Supporting Information

for

Secretion of metal-binding proteins by a newly discovered OsmY homolog in C*upriavidus metallidurans* for the biogenic synthesis of metal nanoparticles

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Materials and Methods

Bacterial strains, growth conditions, and cloning

E. coli cells were cultivated in lysogeny broth (LB) medium at 37°C with 250-rpm shaking. *C. metallidurans* cells were cultured in LB at 30°C (200 rpm). The nutritionally rich medium, LB, was used in RFP characterization and SDS-PAGE studies. For *E. coli*, M9 broth medium (AMRESCO, supplemented with 0.4% glucose) was used in biosynthesis. For *C. metallidurans*, H-3 medium (DSMZ 81 medium supplemented with 0.3% sodium benzoate) was used instead. Strains, plasmids, and primers used are listed in Table S1, S2 and S3.

RFP characterization

The overnight *E. coli* culture was diluted in LB media until reaching OD_{600} of 0.4~0.6. Then, 0.2% arabinose was used to induce the expression of fusion proteins. For *E. coli*, several time points after induction were picked for monitoring: 4, 8, 12, 16, 20, 24, and 50 h. 200 μL cell culture was centrifuged (12,225 g, 1 min). After centrifugation, the pellet was washed and resuspended in 100 μL PBS buffer. The supernatant and the resuspended pellet were transferred to microplate (Synergy HT, BioTeK) to measure RFP intensity (excitation/emission filters: 530 nm/590 nm), and OD_{600} for normalization. *C. metallidurans* culture was prepared same as the procedure above at 4, 8, 12, 16, 20, 24, 41, 48 and 72 h. Experiments were performed in biological quadruplicate.

SDS-PAGE and Western Blot

Samples were mixed with 4X Laemmli loading buffer (AMRESCO). After transferring to PVDF membrane, 1st Ab (anti-His rabbit polyclonal antibody, 1:5000) and 2nd Ab (goat anti-rabbit, HRP conjugate, 1:100,000) were sequentially incubated. Chemiluminescence were detected by ImageQuant LAS 4000 mini (GE Healthcare). Certain samples were ten-fold concentrated (10X) using centrifugal vacuum concentrator (miVac Duo concentrator, Genevac).

Preparation of supernatants

E. coli strains were diluted (2%) in M9 medium. 100 mL culture was harvested. When reaching OD_{600} of 0.4~0.6, 0.2% arabinose was added. After 24 h, the culture was centrifuged (4°C, 8150 g, 30 min) and the supernatant was reserved. It is two-fold concentrated (2X) by vivaspin sample concentrators (3000 MWCO, GE Healthcare). To eliminate background in TEM imaging, the solvent were exchanged with either 0.1 M sodium phosphate or 25 mM HEPES buffer using the same apparatus. Instead of M9, same procedure was applied to *C. metallidurans* with H-3 medium.

Biosynthesis and characterization of metal nanoparticles

Diverse metal salts were added to as-prepared supernatants with final concentration 1 mM (including $HAuCl_4$ (Acros organics), $AgNO_3$ (Acros organics), $CuSO_4$ (Acros organics), $H_2PtCl_6 \cdot H_2O$ (Acros organics) and Na_2PdCl_4 (Acros organics)). The reaction mixture was incubated at room temperature. The sample was dropped on the copper grid for TEM/HR-TEM analysis (JEOL JEM-1200 EXII/FEI Tecnai G2). To get the powder for SEM/EDS and XPS analysis, the aforementioned liquid was dialyzed through ultrapure water for desalting (mini dialysis kit 1kDa cut-off, GE Healthcare) and lyophilized by VirTis Freeze Dryer 5SL. Selected images were imported into ImageJ for particle size analysis as previously described.[1]

UV-Vis spectroscopy

100 μL samples were loaded into 96-well microplate. Solvent (sodium phosphate) with 1 mM Au(III) ion was taken as blank for obtaining spectra. The data was recorded from 200 to 800 nm with a resolution of 1 nm. Extinction coefficient as 8.78 x 10^8 M^{-1} cm^{-1} was used to calculate the concentration of gold nanoparticles.[2]

Protein purification and quantitation

The overnight *E. coli* BL21 culture (YCY_310-2) was rediluted (2%) in LB medium. When reaching OD_{600} of 0.4~0.6, IPTG (Isopropyl β-D-1-thiogalactopyranoside, AMRESCO) was added with final concentration of 1 mM. After cultivating at 18 °C for 22 h, the cells were harvested and lysed through sonication. CupC(s) was extracted using Ni-NTA resin (Thermo Scientific). The concentration of CupC(s) stock solution was estimated to be 0.26 mg/mL by Bradford assay (AMRESCO).

Synthesis of AuNP-Ab-HRP complex

1 mL synthesized Au nanoparticles (0.89 nM) was mixed with 100 μL HRP-conjugated Antibody of 100 μg/mL. After vortexing 30 min, particles were spun down (RT, 4471 g, 10 min) and washed 3 times with wash buffer (0.05% (v/v) Tween 20 in PBS). Finally, 1 mL wash buffer was used to resuspend AuNP-Ab-HRP complex and characterized by UV-Vis spectroscopy before use. The final AuNP-Ab-complex was estimated to be 0.026 nM.

Indirect ELISA

Antigen (purified CupC(s) or Cup(s) without metal binding peptide) was serially diluted in coating buffer (0.1 M sodium carbonate/bicarbonate buffer, pH 9.6) and coated on plates at 4°C overnight. After decanting the plate, it was washed two times with wash buffer. 5% non-fat milk in PBS was used in blocking (1 h, RT). After being

washed 3 times, detection antibody (anti-6X His tag rabbit polyclonal antibody) and HRP-conjugated Ab (polyclonal goat anti-rabbit)/AuNP-Ab-HRP complex were sequentially injected into wells (1 h, RT), with washing 6 times between each step. The final washing extended to 8 times to ensure minimum retaining of unbound antibodies. Finally, 0.1X ECL was added as HRP substrate and the signal of chemiluminescence was recorded each 30 s in total 10 min (integration time 0.2 s and gain= 100).

