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Influence of triethyl phosphate on phosphatase activity in shooting range soil: Isolation of a zinc-resistant bacterium with an acid phosphatase

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ABSTRACT

Phosphatase-mediated hydrolysis of organic phosphate may be a viable means of stabilizing heavy metals via precipitation as a metal phosphate in bioremediation applications. We investigated the effect of triethyl phosphate (TEP) on soil microbial-phosphatase activity in a heavy-metal contaminated soil. Gaseous TEP has been used at subsurface sites for bioremediation of organic contaminants but not applied in heavy-metal contaminated areas. Little is known about how TEP affects microbial activity in soils and it is postulated that TEP can serve as a phosphate source in nutrient-poor groundwater and soil/sediments. Over a 3-week period, TEP amendment to microcosms containing heavy-metal contaminated soil resulted in increased activity of soil acid-phosphatase and repression of alkaline phosphatase, indicating a stimulatory effect on the microbial population. A soil-free enrichment of microorganisms adapted to heavy-metal and acidic conditions was derived from the TEP-amended soil microcosms using TEP as the sole phosphate source and the selected microbial consortium maintained a high acid-phosphatase activity with repression of alkaline phosphatase. Addition of 5 mM zinc to soil-free microcosms had little effect on acid phosphatase but inhibited alkaline phosphatase. One bacterial member from the consortium, identified as *Burkholderia cepacia sp.*, expressed an acid-phosphatase activity uninhibited by high concentrations of zinc and produced a soluble, indigo pigment under phosphate limitation. The pigment was produced in a phosphate-free medium and was not produced in the presence of TEP or phosphate ion, indicative of purple acid-phosphatase types that are pressed by bioavailable phosphate. These results demonstrate that TEP amendment was bioavailable and increased overall phosphatase activity in both soil and soil-free microcosms supporting the possibility of positive outcomes in bioremediation applications.

1. Introduction

A soil polluted with heavy metals alters the microbial community structure and biomass through the inhibition of microbial activity (Sas-Nowosielska et al., 2004; Kucharski et al., 2011; Kandeler et al., 1996; Utobo and Tewari, 2015). One of the common methods for remediation of metal-contaminated soils involve pump and treatment with chelating agents to enhance mobilization of heavy metals (Vulava and Seaman, 2000) and harnessing microbial-reductive processes to precipitate of heavy metals in anaerobic environments (Lovely and Coates, 1997). Drawbacks to these methods include the uncontrolled spread of metals/radionuclides and the alteration of soil chemistry involving removal of non-target elements, such as iron, needed for microbial metabolism.

Microorganisms serve as the foundation for nutrient cycling in any environment. Phosphorus is one of the key nutrients essential to all living things and though phosphorus is ubiquitously distributed, it is often biologically unavailable in an insoluble, inorganic form or organically bound, and must be released as phosphate ion via phosphatases. Phosphate comes from both natural and anthropogenic sources, and environmental phosphatases can play a major role in the phosphorus cycling and breakdown of synthetic organophosphates (Pansu and Gautheyrou, 2006). Microbial enzyme activity in soil can be used as one measure of soil quality (Amador et al., 1997; Kucharski et al., 2011; Utobo and Tewari, 2015). For example, soils exposed to tert-butylphenyl-diphenyl phosphate altered soil microbial phosphatase observed as an increase in monophosphoesterase activity (Heitkamp et al., 1986). However, laboratory and field studies have demonstrated that phosphatases are inhibited by low levels of heavy metals such as zinc (Kucharski et al., 2011; Huang and Shindo, 2000). Where Kucharski et al. (2011) found that compared to alkaline soil, an acidic soil imposes greater metal toxicity to phosphatases than all other soil enzymes tested.

In light of microbial sensitivities to heavy metals, phosphate amendments have been shown to decrease heavy-metal or radionuclide

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bioavailability (Wilson et al., 2006; Knox et al., 2008). For example, phosphatase-mediated immobilization of uranium by microbial activity has been demonstrated in a *Citrobacter sp.* that utilized tributyl phosphate (TBP) as a sole phosphate source (Montgomery et al., 1995). In another study, lead provided as $Pb(NO_3)$ has been shown to be immobilized via phosphatase-mediated precipitation as $Pb(PO_4)$ on microbial surfaces (Levinson and Mahler, 1998). Recently, phosphatase-mediated bioprecipitation of lead by soil fungi can be applied for Pb recovery (Liang et al., 2016).

At many sites the subsurface saturated zones are nutrient-poor, depleted of phosphorus and contaminated with organics and heavy metals. Triethyl phosphate (TEP) has been applied through biosparging, serving both as a carbon and phosphorus source to stimulate endogenous microorganisms, with promising results to remediate organic-groundwater contaminants (Hazen et al., 1996). Although mildly toxic and a corrosive irritant, TEP is one of the safest phosphorus compounds known that can readily be gasified (Bogan et al., 2001). Biosparging employs forced air and gaseous distribution of TEP (carbon/nitrogen source), methane (carbon source) and nitrous oxide (nitrogen source) into the saturated subsurface zone as an effective means to stimulate anaerobic-driven bioremediation (Pfiffner et al., 1997). TEP enhanced the hydrocarbon degradation *in situ* and laboratory groundwater microcosms (Santo Domingo et al., 1997a, 1997b), yet the mechanism by which TEP stimulates microorganisms is unclear. Additionally, the use of TEP as an amendment to heavy-metal remediation has yet to be investigated. TEP absorbs molecularly on both wet and dry iron-hydr(oxide) nanoparticles to the longer aliphatic chain, stabilizing the loss of charge on the methoxy-CO bonds by charge redistribution upon phosphoryl-O coordination to Fe surface atoms (Mäkie et al., 2013). By absorption in soils and sediments, TEP is less likely to wash or leach out and thus more likely to remain biologically available as a nutrient.

