

Isolation of the Genome Sequence Strain *Mycobacterium avium* 104 from Multiple Patients over a 17-Year Period

Kathleen L. Horan,^{1*} Robert Freeman,¹ Kris Weigel,¹ Makeda Semret,² Stacy Pfaller,³ Terry C. Covert,³ Dick van Soolingen,⁴ Sylvia C. Leão,⁵ Marcel A. Behr,² and Gerard A. Cangelosi¹

Seattle Biomedical Research Institute, Seattle, Washington,¹ McGill University Health Center, Montreal, Canada,² U.S. Environmental Protection Agency, National Exposure Research Laboratory, Cincinnati, Ohio,³ National Institute of Public Health and the Environment, Bilthoven, The Netherlands,⁴ Departamento de Microbiologia, Immunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina, UNIFESP-EPM, São Paulo, Brazil⁵

ABSTRACT

The genome sequence strain 104 of the opportunistic pathogen *Mycobacterium avium* was isolated from an adult AIDS patient in Southern California in 1983. Isolates of non-paratuberculosis *M. avium* from 207 other patients in Southern California and elsewhere were examined for genotypic identity to strain 104. This process was facilitated by the use of a novel two-step approach. In the first step, all 208 strains in the sample were subjected to a high-throughput, large sequence polymorphism (LSP)-based genotyping test, in which DNA from each strain was tested by PCR for the presence or absence of 4 hypervariable genomic regions. Nineteen isolates exhibited an LSP type that resembled that of strain 104. This subset of 19 isolates was then subjected to high-resolution repetitive sequence-based PCR typing, which identified 10 isolates within the subset that were genotypically identical to strain 104. These isolates came from 10 different patients at 5 clinical sites in the western United States, and they were isolated over a 17-year time span. Therefore, the sequenced genome of *M. avium* strain 104 has been associated with disease in multiple patients in the western United States. Although *M. avium* is known for its genetic plasticity, these observations also show that strains of the pathogen can be genotypically stable over extended time periods.

INTRODUCTION

The *Mycobacterium avium* complex (MAC) includes the most clinically significant of the environmental mycobacteria that opportunistically infect susceptible humans. Sequencing of the genome of *M. avium* strain 104 is nearing completion by The Institute for Genomic Research (TIGR). Strain 104 was isolated in the mid-1980s from an adult patient with AIDS in Southern California. Like many genome sequence strains, its laboratory characteristics are amenable to genetic analysis, but there is little clinical or epidemiologic information regarding its incidence in human MAC disease. The draft sequence of strain 104 has 4,480 predicted open reading frames (ORFs). Annotation of the strain 104 genome identified genes that are shared with other virulent mycobacteria as well as genes that are unique to MAC (23). Whole-genome DNA microarray and PCR evaluation of 43 clinical isolates of *M. avium* relative to strain 104 revealed a 13.5% polymorphism rate between isolates (23). In comparison to *M. tuberculosis*, this represents an eightfold-greater strain-to-strain variability on the genomic level. In view of this

genomic heterogeneity, the extent to which strain 104 is representative of virulent MAC isolates is an open question.

To assess the clinical incidence of infections involving strain 104, we examined a sample of clinical isolates of *M. avium* from Southern California and elsewhere for genotypic identity with the genome sequence strain. Although these isolates were initially classified as *M. avium* subsp. *avium*, new data suggest that true *M. avium* subsp. *avium* strains infect a narrow spectrum of avian hosts and are described genotypically by a characteristic restriction fragment length polymorphism with IS1245, by the presence of IS901, and by a limited temperature range in which they can be grown (15). Mijs and colleagues recommended the subspecies name "*hominissuis*" for the more genetically polymorphic *M. avium* strains that grow in a wider temperature range of 24 to 45°C and classically infect pigs and humans. The *M. avium* isolates used in this study are phenotypically consistent with *M. avium* subsp. *hominissuis* (15). However, because most of the isolates were not assessed to determine whether they have the genotypic characteristics of *M. avium* subsp. *hominissuis*, they are referred to in this study by the umbrella term "*M. avium*."

M. avium isolates are known for their genotypic polymorphism (1-3, 8, 12, 19, 20, 24). High-resolution pathogen genotyping approaches, such as pulsed-field gel electrophoresis and restriction fragment length polymorphism (RFLP) with IS1245, have rarely linked MAC isolates outside of geographically restricted samples. Because these methods are time consuming, labor intensive, and not portable (i.e., difficult to standardize between laboratories), few molecular epidemiologic studies of MAC have been conducted that encompass broader geographic samples. Recently, a commercial repetitive sequence-based PCR (rep-PCR) system was shown to deliver resolving power equivalent to that of RFLP with IS1245 (6). This method is rapid and reproducible enough for analysis of large samples, but its cost can be restrictive. To stratify strains of interest for high-resolution typing, preliminary analysis with a moderate resolution typing method can be cost effective. For example, a two-tier approach was used for many years in molecular epidemiological analysis of *Mycobacterium tuberculosis*. Spoligotyping was used to presumptively link isolates prior to high-resolution typing by the more labor-intensive RFLP method (4, 10, 26). To search a large strain sample for genotypic identity to strain 104, a high-throughput tool was needed that could be applied like spoligotyping to presumptively link isolates prior to high-resolution typing.

