

Supplementary Material

Linking Cytochrome P450 enzymes from *Mycobacterium tuberculosis* to their cognate ferredoxin partners

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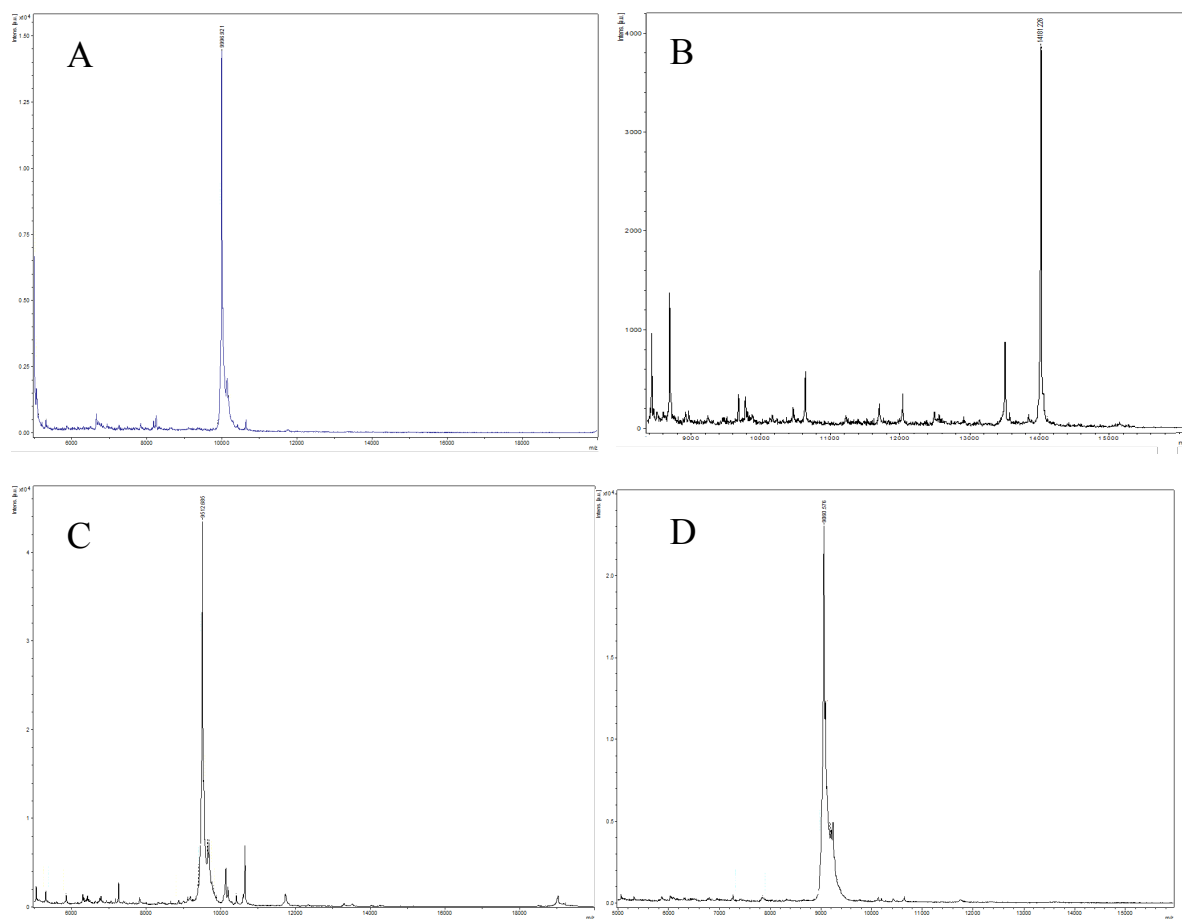


Fig. S1. Identification of recombinant *Mtb* ferredoxins by MALDI-TOF MS analysis. (A) Fdx, approximately 10,000 Da. (B) FdxA, approximately 14,000 Da. (C) FdxD, approximately 9,100 Da. (D) Rv1786/FdxE, approximately 9,500 Da was obtained. Samples were analyzed as described in Experimental Procedures.

