

Studies on a 40kDa Protein Antigen of
IS901/902-Positive *Mycobacterium avium*

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“This thesis is dedicated to Douglas Duncan,
16th July 1959 - 21st April 1979.”

ABSTRACT

The work presented in this thesis was undertaken as part of a larger research initiative in the field of ruminant paratuberculosis (Johne's disease). One of the group's main research objectives was the identification and characterisation of subspecies-specific genes and proteins of the two members of the *Mycobacterium avium* complex (MAC) known to have a causal role in ruminant paratuberculosis, namely *Mycobacterium avium* spp. *paratuberculosis* (*M.a.paratuberculosis*) and IS901/902-positive strains of *Mycobacterium avium* (*M.avium*).

A protein antigen of 40kDa (p40) was identified in an IS901/902-positive strain of *M.avium*, but could not be detected in *M.a.paratuberculosis*, IS901/902-negative *M.avium*, or in any of 13 other species of *Mycobacterium* tested. Examination of 19 further MAC field isolates confirmed the absolute association between p40 and IS901/902, suggesting that p40 is a novel, subspecies-specific protein which may potentially be of value as a diagnostic antigen.

Soluble p40 antigen was precipitated from cleared cell lysates and purified to homogeneity using a series of chromatographic separations. Chemical cleavage and Edman degradation of the purified p40 antigen provided both amino-terminal and internal amino acid sequence data which showed no sequence identity with any protein sequence currently in the OWL database. Translations of IS901/902 in all six reading frames revealed amino acid sequences which confirmed that the genomic insertion sequence does not encode the p40 antigen. A second protein of 30kDa (p30) was purified simultaneously, and analysis of the first 10 amino-terminal amino acid residues revealed up to 90% sequence identity with the mature secretory antigens of the "antigen 85 complex" of eight other species of *Mycobacterium*.

Experiments were designed to ascertain whether the p40 antigen was expressed *in vivo* and to assess whether any immune response to the p40 could be exploited in the development of an immune-based assay to differentiate between animals infected with *M.a.paratuberculosis*, and others infected with IS901/902-positive strains of *M.avium*. Two groups of sheep were infected orally with either *M.a.paratuberculosis* or IS901/902-positive *M.avium*. In assays measuring secondary T-lymphocyte proliferation and IFN- γ production, the p40 antigen stimulated responses only in the sheep infected with IS901/902-positive *M.avium*. As such, the p40 antigen is the first protein molecule to be identified which can be used to distinguish between the two members of the *M.avium* complex responsible for causing chronic inflammatory bowel disease in ruminants. In addition, immune responses to the p30 secretory antigen were observed in both groups of infected sheep, but not in a third group of sheep immunised with killed *M.a.paratuberculosis*. This suggests that immune responses to the p30 secretory antigen have the potential to distinguish between vaccinated animals and those harbouring active mycobacterial infections.

Hyperimmune antisera against the p40 antigen were raised in rabbits. These reagents were used to immunoscreen an amplified library prepared from randomly sheared IS901/902-positive *M.avium* genomic DNA in the λ gt 11 expression vector. Five expressing clones were identified and plaque-purified from a total of 1.6×10^6 pfu screened. Subsequent analysis and characterisation of these clones revealed that four contained identical insert fragments of 7.1kb and expressed a full length subunit of bacterioferritin. The remaining clone was shown to contain an insert fragment of 2.3kb and expressed a protein which was recognised by two anti-hsp65 monoclonal antibodies. No antigenic cross-reactivity could be demonstrated between either of these recombinant proteins and the p40 antigen. The identification of recombinants expressing bacterioferritin was attributed to the presence of a trace impurity in the p40 inoculum used to immunise the rabbits, while antibodies to hsp65 proteins may have been intrinsic to the rabbits themselves. Further immunoscreening of the amplified library with serum absorbed exhaustively with lysates of *M.a.paratuberculosis*, yielded 15 additional expressing clones. All of these were shown to contain a 2.3kb insert fragment identical to that of the putative heat shock recombinant. A second expression library was immunoscreened without amplification and in its entirety (6.4×10^5 pfu) using antiserum which recognised only p40 on Western blots of IS901/902-positive *M.avium*. No clones expressing p40 were detected.

Degenerate oligonucleotides deduced from p40 amino acid sequence data were unsuccessful in detecting homologous sequences in the amplified library. Attempts to amplify p40 gene sequences by PCR were unsuccessful also, regardless of whether template DNA was prepared from the amplified library or from cultured IS901/902-positive *M.avium* cells. One 21mer oligonucleotide (M5018) hybridised with three *EcoRI* fragments (5.2kb, 4.5kb and 4.0kb) common to both IS901/902-positive *M.avium* and *M.a.paratuberculosis* on Southern blots of restricted genomic DNA, and two additional *EcoRI* fragments (3.7kb and 450bp) which were unique to IS901/902-positive *M.avium*. All five IS901/902-positive *M.avium* *EcoRI* fragments were cloned in plasmid vectors and sequenced across the region homologous to oligonucleotide M5018. Analysis of the homologous genomic sequences revealed identity and similarity values ranging between 52.4%-76.2% and 61.9%-90.5% respectively. Translations over the five homologous 21bp regions were compared with the sequence of seven contiguous amino acid residues from which oligonucleotide M5018 was originally deduced. Levels of amino acid sequence identity ranging between a single mismatch, and two mismatches plus two conservative substitutions were revealed. However, the sequences flanking the seven amino acid residues used to deduce oligonucleotide M5018 bore no resemblance to those of any of the five translated genomic clones.

The gene encoding the p40 antigen remains to be cloned and characterised. The relationship between p40 and IS901/902 is not direct and the precise nature of their close association has yet to be established.

DECLARATION

The work presented in this thesis was undertaken by me as part of an integrated research initiative on ruminant paratuberculosis at the Moredun Research Institute. I declare that experimental design, execution and interpretation of results are my own unless stated otherwise. All contributions made by other people are fully acknowledged in the text.

Neil Fraser Inglis

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APPENDIX 5.

Publications Arising From Work Presented in This Thesis

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ABBREVIATIONS SYMBOLS AND FORMULAE

| | |
|--------------------------------------------------------------------------------------|-------------------------------------------------------------------|
| α | alpha |
| aa..... | amino acid |
| A..... | ampere |
| ABTG..... | p-aminobenzyl 1-thio- β -D-galactopyranoside |
| AGID..... | agarose gel immunodiffusion |
| Amp..... | ampicillin |
| ATP..... | adenosine triphosphate |
| ~..... | approximately |
| \cong | approximately equal to |
| β | beta |
| BAP..... | bovine alkaline phosphatase |
| BCA..... | bicinchinoic acid |
| BCG..... | Bacillus Calmette-Guerin |
| bfr..... | bacterioferritin |
| <i>bfr</i> | gene encoding bacterioferritin |
| β -gal..... | betagalactosidase |
| bp..... | base pairs |
| BSA..... | bovine serum albumin |
| CaCl ₂ | calcium chloride |
| CCTB..... | calcium chloride transformation buffer |
| CEC..... | Commission of the European Communities |
| CF..... | complement fixation |
| cfu..... | colony forming units |
| C ₂ HCl ₃ O ₂ | trichloroacetic acid |
| C ₆ H ₈ O ₇ | citric acid |
| C ₆ H ₅ Na ₃ O ₇ .2H ₂ O..... | trisodium citrate |
| Ci..... | curie |
| CIP..... | calf intestinal phosphatase |
| CMI..... | cell mediated immunity |
| CMN group..... | <i>Corynebacterium</i> , <i>Mycobacterium</i> and <i>Nocardia</i> |
| CNBr..... | cyanogen bromide |
| con A..... | concanavalin A |
| Co(NH ₃) ₆ Cl ₃ | hexamine cobalt III chloride |
| DAB..... | diaminobenzidine |
| DAP..... | dual array processor |
| DDA..... | diethyldioctadecyl ammonium chloride |
| DMSO..... | dimethyl sulphoxide |
| DNA..... | deoxyribonucleic acid |
| dNTP:- | deoxynucleotide triphosphate |
| dATP..... | deoxyadenosine triphosphate |
| dCTP..... | deoxycytosine triphosphate |
| dGTP..... | deoxyguanosine triphosphate |

| | |
|-----------------------------------------------------|--------------------------------------------------------|
| dTTP..... | deoxythymidine triphosphate |
| ddATP..... | dideoxyadenosine triphosphate |
| ddCTP..... | dideoxycytosine triphosphate |
| ddGTP..... | dideoxyguanosine triphosphate |
| ddTTP..... | dideoxythymidine triphosphate |
| d/s..... | double stranded |
| DTH..... | delayed type hypersensitivity |
| DTT..... | dithiothreitol |
| DW..... | distilled water |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| ELISA..... | enzyme-linked immunosorbent assay |
| EMBL..... | European Molecular Biology Laboratory |
| FIGE..... | field inversion gel electrophoresis |
| γ | gamma |
| g..... | gram |
| g..... | gravitational force |
| G+C..... | guanosine + cytosine content |
| GPL..... | glycopeptidolipid |
| GST..... | glutathione-S transferase |
| GTP..... | guanosine triphosphate |
| HBSS..... | Hanks balanced salt solution |
| HCl..... | hydrochloric acid |
| <i>hed</i> | (host expression dependent) gene encoding Hed protein |
| Hed..... | (host expression dependent) protein |
| HEPES..... | (N-[hydroxyethyl]piperazine-N'-[ethanesulphonic acid]) |
| HETB..... | high efficiency transformation buffer |
| H ₂ O ₂ | hydrogen peroxide |
| HPLC..... | high performance liquid chromatography |
| hr..... | hour |
| HRP..... | horseradish peroxidase |
| H ₂ SO ₄ | sulphuric acid |
| hsp..... | heat shock protein |
| <i>hsp</i> | gene encoding heat shock protein |
| IFN- γ | interferon gamma |
| IPTG..... | isopropyl β -D-thiogalactopyranoside |
| IS..... | insertion sequence |
| iu..... | international units |
| IUT..... | International Union against Tuberculosis |
| kb..... | kilobase pairs |
| KC ₂ H ₃ O ₂ | potassium acetate |
| kDa..... | kilodaltons |
| kg..... | kilogram |
| K ₂ HPO ₄ | dipotassium hydrogen orthophosphate |
| KH ₂ PO ₄ | potassium dihydrogen orthophosphate |
| λ | lambda |

| | |
|-------------------------------------------------------|--------------------------------------------------------|
| λ gt11..... | bacteriophage lambda gt11 |
| L..... | litre |
| LAM..... | lipoarabinomannan |
| L-JP..... | Lowenstein-Jensen pyruvate medium |
| LMP..... | low melting point |
| LRF..... | large restriction fragment |
| M..... | molar |
| <i>M.</i> | <i>Mycobacterium</i> |
| mA..... | milliampere |
| mab..... | monoclonal antibody |
| MAC..... | <i>Mycobacterium avium</i> complex |
| MAIS..... | <i>Mycobacterium avium/intracellulare/scrofulaceum</i> |
| MBq..... | Mega-Becquerel |
| MES..... | (2-[morpholino]ethanesulphonic acid |
| mg..... | milligram (10^{-3} gram) |
| MHC..... | major histocompatibility complex |
| MgCl ₂ | magnesium chloride |
| MgSO ₄ | magnesium sulphate |
| min..... | minute(s) |
| ml..... | millilitre (10^{-3} litre) |
| mM..... | millimolar (10^{-3} molar) |
| MnCl ₂ | manganese chloride |
| MOTT..... | mycobacteria other than tuberculosis |
| MRI..... | Moredun Research Institute |
| M.wt..... | molecular weight |
| NaCl..... | sodium chloride |
| Na ₂ CO ₃ | sodium carbonate |
| Na ₂ EDTA..... | ethylenediaminetetraacetic acid disodium salt |
| NaHCO ₃ | sodium bicarbonate |
| Na ₂ HPO ₄ | disodium hydrogen orthophosphate |
| NaH ₂ PO ₄ | sodium dihydrogen orthophosphate |
| NaOH..... | sodium hydroxide |
| NCTC..... | National Collection of Type Cultures |
| ng..... | nanogram (10^{-9} gram) |
| NH ₄ Cl..... | ammonium chloride |
| (NH ₄) ₂ SO ₄ | ammonium sulphate |
| NK cells..... | natural killer cells |
| Nonidet-P40..... | nonionic detergent-P40 |
| OAA..... | oxaloacetate |
| OD..... | optical density |
| ORF..... | open reading frame |
| PAGE..... | polyacrylamide gel electrophoresis |
| PBMC..... | peripheral blood mononuclear cells |
| pBS..... | plasmid Bluescript |
| PBS..... | phosphate buffered saline |

| | |
|-------------------------|--------------------------------------------------------|
| PCR..... | polymerase chain reaction |
| PDIM..... | phthiocerol dimycocerosate |
| PEG..... | polyethylene glycol |
| PEP..... | phosphoenolpyruvate |
| PEPCK..... | phosphoenolpyruvate carboxykinase |
| PFGE..... | pulsed-field gel electrophoresis |
| pfr..... | prokaryotic ferritin |
| pfu..... | plaque forming unit |
| pGEX 1 λ T..... | plasmid (GST expression vector) |
| PGL..... | phenolic glycolipids |
| PIM..... | phosphatidylinositol manoside |
| pmol..... | picomole (10^{-12} moles) |
| PMSF..... | phenylmethylsulphonyl fluoride |
| PPD..... | purified protein derivative |
| PPEM..... | potentially pathogenic environmental mycobacteria |
| psi..... | pounds per square inch |
| pUC..... | plasmid |
| PVDF..... | polyvinylidene difluoride |
| p30..... | 30kDa secretory antigen of <i>M.a.paratuberculosis</i> |
| p40..... | 40kDa protein antigen of <i>M. avium</i> type A/I |
| RBS..... | ribosome binding site |
| RE..... | restriction endonuclease |
| RFLP..... | restriction fragment length polymorphism |
| RNA:-..... | ribonucleic acid |
| mRNA..... | messenger ribonucleic acid |
| rRNA..... | ribosomal ribonucleic acid |
| tRNA..... | transfer ribonucleic acid |
| RPMI..... | Rosewell Park Memorial Institute |
| <i>rrn</i> | ribosomal RNA operon |
| SDS..... | sodium dodecyl sulphate |
| SERC..... | Scientific and Engineering Research Council |
| SI..... | stimulation index |
| SMRL..... | Scottish Mycobacteria Reference Laboratory |
| sp.act..... | specific activity |
| TB-complex..... | tuberculosis complex |
| T_m | melting temperature |
| TMB..... | tetramethylbenzidine dihydrochloride |
| μ Ci..... | microcurie (10^{-6} curie) |
| μ g..... | microgram (10^{-6} gram) |
| μ l..... | microlitre (10^{-6} litre) |
| μ M..... | micromolar (10^{-6} molar) |
| UWGCG..... | University of Wisconsin Genetics Computer Group |
| V..... | volt |
| v/v_v | volume to volume |
| V_t | terminal volume |

| | |
|------------|---------------------------------------------------------|
| W..... | watt |
| wt/v..... | weight to volume |
| wt/wt..... | weight to weight |
| X-gal..... | 5-bromo-4-chloro-3-indoly- β -D-galactopyranoside |

CHAPTER 1

INTRODUCTION

1.1 The Genus *Mycobacterium*

The genus *Mycobacterium* represents the only genus in the family *Mycobacteriaceae* and is placed in a taxonomic group which includes the genera *Corynebacterium*, *Mycobacterium* and *Nocardia* (the CMN group) and *Rhodococcus* (Barksdale and Kim, 1977). Referring to the mould-like appearance of *M.tuberculosis* in liquid culture, the generic name *Mycobacterium* (“fungus bacterium”) was proposed by Lehmann and Neumann (1896). The group comprises straight or gently curved bacilli, although coccobacillary, filamentous and branched forms also have been described. The cells are gram positive although all members share a highly impermeable and lipid-rich cell wall structure which negates the routine use of conventional gram staining. All members are aerobic or microaerophilic, non-motile and non-sporing. The genus includes photochromogens, scotochromogens and non-chromogens, and the number of recognised species and subspecies names currently exceeds seventy (Table 1.). The nutritional requirements and optimal growth temperatures for different species vary considerably and not all members can be cultivated on laboratory growth medium. The cultivable members of the genus have been divided into two categories; the rapid growers and the slow growers. Although predominantly a genus of saprophytes, living freely in the environment, the mycobacteria include pathogens of many diverse hosts ranging from marine sponges and fishes, to birds and mammals. These include the two major mammalian pathogens *M.leprae* (Hansen, 1874) and *M.tuberculosis* (Koch, 1882), the aetiological agents of leprosy and tuberculosis respectively. References to these scourges in the Bible, classic literature and opera demonstrate that they have been recognisable to the general public through the centuries, and highlight the continuity of man’s struggle against mycobacterial disease.

Table 1.1
 Alphabetical List of Currently known Species of the
 Genus *Mycobacterium*

| Species | Subspecies | Pathogen | Citation |
|----------------------------|-------------------------|----------|--------------------------------------|
| <i>M. aichiense</i> | | - | Tsukamura <i>et al.</i> , 1981 |
| <i>M. africanum</i> | | + | Castets <i>et al.</i> , 1969 |
| <i>M. agri</i> | | - | Tsukamura, 1972a |
| <i>M. alvei</i> | | - | Ausina <i>et al.</i> , 1992 |
| <i>M. asiaticum</i> | | + | Weiszfeiler <i>et al.</i> , 1971 |
| <i>M. aurum</i> | | - | Tsukamura and Tsukamura, 1966 |
| <i>M. austroafricanum</i> | | - | Tsukamura <i>et al.</i> , 1983c |
| <i>M. avium</i> :- | <i>avium</i> | + | Chester, 1901 |
| | <i>paratuberculosis</i> | + | Bergey <i>et al.</i> , 1923 |
| | <i>silvaticum</i> | + | Thorel <i>et al.</i> , 1990 |
| <i>M. bovis</i> | | + | Karlson and Lessel, 1970 |
| <i>M. branderi</i> | | +/- | Koukila-Kahkola <i>et al.</i> , 1995 |
| <i>M. celatum</i> | | + | Butler <i>et al.</i> , 1993 |
| <i>M. chelonae</i> :- | <i>abscessus</i> | + | Kubica <i>et al.</i> , 1972 |
| | <i>chelonae</i> | + | Bergey <i>et al.</i> , 1923 |
| <i>M. chitae</i> | | - | Tsukamura, 1967a |
| <i>M. chlorophenolicum</i> | | - | Haggeblom <i>et al.</i> , 1994 |
| <i>M. chubuense</i> | | - | Tsukamura <i>et al.</i> , 1967 |
| <i>M. confluentis</i> | | - | Kirschner <i>et al.</i> , 1992 |
| <i>M. conspicuum</i> | | + | Springer <i>et al.</i> , 1995 |
| <i>M. cookii</i> | | - | Kazda <i>et al.</i> , 1990 |
| <i>M. diernhoferi</i> | | - | Tsukamura <i>et al.</i> , 1983c |
| <i>M. duvalii</i> | | - | Stanford and Gunthorpe, 1971 |
| <i>M. fallax</i> | | - | Levy-Frebault <i>et al.</i> , 1983 |
| <i>M. farcinogenes</i> | | + | Chamoiseau, 1979 |
| <i>M. flavescens</i> | | - | Bojalil <i>et al.</i> , 1962 |
| <i>M. fortuitum</i> :- | <i>acetamidolyticum</i> | + | Tsukamura <i>et al.</i> , 1986b |
| | <i>fortuitum</i> | + | da Costa Cruz, 1938 |
| | <i>peregrinum</i> | | Runyon <i>et al.</i> , (1974) |
| <i>M. gadium</i> | | - | Casal and Calero, 1974 |
| <i>M. gastri</i> | | - | Wayne, 1966 |
| <i>M. genavense</i> | | + | Bottger <i>et al.</i> , 1993 |
| <i>M. gilvum</i> | | - | Stanford and Gunthorpe, 1971 |
| <i>M. gordonae</i> | | +/- | Bojalil <i>et al.</i> , 1962 |
| <i>M. haemophilum</i> | | + | Sompolinsky <i>et al.</i> , 1978 |
| <i>M. hiberniae</i> | | - | Kazda <i>et al.</i> , 1993 |
| <i>M. hodleri</i> | | - | Kleespies <i>et al.</i> , 1996 |
| <i>M. interjectum</i> | | + | Springer <i>et al.</i> , 1993 |
| <i>M. intermedium</i> | | + | Meier <i>et al.</i> , 1993 |
| <i>M. intracellulare</i> | | + | Runyon, 1967 |
| <i>M. kansasii</i> | | + | Hauduroy, 1955 |
| <i>M. komossense</i> | | - | Kazda and Muller, 1979 |
| <i>M. leprae</i> | | + | Lehmann and Neumann, 1896 |
| <i>M. lepraemurium</i> | | + | Marchoux and Sorel, 1912 |

Table 1.1 continued

| Species | Subspecies | Pathogen | Citation |
|----------------------------|--------------------|----------|---------------------------------|
| <i>M.lentiflavum</i> | | + | Springer <i>et al.</i> , 1996 |
| <i>M.madagascariense</i> | | - | Kazda <i>et al.</i> , 1992 |
| <i>M.mageritense</i> | | +/- | Domenech <i>et al.</i> , 1997 |
| <i>M.malmoense</i> | | + | Schroder and Juhlin, 1977 |
| <i>M.marinum</i> | | + | Aronson, 1926 |
| <i>M.microti</i> | | + | Breed <i>et al.</i> , 1957 |
| <i>M.moriokaense</i> | | - | Tsukamura <i>et al.</i> , 1986a |
| <i>M.mucogenicum</i> | | | Springer <i>et al.</i> , 1995 |
| <i>M.neoaurum</i> | | - | Tsukamura, 1972b |
| <i>M.nonchromogenicum</i> | | - | Tsukamura, 1965 |
| <i>M.obuense</i> | | - | Tsukamura <i>et al.</i> , 1981 |
| <i>M.parafortuitum</i> | | - | Tsukamura, 1966a |
| <i>M.phlei</i> | | - | Lehmann and Neumann, 1899 |
| <i>M.porcinum</i> | | + | Tsukamura <i>et al.</i> , 1983b |
| <i>M.poriferae</i> | | + | Padgitt and Moshier, 1987 |
| <i>M.pulveris</i> | | - | Tsukamura <i>et al.</i> , 1983a |
| <i>M.rhodesiae</i> | | - | Tsukamura <i>et al.</i> , 1981 |
| <i>M.scrofulaceum</i> | | + | Prissick and Masson, 1956 |
| <i>M.senegalense</i> | | + | Chamoiseau, 1979 |
| <i>M.shimoidei</i> | | + | Tsukamura, 1982 |
| <i>M.simiae</i> | | + | Karassova <i>et al.</i> , 1965 |
| <i>M.smegmatis</i> :- | <i>smegmatis</i> | - | Lehmann and Neumann, 1899 |
| | <i>lacticolum</i> | - | Tsukamura, 1967b |
| <i>M.sphagni</i> | | - | Kazda, 1980 |
| <i>M.szulgai</i> | | + | Marks <i>et al.</i> , 1972 |
| <i>M.terrae</i> | | - | Wayne, 1966 |
| <i>M.thermoresistibile</i> | | + | Tsukamura, 1966b |
| <i>M.tokaiense</i> | | - | Tsukamura <i>et al.</i> , 1981 |
| <i>M.triplex</i> | | + | Floyd <i>et al.</i> , 1996 |
| <i>M.triviale</i> | | - | Kubica <i>et al.</i> , 1970 |
| <i>M.tuberculosis</i> | | + | Lehman and Neumann, 1896 |
| <i>M.ulcerans</i> :- | | + | Fenner, 1952 |
| | <i>shinshuense</i> | + | Tsukamura, <i>et al.</i> , 1989 |
| <i>M.vaccaae</i> | | +/- | Bonicke and Juhansz, 1964 |
| <i>M.xenopi</i> | | + | Schwabacher, 1959 |

1.2 The Mycobacterial Cell Envelope

The mycobacterial cell envelope comprises the cytoplasmic membrane and the cell wall complex.

1.2.1 The cytoplasmic membrane

Electron micrographs have shown that the cytoplasmic membrane is asymmetrical in ultra-thin sections of mycobacterial cells, with the outermost of two electron-dense layers appearing visibly thicker (Paul and Beveridge, 1992). This feature has been attributed to the preferential association of carbohydrate, in the form of phosphatidylinositol mannosides (PIMs), with the outer layer of the membrane (Brennan and Nikaido, 1995). PIMs are major components of the cytoplasmic membrane and are unique to Actinomycetes. Carotenoid compounds, which protect the cells against photolytic damage and confer the characteristic yellow/orange pigmentation upon photochromogenic species such as *M.gordonae* and *M.kansasii*, are associated with the cytoplasmic membrane also.

1.2.2 The mycobacterial cell wall complex

The mycobacterial cell wall lies external to the cytoplasmic membrane. It is a physically resilient, chemically complex structure of exceptionally low permeability which confers characteristic acid and alcohol fastness upon the cell, as well as resistance to many commonly used antibiotics and chemical disinfectants (Jarlier and Nikaido, 1994). The currently favoured model of the mycobacterial cell wall complex is that of an asymmetric lipid bilayer (Minnikin, 1982; Nikaido *et al.*, 1993). In this model, long-chain (C₆₀-C₉₀) mycolic acids in the inner leaflet are tightly packed in a parallel array pointing in a direction perpendicular to the plane of the cell surface. The inner leaflet is thought to be covered by a layer of solvent-extractable lipids forming the outer leaflet of the bilayer. Evidence in support of the lipid bilayer has been provided by X-ray diffraction studies of the *M.chelonae* cell wall (Nikaido *et al.*, 1993). The X-ray data demonstrated that the hydrocarbon chains contained within the inner leaflet of the bilayer are packed in a near crystalline

array, a structural arrangement suggesting very low fluidity and low permeability. Increasingly diffuse diffraction towards the cell wall surface suggested that the fluidity of the outer leaflet was much greater and reflected its chemical composition and physical organisation (see below). Additional evidence was provided by electron microscopic demonstration of a freeze-fracture plane within the cell wall complex, a feature which is highly consistent with the lipid bilayer model (Brennan and Nikaido, 1995).

Three basic sub-structures, peptidoglycan, arabinogalactan and mycolic acids, are covalently associated to form the mycobacterial inner cell wall complex. The innermost layer, the peptidoglycan, is thought to function as a skeletal framework to which arabinogalactan polysaccharides are attached via phosphodiester bonds. The arabinogalactan polysaccharides are esterified with mycolic acids at their distal ends via a terminal penta-arabinose unit to form a mycolyl-arabinogalactan-peptidoglycan complex. This complex forms the major part of the bilayer inner leaflet and connects the inner and outer leaflets of the bilayer.

The outer leaflet is thought to comprise a number of loosely associated solvent-extractable lipid compounds such as the PIMs, including lipoarabinomannan (LAM). Although not considered to be important for the structural integrity of the outer cell wall, LAM has been reported to play a significant role in the immunopathogenesis of *M.tuberculosis* and *M.leprae* (Chatterjee *et al.*, 1992), and *M.a.paratuberculosis* (McNab *et al.*, 1991a; Sugden *et al.*, 1987, 1989 and 1991). Several classes of free lipids and glycolipids including phenolic glycolipids (PGLs) and glycopeptidolipids (GPLs) are associated with the outer cell wall. The GPLs form a group of immunogenic cell surface antigens which have been used to type isolates of *M.avium*, *M.intracellulare* and *M.scrofulaceum* on the basis of serotypic identity (see section 1.4.1). Phthiocerol dimycocerosates (PDIMs) are long-chain multimethyl-branched waxes whose relative abundance in the outer cell wall suggests they may act as structural components (Minnikin *et al.*, 1985). Chemical analysis of highly

purified *M.chelonae* cell wall preparations has confirmed that the outer cell wall also contains large quantities of glycerophospholipid, a compound previously thought to be associated exclusively with the cytoplasmic membrane (Nikaido *et al.*, 1993). Finally, cell wall associated proteins such as MMP II, a bacterioferritin in *M.leprae* (Hunter *et al.*, 1990; Pessolani *et al.*, 1994) and the porin proteins of *M.chelonae* (Trias *et al.*, 1992) and *M.smegmatis* (Trias and Benz, 1994) lie embedded in this lipid matrix.

The preference of any given species of *Mycobacterium* for a particular environment or host is thought to reflect a process of selection during which the structure of the cell wall has been optimised for survival by exposure to the conditions which prevail in that environment. Thus free-living saprophytic species, which are exposed to naturally occurring antibiotics in soil and water, are inherently less permeable to these molecules than the obligate pathogens. For example, *M.tuberculosis* has been shown to be more permeable to cephalosporins than the saprophyte *M.chelonae* (Connell and Nikaido, 1994) and *M.avium*, another environmental species, has been shown to be almost uniformly resistant to most commonly used antibiotics (Barrow, 1991). It has been suggested that these differences in permeability could be at least partly attributable to the relative proportions and arrangement of the three structural types of mycolic acids (α , keto and methoxy) in the inner leaflet of the cell wall (Brennan and Nikaido, 1995). Interestingly, lipophilic antibiotics, such as rifamycins, tetracyclines, macrolides, and fluoroquinolones, are more active against mycobacteria than their hydrophilic counterparts. For example, fluoroquinolones modified for greater lipophilicity such as sparfloxacin and alkylated-ciprofloxacin have been shown to be more active against *M.tuberculosis* and *M.avium* than their parent molecules (Yajko *et al.*, 1990; Haemers *et al.*, 1990). Such compounds are thought to traverse the cell wall directly by virtue of their lipophilicity, while other more hydrophilic molecules probably enter and leave the cell via water-filled porin channels (Trias *et al.*, 1992; Trias and Benz, 1994).

Much work has been done to unravel the chemical and structural complexities of the mycobacterial cell wall and current knowledge of its composition and architecture is at an advanced stage. However, a deeper understanding of the biological functions of the cell wall and the genes that govern its biosynthesis will help resolve aspects of mycobacterial pathogenesis, such as intraphagocytic survival, and assist in the identification of new drug targets and antigens of immunoprophylactic potential.

1.3 Pathogenic Species of *Mycobacterium* and Related Diseases

The genus *Mycobacterium* comprises a number of obligate pathogens, opportunistic pathogens and many saprophytic species which can be found living freely in the environment.

Of those species which are regarded as obligate pathogens, the aetiological agents of tuberculosis (*M.tuberculosis*) and leprosy (*M.leprae*) are certainly the best known. However, other species also can be regarded as obligate pathogens, such as *M.bovis*, *M. africanum* and *M.microti*, the other three members of the *M.tuberculosis* complex, *M.lepraemurium*, the agent of murine leprosy, *M.a.paratuberculosis*, the agent of paratuberculosis or Johne's disease of ruminants and possibly *M.farcinogenes*, the agent of bovine farcy. Characteristically, the obligate pathogens have been isolated only from infected host species and never traced to any environmental reservoir, although *M.a.paratuberculosis* has been known to survive for prolonged periods in infected faeces. The organisms are transmitted by direct contact with an infected host or contaminated body fluids and the resulting diseases are normally highly contagious.

A number of free-living environmental species of *Mycobacterium*, collectively termed "atypical", "opportunistic", "tuberculoid", "MOTT" (Mycobacteria Other Than Tuberculosis) and "PPEM" (Potentially Pathogenic Environmental Mycobacteria), also have been shown to be responsible for diseases in mammals. Unlike the obligate pathogens, the diseases caused by PPEM are not highly

contagious and host to host transmission generally does not occur. These species are ubiquitous in the environment and their pathogenic potential for mammalian hosts varies from harmless colonisation to invasive disease. The opportunistic nature of such infections usually requires certain pre-disposing factors before tissue invasion and disease can occur. For example, chronic lung disease due to an environmental species of *Mycobacterium* is often secondary to existing parenchymal damage (Contreras *et al.*, 1988). Similarly, the risk of contracting a wide range of mycobacterial infections and clinical manifestations is greatly increased in individuals with malignancies (Feld *et al.*, 1976), recipients of organ or tissue transplants (Patel *et al.*, 1994) and patients receiving immunosuppressive drug therapy (Skogberg *et al.*, 1995). However, such opportunistic infections are generally less severe in these groups than in patients with the acquired immunodeficiency syndrome (AIDS), even when CD4/CD8 ratios are seen to be in the range expected for AIDS (Peters *et al.*, 1989). AIDS patients are particularly at risk of disseminated infection by certain strains of *M.avium* (Horsburgh and Selik, 1989) (see below).

Wayne and Sramek (1992) divided the commonly encountered PPEM into three categories: “*Well-documented species of slowly-growing PPEM*” (*M.kansasii*, *M.marinum*, *M.scrofulaceum*, *M.simiae*, *M.szulgai*, *M.ulcerans*, *M.xenopi* and some members of the *M.avium* complex), “*Well documented species of rapidly-growing PPEM*” (members of the *M.fortuitum/M.cheloni* complex) and “*Newly characterised mycobacteria*” (*M.asiaticum*, *M.haemophilum*, *M.malmoense* and *M.shimoidei*). Although these organisms have been isolated from patients with different clinical manifestations, most give rise to tuberculous pulmonary disease. Exceptions include *M.marinum*, originally isolated from marine fish (Aronson, 1926), and *M.ulcerans*, both of which can cause necrotic skin lesions in humans. More recently, the list of PPEM has been extended to include a number of new species of *Mycobacterium*: *M.intermedium* (Meier *et al.*, 1993), *M.genavense* (Bottger *et al.*, 1993), *M.interjectum* (Springer *et al.*, 1993; Emler *et al.*, 1994), *M.celatum* (Bull *et al.*,

1995a; Dahl *et al.*, 1995), *M.branderi* (Koukila-Kahkola *et al.*, 1995), *M.conspicuum* (Springer *et al.*, 1995b), *M.mucogenicum* (Springer *et al.*, 1995a) *M.lentiflavum* (Springer *et al.*, 1996) and *M.triplex* (Floyd *et al.*, 1996). These organisms have been associated with a variety of clinical conditions including pulmonary disease, lymphadenopathy, lymphadenitis, spondylodiscitis, skin disorders such as granulomatous lesions and subcutaneous abscesses, and disseminated infections in immunocompromised individuals. The list of PPEM continues to expand and some newly identified species such as *M.poriferae* (Tortoli *et al.*, 1996), *M.hassiacum* (Schroder *et al.*, 1997) and *M.mageritense* (Domenech *et al.*, 1997) have been isolated from tuberculous lesions in snakehead fish and the human urinary and respiratory tracts respectively. There is no evidence to suggest that any of these species is pathogenic in mammals.

An unusually high susceptibility to infection with PPEM is a striking feature of patients with AIDS (Wayne and Sramek, 1992). However, even though many of the PPEM mentioned above have been associated with disseminated infections in AIDS patients, approximately 90% of AIDS-related mycobacterioses are attributable to infection with either *M.tuberculosis* or *M.avium* (Peters *et al.*, 1989). It has been suggested that AIDS-related tuberculosis could be the result of reactivation rather than that of primary exposure, but *M.avium* infections are thought to arise from unavoidable continuous environmental exposure. Because of the ubiquitous nature of *M.avium*, it is possible that primary colonisation of the gastrointestinal tract may follow normal ingestion of organisms (Jacobson *et al.*, 1991). It has been proposed that the HIV-related immunosuppression in AIDS patients may provide the opportunity for some strains of *M.avium* to disseminate from the primary colonisation site and cause invasive disease (Peters *et al.*, 1989). Potential environmental sources of acquired *M.avium* infection include soil, water, vegetation, animal products and processed organic materials (Horsburgh, 1994), but the precise reservoirs of infection are not known. In cases of cervical lymphadenitis, it is presumed that organisms from soil or water are ingested and enter the lymphatics via

mucosal associated lymphoid tissue (von Reyn *et al.*, 1996). The isolation of MAC from cigarette tobacco (Eaton *et al.*, 1995) and of other PPEM from bronchoscopes (Spach *et al.*, 1993) point to potential sources of pulmonary infection. In addition, the recovery of MAC from hospital tap water (von Reyn *et al.*, 1994; Peters *et al.*, 1995) highlights the potential for nosocomial infection of immunocompromised patients.

The prevalence of MAC-related diseases, especially in AIDS, emphasises the need for further research into the pathogenesis of this important group of organisms, and for a deeper understanding of the molecular basis of virulence. The following sections provide a description of the members of the MAC and a brief overview of their molecular characteristics.

1.4 The *M.avium* Complex (MAC)

The MAC comprises a group of closely related species including *M.avium*, *M.intracellulare*, *M.a.paratuberculosis*, the wood pigeon bacillus (see section 1.4.3) and *M.lepraemurium*.

1.4.1 *M.avium* and *M.intracellulare*

Infection by an organism similar to, but distinct from, the then recently discovered “Tuberkelbacillus” was shown to be the cause of tuberculosis in chickens (Maffucci, 1890). The causative organism was shown to differ from those associated with tuberculosis in humans and other animals and was later named *Mycobacterium avium* (Chester, 1901). *M.avium* was considered indistinguishable from *M.intracellulare* in terms of cultural and biochemical characteristics and the two species were separated only on the basis of their pathogenicity in chickens. Isolates which killed experimentally infected birds were regarded as *M.avium* and those which did not as *M.intracellulare* (Schaefer, 1980). Early serological studies on *M.avium* and *M.intracellulare* revealed the presence of two agglutinating *M.avium* serotypes, (1 and 2), with the latter predominating in the USA (Schaefer, 1965). A third *M.avium*

serotype (3) was isolated in Europe a few years later (Marks *et al.*, 1969) and *M.intracellulare* isolates comprised an additional 17 serotypes designated by Roman numerals or author's surnames (Schaefer, 1980). In adopting the convention used for *M.avium*, all *M.intracellulare* serotypes are currently designated by Arabic numerals and the two species are now represented by serotypes 1-28.

For many years, a set of clearly defined criteria on which *M.avium* and *M.intracellulare* could be distinguished was not available and the two species were grouped together in the MAC on the grounds of close phenotypic similarity and serotypic identity (Meissner *et al.*, 1974). It had been observed during earlier studies that *M.intracellulare* serotypes 4, 5, 6, 8 and 9 were lethal for experimentally infected chickens (Anz and Meissner, 1972), and therefore were considered to be more like *M.avium* than *M.intracellulare*. Subsequent DNA hybridisation studies (see section 1.5.1) confirmed that a number of these *M.intracellulare* serotypes were genetically indistinguishable from *M.avium* (Baess, 1983; Saito *et al.*, 1990). These revelations led to a redistribution of agglutinating serotypes, but disagreement over precisely which serotypes belong to *M.avium* or *M.intracellulare* continues to fuel debate between independent authors (Baess, 1983; Roberts *et al.*, 1987; Peterson *et al.*, 1989; Yakrus and Good, 1990; Saito *et al.*, 1990). The current serotypic groupings, as detailed by Wayne and Sramek (1992), acknowledge and take into consideration the results of DNA homology studies. Serotypes 1-6 and 8-11 have been assigned to *M.avium* and serotypes 7, 12-17, 19, 20 and 25 to *M.intracellulare*. The remaining serotypes do not fit satisfactorily into either category. Some isolates of serotype 18 type as either *M.intracellulare* or *M.simiae*, while others cannot be assigned to any species. Other serotypes (22, 23, 26 and 28) which are phenotypically compatible with the MAC are not recognised by DNA probes which are specific for either *M.avium* or *M.intracellulare*.

1.4.2 *M.scrofulaceum*

The grouping of the 3 currently known serotypes of *M.scrofulaceum* (serotypes 41, 42 and 43) (Schaefer, 1968) together with the members of the MAC has exacerbated confusion which continues to surround classification within the complex.

In 1977, Hawkins referred to strains of mycobacteria which exhibited pigment, urease and catalase patterns that were inconsistent with *M.scrofulaceum*, *M.avium* or *M.intracellulare*, but which appeared to belong to one or other of these species on the basis of other characteristics. In the absence of criteria to enable a more precise classification, it was proposed that these unusual isolates be regarded as unspciated *M.avium/intracellulare/scrofulaceum* (MAIS) intermediates. However, the original proposal has been misconstrued by some authors and references to a “MAIS complex” are common in the literature. The “MAIS” acronym includes well-defined strains of all three species, but is misleading when used in combination with “complex” because a close taxonomic relationship is implied. Definitive evidence based on numerical taxonomy (Meissner *et al.*, 1974), reciprocal intradermal skin testing (Magnusson, 1980), immunodiffusion (Stanford and Grange, 1974), immunological distance of catalases (Wayne and Diaz, 1979 and 1982) and DNA homology (Baess and Weis-Bentzon, 1978; Baess, 1979, 1983 and 1984; Hurley *et al.*, 1988) has demonstrated that *M.scrofulaceum* is a distinct species and that its inclusion with *M.avium* and *M.intracellulare* in a “MAIS complex” is inappropriate.

1.4.3 *M.a.paratuberculosis* and the “wood pigeon bacillus”

While *M.avium* serotypes 1, 2 and 3 are those most commonly associated with infections in birds and animals (Meissner and Anz, 1977), the closely related *M.a.paratuberculosis* has been identified as the aetiological agent of Johne’s disease (see section 1.8) or paratuberculosis of ruminants (Chiodini *et al.*, 1984a). *M.a.paratuberculosis* also has been isolated from humans suffering from Crohn’s disease (see section 1.11) (Chiodini *et al.*, 1984b; McFadden *et al.*, 1987b) although the aetiological significance of its presence in diseased gut tissue is still the subject of

ongoing research. Although indistinguishable from *M.avium* on biochemical and serological criteria, characteristic features of *M.a.paratuberculosis* include slow growth rate *in vitro* and fastidious nutritional requirements. The organism may take up to 4 months to form visible colonies on primary isolation and then only when provided with an exogenous supply of the iron-chelating growth factor mycobactin (Merkal and Curran, 1974). Unlike most other species of *Mycobacterium*, *M.a.paratuberculosis* is a mycobactin auxotroph unable to synthesise its own mycobactin. However, *M.a.paratuberculosis* can be propagated on mycobactin-free medium supplemented with iron at concentrations of $\leq 100\mu\text{M}$ at pH5.0, or $>100\mu\text{M}$ at pH6.8 (Lambrecht and Collins, 1992). The identification of *M.a.paratuberculosis* on the basis of growth requirements and pathogenicity in ruminants is no longer considered reliable, since certain strains of *M.avium* such as the “wood pigeon bacillus” (Matthews and McDiarmid, 1979) have been shown to require mycobactin also, particularly on primary isolation. In addition, experimental infection of bovine calves with the “wood pigeon bacillus” resulted in a disease resembling clinical paratuberculosis (Matthews and McDiarmid, 1979; Thorel *et al.*, 1984; Collins *et al.*, 1985). Subsequent analysis at the genomic level (see section 1.5) has demonstrated a close genetic relationship between *M.avium*, *M.a.paratuberculosis* and “the wood pigeon bacillus”.

1.4.4 *M.lepraemurium*

Unlike most other members of the MAC, the aetiological agent of “rat leprosy”, *M.lepraemurium*, has proved extremely difficult to cultivate *in vitro* and only sparse growth has been achieved on solid inspissated egg yolk medium (Ogawa and Motomura, 1970). However, analyses of *M.lepraemurium* propagated in mice provided evidence that the chemical composition and ultrastructure of the cell wall (Kanetsuna *et al.*, 1968; Draper, 1971), biochemical patterns (Saito *et al.*, 1976) and serological cross reactivity with other mycobacteria (Kronvall *et al.*, 1977) were characteristic of the genus *Mycobacterium*. Molecular characterisation of the *M.lepraemurium* genome (see section 1.5) showed that the M.wt and G+C content

were in the range predicted for mycobacteria and revealed a close genetic relationship with *M.avium* and *M.a.paratuberculosis* (Imaeda *et al.*, 1982).

1.5 Molecular Characterisation of the MAC

1.5.1 DNA-DNA hybridisation

DNA homology determinations have made a major contribution to mycobacterial phylogeny, particularly where studies have focused on clusters of closely related species such as the members of the *M.tuberculosis* and *M.avium* complexes (Baess, 1979). The technique functions on the principal that, under appropriate conditions of temperature and salt concentration, two different species of genomic DNA will hybridise at a rate and to an extent that is proportional to the concentration of homologous nucleotide sequences.

Determinations on DNA homology between the members of the MAC have shown that the genomes of *M.avium*, *M.a.paratuberculosis*, the “wood pigeon bacillus” and some strains of *M.intracellulare* are 72-100% similar (Hurley *et al.*, 1988). When *M.intracellulare* was excluded from the comparisons, levels of DNA homology between *M.a.paratuberculosis*, *M.avium* and the “wood pigeon bacillus” were much higher at 95-100% (McFadden *et al.*, 1987a; Hurley *et al.*, 1988; Saxegaard and Baess, 1988; Yoshimura and Graham, 1988). Comparisons of DNA species which differ by less than ~5% are beyond the resolving capabilities of the technique however, and these species are considered indistinguishable by this method (McFadden *et al.*, 1990). In a separate study, McFadden *et al* (1987c) reported <2% base substitution between strains of *M.avium* and *M.a.paratuberculosis*, but >15% base substitution between strains of *M.intracellulare*. The observed heterogeneity amongst *M.intracellulare* strains prompted the authors to question whether these strains should remain as a single species and to suggest that the “*M.intracellulare* complex” might be a more appropriate term for this particular group of mycobacteria. *M.lepraemurium* has been shown to be more closely related to *M.avium* strains than to any other mycobacterial species. DNA homology levels of 74-93% (Imaeda *et al.*,

1982) and 85-87% (Athwal *et al.*, 1984) have been measured between the genomes of *M.lepraemurium* and *M.avium*. An even closer relationship between these species was reported by McFadden *et al* (1987c) who calculated DNA base substitutions of only 2% between *M.lepraemurium* and *M.a.paratuberculosis* (ATCC 19689) and 3% between *M.lepraemurium* and *M.avium* (serotype 2 strain).

Despite some clear phenotypic similarities, it has been shown that *M.scrofulaceum* shares only 34-41% genomic DNA homology with *M.avium* (serotype 2 strain) (Baess, 1979 and 1983), 11-24% homology with *M.a.paratuberculosis* (Hurley *et al.*, 1988) and 33-36% homology with *M.intracellulare* (serotype 16) (Baess, 1983). The difference in DNA homology observed between *M.scrofulaceum* and *M.avium* (serotype 2), and *M.scrofulaceum* and *M.a.paratuberculosis* is surprising since *M.avium* (serotype 2) strains and *M.a.paratuberculosis* are considered indistinguishable on the basis of genomic hybridisation (McFadden *et al.*, 1990). In any case, most authors now regard *M.scrofulaceum* as a species which is separate and distinct from the members of the MAC (see section 1.4.2).

Within the limitations of the technique, DNA hybridisation studies have proved invaluable in determining levels of genetic similarity between the members of the MAC. However, the results of these studies must be interpreted with caution as high levels of genomic DNA homology may not necessarily reflect true taxonomic status. For example, Brenner (1972) showed that under stringent conditions, genomic DNA homology between *E.coli* K12 and *Shigella* spp. (71-85%) could, in some cases, be higher than that observed between different strains of *E.coli* (72-100%). Therefore, if the degree of nucleotide sequence divergence tolerable within a species is accepted at $\leq 40\%$ (Wayne, 1978), it could be argued that the *Shigellae* be included as one or more species within the genus *Escherichia*. Consequently, in assigning an organism to a genus or a species on the basis of genomic homology measurements, it is important that phenotypic expression is taken into consideration also.

1.5.2 Restriction Endonuclease (RE) profiling and Restriction Fragment Length Polymorphism (RFLP) analysis

Small differences (i.e. single base pair changes) between single genes or entire genomes can be exploited to differentiate between closely related species which are indistinguishable by DNA-DNA hybridisation. Provided that these differences occur within the specific recognition sequence of a given restriction endonuclease, enzymatic cleavage of the DNA followed by electrophoretic separation of the resulting fragments should reveal discernible differences in banding pattern. These differences are known as restriction fragment length polymorphisms (RFLP).

Direct comparison of RE-digested genomic DNA on agarose gels has been used to demonstrate polymorphisms between the members of the MAC (Patel *et al.*, 1986, Collins and deLisle, 1986, Whipple *et al.*, 1987). Overall, distinctions based on differences observed between the individual members of the MAC were seen to concord with the results of genomic DNA homology studies. Easily distinguishable RE profiles supported the status of *M.avium*, *M.intracellulare* and *M.scrofulaceum* as separate and distinct species (Patel *et al.*, 1986). In addition, the banding patterns observed for strains of *M.avium* and *M.intracellulare* were largely in agreement with current serotypic groupings (Whipple *et al.*, 1987). With the notable exception of “strain 18”, all *M.a.paratuberculosis* isolates examined were shown to share an identical RE profile (Collins and deLisle, 1986; Whipple *et al.*, 1987; Wards *et al.*, 1987) which was distinct from those of serotype 2 strains of *M.avium* and the wood pigeon bacillus (Whipple *et al.*, 1987). *M.a.paratuberculosis* strain 18 has since been reclassified (see section 1.6).

The value of direct comparison of two or more genomic RE profiles, as visualised in stained agarose gels, often can be limited by the complexity of the banding pattern and the resolving capabilities of the gel system in use. Simpler and more interpretable banding patterns are obtained when a labelled DNA probe is hybridised to a few homologous fragments on Southern blots of RE-digested genomic DNA.

McFadden *et al* (1987b and c) applied this technology to the identification of RFLP types which could be used to distinguish between isolates of *M.avium* and *M.a.paratuberculosis*. Ten random genomic DNA clones, derived from a Crohn's disease isolate (strain Ben), were used as probes and one of these (clone pMB22) was shown to produce RFLPs which resolved the two species. In concordance with the results of RE analysis, all isolates of *M.a.paratuberculosis*, including strain Ben, were shown to share an identical RFLP type, except "strain 18" which was identical to *M.avium* serotype 2 strains. RFLP types within *M.avium* isolates were noted to be more heterogeneous.

Subsequent characterisation of clone pMB22 revealed the presence of the genomic insertion sequence IS900, of which there are an estimated 15-20 copies per *M.a.paratuberculosis* genome (Green *et al.*, 1989). Identification of a short species-specific region at the 5' end of the insertion element provided a probe which did not hybridise to genomic DNA from any other species of *Mycobacterium* tested, including the members of the MAC. In combination with multiple homologous target sequences, the IS900-derived probe yielded simple yet informative banding patterns on Southern blots of RE-digested *M.a.paratuberculosis* DNA. On the basis of these RFLP types, *M.a.paratuberculosis* isolates have been reported to fall into three groups. The first of these comprised mainly New Zealand cattle strains, but also included some sheep and goat isolates (Collins *et al.*, 1990); the second group comprised predominantly sheep isolates from New Zealand and the Faeroe Islands (deLisle *et al.*, 1993), while the third group included sheep isolates from South Africa (deLisle *et al.*, 1992), Iceland and Canada (Collins *et al.*, 1990). In addition, of 20 *M.a.paratuberculosis* isolates recovered from farmed deer, 17 were shown to be identical to the cattle isolates while the remaining 3 were the same as the New Zealand and Faeroe Island sheep isolates (deLisle *et al.*, 1993). In a further study which examined genomic DNA from 90 *M.a.paratuberculosis* isolates of bovine, ovine, caprine and human origin, *Pst*I digestion and hybridisation with an IS900-derived probe revealed three RFLP types, but could not correlate RFLP type with

host preference (Pavlik *et al.*, 1995). More recently, Bauerfeind *et al* (1996) demonstrated five distinct *PvuII* RFLP types amongst 42 *M.a.paratuberculosis* isolates collected from sheep, goats and cattle in Morocco, South Africa, the USA and Germany. Although individually distinct, the RFLP types could be divided into two major clusters on the basis of similarity of profile. Moreover, complete concordance was observed between RFLP cluster and *in vitro* growth rate, with all sheep isolates falling into cluster I (extremely slow growth) and all goat and cattle isolates falling into cluster II (moderately slow growth). In support of the earlier studies, the authors concluded that in spite of the obvious similarity between isolates of *M.a.paratuberculosis*, there is evidence to support the existence of limited genetic heterogeneity within the subspecies. However, the relationship between RFLP type and host preference remains unclear (Thoresen and Olsaker 1994; Pavlik *et al.*, 1995; Bauerfeind *et al.*, 1996).

Chiadini (1990) used a probe consisting of the entire *E.coli* 5S rRNA gene and ~200bp of the adjacent 23S rRNA gene to identify RFLPs within the rRNA gene region of the members of the MAC. The presence of just one copy of the 5S rRNA gene in the slow growing mycobacteria yielded banding patterns of either a single fragment, or two fragments if the polymorphism was present within the probe target sequence. None of the 8 REs used, including *Ava I*, *Bam HI*, *Bgl II*, *EcoRI*, *EcoRII*, *HindIII*, *PvuII* and *XhoI*, revealed polymorphisms among the 19 *M.a.paratuberculosis* isolates tested. Differences between *M.a.paratuberculosis* and *M.avium* serotypes 2, 4 and 9 were revealed by digestion with *Ava I*, *Bam HI*, *HindIII*, *PvuII* and *SstI*, while differences between the 3 *M.avium* serotypes were apparent on digestion with *AvaI*, *PvuII* and *SstI*. *M.intracellulare* and *M.lepraemurium* were not included in this study.

1.5.3 Pulsed-field and field-inversion gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) and field-inversion gel electrophoresis (FIGE) represent alternative methods of simplifying the banding patterns obtained by

electrophoretic separation of fragments resulting from restriction endonuclease digestion of genomic DNA. The basic technique, which was developed originally to separate yeast chromosomes (Schwartz *et al.*, 1983), entails digesting DNA with restriction endonucleases that cleave only at rarely occurring scission sites. The resulting large restriction fragments (LRFs) are then resolved in agarose gels in a specially devised electrophoresis cell which delivers forward and reverse electric pulses of varying duration.

FIGE analysis of *DraI*-digested genomic DNA from isolates of *M.a.paratuberculosis*, the wood pigeon bacillus, *M.avium* and *M.intracellulare* demonstrated a high level of homogeneity between the LRF patterns obtained for *M.a.paratuberculosis* isolates (Levy-Frebault *et al.*, 1989). LRF patterns obtained for isolates of the wood pigeon bacillus were observed to share a high degree of similarity also, but were distinct from those of *M.a.paratuberculosis* and the more heterogeneous patterns obtained for *M.avium* and *M.intracellulare*. These results corroborated the findings of earlier observations, based on restriction endonuclease analysis, which demonstrated genetic homogeneity among isolates of *M.a.paratuberculosis*, and heterogeneity among isolates of *M.avium* (Collins and deLisle 1986; Wards *et al.*, 1987; Whipple *et al.*, 1987).

In a later study, Coffin *et al.* (1992) used PFGE to analyse *Ssp I*-digested genomic DNA from a number of MAC type strains and field isolates. These authors divided strains between four groups on the basis of their LRF patterns: group 1 contained 2 bovine isolates of *M.a.paratuberculosis*, 1 human isolate from Crohn's disease and the *M.a.paratuberculosis* neotype strain ATCC 19698; group 2 contained 1 isolate from a case of bovine paratuberculosis, *M.avium* strain ATCC 35712 (serotype 2) and "Strain 18"; group 3 contained *M.intracellulare* type strain ATCC 13950 (serotype 16); and group 4 contained *M.intracellulare* ATCC 35771 (serotype 8) and an isolate from a case of bovine paratuberculosis. The results of this study seem somewhat spurious in light of previous data and would have benefited from proper

and thorough identification of the isolates under analysis. Firstly, because all group 1 isolates share an LRF with the *M.a.paratuberculosis* neotype strain ATCC 19698, all are probably correctly identified as *M.a.paratuberculosis*, although screening for the presence of the genomic insertion sequence IS900 would have confirmed this (see sections 1.5.2 and 1.5.5.1). Secondly, because all the isolates in group 2 share an LRF with “Strain 18” (an IS901/902 positive strain of *M.avium*), it is likely that all are strains of *M.avium* containing the insertion sequence. Once again, this could have been confirmed by screening for the presence of the IS901/902 by PCR. Thirdly, group 4 contains an isolate designated *M.intracellulare* (serotype 8) and a field strain designated *M.a.paratuberculosis*. Given that serotype 8 isolates are now considered to be strains of *M.avium* (see section 1.4.1) and that both isolates in this group share an identical LRF pattern, it is likely that both organisms in this group are strains of *M.avium* which lack IS901/902. Yet again, this could have been confirmed by screening for the presence/absence of the insertion sequence.

PFGE has since been used to demonstrate LRF polymorphism among MAC isolates of identical serotype (Mazurek *et al.*, 1993), to highlight genetic diversity among strains isolated from AIDS patients harbouring monoclonal and polyclonal *M.avium* infections (Arbeit *et al.*, 1993; Slutsky *et al.*, 1994; Burki *et al.*, 1995) and to draw genotypic comparisons between strains of *M.avium* isolated from humans and animals (Bono *et al.*, 1995; Feizabadi *et al.*, 1996). In addition, the technique has been applied usefully to studying genetic heterogeneity among other species of *Mycobacterium*, including *M.tuberculosis*, *M.terrae* complex, *M.gordonae*, *M.avium-M.intracellulare* complex and *M.fortuitum* (Kim *et al.*, 1996).

1.5.4 Ribosomal RNA gene sequences

Comparative analysis of ribosomal RNA (rRNA) subunits or their encoding genes has been useful for the phylogenetic classification of bacteria (Woese, 1977). Studies based on mycobacterial rRNA sequences have correlated the classical division of the genus into slow- and fast-growing species, with the presence of either

one or two copies respectively of the rRNA operon (*rrn*) (Bercovier *et al.*, 1986, Stahl and Urbance, 1990). The order of genes along the mycobacterial *rrn* has been demonstrated as -5'-16S-23S-5S-3'- with intergenic spacer regions 1 and 2 separating 16S and 23S, and 23S and 5S rRNA subunit genes respectively (Bercovier *et al.*, 1986). It has been suggested that the low *rrn* copy number in mycobacteria might be at least partly responsible for their slow rate of growth relative to other bacteria which possess higher copy numbers (Bercovier *et al.*, 1986).

Examination of variable regions within mycobacterial 16S rRNA genes has revealed species-specific nucleotide sequences which can be used for genotypic identification of mycobacterial species (Bottger, 1989, Edwards *et al.*, 1989). Direct sequence determination of 16S rRNA gene fragments amplified by the polymerase chain reaction (PCR) has been used to identify mycobacteria in clinical specimens (Rogall *et al.*, 1990a, Kirschner *et al.*, 1993). These studies have shown that while the member species of the tuberculosis complex are indistinguishable by this methodology, many other species, including *M.avium* and *M.intracellulare* possess unique signature sequences. However, while it is generally accepted that a particular 16S rRNA signature sequence is associated with a single species, it should be noted that microheterogeneity between 16S rRNA gene sequences of different isolates of *M.gordoniae* has been reported (Kirschner and Bottger, 1992). In addition, a slow-growing *Mycobacterium* "Strain X" was shown to contain two 16S rRNA genes which differed in 18 positions (Ninet *et al.*, 1996). Phylogenetically, one gene sequence shared a common root with *M.terrae*, while the second was placed entirely separately. The presence of two such markedly different 16S rRNA gene sequences on the same bacterial chromosome has obvious implications for the reliability of methodologies based on species-specific signature sequences. However, because the members of the *M.tuberculosis* complex, *M.leprae* and the members of the MAC possess only a single copy of the *rrn*, their identification on the basis of 16S rRNA signature sequences should remain unaffected.

The identification of specific 16S rRNA sequences led to the development of a diagnostic test kit which is marketed commercially by Gen-Probe® Inc., San Diego, California, USA. The Gen-Probe® AccuProbe system has been shown to be useful for the identification of the tuberculosis complex, the MAC, *M.gordonae* and *M.kansasii* (Reissner *et al.*, 1994). The Gen-Probe® Rapid Diagnostic System for the MAC contains two ¹²⁵I-labelled oligonucleotide probes, each specific for either *M.avium* or *M.intracellulare* 16S rRNA signature sequences. This system has been used successfully to discriminate between the two species in both medical (Saito *et al.*, 1990) and veterinary (Thoresen and Saxegaard, 1993) laboratories. Both of these studies reported results which were in concordance with currently accepted serotypic divisions.

The use of DNA probes based on 16S rRNA gene sequences has been unsuccessful in distinguishing *M.a.paratuberculosis* from other subspecies or serotypes of *M.avium* (Thoresen and Saxegaard, 1991, van der Giessen *et al.*, 1992a). This can be attributed to the high degree of relatedness (99.9%) between the 16S rRNA genes of this group of organisms (Rogall *et al.*, 1990b, Kirschner, *et al.*, 1993). Although a single base pair substitution has been shown to exist at position 135 in the variable region of the 16S rRNA gene, demonstration of the substitution can be achieved only by direct nucleotide sequencing of PCR-amplified 16S rRNA gene fragments (Rogall *et al.*, 1990a).

An assay based on the amplification of 16S rRNA gene sequences and which was originally designed to detect *M.a.paratuberculosis* infection in cattle, has been shown to detect *M.avium* specifically and is in full agreement with current serotypic groupings (van der Giessen *et al.*, 1993). Applied directly to diagnostic specimens, the assay enabled the rapid detection of *M.avium*, wood pigeon mycobacteria and *M.a.paratuberculosis* with no cross-reactivity with any of the other mycobacterial species tested, including *M.intracellulare*.

For the purpose of sequence-based species differentiation, the intergenic spacer 1 regions and 23S rRNA genes have been shown to exhibit greater variability than 16S rRNA genes (Frothingham and Wilson, 1993, van der Giessen *et al.*, 1994). The complete nucleotide sequence of the intergenic spacer 1 region (~280bp) was determined for each of 35 MAC isolates, revealing 12 distinct sequence variants (Frothingham and Wilson, 1993). Of these, 4 variants were noted to correspond to *M.avium*, 1 to *M.intracellulare* and a further 7 variants were observed to vary markedly, both with each other and with those deemed to correspond to *M.avium* and *M.intracellulare*. Although consistent with Gen-Probe[®] MAC typing (see above), the results of intergenic spacer 1 region sequencing correlated poorly with serotypic groupings. Identical sequence variants were shown to belong to different serotypes and some isolates of the same serotype were shown to belong to different sequence variants (Frothingham and Wilson, 1993). van der Giessen *et al* (1994) reported only 9 mismatches (99.7% sequence identity) between the complete 23S rRNA genes (~3.1kb) of *M.avium* and *M.a.paratuberculosis*, and only 2 mismatches between their respective intergenic spacer 1 regions (278bp). Consequently, such high levels of sequence homology over the 16S, 23S and intergenic spacer 1 regions effectively preclude their use as target sites for DNA probe-based differentiation of *M.avium* and *M.a.paratuberculosis*.

1.5.5 Genomic insertion elements

The isolation and characterisation of IS900 in *M.a.paratuberculosis* was the first report of a genomic insertion element in the genus *Mycobacterium* (Green *et al.*, 1989). IS900 is considered atypical in that, unlike classical insertion sequences, it lacks inverted terminal repeats and flanking direct repeats. Consequently, IS900 is classed in a family of insertion elements which includes IS110 of *Streptomyces coelicolor* A3(2) (Brunton and Chater, 1987), IS116 of *Streptomyces clavuligerus* (Leskiw *et al.*, 1990), IS901/902 of *M.avium* (Kunze *et al.*, 1991; Moss *et al.*, 1992a) and IS1110 of *M.avium* (Hernandez Perez *et al.*, 1994). Two further mycobacterial insertion sequences, designated IS1245 (Guerrero *et al.*, 1995) and IS1311 (Guerrero

et al., 1994, unpublished database submission) have been found in *M.avium* also, but are unrelated and will be considered separately (see section 1.5.5.4). Lastly, some strains of *M.intracellulare* have been reported to contain an insertion element designated IS1141 (Via and Falkinham, 1993).

1.5.5.1 IS900 of *M.a.paratuberculosis*

IS900 is 1451bp in length with a nucleotide composition consisting of 66% G+C (Green *et al.*, 1989). Comparisons of IS900 and IS110 at the nucleotide level revealed an overall homology of 52%, although this was observed to be significantly higher at the 3' ends. Typically, between 15 and 20 copies of IS900 are found per *M.a.paratuberculosis* genome and chromosomal insertion has been shown to be site-specific, conforming to the consensus sequence 5'-CATG(N)₍₄₋₆₎*CNCCTT-3' where the asterisk denotes the site of insertion (Green *et al.*, 1989). Originally, IS900 was reported to contain a single open reading frame (ORF) of 1197bp encoding a putative transposase of 399 amino acids (M.wt 43kDa). More recently however, a second ORF has been identified on the complementary strand (Doran *et al.*, 1994a, 1994b and 1997). This gene, designated "*hed*" (host expression dependent), utilises a promoter, ribosome-binding site (RBS) and termination codon acquired upon insertion into the *M.a.paratuberculosis* genome. This is achieved by insertion between the RBS and start codon of a putative host gene. Insertion aligns the *hed* initiation codon adjacent to a host RBS and possibly downstream of an active promoter sequence (e.g. "P_{AN}"). P_{AN} has been located proximal to the 3' end of a copy of IS900 (Murray *et al.*, 1992). Expression of both ORF1 and ORF2 in IS900 has been reported (Tizard *et al.*, 1992; Doran *et al.*, 1997), although the effects of their expression on *M.a.paratuberculosis* virulence remain unknown. The use of IS900 to mediate stable integration of a kanamycin resistance gene into the *M.smegmatis* genome highlights the potential of such mobile genetic elements for use in transposon-mutagenesis experiments (England *et al.*, 1991).

1.5.5.2 IS901/902 of *M.avium*

IS901 in *M.avium* RFLP type A/I (Kunze *et al.*, 1991) and IS902 in *M.avium* subsp. *silvaticum* (Moss *et al.*, 1992a) are actually the same insertion sequence isolated and characterised independently by two different research groups. However, descriptions of the two insertion sequences are not absolutely identical and they are reported to share 98% homology at the nucleotide level. IS901 has a nucleotide sequence of 1472bp, is composed of 62% G+C and contains a single ORF of 1203bp which encodes a protein of 401 amino acid residues (M.wt 44kDa) (Kunze *et al.*, 1991). IS902 is reported to be 1470bp in length, 62% G+C and to contain an ORF of 1200bp encoding a protein of 400 amino acid residues (M.wt 43.9kDa) (Moss *et al.*, 1992a). The translation products of IS901/902 share a high level of sequence identity with that of IS900, although overall sequence homology between IS901/902 and IS900 at the nucleotide level is reported at only 60% (Kunze *et al.*, 1991). However, higher levels of nucleotide homology at the 3' ends of the insertion elements allowed probes derived from IS900 to detect multiple copies of IS901/902 in Southern blots of *M.avium* (wood pigeon bacillus) genomic DNA (McFadden *et al.*, 1987c). Multiple copies (10-12 per genome) of IS901/902 have been reported to occur in the *M.avium* genome (Moss *et al.*, 1992a) and like IS900 of *M.a.paratuberculosis*, chromosomal insertion appears to be site-specific conforming to the consensus sequence 5'-CAT(N)₍₇₎*TTCCNTTC-3' (Kunze *et al.*, 1991) or 5'-CAT(N)₍₇₋₈₎*CCTTC-3' (Moss *et al.*, 1992a) where the asterisk denotes the site of insertion. A second ORF on the complementary strand of IS901/902 encodes a homologue to the IS900 Hed protein. However, unlike the IS900 *hed* gene, ORF2 of IS901/902 encodes a termination codon resulting in a hypothetical peptide of 477 amino acid residues (Doran *et al.*, 1997). To date, no evidence for expression of either ORF in IS901/902 has been reported.

