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Evaluation of the immunogenicity of recombinant stress-associated proteins during *Mycobacterium avium* subsp. *paratuberculosis* infection: Implications for pathogenesis and diagnosis

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ABSTRACT

The aim of this study was to assess the immunogenicity of recombinant stress-associated proteins of *Mycobacterium avium* subsp. *paratuberculosis* in sheep infected with the organism compared to control sheep. Five proteins – MAP2411, ClpP, Ppa, MAP0593c and GreA – which were identified previously in *in vitro* stress or dormancy responses of *M. paratuberculosis* to hypoxia, nutrient starvation and heat, were cloned, expressed and purified as His-tag recombinant proteins from the pET-15b vector in a BL21(DE3)pLysS strain of *E. coli*. The immunogenicity of MAP2411 did not differ between infected and control sheep. However, the serological reactivity of the other recombinant antigens, and combinations of them, varied according to the histological stage of paratuberculosis. Interestingly, the sera from some animals with paucibacillary lesions, which were not immunoreactive in a commercial paratuberculosis ELISA that was based on non-defined native antigens, recognised the recombinant antigens. We infer from their differential immunogenicity in infected and control sheep that four of the stress-associated proteins were expressed by *M. paratuberculosis in vivo*. These data provide fundamental information on host–mycobacterial interactions and have conceptual implications for the development of future diagnostic tests for early immune responses in animals infected with mycobacteria.

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1. Introduction

Johne's disease is a chronic inflammatory disease of the bowel of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) that is associated with economic losses worldwide. The incidence of Johne's disease can be reduced by good management but eradication is dependent on detection and culling of all infected animals. Eradication programs for Johne's disease are not successful because of the lack of simple and specific diagnostic tests for the early detection of disease (Sockett et al., 1992). Early diagnosis is important to identify and remove potential faecal shedders of *M. paratuberculosis* to prevent the spread of Johne's disease, and requires the development of novel, sensitive and specific diagnostic tests. The advantage of using an antibody-based assay instead of a cell-mediated immune response is that a serological test can be developed into a simple and robust kit (Weldingh et al., 2005). Current immuno-diagnostic tests for *M. paratuberculosis* are based on crude antigen mixtures but may benefit from inclusion of defined antigens. A number of these have been characterized previously including the cell wall derived glycolipid lipoarabinomannan (Jark et al., 1997), 35 kDa antigen (el-Zaatari et al., 1997), 34 kDa protein of A36 complex (De Kesel et al., 1992), 65 kDa protein of GroEL family (el-Zaatari et al., 1995), 85 complex (85A, 85B and 85C) and superoxide

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dismutase (SOD) (Shin et al., 2004), 22 kDa exported protein (Dupont et al., 2005), 70 kDa (Hsp70) (Stevenson et al., 1991), MAP2609, MAP2492c and MAP0210c (Willemsen et al., 2006) and 14 culture filtrate proteins of *M. paratuberculosis* (Cho et al., 2007).

In tuberculosis, a feature of *M. tuberculosis* is its ability to exist in a dormant state in the granuloma of an asymptomatic host and to evade the immune system. *M. tuberculosis* can subsequently reactivate to cause progressive disease (Parrish et al., 1998). Compared to other components of this organism, stress-associated proteins have received little attention as antigens for diagnosis and vaccine development against tuberculosis but a strong cellular and humoral immune response to the 16-kDa α-crystallin protein was observed in latently infected individuals (Davidow et al., 2005; Demissie et al., 2006; Khera et al., 2005). This protein is the hallmark of the dormancy response in *M. tuberculosis* (Boon and Dick, 2002). There is no published information on the immunogenicity of these proteins in animals with *M. paratuberculosis* infection and standardized sources of antigen are not available to conduct immunological studies. During *M. paratuberculosis* infection, host defenses either clear the infection or drive the host into different stages of disease and it is uncertain whether there is a dormant phase (Stabel, 2000). However, dormancy has been reported to occur when *M. paratuberculosis* enters the soil/pasture environment (Whittington et al., 2004) and dormancy may be induced under stress conditions *in vitro* (Gumber et al., 2008; Gumber and Whittington, 2009). Therefore, it is possible that *M. paratuberculosis* may enter into a dormant state in the host. We hypothesized that early stage or latently infected animals might have antibodies specific for *M. paratuberculosis* stress or dormancy-associated proteins which can be detected by a serological test. In this novel study, we performed serological screening using stress-associated recombinant proteins and confirmed that some of these proteins elicit an immune response in naturally infected animals that have relatively mild and paucibacillary lesions; rarely have such animals been detected using serological tests.

