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Expression and localization of the *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpA

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Abstract

The *Mycobacterium tuberculosis* open reading frame Rv2234 encodes a low molecular weight tyrosine phosphatase named PtpA. Kinetic analyses of PtpA activity reveal that it is capable of dephosphorylation of p-nitrophenyl phosphate, as well as a variety of phosphotyrosine-containing substrates. In contrast, PtpA showed no detectable activity towards substrates containing phosphoserine or -threonine residues. Transcriptional analysis reveals that the *M. tuberculosis ptpA* promoter is expressed in the slow-growing *Mycobacterium* species *M. bovis* BCG, but not in the fast-growing species *M. smegmatis*. Furthermore, *ptpA* expression is upregulated upon entry of BCG cultures into stationary phase and increases upon infection of human monocytes. We also show that, despite the lack of a general export pathway signal sequence, the *M. tuberculosis* PtpA protein can be released from both *M. tuberculosis* and *M. smegmatis* during growth.

Keywords: Mycobacteria; Protein phosphatase

1. Introduction

There are four major eukaryotic protein phosphatase families. Sequence homology suggests that the *Mycobacterium tuberculosis* tyrosine phosphatase PtpA belongs to the low molecular weight tyrosine phosphatase group, also called low MW PTPs. Low MW PTPs have been identified in a limited number of other bacteria [Reviewed in 13,18]. For example, the genomes for *Erwinia amylvora*, *Klebsiella pneumoniae*, and *Pseudomonas solancearum* have putative low MW PTP genes that cluster with genes involved in exopolysaccharide synthesis. A multiple sequence alignment of PtpA with other LW PTPs shows that its closest homolog is the PtpA protein from *Streptomyces coelicolor.* The *S. coelicolor* PtpA has been purified and shown to hydrolyze free phosphotyrosine residues in vitro, and it has been speculated to be involved in regulation of sulfur amino acid metabolism based on the surrounding genetic loci [15]. In contrast, several bacterial pathogens produce eukaryotic-like protein phosphatases that have been implicated in virulence. For example, the tyrosine phosphatase YopH of *Yersinia pseudotuberculosis* and the *Salmonella typhimurium* tyrosine phosphatase SptP have been shown to be translocated into host cells via a type III secretion system, resulting in disruption of the host cell cytoskeleton [1,8,9].

Analysis of the complete genome sequence of *Mycobacterium tuberculosis* predicts the presence of two putative tyrosine phosphatases, named PtpA and PtpB [4,14]. Surprisingly, this analysis also revealed a conspicuous absence of any predicted protein tyrosine kinases, although there is one report that demonstrates the presence of a major 55-kDa tyrosine-phosphorylated protein in cell extracts from virulent but not avirulent *Mycobacterium* strains [3]. Recently, PtpA and PtpB have been shown, using a qualitative method, to have tyrosine phosphatase activity towards the artificial substrate myelin basic protein (MBP), and to lack apparent activity towards serine and threonine-phosphorylated residues in MBP [14]. However, the *M. tuberculosis* genome has 12 putative serine/threonine kinases, with only one corresponding predicted serine/threonine phosphatase. In addition, some tyrosine phosphatases, such as IphP of *Nostoc commune*, exhibit dual specificity for phosphotyrosine as well as phosphoserine and threonine residues [13,18]. These phosphatases contain a conserved CXXXXXRS/T motif at their catalytic site that is also found in low MW PTPs [13,18]. This observation raises the question as to the specificity of PtpA and PtpB with respect to other phospho-

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rylated substrates, especially those that are smaller than the MBP protein and may present less of a steric challenge in an artificial system.

In light of these findings, a detailed analysis of the substrate specificity of PtpA, as well as the expression of the *ptpA* gene, is important to the understanding of the function of PtpA and its possible role in virulence of *M. tuberculosis*.

Here we show that PtpA is specific for substrates containing phosophotyrosine residues, and that expression from the *M. tuberculosis ptpA* promoter only occurs when cloned into the slow-growing *M. bovis* BCG, but not the fast-growing saprophytic species *M. smegmatis*.

2. Materials and methods

2.1. Strains and plasmids

The bacterial strains, plasmids, and oligonucleotides used in this study are given in Table 1. *Escherichia coli* was grown in Luria-Bertani medium at 37 °C. Ampicillin (100 μg/ml) and hygromycin (150 μg/ml) were used whenever required. *M. smegmatis* mc^2155 and *M. bovis* BCG were grown in 7H10 medium with 10% OADC and 0.05% Tween-20. Hygromycin (50 μg/ml) was used when required. The oligonucleotides, shown in Table 1, were synthesized by Gibco-BRL Laboratories. Inhibitors and amino acids were purchased from Sigma Chemicals (Sigma, St. Louis, MO).

