

Supplementary Materials for

**Identification and evolution of a natural TetR protein based on molecular docking and development of a fluorescence polarization assay for multi-detection of 10 tetracyclines in milk**

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**Table S1.** The nine unique peptide fragments of the natural TetR protein.

No.	Amino Acid Sequence	Length	Mass	N-Term Cleavage Window	C-Term Cleavage Window
1	ALLDALAIEMLDR	13	1442.78	YWHVKNKRALLDALAI	LAIEMLDRHHTHFCPL
2	CALLSHR	7	855.4385	RNNAKSFRCALLSHRD	RCALLSHRDGAKVHLG
3	CALLSHRDGAK	11	1226.619	RNNAKSFRCALLSHRD	LSHRDGAKVHLGTRPT
4	EERETPTTDSMPPLLR	16	1870.91	DQEHQVAKEERETPTT	DSMPPLLRQAIELFDH
5	ETPTTDSMPPLLR	13	1456.723	HQVAKEERETPTTDSM	DSMPPLLRQAIELFDH
6	LGVEQPTLYWHVK	13	1568.835	TTRKLAQKLGVEQPTL	PTLYWHVKNKRALLDA
7	LGVEQPTLYWHVKNKR	16	1967.074	TTRKLAQKLGVEQPTL	YWHVKNKRALLDALAI
8	SFRCALLSHR	10	1245.64	DFLRNNAKSFRCALLS	RCALLSHRDGAKVHLG
9	SFRCALLSHRDGAK	14	1616.821	DFLRNNAKSFRCALLS	LSHRDGAKVHLGTRPT

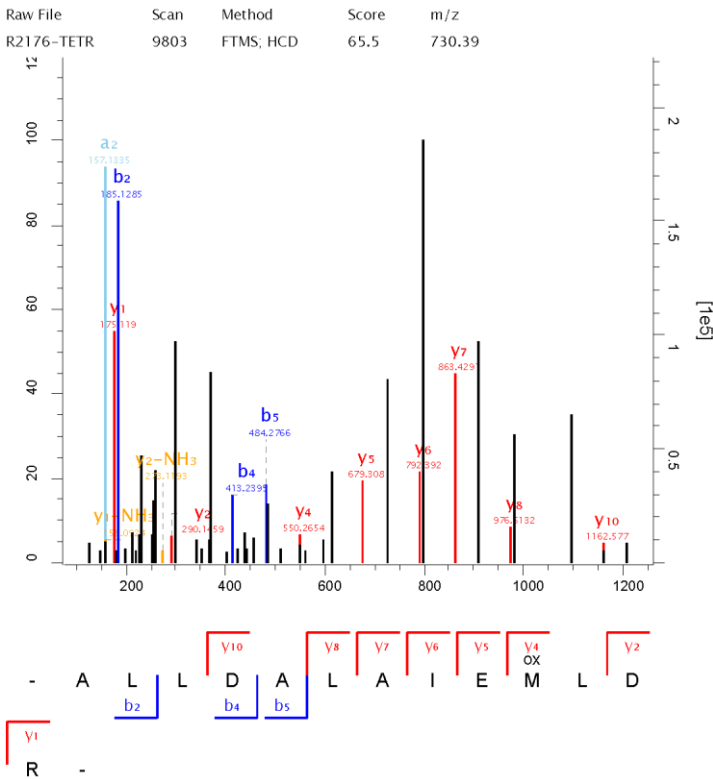
**Table S2.** Affinity parameters of the natural TetR and the mutant for the 10 TCs.

