A FHA domain-containing ABC transporter interacts with a serine-threonine protein kinase and is required for growth of *Mycobacterium tuberculosis* in mice

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ABSTRACT

FHA (forkhead-associated) domains are modular phosphopeptide recognition motifs with a striking preference for phosphothreonine-containing epitopes. FHA domains have been best characterised in eukaryotic signalling pathways but have been identified in six proteins in *Mycobacterium tuberculosis*, the causative organism of tuberculosis. One of these, coded by gene Rv1747, is an ABC transporter and the only one to contain two such modules. A deletion mutant of Rv1747 is attenuated in a mouse intravenous injection model of tuberculosis where the bacterial load of the mutant is ten-fold lower than that of the wild type in both lungs and spleen. In addition, growth of the mutant in mouse bone marrow-derived macrophages and dendritic cells is significantly impaired. In contrast, growth of this mutant *in vitro* was indistinguishable from that of the wild type. The mutant phenotype was lost when the mutation was complemented by the wild type allele, confirming that it was due to mutation of Rv1747. Using yeast two-hybrid analysis, we have shown that the Rv1747 protein interacts with the serine-threonine protein kinase PknF. This interaction appears to be phospho-dependent since it is abrogated in a kinase-dead mutant and by mutations in the presumed activation loop of PknF and in the first FHA domain of Rv1747. These results demonstrate that the protein coded by Rv1747 is required for normal virulent infection by *M. tuberculosis* in mice, and, since it interacts with a serine-threonine protein kinase in a kinase-dependent manner, indicate that it forms part of an important phospho-dependent signalling pathway.

INTRODUCTION

Tuberculosis (TB) was declared a global emergency by the World Health Organisation in 1993. Despite this, estimates suggested that 8.2 million new cases were still occurring world-wide in the year 2000, with 2-3 million people dying from the disease, and a third of the world's population are infected (39). There has been enormous progress in combating the disease including the publication of the complete genome of its causative agent, the bacterium *Mycobacterium tuberculosis* (7). However, the situation has deteriorated in a number of regions due to synergy between TB and AIDS (21). The only vaccine for TB, *Mycobacterium bovis* BCG, developed in the 1920s, does not provide adequate levels of protection in Africa, India and some parts of the USA (12, 13). Moreover, the rise in multi-drug resistant tuberculosis makes the identification of new drug targets and vaccines imperative (39).

The identification of virulence determinants is one approach to the development of novel disease intervention strategies that could be used to develop new therapeutic agents or attenuated vaccine strains. One likely class of proteins for the search for such virulence determinants is in regulatory proteins (eg. 27, 35, 38, 40). In particular, protein kinases have, perhaps surprisingly, emerged as major targets for pharmaceutical intervention over the last few years. *Mycobacterium tuberculosis* faces complex environmental and metabolic choices as it moves from the environment to infection of macrophages, dormancy and growth in the host. It is therefore expected to have an extensive repertoire of regulatory proteins with over 100 predicted in the genome (7). Phosphorylation on serine, threonine or tyrosine is the most common post-translational modification utilised in cellular signalling processes in eukaryotes where the interplay of protein kinases and phosphatases provides for rapid reversibility. In prokaryotes, phosphorylation-dependent signalling is manifested in the use of so-called two-component systems where phosphate is transferred from a histidine on a sensor kinase to a glutamate or aspartate on a response regulator. *M. tuberculosis* has only eleven two-component networks

compared with *Bacillus subtilis* and *Escherichia coli* which each contain over thirty. Nevertheless, it does contain eleven putative Ser-Thr protein kinases. Interestingly, four of these genes are linked in the genome to genes coding for proteins that have the forkhead-associated (FHA) domain that fulfils the important role of modular phosphothreonine binding motif (48). Indeed, the presence of a FHA domain is now generally taken to indicate that the protein is likely to interact with a phosphorylated protein partner (10) although such interactions have been demonstrated in only a relatively limited cohort of biological systems.

The *M. tuberculosis* gene Rv1747 encodes a 92 kDa protein containing an ABC transporter domain and two FHA domains. Although its function is unknown it has been suggested that it functions as an exporter (5). It is one of two genes in a putative operon (7) where the first encodes a eukaryotic-like serine/threonine protein kinase, Rv1746 (*pknF*) (See Fig. 1). Taken together, these observations suggest that Rv1747 is not only likely to function as an exporter with potential importance in virulence, but that it may be part of a complex signalling system involving a eukaryotic-like STK pathway. Here we have investigated the role of Rv1747 in microbial pathogenesis and further examined the potential for regulatory interactions between the FHA domains of Rv1747 and PknF.

