

# Virulence attenuation of two Mas-like polyketide synthase mutants of *Mycobacterium tuberculosis*

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The cell envelope of pathogenic mycobacteria is highly distinctive in that it contains a large number of structurally related very long multiple methyl-branched fatty acids. These complex molecules are thought to play important roles in cell envelope organization and virulence. The genetic and enzymic characterization of the polyketide synthase Mas, which is responsible for the synthesis of one such family of fatty acids (the mycocerosic acids), paved the way towards the identification of other enzymes involved in the synthesis of methyl-branched fatty acids in *M. tuberculosis*. In an effort to elucidate the origin of these complex fatty acids and their possible involvement in pathogenesis, the two *mas*-like polyketide genes *pks5* and *pks7* were disrupted in *M. tuberculosis* and the effects of their inactivation on fatty acid composition and virulence were analysed. While the disruption of *pks7* resulted in a mutant deficient in the production of phthiocerol dimycocerosates, the cell envelope composition of the *pks5* mutant was found to be identical to that of the wild-type parental strain *M. tuberculosis* H37Rv. Interestingly, both the *pks5* and *pks7* mutants displayed severe growth defects in mice.

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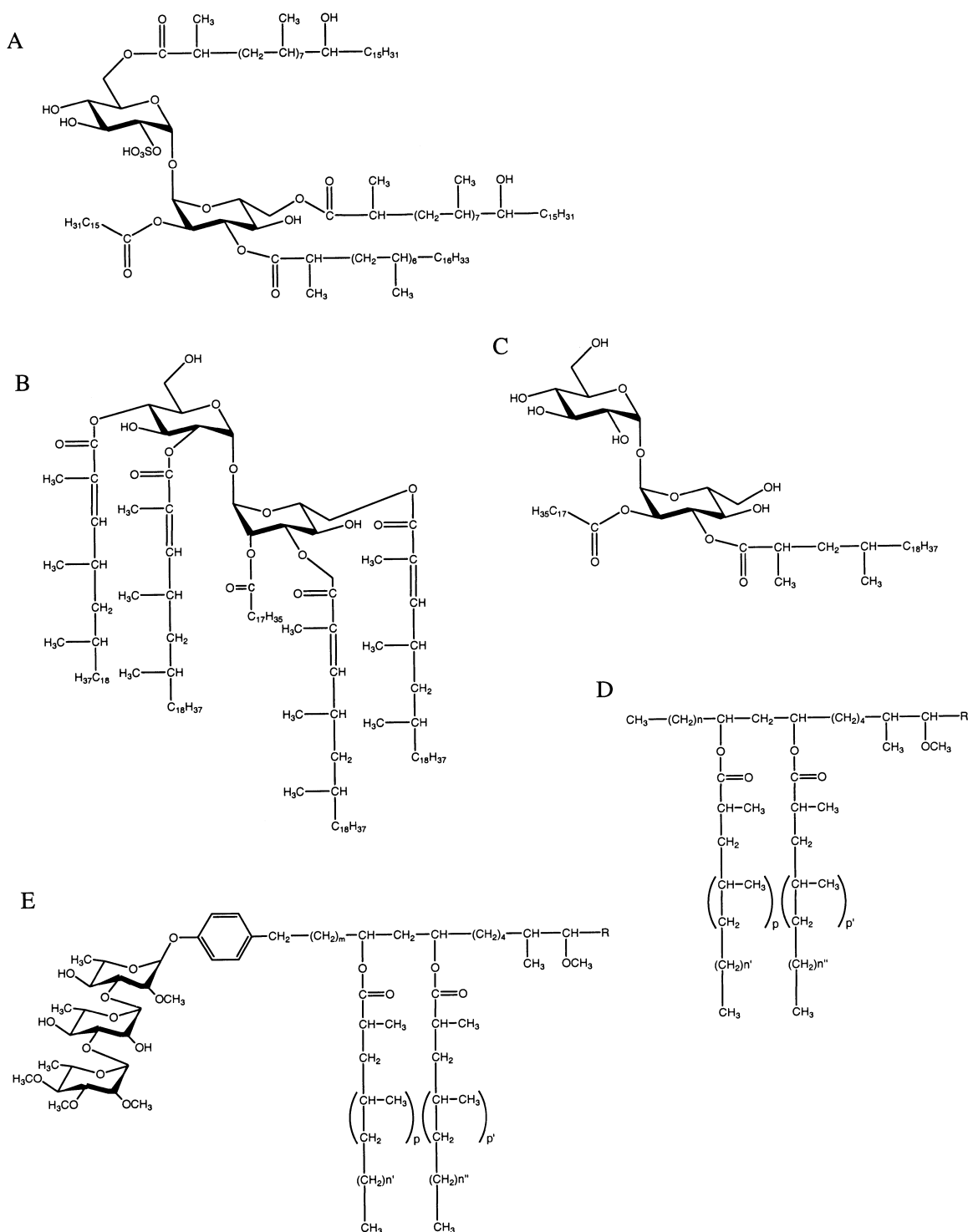
## INTRODUCTION

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* in humans, remains an important cause of mortality and morbidity worldwide (World Health Organization, 2002). Part of the success in combating this disease will involve the development of new antimycobacterial therapies enabling the control of multidrug-resistant tuberculosis. The complex cell envelope surrounding *M. tuberculosis* is one of the major causes of the resistance of this pathogen to host defence mechanisms (Daffé & Draper, 1998) and to therapeutic agents (Jarlier & Nikaido, 1994). It is also the site of action of many front-line anti-TB drugs (Chopra & Brennan, 1998). The identification of unique processes involved in the synthesis of cell envelope components of pathogenic mycobacteria may bring to light attractive drug targets for the development of new anti-TB drugs.

The existence of a large number of very long multiple methyl-branched fatty acids in pathogenic mycobacteria led to the suggestion that these molecules may play a significant

role in virulence. In *M. tuberculosis*, lipids esterified with multiple methyl-branched fatty acyl substituents include sulfolipids (SL) (Goren & Brennan, 1979), di- and triacylated trehaloses (DAT and TAT) (Lemassu *et al.*, 1991; Besra *et al.*, 1992; Muñoz *et al.*, 1997), polyacyltrehaloses (PAT) (Minnikin *et al.*, 1985; Daffé *et al.*, 1988) and phthiocerol dimycocerosates (DIM) (Daffé & Lanéelle, 1988) (Fig. 1). Although the last 50 years have seen considerable effort devoted to studying the possible roles of these molecules in pathogenesis (Goren & Brennan, 1979), we still know little about their biosynthesis. Lipids of the DIM family have received the most attention. In these lipids, 2,4,6,8-tetramethyl C<sub>32</sub> fatty acids and homologues (mycocerosic acids) are esterified to a long-chain  $\beta$ -diol, the phthiocerol (Fig. 1). The genetics and enzymology of the *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) polyketide synthase Mas, which is involved in the synthesis of mycocerosic acids, have been studied in detail (for a review, see Kolattukudy *et al.*, 1997). Moreover, DIM were recently shown to contribute to the impermeability of the cell envelope of *M. tuberculosis* (Camacho *et al.*, 2001; Sirakova *et al.*, 2001) and to the replication of this bacterium in the lungs of mice (Camacho *et al.*, 1999; Cox *et al.*, 1999). Analysis of the *M. tuberculosis* genome sequence has revealed the existence of several polyketide synthase genes (*pks*), seven of which are very similar to *mas* in terms of size, functional domains and relative positions of the domains

Abbreviations: BCG, Bacille Calmette–Guérin; DAT, diacyltrehaloses; DIM, dimycocerosyl phthiocerol; Mas, mycocerosic acid synthase; PAT, polyacyltrehaloses; PGL, phenolglycolipid; radio-GC, gas chromatography by radioactivity detection; SL, sulfolipids; TAT, triacyltrehaloses; TB, tuberculosis; Ts, temperature-sensitive.



