Supplemental Material

A molecular toolbox for the genetic manipulation of the stalked budding bacterium *Hyphomonas neptunium*

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SUPPLEMENTAL METHOD

Operon mapping

An overnight culture of *H. neptunium* was diluted into MB medium. When the culture reached an OD_{600} of approx. 0.5, $CuSO_4$ was added to a final concentration of 0.5 mM. After another 30 min of incubation, a 2 ml sample was taken and immediately centrifuged at 9,500 g for 10 min. To extract total RNA, the sediment was resuspended in 1 ml TRIzol® reagent (Invitrogen, Germany) per 1 x 10^7 bacterial cells. Cells were lysed by repeated up-and-down pipetting and incubated for 10 min at 65°C. After the addition of 200 µl chloroform per 1 ml TRIzol® reagent, the sample was mixed by shaking the tube vigorously by hand and was then incubated at room temperature for 5 min with agitation. The mixture was then transferred to a phase-lock tube (5PRIME, Germany) and centrifuged for 15 min at 12,000 g and 4°C. The upper, aqueous phase was transferred to a fresh tube, and the RNA was purified using the PureLink™ RNA Mini Kit (Invitrogen, Germany) according to the manufacturer's instructions. Contaminating DNA was digested using the Turbo DNA-free Kit (Ambion). Total RNA was reverse-transcribed into cDNA using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany) according to manufacturer's instructions. To test for genomic DNA contamination, a control without reverse transcriptase was prepared for every cDNA synthesis reaction. Subsequently, PCR reactions with different primer pair combinations (see Figure S5A and Table S1) were performed with cDNA as a template. In addition, a positive control reaction with genomic DNA as a template was performed for every primer pair.

SUPPLEMENTAL TABLE

Table S1. Oligonucleotides used for operon mapping.

Name	Sequence
HNE_1483-1484 fw	gcggtgcggggtgtctgccttcg
HNE_1483-1484 rv	cggggtcaactggaaagcctcaactgg
HNE_1484-1485 fw	gcagcagagcaggcgcgtcatcac
HNE_1484-1485 rv	agcctgccgaacgcctgtcctttgatg
HNE_1485-1486 fw	ggatgagcgggccgggcagggttc
HNE_1485-1486 rv	ggctgggacgcaatgacgatggacttc
HNE_1482-1483 fw_neu	ctcggccagcaggaactcgggataaag
HNE_1482-1483 rv	gccctgtcttctgtctccgtgatcgc
HNE_1486-1487 fw	taggggcgtgatcgattgtggcttttg
HNE_1486-1487 rv	tcaatgcaataccggccttcctcaac

SUPPLEMENTAL FIGURES

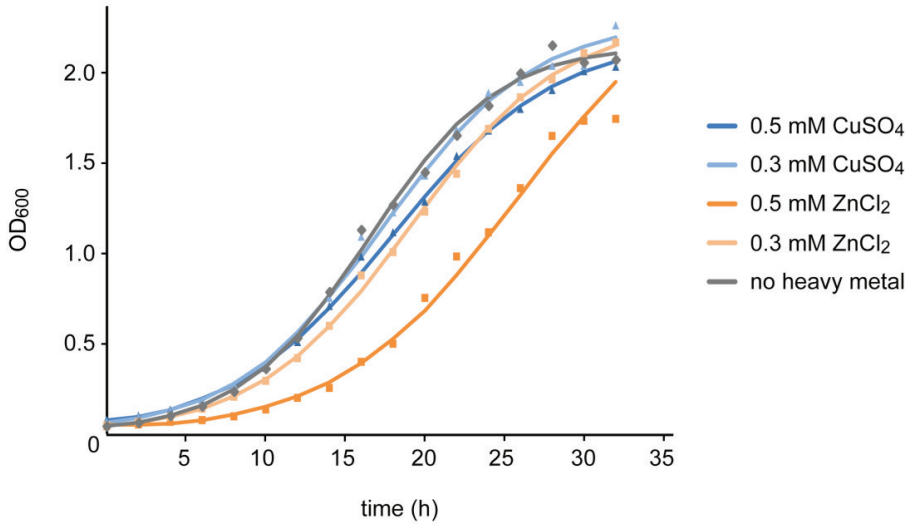

Figure S1. Growth rates of *H. neptunium* treated with varying concentrations of different heavy metals. A pre-culture was diluted into 20 ml MB medium, supplemented with $ZnCl_2$ or $CuSO_4$, respectively, at the indicated concentrations, to obtain an initial OD_{600} of ~0.1. Cell growth was then monitored over 32 h. The data were fitted as described previously (Huang, 2011; see main text).

