Supplemental material

N-acetylglucosamine-1-phosphate transferase WecA as a validated drug target in *M. tuberculosis* for development of new drugs against tuberculosis

Stanislav Huszár,[a§] Vinayak Singh,[b§]* Alica Polčicová,[a] Peter Baráth,[d] María Belén Barrio,[c] Sophie Lagrange,[c] Véronique Leblanc,[c] Carol Nacy,[e] Valerie Mizrahi,[b#] Katarína Mikušová[a#]

Comenius University in Bratislava, Faculty of Natural Sciences, Department of Biochemistry, Bratislava, Slovakia[a]; MRC/NHLS/UCT Molecular Mycobacteriology Research Unit & DST/NRF Centre of Excellence for Biomedical TB Research, Institute of Infectious Disease and Molecular Medicine and Division of Medical Microbiology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa[b]; Infectious Disease Therapeutic Strategic Unit, Sanofi R&D, Toulouse, France[c]; Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia[d]; Sequella, Inc., Rockville, Maryland, USA[e]

Contents

Table S1 **Oligonucleotides used in this study**

Name	Sequence (5'-3')[a]	Application	Remark
Rv1302c-Fwd	GCACCA**CATATG**CAGTACGGTCTCGAGGTG	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a and pET29a plasmid	This study
Rv1302c-NRev	GGG**AAGCTT**CTAGTCCAGGTCCGGGTCG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a plasmid	This study
Rv1302c-CRev	GGG**AAGCTT**GTCCAGGTCCGGGTCGTAG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET29a plasmid	This study
Rv1302-pJAM-Fwd	AAG**GGATCC**GTGCAGTACGGTCTCGAGG	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pJAM2 plasmid	This study
Rv1302-pJAM-Rev	GGC**TCTAGA**GTCCAGGTCCGGGTCGTAG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pJAM2 plasmid	This study
Rv1302SUMOf	ATGCAGTACGGTCTCGAG	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
Rv1302SUMOr	CTAGTCCAGGTCCGGGTC	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
MSM_4947-Fwd	CCACCA**CATATG**GACAGTCAGGTGGTCCTC	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a and pET29a plasmid	This study
MSM_4947-NRev	GGG**AAGCTT**TCACGGCTCCTGCGCACCG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a plasmid	This study

MSM_4947-CRev	GGG**AAGCTT**CGGCTCCTGCGCACCGTGG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET29a plasmid	This study
MSMEG_4947-pJAM-Fwd	AAA**GGATCC**ATGGACAGTCAGGTGGTCCTC	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pJAM2 plasmid	This study
MSMEG_4947-pJAM-Rev	GGG**TCTAGA**CGGCTCCTGCGCACCGTC	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pJAM2 plasmid	This study
MSMEG_4947SUMOf	TTGCTGCAGTACGGTGCT	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
MSMEG_4947SUMOr	TCACGGCTCCTGCGCACC	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
WecA_Mth_SUMOFwd	ATGCAGTACGGTTCAGCGGTG	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
WecA_Mth_SUMORev	CTACTTCGAGTCGTACATCTGC	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pETSUMO plasmid	This study
pET28_9_WecAMth_F	GGA**CATATG**CAGTACGGTTCAGCGGTGG	Forward primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a and pET29a plasmid	This study
pET28_WecAMth_NR	GGG**AAGCTT**CTACTTCGAGTCGTACATCTG	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET28a plasmid	This study
pET29_WecAMth_CR	GGG**AAGCTT**CTTCGAGTCGTACATCTGCTC	Reversed primer used to amplify a 5'-fragment of the *wecA* gene cloned into pET29a plasmid	This study

