An ABC transporter Wzm–Wzt catalyzes translocation of lipid-linked galactan across the plasma membrane in mycobacteria

Karín Savková,1* Stanislav Huszár,1* Peter Baráth,2* Zuzana Pakanová,2 Stanislav Kozmon,2 Marie Vancová,3 Martina Tesařová,2 Jaroslav Blaško,2 Michal Kaliňák,2 Vinayak Singh,5* Jana Korduláková,2* and Katarina Mikušová2,2*

1Department of Biochemistry, Faculty of Natural Sciences, Comenius University in Bratislava, 842 15 Bratislava, Slovakia; 2Institute of Chemistry, Slovak Academy of Sciences, 845 38 Bratislava, Slovakia; 3Biological Centre, Institute of Parasitology, Czech Academy of Sciences, 370 05 Ústí nad Labem, Czech Republic; 4Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University in Bratislava, 842 15 Bratislava, Slovakia; 5Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, 812 37 Bratislava, Slovakia

Edited by Clifton E. Barry III, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and accepted by Editorial Board Member Carl F. Nathan March 17, 2021 (received for review November 14, 2020)

Mycobacterium tuberculosis, one of the deadliest pathogens in human history, is distinguished by a unique, multilayered cell wall, which offers the bacterium a high level of protection from the attacks of the host immune system. The primary structure of the cell wall core, composed of covalently linked peptidoglycan, branched heteropolysaccharide arabinogalactan, and mycolic acids, is well known, and numerous enzymes involved in the biosynthesis of its components are characterized. The cell wall biosynthesis takes place at both cytoplasmic and periplasmic faces of the plasma membrane, and only recently some of the specific transport systems translocating the metabolic intermediates between these two compartments have been characterized [M. Jackson, C. M. Stevens, L. Zhang, H. I. Zgurcová, M. Niederweis, Chem. Rev., 10.1021/acs.chemrev.0c00869 (2020)]. In this work, we use CRISPR interference methodology in Mycobacterium smegmatis to functionally characterize an ATP-binding cassette (ABC) transporter involved in the translocation of galactan precursors across the plasma membrane. We show that genetic knockdown of the transmembrane subunit of the transporter results in severe morphological changes and the accumulation of an aberrantly long galactan precursor. Based on similarities with structures and functions of specific O-antigen ABC transporters of gram-negative bacteria [C. Whitfield, D. M. Williams, S. D. Kelly, J. Biol. Chem. 295, 10593-10609 (2020)], we propose a model for coupled synthesis and export of the galactan polymer precursor in mycobacteria.

The core of the mycobacterial cell wall is a covalent complex of peptidoglycan, the branched heteropolysaccharide arabinogalactan, and mycolic acids, which are extremely long (C70 to C90) α-alkyl β-hydroxy fatty acids forming a basis for the mycobacterial outer membrane (8). Attachment of arabinogalactan to peptidoglycan is accomplished by the “linkage region” composed of rhamnose-N-acetyl glucosamine-1-phosphate (Rha-GlcNAc-1-P) (9), which also serves as an initiation point for the synthesis of arabinogalactan (10). The principal steps in the construction of this large and rather complicated structure counting around 100 monosaccharide units are well understood. Synthesis of arabinogalactan begins with the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to decaprenylphosphate (decaprenyl-P) catalyzed by the WecA enzyme, followed by addition of Rha from dTDP-Rha by rhamnosyltransferase WbbL, giving rise to the decaprenol-linked “linkage region” (decaprenyl-P-P-GlcNAc-Rha, glycolipid 2, GL2) (11, 12). Synthesis of galactan is catalyzed by two bifunctional enzymes, WbbL and WcbP, which transfer galactose precursors across the cell membrane into the periplasm and into the cell wall core where they are polymerized. The galactan polymer is then translocated out of the cell by the ABC transporter Wzm–Wzt. This ABC transporter is involved in the export of galactan polymer produced by cytoplasmic enzymes across the plasma membrane, as well as translocation of metabolic intermediates from the cytoplasm to the periplasmic space. In this work, we characterized an ABC transporter involved in the export of galactan polymer produced by cytoplasmic enzymes across the plasma membrane, providing an important insight into the biosynthesis of a structure critical for the pathogen survival.

The cell envelope of Mycobacterium tuberculosis serves as a primary protective barrier of the pathogen, which claims more than a million lives each year. Its basis, the unique mycobacterial cell wall core, is composed of covalently linked peptidoglycan, branched heteropolysaccharide arabinogalactan, and mycolic acids. Construction of this complex structure located on the bacterial surface requires an array of enzymes acting on both sides of the plasma membrane, as well as translocation of metabolic intermediates from the cytoplasm to the periplasmic space. In this work, we characterized an ABC transporter involved in the export of galactan polymer produced by cytoplasmic enzymes across the plasma membrane, providing an important insight into the biosynthesis of a structure critical for the pathogen survival.

Significance

The cell envelope of Mycobacterium tuberculosis serves as a primary protective barrier of the pathogen, which claims more than a million lives each year. Its basis, the unique mycobacterial cell wall core, is composed of covalently linked peptidoglycan, branched heteropolysaccharide arabinogalactan, and mycolic acids. Construction of this complex structure located on the bacterial surface requires an array of enzymes acting on both sides of the plasma membrane, as well as translocation of metabolic intermediates from the cytoplasm to the periplasmic space. In this work, we characterized an ABC transporter involved in the export of galactan polymer produced by cytoplasmic enzymes across the plasma membrane, providing an important insight into the biosynthesis of a structure critical for pathogen survival.

Author contributions: S.H., P.B., S.K., M.V., V.S., J.K., and K.M. designed research; K.S., S.H., P.B., Z.P., M.V., M.T., J.B., M.K., and K.M. performed research; S.H., P.B., S.K., M.V., J.B., M.K., and K.M. analyzed data; and K.S., S.H., P.B., Z.P., M.V., M.T., J.B., M.K., and K.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. C.E.B. is a guest editor invited by the Editorial Board.

