Supplemental Information

Supplemental Tables and Figures

Supplemental Table 1: Kinetic characterization of wild-type Rv0045c against fluorogenic ester substrates (Figure 1A, 1B).

Substrate	k_{cat} (10^{-3} s^{-1})[a]	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
1	0.01 ± 0.001	2.2 ± 0.2	5.0 ± 0.4
2	0.50 ± 0.06	4.0 ± 2.1	120 ± 70
3	0.36 ± 0.02	1.0 ± 0.2	350 ± 90
4	0.10 ± 0.02	2.3 ± 0.1	45 ± 2
5	0.032 ± 0.001	4.4 ± 0.4	7.3 ± 0.7
6	11 ± 1	1.6 ± 0.5	6600 ± 2100
7	1.1 ± 0.1	3.7 ± 1.9	290 ± 150
8	22 ± 1	8.9 ± 1.3	2500 ± 400
9	0.011 ± 0.001	3.3 ± 0.3	3.4 ± 0.3
10	0.006 ± 0.003	0.52 ± 0.08	13 ± 2
11	0.16 ± 0.01	6.8 ± 0.5	24 ± 2
12	0.065 ± 0.003	3.59 ± 0.64	18 ± 3
13	0.40 ± 0.02	8.7 ± 1.1	48 ± 6
14	0.0055 ± 0.0004	1.4 ± 0.3	3.9 ± 1
15	ND[b]	ND	ND
16	0.026 ± 0.002	2.7 ± 0.5	9.8 ± 2.1
17	0.050 ± 0.001	5.6 ± 0.2	8.9 ± 0.3
18	0.011 ± 0.001	4.1 ± 0.2	2.6 ± 0.2
19	0.0020 ± 0.0001	8.5 ±1.6	270 ± 50
20	ND	ND	ND
21	ND	ND	ND

[a]Kinetic constants for fluorogenic enzyme substrates (Figure 1) were determined by measuring the increase in fluorogenic enzyme substrate fluorescence over time. Data were fitted to a standard Michaelis-Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times and the values are given ± SE.

[b] Not determinable. Values were below the detection limit for the assay, determined based on the catalytic activity of S154A Rv0045c.

Supplemental Table 2: Biochemical characterization of Rv0045c and Rv0045c loop variants.

Rv0045c loop variant	k_{cat} (10^{-3} s^{-1}) [a]	K_m (µM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	T_m (°C)[b]
WT	11 ± 1	1.6 ± 0.5	6600 ± 2100	43 ± 0.5
Q194A	0.75 ± 0.08	0.27 ± 0.16	2800 ± 1700	42 ± 0.5
R195A	2.1 ± 0.1	0.34 ± 0.11	6200 ± 2100	42 ± 0.5
G196A	7.2 ± 0.4	0.82 ± 0.24	8700 ± 2600	42 ± 0.5
T197A	3.0 ± 0.1	0.72 ± 0.13	4100 ± 800	43 ± 0.5
V198A	0.45 ± 0.02	0.62 ± 0.16	730 ± 190	43 ± 0.5
L200A	0.29 ± 0.02	0.29 ± 0.09	1000 ± 300	42 ± 0.5
M201A	5.9 ± 0.5	0.87 ± 0.39	6800 ± 3100	42 ± 0.5
H202A	1.1 ± 0.1	0.35 ± 0.11	3200 ± 1000	43 ± 0.5
G203A	0.76 ± 0.04	0.36 ± 0.10	2100 ± 600	43 ± 0.5
E204A	4.2 ± 0.2	0.61 ± 0.12	6900 ± 1400	40 ± 0.5

[a]Kinetic constants were determined by measuring the increase in fluorescence over time for Rv0045c or its loop variants against substrate **6** (*21*). Data were fitted to a standard Michaelis-Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times and the values are given ± SE.

[b]Values for T_m were determined by following the change in Sypro Orange fluorescence with increasing temperature. Melting curves were repeated three times for each variant and the T_m values reported ± SE.

