MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

Research and Development

# Final Project Report

(Not to be used for LINK projects)

**Date project completed:**

**31/3/99**

## Section 1 : Identification sheet

1. (a) MAFF Project Code: **SE3205**

(b) Project Title: Development of vaccine candidates for protection of cattle and badgers against infection with Mycobacterium bovis

(c) MAFF Project Officer: ____

(d) Name and address of contractor:
Veterinary Laboratories Agency
Woodham Lane, New Haw
ADDLESTONE
Surrey Postcode KT15 3NB

(e) Contractor's Project Officer: Dr Glyn Hewinson____

(f) Project start date: 1/4/98 Project end date: 31/3/99

(g) Final year costs:

| | |
|---|---|
| **approved** expenditure | £248,572 |
| **actual** expenditure | £225,543 |

(h) Total project costs / total staff input:

| | |
|---|---|
| **approved** project expenditure | £248,572 |
| **actual** project expenditure | £225,543 |
| ***approved** staff input | 4.Iy |
| ***actual** staff input | 3.7y |

(i) Date report sent to MAFF: 2/2/00

(j) Is there any Intellectual Property arising from this project ? NO

***staff years of direct science effort**

## Section 2 : Scientific objectives / Milestones

2. Please list the scientific objectives as set out in CSG 7 (ROAME B). If necessary these can be expressed in an abbreviated form. Indicate where amendments have been agreed with the MAFF Project Officer, giving the date of amendment.

- To generate mutant libraries of BCG and *M. bovis* by transposon mutagenesis.
- To identify and characterise auxotrophic mutants from the BCG library for use as vaccine candidates.
- To remove antigens immunodominant in cattle from *M. bovis* homologous recombination.
- To produce new and improved naked DNA constructs encoding antigens that are serodominant in badgers, i.e. MPB83 and MPB70.
- To construct a DNA vaccine encoding Ag85.
- To establish a mechanism for co-ordination between human and bovine TB vaccine development.
- To define antigens upregulated under physiologically relevant conditions (including the host and host macrophage) and to define the mechanisms of gene control for one of these antigens, MPB83.
- To produce final progress report and five manuscripts for publication.

3. List the primary milestones for the final year.

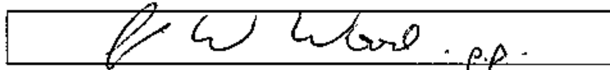
**It is the responsibility of the contractor to check fully that ALL primary milestones have been met and to provide a detailed explanation if this has not proved possible**

| Milestones | | Target date | Milestones met? | |
|---|---|---|---|---|
| Number | Title | | in full | on time |
| NB | PLEASE SEE AFTER SECTION 4 FOR COMPLETE LIST OF MILESTONES FOR THE YEAR / PERIOD UNDER REPORT | — | — | — |
| — | — — | — | — | — |
| — | — — | — | — | — |
| — | — — | — | — | — |
| — | — — | — | — | — |

**If any milestones have not been met in the final year, an explanation should be included in Section 5.**

## Section 3 : Declaration

4. I declare that the information I have given in this report is correct to the best of my knowledge and belief. I understand that the information contained in this form may be held on a computer system.

Signature  Date 31/01/00

Name Dr Glyn Hewinson

Position in Organistation Head of TB Research Group, Department of Bacterial Diseases. (SROII)

## Section 4 : Executive summary

The recently published 'Krebs' Report' on bovine tuberculosis in cattle and badgers recommended the development of vaccines to protect cattle against infection with *Mycobacterium bovis* as a long term strategy to control the disease in cattle. It was also recommended that the option of badger vaccination should be retained and could be pursued if immunological reagents and a challenge model become available for the badger. The aim of this proposal was to apply recent advances in mycobacterial genetics and vaccinology to the development of TB vaccine candidates

Since it cannot be predicted with certainty which approach to vaccine development will yield the best candidate vaccine against *M. bovis* two of the most promising avenues of vaccine research were pursued. The first avenue was to produce live attenuated vaccines based on *M. bovis* and *M. bovis* BCG. For a successful vaccine of this type the disabled micro-organism should be sufficiently robust to induce a protective immune response but be contained or eliminated such that the disease process is self-limited. In this project key genes essential for the growth and survival of *M. bovis* within the host were inactivated by transposon mutagenesis. Techniques were also developed to identify further targets for disabling *M. bovis* namely those genes that are expressed by *M. bovis* inside its host.

The second approach to generating candidate vaccines for *M. bovis* was based on one of the most promising areas of subunit vaccine development, DNA vaccination. This approach involved the introduction of *M. bovis* DNA encoding proteins that stimulate protective immunity into appropriate target cells within the host. Transient expression of the DNA inside the host cell was found to trigger a protective response.

The development of a TB vaccine is an ambitious programme and it was therefore essential that a mechanism be established to co-ordinate R & D efforts between the bovine and human vaccine programmes. A further objective of this project was to establish such a mechanism.

**Main Findings of the project:**

- Transposon mutagenesis using phage delivery systems was found to be more efficient for *M. bovis* BCG than for wild type *M. bovis* (AF2122/97).
- A library of BCG transposon mutants was produced and screened for strains that had been disabled in key metabolic pathways.
- Techniques for the rapid genetic characterisation of transposon mutants were developed.
- One mutant in particular, which required leucine for growth, showed much promise as a vaccine candidate. This strain conferred significant protection against *M. bovis* infection in guinea pigs and did not compromise tuberculin skin testing in either guinea pigs or cattle.
- DNA vaccines based on the *M. bovis* antigens MPB83 and MPB70 were produced which gave significant protection against *M. bovis* in mice and guinea pigs and stimulated strong T cell responses in immunised cattle without compromising tuberculin skin testing. These vaccines were more effective than a DNA vaccine based on Ag85 that had shown much promise against *M. tuberculosis* in mice and guinea pigs.
- A DNA vaccine encoding MPB70 was shown to exert a therapeutic effect on mice heavily infected with *M. tuberculosis*, especially in the lungs.
- The vaccines produced or identified in this project rank among the most effective so far developed against *M. bovis* and *M. tuberculosis*.
- An *in vitro* model was developed which induced the expression of genes that are usually expressed inside bovine macrophages and was used to identify other proteins that are upregulated under such conditions.
- A technique for harvesting *M. bovis* directly from the lungs of guinea pigs was developed.
- A mechanism was established to facilitate co-ordination of R & D efforts between the bovine and human TB vaccine programmes *via* the Animal Models Task Force of the WHO Steering Committee on the Immunology of Mycobacterial Diseases (IMMYC).

VLA Weybridge incorporated the main implications of this research into their CSG 1999-2000 Research Proposals.

## Section 2 (cont.): Primary milestones for the final year

3. List the milestones for the final year.

**It is the responsibility of the contractor to check fully that ALL milestones have been met and to provide a detailed explanation if this has notproved possible**

| Milestones | | Target date | Milestones met? | |
|---|---|---|---|---|
| Number | Title | | in full | on time |
| | | dd/mm/yy | | |
| 01/04 | Generation of transposon mutant libraries in BCG and M. bovis | 31/06/98 | Yes | Yes |
| 01/08 | Isolate mutant libraries of BCG and M. bovis and store | 31/09/98 | Yes | Yes |
| 02/01 | Auxanographic analysis of BCG mutant library | 31/06/98 | Yes | Yes |
| 02/02 | Supply 3 auxotrophs for testing in animal models to assess protective efficacy, attenuation and potential for differential diagnosis. | 31/07/98 | Yes | Yes |
| 02/03 | DNA sequencing of genomic regions adjacent to transposon inserts. | 31/09/98 | Yes | Yes |
| 02/04 | Cloning and complementation of disrupted genes | 31/12/98 | Yes | Yes |
| 03/04 | Introduction of disrupted genes (*mpb*70 and *mpb*83) into suitable suicide vectors | 31/06/98 | Yes | Yes |
| 03/05 | Select knock-out mutants of *M. bovis* using either *sac*B or *rps*L selection | 31/12/98 | Yes | Yes |
| 03/06 | Confirm that genes have been knocked out by Southern and Western blotting | 31/03/99 | No | No |
| 04/04 | Test for expression of mutated MPB83 in COS cells using (DNA vaccine vector) | 30/04/98 | Yes | Yes |
| 04/05 | Supply naked DNA vectors for testing in animal model ROAME | 30/06/98 | Yes | Yes |
| 06/01 | Establish mechanism for co-ordination between human and bovine TB vaccine development | 01/04/98 | Yes | Yes |
| 07/01 | Identify proteins expressed under physiological relevant conditions by Proteomics | 01/08/98 | Yes | Yes |
| 07/02 | Establish conditions for isolation of *M. bovis* from guinea pig lesions | 01/11/98 | Yes | Yes |
| 07/03 | Identify proteins expressed in guinea pig lesions by Proteomics | 01/02/99 | No | No |
| 07/04 | Identify regulatory genes for mpb83. | 01/03/99 | No | No |
| 08/02 | Prepare final report | 31/03/99 | Yes | No |
| 08/03 | Prepare five papers: | 31/03/99 | Yes | No |

**If any milestones have not been met in the final year, an explanation should be included in Section 5.**

## Section 5 : Scientific report

Please see attached document for scientific report.