PNAS 2021 Vol. 118 No. 17 e2023663118

https://doi.org/10.1073/pnas.2023663118
galactofuranosyltransferases, GlfT1 (n=3782 gene product in Mtb H37Rv) and GlfT2 (n=3808c gene product in Mtb H37Rv). GlfT1 attaches the first two galactofuranosyltransferases (GalP) to GL2, forming GalP-β(1→4)-Rhap and GalP-β(1→5)-Gal glycogenic bonds. The processive galactolysotransferase GlfT2 then extends the product of GlfT1 reaction, glycolipid 4 (GL4), by adding alternating β(1→5) and β(1→6) linked Galfs in a linear fashion up to about 30 galactose units, producing lipid-linked galactan polymer (decaprenyl-P-P-GlcNAc-Rha-Gal-α1). GalP residues for transference reactions are donated by UDP-D-Gal, which is produced from UDP-D-Galp by the action of UDP-galactopyranosyl mutase (Guf, n=3809c; gene product in Mtb H37Rv) (14, 15). Branched arabinan domains are built by an array of arabinofuranosyltransferases AftA, AftB, AftC, AftD, EmbA, and EmbB, which utilize decaprenylphosphoryl arabinose as an activated sugar donor (16). While all these arabinofuranosyltransferases belong to the so-called GT-C family of enzymes with multiple transmembrane domains (17, 18), galactolysotransferases GlfT1 and GlfT2 are considered to be soluble enzymes. However, their acceptor substrates have lipidic nature, and the association of GlfT2 with a plasma membrane was proposed based on its crystal structure (19).

It is presumed that arabinogalactan synthesis is initiated on the cytosolic face of the plasma membrane by enzymes relying on sugar nucleotide donors, thus at some point, the arabinogalactan precursor must be translocated across the plasma membrane before its attachment to peptidoglycan by the recently identified ligase(s) (20, 21). Previously, we and others proposed an ATP-binding cassette (ABC) transporter from the conserved mycobacterial arabinogalactan biosynthetic cluster (22) to carry out this function (17, 23, 24). In Mtb, as well as in other pathogenic or environmental mycobacteria, the gene region encoding the transporter has an unusual structure, in that the genes encoding a nucleotide-binding subunit (n=3781 in Mtb H37Rv) and a membrane-spanning subunit (n=3783 in Mtb H37Rv) are separated by the gene encoding the galactolysotransferase GlfT1 (n=3782 in Mtb H37Rv) (Fig. 1A), which pointed out to a role of this transporter in galactan biosynthesis (17, 23, 24). We showed that orthologs of these three genes in Mycobacterium smegmatis mc'155 (Msm) are transcriptionally linked and that the gene encoding the nucleotide-binding subunit is essential (24). Next, we prepared a mutant strain with a disrupted ortholog of the membrane-spanning subunit and performed its phenotypic characterization, but these experiments did not bring conclusive results regarding the precise function or substrate of this ABC transporter (24).

Available of the CRISPR-Cas technology for regulated silencing of the genes in mycobacteria (25) encouraged us to revisit our investigation of the function of this ABC transporter in Msm. We provide evidence that genetic knockdown of the transmembrane subunit, MSMEG_6369, as well as the nucleotide-binding subunit, MSMEG_6366, resulted in the accumulation of lipid-linked galactan polymer (LLG) in the cells and propose that this is a substrate of the studied ABC transporter. Given the similarities of mycobacterial galactan synthesis and transport with those of specific O-antigen polysaccharides in gram-negative bacteria (26, 27), we propose to adopt the terminology from this field (28) and name the nucleotide-binding subunit (Rv3781 ortholog) Wzt and the transmembrane subunit (Rv3783 orthologs) Wzm.

Results

Silencing of wzm and wzt in M. smegmatis Leads to Growth Defects and Morphological Changes, and Points to an Interference with Arabinogalactan Synthesis. For silencing of the genes encoding Wzt (MSMEG_6366, wztSM) and Wzm (MSMEG_6369, wztSM) in Msm, we constructed plasmids derived from PLJR962, which contained fragments of the target genes (SI Appendix, Table S1) and allowed CRISPR interference (CRISPRi) inducible by anhydrotetracycline (ATc) (25). The culturing of the Msm transformants carrying these constructs on solid media confirmed that these were severely compromised for growth in the presence of ATc (SI Appendix, Fig. S1A).

For the initial phenotypic characterization of the effects caused by silencing of the target genes, we cultivated the CRISPRi strains in liquid media in the absence or presence of 100 ng/mL ATc. Monitoring of the OD600 indicated severe growth inhibition of ATc-treated Msm PLJR962-wztSM and Msm PLJR962-wztSM (WZT), and Msm PLJR962-wztSM (WZM) strains grown in the absence or presence of ATc (100 ng/mL). The lipids were separated on silica gel plates in CHCl3/CH3OH/H2O (20:40:5, vol/vol/vol), and visualized with cupric sulfate. TDM—trehalose dimycoclated, TMM—trehalose monomycoclated, and PE—phosphatidyl ethanolamine. (Shown is a representative image corresponding to Experiment 2 specified in SI Appendix, Fig. S1C). (C) DiDPCR analysis of wzt, glfT1, and wzm expression in each of the strains Msm PLJR962 (CON), Msm PLJR962-wztSM (WZT), and Msm PLJR962-wztSM (WZM) strains grown in the absence or presence of ATc (100 ng/mL). The gene expression is normalized to sigA. The error bars are SDs of three technical replicates.
effects within the operon by measuring the messenger RNA copies using droplet digital PCR (ddPCR). The ATc addition reduced the wzmSM expression to almost negligible levels in Msm PLJR962-wztSM, while the repression of the target gene in Msm PLJR962-wzmSM was less severe, reaching ~46% (Fig. 1C). However, in Msm PLJR962-wztSM, transcriptional repression of the target gene caused a considerable reduction in the expression of the downstream genes (Fig. 1C). Therefore, for the functional characterization of the ABC transporter encoded by wztSM and wzmSM, we decided to use Msm PLJR962-wzmSM, where expression of the critical upstream genes of the operon was not inhibited, and phenotypic changes characteristic for arabinogalactan inhibition after silencing of the target gene were obvious. These were further exemplified by examination of this strain using scanning electron microscopy. The images obtained after 24 and 48 h of ATc treatment revealed severe morphological changes of the downstream genes cultured, particularly at the longer exposure. The most frequent phenotype was swelling of the classically rod-shaped bacteria, predominantly at the septum (Fig. 2).