MATERIALS AND METHODS

Strains, growth conditions, and reagents. The wild type strain used was *Mycobacterium tuberculosis* H37Rv Paris (kindly provided by S. Cole). All strains were grown at 37°C in Dubos broth supplemented with 0.05% (v/v) Tween 80, 0.2% (v/v) glycerol and 4% (v/v) Dubos medium albumin (Becton Dickinson), either 100 ml in a Bellco roll-in incubator (2 r.p.m.) or 10 ml in static universals. Mutant strains of *M. tuberculosis* were plated onto Middlebrook 7H11 agar supplemented with 4% (v/v) Dubos medium albumin, and, as noted, with hygromycin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), X-Gal (50 μg ml^{-1}), or sucrose (2% w/v). The PCR products

were cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Adult (8 week) female Balb/C mice were obtained from the specific pathogen free animal unit at NIMR.

Isolation of nucleic acids from mycobacteria. Extraction of genomic DNA used a modification of the method of Belisle *et al.* (3), adapted from Whipple *et al.* (47). For RNA extraction, rolling cultures of *M. tuberculosis* were harvested by centrifugation, and RNA extracted by mechanical lysis using the Fast RNA Pro Blue kit (Q-BIOgene-ALEXIS). The RNA had DNA removed by DNase digestion.

Sequence analysis. Sequencing was carried out using the BigDye™ Terminator Cycle Sequencing kit on an ABI® Prism 377 DNA analyzer, both from Applied Biosystems. The results were compared with the published *M. tuberculosis* sequence (7) using the BLAST facility on the website http://genolist.pasteur.fr/TubercuList/. Sequencing to check the constructs for the Rv1747 knockout was carried out using primers 5'-TTTGCACCTCGCGTTCT-3' and 5'-ACCAACACCACCATCTG-3' for the *Bam*HI insert, and primers 5'-GTGATGCTGTCGAGCTT-3' and 5'-TTGAGCACCTTGTGTGT-3' for the *Not*I insert. The complement was also sequenced entirely.

Microarrays. Microarrays of the *M. tuberculosis* genome were obtained from Dr. J. Hinds and Prof. P. Butcher, Bacterial Microarray Group, St. Georges Hospital Medical School, London. The arrays were scanned using an Axon 4000A together with GenePix software.

Construction of Rv1747 null mutant. Plasmid constructs were made by inserting 2 kb regions of amplified H37Rv Paris DNA flanking each side of the knockout gene into the vector p2NIL (36) (See Fig. 1). The primers for the flanking regions were: 5'-GGATCC-GTAACATCGCGCACGAATTG-3' and 5'-GGATCC-CAGCCGTTGCTTCTGCGAAT-3' (*Bam*HI insert), and 5'-GCGGCCGC-GTGACGTGCGACTGATTCTG-3' and 5'-GCGGCCGC-TTGCGAGCAGGTGACACCTT-3' (*Not*I insert), both amplified with the enzyme Elongase (Promega). The *hyg* cassette was cut from pUC-HY vector (26, 41) using *Kpn*I and inserted

between the 5' and 3' flanking regions. Finally, the *sacB* gene and the *lacZ* gene were inserted from the plasmid pGOAL17 (36) using *Pac*I. The plasmid constructs were pretreated with 100 mJ UV light cm^{-2} (19) and used to electroporate *M. tuberculosis* (34). This was plated onto 7H11 plates supplemented with X-Gal, kanamycin and hygromycin and left to grow for 3 weeks. Single crossover events, seen as blue colonies, were streaked onto 7H11 supplemented with hygromycin and grown for a further 3 weeks before serially diluting and streaking onto 7H11 supplemented with sucrose and hygromycin. The resulting colonies were patch tested on 7H11 plates containing hygromycin, with X-Gal or kanamycin, to check for white, kanamycin-sensitive colonies, indicating a double crossover event.

Check of Rv1747 null mutant. The Rv1747 null mutant was checked by PCR, Southern blot and genomic DNA microarray to ensure the gene had been knocked out. For PCR, the following primer pairs were used: 5'-GCGTTGACCTGCGTGTT-3' from *M. tuberculosis* 5' of the *Bam*HI insert and 5'-CGTTCGAACGCGGCTAC-3' from the p2NIL multiple cloning site; 5'-GGTCAGCGAACCAATCA-3' from the p2NIL multiple cloning site and 5'-ACAGGACACCTGCTATG-3' from *M. tuberculosis* 3' of the *Not*I insert. The fragments were amplified with an annealing temperature of 55ºC using Hotstar Taq. For Southern blots genomic DNA was digested with the restriction enzyme *Asc*I, and blots were probed with DNA from outside the knock-out construct, one from *pknE* (derived by PCR using the primers 5'-GGATCC-TTCAACGAACCCATCTGTCC-3' and 5'-TCGAATAGTTCTTGCTG-3') and the other from Rv1750c (*fad1*) (derived using primers 5-CATAGCAGGTGTCCTGTT-3' and 5'-GGCTGAATTGGTGGCTG-3'). An *Asc*I site is lost when the Rv1747 gene was deleted so that an approx. 7kb fragment that hybridized with the *pknE* probe and an approx. 10kb fragment that hybridized with the *fad1* probe were replaced by an approx. 15kb fragment (data not shown). Genomic DNA was isolated and used to probe *M. tuberculosis* DNA microarrays as described previously (14).