**SECTION 5: SCIENTIFIC REPORT FOR SE3205**

**GENERATION OF LIVE ATTENUATED VACCINES AGAINST *MYCOBACTERIUM BOVIS* (OBJECTIVES 01-03)**

**General Introduction.**
To date, the most effective vaccines against pathogenic intracellular bacteria have been live, attenuated strains of the organism. For example, the current vaccine against TB is a live, attenuated strain of *M. bovis* - BCG. However, the efficacy of BCG in protecting against infection with either *M. tuberculosis* or *M. bovis* is extremely variable and, moreover, vaccination with BCG compromises the tuberculin skin test. Recent studies in possums indicate that although BCG may confer a degree of protection against *M. bovis* infection if given by the subcutaneous or intratracheal route, it does not protect against challenge with *M. bovis* if given via the intragastric route. In New Zealand, vaccination of cattle with either of two attenuated strains of *M. bovis* derived by chemical mutagenesis resulted in significantly fewer animals developing tuberculous lesions after challenge with virulent *M. bovis* than cattle vaccinated with BCG. Therefore it may be possible to generate a vaccine which is better than BCG by attenuating M. *bovis.* A clear strategy for the production of an improved vaccine against TB is therefore to genetically modify *M. bovis* to produce live attenuated vaccines. This procedure involves disrupting specific target genes, which prevent the organism from causing disease but allow it to persist and be presented to the host's immune system in the correct manner so that a protective response is stimulated. Although BCG remains our gold standard against which the protective efficacy of our alternative vaccine candidates will be measured, it seems likely that improved vaccines may be developed in the longer term. It is envisaged that these vaccines may be better than BCG in one of two ways. Such vaccines may either be more efficacious or they will confer similar levels of protection against *M. bovis* infection but will allow the differentiation of vaccinated from infected animals (for example by not compromising the tuberculin skin test). The methods traditionally used for the generation of rationally attenuated mutants are homologous recombination and transposon mutagenesis. Until recently these techniques were not available for the slow-growing mycobacteria. However, significant progress has been made in recent years. Experience in the latest techniques for genetic manipulation and mutagenesis of both slow and rapid growing mycobacteria has been acquired at VLA Weybridge through active collaborations with leading groups in the *M. tuberculosis* field (especially with the laboratory of Dr W R Jacobs Jr) and has culminated in the development of a mycobacterial phage system for the delivery of transposons to BCG and *M. tuberculosis* [Bardarov S et al. (1997) *Proc Natl Acad Sci U S A.* 94: 10961-10966].

**<u>OBJECTIVE 01:</u> GENERATION OF TRANSPOSON MUTANT LIBRARIES IN BCG AND *M. BOVIS.***

**Introduction:**
Transposon mutagenesis of BCG was first demonstrated by R A McAdam *et al.* [*Infect Immun* **63,** 1004-12 (1995)] using transposons Tn5366, Tn5367 and Tn5368, based on the insertion element, IS1096. Three auxotrophic strains were isolated from these experiments, exhibiting two different amino acid requirements, leucine and methionine. The leucine auxotrophs (*leu*D1 and *leu*D2) were cleared rapidly from the mouse model and, furthermore, all three were still able to protect both normal and SCID mice from challenge with virulent *M. tuberculosis* [Guleria, I *et al.* (1996) *Nature Medicine* **2,** 334-38]. These experiments in BCG demonstrated that transposon mutagenesis in slow-growing mycobacteria is feasible and that these attenuated strains retained their protective potency. In order to improve the efficiency of delivery of the transposon to the bacterium, conditionally replicating phage delivery vehicles phAE77 and phAE94 were developed in the laboratory of Dr Jacobs in collaboration with VLA Weybridge [Bardarov, S *et al.* (1997) *Proc Natl Acad Sci U S A.* 94: 10961-10966]. One of the great advantages of a phage delivery system is that potentially every cell in the bacterial population can be infected with the transposon-carrying phage, generating large numbers of independent mutants. Here we have employed the same transposon delivery systems to generate transposon mutants in BCG and *M. bovis.*

**Materials and Methods**

**Bacterial Strains, Media and Culture Methods:** The mycobacterial strains used in this study were *M. smegmatis* mc$^2$155, the Pasteur strain of *M. bovis* BCG and *M. bovis* AF2122/97. *M. smegmatis* mc$^2$155 was grown in Luria-Bertani broth plus 0.05 % Tween 80. BCG was grown in Middlebrook 7H9 broth enriched with 0.5 % albumin (fraction V), 0.2% dextrose, 0.85 % sodium chloride and 0.05% Tween 80 (M-ADC-TW) broth. *M. bovis* was propagated at 37°C in Middlebrook 7H9 broth containing 4.16 g/l sodium pyruvate and 0.05% (v/v) Tween-80 supplemented with 10 % (v/v) Middlebrook ADC enrichment. For transposon delivery experiments, BCG was grown in M-ADC-TW broth without glycerol. For the production of a transposon mutant library a novel growth medium containing over twenty growth supplements (luxury medium) was developed. This medium was designed to support the growth of bacteria with mutations in genes encoding enzymes involved in a diversity of metabolic pathways including amino acid synthesis, fatty acid synthesis, and nucleic acid synthesis. Luxury medium contains the following ingredients: Middlebrook 7H10 (for solid) or 7H9 (for liquid) medium plus glycerol for BCG or pyruvate for *M. bovis*, ADC (0.5 % albumin (fraction V), 0.2% dextrose, 0.85 % sodium chloride), 0.01% (v/v) cyclohexamide, 1% (v/v) casamino acids, 10mg/l kanamycin, 100 mg/l diaminopimelic acid, 40 mg/l shikimic acid, 20 mg/l DL-phenylalanine, 20 mg/l DL-tryptophan, 10 mg/l adenine, 10 mg/l hypoxanthine, 10 mg/l cytosine, 10 mg/l thymine, 10 mg/l uracil, 10 mg/l guanine hydrochloride, 16 μg/l folic acid, 30 μg/l DL-pantothenic acid, 400 μg/l thiamine, 80 μg/l riboflavin, 400 μg/l *p*-aminobenzoic acid, 800 μg/l NAD, 5 g/l choline chloride, 5 mg/l DL-ornithine, 5 mg/l *myo*-inisitol, 30 μg/l coenzyme B12, 50 μg/l DL-thioctic acid (reduced form) and 30 μg/l menadione sodium

bisulfite.

**Transposon Mutagenesis.** BCG Pasteur and M. bovis AF2122/97 were grown for 7-10 days after inoculation of 1 ml of the starter culture into a 100-ml roller bottle culture ($OD_{600}$=1.0) in M-ADC-TW with glycerol or pyruvate as appropriate. The cells were pelleted by centrifugation and resuspended to 3 x $10^{10}$ cfu in MP buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2mM $CaCl_2$). The cells were prewarmed at the non permissive temperature (38.5°C) and then mixed with phAE77 or phAE94 phage suspensions (2 x $10^{10}$ - 2 x $10^{12}$ pfu/ml) to give a range of multiplicity's of infection (Multiplicity of infection, MOI, =1-100). The cell-phage mixture was incubated at 38°C for 2.5 h. After completion of the adsorption time, 1.5 ml of prewarmed adsorption-stop buffer (M-ADC-Tw plus 10 mM sodium citrate and 0.1 % Tween 80) was added to prevent further phage infections. The mixture was plated onto luxury agar plates containing 10 $\mu g.ml^{-1}$ kanamycin and incubated 6-8 weeks at 37°C. Colonies comprising putative recombinants were picked and arrayed into 96-well plates containing luxury medium with 10 $\mu g.ml^{-1}$ kanamycin. The plates were incubated at 37°C for 6-8 weeks. The library was stored at -80°C.

