Research article

Analysis of stress- and host cell-induced expression of the *Mycobacterium tuberculosis* inorganic pyrophosphatase

James A Triccas*[1,2] and Brigitte Gicquel[1]

Address: [1]Unité de Génétique Mycobactérienne, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France and [2]Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag No 6, Newtown, NSW, 2042, Australia

E-mail: James A Triccas* - J.Triccas@centenary.usyd.edu.au; Brigitte Gicquel - bgicquel@pasteur.fr
*Corresponding author

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Abstract

Background: The gene encoding the inorganic pyrophosphatase (PPase) of the intracellular pathogen *Legionella pneumophila* is induced during intracellular infection, but is constitutively expressed in *Eschericia coli*. The causative agent of tuberculosis, *Mycobacterium tuberculosis,* contains a well conserved copy of PPase. We sought to determine if expression of the *M. tuberculosis* PPase is regulated by the intracellular environment.

Results: A strain of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) was constructed in which the *Aequoria victoria* green fluorescent protein (GFP) is controlled by the promoter of the *M. tuberculosis ppa* gene. After prolonged exposure of the recombinant BCG strain within murine bone-marrow-derived macrophages, there was no observed increased activity of the *ppa* promoter. Furthermore, there was no change in promoter activity after exposure to various stress stimuli such as reduced pH, osmotic shock, nutrient limitation or oxidative stress.

Conclusions: These results suggest that macrophage induction of *ppa* is not a general phenomenon among intracellular pathogens.

Background

Pathogenic bacteria such as *Mycobacterium tuberculosis* respond to conditions within the host by coordinate regulation of gene expression. The resulting production of specific gene products permits persistence and multiplication resulting in the manifestation of disease. Studies of facultative intracellular pathogens, who typically survive and replicate within phagocytic cells of the host anti-microbial defence system, indicate that the major genes expressed *in vivo* are in response to conditions encountered within the host cell [1]. These include genes required for the acquisition of metals, DNA repair, thermotolerance, osmotic tolerance and acid tolerance. Genes encoding proteins of an identical or similar function are often expressed *in vivo* by numerous bacteria, suggesting that responses to certain conditions are conserved among pathogens [1, 2].

The microbial inorganic pyrophosphatase (PPase) plays an important role in macromolecular biosynthesis and is essential for the viability of *Escherichia coli* and yeast [3, 4]. The protein appears to be ubiquitous and is highly conserved amongst differing species. In *Legionella pneumophila*, the *ppa* gene is induced during intracellular infection of U937 macrophage-like cells [5] and represents the first example of a regulated *ppa* gene in response to environmental stimuli. In order to determine if induction of *ppa* is a general phenomenon among

intracelluar pathogens, we have analysed the activity of the *M. tuberculosis ppa* promoter upon exposure to the intracellular environment.

Results

Identification of a M. tuberculosis inorganic pyrophosphatase (PPase)

Analysis of the M. tuberculosis genome revealed an open reading frame (Rv3628) that was highly similar to L. pneumophila PPase [5]. The open reading frame consisted of 486 bp and was predicted to encode a protein that was 44% identical to L. pneumophila PPase [5] and 38% identical to E. coli PPase [6] (figure 1). Many of the residues that have been shown to be crucial for the structural and catalytic activity of the enzyme in E. coli are conserved in both M. tuberculosis and L. pneumophila [5, [7–11]]. These data suggest that the product of Rv3628 is a PPase.

The *ppa* promoter region was amplified from *M. tuberculosis* genomic DNA and cloned into the vector pJFX2 [12]. The resulting vector, pJIN6, contains the *ppa* promoter controlling a strongly fluorescent version of the *Aequoria victoria* green fluorescent protein (GFP) [13]. Transformation of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) with pJIN6 resulted in fluorescent bacterial colonies as assessed by fluorescence microscopy (data not shown).

