Lack of Insertional-Deletional Polymorphism in a Collection of *Mycobacterium ulcerans* Isolates from Ghanaian Buruli Ulcer Patients[▽]

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Mycobacterium ulcerans causes the devastating infectious skin disease Buruli ulcer and has a monomorphic population structure. The resolution of conventional genetic fingerprinting methods is therefore not sufficient for microepidemiological studies aiming to characterize transmission pathways. In a previous comparative genomic hybridization analysis with a microarray covering part of the *M. ulcerans* genome, we have found extensive insertional-deletional sequence polymorphisms among *M. ulcerans* isolates of diverse geographic origins that allowed us to distinguish between strains coming from different continents. Since large numbers of insertion sequences are spread over the genome of African *M. ulcerans* strains, we reasoned that these may drive large sequence polymorphisms in otherwise clonal local mycobacterial populations. In this study, we used a printed DNA microarray covering the whole genome of the Ghanaian *M. ulcerans* reference strain Agy99 for comparative genomic hybridization. The assay identified multiple regions of difference when DNA of a Japanese *M. ulcerans* strain was analyzed. In contrast, not a single insertional-deletional genomic variation was found within a panel of disease isolates coming from an area of Ghana where Buruli ulcer is endemic. These results indicate that, despite the expectations deduced from other mycobacterial pathogens, only analyses of single nucleotide polymorphisms will have the potential to differentiate local populations of *M. ulcerans*.

Buruli ulcer (BU) is a severe skin disease characterized by large ulcerative lesions, often leading to deformity and the crippling of extremities. While this neglected tropical disease occurs in more than 30 countries worldwide, it currently has its major focus in West Africa, where the prevalence is greater than that of leprosy, and incidence rates still may be underestimated (61). The social impact of BU endemicity in rural settings of Africa, where children are most affected, is dramatic. Patients often have only limited access to health care and tend to report to health care facilities at advanced stages of disease (20, 49). BU is often concentrated in areas close to stagnant or slow-moving waters. The mode of transmission is not fully understood (13, 36, 44–46, 59, 60), partly because conventional molecular fingerprinting methods have not sufficiently high resolution for microepidemiological analyses (1–4, 10, 11, 23, 24, 27, 33, 51, 53, 55–57).

Mycobacterium tuberculosis and related pathogenic mycobacteria exhibit major large sequence polymorphism (LSP)-based interstrain genomic variations (5, 9, 12, 34, 58). From these genomic insertional-deletional (InDel) markers, evolutionary scenarios were drawn for *M. tuberculosis*, the *M. tuberculosis* complex, *Mycobacterium bovis*, the *M. bovis* BCG vaccine strains, and mycolactone-producing mycobacteria related to

Mycobacterium ulcerans (6–8, 25, 27, 38, 50). In recent years, proposed phylogenies were confirmed and refined by whole-genome sequence analysis identifying single nucleotide polymorphisms (SNPs) (16, 17, 21, 22). Combinations of all identified genomic polymorphisms are currently being used for studying mycobacterial evolution and transmission pathways, helping in the prevention of epidemics (34, 37).

Comparative genomic hybridization (CGH) analysis of *M. ulcerans* clinical isolates of diverse geographic origins also revealed extensive LSPs (47). The identified transposable element-associated InDel recombination events are indicative of progressive genome shrinking in *M. ulcerans*, which has emerged from the environmental mycobacterium *Mycobacterium marinum* by acquisition of a large virulence plasmid (52). An in-depth comparison of the recently published *M. ulcerans* genome of strain Agy99 to the progenitor species strain *M. marinum* M revealed LSPs, named *M. ulcerans* regions of difference (MURDs) (54). Analysis of large InDel polymorphisms allowed us to distinguish between two distinct lineages: (i) the "classical" lineage representing the most pathogenic genotypes, consisting of those that come from Africa, Australia, and Southeast Asia; and (ii) an "ancestral" lineage comprising strains from China and Japan, South America, and Mexico (29). A total of 209 copies of the insertion sequence (IS) IS*2404* and 83 copies of IS*2606* have been found interspersed in the genome of the *M. ulcerans* reference strain Agy99 from Ghana (54). Due to achievements using LSPs in other mycobacterial pathogens, we reasoned that this high abundance of transposable elements may destabilize the gen-