**DNA extraction and Southern Analysis.**

High molecular weight chromosomal DNA was extracted and purified as described previously by V. Balasubramanian *et al.* (1996; *J Bacteriol* **178,** 273-279). Chromosomal DNA was digested overnight at 37°C with 30 units of *BamHI* and the resulting fragments resolved by agarose (1%) gel electrophoresis. Southern blotting was performed using the alkali-denaturing procedure. DNA was transferred to Hybond N+ nylon membranes (Amersham) by the capillary method. The Megaprime random-primed labelling kit (Amersham) and 5 μCi of [α-$^{32}$P]dCTP were used to label the probe (the *aph* gene). Prehybridisation and hybridisation were carried out at 65°C using rapid hybridisation buffer (Amersham) as recommended by the manufacturer. Serial 15 min washes were performed at 65°C as recommended by the manufacturer. BioMax MS X-ray film (Kodak) was exposed to the membrane over night at -80°C

## RESULTS

**01/02 Standardisation of phage propagation in BCG and *M. bovis.***

Two recombinant phage containing Tn*5367* (carrying the *aph* kanamycin resistance gene), phAE77 and phAE94 were tested for their ability to infect *M. bovis* BCG and *M. bovis*. Protocols were optimised such that the multiplicity of infection was 1000:1, with an infection time of 2.5 hours at 38°C. Kanamycin-resistant recombinant bacilli were obtained for phAE77 with *M. bovis* BCG but not *M. bovis*, while phAE94 appeared capable of infecting *M. bovis* but not *M. bovis* BCG.

**01/03 Standardisation of transposon delivery in BCG and *M. bovis.***

Successful transposon delivery by the mycobacteriophage delivery system requires loss of the conditionally replicating phage from the bacilli. Therefore the temperature-sensitive origin of replication in phAE77 and phAE94 is inactivated at the non-permissive temperature of 38°C. Maintenance of this temperature during infection of the bacilli appeared sufficient to cause loss of the phage, allowing selection of kanamycin resistant bacilli that had resulted from transposition into the host genome.

**01/04 Generation of transposon mutant libraries in BCG and *M. bovis.***

Oligonucleotide primers directed against the *aph* kanamycin resistance gene carried by Tn*5367* were used in PCR reactions to screen selected *M. bovis* and BCG colonies obtained following transposon mutagenesis to determine if resistance was due to spontaneous mutations or to the presence of Tn*5367*. All *M. bovis* and BCG colonies tested gave positive PCR products, indicating that Tn*5367* was present. However, Southern blot analysis of genomic DNA from these selected colonies revealed that while an internal fragment of the *aph* gene gave positive signals with all *M. bovis* BCG clones, only ~10% of *M. bovis* clones were positive for the presence of the transposon. This suggests that in *M. bovis* kanamycin resistant colonies had arisen by spontaneous mutation and that the positive PCR results were probably due to the presence of a small population of pseudolysogens in the colony that could be detected by PCR but not by blotting. Hence, the phage-based transposon delivery system appeared not to be ideal for use with *M. bovis*.

**01/08 Isolate mutant libraries of BCG and *M. bovis* and store.**

As the phage delivery system was successful only in BCG this library was replica plated by picking colonies from the surface of the agar plates. Seven thousand colonies were picked into luxurious growth medium containing kanamycin, grown at 38°C, and stored frozen in culture medium at -80°C.

## OBJECTIVE 02: THE IDENTIFICATION AND CHARACTERISATION OF AUXOTROPHIC MUTANTS FROM THE BCG TRANSPOSON LIBRARY FOR USE AS VACCINE CANDIDATES.

Introduction:

**Experience with the development of live attenuated vaccines of salmonella has shown that it is difficult to predict the best targets for rational attenuation. Therefore, it is likely that a panel of live attenuated *M.***

***bovis* vaccine candidates will be required for testing in relevant animal models. Potential target genes include those that would generate auxotrophic mutants, i.e. those encoding metabolic enzymes (e.g. amino acid synthesis, carbohydrate metabolism, etc.), those involved in the stress responses of the organism and those expressed *in vivo*. Here we describe the identification of auxotrophic mutants of BCG from the BCG transposon library. These mutants were supplied for testing in ROAMEs SE3202 and SE3203. It was shown that at least one of the auxotrophic strains tested (*leu*D-) did not compromise tuberculin skin testing in guinea pigs or cattle but conferred significant protection to against *M. bovis* challenge in guinea pigs (See SE3202 final report).**

**Materials and Methods:**

**Screening the library for auxotrophic mutants.** Colonies from the transposon mutant library were replica plated onto luxury medium with 20 μg. ml $^{-1}$ kanamycin and basal 7H10 agar with 20 μg. ml $^{-1}$ kanamycin. Plates were incubated for 4 - 10 weeks at $38^{o}$C. Those colonies that failed to grow on the basal medium were identified as putative auxotrophs. This process was repeated for those putative auxotrophs to confirm phenotype.

**DNA sequencing of genomic regions adjacent to transposon inserts.**

The technique of ligation mediated PCR (LMPCR), as described by Prud'hom *et al.* (1998; *FEMS Microbiol Lett* 158: 75-81) was used to amplify the genomic DNA flanking the Tn*5367* insertions. Sequencing of the resulting amplicons allowed the mapping of 10 insertions on the genome of *M. bovis* BCG and *M. bovis* AF2122/97.

Results:

02/01 Auxanographic analysis of the BCG mutant library.

One thousand five hundred colonies from the transposon mutant library were replica plated onto luxury medium with 10 μg. ml $^{-1}$ kanamycin and basal 7H10 agar with 10 μg. ml $^{-1}$ kanamycin. Plates were incubated for 4 - 10 weeks at $38^{o}$C. Those colonies that failed to grow on the basal medium were identified as putative auxotrophs. The putative auxotrophs were picked and restreaked onto luxury and 7H10 agar plates to confirm phenotype. Five auxotrophs were obtained. Two mutants that failed to grow in medium lacking methionine have been isolated, along with one alanine auxotroph. Southern blotting of chromosomal DNA extracted from these colonies indicated that the transposon had inserted into different regions of the chromosome in these mutant strains.

**02/03 DNA sequencing of genomic regions adjacent to transposon inserts.**

Ligation mediated PCR (LMPCR) was used to amplify the genomic DNA flanking the Tn*5367* insertions. Sequencing of the resulting amplicons allowed the mapping of 10 insertions on the genome of *M. bovis* BCG and *M. bovis* AF2122/97. The results of this work are summarised in Table 1. Using LMPCR the insertion site of one methionine auxotroph was mapped to *cysA1*, whilst alanine auxotrophy was found to have been generated by insertion of the transposon into the gene Rv1337 whose function has not yet been elucidated. These results demonstrate that it is feasible to generate an ordered transposon-mutant library of *M. bovis* BCG and *M. bovis* AF2122/97.

**Table 1**

| *Strain* | *Mutant name* | *Phage* | *Genotype* | *Phenotype* |
|---|---|---|---|---|
| BCG Pasteur | 44 | phAE77 | *cysA*::Tn*5367* | methionine auxotroph |
| BCG Pasteur | 22 | phAE77 | ? | methionine auxotroph |
| BCG Pasteur | 3 | phAE77 | Rv1337::Tn*5367* | Alanine auxotroph |
| *M. bovis* | 2 | phAE94 | *umaA1*::Tn*5367* | ? |
| *M. bovis* | 7 | phAE94 | *moaB*::Tn*5367* | ? |
| *M. bovis* | 25 | phAE94 | Rv0498::Tn*5367* | ? |
| *M. bovis* | 40 | phAE94 | Rv2972c::Tn*5367* | ? |

### 02/02 Supply 3 auxotrophs for testing in animal models to assess protective efficacy, attenuation and potential for differential diagnosis.

The leucine and methionine auxotrophs were supplied for efficacy testing in the guinea pig *M. bovis* aerosol challenge model along with a *lys*R mutant obtained by Dr W R Jacobs. (For full details of the results obtained please see the final report for SE3202.) In summary, the leucine auxotroph of BCG conferred significant protection to infection with both *M. bovis* and *M. tuberculosis* in the absence of a cutaneous hypersensitivity reaction to tuberculin. This result indicates that a new generation of vaccines based on BCG may be developed that are protective, safe for use in the immunocompromised, and do not preclude the use of the tuberculin skin test in both man and animals. The methionine auxotroph and the *lys*R auxotroph gave greater protection than the leucine auxotroph against challenge but both compromised the tuberculin skin test in guinea pigs.
The other auxotrophic mutants are now being tested for their *in vivo* growth characteristics in small animal models. Initial experiments are focused on determining whether the mutant BCG auxotrophs show a faster clearance from infected mice.
The *leu*D mutant was also supplied for testing its ability to induce T cell responses in cattle (for full details of the results please see final report for ROAME SE3203). Briefly, vaccination with both BCG and the BCG (*leu*D) auxotrophic mutant induced systemic immune responses as measured by proliferation and IFN-γ production in all (3/3) and in 2/3 animals respectively. Following the primary vaccination BCG induced strong T cell responses, whilst weaker responses were observed in BCG *leu*D vaccinated animals. However, following the first booster vaccination, we observed strong anamnestic responses against tuberculin *in vitro*, particularly in the BCG *leu*D vaccinated calves which indicated the presence of readily available memory T cells. The BCG (Pasteur) vaccinated animals exhibited a strong positive tuberculin skin test reaction, characterised by a larger PPD-M reaction size compared to PPD-A 4 weeks after the primary vaccination. Similar positive reactions were observed 4 or 5 weeks after the second and third vaccination, respectively. In contrast, vaccination with the BCG *leu*D auxotroph did not sensitise the calves for a positive tuberculin skin test reaction, even when measured after the second boost.

