The Effects of Cupriavidus *Metallidurians* on Plant Growth in Areas with Heavy Metal Runoff

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ABSTRACT

This project measures the effects of *C. metallidurians* on agricultural plant growth in environments with heavy metal runoff. Heavy metals have been known to negatively impact the environment specifically in the form of runoff. Heavy metals usually accumulate plant roots and inhibit nutrient and water transportation within plants. lIn this project, heavy metals being tested include Zinc, which prevents nutrients from being transported to the leaves; Nickel, which inhibits transpiration and photosynthesis and impairs metabolism; and Copper, which can cause protein denaturation and corrupt soil viability. *C. metallidurians* are a type of bacteria known to have heavy metal resistant plasmids and plasmid determinants specifically directed towards a different heavy metal each, with the cnr being resistant to copper and nickel, chr being resistant to copper and zinc, and cop being resistant to specifically copper. *C. metallidurans* have previously been known to detoxify high concentrations of copper and gold. They more famously have been known to pump out gold nuggets from exposure to copper metals. Since the effects of other heavy metals on *C. metallidurans* are not as well studied, this project aims to study the effects of those other heavy metals such as Zinc, Nickel, and Copper and apply this interaction to a real-life scenario of agriculture to see if this bacteria may be able to minimize the damage of Heavy Metal Runoff pollution.

I. Introduction

I. Background

Heavy metal pollution mainly comes from industry, agriculture, and mining. This heavy metal pollution has been affecting the agricultural sector by accumulating in the soil and its uptake in plants. Heavy metals are categorized as from the safest to the most dangerous based on its permissible limits. (In plants, the limit of Cu is the highest (up to 25 mg/kg), followed by Zn and Ni (Cruz et al., 2022).) Heavy metals severely damage the soil and plants in high concentrations. Therefore, it is considered a toxicant.

Nickel is absorbed through the plant roots and taken up through the soil. Nickel pollution has increasingly become a problem. Nickel is transported through the xylem and accumulates in the neonatal parts of the plant (Ahmad & Ashraf, 2011). Nickel is important for plant metabolism and growth. However, an excess amount of Nickel can decrease the germination rates greatly and affect nutrient absorption rates like most heavy metals.

Zinc is a metal that is important for all living systems. They help with enzymic activities and photosynthetic redox reactions within the plant. However, when plants have an excess amount of zinc, the accumulation of zinc in plant roots or shoots can cause severe damages. When this occurs, Zinc results in high plant agitation and eventually plant death. A study showed that an excessive amount of zinc within the plant led up to chlorosis (lack of chlorophyll in leaves) for younger leaves during early exposure (Ebbs et al., 2016). Zinc in the root system prevents nutrients from being transported to the leaves and dry matter (feed after water evaporates from it) productivity is negatively affected. Zinc is transported to the roots through divalent cation channels.

Copper is the least deadly heavy metal, but can still cause negative effects in plants when exposed to great amounts. Copper availability is affected by soil pH. The natural range of Cu concentration in the soil is between 5 and 30 mg/kg. An effect of Cu on an enzyme was reported to be a negative correlation between Cu and urease activity (Caetano et al, 2015) . Urease is an extracellular enzyme that can break down the organic matter of soil. These results were similar to a study done by (Gulser et al, 2013), which showed that Cu toxicity can destroy a cell membrane and cause protein denaturation.

To minimize the damage done on agricultural plants through HMR, Cupriavidus metallidurans will be used as it is found in nature to be able to process heavy metals. C. metallidurans is known to be able to process copper in nature using its CopA and CupA enzyme; However, its effect on other heavy metals are not as well studied.
C. metallidurans are known to have copper resistant enzymes. One of the enzymes is known as CupA. This enzyme pumps out excess copper ions from the bacteria. A study showed the long term survival of C. metallidurans in wet contact with metallic copper (Maertens et al, 2019). Viability and membrane permeability was examined through viable counts and flow cytometry. Flow cytometry is a method using dyes to view the stages of the cell cycle. When exposed to copper metal concentrations, no recovery was observed, but the recovery was observed when copper resistance mechanisms were pre-induced. The results showed long-term bacterial survival in presence of copper is possible when induction of metal resistant mechanisms occurred.