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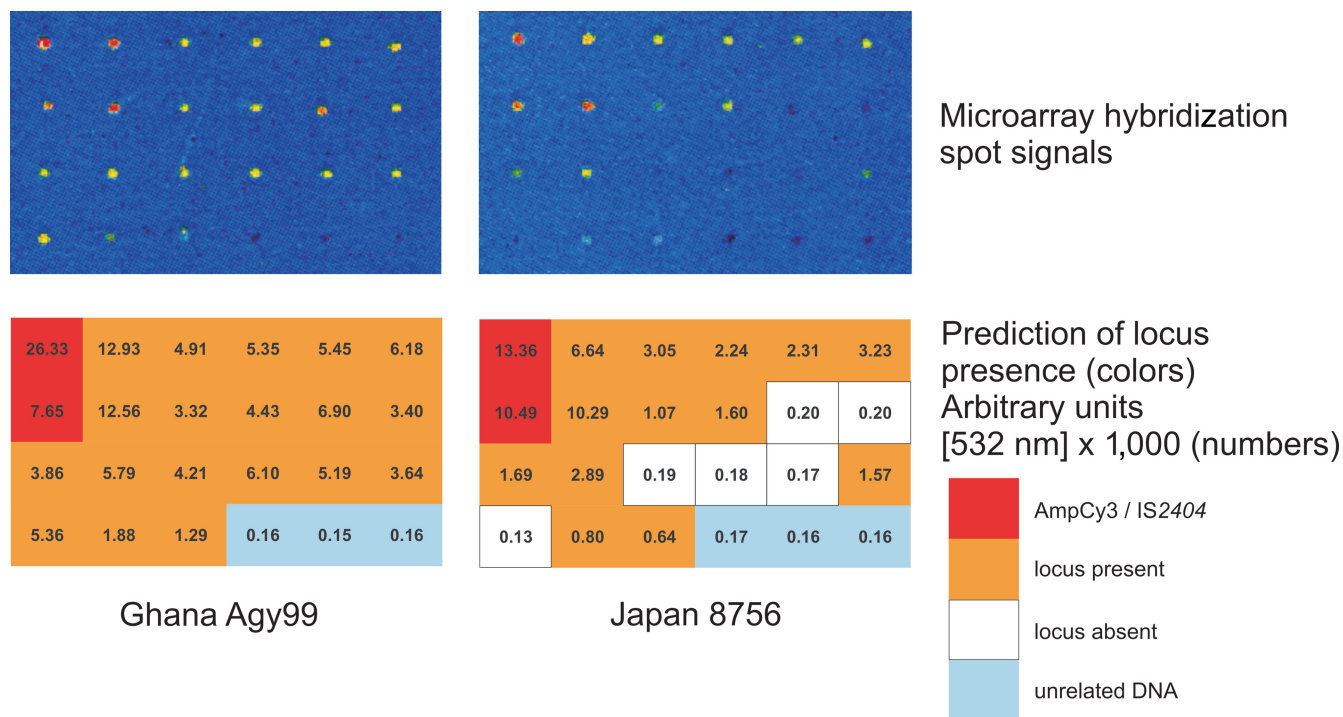
FIG. 1. Setup of comparative genomic hybridization conditions. PCR products sized between 0.6 and 2.3 kb in length were selected to establish spotting and hybridization conditions with the required sensitivity and reliability. PCR products comprising a β-lactamase fragment linked with Cy3 or DNA stretches of IS2404 and IS2606 present in all *M. ulcerans* strains (red) and DNA regions known to be present in both *M. ulcerans* strains Ghana 970483 and Japan 8756 (orange) served as positive controls. PCR products of DNA from unrelated species (*N. meningitidis* and *P. falciparum*; light blue) and regions known to be absent from strain Japan 8756 only (white) were used as negative controls.

eral architecture of the genome of African *M. ulcerans* strains, resulting in a rapid accumulation of LSPs in otherwise monomorphic populations of the pathogen. To assess this experimentally, we performed CGH analyses using a comprehensive whole-genome microarray. Specifically, we tried to identify LSPs within a panel of *M. ulcerans* strains isolated from the BU lesions of 37 patients from several districts in Ghana between the years 2002 and 2004.