Examination of *M.avium* strains from human, animal and environmental sources has demonstrated that IS901/902 is associated almost exclusively with pathogenic animal isolates. Conversely, most human and all AIDS-related *M.avium* isolates have been

observed to lack IS901/902 (Kunze *et al.*, 1992). Interestingly, strains of *M.avium* in possession of IS901/902 (RFLP type A/I) were observed to be more virulent in experimentally infected BALB/c mice than strains lacking the insertion sequence (RFLP type A) (Kunze *et al.*, 1991).

1.5.5.3 IS1110 of *M.avium*

IS1110 is a highly mobile insertion element which was first detected in *M.avium* strain LR541 following an observed increase in the size of a plasmid pLR20 (Hernandez Perez *et al.*, 1994). Subsequent analysis of the enlarged plasmid (pLR20') revealed the acquisition of a 1457bp insertion sequence which shared approximately 60% nucleotide homology with IS900 of *M.a.paratuberculosis*, IS901/902 of *M.avium* and IS116 of *Streptomyces clavuligerus*. Nucleotide sequence analysis of the insertion element revealed the presence of an ORF, although unlike ORF1 of the related insertion elements, a termination codon was absent from the reading frame. From flanking-sequence data, the authors hypothesised that if the nearest downstream termination codon was utilised, the putative transposase encoded by ORF1 would have a C-terminus extended by 46 amino acid residues, yielding a translation product of 50.2kDa. Unlike IS900 and IS901/902, an in-frame stop codon in ORF2 on the complementary strand of IS1110 resulted in early termination of translation and a hypothetical peptide of only 12 amino acid residues. Sites of IS1110 insertion were similar to the consensus sequences observed for related insertion elements, although genomic copy numbers were observed to be lower. In addition, IS1110 was not found in any other species of *Mycobacterium* other than *M.avium* and the presence of IS1110 in only 2 of the 24 isolates examined would suggest that it occurs relatively infrequently.

1.5.5.4 IS1245 and IS1311 of *M.avium*

Some isolates of *M.avium* from human, animal and avian sources have been shown to harbour the insertion element IS1245 (Guerrero *et al.*, 1995). IS1245 shows no significant sequence homology with any of the insertion elements described above

and differs again in its possession of two imperfect inverted repeats at its extremities. IS1245 is 1313bp in length and contains an ORF of 1233bp encoding a putative transposase which shares 64% amino acid sequence identity with that of IS1081 of *M.bovis*. Both IS1245 and IS1081 have been classed among the *Staphylococcus aureus* IS256 family of insertion elements. IS1245 has been shown to be present in multiple copies in strains of *M.avium* isolated from various species. RFLP analysis using an IS1245-specific DNA probe revealed high copy numbers (>8 and up to 27) and highly polymorphic banding patterns among human and porcine isolates. Some avian isolates contained characteristically low copy numbers (2-3), while isolates from other non-human sources were observed to differ widely in IS1245 copy number and banding pattern.

IS1245 has been shown to share 83% sequence identity with another *M.avium* genomic insertion sequence, IS1311 (Guerrero *et al.*, unpublished database submission). IS1311 is 1317bp in length, contains an ORF of 1233bp and encodes a putative transposase similar to that of IS1245. Probes based on IS1311 internal nucleotide sequences have been used to identify up to 19 different RFLPs among 75 clinical isolates of *M.avium* (Roiz *et al.*, 1995). In addition, PCR amplification of sequences located between copies of IS1245 and IS1311 on the same genome yielded reproducible banding patterns and provided the basis for an alternative method to RFLP analysis for typing *M.avium* strains (Picardeau and Vincent, 1996).

1.5.5.5 IS1141 of *M.intracellulare*

IS1141 was identified in the 68kb plasmid of *M.intracellulare* strain Va14 and is the only insertion element known in this species (Via and Falkinham, 1993). This element is 1595bp in length and lacks terminal inverted repeats and short direct repeats in common with several other mycobacterial insertion elements (see above). An ORF of 1002bp encodes a putative transposase which has been observed to share 29% amino acid sequence identity with that of IS916 of the *E.coli* IS3 family of insertion elements. IS1141 is present in 3-6 copies in colonial variants of some

strains of *M.intracellulare*. Colonial variation, as defined by opaque or transparent morphology, has been observed to concord with different RFLP types using IS1141-derived DNA probes. To date, IS1141 has not been detected in any other species of *Mycobacterium* including *M.avium* ssp. and *M.scrofulaceum*.

1.5.6 Plasmids

Since the first mycobacterial plasmids were described by Crawford and Bates (1979), research on their distribution and characterisation has focused largely on those identified in *M.avium*, *M.intracellulare*, *M.scrofulaceum* and the *M.fortuitum* complex. Plasmids are common in these species, with some strains harbouring multiple plasmids.

The plasmids found in the MAC fall predominantly into one of two categories. Small (<30kb) or large (>150kb) plasmids are the most frequently isolated, although a range of intermediate sized plasmids spanning 60-90kb also have been isolated from some strains (Crawford and Falkinham, 1990). The small plasmids have been divided into two groups which are not related at the nucleotide level (Jucker and Falkinham, 1990). Group 1 is represented by pVT2, a 12.9kb plasmid carried by some strains of *M.avium* (Jucker and Falkinham, 1990), while group 2 is represented by pLR7, a 15.3kb plasmid carried by *M.avium* strain LR113 (serotype 4) (Crawford and Bates, 1984).

The *M.avium* plasmid pLR7 is the most extensively studied of the small MAC plasmids. Hybridisation studies have shown that pLR7 exhibits significant nucleotide sequence homology with another MAC plasmid pLR1 (17.2kb), but restriction maps of the two plasmids show that there is considerable divergence of restriction sites despite their similarity (Crawford and Bates, 1984; Crawford and Falkinham, 1990). More recently, the replication region of pLR7 was located to a 1.8kb *Pvu* II to *Sma* I restriction fragment and an ORF of 1068bp, encoding a putative Rep protein of 39.4kDa, was identified. The putative Rep protein was

shown to contain a potential helix-turn-helix motif consistent with other DNA-binding proteins and 43% amino acid sequence similarity with the Rep protein of the *M.scrofulaceum* plasmid pMSC262. The pLR7 origin of replication was used to construct a shuttle vector (pMB351) which was used subsequently to transform *M.avium*, *M.tuberculosis* and *M.bovis* at an efficiency of 10^5 cfu/ μ g DNA (Beggs *et al.*, 1995).

Plasmids pVT2 and pLR20 are closely related and both of these have been shown to hybridise to a region of the 28kb plasmid pLR2 (Crawford and Falkinham, 1990).

DNA probes derived from pVT2 and pLR7 have been used in a number of studies to establish relationships between different plasmids and to assess the distribution of plasmids in MAC strains recovered from human (both AIDS and non-AIDS patients), veterinary and environmental sources. Maskai *et al* (1989) showed that the small plasmids recovered from porcine MAC isolates were homologous to those recovered from human strains. Jucker and Falkinham (1990) showed that a pVT2-derived DNA probe hybridised with every plasmid of ≤ 13.5 kb from MAC isolates of environmental, AIDS and non-AIDS origin. The authors suggested that the observed relatedness between plasmids from environmental and clinical MAC isolates supports the hypothesis that the environment is the most likely source of human infection. In the same study, a second DNA probe derived from pLR7 was observed to hybridise with larger plasmids (15.3-25kb) from environmental, AIDS-associated and non-AIDS related MAC isolates. Another study compared rates of plasmid carriage in both AIDS-associated and non-AIDS related MAC strains from the UK and the USA (Hellyer *et al.*, 1991). Higher rates of plasmid carriage were observed in American AIDS-associated isolates than in non-AIDS strains, whereas no significant difference was observed among UK isolates. However, regardless of geographic origin, pLR7-related plasmids were noted to occur more frequently in MAC serotypes 4 and 8 which are closely associated with AIDS-related infections (Hellyer *et al.*, 1991). The higher frequency of plasmids among these strains

corroborated previous observations (Crawford and Bates, 1986; Meissner and Falkinham, 1986; Jucker and Falkinham, 1990) and suggested that their presence may be associated with virulence. However, curing MAC strains of plasmids has proved difficult and many failed attempts have delayed further investigation of this hypothesis (Falkinham and Crawford, 1994).

Bacterial plasmids often contain genes whose expression products confer new phenotypic characteristics and enhanced chances of survival upon the host organism. In order to determine the phenotype conferred upon an organism by any given plasmid, it is necessary to compare strains which are isogeneic apart from the plasmid DNA. This type of comparison allows different phenotypic traits, e.g. antibiotic resistance, to be identified and attributed to the presence of the plasmid. Unfortunately, because of the difficulties encountered in attempting to cure strains of plasmids, and also because the genetic manipulation of mycobacteria was achieved only relatively recently, the functions of many mycobacterial plasmids have yet to be elucidated. However, some plasmid-encoded characteristics have been identified and examples include a 173kb plasmid (pVT1) in *M.scrofulaceum* strain W262 which confers resistance to mercury and copper (Meissner and Falkinham, 1984; Erardi *et al.*, 1987).

1.6 Taxonomy of the MAC

Attempts to establish clear taxonomic divisions within the MAC have been hampered by the close genetic and phenotypic relationships shared between the members of the group. Serotyping schemes have allowed only a crude differentiation between *M.avium* and *M.intracellulare*, but this is partly because standardised antisera are not generally available for seroagglutination typing. Laboratories utilising this assay must generate their own antisera by immunising rabbits with strains which are considered to be representative of known serovars (Wayne *et al.*, 1993). Not surprisingly, this lack of uniformity has resulted in disagreement between laboratories participating in the characterisation of strains and has exacerbated the

confusion surrounding classification within the complex. Although comparison of different serotypes at the genomic level has helped to assign most strains to either *M.avium* or *M.intracellulare*, species assignments for some serotypes remain unresolved (see section 1.4.1).

Further difficulties in defining clear taxonomic boundaries within the MAC stem from the remarkably close genetic and phenotypic similarities shared between *M.avium*, *M.a.paratuberculosis* and the “wood pigeon bacillus” (see section 1.5). It has not been possible to differentiate between these organisms on biochemical or serological criteria, and unlike *M.avium* and *M.intracellulare*, they cannot be resolved by genomic DNA homology determinations (Hurley *et al.*, 1988) or DNA probes targeting 16S rRNA genes (Thoresen and Saxegaard, 1991, van der Giessen *et al.*, 1992a). However, they can be separated by RFLP analysis (McFadden *et al.*, 1987c) and this has been facilitated by the identification of the genomic insertion elements IS900 in *M.a.paratuberculosis* (Green *et al.*, 1989) and IS901/902 in the wood pigeon bacillus and some other strains of *M.avium* (Kunze *et al.*, 1991; Moss *et al.*, 1992a). Short species-specific regions situated at the 5' end of each insertion element have provided DNA probes and oligonucleotides which allow the three species to be resolved by Southern blotting or PCR respectively (Kunze *et al.*, 1992).

Currently, there are two schools of thought regarding taxonomy within the MAC. The first of these is based on a numerical taxonomic study conducted by Thorel *et al* (1990). This study assigned each of 38 MAC strains to one of four clearly defined clusters on the basis of their reactions in 22 out of 51 separate cultural and biochemical tests. Cluster 1 contained *M.a.paratuberculosis* strains; cluster 2 contained *M.avium* and *M.intracellulare* reference strains; cluster 3 contained wood pigeon isolates; and cluster 4 contained only *M.a.paratuberculosis* strain 316F, a vaccine strain used for antigen production. The results of this study were observed to concord with those of other studies based on FIGE banding patterns (Levy-Frebault *et al.*, 1989) and pathogenicity for bovine calves and chickens (Matthews and

McDiarmid 1979; Thorel *et al.*, 1984; Collins *et al.*, 1985). From the collective results of genotypic and phenotypic studies, Thorel *et al* suggested that *M.avium*, *M.a.paratuberculosis* and the wood pigeon bacillus be reduced to subspecies of *M.avium*, and proposed the following designations: *M.avium* subsp. *avium*, *M.avium* subsp. *paratuberculosis* and *M.avium* subsp. *silvaticum*, respectively.

Further comment on the taxonomic divisions within the MAC stems from the results of a study on the distribution of genomic insertion elements among clinical and environmental MAC isolates (Kunze *et al.*, 1992). Strains containing IS900 were identified as *M.a.paratuberculosis*, while *M.avium* strains were divided into two subgroups depending on whether IS901/902 was absent or present. Strains lacking the insertion sequence were designated “*M.avium* RFLP type A” (environmental organisms rarely associated with animal disease but frequently associated with opportunistic infections in AIDS patients), while those which contained IS901/902 were designated “*M.avium* RFLP type A/I” (rarely associated with human infections but frequently associated with Johne’s disease of ruminants and tuberculosis of birds and mammals). This division was further supported by differences in plasmid content and serotype antigens. The final conclusions of this study differed from those based on numerical analysis in a number of respects. Firstly, *M.a.paratuberculosis* is described as a slow-growing, mycobactin dependent organism which is isolated from animals with clinical Johne’s disease. This description fits both *M.a.paratuberculosis* and *M.avium* RFLP type A/I, which can be considered distinct on the basis of their different insertion elements, their ability to cause tuberculosis in birds and mammals and on the stability of their dependence on mycobactin. Secondly, whereas Thorel *et al* placed *M.a.paratuberculosis* strain 316F separately in cluster 4, Kunze *et al* demonstrated the presence of IS900 and confirmed its identity as typical *M.a.paratuberculosis*. Thirdly, all wood pigeon-derived isolates examined by Kunze *et al* were shown to possess IS901/902 and were typed therefore as *M.avium* RFLP type A/I. However, *M.avium* RFLP type A/I did not agree fully with *M.avium* subsp. *silvaticum*, because the former includes strain 18

and *M.a.paratuberculosis* 2103, both of which were placed in cluster 2 by Thorel *et al* and therefore were considered to be *M.avium* subsp. *avium*. Finally, although there was broad agreement between *M.avium* RFLP type A and *M.avium* subsp. *avium* (cluster 2 of Thorel *et al*), the exclusion of strain 18 (IS901/902 positive) from *M.avium* RFLP type A highlighted a major discrepancy between the results of the two studies. It should be noted that the former working strain of *M.a.paratuberculosis* “strain 18” has been observed to be more closely related to *M.avium* on the basis of cultural characteristics (Merkal, 1979), FIGE banding pattern (Levy-Frebault *et al.*, 1989) and RFLP analysis (McFadden *et al.*, 1987a). Strain 18 has since been identified as a serotype 2 strain of *M.avium* and its abolition as a strain of *M.a.paratuberculosis* was recently proposed by Chiodini (1993).

For the purposes of this thesis, *Mycobacterium paratuberculosis* will be referred to as “*M.a.paratuberculosis*” in accordance with the taxonomic divisions proposed by Thorel *et al* (1990), and as accepted in the *International Journal of Systematic Bacteriology*. However, because the divisions separating different strains of *Mycobacterium avium* remain unclear, IS901/902-positive *M.avium* will be referred to as *M.avium* type A/I in accordance with the nomenclature used by Kunze *et al* (1992).

1.7 Previously Characterised Genes and Proteins of *M.a.paratuberculosis* and *M.avium*

Pioneering work on the identification and characterisation of mycobacterial genes and their expression products was presented to the scientific community when Young *et al.* (1985a and 1985b) prepared libraries of *M.tuberculosis* and *M.leprae* genomic DNA in the λ gt11 expression vector and screened for expression of particular antigens using a range of monoclonal antibodies. Since then, libraries have been prepared from genomic DNA of many other species of *Mycobacterium*, including *M.avium* and *M.a.paratuberculosis*, and a continually expanding catalogue of genes and gene products is now readily accessible. Some of these, such as bacterioferritins

(Brooks *et al.*, 1991; Pessolani *et al.*, 1994; Inglis *et al.*, 1994b), and the alpha secretory proteins of the antigen 85 complex (Matsuo *et al.*, 1988; Wiker *et al.*, 1990; de Mendonca Lima *et al.*, 1991; Matsuo *et al.*, 1990a; Ohara *et al.*, 1993; Kitaura *et al.*, 1993; Takano *et al.*, 1994) are common to more than one species of *Mycobacterium*, while others, such as heat shock proteins, are conserved across the phylogenic spectrum.

Catalogues of previously characterised genes and proteins of *M.avium* and *M.a.paratuberculosis* are presented in tables 1.2 and 1.3 respectively.

Table 1.2
Previously characterised genes and proteins of *Mycobacterium avium*

| Antigen | Gene or clone | EMBL accession number | Function | Features | References |
|----------------------------------|-------------------------------------------|-----------------------|-------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|
| 117.3kDa 114.6kDa | <i>embA</i> <i>embB</i> | U66560 | arabinosyl transferase | Involved in cell wall arabinan biosynthesis, target for ethambutol | Belanger <i>et al.</i> , 1996 |
| ~91kDa | <i>katE</i> | L41246 | catalase HP11 | Strongly similar to <i>E.coli katE</i> and eukaryotic catalases, present in several species of <i>Mycobacterium</i> | Milano <i>et al.</i> , 1996 |
| 70 kDa | <i>hsp70</i> | S76750 | Role in protein folding and translocation | Heat shock protein homologous with <i>E.coli</i> DnaK | Little <i>et al.</i> , 1994 |
| 68 kDa | | | Binds to macrophages | Surface antigen, homologous with the 65kDa heat shock protein | Rao <i>et al.</i> , 1994 |
| 66 kDa | | | Possible virulence factor | SmT66, cytosolic protein possibly associated with smooth transparent morphotype | Prinzis <i>et al.</i> , 1994 |
| 65.8 kDa 18.1 kDa 36.0 kDa | <i>ilvB</i> <i>ilvN</i> <i>ilvC</i> | L49392 | acetohydroxy acid synthase and acetohydroxy acid isomeroreductase | <i>ilvBNC</i> gene cluster involved in branched chain amino acid synthesis | Gusberti <i>et al.</i> , 1996 |
| 65 kDa | <i>hsp65</i> | S76642 | Role in protein folding and translocation | Heat shock protein homologous with <i>E.coli</i> GroEL | Hidaka <i>et al.</i> , 1995 |
| 43.9 kDa | IS901/ IS902 | X59272 X58030 | Putative transposase | Specific for some strains of <i>M.avium</i> | Kunze <i>et al.</i> , 1991 Moss <i>et al.</i> , 1992 |
| 40 kDa | | | Unknown | Protein concurs with IS901/902. Absent from <i>M.a. paratuberculosis</i> | Inglis <i>et al.</i> , 1994a; Burrells <i>et al.</i> , 1995 |
| 38.2kDa | <i>fapA</i> | | fibronectin-binding protein | proline and alanine rich protein | Schorey <i>et al.</i> , 1996 |

Table 1.2 continued

| Antigen | Gene or clone | EMBL accession number | Function | Features | References |
|---------|--------------------------|-----------------------|-------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| 35 kDa | | | Unknown | Homologue of the gene encoding the immunogenic 35 kDa membrane protein of <i>M.leprae</i> | Winter <i>et al.</i> , 1995 |
| 33 kDa | 3954B12 | | Unknown | Induces a weak DTH response in sensitised guinea-pigs | Rouse <i>et al.</i> , 1991 |
| 31 kDa | | X63437 | Unknown | α Antigen, secretory protein | Ohara <i>et al.</i> , 1993 |
| 27 kDa | <i>ahpC</i> | M74232 U18263 | Alkyl hydroperoxidase | Avi-3 antigen, specific for <i>M.avium</i> | Yamaguchi <i>et al.</i> , 1992; |
| 27 kDa | | | May facilitate intracellular survival in host | Expression induced by phagocytosis | Plum & Clark-Curtiss, 1994 |
| 26 kDa | 3808C3 | | Unknown | Induces a DTH response in sensitised guinea-pigs | Rouse <i>et al.</i> , 1991 |
| 23kDa | <i>sodA</i> | | superoxide dismutase | Mn SOD exposed at cell surface | Escuyer <i>et al.</i> , 1996 |
| 20 kDa | R692 | X76906 | Bacterioferritin | Bacterioferritin conserved sequences | Inglis <i>et al.</i> , 1994b |
| 19 kDa | | L12235 | Stress protein | | Booth <i>et al.</i> , 1993 |
| 18 kDa | | L12237 | Stress protein | | Booth <i>et al.</i> , 1993 |
| | <i>ask</i> <i>asd</i> | | Biosynthesis of diamino-pimelic acid in cell wall synthesis | The aspartokinase gene (<i>ask</i>) and the aspartate semialdehyde dehydrogenase gene (<i>asd</i>) are in an operon | Cirillo <i>et al.</i> , 1994 |
| | <i>dnaA</i> | U19185 | Initiation of replication | | Rajagopalan <i>et al.</i> , 1995 |

Table 1.2 continued

| Antigen | Gene or clone | EMBL accession number | Function | Features | References |
|----------|---------------|-----------------------|---------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|
| | <i>dnaJ</i> | | | Stress protein-coding gene. Partial sequence | Takewaki <i>et al.</i> , 1994 |
| | <i>gyrA</i> | X76710 | Quinolone resistance | | Cambau <i>et al.</i> , 1994 |
| | <i>mce</i> | U26017 | Associated with entry and survival in macrophages | Homologous to <i>mce</i> gene of <i>M.tuberculosis</i> (X70901) | database submission |
| | <i>rpoB</i> | | Rifampicin resistance | | Guerrero <i>et al.</i> , 1994 |
| S12 | <i>rpsL</i> | X80120 | Streptomycin resistance | | Honore & Cole, 1994 |
| | <i>ser2</i> | | Biosynthesis of glycopeptido-lipid | Gene cluster responsible for the biosynthesis of the serospecific glycopeptidolipid of <i>M.avium</i> serovar 2 | Belisle <i>et al.</i> , 1991; Mills <i>et al.</i> , 1994 |
| | <i>sod</i> | X81384 | Superoxide dismutase | | Zolg & Philippi-Schultz, 1994 |
| IS1110 | | Z23003 Z28969 | Unknown | Highly mobile | Hernandez Perez <i>et al.</i> , 1994 |
| IS1245 | | L33879 | Putative transposase | Specific for <i>M.avium</i> species but not subspecies specific | Guerrero <i>et al.</i> , 1995 |
| IS1311 | | U16276 | Putative transposase | Similar to protein encoded by L33879 | database submission |
| 16S rRNA | | X52918 | 30S subunit | | Rogall <i>et al.</i> , 1990a |
| 23S rRNA | | X74494 | 50S subunit | | Van der Giessen <i>et al.</i> , 1994 |

Table 1.2 continued

| Antigen | Gene or clone | EMBL accession number | Function | Features | References |
|---------|---------------|-----------------------|----------|------------------------------------------------------------------------|--------------------------------------|
| | DT1 | L04543 | Unknown | Sequence hybridizes specifically to serovars 2, 3, 7, 12 to 20, 23, 25 | Thierry <i>et al.</i> , 1993a; 1993b |
| | DT6 | L04542 | Unknown | Sequence hybridises specifically to serovars 1, 6, 8 to 11, 21 | Thierry <i>et al.</i> , 1993a; 1993b |

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Table 1.3
Previously characterised genes and proteins of
Mycobacterium avium ssp. *paratuberculosis*

| Antigen | Gene or clone | EMBL accession number | Function | Features | References |
|----------|---------------|-----------------------|-------------------------------------------|---------------------------------------------------------------------------|---------------------------------|
| >100 kDa | S5 | | Unknown | Induces humoral immunity in sheep | Stevenson, unpublished |
| 70 kDa | HSP70 | X59437 | Role in protein folding and translocation | Heat shock protein homologous with <i>E.coli</i> DnaK | Stevenson <i>et al.</i> , 1991 |
| 65 kDa | HSP65 | X74518 | Role in protein folding and translocation | Heat shock protein, homologous with <i>E.coli</i> GroEL | Colston <i>et al.</i> , 1994 |
| 43 kDa | IS900 | X16293 | Putative transposase | Specific for <i>M.a. paratuberculosis</i> | Green <i>et al.</i> , 1989 |
| ORF2 | IS900 | X69575 | Unknown | ORF2 on complementary strand of IS900 | Murray <i>et al.</i> , 1992 |
| 35kDa | pMptb # 40 | | Unknown | Potentially subsp.-specific antigen for serodiagnosis of paratuberculosis | El Zaatari <i>et al.</i> , 1997 |
| 34 kDa | S8 | Z23092 | Putative serine protease | Signal peptide, serine protease active site | Cameron <i>et al.</i> , 1994 |
| 34 Da | | X68102 | Unknown | B cell epitopes specific for <i>M.a. paratuberculosis</i> | Gilot <i>et al.</i> , 1993 |
| 34 kDa | S1 | | Unknown | Induces humoral and CMI responses in ruminants | Stevenson, unpublished |
| 31 kDa | | A44892* | Unknown | Antigen A | Sugden <i>et al.</i> , 1991 |
| 20 kDa | | A44893 | Bacterioferritin | Antigen D | Sugden <i>et al.</i> , 1991 |
| | 16S rRNA | M29569 | 30S subunit | | Stahl & Urbance, 1990 |

Table 1.3 continued

| Antigen | Gene or clone | EMBL accession number | Function | Features | Citation |
|---------|---------------|-----------------------|-------------------------|--------------------------------------------------------|--------------------------------------|
| | 23S rRNA | X74495 | 50S subunit | | Van der Giessen <i>et al.</i> , 1994 |
| | F57 | X70277 | Unknown | DNA sequence specific for <i>M.a. paratuberculosis</i> | Poupart <i>et al.</i> , 1993 |
| | <i>dnaJ</i> | | Stress protein | Partial sequence only | Takewaki <i>et al.</i> , 1994 |
| | <i>sod</i> | Z48212 | Mn Superoxide dismutase | | Bull <i>et al.</i> , 1995b |

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1.8 Johne's Disease

1.8.1 Aetiology

As early as 1826, an enteric disease of cattle with chronic diarrhoea was reported by d'Aroval. The cattle dying from this condition were observed to be emaciated, passing blood in the faeces and at post-mortem examination, the intestinal mucosa was noted to be thickened and highly corrugated. It was not until 1895 however, that Johne and Frothingham demonstrated the presence of acid-fast bacilli in sections of diseased intestine and described the disease as "a peculiar case of tuberculosis". Ten years later, Bang (1906) recognised that the disease was not tuberculosis and named it "pseudo-tuberculosis enteritis" or "Johne's disease". Shortly afterwards the name of "paratuberculosis" was coined and the causative agent was isolated and used to reproduce the disease in laboratory animals (Marek, 1910; Twort and Ingram, 1912; Twort, 1914). The organism was renamed "*Mycobacterium enteritidis chronicae pseudotuberculosis bovis johne*" (Twort, 1914), but later became known as *Mycobacterium johnei*, *Mycobacterium paratuberculosis* and more recently, *Mycobacterium avium* subsp. *paratuberculosis* (Thorel *et al.*, 1990) (see section 1.6).

Although *M.a.paratuberculosis* is the predominant pathogen in Johne's disease, wood pigeon strains of *M.avium* (RFLP type A/I) are also known to cause paratuberculosis in ruminants (see section 1.6).

1.8.2 Infection

The primary route of paratuberculosis infection appears to be faecal/oral. Infected animals may excrete the organism in the faeces for up to 18 months prior to developing visible signs of infection and clinical cases may shed as many as 5×10^{12} organisms/day (Chiodini *et al.*, 1984a). Young animals may contract the infection via sucking on teats contaminated with faeces, ingesting infected colostrum or milk or grazing on contaminated pasture. Following oral ingestion, the organism is thought to become localised in the mucosa of the small intestine, its associated

lymph nodes and to a lesser extent, in the tonsils and suprapharyngeal lymph nodes. The organism traverses the intestinal mucosa via M cells in the dome epithelium of ileal Peyer's patches and subsequently is phagocytosed by subepithelial macrophages (Momotani *et al.*, 1988). Evidence supporting the role of ileal Peyer's patches in the uptake of the organism is provided by histopathological observations in experimentally infected calves (Payne and Rankin, 1961; Gilmour *et al.*, 1965; Momotani *et al.*, 1988) and sheep (Kluge *et al.*, 1968; Gilmour *et al.*, 1978; Perez *et al.*, 1996; Perez *et al.*, 1997).

The isolation of organisms from bovine placental tissue, uterus and foetal tissues (Seitz *et al.*, 1989; Sweeney *et al.*, 1992) provides evidence of intrauterine infection of the foetus. However, to date, lesions associated with paratuberculosis have not been demonstrated in the foetus. Contamination of the male reproductive tract and seminal fluid also has been reported (Larsen and Kopecky, 1970), but sexual transmission of the infection has not been demonstrated.

Paratuberculosis has been reproduced experimentally by oral inoculation of calves (Payne and Rankin, 1961; Gilmour *et al.*, 1965), lambs (Nisbet *et al.*, 1962; Kluge *et al.*, 1968; Allen *et al.*, 1974; Gilmour *et al.*, 1978; Garcia Marin, *et al.*, 1992) and deer (Nyange, 1990). Experimental infection, resulting in lesions in the intestine and other organs, also has been transmitted successfully in sheep via intratracheal and intravenous routes (Kluge *et al.*, 1968). Attempts to transmit the disease by experimental intramammary and intrauterine inoculation were unsuccessful (Larsen and Miller, 1978; Merkal *et al.*, 1982).

1.8.3 Prevalence and economic impact

Paratuberculosis is a global disease problem whose prevalence has been reported to be increasing in some countries. Results of studies on bovine paratuberculosis have provided evidence of infection in 15% and 18% of cattle in the UK and USA respectively (Chiodini *et al.*, 1984a; Chiodini and Van Kruiningen, 1986; Braun *et*

al., 1990). Prevalence rates as high as 30% have been reported among beef cattle herds in Louisiana (Turnquist *et al.*, 1991), while in other areas of the USA and Canada, rates may be as low as 1.6%-5% (Merkal *et al.*, 1987; McNab *et al.*, 1991). Rates of mortality are normally <1%, but have been reported to be as high as 10% (Radostits *et al.*, 1994). Recently, the UK Veterinary Investigation Centres reported that the annual incidence of ruminant paratuberculosis in the UK has been rising since 1985. The number of ovine cases is only around 10% of the number seen in cattle, but the geographical distribution is disproportionate with 40% of bovine cases, 75% of ovine cases and 42% of caprine cases being recorded in Scotland. In the Tayside region alone, the incidence of ruminant paratuberculosis has increased over the last three years to constitute 42% of the Scottish total (Grieg, pers.comm.).

It has been estimated that paratuberculosis costs the US agricultural industry in excess of \$1.5 billion annually (Chiodini *et al.*, 1984a; Jones, 1989). Losses totalling approximately \$US 15.4 million have been reported in the cattle industry alone in New England, Australia (Chiodini and Van Kruiningen, 1986; Gill, 1989). Although paratuberculosis is widespread in New Zealand, in common with many other countries throughout the world, there are no reliable estimates on the national prevalence of the disease (deLisle and Milestone, 1989). The absence of reliable data has made the global economic impact of paratuberculosis difficult to calculate and it is likely that the true cost of the disease is considerably higher than currently accepted estimates. In an Australian study, losses attributed to paratuberculosis were arbitrarily classified as being due to direct, indirect and inapparent costs (Gill, 1989). Direct costs include 1. reduced productivity due to clinical and subclinical disease; 2. increased susceptibility of infected cattle to disease and infertility; and 3. the costs of implementing control measures. Indirect costs include; 1. export testing of live animals; 2. testing of animals moving interstate; and 3. funding for paratuberculosis research. Inapparent costs are less specific and are difficult to calculate. An example would be the protection of the export market afforded by government and agricultural industry efforts in implementing paratuberculosis control measures, and

maintaining an effective and credible animal health infrastructure. The costs of running these programmes in respect of paratuberculosis alone are unquantifiable since the service provides other important functions also.

In bovine calves, the risk of infection with *M.a.paratuberculosis* appears to be higher in young animals under four weeks of age, but adult animals exposed to the organism for the first time may develop the disease (Larsen *et al.*, 1975). Results of experimental transmission studies have shown that infection is achieved most successfully by inoculating young calves orally with large numbers of organisms (Larsen *et al.*, 1975). Factors regarded as predisposing animals to infection include intensive farming systems, acid soils, low dietary intake, stress and immunosuppression due to infection with bovine virus diarrhoea virus (Clarke, 1997). Exposure to infected faeces is probably the single most important factor in disease transmission, and may explain why paratuberculosis has been observed to be more prevalent in dairy cattle than beef cattle (Braun *et al.*, 1990). Natural transmission of the disease between different species remains an unresolved issue, and the significance of experimental inter-species infections in relation to naturally occurring paratuberculosis has yet to be established. However, the potential for transmission of the disease from wild rabbits to domestic ruminants is the subject of ongoing research (Greig *et al.*, 1997).

1.9 Immunological Aspects of Johne's Disease

Immunological parallels have been drawn between Johne's disease and leprosy in humans (Cocito *et al.*, 1994). Both mycobacterioses are characterised by an early stage, where a strong cell mediated immune (CMI) response limits proliferation of the organism, and a terminal anergic stage, where CMI is no longer detectable and serum antibody levels are elevated. As in other mycobacterioses, CMI is considered to be the most important immune mechanism for clearing *M.a.paratuberculosis* infection. Although high titres of circulating antibody are characteristic of advanced clinical Johne's disease, the humoral arm of the immune response to infection is

ineffective against mycobacteria which occupy, and replicate in, an intracellular environment (Chiodini *et al.*, 1984a; Clarke, 1994).

1.9.1 Cell mediated immunity

Detectable CMI responses to *M.a.paratuberculosis* appear early in the infection and in some cases are sufficient to clear the invading organism and render the host animal resistant to reinfection (Gilmour *et al.*, 1978). However, CMI may weaken progressively over the protracted period of a persistent infection resulting in bacterial proliferation and disease associated with multibacillary (lepromatous) lesions. Alternatively, persistent and chronic inflammatory disease associated with a paucibacillary (tuberculoid) state may result from prolonged CMI mounted in response to organisms which resist killing (Chiodini *et al.*, 1984a; Clarke, 1997).

T-lymphocytes are responsible for activating macrophages and play a central role in orchestrating the CMI response to mycobacterial infections. The importance of T-lymphocytes in clearing infection with *M.a.paratuberculosis* has been demonstrated in a study where experimentally infected athymic mice were observed to be incapable of impeding mycobacterial proliferation and preventing the development of intestinal lesions (Hamilton *et al.*, 1989). Class I and II major histocompatibility complex (MHC) molecules on the surface of macrophages and other antigen presenting cells, play a central role in delivering mycobacterial protein antigens to T-lymphocytes and triggering their activation and clonal proliferation (Kaufmann, 1993; Rock, 1996). In addition, CD1-restricted T-lymphocyte recognition of mycobacterial lipid antigens, including those of *M.avium*, has been demonstrated in humans (Sieling *et al.*, 1995).

T-lymphocytes carrying $\alpha\beta$ T-cell receptors, including $CD4^+$, $CD8^+$ and $CD4^-$, $CD8^-$ subsets, and $\gamma\delta$ T-cell receptors are known to be active in mycobacterial infections. Experiments where $CD4^+$ T-cell deficient “knockout mice” were observed to succumb to challenge with *M.tuberculosis* have shown that this particular subset is of paramount importance in arresting the progression of mycobacterial disease (Boom

et al., 1991). Th1 and Th2 subsets of murine CD4⁺ T-lymphocytes can be distinguished on the basis of their characteristic cytokine production patterns. Th1 cells secrete interferon gamma (IFN- γ), interleukin-2 (IL-2) and IL-12, and promote activation of macrophages and delayed type hypersensitivity (DTH) responses. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, and promote B-cell differentiation and antibody production (Mosman *et al.*, 1986; Mosmann and Moore, 1991). A balance between the effects of Th1 and Th2 activity is achieved through reciprocal antagonism with IL-4 and IL-10 exerting a suppressive effect on Th1 responses, and IFN- γ having a similar suppressive effect on Th2 responses (Mosmann and Moore, 1991). These mechanisms have been observed in mycobacterial diseases such as tuberculosis and leprosy where IFN- γ production, strong DTH reactions and low numbers of mycobacteria are associated with Th1 responses, and depressed IFN- γ production, high antibody concentrations and large numbers of mycobacteria are associated with Th2 responses (Orme *et al.*, 1992; Barnes *et al.*, 1993). A similar immunological picture has emerged from studies on paratuberculosis in sheep and cattle. During the course of the disease, high CMI, which is characteristic of the early phase of infection, begins to fall and is replaced by an increase in circulating immunoglobulin (see below). This suggests that a Th1-Th2 balance operates in paratuberculosis also. Evidence supporting early CMI responses is provided by measurement of *in vitro* secondary T-lymphocyte proliferation (Lepper *et al.*, 1989; Perez *et al.*, 1994; Burrells *et al.*, 1995) and intradermal skin testing, where positive DTH reactions seen early in infection were observed to weaken progressively in animals with chronic disease (Gilmour *et al.*, 1965; Davies *et al.*, 1974). In addition, stimulation with mycobacterial antigens evoked only weak *in vitro* secondary proliferative responses in T-lymphocytes taken from cattle with advanced paratuberculosis (Kreeger and Snider, 1992). Electron microscopic examination of intestinal macrophages of sheep with paratuberculosis revealed a correlation between strong DTH skin reactions and small numbers of morphologically damaged intracellular mycobacteria (Gilmour *et al.*, 1978). In the same study, intestinal

macrophages of other animals with depressed DTH responses were observed to harbour large numbers of apparently intact intracellular organisms.

To date, descriptions of the immunological course of paratuberculosis have focused primarily on peripheral responses rather than those occurring locally at the intestinal mucosa. Observations following intravenous inoculation of cattle with killed *M.a.paratuberculosis* showed that early CD4⁺ T-lymphocyte and DTH responses preceded an anergic phase and subsequent increases in antigen specific-reactivity of $\gamma\delta^+$ and CD8⁺ T-lymphocytes (Chiodini and Davis, 1992 and 1993). These observations prompted the authors to suggest that these cells may have a modulatory effect on the CD4⁺ response. Shortly afterwards, experimental infections in susceptible (strain C57BL/6) and resistant (strain C3H/He) mice demonstrated both CD4⁺ T-lymphocyte depletion and reduced CD25 expression in the intestines of susceptible animals, whereas no such changes were observed to have occurred in the resistant strain (Veazey *et al.*, 1994). In addition, the susceptible mice were noted to possess increased numbers of antigen-reactive CD8⁺ and $\gamma\delta^+$ T-lymphocytes, although this was not seen to confer any obvious immunological advantage as the development of characteristic granulomatous multibacillary lesions progressed unhindered. These observations contrast with the situation in the murine lung where CD8⁺ cells have been shown to afford protection against infection with *M.tuberculosis*. The specific role of CD8⁺ lymphocytes in the lung and the reasons why they appear to be less important in protecting other organs against mycobacterial infection are unknown. However, the results of a recent study suggest that the protective mechanism of CD8⁺ lymphocytes in the murine lung is one of cytokine production rather than lysis of infected macrophages (Cooper *et al.*, 1997). Further studies conducted in sheep showed that animals with multibacillary lesions and weak CMI responses were depleted in numbers of both CD4⁺ and CD8⁺ intestinal T-lymphocytes, while those with paucibacillary lesions and strong CMI responses were noted to possess increased numbers of both CD4⁺ and $\gamma\delta^+$ intestinal T-lymphocytes (Little *et al.*, 1996). Support for these findings comes from the results of recent

studies which correlated strong peripheral and local antigen-specific lymphocyte reactivity in paucibacillary paratuberculosis with increased Th1 cytokine (IFN- γ and IL-2) production. Conversely, conditions likely to favour mycobacterial persistence, such as depressed lymphocyte reactivity and Th1 cytokine production, were observed to be characteristic of multibacillary paratuberculosis (Burrells, pers. comm.).

Finally, the role of natural killer (NK) cells in the ruminant immune response to paratuberculosis has yet to be described fully. IFN- γ production in response to mycobacterial infection has been observed in NK cells (Moreno and Rees, 1993). More recently, experimental infection of NK cell-depleted beige (highly susceptible) and C57BL/6 (control) mice showed that growth of virulent strains of *M. avium* was not affected in either group (Florido *et al.*, 1997). The authors concluded that lack of NK cell activity is not responsible for the increased susceptibility of beige mice to pulmonary *M. avium* infection.

1.9.2 Humoral immunity

Antibody production in animals with paratuberculosis appears later during the course of infection than corresponding CMI responses, and has been noted to coincide with depressed antigen-specific T-lymphocyte reactivity or even anergy (Chiodini *et al.*, 1984a; Burrells *et al.*, 1995). It has been suggested that intracellular mycobacteria proliferate in the face of decreasing CMI, and that the release of organisms by the lysis of infected cells serves as the stimulus for antibody production (Clarke, 1997). Thus, higher circulating antibody levels are characteristic of animals with multibacillary lesions, whereas lower antibody levels are associated with paucibacillary cases (Sockett *et al.*, 1992a; Clarke *et al.*, 1996). However, circulating antibody is ineffective against remaining intracellular organisms and of no value in arresting the progression of clinical disease.

1.10 Diagnosis of Johne's Disease

Historically, the absence of accurate and reliable methods for the diagnosis of paratuberculosis has been a major limitation to effective control of the disease. Unlike most other bacterial diseases, a long and protracted disease course and late humoral immune response (see section 1.9) preclude early serological detection of animals infected with *M.a.paratuberculosis*. In addition, unavoidable exposure to antigenically related environmental mycobacteria generates a background of non-specific host immunity, against which specific immune-based diagnosis of paratuberculosis may become discretionary and based on quantitative rather than qualitative measurements. More direct methods of detection such as faecal smearing do not discriminate between different species of *Mycobacterium*, and cultural isolation of the organism from clinical specimens requires laboratory confirmation of species identity. However, DNA technology has provided new approaches to the identification of *M.a.paratuberculosis* and, in conjunction with more traditional techniques, can be used to circumvent the problems of specificity and sensitivity associated with many of the currently used diagnostic procedures.

1.10.1 Microscopic detection of *M.a.paratuberculosis* in faeces

Successful detection of mycobacteria in faeces by direct microscopic examination of stained smears depends on the number of organisms present in the specimen at the time of sampling and on the extent to which they may be clustered together. Overall sensitivity is poor on an individual basis because faecal shedding of the organism at levels detectable by smearing can be irregular and may not occur during the early stages of infection (Chiodini *et al.*, 1984a; Merkal, 1984). Therefore, animals in the early or asymptomatic stages of the disease are often missed at a time when a positive diagnosis would be of greatest value for the purpose of disease management. Conversely, animals with more advanced paratuberculosis are scored positive using this technique, but as animals in the later stages of the disease can usually be identified on clinical criteria alone, laboratory confirmation is unnecessary. The sensitivity of faecal smearing can be improved by centrifugal concentration of

specimens and/or replacing acid-fast staining with immunofluorescent staining of organisms (Abbas *et al.*, 1983a). However, problems with specificity due to antigenic cross-reactivity between *M.a.paratuberculosis* and ubiquitous environmental mycobacteria negates the routine use of this procedure.

1.10.2 Cultural isolation of *M.a.paratuberculosis* from clinical specimens

Faecal culture is considered to be more sensitive than microscopic examination of smears, but is very slow and often difficult due to the fastidious nature and long generation time of *M.a.paratuberculosis*. In addition, false negatives due to reduced mycobacterial viability may result from chemical decontamination of the sample prior to culture (Ridge, 1993). Also, some strains of *M.a.paratuberculosis*, especially those of ovine origin, appear to be non-cultivable (Chiodini *et al.*, 1984a).

Cultural isolation of *M.a.paratuberculosis* from biopsy and necropsy tissues such as mesenteric and ileocecal lymph nodes has been used as an alternative, or in addition to faecal culture (Smith, 1954; Rac, 1955; Benedictus and Bosma, 1985; Benedictus and Haagsma, 1986). The rate of isolation of *M.a.paratuberculosis* from tissues is generally higher than from faeces, especially in animals shedding low numbers of organisms (Merkal *et al.*, 1987). However, identification of *M.a.paratuberculosis* on cultural characteristics such as slow rate of growth and mycobactin dependence on primary isolation cannot be assumed, as these characteristics are shared by some strains of *M.avium* which also can cause paratuberculosis in ruminants (Matthews and McDiarmid, 1979). The actual identity of any such isolate should be confirmed by PCR-amplification of the subspecies-specific nucleotide sequences within IS900 for *M.a.paratuberculosis* and IS901/902 for *M.avium* spp. (see section 1.10.6).

1.10.3 Histopathological detection of mycobacteria in tissue biopsies

Histopathological examination of biopsy and necropsy tissues has provided a rapid and accurate means of detecting mycobacteria in the mesenteric lymph node and terminal ileum of infected cattle (Buergelt *et al.*, 1978b; Pemberton, 1979;

Benedictus and Haagsma, 1986). Again however, differentiation between *M.a.paratuberculosis* and other species of *Mycobacterium* is not possible using this technique alone.

1.10.4 Serological detection of *M.a.paratuberculosis* infection

Many different assays for the serological diagnosis of paratuberculosis have been described. Examples include complement fixation (CF), agarose gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA). All are affected by a lack of specificity and none afford the ability to detect animals in the early stages of the disease when circulating antibody levels are either below detectable levels or absent altogether (Chiodini *et al.*, 1984a; Cocito *et al.*, 1994). Of these, ELISA is the most sensitive, but like other serological assays, it suffers from a lack of specificity when crude or even partially purified protein extracts are used as capture antigens (Abbas *et al.*, 1983b; Yokomizo *et al.*, 1985; Collins *et al.*, 1991; Molina *et al.*, 1991). Although background noise may be reduced by pre-absorption of test sera with *M.phlei* cell lysates prior to analysis (Yokomizo *et al.*, 1985; Molina *et al.*, 1991; Bech-Nielsen *et al.*, 1992), this step has not circumvented the problem of cross-reactivity between *M.a.paratuberculosis* and strains of *M.avium*. Earlier confusion surrounding the actual identity of “*M.paratuberculosis* strain 18” (see section 1.6) served only to exacerbate the problem of distinguishing between these two closely related organisms. Ironically, this problem was emphasised by the introduction of a commercially available ELISA (Allied ELISA, Allied Monitor Inc., Fayette, Mo.) which was based on partly purified antigens prepared from “*M.paratuberculosis*” strain 18, an isolate now known to be a serotype 2 strain of *M.avium* (see section 1.6). Another ELISA, this time based on glycolipid (LAM) rather than protein antigens, also was shown to lack specificity due to extensive cross-reactivity between different mycobacterial and corynebacterial LAM preparations (Sugden *et al.*, 1989; McNab *et al.*, 1991a & 1991b). More recently, a clone isolated from a λ gt11 expression library was shown to encode a peptide (a362) which carried subspecies-specific B-cell epitopes corresponding to a 34kDa protein

antigen of *M.a.paratuberculosis* (De Kesel *et al.*, 1993). A peptide derived from the carboxyl terminus of a362 was used to develop an ELISA which the authors claimed was capable of detecting cattle harbouring *M.a.paratuberculosis* infection specifically, and against a background of antibody known to cross-react with strains of *M.avium* (Vannuffel *et al.*, 1994a).

1.10.5 CMI-based detection of *M.a.paratuberculosis* infection

The major advantage of techniques which measure cell mediated rather than humoral immunity is the ability to detect animals in the early or asymptomatic stages of the disease. Cell mediated immune responses to infection with *M.a.paratuberculosis* have been measured *in vivo* using the intradermal skin test (Kormendy, 1988), and *in vitro* using the secondary T-lymphocyte proliferation assay (Buergelt *et al.*, 1977 & 1978a) and the migration inhibition test (Bendixen, 1977). Cutaneous testing entails intradermal inoculation of tuberculin-like preparations of partially purified *M.a.paratuberculosis* or *M.avium* antigens, and measuring the diameter of any subsequent DTH response at the site of inoculation. These antigen preparations elicit cross-reactive as well as specific inflammatory responses and comparative testing of positive animals with both avian and bovine tuberculin is necessary in EEC countries where tuberculosis testing of cattle is compulsory. Animals which react strongly to bovine tuberculin but weakly to avian tuberculin are considered tuberculous and removed from the herd for slaughter. However, the intradermal skin test for paratuberculosis lacks specificity and false positives can result from antigenic cross-reactivity with other species of *Mycobacterium*, and also with *Actinomyces* spp., *Dermatophilus* spp., *Nocardia* spp., *Streptomyces* spp., *Corynebacterium* spp. and certain species of fungi (Chiodini *et al.*, 1984a). In addition, the intradermal skin test cannot distinguish between diseased animals, vaccinates, or others which have successfully cleared the infection and are immune to re-infection. More recently, the correlation between lymphocyte blastogenesis and the production of IFN- γ was used to develop an ELISA designed to detect IFN- γ released by short term whole blood cultures in response to an antigenic stimulus (Rothel *et al.*, 1990; Wood *et al.*, 1991).

This assay was shown to be more sensitive than any of those mentioned above for the diagnosis of tuberculosis in cattle.

1.10.6 Nucleic acid-based detection of *M.a.paratuberculosis*

PCR amplification of specific genomic DNA sequences has been used in the diagnosis of a number of mycobacterial infections, and several target sequences have been exploited for this purpose (Hawkey, 1994). The identification and characterisation of IS900 in *M.a.paratuberculosis* and IS901/902 in certain strains of *M.avium* (see sections 1.5.5.1 and 1.5.5.2), has enabled not only definitive strain differentiation, but a highly specific and sensitive means of detecting the presence of these organisms in pathological specimens by PCR (Vary *et al.*, 1990; Collins *et al.*, 1993a; Challans *et al.*, 1994; Ahrens *et al.*, 1995). A number of studies evaluating the performance of PCR-based diagnosis of *M.paratuberculosis* in clinical specimens have been conducted (van der Giessen *et al.*, 1992b; Sockett *et al.*, 1992b; Whipple *et al.*, 1992; Collins *et al.*, 1993a & 1993b), but a lack of uniformity in sampling and template-DNA extraction techniques precludes any direct comparison. However, PCR-based diagnosis of *M.paratuberculosis* in bovine faeces was found generally to be less sensitive than culture, requiring at least 10^4 organisms/g faeces for positive detection. Conversely, PCR-based detection of *M.a.paratuberculosis* in ovine faeces was found to be more sensitive than culture (Collins *et al.*, 1993b) and could be due to the extremely fastidious nature of some ovine strains which so far have proved impossible to culture in the laboratory. Higher levels of sensitivity and greater reliability have been achieved using tissues obtained from necropsies as opposed to faeces (Stevenson and Sharp, 1997).

In addition to IS900, a second genomic sequence (F57) with *M.a.paratuberculosis* specificity has been cloned and sequenced (Poupart *et al.*, 1993). F57 is 620bp in length, composed of 59% G +C and although an ORF appears to run through the entire fragment, it does not share significant homology with any other sequence currently held in the EMBL database. Labelled RNA transcripts from the F57

fragment were shown to hybridise to genomic DNA from all strains of *M.a.paratuberculosis* tested, but not to the genomes of any other mycobacteria included in the study. Although F57 appears to have diagnostic potential, its use is not widespread and current DNA-based techniques for the detection and identification of *M.a.paratuberculosis* rely on IS900. Very recently however, a multiplex PCR, utilising simultaneous amplification of both IS900 and F57 genomic markers, was applied to the detection of *M.a.paratuberculosis* DNA in Crohn's disease tissue biopsies (Al-Shamali *et al.*, 1997).

1.11 The Association of *M.a.paratuberculosis* With Crohn's Disease in Humans

Crohn's disease is a chronic inflammatory bowel disease, typically of the terminal ileum, but which often affects the entire alimentary canal and extends to involve other tissues such as muscle, bone and skin. The similarities between Crohn's disease and ruminant paratuberculosis were noted by Dalziel (1913) and Crohn *et al.* (1932). Both conditions are diseases of young adults which share similar pathological features and are characterised clinically by intractable diarrhoea and loss of body weight. Crohn's disease lesions are characteristically associated with thickening of the intestinal wall, primarily due to oedema and inflammation of the submucosa, but the lesions are often seen to affect the local lymphatics also. The inflammatory changes often result in fibrosis of the bowel wall leading to intestinal stenosis, and ulceration which often develops to form fistulae (Clarke, 1997).

The precise aetiology of Crohn's disease has yet to be elucidated and a number of potential contributory factors have been suggested (Thompson, 1994). These include host genetic predisposition, environmental factors, immunological influences and microbial factors of which *M.a.paratuberculosis* has been the most commonly implicated.

Occasionally, it has been difficult to detect acid-fast organisms in paratuberculosis lesions, a feature which also is characteristic of Crohn's disease. However, in a proportion of Crohn's cases both electron microscopic examination and prolonged tissue culture of gut biopsies have revealed the presence of cell wall-deficient, non-acid fast spheroplasts (Chiodini *et al.*, 1986; Graham *et al.*, 1987; Gitnick *et al.*, 1989). By way of a corollary, electron microscopic examination of paucibacillary gut lesions in cattle with subclinical paratuberculosis has revealed the presence of similar spheroplastic organisms (Condrón *et al.*, 1994). Also, tuberculoid lesions consistent with paratuberculosis and corresponding anti-mycobacterial immune responses have been observed in sheep in the absence of detectable acid fast bacilli (Clarke and Little, 1996; Perez *et al.*, 1996). PCR amplification of DNA extracted from diseased gut tissue revealed the presence of *M.a.paratuberculosis* DNA sequences and suggested that cell wall-deficient organisms may be involved in the paucibacillary form of paratuberculosis (Clarke and Little, 1996).

The identification of mycobacteria recovered from Crohn's disease patients has been difficult. In combination with the rather imprecise taxonomic divisions within the MAC, *M.a.paratuberculosis* has been incorrectly diagnosed and implicated on a number of occasions (Chiodini, 1989). Early attempts to establish a microbial aetiology for Crohn's disease by experimental inoculation of laboratory animals with material derived from Crohn's disease gut tissue were inconclusive. However, experimental infection of goats with a *M.a.paratuberculosis* strain isolated from a case of Crohn's disease resulted in a disease with pathology consistent with that of ruminant paratuberculosis (Van Kruiningen *et al.*, 1986). Significantly, pathology characteristic of Crohn's disease was reported in a colony of stump-tail macaque monkeys infected with *M.a.paratuberculosis* (McClure *et al.*, 1987). These findings confirm that the organism is capable of causing a chronic inflammatory enteritis in primates and lend support to the possibility that *M.a.paratuberculosis* may play a role in the aetiology of Crohn's disease in humans.

Immunological evidence for an aetiological association of *M.a.paratuberculosis* with Crohn's disease has been inconclusive. Serum antibodies which react with *M.a.paratuberculosis* PPD have been demonstrated in Crohn's disease patients (Thayer, 1984), but the specificity and significance of these remain uncertain given the undefined antigenic composition of PPD and the close relationship between *M.a.paratuberculosis* and ubiquitous environmental mycobacteria. The results of other studies have failed to demonstrate any association between Crohn's disease and antibodies to *M.a.paratuberculosis* (Cho *et al.*, 1986; Tanaka *et al.*, 1991; Brunello *et al.*, 1991). More recently however, antibodies to *M.a.paratuberculosis*-specific epitopes on the carboxyl terminus of a 34kDa protein were reported in 36% of Crohn's disease patients (Vannuffel *et al.*, 1994b).

The identification of species-specific sequences on the genomic insertion sequences IS900 and IS901/902, and the application of PCR has enabled rapid and specific detection of *M.a.paratuberculosis* and *Mycobacterium avium* (wood pigeon strains) genomic DNA in Crohn's disease gut biopsies (Moss *et al.*, 1992b; McFadden *et al.*, 1992). Using this methodology, up to 72% of Crohn's disease patients tested have been shown to harbour *M.a.paratuberculosis* (Moss *et al.*, 1992b; Sanderson *et al.*, 1992; Wall *et al.*, 1993; Dell'Isola *et al.*, 1994; Fidler *et al.*, 1994; Erasmus *et al.*, 1994). However, these findings do not provide unequivocal proof of a causal role for *M.a.paratuberculosis* in Crohn's disease. The results of other studies, which failed to detect *M.a.paratuberculosis* DNA in any of the patients tested, have cast doubt over the association of *M.a.paratuberculosis* with Crohn's disease (Rosenberg *et al.*, 1991; Wu *et al.*, 1991; Dumonceau *et al.*, 1995; Rowbotham *et al.*, 1995; Al-Shamali *et al.*, 1997)). In any case, it remains to be established whether *M.a.paratuberculosis* is of primary aetiological significance in Crohn's disease, or present in lesions merely as a secondary opportunistic invader.

1.12 Identification of Subspecies-Specific Protein Antigens of *M.avium* and *M.a.paratuberculosis*

The search for proteins carrying subspecies-specific epitopes has been driven primarily by the paucity of currently available antigens affording specific and unambiguous immune-based diagnosis of mycobacterial enteritis. Antigenic similarity, even between phylogenetically distant species, is a characteristic feature of the genus *Mycobacterium*, but is exemplified by the members of the MAC which can be up to 98% homologous at the genomic level (McFadden *et al.*, 1987a, 1987c and 1990; Yoshimura and Graham, 1988). That such close genetic relatedness is reflected at the antigenic level is not surprising, and that the host immune response to MAC infection lacks species or subspecies specificity is a natural consequence of phenotypic similarity within the group.

In terms of relative abundance, any potential subspecies-specific antigen may exist as a minor antigenic component and its presence could be masked by an overwhelming number of chemically diverse and possibly more immunogenic cellular antigens. However, monoclonal antibody (mab) technology has provided a means through which the specificity of individual antigens and epitopes can be assessed and compared between species. Efforts to produce mabs against *M.avium* cell wall components have been successful in identifying epitopes on the glycopeptidolipid antigens which confer serotypic identity upon different MAC isolates (Kolk *et al.*, 1989; Rivoire *et al.*, 1989). In addition, a number of mabs raised against mycobacterial cell lysates have highlighted MAC-specific epitopes on two protein antigens of 20kDa and 34kDa, and putative *M.avium*-specific epitopes on a further four proteins of 30, 33, 35 and 36kDa (Rouse *et al.*, 1991). However, as the mabs produced in this study were not screened against *M.a.paratuberculosis*, the claimed specificity of these reagents remains unconfirmed. To date, the only mab with confirmed subspecies-specificity is Avi-3 which recognises an epitope on a 27kDa protein antigen (alkyl hydroperoxidase) of *M.avium* (Abe *et al.*, 1989). Identification of *M.a.paratuberculosis*-specific protein antigens using mab technology has been

attempted as part of a CEC-funded collaboration conducted between the Moredun Research Institute, Edinburgh and the Danish Veterinary Laboratory, Copenhagen. Unfortunately, none of the reagents generated were shown to exhibit specificity for *M.a.paratuberculosis* (Stevenson and Sharp, 1997).

Techniques such as high resolution sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis and Western immunoblotting have been used to identify a number of MAC protein antigens with potential subspecies-specificity (Bech-Nielsen *et al.*, 1985; Valentin-Weigand *et al.*, 1991; Valentin-Weigand and Moriarty, 1992; Deshpande *et al.*, 1992; Elsaghier *et al.*, 1992; Gilot *et al.*, 1992; White *et al.*, 1994). The 34kDa protein of *M.a.paratuberculosis* described by De Kesel *et al.* (1993) represents an example of an antigen carrying subspecies-specific carboxyl-terminal epitopes. The latter has been incorporated into a diagnostic ELISA for the detection of antibodies in *M.a.paratuberculosis* infected cattle (Vannuffel *et al.*, 1994a) and in patients with Crohn's disease (Vannuffel *et al.*, 1994b) (see sections 1.10.4 and 1.11). Additional examples include two *M.avium* proteins of 38.3kDa and 48.8kDa (Gilot and Cocito, 1993), although the subspecies-specificity of these antigens remains to be confirmed. Similarly, the expression products of ORF 1 (p43) (Tizard *et al.*, 1992) and ORF 2 (Hed protein) (Doran *et al.*, 1997) of the *M.a.paratuberculosis*-specific insertion element IS900 have yet to be evaluated for subspecies-specificity and their potential as diagnostic antigens.

More recently, a clone encoding a potentially subspecies-specific protein antigen of 35kDa (p35) was isolated from a *M.a.paratuberculosis* genomic expression library (El-Zaatari *et al.*, 1997). While the DNA fragment containing the p35 gene was observed to hybridise to all members of the MAC tested, sero-reactivity to the protein was confined to animals infected with *M.a.paratuberculosis*. The authors claimed that overall sensitivity and specificity of an immunoblotting assay based on

the p35 was superior to those of commercially available diagnostic tests for paratuberculosis.

1.13 Aim and Topic of This Thesis

The main topic of this thesis is a 40kDa protein antigen (p40), present in IS901/902-positive strains of *M.avium*, which cannot be detected in either *M.a.paratuberculosis* or in strains of *M.avium* which lack IS901/902. The p40 potentially represents the first example of an entire protein molecule carrying subspecies-specificity for a pathogenic member of the MAC. As such, the p40 is a unique protein and merits characterisation and evaluation of its role in the life cycle and pathogenesis of IS901/902 positive *M.avium*. The following chapters describe the chromatographic purification of p40, its evaluation as a subspecies-specific diagnostic antigen and experiments aimed at its molecular characterisation.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Culture of Mycobacteria

2.1.1 *M.avium* type A/I and *M.a.paratuberculosis*

M.avium type A/I (strain JD88/118) and *M.a.paratuberculosis* (strain JD88/107) (Appendix 3) were isolated from commercially farmed red deer (*Cervus elaphus*) (Nyange, 1990) and propagated on solid Stuart's medium (Stuart, 1965) or modified 7H11+ medium (Appendix 4). Cultures were incubated at 37°C for 4-6 weeks for *M.avium* type A/I and 8-10 weeks for *M.a.paratuberculosis*.

2.1.2 *M.avium* type A

M.avium type A (strain T133) (Appendix 3) was provided by Dr. J.J. McFadden, Molecular Biology Group, Dept. of Microbiology, University of Surrey. On receipt the organism was sub-cultured and maintained subsequently on solid normal 7H11 medium (McClatchy (1976) (Appendix 4). Cultures were incubated at 37°C for 6-8 weeks.

2.1.3 MOTT strains

MOTT including *M.fortuitum*, *M.gordonae*, *M.kansasii*, *M.malmoense* and *M.marinum* (Appendix 3) were supplied on slopes of IUT medium (Appendix 4) by the Scottish Mycobacteria Reference Laboratory (SMRL), City Hospital, Edinburgh. Others including *M.avium* (chicken isolate), *M.intracellulare*, *M.scrofulaceum* and *M.smegmatis* (Appendix 3) were purchased from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London. *M.phlei* (Appendix 3) was isolated on modified 7H11+ medium (Appendix 4) at the Moredun Research Institute (MRI), Edinburgh. All of the above organisms were propagated and maintained on solid modified 7H11+ medium at 37°C.

2.1.4 TB-complex

Organisms of the TB-complex were provided by the SMRL. *M.africanum* and *M.tuberculosis* (Appendix 3) were supplied on slopes of IUT medium (Appendix 4), while *M.bovis* (Appendix 3) was supplied on a slope of L-JP medium (Appendix 4). Cultures were propagated and maintained on their respective media at 37°C for 4-6 weeks.

2.2 Disruption of Mycobacterial Cells

2.2.1 By Eaton press

Cells were scraped from the surface of solid growth medium and transferred to a universal bottle. The cells were washed three times in fresh PBS (Appendix 2) by vortexing and centrifugation at 4,800 x g for 10min. Approximately 1g of washed mycobacterial cells was resuspended in 10ml of PBS containing PMSF at 1mM final concentration. The cell suspension was transferred to the top chamber of an Eaton press (Eaton, 1962) pre-chilled to -70°C and allowed 3-4min to freeze. The Eaton press assembly was placed in a floor-standing hydraulic press (Neil Inglis Engineering) and the frozen cells disrupted at 10,000psi. The frozen lysate was thawed and transferred to a universal bottle on ice. The viscous nature of cell lysates prepared by Eaton press was attributable to the presence of high M.wt genomic DNA. The viscosity of the lysate was reduced by 2 x 15sec rounds of sonication at 50% amplitude in a Vibra-cell model VCX 600 sonicator (Sonics & Materials), allowing 1min cooling on ice between each sonication.

2.2.2 By French pressure cell

Approximately 250-300mg of washed mycobacterial cells were resuspended in 3ml of PBS containing PMSF at 1mM final concentration. The cells were transferred to the pressure chamber of a Mini French Pressure-Cell (SLM-Aminco) and disrupted at 1500psi in a floor-standing hydraulic press. The lysate was collected and passed through the pressure cell for a second and third time.

2.2.3 By zirconium bead-beating

Approximately 100mg of washed mycobacterial cells were resuspended in 0.4ml of PBS containing PMSF at 1mM final concentration. The cells were transferred to a screw-cap Eppendorf tube containing 1ml of washed zirconium beads (0.1mm diameter). Cells were disrupted by bead-beating in a Mini Bead-Beater (BioSpec Products) (Hurley *et al.*, 1987). Five beating cycles of 1min each were separated by 1min cooling on ice to avoid overheating and possible denaturation of the antigen preparation.

2.3 Determination of Soluble Protein Concentrations

Soluble protein concentrations were determined using the Micro BCA* Protein Assay Reagent (Pierce-Warriner) in accordance with the manufacturer's recommendations. A set of protein concentrations ranging from 0-20 μ g/ml BSA was prepared in the same diluent used for the unknown samples. Equal volumes (0.5ml) of protein standard and protein assay reagent were mixed in borosilicate glass tubes and incubated at 60°C for 1hr. The spectrophotometer was zeroed against equal volumes of sample diluent and protein assay reagent and the optical densities of standard and unknown protein samples was measured at 562nm. A standard curve prepared by plotting OD⁵⁶² against protein concentration (μ g/ml) was used to read off the protein concentrations of each unknown sample. Samples whose values fell outside the linear working range of the assay (0.5-20 μ g/ml) were re-tested after adjusting the sample dilution accordingly.

2.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis of proteins was carried out using the discontinuous buffer system of Laemmli, (1970). Stacking gels of 3% (^{wt/v}) acrylamide, 0.08% (^{wt/v}) bis-acrylamide in 1 x LSGB (Appendix 2) were layered over resolving gels of 10% (^{wt/v}) acrylamide, 0.26% (^{wt/v}) bis-acrylamide in 1 x LRGB (Appendix 2). Mini gels (80 x 70 x 0.7mm) were prepared in a Mini Protean™ II Dual Slab Cell (Bio-Rad Laboratories) and run at 200V (constant voltage) for 45-

50min in LEB. Large gels (160 x 140 x 0.75mm) were prepared in a Vertical Slab Gel Unit SE600 (Hoefer Scientific Instruments) and run at 20mA (constant current) for 2-3hr in LEB (Appendix 2). For Coomassie blue-stained gels (see section 2.5.1), loadings ranged between 1µg/lane, for a highly purified protein and 20µg/lane for a complex mixture of proteins. However, loadings were reduced ~10-50 fold where silver staining (see section 2.5.2) was used. Protein molecular weight markers spanning size ranges of 12.3-78kDa (cytochrome c 12.3kDa, myoglobin 17.2kDa, carbonic anhydrase 30kDa, ovalbumin 42.7kDa, BSA 66.25kDa and ovotransferrin 76-78kDa) and 42.7-200kDa (ovalbumin 42.7kDa, glutamate dehydrogenase 55.5kDa, ovotransferrin 76-78kDa, phosphorylase b 97.4kDa, β.galactosidase 116.3kDa and myosin 200kDa) (Merck) were used.

2.5 Post-Electrophoresis Staining of Proteins Resolved in SDS-PAGE Gels

2.5.1 By Coomassie brilliant blue staining

Gels were incubated in Coomassie Blue stain (Appendix 2) for 30min with gentle agitation on an orbital shaker. Background staining was eliminated by incubating the stained gel in Coomassie destaining solution (Appendix 2) until the desired level of destaining was attained. The destaining process was assisted by placing a small wick of tissue paper at one end of the destaining dish to absorb excess stain.

