Nanoparticle Interactions with Bacteria:
Toxicity and Chemotaxis

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Abstract

Two projects are presented in this report. First, a silver resistant strain of *Pseudomonas sp.* (closest relatives *Pseudomonas putida and Pseudomonas plecoglossicidaI*) was isolated from Town Neck Beach in Sandwich, MA. This strain could grow in 2ppm $AgNO_3$ and at silver nanoparticle concentrations of 2000ppm. The lack of a toxic effect of high silver nanoparticle concentrations and the formation of precipitates in the colonies indicates that the strain may be tolerant of solid silver. Attempts were made to extract and sequence a silver resistance plasmid. Second, chemotaxis experiments using nanoparticles as chemoattractants/repellants were performed using the silver resistant *Pseudomonas*, *Vibrio gazogenes,* and swamp water from Cedar Swamp. It was demonstrated that there was no chemoattractant effect of reactive iron nanoparticles in Cedar Swamp water despite the generation of hydrogen, which could be a potentially very strong chemoattractant. Additionally it was shown that while *V. gazogenes* did not see silver as a chemorepellant, the silver resistant *Pseudomonas sp.* was repelled by 50 nm silver nanoparticles.

Silver resistance and the toxicity of silver nanoparticles

The amount of nanoscale silver is increasing rapidly in consumer products the ability to synthesize these particles on a large scale improves. Silver nanoparticles (NPs) are toxic to bacteria, and are currently used in everything from medical devices to sport socks and washing machines to deter microbial growth. Silver is a particularly toxic heavy metal as it interferes with the electron transport chain and binds to DNA (Thurman, 1989)The existence of silver resistant bacteria however is well known, and these bacteria may be less impacted by the presence of silver nanoparticles. Certain bacteria have been shown to accumulate or precipitate silver into silver metal or other insoluble form. To date, studies of silver nanoparticle toxicity have focused on strains of bacteria that lack silver resistance. Therefore it is interesting to study the toxicity of silver nanoparticles to silver resistant bacteria.

Materials and Methods

Isolation of silver resistant bacteria

Samples of sand and water were obtained from Town Neck Beach in Sandwich, MA. Sea water was concentrated by centrifuging 50 ml of seawater for 20 minutes at 4100 rpm. The supernatant was removed and the cells were resuspended in 1 ml of seawater. Concentrated seawater (100 microliters) was plated on nutrient agar and R2A plates containing 0.2, 2, and 20 ppm silver (in the form of silver nitrate). Plates were incubated overnight at 30^oC and 6 colonies appearing on the 20 ppm NA plates were streaked for isolation on 20 ppm silver nutrient agar plates. Isolates were identified by colony PCR of the 16S rRNA gene. Briefly, colonies were picked and placed in 20 microliters of 1% Nonidet P-40, which was boiled for 5 minutes at 100^oC. A fraction (0.5 microliters) of this extract was added to a PCR reaction using primers 8F and 1492R (Lane, 1991). PCR product was treated with ExoSAP and directly sequenced. Sequences were uploaded and compared against the ribosomal database project for identification.

Plasmid extraction and attempted sequencing

500 ml of silver resistant *Pseudemonas* strain 3 was grown in nutrient broth with 20 μg/L silver (in the form of AgNO3). Cells were pelleted by centrifugation, and plasmid DNA was extracted using a Qiagen Midiprep large scale plasmid purification kit. DNA was quantified using the nanodrop spectrophotometer. 50 microliters of DNA was sheared by repeatedly pipetting up and down using a 20 microliter pipette. These 50 microliters were concentrated via ethanol precipitation, and resuspended in 1 μl sterile molecular grade water. One microliter of 2x master mix was added to the concentrated DNA, and incubated at 72°C for 10 minutes to add A overhangs to the ends of the sheared DNA. This mixture was ligated into the TOPO cloning vector (Invitrogen) and cloned into One Shot chemical competent *E. coli*. Growth was slow, and after 30 hours approximately 70 clones were picked for sequencing.

Growth curves

A 1:1000 dilution of an overnight culture of silver resistant *Pseudomonas* was aliquoted in 200 μl volumes into 96 well clear polystyrene culture plate. $AgNO_3$ or 10 nanometer PVP modified silver nanoparticles were added to the wells in increasing concentrations (triplicate samples for each concentration). The absorbance at 600 nm was monitored approximately hourly for 14 hours.

Toxicity assay

An overnight culture of silver resistant *Pseudomonas* was pelleted by centrifugation and resuspended in an equal volume of sea water base. This suspension was aliquoted in 100μl volumes into a black costar 96 well plate. Increasing concentrations of AgNO3 or 10 nanometer PVP modified silver nanoparticles were added to each well (in triplicate), and the plates were shaken at room temperature for 20, 40, or 60 minutes, at which point 100 μl of 2x BacLight assay (propidium iodide and SYTO 9, Invitrogen) was added to each well. The samples were incubated for 15 minutes at which point fluorescence was quantified with an excitation of 485nm and an emission of both 530 and 630 nanometers. Calibration samples were made with equal concentration live and killed (with isopropanol)

suspensions, and a calibration curve was made using the ratio of fluorescence at 530 nm to 630 nm according to manufacturers protocol.

PCR and quantitative PCR amplification of silver resistance genes

An attempt was made to quantify environmental silver resistance genes from Eel Pond sediment using quantitative PCR. The silver resistance primers described by Woods et. al. were used with BioRad SYBR green 2X master mix. Primers for each gene were as follows: silE forward 5'-aggggaaacggtctgacttc-3', silE reverse 5'-atatccatgagcgggtcaac-3', silRS forward 5'-ggcaatcgcaatcagatttt-3', silRS reverse 5'-gtggaggatactgcgagagc-3', silCBA forward 5'-cgggaaacgctgaaaaatta-3', silCBA reverse 5'-gtacgttcccagcaccagtt-3', silF forward 5'-cgatatgaatgctgccagtg-3', silF reverse 5'- attgccctgctgaataaacg-3', silB forward 5'-caaagaacagcgcgtgatta-3', silB reverse 5'-gctcagacattgctggcata-3', silA forward 5'-cttgagcatgccaacaagaa-3', silA reverse 5'-cctgccagtacaggaaccat-3', silP forward 5'-cctgggtttacagcgtcatt-3', and silP reverse 5'-atggcacctgaggtttgttc-3'. Standard PCR cycles were 3 min at 95 °C followed by 35 cycles of 95 °C for 40 s, 55°C for 40 s and 72 °C for 40 s with a final elongation step was performed at 72 °C for 10 min (Woods, 2009). Quantitative PCR conditions for the SilA and SilE genes with the same primers. Cycling parameters were an initial activation at 95°C for 10 minutes followed by 50 cycles of 1 minute at 55°C followed by 15 seconds of 95°C. Detection was at the end of the extension. Calibration curves were constructed using serial dilutions of silver resistant *Pseudomonas* strain 3 DNA.