Complementation of the Rv1747 mutation. The genes Rv1747, Rv1746 and 609 bp of Rv1745 (See Fig. 1) were amplified by PCR using the primers 5'-AAGCTT-GCACGCCTTGAGGCGAATCT-3' (5' *Hind*III restriction site) and 5'-GAATTC-GTAACATCGCGCACGAATTG-3' (5' *Eco*RI restriction site) and the Expand Long Template PCR system. The PCR product was sequenced to check there were no base changes. After restriction enzyme digestion, the PCR product was then cloned into the integrating vector pMV306 (43) and used to electroporate the *M. tuberculosis* Rv1747 knockout. This was plated onto 7H11 plates supplemented with kanamycin and hygromycin and left to grow for 3 weeks. The resulting colonies were harvested, the DNA extracted, and checked for complementation by PCR and microarrays.

In vitro **growth determination.** Wild type H37Rv Paris and the Rv1747 knockout mutant and its complement strain were each initially removed from storage at -80°C, grown in 10 ml Dubos medium as static cultures, and then at 37ºC in a rolling incubator as described above. Once they each reached an OD_{600} of approximately 0.5, 1 ml of each culture was subcultured to a fresh bottle of 100 ml Dubos medium and incubation continued in the rolling incubator. Aliquots of 1 ml of each culture were removed for optical density readings at 600 nm at 24 h intervals.

Growth of bacteria in mice. Stock cultures of *M. tuberculosis* H37Rv Paris, Rv1747 knockout and Rv1747 complement were grown in 10 ml Dubos medium standing cultures at 37ºC for 14 days. The cultures were diluted in phosphate-buffered saline to an OD_{600} of 0.02. Infection was induced by injecting 0.2 ml viable *M. tuberculosis* (5 x 10^5 CFUs) into a lateral tail vein of 8 week old adult female Balb/C mice. The infection was monitored by removing the lungs and spleens of infected mice at various intervals, homogenising the tissues and plating 10-fold dilutions to determine numbers of colony forming units of *M. tuberculosis* (45). The growth curves were compared by graph and statistical analysis. The results for each time point are the means of CFU determinations performed on organs from three mice, and the error bars show the standard deviations. The asterisk indicates that the result is statistically significantly different

from that of the wild type by the two-tailed Student's t test for groups of unequal variance ($p <$ 0.01$), as well as by single-factor analysis of variance ($p < 0.01$).

Growth of bacteria in mouse cell culture. Bone marrow cells were extracted from the hind legs of 8 week old adult female Balb/C mice as described previously (44). The cells were resuspended in IMDM+5% FCS, 2mM L-glutamine, 80uM β-mercaptoethanol to which was added, for macrophages, 10% by volume of supernatant from L929 cells that produce M-CSF, or for dendritic cells, 10% supernatant from X-63 cells transfected with GM-CSF cDNA. The cells were plated in 6 well plates and incubated at 37ºC and 5% CO_2 atmosphere for 24 h. At day 2 the cells were washed with fresh pre-warmed medium, and non-adherent dendritic cells were plated in new 6 well plates. Fresh medium was added to the plates containing the adherent bone marrow macrophage cells. The cells were cultured for a further 3 days and at day 5 they were infected for 6 h at a ratio of 2:1 cells:acid fast bacilli. After infection the medium was removed and replaced with fresh medium. The bone marrow macrophages and dendritic cells were lysed with 2% saponin and incubated for 1 h at 37°C. Growth was determined by viable counts on Middlebrook 7H11 agar plates containing Middlebrook OADC supplement at days 0 (6 h post-infection), 4, 7 and 11 post-infection. The growth curves were compared by graph and statistical analysis. The results for each time point are the means of CFU determinations from at least 3 wells, and the error bars show the standard deviations. The asterisk indicates that the result is statistically significantly different from that of the wild type by the two-tailed Student's t test for groups of unequal variance ($p < 0.02$), as well as by single-factor analysis of variance ($p < 0.02$).

Yeast two-hybrid analysis. The *pknF* and Rv1747 genes were amplified by PCR using the proofreading polymerase *Pfu* turbo (Stratagene), and template chromosomal DNA from *M. tuberculosis* H37Rv. For *pknF* the amplification primers were: 5'-CTGCAG-CATGCCGCTCGCGGAAGGTT-3' (forward primer) and 5'-CTGCAG-CGGCCAGCCGTTGCTTCTGC-3' (reverse primer) both carrying a *Pst*I site at the 5' end. For Rv1747 the primers were: 5'-GGATCC-GTGCCGATGAGCCAACCAGC-3' and 5'-GGATCC-

GCACGCCTTGAGGCGAATCT-3' both carrying a 5'BamHI site. The restriction sites were used to clone the genes into the yeast two-hybrid Gal4 activation domain vectors pGAD-C3 and pGAD-C1 respectively, and the Gal4 binding domain vectors pGBD-C3 and pGBD-C1 respectively (22). Constructs were confirmed by sequencing. *Saccharomyces cerevisiae* Y187 (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met⁻, gal80Δ, URA3 : : GAL1ᵤₐₛ -GAL1ₜₐₜₐ –lacZ*) (17) were co-transformed with Gal4 activation domain and Gal4 binding domain vectors according to the LiAc TRAFO method (16), and plated onto minimal Difco plates lacking Leu and Trp for selection of transformants. Protein interactions were measured by assaying for *lacZ* expression using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (28) and expressed as units/min./mg. protein. All *pknF* and Rv1747 Gal4 constructs were co-transformed with either empty pGAD or pGBD vectors to eliminate false positives.

