**Supporting Information**

# Partial Saturation of Menaquinone in *Mycobacterium tuberculosis*: Function and Essentiality of a Novel Reductase, MenJ

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**Methods.**

**Construction of Deletion Mutants.** The Ts/*sacB* method[(1)] was used to achieve allelic replacement at the *MSMEG1132* and *Rv0561c* locus of *M. smegmatis* $mc^2$ 155 and *M. tuberculosis* H37Rv respectively. The *MSMEG1132* and *Rv0561c* genes with flanking regions were PCR amplified from their respective genomic DNA with primers Smeg1132KO F/R and Rv0561cKO F/R, subcloned in pGEMT easy vectors (Supplementary Table 2). The disrupted alleles, *MSMEG1132*::*kan* and *Rv0561c*::kan were obtained by inserting the *Tn903* kanamycin resistance cassette obtained from pUC4K-*kan* at the *Nar*I restriction site of pBK*MSMEG1132* and *Age*I/*Nhe*I site of pBK*Rv0561c*. Both disrupted alleles were then cloned into the *Xba*I site of pPR27-*xylE*[(2)], yielding pPR27*MSMEG1132*::*kan* and pPR27*Rv0561*::*kan*.

**Transformation of Mycobacteria Strains.** Plasmids *pPR27MSMEG1132*::*kan* and pPR27*Rv0561*::*kan* were introduced into *M. smegmatis* $mc^2$ 155 or *M. tuberculosis* H37Rv by electroporation, and transformants were selected at 30 °C on 7H10- containing 50 μg/ml kanamycin for *M. smegmatis* and 32 °C for *M. tuberculosis*. In order to select for allelic exchange, cultures were plated onto 7H10 agar plates containing 2% sucrose and kanamycin (25 μg/ml) at different dilutions and incubated at 42 °C (*M. smegmatis*) or 39 °C (*M. tuberculosis*). X*ylE*$^-$, $Kan^R$ gentamicin-sensitive and sucrose - resistant allelic exchange candidates[(2)] were confirmed by PCR using the primers, SmKOconf F/R and Rv0561conf F/R. PCR primers were designed from outside the genomic regions of the PCR fragment used as the allelic exchange substrate.

**Complementation of Deletion Mutants.** *M. smegmatis* Δ*MSMEG1132* was complemented with pVV16*Rv0561c* and *M. tuberculosis* H37Δ*Rv0561c* with pNIP40b*Rv0561c*. Plasmid pNIP40b*Rv0561c* was constructed by amplifying *Rv0561c* under control of the phsp60 promoter

from pVV16*Rv0561c* using primers Rv0561NIPpvv F/R and cloned at the *Xba*I site of pNIP40b[3].

**Aerobic growth curves.** Cultures were prepared with 7H9 medium supplemented with OADC and 0.05% (w/v) Tween-80 in triplicate for each strain. Optical density was taken at every 2 h for *M. smegmatis* stains and on alternate days for *M. tuberculosis* strains. Bacterial growth in culture was followed by measuring the $OD_{600nm}$ as a function of time and CFU were determined by plating culture dilutions on 7H10 agar supplemented with appropriate antibiotics.

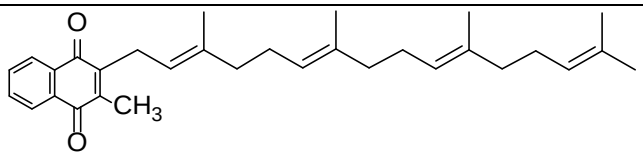
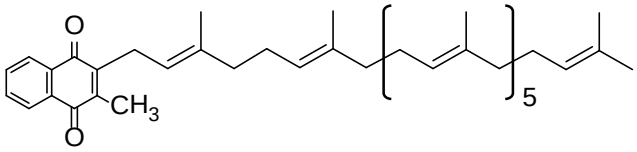
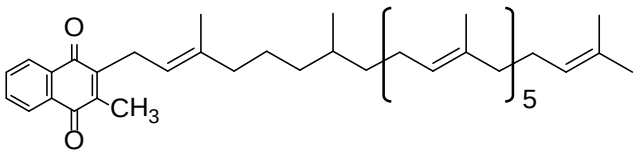
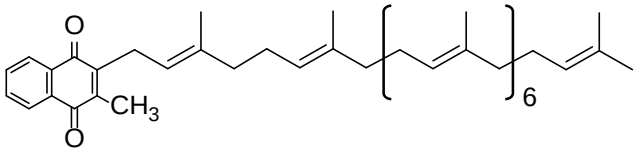
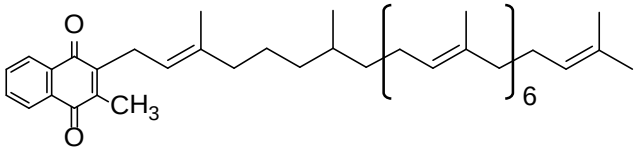
**Hypoxic growth curves and oxygen consumption.** Bacterial growth and oxygen consumption rates were determined by growing the bacteria in sealed tubes using a modified Wayne Model[4] of hypoxic mycobacterial growth. Cultures were grown in glass tubes containing 7H9 medium with or without the addition of methylene blue (1.5 µg/ml) and a headspace ratio of 0.5. Tubes were capped, parafilm-sealed and incubated at 37° C with stirring at 100 rpm. Growth of the bacteria was monitored at $OD_{600nm}$ and oxygen consumption was determined by following the decolorization of methylene blue at $OD_{670nm}$. Oxygen consumption was calculated as previously described[4] and rates were determined under conditions where the decrease in absorbance was linear with regard to time.