Results and Discussion

Isolation and characterization of a silver resistant Pseudomonas strain

Six colonies were chosen for further investigation from the initial plating. Of the six chosen isolates, four had a closest relative as an *Acinetobacter sp.* and two were members of the genus *Pseudomonas*. Of the six strains, the two *Pseudomonas sp.* strains grew more effectively on silver amended plates. Silver resistant strain three, a *Pseudomonas sp.* most closely related to *Pseudomonas putida,* a common soil bacterium, and *Pseudomonas plecoglossicidal,* a fish pathogen, was the easiest to

grow on both silver plates, and could also be grown in liquid media in the presence of silver nitrate, and
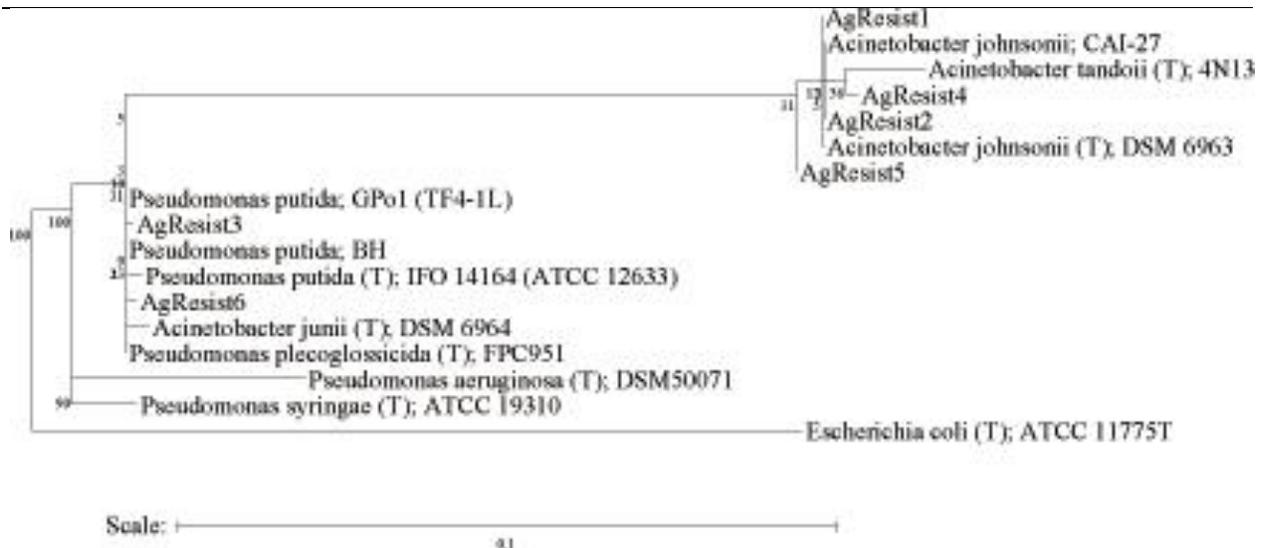
was therefore chosen for further studies.

Figure 1. Six silver resistant isolates and their nearest neighbors as determined by the Weighbor weighted neighbor-joining tree building algorithm.

Silver resistant *Pseudomonas* strain 3 were motile gram negative rods which form smooth sticky

colonies on nutrient agar. When strain 3 was plated on nutrient agar with increasing concentrations of

silver, from 20mg/l to 100mg/L to 200mg/L, the underside of the colonies became significantly darker,

and the agar surrounding the colonies turned brown with increasing silver concentrations. In early

streaks, the formation of precipitates in the center underside of the colonies was visible. This is

consistent with the results of Haefeli et. al. showing that *Pseudomonas sp.* can precipitate silver ions

although further characterization is needed to identify the precipitate in this study (Haefeli, 1989). Likely

