DNA ANALYSIS OF TUBERCULOSIS:

DIAGNOSIS AND PREVENTION

A Senior Thesis

By

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DNA Analysis of Tuberculosis: Diagnosis and Prevention

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ABSTRACT

DNA Analysis of Tuberculosis: Diagnosis and Prevention.

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The continued threat of tuberculosis (TB) to animals and humans is complicated by inefficient or ineffective methods of diagnosis and prevention. The high similarity among mycobacteria makes differentiation of strains and species difficult and laborious. Members of the *Mycobacterium tuberculosis* complex, which include the primary agents of human and bovine tuberculosis, share over 90% DNA identity and are indistinguishable by most modern methods. Additionally, the current vaccine for TB is of questionable efficacy. In this study we demonstrate the use of Random Amplified Polymorphic DNA (RAPD) as a means of typing TB isolates and as the potential source of a *Mycobacterium bovis*-specific PCR assay, arising from a species-specific polymorphism upstream of an acyl-CoA synthase. We also have developed a technique for the identification of antigenic recombinant polypeptide sequences from *M. bovis* that may be of potential worth as vaccines, on the basis of delayed-type hypersensitivity (DTH) responses by infected cattle.

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ACKNOWLEDGMENTS

I would like to thank Dr. Allison Ficht for her help, guidance and understanding during the course of this research. Also, my deepest thanks and appreciation go out to Dr. Roger Smith III for his work with the skin testing and biopsies and providing the impetus for our antigen research; to Dr. L. Garry Adams for all his help and resources in the RAPD experiments; to Dr. David N. McMurray for his guidance and motivation, and for the mycobacterial isolates; to Dr. Thomas Ficht, for his advice and assistance; to Veera Shankar Perumaalla, for all his help with RAPD; and to Dr. Barbara Ruef, for answering all my technical questions. Thanks to the Fellows Program for the opportunity to develop this project in this context.

INTRODUCTION

RESURGENCE AND PATHOGENESIS OF TUBERCULOSIS

Tuberculosis (TB) affects over one third of the world's population and is the leading cause of death from a single infectious agent. It is responsible for 3 million deaths annually, and is especially prevalent in developing nations (*1*). The AIDS epidemic has accompanied a correlated resurgence of TB worldwide (*2*), as have new drug resistant strains. Besides human hosts, TB occurs in cattle, deer, birds, pigs, possums and badgers, and represents a tremendous cost to ranchers.

TB is caused by a number of bacterial species of the genus *Mycobacterium*, which also includes the bacterium responsible for leprosy. Mycobacteria are slow-growing, fastidious acid-fast bacilli, characterized by a high mycolic acid content in the cell wall. Among the mycobacteria, the four members of the *Mycobacterium tuberculosis* complex are highly similar: *M. tuberculosis, M. bovis, M. africanum*, and *M. microti* are genetically nearly identical and are difficult to distinguish. Most commonly, *M. bovis* causes TB in cattle, and *M. tuberculosis* infects humans, but the two can cross-infect, further complicating source identification.

Transmission of *M. tuberculosis* is usually accomplished by aerosol infection, mediated by the droplet nucleus, which consists of two or three viable bacteria. *Mycobacterium bovis* is most commonly spread from cattle to humans through ingestion of infected (unpasteurized) milk or to other cattle by aerosolization. Human-to-cattle transmission may also occur via urinary tract transmission, commonly as a contaminant of hay (*2*). Human-to-human transmission of *M. bovis* is relatively well understood. Additionally, mycobacteria viable in the soil may cause infection, implying a commensal relationship with birds, perhaps

through *M. avium* transmission at the feedlot. We have applied a method of elucidating DNA polymorphisms to the problem of identifying the source species of a TB isolate.

The pathogenesis of pulmonary TB is a complicated process (*3*). Following primary infection, *M. tuberculosis* initially resides in the alveolar spaces of the lung. By recruiting macrophages and other monocytes with chemotactic factors, tubercle bacilli are ingested by phagocytic cells of the host and are transported to the lymph nodes, where they are released as the monocytes die. A stage of logarithmic growth ensues, during which non-specifically activated macrophages harbor multiplying mycobacteria. It has been shown that infection with *M. tuberculosis* induces apoptosis (programmed cell death) of alveolar macrophages by interference with cellular signaling, and that this induction decreases with increased virulence, suggesting that the ability to avoid triggering apoptosis could be a mycobacterial virulence factor (*4*). Cell-mediated immunity and delayed-type hypersensitivity (DTH) begin about three weeks after infection; activated macrophages are finally able to destroy some mycobacteria, ending logarithmic growth. At this point, primary infection is concluded and usually does not progress to disease. This is the state of approximately one third of the world's population; injection of purified protein derivative (PPD; see below) at this stage results in the swelling and induration known as a positive tuberculin test. Secondary infection may occur in two ways: *reactivation* tuberculosis may occur spontaneously years to decades later, or exposure to exogenous mycobacteria may lead to reinfection and disease (*2*).

Antigen-Specific Immunity

"Tuberculin" was identified by Koch in 1891 as an attempt to isolate the released components of *M. tuberculosis* in culture (*1*). His heat-inactivated concentrate of excreted factors elicited a characteristic delayed reaction upon subcutaneous injection in tuberculous patients. The active ingredients of tuberculin were later identified as the ammonium sulfate-

precipitable protein fraction of the culture filtrate, or "purified protein derivative", PPD. Today, PPD remains the standard skin test antigen used to detect exposure to tuberculosis in humans and animals.

During DTH, previously sensitized T cells are mobilized to the site of injection. Proliferation of T cells occurs in response to mycobacterial antigens at the skin test site and produces predominantly Th1 cytokines (IL-1, IL-10, TNF, TGF-ß, etc.). Inflammation and induration result from the release of vasoactive factors from mast cells and basophils under the influence of factors released from the sensitized lymphocytes and is characterized by infiltration of the site with mononuclear cells (*5*). DTH does occur with protective immunity, but is not a definitive measure of protection; it is, however, a good indication of protective potential.

The T cell response is what is referred to as the "cellular" response, as opposed to "humoral" reaction of B cells, or "antibody" response. In terms of vaccination against TB, the cell-mediated response is the important reaction, mobilizing mononuclear phagocytes and T lymphocytes. Vaccination against TB has been performed traditionally with a live, attenuated strain of *Mycobacterium bovis*, known as bacille Calmette-Guérin (BCG), although the actual efficacy of BCG vaccination is variable (*1*).

Immunological protection against TB has been induced in laboratory animals using surface antigens or heat shock proteins of the virulent mycobacteria (*6*). Many of the antigens of mycobacteria, especially the heat shock family, are highly conserved throughout the genus and especially among the *M. tuberculosis* complex, complicating attempts at immunization against a particular species. We have developed a screen for T cell antigens on the basis of DTH reactivity to identify polypeptide sequences from *M. bovis* that may present new options for conferring immunity to TB.

Antibody formation in response to introduction of protein molecules is directed by the conformational aspects of the antigen; i.e., the intact or native molecule. T cell mediated reactions, such as DTH, respond to sequential determinants of the antigen (8-14 amino acids), which are produced after denaturation by macrophage processing (*9*). This implies that a partial sequence of a polypeptide antigen may be sufficient to also elicit a T cell response, whereas the complete sequence would probably be necessary to direct folding into the B cell-recognizable conformation.

In order to devise a screen for T cell antigens, then, complete gene products are not required; in fact, completely synthetic poly- or oligopeptides may be used. We have adapted to this problem a technique which relies on the production of polypeptide sequences by the translational machinery of bacteria; the translation products are coded for by DNA from a library of clones representing the genome or cDNAs of an organism. By inducing translation of genomic clones or pools of clones isolated on an agar plate and transferring these products to a nitrocellulose membrane, we can isolate expression products, dissolve the nitrocellulose, and present a solution containing arbitrary polypeptides from the *M. bovis* genome to TB-infected cattle and test for immunoreactivity.

Polypeptide Expression and Presentation

The ZAP Express™ Vector Kit (Stratagene®, La Jolla, CA) allows DNA of interest to be ligated into the DNA of an engineered phage λ, and then packaged into virulent phage particles. By ligating size-selected fragments of a genome into the ZAP Express™ vector, a genomic library of an organism may be prepared, consisting of arbitrary, "gene-sized" clones. Phage can then be used to infect *Escherichia coli*, from which expression of the DNA clones is performed by the machinery of the bacterial cell prior to lysis by the phage. The *lac* promoter (P_{lac}) drives expression of the cloned DNA in prokaryotic cells; presence

of the LacI protein represses expression, but can be overcome by selective induction with isopropylthio-ß-D-galactoside (IPTG).

As a means of screening for recombinant phage DNAs (arms plus insert), the cloning site of ZAP Express™ lies in the middle of the *lacZ'* gene which, in the absence of cloned DNA, is uninterrupted and produces the N-terminal region (α fragment) of the enzyme ß-galactosidase (ß-gal). The strain of *E. coli* chosen as the host expresses the C-terminal fragment of ß-gal; the two portions of the enzyme join and operate on the substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) to produce a chromogenic product, which appears blue. If the *lacZ'* gene is interrupted by a DNA insert, the open reading frame (ORF) of *lacZ'* is altered, and the expression of the α fragment of ß-gal either includes foreign/inappropriate amino acids or leads to premature termination of the reading frame (incomplete expression). In either case the result is usually non-functional ß-gal, so that upon induction with IPTG in the presence of X-gal these clones fail to produce the blue product and appear normal (white, in the case of bacterial colonies, or clear, in the case of phage plaques). Thus, selection of clear plaques or white colonies is a primary screen for recombinant clones.

Additionally, if the inserted DNA sequence contains an ORF, and if the ORF falls in-frame in relation to the *lacZ'* ORF, then the appropriate amino acid sequence is produced by the bacterial translation machinery. This polypeptide still contains a portion of the α fragment of ß-gal at its N terminus, however (it is technically a *fusion* protein), and may be truncated (with respect to the complete product of the gene from which this clone is derived). For the purpose of this study, since the sequence of the amino acid chain from *M. bovis* is of primary importance, the expression system described above is sufficient for polypeptide preparation.