The spatial dependence of phosphatase activity in soils has important implications for evaluating this enzyme activity (Amador et al., 1997). The application of TEP can overcome these spatial heterogeneities, since it can be widely distributed in gas phase and liquid phase and therefore stimulate microbial phosphatase in the area of interest. In this way, remediation of heavy-metals/radionuclides via microbially-mediated release of excess phosphate in subsurface soil could result in immobilization as metal phosphate.

We postulate that microorganisms adapt upon long-term exposure to heavy metals in an acidic environment by upregulation or production of phosphatases in response to toxic heavy-metal exposure and, given an adequate phosphate source in the form of TEP in a nutrient-limited environment, the microbial population adapts to get the required phosphorus nutrient and protection from heavy-metal exposure. Here will investigate the selective impact of TEP on two major classes of microbial phosphatase and its effects on the soil microbiota. We propose that specific classes of phosphatases can indirectly relieve the toxic effects of heavy metals via chemical precipitation as metal phosphate with acquisition of the essential phosphorus nutrient, increasing microbial activity.

2. Material and methods

2.1. Soil sampling

A soil sample consisting of approximately 10 kg was collected at the DoE's Savannah River Site, Aiken, SC, from its Small Arms Training Area (SATA) firing range, an area with high levels of heavy-metal contamination (Wilson et al., 2006). Surface soil was collected to a depth of 30 cm from the range center with a stainless-steel coring device and transported to the laboratory. Soils from the SATA area are generally considered to be within the Udorthents, Great Group (Rogers and Herren, 1990; USDA-NRCS, 2005). Soil samples were processed the same day as collection. One portion (100 g) was assayed for acid and alkaline phosphatase; another was put on a tray to air-dry for elemental analysis. The air-dried soil was sieved through 4 mm mesh screening. Sieved soil was mixed and distributed into 100 mg aliquots for analysis of lead levels using multi-element X-Ray Fluorescence (XRF). The accuracy of the XRF analyses was verified by comparing results from selected samples using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Vulava and Seaman, 2000). Soil pH was averaged to a pH of 5.1, which was determined by a standard method previously described (Wilson et al., 2006).

2.2. Ion chromatography

Phosphate concentrations were measured using a Dionex DX500 Ion Chromatograph (IC), operating at ambient temperatures and equipped with a conductivity detector and 250 mm Dionex IonPac AS14 Analytical Column (4 mm ID, 16 µm bead; Dionex Corp., Sunnyvale, CA). A 3.5 mM sodium carbonate/1 mM sodium bicarbonate buffer solution was used as the eluent (1.2 mL/min). Samples were taken from the supernatant of a solution consisting of: water, media or 5 g of dry soil (dried at 121 °C for 24 h), and 5 mL of deionized water; vortexed for 1 min and then centrifuged for 5 min at 2500 rpm. The ion detection limit for ions measured on this IC were 0.5 µg/L.

2.3. Soil phosphomonoesterase activities

All phosphatase assays were measured in triplicate on both soil and soil free treatments. Based on the method of Eivazi and Tabatabai (1977), p-nitro phenyl phosphate was used to assay soil acid and alkaline phosphomonoesterase, bis-p-nitro diphenyl phosphate for phosphodiesterase, and tris-p-nitro triphenylphosphate for phosphotriesterase. A modified, universal buffer (TRIS-maleate) was used to maintain the pH for the assay of acid phosphatase (pH 6), alkaline phosphatase (pH 8), and phosphodiesterase and phosphotriesterase (pH 10). Absorbance units were converted to µmol p-nitrophenol (p-NP) produced per mL or gm soil per h. Saturating substrate at 3.0 mM was determined empirically. The assay concentration of p-NP released was calculated from a standard curve prepared with p-NP standards. Controls without substrate were extracted as described above, and values were subtracted as background. The apparent Michaelis-Menton enzyme constant (K_m) and maximum enzyme reaction velocity (V_{max}) were calculated from the classical Lineweaver-Burk plot for all enzyme assays.

2.4. Microbial growth media

Phosphate-free medium used in soil slurries and soil-free cultures was prepared in g/L: TRIS base, 12.0; $(NH_4)_2SO_4$, 0.96; KCl, 0.62; $MgSO_4$, 0.063; $FeSO_4 \cdot 7H_2O$. Culture medium was sometimes amended with 1% glucose (w/v), 1–10% TEP (v/v), 10 mM glycerol phosphate or 10 mM potassium phosphate. The pH of the medium was adjusted to 6.0 or 8.0 with NaOH or HCl. Triplicate sets of soil slurries consisted of 100 mL medium and 10 g soil, with a one-time amendment of 10% TEP and aerated on a shaking incubator at 25 °C. Sampling for phosphatase assays was performed in triplicate. For soil-free microcosms, 10 mL of soil-slurry supernatant was transferred to 40 mL, fresh TRIS base medium (pH adjusted to 6 and 8), with or without 10% TEP and 1% glucose. Zinc chloride was added (5 mM) to the soil-free consortium to observe the effect of metal amendment on phosphatase activity. Tryptone-peptone broth (TSB) or Tryptic Soy Agar (TSA) was purchased from Difco, Inc. (Detroit, MI) and prepared as per the manufacturer. TBP was added at 10% final concentration. Dilutions of microcosm solutions were made with 0.2 µm filter-sterilized, phosphate-buffered saline (PBS) and plated onto 1% TSA medium to determine cultivable abundance and diversity. All bacterial growth conditions were done in triplicate and phosphatase assays were performed in triplicate as well.