Electronic Supplementary Material (ESI) for Metallomics

Supporting Information

Mutations on Asn11

To study the metal binding properties of TroR in detail, three mutations focus on site 11 (Asn) including N11A, N11M and N11D were constructed based on the template pTroR4 plasmid. The primer pairs (P8, P11), (P9, P11) and (P10, P11) were used to replace Asn11 with Ala, Met and Glu respectively by Site-Directed Mutation Kit (Toyobo).

P8: 5'-GCTTATTTGAAGACAGTGGTAAAGGC-3'

P9: 5'-ATGTATTTGAAGACAGTGGTAAAGGC-3'

P10: 5'-GATTATTTGAAGACAGTGGTAAAGGC-3'

P11: 5'-CTCTGCTGCAATATCTGACACTAACG-3'

Expression and Purification of His-TroR with Plasmid pTroR1

His-TroR was overexpressed *in E.coli* XL-Blue harboring pTroR1 in 1 liter of LB medium containing 100 μg/ml ampicillin at 37°C with vigorous shaking. When the cells reached an OD_{600} of 0.8, IPTG was added to the culture to a final concentration of 0.5 mM, and the incubation was continued for an additional 6 h at 25°C. After induction, the cells were harvested by centrifugation (11,000 x g, 30 min, 4°C) and stored at -80°C until needed. Under these conditions, the TroR was overexpressed but almost localized to inclusion bodies as determined by

SDS/PAGE (Figure S1). Based on these results, the denature/refolding method with some modifications was used to purify the His-TroR. The cell lysate was treated with 8 M urea and applied to a Ni-NTA column equilibrated with buffer A (50 mM Na-Pi, 300 mM NaCl, 5 mM β-mecaptoethanol (β-Me), 20 % glycerol, pH 7.4). The protein was eluted with 250 mM imidazole in 8 M urea, fraction containing His-TroR was pooled, concentrated and dialyzed against in buffer B (20 mM Tris-HCl, 200 mM NaCl, 5 % glycerol, pH 7.4) plus 0.2% Sarkosyl. The purified His-TroR was obtained about 80% purity according to the SDS/PAGE result (Figure S2).

Protein Purification and Digestion by Proteases.

The fusion protein MBP-TroR expressed by plasmid pTroR2 was purified as follows: the frozen cell pellets were resuspended in Buffer A with 1 mM phenyl methyl sulfonyl fluoride (PMSF), and lysed with 100 μg/ml lysozyme, 1μg/ml Dnase I on ice for 20 min. Cells were sonicated for 5 min on ice, and the suspension was centrifuged at 4°C (12,000 rpm for 30 min). The supernatant from this centrifugation was loaded onto a Ni-NTA column (2.5 x 20 cm) equilibrated in buffer A. The column was washed with 10 column volume buffer A containing 10 mM imidazole and then the protein was eluted with 4 column volume buffer containing 200 mM imidazole. The imidazole in the elution was removed by dialyzing with a large amount of buffer A three times per 3 h at 4°C. After

cleavage of the N-terminal MBP tag by His-TEV protease (1:100, w/w) overnight at 16°C, the protein solution was subject to Ni-NTA column again to remove the MBP tag by binding it to Ni-NTA. The eluted fraction was collected, concentrated using an Amicon concentrator equipped with an YM-10 membrane (Millipore, Billerica, MA) to load onto a Superdex^{TM} 200 Hi-load 16/60 gel filtration column equilibrated with buffer B. The fusion protein Smt3-TroR expressed by pTroR3 was expressed well but almost localized to inclusion bodies as determined by SDS/PAGE (Figure S3), thus failed to be further purification.

Preparation of Metal-Free Labware and Buffers.

Clear and colorless plastic tips and microcentrifuge tubes were used because often the colored plastics contained traced metal. To prepare the metal-free water, the ultra-pure water collected from Milli Q was loaded onto a column filled with Chelex$^{@}$ 100 resins (Bio-Rad). Glassware was washed with 10% nitric acid and rinsed with metal-free buffer.

Purification of His-TroR with pTroR1.

