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Soil components mitigate the antimicrobial effects of silver nanoparticles towards a beneficial soil bacterium, *Pseudomonas chlororaphis* O6

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ABSTRACT

Silver nanoparticles (Ag NPs) are widely used for their antimicrobial activity and consequently the particles will become environmental contaminants. This study evaluated in sand and soil matrices the toxicity of 10 nm spherical Ag NPs (1 and 3 mg Ag/L) toward a beneficial soil bacterium, *Pseudomonas chlororaphis* O6. In sand, both NP doses resulted in loss in bacterial culturability whereas in a loam soil, no cell death was observed. Amendments of sand with clays (30% v/v kaolinite or bentonite) did not protect the bacterium when challenged with Ag NPs. However, culturability of the bacterium was maintained when the Ag NP-amended sand was mixed with soil pore water or humic acid. Imaging by atomic force microscopy revealed aggregation of single nanoparticles in water, and their embedding into background material when suspended in pore water and humic acids. Zeta potential measurements supported aggregation and surface charge modifications with pore water and humic acids. Measurement of soluble Ag in the microcosms and geochemical modeling to deduce the free ion concentration revealed bacterial culturability was governed by the predicted free Ag ion concentrations. Our study confirmed the importance of Ag NPs as a source of ions and illustrated that processes accounting for protection in soil against Ag NPs involved distinct NP- and ion-effects. Processes affecting NP bioactivity involved surface charge changes due to sorption of Ca^{2+} from the pore water leading to agglomeration and coating of the NPs with humic acid and other organic materials. Removal of bioactive ions included the formation of soluble Ag complexes with dissolved organic carbon and precipitation of Ag ions with chloride in pore water. We conclude that mitigation of toxicity of Ag NPs in soils towards a soil bacterium resides in several interactions that differentially involve protection from the Ag NPs or the ions they produce.

1. Introduction

Nanotechnology is increasingly gaining interest and attention with investments of $1 trillion by 2015 (Navarro et al., 2008a). Kahru and Dubourguier (2010), using data based on organisms in the lower food web (bacteria, algae, crustaceans, ciliates, fish, yeasts and nematodes), indicate that NPs of Ag, ZnO and CuO are among the most toxic of the different classes of NPs. The use of silver nanoparticles (NPs) is high in medically related applications due to their antibacterial and antiviral properties (Cumberland and Lead, 2009; Akaighe et al., 2011). Antimicrobial effects of Ag NPs on planktonic cells of pathogenic bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Sondi and Sondi, 2004; Morones et al., 2005) show dose dependency; moreover toxic levels differ significantly depending on bacteria, NP shape, size, and the extent to which they release Ag ions (Pal et al., 2007; Sotiriou and Pratsinis, 2010).

Ag NPs are increasingly used in consumer products including textiles, cosmetics, soaps, water purifiers, food preparation and packaging surfaces, linings in dishwashers and washing machines, and coolants for refrigerators and air conditioners. Consequently, Ag NPs will likely contaminate the environment (Lin et al., 2010) and safe use practices and toxicity thresholds need to be established to minimize impact on beneficial bacteria, animals, and the food chain (Navarro et al., 2008a; Kahru and Dubourguier, 2010; Ma et al., 2010). Research on Ag NPs, such as that discussed by Gottschalk et al. (2009), focuses on impacts in wastewater treatment plants, wastewater effluent, biosolids, and surface waters. However, soils will be contaminated from on-site wastewater management systems,

Abbreviations: Ag NP(s), silver nanoparticle(s); AFM, atomic force microscopy; CEC, cation exchange capacity; DOC, dissolved organic carbon; DLS, dynamic light scattering; *Pc*O6, *Pseudomonas chlororaphis* O6.

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biosolids application, improper disposal, accidental spills, as well as through application of Ag NPs as an "organic" fertilizer/pesticide (Walters, 2011).

To understand the effects Ag NPs have on bacteria in soils, it is important to study how physico-chemical variables in natural systems influence the toxicity of NPs. Under aqueous conditions, humic acids cause partial disaggregation and stabilization of Ag NPs and reduce their antimicrobial effects against *Pseudomonas fluorescens* (Fabrega et al., 2009a, 2009b). Solution properties, such as pH, ionic strength and background electrolytes, alter the surface charge and aggregation of Ag NPs (El Badawy et al., 2010) resulting in altered toxicity (Jin et al., 2010; Yang et al., 2012).

Tests with planktonic cells demonstrate that Ag NPs are antimicrobial towards different strains of soil-dwelling pseudomonads (Morones et al., 2005; Fabrega et al., 2009a, 2009b; Gajjar et al., 2009; Dimkpa et al., 2011a). In this paper, we investigate in solid matrices the antimicrobial impact of Ag NPs on the beneficial soil bacterium, *Pseudomonas chlororaphis* O6 (*Pc*O6). Pseudomonads are model bacterial species because their versatility in metabolism makes them a dominant class of microbes globally. Root colonization by *Pc*O6 induces systemic tolerance to abiotic and biotic stresses in the plant (Spencer et al., 2003; Cho et al., 2008). Challenge of *Pc*O6 with CuO and ZnO NPs differentially affects cell culturability and, when sublethal levels are used, alters secondary metabolic pathways (Dimkpa et al., 2011b, 2011c, 2012a, 2012b). Thus, studying Ag NP-induced changes at lethal and sublethal levels with *Pc*O6 provides useful insights on potential environmental impacts of NPs.