**Genetic Knockdown of wzm in M. smegmatis Triggers the Accumulation of LLG.** To get a better insight into metabolic changes caused by the genetic knockdown of wzmSM, we performed [14C]-glucose labeling of the CRISPRi strain and the control strain containing the empty PLJR962 plasmid grown with and without ATc. Given the hypothesis that the studied ABC transporter is involved in the translocation of LLG across the plasma membrane, inhibition of wzmSM expression could cause the accumulation of these metabolic intermediates in the cells. In search of such molecules in the radiolabeled cells, we developed the extraction procedure inspired by the experimental scheme utilized for obtaining LLG synthesized by the cell-free system (10, 33). The first step included incubation of the washed harvested cells in hot ethanol (34). The pellets obtained by centrifugation of the ethanolic cell suspensions were extracted with hot solvents CHCl3/CH3OH/H2O (10:10:3) and E-soak. Intriguingly, in this experiment, silencing of wzmSM caused about two to threefold accumulation of the radiolabeled material in both CHCl3/CH3OH/H2O (10:10:3) and E-soak extracts. Since radioactive LLG produced by cell-free reactions can be analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by the transfer of the separated molecules to nitrocellulose and their visualization by autoradiography, we examined the material extracted from the radiolabeled cells in the same way. In addition, we tested the sensitivity of the extracted products toward mild acid and mild alkali hydrolys. Stability in mild alkali conditions and lability in mild acid conditions are indicative of the polyprenylphosphate-based molecules, and these properties were confirmed for LLG produced in the cell-free reactions (33). These analyses point to the presence of presumably two different polymers extracted by CHCl3/CH3OH/H2O (10:10:3) and E-soak, respectively, which have properties resembling lipid-linked oligosaccharides (Fig. 3A). Next, we analyzed the monosaccharide composition of these extracts and discovered that the major radiolabeled monosaccharide in all extracts is [14C]-glucose, except for the E-soak fraction obtained from Msm PLJR962-wzmSM grown in the presence of ATc. In this case, radioactive galactose was the main monosaccharide (Fig. 3B). Traces of arabinose in this sample could be related to the presence of lipoarabinomannan, which was found in E-soak extract (Fig. 3A). To ensure that the observed changes were due to the on-target effects, we examined two additional CRISPRi strains, bearing PLJR962-wzmSM plasmids with different single guide RNAs for the target gene. We found that genetic knockdown of wzmSM in all tested strains caused overproduction of LLG, as well as of TMM and TDM, as described above (SI Appendix, Fig. S4). Collectively, these data suggest that wzmSM silencing leads to the accumulation of LLG in mycobacteria.

Our previous examination of the Msm with disrupted wzmSM revealed increased cell-free production of LLG compared with

![Fig. 2. Genetic knockdown of wzmSM causes severe morphological changes of Msm. Scanning electron microscopy images of Msm PLJR962 (CON) and Msm PLJR962-wzmSM (WZM) grown in the absence or presence of ATc (100 ng/mL) for indicated times (Scale bar, 1 μm).](https://doi.org/10.1073/pnas.2023663118)
The control strain (24). We thus decided to subject *M. smegmatis* PLJR962 (CON) to a similar study, along with the control strain containing the empty plasmid PLJR962. Cell envelope fractions prepared from these strains grown with and without ATc served as a source of enzymes and decaprenyl-P, the acceptor for galactan polymerization. Cell-free production of LLG was initiated by the addition of sugar nucleotides necessary for the synthesis of the linkage region, i.e., UDP-GlcNAc and TDP-Rha, and UDP-[14C]-Gal as a tracer for monitoring of galactan polymerization. We observed almost three to seven times higher incorporation of [14C]-Gal into LLG in the reactions containing the enzymes prepared from ATc-treated *M. smegmatis* PLJR962-wzmSM compared with the untreated cells (SI Appendix, Fig. S5A). We then asked whether the increase is due to more efficient de novo synthesis of these metabolic intermediates or if it could be attributed to a mere extension of the preformed LLG, which accumulates in bacteria as a result of wzmSM knockdown. To answer this, we included tunicamycin in the reaction mixtures. This drug inhibits WeCA, an enzyme initiating the synthesis of LLG by attaching GlcNAc-1-P from UDP-GlcNAc to decaprenyl-P (10, 12). As shown in Fig. 3C, tunicamycin almost completely inhibited the radiolabeled LLG synthesis when the enzyme fraction isolated from the untreated *M. smegmatis* PLJR962-wzmSM strain was used. On the other hand, the cell envelope fraction prepared from ATc-treated *M. smegmatis* PLJR962-wzmSM strain produced radiolabeled LLG very efficiently (Fig. 3C and SI Appendix, Fig. S5A). We thus concluded that the increase in the [14C]-Gal incorporation into LLG, in this case, is due to the extension of galactan precursors, which are stalled from further processing because of the missing functional transport system. Furthermore, we observed decreased cell-free production of [14C]-GlcNAc-labeled GL1 and GL2 in reactions using the enzyme fractions prepared from ATc-treated *M. smegmatis* PLJR962-wzmSM compared with the bacteria cultivated without ATc. This observation can be explained by the lack of decaprenyl-P, which is bound in the arrested LLG (SI Appendix, Fig. S5B).

*M. smegmatis* with Genetically Silenced *wzm* Produces Oversized Galactan Precursors. Accumulation of galactose-containing material with properties corresponding to LLG in the CRISPRi strain with the genetically silenced expression of wzmSM (Fig. 3) encouraged us to obtain this substance from these cells and to subject it to structural characterization. To this end, we treated the E-soak extracts obtained from the cell envelope fractions of *M. smegmatis* PLJR962-wzmSM grown with and without ATc with proteinase K and subjected this material to mild acid and mild alkaline hydrolyses. E-soak efficiently extracted carbohydrate-positive material, which was separated by SDS-PAGE and visualized using silver staining (Fig. 4A). In the ATc-treated culture, we observed accumulation of the material that appeared to be resistant to mild alkaline hydrolysis. However, the majority of the extracted carbohydrate matter was labile under these conditions and resistant to mild acid, which resembles the properties of lipoarabinomannan (LAM)/lipomannan (35). This assumption was confirmed by Western blotting and probing the nitrocellulose membrane with anti-LAM–specific antibodies CS-35 (36, 37) (Fig. 4B). Next, mild acid hydrolysis was applied to release the oligosaccharide part from the predicted LLG extracted from ATc-treated *M. smegmatis* PLJR962-wzmSM, which was then separated by gel filtration on Bio-Gel P-100, as described previously (33). Based on the elution volume, the size of this material corresponded to the radiolabeled galactan polymer released from the similarly treated E-soak extract obtained from [14C]-glucose–labeled ATc-treated *M. smegmatis* PLJR962-wzmSM (SI Appendix, Fig. S5B). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) of the individual nonradioactive carbohydrate–containing...
Fig. 4. Characterization of LLG from *Msm* with genetically repressed wzm. (A) SDS-PAGE and Western blot analyses of deproteinized E-soak extracts subjected to mild acid (H\(^+\)) and mild alkali (OH\(^-\)) hydrolyses. The gel was stained with silver; the blot was probed with anti-LAM antibodies CS-35 and visualized with secondary antibodies conjugated to alkaline phosphatase. The red arrow points to the accumulated LLG. UN—untreated samples. (B) MALDI-TOF MS analysis of fractions 46 to 51 obtained by gel filtration of mild acid–treated LLG. Square—GlcNAc; triangle—Rha; circle—Gal. (C) GC/MS analyses of alditol acetates prepared from the combined galactose-rich fractions from Bio-Gel P-100 (Left) and cell walls obtained from *Msm* PLIR962-wzmSM (WZM) grown in the absence and presence of ATc (100 ng/mL) (Right). (D) gHSQCAD spectrum of the combined galactose-rich fractions from Bio-Gel P-100 showing the assignments of 5-Galf (black) and 6-Galf (red), respectively. For chemical shifts, see SI Appendix, Fig. S10A.
samples revealed the presence of polysaccharides from almost 10 to about 6 kDa in the fractions with most carbohydrates (Fig. 4B and SI Appendix, Fig. S6B). To verify their internal structure, we performed a fragmentation MS/MS experiment on precursor signals (e.g., m/z 8,149). The results showed the presence of oligohexose structures. Terminal N-acetyl hexosamine (in its anhydro form) and deoxyhexose structures were further verified in pseudo-MS² (T3) experiment that uses fragmentation of the fragment signals generated by the in-source decay (38). Among dominant oligohexose fragments found at the low m/z range of the MS spectrum, we were able to detect minor fragments with a mass corresponding to the HexNAc-DeoxyHex-Hex5 structure (SI Appendix, Fig. S7), which corresponds to the expected GlcNAC-Rha-Gal4 oligosaccharide. Intriguingly, the sum of the presumed galactomannol monomers, as established by MALDI-TOF MS, ranged from ~40 to more than 50 residues, which largely exceeds the number of Gal/units in the mature mycolyl-arabinogalactan complex recently established to contain ~35 Gal residues in Msm (39). On the other hand, an examination of the E-soak extract from Msm PLJR962-wzmSM grown without ATc showed almost negligible amounts of the α-naphthol-positive material in the fractions from gel filtration. Their MALDI-TOF MS analysis revealed that in this case, no hexose-containing polymer is present (SI Appendix, Fig. S8).

