Mycobacterium tuberculosis Antigen 85B and ESAT-6 Expressed as a Recombinant Fusion Protein in *Mycobacterium smegmatis* Elicits Cell Mediated Immune Response in a Murine Vaccination Model

Anthony G. Tsolaki[a,*], Judit Nagy[b], Sergio Leiva[c], Uday Kishore[a] , Ida Rosenkrands[d] and Brian D. Robertson[b]

[a]Centre for Infection, Immunity and Disease Mechanisms, Biosciences, School of Health Sciences and Social Care, Brunel University, London, UB8 3PH, United Kingdom

[b]MRC Centre for Molecular Bacteriology & Infection, Department of Medicine, Flowers Building, Imperial College London, South Kensington, London SW7 2AZ, United Kingdom

[c]Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile

[d]Statens Serum Institut, 5 Artillerivej, DK-2300 Copenhagen S, Denmark

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Abbreviations: ESAT-6, early secretory antigenic target 6; Ag85, antigen 85; TB, tuberculosis; BCG, Bacillus Calmette-Guerin; LPS, lipopolysacharide.

***Corresponding author:** Dr. Anthony G. Tsolaki (anthony.tsolaki@brunel.ac.uk; Phone: +44 1895 266077

Abstract

In this study, we investigated the potential molecular and immunological differences resulting from production of the recombinant fusion proteins (Hybrid-1, comprising of the immunodominant antigens Ag85B and ESAT-6 from *Mycobacterium tuberculosis*) in two different expression systems, namely *M. smegmatis* and *Escherichia coli*. The fusion protein was successfully expressed and purified from both bacterial hosts and analyzed for any host-dependent post-translational modifications that might affect the immunogenicity of the antigen. We investigated the immunogenicity from both from *E. coli*-derived Hybrid-1 fusion and *M. smegmatis*-derived Hybrid-1 fusion in a murine vaccination model, together with a reference standard Hybrid-1 (expressed in *E. coli*) from the Statens Serum Institut. No evidence of any post-translation modification was found in the *M. smegmatis*-derived Hybrid-1 fusion protein, nor were there any significant differences in the T-cell responses obtained to the three antigens analyzed. In conclusion, the Hybrid-1 fusion protein was successfully expressed in a homologous expression system using *M. smegmatis* and this system is worth considering as a primary source for vaccination trails, as it provided protein of excellent yield, stability and free from lipopolysaccharide.

1. Introduction

Tuberculosis (TB) is still a major cause of morbidity and mortality in the world today. Currently, it is estimated that 2-3 million deaths occur worldwide from active TB and there are 8-10 million new cases per year, whilst a third of the world population are latently infected with the causative agent, *Mycobacterium tuberculosis* (Dye, 2006; Maher et al., 2007). During latency, M. tuberculosis is able to survive and persist as an intracellular pathogen for years, by being able to modulate phagosomal maturation, preventing phagosomal-lysosome fusion and reducing acidity within the phagosome (Gupta et al., 2012; Russell, 2001). *M. tuberculosis* also has significant interaction with components of the innate immune system e.g. toll-like receptors (TLRs), complement and lung surfactant proteins SP-A and SP-D, which play important roles in shaping adaptive immunity to TB infection in the host (Tsolaki, 2009). The only licensed vaccine being used currently against M. tuberculosis is *M. bovis* Bacillus Calmette-Guerin (BCG), which has been used worldwide since the early 1900s. Whilst BCG has been shown to be protective against childhood forms of TB, its efficacy against adult active TB varies greatly, ranging from 0 to 85% (Andersen and Doherty, 2005; Fine, 1995). Improved vaccines are urgently needed, particularly to target the global epidemic of adult active TB, which is the most infectious form of the disease (Hanekom et al., 2008). During the last few years, a number of new candidate vaccines against TB have now been trailed, with several vaccines showing improved efficacy (Andersen, 2007). Among these candidates is the fusion protein Hybrid-1 or H1, which is based on the immunodominant antigens Antigen 85B (Ag85B) and the Early Secretory Antigenic Target (ESAT-6) from M. tuberculosis. The Hybrid-1 fusion protein is at the forefront of candidate vaccines against TB and has been extensively tested in a number of studies (Agger et al., 2008; Dietrich et al., 2007; Langermans et al., 2005; Olsen et al., 2004; van Dissel et al., 2010; van Dissel et al., 2011; Weinrich Olsen et al., 2001). A recent variant has also been fused to the latency associated protein Rv2660c (H56) that promises efficacy against pre- and post exposure (latent) TB (Aagaard et al., 2011). The majority of tested vaccine candidate antigens are heterologously expressed in various strains of the common laboratory organism *Escherichia coli* (*E. coli*), in spite of the fact that the protein folding and post-translational modification mechanism in the pathogen might be very different, compared to the host mycobacterium. Protein lacking the native mycobacterial milieu

and modifications may be less immunologically active (Triccas et al., 1996). Post-translational modifications such as methylation have been shown to alter the immunogenicity of recombinant proteins when comparisons were made between native mycobacterial protein versus the recombinant protein expressed from *E. coli* (Menozzi et al., 1998; Pethe et al., 2002; Temmerman et al., 2004). The purification of native proteins from M. tuberculosis or M. bovis BCG for biochemical and immunological analysis is a complex and laborious process, often resulting in poor yields of protein (Delogu and Brennan, 1999; Menozzi et al., 1996). Recombinant expressions systems have been developed recently that can be used in fast-growing non-pathogenic mycobacteria. These systems have made use of modified mycobacterial plasmids that have been engineered to over-express protein, via the hsp60 promoter (Curcic et al., 1994; Delogu et al., 2004; DeMaio et al., 1997; Dziadek et al., 2002; O'Donnell et al., 1994). Inducible systems for mycobacteria expression have also been developed, using tetracycline induction, which provide controlled amounts of protein expression (Blokpoel et al., 2005; Carroll et al., 2005; Ehrt et al., 2005; Triccas et al., 1998). Using such systems has potential in producing recombinant proteins with features that are native to those in M. tuberculosis, free from lipopolysaccharide (LPS) contamination, thus facilitating its use as an optimum vaccine candidate or new diagnostic marker (Masungi et al., 2002).

