Differentiation of Strains in *Mycobacterium tuberculosis* Complex by DNA Sequence Polymorphisms, Including Rapid Identification of *M. bovis* BCG

RICHARD FROTHINGHAM*

Infectious Diseases Section, Durham Veterans Affairs Medical Center, Durham, North Carolina 27705, and Division of Infectious Diseases, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

Received 28 October 1994/Returned for modification 15 December 1994/Accepted 20 January 1995

The *Mycobacterium tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*. Seven strains of the *M. tuberculosis* complex were sequenced in a region of about 300 bp which contains multiple 15-bp tandem repeats and which is part of a 1,551-bp open reading frame. Four distinct sequences were obtained, each defining a sequevar. A sequevar includes the strain or strains with a given sequence. The type strain *M. tuberculosis* TMC 102 (H37Rv) was designated sequevar MED-G. When compared to MED-G, sequevar LONG had an insertion of one 15-bp tandem repeat and sequevar SHORT had a deletion of one tandem repeat. Sequevar MED-C had a G→C substitution, coding for the conservative change Ser→Thr. *BanI* cuts only sequevar MED-C at the site of the substitution. PCR-restriction enzyme analysis was used to determine the sequevars of 92 *M. tuberculosis* complex strains. All 23 *M. bovis* BCG strains belonged to sequevar MED-C. The *M. africanum* type strain was sequevar SHORT. The remaining 68 strains of *M. tuberculosis*, *M. bovis* (not BCG), and *M. microti* were sequevars LONG (3 strains) or MED-G (65 strains). PCR-restriction enzyme analysis was applied to reference strains and clinical isolates with a worldwide distribution. This method provides rapid, sensitive, and specific identification of the important vaccine strain *M. bovis* BCG.

Nucleic acid sequence analysis has yielded useful phylogenetic trees at the strain level for a variety of viruses (human immunodeficiency virus [HIV], hepatitis C virus, hantaviruses, rabies, and poliovirus, etc.). These phylogenetic analyses have enhanced our understanding of epidemiology, pathogenesis, and treatment. Sequence-based strain differentiation has also been applied to bacteria. Within the *Mycobacterium avium* complex, strains associated with AIDS are phylogenetically distinct on the basis of sequence analysis (19).

The *M. tuberculosis* complex includes the four species, *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*, and causes human and animal tuberculosis. These four species have high DNA-DNA relatedness and have completely conserved DNA sequences in several gene regions (16S rDNA, the 16S-to-23S rDNA internal transcribed spacer, and *dnaJ* [17, 26, 31]). This has limited the use of sequence analysis for strain differentiation.

The major polymorphic tandem repeat (MPTR [22]) was chosen as a target for comparative sequence analysis. The MPTR is a repetitive DNA found in several mycobacterial species. It makes up the majority of repetitive DNA in *M. tuberculosis* on the basis of Southern blot analysis (22). Each 15-bp repeat unit consists of a 10-bp conserved repeat with a 5-bp spacer. They are found in regions of 15 to 20 tandem repeat units. Since adjacent repeats vary substantially in sequence, the likelihood of strain-to-strain sequence variability appeared high. A particular MPTR-containing region was amplified and sequenced. The region chosen is about 300 bp in length and is part of a 1,517-bp open reading frame (28).

(This work was presented in part at the 32nd Annual Meeting of the Infectious Diseases Society of America, Orlando, Fla., 7 to 9 October 1994 [16]).

* Mailing address: Durham VA Medical Center, 508 Fulton St., Building 4, Room 210, Durham, NC 27705. Phone: (919) 286-0411, extension 6566. Fax: (919) 286-6824.

MATERIALS AND METHODS

PCR. Bacterial colonies were suspended in TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA), heat killed, and lysed by agitation with glass beads as previously described (18). PCR was done with 10 µl of the crude lysate in a total volume of 50 µl of PCR mix. The PCR mix contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2.0 mM MgCl₂, 100 nM each primer, 200 µM each of the four deoxynucleoside triphosphates, and 1.25 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.). The following primers were used: Mt MPTR.541p, 5'-GGTTACCACTTCGATGCGTCTGCG-3'; and MtMPTR.883n, 5'-AGCCGCCGAAACCCATC-3'. The primers were based on published sequence (28) and flank an MPTR-containing region (see Fig. 1). The mixture was denatured for 3 min at 95°C and cycled 30 times to 94°C for 30 s (denaturation) and 60°C for 1 min (annealing and extension), followed by a final 10-min extension at 72°C. Multiple interspersed negative controls (reagents only, no DNA) were included each time that PCR was performed. The positive control was 10 ng of chromosomal DNA from *M. tuberculosis* TMC 102^T (the T indicates type strain). The presence and size of each PCR product was determined by electrophoresis on an ethidium bromide-stained 3% agarose gel.