2.2. Cloning of the ptpA *gene from* M. tuberculosis

The open reading frame (ORF) Rv2234 encoding PtpA was amplified from *M. tuberculosis* H37Rv DNA using the primers P1 and P2 (Table 1): P1 and P2 contained *Nco*I and *Xho*I restriction sites, respectively. PCR was performed with Taq polymerase obtained from Gibco BRL, using 1.5 mM $MgCl_2$ and 5% dimethyl sulfoxide (DMSO). 30 cycles of PCR included denaturation at 94°C for 40 s, annealing at 55°C for 1 min and amplification at 72°C. The appropriate PCR product was ligated into the vector pCR2.1 of the TA cloning kit (Invitrogen) and transformed into *E. coli* DH5α by standard chemical transformation procedure. Clones containing the vector were digested with *Nco*I and *Xho*I (Fermentas). Restriction enzyme-digested plasmids were isolated with a QIAquick gel extraction kit (Qiagen Ltd.). A corresponding digestion was also applied to the plasmid pET-22b and the two products were ligated together with T4 DNA ligase to obtain the plasmid pYA150, which adds a PelB leader sequence to the N-terminus of the expressed protein. To ensure that this 26-amino acid leader does not affect PtpA activity, the *ptpA* gene from pYA150 was excised using *Nco*I and *Xho*I and ligated to an aliquot of *Nco*I and *Xho*I cut pET16b to generate the plasmid pRB1. For both pYA150 and pRB1, protein expression was induced using 0.4 mM IPTG (isopropyl-β-D-thiolgalactopyranoside), and the resulting inclusion bodies were isolated from 1 l of induced *E. coli* BL21 culture as previously described [2]. Washed inclusion bodies were denatured by drop-wise addition of a urea/DTT solution to give a final concentration of 4 M urea and 0.5 M DTT (dithiothrietol). The urea and DTT were removed by dialysis in TBS (Tris-buffered saline), and the resulting renatured protein was tested for activity. The protein obtained from the inclusion bodies (from both pET16b and pET22b) was subjected to N-terminal sequencing; for the protein expressed from pET22b, the first 26 amino acids corresponded to the PelB leader, and the next 11 amino acids were identical to the amino acid sequence of the *ptpA* gene product derived from the *M. tuberculosis* genome sequence database. The N-terminal 10 amino acids of the recombinant PtpA protein expressed from pET16b were also found to be identical to the amino acid sequence of the *ptpA* gene product derived from the *M. tuberculosis* genome sequence database. Both proteins were further examined for purity by 16.3% Tricine SDS-PAGE. The recombinant PtpA proteins isolated from pET22b (with a PelB leader) and pET16b (without a PelB leader) both performed identically in activity assays; the quantities of recombinant PtpA obtained from pET22b were found to be more abundant, and thus this recombinant protein was chosen for further experiments.

2.3. Characterization of the PtpA phosphatase

With p-nitrophenyl phosphate (pNPP) as a substrate, PtpA phosphatase activity was determined by adding 2.95 μg of the purified PtpA to the reaction buffer (6.7 mM PIPES buffer, pH 6.5, 4 mM $MgCl_2$, 15 mM DTT, and 1 mM pNPP). The absorbance at 410 nm was followed over time using a Dynatek MR5000 ELISA plate spectrophotometer. The assay was optimized with respect to time and protein concentration so that the reaction was performed within the linear region. Optimal concentrations of Mg^{2+}, Mn^{2+}, and Ca^{2+} were determined using the same method. The optimal pH (6–7) was determined using the above method, but the PIPES buffer was replaced with either MES or Tris-aminomethane for the appropriate pH range. The effect of sodium vanadate (200 μM, Sigma, St. Louis, MO) was also tested according to a similar procedure. Phosphatase assays involving the substrates O-phospho-tyrosine, O-phosphoserine, O-phospho-threonine, and the commercial tyrosine phosphopeptide RRLIEDAEpYAARG or serine phosphopeptide RRApSVA (Upstate Biotechnologies, Inc.) were performed using a malachite green assay to measure the release of free inorganic phosphate according to the manufacturer's instructions (Upstate Biotechnologies, Inc.). In each case, the reaction buffer contained 6.7 mM PIPES buffer, pH 6.5, 4 mM $MgCl_2$, and 15 mM DTT in addition to 2.95 μg of PtpA. Identical reactions run in parallel but lacking PtpA were performed to assess background levels of inorganic phosphate. All reaction ingredients were tested for the presence of inorganic phosphate using malachite green prior to use. PtpA phosphatase activity using

Table 1
Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, oligonucleotide	Characteristics[a]	Source or reference
Strains		
M. tuberculosis	H37Rv	
M. smegmatis	mc^2155	W.R. Jacobs
M. bovis BCG	Pasteur strain	ATCC 35374
E. coli BL21	$F^- ompT\ hsdS_B(r_B^- m_B^-) gal dcm$	Novagen
E. coli DH5α	$F^- rec$A1 *hsd*R17 *thi*-1 *gyr*A96 *sup*E44 *end*A1 *rel*A1 *rec*A1 *deo*R Δ(*lac*ZYA-*arg*F)U169 (ϕ80 *lac*Z ΔM15)	
Plasmids		
pET22b	T7 expression vector, Amp^r	Novagen
pET16b	T7 expression vector, Amp^r	Novagen
pCR2.1	TA cloning vector, Amp^r	Invitrogen
pSC301	*gfp* transcriptional fusion vector, Hyg^r	[5]
pSC301b	Background fluorescence control for pSC301; pSC301 in which the SOD promoter was excised.	[5]
pSC601	Transcriptional fusion to *gfp*; pSC301 containing the *M. tuberculosis ptpA* gene upstream 1415bp, Hyg^r	This study
pSC801	Transcriptional fusion to *gfp*; pSC301 containing the *M. tuberculosis pknH* gene upstream 636bp, Hyg^r	[5]
pSC300	Translational *gfp* fusion vector, Hyg^r	[5]
pSC120	Translational fusion to *gfp*; pSC300 containing the first 95 codons of the *M. tuberculosis cho*D gene, Hyg^r	[5]
pSC720	Translational fusion to *gfp*; pSC300 containing all but the last 4 codons of the *M. tuberculosis ptpA* gene, Hyg^r	This study
pYA150E	pET22b containing the *ptpA* ORF in the *Nco*I/*Xho*I sites	This study
pRB1	pET16b containing the *ptpA* ORF in the *Nco*I/*Xho*I sites	This study
Oligonucleotides		
P1	5′-TAGCCATGGTGTCTGATCCGCTGCACG-3′	This study
P2	5′-TAGCTCGAGGCACAGGTAGGTGAACGCGAC-3′	This study
SC1	5′-ACTAGTCGACATCACAGGCAGCTAA-3′	This study
SC2	5′-GATATCTCAGACACCTAGCGCCTCC-3′	This study
SC3	5′-TCTAGAACCTGCACGACATCGCCTC-3′	[5]
SC4	5′-GATATCGTCCTGTGCGTCGCTCATC-3′	[5]
SC5	5′-AGATCTCCACCACCGTCGTGACGCA-3′	This study
SC6	5′-GATATCCGTTCCGCGCGAGACGTTC-3′	This study