MATERIALS AND METHODS

PCR product-based DNA microarray. A shotgun clone library of *M. ulcerans* strain Agy99 DNA, comprising 1,536 *Escherichia coli* plasmids (pCDNA2.1; Invitrogen, Basel, Switzerland) was used, with each plasmid containing an *M. ulcerans* fragment of ~2.3 to 2.7 kb (54). Plasmids were selected so that the contigs covered the entire genome at least once, with particular regard to all coding sequences (CDSs), whereas sequence redundancies were excluded. Given a genome size of 5.8 Mb and after exclusion of redundant fragments, this set of plasmid inserts represents a theoretical coverage of >75% of the *M. ulcerans* genome. Plasmid DNA was prepared by using a Biomek 2000 Workstation (Beckman Coulter, Krefeld, Germany) and dissolved at a concentration of 150 ng/μl in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). For all plasmids, PCR was performed using primers pCDNA2_F (5′-TAAAACGACG GCCAGTGAAT-3′) and pCDNA2_R (5′-CGAGCTCGGATCCACTAGTAA-3′), with binding in the multiple cloning sites outside of the inserted regions. PCR products were checked by gel electrophoresis using 1% agarose gels, purified using a MinElute 96 ultracentrifugation PCR purification kit (Qiagen, Hombrechtikon, Switzerland), and dissolved at a concentration of 400 ng in 3× SSC. The resulting 1,520 PCR products were loaded on a nanoliter dispenser printhead in a 96-well format (Topspot, Biofluidix, Freiburg, Germany). A piezo-actuator-driven pressure pulse was applied for spotting of 1-nl droplets onto glass slides coated with poly-L-lysine (Superfrost Plus, Menzel, Braunschweig, Germany). The spots had an average diameter of 150 μm, with a distance of 500 μm

to each other, and were arranged in "standard packing" onto the arrays for establishing and evaluating hybridization conditions (Fig. 1). In the final microarray, arranging spots in "orange packing" allowed for a microarray layout of 1,520 spots in triplicates on one glass slide. The microarray slides were incubated at 4°C overnight and under 60% humidity for 1 h in an incubator, and DNA was fixed by exposure to UV light at 650 mJ in a Stratalinker 2400 (Strategene, La Jolla, CA). Controls spotted included 3× SSC buffer only, a β-lactamase gene fragment labeled with either Cy3 (spotting control) or biotin (colorization control), PCR products comprising IS2404 and IS2606 stretches (hybridization controls), and PCR products from the unrelated species *Neisseria meningitidis* and *Plasmodium falciparum* (nonhybridization controls).

Bacterial strains. *M. ulcerans* strains were isolated from lesions of BU patients as described earlier (62). Isolates used for microarray hybridization were as follows: Agy99; Japan 8756; strains NM01/03, NM14/02, NM18/02, NM21/02, NM23/02, NM27/02, NM28/02, NM32/02, NM33/02, NM34/02, NM40/02, NM41/02, NM43/02, NM44/02, NM46/02, NM47/02, NM49/02, NM50/02, NM51/02, NM52/02, NM54/02, NM56/02, NM59/02, NM60/02, NM61/02, NM62/02, NM68/02, NM69/02, NM72/03, NM89/03, and NM90/03 from the Greater Accra region, Ghana; strains NM22/02, NM37/02, and NM38/02 from the Ashanti region, Ghana; and strains NM42/02 and NM53/02 from the Eastern Region, Ghana (for a distribution map see reference 24). DNA from clinical isolates *M. marinum* M (ATCC BAA-535 from LGC Standards; http://www.lgcstandards-atcc.org /ATCCCulturesandProducts/tabid/979/Default.aspx), *N. meningitidis* N3221, and *P. falciparum* 3D7 were used as controls.

DNA extraction and biotinylation of *M. ulcerans* genomic DNA fragments. Genomic DNA was extracted from bacterial pellets using an optimized method for mycobacterial DNA preparation (30). Bacterial pellets of about 20 mg (wet weight) were heat inactivated for 1 h at 95°C, followed by cell wall disruption and digestion. DNA was extracted from the supernatants by phenol-chloroform (Fluka, Buchs, Switzerland) extraction and subjected to ethanol precipitation as described previously (30). DNA was measured by the optical density at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher, Waltham, MA). Digestion and biotinylation of genomic DNA were optimized to yield high-quality labeling as follows: 1 μg of genomic DNA was digested with 8 U of

Sau3AI (New England Biolabs, Hitchin, United Kingdom) in a volume of 60 μl, incubated for 75 min at 37°C, and ethanol precipitated, followed by measurement of the DNA concentration. Of the digested DNA, 250 ng was biotinylated using a BioPrime DNA Labeling System (Invitrogen, Basel, Switzerland) for 7 h at 37°C, ethanol precipitated, and eluted in water. DNA concentration measurement showed average yields of 25 μg of biotinylated DNA.