The gene coding TroR protein was amplified by PCR from *T.pallidum* genomic DNA by using primers P3 and P4, and the product was digested with *Bam*HI plus *Hind* III sites of expression vector pQE-30 generating plasmid pTroR1. The pTroR1 overexpressed a 19-kDa

his-TroR fusion protein in *E.coli* XL- Blue. However, the His-TroR localized near 95% in the insoluble fraction of the cell lysate (Figure S1). Following the procedure described in the *Materials and Methods*, the His-TroR was obtained about 80% purity based on the SDS/PAGE result (Figure S2). However, this His-TroR is a denatured-refolding protein containing minimum 0.2% sarkosyl rather than native proteins, which may result in various difficulties for biochemical and crystallographic experiments. To date, there is no method to obtain soluble recombinant TroR. Thus, we tried to obtain soluble TroR by different fusion expression systems, and successively constructed the expression plasmids pTroR2, pTroR3 and pTroR4.

Purification of TroR with pTroR2.

To obtain soluble recombinant TroR from *E.coli* expression systems, we have tried lots of fusion tags, including glutamine (GST), thioredoxin (data not shown), Smt3 and MBP. However, only the TroR with MBP fusion tag was in the soluble lysate. As well known, the MBP-tag expression system has many advantages, including high expression level, diverse purification methods and high-quality purification of target proteins .

The fusion protein MBP-TroR harboring pTroR2 was overexpressed in *E.coli* Rosseta (DE3) pLysS cells, yielding the TroR in fusion with a

MBP and 8 x His-tag (Figure 1A). After lysis the cells, the lysate containing soluble MBP-TroR was loaded onto a Ni-NTA column equilibrated with buffer A. The interest protein was eluted by buffer A containing 200 mM imidazole, and then the imidazole in the elution was removed by dialyzing with a large amount of buffer A three times per 3 h at 4°C. After this procedure, the fusion protein MBP-TroR was ~ 90% purity based on the SDS/PAGE result (Figure S4). The N-terminal MBP fusion tag was cleavage with His-TEV protease (1:100, w/w) overnight at 16°C. The efficiency of the digestion process was good as shown in Figure S5. Two new protein bands migrated with apparent molecular mass ~ 43 kDa and ~ 18 kDa, which corresponding to MBP and target protein TroR, respectively. Then the protein solution was subject to Ni-NTA column again to remove the MBP tag and a little amount of undigested fusion proteins by binding them to Ni-NTA. The elution was collected, concentrated and loaded onto a Superdex^{TM} 200 Hi-load 16/60 gel filtration column equilibrated with buffer B. The FPLC result presented two shape peaks (shown in Figure S6); the fractions of the two peaks were collected and detected by SDS/PAGE. Unfortunately, the target protein TroR came out in peak 1, which was mixed with some undigested fusion protein and a few MBP-tag in aggregation (Figure S6, insert). Furthermore, we had tried lots of columns to separate the target soluble TroR from the mixed fractions, such as DEAE, Mono Q and

amylose resin columns (Data not shown). However, no positive results were obtained. To explain it, we believed that the TroR after cleavage from the MBP fusion proteins would have a strong interaction with MBP-tag plus some undigested fusion protein (shown in Figure 2, top). The predicted pI value of TroR and MBP is 8.83 and 5.51, respectively; therefore, a strong electrostatic interaction may exist between them, making the separation of them in great difficult.

Co^{2+} Titration Experiments for WT TroR and TroR Mutations

In accurate metal titration studies for Co^{2+}, the metal stock solutions (from 0.5-1000 μM) were titrated into the ANS/apo-TroR mixture. The fluorescence emission intensity at 486 nm was plotted as a function of metal ions concentration, and data were fitted with 1:1 binding isotherm by using equation 3:

$$Y = A*X / (K_d + X) \quad (3)$$

Where Y represents fluorescence quenching ration, X represents a final concentration of metal ions in buffer, A stands for the maximum quenching ratio and K_d stands for the dissociation constant, respectively.

