The antibacterial prodrug activator Rv2466c is a mycothiol-dependent reductase in the oxidative stress response of *Mycobacterium tuberculosis*

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SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

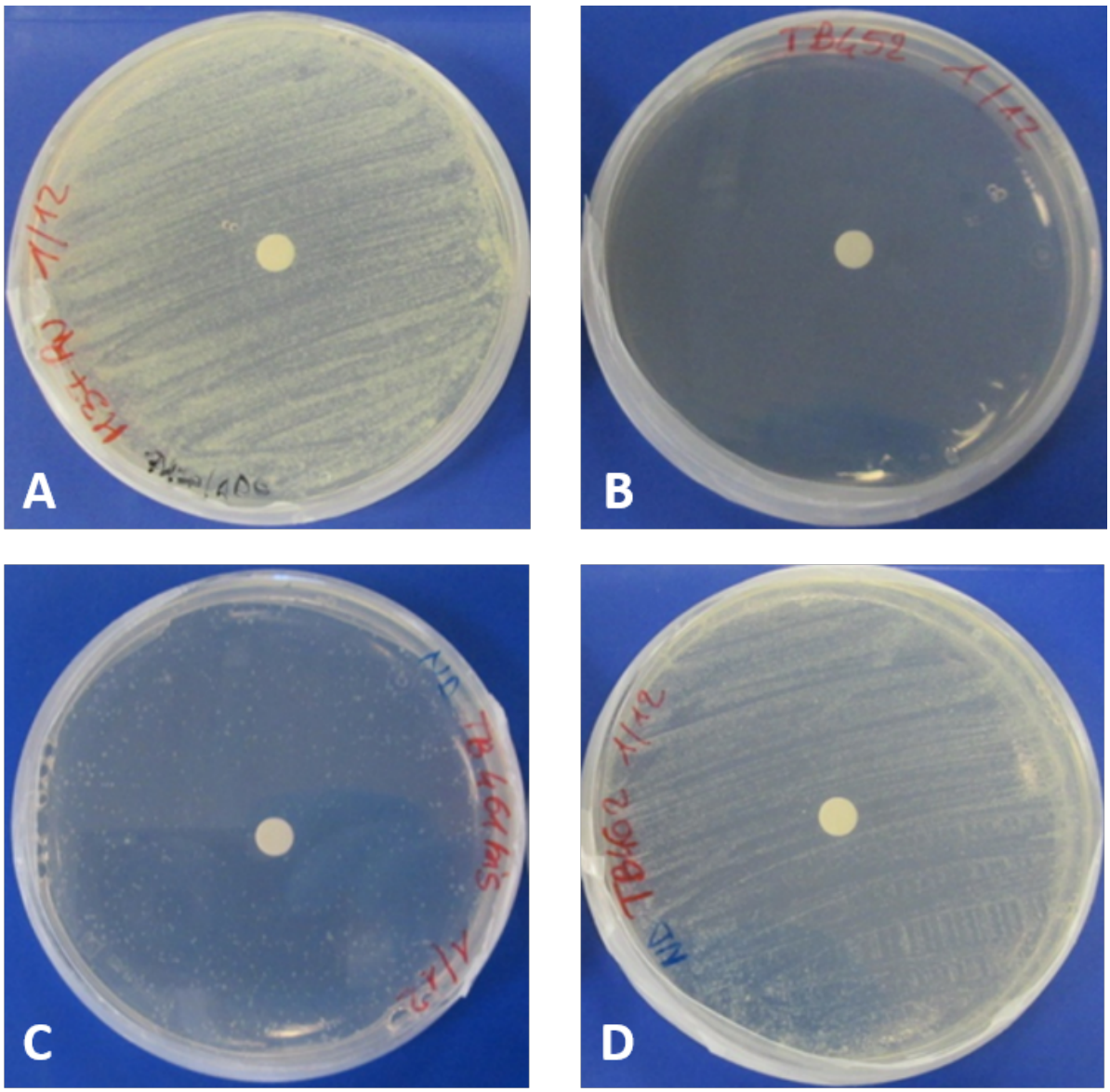

Fig. S1. Rv2466c is involved in coping with diamide stress. One hundred microliters of a bacterial culture growing in early exponential phase (about 3 x 10^6 cfu) were spread on each plate. A paper disk was than placed in the center of the plate and soaked with 10 µl of a 0.5 M diamide stock solution. Plates were incubated at 37°C for 15 days. (A), H37Rv; (B) *sigH* mutant ST49; (C), *rv2466c* mutant TB461b; (D), *rv2466c* complemented strain TB462.

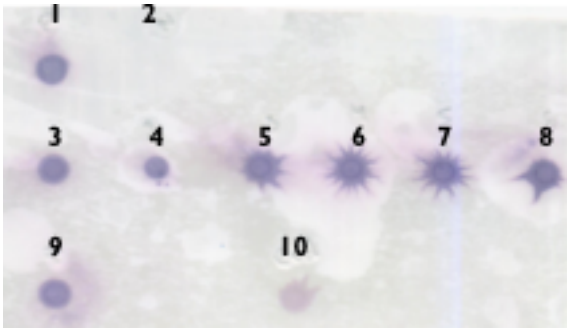

Fig. S2. Rv2466c forms a stable mycothiolated complex. Mycothiolation assessed by dot blot containing anti-mycothiol antibody. As a positive control, the *M. tuberculosis* AhpE enzyme previously described as a target for mycothiolation was used (1). Dot 1, AhpE S-mycothiolation as a positive control (1 min H_2O_2 oxidation); Dot 2, BSA S-mycothiolation as a negative control (1 min H_2O_2 oxidation); Dot 3 to 9, Rv2466c S-mycothiolated in different H_2O_2 exposition time points; respectively 0.5, 1, 1.5, 2, 4, 6 and 10 min; Dot 10, Rv2466c without previous treatment.

