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Purification and characterization of the acyltransferase involved in biosynthesis of the major mycobacterial cell envelope glycolipid - monoacylated phosphatidylinositol dimannoside

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Abstract

Phosphatidylinositol mannosides are essential structural components of the mycobacterial cell envelope. They are implicated in host-pathogen interactions during infection and serve as a basis for biosynthesis of other unique molecules with immunomodulatory properties- mycobacterial lipopolysaccharides lipoarabinomannan and lipomannan. Acyltransferase Rv2611 is involved in one of the initial steps in the assembly of these molecules in *Mycobacterium tuberculosis* - the attachment of an acyl group to position-6 of the 2-linked mannosyl residue of the phosphatidylinositol mannoside anchor. Although the function of this enzyme was annotated 10 years ago, it has never been completely biochemically characterized due to lack of the pure protein. We have successfully overexpressed and purified *MSMEG_2934*, the ortholog of *Rv2611c* from the non-pathogenic model organism *M. smegmatis* mc²155 using mycobacterial pJAM2 expression system, which allowed confirmation of its *in vitro* acyltransferase activity, and establishment of its substrate specificity.

Keywords

Mycobacterium tuberculosis; mannosylated glycoconjugates; expression system

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Introduction

The cell surface of *Mycobacterium tuberculosis* is responsible for initial interaction of the bacillus with the host immune system and it therefore plays a crucial role in the pathogenesis of tuberculosis. From a large number of mycobacterial immunomodulators the mannosylated glycoconjugates of the mycobacterial cell envelope - lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIMs), represent the most prominent molecules proposed to significantly affect the course of infection [1–3].

Monoacylated phosphatidylinositol dimannoside (Ac₁PIM₂) is the most abundant mycobacterial cell envelope lipid from the family of PIMs [4] – phospholipids derived from 1,2-diacyl-*sn*-glycero-3-phospho-1-D-*myo*-inositol (PI), which is decorated with one to six mannosyl residues and one or two additional acyl groups. This molecule represents both an end product and an intermediate in the metabolic pathway leading to LAM and LM [3]. Biosynthesis of Ac₁PIM₂ in *M. tuberculosis* involves an action of three essential enzymes [5, 6], two mannosyltransferases – PimA (Rv2610c) [7] and PimB (previously known as PimB') (Rv2188c) [8], and acyltransferase Rv2611c [9] (Fig. 1). PimA catalyzes the transfer of the mannosyl residue from GDP-mannose to position-2 of the *myo*-inositol ring of PI producing phosphatidylinositol monomannoside (PIM₁). A second mannose is added to position-6 of the *myo*-inositol ring of PIM₁ by PimB enzyme, giving rise to phosphatidylinositol dimannoside (PIM₂). *M. smegmatis* mc²155 counterparts of both these mannosyltransferases have been successfully produced and purified from *E. coli* hosts. This allowed thorough structural and mechanistic characterization of PimA protein [10–13] and in-depth investigation of PimB enzyme activity [8]. In contrast, acyltransferase Rv2611c has been only partially characterised. The enzyme was shown to attach palmitoyl group from its CoA carrier to position-6 of the 2-linked mannosyl residue in both PIM₁ and PIM₂ in the cell free assays performed with the crude mycobacterial membrane fractions [9], but the latter substrate was later proposed to be the preferred one [8]. Closer investigation of this acyltransferase has been precluded due to lack of the pure and active enzyme. In our previous work we attempted to produce Rv2611c using several expression systems in *E. coli* hosts (unpublished results), as well as from *M. smegmatis* strain constitutively expressing the recombinant His-tagged Rv2611c protein [9]. Since our efforts to obtain sufficient amounts of highly purified enzyme, suitable for further biochemical characterization from these sources failed, we decided to clone, express and purify an ortholog of Rv2611c from *M. smegmatis* mc²155 - MSMEG_2934 protein.