Presentation of polypeptide antigens to tuberculous cattle was done in a BL3 containment facility by subcutaneous injection of a solution containing the polypeptide. DTH was estimated by measuring the swelling of the injection site 72 hours after injection; comparison to a background control (parental λZAP) and to PPD as a positive control allowed for identification of polypeptides eliciting a cellular immune response. In this way, we have tested the arbitrary translation products of segments of the *M. bovis* genome for potential as immunoreactive antigens.

DNA POLYMORPHISMS

The members of the *Mycobacterium tuberculosis* complex share 85 - 100% DNAsequence homology by hybridization (*7*). Direct sequencing of DNA regions known to have high rates of polymorphism show no nucleic acid substitutions within the complex (*8*). These levels of similarity are in fact lower than those widely accepted for many single species of bacteria. While the members of the *M. tuberculosis* complex differ in epidemiology and may be distinguished based on morphological characteristics, the occurrence of cross-infection and the very slow doubling time of mycobacteria in culture make these methods of differentiation laborious and impractical. Serological diagnostic methods of speciation have also failed, due to cross-reactivity of surface antigens. More sensitive, specific and efficient methods of differentiation are needed.

As mentioned above, even direct sequencing of mycobacterial DNA often cannot identify species, and requires careful preparation of specific, predetermined regions of the genome for analysis. Some success has been reported with RFLP (restriction fragment length polymorphism) analysis of mycobacterial DNA; for example, using the number of copies of a direct repeat region of the genome and/or the location of translocatable insertion elements, important epidemiological studies have been done to type TB infections and trace the spread of bovine TB (*9*). In this study we applied the technique of Random Amplified

Polymorphic DNA (RAPD) to the problem of differentiation of strains and species of mycobacteria, especially within the *Mycobacterium tuberculosis* complex.

RAPD is a variation of the Polymerase Chain Reaction (PCR), which utilizes a DNA polymerase to amplify regions of DNA. DNA polymerization requires initiation of synthesis by a primer, which must bind by basepair complementarity to a site on the template DNA to provide a free 3' hydroxyl. Through a series of controlled temperature changes and in the presence of free nucleotides, complementary strands of a denatured template DNA are created sequentially, requiring the binding of the primer in two sites to provide synthesis of a region of the template in the 5' and 3' directions. RAPD uses only one primer, which is relatively short (10-15nt) and the sequence of which is chosen arbitrarily; that is, a RAPD primer possesses no known homology to the template DNA, and binding sites are not predicted. Therefore, the results of RAPD are usually numerous double stranded DNAs of various, unpredictable sizes. The diagnostic power of RAPD arises from the fact that subtle polymorphisms in the nucleotide sequences of two template DNAs will often give rise to distinctly different amplification products, or "fingerprints" of bands on an agarose gel following electrophoresis. By testing numerous primers, we have identified several that can distinguish between strains of mycobacteria and one that can differentiate on a species level.

In itself, RAPD is a quick and sensitive diagnostic assay when a chosen primer is able to produce variable patterns of bands from different DNAs. Only small amounts of mycobacterial DNA are required, and the PCR reaction can be performed and products analyzed in 6 - 8 hours. However, the nature of RAPD entails certain inconsistencies and ambiguities that make it unsuitable as a purely diagnostic assay. Perhaps most importantly, the progress of the RAPD reaction has been shown to be unpredictably dependent on factors such as the volume of the PCR reaction, the concentration of magnesium (Mg^{2+}),

and the primer:template ratio of DNA concentration. It is important to remember that the sites of binding of the RAPD primer often involve mismatch base pairing, which is allowed by the low annealing temperature of the PCR reaction, so that the primer binding event may be imperfect and inherently inconsistent. As a result, from one trial to the next the pattern of RAPD bands produced may vary even from the same template.

RAPD is an important means of elucidating polymorphisms, and the knowledge of these makes available the development of more sensitive and consistent diagnostic assays. Rodriguez et al. found a RAPD product generated only from *M. bovis* templates. When the product was isolated, radiolabeled, and used as a probe in Southern analysis of genomic DNA digests, RFLP polymorphisms were obtainable. By sequencing the ends of the RAPD product, two standard PCR primers were created, which provided an extremely sensitive and consistent assay to identify the source of a TB infection; as little as 10 fg of DNA was detectable, which is estimated to be the equivalent of two bacilli of *M. bovis* (*10*).

In this study we report the preliminary results of a TB antigen detection system for identifying antigenic polypeptide sequences in *Mycobacterium bovis*. We also describe the characterization of a species-specific polymorphism between *M. bovis* and *M. tuberculosis* identified by RAPD PCR.

METHODS

ISOLATION OF GENOMIC DNA FROM MYCOBACTERIA

Heat-killed cells from various *M. tuberculosis* isolates were obtained from the lab of Dr. David McMurray, Texas A&M University Health Science Center. Lysozyme was added to a final concentration of 1.1 mg/ml and the mixture was vortexed and incubated at 37°C overnight. An SDS/proteinase K mix was added to final concentrations of 1.5% and 15 µg/ml, respectively. The mixture was vortexed and incubated at 65°C for 10 min. NaCl was added to 1 M in a final volume of 625 µl. 100 µl of a CTAB/NaCl solution, prewarmed to 65°C, was added and the solution was vortexed and incubated at 65°C for 10 min. [CTAB is N-cetyl-N,N,N,-trimethyl ammonium bromide.] The mixture was extracted with chloroform/isoamyl alcohol and isopropanol precipitated at -20°C for 30 min. The solution was centrifuged at 12,000xg for 15 min in a microcentrifuge and the supernatant removed. The pellet was washed in 70% ethanol, air dried, and resuspended in 20 µl of TE buffer. [TE buffer is 100 mM TrisHCl, pH 8.0, 100 mM EDTA.]

DNA from *M. bovis* isolates were obtained from the lab of Dr. L. Garry Adams, Dept. Veterinary Pathobiology, Texas A&M University.

RAPD AMPLIFICATION

Per 10 µl reaction:

40 ng template DNA

0.5 Units Taq polymerase

0.24 µM primer

1 X PCR Buffer (without Mg^{2+})

2.0 - 2.3 mM Mg^{2+}

0.1 mM each dNTP

PCR conditions:

Denaturation: 94˚C, 1 min

Annealing: 37˚C, 2 min

Extension: 72˚C, 2 min

42 cycles

PCR reactions were done in 0.6 ml microcentrifuge tubes, with one drop of mineral oil overlaid. Amplification was done in a Precision GTC-1 thermal cycler.

Plasmid DNA Isolation

The plasmid preparation technique used was the plasmid mini boiling prep, adapted from Holmes and Quigley (*11*). 3 ml overnight cultures of the *E. coli* containing the plasmid of interest were grown at 37°C with shaking at 300 rpm in Luria-Bertani broth (10 mg/ml NaCl, 10 mg/ml tryptone, 5 mg/ml yeast extract, pH 7.0) without antibiotic. A pellet of cells was formed by centrifuging in 1.5 ml portions in a microcentrifuge for 30 sec. The pellet was resuspended in 200 µl STETL (8% sucrose, 5% Triton X-100, 50 mM Tris pH 8.0, 50 mM EDTA, 0.5 mg/ml lysozyme) and boiled for 30 seconds. The mixture was centrifuged for 15 minutes at 4°C, and the resulting pellet removed with a sterile toothpick and discarded. RNAse A was added to 50 µg/ml; the mixture was incubated at 37°C for 15 minutes. 200 µl isopropanol was added and the preparation was immediately centrifuged for 20 minutes at room temperature, followed by resuspension in 40 µl of TE buffer. This DNA solution was used for restriction digest analysis to check for recombinant plasmids. If sequencing or PCR were to follow, two phenol:chloroform extractions, one chloroform extraction and sodium acetate and ethanol precipitation would be used to purify the DNA for these techniques.

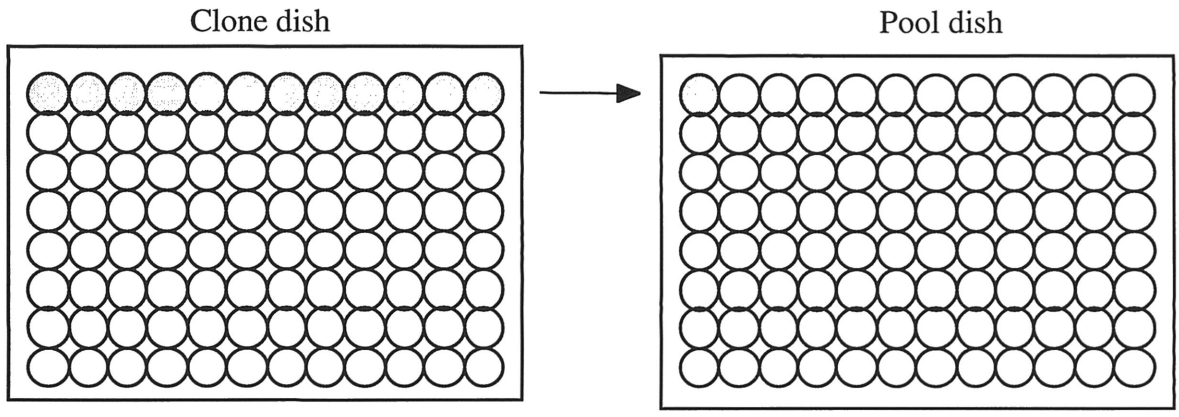


Figure 1. The pooling procedure for consolidating clones from the *Mycobacterium bovis* genomic DNA library into pools of 12. 96-well microtiter dishes were filled with isolated phage plaques, such that each well of a clone dish contains a clonal population of recombinant virus in 200 µl SM buffer. Each row from a clone dish was pooled into one well of a pool dish by transferring 10 µl from each well in the row to the well of the pool dish. The order of rows was conserved. Each column in the pool dish represents a clone dish.

