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Identification and characterization of an 8.4 kDa protein antigen of *Mycobacterium bovis*.

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

at Massey University, Palmerston North, New Zealand

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ABSTRACT.

The culture filtrate (CF) derived from a *M. smegmatis* subclone transformed with the mycobacteria/*E. coli* plasmid shuttle vector pSU4511 containing a 4.3 kb fragment of *M. bovis* DNA (*M. smegmatis* pSU151.43), was observed to stimulate PBMC from a steer vaccinated with *M. bovis* BCG to proliferate and produce IFN-γ. To identify the source of immunoreactivity, the proteins in CF derived from *M. smegmatis* pSU151.43 were separated by fast protein liquid chromatography (FPLC) and the fractions were screened in whole blood IFN-γ assays. A stimulatory protein was purified that had a molecular mass of 8335 Da and the N-terminal amino acid sequence: DPVDAVINTI. Polyclonal antisera were raised against the purified recombinant antigen in rabbits and used for Western blotting.

The nucleotide sequence of the 4.3 kb insert of *M. bovis* DNA was determined, and the open reading frame (ORF) coding for the 8.4 kDa protein was identified. Computer analysis of the deduced amino acid sequence with the programme PSORT predicted that the nascent protein consisted of a 28 amino acid export signal sequence followed by an 82 amino acid mature protein. It was also found that *M. avium* possesses a nucleotide sequence that potentially codes for a protein with a high degree of homology to the 8.4 kDa antigen of *M. bovis*.

A segment of the 4.3 kb insert of *M. bovis* DNA adjacent to the gene coding for the 8.4 kDa antigen was found to be polymorphic between the strain of *M. bovis* from which the cosmids library was constructed and the published sequence of *M. tuberculosis* H37Rv (Cole et al. 1998). The *M. bovis* sequence contained 1.7 copies of a 62 bp exact tandem repeat and the *M. tuberculosis* sequence contained 2.7 copies. The species distribution of the 62 bp exact tandem repeat (ETR) locus was characterized by polymerase chain reaction (PCR) and Southern blotting. The 62 bp ETR was found to occur only in *M. tuberculosis* complex species and may be a useful genetic marker for differentiating between *M. bovis* and *M. tuberculosis*.

Lymphocyte proliferation and IFN-γ assays were used to measure the responses of ten BCG vaccinated and ten unvaccinated calves the 8.4 kDa antigen, PPD-B and PPD-A tuberculin, both before and after intratracheal challenge infection with virulent *M. bovis.*
The results provided evidence that vaccination of cattle with *M. bovis* BCG but not infection with *M. bovis* appeared to elicit an immune response to the 8.4 kDa antigen of *M. bovis*.

To obtain greater quantities of recombinant 8.4 kDa antigen, the gene that codes for the protein was cloned into *E. coli* and *M. smegmatis* expression plasmids. The 8.4 kDa antigen was overexpressed and secreted with an N-terminal 6 x Histidine tag by *M. smegmatis*. Approximately 500 µg of 6 x Histidine tagged 8.4 kDa Ag were purified / litre of CF in one step by metal chelate affinity chromatography. The recombinant protein was shown to elicit specific IFN-γ responses *in vitro.*
ACKNOWLEDGEMENTS.

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In common with many others who have embarked on Ph.D studies I have found it to have been a tremendous growth process, both professionally and personally. I hereby acknowledge the primary contribution made by my supervisors. My chief supervisor, Dr. Alan Murray took a chance by engaging a veterinarian with limited research experience, had a project with definite objectives and gave me a free rein, but provided useful criticism of often overlooked details. Dr. Murray also performed the PCR analysis described in Chapter 5. Dr. Brigitte Gicquel must be thanked for enabling this work to be conducted at the Pasteur Institute, Paris. My second supervisor, Dr. John Lumsden provided critical input into chromatographic and SDS-PAGE protein separation, Chapters 3 and 6, insight into the analysis of immunological assays, and from time to time - pastoral care.

Much of the work reported in this thesis was only made possible by the assistance of collaborators and commercial service providers. The nucleotide sequence of the 4.3 kb insert of *M. bovis* and other DNA sequences was determined by Dr. Kathryn Stowell and Lorraine Berry at the Massey University DNA Analysis Service. The species distribution of the 62 bp ETR locus was investigated in many more isolates of mycobacteria than available at Massey University by Dr. Cristina Gutierrez at the Pasteur Institute, Paris. Mass spectrometry and N-terminal sequencing was performed by Dr. Gill Norris and Mr. Trevor Loo at Massey University MasSpec, and Protein Sequencing Services. The experimental animals used at Massey University were grazed at the Large Animal Teaching Unit, and thanks must be extended to Mr. Robin Whitson and Odine Johnstone for their willing assistance with blood sampling.