Table S1. Bacterial Strains Used in This Study

Strains	Relevant genotype	Source
***E. coli* DH5α**	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169 hsdR17(rK-mK+) λ^-	Purchased from Protech Technology
***E. coli* BL21 (DE3)**	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Invitrogen
***C. metallidurans* CH34**	Wild type	Purchased from BCRC (Bioresource Collection and Research Center, Taiwan)
***Pseudomonas putida* mt-2**	Wild type	Purchased from BCRC (Bioresource Collection and Research Center, Taiwan)
YCY_265	*E. coli* DH5α *pBAD-osmY-rfp*	pYCY_265 transformed into *E. coli* DH5α.
YCY_262	*E. coli* DH5α *pBAD-Rmet_4872-rfp*	pYCY_262 transformed into *E. coli* DH5α.
YCY_266	*E. coli* DH5α *pBAD-Rmet_3428-rfp*	pYCY_266 transformed into *E. coli* DH5α.
YCY_305	*E. coli* DH5α *pBAD-osmY-mt-his tag*	pYCY_305 transformed into *E. coli* DH5α.
YCY_306	*E. coli* DH5α *pBAD-Rmet_4872-mt-his tag*	pYCY_306 transformed into *E. coli* DH5α.
YCY_307	*E. coli* DH5α *pBAD-Rmet_3428-mt-his tag*	pYCY_307 transformed into *E. coli* DH5α.
YCY_308	*E. coli* DH5α *pBAD-osmY-cupC(s)-his tag*	pYCY_308 transformed into *E. coli* DH5α.
YCY_309	*E. coli* DH5α *pBAD-Rmet_4872- cupC(s)-his tag*	pYCY_309 transformed into *E. coli* DH5α.

Table S1. *Continued.*

Strains	Relevant genotype	Source
YCY_310-1	*E. coli* DH5α *pBAD-Rmet_3428- cupC(s)-his tag*	pYCY_310-1 transformed into *E. coli* DH5α.
YCY_310-2	*E. coli* BL21 (DE3) *pET-29b(+)-Rmet_3428-cupC(s)-his tag*	pYCY_310-2 transformed into *E. coli* BL21 (DE3).
YCY_286	*C. metallidurans* CH34 *pBAD-osmY-rfp*	pYCY_265 conjugated into *C. metallidurans* CH34.
YCY_287	*C. metallidurans* CH34 *pBAD-Rmet_4872-rfp*	pYCY_262 conjugated into *C. metallidurans* CH34.
YCY_288	*C. metallidurans* CH34 *pBAD-Rmet_3428-rfp*	pYCY_266 conjugated into *C. metallidurans* CH34.
YCY_408	*C. metallidurans* CH34 *pBAD-Rmet_3428- cupC(s)-his tag*	pYCY_310 conjugated into *C. metallidurans* CH34.

Table S2. Plasmids Used in This Study

Plasmids	Relevant genotype	Construction and source
pYCY_037	pBBR1MCS plasmid with *pBAD-rfp*, Kan^R	
pYCY_265	pBBR1MCS plasmid with *pBAD-osmY-rfp*, Kan^R	PCR fragments of OsmY were amplified with primers 147 and 148, from *E. coli* DH10B chromosome as the template. The PCR products were treated with BglII and NdeI, and cloned into plasmid pYCY_037.
pYCY_262	pBBR1MCS plasmid with *pBAD-Rmet_4872-rfp*, Kan^R	PCR fragments of Rmet_4872 were amplified with primers 149 and 150, from *C. metallidurans* CH34 megaplasmid as the template. The PCR products were treated with EcoRI and NdeI, and cloned into plasmid pYCY_037.

Table S2. *Continued.*

Plasmids	Relevant genotype	Construction and source
pYCY_266	pBBR1MCS plasmid with *pBAD-Rmet_3428-rfp*, Kan^R	PCR fragments of Rmet_3428 were amplified with primers 151 and 152, from *C. metallidurans* CH34 chromosome as the template. The PCR products were treated with BglII and NdeI, and cloned into plasmid pYCY_037.
pYCY_305	pBBR1MCS plasmid with *pBAD-osmY-mt-his tag*, Kan^R	PCR fragments of MT were amplified with primers 176 and 177, from *P. putida* mt-2 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_265.
pYCY_306	pBBR1MCS plasmid with *pBAD-Rmet_4872-mt-his tag*, Kan^R	PCR fragments of MT were amplified with primers 176 and 177, from *P. putida* mt-2 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_262.
pYCY_307	pBBR1MCS plasmid with *pBAD-Rmet_3428-mt-his tag*, Kan^R	PCR fragments of MT were amplified with primers 176 and 177, from *P. putida* mt-2 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_266.
pYCY_308	pBBR1MCS plasmid with *pBAD-osmY-cupC(s)-his tag*, Kan^R	PCR fragments of truncated CupC were amplified with primers 178 and 179, from *C. metallidurans* CH34 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_265.

Table S2. *Continued.*

Plasmids	Relevant genotype	Construction and source
pYCY_309	pBBR1MCS plasmid with *pBAD-Rmet_4872- cupC(s)-his tag*, Kan^R	PCR fragments of truncated CupC were amplified with primers 178 and 179, from *C. metallidurans* CH34 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_262.
pYCY_310-1	pBBR1MCS plasmid with *pBAD-Rmet_3428- cupC(s)-his tag*, Kan^R	PCR fragments of truncated CupC were amplified with primers 178 and 179, from *C. metallidurans* CH34 chromosome as the template. The PCR products were treated with NdeI and BamHI, and cloned into plasmid pYCY_266.
pYCY_310-2	pET-29b(+) expression system with *Rmet_3428-cupC(s)-his tag*	pYCY_310-1 was digested with NdeI and BamHI, and the fragment was inserted into pET-29b(+).