Genomic Library Preparation

The λZAP Express™ (Stratagene, La Jolla, CA) genomic library was prepared according to manufacturer's instructions using DNA from *M. bovis* ATCC 35720. Genomic DNA of size 400-2000bp was size-fractionated by electroelution fragments from a partial DNAse I digestion.

In Vivo Polypeptide Expression and Isolation

From the *M. bovis* genomic library, individual clones were isolated by growing the phage on a lawn of *E. coli* strain XL1-Blue MRF', which were grown on NZY agar (100 mM NaCl, 10 mM $MgSO_4 \cdot H_2O$, 5 mg/ml yeast extract, 10 mg/ml NZ amine (casein hydrosylate), 15 mg/ml agar) in the presence of maltose to facilitate phage binding. Plaques of lysed bacterial cells were picked using a 1000 µl micropipet with wide-bore tips to approximate the diameter of the plaques. By picking a core of agar including the plaque and the media beneath it, a clonal population of phage was isolated. Picked plaques were transferred to 96-well microtiter dishes and eluted in 200 µl SM buffer (100 mM NaCl, 10

mM $MgSO_4 \cdot H_2O$, 50 mM Tris-HCl pH 7.5, .01% gelatin) by shaking the dishes at room temperature overnight. 1 drop 7% DMSO was added to each well and the phage stocks were frozen at -20°C.

Phage stocks were pooled by 12 by transferring 10 µl of phage eluate from each well of a row from a stock dish to one well of a pool dish. That is, 12 clones (from one row of an 8 by 12 dish) were combined into one well of a new pool dish. In this way, 12 clone dishes were pooled into one dish (Figure 1).

Phage pools were grown for polypeptide expression by using an 8 by 6 stamp to transfer about 2 µl liquid phage eluate onto a bacterial lawn. After 6-8 hr of growth at 37°C pools of plaques could be seen; at this point, a nitrocellulose membrane (Protran™ BA85, 132 mm, Schleicher & Schuell, Keene, NH), previously soaked in 10 mM IPTG and marked with a pencil to reflect the "grid" pattern of the stamp, was overlaid on the agar plate containing the growing phage pools. The plates were then incubated at 37°C overnight to allow proteins from the lysates of infected cells to adhere to the membrane.

The nitrocellulose membranes were peeled from the agar plates and immediately washed in 1 X PBS for 2-3 hours. Washes were repeated 2 to 3 times. The membranes were then dried under a heat lamp, and 3 µl of 2 µg/ml polymyxin B sulfate, an antibiotic, was added to each square on the membrane (i.e., to each pool lysate). The membranes were dried again and then the squares cut out and transferred to labeled microcentrifuge tubes. Nitrocellulose squares were stored at room temperature until injection. Control pools of ZAP Express with no insert (identified by blue-white ß-gal screening) were prepared alongside the others.

500 µl DMSO was added to each tube containing 4 nitrocellulose squares (from the same clones) and the tube was vortexed immediately for several minutes to dissolve all the nitrocellulose. The resulting (very viscous) solution was drawn into 0.5 ml syringes (27G1/2, 0.40 mm x 13 mm) and used to inject 4 cattle infected with *M. bovis* (100 µl per cow). These cattle were shaven from the shoulder to the haunch and injections were done in a grid pattern. 72 hr after injection, swelling of the skin surrounding the injection site was measured with a caliper and punch biopsies were taken to examine the site for mononuclear infiltration.

Based on the caliper measurements and the biopsy results, pools of clones were selected that showed a consistent response above background. Each individual from the selected pools was then tested in the same manner described above, to determine which clone(s) elicited the pooled response.

In Vivo Excision of the pBK-CMV Plasmid

Excision of a plasmid from λZAP Express™ phage was accomplished with the use of a "helper" phage, which recognizes sequences in the λZAP DNA and reproduces a single-stranded version of the pBK-CMV phagemid and the insert, if any. The is secreted along with helper phage from the *E. coli* cell, which was isolated by heat-killing the λ phage and *E. coli* at 70°C and infecting fresh *E. coli* with the helper virus, which then creates double-stranded phagemid. The exact protocol was followed from the ZAP Express™ Vector Kit instruction manual (Stratagene, La Jolla, CA).

Probe Preparation and Screening

The 2100bp RAPD product was radiolabeled by the random primer method. 10 ng of DNA was mixed with 0.025 mM dCTP, dTTP, and dGTP, 4 units Klenow enzyme, 5 µl α^{32}P-dATP, and a random hexamer/reaction buffer mix at 37°C for 30 minutes. Free nucleotides were removed by passing the mixture through a Sephadex® G-25 column equilibrated with an oligo stop mix (20 mM NaCl, 20 mM Tris pH 7.5, 2 mM EDTA, 0.25% SDS).

Clones representing the *M. bovis* genomic library were grown in XL1-Blue plated on large NZY agar plates overnight at 37°C. Nitrocellulose membranes were overlaid on the plates for 2 minutes, during which time the orientation of the filter on the plate was marked with a needle and ink. The membranes were then soaked for 2 minutes in 0.5 M NaOH / 1.5 M NaCl, for 5 minutes in 1.5 M NaCl / 0.5 M Tris pH 8.0, and 30 seconds in 0.2 M Tris pH 8.0 / 2 X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). DNA was crosslinked to the nitrocellulose by irradiation with 150 mJ UV light for 30 sec.

Hybridization was performed in 4 X SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 2 X Denhardt's solution, 25 µg/ml herring sperm DNA at 65°C in a shaking water bath. Post-hybridization washes were done with 4 X SSC and 2 X SSC at 50°C for 30 minutes each. Membranes were then exposed to X-ray film (Hyperfilm™-MP, Amersham) at -80°C.

RESULTS

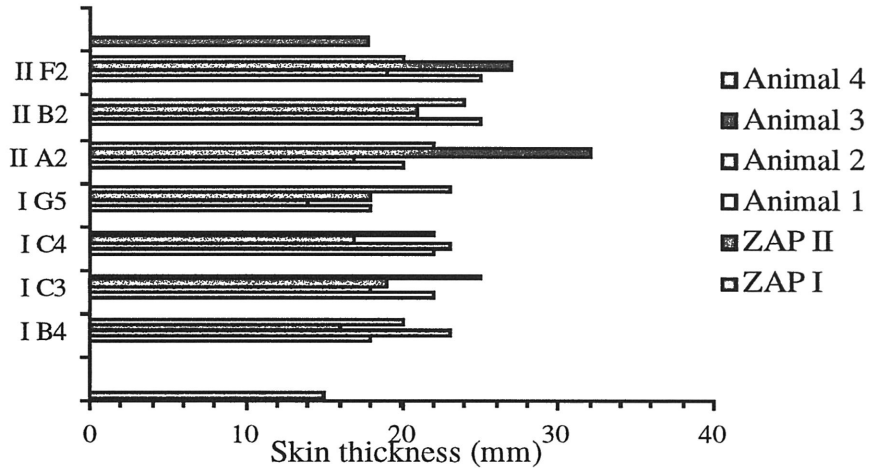
IDENTIFYING ANTIGENIC *M. BOVIS* POLYPEPTIDES

Outbred heifers previously infected with *M. bovis* were the subjects of the DTH testing described here. Pooled antigens were injected and the size of induration of injection sites 72 hr after injection. Pools of clones were chosen that elicited induration greater than that observed in the control (phage without insert) injection sites in at least 2 of the 4 cows. Biopsies of these sites were taken to examine the histologic appearance of the site. All sites had nonspecific inflammation (neutrophils and edema) and necrosis, probably from the nitrocellulose. The sites we selected also showed areas of mononuclear cell infiltrates (lymphocytes and macrophages), predominantly around small blood vessels in the superficial dermis. Figure 2 shows data from injection site measurements and an example of biopsy results.

Complicating identification of clones which elicit DTH was the tissue damage at the injection site, presumably caused by the nitrocellulose, which precipitates from the DMSO adjuvant upon injection in an aqueous environment. This led to non-specific swelling of the skin and to the recruitment of cells to the site as a response to damage, rather than to the introduction of antigen. The results in Figure 3 were obtained in part as an attempt to explore other ways of nitrocellulose-mediated presentation of antigen. The addition of an equal volume of 1 M sodium bicarbonate to a sample of nitrocellulose dissolved in DMSO causes precipitation of the nitrocellulose in an exothermic reaction. However, the supernatant of this reaction contains little or no protein; the nitrocellulose precipitate, when redissolved in DMSO, shows the most protein (Figure 3A).

We have identified 7 pools of clones which elicit induration above background and which may elicit DTH based on biopsy results. We will test each individual from these pools (84

A.



B.