The investigation of immune responses of BCG vaccinated and *M. bovis* challenged animals to the 8.4 kDa antigen was only possible because Dr. Bryce Buddle extended an invitation to ‘piggy-back’ on a trial conducted at AgResearch Wallaceville. I am indebted to him and Denise Keen for supplying blood from the calves, despite sometimes inclement weather. Acknowledgement must also be made of the input Dr. Bryce Buddle provided towards analysis and presentation of the data. Also Mr. Duncan Hedderley from the
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ABBREVIATIONS.

2D-PAGE  two dimensional polyacrylamide gel electrophoresis.
2-ME     2-mercaptoethanol.
2xSLB    2 x sample loading buffer.
6 x His  6 x Histidine.
A_{280}  absorbance at 280 nm.
Ag       antigen.
AHB      Animal Health Board.
APC      antigen presenting cell.
APS      ammonium persulfate.
ATCC     American Type Culture Collection.
BCG      M. bovis bacillus Calmette-Guérin.
BLAST    basic local alignment search tool.
bp       nucleotide base pairs
BSA      bovine serum albumin.
CCT      comparative cervical test.
CD       cluster of differentiation.
CF       culture filtrate.
Cos151   M. smegmatis cosmid library clone 151.
ConA     concanavalin A.
dH_{2}O  double distilled water.
c.p.m    counts per minute.
Δ c.p.m  difference in counts per minute.
Δ OD_{450} difference in optical density at 450 nm.
DAB      3, 3’-Diaminobenzidine.
DIG-dUTP Digoxigenin-11-2’-deoxy-uridine-5’-triphosphate.
DMSO     dimethyl sulfoxide.
DNA      deoxyribonucleic acid.
dsDNA    double stranded DNA.
dNTP     deoxynucleoside triphosphate.
DR       direct repeat.
DTH      delayed type hypersensitivity.
DTT      dithiothreitol.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>EIA</td>
<td>enzyme immunoassay.</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay.</td>
</tr>
<tr>
<td>ERMA</td>
<td>Environmental Risk Management Authority.</td>
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<tr>
<td>ETR</td>
<td>exact tandem repeat.</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations.</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum.</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography.</td>
</tr>
<tr>
<td>g</td>
<td>gravity (a force of ~ 10 N).</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase.</td>
</tr>
<tr>
<td>HRP</td>
<td>horesradish peroxidase.</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule.</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund’s Adjuvant.</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon.</td>
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<tr>
<td>IFN-γ</td>
<td>interferon gamma.</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin.</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-galactoside.</td>
</tr>
<tr>
<td>IS</td>
<td>insertion sequence.</td>
</tr>
<tr>
<td>IU</td>
<td>international units.</td>
</tr>
<tr>
<td>IUATLD</td>
<td>International Union Against Tuberculosis and Lung Disease.</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemists.</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs.</td>
</tr>
<tr>
<td>LB</td>
<td>Lauria-Bertani.</td>
</tr>
<tr>
<td>LpP</td>
<td>lymphocyte proliferation.</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex.</td>
</tr>
<tr>
<td>MIRU</td>
<td>mycobacterial interspersed repetitive unit.</td>
</tr>
<tr>
<td>MPTR</td>
<td>major polymorphic tandem repeat.</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight.</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off.</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information.</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer T-lymphocyte.</td>
</tr>
<tr>
<td>NVL</td>
<td>no visible lesions.</td>
</tr>
<tr>
<td>OD</td>
<td>optical density.</td>
</tr>
<tr>
<td>OIE</td>
<td>Office International des Epizooties.</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame.</td>
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PBMC  peripheral blood mononuclear cells.
PBS  phosphate buffered saline.
PCR  polymerase chain reaction.
PGRS  polymorphic GC rich repetitive sequence.
PMSF  phenylmethylsulfonyl fluoride.
PO₄SB  phosphate start buffer.
PO₄SB  phosphate wash buffer.
PPD  purified protein derivative.
PPD-A  PPD derived from *M. avium*.
PPD-B  PPD derived from *M. bovis*.
RFLP  restriction fragment length polymorphism.
RNA  ribosomal nucleic acid.
r.p.m  revolutions per minute.
SDS-PAGE  sodium dodecyl sulphate - polyacrylamide electrophoresis.
SIT  single intradermal test.
TAE  Tris-acetate.
TB complex  *Mycobacterium tuberculosis* complex.
TBE  Tris-borate.
TIGR  The Institute for Genomic Research.
TEMED  N, N, N’, N’-tetramethylethylenediamine.
Th 1/Th 2  T-helper cell phenotype Type 1/Type 2.
TNF  tumour necrosis factor.
TTBS  Tween Tris-buffered saline.
U  units.
UV  ultraviolet.
V  volts.
VNTR  variable number of tandem repeats.
WHO  World Health Organization.
w/v  weight for volume.
X-Gal  5-bromo-4-chloro-3-indoyl-β-D-galactoside.