Sequencing and annotation of multiple mycobacterial genomes has helped to identify large sequence polymorphisms (LSPs), defined as insertions or deletions comprising at least one ORF. Varying patterns of LSP occurrence offer an alternative method of typing mycobacteria in a quick, PCR-based method (7, 9). LSPs are thought to be relatively stable and unique events, and they have been used to identify genetic linkages within and between *Mycobacterium* species (11, 16, 25). As a simple, inexpensive method to interrogate strains for stable polymorphisms, LSP typing by PCR can streamline the molecular epidemiological analysis of a large sample, especially when the sample set is screened for isolates that are genotypically related to a reference strain. It can be expected that isolates that share the reference strain's LSP type are more likely than other isolates to share the reference strain's high-resolution DNA fingerprint (e.g., IS1245 RFLP or rep-PCR patterns). We used this strategy to screen a large clinical isolate sample set for epidemiologically unlinked isolates that share genotypic identity with the genome sequence strain *M. avium* strain 104.

MATERIALS AND METHODS

***M. avium* strains.** The analysis was conducted on strain 104 and 207 additional archived human clinical isolates of *M. avium* from 6 geographical regions: Southern California (75 strains); Seattle, Washington (22 strains); Little Rock, Arkansas (5 strains); The Netherlands (61 strains); São Paulo, Brazil (22 strains); and Montreal, Quebec, Canada (23 strains). Each isolate originated from a single, anonymous patient. Sites of isolation included sputum, bronchoalveolar lavage fluid, blood, lymph node biopsy, and unknown sites. The Southern California and The Netherlands isolates came from patients at multiple clinical sites (6 and 35, respectively), whereas the isolates from the other 4 regions originated from patients in 1 or 2 clinical sites each. Human immunodeficiency virus coinfection information was available for some, but not all, of the isolates. The isolates are summarized in Table 1 and were originally isolated and archived from other studies (2, 5, 6, 13, 17, 18, 23).

TABLE 1. *M. avium* subsp. *avium* clinical isolates ($n = 208$)

Location	No. of isolates	No. of clinical sites	Yr of collection	Reference(s)
Southern California	75	6	1982-1994	2, 5
The Netherlands	61	35	1994-1996	13
Brazil	22	1	1998	18
Seattle, Wash.	22	2	1999-2001	5, 6, 17
Montreal, Canada	23	1	1999-2003	23
Arkansas	5	1	Prior to 2000	17

For most isolates, DNA was extracted at collaborating sites and shipped in pure form to Seattle Biomedical Research Institute (SBRI) for analysis. In some cases, the isolates were delivered to SBRI as viable bacterial cells. In those cases, *M. avium* colonies were grown on Middlebrook 7H10 agar plates (Fisher Scientific International, Hampton, NJ) containing oleic acid-albumin-dextrose-catalase at 37°C, and DNA was extracted using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA). All DNA samples were diluted to 10 µg/ml in sterile water for analysis.

LSP typing PCR strategy. The 4 LSPs examined in this study were designated LSP2, LSP7, HSD, and LSPP5. This nomenclature was consistent with prior publications (14, 22, 23). Each consisted of a genomic region of ≥ 2.5 kb that was present in some *M. avium* strains and absent in others. Criteria for their selection are described in Results. PCR primers used to interrogate genomic DNA for the presence or absence of each region are summarized in Table 2. Genomic DNA from each isolate was interrogated with 4 multiplex PCRs, each of which tested for one LSP.

TABLE 2. PCR primers used for LSP typing

Primer ^a	Sequence	Reference
LSP2 LFP	TTTATCCGCCTGATCGAAAC	23
LSP2 RFP	ATTCGTTTCGTGTCGTCGTC	23
LSP2 LIP	CACGATCTTGGCTTCCTCAG	23
LSP7 LFP	GAGGTGGTGGACCTGCTG	23
LSP7 RFP	CATCCGGTCTTCCAGCAC	23
LSP7 LIP	CGGATCGCCCTACAGATAAC	23
HSD LFP	AGGTCCATCTGCCCTGTTG	This study
HSD LIP	CCGAATACGCAAATCACCTT	This study
HSD BP	CAGGGCGTTTCACGGTC	This study
LSPP5 RFP	GTCATACGGCTGTCCTGGTT	This study
LSPP5 LFP	GGCAGCAGCACTATTTCAT	This study
LSPP5 RIP	TGCTCAGGCCAACCCGCGC	This study
LSPP5 LIP	TACTGGGCGGCGCACCCGAC	This study

^a Abbreviations: LFP, left flanking primer; RFP, right flanking primer; LIP, left internal primer; RIP, right internal primer; BP, bridging primer.