Table S3. Primers Used in This Study

Primers	Sequence (5' → 3')
0147_Ecoli OsmY 5' BglII	TTTTAGATCTTTTAAGAAGGAGATATAatgacta tgacaagactgaagatttcgaaa
0148_Ecoli OsmY 3' NdeI	TTTTCATATGcttagttttcagatcatttttaacgcttttc
0149_Cm Rmet_4872 5' EcoRI	TTTTGAATTCTTTAAGAAGGAGATATAatgtccc cggccgacgc
0150_Cm Rmet_4872 3' NdeI	TTTTCATATGactgctcagggcgatgtgattg
0151_Cm Rmet_3428 5' BglII	TTTTAGATCTTTTAAGAAGGAGATATAatgacg aacgtgaccaagactaacc
0152_Cm Rmet_3428 3' NdeI	TTTTCATATGcggcaggttgcggccc
0176_ MT 5' SmaI NdeI	TTTTCATATGGAAAACCTGTATTTTCAGGGC CCCGGGaacgatcaacgctgcgcgt
0177_MT 3' His Tag BamHI	TTTTGGATCCTTAGTGGTGGTGGTGGTGGTG gggcgagatcggatcactcg
0178_CupC(s) 5' SmaI NdeI	TTTTCATATGGAAAACCTGTATTTTCAGGGC atccagttccaagtcgaaggcat
0179_CupC(s) 3' His Tag BamHI	TTTTGGATCCTTAGTGGTGGTGGTGGTGGTG gcttgccgacttgaccgggt

Table S4. Intra-assay Precision of ELISA Performed in the Same Day and Using the Same AuNP-Ab-HRP Complex Solution

Purified CupC(s) (μg/mL)	Mean (RLU)	±SD (N=4)	% CV
0	22,173	1,423	6
0.161	19,486	1,579	8
0.321	25,169	3,911	16
0.482	28,640	1,742	6
1.44	34,368	2,991	9
2.89	41,534	3,469	8
4.33	41,568	1,045	3
LOD		**LOQ**	
0.41 μg/mL		1.88 μg/mL	

* RLU: relative luminescence units; SD: standard deviation; CV: coefficient of variation.

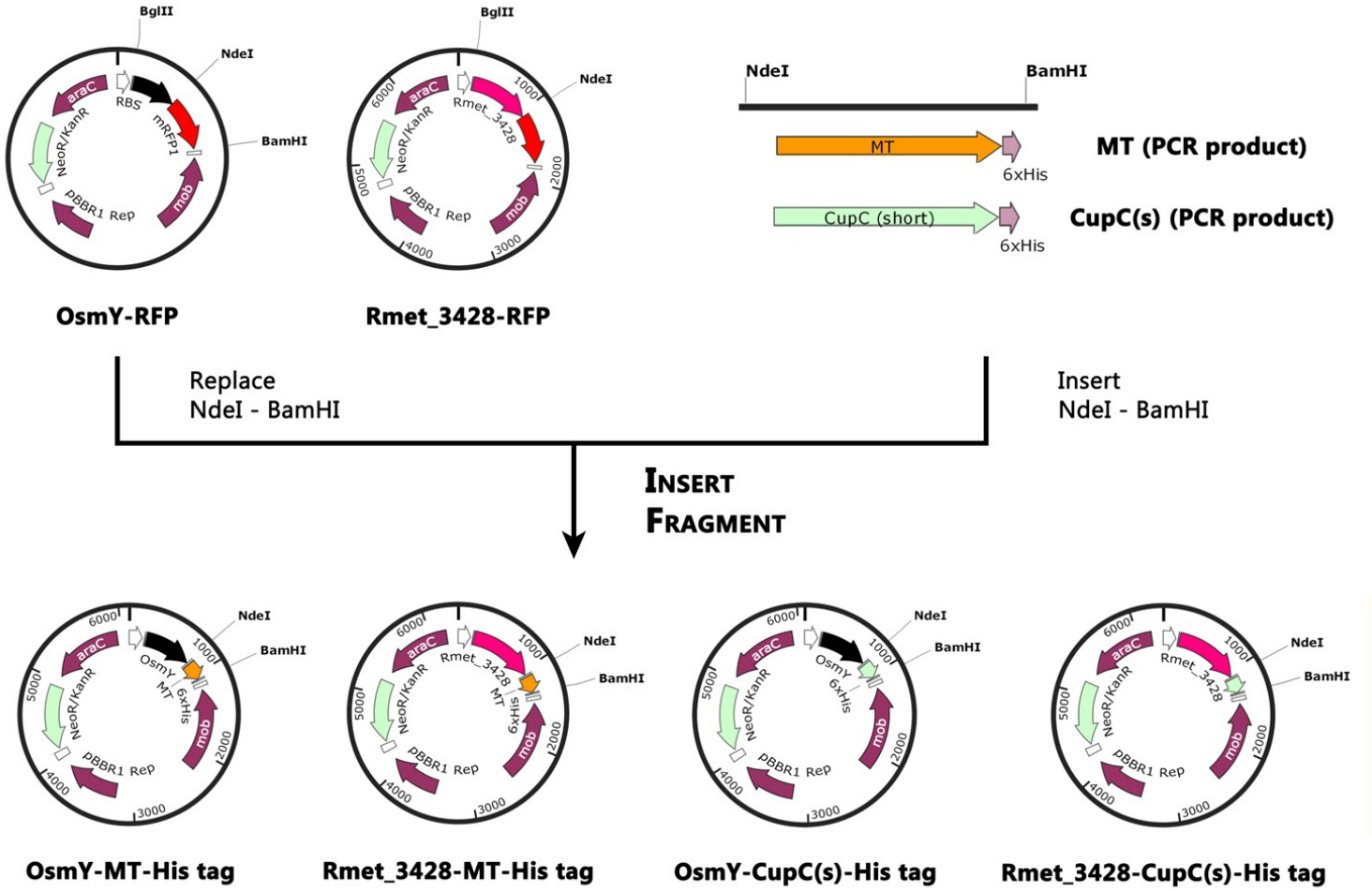


Figure S1. Cloning history of constructs with carrier-MT/CupC(s) fusions.

