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Investigation of ABC transporter from mycobacterial arabinogalactan biosynthetic cluster

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Abstract. Two genes from the "mycobacterial arabinogalactan biosynthetic cluster" spanning the region from *Rv3779* to *Rv3809c* in the genome of *Mycobacterium tuberculosis* H37Rv were annotated as possible components of the ATP-binding cassette transporter. *Rv3781* encodes a nucleotide-binding domain and *Rv3783* determines production of a membrane-spanning domain. We have examined possible roles of these genes in mycobacterial cell wall biosynthesis through inactivation of their respective orthologs in *Mycobacterium smegmatis* mc^{2}155, phenotypic characterization of the mutant strains *via* metabolic labeling with [U-^{14}C]-glucose, cell-free reactions with UDP-[U-^{14}C]-galactose monitoring galactan build-up and transcriptional analysis. Several lines of evidence suggest that this ABC transporter is involved in biosynthesis of arabinogalactan, although more investigation is needed to establish its precise role or the transported substrate.

Key words: *Mycobacterium* — Cell wall — ABC transporter

Abbreviations: ABC transporter, ATP-binding cassette transporter; AG, arabinogalactan; DEPC, diethylpyrocarbonate; GAS medium, glycerol/alanine/salts containing medium; GL, glycolipid; GT, glycosyltransferase; Km, kanamycin; LAM, lipoarabinomannan; LM, lipomannan; NBD, nucleotide binding domain; PBS, phosphate/saline buffer.

Introduction

The mycobacterial cell wall core composed of covalently linked peptidoglycan, heteropolymeric arabinogalactan (AG) and the branched-chain mycolic acids is the site of action of many of the current anti-tuberculosis drugs and instrumental in the viability and pathogenesis of *Mycobacterium tuberculosis* in the intracellular environment (McNeil and Brennan 1991). Within the biogenesis of AG – the intricate structure composed of approximately 100 monosaccharide units, a number of fundamental glycosyltransferases have been discovered (for review, see Berg et al. 2007; Kaur et al. 2009). Nevertheless, the questions of topology of the events leading to the production of full-length AG in the pseudo-outer layer of mycobacteria remain open.

The initial acceptor for synthesis of the cell wall core is the carrier lipid, decaprenyl-P (C_{50}-P), upon which the cell wall linker unit (Rha-GlcNAc-P-) is constructed (Mikusova et al. 1996). These steps are catalyzed by the subsequent actions of the GlcNAc-1-phosphate transferase WecA (Jin et al. 2010) and rhamnosyltransferase WbbL (Mills et al. 2004) giving rise to glycolipid intermediates GL1 (C_{50}-P-P-GlcNAc) and GL2 (C_{50}-P-P-GlcNAc-Rha). Addition of the first two galactofuranose (Gal*f*) units from UDP-Gal*f* to GL2 carrier is assured by galactofuranosyl transferase

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GlfT1, the *Rv3782* gene product. Further polymerization of galactofuran continues by the action of GlfT2, the Rv3808c enzyme (Belanova et al. 2008), resulting in the production of more glycosylated intermediates C_{50}-P-P-GlcNAc-Rha-$(Gal)_{3-n}$, where n may represent the full galactan polymer. The genetics and enzymology of *de novo* arabinan synthesis have also been largely developed. Presumably, it takes place on the outside of the plasma membrane on account of dependency on the lipidic substrate decaprenyl-P-Ara*f* as a sugar donor and arabinosyltransferases belonging to the membranous GT-C class (Alderwick et al. 2006; Seidel et al. 2007; Birch et al. 2008; Skovierova et al. 2009). Subsequent events, notably growth of arabinan on the preformed C_{50}-P-P-GlcNAc-Rha-galactan, ligation to peptidoglycan and attachment of mycolates, are undefined (Berg et al. 2007; Kaur et al. 2009). Likewise, topology of the galactan build-up and transposition to the extra-membrane compartment has not been answered. It is expected that the initial steps in galactan synthesis take place on the cytosolic face of the plasma membrane because of their reliance on nucleotide sugar donors and soluble glycosyl transferases of the GT-A class (Berg et al. 2007). At some point the C_{50}-P-P-GlcNAc-Rha-galactan must be translocated to the outer leaflet of the membrane to facilitate arabinan attachment and ligation to peptidoglycan; however, neither the nature of the transported intermediates nor the proteins involved in these processes are known.

The initiating *glfT1* gene, encoded by *Rv3782* in *M. tuberculosis* H37Rv, is located in a highly conserved "AG biosynthetic cluster" (Belanger and Inamine 2000) that is found in all sequenced mycobacterial genomes. Interestingly, the two neighboring genes, *Rv3781* and *Rv3783,* appear to encode components of the predicted ABC transporter. As pointed by Braibant et al. (2000), among at least 37 ABC transporters identified in *M. tuberculosis,* $(Rv3781)_2/(Rv3783)_2$ is the only candidate suggested to be involved in the export of polysaccharides to the cell surface of *M. tuberculosis* based on significant similarities in amino-acid sequences of the two gene products with corresponding proteins involved in the translocation of capsular polysaccharides and O-antigens, respectively, from *Yersinia enterocolitica* (Zhang et al. 1993), *Brucella melitensis*, *Vibrio cholerae* (Manning et al. 1995), and *Escherichia coli* (Pavelka et al. 1994), and teichoic acids from *Bacillus subtilis* (Lazarevic and Karamata 1995). Rv3781 represents nucleotide binding domain (NBD) of ABC transporter with typical motifs such as Walker A, ABC signature, Walker B and Linton and Higgins motif (Braibant et al. 2000). Rv3783, on the other hand does not possess the characteristic motifs of membrane spanning domains, but its location in proximity to NBD encoding gene *Rv3781* and the presence of six transmembrane domains allows to predict that the protein is part of the putative ABC transporter (Braibant et al. 2000).

In the present study we focused on the investigation of the functions of *MSMEG_6366* and *MSMEG_6369,* orthologs of *Rv3781* and *Rv3783,* in non-pathogenic *Mycobacterium smegmatis* mc^2155 through transcriptional analysis, gene inactivation and phenotypic characterization of mutant strains.

Materials and Methods

RT-PCR of MSMEG_6366-MSMEG_6369 region

For RNA extraction *M. smegmatis* was incubated in 50 ml of Luria-Bertani medium containing 0.05% Tween 80 at 37°C until $O.D._{600}$ ~ 0.6–0.9. The culture was then transferred to the pre-chilled tube, the cells were harvested by centrifugation and washed with phosphate/saline buffer (PBS). The pellet was suspended in 3 ml of Trizol (Invitrogen), divided to three aliquots and disintegrated by 4 cycles of 20 s pulses in the bead beater (FastPrep-24, MP Biochemicals) using the tubes pre-filled with Lysing Matrix B (MP Biochemicals). Following the 4 min incubation at room temperature, 200 μl of chloroform : isoamylalcohol (24 : 1) were added, the mixture was incubated 3 min on ice and centrifuged 10 min at 16,000 × *g* at 4°C. Upper water phase containing RNA was removed and RNA was precipitated by addition of 500 μl isopropanol and incubation at –20°C for 4 h. The mixture was then centrifuged as above, the RNA pellet was washed two times with 80% ethanol, and suspended in 50 μl of deionized water treated with diethylpyrocarbonate (DEPC). The sample was further incubated with DNase (20 U, New England Biolabs) and RNase inhibitor (40 U, New England Biolabs) in the final volume of 60 μl for 40 min at 37°C. The reaction was stopped by adding 60 μl of DEPC-treated water and 120 μl of chloroform : isoamylalcohol (24 : 1). Following 3 min incubation at room temperature the mixture was centrifuged as above, water phase was taken out and supplemented with 1/10 of its volume with 2.5 M NaCl and 2.5-fold of its volume with 96% ethanol. RNA was then precipitated from the mixture at –20°C overnight. The final RNA pellet obtained by centrifugation and washing with 80% ethanol was dissolved in 50 μl of DEPC-water with RNase inhibitor (20 U, New England Biolabs). Concentration of RNA was established with Qubit RNA kit (Invitrogen). RT-PCR was performed with 300 ng of RNA using RT-PCR One step protocol kit (Roche) and the following primers: (smg) 3782. 1 (5'-GGGTCTAGACGCGCAAGCAGATGCTGG-3), (smg) 3781. 2 (5'-GCCTCTAGATGCCCGGCAGCAGATCCC-3), (smg) 3782. 5 (5'-GTGCAACCTCGACGATCCG-3), (smg) 3783. 4 (5'-CCTCGTTGGCGATGAACAC-3'), (smg) 3781. 5 (5'- CTACGAGGGTGAGGACGC -3'), (smg) 3783. 6 (5'-GGCTCTGGCCATGGTCTTC-3'), sigA3-F (5'-GCCGAAGAAGAGGTGGAGC-3'), sigA-R (5'-TAGTCGCGCACGACCTGC-3').

