Ppm1, a novel polyprenol monophosphomannose synthase from *Mycobacterium tuberculosis*

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Dolichol monophosphomannose (DPM) is an ever-present donor of mannose (Man) in various eukaryotic glycosylation processes. Intriguingly, the related polyprenol monophosphomannose (PPM) is involved in the biosynthesis of lipomannan and lipoarabinomanan, key bacterial factors termed modulins that are found in mycobacteria. Based on similarities to known DPM synthases, we have identified and characterized the PPM synthase of *Mycobacterium tuberculosis*, now termed *Mt*-Ppm1. In the present study, we demonstrate that *Mt*-Ppm1 possesses an unusual two-domain architecture, by which the second domain is sufficient for PPM synthesis. However, when overexpressed separately in mycobacteria, domain 1 of *Mt*-Ppm1 appears to increase the synthesis of PPM. Interestingly, other mycobacteria such as *M. smegmatis*, *M. avium* and *M. leprae* produce two distinct proteins, which are similar to the two domains found in *Mt*-Ppm1. Using an *in vitro* assay, we also demonstrate that *Mt*-Ppm1 transfers Man from GDP-Man to a structurally diverse range of lipid monophosphate acceptors. The identification of the PPM synthase as a key enzyme in lipoarabinomannan biosynthesis now provides an attractive candidate for gene disruption to generate mutants for subsequent immunological studies. PPM synthase can also be exploited as a target for specific inhibitors of *M. tuberculosis*.

Key words: GDP-mannose, lipoarabinomannan, mannosyltransferase.

INTRODUCTION

Survival of *Mycobacterium tuberculosis* within the host macrophage is dependent on the basic structure of lipomannan (LM) and lipoarabinomannan (LAM) [1,2]. LAM belongs to the class of bacterial factors termed modulins and operates by inducing the expression of several 'early' genes, which are involved in macrophage activation [3], synthesis of host cytokines such as tumour necrosis factor-α [4] and production of nitric oxide, synergistically with interferon-γ [5]. LAM is also implicated in the phagocytosis of mycobacteria by phagocytic cells [6]. Phosphatidyl-*myo*-inositol mannosides (PIMs), known to be precursors of LM and LAM, have been recently proposed to recruit natural killer T cells, which play a primary role in the granulomatous response [7,8].

Although the structure of LAM has been well documented [2,9,10], the genetics of its biosynthesis still remains largely ill-defined. It involves the addition of mannose (Man) residues to phosphatidyl-*myo*-inositol to produce both the short PIMs (2–5 Man residues) and LM, which is further glycosylated with arabinan to form LAM. The biosynthetic relationship of phosphatidyl-*myo*-inositol → PIMs → LM → LAM has recently been supported by biochemical [11,12] and genetic studies [13]. Besra et al. [12] demonstrated that triacylated PIM_2 (phosphatidyl-*myo*-inositol dimannoside) is specifically extended by the addition of Man*p* residues from the alkali-stable sugar donor, polyprenol monophosphomannose (PPM), to form higher PIMs (at least triacylated PIM_3 and PIM_4) and linear LM. PPM is generated from GDP-Man and the corresponding polyprenol phosphate. Therefore the PPM synthase is a key enzyme in the generation of PPM, which is subsequently utilized in the biosynthesis of LM and LAM.

As an approach to studying the biosynthesis and immunological function of various mannan-containing glycoconjugates (LM and LAM) in mycobacteria, we have exploited the recently available *M. tuberculosis* genome sequence [14]. By comparison with a variety of published eukaryotic dolichol monophosphomannose (DPM) synthase sequences, we identified a homologous synthase in *M. tuberculosis*, now termed as *Mt*-Ppm1. Surprisingly, the protein identified is much larger than the eukaryotic or bacterial homologues and is composed of two domains. The catalytic DPM-homologous domain is located in the C-terminus of the protein, whereas the N-terminal domain possesses a low degree of similarity with a variety of bacterial acyltransferases. Interestingly, related mycobacterial species such as *M. leprae*, *M. avium* and *M. smegmatis* possess the equivalent locus, but it is organized as an operon with two distinct genes encoding proteins that display a high degree of sequence similarities to the C- and N-domains of *Mt*-Ppm1. The identification of the PPM synthase as a key enzyme in LAM biosynthesis now provides an interesting target to be exploited, possibly in the generation of LAM-deficient mutants for subsequent immunological studies, or

Abbreviations used: DPM, dolichol monophosphomannose; LAM, lipoarabinomannan; LM, lipomannan; Man, mannose; PIM, phosphatidyl-*myo*-inositol mannoside; PPM, polyprenol monophosphomannose.

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alternatively for the validation of the mycobacterial PPM synthase as a possible drug target.

EXPERIMENTAL

Bacterial strains and growth conditions

All cloning steps were performed in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, U.S.A.). *M. smegmatis* mc²155 was a gift from W. R. Jacobs (Albert Einstein College of Medicine, Bronx, New York, U.S.A.) [15]. *M. smegmatis* mc²155 was transformed as described previously [16], and recombinant clones were selected on Middlebrook 7H10 agar supplemented with oleic acid/albumin/dextrose/catalase enrichment (Difco, Detroit, MI, U.S.A.) containing 25 μg/ml kanamycin (Sigma). Liquid cultures of *M. smegmatis* (pMV261) and *M. smegmatis* (pMV261–*Mt-ppm1*) [also *M. smegmatis* (pMV261–*Mt-ppm1/D1*) and *M. smegmatis* (pMV261–*Mt-ppm1/D2*)] were grown at 37 °C in Luria–Bertani broth (Difco) supplemented with 25 μg/ml kanamycin and 0.05 % (v/v) Tween 80. Liquid cultures of *E. coli* (pUC8) and *E. coli* (pUC8–*Mt-ppm1/D2*) were grown in Luria–Bertani broth at 37 °C with 100 μg/ml ampicillin to A_{600} 0.4 and induced for 4 h with 1 mM isopropyl β-D-thiogalactopyranose. Large-scale cultures of bacteria were grown as described above, harvested by centrifugation, washed with PBS and stored at −20 °C until further use.

Plasmids and DNA manipulation

The *E. coli*-mycobacterial shuttle vector pMV261, containing the *hsp60* promoter, was used as described previously [17]. With the electroduction of *E. coli* as described in [18], analysis of plasmids from mycobacteria was achieved. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany) and Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA, U.S.A.). The manipulations of all DNA were performed using standard protocols.

