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# Heavy Metal Pollution and Use of Microorganisms for Bioremediation

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# Heavy Metal Pollution and Use of Microorganisms for Bioremediation

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## Abstract

*Rhodobacter sphaeroides* belongs to α-3 subdivision of the *Proteobacteria* that is metabolically capable of tolerating high levels of toxic heavy metals including lead, zinc, gold and mercury. These heavy metals constitute a major pollution that is contributed to by a variety of sources, such as industrial effluents, leaching out metal ions from the soil, and acid rain. These pollutions pose a serious problem to human health and therefore require bioremediation of such toxic metals from the streams, rivers, and soils. Previous studies have shown that some bacterial species tolerate varying levels of heavy metals in their environments. Gene homologs of previously identified genes involving metal tolerance in *Pseudomonas putida* were identified in the genome of *R. sphaeroides*; these genes include sensor kinases, membrane bound transporters, and enzymes involved in carotenoid biosynthesis. The objective of this study is to examine the resistance of *R. sphaeroides* against gold by growing these bacteria with varying concentrations of gold contaminated media under aerobic and photosynthetic growth conditions. Analyses of growth characteristics, cell survival and colony morphology reveal that *R. sphaeroides* is resistant against moderately high levels of gold, and its growth in photosynthetic growth condition shows increase level of resistance. Results of the current study will have an array of applications to scavenge heavy metals from polluted environments at a larger scale.

**Keywords:** Bioremediation, heavy metal pollution, Rhodobacter sphaeroides.

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## Introduction

The definition of heavy metals has differed over the years, beginning with defining heavy metals as metals with a density of five times greater than water [1] and then as metals with densities above 4-5 g/cm$^3$ [2, 3]. The definition of heavy metals has also included the elements with atomic weight between 63.546 and 200.590 [4]. Heavy metal pollution refers to heavy metals such as zinc (Zn), mercury (Hg), gold (Au), lead (Pb), cadmium (Cd), and chromium (Cr), which have densities that are greater than 5g/cm$^3$ [5].

### *Sources of Heavy Metal Contamination*

Heavy metal contamination, particularly in the air, soil and rivers, is a major problem for their toxic effects worldwide [6,7]. The toxic contaminants come from a variety of sources, including industrial effluents, gold mines, acid rain, and metal ions leaching out into the soil. For example, a major source of copper in the agricultural processes comes from fertilizers that use the metal to enrich the soils around the crops, as well as pesticides that are used widely in agriculture [8]. Metals can also enter the environment through animal waste that is used as fertilizers; the pig and poultry industries use various metals, Cu and Zn, to promote growth of the animals, and the metals accumulate within the waste [8]. A significant source of gold contamination comes from gold mines that have been abandoned and left unregulated where it was reported that platinum and gold are the most environmentally damaging metals [9]. Heavy metals are also used in manufacturing processes which end up as industrial waste. The metals then leach into the water supplies and water cycle, enter the food chain and ultimately accumulate to toxic levels [10].

### *Implications of Metal Contamination*

Heavy metal pollution is irreversible, but can be managed through bioremediation tactics [11]. The implications of the metal contamination include degradation of food crops, water sources and atmospheric changes [12]. Industrial wastewater is commonly used in third world countries, and when the presence of heavy metals reaches the toxic concentration, there are serious impacts on agricultural products [13]. These toxic pollutants pose serious health risks to humans, including bone loss [14], kidney damage [15], neurological damage [16], skin cancer [17], and lung cancer [18]. Some of these metals, such as chromium, cobalt and nickel, not only play a vital role in metabolic processes as essential micronutrients, stabilizing molecules [19] and catalysts in enzymatic reactions [20], they also help regulate osmotic balance [9], and are used in redox reactions [21].

