of many infectious diseases, leprosy, caused by the obligate intracellular organism *Mycobacterium leprae*, has not benefited to the same extent. A primary reason is the inability to cultivate *M. leprae* and analyze whole-genomic DNA directly from clinical samples, particularly for the paucibacillary form of the disease.

The current approach to molecular typing largely involves locus-to-locus comparisons, such as microsatellite analysis. However, we need to move beyond examination of small subsets of loci because the application of higher-resolution analysis to closely related bacterial species, strains, and isolates is more likely to enhance our understanding of disease. Unlike locus-directed methods of genotyping, current advances in technology do allow insertion, deletion, and single-nucleotide polymorphism detection on a genome-wide basis. While new whole-genome sequencing methods are nearing fruition, the performance of such methods will be possible only if there is access to ample amounts of complete genomic DNA. For bacterial organisms that are not cultivable, it is difficult to obtain sufficient amounts of genomic DNA, particularly from clinical material.

PCR-based amplifications methods have been developed to overcome the problem of limited sources of genomic DNA. In general, these methods are sufficient for end-point molecular evolutionary studies (16, 26). However, experimental comparative genomic approaches based on whole-genome hybridizations can be compromised by the bias introduced during geometric amplification. In addition, PCR-based methods are not useful for serial amplification of nucleic acids with whole-genome coverage. This fact prevents PCR-based methods from being used with limited amounts of archived clinical samples where whole-genome coverage is required. In order to overcome the limitations associated with PCR-based amplification, several methods have been developed, including balanced PCR (13). One limitation of this approach is that small segments of genomic DNA are amplified preferentially, leading to incomplete genome representation. The recent development of rolling-circle isothermal amplification has provided a means to linearly replicate genomic DNA (2). One such method, restriction and circularization-aided rolling-circle amplification, shows promise for the in vitro replication of genomic DNA from fixed tissues (24). However, isothermic amplification has yet to be applied to genomic DNA from archived infected tissues harboring noncultivable etiological agents and evaluated in regard to genome coverage and amplification bias.

Amplification of source DNA is a particularly attractive approach for research on an obligate intracellular organism such as *M. leprae*, which is derived solely from human lesions, both fresh and archived mouse footpads, and armadillos (1). Rolling-circle amplification, which uses the ϕ29 DNA polymerase,

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has previously been used to amplify plasmid and phage DNA, for mutation detection, and for sequencing (2–5, 8, 11, 12, 14, 15, 26). The objective of this study was to evaluate isothermal genomic amplification for preparation of *M. leprae* genomic DNA from archived sources before subjection to error-sensitive molecular typing.

MATERIALS AND METHODS

Origins of *M. leprae* strains and biopsy samples. *M. leprae* strains NHDP-63 (Louisiana), NHDP-98 (Mexico), and Thai-53 (Thailand) were obtained from Richard Truman, National Hansen's Disease Programs, Louisiana State University, Baton Rouge. Clinical biopsy samples containing *M. leprae*, designated 3044/323, 3045/336, 3044/329, 3045/334, 3044/325, 3044/333, and 3030/267, were obtained from India circa the 1970s. The biopsy samples were originally housed at the National Institute of Medical Research at Mill Hill, London, United Kingdom, and were then transferred to the *M. leprae* repository at Colorado State University and maintained under NIH, NIAID, contract NO1 AI-25469.

Genomic DNA purification. *M. leprae* was isolated from the livers of armadillos as described previously (19), washed with Tris-EDTA buffer, and concentrated by centrifugation at 600 × *g* for 10 min. The recovered bacteria were subjected to a freeze-thaw cycle, suspended in TE (Tris-EDTA) buffer, and extracted with $CHCl_3$-CH_3OH (2:1) to yield a biphasic mixture. Centrifugation at 600 × *g* for 20 min separated the aqueous and the organic layers, leaving a tight band of whole cells at the interface. Both the organic and the aqueous phases were removed by aspiration; the resulting cellular material was resuspended in 500 μl of TE buffer and 50 μl of 1 M Tris-HCl (pH 9.0) and subjected to a temperature of 55°C for 15 min. The cells were again concentrated by centrifugation and resuspended in 500 μl TE buffer with lysozyme and RNase and incubated for 12 h at 37°C. Upon completion, 55 μl of 10% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K were added to the cell lysate and the mixture was incubated for an additional 3 h at 55°C, followed by the addition of 500 μl of phenol, chloroform, and isoamyl alcohol (25:24:1). The aqueous DNA-containing phase was separated from the organic phase by centrifugation at 12,000 × *g* for 30 min at 4°C. Genomic DNA was precipitated by the addition of 100 μl of 3 M sodium acetate (pH 5.2) and 500 μl of isopropanol and incubation at 4°C for 1 h. Genomic DNA was recovered by centrifugation at 12,000 × *g* for 30 min at 4°C.