Sequencing. PCR products were sequenced directly with the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit and the 373A DNA Sequencer (both from Applied Biosystems, Foster City, Calif.) as previously described (17). The PCR primers were used as sequencing primers. Sequences from the two strands were combined; discrepancies were resolved by examination of chromatographs generated by the automated sequencer. PCGENE (IntelliGenetics, Mountain View, Calif.) was used for sequence assembly and comparison.

Restriction enzyme analysis. The restriction enzyme *BanI* (New England Biolabs, Beverly, Mass.) was diluted in the buffer provided by the manufacturer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol [pH 7.9]). Buffer (8 µl) with 10 U of *BanI* was added to 4 µl of each PCR product (about 100 ng) and incubated for 1 h at 37°C. PCR products from *M. tuberculosis* TMC 102^T and *M. tuberculosis* TMC 107 were used as positive controls for restriction enzyme digestion. The restriction fragment sizes were determined by electrophoresis on an ethidium bromide-stained 3% agarose gel.

Nucleotide sequence accession number. The region shown in Fig. 1 corresponds to nucleotides 3408 to 3066 of GenBank accession number M15467 (28).

RESULTS

PCR. All 92 *M. tuberculosis* complex strains were amplified successfully by 30 cycles of PCR. Beginning with 500 pg to >250 ng of chromosomal DNA per reaction, 30 cycles of PCR generated about 1 µg of the desired PCR product (about 330 bp), producing a bright band on the ethidium bromide-stained

FIG. 1. Four DNA sequences found in eight *M. tuberculosis* complex strains. The region shown comprises nucleotides 541 to 883 of a 1,551-bp open reading frame (28) and contains the MPTR (22). The 17 tandem DNA repeats are underlined. Each 10-bp repeat has 6 to 10 bp in common with the consensus sequence 5'-CAACACCGGC-3'. The conserved repeats are separated by variable 5-bp spacers. Sequence LONG is given in full with the deduced amino acid sequence. The dots represent sequence identity with sequence LONG; the dashes represent deletions. The shaded regions are recognition sites for the restriction enzyme *BanI* (5'-G¹GYRC₁C-3', where Y = C or T and R = A or G; bars indicate points of cleavage). The sites used for PCR primers are underlined twice. The sequences between the primers were determined for all eight strains, but the sequences of the primer sites are known only for *M. tuberculosis* TMC 107 (28).

agarose gel. By using 45 cycles of PCR, products were visible with as little as 500 fg of starting DNA. None of the interspersed negative controls (reagents only, no DNA) yielded a visible PCR product with 30 or 45 cycles.

Sequencing results. Seven *M. tuberculosis* complex strains were sequenced. Sequence for another strain is published (28). These eight *M. tuberculosis* complex strains yielded four sequences (Fig. 1 and Table 1). Each sequence defines an infraspecific taxon, a sequevar. Just as a serovar contains the strain or strains which react with a given serum, a sequevar contains the strain or strains with a given sequence. *M. tuberculosis* TMC 102^T (H37Rv) was designated sequevar MED-G. When compared to MED-G, sequevar LONG had an insertion of one 15-bp repeat unit and sequevar SHORT had a deletion of one repeat unit. MED-C had a G→C substitution, coding for the conservative change Ser→Thr.

