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Inactivation of polyketide synthase and related genes results in the loss of complex lipids in *Mycobacterium tuberculosis* H37Rv

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ABSTRACT

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Aims: Phthiocerol dimycocerosate (PDIM) waxes and other lipids are necessary for successful *Mycobacterium tuberculosis* infection, although the exact role of PDIM in host–pathogen interactions remains unclear. In this study, we investigated the contribution of *tesA*, *drrB*, *pks6* and *pks11* genes in complex lipid biosynthesis in *M. tuberculosis*.

Methods and Results: Four mutants were selected from *M. tuberculosis* H37Rv transposon mutant library. The transposon insertion sites were confirmed to be within the *M. tuberculosis* open reading frames for *tesA* (a probable thioesterase), *drrB* (predicted ABC transporter), *pks11* (putative chalcone synthase) and *pks6* (polyketide synthase). The first three of these transposon mutants were unable to generate PDIM and the fourth lacked novel polar lipids.

Conclusions: *Mycobacterium tuberculosis* can be cultivated *in vitro* without the involvement of certain lipid synthesis genes, which may be necessary for *in vivo* pathogenicity.

Significance and Impact of the Study: The use of transposon mutants is a new functional genomic approach for the eventual definition of the mycobacterial 'lipidome'.

Keywords: cell wall, lipids, mutants, *Mycobacterium tuberculosis*, polyketide synthases.

INTRODUCTION

The World Health Organisation (1993), declared tuberculosis as a global health emergency (Najima 1996). Ten years on, it has been estimated that the global incidence rate of tuberculosis is growing at *c.* 0·4% per year (WHO Report 2003). Many front line drugs target the cell wall of *Mycobacterium tuberculosis*. Understanding the role of cell envelope components may indicate new drug targets.

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Phthiocerol dimycocerosate (PDIM) waxes consist of two multi-methyl branched mycocerosic acids esterified to phthiocerol and they occur in a limited group of pathogenic mycobacteria. The related phenolphthiocerol dimycocerosates are the lipid core of the glycosyl-phenolphthiocerol dimycocerosates, known as phenolic glycolipids (PGL) (Minnikin *et al.* 2002). PGL are present in *Mycobacterium bovis* and smooth 'Canetti' variants of the tubercle bacillus but are absent from many *M. tuberculosis* strains (Constant *et al.* 2002; Minnikin *et al.* 2002).

Rainwater and Kolattukudy (1985) demonstrated the elongation of *n*-fatty acyl-CoA substrates, using methyl-malonyl-CoA to generate mycocerosic acids. The gene encoding this methylmalonyl-specific elongation in *M. bovis* BCG is the type I polyketide synthase, *mas*-mycocerosic

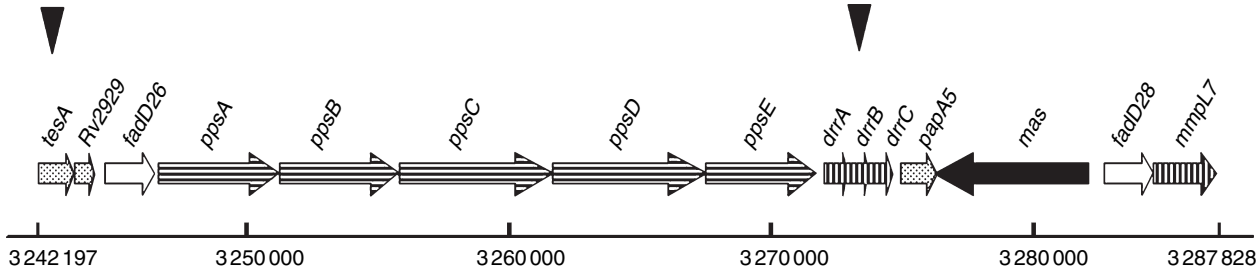

Fig. 1 The PDIM gene cluster in *Mycobacterium tuberculosis*. Insertion sites of the transposon mutants analysed in this investigation are marked with black triangles