No.	Receptor	Ligand	Ka (1/Ms)	Kd (1/s)	KD (M)	KA (Log2(KD))
1	TetR	Tetracycline	2.03E+02	1.56E-03	7.69E-06	16.988
2	TetR	Chlortetracycline	2.97E+03	1.48E-02	4.99E-06	17.612
3	TetR	Minocycline	4.54E+03	5.07E-04	1.12E-07	23.093
4	TetR	Tigecycline	1.01E+03	1.04E-05	1.04E-08	26.525
5	TetR	Doxycycline	5.34E+04	1.13E-03	2.11E-08	25.496
6	TetR	Demecycline	2.77E+03	2.06E-04	7.44E-08	23.680
7	TetR	Metacycline	1.44E+03	1.60E-04	1.11E-07	23.101
8	TetR	Lymecycline	3.17E+03	1.16E-03	3.66E-07	21.380
9	TetR	Oxytetracycline	2.06E+03	1.99E-03	9.65E-07	19.983
10	TetR	Sancycline	2.14E+03	1.27E-04	5.95E-08	24.003
11	TetR+Mg <sup>2+</sup>	Tetracycline	2.78E+02	1.33E-03	4.79E-06	17.672
12	TetR+Mg <sup>2+</sup>	Chlortetracycline	4.43E+03	1.13E-02	2.56E-06	18.578
13	TetR+Mg <sup>2+</sup>	Minocycline	7.27E+03	5.11E-04	7.03E-08	23.763
14	TetR+Mg <sup>2+</sup>	Tigecycline	6.33E+02	1.78E-05	2.82E-08	25.079
15	TetR+Mg <sup>2+</sup>	Doxycycline	4.39E+04	1.80E-03	4.11E-08	24.538
16	TetR+Mg <sup>2+</sup>	Demecycline	4.61E+03	1.27E-04	2.76E-08	25.110
17	TetR+Mg <sup>2+</sup>	Metacycline	1.33E+03	1.62E-04	1.22E-07	22.961
18	TetR+Mg <sup>2+</sup>	Lymecycline	4.59E+03	1.21E-03	2.63E-07	21.856
19	TetR+Mg <sup>2+</sup>	Oxytetracycline	3.52E+03	1.44E-03	4.09E-07	21.221
20	TetR+Mg <sup>2+</sup>	Sancycline	2.31E+03	1.62E-04	7.02E-08	23.764
21	TetR mutant	Tetracycline	2.03E+02	1.56E-04	7.68E-07	20.312
22	TetR mutant	Chlortetracycline	5.05E+04	8.22E-04	1.63E-08	25.871
23	TetR mutant	Minocycline	1.36E+04	4.23E-05	3.10E-09	28.265
24	TetR mutant	Tigecycline	9.09E+03	9.45E-07	1.04E-10	33.163
25	TetR mutant	Doxycycline	2.67E+05	6.65E-05	2.49E-10	31.903
26	TetR mutant	Demecycline	5.54E+03	1.72E-05	3.10E-09	28.265
27	TetR mutant	Metacycline	1.44E+03	5.71E-06	3.97E-09	27.908
28	TetR mutant	Lymecycline	2.85E+04	7.25E-05	2.54E-09	28.553
29	TetR mutant	Oxytetracycline	4.53E+04	7.96E-05	1.76E-09	29.082
30	TetR mutant	Sancycline	1.07E+04	6.35E-06	5.93E-10	30.651
31	TetR mutant+Mg <sup>2+</sup>	Tetracycline	1.95E+03	1.21E-04	6.21E-08	23.941
32	TetR mutant+Mg <sup>2+</sup>	Chlortetracycline	4.43E+04	4.04E-04	9.11E-09	26.710
33	TetR mutant+Mg <sup>2+</sup>	Minocycline	1.67E+05	3.19E-05	1.91E-10	32.286
34	TetR mutant+Mg <sup>2+</sup>	Tigecycline	1.71E+04	1.78E-06	1.04E-10	33.163
35	TetR mutant+Mg <sup>2+</sup>	Doxycycline	1.76E+05	1.13E-04	6.41E-10	30.539
36	TetR mutant+Mg <sup>2+</sup>	Demecycline	5.07E+04	4.70E-06	9.28E-11	33.327
37	TetR mutant+Mg <sup>2+</sup>	Metacycline	1.86E+04	5.59E-06	3.00E-10	31.634
38	TetR mutant+Mg <sup>2+</sup>	Lymecycline	1.01E+05	1.10E-04	1.09E-09	29.773
39	TetR mutant+Mg <sup>2+</sup>	Oxytetracycline	8.10E+04	1.20E-04	1.48E-09	29.332
40	TetR mutant+Mg <sup>2+</sup>	Sancycline	1.39E+04	5.79E-06	4.17E-10	31.159

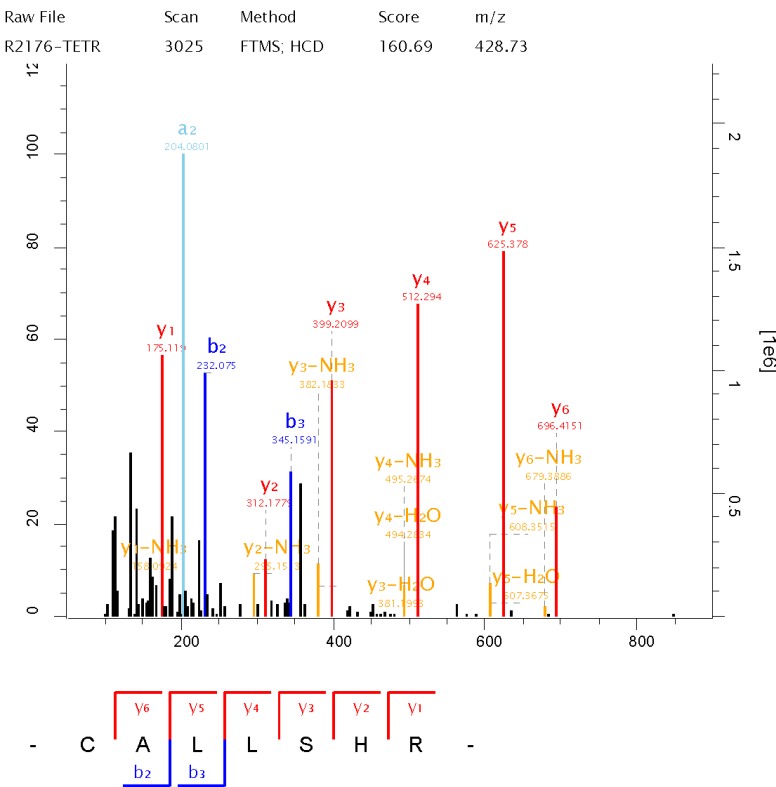
Ka, association constant; Kd, dissociation constant; KD = Kd/Ka, equilibrium dissociation constant; KA = Abs (Log2(KD)), absolute affinity constant.

