Detection of the pathogenicity factor PtpA in *Mycobacterium bovis* BCG

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ABSTRACT

PtpA is a tyrosine phosphatase that is secreted by *Mycobacterium tuberculosis* in early infection of macrophages. This protein has been previously shown to be expressed in *M. bovis* BCG (Cowley 2002), but it is unknown whether BCG secretes this protein. In this study, we used antibody based assays (western blotting and immunoprecipitation) to investigate the secretion of PtpA by wt *M. bovis* BCG *in vitro*. The protein could not be detected *in vitro* using polyclonal αPtpA, which could indicate that it is produced in very small amounts prior to infection. We also constructed a BCG recombinant strain which overexpresses PtpA to study the protein's secretion. Further studies will explore the post-infection secretion of this molecule and attempt to detect it in the serum of infected animals.

INTRODUCTION

Mycobacterium bovis, the causative agent of bovine tuberculosis (bTB), has an important economic impact on the cattle industry due to loss of production and early culling (Amanfu, 2006). Additionally, bTB represents an important zoonotic risk in developing countries and those who drink unpasteurized milk (Amanfu, 2006). Despite control procedures in place, the incidence of bTB has been steadily increasing in the UK since 1988 (Vordermeier *et al.,* 2002). The only available vaccine is the attenuated bacillus Calmette-Guerin (BCG) strain of *Mycobacterium bovis*, which is banned from use in the EU due to the inability to differentiate BCG vaccinated animals and *M. bovis* infected animals (Truss, 2013). Therefore, research in the last 10 years has been highly focused on the development of a so-called DIVA (differentiating infected and vaccinated animals) test, which would allow the re-introduction of the BCG vaccine to the UK.

It has been shown that *M. bovis* BCG lacks a secretion system, called ESX-1 in *M. tuberculosis*, which is necessary for the secretion of several proteins, including ESAT-6 and CFP-10. These proteins have become the most promising targets of research into development of the DIVA test, as they cause a strong antigenic response TB-infected cattle but not in BCG-vaccinated cattle (Cockle *et al.* 2002; Whelan *et al.,* 2010; Vordermier *et al.,* 2011).

Additionally, the BCG vaccine has a variable efficacy, averaging only 56-68% (Ameni *et al.,* 2010). The most promising strategy for increasing vaccination efficacy is based on BCG vaccination followed by boosting with protective antigens, such as cellular filtrate proteins (Vordermeier *et al.,* 2011, Wedlock *et al.,* 2005). Studies in *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, have identified protein tyrosine phosphatase A (PtpA) as a likely candidate for a subunit vaccine that activates cell-mediated immunity and therefore has an increased efficacy against mycobacterial infection (Merly *et al.,* 2012).

PtpA has been shown to be necessary for *M. tuberculosis* infection of the macrophage (Bach 2008). It is secreted into the cytoplasm in early infection, where it binds to VSP33B of the lysosome and subunit H of the phagosome ATPase, preventing lysosome fusion and phagosome acidicification (Wong *et al.,* 2011; Bach *et al.,* 2008). The ptpA gene has been shown to be present in several related organisms, including *M.*

bovis and *M. avium* subspecies *paratuberculosis*, the causative agents of bovine tuberculosis and ovine Johne's disease respectively (Bach *et al.,* 2006). PtpA has also been shown to have increased expression upon infection of macrophages with *M. bovis* BCG (Cowley *et al.,* 2002) and has been detected in the serum of sheep with Johne's disease (Gurung *et al.,* 2014).

The goal of this study was to investigate whether PtpA is secreted by *M. bovis* BCG. Because *M. bovis* BCG lacks the ESX-1 secretory system, we hypothesize that PtpA is expressed but not secreted. To investigate this we used a western blot to detect PtpA in cell lysates and in the supernatant. Since the western blot was unable to detect the molecule in either the cell lysate or the media, we used an immunopreciptation assay to concentrate PtpA in the sample in order to prevent interference from other cellular proteins. After immunoprecipitation, the protein could not be detected in the cell lysate or supernatant, so we constructed a BCG recombinant strain which overexpresses the PtpA molecule, which will be used to analyze secretion *in vitro* and *in vivo*.