**Fig. 1.** Structures of some methyl-branched fatty acid-containing lipids of *M. tuberculosis*. A, The major sulfolipid SL-I; trehalose is sulfated at the 2' position and esterified with palmitic acid and the multimethyl-branched phthioceranic and hydroxyphthioceranic acids. B, Polyacyltrehalose (PAT); trehalose is esterified with stearic acid and the multimethyl-branched mycolipenic acids. C, 2,3-Di-O-acyltrehalose (DAT); trehalose is esterified with stearic acid and the multimethyl-branched mycosanoic acid. D, Phthiocerol dimycocerosates (DIM); the long-chain  $\beta$ -diol (phthiocerol moiety) is esterified with two mycocerosic acids;  $n = 20-22$ ;  $n', n'' = 16, 18$ ;  $p, p' = 2-5$ ;  $R = -CH_2-CH_3$  or  $-CH_3$ . E, Phenolic glycolipid isolated from *M. tuberculosis* Canetti (PGL);  $m = 15-17$ .

(Sirakova *et al.*, 2001). In an attempt to determine the function of mycobacterial *pks* genes, mutants deficient in the expression of the *ppsB/ppsC*, *pks2*, *pks3/4*, *pks10* and *pks15/1* genes were constructed by allelic replacement in *M. tuberculosis* or *M. bovis* BCG. Disruption of the *pks2* gene in *M. tuberculosis* H37Rv resulted in a mutant that does not produce the hepta- and octamethyl-branched phthioceranic and hydroxyphthioceranic acids and is therefore SL-deficient (Sirakova *et al.*, 2001) (Fig. 1). Disruption of the *pks3/4* genes in *M. tuberculosis* H37Rv resulted in a mutant deficient in the production of the di- and trimethyl-branched fatty acids mycolipenates, mycolipodienates and mycosanoates required for the synthesis of some forms of PAT and DAT (Dubey *et al.*, 2002; Rousseau *et al.*, 2003) (Fig. 1). Disruption of *ppsB* and *ppsC* in *M. bovis* BCG led to a mutant unable to synthesize the phthiocerol and phenolphthiocerol derivatives required for the production of DIM and the *M. bovis*-specific phenolglycolipids (PGL) (Azad *et al.*, 1997). Interestingly, disruption of the *pks1* gene abolished the production of PGL but not that of DIM in *M. bovis* BCG (Constant *et al.*, 2002), whereas in *M. tuberculosis* H37Rv (a strain naturally deficient in PGL production), a similar mutation in *pks1* led to DIM deficiency (Sirakova *et al.*, 2003). In *M. bovis* BCG, it was proposed that the fused *pks15-pks1* ORFs are involved in the elongation of *p*-hydroxybenzoic acid derivatives to form *p*-hydroxyphenylalkanoates which are in turn converted into phenolphthiocerol derivatives (Constant *et al.*, 2002). In *M. tuberculosis* H37Rv, the two adjacent separate ORFs *pks15* and *pks1* are apparently unable to catalyse such an elongation reaction (Constant *et al.*, 2002), and are instead thought to be involved in the synthesis of phthiocerol derivatives (Sirakova *et al.*, 2003). Finally, the *pks10* gene, disruption of which in *M. tuberculosis* H37Rv also caused DIM deficiency without affecting the ability of the mutant strain to synthesize mycocerosic acids, is thought to be involved in the production of phthiocerol derivatives (Sirakova *et al.*, 2003). As for *pks15-pks1* in *M. tuberculosis* H37Rv, the precise function of *pks10* is currently unclear.

In order to elucidate the role of other *mas*-like genes in *M. tuberculosis*, we constructed *M. tuberculosis* H37Rv mutants deficient in the expression of the *mas*-like genes *pks5* and *pks7* and undertook their characterization. Of all the *M. tuberculosis* *pks* genes, *pks5* is the most similar to *mas* (66% amino acid identity). *pks7* is part of a four-*pks* gene cluster consisting of *pks7*, *pks8*, *pks17* and *pks9*, flanked by two chalcone-synthase-like genes, *pks10* and *pks11*. *pks7* shares 31% amino acid identity with *Mas*. We report here the construction and biochemical characterization of these two mutants and the analysis of their growth characteristics in cellular and animal models.

## METHODS

**Bacterial strains and growth conditions.** *Escherichia coli* XL-1 Blue and DH5 $\alpha$ , the strains used for cloning experiments, were routinely propagated in Luria-Bertani (LB) broth and LB agar (Difco)

at 37 °C. *Mycobacterium smegmatis* mc<sup>2</sup>155 was cultured at 37 °C in liquid LB medium supplemented with 0.05% Tween 80 for competent cell preparation and in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 for transduction. *M. tuberculosis* H37Rv (ATCC 25618) was cultured in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC enrichment (Difco) and 0.05% Tween 80 or on agar Middlebrook 7H11 medium (Difco) supplemented with OADC (Difco). Kanamycin (20  $\mu$ g ml<sup>-1</sup>), hygromycin B (50  $\mu$ g ml<sup>-1</sup>), ampicillin (100  $\mu$ g ml<sup>-1</sup>) and sucrose (2%) were added to the medium when required.

**Electrotransformation, cloning and Southern blot analysis.** Molecular cloning, restriction endonuclease digestions and electrotransformation were performed by standard techniques. Southern blot analysis was performed as previously described (Pelicic *et al.*, 1997).

**Generation of *M. tuberculosis pks5* and *pks7* mutants by allelic replacement.** A *pks5* mutant of *M. tuberculosis* H37Rv was constructed by allelic replacement using the Ts/*sacB* procedure described by Pelicic *et al.* (1997). The vector used for gene inactivation was constructed as follows. A *M. tuberculosis* H37Rv 5418 bp *Bam*HI restriction fragment carrying part of the *pks5* coding sequence was excised from the integrative cosmid I380 (kindly provided by Professor S. T. Cole, Institut Pasteur, Paris, France) and inserted into the *Bam*HI site of the pBluescript II KS(-) vector. An internal 1746 bp *Sph*I restriction fragment from the *pks5* gene was then excised from the vector and replaced with the 1.2 kb kanamycin-resistance cassette (*km*) from pUC4K, such that *km* was flanked by 1.7 and 2.0 kb segments of *pks5*. The resulting disrupted *pks5::km* allele was then excised from the pBluescript II KS(-) vector and inserted together with the *xyIE* gene into pPR27 (Pelicic *et al.*, 1997), yielding p27P5KX, which was used in the allelic exchange experiment.