Cloning of *Mt-ppm1*, *Mt-ppm1/D1* and *Mt-ppm1/D2*

Sequences for *Rv2051c* (*Mt-ppm1*) and the sequences corresponding to the N- and C-terminal domains of the *Rv2051c* gene product (*Mt-ppm1/D1* and *Mt-ppm/D2* respectively), required for cloning into pUC8 [19] and/or pMV261 [17], were obtained by PCR using Vent polymerase (New England Biolabs). The complete *Rv2051c* DNA fragment was amplified using the primer pairs 145 and 146. The 2740 bp PCR product was then digested with *Eco*RI and cloned into *Eco*RI–*Msc*I-restricted pMV261, giving rise to pMV261–*Mt-ppm1*. *Mt-ppm1/D1* and *Mt-ppm1/D2* were generated by PCR using pMV261–*Mt-ppm1*. *Mt-ppm1/D1* was amplified using primer pairs 146 and 152, whereas *Mt-ppm1/D2* was amplified using primer pairs 134 and 135. After restriction with *Eco*RI, the fragments were cloned into pMV261, previously digested with *Msc*I–*Eco*RI to create pMV261–*Mt-ppm1/D1* and pMV261–*Mt-ppm1/D2* respectively. For expression in *E. coli*, *Mt-ppm1/D2* was cloned into pUC8 cut by *Sma*I generating pUC8–*Mt-ppm1/D2*. The DNA of each construct was sequenced to verify the coding sequence of *Mt-ppm1* (also *Mt-ppm1/D1* and *Mt-ppm1/D2*) as well as its junction with the *hsp60* or *Plac* promoters. Oligonucleotides: no. 145: 5′-ACCGAATTCGAGGACGACCTGCCCGAGCC-3′; no. 146: 5′-TGAAGCTTGGCGCATGGGTGGCAGC-3′; no. 152: 5′-GGGAATTCTGGCTGGGACGGTTACC-3′; no. 134: 5′-GGAATTCCGGTCGTGCGCGTGCGTGGTC-3′; no. 135: 5′-GGAATTCGCCTCGAGCTCATTCGGTCACG-3′.

Cloning and sequencing of the *M. smegmatis ppm1* locus

The DNA sequence encoding the orthologue of Ppm1 in *M. smegmatis* (*Ms*-Ppm1) was identified from a genomic cosmid library by colony-blot hybridization. The cosmid library referred as pYUB415::155 has been described by Yu et al. [20] and was provided by W. R. Jacobs (Albert Einstein College of Medicine, New York, NY, U.S.A.). The library was used to transform *E. coli* TOP-10 cells (Invitrogen). For isolation of *Ms-ppm1*, approx. 600 individual clones were screened with the labelled 3′-end of *Mt-ppm1*. The probe was labelled with digoxigenin (DIG)-dUTP5′ using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim) with the following specific oligonuceotides: no. 174: 5′-GGAATTCGCCTCGAGCTCATTCGGTCACG-3′; no. 175: 5′-CCACAGGACAGCCGGCGCCCCCAGC-3′.

Prehybridization, hybridization, post-hybridization washes and detection were performed according to the manufacturer's recommendations. From four positive clones, cosmid DNA was prepared and further analysed by restriction digests and Southern-blot hybridization. *Bam*HI restriction of one of these cosmids produced a 5-kb fragment reacting strongly with the probe by Southern-blot hybridization. This fragment was subsequently purified, cloned into pUC18 and analysed by DNA sequencing.

Preparation of enzyme fractions

M. smegmatis (pMV261), *M. smegmatis* (pMV261–*Mt-ppm1*), *M. smegmatis* (pMV261–*Mt-ppm1/D1*), *M. smegmatis* (pMV261–*Mt-ppm1/D2*), *E. coli* (pUC8) and *E. coli* (pUC8–*Mt-ppm1/D2*) were grown as described above, harvested by centrifugation, washed with PBS and stored at −20 °C until further use. Mycobacterial cells (10 g wet wt) were washed and resuspended in 30 ml of buffer A, containing 50 mM Mops (adjusted to pH 8.0 with KOH), 5 mM β-mercaptoethanol and 10 mM $MgCl_2$ at 4 °C and subjected to sonication (1 cm probe; Soniprep 150, MSE Sanyo Gallenkamp, Crawley, Sussex, U.K.) for a total time of 10 min using 60 s pulses and 90 s cooling intervals. *E. coli* cells were disrupted in a similar way using 30 s pulses and 45 s cooling intervals. The sonicated materials were centrifuged at 27000 ***g*** for 60 min at 4 °C. The supernatant fractions from *E. coli* (pUC8) or *E. coli* (pUC8–*Mt-ppm1/D2*) were used in all subsequent PPM experiments. Membrane fractions from *M. smegmatis* harbouring pMV261, pMV261–*Mt-ppm1*, pMV261–*Mt-ppm1/D1* or pMV261–*Mt-ppm1/D2* were obtained by further centrifugation of the 27000 ***g*** supernatant at 100000 ***g*** for 1 h at 4 °C. The supernatant was carefully removed and the membranes were gently resuspended in buffer A at a protein concentration of 20 mg/ml. Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce Europe, Oud-Beijerland, The Netherlands).

PPM synthase assay

Reaction mixtures for assessing [^{14}C]Man incorporation consisted of 2.4 μM GDP-[U-^{14}C]Man (321 mCi/mmol, 0.25 μCi; DuPont–New England Nuclear), 62.5 μM ATP, 10 μM $MgCl_2$ and membrane preparations corresponding to 5–25 μg protein in a final vol. of 50 μl. In some cases, exogenous lipid monophosphate substrates (C_{10}, geranyl monophosphate; C_{15}, farnesyl monophosphate; C_{20}, geranylgeranyl monophosphate; C_{35}DOL, α-dihydroheptaprenyl monophosphate; C_{35}, heptaprenyl monophosphate; C_{40}, octaprenyl monophosphate; C_{50}, decaprenyl monophosphate; C_{60}, dodecaprenyl monophosphate; C_{75}, pentadecaprenyl monophosphate; C_{95}, dolichol monophosphate)

