RESEARCH LETTER

An attenuated mutant of the Rv1747 ATP-binding cassette transporter of *Mycobacterium tuberculosis* and a mutant of its cognate kinase, PknF, show increased expression of the efflux pump-related iniBAC operon

Vicky L. Spivey1, Rachael H. Whalan1, Elizabeth M. A. Hirst2, Stephen J. Smerdon3 & Roger S. Buxton1

1Division of Mycobacterial Research, MRC National Institute for Medical Research, London, UK; 2Division of Developmental Neurobiology, MRC National Institute for Medical Research, London, UK and 3Division of Molecular Structure, MRC National Institute for Medical Research, London, UK

Correspondence: Roger S. Buxton, Division of Mycobacterial Research, MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK. Tel.: +44 020 8816 2225; fax: +44 020 8906 4477; e-mail: rbuxton@nimr.mrc.ac.uk

Present address: Vicky L. Spivey, Horizon Discovery Ltd., Building 7100, IQ Cambridge, Waterbeach, Cambridge CB25 9TL, UK

Received 19 June 2013; revised 24 July 2013; accepted 24 July 2013. Final version published online 23 August 2013.

DOI: 10.1111/1574-6968.12230

Editor: Anthony George

Keywords
mycobacteria; virulence; serine/threonine protein kinase; transcriptomics; DNA microarray; isoniazid.

Abstract

The ATP-binding cassette transporter Rv1747 is required for the growth of *Mycobacterium tuberculosis* in mice and in macrophages. Its structure suggests it is an exporter. Rv1747 forms a two-gene operon with *pknF* coding for the serine/threonine protein kinase PknF, which positively modulates the function of the transporter. We show that deletion of Rv1747 or *pknF* results in a number of transcriptional changes which could be complemented by the wild type allele, most significantly up-regulation of the iniBAC genes. This operon is inducible by isoniazid and ethambutol and by a broad range of inhibitors of cell wall biosynthesis and is required for efflux pump functioning. However, neither the Rv1747 or *pknF* mutant showed increased susceptibility to a range of drugs and cell wall stress reagents including isoniazid and ethambutol, cell wall structure and cell division appear normal by electron microscopy, and no differences in lipoarabinomannan were found. Transcription from the *pknF* promoter was not induced by a range of stress reagents. We conclude that the loss of Rv1747 affects cell wall biosynthesis leading to the production of intermediates that cause induction of iniBAC transcription and implicates it in exporting a component of the cell wall, which is necessary for virulence.

Introduction

The increase in multidrug and extensively drug-resistant *Mycobacterium tuberculosis* strains has made the search for new TB drugs ever more important. Deciphering the function of important *M. tuberculosis* proteins is a key strategy to identify potential new drug targets. Rv1747 is an ATP-binding cassette (ABC) transporter that is required for the growth of *M. tuberculosis* in macrophages, dendritic cells and mice (Curry et al., 2005). A total of 37 ABC transporters have been identified in *M. tuberculosis*; 16 have been categorised as importers and 21 as exporters (Braibant et al., 2000).

ABC transporters are integral membrane proteins comprising two transmembrane domains and two cytoplasmic nucleotide-binding domains; they bind and hydrolyse ATP providing energy for uptake or export of substrates across cell membranes. Functions include the uptake of nutrients into the cells and the export of virulence factors and toxins (Holland et al., 2003). Bacterial ABC importers are typically formed from four polypeptide chains that are often separately encoded (Saurin et al., 1999) and require an external binding protein, which functions to deliver the substrate to the transporter (Dawson et al., 2007). In contrast, bacterial exporters are produced as one polypeptide where a single gene usually encodes both
the transmembrane domain and a nucleotide-binding domain (Saurin et al., 1999). The presence of fused nucleotide-binding and transmembrane domains is a strong indicator of an ABC exporter (Dawson et al., 2007; Davidson et al., 2008). Based on its amino acid sequence, Rv1747 belongs to the G subfamily of ABC transporters; this family consists of half-transporters, which oligomerise to form the functional transporter. Furthermore, this protein encodes both the transmembrane and nucleotide-binding domains in one polypeptide and is thus probably an exporter. Rv1747 forms a two-gene operon with its upstream adjacent gene, the serine–threonine protein kinase (STPK) pknF (Spivey et al., 2011).