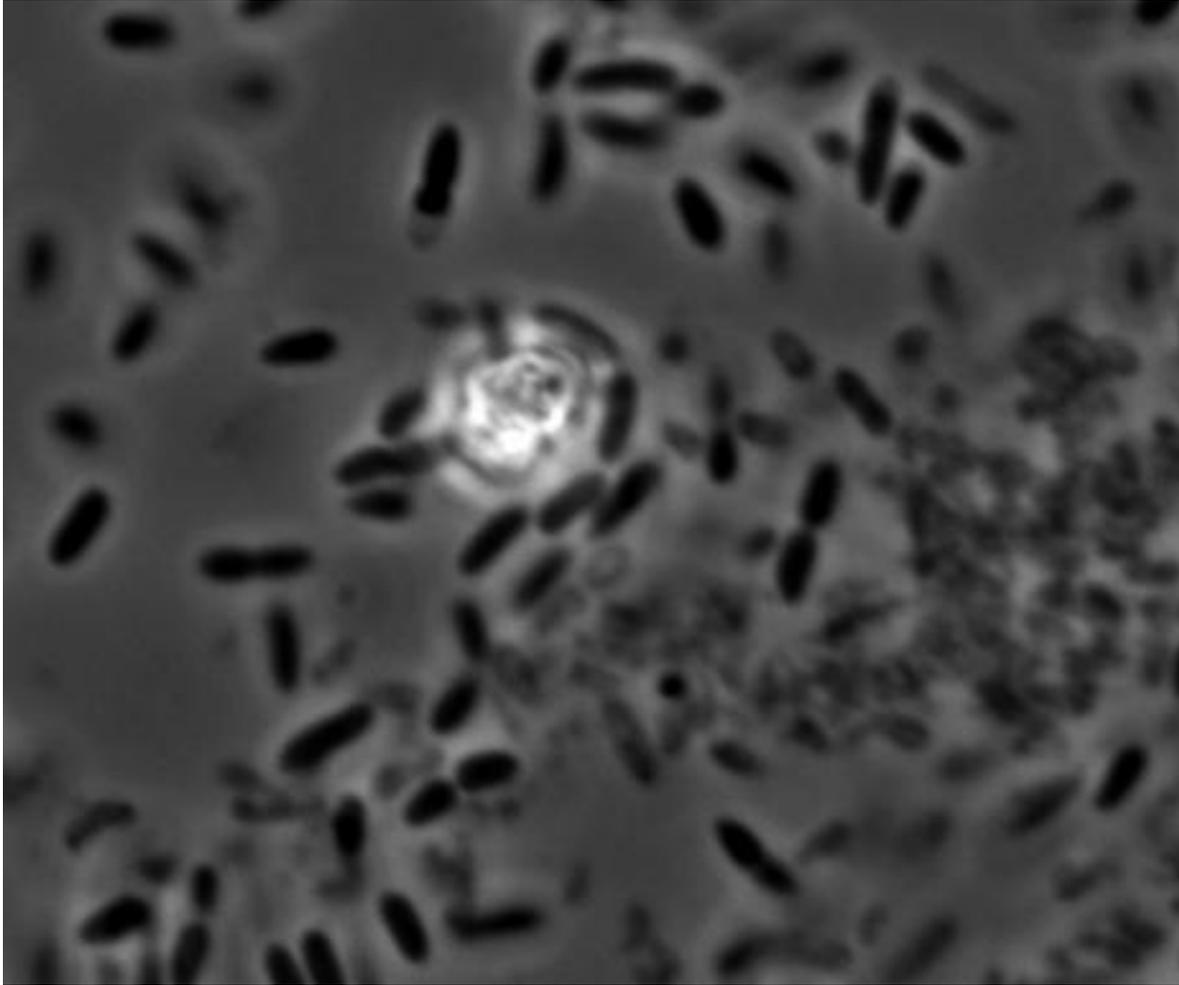as it increases with silver concentration it is a silver compound.

Figure 2. Phase contrast micrograph of silver resistance *Pseudomonas sp.* surrounding a precipitate from 200 mg/L silver nutrient agar plate
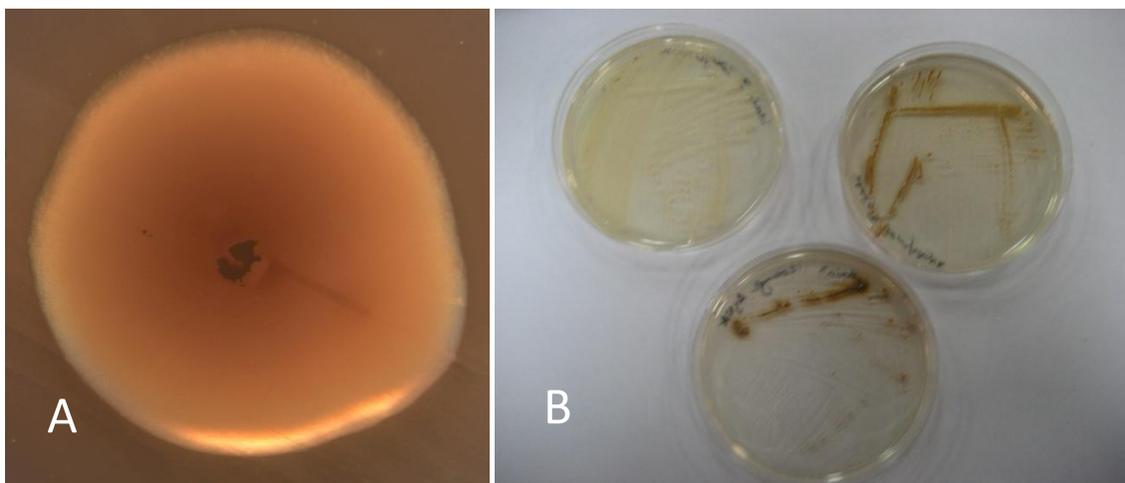

Figure 3. Colony morphology (A) of silver resistant *Pseudomonas sp.* on a nutrient agar plate amended with 200mg/L silver. Darkening of colonies with increase in silver concentration (B), clockwise from top left 20mg/L Ag, 100 mg/L Ag, 200 mg/L Ag.

Pseudomonas sp. silver resistant strain 3 was able to be transferred to liquid culture and grew in both the presence of silver nitrate and silver nanoparticles. The measured minimum inhibitory concentration of $AgNO_3$ was 20ppm Ag+, and the strain was still able to grow in the presence of 2000ppm 10 nanometer silver nanoparticles.
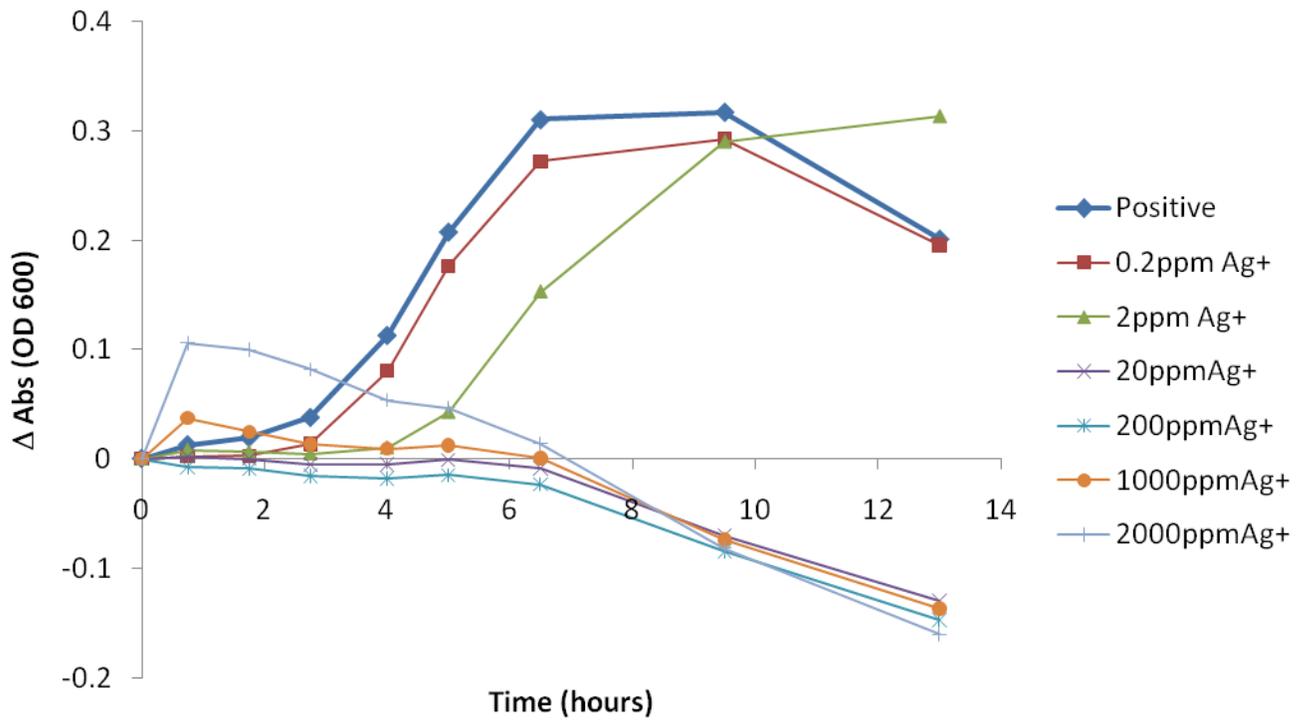