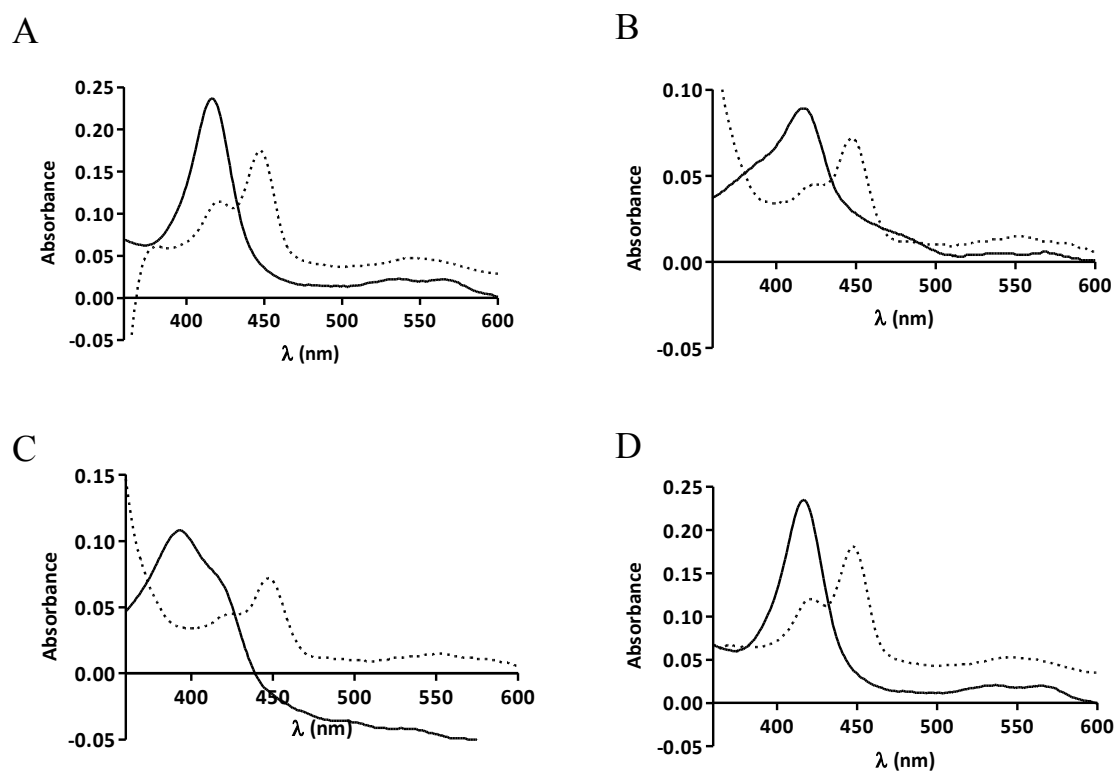


Fig. S2: Characterization of recombinant (A) CYP121A1, (B) CYP124A1, (C) CYP125A1 and (D) CYP142A1. *Black line*, P450 absorbance spectra in the ferric state; and *dotted line*, ferrous CO-complexed state. Samples were analyzed as described in Experimental Procedures.