Isothermic amplification of *M. leprae* genomic DNA. Multiple-primed, isothermal amplification of *M. leprae* genomic DNA was performed according to the supplier's recommendations (Genomiphi; Amersham, Piscataway, NJ). Specifically, 1 μg of *M. leprae* genomic DNA (template) and 9 μl of commercially supplied amplification buffer containing N^6 primers and deoxynucleoside triphosphates were combined and heated to 95°C for 3 min. Isothermic amplification was achieved by the addition of 1 μl of ϕ29 polymerase and incubation for 16 h at 30°C. The reaction was stopped by inactivation of the polymerase at 65°C for 10 min. An identical protocol was performed with DNA obtained from biopsy samples.

Size determination by use of CHEF Mapper XA system. Native and synthetic genomic DNAs were analyzed by comparative electrophoretic migration on 0.8% certified megabase agarose-TAE (Tris-acetate-EDTA) gel (Bio-Rad, Hercules, CA) at 14°C with a CHEF Mapper XA system. The run time was 48 h at 2 V/cm (70 V) with a 20- to 30-min switch time ramp at an included angle of 106°. In addition to the bacteriophage lambda ladder-PFG marker (New England Biolabs, Beverly, MA), *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomal DNAs of known size were included.

Molecular quantification by PCR. Quantification of DNA upon amplification was achieved by quantitative real-time PCR (qRT-PCR) with SYBR green. All fluorescent measurements were made with a Bio-Rad iCycler iQ instrument. The primer sequences used routinely in the laboratory for qRT-PCR were 5′GATG ATACAGGCGTGGCACTT and 3′CGATCTCGTGCGTGCGTATTC, which correspond to a region of *ml0049* unique to *M. leprae*. The thermocycler was programmed to begin with a 55°C cycle for 2 min. After the DNA was denatured at 94°C for 2 min, real-time PCR was carried out for 45 cycles, which consisted of a denaturation step at 95°C for 15 s, an annealing step starting at 60°C for 30 s, and finally, an extension at 72°C for 45 s, with a final extension at 72°C for 5 min. The final extension step at 72°C for 5 min was included in the thermocycling protocol to allow gel electrophoresis (2% agarose gel) and melting curve analysis from a single locus. The fluorescence was monitored throughout the thermocycling protocol, and all data collected were analyzed by using the accompanying analysis software (version 7.1.1.1).

STR locus analysis. In order to determine the fidelity of the amplification process at the nucleotide level, six short tandem repeat (STR) loci identified in coding and noncoding regions of the *M. leprae* genome (http://genolist.pasteur.fr/Leproma/) were evaluated as described previously (6). The PCR mixture (23 μl) consisted of 20 μl Platinum PCR SuperMix (Invitrogen Corporation, Carlsbad, CA), 1 μl of both 10 μM primer stocks, and 1 ng of genomic DNA template. After denaturation of the DNA at 94°C for 1 min, PCR was carried out for 35 cycles, which consisted of a denaturation step at 94°C for 30 s, an annealing step starting at 65°C for 30 s (incrementally decreasing every subsequent cycle by 1°C until 55°C was reached), and finally, an extension at 72°C for 30 s, with a final extension at 72°C for 5 min in a thermocycler. Visualization was performed by 2% agarose gel electrophoresis.