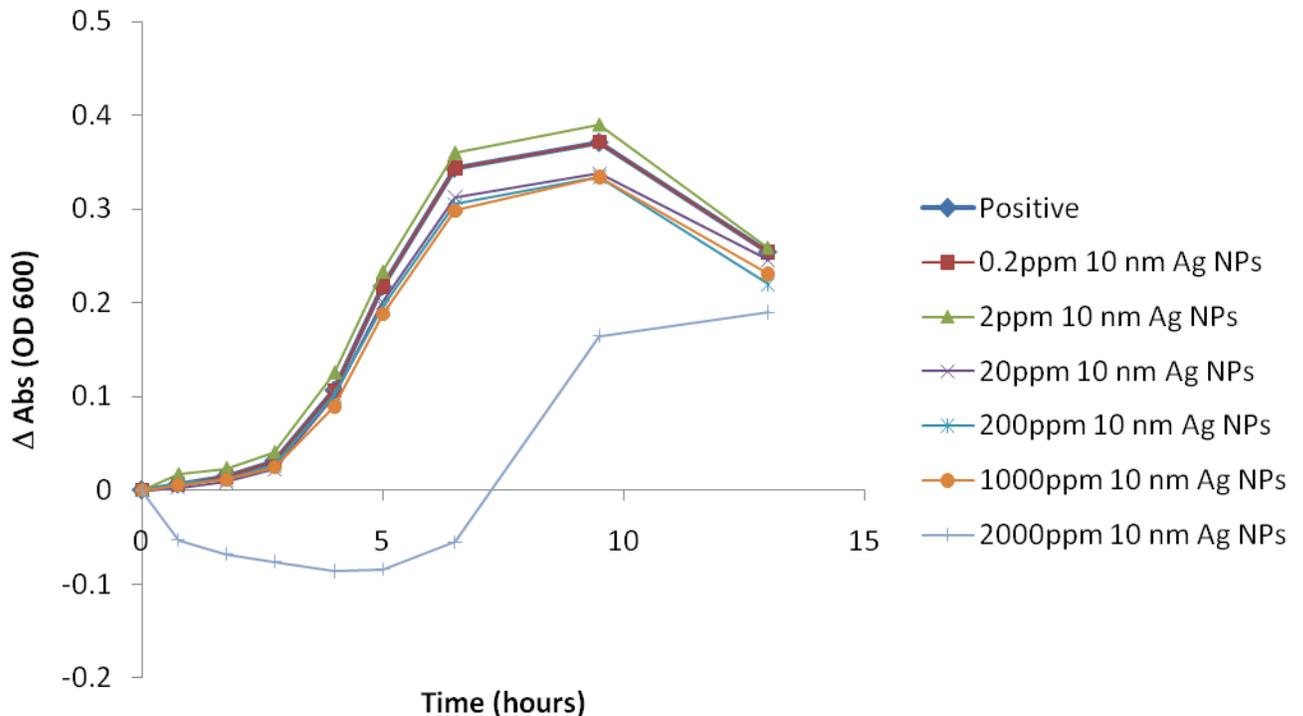**Figure 4. Growth of silver resistant *Pseudomonas sp.* in the presence of silver nitrate.**

Figure 5. Growth of silver resistant *Pseudomonas sp.* in the presence of 10 nm PVP coated silver nanoparticles

Silver nanoparticles were less toxic to stationary phase bacteria at similar silver concentrations than the dissolved ion. The toxicity of silver nanoparticles has been proposed to be a combination of three methods: dissolution of Ag+ ions, the stimulation or reactive oxygen species production, and the disruption of membrane integrity (Thurman, 1989). Concentrations of 20 ppm silver nitrate was enough to see a decrease in the percent live bacteria after a 20 minute exposure, whereas live bacteria did not decrease significantly over the course of an hour with nanoparticle concentrations up to 1000ppm. The solubility of silver increases with pH and although the dissolved silver concentrations in the nanoparticle suspensions could not be measured, it can be imagined that it would be close to 1% of the silver nanoparticle concentration (Fabrega, 2009). This would result in the 10ppm dissolved ion concentration in the 1000 ppm nanoparticle suspensions which would be close to the MIC. Therefore since no toxic