#### 02/04 Cloning and complementation of disrupted genes.

The *cysA1* wild-type locus from *M. tuberculosis* H37Rv was amplified by PCR and cloned into a mycobacterial expression vector under the control of the *hsp70* promoter. This construct was transformed by electroporation into the BCG *cysA1*::Tn*5367* mutant. Complementation was confirmed by demonstrating reversion to the *cysA*$^{+}$ phenotype by growth of the recombinant on media lacking methionine.

### Future work: Construction of live attenuated vaccines.

This work has demonstrated that it is possible to generate transposon libraries of *M. bovis* and has developed screening procedures with which to obtain auxotrophic mutants. However, due to the high frequency of spontaneous kanamycin resistance observed for *M. bovis*, this is a relatively inefficient process. In future it will be necessary to use a more suitable antibiotic resistance marker such as hygromycin for transposon mutagenesis of *M. bovis*. Moreover, it has been demonstrated recently that signature tagged mutagenesis is a feasible approach to identify virulence factors for *M. tuberculosis* [Cox JS, *et al.* Nature 1999 402(6757): 79-83; Camacho LR, *et al.* Mol Microbiol. 1999 34:257-67]. Signature-tagged mutagenesis allows the identification of individual mutants within complex pools of mutants. Large numbers of mutants can be analysed in a parallel manner for negative phenotypes like loss of function or attenuation of virulence. Further analysis of mutations identified by a negative selection procedure can be performed by linking the position of the respective mutations to genes identified by whole genome sequencing of microbes. Future work should therefore concentrate on generating a signature-tagged transposon mutant library of *M. bovis* using hygromycin as the selective marker.

## <u>OBJECTIVE 03:</u> TO REMOVE ANTIGENS IMMUNODOMINANT IN CATTLE FROM *M. BOVIS* HOMOLOGOUS RECOMBINATION.

#### Introduction:

Use of BCG as a cattle vaccine is effectively prohibited by current EU legislation because it would compromise the tuberculin skin test. It is therefore essential to develop a specific diagnostic test that can detect and differentiate vaccinated cattle from those infected with virulent *M. bovis*. Our goal is therefore to develop live vaccines by generating attenuated strains of *M. bovis* lacking genes encoding antigens which are immunodominant in cattle so that these antigens may be used for the immunodiagnosis of *M. bovis* infection. The *M. bovis* antigens MPB83 and MPB70 are recognised by B and T cells of *M. bovis*-infected cattle and can also elicit a DTH response in *M. bovis* sensitised guinea pigs. Since these antigens are strongly recognised in cattle infected with *M. bovis*, they may be used for immunodiagnosis following vaccination with vaccine strains of *M. bovis* lacking these antigens. A further advantage of this objective was that the technologies developed for homologous recombination to remove *mpb*83 and *mpb*70 from the genome may be generally applicable to the rational attenuation of *M. bovis*.

**Methods and Results:**

**03/01** An *M. bovis* BCG library was prepared in the pacI cosmid library pYUB412. **03/02** Three clones were identified from the pYUB412 library that encoded chromosomal regions of the genome encompassing mpb70 and mpb83.
**03/03 Disruption of *mpb70* and *mpb83* by kanamycin-resistance gene or other suitable marker.**
The flanking regions of *mpb70* and *mpb83* were amplified by PCR from cosmid pA3 (Hewinson *et al.*, Scand J Immunol. 1996. 435;490-9.) and the resulting amplicon was cloned into the *Hind*III and *Xba*I sites of pBluescript (Stratagene) to produce plasmid pSM88. The kanamycin resistance cassette from pUCKAN$^r$ (Pharmacia) was amplified by PCR and cloned into internal *Nco*I sites of pSM88 to disrupt the *mpb83* ORF, generating plasmid pSM91.

**03/04 Introduction of disrupted genes into suitable suicide vectors.**
The *mpb83-mpb70* cloned flanking sequence carrying a kanamycin resistance marker was released from pSM91 by *Xba*I digestion and cloned into the unique *Xba*I site of the pPR27, the conditionally replicating shuttle vector that carries the *sacB* selective marker (Pelicic *et al.*, Proc Natl Acad Sci U S A. 1997. 94:10955-60). This construct was designated pSM94 and its identity was confirmed by restriction digestion mapping and DNA sequence analysis.
**03/05 Select knockout mutants of *M. bovis* using either *sacB* selection.**
Construct pSM94 was introduced by electroporation into *M. bovis* BCG Tokyo and recombinants selected by the method of Pelicic et al., (1997; Proc Natl Acad Sci USA 94:10955-60). Double cross over events were selected by replica plating kanamycin resistant transformants on 10% sucrose. The presence of *sac*B is lethal to the recombinant bacteria under these conditions so that those recombinants which grew on media containing kanamycin and 10% sucrose were theoretically double cross over mutants. A number of transformants (20) resistant to both kanamycin and sucrose were obtained. Confirmation of homologous recombination by Southern and Western blotting of transformants is in progress. If this approach is successful in BCG, the pSM94 construct will be used to inactivate *mpb83-mpb70* in *M. bovis*AF2122/97.

**03/06 Confirm that genes have been knocked-out by Southern and Western blotting.** This is in progress.

## <u>OBJECTIVE 04 and 05:</u> To produce DNA Vaccines against Mycobacterium bovis based on MPB70, MPB83 (04) and Ag85 (05).

**Introduction:**
One of the most exciting new developments in vaccinology is the use of naked DNA as a vaccine. The concept of naked DNA vaccines is simple. A plasmid vector is constructed to carry a promoter that is turned on when the plasmid enters mammalian cells. A gene or complexes of several genes encoding protective antigens are cloned using standard techniques into the DNA vector such that the genes will be expressed when the plasmid is inside the host cell. Naked DNA is taken up rapidly by eukaryotic host cells and may be delivered by direct intra-muscular injection or by an attenuated intracellular bacterium which, once inside the host, is killed, thus releasing the plasmid to express its genes inside the host. The recombinant protein is expressed inside the cytoplasm of antigen-presenting cells, which processes the protein in a much more efficient and effective way than if the protein is injected directly into the host. DNA immunisation elicits a wide range of immune responses against the antigen encoded by the plasmid and gives long-lasting immunity (>2 years in mice). It has been reported that intra-muscular immunisation of mice with 50 μg of the gene encoding mycobacterial HSP65 or Ag85 protected mice challenged with virulent *M. tuberculosis* to the same extent as BCG.
As yet the extent to which small animal vaccine data can be extrapolated between *M. bovis* and *M. tuberculosis* is unclear. The aim of objectives 05 was to produce a DNA vaccine based on Ag85, a well characterised vaccine which has been shown to be protective against *M. tuberculosis,* in order to assess its protective efficacy against *M. bovis*. The antigens MPB83 and MPB70 were chosen as target antigens for DNA vaccine development (objective 04) since these closely related antigens have been shown to be immunodominant during *M. bovis* infection in cattle and badgers.

**Methods and Results:**
**04/01and 05/01 Construction of naked DNA vectors:**
Plasmids expressing antigen consisted of pcDNA3 containing the promoter of the cytomegalovirus immediate early gene plus the first intron (IE-1) and additional polyA sequences from SV40 (pCMV4) flanking mycobacterial sequences encoding MPB70 or MPB83. Protein expression was directed from a Kozak site (eukaryotic ribosome binding site) which was introduced at the optimum distance from the ATG start site by incorporating the sequence into the 5' PCR primer used to amplify the genes encoding the target antigens. The plasmid pCMV-83 was made by inserting the *mpt*83 gene from *Mycobacterium tuberculosis* H37Rv into the plasmid backbone pCMV4. The *mpt*83 gene was obtained as a 700bp product by performing PCR on genomic DNA from *Mycobacterium tuberculosis* H37Rv using
5'-ATTGGATCCGCCATGATCAACGTTCAG-3'
and 5'-TATGCGGCCGCCGAACGTTACTGT-3' as forward and reverse primers, respectively. The product was cloned into the *Bam*HI and *Not*I restriction sites of pCMV4. The control plasmid, pCMV-link, was made by removing *mpt*83 from pCMV4.83 using *Xba*I and *Bam*HI and replacing it with a polylinker containing *Bam*HI, *Eco*RI, *Pst*I, *Hind*III, and *Xba*I restriction sites. The plasmid encoding MPB70, pCMV-70, was produced by cloning the *mpb*70 gene with upstream Kozak site into the *Bam*HI and *Xba*I sites of pCMV-link. The Ag85A expressing plasmid was produced as described earlier [Huygen, K *et al.* (1996) *Nature Medicine* **2,** 893-898]. The recombinant plasmids were sequenced to ensure fidelity of the PCR before use as DNA vaccines.