**Figure S1.** The mass spectrometry results of the nine unique peptides (the number sequence was consistent with that of in Table S1).

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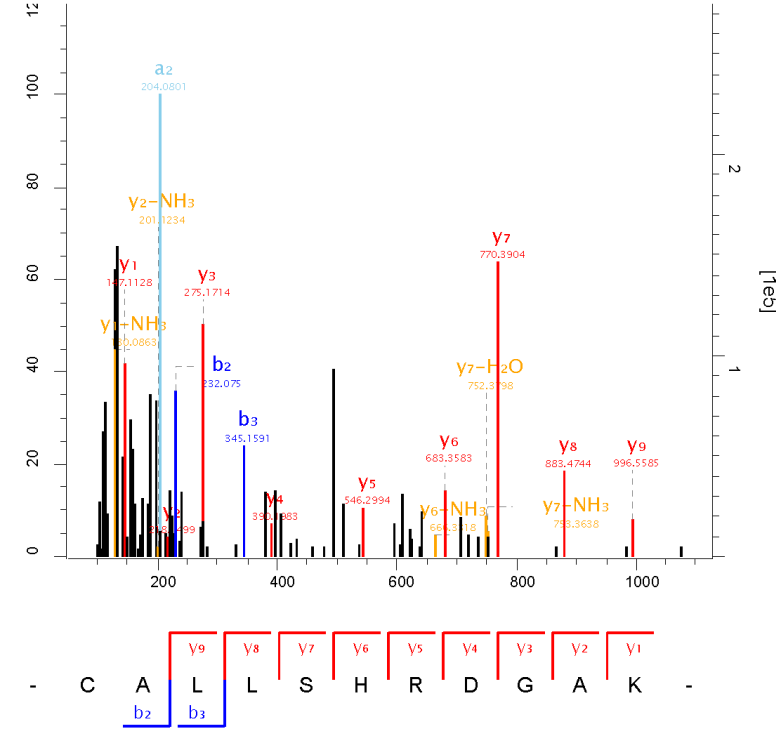