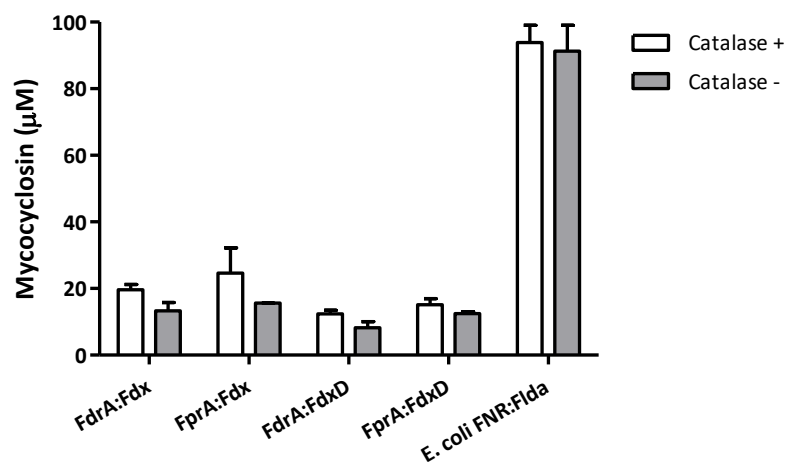


Fig. S3: Mycocyclosin formation catalyzed by CYP121A1 (5 μ M) supported by cognate redox-partners in the presence or absence of catalase (10 μ g/mL). Incubations were conducted in 1:5:10 molar ratio. Error bars represent the variability of duplicates.

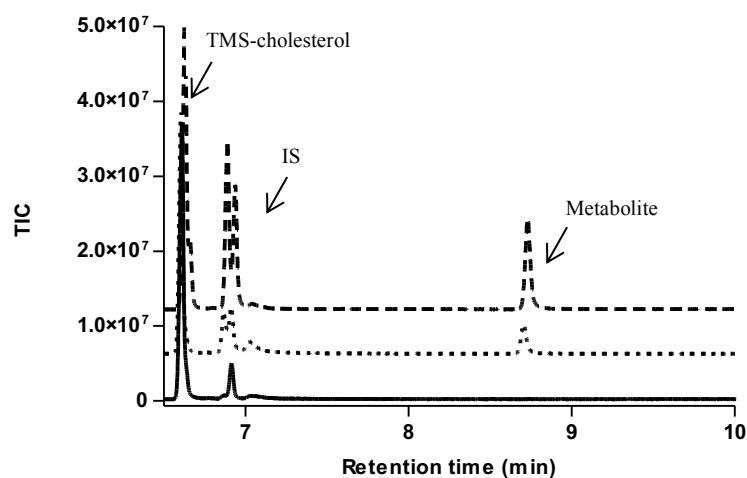
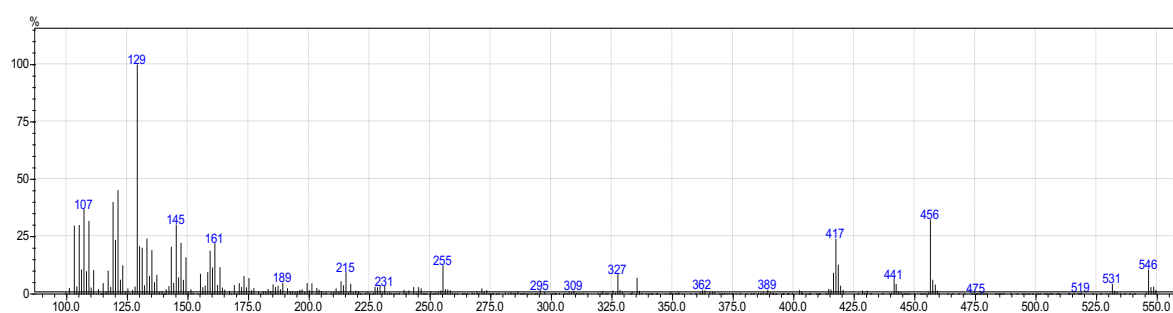
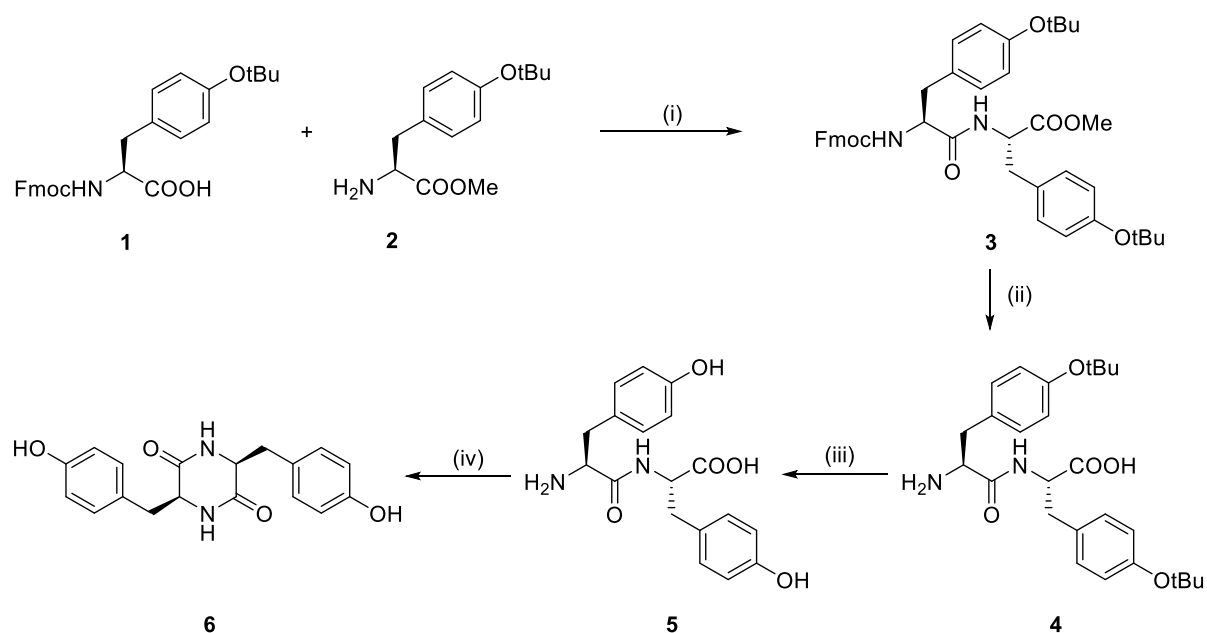
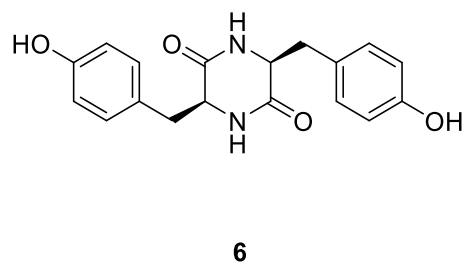
A**B**