The method used to disrupt *pks7* in *M. tuberculosis* H37Rv relies upon the use of a conditionally replicating mycobacteriophage (Bardarov *et al.*, 1997). A DNA fragment containing part of the *pks7* gene (bp 1044–4639 of the coding sequence) was amplified from genomic *M. tuberculosis* H37Rv DNA, using the following primers: sense primer, 5'-GGATCCATTGAGGCGCAGGCG-3' (A), antisense primer, 5'-GGATCCGTCAAAACCCATCGCC-3' (B), introducing *Bam*HI sites at the 5' and 3' ends of the sequence. The 3595 bp PCR product was inserted into the *Bam*HI-digested pUC19 vector and a 1577 bp *Mlu*I fragment was replaced by a hygromycin-resistance gene cassette (*hyg*), such that *hyg* was flanked by 1.3 and 0.9 kb segments of *pks7*. The resulting disrupted *pks7* gene and flanking regions were cloned into the *cos* vector pYUB572 and the recombinant cosmid was digested with *Pac*I and ligated into *Pac*I-digested pAE87 DNA. The ligation mixture was packaged using lambda *in vitro* packaging mix (Gigapack III, Stratagene), transduced into *E. coli* HB101 and plated on LB plates with hygromycin. DNA from several phasmid clones was isolated, confirmed by restriction digestion, electroporated into *M. smegmatis* strain mc<sup>2</sup>155 and plated for plaques at 30 °C. Individual plaques were tested for thermosensitivity, amplified and used to infect *M. tuberculosis* H37Rv as previously described (Sirakova *et al.*, 2001).

**Biochemical analysis of cell envelope lipids in the wild-type and *pks5*- and *pks7*-disrupted mutants of *M. tuberculosis*.** [1-<sup>14</sup>C]Propionate (50  $\mu$ Ci; specific activity 55 Ci mol<sup>-1</sup>, 2035 GBq mol<sup>-1</sup>) and [1-<sup>14</sup>C]acetate (50  $\mu$ Ci; specific activity 56.7 Ci mol<sup>-1</sup>, 2098 GBq mol<sup>-1</sup>) (American Radiolabelled Chemicals) were added to 30 ml of 10–12-day-old cultures (OD<sub>600</sub> 1.6–1.8) of *M. tuberculosis* H37Rv and its *pks5*- and *pks7*-disrupted mutants, and incubation was continued for a further 24 h at 37 °C in roller bottles. [1-<sup>14</sup>C]Propionate is a precursor molecule known to be incorporated into methyl-branched fatty acids. [1-<sup>14</sup>C]Acetate is incorporated into all classes of fatty acids, allowing the analysis of all lipid types. Cells

**Table 1.** Solvent systems for TLC analysis of lipids

Solvent system	Components*	Types of lipids resolved†
A	1. Petroleum ether/acetone (92:8) ( $\times 3$ ) 2. Toluene/acetone (95:5) ( $\times 1$ )	FA, PAT
B	1. Chloroform/methanol/water (100:14:0.8) ( $\times 1$ ) 2. Chloroform/acetone/methanol/water (50:60:2.5:3) ( $\times 1$ )	TDM, SL, DAT
C	1. Chloroform/methanol (96:4) ( $\times 1$ ) 2. Toluene/acetone (80:20) ( $\times 1$ )	FA, G
D	1. Petroleum ether/ethyl acetate (98:2) ( $\times 3$ ) 2. Petroleum ether/acetone (98:2) ( $\times 1$ )	DIM, TAG, MQ
E	Chloroform/methanol/water (90:10:1) ( $\times 1$ )	TDM, SL, DAT
F	Chloroform/methanol/water (65:25:4) ( $\times 1$ )	PI, PE, CL, PIM, TMM, TDM, DAT
G	Chloroform/methanol/water (20:4:0.5) ( $\times 1$ )	DAT, TDM, TMM, PE
H	Chloroform/methanol (95:5) ( $\times 1$ )	M

\* ( $\times n$ ) indicates the number of independent developments with the indicated solvent system. 1 refers to the solvent used in the first dimension of silica gel TLC plates, 2 to the second dimension. The boiling point range of the petroleum ether was 60–80 °C.

† CL, cardiolipin; DAT, diacyltrehaloses; DIM, dimycocerosyl phthiocerol; FA, fatty acids; G, glycosides; M, mycolic acids; MQ, menaquinone; PAT, polyacyltrehaloses; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; SL, sulfolipids; TAG, triacylglycerides; TDM, trehalose dimycolates; TMM, trehalose monomycolates.

were collected and total extractable lipids from bacterial pellets and culture supernatants were extracted as described by Sirakova *et al.* (2001). Total cellular and extracellular [1-<sup>14</sup>C]propionate- and [1-<sup>14</sup>C]acetate-labelled lipids from H37Rv wild-type and *pks* mutants were subjected to one- and two-dimensional thin-layer chromatography (TLC) on silica gel G plates in a variety of solvent systems to resolve lipids of various polarities (Table 1), and the lipids were detected by autoradiography. Labelled lipids recovered from TLC plates were eluted from the silica gel with chloroform/methanol (2:1, v/v). Fatty acid methyl esters were prepared from total lipids derived from [1-<sup>14</sup>C]propionate and from DIM, PAT, SL and DAT fractions isolated by TLC. Methyl esters of fatty acids and acetylated hydroxy-fatty acids were subjected to radio-GC analysis (Sirakova *et al.*, 2001; Dubey *et al.*, 2002). The methyl esters of fatty acids from the total [1-<sup>14</sup>C]propionate-derived lipids of both the wild-type strain and the *pks* mutants were also separated by argentation-TLC (5% AgNO<sub>3</sub> in silica gel) using hexane/ethyl ether (9:1, v/v) as the developing solvent and each separated fatty acid methyl ester fraction was analysed by radio-GC using conditions similar to those previously described (Dubey *et al.*, 2002). Mycolic acid methyl esters prepared from cells that had incorporated [1-<sup>14</sup>C]acetate were analysed as previously described (Kremer *et al.*, 2002).