PCR-restriction enzyme analysis (PCR/REA). The 15-bp insertions or deletions were readily identified as length polymorphisms in the PCR products (Table 2 and Fig. 2A). PCGENE was used to search for a restriction enzyme with specificity for the nucleotide substitution. *BanI* was identified (recognition sequence, 5'-G¹GYRC₁C-3'), which cuts only sequevar MED-C at the site of the substitution (Fig. 1). *BanI* cuts all four sequevars at two other sites (Fig. 1). On the basis of the sizes of the PCR products and their *BanI* restriction fragments (Table 2 and Fig. 2B), the sequevars of 92 *M. tuberculosis* complex strains were determined. The strains are listed in

Table 3, and the results are summarized in Table 4. All 23 *M. bovis* BCG strains belonged to sequevar MED-C (Table 4). Only the *M. africanum* type strain was sequevar SHORT (Table 4). The remaining 68 strains of *M. tuberculosis*, *M. bovis* (not BCG), and *M. microti* were sequevars LONG or MED-G (Table 4). Sequevar determinations by PCR/REA were unequivocal and reproducible. Isolates of several reference strains were obtained from more than one source (Table 3). In every case, they yielded identical PCR/REA results.

Identification of clinical isolates by PCR/REA. Studies of reference strains demonstrated that sequevar MED-C was found only in *M. bovis* BCG (Table 3). I received a set of 30 coded human *M. bovis* isolates from M. Donald Cave, University of Arkansas for Medical Sciences, Little Rock, Ark. The strains were originally isolated and characterized in San Diego, Calif. (9). The cases associated with some of these isolates are described in reference 9. PCR/REA identified 28 of these strains as sequevar MED-G and 2 of these strains (B140 and B142) as sequevar MED-C (Table 3). After PCR/REA was completed, strains B140 and B142 were shown to be *M. bovis* BCG by high-performance liquid chromatography (HPLC [13]). B140 was isolated from the urine of a man who was previously treated with intravesical BCG for bladder cancer. B142 was isolated from a spinal epidural abscess in a boy with leukemia and corresponds to patient 73 in reference 9. A third human isolate, suspected to be *M. bovis* BCG, was obtained from Kathryn M. Edwards, Vanderbilt University, Nashville, Tenn. The strain was isolated from the blood of a child with HIV infection (12). This isolate was sequevar MED-C (Table 3). It was shown to be *M. bovis* BCG by HPLC (13).

PCR/REA of nontuberculous mycobacteria. The specificity of the PCR/REA assay for the *M. tuberculosis* complex was tested by using DNA from the following 14 strains of nontuberculous mycobacteria: *M. avium* ATCC 25291^T, *M. avium* TMC 706, *M. avium* subsp. *paratuberculosis* ATCC 19698^T, *M. intracellulare* ATCC 13950^T, *M. scrofulaceum* ATCC 19981^T, *M. kansasii* ATCC 12478^T, *M. goodii* ATCC 14470^T, *M. xenopi* ATCC 19250^T, *M. leprae* ML889 (39), an *M. celatum* clinical isolate (19), an *M. szulgai* strain from a proficiency survey (College of American Pathologists, Northfield, Ill.), *M. chelonae* subsp. *abscessus* ATCC 19977^T, *M. fortuitum* subsp. *fortuitum* ATCC 6841^T, and *M. phlei* ATCC 11758^T. The DNA samples were subjected to 30 cycles of PCR using conserved

TABLE 1. Sequence results for eight *M. tuberculosis* complex strains

Species	Strain(s) ^a	Sequence ^b
<i>M. tuberculosis</i>	TMC 107, T5	LONG
<i>M. bovis</i> (not BCG)	TMC 401	LONG
<i>M. tuberculosis</i>	TMC 102 ^T , T2	MED-G
<i>M. bovis</i> (not BCG)	TMC 410 ^T	MED-G
<i>M. bovis</i> BCG	TMC 1024	MED-C
<i>M. africanum</i>	ATCC 25420 ^T	SHORT

^a Abbreviations: TMC, Trudeau Mycobacterial Culture Collection; ^T, type strain; BCG, bacillus Calmette-Guérin; ATCC, American Type Culture Collection.

^b Sequences are shown in Fig. 1. The sequence for TMC 107 is published (28). The other seven sequences were determined as part of this work.

TABLE 2. Predicted sizes of PCR products^a and of fragments of PCR products after digestion with restriction enzyme *Ban*I

Sequevar ^b	Size (bp)	
	PCR product	Fragments of PCR product
LONG	343	157, 143, 43
MED-G	328	143, 142, 43
MED-C	328	142, 128, 43, 15
SHORT	313	142, 128, 43

^a Products of PCR amplification with primers MtMPTR.541p and MtMPTR.883n.