Fig. S4: GC-MS separation of the extracts from the CYP142A1 incubations with cholesterol substrate. **(A)** GC-MS chromatogram for the incubation of CYP142A1 supported by; *black line*; no CYP142A1; *dotted line*, CYP142A1:FNR:Flda from *E. coli* in 1:5:10 ratio; and *dashed line*; CYP142A1:FdrA:FdxD from Mtb in 1:5:10 ratio. **(B)** Mass spectrum of the major product from the incubation with CYP142A1-FdrA-FdxD catalytic system following GC separation, showing key peaks at m/z values 417, 456, and 546 that are diagnostic of the methyl ester-TMS ether of the 27-hydroxycholesterol product. Samples were analyzed as described in Experimental Procedures.

A



B



$^1\text{H NMR}$ (500 MHz, DMSO-d_6) δ = 6.69 (d, J = 8.2 Hz, 4H), 6.53 (d, J = 8.2 Hz, 4H), 3.74 – 3.68 (m, 2H), 2.36 (d, J = 3.3 Hz, 6H). $^{13}\text{C NMR}$ (126 MHz, DMSO-d_6) δ = 166.46, 156.19, 130.89, 126.62, 115.15, 55.82, 38.92.

Fig. S5: Schematic overview of cyclo(L-tyr-L-tyr) synthetic pathway. (A) Synthesis of cyclo(L-tyr-L-tyr) (6). (i) EDC-HCl, HOBT, DIPEA, DCM, room temperature, 7.25 h; (ii) LiOH, THF:H₂O, 0 °C, 0.5 h; (iii) formic acid, room temperature, 2 h; (iv) sec-butanol:toluene, 105 °C, 5 h. The Synthesis of Cyclo(L-tyr-L-tyr) 6 was performed according to adapted protocols from Cochrane et al. 2012 (B) NMR Data of the final product 6, cyclo(L-tyr-L-tyr).