**ΔpH measurements**. Determination of ΔpH was performed using $^{31}P$ NMR as previously described[5, 6]. Cells were grown to a density of $10^8$ cells/mL in 500 mL of 7H9 medium. Cells were concentrated by centrifugation and washed twice with 5 mM phosphate buffer at pH 6.8. The cell pellet was resuspended in 200 µL of the same buffer and 500 µL of the cell slurry was transferred to a 5 mm NMR tube. A capillary tube containing 85% phosphoric acid in $D_2O$ was inserted in the NMR tube to be used as standard on a Varian INOVA 300, at a frequency of 121.5 MHz. A proton-decoupled pulse angle of 60 degrees and a relaxation delay of 1 s, with a observe pulse of 68 µs, a receiver gain of 46 dB were used. Spectra were obtained within a spectral width of -50 to 50 ppm and with 1024 scans and analyzed as described elsewhere[6]

with minor modification. No oxygen or nitrogen bubbling was applied. For spectra processing data was binned every 0.02 ppm. This resulted in spectra with ~5000 data points increasing the S/N ratio. The pH was calculated using the peak corresponding to the α-phosphate of ATP (-10.5 ppm) and the inorganic phosphate peaks of interest (in the region of 0-1.5 ppm), using the equation, $\mathrm{pH} = 6.75 + \log\frac{\mathrm{d}-10.85}{13.25-\mathrm{d}}$ where d is the distance between the α-phosphate of ATP and the inorganic phosphate peak, in ppm[6]. The antibiotics valinomycin and nigericin were used as control compounds that dissipate ΔΨ and ΔpH, respectively, in many bacteria including mycobacteria [7].

**Fluorometric measurement of membrane potential dissipation (ΔΨ).** The membrane potential dissipation was measured fluorimetrically using the ΔΨ-sensitive fluorescent dye DiSC3(5) as previously described[5, 8, 9] with minor modifications. Assays were performed in black walled 96 well plates with a reaction volume of 200 µl using a BioTek® Synergy HT Multi-Mode Micro Plate Reader with excitation at 600 nm, emission at 645 nm and a slit width of 40 nm for detection. Dissipation of ΔΨ at the indicated concentrations of valinomycin was measured and the effective concentrations for 50% dissipation of ΔΨ ($EC_{50}$) were calculated[5].

**Table S1.** Structures and calculated monoisotopic masses for menaquinones described in this study.

| Menaquinone | Calculated monoisotopic mass | Structure |
|---|---|---|
| MK-4 | 444.3028 |  |
| MK-8 | 716.5532 |  |
| *MK-8(II-$H_2$) | 718.5689 |  |
| MK-9 | 784.6158 |  |
| *MK-9(II-$H_2$) | 786.6315 |  |

*position of saturated double bond is drawn to be consistent with previous reports for *Mycobacterium phlei* and various other Gram-positive organisms [(10-13)].