Activity of the M. tuberculosis ppa promoter within murine macrophages

In order to determine if the ppa promoter displayed enhanced activity within host cells, murine bone-marrow-derived macrophages were infected with BCG/pJIN6, and the level of fluorescence of recovered bacteria compared by flow cytometry after 0 or 6 days of infection. Bacteria displayed equivalent levels of fluorescence at both time points, suggesting that there was no increased activity of the ppa promoter after exposure to the intracellular environment (figure 2a). Analysis of GFP levels after 3 days of infection also showed no change in ppa promoter activity (data not shown). This was in contrast to BCG harbouring pJIN10, in which GFP is controlled by the M. tuberculosis sigE promoter, known to be active within macrophages [14]. BCG containing pJIN10 displayed approximately 2 times the level of fluorescence after 6 days of infection compared to the initial inoculum (figure 2b). Due to the relative stability of the GFP used in this study [15] it is unlikely that transient induction of the ppa promoter was not detected due to rapid breakdown of the reporter protein.

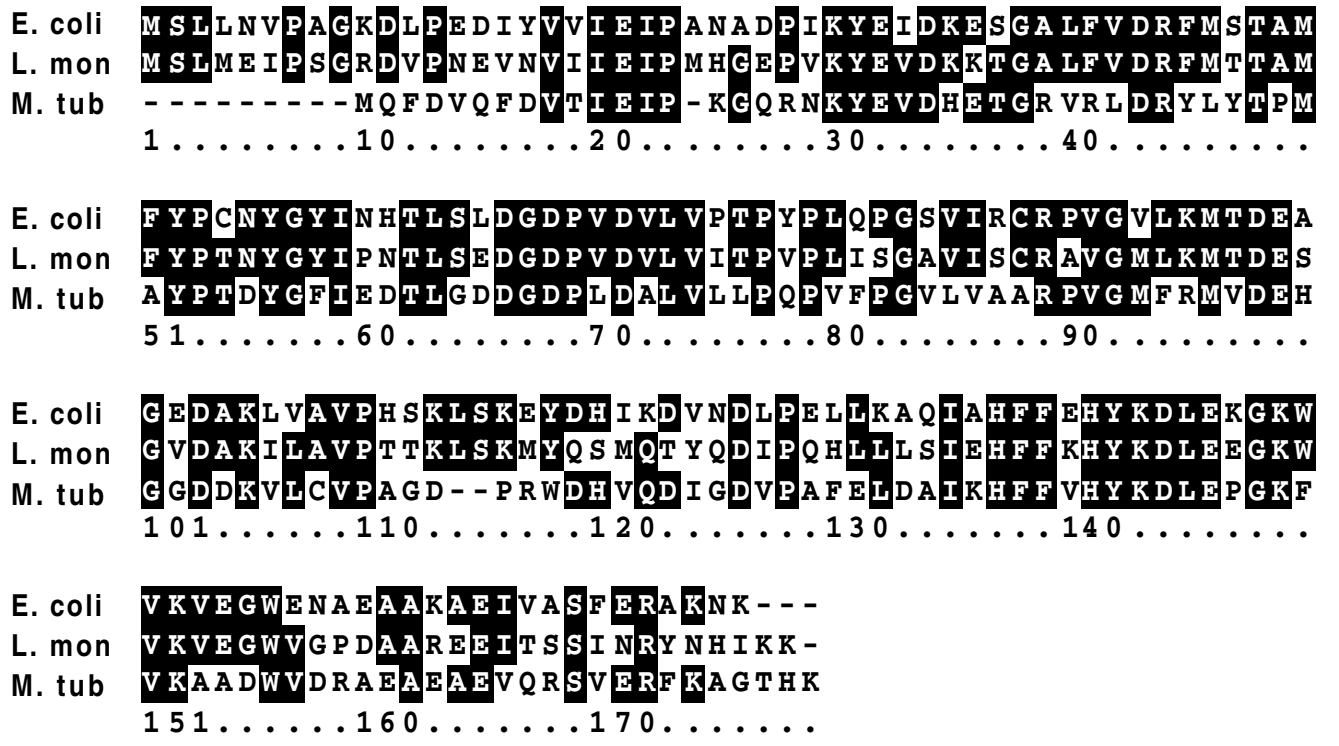

Figure 1
Comparison of the PPase proteins of *E. coli* (E. coli), *L. pneumophila* (L. mon) and *M. tuberculosis* (M. tub). Identical amino acids shared between any 2 proteins are shaded.