effect is seen at a 1000 ppm nanoparticle concentration, it can be imagined that there is not a nanoparticle effect observed in this case. This could be the result of the PVP stabilizing coating on the nanoparticles which electrosterically repels the bacteria, or it can be imagined that since the bacteria appears to be precipitating silver, it is tolerant to living in close relation to silver particles, rendering them less toxic than to an unadapted organism.
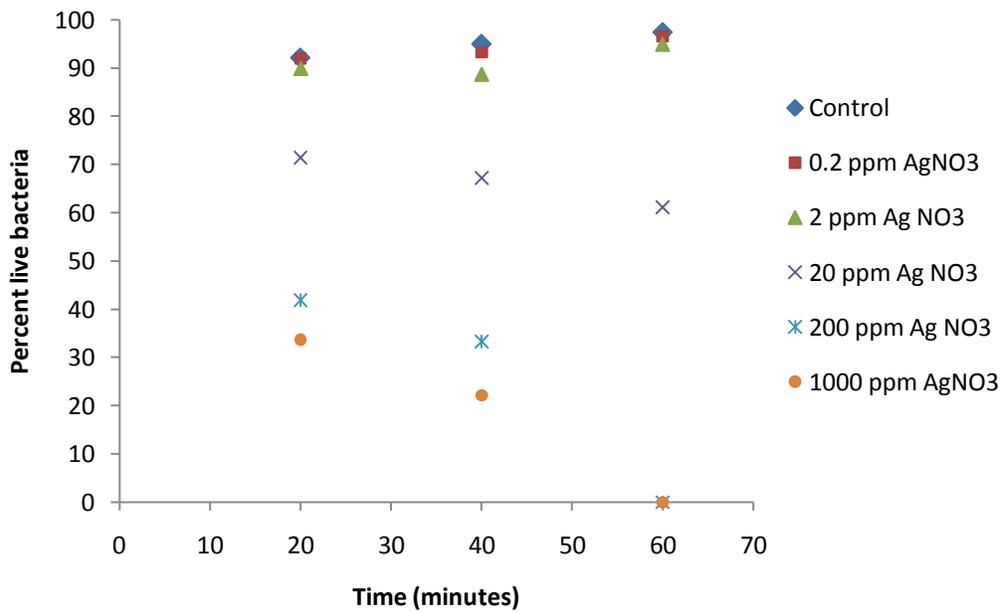

Figure 6. Toxicity of silver nitrate on suspensions of silver resistant *Pseudemonas* strain 3 over a one hour exposure period.
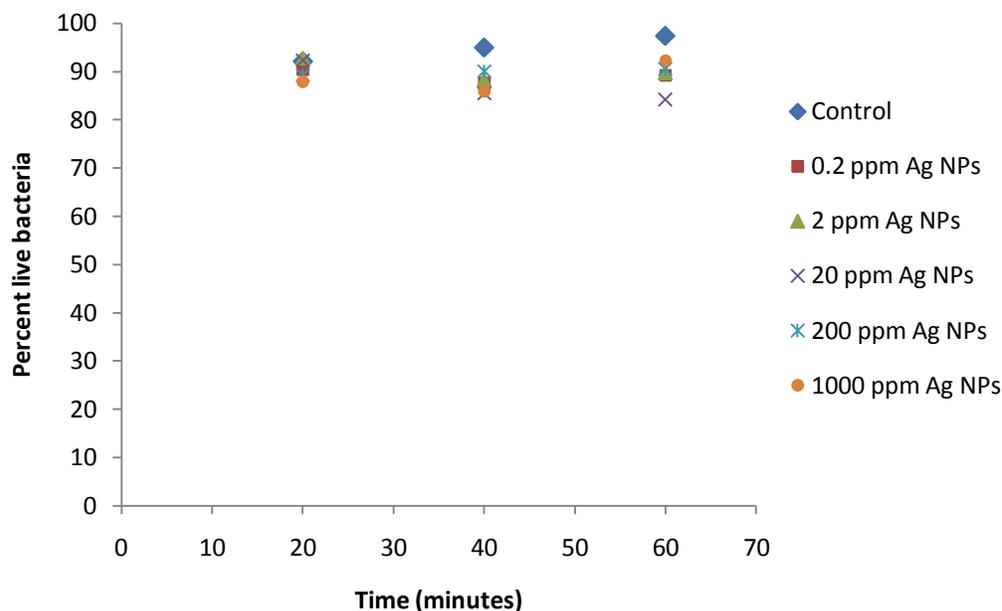
Figure 7. Toxicity of 10 nanometer silver on suspensions of silver resistant _Pseudemonas_ strain 3 over a one hour exposure period.

Attempted sequencing of plasmid DNA

A reasonably large number of metal resistance genes are plasmid mediated. Because of this it was attempted to extract, clone and sequence plasmid DNA from a large culture of the silver resistant _Pseudomonas_. A total of 5.4μg of DNA was extracted and the sheared DNA had a size of approximately 1.5 kbp according to agarose gel electrophoresis. The clones from the Invitrogen TOPO cloning kit grew particularly slowly. Sequencing failed, however PCR using M13 primers revealed that the vector had inserts between about 1.5 and 8 kpb. It was eventually determined that low copy numbers of targets. In the future sequencing may be attempted on this clone library by first amplifying the insert by PCR and then direct sequencing.

PCR amplification of silver resistance genes and qPCR of silver resistance in the environment

Positive amplification of SilA, SilE, Sil B and SilF were obtained using template DNA from the silver resistant _Pseudomonas_ isolate, however multiple products were obtained for the SilE and SilF

genes, possibly because of a low annealing temperature, as Primer BLAST did not indicate additional PCR

products from known genome sequences. Because of the strong amplification, SilA and SilE were used in

quantitative PCR. Quantitative PCR results showed DNA extracted from Eel Pond sediment and Eel pond

sediment exposed to silver nanoparticles had larger copy numbers of SilA and SilE genes than the total

DNA of the silver resistant isolate. This larger than expected result could indicate that the quality of the

standard DNA is not satisfactory, or that the primers are not targeting the desired genes. Studies of

silver resistant isolates from medical environments indicate that the sil genes are only present in 5-10%

of isolates (Fabrega, 2009). This number could increase in sediment, but it still appears that the assay