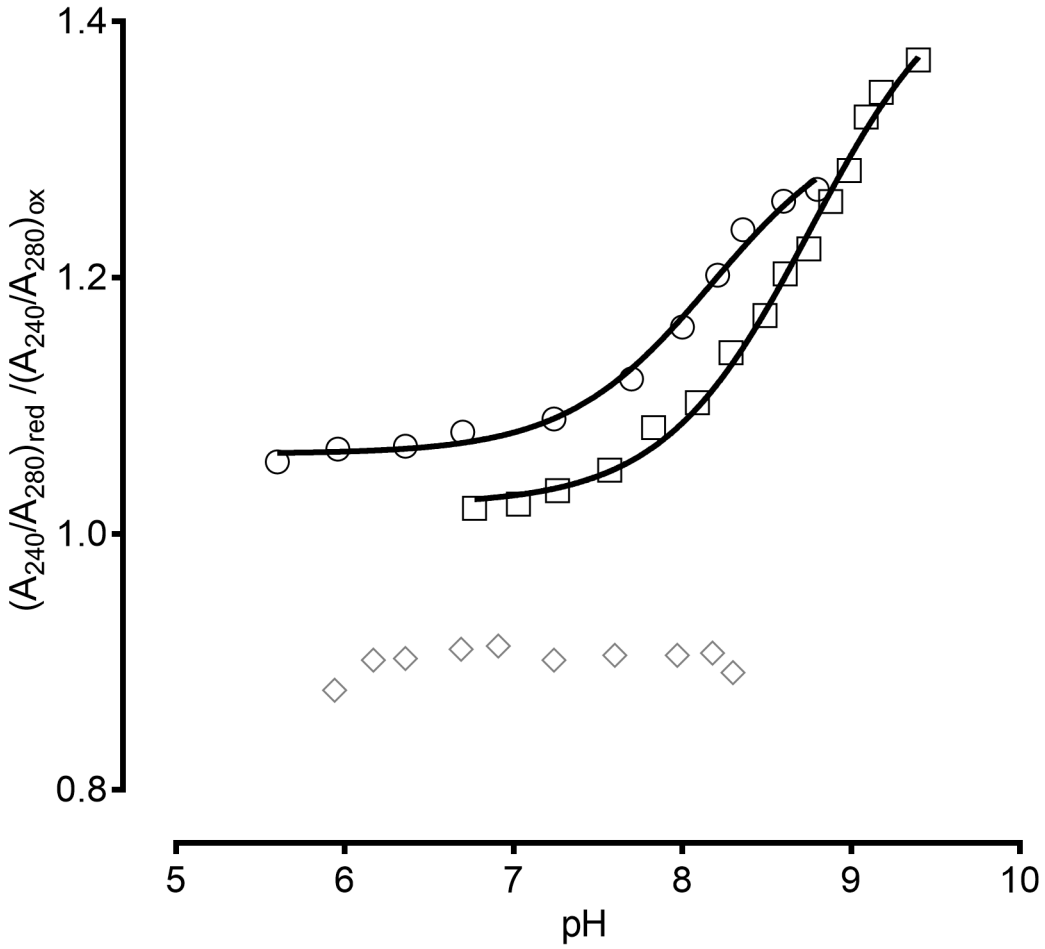

Fig. S3. Cys19 is the nucleophilic cysteine of Rv2466c. The pK_a measurement of the active site cysteines indicates that Cys19 has an unusual pK_a value lower than 6 and that Cys22 is the resolving cysteine with a pK_a of 8.17. Ionized thiol groups (R-SH) extinction coefficient at 240 nm was utilized to measure pK_a values of cysteines of Rv2466c WT (○) and the mutants C22S (◇) and C19S (□). The ratio composed by $240/280_{red}$ and $240/280_{ox}$ in a pH range of 5.8 to 9.5 was fitted with the Henderson-Hasselbalch equation.