Interestingly, Rv1747 also contains two forkhead-associated (FHA) domains; these are modular phosphopeptide recognition motifs, and their presence is taken to indicate that the protein is likely to interact with a phosphorylated protein partner (Durocher & Jackson, 2002; Spivey et al., 2011). Rv1747 exhibits ATPase activity and is a substrate for PknF in vitro; furthermore, both FHA domains of Rv1747 are required for this interaction in a yeast two-hybrid assay (Molle et al., 2004; Curry et al., 2005). More recently, we have identified specific threonine residues on Rv1747 that are phosphorylated, at least in vitro, by PknF and which have in vivo modulatory effects on the function of the Rv1747 ABC transporter (Spivey et al., 2011).

Bacterial ABC exporters transport many different substances including cell surface components such as lipopolysaccharides, lipids, proteins involved in pathogenesis, peptides, drugs and siderophores (Dassa, 2003). In M. tuberculosis, one ABC transporter, DrrABC and an RND family transmembrane protein, MmpL7, are required for the translocation to the cell surface of phthiocerol dimycocerosates (PDIMs), complex lipids required for virulence (Cox et al., 1999; Camacho et al., 2001); interestingly, MmpL7 is a potential substrate of another STPK, PknD (Pérez et al., 2006). Rv1747 could export any one of these molecules, which would make the function of the transporter important for growth in vivo. Rv1747 falls into a subclass of M. tuberculosis ABC transporters, which have an unknown function (Braibant et al., 2000). Similarity was found to the White protein from Drosophila melanogaster, a permease necessary for the transport of pigment precursors responsible for eye colour, and to NoDI from Rhizobium strains, a protein implicated in the nodulation process by export of a polysaccharide (Braibant et al., 2000).

We have investigated the phenotypic consequences of the loss of the Rv1747 and PknF proteins in deletion mutants. Significantly, using transcriptional profiling, we demonstrate that the expression of the iniBAC operon is up-regulated in both mutants.

Materials and methods

Bacterial strains and growth conditions

Mycobacterium tuberculosis H37Rv and Escherichia coli K-12 strains are described in Table 1. Growth conditions have been described previously (Spivey et al., 2011).

Generation of the pknF and Rv1747 deletion and complementing strains

The construction of the pknF (Rv1746) null strain (ApknF) was described previously (Spivey et al., 2011); this has an in-frame unmarked deletion to avoid downstream polar effects on the Rv1747 gene. For complementation of the pknF deletion, the genes pknF, 609 bp of Rv1745c and 20 bp of Rv1747 were amplified by PCR (Spivey et al., 2011) and the product cloned into the vector pKP186 (Rickman et al., 2005), a pMV306 (Kong & Kunimoto, 1995) derivative lacking the integrase gene and electroporated into the ApknF mutant along with the mycobacterial suicide vector, pPS-Int containing the integrase gene (Springer et al., 2001; Curry et al., 2005). Construction of the hygromycin-marked Rv1747 deletion mutant was described previously (Curry et al., 2005). For complementation of the Rv1747 deletion, the genes Rv1747, Rv1746 (pknF), and 609 bp of Rv1745c were amplified by PCR (Curry et al., 2005), cloned into the vector pKP186 (Rickman et al., 2005) and transformed into the ΔRv1747 mutant.

cDNA labelling and microarray analysis

RNA isolation from M. tuberculosis liquid cultures was described previously (Spivey et al., 2011). Whole genome DNA microarrays of M. tuberculosis (version 2) were provided by the BuG@S group (St. George’s, University of London). cDNA labelling and RNA–DNA microarray hybridisations were described previously (Rickman et al., 2005). Microarray slides were scanned as previously (Hunt et al., 2008), grids were fitted using BLUEFUSE software and analysed using GENESPRING, version 10 (Agilent). Three biological replicates were performed for each condition, carried out in duplicate for dye swaps. The genes described only include those whose differential gene regulation was restored to wild type (WT) in complemented mutants. The array design is available in ArrayExpress (Accession No. A-BUGS-347). The full gene expression dataset has been deposited in BujG@Sbase (accession number E-BUGS-149).
Quantitative real-time PCR (qRT-PCR)

cDNA was generated from 1 μg RNA using the Quantitect reverse transcription kit (Qiagen). Primers (Table 2) were designed using PRIMER EXPRESS 3.0 (Applied Biosystems). Real-time quantitative PCR analysis on this cDNA was performed using the ABI Prism 7500 using the Fast SYBR green master mix (Applied Biosystems). Data were normalised to sigA expression.