2.5.2 By silver staining

Gels were silver-stained using the method described by Morrisey (1981) with minor modifications. Polyacrylamide gels were incubated for 20min in each of the three following solutions sequentially: 1. 50% (v/v) methanol, 10% (v/v) glacial acetic acid; 2. 5% (v/v) methanol, 7% (v/v) glacial acetic acid; 3. 10% (v/v) glutaraldehyde. Gels were then washed in 8-10 x 500ml changes of DW over a period of 3-4hr or soaked overnight in a large volume (2-3L of DW). Washed gels were then incubated sequentially for 20min in each of the following two freshly made solutions: 1. 5µg/ml DTT; 2. 6mM silver nitrate. Silver-equilibrated gels were rinsed briefly in

DW and then twice in freshly made developer solution (0.28M sodium carbonate containing 0.05% (v/v) formaldehyde 37-42% solution). Gels were then incubated in developer solution until the desired level of staining was attained. The colour development reaction was stopped by the addition of 2.3M citric acid (5ml/100ml of developer solution) and continuing agitation for a further 20min. Typically, 200ml (large gels) or 50ml (mini-gels) of each solution was used at each stage of the staining procedure. All stages of the procedure were carried out with gentle agitation on an orbital shaker.

2.6 Preservation of Stained SDS-PAGE Gels

Stained gels were soaked in 4% (v/v) glycerol for 30-40min prior to drying on to 3MM filter paper (Whatman Scientific) in a slab gel dryer (Hoefer Scientific Instruments).

2.7 Western Transfer and “Immunoblotting”

2.7.1 Western transfer

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose membranes was carried out in accordance with the method of Herring and Sharp (1984). The procedure was based on the method previously described by Burnette (1981) using TGBB (Appendix 2). Proteins resolved in mini gels were transferred in a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad Laboratories) at 100V/250mA (constant voltage) for 1hr. Proteins resolved in large gels were transferred in a Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad Laboratories) at 100V/1.0A (constant voltage) for 1hr, or 15V/100mA (constant voltage) for 16hr.

2.7.2 Immunoblotting

Immunoblotting was carried using a modification of the method of Herring and Sharp (1984). Membranes were blocked by washing in WFB (Appendix 2) in the absence of any extraneous protein e.g. BSA or horse serum. The blotted proteins were probed in appropriate dilutions of primary antibody in WFB for 1hr at RT.

Excess primary antibody was removed by 3 x 5min washes in WFB. Membranes were then incubated in secondary antibody (HRP-conjugated anti species IgG) (Dako) diluted $1/1000$ in WFB for 1hr at RT. Excess secondary antibody was removed by a further 3 x 5min washes in WFB. Proteins recognised by the primary antibody were visualised by incubating the filters in a substrate solution containing 100mM Tris/HCl pH 7.5, 1mM DAB and 60mM H₂O₂. The colour reaction was stopped by washing the membrane thoroughly in running tap water.

2.8 Visualisation of Nitrocellulose-Bound Proteins

2.8.1 By Ponceau S staining

Freshly transferred proteins were stained reversibly by incubating the membrane in Ponceau S solution (Sigma-Aldrich) for 5min. Excess stain was removed by rinsing briefly in DW. The positions of M.wt marker proteins were marked with a black ball-point pen and the membrane was processed as described in section 2.7.2.

2.8.2 By Coomassie brilliant blue staining

Freshly transferred proteins were stained irreversibly by incubating the membrane in Coomassie Brilliant Blue stain (Appendix 2) diluted $1/25$ in blot destain solution (30% (v/v) methanol, 5% (v/v) glacial acetic acid) for 1-2hr. Background staining was reduced by soaking the membrane in Coomassie destain solution as described in section 2.5.1.

2.9 Immuno-Dot Blotting

Protein dot-blots were prepared in a Bio-Dot 96 well Microfiltration Apparatus (Bio-Rad Laboratories). A sheet of nitrocellulose membrane (Schleicher and Schuell) was cut to the size of the microfiltration unit and wetted in 1 x PBS (Appendix 2) prior to assembling the apparatus. Approximately 0.5-1.0 μ g of highly purified protein, or up to 10 μ g of a complex mixture of proteins, was applied to the membrane in 100 μ l of 1 x PBS. Samples were drawn on to the membrane under gentle vacuum and washed

with a further 200µl of 1 x PBS. The membrane was removed from the apparatus and processed as described in section 2.7.2.

2.10 Confirmation of Strain Identity by Polymerase Chain Reaction

2.10.1 Detection of IS900 in *M.a.paratuberculosis*

Cultured mycobacterial cells were mechanically disrupted by bead beating (see section 2.2.3). Lysates were centrifuged at 13,000 x g for 5min and the supernatants transferred to fresh tubes. 5µl of lysate was added to pre-prepared reactions mixtures of 50µl (final volume) containing 67mM Tris/HCl pH8.8, 3mM MgCl₂, 200µg/ml gelatin, 25mM each of dATP, dCTP, dGTP and dTTP, 20pmol of primers (forward primer 5'-GTT CGG GGC CGT CGC TTA GG-3') (positions 22-41), (reverse primer 5'-CCC ACG TGA CCT CGC CTC CA-3') (position 410-391), and 1 unit of *Taq* polymerase (Boehringer Mannheim). The reactions were covered with 100µl of mineral oil (Sigma) to prevent evaporation. PCR conditions consisted of an initial denaturation step of 5min at 94°C and 30 cycles of annealing at 55°C for 30sec, extension at 72°C for 60sec and denaturation at 94°C for 30sec. The final cycle included an extension at 72°C for 120sec. Amplification products were analysed by agarose gel electrophoresis, and a single band of 389bp confirmed species identity as *M.a.paratuberculosis*.

2.10.2 Detection of IS901/902 in *M.avium* type A/I

Template DNA was prepared from cultured cells as described in section 2.10.1. 5µl of lysate was added to pre-prepared reactions mixtures of 50µl (final volume) containing 67mM Tris/HCl pH8.8, 3.7mM MgCl₂, 200µg/ml gelatin, 25mM each of dATP, dCTP, dGTP and dTTP, 20pmol of primers (forward primer 5'-CTG ATT GAG ATC TGA CGC-3') (positions 132-149), (reverse primer 5'-TTA GCA ATC CGG CGC CCT-3') (position 383-365), and 1 unit of *Taq* polymerase (Boehringer Mannheim). PCR conditions were as described for amplification of IS900 (see section 2.10.1). Amplification of a single band of 252bp confirmed species identity as IS901/902-positive *M.avium*.

CHAPTER 3

IDENTIFICATION AND PURIFICATION OF A 40kDa PROTEIN OF *Mycobacterium avium*

3.1 Introduction

During a study on the prevalence and pathogenesis of *M.a.paratuberculosis* in red deer, it was observed that some of the strains isolated from clinical cases differed at the polypeptide level by the presence of a prominent band of ~40kDa as visualised in polyacrylamide gels (Fig. 3.1) (Nyange, 1990). Like *M.a.paratuberculosis*, the organisms isolated from these animals were characteristically slow growing and exhibited mycobactin dependence on primary isolation. However, unlike *M.a.paratuberculosis* they could be adapted to propagate on mycobactin-free growth medium and required only 4-6 weeks to reach confluence, as opposed to the 8-12 weeks required by *M.a.paratuberculosis*. Experimental infection of deer calves with these organisms produced a disease clinically and pathologically similar to paratuberculosis (Nyange, 1990). However, the disease was observed to be one of greater severity, following a more rapid progression with the infection disseminating to involve organs other than the intestine. In spite of obvious similarities between the two organisms, genomic DNA extracted from these mycobacteria was not recognised in a PCR based assay designed to amplify a 389bp (see section 2.10.1) *M.a.paratuberculosis*-specific region of the genomic insertion element IS900. The isolates were identified subsequently as strains of *M.avium* type A/I (Stevenson, unpublished observation).

The presence of the p40 antigen in strains of *M.avium* type A/I represented a distinct difference between two pathogenic mycobacteria, which share a high degree of genotypic and phenotypic similarity (Inglis *et al.*, 1994a). At the outset of this study, immune based differentiation of *M.a.paratuberculosis* and *M.avium* had not been

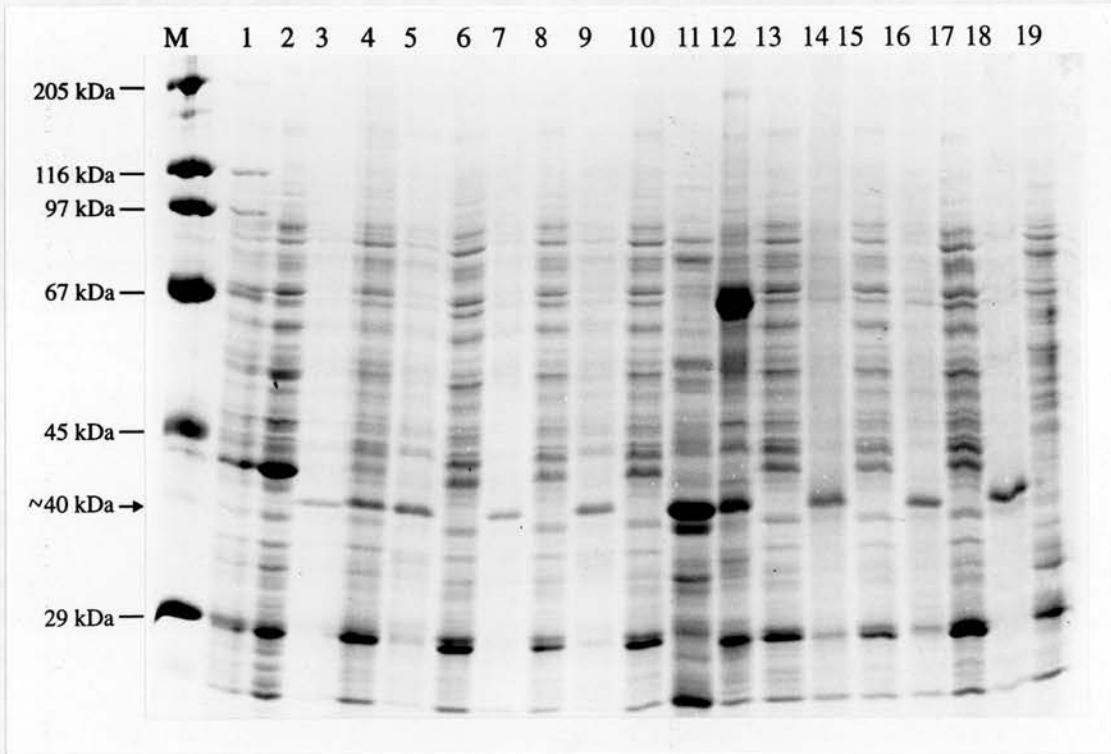


Fig. 3.1 Coomassie blue stained polyacrylamide slab gel showing the polypeptide profiles of nineteen isolates of *M.a.paratuberculosis* and *M.avium*. Lanes 1,2,6,8,10,13,15,17 and 19 contain isolates of *M.a.paratuberculosis* as confirmed by mycobactin dependence and the presence of IS900. Lanes 3,4,5,7,9,11,12,14,16 and 18 contain isolates of IS901/902-positive *M.avium* as confirmed by PCR. The lane marked M contains M.wt markers. Note the presence of a prominent band of ~40kDa in the *M.avium* isolates which is absent in *M.a.paratuberculosis*. The author is indebted to Dr. John F.C. Nyange for providing all the material shown in this illustration.

achieved and the p40 antigen was identified as one which might potentially form the basis of an assay to distinguish between the two.

As an initial objective, the extraction and purification of p40 from *M. avium* type A/I cell lysates was of fundamental importance to the work presented in subsequent chapters. The work presented in this chapter describes the optimisation of a serial chromatographic procedure to enable the production of milligram quantities of high purity p40.

3.2 Materials and Methods

Methods and protocols used for *in vitro* culture of mycobacterial strains, mechanical disruption of mycobacterial cells, determination of soluble protein concentration, SDS-PAGE, western transfer and “immunoblotting” are described fully in sections 2.1-2.4, 2.7.1 and 2.7.2 respectively of Chapter 2.

3.2.1 Ammonium sulphate precipitation of soluble proteins

Intact cells and insoluble material were removed from *M. avium* type A/I cell lysates by centrifugation at 100,000 x *g* for 60 min at 4°C. Soluble proteins in the resulting supernatant were precipitated in a stepwise manner using ammonium sulphate [(NH₄)₂ SO₄] in accordance with the method of Scopes (1984). Precipitated proteins in fractions selected for further study were redissolved in 1ml of PBS and passed through a 0.22µm membrane filter to remove insoluble material and particulate matter.

3.2.2 Purification of p40 by high performance liquid chromatography (HPLC)

All chromatographic separation steps were performed on a System Gold HPLC Apparatus (Beckman Instruments).

3.2.2.1 Size exclusion HPLC

Size exclusion HPLC was performed using a TSK-G3000-SW (8 x 300mm) silica gel column pre-equilibrated with PBS (Appendix 2). Five individual injections of 200µl were applied to the column and proteins were resolved over a period of 40 min at a flow rate of 0.5ml/min. The proteins, which eluted from the column, were monitored spectrophotometrically at a wavelength of 280nm. Fractions of 0.5ml were collected and examined for the presence of p40 by SDS-PAGE and silver-staining. Fractions containing p40 were pooled (2ml x 5 runs = 10ml total) and dialysed against 1000ml of BTN buffer (Appendix 2) for 16hr at 4°C.

3.2.2.2 Anion exchange HPLC

Anion exchange HPLC was performed using a TSK-DEAE-5PW (8 x 75mm) column pre-equilibrated with BTN buffer. Following dialysis the entire sample was applied to the column in a series of 1ml injections. Sufficient time was allowed after the final injection for non-absorbed material to pass through the column and for the baseline to stabilise. Absorbed proteins were eluted from the anion exchange matrix over a period of 60 min by the application of a stepped NaCl gradient at a flow rate of 0.5ml/min. NaCl concentration was increased linearly from 0.15M to 0.3M between 4 min and 14min, 0.3M to 0.5M between 19 min and 49 min and 0.5M to 1.5M between 49 min and 54 min. Fractions of 0.5ml were collected and examined for the presence of p40 by SDS-PAGE and silver-staining.

3.2.2.3 Hydrophobic interaction HPLC

Hydrophobic interaction HPLC was performed using a TSK-phenyl-5PW (8 x 75mm) hydrophobic interaction column, pre-equilibrated with BTA buffer (Appendix 2). Anion exchange fractions containing p40 were diluted ten fold in BTA buffer and applied to the column in a series of 1ml injections. Sufficient time was allowed after the final injection for non-absorbed material to pass through the column and for the baseline to stabilise. Absorbed proteins were eluted from the column matrix over a period of 30 min by the application of a linear $(\text{NH}_4)_2 \text{SO}_4$ gradient, decreasing in concentration from 1.5M to 0.0M, at a flow rate of 0.5ml/min. The quality of fractions containing p40 was assessed visually by SDS-PAGE and silver-staining.

3.2.3 Chromatographic purification of a 30kDa (p30) antigen

Size exclusion HPLC was performed as described for p40 and the resulting fractions were examined for the presence of p30 by SDS-PAGE and silver staining. Fractions containing p30 (~24-25 min) were collected and pooled. Following overnight dialysis against BT buffer (Appendix 2), p30 was purified further by anion exchange HPLC. The column was equilibrated with BT buffer and the sample applied in a

series of 1ml injections. Bound proteins were eluted over a period of 30 min by the application of a linear NaCl gradient increasing in concentration from 0.0M to 0.5M. Resulting fractions were examined for the presence of pure p30 by SDS-PAGE and silver-staining.

3.2.4 Production of immune reagents

Both polyclonal and monoclonal immune reagents were raised against HPLC purified p40 antigen:-

(i) Generation of anti p40 polyclonal antibodies in rabbits

Each of two 10 week old NZ/Lop cross female rabbits were inoculated intramuscularly with 50µg of purified p40 emulsified in incomplete Freund's adjuvant (IFA). Primary inoculations were followed by two further boosting doses of 20µg, 2 and 6 weeks later. Seroconversion in response to the p40 antigen was demonstrated by reacting a $1/80$ dilution of pre- and post-immunisation rabbit serum against western blotted *M.avium* type A/I cell lysate.

(ii) Generation of anti p40 monoclonal antibodies

As part of a Commission of the European Communities (CEC) funded collaboration, highly purified p40 was sent to the Danish Veterinary Laboratory, Copenhagen, Denmark, where the protein was used to produce eleven Mabs (Ahrens *et al.*, 1995; Klausen *et al.*, 1997). Briefly, eight to ten week old BALB/c mice were inoculated intraperitoneally on three occasions, at two week intervals, with p40 purified from *M.avium* type A/I (strain JD88/118). The mice were boosted three days before fusion of their spleen cells with the myeloma cell line P3 X63 Ag.8.6.5.3 using standard procedures (Goding, 1983). Hybridoma culture supernates were screened by ELISA in microtitre plates coated with p40.

3.3 Results

The various techniques and procedures used during the serial chromatographic purification of p40 and p30 are summarised schematically in Fig. 3.2.

3.3.1 Salt Precipitation of Proteins in Mycobacterial Cell Lysates

Although most of the proteins resulting from stepwise ammonium sulphate precipitation could be re-solubilised in aqueous buffer, some material remained insoluble, presumably resulting from irreversible denaturation of protein tertiary structure. However, SDS-PAGE analysis of the soluble and insoluble fractions of the precipitates revealed a major band of 40kDa in the soluble 60% ammonium sulphate fraction (Fig. 3.3). Some p40 was present in the soluble 40% and 80% ammonium sulphate fractions also, but insufficient quantities of pure protein were recovered from these to justify the lengthy purification procedure. Therefore, efforts towards obtaining usable quantities of p40 focused upon the soluble 60% fraction which contained the majority of the protein. The p40 in the soluble 60% ammonium sulphate fraction redissolved readily in PBS facilitating subsequent chromatographic procedures. No p40 was detected in any of the insoluble fractions.

3.3.2 Size Exclusion HPLC of Salt-Precipitated p40

Size exclusion chromatography of proteins recovered from the soluble 60% ammonium sulphate fraction (see Fig. 3.3) revealed a series of eight peaks between 10.13 and 24.3 min, with a major peak at 17.67 min (Fig. 3.4). Subsequent analysis of the resulting fractions by SDS-PAGE and silver staining (Fig. 3.5) revealed that the major peak contained the p40, most of which eluted from the silica gel column between 17 and 18 min. Smaller quantities of p40 could be seen in the fractions corresponding to elution times of 19-21 min, but these were not used for further purification. The p40 which eluted between 17 and 18 min was collected, pooled and dialysed against BTN buffer (Appendix 2) prior to anion exchange HPLC.

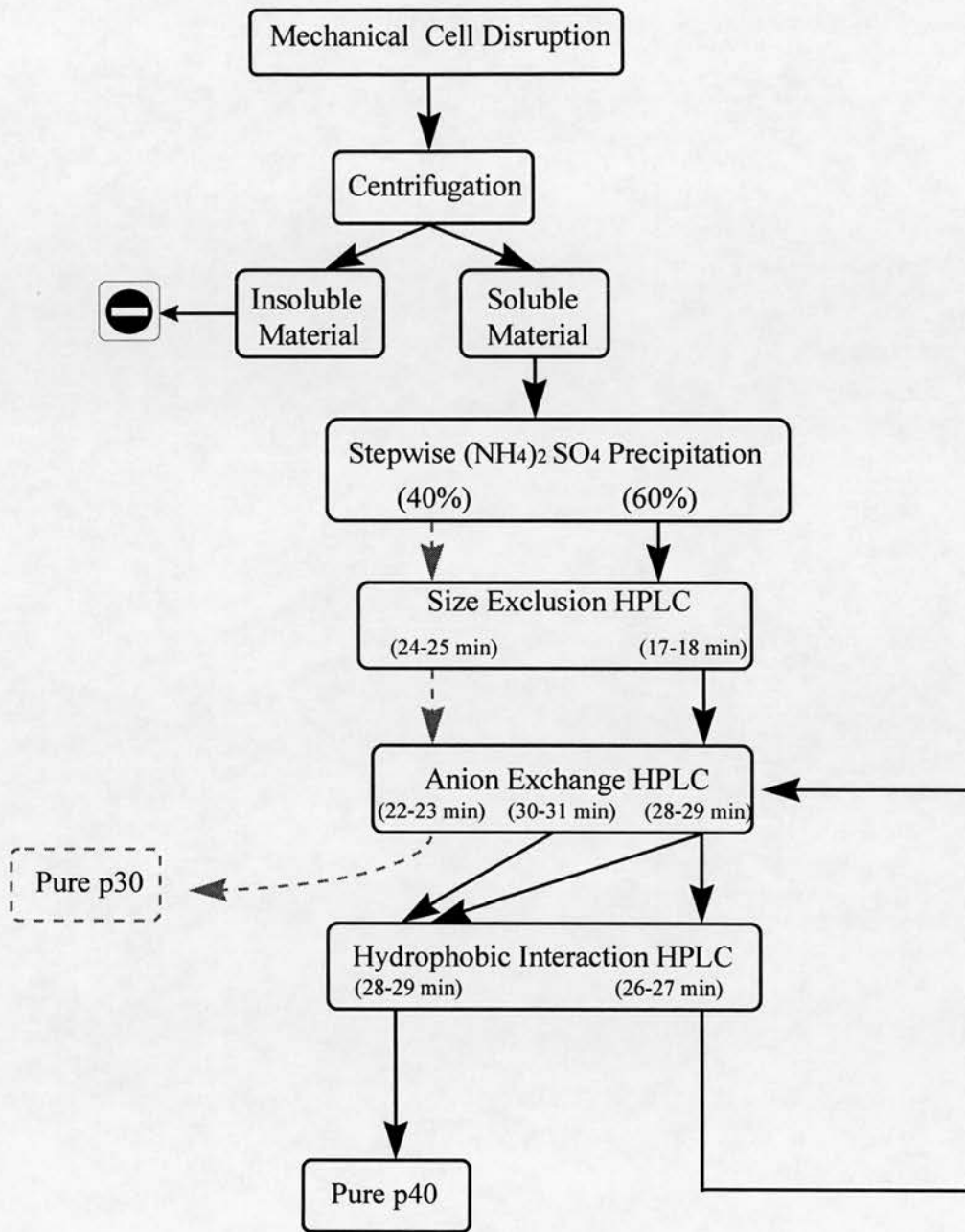


Fig 3.2 Schematic representation of procedures and serial chromatographic separations leading to the purification of p30 and p40 from mycobacterial cell lysates.

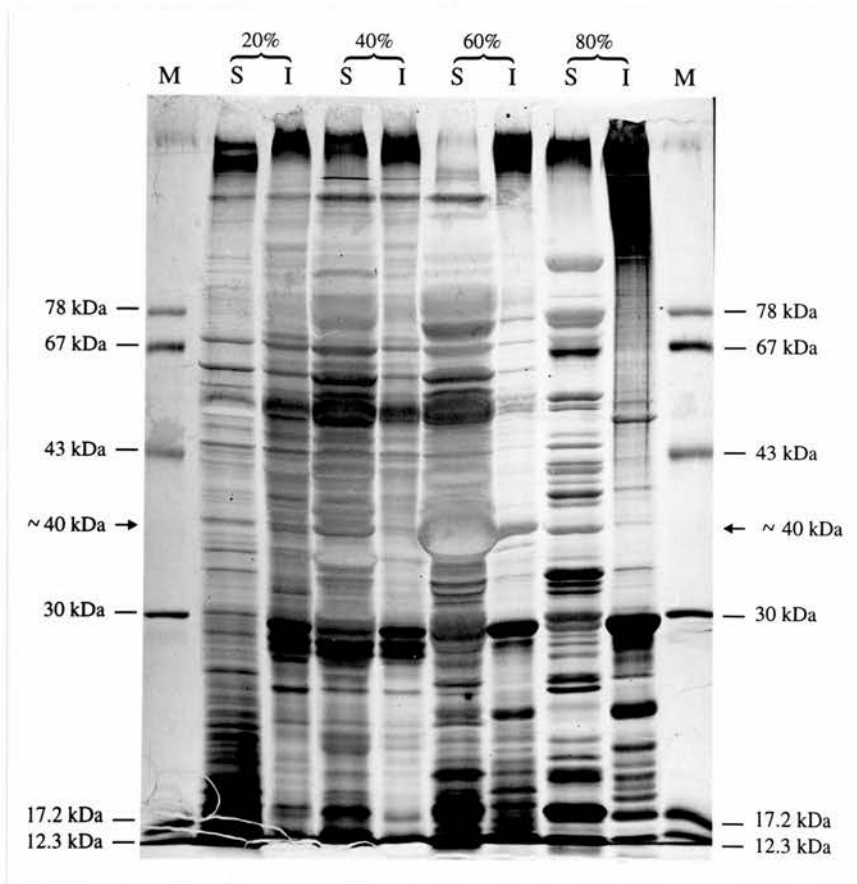


Fig. 3.3 Silver stained SDS-PAGE gel showing proteins which were precipitated out of the soluble phase of *M.avium* type A/I cell lysates at 20%, 40%, 60% and 80% saturation with ammonium sulphate. Lanes are in pairs showing the material which was re-solubilised in buffer after salt precipitation (S) and that which was rendered insoluble and lost (I). Lanes marked M contain M.wt markers. Note that the majority of the p40 is contained in the soluble 60% ammonium sulphate fraction.

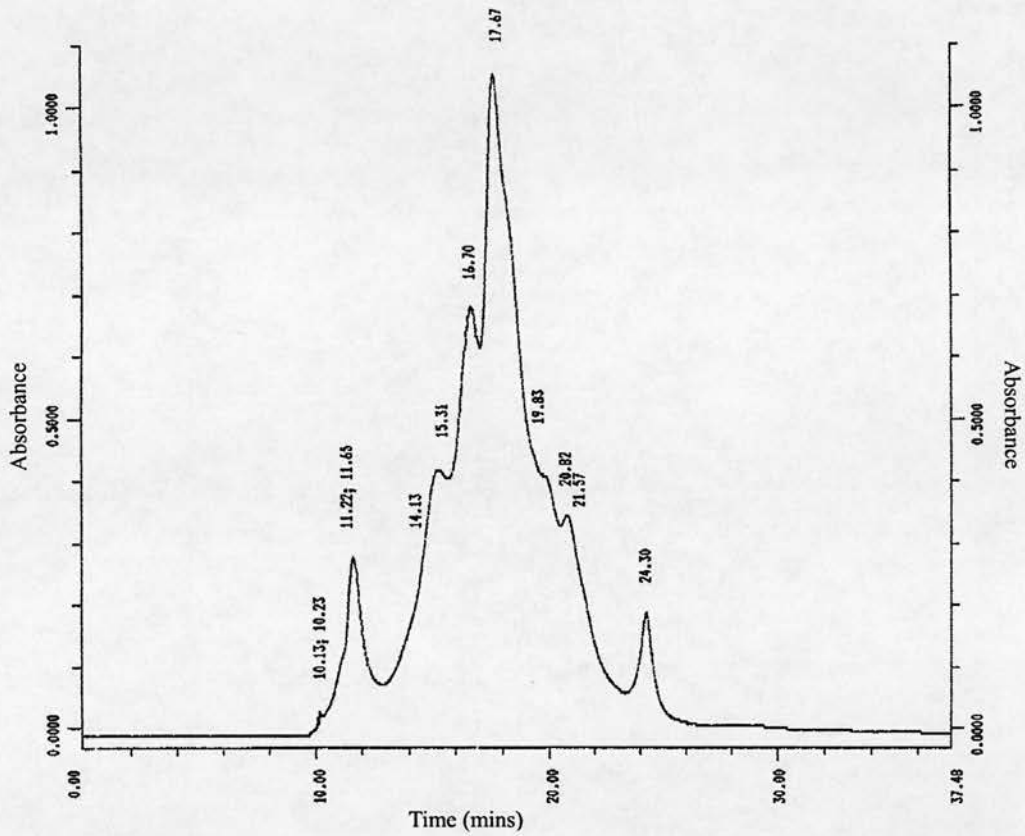


Fig. 3.4 Chromatogram resulting from size exclusion HPLC of proteins contained in the soluble 60% ammonium sulphate fraction (see Fig. 3.3). The peak of U.V absorbing material at 17.67 min was shown to contain p40 (see Fig. 3.5). The smaller peak at 24.3 min, which coincides with the column V_t (see Fig. 3.6), contained a protein which was shown subsequently to be a member of the “antigen 85 complex” of mycobacterial secretory antigens (see main text).

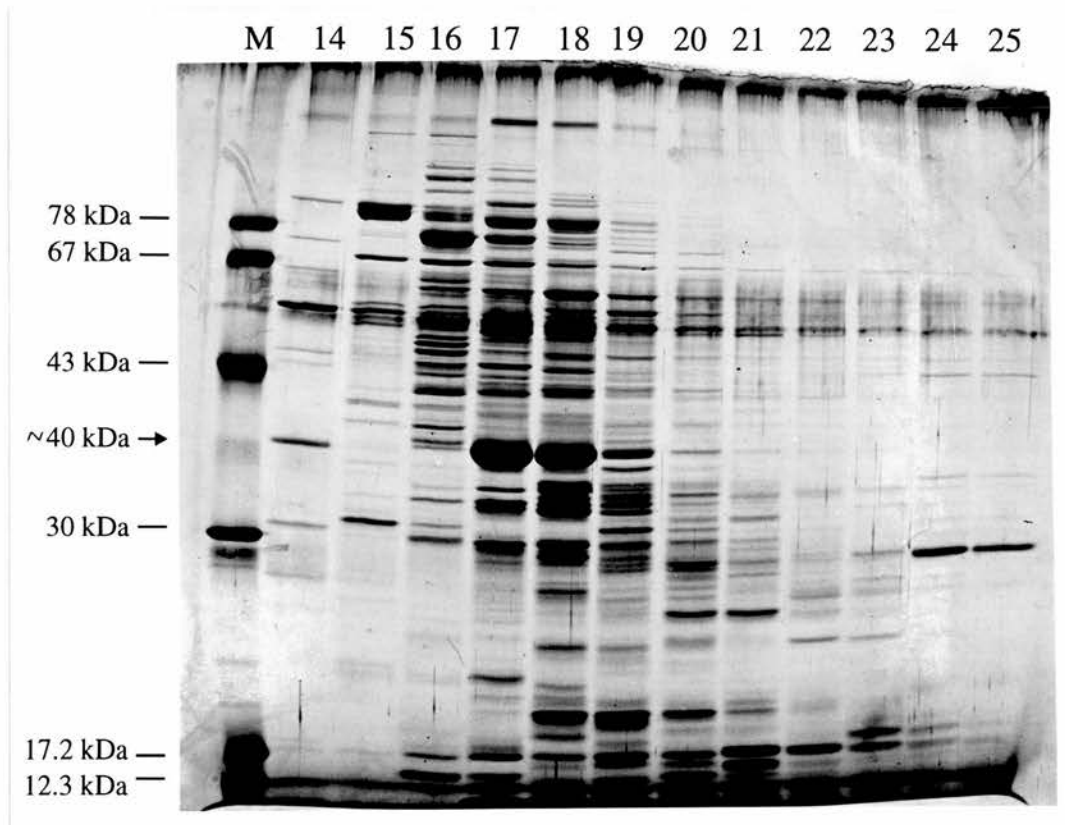


Fig. 3.5 Silver stained SDS-PAGE gel showing proteins contained in the soluble 60% ammonium sulphate fraction as resolved by size exclusion HPLC. Lanes marked 14-25 correspond to retention times (min). The lane marked M contains M.wt markers. Note that the majority of the p40 eluted from the size exclusion column between 17 and 18 min and that a protein of ~30kDa eluted at a time coinciding with the column V_t (see Fig. 3.6).

3.3.3 Anion Exchange HPLC of Size Exclusion Fractions

Anion exchange chromatography of the proteins that eluted from the size exclusion column at 17-18 min (see Fig. 3.5) revealed approximately 20 peaks corresponding to NaCl concentrations ranging between ~0.3M-1.5M NaCl (Fig. 3.7). Analysis of the resulting fractions by SDS-PAGE and silver staining revealed that a dominant peak between 0.35M-0.39M NaCl (Fig. 3.7) and which corresponded to an elution time of 28-31 min, consisted largely of p40 (Fig. 3.8). The p40 collected between 30 and 31 min was in a higher state of purity than the majority of the protein which eluted between 28 and 29 min. Separate pools of p40, corresponding to 28-29 min and 30-31 min, were prepared and stored individually for the purpose of further purification by hydrophobic interaction chromatography.

3.3.4 Hydrophobic Interaction HPLC of Anion Exchange Fractions

Hydrophobic interaction chromatography (HIC) of pools of anion exchange-purified p40 revealed a single peak (Fig. 3.9), regardless of whether the 28-29 min, or 30-31 min anion exchange pool (see above) was applied to the column. Subsequent SDS-PAGE analysis of fractions resulting from HIC purification of the 28-29 min anion exchange pool, revealed that p40 eluted from the column between 26 and 29 min at a salt concentration of ~0.37-0.32M ammonium sulphate (Fig. 3.10). The 26 and 27 min HIC fractions contained the majority of the p40, but were observed to contain some additional protein components ranging between ~12-38kDa. The p40 which eluted at 28-29 min appeared to be more highly purified. The p40 fractions which contained extraneous proteins were re-dialysed against BTA to remove ammonium sulphate and both anion exchange and HIC separations were repeated as described above. Repeating the separations in this way increased the total yield of highly purified p40 (Fig. 3.11), which was collected and stored as HIC fractions at 4°C. Up to 1mg of p40 could be purified from 1g (wet weight) of *M.avium* type A/I cells. Ammonium sulphate was removed by dialysis immediately prior to use. This pure material was used for all subsequent procedures and experiments.

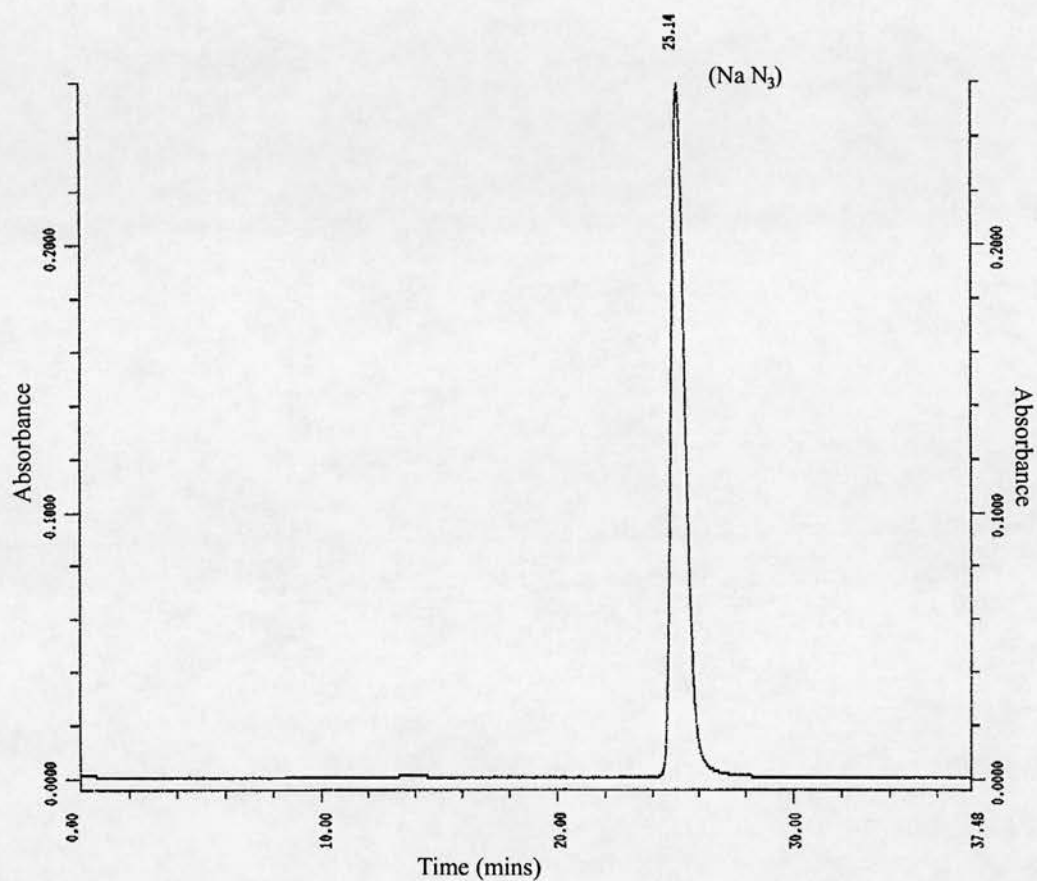


Fig. 3.6 Chromatogram illustrating the V_t of the size exclusion column used for the serial chromatographic purification of p40. A 200 μ l sample of a 0.9% sodium azide solution was injected onto the column and run under the same conditions used to resolve mycobacterial proteins. The peak of U.V absorbing material at 25.14 min marks the point at which sodium azide eluted from the column. At a flow rate of 0.5ml/min, the column V_t was calculated at 12.57ml.

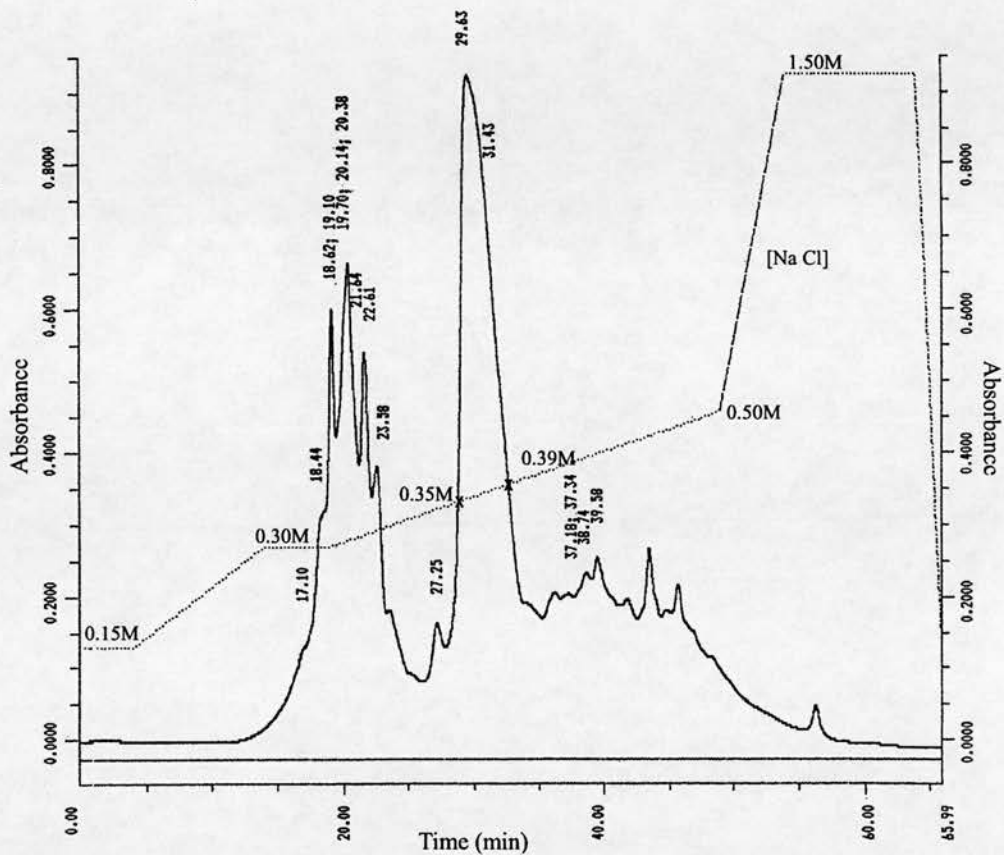


Fig. 3.7 Chromatogram resulting from anion exchange HPLC of proteins which had retention times of between 17 and 18 min on the size exclusion column (see Fig. 3.5). A large peak at 29.63 min, which represents material which eluted from the column between 0.35M and 0.39M NaCl (marked by X-X), was shown subsequently to contain p40 (see Fig. 3.8).

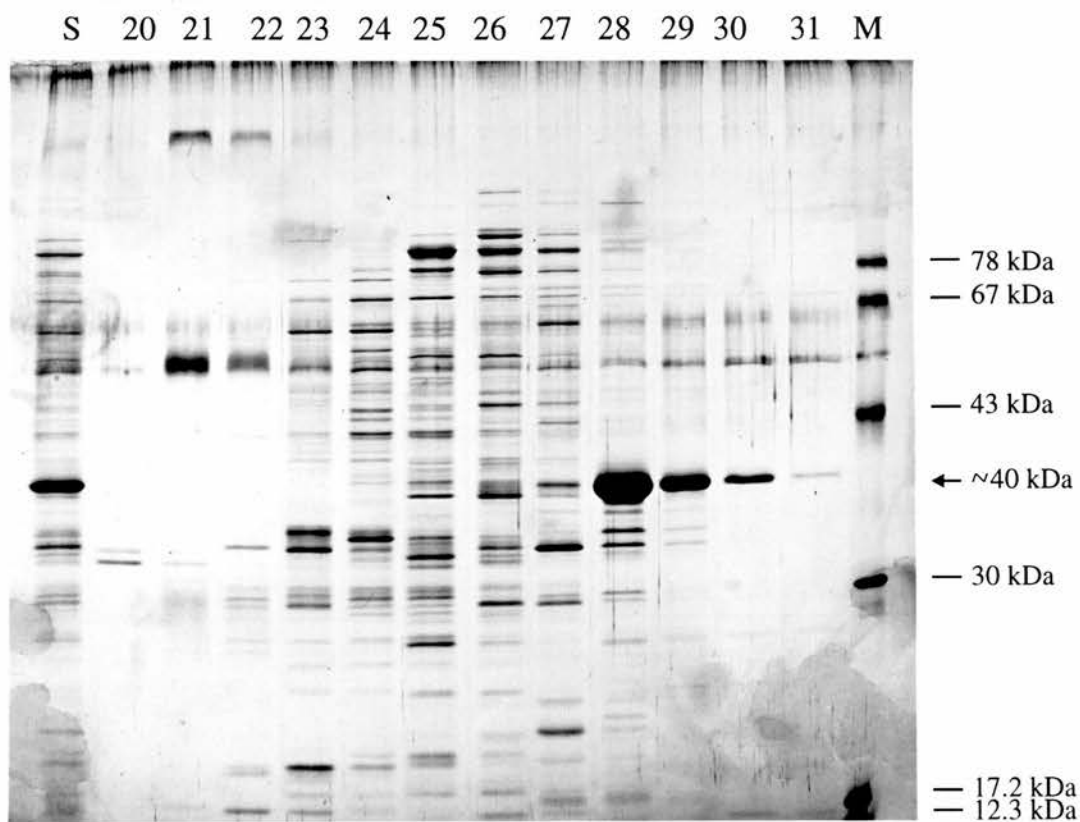


Fig. 3.8 Silver stained SDS-PAGE gel showing fractions resulting from anion exchange HPLC of proteins which had retention times of between 17 and 18 min on the size exclusion column (see Fig. 3.5). The lane marked S contains a sample of the pooled starting material. Lanes marked 20-31 correspond to elution times (min) and the lane marked M contains M.wt markers. The p40 component eluted between 0.35M and 0.39M NaCl (see Fig. 3.7) with the majority of the protein being collected between 28 and 29 min. A smaller quantity of high purity p40 was collected between 30 and 31 min.

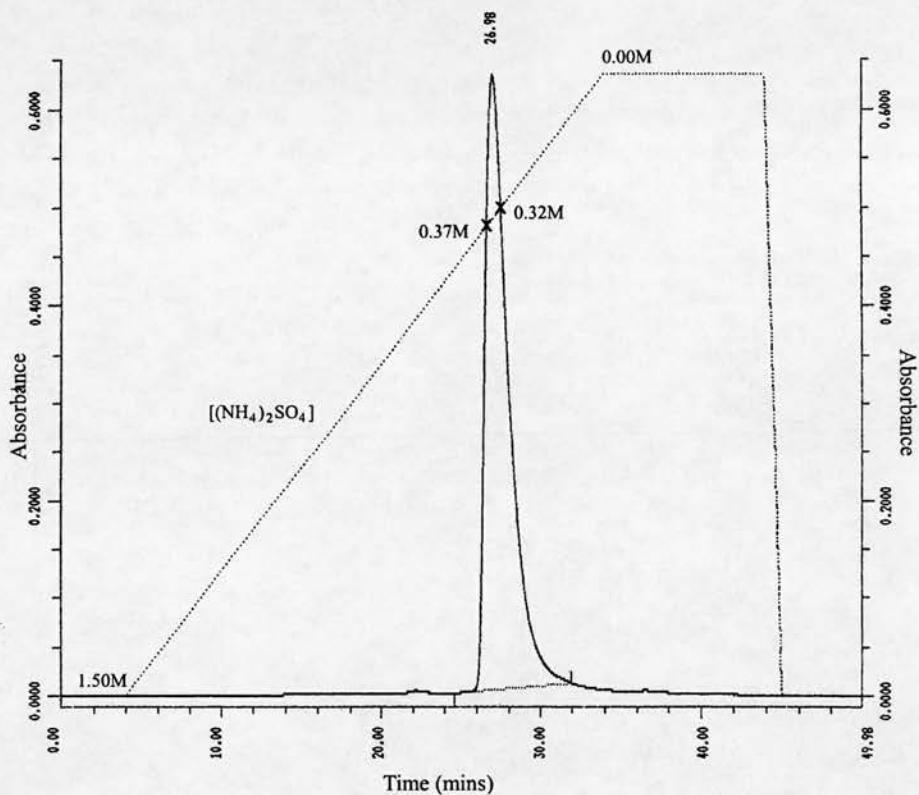


Fig. 3.9 Chromatogram resulting from hydrophobic interaction HPLC of proteins which eluted from the anion exchange column between 0.35M and 0.39M NaCl (see Figs. 3.7 and 3.8). The p40, which was the most abundant component, eluted from the column between 0.37M and 0.32M ammonium sulphate (marked by X-X) and is represented by a single peak of U.V absorbing material at 26.98 min.

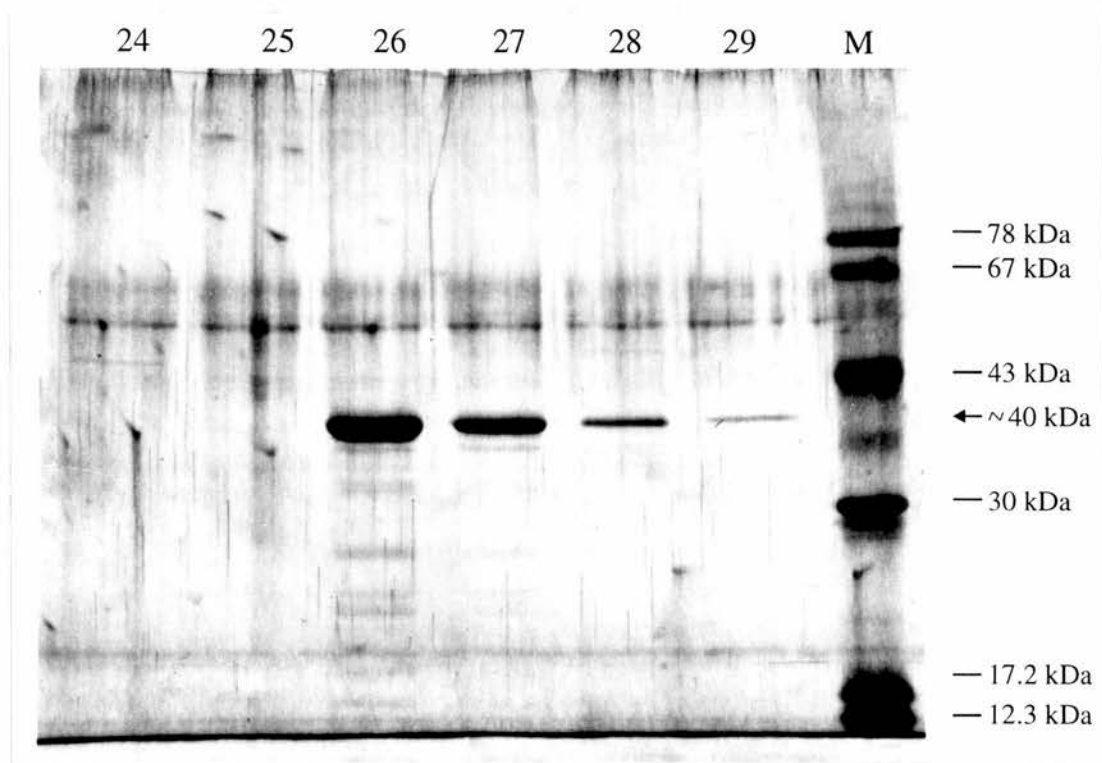


Fig. 3.10 Silver stained SDS-PAGE gel showing fractions resulting from hydrophobic interaction HPLC of proteins which eluted from the anion exchange column between 0.35M and 0.39M NaCl (see Figs. 3.7 and 3.8). Lanes marked 24-29 correspond to elution times (min). The lane marked M contains molecular weight markers. p40 eluted from the column between 0.37M and 0.32M ammonium sulphate (see Fig. 3.9). The majority of the p40 was collected between 26 and 27 min whilst a smaller quantity of highly purified material was collected between 28 and 29 min. Further anion exchange and hydrophobic interaction separations increased the total yield of pure p40 (see Fig. 3.11).

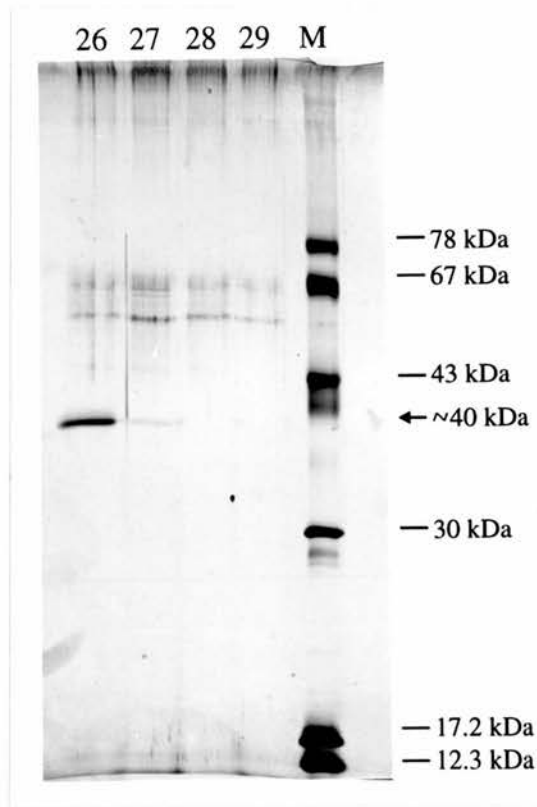


Fig. 3.11 Silver stained SDS-PAGE gel showing highly purified p40. Material collected from hydrophobic interaction HPLC between 26 and 27 min (see Fig. 3.10) was further purified by additional anion exchange and hydrophobic interaction HPLC separations. Lanes marked 26-29 correspond to elution times (min) from the hydrophobic interaction column. The lane marked M contains M.wt markers. This highly purified p40 was used for subsequent experiments and analyses. NB The bands visible in lanes 26-29 at ~55-67kDa are artefactual and originate from the 2- β -mercaptoethanol used in the sample denaturing buffer (Tasheva and Dessev, 1983).

3.3.5 Simultaneous purification of p30

During the serial chromatographic purification of p40, it was observed that a peak of UV absorbing material eluted from the size exclusion column at a time which coincided with the column V_t (24-25 min) (Figs. 3.4 and 3.6). Analysis of these fractions by SDS-PAGE and silver staining revealed the presence of p30 in a relatively pure form (Fig. 3.5, 23-25 min). The delayed elution of a 30kDa molecule was unexpected and contrasted with the migration rates of standard proteins ovalbumen (42.7kDa) and chymotrypsinogen (25kDa), whose respective elution times were recorded at 19.22 min and 22.09 min (Fig. 3.16). The p30 was collected for further purification and analysis. Retrospective analysis of the other ammonium sulphate fractions showed that larger quantities of the protein could be obtained from the soluble 40% fraction than the 60% fraction. Further purification of p30 therefore utilised this soluble 40% fraction as starting material. Anion exchange HPLC yielded a peak of material at 0.15M NaCl (22-23 min) which contained pure p30 (data not shown). Up to 150 μ g of p30 could be purified from 1g (wet weight) of either *M.avium* type A/I or *M.a.paratuberculosis* cells. P30 was stored at -20°C for future use (see Chapter 4). Analysis of the first ten amino-terminal amino acid residues by Edman degradation yielded the following sequence:-

| | | | | | | | | | |
|-----|-----|---------|-----|-----|-----|-----|-----|-----|-----|
| F | S | P/R | P | G | L | P | V | E | Y |
| Phe | Ser | Pro/Arg | Pro | Gly | Leu | Pro | Val | Glu | Tyr |

3.3.6 Production of Polyclonal Antibodies in Rabbits

Pre-bleeds taken from the rabbits immunised with HPLC purified p40 demonstrated that neither animal had had any previous immunological experience of *M.avium*. Post-immunisation bleeds taken after a second boosting dose reacted strongly with the p40 antigen and weakly with a number of other protein bands on western blots of *M.avium* cell lysates (Fig. 3.12).

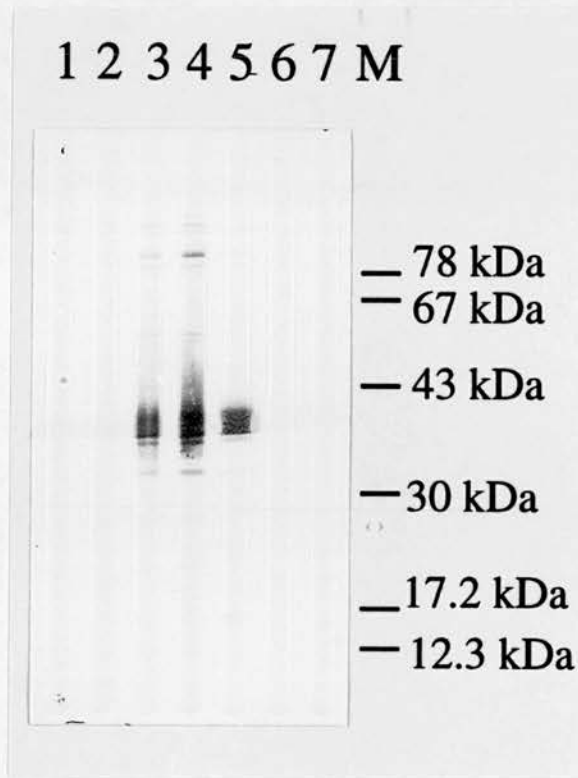


Fig. 3.12 Strips of Western blotted *M.avium* type A/I cell lysate probed with polyclonal and monoclonal antibodies raised against HPLC purified p40. Lane 1., rabbit R692 pre-bleed; lane 2., rabbit R696 pre-bleed; lane 3., rabbit R692 6wk post-immunisation bleed; lane 4., rabbit R696 6wk post-immunisation bleed; lane 5., Mab 6.66; lane 6., donkey anti rabbit-HRP conjugate control; lane 7., sheep anti mouse-HRP conjugate control. The lane marked M contains M.wt markers. Note that the hyperimmune rabbit sera recognised mycobacterial proteins in addition to p40.

3.3.7 Production of Monoclonal Antibodies

Eleven Mabs were raised against p40 and all were classified as IgG₁. In ELISA, all 11 Mabs were shown to react with every strain of IS901/902-positive *M.avium* tested, but not with IS901/902-negative strains of *M.avium* or *M.a.paratuberculosis*. Only five of the 11 Mabs were shown to react strongly with p40 in western blots of *M.avium* cell lysates (Fig. 3.13).

3.3.8 Expression of p40 in Mycobacteria Other Than *M.avium* Type A/I

Western blotted cell lysates of 15 different species of *Mycobacterium* (Table 3.1) were immunoscreened with rabbit anti-p40 polyclonal antibody. Only strains of *M.avium* which tested positively for IS901/902 by PCR were observed to express the p40 antigen (Fig. 3.14a,b and c). Interestingly, *M.avium* strain T133 tested negatively for both IS901/902 and p40.

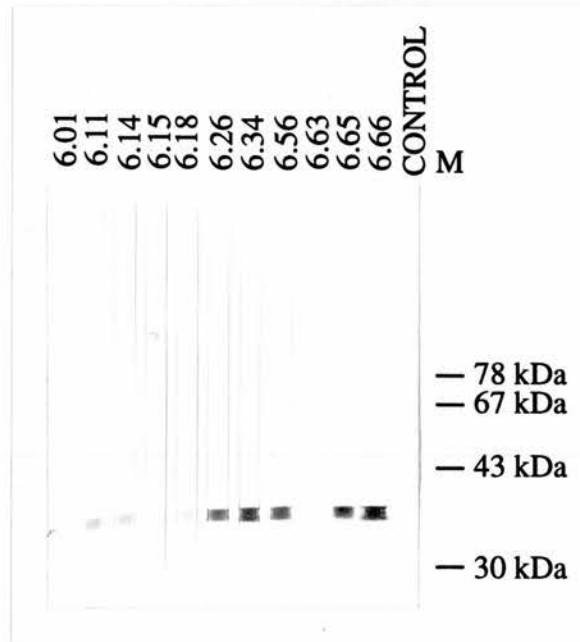


Fig. 3.13 Strips of Western blotted HPLC purified p40 each probed with one of eleven Mabs raised against the antigen. Lanes are marked with Mab identification numbers with the exception of the lane marked “control” which was probed with the sheep anti mouse-HRP conjugate. The lane marked M contains M.wt standards. Five Mabs, 6.26, 6.34, 6.56, 6.65 and 6.66 were observed to react strongly with p40.

| Species | IS901/902 | p40 |
|--------------------------------------------|-----------|-----|
| <i>M. avium</i> ^a | + | + |
| <i>M. avium</i> ^b | + | + |
| <i>M. avium</i> ^c | - | - |
| <i>M. africanum</i> | - | - |
| <i>M. bovis</i> | - | - |
| <i>M. fortuitum</i> | - | - |
| <i>M. gordonae</i> | - | - |
| <i>M. intracellulare</i> | - | - |
| <i>M. kansasii</i> | - | - |
| <i>M. leprae</i> ^d | - | - |
| <i>M. malmoense</i> | - | - |
| <i>M. marinum</i> | - | - |
| <i>M. a. paratuberculosis</i> ^e | - | - |
| <i>M. phlei</i> | - | - |
| <i>M. scrofulaceum</i> | - | - |
| <i>M. smegmatis</i> | - | - |
| <i>M. tuberculosis</i> | - | - |

Table 3.1 Mycobacterial species tested for the presence of IS901/902 by PCR and expression of p40 by immunoscreening Western blots of cell lysates. ^a*M. avium* NTCC chicken isolate; ^b*M. avium* strain JD88/118; ^c*M. avium* strain T133 (a gift from Dr. J. J. McFadden); ^d*M. leprae* cell sonicate (a gift from Dr. M. J. Colston); ^e*M. a. paratuberculosis* strain JD88/107.

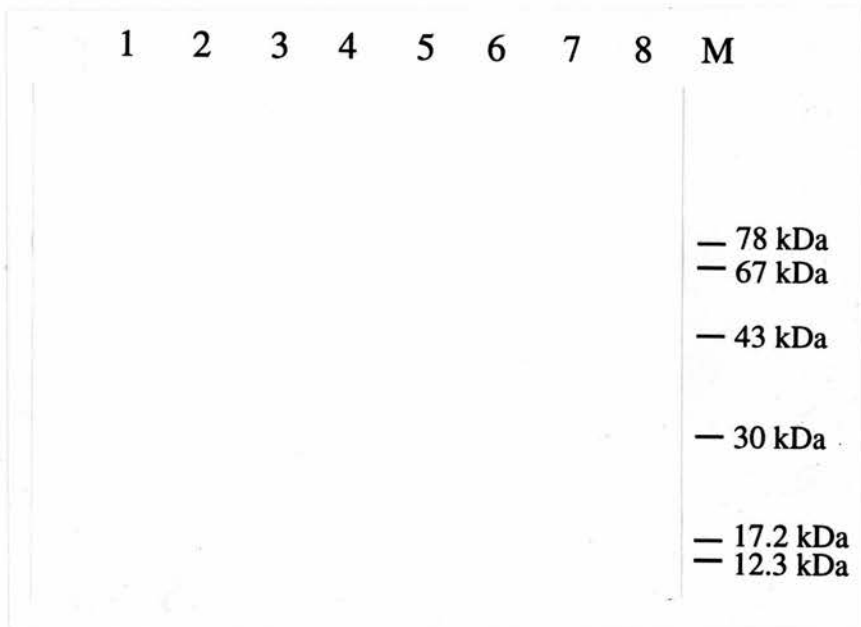
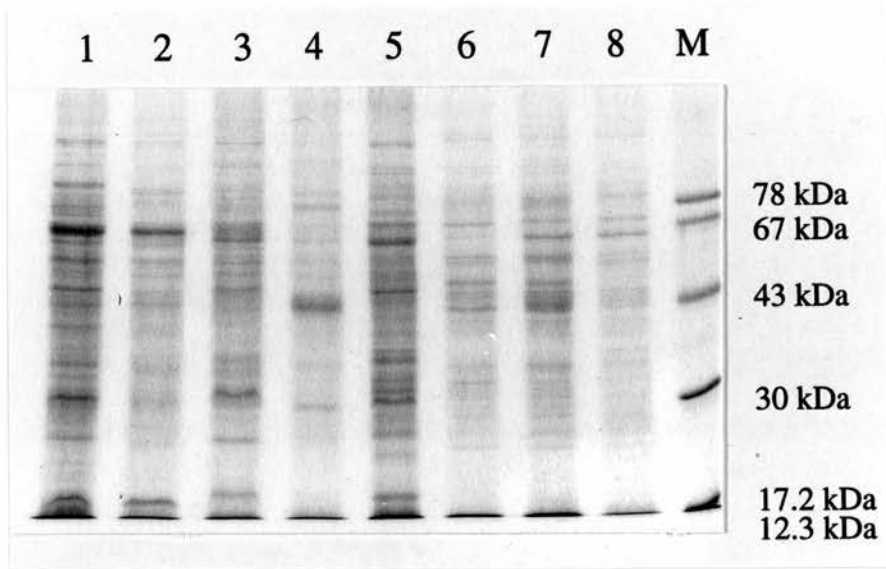


Fig. 3.14a Coomassie blue stained SDS-PAGE gel and Western blot of cell lysates of:- lane 1., *M.gordonae*; lane 2., *M.marinum*; lane 3., *M.kansasii*; lane 4., *M.malmoense*; lane 5., *M.fortuitum*; lane 6., *M.africanum*; lane 7., *M.bovis* and lane 8., *M.tuberculosis*. The lane marked M contains M.wt markers. The blot was probed with anti p40 hyperimmune rabbit serum. None of the above species could be shown to express p40 or any cross-reacting protein.

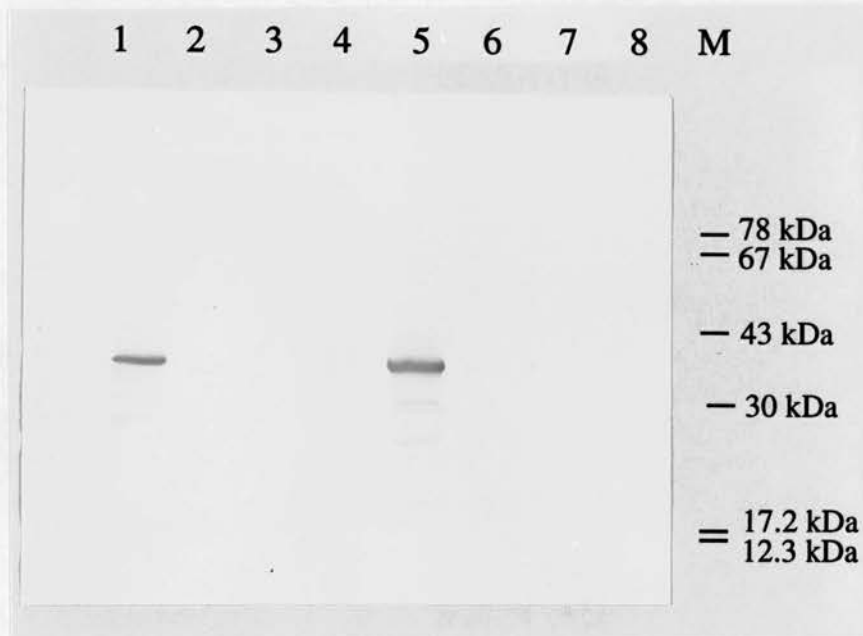
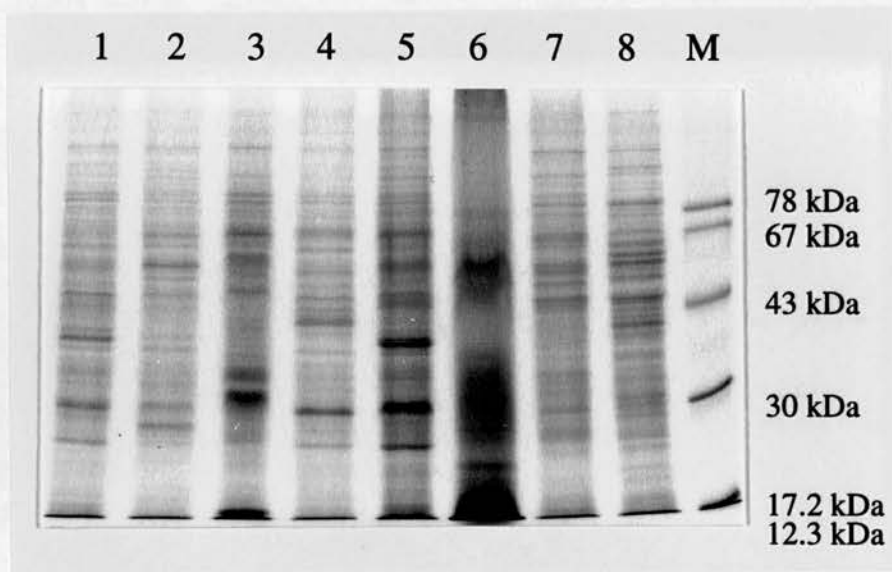


Fig. 3.14b Coomassie blue stained SDS-PAGE gel and Western blot of cell lysates of:- lane 1., *M.avium* type A/I (chicken isolate, NTCC); lane 2., *M.intracellulare*; lane 3., *M.scrofulaceum*; lane 4., *M.a.paratuberculosis* (strain JD 88/107); lane 5., *M.avium* type A/I (strain JD 88/118); lane 6., *M.leprae* (cell sonicate); lane 7., *M.smegmatis* and lane 8., *M.phlei*. The lane marked M contains M.wt markers. The blot was probed with anti p40 hyperimmune rabbit serum. Only IS901/902-positive strains of *M.avium* (lanes 1 and 5) could be shown to express p40.