The mutated proteins, including N11A, N11M and N11D, focus on one of the putative metal binding sites in TroR, were also applied to Co^{2+} titration study. The data of these mutants were fitted with equation 3 except for N11M, which was fitted with 1:2 binding isotherm (eq.4)

instead.

$$Y = A_1 * X / (K_{d1} + X) + A_2 * X / (K_{d2} + X) \quad (4)$$

The initial metal addition experiments indicated that up to 100 μM of Co^{2+} could stabilize the TroR (wt). Thus to determine the binding affinity of Co^{2+} for wild-type TroR, the Co^{2+} solutions were added to wild-type TroR (2.5 μM)/ANS (500 μM) mixture to final concentrations 2 μM-100 μM. The fluorescence intensity at 486 nm was recorded and converted to quenching ratio by the formula: $A = (I_o - I) / (I_F - I_o)$, Where I is the fluorescence intensity at 486 nm, I_F is the final intensity, and I_o is the initial intensity. The data was fitted with 1:1 binding isotherm by equation: $Y=A*X/(K_d+X)$ using Origin 8.0 software, and the value of K_d was 3.9±0.4 μM (Figure S8). From the sequence alignments of TroR to its homologies (Figure 7) and bioinformatic analysis, the Asn 11 was predicted to be one of the binding residues for TroR, and it was distinct from the corresponding residues in MntR and DtxR, which is Met 10 and Glu 8, respectively. Thus, we designed and constructed three single-site mutants, including N11A, N11M and N11D, to further investigate the binding property of TroR compare to its homology DtxR and MntR.

For variant N11A, the putative binding residue Asn was replaced with Ala, which has no coordinated atoms in the side chain. The K_d value of N11A was 46.1±2.8 μM, having a dramatically decrease of about one order of magnitude over WT TroR, which proved that Asn indeed takes

part in metal coordination according to the assumption. By comparison, the calculated K_d of N11D was 25.8±1.9 μM, which was about six fold of WT TroR and two fold of MntR monitored by ANS, indicating that Asn indeed has an important role on metal binding for TroR and the variant N11D was structurally and functionally deficient. The most interesting result was for N11M, in which the binding residue Asn was replaced with the corresponding residue Met in DtxR. The data of this variant cannot be fitted with 1:1 binding model well (with a K_d value 14 ±4 μM), but was in great agreement with 1:2 binding isotherm by equation: $Y= A_1*X/(K_{d1}+ X) + A_2*X/(K_{d2}+ X)$. Accordingly, the K_{d1} and K_{d2} value was 1.0±0.2 μM and 91.0±10 μM, respectively. Meanwhile, the ITC experiment, for Co^{2+} titration to the three variants, was also performed to confirm that the vital role of Asn 11 in TroR. The results shown in Figure S9, likewise, indicated that substitution of Asn to Ala and Glu decrease the binding affinity of Co^{2+} compares to wild-type TroR. For variant N11M, its titration curve was shown as a two-phase pattern, consist with the ANS titration result, indicating that substitution of Asn to Met would alter the binding property of TroR to a DtxR-like model.

In Co^{2+} reconstructed DtxR, two distinct metal binding sites have been intensified: the ancillary metal binding site (site 1: H79, E83, H98, E170) is playing a critical role in stabilization of the repressor and allow metal ions binding at the primary site (site2: M10 and C102, E105, H106),

which is essential for recognition of the target DNA repressor. Substitution of Asn to Met significantly alter the metal binding properties of TroR, suggesting that although the TroR is predicted to be one of the members of DtxR family, it has a distinct metal binding and metal-induced activation mechanism.

Figures and Figure Legends

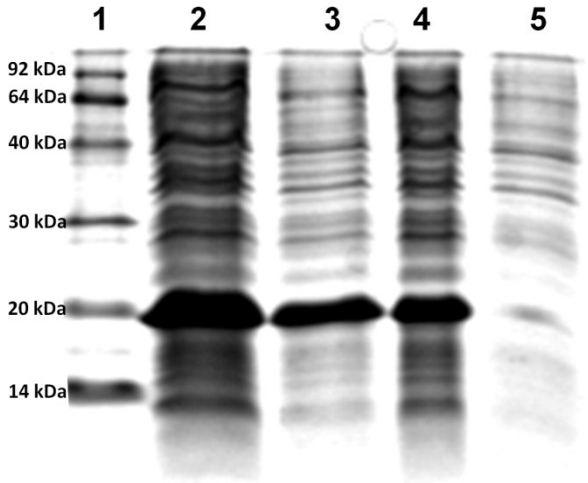

Figure S1. Production of recombinant TroR using plasmid pTroR1 in *E.coli* XL-blue cells. SDS-PAGE analysis is conducted in a 15% gel. Lane 1: Molecular weight markers; lane 2: overexpression of TroR after 7 hours since adding 0.5 mM IPTG; lane 3: overexpression of TroR after 3 hours since adding 0.5 mM IPTG; lane 4: the lysate of cell pellets from induced cells; lane 5: cell-free supernatant from induced cells. The MW of the target protein TroR is 19 kD.