[a] Amp^r, ampicillin resistance; Hyg^r, hygromycin resistance; SOD, superoxide dismutase; ORF, open reading frame.

tyrosine-phosphorylated myelin basic protein (MBP) radiolabelled with AbI tyrosine kinase as a substrate was determined using the PTP assay system according to the manufacturer's instructions (New England BioLabs, Inc.). The precipitated protein was resolubilized in reaction buffer and subjected to electrophoresis on a 12.5% SDS-PAGE, followed by autoradiography of the dried gel. All reactions involving PtpA were performed at 37 °C.

2.4. Green fluorescent protein fusion (Gfp) plasmid construction

The *ptpA-gfp* transcriptional fusion was made by PCR amplification of a sequence that includes 7 nucleotides past the *ptpA* start codon and the sequences including the upstream 1415 bp from the *ptpA* gene. This fragment was amplified from H37Rv genomic DNA using primers SC1 and SC2 (Table 1) designed to result in a 5′ *Xba*I site and a 3′ *Eco*RV site. Similarly, the control *pknH* transcriptional fusion was made by PCR amplification of a sequence that includes 17 nucleotides past the *pknH* start codon and the sequences including the upstream 636 bp from the gene. This fragment was amplified from H37Rv genomic DNA using primers SC3 and SC4 (Table 1) designed to result in a 5′ *Xba*I site and a 3′ *Eco*RV site. In the case of both the *ptpA* and *pknH* transcriptional fusions, the PCR fragments were TA cloned into pCR2.1, and subsequently excised with *Xba*I and *Eco*RV, and ligated to *Xba*I and *Eco*RV cut pSC301 from which the SOD promoter had been excised to yield plasmids pSC601 and pSC801, respectively. The *ptpA-gfp* translational fusion was constructed by PCR amplification of a sequence from *M. tuberculosis* H37Rv genomic DNA that includes the *ptpA* start codon as well as the entire coding sequence of the gene minus the last 4 codons. This was done using primers SC5 and SC6 (Table 1) that had been designed to result in a 5′ *Bgl*II site and a 3′ *Eco*RV site, and the PCR product was TA cloned into pCR2.1. To create the PtpA-Gfp fusion plasmid, pSC720, this PCR product was excised from pCR2.1 using *Bgl*II and *Eco*RV, and cloned into the corresponding *Bam*HI and

*Eco*RV sites in pSC300. The Gfp translational fusion vector pSC300 and the *choD-gfp* translational fusion (pSC120) have been previously described [5]. These constructs were electroporated into *M. smegmatis* or *M. bovis* BCG as previously described [5].

2.5. Gfp transcriptional fusion assays

Gfp fluorescence of bacterial cultures was quantified using a spectrofluorometer (BioRad VersaFluor^{TM}). Cells were grown as described, and at the indicated time intervals 100 μl of each culture was removed, diluted into 1–2 ml of PBS, and the relative fluorescence units (RFU) were measured using a 490/10 excitation filter and a 520/10 emission filter. The OD at 600 nm of each culture was measured for each culture in a Jenway 6405 UV/Vis. spectrophotometer. Background fluorescence due to readthrough transcription of the transcriptional fusion vector was determined by measuring the RFU of strains subjected to the same conditions harboring the pSC301b plasmid (Table 1). Special conditions used to test promoter expression include exposure to microaerophilic conditions for 10 days as previously described [6], heat shock (incubation at 45 °C), cold shock (incubation at 4 °C or room temperature), oxidative stress (exposure to 10 mM H_2O_2), nutrient stress (incubation in water), and acid stress (7H9 medium adjusted to a pH of 5.56). Early logarithmic phase cultures were exposed to the indicated conditions for a minimum of 5 h prior to removal of a 100-μl aliquot and measurement of RFU and OD at 600 nm.

2.6. Gfp translational fusion assays

The optical density at 600 nm was measured for triplicate cultures grown for 16 h 7H9 broth media, and the ODs were adjusted to 0.5 for each culture using additional 7H9. Cells from the OD-adjusted cultures were separated from the supernatant by centrifugation at 20 000 *g* for 15 min. The resulting separated cells were then resuspended in a volume of fresh broth media that was equivalent to the volume of the supernatant collected for RFU measurement. The percent fluorescence of the cells was calculated by dividing the total fluorescence of the OD-adjusted culture by the fluorescence measured for the separated cells alone, and then multiplied by 100%. The fluorescence intensity of the fractionated cells and supernatant was determined by spectrofluorometry using a BioRad VersaFluor^{TM} fluorometer.