MATERIALS AND METHODS

Table 1. Strains and plasmids

Strain or plasmid	Characteristic(s)	Source or Reference
	Strains	
E. coli BL21		Novagen
M. smegmatis	mc^2155	W.R. Jacobs
M. bovis BCG	Pasteur strain	ATCC 35374
	Plasmids	
pALACE ptpA	Hygromycin resistant, *M. tuberculosis* PtpA under *ace* promoter control	Bach *et al.,* 2008
pET30 ptpA	Kanamycin resistant, ptpA under *Lac* promoter control	Novagen

Growth conditions and preparations of bacteria

Mycobacteria were grown in Middlebrook 7H9 + ADS + 0.05% Tween 80 at 37°C unless otherwise specified. Cell lysates were created via mechanical disruption of membranes using a "Mini-Beadbeater" apparatus (Glen Mills). The protein contents of media corresponding to cell cultures ("supernatant") was concentrated via TCA and acetone precipitation and re-suspended in 6x SDS-PAGE sample buffer.

DNA Manipulation

Plasmids were isolated using a mini-prep kit (Quiagen) and re-suspended in sterile distilled water. *M. bovis* BCG and *M. smegmatis* were grown to an OD600 of 0.5 in Middlebrook 7H9 + ADS + 0.05% Tween 80, then transformed with pALACE PtpA via electroporation. Transformants were grown on Middlebrook 7H10 agar + OADC + 50µg/µl hygromycin.

M. smegmatis transformants were grown as above; then plasmids were extracted using a modified miniprep isolation (AbCam Protocols). Plasmid identity was confirmed using polymerase chain reaction and agarose gel electrophoresis (Figure S2).

Immunoprecipitation

2mg samples of BCG cell lysate and supernatant from the same culture were added to rabbit αPtpA polyclonal antibody (YenZym Antibodies, LLC) at 1:100 dilution for 1hr at room temperature. After washing with 25mM Tris buffer and overnight blocking with 0.5% BSA, Affi-gel protein A agarose resin (Bio-rad) was added to the samples and incubated 2h room temperature with shaking. The resin was washed with PBS and purified antigen was eluted with SDS sample buffer. The resulting samples were resolved by SDS-Page and analyzed by western blot as indicated.

Western Blots

Samples were separated by 12% SDS-Page gel, then transferred to a 0.45 um nitrocellulose membrane. PtpA was detected by rabbit α-PtpA (Wong 2011), which was

then detected by goat α-rabbit Fluor 680 (Invitrogen) at a dilution of 1/3500. Blot was visualized using an Odyssey CLX infrared imaging system.

RESULTS

Wild type *M. bovis* BCG experiments

PtpA could not be detected in either cell lysates or supernatants of wild type *M. bovis* BCG (Figure 1). When the PtpA in the sample was concentrated by immunoprecipitation before blotting (Figure 2), the protein still could not be detected. In each of these figures, recombinant PtpA purified from E. coli BL21 containing an expression plasmid (pET30), as detailed in supplementary data, was used as a positive control (Figure 1: lane 4; Figure 2: lane 8). In Figure 2, the immunoprecipitation method was used on a sample of purified PtpA as a positive control for the immunoprecipitation method. In this figure, extraneous bands between 25 and 50kDa represent non-specific binding of the secondary antibody to light and heavy chain molecules of the primary antibody used for immunoprecipitation.

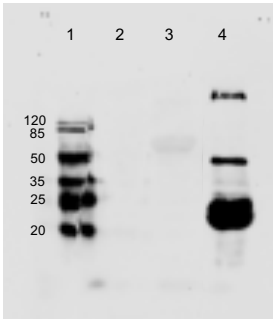

Figure 1. Western blot of PtpA. Samples were prepared as described in Materials and Methods. Lanes: 1, pre-stained molecular weight marker (Thermo Scientific); 2, *M. bovis* BCG cell lysate; 3, protein contents of media corresponding to BCG culture; 4, recombinant PtpA

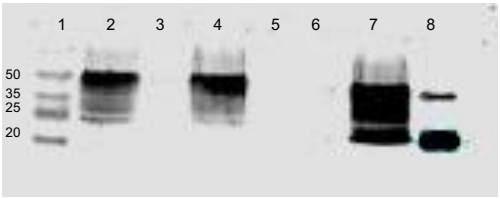

Figure 2. Immunoprecipitation of PtpA. Samples prepared as described in Materials and Methods and detected by western blot. Lanes: 1, pre-stained molecular weight marker (Thermo Scientific); 2,

immunoprecipitate of *M. bovis* BCG cell lysate; 3, empty; 4, immunoprecipitate of protein contents of media corresponding to BCG culture; 4 and 6, empty; 7, immunoprecipitate of recombinant PtpA; 8, recombinant PtpA.