^b A sequevar contains the strain or strains with a given sequence.

primers flanking the 16S-to-23S rDNA internal transcribed spacer (18). All 14 samples yielded PCR products of the expected sizes which appeared as bright bands on an ethidium bromide-stained agarose gel. The same DNA samples were subjected to 45 cycles of PCR in the MPTR-containing region, followed by digestion with *Ban*I. The nontuberculous mycobacteria yielded PCR products of multiple sizes which were weakly visualized on the agarose gel, probably representing arbitrary primer annealing. After digestion with *Ban*I, none of the nontuberculous mycobacteria had patterns resembling any of the four *M. tuberculosis* complex sequevars shown in Fig. 2B. As expected, PCR/REA in this MPTR-containing region was specific for the *M. tuberculosis* complex.

DISCUSSION

***M. bovis* BCG.** PCR/REA provides a rapid, sensitive, and specific test for the identification of BCG. All 23 *M. bovis* BCG strains were sequevar MED-C (Table 4). These strains included 20 reference BCG strains (27) from 12 countries (Australia, Brazil, Canada, Czechoslovakia, Denmark, England, France, Mexico, Japan, Russia, Sweden, and the United States). Fomukong et al. (14) have identified two groups of BCG strains which differ in antigen secretion, lipid structure, and IS6110 copy number. Both BCG groups were included in this study. None of the 55 *M. bovis* (not BCG) or 14 other *M. tuberculosis* complex strains were MED-C (Table 4). The *M. bovis* (not BCG) strains included isolates from humans and cattle, strains with a wide variety of IS6110 fingerprints, and strains from many geographic regions.

IS6110 fingerprinting is the most widely used method for the differentiation of *M. tuberculosis* complex strains. Though useful for tuberculosis epidemiology, IS6110 fingerprinting does not identify BCG specifically. BCG strains have two IS6110 patterns; one of these patterns is found commonly in *M. bovis* (not BCG) strains and rarely in *M. tuberculosis* strains (4, 5, 7, 14, 34). *M. bovis* BCG can be identified by HPLC and by IS1081 fingerprinting (13, 35).

Live BCG vaccine is given to newborn infants throughout the developing world. Recombinant BCG is also a potential vaccine vehicle for presentation of antigens from multiple pathogens (30). Several cases of disseminated BCG infection with bacteremia have been reported in HIV-infected patients (2, 6, 12). The prevalence of systemic BCG disease is not known, because few laboratories are capable of specifically identifying BCG. The widely used AccuProbe test (Gen-Probe, Inc., San Diego, Calif.) identifies the *M. tuberculosis* complex as a whole. MPTR PCR/REA may be useful in determining the frequency of BCG in collections of *M. tuberculosis* complex isolates from countries which use the vaccine.

M. bovis BCG was derived by serial passage of an *M. bovis*

FIG. 2. Ethidium bromide-stained 3% agarose gel of PCR products (A) and fragments of PCR products after digestion with *Ban*I (B). The strains shown are *M. tuberculosis* TMC 102^T (sequevar MED-G), *M. africanum* ATCC 25420^T (SHORT), *M. bovis* BCG Glaxo (MED-C), and *M. tuberculosis* TMC 107 (LONG). Sequevars were determined by the sizes of the PCR products and their major fragments (Table 2). The two fragments of the sequevar MED-G PCR product (143 and 142 bp) were not resolved and appear as a single bright band. The smaller fragments (43 and 15 bp, not shown) were not used for sequevar determination. The PCR products and restriction fragments from *M. tuberculosis* TMC 102^T (sequevar MED-G) and *M. tuberculosis* TMC 107 (LONG) were used in other gels as size standards.

strain (14). The parent strain is not available today (36). On the basis of MPTR sequence analysis (Fig. 1), IS1081 fingerprinting (35), and HPLC (13), the number of genetic events separating BCG from other *M. bovis* strains equals or exceeds the number of events separating all the other members of the *M. tuberculosis* complex.