Analysis of genome coverage and amplification bias by use of microarray hybridization technology. Whole-genome DNA-based *M. leprae* arrays were used to evaluate total genome coverage and the amplification bias for each generation of amplified genomic DNA. For this analysis, we printed and used an *M. leprae*-specific oligonucleotide-based microarray that represented a total of 2,747 genes consisting of 1,614 coding sequences and 1,133 pseudogenes annotated as described by the Pasteur Institute on the Leproma website (http://genolist.pasteur.fr/Leproma/). The microarrays were printed on commercially available polylysine activated glass slides with SMP3 printing pins and with each oligonucleotide at a concentration of 40 μM. The printed slides were postprocessed by the postprocessing protocol of Brown and colleagues (http://cmgm.stanford.edu/pbrown/protocols/index.html). Briefly, the arrays were rehydrated by exposure to humidity and snap dried on a hot plate, followed by UV cross-linking at 65 mJ. Following cross-linking, the slides were blocked by subjecting them to blocking solution consisting of 15 ml 1 M sodium borate (pH 8.0) and 6 g succinic anhydride in 350 ml 1-methyl-2-pyrrolodinone. After 15 min, the slides were removed and washed in 95°C water and then in 95% ethanol for 1 min. The slides were dried by centrifugation. Template DNA (2 μg), 15 μg of N^6 primers, and reaction buffer were combined and diluted to a final volume of 41 μl and heated to 95°C for 5 min. The mixture was cooled to 4°C and combined with a 1.2 mM dATP, dCTP, and dGTP and 0.6 mM dTTP mixture; 3 μl of a 1 mM stock of either Cy5-dUTP or Cy3-dUTP dye; and 50 U of the Klenow fragment, which resulted in a final reaction volume of 50 μl that was allowed to incubate at 37°C for 2 h. After the labeling was complete, the reaction was stopped by adding 5 μl of 0.5 M EDTA; and the mixture was filtered through a Micron-30 filter into a clean tube and lyophilized. The probes were resuspended with 24 μl of hybridization buffer consisting of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1% SDS. Three microliters of a 5 mg/ml tRNA stock was added to each reaction mixture. The probe mixture was heated to 95°C for 3 min and snap cooled on ice for 30 s. Hybridization was carried out in a 42°C water bath for 16 h. Upon completion, the slides were washed with low-stringency wash buffer containing 1× SSC and 0.2% SDS, then with high-stringency wash buffer containing 0.1× SSC and 0.2% SDS, and finally, with 0.1× SSC. The slides were dried by centrifugation and were scanned immediately after completion of the hybridization. The slides were scanned by using a VersArray chip reader (Bio-Rad), and SpotFinder software (TIGR) was used for spot quantification and filtering. Global normalization of each feature was performed across each channel and across each experiment. The normalized intensities were used to calculate a feature index based on the average intensity of the channel. Concordance analysis was performed based on the index for each feature per data set by using Analyze-It software (Analyze-It Software, Ltd., Leeds, United Kingdom).

RESULTS

Isothermal amplification of genomic DNA. Genomic DNA isolated from well-characterized *M. leprae* clinical strains Thai-53, NHDP-63, and NHDP-98, which were grown in vivo in armadillos, was used to evaluate and standardize serial whole-genome isothermal amplification for the production of genomic DNA from complex biological samples. Since genomic DNA isolated from bacteria obtained from in vivo sources is limited in quantity due to variability in recoveries, template DNA was quantified by qRT-PCR to determine the effects of different amounts of starting material on product yield. Amplification reactions with greater than 1 pg genomic DNA were found to yield similar amounts of high-molecular-weight material, while amplifications that were initiated with less than 1 pg of genomic