Other methods are described in Supporting information, Appendix S1.

Results

Transcriptional microarray analysis of the ΔpknF and ΔRv1747 mutants

Transcriptional microarray analysis was performed to compare gene expression in WT H37Rv vs. ΔRv1747, WT vs. ΔpknF, WT vs. the Rv1747 complemented mutant and WT vs. the pknF-complemented mutant. The top 10 most highly regulated genes whose differential expression was restored by complementation are shown in Table 3. As expected, expression of Rv1747 was 29-fold down-regulated in the mutant (compared with WT). In the Rv1747 complemented strain, expression of Rv1747 was 1.3-fold up-regulated compared with WT, confirming restoration of gene expression.

The gene most up-regulated (3.8-fold) in the ΔRv1747 strain was the isoniazid-inducible gene iniB, whilst the second most up-regulated was iniA (3.2-fold). iniB forms an operon with iniA and iniC, which was up-regulated 1.6-fold. Other genes in the top 10 list of up-regulated

Table 1. Bacterial strains and plasmids

| Strains or plasmids | Genotype or description | Source or reference |
|---------------------|-------------------------|---------------------|
| **E. coli strains** |                         |                     |
| E. coli TOP10       | F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG; used for general cloning | Invitrogen |
| **M. tb. strains** |                         |                     |
| H37Rv               | M. tuberculosis WT strain | Oatway & Steenken (1936) |
| ΔpknF               |                         | Spivey et al. (2011) |
| pknF complement     |                         | Curry et al. (2005) |
| ΔRv1747             |                         | Curry et al. (2005) |
| Rv1747 complement   |                         | Curry et al. (2005) |

**M. tuberculosis shuttle plasmids**

- p2Nil: Suicide gene delivery vector, oriE, KanR
- pKF186: Integrase negative derivative of the integrating vector pMV306, KanR
- pBS-Int: Suicide vector containing integrase, AmpR
- pEJ414: shuttle plasmids
- pRW69: Suicide gene delivery vector, oriE
- pRW76: Suicide gene delivery vector, oriE
- pRW95: Suicide gene delivery vector, oriE
- pVS_01: Suicide gene delivery vector, oriE

Table 2. Primers used for qRT-PCR

| Primer name | Sequence (5′-3′) |
|-------------|------------------|
| pknF F      | CACGAACTCGGCTGTTG |
| pknF R      | GAGCATCAGTGAATCAGGATG |
| Rv1747 F    | TACGCTGACGCTGAATTG |
| Rv1747 R    | GGCTGCGGTGTGGA |
| iniA F      | TTGACCTCCTGTTGTG |
| iniA R      | TTATCGATTACATCCTGAGCCTG |
| iniB F      | CGAGCCCAGCACGAAGAA |
| iniB R      | ACTCCTCAATCAAGCTTTT |
| iniC F      | CAGCGACCAGCATTCCGCT |
| iniC R      | GGAAGAGCGGTGTG |
| ethA F      | GGCAAGCACAGCATCAG |
| ethA R      | CGATGATGCTGTG |
| pknD F      | TGTTCATAGGCGGCCGA |
| pknD R      | TTGACCTCCTGTTG |
| sigA F      | CGGGGCGCTCCCTCT |
| sigA R      | GGCTAGCTGACCTCCT |

FEMS Microbiol Lett 347 (2013) 107–115 © 2013 The Authors. FEMS Microbiology Letters published by John Wiley & Sons Ltd on behalf of the Federation of European Microbiological Societies.
Table 3. Microarray data for the 10 Mycobacterium tuberculosis genes most highly up- and down-regulated upon Rv1747 deletion