The aim of the present study was to develop a system of expression in *M. smegmatis* that would allow the expression of antigens from M. tuberculosis and their efficient purification, with the resultant recombinant protein resembling the native antigen. We therefore describe the expression of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis*, using mycobacterial-shuttle plasmid vectors (Blokpoel et al., 2005; Wiles et al., 2005). The expresssion vector used here contains the *tetRO* region from the *Corynebacterium glutamicum* TetZ, making expression of genes cloned downstream of *tetRO* responsive to tetracycline We demonstrate the purification of the recombinant Hybrid-1 proteins from both bacterial hosts and analysis of their biochemical and immunological characteristics, in order to determine whether there are any differences in their immunogenicity.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

Mycobacterium smegmatis mc^2-155 was grown at 37^{o}C, in Middlebrook 7H9 liquid broth or on Middlebrook 7H11 solid media, prepared according to the manufacturer's instructions, and supplemented with OADC (Difco), 0.08% glycerol and 0.05% Tween-80 (Sigma). All *Escherichia coli* strains were grown on LB-agar plates or in LB broth. When needed, kanamycin was added at a final concentration of 50μg/ml for *E. coli* and hygromycin was added at a concentration of 50 μg/ml for *M. smegmatis*.

2.2 Cloning of Hybrid1fusion into mycobacteria shuttle vectors

The pMCT6 plasmid containing the gene fusion Ag85B and ESAT-6 (Hybrid-1) was constructed as previously described (Weinrich Olsen et al., 2001). The hybrid-1 fusion was amplified from pMCT6 using, Ag85B-HB-forward primer (5'-CGC GGA TCC ATG CAC CAC CAC CAC CAC CAC TTC TCC CGG CCG GGG CTG CC-3') and ESAT-6-SpeI-reverse primer (5'-GGA CTA GTC TAT GCG AAC ATC CCA GTG ACG TTG CCT TC-3'). The forward primer contained a BamHI restriction site and the reverse primer contained a SpeI restriction site (underlined). The forward primer also contains a HIS-tag to facilitate purification. Amplification was carried out for 30 cycles with denaturation at 94^{o}C for 1min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 90 secs, using Biolase Taq polymerase (Bioline Reagents, UK). PCR products were purified (Qiagen, UK) and digested by BamHI and SpeI enzymes (New England Biolabs, UK) and positionally cloned into the tetracycline-inducible vector pMind, described previously (Blokpoel et al., 2005) and the pSHKLx1 shuttle vector, (Wiles et al., 2005). Both plasmids pMind and pSHKLx1 are E.coli–mycobacteria shuttle plasmids containing kanamycin and hygromycin selection markers. The pSHKLx1 also contains the constitutively expressed promoter hsp60 from M. tuberculosis H37Rv (Wiles et al., 2005). The luxAB reporter genes in pSHKLx1 were replaced with the Hybrid-1 fusion, via the BamHI and SpeI sites. Both pMind (Hybrid-1) and pSHK (Hybrid-1) were transformed into *E. coli* strains DH5a and BL21 (Invitrogen, UK), using the $CaCl_2$ method (Sambrook, 2000) and pSHK (Hybrid-1) expressed in *E. coli* strain BL21 (Invitrogen, UK). Vectors pMind

(Hybrid-1) and pSHK (Hybrid-1) were introduced into *M. smegmatis* by electroporation, using the protocol previously described (Parish and Stoker, 1998).

2.3. Expression conditions

For *M. smegmatis* starter cultures were set up consisting of pMind(Hybrid-1) transformants in triplicate 20 ml cultures of Middlebrook 7H9 liquid broth, supplemented with OADC, 0.08% glycerol and 0.05%Tween-80 at 37^{o}C, in 125 ml conical flasks and shaken at 200 rpm in an orbital shaker. Mycobacterial cultures were then sub-cultured at log phase, in 800ml in 2L flasks overnight. Induction of expression was achieved by adding 20ng/ml of tetracycline. Cells were harvested after 4 hours of induction. For *E. coli*, starter cultures were performed using triplicate 20 ml cultures in LB-broth, in 125 ml conical flasks, shaken at 200 rpm in an orbital shaker. *E. coli* cultures were then sub-cultured at log phase in 400ml of 1L LB and incubated overnight at 37^{o}C before harvesting. Samples were processed for protein purification as described below.

2.4. Protein purification

M. smegmatis cultures were harvested at 4400×g and the pellet washed twice with ice cold PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). Cells were resuspended in PBS containing 0.05% Tween 80 (Sigma-Aldrich), protease inhibitor (P-8849, Sigma-Aldrich), lysozyme (Sigma-Aldrich, at a final concentration of 2.4 mg/ml) and incubated on ice for 30 min. Cells were disrupted twice with a cell disrupter French press machine (Constant Systems Ltd) at a pressure of 1.1×108 Pa. *E. coli* cultures were harvested at 10,000 x g for 10 min and the pellet resuspended in BugBuster Reagent (5 mL/g wet pellet) (Novagen), Benzonase nuclease (Novagen) (25 U per 1 mL BugBuster), Lysozyme (Sigma) (5 KU/g wet pellet) and protease inhibitor (P-8849, Sigma-Aldrich). The cell suspension was then incubated for 30 mins at room temperature with gentle shaking. Insoluble debris was removed by centrifugation at 16,000 x g for 20 min at 4°C.