Heavy metals are also expelled into the air through the process of mining and other industrial effluents. The changes in energy use and energy generation will have huge impacts on the atmosphere and lead to increases in the metal emission. A study done on the energy matrix of Brazil predicted a 100% increase of heavy metals into the atmosphere two years after a

major policy on energy production was passed [22]. Metals within the atmosphere add to the pollution that the World Health Organization estimates to be the cause of death of nearly two million people per year. There is a serious need for the bioremediation of the metal contaminants from not only the soil and water samples, but also the atmosphere. Several organisms are being utilized as a biomonitoring system to detect and monitor the amount of metal within the atmosphere in certain regions and locations.

The byproducts of heavy metal contamination can be useful in a variety of applications. An example is bio nanoparticles that are formed when microorganisms cannot remove the heavy metals from their cells. The metals are transformed into less toxic bio-nanoparticles that have a wide array of uses. The unique features of gold bio nanoparticles allow them to be used as transporters for therapeutic agents, as basic components of nanocomposite preparations [23], in electronic plating [24], and in cancer therapy designed to target tumors [25].

## Heavy Metal Bioremediation

Bioremediation is the use of microorganisms to clean up contaminated water or soil, whereby the microorganisms use the contaminant as a source of energy or as nutrients [26]. Within the process of bioremediation, there are three main components necessary to successfully implement the methodology. The three components include the microorganisms, a food source, and nutrients [27]. The use of microorganisms for bioremediation was analyzed by Nies [3] and three potential uses were identified: 1) Biotechnological processes can be facilitated through the addition of metal resistance to microorganisms. 2) Expensive metals could be extracted from environmental sources through bioleaching with metal resistant bacteria. 3) Bioremediation of metal-contaminated environments could be possible through metal tolerant microorganisms. Many bacterial strains are found to be useful in bioremediation processes, however some are only found to be successful in laboratory conditions. The limiting factors for bacteria to be used as a bioremediation tool include pH, soil structure, nutrient availability, temperature, and the presence of other toxins and contaminants [28]. In the laboratory setting, these factors are easily controlled and accounted for in the optimization of bacteria in the use of bioremediation. However, in the environmental setting, these factors are no longer controlled, which will alter the performance of a microorganism in the tolerance and bioremediation of toxic heavy metals.

### *Tolerance Mechanisms*

Microorganisms have a wide array of different tolerance mechanisms depending on the organism as well as the heavy metal involved; each mechanism is specific to particular metals or group of metals. Whether essential or non-essential, heavy metals become toxic to organisms at high levels, resulting in bioaccumulation, modifications of conformational

structure of nucleic acids and proteins, damage to the DNA and cell membrane, and interference with the oxidative phosphorylation and osmotic balance [9]. Resistance mechanisms to heavy metals have been identified including intracellular and extracellular sequestration, exclusion by permeability barrier, efflux pumps, active transport, reduction of heavy metal ions and cellular targets, and enzymatic detoxification.

Heavy metals and their byproducts are generally detoxified by the oxidative coupling, which is mediated by oxidoreductase enzymes. Transformation of the heavy metals and metalloids into bio-nanoparticles can occur in microorganisms by oxidation, reduction, methylation, and dealkylation [29]. Studies have shown that there are many algae, fungi, bacteria have the ability to reduce the metal ions into elemental metallic form; for example, reduction of Au(III) to the elemental Au(0) as well as Ag(I) to Ag(0) [30]. Moreover, some heavy metals and metalloids have multiple oxidation states that can be reduced, as they provide electrons to the terminal electron acceptors in the electron transport chain of the heterotrophic bacteria.