Primers for LSP2 and LSP7 were described previously by Semret et al. (23). For each of these LSPs, a left flanking primer (LFP) and a right flanking primer (RFP) were designed. A third primer hybridized internally near the left end of each region (left internal primer [LIP]). The presence of each region was indicated by a PCR amplicon primed by the LFP and LIP. The absence of each region was indicated by an amplicon primed by the two flanking primers.

The presence of the HSD region was detected in a similar manner. When the HSD region was present, an amplicon was primed by the LFP and LIP, as in LSP2 and LSP7. When the HSD region was absent, an amplicon was primed by the LFP and an opposing primer that spanned the deletion junction (bridging primer).

Preliminary analysis of LSPP5 found multiple polymorphisms in both border regions. Therefore, a set of 4 primers was used to detect the presence or absence of this LSP. When the region was present, two internal primers (LSPP5 LIP and right internal primer) amplified an internal segment. When it was absent, flanking primers (left and right) amplified across the junction as in LSP2 and LSP7.

Amplicon sizes expected from the multiplex PCRs testing for the presence and absence of each region were as follows: LSP2, 954 bp (present) and 834 bp (absent); LSP7, 181 bp (present) and 329 bp (absent); HSD, 480 bp (present) and 343 bp (absent); LSPP5, 794 bp (present) and 728 bp (absent).

PCR conditions. Except for HSD, the PCR was performed by combining 5 μ l of 5x GC-rich PCR buffer, 2 μ l of 10 mM nucleotides, 1.5 μ l of 10 mM primer mix, 2.5 μ l of GC-rich resolution solution (Roche Diagnostics, Basel, Switzerland), 1 μ l of 10 μ g/ml template, 1 U of *Taq* enzyme, and sterile water to make 25 μ l per reaction mixture. The HSD PCR mixture required the addition of 0.6 μ l of 2.5 mM MgCl₂, which lowered the GC-rich resolution solution to 2 μ l per reaction. PCR was performed with a PTC-200 Peltier thermocycler (MJ Research, Boston, MA). Both LSP7 and LSPP5 used an initial denaturation step of 95°C for 3 min, followed by 36 cycles of 1 min at 58°C, followed by 1 min at 72°C. PCR conditions for HSD began with an initial denaturation step at 95°C for 3 min followed by 36 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. PCR conditions for LSP2 included an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 45 s each at 94°C, 57°C, and 72°C.

Amplicons were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining in comparison to a 1-kb ladder (Sigma-Aldrich Corp., St. Louis, MO). Sizing was confirmed by the ladder as well as by correspondence to well-characterized positive- or negative-control strains (*M. avium* strains 104 and HMC02, and *M. avium* subsp. *paratuberculosis* strain K10).

LSP typing nomenclature. An amplification result corresponding precisely in size to the presence of a region was denoted with the number "2." A result corresponding precisely in size to the absence of a region was denoted with the number "1." If no amplification or anomalous bands were observed, the locus was assigned an "X" (indeterminate). Thus, each isolate was assigned a 4-digit LSP type, with results for each locus ordered as follows: HSD, LSP2, LSP7, LSPP5. For example, a strain lacking all 4 regions was denoted "t1111," whereas a strain with genomic material at LSP2 and LSP7, but not at the other two LSPs, was denoted "t1221."

High-resolution strain typing by rep-PCR. The DiversiLab System (Bacterial Barcodes, Athens, GA) was used for high-resolution strain typing, following the manufacturer's protocols. rep-PCR takes advantage of repetitive elements interspersed throughout bacterial genomes. When amplified by PCR, these elements generate highly discriminative genomic fingerprints. The DiversiLab *Mycobacterium* kit has been shown to deliver resolving power equivalent to that of restriction fragment length polymorphism targeting IS1245 in MAC (5). rep-PCR required 2 μ l of 25 to 50 ng/ μ l genomic DNA. Dilutions were performed with sterile water.

RESULTS

Selection of LSPs. The four LSPs used in this study were chosen on the basis of their variable occurrence among human clinical isolates of *M. avium*. LSP2 and LSP7 were identified by in silico comparison of the genomes of *M. avium* strain 104 and *M. avium* subsp. *paratuberculosis* strain K10, followed by microarray and PCR analysis of genomic DNA of multiple isolates of both subspecies (23). LSP2 encompasses 22,038 bp and 17 ORFs, and LSP7 encompasses 31,474 bp and 25 ORFs. Both of these regions are present in the 5.48-Mb genome of *M. avium* strain 104 and absent in the 4.83-Mb genome of *M. avium* subsp. *paratuberculosis* strain K10.

