A study on secreted proteins of *Mycobacterium avium* subspecies *paratuberculosis* vaccine strain 316F

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) strain 316F is the organism in the live attenuated vaccine Neoparasec™ which has been used to control paratuberculosis or Johne's disease in cattle and sheep.

The aim of this study was to identify novel exported proteins of MAP strain 316F, with a view to identifying immunogens that may have application in diagnostic tests. Potentially exported proteins were identified using alkaline phosphatase gene fusion technology. A partial digest of the MAP strain 316F genomic DNA was cloned into the vector pJEM11, and expressed in the surrogate hosts *E. coli* and *M. smegmatis*. The DNA inserts from selected alkaline phosphatase positive clones were partially sequenced and the sequences were analysed using public databases to identify and obtain full gene sequences and to predict the potential function of the identified proteins.

The genes from three putative exported proteins: glutamine binding protein (*glnH*, MAP3894c), sulphate binding protein (*subI*, MAP2213c) and a hypothetical protein (MAP3273c), were selected for preliminary investigation. The open reading frame of each gene was obtained by PCR amplification and was cloned into the *E. coli* expression vector pET-26b (+) for the expression of C-terminal histidine-tagged fusion proteins. The recombinant proteins were prepared and purified by immobilized-metal affinity chromatography.

Following SDS-PAGE, the three antigens were screened by Western blot analysis using sera from sheep vaccinated with Neoparasec™ and from control pre-vaccinated animals. Western blot analysis indicated that whilst antibodies could be detected in vaccinated animals to subI and the hypothetical protein, cross reactive antibodies could also be detected in some sera taken prior to vaccination. However, five out of eight animals had a strong antibody responses to glnH following vaccination with Neoparasec™ compared with one out of eight

in the pre-vaccinated control animals suggesting that this was an immunogenic protein expressed in the native host. GlnH was therefore selected for further characterisation.

Investigation into the presence of the *glnH* gene in other mycobacterial species revealed that *glnH* has a 99% identity with the extracellular solute-binding protein of *Mycobacterium avium* subspecies *avium* and similar genes exist in *M. bovis* & *M. tuberculosis* (85.1% identity), *M. ulcerans* (83.4% identity), *M. vanbaalenii* (78.9% identity), *M. smegmatis* (78.1% identity) and *M. gilvum* (78% identity).

An antibody raised in a rabbit to glnH and used in immunofluorescence and transmission electron microscopy studies for protein localisation in MAP strain 316F cells suggested that glnH is located on the surface of the native host.

In addition to antibody against glnH being detected in the sera of sheep vaccinated with Neoparasec™, Western blot analysis also showed that antibody could be detected in the sera of sheep and deer naturally infected with MAP. In order to quantify these responses, an ELISA was developed and a pilot study undertaken that confirmed that there was a significant difference ($p < 0.05$) in antibody responses to glnH between the vaccinated sheep and the unvaccinated controls. Also, serum samples collected from sheep and deer naturally infected with MAP were found to have significant ($p < 0.05$) levels of antibody to glnH compared to uninfected control animals.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

The abbreviations commonly used are presented in the following list:

ABC	ATP binding cassette
ADC	Albumin-D-glucose/dextrose-Catalase
EDTA	Ethylenediamine tetraacetic acid
AGID	Agar gel immunodiffusion test
ATP	Adenosin triphosphate
BCIP	5-bromo-4-chloro-indolyl phosphate
BLAST	Basic local alignment search tool
bp	base pair (s)
CD	Crohn's disease
CIAP	Calf Intestinal alkaline phosphatase
CFT	Complement fixation test
CFU	Colony forming unit
CMI	Cell-mediated immune response
ConA	Concavalin-A
Cat. No.	Catalog number
cm	Centimetre(s)
^{o}C	Degrees Celcius
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTH	Delayed type hypersensitivity
EPB	Electroporation buffer
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GTE	Glucose-Tris-EDTA
g	Acceleration due to gravity
IgG	ImmunoglobulinG
IMAC	Immobilised metal affinity chromatography
IS*900*	Insertion segment-*900*
IFN-gamma	Interferon-gamma
IM	Inner membrane
IPTG	Isopropyl-β-δ-thiogalactopyranoside
kDa	Kilo dalton
kb	Kilo base pair(s)
L	Litre(s)
LB	Luria-Bertani broth

LBA	Luria-Bertani agar
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MW	Molecular weight
Mb	Mega base pair(s)
M	Molar
µM	Micromolar
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
mM	Millimolar
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
min	Minute(s)
nm	Nanometre(s)
ng	Nanogram(s)
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PBS	Phosphate-buffered saline
PhoA	Alkaline phosphatase
PFC	Pooled faecal culture
PVDF	Polyvinylidene difluoride membrane
PPDA	Avian purified-protein derivative
PCR	Polymerase chain reaction
rpm	Revolutions per minute
SBP	Substrate binding protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	Super optimal broth
Tm	Melting temperature
TAE	Tris-acetate-EDTA
TE	Tris-HCl-EDTA
v/v	Volume/volume
w/v	Weight/volume
7H9-B	Middlebrook 7H9 broth
7H10-A	Middlebrook 7H10 agar

Nucleotides

A	Adenine
C	Cytidine
G	Guanosine
T	Thymidine

Amino acids

A	Alanine	I	Isoleucine	R	Arginine
C	Csyteine	K	Lysine	S	Serine
D	Aspartic acid	L	Leucine	T	Threonine
E	Glutamic acid	M	Methionine	W	Tryptophan
F	Phenyl alanine	N	Asparagine	V	Valine
G	Glycine	P	Proline	Y	Tyrosine
H	Histidine	Q	Glutamine		