**Extraction of RNA from *M. tuberculosis* and RT-PCR experiments.** Cultures of *M. tuberculosis* were grown in 7H9 medium to OD<sub>600</sub> 0.6. For analysing the expression of the *pks5* gene, bacteria

were harvested and broken in 1 ml Trizol solution (Life Technologies) with mini glass beads using a Bead Beater apparatus (Polylabo) set at maximum speed. RNA was extracted with 200 µl chloroform/isoamyl alcohol (Ready Red, Appligene-Oncor). After 15 min centrifugation at 12 000 g, the aqueous phase was transferred to a tube containing 500 µl 2-propanol. Total RNA was precipitated for 10 min at room temperature, centrifuged for 10 min at 12 000 g and washed with 1 ml of a 75% ethanol solution before resuspension in DEPC-treated water (Ambion). Contaminating DNA was removed by digestion with DNase I according to the manufacturer's instructions (Ambion). The same protocol was used to extract RNA from *M. tuberculosis* infecting resting mouse bone-marrow macrophages. Macrophages were infected at an m.o.i. of five bacteria per cell. Sixteen hours post-infection, cells were washed three times with fresh medium and then lysed with cell culture lysis reagent (Promega). RNA was extracted from the lysate as described above. Reverse transcription was performed using the Expand Reverse Transcriptase (Roche) with 2 µg total RNA and 50 pmol antisense primers specific for *pks5* and *sigA* (Table 2). Quantitative PCR was performed on a Light Cycler apparatus (Roche), using the SYBR Green technique (Fast start DNA Master SYBR Green I, Roche) with specific primers for *pks5* and *sigA* (Table 2). The number of cDNA copies present in each sample was determined as previously described (Manganelli *et al.*, 1999). The number of *pks5* mRNA copies in bacteria grown axenically and inside macrophages was compared to the number of copies of *sigA* mRNA in the same

**Table 2.** Primers used for RT-PCR experiments

Gene	Forward primer (5'–3')	Antisense primer (5'–3')
<i>sigA</i>	GGCCAGCCGCGCACCCCTTGAC	GTCCAGGTAGTCGCGCAGGACC
<i>pks5</i>	CCGACTGGAACCTCTTGCCA	TCAACCGATACACCGTGCTC
<i>pks7</i>	CGGCGCTACGGGTGAACCG	CCGCACCCCATCTGCCGCGC
<i>pks8</i>	TGACGTTGGCATCATGCCC	CATCTCGATGAAGCGACCG
<i>pks17</i>	ATTGCGCAGATGCTGGCC	GTCCACCAATGCTGCGAC
<i>pks10</i>	GCCGTGGTTCGAGCAGTATCTG	CACATGCAGCACCGACGCTGA

sample (Manganelli *et al.*, 1999). RT-PCR experiments were performed on the RNA extracted from three independent 7H9 cultures of *M. tuberculosis* and from three independent preparations of macrophages infected with *M. tuberculosis*. Analysis of the *pks7*, *pks8*, *pks17* and *pks10* transcripts in the wild-type H37Rv and *pks7* mutant strains was performed as follows. RNA was isolated from bacteria grown to mid-exponential phase. Chilled bacterial cells collected by centrifugation were resuspended in RNeasy lysis buffer (Qiagen), transferred to a 2 ml tube containing ceramic and silica beads (FastRNA blue) and disrupted using a FastPrep F120 instrument (Q.BIOgene). The extract collected by centrifugation was used to isolate total RNA using RNeasy kit (Qiagen) according to the protocol provided by the manufacturer. Reverse transcription was performed using random primers and SuperScript RNase H reverse transcriptase (Life Technologies). PCR on the cDNA was done with Platinum Taq DNA polymerase (Invitrogen) and the sets of specific primers listed in Table 2. A control without the reverse transcriptase verified the absence of DNA contamination.

**Preparation and infection of murine bone-marrow macrophages.** Bone-marrow macrophages were prepared as previously described (Jackson *et al.*, 1999) and infected at an m.o.i. of 0.5 bacterium per cell for 4 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. For experiments with activated macrophages, 100 U IFN $\gamma$  ml<sup>-1</sup> and 10 ng TNF $\alpha$  ml<sup>-1</sup> were added to the medium 24 h before infection, and maintained throughout the infection process. On days 0 (4 h), 3, 5 and 7 the infected macrophage monolayers (3 wells per strain) were lysed in 200  $\mu$ l cell culture lysis reagent (Promega) and the number of viable intracellular c.f.u. was evaluated by plating the lysed cell suspensions on 7H11, 7H11-Km or 7H11-Hyg plates. Infection experiments were carried out in duplicate using two independent batches of bacterial stocks and cell preparations.

***M. tuberculosis* growth and persistence in mice.** *M. tuberculosis* H37Rv wild-type and mutant strains were used to infect female BALB/c mice (6–8 weeks old) via the respiratory route. *M. tuberculosis* aerosols were generated from bacterial suspensions consisting of 10<sup>7</sup> c.f.u. ml<sup>-1</sup> in phosphate-buffered saline (PBS) (pH 7.4) with 0.05% Tween 80. Mice were exposed to the aerosols for 15 min. Four to six mice were used per experimental point and per strain. At various time points after infection (days 1, 7, 14, 20, 42 and 98 for the *pks5* mutant; days 1, 7, 14, 22, 43, 64 and 100 for the *pks7* mutant), the lungs and spleen were removed aseptically and homogenized in Sauton's medium diluted 1:4 and supplemented with Middlebrook OADC. The viable bacteria in the organs of infected animals were counted by plating serial dilutions of the organ homogenates on 7H11, 7H11-Km or 7H11-Hyg agar.