Transformation of BCG

pALACE ptpA was inserted into *M. bovis* BCG with a transformation efficiency of 38.0 cfu/µL. These transformants will be used for future experiments to detect PtpA secretion.

Table 2: Transformation efficiency of *Mycobacterium bovis* BCG with pALACE DNA

Plasmid insert	Amount DNA (µg)	Total cell Volume (µL)	Volume Plated (µL)	Number of colonies	Transformation efficiency (cfu/µg)
pALACE	3.452	1300	200	9,3,1,3*	7.52 +/- 5.64
pALACE ptpA	4.187	1300	200	54,4,16,8,7,58	38.0 +/- 35.1

* two plates were destroyed due to drying in the incubator.

DISCUSSION

We were unable to detect PtpA in either the cell-free filtrate or the supernatant of *Mycobacterium bovis* BCG using either western blot or immunoprecipitation. Though it has been shown that the PtpA gene is expressed by *M. bovis* BCG (Cowley, 2002), it is possible that it is not translated into a functional protein, or that post-translational modifications make the protein unable to be detected using antibodies. It has also been shown that expression of the gene is increased upon infection of macrophages (Cowley *et al.,* 2002), so it is possible that PtpA may be detected with greater success in an infection assay. If antibody-based methods continue to be unsuccessful, it may be necessary to use alternative methods to detect PtpA, such as gfp-fusion constructs. It will also be necessary to investigate wild type *M. bovis* to determine if PtpA can be detected in the infectious strain.

Western blotting with immunofluorescence detection has been shown to detect as little as 1.2pg of protein (Schutz-Geschwender et al., 2004), and the immunoprecipitation method is used to further concentrate the protein in a solution to aid detection (Weiser and Schweiger, 1986), so it is unlikely that the lack of detection is due to low concentration. To ensure that this is not the case, we created an overexpression mutant,

M. bovis BCG pALACE ptpA. Unfortunately, due to the low transformation efficiency of *M. bovis* BCG (Table 2) and its slow growth (13-20h doubling time), we were unable to test the overexpression mutant within the timeframe of this project.

The transformation protocol was successful for the fast-growing *M. smegmatis* (Table S1) but the transformation efficiency for *M. bovis* BCG was very low (Table 2). This is likely due to the slow growth of BCG, which allowed the plates to start to dry out, and the tendency of BCG to clump, creating a smaller surface area for DNA to be taken up by BCG competent cells (Wards and Collins, 1996). Additionally, electroporation is difficult to perform in all mycobacterial species due to their complex cell wall (Wards and Collins, 1996). Transformation efficiency may also have been decreased due to the relatively large amount of plasmid DNA used ([DNA] in excess). Greater transformation efficiency has been shown using 100ng DNA/cuvette (Cho *et al.*, 1998). Interestingly, it has been shown that while *M. smegmatis* have a high transformation efficiency at 0°C (as used in this experiment), *M. bovis* transformation efficiencies are increased when the temperature is raised to 37°C (Wards and Collins, 1996). Future experiments will raise the cell temperature to 37°C and use the electroporation settings and DNA concentration specific to BCG (Wards and Collins, 1996, Cho *et al.,* 1998). Following electroporation, the cells will be grown in sealed plated in a well humidified incubator to prevent plate drying.

Continuing experiments will be focused on the detection of PtpA from overexpression in recombinant BCG strains, and determining whether our inability to detect PtpA from wild type strains is due to low expression in the wild type cell or post-expression effects. Additionally, if overexpression or infection proves to allow successful detection of PtpA, the next logical step is to investigate ptpA secretion in infectious *M. bovis* using similar *in vitro* experiments and infection of macrophages. Finally, it should be determined if PtpA can be isolated from the sera of infected cattle.

Though we were unable to detect the protein in this *in vitro* study, the evidence we have presented suggests that PtpA secretion patterns may differ in BCG versus infectious *M. bovis*, making it an interesting topic of further investigation.

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SUPPLEMENTAL DATA

1. Purification of PtpA

A single colony of E. coliBL21 his-ptpA were grown overnight at 37 °C with shaking in LB + kanamycin, then diluted 1/50 in fresh LB + kanamycin and incubated in the same conditions until OD600 = 0.648 and 0.705, then induced with 0.4 mM IPTG overnight (37 °C shaking). Cells were harvested by centrifugation and re-suspended in lysis buffer + PMSF. Cells were lysed by sonication and his-ptpA was purified using a Ni2+ column and dialysed against PtpA storage buffer (20mM Tris-HCl pH 7.4 150mM NaCl 1mM DTT and 10% glycerol). Protein concentration was determined by a Bradford assay and confirmed with a SDS-Page gel.