Table S1: Sequence alignment of the amino acid sequences of ferredoxins in *Mtb* H37Rv strain genome. Fdx (*Rv0763c*), 69 aa; FdxA (*Rv2007c*), 114 aa; FdxC (*Rv1177*), 108 aa; FdxD (*Rv3503c*), 63 aa; and Rv1786/FdxE, 67 aa.

	Fdx	FdxA	FdxC	FdxD	FdxE
Fdx	100%	15%	17%	32%	29%
FdxA		100%	58%	21%	13%
FdxC			100%	21%	13%
FdxD				100%	32%
FdxE					100%

Abbreviations: aa, amino acid.

Table S2: Kinetic parameters for the DCPIP reductase reaction of FdrA and FprA.

	NADH			NADPH		
	k_{cat} (min ⁻¹)	K_m^{DCPIP} (μM)	K_m^{NADH} (μM)	k_{cat} (min ⁻¹)	K_m^{DCPIP} (μM)	K_m^{NADPH} (μM)
FdrA	33 ± 1	24 ± 1	92 ± 1	NA	NA	NA
FprA	15 ± 1	51 ± 1	85 ± 1	18 ± 1	49 ± 1	1.2 ± 0.1

NA: No activity

Table S3: Chemical assignments of ions identified in the fragmentation patterns from GC-MS analysis of the major metabolite, 27-hydroxycholesterol.

m/z	Description
546	M ⁺ (molecular ion)
531	M ⁺ - 15 (loss methyl group)
456	M ⁺ - 90 (loss TMS group)
441	M ⁺ - (90 + 15) (loss methyl + TMS group)
417	M ⁺ - 129 (loss (CH ₃) ₃ SiOCHCH=CH ₂) (Brooks et al. 1968)
327	M ⁺ - (129 + 90) (loss 129 fragment + TMS group)
255	M ⁺ - (ABCD/androstane skeleton, loss TMS group + sidechain (including TMS group))
129	129 fragment, characteristic for TMS-ethers of 3-hydroxyl, δ^5 sterols (Brooks et al. 1968)

Table S4: Characterization of cholesterol hydroxylation by selected Mtb CYPs supported by surrogate and cognate redox-partners at different molar ratios.

TN				
FdrA	Molar ratio (CYP:FNR:Fd)	CYP124A1	CYP125A1	CYP142A1
FdrA:Fdx	1:1:2	NA	NA	NA
	1:2:5	NA	NA	NA
	1:5:10	NA	NA	NA
FdrA:FdxA	1:1:2	NA	NA	NA
	1:2:5	NA	NA	NA
	1:5:10	NA	NA	NA
FdrA:FdxD	1:1:2	58.3 ± 1.2	33.9 ± 0.9	73.7 ± 2
	1:2:5	57.6 ± 0.9	26.7 ± 2.7	78.7 ± 1.3
	1:5:10	63.7 ± 1.3	65.4 ± 2.0	81.3 ± 1.5
FdrA:FdxE	1:1:2	1.9 ± 0.2	3.7 ± 0.7	13.5 ± 2
	1:2:5	2.2 ± 0.6	15.1 ± 3.8	20.7 ± 2.4
	1:5:10	6.3 ± 1.3	15.8 ± 3.1	30.3 ± 3.1
FprA	Molar ratio (CYP:FNR:Fd)	CYP124A1	CYP125A1	CYP142A1
FprA:Fdx	1:1:2	NA	NA	NA
	1:2:5	NA	NA	NA
	1:5:10	NA	NA	NA
FprA:FdxA	1:1:2	NA	NA	NA
	1:2:5	NA	NA	NA
	1:5:10	NA	NA	NA
FprA:FdxD	1:1:2	2.2 ± 0.8	55.8 ± 0.5	68.2 ± 1.3
	1:2:5	13.0 ± 1.5	64.8 ± 2.7	82.2 ± 0.68
	1:5:10	28.1 ± 0.6	62.9 ± 1.3	75.65 ± 1.2
FprA:FdxE	1:1:2	NA	NA	7.3 ± 0.7
	1:2:5	3.5 ± 0.1	2 ± 1.2	8.5 ± 2.1
	1:5:10	3 ± 0.1	5.5 ± 0.7	5.1 ± 1.0
	Molar ratio (CYP:FNR:Flda)	CYP124A1	CYP125A1	CYP142A1
<i>E. coli</i> FNR/Flda	1:1:2	25 ± 2.0	69.1 ± 1.8	75.9 ± 2.5
	1:2:5	47.2 ± 2.1	78.4 ± 1.4	77 ± 1.9
	1:5:10	61.3 ± 1.7	73.7 ± 0.8	76.3 ± 3.2