There has been considerable interest for the identification of tolerance mechanisms for specific heavy metals in various microorganisms. Cadmium is one of the most well-known toxic heavy metals, and is commonly used in paints and plastics, as well as steel coatings [31]. It appears that an overall trend in cadmium resistance in bacteria is a result of cadmium efflux. A mutation in the DsbA product in *Escherichia coli* impairs resistance against cadmium to the bacterium [32]. Chromium has serious toxic effects on human kidney and livers. It has previously been determined that chromate enters bacterial cells through a sulfate system that is mirrored in many other microorganisms to uptake chromium and other metal ions [3]. It has been suggested that the resistance to chromate is a result of chromate reduction and efflux, which allow other bacteria including *Pseudomnas fluorescens* strain LB300 [33] as being resistant to chromate. Copper is commonly found in many industrial processes and pesticides, and that damages the nucleic acids of microorganisms, and as a result there are many mechanisms that microorganisms utilize in order to minimize the damage done by the copper. Microorganisms utilize various mechanisms, which include detoxification and sequestration to mediate copper resistance to the cell [34]. Copper resistance has been shown across species, including *Escherichia coli* and *Pseudomonas*, however there are phenotypic differences within the copper-resistant bacteria. It has been shown that the *E. coli* remains colorless while *Pseudomonas* strains exhibit a blue morphology when in contact with high copper medium as a result of accumulation within the outer membrane [35, 36]. This type of phenotypic difference may be implemented as a means of quantifying the amount of copper contamination within environmental samples.

Mercury is perhaps one of the most toxic heavy metals with no beneficial use to microorganisms. Due to the widespread prevalence of the metal, microorganisms have mercury resistant genetic determinants (mer system) are found in many bacterial species [37]. It has been characterized that MerP protein binds the toxic cation and delivers it to a mercury transporter, MerT, which then transports the cation to the cytoplasm of the

cell [38]. This system is tightly regulated by the MerR protein, which binds to the *mer* promoter to prevent transcription unless $Hg^{2+}$ is present. To ensure the induction of the *mer* promoter, MerD is a secondary regulator that is involved in order to initiate transcription. The *mer* system has been extensively studied and has been successfully transferred to other bacterial systems in order to promote the tolerance of mercury contamination within species of bacterial equipped to tolerate other metals. Nickel is useful in many bacteria for enzymes like dehydrogenase, but as with most heavy metals, an excess of nickel is toxic to the cells [39]. Nickel tolerance follows similar mechanisms to other metals and is primarily believed to be through sequestration and transport/efflux, although the transport may be mediated by a chemiosmotic driven gradient. *Ralstonia eutrophus*, a member of the proteobacteria is one of the best known organisms with nickel resistance. Two early tolerance mechanisms were described as a nickel-cobalt resistance (Cnr) as well as a nickel-cobalt-cadmium resistance (Ncc) [40]. Zinc is a very essential metal for the growth of bacteria and other microorganisms, as it is found as cofactor in a variety of enzymes and DNA-binding proteins [41]. The zinc transport is not strictly limited to "zinc only transport", but the metal may be transported through other systems, such as magnesium transporters, when there is an excess of zinc. The three transport groups that are seen within these magnesium/zinc systems include CorA, found in *Saccharomyces cervisiae* as well as many other bacteria and archaea, MgtE and MgtA transporters found in *Salmonella typhimurium*. There are a few zinc only transporters that have been identified, including ZntA, which is found in *E. coli*, and ZiaA, which is found in the *cyanobacterium Synechocystis* [42].

Gold averages about 1-5ppt in natural water [43], but has been found to reach more than 100ppb in soils [44]. Free gold ions are found in high abundance in aqueous media since the redox potential of Au (I) and Au (III) exceeds water, which could lead to the toxicity of gold on organisms [43]. To combat the toxicity, bacteria actively transports the gold out of the cytoplasm through means of efflux pumps. *Salmonella* contains a *gol*-gene cluster, which codes for a metal exporter, GolT, a transcriptional regulator, GolS, and GolB, which is a metal binding protein. This cluster is responsible for the resistance of gold, and while it is found in two species of *Salmonella*, it is not found in other enteric bacteria; this finding suggests that it is not a required component but possibly arose through horizontal gene transfer to allow the bacteria to survive the metal contamination. GolS, along with CupR, belong to the MerR family of regulators that are activated in the presence of heavy metal ions, ranging from the essential ions such as Zn (II) or Cu (I), to the toxic Pb (II) and Au (I) ions. Studies have shown the formation of biofilms, such as *Cupriavidus*, on deposits of Au, which suggests that Au-specific resistance mechanisms are the mechanisms to survive against gold toxicity. Arsenic is found in soil and water in two main forms, As (III) and As (V). It has been shown to be a carcinogen that leads to skin, bladder, kidney and lung cancers. Two main mechanisms of arsenic resistance have been identified so far for As (V), including the reduction of arsenate, which was initially identified and characterized in *Sulfursopirillum arsenophilum* [45]. The second arsenic tolerance mechanism involves ArsC