C. metallidurans are not only highly resistant to heavy metals but they reduce the heavy metal complexes by biominerizing them into nanoparticles like gold nanoparticles. The pMOL30 and pMOL28 plasmids are found within the CH34 strain (see other required procedures). Through the cloning and sequencing of multiple fragments from the plasmids, many metal resistant determinants were found such as czc and pbr on the pMOL30 plasmid and the cnr and chr on the pMOL28 plasmid.

Three putative genomic islands on pMOL28 and pMOL30 plasmids with metal resistant operons flanked by mobile genetic elements. Metal-mediated upregulation of 83 genes on pMOL28 and 143 on pMOL30. Through cloning and sequencing, determinants of resistance (cnr, chr, and mer on pMOL28 and czc, pbr, mer, sil, and cop for pMOL30) were found (Monchy et al., 2007). Mechanisms of resistance included chemiosmotic efflux of cations with proton antiporters (secondary transport of two or more molecules/ions across a membrane) in the (HME-RND family (Heavy Metal Efflux of the Resistance Nodulation Division, which are a category of bacterial pumps in the cytoplasmic membrane)), cation diffusion facilitators (transmembrane proteins that provide tolerance to divalent metal ions by removing them), and P-type ATPases (ion and lipid pumps found in bacteria using ATP hydrolysis to pump across a membrane) for cytoplasmic detoxification.

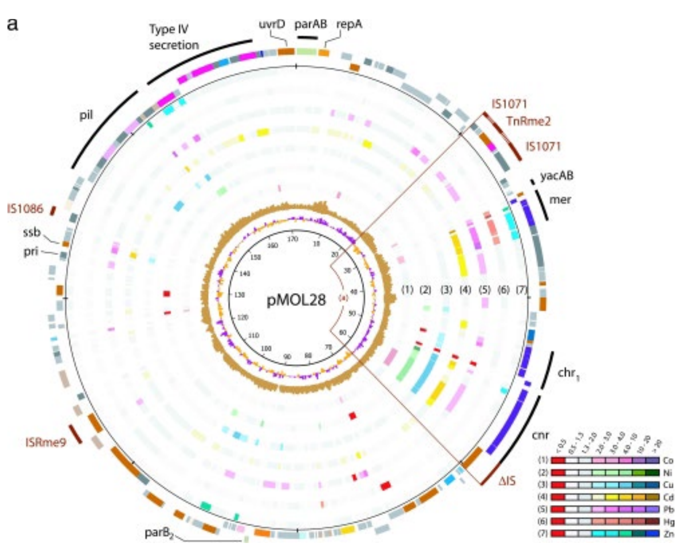


Figure 1: Plasmid map for pMOL28 plasmid (Monchy et al., 2007)

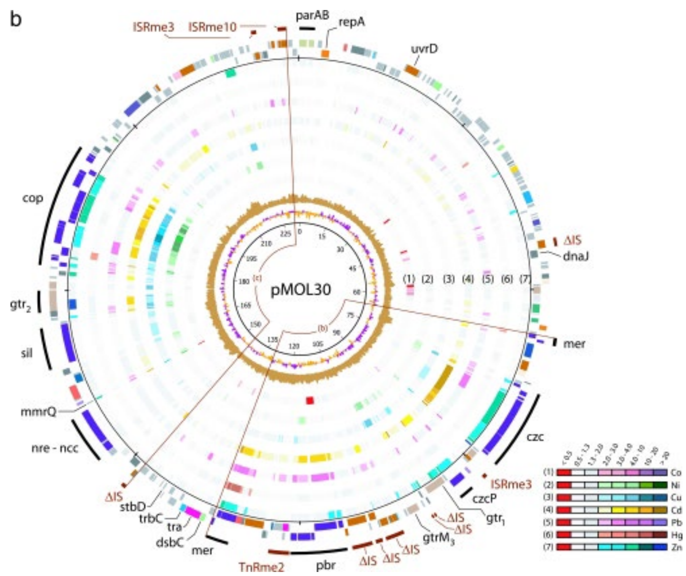


Figure 2: Plasmid map for pMOL30 plasmid (Monchy et al., 2007)