**04/02 Transfection of COS-7 cells with DNA vaccines encoding MPB70 and MPB83.**
Recombinant plasmids encoding MPB70 and MPB83 or control vector pCMV-link, were transfected into COS-7 cells. 48 hours later, expression of MPT83 or MPB70 in the transfected cells was determined by indirect immunofluorescence using monoclonal antibodies (CVL: 12/6/1 or SB10) that specifically recognise MPB83 and MPB70 respectively. The distribution of fluorescence was found to antigen dependant. For MPB83, a bright juxtanuclear staining was observed against more diffuse staining throughout the cytoplasm of the cell. Preliminary examination of these COS-7 cells using confocal microscopy has shown the juxtanuclear concentration of MPB83 expression to overlay the golgi apparatus. For MPB70, there was no juxtanuclear staining, rather staining was observed as discrete bodies distributed throughout the cytoplasm with a morphology and distribution similar to that observed following lysosomal staining. No fluorescence was observed in cells transfected with pCMV4.link.

**04/03: Construct DNA vaccine encoding mutated glycosylation motifs.**

## Introduction:

It had been observed previously (Dr Lowrie - personal communication) that, for the antigen 85A complex of *M. bovis*, the immunogenicity of the antigens expressed as DNA vaccines was inversely proportional to the degree of glycosylation of the molecules by the eukaryotic host cell. It was therefore postulated that glycosylation may have profound effects on the immune response of the host to the glycosylated protein. In order to test this hypothesis we sought to compare the antigenicity of MPB83, a glycosylated protein of *M. bovis*, with the immunogenicity of a non-glycosylated form of the antigen in which the glycosylation motifs had been removed by site directed mutagenesis.

**Methods:** Site directed mutagenesis of MPB83 was accomplished using PCR. PCR amplifications for SDM were performed using the high fidelity proof-reading enzyme *pfu* DNA polymerase (Sratagene) in place of *Taq* DNA polymerase. Complementary forward and reverse oligonucleotide primers containing the desired mutation were used in conjunction with oligonucleotide primers for the 3'- end and 5'- end of the target gene respectively to generate two PCR products with an overlap of 30 bp which contained the desired mutation. The two PCR products were then combined and used as substrate for a further PCR amplification reaction using the oligonucleotide primers for the 5' and 3' end of the entire gene. The resulting product gave the gene with the desired mutation. For this study MPB83 was mutated at residues 48 and 49 (TT to VV) and at residue 192 (N to G). This construct was then cloned into pCMV-link along with an upstream Kozak site introduced into the 5' PCR primer.

## 04/04 Test for expression of mutated MPB83 in COS-7 cells.

Mutation of MPB83 resulted in the abolition of binding of the monoclonal antibody 12/6/1 when the mutant protein was expressed in transfected COS-7 cells. We were therefore unable to determine whether transfected COS-7 cells expressed this construct. However, it is now thought that the differences observed in the immune responses to DNA immunisation with the three members of the Ag85 family were due to differences in the number of CpG motifs within the three genes rather than differences in glycosylation of the three molecules. It is likely that the differences in the number of CpG motifs within the DNA sequences of the three genes resulted in differences in the levels of IL-12 induced following immunisation with these constructs.

**04/05 Supply DNA vaccines for testing in animal models (ROAME SE0302 and SE3203)**

**Methods:**

**Preparation of DNA vaccines for immunisation studies:** DNA for immunisation was prepared using a QIAGEN-tip 10000 plasmid extraction kit and endotoxin-free buffers (QIAGEN Ltd, Dorking, UK), as per manufacturer's instructions. The DNA was resuspended in endotoxin-free PBS (Sigma, Poole, UK) prior to injection.

The vaccines were supplied to ROAME SE3202 for testing in guinea pigs, to SE3203 for testing in cattle and to collaborators at NIMR, Mill Hill to assess the potential of the constructs as therapeutic vaccines.

## Results:

**Testing of DNA vaccines in guinea pig aerosol challenge model:** (For full details see final report for SE3202). Briefly, it was found that DNA vaccination had little effect in controlling the numbers of *M. bovis* organisms that spread from the lung to the spleen. However, DNA vaccination of guinea pigs with MPB83 but not Ag85 reduced the pathology of the disease within the lung.
**Testing of DNA vaccines expressing the mycobacterial antigens MPB70, MPB83, and Ag85A in cattle:** (For full details see final report for SE3203). All calves vaccinated with the MPB83 expressing plasmid demonstrated potent cellular immune responses, characterised by T cell proliferation and interferon-gamma production as well as humoral immunity characterised by IgGI biased specific antibodies. Vaccination with MPB70 was less effective because we could demonstrate systemic immune responses only in half of the vaccinated animals. Vaccination with Ag85A resulted in interferon-gamma responses in only half of the animals but no proliferative or antibody responses.
**Testing of vaccine candidates as immunotherapeutic reagents:** The use of DNA vaccines as therapeutic vaccines to cure pre-existing infections is a particularly attractive approach for resolving *M. bovis* infection in badgers. In order to assess the feasibility of

this approach, the plasmid encoding MPB70 was supplied to our collaborator (Dr D Lowrie at NIMR, Mill Hill) to test whether this vaccine, along with a panel produced by Dr Lowrie, could resolve infection in mice infected with *M. tuberculosis*. Female Balb/c mice aged 6 to 8 weeks were infected intraperitoneally with $10^6$ live *M. tuberculosis* H37Rv. At weeks 8, 10, 12 and 16 of infection they received 50 µg of plasmid DNA in saline into the quadriceps muscle of each hind leg. Control mice received empty pcDNA3 or saline injections only or a single subcutaneous injection of $10^5$ live BCG in 50 ul at week 8. Groups of 5 animals were killed 2 and 5 months after the first DNA treatment and the numbers of live bacteria in spleens and lungs were determined as colony forming units (c.f.u.) on 7H11 medium. The control mice that were untreated or received empty plasmid began dying from advanced tuberculosis. None of the mice vaccinated with DNA expressing IL-12, hsp65 or MPB70 died. Plasmid expressing MPB70 appeared to be of greater benefit than plasmid expressing hsp65, particularly against bacteria in the lung. Therefore in heavily infected mice, the immune response could be caused to switch from one that gives bacterial stasis to one that kills the bacteria, simply by giving DNA vaccination therapeutically. This work was recently published in Nature and full details may be found in this paper (Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, Colston MJ, Hewinson RG, Moelling K, Silva CL. Therapy of tuberculosis in mice by DNA vaccination. Nature. 1999 Jul 15;400(6741):269-71).

**OBJECTIVE 06: Establish mechanism for co-ordination between human and bovine TB vaccine development.**

Dr Hewinson attended two annual Meetings of the Animal Models Task Force of IMMYC, the TB arm of the Global Programme for Vaccines and Immunisation, Vaccine Research and Development, WHO in Copenhagen in March 1998 and February, 1999. A report of the 1998 meeting was compiled and supplied to Dr Reynolds at CVOG. In 1998, Dr J Colston, consultant to the *M. bovis* vaccine programme in GB, accompanied Dr Hewinson to the Task Force meeting. Presentations to the Task Force resulted in recommendations to intensify collaborations with developers of veterinary TB vaccines (see pp 64-68, Report of the Technical Review Group Meeting, 7-8 June 1998, Global Programme for Vaccines and Immunization, WHO, Geneva. Ordering Code WHO/VRD/GEN/98.02). Dr Hewinson was also invited to become a regular member of the Task Force to represent veterinary TB Vaccine R&D and to present a paper to the Steering Committee on the Immunology of Mycobacterial Diseases entitled 'Collaboration with Veterinary TB Vaccine Research' April 20-21 1999. At the 1998 meeting a system was established whereby owners of promising vaccine candidates would be informed of MAFF's vaccine development programme for bovine tuberculosis and invited to submit their vaccine candidates for testing against *M. bovis*.