Supplemental Table 3: Substitutions among hydrolases similar to Rv0045c.

Part 1: Substitutions in substrate binding and catalytic residues among hydrolases similar to Rv0045c.

Organism	**90**	**92**	**153**	**154**	**155**	**178**	**184**	**187**	**252**	**255**	**282**	**309**	**% Identity**
M. tuberculosis H37Rv	G	Q	M	S	L	D	L	H	I	F	F	H	-
M. marinum													79
M. ulcerans													78
M. avium													77
M. vanbaalenii							P			G			66
M. rhodesiae							P		F	G			65
M. smegmatis							P		F	G			63
G. polyisoprenivorans							P	A		A			57
R. jostii							H		L	G			52
N. aromatocivorans			A				-	L	D	S	I		25
G. proteobacterium			A		M		-	L	S	S	V		25
A. xylosoxidans	I	G	H		M		-	F	P	A	Y		25
R. slithyformis			A		M		-	A	T	Q	V		24
B. bacteriovorus	L	G	H		M		-	N	V	G	E		22
H. influenzae	L	G	H		M		-	Y	L	L	Y		20
V. cholerae	L	G	H		M		-	Y	V	L	Y		19
E. coli	L	G	H		M		-	Y	V	L	Y		17

Part 2: Substitutions in flexible loop among hydrolases similar to Rv0045c.

Organism	**194**	**195**	**196**	**197**	**198**	**200**	**201**	**202**	**203**	**% Identity**
M. tuberculosis H37Rv	Q	R	G	T	V	L	M	H	G	-
M. marinum										79
M. ulcerans										78
M. avium		Q								77
M. vanbaalenii		K					V	Q		66
M. rhodesiae		K					V	Q		65
M. smegmatis		M					V	R	E	63
G. polyisoprenivorans	A	R					I	R		57
R. jostii					T		I	N		52
N. aromatocivorans	V	E	R	I		F		T	A	25
G. proteobacterium	V		R	I	I	F		R	R	25
A. xylosoxidans			E	L	R	T	P	A	S	25
R. slithyformis	I	E	R	I	F	F		S	A	24
B. bacteriovorus	E	Y	L	L	N	V	P	S	P	22
H. influenzae	N	G	L	F	A	K	N	A	K	20
V. cholerae	N	G	L	R	A	I	E	E	Q	19
E. coli	A	A	I	N	A	S	E	S	D	17

[a]The amino acid sequence of Rv0045c was aligned using ClustalW [45]. The amino acid numbering corresponds to the amino acid numbering in Rv0045c. The sequences used in the alignment were from *Mycobacterium tuberculosis* H37Rv (NP_214559.1),

Supplemental Table 4: Biochemical characterization of Rv0045c and Rv0045c active site variants.

Rv0045c active site variant	k_{cat} (10^{-3} s^{-1}) [a]	K_m (µM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	T_m (°C)[b]
WT	11 ± 1	1.6 ± 0.5	6600 ± 2100	43 ± 0.5
G90A	7.0 ± 0.3	0.95 ± 0.21	7400 ± 1700	46 ± 0.5
Q92A	1.5 ± 0.2	0.64 ± 0.28	2300 ± 1100	38 ± 0.5
M153A	1.7 ± 0.1	0.50 ± 0.15	3500 ± 1100	46 ± 0.5
S154A	0.020 ± 0.001	0.32 ± 0.10	63 ± 19	44 ± 0.5
L155A	1.0 ± 0.5	0.51 ± 0.11	2000 ± 400	37 ± 0.5
D178A	0.20 ± 0.02	0.93 ± 0.43	210 ± 100	36 ± 0.5
L184A	0.64 ± 0.03	0.49 ± 0.08	1300 ± 200	45 ± 0.5
H187A	16 ± 1	1.6 ± 0.3	9900 ± 1900	42 ± 0.5
I252A	4.8 ± 0.3	0.51 ± 0.16	9500 ± 2900	41 ± 0.5
F255A	6.7 ± 0.5	0.14 ± 0.06	4900 ± 2300	41 ± 0.5
F282A	0.19 ± 0.06	0.40 ± 0.06	490 ± 90	40 ± 0.5
H309A	0.062 ± 0.003	0.25 ± 0.11	250 ± 110	38 ± 0.5
G90A H187A	22 ± 2	6.8 ± 1.3	3300 ± 600	45 ± 0.5

[a]Kinetic constants were determined by measuring the increase in fluorescence over time for Rv0045c or its loop variants against substrate **6** (*21*). Data were fitted to a standard Michaelis-Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times and the values are given ± SE.