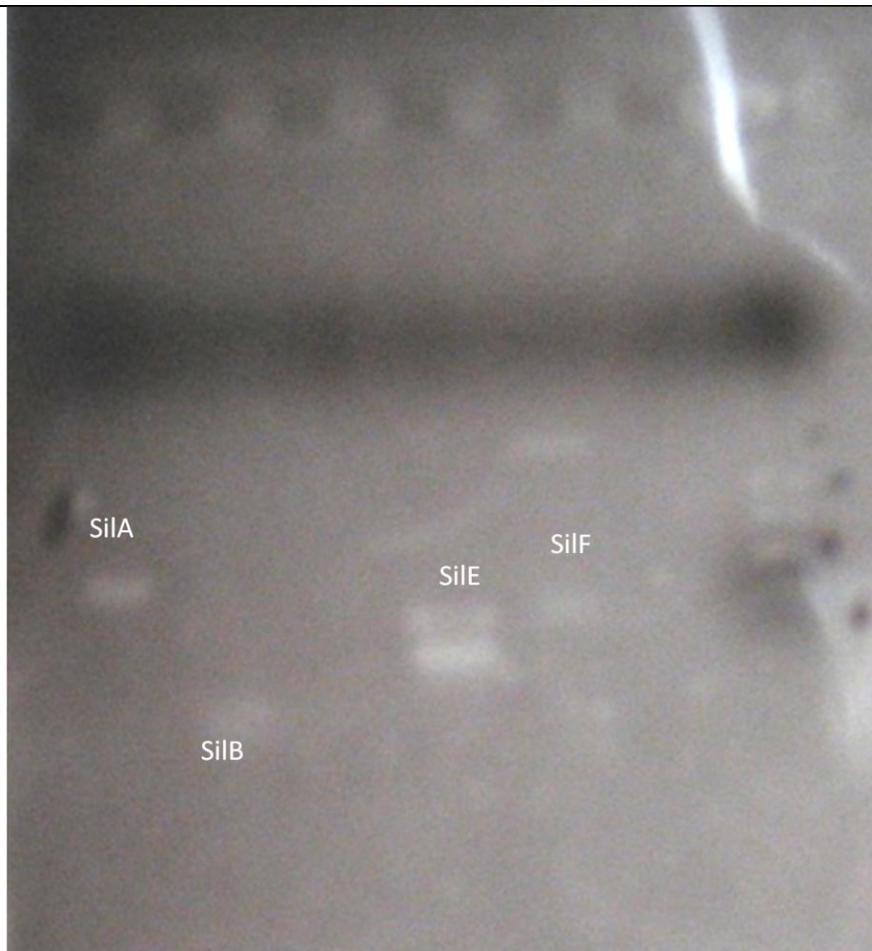must be optimized prior to quantification of genes from the environment.

Figure 8. Amplification of silver resistant genes from silver resistant *Pseudomonas* strain 3. SilCBA, and SilP primers gave negative results.

In conclusion, a silver resistant *Pseudomonas* strain was isolated, and its silver resistance was characterized. The strain was able to grow in the presence of 20ppm silver nitrate, and 1000 ppm silver nanoparticles. The lack of a toxic effect at these high nanoparticle concentrations implies that there may not be specific nanoparticle toxicity either because of nanoparticle coating, or because of the ability of the bacteria to survive in the presence of colloidal silver. Silver resistance genes SilA, SilB, SilE and SilF were amplified from the *Pseudomonas sp.* Optimizing the PCR and quantitative PCR assay would be interesting as there has been little work on detecting silver resistance genes in the environment which could give an idea of how affected an area has been from a release of silver or silver nanoparticles.

Chemotaxis towards nanomaterials

Nanoparticles often have interesting characteristics, including toxicity, that may attract or deter motile bacteria from encountering them. Nanomaterials could potentially act as chemoattractants by creating favorable environments for hydrogenotrophic microorganisms, or could be chemorepellents because of the release of toxic ions. One interesting nanomaterial that could potentially act as a chemoattractant is nanoscale zero-valent iron. These 50-100 nm particles have a zero-valent iron core and a magnetite shell. When in water, they react rapidly to not only reduce the environment, but also to produce hydrogen. This could attract sulfate reducers or methanogens in anaerobic environments, or other organisms that like hydrogen in aerobic environments. A possible chemorepellent would be silver nanoparticles, as silver ion is released from these. In this project, RNIP was studied as a possible chemoattractant, using natural samples in anoxic conditions. Silver nanoparticles were studied as a possible chemorepellent using pure cultures of *Vibrio gazogenes* and *Pseudomonas putida.*

Materials and Methods

Sampling site

Water and sediment samples were obtained for chemotaxis experiments from Cedar Swamp. Nalgene sampling bottles were capped underwater to minimize exposure to oxygen.

Nanoparticles

Reactive nano-iron particles (RNIP 10 DS) were obtained from Toda Kogoya company (Japan) and stored in an inert atmosphere. Silver nanoparticles (10 and 50 nm, coated with polyvinylpyrrolidone) (NanoAmor) were dispersed at a concentration of 10 mg/ml in sterile DI water using a 1/8th inch ultrasonic probe (Heat Systems Ultrasonics, now Misonix), at power 3 for 10 minutes on ice. Particle suspensions were then diluted appropriately in filter sterilized swamp water, or sea water base, and under Nitrogen in the case of RNIP

Chemotaxis apparatus

Chemotaxis experiments were performed in sterilized modified Meplats bottles (see figure x) according to the procedures of Jörg Overmann{Overmann, 2005 #1}. Bottles were filled with either water from Cedar Swamp or a 1:10 dilution of an overnight culture in the case of *V. gazogenes* and the silver resistant *Pseudomonas sp.* Eight capillaries of each test substance were filled with nanoparticle suspensions.