To verify the nature of the monosaccharides in the Bio-Gel P-100-isolated fractions, we subjected the selected samples to gas chromatography-MS (GC/MS). Indeed, Gal was the major monosaccharide in the fractions containing the predicted galactan polymer, and we also confirmed the presence of the expected Rha (saccharide in the fractions containing the predicted galactan polymer). The ratio of 46 Gal per 1 Rha, which corresponds to an average size of galactan after wzmSM knockdown, was ~1:1, whereas minor peaks were attributed to the above-mentioned Rha/GlcNAc or arabino, mannose, and glucose-containing impurities.

Genetic Knockdown of wztSM Phenocopies Transcriptional Silencing of wzmSM. The unique phenotype of the Msm strain with transcriptionally silenced wzmSM encouraged us to search for the identified changes in the strain targeting the nucleotide-binding subunit of the studied transporter, Msm PLJR962-wzmSM. To overcome the polar effect of ATc-induced silencing of wztSM on the downstream gene gfft1SM (Fig. 1), we complemented Msm PLJR962-wztSM with the plasmid pCG76-gfft1SM. Both the parent strain transformed with empty plasmid pCG76 as well as the complemented strain were severely inhibited in the growth in the presence of ATc (SI Appendix, Fig. S14A). Proteomic analysis showed that under these conditions, the amounts of Wzt were drastically reduced in both strains. On the contrary, the Gft1 protein was hardly detectable in the parent strain but was produced efficiently in the complemented strain (SI Appendix, Table S2). We next examined the lipid composition of these two strains grown with and without ATc, as well as their ability to produce LLG. Thin layer chromatography (TLC) analysis of the organic extracts revealed accumulation of TDM and TMM in both ATc-treated strains (SI Appendix, Fig. S11B). Radio-labeling of the cultures with [14C]-glucose and analysis of the material released with CHCl₃/CH₃OH/H₂O (10:10:3) and E-soak disclosed profiles similar to those obtained with CRISPRi strain targeting wzmSM (SI Appendix, Fig. S11C), although it appeared that Lam was extracted in a relatively higher proportion (SI Appendix, Fig. S11D). As expected, in the cultures grown with ATc, the amount of [14C]-LLG was lower in the parent strain (SI Appendix, Fig. S11 C and E) due to the lack of Gft1, which synthesizes GL4 serving as an acceptor for galactan polymerization. The structure of the putative glucose-containing polymer found in CHCl₃/CH₃OH/H₂O (10:10:3) extracts of all analyzed CRISPRi strains grown with ATc (Fig. 3 and SI Appendix, Figs. S4 and S11) is currently unknown—its amount is much lower than that of LLG, which precluded its structural analysis. This finding is corroborated by no signals in the area corresponding to [14C]-labeled CHCl₃/CH₃OH/H₂O (10:10:3) extract on silver-stained SDS-PAGE gel (SI Appendix, Fig. S12).

Molecular Modeling of WzmSM–WztSM Suggests Structural Features Similar to those of a Channel-Forming O-Antigen Polysaccharide ABC Transporter from Aquifex aeolicus. The crystallographic structure of the Wzm–Wzt domains from A. aeolicus (PDB ID: 6M96) (41) was used as a template for WzmSM–WztSM homology model preparation (Fig. 5A). The alignment of primary sequences of the two proteins shows reasonable identities and homologies for both domains, reaching 30 and 55% for Wzt and 17 and 42% for Wzm, respectively (Fig. 5B). These comparisons proved that sequence homologies between the Wzt and Wzm from Msm and A. aeolicus are sufficient to produce an accurate homology model, which was generated using the MODELER program (42). Models of WztSM and WzmSM domains were generated as two separate heterodimeric complexes. Five models and additional five loop models for each model, altogether 30 models, were produced and their probability density function (PDF) energies were calculated (SI Appendix, Figs. S4 and S11) is currently unknown. Five models and additional five loop models for each model, altogether 30 models, were produced and their probability density function (PDF) energies were calculated (SI Appendix, Table S3). The WzmSM–WztSM model with the lowest PDF energy was chosen for further studies (MSMEG3636.MM-MSEMG6369.BL00030001 model) and its alignment with the template X-ray structure is presented in Fig. 5A.

The WzmSM homology model structure consists of six transmembrane helices TM1 to TM6, two periplasmic gate helices PG1 and PG2, and an interface helix (IF). The alignment of the Wzm secondary structures from Msm and A. aeolicus showed a very good correlation (Fig. 5 A and B), with only a few small differences. Namely, WzmSM TM2 helix is shorter, and TM4 and TM6 helices are split into two shorter helices. In case of the WztSM model, the secondary structure alignment showed an almost identical overlay with the Wzt 6M96 domain from A. aeolicus. All secondary structure elements followed the template structure with minimal differences. The important motifs, such as beta-sheet structures β1 and β2, cytosolic gate helix GH, Walker A and Walker B domains, and H-loop and Signature loops have the same lengths (Fig. 5) in both parent structure and the model. Moreover, these structural elements also show high sequence identities.

