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Identification of a Polymorphic Nucleotide in *oxyR* Specific for *Mycobacterium bovis*

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Automated sequence analysis of a 410-bp region of the *oxyR* gene in 105 *Mycobacterium tuberculosis* complex isolates identified a polymorphic nucleotide that differentiated *Mycobacterium bovis* isolates from other complex members. All 29 *M. bovis* isolates sequenced had an adenine residue at nucleotide 285, whereas all 76 other complex isolates had a guanine residue. PCR-restriction fragment length polymorphism analysis of *oxyR* with restriction endonuclease *Alu*I in an additional 255 complex isolates from widespread intercontinental sources confirmed and extended the unique association of adenine at position 285 with *M. bovis* isolates.

The four members of the *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*) share 85 to 100% DNA-DNA relatedness (9). Differentiation among the members is currently based mainly on analysis of phenotypic characteristics such as colony morphology, growth rate, and biochemical tests (7, 14). In part because of their close genetic relatedness and slow growth, species assignment is time-consuming and difficult.

Several phenotypic and genotypic strategies have been used in an attempt to differentiate among members of the *M. tuberculosis* complex. Although characterization of mycolic acid profiles by high-performance liquid chromatography has sometimes been useful for the differentiation of *M. bovis* BCG isolates from *M. bovis* non-BCG isolates and other members of the complex (4), this technique requires substantial bacterial growth, can be labor intensive, and is not widely available. In addition, the technique does not distinguish among the four species of the *M. tuberculosis* complex. Several nucleic acid-based strategies such as restriction fragment length polymorphism (RFLP) analysis or sequencing of target genes (5, 10, 16, 18) or commercially available test systems (Accuprobe; Gen-Probe, San Diego, Calif.) are unable to unambiguously differentiate among *M. tuberculosis* complex species. More recently, differentiation among the members of *M. tuberculosis* complex has been attempted by using random amplified polymorphic DNA analysis (17) and amplification of *M. tuberculosis*-specific sequences (20). Although some success has been achieved in distinguishing *M. tuberculosis* (strict sense) (20) and *M. bovis* (17) from other *M. tuberculosis* complex organisms, relatively few samples have been analyzed. Inasmuch as it is sometimes important for public health reasons to distinguish *M. bovis* isolates from other *M. tuberculosis* complex species, a rapid and unambiguous differentiation strategy would be useful.

In *Escherichia coli* and *Salmonella typhimurium*, *oxyR* functions as a sensor and a transcriptional regulator of proteins involved in the oxidative stress response (3). The homolog of the *oxyR* gene in *M. tuberculosis* complex organisms contains numerous deletions and frameshift mutations (2, 19). Because *oxyR* in *M. tuberculosis* isolates probably does not encode a functional protein (2, 19), it is referred to as a pseudogene. Inasmuch as pseudogenes accumulate mutations at an increased rate compared with functional genes (12, 13), we thought it possible that *oxyR* might contain polymorphic nucleotide sites useful for differentiating among the closely related members of the *M. tuberculosis* complex (11). In this report we describe a polymorphic nucleotide located at position 285 of the *oxyR* gene that differentiates *M. bovis* from other complex members.

Bacterial strains. Three hundred sixty isolates of the *M. tuberculosis* complex were analyzed, including samples of *M. microti* ($n = 9$ isolates), *M. africanum* ($n = 11$), *M. bovis* ($n = 124$), and *M. tuberculosis* strict sense ($n = 214$). Two *M. bovis* BCG strains were also studied. The *M. bovis* isolates were recovered in California ($n = 23$ isolates), Colorado ($n = 2$), Hawaii ($n = 1$), Kansas ($n = 1$), Maryland ($n = 2$), Montana ($n = 5$), New Mexico ($n = 2$), New York ($n = 3$), Texas ($n = 24$), Virginia ($n = 1$), Canada ($n = 22$), Mexico ($n = 13$), The Netherlands ($n = 5$), Switzerland ($n = 6$), and unknown localities ($n = 14$). These organisms were cultured from a variety of hosts, including humans ($n = 47$ isolates), bison ($n = 6$), cattle ($n = 52$), cougars ($n = 2$), domestic cats ($n = 2$), seals ($n = 2$), elk ($n = 7$), and deer ($n = 6$). The *M. bovis* sample included organisms with a range of IS*6110* copy numbers (0 to 19 copies) and patterns. The *M. microti* specimens were recovered from voles ($n = 7$), a pig ($n = 1$) in The Netherlands, and a rock hyrax ($n = 1$) in South Africa. The *M. africanum* isolates