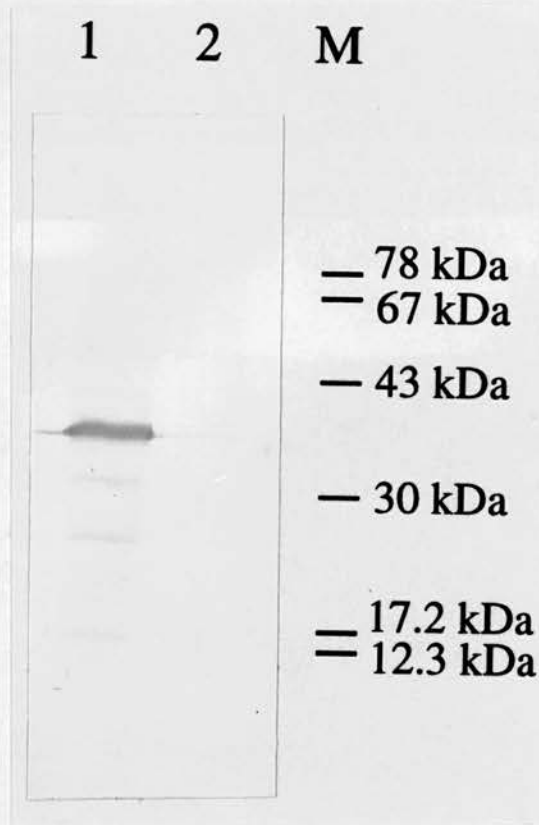


Fig. 3.14c Western blot of cell lysates of:- lane 1., *M. avium* type A/I (strain JD88/118) and lane 2., *M. avium* type A (strain T133). The lane marked M contains M.wt markers. Only strain JD88/118, which was positive for IS901/902, could be shown to express p40. Strain T133 tested negatively for IS901/902 and could not be shown to contain p40 or any cross-reacting protein.

3.4 Discussion

The MAC comprises a group of closely related organisms (Meissner *et al.*, 1974) which show not only phenotypic similarity, but share up to 98% homology at the genomic level (McFadden *et al.*, 1987a and 1987c). Among the many attempts to distinguish between the members of the MAC, seroagglutination (see section 1.4.1) and genetic analysis (see section 1.5) have allowed differentiation between *M.avium* and *M.intracellulare*. However, it was not until the identification of the genomic insertion elements IS900 in *M.a.paratuberculosis* (Green *et al.*, 1989) and IS901/902 in some strains of *M.avium* (Kunze *et al.*, 1991; Moss *et al.*, 1992) that PCR-based techniques were developed to differentiate between these two organisms and strains of *M.avium* type A. While the search for species- or subspecies-specific proteins with diagnostic potential has enjoyed limited success (see section 1.12), to date only the carboxy terminus of a 34kDa protein of *M.a.paratuberculosis* appears to contain specific epitopes that may be applied usefully to the detection of humoral immune responses in cattle with paratuberculosis (DeKesel *et al.*, 1993; Vannuffel *et al.*, 1994a). The results presented in this chapter describe the identification and purification of a novel 40kDa protein which appears to concur with the presence of IS901/902 in some strains of *M.avium*. The p40 provides a marker which allows both visual and immunological discrimination between *M.a.paratuberculosis* and strains of *M.avium* type A/I (Inglis *et al.*, 1994a).

The p40 protein was shown to partition to the soluble phase of *M.avium* type A/I cell lysates. Following ammonium sulphate precipitation at 60% saturation, the p40 component of the precipitated material was readily resolubilised in buffer without incurring any measurable losses (Fig. 3.3). Frozen storage of the solubilised proteins was observed to result in the irreversible denaturation and consequent precipitation of some protein components. However, analysis of the precipitated material by SDS-PAGE revealed that p40 was absent. Indeed a single freeze/thaw cycle was considered advantageous in that some non-p40 proteins were selectively removed from the preparation whilst leaving the p40 molecule intact. There can be no doubt

that the inherently soluble nature of p40 simplified its chromatographic purification by allowing non-denaturing conditions to be applied throughout.

The term "p40" seemed appropriate for a protein whose M.wt was estimated at ~40kDa from its migration through SDS-PAGE gels. However, a retention time of ~17 min during size exclusion chromatography (Fig. 3.15) suggested the protein may in fact be considerably larger than 40kDa. Calibration of the size exclusion column showed that BSA (67kDa), ovalbumin (42.7kDa), chymotrypsinogen (25kDa), lysozyme (14.6kDa) and uridine (~300Da) had retention times of 18.5, 19, 22, 24 and 25.5 min respectively (Fig. 3.16). The largest of these standard molecules (BSA, 67kDa) was observed to elute from the column 1 min later than p40 (Fig. 3.17) and suggested that the latter could possibly exist naturally as a dimer of ~80kDa. Alternatively, it is conceivable that p40 dimer formation may occur artefactually and as a consequence of purification. It is possible also, that p40 may complex with another protein or proteins to form a multimer of ~80kDa. This possibility is supported by the observation that, even after rigorous serial chromatographic purification, extraneous proteins can be seen contaminating the majority of the HIC-purified p40 (see Fig. 3.10, 26-27 min). These two apparently conflicting results can be reconciled however. Amino-terminal amino acid sequence determinations carried out on purified p40 (see Chapter 5) provided a clean and unambiguous sequence with no contaminating peaks. Protein for Edman degradation was obtained by western transfer of SDS-PAGE-resolved p40 onto PVDF membrane, and precise excision of the Coomassie blue-stained p40 band. Other than molecules of equal M.wt, which would co-migrate with p40, all contaminating proteins are removed at this stage. It is unlikely therefore that there is covalent association between p40 and any other protein, although covalent bonding between two p40 molecules, or the existence of weaker interactions between p40 and other protein molecules cannot be ruled out. Inter-molecular interactions such as electrostatic attraction, van der Waals forces, disulphide bridging or hydrogen-bonding would remain intact under the non-denaturing conditions applied throughout the purification of p40, but would be

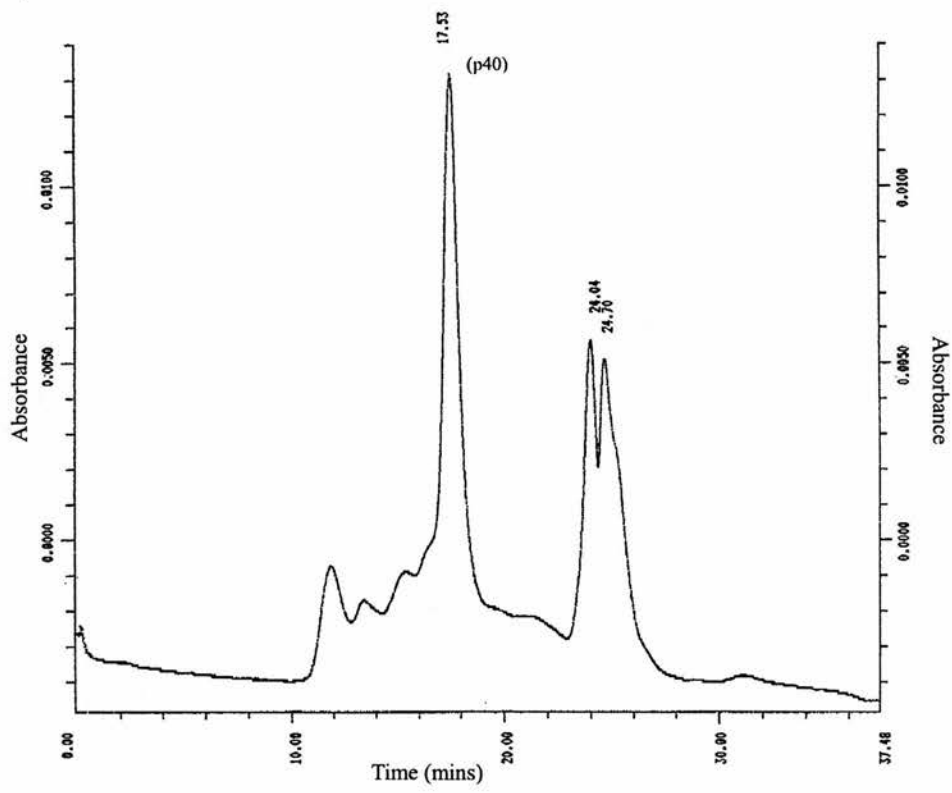


Fig. 3.15 Chromatogram illustrating the retention time of partially purified p40 on the size exclusion column. The peak of U.V absorbing material at 17.53 min represents the p40 component in a sample of first run HIC-purified protein (see Fig. 3.10, 26 min). The observed retention time of ~17.5 min was not consistent with that predicted for a molecule of 40kDa (compare with Figs. 3.16 and 3.17).

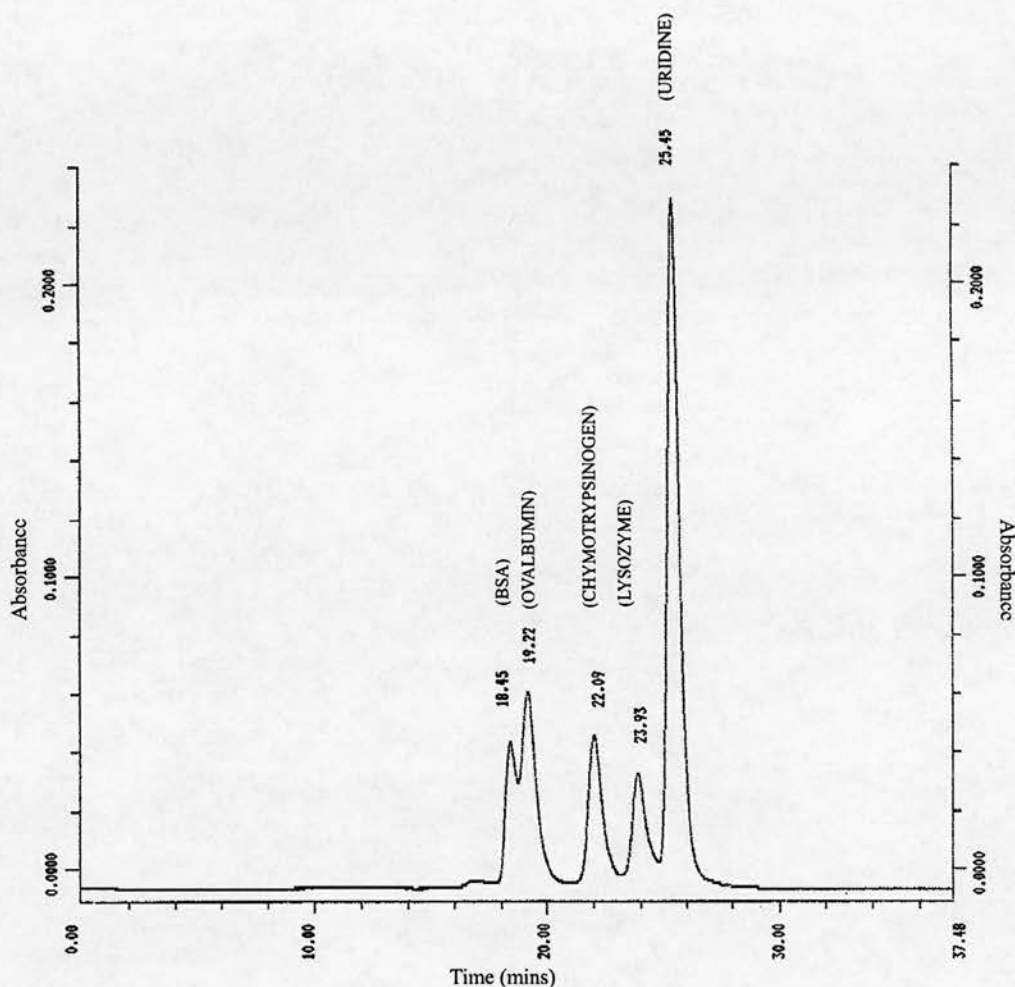


Fig. 3.16 Chromatogram showing the results of a calibration run on the size exclusion column. A mixture containing five molecules of known concentration and M.wt was injected onto the column and run under the same conditions used to resolve mycobacterial proteins. Peaks of U.V absorbing material at 18.45 min, 19.22 min, 22.09 min, 23.93 min and 25.45 min represent 40 μ g BSA (67kDa), 40 μ g ovalbumin (42.7kDa), 40 μ g chymotrypsinogen (25kDa), 20 μ g lysozyme (14.6kDa) and 40 μ g uridine (~300Da) respectively.

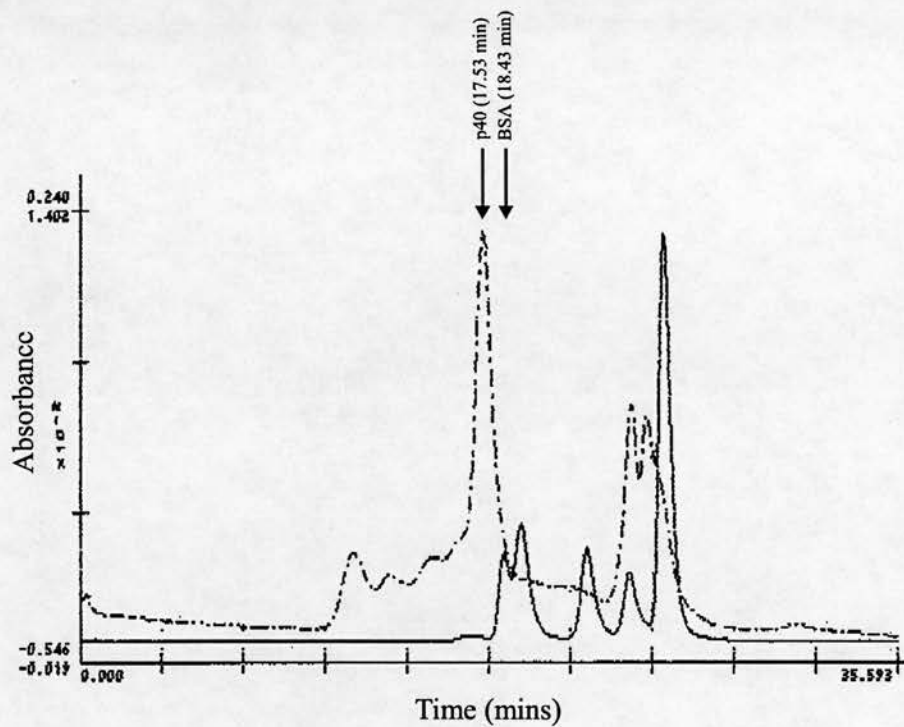


Fig. 3.17 Superimposed chromatograms (Figs. 3.15 and 3.16) to illustrate the retention time of p40 relative to those of five standard molecules. The p40 was observed to elute from the size exclusion column ~1 min earlier than the largest standard molecule (BSA at 67kDa) suggesting that the former may exist as a dimer of ~80kDa.

disrupted by heating in the presence of SDS and 2- β -mercaptoethanol prior to SDS-PAGE analysis. A fourth possibility is that the migration of p40 through the size exclusion column may have been retarded by reversible non-specific interaction of surface groups with the column matrix. An example of this type of retarded migration is provided by a protein of ~30kDa which eluted from the silica gel size exclusion column at a time coinciding with the V_t (Figs. 3.4 and 3.5).

A number of common pathogenic and non-pathogenic species of *Mycobacterium*, including three different isolates of *M.avium*, were examined by PCR and immunoblotting for the presence of IS901/902 and expression of p40 respectively (Table 3.1). Only those strains of *M.avium* which contained IS901/902 could be shown to express p40. A single strain of *M.avium* (T133) could not be shown to harbour either marker. This result was in agreement with the original observation that only p40 positive isolates of *M.avium* could be shown to react positively for IS901/902 (Inglis *et al.*, 1994a). However, it was clear that a further and more extensive study would be required to corroborate the association between p40 and IS901/902. In order to extend and confirm these observations, and examine a greater number of field isolates, a collaborative study was initiated with the Danish Veterinary Laboratory, Copenhagen, Denmark. In this study a total of 184 MAC type and field isolates were examined for 1. the presence of IS901/902 by PCR, 2. the expression of p40 using an ELISA incorporating Mabs raised against HPLC-purified p40 and 3. serotypic identity (Ahrens *et al.*, 1995). Of the 184 isolates examined, only 47 were shown to possess both p40 and IS901/902. None of remaining 137 isolates could be shown to contain either marker alone. In addition, of the marker positive isolates, >89% were typed as serotype 2 isolates and the majority of these (74%) were of porcine origin. This latter observation contrasts with the results of a Japanese study where none of 110 porcine MAC isolates examined by PCR could be shown to contain IS901/902 (Nishimori *et al.*, 1995). The authors concluded that the absence of IS901/902 might be characteristic of porcine *M.avium* isolates.

A 30kDa protein, which was purified unexpectedly during size exclusion chromatography of salt-precipitated *M.avium* proteins, was sent for amino-terminal aa sequence analysis. A search of the OWL protein database revealed that the ten residue sequence obtained shared up to 90% identity with the mature secretory proteins of the "antigen 85 complexes" of several mycobacterial species (Fig. 3.18). The antigen 85 complex comprises a group of closely related secretory molecules which range in size from 28-32kDa (Matsuo *et al.*, 1988) following endoproteolytic removal of a hydrophobic signal peptide (von Heijne 1984). The mature secretory proteins are transported into the extracellular environment by actively metabolising mycobacteria (Abou-Zeid *et al.*, 1988b; Nagai *et al.*, 1991). Three individual components of the complex designated 85-A, 85-B and 85-C (Wiker *et al.*, 1990) have been characterised and the corresponding genes cloned and sequenced for *M.leprae* (de Mendonca Lima *et al.*, 1991; Rinke de Wit *et al.*, 1993) and *M.tuberculosis/M.bovis* (Matsuo *et al.*, 1988; Borremans *et al.*, 1989; De Wit *et al.*, 1990; Content *et al.*, 1991; Nagai *et al.*, 1991). The genes encoding the 85-B antigens of *M.kansasii* (Matsuo *et al.*, 1990a), *M.avium* (Ohara *et al.*, 1993), *M.intracellulare* (Kitaura *et al.*, 1993) and *M.scrofulaceum* (Takano *et al.*, 1994) also have been cloned and sequenced. The proteins of the antigen 85 complex are known to bind fibronectin (Abou-Zeid *et al.*, 1988a; Thole *et al.*, 1992) and have been shown to be potent inducers of both humoral and cellular immune responses (Huygen *et al.*, 1988; Rumschlag *et al.*, 1988; Turneer *et al.*, 1988; Andersen *et al.*, 1991; Horwitz *et al.*, 1995; Burrells *et al.*, 1995). The close antigenic relatedness between the antigen 85 complex molecules of different species is reflected by their serological cross-reactivity (Wiker *et al.*, 1986a). However, serological cross-absorption studies have demonstrated species-specific antigenic determinants in *M.kansasii*, *M.marinum*, *M.scrofulaceum*, *M.gordoniae* and *M.szulgai* and group-specific antigenic determinants within the *M.tuberculosis* complex and the MAC (Tasaka *et al.*, 1983; Tasaka and Matsuo 1984; Tasaka *et al.*, 1985). The proteins of the antigen 85 complex have been usefully exploited in ELISAs designed to enable the serodiagnosis of tuberculosis in humans (Sada *et al.*, 1990), leprosy (Das *et al.*, 1990;

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|----------------------|----|----|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| <i>M.leprae</i> 85-A | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | P | S | M | G | R |
| <i>M.bovis</i> 85-A | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | P | S | M | G | R |
| <i>M.tuber</i> 85-A | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | P | S | M | G | R |
| <i>M.leprae</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | E | A | M | G | R |
| <i>M.bovis</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | P | S | M | G | R |
| <i>M.avium</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | G | M | G | R |
| <i>M.intra</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | G | M | G | R |
| <i>M.para</i> Ag A | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | G | M | G | R |
| <i>M.para</i> p30 | F | S | P/R | P | G | L | P | V | E | Y | - | - | - | - | - | - | - | - | - | - |
| <i>M.scrof</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | G | M | G | R |
| <i>M.kans</i> 85-B | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | A | M | G | R |
| <i>M.leprae</i> 85-C | F | S | K | P | G | L | P | V | E | Y | L | Q | V | P | S | P | S | M | G | H |
| <i>M.tuber</i> 85-C | F | S | R | P | G | L | P | V | E | Y | L | Q | V | P | S | A | S | M | G | R |
| | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| <i>M.leprae</i> 85-A | D | I | K | V | Q | F | Q | N | G | G | A | N | S | P | A | L | Y | L | L | D |
| <i>M.bovis</i> 85-A | D | I | K | V | Q | F | Q | S | G | G | A | N | S | P | A | L | Y | L | L | D |
| <i>M.tuber</i> 85-A | D | I | K | V | Q | F | Q | S | G | G | A | N | S | P | A | L | Y | L | L | D |
| <i>M.leprae</i> 85-B | T | I | K | V | Q | F | Q | N | G | G | N | G | S | P | A | V | Y | L | L | D |
| <i>M.bovis</i> 85-B | D | I | K | V | Q | F | Q | S | G | G | N | N | S | P | A | V | Y | L | L | D |
| <i>M.avium</i> 85-B | D | I | K | V | Q | F | Q | S | G | G | N | G | S | P | A | V | Y | L | L | D |
| <i>M.intra</i> 85-B | D | I | K | V | Q | F | Q | S | G | G | N | G | S | P | A | V | Y | L | L | D |
| <i>M.para</i> Ag A * | D | I | I | V | Q | F | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| <i>M.para</i> p30 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| <i>M.scrof</i> 85-B | N | I | K | V | Q | F | Q | S | G | G | N | N | S | P | A | V | Y | L | L | D |
| <i>M.kans</i> 85-B | S | I | K | V | Q | F | Q | S | G | G | D | N | S | P | A | V | Y | L | L | D |
| <i>M.leprae</i> 85-C | D | I | K | I | Q | F | Q | G | G | G | Q | H | A | V | Y | L | L | D | G | L |
| <i>M.tuber</i> 85-C | D | I | K | V | Q | F | Q | G | G | G | P | H | A | V | Y | L | L | D | G | L |

Fig. 3.18 Multiple alignment comparing the first 10 amino-terminal amino acids of *M.a.paratuberculosis* p30 (*M.para* p30) with the first 40 residues of the “mature” antigen 85 complex molecules currently listed in the OWL protein database. 85-A, 85-B and 85-C antigens are shown in three separate blocks. Black boxing indicates complete identity, shaded boxing indicates conserved substitutions. Note that an apparent deletion of two amino acid residues from the 85-C antigens causes an overall misalignment with the 85-A and 85-C antigens from position 30 onwards. The sequences compared are as follows, with database accession numbers in parenthesis:- the 85-A antigens of *M.leprae* (Q05861), *M.bovis* (P17996) and *M.tuberculosis* (P17944), the 85-B antigens of *M.leprae* (P31951), *M.bovis* (P12942), *M.avium* (Q06947), *M.intracellulare* (JN0897), *M.a.paratuberculosis* (A44892), *M.scrofulaceum* (D26187) and *M.kansasii* (P21160) and the 85-C antigens of *M.leprae* (Q05862) and *M.tuberculosis* (P31953). * Only the first 26 amino acid residues were available for *M.a.paratuberculosis* antigen A (Sugden *et al.*, 1991).

Pessolani *et al.*, 1991; Yin *et al.*, 1994) and paratuberculosis in sheep (Sugden *et al.*, 1991). Similarly, lymphoproliferation and IFN- γ release have been used to measure the response of peripheral blood mononuclear cells from cases of leprosy (Launois *et al.*, 1994), tuberculosis in humans (Launois *et al.*, 1994; Roche *et al.*, 1994) and paratuberculosis in sheep (Burrells *et al.*, 1995). By using a series of overlapping synthetic oligopeptides representing the entire molecule, the T-cell epitopes responsible for stimulating lymphocyte proliferation and cytokine release have been mapped for the 85-A and 85-B antigens of *M.tuberculosis* and *M.bovis* BCG (Leao *et al.*, 1993; Huygen *et al.*, 1994; Launois *et al.*, 1994; Roche *et al.*, 1994; Silver *et al.*, 1995). In addition, peptide mapping studies have identified the fibronectin-binding domains of the *M.tuberculosis* 85-A antigen (Leao *et al.*, 1993) and helped to elucidate the mechanism of interaction between human fibronectin and the 85-B antigen of *M.bovis* (Peake *et al.*, 1993). Analysis of secretory proteins purified from mycobacterial culture filtrates has shown that the mature secretory molecules of the antigen 85 complex can account for up to 30% of the protein secreted in culture (Abou-Zeid *et al.*, 1988a; De Bruyn *et al.*, 1987; Wiker *et al.*, 1986b). Subunit vaccines incorporating purified secretory antigens have been shown to induce a CMI response and significant protective immunity against pulmonary tuberculosis in mice (Hubbard *et al.*, 1992) and guinea pigs (Pal and Horwitz 1992) which were later challenged with aerosolised *M.tuberculosis*. Similarly, purified secretory proteins in combination with dimethyl dioctadecyl ammonium chloride (DDA) were shown to confer a level of protective immunity comparable with that afforded by bacillus BCG in mice subsequently challenged parenterally with virulent *M.tuberculosis* (Andersen 1994). In addition, this study demonstrated that the protection conferred upon vaccinated mice was adoptively transferable to naive mice via CD4⁺ T-cells. More recently, it was shown that vaccination with a single homogeneous 30kDa protein of the antigen 85 complex can afford guinea pigs partial protection against challenge with aerosolised *M.tuberculosis* (Horwitz *et al.*, 1995). Very recently, naked DNA vaccines, utilising the gene encoding the *M.tuberculosis* 85-A antigen, have successfully stimulated protective immunity in mice challenged subsequently with

live *M.tuberculosis* (Huygen *et al.*, 1996; Montgomery *et al.*, 1997). The high immunoprophylactic efficacy of secreted antigens is intriguing, but might be explained in terms of their earlier accessibility over somatic antigens. Because mycobacteria are intracellular organisms and reside unchallenged in host macrophages, somatic antigens are not available for antigen processing until macrophage activity is triggered. The activation of macrophages however, is a direct consequence of the antigenic stimulus of T-lymphocytes, which in the case of mycobacterial infections is likely to result from exposure to secretory rather than somatic antigens.

While the antigens of the complex themselves are of proven immunotherapeutic potential, the mechanism by which they are actively exported out of the cell represents a naturally occurring bacterial protein secretory system and a novel approach to the construction of recombinant vaccines. To this end, bacillus BCG and the antigen 85 complex secretory mechanism have been usefully exploited in the construction of a recombinant vaccine vehicle and antigen delivery system. Bacillus BCG transformants, containing recombinant *E.coli-Mycobacterium* shuttle plasmid constructs, have been shown to express and actively secrete the *M.kansasii* 85-B antigen and a 12 aa B-cell epitope of HIV type 1 p17^{gag} as a chimeric fusion protein (Matsuo *et al.*, 1990b). More recently, a similar BCG transformant expressing the *M.kansasii* 85-B antigen fused to a 15 aa peptide of HIV type 1 *Env* protein V3 region, has been shown to elicit a cytotoxic T-cell response towards the viral antigen in recipient mice (Kameoka *et al.*, 1994).

The work undertaken in this chapter resulted in the purification of two protein components of an isolate of *M.avium* type A/I. The first of these, an antigen of ~40kDa, appears to be specific to strains of *M.avium* type A/I. As such, p40 represents a molecule which allows the two pathogenic and mycobactin dependent members of the MAC to be distinguished from each other and from strains of *M.avium* which do not harbour the IS901/902. The second is a known secretory

antigen of ~30kDa which does not provide a species-specific immunological marker, but is produced and secreted in significant quantities only by actively metabolising mycobacteria. Therefore, the p40 and p30 antigens potentially provide the antigenic basis of tests which could be used to distinguish between animals infected with one or other organism and also between animals which are actively infected and those which have received vaccination. In the following chapter, the two purified mycobacterial proteins are used in assays designed to measure the specific reactivity of T-lymphocytes taken from sheep experimentally infected with strains of *M.avium* type A/I and *M.a.paratuberculosis*.

CHAPTER 4

OVINE T-CELL RESPONSES TO TWO DEFINED MYCOBACTERIAL ANTIGENS IN ANIMALS INFECTED WITH *M.avium* TYPE A/I AND *M.a.paratuberculosis*

4.1 Introduction

Immune-based methodologies for the diagnosis of paratuberculosis remain unreliable (reviewed by Chiodini *et al.*, 1984a; Cocito *et al.*, 1994; Collins, 1994). This is partly attributable to the sequence of immunological events which follow exposure to the organism and the onset of the disease process, but also to the lack of specific immunodiagnostic reagents.

Two expressions of clinical paratuberculosis have been described; a paucibacillary (tuberculoid) form and a multibacillary (lepromatous) form and in this respect the disease is analogous to leprosy (Stamp and Watt, 1954; Gilmour *et al.*, 1965; Gilmour *et al.*, 1978; Clarke *et al.*, 1996). These two disease states lie characteristically at extreme ends of the immunological spectrum with the former marked by strong CMI responses and low levels of circulating immunoglobulin, and the latter characterised by anergy and high levels of circulating immunoglobulin (Merkal *et al.*, 1970; Davies *et al.*, 1974; Bendixen, 1978; de Lisle and Duncan, 1981; Paliwal *et al.*, 1985; Lepper *et al.*, 1989). The clinical outcome of both forms of paratuberculosis is the same however, with early CMI and late humoral immune responses representing characteristic immunological features of the disease.

Accurate serological detection of paratuberculosis infection has been hampered by a number of factors. Animals in the early stages of infection have little or no circulating antibody and serological diagnosis of infection with *M.a.paratuberculosis* is therefore of limited value. In addition, high levels of circulating antibody do not

discriminate between actively and sub-clinically infected animals or those which have recovered and acquired protective immunity. Continuous exposure to *M.a.paratuberculosis* and related *Mycobacterium* spp. ensures that resistant animals remain immunologically reactive on subsequent testing, with some exhibiting greater reactivity than animals which are actively infected (Chiodini *et al.*, 1984a). The inevitable exposure of animals to ubiquitous environmental strains of non-pathogenic *M.avium* often results in cross-reactive immunity and false positive diagnoses are commonplace. In addition, vaccinated animals also are likely to have serum antibodies which cross-react with components of the relatively crude antigen preparations used in some serological assays. Cross-reactivity is not limited to antigens of other *Mycobacterium* spp., but extends to include *Actinomyces* spp., *Dermatophilus* spp., *Nocardia* spp., *Streptomyces* spp., *Corynebacterium* spp. and a number of fungi (Pier *et al.*, 1968; Gilmour and Goudswaard, 1972; McKenzie and Ward, 1981; Wilks *et al.*, 1981; Chiodini *et al.*, 1984a; Pepin *et al.*, 1987; Cocito *et al.*, 1994).

Serological tests including complement fixation (CFT), agarose gel immunodiffusion (AGID), fluorescent antibody (FA), haemagglutination (HA), haemagglutination-lysis (HA-lysis) (Goudswaard *et al.*, 1976) and more recently enzyme-linked immunosorbent assay (ELISA) have been used in the serodiagnosis of paratuberculosis and other mycobacterial infections. Of these, ELISA has proven to be the more sensitive and has been developed for the diagnosis of human tuberculosis (Daniel and Debanne, 1987; Sada *et al.*, 1990), leprosy (Pessolani *et al.*, 1991) and paratuberculosis in cattle (Bech-Nielsen *et al.*, 1991; Cox *et al.*, 1991; McNab *et al.*, 1991a; Ritacco *et al.*, 1991; De Kesel *et al.*, 1993; Vannuffel *et al.*, 1994a), sheep (Brooks *et al.*, 1988; Sugden *et al.*, 1991) and goats (Molina *et al.*, 1991).

The earliest detectable immune responses to mycobacterial infection are cell-mediated, therefore an assay intended for identification of animals in early and subclinical phases of disease should be designed to measure cellular immunity.

Assays including the intradermal skin test (Kormendy, 1988), the secondary T-cell proliferation assay (Buergelt *et al.*, 1977 and 1978a) and the migration inhibition test (Bendixen, 1977) have been used to measure the CMI responses of animals infected with *M.a.paratuberculosis*. These assays offer variable levels of sensitivity, but again the undefined antigen preparations used to stimulate lymphocytes result in an overall lack of specificity. Recently, a λ gt11 clone (a362) was isolated from a *M.a.paratuberculosis* genomic DNA library. This clone expressed a carboxy-terminal portion of a 34kDa protein antigen and appeared to carry *M.a.paratuberculosis*-specific B-cell epitopes (De Kesel *et al.*, 1993) (see section 1.12). The peptide encoded by clone a362 was used to develop an ELISA which identified *M.a.paratuberculosis* infected animals and appeared to be specific in the presence of serum antibody which cross-reacted with strains of *M.avium* (Vannuffel *et al.*, 1994a). However, when this peptide was tested using PBMC isolated from experimentally infected sheep and red deer, the cells did not respond to stimulation in either lymphocyte stimulation or interferon gamma (IFN- γ) release assays (Burrells and Stevenson, unpublished observations). Therefore, the recombinant a362 peptide does not appear to be useful in assays designed to measure cell-mediated immune responses.

The condition known as paratuberculosis was first described over a century ago (Johne and Frothingham 1895), yet the immune-based identification of animals in the early phase of the disease continues to present a challenge. This is partly due to a background of cross-reactive immunity against related environmental species of *Mycobacterium*, but also to the lack of species-specific immunodiagnostic reagents as described above. Meanwhile, the percentage of cases of ruminant paratuberculosis caused by infection with *M.avium* type A/I, rather than *M.a.paratuberculosis*, remains unknown. The work presented in this chapter describes a means by which this question might be addressed however. Two highly purified protein antigens; a 30kDa secretory protein (p30) from *M.a.paratuberculosis* and a 40kDa protein (p40) from a strain of *M.avium* type A/I, were compared with

johnin PPD (JPPD) to assess their immunodiagnostic potential. The antigens were used in assays measuring secondary T-cell proliferation (Burrells and Wells, 1977) and release of IFN- γ (Rothel *et al.*, 1990).

4.2 Materials and Methods

4.2.1 Culture of mycobacteria for the preparation of antigens

M.a.paratuberculosis (strain JD88/107) and *M.avium* type A/I (strain JD88/118) were cultured *in vitro* as described in Chapter 2.

4.2.2 Preparation of crude non-viable mycobacterial cell lysate for immunisation

Approximately 100mg of cultured *M.a.paratuberculosis* (strain JD88/107) was scraped from the surface of solid growth medium and washed three times in DW before finally resuspending in 400µl of DW containing the protease inhibitor PMSF at a final concentration of 1mM. Cells were disrupted by zirconium bead beating as described in section 2.2.3. After cooling on ice the disrupted cells were transferred to a fresh Eppendorf tube. Surviving cells and cellular debris were removed by centrifugation at 20,000 x g in a refrigerated microcentrifuge (Ole Dich Instruments). Protein concentrations were determined using the Micro BCA* Protein Assay Reagent (see section 2.3). The final concentration of the antigen preparation was adjusted to 2mg/ml protein and aliquots were stored at -80°C until required.

4.2.3 Antigens used to stimulate PBMC

Concanavalin A (Con A) (ICN-Flow Laboratories) at 75µg/ml.

M.a.paratuberculosis (johnin) PPD (JPPD) (Central Veterinary Laboratory, Weybridge, UK) at 62.5µg/ml.

HPLC-purified p30 from *M.a.paratuberculosis* (strain JD88/107) at 25µg/ml.

HPLC-purified p40 from *M.avium* type A/I (strain JD88/118) at 25µg/ml.

4.2.4 Experimental animals

Eleven female Greyface lambs were divided into the following groups:-

Group (A) Five lambs (V1-V5) received subcutaneous inoculations of 1.0mg crude *M.a.paratuberculosis* cell lysate in 10% aluminium hydroxide at 12 and 21 weeks of age;

Group (B) Four lambs received oral doses of 10^8 live organisms at 24hr post-parturition and then weekly for nine weeks: two lambs (P1 and P2) received *M.a.paratuberculosis* (strain JD88/107), while the remaining two (S1 and S2) received *M.avium* type A/I (strain JD88/118);

Group (C) Two untreated control lambs (C1 and C2).

4.2.5 Lymphocyte stimulation assay

Secondary T-cell proliferation assays were performed as described by Burrells and Wells (1977).

(i) Blood collection and preparation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected from the jugular vein by venepuncture into sterile silicone-coated vacutainer tubes (Becton-Dickinson) containing 100iu of preservative-free heparin (Sigma-Aldrich). Heparinised blood was layered over 10ml of Lymphoprep (Nycomed) in sterile plastic universal bottles. PBMC were separated from erythrocytes and granulocytes by centrifugation at $400 \times g$ for 30 min at room temperature. Purified PBMC were removed from the plasma/Lymphoprep interface using a sterile disposable pipette and transferred to a fresh universal bottle containing 15ml of wash solution (HBSS containing heparin at 5 iu/ml) (Appendix 2). PBMC were pelleted at $200 \times g$ for 10 min and the wash step repeated. The cell pellet was resuspended in 1ml of RPMI-1640 tissue culture medium (Life Technologies) (Appendix 4) and the number of viable cells determined by staining with Trypan blue and counting in an improved Neubauer haemocytometer. The final concentration of the cell suspension was adjusted to 1×10^6 cells/ml.

(ii) Lymphocyte stimulation assay

All tests and control cultures were performed in triplicate in sterile, flat bottomed, 96 well, tissue-culture grade microplates (Nunc). Aliquots of lymphocyte suspension (200µl) were added to sample wells containing:-

- (a) 20µl of RPMI-1640 culture medium (negative control);
- (b) 20µl of con A at a final concentration of 7.5µg/ml (positive control);
- (c) 20µl of *M.a.paratuberculosis* JPPD at a final concentration of 6.25µg/ml;
- (d) 20µl of HPLC-purified p30 from *M.a.paratuberculosis* (strain JD88/107) at a final concentration of 2.5µg/ml;
- (e) 20µl of HPLC-purified p40 from *M.avium* type A/I (strain JD88/118) at a final concentration of 2.5µg/ml.

Cultures were incubated for 90hr at 37°C in a controlled moist atmosphere of 5% CO₂/95% air. After 90hr the cultures were pulsed by the addition of 25µl of RPMI medium containing 1µCi (0.037MBq) of methyl-[³H]-thymidine (Amersham International) to each sample well and the plates returned to the incubator for a further 16-18hr. Lymphocytes were harvested on to glass fibre discs using a semi-automatic cell harvester (Dynatech). Discs were washed three times with DW and allowed to dry completely before transferring each to a clean scintillation vial containing 1ml of liquid scintillation fluid ES299 (Canberra-Packard). Incorporation of labelled thymidine was measured by liquid scintillation counting in a Packard 2500TR Liquid Scintillation Analyzer (Canberra-Packard). Individual samples were counted for 10min and the mean, expressed as counts per minute (cpm), was calculated for each triplicate sample. Final figures were expressed as stimulation indices (SI):-

$$SI = \frac{\text{mean cpm stimulated cultures} - \text{mean cpm blanks}}{\text{mean cpm control cultures} - \text{mean cpm blanks}}$$

4.2.6 Interferon gamma (IFN- γ) assay

IFN- γ was detected in culture supernatant by using a sandwich ELISA (Rothel *et al.*, 1990) as follows.

Peripheral blood was collected from animals as described above. Undiluted heparinised blood was cultured in duplicate 200 μ l aliquots in the presence of 20 μ l of RPMI medium (unstimulated control), 20 μ l of JPPD, 20 μ l of HPLC-purified p30 or 20 μ l of HPLC-purified p40 as described for the lymphocyte stimulation assay. Cultures were maintained for 24hr in a moist 5% CO₂/95% air atmosphere. After incubation, cells were separated from the plasma by centrifugation at 450 x g for 20min. Duplicate plasmas were removed, pooled and stored at -20°C until required.

Two Mabs (IFN9 and IFN2) raised against bovine IFN- γ were kindly provided by Drs Paul Wood and Jim Rothel, CSIRO, Division of Animal Health, Animal Health Research Laboratory, Victoria, Australia. ELISA plates were coated with Mab IFN9 by incubating 100 μ l per well of Mab at 5 μ g/ml in CAB (Appendix 2) for 18hr at 4°C. Excess coating antibody was removed by washing x 3 with PWB (Appendix 2). Supernatant plasmas (100 μ l) were added in triplicate to pre-coated ELISA plate sample wells and incubated for 60min at 20°C. Following a further three washes in PWB, 100 μ l of HRP-conjugated Mab IFN2 at 5 μ g/ml was added to each test well and the plates were incubated for 30min at 20°C. Excess conjugate was removed by washing x 3 with PWB and 100 μ l of ELISA substrate (Appendix 2) was added to each well. The colour reaction was allowed to develop over 30min at 20°C before being stopped by the addition of 50 μ l of 0.5M H₂SO₄ per test well. Optical densities (OD) were measured at 450nm using a Titretrek Multiskan ELISA plate reader (ICN-Flow Laboratories). Final values were expressed as corrected OD₄₅₀ (OD₄₅₀ test antigen - OD₄₅₀ medium control).

4.3 Results

4.3.1 Lymphocyte stimulation

PBMC from all sheep proliferated in response to stimulation with the T-cell mitogen con A. Responses were generally high and fell within the values previously reported for sheep (mean cpm of unstimulated cultures = 4.9×10^3 ; mean cpm of con A stimulated cultures = 227.9×10^3) (Burrells and Wells 1977).

Responses to JPPD were greater in three of the infected animals (group B; S1, S2 and P1) than in the immunised animals (group A; V1-V5) (Fig. 4.1). PBMC isolated from the animals in the control group (group C) did not respond to stimulation with JPPD.

PBMC isolated from three of the infected animals (S1, S2 and P1) proliferated in response to stimulation with purified p30. One of the immunised animals (V5) responded to p30 (SI = 3.0). PBMC from the control animals did not respond to p30.

None of the immunised sheep (group A) yielded PBMC which proliferated in response to stimulation with purified p40. Similarly, PBMC isolated from animals infected with *M.a.paratuberculosis* (group B) failed to respond to stimulation with p40. Only PBMC isolated from sheep infected with *M.avium* type A/I (group B) proliferated upon stimulation with p40. Stimulation indices of 38.0 and 93.7 were recorded for lambs S1 and S2 respectively. Animals in the control group were non-reactive (SI \leq 2.0).

4.3.2 IFN- γ production

JPPD induced the production of IFN- γ in cultures of whole blood prepared from all experimental animals irrespective of whether they received non-viable mycobacterial antigen or live organisms (Fig. 4.2).

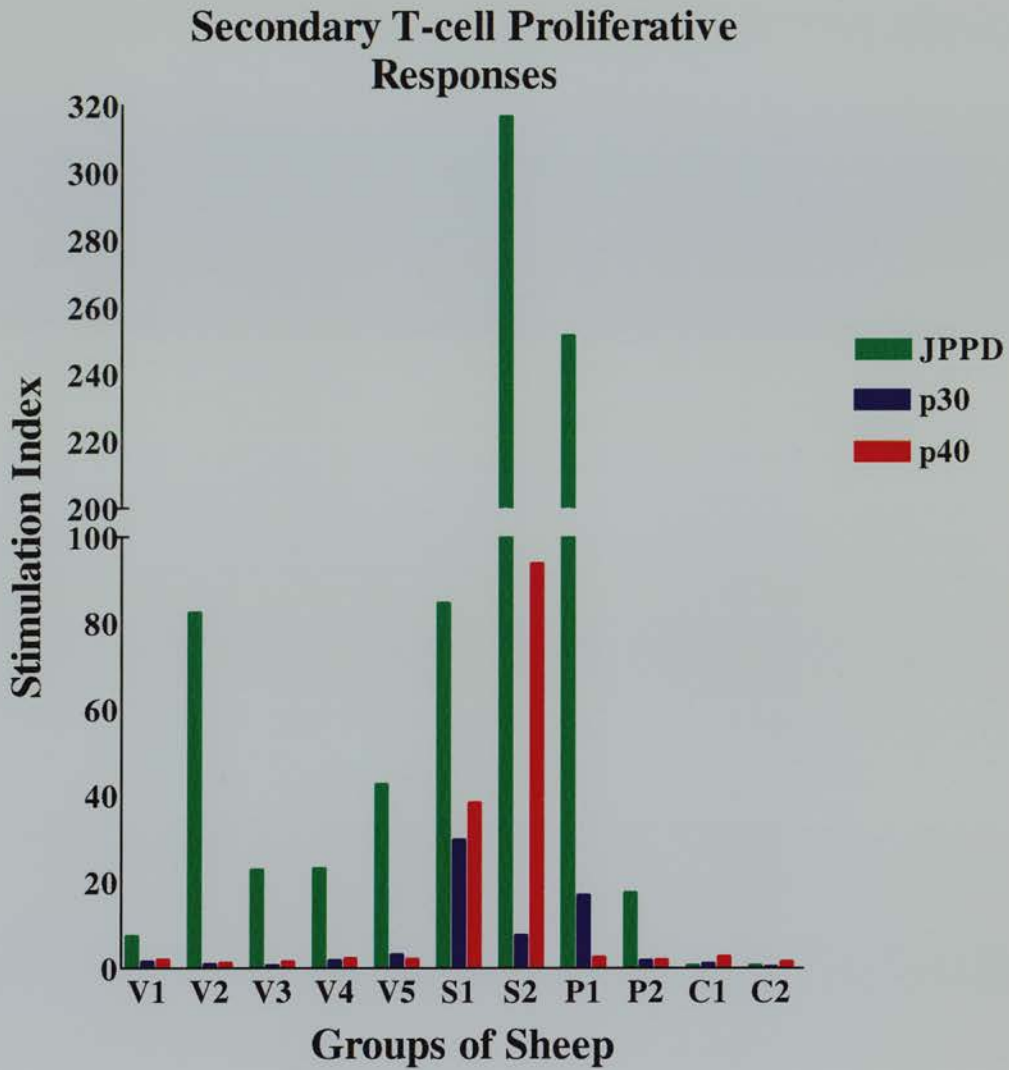


Fig. 4.1 Bar chart illustrating proliferation of PBMC isolated from three different groups of sheep in response to stimulation with JPPD, HPLC-purified p30 from *M.a.paratuberculosis* and HPLC-purified p40 from *M.avium* type A/I. Sheep in group A (V1-V5), group B (S1, S2, P1 and P2) and group C (C1 and C2) are as described in section 4.2.4.

IFN- γ Production

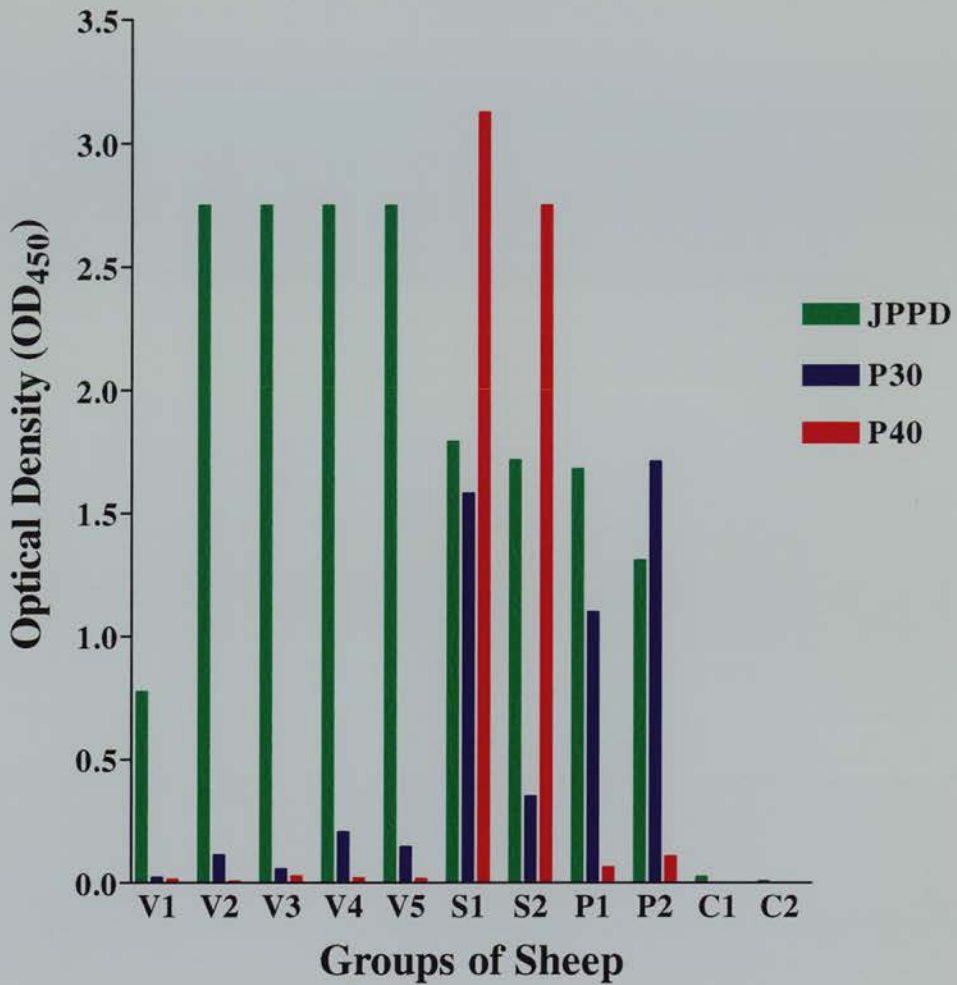


Fig. 4.2 Bar chart illustrating IFN- γ release in whole blood cultures from three different groups of sheep in response to stimulation with JPPD, HPLC-purified p30 from *M.a.paratuberculosis* and HPLC-purified p40 from *M.avium* type A/I. Sheep in group A (V1-V5), group B (S1, S2, P1 and P2) and group C (C1 and C2) are as described in section 4.2.4.

Stimulation with purified p30 induced IFN- γ release by PBMC from all infected animals (group B). PBMC from four of the immunised animals (group A) produced levels of IFN- γ which were below the cut-off value (cut-off OD₄₅₀ <0.200). IFN- γ released by PBMC from a single immunised animal (V4) was measured at OD₄₅₀ = 0.205.

Only PBMC isolated from the sheep infected with *M.avium* type A/I (group B; S1 and S2) released IFN- γ in response to stimulation with purified p40.

PBMC from the control lambs did not produce IFN- γ in response to stimulation with any of the three antigens tested.

4.4 Discussion

The diagnostic value of two defined mycobacterial antigens (p40 and p30) has been demonstrated. Of these, p40 appears to concur with the presence of IS901/902 in certain strains of *M.avium*, and is capable of distinguishing between animals infected with *M.avium* type A/I and *M.a.paratuberculosis*. As such, p40 is the first entire protein molecule to be identified which has been shown subsequently to be specific to a *Mycobacterium* species implicated in the aetiology of ruminant paratuberculosis. The second of these defined antigens, p30, appears to stimulate CMI responses in animals harbouring active and ongoing mycobacterial infections, but not in animals which have received vaccination with killed organisms.

The results illustrated in Figs. 4.1 and 4.2 highlight the advantages of using defined mycobacterial antigens as opposed to a crude antigenic extract to stimulate lymphocytes in both secondary T-cell proliferation and IFN- γ release assays. While JPPD induced T-cell proliferation and IFN- γ production in PBMC isolated from both infected and immunised animals, the purified p30 antigen could be used to distinguish between animals immunised with a non-viable cell lysate and those harbouring an active infection. This could be explained by the fact that the p30 is a secretory antigen (see section 3.4) which is produced and released into the extracellular environment by actively metabolising mycobacteria. As such the protein may not be present in cell lysates in quantities sufficient to induce significant levels of cellular immunity. Conversely, animals harbouring active infections are continuously exposed to the protein as it is synthesised and secreted by metabolising cells. Infected animals are therefore more likely to mount a measurable immune response against p30. Stimulation with purified p30 antigen did not appear to induce a subspecies-specific immune response however, and could not distinguish between animals infected with either *M.avium* type A/I or *M.a.paratuberculosis*. Species-specific epitopes have been described for some members of the mycobacterial antigen 85 complex (Tasaka *et al.*, 1983; Tasaka and Matsuo, 1984; Tasaka *et al.*, 1985). However, the wide distribution and highly conserved nature of these

secretory proteins precludes their use as markers of infection with any particular organism. In addition, the deduced amino-terminal region of the PS1 secretory protein of *Corynebacterium glutamicum* has been shown to share up to 33% sequence identity (52% similarity) with the proteins of the mycobacterial antigen 85 complex (Joliff *et al.*, 1992). It is conceivable therefore, that cross-reactivity between *Corynebacterium* spp. and members of the MAC could continue to contribute to false diagnoses, even when using highly purified p30.

Unlike the p30 antigen which stimulated T-cell responses in PBMC from all infected animals, the p40 antigen was observed to stimulate T-cell responses specifically in lambs infected with *M.avium* type A/I. Expression of p40 was not detected in any of 14 other species of *Mycobacterium* examined (see section 3.3.8 and Table 3.1) and it is unlikely therefore, that exposure to environmental mycobacteria would generate a background of cross-reactive immunity. Recently, a monoclonal antibody raised against p40 was used successfully in a blocking ELISA designed to distinguish between sheep infected with *M.avium* type A/I and *M.a.paratuberculosis* (Klausen *et al.*, 1997). The results of the study corroborate the findings presented here and in Burrells *et al.* (1995), and provide further evidence that the immune response to *in vivo* expression of p40 can be exploited as a subspecies-specific marker for the detection of animals harbouring *M.avium* type A/I infections. In addition, serological detection of p40 indicates that the protein carries B-cell as well as T-cell epitopes. The blocking ELISA could be used to monitor the prevalence of *M.avium* type A/I infection in conjunction with existing diagnostic assays for *M.a.paratuberculosis*. Such a venture would go some way towards assessing the aetiological significance of *M.avium* type A/I infection in paratuberculosis of domestic ruminants.

The detection of IFN- γ produced by T-cells in response to antigen-specific stimulation has been shown to correlate with secondary proliferative responses (D'Andrea *et al.*, 1986) and to be more sensitive (D'Andrea *et al.*, 1986; Hao *et al.*, 1986). The data presented here confirm these findings. Despite exhibiting no, or

only weak proliferative responses, PBMC isolated from lamb P2 produced IFN- γ in response to stimulation with johnin PPD and purified p30. Cellular anergy is characteristic of advanced paratuberculosis (Merkal *et al.*, 1970; Bendixen, 1978; Chiodini *et al.*, 1984b) and animals with suppressed lymphocyte activity cannot be identified reliably using assays which measure T-cell proliferation.

As a diagnostic tool the IFN- γ release assay has several advantages over the lymphocyte stimulation assay. It is comparatively inexpensive, technically robust and simple in operation. This makes screening the numbers of animals involved at the herd or flock level logistically feasible. Using p40 as antigen, the assay represents the first immune-based diagnostic test which has the potential to identify animals in early and advanced stages of infection with *M.avium* type A/I. Unfortunately, *M.a.paratuberculosis*-specific antigens capable of stimulating cellular immune responses have not yet been identified and work towards their identification continues. Once such antigens have been identified and their genes cloned and expressed, it is conceivable that recombinant immunodiagnostic reagents, including p40, will become available commercially. By identifying subclinically infected animals early, the use of such recombinant antigens in conjunction with the IFN- γ release assay would have a significant impact on the management of paratuberculosis-infected herds. Tighter controls on the movement of infected livestock could be implemented and new stocks of disease-free animals established and maintained.

The p40 is of interest not only as a diagnostic antigen, but also as one which may prove to be of immunoprophylactic value. As such, p40 clearly merits further study and the following chapter documents experiments undertaken to clone and characterise the p40 gene.

CHAPTER 5

EXPERIMENTS TO ISOLATE AND CHARACTERISE THE GENE ENCODING p40

5.1 Introduction

The primary objective of the work presented in this chapter was to clone and characterise the gene encoding the p40 antigen. This task was undertaken with a view to obtaining nucleotide sequence information, an essential step towards identifying a possible function for the protein and for generating specific DNA probes to enable the distribution of the gene amongst other mycobacteria to be assessed.

Since 1985, genes of a number of pathogenic mycobacterial species have been cloned in *E.coli* using both plasmid and bacteriophage vectors (Clark-Curtiss *et al.*, 1985 and 1990; Young *et al.*, 1985a&b; Thole *et al.*, 1985; Jacobs *et al.*, 1986; Khandekar *et al.*, 1986; Morris *et al.*, 1988; Rouse *et al.*, 1991; Stevenson *et al.*, 1991; DeKesel *et al.*, 1993; Cameron *et al.*, 1994 and Inglis *et al.*, 1994b). One particularly successful strategy entailed the use of the bacteriophage vector, λ gt11, to clone and express antigens of *M.tuberculosis* (Young *et al.*, 1985a) and *M.leprae* (Young *et al.*, 1985b). This approach has allowed the isolation of foreign genes expressed in *E.coli* when specific antisera were the only means of detection available. In this system, randomly-generated genomic DNA fragments of up to 7.2kb are cloned into a unique *EcoRI* site situated in the carboxy-terminus of the *E.coli* β -gal gene. The use of randomly-generated fragments, as opposed to those produced by partial restriction, is advantageous when constructing a DNA expression library, since it is important to ensure that all the protein encoding sequences in the genome are expressed if the library is to be truly representative. This requires the generation of a large number of fragments with endpoints which occur at random and ideally at every base pair throughout the genome. Provided the insert fragments are positioned in the correct

orientation and in frame with *lacZ*, expression of the chimeric genes as β -gal fusion proteins can be induced in a suitable *supF* host strain under the control of the native *E.coli* upstream promoter region. Expressed antigens bound to a solid support are then detected immunologically using specific primary antibody in combination with an appropriate labelled secondary antibody. The λ gt11 expression vector was an appropriate choice of cloning vehicle for the gene encoding the p40 antigen, because hyperimmune antisera already had been raised against HPLC purified protein (see section 3.2.4).

5.2 Materials and Methods

5.2.1 Disruption of *M.avium* Type A/I Cells and Purification of Genomic DNA

M.avium type A/I cells were propagated on 7H11+ medium. Washed cells (0.7g) were pelleted and resuspended in 10ml of 4M GIT (Appendix 2) (Sambrook *et al*, 1989). The cells were disrupted mechanically in an Eaton press (Eaton 1962) as described in section 2.2.1. The lysate was thawed and layered onto 5ml cushions of 40% (^{wt}/_{wt}) CsCl in TE buffer (Appendix 2). Nucleic acid was pelleted through the CsCl at 113,000 x g in a Beckman SW40ti rotor (Beckman Instruments) for 16hr at 20°C. Each of the resulting nucleic acid pellets was collected and resuspended in 5ml of TE buffer. Excess CsCl was removed by dialysis against 500ml of TE buffer for 16hr at 4°C. Cellular RNA was removed by digesting with RNase A (Sigma-Aldrich) at 20µg/ml for 60 min at 37°C. The DNA was extracted once with an equal volume of Tris-buffered phenol pH7.5 and twice with equal volumes of chloroform. Salt concentration was raised by the addition of sodium acetate to 0.3M and the DNA was precipitated by the addition of two volumes of chilled ethanol and overnight incubation at -20°C. Precipitated DNA was pelleted at 4800 x g and washed twice in wash ethanol (Appendix 2). The pellets were desiccated in a vacuum chamber and redissolved in TE buffer. After spectrophotometric quantitation at 260nm, the DNA concentration was adjusted to 330µg/ml. This extraction procedure yielded a total of 1.9mg of *M.avium* type A/I genomic DNA.

5.2.2 Construction of *M.avium* Type A/I Genomic DNA Expression Libraries in λgt11

(Method 1)

Preparation and Modification of Genomic DNA Fragments Using the Method of Young *et al.* (1985a).

1. Generation of randomly sheared genomic DNA fragments

Randomly sheared genomic DNA fragments were prepared by repeated passage of DNA through hypodermic needles. A total of 300µg of genomic DNA in 2ml of TE buffer was forced through 27 gauge and 30 gauge needles 100 times each to generate

smaller fragments. The sheared DNA was ethanol precipitated as described in section 5.2.5, desiccated and redissolved in 200 μ l of TE buffer. The DNA fragments were then size-selected by rate-zonal sucrose density gradient centrifugation as described in section 5.2.6. The size distribution of DNA fragments was analysed by agarose gel electrophoresis, and gradient fractions containing fragments ranging between ~3.5 - 8.0kb in size were selected for the purpose of preparing a library.

2. Methylation of internal *EcoRI* sites

1 μ g of size-selected genomic DNA fragments was methylated in a 20 μ l reaction mixture containing 50mM Tris/HCl pH7.5, 1mM Na₂ EDTA, 5mM dithiothreitol (DTT), 10 μ M S-adenosyl-L-methionine (SAM) (Boehringer Mannheim) and 30 units of *EcoRI* methylase (Northumbria Biologicals). DNA was methylated over 30 min at 37°C and the reaction was terminated by heating at 70°C for 10 min. The reaction was controlled by methylating an equal quantity of wild type λ DNA under identical conditions and demonstrating resistance to digestion with *EcoRI*.

3. Blunt-ending

Overhangs on DNA fragments were blunt-ended by the addition of 2.5 μ l of 100mM MgCl₂, 2.5 μ l of 200mM dNTPs and 5 units of T4 DNA polymerase (Boehringer Mannheim) to the 20 μ l methylation reaction. Ends were blunted over 30 min at 37°C and the reaction was terminated by increasing the Na₂EDTA concentration to 15mM and phenol/chloroform extraction. Sodium acetate was added to 0.3M and the DNA was precipitated by the addition of two volumes of chilled ethanol and overnight incubation at -20°C. Precipitated DNA was pelleted at 30,000 x g, washed twice with wash ethanol and desiccated. In a control reaction run in parallel, DNA fragments resulting from *Hind* III restriction of wild-type λ DNA were blunt-ended in the presence of [α -³²P]-dCTP (Amersham International). Incorporation of labelled dCTP was confirmed by TLC and autoradiography (see section 5.2.16).

4. Ligation of *Eco*RI linkers to modified DNA fragments

The desiccated DNA was redissolved in 4.5 μ l of 50mM Tris/HCl pH 7.5, 10mM MgCl₂, 10mM DDT, 1.5mM ATP containing phosphorylated 8-mer *Eco*RI linkers (Boehringer Mannheim) at 100 μ g/ml. Once the DNA had dissolved, 5 units of T4 DNA ligase (Boehringer Mannheim) was added to the mixture and the reaction was incubated at 12°C for 16hr. The enzyme was inactivated by heating at 70°C for 10 min. Enzyme activity was confirmed in a control reaction where linker molecules were ligated to produce a ladder effect as visualised in a silver-stained 7.5% continuous polyacrylamide gel.

5. Removal of excess *Eco*RI linkers from modified DNA fragments

Buffering conditions were altered to favour *Eco*RI digestion by the addition of 5 μ l of conversion buffer (50mM Tris/HCl pH 7.5, 10mM MgSO₄, 200mM NaCl). Concatenated linker molecules were digested to completion by the addition of 5 units of *Eco*RI (Boehringer Mannheim) and incubation at 37°C for 3hr. The enzyme was heat inactivated at 70°C for 10 min and the reaction mixture was increased to 200 μ l with TN buffer (Appendix 2). The whole sample was injected onto a Beckman Ultraspherogel SEC-3000 (7.5 x 300mm) size exclusion HPLC column (Beckman Instruments) pre-equilibrated with TN buffer. Linkers were separated from DNA fragments over 30 min at a flow rate of 0.5ml/min. Material which eluted from the column was monitored at 260nm. The DNA fragments were collected from the column in 1ml as a single sharp peak at 10.4min. Salt concentration was increased to 0.5M NaCl and 1 μ g of muscle glycogen was added prior to precipitation with two volumes of chilled ethanol and overnight incubation at -20°C. The DNA was pelleted at 30,000 x g, washed twice in wash ethanol and desiccated.

6. Ligation of modified fragments into λ gt11 arms

The DNA pellet was redissolved in 4 μ l of T4 DNA ligase buffer (Boehringer Mannheim) containing 1 μ g of *cos*-ligated, *Eco*RI digested, dephosphorylated λ gt11

arms (Stratagene) and 10mM ATP. Once the DNA had dissolved, 5 units of T4 DNA ligase were added and the reaction was incubated at 12°C for 16hr.

7. *In vitro* packaging and amplification of the library

The library was packaged into phage heads using Gigapack® II Gold (Stratagene) packaging extracts and amplified on *E.coli* host strain Y1088 in accordance with the manufacturer's recommendations.

8. Titration of the library

Pre- and post-amplification titres were obtained from the newly prepared library. Log¹⁰ dilutions were prepared in SM buffer (Appendix 2). Equal volumes (100µl) of library dilution and plating cells (see section 5.2.3) were mixed and incubated at 37°C for 15 min. The infected cells were added to supplemented L-B top agarose (Appendix 4), spread out and incubated as described in section 5.2.4. L-B top agarose contained 5mM IPTG and 2.5mM X-gal to allow differentiation between recombinant (clear) and non-recombinant (blue) plaques. The titre of the library and percentage of recombinants were calculated from the total number of pfu and the ratio of blue to clear plaques. Confirmation of the number of recombinant phage in the library was obtained by hybridising [α -³²P]-dCTP-labelled genomic DNA fragments to a plaque lift taken from the same plate. The plate was chilled to 4°C and overlaid with a 90mm nitrocellulose circle (Schleicher & Schuell). After 2min the membrane was lifted and placed in a petri-dish containing denaturing solution (Appendix 2) for 1min. The membrane was transferred to a second petri-dish containing neutralising solution (Appendix 2) for a further 1min before rinsing gently in 6 x SSC. Excess moisture was removed and phage DNA was fixed to the membrane under vacuum at 80°C for 2hr. The plaque lifts were pre-hybridised and hybridised against labelled genomic DNA fragments as described in section 5.2.18.

(Method 2)

Preparation and Modification of Genomic DNA Fragments Using the RiboClone[®] *Eco*RI Adapter System (Promega)

1. Generation of randomly sheared genomic DNA fragments

Genomic DNA was mechanically sheared by sonication. Randomly generated fragments of ~1-3kb resulted from sonication at 25% amplitude for 1 second using a Vibra-cell model VCX 600 sonicator (Sonics & Materials).

2. Blunt-ending

DNA fragments were blunt-ended in a T4 DNA polymerase reaction as described in Method 1.

3. Ligation of *Eco*RI adapters to blunt-ended DNA fragments

*Eco*RI adapters were blunt-end ligated to genomic DNA fragments in a 30 μ l reaction containing 30mM Tris/HCl pH 7.8, 10mM MgCl₂, 10mM DTT, 5mM ATP, 100 μ g/ml acetylated BSA, a 20-fold molar excess of adapter molecules, 250ng of blunt-ended genomic DNA fragments and 2.5 units of T4 DNA ligase. The reaction was incubated at 15°C for 18hr. Ligase was heat inactivated at 70°C for 10min.

4. Phosphorylation of 5' ends of adapter molecules

Adapter molecules were phosphorylated by adding the following to the above heat-inactivated ligation reaction; 4 μ l of 10 x T4 PNK buffer (Appendix 2), ATP to 0.1mM, 10 units of T4 PNK (Boehringer Mannheim) and DW to a final volume of 40 μ l. The reaction was incubated at 37°C for 30min prior to phenol and chloroform extractions.

5. Removal of excess *EcoRI* adapter molecules

Excess adapter molecules were separated from modified genomic DNA fragments by size exclusion chromatography using a 1ml Sephacryl S-400 spin-column in accordance with the manufacturer's instructions.

6. Ligation of modified fragments into λ gt11 arms

Ligation of modified genomic fragments into λ gt11 arms, *in vitro* packaging reactions and titration of the library were performed as described in Method 1.

5.2.3 Preparation of Plating Cells

E. coli host strains Y1088 and Y1090 used for the propagation of λ gt11 were grown on L-B media supplemented with maltose at 0.2% (^{wt}/_v), MgSO₄ at 10mM and ampicillin at 50 μ g/ml final concentrations. An overnight culture of an appropriate *E. coli* host strain was sub-inoculated into fresh pre-warmed (37°C) supplemented L-B broth (Appendix 4) and incubated in an Aerotron shaking incubator (Infors) at 37°C until the OD₆₀₀ reached a value of 0.6. Cells were pelleted and resuspended in ice cold 10mM MgSO₄ to yield an OD₆₀₀ of 0.5. The cell suspension was stored at 4°C and used within 48hr.