Inactivation of MSMEG_6366 and MSMEG_6369 in M. smegmatis

The ts-sacB method was used to achieve allelic replacement at the *MSMEG_6366* and *MSMEG_6369* loci of *M. smegmatis* (Pelicic et al. 1997; Jackson et al. 2000). For disruption of the first gene 2.4 kb fragment containing *MSMEG_6366* and flanking regions was amplified from *M. smegmatis* chromosomal DNA with primers 5´-TTTTCTAGACCGACCGT-GCGTCTCGG-3´ (*Xba*I site underlined) for forward direction and 5´-GCCTCTAGATGCCCGGCAGCAGATCCC-3´ (*Xba*I site underlined) for reverse direction and ligated into *Hinc*II (blunt ended) site of pUC18 vector. 1.2-kb kanamycin (Km) resistance cassette was cut out with *Hinc*II from plasmid pUC4K (Amersham) and ligated into pUC18-*MSMEG_6366* digested with *Avr*II and blunt ended with T4 DNA polymerase yielding a plasmid pUC18-*MSMEG_6366*::Km. A 3.6-kb *MSMEG_6366*::Km fragment was excised from pUC18-*MSMEG_6366*::Km with *Xba*I and inserted to the *Xba*I site of pPR27-*xylE*. Plasmid pPR27-*xylE*-*MSMEG_6366*::Km was used to achieve allelic replacement at the *MSMEG_6366* locus of *M. smegmatis*. Our attempt to achieve double crossover resulting in the disruption of *MSMEG_6366* by this approach was unsuccessful suggesting the gene is essential. We thus proceeded with preparation of a conditional mutant from the clone, in which single crossover event was confirmed by Southern blot and hybridization using DIG High Prime DNA labeling and detection starter kit II (Roche). We have prepared a rescue plasmid pCG76 with *MSMEG_6366* gene under the control of *hsp60* promotor. First we have amplified *MSMEG_6366* from *M. smegmatis* chromosomal DNA with the forward primer 5´-GCACCAACATATGACATCCTCT-TCTGACCGCG-3´ (*Nde*I site underlined) and the reverse primer 5´-CCCAAGCTTTCAGTGTGCGTCACGCGCGG-3´ (*Hin*dIII site underlined), digested the PCR product with *Nde*I and *Hin*dIII and ligated it into similarly cut pET23b (Novagen) containing *hsp60* promotor. The fragment containing *MSMEG_6366* gene with *hsp60* promotor was excised from the resulting plasmid with *Xba*I and *Hin*dIII, blunt ended and inserted into *Xba*I site (blunt ended) of pCG76 yielding plasmid pCG76-*MSMEG_6366*. Conditional mutant was obtained by transformation of the single cross-over strain with pCG76-*MSMEG_6366*. Genotype of the selected clones with the required $xylE^-$ and $sacB^-$ phenotypes was confirmed by Southern analysis as above. The conditional mutant *M. smegmatis ΔMSMEG_6366*/pCG76-*MSMEG_6366* was grown in the presence of streptomycin (20 μg/ml) and Km (20 μg/ml).

For construction of the *M. smegmatis* strain with a disrupted copy of *MSMEG_6369*, 1.7 kb fragment containing the gene and flanking regions was PCR amplified from *M. smegmatis* genomic DNA using the primers 5'-CCTCTAGAGT-GTGCAACCTCGACGATCCGC-3' (*Xba*I site underlined) and 5'-CCTCTAGAGGCCCGGTGTCAGTGCTCAGC-3' (*Xba*I site underlined) and the disrupted allele, *MSMEG_6369*::Km, was obtained by inserting the Km resistance cassette from pUC4K (Amersham) into the blunt-ended *Nco*I restriction site of *MSMEG_6369*. *MSMEG_6369*::Km was then cloned into *Xba*I-cut pPR27-*xylE* to obtain pPR27-*xylE*-*MSMEG_6369*::Km, the construct used for allelic replacement. Southern blot analysis revealed successful disruption of a chromosomal copy of *MSMEG_6369* and the resulting strain is further referred to as *M. smegmatis ΔMSMEG_6369* and grown in presence of 20 μg/ml of Km.

Complementation of M. smegmatis ΔMSMEG_6369

For complementation of the strain with disrupted *MSMEG_6369* gene we prepared plasmid pVV2-*MSMEG_6369* allowing constitutive expression of the cloned gene in mycobacteria (Dhiman et al. 2004). The gene was amplified from genomic *M. smegmatis* DNA using the primers: 5'- GGTGGAACATATGGATTCGGCTGCGGCGGTTG -3' and 5'-CACAAGCTTTCAGACCCAGTAGGGCACGCG - 3' containing *Nde*I and *Hin*dIII restriction sites, respectively (underlined). The PCR fragment was digested with *Nde*I and *Hin*dIII, and ligated into the pVV2 vector cut with the same enzymes. Recombinant plasmids were obtained from *E. coli* DH5*α* cells (Invitrogen) that were used for cloning. The resulting pVV2-*MSMEG_6369* construct was electroporated into the competent *M. smegmatis ΔMSMEG_6369*. The complemented strain *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369* was grown in media supplemented with Km (20 μg/ml) and hygromycin (50 μg/ml).

In vivo [^{14}C]-glucose metabolic labeling, fractionation of the cells and analysis of the radiolabeled products

Metabolic labeling of the wild type *M. smegmatis*, mutant *M. smegmatis ΔMSMEG_6369* and complemented strain *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369* was performed in two experimental conditions. In the first case, the cultures were grown at 30°C in 25 ml of Sauton media (Sauton 1912) with appropriate antibiotics to OD_{600}~0.8. D-[U-^{14}C]-glucose (Amersham, specific activity 287 mCi/ml) was added to the cultures to final concentration 0.2 μCi/ml and labeling was carried out for 8 h. In the second case, 25 ml of glycerol-alanine-salts (GAS) medium (Takayama et al. 1975) supplemented with antibiotics were inoculated with saturated cultures grown in Sauton media (Sauton 1912) to achieve initial OD_{600}~0.02. Bacteria were cultivated at 30°C for 18 h and then labeled with 0.5 μCi/mL of D-[U-^{14}C]-glucose (as above) for 3 h. The cells were harvested by centrifugation, washed with PBS and subjected to series of extractions with $CHCl_3$: CH_3OH (2 : 1; 6 ml), 50% CH_3OH in H_2O containing 0.9% NaCl (1 ml), 50% CH_3OH in H_2O

(1 ml), 100% CH_3OH (1 ml), the solvent "TT3" ($CHCl_3$: CH_3OH : H_2O; 10 : 10 : 3; 1 ml) and "E-soak" (water/ethanol/diethyl ether/pyridine/concentrated ammonium hydroxide; 15 : 15 : 5 : 1 : 0.017; 1 ml) as described for obtaining lipid-linked galactan intermediates (Mikusova et al. 2000). The $CHCl_3$: CH_3OH (2 : 1) extract was subjected to biphasic Folch wash (Folch et al. 1957). The bottom phase was dried under a stream of N_2 at room temperature, redissolved in 500 µl of Solvent I ($CHCl_3$: CH_3OH : H_2O : NH_4OH (65 : 25 : 3.6 : 0.5)). Part of the extract was hydrolyzed in mild alkali conditions as described (Mikusova et al. 1996), occasionally followed by mild acid hydrolysis (Mikusova et al. 2000). Thin layer chromatography (TLC) of the $CHCl_3$: CH_3OH (2 : 1) extracts was performed on silica gel plates (Merck) in the Solvent I or Solvent II ($CHCl_3$: CH_3OH : NH_4OH : 1 M ammonium acetate : H_2O (180 : 140 : 9 : 9 : 23)) and the radiolabeled bands were visualized by autoradiography using Biomax MR-1 film (Kodak).