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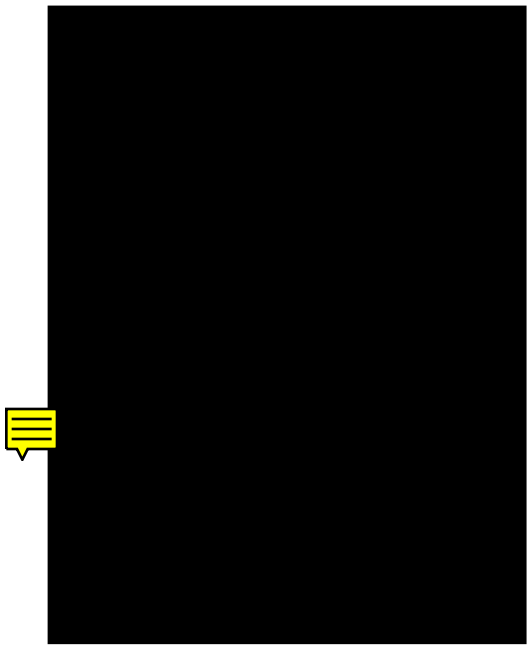

FIG. 1. Identification of polymorphism at *oxyR* nucleotide 285. (A) DNA sequencing chromatogram of the region with a polymorphism located at nucleotide 285 in *oxyR*. The numbers noted on top of the peak tracings and below the nucleotides in the chromatograms are arbitrary marker positions and have no relevance to the actual nucleotide positions of *oxyR*. The *M. bovis* isolates studied had an adenine at nucleotide 285, whereas all other *M. tuberculosis* complex isolates studied had a guanine. (B) Results of a PCR-RFLP strategy to detect variation at *oxyR* nucleotide 285. A 548-bp segment of *oxyR* was amplified by PCR and was digested with *Alu*I. Even-numbered lanes contain the amplified gene segment, and odd-numbered lanes have the same samples treated with *Alu*I. Lanes 1 and 3, digested DNAs from a representative *M. bovis* isolate and *M. bovis* BCG (Brazil), respectively. Lanes 5, 7, 9, 11, and 13, digested DNAs from H37Ra, H37Rv, a clinical isolate of *M. tuberculosis*, *M. microti*, and *M. africanum*, respectively.

were recovered from humans with tuberculosis in Sierra Leone.

The 214 *M. tuberculosis* strains were obtained from widespread global localities including Brazil ($n = 9$ isolates), Chile ($n = 14$), Ecuador ($n = 1$), Mexico ($n = 14$), Venezuela ($n = 8$), Trinidad-Tobago ($n = 2$), India ($n = 1$), Japan ($n = 3$), Mongolia ($n = 3$), Nepal ($n = 1$), Philippines ($n = 6$), South Korea ($n = 3$), Thailand ($n = 4$), Tibet ($n = 1$), Vietnam ($n = 3$), Germany ($n = 1$), Italy ($n = 1$), The Netherlands ($n = 5$), Portugal ($n = 1$), Romania ($n = 9$), Spain ($n = 3$), Switzerland ($n = 4$), former Yugoslavia ($n = 2$), Israel ($n = 10$), Turkey ($n = 2$), Algeria ($n = 1$), Guinea ($n = 2$), Kenya ($n = 20$), Somalia ($n = 1$), Yemen ($n = 4$), Zaire ($n = 1$), and different regions of the United States including California ($n = 4$ isolates), Colorado ($n = 3$), New York City ($n = 20$), and Texas ($n = 47$). This collection of *M. tuberculosis* isolates represents the range of IS*6110* fingerprints diversity recently described in the species (8, 20a) and includes organisms recovered from patients with pulmonary and extrapulmonary tuberculosis. The organisms in the sample had from 0 to 21 copies of IS*6110* (22). In addition, the isolates include members of three genetic categories into which all *M. tuberculosis* isolates can be assigned on the basis of sequence polymorphisms located in *katG* and *gyrA* (21). Moreover, organisms classified into several groups on the basis of a multiplex PCR analysis (15) were included in the analysis.

Species assignment of isolates of *M. tuberculosis* strict sense, *M. bovis*, and *M. microti* were made on the basis of analysis of accepted phenotypic criteria (7, 14). Some of the *M. bovis* isolates recovered from humans have been described previously (1). No simple set of phenotypic features that can be used to differentiate *M. africanum* from other *M. tuberculosis* complex members is generally accepted. The organisms that we studied were identified as *M. africanum* on the basis of the results of an extensive panel of phenotypic criteria used at the Armauer-Hansen-Institut, Wurzburg, Germany. In addition to other criteria used for classification, these organisms did not grow in the presence of 1 mg of thioxyphene-2-carboxylic acid hydrazide per ml and were susceptible to pyrazinamide (24).