Lysates from both *M. smegmatis* and *E. coli* were then size-fractionated using gel-filteration S-100 and S-10 spin columns (Amicon, Millipore) to exclude protein outside the range of 10-100kDa. This fractionated lysate was then passed over a 1 ml

HisTrap HP Ni-affinity sepharose column (GE Healthcare) and eluted with 500mM imidazole. Eluted fractions were analyzed on SDS-PAGE and Coomassie staining. Positive fractions were then further purified by FPLC (AKTA, GE healthcare) by loading onto a 1 ml HisTrap HP column (GE Healthcare). For the purification a gradient of imidazole concentration was used, from 10 mM (wash buffer, 20 mM sodium phosphate, 0.5 M NaCl and 10 mM imidazole, pH 7.4) to 0.5 M (elution buffer, 20 mM sodium phosphate, 0.5 M NaCl and 0.5 M imidazole, pH 7.4). Eluted fractions were tested for the presence of the Hybrid-1 fusion protein, by immunoblotting. Protein concentration of the final pooled purified sample of hybrid 1 was determined using the BCA method (Piercenet). H1 (EC, SSI) was produced and purified as previously described (Weinrich Olsen et al., 2001).

2.5. SDS-PAGE and immunoblots

Cell fractions were analyzed with the PhastSystem (Amersham) using 12% SDS-PAGE gel and transfer to nitrocellulose membrane (Laemmli, 1970). This was further interrogated using anti-His tag (Invitrogen), anti-Ag85-TD17 and anti-ESAT-6 - HYB76-8 antibodies (obtained from SSI).

2.6. Post-translational modification assays

Protein fractions on Immunoblots were analyzed for glycosylation using the GLYCO-PRO kit (Sigma-Aldrich) and for phosphorylation by the PhosDecor kit (Sigma-Aldrich).

2.7. Mass spectrometry

Purified proteins were excised from SDS-PAGE gels and subjected to MALDI-TOF mass spectrometry, using MASCOT to identify the peptides. This was performed at Hammersmith Hospital, Imperial College, London.

2.8. Immunization study

Immunogenicity of the *M. smegmatis* derived (H1 (MS, IC)) and *E. coli* derived (H1 (EC, IC)) Hybrid1 proteins were tested, together with an *E. coli* Hybrid-1 preparation

previously published (H1 (EC, SSI)) (Weinrich Olsen et al., 2001). The experiments on all three antigens were done using the methods outlined recently (Agger et al., 2008). Briefly, mice were immunized three times, intramuscularly, at 3-week intervals, using a volume of 200 µl. All mice received a dose of adjuvant (250µg Dimethyldioctadecylammonium (DDA)/50 µg a,a'-trehalose 6,6'-dibehenate (TDB)) with 2µg of Hybrid1 antigen preparation.

2.9. Splenocyte cultures

Mice were rested for 3 weeks following the third immunisation, after which they were culled and spleen harvested. Cultures of splenocytes were obtained by homogenizing the organ into complete RPMI (RPMI 1640 supplemented with 5×10^{-5} M 2-mercapthoethanol, 1 mM glutamine, 1% penicillin-streptomycin, 1% HEPES and 10% fetal calf serum all from Gibco (Invitrogen, Carlsbad, CA). Subsequently, cells were washed twice and adjusted to a final concentration of 2.6×10^5 cells/well in a total volume of 200 µl/well. Splenocytes were re-stimulated with purified antigen at 5µg/ml, whereas Concanavalin A (Con A) at a concentration of 5µg/ml was used as a positive control for cell viability and medium used as a negative control. Ag85B and ESAT-6 antigen peptides were tested (E6-P1 and 85B-P63), as well as recombinant Ag85B, the ESAT-6 dimer protein dESAT-6, and the three hybrid1 antigens (H1 (EC, IC), H1 (MS, IC) and H1 (EC, SSI). Culture supernatants were harvested from parallel cultures after 72 h of incubation for the investigation of IFN-γ by ELISA performed as previously described (Dietrich et al., 2007).

3. Results

3.1. Successful expression of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis*

The Hybrid-1 (Ag85B-ESAT-6) fusion gene fragment was successfully cloned into the vectors pMind and pSHK, with an additional histidine tag at the N-terminal end of the protein. Cloning the Hybrid-1 fusion protein into the plasmid pMind allowed the controlled expression of this protein in *M. smegmatis* under the tetracycline inducible promoter. The histidine tag facilitated purification. Expression of the Hybrid-1 fusion protein is shown in Figure 1. In both *E. coli* and *M. smegmatis* hosts, the expected size of the 35 KDa Hybrid-1 fusion proteins was obtained. In *E. coli*, expression did not require induction, since the pSHK (Hybrid-1) was under the control of the constitutive promoter hsp60. After 1hr, expression was detected and this was increased after overnight incubation. In *M. smegmatis*, the expression of the Hybrid-1 fusion was also detected after just 1 hr of induction with 20ng/ml of tetracycline. There did not appear to be significant increase in the levels of expression after 4hrs. The 35KDa band obtained was confirmed to be the Hybrid-1 fusion protein by immunoblotting, using anti-His tag antibody (results not shown).

3.2. Purification of the histidine-tagged Hybrid-1 fusion protein in *E. coli* and *M. smegmatis*

We next determined whether our expression of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis* allowed for the efficient purification of the 35KDa protein using the histidine tag on its N-terminus. A similar approach has been used before to purify proteins from mycobacteria successfully, without the histidine tag compromising the function or immunogenicity of the protein (Crowe et al., 1994; Triccas et al., 1998). Lysates of the *M. smegmatis* were obtained using a French Press cell disrupter and then size fractionated. These fractions were then past over a HIS-Trap column with varying amounts of imidazole (10mM, 20mM and 50mM), before bound protein was washed off with 250mM of imidazole. The same process was also used for the *E. coli* Hybrid-1 fusion protein purification, except that *E. coli* cells were disrupted using Bugbuster lysis buffer. Purified fractions from the His-trap from *E. coli* and *M. smegmatis* were run on a 12% SDS-PAGE gel, in order to examine the purity of the protein extracts. Although a distinct 35KDa band was observed (confirmed by

immunoblot with anti-HIS tag antibody), there were also contaminating proteins still present (results not shown). Pooled fractions of HIS-trap purified protein were therefore subjected to further standard gel filtration fractionation, using an automated AKTA FPLC system, in order to remove contaminating host proteins (Figure 2). The presence of HIS-tagged fusion protein was confirmed using anti-HIS tag antibody. The yield of protein obtained for the *M. smegmatis*-derived Hybrid-1 is 5mg/L of culture, whilst for *E. coli*-derived Hybrid-1 it was 0.5mg/L of culture.