Site directed mutagenesis. Site directed mutagenesis was carried out according to the Stratagene QuickChange® XL Site-Directed Mutagenesis manual, using SoloPack Gold Supercompetent *E. coli* for transformation. Primers used for mutagenesis are described in Table 1. A mutation was introduced into the PknF active site, where Lys-41 was substituted by Ala. Mutation of the activation loop of PknF was created by individual substitution of Thr-173, -175 and -178 with alanines. Rv1747 was mutated in FHA domain-1, where Ser-47 was substituted by Ala, and a second mutant was created where Ser-248 in FHA domain-2 was also substituted by Ala. The presence of the desired mutations was confirmed by sequencing.

RESULTS

Construction of a Rv1747 knockout. The serine-threonine protein kinase *pknF* is adjacent to and probably in the same operon as Rv1747, an ABC transporter protein with two forkhead-associated domains (Fig. 1). Most of the coding region of gene Rv1747 was successfully deleted from *M. tuberculosis* H37Rv using homologous recombination, and this was confirmed by PCR, Southern blot and DNA microarray (see Materials and Methods). A complement strain was

constructed using the region of DNA including the promoter, Rv1746 and Rv1747 to demonstrate that phenotypic effects were due to deletion of Rv1747 and not to downstream polar effects of the mutation on Rv1748. Microarray experiments using DNA from the complement strain showed that the Rv1747 gene had indeed been replaced and that there was an additional Rv1746 gene (data not shown).

Growth of the Rv1747 mutant is normal *in vitro* but attenuated *in vivo*. Growth, as measured by optical density readings at 600 nm, of the Rv1747 mutant and its complement compared to growth of the H37Rv wild type in 100 ml Dubos rolling culture was not statistically different (Fig. 2). However, mouse infection with the Rv1747 mutant resulted in severely impaired growth of the mutant compared with the wild type H37Rv. This was demonstrated in both the lungs (by t-test, $p = 6.4 \times 10^{-4}$) and spleen ($p = 3.5 \times 10^{-4}$) of mice (Fig. 3). The mutant was also significantly different to the complement ($p = 1.4 \times 10^{-3}$ and 4.4×10^{-5} in lungs and spleen respectively). The mutant strain did however persist within the animals. In mouse bone marrow-derived macrophages growth of the Rv1747 knockout was impaired compared to both the H37Rv wild type and Rv1747 complement by t-test (Fig. 4, $p = 4.5 \times 10^{-4}$ and 3.9×10^{-4} respectively). In bone marrow-derived dendritic cells the Rv1747 complement did not grow as well as the H37Rv wild type but the difference was not significantly different (see Fig. 4), whereas the Rv1747 mutant was again significantly impaired in growth compared to the H37Rv wild type by t-test ($p = 0.019$). The mutant was also significantly different to the complement ($p = 0.034$).

Interaction between PknF and Rv1747 is phospho- and FHA domain-dependent. Using yeast two-hybrid analysis, a strong interaction was observed between PknF and Rv1747 (Fig. 5, bar 5). This interaction was abolished upon mutation of Lys-41 to Ala in the active site of PknF (23) (Fig. 5, bar 8) strongly suggesting that the interaction between PknF and Rv1747 is phosphorylation-dependent.

Interaction of PknF with Rv1747 was abolished by mutation to Ala of Thr-173 in the activation loop of PknF (Fig. 5, bar 9). Mutation of Thr-175 or Thr-178 (Fig. 5, bars 10 and 11) reduced the interaction by less than 3-fold, showing that all three threonines are involved in the interaction, but that only Thr-173 is essential for recognition by the FHA-containing Rv1747.

The X-ray structure of FHA1 of Rad53p in complex with a phospho-threonine peptide has indicated that there are six highly conserved residues in the FHA domain (11). Five are located around the peptide binding site, three of which make interactions with the peptide. Of these three, only two, Arg-70 and Ser-85, bind directly to the pThr residue itself (Fig. 6). We have therefore mutated the equivalent serine residues in the two FHA domains of Rv1747, Ser-47 in FHA-1 and Ser-248 in FHA-2. The interaction of Rv1747 with PknF was reduced by greater than 99% by substitution of Ser-47 by Ala in FHA domain 1 of Rv1747 (Fig. 5, bar 6), and was reduced by ~60% by the mutation of Ser-248 to Ala in FHA-2 (Fig. 5, bar 7), suggesting that both FHA domains are involved in the interaction with PknF, but only FHA-1 is essential for this process. These data complement and extend previous observations that the level of phosphorylation of Rv1747 by PknF is dependent on both FHA domains (31). A weak but measurable interaction was noted when Rv1747 was present in both the activation and binding domains of Gal4 showing that this protein interacts with itself (Fig. 5, bar 4). This is consistent with the fact that ABC transporters are generally active as dimers or higher oligomers. Nevertheless, this weak interaction was not affected by either of the mutations in the Rv1747 FHA domains (data not shown).