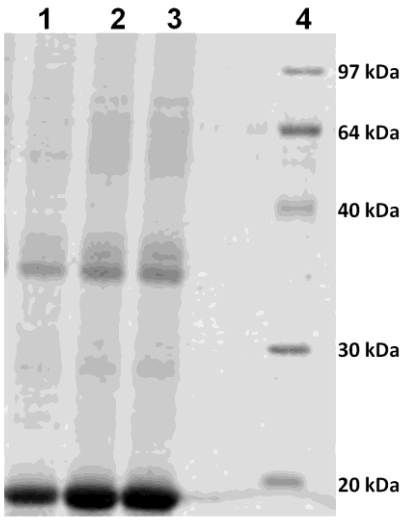

Figure S2. Production of recombinant TroR using plasmid pTroR1 in *E.coli* XL-blue cells. SDS-PAGE analysis is conducted in a 15% gel. Lane 1-3: Fractions containing 8 M urea eluted from Ni-NTA column with 100, 250 and 500 mM imidazole in buffer B respectively. Lane 4: molecular weight markers. The MW of the target protein TroR is 19 kD.

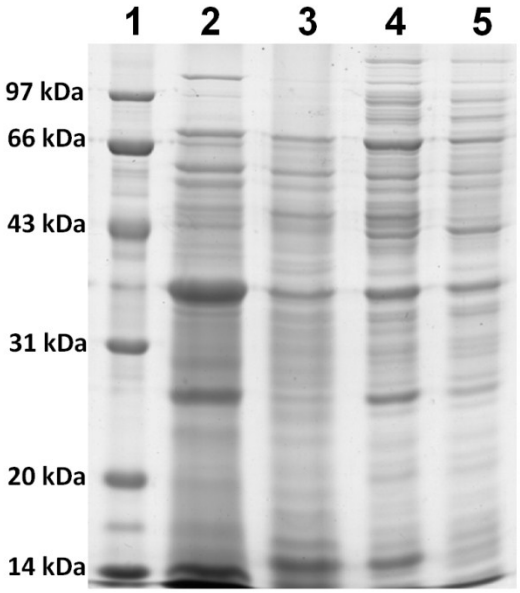

Figure S3. Production of recombinant TroR with a fusion Smt3 tag using plasmid pTroR3 in *E.coli* Rosseta (DE3)pLys cells. SDS-PAGE analysis is conducted in a 15% gel. Lane 1: molecular weight markers; lane 2: the lysate of cell pellets from induced cells; lane 3: cell-free supernatant from induced cells; lane 4: cells with pTroR3 after 0.1 mM IPTG induction; lane 5: cells with pTroR3 before IPTG induction. The MW of the fusion protein Smt3-TroR is ~ 33 kD.

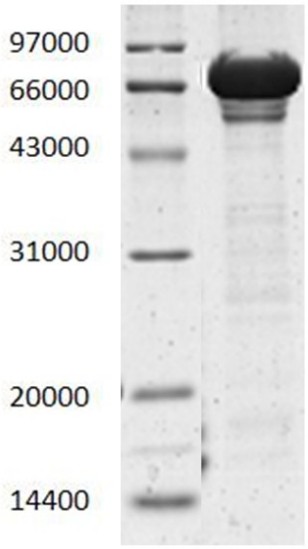

Figure S4. Production of recombinant TroR with a fusion MBP tag using plasmid pTroR2 in *E.coli* Rosseta (DE3)pLys cells. The MW of the fusion protein MBP-TroR is ~ 60 kD.