| Rv number | Gene name | Gene product | Fold regulated | P-value |
|-----------|-----------|--------------|---------------|---------|
| Rv0005    | gyrB      | DNA gyrase subunit B | 2.0 up | 0.001 |
| Rv0046c   | ino1      | Myo-inositol-1-phosphate synthase Ino1 | 2.0 up | 3.00E-04 |
| Rv0047c   | Rv0047c   | Conserved hypothetical protein | 2.2 up | 4.10E-05 |
| Rv0341    | inIB      | Isoniazid-inducible gene protein IniB | 3.8 up | 2.40E-04 |
| Rv0342    | inIA      | Isoniazid-inducible gene protein IniA | 3.2 up | 8.10E-05 |
| Rv0822c   | Rv0822c   | Conserved hypothetical protein | 2.0 up | 4.00E-05 |
| Rv1040c   | PE9       | PE family protein | 2.1 down | 0.013 |
| Rv1380    | pyrB      | probable aspartate carbamoyltransferase PyrB | 2.2 down | 0.001 |
| Rv1382    | Rv1382    | probable export or membrane protein | 2.2 down | 6.20E-04 |
| Rv1747    | Rv1747    | probable ABC transporter | 29.2 down | 8.90E-06 |
| Rv1999c   | Rv1999c   | probable conserved integral membrane protein | 2.2 down | 0.004 |
| Rv2007c   | fdxA      | probable ferredoxin FdxA | 2.1 up | 0.005 |
| Rv2265    | Rv2265    | possible conserved integral membrane protein | 2.1 down | 0.005 |
| Rv2396    | PE_PGRS41 | PE-PGRS family protein | 2.0 up | 7.00E-04 |
| Rv2415c   | Rv2415c   | Conserved hypothetical protein | 2.3 down | 7.90E-04 |
| Rv2528c   | mrr       | probable restriction system protein MrR | 2.1 down | 1.90E-04 |
| Rv2577    | Rv2577    | Conserved hypothetical protein | 2.1 down | 0.015 |
| Rv2814c   | Rv2814c   | probable transposase | 2.2 down | 0.011 |
| Rv3140    | fadE23    | probable acyl-CoA dehydrogenase FadE23 | 2.1 up | 0.001 |
| Rv3854c   | ethA      | Monoxygenase EthA | 2.0 up | 0.003 |

Comparison of the WT and ApkF strains yielded only 12 genes differentially regulated at least twofold, all down-regulated in the mutant. This number increased to 72 with a 1.5-fold cut-off, of which only 12 were up-regulated. The microarray data for the 10 most up- and down-regulated genes in the pknF deletion strain are presented (Table 4). The pknF gene itself did not appear in the microarray results list because it did not pass the filtering stages within the analysis.

Interestingly, the gene most up-regulated in the ApkF strain was also iniB (1.8-fold). iniA was also up-regulated 1.5-fold in the pknF deletion strain. Other genes present in the ApkF list that were also up-regulated in the Rv1747 null strain were fdxA (1.8-fold) and ethA (1.7-fold). Other genes up-regulated in the ApkF mutant were glpQ1, a probable glycerophosphoryl diester phosphodiesterase (1.8-fold), the conserved hypothetical proteins Rv3850 (1.7-fold) and Rv1738 (espE), an Esx1 secretion-associated protein (1.6-fold), Rv3727 (probably involved in cellular metabolism), Rv3728 (probably involved in an efflux system) and Rv0175 (a probable conserved Mce-associated membrane protein; all 1.5-fold). The top 10 list of genes down-regulated in the ApkF strain comprised seven possible transposases; Rv2480c (2.4-fold), and Rv1380, Rv2106, Rv3640c, Rv2815c, Rv2167c, Rv0796 (all 2.0-fold). The remaining three down-regulated genes were Rv1371 (2.4-fold; conserved membrane protein) and Rv1372 (2.2-fold) and Rv2515c (2.0-fold), both annotated as conserved hypothetical proteins.

Confirmation of the microarray results for selected genes was obtained using qRT-PCR. The results (Fig. 1) confirmed that expression of Rv1747 in the deletion mutant was virtually undetectable and transcription was restored to WT levels in the complementing strain. Expression of pknF did not increase in the ΔRv1747 mutant, but did increase in the complementing strain, as mentioned above.