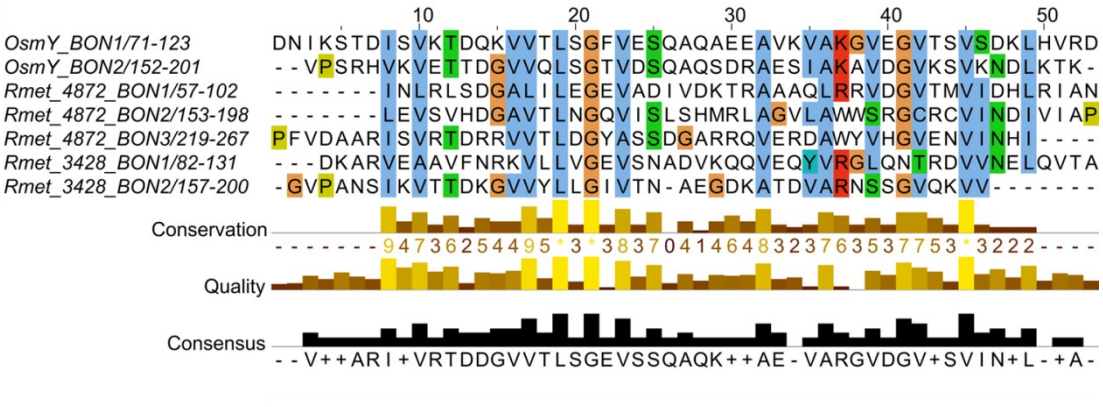


Figure S2. Alignment of BON domains in each carrier. BON domains were extracted from the sequences downloaded from NCBI, according to annotations labeled by conserved domain database (CDD). Alignment was performed in UGENE[3] with package of Clustal W[4], and displayed by Jalview[5] and ClustalX coloring. Sequences were named in following format: gene (or locus tag)_the order of BON from N-terminus/the location from parent sequences.

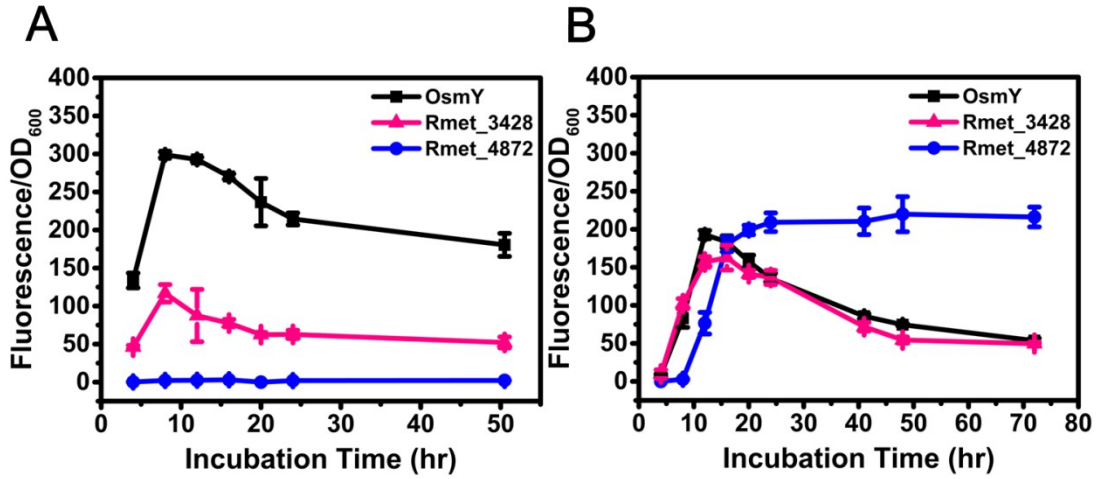


Figure S3. Normalized fluorescent intensity of cell pellets in (A) *E. coli* DH5α, and (B) *C. metallidurans* CH34. Incubation time is the time interval after adding arabinose.

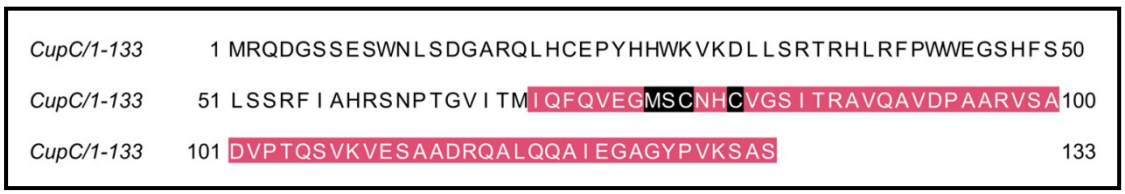


Figure S4. Amino sequence of CupC (GenBank ABF10397.1). Residues shaded in black and pink are metal-binding motif and truncated part, respectively. The truncated form, CupC(s), reserves only 65 residues.