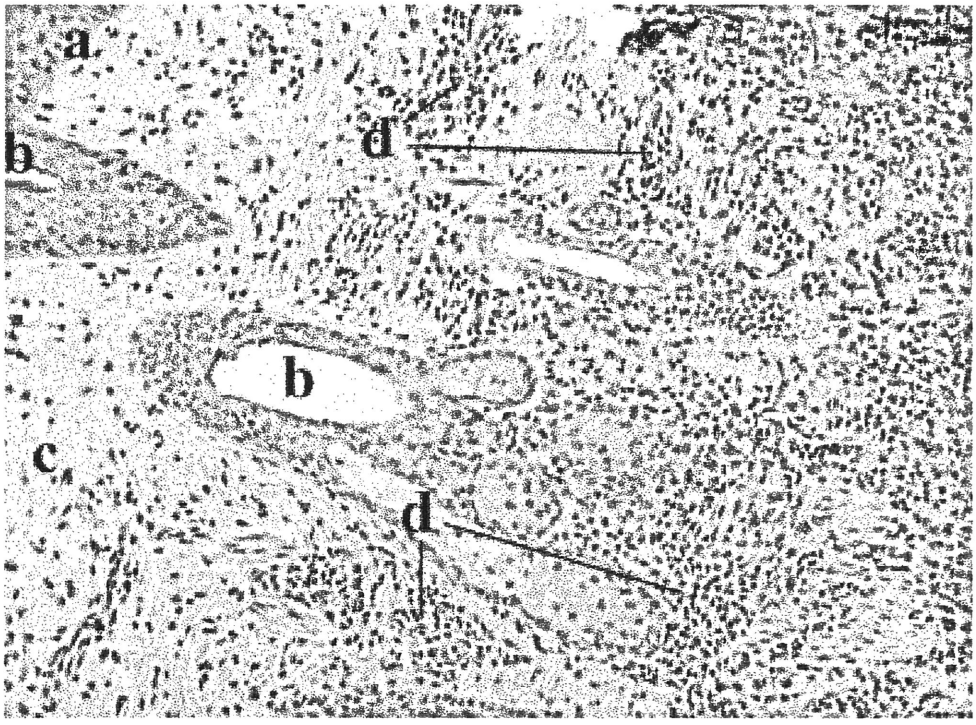


Figure 2. Skin test results from injection of recombinant polypeptides. (**A**) Measurements of the swelling of the skin surrounding injection sites 72 hours after injection. Two sets of 96 pools (I and II) were tested on separate dates; measurements from 4 cows are given here. Animal 1 from set I is not the same as Animal 1 from set II. Average values of the reaction from empty vector (ZAP) for both sets is given. 6 of these 7 pools were chosen for further study based on biopsy results. (**B**) 100X magnification of punch biopsy of calf #12 (Animal 4 from above set I), pool C3. Labeled are: (a) cell surface (epidermis), (b) hair follicles, (c) normal dermis tissue, and (d) mononuclear cells (macrophages and lymphocytes) infiltrating the site. Pink material is collagen fibers; small purple cells are mononuclear cells. (Picture and data kindly provided by Dr. Roger Smith)

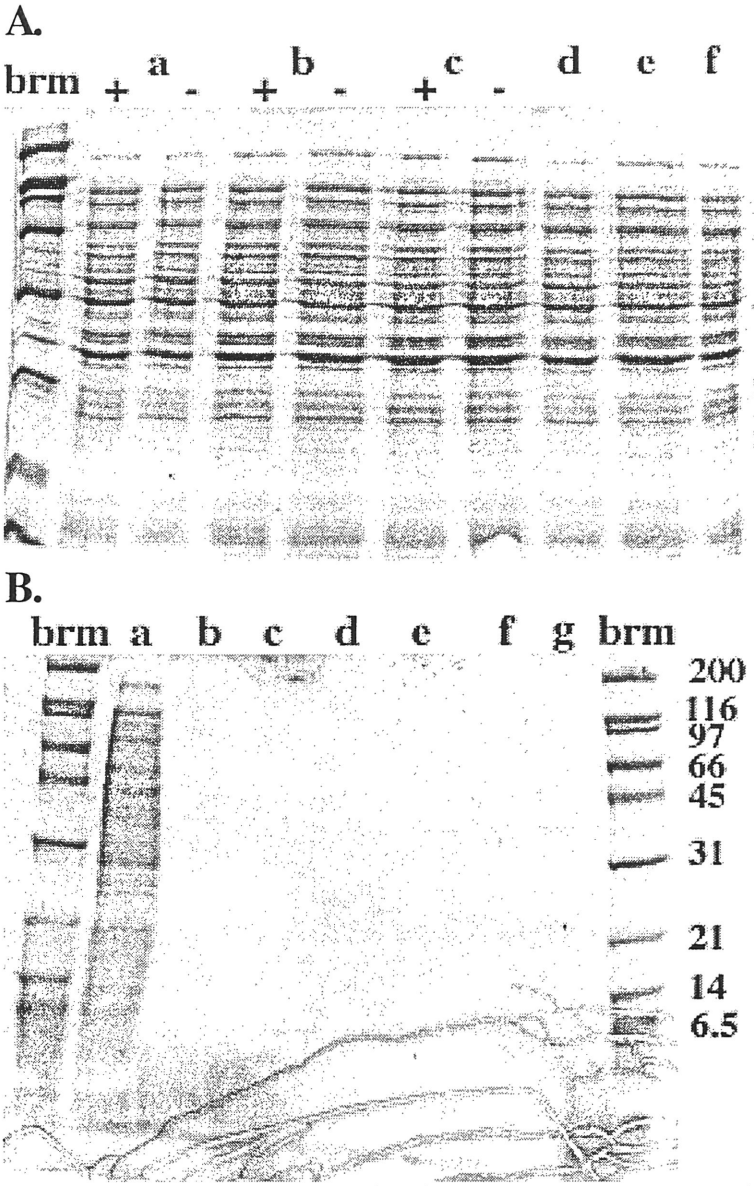


Figure 3. Coomassie-stained SDS-PAGE gels showing proteins expressed by λZAP Express-infected *E. coli*. Broad-range molecular weight markers give sizes in kDa at right (brm). (**A**) Lysates from induction time points of *E. coli* liquid cultures infected with λZAP non-recombinant phage. (a through c) 1, 2, and 3 hr time points, respectively, after induction with IPTG (+) or without induction (-). (d through f) A recombinant clone from the *M. bovis* genomic library, 1, 2, and 3 hr after induction with IPTG. This clone elicited DTH when grown on solid media, induced with IPTG, and transferred to nitrocellulose and injected in cattle infected with *M. bovis*. (**B**) Lysate from uninduced *E. coli* in the absence of phage infection (a) shows total bacterial proteins. Low molecular weight proteins were transferred to nitrocellulose by phage lysis overlays and dissolved in DMSO (b). (c) Precipitate from solution in (a) after addition of 1 M sodium bicarbonate, redissolved in DMSO, shows a large fraction of protein still present in the precipitate. (d) The supernatant after precipitation with sodium bicarbonate, (e) the supernatant after centrifugation at high rpm of precipitation supernatant, and (f) the pellet from centrifugation to give (d), resuspended in PBS, show no or little protein. Non-resuspendable pellet from high-speed centrifugation of (d), dissolved in DMSO (g), shows some low molecular weight protein.

		Tm
G3	5′ GAGCCCTCCA 3′	34°C
G19	5′ GTCAGGGCAA 3′	32°C
H3	5′ AGACGTCCAC 3′	32°C
H4	5′ GGAAGTCGCC 3′	34°C

Table 1. 10nt RAPD primers used to differentiate mycobacteria. These oligonucleotides were selected from a bank of primers on the basis of the patterns of RAPD products produced from mycobacterial templates and the potential of these results for differentiating species of mycobacteria. RAPD primers have at least 50% GC content to provide a substantial melting temperature and allow annealing at low temperature PCR conditions.

clones) in the same manner to identify the source of the positive response. Positive clone inserts will be characterized by sequencing and database comparison.

Testing RAPD Primers for Differentiation

RAPD was done with primers from a bank of 10nt oligonucleotides in order to identify a primer that could distinguish *M. bovis* from *M. tuberculosis* and from *M. avium*, a mycobacterium outside the *M. tuberculosis* complex. DNA from isolates of these species was isolated from heat-killed cells and used as templates for RAPD PCR. Four primers were identified that gave relatively consistent product patterns which differed significantly depending on the template identity. Figure 4 shows amplification products from mycobacterial templates separated on an agarose gel. Different patterns of products are generated from different mycobacterial species templates, allowing differential determination from amplification products; most molecular techniques give identical results from *M. bovis* and *M. tuberculosis.* However, these RAPD patterns varied occasionally from reaction to reaction from the same template. The sequences of these primers are shown (Table 1).

Additionally, several of a bank of 10nt primers from Operon Technologies (Alameda, CA) were tested with multiple isolates of *M. bovis* and *M. tuberculosis* as templates. One primer, opA2 (5' TGCCGAGCTG 3'), gave a characteristic 2100bp product only from *M. bovis* templates (Figure 5); other RAPD products were conserved between the two species. We cloned this species-specific product to determine the source of the polymorphism and to analyze its potential as a means of species differentiation.

The products of all PCR reactions contain an overhanging adenosine (A) as the 3' base of both strands, regardless of the template sequence. This allows cloning of PCR products into commercial plasmid vectors, such as pCRII™ (Invitrogen, San Diego, CA), which have an overhanging thymine (T) on each strand. We cloned the 2100bp RAPD product into pCRII by pooling the product from several 10 µl amplification reactions and purifying it by electroelution, then ligating into the linear plasmid and transforming competent *E. coli* with the resulting DNA. Cloning was verified by restriction analysis following blue/white ß-gal screening of colonies. Figure 8 shows the RAPD reactions used to generate the 2100bp product for cloning, and the restriction digest results of the cloned product.