## RESULTS

### Disruption of the *pks5* and *pks7* genes by allelic replacement

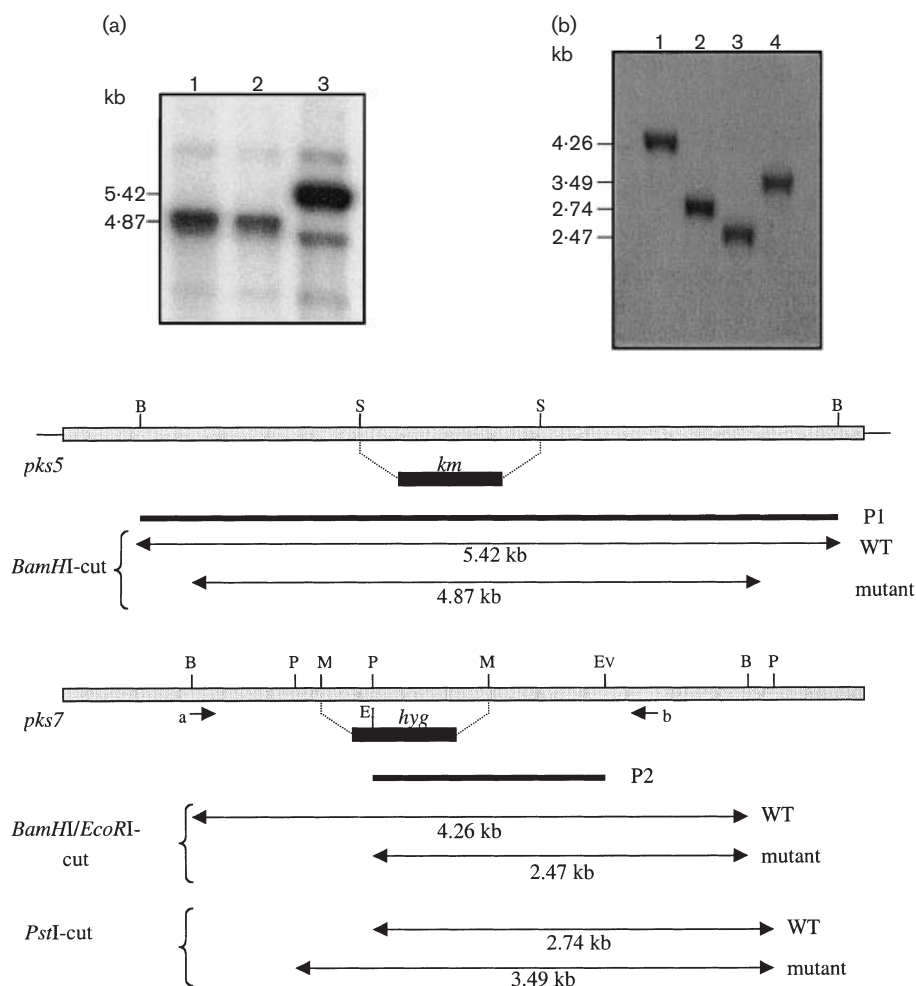
A *pks5* and a *pks7* mutant of *M. tuberculosis* H37Rv were constructed by allelic replacement using the Ts/*sacB* method described by Pelicic *et al.* (1997) and the temperature-sensitive mycobacteriophage system developed by Bardarov *et al.* (1997), respectively. Disrupted alleles of the *pks5* and *pks7* genes were generated by replacing 1746 bp of the *pks5* coding sequence and 1577 bp of the *pks7* coding sequence with a kanamycin- and a hygromycin-resistance cassette, respectively. In *pks5*, this deletion resulted in the loss of the regions encoding the acyl transferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR) and acyl-carrier protein (ACP) domains. In *pks7*, the deletion

resulted in the loss of the AT, DH and ER domains. Genomic DNA from two *pks5* and three *pks7* mutant candidates was analysed by Southern blotting and all mutants gave hybridization signals consistent with allelic replacement at the *pks5* or *pks7* locus (Fig. 2).

As the *pks7* gene is part of a cluster of four *pks* genes – *pks7*, *pks8*, *pks17* and *pks9* – separated by short intergenic spaces (23 bp between *pks7* and *pks8*, 3 bp between *pks8* and *pks17*, 9 bp between *pks17* and *pks9*) and flanked by two chalcone-synthase-like genes (*pks10* and *pks11*), we could not exclude the possibility that the mutation in *pks7* had a polar effect on the expression of neighbouring genes. To address this question, we examined the *pks7* mutant for *pks8*, *pks17* and *pks10* transcripts using a gel-based RT-PCR assay. *pks8*, *pks17* and *pks10* transcripts were detected in the *pks7* mutant (Fig. 3), indicating that the insertion of a hygromycin cassette within *pks7* had not abolished the expression of neighbouring genes, although their levels of expression and regulation might differ from those in the wild-type H37Rv strain.

### Biochemical characterization of the *pks5* and *pks7* mutants

We investigated the biochemical consequences of *pks5* and *pks7* gene disruption by labelling lipids with [1-<sup>14</sup>C] propionate, a precursor molecule known to be incorporated into multimethyl-branched fatty acids. The wild-type strain and the two *pks* mutants incorporated similar amounts (20–25%) of <sup>14</sup>C into lipids. Radio-GC analysis of the methyl esters of fatty acids from total lipids showed that both *pks* mutants generated all the classes of fatty acids produced by the wild-type strain. No difference was observed in the fatty acid profiles of the wild-type strain and the *pks5* mutant, but lower levels of mycocerosic acid labelling were observed in the *pks7* mutant than in the wild-type strain (Fig. 4). Argentation-TLC of fatty acid methyl esters from total lipids, followed by radio-GC analysis of individual fatty acid fractions purified from the TLC plate, showed that both *pks* mutants produced all classes of fatty acids but that the labelling of the mycocerosic-acid-containing fraction was much less in the *pks7* mutant than in the wild-type H37Rv and the *pks5* mutant (data not shown). When total lipids were separated by TLC using 10% ethyl ether in hexane as the solvent, about 30% of the incorporated <sup>14</sup>C was found in the dimycocerosyl phthiocerol (DIM) fraction in the wild-type strain, 40% was found in the DIM fraction of the *pks5* mutant, and the DIM fraction of the *pks7* mutant was barely labelled (Fig. 5). The higher degree of labelling of DIM in the *pks5* mutant affected both forms of DIM, the dimycocerosates of phthiocerol (DIM A) and the dimycocerosates of phthiodiolone (DIM B) (Fig. 5). The absence of DIM in the *pks7* mutant was confirmed by treating the plate with dichromate/H<sub>2</sub>SO<sub>4</sub> followed by heating. The charred band at R<sub>F</sub> 0.6 for the wild-type strain was not observed for the mutant (data not shown). Similarly, DIM was absent from the culture filtrates of the *pks7* mutant (data not shown). Thus, the *pks7* mutant



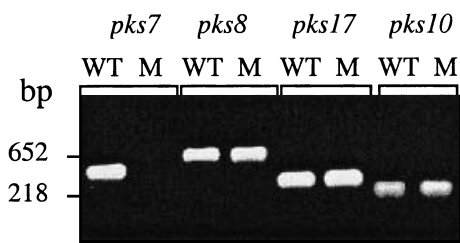
**Fig. 2.** Southern blot analysis of *M. tuberculosis pks5* and *pks7* knock-out strains. (a) Expected hybridization profile and Southern blot analysis of *M. tuberculosis pks5* knock-out strains. Genomic DNA from *pks5* mutant candidates was digested with *Bam*HI. Lanes 1–2, *pks5* mutants; lane 3, wild-type H37Rv. The specific probe P1 used to reveal the blot corresponds to the 5.42 kb *Bam*HI restriction fragment carrying *pks5* excised from cosmid I380. S, *Sph*I; B, *Bam*HI. (b) Expected hybridization profile and Southern blot analysis of a *M. tuberculosis pks7* knock-out strain. Genomic DNA from the *pks7* mutant candidate was digested with *Bam*HI/*Eco*RI (lanes 1 and 3) and *Pst*I (lanes 2 and 4). Lanes 1–2, wild-type H37Rv; lanes 3–4: *pks7* mutant. The specific probe P2 used to reveal the blot corresponds to the 1.82 kb *pks7 Pst*I–*Eco*RV restriction fragment containing part of the gene segment deleted in making the construct and 801 bp DNA 3' of the deletion. Primer pair a/b was used to amplify the *pks7* region chosen to generate the disruption construct. P, *Pst*I; B, *Bam*HI; M, *Mlu*I; EV, *Eco*RV; Ei, *Eco*RI.

is deficient in the production of DIM but capable of generating mycocerosic acids.