**Table S2.** Bacterial strains and plasmids used in this study.

| Strains/ plasmids | Relevant characteristics/ Description | References |
|---|---|---|
| *Escherichia coli* DH5α | F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-, mK+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 | Invitrogen |
| *Escherichia coli* BL21 (DE3) pLysS | F- *omp*T *hsd*SB(rB-, mB-) *gal dcm* (DE3) pLysS ($Cam^R$) | Invitrogen |
| *M. smegmatis* $mc^2$ 155 | Parent strain | *(14)* |
| *M. smegmatis* Δ*MSMEG1132* | *M. smegmatis* $mc^2$ 155 knock out strain with deletion and insertion of $kan^r$ gene in *MSMEG1132* | This study |
| *M. tuberculosis* H37Rv | Parent strain H37Rv TMC 102 | ATCC 27094 |
| *M. tuberculosis* H37ΔRv0561c | *M. tuberculosis* H37Rv knock strain with deletion and insertion of $kan^r$ gene in *Rv0561c* | This study |
| pGEM®-T Easy | 2.9 kb *E. coli* cloning vector*, lacZ*, *amp*, f1 origin | Promega |
| pBluescript SK+ | Cloning vector; ColE1 replicon; *amp* (2.9 kb) | Stratagene |
| pPR27-*xylE* | Derivative of pPR27 carrying the *xylE* gene. *E. coli*- Mycobacterial shuttle vector, *ts* oriM, *sacB*, $gm^r$, *xylE* (~10.7kb) | *(1)* |
| pUC4K-kan | 3.9 kb *E. coli* vector, source of $kan^r$ gene | Pharmacia Biotech |
| pVV16 | $Kan^r$; $Hyg^r$; expression vector used for constitutive protein expression from a p*hsp60* promoter (~5.9 kb) | *(15)* |
| pNIP40b | $Hyg^r$; Integrative vector | *(3, 16)* |
| pGEKO*MSMEG1132* | 621 bp upstream and 751 bp downstream region of *MSMEG1132* gene from *M. smegmatis* $mc^2$ 155 cloned in pGEM®-T Easy vector (~5.5 kb) | This study |
| pBKO*MSMEG1132* | *MSMEG1132* gene obtained from pGEKO*MSMEG1132*, cloned at *Xba*I in pBluescript SK+ vector (~5.5 kb) | This study |
| pBKKO*MSMEG1132*::*kan* | pBKO*MSMEG1132* containing $kan^r$ gene at *Nar*I site (~6.7 kb) | This study |

| | | |
|---|---|---|
| pPR27KO*MSMEG1132*::*kan* | *MSMEG1132*::*kan* obtained from pBKO*MSMEG1132*::*kan* cloned into pPR27-*xylE* at *Xba*I site (~14.5 kb). | This study |
| pGEMKO*Rv0561c* | 830 bp upstream and 858 bp downstream region of Rv0561c gene from *M*. *tuberculosis* H37Rv cloned in pGEM®-T Easy vector (~5.8 kb) | This study |
| pBKKO*Rv0561c* | *Rv0561c* gene obtained from pGEMKO*Rv0561c*, cloned at *Xba*I in pBluescript SK+ vector (~5.8 kb) | This study |
| pBKKORv0561c::*kan* | pBKO*Rv0561c* containing *kan*$^r$ gene at *Age*I/ *Nhe*I site (~7.0 kb) | This study |
| pPR27KO*Rv0561c*::*kan* | *Rv0561c*::*kan* obtained from pBKO*Rv0561c*::*kan* cloned into pPR27-*xylE* at *Xba*I site (~17.7 kb) | This study |
| pVV16*Rv0561c* | *Rv0561c* gene cloned in pVV16 vector (~7.1 kb) | This study |
| pNIP40b*Rv0561c* | Construct containing promoter regions of pVV16 with *Rv0561c* gene from pVV16*Rv0561c*, cloned into pNIP40b at *Xba*I site (~1.5 kb), *hyg*$^r$ | This study |

**Table S3.** PCR primers used in this study.

| Primers | Sequence (5'-3') | Restriction site |
|---|---|---|
| Smeg1132 F | ATTCATATGAACACCCGAGCGGATGTGGTC | *Nde*I |
| Smeg1132 R | TATAAGCTTTCAGCTGAACGGCACCCGCTG | *Hind*III |
| Rv0561c F | ATTCATATGAGCGTGGATGACAGTGCCGAC | *Nde*I |
| Rv0561c R | TATAAGCTTTCAGCTGAACGGCGGTCGTCG | *Hind*III |
| Smeg1132KO F | ATATCTAGAGGAGAACGTCGCACCGAACCG | *Xba*I |
| Smeg1132KO R | ATATCTAGAAGGATCAGCCCGGTGGAGGGC | *Xba*I |
| Rv0561cKO F | ATATCTAGACCGTTCAGCAGTGCGCGCA | *Xba*I |
| Rv0561cKO R | ATATCTAGATGCGCCGGCGCTGCCAAAC | *Xba*I |
| Rv0561conf F | CCGTCCCGGGACGTCGGGCAG | - |
| Rv0561conf R | AAGCCAAGCGTTGTAGTCCTG | - |
| SmKOconf F | GGGCGTCTTGCCGGACTCGTC | - |
| SmKOconf R | CTACGTGGGGACCGTGGGTGC | - |
| Rv0561NIPpvv F | ATATCTAGACGAATCGCTCATCACCTCGTC | *Xba*I |
| Rv0561NIPpvv R | ATATCTAGAAGTCATGTGTTCATATATATC | *Xba*I |
| Rv0561confNIP F | GTGCGTGCTGGTGTCGCGCGA | - |
| Rv0561confNIP F | CGGCCGGCAACCAAGGGTGCG | - |
| Rv0561complconf F | ATGCTACTTGGCGCTAAAGCC | - |
| Rv0561complconf R | AACCGAGAAGCCGCGACCGTA | - |