In order to produce enough of the product for purification and subsequent cloning, we tried to increase the volume of the RAPD PCR reaction. A direct scale-up of the reaction led to a loss of higher molecular weight products, including the 2100bp product (Figure 6A). We then attempted to amplify the 2100bp product by PCR using opA2 as the primer; this reaction was done under RAPD conditions (annealing temperature 37°C) to allow priming with opA2. Multiple products resulted, all smaller than the full 2100bp template, and most different than the other smaller products of *M. bovis* RAPD (Figure 6B). Additionally, hybridization of the radiolabeled 2100bp product to a Southern blot of an opA2 RAPD gel shows that smaller RAPD products hybridize to the 2100bp product (Figure 7). These

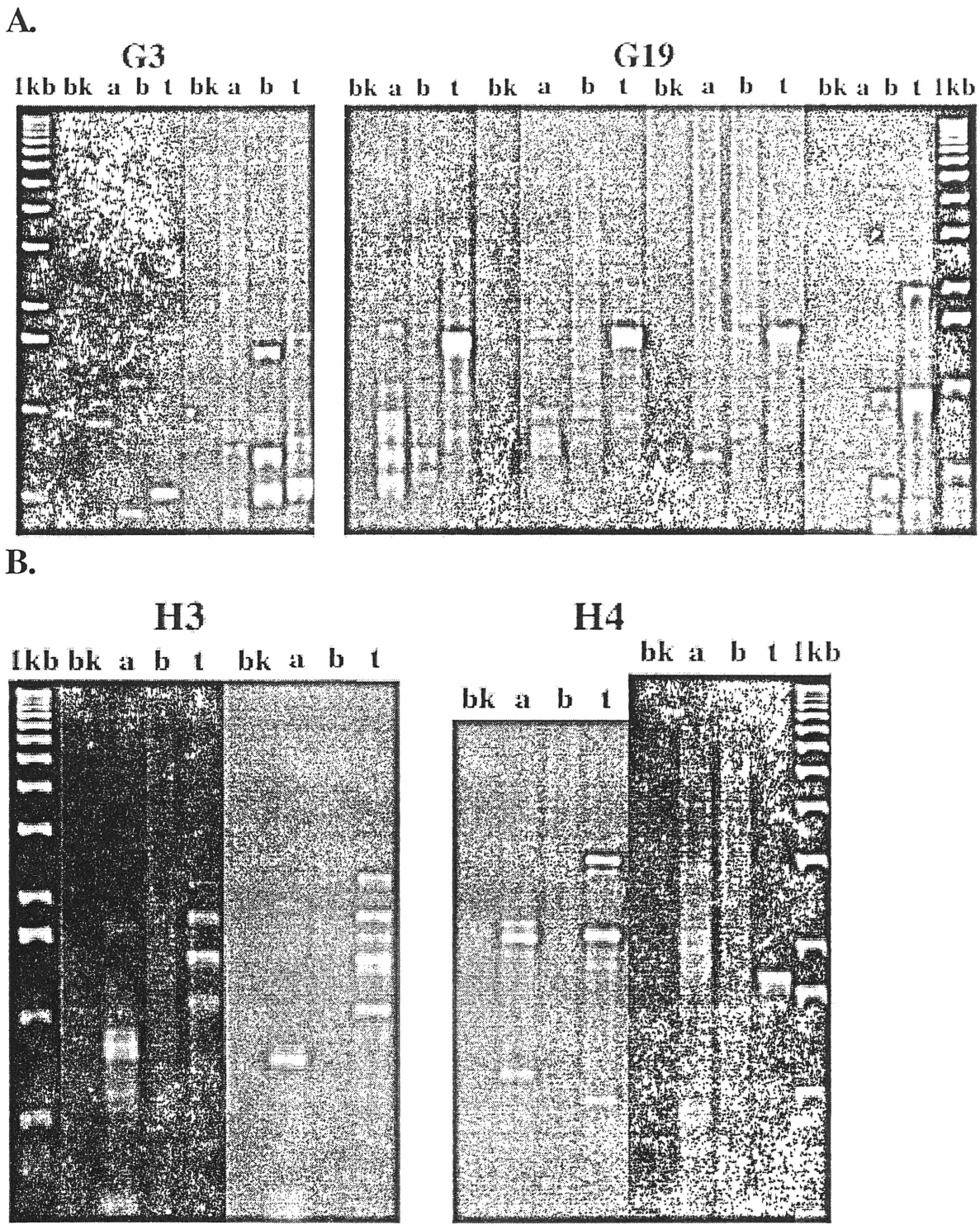


Figure 4. RAPD products with primers (**A**) G3 and G19, (**B**) H3 and H4 separated on a 1.4% agarose gel with ethidium bromide. Template DNAs were from *Mycobacterium avium* ATCC 25291 (a), *M. bovis* ATCC 35720 (b), and *M. tuberculosis* H37Rv (t), respectively. 1kb DNA ladder (Gibco-BRL) is in the outside lanes (1kb) and lanes labeled (bk) are reactions with no template DNA.

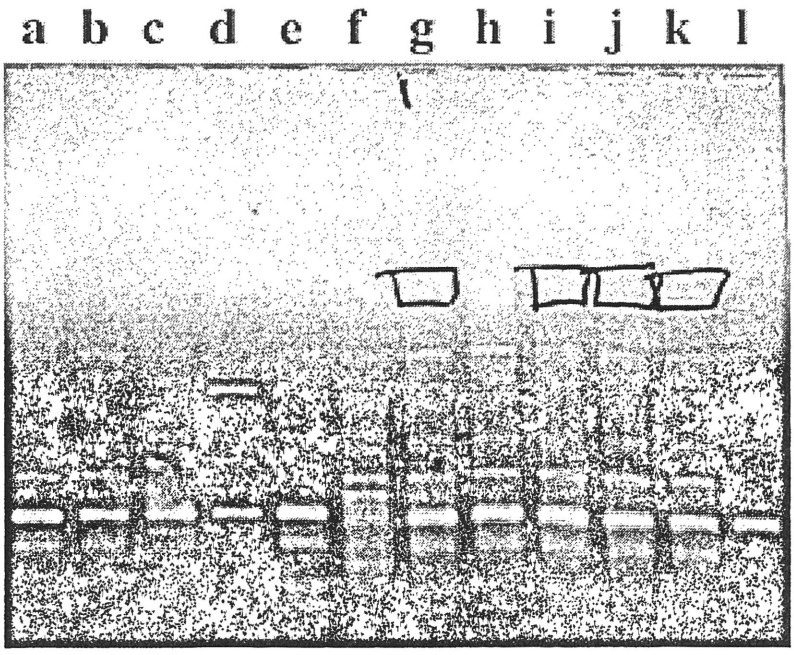


Figure 5. RAPD of *M. tuberculosis* and *M. bovis* genomic DNA with primer opA2. Products from templates from *M. tuberculosis* isolates (a through f). Products from templates from *M. bovis* isolates (g through l). The squared product is present only in reactions from *M. bovis* templates.

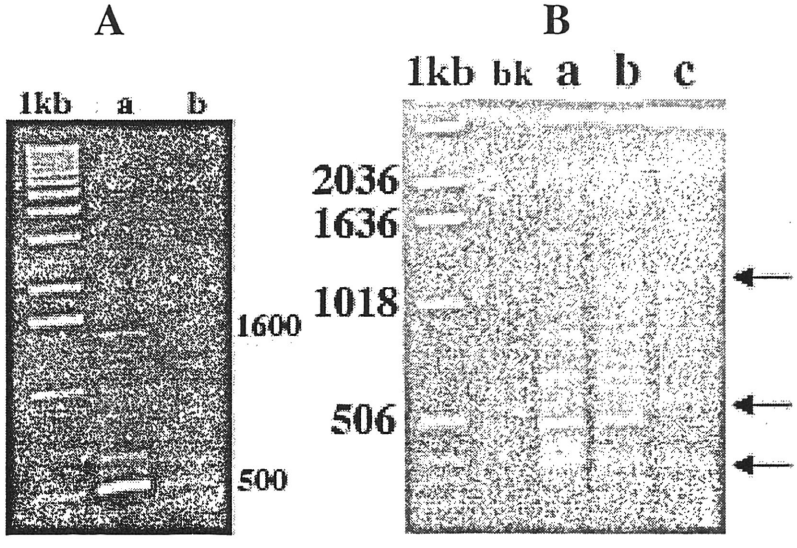


Figure 6. (**A**) Direct scale-up amplification of the *M. bovis* RAPD with opA2. 20 µl RAPD reactions from *M. bovis* templates (a and b) gave fewer, lower molecular weight products than the 10 µl reaction. The *M. bovis*-specific 2100bp product is not produced at this volume, based on sizes of the 1kb DNA ladder (1kb). Sizes of two of the products are given at right in bp. (**B**) RAPD using primer opA2 with the purified 2100bp DNA as template (c). Arrows show multiple amplification products from the single template. Products from genomic *M. bovis* DNA from two isolates (a and b) included the high molecular weight product (not visible in figure).

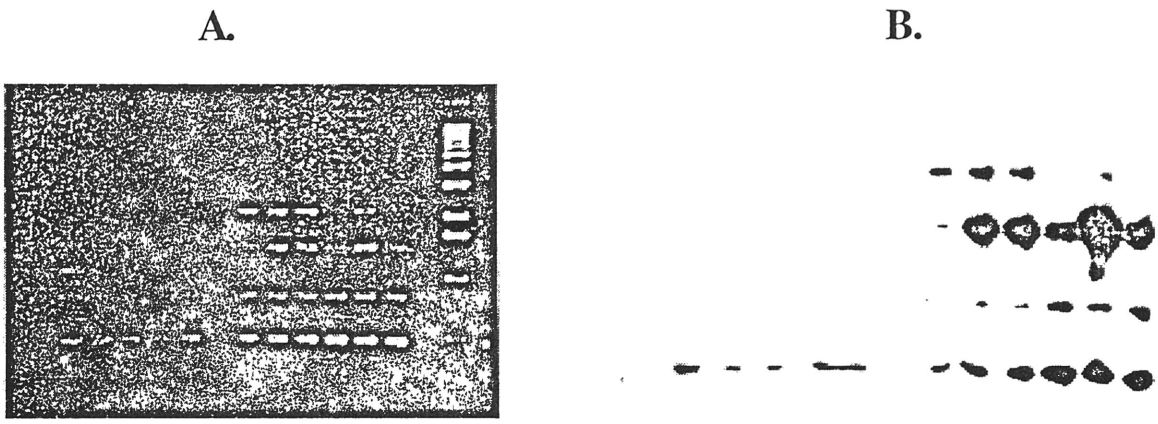


Figure 7. Hybridization of the 2100bp RAPD product to other RAPD products from the same reaction. The 2100bp product was purified, labeled and used to probe a Southern blot (**B**) of a RAPD gel with *M. tuberculosis* and *M. bovis* templates (**A**). The first six reactions (left to right) are *M. tuberculosis*, while the last six are *M. bovis.*