RNIP and Cedar Swamp

Capillaries were loaded with three different concentrations of RNIP. In addition, 8 capillaries were filled with filter sterilized Cedar Swamp water as a control. The chemotaxis bottle was filled under a steady stream of nitrogen, and was incubated overnight in an anaerobic jar with a nitrogen atmosphere. After the incubation the capillaries were pooled into microcentrifuge tubes. Volumes of 0.5 ml of the pooled samples were filtered onto a 0.2 micron Isopore filter. The filters were allowed to dry, and were stained with 1 μg/ml DAPI. A total of 8 fields were counted on each filter under 100x magnification.
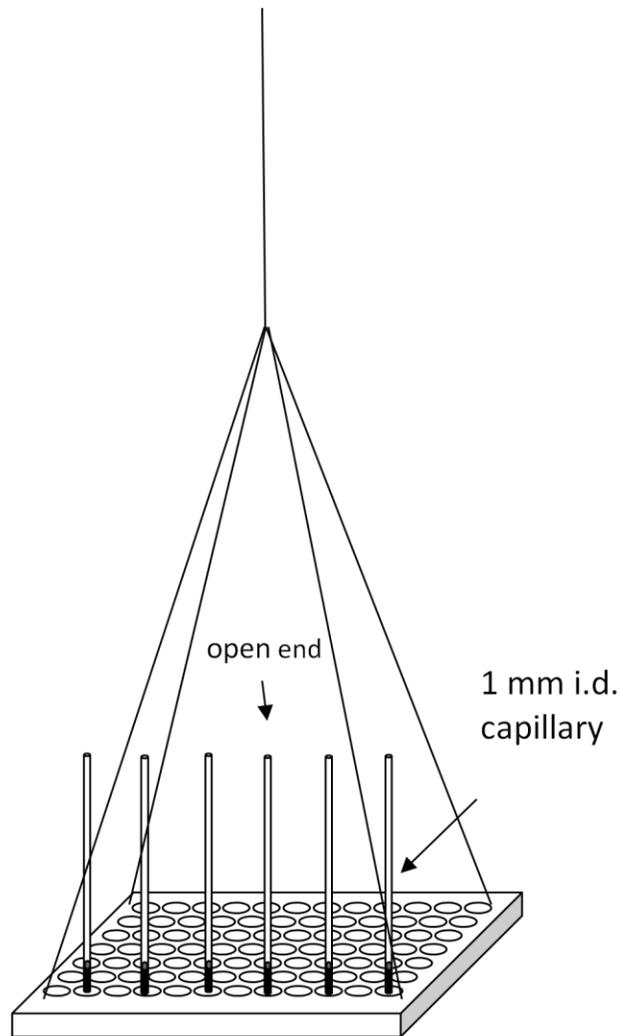
Figure 9. Apparatus for the in-situ measurement of chemotaxis to nanomaterials.

Silver and pure culture chemotaxis experiments

      Vibrio gazogenes was obtained from Alex Petroff who isolated the strain from Stoney Beach. A

silver resistant *Pseudomonas pudita* was isolated from the Cape Cod Bay and the details of isolation and

characterization are above in the first project. Silver nanoparticles (50nm diameter) were added to

capillaries at concentrations of 0.005, 0.05, and 0.5 mg/ml. Eight capillaries of each suspension were

incubated for 30 minutes to 1 hour after which they were emptied into microcentrifuge tubes and

quantified by a bulk fluorescence assay described below.

Quantification of Chemotaxis

Calibration curves were constructed by diluting the 1:10 overnight dilution to five different

concentrations. Two hundred microliters of sample or standard (in triplicate) were added to wells on a

costar black 96 well plate. Each sample was fixed with a final concentration of 1% paraformaldehyde.

Samples were incubated at 37°C for 15 minutes. After fixing the cells, 1 microliter of 1000x SYBR green

was added to each well. The plate was incubated for another 15 minutes at 37°C. After the incubation

the fluorescence was measured with an excitation of 494nm and an emission of 521nm. Calibration
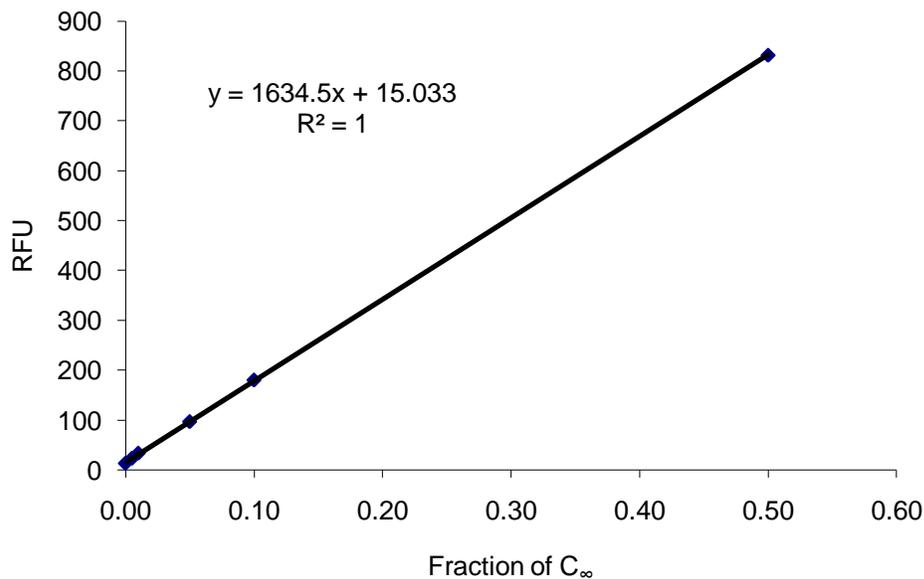
curves were linear as shown in Figure 9.

$$y = 1634.5x + 15.033$$
$$R^2 = 1$$

Figure 10. Calibration curve for the SYBR green quantification of *Vibrio gazogenes*.

Results

RNIP and Natural Samples

Cedar Swamp was chosen as an appropriate natural sample for the RNIP chemotaxis

experiments. The swamp sediment is anaerobic, and the water has a pH of approximately 4.5. This pH

would likely give the RNIP a half life of less than two weeks, and would result in the largest hydrogen

and dissolved iron. Cell counts of settled swamp water were 1.8×10^7 cells per ml. The chemotaxis experiment revealed no chemoattractant or repellant qualities of the RNIP, although aggregates of cells were visible on the aggregates of nanomaterials under the microscope. It would be interesting to extend this technique to pure cultures of hydrogenotrophic bacteria such as motile sulfate reducers and methanogens, as the cell density in a pure culture would make chemoattractive effects easier to measure.

