Two polyketide-synthase-associated acyltransferases are required for sulfolipid biosynthesis in *Mycobacterium tuberculosis*

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The methyl-branched fatty acyl components of sulfolipid-I (SL-I), a major glycolipid of the human pathogen *Mycobacterium tuberculosis*, are synthesized by the polyketide synthase Pks2. *Rv3824c* (*papA1*), located downstream of *pks2*, encodes a protein that belongs to a subfamily of acyltransferases associated with mycobacterial polyketide synthases [polyketide synthase-associated proteins (PAPs)]. The presence of a conserved acyltransferase motif (HX₃DX₁₄Y) suggested a role for PapA1 in acylation of sulfated trehalose to form SL-I. Targeted deletion of the H37Rv *papA1* resulted in loss of SL-I, demonstrating its role in mycobacterial sulfolipid biosynthesis. Furthermore, SL-I synthesis was restored in the mutant strain following complementation with *papA1*, but not with mutant alleles of *papA1* containing alterations of key residues in the acyltransferase motif, confirming that PapA1 was an acyltransferase. While other *M. tuberculosis* *pks* clusters are associated with a single PAP-encoding gene, it was demonstrated that another open reading frame, *Rv3820c* (*papA2*), located 5.8 kb downstream of *papA1* is also an acyltransferase gene involved in SL-I biosynthesis: deletion of *papA2* abolished SL-I production. The absence of any partially acylated intermediates in either null mutant indicated that both PapA1 and PapA2 were required for all acylation steps of SL-I assembly.

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, has a distinct, lipid-rich cell wall that plays an important role in virulence (Brennan & Nikaido, 1995; Puzo, 1990). Sulfolipids are one of the major *M. tuberculosis* cell wall lipids, of which sulfolipid-I (SL-I) is the most abundant (Goren, 1970a, b; Goren *et al.*, 1976; Middlebrook *et al.*, 1959). The presence of sulfolipids in virulent strains led to the speculation that this class of polar lipids plays an important role in pathogenesis. However, there have been mixed reports about the role of sulfolipids in virulence, with various modulatory effects being observed with purified sulfolipids (Okamoto *et al.*, 2006; Pabst *et al.*, 1988; Zhang *et al.*, 1988), but no attenuation observed with mutant strains in mouse and guinea pig models of infection (Rousseau *et al.*, 2003). SL-I consists of a tetra-acylated trehalose sulfate (Fig. 1): of the four acyl groups that are esterified to trehalose, one is palmitate while the remaining three are complex methyl-branched fatty acids, termed phthioceranic and hydroxyphthioceranic acids (Goren *et al.*,

1971). The sulfation of trehalose is presumed to be the first step in SL-I assembly and is catalysed by the sulfotransferase Stf0 (Mougous *et al.*, 2004). The palmitate is fatty acid synthase-I (FAS-I)-derived, the methyl-branched moieties, phthioceranic and hydroxyphthioceranic acids, are synthesized by a polyketide synthase, Pks2 (Sirakova *et al.*, 2001). Present in the same cluster as *pks2* is *mmpL8*, a gene encoding a transmembrane protein involved in transport (Converse *et al.*, 2003; Domenech *et al.*, 2004). Though the genes involved in the biosynthesis of individual components of SL-I are well studied, not much is known about the assembly of SL-I, vis-à-vis the components and sequence of events involved in acylation of trehalose-2-sulfate to SL-I. Located immediately downstream of *pks2* is *Rv3824c* (*papA1*; Fig. 2a), which encodes a protein that belongs to a subfamily of acyltransferases associated with mycobacterial polyketide synthases [polyketide synthase associated proteins (PAPs)]. The presence of a characteristic HX₃DX₁₄Y acyltransferase motif suggested a role for PapA1 in the acylation steps of SL-I assembly. Indeed, PapA5, another PAP, has been shown to be an acyltransferase involved in the esterification of phthiocerol with methyl-branched mycocerosic fatty acids to form the virulence lipid phthiocerol dimycocerosate (PDIM) (Onwueme *et al.*, 2004;

Abbreviations: FAS-I, fatty acid synthase-I; PAP, polyketide synthase-associated protein; PDIM, phthiocerol dimycocerosate; SL-I, sulfolipid-I.