TN: Turnover (concentration metabolite over IS (μM)/concentration CYP (μM))

NA: No activity

Table S5: Oligonucleotide primers used in plasmid construction.

Direction	Primer sequence	Restriction site
F-FprA	5'- catatg cggtccctattacatcgt-3'	<i>NdeI</i>
R-FprA	5'- aagctt tcagccgagcccaatcc-3'	<i>HindIII</i>
F-FdrA	5'- catatg aacgcacacgtgacca-3'	<i>NdeI</i>
R-FdrA	5'- aagctt ctaggcctgagtttggtct-3'	<i>HindIII</i>
F-Fdx	5'- gctagc atgggctatcgagtcgaag-3'	<i>NheI</i>
R-Fdx	5'- aagctt ttactctcccgtttctcgg-3'	<i>HindIII</i>
F-FdxA	5'- catatg gtgacatgatgatcggtta-3'	<i>NdeI</i>
R-FdxA	5'- aagctt ctaagggcactccaccg-3'	<i>HindIII</i>
F-FdxC	5'- catatg gtgacgtacacgatcgcc-3'	<i>NdeI</i>
R-FdxC	5'- aagctt tcaggcgtcctcgctct-3'	<i>HindIII</i>
F-FdxD	5'- catatg gtgcgggtgatcggtga-3'	<i>NdeI</i>
R-FdxD	5'- aagctt ctactcaccacgcgacaa-3'	<i>HindIII</i>
F-Rv1786/FdxE	5'- catatg gtgaaagtcgctcgcgtc-3'	<i>NdeI</i>
R-Rv1786/FdxE	5'- aagctt tcagtcgcgcgtcgcct-3'	<i>HindIII</i>
F-CYP121A1	5'- catatg accgcgaccgttctg-3'	<i>NdeI</i>
R-CYP121A1	5'- aagctt atcctaccagagcaccgg-3'	<i>HindIII</i>
F-CYP124A1	5'- catatg gggctcaacacggc-3'	<i>NdeI</i>
R-CYP124A1	5'- aagctt tcaggaccacgtaactgg-3'	<i>HindIII</i>
F-CYP125A1	5'- catatg gtgtcgtggaatcaccagt-3'	<i>NdeI</i>
R-CYP125A1	5'- tctag attagtgagcaaccgggcat-3'	<i>XbaI</i>
F-CYP142A1	5'- catatg actgaagctccggac-3'	<i>NdeI</i>
R-CYP142A1	5'- aagctt tcagcccagcggcg-3'	<i>HindIII</i>

In bold text, the restriction sites are depicted.

References

- Brooks CJ, Horning EC, Young JS (1968) Characterization of sterols by gas chromatography-mass spectrometry of the trimethylsilyl ethers. *Lipids* 3:391-405. doi: 10.1007/BF02531277
- Cochrane JR, White JM, Wille U, Hutton CA (2012) Total Synthesis of Mycocyclosin. *Organic Letters* 14:2402- 2405. doi: 10.1021/ol300831t