Automated DNA sequencing of the *oxyR* locus. The DNA sequence of a 410-bp region of *oxyR* was analyzed in 105 organisms, including *M. tuberculosis* ($n = 60$ isolates), *M. bovis* ($n = 29$), *M. microti* ($n = 9$), and *M. africanum* ($n = 7$). A 548-bp segment of *oxyR* was amplified with the following oligonucleotide primers: forward primer, 5′-GGTGATATATCA CACCATA-3′; reverse primer, 5′-CTATGCGATCAGGCGT ACTTG-3′. A GeneAmp System 9600 thermocycler (Perkin-Elmer Corp, Foster City, Calif.) was used with the following parameters: annealing temperature of 55°C for 21 s, extension at 72°C for 22 s, and a denaturation step at 94°C for 21 s. Each reaction was preceded by an initial denaturation step at 94°C for 60 s and was terminated with a final extension step at 72°C for 5 min. DNA sequencing reactions were performed with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.), and data were generated with an ABI 373A automated instrument. The sequence data were assembled and edited electronically with the EDITSEQ, ALIGN, and MEGALIGN programs (DNASTAR, Madison, Wis.) and were compared with a published sequence (19) for *oxyR* (GenBank accession number U18263).

The *oxyR* analysis showed that all 29 *M. bovis* isolates had an adenine at position 285. In striking contrast, all 76 other *M. tuberculosis* complex (*M. tuberculosis*, *M. africanum*, and *M. microti*) strains sequenced had a guanine at position 285 (Fig. 1A). With the exception of an occasional rare nucleotide substitution, no additional sequence variation was found in the 410-bp region studied.

PCR-RFLP analysis of allelic variation at *oxyR* nucleotide position 285. Inasmuch as the sequence data showed that all *M. bovis* isolates had an adenine, whereas other *M. tuberculosis* complex isolates had a guanine, at position 285 of *oxyR*, a PCR-RFLP strategy was designed to simplify the differentiation of allelic variation at this polymorphic site. A 548-bp fragment of *oxyR* containing nucleotide 285 was amplified by PCR as described above. The PCR product (10 μl) was digested with 4 U of *Alu*I (New England Biolabs, Beverly, Mass.), a restriction enzyme that cleaves at an AGCT sequence. The *oxyR* gene of *M. bovis* has an AGCT sequence beginning at the adenine residue located at nucleotide 285. Digestion was carried out at 37°C for 90 min, and the resulting DNA fragments were electrophoretically separated with a 1.8% agarose gel containing 0.03% ethidium bromide at a constant voltage of 90 V for approximately 2 h. Digested and undigested samples from each strain were electrophoresed in adjacent lanes. The DNA bands were visualized with a UV transilluminator and were documented by photography. There are three *Alu*I restriction sites in the 548-bp sequence of *oxyR* in *M. tuberculosis* complex organisms with a guanine at nucleotide 285. Digestion with *Alu*I should yield four DNA fragments of 236, 227, 55, and 30 bp. However, the electrophoretic conditions used in the present study were formulated to identify only one band at about 230 bp for DNA samples from *M. tuberculosis* complex organisms. This band represents both the

236-bp and the 227-bp *oxyR* DNA fragments. The *M. bovis* isolates (with an adenine at nucleotide 285) have four restriction sites for *Alu*I in the amplified *oxyR* gene segment, and therefore, five DNA fragments (236, 148, 79, 55, and 30 bp) should be produced. However, the electrophoretic conditions used identified a three-band pattern of 236, 148, and 79 bp for all *M. bovis* isolates (Fig. 1B).

A total of 255 *M. tuberculosis* complex strains were analyzed by this method, including 95 isolates of *M. bovis*, 2 strains of *M. bovis* BCG, 154 isolates of *M. tuberculosis*, and 2 isolates each of *M. africanum* and *M. microti*. All *M. bovis* isolates, including the two *M. bovis* BCG strains, had the expected three-band pattern (Fig. 1B). In contrast, all other *M. tuberculosis* complex isolates had the expected one-band pattern.

In preliminary studies, isolates in five early BACTEC (Becton-Dickinson, Sparks, Md.) cultures with growth indices of between 50 and 120 were also analyzed for the PCR-RFLP pattern of *oxyR*. DNA was extracted from these cultures, and isolates were assigned to the *M. tuberculosis* complex by *hsp65* sequence analysis as described previously (10). The isolates in all BACTEC cultures studied yielded the one-band pattern.