10,000 dpm of "TT3" and "E-soak" fractions were dried, treated overnight with 1 µg of proteinase K (Sigma) in 10 µl of 25 mM Tris-HCl buffer (pH 7.5) and analysed by SDS-PAGE in 10–20% Novex Tricine gels (Invitrogen), Western blotting and autoradiography, as described Mikusova et al. (2000). Monosaccharide composition of the resulting pellets was analyzed by TLC in pyridine/ethyl acetate/glacial acetic acid/water (5 : 5 : 1 : 3) developed twice, in the samples hydrolyzed with 2 M CF_3COOH for 2 h at 120°C. Same approach was applied for the analysis of the sugar content of the compound X that was isolated by preparative TLC in the Solvent II from mild alkali stable $CHCl_3$: CH_3OH (2 : 1) extract obtained from radiolabeled *M. smegmatis ΔMSMEG_6369* grown in GAS medium.

In vitro reactions with UDP-[^{14}C]-galactose

Preparation of the enzyme fractions used in the *in vitro* reactions has been slightly modified from the described protocol (Mikusova et al. 1996), in that the membrane fraction corresponds to 100,000 × *g* pellet of the 15,500 × *g* supernatant obtained from mycobacteria disintegrated by probe sonication; the cell envelope fraction was purified from the corresponding 15,500 × *g* pellet using centrifugation in 60% Percoll (Amersham). Both enzyme fractions were suspended in the Buffer A (50 mM MOPS buffer (pH 7.9), containing 5 mM 2-mercaptoethanol and 10 mM $MgCl_2$). Reaction mixtures for evaluation of *in vitro* galactan build up in the wild type strain *M. smegmatis* and in the mutant strain *M. smegmatis ΔMSMEG_6369* contained 320 µg of membrane and 160 µg of cell envelope protein, 62.5 µM ATP, 2.5 mM NADH, 187.5 µM UDP-GlcNAc and dTDP-Rha (prepared as described Mikusova et al. 2000) and UDP-[U-^{14}C]Gal*p* (Amersham; 285 mCi/mmol, 0.25 µCi) in the final volume 80 µl adjusted with buffer A. After 1 h incubation at 37°C the reaction products were obtained by a series of extractions with 1.5 ml of $CHCl_3$: CH_3OH (2 : 1) and 0.5 ml each of solvents described above for obtaining lipid-linked galactan polymer (Mikusova et al. 2000). $CHCl_3$: CH_3OH (2 : 1) extract was further fractionated by Folch wash (Folch et al. 1957). The bottom phase was recovered, dried under N_2 and dissolved in 50 µl of Solvent I. The incorporation of the radioactive label to the products extracted with $CHCl_3$: CH_3OH (2 : 1), "TT3" and "E-soak" was established by scintillation counting.

Results

Orthologs of Rv3781 and Rv3783 are highly conserved in mycobacteria

Conservation of the orthologs of Rv3781 and Rv3783 in mycobacteria is extensive. There is 100% sequence identity in protein sequences of Rv3781 orthologs among all *M. tuberculosis* and *M. bovis* strains sequenced so far, similar evaluation of primary structures of Rv3783 orthologs from these strains shows about 96% sequence identity (http://genolist.pasteur.fr/TubercuList/). Even comparison of rather distant species of *M. tuberculosis* H37Rv and *M. smegmatis* mc^{2}155 reveals 88% and 78.2% identical residues in protein sequences of Rv3781 and Rv3783 orthologs, respectively. This observation allowed us to investigate the function of these proteins in the latter, non-pathogenic species.

MSMEG_6366 and MSMEG_6369 are co-transcribed in M. smegmatis

Genetic organization of the *Rv3781-Rv3783* region differs from all other ABC transporters identified in *M. tuberculosis* (Braibant et al. 2000), in that the two genes are separated by another gene, namely *glfT1* (*Rv3782*), encoding galactofuranosyl transferase catalysing initial steps of galactan polymerisation (Mikusova et al. 2006; Belanova et al. 2008). Sequence analysis of the region encoding the studied ABC transporter in *M. tuberculosis* shows a 4 nt overlap between *Rv3781* and *Rv3782*, and a similar sized overlap between *Rv3782* and *Rv3783*. Likewise, in *M. smegmatis* there is a 14 nt overlap between the first two genes, *MSMEG_6366* and *MSMEG_6367* (*glfT1*) and a 4 nt overlap between the last two genes, *MSMEG_6367* and *MSMEG_6369*. The organization of the region encompassing *MSMEG_6366 – MSMEG_6369* thus suggests that they are co-transcribed. With only 7 bp separating *MSMEG_6366* from the conserved *MSMEG_6365* gene (orthologous to *Rv3780* from *M. tuberculosis*; 3 bp spacing in this species), it is possible that it also belongs to the same transcriptional unit. However, its function is unknown and closer investigation of this gene was beyond the scope of this paper. The organization of further genes surrounding this cluster – *MSMEG_6364* (a pseudogene)

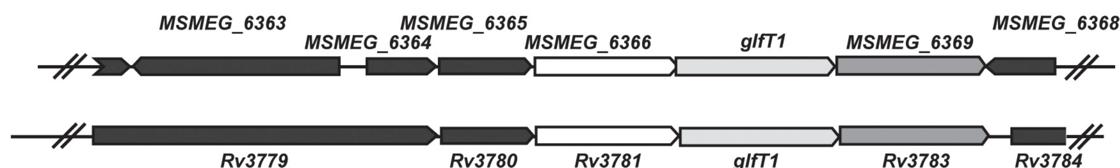

Figure 1. Genome region of *Rv3781-Rv3783* and their orthologs in *M. smegmatis* mc^{2}155. Drawn according to the image from http://genolist.pasteur.fr/TubercuList/.

and *MSMEG_6368* (encoding a putative DNA-binding protein) – is not conserved among *Mycobacterium* species (Fig. 1). In order to affirm the co-transcription of the ABC-transporter genes with *glfT1*, we performed RT-PCR of mRNA template from *M. smegmatis* with primers designed to amplify regions overlapping the intergenic areas between each two neighboring genes. Indeed, the three genes, *MSMEG_6366-MSMEG_6367-MSMEG_6369*, are co-transcribed in *M. smegmatis* (Fig. 2). In bacteria evidence of operon structure is often indicative of coexpression of the protein products for involvement in a common biochemical pathway. It is thus tempting to suggest that MSMEG_6366 and MSMEG_6369, along with GlfT1, take part in the galactan biogenesis.

MSMEG_6366 is essential; M. smegmatis ΔMSMEG_6369 strain demonstrates growth defects

In order to establish the roles of MSMEG_6366 and MSMEG_6369 we decided to construct the mutants, or conditional mutants, in case the genes are essential, in *M. smegmatis*, which would allow investigation of the affected metabolic pathways through phenotypic characterization of the obtained mutant strains. A two-step homologous recombination procedure (Pelicic et al. 1997), using temperature-sensitive replicative vectors derived from pPR27*xylE* (Jackson et al. 2000), allowed the successful construction of a deletion strain in *MSMEG_6369* confirmed by Southern blot (Fig. 3A) and PCR analysis (data not shown). However, the *MSMEG_6366* gene could not be knocked out without the presence of the rescue plasmid, pCG76-*MSMEG_6366*, carrying a functional copy of the gene. Increase in cultivation temperature to 42°C causing the loss of the temperature-sensitive rescue plasmid resulted in the complete abolition of the growth of the mutant (Fig. 3B). Thus *MSMEG_6366* is essential in *M. smegmatis.* Since *MSMEG_6366* alone, provided on the rescue plasmid, was sufficient for complementation, polar effects of *MSMEG_6366* inactivation on downstream genes are unlikely. The knockout strain *M. smegmatis ΔMSMEG_6369* did not show any growth defects in nutritionally rich media, such as LB, but was significantly growth retarded in the minimal Sauton medium (Sauton 1912); in particular, it displayed an extended lag period which preceded an apparently normal log phase (Fig. 4). That this extended lag period is due to a polar effect of *MSMEG_6369* disruption on the expression of adjacent genes is unlikely given that the gene located immediately downstream, *MSMEG_6368*, is transcribed in the opposite direction.