**Table S4.** Comparison of ΔpH in WT *M*. *smegmatis* $mc^2$ 155 and *M*. *smegmatis*Δ*MSMEG1132* treated with various ionophores.

| **Strains** | **Drugs (μM)** | **$pH_{out}$** | **$pH_{in}$** | **ΔpH** |
|---|---|---|---|---|
| *M*. *smegmatis* $mc^2$ 155 | No drug | 6.82 | 7.08 | 0.26 |
| | Valinomycin (33 μM) | 6.83 | 7.07 | 0.24 |
| | Nigericin (5 μM) | 6.82 | 6.82 | - |
| *M*. *smegmatis* Δ*MSMEG1132* | No drug | 6.80 | 7.06 | 0.26 |
| | Valinomycin (33 μM) | 6.84 | 7.08 | 0.26 |
| | Nigericin (5 μM) | 6.83 | 6.83 | - |

**Figure S1. Sequence alignment of MenJ with similar proteins from representative mycobacteria spp.**

Amino acid sequence alignment of *Rv0561c* and *MSMEG1132* with similar proteins from representative *Mycobacterium* species. Alignment was done on the MultAlin website[17]. White letters on red = high consensus, red letters on white = low consensus, black letters = neutral. Figure prepared using ESPript 3.0[17].