and other arsenic resistance genes [46] which have arisen by convergent evolution [47] and includes the following components: 1) ArsC, which is the arsenate reductase, 2) ArsB, which is the arsenite-specific efflux pump, and 3) a group of reducing agents such as thioredoxin or glutaredoxin [45, 46, 47].

As previously mentioned, there is no single mechanism of metal tolerance found within bacteria. This presents a challenge as each metal has a different mechanism found in the different group of bacteria. The main mechanisms of heavy metal tolerance that have been identified in microorganisms to date includes transport of the metal ions out of the cell, through efflux pumps, or detoxification and sequestration to form bio-nanoparticles. In order to design and synthetically construct an effective bioremediation tool, the mechanisms of metal transport and the use of efflux pumps needs to be better characterized in a choice of microorganism. As mentioned above, the transport systems are not always limited to a single metal, but may be activated in the presence of several metals. This type of system would be beneficial for use to implement into organisms to allow a wide array of metal tolerance and bioremediation.

## Current Study

*Rhodobacter sphaeroides* belongs to α-3 subdivision of the *Proteobacteria* that is metabolically capable to tolerate high levels of several toxic heavy metals. This study focuses to understand the growth characteristics, colony morphology and tolerance mechanisms of *R. sphaeroides* in gold-contaminated minimal media under aerobic and photosynthetic growth conditions. These are the following hypotheses: *R. sphaeroides* is capable to grow in gold-contaminated minimal media; Photosynthetic growth condition is more suited for the gold resistance and possibly of its bioremediation; and (3) the resistance is mediated by gene mutation and selection.

## Materials and Methods

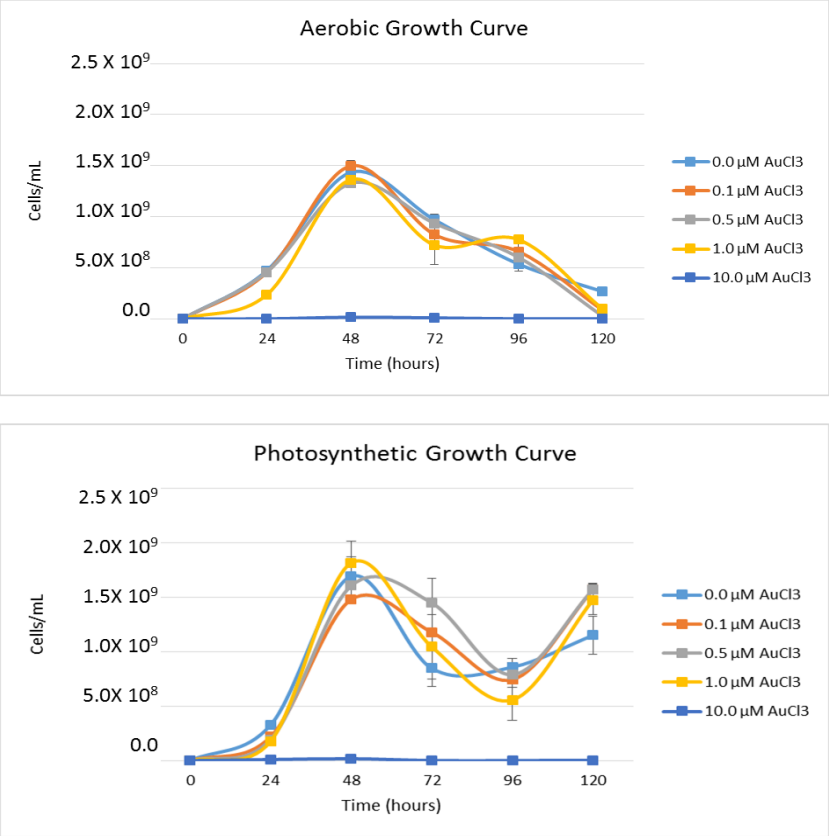
*R. sphaeroides* 2.4.1 was grown in Sistrom (SIS) minimal media under aerobic and photosynthetic conditions. Aerobic growth condition constitutes of growing the cultures with ~20% $O_2$ in a 30˚C shaker under natural light conditions. Photosynthetic cultures were grown under anaerobic with constant 3 Watt/$cm^3$ light conditions. The cultures were grown to the log phase of growth (0.6-0.8 optical densities at 600nm for *R. sphaeroides*) in order to be used for the experimental conditions; the log phase of growth has been identified as the best to study the metal tolerance (48). The starting bacterial culture was contaminated with varying concentrations of gold chloride, 0.1μM. 0.5μM, 1.0μM and 10.0μM under both growth conditions. Optical Density (OD) readings were taken every 24 hours for 120 hours to analyze growth characteristics and kinetics. Cell samples were diluted at different serial dilutions and grown on SIS plus/minus gold contamination