2.7. Macrophage studies

The human monocytic cell line THP-1 was used for all experiments. Determination of the relative fluorescence units of mycobacteria expressing Gfp and grown inside macrophage cultures was performed as described previously [21]. Briefly, THP-1 cells were grown in RPMI 1640 medium (Stem Cell Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Stem Cell Technologies, Inc.) and 0.05 mM 2-mercaptoethanol. Cells were seeded into 6-well tissue culture plates at a density of 5×10^6 cells per well and differentiated overnight at 37 °C with 20 ng/ml phorbol myristic acid (PMA). The following morning, the medium was washed away and replaced with fresh medium, and the cells were allowed to recover for 4 h prior to the experiment. Macrophages were infected with the *M. smegmatis* or *M. bovis* BCG strains of interest at a density of 10 or 50 bacteria per macrophage, respectively. Phagocytosis was allowed to occur for one h followed by 3 washes with prewarmed PBS. After the final wash, the macrophages were incubated at 37 °C for the indicated time periods in RPMI 1640 containing 10% FBS and 0.05 mM 2-mercaptoethanol. At the indicated times of lysis, the monolayers were washed 3 times with PBS, and disrupted using ice cold water followed by vigorous pipetting. The lysed macrophage mixture was assessed for bacterial numbers by dilution of an aliquot of this mixture and plating on 7H10 media containing 10% OADC, 0.5% Tween-80, and 50 μg/ml hygromycin. The lysis mixture was then subjected to centrifugation at 200 *g* for 10 min to remove macrophage nuclei and cell debris. The supernatant was then collected and the bacteria were obtained following a centrifugation at 2500 *g* for 15 min at 4 °C. The bacteria were resuspended in a solubilization buffer (PBS with 0.1% SDS and 0.1% Triton X-100), and subjected to centrifugation through a 15% sucrose cushion at 2500 *g* for 15 min at 4 °C to remove any residual macrophage debris. The final bacterial pellet was resuspended in 2 ml PBS and the RFU and absorbance at 600 nm was determined as described.

3. Results

3.1. PtpA is specific for phosphotyrosine-containing amino acid substrates

In order to characterize the ability of PtpA to dephosphorylate substrates containing phosphoserine, -threonine, and -tyrosine residues, we quantified PtpA activity during incubation with a panel of various artificial substrates. We cloned and overexpressed the *M. tuberculosis ptpA* gene using the *E. coli* expression vector pET22b. The PtpA protein was then purified from inclusion bodies according to a previously described method [2]. The phosphatase substrate pNPP was used to confirm that our purified PtpA is a functional phosphatase. The specific activity of the purified recombinant PtpA, using pNPP as a substrate, was found to be 556 nmoles pNP/min/mg of protein. This activity was inhibited by the phosphatase inhibitor sodium orthovanadate, and was not present in cell extracts from a vector control culture containing pET22b and lacking the *ptpA* gene, thus confirming that dephosphorylation of pNPP was due to phosphatase activity of PtpA (data not shown). This finding also demonstrates that, similar to other low MW PTPs, PtpA can efficiently dephosophorylate pNPP.

Purified PtpA was incubated with a variety of phosphorylated substrates that ranged from an entire protein, to phosphorylated peptides and phosphoamino acids (Fig. 1 and Table 2). The K_m and V_{max} was determined for each of these substrates (Table 2). Similar to other low molecular weight tyrosine phosphatases, substrates containing O-phosphoserine or O-phosphothreonine residues were not dephosphorylated by PtpA at a detectable level. Conversely, O-phosphotyrosine-containing substrates (O-P-Y) were dephosphorylated to varying degrees (Fig. 1). The V_{max} value for the substrate O-P-Y was determined to be 1.688 μmoles/min/mg, and was found to be similar to the value obtained for the *S. coelicolor* PtpA [15]. In each case, phosphatase activity was abolished by addition of 200 μM of the phosphatase inhibitor sodium vanadate (data not shown).

Table 2
Kinetic studies of PtpA using artificial substrates

Substrate[a]	Km [mM]	V_{max}[b]
Free O-P-Y	0.402	1.688
Free O-P-S	undetectable	–
Free O-P-T	undetectable	–
O-P-Y peptide	0.131	0.095
O-P-S peptide	undetectable	–
MBP	0.026	0.03

[a] Abbreviations: O-P-Y, O-phosphotyrosine; O-P-S, O-phosphoserine; O-P-T, O-phosphothreonine; MBP, myelin basic protein.

[b] V_{max} values are reported as μmol phosphate/min/mg protein for all substrates.

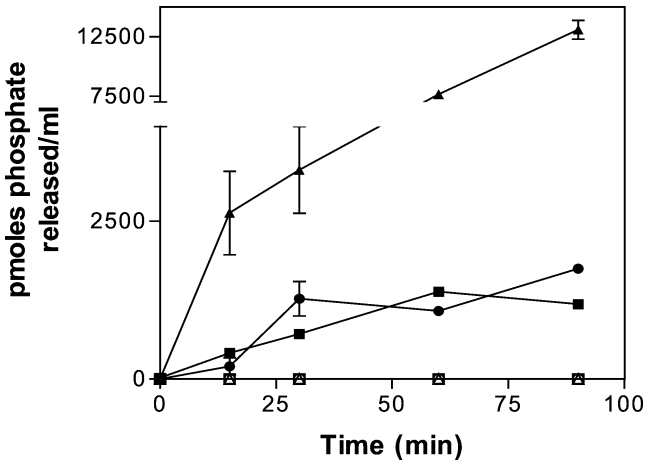

Fig. 1. Ability of *M. tuberculosis* PtpA to dephosphorylate various serine, threonine, and tyrosine-phosphorylate substrates. Symbols: 25 μM tyrosine-phosphorylated MBP (closed squares), 250 μM tyrosine-phosphorylated peptide (closed circles), 150 μM O-phosphotyrosine (closed triangles), 0.63 mM O-phosphoserine (open circles), 0.63 mM O-phosphothreonine (open squares), 250 μM serine-phosphorylated peptide (open triangles). Release of orthophosphate was quantified using either a radioactive assay [MBP] or a malachite green assay, and in each case background free phosphate was subtracted from the results shown. Each data point is the result of triplicate assays and is expressed as the mean ± standard error.