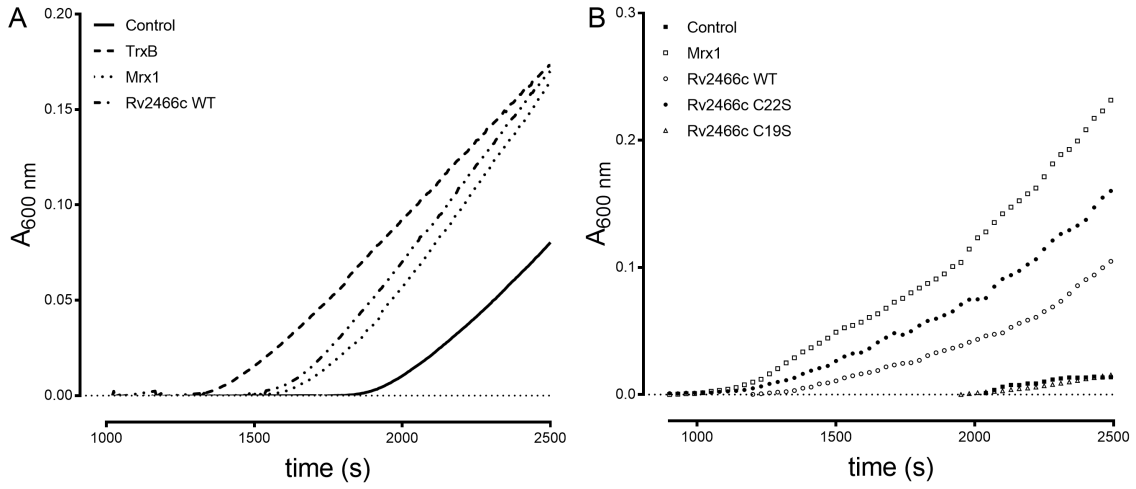

Fig. S4. Reduction of intramolecular disulfide bonds is catalyzed by Rv2466c. Rv2466c is a catalyst for the reduction of insulin intramolecular disulfide bonds. The insulin reduction catalyzed by Rv2466c, TrxB and Mrx1 is shown. (A), Reaction mixtures containing Rv2466c, Mrx1 or TrxB as catalysts and DTT as electron donor. (B), Reaction mixtures containing Rv2466c, Mrx1, C22S or C19S as catalysts and MSH/Mtr/NADPH as electron donor. Reactions were monitored at 600 nm during 2500 s and control measurements were performed in the absence of enzyme.