of appropriate concentrations. The colony forming units (cfu) and colony morphology were determined. The aerobic plates were left in the 30°C incubator while the photosynthetic plates were sealed with Parafilm and left in the photosynthetic condition with no oxygen and constant 3W/cm$^3$.

## Results and Discussion

The results of the growth kinetics, seen in Figure 1, suggest two different tolerance mechanisms of *R. sphaeroides* in the presence of gold. The aerobic condition depicts a significant lag phase, up to 24 hours, at the higher concentration (1.0μM) of gold. There is no significant difference of bacterial growth between the control and the contaminated samples in the photosynthetic condition, which suggests the bacteria is metabolically equipped to tolerate the metallic stress under that condition. The 10.0μM concentration showed to be toxic under both growth conditions for this bacterium.

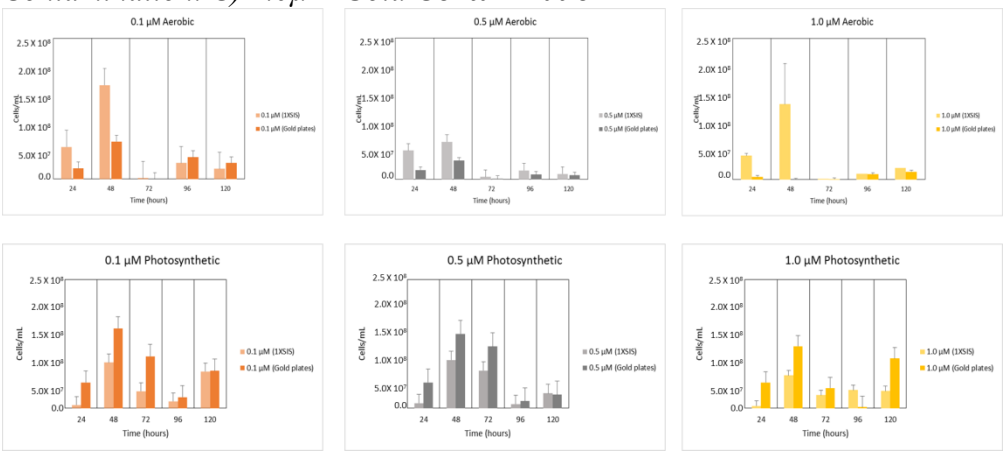
**Figure 1.** *Growth Kinetics of R. Sphaeroides when Contaminated with Varying Concentrations of Gold Chloride. A) Aerobic Growth Kinetics. B) Photosynthetic Growth Kinetics*