Fig. 1. Structure of sulfolipid-I (SL-I) from *M. tuberculosis*.

Trivedi *et al.*, 2005). In general, *pks* clusters in *M. tuberculosis* are associated with only one PAP-encoding gene (Cole *et al.*, 1998). Interestingly, *Rv3820c* (*papA2*), a gene located 5.8 kb downstream of *papA1* (Fig. 2a), also encodes a protein with an acyltransferase motif, indicating that SL-I biosynthesis may involve more than one PAP. A similar arrangement of homologues of *pks2*, *papA1* and *papA2* is also seen in the genome of the bovine pathogen *Mycobacterium bovis* (Garnier *et al.*, 2003), although the *M. bovis*-derived vaccine strain BCG does not synthesize SL-I (Rivera-Marrero *et al.*, 2002). In these studies we have used a genetic approach to investigate whether *papA1* and *papA2* play a role in SL-I biosynthesis. Individual null mutants of H37Rv *papA1* and *papA2* failed to produce SL-I. However, no intermediates were observed in either of the mutants, indicating an essential role for each acyltransferase in assembly of complete SL-I.

METHODS

Plasmids, phages and culture conditions. Plasmids and phages used in this study are listed in Table 1. *Mycobacterium tuberculosis* H37Rv was cultured in 7H9 broth (Difco) containing 10% Middlebrook OADC enrichment and 0.05% Tween 80, or on Middlebrook 7H10 agar (Difco); *Escherichia coli* strains were cultured in LB broth. The concentrations of antibiotics used were 75 µg hygromycin ml⁻¹ and 20 µg kanamycin ml⁻¹ for H37Rv, and 150 µg hygromycin ml⁻¹ and 40 µg kanamycin ml⁻¹ for *E. coli*.

Construction of null mutants. For generating an allelic-exchange construct designed to replace the *papA1* gene with a hygromycin resistance cassette (*hyg*), ~1 kb sequences flanking the left and right of the *M. tuberculosis* H37Rv *papA1* gene were PCR amplified from genomic DNA using the primer pairs *papA1L1* (5'-CGCACTAGTGTTCTCCAGCAGCAACGG-3')/*papA1L2* (5'-AGCAGATCTCGAGGTGTAAGTCTGCTGCC-3') and *papA1R1* (5'-TGCTCTAGAGAAAGGATGACATGGCGGTC-3')/*papA1R2* (5'-AGACTTAAGGCAACGAGGCGCTGTGCAAC-3') respectively. Following cloning into pCR2.1-TOPO and sequencing, the cloned PCR fragments were excised using the primer-introduced restriction sites and cloned into the allelic-exchange plasmid vector pJSC347 (Table 1). The resultant plasmid, pYUB2418, was then packaged into the temperature-sensitive phage phAE159 (J. Kriakov & W. R. Jacobs Jr., unpublished results) as described previously (Bardarov

et al., 2002) to yield the *papA1*-knockout phage phAE405. In a similar fashion the primer pairs *papA2L1* (5'-CGGACTAGTCAGCTTATGCGCACCAC-3')/*papA2L2* (5'-AATCTCGAGCGGTGATGCGTGCCAAC-3') and *papA2R1* (5'-CGGTCTAGAGTCGCCCAATTACATCGCG-3')/*papA2R2* (5'-AATGGTACCACTGGCGCTCTGGACGACG-3') were used to PCR amplify ~1 kb sequences flanking the *papA2* gene. Following sequence confirmation, the flanks were used to construct the allelic-exchange plasmid pYUB2419, which was then packaged into phAE159 to yield the *papA2*-knockout phage phAE406. The phages were used to generate allelic-exchange mutants using specialized transduction as described previously (Bardarov *et al.*, 2002).