OBJECTIVE 07:

**07/01 Identify proteins expressed under physiologically relevant conditions by Proteomics**

## Scientific rationale and background

Mycobacteria, including the strains that cause bovine tuberculosis, are able to invade their hosts and then survive and grow inside the host macrophages - host cells that are normally designed to kill invading organisms. Experience with the development of live attenuated vaccines against other pathogenic intracellular bacteria has shown that it is difficult to predict the best targets for rational attenuation. However, some of the most promising targets for rational attenuation are those genes that are expressed *in vivo*. We have previously described the increase in expression of a set of antigens in BCG-Pasteur when grown inside bovine alveolar macrophages. Using one dimensional PAGE and immunoblotting with various poly- and mono-clonal sera, two immunodominant antigens of *M. bovis*, MPB83 and MPB70, and a 95 kDa were shown to be up-regulated in intracellularly grown BCG Pasteur (Hewinson et al., 1995). The rationale of this objective was to identify proteins of *M. bovis* that are possible virulence factors expressed *in vivo* using Proteomics. Proteomics involves running 2-dimensional gels to resolve all the proteins expressed by the cell and making comparisons between the proteins produced in conditions relevant to pathogenicity with other conditions such as growth on artificial growth culture medium. Proteins that are upregulated in the relevant conditions are possible virulence factors and can be sequenced from the gels and compared with a translated genome database (if the genome of the organism has been sequenced as in the case for *M. tuberculosis*) to deduce their identity.

**Methods**

***In Vitro* Growth Conditions:** Initially 0.2ml of frozen stocks of each culture were inoculated into 10ml of Middlebrook 7H9 broth supplemented with ADC, 0.05% Tween 80 and 0.4% sodium pyruvate (MADCTwP) and grown statically at 37$^0$C for 10 days (passage 1). They were then diluted 1 in100 into MADCTwP and allowed to grow at 37$^0$C for 20 days (statically) or 7 days (roller bottles) for exponential phase cultures and for 40 - 60 days (statically) for late stationary phase cultures. Cultures (100ml) grown in microbiological medium were then exposed to growth in physiological medium, RPMI-1640 containing 25mM HEPES buffer, 2 mM L-glutamine and supplemented with 10% foetal bovine serum (RPMIFBS), over a 24 hour period. Bacterial cultures were centrifuged at 4500 x g for 15 min., washed twice in RPMIFBS, resuspended in 100ml RPMIFBS and incubated at 37$^0$C, 5% $CO_2$ for 24 hrs.

**Antigen Preparation:** Mycobacteria grown *in vitro* were harvested by centrifuging at 4500 xg for 15 min, washed 2x in phosphate buffered saline (PBS) and resuspended in 3 ml PBS. Bacterial cells were sonicated and the extracts filtered 2x through 0.2µm filters. Aliquots were retained for protein estimation using the bicinchoninic acid (BCA) protein assay (Pierce and Warriner, Chester, UK) with bovine serum albumin as the reference standard. Equal sample concentrations (40µg total protein per gel) were solubilized in 40µl 2D-gel electrophoresis buffer I (0.3 % SDS, 200mM DTT, 28mM Tris-HCl, 22mM Tris-base, pH 8.0) and heated at 80$^0$C for 10 min. Incubation was continued on ice for 10 min. with 4 µl of sample buffer II (24mM Tris-base, 476mM Tris-HCl, pH 8.0, 50mM magnesium chloride, 1 mg/ml DNAse I, 0.25 mg/ml RNAse A). Finally, 160 µl of sample buffer III (9.9M urea, 4% Triton X-100, 2.2% ampholytes (pH 4-8), 100mM DTT) was added and the samples stored at -70$^0$C.
**Gel Electrophoresis:** The protein sample (40µg) was prepared as described above and applied to a polyacrylamide isoelectric focusing tube gel (1mm x 26cm ) containing 4.1% acrylamide, 9.5M urea, 2% (v/v) Triton X-100, 5mM CHAPS and 5.8% Pharmalytes ( pH 4-8, Esa Inc.). The proteins were focused for 2 hrs. at 1.5 kV with 10mM $H_3PO_4$ and 100mM NaOH as the anolyte and catholyte, respectively. The tube gels were subsequently immersed in equilibration buffer (0.3M Tris-base, 0.075M Tris-HCl, 3% SDS, 50mM DTT & 0.01% bromophenol blue) for 2 min. and placed on a 12.5% SDS-polyacrylamide gel (22cm x 22cm x 1mm). Electrophoresis in the second dimension was carried out at 40 mA per gel for 5-6 hrs.
**Staining methods:** Gels were silver stained according to the manufacturers' protocol (Millipore) using a carbonate-formaldehyde developer.
**Computer assisted analysis of 2D gels:** Silver stained 2-D PAGE gels were scanned and images analysed with an Omnimedia XRS scanner and the Investigator software package (Bioimage) using a Sun SPARK station computer.
***In situ* tryptic digestion of protein spots:** Spots of interest were excised from the silver-stained polyacrylamide gel, reduced, carboxyamidated and digested with trypsin (bovine sequencing grade, Boehringer Mannheim UK Ltd., East Sussex, UK) overnight. Samples were then spun down and aliquots of the digest mixture taken directly for MALDI analysis. For nanoelectrospray, tryptic peptides were extracted with 25 mM $NH_4HCO_3$ and acetonitrile, followed by 5 % (v/v) formic acid and acetonitrile and concentrated to dryness by lyophilisation.
***Matrix-Assisted Laser Desorption-Ionization (MALDI) mass spectrometry*:** MALDI mass spectrometry was performed on a VG TofSpec SE time-of-flight (TOF) mass spectrometer equipped with a delayed extraction ion source and operating in the reflector mode (Micromass, Manchester, UK). A saturated solution of a-cyano-4-hydroxycinnamic acid in acetone was mixed in a 4:1 ratio (v/v) with a 10 g/L solution of nitrocellulose in acetone/2-propanol (1:1, v/v), and 0.6 ml aliquots of this solution were deposited on the stainless-steel target. Aliquots (5 ml) were taken from the digest mixtures and loaded onto the target. Samples were air-dried and washed with 10 ml of 5 % (v/v) formic acid prior to insertion into the instrument. Spectra were internally calibrated using a matrix ion-related signal (*m/z* 1060.10) and trypsin autolysis peaks (MH+ 2163.06, MH+ 2289.15). Monoisotopic masses were assigned and used to search the non-redundant protein database by the PeptideSearch software package.
**Nanoelectrospray (NanoES) mass spectrometry:** Needles for nanoelectrospray mass spectrometry were made using a micropipette puller (Sutter Instrument Co., Novato, CA) from borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, reading, UK) and were gold-coated in a vapour desorption instrument. Dried tryptic digests were dissolved in 0.5 % (v/v) formic acid and desalted in a pulled glass capillary packed with ~5 ml of POROS R2 sorbent slurry (PerSeptive Biosystems, Cambridge, MA) by washing with 5 ml of 0.5 % (v/v) formic acid and eluting with 3 ml of 50 % (v/v) MeOH, 1 % (v/v) formic acid. 1 ml was inserted into the spraying needle. Electrospray mass spectra were acquired on an API III triple quadrupole instrument (PE Sciex, Ontario, Canada) equipped with a nanoES ion source. $Q_1$ scans were performed with 0.1 Da mass steps. For operation in MS/MS mode, $Q_1$ was set to transmit a window of 2 Da for both parent ion and product ion scans and spectra were accumulated with 0.2 Da mass steps. The dwell time was 1 ms. for all scans except for parent ion scans, where it was 3 ms. Resolution was set so that masses could be assigned to better than 1 Da. Collision energy was tuned individually for each peptide for optimum MS/MS spectra. A new needle was used for each experiment. Peptide sequence tags were constructed from the product ion scan spectra and used to search the non-redundant protein database using the PeptideSearch software.