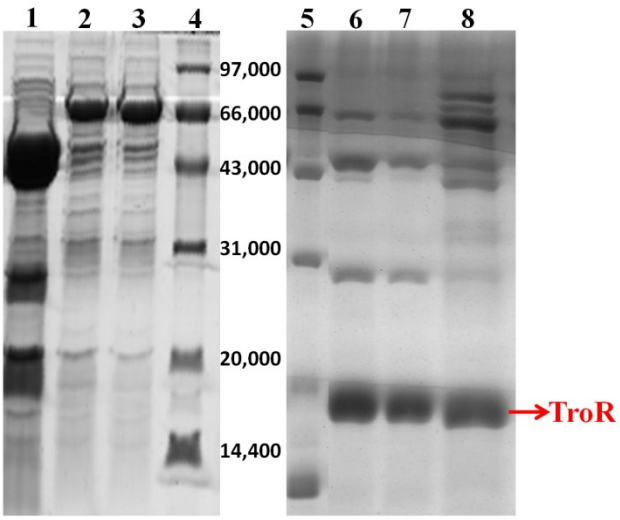

Figure S5. Expression, purification and digestion of MBP-Smt3-TroR fusion protein using vector pTroR4. SDS-PAGE analysis in a 15% gel. Lane 1: fusion protein after digestion with by His-SUMO protease (1:50, w/w) for 2 h at 16°C. Lane 2-3: the MBP-Smt3-TroR fusion protein after purification with a Ni-NTA column. Lane 4-5: molecular weight markers. Lane 6: the elution of lane 1 after an amylose resin column. Lane 7: the elution of lane 6 after an amylose resin column. Lane 8: the elution of lane 1 after a Ni-NTA column. The MW of the fusion protein MBP-Smt3-TroR is ~ 66 kD; the MW of the MBP-Smt3-tag is ~ 51 kD; the MW of MBP-tag is ~ 43 kD; the His-SUMO protease is ~ 28 kD; and the target protein TroR is 19 kD.

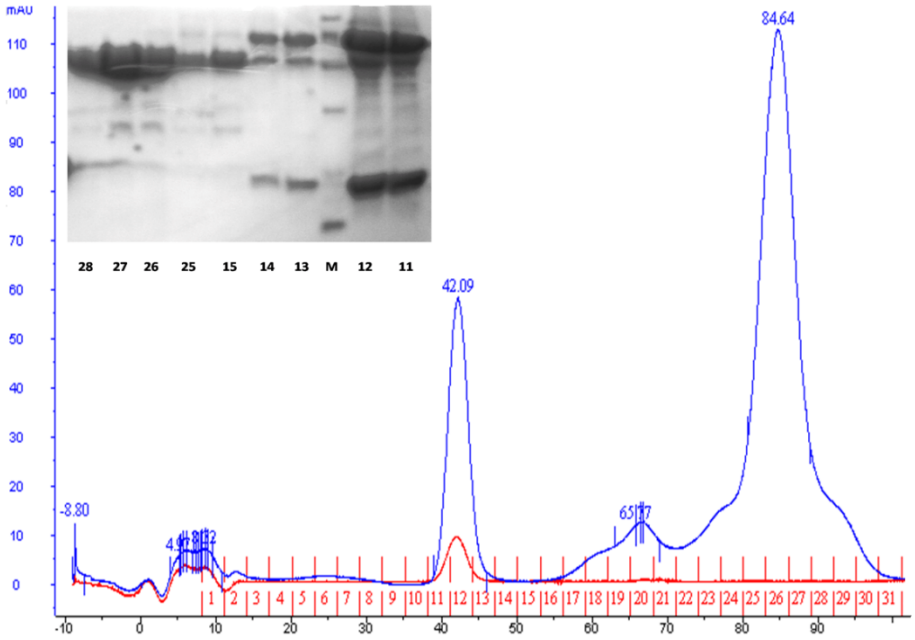

Figure S6. Elution profile of TroR from Superdex^{TM} S200 Hiload 16/60 gel filtration column of AKTA explorer (Amersham). Insert figure shows the SDS-PAGE analysis of the collected fraction according to the number of tube accordingly.