Unlike the microarray data, the level of pknF transcript did not increase in the ΔRv1747 strain when determined by qRT-PCR. The transcriptional profiles of iniA, iniB, iniC, ethA and pknD all follow the same expression pattern as shown by the microarray results: transcript levels were increased in the Rv1747 mutant strain and were complemented when the Rv1747 gene was replaced. This was particularly striking for iniB and iniA where there was approximately three times as much transcript present in the mutant strain compared with the WT.
The results of qRT-PCR for the WT, ΔpknF and the pknF-complemented strain (Fig. 2) confirmed that expression of pknF in the ΔpknF mutant was undetectable and transcription was restored to almost WT level in the complementing strain. Expression of Rv1747 was the same in all three strains; thus, the transcriptional changes seen in the ΔpknF mutant are not due to changes in Rv1747 expression. The transcriptional profiles of iniB, iniA and iniC (Fig. 2c–e) follow the same pattern as shown by the microarray data. The iniC gene did not appear in the gene lists generated by microarrays in the pknF mutant or complement strain, but Fig. 2e clearly shows that the level of iniC transcript did not change in the ΔpknF mutant strain but was slightly decreased in the pknF-complemented strain.

Table 4. Microarray data for the 10 Mycobacterium tuberculosis genes most highly up- and down-regulated upon pknF deletion

| Rv number   | Gene name | Gene product                                  | Fold regulated | P-value |
|-------------|-----------|-----------------------------------------------|----------------|---------|
| Rv0175      | Rv0175    | Probable conserved Mce-associated membrane protein | 1.5 up         | 0.002   |
| Rv0341      | iniB      | Isoniazid-inducible gene protein IniB          | 1.8 up         | 0.001   |
| Rv0796      | Rv0796    | Putative transposase for insertion sequence element IS6110 | 2.0 down     | 0.035   |
| Rv1370c     | Rv1370c   | Putative transposase for insertion sequence element IS6110 | 2.0 down     | 0.042   |
| Rv1371      | Rv1371    | Probable conserved membrane protein            | 2.4 down       | 0.042   |
| Rv1372      | Rv1372    | Conserved hypothetical protein                 | 2.2 down       | 0.037   |
| Rv1738      | Rv1738    | Conserved hypothetical protein                 | 1.6 up         | 0.005   |
| Rv2007c     | fdxA      | Probable ferredoxin fdxA                       | 1.8 up         | 0.023   |
| Rv2106      | Rv2106    | Probable transposase                           | 2.0 down       | 0.029   |
| Rv2167c     | Rv2167c   | Probable transposase                           | 2.0 down       | 0.015   |
| Rv2480c     | Rv2480c   | Possible transposase for insertion sequence element IS6110 | 2.4 down     | 0.003   |
| Rv2515c     | Rv2515c   | Conserved hypothetical protein                 | 2.0 down       | 0.034   |
| Rv2815c     | Rv2815c   | Probable transposase                           | 2.0 down       | 0.044   |
| Rv3640c     | Rv3640c   | Probable transposase                           | 2.0 down       | 0.036   |
| Rv3727      | Rv3727    | Possible oxidoreductase                         | 1.5 up         | 0.008   |
| Rv3728      | Rv3728    | Probable conserved two domain membrane protein | 1.5 up         | 0.006   |
| Rv3842c     | glpQ1     | Probable glycerophosphoryl diester phosphodiesterase GlpQ1 | 1.8 up     | 0.006   |
| Rv3850      | Rv3850    | Conserved hypothetical protein                 | 1.7 up         | 0.002   |
| Rv3854c     | ethA      | Monoxygenase EthA                              | 1.7 up         | 0.006   |
| Rv3864      | espE      | Esx-1 secretion-associated protein EspE        | 1.6 up         | 0.003   |

The cell wall of the ΔpknF and ΔRv1747 mutants appeared normal by drug sensitivity assays, electron microscopy and lipoarabinomannan content

Many of the genes which were differentially regulated between WT and ΔpknF and ΔRv1747 mutants are involved in cell wall processes. We therefore tested whether the mutants were any more susceptible to cell wall and other stresses, viz. isoniazid, ethambutol, streptomycin, ciprofloxacin, ofloxacin, hydrogen peroxide, S-nitrosoglutathione, ethidium bromide, mitomycin C and sodium dodecyl sulphate, using a microplate Alamar Blue assay. However, no differences were observed in MICs between the mutants and the WT (Fig. S1).