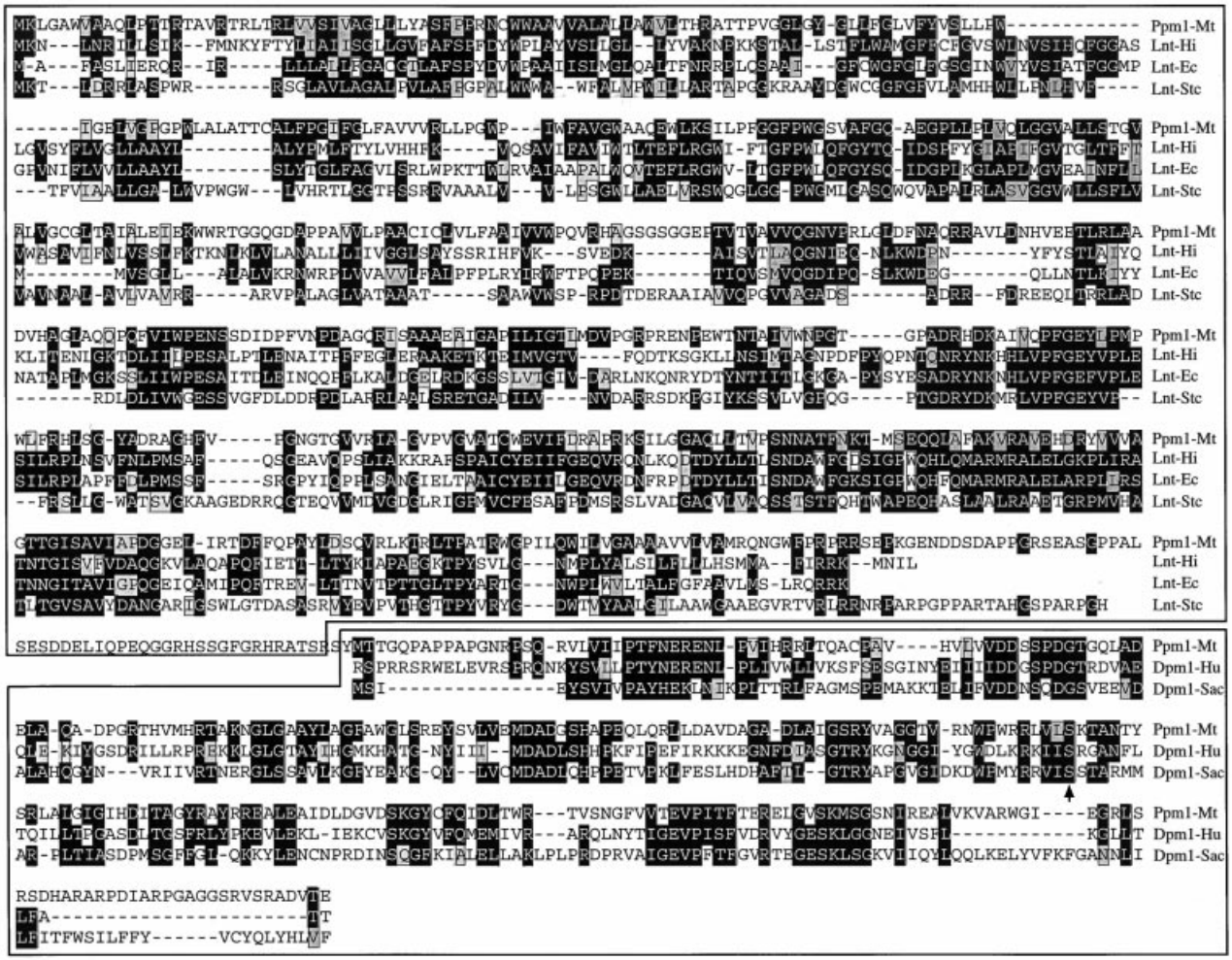

Figure 1 Sequence alignment of *Mt*-Ppm1 homologues: major homologues to *Mt*-Ppm1 (*Rv2051c*) identified by BLAST were aligned with Multalin (http://www.toulouse.inra.fr/multalin.html) [32]

The gene products shown are from *M. tuberculosis* H37Rv (Ppm1-*Mt*), *H. influenzae* (Lnt-*Hi*), *E. coli* (Lnt-*Ec*), *Streptomyces coelicolor* A3 (Lnt-*Stc*), human (Dpm1-*hu*) and *S. cerevisiae* (Dpm1-*Sac*). Conserved (black) or related (grey) residues are boxed. The two domains of *Mt*-Ppm1 (*Mt*-Ppm1/D1 and *Mt*-Ppm1/D2) are shown separately with their respective homologues. The arrow indicates the conserved serine residue proposed to be phosphorylated in eukaryotic DPM synthases [33].

were added to the reaction mixtures at a final concentration of 0.25 mM in 0.25 % (v/v) CHAPS. The reaction mixtures were then incubated at 37 °C for 30 min.

Extraction and characterization of [^{14}C]Man-labelled products from reaction mixtures

The enzymic reactions were terminated by the addition of $CHCl_3/CH_3OH$/0.8 M NaOH (10:10:3, by vol.) (4 ml/50 μl of reaction mixture) followed by further incubation at 50 °C for 20 min. The mixtures were then allowed to cool; 1.75 ml of $CHCl_3$ and 0.75 ml of water were added. The mixture was vortexed and centrifuged, and the upper aqueous phase discarded. The organic phase was washed three times with 2 ml of $CHCl_3/CH_3OH/H_2O$ (3:47:48, by vol.), dried to yield an organic fraction, which contained exclusively the mild-alkali stable family of PPMs or DPMs. Alternatively, after base treatment, the reaction mixtures possessing exogenous substrates C_{10} and C_{15} monophosphates were dried and partitioned between the phases arising from butan-1-ol and water. The butan-1-ol-saturated water layer was recovered, backwashed twice with water saturated with butan-1-ol and dried. The resulting PPMs and DPMs were resuspended in 200 μl of either $CHCl_3/CH_3OH$ (2:1, v/v) (C_{20}–C_{95}) or butan-1-ol-saturated water (C_{10} and C_{15}), and an aliquot (20 μl) was dried under a stream of argon in a scintillation vial before scintillation counting using 10 ml of Ecolume™ (ICN Biomedicals, Costa Mesa, CA, U.S.A.). TLC of the reaction products, usually aliquots representing 10 % of the reaction mixtures, was conducted on aluminium-backed plates of silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.4:3.6, by vol.). Autoradiograms were obtained by exposing chromatograms to Kodak X-Omat AR films at −70 °C for 4–5 days. Large-scale reaction mixtures containing unlabelled GDP-Man (80 mM) and the other components were prepared and processed as described above. The chemical identity of mycobacterial PPMs was confirmed following preparative TLC [12] and analytical electrospray ionization MS (Quattro II triple quadrupole mass spectrometer; Micromass, Beverly, MA, U.S.A.). Electrospray MS was also performed on crude reaction products resulting from the

utilization of exogenous polyprenol and dolichol monophosphate substrates and on enzymic extracts prepared from *E. coli* (pUC8–*Mt-ppm1/D2*).

RESULTS

Identification of *Mt-ppm1*

In eubacteria, biosynthetic lipid intermediates based on polyprenols are usually involved in peptidoglycan and O-antigen biosynthesis and are generally of the polyprenol–pyrophosphate–oligosaccharide type [21]. By contrast, mycobacterial polyprenol phosphates used as carriers during LAM biosynthesis contain single Man residues linked to the lipid prenol moiety through a monophosphate group [12]. Since similar carriers, such as DPM, are found in eukaryotes, we have used the amino-acid sequences of various DPM synthases to blast the *M. tuberculosis* H37Rv genome database. One strong match was found for the mycobacterial gene *Rv2051c*. As polyprenol phosphates, and not dolichol phosphates, are the acceptors of Man from GDP-Man in mycobacteria, we named the gene *Mt-ppm1*. Interestingly, only the C-terminal region of the predicted *Mt*-Ppm1 protein is similar to the human Dpm1 (Figure 1). The N-terminal domain of *Mt*-Ppm1 shows some similarities to various putative apolipoprotein *N*-acyltransferases (Lnt). A computer-assisted analysis revealed several putative transmembrane regions in this domain of *Mt*-Ppm1 (Figure 2). In addition, the presence of a predicted transmembrane α-helix, followed by a stretch of prolines at the hinge of the two domains, suggests that these two regions of the protein are located on opposite sites of the membrane (Figure 2). Thus based on similarity and hydrophobicity profiles,