wecA_*Sph*I F	cgctgg**GCATGC**agtgttcagctctcaggt	Forward primer used to amplify a 5'-fragment of the *wecA* gene	This study
wecA_*Not*I R	gtccaa**GCGGCCGC<u>TCA</u>**cggtggaatagacgaacc	Reverse primer used to amplify a 5'-fragment of the *wecA* gene	This study
wecA SCO F	ctagccgccgaacgtgca	To confirm the SCO	This study
wecA SCO R	tctagccgcaccgtacca	To confirm the SCO	This study
wecARTF	GGGCTTTCTGCCACACAACT	Forward primer for ddPCR analysis of *wecA*	This study
wecARTR	CCGGAATCGCCCATGAA	Reverse primer for ddPCR analysis of *wecA*	This study
wecAprobe	CACCGGGCCAAGAT	TaqMan (FAM) probe for ddPCR of *wecA*	This study
sigARTF	cgagccgatctcgttgga	Forward primer for ddPCR analysis of *sigA*	Suppl. Ref. 1
sigARTR	ttcgatgaaatcgccaagct	Reverse primer for ddPCR analysis of *sigA*	Suppl. Ref. 1
sigAProbe (VIC)	acgagggcgacagc	TaqMan (VIC) probe for ddPCR of *sigA*	Suppl. Ref. 1
pSE100 F	TGAGCTCTACGCCATCCCG	Primers to verify the cloned gene in pSE100 and to confirm homologous recombination of pWecA-SCO	Suppl. Ref. 1
pSE100 R	TCTCCGGCTTCACCGATCC		

[a] Restriction sites are shown in bold; in-frame stop codon is underlined.

Supplemental Materials and Methods

Purification of *E. coli* MurF protein

E. coli strain T7 express (C2566) harboring recombinant plasmid pTYB21 *murF* (kind gift from D. Crick, Colorado State University) was grown at 37 °C in 1 liter of LB-ampicillin (100 μg/ml) medium until the OD_{600} reached ~ 0.6. The cultures were cooled at 4 °C for 30 min, followed by further growth at 15 °C with 0.4 mM IPTG for 18 hours. The cells were harvested by centrifugation (4,500 × g, 10 min, 4 °C) and washed 2 times with 20 mM HEPES pH 8.5. 1.2 g (wet weight) of cells were suspended in 10 ml of Column buffer (20 mM HEPES pH 8.5, 500 mM NaCl) and disrupted by sonication (15-s pulses with 40-s cooling intervals, 10 cycles) at 4 °C. The lysate was centrifuged (15,000 × g, 20 min, 4 °C) and ½ of the supernatant was used for purification. *E. coli* MurF protein was then purified according to IMPACT Kit Manual (NEB) using chitin resin (NEB cat. # S6651S). The cleavage of the intein-tag was done by incubation with 50 mM dithiothreitol for 40 hours at 20 °C on the column (Poly-Prep® Chromatography Column, Bio-RAD). Purified MurF protein was then eluted from the column, concentrated to 3.5 mg/ml (Amicon® Ultra 0.5 ml Centrifugal Filter, 10 000 MWCO) and used in enzymatic synthesis of [^{14}C]-labeled UDP-MurNAc-pentapeptide.

Enzymatic synthesis of [^{14}C]-UDP-MurNAc-pentapeptide

The reaction mixture contained 100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 30 mM $MgCl_2$, 10 mM ATP, 1 mM UDP-MurNAc-tripeptide (BacWAN facility, University of Warwick) and 16.5 μCi [1-^{14}C] D-Ala-D-Ala (specific activity 55 mCi/mmol, ARC) in the final volume of 300 μl. The reaction was initiated by the addition of purified *E. coli* MurF protein (100 μg) and the mixture was incubated 2 hours at 37°C. The MurF protein was then separated from the reaction mixture by Amicon® Ultra 0.5 ml Centrifugal Filter (10,000 MWCO). The final product was analyzed by TLC on aluminium-coated silica 60 F_{254} plate (Merck) developed in 2-propanol/conc. NH_4OH/H_2O (6:3:1), followed by autoradiography using Kodak Bio-Max MR films. Conversion of [1-^{14}C] D-Ala-D-Ala to [^{14}C]-UDP-MurNAc-pentapeptide reached about 93-97% and thus no further purification of the reaction product was performed.