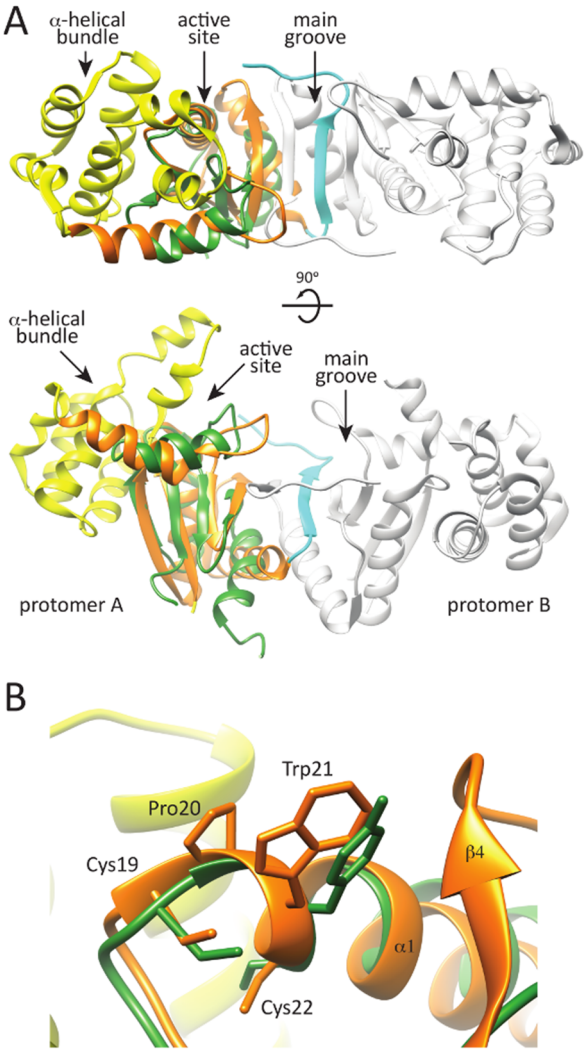

Fig. S5. Rv2466c is structurally similar to MtMrx1. (A). Structural alignment of Rv2466c (α-helical bundle is in yellow, thioredoxin-fold in orange and the swapping β-strand in cyan) and the NMR solution structure of Mrx1 (green, PDB ID 2LQO) (B). Overlay of the CPWC motif of Rv2466c (orange) with the CGYC motif of Mrx1 (green).