The most successful approach, which we describe in this report, takes advantage of the acetamide-inducible expression vector pJAM2 designed for efficient production of recombinant C-terminally His-tagged proteins in *M. smegmatis*. The plasmid, derived from the *Escherichia coli* – mycobacteria shuttle vector pJEM12 [14], contains 1.5 kb upstream of the acetamidase coding region from *M. smegmatis* NCTC8159, DNA encoding the first six amino acids (Met, Pro, Glu, Val, Val, Phe) of the acetamidase gene, the sites for the restriction enzymes *Bam*HI, *Sca*I, *Xba*I and a sequence for 6-histidine residues [15]. This expression system, described already in 1998 [15], has been used for a number of applications, but as far as we are aware, this is the first report of its exploitation for successful purification of the mycobacterial protein since the original publication. The

identity and integrity of the produced protein was confirmed by mass-spectrometry and its activity, as well as specificity was examined with a range of anticipated natural substrates.

Material and Methods

Bacterial strains and culture conditions

Escherichia coli XL1-Blue (Stratagene) used in cloning experiments was routinely grown in Luria-Bertani (LB) medium at 37°C. *M. smegmatis* mc²155 was cultivated in the LB medium supplemented by 0.05 % Tween 80 at 37°C. The mycobacterial mutant strain MYC1573 [9] was grown in LB medium supplemented by 0.05 % (v/v) Tween 80 and 20 µg/ml kanamycin at 37°C. The overproducing strain *M. smegmatis* mc²155 pJAM2msmeg_2934 was cultivated at 37°C in MM63 medium [15 mM (NH₄)₂SO₄, 10 mM KH₂PO₄, 18 µM FeSO₄·7H₂O, pH 7] supplemented with 1 mM MgSO₄, 0.025 % (v/v) tyloxapol and 0.2 % (w/v) succinate and 20 µg/ml of kanamycin (modification from [15]). After the culture reached OD₆₀₀ = 0.6 the expression of the cloned gene in the strain *M. smegmatis* mc²155 pJAM2msmeg_2934 was induced by 0.2 % acetamide treatment for 16 hrs at 37°C. The induced cells were harvested by centrifugation at 4,000 × g for 10 min at 4°C and washed with buffer A (20 mmol/l Tris-HCl, pH 7.5) with the typical yield of approximately 3 g of the cells (wet weight) per 1 l of the induced culture.

Amplification and cloning of msmeg_2934 gene from *M. smegmatis* mc²155

The genomic sequence of msmeg_2934 (JCVI CMR, U.S.A.) was amplified from *M. smegmatis* mc²155 DNA by PCR using forward primer AT.fw-1 (5'-AAGGGATCCGTGACGGACTTGGGGTATGCGG-3') and reverse primer AT.rv (5'-GGCTCTAGAGGTTCCCAACCGTGCGCGGC-3'). PCR amplification of the gene consisted of the initial denaturation step (95°C, 10 min) followed by 35 cycles of denaturation (95°C, 60 s), annealing (67°C, 60 s) and primer extension (72°C, 70 s) followed by final extension at 72°C for 10 min. The PCR product was cloned directly into the *E. coli* - mycobacterial shuttle pJAM2 vector [15] between *Bam*HI and *Xba*I restriction sites providing the recombinant protein with a six-histidine tag on the carboxyl terminus. Positive transformants were isolated by growth at 37°C on LB agar supplemented by 20 µg/ml kanamycin.

Subcellular localization of MSMEG_2934 in the mycobacterial cells

Cells were suspended in buffer A (1 g of cells/5 ml of buffer A) and disrupted by sonication in 10 cycles of 60 s pulses with 90 s cooling intervals between the pulses. Unbroken cells and cell debris were removed by centrifugation of the cell lysates at 11,000 × g and 4°C for 20 min. The supernatant was further centrifuged at 100,000 × g and 4°C for 1 hr to sediment the membranes. These were then gently suspended in buffer A to obtain the membrane fraction with protein concentration of around 80 mg/ml. The cell debris was re-suspended in buffer A in ratio of 1 g of cells (the initial wet weight) per 2 ml of the final suspension and centrifuged at 23,000 × g and 4°C for 1 hr in a gradient formed from 60 % (v/v) Percoll (GE Healthcare). The upper white layer representing the “cell envelope” fraction was collected and washed three times with buffer A by centrifugation at 23,000 × g for 10 min and then re-suspended in buffer A to a final protein concentration of around 15 mg/ml. Localization of

the recombinant protein was determined by SDS-PAGE followed by Western blotting and immunodetection, as described below.