Supplemental Figures

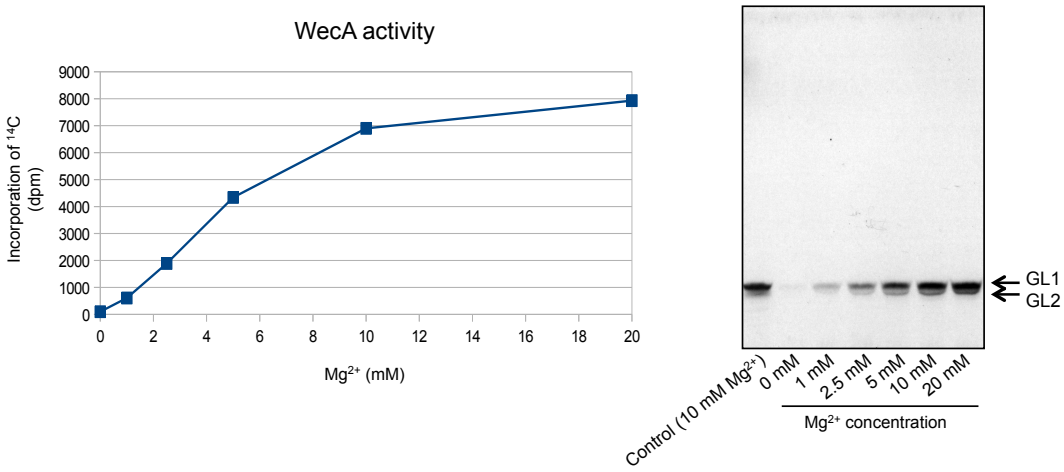


Figure S1. Effect of Mg^{2+} on activity of WecA from *M. smegmatis* mc^{2}155. Enzymatic reactions contain membrane fraction (300 µg protein) isolated from *M. smegmatis* mc^{2}155, radiolabeled substrate UDP-[^{14}C]GlcNAc (0.25 µCi) and different concentrations of $MgCl_2$. The amount of radiolabeled product was quantified by scintillation spectrometry. 20% of the lipid extract was loaded on Silica-gel TLC plate, developed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.5:3.6) and then exposed to autoradiography film for 3 days.

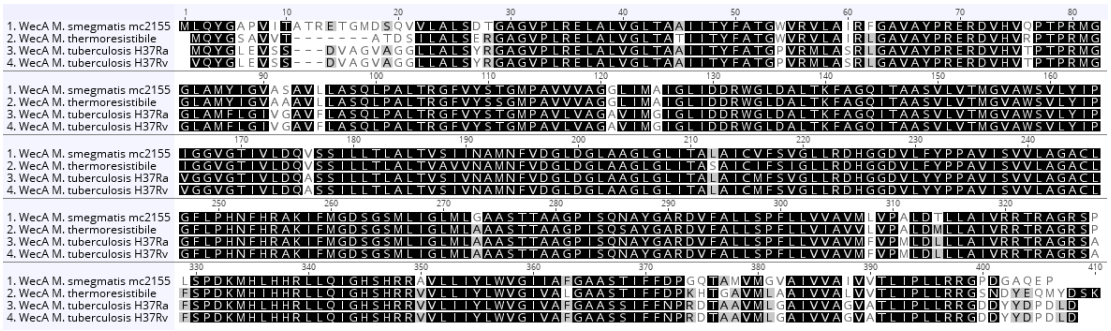


Figure S2. Protein identity of mycobacterial WecA orthologs. Protein identity was compared by Geneious software (Geneious version 7.1 created by Biomatters. Available from http://www.geneious.com)