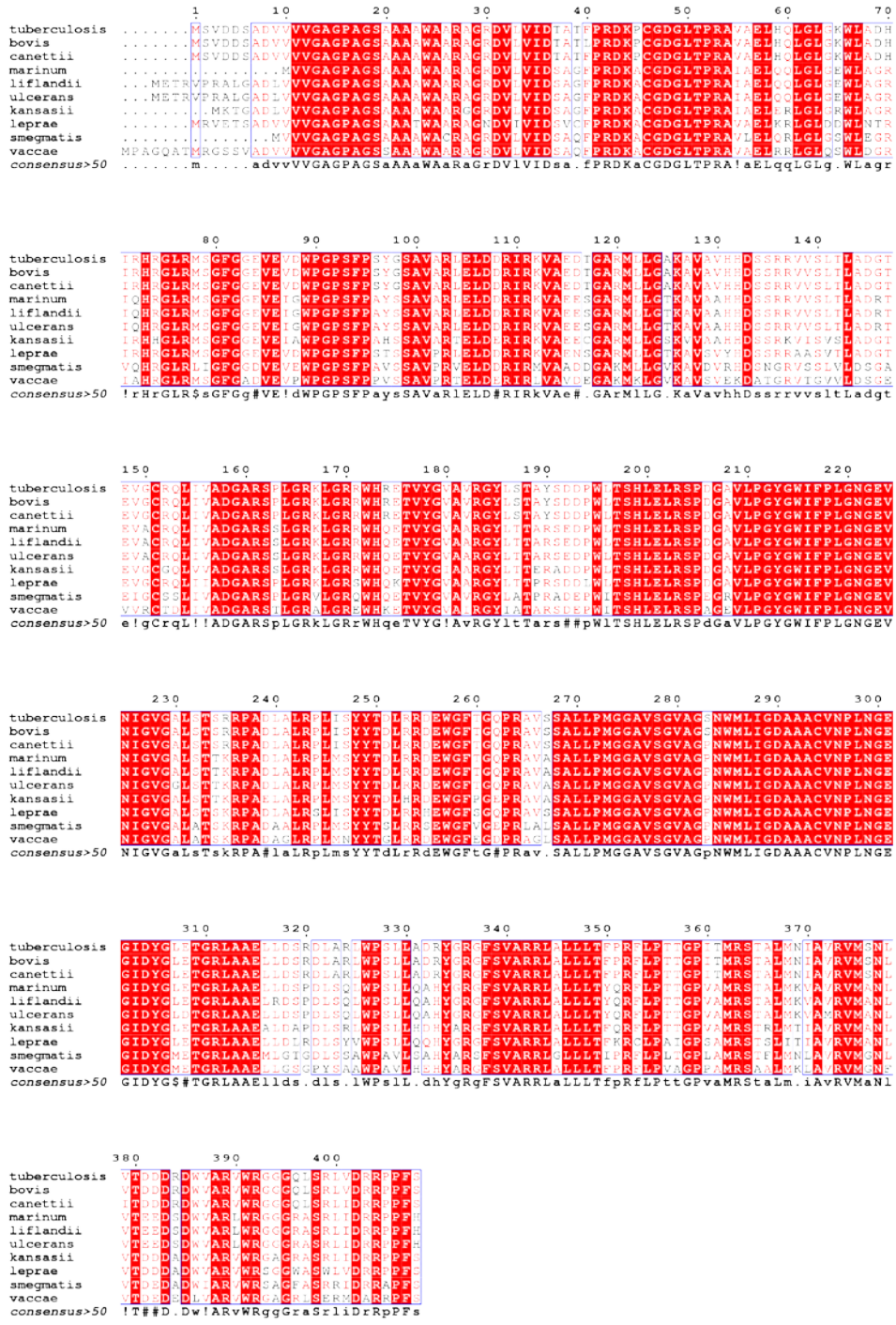


**Figure S2. Tandem MS analysis of menaquinone from recombinant *E. coli* expressing *MSMEG1132.***

LCQ ion-trap tandem MS analysis of MK-8 from WT *E. coli* (Panel A) and MK-8(II-$H_2$) from recombinant *E. coli* expressing *MSMEG1132* (Panel B). The insets show the inferred fragmentation which probably occurs with internal hydrogen migration to form a conjugated double bond system.