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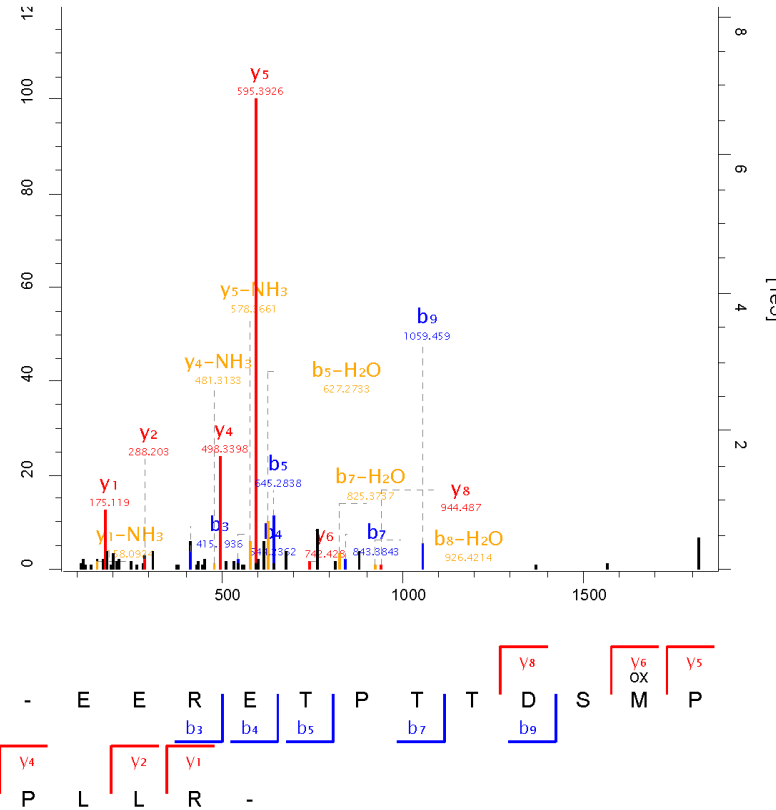
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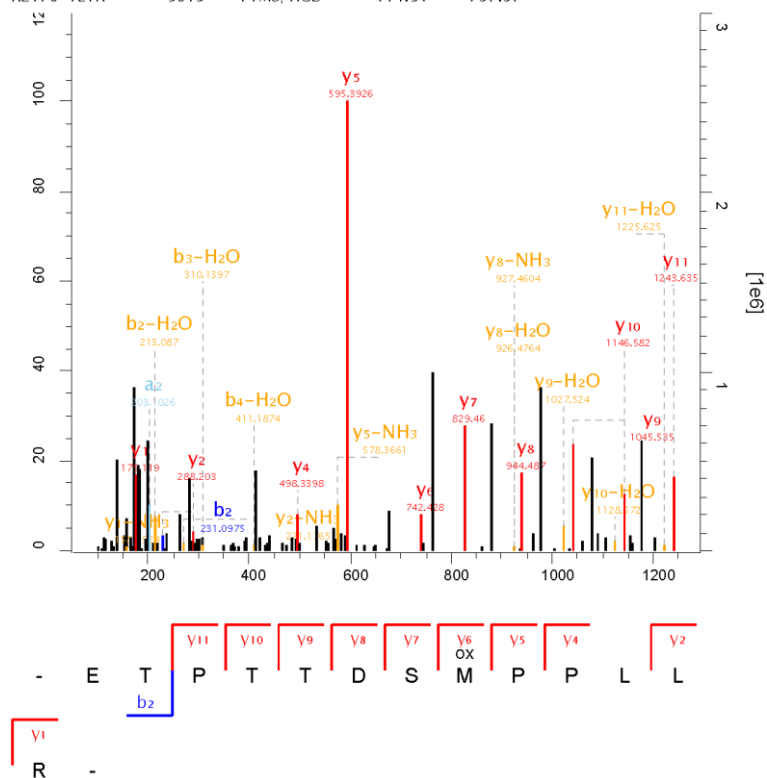
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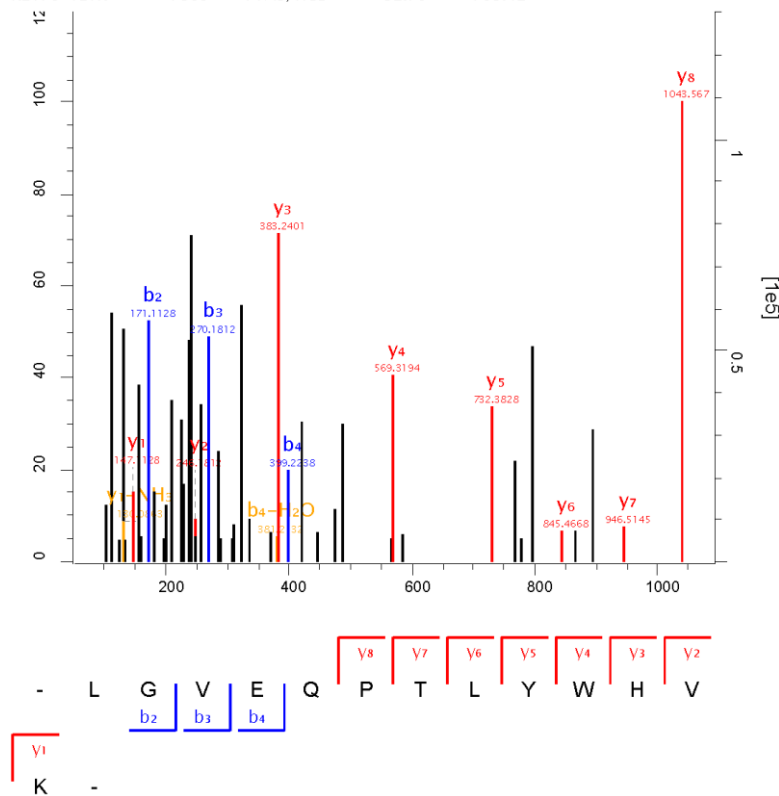
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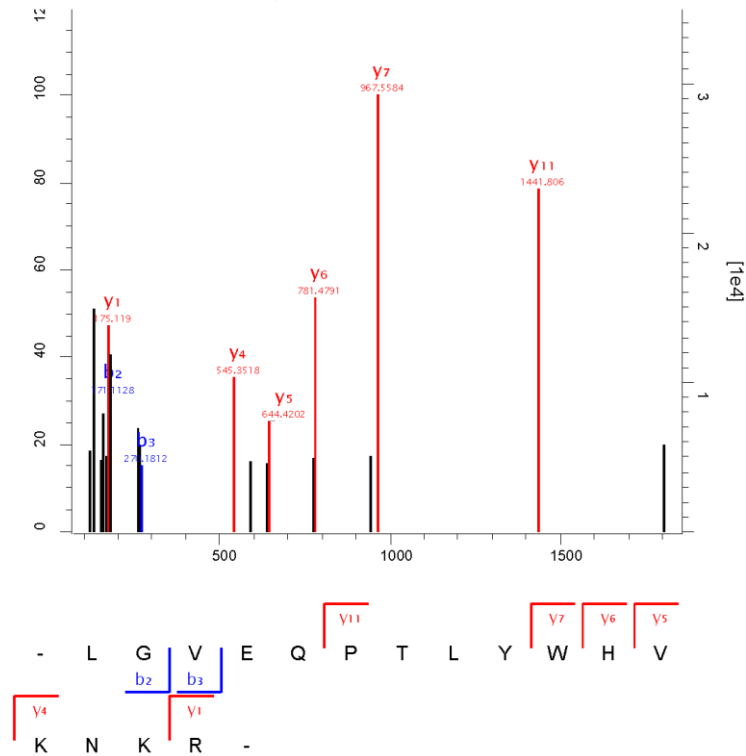
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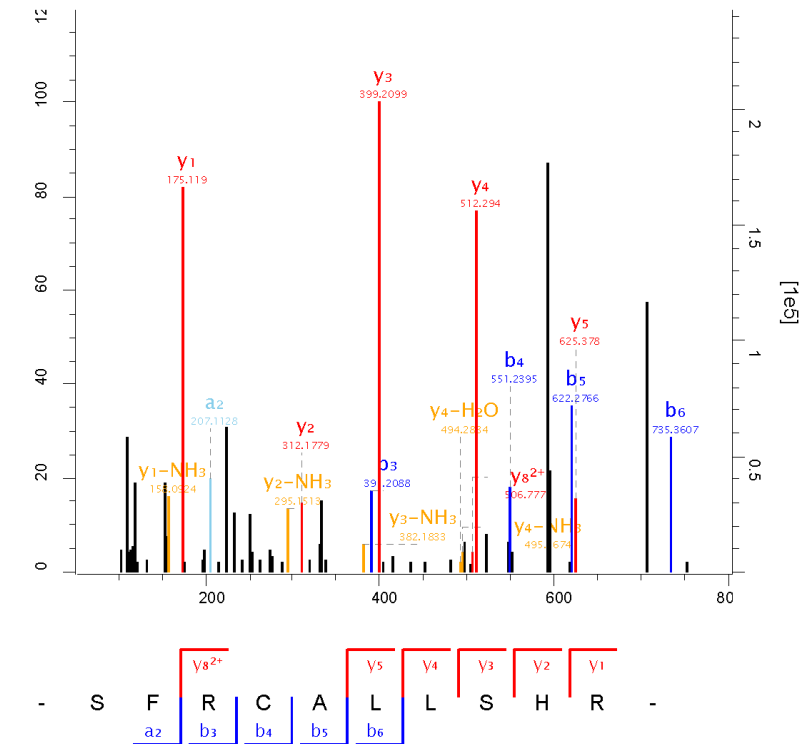
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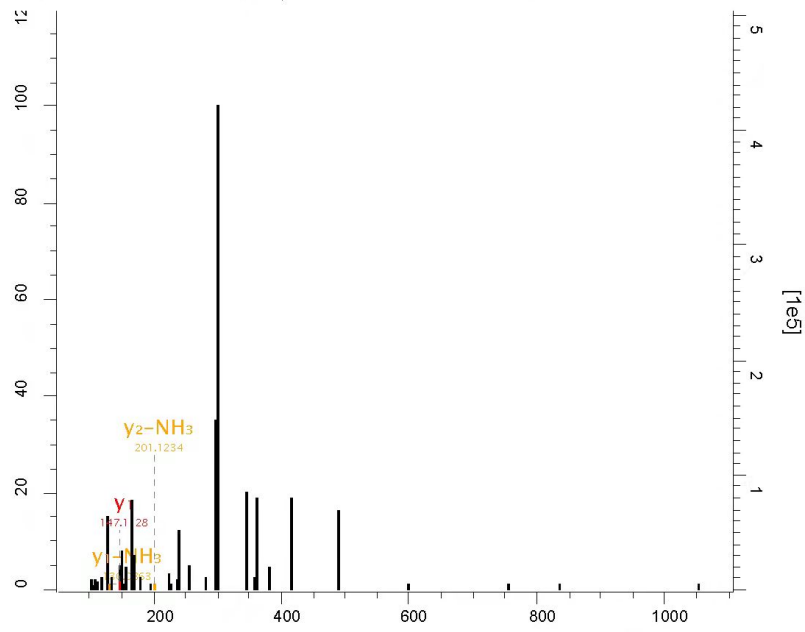
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No. 9