Complementation of mutant strains. The primers *papA1-F* (5'-CATGAATTCGTGCGAATAGGACC-3') and *papA1-R* (5'-GGCGTTAACCTAAGCTTCTCTATC-3') were used to PCR amplify the *papA1* gene from H37Rv genomic DNA. Following cloning into pCR2.1-TOPO and sequencing, the cloned PCR fragment was excised by digestion of the primer-introduced *EcoRI* and *HpaI* sites and cloned downstream of the *hsp60* promoter in the integrative vector pMV361 (Stover *et al.*, 1991). The resultant plasmid pMV361*papA1* was introduced into Δ *papA1* by electroporation to yield the complemented strain Δ *papA1*::pMV361*papA1*. Similarly, the primers *papA2-F* (5'-CTAAGCTTGTGTTTAGCATTACAAC-3') and *papA2-R* (5'-CGATCGATTATGTCCTGGTTTAAG-3') were used to PCR amplify the *papA2* gene. Following subcloning and sequence confirmation, the PCR product was excised and cloned as a *HindIII*–*Clal* fragment into pMV361 to yield the complementing plasmid pMV361*papA2*, which was then electroporated into Δ *papA2* to yield the complemented strain Δ *papA2*::pMV361*papA2*.

The acyltransferase motif of *papA1* was mutated using the Stratagene Quik Change site-directed mutagenesis kit, with pMV361*papA1* as template DNA and the primer pairs *papA1HA-F* (5'-CAGCATCGATGCTCTGCATGCG-3')/*papA1HA-R* (5'-CGCATGCAGAGCATCGATGCTG-3') or *papA1DA-F* (5'-TCTGCATGCGCCGGTCAGTTCG-3')/*papA1DA-R* (5'-CGAACTGACCGGCCGATGCAGA-3'). The resultant plasmids, pMV361*A1-HA* or pMV361*A1-DA*, were then electroporated into Δ *papA1*. In a similar fashion, the plasmids pMV361*A2-HA* and pMV361*A2-DA* were constructed using the primer pairs *papA2HA-F* (5'-GAGTATCGTCTGCTCTGTGTC-3')/*papA2HA-R* (5'-GACACAGAGAGCAGCGATACTC-3') and *papA2DA-F* (5'-CTCTGTGTCGCCCGATGATTG-3')/*papA2DA-R* (5'-CAATCATCGGGGCGACACAGAG-3') respectively, and then electroporated into Δ *papA2*.

Fig. 2. Generation of *M. tuberculosis* *papA1* and *papA2* null mutants. (a) Maps of the *M. tuberculosis* *pks2* region in wild-type (H37Rv), $\Delta papA1$ and $\Delta papA2$ strains: [α^{32} P]dCTP-labelled probes were derived from ~1 kb upstream and downstream flanking sequences that were used to construct the knockout plasmids, and are indicated by thick lines with diamonds at each end. The fragments expected to hybridize with the probes in a Southern blot are indicated by gapped lines with sizes. *res*, $\gamma\delta$ -resolvase site; *hyg*, hygromycin resistance gene. (b) Southern blot of *KpnI*-digested genomic DNA from H37Rv and $\Delta papA1$ strains. (c) Southern blot of *BbsI*-digested genomic DNA from H37Rv and $\Delta papA2$ strains.

Biochemical analysis of mutant strains. Labelling of *M. tuberculosis* cultures with [14 C]acetate, [14 C]propionate or $\text{Na}_2^{35}\text{SO}_4$ was done as described previously (Sirakova *et al.*, 2001). Total lipids were extracted from strains using previously described protocols (Dobson *et al.*, 1985) and separated on a TLC plate using chloroform/methanol/water (100:14:0.8, by vol.) in the first dimension and chloroform/acetone/methanol/water (50:60:2.5:3, by vol.) in the second dimension. The TLC plates were exposed to X-ray films for 24 h.

RESULTS

Generation of *papA1* and *papA2* null mutants of *M. tuberculosis*

We used specialized transduction (Bardarov *et al.*, 2002) to replace *papA1* in the *M. tuberculosis* H37Rv genome with a hygromycin resistance marker (*hyg*) (Fig. 2a). Genomic DNA obtained from Hyg^R *M. tuberculosis* H37Rv colonies following transduction with phAE405 was digested with *KpnI* and analysed by Southern blotting (Fig. 2b). The band pattern obtained confirmed that *papA1* was replaced by *hyg*. Similarly, the knockout phage phAE406 was used to obtain a *papA2* null mutant of H37Rv (Fig. 2c). The *papA1* and

papA2 null mutants are referred to as $\Delta papA1$ and $\Delta papA2$ respectively.