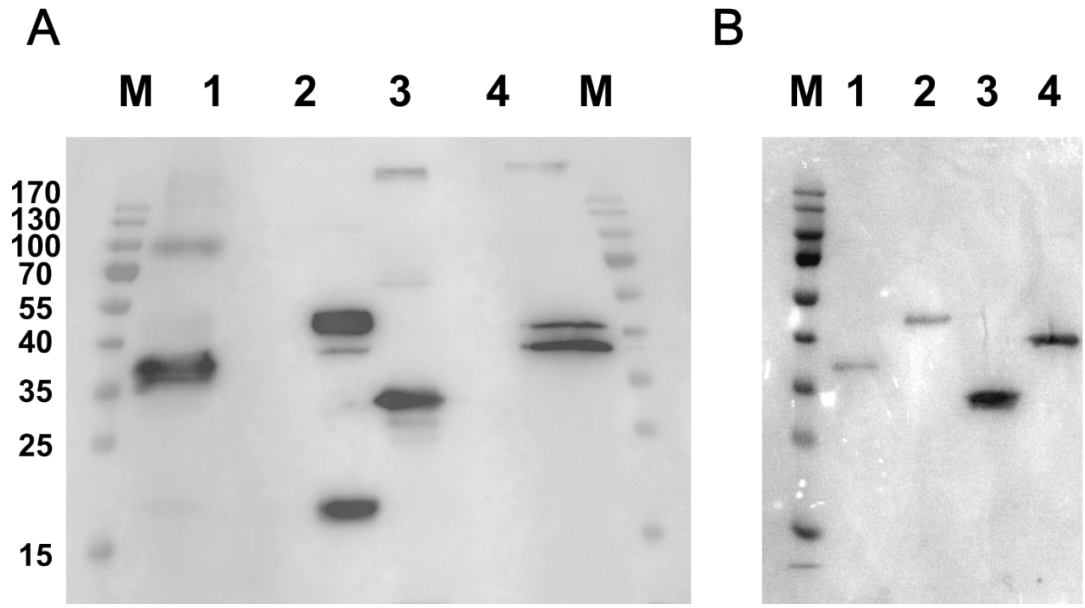


Figure S5. Western blot of (A) 10X supernatants of *E. coli* DH5α and (B) 1X supernatants of *C. metallidurans* CH34 expressing fusion proteins in LB medium. Cell culture was harvested at 24 h. **M**: protein marker, **1**: OsmY-MT (30.93), **2**: Rmet_3428-MT (39.11), **3**: OsmY-CupC(s) (29.73), and **4**: Rmet_3428-CupC(s) (37.91). Estimated molecular weight (kDa) is shown in parentheses.

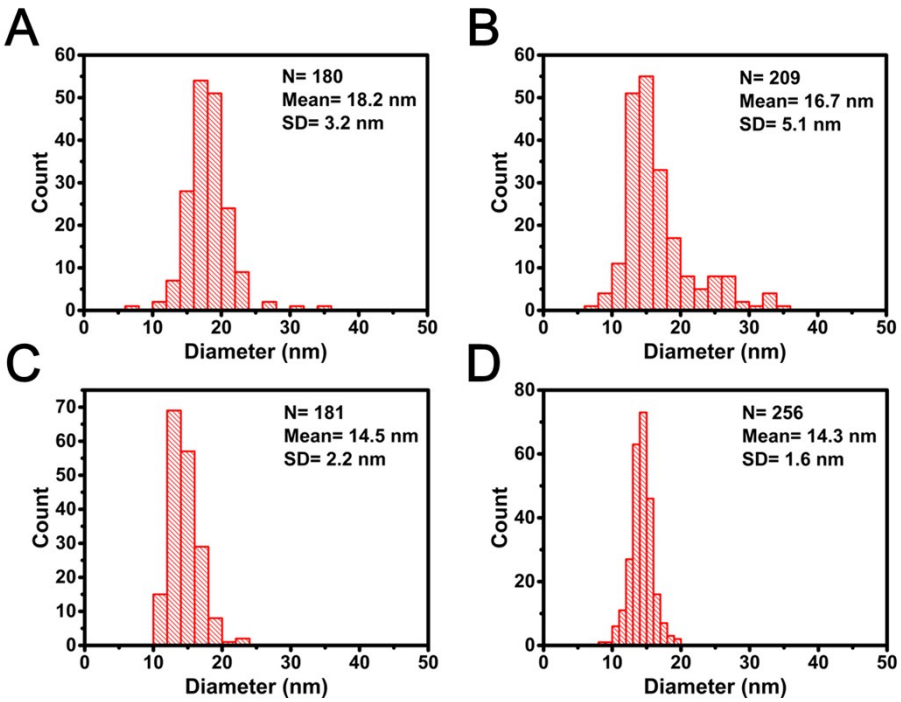


Figure S6. Histograms of diverse metal nanoparticles synthesized by supernatants of *E. coli* expressing Rmet_3428-MT. (A) Au, (B) Ag, (C) Pt, and (D) Pd NPs.