Raw File	Scan	Method	Score	m/z
R2176-TETR	6475	FTMS; HCD	0	539.95



- S F R C A L L S H R D G

A  $y_1$   
K -

**Figure S2.** Amino acid sequences of the natural TetR and the TetR from *Escherichia coli*. (NCBI ID: WP\_000088605.1), *Enterobacter hormaechei* (NCBI ID: WP\_058686930.1), *Morganella morganii* (NCBI ID: WP\_052927103.1), *Salmonella* (NCBI ID: WP\_050959760.1), and *Acinetobacter baumannii* (NCBI ID: WP\_185936927.1). The highlighted amino acids are the 100% conserved residues.

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our natural TetR.seq      MSRLDKSKVINSALELLNEVGIEGLTTEKLAQKLGVEQTLYWHVKNKRA 50
Escherichia coli.seq     MSRLDKSKVINSALELLNEVGIEGLTTEKLAQKLGVEQTLYWHVKNKRA 50
Enterobacter hormaechei.seq MNKLQREAVIRTALELLNDVMEGLTTERLAERLGVQQFALYWHFKNKRA 50
Morganella morganii.seq  MARLNRESVIDAALELLNETSIDGLTTEKLAQKLGIEQTLYWHVKNKRA 50
Salmonella.seq          MTKLDKGTVIAAALELLNEVGMDSLTTEKLAERLKVQQFALYWHFQNKRA 50
Acinetobacter baumannii.seq -----LLNEVGVDGLTTEKLAERLGVQQFALYWHFRNKRA 35