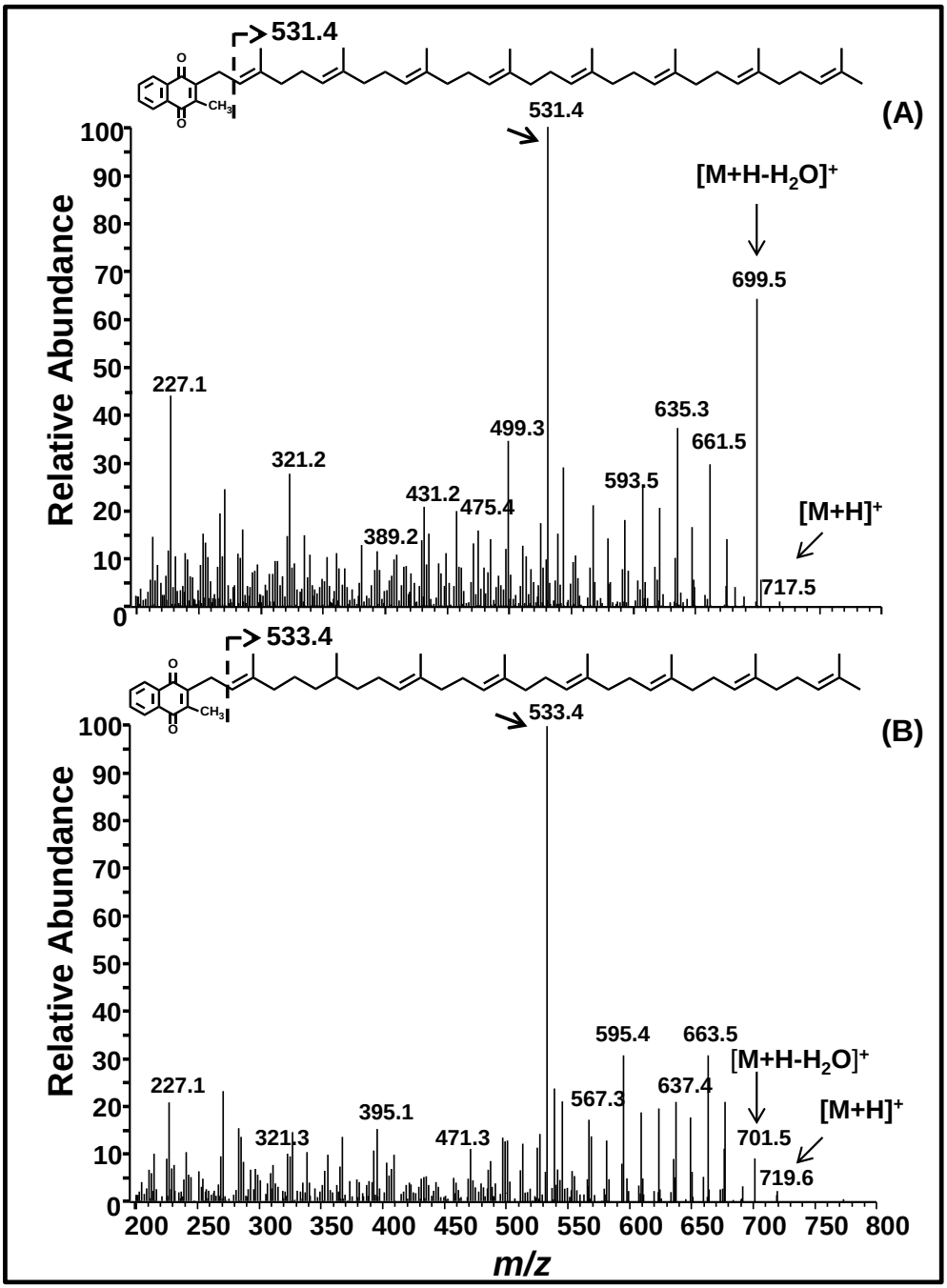
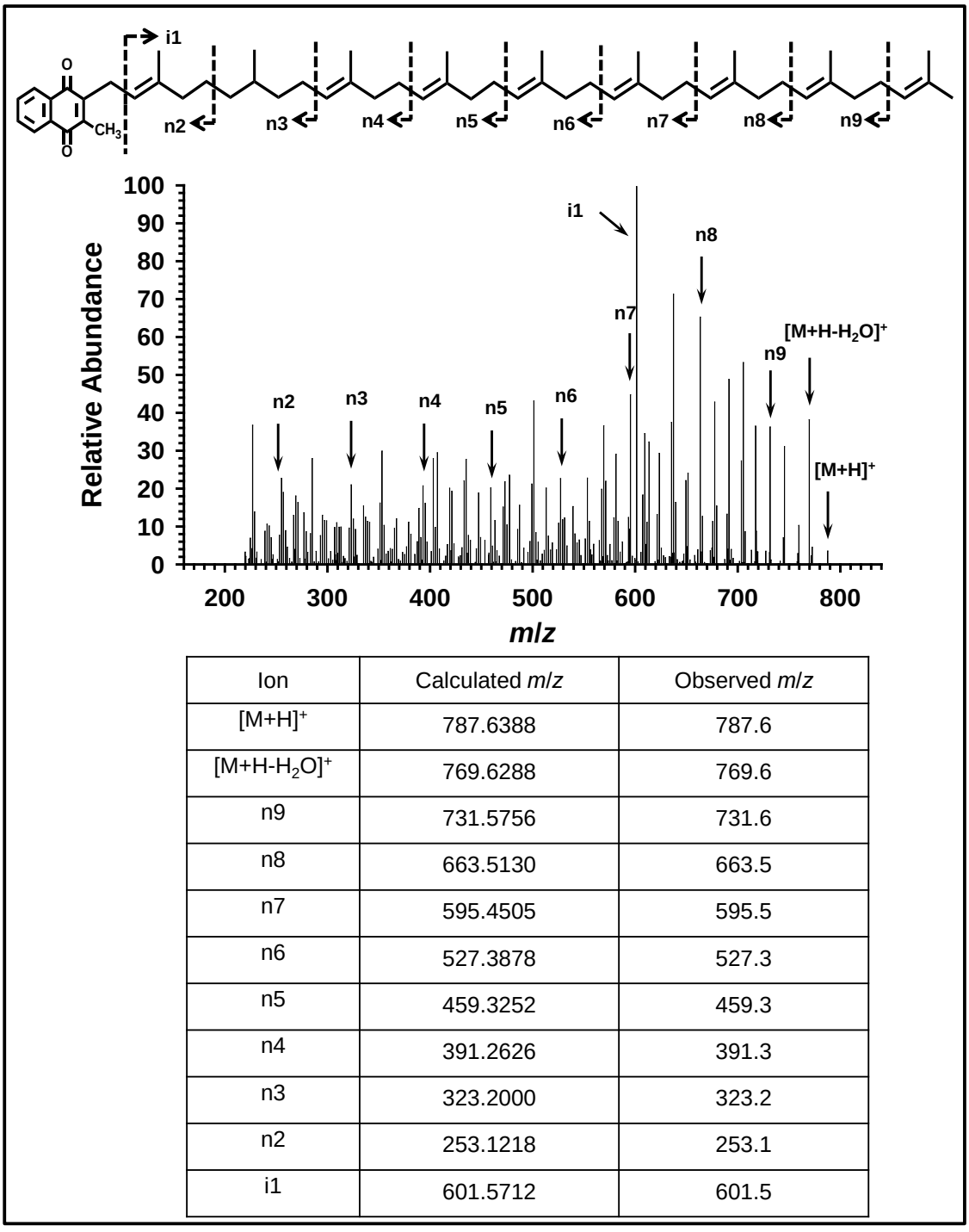