Purification of recombinant MSMEG_2934

Growth, induction, harvest and ultrasonic disintegration of *M. smegmatis* mc²155 pJAM2msmeg_2934 were performed as described in the previous sections. Following the sonication of 1 g of cells in 5 ml of buffer A, recombinant MSMEG_2934 was released from the cellular structures by addition of 2 mM CHAPS, 300 mM NaCl and 10 % (v/v) glycerol to the cell lysates, which were then gently stirred at 4°C for 1 hr. Unbroken cells and cell wall debris were removed by centrifugation at 11,000 × g and 4°C for 20 min. Imidazole was added to the 11,000 × g supernatant to a final concentration of 10 mM and recombinant proteins were allowed to bind to 1 ml TALON Co²⁺ affinity resin (Clontech) in the Falcon tube with gently stirring on rotator at 4°C for 1 hr. The resin was then transferred to the column and washed with 20 ml of 10 mM imidazole solution in buffer BE (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 % glycerol). The proteins were then eluted from the column by gravity flow with the step-wise gradient of 50, 200 and 1,000 mM imidazole in 5 ml of buffer BE. Collected samples were analysed by SDS-PAGE. Fractions with the largest amount of MSMEG_2934 protein were combined, dialysed against 5 l of buffer A for 12 hrs at 4°C, and stored at -20°C in buffer A containing 10 % (v/v) glycerol. The procedure typically yielded 1 mg of pure protein from 1 g of the cells (wet weight).

Preparation of radioactive substrates for the acyltransferase assay

[¹⁴C]Ac₁PIM₂ was obtained from the lipid extract of radiolabeled *M. smegmatis* mc²155; [¹⁴C]PIM₂ was extracted from radiolabeled cells of the mutant strain MYC1573, which accumulates this lipid [9]. Both strains were cultivated as specified above until OD₆₀₀ of the cultures reached 0.5. Then [1, 2- ¹⁴C]-acetate (specific activity of 106 mCi/mmol, ARC Inc.) was added in the final concentration of 0.5 μCi/ml and the cells were grown for further 4 hrs at 37°C. These radiolabeled cells were harvested by centrifugation at 4,000 × g for 10 min at 4°C and washed with buffer A. Each cell pellet was incubated for 15 min at 70°C in 2 ml ethanol with constant stirring. The extracts were separated from the mixtures by centrifugation at 1,000 × g and 25°C for 5 min and the pellets were re-extracted twice with 2 ml of CHCl₃/CH₃OH (2:1, v/v) at 56°C for 2 hrs with constant stirring. After centrifugation of the mixtures, supernatants were removed, all extracts were pooled, dried under the stream of nitrogen at 25 °C and dissolved in 2 ml of CHCl₃/CH₃OH/H₂O (4:2:1). Centrifugation of the mixture at 1,000 × g for 5 min at 25°C resulted in formation of two phases. Bottom organic phase was removed, dried under the stream of nitrogen at 25°C and re-dissolved in CHCl₃/CH₃OH (2:1). Radiolabelled lipids were analysed by TLC on aluminium-coated silica 60 F₂₅₄ plate (Merck) developed in CHCl₃/CH₃OH/conc. NH₄OH/H₂O (65:25:0.5:4), followed by autoradiography using Kodak Bio-Max MR films.

The lipid extracts enriched with [¹⁴C]PIM₁ and [¹⁴C]Ac₁PIM₁ were generated enzymatically in reaction mixtures containing GDP-[¹⁴C]mannose and *M. smegmatis* membranes supplemented with purified PimA for the former substrate and with both PimA and MSMEG_2934 for the latter substrate, as described below.