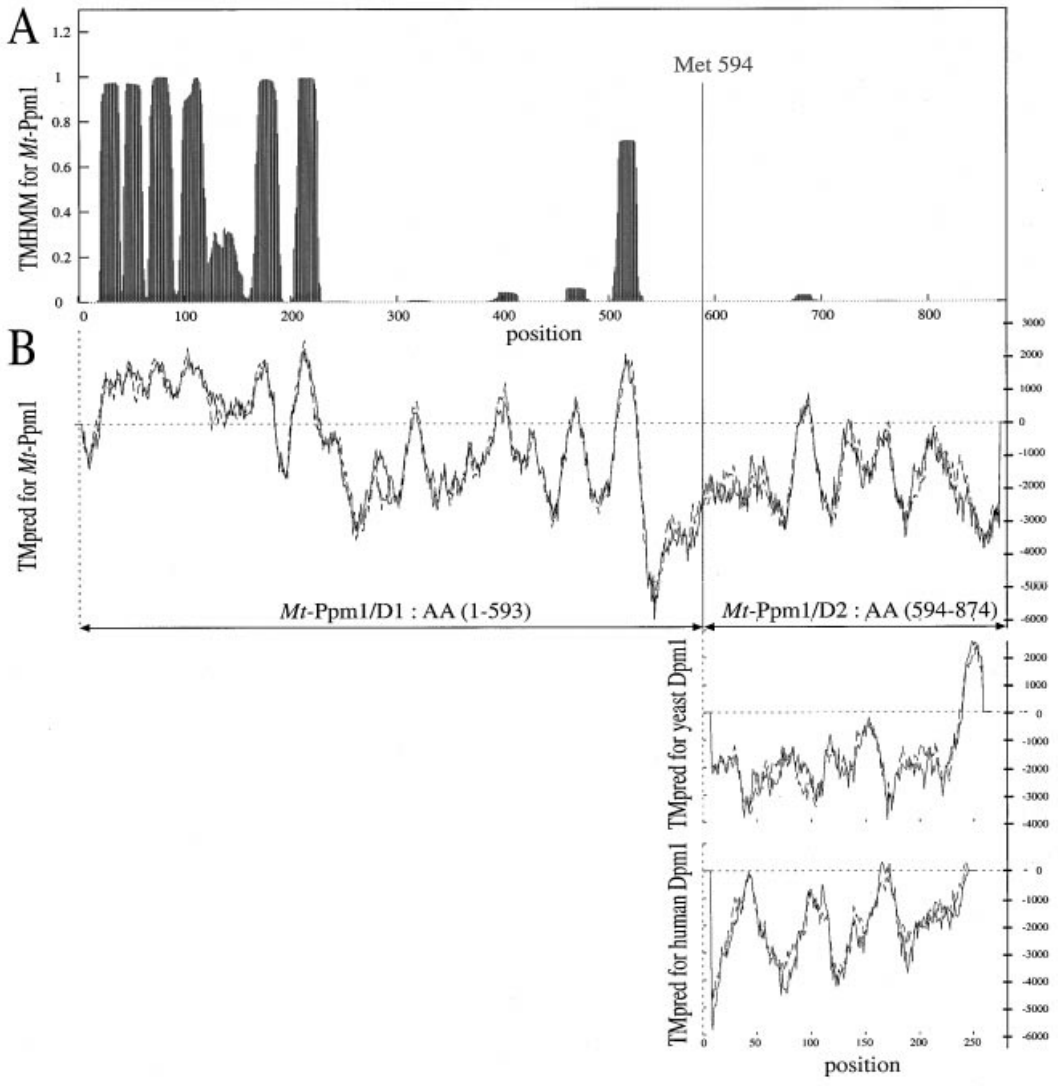

Figure 2 Comparative prediction model of the location of α-helices in the *Mt*-Ppm1, the yeast and the human DPMs

(**A**) Graphical representation of *Mt*-Ppm1 as predicted by TMHMM1.0 software (http://www.cbs.dtu.dk/services/TMHMM-1.0/). A minimum of seven transmembrane domains were predicted in *Mt*-Ppm1, all of them in *Mt*-Ppm1/D1. The last one is located just before the beginning of the second domain (*Mt*-Ppm1/D2). (**B**) A more detailed model drawn using TMpred (http://www.ch.embnet.org/software/TMPRED-form.html) and confirming the predictions obtained with TMHMM1.0. The predicted amino-acid sequence of *Mt-ppm1/D2* lacks the C-terminal hydrophobic sequence present in the DPMs of *L. mexicana*, *T. brucei*, *U. maydis* and *S. cerevisiae*, and, in this regard, is more related to the DPM synthases of *Schizosaccharomyces pombe*, *Caenorhabditis briggsiae* and mammals.

Table 1 Incorporation of [^{14}C]Man from GDP-[^{14}C]Man utilizing either endogenous mycobacterial polyprenol monophosphates (C_{35}–C_{40}–C_{50}) and membrane extracts prepared from *M. smegmatis* (pMV261), *M. smegmatis* (pMV261–*Mt-ppm1/D2*), or exogenous C_{50} polyprenol monophosphate with enzymic extracts prepared from *E. coli* (pUC8–*Mt-ppm1/D2*)

Incubations were carried out for 30 min at 37 °C and the PPMs were isolated after base treatment as described in the Experimental section before scintillation counting. The above results represent means of duplicates and are representative of several independent experiments.

	Protein (μg)	Specific activity (pmol/min)
M. smegmatis (pMV261)	10	2.95
	25	5.81
M. smegmatis (pMV261–*Mt-ppm1/D2*)	10	4.19
	25	8.09
E. coli (pUC8)	5	–*
	10	–*
	25	–*
E. coli (pUC8–*Mt-ppm1/D2*)	5	3.39
	10	5.94
	25	11.68

* Experimental values obtained were similar to control assays (< 0.005 pmol/min) performed either in the absence of enzyme or in the presence of heat-inactivated enzymes.

we postulated that the catalytic domain of *Mt*-Ppm1 is located in the second domain. These analyses also suggested that the two domains of the proteins could be artificially disconnected and studied separately. Hence, in addition to the complete *Mt-ppm1* open reading frame, two regions coding for the putative *Mt*-Ppm1 domains, Met^{1}–Tyr^{593} and Met^{594}–Glu^{874}, were amplified by PCR and cloned in frame with the initiation codon of *hsp60* into pMV261 to yield pMV261–*Mt-ppm1*, pMV261–*Mt-ppm1/D1* and pMV261–*Mt-ppm1/D2*. Also, *Mt-ppm1/D2* was fused to *lacZ* in pUC8 for expression in *E. coli*.

Incorporation of GDP-[^{14}C]Man by enzymic extracts from *M. smegmatis* (pMV261–*Mt-ppm1*), (pMV261–*Mt-ppm1/D2*) and *E. coli* (pUC8–*Mt-ppm1/D2*)

To establish the relationship between *Rv2051c* and PPM synthesis, we examined extracts prepared from bacteria expressing *Mt-ppm1* and/or *Mt-ppm1/D2*. *M. smegmatis* and *E. coli* were transformed with plasmids pMV261–*Mt-ppm1/D2* and pUC8–*Mt-ppm1/D2* respectively to overproduce the putative catalytic domain *Mt*-Ppm1/D2. The relative PPM transferase activities within these preparations were compared with those of extracts prepared from *M. smegmatis* and *E. coli* transformed with pMV261 and pUC8 respectively (Table 1). The PPM synthase activity from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) membrane extracts was significantly increased compared with that of *M. smegmatis* (pMV261) membrane extracts (Table 1). *E. coli* (pUC8) was found to possess no PPM transferase activity in the presence of exogenous C_{50}-polyprenol monophosphate lipid substrate. However, *E. coli* overexpressing *Mt-ppm1/D2* possessed considerable PPM transferase activity using exogenous C_{50}-polyprenol monophosphate lipid as substrate (Table 1), confirming that *Rv2051c* encodes a PPM synthase and that the GDP-Man:polyprenol phosphate mannosyltransferase activity is catalysed by the second domain of the protein.