Our analysis of polymorphic nucleotide 285 in the *oxyR* genes of 360 *M. tuberculosis* complex organisms from widespread geographic sources and host species was 100% sensitive and specific for distinguishing *M. bovis* isolates from other complex members. The data indicate that use of either direct sequence analysis or a PCR-RFLP strategy to identify polymorphism at nucleotide 285 in *oxyR* reliably differentiates *M. bovis* from other *M. tuberculosis* complex members. Analysis of *oxyR* nucleotide 285 variation does not differentiate *M. bovis* BCG isolates from *M. bovis* non-BCG isolates. Although our data show 100% specificity of the nucleotide at position 285 for *M. bovis* among the 360 strains studied, it is possible that analysis of larger numbers of samples will identify organisms for which this association breaks down. No reasonable amount of strain sampling can permit us to rule out this possibility. However, it is clear that these organisms will be relatively rare or confined to restricted geographic localities or host species.

Epidemiologic investigations (6, 23) and clinical decisions are sometimes based on phenotypic classification of *M. tuberculosis* complex organisms. Molecular strategies for assignment of *M. tuberculosis* complex organisms to a particular species have largely been unsuccessful because of their lack of resolution and requirement for sophisticated and expensive equipment (4, 10, 18). Other *M. bovis*-specific (17) and *M. tuberculosis* (strict sense)-specific (20) methods have been described, but they have not been applied to analyses of large samples of isolates from diverse geographic localities. The PCR-RFLP method used to identify the *M. bovis*-specific *oxyR* nucleotide 285 is inexpensive, rapid, highly sensitive, and specific. Preliminary results indicate that the methodology is also applicable to DNA extracted from early BACTEC culture specimens. Although our study did not analyze other specimen types, it is possible that this method could also be successfully applied to some primary clinical specimens, but additional studies are required to address this issue. Analysis of *oxyR* nucleotide 285 polymorphism could also be used to obtain precise measures of prevalence and incidence rates for human infections caused by *M. bovis* isolates. The strategy would be especially useful in areas of Africa, South and Central America, Southeast Asia, and other regions where the transmission of *M. bovis* from animals to humans and vice versa can occur or is a suspected problem. The ability of this method to distinguish *M. bovis* BCG strains from *M. tuberculosis* (strict sense) isolates will also be useful in the characterization of slowly growing mycobacteria recovered from patients treated with *M. bovis* BCG for bladder cancer or some BCG-immunized immunocompromised patients with tuberculosis.