SL-I is not synthesized in $\Delta papA1$

To determine changes in the lipid profiles of the $\Delta papA1$ mutant, we first monitored differences in the [14 C]acetate-labelled lipids from wild-type and $\Delta papA1$ strains by TLC. In a solvent system designed to separate *M. tuberculosis* glycolipids (Dobson *et al.*, 1985), a [14 C]-labelled lipid, which was clearly present in the wild-type but missing in the mutant strain, migrated in a manner similar to an authentic SL-I standard (Fig. 3a). Three of the four acyl components of SL-I are methyl-branched fatty acids (phthioceranate and hydroxyphthioceranate) which can be labelled using [14 C]propionate. Indeed, the lipid missing in $\Delta papA1$, but present in WT showed high incorporation of [14 C] label when propionate was used as the radiolabel (Fig. 3b). Finally, this lipid also showed incorporation of [35 S] from $\text{Na}_2^{35}\text{SO}_4$, confirming that the species was indeed SL-I (Fig. 3b). Introduction of pMV361*papA1*, a single copy integrative vector containing *papA1* cloned downstream of

Table 1. Plasmids and phages

Plasmid or phage	Description	Reference/source
Plasmids		
pCR2.1-TOPO	PCR product cloning vector	Invitrogen Life Technologies
pJSC347	Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains λ phage <i>cos</i> site and Hyg ^R marker	Sambandamurthy <i>et al.</i> (2002)
pYUB2418	Derivative of pJSC347 designed for allelic exchange of <i>M. tuberculosis papA1</i>	This work
pYUB2419	Derivative of pJSC347 designed for allelic exchange of <i>M. tuberculosis papA2</i>	This work
pMV361	Single-copy mycobacterial integrative vector with <i>hsp60</i> promoter	Stover <i>et al.</i> (1991)
pMV361 <i>papA1</i>	<i>M. tuberculosis papA1</i> cloned in pMV361	This work
pMV361A1-HA	His-171→Ala-171 mutant of <i>papA1</i> in pMV361	This work
pMV361A1-DA	Asp-175→Ala-175 mutant of <i>papA1</i> in pMV361	This work
pMV361 <i>papA2</i>	<i>M. tuberculosis papA2</i> cloned in pMV361	This work
pMV361A2-HA	His-166→Ala-166 mutant of <i>papA2</i> in pMV361	This work
pMV361A2-DA	Asp-170→Ala-170 mutant of <i>papA2</i> in pMV361	This work
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4	Gift from J. Kriakov*
phAE405	Derivative of phAE159 obtained by cloning pYUB2418 in its unique <i>PacI</i> site	This work
phAE406	Derivative of phAE159 obtained by cloning pYUB2419 in its unique <i>PacI</i> site	This work

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the *hsp60* promoter, into $\Delta papA1$ fully restored SL-I biosynthesis, indicating that the observed phenotype in $\Delta papA1$ was solely due to deletion of *papA1* (Fig. 3a, b). These results demonstrated that *papA1* function was necessary for SL-I biosynthesis.

PapA2 is also required for SL-I biosynthesis

To investigate the role of *papA2* in SL-I biosynthesis, an approach similar to that used for the $\Delta papA1$ strain was followed. An initial analysis of total lipids extracted from cultures of H37Rv and $\Delta papA2$ labelled with [¹⁴C]acetate indicated that SL-I was missing in the $\Delta papA2$ strain (Fig. 4a). This was then confirmed using [¹⁴C]propionate or Na₂³⁵SO₄ as the labelling reagents (Fig. 4b). SL-I biosynthesis could be restored in $\Delta papA2$ following complementation with pMV361*papA2*, a single-copy integrative vector containing *papA2* cloned downstream of the *hsp60* promoter (Fig. 4a, b). Thus, like *papA1*, *papA2* was also essential for SL-I biosynthesis.