our natural TetR.seq      LLDALAIEMLDRHHTHFCFLEGESWQDFIRNNAKSFRCALLSHRDGAKVH 100
Escherichia coli.seq     LLDALAIEMLDRHHTHFCFLEGESWQDFIRNNAKSFRCALLSHRDGAKVH 100
Enterobacter hormaechei.seq LLDALAEAMLTINHTHSTERRDDDDWRSFLKGNACSFRRALLAYRDGARIH 100
Morganella morganii.seq  LLDALAVEILARHHDYSLPAAGESWQSFIRNNAMSFRRALLYRDGAKVH 100
Salmonella.seq          LLDALAEAMLAERHTRSLPEENEDWRVFLKENALSFRTALLSYRDGARIH 100
Acinetobacter baumannii.seq LLDALAEAMLAENHTHSVERADDDWRSFLIGNARSFRRQALLAYRDGARIH 85

our natural TetR.seq      LGTRFTEKQYETLENLAFLCQQSFSLLENALYALSAVGHFTLGCVLEDQE 150
Escherichia coli.seq     LGTRFTEKQYETLENLAFLCQQSFSLLENALYALSAVGHFTLGCVLEDQE 150
Enterobacter hormaechei.seq AGTRFAAPQMEKADALRLCDAGFSAGDATYALMAISYFTVGAIVLEQQA 150
Morganella morganii.seq  LGTRFDEKQYDTVETQLRFMTDNGFSLRDGLYALSAVSHFTLGAIVLEQQA 150
Salmonella.seq          AGTRFTEPIFCSAETQLRFLCAEGFCPKRAVWALRAVSHYVVGSVLEQQA 150
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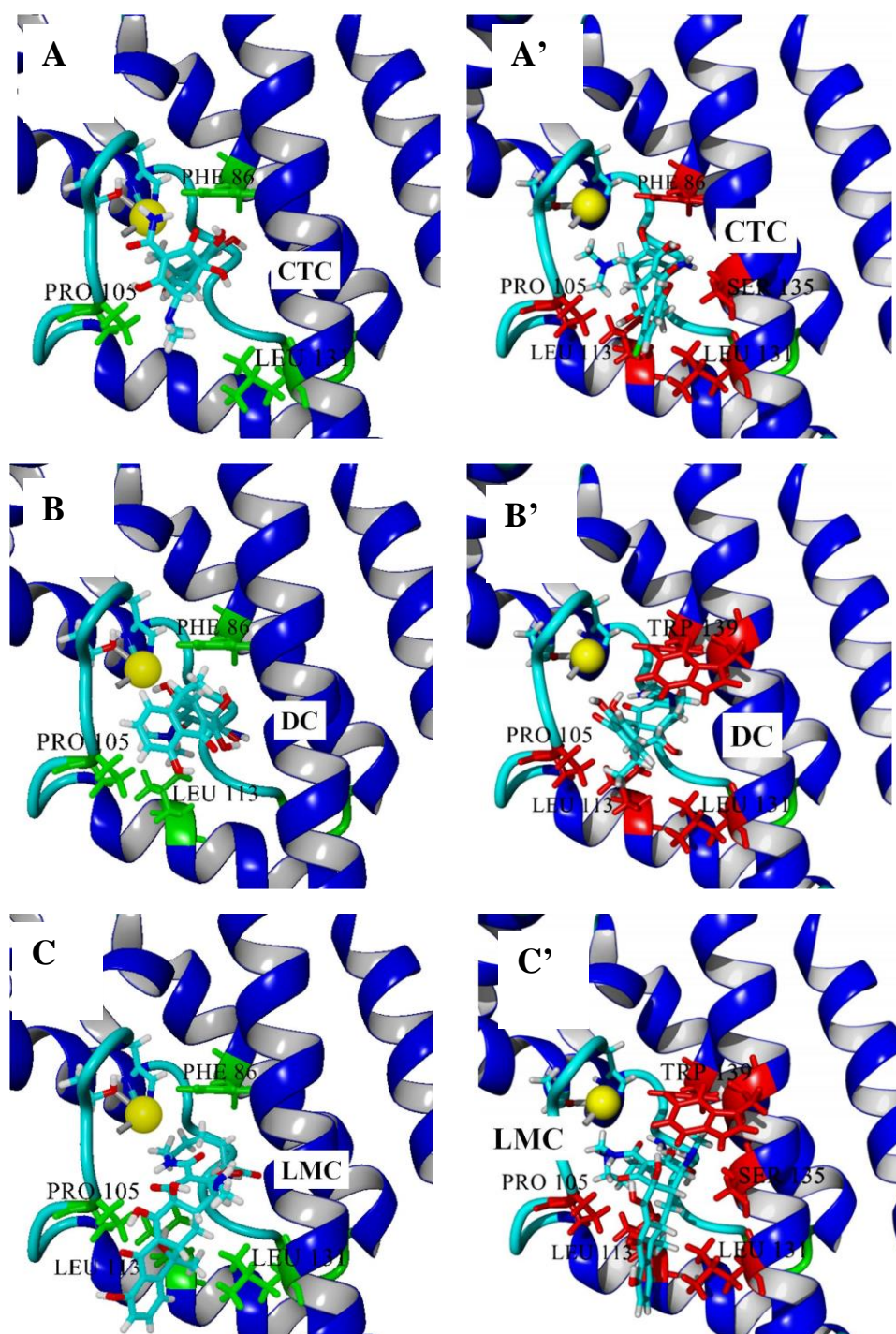
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Escherichia coli.seq     HQVAKEERE----TPTTDSMPPLLRQAIELFDHQGAEPALFGLLELIICG 196
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Morganella morganii.seq  HSAALADSS----ATPDENLPPLIREALHIMSDDGEQAFHGLLESILIG 196
Salmonella.seq          S--DADERVPDRPDVSEQAPSSFLHDLFHELETGMDAAFNFGLDSLIAG 198
Acinetobacter baumannii.seq GDSDAGEEGG---TVEQAPLSPLRAAIDAFDEAGPDAAFEQ 174