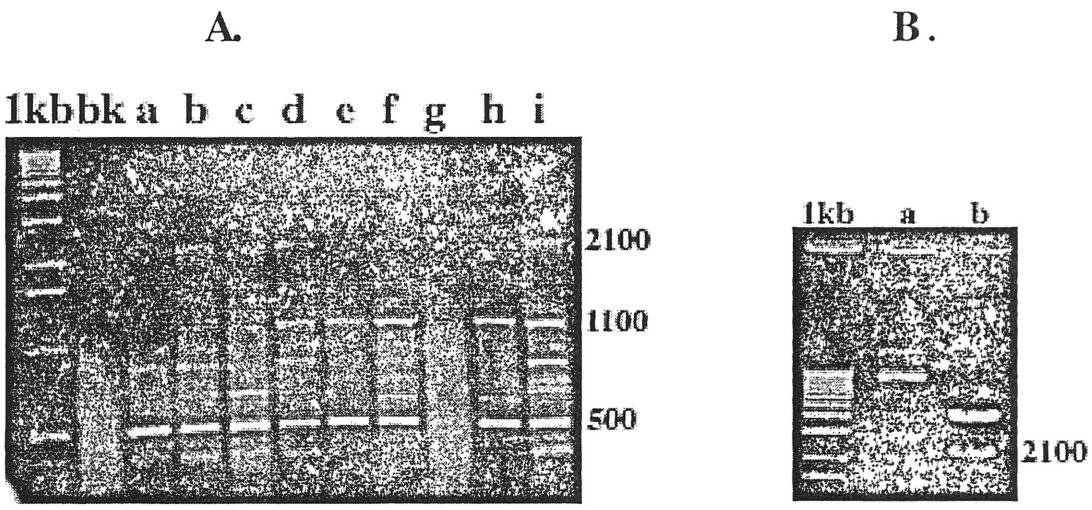
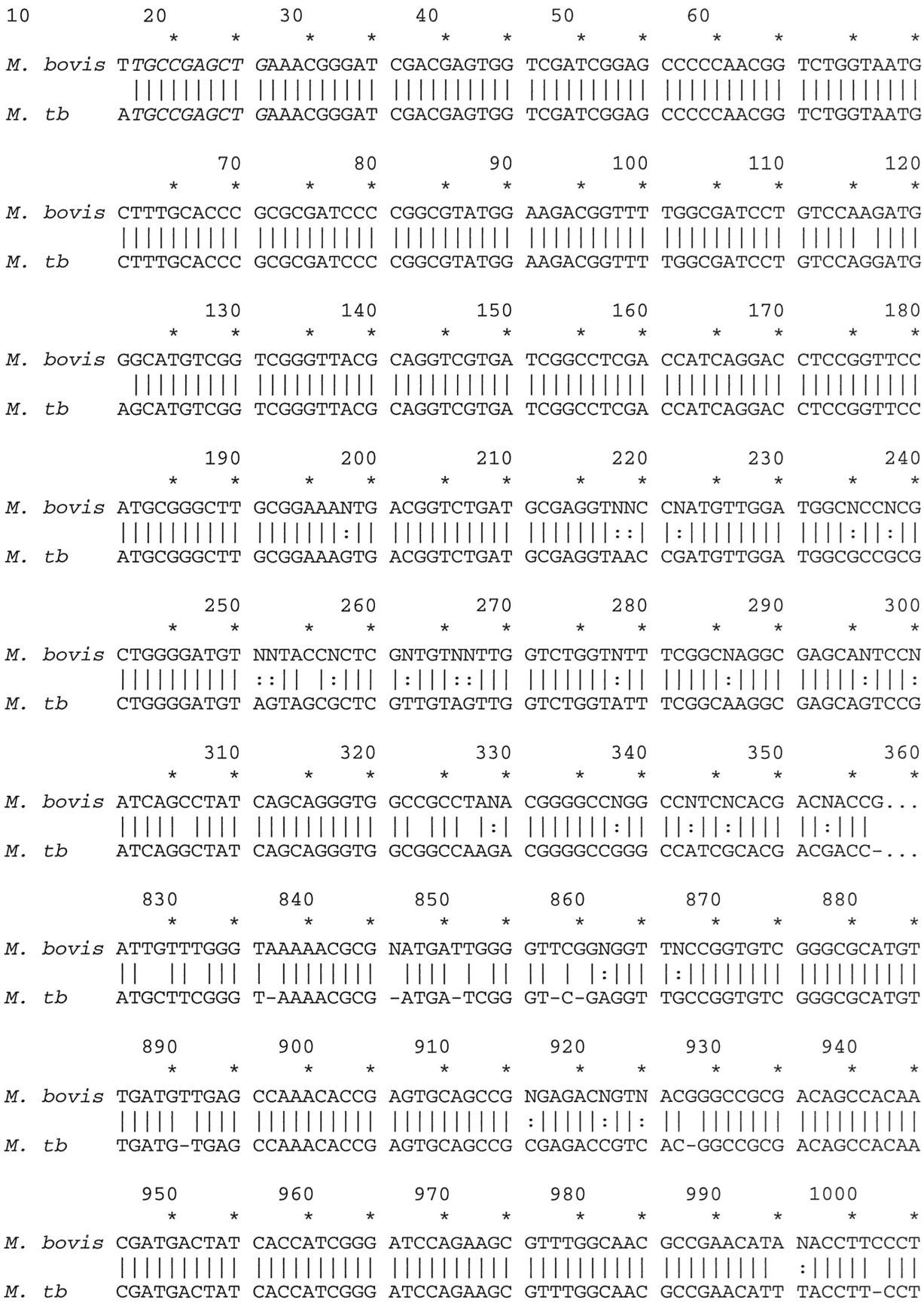


Figure 8. (**A**) Multiple amplifications of the *M. bovis*-specific RAPD product for purification. Templates were from isolates w221 (a through e) and w225 (f through i). Products were separated on a 0.8% agarose gel and the 2100bp product was cut out of the gel with a scalpel, pooled, and purified by electroelution into one chamber. (**B**) Cloning of the 2100bp *M. bovis*-specific RAPD product by T/A ligation. Recombinant pCRII™ (Invitrogen, San Diego, CA) containing the 2100bp insert (a) was cut with *Eco*RI to release the *M. bovis*-specific product (b).

results have interesting implications regarding the source of the species-specific product (see Discussion).

A partial sequence of the cloned product was determined (Figure 9). When this sequence was compared to sequences in GenBank and in the mycobacterial database (http://kiev.physchem.kth.se/MycDB/seqsearch_form.html), two separate cosmid sequences from *M. tuberculosis* H37Rv were found which displayed essentially 100% homology to the cloned product. The source of the RAPD polymorphism, based on this comparison, is a single nucleotide difference in a highly conserved region, which happens to fall in the opA2 primer binding site and therefore prevents priming from *M. tuberculosis* templates. Comparison of the two H37Rv cosmid sequences showed in one sequence a guanine insertion one basepair 5' of the opA2 substitution; this nucleotide was deleted in the other sequence (Figure 9).

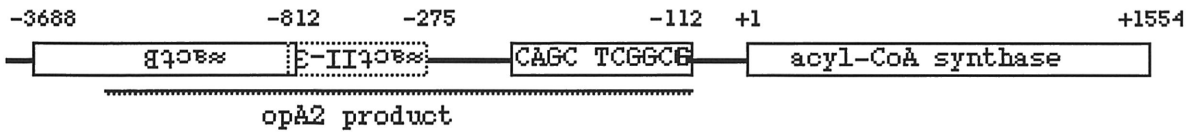
Based on database homologies, we believe the opA2 polymorphism lies in a highly variable site within an otherwise strictly conserved untranslated region upstream of a acyl-CoA synthase gene in mycobacteria. Figure 10 depicts a map of the polymorphic site in relation to its nearest ORFs (A), and gives the amino acid similarity of the proposed gene acyl-CoA synthase gene product to the acyl-CoA synthase MasC (B). MasC is associated with Mas, a mycocerosic acid synthase involved in synthesis of long-chain fatty acids that become part of the mycobacterial cell wall and are implicated in pathogenesis and antibiotic resistance.