**Figure S3. Tandem MS of MK-9(II-$H_2$).**

LCQ ion-trap tandem MS analysis of MK-9(II-$H_2$) extracted from WT *M. smegmatis* showing the structure with inferred fragmentation pattern, a typical chromatogram and table showing observed *m*/*z* values for the indicated ions.




| Ion | Calculated *m*/*z* | Observed *m*/*z* |
|---|---|---|
| $[M+H]^+$ | 787.6388 | 787.6 |
| $[M+H-H_2O]^+$ | 769.6288 | 769.6 |
| n9 | 731.5756 | 731.6 |
| n8 | 663.5130 | 663.5 |
| n7 | 595.4505 | 595.5 |
| n6 | 527.3878 | 527.3 |
| n5 | 459.3252 | 459.3 |
| n4 | 391.2626 | 391.3 |
| n3 | 323.2000 | 323.2 |
| n2 | 253.1218 | 253.1 |
| i1 | 601.5712 | 601.5 |

**Figure S4. PCR confirmation of different strains generated in the study.**

PCR amplification using the SmKNconf F/R and Rv0561conf F/R primers (Table S3) confirmed the knock outs for *M*. *smegmatis* Δ*MSMEG1132* (gel A) and *M*. *tuberculosis* H37Δ*Rv0561c* (gel B) strains, respectively. Amplicons from the KO strains contain an insertion of a 1.2 kb kan$^r$ gene. Confirmation of *Rv0561c* complementation in *M*. *tuberculosis* H37Δ*Rv0561c* (gel C) was done using primers Rv0561confNIP F/R designed from the deleted regions of *Rv0561c* from *M*. *tuberculosis* H37Δ*Rv0561c*. *M*. *tuberculosis* H37Δ*Rv0561c*, containing the kan$^r$ gene, did not amplify but when complemented with *Rv0561c* generated an amplicon similar to WT. The lane labelled L contains DNA size markers.