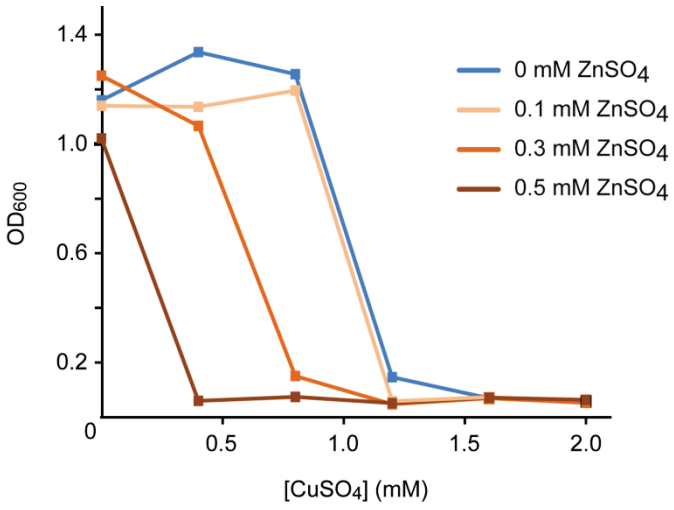

Figure S2. Combined effect of copper and zinc on the growth of *H. neptunium*. MB medium supplemented with the indicated concentrations of $CuSO_4$ and $ZnSO_4$ was inoculated with a pre-culture of *H. neptunium* (initial OD_{600} = 0.05). Growth was assessed spectrophotometrically after 24 h of incubation.