Remarkable changes were observed also in the appearance of the cultures grown in minimal GAS media (Takayama

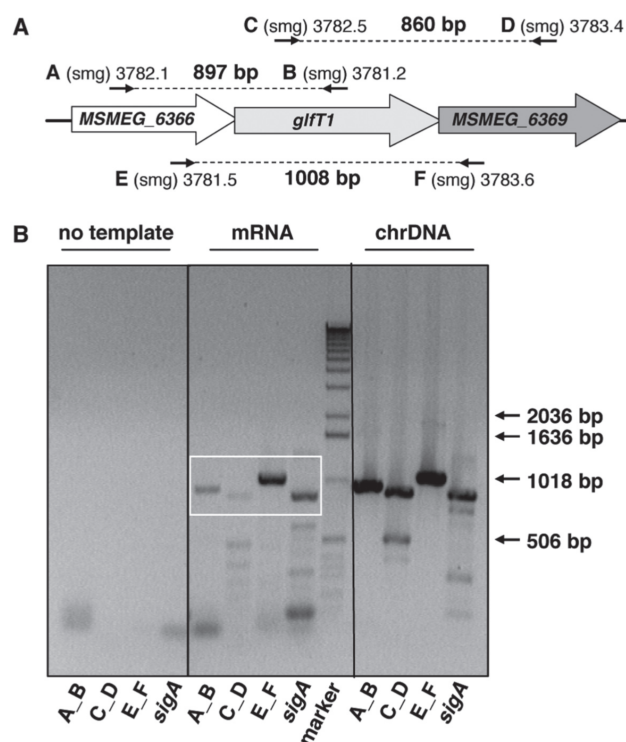

Figure 2. Evidence for cotranscription of *MSMEG_6366-MSMEG_6369.* **A.** Schematic representation of the amplified regions with specified primers and expected lengths of the PCR products. **B.** Analysis of the RT-PCR products amplified from *M. smegmatis* RNA by electrophoresis in 0.7% agarose gel. First panel corresponds to the reactions without any template for verification of the purity of materials used in the RT-PCR reaction. Second panel shows RT-PCR products of corresponding lengths obtained with combination of the primers specified at the individual lanes; SigA served as the internal control. Third panel represents the control reaction performed with the genomic DNA from *M. smegmatis.* RT-PCR with mRNA templates was preceded by conventional PCR to exclude the possibility of DNA contamination.

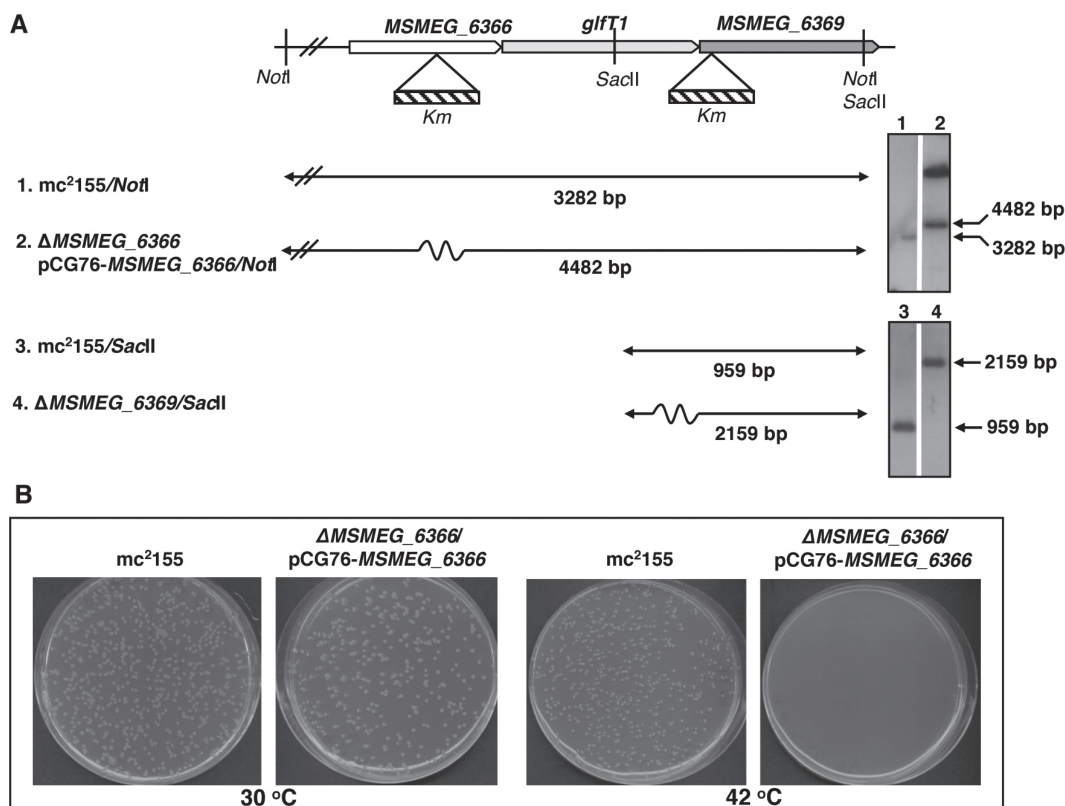

Figure 3. Inactivation of *MSMEG_6366* and *MSMEG_6369*. **A.** Illustration of the expected profiles of the strains with inactivated genes *MSMEG_6366*, or *MSMEG_6369*, respectively, and Southern blot analysis of the conditional mutant *M. smegmatis ΔMSMEG_6366*/pCG76-*MSMEG_6366* and mutant *M. smegmatis ΔMSMEG_6369*. 2.4 kb *Xba*I fragment used for *MSMEG_6366* inactivation, as described in Materials and Methods, and 0.99 kb *Sal*I fragment cut from pBS KS- *MSMEG_6369*, served as probes for Southern blotting in the respective strains. Unlabeled upper band in the lane 2 probably results from the presence of the *pCG67-MSMEG_6366* in the analyzed cells. **B.** Growth characteristics of the conditional mutant *M. smegmatis ΔMSMEG_6366*/pCG76-*MSMEG_6366.* Wild type and mutant strains were incubated at 30°C and 42°C on 7H11 agar plates, supplemented in case of the latter strain with kanamycin.

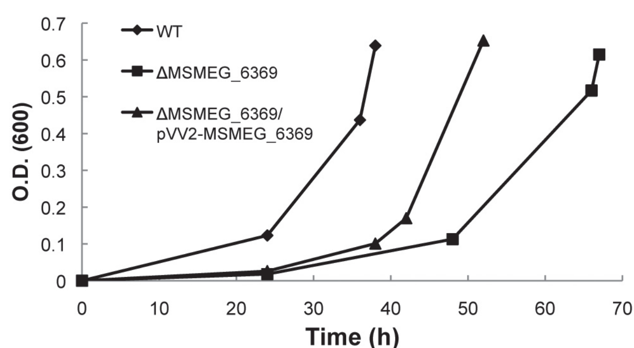

Figure 4. Growth curves of the wild type *M. smegmatis*, mutant *M. smegmatis ΔMSMEG_6369* and complemented strain *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369*. The bacteria were cultivated in Sauton media with appropriate antibiotics at 30°C and 130 rpm. *M. smegmatis* (diamonds), *M. smegmatis ΔMSMEG_6369* (squares), *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369* (triangles). O.D., optical density.

et al. 1975). In this case, wild type *M. smegmatis* formed distinct clumps, which are characteristic for the growth of mycobacteria in this type of media, while the *M. smegmatis ΔMSMEG_6369* culture looked significantly more turbid implying disintegration of the clumps that could be associated with the defect in producing the surface structures.