3.2. The M. tuberculosis *PtpA protein can be released from* M. smegmatis *during growth*

It has previously been shown using Western analysis with an anti-PtpA antibody that the PtpA protein is detectable in the supernatant of *M. tuberculosis* cultures [14]. In order to confirm the cellular location of PtpA in *M. tuberculosis* H37Rv, we generated a rabbit polyclonal antibody to PtpA and used this antibody in western immunoblots of *M. tuberculosis* cellular fractions obtained from Colorado State University (membrane, cytosol, and supernatant). The ratio of protein concentrations from the average culture grown to obtain these samples was 1 : 0.55 : 4.86 (culture supernatant : membrane : cytosol). Thus, we loaded these fractions according to this protein ratio in order to compensate for the relative amounts of each fraction found in the average *M. tuberculosis* culture. As seen in Fig. 2A, it is evident that the majority of the PtpA protein is found in the *M. tuberculosis* culture filtrate, and suggests that this protein is either secreted or released from *M. tuberculosis* during growth in bacteriological media.

It is interesting to note that PtpA does not exhibit a general export pathway signal sequence at its N-terminus.

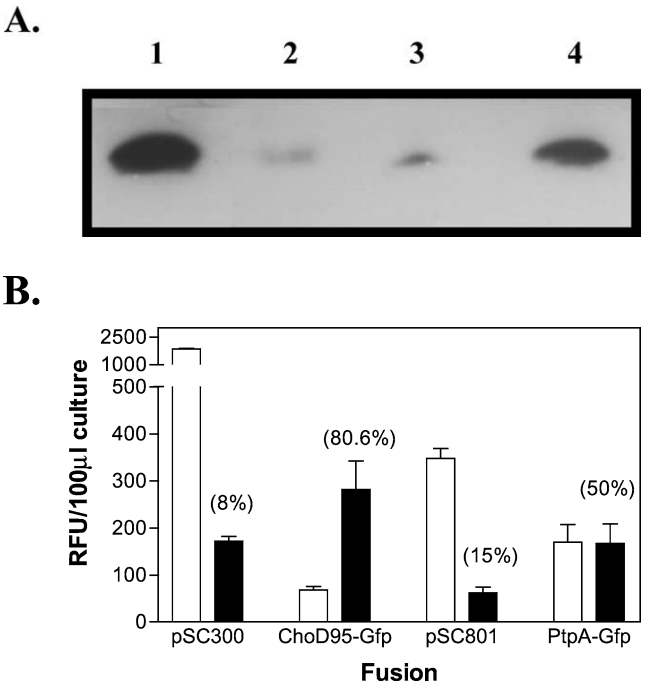

Fig. 2. Localization of PtpA in *M. tuberculosis* and *M. smegmatis* cultures **A.** Western immunoblot of *M. tuberculosis* cell fractions (Colorado State University) using anti-PtpA rabbit polyclonal antibody. Lanes 1, 1 μg purified PtpA; 2, cytosolic fraction (15.4 μg total protein); 3, membrane fraction (23.9 μg total protein); 4, culture filtrate fraction (74.8 μg total protein). **B.** Analysis of PtpA-Gfp fusion protein localization in *M. smegmatis* cultures. Spectrofluorometry of cells (white bars) and supernatant (black bars) from *M. smegmatis* cultures harboring transcriptional or translational *gfp* fusions as indicated. The percent of Gfp located in the supernatant for each culture is shown in parentheses. Data is representative of several experiments, and results are expressed as the averages of three determinations ± standard error.

The extracellular high levels of *M. tuberculosis* PtpA protein may be due to high expression and leakage, such as that used by the *M. tuberculosis* superoxide dismutase and glutamine synthetase. These two proteins also lack a general export pathway signal sequence, but are found in *M. tuberculosis* growth culture [10–12,20]. Interestingly, expression of both of these *M. tuberculosis* proteins in *M. smegmatis* results in their secretion into the growth medium, thus demonstrating that *M. smegmatis* functions as a suitable host for secretion studies of these proteins [11,12]. To determine whether the *M. tuberculosis* PtpA protein can also be released from *M. smegmatis*, we used an alternative approach. We created a Gfp translational fusion to the *ptpA* gene in the fusion vector pSC300, and introduced this construct (named pSC720) into *M. smegmatis*. The resulting plasmid is expected to drive high levels of expression of a PtpA-Gfp fusion protein in *M. smegmatis* via the highly active promoter for the Superoxide dismutase gene [5].

Cultures expressing the PtpA-Gfp fusion protein were grown in liquid media for only 16 h in order to minimize cell death and lysis. As shown in Fig. 2B, cultures expressing the PtpA-Gfp fusion construct contained 50% of the fluorescence in the growth media, while the negative secretion controls pSC300 and pSC801 revealed no more than 8% and 15%, respectively. The negative controls pSC300 and pSC801 both express Gfp without a protein fusion (Table 1), and therefore should not be released or secreted. In contrast, the positive secretion control ChoD95-Gfp (plasmid pSC120; Table 1) exhibited a high level of extracellular Gfp, with as much as 80% of the fluorescence present in the culture supernatant. ChoD95-Gfp is a fusion between the first 95 amino acids of the *M. tuberculosis* protein cholesterol oxidase and Gfp, that we have previously shown to be secreted using this system [5]. From these results, it appears that when expressed in *M. smegmatis*, PtpA is released to the growth media.