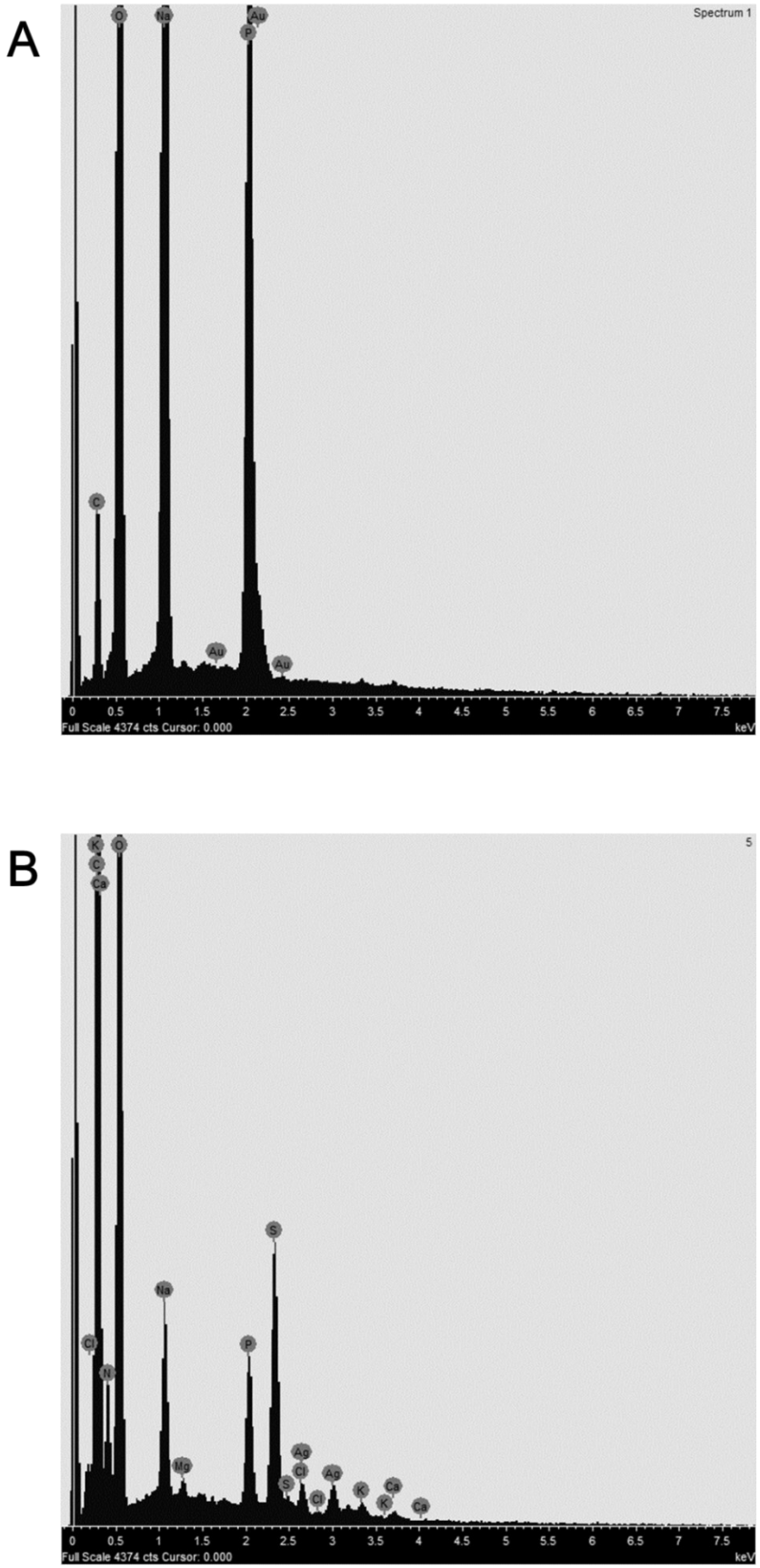
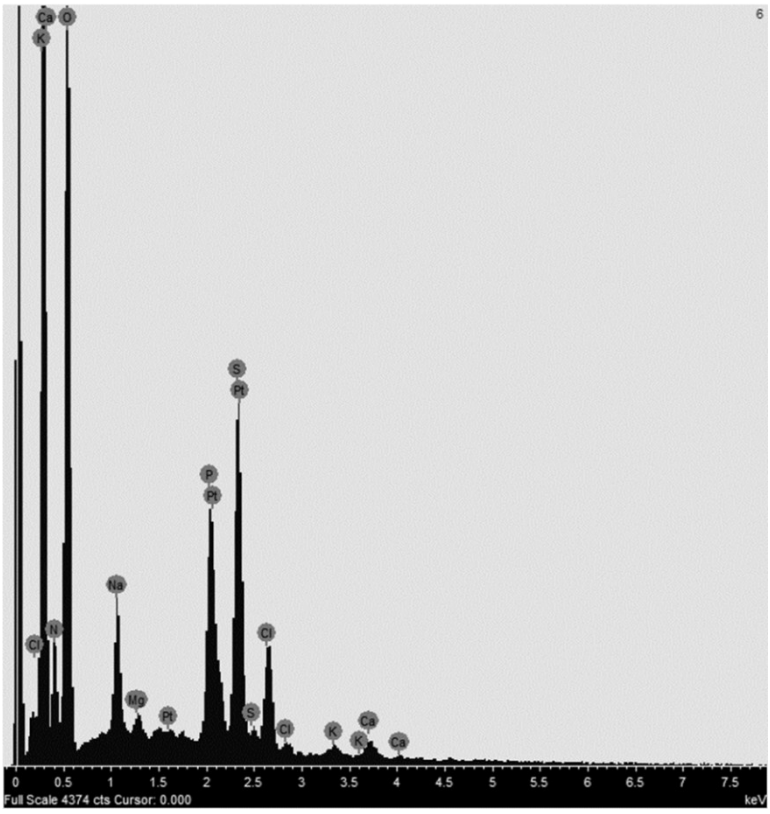


Figure S7. EDS spectra show the presence of (A) Au in Figure 2A, (B) Ag in Figure

2B, (C) Pt in Figure 2C, (D) Pd in Figure 2D, (E) Au in Figure 3A, and (F) Cu in Figure 3B.