Both plasmid sequences begin at the ATG codon of the parA gene (essential for plasmid partition). Layers 1-7 represent the gene expression for cobalt, copper, nickel, cadmium, lead, mercury, and zinc. The a on the pMOL28 plasmid represents the cnr-chr-mer genomic island while the b and the c on the pMOL30 plasmid represent the mer-pbr-czc island and the copper island respectively.

II. Scientific Goals

The goal of this experiment is to hopefully find out that *C. metallidurans* will help agricultural plants grow more efficiently in the event of heavy metal pollution in the soil. This will help the agricultural field as *C. metallidurans* might be able to be used in heavy metal polluted environments.

III. Hypotheses

- Null: If *C. metallidurans* are put in the soil around plants in a heavy metal concentrated environment, then the concentration of heavy metals in plants will not decrease compared to an environment of no heavy metals
- Alternative: If the *C. metallidurans* are placed in the soil surrounding plant then the resulting heavy metal concentrations in plants exposed to C. metallidurans will be significantly less than those not exposed to *C. metallidurans*

II. Materials and Procedures

Prior to any procedures PPE will be worn to avoid harmful contact with contaminants and bacteria. Chemical mixtures will be worked with in the fume hood. *C. metallidurans* are the main bacteria used in the study. An incubation period occurred on Nutrient Broth Agar plates and through Nutrient Broth and an appropriate concentration of heavy metals to induce proper resistance on the bacteria.

I. Plant growth and bacteria induction

Arabidopsis are grown in a 3x4 growing grid with 4 seeds put into each box. Plants are watered by covering the bottom of the containers with water for 5 min. Plants are grown to mid-age. Proper concentrations of each metal are measured (.005843g for CuSO4, .006466g for ZnCl2, and .02569g for NiCl2). These concentrations exceed the normal threshold for metal concentration by around 100 mg/kg. 10mL of water is combined with the measured out concentration of metal and watered on plants 2 times a week for 2 weeks. Half of the boxes in each grid were added with *C. metallidurians* while the other half wasn't.

II. Aqua Regia Digestion Method

1g of soil was grinded and transferred into a 500mL beaker. 10 mL of nitric added then heated for 10 min followed by 10 min of cooling. Another 5mL nitric acid is added and heated for 30 min followed by 10 min of cooling. 1mL of hydrogen peroxide was added until the solution stopped bubbling. Liquid was heated until volume reached 1mL, then 10mL of hydrochloric acid was added.The solution was diluted to 100mL using distilled water. Then each solution was filtered and transferred to another container.The filtered solution is put into the mass spectroscopy to find the concentration of metals in the soil.