3.3. Characterization of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis*

Anti-Ag85B or ESAT-6 specific monoclonal antibodies of well-defined specificity (obtained from the SSI, Figure 3), were used to identify recombinant proteins via immunoblotting. This showed that the *M. smegmatis*-derived Hybrid-1 fusion protein was intact (Figure 4). However, using the same monoclonal antibodies, the *E. coli* Hybrid-1 protein was shown to have lost the ESAT-6 epitope recognised by the monoclonal antibody HYB76-8 (Figure 4B). Positive signals for monoclonal antibody TD17 indicated that a full length Ag85B was present. The epitope for HYB76-8 has been mapped to the start of the ESAT-6 molecule suggesting that the whole of the ESAT-6 portion has been deleted from the *E. coli*-derived Hybrid-1.

In order to further investigate this truncation of the *E. coli*-derived Hybrid-1, we carried out MALDI-TOF mass spectrometry, using MASCOT to identify the peptides obtained. Peptides were obtained that were identified as Ag85B (Figure 3), being as follows: MS-pep1 (VQFQSGGNNSPAVYLLDGLR) and MS-pep2 (AADMWGPSSDPAWERNDPTQQ). No ESAT-6 peptides were identified and this was consistent with the pattern of antibody staining obtained (Figure 4B).

Post-translational modifications were sought using commercially available gel staining kits, but there was no evidence of either glycosylation or phosphorylation in the hybrid-1 fusion from *M. smegmatis* (results not shown).

3.4. Immunogenicity of the Hybrid-1 fusion protein in *E. coli* and *M. smegmatis* in mice

In order to compare the immunogenicity of the *M. smegmatis*- and *E. coli*-derived Hybrid-1 proteins, all three antigens were evaluated using 3 subcutaneous injections at 3 weeks intervals of 2µg of protein in DDA-TDB adjuvant CAF01. In addition, a reference Hybrid-1 fusion also expressed in *E. coli* and obtained from the SSI (H1, (EC, SSI)) was included for comparison. Animals were rested for 3 weeks after the third immunization, after which they were culled and the spleens were harvested. Splenocytes were re-stimulated with antigen at 5µg/ml for 72 hours, and then IFN-γ levels in the culture supernatants were measured by ELISA in the culture supernatants. A range of Ag85B and ESAT-6 peptides were tested, as well as the whole antigens from the various sources, and the levels of IFN-γ obtained are shown for a selection (Figure 5). There was little difference in the pattern of reactivity observed from all three proteins analyzed, with even the truncated protein (H1 (EC, IC)) showing very similar levels of reactivity to the two full length proteins H1 (MS, IC) and H1 (EC, SSI). Immunisation with all three H1 proteins led to recognition of the Ag85B recombinant protein and the previously identified CD4 epitope QDAYNAAGGHNAVFN, represented by the Ag85B-P63 peptide (Bennekov et al., 2006). However, the Hybrid-1 protein from *E. coli* (H1 (EC, IC)), missing the ESAT-6 portion of the fusion, did not react in the stimulation assay to either the ESAT-6 dimer (dESAT-6), or to the peptide E6-P1, which maps to the deleted region. This further confirms the loss of this region in the fusion protein.

4. Discussion

Expression of recombinant proteins in fast-growing non-pathogenic mycobacteria has been used extensively, as a result of the recent developments in Mycobacterial shuttle vectors. In the present study, our goal was to express recombinant fusion protein Hybrid-1 (Ag85B-ESAT-6) in the mycobacterial host *M. smegmatis* to test if expression in a mycobacterial host resulted in a vaccine candidate fusion protein with intact or improved immunogenicity as well as possible post-translational changes. We have successfully cloned the Hybrid-1 fusion into a mycobacterial vector pMind and expressed the protein in *M. smegmatis*, using a Tetracycline-inducible expression system. The yield obtained was 5mg/L of *M. smegmatis*-derived Hybrid-1 protein, compared to 0.5mg/L of *E. coli*-derived Hybrid-1, giving sufficient soluble protein for further analysis and animal trials. We have also demonstrated successful expression and purification of the Hybrid-1 fusion protein by cloning it into the plasmid pSHK, with the constitutively expressed promoter hsp60.

M. smegmatis protein reacted with all the available Hybrid-1 component specific antibodies. However, the *E. coli*-derived Hybrid-1 protein lacked the ESAT-6 epitope recognized by the antibody HYB76-8, and MALDI-TOF analysis provided confirmation that part of the Hybrid-1 molecule had been lost. The mechanism for this is unknown, but may have involved some post translation cleavage of the protein within the host *E. coli*. It is also possible that the overnight expression of the soluble protein in *E. coli* led to protein cleavage of the ESAT-6 fragment. In the original report describing the expression of the Hybrid-1 fusion, the authors used *E. coli* XL-1 blue (Weinrich Olsen et al., 2001), which differs in genotype to *E. coli* BL21 and may give some insights into how the truncation of Hybrid-1 occurred. It is possible that *E. coli* BL21 has cleaved the expressed fusion in order to keep the protein soluble. It is interesting to note that truncated forms of ESAT-6 have been identified from M. tuberculosis culture filtrate, showing loss of 11 residues at the C-terminal end of the antigen, but this did not affect its binding to the 10 KDa culture filtrate protein (CFP10) (Okkels et al., 2004).