To evaluate the possible role of domain 1 of *Mt*-Ppm1, enzymic assays were performed with membrane fractions (5 μg each) prepared from *M. smegmatis* harbouring pMV261, pMV261–*Mt-ppm1* and pMV261–*Mt-ppm1/D2*. Membranes prepared from *M. smegmatis* pMV261–*Mt-ppm1* in several independent experiments consistently catalysed a 3-fold higher PPM production (7.48 ± 0.29 pmol/min) compared with membrane fractions prepared from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) (2.34 ± 0.09 pmol/min) and an approx. 5-fold higher activity compared with membrane fractions prepared from *M. smegmatis* (pMV261) (1.46 ± 0.08 pmol/min). Values are given as means $\pm$ S.E.M. (Figure 3). These results suggest either that full-length Ppm1 and/or its mRNA is more stable than Ppm1/D2 and/or its mRNA, or that domain 1 exerts a positive influence on the catalytic activity of domain 2. Quantification of PPM synthesis in extracts prepared from *E. coli*, harbouring both *Mt-ppm1/D1* and *Mt-ppm1/D2* on compatible plasmids, would allow discrimination between these two possibilities. Unfortunately, several attempts to express *Mt-ppm1/D1* (or *Mt-ppm1*) in *E. coli* failed (results not shown). This observation and the earlier prediction of several putative transmembrane regions in domain 1 (Figure 2) suggest that production of *Mt*-Ppm1/D1 can be toxic for *E. coli*. We therefore decided to turn to a mycobacterial expression system.

Identification of the *M. smegmatis* PPM locus and role of *Mt-ppm1-D1* in *M. smegmatis* PPM synthase activity

M. avium and *M. leprae* potentially produce two distinct proteins, which are similar to the two domains found in *Mt*-Ppm1 respectively. The sequence and structure of the homologous *ppm1* locus of *M. smegmatis* was examined. Sequence analysis of the locus revealed that the homologue of *Mt-ppm1* is encoded by two separate and adjacent genes, one of 1785 nt (gene homologous with *Mt-ppm1/D1*, now termed *Ms-ppm2*) and one of 797 nt (gene homologous with *Mt-ppm1/D2*, now termed *Ms-ppm1*) respectively (GenBank® Nucleotide Sequence Database accession no. AJ294477). An intergenic region of 9 nt containing a putative ribosome-binding site separates the two genes (Figure 4).

To evaluate the possible role of Mt-Ppm1/D1 on PPM synthase activity in *M. smegmatis*, membrane fractions (5 μg each) were prepared from *M. smegmatis* (pMV261–*Mt-ppm1/D1*) and were found to consistently increase PPM synthesis (2.31 ± 0.08 pmol/min) in several independent experiments compared with membrane fractions prepared from *M. smegmatis* (pMV261) (1.46 ± 0.08 pmol/min). Values are given as means $\pm$ S.E.M. This activity was similar to that expressed by *M. smegmatis* (pMV261–*Mt-ppm1/D2*) (2.34 ± 0.09 pmol/min). In the light of the structure of the *M. smegmatis ppm2–ppm1* locus (see Figure 4), the above biochemical experiments suggest a possible role of *Mt-Ppm1/D1* in PPM synthesis at a protein level. In addition, these results suggest that domains 1 and 2 do not need to be produced as a single protein, even if both are involved in PPM synthesis.

Identification and characterization of a novel C_{40}-PPM from *M. smegmatis*

An examination of the mycobacterial PPM family by TLC/autoradiography (Figure 3) indicated that all members of the mycobacterial PPM family were overproduced in *M. smegmatis* harbouring pMV261–*Mt-ppm1*, pMV261–*Mt-ppm1/D2* or pMV261–*Mt-ppm1/D1* (results not shown). This result suggests that the intact Ppm1 or the truncated versions are unable to discriminate between subtle chain-length variations found within the endogenous pool of polyprenol monophosphates. It is well documented that the major PPM in *M. smegmatis* is a C_{50}-PPM [22], and our earlier studies [12] along with others [23] have identified a novel C_{35}-PPM in which four of the possible seven isoprenoid double bonds were saturated [23,24]. The presence of

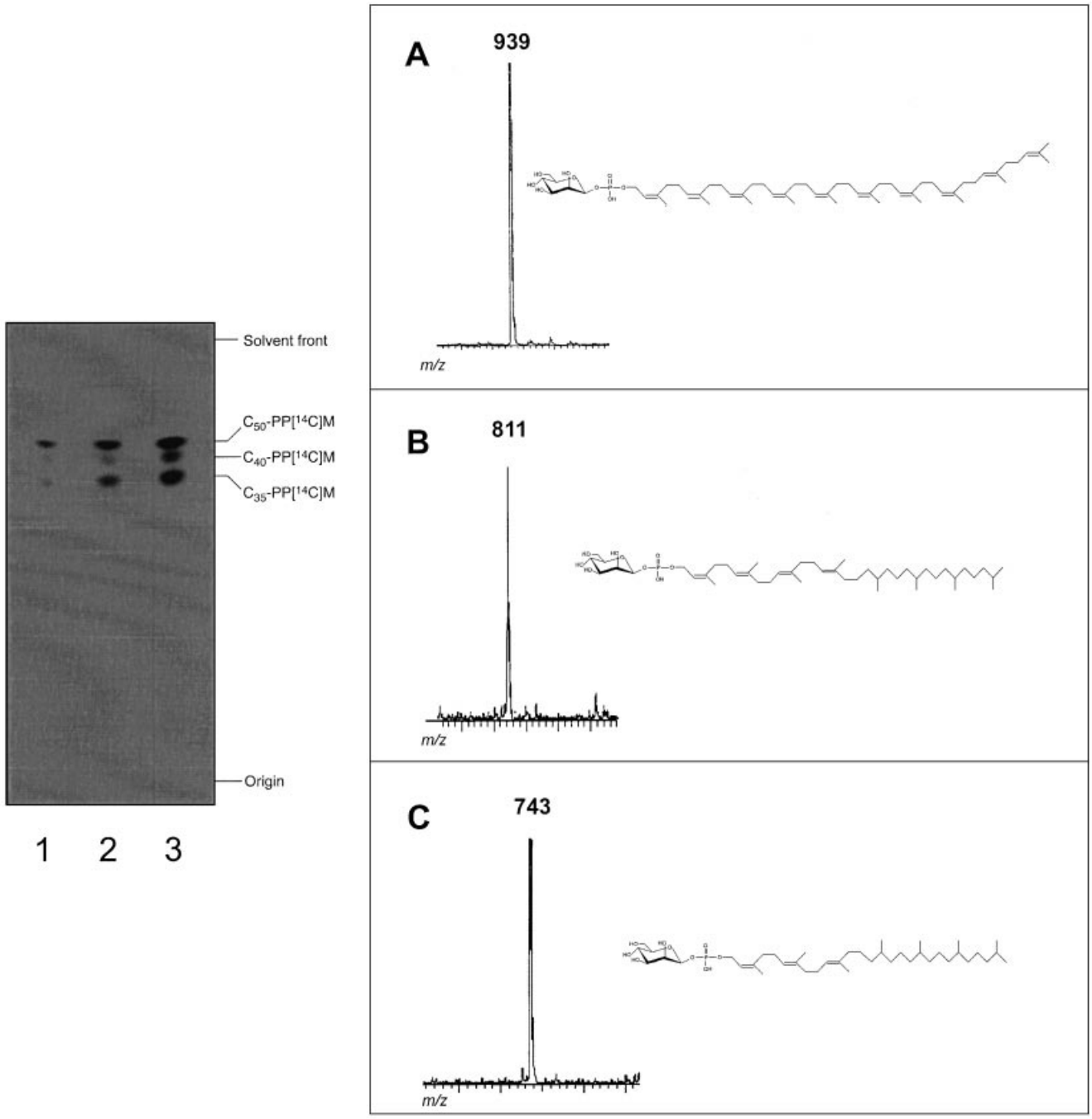

Figure 3 Thin-layer chromatogram of labelled endogenous polyprenol monophosphates (C_{35}–C_{40}–C_{50}) using GDP-[^{14}C]Man and membrane extracts from *M. smegmatis* harbouring pMV261, pMV261–*Mt-ppm1/D2* or pMV261–*Mt-ppm1*