In this paper we performed studies in microcosms with sand or soil as a solid matrix. Most reported studies expose soil-dwelling bacteria to the NPs in liquid cultures and consequently these findings do not adequately model the effects that will be seen in the real environment. Sand amended with clays was studied because clay minerals act as high surface area sorbents with cation exchange capacity (CEC). Two clay types, kaolinite, a 1:1 clay mineral with low CEC, and the 2:1 clay bentonite with higher CEC, were used as amendments. Humic acid is part of the aromatic complex present in soils (Galeska et al., 2001) and reduces the antimicrobial activity of Ag NPs (Fabrega et al., 2009a, 2009b). Responses with humic acid were compared to amendments with pore water generated from soil. Soil pore water contains a mix of nutrients, inorganic ions and dissolved organic carbon (DOC) that contains phenolic materials.

Toxicity of the Ag NPs arises in part from release of Ag ions (Tolaymat et al., 2010). Factors affecting release include addition of humic acid or excess citrate, the reduction of temperature, and increase in pH (Liu and Hurt, 2010). Thus, responses to the Ag NPs were compared to responses caused by Ag ion amendments. Atomic force microscopy, dynamic light scattering and zeta potential were used to characterize NPs.

2. Materials and methods

2.1. Sources of chemicals

The commercial Ag NP suspension was obtained from ATTOSTAT Inc. (West Jordan, UT, USA) with a manufacturer-reported particle size of 10 nm (Dimkpa et al., 2011a). Further information from the manufacturer indicates that the NPs were made by a laser-based technology and have no surface coatings. The concentration of Ag in the stock suspension was 22.4 mg/L (Dimkpa et al., 2011a). Ag ions (as $AgNO_3$) were obtained from Alfa Aesar (Ward Hill, MA, USA). Humic acid was product number H16752 from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). The clays, kaolinite (Na) and bentonite (Na) were from Ward Natural Science (Rochester, NY, USA) and Fisher Scientific (Pittsburgh, PA, USA), respectively. Sterile, deionized distilled (dd) water was used in all studies.

2.2. Growth of *Pc*O6

Inocula, from frozen stocks of *Pc*O6 cells in 15% glycerol at −80 °C were transferred into minimal medium (MM) with sucrose and citrate as described by Gajjar et al. (2009). Cells were grown for 14 h, transferred to new MM, and grown until log phase ($OD_{600\ nm} = 0.1 = 10^8$ cells/mL). Cells were pelleted by centrifugation at 10,000 g for 10 min and resuspended in the same volume of sterile dd water with known concentrations of additives for the different assays as described below.

2.3. Antibacterial activity of Ag NPs and Ag ions in sand or soil

White silica sand, obtained from UNIMIN Corp., ID, USA, was washed with dd water and dried before use. To eliminate culturable microbes from the solid matrices, the sand and an agricultural soil (a mixed mesic Entric Haploxeroll, Steed gravelly loam) were autoclaved twice for 40 min. Water washes of the sand were assayed by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500c) for elements that potentially could harm the bacteria. Kaolinite and bentonite were sterilized in dry powder form by microwave exposure (3 min at 1000 W).

Microcosms were established with 10 mL (12 g) sand, or 7 mL (8.5 g) of sand mixed by wrist-action shaking with 3 mL (2 g) of clay or 10 mL (10 g) soil in sterile tubes. Each tube received 3 mL of one of seven treatments: 1) water; 2) *Pc*O6 cell suspension (10^8 cells per tube); 3 and 4) *Pc*O6 cells plus 1 or 3 mg/L Ag NPs; and 5 to 7) *Pc*O6 cells plus 0.3, 1 and 3 mg/L Ag ions. The NP concentrations were selected based on the findings that planktonic *Pc*O6 cells (10^8 cells/mL) required over 1 mg/L Ag NPs for total loss of culturability (Dimkpa et al., 2011a). Thus, the present study was conducted with both lethal and sublethal doses. The contents of each tube were mixed thoroughly after each of these additions. The bacterial inoculum was the last added component. For the pore water and humic acid studies, the sand microcosms were amended with either 1 mL undiluted pore water or 1 mL of 100 mg/L humic acid.

The microcosms were incubated at 28 °C for four days and then one g of sand, or soil or sand/clay from the different treatments was suspended in 10 mL of sterile dd water. The samples were shaken on a vortex for 30 s. Serial dilutions were prepared and aliquots of 100 μL were plated onto Luria Broth (LB) medium, lacking NaCl, to determine cfu/g sand or soil, or sand/clay.

2.4. Characterization of sand matrix

To determine the presence of soluble components that might influence NP fate, 20 g sand was extracted in triplicate with 40 mL of dd water for 24 h on a reciprocal shaker. The aqueous fraction obtained by filtration with a 0.2 μm filter was analyzed by ion chromatography (Dionex ICS-3000) for major cations and assayed by ICP-MS for elements that potentially could harm the bacteria. Total organic and inorganic carbon was determined by combustion and IR detection using a Skalar Promacs SLC TOC Analyser (Skalar Analytical, Netherlands).

2.5. Soil characterization and pore water extraction and characterization of pore water and humic acid

The agricultural soil, obtained from the top 15 cm of a previously cropped area, was sieved and stored in a closed container at 4 °C. The soil was characterized by standard methods for particle size distribution as determined by hydrometry (Klute, 1986). pH was determined from the soil paste (Sparks, 1996).

To prepare pore water, the native soil was autoclaved twice for 40 min and sterile dd water was added until saturation but without standing water (Rhodes, 1996). The saturation paste was incubated