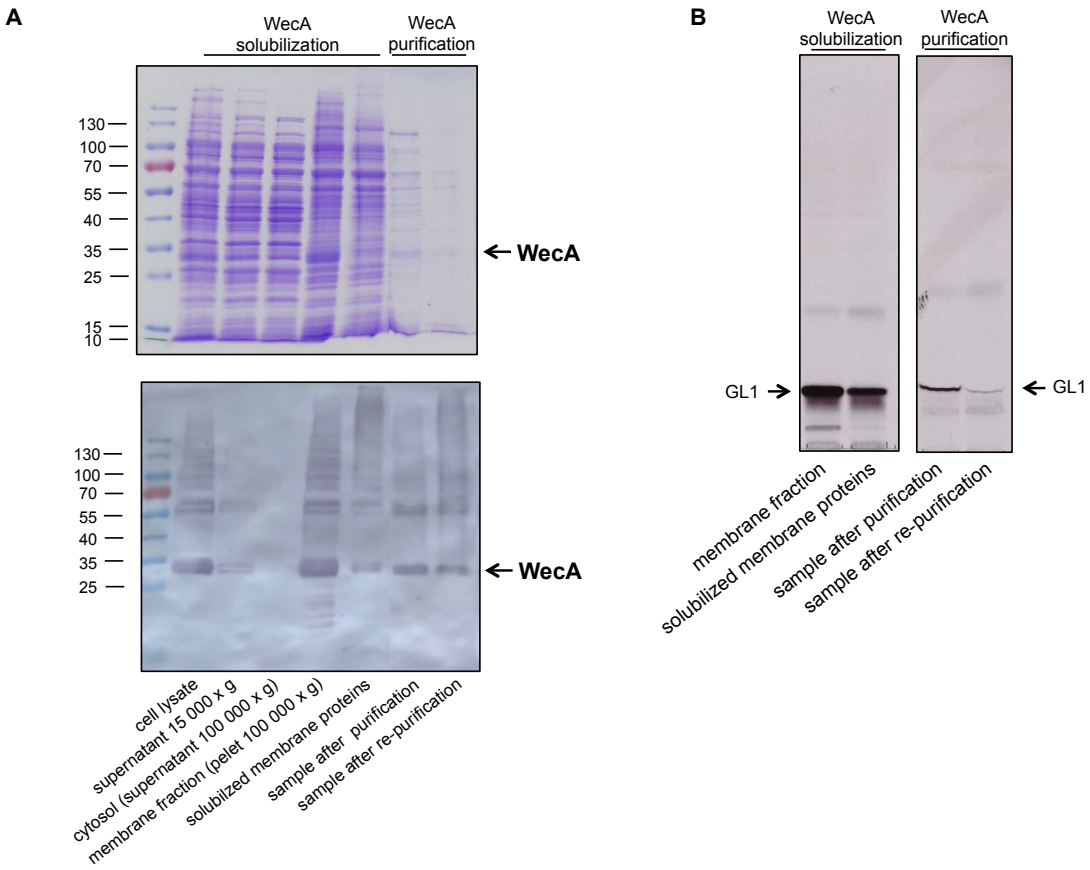


Figure S3. Solubilization, purification and activity of WecA from *M. thermoresistibile*.
A. HisTagged recombinant WecA from *M. thermoresistibile* was solubilized with CHAPS detergent (136 mM) from membrane fraction isolated from overproducing strain *E. coli* C43(DE3)/pET28a-$wecA_{Mth}$. Purification was performed in two steps using TALON Co^{2+}resin. Samples after solubilization and purification were analyzed by SDS-PAGE (upper panel) and Western blot (bottom panel).
B. Autoradiogram of TLC analysis of WecA reacion products. Reaction mixture contained 50 mM TRIS HCl pH 8.0, 40 mM $MgCl_2$, 50 mM sucrose, 10% glycerol, 5 mM β-mercaptoethanol, 0.5% CHAPS, 50 µM decaprenyl phosphate and 0.25 µCi of UDP-[^{14}C]-GlcNAc in total volume 80 µl. Reactions were initiated by addition of crude membrane fraction (200 µg of proteins) or solubilized membrane proteins (200 µg of proteins) or sample after purification (20 µg of proteins) or sample after re-purification (20 µg of proteins), respectively from *E. coli* C43(DE3)/*pET28a-*$wecA_{Mth}$ strain. Reactions were incubated 1 hour at 37 °C. [^{14}C]-labeled glycolipid 1 (GL1) was extracted by Folch wash as described in Materials and Methods and the extraction procedure was repeated one more time. Organic phase was finally dried under nitrogen stream and dissolved in 50 µl of $CHCl_3$/CH_3OH/conc. NH_4OH/H_2O (65:25:0.5:3.6). 20% of the organic phase was measured by scintillation counting and 40% was loaded on Silica-gel TLC plate followed by developing in $CHCl_3$/CH_3OH/conc. NH_4OH/H_2O (65:25:0.5:3.6). TLC plate was exposed on autoradiography film for 36 days.