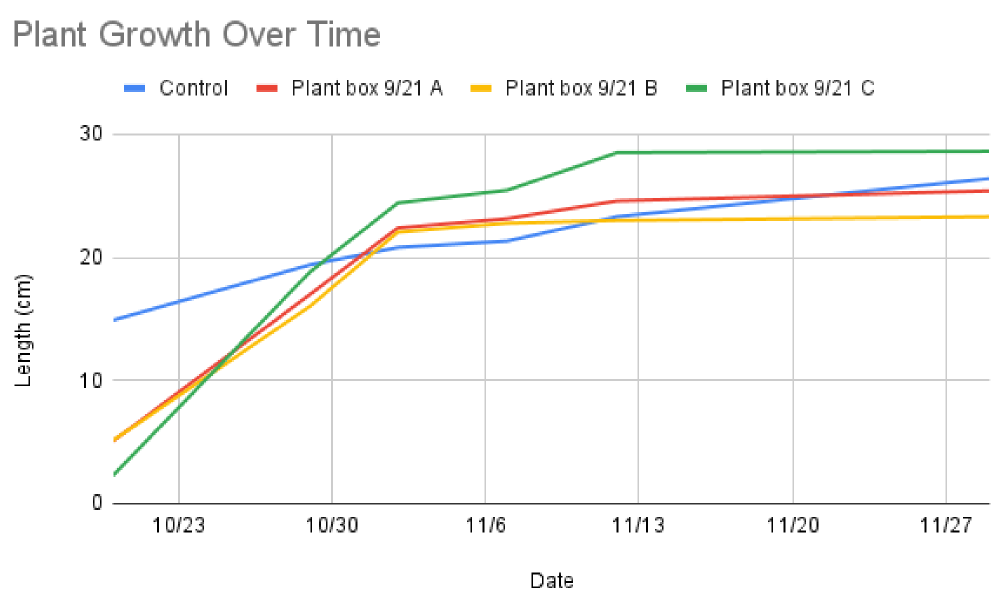
III. Dry Mass Measurement

Original mass of plant measured. At least 3 plants from each environment were taken. Dry the plants in a low heat oven (100 degrees F) overnight. Final mass of the plant was measured.

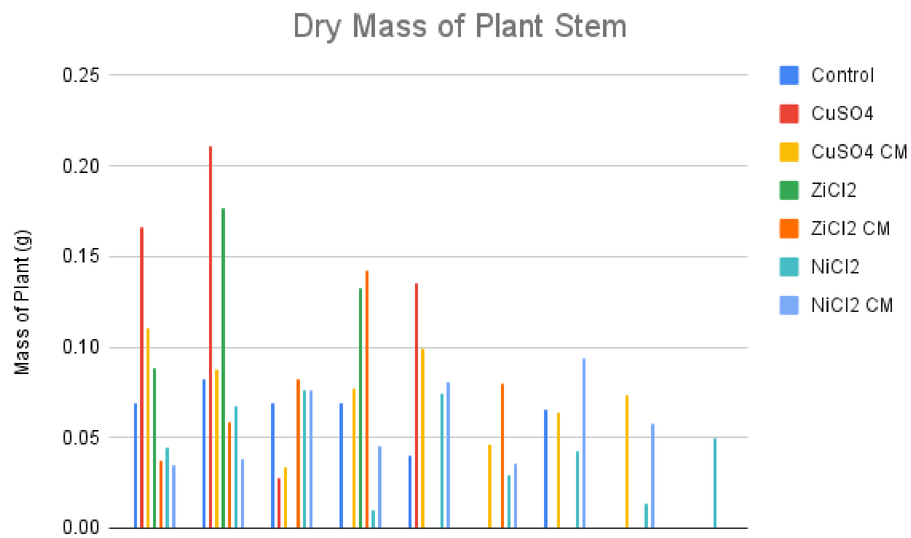
III. Absorption Assay

Arabidopsis leaves are mixed with 30 mL of 70% ethanol and grinded with mortar and pestle. Solution is filtered through filter paper and poured into the beaker. Solution in the beaker is poured into cuvettes and absorbance is measured in the spectrometer at wavelengths of 450 to 690 at intervals of 40 nm.

III. Results and Conclusions

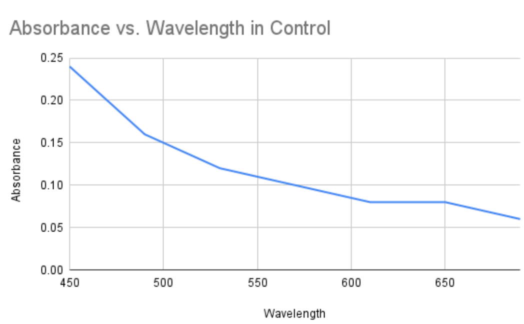


Graph 1: Plant growth shows the control growing the best compared to the plants being exposed to heavy metals which is expected.