5.2.4 Immunoscreening of Genomic DNA Expression Libraries

Libraries were immunoscreened for specific antigen-producing recombinant phage using a modification of the method described by Young and Davies (1983a). Approximately 5×10^4 pfu were plated on lawns of *E. coli* host strain Y1090 in 20 x 20cm culture plates by the top agarose method (Sambrook *et al*, 1989). Plates were incubated at 42°C until pin-prick size plaques became visible. Plates were overlaid with dry 20 x 20cm nitrocellulose (Schleicher & Schuell) impregnated with 10mM IPTG and incubated at 37°C for a further 16hr. Plates were chilled to 4°C and positions of filters marked by needle stabs. Filters were removed and washed in several changes of WFB (Appendix 2) over 60min. Filters were incubated in primary antibody (rabbit anti p40) diluted $1/80$ in WFB for 60min at RT. Excess

primary antibody was removed by 3 x 5min washes in WFB prior to incubating in secondary antibody (swine anti rabbit-HRP conjugate) (Dako) diluted $1/1000$ in WFB for 60min at RT. Excess secondary antibody was removed by a further 3 x 5min washes in WFB. Recombinants expressing antigens recognised by the primary antibody were visualised by incubating the filters in a solution containing 100mM Tris/HCl pH 7.5, 1mM DAB and 60mM H₂O₂. Filters were aligned with their corresponding plates and agarose plugs containing clones of interest were collected into 1ml each of SM buffer containing 50µl of chloroform. Recombinant phage in this suspension were re-plated and immunoscreened in an identical manner on 90mm plates. Approximately 5×10^2 pfu per 90mm plate were screened and the process was repeated picking an individual expressing clone each time until all the plaques on subsequent filters were positive. These “plaque purified” clones were used to prepare high titre stocks of recombinant phage by either plate lysate or liquid lysate methods as described by Sambrook *et al*, 1989 (see section 5.2.5).

5.2.5 Preparation of recombinant phage DNA

1. Large scale preparation of lambda DNA

Quantities of lambda DNA ranging between 30µg and 70µg were extracted from recombinant phage harvested from plate or liquid lysates and purified by isopycnic ultracentrifugation using CsCl density gradients (Sambrook *et al.*, 1989).

(i) Plate culture method:-

Approximately 1×10^5 pfu were mixed with 1ml of fresh *E.coli* host strain Y1090 plating cells and incubated at 37°C for 20min. Infected cells were mixed with 33ml of supplemented L-B top agarose cooled to 45°C and spread evenly over the surface of a pre-warmed (37°C) 20 x 20cm L-B agar plate. Once the L-B top agarose had solidified, plates were inverted and incubated overnight at 37°C. Each plate was overlaid with 30ml of SM buffer and incubated at RT for 3hr on a rocking platform. The phage eluate was transferred to a 50ml centrifuge tube by pipette and cleared of bacteria and other debris at 4800 x g at 4°C for 20min. The supernatant was

transferred to a fresh tube and chloroform added to 0.5% (v/v). The lysate was stored at 4°C until required.

(ii) Liquid culture method:-

A single well-isolated plaque was picked from an L-B agar plate and transferred to a 1.5ml tube containing 100 μ l of SM buffer. After overnight incubation at 4°C, 20 μ l of the phage eluate was mixed with 100 μ l of fresh *E.coli* host strain Y1090 plating cells and incubated at 37°C for 20min. The infected cells were used to inoculate 100ml of pre-warmed (37°C) supplemented L-B broth and the culture was incubated in a shaking incubator at 37°C for 5-7hr. Chloroform was added to 0.5% (v/v) and shaking was continued for a further 15min. The lysate was cleared of debris by centrifugation at 4800 x g for 20min at 4°C and the supernatant transferred to chloroform-resistant plastic tubes. Chloroform was added to 0.5% (v/v) and the lysate stored at 4°C until required.

(iii) Purification of phage by isopycnic ultracentrifugation:-

Recombinant phage in either plate or liquid lysates was pelleted in a type 45 ultracentrifuge rotor (Kontron Instruments) at 100,000 x g for 60min at 4°C. Pellets were resuspended in 3ml of SM buffer and layered carefully onto a 3-step density gradient consisting of 3ml each of 90%, 60% and 40% (w/v) CsCl in SM buffer. Phage particles were banded at 61120 x g for 2hr at RT in an SW40ti ultracentrifuge rotor (Beckman Instruments). A band of phage was collected from the interface separating the 90% and 60% layers of the gradient by puncturing the tube with a 21-gauge hypodermic needle. The preparation was diluted to 5ml with SM buffer and phage particles were pelleted at 100,000 x g in a SW55ti ultracentrifuge rotor for 60min at 4°C.

(iv) Extraction of DNA from purified phage particles:-

The phage pellet was resuspended in 500 μ l of SDS extraction buffer (Appendix 2) containing 50 μ g/ml Proteinase K (Sigma-Aldrich) and incubated at 37°C for 30min.

The preparation was extracted once with an equal volume of Tris-buffered phenol pH7.5 and twice with equal volumes of chloroform. Sodium acetate was added to 0.3M and the lambda DNA was precipitated by the addition of 2.5 volumes of chilled ethanol and overnight incubation at -20°C. Precipitated DNA was pelleted in a refrigerated microcentrifuge (Ole Dich Instruments) at 20,000 x g for 10min and desiccated prior to dissolving in TE buffer. DNA concentration was determined spectrophotometrically at 260nm and adjusted to 3µl/µg.

2. Small scale preparation of lambda DNA

Smaller quantities of recombinant lambda DNA (1-5µg) were prepared using the Wizard™ Lambda Preps DNA Purification System (Promega) in accordance with the manufacturer's recommendations.

5.2.6 Size Selection of DNA Fragments by Rate-Zonal Centrifugation

Size selection of genomic DNA fragments generated by mechanical shearing or restriction endonuclease digestion was achieved by rate-zonal ultracentrifugation using continuous sucrose density gradients as described by Nevins and Darnell, (1978). In a final volume of 200µl, 50µg of genomic DNA fragments was layered onto a 12ml sucrose gradient of 5-20% (^{wt/v}) in 1 x TE buffer. DNA fragments were separated at 78,925 x g over 18hr at 4°C in an SW40ti rotor (Beckman Instruments). Gradients were fractionated into 24 x 0.5ml fractions using a Model 185 Density Gradient Fractionator, a Model UA-5 Absorbance Monitor and a Model 1200 Golden Retriever Fraction Collector (Instrumentation Specialities Company). Gradients were pumped out from the bottom using 30% (^{wt/v}) sucrose at a flow rate of 0.5ml/min at a sensitivity of 0.5 full scale deflection. The size distribution of DNA fragments was analysed by agarose gel electrophoresis.

5.2.7 Agarose Gel Electrophoresis

DNA fragments were resolved in horizontal agarose slab gels as described by Sambrook *et al.* (1989). Gels of 75 x 50 x 6.5mm (mini-gels), 140 x 110 x 8.0mm

(midi-gels) and 247 x 200 x 10mm (maxi-gels) were cast in “Agarose Gel Electrophoresis Apparatus”, Models H6, H5 and H4 (Life Technologies) respectively. Gels of 0.6%-1.0% (wt/v) nucleic acid grade “Ultrapure” agarose (Life Technologies) and 0.5µg/ml ethidium bromide were prepared and run in 1 x TBE buffer (Appendix 2). Gels, from which DNA fragments were later recovered, were prepared from nucleic acid grade “Low Melting Point” (LMP) agarose (Life Technologies) (see section 5.2.12). DNA samples were mixed with 0.1 volume of gel loading buffer (Appendix 2) prior to electrophoresis. The duration of electrophoretic separation and the precise current densities were adjusted to allow for gel format, agarose concentration and the size of the DNA fragments under analysis. Molecular weight markers used were *Hind* III-digested phage-λ DNA and/or a 1kb DNA ladder (Life Technologies). Resolved DNA fragments were visualised and photographed over a Model TM20 short wave ultraviolet transilluminator (Ultra Violet Products).

5.2.8 Polyacrylamide Gel Electrophoresis of DNA Fragments

Polyacrylamide gel electrophoresis of small PCR-generated DNA fragments was carried out using a continuous TBE buffer system. Mini gels (see section 2.4) of 7.5% (wt/v) acrylamide and 0.2% (wt/v) bis-acrylamide in 1 x TBE were prepared. Typically, gel loadings ranged between 10-100ng of DNA. DNA samples were mixed with 0.1 volume of gel loading buffer (Appendix 2) prior to electrophoresis. Bands were visualised using a highly sensitive silver staining procedure (see section 5.2.9).

5.2.9 Silver Staining of DNA Fragments Resolved in Polyacrylamide Gels

Visualisation of DNA fragments resolved in polyacrylamide gels was carried out using a modification of the method of Herring *et al.* (1982). The procedure was based on the method previously described by Sammons *et al.* (1981). Following electrophoresis, gels were soaked in 10% (v/v) methanol, 0.5% (v/v) glacial acetic acid for 20min. The gels were then equilibrated in 12mM silver nitrate for a further

20min. After a brief rinse in DW, gels were soaked in developer solution (0.75M sodium hydroxide containing 0.75% (v/v) formaldehyde 37-42% solution) until the desired level of staining was attained. The developer was discarded and the colour reaction was stopped by soaking the gels in 70mM sodium carbonate for 30min. Typically, 50ml of each solution was used at each stage of the staining procedure. All stages of the procedure were carried out with gentle agitation on an orbital shaker. Gels were preserved as described in section 2.6.

5.2.10 Southern Transfer

Southern transfer of DNA fragments from agarose gels to Hybond nylon membrane (Amersham International) was carried out in accordance with the method described by Sambrook *et al.* (1989). The procedure was based on the method devised by and named after Southern (1975). Prior to transfer, d/s DNA fragments were reduced to single strands by soaking gels in denaturing and neutralising solutions (Appendix 2) sequentially for 1hr in each at RT. Gels were inverted and placed on a 3MM filter paper (Whatman Scientific) wick connected at both ends to a reservoir of 10 x SSC (Appendix 2). Peripheral areas of the wick not covered by the gel were blanked off with strips of polythene in order to avoid short-circuiting the transfer. A sheet of Hybond cut to the exact size of the gel was wetted in 10 x SSC and placed directly on top of the gel taking care to avoid trapping air bubbles. Positions of sample wells were marked using a black ball-point pen. Two sheets of 3MM filter paper, also cut to the size of the gel, were placed on top of the membrane. A stack of paper towels ~15cm thick was then positioned over the filter paper and a weight of ~1kg was placed on top to compress the towels. Transfer was allowed to take place overnight at RT. Membranes were removed, rinsed gently in 6 x SSC for a few seconds and the transferred DNA was fixed to the nylon by baking at 80°C in a vacuum oven.

5.2.11 DNA Dot-Blotting

DNA dot-blots were prepared in a Bio-Dot 96 well Microfiltration Apparatus (Bio-Rad Laboratories). A sheet of Hybond (Amersham International) nylon membrane

was cut to the size of the microfiltration unit and wetted in 6 x SSC prior to assembling the apparatus. Approximately 10-25ng of plasmid DNA, or up 1µg of genomic DNA, was applied to the membrane in 100µl of 6 x SSC. Samples were drawn onto the membrane under gentle vacuum and washed with a further 200µl of 6 x SSC. The membrane was removed and the DNA fixed to the nylon in a vacuum oven at 80°C for 2hr. Hybridisation was performed as described in section 5.2.18.

5.2.12 Recovery of DNA Fragments From LMP-Agarose Gels

Fragments of interest were excised from 0.8% LMP-agarose gels under ultraviolet illumination using a clean disposable scalpel. Excess agarose was trimmed away and the block was transferred to a pre-weighed plastic tube. The weight of the block was determined and 0.04 volumes of 25 x Agarase buffer (Appendix 2) added to the tube. The tube and its contents were incubated at 65°C until the agarose was completely molten. After cooling to 45°C, two units of Agarase (Boehringer Mannheim) were added for each 100mg of agarose and the digestion was incubated at 45°C for 2-3hr. Enzyme protein and remaining oligosaccharides were removed by extracting once with Tris-buffered phenol pH7.5 and twice with chloroform. Salt concentration was raised by the addition of sodium acetate to 0.3M and the DNA was precipitated by the addition of 2 volumes of chilled ethanol and overnight incubation at -20°C.

5.2.13 DNA Manipulations

Recombinant phage genomic DNA was prepared and sub-cloned into the plasmid vectors pMS1S, pGEX1λT, pBS and pUC 18 (Appendix 3) using standard laboratory procedures (Sambrook *et al.*, 1989). Sub-cloning directly from λgt 11 into pMS1S or pGEX1λT conserved the ORF and enabled expression of cloned sequences as β-gal or GST fusion proteins respectively.

5.2.14 Preparation of Plasmid DNA

1. Large scale preparations

Plasmid DNA was extracted from *E.coli* host cells using the alkaline-SDS procedure and purified subsequently by isopycnic ultracentrifugation using CsCl-ethidium bromide density gradients as described by Sambrook *et al.* (1989).

2. Small scale preparations

Small scale preparations “minipreps” of plasmid DNA were prepared from *E.coli* host cells using the QIAprep™ Spin Plasmid Miniprep Kit (Qiagen) or Wizard™ Plus Minipreps Plasmid DNA Purification System (Promega). Both kits were used in accordance with the manufacturer’s recommendations.

5.2.15 Preparation and Transformation of Competent *E.coli* Host Cells

The preparation and transformation of competent *E.coli* host cells were carried out in accordance with protocols described by Hanahan (1985).

1. Calcium chloride method

A single colony of an appropriate host strain was picked from an L-B agar plate and inoculated into 10ml of L-B broth. The culture was incubated overnight at 37°C in a shaking incubator. The overnight culture was diluted $\frac{1}{10}$ in 10ml of fresh L-B broth and grown to an OD₆₀₀ of 0.5 at 37°C in a shaking incubator. The culture was chilled on ice for 15min and the cells pelleted at 1500 x g for 10min at 4°C. The cells were resuspended in 3ml of chilled CCTB (Appendix 2) and incubated on ice for 1hr. The cells were pelleted as before and resuspended in 0.8ml of chilled CCTB. Aliquots of 200µl of competent cells were transferred to pre-chilled tubes and transforming DNA was added in a volume not exceeding 20µl. The cells and DNA were mixed by gentle swirling and the tubes incubated on ice for a further 1hr. The cells were heat shocked in a water bath for 90 seconds at 42°C, then immediately re-chilled on ice. Each transformation was mixed with 800µl of pre-warmed (37°C) SOC broth (Appendix 4) and incubated at 37°C for 1hr in a shaking incubator. Aliquots of

200µl of each transformation were spread out on each of five 90mm plates of an appropriate selective medium and incubated overnight at 37°C.

2. High efficiency method

A single colony of an appropriate host strain was picked from an SOB agar plate (Appendix 4) and inoculated into 10ml of SOC broth (Appendix 4). The culture was incubated overnight at 37°C in a shaking incubator. The overnight culture was diluted $1/10$ in 10ml of fresh SOC broth and grown to an OD_{600} of 0.5 at 37°C in a shaking incubator. The culture was chilled on ice for 15min and the cells pelleted at 1500 x g for 10min at 4°C. The supernatant was drained thoroughly and the cells resuspended in 3ml of HETB (Appendix 2) prior to incubating on ice for 15min. The cells were pelleted as before and resuspended in 0.8ml of HETB. Exactly 28µl of DnD (Appendix 2) was mixed gently into the cell suspension before incubating the tubes on ice for 10min. A second 28µl aliquot of DnD was added to the cells and the tubes returned to ice for a further 20min. Aliquots of 210µl were transferred to pre-chilled tubes and transforming DNA was added in a volume not exceeding 20µl. The tubes were incubated on ice for 40min before heat shocking the cells at exactly 42°C for 90 seconds in a water bath. The cells were transferred immediately to ice for 2min before adding 800µl of pre-warmed (37°C) SOC broth and incubating at 37°C for 1hr in a shaking incubator. Aliquots of 200µl of each transformation were spread out on each of five 90mm plates of an appropriate selective medium and incubated overnight at 37°C.

3. Commercially prepared competent cells

Commercially prepared competent *E.coli* host strains were used in accordance with the manufacturer's recommendations.

5.2.16 Radio-Isotopic Labelling of DNA

1. Random priming of double-stranded DNA fragments

Double-stranded DNA was labelled to high specific activity in accordance with the method described by Feinberg and Vogelstein, (1983 and 1984). The DNA to be labelled was denatured to single strands by heating at 100°C for 10min and subsequent cooling on ice. The following labelling reaction was assembled on ice. In a total reaction volume of 20µl, 200ng of denatured DNA was mixed with dATP, dGTP and dTTP (Boehringer Mannheim) at 0.5mM each, 400ng of random hexanucleotides (Oswel DNA Service), 25µCi (0.925MBq) of [α -³²P]-dCTP (sp.act. >3000Ci/mmole) (Amersham International) and 2 units of DNA polymerase 1 Klenow fragment (Boehringer Mannheim) in 50mM Tris/HCl pH7.2, 10mM MgCl₂, 0.1mM DTT, 0.2µg/ml BSA. The reaction was incubated at 37°C for 30min. Incorporation of labelled dCTP was monitored by TLC and autoradiography (see below). Labelled DNA was separated from unincorporated [α -³²P]-dCTP by size exclusion chromatography using disposable Sephadex[®] G-50 (NICK[™]) columns (Pharmacia Biotech) in accordance with the manufacturer's recommendations. Double stranded DNA probes were heat-denatured as described above immediately prior to use.

2. End-labelling of synthetic oligonucleotides

Synthetic oligonucleotides were labelled by transfer of the terminal phosphate group of [γ -³²P]-ATP to the 5' hydroxylated terminus of single-stranded DNA. The following labelling reaction was assembled on ice. In a total reaction volume of 50µl, ~20pmol of 5'-OH-termini (\cong 150ng of 21mer oligonucleotide) (Oswel DNA Service) was mixed with 16pmol (= 50µCi or 1.85MBq) of [γ -³²P]-ATP (sp.act. >3000Ci/mmole) (Amersham International) and 10 units of T4 PNK (Boehringer Mannheim) in 1 x T4 PNK buffer (Appendix 2). The reaction was incubated at 37°C for 30min. Incorporation of radioactive phosphate was monitored by TLC and autoradiography (see below). Unincorporated isotope was removed on Sephadex[®] G-50 columns as described above.

3. Monitoring of isotope incorporation by TLC and autoradiography

Approximately 0.2 μ l of the labelling reaction mixture was applied as a single spot to one end of a strip (10 x 1.5cm) of Polygram[®] CEL (0.1mm) 300 PEI cellulose MN300 TLC sheet (Macherey-Nagel). The strip was held vertically in a beaker containing TLCB (Appendix 2) to a depth ~5mm below the sample spot and separation was allowed to proceed for 20min at RT. The chromatogram was removed and blotted dry before wrapping in cling-film. The chromatogram was then placed in an X-ray cassette (B. Rosenberg) containing a Hi-Speed-X blue-emitting intensifying screen (Genetic Research Instrumentation) and exposed to a 13 x 18cm sheet of X-Omat S film (Kodak) for 20min at RT. Autoradiographs were processed using D-19 developer and Unifix liquid-fixer (Kodak) in accordance with the manufacturer's recommendations.

5.2.17 Colony Array Screening of Bacterial Transformants

Duplicate plates of bacterial transformants were prepared in grid-arrays of 100 transformants per 90mm plate by picking and streaking individual colonies with sterile cocktail sticks. One of the plates was overlaid with a sterile 90mm Hybond (Amersham International) circle and the plates were incubated overnight at 37°C. The identification of recombinants of interest was achieved by oligonucleotide hybridisation in combination with a rapid procedure which allowed colony lysis, DNA denaturation and fixation of DNA to the nylon membrane in a single step (Buluwela *et al.* 1989). Membranes were removed from plates and placed face down onto 3MM filter paper (Whatman Scientific) soaked in colony lysis solution (Appendix 2). Once the colonies became visibly mucoid in appearance (~2-4min), the filter paper and membranes were transferred to the glass turntable of a Matsui Model M180 TC microwave oven and irradiated for 2-3min at full power. The membranes were then washed in membrane washing solution (Appendix 2) for 2hr at 42°C in a shaking water bath. Any residual material was removed by wiping the surface of each membrane manually with a wad of tissues soaked in the wash

solution. The filters were then pre-hybridised and hybridised as described in section 5.2.18.

5.2.18 Hybridisation of Labelled DNA Probes to Membrane-Bound Target DNA

Membranes from Southern blots, dot blots, plaque lifts or colony arrays were wetted in 6 x SSC and pre-hybridised for 2hr in pre-hybridisation solution (Appendix 2). Pre-hybridisation fluid was discarded and replaced with 5-10ml (depending on the size of the membrane) of hybridisation fluid (Appendix 2) containing ~40ng/ml of labelled probe DNA (see section 5.2.16). Hybridisation took place over 18hr in sealed cylindrical Pyrex flasks in a hybridisation oven equipped with a rotisserie (Hybaid). Pre-hybridisation and hybridisation temperatures varied depending on whether a d/s DNA probe or oligonucleotide probe was in use. Generally, both pre-hybridisation and hybridisation were carried out at 65°C if a d/s DNA probe was used. However, in situations where an end-labelled oligonucleotide was the probe of choice, temperatures 10°C below the T_m of individual oligonucleotides were calculated. Following hybridisation, excess probe was removed initially, and in all cases, by 3 x 5min washes in 1 x SSC, 0.1% (wt/v) SDS at RT. For d/s DNA probes, initial washes were followed by 3 x 15min washes in 0.1 x SSC, 0.1% (wt/v) SDS at 42°C. For oligonucleotide probes, additional washes were carried out in 1 x SSC, 0.1% (wt/v) SDS at 10°C below the T_m . If non-specific hybridisation was observed after an initial autoradiographic exposure, further washes were carried out, increasing the temperature by increments of 2-3°C between subsequent autoradiographic exposures.

5.2.19 Nucleotide Sequencing

All clones were sequenced on both strands by the dideoxy-chain-termination method (Sanger *et al.*, 1977) using Sequenase Version 2.0 (Amersham International) in accordance with the manufacturer's recommendations. dCTP labelling mix and [³⁵S]-dCTPαS were chosen in order to achieve higher levels of incorporation into the G-C rich mycobacterial DNA. The Sequenase enzyme was used routinely at a

dilution of $1/4$, but the concentration was increased to $1/2$ where compressions were encountered. In order to help overcome problems associated with compressions, sequencing reactions were resolved in polyacrylamide gels consisting of 7.6% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide, 40% (v/v) deionised formamide and 7M urea in GTSB (Appendix 2). Electrophoretic conditions were set at 2980V, 40mA (constant current) and 149W. Sequencing reactions were primed using M13 universal forward and reverse primers, a pMS1S specific primer and a series of internal 18-21mer oligonucleotides. Gels were fixed in 20% (v/v) methanol, 7% (v/v) glacial acetic acid for 20min prior to lifting onto a sheet of 3MM filter paper (Whatman Scientific) and drying in a slab gel drier (Hoefer Scientific Instruments). Autoradiography was performed at RT in an X-ray cassette (B. Rosenberg) with the dried gel in direct contact with a 35 x 43cm sheet of RX medical X-ray film (Fuji Photo Film). Exposure times varied between 18hr and several days depending on the band intensities of initial autoradiographs.

5.2.20 Cyanogen Bromide (CNBr) Cleavage of HPLC Purified p40

Chemical cleavage of p40 was performed using CNBr as described by Matsudaira, (1990). Approximately 100 μ g of HPLC purified p40 was dialysed against DW and lyophilised in a Super Modulyo freeze dryer (Edward's High Vacuum). Working in a fume cupboard, the lyophilised protein was solubilised in 200 μ l of 70% (v/v) formic acid and a single crystal of CNBr (Janssen Pharmaceuticals) was dissolved in the solution. Oxygen was removed from the tube by flushing with nitrogen and the capped tube was incubated in total darkness for 18hr. The formic acid concentration was reduced to 7% by diluting with DW and the sample was lyophilised a second time. Cleaved lyophilised protein was stored at RT in a nitrogen flushed tube until required.

5.2.21 Amino-Terminal Amino Acid Sequencing

Approximately 20 μ g of HPLC purified protein was run into a 16 x 14cm 10% SDS-PAGE gel under standard electrophoretic conditions (see section 2.4). Gels were

equilibrated in sodium borate blot buffer (Appendix 2) for 20min prior to electrophoretic transfer of protein (see section 2.7.1) onto PVDF membrane in fresh borate blot buffer. Transfer took place over 1hr at 100V/1.0A (constant voltage). Membrane-bound protein was visualised by staining with Coomassie Brilliant Blue (see section 2.8.2). Bands of interest were excised and amino-terminal amino acid sequence was determined by sequential Edman degradation at the Microchemical Facility, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK.

5.2.22 Preparation of Fusion Proteins

1. Expression and purification of GST-fusion proteins

Expression and purification of GST-fusions was carried out essentially as described by Smith and Johnson, (1988). A fresh overnight culture of *E.coli* host strain AD202 transformed with a pGEX1 λ T construct of interest was diluted $1/10$ in fresh pre-warmed (37°C) L-B broth supplemented with Amp at 50 μ g/ml final concentration. (*E.coli* host strain AD202 carries a mutation in the *ompT* outer membrane associated protease (Nakano *et al.*, 1994) and has been reported to yield higher quantities of some GST-fusion proteins.) The sub-culture was incubated at 37°C for 1hr in a shaking incubator before IPTG (Boehringer Mannheim) was added to a final concentration of 1mM. Incubation was continued for a further 4hr and the cells were harvested by centrifugation at 4°C for 20min at 4,800 x g. The cell pellet was resuspended in $1/10$ volume of NETN buffer (Appendix 2). The cells were lysed on ice by sonication at 50% amplitude for 8 x 15 seconds using a Vibra-cell model VCX 600 sonicator (Sonics & Materials), allowing 1min cooling on ice between each round of sonication. The sonicate was cleared of intact cells and cell debris by centrifugation at 10,000 x g for 5min at 4°C. Glutathione-Sepharose[®] (Pharmacia Biotech) was added to the bacterial supernatant at 25 μ l/1ml and incubated at 4°C for 1hr on a rocking platform. The sepharose beads were pelleted at 1500 x g, washed x 3 in NETN buffer and finally resuspended in 1 volume of NETN buffer containing 0.5% (^w/_v) powdered milk. Bound fusion protein was analysed by PAGE after

heating a sample of the beads at 100°C for 90 seconds in 1 x LDB (Appendix 2). Proteins were visualised by Coomassie Blue or silver staining.

2. Expression and purification of β -gal fusion proteins

A fresh overnight culture of an *E.coli* host strain transformed with a pMS1S construct of interest was diluted $1/100$ in fresh pre-warmed (37°C) L-B broth supplemented with Amp at 50 μ g/ml final concentration. The sub-culture was incubated at 37°C in a shaking incubator until the OD₆₀₀ measured 0.5-0.6. IPTG was added to a final concentration of 10mM and incubation was continued for a further 4hr. Cells were pelleted at 4,800 x g for 20min at 4°C and resuspended in Buffer-A (Appendix 2). The cells were disrupted in a Mini French Pressure Cell as described in section 2.2.2 and the lysate was cleared of debris by centrifugation at 10,000 x g for 10min at 4°C. β -gal fusion protein was purified from the cleared cell lysate by affinity chromatography on p-aminobenzyl 1-thio- β -D-galactopyranoside (ABTG) agarose (Sigma-Aldrich) (Steers *et al.* 1971). The cleared cell lysate was applied to a 2ml Econo-Column (Bio-Rad Laboratories) packed with ABTG-agarose and equilibrated with Buffer-A. The column was washed sequentially with 10ml of Buffer-A, 10ml of Buffer-B (Appendix 2) and 10ml of Buffer-C (Appendix 2). Bound fusion protein was eluted from the affinity matrix with 10ml of 100mM sodium borate pH 10.0 and collected into a tube containing 1ml of 2M Tris/HCl pH7.0. The column eluate was dialysed against 10mM Tris/HCl pH8.0 overnight at 4°C. The fusion protein was concentrated by placing the dialysis bag on a bed of PEG 20,000 (Merck) until the volume had reduced to ~2ml. Protein concentration was determined as described in section 2.3.

5.2.23 Affinity Purification of Mono-Specific Polyclonal Antibodies

1. From western blotted antigens

Mono-specific polyclonal antibodies were affinity-purified from sera as described by Beall and Mitchell (1986). Approximately 200-250 μ g of absorbing antigen was run into a 16 x 14cm 10% SDS polyacrylamide gel (see section 2.4) using a single large

sample well. Following electrophoresis, the antigen was blotted onto nitrocellulose (Schleicher and Schuell) by western transfer (see section 2.7.1). The blotted antigen was visualised by staining with Ponceau-S solution (Sigma-Aldrich) and bands of interest were cut out as individual strips. Up to 4 individual strips of absorbing antigen were prepared in this way for each affinity purification run. Strips were washed for 60min in WFB (Appendix 2) prior to incubating for 3hr at RT in 10ml of serum diluted $1/40$ in WFB. Excess antibody was removed by 3 x 5min washes in fresh WFB, 1 x 30min wash in 0.1M boric acid, 0.5M NaCl pH8.0 and 1 x 30min wash in 1 x PBS (Appendix 2). Specifically bound antibody was eluted from the absorbing antigen by incubating the strips in 2ml of 0.1M glycine, 0.15M NaCl pH2.6 for 3min. The strips were removed and the pH of the eluate was immediately adjusted to 8.0 by the addition of 200 μ l of 2M Tris/HCl pH8.0. The elution step was repeated and the two resulting eluates were pooled. Sodium azide was added to 0.02% (w/v) and the affinity-purified antibody was stored at 4°C until required.

2. From plaque lifts of induced recombinant λ gt11 clones

Approximately 10^3 pfu of a plaque-purified recombinant λ gt11 clone were mixed with 100 μ l of fresh *E.coli* host strain Y1090 plating cells and plated out on a 90mm L-B agar plate using the top agarose method as described in section 5.2.4. Plates were incubated at 42°C until pin-prick plaques became visible. Expression of β -gal fusion protein was induced by overlaying each plate with a nitrocellulose circle (Schleicher & Schuell) impregnated with 10mM IPTG (Boehringer Mannheim) and incubating at 37°C overnight. Filters were removed and washed in several changes of WFB over 60min. Incubation in serum and antibody elution were carried out as described above.

5.2.24 Polymerase Chain Reaction

1. Amplification of a 311bp internal fragment of *M.avium* type A/I *bfr*

25ng of template plasmid DNA (clone pBS/R692/3.1/2) was added to pre-prepared reaction mixtures of 50 μ l (final volume) containing 10mM Tris/HCl pH8.8, 50mM

KCl, 2.5mM MgCl₂, 200µg/ml gelatin, 25mM each of dATP, dCTP, dGTP and dTTP, 20pmol primers (forward primer, 5'-GCA CTC CAA GAT GCA GGA CAA-3'; reverse primer, 5'-TGT GCT TTT CCT CGT CGG-3') (see Fig. 5.3 for positions), and 1 unit of *Taq* polymerase (Boehringer Mannheim). The reactions were covered with 100µl of mineral oil (Sigma) to prevent evaporation. PCR conditions consisted of an initial denaturation step of 5min at 94°C and 30 cycles of annealing at 55°C for 30sec, extension at 72°C for 60sec and denaturation at 94°C for 30sec. The final cycle included an extension at 72°C for 120sec.

2. Amplification of the 5' end of the p40 gene from genomic DNA

100ng of *M.avium* type A/I genomic DNA was added to pre-prepared reaction mixtures of 50µl (final volume) containing 10mM Tris/HCl pH8.8, 50mM KCl, 200µg/ml gelatin, 25mM each of dATP, dCTP, dGTP and dTTP, and 1 unit of *Taq* polymerase (Boehringer Mannheim). In a series of reactions designed to determine the optimum conditions for amplification of the target sequence, MgCl₂ concentrations were adjusted between 0.5-4.0mM in increments of 0.5mM, and primer concentrations were varied between 0.2-4.0µM. The reactions were covered with 100µl of mineral oil (Sigma) to prevent evaporation. PCR conditions were varied, and annealing temperatures were dictated by the T_m of chosen pairs of primers. Positions of primers, as predicted from a hypothetical CNBr cleavage map of p40, and their nucleotide sequences, as deduced from amino acid sequence data, are shown in Figs. 5.12c and 5.13 respectively. Generally, PCR conditions consisted of an initial denaturation step of 5min at 94°C and 30-35 cycles of annealing between 40-55°C for 30sec, extension at 72°C for 60sec and denaturation at 94°C for 30sec. The final cycle included an extension at 72°C for 120sec. Many permutations of MgCl₂ and primer concentration were used in combination with different annealing temperatures and primer pairs in both conventional and hemi-nested PCR experiments. The reader is referred to tables 5.3a and b (see section 5.3.14) for precise details of experimental conditions.

3. Amplification of the 5' end of the p40 gene from the amplified λ gt11 library

100ng of recombinant DNA, extracted from the amplified λ gt11 library, was added to pre-prepared reactions mixtures of 50 μ l (final volume) containing 10mM Tris/HCl pH8.8, 50mM KCl, 200 μ g/ml gelatin, 25mM each of dATP, dCTP, dGTP and dTTP, and 1 unit of *Taq* polymerase (Boehringer Mannheim). MgCl₂ and primer concentrations were varied as described above. The λ gt11 forward primer (5'-GGT GGC GAC GAC TCC TGG AGC CCG-3') (Promega) was used in combination with the p28-derived (M5018) reverse primer in conventional PCR experiments, or with the p31-derived (M5806) reverse primer in hemi-nested reactions. PCR conditions were similar to those described above, consisting of an initial denaturation step of 5min at 94°C and 30-35 cycles of annealing between 55-65°C for 30sec, extension at 72°C for 60sec and denaturation at 94°C for 30sec. The final cycle included an extension at 72°C for 120sec.

5.3 Results

5.3.1 Construction of a λ gt11 Expression Library (Method 1)

Following amplification in *E. coli* host strain Y1088, a pre-amplification titre of 8.5×10^4 pfu in total was increased to 1.5×10^{10} pfu/ml. A sample of 5×10^2 pfu was plated out in the presence of the chromogenic substrate X-gal and shown to comprise 82.6% recombinant phage by blue/white colour differentiation. Confirmation of this value was obtained by hybridising a plaque lift of the same 5×10^2 pfu with a [α - 32 P]-dCTP-labelled DNA probe prepared from the genomic DNA fragments used to construct the library. A mean insert fragment length of 3.96kb was calculated on the basis of sizing twenty randomly selected recombinant phage genomes by *Eco*RI digestion and agarose gel electrophoresis (data not shown).

5.3.2 Immunoscreening the Amplified Library

A total of 4×10^5 pfu and 1.2×10^6 pfu were screened using antisera from rabbits R696 and R692 respectively. Immunoscreening yielded a total of five expressing clones, λ R696/1 and λ R692/1-4. All five recombinants were plaque purified and high titre stocks prepared. Digestion with *Eco*RI and electrophoretic analysis of DNA prepared from these clones revealed that clone λ R696/1 contained an insert fragment of 2.3kb and that all four λ R692 recombinants contained an insert fragment of 7.1kb. The larger inserts each contained two internal *Eco*RI sites resulting in cleavage fragments of 3.1, 2.5 and 1.5kb (Fig. 5.1).

5.3.3 Selection of a Clone for Further Study

The expression products of all five λ clones were screened with a panel of five mabs raised against HPLC purified p40 (see section 3.2.4). The results of screening plaque lifts of each recombinant are shown in Table 5.1. On the basis of the results of mab screening, clone λ R692/2 was selected for further analysis. In order to assign the expressing region (5' end) of the 7.1kb insert to one of the three restriction fragments resulting from digestion with *Eco*RI, the individual fragments were recovered from LMP agarose blocks and subcloned into the plasmid expression vector pMS1S.

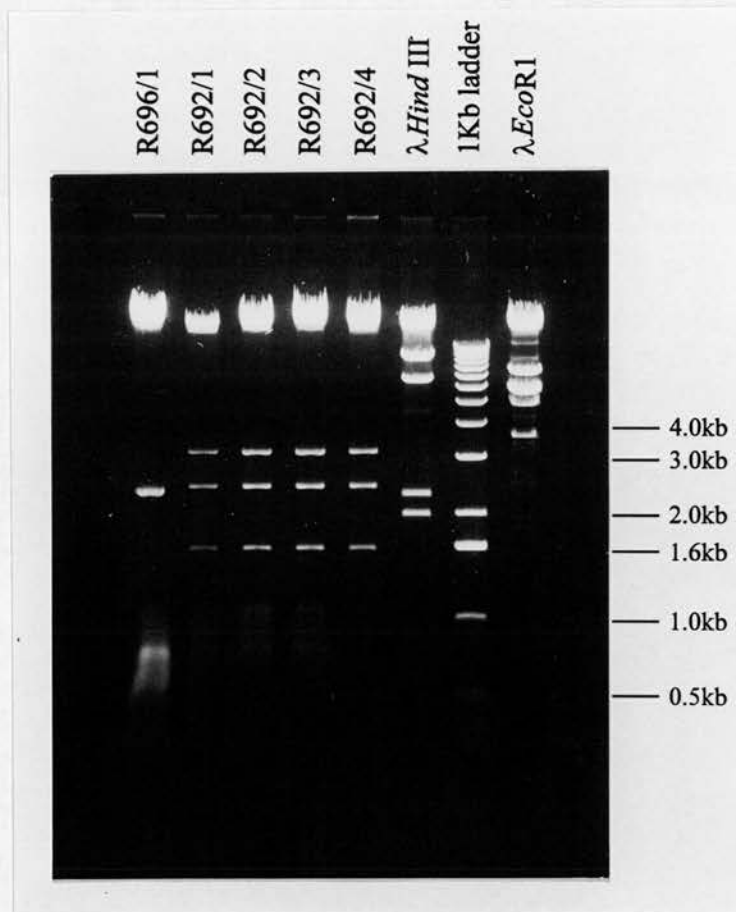


Fig. 5.1 Agarose gel showing restriction digests of DNA prepared from each of five expressing λ gt11 clones. Cleavage of the recombinant genomes with *EcoRI* revealed a single fragment of ~ 2.3 kb in clone R696/1 and identical inserts of ~ 7.1 kb in clones R692/1-4. The ~ 7.1 kb insert contained two internal *EcoRI* sites yielding fragments of ~ 3.1 , ~ 2.5 and ~ 1.5 kb. M.wt markers include a 1kb ladder and wild type λ DNA digested with 1. *Hind* III and 2. *EcoRI*.

| Monoclonal Antibody | Clone Identification No. | | | | |
|---------------------|--------------------------|------------------|------------------|------------------|------------------|
| | λ R696/1 | λ R692/1 | λ R692/2 | λ R692/3 | λ R692/4 |
| 6.26 | -ve | +/-ve | +ve | +/-ve | -ve |
| 6.36 | -ve | -ve | -ve | -ve | -ve |
| 6.56 | -ve | -ve | -ve | -ve | -ve |
| 6.65 | -ve | -ve | -ve | -ve | -ve |
| 6.66 | -ve | -ve | -ve | -ve | -ve |

Table 5.1 Results of screening plaque lifts of clones λ R696/1 and λ R692/1-4 with a panel of five mabs raised against HPLC purified p40. Signals observed to score positive are marked by +ve, while weaker or negative signals are marked +/-ve and -ve respectively.

Transformation of competent *E.coli* host strain NM522 cells yielded five transformants containing the 3.1kb fragment, two containing the 2.5kb fragment and four containing the 1.5kb fragment. Cell lysates of induced bacterial transformants were western blotted and immunoscreened with the same antiserum used to identify the original λ clone (Fig. 5.2). Two clones, both containing 3.1kb insert fragments, were shown to express β -gal fusion proteins recognised by R692 antiserum. This result indicated that the 3.1kb *EcoRI* fragment was situated at the 5' end of the parent clone. One of these clones (pMS1S/692/3.1) was selected for nucleotide sequencing.

5.3.4 Nucleotide Sequencing of Clone pMS1S/692/3.1

A study which compared the quantity and quality of sequencing template obtained from a number of different *E.coli* host strains revealed inconsistencies in the quality of template DNA prepared from different host strains (Taylor *et al.*, 1993). Template DNA amplified in strains NM522 and TG1 was noted to suffer from abnormally high backgrounds and band shadowing on sequencing gels, while that obtained from strains DH5 α , JM109 and SURE was of high quality. The study concluded that strains NM522 and TG1 should be avoided for the purpose of generating sequencing template.

Initial attempts to obtain nucleotide sequence for clone pMS1S/692/3.1 were hampered by problems similar to those described by Taylor *et al* (1993) and low yields of template DNA from both small and large scale plasmid preparations. In an attempt to circumvent these difficulties, the 3.1kb insert fragment was subcloned into the high copy number plasmid Bluescript (pBS) and the new construct (clone pBS/692/3.1) was amplified in *E.coli* host strain JM109 instead of NM522. The implementation of these changes enabled the first 725bp of the 5' end of clone pBS/692/3.1 to be determined for both strands using the dideoxy-chain termination method (Sanger *et al.*, 1977).

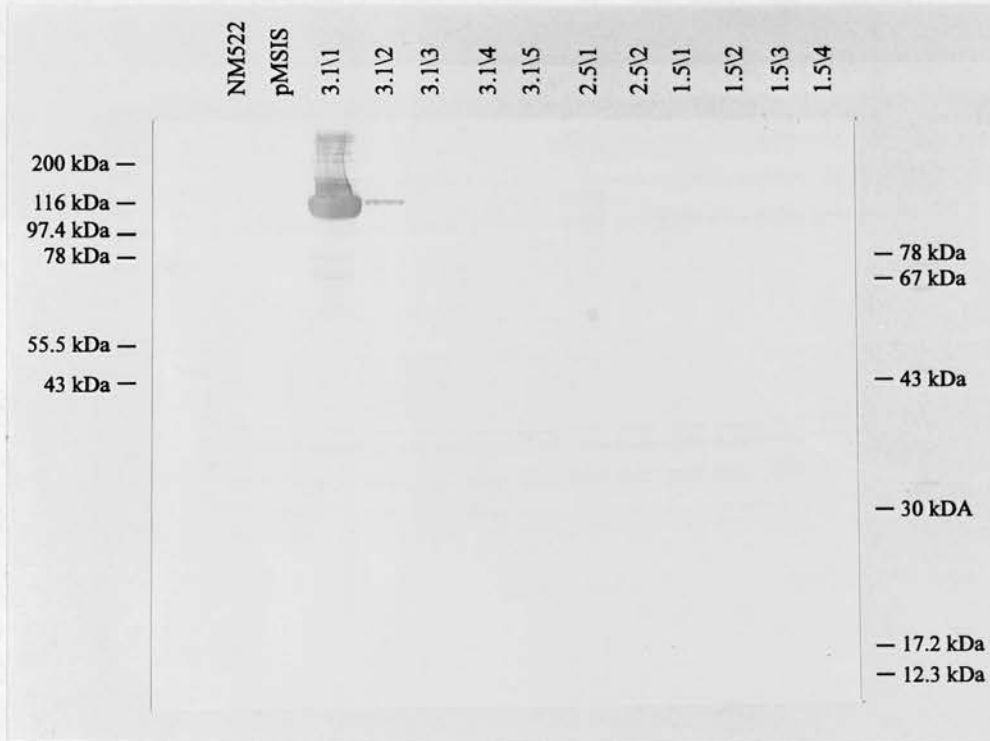


Fig. 5.2 A western blot showing cell lysates of induced pMS1S transformants immunoscreened for expression of β -gal/fusion protein using NM522-absorbed R692 antiserum. Five clones (3.1/1-5) contained a 3.1kb fragment, two clones (2.5/1-2) contained a 2.5kb fragment, and a further four clones (1.5/1-4) contained a 1.5kb fragment resulting from *Eco*RI digestion of the parent 7.1kb insert of clone λ R692/2. Two expressing clones, 3.1/1 and 3.1/2, were detected indicating that the 3.1kb *Eco*RI fragment was situated at the 5' end of the parent clone. No background signal was detected in control lysates of induced NM522 cells or transformed NM522 cells containing the empty pMS1S vector.

5.3.5 Nucleotide Sequence Analysis and Identification of a Gene Encoding the bfr Subunit of *M.avium* Type A/I

Analysis of the sequence revealed an ORF of 477bp that translated to a peptide of 159 aa residues with a theoretical mass of 17.4kDa. The predicted ATG initiation codon was preceded by a potential Shine-Dalgarno sequence from nucleotide 66-71 (Fig. 5.3).

A FASTA search of the EMBL database revealed that the 725bp cloned sequence (EMBL Acc.No. X76906) shared 80% homology with 500bp of a *M.leprae* genomic DNA sequence contained in cosmid mlb38 (Acc.No. L01095), 59% homology with 455bp of the gene encoding an iron storage protein of *Azotobacter vinelandii* (Acc.No. M83692) and 56% homology with 598bp of the *E.coli* bfr (Acc.No. M27176). DAP and SWEEP searches of the OWL database using the deduced 159 aa sequence of the cloned DNA revealed sequence identity with six bacterial proteins, the bfr subunits of *Azotobacter vinelandii* (Andrews *et al.*, 1989a), *Nitrobacter winogradskyi* (Kurokawa *et al.*, 1989), *Escherichia coli* (Andrews *et al.*, 1989b) *M.a.paratuberculosis* (antigen D) (Brooks *et al.*, 1991) *Synechocystis* P.C.C. 6806 (Laulhere *et al.*, 1992) and *Brucella melitensis* (Denoel *et al.*, 1995). Three additional bacterial proteins which are not yet available in the databases were also shown to share sequence similarity, the bfr subunit of *Rhodobacter capsulatus* (Ringeling *et al.*, 1994) and the α and β bfr subunits of *Pseudomonas aeruginosa* (Moore *et al.*, 1994). The deduced aa sequence of clone pBS/692/3.1 also shared 92% sequence identity with 162 aa residues of the translated *M.leprae* sequence (positions 20960-21439), reverse strand on cosmid mlb38). There was only a single aa substitution between this translated cosmid sequence and the first 14 amino-terminal aa residues reported for the *M.leprae* 22kDa major membrane protein (MMP-II) (Hunter *et al.*, 1990). The identity of the *M.leprae* MMP-II has been confirmed recently as a bfr (Pessolani *et al.*, 1994; Deshpande *et al.*, 1995). The first 42 amino-terminal aa residues of the nine bfr proteins listed above are compared with

10

30

50

CCCCAGGTTTCGTGCGCTCCAGCCC GGCGCACCGACCGCGGCCGAACAGCCAGCCGCTCAC

70

90

110

ACCGTAGGAGTGAAAATGCAAGGGGATCCGGAAGTTTTGCGTCTGCTCAACGAGCAGCTG

M Q G D P E V L R L L N E Q L

130

150

170

ACCACGCAACTCACCGCGATCAACCAGTACTTCTGCACTCCAAGATGCAGGACAACTGG

T T Q L T A I N Q Y F L H S K M Q D N W

190

210

230

GGGTTACCCGAGTTAGCTGAGCACACCCGGGCGGAGTCCTTCGACGAGATGCGCCACGCC

G F T E L A E H T R A E S F D E M R H A

250

270

290

GAGGCGATCACCGACCGCATCCTGCTGCTCGACGGGTTGCCGAACATATCAGCGCCTGTTT

E A I T D R I L L L D G L P N Y Q R L F

310

330

350

TCGCTGCGCATCGGCCAGACGCTGCGCGAGCAGTTCGAGGCCGACCTAGCCATCGAATAC

S L R I G Q T L R E Q F E A D L A I E Y

370

390

410

GAGGTGATGGACCGGCTCAAGCCGGCCATCATCCTGTGCCGGGAGAAGCAGGACTCCACC

E V M D R L K P A I I L C R E K Q D S T

430

450

470

ACCGCCACGCTTTTCGAGCAGATCGTCGCCGACGAGGAAAAGCACATCGACTACCTGGAG

T A T L F E Q I V A D E E K H I D Y L E

490

510

530

ACGCAGCTGGAGTTGATGGACAAGCTGGGCGTGGAGCTCTACTCGGCGCAGTGCCTGTCCG

T Q L E L M D K L G V E L Y S A Q C V S

550

570

590

CGGCCACCGAGCTGATGCCGGGGCGGCACGGCGCGCCCTCGGGGTGACGGGCTGACAGG

R P P S *

610

630

650

TCCACGCCGACAGCTGGGTACTCATAGCCGACGCCGACGCGACGGAAGGCAGCCATGACG

670

690

710

ACCCCGAGAGCAGCAGTGAATGCACCGGCCCGCGCCGATACCGGATCCGGCGGGGAGCGG

725

ATCAG

Fig. 5.3 Nucleotide sequence of the first 725bp of clone pMSIS/692/3.1 (λ R692/2), showing an ORF of 477bp (76-552) translated to a peptide of 159 aa residues. The predicted ATG initiation codon is preceded by a potential Shine-Dalgarno sequence (double-underlined) from nucleotide 66-71. Forward (157-177) and reverse (467-450) priming positions, used for PCR-amplification of a 311bp internal fragment of the *bfr* (see section 5.2.24), are underlined.

the deduced sequences of the *M.leprae* genomic sequence L01095 and clone pBS/692/3.1 (Fig. 5.4).

Additional evidence for an antigenic relationship between the expression product of clone pBS692/3.1 and the bfr proteins of mycobacteria was provided by a serological cross-reactivity study. Antibody from the serum of a sheep with clinical paratuberculosis was affinity-purified on strips of western blotted pBS692/3.1 fusion protein (see section 5.2.23). When used to probe a western blot, the affinity-purified antibody was observed to recognise purified *M.a.paratuberculosis* antigen D (~20kDa) (Brooks *et al.*, 1991), cellular antigens of ~20kDa and ~400kDa in lysates of *M.a.paratuberculosis* and *M.avium* type A/I (Inglis *et al.*, 1994b), and an antigen of approximately 20-22kDa in a *M.leprae* cell sonicate (Pessolani *et al.*, 1994; Deshpande *et al.*, 1995) (Fig. 5.5).

5.3.6 Are bfr and p40 Antigenically Related?

The isolation of clones encoding bfr using an antiserum raised against HPLC purified p40 suggested that the two proteins might be related. In order to establish whether this apparent relationship between bfr and p40 was one resulting from genuine antigenic similarity or artefactual, specific polyclonal R692 antiserum was prepared by affinity purification on western blots of β -gal/bfr fusion protein and HPLC purified p40. The antibodies eluted from these proteins were used to probe western blots of *M.avium* type A/I and *M.a.paratuberculosis* cell lysates. Antibody eluted from the β -gal/bfr fusion protein reacted with proteins of ~20kDa in both *M.avium* type A/I and *M.a.paratuberculosis*, but did not recognise the p40 antigen (Fig. 5.6a). Antibody eluted from HPLC purified p40 reacted only with the 40kDa protein in *M.avium* type A/I with no evidence of any specific cross-reactivity with any other protein (Fig. 5.6b). In addition, neither of these affinity-purified antibodies recognised the β -gal fusion protein expressed by the 2.3kb insert contained in clone λ R696/1. This result suggested that clone λ R696/1 encoded a protein antigen

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|-----------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| <i>λR692</i> | M | Q | G | D | P | E | V | L | R | L | L | N | E | Q | L | T | T | Q | L | T | A |
| <i>mlb38cos</i> | M | Q | G | D | P | D | V | L | R | L | L | N | E | Q | L | T | S | E | L | T | A |
| <i>M.ptb</i> | M | Q | G | D | P | E | V | L | R | L | L | N | E | Q | L | T | S | E | L | T | A |
| <i>Nit.wi</i> | M | K | G | D | P | K | V | I | D | Y | L | N | K | A | L | R | H | E | L | T | A |
| <i>Syny. 3</i> | M | K | G | K | P | A | V | L | A | Q | L | H | K | L | L | R | G | E | L | A | A |
| <i>Azo.vi</i> | M | K | G | D | K | I | V | I | Q | H | L | N | K | I | L | G | N | E | L | I | A |
| <i>E.coli</i> | M | K | G | D | T | K | V | I | N | Y | L | N | K | L | L | G | N | E | L | V | A |
| <i>B.meli</i> | M | K | G | E | P | K | V | I | E | R | L | N | E | A | L | F | L | E | L | G | A |
| <i>R.caps</i> | M | K | G | D | A | K | V | I | E | F | - | - | - | - | - | - | - | - | - | - | - |
| <i>P.aeru α</i> | M | Q | G | H | P | E | V | I | D | Y | L | N | T | L | L | T | G | E | L | A | A |
| <i>P.aeru β</i> | M | K | G | D | K | K | V | I | Q | H | L | N | K | I | L | G | N | E | L | I | A |

| | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| <i>λR692</i> | I | N | Q | Y | F | L | H | S | K | M | Q | D | N | W | G | F | T | E | L | A | E |
| <i>mlb38cos</i> | I | N | Q | Y | F | L | H | S | K | M | Q | E | N | W | G | F | T | E | L | A | E |
| <i>M.ptb</i> | I | N | Q | Y | F | L | H | S | K | M | Q | D | N | W | G | F | T | E | L | A | E |
| <i>Nit.wi</i> | I | N | Q | Y | W | L | H | Y | R | L | L | D | N | W | G | I | K | D | L | A | K |
| <i>Syny. 3</i> | R | D | Q | Y | F | I | H | S | R | M | Y | Q | D | W | G | L | E | K | L | Y | S |
| <i>Azo.vi</i> | I | N | Q | Y | F | L | H | A | R | M | Y | E | D | W | G | L | E | K | L | G | K |
| <i>E.coli</i> | I | N | Q | Y | F | L | H | A | R | M | F | K | N | W | G | L | K | R | L | N | D |
| <i>B.meli</i> | V | N | Q | Y | W | L | H | Y | R | L | L | N | D | W | G | Y | T | R | L | A | K |
| <i>R.caps</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| <i>P.aeru α</i> | R | D | Q | Y | F | I | H | S | R | M | Y | E | D | W | G | F | S | K | L | Y | E |
| <i>P.aeru β</i> | I | N | Q | Y | F | L | H | S | R | M | W | N | D | W | G | L | K | R | L | Y | A |

Fig. 5.4 Multiple alignment of the first 42 amino-terminal amino acid residues of known bacterioferritins. Black boxing indicates complete identity, shaded boxing indicates conserved amino acids. Accession numbers for the OWL database are given in parenthesis. Symbols: *λR692*, translation of the *M.avium* type A/I genomic sequence contained in clone *λR696/2* (X76906); *mlb38cos*, translation of the *M.leprae* genomic sequence L01095, reverse strand; *M.ptb*, *M.a.paratuberculosis* (A44893); *Nit.wi*, *Nitrobacter winogradskyi* (P13570); *Syny. 3*, *Synechocystis* P.C.C. 6803 (P24602); *Azo.vi*, *Azotobacter vinelandii* (P22759); *E.coli*, *Escherichia coli* (P11056); *B.meli*, *Brucella melitensis* 16M strain (U19760); *R.caps*, *Rhodobacter capsulatus* (no accession number available); *P.aeru α* and *P.aeru β*, *Pseudomonas aeruginosa* Bfr alpha and beta subunits (no accession numbers available).

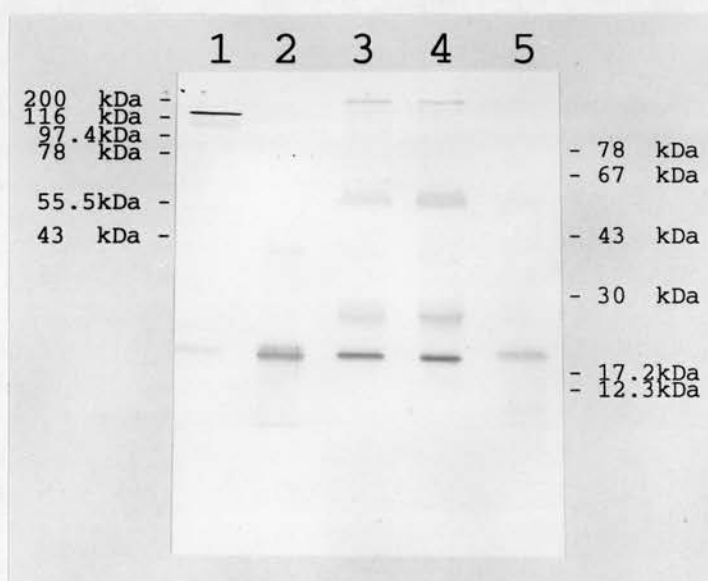


Fig. 5.5 Western blot of mycobacterial proteins reacted with affinity purified ovine antibody. Lane 1, purified λ R692/ β -gal fusion protein; lane 2, purified *M.a.paratuberculosis* antigen D; lane 3, *M.a.paratuberculosis* cell lysate; lane 4, *M.avium* type A/I cell lysate; lane 5, *M.leprae* cell sonicate. Bands of ~20kDa and ~400kDa in lanes 3 and 4 probably represent the monomeric and multimeric forms of bacterioferritin respectively. The additional faint bands visible at ~25kDa and ~55kDa in lanes 3 and 4 are artefacts of the antibody elution protocol. They appear consistently and regardless of the antigenic identity of the recombinant protein used for affinity purification.

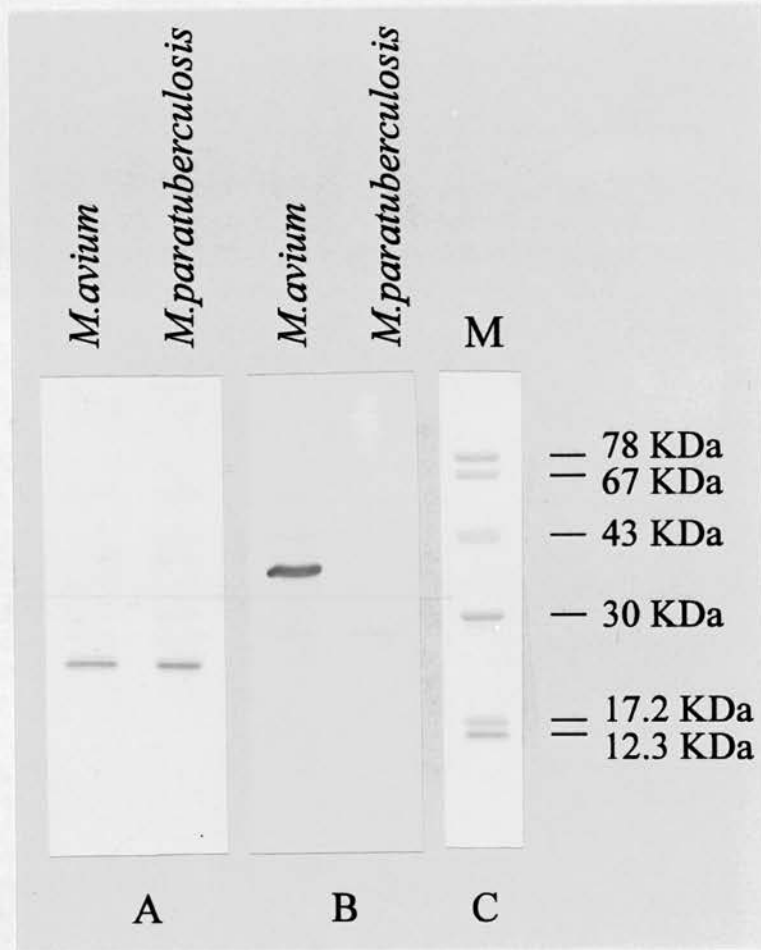


Fig. 5.6 Identical western blots showing cell lysates of *M. avium* type A/I and *M. a. paratuberculosis* probed with antibodies affinity purified on R692/2 fusion protein or HPLC purified p40. Block A, a band of ~20kDa, corresponding to the bfr subunit, was recognised by R692/2-purified antibody in both *M. avium* type A/I and *M. a. paratuberculosis*. Block B, a single band of 40kDa was recognised by p40-purified antibody in *M. avium* type A/I only. Block C, molecular weight markers stained with Ponceau S. No evidence to support any antigenic relatedness between the bfr subunit and p40 was obtained.

other than the *bfr* encoded by clones λ R692/1-4, but yet another which did not appear to be antigenically related to p40 (see sections 5.3.8 and 5.3.9).

5.3.7 Do All Five λ Clones Contain the *bfr*?

A PCR was designed to amplify a 311bp internal region of the 477bp *bfr* ORF. The resulting amplification product was labelled with [α -³²P]-dCTP by random priming and used to probe a dot blot containing DNA prepared from all five λ clones. Clones λ R696/1-4 reacted positively whilst clone λ R696/1 was negative. Both *M.avium* type A/I and *M.a.paratuberculosis* genomic DNA reacted positively whilst wild type λ DNA and a clone containing the *M.a.paratuberculosis hsp70* (Stevenson *et al.*, 1991), which was included as a G/C rich control, were negative (data not shown).

5.3.8 Further Immunoscreening of the Genomic DNA Library Using Antiserum Absorbed With a *M.a.paratuberculosis* Cell Lysate

Exhaustive absorption of R696 antiserum with crude cell lysates of *E.coli* host strain Y1090 and *M.a.paratuberculosis* yielded an immune reagent which could not be seen to react with any proteins other than p40 in western blots of *M.avium* type A/I and *M.a.paratuberculosis* (data not shown). Further immunoscreening of the library resulted in the isolation of 15 additional expressing clones (λ R696/2-16). In order to establish the M.wt. of the corresponding *M.avium* type A/I antigens encoded by these clones, antibody eluted from plaque lifts of each of the 15 recombinants was reacted against strips of western blotted *M.avium* type A/I cell lysate. The results of this experiment were inconclusive, as multiple faint bands, covering a broad size range, were observed (Fig. 5.7).

In order to eliminate the possibility that further *bfr* clones had been isolated, the 311bp *bfr*-specific PCR fragment was hybridised against a dot-blot of DNA prepared from all 15 recombinants. None of the clones were recognised by the *bfr*-specific

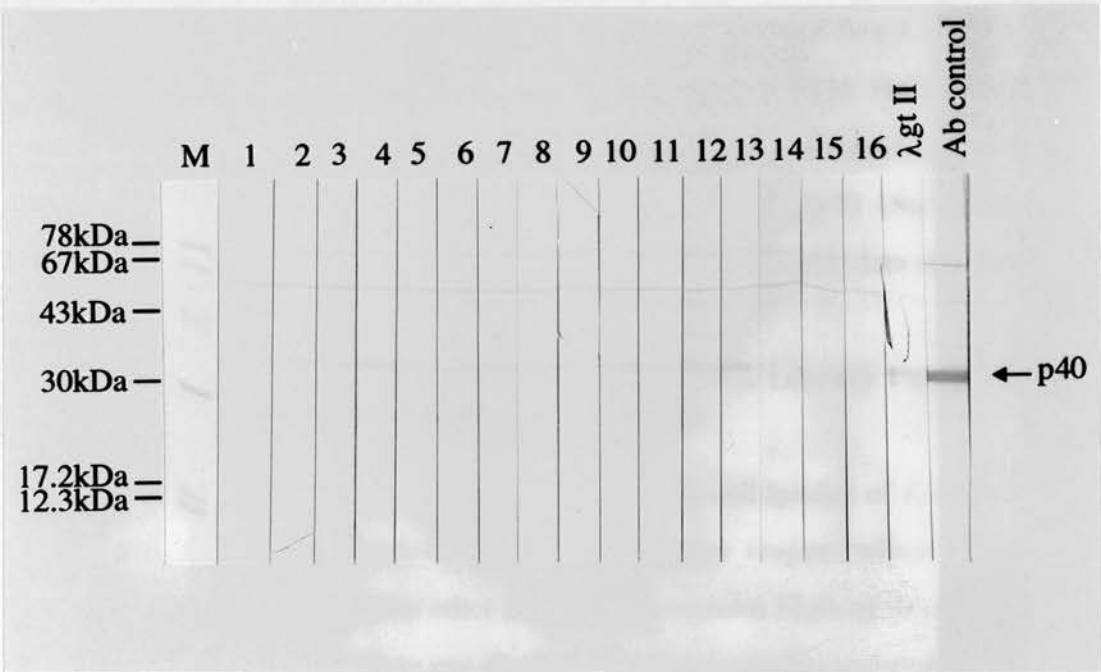


Fig. 5.7 Western blotted *M. avium* type A/I cell lysate cut in to strips and probed with one each of 16 individual affinity-purified antibodies prepared on plaque lifts of each of 16 induced λ gt11 recombinants (λ R696/1-16). Antibody affinity-purified on native (non-recombinant) λ gt11, and a $1/80$ dilution of whole R696 antiserum were included as negative and positive controls respectively. Multiple faint bands were observed on all strips, including the λ gt11 control. Only the R696 antiserum control was observed to recognise the p40 antigen.

probe, whereas clone pBS/692/3.1 and *M.avium* type A/I genomic DNA reacted positively (data not shown).

Digestion with *EcoRI* and electrophoretic analysis of the 15 recombinant genomes revealed that all contained a single insert fragment of ~2.3kb, the same size of insert fragment as that contained in clone λ R696/1 (see Fig.5.1). Insert DNA from λ R696/1 was purified and hybridised against a dot-blot of DNA prepared from clones λ R696/2-16. All 15 clones reacted positively indicating that the clones λ R696/1-16 contained identical insert fragments (data not shown).

The insert fragment from clone λ R696/1 was subcloned into plasmid pGEX1 λ T (clone pGEX/696/2.3) and expressed as a GST fusion protein in *E.coli* host strain AD202 (see section 5.2.22). Purified GST fusion protein (~80kDa) was western blotted and used to affinity-purify antibody from R696 antiserum. A series of cross-reactivity experiments was designed, primarily to ascertain whether an antigenic relationship between p40 and the λ R696/1 expression product could be demonstrated, but also to identify the corresponding natural antigen in western blots of *M.avium* type A/I cell lysates:-

1. antibody affinity-purified on pGEX/696/2.3 GST/fusion protein was used to probe a western blot containing *M.avium* type A/I and *M.a.paratuberculosis* cell lysates, and pGEX/696/2.3 GST/fusion protein (Fig. 5.8a). The affinity-purified antibody did not recognise HPLC purified p40 in either denatured (western blotted *M.avium* type A/I cell lysate) or non-denatured (dot blotted HPLC-purified p40) states, but did detect a cellular protein of ~65kDa on western blots of both *M.avium* type A/I and *M.a.paratuberculosis* cell lysates.