The three-dimensional (3D) structure alignment of the WzmSM–WztSM complex with 6M96 template structure displays a high level of similarity (Fig. 5A). The measured RMSD of atomic positions on the common backbone atoms revealed a favorable value of 0.4 Å.
The main difference can be seen for the TM2 helix, which is shorter compared with the template. However, TM1b and TM2 interconnecting loop is long enough to place the TM2 in the same space as in the 6M96 template. The quality of the 3D structure of the WzmSM–WztSM complex homology model was also evaluated by the Ramachandran plot, and nine amino acid residues were found in the unfavorable regions (SI Appendix, Fig. S13). Considering that the template 6M96 X-ray structure contains three amino acid residues in the unfavorable regions, this result is in accordance with a very good quality of the produced homology model.

**Discussion**

Biosynthesis of mycobacterial arabinogalactan shares certain features with the assembly of specific O-antigen polysaccharides (O-PS) in gram-negative bacteria (27), as well as of cell wall teichoic acids (WTA) in gram-positive bacteria (43). Probably the closest example is the O2-antigen of *Klebsiella pneumoniae*. It is characterized by the presence of D-galactan I, containing the alternating D-Galf and D-Galp in the repeating unit \(\rightarrow 3)\beta-D-Galp-(1 \rightarrow \) (44). Its biosynthesis is initiated on an undecaprenylphosphate by the WecA-catalyzed transfer of GlcNAc-1-P from UDP-GlcNAc (45). This step is followed by activities of galactosyltransferases WbbN and WbbO, forming an adapter region, which is extended by bifunctional galactosyltransferase WbbM (46). The genes for the synthesis of the three galactosyltransferases form a cluster with the *wzm* and *wzt* genes encoding the subunits of an ABC transporter responsible for translocation of the O2-antigen across the plasma membrane (26, 47). Among the intriguing features of this transporter is its proposed capacity to control the length of the D-galactan I; however, the mechanistic principles of this regulation are not currently known (26, 27). Yet, the recently solved structures of the Wzm–Wzt homolog from *A. aeolicus* substantially contribute to the understanding of the long polysaccharide translocation across the bacterial plasma membrane (41, 48). The versions of the ABC transporter used for crystallization lack the carbohydrate-binding domain (CBD) in Wzt protein, which is responsible for the recognition of the terminal structures of the translocated O-antigen polymers. Examples of such capping residues include methyl and phosphate groups in *Escherichia coli* O9a (49, 50) or 3-keto-D-manno-oct-2ulosonic acid in *K. pneumoniae* O12 (51), respectively. Nevertheless, as noted by Bi et al. (48) even such truncated forms of Wzt were shown to be completely functional in both of these strains if the CBD domain was supplemented in *trans* (52, 53), so the obtained structures are relevant for the functional studies. Intriguingly, CBD is naturally...
missing in the Wzt proteins of both O2-antigen transporter of *K. pneumoniae* and the mycobacterial ABC transporter of our investigation, which correlates with the lack of termination signals in the respective galactan polymers (26, 54, 55). These common features open the possibility that, similar to O2 antigen, the control of mycobacterial galactan length is linked to translocation of its precursor across the plasma membrane. Such a mechanism for O-antigen ABC transporter was described for the first time in 2009 by Whitfield and coworkers (26). They showed that in *K. pneumoniae* O2 overexpression of *wzm* and *wzt* resulted in the production of shorter forms of D-galactan I–containing smooth LPS (S-LPS) compared with the strain with natural levels of this transporter. On the other hand, lower expression of *tramer* is the presence of a hollow funnel-shaped pore with a diameter over 40 Å at the part interacting with the membrane and of less than 10 Å at the opposing face. It was suggested that the volume of the cavity regulates the size of the produced galactan, as it can accommodate about 100 to 150 residues, corresponding to four chains each containing about 30 GalF residues (19). In a recent study focusing on galactan length control, Kiesling and coworkers compared the enzymatic products of *Glt2* from *Corynebacterium diphteriae* and *Mtb* and concluded that *Glt2* itself can regulate the galactan length, as the former enzyme produced shorter galactan polymer than the latter one, which appears to be in accordance with the galactan sizes in the two bacterial species (57). This idea was further corroborated in a follow-up work by the same group, pointing out different sizes of galactan polymer in *Msm* strains in which native *gltF2* was replaced with orthologs from *C. diphteriae* or *Nocardia brasiliensis* (39). Our isolation of *LLG* from *Msm* cells after transcriptional repression of *wzmSM* and its thorough chemical analysis not only provided evidence about the substrate of the studied ABC transporter but also led us to suggest an alternative mechanism for the galactan length control linked to its transport. Indeed, the size of the isolated LLG was unexpectedly large, reaching more than 50 GalF residues, far exceeding the number of ~30 GalF residues found in the mature cell wall galactan. However, an aberrantly long galactan polymer reaching up to 48 units was also produced by purified recombinant mycobacterial *Glt2* when a specific long (C15) alkene phenoxy acceptor substrate [19-phenoxy-nondec-2-enyl β-D-galactofuranosyl-(1→6)-β-D-galactofuranoside] was used (56). Similarly, the polymerizing galactosyltransferase *WbbM* from *K. pneumoniae* was found to generate abnormally long products in the cell-free system (46), as well as in the bacteria in the absence of the transporter (26). Thus, the chain-length distribution in both O2a O-PS biosynthesis and in the mycobacterial cell wall galactan precursor production appears to be influenced by the stoichiometry between export and biosynthesis proteins.