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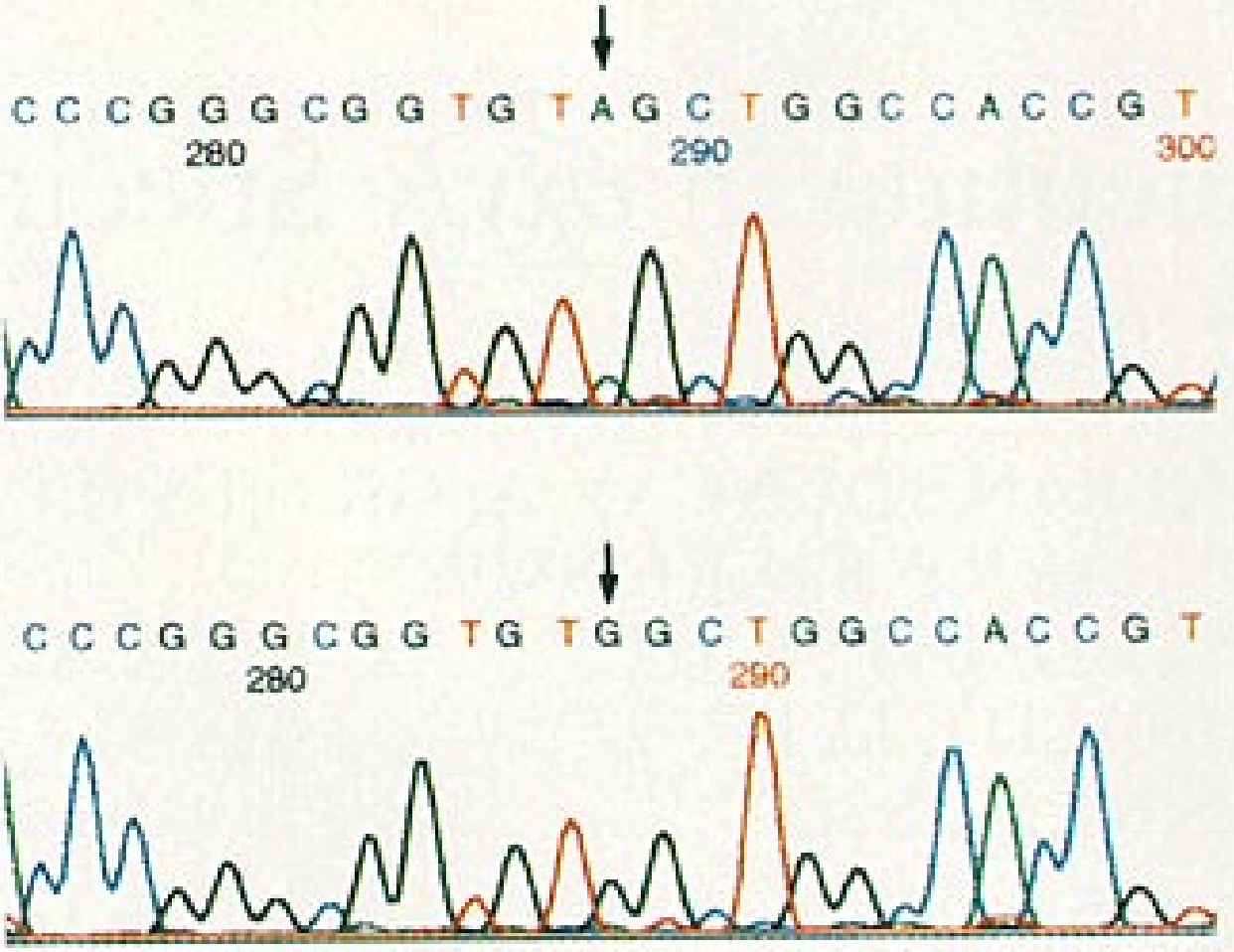
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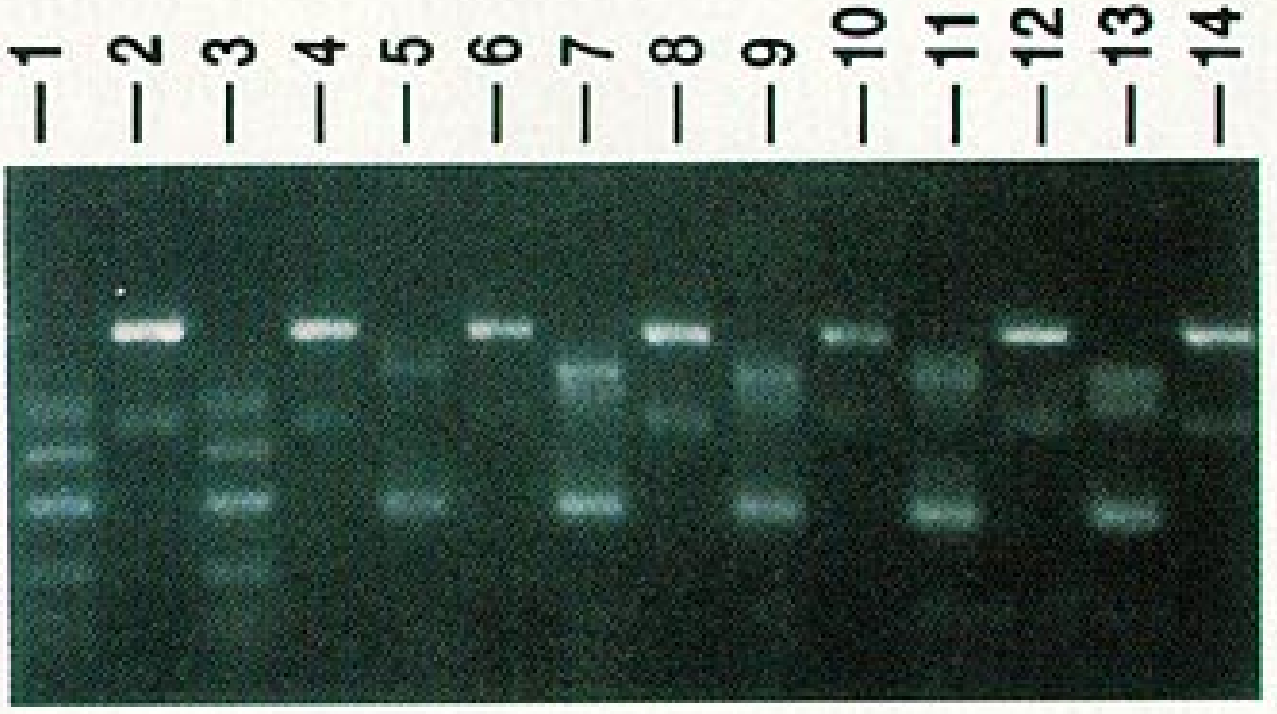
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