acid synthase (Mathur and Kolattukudy 1992). The biosynthesis of phthiocerol and phenolphthiocerol segments of PDIM, is catalysed by products of the *ppsA-E* gene cluster (Azad *et al.* 1997). Many of the genes responsible for the synthesis of PDIM (*mas* and *ppsA-E*) are located in a gene cluster of upto 15 genes, >45 kb in length (Cole *et al.* 1998) (Fig. 1). Mutagenic loss of PDIM results in the attenuation of *M. tuberculosis* in mouse lung (Camacho *et al.* 1999; Cox *et al.* 1999). However, the detailed functional significance of PDIM, during *M. tuberculosis* infection, is unknown. This cluster also has genes hypothesized to be involved in the production and transportation of PDIM. Genes coding for putative acyl-CoA/AMP synthases (*fadD26* and *fadD28*) have been implicated in translocation during synthesis (Fitzmaurice and Kolattukudy 1998; Trivedi *et al.* 2004). The *drrA/B/C* genes (coding for ABC-like transporters) and *mmpL7* (coding for a large conserved membrane protein) may also be involved in the transport of PDIM (Cox *et al.* 1999; Camacho *et al.* 2001). The roles of the remaining genes, in the PDIM gene cluster, remain to be determined. Interestingly, other members of the polyketide synthase family have been implicated in the generation of PDIM and PGL. For instance, *pks1/15* (*msl7*), located downstream of the PDIM gene cluster in *M. tuberculosis*, is involved in the biosynthesis of PGL, and *pks10* (a chalcone-like synthase) has been demonstrated to be necessary for successful PDIM synthesis (Constant *et al.* 2002; Sirakova *et al.* 2003a). Recently, the polyketide synthases coded by *pks12* and *pks7* have also been identified to be required for PDIM synthesis in *M. tuberculosis* H37Rv (Rousseau *et al.* 2003; Sirakova *et al.* 2003b).

Libraries of *M. tuberculosis* mutants derived from transposon mutagenesis provide a convenient source of strains for studying the role of particular lipids. The temperature-sensitive bacteriophage phAE87 was used to deliver the Tn5370 mini-transposon to generate a library of *M. tuberculosis* mutants, from which the insertion points in 351 genes were defined (McAdam *et al.* 2002). We report here lipid analysis from *tesA* (coding for a probable thioesterase), *drrB* (encoding a predicted ABC transporter), *pks6* (coding for a putative polyketide synthase) and *pks11* (a putative chalcone synthase) transposon mutants.

MATERIALS AND METHODS

Transposon library, PCR and sequencing

The *tesA*, *drrB*, *pks6* and *pks11* transposon mutants (plate numbers 14A4, 49C4, 20F1 and 2F8 respectively) were selected from the GlaxoSmithKline *M. tuberculosis* H37Rv transposon mutant library (McAdam *et al.* 2002), and grown to mid-late log phase at 37°C in Dubos liquid medium, supplemented with Bacto Dubos medium albumin. The culture media for the transposon mutants were supplemented with 100 μg ml^{-1} hygromycin.

PCR was performed for the presence of Tn5370 targeting the hygromycin resistance gene using the primers 5′-CGG CCC GTA CCC TGT GAA TA-3′ (forward) and 5′-CAC CAG GCT GTA GCG GGA GT-3′ (reverse). PCR verification of the transposon insertion site was conducted using the forward hygromycin primer with the gene-specific primers *tesA* (5′-GCC AGC TAC ATC CGG AGC AT-3′), *drrB* (5′-CTG TGG ACC GAT GCT TCA CG-3′), *pks6* (5′-GGG TCG CGG CAT TTT CAG TA-3′) and *pks11* (5′-GGG GTC GAA CTA CGC AAT CG-3′). PCR products suitable for sequencing were generated using the SP3 primer (McAdam *et al.* 2002) 5′-GCA CAC CCA AGC CAA CCA GAC C-3′ with the *tesA*, *drrB*, *pks6* and *pks11* gene specific primers described above. The PCR products were sequenced from the SP3 primer using the Sanger dideoxy method of sequencing, using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Lipid extractions

Apolar and polar lipid extracts were prepared as detailed by Dobson *et al.* (1985). Apolar lipids were analysed by two-dimensional thin layer chromatography (2D-TLC) using four different solvent systems (A–D) and polar lipids by two different systems (D and E) according to Dobson *et al.* (1985).