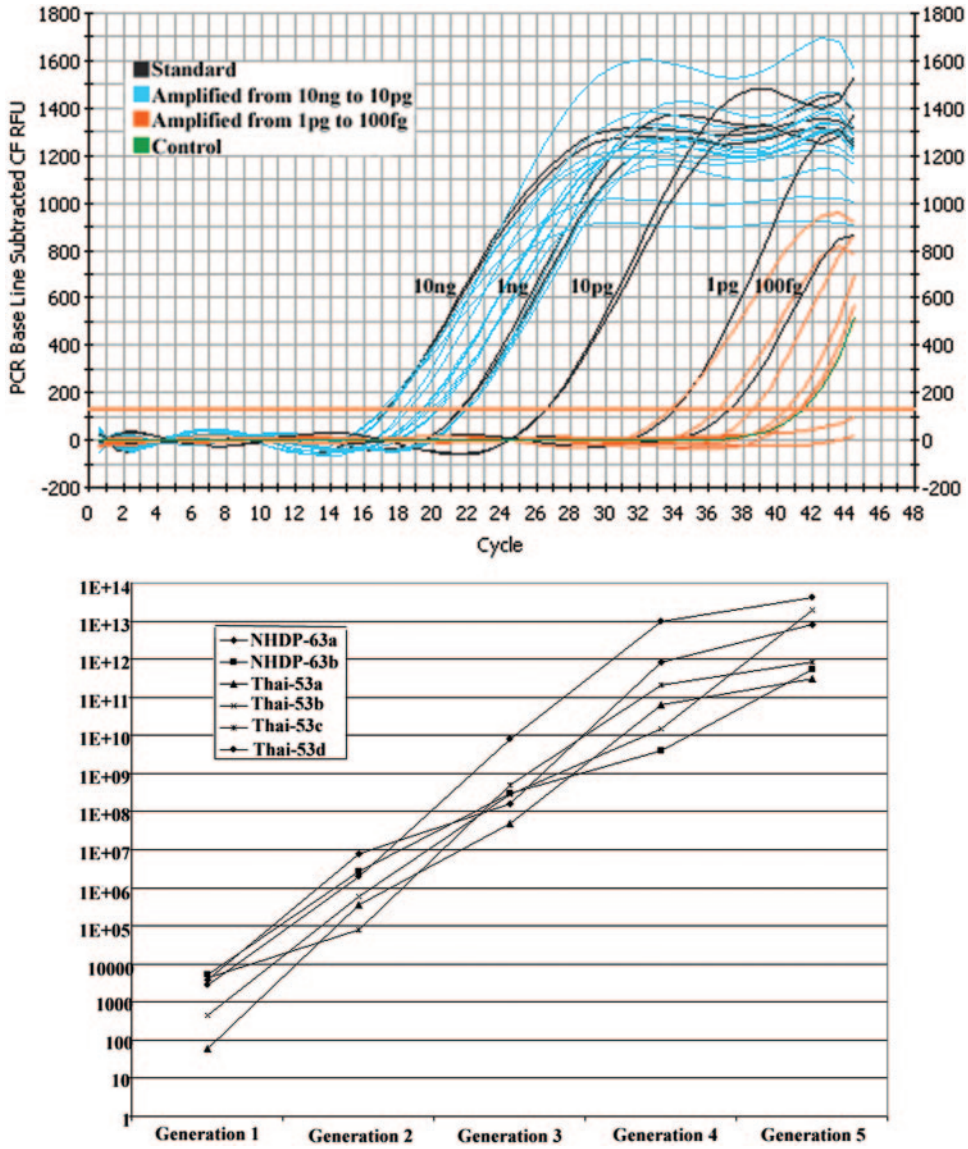

FIG. 1. Determination of the lower limit of *M. leprae* genomic material required for amplification, amplification kinetics, and total amplification yield. (Top) Quantification of isothermal amplification genomic material. qRT-PCR was performed directly from amplified genomic material and was initiated by using different amounts of starting template from 10 pg to 10 ng. CF RFU, curve fit relative fluorescence unit. (Bottom) Total DNA material yield over five serial amplifications. The linear increase in the amount of genomic material as a result of serial amplification is shown. Data represent the results of independent duplicate experiments with DNA obtained from three different *M. leprae* strains.

DNA template demonstrated a dramatic decline in overall amplification yield (Fig. 1, top). To ensure that the total DNA yield was the result of whole-genome amplification rather than the effects of polymerase on specific regions of the genome, the kinetics of serial DNA synthesis over five consecutive amplifications was further evaluated by quantitative real-time PCR. As expected, DNA produced from isothermal amplification was linear, which in theory could yield a greater than 1×10^{13}-fold increase in DNA after just five reactions (Fig. 1, bottom). However, it should be noted that the yield from the fifth amplification was consistently lower than the average from the prior amplifications.