Membrane fractions (5 μg of protein) were incubated in buffer A with GDP-[^{14}C]Man (0.25 μCi) in a total vol. of 50 μl for 30 min and base treated. Aliquots (10%) were taken for scintillation counting and TLC/autoradiography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.5:3.6). Electrospray MS of mycobacterial C_{50}-, C_{40}- and C_{35}-PPM are shown in (**A**–**C**) respectively. Lane 1, *M. smegmatis* (pMV261); lane 2, *M. smegmatis* (pMV261–*Mt-ppm1/D2*); and lane 3, *M. smegmatis* (pMV261–*Mt-ppm1*). Assays were performed in triplicate using freshly prepared membranes, with the thin-layer chromatogram being a representative example of a number of samples from independent experiments.

a third, previously unknown member of the PPM family was surprising and probably reflects the improved TLC resolution in the present study, compared with previous studies [22–24]. Electrospray MS of the individual PPMs generated from unlabelled GDP-Man after preparative TLC purification [12,24] yielded intense $(M-H)^-$ ions, confirming the presence of the C_{50}-PPM ($m/z = 939$, M_r 940) and the C_{35}-PPM ($m/z = 743$, M_r 744) (see Figures 3A and 3C). An intense $(M-H)^-$ ion was also obtained at $m/z = 811$, M_r 812, which corresponds to the addition of a C_5-isoprene ($m/z = 68$) to the mycobacterial C_{35}-PPM, suggesting that the unknown PPM is a C_{40}-PPM that is structurally related to the mycobacterial C_{35}-PPM (see Figure 3B) [24].

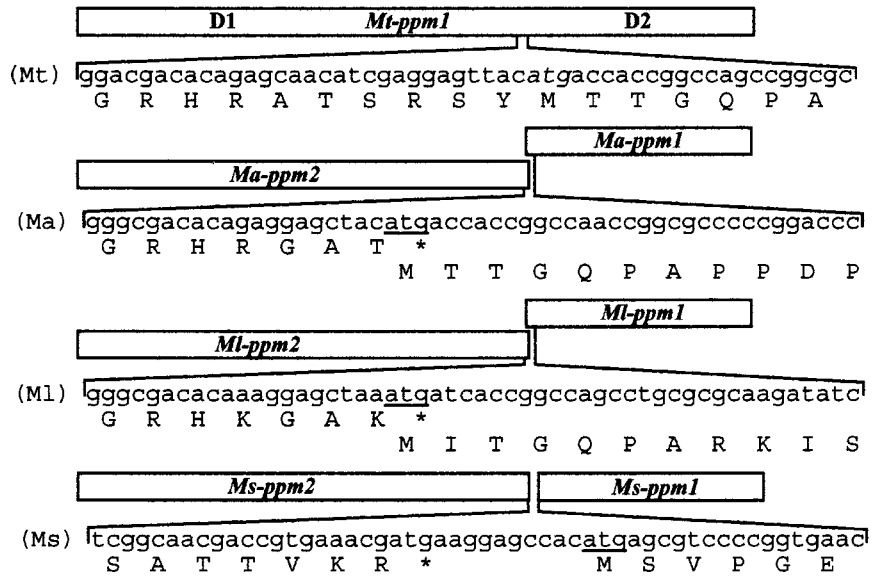

Figure 4 Structure of the *ppm1* locus and detailed junction regions in *M. tuberculosis*, *M. avium*, *M. leprae* and *M. smegmatis*

Asterisks indicate 'stop' codons. Initiation codons are underlined. For *M. tuberculosis*, italicized nucleotides (*ATG*) correspond to the first codon of *ppm1/D2*.

Table 2 Incorporation of [^{14}C]Man from GDP-[^{14}C]Man and exogenous polyprenol and dolichol monophosphates using enzymic extracts prepared from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) [I] and *E. coli* (pUC8–*Mt-ppm1/D2*) [II]

The reaction mixtures contained various lipid monophosphate substrates at a final concentration of 0.25 mM in 0.25% (w/v) CHAPS, membranes (25 μg) from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) or enzymic extracts (5 μg) from *E. coli* (pUC8–*Mt-ppm1/D2*) in a total vol. of 50 μg. Incubations were performed for 30 min at 37 °C and the PPMs/DPMs isolated after base treatment before scintillation counting. The results represent means of duplicates.

	Specific activity (pmol/min)	
	I	II
No exogenous substrate	2.82	0.84
C_{10}-P	1.43	0.47
C_{15}-P	2.63	1.21
C_{20}-P	9.61	5.60
C_{35}-P (α-dihydroheptaprenyl)	5.80	3.50
C_{35}-P	7.37	3.98
C_{50}-P	9.11	3.73
C_{60}-P	18.89	5.35
C_{75}-P	20.48	6.33
C_{95}-P (dolichol)	17.97	6.22

Incorporation of GDP-[^{14}C]Man by membrane fractions from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) and enzymic extracts from *E. coli* (pUC8–*Mt-ppm1/D2*) using various lipid monophosphate substrates

The chain-length specificity of *Mt*-Ppm1/D2 in relation to other non-mycobacterial monophosphate isoprene lipid substrates was examined by the use of commercially available polyprenol/dolichol monophosphates and membrane fractions from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) or extracts from *E. coli* (pUC8–*Mt-ppm1/D2*). Incubation of membrane fractions from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) in the absence of exogenous substrates led to a typical profile of C_{35}-, C_{40}- and C_{50}-PPMs (Table 2; Figure 5A, lane 10). The *E. coli* (pUC8–*Mt-ppm1/D2*) extract expressed a moderate level of activity in the absence of substrate (Table 2), which by TLC/autoradiography (Figure 5B, lane 10) was attributed to the utilization of the endogenous *E. coli* C_{55}-PPM pool. A similar observation by Orlean et al. [25] using recombinant yeast Dpm1 produced in *E. coli* suggested a lack of specificity with this family of glycosyltransferases. Interestingly, untransformed *E. coli* possessed no enzymic activity, a somewhat surprising observation given that alignment algorithms indicated the presence of an orthologue to *Mt*-Ppm1/D2 in the *E. coli* genome (P77757). Thus the putative *E. coli* gene may be inactive or may possess an unexpected substrate requirement, which is yet to be identified. C_{10}-geranyl monophosphate was inactive as a substrate for extracts from either *M. smegmatis* (pMV261–*Mt-ppm1/D2*) or *E. coli* (pUC8–*Mt-ppm1/D2*) and showed a slight inhibitory effect (Table 2; Figures 5A and 5B, lane 1). C_{15}-farnesyl monophosphate was weakly active. Increasing levels of PPM synthesis in place of endogenous PPM synthesis were observed with increasing chain lengths of the lipid monophosphate substrates (Table 2; Figures 5A and 5B, lanes 2–9). The newly synthesized products, resulting from the incubation of the lipid substrates (C_{35}–C_{95} monophosphate), unlabelled GDP-Man and *E. coli* (pUC8–*Mt-ppm1/D2*) extracts, were further characterized using electrospray MS. The reaction products yielded intense $(M-H)^-$ ions for the precursor lipid phosphates and, more importantly, the addition of m/z 162 of a hexose unit (Man), confirming glycosylation of the lipid phosphates (see Figures 6A–6F). Thus *Mt*-Ppm1/D2 seems to prefer substrates $> C_{15}$ and tolerates additional changes in the lipid moiety, such as saturation of the first isoprene unit for both the synthetic C_{35}-dolichol monophosphate and the C_{95}-dolichol monophosphate isolated from human livers. In addition to the saturation of the α-isoprene, other modifications such as the polysaturation of the mycobacterial C_{35}- and C_{40}-polyprenol monophosphates are also tolerated by the *Mt*-Ppm1/D2 synthase, suggesting that chain length is possibly the only key