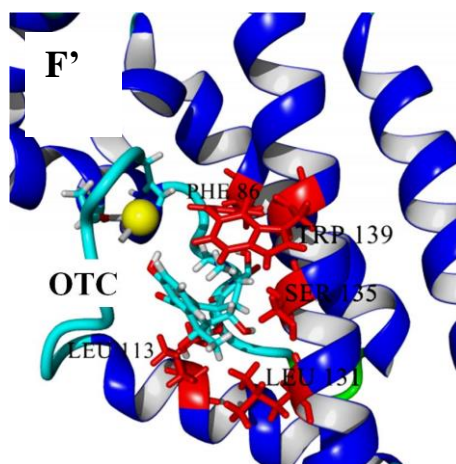
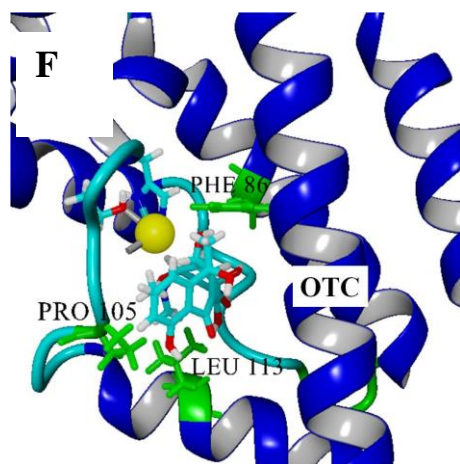
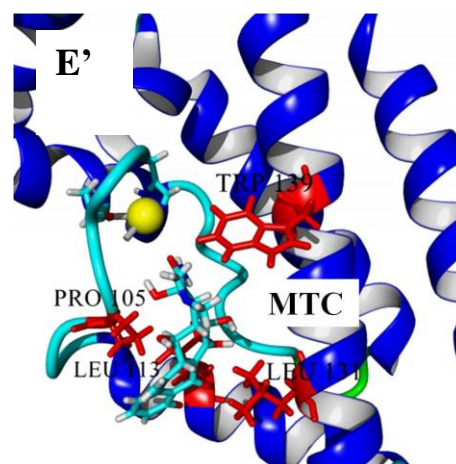
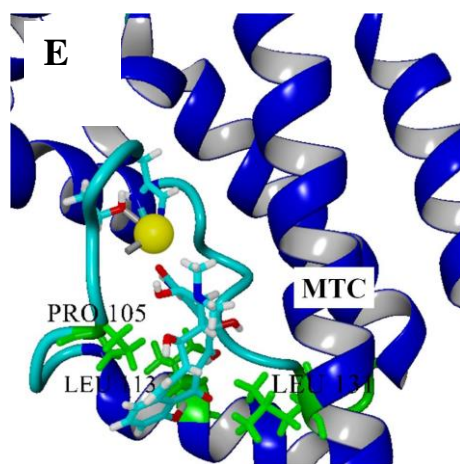
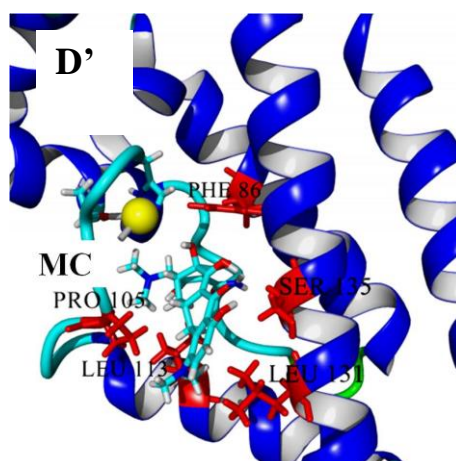
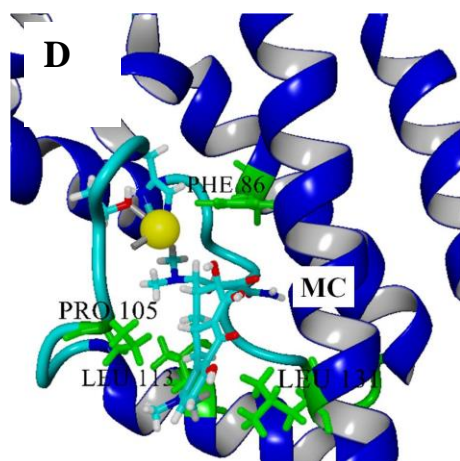
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Escherichia coli.seq     LEKQLKCESGS 207
Enterobacter hormaechei.seq LEKMRLTTNDIEVLKNVDE 219
Morganella morganii.seq  FEVQLTHSGQSPQ 209
Salmonella.seq          FERLRSSTTD 208
Acinetobacter baumannii.seq 174