papA1 and papA2 encode acyltransferases

The presence of a conserved acyltransferase motif (HX₃DX₁₄Y) had suggested a role for PapA1 and PapA2 in acylation of either trehalose or sulfated trehalose, to form SL-I. The histidine and aspartate residues of the motif are considered essential for acyltransferase activity (Bergendahl *et al.*, 2002; Lewendon *et al.*, 1988). To demonstrate that *papA1* and *papA2* do encode acyltransferases, we tested the ability of mutant alleles of the genes containing alterations in either of these key residues to restore SL-I biosynthesis in the mutant strain. First, single-copy-integrative plasmids

containing two mutant alleles of *papA1* containing separate point mutations in the HX₃DX₁₄Y motif were constructed using site-directed mutagenesis (Fig. 5). The mutant constructs pMV361A1-HA (His-171→Ala-171) and pMV361A1-DA (Asp-175→Ala-175) were then introduced separately into $\Delta papA1$ by electroporation and the resultant strains, $\Delta papA1::pMV361A1-HA$ and $\Delta papA1::pMV361A1-DA$, were tested for functional complementation. Analysis of [¹⁴C]propionate- or ³⁵S-labelled total lipids from the transformed strains revealed that neither construct reconstituted SL-I biosynthesis in $\Delta papA1$ (Fig. 3b). Similarly, the plasmids pMV361A2-HA (His-166→Ala-166) and pMV361A2-DA (Asp-170→Ala-170) failed to restore SL-I biosynthesis in $\Delta papA2$ (Fig. 4b). Thus, the key residues in the putative acyltransferase motifs of PapA1 and PapA2 were essential for functional complementation of the corresponding SL-I deficient mutants, indicating that PapA1 and PapA2 are acyltransferases involved in SL-I biosynthesis.

DISCUSSION

The sequencing of the *M. tuberculosis* H37Rv genome revealed five open reading frames, *papA1*–*papA5*, that were annotated as genes for polyketide synthase-associated proteins (PAPs) because of their linkage to genes encoding enzymes involved in polyketide biosynthesis (Cole *et al.*, 1998). Based on the presence of an acyltransferase motif, it was speculated that PAPs may be acyltransferases involved in the assembly of complex lipid metabolites synthesized by the associated *pks* cluster. Experimental proof for this came first from *in vitro* experiments using purified PapA5, a PAP

Fig. 3. Sulfolipid analysis of the $\Delta papA1$ mutant by 2D-TLC. X-ray film exposed to TLC plates following 2D-TLC of total lipid samples extracted from cultures labelled with (a) $[^{14}C]$ acetate or (b) $[^{14}C]$ propionate or $Na_2^{35}SO_4$. The spot corresponding to SL-I is indicated in the images. GMM, glucose monomycolate; DAT, diacyltrehalose; TMM, trehalose monomycolate; TDM, trehalose dimycolate.

involved in PDIM biosynthesis (Onwueme *et al.*, 2004; Trivedi *et al.*, 2005). PDIM is made up of two mycocerosic acid residues esterified to the hydroxyl groups of a diol (phthiocerol). Using purified proteins and artificial substrates, Onwueme *et al.* (2004) first showed that PapA5 could catalyse esterification of an alcohol with an acyl group. Trivedi *et al.* (2005) then demonstrated that purified PapA5 catalysed trans-esterification of mycocerosic acid synthase-bound mycocerosic acids to dodecanol.

Of all the *pks* genes linked to PAPs, *pks2* is the only one located next to two *pap* genes (*papA3* and *pks3/4* are involved in the biosynthesis of polyacylated trehalose, and the metabolite synthesized by *papA4* and *pks5* has not yet been identified). Deletion of *pks2* resulted in loss of the

principal sulfolipid SL-I in *M. tuberculosis* H37Rv (Sirakova *et al.*, 2001). The absence of SL-I in $\Delta papA1$ and in $\Delta papA2$, the two mutants generated in this study, demonstrated that PapA1 and PapA2 were indeed functionally associated with Pks2. Phthioceranic acid and hydroxyphthioceranic acid, the methyl-branched products of Pks2, are found esterified to positions 3, and 6 and 6', respectively, of sulfated trehalose in the principal sulfolipid SL-I, while position 2 is substituted with FAS-I-derived palmitate. It was thus tempting to speculate that PapA1 and PapA2 may differ in their substrate specificities, with one PAP involved in the transfer of the methyl-branched acyl chains, and the other in that of palmitate. Another possibility was that one of the *pks2*-associated PAPs was specific for transfer of phthioceranic acid and the other for hydroxyphthioceranic acid, with