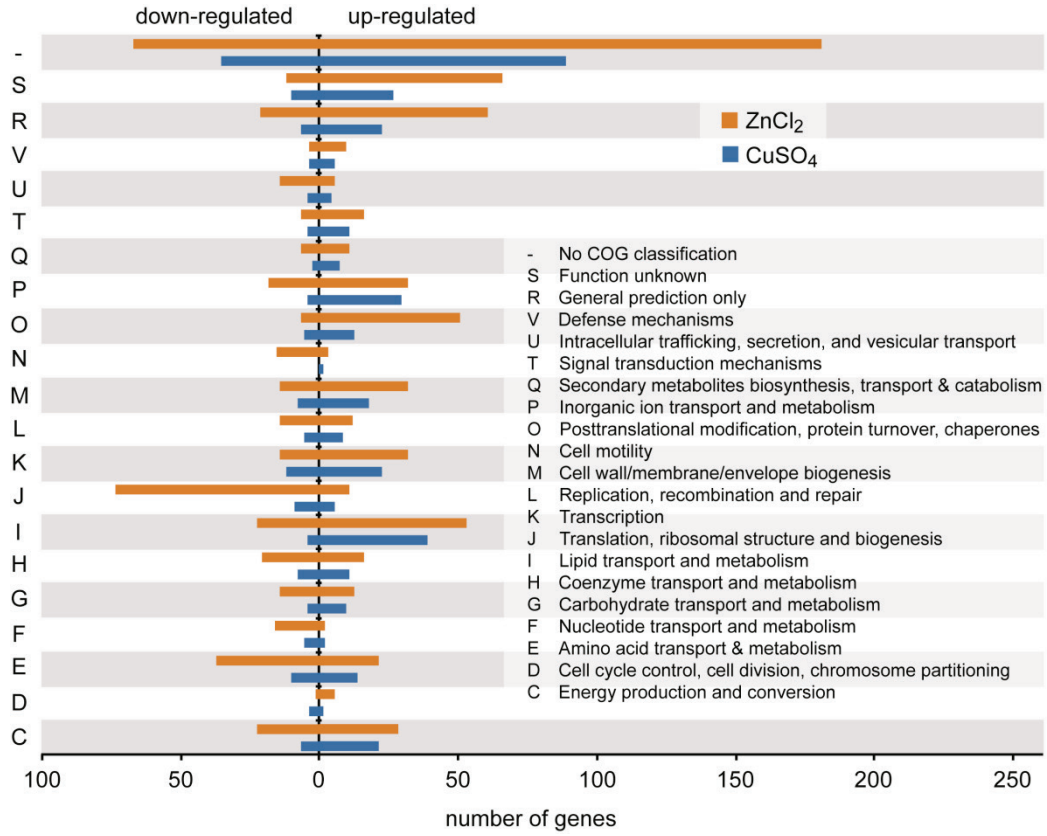

Figure S3. Genes significantly regulated in conditions of heavy metal stress. Functional classification of genes that were significantly regulated in cells treated for 60 min with $ZnCl_2$ and $CuSO_4$, respectively, according to their COG categories.

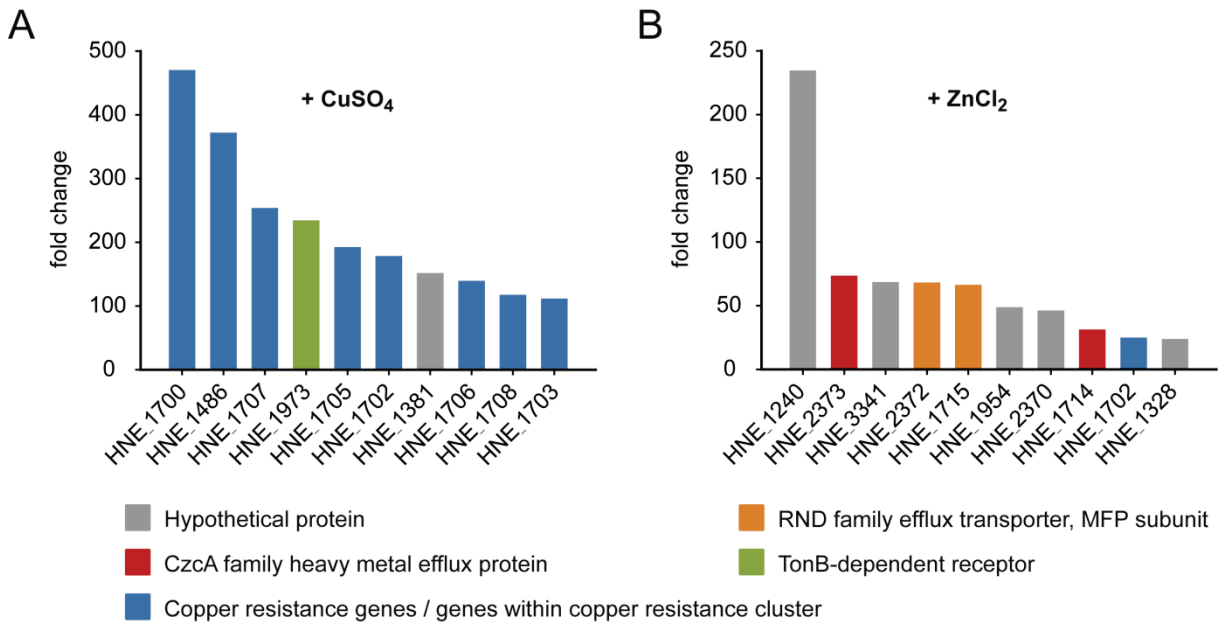

Figure S4. Genes most highly up-regulated under heavy metal stress. The graph shows the changes observed in the transcript levels of the ten genes that are most highly up-regulated after a 20-min pulse of $ZnCl_2$ or $CuSO_4$ (0.5 mM), respectively. The color of the bars indicates the putative function of the corresponding proteins.

