Identification and characterisation of an exported immunogenic protein of *Mycobacterium avium* subspecies *paratuberculosis*

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

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Thesis Title: Identification and Characterisation of an Exported Immunogenic Protein of M. avium Subspecies paratuberculosis
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Abstract

Exported proteins of mycobacteria are available to interact with the immune system at an early stage of infection and are potent inducers of immune responses. Potentially exported proteins of Mycobacterium avium subspecies paratuberculosis were identified using alkaline phosphatase gene fusion technology. A library of partial gene fusions from a New Zealand clinical isolate of M. a. paratuberculosis was constructed in the shuttle vector pJEM11 and expressed in the surrogate hosts E. coli and M. smegmatis. The DNA inserts from a portion of the resulting clones expressing alkaline phosphatase-positive fusion proteins were partially sequenced to identify the proteins. Eleven proteins not previously described for M. a. paratuberculosis were identified as containing signal sequences for export. One of these, a putative lipoprotein named P22 was selected for further study. The full nucleic acid sequence of the p22 gene was determined and the open reading frame was cloned into the mycobacterial expression vector pMIP12. This enabled P22 to be produced as a polyhistidine-tagged protein in M. smegmatis and facilitated purification by chromatography. N-terminal sequencing of the recombinant protein confirmed cleavage of an N-terminal signal sequence. Native P22 was detected in culture supernatants and cell sonicates of M. a. paratuberculosis strain 316F using rabbit antibody raised to P22. Investigation of the presence of genes similar to p22 in other mycobacterial species, revealed p22 was present in Mycobacterium avium subspecies avium and similar genes existed in M. intracellulare (88.5% identity) and M. saofulaceum (87.7% identity). Database searches showed P22 belonged to the LppX/LprAFG family of mycobacterial lipoproteins also found in M. leprae and in members of the M. tuberculosis complex. P22 shared less than 75% identity to these proteins. Recombinant P22 was able to elicit significantly increased interferon-gamma secretion in blood from a group of eight sheep vaccinated with a live, attenuated strain of M. a. paratuberculosis (strain 316F) compared to a group of five unvaccinated sheep. Antibody to P22 was detected by Western blot analysis in 10 out of 11 vaccinated sheep, in two out of two clinically affected cows and in 11 out of 13 subclinically infected cows.
Acknowledgements

Much of the work reported in this thesis was made possible by the assistance of many people and service providers. DNA sequencing was done by Lorraine Berry at the Massey DNA sequencing facility. N-terminal sequencing of P22 was done by Trevor Loo at Massey University Protein Sequencing Services. Faecal culture and ELISA tests for the cattle used, were carried out by AgResearch, Wallaceville. The serum samples from these cattle were generously donated by Dr. Cord Heuer and Solis Norton. *M. a. paratuberculosis* DNA samples used for PCR analysis of strain distribution were a gift from Dr. Desmond Collins. The experimental sheep were housed at Massey University Agricultural Services. I'd especially like to thank Margaret Brown and Geoff Warren for wrangling sheep and collecting blood samples and watching over the animals that were so important to this study. The mycobacterial shuttle vectors pJEM11 and pMIP12, were kindly supplied by Professor Brigitte Gicquel, Unite de Genetique Mycobacterienne, Pasteur Institute, Paris, France. Sue Copland assisted with the Southern blots used in this study and Dr. Jeremy Rae contributed in the preparation of *M. a. paratuberculosis* culture filtrates and isocitrate dehydrogenase assays. Support for this work was provided by Meat New Zealand, WoolPro and the IVABS post-graduate student support fund.

The people who have influenced me in the decision to embark on a career in scientific research deserve more thanks than I can mention here. My chief supervisor, Dr. Alan Murray has provided unconditional guidance and support over the years. He has a gift of always finding some positive aspect in every result and I could always count on him for encouragement through rough times. My second supervisor Dr. Keith Thompson always managed to find time for me (and my sheep). His wisdom and experience as a scientist and teacher has been greatly appreciated and admired. My third supervisor Dr. John Tweedie gave essential criticism and advice in regards to this thesis and was always willing to help and support me. Dr. Catherine Day introduced me to the wonderful world of protein expression and encouraged me in my decision to undertake PhD studies, even though it meant losing her only technician. My lab-mates Dr. Jane
Oliaro, Dr. Sandy Maclachlan, Miho Minamikawa and Dr. Becky Davies have been excellent company over the years.

Special thanks must be said to members of my family whose support has been a significant factor in my enjoyment of this work. Because of my insistence in pursuing this path, my family in Canada have suffered the (temporary) loss of their daughter and grand children. Despite this, they constantly offered their support. My husband Dr. John Lumsden who quietly encouraged me through this endeavour and always managed to help me keep things in perspective. Last but not least, my children Jamie and Genna who handled my "absence" with great maturity. I'm sure all the nights of take-aways for dinner contributed to their understanding.

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Construction of the M. a. paratuberculosis PhoA fusion library and characterisation of SodC from this study have been published (Dupont, C. & Murray, A. (2001). Identification, cloning and expression of sodC from an alkaline phosphatase gene fusion library of Mycobacterium avium subspecies paratuberculosis. Microbios 106 S1: 7-19).
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List of Abbreviations

A$_{280}$nm absorbance at 280 nm
ATCC American type culture collection
Avian PPD Purified protein derivative from M. a. avium
BCG bacillus Calmette-Guerin
BCIP 5-bromo-4-chloro-3-indolyl phosphate
BLAST basic local alignment search tool
ConA concavalin A
dTTP deoxythymidine triphosphate
dUTP deoxyuridine triphosphate
DIG digoxigenin
DNA deoxyribonucleic acid
EDTA ethylenediamine tetraacetic acid
ELISA enzyme-linked immunosorbent assay
HPLC high pressure liquid chromatography
IFN-γ interferon-gamma
Johnin PPD purified protein derivative from M. a. paratuberculosis
kan kanamycin
kb kilobase pairs
kDa kilodalton(s)
LAM lipoarabinomannan
LB Luria-Bertani
OD optical density
ORF open reading frame
PBS phosphate-buffered saline
PCR polymerase chain reaction
PhoA alkaline phosphatase
POD peroxidase
PVDF polyvinylidene difluoride
RBS ribosome binding site
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE Tris-acetate, EDTA
UV ultraviolet

Amino acids

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</tbody>
</table>