Reverse transcriptase-PCR

Reverse transcriptase (RT)-PCR protocols were adapted from Butcher *et al.* (1998). DNaseI-treated RNA plus 10 μM reverse primer were incubated at 85°C for 5 min, and the samples snap-cooled. RT master mix containing: 5X RT Buffer, dithiothreitol, dNTPs, RNaseOUT^{TM} and SuperScript^{TM} II Reverse Transcriptase (Invitrogen, Paisley, UK) was added, and the samples incubated for 1 h at 42°C. Each sample was prepared in duplicate; the repeat lacked RT. Negative controls contained no template or template-RNA treated with RNase A. To amplify the first strand products, PCR was conducted using 1–2 μl of the first strand synthesis product as template DNA. Positive (*M. tuberculosis* genomic) and negative (no template DNA) PCR controls were also performed. Products were analysed by gel electrophoresis on 0·8% TBE/agarose gels using Hyperladder I (Bioline, London, UK) electrophoresis markers. Primers were designed to cross the intergenic region between the *tesA* and *Rv2929* open reading frames, forward (5′-ACA GTA TGG GCG GAA TGC TA-3′), reverse (5′-CAC CCG AGA TGG TGC ATA C-3′) to generate a product of 680 bp. Control primers were designed to amplify a 975 bp intragenic region of *fadD26* (located in the PDIM gene cluster, Fig. 1), forward (5′-AGT TCA GAC CGG CAC GTT TG-3′), reverse (5′-GCC ATG TCC TGG TCA GAT G-3′).

RESULTS

Confirmation of the transposon insertion sites

The transposon insertion sites (McAdam *et al.* 2002) were verified by three PCR strategies and sequencing. Firstly, primers were designed to amplify an 801-bp fragment of the hygromycin resistance gene present on the transposon. A product of the correct size was generated with genomic DNA from *tesA*Δ, *drrB*Δ, *pks6*Δ and *pks11*Δ; there was no amplification using *M. tuberculosis* H37Rv genomic DNA. This established that Tn5370 had indeed inserted into the transposon mutant genomes. Secondly, primers were designed *c.* 500 bp on either side of the suspected transposon insertion sites. PCR was conducted using each of the *tesA*, *drrB*, *pks6* and *pks11* specific primers with the hygromycin specific primers. PCR products of the correct size were generated with transposon mutant genomic DNA; only nonspecific products were generated using *M. tuberculosis* genomic DNA. The use of transposon-specific, in combination with gene-specific, primers verified that the transposon insertion sites were within the *tesA*, *drrB*, *pks6* and *pks11* open reading frames, as predicted. Finally, the PCR products (generated using the SP3 primer designed at GSK to extend outwards from Tn5370 and the gene-specific primers described above) from transposon mutant single colonies picked from 7H10 plates (containing 100 μg ml^{-1} hygromycin) were sequenced and compared by homology searching to the *M. tuberculosis* H37Rv genome. This confirmed that the transposons were inserted into the open reading frames of the probable thioesterase *tesA*, the predicted ABC transporter *drrB* and the putative polyketide synthases, *pks6* and *pks11*.

Lipid TLC

Apolar and polar lipid fractions were extracted and compared with WT *M. tuberculosis* H37Rv by 2D TLC (Dobson *et al.* 1985). PDIM were missing from the *tesA*Δ, *drrB*Δ and *pks11*Δ apolar lipid fractions (Fig. 2) but present in *pks6*Δ. Unknown WT *M. tuberculosis* polar lipids were absent in the *pks6*Δ (Fig. 2) but retained in the other mutants (data not shown).