## Results

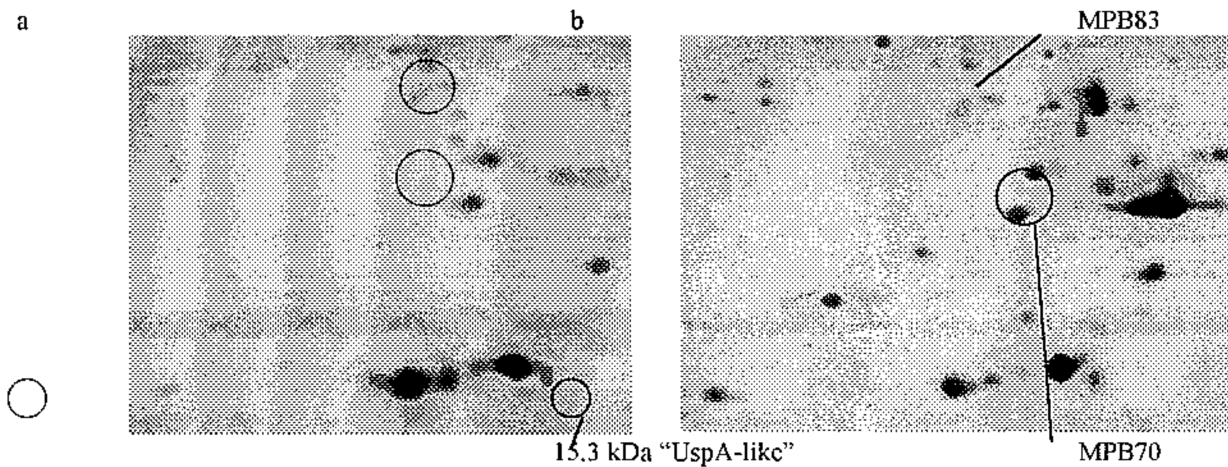
### 1D-gels and immunoblotting establish physiologically relevant conditions:

Sonicates, corresponding to all the protein of the bacteria, were used in all these experiments. Initially, blotting 1D-gels showed us that physiologically relevant conditions had been achieved by growing BCG-Pasteur for 56 days in MADCTwP (a standard, 7H9 Middlebrook Medium; Difco, supplemented with Pyruvate). The 56 day BCG-Pasteur alone displayed some of the up-regulated profile seen on growth inside bovine macrophages observed previously, especially in a region corresponding to 32-42 kDa. However, when these cultures were transferred to the physiological medium used for cell culture, RPMIFBS, and incubated for 24 h, the profile closely resembled that of exponential phase BCG-Pasteur grown inside bovine macrophages. This intracellular-like profile was not seen if the 56 day old cultures were transferred to fresh MADCTwP. Expression of a 95 kDa antigen was observed only with the intracellularly grown BCG-Pasteur.
Immunoblotting with the monoclonal antibody 12/6/1 which specifically recognises MPB83 revealed that the level of up-regulation of this antigen was 3 to 5 -fold higher in 56 day cultures incubated in RPMIFBS for 24 hours than 7 day old BCG-Pasteur similarly incubated in RPMIFBS. In contrast, old cultures of BCG-Tokyo appeared to express similar levels of MPB83 in the presence of microbiological or physiological medium (results not shown) and the high level of MPB83 observed with BCG-Pasteur was equivalent to that of the high producing BCG-Tokyo strain. This finding is consistent with the Tokyo strain being a constitituively high-producer of MPB83.

**Characterisation of up-regulated antigens by Proteomics**

Having established *in vitro* physiologically relevant conditions which upregulate proteins known to be upregulated inside bovine alveolar msacrophages, we further characterised up-regulated antigens by amino acid sequencing proteins identified by 2-D gel electrophoresis as described above. The protein spots were analysed using MALDI mass spectrometry by our collaborator, Joanna Betts at GlaxoWellcome, Stevenage. BCG-Pasteur grown for 56 days then incubated for 24h in RPMIFBS up-regulated a sub-set of proteins not seen in controls transferred to MADCTwP and incubated for 24h. (Fig.1a). Seven additional spots were evident in the RPMIBFS- incubated bacteria (Fig. 1b). One of these proteins was identified as a hypothetical 15.3 kDa protein from cosmid MTCY01B2 of *M. tuberculosis* (TREMBL 006153) that has analogy to UspA of *E.coli*. The peptide yield from the remaining spots was not sufficient for MALDI mass spectrophotometry-based identification so these samples were analysed by nanoelectrospray mass spectrometry. Database searching of the nanoES data using the sequence tag approach identified two spots as MPB70 (D38229) and MPT83 (Q10790), respectively. The four other spots could not be identified by MALDI or nanoES mass spectrometry due to low peptide yield.

**Figure 1. Upregulated proteins of BCG-Pasteur in RPMIFBS, an *in vitro* model for macrophage infection**



**Regulation of MPB83 in a virulent *M. bovis* strain AN5**

Since expression of both MPB70 and MPB83 in *vitro* grown substrains of BCG is linked, with BCG-Pasteur and BCG-Tokyo representing low and high expression strains, respectively, we decided to investigate their expression in a more virulent laboratory adapted *M. bovis* strain, AN5. A 56 day old culture of strain AN5 was grown in MADCTwP and then incubated in RPMIFBS for 24h to reproduce the conditions that led to the up-regulation observed above with BCG-Pasteur. All 3 antigens that were up-regulated in BCG-Pasteur, i.e. MPB70, MPB83 and the hypothetical 15.3 kDa protein, were also found to be up-regulated in strain AN5.

**Discussion of the results and their reliability**

One of the main antigenic differences between *M. bovis* and *M. tuberculosis* is the constitutive expression of MPB83 and MPB70 by *M. bovis*. Indirect evidence that these antigens are upregulated during *M. tuberculosis* infection in mice has been reported previously (Hewinson et al., 1996). It is possible that the increased virulence of *M. bovis* for cattle is due to the constitutive expression of proteins that are only upregulated inside the host upon infection by *M. tuberculosis*. Thus one could envisage that *M. bovis* is pre-primed for virulence in cattle by expressing its virulence factors constitutively.

The principle that proteins other than the immunodominant antigens would be revealed by Proteomics has been proved with the identification of an UspA-like protein. If UspA proves to be the identity of the 15.3 kDa protein, we will have shown up-regulation of a key regulatory protein since *upsA* is part of the *fadR* regulon. In *E.coli*, UspA is induced in response to inhibtion of balanced, unrestricted growth and regulates pathways of carbon metabolism amongst many functions. It would be of great interest to investigate if UpsA or other members of the *fadR* regulon upregulate MPB70 and MPB83 in the *M. tb* complex, and any whether the expression of these proteins can be linked with virulence.

Now that a model for upregulation of proteins in physiologically relevant conditions has been achieved for BCG grown *in vitro*, it is necessary to define the conditions carefully to show if the model is reliable, to define the components of RPMIFBS that induce upregulation of the mycobacterial proteins and to determine whether the incubation time for these experiments can be reduced.

**07/02 Establishing conditions for isolation of *M. bovis* from guinea pig lesions**

**Scientific rationale**

In vitro assays contribute greatly to our understanding of bacterial pathogenesis, but they frequently cannot replicate the complex environment encountered by pathogens during infection. The information gained from such studies is therefore limited. In vivo models, on the other hand, can be difficult to use, and this has to some extent diminished the incentive to perform studies in living animals. The purpose of this work was to establish conditions for obtaining sufficient bacteria from aerogenically infected guinea pigs to be able to run Proteomics gels. As a guide, about $10^9$ cfu are required to give sufficient bacterial protein to run a single Proteomics gel, so several times this number is required for replicates to show that the gels are reproducible. In order to show the important proteins for virulence of bovine tuberculosis, a comparison needs to be made with a strain that is not virulent for cattle, so an attempt was made to establish conditions for the human pathogen, *M.tuberculosis* H37Rv, in parallel.

**Methods**

The *M. bovis* was propagated at 37°C in Middlebrook 7H9 broth containing 4.16 g/l sodium pyruvate and 0.05% (v/v) Tween-80 supplemented with 10 % (v/v) Middlebrook ADC enrichment. Prior to challenge the inoculum of *M. bovis* was washed, resuspended in endotoxin-free PBS and dispersed by brief sonication using a Vibracell control unit plus CV18 converter fitted with a 3 mm diameter tip (Sonics & Materials Inc., Danbury, CT). Eight guinea pigs were exposed to $10^7$ cfu/ml bacteria as an aerosol spray in the low-dose guinea pig aerosol challenge model developed with CAMR as part of SE0130 and SE2302. Two strains of bacteria were used:

| Mycobacterium | Strain | Virulent in: |
|---|---|---|
| *M. bovis* | 2122/97 | Cattle, (humans?) |
| *M. tuberculosis* | H37Rv | Humans |

*M.bovis*-infected animals were sacrificed by euthanasia 3 weeks after infection while *M.tuberculosis*- infected animals were sacrificed 5 weeks after infection. Spleens and lungs were homogenised in 5ml sterile distilled water using a rotating blade macerator system. The organisms were separated from tissue by percoll gradient centrifugation. Organisms were harvested from the gradient and viable counts were performed by serial dilution in sterile distilled water and plating of dilutions onto Middlebrook 7H10 agar containing 4.16mg/ml sodium pyruvate and 10% (v/v) Middlebrook ADC enrichment. Plates were incubated at $37 \pm 2^{\circ}$C and examined after 4 weeks for growth of *M. bovis*. The number of colonies on each plate containing between 30 and 300 colonies was counted and recorded. The remainder of the cells were frozen and stored at –80°C.

**Results of Research**

Lesions were clearly observed in the spleen and lungs of all infected animals but were more severe in those animals infected with *M. bovis*.