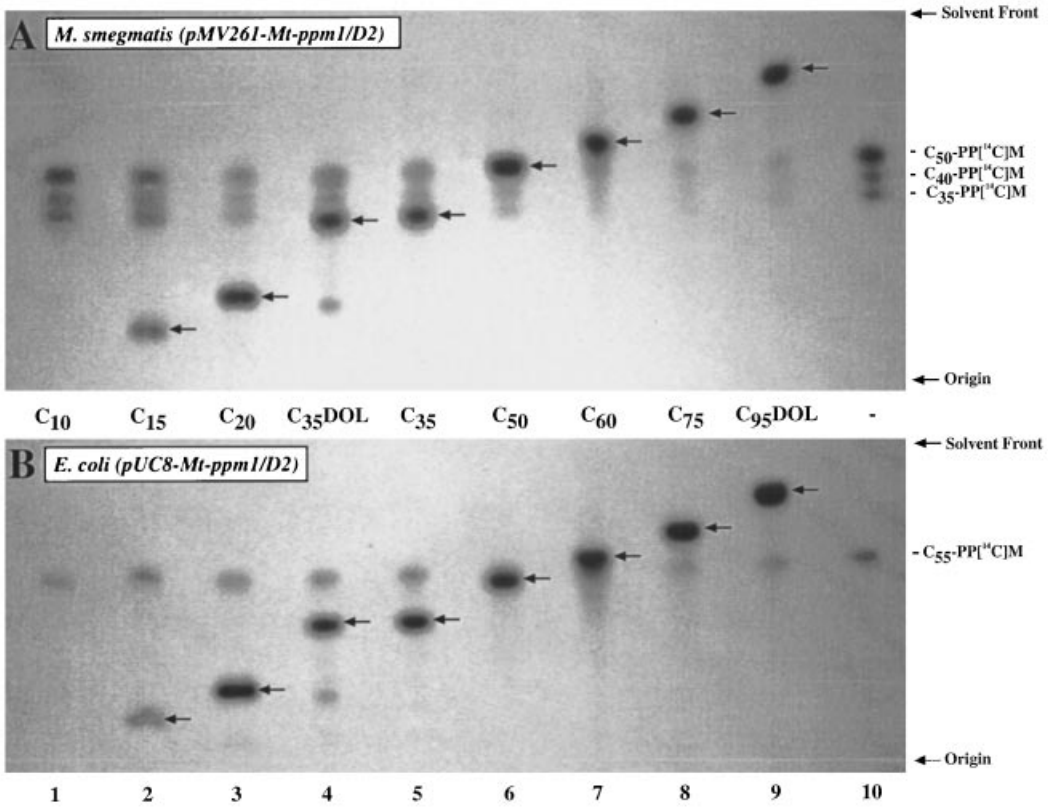

Figure 5 Incorporation of [^{14}C]Man from GDP-[^{14}C]Man into exogenous polyprenol and dolichol monophosphates using extracts from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) and *E. coli* (pUC8–*Mt-ppm1/D2*)

The standard reaction mixture contained the indicated lipid monophosphate substrates at a final concentration of 0.25 mM in 0.25% (w/v) CHAPS. Incubations were performed for 30 min at 37 °C with extracts from *M. smegmatis* (pMV261–*Mt-ppm1/D2*) (**A**) or *E. coli* (pUC8–*Mt-ppm1/D2*) (**B**), and the PPMs/DPMs were isolated after base treatment. An aliquot (10%) was subjected to scintillation counting (see Table 2). A second aliquot (10%) was subjected to TLC and autoradiography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.4:3.6). Arrows indicate the different PPMs/DPMs corresponding to the various chain lengths. Lane 1, C_{10}, geranyl monophosphate; lane 2, C_{15}, farnesyl monophosphate; lane 3, C_{20}, geranylgeranyl monophosphate; lane 4, C_{35}DOL, α-dihydroheptaprenyl monophosphate; lane 5, C_{35}, heptaprenyl monophosphate; lane 6, C_{50}, decaprenyl monophosphate; lane 7, C_{60}, dodecaprenyl monophosphate; lane 8, C_{75}, pentadecaprenyl monophosphate; lane 9, C_{95}, dolichol monophosphate; and lane 10, no exogenous substrate.

feature regarding the specificity of the mycobacterial PPM synthase.

DISCUSSION

Over the last decade, several studies have emphasized the role of LAM as a key factor in the major steps of tuberculosis immunopathogenesis and, paradoxically, also in protective immunity. Despite the importance of this immunomodulator, the biosynthetic origin of these important lipoglycans has largely been ignored until recently. The biosynthesis of PIMs is initiated by distinct α-mannosyltransferase activities that utilize GDP-Man as a sugar donor, transferring initially a Man*p* residue to the 2-position of *myo*-inositol to form phosphatidyl-*myo*-inositol monomannoside (PIM_1), followed by a further addition at the 6-position of *myo*-inositol to generate PIM_2 [11]. The latter α-mannosyltransferase activity has been shown to be catalysed by the *pimB* gene product from *M. tuberculosis* [13]. The addition of Man*p* residues to PIM_2 to form more complex PIMs also requires GDP-Man, according to the earlier studies of Brennan and Ballou [26]. However, Besra et al. [12] demonstrated that triacylated-PIM_2 is specifically extended by the addition of Man*p* residues from the alkali-stable sugar donor, PPM (C_{35}- and C_{50}-P-Man), which is generated from GDP-Man and the corresponding C_{35}- and C_{50}-polyprenol phosphate. These additions yield complex PIMs (at least triacylated-PIM_3 and triacylated-PIM_4) and linear LM possessing an $\alpha(1\rightarrow6)$-linked Man*p* backbone, ultimately leading to mature LAM.