[^{14}C]-glucose metabolic labeling of M. smegmatis ΔMSMEG_6369 in Sauton media did not reveal changes in the lipid or cell wall components

For characterization of the essence of the altered growth of *M. smegmatis ΔMSMEG_6369* we performed [^{14}C]-glucose metabolic labeling in the restrictive conditions provided by Sauton medium. Due to the anticipated roles of the *MSMEG_6366* and *MSMEG_6369* in the transport of lipid-linked galactan intermediates during the mycobacterial cell wall biosynthesis, the fractionation scheme of the radiolabelled bacteria followed the procedure that we developed for obtaining these molecules

from the cell-free reactions (Mikusova et al. 2000). It includes step-wise extractions of the cell pellets with the organic solvents of increasing polarity. Galactan intermediates containing up to five monosaccharide units (glycolipids 1-5, GL1-5) are extracted with $CHCl_3 : CH_3OH$ (2 : 1), whereas more galactosylated products (lipid-linked galactan polymer) extract into the solvents of higher polarity – "TT3" and "E-soak". In these fractions we searched particularly for the compounds with properties characteristic for the lipid-linked galactan, such as stability in mild alkali conditions and sensitivity to mild acid hydrolysis. Although this kind of intermediates could not be found in the whole *M. smegmatis* cells before (our unpublished data), inactivation of the gene responsible for their transport across the plasma membrane could potentially lead to their accumulation. TLC examination of the lipid extracts, however, did not reveal any changes between the wild type *M. smegmatis* and the mutant strain *M. smegmatis ΔMSMEG_6369* in the total lipids or mild alkali stable and mild acid labile population (data not shown).

Comparison of the "TT3" and "E-soak" extracts from the [^{14}C]-glucose radiolabeled cells disclosed mostly the presence of mycobacterial lipoglycans, lipoarabinomannan (LAM) and lipomannan (LM) with no significant qualitative changes between the examined wild type strain *M. smegmatis* and the mutant strain *M. smegmatis ΔMSMEG_6369*. Similarly, examination of monosaccharide composition of the radiolabeled cell wall residues obtained after the extractions did not reveal any changes between these strains (data not shown). Therefore it appears that the function of MSMEG_6369 in the mutant strain *M. smegmatis ΔMSMEG_6369* is compensated in these conditions.

M. smegmatis ΔMSMEG_6369 grown in GAS media produces an unknown compound

Similar [^{14}C]-glucose metabolic labeling and cell fractionation as above were carried out with the cultures of the wild type *M. smegmatis*, mutant *M. smegmatis ΔMSMEG_6369* and complemented *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369* grown in GAS media. Most striking difference between the three analyzed strains was the accumulation of a mild alkali stable and mild acid labile compound, migrating on TLC in the area of glycolipids GL3-5 in the mutant strain (Fig. 5A). In the attempt to characterize the sugar

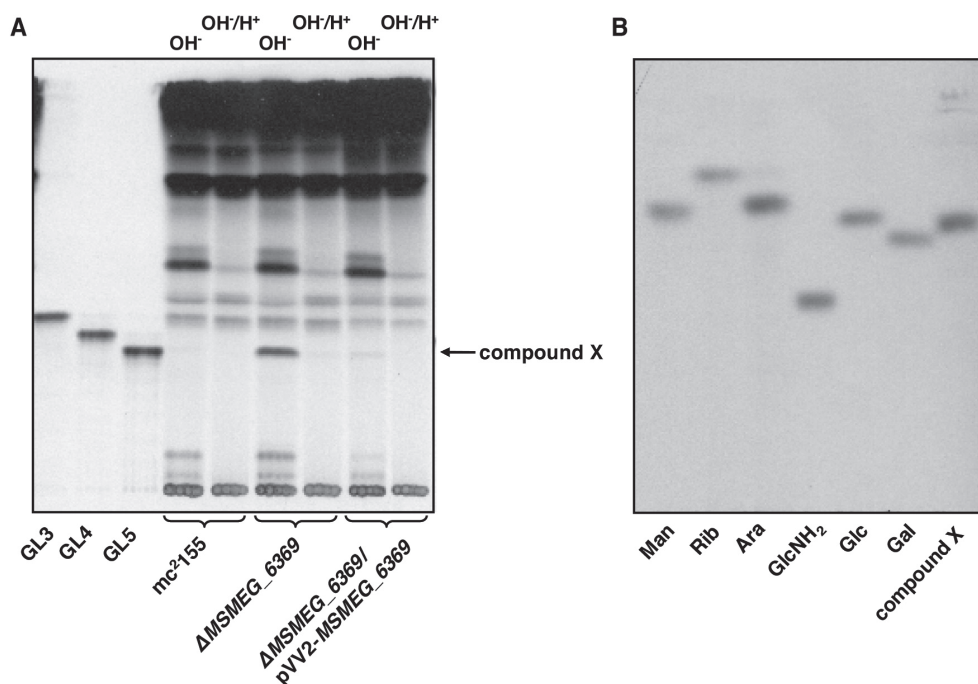

Figure 5. Examination of mild alkali stable lipids from [^{14}C]-glucose labeled *M. smegmatis, M. smegmatis ΔMSMEG_6369* and *M. smegmatis ΔMSMEG_6369*/pVV2-*MSMEG_6369* cultivated in GAS medium. **A.** From each sample two aliquots of 70 000 dpm of the $CHCl_3 : CH_3OH$ (2 : 1) extracts were subjected to mild alkali hydrolysis; one aliquot was further hydrolyzed in mild acid conditions. Resulting samples were resolved on TLC in Solvent II and visualized by autoradiography (Biomax MR-1 film, Kodak, –70°C, 26 days). 100 dpm each of GL-3, GL-4 and GL-5 that were prepared by preparative TLC from the *in vitro* reactions with UDP-[^{14}C]Gal served as standards. **B.** Monosaccharide composition of compound X was analyzed from the sample purified by preparative TLC and subjected to 2 h hydrolysis in 2 M CF_3COOH at 120°C. 400–600 dpm of [^{14}C] monosaccharides were used as standards. The TLC plate was chromatographed in pyridine/ethyl acetate/glacial acetic acid/water (5 : 5 : 1 : 3) and visualized by 5 days exposure to Biomax MR-1 film (Kodak) at –70°C.

composition of this compound, the corresponding band was purified by preparative TLC from the radiolabeled $CHCl_3$: CH_3OH (2 : 1) fraction, subjected to strong acid hydrolysis and resulting free sugars were separated by TLC. The profile presented in Fig. 5B confirms the presence of a major radioactive band located between the standards corresponding to Glc and Gal. Another, much lighter upper band migrated in the region of cold Rha standard. These findings, however, do not allow us to draw any conclusions about the identity of the compound X at present.

Disruption of MSMEG_6369 affects in vitro galactan synthesis

Most of basic information regarding polymerization of galactan on its decaprenol carrier has been accumulated through cell free experiments using membrane and/or cell wall fractions of *M. smegmatis* as the source of the enzymes and UDP-[^{14}C]-Gal as a tracer (Mikusova et al. 1996, 2000). We thus used this approach for investigation of the effects of *MSMEG_6369* disruption on the *in vitro* galactan production. Following the incubation of the reaction mixtures that contained also UDP-GlcNAc and TDP-Rha for *in situ* synthesis of the galactose acceptor GL2 (decaprenyl-P-P-GlcNAc-Rha), we extracted the lipid-linked galactan intermediates with $CHCl_3$: CH_3OH (2 : 1) and more polar solvents, as mentioned above. In three separate experiments quantification of radioactivity in the obtained fractions revealed 26–78% increase in the incorporation of the radioactive label to the lipid-linked galactan polymer (combined "TT3" and "E-soak" fractions) in the *M. smegmatis ΔMSMEG_6369* strain compared to the wild type *M. smegmatis* suggesting possible association of MSMEG_6369 with the metabolism of the galactan precursors.

Discussion

Translocation of polysaccharides exposed on the surface of bacteria belongs to crucial and the least understood steps in the assembly of the cell wall structures. Braibant et al. (2000) identified $(Rv3781)_2/(Rv3783)_2$ as the only ABC transporter probably involved in the transfer of polysaccharides in *M. tuberculosis* and suggested that lipoglycans LAM/LM serve as the possible substrates. This case was excluded by Pitarque et al. (2008) who investigated lipoglycan distribution in the wild type *M. smegmatis* cells and the strain with inactivated copy of *Rv3783* ortholog. They found that the mutant bacteria exposed similar amounts of lipoglycans on the surface as the wild type cells and also secreted comparable amounts of glucan, and the LAM- and LM- derived glycans – arabinomannan and mannan (Pitarque et al. 2008).