Hybridization and colorization of microarray slides. Hybridization was done under identical conditions for all strains. Nine nanograms of biotinylated DNA was mixed with 16.5 μg of human Cot-1 DNA (Roche Applied Science, Indianapolis, IN) and 90 μg of yeast (*Saccharomyces cerevisiae*) tRNA (Gibco/BRL, Gaithersburg, MD). The hybridization mix was concentrated with a Speed Vac Concentrator System (Eppendorf, Basel, Switzerland), resuspended in 20 μl of hybridization buffer (30% formamide, 5× SSC, 0.1% sodium dodecyl sulfate, 100 ng/μl double-stranded herring sperm DNA), denatured for 2 min at 95°C, and applied to the preheated (5 min at 95°C) microarray slide. Following hybridization at 60°C for 15 h, slides were washed once with 2× SSC–0.1% sodium dodecyl sulfate for 5 min at 60°C, three times with 0.1× SSC for 5 min at room temperature, and twice with 0.01× SSC for 10 min at room temperature. The colorization step was performed with 2 ml of staining solution containing 50% caseine (Roche Applied Science, Basel, Switzerland), 1× maleic acid buffer (Roche Applied Science, Basel, Switzerland), and 2 μl of streptavidin Cy3 fluorolink (Amersham, Piscataway, NJ) for 30 min at room temperature under slow agitation, followed by additional washing steps (twice with 0.1× SSC for 5 min, twice with 0.01× SSC for 5 min, and once with water) before slides were dried by centrifugation at 900 × *g* for 1 min.

Microarray scanning and data evaluation. Images of the microarrays were retrieved using the laser scanner GenePix 4100 A (Axon Instruments Inc., Foster City, CA) with an excitation wavelength of 532 nm, an emission wavelength of 570 nm, and standardized measurement parameters (laser power of 100%; photomultiplier tube gain of 600). The resulting images were analyzed using GenePix Pro, version 6.0, software (Axon Instruments Inc., Foster City, CA) for background subtraction and calculation of spot intensities (fluorescence signal intensity units). A total of 170 spots with insufficient amounts of DNA spotted were excluded, as were 7 spots for which the coefficient of variation in Agy99 was >30% between the replicates. Control strains (Agy99 and *M. marinum* M) were hybridized in duplicates, yielding six values per spot (each), and test strains yielded three values per spot. For each spot, the background signal was subtracted, and mean values were determined. To equalize values across hybridization experiments, the values were normalized for the fluorescence signal of a spotted control DNA on each array. In order to determine outlier fluorescence signals that are much lower than signals in the reference strain or absent from the reference strain, we calculated fractions for each spot as follows: number of fluorescence units of Agy99/number of fluorescence units of the test strain. Thus, spots with signal intensities similar to the reference DNA gave values close to 1, whereas spots with no or weak fluorescence signals in the test strain resulted in high fluorescence intensity ratios. These outlier signal ratios suggested absent genomic regions compared to the Agy99 genome. Raw data were sorted and visualized as outlined (see the legend of Fig. 2) using the data computational program Spotfire (Spotfire DecisionSite FunctionalGenomics; Tibco Spotfire, Göteborg, Sweden).

Verification of microarray data. Spots that indicated the presence of a deletion compared to strain Agy99 were analyzed for verification. First, the spotted genome locus was identified by sequencing of the PCR products spotted on the respective positions using primers pCDNA_F and pCDNA_R. Second, the defined region was identified on the Agy99 genome sequence (BuruList server; http://genopole.pasteur.fr/Mulc/BuruList.html), and primers complementary to these regions were designed that allowed scanning of the respective DNA stretch in steps of a maximum of 1 kb. Using these primer sets, a test PCR and sequencing were performed for each strain detected by the microarray data including control strains at conditions that allowed for identification of deletions as small as 50 bp, thus verifying the spot signals as true- or false-positive hits.