RT-PCR across the *tesA-Rv2929* junction

RT-PCR was performed on RNA, extracted from *in vitro* mid-log phase WT *M. bovis* BCG, to investigate the transcriptional organization of *tesA* and *Rv2929*, located in the PDIM gene cluster depicted in Fig. 1. Controls, ensuring that no products were generated from contaminating genomic DNA, were negative. An RT-PCR product of the correct size was detected, using primers designed to identify a *tesA-Rv2929* intergenic sequence (Fig. 3).

DISCUSSION

The *M. tuberculosis tesA* transposon mutant was unable to generate PDIM. This thioesterase is likely to be involved in the chain termination and release of lipid products from the multifunctional enzyme complexes in mycocerosic acid and/or phthiocerol generation (Minnikin *et al.* 2002). The product of *tesA* may therefore provide the thioesterase activity proposed to be lacking in the PPSA-E and MAS enzyme complexes. This is the first experimental confirmation of the involvement of *tesA* in PDIM production in mycobacteria. The experimental strategy also defines a novel approach, using a transposon mutant library to investigate the contribution of unknown genes in the generation of complex lipids in *M. tuberculosis*. Further to the work by Camacho *et al.* (2001) describing the transcriptional units of the PDIM-gene cluster, there is an evidence from RT-PCR (Fig. 3) that *tesA* may be transcriptionally linked with *Rv2929* (unknown function), located upstream of *fadD26* and the *ppsA-E* genes in the PDIM gene cluster (Fig. 1). This RT-PCR analysis, to define the transcriptional organization of *tesA*, does not take into account the phenomenon of transcriptional read-through.