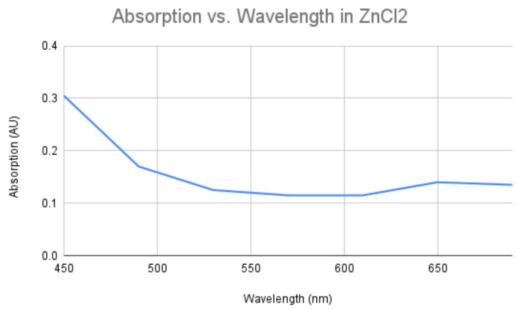


Graph 2: Dry mass shows that nickel and zinc metals cause plants to lose more water, resulting in an increase in dry mass change.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.02710943073	6	0.004518238455	3.100297202	0.01804270737	2.432434105
Within Groups	0.04226334015	29	0.001457356557			
Total	0.06937277087	35				



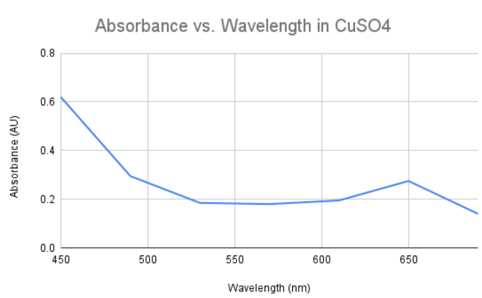
Graph 3: Plants in the control have the most absorption at wavelength of 450 and becomes less as wavelength increases



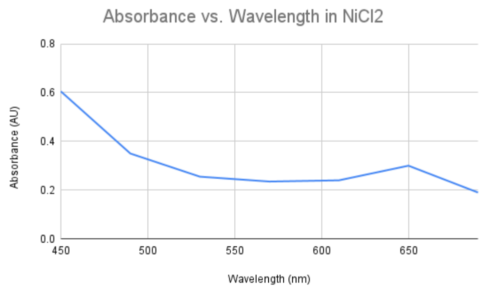
Graph 4: Zinc behaves similarly to the control but absorbed more after the 650 nm wavelength

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1136671.25	1	1136671.25	304.4653558	0.0000000006817437725	4.74722522
Within Groups	44800.0232	12	3733.335267			
Total	1181471.274	13				

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1136520.237	1	1136520.237	304.4248774	0.00000000068 2268686	4.74722522
Within Groups	44800.02739	12	3733.335616			
Total	1181320.265	13				



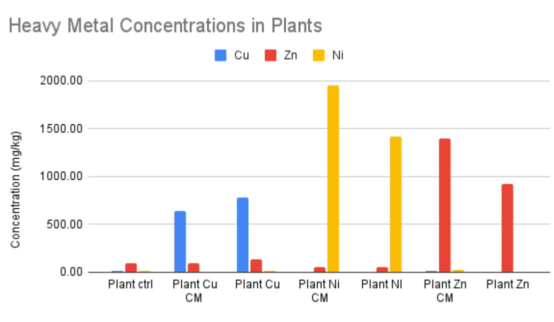
Graph 5: Plants exposed to copper absorbed lengths significantly more than the control and there's an irregular peak at the 650 nm wavelength



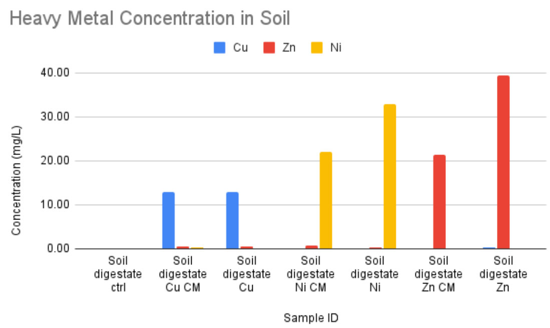
Graph 6: Nickel behaves similarly to copper and the absorption pattern is almost exactly the same.