2. antibody affinity-purified on western blots of HPLC-purified p40 was used to probe a western blot containing pGEX/696/2.3 GST/fusion protein and HPLC-purified p40. The affinity-purified antibody did not react with pGEX/696/2.3

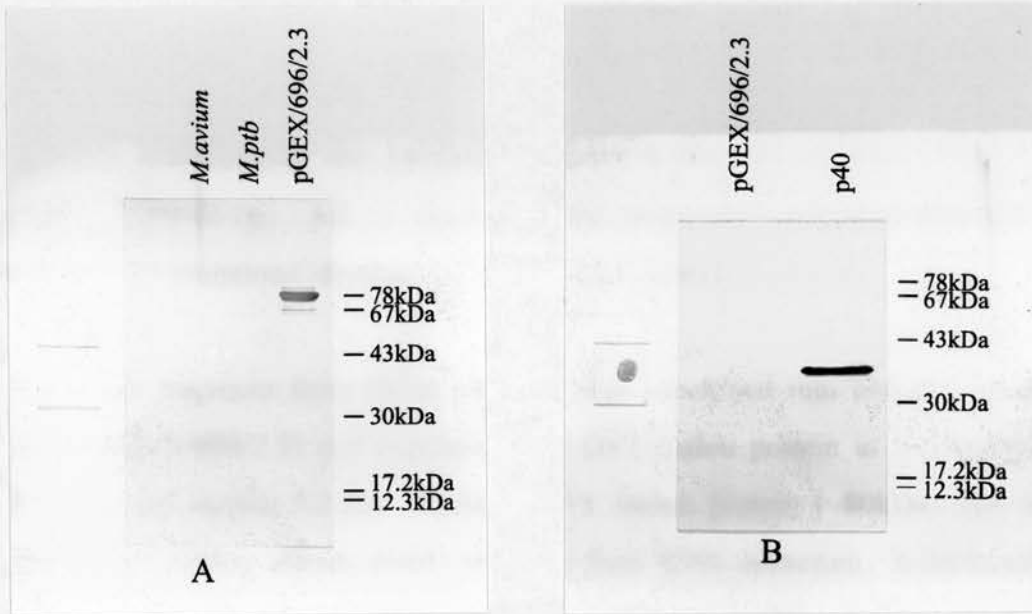


Fig. 5.8 Western and dot-blots to establish whether serological cross-reactivity exists between p40 and the recombinant protein of clones λ R696/1-16, and to identify a corresponding cellular antigen in mycobacterial lysates.

a) Western blot of *M. avium* type A/I and *M. a. paratuberculosis* cell lysates, and pGEX/696/2.3 GST/fusion protein probed with R696 antiserum affinity-purified on pGEX/696/2.3 GST fusion protein. The affinity-purified antibody recognised the GST/fusion protein (positive control) and a cellular antigen of ~65kDa in both *M. avium* type A/I and *M. a. paratuberculosis*, but did not react with p40 in the *M. avium* type A/I cell lysate or in a dot blot of non-denatured HPLC purified p40.

b) Western blot of HPLC purified p40 and pGEX/696/2.3 GST fusion protein probed with R696 antiserum affinity-purified on western blots of HPLC purified p40. The affinity-purified antibody recognised both denatured (western blotted) and non-denatured (dot blotted) p40, but did not react with the GST fusion protein.

GST/fusion protein, but detected p40 in both denatured (western blotted) and non-denatured (dot-blotted) states (Fig. 5.8b).

5.3.9 Do Clones λ R696/1-16 Encode a *M.avium* type A/I Heat Shock Protein?

In order to investigate whether the 65kDa cellular antigen recognised by pGEX/696/2.3 affinity-purified antibody was a hsp, four anti-hsp mabs, CosII and Hat 3 (hsp71, *M.tuberculosis*) and Y1.2 and D7C (hsp65, *M.leprae*), were reacted against western blotted pGEX/696/2.3 GST/fusion protein. Two of the four mabs (Y1.2 and D7C) detected the fusion protein (Fig. 5.9), indicating that clones λ R696/1-16 encoded a protein related to the *M.avium* type A/I hsp65. To support this possibility a 20mer oligonucleotide (982J), derived from the 5' end (positions 240-259) of the *M.avium* hsp65 (Hance *et al.*, 1989), was end-labelled and hybridised against the R696/1 insert fragment. However, oligonucleotide 982J failed to hybridise with R696/1 DNA, but reacted positively with *M.avium* type A/I genomic DNA (data not shown). In addition, none of the four anti-hsp mabs could be shown to recognise HPLC-purified p40 (data not shown). In view of the lack of demonstrable identity between p40 and the expression product of clone pGEX696/2.3, the discrepancy between the mab and hybridisation results was not investigated further as it was considered to lie beyond the remit of this project.

5.3.10 Preparation and Immunoscreening of a Second "Unamplified" *M.avium* Type A/I Genomic DNA Expression Library in λ gt11

In an attempt to circumvent the possibility that clones expressing p40 could be toxic to the host cell and lost or underrepresented following amplification of the library, a second genomic DNA expression library was prepared in λ gt11 using randomly generated fragments ranging from ~1-3kb in size. It was established that this library contained a total of 6.38×10^5 pfu. The entire library was immunoscreened without amplification using an *M.a.paratuberculosis*-absorbed antiserum which recognised only p40 in western blots of *M.avium* type A/I cell lysates. No clones expressing the p40 antigen were detected.

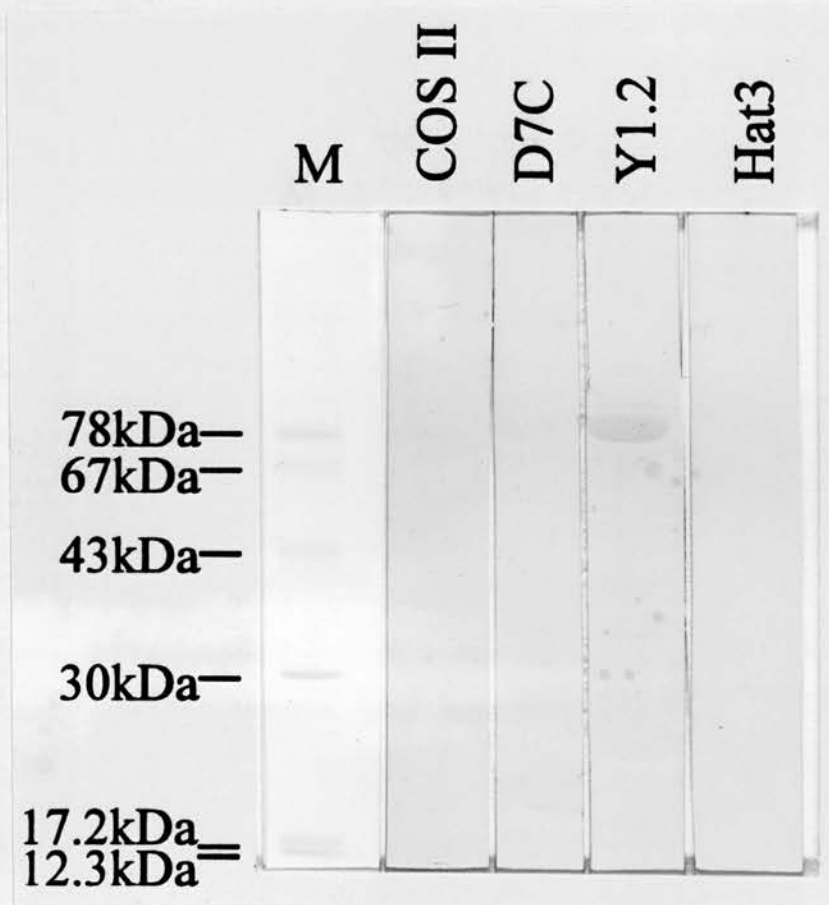


Fig. 5.9 Strips of Western blotted pGEX692/2.3 GST fusion protein each probed with one of four anti-hsp mabs:- Cos II and Hat 3, anti *M. tuberculosis* hsp71; Y1.2 and D7C, anti *M.leprae* hsp65. Two of the four mabs, Y1.2 and D7C, recognised the recombinant protein although the reaction between mab D7C and the fusion protein was weak.

5.3.11 Chemical Cleavage of HPLC Purified p40

The lack of success in immune-based detection of recombinants expressing p40 necessitated consideration of different cloning strategies, one of which was detection by nucleic acid hybridisation. Unfortunately, the absence of any nucleotide sequence data for the p40 gene precluded the design and synthesis of suitable DNA probes. However, together with knowledge of mycobacterial codon usage, the deduction of nucleotide sequences from aa sequence data represented a means by which the absence of nucleotide sequence data could be circumvented. To this end, purified p40 was cleaved into a series of smaller peptides and both amino-terminal and internal aa sequences obtained by Edman degradation.

Protein molecules, denatured by formic acid treatment, can be cleaved specifically on the carboxyl side of methionine residues by CNBr (Matsudaira, 1990). As the average number of methionine residues in any given protein is relatively low (~1-2%), CNBr cleavage normally yields a smaller and more manageable number of peptides than would result from proteolytic digestion with trypsin, for example. CNBr treatment of p40 resulted in the generation of a number of smaller fragments, including three at ~12kDa (p12), ~28kDa (p28) and ~31kDa (p31), which were recovered and submitted for aa sequencing, and a faint band of ~15kDa which was not present in quantities sufficient for sequencing (Fig. 5.10). Amino acid sequence was obtained for the p12, p28 and p31 peptides by Edman degradation (Fig. 5.11). One of these (p12) shared an identical sequence with that of uncleaved p40. However, none of the three aa sequences obtained were shown to share any significant degree of sequence identity with any peptide sequence available in the OWL database at that time, including the ORF contained within the genomic insertion sequence IS901/902.

A hypothetical map of p40 was postulated on the basis of the sequence data obtained and the CNBr cleavage profile. (Fig. 5.12).

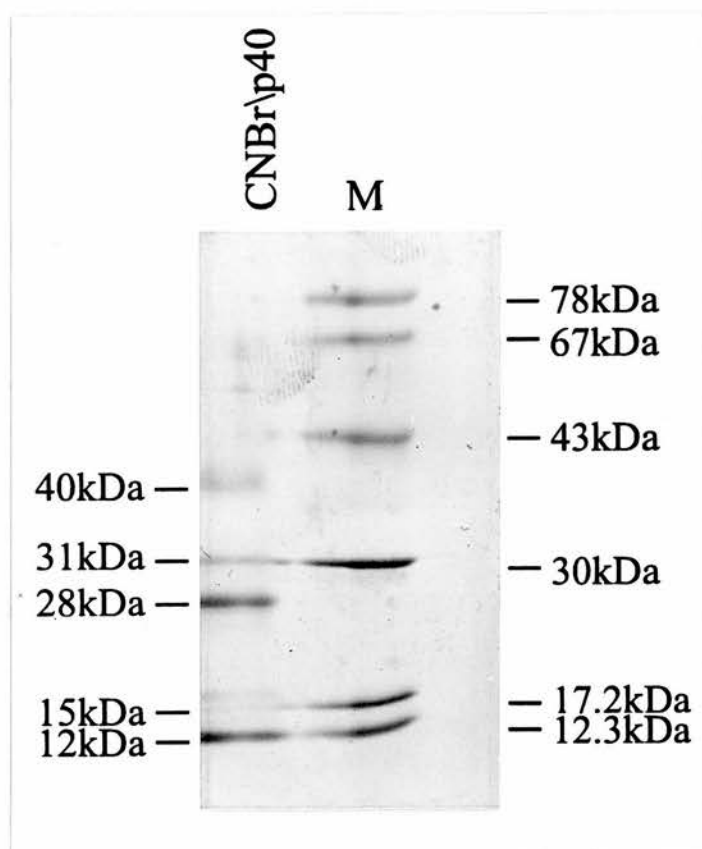


Fig. 5.10 Coomassie blue stained Western blot of HPLC purified p40 following chemical cleavage with CNBr. Cleavage peptides can be seen at ~31, ~28, ~15 and ~12kDa.

p40 T S D G Y A R I R E G G P Y F D D L S V (20)
P12 T S D G Y A R I R E (10)
P28 L P A S P E F T A E A G S V (14)
P31 I T I D Q A D Q L V P D F Y R G G G L P (20)

Fig. 5.11 Amino terminal amino acid sequences obtained by Edman degradation for p40 and the p12, p28 and p31 CNBr cleavage peptides. Stretches of seven contiguous residues used for the deduction of oligonucleotides are underlined.

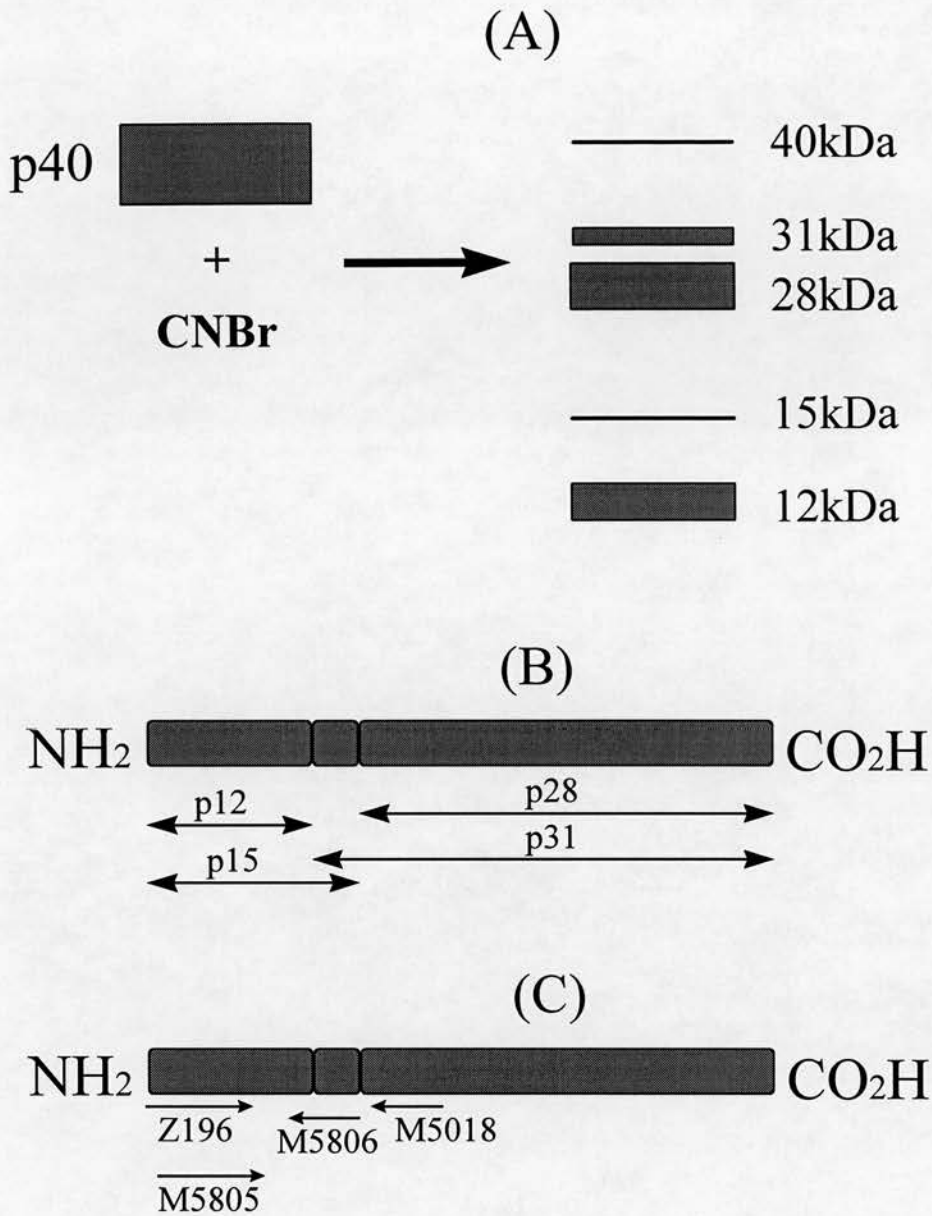


Fig. 5.12 (A) Diagrammatic representation of the peptides resulting from CNBr cleavage of HPLC purified p40. (B) A hypothetical map of p40 was formulated on the distribution of the fragments and amino acid sequence data obtained on the p40 amino-terminus and the p12, p28 and p31 cleavage products. (C) Arrows indicate positions and 5'-3' direction of four oligonucleotides deduced from amino terminal and internal amino acid sequence data (see Fig. 5.13).

5.3.12 Deduction of Oligonucleotides From Amino-Terminal and Internal Amino Acid Sequences

A mycobacterial codon usage table (Table 5.2) was compiled using information derived from the translations of 19 MAC gene sequences currently held in the EMBL database, and the UWGCG program "Codon Frequency" available on the SEQNET node, SERC, Daresbury laboratory. One non-degenerate (Z196) and three degenerate (M5805, M5806 and M5018) oligonucleotides of 20 or 21bp in length were deduced from the amino-terminal and internal amino acid sequences obtained (see Figs. 5.12c and 5.13). These oligonucleotides were used subsequently :-

1. as end-labelled probes to screen the amplified λ gt11 library by DNA hybridisation,
2. as primers in reactions designed to amplify the 5' end of the p40 gene from genomic DNA by PCR,
3. as end-labelled probes to screen Southern blots of restriction endonuclease digested genomic DNA for fragments carrying all or part of the p40 gene.

5.3.13 Screening of the Genomic DNA Library By Hybridisation Using End-Labelled Oligonucleotide Probes

A further 2.4×10^6 pfu of the amplified library were screened with individual end-labelled oligonucleotides, but no hybridising clones were detected.

5.3.14 Experiments To Amplify the 5' End of the p40 Gene From Genomic DNA by PCR

Oligonucleotide primers deduced from internal aa sequences, have been used in combination with PCR to isolate the *M.tuberculosis* ribonucleotide reductase gene from genomic DNA (Yang *et al.*, 1994) and the *M.leprae bfr* from one of a series of overlapping cosmid clones (Pessolani *et al.*, 1994). This type of approach was adopted in an attempt to amplify the 5' end of the p40 gene from *M.avium* type A/I genomic DNA in a series of PCR experiments which spanned a broad range of different reaction conditions. All the reactions used combinations of forward and reverse primers whose positions on the p40 gene were presumed from a hypothetical

| a.a. | codon | <i>E.coli</i> | MAC | <i>M.tb</i> | <i>M.lep</i> | a.a. | codon | <i>E.coli</i> | MAC | <i>M.tb</i> | <i>M.lep</i> |
|------|-------|---------------|------|-------------|--------------|------|-------|---------------|------|-------------|--------------|
| Phe | TTT | 0.26 | 0.06 | 0.08 | 0.20 | Ser | TCT | 0.36 | 0.02 | 0.04 | 0.19 |
| Phe | TTC | 0.74 | 0.94 | 0.92 | 0.80 | Ser | TCC | 0.34 | 0.25 | 0.22 | 0.19 |
| Leu | TTA | 0.03 | 0.00 | 0.01 | 0.01 | Ser | TCA | 0.02 | 0.03 | 0.04 | 0.16 |
| Leu | TTG | 0.04 | 0.10 | 0.18 | 0.20 | Ser | TCG | 0.05 | 0.35 | 0.35 | 0.20 |
| Leu | CTT | 0.06 | 0.05 | 0.02 | 0.09 | Pro | CCT | 0.10 | 0.04 | 0.06 | 0.17 |
| Leu | CTC | 0.08 | 0.27 | 0.19 | 0.17 | Pro | CCC | 0.01 | 0.37 | 0.28 | 0.18 |
| Leu | CTA | 0.01 | 0.01 | 0.04 | 0.10 | Pro | CCA | 0.12 | 0.10 | 0.08 | 0.17 |
| Leu | CTG | 0.79 | 0.57 | 0.57 | 0.44 | Pro | CCG | 0.77 | 0.49 | 0.58 | 0.47 |
| Ile | ATT | 0.20 | 0.09 | 0.19 | 0.23 | Thr | ACT | 0.36 | 0.04 | 0.06 | 0.15 |
| Ile | ATC | 0.79 | 0.88 | 0.77 | 0.66 | Thr | ACC | 0.48 | 0.67 | 0.64 | 0.46 |
| Ile | ATA | 0.01 | 0.02 | 0.04 | 0.11 | Thr | ACA | 0.05 | 0.05 | 0.06 | 0.15 |
| Met | ATG | 1.00 | 1.00 | 1.00 | 1.00 | Thr | ACG | 0.10 | 0.24 | 0.24 | 0.23 |
| Val | GTT | 0.44 | 0.06 | 0.10 | 0.11 | Ala | GCT | 0.37 | 0.08 | 0.09 | 0.18 |
| Val | GTC | 0.09 | 0.44 | 0.43 | 0.47 | Ala | GCC | 0.10 | 0.50 | 0.49 | 0.43 |
| Val | GTA | 0.27 | 0.06 | 0.05 | 0.11 | Ala | GCA | 0.25 | 0.07 | 0.09 | 0.13 |
| Val | GTG | 0.19 | 0.43 | 0.42 | 0.31 | Ala | GCG | 0.28 | 0.34 | 0.33 | 0.26 |

| a.a. | codon | <i>E.coli</i> | MAC | <i>M.tb</i> | <i>M.lep</i> | a.a. | codon | <i>E.coli</i> | MAC | <i>M.tb</i> | <i>M.lep</i> |
|------|-------|---------------|------|-------------|--------------|------|-------|---------------|------|-------------|--------------|
| Tyr | TAT | 0.26 | 0.15 | 0.14 | 0.26 | Cys | TGT | 0.36 | 0.27 | 0.26 | 0.44 |
| Tyr | TAC | 0.74 | 0.85 | 0.86 | 0.74 | Cys | TGC | 0.64 | 0.73 | 0.74 | 0.56 |
| His | CAT | 0.20 | 0.31 | 0.24 | 0.19 | Trp | TGG | 1.00 | 1.00 | 1.00 | 1.00 |
| His | CAC | 0.80 | 0.69 | 0.76 | 0.81 | Arg | CGT | 0.67 | 0.10 | 0.18 | 0.27 |
| Gln | CAA | 0.18 | 0.17 | 0.18 | 0.27 | Arg | CGC | 0.30 | 0.49 | 0.46 | 0.47 |
| Gln | CAG | 0.82 | 0.83 | 0.82 | 0.73 | Arg | CGA | 0.01 | 0.09 | 0.10 | 0.06 |
| Asn | AAT | 0.08 | 0.15 | 0.13 | 0.22 | Arg | CGG | 0.00 | 0.29 | 0.21 | 0.16 |
| Asn | AAC | 0.92 | 0.85 | 0.88 | 0.78 | Ser | AGT | 0.04 | 0.05 | 0.04 | 0.04 |
| Lys | AAA | 0.71 | 0.14 | 0.10 | 0.25 | Ser | AGC | 0.19 | 0.30 | 0.31 | 0.22 |
| Lys | AAG | 0.29 | 0.86 | 0.90 | 0.75 | Arg | AGA | 0.01 | 0.01 | 0.00 | 0.02 |
| Asp | GAT | 0.36 | 0.19 | 0.17 | 0.29 | Arg | AGG | 0.00 | 0.02 | 0.05 | 0.02 |
| Asp | GAC | 0.64 | 0.81 | 0.83 | 0.71 | Gly | GGT | 0.54 | 0.22 | 0.25 | 0.35 |
| Glu | GAA | 0.76 | 0.28 | 0.19 | 0.31 | Gly | GGC | 0.42 | 0.60 | 0.54 | 0.48 |
| Glu | GAG | 0.24 | 0.72 | 0.81 | 0.69 | Gly | GGA | 0.01 | 0.07 | 0.07 | 0.06 |
| | | | | | | Gly | GGG | 0.03 | 0.12 | 0.14 | 0.10 |

Table 5.2 A comparison of codon usage in genes of members of the MAC, *M.tuberculosis*, *M.leprae* and *E.coli*. The data for *E.coli* were based on those of Grosjean and Fiers (1982) and the values shown are for genes which are known to be highly expressed, namely the RNA polymerase subunits, the ribosomal proteins, the translation elongation factor EF-Tu, the RecA protein and a number of outer membrane proteins. The data for the MAC members were compiled using information derived from the translations of MAC genes currently in the EMBL database and the UWGCG program "Codon Frequency" available on the SEQNET node, SERC, Daresbury laboratory. The data for *M.tuberculosis* and *M.leprae* are based on the codon usage of nine and five genes respectively and are quoted directly from Dale and Patki (1990). Frequency of codon usage is expressed as a fraction of the total number of occurrences of each triplet.

| | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------|------------|------------|------------|------------|------------|------------|------------|
| (p40/p12) | Asp | Gly | Tyr | Ala | Arg | Ile | Arg |
| | GAC | GGC | TAC | GCC | CGC | ATC | CGC |
| | T | T | T | G | G | T | G |
| | | G | | T | T | A | T |
| | | A | | A | A | | A |
| | | | | | A G | | A G |
| | | | | | A A | | A A |
| Z196 | 5' GAC | GGC | TAC | GCC | CGC | ATC | CGC 3' |
| M5805 | 5' GAY | GGY | TAY | GCS | CGI | ATY | CG 3' |

| | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|--------------|------------|------------|------------|------------|------------|------------|------------|
| (p31) | Asp | Phe | Tyr | Arg | Gly | Gly | Gly |
| | GAC | TTC | TAC | CGC | GGC | GGC | GGC |
| | T | T | T | G | T | T | T |
| | | | | T | G | G | G |
| | | | | A | A | A | A |
| | | | | A G | | | |
| | | | | A A | | | |
| M5806 | 3' CTG | AAG | ATG | GCS | CCI | CCI | CC 5' |

| | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|--------------|------------|------------|------------|------------|------------|------------|------------|
| (P28) | Pro | Glu | Phe | Thr | Ala | Glu | Ala |
| | CCG | GAG | TTC | ACC | GCC | GAG | GCC |
| | C | A | T | G | G | A | G |
| | A | | | A | T | | T |
| | T | | | T | A | | A |
| M5018 | 3' GGS | CTC | AAG | TGG | CGS | CTC | CGS 5' |

Fig. 5.13 Deduction of oligonucleotides (20 or 21mers) from aa sequence data. Stretches of seven contiguous residues were selected from the amino-terminal aa sequences obtained for p40 and the p31, p28 and p12 CNBr cleavage peptides (see Fig. 5.12). Preferred codons (see Table 5.2) and degeneracy are shown below the amino acids. Non-degenerate oligonucleotides were designed from preferred codons only, while degenerate oligonucleotides were based on preferred codons but incorporated "S" where either C or G was the preferred third position, "Y" where C or T was the preferred third position and "I" (inosine) where the third position exhibited four fold degeneracy and C or G were not the two preferred bases.

CNBr cleavage map of the purified protein (see Fig. 5.12). Various permutations of primer, Mg^{2+} and template concentration and different annealing temperatures were used in both conventional and hemi-nested PCRs. Multiple bands were observed in reactions using primers derived from the p40 amino-terminus and p28 internal region, especially in those where degenerate oligonucleotide M5805 ($T_m = 43^\circ C$ in 0.1M NaCl) was used, and annealing temperatures were set in favour of the primer with the lower T_m (see Table 5.3a and b). Based on the assumption that the CNBr cleavage map of p40 (Fig. 5.12b) was correct, Southern blotting experiments to confirm the identity of the PCR products were undertaken using oligonucleotide M5806, (p31, degenerate), a primer predicted to be internal to the region amplified by the p40- and p28-derived primers (Fig 5.12c). However, Southern blotting analysis did not detect any amplified product that hybridised to oligonucleotide M5806, including two prominent bands of 300-350bp (Table 5.3a) and 200bp (Table 5.3b) which were observed in some reactions. Similarly, hemi-nested PCR, using primers derived from the p40 and p31 regions, was unsuccessful in further amplifying product from first round amplifications using primers derived from p40 and p28 regions of the protein (data not shown). Particular sets of reaction conditions (see Table 5.3a) were observed to produce only two or three bands as observed by PAGE and silver staining. However, all of these amplification products were larger than the ~300bp and ~400bp fragments expected to correspond to peptides of 12kDa and 15kDa respectively (see Fig. 5.12c).

The results of PCR experiments, aimed at amplifying the 5' end of the p40 gene from recombinant λ gt11 DNA, were similar to those described above. Analysis of amplified products resulting from reactions based on a combination of λ gt11-derived forward and p28-derived reverse primers, revealed multiple bands which decreased in number as the annealing temperature was increased from 55°C to 65°C. Once again however, Southern blotting analysis did not reveal any amplified product that hybridised to oligonucleotide M5806. Similarly, hemi-nested PCR, using λ gt11- and

| Mg ²⁺ (mM) | Temp (°C) | Primers [μ M] | | | | | | | | | |
|-----------------------|-----------|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 4.0 |
| 0.5 | 55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | 55 | 0 | 0 | 5 | ≥ 10 | ≥ 10 | ≥ 10 | 0 | ≥ 10 | ≥ 10 | ≥ 10 |
| 1.5 | 55 | 0 | 7 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 2.0 | 55 | 3 | 6 | 8 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 2.5 | 55 | 2 | 6 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 3.0 | 55 | 2 | 8 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 3.5 | 55 | 6 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 4.0 | 55 | 6 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |

Table 5.3a Results of PCR experiments using different permutations of Mg²⁺ and primer concentration. Forward and reverse primers were Z196 (T_m = 74°C) and M5018 (T_m = 57°C) respectively. Annealing temperature was fixed at 55°C and genomic DNA template concentration was 100ng/50 μ l reaction. Numbers shown indicate the number of amplified fragments per reaction as analysed by PAGE and silver staining. Shaded values indicate reactions which amplified a prominent band of ~300-350bp.

| Mg ²⁺ (mM) | Temp (°C) | Primers [μ M] | | | | | | | | | |
|-----------------------|-----------|--------------------|-----|-----|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| | | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 4.0 |
| 0.5 | 40 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | 40 | 0 | 0 | 7 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 | ≥ 10 |
| 1.5 | 46 | 0 | 0 | 0 | 4 | 8 | 8 | 8 | 8 | 8 | 8 |
| 1.5 | 50 | 0 | 0 | 0 | 0 | 3 | 4 | 3 | 3 | 3 | 3 |
| 1.5 | 55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 5.3b Results of PCR experiments using different permutations of Mg²⁺ concentration, primer concentration and annealing temperature. Forward and reverse primers were M5805 (T_m = 43°C) and M5018 (T_m = 57°C) respectively. Annealing temperature was varied between 40-55°C while genomic DNA template concentration was fixed at 100ng/50 μ l reaction. Numbers shown indicate the number of amplified fragments per reaction as analysed by PAGE and silver staining. Shaded values indicate reactions which amplified a prominent band of ~200bp.

p31-derived primers, failed to further amplify any products from first round amplifications (data not shown).

5.3.15 Identification of *M.avium* Type A/I Genomic DNA Restriction Fragments Recognised By End-Labelled Oligonucleotides

At this juncture neither immunoscreening or oligonucleotide hybridisation had succeeded in detecting λ gt11 recombinants encoding the p40 in either of two genomic DNA libraries. Similarly, attempts to amplify the 5' end of the gene by PCR had yielded products which could not be proven to contain p40 sequences. Therefore the alternative strategy of probing Southern blots of restriction endonuclease digested genomic DNA with labelled oligonucleotides was adopted in an attempt to identify restriction fragments which could be targeted for cloning. Table 5.4 shows the results of washing four strips of *Eco*RI digested *M.avium* type A/I DNA at three different temperatures following hybridisation with one of four end-labelled oligonucleotide probes. Only one of the four available oligonucleotide probes (M5018) continued to emit a detectable signal after 10min at 65°C in 1 x SSC and 15hr exposure at -70°C. Five bands at ~5.2kb, ~4.5kb, ~4.0kb, ~3.7kb and ~450bp were visible in *M.avium* type A/I DNA, but only the largest three of these were common to the *M.a.paratuberculosis* genome (Fig. 5.14).

5.3.16 Experiments To Clone *Eco*RI Restriction Fragments of *M.avium* Type A/I Genomic DNA Recovered From LMP agarose

Initial efforts to clone the *Eco*RI restriction fragments recognised by oligonucleotide M5018 focused on those that emitted the strongest signals on Southern blots, i.e. the 5.2 and 3.7kb fragments. *M.avium* type A/I genomic DNA was digested to completion with *Eco*RI and the resulting fragments resolved on a LMP agarose gel. Restriction fragments ranging from ~3.5kb to ~6.5kb were excised from the gel in seven contiguous blocks. The DNA was recovered by agarase digestion and analysed by further agarose gel electrophoresis and Southern blotting prior to ligation into plasmid vectors (Figs. 5.15a and b).

| Oligonucleotide Identification No. | Washing Temperature | | |
|------------------------------------|---------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| | 42°C | 60°C | 65°C |
| Z196 (p40/p12) | multiple bands | single band at ~20kb | -ve |
| M5805 (p40/p12) | multiple bands | -ve | -ve |
| M5806 (p31) | multiple bands | -ve | -ve |
| M5018 (p28) | black smear | multiple bands including five strong signals at 5.2, 4.5, 4.0 and 3.7kb and 450bp | five bands, two strong signals at 5.2 and 3.7kb and three weaker signals at 4.5 and 4.0kb and 450bp |

Table 5.4 Results of washing Southern blots of *Eco*RI digested *M. avium* genomic DNA sequentially at 42°C, 60°C and 65°C following hybridisation with four individual end-labelled oligonucleotide probes. All Southern blots were washed in 1 x SSC/0.1% SDS for 10min at the specified temperature and exposed for 15hr at -70°C.

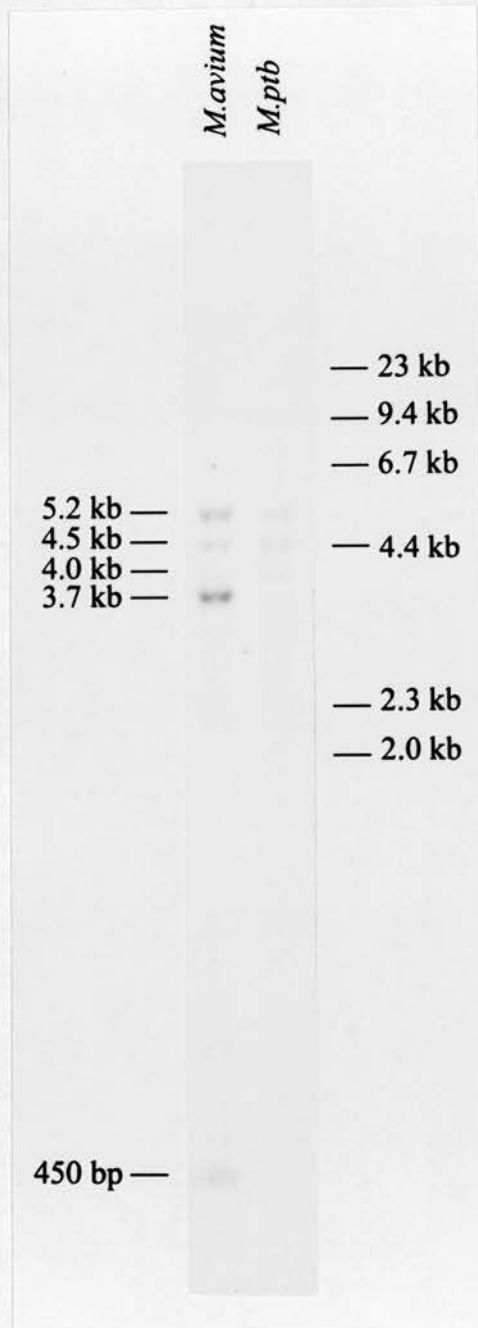


Fig. 5.14 Autoradiograph of *Eco*RI digested *M.avium* and *M.a.paratuberculosis* genomic DNA following Southern transfer and hybridisation with end labelled oligonucleotide M5018. The Southern blot was washed in 1 x SSC, 0.1% SDS at 65°C for 10min and autoradiography took place at -70°C over 15hr. Fragments of ~5.2, ~4.5 and ~4.0kb were seen in both *M.avium* and *M.a.paratuberculosis*, but additional bands of ~3.7kb and ~450bp were seen only in *M.avium*.

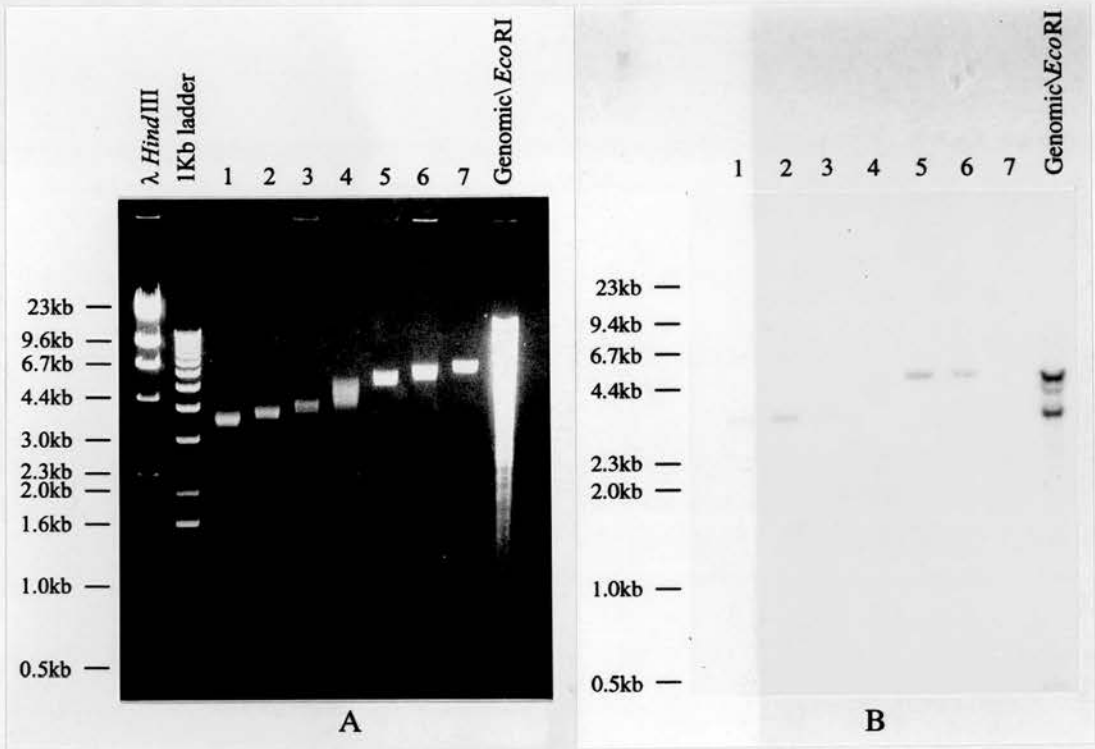


Fig. 5.15a and b Agarose gel (A) and autoradiograph (B) showing *M. avium* type A/I genomic DNA fragments recovered from LMP agarose by digestion with agarase. DNA was digested to completion with *EcoRI* and the resulting fragments resolved electrophoretically. Restriction fragments ranging from ~3.5kb to ~6.5kb were excised from the gel in seven contiguous blocks. A Southern blot of the recovered DNA probed with oligonucleotide M5018 revealed the fragments of interest at ~3.7, ~4.0, ~4.5 and ~5.2kb and their corresponding bands in whole genomic DNA digested with *EcoRI*. M.wt. markers include a 1kb ladder and wild type λ DNA digested with *Hind III*.

Initial experiments to clone the genomic DNA fragments directly into the plasmid vectors pBS and pUC18 were unsuccessful. This was in spite of the fact that all the usual controls were noted to be satisfactory; vector DNA (pBS or pUC18 linearised with *EcoRI* and dephosphorylated with CIP) produced a background transformant level of ~2 cfu/50ng DNA and could not be seen to ligate to itself as viewed by agarose gel electrophoresis; wild type λ DNA digested with *Hind* III was observed to ligate to itself indicating the efficacy of the T4 DNA ligase and the reaction buffer supplied by the manufacturer (Boehringer Mannheim); transformation controls using empty circular plasmid DNA yielded approximately 5×10^3 cfu/50ng DNA ($\sim 10^5$ cfu/ μ g) and the competent cells in use (*E. coli* host strain DH1 prepared by the CaCl_2 method) were shown to be sensitive to Amp containing medium.

The use of different vector to insert ratios failed to increase the efficiency of transformation from zero. The CIP treated pBS and pUC18 vectors were replaced with pGEX1 λ T (Pharmacia), which was supplied ready to use linearised with *EcoRI* and dephosphorylated with BAP. The use of this vector improved transformation efficiencies only slightly, resulting in values of 5-10 cfu/50ng DNA. Complete DNA ligase buffers from two other commercial sources (Promega and Invitrogen) and an "in house" buffer, using ATP concentrations ranging from 5-20mM, failed to improve on the efficiencies achieved using the original buffering conditions. Interestingly however, ATP concentrations in excess of 5mM were noted to inhibit rather than enhance ligation efficiency (data not shown). The use of competent host cells prepared using HETB rather than CCTB increased transformation efficiencies marginally to ~75 cfu/50ng DNA.

Electrophoretic analysis of a test ligation designed to ascertain whether the agarose-recovered *EcoRI* fragments were of a quality suitable for ligation showed that only a small percentage of the DNA had undergone a noticeable shift in migration and that there was little visible difference between ligated test DNA and unligated control DNA. Conclusive evidence that the genomic DNA fragments could not be ligated

efficiently was obtained when a commercially prepared *EcoRI*-ended test insert fragment of 3.5kb (Amersham International) was successfully ligated into pGEX1 λ T using the same reaction conditions and vector to insert ratios. Transformation of HETB-prepared DH1 cells using these constructs was achieved at an efficiency of $5.4 \times 10^5/\mu\text{g}$ DNA.

5.3.17 Sucrose Gradient Purification of Genomic DNA Restriction Fragments

In an attempt to generate fragments of a quality suitable for ligation, *EcoRI* digested genomic DNA was purified by sucrose density gradient centrifugation (Nevins and Darnell, 1978). Fractionation of the gradient yielded 24 x 0.5ml fractions containing a series of overlapping fragment sizes (Fig. 5.16). Although all the fractions between 9 and 16 were seen to contain a range of fragment sizes which included the 3.7-5.2kb fragments of interest, fraction 16 was selected because it contained fewer low M.wt. DNA fragments than any of the preceding fractions. Smaller DNA fragments tend to be ligated preferentially over larger ones, thus the selection of a fraction containing a minimum fragment size of ~3kb theoretically increased the likelihood of cloning the fragments of immediate interest. Fraction 5 was the only one observed to contain fragments in the 400-600bp size range and was selected for the purpose of cloning the ~450bp fragment recognised by oligonucleotide M5018.

Confirmation of the quality of the sucrose-purified restriction fragments was obtained when a sample of the DNA purified from fraction 16 was ligated to itself and electrophoretic analysis revealed an obvious shift in migration (Fig. 5.17).

5.3.18 Cloning and Sequencing of Genomic *EcoRI* Fragments Purified By Sucrose Density Gradient Centrifugation

Sucrose purified *EcoRI* fragments from fraction 16 were ligated into pGEX1 λ T at a vector to insert ratio of 1:1. The resulting constructs transformed high efficiency competent *E.coli* host strain HB101 cells (Promega) at an efficiency of 5.2×10^3 cfu/50ng (10^5 cfu/ μg). A total of 5195 transformants, screened by colony array

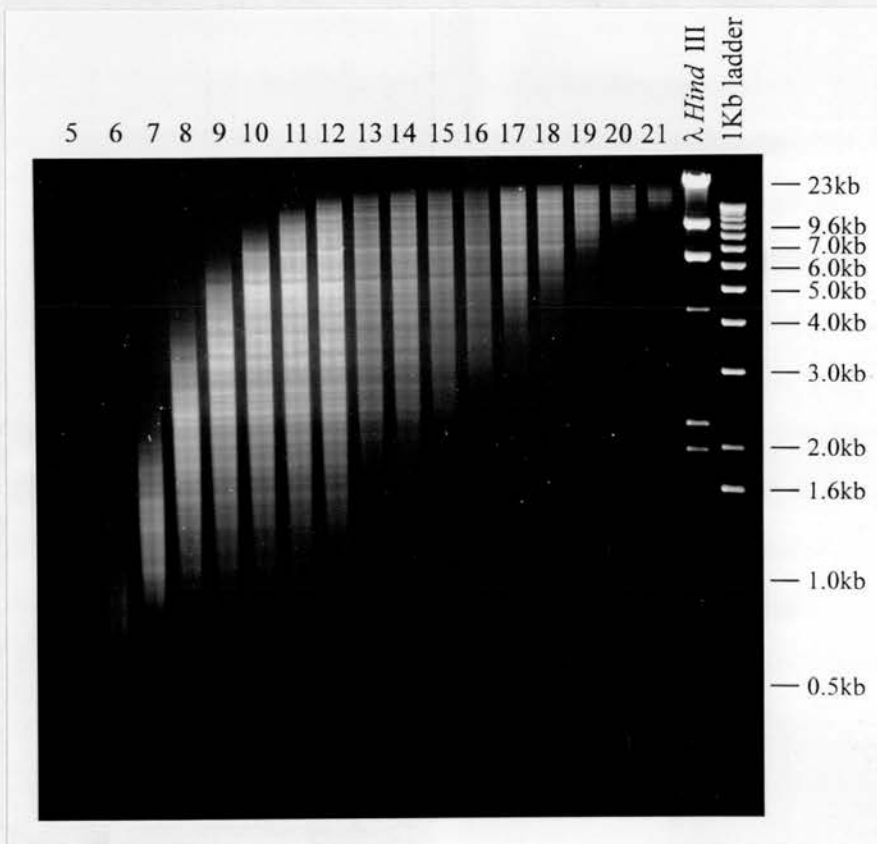


Fig. 5.16 Agarose gel showing a series of fractions resulting from the purification of *Eco*RI digested *M.avium* type A/I genomic DNA by sucrose density gradient centrifugation. Fractionation of the gradients yielded 24 x 0.5ml fractions, 17 of which (5-21) were shown to contain restriction fragments of overlapping sizes. M.wt. markers include a 1kb ladder and wild type λ DNA digested with *Hind* III.

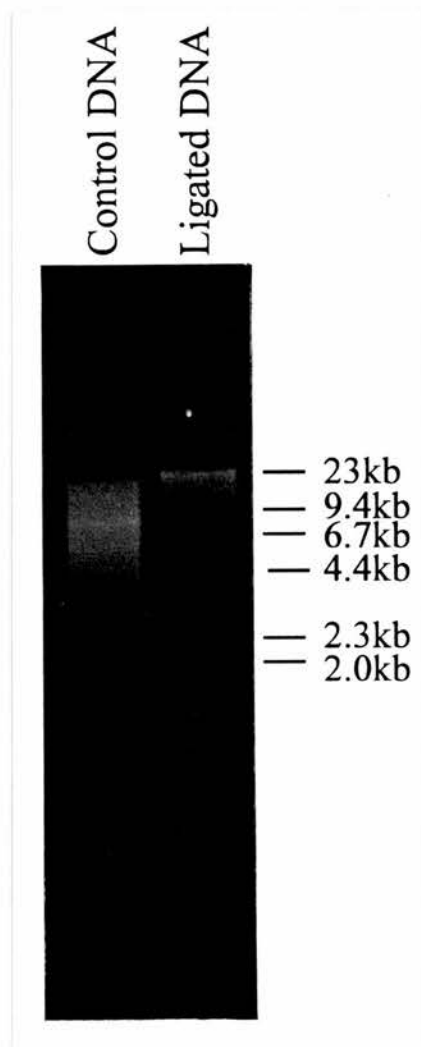


Fig. 5.17 Agarose gel demonstrating the quality of sucrose purified DNA fragments for the purpose of ligation. Ligated *EcoRI* fragments from gradient fraction 16 showed a marked shift in migration when compared to non-ligated control DNA. Bars representing M.wt. are derived from wild type λ DNA digested with *Hind* III.

hybridisation using oligonucleotide M5018, yielded four clones containing the 5.2kb fragment, a single clone containing the 4.5kb fragment, five clones containing the 4.0kb fragment and three clones containing the 3.7kb fragment (Figs. 5.18a and b). A single clone containing the ~450bp *EcoRI* fragment was isolated in an identical manner after screening a further 500 transformants (data not shown).

In order to determine whether any of the cloned *EcoRI* fragments contained the p40 gene, it was necessary to obtain sequence information across the M5018 recognition site for a representative clone containing each insert (Table 5.5). The sequencing procedure was simplified for clones pGEX16/84 (~3.7kb insert) and pGEX22/41 (~4.0kb insert) by virtue of the fact that oligonucleotide M5018 was capable of priming an initial sequencing reaction on these inserts. Further primers designed from new sequence data were then used to prime second and third reactions on opposite strands (Fig. 5.19). This meant that sequence data over the recognition site and flanking regions was obtained in both directions.

Sequencing reactions on clones pGEX3/14 (~5.2kb insert), pGEX42/70 (~4.5kb insert) and pUC1/28 (~450bp insert) could not be primed by oligonucleotide M5018. As the insert fragments of clones pGEX3/14 and pGEX42/70 were considered too large to sequence in their entirety, insert DNA was purified from each clone and digested to completion with *Sau3a*. Southern blots probed with oligonucleotide M5018 revealed *Sau3a* cleavage fragments of ~500bp and ~300bp from clones pGEX3/14 and pGEX42/70 respectively. Two subclones, pUC3/46 (~500bp) and pUC4/33 (~300bp) were selected from a total of eight produced by shotgun-ligating *Sau3a* digests of purified 5.2kb and 4.5kb fragments into pUC18/*Bam*H1/BAP (Pharmacia) and transforming high efficiency *E.coli* host strain HB101 cells (Promega). Clones pUC3/46, pUC4/33 and pUC1/28 were then sequenced in both directions using M13 -40 and reverse primers.

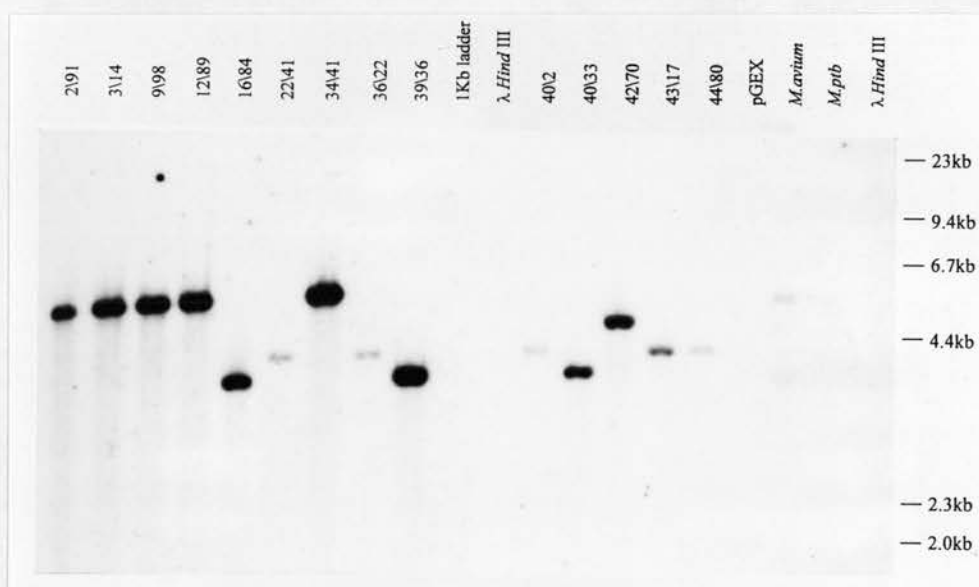
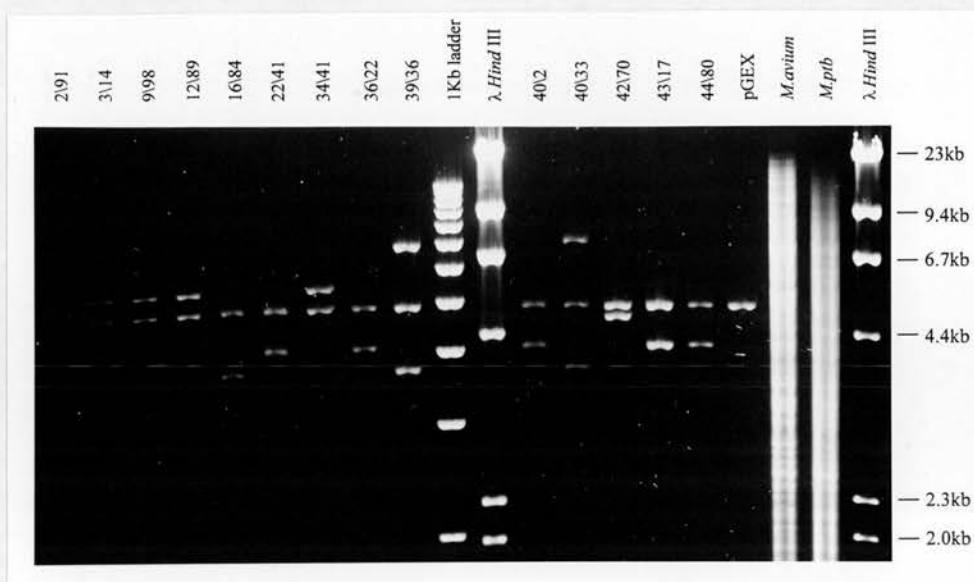


Fig. 5.18a and b Agarose gel and autoradiograph showing *Eco*RI digests of 14 pGEX1λT recombinants containing *M.avium* type A/I genomic *Eco*RI fragments identified by hybridisation with oligonucleotide M5018. Clones 2/91, 3/14, 9/98, 12/89 and 34/41 contain an insert fragment of ~5.2kb, clone 42/70 contains an insert fragment of ~4.5kb, clones 22/41, 36/22, 40/2, 43/17 and 44/80 contain an insert fragment of ~4.0kb and clones 16/84, 39/36 and 40/33 contain an insert fragment of ~3.7kb. In addition to a reactive fragment of ~3.7kb, clones 39/36 and 40/33 also contain a larger fragment of ~8.0kb which was not recognised by oligonucleotide M5018. Bands corresponding to the cloned *Eco*RI fragments can be seen in *Eco*RI digests of *M.avium* type A/I and *M.a.paratuberculosis* genomic DNA. M.wt markers include a 1kb ladder, empty pGEX1λT linearised with *Eco*RI (4.7kb) and wild type λ DNA digested with *Hind* III.

| Genomic Clone (<i>EcoRI</i> frag.) | Insert Size (kb) | Sub-clone (<i>Sau3a</i> frag.) | Insert Size (kb) |
|----------------------------------------|---------------------|------------------------------------|---------------------|
| pGEX3/14 | 5.2 | pUC3/46 | 0.5 |
| pGEX42/70 | 4.5 | pUC4/33 | 0.3 |
| pGEX22/41 | 4.0 | | |
| pGEX16/84 | 3.7 | | |
| pUC1/28 | 0.45 | | |

Table 5.5 Five recombinant plasmids containing *M. avium* type A/I genomic *EcoRI* fragments identified by hybridisation with oligonucleotide M5018. Cloning vectors used and insert sizes are shown. The insert fragments of clones pGEX3/14 (5.2kb) and pGEX42/70 (4.5kb) were digested to completion with *Sau3a* and sub-cloned into pUC18/*Bam*HI/BAP. Subclones containing 500bp and 300bp *Sau3a* insert fragments of interest were identified by hybridising with M5018.

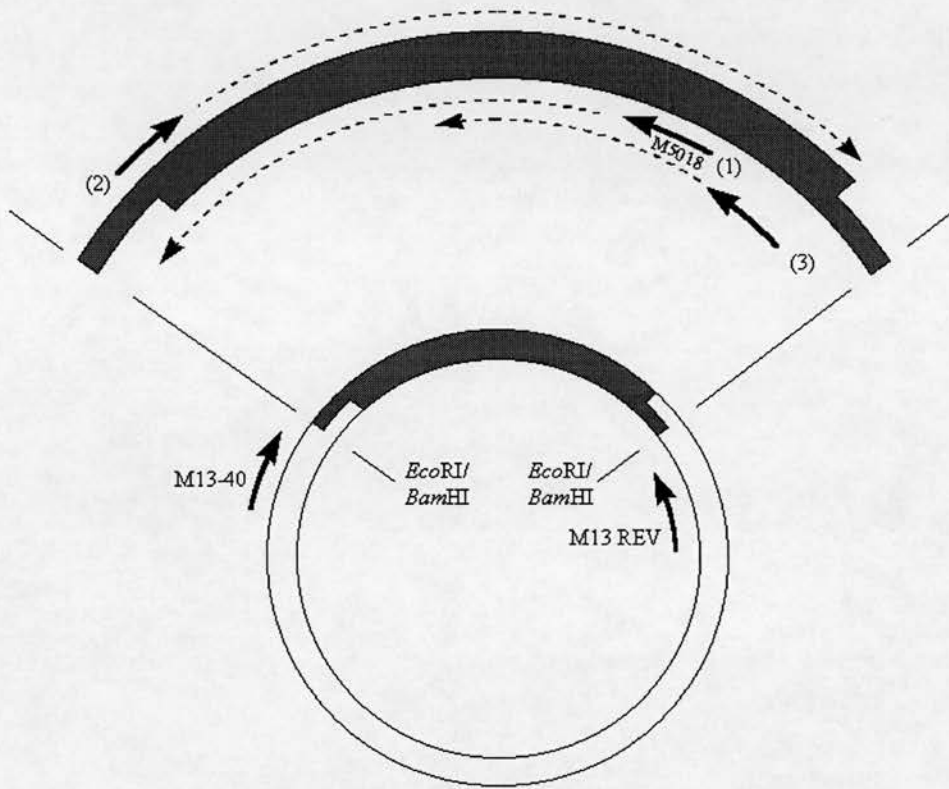


Fig. 5.19 Diagrammatic representation of strategies used to obtain bi-directional nucleotide sequence data for five *M. avium* type A/I genomic DNA fragments :- 1. two pGEX/*EcoRI* constructs (pGEX16/84 and pGEX22/41) were sequenced in three steps, using three oligonucleotide primers, beginning with M5018; 2. the insert fragments of two further pGEX/*EcoRI* constructs were further digested with *Sau*3a, ligated into pUC18/*Bam*HI/BAP generating two pUC subclones (pUC3/46 and pUC4/33) and sequenced using M13 -40 and reverse primers; 3. a single pUC construct (pUC1/28), containing an *EcoRI* fragment of ~450bp, was sequenced using M13 -40 and reverse primers also.

The five sequences generated were matched against the sequence of oligonucleotide M5018 using the UWGCG program "BESTFIT". A multiple alignment of the homologous sequences and comparison with oligonucleotide M5018 is shown in Fig. 5.20. Table 5.6 shows values of percentage similarity and percentage identity between the degenerate sequence of oligonucleotide M5018 and the homologous sequences on each of the five clones. Similarity values ranging between 61.9% and 90.5% were calculated awarding a full match where "S" (C/G) aligned with either "C" or "G". Identity values between 52.4% and 76.2% were calculated on full matches only, making no allowance for degenerate matches. In frame translations of the five homologous 21bp nucleotide sequences and their immediate flanking regions were compared with the 14 aa sequence of the p28 CNBr cleavage peptide (Fig. 5.21). Translations over the seven contiguous amino acid residues, from which oligonucleotide M5018 was originally deduced, revealed levels of amino acid sequence identity ranging between a single mismatch, and two mismatches plus two conservative substitutions. However, the sequences flanking the seven amino acid residues bore no resemblance to those of any of the five translated genomic clones.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| M5018 | C | C | S | G | A | G | T | T | C | A | C | C | G | C | S | G | A | G | G | C | S |
| pGEX3/14 | A | C | C | G | A | G | T | T | C | A | C | C | G | C | G | G | T | G | G | C | C |
| pGEX42/70 | G | A | C | G | A | G | T | T | C | A | C | C | G | C | C | G | A | G | G | T | G |
| pGEX22/41 | C | C | C | G | A | G | G | C | C | A | C | C | G | C | C | G | A | G | G | C | C |
| pGEX16/84 | C | C | C | G | A | G | T | T | C | A | C | C | G | A | C | G | A | G | C | C | A |
| pUC18-1/28 | G | C | C | A | C | C | G | T | G | A | C | C | G | C | C | G | A | G | G | G | A |
| Consensus | - | C | C | G | A | G | T | T | C | A | C | C | G | C | C | G | A | G | G | C | - |

Fig. 5.20 Multiple alignment of homologous sequences obtained from five cloned genomic *Eco*RI fragments and comparison with the 21bp complement of oligonucleotide M5018. Black boxing indicates complete identity, shaded boxing indicates degenerate matches where “C” or “G” aligns with “S” on the M5018 sequence.

| Clone Identification No. | Insert Fragment Size (kb) and Type | No. Base Pairs Sequenced | No. Complete Mismatches | No. Degenerate Matches | Percentage Similarity Over 21bp of M5018 | Percentage Identity Over 21bp of M5018 |
|--------------------------|------------------------------------|--------------------------|-------------------------|------------------------|------------------------------------------|----------------------------------------|
| pUC3/46* | 500bp <i>Sau3a</i> | 174 | 2 | 3 | 90.5% | 76.2% |
| pGEX16/84 | 3.7kb <i>EcoRI</i> | 230 | 3 | 2 | 85.7% | 76.2% |
| pUC4/33** | 300bp <i>Sau3a</i> | 222 | 3 | 3 | 85.7% | 71.4% |
| pGEX22/41 | 4.0kb <i>EcoRI</i> | 219 | 2 | 3 | 90.5% | 76.2% |
| pUC1/28 | 450bp <i>EcoRI</i> | 477 | 8 | 2 | 61.9% | 52.4% |

Table 5.6 *M. avium* type A/I genomic DNA fragments cloned in plasmid vectors pGEX1 λ T and pUC18. Constructs pUC3/46* and pUC4/33** are subclones of pGEX3/14 (5.2kb *EcoRI* insert) and pGEX42/70 (4.5kb *EcoRI* insert) respectively. Approximate sizes of insert fragments and length of sequence obtained are shown. Percentage identity and similarity values were calculated from numbers of complete mismatches and degenerate matches over the 21bp sequence of oligonucleotide M5018.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|----------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|
| p28 N.terminus | L | P | A | S | P | E | F | T | A | E | A | G | S | V |
| pGEX16/84 | L | D | A | I | P | E | F | T | D | E | P | F | E | L |
| pGEX3/14 | D | A | A | Q | T | E | F | T | A | V | A | Q | R | A |
| pGEX22/41 | A | A | F | T | P | E | A | T | A | E | A | D | G | T |
| pGEX42/70 | L | D | S | S | D | E | F | T | A | E | V | V | A | S |
| pUC18-1/28 | C | A | D | D | A | T | V | T | A | E | G | I | R | Q |
| Consensus | - | - | - | - | - | E | - | T | A | E | - | - | - | - |

Fig. 5.21 Multiple alignment of translations across the M5018 hybridisation site of five cloned genomic *EcoRI* fragments and comparison with the 14 amino-terminal residues of the p28 CNBr cleavage peptide. Residues 5-11 represent the 7 contiguous amino acids from which oligonucleotide M5018 was derived. Black boxing indicates complete identity, shaded boxing indicates conserved amino acids.

5.4 Discussion

The λ gt11 expression vector has been a particularly useful cloning vehicle in situations where nucleotide sequence information on a given gene has yet to be established and immune reagents represent the only available means of detecting clones (Young and Davis, 1983a). A number of different immunoscreening strategies have been used to target mycobacterial genes expressed in λ gt11 libraries. These include mabs raised against the antigens comprising crude or partially purified cell lysates (Young *et al.*, 1985b; Morris *et al.*, 1990; Rouse *et al.*, 1991); convalescent sera taken from naturally infected humans or animals to enable the identification of proteins expressed *in vivo* which may be important in the host immune response, or whose expression might be down regulated during propagation on artificial culture media (Clark-Curtiss *et al.*, 1990; Sathish *et al.*, 1990; DeKesel *et al.*, 1993; Cameron *et al.*, 1994; Wieles *et al.*, 1994); hyperimmune polyclonal antisera raised against either intact or disrupted cells (Thole *et al.*, 1985; Khandekar *et al.*, 1986; Stevenson *et al.*, 1991; DeKesel *et al.*, 1993); and specific hyperimmune polyclonal antisera raised against highly purified individual antigens (Inglis *et al.*, 1994b).

The strategy adopted in this study entailed the extraction and chromatographic purification of a 40kDa antigen from disrupted *M.avium* type A/I cells, raising specific hyperimmune polyclonal antisera against the purified antigen in rabbits and using the newly generated immune reagents to screen for expression of the antigen in a λ gt11 library.

5.4.1 Detection and Characterisation of a λ gt11 Clone Encoding the bfr Subunit of *M.avium* Type A/I

Using antiserum pre-absorbed with *E.coli* host strain Y1090, five expressing clones were isolated from the original amplified λ gt11 library. Analysis of these revealed that four clones (λ R692/1-4) contained a 477bp ORF representing the complete nucleotide sequence of the gene encoding the 159 aa residues of the *M.avium* type A/I bfr subunit.

Bacterioferritins and ferritins are proteins which are involved in the storage of iron in prokaryotic and eukaryotic organisms respectively, although prokaryotic ferritins (pfr) also have been reported to occur in *Escherichia coli* (Izuhara *et al.*, 1991), *Bacteroides fragilis* (Rocha *et al.*, 1992) and *Helicobacter pylori* (Frazier *et al.*, 1993). Both proteins are multimeric molecules of ~400kDa which are assembled from 24 subunits of ~17-22kDa each to form a rhombic dodecahedral protein shell surrounding a central iron storage cavity (Mann *et al.*, 1987). Amino acid sequence analysis of the subunits has revealed a high degree of identity between the bfrs of different bacterial genera. Also, all the key aa residues, which specify the unique structural motifs of eukaryotic ferritin, are conserved or conservatively substituted in bfr (Grossman *et al.*, 1992) in spite of an otherwise complete lack of identity between the primary structures of bfr and ferritin. Thus the subunits of both bfr and ferritin share a common tertiary structure consisting of four α -helices which come together to form a bundle (Andrews *et al.*, 1991; Smith, 1991; Stiefel *et al.*, 1994). Both bfr and ferritin contain ferric iron, although the former also contains haem groups in the form of protoporphrin IX (Moore *et al.*, 1986).

Bacterioferritin, also known as cytochromes b₁ and b_{557.5}, (Smith *et al.*, 1988) has been reported in at least twelve diverse genera to date. These include *Azotobacter* (Bulen *et al.*, 1973; Stiefel and Watt, 1979; Chen and Crichton, 1982), *Brucella* (Denoel *et al.*, 1995), *Cyanobacterium* (Laulhere *et al.*, 1992), *Escherichia* (Yariv *et al.*, 1981; Andrews *et al.*, 1989b), *Mycobacterium* (Brooks *et al.*, 1991; Pessolani *et al.*, 1994; Inglis *et al.*, 1994b), *Nitrobacter* (Kurokawa *et al.*, 1989), *Pseudomonas* (Moore *et al.*, 1994), *Rhodobacter* (Ringeling *et al.*, 1994), *Rhodopseudomonas* (Meyer and Cusanovich, 1985), *Rhodospirillum* (Bartsch *et al.*, 1971), *Streptomyces* (Inoue and Kubo, 1965) and *Yersinia* (Perry *et al.*, 1993).

The fact that an ORF of 477bp represented approximately half the coding capacity required for the synthesis of a 40kDa protein raised a number of possibilities. The first of these was that p40 might be a dimer composed of two bfr subunits and that its

apparent M.wt of 40kDa is artefactual and a direct consequence of routine denaturation prior to PAGE and western transfer. However, this theory could not be supported experimentally and bands of ~400kDa (multimeric form) and ~17-22kDa (monomeric form) were the only two forms of the protein visible on western blots probed with affinity-purified anti-bfr antibody (see Fig. 5.5). These findings were in complete concordance with those currently in the literature where there are no reports of the occurrence of any intermediate between the monomeric and complete multimeric forms of bfr. A second possibility was that one or more epitopes were common to both proteins resulting in the recognition of clones encoding and expressing bfr. Although the likelihood of shared epitopes was supported by the recognition of clone λ R692/2 by one of five mabs raised against p40, the results of serological cross-reactivity experiments using two different affinity-purified antibodies strongly suggested that the two proteins were unrelated. Thirdly, it was conceivable that the HPLC purified p40 used to immunise rabbits was not homogeneously pure, but contaminated with other mycobacterial antigens at a level below the sensitivity of the silver staining technique used to visualise purified proteins resolved in polyacrylamide gels. The presence of such contaminants could have resulted in the recipient rabbits mounting a humoral immune response to antigens other than p40 and would explain why clones encoding bfr were isolated from the library. This third possibility was supported by a complete lack of cross-reactivity between the expression product of clone λ R696/1 and either p40 or the recombinant bfr encoded by clones λ R692/1-4. In addition, the recognition of the λ R696/1 fusion protein by two of four anti-hsp mabs suggested a possible relationship between this clone and the *hsp65* of *M.avium* type A/I.