10 20 30 40 50 60
M. bovis TTGCCGAGCT GAAACGGGAT CGACGAGTGG TCGATCGGAG CCCCCAACGG TCTGGTAATG
M. tb ATGCCGAGCT GAAACGGGAT CGACGAGTGG TCGATCGGAG CCCCCAACGG TCTGGTAATG
70 80 90 100 110 120
M. bovis CTTTGCACCC GCGCGATCCC CGGCGTATGG AAGACGGTTT TGGCGATCCT GTCCAAGATG
M. tb CTTTGCACCC GCGCGATCCC CGGCGTATGG AAGACGGTTT TGGCGATCCT GTCCAGGATG
130 140 150 160 170 180
M. bovis GGCATGTCGG TCGGGTTACG CAGGTCGTGA TCGGCCTCGA CCATCAGGAC CTCCGGTTCC
M. tb AGCATGTCGG TCGGGTTACG CAGGTCGTGA TCGGCCTCGA CCATCAGGAC CTCCGGTTCC
190 200 210 220 230 240
M. bovis ATGCGGGCTT GCGGAAANTG ACGGTCTGAT GCGAGGTNNC CNATGTTGGA TGGCNCCNCG
M. tb ATGCGGGCTT GCGGAAAGTG ACGGTCTGAT GCGAGGTAAC CGATGTTGGA TGGCGCCGCG
250 260 270 280 290 300
M. bovis CTGGGGATGT NNTACCNCTC GNTGTNNTTG GTCTGGTNTT TCGGCNAGGC GAGCANTCCN
M. tb CTGGGGATGT AGTAGCGCTC GTTGTAGTTG GTCTGGTATT TCGGCAAGGC GAGCAGTCCG
310 320 330 340 350 360
M. bovis ATCAGCCTAT CAGCAGGGTG GCCGCCTANA CGGGGCCNGG CCNTCNCACG ACNACCG...
M. tb ATCAGGCTAT CAGCAGGGTG GCGGCCAAGA CGGGGCCGGG CCATCGCACG ACGACC-...
830 840 850 860 870 880
M. bovis ATTGTTTGGG TAAAAACGCG NATGATTGGG GTTCGGNGGT TNCCGGTGTC GGGCGCATGT
M. tb ATGCTTCGGG T-AAAACGCG -ATGA-TCGG GT-C-GAGGT TGCCGGTGTC GGGCGCATGT
890 900 910 920 930 940
M. bovis TGATGTTGAG CCAAACACCG AGTGCAGCCG NGAGACNGTN ACGGGCCGCG ACAGCCACAA
M. tb TGATG-TGAG CCAAACACCG AGTGCAGCCG CGAGACCGTC AC-GGCCGCG ACAGCCACAA
950 960 970 980 990 1000
M. bovis CGATGACTAT CACCATCGGG ATCCAGAAGC GTTTGGCAAC GCCGAACATA NACCTTCCCT
M. tb CGATGACTAT CACCATCGGG ATCCAGAAGC GTTTGGCAAC GCCGAACATT TACCTT-CCT

```
           1010       1020       1030       1040       1050       1060
              *    *     *    *     *    *     *    *     *    *     *    *
M. bovis GATTCCATNG CTTCAACAAG CCGCCGCGTG AGGACGAACC CTACCGGGGA GACGCCANTC
         ||||||||:| |||||||||| |||||||||| |||||||||| |||||||||| |||||||:||
M. tb    GATTCCATCG CTTCAACAAG CCGCCGCGTG AGGACGAACC CTACCGGGGA GACGCCACTC

           1070       1080       1090       2000       2010       2020
              *    *     *    *     *    *     *    *     *    *     *    *
M. bovis GTTGGGGCAG TTTNGNACAC TCCGTTNACA TCGTTTACGG CGAGGTCAAA AAATTTCGGT
         |||||||||| |||:|:|||| ||||||:||| |||||||||| |||||||||| ||||||||||
M. tb    GTTGGGGCAG TTTTGTACAC TCCGTTTACA TCGTTTACGG CGAGGTCAAA AAATTTCGGT

           2030       2040       2050       2060       2070       2080
              *    *     *    *     *    *     *    *     *    *     *    *
M. bovis TAATCGTACA GGCTGCCGCT CGGTCATCTA TAGTCATCGA TCCAGAGCCG NTTCGACCAG
         |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| :|||||||||
M. tb    TAATCGTACA GGCTGCCGCT CGGTCATCTA TAGTCATCGA TCCAGAGCCG CTTCGACCAG

           2090       2100       2110       2120       2130       2140
              *    *     *    *     *    *     *    *     *    *     *    *
M. bovis CCTGTGGTCG AAGCGGATCA GTTGAACCGG AGGAGTGGAA ACATGAGCGG CCCGACGGGA
         |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
M. tb    CCTGTGGTCG AAGCGGATCA GTTGAACCGG AGGAGTGGAA ACATGAGCGG CCCGACGGGA

           2150       2160       2170
              *    *     *    *     *
M. bovis AATTCGATGC CCAGACAGCT CGGCA
         |||||||||| |||||||||| ||||
M. tb    AATTCGATGC CCAGACAGCT CGGCG-CCTG GTGGCCAGGA TCGTTACCGG
         |||||||||| |||||||||| ||||| |||| |||||||||| ||||||||||
MSGY423  AATTCGATGC CCAGACAGCT CGGCGGCCTG GTGGCCAGGA TCGTTACCGG
```

Figure 9. Source of the opA2 polymorphism. Shown is the partial sequence of the *M. bovis* 2100bp RAPD product, aligned with the corresponding sequence from a published *M. tuberculosis* H37Rv cosmid sequence (GenBank MTCY4D9, accession number Z84725). Sites of opA2 priming are italicized. The source of the RAPD polymorphism is in bold at position 2170. Partial alignment to another *M. tuberculosis* cosmid sequence (GenBank MSGY423) is shown on the bottom line. Misalignments may be due to sequencing errors. T at position 1 in *M. bovis* sequence is the 5' vector overhang used for T/A cloning and is a PCR artifact.

A.



B.

```
                                        10          20          30
                                      *    *     *     *     *     *     *
BCG acyl-CoA synthase (MasC)  MDYEQDWD GVAITLTWSQ LYRRTLNVAR ELSRCGSTGD
                              |||| : | |    ||||| ||:|: :||   | ::|: ||
H37Rv acyl-coA synthase       MDYDTKTG DQIDRMTWSQ LYSRVTAVSA YLISYGRHAD

     40          50          60          70          80          90
      *    *     *    *     *    *     *    *     *    *     *    *
      --R-VVISRP QGLEYVVAFL GALQAGRIAV PLSVPQGGVT DERSDSVLSD SSPVAILTTS
        | ::|| | ||||||:||| ||| |||:|| ||| | |||  |:||| :| | :|| :|||||
      RRRTAAISAP QGLDYVAGFL GALCAGWTPV PLPEPLGSLR DKRTGLAVLD CAADVVLTTS

    100         110         120         130         140         150
      *    *     *    *     *    *     *    *     *    *     *    *
      SAVDDVVQHV ARRPGESPPS IIEVDLLDLD APNGYTFKED EYPSTAYLQY TSGSTRTPAG
       | : | : | | | |: ::| ||:|| ||   | |:::|::|     |||||| ||||| :| |
      QAETRVRATI ATHGASVTTP VIALDTLDEP SGDNCDLDSQ LSDWSSYLQY TSGSTANPRG

    160         170         180         190         200         210
      *    *     *    *     *    *     *    *     *    *     *    *
      VVMSHQNVRV NFEQLMSGYF ADTDGIPPPN SALVSWLPFY HDMGLVIGIC APILGGYPAV
      |||| |||   | ||||::||  |:|| |:   |||||||||| |||||||||   ||| |:|:|
      VVLSMRNVTE NVDQIIRNYF RHEGGAPRLP SSVVSWLPLY HDMGLMVGLF IPLFVGCPVI

    220         230         240         250         260         270
      *    *     *    *     *    *     *    *     *    *     *    *
      LTSPVSFLQR PARWMHLMAS DFHAFSAAPN FAFELAARRT TDDDMAGRDL GNILTILSGS
      |||| ||||| |||||||||: |  ||||||| ||||||: || ||||||| || ||| ||||||
      LTSPEAFIRK PARWMQLLAK HQAPFSAAPN FAFDLAVAKT SEEDMAGLDL GHVNTIINGA

    280         290
      *    *     *
      ERVQAATIKP F
      |||||:||:  |
      EQVQPNTITK F
```

Figure 10. (**A**) Location of the opA2 polymorphic region 5' of an acyl-CoA synthase in *M. tuberculosis* H37Rv. Two ORFs with homology to antibiotic transport proteins, actII-3 and actB, are 5' of the polymorphism in the sense of the minus strand. The approximate position of the opA2 RAPD product is shown. (**B**) Alignment of the acyl-CoA synthase from the Mas region of *M. bovis* BCG to the *M. tuberculosis* H37Rv acyl-CoA synthase downstream of the opA2 polymorphism. Homologies and identities are based on the pam250 scoring matrix (MacVector™ 5.0, Kodak Scientific Imaging Systems).

DISCUSSION

IN VIVO ANTIGENIC ASSAY

The ability to translate polypeptides that retain their antigenic character in an engineered expression system relies on several factors that are not guaranteed in our system. Most obviously, translation from genomic clones requires that the coding DNA be in frame with the ß-gal α peptide, which statistically will occur in one of six clones containing ORFs. Additionally, the antigenic character of some mycobacterial proteins may require post-translational processing by the mycobacterium, which may not occur in the *E. coli* host. The converse also holds: if the *E. coli* process recombinant translation products in a manner different from the mycobacterium, the result may not be antigenic. Furthermore, *E. coli* contain proteolytic enzymes that destroy proteins.

We have not clearly shown which proteins are successfully isolated by transferring to nitrocellulose. Figure 3 suggests that only low molecular weight proteins (< 6.5 kDa) are isolated by this method. Nor has it been shown that induction of *E. coli* infected with recombinant λ ZAP Express from our library will produce a fusion protein, even in liquid culture. In fact, the only evidence that our system for producing recombinant T cell antigens is successful has been the development of DTH in cattle injected with our polypeptides, and the fact that there are responses above background, with certain individual clones from "positive" pools also causing DTH. Moreover, in past experiments "positive" individual clones appeared to be non-recombinant, based on restriction mapping of excised phagemids (data not shown).

Perhaps a more efficient and less toxic presentation could be achieved with phage expression in liquid culture. Such a technique was avoided in favor of a nitrocellulose-

mediated transfer because of the presence of bacterial endotoxins in a liquid lysate, which complicate skin testing by affecting cell recruitment at the injection site. We will examine different methods of preparing the antigen to lessen or avoid the effects of nitrocellulose.

Also complicating skin testing was an apparent trend of increasing induration at injection sites in a gradient from the rear of the animals to the shoulder in initial injections. This may simply be due to the relative proximity of the lymph nodes to the injection sites. In any case, potential effects of this pattern on results were minimized by placing control (empty vector) injections at various points along the "gradient" ; previously, control injections had been done first, or nearest to the neck, and accordingly high reactions were observed.

Mustafa (*12*) identified recombinant proteins from pathogenic mycobacteria originally isolated as B cell epitopes which also stimulate T cell proliferation. Furthermore, several of these recombinant mycobacterial antigens activated T cells of a protective phenotype and these cells were represented in the memory T cell repertoire years after vaccination. Similar studies have identified polypeptide antigens that activate T cells (Table 2). Heat shock proteins tend to dominate the classification of mycobacterial antigens.