***M. tuberculosis* and *M. bovis* (not BCG).** A bacterial species generally includes strains with 70% or greater DNA-DNA relatedness (37). By this criterion, all the members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. africanum*) belong to one species since they share a DNA-DNA relatedness of 85 to 100% (24). However, *M. tuberculosis* and *M. bovis* differ by their biochemical phenotypes and their pathogenicities for various hosts (3, 27, 36). *M. tuberculosis* and *M. bovis* are usually considered to be distinct taxa, either as separate species, subspecies, or biovars (3, 23, 36, 38).

Several DNA fingerprinting methods are available which differentiate *M. tuberculosis* strains (33). Surprisingly, these methods do not segregate strains of *M. tuberculosis* and *M. bovis* by species. Many *M. bovis* strains have one or two copies of IS6110, and most *M. tuberculosis* strains have six or more copies (4, 34). However, recent reports have shown that low-IS6110-copy-number *M. tuberculosis* strains (29, 33, 40) and high-copy-number *M. bovis* strains (32) are common in certain populations. Strains of *M. tuberculosis* and *M. bovis* (not BCG) do not segregate by their IS1081 fingerprints (35) or their HPLC patterns (13). Two PCR-based tests were recently proposed as *M. tuberculosis* specific. These reports only included one or two strains of *M. bovis* and must be validated (10, 11).

M. tuberculosis and *M. bovis* (not BCG) did not segregate on the basis of MPTR sequence analysis. Strains of both species belonged to both sequevars LONG and MED-G (Table 1 and Table 4), which differ by a 15-bp insertion or deletion. Either this genetic event has occurred more than once or the transition between *M. tuberculosis* and *M. bovis* has occurred more than once. *M. tuberculosis* may be a variant of *M. bovis* adapted to humans (3). *M. tuberculosis* may have arisen more than once, as various *M. bovis* strains originally acquired from cattle have propagated for many generations in humans. Many *M. bovis* isolates from humans have high IS6110 copy numbers (5, 32); these isolates may be in transition from *M. bovis* to *M. tuberculosis*. Some recent reports have identified a continuum of

TABLE 3. Strains of the *M. tuberculosis* complex analyzed by PCR/REA

Species	Strain(s) ^a	Reference
Sequevar LONG ^b		
<i>M. tuberculosis</i>	TMC 107	27
	Arkansas human isolate T5	4
<i>M. bovis</i> (not BCG)	TMC 401 (two sources)	27
Sequevar MED-G		
<i>M. tuberculosis</i>	TMC 102 ^T (two sources), 201 (three sources)	27
	Arkansas human isolates T1, T2, T3, T4	4
	Korea human isolates CSU-22, CSU-23, CSU-24, CSU-28	None ^c
<i>M. bovis</i> (not BCG)	TMC 409, 410 ^T , 412	27
	El Paso, Tex., area bovine isolates B97, B98, B99, B100, B102, B103, B107, B108, B109, B111, B112, B113	None ^d
	Other U.S. bovine isolates 485, 558, 1700, 2262, 2295, 2428	5
	San Diego, Calif., human isolates B131, B132, B133, B134, B135, B136, B137, B138, B139, B141, B143, B144, B145, B146, B147, B148, B149, B150, B151, B152, B153, B154, B155, B156, B157, B158, B159, B161	9
	Switzerland human isolates 243, 2072, 4306, 4401, 10983	5
<i>M. microti</i>	ATCC 19422 ^T	1
Sequevar MED-C		
<i>M. bovis</i> BCG	TMC 1002, 1009, 1010, 1012, 1013, 1019, 1020, 1021, 1022, 1024 (2 sources), 1025, 1028, 1029, 1030, 1101, 1103, 1108	27
	ATCC 19274, 27290, 35734 (two sources)	1
	San Diego, Calif., human isolates B140, B142	9
	Nashville, Tenn., human isolate KE1003	12
Sequevar SHORT		
<i>M. africanum</i>	ATCC 25420 ^T	1

^a As indicated, some of the reference strains were obtained from multiple sources. Strains from multiple sources were counted as single strains. *M. bovis* BCG is actually a strain, not a species. The BCG strains listed could be considered "substrains."

^b Strains are grouped by MPTR sequevars as determined by PCR/REA.

^c Obtained from John T. Belisle and Patrick J. Brennan, Colorado State University.