To avoid bias toward regions that are present in strain 104, two additional regions were identified that are absent in strain 104 relative to other strains. One such region, HSD, was characterized in a previous genetic analysis of a Seattle clinical isolate, HMC02 (14). It is \approx 2,631 bp long and codes for a cytochrome P450-like gene and two type I restriction-modification genes, *hsdR* and *hsdM*, which are disrupted by insertion sequences in some clones. In strain 104, there is a deletion of this region that starts within the P450-like gene and extends beyond *hsdR* and *hsdM* to a right terminus that has not yet been mapped. Preliminary analysis showed the mapped terminus within the P450-like gene to be well conserved with almost no polymorphism between strains that carry the deletion.

The fourth LSP, LSPP5, was identified for this study by in silico analysis. It is a 14,471-bp region that was found by CROSS_MATCH analysis to be present in *M. avium* subsp. *paratuberculosis* strain K10 but absent in strain 104. It carries 15 ORFs, including multiple transposase genes, indicative of insertion elements. As preliminary PCR analysis revealed the bordering regions to be variable between *M. avium* strains, PCR primers were designed to hybridize to the conserved internal and external regions within and outside the variable border regions.

The 2 LSPs present in strain 104, LSP2 and LSP7, mapped at positions 5,240,295 to 5,263,079 and 3,210,765 to 3,242,533, respectively, in the most recent draft of the *M. avium* 104 genome sequence (www.tigr.org). The genomic region that amplified between the flanking primers in LSP5 corresponded to positions 2,622,423 to 2,623,150 in the 104 genome. The HSD deletion junction mapped to base pair 1,537,166.

Frequency of precise LSP types. Strain 104 plus 207 additional human clinical isolates of *M. avium* were evaluated by PCR for the 4 LSPs. Each isolate was assigned an "LSP type" that consisted of 4 binary scores for the presence (coded as "2") or absence (coded as "1") of the 4 loci. The scores were listed in the order HSD, LSP2, LSP7, and LSP5. Of the 16 possible LSP types, 14 were found in the sample. Of the 208 isolates, 174 (84%) yielded amplicons at all four sites of the precise sizes expected for "present" or "absent" results (Fig. 1). The other 34 strains yielded indeterminate results at one or more loci. Most indeterminate results consisted of failure to amplify at one or more loci or the occurrence of multiple bands at one or more loci. Indeterminate results occurred relatively frequently at LSP2 (7.2%) and LSP5 (6.3%) and less frequently at LSP7 (4.8%) and HSD (3.8%). The largest geographical sample sets had the greatest variety of LSP types. The Southern California and The Netherlands samples exhibited 10 and 8, respectively, of the 16 possible LSP types.

LSP Type	Arkansas	Montreal	Brazil	Netherlands	California	Seattle
1111		1	1	38		
1112				1	3	
1121			2	3	8	2
1122						
1211				4	12	
1212					1	
1221			1	1	12	5
1222					1	
2111	1		2	1		
2112	1	15	7	2	9	10
2121			1			
2122						
2211				3	6	2
2212			2	1	10	1
2221					2	
2222	1					1
Indeterminate	2	7	6	7	11	1
Total	5	23	22	61	75	22

FIG. 1. Distribution of LSP types. The number of isolates with each LSP type is shown. Blanks indicate that no isolates were found with a given LSP type at a given site. The t1221 LSP types are highlighted by the box.

Only one LSP type, t2112, was found in all geographical sites examined (Fig. 1). This was also the most common LSP type overall, accounting for 44 (21%) of the 208 strains examined. The incidence of the t2112 LSP type, and its occurrence in all geographical regions examined, may reflect an ancestral configuration at most or all of the 4 genomic positions.

Identification of t1221 isolates. Strain 104 exhibited the t1221 LSP type. In total, 19 t1221 isolates were identified, 12 (63%) of which came from Southern California, a region that provided 75 (36%) of the 208 isolates examined. Of the remaining 7 t1221 isolates, 5 came from Seattle, and 1 (each) came from The Netherlands and Brazil (Fig. 1). The United States t1221 isolates originated from 4 of the 6 Southern California sites and 1 of the 2 Seattle sites. In addition to the

19 t1221 strains, a single indeterminate strain from Southern California, W214 (t1X21), had an LSP type identical to strain 104 at 3 of the 4 loci but failed to produce an amplicon for LSP2.

High-resolution linkages within LSP types. To identify t1221 isolates that were genotypically identical to strain 104, the 18 additional t1221 strains plus the t1X21 indeterminate strain W214 were typed by rep-PCR, a high-resolution method (5). Nine t1221 isolates exhibited $\geq 92\%$ similarity to strain 104 (Fig. 2), a level of similarity that was previously established as the threshold for strain identity within MAC (5). Strain W214 also fell into this cluster. The remaining eight t1221 isolates, including isolates from Brazil and The Netherlands, showed $< 92\%$ similarity on rep-PCR.