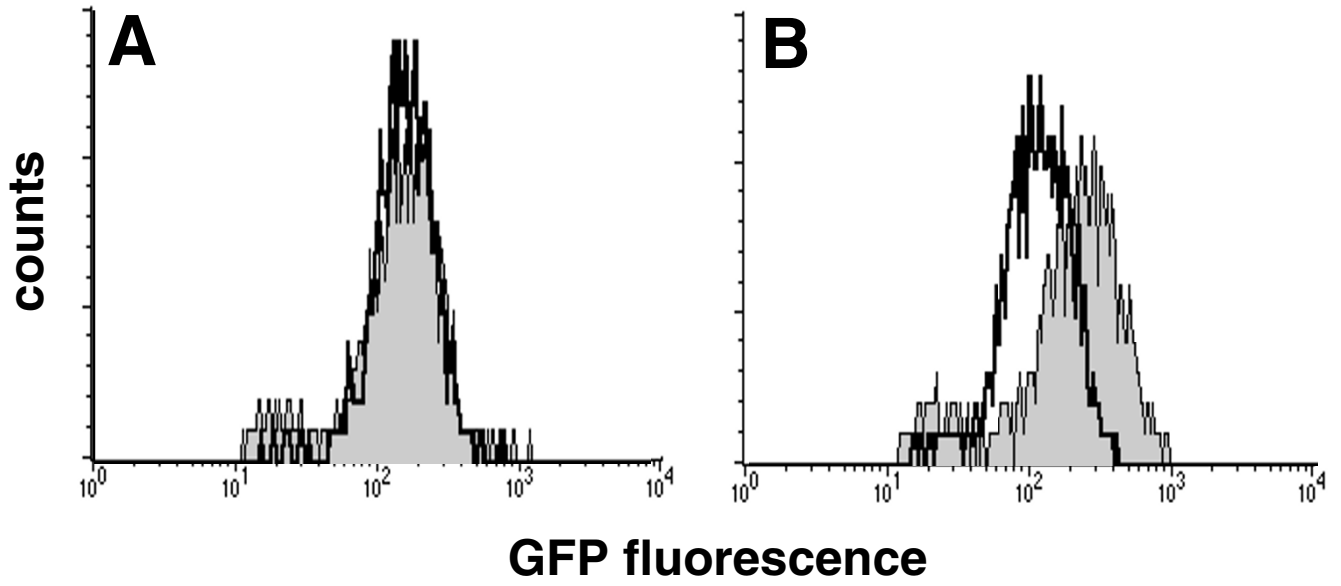

Figure 2
Activity of the *M. tuberculosis ppa* promoter in macrophages. Murine bone marrow-derived macrophages were infected with A) BCG/pJIN6 (*gfp* controlled by *ppa* promoter) or B) BCG/pJIN10 (*gfp* controlled by *sigE* promoter). After 6 days, macrophages were lysed and the fluorescence of recovered bacteria assessed by flow cytometry (grey histogram) and compared to the fluorescence of the initial inoculum (white histogram).

Effect of stress stimuli on M. tuberculosis ppa promoter activity

While the *ppa* promoter of *L. pneumophila* is induced within the intracellular environment, it is not induced after exposure to stress stimuli such as acid shock, osmotic shock or oxidative stress [5]. BCG/pJIN6 (*ppa* promoter) displayed no change in fluorescence after exposure to H_2O_2 (oxidative stress), high NaCl concentrations (osmotic shock), deprivation of iron by 2,2'-dipridyl treatment (nutrient limitation) or a reduction in pH (acid shock) (figure 3). The conditions used did permit analysis of *in vitro* stress as the *sigE* promoter of BCG/pJIN10 displayed increased activity due to acid shock, nutrient limitation and osmotic shock (figure 3). These data suggest that expression of the *M. tuberculosis ppa* is not regulated by the stress stimuli tested here.