Detailed analysis of the more polar lipids labelled by [ $^{14}\text{C}$ ]propionate showed that no other lipid class was affected by the two *pks* gene disruptions. The distribution of  $^{14}\text{C}$ -radioactivity in the PAT, SL, and DAT fractions was very similar in the wild-type strain and in both *pks* mutants, except that the *pks7* mutant incorporated 67% more radioactivity into SL than did the wild-type strain (data not shown). Further detailed one- and two-dimensional TLC analyses of [ $^{14}\text{C}$ ]propionate- and [ $^{14}\text{C}$ ]acetate-derived lipids from cell pellets and culture filtrates of the

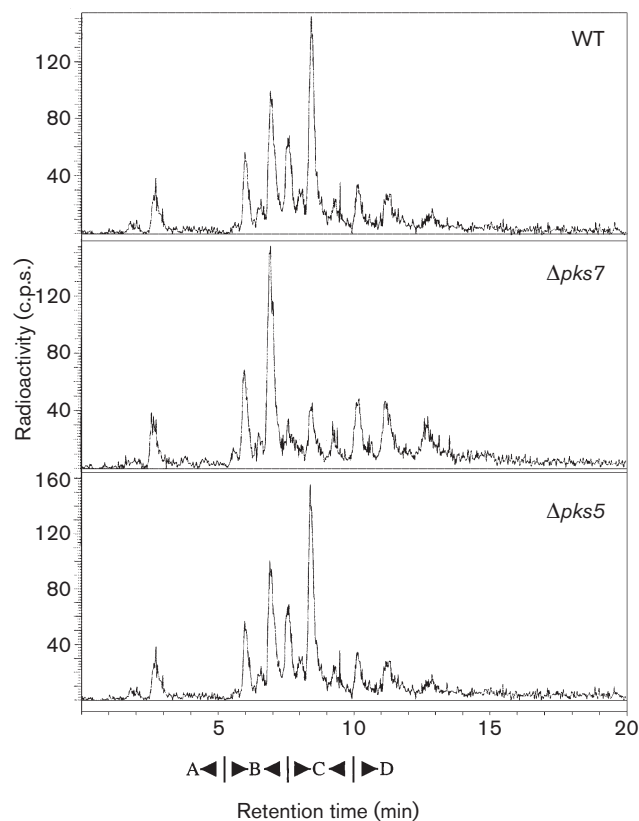
H37Rv wild-type strain and the *pks5* mutant collected at various growth stages (early and late exponential phase) were performed in a variety of solvent systems (Table 1). This allowed lipids of various polarities to be analysed, including all classes of phospholipids and phosphatidylinositol mannosides, acyltrehaloses (trehalose mono- and di-mycolates, SL, PAT, DAT), DIM, glycosides, triacylglycerides, menaquinones and free fatty acids and mycolic acids. No difference between the two strains was found.

The fatty acids derived from [ $^{14}\text{C}$ ]propionate present in the individual TLC fractions, DIM, PAT, SL and DAT, were also analysed by argentation-TLC and radio-GC as

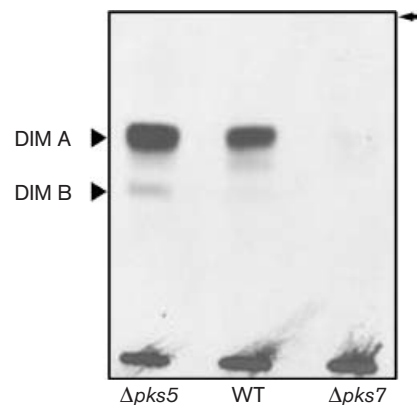


**Fig. 3.** RT-PCR analysis of the *in vitro* expression of the *pks7*, *pks8*, *pks17* and *pks10* genes in wild-type *M. tuberculosis* H37Rv and in the *pks7* mutant. The PCR products were of the size expected from the primers used (see Table 2) and there were no products when reverse transcriptase was omitted. WT, wild-type H37Rv; M, *pks7* mutant.

previously described (Dubey *et al.*, 2002), in order to define the methyl-branched fatty acid composition of each of these individual lipids. These analyses revealed a stronger  $^{14}\text{C}$ -labelling of hydroxyphthioceranic acids in the *pks7* mutant



**Fig. 4.** Radio-GC analysis of total fatty acid methyl esters derived from  $[1-^{14}\text{C}]$ propionate in *M. tuberculosis* H37Rv (top), *pks7* (middle) and *pks5* (bottom) mutants. Retention time ranges for branched short-chain acids (A) mycolipanoic and mycolipenic acids (B), mycocerosic acids (C) and phthioceranic acids (D) are indicated.



**Fig. 5.** Autoradiograms of thin-layer chromatograms of lipids derived from  $[1-^{14}\text{C}]$ propionate in *M. tuberculosis* H37Rv (WT) and two *pks*-disrupted mutants ( $\Delta pks5$  and  $\Delta pks7$ ). Total lipids were subjected to TLC on silica gel G with 10% ethyl ether in *n*-hexane as the solvent. DIM A (dimycocerosates of phthiocerol) and DIM B (dimycocerosates of phthiodiolone) are indicated by arrows. The arrow at the top of the right-hand side shows the solvent front.

than in the wild-type strain, probably accounting for the stronger labelling of SL in this mutant. No other significant difference in the fatty acid composition of individual  $^{14}\text{C}$ -propionate-labelled lipids between the wild-type strain and the *pks7* mutant was observed (data not shown). The relative amounts of  $^{14}\text{C}$  incorporated from  $[1-^{14}\text{C}]$ propionate into the different methyl-branched fatty acids present in PAT, SL and DAT in the *pks5* mutant (including  $\text{C}_{16}$ -,  $\text{C}_{18}$ -branched fatty acids and mycolipenic, mycosanoic, mycolipodienic, mycolipanoic, phthioceranic, hydroxyphthioceranic and mycocerosic acids) were identical to those found in the wild-type strain.

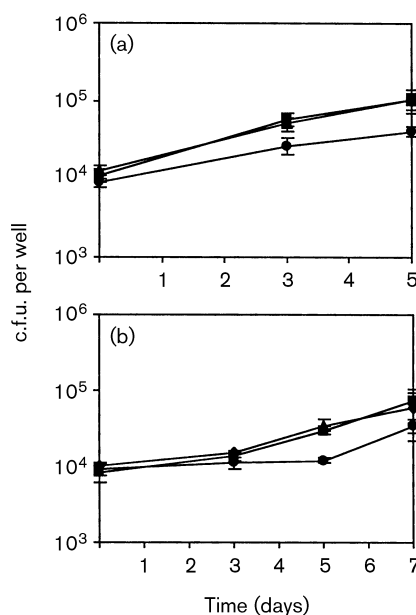
The delipidated cells remaining after soluble lipid extraction were also saponified in 2-methoxyethanol containing 12%  $\text{H}_2\text{O}$  and 5% KOH and methylated as previously described (Sirakova *et al.*, 2001) for analysis of potential cell-wall-linked methyl-branched fatty acids. TLC analysis of the fatty acid methyl esters prepared by this technique using hexane/ethyl ether (9:1, v/v) as the solvent system showed no difference between the wild-type H37Rv strain and the *pks5* mutant (data not shown). Finally, to investigate differences in cell-wall-bound mycolates, the mycolic acid methyl esters from  $[1-^{14}\text{C}]$ acetate-labelled delipidated cells were prepared and analysed as described elsewhere (Kremer *et al.*, 2002). Again, no significant difference was observed between the wild-type strain and the two *pks* mutants (data not shown).