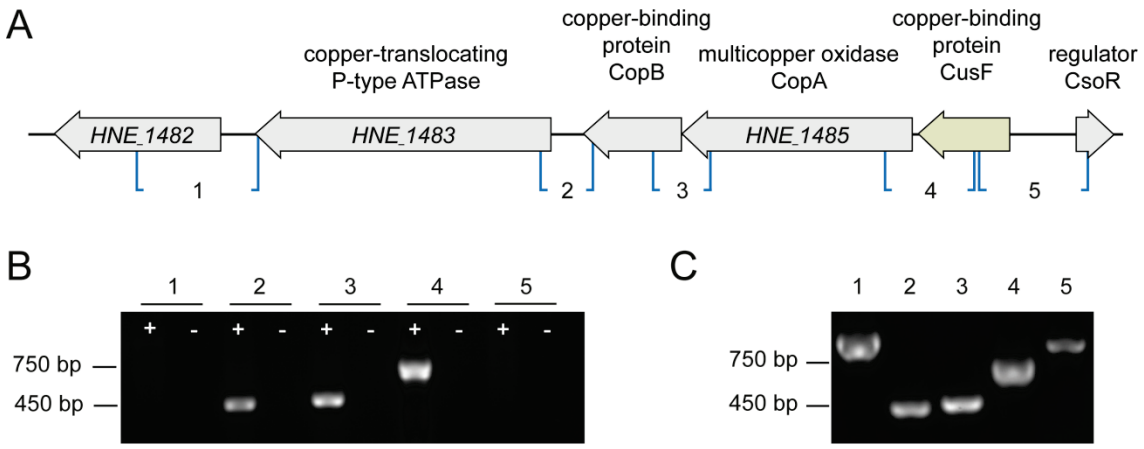

Figure S5. Mapping of the *HNE*_1486-*HNE_1483* operon. (A) Genomic context of *HNE_1486*. Blue bars indicate the annealing sites of PCR primers (see Table S1). Numbers between brackets refer to the identity of the PCR products shown in (B) and (C). **(B)** Operon mapping. Cells were induced for 30 min with 500 µM $CuSO_4$. Total RNA was extracted and reverse-transcribed into cDNA, which was then used as a template for PCR reactions with primer pairs flanking all intergenic regions in the cluster (+). PCR reactions on samples, in which no reverse transcriptase had been added in the cDNA synthesis step served as negative controls (-). PCR products were analysed on a 1% agarose gel. **(C)** Positive controls for the PCR reactions. Shown are the products of PCR reactions performed with the same primers as in (B) and chromosomal DNA of *H. neptunium* as a template.

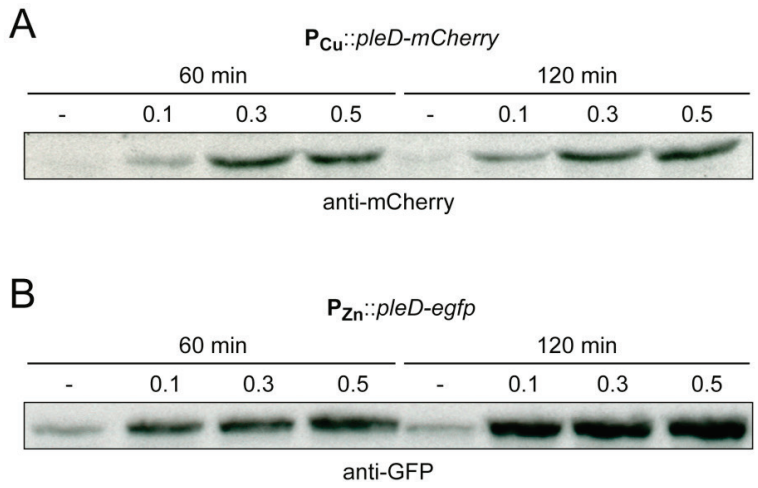

Figure S6. Dependence of P_{Cu} and P_{Zn} activity on the heavy metal concentration. (A) Analysis of P_{Cu}. Cells of strain EC85 (P_{cu}::P_{cu}-*HNE_2284-mCherry*) were grown to exponential phase in MB medium and induced with the indicated concentrations (in mM) of $CuSO_4$. At the indicated time points, samples were taken and subjected to immunoblot analysis with an anti-mCherry antibody. **(B)** Analysis of P_{Zn}. Cells of strain SE107 (P_{zn}::P_{zn}-*HNE_2284-egfp*) were induced with the indicated concentrations of $ZnSO_4$ and analyzed by immunoblotting with an anti-GFP antibody.