All radiolabeled substrates were purified from the lipid extracts by preparative TLC performed on the aluminium-coated silica 60 F₂₅₄ plates (Merck) developed in CHCl₃/CH₃OH/conc. NH₄OH/H₂O (65:25:0.5:4). The bands corresponding to [¹⁴C]PIM₁, [¹⁴C]Ac₁PIM₁, [¹⁴C]PIM₂ and [¹⁴C]Ac₁PIM₂ revealed by autoradiography using Kodak Bio-Max MR films, were scraped off the TLC plates and extracted from silica with CHCl₃/CH₃OH (2:1) by repeated vortexing. The extracts were dried under a stream of nitrogen at 25°C, re-dissolved in CHCl₃/CH₃OH (2:1) and quantified by scintillation spectrometry.

Acyltransferase activity assays

Initially, the activity of recombinant MSMEG_2934 was assessed in cell-free'' reactions with crude membranes from *M. smegmatis* mc²155, which were incubated with GDP-[¹⁴C]mannose to produce putative MSMEG_2934 substrates *in situ*. The synthesis of [¹⁴C]PIM₁ in the reaction mixture was enhanced by an addition of mannosyltransferase PimA from *M. smegmatis* mc²155 (PimA_{SM}) [10]. These enzymatic reactions consisted of 10 µg of purified MSMEG_2934, 1.2 µg of purified PimA_{SM} [16] and 250 µg of membrane proteins from *M. smegmatis* mc²155, 0.1 µCi GDP-[¹⁴C]mannose (specific activity of 55 mCi/mmol, ARC Inc.), 0.12 mM palmitoyl-CoA (Sigma-Aldrich) in DMSO with final concentration in the reaction mixture 2 % (v/v), 62 µM ATP, 10 mM MgCl₂, and buffer A in the final volume of 50 µl. After 1 hr incubation at 37°C reactions were stopped by an addition of 300 µl of CHCl₃/CH₃OH (2:1). Organic phase containing the radioactive reaction products was separated from the mixture by centrifugation at 1,000 × g and 25 °C and half of it was analysed by TLC, followed by autoradiography using Kodak Bio-Max MR film. Because this assay relies entirely on commercially available substrates - GDP-[¹⁴C]mannose (as a tracer) and palmitoyl-CoA, we routinely used it for monitoring the activity of purified MSMEG_2934.

An alternative assay employing purified MSMEG_2934 and a range of the putative lipid substrates was used to assess substrate specificity of the recombinant enzyme. Here, reaction mixtures consisted of 650 dpm of the radioactive substrate, 10 µg of purified MSMEG_2934, 0.12 mM palmitoyl-CoA in DMSO with a final concentration of 2 % (v/v), 62 µM ATP, 10 mM MgCl₂, and buffer A in a final volume of 50 µl. After 1 hr incubation at 37 °C the samples were processed as described above. The intensity of the bands on the autoradiogram corresponding to the substrates and the products of the reaction were quantified by IMAGEJ (NIH) software.

In Source Decay (ISD) characterization of recombinant acyltransferase MSMEG_2934

Purified protein (1 µl, 10 mg/ml) has been mixed with 2 µl of saturated 1,5-diaminonaphthalene in TA50 (0.1% trifluoroacetic acid: acetonitrile, 1:1) and spotted on the MALDI target plate. In-source decay data was collected using utrafleXtreme MALDI-TOF/TOF instrument (Bruker). N- and C-terminal fragments were identified by BioTools software (Bruker) using theoretical sequence of recombinant MSMEG_2934 protein.