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1136072.955	1	1136072.955	304.3041623	0.000000000683836876	4.74722522
Within Groups	44800.161	12	3733.34675			
Total	1180873.116	13				

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1135910.588	1	1135910.588	304.2609722	0.00000000068 43989819	4.74722522
Within Groups	44800.11667	12	3733.343056			
Total	1180710.705	13				



Graph 7: Heavy metal concentrations can be seen being much greater in those plants exposed to *C. metallidurans* and metals compared to just metals in all metals besides copper.



Graph 8: *C. metallidurans* seem to decrease the concentration of heavy metals in the soil of all three heavy metals.

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	287180.5104	2	143590.2552	0.3899978767	0.6826422097	3.554557146
Within Groups	6627278.63	18	368182.1461			
Total	6914459.14	20				

ANOVA						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	106.2383782	2	53.11918911	0.3424200976	0.7145753907	3.554557146
Within Groups	2792.316837	18	155.1287132			
Total	2898.555215	20				

IV. Discussion

I. Conclusion

C. metallidurans seem to have a positive effect on the plant in a heavy metal concentrated environment. The dry mass assay shows that *C. metallidurans* could possibly negate the effects metals have on the plant as plants with *C metallidurans* and metals behave more similarly to the control than the plants exposed to metals.

The absorption assay shows that heavy metals provide and increase absorption in plants at multiple wavelengths showing a possible positive impact on how well plants can absorb lights of multiple wavelengths when exposed to these heavy metals. To test the extent of this relationship, chlorophyll concentrations could be measured from the leaves in future testing. The final mass spectroscopy assay shows that although these bacteria didn't prevent metals from entering the plant as well as hoped, it could be useful in these metal deficient environments as it helps the plants absorb Nickel and Zinc as shown from the graphs. Further testing is needed on the usefulness of these bacteria in a metal deficient environment as this project's aim was to test bacteria usefulness on a heavy metal excess environment.

II. Applications

The results of this experiment could be mainly applied to the agricultural field. One of the main problems in agriculture when located next to industrial buildings is heavy metal pollution. These heavy metals tend to stay in the soil and when plants are planted, they absorb this heavy metal which negatively affects the plant. *C. metallidurans* seems to be able to sufficiently remove heavy metals from the soil allowing farmers whose crops are exposed to heavy metal pollution in the soil to be placed in less polluted environments.

III. Limitations and Error Analysis

One of the principal errors of this experiment was that the plants used were very old by the time they were exposed to heavy metals. This was due to the bacteria not arriving on time and the plants being planted too early. This could be different from the way younger plants respond to the bacteria and heavy metals. The concentration of heavy metals could have also been measured incorrectly as the mass of the required heavy metal concentration is very small.

Limitations of this experiment include the different types of metal used. While the three types of metals used are very commonly seen in heavy metal pollution, these are not the only metals. Since the *C. metallidurans* reacted with copper differently than zinc and nickel, maybe C. metallidurans will react better to a different type of metal that was not tested in the experiment.

IV. Future Research

For the absorbance assay, plants exposed to heavy metals generally have a greater absorbance rate than their *C. metallidurans* added counterparts as evidently shown by the absorbance value from the graphs. This shows that the plants being exposed to these heavy metals actually might have beneficial effects on plant light absorbance. Further testing could prove if these heavy metals increase absorbance by increasing chlorophyll response or another method. Based on the mass spectroscopy assay, it can be seen that the plants being exposed to the bacteria actually absorbed more heavy metals than those not exposed to the bacteria when tested with zinc and nickel. This shows a possibility of the bacteria helping the plant absorb these heavy metals. Although this may not be helpful in heavy metal excess environments, they could be useful in heavy metal excess environments and future testing can be used to confirm this.