[b]Values for T_m were determined by following the change in Sypro Orange fluorescence with increasing temperature. Melting curves were repeated three times for each variant and the T_m values reported ± SE.

Supplemental Table 5: Biochemical characterization of His187 Rv0045c variants against different fluorogenic substrates.

Rv0045c His187 variant	Fluorogenic Substrate (k_{cat}/K_m ($M^{-1}s^{-1}$))				
	3	**6**	**13**	**14**	**18**
WT	100 ± 40	6600 ± 2100	4.9 ± 2.0	12 ± 1	6.7 ± 0.8
G90A	250 ± 100	7400 ± 1700	27 ± 6.6	17 ± 2	25 ± 5
H187A	650 ± 80	9900 ± 1900	39 ± 8	17 ± 4	9.0 ± 0.5
H187Y	2100 ± 800	53000 ± 6000	110 ± 10	22 ± 4	22 ± 4
G90A/H187A	20 ± 2	3300 ± 600	3.6 ± 0.4	2.6 ± 0.1	4.6 ± 0.8

[a]Kinetic constants were determined by measuring the increase in fluorescence over time for Rv0045c or its catalytic variants against various substrates (Figure 1) (*21*). Data were fitted to a standard Michaelis-Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times and the values are given ± SE.

Supplemental Table 6: Biochemical characterization of His187 Rv0045c variants.

Rv0045c His187 variant	k_{cat} (10^{-3} s^{-1}) [a]	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	T_m (°C)[b]
WT	11 ± 1	1.6 ± 0.5	6600 ± 2100	43 ± 0.5
H187D	3.9 ± 0.2	1.4 ± 0.3	2900 ± 600	39 ± 0.5
H187F	6.8 ± 0.2	3.2 ± 0.3	2200 ± 200	41 ± 0.5
H187G	13 ± 1	1.4 ± 0.2	8200 ± 2200	39 ± 0.5
H187K	6.9 ± 0.3	2.2 ± 0.5	3200 ± 700	39 ± 0.5
H187N	2.0 ± 0.1	1.0 ± 0.1	1900 ± 300	40.5 ± 0.8
H187P	0.90 ± 0.01	0.60 ± 0.43	1400 ± 1000	41 ± 0.5
H187S	5.4 ± 0.2	0.60 ± 0.10	9100 ± 1500	39 ± 0.5
H187V	10 ± 1	1.6 ± 0.8	6200 ± 2900	40 ± 0.5
H187W	14 ± 1	6.0 ± 1.7	2400 ± 700	43 ± 0.5
H187Y	11 ± 1	0.21 ± 0.03	53000 ± 6000	41.5 ± 0.8

[a]Kinetic constants were determined by measuring the increase in fluorescence over time for Rv0045c or its loop variants against substrate **6** (*21*). Data were fitted to a standard Michaelis-Menten equation to determine the values for k_{cat}, K_M, and k_{cat}/K_M. Kinetic measurements for each substrate were repeated three times and the values are given ± SE.