Thus, *pks5* disruption did not seem to alter the fatty acid and lipid compositions of *M. tuberculosis*. The *pks5* gene was also disrupted in *M. bovis* BCG and thorough analyses of soluble and cell-wall-bound lipids derived from  $[1-^{14}\text{C}]$ propionate

and [ $^{14}\text{C}$ ]acetate showed no detectable difference in labelling patterns (data not shown).

### Growth kinetics of the *pks5* and *pks7* mutants *in vitro* and in mouse bone-marrow macrophages

The growth rates of the two mutants were similar to that of wild-type *M. tuberculosis* H37Rv in 7H9 broth (data not shown), suggesting that the changes affecting their cell envelope composition or structure had no effect on growth in this medium. We then analysed the ability of the two mutants to replicate and to persist within resting and activated mouse bone-marrow macrophages. The *pks5* mutant displayed a similar rate of intracellular growth to the parental strain H37Rv in both resting and activated macrophages (Fig. 6). Therefore, *pks5* is unlikely to be involved in the survival of *M. tuberculosis* within murine phagocytic cells, or in the resistance of mycobacteria to the respiratory burst that accompanies macrophage activation. In contrast, the *pks7* mutant replicated 1.7-fold less than did the wild-type strain in resting macrophages over a 5 day period. It also replicated 1.6-fold less than did the wild-type strain in activated macrophages over a 7 day period (Fig. 6). However, this difference in growth between the two strains was not statistically significant in Student's *t* test ( $P < 0.05$ ).



**Fig. 6.** Multiplication and persistence of *M. tuberculosis* H37Rv wild-type (▲), *pks5* (■) and *pks7* (●) mutant strains in resting (a) and activated (b) murine bone-marrow macrophages. The reported values are the mean and standard deviation of data obtained from three independent wells in one typical experiment. This experiment was executed twice using independent cell and bacterial cultures.

### Growth kinetics of the *pks5* and *pks7* mutants in an *in vivo* mouse model

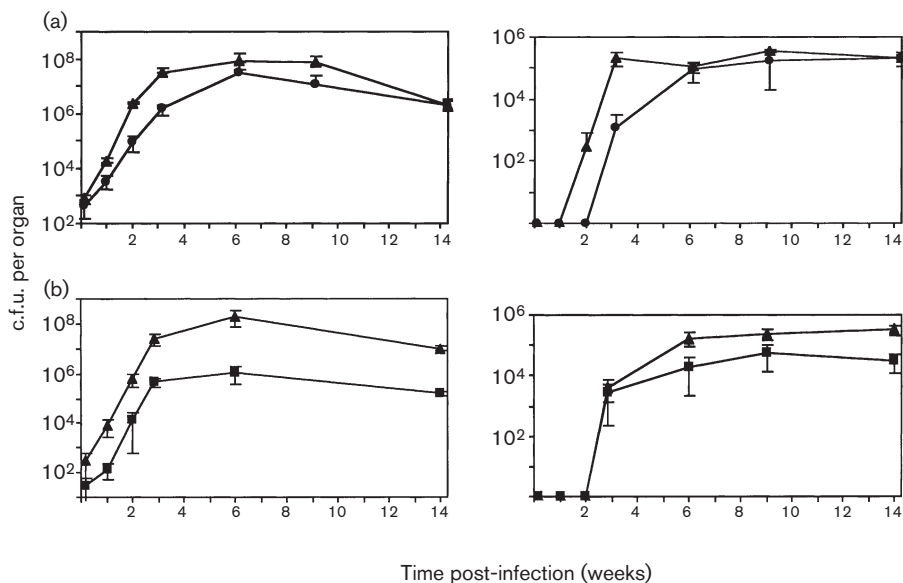
We then assessed the virulence of the *pks5* and *pks7* mutants in mice infected via the respiratory route. Consistent with the demonstrated role of DIM in the *in vivo* replication of *M. tuberculosis* (Camacho *et al.*, 1999; Cox *et al.*, 1999), the DIM-deficient *pks7* mutant showed attenuated growth during the acute phase of infection in both organs examined (Fig. 7a). One-twentieth the number of c.f.u. was recovered from the lungs of mice infected with the *pks7* mutant than from those of H37Rv-infected mice 21 days post-infection. The difference between the mutant and the wild-type strain was even more marked in the spleen, with 180 times more wild-type than mutant c.f.u. recovered at the same time point. These differences in c.f.u. counts were statistically significant in Student's *t* test ( $P < 0.05$ ) on days 7, 14 and 22 in the lungs and on day 21 in the spleen. At later stages of the infection, differences tended to lessen, suggesting that *pks7* plays a less important role in virulence during the persistent phase of infection.

The *pks5* mutant also multiplied much less extensively than did the parental strain in the lungs and in the spleen during the acute phase of infection (Fig. 7b). This difference between the two strains was especially obvious between days 21 and 42. During these 3 weeks, the rates of multiplication of the *pks5* mutant were about one-third and one-fifth of those of the wild-type strain in the lungs and in the spleen, respectively. There were no statistical differences between c.f.u. counts on day 1. However, differences in c.f.u. counts were statistically significant on days 7, 20, 42 and 98 in the lungs and on days 42 and 98 in the spleen ( $P < 0.05$ ).

### Expression of the *pks5* gene in 7H9-grown and intracellular-grown *M. tuberculosis*

The virulence attenuation of the *pks5* mutant in mice despite its apparent lack of biochemical phenotype when grown under axenic conditions led us to question the regulation of the expression of *pks5* during *in vitro* growth and host infection. We used a quantitative real-time RT-PCR assay to compare the level of transcription of *pks5* when *M. tuberculosis* is grown axenically or inside mouse bone-marrow macrophages. The amount of cDNA produced, which is proportional to the amount of the specific transcript present in the original RNA sample, was measured. In all experiments, *sigA* was used as an internal standard. *sigA* is an essential housekeeping sigma factor in *M. tuberculosis*, and the amount of *sigA* mRNA remains constant in different growth conditions (Manganelli *et al.*, 1999) and during macrophage infection (Manganelli *et al.*, 2001). This experiment, performed on three RNA preparations for each culture condition, indicated that *pks5* was expressed both in 7H9 medium and inside macrophages. The amount of *pks5* transcripts was found to be  $0.355 \pm 0.048$  that detected for *sigA* in *M. tuberculosis* grown in 7H9 medium, and  $0.221 \pm 0.056$  that detected for *sigA* in *M. tuberculosis* grown intracellularly. This difference in expression of the *pks5* gene





**Fig. 7.** Multiplication and persistence of *M. tuberculosis* H37Rv wild-type, *pks5* and *pks7* mutant strains in the lungs and spleen of mice infected via the respiratory route. (a) *M. tuberculosis* H37Rv wild-type (▲) and *pks7* mutant (●). (b) *M. tuberculosis* H37Rv wild-type (▲) and *pks5* mutant (■). The multiplication and persistence of the wild-type and mutant strains in the lungs (left panels) and spleens (right panels) of infected animals was followed by counting bacteria in these organs over a 100-day period. Results are expressed as means and standard deviations of c.f.u. counts for four to six infected mice.

under axenic and intracellular conditions was not statistically significant in Student's *t* test ( $P < 0.05$ ).