3.3. *The* M. tuberculosis ptpA *gene is expressed in* M. bovis *BCG but not* M. smegmatis

Analysis of the chromosomal region surrounding *ptpA* reveals that it is likely to be the third gene in a 4-gene operon (Fig. 3A). In order to clone the promoter controlling *ptpA* expression and analyze the transcriptional activity of *ptpA*, we cloned the promoter region of the *ptpA* gene from the *M. tuberculosis* H37Rv genome into the *Mycobacterium–E. coli* shuttle vector pSC301 upstream of a promoterless green fluorescent protein (*gfp*) gene [5]. The resulting plasmid (named pSC601) was introduced into *M. bovis* BCG as well as *M. smegmatis* in order to monitor transcriptional activity of the *M. tuberculosis ptpA* gene in these species. As seen in Fig. 3B, transcription from the *M. tuberculosis ptpA* promoter is undetectable in *M. smegmatis* cultures grown in bacteriological media over the entire course of the growth curve. However, transcriptional activity of another *M. tuberculosis* promoter, *pknH*, was readily detectable in

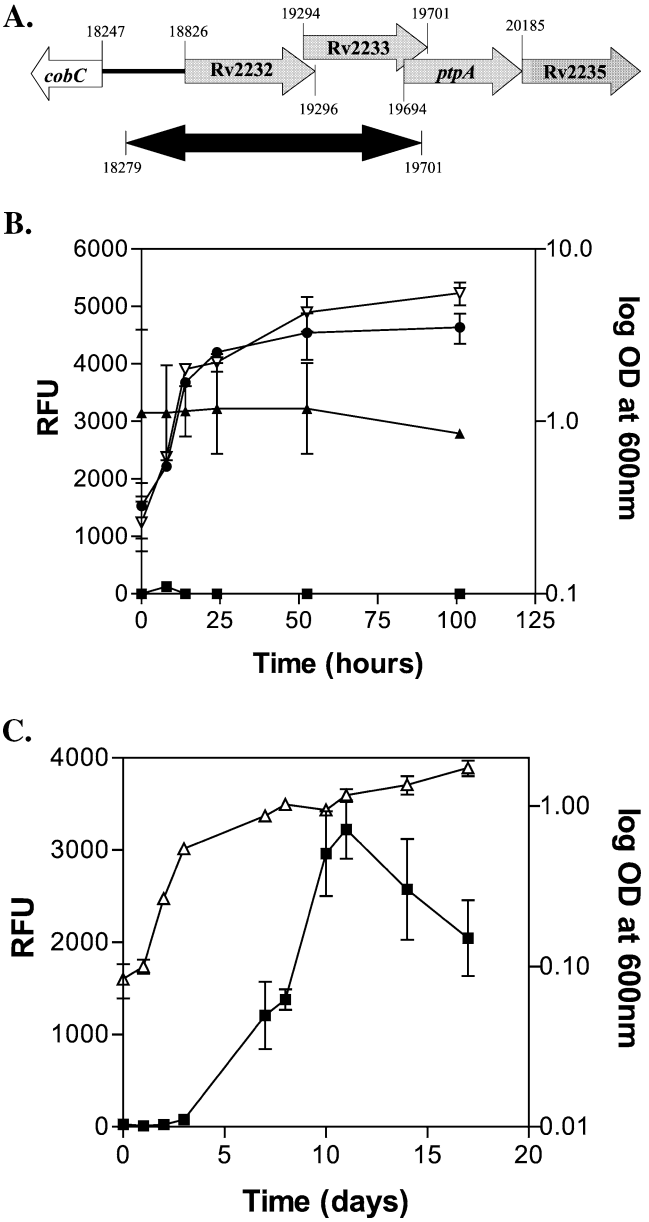

Fig. 3. Analysis of expression of the *ptpA* gene using *ptpA-gfp* promoter fusions. **A.** The genetic region surrounding the gene for the low molecular weight tyrosine kinase PtpA in the *M. tuberculosis* genome. The region cloned for construction of the *ptpA-gfp* promoter fusion is shown beneath as a black arrow. **B.** Transcriptional activity of the *ptpA-gfp* promoter fusion in *M. smegmatis*. **C.** Transcriptional activity of the *ptpA-gfp* promoter fusion in *M. bovis* BCG. Relative fluorescence units (RFU) indicating Gfp levels are plotted on the left y-axis, and the optical density at 600 nm of the culture is plotted on the right y-axis. Symbols: *ptpA-gfp* strain RFU (closed squares), *pknH-gfp* strain RFU (closed triangles), *ptpA-gfp* strain OD at 600 nm (open triangles), *pknH-gfp* strain OD at 600 nm (closed circles). All measurements were carried out on triplicate cultures (± standard error) and were corrected for background fluorescence by subtracting the RFU for control strain pSC301b incubated under identical conditions.

M. smegmatis by this method (plasmid pSC801). Fig. 3C reveals that introduction of the same *M. tuberculosis ptpA-gfp* transcriptional fusion plasmid into BCG yields entirely different results. Using BCG as a host, *ptpA* expression is

easily detectable and becomes maximal upon entry of the organism into stationary phase.