**Yields of bacteria from infected tissues, expressed per guinea pig**

| Mycobacterium | In lungs: | In spleen: |
|---|---|---|
| *M. bovis* AF 2122/97 | $10^9$ cfu | $10^8$ cfu |
| *M. tuberculosis* H37Rv | $10^6$ cfu | $10^6$ cfu |

**Discussion and reliability of results**
The yields reported in the table above were reproducible in each of the guinea pigs. The difference in our success with the two strains reflects our greater experience with the bovine pathogen. In any future attempt to obtain sufficient *M. tuberculosis* we will use the H37Rv strain as it is known to be virulent in the guinea pig model, but we will increase the infective dose and kill the guinea pigs after longer periods of infection.
**Extent to which objective 07/02 was met:** The objective has been clearly achieved as stated for *M. bovis*.
**Future research:** Proteomics will be performed on the *M. bovis* derived from guinea pigs when sufficient material is obtained to ensure reproducibility of the techniques.

**07/03 Identification of proteins expressed in guinea pig lesions by Proteomics**

As stated above, sufficient *M. bovis* but not *M. tuberculosis* was obtained for proteomics gels. Since our reason for doing Proteomics was to make comparisons between strains virulent and avirulent for cattle, we have stored the virulent *M. bovis* until we have sufficient *M. tuberculosis* H37Rv (which is avirulent for cattle). The plan for obtaining sufficient H37Rv is outlined in the report on milestone 07/02.
**Future work:** The discovery and characterisation of genes specifically induced in vivo upon infection and/or at a specific stage of the infection will be the next phase in studying bacterial virulence at the molecular level. Genes isolated are most likely to encode virulence-associated factors or products essential for survival, bacterial cell division and multiplication in situ. Identification of these genes is expected to provide new means to prevent infection as well as new insights into the infection process. Analysis of genes and their sequences initially discovered as induced in vivo might be revealed by functional and comparative genomics. The new field of virulence genomics makes feasible their in-depth analysis. Application of new technologies such as in vivo expression technologies, signature-tagged mutagenesis, differential fluorescence induction, differential display using polymerase chain reaction coupled to bacterial genomics is expected to provide a strong basis for studying in vivo induced genes, and a better understanding of bacterial pathogenicity in vivo.

## 07/04 Identification of Regulatory Genes of *mpb*83

### Scientific Rationale

The identification of genes that are differentially regulated under *in vivo* conditions also allows for the identification of the genes responsible for their control. These are excellent targets for rational attenuation. For example, Collins, DM *et al.*, (1995: *Proc Natl Acad Sci U S A.* 92: 8036-8040) identified an *rpo*V mutant of *M. bovis* which is completely avirulent in guinea pigs. As described above, one of the genes identified as being differentially regulated within the TB complex is *mpb*83. The aim of this objective was to identify genes involved in the regulation of *mpb*83.

### Methods and Results:

The promoter region for *mpb*83 (1.1 kb of DNA upstream from the start codon of mpb83) was obtained by PCR and cloned upstream of promoterless *leu*C and *leu*D genes in the IVET vector pYUB509. In this vector fflux and beta-galactosidase are linked to the expression of *leu*CD by translational coupling and allows levels of expression to be measured using these reporter genes. This construct was confirmed by DNA sequencing and designated pSLIVET. The construct was introduced into the chromosome of a leucine mutant of BCG using the attP site encoded by pSLIVET. Resulting hygromycin resistant mutants were selected and insertion confirmed by Southern blotting. Wild type BCG, BCG leucine mutant and BCG leucine mutant encoding the complementing *leu*C and *leu*D genes under the control of the mpb83 promoter were tested for retained auxotrophy *in vitro* by growth on minimal 7H9 agar. Growth of the BCG leucine mutant encoding the complementing leuC and leuD genes under the control of the mpb83 promoter was severely retarded in comparison to wild type BCG indicating that although the *mpb*83 promoter was not completely repressed *in vitro*, expression was extremely low. In order to identify a repressor of *mpb*83, transposon mutagenesis of this recombinant BCG::pSLIVET was performed as described in Objective 01. It was envisaged that recombinants that were fast growing and blue on X-gal-containing plates would be those in which the repressor protein of *mpb*83 had been inactivated by insertion of the transposon. Unfortunately read through from the $tRNA^{gly}$ promoter upstream of the attP integration site within the M. bovis genome was too high to differentiate wild type cells from those derepressed for MPB83 expression.

**Future Work:** A strong terminator should be placed in front of the cloning site of pYUB509 in order to prevent transcriptional read through directed by the tRNA$^{gly}$ promoter upstream of the *att*P integration site.
An alternative approach to identifying the control proteins of mpb83 was also pursued. The expression of Green Fluorescent Protein (GFP) was placed under the control of the *mpb*83 promoter and the resulting construct, pJK83 expressed in BCG Tokyo and BCG Pasteur. The reporter substrate (GFP) was expressed at high levels in BCG Tokyo but not in BCG Pasteur indicating that the difference between the expression of MPB83 in the two strains was not due to sequence differences in the promoter region of the two strains of BCG but rather to differences in other regulatory genes. In order to identify these genes, cosmid libraries BCG Tokyo and BCG Pasteur were prepared for introduction into the heterologous GFP BCG recombinants. The rationale for this approach was to identify those cosmids, which decreased the level of GFP expression in BCG Tokyo or increased the level of GFP in BCG Pasteur. If this was found to be the case, the control genes for *mpb*83 could be identified by subcloning from the relevant cosmids. To date a cosmid encoding a region of DNA present in BCG Tokyo but absent from BCG Pasteur (the so-called RD2 region [Mahairas, GG *et al.*, 1996; *J. Bacteriol.* 178, 1274-82.]) has been introduced into BCG Pasteur expressing GFP under the control of the mpb83 promoter. Introduction of this cosmid did not result in the upregulation of GFP indicating that RD2 does not contain the regulatory genes for *mpb*83.
**Future work:** A systematic screening of the cosmid libraries using the approach detailed above is required to identify the regulatory genes of *mpb*83. If the regulatory genes can be identified this may lead to the production of attenuated vaccine candidates by disrupting these control genes. Moreover, the elucidation of mechanisms of in vivo regulation may lead to the ability utilise in vivo expression technology to identify other genes expressed *in vivo* in *M. bovis*.

**<u>OBJECTIVE 08:</u> To produce five scientific papers for submission to peer-group reviewed journals and one final report.**

Delays have been incurred in the writing of the final report and production of papers for publication. This is due to the exceptional demands placed upon the project leader over the past year. These include: (1) The presentation of TB vaccine development to the Agricultural Select Committee, (2) The re-writing of 9 CSG ROAME proposals for 1999-2000, (3) The enforced completion of all existing ROAME proposals (five in total) resulting in the requirement for five final reports in the same year, (4) Responding to CSG Research Requirements for 2000-2001. Some of the papers envisaged at the beginning of the project are no longer valid. However, five manuscripts have been prepared for publication, 3 of which have been submitted, 2 of which have been accepted for publication, and two are now in an advanced draft form ready for submission. Details of the manuscripts are listed below:

**Those submitted:**


1. Lowrie DB, Tascon RE, Bonato VL, Lima VM, Faccioli LH, Stavropoulos E, Colston MJ, Hewinson RG, Moelling K, Silva CL. Therapy of tuberculosis in mice by DNA vaccination. Nature. 1999 Jul 15;400(6741):269-71.
2. M.A. Chambers, H-M. Vordermeier, N. Commander, R. Tascon, D. Lowrie, and R.G. Hewinson. Vaccination of Mice and Cattle with Plasmid DNA Encoding the *Mycobacterium bovis* antigen MPB83. Accepted by *Clin. Infect. Dis.*
3. Mark A. Chambers, Ann Williams, Dolores Gavier-Widen, Adam Whelan, Graham Hall, Philip D. Marsh, William R. Jacobs, and R. Glyn Hewinson. Identification of BCG auxotrophic mutants that protect guinea pigs against tuberculosis without sensitising to tuberculin. Submitted to Infection and Immunity


**To be submitted:**


H. M. Vordermeier, P. J. Cockle, A. O. Whelan, S. Rhodes, M.A. Chambers, D. Clifford, K. Huygen, D. Lowrie, R. Tascon, R. G. Hewinson. Nucleic acid vaccination of cattle with the mycobacterial antigens MPB83 and MBP70 does not compromise the specificity of the tuberculin skin test. In preparation.

J Inwald, S V Gordon, P Wheeler, I Monaghan, P D Butcher, A Shiekh and R G Hewinson. Differential expression of proteins during stationary phase growth of *Mycobacteium bovis* BCG. In Preparation.