Possible involvement of Rv3781 and Rv3783 proteins in the transport of AG intermediates across the plasma membrane occurred to us in conjunction with recognition of Rv3782, as the enzyme initiating galactose polymerization encoded by a gene located between the two studied genes (Mikusova et al. 2006). Just very recently, the same role for the two proteins under investigation was hypothesized by Cuthbertson et al. (2010) in the excellent review focused on ABC transporters involved in the export of cell surface glycoconjugates. We have now confirmed that the three genes are co-transcribed in *M. smegmatis*, which suggests that they are involved in a common metabolic pathway. As we pointed out before (Mikusova et al. 2006), the sequence of the initial events in mycobacterial AG biosynthesis is strikingly similar to the ABC transporter-dependent build-up of lipopolysaccharide O-antigens in several species of Gram-negative bacteria. Prototype models are those for polymannan biosynthesis in *E. coli* Serotypes O8 and O9a, and D-galactan I biosynthesis from *Klebsiella pneumoniae*. In these bacteria, as well as in mycobacteria the first reaction is catalyzed by WecA enzyme attaching GlcNAc-P from UDP-GlcNAc to C55/C50 – P carrier (Meier-Dieter et al. 1992; Alexander and Valvano 1994; Rick et al. 1994; Clarke et al. 1995; Jin et al. 2010). This event is followed by the action of dedicated glycosyltransferases, which attach several sugar residues forming the adapter. These enzymes are mannosyltransferases WbdB and WbdC for *E. coli* O8/O9a (Kido et al. 1995), galactosyltransferase WbbO (Clarke et al. 1995; Guan et al. 2001) for production of D-galactan I in *K. pneumoniae* and rhamnosyltransferase WbbL (Mills et al. 2004) and galactosyltransferase GlfT1 (Mikusova et al. 2006; Belanova et al. 2008) in mycobacteria. Enzymes WbdA (Kido et al. 1998), WbbM (Guan et al. 2001) and GlfT2 (Mikusova et al. 2000; Kremer et al. 2001; May et al. 2009), in the respective species, then catalyze elongation of the polymers. Polymannan O-antigens O8 and O9a are further modified by WbdD enzyme catalysing methylation (O8), or both methylation and phosphorylation (O9a) of the non-reducing end residue, which serves as a termination signal (Clarke et al. 2004), while D-galactan I does not possess any specific chain-terminating residue (Vinogradov et al. 2002). This difference is reflected also in the mechanism of transfer of these O-antigens across the plasma membranes by ABC transporter Wzt/Wzm. In case of O8 and O9a antigens the modified terminal residue is recognized as the export signal by the C-terminal domain of NBD protein Wzt (Clarke et al. 2004). In the absence of the termination signal in D-galactan I translocation, export and O-antigen polymerization are strictly coupled and this process also establishes the chain length (Kos et al. 2009). Comparison of the Wzt proteins further confirms the difference in the two mechanisms of export – Wzt from *E. coli* Serotype O9a is 431 amino acids long, in which C-terminal domain is responsible for specificity of the transported substrate, while its N-terminal domain is highly similar to significantly shorter Wzt protein from *K. pneumoniae*

composed of 246 amino acids (Cuthbertson et al. 2007). Mycobacterial orthologs of Rv3781 protein are similar to the latter case with their lengths 274 and 269 amino acids for *M. tuberculosis* and *M. smegmatis*, respectively. Moreover, similar to D-galactan I, mycobacterial galactan polymer of AG does not appear to contain any specific termination signal at the non-reducing end (Bhamidi et al. 2008), so we could speculate that transfer of AG precursors across the plasma membrane occurs in a way similar to that suggested for O2 antigen from *K. pneumoniae*. As stated in Kos at al. (2009), this mechanism is facilitated by an interaction between the transporter and glycosyltransferase, although by so-far unspecified way. Our unpublished preliminary data obtained by a "pull-down" method suggest a direct interaction between Rv3781 (putative Wzt) and galactosyltransferase GlfT1, which implies that the proposed participation of the mycobacterial transporter Rv3781-Rv3783 in galactan build up might be correct. These proteins could be a part of a large multiprotein molecular machine responsible for biosynthesis and export of AG intermediates across the plasma membrane, which may include also other enzymes involved in the synthesis of mycobacterial galactan, such as WecA, rhamnosyltransferase WbbL and polymerizing galactosyltransferase GlfT2 (Fig. 6).

In this report we attempted to address the question of a possible substrate of mycobacterial ABC transporter composed of Rv3781 and Rv3783 orthologs by inactivation of the corresponding genes in *M. smegmatis*. We anticipated that metabolic intermediates directed for transport would accumulate in such strains. We have successfully disrupted *MSMEG_6369* (*Rv3783* ortholog) gene and showed that *MSMEG_6366* (*Rv3781* ortholog) is indispensable for growth of *M. smegmatis*. Similarly, essentiality was suggested for *Rv3781* (Sassetti et al. 2003) from *M. tuberculosis* by transposon mutagenesis approach, while *Rv3783* was found non-essential (Lamichhane et al. 2003). *In vivo* studies comparing phenotypes of wild type *M. smegmatis* and *M. smegmatis ΔMSMEG_6369*, in the restrictive growth conditions in Sauton medium by the [^{14}C]-glucose labeling did not reveal any changes in the lipid population, LAM/LM fraction or AG composition, so it appears that the function of *MSMEG_6369* in the mutant strain was compensated under our experimental conditions. Major phenotypic change associated with the inactivation of *MSMEG_6369* was observed in the cells grown in GAS medium, which accumulated compound X migrating on the TLC in the region of GL4–GL5. So far, our efforts for structural characterization of this molecule were unsuccessful. The reasons could be its unusual structure, properties or its very low content in the cells.

Noteworthy changes, i.e. accumulation of the lipid-linked galactan polymer in the strain with inactivated copy of *MSMEG_6369* compared to the wild type *M. smegmatis*, were

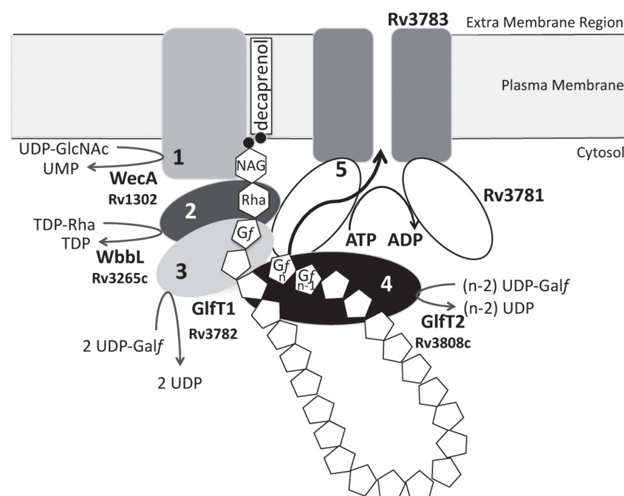

Figure 6. Proposed pathway for the biosynthesis and export of mycobacterial galactan. Polymerization proceeds in a series of Steps 1–4 by the action of the respective membrane bound WecA or cytosolic WbbL, GlfT1 and GlfT2. Transport of galactan (Step 5) through the membrane takes place while lipid/C_{50}-P-bound at the fully or partially polymerized state. NAG, N-acetylglucosamine; Rha, rhamnose; G*f*, galactofuranose.

observed in the *in vitro* experiments. Attractive hypothesis would be that disruption of the gene *MSMEG_6369*, which is the part of the ABC transport system leads to abolition of further processing of the galactan intermediates, which results in their accumulation. This could be expected if the reactions take place in closed compartments, such as vesicles formed from the mycobacterial membranes and cell walls. Such structures were identified by electron microscopy in comparable enzymatic fractions used for examination of localization of phospholipid biosynthesis in mycobacteria (Morita et al. 2005), and we anticipate presence of such vesicles also in enzymatic fractions tested for galactan biosynthesis.