^d Obtained from M. Donald Cave, University of Arkansas for Medical Sciences.

phenotypes in human isolates ranging from classical *M. bovis* to classical *M. tuberculosis* with intermediate phenotypes (8, 23).

***M. africanum*.** The species *M. africanum* is poorly defined with no phenotypic features clearly setting it apart from the other members of the *M. tuberculosis* complex (27, 36). Some authors have questioned its status as a separate species (27, 38). *M. africanum* ATCC 25420^T was isolated from the sputum of a Senegalese patient with tuberculosis (38). By MPTR sequence analysis and PCR/REA, it belongs to sequevar SHORT, a sequevar not found in 91 other strains of the *M. tuberculosis* complex. PCR/REA may be useful in providing a species definition for *M. africanum*. This result needs to be confirmed in other *M. africanum* strains, and it should also be supported by phenotypic characteristics (37).

MPTR. The *M. tuberculosis* genome has about 80 MPTR-containing regions (22). The function of this common repetitive DNA is not known. The region studied is part of a large open reading frame. All three mutations identified (Fig. 1) preserve the open reading frame. It is not known whether this

open reading frame is expressed as a protein (28). The most common mutations were insertions or deletions of 15-bp tandem repeat units (Fig. 1). Each insertion or deletion was flanked by a repeat unit with identical or nearly identical DNA sequence (0 to 2 mismatches out of 15). In contrast, adjacent repeat units in other portions of this MPTR region varied substantially (up to 9 mismatches out of 15). Similarly, insertions of 3 and 6 bp in the *M. tuberculosis rpoB* gene are each flanked by identical sequence (25). These events are consistent with strand slippage, though homologous recombination is an alternate explanation (20).

Other bacterial genes contain repetitive DNA and have similar polymorphisms in the numbers of repeat units (15, 21). Length polymorphisms in repetitive DNA regions may be generally useful for bacterial strain differentiation. Such polymorphisms have been associated with virulence phenotypes in a *Xanthomonas campestris* pathovar vesicatoria avirulence gene (21) and in the *Staphylococcus aureus* protein A gene (15). The *M. tuberculosis* complex strains analyzed in the present study have varied virulence phenotypes (27), but these did not appear to be associated with the number of MPTR units.

The sequence variability among *M. tuberculosis* complex strains was not sufficient for formal phylogenetic analysis. MPTR-containing regions which are not part of open reading frames may have more sequence variability.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI07392 and AI35230, the Durham VA Medical Center's Research Center on AIDS and HIV Infection, and the Department of Veterans Affairs.

I thank the following individuals who provided bacterial strains or bacterial DNA used in this study: M. Donald Cave and Kathleen D. Eisenach, University of Arkansas for Medical Sciences; Suzanne Glickman and W. Ray Butler, Centers for Disease Control and Prevention;

TABLE 4. Summary of MPTR sequevars of 92 *M. tuberculosis* complex strains, as determined by PCR/REA

Species (no. of strains)	No. of strains with MPTR sequevar			
	LONG	MED-G	MED-C	SHORT
<i>M. tuberculosis</i> (12)	2	10	0	0
<i>M. bovis</i> (not BCG) (55)	1	54	0	0
<i>M. microti</i> (1)	0	1	0	0
<i>M. bovis</i> BCG (23)	0	0	23	0
<i>M. africanum</i> (1)	0	0	0	1
Total (92)	3	65	23	1

John T. Belisle and Patrick J. Brennan, Colorado State University; Kathy H. McDonald, Nancy Myers, and Peter Zwadyk, Jr., Durham VA Medical Center; Celeste M. McKnight and Robert P. Gruninger; Duke University Medical Center; Phyllis Pienta, American Type Culture Collection; Kathryn M. Edwards, Vanderbilt University; Rodrick J. Chiodini, Brown University; and Thomas P. Gillis and Diana L. Williams, GWL Hansen's Disease Center at Louisiana State University. I thank Margaret M. Floyd and W. Ray Butler, Centers for Disease Control and Prevention, for performing HPLC to identify the clinical *M. bovis* BCG isolates. I thank Kenneth H. Wilson for helpful discussions and advice.

ADDENDUM IN PROOF

Similar work was recently published (M. Goyal, D. Young, Y. Zhang, P. A. Jenkins and R. J. Shaw, *J. Clin. Microbiol.* 32:3070–3071, 1994). Length polymorphisms in a region of repetitive DNA upstream from the *katG* gene were used to differentiate *M. tuberculosis* strains.

