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

Doctor of Philosophy

at Massey University, Palmerston North, New Zealand

Christine Dupont

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Name of Chief Supervisor: Dr Alan Murray Telephone Ext: 7895

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Part of the work is being included in a patent application which is currently being prepared.

The patent applicants will be Massey University and the Pasteur Institute in Paris.

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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. intracellularae* (88.5% identity) and *M. scrofulaceum* (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, Wool*Pro* 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

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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.

I would also like to express my thanks to the sheep used in this study. Approval for the use of experimental animals was granted by the animal ethics committee of Massey University, protocols 99/155 and 00/87. Permission to carry out genetic manipulations of *E. coli* and *M. smegmatis* was granted by Massey University Genetic Technology Committee under Section 40 of the Hazardous Substances and New Organisms Act 1996, GTC application GM099/MU/179.

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 tetraacedic 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				
	A	alanine	C	cysteine
	D	aspartic acid	E	glutamic acid
	F	phenylalanine	G	glycine
	H	histidine	I	isoleucine
	K	lysine	L	leucine
	M	methionine	N	asparagine
	P	proline	Q	glutamine
	R	arginine	S	serine
	T	threonine	W	tryptophan
	V	valine	Y	tyrosine

Nucleic acids	**A**	adenosine
	T	thymidine
	C	cytidine
	G	guanosine