The question of mycobacterial galactan length regulation was addressed in several studies. In 2009, Kiesling and coworkers studied the galactan-polymerizing enzyme, *Glt2*, and proposed a tethering mechanism for the length control of its product (56). They suggest that a specific acceptor lipid anchoring site on the enzyme is important for the regulation of the extent of polymerization. In this model, both ends of the GL4 acceptor substrate, i.e., the decaprenyl portion, as well as the oligosaccharide part, need to directly interact with *Glt2* to achieve efficient galactan polymerization (56). Subsequent resolution of the *Glt2* structure by Ng and Lowary with collaborators proposed that most of the decaprenyl chain is embedded in the plasma membrane and that GL4 diffuses freely in the membrane to deliver its nonreducing end of the polymer and is driven by ATP hydrolysis occurring in the Wzt subunit (Step 3). Lack of *Wzm–Wzt* transporter results in excessively long galactan precursors open the possibility that, similar to O2 antigen, the control of mycobacterial galactan length is linked to translocation of its precursor across the plasma membrane. Such a mechanism for O-antigen ABC transporter was described for the first time in 2009 by Whitfield and coworkers (26). They showed that in *K. pneumoniae* O2 overexpression of *wzm* and *wzt* resulted in the production of shorter forms of D-galactan I–containing smooth LPS (S-LPS) compared with the strain with natural levels of this transporter. On the other hand, lower expression of *tramer* is the presence of a hollow funnel-shaped pore with a diameter over 40 Å at the part interacting with the membrane and of less than 10 Å at the opposing face. It was suggested that the volume of the cavity regulates the size of the produced galactan, as it can accommodate about 100 to 150 residues, corresponding to four chains each containing about 30 GalF residues (19). In a recent study focusing on galactan length control, Kiesling and coworkers compared the enzymatic products of *Glt2* from *Corynebacterium diphteriae* and *Mtb* and concluded that *Glt2* itself can regulate the galactan length, as the former enzyme produced shorter galactan polymer than the latter one, which appears to be in accordance with the galactan sizes in the two bacterial species (57). This idea was further corroborated in a follow-up work by the same group, pointing out different sizes of galactan polymer in *Msm* strains in which native *gltF2* was replaced with orthologs from *C. diphteriae* or *Nocardia brasiliensis* (39). Our isolation of *LLG* from *Msm* cells after transcriptional repression of *wzmSM* and its thorough chemical analysis not only provided evidence about the substrate of the studied ABC transporter but also led us to suggest an alternative mechanism for the galactan length control linked to its transport. Indeed, the size of the isolated LLG was unexpectedly large, reaching more than 50 GalF residues, far exceeding the number of ~30 GalF residues found in the mature cell wall galactan. However, an aberrantly long galactan polymer reaching up to 48 units was also produced by purified recombinant mycobacterial *Glt2* when a specific long (C15) alkene phenoxy acceptor substrate [19-phenoxy-nondec-2-enyl β-D-galactofuranosyl-(1→6)-β-D-galactofuranoside] was used (56).

Similarly, the polymerizing galactosyltransferase *WbbM* from *K. pneumoniae* was found to generate abnormally long products in the cell-free system (46), as well as in the bacteria in the absence of the transporter (26). Thus, the chain-length distribution in both O2a O-PS biosynthesis and in the mycobacterial cell wall galactan precursor production appears to be influenced by the stoichiometry between export and biosynthesis proteins.

It should be noted that until now, LLG molecules were not isolated from mycobacteria, although their involvement in the biosynthesis of the mycobacterial cell wall is widely accepted. Indeed, the key enzymes responsible for their production are known, and they were thoroughly studied (58). However, initially, the pathway was proposed based merely on the characterization of the radioactive enzymatically produced metabolic intermediates, in which structural features typical for mycobacterial arabinogalactan

![Hypothetical model of the coupled galactan synthesis and transport in mycobacteria. Adapter synthesis ensured by the stepwise actions of N-acetyl glucosaminy1-1-P-transferase WecA, rhamnosyltransferase WbbL, and galactosyltransferase GltF1 (Step 1) is followed by galactan polymerization catalyzed by galactosyltransferase GltF2 (Step 2). Translocation of galactan polymer across the plasma membrane through the Wzm–Wzt transporter starts from the reducing end of the polymer and is driven by ATP hydrolysis occurring in the Wzt subunit (Step 3). Lack of Wzm–Wzt transporter results in excessively long galactan intermediates. The proposed protein–protein interactions are only hypothetical, and they were not experimentally confirmed. The image was created with Biorender.com.](https://doi.org/10.1073/pnas.2033613118)
were recognized (10, 33). This included confirmation of the presence of the linkage region monosaccharides GlcNAc and Rha, as well as Gal and even arabinose in the LLG. In fact, the occurrence of arabinose in the enzymatically produced galactan intermediate was unexpected because polymerization of galactan and arabinian were predicted to be spatially separated—the former taking place in the cytoplasm and the latter in the periplasm. However, methylation analysis of the radioactive LLG produced in cell-free reaction revealed the presence of 5.6-linked [14C]-Gal, confirming the presence of arabinosyl residues (33). Our current findings, particularly the MALDI-TOF MS analysis and 2D NMR analyses do not support the presence of arabinose in LLG produced in the cells with transcriptionally repressed \textit{wzm}$_{SM}$. This is in accordance with the distinct and well-separated sites of galactan and arabian build-up in the intact cells.

Molecular modeling suggests a very good correlation of \textit{Wzm–Wzt} from \textit{Msm} with the resolved structures of the channel-forming O-antigen polysaccharide ABC transporter from \textit{A. aeolicus} (41, 48). Accordingly, we propose a similar hypothetical mechanism of LLG translocation across the plasma membrane (Fig. 6). In this model, synthesis of the adapter region by the action of WecA, WbbL, and GltF1 (Step 1) is followed by GltF2-catalyzed polymerization (Step 2). The GlcNAc pyrophosphate group at the non-reducing end in LLG is recognized by the \textit{Wzt} transporter (59) and it is engaged in translocation. The forward movement of the polymer is assured by ATP hydrolysis, inducing conformational changes in \textit{Wzm–Wzt}. The structural analysis of the GltF2 homotetrramer indicates the position of the groove responsible for the entrance of the GltF2 acceptor substrate, GL4. It is located at the membrane-interacting bottom side of GltF2 between its monomeric units (\textit{SI Appendix}, Fig. S14). The groove is ~12 Å wide in the middle, providing enough space for the entry of the GL4 substrate. Moreover, three positively charged amino acid residues that are present in the groove, Lys500, Lys510, and Arg543, can possibly interact with the di-phosphate moiety of GL4 and thus be responsible for bringing the substrate into the inner space of the GltF2 tetramer. We propose that the grooves between the subunits of the tetramer serve not only as the entry points for the GL4 substrates but also as the exit gates for LLG, which is produced by the enzyme (Step 3). This arrangement would allow initiation of the galactan translocation during its synthesis and thus perhaps also regulation of its length.

Historically, the determination of the galactan size in \textit{Mtb} cell wall was challenging, and the reported values differ by about 10 Gal/units, ranging from 22 to ~30 Gal residues (54, 55). Indeed, the presumed heterogeneity of both galactan and arabian chains was mentioned as one of the limitations of the proposed mycolyl-arabinogalactan-peptidoglycan model (54). In view of our current findings, such heterogeneity seems to be the intrinsic property of the mature galactan. This conclusion is supported by the precise evaluation of the structure of arabinogalactan from a mutant of \textit{Corynebacterium glutamicum} with a disrupted \textit{emb} gene, in which only three arabinoses attached by the initiating arabinosyltransferase AftA were present (60). MALDI-TOF MS examination of the methylated cell wall from this mutant revealed arabinogalactan species containing increasing numbers of Gal residues: Ara$_2$Gal$_4$Rha, Ara$_2$Gal$_5$Rha, Ara$_2$Gal$_4$Rha, and Ara$_2$Gal$_5$Rha, pointing to heterogeneity of the galactan domain size in the intact cells (60).