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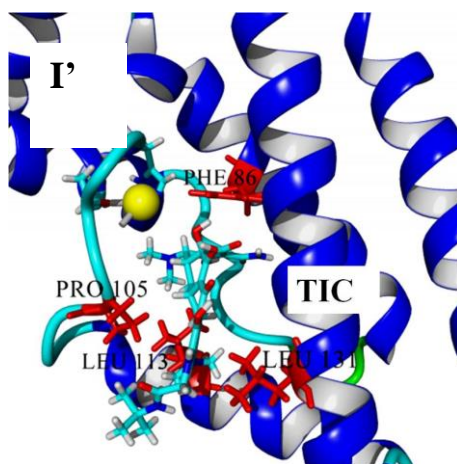
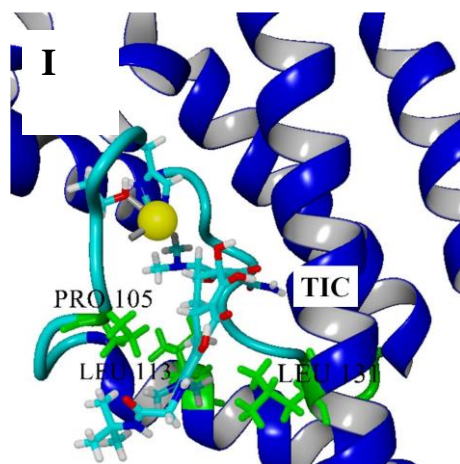
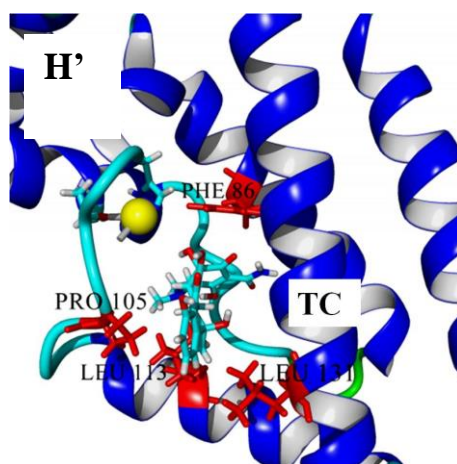
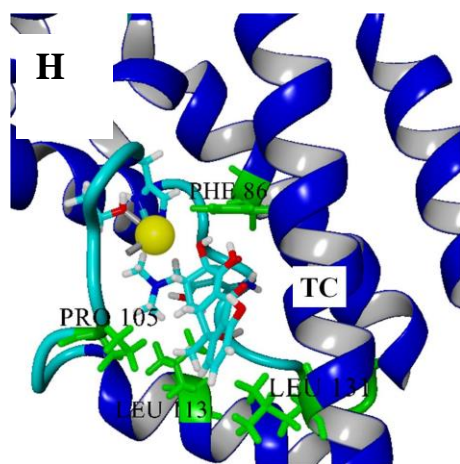
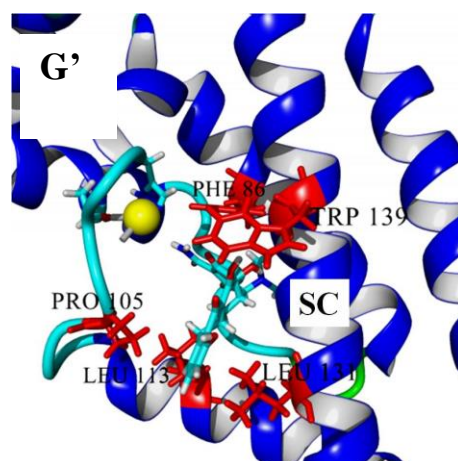
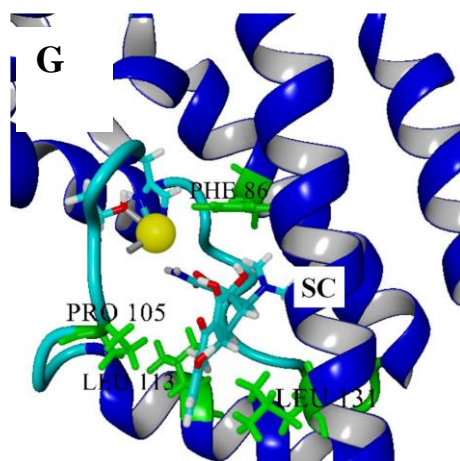


**Figure S3.** Docking complexes of the natural TetR (A-I) and the mutant (A'-I') with other 9 TCs.









**Figure S4.** Results for optimization of (A) FA-MC/mutant concentrations ( $\text{MgCl}_2$  5 mM, incubation 5 min), (B)  $\text{MgCl}_2$  concentration (FA-MC 1:500, mutant 1:2000, incubation 5 min), and (C) incubation time (FA-MC 1:500, mutant 1:2000,  $\text{MgCl}_2$  8 mM) by using 100 ng/mL of DMC (n=5).