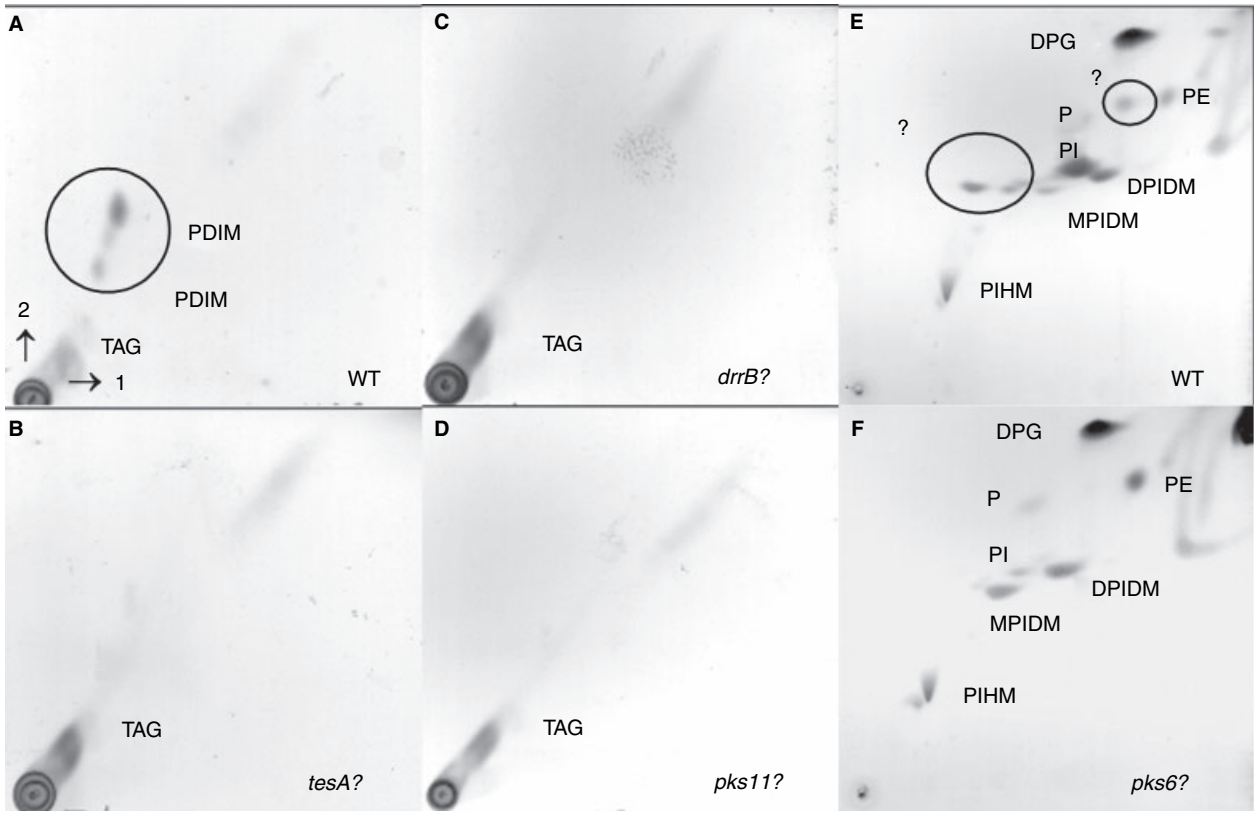

Fig. 2 TLC of lipid extracts; nonpolar WT (A), *tesA*Δ (B), *drrB*Δ (C), *pks11*Δ (D); polar WT (E), *pks6*Δ (F). Plates were developed with solvent system A (A–D) or E (E–F), according to Dobson *et al.* (1985), and sprayed with 5% ethanolic molybdophosphoric acid followed by charring. TAG, triacylglycerol; ?, uncharacterised polar lipids; P, unknown phospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; MPIDM and DPIDM, mono- and diacyl-phosphatidylinositol dimannosides; PIHM, phosphatidylinositol hexamannosides

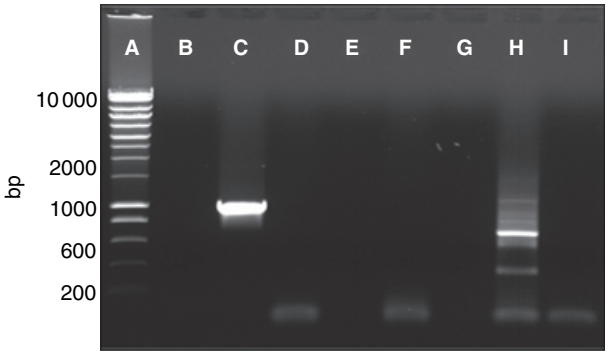

Fig. 3 Ethidium bromide stained 0·8% agarose electrophoresis gel of products from the RT-PCR/PCR control reactions to determine the presence of RNA between the *tesA* and *Rv2929* open reading frames. A: Hyperladder I electrophoresis markers from 10 000 to 200 bp; B, C: PCR control reactions, using primers for an intragenic 975 bp product in *fadD26* (used in all control reactions) ∓ *Mycobacterium tuberculosis* DNA template; D, E: RT-PCR control reactions ± RT containing no template RNA; F, G: RT-PCR control reactions ± RT containing RNase-treated RNA; H, I: RT-PCR across the *tesA-Rv2929* junction ± RT. A product of 680 bp was generated with the addition of RT only

Disruption of *drrB* resulted in the loss of PDIM production in *M. tuberculosis*. The *drrA/B/C* genes are highly similar to daunorubicin ABC transporters found in *Streptomyces* species; *drrA* codes for a nucleotide binding domain and *drrB/C* encodes membrane-spanning domains (Braibant *et al.* 2000). Camacho *et al.* (2001) described a *drrC* mutant that was only reduced in PDIM production. The PDIM in this mutant was localized mainly in the cytosol and not in the cell wall or culture medium. The total loss of PDIM in the *drrB*Δ, described here, may be explained by the compensation of *drrC* function by the products of *drrA* and *drrB*, which are located directly upstream of *drrC* (Fig. 1). Indeed, increased resistance was conferred to a number of antibiotics in *Mycobacterium smegmatis* by the simultaneous expression of *M. tuberculosis drrA/B* alone (Choudhuri *et al.* 2002). It is unclear why the interruption of these transport-related genes affects the generation of PDIM, when the disruption of *mmpL7*, which codes for a conserved PDIM translocation membrane protein does not (Cox *et al.* 1999). However, a polar effect of the transposon insertion on the expression of the *ppsA-E* gene products cannot be excluded.