DNA fragment amplification and sequencing. PCR was performed using the FirePol 10× BD buffer and 0.5 μl of FirePol *Taq* polymerase (Solis BioDyne, Tartu, Estonia) with 5 ng of genomic DNA, 0.6 μM (each) forward and reverse primer, 1.7 mM MgCl₂, and a 0.3 mM concentration of each deoxynucleoside triphosphate in a total volume of 30 μl. PCRs were run in a GeneAmp PCR System 9700 PCR machine. The thermal profile for PCR amplification of *E. coli* plasmids and *M. ulcerans* genomic DNA included an initial denaturation step of 95 to 98°C for 3 min, followed by 32 cycles of 95°C for 20 s, annealing at 58 to 65°C for 20 s, and elongation at 72°C for 30 s up to 2 min. The PCRs were finalized by an extension step at 72°C for 10 min. PCR products were analyzed on 1% agarose gels by gel electrophoresis using ethidium bromide staining and an AlphaImager illuminator (Alpha Innotech, San Leandro, CA). Primers (Sigma-Aldrich, Steinheim, Germany) were designed using Primer3 software, version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequencing was performed using a Big Dye kit and an ABI Prism 310 genetic sequence analyzer (Perkin-Elmer, Waltham, MA).

RESULTS

In order to detect new LSPs in *M. ulcerans* by CGH, we developed a PCR product-based printed DNA microarray covering a large proportion of the genome of *M. ulcerans* strain Agy99. Test hybridizations were performed with spotted DNA derived from regions of difference (RDs) identified in previous studies (29, 47). Using DNA of strains differing in these target sequences, we established spotting and hybridization conditions that yielded the predicted hybridization signals in a reliable and reproducible manner (Fig. 1). CGH was performed with genomic DNA of 37 Ghanaian *M. ulcerans* isolates, one Japanese *M. ulcerans* strain representing the ancestral lineage, and the *M. marinum* strain M (Fig. 2). Seventy-five of the spotted PCR products (Fig. 2) yielded a total of 321 outlier signals that were >100-fold lower in intensity than the corresponding signal of the reference strain Agy99. Thirty-five of these were >1,000-fold lower.

None of the negative control spots showed a significant outlier signal. Instead, probes containing IS*2404*/IS*2606* fragments showed an outlier ratio in *M. marinum*, as expected due to its lack of either IS element (ISE). For *M. marinum* M, other outlier signals >1,000-fold lower than the reference were found that corresponded either to non-ISEs not present in the *M. marinum* M genome or to regions in the *M. ulcerans* plasmid pMUM001, which is also not present in *M. marinum* M. In addition, outlier signals of 100-fold intensity difference yielded two regions from within ISEs and three regions in close vicinity to either a MURD or to genome inversions between the sequences of *M. marinum* M and *M. ulcerans* Agy99, which likely results in steric hindrance of DNA-DNA hybridization. These results confirm the reliability established in the above test hybridizations.