The microscopic inspection of the \textit{Msm} CRISPRi strain with the silenced expression of \textit{wzm}$_{SM}$ disclosed severe morphological changes consistent with the inhibition of the cell wall in a subset of the affected cells. Indeed, similar “lemon-shape” phenotypes were observed in \textit{Msm} strains after transcriptional repression of the genes involved in the terminal cytosolic steps of cell wall synthesis—\textit{murG}, encoding glycosyltransferase producing the peptidoglycan precursor lipid II, galactosyltransferase \textit{glt2}, and \textit{pkls} that is necessary for the synthesis of mycolic acids (29). These enzymes, as well as the studied ABC transporter \textit{Wzm–Wzt}, play critical roles in mycobacterial cell wall biosynthesis. It is expected that the discovery of transporters important for biogenesis and maintenance of mycobacterial cell wall will open further possibilities for the TB drug development (61).

Recently, cryogenic electron microscopy of an ABC transporter \textit{TarGH} from \textit{Alicyclovibacillus herbarius}, which translocates WTA precursors from the cytoplasm into extracellular space, revealed the binding site and an inhibitory mechanism of Targocil, a drug active against methicillin-resistant \textit{Staphylococcus aureus} (62). We believe that elucidation of the function of the \textit{Wzm–Wzt} transporter from mycobacteria will lead to the exploration of its potential in finding new medicines against TB.

Materials and Methods

The CRISPRi strains derived from \textit{M. smegmatis} mc$^{+}$155, which allow ATc-induced silencing of \textit{wzm} and \textit{wzt} expression, were prepared by the procedure described by Rock et al. (25) using the plasmid PLR962 (Addgene). Gene expression analysis in the constructed strains was performed by qPCR. Cultivation of the strains in liquid media was performed by shaking in glycerol–alanine–salts medium supplemented with tyloxapal (0.025%) and kanamycin (25 µg/mL), with and without ATC (100 ng/mL) at 37 °C. Radiolabeling of the CRISPRi and control strains with [14C]-D-glucose was performed essentially as described (30). The radiolabeled cells were extracted step-wise with 96% ethanol for 20 min at 70 °C, CH$_3$COCH$_2$OH and H$_2$O (10:10.3) and E-soak $M$$_2$O$_5$CH$_2$OHsalicyl ether/pyridine/NH$_3$OH (15:15:1:0.017) both for 30 min at 70 °C. The latter two extracts were subjected to mild acid and mild alkaline hydrolyses as described (33), analyzed by SDS-PAGE followed by blotting to nitrocellulose, and autoradiography. LLG was identified that the cell envelope extract and isolated in the same fashion from the cell envelope fraction corresponding to 100,000 × g pellet of the cell lysate obtained by sonication (33). Oligosaccharide part of the lipid-linked galactan was isolated from the deproteinized, mild-acid–treated E-soak fraction extracted from ATc-treated \textit{M. smegmatis} PLR962–\textit{wzm}$_{SM}$–\textit{murG}–\textit{glt2} (100 ng/mL ATc) by gel filtration on Bio-Gel P100 (BioRad) in 50 mM ammonium bicarbonate (33). The carbohydrate-positive fractions were analyzed by MALDI-TOF MS, NMR, and GC analyses. Cell-free reactions using cell envelope fraction as an enzyme source and analysis of the reaction products were performed as described previously (33). Full details of the methods are described in \textit{SI Appendix}.

Data Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. This work was supported by the Slovak Research and Development Agency (Grant APVV-15-0515, Grant APVV-18-0282), by the Ministry of Education, Science, Research and Sport of the Slovak Republic (Grant 2013/20 and Grant KEGA 0375UL-4/2020); by the Ministry of Education, Youth and Sports of the Czech Republic (Grant MEYS CR –LM2018129 Czech Bioimaging); and by the Operational Program Integrated Infrastructure for the project: Center for Biomedical Research - BIOMEDIIRES – II. stage, ITMS: 3130101428, cofinanced by the European Regional Development Fund. V.S. acknowledges support from the South African Medical Research Council, South Africa. The following reagent was obtained through BEI Resources, NIAID, NIH: monoclonal anti-\textit{Mycobacterium tuberculosis} LAM, clone CS-35 (produced in vitro), NR-13811. PLR962 was a gift from Sarah Fortune (Addgene plasmid \\#115162). http://n2t.net/addgene:115162; RRID: Addgene_115162).

1. R. M. Houben, P. J. Dodd, The global burden of latent tuberculosis infection: A re-estimation using mathematical modelling. PLoS Med. 13, e1002152 (2016).
2. “Global tuberculosis report 2020” (Geneva, World Health Organization, 2020); Licence: CC BY-NC-SA 3.0 IGO.
3. T. Togun, B. Kampmann, N. G. Stoker, M. Lipman, Anticipating the impact of the COVID-19 pandemic on TB patients and TB control programmes. Ann. Clin. Microbiol. Antimicrob. 19, 21 (2020).
4. C. E. Baer, E. J. Rubin, C. M. Sassetti, New insights into TB physiology suggest un-derplayed therapeutic opportunities. Immunol. Rev. 264, 327–343 (2015).
5. R. Abreu, P. Giri, F. Quinn, Host-pathogen interaction as a novel target for host- directed therapies in tuberculosis. Front. Immunol. 11, 1553 (2020).
6. A. Garcia-Vilanova, J. Chan, J. B. Torrelles, Underestimated manipulative roles of \textit{Mycobacterium tuberculosis} cell envelope glycolipids during infection. Front. Immunol. 10, 2909 (2019).

Savková et al.

An ABC transporter \textit{Wzm–Wzt} catalyzes translocation of lipid-linked galactan across the plasma membrane in mycobacteria

https://doi.org/10.1073/pnas.2023663118
31. L. Cuthbertson, V. Kos, C. Whitfield, ABC transporters involved in export of cell surface antigens.

23. L. Cuthbertson, V. Kos, C. Whitfield, The glycosyltransferases of Mycobacterium tuberculosis-Roles in the synthesis of arabinogalactan, lipooligosaccharide, and other glycoconjugates. Glycobiology 17, 35-56 (2007).