In conclusion, this is the first report on the investigation of function of the putative mycobacterial ABC transporter composed of Rv3781 and Rv3783 through their *M. smegmatis* homologs. Already these have been proposed to play a role in the export of polysaccharides (Braibant et al. 2000; Cuthbertson et al. 2010). To date, characterized mycobacterial ABC transporters have been mostly involved in uptake or efflux of small molecules (Choudhuri et al. 2002; Pasca et al. 2004; Gebhard et al. 2006; Price et al. 2008; Ryndak et al. 2010). Our data imply the participation of *MSMEG_6366-MSMEG_6369/Rv3781-Rv3783* in the galactan polymerization/deposition based on two observations: i) confirmation of the co-transcription of the genes forming ABC transporter *MSMEG_6366* and *MSMEG_6369* with the gene encoding galactosyltransferase GlfT1 in *M. smegmatis*; and ii) effects

of inactivation of the *MSMEG_6369* gene encoding membrane-spanning part of the transporter on the *in vitro* galactan biosynthesis in *M. smegmatis*. Our finding that the ATP-binding component, MSMEG_6366, is essential for the viability of *M. smegmatis* demonstrates its crucial role in the physiology of mycobacteria. Recently, Gronenberg and Kahne proposed that ATPase components of the essential bacterial ABC transporters, and particularly those participating in cell wall biogenesis, are highly vulnerable drug targets (Gronenberg and Kahne 2010). Although more work is required to unequivocally confirm the suggested function of the ABC transporter Rv3781-Rv3783 and to characterize its substrate, our current study provides the tools and opens new perspectives for investigation of the next important chapter in mycobacterial cell wall biogenesis that could lead to the revelation of attractive targets for development of new antituberculosis drugs.

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References

Alderwick L. J., Seidel M., Sahm H., Besra G. S., Eggeling L. (2006): Identification of a novel arabinofuranosyltransferase (AftA) involved in cell wall arabinan biosynthesis in Mycobacterium tuberculosis. J. Biol. Chem. **281,** 15653–5661
doi:10.1074/jbc.M600045200

Alexander D. C., Valvano M. A. (1994): Role of the rfe gene in the biosynthesis of the Escherichia coli O7-specific lipopolysaccharide and other O-specific polysaccharides containing N-acetylglucosamine. J. Bacteriol. **176,** 7079–7084

Belanger A. E., Inamine J. M. (2000): Genetics of cell wall biosythesis. In: Molecular Genetics of Mycobacteria. (Eds. G. F. Hatfull and W. R. Jacobs Jr.), pp. 191–202, ASM Press, Washington, D.C.

Belanova M., Dianiskova P., Brennan P. J., Completo G. C., Rose N. L., Lowary T. L., Mikusova K. (2008): Galactosyl transferases in mycobacterial cell wall synthesis. J. Bacteriol. **190,** 1141–1145
doi:10.1128/JB.01326-07

Berg S., Kaur D., Jackson M., Brennan P. J. (2007): The glycosyltransferases of Mycobacterium tuberculosis – roles in the synthesis of arabinogalactan, lipoarabinomannan, and other glycoconjugates. Glycobiology **17,** 35–56R
doi:10.1093/glycob/cwm010

Bhamidi S., Scherman M. S., Rithner C. D., Prenni J. E., Chatterjee D., Khoo K. H., McNeil M. R. (2008): The identification and location of succinyl residues and the characterization of the interior arabinan region allow for a model of the complete primary structure of Mycobacterium tuberculosis mycolyl arabinogalactan. J. Biol. Chem. **283,** 12992–13000
doi:10.1074/jbc.M800222200

Birch H. L., Alderwick L. J., Bhatt A., Rittmann D., Krumbach K., Singh A., Bai Y., Lowary T. L., Eggeling L., Besra G. S. (2008): Biosynthesis of mycobacterial arabinogalactan: identification of a novel alpha(1-->3) arabinofuranosyltransferase. Mol. Microbiol. **69,** 1191–1206

Braibant M., Gilot P., Content J. (2000): The ATP binding cassette (ABC) transport systems of Mycobacterium tuberculosis. FEMS Microbiol. Rev. **24,** 449–467
doi:10.1111/j.1574-6976.2000.tb00550.x

Choudhuri B. S., Bhakta S., Barik R., Basu J., Kundu M., Chakrabarti P. (2002): Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drrA and drrB of Mycobacterium tuberculosis. Biochem. J. **367,** 279–285
doi:10.1042/BJ20020615

Clarke B. R., Bronner D., Keenleyside W. J., Severn W. B., Richards J. C., Whitfield C. (1995): Role of Rfe and RfbF in the initiation of biosynthesis of D-galactan I, the lipopolysaccharide O antigen from Klebsiella pneumoniae serotype O1. J. Bacteriol. **177,** 5411–5418

Clarke B. R., Cuthbertson L., Whitfield C. (2004): Nonreducing terminal modifications determine the chain length of polymannose O antigens of Escherichia coli and couple chain termination to polymer export via an ATP-binding cassette transporter. J. Biol. Chem. **279,** 35709–35718
doi:10.1074/jbc.M404738200

Cuthbertson L., Kimber M. S., Whitfield C. (2007): Substrate binding by a bacterial ABC transporter involved in polysaccharide export. Proc. Natl. Acad. Sci. U.S.A. **104,** 19529–19534
doi:10.1073/pnas.0705709104

Cuthbertson L., Kos V., Whitfield C. (2010): ABC transporters involved in export of cell surface glycoconjugates. Microbiol. Mol. Biol. Rev. **74,** 341–362
doi:10.1128/MMBR.00009-10

Dhiman R. K., Schulbach M. C., Mahapatra S., Baulard A. R., Vissa V., Brennan P. J., Crick D. C. (2004): Identification of a novel class of omega,E,E-farnesyl diphosphate synthase from Mycobacterium tuberculosis. J. Lipid Res. **45,** 1140–1147
doi:10.1194/jlr.M400047-JLR200

Folch J., Lees M., Sloane Stanley G. H. (1957): A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226,** 497–509

Gebhard S., Tran S. L., Cook G. M. (2006): The Phn system of Mycobacterium smegmatis: a second high-affinity ABC-transporter for phosphate. Microbiology **152,** 3453–3465
doi:10.1099/mic.0.29201-0

Gronenberg L. S., Kahne, D. (2010): Development of an activity assay for discovery of inhibitors of lipopolysaccharide export. J. Am. Chem. Soc. **132,** 2518–2519
doi:10.1021/ja910361r

Guan S., Clarke A. J., Whitfield C. (2001): Functional analysis of the galactosyltransferases required for biosynthesis of D-galactan I,

a component of the lipopolysaccharide O1 antigen of Klebsiella pneumoniae. J. Bacteriol. **183,** 3318–3327
doi:10.1128/JB.183.11.3318-3327.2001

Jackson M., Crick D. C., Brennan P. J. (2000): Phosphatidylinositol is an essential phospholipid of mycobacteria. J. Biol. Chem. **275,** 30092–30099
doi:10.1074/jbc.M004658200

Jin Y., Xin Y., Zhang W., Ma Y. (2010): Mycobacterium tuberculosis Rv1302 and Mycobacterium smegmatis MSMEG_4947 have WecA function and MSMEG_4947 is required for the growth of M. smegmatis. FEMS Microbiol. Lett. **310,** 54–61
doi:10.1111/j.1574-6968.2010.02045.x

Kaur D., Guerin M. E., Skovierova H., Brennan P. J., Jackson M. (2009): Chapter 2: Biogenesis of the cell wall and other glycoconjugates of Mycobacterium tuberculosis. Adv. Appl. Microbiol. **69,** 23–78
doi:10.1016/S0065-2164(09)69002-X

Kido N., Sugiyama T., Yokochi T., Kobayashi H., Okawa Y. (1998): Synthesis of Escherichia coli O9a polysaccharide requires the participation of two domains of WbdA, a mannosyltransferase encoded within the wb* gene cluster. Mol. Microbiol. **27,** 1213–1221
doi:10.1046/j.1365-2958.1998.00765.x