Discussion

Bacterial pathogens regulate gene expression in response to environmental stimuli encountered within the host and often contain related genes whose expression are influenced by the macrophage environment [1,2]. In this report, we have investigated the intracellular expression of the *M. tuberculosis ppa* gene, whose counterpart in *L. pneumophila* is induced within the macrophage. While *M. tuberculosis* contains a well conserved homologue of the *L. pneumophila* PPase, we observed no increased activity of the *M. tuberculosis ppa* promoter within macrophages or by exposure to stress stimuli *in vitro*. This implies that the *M. tuberculosis ppa* promoter is not responsive to any specific intracellular triggers that influence the *L. pneumophila* counterpart. This may in part be due to differences in the intracellular microenvironment encountered by these different pathogens [16]. Alternatively, induction of the *L. pneumophila* promoter may occur independently of any intracellular signal and could be a by-product of an increased requirement for PPase *in vivo*, possibly a consequence of the greater growth rate of *L. pneumophila* within macrophages compared to *in vitro*-grown bacteria [17]. The results of this study suggest that the *M. tuberculosis ppa* is more characteristic of the constitutively expressed *ppa* of *E. coli* [18] rather than the *in vivo*-induced homologue of *L. pneumophila*, implying that induction of *ppa* does not appear to be an event common to intracellular pathogens.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli DH5α was grown routinely on liquid or solid Luria-Bertani medium. *M. tuberculosis* 103 (isolated directly from an tuberculosis patient; laboratory collection) and *M. bovis* BCG Pasteur were grown in liquid Middlebrook 7H9 medium (Difco laboratories, Detroit, USA) supplemented with ADC enrichment (Difco) or solid Middlebrook 7H10 medium (Difco) supplemented