REFERENCES

1. American Type Culture Collection. 1992. Catalogue of bacteria and bacteriophages, 18th ed. American Type Culture Collection, Rockville, Md.
2. Armbruster, C., W. Junker, N. Vetter, and G. Jaksch. 1990. Disseminated bacille Calmette-Guérin infection in an AIDS patient 30 years after BCG vaccination. *J. Infect. Dis.* 162:1216. (Letter.)
3. Bates, J. H., and W. W. Stead. 1993. The history of tuberculosis as a global epidemic. *Med. Clin. North Am.* 77:1205–1217.
4. Cave, M. D., K. D. Eisenach, P. F. McDermott, J. H. Bates, and J. T. Crawford. 1991. IS6110: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. *Mol. Cell. Probes* 5:73–80.
5. Cave, M. D., K. D. Eisenach, M. Salfinger, J. H. Bates, and J. T. Crawford. 1992. Usefulness of IS6110 in fingerprinting DNA of *Mycobacterium bovis*. *Med. Microbiol. Lett.* 1:96–102.
6. Centers for Disease Control. 1985. Disseminated *Mycobacterium bovis* infection from BCG vaccination of a patient with acquired immunodeficiency syndrome. *Morbidity Mortal. Weekly Rep.* 34:227–228.
7. Chevreil-Dellagi, D., A. Abderrahman, R. Haltiti, H. Koubaji, B. Gicquel, and K. Dellagi. 1993. Large-scale DNA fingerprinting of *Mycobacterium tuberculosis* strains as a tool for epidemiological studies of tuberculosis. *J. Clin. Microbiol.* 31:2446–2450.
8. Collins, C. H., M. D. Yates, and J. M. Grange. 1982. Subdivision of *Mycobacterium tuberculosis* into five variants for epidemiological purposes: methods and nomenclature. *J. Hyg. Camb.* 89:235–242.
9. Dankner, W. M., N. J. Waecker, M. A. Essey, K. Moser, M. Thompson, and C. E. Davis. 1993. *Mycobacterium bovis* infections in San Diego: a clinico-epidemiologic study of 73 patients and a historical review of a forgotten pathogen. *Medicine* 72:11–37.
10. del Portillo, P., L. A. Murillo, and M. E. Patarroyo. 1991. Amplification of a species-specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. *J. Clin. Microbiol.* 29:2163–2168.
11. de Wit, D., L. Steyn, S. Shoemaker, and M. Sogin. 1990. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. *J. Clin. Microbiol.* 28:2437–2441.
12. Edwards, K. M., R. Voladri, B. Menzies, and D. S. Kernodle. 1994. How safe is routine BCG immunization in HIV-infected children? *Clin. Infect. Dis.* 19:605, abstr. 252.
13. Floyd, M. M., V. A. Silcox, W. D. Jones, Jr., W. R. Butler, and J. O. Kilburn. 1992. Separation of *Mycobacterium bovis* BCG from *Mycobacterium tuberculosis* and *Mycobacterium bovis* by using high-performance liquid chromatography of mycolic acids. *J. Clin. Microbiol.* 30:1327–1330.
14. Fomukong, N. G., J. W. Dale, T. W. Osborn, and J. M. Grange. 1992. Use of gene probes based on the insertion sequence IS986 to differentiate between BCG vaccine strains. *J. Appl. Bacteriol.* 72:126–133.
15. Frénay, H. M. E., J. P. G. Theelen, L. M. Schouls, C. M. J. E. Vandenbroeke-Grauls, J. Verhoef, W. J. van Leeuwen, and F. R. Mooi. 1994. Discrimination of epidemic and non-epidemic methicillin-resistant *Staphylococcus aureus* strains on the basis of protein A gene polymorphism. *J. Clin. Microbiol.* 32:846–847.
16. Frothingham, R. 1994. Sequence-based strain differentiation in the *Mycobacterium tuberculosis* complex, including rapid identification of *M. bovis* BCG. *Clin. Infect. Dis.* 19:565, abstr. 10.
17. Frothingham, R., H. G. Hills, and K. H. Wilson. 1994. Extensive DNA sequence conservation throughout the *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* 32:1639–1643.
18. Frothingham, R., and K. H. Wilson. 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *J. Bacteriol.* 175:2818–2825.
19. Frothingham, R., and K. H. Wilson. 1994. Molecular phylogeny of the *Mycobacterium avium* complex demonstrates clinically meaningful divisions. *J. Infect. Dis.* 169:305–312.