Since isolation and purification of *M. leprae* genomic DNA from biopsy samples are harsh processes that result in much fragmentation, the consequences of low-molecular-weight genomic DNA on the overall amplification yield was evaluated. High-molecular-size DNA is considered to have an estimated electrophoretic mobility of up to ~300 kb compared to that of low-molecular-size DNA, which is considered DNA with an estimated molecular size of less than 25 kb. In general, the amplification efficiencies for high- and low-molecular-size DNA were similar. However, our studies consistently demonstrated amplification of high-molecular-size DNA on the order 6×10^3- to 8×10^3-fold, whereas low-molecular-size DNA yielded from 2×10^2- to 2×10^3-fold amplification. Although various sources of genomic DNA led to different amplification yields, the overall average yield was $\sim 2 \times 10^3$-fold.

A potential artifact of whole-genome amplification is the

magnification of small select regions of DNA, leading to fragmentation and an overall loss of high-molecular-weight DNA. In order to investigate the extent to which serial amplification leads to less high-molecular-weight genomic DNA, total DNA from five serial amplifications was subjected to gel migration analysis. No significant alterations or loss of high-molecular-weight DNA compared to the amount of unamplified DNA was detected. However, there was an increase in low-molecular-weight DNA (Fig. 2), indicating that isothermal amplification did not lead to a significant reduction in genomic material, nor was it capable of restoring high-molecular-weight DNA. Overall, the serially amplified DNA was of a predominant molecular weight similar to that of the starting template and contained lower-molecular-weight DNA species.

Amplification fidelity. Fidelity at the single-nucleotide level was evaluated by the analysis of 10 clinically relevant STR loci in strains Thai-53, NHDP-63, and NHDP-98 (Table 1). Gel migrations and sequence analysis revealed no alterations in any of the STR loci upon amplification. In order to investigate fidelity upon repeated amplifications, an analysis of at least seven STR loci from five serial amplifications was performed (Table 2). No polymorphisms were detected in any of the loci analyzed after five serial amplifications, based on gel migration patterns and comparison to the native sequence, confirming the fidelity of serial whole-genome amplification at the single-nucleotide level. Consequently, consecutive amplification enables STR locus examination and molecular epidemiology analysis from archived clinical samples with limited starting material for a wide array of markers.

An analysis of macrogenic loci distributed equally around the genome was performed to determine if there were gross genome rearrangements as a result of serial amplification. Each locus examined from the serial amplifications of Thai-53, NHDP-63, and NHDP-98 DNA was found to be identical to the others, indicating that local genomic loci were preserved through each amplification step and that there were no large genomic rearrangements due to repeated priming and amplification (data not shown). Together, these analyses did not reveal any mutations, deletions, slip-strand alterations, or genomic rearrangements in the DNA derived from the three independent strains that could be attributed to isothermal amplification.

Evaluation of genome coverage and amplification bias by microarray analysis. To establish if DNA microarray technology provides an accurate means for the identification of amplification bias across a whole genome, native purified *M. leprae* genomic DNA was labeled and hybridized. Analysis of independent technical replicates revealed a high similarity (average $R^2 = 0.92$), proving that the microarray technology is an efficient means of identifying amplification alterations. To validate the application of serial isothermal amplification for generating whole-genome genetic material for molecular epidemiology studies, DNA from five serial amplifications was screened in independent microarray experiments. Linear regression analysis of the first amplification and comparison to that of unamplified material indicated that while the majority of the genome was amplified equivalently, some regions were amplified better than others (average $R^2 > 0.56$). Analysis of the DNA material generated from each consecutive amplification showed higher concordance, with R^2 values ranging from 0.77 to 0.59 for amplifications 1 to 4 (Table 3). The concordance for the fifth amplification was lower, in general, with R^2 values ranging from 0.70 to 0.35. Kendall tau analysis of unamplified and amplified DNA material from each serial amplification revealed that ~56% of the genome features were similarly amplified. Comparison of reference DNA to DNA from the other serial amplifications showed a similar trend, with from ~50 to 66% of the material amplified for amplifications 2 through 5 (Table 3). Knowing that the quality of the DNA isolated from *M. leprae* is generally poor, the relatively high concordances ($R^2 = 0.77$ to 0.59) observed in this study illustrate that isothermal amplification is uniquely suited for use in these applications. It has previously been reported that concordances in amplification of DNA were as low as <0.1, depending on the source of DNA and the extent of degradation (24).