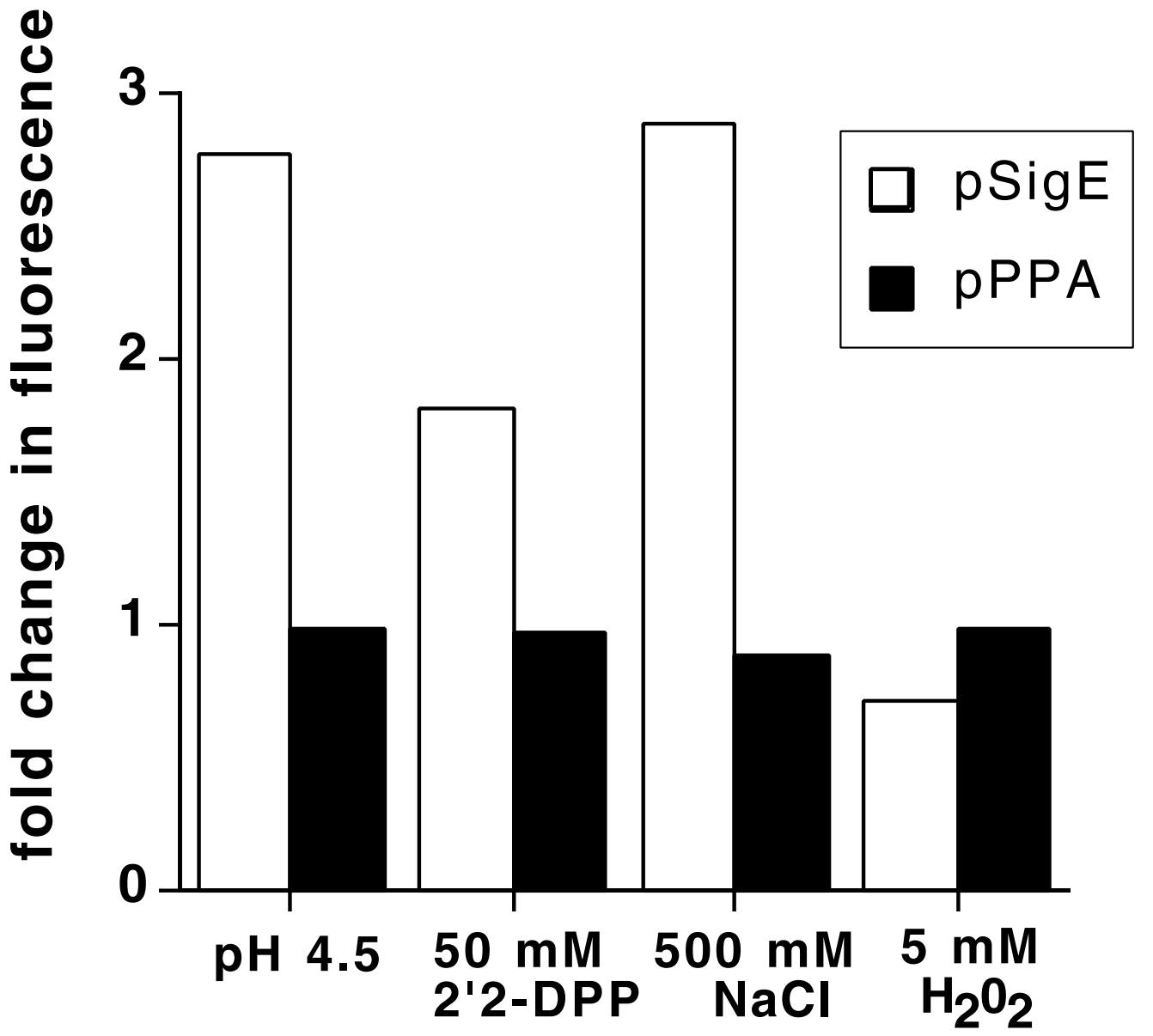

Figure 3
Effect of stress stimuli on *M. tuberculosis ppa* promoter activity. BCG harbouring pJIN6 (*gfp* controlled by *ppa* promoter; hatched bars) or pJIN10 (*gfp* controlled by *sigE* promoter; white bars) were grown for 24 hours in 7H9 medium of pH 4.5 or containing either 50 mM 2,2'-dipyridyl, 500 mM NaCl or 5 mM H_2O_2. The fold change in fluorescence represents the fluorescence value of bacteria after 24 hour exposure to stress conditions divided by the fluorescence level of the same bacteria grown in the absence of stress stimuli.

with OADC enrichment (Difco). When required, the antibiotic kanamycin was added at a concentration of 25 μg ml^{-1} for both *E. coli* and mycobacteria. For the analysis of stress induced responses, 7H9 medium was supplemented with either 500 mM NaCl (Sigma Chemical Co. St Louis, USA), 5 mM H_2O_2 (Sigma) or 50 mM 2,2'-dipyridyl (Aldrich Chemical Co. Milwauke, USA).

Vector construction

The *ppa* gene was located on the *M. tuberculosis* genome ([19] www.sanger.ac.uk/Projects/M_tuberculosis/) and 500 bp upstream of the initiation codon amplified from *M. tuberculosis* genomic DNA using the primers PPA.for (5'-GTCGGAGTACTAAACGCCGAAGCGT) and PPA.rev (5'-GAATTGGATCCGTCGGCTCCTTCAG). The product

was cloned into the *E. coli*/mycobacterial shuttle vector pJFX2 [12] to yield plasmid pJIN6. The *sigE* promoter was amplified from the *M. tuberculosis* genome using primers SIGE.for (CCGCAAGTACTCGGCGACG-TAATCT) and SIGE.rev (CGAGGGGATCCATGGGAAT-TACCGT) and cloned into pJFX2 to yield plasmid pJIN10. Preparation of competent cells and electroporation of mycobacteria was carried out as described previously [1920].

Macrophage preparation and infection

Murine bone-marrow-derived macrophages were prepared as described previously [12]. Macrophage monolayers were infected with bacteria at a multiplicity of infection (MOI) of 1:1. After 4 hours of infection, extracellular bacteria were removed by washing 4 times with PBS, and incubation continued at 37°C in 5% CO_2. After 6 days of infection, BCG infected-macrophages were washed 3 times in PBS, centrifuged and lysed in water plus 0.1% Tween 80. Recovered bacteria were analysed directly by flow cytometry. For the estimation of initial levels of fluorescence, bacteria were added to macrophage monolayers and immediately recovered and treated as above for flow cytometric analysis.

Flow cytometric analysis of fluorescent bacteria

Bacteria were analysed with a FACScan (Becton-Dickinson Immunocytometry Systems, Franklin Lake, USA). After growth in 7H9 media or macrophages, BCG cells were resuspended in 1 ml of PBS (approximately 1 x 10^6 bacteria), and a total of 2000 bacteria were analysed as a function of side scatter and GFP fluorescence. Quantitation of fluorescence levels was determined by the use of the Lysis II program (Becton-Dickinson).

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