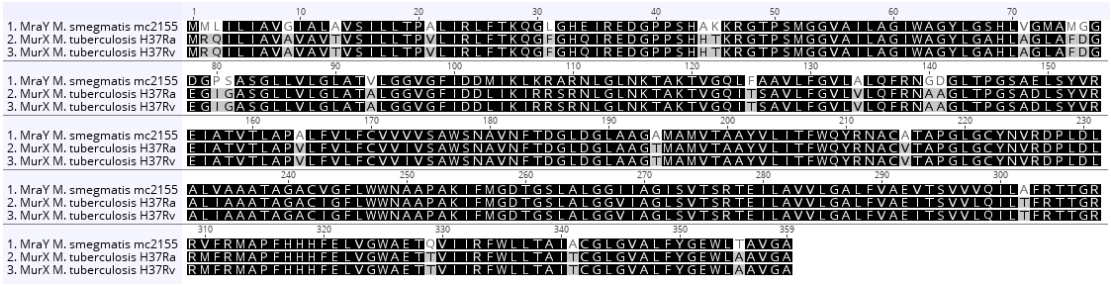


Figure S4. Protein identity of mycobacterial translocase I orthologs. Protein identity was compared by Geneious software (Geneious version 7.1 created by Biomatters. Available from http://www.geneious.com)

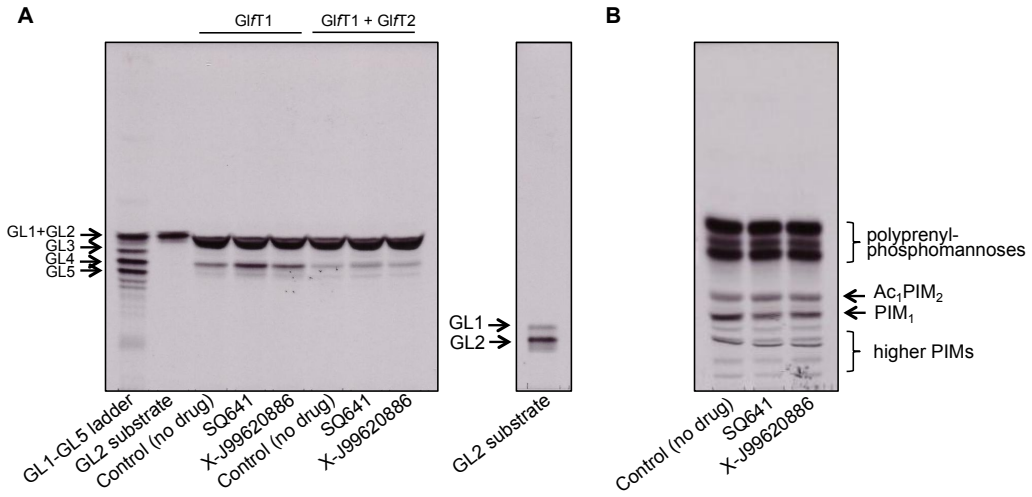
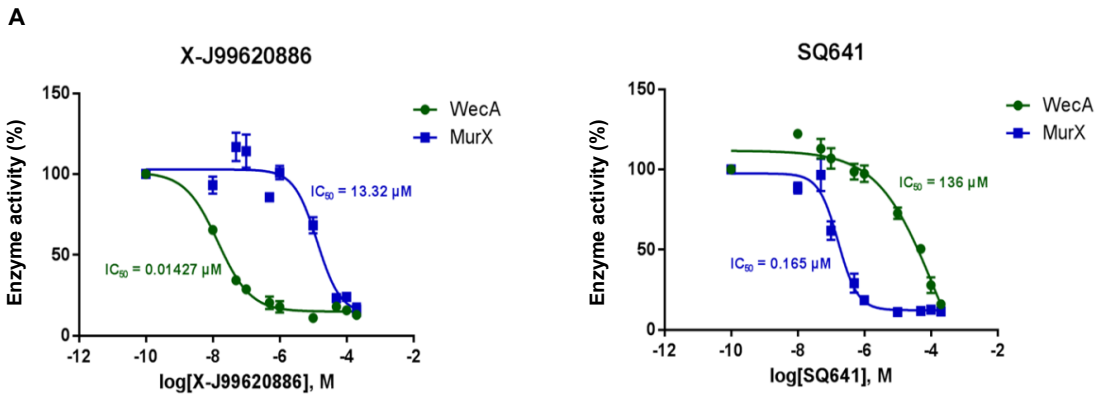


Figure S5. Effects of SQ641 and X-J99620886 on mycobacterial galactofuranosyltransferases and mannosyltransferases.