Other procedures

Protein concentration was measured with fluorescent Qubit® Protein Assay kit (Invitrogen). Production and purity of the recombinant protein were analysed by SDS-PAGE using 4–12% Bis-Tris polyacrylamide gels (Novex) followed by dry Western blotting to nitrocellulose membrane (Invitrogen) and immunochemical detection with mouse primary anti-His antibodies (Sigma-Aldrich) diluted 1:2,000. Goat anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) were used as secondary antibodies in a dilution of 1:8,000 to provide chromogenic reaction with BCIP/NBT substrates (Promega).

Results and discussion

Recombinant acyltransferase MSMEG_2934 can be overproduced in *M. smegmatis* and purified to homogeneity by affinity chromatography

Biochemical characterization of the enzymes with novel functions is often hampered by the unavailability of pure and active proteins. Although many efficient systems have been described for production of foreign proteins in *E. coli*, very frequently, expression of proteins from *Mycobacterium* sp. in this host results in production of insoluble inclusion bodies [17]. This well-known phenomenon proved to be the case also for mycobacterial enzyme acylating the 6-position of the 2-linked mannosyl residue of PIMs. The importance of investigation of this enzyme is underlined by the fact that it is distinct from any other bacterial acyltransferase described so far [18]. In addition, the acylation pattern of mycobacterial LM determines anti-inflammatory *versus* pro-inflammatory effects of LM, which could represent the means of regulation of the host innate immunity by mycobacteria [19]. Our attempts to produce recombinant acyltransferase MSMEG_2934 included expression vectors pET15b, pET28a, pET29a, pMALc2X in *E. coli* BL₂₁(DE3), *E. coli* BL₂₁(DE3)pLysS, *E. coli* Rossetta-gami (DE3)pLysS, C41(DE3) and a range of conditions for the induction of gene expression, such as different temperatures (16°C, 25°C, 37°C) or various cultivation media (LB, auto-inducing medium [20]) (data not shown). However, none of these approaches was successful. To overcome the problem with the solubility of mycobacterial proteins produced in *E. coli*, several procedures have been designed for efficient expression and purification of such proteins from *M. smegmatis* host [17, 21, 22], including those which exploit plasmids harbouring the promoter region of the acetamidase gene [15, 23–25]. Based on our previous laboratory experience we have chosen to use one of these plasmids, *E. coli*-mycobacterial shuttle vector pJAM2 [15]. Compared to the expression vectors pVV2 [26] and pVV16 [27], which we widely use for constitutive expression of His-tagged proteins of interest in mycobacteria, pJAM2 appeared to be superior for purification purposes in that the level of expression was usually significantly higher, purification was more efficient and also it allowed expression of proteins which are toxic during the constitutive expression (data not shown).

Overexpression of MSMEG_2934 gene in *M. smegmatis* provided a large amount of the protein with the expected size (~34 kDa) carrying a C-terminal histidine tag. Our initial purification trials from the cell lysates prepared from the induced *M. smegmatis* pJAM2_{msmeg_2934} cells using buffer A (20 mmol/l Tris-HCl pH 7.5) resulted in low yields of insufficiently purified protein. Since the recombinant protein localized to cytosolic,

membrane and cell envelope fractions (Fig. 2), we have examined several conditions for its efficient release from cellular structures, specifically the addition of 300–500 mM NaCl and/or 2–8 mM CHAPS (data not shown). The best results were achieved by treating the crude lysates with both 2 mM CHAPS and 300 mM NaCl. Isolation of MSMEG_2934 was then performed on Co^{2+} affinity resin as described in *Materials and methods* and resulted in obtaining highly purified protein, as confirmed by SDS-PAGE (Fig. 3). The intactness of the protein was confirmed by ISD (Fig. 4). The recombinant MSMEG_2934 protein carried the first six amino acids of the acetamidase gene and two amino acids from the *Bam*HI restriction site on its N-terminus, as well as two amino acids from the *Xba*I restriction site and 6-histidine residues derived from the pJAM2 vector on its C-terminus. However, these additions did not seem to affect the protein acyltransferase activity, which was corroborated in the cell free reactions.