20. Groenen, P. M. A., A. E. Bunschoten, D. van Soolingen, and J. D. A. van Embden. 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Mol. Microbiol.* 10:1057–1065.
21. Herbers, K., J. Conrads-Strauch, and U. Bonas. 1992. Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. *Nature (London)* 356:172–174.
22. Hermans, P. W. M., D. van Soolingen, and J. D. A. van Embden. 1992. Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium goodii*. *J. Bacteriol.* 174:4157–4165.
23. Hoffner, S. E., S. B. Svenson, R. Norberg, F. Dias, S. Ghebremichael, and G. Källenius. 1993. Biochemical heterogeneity of *Mycobacterium tuberculosis* complex isolates in Guinea-Bissau. *J. Clin. Microbiol.* 31:2215–2217.
24. Imaeda, T. 1985. Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. *Int. J. Syst. Bacteriol.* 35:147–150.
25. Kapur, V., L.-L. Li, S. Iordanesco, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser. 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase β subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J. Clin. Microbiol.* 32:1095–1098.
26. Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. C. Bange, and E. C. Böttger. 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J. Clin. Microbiol.* 31:2882–2889.
27. National Institutes of Health. 1980. *Mycobacterial culture collection*. NIH publication no. 80-289. National Institutes of Health, Bethesda, Md.
28. Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J. Bacteriol.* 169:1080–1088.
29. Small, P. M., P. C. Hopewell, S. P. Singh, A. Paz, J. Parsonnet, D. C. Ruston, G. F. Schecter, C. L. Daley, and G. K. Schoolnik. 1994. The epidemiology of tuberculosis in San Francisco. *N. Engl. J. Med.* 330:1703–1709.
30. Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom. 1991. New use of BCG for recombinant vaccines. *Nature (London)* 351:456–460.
31. Takewaki, S.-L., K. Okuzumi, I. Manabe, M. Tanimura, K. Miyamura, K.-I. Nakahara, Y. Yazaki, A. Ohkubo, and R. Nagai. 1994. Nucleotide sequence comparison of the mycobacterial *dhx1* gene and PCR-restriction fragment length polymorphism analysis for identification of mycobacterial species. *Int. J. Syst. Bacteriol.* 44:159–166.
32. van Soolingen, D., P. E. W. de Haas, J. Haagsma, T. Eger, P. W. M. Hermans, R. Ritacco, A. Alito, and J. D. A. van Embden. 1994. Use of various genetic markers in differentiation of *Mycobacterium bovis* strains from animals and humans and for studying epidemiology of bovine tuberculosis. *J. Clin. Microbiol.* 32:2425–2433.
33. van Soolingen, D., P. E. W. de Haas, P. W. M. Hermans, P. M. A. Groenen, and J. D. A. van Embden. 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 31:1987–1995.
34. van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden. 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J. Clin. Microbiol.* 29:2578–2586.
35. van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, and J. D. A. van Embden. 1992. Insertion element IS1081-associated restriction fragment length polymorphisms in *Mycobacterium tuberculosis* complex species: a reliable tool for recognizing *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* 30:1772–1777.
36. Wayne, L. G. 1982. Microbiology of tubercle bacilli. *Am. Rev. Respir. Dis.* 125(Suppl.):31–41.
37. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37:463–464.
38. Wayne, L. G., and G. P. Kubica. 1986. The mycobacteria, p. 1435–1457. In P. H. A. Sneath and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore.
39. Williams, D. L., T. P. Gillis, and F. Portaels. 1990. Geographically distinct isolates of *Mycobacterium leprae* exhibit no genotypic diversity by restriction fragment-length polymorphism analysis. *Mol. Microbiol.* 4:1653–1659.
40. Yuen, L. K. W., B. C. Ross, K. M. Jackson, and B. Dwyer. 1993. Characterization of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization. *J. Clin. Microbiol.* 31:1615–1618.