VI. References

Alengybawe, A., Adelkhalek, S. T., Qureshi, S. R., & Wang, M. Q. (2021). Heavy Metals and Pesticides Toxicity in Agricultural Soil and Plants: Ecological Risks and Human Health Implications. *Toxics.*, *9*(3). https://doi.org/10.3390/toxics9030042

Ali, M. M., Provoost, A., Maertens, L., Leys, N., Monsieurs, P., Charlier, D., & Houdt, R. V. (2019). Genomic and transcriptomic changes that mediate increased platinum resistance in Cupriavidus metallidurans. Genes, 10(1), 63. https://doi.org/10.3390/genes10010063

Grass, G., Grobe, C., & Nies, D. H. (2000). Regulation of the cnr Cobalt and Nickel Resistance Determinant from Ralstonia sp. Strain CH34. Journal of Bacteriology, 182(5). https://doi.org/10.1128/JB.182.5.1390-1398.2000

Lal, D., Nayyar, N., Kohil, P., & Lal, R. (2013). Cupriavidus metallidurans: A Modern Alchemist. Indian J Microbiol, 53(1), 114-115. https://doi.org/ 10.1128/JB.00375-07

Maertens, L., Coninx, I., Claesen, J., Leys, N., Matroule, J.-Y., & Van Houdt, R. (2020). Copper resistance mediates long-term survival of cupriavidus metallidurans in wet contact with metallic copper. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01208

Monchy, S., Benotmane, M. A., Janssen, P., Vallaeys, T., Taghavi, S., Lelie, D., & Mergeay, M. (2007). Plasmids pMOL28 and pMOL30 of Cupriavidus metallidurans Are Specialized in the Maximal Viable Response to HeavyMetals. *J Bacteriol*, *189*(20), 7417-7425. https://doi.org/10.1128/JB.00375-07

Rensing, C., Fan, B., Sharma, R., Mitra, B., & Rosen, B. P. (2000). CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. *Proc Natl Acad Sci U SA.*, *97*(6), 652-656. https://doi.org/10.1073/pnas.97.2.652

Scherer, J., & Nies, D. H. (2009). CZCP is a novel efflux system contributing to transition metal resistance in*cupriavidus metallidurans*CH34. *Molecular Microbiology*, *73*(4), 601–621. https://doi.org/10.1111/j.1365-2958.2009.06792.x

Sota, M., Yano, H., Nagata, Y., Ohtsubo, Y., Genka, H., Anbutsu, H., Kawasaki, H., & Tsuda, M. (2006). Functional analysis of Unique Class II insertion sequence is 1071. Applied and Environmental Microbiology, 72(1), 291–297. https://doi.org/10.1128/aem.72.1.291-297.2006

Wiesemann, N., Butof, L., Herzberg, M., Hause, G., Bethold, L., Etschmann, B., Brugger, J., Martinez-Criado, G., Dobritzsch, D., Baginsky, S., Reith, F.,& Nies, D. H. (2017). Synergistic Toxicity of Copper and Gold Compounds in Cupriavidus metallidurans. *Appl Environ Microbiol*, *83*(23).

Safety Data Sheet - Fisher SCI. (n.d.). Retrieved January 16, 2023, from https://www.fishersci.com/msds?productName=AC422870050

Safety Data Sheet - Fisher SCI. (n.d.). Retrieved January 16, 2023, from S25635.pdf (fishersci.com)

Material Safety Data Sheet - Fisher SCI. (n.d.). Retrieved January 16, 2023, from https://fscimage.fishersci.com/msds/16310.htm

Cupriavidus metallidurans (Goris et al.) Vandamme and Coenye. ATCC. (n.d.). Retrieved January 15, 2023, from https://www.atcc.org/products/43123