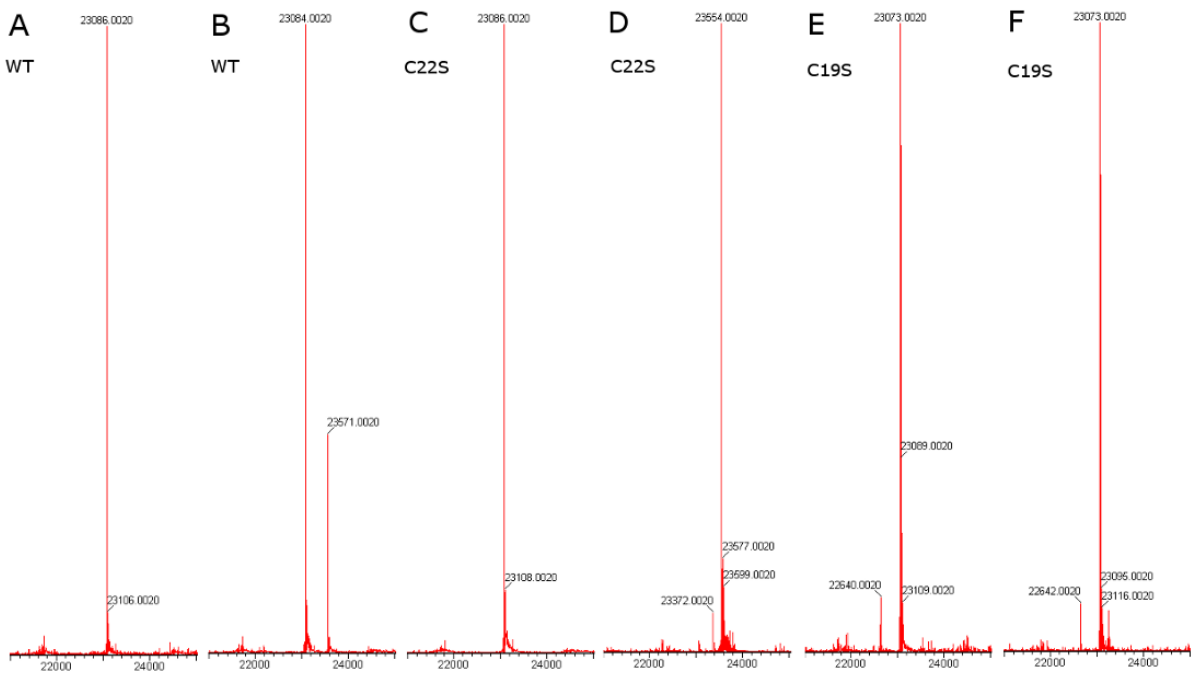

Fig. S6. Liquid chromatography coupled mass spectrometry revealed reaction species. LC-MS was performed by electrospray ionization mass spectrometry on a Micromass Q-Tof micro system coupled to a Waters Breeze analytical HPLC. (A), Apo Rv2466c WT shown a molecular mass of 23086 Da. (B), In the presence of MSH/Mtr/NADPH and TP053 two species could be found, 23084 Da and 23571 Da corresponding to unbound and mycothiolated Rv2466c. (C), Apo Rv2466c C22S revealed a molecular mass of 23086 Da, indicating that C19 is found in a sulfenic acid form (+16 Da). (D), In the presence of MSH/Mtr/NADPH and TP053 two mycothiolated protein species were detected, 23554 Da and 23372 Da (MSH lacking the inositol portion). No apo Rv2466c C22S was detected. (E and F), Apo Rv2466c C19S and in the presence of MSH/Mtr/NADPH and TP053 demonstrated only the molecular mass corresponding to apo enzyme, indicating no mycothiolation of C22 (E and F). A consistent increase of 5 Da was found for every measurement, indicating a systematic variation due to methodological approach and $[H^+]$ from ionization. In the process of His-tag digestion by TEV protease, a Gly residue remains at the N-terminus, increasing the total mass with 57 Da.