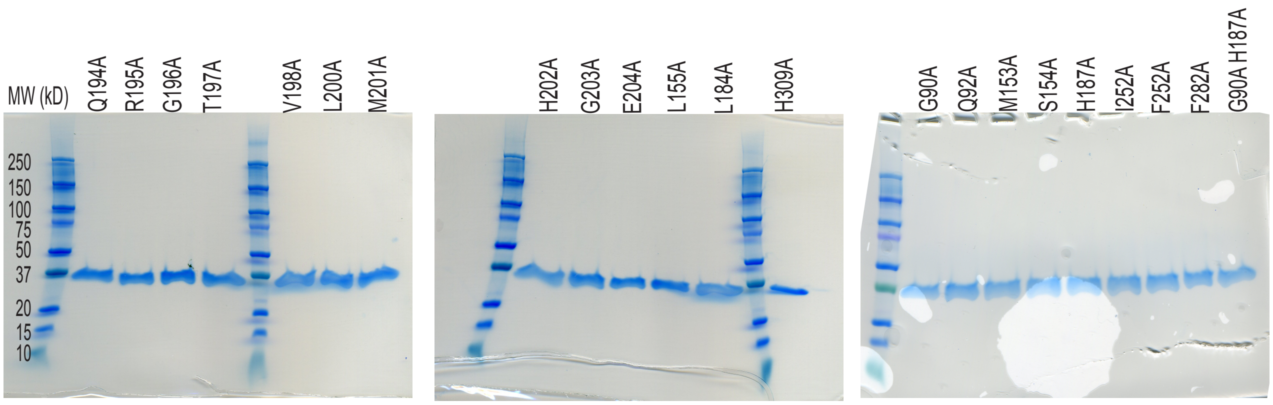
[b]Values for T_m were determined by following the change in Sypro Orange fluorescence with increasing temperature. Melting curves were repeated three times for each variant and the T_m values reported ± SE.

Supplemental Table 7: PCR primers used for cloning and site-directed mutagenesis.[a]

Rv0045c Variant	Primer nucleotide sequence
G90A	5'-GGT GAT CTT TCT GCA CGG CGC CGG ACA GAA CGC CCA TAC C-3'
Q92A	5'-CTG CAC GGC GGC GGA GCG AAC GCC CAT ACC TGG G-3'
M153A	5'-GCC GAA TTC GTG GTC GGC GCG TCG CTG GGC GGG TTG-3'
S154A	5'-GCC GAA TTC GTG GTC GGC ATG GCG CTG GGC GGG TTG ACT GCG-3'
L155A	5'-CGT GGT CGG CAT GTC GGC GGC GGG CGG GTT GAC TGC G-3'
D178A	5'- CGG CGA ACT CGT TCT CGT CGC CGT CAC CCC GTC GGC ATT GC-3'
L184A	5'- CGT CAC CCC GTC GGC AGC GCA ACG GCA CGC CGA GC-3'
H187A	5'- CCG TCG GCA TTG CAA CGG GCC GCC GAG CTG ACC GCC G-3'
H187D	5'-CCG TCG GCA TTG CAA CGG GAC GCC GAG CTG ACC GCC G-3'
H187F	5'-CCG TCG GCA TTG CAA CGG TTC GCC GAG CTG ACC GCC G-3'
H187G	5'-CCG TCG GCA TTG CAA CGG GGC GCC GAG CTG ACC GCC G-3'
H187K	5'-CCG TCG GCA TTG CAA CGG AAG GCC GAG CTG ACC GCC G-3'
H187N	5'- CCG TCG GCA TTG CAA CGG AAC GCC GAG CTG ACC GCC G-3'
H187P	5'-CCG TCG GCA TTG CAA CGG CCC GCC GAG CTG ACC GCC G-3'
H187S	5'-CCG TCG GCA TTG CAA CGG AGC GCC GAG CTG ACC GCC G-3'
H187V	5'-CCG TCG GCA TTG CAA CGG GTC GCC GAG CTG ACC GCC G-3'
H187W	5'- CCG TCG GCA TTG CAA CGG TGG GCC GAG CTG ACC GCC G-3'
H187Y	5'-CCG TCG GCA TTG CAA CGG TAT GCC GAG CTG ACC GCC G-3'
Q194A	5'-CCG AGC TGA CCG AGG CGC GCG GCA CGG TGG CG-3'
R195A	5'-GAG CTG ACC GCC GAG CAG GCC GGC ACG GTG GCG C-3'
G196A	5'-ACC GCC GAG CAG CGC GCC ACG GTG GC-3'
T197A	5'-GCC GAG CAG CGA GGT GCA GTG GCG CTG ATG CAC GG-3'
V198A	5'-CGC GGC ACG GCT GCG CTG ATG CAC GGC G-3'
L200A	5'-GGC ACG GTG GCG GCA ATG CAC GGC GAG CGG-3'
M201A	5'-GCG GCA CGG TGG CGC TGG CAC ACG GC-3'
H202A	5'-GCA CGG TGG CGC TGA TGG CGG GCG AGC GGG AAT TCC-3'
G203A	5'-GGT GGC GCT GAT GCA CGC CGA GCG GGA ATT CCC C-3'
E204A	5'-GCG CTG ATG CAC GGC GCG CGG GAA TTC CCC-3'
I252A	5'- GGG TGT GGC GCT ATG ACG CGG CCC GCA CGT TCG GAG ATT TCG C-3'
F255A	5'- GAC GCG ATC CGC ACG GCC GGA GAT TTC GC-3'
F282A	5'- GCG CGG CGG CTC GTC GGG CGC AGT CAC CGA CCA GGA CAC CGC-3'
H309A	5'- CGT CGA GAA GTC AGG CGC CTC GGT GCA AAG TGA CC-3'