A. An autoradiogram of TLC analysis monitoring polymerization of mycobacterial galactan using glycolipid 2 ([^{14}C]-GL2 as a radiolabeled substrate. **B.** An autoradiogram of TLC analysis monitoring mannolipid biosynthesis using GDP-[^{14}C]Man as a substrate. [^{14}C]-GL2 was prepared as described (2), and analyzed by TLC (A, right panel). All assays were performed in 50 mM MOPS pH 7.9, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol in total volume 80 µl. SQ641 and X-J99620886 were tested in 200 µM final concentrations. The final concentration of DMSO in assays was 2% (v/v). Reaction mixtures containing [^{14}C]GL2 (2 000 dpm) were supplemented with crude GlfT1 and GlfT2 extracts (15,000 x g supernatant of lysate, 200 µg of protein) from *E. coli* BL21(DE3)/pET28a-*msmeg_6367 (glfT1)* and *E. coli* C41(DE3)/pET15b-*rv3808c (glfT2)* as described (3). Reaction mixtures containing GDP-[^{14}C]Man (0.25 µCi; specific activity 305 mCi/mmol, ARC) contained membrane fraction (300 µg protein) isolated from *M. smegmatis* mc^{2}155 as described (4). 10% of the organic phase after Folch wash extraction was loaded on Silica-gel TLC plate followed by developing in $CHCl_3$/CH_3OH/conc. NH_4OH/1 M CH_3COONH_4/H_2O (65:25:0.5:3.6) (A) or $CHCl_3$/CH_3OH/conc. NH_4OH/H_2O (65:25:0.5:4) (B). TLC plate was exposed on autoradiography film for 19 days (A) or 2 days (B).