CGH analysis of the *M. ulcerans* strain Japan 8756 yielded 11 outlier signals that were >1,000-fold lower. All of these hits were associated with deletions in the corresponding genomic loci (Fig. 3). Thereby, all previously described deletions of the Asian haplotype in the RDs 9/15, 11, and 12 (28) were traced, and five additional deletions were newly identified (Fig. 3B). PCR analyses showed that all five of these deletions are larger than 2 kb. Moreover, hits relating to the same RDs but derived from different spotted probes demonstrated the reliability of the microarray-based CGH technology. Of the spotted probes associated with outlier signals with only a 130- to 160-fold reduction in signal intensity, one was related to a new RD. Furthermore, one spotted sequence directly adjacent to MURD 87 (and thus prone to reduced hybridization) yielded outlier signals with both *M. ulcerans* Japan 8756 and *M. marinum* M DNA. The newly identified deletions in the Japanese strains affected two IS transposition genes and CDSs of the following functional categories: PE/PPE family (three CDSs), conserved hypotheticals (eight CDSs), regulatory proteins (one CDS), cell wall/cell processes (three CDSs), and intermediary metabolism/respiration (six CDSs), the latter including a cytochrome P450 and dehydrogenase cluster.
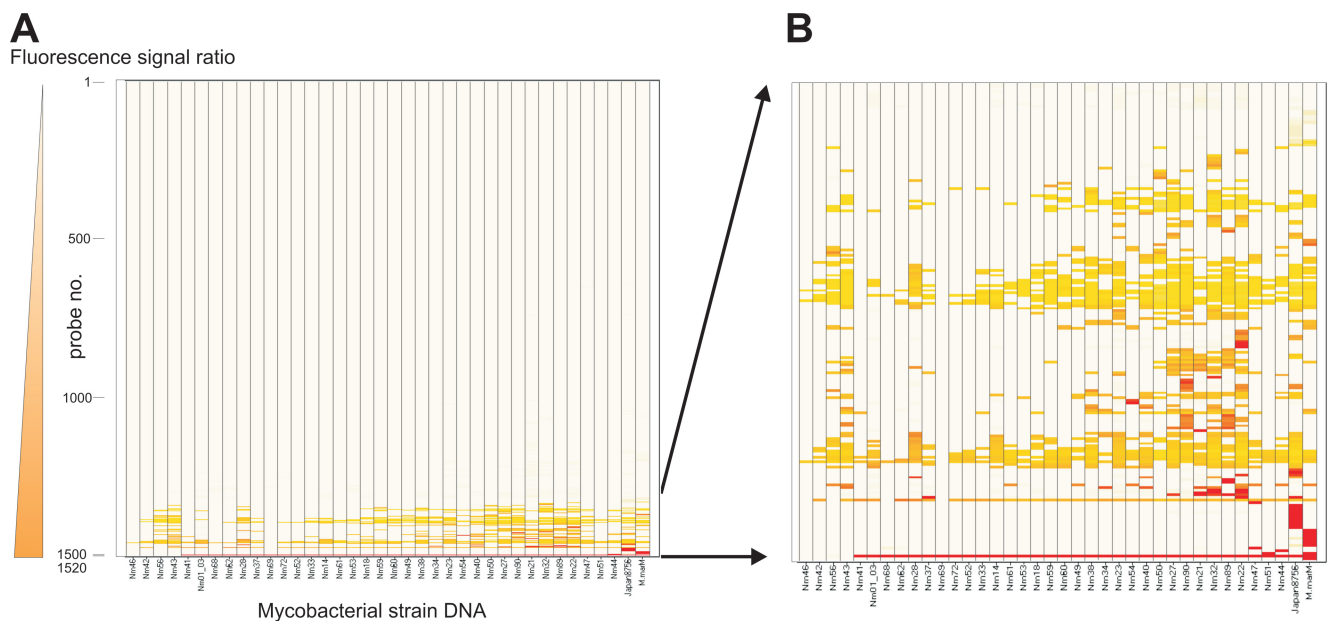
FIG. 2. Heat map representation of microarray hits. In the heat map, each row represents the fluorescence signal ratio (reference strain Agy99/test strain) of one of the 1,520 immobilized insert probes, and each column represents a mycobacterial strain DNA sample. Outlier ratios of normalized fluorescence signals were sorted using an algorithm combining both the ratio value (color ranges from light yellow for low values to dark red for high values) and the number of affected strains (Spotfire DecisionSite). Panel A shows all 1,520 spots, and panel B shows a zoomed-in version of the 75 hit-generating spots across the 37 patient isolates and control strains. M.marM, *M. marinum* M.

CGH with DNA of 37 disease isolates from Ghana resulted in 235 hits with signals >100-fold lower than the reference. These were associated with 40 of the 1,520 printed PCR products (Fig. 3A). Only 15 of the outlier signals were >1,000-fold weaker than the corresponding Agy99 signal. When the genome regions of the 37 Ghanaian patient isolates, corresponding to the 40 implicated spotted probes, were scanned by PCR for the presence of deletions, no LSP was found.

DISCUSSION

A thorough knowledge of the epidemiology of a pathogen is of great importance for public health interventions and the identification of targets for diagnostic tests and drug and vaccine development (17). Determining genetic variability is a prerequisite for genomic fingerprinting and microepidemiological analyses. In various mycobacterial species, LSPs represent robust and accurate markers for mapping of chromosomal imbalances. In *M. tuberculosis* (15, 32, 58), *Mycobacterium africanum* (40), *M. bovis* (39), the vaccine strain *M. bovis* BCG (6, 19), and in other mycobacteria (18, 35, 42, 43, 48), most known LSPs have been identified using microarray technologies. Previous identification of RDs in a collection of *M. ulcerans* patient isolates originating from different regions worldwide where BU is endemic suggested that a whole-genome microarray might lead to the identification of LSPs also at a regional level (47).