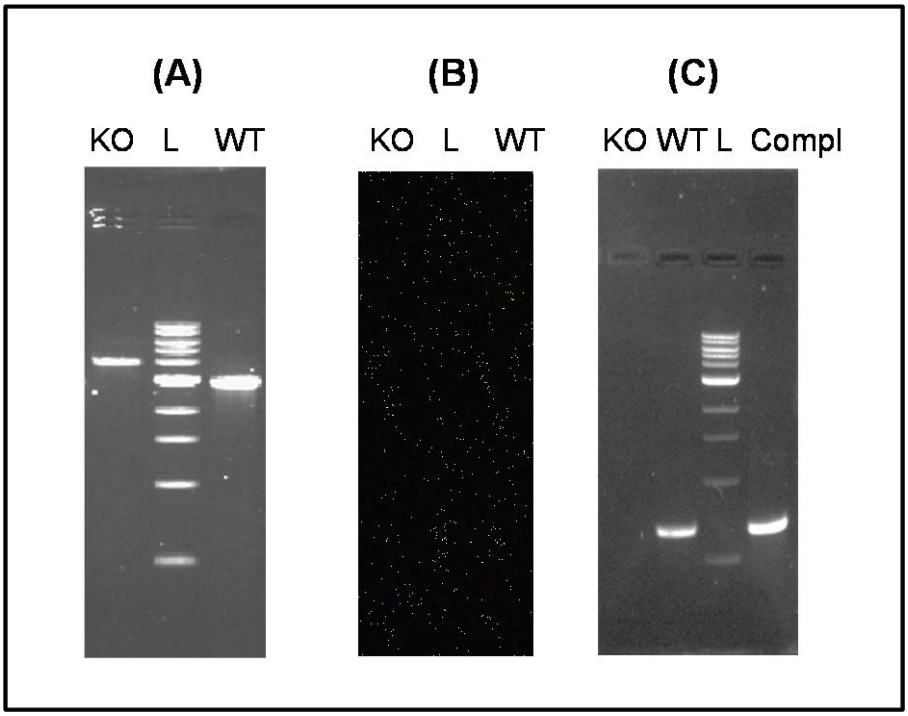
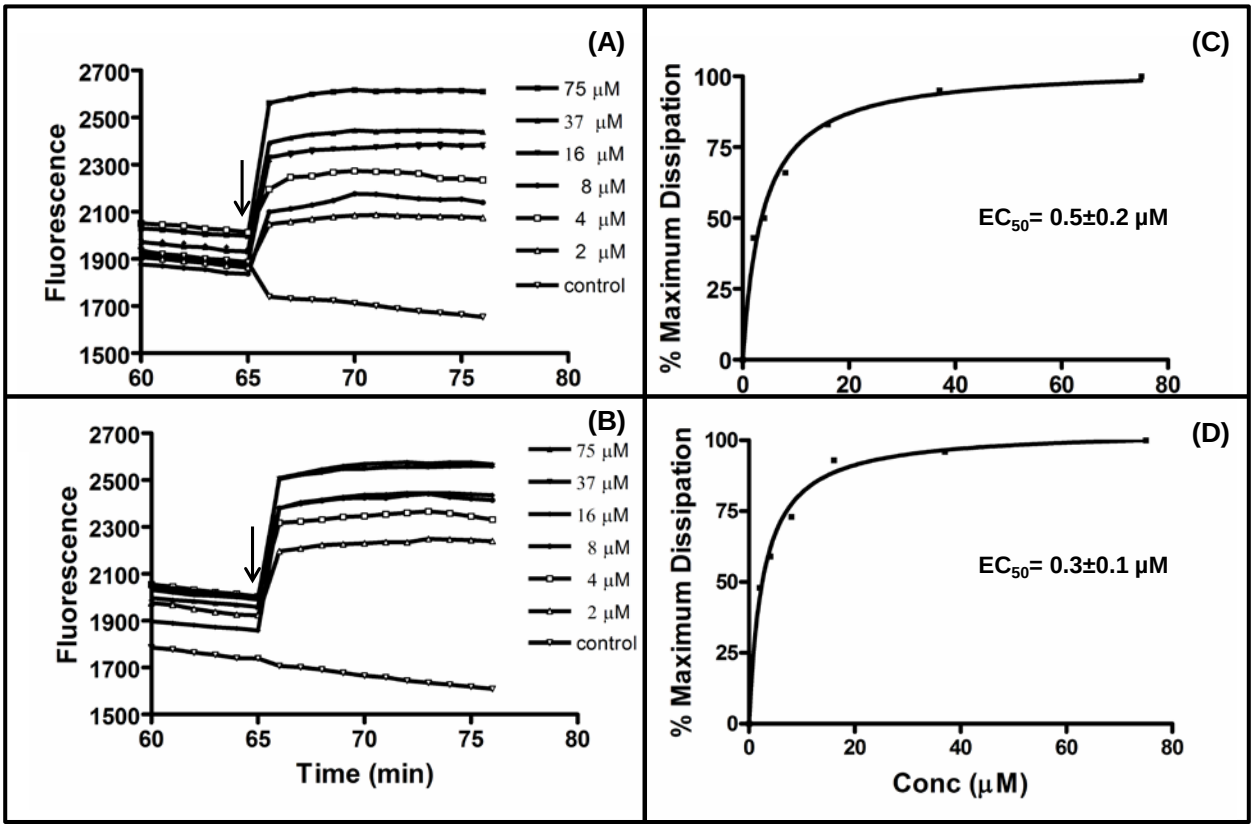


**Figure S5. Fluorometric measurement of membrane potential (ΔΨ) dissipation.**

Fluorimetric measurement of membrane potential dissipation (ΔΨ) by the indicated concentrations of valinomycin in WT *M*. *smegmatis* (Panel A) and *M*. *smegmatis* Δ*MSMEG1132* (Panel B) using DiSC3(5). Arrows indicate addition of valinomycin. $EC_{50}$ values for valinomycin dissipation of ΔΨ in WT (Panel C) and Δ*MSMEG1132 M. smegmatis* (Panel D) were calculated as % of maximally observed dissipation using the data shown in Panels A and B. $EC_{50}$ values, ± standard error, were calculated using GraFit 5.0.13 (Erithacus Software Ltd.).



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