	IC_{50} (µM)	
	SQ641	X-J99620886
WecA	136.00	0.0143
MurX	0.1650	13.320

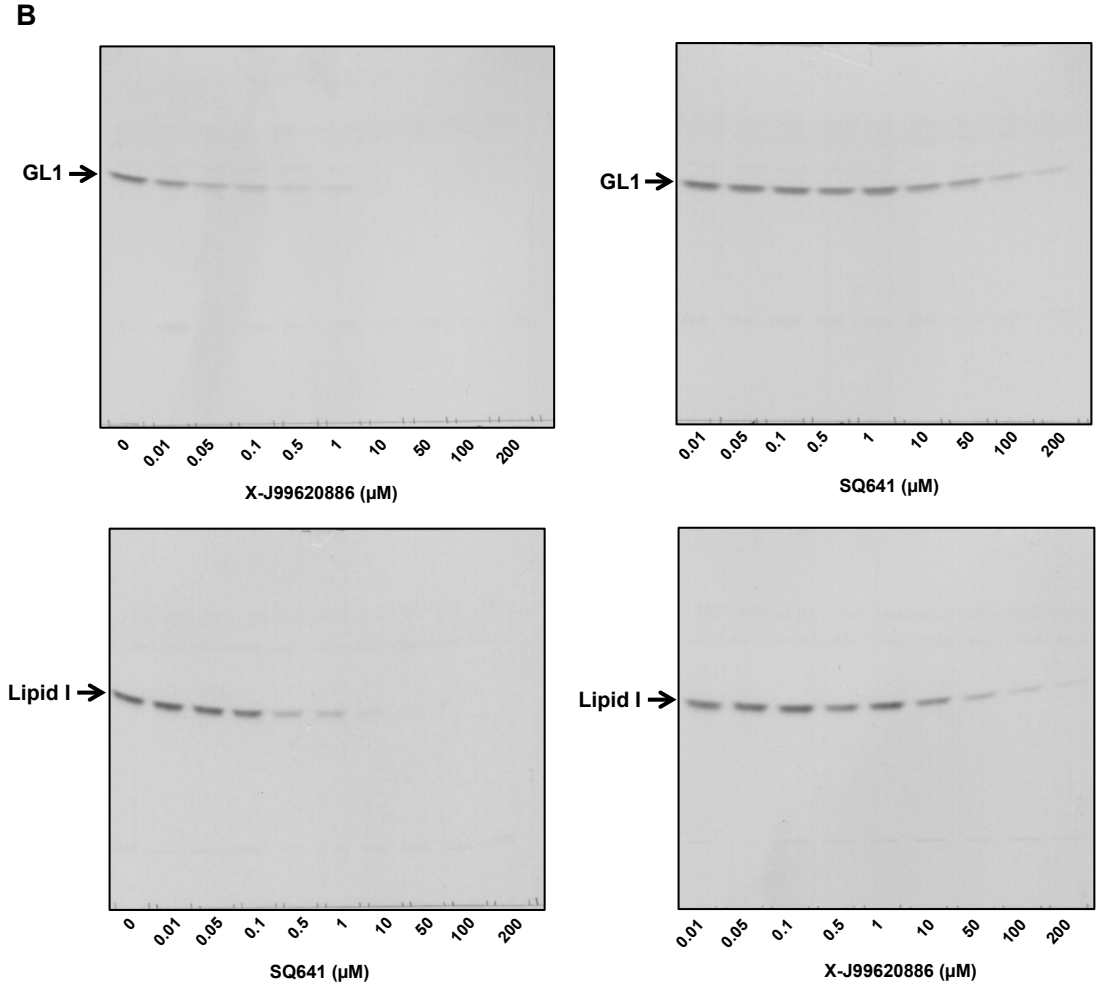


Figure S6. Determination of IC_{50} values for SQ641 and X-J99620886. Selectivity of SQ641 and X-J99620886 towards WecA and MurX of *M. tuberculosis* H37Ra was investigated by dose-dependent inhibition study using radiometric assays. Nonlinear regression analysis was used to calculate IC_{50} values by GraphPad Prism 6 software. **A.** Log dose *vs.* response curves with IC_{50} values. **B.** Products of the reaction mixtures (GL1 or lipid I) after visualization by autoradiography.