In this study, we have used the largest collection thus far of regional strains, isolated between 2002 and 2004 from patients living in different districts of Ghana, including the Greater Accra, the Ashanti, and the Eastern regions. It was thought that such a panel of strains would show LSPs in a screening of the whole genome. Based on the genomic versatility discovered in other mycobacterial species (5, 9, 12, 34, 58), it was commonly assumed that LSPs would provide the key source of diversity, with genetic discriminatory power for microepidemiological purposes. However, no such variation was found across the investigated collection.

Suitability of the microarray assay was evidenced by confirmation of known LSPs, as well as by identification of new RDs in comparison to *M. marinum* M and *M. ulcerans* Japan 9756. Still, absence of PCR-reconfirmed hits in the CGH experiments with the Ghanaian *M. ulcerans* isolates does not strictly prove the absence of any LSPs. Some genomic regions might not be covered by the library; others might have been lost either because Sau3AI restriction sites were possibly overrepresented in the respective sites or because amplification or spotting was incomplete and deletions of <500 bp are not necessarily identified with the microarray setup used. However, the theoretical coverage, the confirmation of already known deletions, and the identification of new LSPs in *M. marinum* and an *M. ulcerans* strain belonging to the ancestral lineage indicate that the number of undetected deletions is expected to be small using this assay.

In contrast, our data suggest that in spite of the presence of numerous transposable ISs in the genome of African *M. ulcerans* strains, genetic changes leading to LSPs appear too rare to provide a basis for genetic fingerprinting of local *M. ulcerans* populations. We conclude that on a regional geographic scale, differentiation within these largely monomorphic local clones might be possible through identification of SNPs. First evidence for a SNP-based genetic discrimination between and within African countries has already been provided (31).

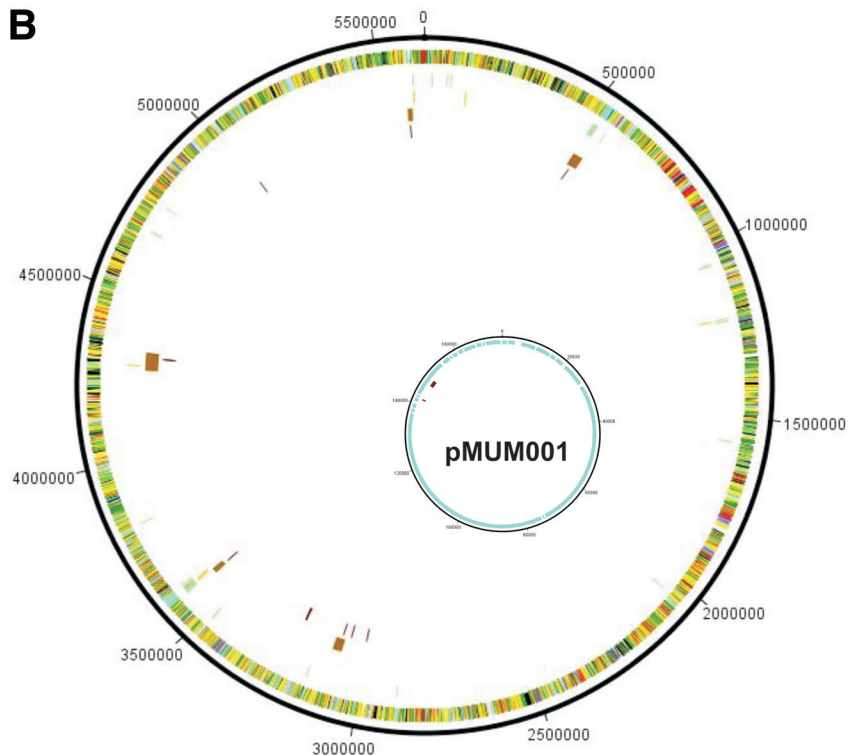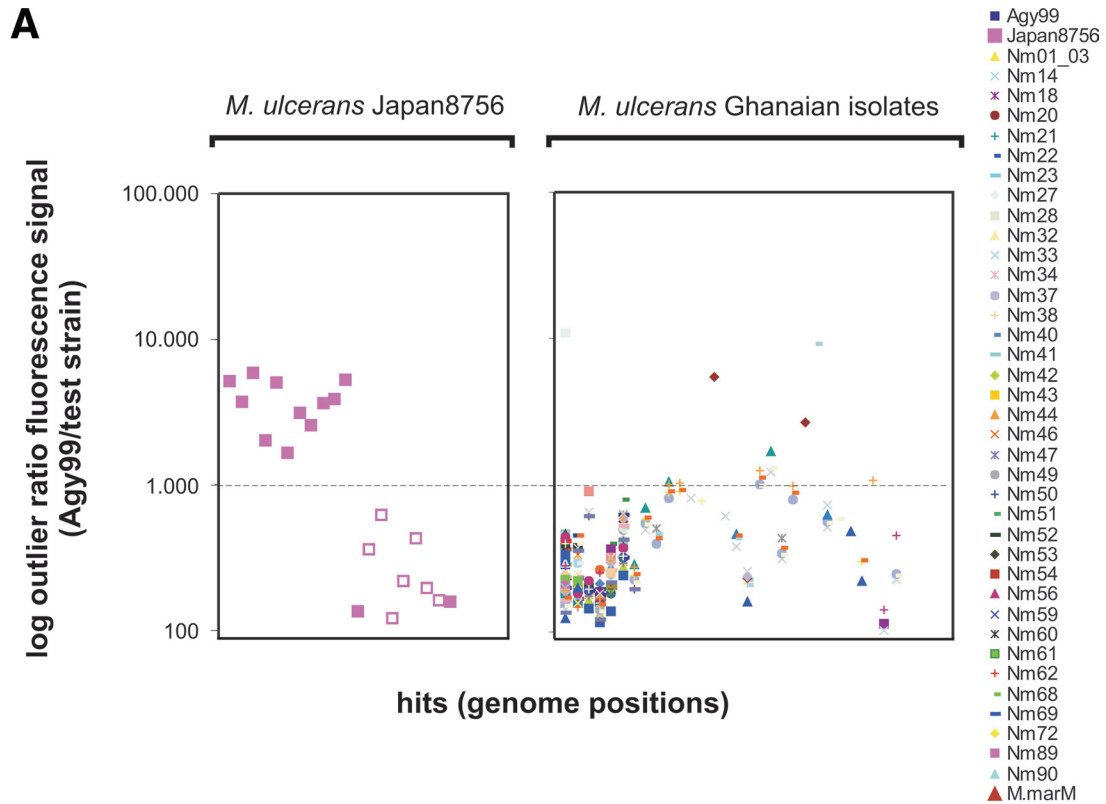The *M. ulcerans* classical lineage, responsible for the vast