DISCUSSION

Reversible phosphorylation is a ubiquitous mechanism of signalling transduction. In eukaryotes, proteins are phosphorylated on either tyrosine residues or serine/threonine residues by two distinct but structurally related families of kinases. In contrast, prokaryotes utilise the phosphorylation of histidine residues and subsequent phosphoryl transfer to aspartate residues in so-called two-component phosphorelay systems. Somewhat surprisingly, the genome sequence of

M. tuberculosis revealed a number of putative two-component histidine kinases but also a complement of 11 eukaryotic-like serine-threonine kinases together with a number of phosphatases (7). It has been postulated that these kinases are likely to play regulatory roles (1) and some of them have been investigated biochemically (2, 23, 29, 30, 37) and structurally (32, 49). Potential regulatory functions have been have been investigated in some cases (for review see 33). For example, PknA has been implicated in the regulation of morphological changes associated with cell division (6), PknH is differentially expressed under stress conditions (42) and PknG is linked to cellular glutamate levels (8) and is secreted within macrophages and inhibits phagosome-lysosome fusion (46)**.** Inactivation of PknG led to lysosomal localization and mycobacterial cell death due to loss of inhibition of phagosome-lysosome fusion (46).

The FHA domain is a modular phosphopeptide recognition motif (9) showing a striking specificity for phosphothreonine-containing epitopes (11). FHA domains have been best studied in their roles in the eukaryotic DNA damage response, in particular, in the context of the Rad53/Chk2 family of checkpoint kinases. Of particular relevance is the observation that these molecules encode modular proteins each containing an STK domain together with one or more FHA domains. In this context, the FHA domains seem to play a variety of regulatory roles in kinase activation and subcellular localisation. However, relatively little is known about how prokaryotic FHA domains function. Notably, four *M. tuberculosis* STK-encoding genes are linked to genes coding for proteins containing the FHA phosphopeptide recognition domain suggesting, by analogy to Chk2 and Rad53, that they may play a direct role in regulating STK activity. Support for this notion has emerged from studies of PknH which been shown to phosphorylate the FHA-containing EmbR protein, an activity that is abolished by mutations in the FHA domain of EmbR that would be predicted to compromise pThr-binding (30).

In the present study we have demonstrated the interaction of PknF with the product of Rv1747. We have corroborated this result by mutating both the PknF kinase and Rv1747. Thus, this interaction was abrogated by mutation of Lys-41, known to abolish the kinase activity of

PknF (23), providing evidence that the interaction between PknF and Rv1747 is phosphorylation dependent. It was also abolished by mutation of Thr-173 in the activation loop of PknF, but reduced only 3-fold by mutation of Thr-175 or Thr-178. Thr-173 and -175 are homologous with residues Thr-171 and Thr-173 in PknB, identified as autophosphorylation sites in PknB (4). The presence of pThr in FHA domain binding sites has been shown to be essential for interaction (11) suggesting that Thr-173 may be the phosphorylation site of PknF that is recognised by the Rv1747 FHA domains. Alternatively, since mutation of Thr-173 in the activation loop could, potentially, prevent auto-phosphorylation at other sites in PknF, confident assignment of the actual FHA binding site will require further investigation and these experiments are in progress. Lastly, the protein-protein interaction was disrupted by mutation of Ser-47 of Rv1747, which is highly conserved in FHA domains and directly interacts with the phosphothreonine residue in both Rad53 FHA-1 and the FHA domain of Chk2 (11, 24). In contrast, binding was reduced by less than 3-fold upon mutation of Ser-248 in the second putative FHA domain. These results suggest that phospho-dependent interactions between PknF and the FHA domain of Rv1747 form part of a regulatory cascade that involves phosphorylation by PknF that is directed predominantly by binding of FHA-1 to a pThr-containing autophosphorylation site on PknF, possibly located within the kinase activation loop itself.