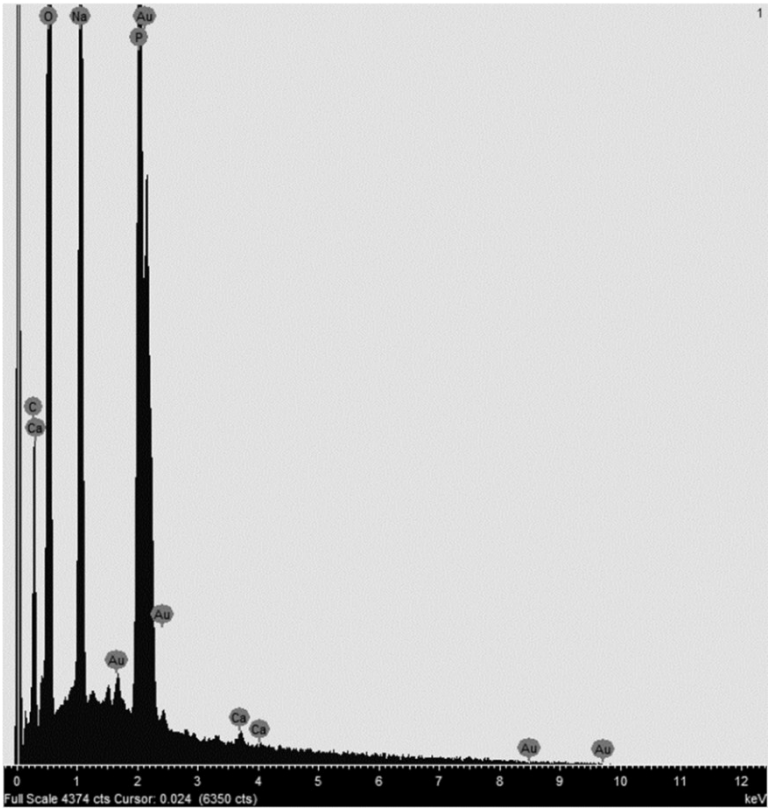
C



D

Figure S7. *Continued.*

E



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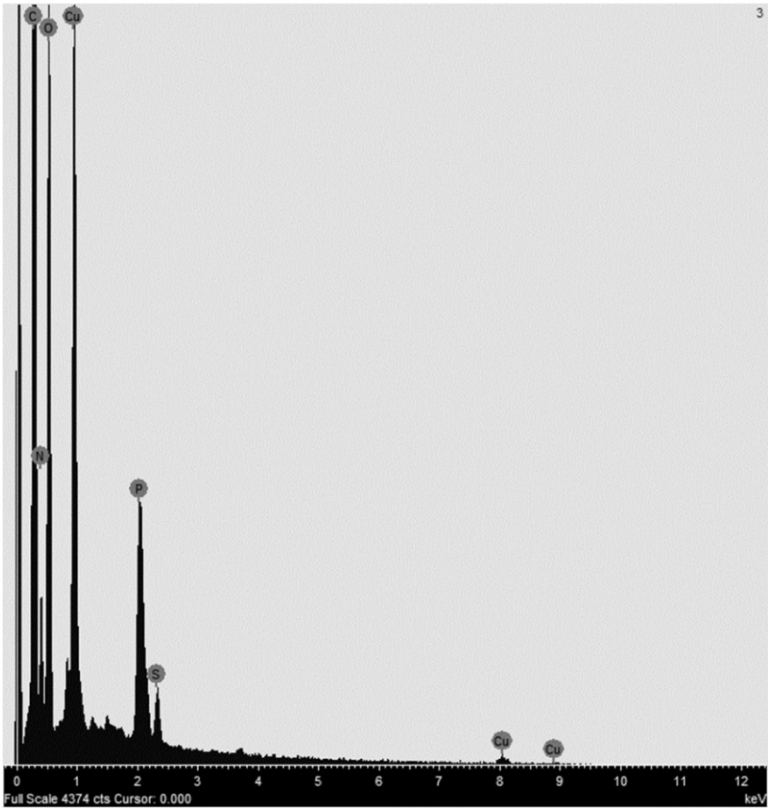

Figure S7. *Continued.*

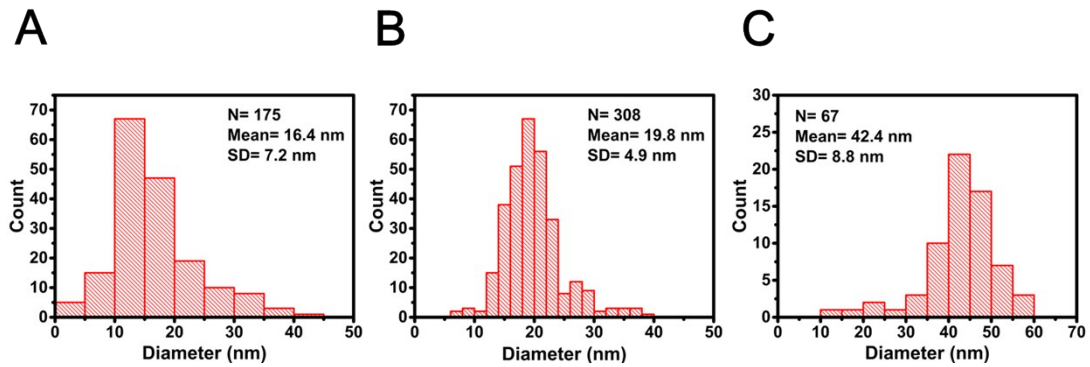

Figure S8. Size control of Au nanoparticles synthesized by *E. coli* expressing Rmet_3428-CupC(s). (A) 0.5 mM, (B) 1 mM, and (C) 2 mM salts with 2X supernatants.

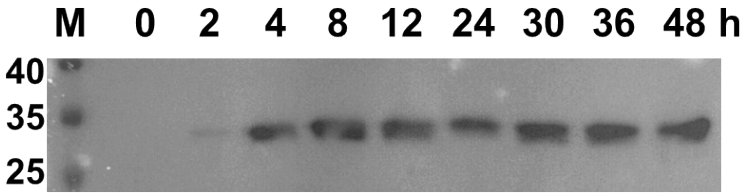

Figure S9. Western blot of 1X supernatants of *C. metallidurans* CH34 expressing 3428-CupC(s) in LB medium. Cell culture was harvested at indicated time points. **M**: protein marker.

