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

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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 chromotography (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: DPVDAVINTT. 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 cosmid 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 tuberculins, 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.*

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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.

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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_2O	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.

EIA	enzyme immunoassay.
ELISA	enzyme linked immunosorbent assay.
ERMA	Environmental Risk Management Authority.
ETR	exact tandem repeat.
FAO	Food and Agriculture Organization of the United Nations.
FCS	foetal calf serum.
FPLC	fast protein liquid chromatography.
g	gravity (a force of ~ 10 N).
GST	glutathione-S-transferase.
HRP	horesradish peroxidase.
ICAM	intercellular adhesion molecule.
IFA	Incomplete Freund's Adjuvant.
IFN	interferon.
IFN-γ	interferon gamma.
IL	interleukin.
IPTG	isopropylthio-β-galactoside.
IS	insertion sequence.
IU	international units.
IUATLD	International Union Against Tuberculosis and Lung Disease.
IUPAC	International Union of Pure and Applied Chemists.
IWGMT	International Working Group on Mycobacterial Taxonomy.
kb	kilobase pairs.
LB	Lauria-Bertani.
LøP	lymphocyte proliferation.
MHC	major histocompatability complex.
MIRU	mycobacterial interspersed repetitive unit.
MPTR	major polymorphic tandem repeat.
MW	molecular weight.
MWCO	molecular weight cut-off.
NCBI	National Center for Biotechnology Information.
NK	natural killer T-lymphocyte.
NVL	no visible lesions.
OD	optical density.
OIE	Office International des Epizooties.
ORF	open reading frame.

PBMC	peripheral blood mononuclear cells.
PBS	phosphate buffered saline.
PCR	polymerase chain reaction.
PGRS	polymorphic GC rich repetitive sequence.
PMSF	phenylmethylsulfonyl flouride.
PO_4SB	phosphate start buffer.
PO_4SB	phosphate wash buffer.
PPD	purified protein deriviative.
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.