FIG. 3. Identification and validation of deletions. (A) Microarray hits with outlier ratios (reference strain Agy99/test strain) of normalized fluorescence signals higher than 100 (accounting for low signal/absence of a genomic region) are indicated as single symbols, as indicated on the figure. Each column represents one genome position that yielded a microarray hit. Hybridization was done with *M. ulcerans* Japan 8756 (filled symbols, confirmed absent genomic loci; open symbols, confirmed present genomic loci) and 37 Ghanaian isolates (all loci confirmed present). M.marM, *M. marinum* M. (B) Mapping of identified deletions to regions known to be absent. The circular view depicts the *M. ulcerans* Agy99 genome (outer circle), positions of non-ISEs not present in *M. marinum* M (http://www.sanger.ac.uk/Projects/M_marinum/) larger than 500 bp (second circle from outside) with their confirmation of absence by the microarray (third circle from outside), and deletions formerly found for the Asian haplotype (fourth circle from outside), with newly identified deletions in strain Japan 9756 (third circle from outside and innermost circle). Inset: regions absent from *M. marinum* M that corresponded to the *M. ulcerans* plasmid pMUM001.

majority of BU cases worldwide, is thought to have undergone massive genome reduction while adapting to a relatively stable ecological niche (14, 28, 29, 47, 54). The nature of some of the genomic changes speaks for an adaptation to an environment which is screened by immune effector mechanisms (14, 26, 28, 41). Genetic monomorphism indicates that this lineage has been forged through a bottleneck situation (29, 54, 63). The genomic stability demonstrated here of this successful clone may be indicative of decreased selective pressure, rendering further genome shrinkage unnecessary. The natural habitat and transmission pathways of *M. ulcerans* remain unknown, and it is not clear whether long-term persistence in ulcerative wounds of mammalian hosts is contributing significantly to the survival of the species. Development of a SNP-based rather than an LSP-based genetic fingerprinting method may help answer these key questions.

ACKNOWLEDGMENTS

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