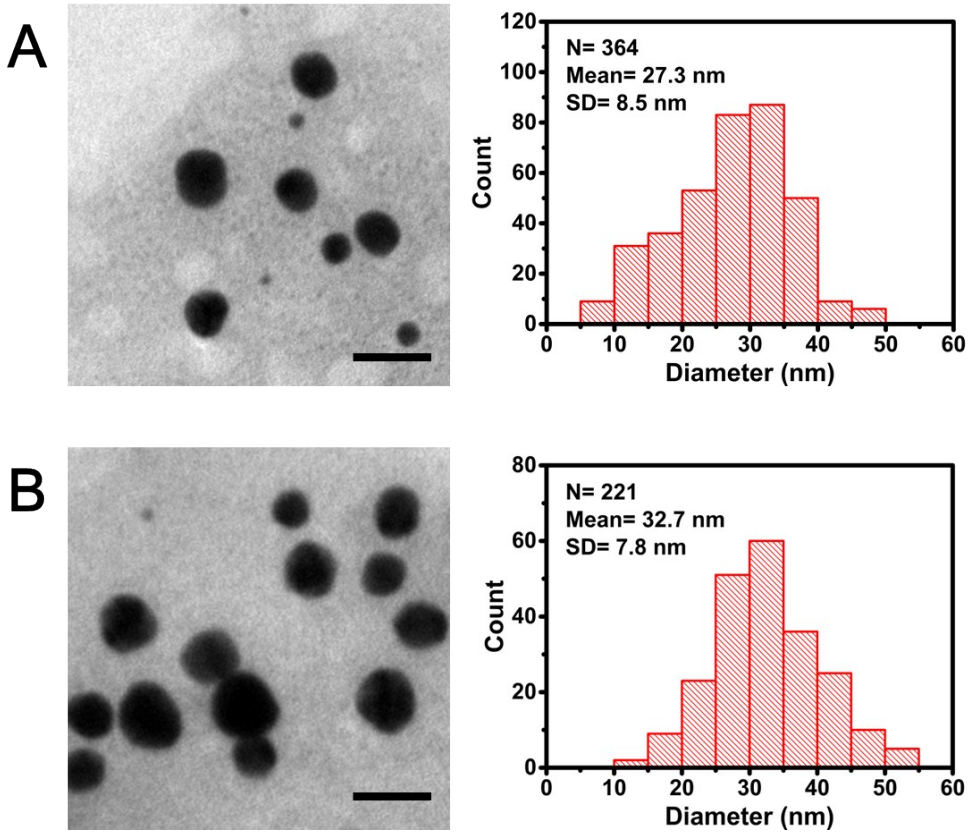

Figure S10. TEM images and corresponding histograms of gold nanoparticles synthesized by supernatants of *C. metallidurans* expressing Rmet_3428-CupC(s). 1 mM Au(III) and phosphate buffer were used. Reaction time: (A) 24 h, and (B) 48 h. Scale bar: 50 nm.

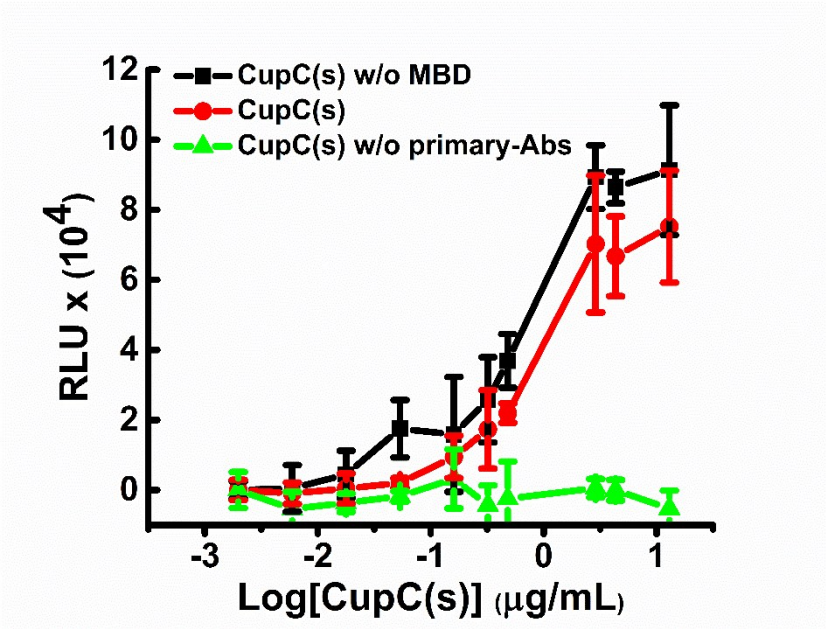

Figure S11. Detection of ELISA. Dose response curves of CupC(s) without metal binding domain (black) and CupC(s) (red) were tested at 0.002, 0.006, 0.018, 0.054, 0.161, 0.321, 0.482, 2.89, 4.33 and 13 μg/mL. Additional control (green line) was perform as the red line, except that the primary antibody was omitted from the procedure.

References:

1. Tsai, Y.-J.; Ouyang, C.-Y.; Ma, S.-Y.; Tsai, D.-Y.; Tseng, H.-W.; Yeh, Y.-C., Biosynthesis and display of diverse metal nanoparticles by recombinant Escherichia coli. *RSC Advances* **2014,** *4* (102), 58717-58719.

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5. Waterhouse, A. M.; Procter, J. B.; Martin, D. M. A.; Clamp, M.; Barton, G. J., Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **2009,** *25* (9), 1189-1191.