Percent genome distribution was determined from the average fluorescent intensity values of each gene for unamplified DNA and DNA from each serial amplification. Selective genomic amplification was the greatest for the first serial amplification, at ~10% of the total genome, and was lowest for the fifth serial amplification, at ~2.5%, further supporting the notion that although slight changes occur, they are limited in the overall impact of total genome coverage and bias over a series of amplifications.

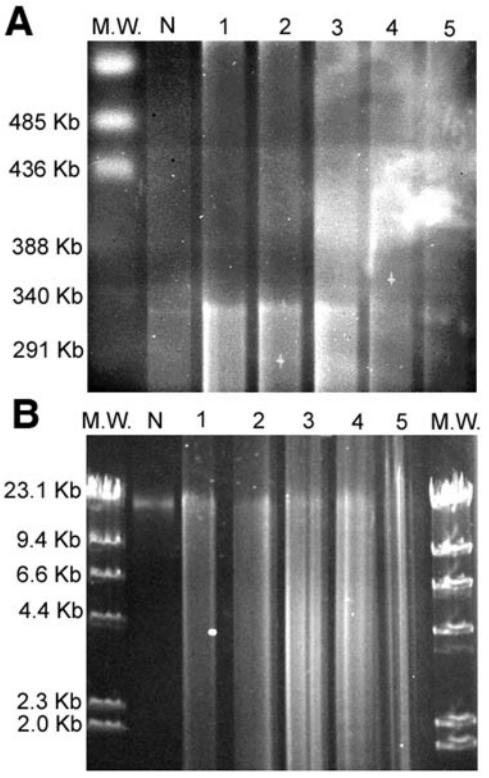

FIG. 2. Electrophoretic migration of amplified genomic DNA from *M. leprae* sources. (A) Contour-clamped homogeneous electric field analysis of high-molecular-weight genomic DNA and (B) agarose gel analysis of low-molecular-weight genomic DNA for native material (lanes N) and each of five serial amplifications (lanes 1 to 5, respectively). Lanes M.W., molecular size markers. Contour-clamped homogeneous electric field analysis was performed with 0.8% certified megabase agarose-TAE gel, and agarose gel analysis was performed with 0.7% certified molecular biology-grade agarose.

TABLE 1. Analysis of STR locus instability in three characterized *M. leprae* strains[a]

Strain	Origin	STR locus[a]	Locus stability (no. of copies)[b]		
			A	B	C
Thai-53	Thailand	GTA-9	9	9	9
		TTC-21	14	14	14
		27-5	5	5	5
		21-3	3	3	3
		12-5	5	5	5
		AT-17	10	10	10
		TA-18	13	13	13
		6-7	6	6	6
		C-20	11	11	11
		AC-9	9	9	9
NHDP-63	Louisiana	GTA-9	10	10	10
		TTC-21	9	9	9
		27-5	4	4	4
		21-3	2	2	2
		AT-17	13	13	13
		C-20	15	15	15
		AC-9	8	8	8
NHDP-98	Mexico	GTA-9	9	9	9
		TTC-21	9	9	9
		27-5	4	4	4
		21-3	2	2	2
		12-5	5	5	5
		AT-17	11	11	11
		TA-18	12	12	12
		6-7	7	7	7
		C-20	13	13	13

[a] Genomic DNA was obtained from *M. leprae* strains Thai-53, NHDP-63, and NHDP-98.
[b] STR locus stability was calculated by comparison of the STR locus sequence to the known sequence (A), the sequence obtained directly from unamplified DNA (B), and the sequence obtained from amplified DNA (C).