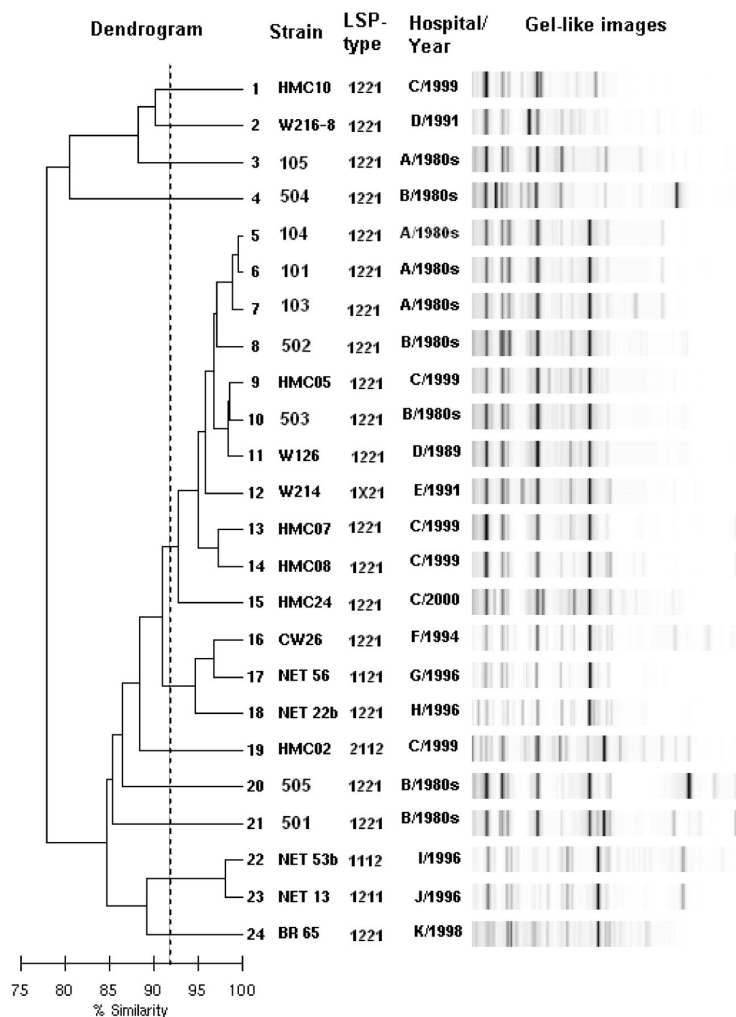


FIG. 2. rep-PCR analysis of t1221 isolates and other clinical isolates. The dendrogram and gel-like images were generated by the DiversiLab software, which also assigned numbers (1 through 24) to the samples in the set. The strain identifications, LSP types, and dates and locations of collection were added by us, as was the vertical dotted line that marks the 92% similarity cutoff. The Southern California sample collection dates spanned a decade, with the 100 and 500 series collected in the mid-1980s. The 104-like cluster included isolates numbered 5 through 15.

The 10 104-like isolates identified in this fashion and strain 104 came from four different hospitals in Southern California and a fifth hospital in Seattle. Isolation dates spanned a 17-year period, from 1983 through 1999.

To assess the occurrence of the 104-like rep-PCR type in strains that did not share the t1221 LSP type, rep-PCR patterns of 65 non-t1221 strains, representing 11 of the other 13 LSP types that occurred within the sample, were compared to that of strain 104. These strains had been typed in separate studies (40 strains) or were chosen at random (25 strains) for this comparison. None of these strains exhibited $\geq 92\%$ similarity to strain 104 (Fig. 3).