As the enzymes in these biochemical steps have not been identified yet, we have embarked on a series of studies to identify mannosyltransferases implicated in the biosynthesis of LAM. The present communication reports on the identification of a PPM synthase from *M. tuberculosis*. Unexpectedly, *Mt*-Ppm1 turns out to be a two-domain enzyme. *Mt*-Ppm1/D2 is responsible for the catalytic activity of *Mt*-Ppm1 and *Mt*-Ppm1/D1 enhances the transferase activity of the protein by a mechanism that still needs to be clarified. Surprisingly, the Ppm1 orthologues in *M. leprae*, *M. smegmatis* and *M. avium* do not show this two-domain architecture. Instead, two separate proteins are coded by neighbouring genes (Figure 4). It is highly probable that *Mt*-Ppm1 is the consequence of a recent genetic fusion between the ancestors of *Mt-ppm1/D1* and *Mt-ppm1/D2*. The conservation of *ppm1* genes in mycobacteria, especially in the highly reduced genome of *M. leprae* [27] suggests that PPM synthase is an essential enzyme in mycobacteria. The results obtained in *M. smegmatis* suggest that *Mt*-Ppm1/D1 could physically interact with *Ms*-Ppm1. The formal demonstration of the interaction between *Mt*-Ppm1/D1 and *Ms*-Ppm1, and by

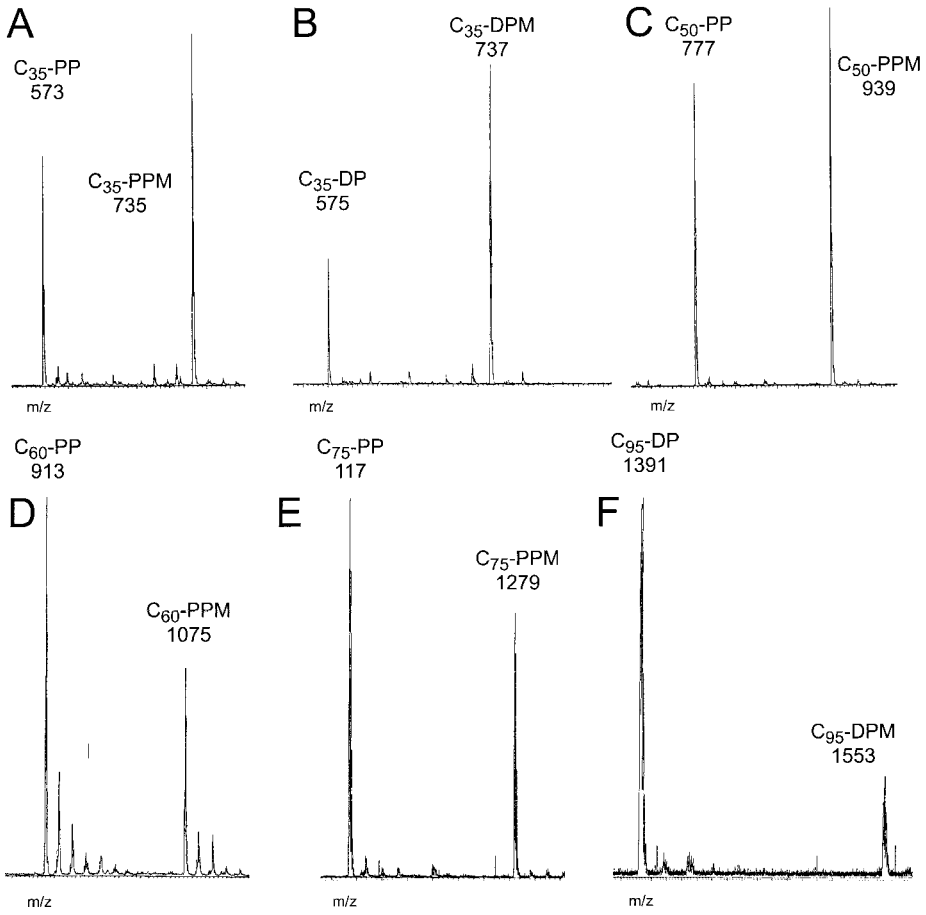

Figure 6 Electrospray MS analysis of reaction products resulting from the utilization of exogenous lipid phosphates and enzymic extracts prepared from *E. coli* (pUC8–*Mt-ppm1/D2*)

Reaction mixtures containing polyprenol and dolichol monophosphates, unlabelled GDP-Man and enzymic extracts from *E. coli* (pUC8–*Mt-ppm1/D2*) were incubated and processed as described in the Experimental section and then analysed by electrospray MS. C_{35}-PP, C_{35}-polyprenol phosphate; C_{35}-DP, C_{35}-dolichol phosphate; C_{50}-PP, C_{50}-polyprenol phosphate; C_{60}-PP, C_{60}-polyprenol phosphate; C_{75}-PP, C_{75}-polyprenol phosphate; and C_{95}-PP, C_{95}-polyprenol phosphate.

analogy between *Ms*-Ppm2 and *Ms*-Ppm1 is currently being investigated.

Based on the existence of two structurally distinct mycobacterial PPMs, Wolucka and de Hoffmann [28] proposed that specific mannosyltransferases would utilize individual polyprenol monophosphates. The rationale was based on the precedence that eukaryotic systems are able to differentiate between polyprenol substrates in terms of chain length and the presence of the α-saturated isoprene subunit. By contrast, the present study demonstrates that *Mt*-Ppm1 catalyses the production of three PPM species (C_{35}-, C_{40}- and C_{50}-PPM), and that *Mt*-Ppm1 synthase does not discriminate between polyprenol substrates in terms of chain length (for the endogenous polyprenol pool) and the nature of the saturated-isoprene subunits.

Eukaryotic DPM synthases have been classified into two families. Similar to the human synthase, *Mt*-Ppm1 lacks the typical C-terminal hydrophobic domain found in Dpm1 from *Saccharomyces cerevisiae* (*Sc*-Ppm1), *Ustilago maydis* and *Trypanosoma brucei* [29]. This hydrophobic terminal domain has been proposed to stabilize these enzymes in membranes [29]. Domain 1 of *Mt*-Ppm1 is rich in predicted transmembrane segments and may therefore play a similar role in the stabilization of the catalytic domain. In addition, various DPM synthases that possess a C-terminal hydrophobic domain were active *in vitro* when expressed in *E. coli*. By contrast, human Dpm1, which lacks this hydrophobic domain, is not active in *E. coli* [29]. In the present study, we show that the second domain of *Mt*-Ppm1 also lacks the terminal domain but is active *in vitro* when expressed in *E. coli*. Based on these criteria, *Mt*-Ppm1 may be considered as the first member of a new class of DPM synthases.

Eukaryotic DPM synthases have been demonstrated to be activated *in vitro* by a cAMP-dependent protein kinase [30,31], suggesting that DPM activity is regulated. It is interesting to note that *Mt*-Ppm1/D2 and its homologues in *M. smegmatis* and *M. leprae* possess a conserved serine residue corresponding to the putative phosphorylated serine of various eukaryotic DPM synthases (Figure 1).

Probable PPM synthases (EC 2.4.1.83) have been identified by homology search in various bacterial genomes (http://www.genome.ad.jp/dbget-bin/www.bget?enzyme+2.4.1.83), but to our knowledge *Mt*-Ppm1 is the first enzyme of this family that has been cloned and studied in bacteria. The identification of the key role of *Mt*-Ppm1/D1 and *Mt*-Ppm1/D2 in the synthesis of LAM will help in clarifying the biological importance of such molecules as immunomodulators and drug targets, and in defining pathogenic events and remedies for them. Further

characterization is required to identify the differences and similarities at a genomic and biochemical level of PPM and DPM synthesis in bacterial and eukaryotic systems in order to determine the precise roles of *Mt*-Ppm1/D1 and *Mt*-Ppm1/D2.

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