Table S1. Protein concentrations of *M. bovis* BCG cell lysates following sonication and Ni^{2+} affinity chromatography as measured via Bradford Assay

Aliquot number	Concentration
1	0.035 mg/mL
2	0.077 mg/mL
3	0.034 mg/mL
4	Nil
5	0.097 mg/mL

Equation of standard curve of known concentrations of BSA (0.02-0.14mg/mL):

$y = 0.75x + 0.042$ $r^2 = 0.948$

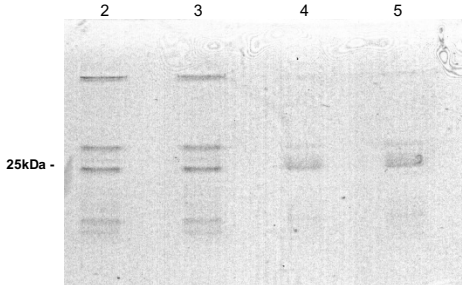

Figure S1. SDS-PAGE of PtpA samples (numbered 2-5) isolated from *M. bovis* BCG cell lysates via Ni^{2+} chromatography. Samples were resolved through a 5% stacking and 15% separating gel and stained with Coomassie blue.

2. Transformation of *M. smegmatis*

M. smegmatis was grown to an OD600 and transformed with pALACE ptpA as a test of the transformation protocol (described above). The plasmid DNA for the experiment was purified from E. coli stocks using a mini-prep system (Quigen) and resuspended in either elution buffer (EB) or sterile double distilled water (ddH_2O). Transformants were grown on 7H10 plates for 3 days, resulting in colony numbers too numerous to count. Single colonies were transferred to separate 7H10 plates and grown 2 days at 37 °C. Colonies were then grown in 7H9 broth and plasmids were extracted using a modified mini-prep protocol. Transformation success was confirmed by extracting the plasmids using a modified mini-prep protocol (AbCam) and polymerase chain reaction (PCR) amplification of the ptpA gene (Figure S2).

Table S2. Transformation efficiency of *M. smegmatis* with pALACE vector.

Plasmid insert	Amount DNA (µg)	Total cell Volume (µL)	Volume Plated (µL)	Number of colonies	Transformation efficiency (cfu/µg)
pALACE ptpA (EB)	4.19	1300	200	est. 384, est. 984, est. 520	976 +/- 398
pALACE ptpA (ddH_2O)	4.87	1300	200	TNTC (>1000)	n/a

Transformation Efficiency was calculated using the following equation:

$$\left[\begin{matrix}\#\ of\ Colonies\\ on\ Plate\end{matrix}\right] \times \left[\frac{Total\ Cell\ Volume\ (\mu l)}{Volume\ Plated\ (\mu l)}\right] \times \left[\frac{1}{\mu g\ transformed}\right] = CFU/\mu g$$

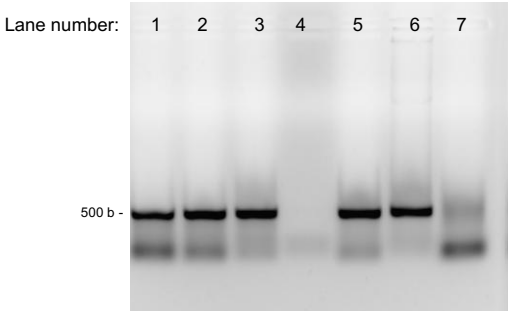

Figure S2. PCR of extracted plasmids after amplifying the ptpA gene. Each sample contained PCR mastermix, 1µL sample and 1µL KOD polymerase. Negative controls (lanes 4 and 7) contained no polymerase and no sample respectively. Positive control (lane 6) contains a sample of isolated

pALACE ptpA plasmid which was used for the transformation. It was separated on a 1% agarose gel at 90V for 40m and visualized with SYBRSafe® DNA gel stain (Life Technologies).

Table S3. Primers used in PCR amplification of ptpA gene isolated from *M. smegmatis* transformants

Primer	Sequence	Source
PtpA F	ATATATGAATTCCGTGTCTGATCCGCTG	Poirier, V. [1]
PtpA R	ATATATCTCGAGTCAACTCGGTCCGTTC	Poirier, V. [1]

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