Purified recombinant MSMEG_2934 is showing acyltransferase enzymatic activity

Palmitoyl groups have been found to be preferentially present in the 6-position of the 2-linked mannosyl residue of Ac_1PIM_2 [4, 28, 29] and therefore palmitoyl-CoA was chosen as a donor of acyl units for MSMEG_2934. The assessment of the MSMEG_2934 activity is complicated by the fact that the proposed preferred acceptor substrate PIM_2 is not commercially available and also due to lipid nature of both substrates. To overcome these difficulties the acceptor substrate can be produced *in situ* in the cell-free reactions with enzymatically active membranes from *M. smegmatis* and GDP- $[^{14}\text{C}]$ mannose as a tracer, as described before [8]. However, under these conditions, PIM_2 is very efficiently converted to Ac_1PIM_2 by the activity of endogenous MSMEG_2934 and thus addition of purified recombinant MSMEG_2934 would very likely not result in the detectable increase of its reaction product Ac_1PIM_2 due to lack of PIM_2 substrate. We have thus produced an alternative MSMEG_2934 substrate $[^{14}\text{C}]\text{PIM}_1$ in the reaction mixture by an addition of the recombinant PimA_{SM} . As shown in Fig. 5, the presence of MSMEG_2934 resulted in a significantly higher synthesis of $[^{14}\text{C}]\text{Ac}_1\text{PIM}_1$ and also in a slight increase of the amount of $[^{14}\text{C}]\text{Ac}_1\text{PIM}_2$ confirming the palmitoyltransferase activity of the purified recombinant protein.

PIM_2 is the preferred substrate for purified recombinant MSMEG_2934

In order to address the acceptor substrate specificity of the purified enzyme we have examined its activity in reaction mixtures containing solely radioactive $[^{14}\text{C}]\text{PIM}_1$ or $[^{14}\text{C}]\text{PIM}_2$, palmitoyl-CoA as the donor substrate, purified MSMEG_2934 and the reaction buffer. After incubation the lipids were extracted from reaction mixtures and separated by TLC. The results presented in Fig. 6 confirm that both $[^{14}\text{C}]\text{PIM}_1$ and $[^{14}\text{C}]\text{PIM}_2$ were acylated by purified recombinant MSMEG_2934, leading to the production of $[^{14}\text{C}]\text{Ac}_1\text{PIM}_1$ and $[^{14}\text{C}]\text{Ac}_1\text{PIM}_2$, respectively. In case of the former substrate, 60% of the total intensity distributed within individual signals can be attributed to the unreacted substrate and the remaining 40% to the reaction product, while with the latter substrate these values are 14% for unreacted substrate and 86% for the reaction product. This finding confirms that PIM_2 is the preferred substrate of purified recombinant MSMEG_2934, consistent with the metabolic pathway proposed by Guerin et al [8]. We have also examined the possible role of MSMEG_2934 in further acylation of monoacylated PIM_1 or PIM_2 at

position-3 of their inositol ring to form diacylated forms of PIMs in an experiment with the [^{14}C]Ac₁PIM₁ and [^{14}C]Ac₁PIM₂. As shown in Fig. 6 these compounds did not serve as substrates for MSMEG_2934 and thus the enzyme catalyzing this modification has yet to be identified.

Concluding remarks

The presented data demonstrate that pJAM2 system allows efficient production and successful purification of the mycobacterial membrane associated recombinant protein from *M. smegmatis* host in the active form. By the described procedure we have produced and characterized the novel mycobacterial acyltransferase responsible for the attachment of the palmitoyl group on position-6 of the 2-linked mannosyl residue of PIM₂ – the modification found in the backbones of higher PIMs, LM and LAM, which significantly affects the immunological response of the host to mycobacterial infection. Based on the confirmed activity of the purified protein we propose to name the mycobacterial genes encoding the homologs of this protein *ptfP1* (palmitoyl transferase acting on PIM₂).