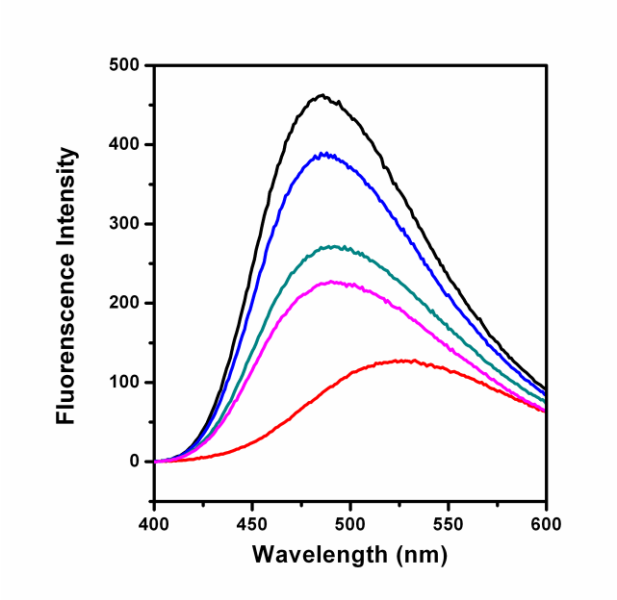

Figure S7. ANS fluorenscene spectra in the presence of wild-type TroR and variable amounts of Mn^{2+}. The red curve is the initial spectrum of ANS (500 μM). Other curves are upon addition of 2.5 μM TroR (black line), 2.5 μM TroR and 0.1 mM Mn^{2+} (blue line), 2.5 μM TroR and 0.5 mM Mn^{2+} (dark cyan line), 2.5 μM TroR and 1 mM Mn^{2+} (magenta). λ_{ex} = 378 nm; Buffer = 20 mM HEPES; pH 7.4; T = 25 °C.

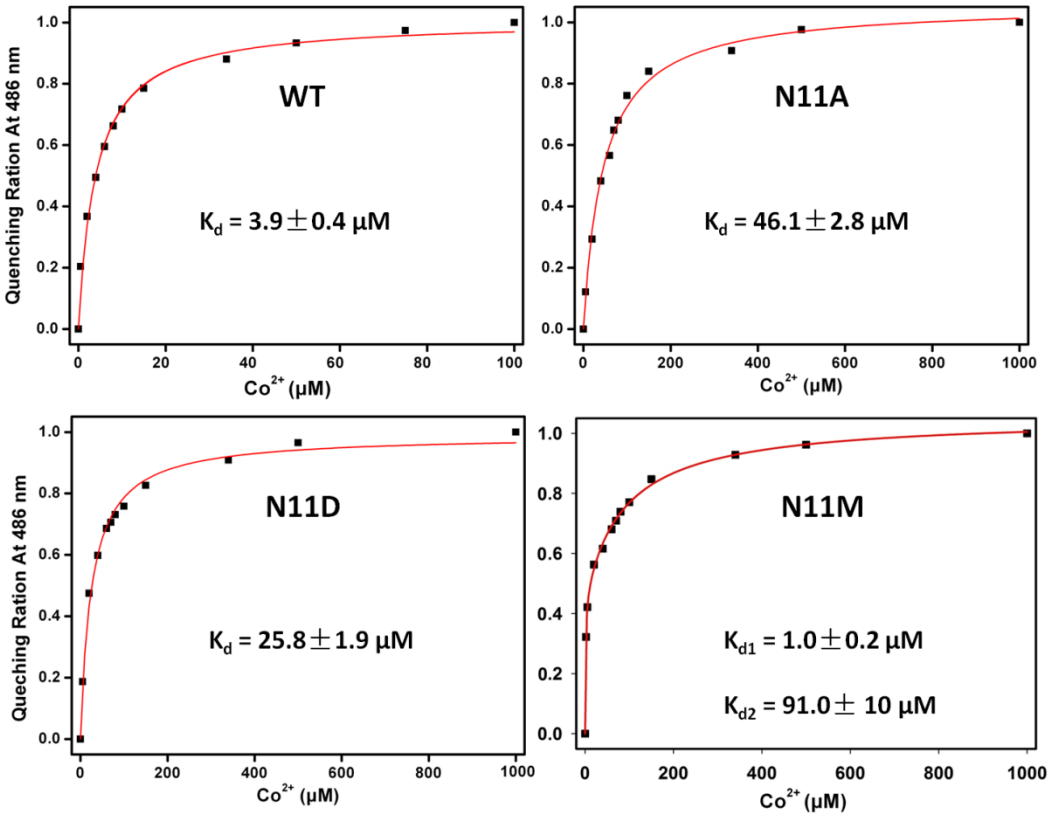

Figure S8. Assay of Co^{2+} titration of wild-type TroR and mutated TroR using ANS fluorescence emission. Data from WT, N11A and N11D were fit with a single-ligand binding model by Origin 8.0, while dada from N11M was fit with a two-ligands binding model by Sigmal Plot 11.