It should be noted that a large amount of time was invested in performing an in-situ chemotaxis experiment in the swamp using an apparatus as shown in Figure 10. Challenges were unique to this project as the addition of nanoparticles did not allow the direct quantification of bacteria by phase contrast or dark-field microscopy. Several capillaries needed to be pooled in order to obtain enough cells for DAPI filter counts, and the movement of particulate in the swamp made every attempt at collecting enough sample fail. In-situ experiments may work better in open water environments, with a strong chemoattractant, and less active sediment.
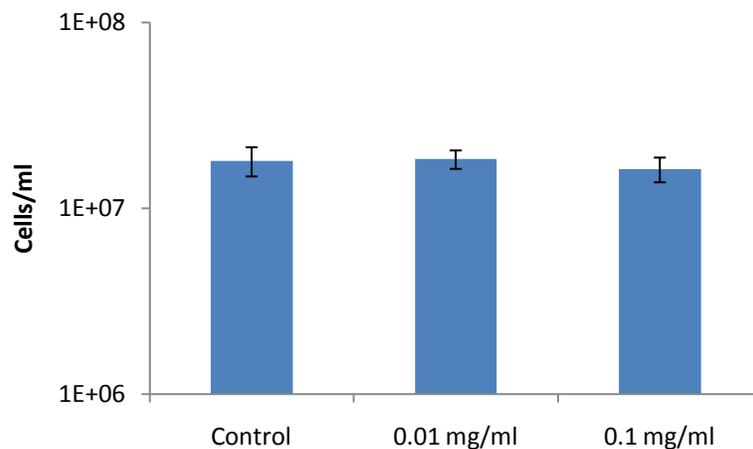

Figure 11. Quantification of capillaries from chemotaxis experiments using Cedar Swamp water and sediment, and reactive iron nanoparticles.

Silver nanoparticle chemotaxis experiments

Initial experiments using culture dependent techniques showed less growth in capillaries with larger amounts of silver nanoparticles. Unfortunately this technique does not distinguish between silver being a chemorepellant, and lack of a chemorepellant effect as nanoparticles are toxic to *V. gazogenes.* Therefore, a bulk fluorescence measurement was used in successive experiments to quantify chemotaxis.
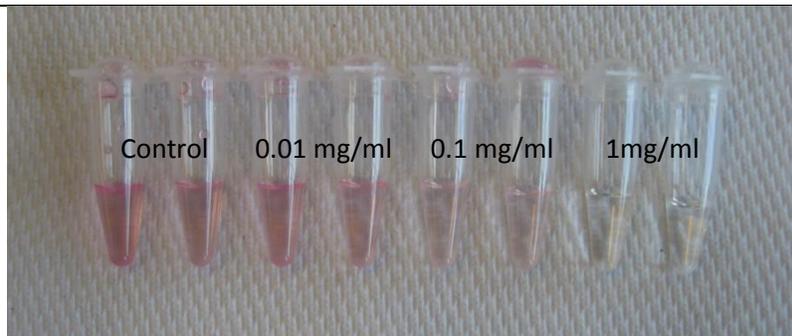

Figure 12. Overnight incubations of *Vibrio gazogenes* from silver nanoparticle chemotaxis experiments in increasing concentrations: 0.01mg/ml, 0.1mg/ml, and 1mg/ml concentrations. Less growth is observed from the capillaries with higher nanoparticle concentrations.

No chemorepellant effect was observed for *V. gazogenes* in the culture independent quantification, however a slight chemorepellant effect was seen for nanoparticle concentrations above 0.005mg/ml for the silver resistant *Pseudomonas* strain. This could be a result of having receptors for silver ions (the SilE binding protein), allowing it to detect silver in the environment. The *V. gazogenes* would have little contact with silver in its typical environment (seawater) as most silver would precipitate out as silver chloride, making silver detection a waste of ATP. It would be interesting to collect data on multiple types of bacteria from multiple sources to see if the correlation extends to other species who are either known to be resistant to silver or susceptible to silver toxicity.
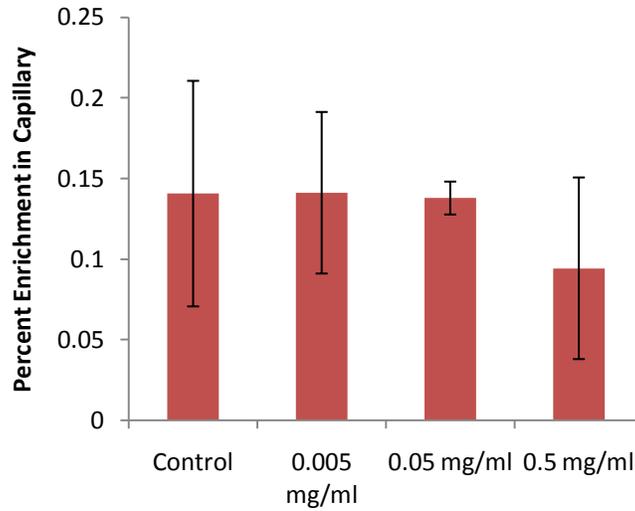
Figure 13. Absense of chemotaxis towards or away from 50 nm silver nanoparticles in a culture of *V. gazogenes*
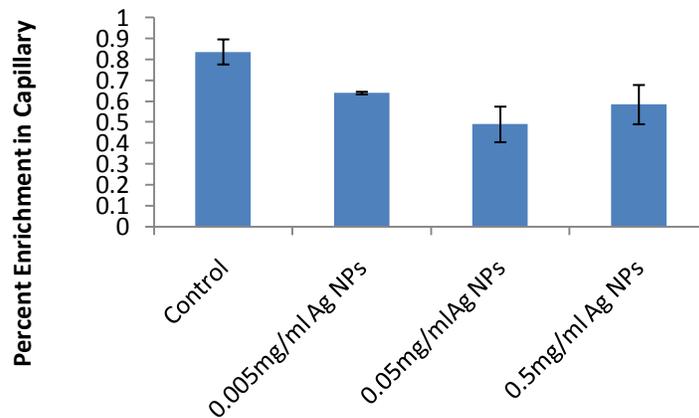

Figure 14. Demonstration of a chemorepellant effect of 50 nm silver nanoparticles in a culture of silver resistant *Pseudomonas*.

In conclusion, no chemoattractant effect of RNIP was observed in Cedar Swamp water.

Nanosilver was a chemorepellant for silver resistant *Pseudomonas*, but not for *V. gazogenes.* These

experiments could be easily extended to test other silver resistant bacteria and silver sensitive bacteria

to determine if perhaps the presence of silver binding proteins has any effect on whether nanoparticles

are a chemorepellant. Reactive iron nanoparticles could also be tested on a suite of pure cultures of

hydrogenotrophic bacteria.

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