5.4.2 Further Immunoscreening of the Amplified Library Using Antiserum Absorbed With *M.a.paratuberculosis* Cell Lysate

Further immunoscreening of the library with R696 antiserum, which had been pre-absorbed with cell lysates of both *E.coli* host strain Y1090 and *M.a.paratuberculosis* to remove extraneous anti-mycobacterial antibody, yielded a further fifteen

expressing clones (λ R696/2-16). However, subsequent analysis revealed that all 15 clones contained an identical insert fragment to that of clone λ R696/1 and encoded the same putative hsp65 protein. It is likely that, for the purpose of antibody pre-absorption, the *in vitro* grown *M.a.paratuberculosis* cells contained insufficient quantities of hsp65 antigen to absorb all traces of anti-hsp65 immunoglobulin, as they had not experienced either nutritional or physical stresses during their propagation. Indeed, antibody affinity-purified on pGEX/696/2.3 GST/fusion protein revealed small but demonstrable quantities of the corresponding cellular protein, possibly hsp65, in lysates of cultured *M.a.paratuberculosis* cells (see Fig. 5.8a). This could explain why residual anti-hsp65 antibody had not been removed from the antiserum during pre-absorption, and why clones encoding a hsp65 were so readily isolated from the library.

5.4.3 Why Was the Immunoscreening Strategy Unsuccessful In Isolating p40 From λ gt11 Expression Libraries?

A number of possible reasons can be put forward to explain why extensive immunoscreening with high titre hyperimmune antisera was unsuccessful in detecting clones encoding p40 in a genomic DNA expression library which was perceived to be representative of the *M.avium* type A/I genome.

1. Is p40 expression toxic to phage or host cell propagation?

It is conceivable that the expression of p40 may be toxic to either phage or host cell propagation resulting in the under-representation of these recombinants in an amplified library. It is accepted that a level of inequality in the growth of λ gt11 recombinants persists even in the presence of the *lacI* repressor which represses transcription from the β -gal promoter in the absence of IPTG (Huynh *et al.*, 1984). An effect of this "leakage" on amplified libraries is a bias towards non-toxic and rapidly growing recombinants which multiply to high titres and tend to be over-represented. With this in mind, a second λ gt11 library was prepared and immunoscreened in its entirety without amplification. However, no clones encoding

the p40 antigen were detected in the unamplified library. Therefore, based on the assumption that the unamplified library represented the genome comprehensively, the possibility of p40 toxicity cannot be ruled out. However, it seems unlikely that all p40-derived peptides should be lethal to either host cell or phage propagation, and alternative explanations must be considered also.

2. Could p40 codon usage be incompatible with isoaccepting tRNA population profiles in *E.coli* host strain Y1090?

A second explanation might be drawn from differences in codon usage between *M.avium* type A/I genes and those of the *E.coli* host strain Y1090 used while screening the expression library. In the “wobble hypothesis” (Crick, 1966) it is proposed that a single tRNA has the ability to respond to more than one codon. In spite of this degeneracy in the genetic code however, it is generally accepted that codon choices, particularly for certain amino acids, can be heavily biased and that the choice of synonymous codons varies between different organisms (Grantham, 1980). Such organism-specific codon preferences are related to the population profiles of the isoaccepting tRNAs for individual species (Ikemura, 1985) and the correlation between the usage frequency of particular codons and the relative abundance of their respective tRNAs is especially evident for highly expressed genes, eg *E.coli* RNA polymerase subunits, ribosomal proteins, the elongation factor EF-Tu, the recA protein and a number of membrane proteins (Grosjean and Fiers, 1982).

Although the choice between synonymous codons does not affect the nature of the protein synthesised, it will influence the translation efficiency of mRNAs transcribed from these genes (Ikemura, 1981). A high degree of codon bias has been reported for mycobacterial genes (Dale and Patki, 1990) and a feature of this bias is the large number of preferred codons which contain C or G in the third position (Table 5.2). This bias is more pronounced amongst members of the MAC and *M.tuberculosis* than it is in *M.leprae*, whose G+C content is markedly lower than those of other mycobacteria, and whose codon usage is more akin to that of *E.coli*. It is reasonable

to assume therefore that the population profiles of isoaccepting tRNAs will reflect codon preference in *M. avium* type A/I and that highly expressed genes, as is the case in *E. coli*, will exhibit a higher degree of codon bias than genes which are expressed at a lower level. Given that p40 would appear to be present in relative abundance in *M. avium* type A/I cell lysates (see Chapter 3), it is conceivable that, as a mycobacterial gene which may be highly expressed in its natural environment, its *lacZ* driven expression in *E. coli* may not be compatible with the isoaccepting tRNA populations of the host cell and that rapid depletion of particular tRNA species results in the early truncation of the foreign polypeptide. Alternatively, if low concentrations of certain tRNAs force ribosomes to pause frequently at particular codons during the translation of p40 messages, the downstream mRNA may be left exposed and thus able to fold into secondary structures. At best, events such as this would slow down translation considerably, but may even cause further transcription of the gene to be aborted (Platt and Bear, 1982).

3. Could post-translational modification of p40 result in masking of protein epitopes?

A humoral immune response biased towards non-protein antigens, which may be added to the p40 protein as post-translational modifications, offers a third possible explanation. Although post-translational events are not yet well characterised in the mycobacteria, both biotinylation (Henrikson and Allen, 1979; Collins *et al.*, 1987; Norman *et al.*, 1994) and glycosylation (Fifis *et al.*, 1991; Garbe *et al.*, 1993; Lopez *et al.*, 1994; Dobos *et al.*, 1995) of proteins have been reported. If, for example, the p40 antigen exists as a heavily glycosylated globular protein, it is possible that antibody raised against non-denatured p40 would be directed predominantly against carbohydrate moieties and only weakly, or not at all, against protein epitopes masked by non-protein antigens. As it is unlikely that post-translational glycosylation of a mycobacterial protein would take place in an *E. coli* host cell, such an antibody might not be capable of recognising clones expressing the protein component of the p40 molecule. However, a study conducted on the immunogenicity of the polysaccharide

components of the thermostable macromolecular antigen (TMA) group of *M.avium*, *M.a.paratuberculosis* and *M.bovis* presents evidence to oppose this argument. A number of immunological laboratory procedures were used to demonstrate a negligible immunogenicity of the glucans examined (that is, “the inability to produce, upon injection, the synthesis of specific immunoglobulins”) and the authors concluded that the high immunogenicity of the TMA complex is attributable to protein and not to carbohydrate (Bruneteau *et al.*, 1992).

4. Could the *M.avium* type A/I genome have been under-represented in the amplified library?

Firstly, at 8.5×10^4 pfu in total, the pre-amplification titre of the library was considerably lower than those described previously (*M.tuberculosis* λ gt11 library at 4×10^6 pfu and *M.leprae* λ gt11 library at 2×10^6 pfu) (Young and Davis, 1985a and b). Secondly, when factors including genome size, average insert size and percentage recombinants are taken into consideration, the *M.tuberculosis* and *M.leprae* genomes are represented ~ 2560 and ~ 1250 times respectively. Even allowing for the fact that the *M.avium* type A/I library contained a higher percentage of recombinants (80%) than either the *M.tuberculosis* (40%) or *M.leprae* (25%) libraries, the *M.avium* type A/I genome was represented only ~ 90 times. Thirdly, given that all the protein-encoding sequences in the genome must be expressed if the library is to be truly representative, it is essential that a large number of genomic DNA fragments, ideally with endpoints which occur at random and at every base pair throughout the genome, are used in the construction of an expression library (Young and Davis, 1985a and b). The relationship between the average insert fragment size of a genomic DNA library and the number of random endpoints attained is reciprocal, and total representation of the protein-encoding regions will be favoured by a large number of smaller insert fragments. Therefore, it is possible that 90 genomic representations, using insert fragments with an average size of 4kb, is insufficient to ensure that all ORFs contained within the genome are expressed.

5.4.4 Why Was Screening the Amplified Library by DNA-Hybridisation Unsuccessful in Detecting Clones Containing the p40 Gene?

Possible explanations as to why the DNA hybridisation screening strategy was unsuccessful in identifying homologous sequences in the amplified library should be considered under the separate headings of library related, and screening related factors.

1. Library related factors

While leakage of a toxic expression product during amplification of the λ gt11 library cannot be ruled out, the other arguments presented above cannot be used to explain why end-labelled oligonucleotides did not detect clones encoding p40 by DNA hybridisation. In contrast to an antibody, a nucleic acid probe does not rely on gene expression and will detect its homologous target sequence in a library regardless of insertional orientation or reading frame in the vector, and thereby offers a six times greater chance of isolating clones of interest. Therefore, unlike an expression library, which requires a large number of small insert fragments to ensure that all ORFs contained within the genome are expressed (see above), a library intended for screening by nucleic acid hybridisation need not be so comprehensively representative. In this particular case, a library of ~ 90 genomic representations should have been adequate for the purpose of screening with an end-labelled oligonucleotide. However, it is of interest that, although clones expressing bfr and a putative hsp65 antigen were isolated from the library (four and sixteen clones respectively) by immunoscreening, in each case an identical clone was isolated several times over. This was surprising given that the library was prepared from randomly sheared genomic DNA fragments and it is clear that the amplified library was heavily biased towards certain recombinants. In addition, in spite of the fact that oligonucleotide M5018 was shown to hybridise to five different fragments on Southern blots of *Eco*RI restricted genomic DNA (see Fig. 5.14), no recombinants containing these homologous sequences were recognised in the amplified λ gt11 library. Based on the assumption that the amplified library contained ~ 90 genomic

representations, it is reasonable to expect that screening with the same oligonucleotide would have identified the same five genomic sequences recognised on Southern blots. These points suggest that the library may have been under-representative of the *M.avium* type A/I genome and sub-optimal for the purposes of screening with either immune- or nucleic acid-based probes.

2. Screening related factors

The oligonucleotides used to screen the library were deduced from small stretches of p40-derived amino-terminal and internal amino acid sequence. Consequently, a considerable degree of redundancy at the third position of certain codons had to be allowed for (see Fig. 5.13). In addition, G+C rich oligonucleotides of only 21bp in length with built in degeneracy were not considered ideal for the purpose of screening a genome which has itself been estimated to be inherently G+C rich at ~65%. It is conceivable therefore, that oligonucleotide M5018 shared greater levels of homology with certain other genomic sequences (see Fig. 5.20) than with its intended p40 target sequence which remains unknown at this time.

5.4.5 Why Did Attempts to PCR-Amplify p40 Gene Sequences From Genomic DNA Yield No Detectable Products?

Degenerate oligonucleotides have been used in combination with PCR to amplify entire or partial gene sequences from genomic DNA. In many cases, such as the response regulator genes *phoB*, *phoP* and *creB* of *Yersinia* spp., *Campylobacter jejuni*, *Listeria monocytogenes*, *M.tuberculosis* and *E.coli* (Wren *et al.*, 1992); the amikacin-resistance “*aac(6')*-I genes” of *Acinetobacter* spp. (Ploy *et al.*, 1994); and the flagellum protein gene “*flII*” of *Legionella pneumophila* (Merriam *et al.*, 1997), the target genes are highly conserved and related sequences are accessible in gene and protein databases. In such situations the design of oligonucleotide primers for the amplification of specific target sequences is facilitated by pre-existing nucleotide sequence data on related genes. However, primer design is more difficult where no related gene sequences are available, and the only available sequence information is a

limited amount of aa sequence data obtained by Edman degradation of a purified protein. Nevertheless, oligonucleotide primers deduced from aa sequences have been used successfully to amplify a portion of the gene encoding the MMPII bacterioferritin subunit of *M.leprae* (Pessolani *et al.*, 1994); a novel 23kDa *in vivo*-expressed protein of *Francisella tularensis* (Golovliov *et al.*, 1997); and the heat shock proteins chaperone-10 (Cpn-10) and Cpn-60 of the groE operon of *Francisella tularensis* (Ericsson *et al.*, 1997). This “reverse genetics” approach was applied to the p40 gene using a MAC-derived codon usage table and pairs of degenerate oligonucleotides, deduced from p40 aa sequences, as forward and reverse primers. However no PCR products, either in the predicted size range or which could be identified by hybridisation using a putative interior oligonucleotide, were detected.

1. Oligonucleotide primer design and related factors

Working within the constraints imposed by limited aa sequence, where most residues were encoded by more than one codon, necessitated a degree of degeneracy in oligonucleotide design. All four oligonucleotides were designed to meet a number of criteria. Each had to be a minimum of 20bp in length, be derived from a stretch of seven aa residues exhibiting the highest degree of codon preference possible, include a 3' G/C clamp for the purpose of PCR and display little or no complementarity. No attempt was made to pre-determine T_m . Working within these constraints required a degree of compromise however. For example, selecting a stretch of seven contiguous aa residues with the highest possible codon bias did not automatically yield an oligonucleotide with a 3' G/C clamp which also could be guaranteed not to fold back on itself. Ultimately, of the four resulting oligonucleotides, whether “best guess” or degenerate, each represented a compromise relative to the set criteria. In addition, the T_m of individual oligonucleotides was observed to range from 59.6° to 74°C in 1M NaCl, a level of disparity which required that PCR annealing temperatures were adjusted in favour of the primer with the lower T_m . In turn, this allowed the primer with the higher T_m to anneal to the genomic template non-specifically and resulted in the amplification of multiple areas of the genome.

2. Presumption of PCR priming sites based on CNBr cleavage of p40

For the purpose of amplifying the 5' end of the p40 gene by PCR, the presumed priming sites of forward and reverse primers were based on a hypothetical CNBr cleavage map of purified p40 (see Fig. 5.12c). Logically, the oligonucleotides derived from the amino-terminal aa sequence were designed as forward primers and those derived from internal aa sequences as reverse primers. Although the proposed CNBr cleavage map can be rationalised, it remains hypothetical and the presumed positions of the two internal aa sequences cannot be guaranteed. This raises the possibility that the target sequence was successfully amplified but that the specific amplified product was not identified by the chosen screening strategies. If the p31-derived oligonucleotide (M5806) used for screening was not interior to the amplified region of the p40 gene as presumed, but exterior, then neither hemi-nested PCR nor DNA hybridisation would have detected the product of the first round amplification.

3. Could a lack of co-linearity between the gene encoding p40 and the mature protein have resulted in the inappropriate selection of PCR priming sites?

Protein splicing has been shown to occur in the post-translational processing of the RecA proteins of *Mycobacterium tuberculosis* (Davis *et al.*, 1992) and *Mycobacterium leprae* (Davis *et al.*, 1994) as well as in a number of other unrelated gene products of such phylogenetically diverse species as *Saccharomyces cerevisiae* (Hirata *et al.*, 1990; Kane *et al.*, 1990), *Thermococcus litoralis* (Perler *et al.*, 1992; Hodges *et al.*, 1992), *Candida tropicalis* (Gu *et al.*, 1993) and *Pyrococcus* spp. strain GB-D (Xu *et al.*, 1993). More recently, a second mycobacterial protein splicing event has been reported to occur in the DNA gyrase A-subunit of *Mycobacterium leprae* and some, but not all, strains of *M.flavescens*, *M.gordonae* and *M.kansasii* (Fsihi *et al.*, 1996). It is conceivable therefore, that protein splicing may be more widespread amongst the mycobacteria than at first thought, and the possibility that the p40 antigen might be processed in this way merits consideration.

In a post-translational modification analogous to the removal of an intron from mRNA, protein splicing involves the precise excision of an embedded in-frame internal protein sequence (intein) from a larger precursor polypeptide and the ligation of the flanking amino- and carboxy-terminal sequences (exteins) to yield a functionally active mature protein. Protein splicing demonstrates that the aa sequence of a protein need not be co-linear with the encoding gene, the mRNA transcript or the primary translation product and that the regions of the gene which encode the mature protein can be more widely separated than could be predicted from the M.wt of the protein alone. Therefore, given that the reverse primers were deduced from two internal aa sequences of the p40 antigen, it is conceivable that one or both of these are internal to the carboxy-terminal extein of the mature protein. This being the case, premature termination of any PCR using either of these reverse primers, in conjunction with an amino-terminus deduced forward primer, may be the consequential result of the additional distance imposed by the intervening region of the gene encoding the spliced intein. Alternatively, a successfully amplified product would not have fallen into the predicted size range and may have been overlooked as one of a number of non-specific amplified products. Finally and as mentioned above, even if a p40 specific gene product had been successfully amplified, it would not have been recognised by either DNA hybridisation or hemi-nested PCR if the third oligonucleotide was exterior to the amplified region of the gene.

5.4.6 Cloning and Nucleotide Sequencing of Genomic *EcoRI* Fragments Which Hybridised With Oligonucleotide M5018

Attempts to clone genomic DNA restriction fragments recovered from LMP agarose gels were unsuccessful in comparison with attempts to clone the same fragments purified on sucrose density gradients. This reflects the difference in quality between agarose and sucrose recovered DNA, where, for the purposes of ligation, sucrose purified DNA was shown to be superior. DNA recovered from agarose was used successfully where large numbers of a single fragment, eg. an insert fragment excised from a recombinant plasmid, were extracted for the purpose of subcloning into an

alternative cloning vehicle. Transformation efficiencies were generally low but could not be attributed to low quality competent cells. However, because all the DNA fragments were identical, very few transformants were required to ensure that one containing a correctly orientated fragment was obtained. Much higher ligation efficiencies were required for shotgun cloning restriction fragments into plasmids however. In this study, two sucrose density gradient fractions containing a broad range of fragment sizes were used to target *EcoRI* fragments of ~5.2kb, 4.5kb, 4.0kb, 3.7kb and 450bp. All five fragments were cloned at the first attempt and sequenced, either partially or completely, in order to determine the level of homology each shared with oligonucleotide M5018. "Bestfit" analyses revealed that the reason why oligonucleotide M5018 failed to prime sequencing reactions on clones pGEX3/14 (~5.2kb fragment) and pGEX42/70 (~4.5kb fragment) was poor homology at the 3' end of the primer. Comparative analysis of the sequence data generated showed that none of the homologous regions on any of the five clones provided an identical match (see Fig 5.20), with identity and similarity values ranging between 52.4%-76.2% and 61.9%-90.5% respectively (see Table 5.6). Translations across the M5018 recognition site and flanking regions of all five clones yielded aa sequences exhibiting varying levels of identity with the original p28 amino-terminus (see Fig 5.21). Over the seven contiguous residues from which oligonucleotide M5018 was deduced, the observed levels of identity ranged between a single mismatch (pGEX22/41) and three full matches plus two conservative substitutions (pUC1/28). Over the flanking regions however, no significant identity could be demonstrated between any one of the five translations and the 14 amino-terminal aa residues of the p28 CNBr cleavage peptide. It was clear that none of the five cloned genomic *EcoRI* fragments identified by oligonucleotide M5018 contained the gene encoding the p40 antigen.

Given that oligonucleotide M5018 identified five similar sequences in *M. avium* type A/I genomic DNA, translations of which exhibited significant levels of identity with the original p28-derived seven aa residue template, it is surprising that the intended

p40 target sequence was not identified also. While the absence of ambiguities in the readings obtained from Edman degradation of the p28 effectively precluded the possibility of an incorrect aa sequence (Barker, pers. comm.), it is conceivable that the codon preference of the p40 gene was inconsistent with the table used to design the four oligonucleotides. If this was indeed the case, then the identification of five similar sequences could be attributed to a combination of degeneracy, high G+C content and the likelihood that oligonucleotide M5018 shares greater homology with other genomic sequences than with the intended p40 target sequence.

5.4.7 Uncovered Areas

While hybridising each of the four p40-derived oligonucleotides to Southern blots of *EcoRI* digested genomic DNA, a single fragment of ~20kb was identified by the non-degenerate or “best guess” oligonucleotide Z196 (see Table 5.4). The 20kb fragment remained visible by autoradiography at a washing stringency of 1 x SSC/0.1% SDS at 60°C, but not at 65°C. Although attractive in that it was the only hybridising band on a Southern blot of total *M. avium* type A/I genomic DNA, the 20kb fragment was overlooked in favour of the five smaller *EcoRI* fragments identified by oligonucleotide M5018 for a number of reasons. Firstly, the degenerate oligonucleotide M5805, which was derived from the same seven amino terminal aa residues as Z196, did not recognise the 20kb fragment even at lower washing stringencies. This observation reduced the likelihood that the apparent homology between Z196 and the 20kb fragment was absolute. Secondly, oligonucleotide M5018 remained hybridised to each of its five homologous *EcoRI* fragments at a stringency 5°C higher than Z196 and the 20kb fragment. This observation indicated that the degree of homology between M5018 and each of the homologous sequences was greater, and therefore more attractive, than the association between Z196 and the 20kb fragment. Thirdly, at 20kb, the Z196 homologous fragment was too large to shotgun-clone into a convenient plasmid vector. However, this problem could have been overcome by shotgun-cloning into the phage lambda vector EMBL3, and screening plaque lifts of recombinant phage with end-labelled Z196 in the same

manner described for screening the amplified λ gt11 library (section 5.2.2, Method 1., subsection 8.).

It is not inconceivable that the 20kb *EcoRI* fragment may still hold the key to the p40 gene, and any future work in this area would almost certainly have to eliminate this possibility before embarking upon alternative cloning strategies.

CHAPTER 6

GENERAL DISCUSSION

The work covered in this thesis focuses primarily on a novel protein of 40kDa (p40) identified in IS901/902-positive strains of *M.avium*, but not in *M.a.paratuberculosis* or in any of 13 other species of *Mycobacterium* examined. Soluble p40 antigen was purified to homogeneity, using a series of chromatographic separations, and used subsequently to generate both polyclonal and monoclonal immune reagents. *In vitro* stimulation of ovine T-cells demonstrated that the p40 is an *M.avium* type A/I-specific immunological marker which can be used to distinguish between animals infected with *M.avium* type A/I or *M.a.paratuberculosis*. The p40 is therefore the first entire protein molecule to be identified in a pathogenic member of the MAC which has subspecies specificity and the potential to form the basis of a diagnostic immunoassay. As such, the p40 merited further study, and experiments were undertaken to clone and characterise the encoding gene. However, in spite of many attempts using a number of different cloning and screening strategies, all efforts to clone the p40 from *M.avium* type A/I genomic DNA were unsuccessful, and several important questions remain unanswered.

6.1 Why was p40 not cloned?

Of the questions left unanswered the first of these is why all attempts to clone the p40 gene in λ gt11 proved unsuccessful, and these include an independent attempt to isolate the gene from a further λ gt11 expression library by immunoscreening with mabs raised against HPLC-purified p40 (Ahrens, pers. comm.). Possible explanations including expression product toxicity, codon incompatibility, post-translational modification and under-representation of the *M.avium* type A/I genome already have been covered in the preceding chapter, but none of these explain why translations of all five genomic *Eco*R1 restriction fragments, identified by oligonucleotide M5018, lacked overall sequence identity with p40 internal amino

acid sequence data. Following the lines of currently accepted dogma, it can be assumed that where a protein is expressed there must also be a gene encoding that protein. The gene in question may be located on the chromosome of the organism, or on an autonomously replicating genetic element such as a plasmid, insertion sequence or transposon. Whichever of these applies in the case of *M. avium* type A/I and p40, the method of total nucleic acid extraction and purification used to prepare DNA was the same for both library construction and genomic Southern blotting. Theoretically, all cellular nucleic acid, including plasmid DNA, is pelleted through a CsCl cushion following cell disruption. Cellular RNA is removed selectively by digestion with ribonuclease A and the resulting DNA preparation should contain all cellular DNA species. It is therefore difficult to reason why a fragment containing the p40 gene was not identified on Southern blots by oligonucleotide M5018 along with the other five *Eco*R1 fragments recognised. However, it could be argued that p40 might be encoded by a gene present on a very low copy-number plasmid, and that its low concentration, relative to the chromosomal DNA, is not detectable on Southern blots using an end-labelled oligonucleotide probe. Alternatively, the nucleotide sequence of oligonucleotide M5018 may have been deduced inaccurately from internal p40 amino acid sequence relative to actual p40 codon usage. Consequently, it is possible that M5018 may hybridise preferentially to sequences which are not actually related to the p40 gene. Indeed, oligonucleotide M5018 was observed to hybridise to many more genomic fragments at lower stringency washes, but only to the five cloned and sequenced here at 65°C in 1 x SSC. Further amino acid sequence data on the p40 antigen would provide templates for a wider range of "best guess" and degenerate oligonucleotides which could be used to highlight and confirm the identity of restriction fragments on Southern blots of genomic DNA. If for example, a genomic DNA fragment could be identified which hybridised with two or more oligonucleotides deduced from different internal amino acid sequences, the techniques and cloning strategy described in this thesis could be usefully applied to its isolation and characterisation.

6.2 What Is The Nature of The Apparent Association Between p40 and The Genomic Insertion Element IS901/902?

The expression of the p40 antigen appears to be inextricably linked to the presence of IS901/902 in *M.avium* type A/I. However, translations of the insertion element in all six reading frames did not yield amino acid sequences which bore any resemblance to the amino terminal or internal amino acid sequences obtained for the p40 antigen. Therefore, the relationship between the insertion element and the protein cannot be direct, and the precise nature of their close association remains to be elucidated. One possibility is that the p40 gene exists in a repressed state and under the control of a gene encoding a repressor protein in IS901/902-negative strains of *M.avium*. Given that genomic insertion of IS901/902 is site-specific, it is conceivable that one copy of the insertion element may recognise an insertion site within the repressor gene. Insertion of IS901/902 at this site would disrupt expression of the repressor protein, which would in turn up-regulate expression of the p40 antigen. The effects of knocking out one gene and activating another may manifest themselves phenotypically and could alter the pathogenic potential of the organism. For example, it has been demonstrated that strains of *M.avium* type A/I are more virulent than strains of *M.avium* type A when inoculated into BALB/c mice (Kunze *et al.*, 1991; Pedrosa *et al.*, 1994). The authors associated the observed increase in virulence with the presence of the IS901/902 in *M.avium* type A/I strains, but did not address differences between the two types of *M.avium* at the polypeptide level. It is conceivable therefore that unrepressed expression of p40 may be partly responsible for the greater virulence observed in strains of *M.avium* type A/I.

The likelihood of such a repressor mechanism existing in *M.avium* type A/I is given credence by recent observations in *M.a.paratuberculosis*. Studies on the genomic regions flanking IS900 insertion have revealed that a gene of ~12kDa is disrupted by a copy of IS900 (Tizard, pers. comm.). Whilst there is no direct evidence that this 12kDa protein has any repressor function, the size of the molecule and limited amino acid sequence identity are apparently consistent with those of other such molecules.

Clearly, if IS900 insertion has indeed inactivated the expression of a putative repressor molecule, its effect on the phenotype of *M.a.paratuberculosis* does not include expression of p40. However, as the respective recognition sequences of IS900 and IS901/902 are not identical, it is possible that the p40 gene may be present in *M.a.paratuberculosis* and the other members of the MAC, but maintained in a repressed state in the absence of IS901/902. Unfortunately, the lack of success in cloning and characterising the p40 gene precluded any opportunity to assess its distribution within the members of the MAC, and among more distantly related species of *Mycobacterium*.

6.3 Is The p40 Gene Widely Distributed Throughout The Members of The Genus *Mycobacterium*, and Is Expression Repressed In Species Other Than IS901/902-Positive Strains of *M.avium*?

Western immunoblotting analysis showed that, of the 15 species of *in vitro*-grown *Mycobacterium* examined, only IS901/902-positive strains of *M.avium* expressed p40 in detectable quantities (see Table 3.1 and Fig. 3.14a,b&c). However, it is not inconceivable that homologous genes may be present, but silent, in other species of *Mycobacterium*. If p40-like genes are indeed present in other species, it would be of interest to establish whether they are maintained in a repressed state, and under what environmental conditions their expression might be induced. In addition, if future studies on IS901/902 flanking sequences reveal putative repressor gene disruption, it would be of interest to ascertain whether reconstruction of the gene in an expression plasmid could restore repression of p40 expression in *M.avium* type A/I transformants. If successful, such transformants could be used to assess the effect of p40 repression on the virulence of *M.avium* type A/I strains in BALB/c mice. However, given that IS901/902 is a mobile genetic element, it is not inconceivable that a copy of the insertion sequence may insert into the vacant site on the reconstructed gene, and care would have to be exercised in monitoring any transformant for reversion to the p40 phenotype.

6.4 Alternative Cloning Strategies

Given that all attempts to clone the p40 gene in an *E.coli*-based expression system were unsuccessful, the future realisation of that objective will necessitate the consideration of alternative cloning strategies. Cloning and expression in a mycobacterial cell such as *M.smegmatis* could eliminate many of the potential problems associated with expression in *E.coli*. Cloning in *M.smegmatis* already has been successfully applied to isolating and characterising *ser2*, a gene cluster responsible for the biosynthesis of the oligosaccharide segment of the *M.avium* serovar 2-specific GPL (Belisle *et al.*, 1991 and 1993). This was achieved by constructing a *M.avium* serovar 2 (strain TMC 724) genomic DNA library in the *E.coli-Mycobacterium* shuttle cosmid pYUB18, and immunoscreening *M.smegmatis* transformants for expression of the *ser2*-specific GPL with the serovar 2-specific mab CS-17. Interestingly, work carried out at the MRI demonstrated that strain TMC 724 was positive for both IS901/902 and p40 (Sharp, pers. comm.). It would be interesting and potentially valuable to screen this *M.avium* serovar 2 library for expression of p40 with the immune reagents generated in this study.

Cloning genes from one species of *Mycobacterium* in another offers a number of advantages. Firstly, codon usage is more likely to be compatible between two species belonging to the same genus than between genera as distantly related as *Mycobacterium* and *E.coli*. This would circumvent the problem of rapid tRNA depletion and early truncation of peptides relying on tRNA species which may be rare in *E.coli*. Secondly, post-translational modifications such as glycosylation (Fifis *et al.*, 1991; Garbe *et al.*, 1993; Lopez *et al.*, 1994; Dobos *et al.*, 1995), biotinylation (Henrikson and Allen, 1979; Collins *et al.*, 1987; Norman *et al.*, 1994) and protein splicing (Davis *et al.*, 1992 and 1994; Fsihi *et al.*, 1996) would be more likely to occur in a mycobacterial host cell than in *E.coli* where these events have not been reported. Thirdly and finally, expression of p40 would be less likely to exert any toxic effects in a mycobacterial host cell. Consequently, and given that the immune reagents generated during this study were not observed to cross-react with any other

antigen in *M.smegmatis*, successful expression of p40 could be detected easily in lysates of mycobacterial transformants.

A second alternative cloning strategy is offered by phage display. This technique entails the expression and display of a foreign peptide, as a fusion with a viral capsid protein, on the surface of the phage particle. The first phage vectors designed for surface display were filamentous phages (Scott and Smith, 1990), but a more versatile system based on the T7 phage has since been developed for the same purpose (Rosenberg *et al.*, 1997). Traditionally, phage display has been used to create libraries of short peptides (~6 amino acid residues), which have been used to help identify cell receptor molecules, enzyme active sites, enzyme inhibitors, antibody binding sites and linear epitopes of various antigens (O'Neil and Hoess, 1995). However, 415 copies of a foreign peptide of up to 50 aa in length can be expressed and displayed on the surface of the T7 phage, a number which corresponds to the total number of capsomeric fusions per recombinant virion. This provides both a useful source of the peptide of interest, and up to 150bp of nucleotide sequence of the encoding gene. The phage vector relies on *E.coli* as a host cell and would not totally overcome the problem of rare tRNA species. However, the short length of the expressed peptides would reduce the likelihood of both early tRNA depletion and expression product toxicity.

6.5 Immunoprophylactic Potential of p40

As a unique antigen of an organism known to cause a paratuberculosis-like disease in ruminants, the p40 antigen merits consideration as a potential component of any future subunit paratuberculosis vaccine. This would necessitate assessing the immunoprophylactic potential of the p40 antigen in combination with different adjuvants and delivery systems. Preliminary results of immunisation experiments have not been encouraging however. Vaccination of BALB/c mice with p40 in incomplete Freund's adjuvant failed to impede bacterial proliferation upon subsequent intravenous challenge with live *M.avium* type A/I (Inglis *et al.*, 1994a).

No significant reductions in the numbers of organisms recovered from the spleens and livers of immunised and placebo mice were observed. Similarly, vaccination with p40 in combination with the adjuvant dimethyl dioctadecyl ammonium bromide (DDA) was ineffective in eliciting protective CMI (Inglis, unpublished observations). However, techniques for presenting defined mycobacterial antigens to the murine immune system have been described which stimulate protective CMI responses comparable with those afforded by vaccination with BCG (Andersen, 1994; Silva and Lowrie, 1994). The success of those experiments serves to illustrate the point that antigens identified as potential vaccine components must be delivered and presented to the immune system appropriately if their true immunoprophylactic value is to be accurately assessed. Such work needs to be repeated with the p40 antigen.

6.6 Are Strains of IS901/902-Positive *M.avium* of Major Aetiological Significance in Ruminant Paratuberculosis?

There is currently no information in the scientific literature which apportions responsibility for the total number of clinical paratuberculosis cases recorded each year, between *M.a.paratuberculosis* and *M.avium* type A/I. This is primarily because reagents enabling simple, rapid and accurate immunodiagnoses have not been generally available. Even among the few potentially subspecies-specific antigens identified to date, the carboxyl terminus on the 34kDa antigen of *M.a.paratuberculosis* (De Kesel *et al.*, 1993; Vannuffel *et al.*, 1994a) does not stimulate *in vitro* secondary T-lymphocyte proliferation in its recombinant form, and is therefore of no value in detecting early stage infections (Burrells and Stevenson, pers. comm.). The p40 antigen is the first entire protein molecule to show subspecies-specificity within the MAC, and as such provides the means to distinguish between animals infected with *M.avium* type A/I or *M.a.paratuberculosis*, even in the early stages of infection when circulating antibody is absent. Consequently, it is conceivable that the aetiology of clinical paratuberculosis, especially in species such as deer and goats, may require re-evaluation.

6.7 Identification of Further Subspecies-Specific Antigens: The Future

Both antigenic complexity and close relatedness between the members of the MAC have made the task of identifying subspecies-specific proteins arduous. In addition, further difficulties in the identification, purification and analysis of candidate antigens arise from the complexing of many mycobacterial protein antigens with other biomolecules to form insoluble cell wall components. However, recent developments in the comprehensive analysis of protein expression “*proteome analysis*” may offer a means by which subspecies-specific proteins of two or more antigenically similar organisms can be readily identified (Pennington *et al.*, 1997). The proteome can be regarded as a comprehensive expression profile of all the protein encoding sequences in the genome of a given organism. There are two phases to proteome analysis. First, all the proteins expressed by a given organism under defined conditions are identified and mapped to positions on a standardised two-dimensional (2-D) gel format. This defines the constitutive proteome, and the information generated can be used to produce a 2-D reference map and protein database for the organism in question. There are several 2-D reference maps and protein databases already available and these are accessible through the world wide web and internet (see Pennington *et al.*, 1997). The 2-D reference maps serve as the foundation on which differences between closely related organisms can be established at the level of protein expression. The second phase of proteome analysis involves measuring both qualitative and quantitative changes in the proteome of an organism in response to alterations in environmental conditions. An example of this would be the comparative analysis of the proteomes of organisms grown on laboratory media, and those recovered from macrophages of an infected host. Alternatively, comparative proteome analysis of pathogenic and non-pathogenic bacterial strains, e.g. *M.tuberculosis* and *M.bovis* BCG, may provide new information leading towards the elucidation of bacterial virulence mechanisms. This type of comprehensive expression product analysis allows the identification of antigens which are selectively expressed under different sets of conditions, the occurrence of, and changes in, post-translational modification events and even

changes in subcellular localisation of proteins where appropriate cellular fractionation has been performed prior to analysis.

In the longer term, it is anticipated that the 2-D reference maps and protein sequence databases will become integrated with genome sequence databases. Once fully developed, such an integrated system should provide a direct link from an unidentified spot on a 2-D gel to a full nucleotide sequence of the corresponding gene and positive identification of the protein in question. Protein identification at this level will revolutionise the search for new species- and subspecies-specific mycobacterial proteins, and the identification of candidate antigens for the diagnosis and effective immunoprophylaxis of paratuberculosis may be on the horizon after more than a century since the disease was first described.

6.8 Addendum

6.8.1 Identification of a p40 homologue

Prior to the submission of this thesis, a final search of the OWL database was performed to check recent submissions for aa sequences related to the p40-derived CNBr cleavage peptides. This last database search revealed two cosmid clones (*M.tuberculosis* MTCY8D5 and *M.leprae* MLCL622), both of which contain positive strand ORFs encoding similar proteins of 337 and 339 aa residues respectively (~38.5kDa). The *M.tuberculosis* protein, for which no identity or biological function has been proposed, is encoded by ORF 11 which is located between positions 13842-14855 on cosmid MTCY8D5 (OWL accession number Z92669). The *M.leprae* protein has been identified as a putative phosphoenolpyruvate carboxykinase (PEPCK), and is encoded by ORF 22 on cosmid MLCL622 between positions 31504-32523 (OWL accession number Z95398).

6.8.2 Alignment of p40-derived peptides with the *M.tuberculosis* and *M.leprae* ORFs supports the hypothetical CNBr cleavage map of p40

Significantly, the p40 amino-terminal aa sequence and both the p31- and p28-derived internal aa sequences were shown to share identity with the translated ORFs in a pattern which corroborated the hypothetical CNBr cleavage map of p40 proposed in the previous chapter (see Figs. 5.12 and 6.1). The p40 amino terminal aa sequence was shown to share 72% ($^{13}/_{18}$) identity and 77% ($^{14}/_{18}$) similarity across 18 amino terminal aa residues of the *M.tuberculosis* hypothetical protein, and 70% ($^{14}/_{20}$) identity and 90% ($^{18}/_{20}$) similarity across 20 residues of the *M.leprae* putative PEPCK. The p31-derived internal aa sequence was shown to share 63% ($^{12}/_{19}$) identity and 73% ($^{14}/_{19}$) similarity across 19 aa residues of the *M.tuberculosis* protein, and 63% ($^{12}/_{19}$) identity and 68% ($^{13}/_{19}$) similarity across the same 19 aa residues of the *M.leprae* protein. Whilst identity and similarity values were observed to be lower for the p28-derived internal sequence, there was visible overlap between the carboxyl terminus of the p31, and the amino terminus of the p28 CNBr cleavage fragments. However, even though the area covered by the overlapping region was conserved between all three proteins, the absence of a methionine residue at the carboxyl terminus of the p31, and immediately preceding the amino terminus of the p28, indicates that the overlap between the two peptides may not be authentic. Even so, it is conceivable that the conserved methionine at position 156 on both *M.tuberculosis* and *M.leprae* proteins is slightly displaced on p40, and that the p31 and p28 peptides represent genuine contiguous sequences.

On the basis of the alignment shown in Fig. 6.1, it was calculated that CNBr cleavage peptides spanning positions 1-138, 139-156 and 157-340 would have respective M.wts of ~15.6kDa, ~2kDa and ~20.8kDa (~38.4kDa). These values compare with ~12kDa, ~3kDa and ~28kDa (~43kDa) as proposed in Fig. 5.12. Such a favourable comparison supports not only the hypothetical cleavage map of p40, but also the PCR strategy used in terms of priming positions relative to the gene. In turn, this emphasises the possibility that the 5' end of the p40 gene was successfully amplified


```

1                                     60
M.tb  M-ASGYGGIRVGGPYFDDLSSKGQVFDWAPGVTLSLGLAAHQSIVGNRLRLALDSDLCAA
M.lep  MTANGHAGRREGGPYFDDLSIGQVFDWAPAVTLTSGMAAVHQAILGDRMRLALDAELSTT
p40    -TSDGYARIREGGPYFDDLSV-----
p31    -----
p28    -----

61                                     120
M.tb  VTGMPGPLAHPGLVCDVAIGQSTLATQRVKANLFYRGLRFHRFPAVGDTLYTRTEVVGLR
M.lep  VIGTHAMLAHPGLVCDVAIGQSTLVTQRVKANLFYRGLTFHRFPVIGDTLYTSTEVVGLQ
p40    -----
p31    -----
p28    -----

121                                     139                                     157                                     170                                     180
M.tb  ANSPKPGRAPTGLAGLRMTTIDRTDRLVLDDFYRCAMLPASPDWKPGAV--PGDDLSRIGA
M.lep  ANSAKPGRPPTGMAALRITTTDQHDLVLDDFYRCAMLPASAAAWHPHDALNRNDDLANVGA
p40    -----
p31    -----ITIDQADQLVPDFYRGGGLP-----
p28    -----LPASPEFTAEAGSV-----

181                                     240
M.tb  DAPAPAADPTAHWDGAVFRKRVPGPPHFDAGIAGAVLHSTADLVSGAPELARLTLNIAATH
M.lep  DAVASASDPTGQWDAAAFRERVPGPPHFDAGITGAVLRSTGDIVSSAPDLARLTLNIASTH
p40    -----
p31    -----
p28    -----

241                                     300
M.tb  HDWRVSGRRLVYGGHTIGLALAQATRLLPNLATVLDWESCDHTAPVHEGDTLYSELHIES
M.lep  HDSRVRGLRLVYGGHTIGLALAQAGRMLPNLATVLNWRSCDHTGPVHEGDTLYSELHVES
p40    -----
p31    -----
p28    -----

301                                     340
M.tb  AQAHADGGVLGLRSLVYAVSDSASEPDRQVLDWRFSALQF
M.lep  AEATEQGGVLGLRSLVYAVS-AAGGSDHLVLDWRFTVLQF
p40    -----
p31    -----
p28    -----

```

Fig 6.1 Multiple alignment showing sequence identity between p40-derived amino acid sequences (p40, p31 and p28) and translations of ORFs contained within *M.tuberculosis* cosmid MTCY8D5 (*M.tb*) and *M.leprae* cosmid MLCL622 (*M.lep*). Amino acids highlighted in bold represent residues which are conserved between all three proteins. Methionine residues, highlighted in red, represent CNBr cleavage sites.

in reactions using lower annealing temperatures, but not detected subsequently. Given that this was indeed the case, it must be assumed that the internal p31-derived degenerate oligonucleotide, M5806, was either inaccurately deduced from the available aa sequence data and codon usage table, or too degenerate to detect specific amplified product by either hemi-nested PCR, or by hybridisation with Southern blots of first-round amplification products. Alternatively, given that the p28-derived oligonucleotide, M5018, was observed to hybridise preferentially to genomic sequences other than the p40 gene, especially at low stringencies, it could be argued that first round amplifications using this particular primer had only a limited chance of success. In this event, screening with a third oligonucleotide, presumed to be internal to a first round amplification product, could not have been expected to detect a sequence of interest.

6.8.3 Distribution and expression of the p40 homologue within the genus *Mycobacterium*

Clearly, a p40-related gene is present in *M.tuberculosis* and *M.leprae*, and this fact provides reason to believe that similar sequences may be present in other species of *Mycobacterium* also. In this study however, 15 different species of *Mycobacterium* were examined, including *M.tuberculosis* and *M.leprae*, but only IS901/902-positive strains of *M.avium* could be shown to express p40. Unless the related genes are silent in other species when propagated *in vitro*, or expressed at a level which cannot be detected by the immune reagents generated in this study, expression of a p40-like protein was not apparent in any of the 14 other species tested. Whilst it is conceivable that the p40-related gene may be expressed *in vivo* by other species of *Mycobacterium*, the data presented in chapter 4 demonstrate that p40-specific T-cell responses can be used to distinguish between one group of sheep infected with IS901/902-positive *M.avium*, and a second group infected with its closest relative, *M.a.paratuberculosis*. Given the close similarity shared by these two organisms, it seems more likely that *M.a.paratuberculosis*, or the other members of the MAC,

would express a p40 homologue than species which are phylogenetically more distant.

6.8.4 Elucidation of the association between p40 and IS901/902

Unfortunately, the identification of a p40 homologue in other species of *Mycobacterium* does not provide any information to help elucidate the nature of the apparent association between p40 and IS901/902. A first step towards that goal will require the cloning and sequencing of the flanking regions of every IS901/902 insertion site in the *M.avium* type A/I genome. Characterisation of these regions and identification of disrupted genes may provide vital clues, especially if the disrupted repressor gene theory, referred to earlier (see section 6.2), is proven correct. The recent proposal to sequence the *M.avium* genome in its entirety will not only complement the existing *M.tuberculosis* and *M.leprae* genome projects, but in the absence of any independent attempt to characterise IS901/902 flanking regions, will provide all the information necessary to perform such an analysis. However, since IS901/902-positive strains of *M.avium* do not appear to be of medical importance, it is more likely that the strain chosen for genomic sequencing will be of human origin and most probably an AIDS associated isolate.

6.8.5 Is p40 a phosphoenolpyruvate carboxykinase?

The translation product of the *M.leprae* p40-homologous gene has been identified as a putative PEPCK. PEPCK is an enzyme which catalyses the decarboxylation and mononucleotide-dependent phosphorylation of oxaloacetate (OAA) to form phosphoenolpyruvate (PEP). This conversion forms part of the gluconeogenic pathway in organisms across the phylogenic spectrum, and represents the second step in a biosynthetic pathway which synthesises glucose from non-carbohydrate precursor molecules such as lactate, pyruvate, citric acid cycle intermediates, most amino acids, and HCO_3^- . In bacteria such as *E.coli*, PEPCK is utilised during gluconeogenic growth at times when sugar levels are low (Goldie and Sanwal, 1980a). Traditionally, PEPCKs have been divided into two groups based on their

mononucleotide-specificity, with the ATP-dependent enzymes found in prokaryotes, yeasts, Trypanomastid parasites and plants, and the GTP-dependent PEPCKs in a wide variety of other eukaryotes and mammals (Utter and Kolanbrander, 1972). Whilst performing the same biological function, there are important differences and similarities between ATP- and GTP-dependent PEPCKs. With the exception of bacterial PEPCKs, which are monomeric (Goldie and Sanwal, 1980b), the ATP-dependent enzymes are predominantly multimeric, with two, four, or six subunits per enzyme. Conversely, all known GTP-dependent PEPCKs, are monomeric (Matte *et al.*, 1997). Although enzymes of either class show significant (40-80%) aa sequence identity within their respective divisions, there is no overall sequence homology between the ATP- and GTP-dependent PEPCKs. However, both groups of enzymes contain similar mononucleotide and OAA binding consensus motifs which are thought to be involved in substrate binding.

There are three conserved regions, within the ATP-dependent PEPCKs, including those of *E.coli* and *S.aureus*, which are known to interact with ATP-Mg²⁺ (Matte *et al.*, 1997). The first of these is the “Kinase-1a” sequence (GLSGTGKT) which interacts with the phosphoryl oxygen of ATP through electrostatic interactions and hydrogen bonds. The second conserved region is the “Kinase 2” sequence (LIGDD) whose specific function is the binding of the Mg²⁺ cation. The third, “Adenine-binding region”, is not as highly conserved as the Kinase 1a and Kinase 2 sequences (RISIKDT in *E.coli*, and RISLHYT in *S.aureus*), and is involved in interactions with the adenine base and ribose sugar. A fourth conserved sequence, the “PEPCK-specific domain”, (GTWYGGEMKK in *E.coli*, and GTEYAGEMKK in *S.aureus*) is thought to form part of the PEPCK active site (Matte *et al.*, 1996). Despite the highly conserved nature of these PEPCK sequences, “best fit” comparisons with the *M.tuberculosis* and *M.leprae* ORFs revealed no significant aa sequence identity. The presumed identity of the *M.leprae* protein is therefore open to question. It is surprising, given the level of sequence identity shared between the *M.tuberculosis* and *M.leprae* proteins, that only the latter was identified as a putative PEPCK. In

addition, at ~38.5kDa, the *M.leprae* protein is considerably smaller than other bacterial PEPCKs which have been reported in *Escherichia coli* K-12 (~60kDa) (Medina *et al.*, 1990), *Rhizobium* NGR234 (58.4kDa) (Osteras *et al.*, 1991), *Staphylococcus aureus* (~59kDa) (Scovill *et al.*, 1996), *Anaerobiospirillum succiniciproducens* (58.7kDa) (Laivenieks *et al.*, 1997) and *Ruminococcus flavefaciens* (66.3kDa) (Schocke and Weimer, 1997).

The possibility that p40 may function as a PEPCK raises new questions regarding its subspecies-specificity. Within the context of “housekeeping proteins”, it is difficult to perceive how such a ubiquitous enzyme could exist in an antigenically distinct isoform in IS901/902-positive strains of *M.avium*. In addition, if future research confirms a housekeeping function for p40, a role in the observed virulence of *M.avium* type A/I for BALB/c mice (Kunze *et al.*, 1991; Pedrosa *et al.*, 1994) will look less likely.

6.9 Future Work

The identification of the *M.tuberculosis* and *M.leprae* cosmid sequences creates new openings in the search for the gene encoding the *M.avium* type A/I p40 antigen. Using primers designed from regions which are conserved between the *M.tuberculosis* and *M.leprae* genes, amplification of DNA fragments from *M.tuberculosis* genomic DNA hopefully will provide a probe which will lead to the identification and isolation of the *M.avium* type A/I p40 gene. With this goal achieved, it will be possible to assess the distribution of the p40 gene, not just within the MAC, but among other species of the genus. Work towards this end, and towards establishing a biological function for p40, is currently underway at the MRI.

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APPENDIX 1.

Suppliers of Laboratory Consumables and Equipment

Allied Monitor
P.O. Box 71
201 Golden Drive
Fayette
Missouri 65248
USA

Amersham International plc.
Amersham Place
Little Chalfont
Bucks
HP7 9BR
UK

Beckman Instruments (UK) Ltd.
Oakley Court
Kingsmead Business Park
London Road
High Wycombe
Bucks
HP11 1JU
UK

Becton-Dickinson
Between Towns Road
Cowley
Oxford
OX4 3LY

Bio-Rad Laboratories Ltd.
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Herts
HP2 7TD
UK

BioSpec Products
P.O. Box 722
Bartlesville
Oklahoma 74005
USA

Boehringer Corporation (London) Ltd.
Boehringer Mannheim House
Bell Lane
Lewes
East Sussex
BN7 1LG
UK

Canberra-Packard
14 Station Road
Pangbourne
Berks
UK

Central Public Health Laboratory
National Collection of Type Cultures
61 Colindale Avenue
London
NW9 5HT
UK

Central Veterinary Laboratory
New Haw
Weybridge
UK

Dako A/S
Produktionsvej 42
DK-2600
Glostrup
Denmark

Difco Laboratories Ltd.
P.O. Box 14B
Central Avenue
West Molesey
Surrey
KT8 2SE
UK

Dynex Technologies
Daux Road
Billinghamurst
Sussex
RH14 9ST
UK

Edwards High Vacuum
Manor Royal
Crawley
Sussex
UK

Fuji Photo Film (UK) Ltd.
Government Markets Department
Professional Photographics Division
Fuji Film House
London
NW3 6JH
UK

Genetic Research Instrumentation Ltd.
Gene House
Dunmow Road
Felsted
Essex
CM6 3LD
UK

Hoefer Scientific Instruments (UK)
Unit 12
Croft Road Workshops
Croft Road
Off Hempstalls Lane
Newcastle under Lyme
ST5 0TW
UK

Hybaid Ltd.
St Clare Business Park
Holly Road
Hampton Hill
Middlesex
TW12 IP2
UK

ICN-Flow Laboratories
Unit 18
Thame Park Business Centre
Thame
Oxford
OX9 3TU
UK

Infors UK Ltd.
Fortune House
10 Bridgeman Terrace
Wigan
Lancs
WN1 1SX
UK

Invitrogen BV
De Schelp 12
9351 NV Leek
The Netherlands

Instrumentation Specialities Company
(ISCO)
4700 Superior
Lincoln
Nebraska
68504
USA

Janssen Pharmaceutical Ltd.
Grove
Wantage
Oxfordshire
OX2 0DQ
UK

Kodak Ltd.
PO Box 66
Kodak House
Station Road
Hemel Hempstead
Herts
HP1 1TU
UK

Kontron Instruments Ltd.
Blackmoor Lane
Croxley Centre
Watford
Herts
WD1 8XQ
UK

Life Technologies Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley
PA4 9RF
UK

Macherey-Nagel
GmbH & Co. KG
Postfach 10 13 52
D-52313 Duren
Germany

Mast Laboratories Ltd.
Mast House
Derby Road
Bootle
Merseyside
L20 1EA
UK

Merck Ltd.
Hunter Boulevard
Magma Park
Lutterworth
Leics
LE17 4XN
UK

Neil Inglis Engineering
Auchanruidh
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APPENDIX 2.

Solutions and Reagents

25 x Agarase Buffer

0.75M Bis-Tris/HCl pH6.0, 0.25M Na₂EDTA

Buffer-A

50mM Tris/HCl pH8.0, 300mM NaCl

Buffer-B

50mM Tris/HCl pH8.0, 600mM NaCl

Buffer-C

50mM Tris/HCl pH8.0

BT Buffer

50mM bis-Tris/HCl pH7.0

BTA Buffer

50mM bis-Tris/HCl pH7.0, 1.5M (NH₄)₂SO₄

BTN Buffer

50mM bis-Tris/HCl pH7.0, 150mM NaCl

Calcium Chloride Transformation Buffer (CCTB)

10mM MES/KOH pH6.2, 100mM KCl, 50mM CaCl₂

Coating Antibody Buffer (CAB)

15mM Na₂CO₃, 35mM NaHCO₃ pH9.6

Coomassie Brilliant Blue Stain

0.04% (wt/v) Coomassie Brilliant Blue R, 62mM C₂HCl₃O₂, 7.4% (v/v) glacial acetic acid, 50% (v/v) methanol

Coomassie Destaining Solution

7% (v/v) glacial acetic acid, 23% (v/v) methanol

Colony Lysis Solution

2 x SSC, 5% (wt/v) SDS

Denaturing Solution

1.5M NaCl, 0.5M NaOH

100 x Denhardt's Solution

2% (wt/v) bovine serum albumin, 2% (wt/v) Ficoll 400, 2% (wt/v) polyvinylpyrrolidone

DMSO and DTT Solution (DnD)

1M DTT, 90% (v/v) DMSO, 10mM $\text{KC}_2\text{H}_3\text{O}_2$ pH7.5

EcoRI Methylase Buffer

50mM Tris/HCl pH7.5, 1mM Na_2EDTA , 5mM DTT, 10 μM S-adenosyl-L-methionine

ELISA Substrate

25mM $\text{C}_6\text{H}_8\text{O}_7$, 50mM Na_2HPO_4 , 416 μM tetramethylbenzidine, 1.8mM H_2O_2

Gel Loading Buffer (GLB)

50mM Na_2EDTA , 0.1% (wt/v) (bromophenol blue, 25% (wt/v) sucrose or Ficoll 400

1 x Glycerol-Tolerant Sequencing Buffer (GTSB)

89mM Tris, 28.5mM taurine, 0.5mM Na_2EDTA
pH correct at 9.0

4M Guanidinium Isothiocyanate Solution (4M GIT)

4M guanidinium isothiocyanate, 50mM Tris/HCl pH7.5, 10mM Na_2EDTA , 2% (wt/v) sarcosyl, 145mM 2- β -mercaptoethanol

Hanks Basic Salt solution (HBSS)

1.3mM CaCl_2 , 5.4mM KCl, 0.5mM KH_2PO_4 , 0.8mM MgSO_4 , 136.9mM NaCl, 0.34mM Na_2HPO_4 , 5.6mM D-glucose, 0.03mM phenol red

High Efficiency Transformation Buffer (HETB)

10mM MES/KOH pH6.2, 100mM KCl, 10mM CaCl_2 , 45mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$

Hybridisation Solution

6 x SSC, 5 x Denhardt's solution, 0.5% (wt/v) SDS, 100 $\mu\text{g/ml}$ denatured salmon sperm DNA, 10% (wt/v) dextran sulphate

4 x Laemmli Denaturing Buffer (LDB)

0.5M Tris/HCl pH6.8, 4% (wt/v) SDS, 1.2 M 2- β -mercaptoethanol, 40% (wt/v) sucrose, 0.1% (wt/v) bromophenol blue

1 x Laemmli Electrode Buffer (LEB)

0.025M Tris, 0.192M glycine, 0.1% (wt/v) SDS
pH correct at 8.3

4 x Laemmli Resolving Gel Buffer (LRGB)

1.49M Tris/HCl pH8.8, 0.4% (wt/v) SDS

2 x Laemmli Stacking Gel Buffer (LSGB)

0.25M Tris/HCl pH6.8, 0.2% (wt/v) SDS

Membrane Washing Solution

5 x SSC, 0.1% (wt/v) SDS

M9-Salts

42mM Na₂HPO₄, 45mM KH₂PO₄, 8.5mM NaCl, 18.7mM NH₄Cl
pH adjusted to 7.5

Neutralising Solution

0.5M Tris/HCl pH8.0, 1.5M NaCl

NETN Buffer

20mM Tris/HCl pH8.0, 100mM NaCl, 1mM Na₂EDTA, 0.5% (v/v) Nonidet-P40

1 x Phosphate Buffered Saline (PBS)

0.137M NaCl, 0.003M KCl, 0.008M Na₂HPO₄, 0.015M KH₂PO₄
pH adjusted to 6.9

Plate Washing Buffer (PWB)

1 x PBS, 0.05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20)

Pre-Hybridisation Solution

6 x SSC, 5 x Denhardt's solution, 0.5% (wt/v) SDS, 100µg/ml denatured salmon sperm DNA

SDS Extraction Buffer

50mM Tris/HCl pH8.0, 1mM Na₂EDTA, 100mM NaCl, 2% (wt/v) SDS

SM Buffer

50mM Tris/HCl pH7.5, 10mM MgSO₄, 100mM NaCl, 0.02% (wt/v) gelatin

Sodium Borate Blot Buffer

50mM boric acid, 20% (v/v) methanol, 3mM 2-β-mercaptoethanol
pH adjusted to 8.0 with NaOH

20 x Standard Saline Citrate (SSC)

3M NaCl, 0.3M C₆H₅Na₃O₇·2H₂O
pH adjusted to 7.0 with citric acid

10 x TBE

0.89M Tris, 0.89M boric acid, 0.025M Na₂EDTA
pH correct at 8.3

1 x TE Buffer

10mM Tris/HCl pH7.5, 1mM Na₂EDTA

1 x Thin Layer Chromatography Buffer (TLCB)

0.75M KH₂PO₄
pH adjusted to 3.5 with phosphoric acid

1 x TNE Buffer

10mM Tris/HCl pH7.5, 100mM NaCl, 1mM Na₂EDTA

Tris Buffered Phenol

Phenol saturated with 10mM Tris/HCl pH7.5

Tris Glycine Blot Buffer (TGBB)

20mM Tris, 153mM glycine, 20% (v/v) methanol
pH correct at 8.0

10 x T4 PNK Buffer

500mM Tris/HCl pH7.6, 100mM MgCl₂, 5mM DTT, 1mM ATP, 1mM Na₂EDTA,
1mM spermidine

Wash Ethanol

10mM Tris/HCl pH7.5, 1mM Na₂EDTA in 70% (v/v) ethanol

APPENDIX 3.

Bacterial Strains and Vectors

***E. coli* host strains:-**

| Strain | Genotype | Reference |
|--------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------|
| AD202* (Dr. T. Saito) | F ⁻ , <i>ompT::kan</i> , <i>relA1</i> , <i>araD139</i> , <i>rbsR</i> , $\Delta(\textit{argF-lac})\text{U169}$, <i>rpsL150(str^r)</i> , <i>flbB5301</i> , <i>deoC1</i> , <i>ptsF25</i> | Akiyama and Ito, 1990 |
| DH1 (Invitrogen) | <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i> , <i>hsd R17(r_k⁻, m_k⁺)</i> , <i>supE44</i> | Hanahan, 1983 |
| HB101 (Invitrogen) | <i>thi-1</i> , <i>hsdS20 (r_B⁻, m_B⁻)</i> , <i>supE44</i> , <i>recA13</i> , <i>ara-14</i> , <i>leuB6</i> , <i>proA2</i> , <i>lacY1</i> , <i>rpsL20(str^r)</i> , <i>xyl-5</i> , <i>mtl-1</i> | Boyer and Roulland- Dussoix, 1969 |
| JM109 (Promega) | <i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> , <i>hsd R17(r_k⁻, m_k⁺)</i> , <i>relA1</i> , <i>supE44</i> , $\Delta(\textit{lac-proAB})$, <i>thi</i> , [F', <i>traD36</i> , <i>proAB</i> , <i>lacIqZ</i> ΔM15] | Yanisch-Perron <i>et al.</i> , 1985 |
| NM522 (Promega) | <i>supE</i> , <i>thi</i> , $\Delta(\textit{lac-proAB})$, $\Delta\textit{hsd5}(r_{k}^{-}, m_{k}^{-})$, [F', <i>proAB</i> , <i>lacIqZ</i> ΔM15] | Gough and Murray, 1983 |
| Y1088 (Stratagene) | <i>e14⁻(mcrA)</i> , $\Delta(\textit{argF-lac})\text{U169}$, <i>supE</i> , <i>supF</i> , <i>hsdR(r_k⁻, m_k⁺)</i> , <i>metB</i> , <i>trpR</i> , <i>tonA21</i> , <i>proC::Tn5(kan^r)</i> , [pMC9, <i>amp^r</i> , <i>tet^r</i> , <i>lacIq</i>] | Young and Davis, 1983b |
| Y1090 (Stratagene) | $\Delta(\textit{argF-lac})\text{U169}$, $\Delta(\textit{lon})?$ <i>araD139</i> , <i>rpsL(str^r)</i> , <i>supF</i> , <i>mcrA</i> , <i>trpC22::Tn10(tet^r)</i> , [pMC9, <i>amp^r</i> , <i>tet^r</i> , <i>lacIq</i>] | Young and Davis, 1983b |

(*A gift from Dr. Takashi Saito, Division of Molecular Genetics, School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, Japan 260.)

***Mycobacterium* species/strains:-**

| <i>Mycobacterium</i> | Source/Details |
|-----------------------------|----------------------------------------------------------|
| <i>M.avium</i> type A/I | MRI, deer isolate, strain JD88/118, IS901/902 +ve |
| <i>M.avium</i> type A/I | NCTC 8559, chicken isolate, IS901/902 +ve |
| <i>M.avium</i> type A** | Strain T133 (IS901/902 -ve), human (HIV patient) isolate |
| <i>M.a.paratuberculosis</i> | MRI, deer isolate, strain JD88/107, IS900 +ve |
| <i>M.intracellulare</i> | NCTC 10425, (ATCC 23068) |
| <i>M.scrofulaceum</i> | NCTC 10803, (ATCC 19981) |
| <i>M.gordonae</i> | SMRL, City Hospital, Edinburgh, human isolate, 38320/93 |
| <i>M.malmoense</i> | SMRL, City Hospital, Edinburgh, human isolate, 35056/93 |
| <i>M.kansasii</i> | SMRL, City Hospital, Edinburgh, human isolate, 34902/93 |
| <i>M. marinum</i> | SMRL, City Hospital, Edinburgh, human isolate, 33811/93 |
| <i>M.fortuitum</i> | SMRL, City Hospital, Edinburgh, human isolate, 41780/93 |
| <i>M.smegmatis</i> | NCTC, 8159 |
| <i>M.phlei</i> | MRI, M/1025 |
| <i>M.leprae</i> *** | <i>M.leprae</i> cell sonicate |
| <i>M.africanum</i> | SMRL, City Hospital, Edinburgh, human isolate, 17205 |
| <i>M.tuberculosis</i> | SMRL, City Hospital, Edinburgh, human isolate, 20963 |
| <i>M.bovis</i> | SMRL, City Hospital, Edinburgh, human isolate, 3433 |

(** A gift from Dr. J.J. McFadden, Molecular Biology Group, Dept. of Microbiology, University of Surrey, Guildford, Surrey, GU2 5XH, UK.)

(*** A gift from Dr. M.J. Colston, MRC National Institute of Medical Research, The Ridgeway, Mill Hill, London NW1 1AA.)

Plasmid and phage vectors:-

| Vector | Reference |
|------------------------------------------|-------------------------------------|
| pBluescript KS ⁻ (Stratagene) | Short <i>et al.</i> , 1988 |
| pGEX 1 λ T (Pharmacia) | Smith and Johnson, 1988 |
| pMS1S **** | Scherf <i>et al.</i> , 1990 |
| pUC18 (Pharmacia) | Yanisch-Perron <i>et al.</i> , 1985 |
| λ gt 11 (Stratagene) | Young and Davis, 1983a |

(**** A gift from J. Scaife and M. Schreiber)

APPENDIX 4.

Laboratory Media

IUT (International Union against Tuberculosis) medium

61% whole hen's egg, 50mM KH_2PO_4 , 25mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.6mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14mM $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, 67mM L.asparagine, 0.2% glycerol, 0.026% malachite green, pH 7.0. Sterilise at 121°C for 15min.

L-B agar

1% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, pH7.5, 1.5% Bacto[®]-agar. Sterilise at 121°C for 15min.

L-B top agarose

1% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, pH7.5, 0.7% agarose. Sterilise at 121°C for 15min.

Lowenstein-Jensen pyruvate medium (L-JP)

64.6% whole hen's egg, 50mM KH_2PO_4 , 25mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 114mM $\text{C}_3\text{H}_3\text{O}_3\text{Na}$, 14mM $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, 0.026% malachite green, 0.006% trypan blue, pH 7.0. Sterilise at 121°C for 15min.

Luria-Bertani (L-B) broth

1% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, pH7.5. Sterilise at 121°C for 15min.

Minimal agar (M-9 medium)

42mM Na_2HPO_4 , 45mM KH_2PO_4 , 8.5mM NaCl, 18.7mM NH_4Cl , pH7.5, 1.5% Bacto[®]-agar. Sterilise at 121°C for 15min and supplement as follows using sterile stock solutions; 2mM MgSO_4 , 0.1mM CaCl_2 , 0.2% glucose, 1mM thiamine-HCl.

Modified 7H11+ medium

2.1% 7H11 agar (Difco), 2.3mM L.asparagine, 2.5% glycerol, 2µg/ml mycobactin J (Allied Monitor), pH 7.0. Sterilise at 121°C for 15min and supplement as follows using sterile stock reagents; 10% Middlebrook OADC enrichment (Difco), 20% heat-inactivated new born calf serum (Life Technologies), *Selectatabs, Code MS 24 (Mast Laboratories).

Normal 7H11 medium

2.1% 7H11 agar (Difco), 0.5% glycerol, pH 7.0. Sterilise at 121°C for 15min and supplement as follows using sterile stock reagents; 10% Middlebrook OADC enrichment (Difco), pH 7.0, *Selectatabs, Code MS 24 (Mast Laboratories).

1 x RPMI-1640 medium

RPMI-1640 medium (Life Technologies) supplemented as follows using sterile stock solutions; 10% heat-inactivated foetal calf serum (Life Technologies), 30mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), 2mM glutamine, 24mM NaHCO₃, 0.74mM 2-mercaptoethanol, 50µg/ml gentamycin, 50iu/ml mycostatin.

SOB agar

2% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, 2.5mM KCl, 5mM MgCl₂.6H₂O, 5mM MgSO₄.7H₂O, 1.5% Bacto[®]-agar, pH7.0. Sterilise at 121°C for 15min.

SOB broth

2% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, 2.5mM KCl, 5mM MgCl₂.6H₂O, 5mM MgSO₄.7H₂O, pH7.0. Sterilise at 121°C for 15min.

SOC broth

2% Bacto[®]-tryptone, 0.5% Bacto[®]-yeast extract, 10mM NaCl, 2.5mM KCl, 5mM MgCl₂.6H₂O, 5mM MgSO₄.7H₂O, pH 7.0. Sterilise at 121°C for 15min and supplement with sterile glucose to 0.36%.