Moreover, the transcription of the pknF-Rv1747 operon was not altered by isoniazid or ethambutanol (or indeed by a number of other stresses: gentamicin, streptomycin, hydrogen peroxide, t-butyl hydrogen peroxide, diamide, mitomycin C, S-nitrosoglutathione, ofloxacin, plumbagin, sodium nitroprusside, pH 7.2, pH 5.5 or in stationary phase; Fig. S2).

Transmission electron microscopy was performed to examine whether there were any differences in the cell wall structure and composition between the WT, ΔpknF, ΔRv1747 and the respective complementing strains. No discernible differences in cell wall structure were evident between the strains (Fig. S3).

The possibility that Rv1747-transported lipoarabinomannan was tested by analysing this molecule using two anti-lipoarabinomannan antibodies, one primarily recognising not only PIM6 but also ManLAM capped with three mannosyl residues (Mab F183-24) and one recognising the Ara6 structure in lipoarabinomannan (Mab F30-5). There were however no differences in the levels of ManLAM between cell suspensions of the WT, ΔpknF, ΔRv1747 and Rv1747 complementing strains (Fig. S4).

Discussion

The substrate of the Rv1747 transporter is presently unknown. Moreover, other than the attenuation of growth of a ΔRv1747 mutant in mice and macrophages, there are presently no further mutant phenotypes known, growth being normal in vitro (Curry et al., 2005). We have used transcriptional microarray analysis to see
whether this would throw light on the nature of \textit{Rv1747} transport function. This has demonstrated significant changes in transcriptional profiles between the \textit{\textless\textit{Rv1747} and \textit{pknF}} mutants and the WT. Interestingly, the genes most up-regulated in both of the mutant strains were in the \textit{iniBAC} operon, identified as isoniazid-inducible (Alland \textit{et al.}, 1998). The \textit{iniA} gene was also induced by ethambutol, another \textit{M. tuberculosis} therapeutic agent that also targets the cell wall but with a different mechanism of action. Using \textit{M. bovis BCG}, the promoter of the \textit{iniBAC} operon was shown to be specifically induced by a broad range of inhibitors to cell wall biosynthesis including antibiotics that inhibited the synthesis of peptidoglycan, arabinogalactans, mycolic acids and fatty acids (Alland \textit{et al.}, 2000). \textit{iniA} is also essential for the activity of an efflux pump, which confers resistance to isoniazid.

\textbf{Fig. 1.} qRT-PCR to confirm the effect of \textit{Rv1747} deletion and complementation on the relative transcription levels of selected genes. Each panel shows the normalised transcription level of each gene investigated in WT, \textit{\textless\textit{Rv1747} and complement strains. Data plotted are the mean of three biological replicates, and the error bars show the standard deviations. Data were normalised to \textit{sigA} expression.
and ethambutol, although IniA does not directly transport isoniazid from the cell (Colangeli et al., 2005). All these findings would be compatible with the Rv1747 transporter exporting a component of the cell wall necessary for growth of the bacillus in vivo. The sensitivity of the ΔRv1747 or ΔpknF mutants was not however altered towards isoniazid or indeed any other of the agents tested. Neither was the transcription of the pknF-Rv1747 operon altered by isoniazid or ethambutol. Moreover, there were no observable changes in morphology of the cell wall as seen by electron microscopy. Thus, Rv1747 does not appear to export any component of the cell wall that is involved in formation of an observable structure.

Other significant changes in expression of cell wall-associated genes were also found in the microarray study. Thus, ethA and ino1 were up-regulated in the ΔRv1747 strain. EthA functions to activate the prodrugs ethionamide, thiacetazone and isoxyl, which all use different mechanisms to inhibit mycolic acid synthesis (Dover et al., 2007). Ino1 is involved in the PI biosynthetic pathway; this phospholipid is also a component of the cell envelope. The list of the top ten down-regulated genes included two genes annotated as being conserved integral membrane proteins, namely Rv1999c and Rv2265, and one gene, Rv1382, annotated as a probable export or membrane protein. Up-regulation of all these genes may be acting as a compensatory mechanism for the loss of the function of Rv1747.

As PknF positively regulates Rv1747 function (Spivey et al., 2011), it may be expected that a ΔpknF mutant would have a similar phenotype to a ΔRv1747 mutant. Significantly, the iniB and iniA were also up-regulated in the ΔpknF mutant, correlating with the demonstration that PknF positively modulates the function of Rv1747.