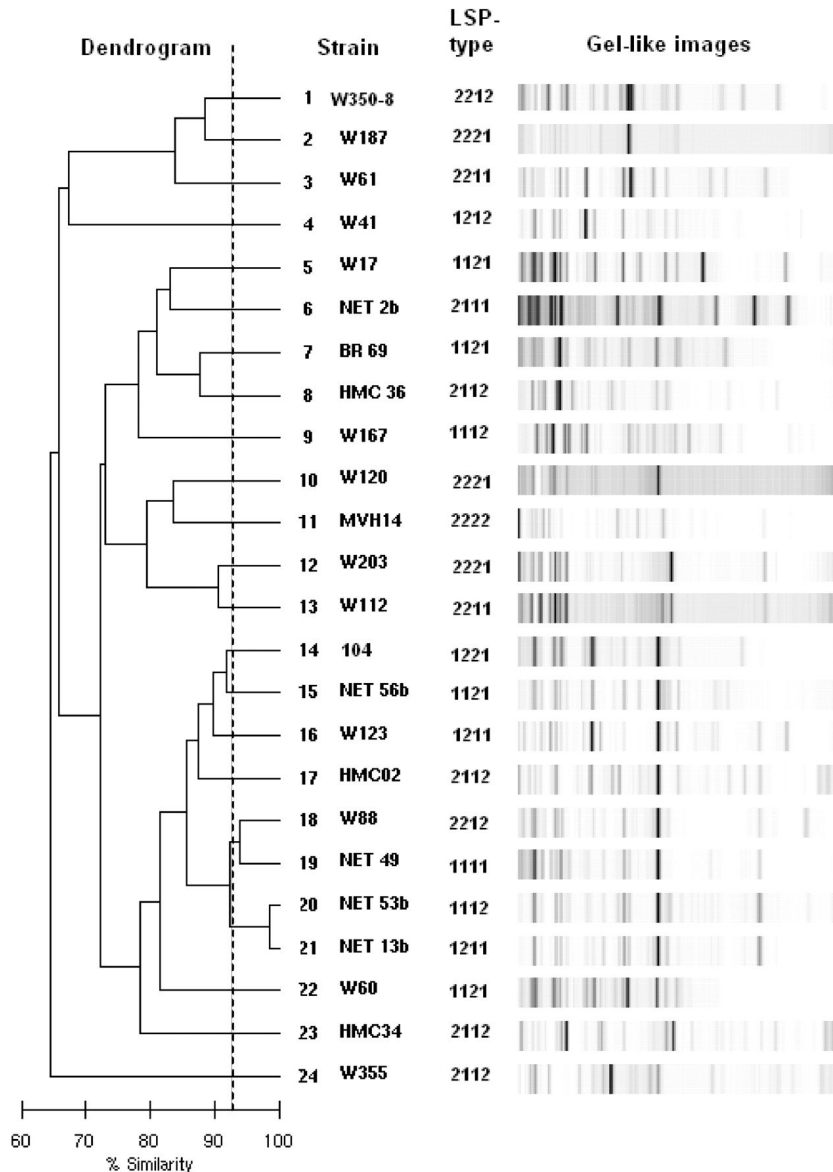


FIG. 3. rep-PCR analysis of non-t1221 isolates compared to strain 104. The dendrogram and gel-like images were generated by the DiversiLab software. The strain identifications, LSP types, and 92% similarity cutoff line were added by us, as for Fig. 2. Strain 104 is isolate number 14 on the figure.

DISCUSSION

LSP typing proved useful as an inexpensive and expeditious way to screen a large number of clinical isolates of *M. avium* for a specific genotype prior to high-resolution typing by the more expensive rep-PCR method. We have reported the successful use of a similar model in tuberculosis outbreak monitoring (7). In the present study, we used this strategy to interrogate a sample of 207 *M. avium* clinical isolates for genotypic similarity with the genome sequence strain 104. Once the 19 isolates with the shared t1221 or t1X21 type were identified, we focused high-resolution typing on those isolates. In this manner, we quickly identified 10 additional 104-like isolates from within a large and diverse sample set. Not surprisingly, two of these isolates (101 and 103) came from separate patients at the same hospital, and during the same time period, as strain 104. Less expected was the isolation of such strains from patients in four other hospitals in Southern California and Seattle. The results show that strain 104 has been isolated from multiple patients over a 17-year period.

The 17-year time span was an unexpected finding. We are not aware of prior reports of this nature, and there are several possible models to explain it. A single environmental source of infection may exist, and the 11 patients may have acquired their infections from that single source at different time points over 17 years. Alternatively, the 104-like strain may have stably colonized multiple environmental sites in the region, resulting in infection of epidemiologically unlinked patients. A third scenario is that all 11 patients were exposed to a single point source in the early 1980s and became stably but asymptotically colonized. Subsequently, over the course of the ensuing 17 years, they may have become immunocompromised and developed MAC disease at differing times and locales. These divergent models highlight an unanswered question in MAC disease. Namely, is the pathogen acquired early in life and asymptotically carried until host immunity fails, or is MAC acquisition transient and cleared unless a preexisting susceptibility exists? To answer these questions and to better define models of MAC acquisition, large numbers of clinical and environmental isolates will have to be analyzed from multiple sites. The PCR-based LSP typing would greatly facilitate such a study.

LSP typing successfully narrowed the search for the 104-like genotype, as indicated by the fact that 104-like isolates were common among t1221 and t1X21 strains but were not found in a sample of 65 strains with other LSP types. However, it was also noted that the non-t1221 sample included strains that appeared similar or identical to each other by rep-PCR but had differing LSP types (Fig. 3). This observation indicates that LSP types can, in some instances, segregate rapidly and independently of other genotypic parameters.

In contrast to a previous LSP-based study conducted on *M. tuberculosis* (7), which targeted LSPs that were unique to a single Beijing family outbreak strain, the LSPs used in this study of *M. avium* strains were broadly heterogeneous among isolates from 3 continents, as evidenced by the variety in LSP types observed. Therefore, the specific LSP tests used in this study can be applied to any molecular epidemiologic analysis of clinical *M. avium*.