12. S. Huszár, M. Belánová, S. Berg, D. Kaur, M. Jackson, P. J. Brennan, The glycosyltransferases of Mycobacterium tuberculosis-Core.

13. M. Belánová, D. E. Minnikin, G. S. Besra, The thick waxy coat of mycobacteria, a pro-}

28. P. R. Reeves, J. M. Rock, J. Bacteriol. 190, 1141-1145 (2008).

19. R. W. Wheatley, R. B. Zheng, M. R. Richards, T. L. Lowary, K. K. Ng, Tetrameric linkage region of the mycobacterial cell wall. J. Biol. Chem. 278, 28312-28313 (2012).

26. A. E. Belanger, L. Chen, E. Mallette, B. R. Clarke, M. S. Kimber, C. Whitfield, Identification of an ATP-binding cassette transport system required for translocation of lipooligosaccharide O-antigens side-chains across the cytoplasmic membrane of Klebsiella pneumoniae serotype O1. J. Biol. Chem. 288, 35709-35718 (2004).

20. L. Cuthbertson, C. Whitfield, 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 (2004).

41. Y. Bi, E. Mann, C. Whitfield, Architecture of a channel-forming O-antigen polysaccharide ABC transporter. Nature 553, 361-365 (2018).

39. A. M. Justen, P. J. Brennan, C. Whitfield, Identification of an ATP-binding cassette transport system required for translocation of lipooligosaccharide O-antigens side-chains across the cytoplasmic membrane of Klebsiella pneumoniae serotype O1. J. Mol. Biol. 815 (1993).

40. C. Whitfield, M. B. Perry, L. L. Maclean, S. H. Yu, Structural analysis of the O-antigen side chain polysaccharides in the lipooligosaccharides of Klebsiella serotype O2a(2a), O2a(2a,b), and O2a(2a,2c). J. Bacteriol. 174, 4913-4919 (1992).

38. B. R. Clarke et al., Role of Rfe and Rfp in the initiation of biosynthesis of D-galactan I, the lipooligosaccharide O antigens from Klebsiella pneumoniae serotype O1. J. Biol. Chem. 177, 5411-5418 (1995).

37. B. R. Clarke et al., A bifunctional O-antigen polysaccharide structure reveals a new glycosyltransferase family. Nat. Chem. Biol. 16, 450-457 (2020).

36. B. R. Clarke, C. Whitfield, Identification of an ATP-binding cassette transport system required for translocation of lipooligosaccharide O-antigen side-chains across the cytoplasmic membrane of Klebsiella pneumoniae serotype O1. J. Mol. Biol. 815 (1993).

35. Y. Bi, E. Mann, C. Whitfield, Architecture of a channel-forming O-antigen polysaccharide ABC transporter. Nature 553, 361-365 (2018).

34. M. Carter, M. B. Perry, L. L. MacLean, S. H. Yu, Structural analysis of the O-antigen side chain polysaccharides in the lipooligosaccharides of Klebsiella serotype O2a(2a), O2a(2a,b), and O2a(2a,2c). J. Bacteriol. 174, 4913-4919 (1992).

33. S. Brown, J. P. Santa Maria Jr, S. Walker, Wall teichoic acids of gram-positive bacteria. Annu. Rev. Microbiol. 67, 313-336 (2013).

32. C. Whitfield, M. B. Perry, L. L. Maclean, S. H. Yu, Structural analysis of the O-antigen side chain polysaccharides in the lipooligosaccharides of Klebsiella serotype O2a(2a), O2a(2a,b), and O2a(2a,2c). J. Bacteriol. 174, 4913-4919 (1992).

31. L. Cuthbertson, V. Kos, C. Whitfield, ABC transporters involved in export of cell surface glycoconjugates. Microbiol. Mol. Biol. Rev. 74, 341-362 (2010).

29. P. Dianiková et al., Investigation of ABC transporter from mycobacterial arabinogalactan biosynthetic cluster. Gen. Physiol. Biophys. 30, 239-250 (2011).

28. J. M. Rock et al., Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. Nat. Microbiol. 2, 1627 (2017).

27. V. Kos, L. Cuthbertson, C. Whitfield, The Klebsiella pneumoniae O2a antigen defines a second mechanism for O antigen ATP-binding cassette transporters. J. Biol. Chem. 284, 2947-2956 (2009).

26. C. Whitfield, M. M. Williams, S. D. Kelly, Lipopolysaccharide O antigens-bacterial glycans made to measure. J. Biol. Chem. 291, 10593-10609 (2020).

25. P. R. Reeves et al., Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol. 4, 495-503 (1996).

24. X. Meniche et al., Subpolar addition of new cell wall is directed by DivIVA in mycobacteria. Proc. Natl. Acad. Sci. U.S.A. 111, E2343-E2351 (2014).

23. K. Mikusová, R. A. Slayden, G. S. Besra, P. J. Brennan, Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob. Agents Chemother. 39, 2484-2489 (1995).

22. A. E. Belanger et al., The embA genes of Mycobacterium avium encode an arabinosyltransferase involved in cell wall arabinosylation that is the target for the antituberculosis drug ethambutol. Proc. Natl. Acad. Sci. U.S.A. 93, 11919-11924 (1996).

21. L. Zhang et al., Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. Science 368, 1211-1219 (2020).

20. K. Mikusová et al., Biosynthesis of the galactan component of the mycobacterial cell wall. J. Biol. Chem. 275, 33880-33887 (2000).

19. B. A. Wolucka, M. R. McNeil, E. de Hoffmann, T. Chojnacki, P. J. Brennan, Recognition of the lipid intermediate for arabinogalactan/lipooligosaccharide biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J. Biol. Chem. 269, 23328-23335 (1994).

18. S. W. Hunter, P. J. Brennan, Evidence for the presence of a phosphatidylinositol anchor on the lipooligosaccharide and lipomannan of Mycobacterium tuberculosis. J. Biol. Chem. 265, 9272-9279 (1990).

17. S. Huszár, D. S. Huszár, M. Belánová, S. Berg, D. Kaur, M. Jackson, P. J. Brennan, Recognition of the lipid intermediate for arabinogalactan/lipooligosaccharide biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J. Biol. Chem. 269, 23328-23335 (1994).

16. S. W. Hunter, H. Gaylord, P. J. Brennan, Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. 261, 12345-12351 (1986).

15. D. Kaur, T. L. Lowary, V. D. Viss, D. C. Crick, P. J. Brennan, Characterization of the epitopes of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans. Microbiology (Reading) 148, 3049-3057 (2002).

14. D. Sukaza et al., A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal. Bioanal. Chem. 376, 952-965 (2003).

13. A. M. Justen et al., Polysaccharide length affects mycobacterial cell shape and anti-}