No evidence was found of either phosphorylation or glycosylation in the *M. smegmatis*-derived Hybrid-1fusion protein. This finding is also reflected in the data from the vaccination experiments, where there were no significant differences in the T-cell responses in vaccinated animals, apart from those differences consistent with the loss of ESAT-6 from the *E. coli*-derived protein. The *E. coli*-derived Hybrid-1 control protein from the SSI, and *M. smegmatis*-derived fusion protein from this study showed identical reactivity. The present study did not analyse the Hybrid-1 fusion proteins for methylation and acetylation, but given the similarity in the immune responses following vaccination this is unlikely to play a major role in immunogenicity. Methylation of the M. tuberculosis heparin-binding hemagglutinin (HBHA) is essential for effective T-cell immunity to this antigen (Temmerman et al., 2004). Moreover, acetylated and non-acetylated forms of ESAT-6, show differential binding to the antigen CFP10 (Okkels et al., 2004).

In M. bovis BCG as a host, expression of recombinant protein was found to be significantly lower with the inducible tetA (present in pMind), than the strong mycobacterial promoter hsp60 (Blokpoel et al., 2005). However, the same study also found that the magnitude of induction of tetA was approximately 50 times higher in fast-growing mycobacteria (*M. smegmatis*), than slow growing mycobacteria (M. tuberculosis, and M. bovis BCG) (Blokpoel et al., 2005). The inducible pMind system described in the present study may not be suitable for over-expression of recombinant antigens for high yields, but is better suited when controlled expression is required in a situation where a gene product may be toxic to the mycobacterial host cell. A better regulated version of this system has recently been published (Williams et al., 2010). Similarly, the inducible pMind system provides a useful system to study mycobacterial pathogenesis, by regulating the expression of mycobacterial genes, using antisense mRNA to knockdown specific gene products (Blokpoel et al., 2005). It is also preferred to express recombinant mycobacterial proteins in mycobacterial hosts, since a number of proteins are post-translationally modified in mycobacteria and therefore cannot be expressed as a native protein in the *E. coli* host.

Previously, other expression systems have been used, that contain alternative promoters such as the *M. smegmatis* acetamidase promoter and the β-lactamase promoter of M. fortuitum (Triccas et al., 1998).

There have been studies showing the advantage of recombinant proteins expressed and purified from mycobacterial hosts versus to *E. coli* (Garbe et al., 1993; Roche et al., 1996; Triccas et al., 1998; Triccas et al., 1996), with one study showing that a recombinant M. leprae antigen expressed in *M. smegmatis* was only recognized by the antisera from leprosy patients (Triccas et al., 1996).

The addition of the HIS-tag allows for a simple and effective purification of the fusion. The HIS-tag on the Hybrid-1 fusion of both the *E. coli*-derived protein and *M. smegmatis* protein also does not appear to have affected the confirmation of the protein and there is little difference in the T-cell responses, when compare to the E coli derived Hybrid-1 protein from the SSI, which lacks the HIS-tag. A similar finding was also demonstrated in the analysis of another mycobacterial antigen (Triccas et al., 1996).

In conclusion, we have provided proof of principle evidence that vaccine candidates from M. tuberculosis can be expressed in a surrogate mycobacterial host and sufficient protein obtained for immunological and biochemical analysis. The expression systems described in this study could be used to test other M. tuberculosis antigens in preparation for obtaining an optimal TB vaccine, facilitating the analysis or their structure, function and antigenicity.

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Figure Legends

Figure 1: Expression of 35kDa Hybrid-1 gene fusion in *E. coli* (Ec) and *M. smegmatis* (Ms). Lysates run on a 12% SDS-PAGE gel with Coomassie staining: Lane 1: Protein standard marker; lane 2, *E. coli* only; lane 3, *M. smegmatis* habouring pMind(Hybrid-1) in the absence of tetracycline; lanes 4 and 5, *E. coli* harbouring pSHK(Hybrid-1) and harvested after 1hr and 16hrs of growth respectively; lanes 6 and 7, *M. smegmatis* habouring pMind(Hybrid-1) in the presence of 20ng/ml of tetracycline, for 1 hour and 4 hours respectively. Comparable amounts of bacterial extracts were loaded in each well.

Figure 2: Purification of Hybrid-1 fusion protein from *M. smegmatis* using FPLC. Semi-purified fractions from HisTrap HP Ni-affinity sepharose column were further purified using FLPC to remove contaminating proteins. Three fractions (Lanes 1, 2 and 3) were obtained and confirmed to be the Hybrid-1 fusion using immunoblotting with the Anti-HIS monoclonal antibody. Results shown are from *M. smegmatis* Hybrid-1. The *E. coli* Hybrid-1 was also purified using this method (not shown).

Figure 3: Schematic representation of the Hybrid-1 fusion protein. The fusion is made up of the antigen 85 and ESAT-6 proteins of M. tuberculosis. There is a histidine-tag at the N-terminal end to aid purification. The position and name of the monoclonal antibodies used for characterization are shown; together with the peptide sequences resolved using mass spectroscopy (MS-pep1 and MS-pep2).

Figure 4: Purified Hybrid-1 fusion protein from *E. coli* and *M. smegmatis* characterized by immunoblotting. A) Lysates run on a 12% SDS-PAGE gel with Coomassie staining: Lane 1: Protein standard marker; lane 2, *E. coli* Hybrid-1 protein; lane 2 *M. smegmatis* Hybrid-1 protein. B) Immunoblots of SDS-PAGE gel: Lanes 1 and 2, *E. coli* Hybrid-1 *M. smegmatis* Hybrid-1 probed with HYB76-8 antibody, respectively; lanes 3 and 4, *E. coli* Hybrid-1 *M. smegmatis* Hybrid-1 probed with TD17 antibody, respectively; lanes 5 and 6, *E. coli* Hybrid-1 *M. smegmatis* Hybrid-1 probed with Anti-HIS antibody, respectively.