In theory, both the resolving power and the throughput of LSP typing can be further improved. Analysis of additional LSPs will improve the resolution of the test, and probes for these LSPs can be incorporated into filter-based, high-throughput formats similar to that described for "deligotyping" of *M. tuberculosis* (9). Alternatively, these four LSP loci could be combined with the recently described *M. avium* variable number of tandem repeats-mycobacterial


interspersed repetitive unit loci (21) for a multimodal PCR-based genotyping method. An advantage of LSP typing is that its binary readout is portable, meaning that results can be expressed in spreadsheet form and compared between laboratories. As illustrated by the identification of 104-like clones, LSP typing can be used as a high-throughput, moderate-resolution tool to expedite searches for shared genotypic identity.

ACKNOWLEDGMENTS

K.L.H. is supported by NIH training grant HL07287. Additional funding and support was provided by the M.J. Murdoch Charitable Trust, the Firland Foundation, and NIH grants R01-AI46226 and R21-AI061006.

Genome sequences were made available by www.tigr.org. We are indebted to Clark Inderlied for providing some of the Southern California isolates and sharing information about their derivation. The United States Environmental Protection Agency, through its Office of Research and Development, collaborated in the research described here. It has been subjected to Agency review and approved for publication.

FOOTNOTES

* Corresponding author. Mailing address: Seattle Biomedical Research Institute, 307 Westlake Avenue N, Suite 500, Seattle, WA 98109. Phone: (206) 256-7200. Fax: (206) 256-7229. E-mail: kthoran@u.washington.edu . 

REFERENCES

1. Arbeit, R. D., A. Slutsky, T. W. Barber, J. N. Maslow, S. Niemczyk, J. O. Falkinham III, G. T. O'Connor, and C. F. von Reyn. 1993. Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. *J. Infect. Dis.* 167:1384-1390.
2. Aronson, T., A. Holtzman, N. Glover, M. Boian, S. Froman, O. G. Berlin, H. Hill, and G. Stelma, Jr. 1999. Comparison of large restriction fragments of *Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. *J. Clin. Microbiol.* 37:1008-1012.
3. Bauer, J., A. B. Andersen, D. Askgaard, S. B. Giese, and B. Larsen. 1999. Typing of clinical *Mycobacterium avium* complex strains cultured during a 2-year period in Denmark by using IS1245. *J. Clin. Microbiol.* 37:600-605.
4. Borsuk, S., M. M. Dellagostin, S. D. Madeira, C. Lima, M. Boffo, I. Mattos, P. E. Almeida da Silva, and O. A. Dellagostin. 2005. Molecular characterization of *Mycobacterium tuberculosis* isolates in a region of Brazil with a high incidence of tuberculosis. *Microbes Infect.* 7:1338-1344.
5. Cangelosi, G. A., R. J. Freeman, K. N. Lewis, D. Livingston-Rosanoff, K. S. Shah, S. J. Milan, and S. V. Goldberg. 2004. Evaluation of a high-throughput repetitive-sequence-based PCR system for DNA fingerprinting of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex strains. *J. Clin. Microbiol.* 42:2685-2693.
6. Cangelosi, G. A., C. O. Palermo, J. P. Laurent, A. M. Hamlin, and W. H. Brabant. 1999. Colony morphotypes on Congo red agar segregate along species and drug susceptibility lines in the *Mycobacterium avium*-intracellulare complex. *Microbiology* 145:1317-1324.
7. Freeman, R., M. Kato-Maeda, K. A. Hauge, K. L. Horan, E. Oren, M. Narita, C. K. Wallis, D. Cave, C. M. Nolan, P. M. Small, and G. A. Cangelosi. 2005. Use of rapid genomic deletion typing to monitor a tuberculosis outbreak within an urban homeless population. *J. Clin. Microbiol.* 43:5550-5554.
8. Garriga, X., P. Cortes, P. Rodriguez, F. March, G. Prats, and P. Coll. 2000. Comparison of IS1245 restriction fragment length polymorphism and pulsed-field gel electrophoresis for typing clinical isolates of *Mycobacterium avium* subsp. *avium*. *Int. J. Tuberc. Lung Dis.* 4:463-472.
9. Goguet de la Salmoniere, Y. O., C. C. Kim, A. G. Tsolaki, A. S. Pym, M. S. Siegrist, and P. M. Small. 2004. High-throughput method for detecting genomic-deletion polymorphisms. *J. Clin. Microbiol.* 42:2913-2918.
10. Gori, A., A. Bandera, G. Marchetti, A. Degli Esposti, L. Catozzi, G. P. Nardi, L. Gazzola, G. Ferrario, J. D. van Embden, D. van Soolingen, M. Moroni, and F. Franzetti. 2005. Spoligotyping and *Mycobacterium tuberculosis*. *Emerg. Infect. Dis.* 11:1242-1248.
11. Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small. 2001. Comparing genomes within the species *Mycobacterium tuberculosis*. *Genome Res.* 11:547-554.
12. Koivula, T., M. Cristea-Fernstrom, E. Chryssanthou, B. Petrini, and G. Kallenius. 2004. Genetic diversity in clinical isolates of *Mycobacterium avium* complex from Guinea-Bissau, West Africa. *Microbes Infect.* 6:1320-1325.
13. Komijn, R. E., P. E. de Haas, M. M. Schneider, T. Eger, J. H. Nieuwenhuijs, R. J. van den Hoek, D. Bakker, F. G. van Zijderveld, and D. van Soolingen. 1999. Prevalence of *Mycobacterium avium* in slaughter pigs in The Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates. *J. Clin. Microbiol.* 37:1254-1259.
14. Laurent, J. P., S. Faske, and G. A. Cangelosi. 2002. Characterization of IS999, an unstable genetic element in *Mycobacterium avium*. *Gene* 294:249-257.