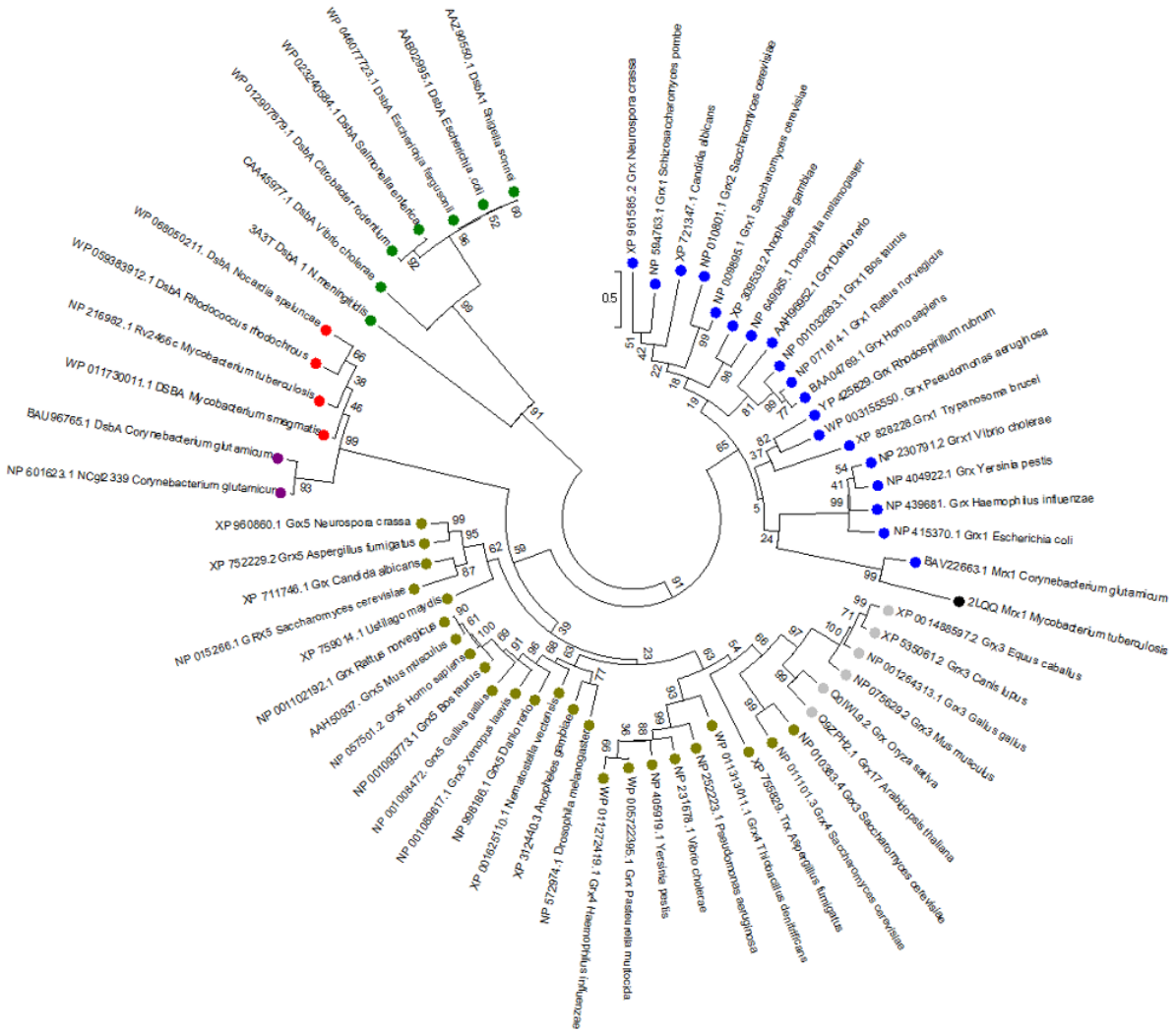

Fig. S7. A phylogenetic reconstruction (phylogram) of mycoredoxins, glutaredoxins and oxidoreductases (DsbA). Color code is as follows: olive = CGFS; gray = three times CGFS; black = CGYC; blue = CPYC; grape = CPFC; red = CPWC and green = CPHC. The final bootstrap values are generated by 2000 replicates. The analysis was performed in Mega 6 software(2).

SUPPLEMENTARY REFERENCES

1. Hugo, M., Van Laer, K., Reyes, A. M., Vertommen, D., Messens, J., Radi, R., and Trujillo, M. (2014) Mycothiol/mycoredoxin 1-dependent reduction of the peroxiredoxin AhpE from *Mycobacterium tuberculosis*. *J Biol Chem* **289**, 5228-5239
2. Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**, 2725-2729