Figure 5: Comparison of the immunogenicity of the *E. coli* and *M. smegmatis* derived Hybrid-1 proteins. Proteins *E. coli* Hybrid-1 and *M. smegmatis* Hybrid-1 were compared with an *E. coli* Hybrid-1 prepared at the SSI, Denmark. C57BL/6J Mice were injected three times (SC) at 3 weeks intervals using 2µg of protein in CAF01 adjuvant. Three weeks after the third immunisation, animals were culled and spleens harvested. Splenocytes were re-stimulated with antigen at 5µg/ml for 72 hours, and then IFN-γ levels were measured by ELISA in the culture supernatants, using Ag85B and ESAT-6 peptides as well as Ag85B, the ESAT6 dimer protein and the H1 antigens from the various sources. H1(EC, IC) : *E. coli* Hybrid-1; H1 (MS, IC): *M. smegmatis* Hybrid-1; H1(EC, SSI) : *E. coli* Hybrid-1 from SSI, Denmark; Con A: Concanavalin A. Results are presented as the mean value ± S.E.M. of 3 mice.

Figure 1:

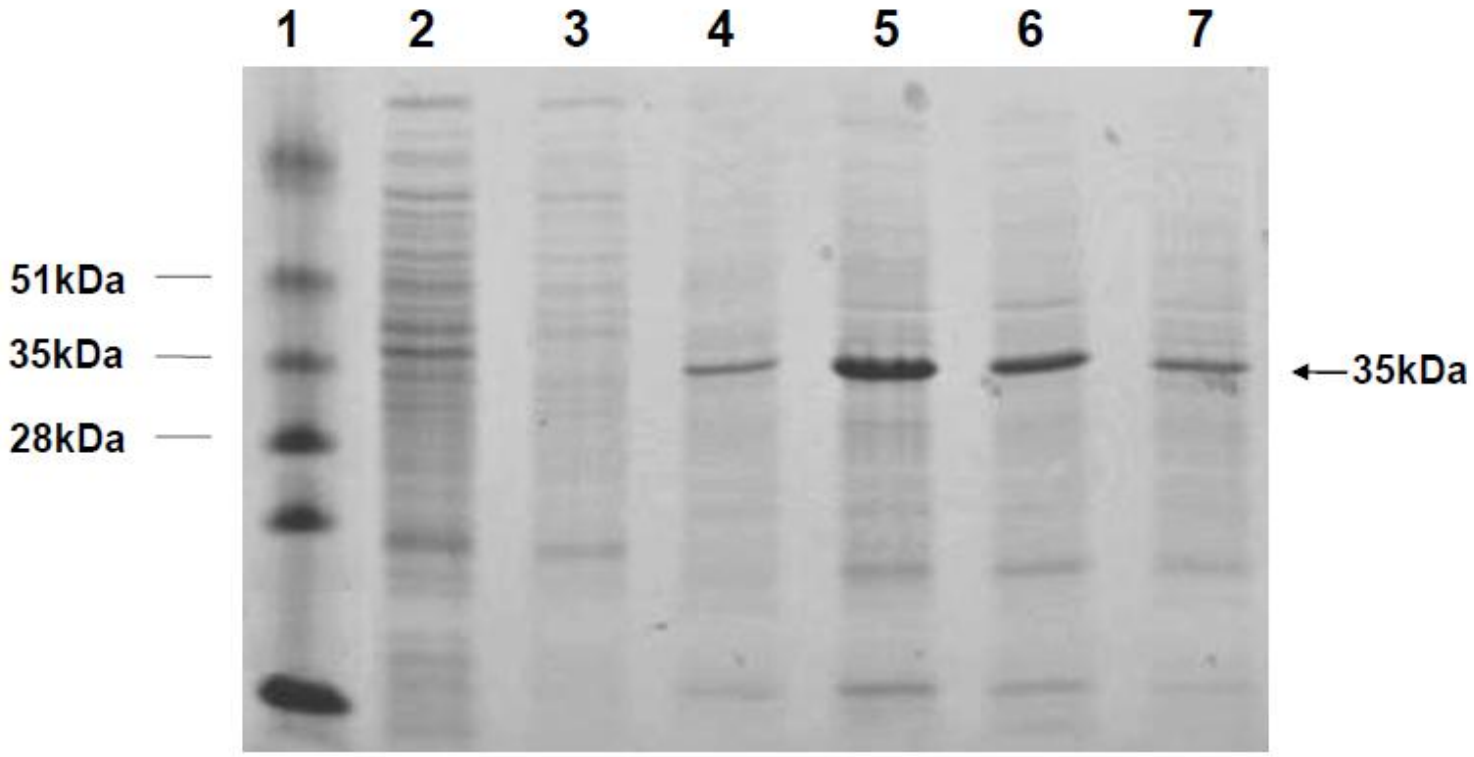

Figure 2:

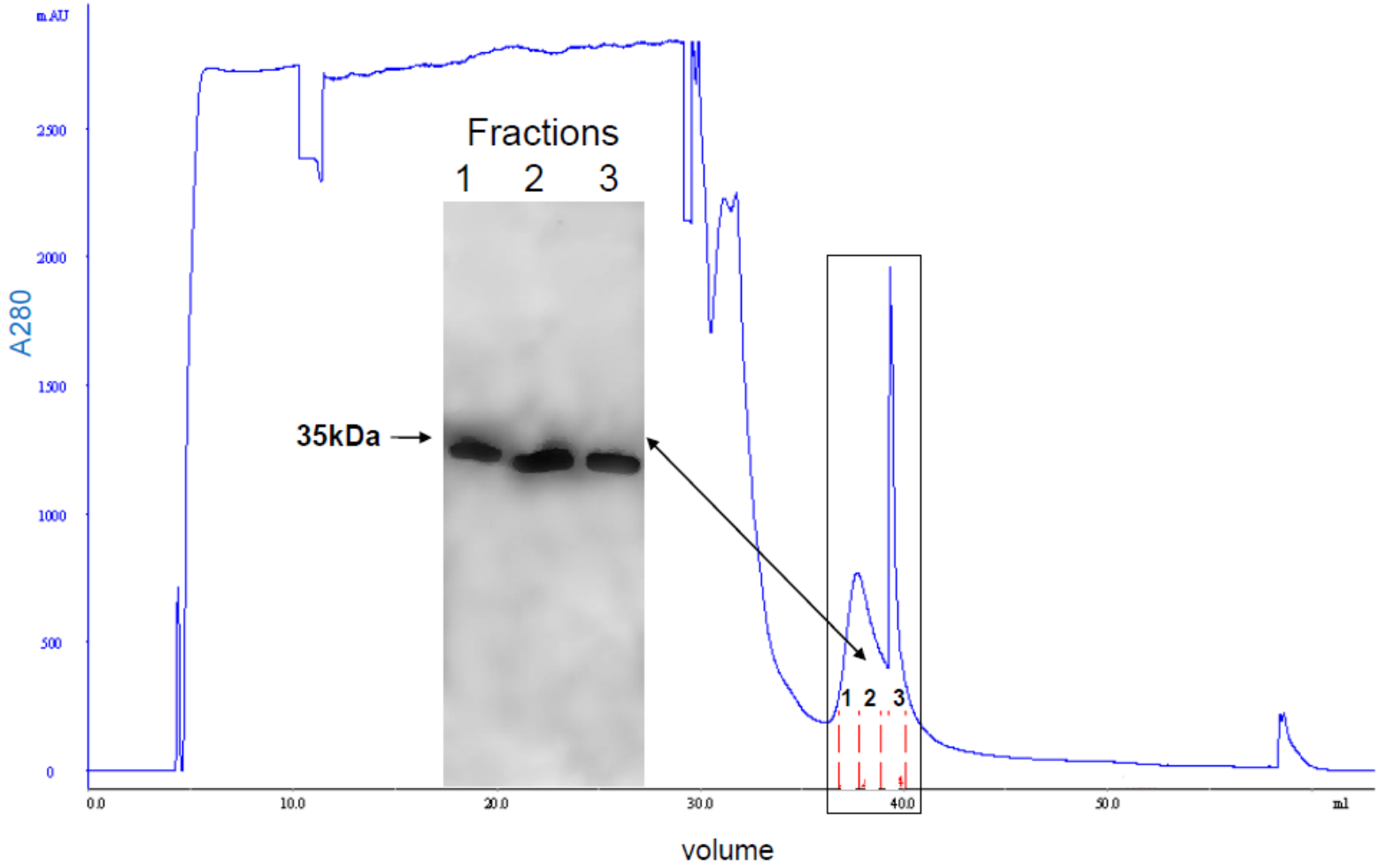
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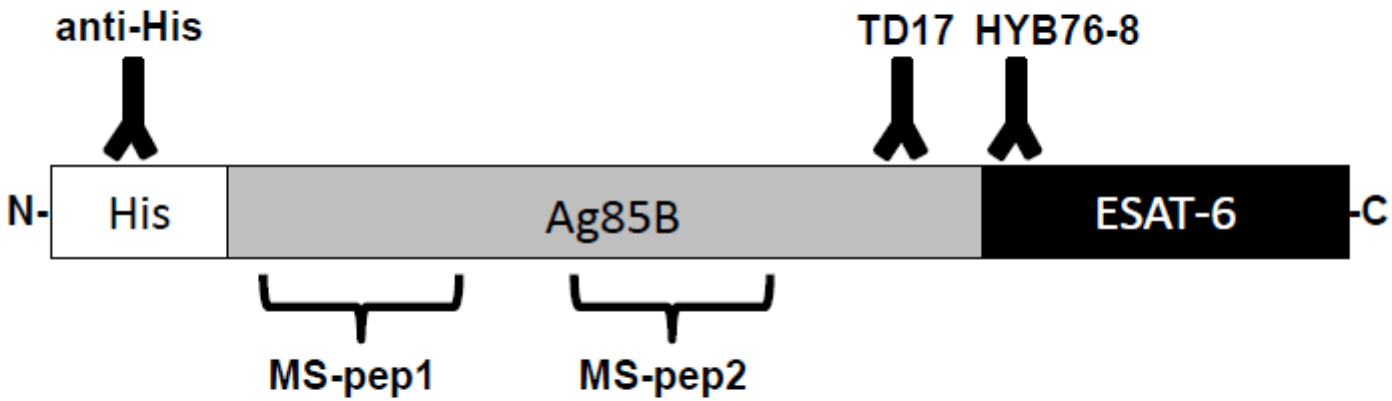
Figure 3:



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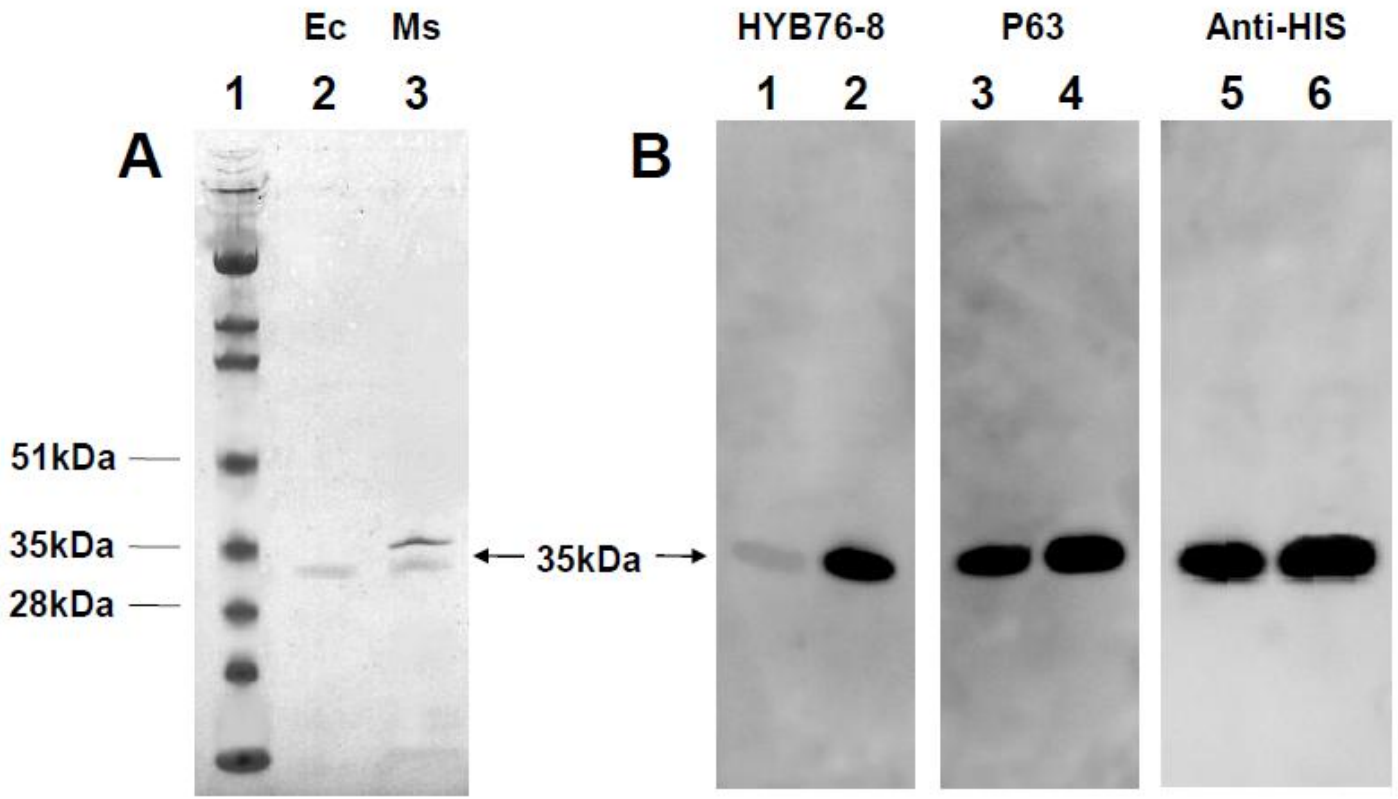
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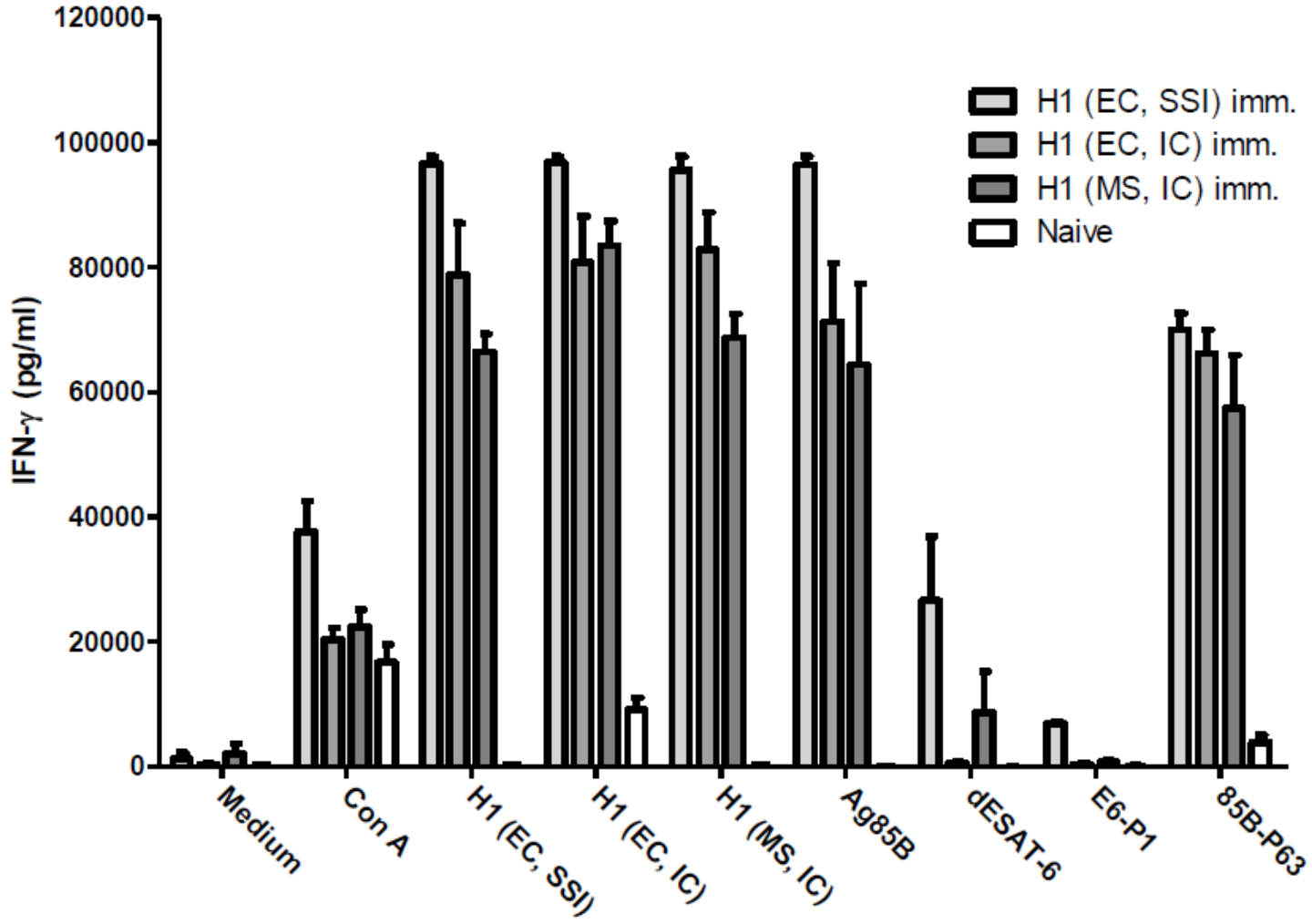
Figure 5: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H1 (EC, IC) imm.
H1 (MS, IC) imm.
Naive
Medium
Con A
H1 (EC, SSI)
H1 (EC, IC)
H1 (MS, IC)
Ag85B
dESAT-6
E6-P1
85B-P63