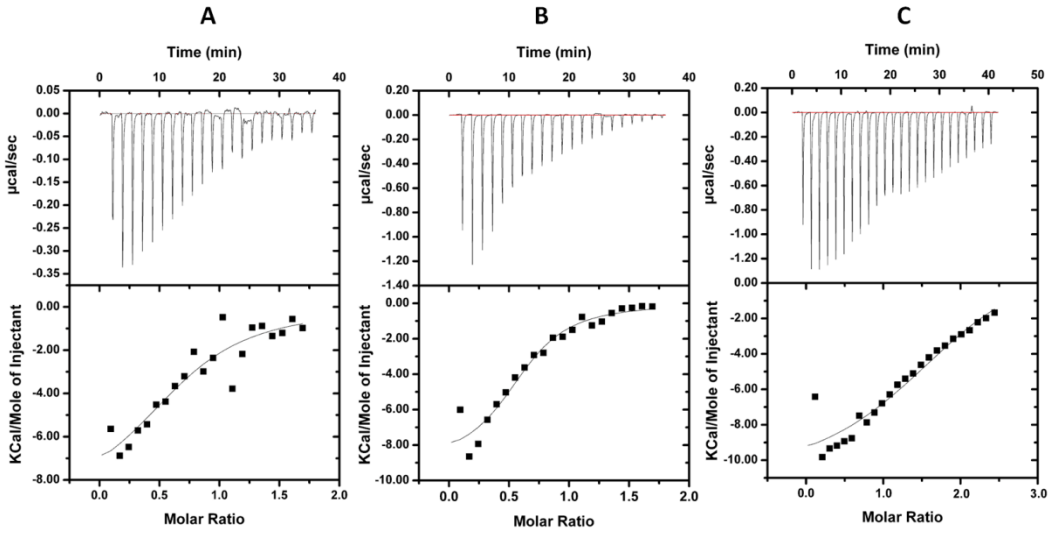

Figure S9. Calorimetric titration results of TroR mutations. (A) Titration of 0.4 mM $CoCl_2$ into a solution of 40 µM N11A at pH 7.4. (B) Titration of 1.0 mM $CoCl_2$ into 100 µM N11D at pH 7.4. (C) Titration of 1.0 mM $CoCl_2$ into 100 µM N11M at pH 7.4. Data from the N11A and N11D were fit with a single-site model, while data from N11M was fit with a two site sequential binding model.

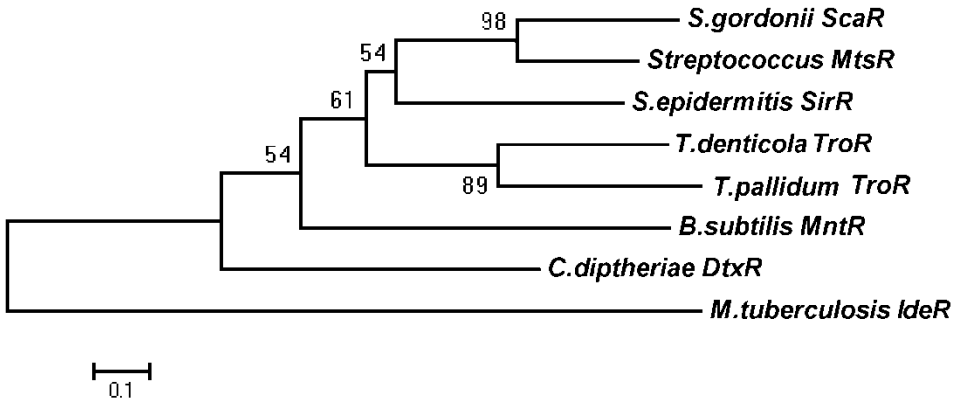

Figure S10. The phylogenetic analysis of TroR and other metal-dependent regulatory proteins using Neighbor-Joining (NJ) method by program MEGA 5.0.