## DISCUSSION

In an effort to define the functions associated with the polyketide synthase genes (*pks*) of *M. tuberculosis* we followed a global genetic approach involving the construction and biochemical characterization of *pks*-deficient mutants (Sirakova *et al.*, 2001; Dubey *et al.*, 2002; Sirakova *et al.*, 2003). In this study, we disrupted the *mas*-like genes *pks5* and *pks7* and analysed the effects of their inactivation on fatty acid composition and virulence in *M. tuberculosis*.

Biochemical analyses of the *pks7* mutant indicated that this strain is deficient in DIM synthesis although it produces mycocerosic acids. Therefore, *pks7*, like the *ppsA-E*, *pks10* and *pks15/1* genes, is probably involved in the synthesis of phthiocerol derivatives (Azad *et al.*, 1997; Sirakova *et al.*, 2003). As the *pks7* gene is part of a cluster of four *pks* genes – *pks7*, *pks8*, *pks17* and *pks9* – separated by short intergenic spaces, we checked whether the mutation in *pks7* had a polar effect on the expression of neighbouring genes using a gel-based RT-PCR assay. Transcripts of the *pks8*, *pks17* and *pks10* genes were detected in the *pks7* knock-out strain, indicating that the insertion of a hygromycin cassette within *pks7* had not abolished the expression of adjacent genes. The two adjacent ORFs *pks8* and *pks17* together contain all the domains required for the synthesis of methyl-branched fatty acids (Sirakova *et al.*, 2001), and Pks8/17 displays 32%

amino acid identity to Mas. Pks9 (1017 amino acids in length) is 42% identical to Pks8 (1603 amino acids in length) on a 809 amino acid overlap. Therefore, Pks7, Pks8/17 and Pks9 appear to belong to the same polyketide synthase family, and could be involved in the same biosynthetic pathway leading to the production of DIM. Although the reason of the existence of such a high number of polyketide synthases dedicated to the production of phthiocerol derivatives and DIM is unclear, the fact that the *pks7*, *pks10*, *pks15/1* and *ppsA-E* mutants fail to synthesize DIM suggests that the polyketide synthase enzymes encoded by these genes serve non-redundant catalytic functions. Like Pks15/1 and Pks10, Pks7 may act upstream from PpsA-E in the pathway, catalysing the formation of precursors that are further elongated by PpsA-E to give the final phthiocerol products. Careful enzymic studies are required to define precisely the reactions catalysed by each of these enzymes.

Wild-type H37Rv and the *pks7* mutant replicated equally well in 7H9 broth, but the mutant grew slightly less well in activated and non-activated mouse bone-marrow macrophages. However, this difference in intracellular growth between the two strains was not statistically significant. This suggests that, although involved in the permeability of the cell envelope (Camacho *et al.*, 2001), DIM do not contribute in a major way to the resistance of the tubercle bacillus to the intracellular environment. We also cannot rule out the possibility that increased amounts of other lipids such as SL compensate for the absence of DIM in the *pks7* mutant. The *pks7* mutant showed reduced virulence during the

acute phase of infection in the lungs and spleen of mice infected via the respiratory route. This result is consistent with previous reports that mutations in other genes that caused DIM deficiency resulted in attenuation (Camacho *et al.*, 1999; Cox *et al.*, 1999). Interestingly, 3 weeks post-infection, *fadD26* and *fadD28* DIM-deficient mutants of *M. tuberculosis* Erdman and Mt103 were found to be five- to tenfold more attenuated for growth in the lungs of BALB/c and C57BL/6 mice infected intravenously (Camacho *et al.*, 1999; Cox *et al.*, 1999), than the *pk5* mutant in our mouse model of infection. As the *pk5* mutant produces mycocerosic acids whereas the *fadD28* mutant does not (Fitzmaurice & Kolattukudy, 1998), it is tempting to speculate that the presence of mycocerosic acids in the *pk5* mutant accounts for its higher virulence. As mentioned above, it is also possible that increased incorporation of the methyl-branched precursors into other lipids, such as SL, in the *pk5* mutant partially restores some virulence in this strain.

In contrast to the situation encountered in all *msh*-deficient mutants examined so far, and despite the high level of sequence similarity between *pk5* and *mas*, the disruption of *pk5* caused no major change in the fatty acid and lipid contents of the mutant strain. The mutant produced all the major methyl-branched fatty acid containing lipids, including DIM, in similar amounts to the wild-type strain. Interestingly, the replication of the *pk5* mutant was unaffected in mouse bone-marrow macrophages, but this mutant replicated much less efficiently than the wild-type strain in the lungs and spleen of BALB/c mice infected via the aerosol route, suggesting an important role for Pks5 or for its polyketide product in the course of infection. These findings provide support for the belief that key cell envelope lipids or polyketides might only be produced under certain culture conditions or during host infection (Minnikin *et al.*, 2002) and raise questions about the expression of *pk5* in *M. tuberculosis* grown under axenic conditions or *in vivo*. A search for *pk5* transcripts in *M. tuberculosis* H37Rv grown in 7H9 medium by quantitative real-time RT-PCR indicated that the *pk5* gene was expressed and that its level of expression was about one-third that of *sigA*. Therefore, *pk5* is expressed *in vitro*. The absence of detectable changes in the polyketide composition of the *pk5* mutant may then be due to the too low level of expression of the *pk5* gene or to the fact that the transcript of this gene is not translated into an active product. Interestingly, the level of expression of *pk5* inside macrophages was found to be similar to that detected *in vitro*. It is possible that the very low level of polyketide produced by *pk5* has an important role in mouse infection but not in macrophage infection. Alternatively, the expression of *pk5* within the host may cause the production of a polyketide that is not produced at detectable levels in culture or inside macrophages, and this polyketide product may be important for virulence. Clearly, more genetic and biochemical analyses are required to determine the level of expression of *pk5* *in vivo* and the effects of the inactivation of this gene on the cell envelope and virulence of *M. tuberculosis*.

In conclusion, our study provides two more examples of *mas*-like polyketide synthase genes, the expression of which is required for the full virulence of *M. tuberculosis* during host infection. Due to their role during host infection and their restricted distribution to pathogenic mycobacterial species, the enzymes of this family constitute interesting drug targets for the development of new anti-TB drugs that could be used in addition to classical treatments.

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