3.4. *Expression of* ptpA *in* M. bovis *BCG in human macrophages*

To determine whether *ptpA* is expressed during a macrophage infection, we infected PMA-differentiated cells from the human monocytic cell line THP-1 with *M. bovis* BCG and *M. smegmatis* harboring the *ptpA-gfp* transcriptional fusion plasmid pSC601. Fig. 4 shows that *ptpA* expression increased continuously over a 72-h period in BCG-infected macrophages, although it was undetectable from *M. smegmatis*-infected macrophages, and not detected from bacteria cultured in RPMI tissue culture medium without macrophages (data not shown). As a positive expression control, THP-1 cultures were also infected with strains carrying the plasmid pSC301, which directs constitutive *gfp* transcription through a superoxide dismutase (SOD) promoter. Control transcriptional activity was detectable at a low but constant level from the SOD promoter in macrophages infected with both *M. bovis* BCG and *M. smegmatis*, thus confirming that Gfp expression is detectable from macrophages infected with both *Mycobacterium* species. These results indicate that *ptpA* is expressed under the conditions found during an intracellular infection.

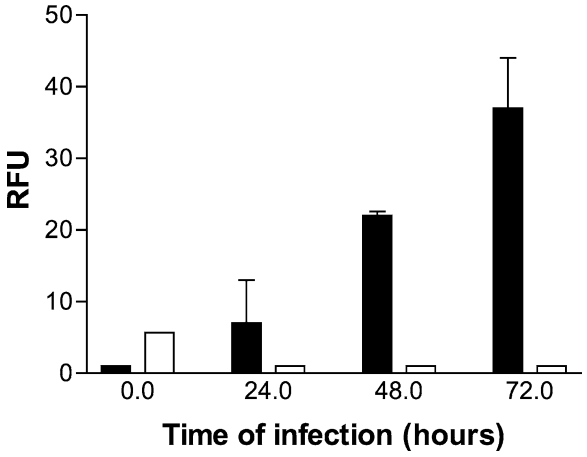

Fig. 4. Transcriptional activity of *ptpA-gfp* promoter fusions during *M. bovis* BCG infection of differentiated human THP-1 cells. Macrophages were infected with bacteria harboring the *ptpA-gfp* fusion (black bars) or a *SOD-gfp* promoter fusion (white bars), lysed at the indicated times, and the RFU was measured on bacteria isolated according to the procedure indicated in Materials and methods. CFUs of *M. bovis* BCG increased from 6.452 ± 0.039 to 6.612 ± 0.045 over the course of the culture period. All measurements were carried out on triplicate cultures ($\pm$ standard error) and were corrected for background fluorescence by subtracting the RFU for control strain pSC301b incubated under identical conditions.

3.5. *Expression of* ptpA *in* M. bovis *BCG and* M. smegmatis *under stress conditions*

To determine the environmental conditions that trigger *ptpA* expression, we exposed cultures of *M. bovis* BCG harboring the *ptpA-gfp* transcriptional fusion plasmid pSC601 to a panel of stress conditions. The stress conditions and times of exposure chosen were based upon studies that detected measurable differences in the levels of expression of various *M. tuberculosis* sigma factors [16,17]. These studies used periods of exposure that ranged from 2–4 h; an additional 2 h were added in our assays to allow for changes in expression of *ptpA*, as well as folding and oxidation of the Gfp reporter, which requires approximately an hour to achieve full fluorescence [19]. We found that most stress conditions had either no effect or a slightly negative effect on *ptpA* promoter activity in BCG as compared to control cultures (Fig. 5). However, it was found that *ptpA* promoter activity consistently increased by a factor of 2 to 5-fold in cultures entering into stationary phase (optical density of 1) as compared to BCG cultures in early exponential phase (optical density of 0.1–0.3). In contrast, expression from the *ptpA* promoter in *M. smegmatis* was undetectable in all conditions tested (data not shown).

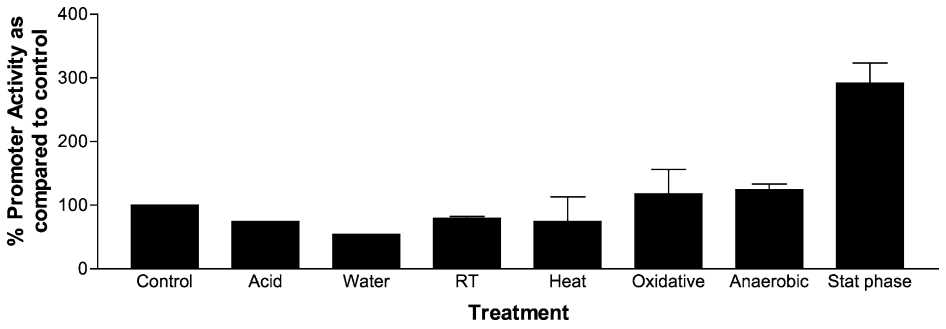

Fig. 5. Effect of various environmental stresses on *ptpA-gfp* promoter activity in *M. bovis* BCG. The values were calculated as a ratio between the RFU values and the OD 600 nm of the culture in order to evaluate promoter activity in relation to the number of bacterial cells present following a 6-h exposure at 37 °C with shaking (unless otherwise noted) to the indicated conditions. These values were then compared to a control culture incubated at 37 °C, which was assigned a value of 100%. Treatments include: acid 97H9 media at pH 5.56, water (sterile distilled water), RT (incubation at room temperature), heat (incubation at 42 °C), oxidative (incubation in 10 mM hydrogen peroxide), Anaerobic (stationary microaerophilic conditions for 10 days), and Stat phase (approximately 10–12 day cultures with an OD at 600 nm of 1–2). The data shown is the mean of three separate experiments ($\pm$ standard error), each done in triplicate. All measurements were corrected for background fluorescence by subtracting the RFU for control strain pSC301b incubated under identical conditions.