Stuart's medium

0.25% casein hydrolysate, 3mM L-asparagine, 18mM Na₂HPO₄, 7mM KH₂PO₄, 5mM Na₃C₆H₅O₇.2H₂O, 2mM MgSO₄.7H₂O, 2.5% glycerol, 0.05% Tween 80 (polyoxyethylenesorbitan monooleate), 2µg/ml mycobactin J (Allied Monitor), pH 7.0, 1.5% Bacto[®]-agar. Sterilise at 115°C for 15min and supplement as follows; heat-inactivated new born calf serum (Life Technologies) to 20% (v/v), *Selectatabs, Code MS 24 (Mast Laboratories).

Supplemented L-B media

All L-B media intended for *E.coli* host strains used to support the propagation of λgt11 was supplemented with maltose at 0.2% (w/v), MgSO₄ at 10mM and ampicillin at 50µg/ml.

*Each Mycobacteria Selectatab, Code MS 24, contains the following antibiotics:-

Polymyxin B.....100,000 units
Carbenicillin.....50mg
Amphotericin B.....5mg
Trimethoprim.....5mg

One Selectatab was used per 500ml medium.

APPENDIX 5.

Publications Arising From Work Presented in This Thesis

Ahrens, P., Giese, S.B., Klausen, J. and **Inglis, N.F.** (1995) Two markers, IS901/902 and p40, identified by PCR and by using monoclonal antibodies, in *Mycobacterium avium* strains. *Journal of Clinical Microbiology* **33**: 1049-1053.

Burrells, C., **Inglis, N.F.**, Davies, R.C. and Sharp, J.M. (1995) Detection of specific T-cell reactivity in sheep infected with *Mycobacterium avium* subspecies *silvaticum* and *paratuberculosis* using two defined mycobacterial antigens. *Veterinary Immunology and Immunopathology* **45**: 311-320.

Inglis, N.F., Davies, R.C., Stevenson, K., Burrells, C., Porter, J.F., Nyange, J.F.C. and Sharp, J.M. (1994a) Identification of an immunogenic 40kDa protein that may be specific for some strains of *Mycobacterium avium*. *In*, Proceedings of the Forth International Colloquium on Paratuberculosis. Chiodini, R.J., Collins, M.T. and Bassey, E.O.E. (eds.). Providence, R.I.: International Association for Paratuberculosis. 225-230.

Inglis, N.F., Stevenson, K., Hosie, A.H.F. and Sharp, J.M. (1994b) Complete sequence of the gene encoding the bacterioferritin subunit of *Mycobacterium avium* subspecies *silvaticum*. *Gene* **150**: 205-206.

Two Markers, IS901-IS902 and p40, Identified by PCR and by Using Monoclonal Antibodies in *Mycobacterium avium* Strains

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The occurrence of two markers, a newly identified 40-kDa protein (p40) and the insertion sequence IS901-IS902, in strains of *Mycobacterium avium* subsp. was evaluated. Analysis of 184 type and field strains of the *M. avium* complex from human, animal, and environmental sources by PCR specific to IS901 and by a monoclonal antibody specific to p40 demonstrated the presence of the two molecular markers in all of the *M. avium* subsp. *silvaticum* strains examined and also in a number of *M. avium* subsp. *avium* strains (the latter isolated mainly from pigs). The appearance of the two markers was completely concurrent in all strains. Further, the marker-positive *M. avium* subsp. *avium* strains were mainly serotype 2, whereas *M. avium* complex strains of serotypes 4, 6, 8, 9, and 10 were marker negative. The *M. avium* subsp. *avium* type strain ATCC 25291 and approximately 50% of the *M. avium* subsp. *avium* field strains isolated from animals contained the markers, while only one strain of human origin was found to be marker positive. Therefore, IS901 and p40 appear to have substantial potential to differentiate among isolates of the *M. avium* complex. This observation raises new issues regarding classification of strains, since the presence of the markers was found to be inconsistent with the present taxonomic grouping of *M. avium* subsp.

The *Mycobacterium avium* complex is a group of mycobacteria that are widespread in animals and the environment. The complex consists of *M. avium*, *M. intracellulare*, and *M. paratuberculosis*, as well as the wood pigeon bacillus. These slowly growing mycobacteria can infect and cause disease in lungs, lymph nodes, intestines, skin, bones, and soft tissues of animals and humans. *M. avium* infections have become the most commonly disseminated bacterial infections in immune-compromised populations such as patients with AIDS (12). *M. paratuberculosis* is the causative organism of paratuberculosis (Johne's disease), a debilitating chronic enteritis in ruminants, and it has been associated with Crohn's disease in humans (7, 25). The wood pigeon bacillus can cause enteritis in ruminants as well as disseminated infection in other hosts (8).

Taxonomic analysis of the *Mycobacterium* genus is complicated, and a number of schemes have been proposed to discriminate and classify organisms within the *M. avium* complex. The use of seroagglutination has enabled discrimination of *M. avium* and *M. intracellulare* (28, 29). Genetic analyses, particularly those examining the 16S rRNA genes, have been useful for discrimination of strains (23, 24) and even identification of new species (4). However, this approach has failed to discriminate among *M. avium*, *M. paratuberculosis*, and the wood pigeon bacillus (27, 32) and has led to suggestions that they were one species. Attempts to discriminate slowly growing mycobacteria with DNA probes have been reported, and considerable advances have followed the identification of the insertion elements IS900 and IS901-IS902 in *M. paratuberculosis* (10) and strains of *M. avium*, respectively (18, 22).

Each of the two insertion elements contains a unique sequence at the 5' end of the gene which has formed the basis for PCR analysis of mycobacteria in several laboratories (6, 35, 36). IS900 has been identified only in *M. paratuberculosis* strains (10), whereas IS901-IS902 has been found in wood

pigeon bacillus or in *M. avium* strains isolated mainly from animals (17, 18, 22).

Thorel et al. proposed *M. avium*, *M. paratuberculosis*, and the wood pigeon bacillus as a single species with three subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (31). This was based on genetic analysis and a number of culture characteristics such as mycobactin dependence, ability to grow on egg medium, and growth stimulation by pyruvate or pH 5.5.

Another grouping of strains was proposed by Kunze et al. (17) on the basis of the insertion elements IS900 and IS901. These authors suggested that *M. paratuberculosis* was defined by the presence of IS900. *M. avium* consisted of two distinct groups, referred to as type A/I, containing IS901 and type A not having this insertion element. The type A/I group corresponded mainly to *M. avium* subsp. *silvaticum* but also included *M. avium* subsp. *avium* strains, while type A corresponded to the remaining *M. avium* subsp. *avium* group. The division in type A and A/I was further supported by differences in plasmid possession and serotype antigens.

Recently, a 40-kDa protein (p40) was identified in a *M. avium* subsp. *silvaticum* field strain but could not be detected in isolates of *M. avium* subsp. *paratuberculosis*. Preliminary sequence analysis of this protein revealed that the amino-terminal 20 amino acids were not homologous to any known protein. Further, polyclonal sera from sheep and deer naturally or experimentally infected with *M. avium* subsp. *silvaticum* reacted with the 40-kDa antigen in immunoblots (5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated that p40 is one of the most abundant antigens in strains in which it is found to be present (12a). The function of the protein is not known. In the present study, 184 mycobacterial strains were examined by PCR specific to IS900 and IS901-IS902, by a monoclonal antibody (MAb) raised against the 40-kDa protein, and by serotyping in order to evaluate the distribution of the two markers IS901 and p40.

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TABLE 1. Source and origin of 184 *M. avium* complex strains used in this study and distribution of IS900, IS901, and p40 in all strains and serotypes of *M. avium* subsp. *avium* type, and reference strains^a

| Strain designation | Total no. | Origin | No. of strains showing the presence of: | | | Serotype ^b | Source |
|------------------------------------------------|-----------|--------------|-----------------------------------------|-------|-----|-----------------------|---------|
| | | | IS900 | IS901 | p40 | | |
| Reference strains | | | | | | | |
| <i>M. avium</i> subsp. <i>paratuberculosis</i> | | | | | | | |
| ATCC 19698 | 1 | Bovine | 1 | 0 | 0 | | |
| <i>M. avium</i> subsp. <i>avium</i> | | | | | | | |
| ATCC 19075 | 1 | Human | 0 | 1 | 1 | 2 | |
| ATCC 25291 | 1 | Avian | 0 | 1 | 1 | 2 | |
| ATCC 15769 | 1 | Avian | 0 | 0 | 0 | 1 | |
| NCTC 8551 | 1 | Porcine | 0 | 1 | 1 | 2 | |
| NCTC 8552 | 1 | Bovine | 0 | 1 | 1 | 2 | |
| NCTC 8558 | 1 | Ovine | 0 | 1 | 1 | 2 | |
| NCTC 8559 | 1 | Avian | 0 | 1 | 1 | 2 | |
| NCTC 8562 | 1 | Avian | 0 | 1 | 1 | 2 | |
| Field strains | | | | | | | |
| <i>M. avium</i> subsp. <i>paratuberculosis</i> | | | | | | | |
| | 25 | Bovine | 25 | 0 | 0 | | NVL |
| | 7 | Cervine | 7 | 0 | 0 | | NVL |
| | 6 | Caprine | 6 | 0 | 0 | | FS |
| <i>M. avium</i> subsp. <i>avium</i> | | | | | | | |
| | 42 | Human | 0 | 1 | 1 | | SSI |
| | 62 | Porcine | 0 | 31 | 31 | | NVL |
| | 6 | Cervine | 0 | 2 | 2 | | NVL |
| | 20 | Environment | 0 | 0 | 0 | | NVL |
| | 1 | Bovine | 0 | 0 | 0 | | NVL |
| | 1 | Avian | 0 | 1 | 1 | | NVL |
| <i>M. avium</i> subsp. <i>silvaticum</i> | | | | | | | |
| | 5 | Wild animals | 0 | 5 | 5 | | FS, NVL |

^a ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; FS, Finn Saxegaard, National Veterinary Institute; NVL, National Veterinary Laboratory; SSI, Statens Seruminstitut.

^b The serotypes of the *M. avium* subsp. *avium* field strains are listed in Table 2.

MATERIALS AND METHODS

Mycobacterial strains and growth conditions. The mycobacterial strains used in this study are listed in Table 1. The collection of strains consisted of a number of type strains from the American Type Culture Collection, Rockville, Md., and the National Collection of Type Cultures, London, United Kingdom; a number of human isolates (provided by Kurt Fuursted, Statens Seruminstitut, Copenhagen, Denmark); and isolates from goats and wild animals (provided by Finn Saxegaard, National Veterinary Institute, Oslo, Norway) as well as a number of field isolates from pigs, cattle, farm deer, and environmental sources collected at the National Veterinary Laboratory, Copenhagen, Denmark. The *M. avium* subsp. *paratuberculosis* strains were isolated from feces, mesenteric lymph nodes, or intestinal mucosa. Other strains from humans or animals were isolated from various organs.

Fecal samples were decontaminated by the method described by Beerwerth (3), while tissue samples were decontaminated with 5% sulfuric acid for 10 min.

Mycobacteria were grown on slopes of modified Löwenstein-Jensen medium (14) or Middlebrook 7H11 medium. The identification of field strains was based on growth rate, colonial morphology, mycobactin dependence, growth stimulation by pyruvate, resistance to isoniazid, and serotyping. Furthermore, 66 of the *M. avium* subsp. *avium* field strains from all sources were tested by the Gen-Probe (San Diego, Calif.) Rapid Diagnostic System according to the manufacturer's recommendations for confirmation of *M. avium* identity.

The five wood pigeon strains have previously been identified as such by F. Saxegaard (26a) and by J. B. Jørgensen (15).

Serotyping. Serotyping was performed as a tube agglutination test with 25 antisera as described by J. B. Jørgensen (13). In cases of cross-reactions, an antibody absorption test was performed (28). No further examination was made of strains that were nonagglutinable or that showed spontaneous agglutination.

Preparation of MAbs. The 40-kDa antigen was purified by high-performance liquid chromatography from a *M. avium* subsp. *silvaticum* field strain (5). BALB/c mice were immunized intraperitoneally with the purified antigen adsorbed to Al(OH)₃. The mice were immunized twice at intervals of 2 weeks and were boosted with the same amount of antigen 3 days before the fusion with the myeloma cell line P3 X63 Ag8-653 (16) by standard procedures (9). Screening of supernatant from growing hybridomas was performed by an enzyme-linked immunosorbent assay (ELISA) essentially as described below. Hybridoma supernatants diluted 1:10 were screened against crude antigen from *M. avium* subsp. *avium* ATCC 25291 and *M. avium* subsp. *paratuberculosis* ATCC 19698. The corresponding hybridomas were cloned by limiting dilution.

ELISA. An ELISA was used for the examination of antigen from different

mycobacterium strains. Microtiter plates were coated with 50 µl of crude antigen diluted in 50 mM sodium carbonate buffer (pH 9.6). Each antigen was tested with the hybridoma supernatant diluted 1:10 and 1:100 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin and was incubated for 2 h at 20°C.

Horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) diluted 1:1,000 was added for 1 h. The substrate-dye solution (0.07% 1,2-phenylenediamine, 0.04% H₂O₂ in 10 mM citrate [pH 5.0]) was allowed to react for 20 min; the reaction was stopped by the addition of 0.5 M H₂SO₄. The optical density at 490 nm was measured with 650 nm as the reference.

In addition to the specific MAb, the ELISA analysis included as a positive control a MAb directed against an epitope present in all *M. avium* subsp. *avium* (16a) and as a negative control a MAb raised against *Actinobacillus pleuropneumoniae*.

Sera from immunized mice were diluted 1:1,000 and were used as mentioned above.

Antigen preparation. Crude antigen was produced from cultured mycobacteria. The mycobacteria were harvested in 10 mM PBS (pH 7.2) containing 0.5% phenol, washed three times in PBS, disrupted with zirconium beads in a bead beater (Biospec Products, Bartlesville, Okla.) four times for 50 s, and clarified by centrifugation for 30 min at 20,000 × g. Finally, the suspension was filtered through a 0.22-µm-pore-size filter.

SDS-PAGE and immunoblotting. Antigens from different mycobacteria strains were separated on 12.5% polyacrylamide gels (19) and transferred onto nitrocellulose membranes by standard procedures (33). The protein concentrations of the individual samples were adjusted according to visual judgment of silver-stained gels (21).

The nitrocellulose membranes were cut into strips and incubated overnight with MAbs or serum and were visualized by peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:1,000 and then by staining with tetramethylbenzidine and hydrogen peroxide.

PCR. Cultured mycobacteria were disrupted by bead beating. A 2-µl sample was inoculated into 48 µl of a prepared reaction mixture containing 65 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, the four deoxyribonucleotide triphosphates (100 µM each), 65 pmol of each of the oligonucleotide primers, and 0.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany, or Perkin-Elmer Cetus, Norwalk, Conn.), and the mixture was covered with 50 µl of paraffin oil. Samples were subjected to an initial denaturation step 94°C for 3 min and then to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min in a thermal cycler.

The primers for IS901 PCR were GCTCGGGATTGGATGTCCTG (primer 901a) and CACCACGTGGTTAGCAATCC (primer 901c), which were derived from positions 19 to 394 of the IS901 sequence (18). Primers for IS900 PCR were CGTCGTTAATAACAATGCAG (primer 11) and GGCCGTGCGTTAGGCTTCGA (primer 36), which were derived from positions 27 to 304 of the IS900 sequence (10). Amplification products were analyzed by PAGE. Five microliters of the reaction product was subjected to electrophoresis through a 7.5% polyacrylamide gel in a Tris-phosphate buffer (pH 7.5). The gel was fixed in 10% ethanol-0.5% acetic acid and was silver stained according to standard protocols (21). Samples were considered IS901-IS902 positive when a single band of DNA, 375 bp in size, was evident in the gels. IS900 was identified by the appearance of a 279-bp band of DNA. Amplification products were digested with the restriction enzymes *MspI* and *RsaI* and were analyzed on a 10% polyacrylamide gel, fixed, and silver stained as mentioned above. Agarose gel electrophoresis and Southern blotting were performed by standard procedures. Nitrocellulose filters were hybridized with a DNA probe labeled with ^{32}S -dATP (Amersham International) as described earlier (1). The hybridization probe was the 375-bp PCR amplification product using DNA from *M. avium* subsp. *avium* ATCC 25291 as a target. DNA cycle sequencing (30) was performed as prescribed by the supplier (CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit; New England Biolabs, Beverly, Mass.).

RESULTS

Sera and MAb. ELISA screening of hybridoma supernatants led to the identification of 10 clones producing MAbs to p40. One of these, MAb 6.34, reacted in ELISA as well as in immunoblotting and was chosen for this study.

The molecular specificity of MAb 6.34 and of murine serum raised against the 40-kDa protein was demonstrated by immunoblot analysis (Fig. 1). The murine serum (Fig. 1A) bound strongly to a 40-kD protein present in the *M. avium* subsp. *avium* serotype 2 strains ATCC 19075 and ATCC 25291 (lanes 3 and 4, respectively), whereas this protein was not identified in the *M. avium* subsp. *avium* serotype 1 strain ATCC 15769 (lane 2) or in *M. avium* subsp. *paratuberculosis* ATCC 19698 (lane 1). Minor reaction was seen with other antigens (especially seen in lane 4). The reaction of MAb 6.34 is displayed in Fig. 1B. This MAb reacted only with a 40-kDa antigen present in the *M. avium* subsp. *avium* strains ATCC 19075 (lane 3) and ATCC 25291 (lane 4) as well as the field strains 42 (*M. avium* subsp. *silvaticum* [lane 5]) and FGR41 (*M. avium* subsp. *avium* serotype 2 [lane 6]). There was no reaction with *M. avium* subsp. *paratuberculosis* (lane 1) and *M. avium* subsp. *avium* ATCC 15769 (lane 2).

Field strains. The specificities of the MAbs were further investigated by ELISA with MAb 6.34 and by immunoblot analyses of field strains. Antigens from 64 strains were subjected to SDS-PAGE, blotted onto a nitrocellulose membrane, and incubated with MAb 6.34. A single 40-kDa band was observed for 15 strains, while 49 strains gave negative results. The ELISA analysis demonstrated that identical results was obtained by the two immunological methods (data not shown).

Antigens from 39 of the above-mentioned 64 strains were also tested by ELISA with murine serum instead of MAb 6.34. Murine serum showed results identical to those obtained with the MAb. This suggested that the specificity of p40 is not confined to a single epitope.

PCR. The published DNA sequences of the insertion sequences IS901 (18) and IS902 (22) were compared for selection of primers for specific amplification of a 375-bp fragment. The specificity of the PCR was examined by amplification with type strains of mycobacteria (Table 1). The insertion sequence was identified in the *M. avium* subsp. *avium* strains ATCC 19075, ATCC 25291, NCTC 8551, NCTC 8552, NCTC 8558, NCTC 8559, and NCTC 8562 by the appearance of a 375-bp fragment, whereas no amplification was observed for strain ATCC 15769 or for the *M. avium* subsp. *paratuberculosis* strain ATCC 19698.

The amplification products were further examined by re-

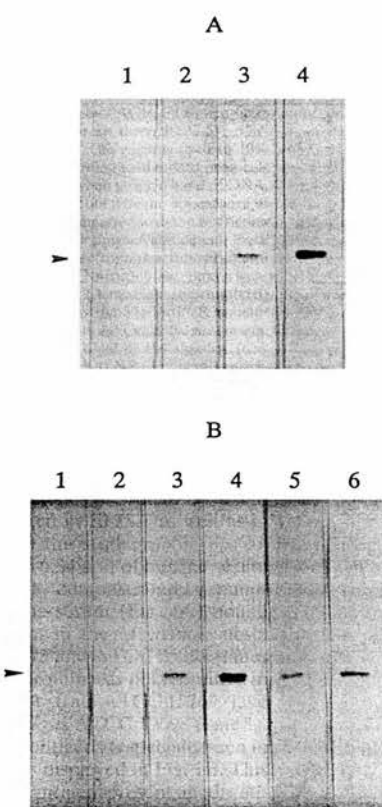


FIG. 1. Immunoblot of antigens from strains of *M. avium* subsp. showing the reactivities of murine serum to p40 (A) and MAb 6.34 (B). Lanes: 1, *M. avium* subsp. *paratuberculosis* ATCC 19698; 2, *M. avium* subsp. *avium* ATCC 15769; 3, *M. avium* subsp. *avium* ATCC 19075; 4, *M. avium* subsp. *avium* ATCC 25291; 5, *M. avium* subsp. *silvaticum* field strain; 6, *M. avium* subsp. *avium* field strain. The arrows indicate the positions of a 40-kDa band.

striction fragment analysis. Amplified DNAs from ATCC 25291 and ATCC 19075 digested with *RsaI* showed fragments of 241 and 134 bp, while digestion with *MspI* showed fragments of 282, 73, and 21 bp, as could be predicted from the published sequence of IS901 (not shown).

For verification of the PCR, the amplification products from 12 IS901 positive strains were examined by digestion with the restriction enzymes *MspI* and *RsaI*, respectively. The amplification products from all strains showed *RsaI* and *MspI* fragments of identical sizes which were indistinguishable from the expected size (*RsaI*, 241 and 134 bp; *MspI*, 282, 73, and 21 bp [data not shown]).

The identities of the amplification products were further confirmed by Southern blotting with purified amplification product from *M. avium* subsp. *avium* ATCC 25291 as probe (not shown). Primers 11 and 36 directed the amplification of a 279-bp fragment with ATCC 19698 but not with any of the other type or reference strains (Table 1).

Partial DNA sequence determinations (average, 166 nucleotides) of the amplification products of ATCC 25291 and ATCC 19698 and six field strains confirmed the specificity of the amplification.

TABLE 2. Serotypes of 132 *M. avium* subsp. *avium* field strains

| Origin | No. of isolates of the following serotypes ^a : | | | | | | | | | | | | | | | Total |
|-------------|-----------------------------------------------------------|---------|-------|----|----|---|----|----|-----|--------|-----|-----|-----|----|----|-------|
| | 1 | 2 | 3 | 4 | 6 | 8 | 9 | 10 | 1/6 | 2/3 | 2/8 | 3/9 | 4/8 | NA | SA | |
| Human | 3 | 3 (1) | 1 | 8 | 5 | 2 | 4 | | | 9 | 1 | | | 4 | 2 | 42 |
| Porcine | 2 (1) | 30 (29) | | 4 | 8 | 3 | 8 | 1 | 1 | 1 (1) | | 3 | 1 | | | 62 |
| Cervine | 1 | 1 (1) | 1 (1) | | | 2 | | | | | | | | 1 | | 6 |
| Environment | | | | 1 | 5 | | 4 | | | | | 4 | | 3 | 3 | 20 |
| Bovine | | | | 1 | | | | | | | | | | | | 1 |
| Avian | 1 (1) | | | | | | | | | | | | | | | 1 |
| Total | 7 (2) | 34 (31) | 2 (1) | 14 | 18 | 7 | 16 | 1 | 1 | 10 (1) | 1 | 7 | 1 | 8 | 5 | 132 |

^a Numbers of IS901- and p40-positive isolates are given in parentheses. NA, not agglutinable; SA, spontaneous agglutination.

Distribution of IS900, IS901, and p40. A total of 184 type and field strains were analyzed by PCR specific to IS901 or to IS900 and by ELISA with MAb.

All of the 38 strains identified by culture as *M. avium* subsp. *paratuberculosis* showed positive reactions with the IS900 PCR, whereas all other strains were found negative. Of the 184 strains analyzed, 47 were IS901 positive and 137 were IS901 negative. All strains that were found to contain IS901 were also shown to be positive by monoclonal ELISA, whereas IS901-negative strains did not react with MAb 6.34. This suggests that the two markers IS901 and the 6.34 epitope do not segregate independently ($P < 0.001$ [χ^2 test]).

Association of IS901 and p40 with serotypes. With the exception of the *M. avium* subsp. *paratuberculosis* strains, all of the isolates were serotyped. Of the five *M. avium* subsp. *silvaticum* strains, two were untypeable, one was serotype 2, and two strains were agglutinated with both type 2 and type 3 sera.

The serotypes as well as the presence of p40 and the insertion elements IS900 and IS901 in type and reference strains are listed in Table 1.

The distribution of serotypes of the 132 *M. avium* subsp. *avium* strains is shown in Table 2. Of 34 field strains identified as serotype 2, 31 were positive for both markers, while 3 strains did not possess any of the two markers. Furthermore, the markers were identified in 2 of 7 serotype 1 isolates, in 1 of 2 serotype 3 isolates, and 1 of 10 serotype 2/3 isolates. All but one of the strains that contained IS901 and p40 were isolated from animals.

DISCUSSION

The *M. avium* complex consists of a group of microorganisms whose members are not readily distinguishable from one another. They are important pathogens in animals and humans, and the AIDS epidemic has drawn increasing attention to this group of bacteria. The establishment of a taxonomic relationship among isolates has proved difficult. Different and incomparable schemes have been used for classification, and this may evoke some confusion.

In this study, a MAb directed against a newly identified protein p40 and a PCR test specific for the insertion sequence IS901-IS902 were used for the analysis of type and field strains of the *M. avium* complex. The insertion sequence appeared concurrently with the MAb 6.34 epitope in all of the analyzed strains. The nature of this close linkage between the two molecular markers remains to be clarified. Only *M. avium* subsp. *silvaticum* strains, most *M. avium* subsp. *avium* serotype 2 strains, and some serotype 1 and serotype 3 strains from animals were found to contain the two markers. The only human marker-positive strain was the reference strain ATCC 19075.

The correct assignment of isolates within the *M. avium* complex is by no means simple. In recent years, the use of DNA probes has gained widespread acceptance as the method of choice. However, these probes do not distinguish among *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*. The insertion sequence IS901-IS902 has previously been suggested to be specific for *M. avium* subsp. *silvaticum* (22). The data presented here demonstrate that this insertion element was present in all strains of *M. avium* subsp. *silvaticum* but also in a number of *M. avium* subsp. *avium* strains. IS901 was identified not only in field strains but also in reference strains, including the type strain of *M. avium* subsp. *avium* (ATCC 25291). The correct assignment of these strains can hardly be questioned. This demonstrates that *M. avium* subsp. *silvaticum* could not be defined by IS901.

Another study suggested that IS901 is a marker for strains differing in host range and virulence from other isolates of the *M. avium* complex (17). In the present study, the majority of the marker-positive strains were isolated from pigs. Nevertheless, about 50% of the porcine strains were found to be marker negative. Marker-positive and marker-negative strains could not be distinguished by cultivation characteristics such as growth rate and colonial morphology.

In the present study, the presence of p40 was identified by ELISA using a specific MAb. Nevertheless, all of the strains analyzed by ELISA using polyclonal serum only confirmed the results obtained by the MAb. Thus, it is likely that not only the MAb 6.34 epitope but also the 40-kDa protein are specific to a certain group of isolates.

The different appearance of IS901 and p40 in human and animal isolates could support the idea that livestock is not the primary source of human infections.

The function of p40 is not known. Partial amino acid sequencing of p40 has failed to demonstrate homology with any known protein, including the putative protein from the open reading frame of IS901 (5). Therefore, we have no leads regarding the apparently different host ranges of the two markers.

In the present study, the majority of the marker positive strains were serotype 2. The finding of variant prevalence of IS901-IS902 in different hosts could simply reflect the variant prevalence of serotype 2. Kunze and coworkers (17) examined a number of strains for the presence of IS901 and found the insertion element present in serotypes 1, 2, 3, 6, and 8. In the present study, the insertion element was found only in serotypes 1, 2, and 3.

A different distribution of serotypes in human and animal isolates has been described previously. Serovars 4, 8, 1, 9, and 6 are the most prevalent among human isolates in Western countries (2, 11, 34), while among animal isolates serotype 2 is

more prominent. The distribution of serotypes among porcine isolates seems to differ considerably among countries. Serotype 2 constituted 7.3% of the isolates in Norway (26), an earlier study found a result of 86.3% in Denmark (13), and in Japan serotype 2 was not found at all (20, 37). This highly variant distribution of *M. avium* subsp. *avium* serotype 2 strains could indicate that the molecular markers p40 and IS901 appear with variant frequencies in different countries.

Taxonomy within the *M. avium* complex has been confused by the lack of uniform characters. The taxonomic scheme proposed by Thorel et al. (31) is not in full agreement with the system based on insertion sequences used by Kunze et al. (17).

The presence of the two markers described in the present paper could be demonstrated without laborious DNA purification or time-consuming biochemical tests. Although further work is needed to elucidate the linkage between the two markers, we believe that the system will prove helpful in the characterization of *M. avium* complex isolates.

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Detection of specific T cell reactivity in sheep infected with *Mycobacterium avium* subspecies *silvaticum* and *paratuberculosis* using two defined mycobacterial antigens

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Abstract

A 30 kDa antigen (P30) from *Mycobacterium avium* ssp. *paratuberculosis* (*M. a. paratuberculosis*) and a 40 kDa (P40) antigen from *Mycobacterium avium* ssp. *silvaticum* (*M. a. silvaticum*) were employed in two different assays to measure the cell-mediated immune reactivity of ovine peripheral blood lymphocytes.

In lymphocyte stimulation assays, proliferative responses to the P30 were observed only with lymphocytes from sheep inoculated with live *M. a. paratuberculosis* or *M. a. silvaticum*. Although this antigen was not subspecies-specific it differentiated between animals given live organisms and those inoculated with an inactive lysate. The P40 protein from *M. a. silvaticum* showed subspecies specificity by eliciting in vitro responses only with lymphocytes derived from sheep inoculated with live *M. a. silvaticum*. Similar results were obtained using an interferon-gamma release assay which proved to be a more rapid and sensitive system.

1. Introduction

Infections with mycobacteria induce complex immune responses in the host involving both cell-mediated and antibody responses. Several antibody assay techniques have been employed and more recently, enzyme-linked immunosorbent assays (ELISA) have been developed as more sensitive tests in the serodiagnosis of mycobacterial infections such as human tuberculosis (Daniel and De-

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banne, 1987; Sada et al., 1990), leprosy (Pessolani et al., 1991) and paratuberculosis (Johne's disease) in cattle (McNab et al., 1991; Ritacco et al., 1991) and sheep (Brooks et al., 1988; Sugden et al., 1991). It is known, however, that the earliest responses and immunological resistance to mycobacterial infection depend upon cell-mediated immune (CMI) mechanisms (Thorns and Morris, 1983; Bloom and Godal, 1989) and that a reciprocal relationship exists between the cellular and humoral responses in respect of the subclinical (resistant) and clinical disease status of the host (Huygen et al., 1988; Ritacco et al., 1991).

The measurement of CMI to *Mycobacterium avium* ssp. *paratuberculosis* (*M. a. paratuberculosis*) infection has been undertaken using the intradermal skin test (Kormendy, 1988) measuring delayed-type hypersensitivity (DTH), the lymphocyte stimulation assay (LSA) (Buergelt et al., 1977, 1978) and the migration inhibition test (MIT) (Bendixen, 1977). These assays exhibit variable sensitivity but also lack specificity because lymphocytes are stimulated with relatively crude and undefined antigen preparations such as tuberculin or their purified protein derivatives (PPD). Consequently, cross-reactions occur as a result of the presence of antigens shared with other *Mycobacterium* spp., *Actinomyces* spp., *Dermatophilus* spp., *Nocardia* spp., *Streptomyces* spp., *Corynebacterium* spp., and fungi (Chiodini et al., 1984).

In a recent development, the correlation between lymphocyte blastogenesis and the production of immune-specific interferon-gamma (IFN- γ) has been exploited by Rothel et al. (1990) and Wood et al. (1991). They showed that the detection of IFN- γ in short-term whole blood cultures by ELISA was the most sensitive means for the diagnosis of bovine tuberculosis.

As part of a wider programme on paratuberculosis of ruminants, studies were undertaken on the host immune response to defined antigens of the causal microorganism, *M. a. paratuberculosis*. Two antigens were studied; a 30 kDa alpha antigen which exists in many mycobacterial species and has been shown to be a secretory protein, and a 40 kDa protein which has been demonstrated only in mycobacteria containing the specific insertion sequence IS901/IS902 (N.F. Inglis et al., unpublished observations, 1993; Ahrens et al., unpublished observations, 1993). This 40 kDa protein may therefore have potential as a specific diagnostic marker for *Mycobacterium avium* ssp. *silvaticum* (*M. a. silvaticum*) infections.

In the present study, the specificities of the two defined proteins were compared with PPD in tests to examine CMI responses of sheep immunised with an inactive lysate of *M. a. paratuberculosis* or infected with *M. a. paratuberculosis* or *M. a. silvaticum*.

2. Materials and methods

2.1. Culture of *M. a. paratuberculosis*

In vitro propagation of a strain (JD88/107) of *M. a. paratuberculosis* isolated from a red deer (*Cervus elaphus*) (Nyange, 1990) was performed on solid me-

dium as previously described (Stuart, 1965) except that crude *Mycobacterium phlei* mycobactin was replaced by purified mycobactin J (Allied Monitor Inc., Fayette, MO) (Merkal and McCullough, 1982). Cultures were incubated at 37°C for 6–8 weeks.

2.2. Preparation of crude lysate for immunisations

Mycobacterium a. paratuberculosis cells scraped from the surface of the growth medium were washed three times and resuspended in distilled water. Mechanical rupture was achieved using zirconium beads in a mini bead-beater as described by Hurley et al. (1987). After separation from the beads, the lysate was centrifuged at 30 000×g for 30 min to remove intact bacteria. Supernates were pooled and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK). Lysates were adjusted to 2 mg ml⁻¹ protein, aliquoted and stored at -80°C.

2.3. Preparation of 30 kDa antigen (P30)

Mycobacterium a. paratuberculosis organisms scraped from the surface of the growth medium were washed three times in TE buffer (0.01 M Tris/HCl pH 7.5 containing 0.001 M EDTA, disodium salt). One gram of washed pelleted organisms was resuspended in 3 ml TEM buffer (TE buffer adjusted to 0.005 M with 2-β-mercaptoethanol containing 0.001 M phenylmethylsulphonyl fluoride (PMSF)) and physically ruptured as above.

Cell lysates were clarified by centrifuging at 100 000×g for 60 min and the soluble supernatant proteins were precipitated sequentially using 20% and 40% saturated ammonium sulphate ((NH₄)₂SO₄). The precipitate was centrifuged at 30 000×g, dissolved in 1 ml TEM buffer, dialysed to remove salts, and proteins fractionated by high performance liquid chromatography (HPLC) gel filtration followed by anion-exchange chromatography.

(i) Gel filtration was carried out using PBS (0.15 M NaCl, 0.01 M phosphate, pH 6.9) containing 0.001 M β-mercaptoethanol on a TSK-G3000-SW silica column (8 mm×300 mm) at room temperature at a rate of 0.5 ml min⁻¹. Fractions containing a band of molecular mass 30 kDa (P30), as determined by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), were pooled.

(ii) Anion exchange chromatography: a pool of P30 was dialysed overnight against 0.05 M bis-Tris/HCl (BT) buffer at pH 7.0 to remove extraneous matter and absorbed to a TSK-DEAE-5PW HPLC column (8 mm×75 mm) equilibrated with BT buffer at 0.5 ml min⁻¹. Bound proteins were eluted by a continuous gradient of 0–0.5 M NaCl in buffer at a rate of 0.5 ml min⁻¹ for 30 min. The peak of material eluting at 0.15 M NaCl was homogeneous P30 protein as confirmed by SDS-PAGE. This P30 in 0.05 M BT buffer, pH 7.0 was then concentrated using a Millipore membrane with a cut-off point of 10 000.

2.4. Preparation of 40 kDa antigen (P40)

Mycobacterium a. silvaticum scraped from the surface of solid 7H11+ medium (McClatchy et al., 1976) was washed twice in PBS before being resuspended in PBS containing 0.001 M PMSF. Cell lysates were prepared by passing a suspension of 100 mg organisms in 3.0 ml PBS through a 'mini-French pressure cell' (SLM Aminco, SLM Instruments Inc., Urbana, IL) at 20 000 psi at room temperature. Cell lysates were clarified as above and proteins were selectively precipitated from the supernates using 20–60% saturated $(\text{NH}_4)_2\text{SO}_4$. Precipitates containing P40 were identified by SDS-PAGE, centrifuged at $30\,000\times g$ and dissolved in 1 ml PBS. Volumes (200 μl) were fractionated by HPLC gel filtration as for P30 except that the PBS did not contain β -mercaptoethanol. Anion exchange chromatography was performed as for P30 and fractions containing the P40 were identified as above. These were diluted tenfold with 2 M $(\text{NH}_4)_2\text{SO}_4$ in BT buffer and clarified through a 0.45 μm Millipore membrane. The filtrate was finally fractionated by hydrophobic interaction HPLC using a TSK-phenyl-5PW (8 mm \times 75 mm) column, equilibrated with 2 M $(\text{NH}_4)_2\text{SO}_4$ in BT buffer at 0.5 ml min⁻¹. Approximately 30% of ultraviolet-absorbing material was bound to the column and was eluted by application of a linear gradient decreasing from 2.0 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in BT buffer over 30 min at 0.5 ml min⁻¹. Pure P40 was eluted in 2 ml as a sharp peak at approximately 0.3 M $(\text{NH}_4)_2\text{SO}_4$.

2.5. Animals

Peripheral blood lymphocytes (PBL) from 12 sheep from various sources were examined for reactivity to *M. a. paratuberculosis* PPD and the two purified proteins:

(A) five lambs (V1–V5) each of which had been injected subcutaneously with 1.0 mg crude *M. a. paratuberculosis* lysate in 10% aluminium hydroxide (AlOH) first at 12 weeks of age and again 9 weeks later;

(B) four lambs each of which had been inoculated orally with 10^8 live mycobacteria first at 1 day of age then weekly for 9 further weeks: two receiving *M. a. silvaticum* (strain JD88/118) (S1–S2) and two receiving *M. a. paratuberculosis* (strain JD88/107) (P1–P2) (Nyange, 1990);

(C) two 12-week-old normal control lambs (C1–C2).

2.6. Lymphocyte stimulation assay

Lymphocyte stimulation assays (LSA) were performed as described previously (Burrells and Wells, 1977; Wells et al., 1979). Volumes (200 μl) of lymphocyte suspensions (10^6 ml⁻¹) were added to triplicate wells of sterile 96-well flat-bottomed microplates (NUNC-GIBCO Ltd., Paisley, UK) containing:

(a) 20 μl whole medium as an unstimulated control culture;

(b) 20 μl concanavalin A (Con A) (ICN Biomedicals, High Wycombe, UK)

at a final concentration of $7.5 \mu\text{g ml}^{-1}$ culture as a positive control of lymphocyte stimulation;

(c) $20 \mu\text{l}$ *M. a. paratuberculosis* PPD ('Johnin', Central Veterinary Laboratory, Weybridge, UK) at a previously determined optimal final concentration of $6.25 \mu\text{g ml}^{-1}$ culture;

(d) $20 \mu\text{l}$ *M. a. paratuberculosis* P30 antigen at a previously determined optimal final concentration of $2.5 \mu\text{g protein ml}^{-1}$ culture;

(e) $20 \mu\text{l}$ *M. a. silvaticum* P40 antigen at a previously determined optimal final concentration of $2.5 \mu\text{g protein ml}^{-1}$ culture.

Following culture, harvesting and liquid scintillation counting results were expressed as stimulation indices (SI)

$$SI = \frac{\text{Mean CPM of stimulated cultures} - \text{Mean CPM of blanks}}{\text{Mean CPM of control cultures} - \text{Mean CPM of blanks}}$$

2.7. Interferon gamma (IFN- γ) assay

A whole blood culture method followed by a sandwich ELISA as described by Rothel et al. (1990) for the detection of bovine IFN- γ and which had been shown to detect ovine IFN- γ (Rothel et al., 1990; G. Entrican and C. Burrells, unpublished data, 1992) was used. Heparinised whole blood was cultured in duplicate $200 \mu\text{l}$ volumes in a 96-well flat-bottomed microplate in the presence of $20 \mu\text{l}$ of medium (control), $20 \mu\text{l}$ of *M. a. paratuberculosis* PPD, $20 \mu\text{l}$ of P30 or $20 \mu\text{l}$ of P40 antigen as used in the LSA. Whole blood cultures were maintained for 24 h in a moist 5% CO_2 /95% air atmosphere after which time microplates were centrifuged at $450 \times g$ to deposit the cells. The supernatant plasmas were harvested and the duplicates pooled and stored at -20°C . A sandwich ELISA to detect ovine IFN- γ in the plasma was performed as described by Rothel et al. (1990). Briefly, volumes ($100 \mu\text{l}$) of plasma samples were added to triplicate wells of ELISA plates pre-coated with a dilution ($5 \mu\text{g ml}^{-1}$) of a monoclonal antibody (IFN 9) to bovine IFN- γ (Wood et al., 1990). Plates were incubated for 1 h at 20°C . After washing, $100 \mu\text{l}$ volumes of horseradish peroxidase (HRP) conjugated second anti-bovine IFN- γ (IFN2) monoclonal antibody (Wood et al., 1990) were added at a pre-determined optimal dilution. After incubation at 20°C for 30 min, plates were again washed prior to the addition of $100 \mu\text{l}$ of substrates (tetramethylbenzidine—TMB and hydrogen peroxide). The reaction was stopped after 30 min by the addition of $50 \mu\text{l}$ of $0.5 \text{ M H}_2\text{SO}_4$ per well and results were obtained using a titertek multiskan plate reader (Flow Laboratories, Irvine, UK) fitted with a 450 nm filter. Results are expressed in this study as 'corrected' optical densities (OD) (OD_{450} with test antigen $- \text{OD}_{450}$ with medium control).

3. Results

3.1. Lymphocyte stimulation assay

The responses of the PBL from all animals to stimulation with the T-cell mitogen Con A were generally high and in the range expected for sheep (mean CPM of unstimulated cultures 4.9×10^3 ; mean CPM of Con A stimulated cultures 227.9×10^3) (Burrells and Wells, 1977).

With the exception of the two control lambs (Group C), all the animals provided PBL which proliferated in response to stimulation with *M. a. paratuberculosis* PPD (Fig. 1). These PPD-driven responses tended to be greater with PBL from animals which had received live organisms (Group B; S1, S2 and P1) than those from sheep given inactive material (Group A; V1, V2, V3, V4 and V5).

Proliferative responses to the purified P30 antigen were obtained with PBL derived from three of the four animals inoculated with live organisms (Group B). PBL from only one of the immunised lambs (V5) responded minimally ($SI=3.0$) to P30 and PBL from the two control lambs (Group C) were not reactive.

None of the PBL from immunised animals (Group A) responded to the P40 antigen. Lymphocytes from the two animals (S1 and S2) given live *M. a. silvaticum* showed marked proliferative responses with stimulation indices of 38.0 and 93.7 respectively whilst PBL from the two lambs infected with *M. a. paratuber-*

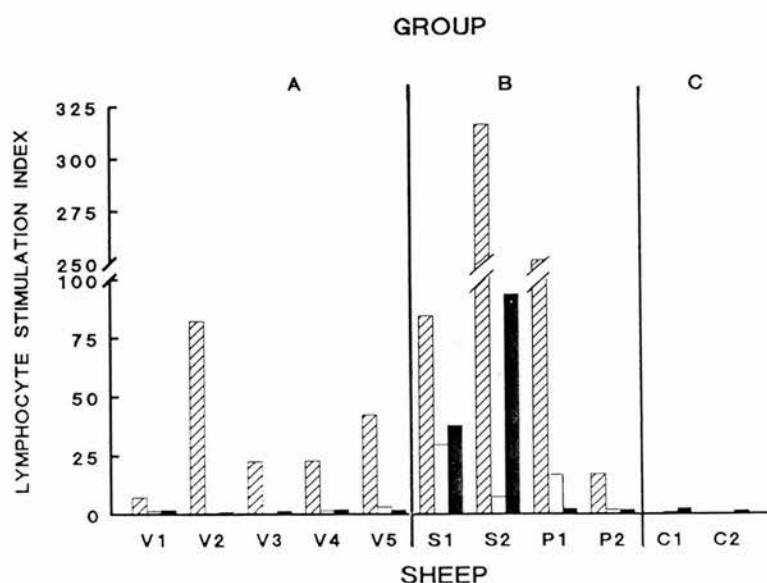


Fig. 1. Proliferation of peripheral blood lymphocytes from groups of sheep in response to PPD (Johnin) (hatched) and P30 (blank) from *M. a. paratuberculosis* and P40 (shaded) from *M. a. silvaticum*: V1-V5, immunised with inactive *M. a. paratuberculosis* lysate; S1-S2, infected with live *M. a. silvaticum*; P1-P2, infected with live *M. a. paratuberculosis*; C1-C2, non-immunised, non-infected normal control lambs.

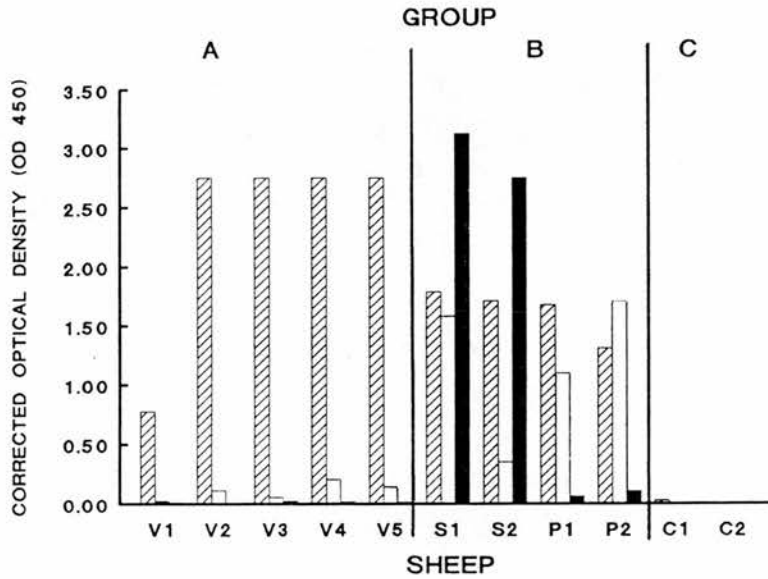


Fig. 2. *Mycobacterium a. paratuberculosis* PPD (hatched) and P30 (blank) and *M. a. silvaticum* P40 (shaded) antigen-stimulated release of interferon- γ in cultures of whole blood from groups of sheep: V1-V5, immunised with inactive *M. a. paratuberculosis*; S1-S2, infected with live *M. a. silvaticum*; P1-P2, infected with live *M. a. paratuberculosis*; C1-C2, non-immunised, non-infected normal control lambs.

culosis (P1 and P2) were not responsive to this protein. No responses were obtained with lymphocytes from the two control animals.

3.2. Production and release of IFN- γ

Mycobacterium a. paratuberculosis PPD induced the release of IFN- γ in cultures of blood from all the experimental animals whether given non-viable mycobacterial antigen or live bacteria (Fig. 2). Although a small amount of the cytokine was released by PBL from one of the immunised lambs (Group A; V4) (cut-off OD₄₅₀ < 0.200) in response to the P30 antigen, responses were much greater with PBL from all the infected animals (Group B). P40-induced IFN- γ release was achieved only with blood from the two lambs (S1 and S2) given live *M. a. silvaticum*. PBL from the two control lambs did not produce IFN- γ in response to any of the three antigens.

4. Discussion

On the basis of the results described, the use of purified mycobacterial antigens together with the IFN- γ release assay provides a test system which is superior to existing assays for CMI in terms of specificity, sensitivity speed, simplicity and cost.

With the exception of the two negative controls, lymphocyte proliferative responses to *M. a. paratuberculosis* PPD were mounted by PBL from all of the animals whilst responses to the two purified antigens were mainly confined to PBL from infected animals, only a very slight response to P30 being observed with one immunised animal (V5). This would suggest that the two purified proteins detected a CMI directed against products secreted by live organisms.

The use of such purified antigens would therefore be advantageous over PPD in proliferation assays due to their abilities to confer specificity and to discriminate between animals infected with live bacteria and those given inactive material.

Evidence to support this is accumulating from several sources. Thus, killed mycobacteria can stimulate the immune system but do not evoke long-term specific CMI (Orme, 1988a,b) and major differences are apparent in the T-cell repertoire stimulated by live and killed mycobacteria (Rook et al., 1986). Consequently, it has been suggested that proteins secreted by actively multiplying mycobacteria may have an important role in protection (Rook et al., 1986; Orme, 1988b).

In accord with the results presented here, Andersen et al. (1991) recently demonstrated that lymphocytes from mice given live *Mycobacterium tuberculosis* responded strongly to a 32–33 kDa secreted antigen whereas mice given killed organisms did not respond at any time. Furthermore, antibodies to the secreted antigen were not detected during the infection. Those authors also suggest a major role for antigens secreted by live mycobacteria in the induction of protective cellular immune responses.

The proliferative responses to the P40 antigen by PBL from only the two animals given live *M. a. silvaticum* enhances the evidence that this protein is specific for *M. a. silvaticum* and can differentiate between animals infected with this organism and those infected with *M. a. paratuberculosis*. Recently, a 40 kDa protein antigen from *M. tuberculosis* was shown to be specific for *M. tuberculosis* and *Mycobacterium marinum* and discriminate between these and other mycobacterial species such as *Mycobacterium avium*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, *Mycobacterium scrofulaceum* and *Mycobacterium szulgai* (Andersen et al., 1992).

The detection of IFN- γ correlates with the demonstration of antigen-specific lymphocyte stimulation (D'Andrea et al., 1986) and can be more sensitive (D'Andrea et al., 1986; Hao et al., 1986). This experience was borne out by the present results particularly with PBL from lamb P2 which showed release of IFN- γ in response to both PPD and P30 despite the slight or negative responses in proliferation assays. In an extensive field comparison the assay for IFN- γ release by lymphocytes in 24 h cultures in response to PPD was shown to be more sensitive than the single intradermal tuberculin test for the diagnosis of bovine tuberculosis (Wood et al., 1991).

While purified antigens are sought which will discriminate between other mycobacterial species, we suggest that the assay system described may be a more accurate indicator of infection, particularly at the subclinical stage or where previous vaccination with dead mycobacteria or intradermal testing could confuse the interpretation of the results of other assays.

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Identification of an immunogenic 40kDa protein that may be specific for some strains of *Mycobacterium avium*

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Summary

Members of the M. avium complex are over 95% homologous and differentiation between them is notoriously slow and difficult. Particular difficulties are encountered in discriminating M. paratuberculosis from other mycobactin-dependent members of the group eg wood pigeon bacillus (syn. M. a. silvaticum). We have identified a 40kDa protein that appears to be expressed in IS901/902+ strains of Mycobacterium avium but not in Mycobacterium paratuberculosis. The amino terminal twenty amino acids of the protein share no significant homology with any proteins currently available in the OWL database. The protein stimulates strong cell-mediated and humoral immune responses in sheep and deer. Such responses are observed only in animals infected with IS901/902+ strains of M. avium. The gene encoding the 40kDa protein has been isolated from a gtl1 expression library of M. avium and is currently being characterized.

Introduction

The *Mycobacterium avium* complex (MAC) comprises a group of closely related organisms (12) that show not only phenotypic similarity, but also share up to 98% sequence identity at the genomic level (10). Although attempts have been made to distinguish between the individual members of the MAC using a number of techniques the criteria and reagents for precise classification are still not available. Recombinant DNA technology has led recently to the identification of two species-specific genetic markers, IS900 in *M.a. paratuberculosis* (7,11) and IS901/902 in mycobactin-dependent strains of *M. avium* and *M.a. silvaticum* (9,13). These genetic elements have been exploited to detect these organisms directly in pathological samples (5,15,17). The search for species-specific proteins that could be employed to detect specific immune responses in infected animals has been less successful. So far, only the carboxy terminus of a 34kDa protein in *M.a. paratuberculosis* appears to contain specific epitopes that may be useful to detect antibodies in cattle with paratuberculosis (6,16).

The present study describes the identification of a novel 40kDa protein which co-segregates with the genomic insertion sequence IS901/902 in *M.a. silvaticum*. The 40kDa antigen provides a marker which allows both visual discrimination between *M.a. silvaticum* and *M.a. paratuberculosis* at the polypeptide level and immunological differentiation between animals infected with these *M. avium* subspecies.

Identification of a 40kDa protein in IS901/902+ strains of *M. avium*

Comparison of the polypeptide compositions of 19 MAC isolates by SDS-PAGE revealed the presence of a prominent protein, with an apparent molecular weight of 40kDa (Figure 1). This protein was

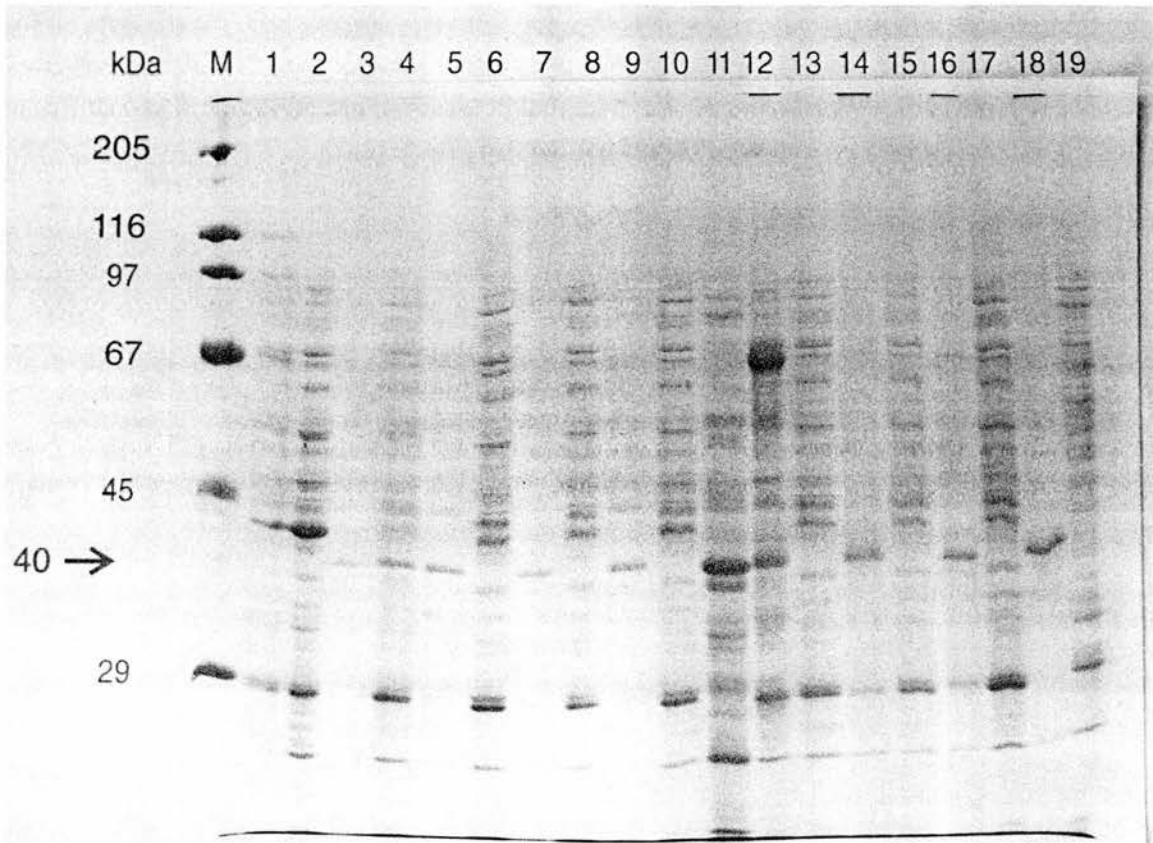


Figure 1: SDS-PAGE showing the polypeptide compositions of 19 MAC isolates. Isolates in lanes 1, 2, 6, 8, 10, 13, 15, 17 and 19 were identified as *M.a. paratuberculosis* by PCR for IS900. Isolates in lanes 3, 4, 5, 7, 9, 11, 12, 14, 16 and 18 were identified as IS901/902 positive by PCR. A prominent 40kDa protein is visible only in IS901/902 positive isolates.

identified only in those isolates containing IS901/902, and was absent in isolates positive for IS900. No isolate was shown to harbor either IS901/902 or the 40kDa antigen alone.

These preliminary observations have since been corroborated in a further and more extensive study in which 33 of 161 MAC type-and field-strains were shown to contain both 40kDa and IS901/902 (1). All 33 of these 40kDa and IS901/902 positive isolates were shown to be serotype 2, although 10% of serotype 2 isolates contained neither 40kDa nor IS901/902. None of the remaining 128 isolates, comprising a number of serotypes, could be shown to harbor either 40kDa or IS901/902 alone.

The 40kDa protein was purified to homogeneity by a series of chromatographic procedures (4) and the first 20 NH₂ terminal amino acids sequenced from western blots by Edman degradation. No significant homology was demonstrated with any protein currently available in the OWL database or with the amino acid sequence deduced from the ORF contained within IS901/902.

Immune response of infected sheep to the 40kDa protein

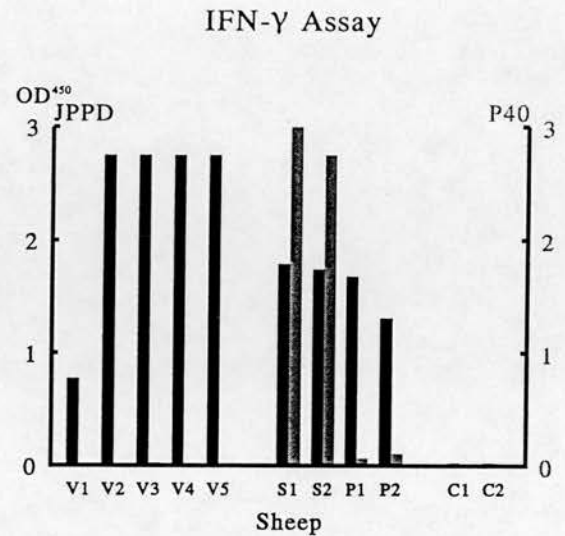
Peripheral blood lymphocytes (PBL) from 12 sheep were examined for reactivity to *M.a. paratuberculosis* PPD and purified 40kDa antigen by both the lymphocyte stimulation assay (LSA) and the δ -interferon (δ -IFN)-ELISA (4). Experimental animals were in three separate groups as follows:

Group A. 5 lambs (V1-V5), each of which was injected subcutaneously with 1.0mg of crude *M.a. paratuberculosis* cell lysate in 10% aluminum hydroxide at 12 and 21 weeks of age.

Group B. 4 lambs, each of which was dosed orally with 10^8 cfu live mycobacteria at 1 day old and then weekly for a further 9 weeks. Two lambs (S1,S2) received *M.a. silvaticum* (strain JD88/118), while the remaining two (P1,P2) received *M.a. paratuberculosis* (strain JD88/107).

Group C. 2 twelve week old uninoculated control lambs (C1,C2).

CMI responses to *M.a. paratuberculosis* PPD were detected by LSA and IFN- δ ELISA in all animals except the uninoculated controls (Figure 2). Higher responses were observed with PBL from the animals receiving live organisms. In contrast, only the two lambs (S1 and S2) given live *M.a. silvaticum* responded to stimulation with the 40kDa protein. These observations demonstrate that the 40kDa protein is expressed *in vivo* as well as *in vitro*. They further highlight the diagnostic potential of the 40kDa protein, particularly its ability to detect specific immune responses in animals infected with IS901/902-containing *M. avium* and discriminate these from those in animals infected with *M.a. paratuberculosis*.



Lymphocyte Stimulation Assay

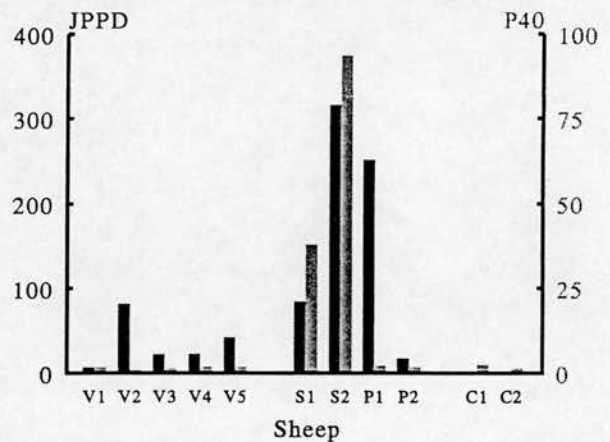


Figure 2: CMI responses to *M.a. paratuberculosis* PPD and the 40kDa protein. Groups of sheep are described in the text. □, response to PPD; ◻ response to 40kDa protein.

Immunization of Mice with 40kDa Protein

Strains of *M. avium* that contained IS901/902 (RFLP type A/I) were noted to be more virulent in mice than strains without the insertion sequence (RFLP type A) (9). Given the complete concordance between IS901/902 and the 40kDa protein, it was postulated that vaccination with this protein could protect recipient mice against subsequent challenge with live *M.a. silvaticum*.

Three groups of 32 newly weaned male BALB/c mice were inoculated as follows:

Group A. 2 μ g of purified 40kDa protein in Freund's incomplete adjuvant (IFA) was inoculated intraperitoneally in a total volume of 100 μ l at 3, 6 and 9 weeks of age.

Group B. 10 μ l of PBS in IFA was inoculated as described for group A.

Group C. 10 μ l of PBS was inoculated as described for group A.

When mice in group A had seroconverted to the 40kDa protein, all 96 mice were inoculated intravenously with 10⁶ cfu live *M.a. silvaticum* in 100 μ l of PBS. Eight mice from each group were killed at 6hr and 30, 60 and 90 days post challenge. Spleens and livers were removed aseptically and the number of viable organisms in each organ calculated.

The results of this experiment showed that the 40kDa protein did not confer protection in that bacterial proliferation was not measurably impeded. There were no significant differences between the numbers of organisms recovered from the spleens or livers of vaccinated and placebo mice (Figure 3). However, recent work has shown that depot adjuvants like IFA and aluminum hydroxide stimulate murine Th₂ cell responses characterized by the release of IL-4 and IL-5, leading ultimately to antibody production (3,8). Since protective immunity to intracellular pathogens like mycobacteria is known to be dependent

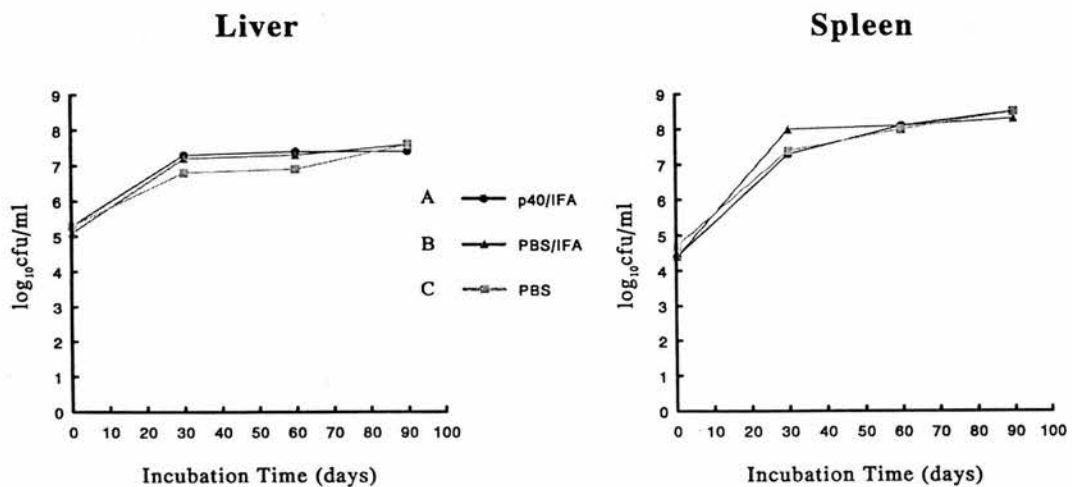


Figure 3: Viable counts of *M.a. silvaticum* recovered from the spleens and livers of vaccinated and placebo mice. Groups of mice are described in the text.

upon a cellular immune response, it is perhaps not surprising that the immunization strategy used in the present study was unsuccessful. Recently, methods have been described to present defined mycobacterial proteins to the murine immune system so that CMI responses are stimulated and even protection induced (2, 14). The success of those experiments illustrates the important point that antigens identified as potential vaccine components must be delivered and presented to the immune system correctly if their true immunogenic value is to be correctly assessed, and such work needs to be repeated with the 40kDa protein.

Acknowledgments

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GENE 08240

Complete sequence of the gene encoding the bacterioferritin subunit of *Mycobacterium avium* subspecies *silvaticum**

(Bacterioferritin; nucleotide sequence; expression; *Mycobacterium avium*; *Mycobacterium leprae*)

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SUMMARY

A gene encoding the bacterioferritin subunit (Bfr) of *Mycobacterium avium* (*Ma*) subspecies *silvaticum* has been cloned, sequenced and expressed. The 477-bp open reading frame codes for 159 amino acids, which were shown to share up to 92% identity with the Bfr of five bacterial genera. The recombinant Bfr exhibits serological cross-reactivity with *Ma paratuberculosis* antigen D, a protein of approx. 20 kDa in cell lysates of *Ma paratuberculosis* and *Ma silvaticum* and a protein of 20–22 kDa in sonicates of *M. leprae*.

Bacterioferritins (Bfr), instrumental in the storage of iron in at least ten diverse bacterial genera, are spherical multimers of approx. 400 kDa, assembled from 24 subunits of 17–20 kDa each (Grossman et al., 1992). Four λ gt11 re-clones were isolated from a *Ma silvaticum* genomic library by screening with Ab from a hyperimmune rabbit (Young et al., 1985). A 3.1-kb insert from one clone (λ /R692) was subcloned into pBluescript (clone pR692) and the sequence of the first 725 nt (EMBL accession No. X76906) revealed a 477-bp ORF (159 aa, 17.4 kDa). The predicted ATG start codon was preceded by a potential Shine-Dalgarno sequence from nt 66 to 71.

A FASTA search of the EMBL database revealed that the 725-bp cloned sequence shared 80% nt sequence identity with 500-bp of *M. leprae* genomic DNA (in

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*On request, the authors will supply detailed experimental evidence for the conclusions reached in this Brief Note.

Abbreviations: aa, amino acid(s); Ab, antibody(ies); Bfr, bacterioferritin(s); *bfr*, gene encoding Bfr; β Gal, β -galactosidase; bp, base pair(s); *E.*, *Escherichia*; kb, kilobase(s) or 1000 bp; *M.*, *Mycobacterium*; *Ma*, *M. avium* subspecies; nt, nucleotide(s); ORF, open reading frame; re-, recombinant; X, any aa.

cosmid mlb38; L01095), 59% with 455 bp of the gene encoding an iron storage protein of *Azotobacter vinelandii* (M83692) and 56% with the 598-bp sequence of the *E. coli bfr* (M27176). DAP and SWEEP searches of the OWL database using the deduced 159-aa sequence revealed homology with five bacterial proteins, the Bfr of *Ma paratuberculosis* (antigen D) (Brooks et al., 1991), *Azotobacter vinelandii* (Andrews et al., 1991), *Escherichia coli* (Andrews et al., 1989), *Nitrobacter winogradskyi* (Kurokawa et al., 1989) and *Synechocystis* PCC 6803 (Laulhere et al., 1992). The deduced aa sequence of clone pR692 was also 92% homologous to 162 aa of the translated *M. leprae* sequence (nt 20960–21439, reverse strand on cosmid mlb38). There is only a single aa substitution between this translated cosmid sequence and the first 14 N-terminal aa reported for the *M. leprae* 22-kDa major membrane protein (MMP-II) (Hunter et al., 1990). Alignment of the first 42 N-terminal aa of the known Bfr (Fig. 1) revealed strong identity, including the Bfr consensus pattern M-X-G-X₃-V-(LIV)-X₂-L-X-K.

Serological confirmation that clone pR692 encoded the mycobacterial Bfr was obtained using affinity purified polyclonal Ab (Beall and Mitchell, 1986). Ab eluted from the induced pR692- β -Gal fusion protein reacted with Western blots of purified *Ma paratuberculosis* antigen D,