PDIM were not detectable in the *M. tuberculosis pks11* transposon mutant. The *pks11* gene codes for a putative type III chalcone-like polyketide synthase. Chalcone synthases catalyse the condensation of one coumaroyl-CoA and three malonyl-CoA moieties to generate chalcone, a secondary metabolite in the production of plant flavonoids and antifungal agents (Jez *et al.* 2001). Recently, purified PKS11 and PKS18 have been demonstrated to catalyse the formation of α-pyrones, using long-chain aliphatic precursors *in vitro* (Saxena *et al.* 2003). The *pks11* ORF is located in a cluster of *pks* genes in the *M. tuberculosis* genome (*pks10/7/8/17/9/11*). Of these genes, *pks10* (a chalcone-like synthase) and *pks7* have been identified to be involved in PDIM generation in *M. tuberculosis* (Rousseau *et al.* 2003; Sirakova *et al.* 2003a) and *pks8/17* (*msl5*) has been implicated in the biosynthesis of monomethyl-branched unsaturated fatty acids (Dubey *et al.* 2003). The loss of PDIM generation in the *pks11* transposon mutant suggests that the gene product of *pks11* is likely to be involved in the production of precursor molecules necessary for successful PDIM generation in *M. tuberculosis*. As with other mutants identified in putative polyketide synthase genes lacking PDIM, *pks10*, *pks7*, *pks12* (Sirakova *et al.* 2003b) and *pks15/1* (Sirakova *et al.* 2003a), *pks11* probably plays a role in the biosynthesis of phthiocerol or phenolphthiocerol. The potential ring structures generated by the products of *pks11* (and putatively by *pks10* and *pks18*) may be a distinct step in the biosynthesis of phenolphthiocerol or it may be that the chalcone-like polyketide synthases *pks10* and *pks11* provide specificity for an additional range of starter molecules during PDIM generation. Interestingly, *Mycobacterium leprae* is able to generate PDIM although a number of the polyketide genes, identified by gene knockouts to be essential for PDIM production in *M. tuberculosis*, do not appear to be functional. Numerous gene inactivation studies demonstrate that PDIM are required for the growth of *M. tuberculosis in vivo* (Camacho *et al.* 1999; Cox *et al.* 1999; Rousseau *et al.* 2003; Sirakova *et al.* 2003a; Sirakova *et al.* 2003b).

The loss of unknown polar lipids in the *pks6*Δ, compared with WT *M. tuberculosis*, is significant, as the expression of *pks6* has been identified to be up-regulated in THP-1 phagocytosed-*M. bovis* BCG (Li *et al.* 2004). Additionally, a *pks6* transposon mutant is attenuated for growth in mouse lung (Camacho *et al.* 1999). The true significance of this finding will have to await the isolation and characterization of these polar lipids from WT *M. tuberculosis* H37Rv. In previous studies, *M. tuberculosis* H37Rv did not produce corresponding lipids when cultivated on less-rich Sauton's medium (Minnikin *et al.* 1986). Growth media can have a profound effect on mycobacterial polar lipid expression, as exemplified by the enhanced production of a polar glycolipid (Minnikin *et al.* 1989) in *Mycobacterium marinum*, when grown on Middlebrook 7H9, in comparison with Sauton's (A.M. Burguière, M. Ridell, D.E. Minnikin and G.S. Besra, unpublished data). Polar glycolipids of this type may include methyl-branched acyl components, as found in the lipo-oligosaccharides from the 'Canetti' variants of *M. tuberculosis* (Daffé *et al.* 1991); *pks6* may be involved in the biosynthesis of such branched fatty acids.

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