Rv1747 codes for an ABC-transporter protein. ATP binding cassette (ABC) transport systems occur in all cells of all living species (18) and in *M. tuberculosis* genes encoding these proteins occupy 2.5% of the genome (5). They are involved in the active transport of solutes across cellular membranes, including the uptake of a large variety of essential nutrients and the secretion of virulence factors, proteases and toxins. The ABC transporter gene Rv1747 contains only one membrane spanning domain and one nucleotide binding domain so is expected to dimerise to function as a transporter (5). There is presently no information as to what substrate it transports. Similarity has been found with the White protein from *Drosophila melanogaster*, a permease necessary for the transport of pigment precursors into pigment cells responsible for eye

colour, and with NodI from *Rhizobium* and *Bradyrhizobium* strains, a protein implicated in the nodulation process by the export of a polysaccharide (5). It has been suggested that the substance transported may be a lipooligosaccharide (http://genolist.pasteur.fr/TuberculList/) and that the Rv1747 product possibly functions as an exporter protein (5). Among eukaryotic ABC transporters with known structures, Rv1747 is most closely related at the sequence level to TAP (transporter associated with antigen processing; PDB ID 1JJ7, E=7e-15) (25) and CTFR (cystic fibrosis transmembrane conductance channel; PDB ID 1R10, E=4e-10) (15), which are both regulated by phosphorylation.

The phenotype of the Rv1747 knockout demonstrated here is the first reported mutation in an FHA domain-containing protein of *M. tuberculosis*, and is intriguing in its very marked reduction in growth in macrophages and in mice. Thus the mutant exhibited greatly reduced bacterial loads in the mouse lungs and spleen compared with infection by the wild-type and therefore falls within the growth *in vivo* (giv) type of mutant defined by Hingley-Wilson *et al.* (20). This ABC transporter system therefore appears to be very important for the normal growth of the bacterium during the multiplication phase in macrophages and in mice, but did not appear necessary for the persistence phase of growth. Clearly, whether the protein involved is importing or exporting a substrate, its absence results in failure of the mycobacterium to thrive in mice but makes no difference to its growth *in vitro*. The fact that Rv1747 interacts with the PknF kinase suggests that its activity is modulated in response to unknown signals that are potentially detected by *pknF* through its extracellular C-terminal region/domain and transduced into the intracellular milieu via the activity of its STK domain.

FIGURE LEGENDS

FIG. 1. Diagram of the genomic region of *M. tuberculosis* containing the *pknF* and Rv1747 operon and adjacent genes, showing the extent of the deletion and the complementing plasmid pRv1747$^+$. The marks on the chromosome are at 1000 bp intervals.

FIG. 2. Growth of the Rv1747 knock-out mutant is unimpaired *in vitro*. The H37Rv wild-type, Rv1747 knock-out and the complemented strain were grown in Dubos medium in rolling bottles as described in Materials and Methods, and optical density measurements were made on 1 ml aliquots taken at 24 hour intervals. There was no statistical difference between the OD measurements from the three strains.

FIG. 3. Growth of the Rv1747 knock-out mutant is impaired in a mouse intravenous infection. BALB/c mice were inoculated intravenously with approximately 5 x 10^5 CFU of each strain. The survival and multiplication of the *M. tuberculosis* strains in the lungs and the spleen were determined by CFU counts and are shown for the H37Rv wild-type (●), Rv1747 knockout mutant (○), and Rv1747 complemented mutant (▼) strains. The results for each time point are the means of CFU determinations performed on organs from 3 to 5 infected mice, and error bars indicate the standard deviations. The asterisks indicates that the result is statistically significantly different from that of the wild type by the two-tailed Student's t test for groups of unequal variance ($p < 0.01$), as well as by single-factor analysis of variance ($p < 0.01$).

FIG. 4. Growth of the Rv1747 knock-out mutant is impaired in *in vitro* infections into mouse bone marrow-derived macrophages and dendritic cells. Macrophages were isolated from BALB/c mice as described in the text and infected at a multiplicity of infection of one bacterium to two macrophages with each strain. The survival and multiplication of the *M. tuberculosis* strains were determined by CFU counts and are shown for the H37Rv wild-type (●), Rv1747 knockout mutant (○), and Rv1747 complemented mutant (▼) strains. The experiment was performed twice with similar results; the results of a representative experiment are shown. The results for each time

point are the means of CFU determinations performed on triplicate infections, and error bars indicate the standard deviations. The asterisks indicates that the result is statistically significantly different from that of the wild type by the two-tailed Student's t test for groups of unequal variance ($p < 0.02$), as well as by single-factor analysis of variance ($p < 0.02$).

FIG. 5. β-galactosidase assay of yeast two-hybrid constructs showing the effect of the PknF K41A, T173A, T175A and T178A mutations, and the Rv1747 S47A and S248A mutations, on the interaction between PknF and Rv1747. *S. cerevisiae* Y187 were co-transformed with plasmids expressing PknF in the Gal4 binding domain and Rv1747 in the Gal4 activation domain. Transformants were selected on minimal Difco –Leu –Trp plates and further tested for *lacZ* expression by β-galactosidase assay using o-nitrophenyl β-D-galactopyranoside as substrate. (1) *S. cerevisiae* [pGAD/pGBD]. (2) *S. cerevisiae* [pGAD Rv1747/pGBD]. (3) *S. cerevisiae* [pGAD/pGBD PknF]. (4) *S. cerevisiae* [pGAD Rv1747/pGBD Rv1747]. (5) *S. cerevisiae* [pGAD Rv1747/pGBD PknF]. (6) *S. cerevisiae* [pGAD Rv1747 S47A/pGBD PknF]. (7) *S. cerevisiae* [pGAD Rv1747 S248A/pGBD PknF]. (8) *S. cerevisiae* [pGAD Rv1747/pGBD PknF K41A]. (9) *S. cerevisiae* [pGAD Rv1747/pGBD PknF T173A]. (10) *S. cerevisiae* [pGAD Rv1747/pGBD PknF T175A]. (11) *S. cerevisiae* [pGAD Rv1747/pGBD PknF T178A].