Fig. 4. Sulfolipid analysis of the $\Delta papA2$ mutant by 2D-TLC. X-ray film exposed to TLC plates following 2D-TLC of total lipid samples extracted from cultures labelled with (a) $[^{14}C]$ acetate or (b) $[^{14}C]$ propionate or $Na_2^{35}SO_4$. The spot corresponding to SL-I is indicated in the images. GMM, glucose monomycolate; DAT, diacyltrehalose; TMM, trehalose monomycolate; TDM, trehalose dimycolate.

the transfer of palmitate being carried out by an unknown acyltransferase. In either case, if the acylation reactions were sequential, partially acylated trehalose sulfate intermediates would be expected to accumulate in either $\Delta papA1$ or $\Delta papA2$. While both $\Delta papA1$ and $\Delta papA2$ did not synthesize SL-I, neither mutant strain showed accumulation of any partially acylated intermediate. This result indicated that in one of the mutants, feedback inhibition of the initial step may have prevented the accumulation of the intermediate. Alternatively, instead of a sequential addition of different acyl groups to sulfated trehalose by PapA1 and PapA2, concurrent presence of both proteins may be required for complete assembly of a tetracylated trehalose sulfate molecule. Some indication that this might be a possibility comes from recent work on the components of the PDIM biosynthetic cluster. Jain & Cox (2005) showed that

polyketide synthase PpsE directly interacted with the transmembrane protein and PDIM transporter MmpL7. This suggested that not only was MmpL7 involved in transport, but that it also probably acted as a scaffold in a tightly integrated system that couples the biosynthesis of PDIM to its secretion from the bacterial cell. In light of these findings it is plausible that MmpL8, the transporter associated with the *pks2* cluster, may play a similar 'scaffolding' role in SL-I assembly through interactions with Pks2, PapA1 and Pap2, wherein PapA1 and PapA2 may carry out localized and simultaneous acylation reactions. In the $\Delta papA1$ or $\Delta papA2$ mutant, loss of one PAP would result in non-functional SL-I assembly machinery and complete loss of SL-I biosynthesis (rather than accumulation of a partially acylated intermediate). However, two independent reports of a *M. tuberculosis mmpL8* null mutant showed that

PapA1	HLHADGQFVGVLMEFQSMY
A1-HA	ALHADGQFVGVLMEFQSMY
A1-DA	HLHAAGQFVGVLMEFQSMY
PapA2	HLCVDPMIVGVLFIEIHMMY
A2-HA	ALCVDPMIVGVLFIEIHMMY
A2-DA	HLCVAPMIVGVLFIEIHMMY
Motif consensus	HXXXDXXXXXXXXXXXXXX

Fig. 5. Mutation of the HX₃DX₁₄Y motif in PapA1 and PapA2. Alignment of the amino acid sequences in the HX₃DX₁₄Y motif of PapA1, PapA2 and the putative proteins encoded by the mutant constructs pMV361A1-HA (A1-HA), pMV361A1-DA (A1-DA), pMV361A2-HA (A2-HA), pMV361A2-DA (A2-DA). The conserved amino acids are indicated in bold and the altered residues are underlined.

the loss of MmpL8 resulted in the accumulation of a diacylated trehalose sulfate intermediate (Converse *et al.*, 2003; Domenech *et al.*, 2004). Nevertheless, we did not detect accumulation of such an intermediate in either the Δ papA1 or the Δ papA2 mutant. Though the absence of any partially acylated intermediates in either Δ papA1 or Δ papA2 made it difficult to assign putative substrate specificities (if any) to PapA1 and PapA2, our results have clearly demonstrated that these two distinct acyltransferases are involved in the biosynthesis of the major sulfolipid SL-I in *M. tuberculosis*.

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