Molecular typing of bacteria from archived samples. To examine whether isothermal amplification is appropriate for application in error-sensitive epidemiology approaches, seven archived samples were processed, amplified, and subjected to microsatellite analysis. Because of the overall low abundance of bacteria in the samples and their degraded nature due to long-term storage, isolation of bacteria directly from the tissue was not performed. Rather, total DNA was isolated from the entire sample, resulting in a complex mixture of bacterial and host DNA. The STR loci chosen for analysis of the clinical isolates were GTA-9 and TTC-21 (6, 9, 10, 20, 23, 25). GTA-9 was chosen because it is a well-established microsatellite used for the genotyping of *M. leprae* species (6, 10, 23). It is also known to be very stable and therefore provides a good reference for analysis of other more polymorphic STR loci (6, 10, 23). TTC-21 was chosen because it is a more polymorphic STR locus and is routinely used for genotyping based on that fact (6, 9, 10, 20, 23, 25). In addition, it is thought that TTC-21 is more susceptible to alteration based on sample preparation methods and thus is an ideal typing locus for the present purposes.

In general, the total DNA recovered for each sample was found to be complex and highly degraded, as determined by electrophoretic migration on agarose gels (data not shown). It was either directly analyzed or isothermally amplified prior to analysis. Of the seven biopsy samples directly examined, 12 of 13 analyses failed (Table 4), presumably due to the low abundance of bacterial DNA (17). After isothermal amplification, analysis was possible for nine of the previous failed reactions, resulting in an improvement of failure rate from 92% to 25% (Table 4). As expected, the STR loci examined showed no instability. Clearly, isothermal amplification is applicable to DNA obtained directly from archived biopsy samples for molecular genotyping approaches.

TABLE 2. Analysis of STR loci stability through five serial amplification reactions

Strain	Origin	STR locus[a]	Locus stability (no. of copies)[b]					
			N	G1	G2	G3	G4	G5
Thai-53	Thailand	TTC-21	14	14	14	14	14	14
		27-5	5	5	5	5	5	5
		21-3	3	3	3	3	3	3
		12-5	5	5	5	5	5	5
		AT-17	10	10	10	10	10	10
		C-20	11	11				
		AC-9	9	9				
NHDP-63	Louisiana	TTC-21	9	9	9	9	9	9
		27-5	4	4	4	4	4	4
		21-3	2	2	2	2	2	2
		AT-17	13	13	13	13	13	13
		C-20	15	15	15	15	15	15
		AC-9	8	8	8	8	8	8
NHDP-98	Mexico	TTC-21	9	9	9	9	9	9
		27-5	4	4	4	4	4	4
		21-3	2	2	2	2	2	2
		12-5	5	5	5	5	5	5
		AT-17	11	11	11	11	11	11
		C-20	13	13				

[a] Genomic DNA obtained from *M. leprae* strains Thai-53, NHDP-63, and NHDP-98 was subjected to five serial amplifications.
[b] The sequences of six highly polymorphic STR loci of unamplified material (N) were compared to the sequences of synthetic material from generation 1 (G1), generation 2 (G2), generation (G3), generation 4 (G4), and generation 5 (G5).

DISCUSSION

While molecular typing approaches are commonly used for epidemiological studies for many pathogens, molecular geno-

TABLE 3. DNA microarray and concordance analysis of serial amplified genomic material[c]

Material[d]	Concordance					
	N	G1	G2	G3	G4	G5
N		0.56[a]	0.55[a]	0.44[a]	0.55[a]	0.66[a]
G1	0.56[b]		0.62[a]	0.58[a]	0.58[a]	0.55[a]
G2	0.46[b]	0.66[b]		0.70[a]	0.63[a]	0.46[a]
G3	0.37[b]	0.59[b]	0.77[b]		0.66[a]	0.43[a]
G4	0.52[b]	0.59[b]	0.67[b]	0.71[b]		0.61[a]
G5	0.70[b]	0.53[b]	0.39[b]	0.35[b]	0.59[b]	

[a] Kendall tau coefficients calculated from normalized fluorescent intensities ($P < 0.0001$).
[b] R^2 values calculated from the rank index order of normalized fluorescent intensities (99% confidence interval).
[c] All values were calculated from at least two independent experiments for 1,043 features.
[d] N and G1 to G5 are as defined in footnote *b* of Table 2.