**Fig. 2.** qRT-PCR to confirm the effect of pknF deletion and complementation on the relative transcription levels of selected genes. Each panel shows the normalised transcription level of each gene investigated in WT, ΔpknF and complement strains. Data plotted are the mean of three biological replicates, and the error bars show the standard deviations. Data were normalised to sigA expression.
There were fewer genes whose expression level changed upon pknF deletion. This may be because of cross-talk between the 11 M. tuberculosis STPKs, which are able to cross-talk and recognize the same substrate in vitro (and probably also in vivo; Greenstein et al., 2005; Grundner et al., 2005; Molle & Kremer, 2010; Prisic et al., 2010). There is also a high degree of cross-reactivity between inhibitors of PknB and PknF

The role of the STPK PknF that controls Rv1747 function has previously been examined in a pknF antisense strain (Deol et al., 2005). These authors described changes in cell morphology, including aberrant septum formation and reported a 16-fold increase in the uptake of \( \alpha \)-glucose in the antisense strain. In contrast, in the present study, we did not find any morphological changes in the \( \Delta pknF \) mutant.

Pitarque et al. (2008) have proposed that an unidentified transporter may be required to translocate the lipoglycans lipoarabinomannan and lipomannan, to the cell surface as the virulence of \( M. \) tuberculosis depends upon the export of these immunomodulatory molecules to the cell surface, as shown for the translocation of PDIMs (Sulzenbacher et al., 2006). The involvement in PDIM transport of an ABC transporter, DrrABC, together with the RND family protein MmpL7 (Cox et al., 1999; Camacho et al., 2001), which is a potential substrate of the STPK PknD (Pérez et al., 2006), is a possible paradigm for Rv1747 and PknF. Thus, the translocation of virulence-critical molecules such as lipoglycans could be a plausible explanation for the attenuation of the \( \Delta Rv1747 \) mutant in mice. However, our limited analysis with two anti-lipoarabinomannan antibodies failed to find any difference in this molecule in the \( \Delta Rv1747 \) mutant.

In this study, we have demonstrated that there are significant increases in gene expression in the efflux pump-related \( iniBAC \) genes in the \( \Delta Rv1747 \) and \( \Delta pknF \) deletion strains. As this operon is inducible by a broad range of inhibitors of cell wall biosynthesis, we conclude that the loss of the Rv1747 transporter system affects cell wall biosynthesis and results in the accumulation of intermediates that may play a role in cell wall processes or biosynthesis, causing an induction of the \( iniBAC \) operon expression and implicates Rv1747 in exporting a component of the cell wall, which is required for virulence.

Acknowledgements

We thank Kathyrn Lougheed for help in setting up the Alamar Blue assays, Ben Appelmelk and Jeroen Geurtsen (VU University Medical Centre Amsterdam) for the gift of anti-lipoarabinomannan antibodies and Arnold Driessen (University of Groningen) for helpful discussion on ABC transporter function. We acknowledge B\( \mu \)G@S (the Bacterial Microarray Group at St. George’s, University of London) and particularly Philip Butcher, Jason Hinds, Kate Gould and Denise Waldron for the supply of the \( M. \) tuberculosis microarrays and advice. This work was supported by an MRC studentship to V.L.S. and by the Medical Research Council (Grant numbers U117585867 and U117584228).

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Growth inhibition assays assessing the susceptibility of WT H37Rv, *ApknF* and *Δrv1747* strains to a range of drug and stress reagents.

**Fig. S2.** β-Galactosidase assays on the *pknF* promoter- lacZ strain and pEJ414 control strain in *Mycobacterium tuberculosis* after a panel of treatments.

**Fig. S3.** Transmission electron micrographs of *Mycobacterium tuberculosis* comparing cell wall structure in (a) WT H37Rv, *Δrv1747* and *rv1747* complement strains, and (b) WT H37Rv, *ApknF* and *pknF* complement strains.

**Fig. S4.** *Mycobacterium tuberculosis* whole cell ELISAs comparing the levels of ManLAM in H37Rv WT, *Δrv1747* and *rv1747* complement strains.

**Appendix S1.** Supplementary Materials and methods.