FIG. 6. Structure of the pThr binding site in FHA domains. The top panel shows the interactions of Ser85 and Arg70 from the N-terminal FHA domain (FHA1) of *Saccharomyces cerevisae* Rad53p checkpoint kinase (11). The lower panel shows a sequence overlap within the core FHA homology region between Rad53p FHA1 and the two FHA domains of *M. tuberculosis* Rv1747. Six of the most highly conserved residues are highlighted (Arg70 and Ser85 in red, Gly69, His88, Asn107 and Asn112 in blue - Rad53 numbering).

TABLE 1. Oligonucleotide primer pairs used for site directed mutagenesis of *pknF* and Rv1747.

Gene	Mutation	Primer sequences 5'-3'[a]
pknF	$K_{41} \rightarrow A$	CGCCAGGACGCGCTC<u>GCG</u>GTACTGCGGGCCG
		CGGCCCGCAGTAC<u>CGC</u>GAGCGCGTCCTGGCG
pknF	$T_{173} \rightarrow A$	CCAAGCGGATTG<u>GCC</u>GCCACAAACATGACTGTGGGC
		GCCCACAGTCATGTTTGTGGC<u>GGC</u>CAATCCGCTTGG
pknF	$T_{175} \rightarrow A$	CCAAGCGGATTGACCGCC<u>GCA</u>AACATGACTGTGGGC
		GCCCACAGTCATGTT<u>TGC</u>GGCGGTCAATCCGCTTGG
pknF	$T_{178} \rightarrow A$	CCGCCACAAACAT<u>GGC</u>TGTGGGCACCG
		CCGTGCCCACA<u>GCC</u>ATGTTTGTGGCGG
Rv1747	$S_{47} \rightarrow A$	CGCACACCCCTGATC<u>GCC</u>CGGGCACACCTGCTGC
		GCAGCAGGTGTGCCCG<u>GGC</u>GATCAGGGGGTGTGCG
Rv1747	$S_{248} \rightarrow A$	CCCGAGGTGTTGGCC<u>GCA</u>CGTCACCACGCCACCC
		GGGTGGCGTGGTGACG<u>TGC</u>GGCCAACACCTCGGG

[a]Underlined bases indicate the change of an amino acid to alanine.