TABLE 4. Analysis of STR locus instability of *M. leprae* clinical strains obtained from archived samples

Clinical isolate[a]	STR locus	Locus stability (no. of copies)[b]		
		A	B	C
3044/323	GTA-9	10	10	
	TTC-21			
3045/336	GTA-9	10		10
	TTC-21	15		15
3044/329	GTA-9	11		11
	TTC-21	15		15
3045/334	GTA-9	10		10
	TTC-21	15		15
3044/325	GTA-9	11		
	TTC-21	15		
3044/333	GTA-9	11		11
	TTC-21	15		15
3030/267	GTA-9	11		
	TTC-21	15		15

[a] All isolates were from India.
[b] STR locus stability was calculated by comparison of the STR locus sequence to the genome sequence (A), the reference obtained directly from unamplified DNA (B), and the sequence obtained from amplified DNA (C).

typing is less routine for obligate intracellular pathogens such as *M. leprae*. A necessity of these technologies is the availability of ample amounts of bacterial genomic DNA derived from clinical samples. Since *M. leprae* cannot be cultivated in vitro, the collection of DNA from clinical sources is difficult. This attribute of *M. leprae* alone prevents routine large-scale epidemiology studies. In addition, the recovery of bacterial DNA from the vast numbers of archived biopsy samples for evolutionary studies is made even more difficult by the variety of storage conditions encountered. These facts make molecular epidemiology and postperspective studies of leprosy nearly impossible. To address this need for an efficient and reliable method for the amplification of whole bacterial genomic material, we have developed an isothermal amplification approach applicable to error-sensitive sequence-based genotyping techniques.

This study is the first to demonstrate that multiple-primed, isothermal amplification is a useful approach for the consistent serial amplification of bacterial genomic DNA. The fidelity of the process was determined by evaluating the instability of the STR loci, which are known to be highly variable and likely subject to slip-strand replication errors. The analysis of these sites revealed that isothermal amplification did not introduce errors, a point further confirmed by direct sequencing of amplified products generated from serially amplified DNA. Neither of these methods detected replication mistakes. Our findings are consistent with the low error rates reported by other laboratories (7, 8, 14). The use of amplification approaches often leads to low genome coverage due to priming bias (5, 14). However, whole-genome hybridization analysis by using microarrays of DNA from serial amplifications revealed concordance among the amplified samples; a large percentage of the genome was amplified in a linear fashion without bias over five serial amplifications.

To determine whether the use of isothermal amplification to amplify bacterial genomic material in the presence of host DNA is appropriate for error-sensitive epidemiology studies, we investigated STR locus stability in isolates obtained from biopsy samples, which are complex biological samples. No evidence of polymorphisms that could be attributed to amplification fidelity was detected. More importantly, however, isothermal amplification enhanced the efficiency of routine PCR-based genotyping procedures. Preisothermal amplification increased the success rate of strain typing from ~25% to 92%, emphasizing that the inclusion of isothermal amplification significantly improves current genotyping procedures.

The need to move beyond the analysis of small subsets of loci in the future will require the ability to amplify genomic material with a high fidelity in a nonbiased fashion, thus allowing a comparison of organisms on a whole-genome level rather than on the basis of a few genomic features. Isothermal amplification will be able to complement emerging advances in whole-genome high-throughput sequencing technologies. Another potentially clinically important application of whole-genome amplification is for the routine preparation of clinical samples for genotyping. It is now recognized that during the development of infection and the progression of disease, bacterial populations contain numerous phenotypically and/or genotypically diverse bacterial groups (18, 21, 22). The regrowth of bacteria from clinical samples before genotyping could potentially lead to the inadvertent selection of a subpopulation of the bacteria. Subsequent genotyping would not fully represent the genotype of the bacterial population involved in the existing disease state.

Overall, this study has demonstrated that serial isothermic amplification of genomic DNA is a simple and robust approach to the amplification and the generation of representative DNA in a serial manner that can be used for error-sensitive genomic studies, including strain typing and evolutionary mapping. This method is particularly useful for amplification of genomic material from noncultivable, intracellular pathogens and may be used to investigate the gene copy numbers or the genotypes of these genomic material sources. It should be considered a routine genomic method for amplifying whole genomic DNA from archived samples for sequence-based applications, such as comparative genomics and molecular epidemiology.

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