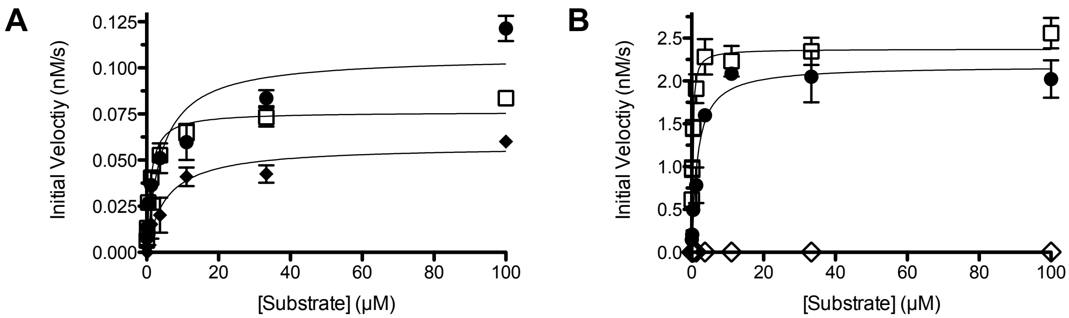
[a]Primers for mutagenesis are one of the two complementary primers used in the mutagenesis reaction. Mutagenic PCR reactions were subjected to the following thermal cycle using a BIO-RAD MyCycler™: 1) initial denaturation at 95°C for 30 s, 2) denaturation at 95°C for 30 s, 3) annealing at temperatures between 52 – 60°C for 60 s, 4) extension at 68°C for 14 min. Steps 2-4 were repeated 16 times.

Supplemental Figure 1



Supplemental Figure 1: Protein purification of variants of Rv0045c. Each Rv0045c variant was constructed and purified as described in the Experimental Section. The expected molecular weight of Rv0045c is 35.5 kDa. Each variant (2 µg) was loaded onto the 4-20% Tris-glycine gel and run at 150 V for 45 minutes. The molecular weight was confirmed by comparison to the Kaleidoscope prestained protein standard (Bio-rad laboratories).

Supplemental Figure 2



Supplemental Figure 2: Kinetic activity of Rv0045c. A) Kinetic activity of wild-type Rv0045c against substrates 2 (closed circles), 3 (open squares), and 4 (closed diamonds). All measurements were completed in triplicate and shown ± SE. Data were fitted to the Michaelis-Menten equation using Graphpad Prism 5.0. B) Kinetic activity of Rv0045c variants. The kinetic activity of S154A (open diamonds), H187A (closed circles), and H187Y (open squares) were determined and plotted the same as part A.