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- we used mycobacterial pJAM2 expression system to obtain acyltransferase MSMEG_2934
- MSMEG_2934 protein purified to homogeneity was intact and active
- MSMEG_2934 attaches palmitoyl group to phosphatidylinositol dimannoside

Figure 1. Metabolic pathway for biosynthesis of Ac_1PIM_2 in mycobacteria

The enzymes catalysing the individual reactions in *M. tuberculosis* H37Rv and *M. smegmatis* mc²155 are shown in the scheme. R_1 , R_3 – $\text{C}_{15}\text{H}_{31}$ (alkyl chain of palmitic acid), R_2 – $\text{C}_{18}\text{H}_{37}$ (alkyl chain of tuberculostearic acid) represent the most abundant substituents at the specified positions [4].

Figure 2. Subcellular localization of recombinant MSMEG_2934

Panel A. 50 µg of the membrane (M), cell envelope (CE) and cytosolic (C) protein from the control strain *M. smegmatis* pJAM2 and the overproducing strain *M. smegmatis* pJAM2msmeg_2934 was separated on SDS-PAGE and visualized by Coomassie Brilliant Blue. *Panel B.* Western blot from the gel described in *Panel A.* Immunodetection was performed with mouse Anti-His antibodies and goat Anti-mouse antibodies conjugated with alkaline phosphatase.

Figure 3. Evaluation of MSMEG_2934 purity by SDS-PAGE

65 μ g of the cell lysate (Lane 1), 65 μ g of the 11,000 $\times$ g supernatant used for purification (Lane 2) and 2.5 μ g of purified MSMEG_2934 (Lane 3) were separated on SDS-PAGE and visualized by Coomassie Brilliant Blue.

A.

B.

PEVVFGSVTDLGYAAGWRLVRAMPEAMAQGVFGAGARYAARNGGPEQLRRNLARVVGKPPAD
 VPDDLIRASLASYARYWREAFRLPAMDHGRLGEQLDVIDIDHLWSALDAGRGAVLALPHSGNWD
 MAGVWLQNYGPFTTVAERLKPESLYRRFVEYRESLGFEVLPLTGGERPPFEVLAERLTDNRPIC
 LMAERDLTRSGVQVDFFGEATRMPAGPAKLAJETGAALFPVHCWFEGDGGWGMRYPELDTSSG
 DVTAITQALADRFAANIATYPADWHMLQPQWIADLSDERRARLGTSRHHHHHH

Figure 4. In-source decay analysis of purified recombinant MSMEG_2934

Panel A. ISD spectrum shows N- and C- terminal peptide fragments (c and z+2 ion series, respectively) of expected molecular masses confirming the presence of intact protein termini. The mass differences between the fragments allow the identification of individual amino acids in the sequence. *Panel B.* Theoretical sequence of recombinant MSMEG_2934 with underlined sequences observed in ISD analysis.

Figure 5. Confirmation of the acyltransferase activity of purified MSMEG_2934 in the crude membrane assay

Reaction mixtures contained crude membranes from *M. smegmatis* mc²155 and GDP-[¹⁴C]-mannose as a tracer (Lane 1), supplemented with PimA_{SM} and palmitoyl-CoA (both Lanes 2 and 3) and purified MSMEG_2934 (Lane 3). The lipids were extracted from reaction mixtures and analysed by TLC and autoradiography as described in Material and Methods section.

Figure 6. Substrate specificity of purified MSMEG_2934

Reaction mixtures contained 650 dpm of each radioactive substrate: $[^{14}\text{C}]\text{PIM}_1$, $[^{14}\text{C}]\text{Acyl}_1\text{PIM}_1$, $[^{14}\text{C}]\text{PIM}_2$, $[^{14}\text{C}]\text{Acyl}_1\text{PIM}_2$. Lane 1 – control reaction mixture without the enzyme and without the donor substrate, Lane 2 – reaction mixture with the enzyme, but without the donor substrate, Lane 3 – reaction mixture containing both the enzyme and palmitoyl-CoA. Following incubation the lipids were extracted from reaction mixtures and analysed by TLC and autoradiography, as described in *Material and Methods*.