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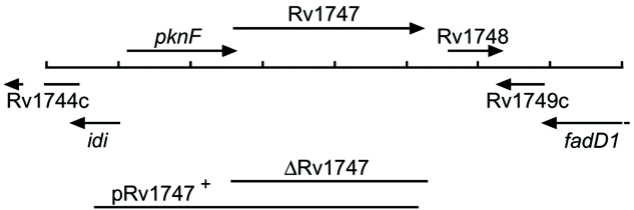
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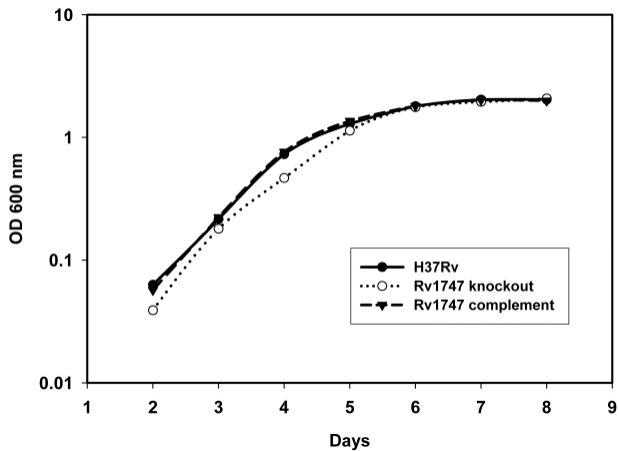
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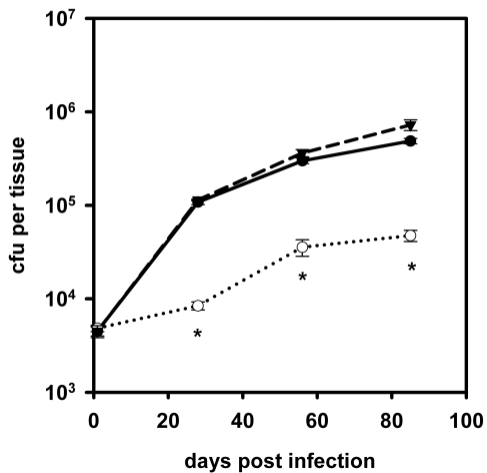
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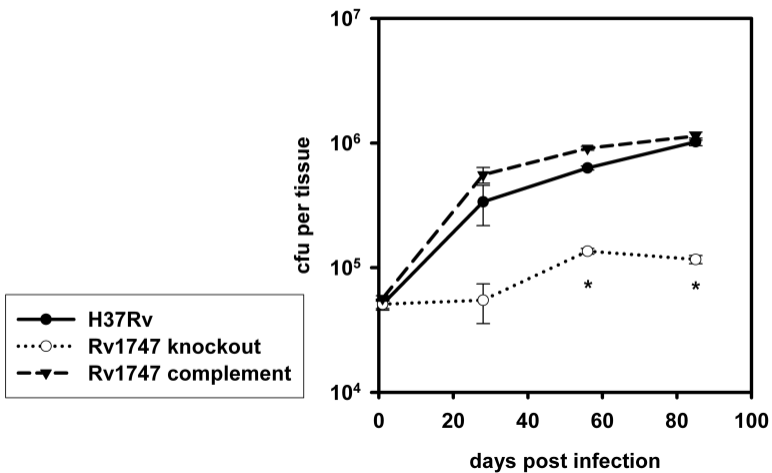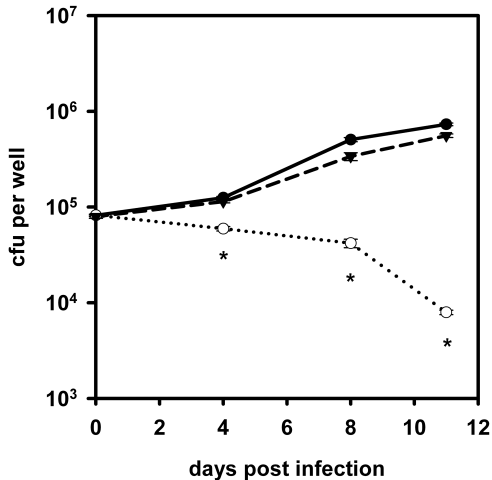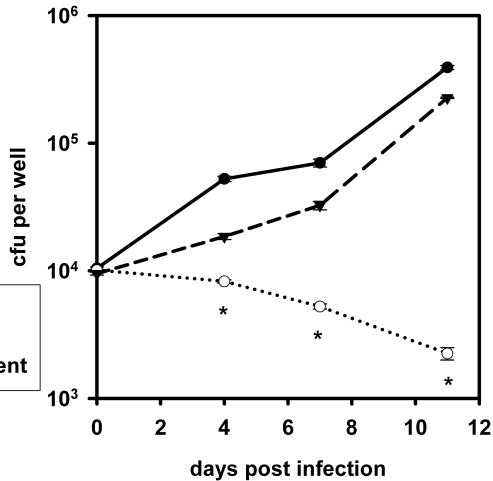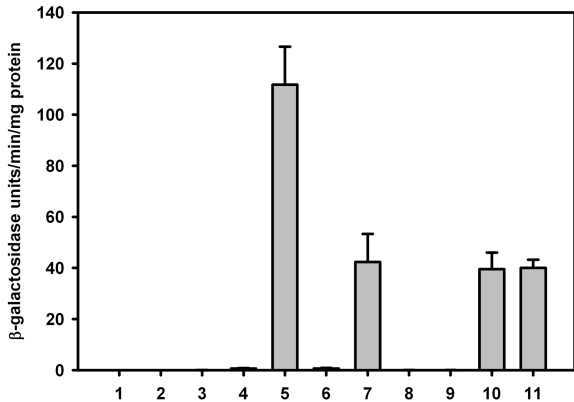
lungs

spleens

Legend:
- H37Rv (solid line, filled circles)
- Rv1747 knockout (dotted line, open circles)
- Rv1747 complement (dashed line, filled triangles)

Axes: cfu per tissue vs days post infection

bone marrow-derived macrophages

bone marrow-derived dendritic cells

H37Rv
Rv1747 knockout
Rv1747 complement
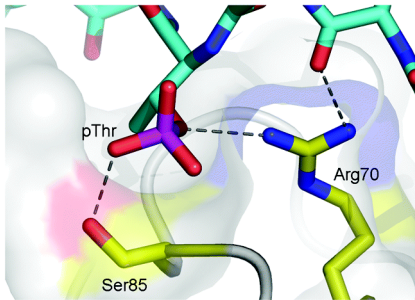
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Rad53   FHA1  VWTFGRNPACDYHLGNISRLSNKHFQILLGEDGN-LLLNDIS-TNGTWLNGQKV
Rv1747  FHA1  DVVVGRDLRADVRVAHPL-ISRAHLLLR--FDQGRWVAIDNS-LNGLYLNNRRV
Rv1747  FHA2  AVRIGRANDNDIVIPEVL-ASRHHATLV--PTPGGTEIRDNRSINGTFVNGARV
```