Name	Organism	Size (kDa)	Function/Homology	Immunological characteristics
?	*M. tuberculosis*	71	HSP, role in protein folding and translocation	Antibody response, T cell proliferative response
DnaK	*M. bovis* BCG	70	>50% sequence identity with *E. coli* DnaK and human *hsp70*	Antibody response, T cell proliferative and cytotoxic response, recognized by some γδ T cells
GroEL	*M. bovis* BCG	65	With GroES, molecular chaperones which assist protein folding after heat shock	Antibody response, T cell proliferative and cytotoxic response, recognized by some γδ T cells
GroES	*M. bovis* BCG	12		Strong T cell proliferative response
PhoS	*M. tuberculosis*	38	Role as 'binding protein' in phosphate transport material	*M. tuberculosis* complex-specific antibody response in smear-positive patients; T cell response in patients and after BCG vaccination
HBT10	*M. tuberculosis*	44	L-alanine dehydrogenase	Antibody distinguishes BCG and *M. tuberculosis*
?	*M. tuberculosis*	10	?	DTH response in guinea pigs
?	*M. tuberculosis*	28	?	Proliferative and cytotoxic T cell response[b]

Table 2. A compilation of mycobacterial antigens identified by recombinant methods, adapted from (*13*).[b]This entry from (*18*).

We hope to identify multiple clones from our pools of *M. bovis* library clones which elicit a DTH response. Characterization of the DNA inserts will allow identification of the source gene and the sequence of the polypeptide itself. Further experiments will then be done to examine the potential for protective immunity of each antigen. Potentially, the DNA itself from positive clones may be used to confer TB resistance, as has been done in mice by injection of purified plasmid DNA containing the sequence of interest (*6*). We may examine this alternative with highly antigenic clones. Additionally, variations in the antigen gene or the character of the antigen may be useful for differentiation of mycobacteria, if a certain antigen elicits DTH only in an animal infected with the same species or strain of mycobacteria.

It would also in theory be very simple to reverse Mustafa's experiment, and to look for B cell epitopes among our T cell epitopes. This would only require screening of the same nitrocellulose membranes from lysis overlays with antibodies against tuberculous cow serum. Pools of clones giving a positive response would contain a DNA insert coding a polypeptide recognized by an antibody: a B cell epitope. However, this would presumably require a long enough polypeptide sequence to fold into a structure recognizable by the antibody; again, such antibody probes may also be useful for species differentiation.

RAPD Polymorphism

Cloning of the 2100bp RAPD product was complicated by an apparent volume dependence of the reaction. A direct scale-up of reaction concentrations to a volume only twice the original prevented amplification of the highest molecular weight product (Figure 6). The fact that the 2100bp probe hybridized to most or all of the products of the RAPD reaction suggests that lower molecular weight products arise from the same region of the genome as the 2100bp product (Figure 7). It could be that under certain RAPD conditions, priming is inconsistent and occurs in multiple places within the 2100bp region to produce the smaller products. This is supported by the smaller products that arise from RAPD with opA2 using the 2100bp product as template. The partial sequence of the product does not contain any "perfect" priming sites. However, under the less stringent hybridization conditions of RAPD amplification, priming may occur at less than perfect sites. However, the fact that a one basepair mismatch at the 5'-most base is enough to prevent priming from *M. tuberculosis* templates under RAPD conditions would seem to preclude this. It is interesting and difficult to interpret the implication that one region of the genome is highly susceptible to opA2 priming, given the arbitrary, short sequence of the primer.

The partial sequence from each end of the cloned *M. bovis*-specific fragment has not yet been confirmed by sequencing both strands; differences from the *M. tuberculosis* H37Rv

cosmid sequence may therefore be due to sequencing errors in either sequence, not actual species-specific variation. Indeed, the only unquestionable sequence polymorphism between the two species is the single base substitution at the opA2 primer binding site, since this polymorphism is partially confirmed in various strains and isolates from each species by the RAPD results (see Figure 5). The most confident conclusion is that the complete opA2 primer site at the polymorphic region does not occur in *M. tuberculosis*, since the 2100bp product was never generated from an *M. tuberculosis* template, and that the primer site polymorphism is probably an A to G substitution in all cases. Interestingly, in a comparison of the region of the polymorphism in the H37Rv cosmid mentioned above to another *M. tuberculosis* cosmid (GenBank MSGY423), we found a one nucleotide insertion immediately 3' of the primer binding sequence (Figure 9). Unless this is a sequencing error, this suggests that this region of the genome is highly polymorphic.

The polymorphic substitution is 5' of an ORF with significant homology to MasC (Figure 10), an acyl-CoA synthase, located 5' of and associated with the *mas* gene in *M. bovis* (*14*). The *mas* gene product, mycocerosic acid synthase, has been cloned, characterized, and shown to be required for the synthesis of branched fatty acids (mycosides) that are present only in slow-growing pathogenic mycobacteria (*15,16*). Two ORFs coded in the minus strand technically contain the polymorphism in their 5' region, but not in as close proximity as the acyl-CoA synthase. The putative gene products of these sequences both share homology with antibiotic transport proteins, actII-3 and actB, respectively.

Polymorphism in fatty acid synthase genes among species of pathogenic mycobacteria has several potential sources. Mycosides are phenolic glycolipids exposed on the cell wall of mycobacteria; they represent the major glycolipid component of mycolic acids, which are unique to mycobacteria and are implicated in resistance to host defenses and multiplication within the host. Indeed, inhibition of cell wall synthesis is the basis for a major strategy of

mycobacterial antibiotics. Isoniazid (INH), the most commonly prescribed antimycobacterial drug, blocks mycolic acid synthesis. It has been shown that the effect of INH on long-chain fatty acid synthesis in *M. tuberculosis* can be reversed by the addition of NADH and NADPH, but this effect cannot be reproduced in *M. bovis* BCG (*17,18*). This may correspond with differences in NAD synthesis between *M. tuberculosis* and *M. bovis.*

The product of the *inhA* gene in mycobacteria is an enoyl-ACP reductase involved in fatty acid biosynthesis and is thought to be the target of isoniazid inhibition(*19*). In INH-resistant *M. bovis* and *M. smegmatis*, a single amino acid substitution in *inhA* may be responsible for resistance, while in *M. tuberculosis* mutations in the promoter region of *inhA* were found in resistant strains (*20*). Perhaps different antibiotic pressures on *M. bovis* and *M. tuberculosis* lead to different mechanisms of resistance, and polymorphisms in the fatty acid biosynthetic pathway or antibiotic transport genes (in gene product or in pattern of expression) are the result of attempts by *M. tuberculosis* to develop resistance in the target pathway of INH. The fact that the *M. tuberculosis* isolates we used for RAPD analysis were from human infections whereas the *M. bovis* isolates were bovine suggests that the species we examined were under different antibiotic selection pressures, as INH is not used to treat bovine TB; we should examine *M. bovis* isolates from humans and *M. tuberculosis* isolates from cattle for a dependence of the polymorphism on antibiotic pressure, rather than species identity.

Cell wall makeup is also important in pathogenesis. Invasion of macrophages, interaction with macrophage signaling and function, and immune response by the host are mediated by exposed cell wall constituents. The role of mycolic acids and other surface molecules in host response may offer an explanation of genetic differences between mycobacterial species and strains in mycolic acid synthesis genes. Host T cell proliferation has been

shown to occur in response to mycobacterial lipids and fatty acids; that is, lipids may act as mycobacterial antigens, and these studies suggest that subtle differences existing in the lipids among mycobacterial species are discriminated by cultured T cell lines (*21*). If *M. tuberculosis* and *M. bovis* have different requirements for or methods of host interaction in pathogenesis, variation in fatty acid synthesis genes would be expected. Indeed, the relative virulence of the two in the same host may differ greatly: rabbits are 100 to 1000 times more resistant to *M. tuberculosis* infection than to *M. bovis* (*22*). The importance of interaction with host macrophages in virulence and a role of mycolic acids therein also may explain species variation in the fatty acid synthetic pathway.

We have screened our *M. bovis* genomic library with the radiolabeled 2100bp RAPD product and have identified two clones. Using primers generated from the sequence of the cloned RAPD product and vector primers, we will sequence the region directly outside the opA2 primer sites, both to determine if further polymorphisms occur and to ensure that the sequence at the opA2 priming site is completely conserved in the genome, and is not a PCR artifact due to imperfect primer hybridization. Also, we will complete the sequence of the opA2 RAPD product to resolve ambiguities, check for further polymorphisms, and search for potential internal opA2 priming sites that could explain our results.

A consistent one nucleotide species-specific polymorphism could be exploited to create a PCR assay for diagnosis. Using the polymorphic nucleotide as the 3' end of a long PCR primer would allow amplification of a DNA fragment of predetermined size only from *M. bovis* templates, assuming that the A at position 2170 (Figure 10) is present only and always in all *M. bovis* templates. For example, PCR from mycobacterial templates with the 22nt forward primer 5' GGTCTGGTAATGCTTTGCACCC 3' and the 32nt reverse primer 5' CTTACCCGGTAACGATCCTGGCCACCAGGCC 3' should amplify a fragment of 2154bp from *M. bovis* templates and no fragment from non-*M. bovis*

templates. This assay would also identify other polymorphisms in this region, if the fragment fails to be amplified in an *M. bovis* template, for example. We will synthesize such primers and test the usefulness of such an assay on typing of mycobacterial isolates.

SUMMARY

We have applied techniques that utilize recombinant mycobacterial DNA to two problems in the control of TB infection. First, we have developed a system for identification of mycobacterial antigens by an *in vivo* assay using infected cattle that may represent an advantage over other systems relying on cultured T cell proliferation, considering the role of other host factors and cell types in the immune response. We have identified 7 pools of recombinant clones which through further testing may yield T cell epitopes useful in the development of new protective strategies against TB. Second, through the use of RAPD we have identified: (1) primers which can differentiate closely-related species of mycobacteria, and (2) the source of an apparently species-specific single nucleotide polymorphism between *Mycobacterium tuberculosis* and *M. bovis* which occurs upstream of a gene in the mycolic acid biosynthetic pathway, and may be used both to develop a diagnostic PCR assay and to elucidate biological differences in the two species.

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