Kido N., Torgov V. I., Sugiyama T., Uchiya K., Sugihara H., Komatsu T., Kato N., Jann K. (1995): Expression of the O9 polysaccharide of Escherichia coli: sequencing of the E. coli O9 rfb gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. J. Bacteriol. **177,** 2178–2187

Kos V., Cuthbertson L., Whitfield C. (2009): The Klebsiella pneumoniae O2a antigen defines a second mechanism for O antigen ATP-binding cassette transporters. J. Biol. Chem. **284,** 2947–2956
doi:10.1074/jbc.M807213200

Kremer L., Dover L. G., Morehouse C., Hitchin P., Everett M., Morris H. R., Dell A., Brennan P. J., McNeil M. R., Flaherty C., Duncan K., Besra G. S. (2001): Galactan biosynthesis in Mycobacterium tuberculosis. Identification of a bifunctional UDP-galactofuranosyltransferase. J. Biol. Chem. **276,** 26430–26440
doi:10.1074/jbc.M102022200

Lamichhane G., Zignol M., Blades N. J., Geiman D. E., Dougherty A., Grosset J., Broman K. W., Bishai W. R. (2003): A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U.S.A. **100,** 7213–7218
doi:10.1073/pnas.1231432100

Lazarevic V., Karamata D. (1995): The tagGH operon of Bacillus subtilis 168 encodes a two-component ABC transporter involved in the metabolism of two wall teichoic acids. Mol. Microbiol. **16,** 345–355
doi:10.1111/j.1365-2958.1995.tb02306.x

Manning P. A., Stroeher U. H., Karageorgos L. E., Morona R. (1995): Putative O-antigen transport genes within the rfb region of Vibrio cholerae O1 are homologous to those for capsule transport. Gene **158,** 1–7
doi:10.1016/0378-1119(95)00124-O

May J. F., Splain R. A., Brotschi C., Kiessling L. L. (2009): A tethering mechanism for length control in a processive carbohydrate polymerization. Proc. Natl. Acad. Sci. U.S.A. **106,** 11851–11856
doi:10.1073/pnas.0901407106

McNeil M. R., Brennan P. J. (1991): Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. **142,** 451–463
doi:10.1016/0923-2508(91)90120-Y

Meier-Dieter U., Barr K., Starman R., Hatch L., Rick P. D. (1992): Nucleotide sequence of the Escherichia coli rfe gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the rfe-rff gene cluster. J. Biol. Chem. **267,** 746–753

Mikusova K., Mikus M., Besra G. S., Hancock I., Brennan P. J. (1996): Biosynthesis of the linkage region of the mycobacterial cell wall. J. Biol. Chem. **271,** 7820–7828
doi:10.1074/jbc.271.13.7820

Mikusova K., Yagi T., Stern R., McNeil M. R., Besra G. S., Crick D. C., Brennan P. J. (2000): Biosynthesis of the galactan component of the mycobacterial cell wall. J. Biol. Chem. **275,** 33890–33897
doi:10.1074/jbc.M006875200

Mikusova K., Belanova M., Kordulakova J., Honda K., McNeil M. R., Mahapatra S., Crick D. C., Brennan P. J. (2006): Identification of a novel galactosyl transferase involved in biosynthesis of the mycobacterial cell wall. J. Bacteriol. **188,** 6592–6598
doi:10.1128/JB.00489-06

Mills J. A., Motichka K., Jucker M., Wu H. P., Uhlik B. C., Stern R. J., Scherman M. S., Vissa V. D., Pan F., Kundu M., Ma Y. F., McNeil M. (2004): Inactivation of the mycobacterial rhamnosyltransferase, which is needed for the formation of the arabinogalactan-peptidoglycan linker, leads to irreversible loss of viability. J. Biol. Chem. **279,** 43540–43546
doi:10.1074/jbc.M407782200

Morita Y. S., Velasquez R., Taig E., Waller R. F., Patterson J. H., Tull D., Williams S. J., Billman-Jacobe H., McConville M. J. (2005): Compartmentalization of lipid biosynthesis in mycobacteria. J. Biol. Chem. **280,** 21645–21652
doi:10.1074/jbc.M414181200

Pasca M. R., Guglierame P., Arcesi F., Bellinzoni M., De Rossi E., Riccardi G. (2004): Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **48,** 3175–3178
doi:10.1128/AAC.48.8.3175-3178.2004

Pavelka M. S., Jr., Hayes S. F., Silver R. P. (1994): Characterization of KpsT, the ATP-binding component of the ABC-transporter involved with the export of capsular polysialic acid in Escherichia coli K1. J. Biol. Chem. **269,** 20149–20158

Pelicic V., Jackson M., Reyrat J. M., Jacobs W. R., Jr., Gicquel B., Guilhot C. (1997): Efficient allelic exchange and transposon mutagenesis in Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U.S.A. **94,** 10955–10960
doi:10.1073/pnas.94.20.10955

Pitarque S., Larrouy-Maumus G., Payre B., Jackson M., Puzo G., Nigou J. (2008): The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. Tuberculosis (Edinb) **88,** 560–565
doi:10.1016/j.tube.2008.04.002

Price C. T., Bukka A., Cynamon M., Graham J. E. (2008): Glycine betaine uptake by the ProXVWZ ABC transporter contributes

to the ability of Mycobacterium tuberculosis to initiate growth in human macrophages. J. Bacteriol. **190,** 3955–3961
doi:10.1128/JB.01476-07

Rick P. D., Hubbard G. L., Barr K. (1994): Role of the rfe gene in the synthesis of the O8 antigen in Escherichia coli K-12. J. Bacteriol. **176,** 2877–2884

Ryndak M. B., Wang S., Smith I., Rodriguez G. M. (2010): The Mycobacterium tuberculosis high-affinity iron importer, IrtA, contains an FAD-binding domain. J. Bacteriol. **192,** 861–869.
doi:10.1128/JB.00223-09

Sassetti C. M., Boyd D. H., Rubin E. J. (2003): Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. **48,** 77–84
doi:10.1046/j.1365-2958.2003.03425.x

Sauton M. B. (1912): Sur la nutrition minérale du bacilli tuberculeux. Comptes Rendus des Séances de l'Académie des Sciences **155,** 860–861

Seidel M., Alderwick L. J., Birch H. L., Sahm H., Eggeling L., Besra G. S. (2007): Identification of a novel arabinofuranosyltransferase AftB involved in a terminal step of cell wall arabinan biosynthesis in Corynebacterianeae, such as Corynebacterium glutamicum and Mycobacterium tuberculosis. J. Biol. Chem. **282,** 14729–14740
doi:10.1074/jbc.M700271200

Skovierova H., Larrouy-Maumus G., Zhang J., Kaur D., Barilone N., Kordulakova J., Gilleron M., Guadagnini S., Belanova M., Prevost M. C., Gicquel B., Puzo G., Chatterjee D., Brennan P. J., Nigou J., Jackson M. (2009): AftD, a novel essential arabinofuranosyltransferase from mycobacteria. Glycobiology **19,** 1235–1247
doi:10.1093/glycob/cwp116

Takayama K., Schnoes H. K., Armstrong E. L., Boyle R. W. (1975): Site of inhibitory action of isoniazid in the synthesis of mycolic acids in Mycobacterium tuberculosis. J. Lipid Res. **16,** 308–317

Vinogradov E., Frirdich E., MacLean L. L., Perry M. B., Petersen B. O., Duus J. O., Whitfield C. (2002): Structures of lipopolysaccharides from Klebsiella pneumoniae. Eluicidation of the structure of the linkage region between core and polysaccharide O chain and identification of the residues at the non-reducing termini of the O chains. J. Biol. Chem. **277,** 25070–25081
doi:10.1074/jbc.M202683200

Zhang L., al-Hendy A., Toivanen P., Skurnik M. (1993): Genetic organization and sequence of the rfb gene cluster of Yersinia enterocolitica serotype O:3: similarities to the dTDP-L-rhamnose biosynthesis pathway of Salmonella and to the bacterial polysaccharide transport systems. Mol. Microbiol. **9,** 309–321
doi:10.1111/j.1365-2958.1993.tb01692.x

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