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**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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MASSEY UNIVERSITY

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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. intracellulae* (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.

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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 <i>M. a. avium</i>
BCG	bacillus Calmette-Guerin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	basic local alignment search tool
ConA	concanavalin 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 <i>M. a. paratuberculosis</i>
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