The cfu's were counted for both the aerobic and photosynthetic conditions and it was found that the mechanisms of tolerance to gold appear to be different between these two conditions. In the aerobic condition, the number of cfu's was higher for the cells grown on the Sis plate compared to

the cells grown on the gold contaminated Sis plates. This suggests that aerobically grown cells slowly adapt to that growth condition. However for the photosynthetic growth condition, there was higher number of cfu's for cells plated on the gold contaminated Sis plates compared to the cells plated on only Sis plate, as seen in Figure 2.

**Figure 2.** *Cfu Counts for Both Growth Conditions in Varying Concentrations of Gold. A.) 0.1μM Gold Contamination. B) 0.5μM Gold Contamination. C) 1.0μM Gold Contamination*




Since the cells that had been exposed to gold grew better on non-gold plates compared to plates with the same gold concentration, it strongly suggests the mechanism of tolerance in the aerobic condition is due to cellular adaptation. In contrast, the cells exposed to gold grew better on plates with the same gold concentration compared to the cells plated on Sis media without gold. Therefore, under the photosynthetic condition, cells with mutations are likely selected or that photosynthetic growth mode help rapid cellular adaptation, which is supported by low oxygen tension light.

## Future Work

Heavy metal tolerance gene homologs have been identified between *R. sphaeroides* and *Psuedomonas putida* in order to select the targets for future study. Since the list of genes is currently limited, total RNA sequencing will be a better approach to identify all genes in *Rhodobacter sphaeroides* that are fully expressed. Profiling the expression levels of the total genes will provide a complete list of genes possibly involved in the gold bioremediation processes. The study will provide the clues about the metabolic pathways and necessary growth mode responsible for the gold bioremediation. Later, these specific target genes will be examined genetically. A gene-knock out library of *R. sphaeroides* has been previously constructed using *Tn5* transposon mutagenesis. A set of genes that are strongly involved in gold bioremediation process can be further augmented and cloned into a native or synthetic plasmids along with species specific promoters, and can be tested using real time PCR (RTPCR) in the transformed bacterial strains under appropriate growth conditions.

The ongoing work has the goal of creating a gene knockout library in order to screen for potential organisms that may be used in the bioremediation of heavy metals, including gold contaminated environments. Implementing an effective system for monitoring the levels of heavy metals within the environmental samples will lead to an effective means of environmental monitoring and cleaning heavily contaminated sites worldwide. Studies have previously demonstrated such transfer systems, such as the *mer* system, to bacteria. This would be useful biotechnological applications capable of metal detoxification and bioremediation of multiple, if not all, metal contaminants.

There is an urgent need to develop a more refined, world-wide monitoring system to control the amount of heavy metals that are being expelled into the water supplies and the environment. Arsenic, for example, has been an area of interest to the World Health Organization as well as the European Union in attempts to reduce the level of arsenic in the free water to 10 μg/L to reduce the negative affects to human health. However, several third world countries are unable to reduce the amount of arsenic below 50 μg/L in the supplies. Monitoring systems such as thermal, pH biosensors, or optical systems have been suggested, especially in regards to arsenic (46). If the concentrations of toxic metals can be effectively monitored worldwide, there can be a reduction in the negative impacts on the environment and human health. A possible global approach could be through the implementation of a Geographic Information Systems (GIS) integrated monitoring system. This system has the ability to capture and store information on many types of spatial data. If an effective monitoring and detection of toxic metals system was utilized and all the data was collected for many types of metals, a global map could be made available in real time of the worldwide distribution of heavy metals. With this type of data and other associated information on human health risks, and events, large scale planning will be set up for bioremediation and implementing effective policies to limit the metal pollution in the future.

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