4. Discussion

The *M. tuberculosis* genome is predicted to encode two tyrosine phosphatases, named PtpA and PtpB. The discovery that there are no predicted tyrosine kinases in the *M. tuberculosis* genome sequence database raises the question as to the function of both these phosphatases in the life cycle of *M. tuberculosis*. Koul et al. made the first step in answering this question through the isolation, purification, and demonstration that PtpA and PtpB are both functional phosphatases capable of dephosphorylating phosphotyrosine residues, but not phosphoserine or phosphothreonine residues, in the artificial substrate MBP [14]. In addition, they found that these proteins were present in both the whole cell lysates and culture filtrates of *M. tuberculosis*, suggesting that they may be secreted.

Here we have further characterized the *M. tuberculosis* tyrosine phosphatase PtpA with respect to its substrate specificity, kinetics, and gene regulation. In this study, we show that PtpA dephosphorylates only phosphotyrosine residues in a variety of substrates. In addition, we use a Gfp translational fusion to provide direct evidence that the leaderless PtpA protein can be released from mycobacterial cells, including *M. smegmatis*. Finally, we demonstrate that the *M. tuberculosis ptpA* gene is expressed only under precise conditions: although the *M. tuberculosis ptpA* promoter is not expressed in the slow-growing saprophyte *M. smegmatis*, expression in *M. bovis* BCG is upregulated upon entry of this organism into stationary phase. Most importantly, *ptpA* expression was found to occur during growth in human monocytes, the primary environment of *M. tuberculosis*.

Recent Southern hybridization studies indicate that there is a copy of the *ptpA* gene in both the BCG and *M. smegmatis* genomes [14]. Examination of the TIGR database revealed that both *M. bovis* and *M. smegmatis* have *ptpA* homologues that exhibit 98% and 71% identity at the deduced amino acid level to the *M. tuberculosis* PtpA, respectively. Similarly, the two genes located upstream of *ptpA* in an apparent operon in the *M. tuberculosis* genome (Rv2232 and Rv2233, see Fig. 3A), also have homologues located immediately upstream of *ptpA* in the genomes of both *M. bovis* and *M. smegmatis*. In the *M. bovis* genome sequence, Rv2232 and Rv2233 exhibit 91% and 100% identity to the *M. tuberculosis* genes, respectively; in *M. smegmatis*, these genes show 53% and 68% identity. Thus, this locus appears to be present in all three organisms. Therefore, one would expect that the proteins involved in regulation of the endogenous *ptpA* homologues found in *M. bovis* BCG and *M. smegmatis* would also regulate the expression of the *M. tuberculosis ptpA-gfp* transgene. In addition, it is possible that the *M. tuberculosis* genes Rv2232 and Rv2233, which were included in our *ptpA-gfp* promoter fusion construct, may be involved in regulation of the apparent *ptpA* operon. However, from the experiments presented here, it is clear that they are not sufficient for expression of the *M. tuberculosis ptpA-gfp* transgene in *M. smegmatis*.

Our finding that expression of the *M. tuberculosis ptpA-gfp* transcriptional fusion was undetectable in *M. smegmatis* suggests that there are promoter elements present in the *M. tuberculosis ptpA* promoter region that are not recognized by *M. smegmatis* under the conditions tested here. Given that the *M. tuberculosis* genome predicts the presence of 13 different sigma factors in this organism [4], it is possible that a sigma factor present in *M. tuberculosis* and *M. bovis*, and lacking in *M. smegmatis*, is responsible for *ptpA* expression. Interestingly, *sigF* is one such sigma factor that is found only in the slow-growing species of mycobacteria, such as *M. tuberculosis* and *M. bovis* BCG, although it is not present in the fast growing mycobacterial species, such as *M. smegmatis*. Similar to the *ptpA* gene, *sigF* gene expression has been shown to be upregulated during entry of BCG cultures into stationary phase [7,16], and thus is an example of a sigma factor that may be required for *ptpA* expression. This is the first demonstration of differential expression of an eukaryotic-like phosphatase in different Mycobacterial species.

It is interesting to note that the *M. tuberculosis* PtpA protein can be released to the growth media from both *M. tuberculosis* and *M. smegmatis*. PtpA does not exhibit a general export pathway signal sequence at its N-terminus, it does lack predicted transmembrane domains and may be released to the growth media in a similar mechanism to that used by the leaderless *M. tuberculosis* proteins, superoxide dismutase and glutamine synthetase [20]. Interestingly, although the glutamine synthetase gene is present in a variety of pathogenic and nonpathogenic mycobacteria, it was only thought to be secreted by pathogenic mycobacteria, where it is thought to play a role in virulence [10,11]. It is relevant to the data presented here to note that when the *M. tuberculosis* glutamine synthetase gene is cloned and expressed in *M. smegmatis*, it is abundantly present in the growth medium, thus demonstrating that *M. smegmatis* functions as a suitable host for protein localization studies of these proteins [11,20]. In a manner similar to glutamine synthetase, cloning and expression of the *M. tuberculosis* superoxide dismutase gene in *M. smegmatis* results in the presence in the growth media of the recombinant *M. tuberculosis* enzyme 26-fold more than the endogenous *M. smegmatis* enzyme [12]. Tullius and Horowitz propose that high extracellular levels of *M. tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than a protein-specific export mechanism [20]. PtpA may use a similar mechanism as these proteins for its release from both *M. tuberculosis* and *M. smegmatis*.

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