2. Materials and methods

2.1. Bacterial strains used during this study

E. coli TOP 10 cells (Invitrogen) F′ *mcr*A, Δ (*mrr-hsd*RMS-*mcr*BC), φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*D139, Δ (*araleu*) 7697, galU, galK, *rps*L (StrR), *end*A1, and *nup*G were used in cloning experiments. *E. coli* BL21(DE3)pLysS cells (Novagen) F′ *ompT*, *hsdSB* (r_B^- m_B^-), *gal dcm* (DE3)pLysS (CamR) were used as the host strain for the pET-15b expression vector. *M. paratuberculosis* S strain (Telford 9.2) was also used. This is a clonal isolate with an IS*1311* S pattern and an IS*900* Type S1 pattern (Marsh and Whittington, 2007).

2.2. Nucleic acid extraction and PCR amplification

DNA from *M. paratuberculosis* was extracted and purified using a modified chloroform/isoamyl alcohol technique as previously described (Choy et al., 1998). To amplify five genes which were expressed during stressful conditions *in vitro* (Gumber et al., 2008; Gumber and Whittington, 2009), primers (Table 1), containing NdeI and BamHI restriction endonuclease sites were designed on the basis of DNA sequence available for the *M. paratuberculosis* K-10 genome (GenBank Accession No. AE016958). DNA amplification was carried out in a 50 μl reaction containing 10 ng *M. paratuberculosis* DNA, 200 μM dNTPs, 0.5 μM each primer and 2U Taq polymerase (Expand High Fidelity PCR System, Roche). Each reaction was subjected to the following conditions: 1 cycle of denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min and extension at 72 °C for 30 s. After 30 cycles of amplification, a final extension was carried at 72 °C for 10 min. PCR products were visualized by 2% agarose gel electrophoresis using ethidium bromide.

2.3. Cloning and expression of recombinant proteins

PCR fragments generated were cloned into pCR2.1 (Invitrogen) for sequencing with M13 universal primers (M13(-20) and M13(-40)). The ORF was then subcloned as an NdeI/BamHI fragment into pET-15b. 6′His-tagged proteins were expressed from the T7 promoter of pET-15b in *E. coli* BL21(DE3) pLysS cells with 1 mM IPTG according to manufacturer's instructions (Novagen). Cell lysis was performed by sonication, and recombinant protein was purified on Talon metal affinity resin (Clontech) or Ni-NTA agarose resin (Invitrogen) under denaturing conditions according to the manufacturers' instructions. Protein-containing fractions were pooled and dialyzed overnight into 50 mM sodium phosphate pH 7.5, 150 mM NaCl, 0.05% Tween20 to refold the proteins before being snap frozen and stored at −80 °C.

Table 1
Primers used for PCR amplification and cloning.

Protein/gene	Accession no.	Forward primer sequence[a]	Reverse primer sequence[a]	Size (base pairs)[b]	Molecular mass (kDa)[b]
MAP2411	41408509	CATATGAAACCCTTCAGCGAATCCG	GGATCCGACATGGTGCTCACCATGC	509	15.5
MAP0593c	41406691	CATATGGCGTCGATCTTCACCAAG	GGATCCAGGGTTGCTGCTTCCG	459	14.7
clpP (MAP2281c)	41408379	CATATGTCTGACATGCGTTCGCC	GGATCCTGGAGTGCTCGATGAACG	657	21.6
ppa (MAP0435c)	41406533	CATATGGAATTCGACGTGACCATCG	GGATCCGGTGTTCTTCATCGGAACGGC	553	18.5
greA (MAP1027c)	41407125	CATATGACGGATACTTCCGTGACC	GGATCCAGTTGATACGCCGAGCGC	520	17.8

[a] Restriction sites used for cloning are underlined. NdeI, CATATG; BamHI, GGATCC.
[b] Expected sizes of PCR amplified products and expressed recombinant proteins.

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