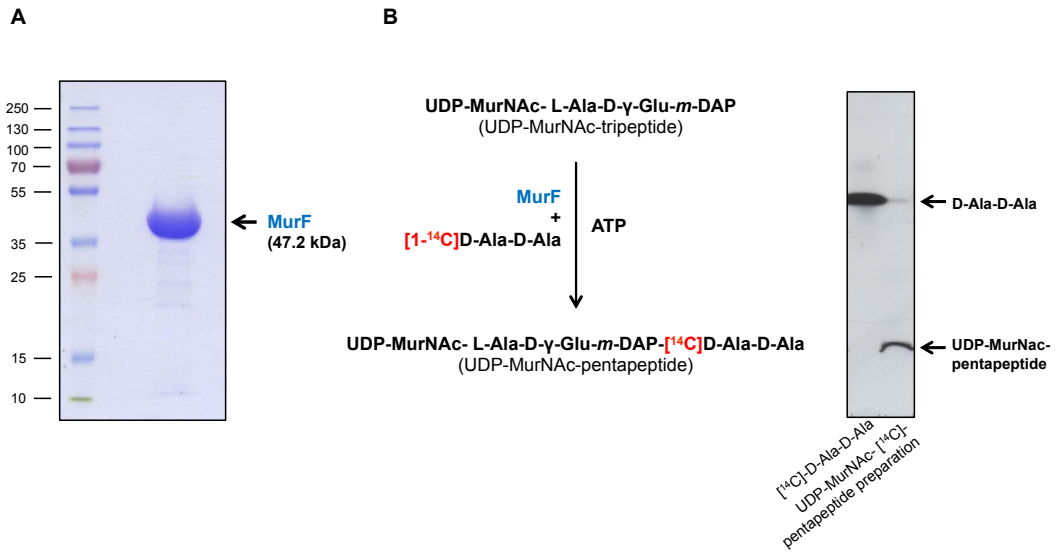


Figure S7. Enzymatic synthesis of UDP-MurNAc-[^{14}C]-pentapeptide (Park´s nucleotide). [^{14}C]-labeled UDP-MurNAc-pentapeptide was enzymaticaly synthetized with purified MurF protein using UDP-MurNAc-tripeptide and [^{14}C]-D-Ala-D-Ala**. A.** Purified MurF protein analyzed by SDS-PAGE**. B.** Enzymatic reaction catalyzed by MurF protein and prepared UDP-MurNac[^{14}C]-pentapeptide analyzed by TLC followed by autoradiography. Silica-gel TLC plate was developed in 2-propanol/conc. NH_4OH/H_2O (6:3:1) and exposed on autoradiography film for 4 days.

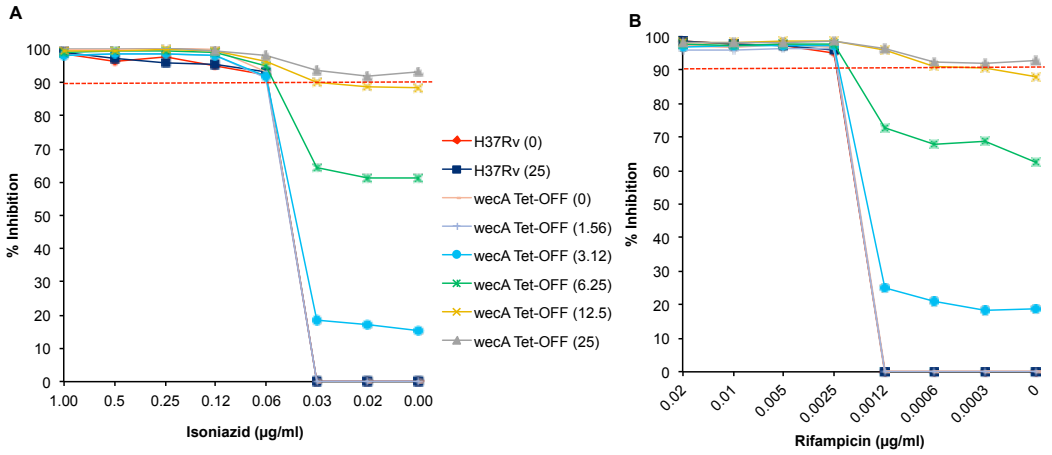


Figure S8. Silencing of *wecA* in *Mtb* showed no effect on sensitivity to Isoniazid and Rifampicin. A two dimensional array of serial dilutions of ATc and Isoniazid and Rifampicin were added to the cultures for determining the susceptibility of H37Rv and *wecA* Tet-OFF strains. Bacterial viability was assessed by the Alamar Blue assay as described in Methods.

References:

1. **Singh V, Donini S, Pacitto A, Sala C, Hartkoorn RC, Dhar N, Keri G, Ascher DB, Mondesert G, Vocat A, Lupien A, Sommer R, Vermet H, Lagrange S, Buechler J, Warner DF, McKinney JD, Pato J, Cole ST, Blundell TL, Rizzi M, Mizrahi V.** 2017. The Inosine Monophosphate Dehydrogenase, GuaB2, Is a Vulnerable New Bactericidal Drug Target for Tuberculosis. ACS Infect Dis **3:**5-17.
2. **Mikusova K, Mikus M, Besra GS, Hancock I, Brennan PJ.** 1996. Biosynthesis of the linkage region of the mycobacterial cell wall. J Biol Chem **271:**7820-7828.
3. **Belanova M, Dianiskova P, Brennan PJ, Completo GC, Rose NL, Lowary TL, Mikusova K.** 2008. Galactosyl transferases in mycobacterial cell wall synthesis. J Bacteriol **190:**1141-1145.
4. **Kordulakova J, Gilleron M, Mikusova K, Puzo G, Brennan PJ, Gicquel B, Jackson M.** 2002. Definition of the first mannosylation step in phosphatidylinositol mannoside synthesis. PimA is essential for growth of mycobacteria. J Biol Chem **277:**31335-31344.