15. Mijs, W., P. de Haas, R. Rossau, T. Van der Laan, L. Rigouts, F. Portaels, and D. van Soolingen. 2002. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and 'M. avium subsp. hominissuis' for the human/porcine type of *M. avium*. *Int. J. Syst. Evol. Microbiol.* 52:1505-1518.
16. Mostowy, S., D. Cousins, J. Brinkman, A. Aranaz, and M. A. Behr. 2002. Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex. *J. Infect. Dis.* 186:74-80.
17. Mukherjee, S., M. Petrofsky, K. Yaraei, L. E. Bermudez, and G. A. Cangelosi. 2001. The white morphotype of *Mycobacterium avium-intracellulare* is common in infected humans and virulent in infection models. *J. Infect. Dis.* 184:1480-1484.
18. Oliveira, R. S., M. P. Sircili, E. M. Oliveira, S. C. Balian, J. S. Ferreira-Neto, and S. C. Leao. 2003. Identification of *Mycobacterium avium* genotypes with distinctive traits by combination of IS1245-based restriction fragment length polymorphism and restriction analysis of hsp65. *J. Clin. Microbiol.* 41:44-49.
19. Olivier, K. N., D. J. Weber, R. J. Wallace, Jr., A. R. Faiz, J. H. Lee, Y. Zhang, B. A. Brown-Elliott, A. Handler, R. W. Wilson, M. S. Schechter, L. J. Edwards, S. Chakraborti, and M. R. Knowles. 2003. Nontuberculous mycobacteria. I. Multicenter prevalence study in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 167:828-834.
20. Pestel-Caron, M., G. Graff, G. Berthelot, J. L. Pons, and J. F. Lemeland. 1999. Molecular analysis of *Mycobacterium avium* isolates by using pulsed-field gel electrophoresis and PCR. *J. Clin. Microbiol.* 37:2450-2455.
21. Romano, M. I., A. Amadio, F. Bigi, L. Klepp, I. Etchechoury, M. N. Llana, C. Morsella, F. Paolicchi, I. Pavlik, M. Bartos, S. C. Leao, and A. Cataldi. 2005. Further analysis of VNTR and MIRU in the genome of *Mycobacterium avium* complex, and application to molecular epidemiology of isolates from South America. *Vet. Microbiol.* 110:221-237.
22. Semret, M., D. C. Alexander, C. Y. Turenne, P. de Haas, P. Overduin, D. van Soolingen, D. Cousins, and M. A. Behr. 2005. Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. *J. Clin. Microbiol.* 43:3704-3712.
23. Semret, M., G. Zhai, S. Mostowy, C. Cleto, D. Alexander, G. Cangelosi, D. Cousins, D. M. Collins, D. van Soolingen, and M. A. Behr. 2004. Extensive genomic polymorphism within *Mycobacterium avium*. *J. Bacteriol.* 186:6332-6334
24. Swanson, D. S., X. Pan, M. W. Kline, R. E. McKinney, Jr., R. Yogev, L. L. Lewis, M. T. Brady, G. D. McSherry, W. M. Dankner, and J. M. Musser. 1998. Genetic diversity among *Mycobacterium avium* complex strains recovered from children with and without human immunodeficiency virus infection. *J. Infect. Dis.* 178:776-782.
25. Tsolaki, A. G., A. E. Hirsh, K. DeRiemer, J. A. Enciso, M. Z. Wong, M. Hannan, Y. O. Goguet de la Salmoniere, K. Aman, M. Kato-Maeda, and P. M. Small. 2004. Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. *Proc. Natl. Acad. Sci. USA* 101:4865-4870.
26. Van Soolingen, D. 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. *J. Intern. Med.* 249:1-26.