Effect of pH on the mobilization of copper and iron by pyoverdin I in artificially contaminated soils

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ABSTRACT: The effect of pyoverdin I, a natural product from a Thai *Pseudomonas aeruginosa* strain, on the solubilization of iron and copper in two different artificially contaminated soils was investigated. The mobilized concentrations of both metals in the samples were determined by atomic absorption spectrometry. From these results, the presence of pyoverdin I can significantly increase the amount of extractable iron and copper compared to the control. The adsorption and desorption of these metals depend not only on the environmental pH but also on the soil type, which results from differences in the quantity of the organic matter. The ability of pyoverdin I to mobilize either metal in the soils indicates its important role in connection with toxicology, as well as for soil extraction and weathering. Studying the mobilization of metals by the bacterial pyoverdin I as a function of pH may provide additional information on the chemical association of metals in soil.

KEYWORDS: pyoverdin I, *Pseudomonas aeruginosa* R′, mobilization, copper, iron, soils

INTRODUCTION

Soil may be contaminated by heavy metals such as Pb, V, Cr, or Cu due to the use of sludge or urban composts, pesticides, fertilizers, and emissions from municipal waste incinerators. The effects of heavy metals on ecosystems vary considerably and are of economic and public health significance. The mechanisms concerning heavy metals accumulation in soil lead to the existence of four major geochemical forms[1]: (i) exchangeable form which is bound to carbonate phase, (ii) bound to iron-manganese oxides, (iii) bound to organic matter, and (iv) residual metal phase. A number of heavy metals are essential as trace elements for microorganisms, but at high levels they become toxic. For example, copper occurs naturally in rock, soil, water, air, and also in plants and animals. The copper ion is a metal cofactor of several enzymes including tyrosinase, ascorbic acid oxidase, and butyryl Co-A dehydrogenase. It is one of the metals concerned with photosynthesis. While copper is an essential micronutrient, exposure to excess copper has a detrimental effect on plant growth, especially on root growth and morphology[2,3]. Thus, copper is considered as a hazardous heavy metal and is included in the Environmental Protection Agency's list of priority pollutants[4]. In soil solution, the copper concentrations are generally extremely low, with more than 98 % of the copper ions bound to soluble organic matter, irrespective of pH[5]. Due to its high affinity to organic matter, copper is not readily leached from the soil profile and tends to accumulate in the surface soil[6].

Iron has a major metabolic role in almost all living systems except the *Lactobacilli* and *Borrelia burgdorferi*. It is poorly soluble since both ferric and ferrous salts rapidly hydrolyse to insoluble polymeric hydroxides at near neutral pH[7,8]. Although ferric ions may be adsorbed by plants, the metabolically active form seems to be the ferrous ion. The concentration of free ferric ions in solution at a neutral pH has been estimated to be 10^{-18} M, a value too low for the microbial and plant requirements[9].

There is a wide range of phenomena from simple to complex interactions which can occur between microorganisms and heavy metals such as adsorption, oxidation/reduction, solubilization, and precipitation. There are numerous studies on complexation of metal ions with organic acids such as citric acid, acetic acid, and oxalic acid which may exist in the soil for a short period of time[4,10,11]. Siderophores are other important natural chelating agents which are synthesized by microorganisms and some plants[12–14] to solubilize external iron. The siderophores are secondary metabolites secreted under iron-deficient conditions, binding to Fe(III) with high affinity. These compounds are also able to bind to other metal ions such as lead, chromium, gallium, cobalt,

and copper with less affinity[7,15,16]. The siderophore pyoverdin is a more complex molecule (1.5 kDa) consisting of an 8-hydroxyquinoline derivative which forms a fluorescent chromophore, and an octapeptide which contains two residues of N^5-hydroxyornithine. Pyoverdin is a very water-soluble molecule and a powerful chelator of ferric iron, which is bound with a stoichiometry of 1:1[17,18]. It may also successfully compete for iron either with other siderophores produced by some microorganisms in the same environment or with the iron binding proteins like transferrin, lactoferrin, and ferritin representing the sole potential sources of iron for pathogenic bacteria developing *in vivo*.

In this study, we focused on the mobilization mechanisms of copper and iron both in the absence and presence of pyoverdin I. The pH condition is of prime importance in determining the mobility of either metal from soil samples. For practical purposes, the possible transfer of the metal from a solid to a liquid phase of soil can be monitored by determining its solubility as a function of pH. Therefore, the effect of pyoverdin I on metal mobility in artificially contaminated soils was studied.

MATERIALS AND METHODS

Soil characterization

Two unpolluted soil samples were used in this study: Yasothon soil series (Y) collected from Khon Kaen province, Thailand, and sea sand (S) purchased from Fluka (Switzerland). Coarse particles were removed from the soil samples using a 2-mm sieve. After drying, both soil samples were homogenized and stored in a plastic container for subsequent experiments. Soil pH was determined from the supernatant of a 1:2 soil to medium (deionized water or 0.01 M $CaCl_2$) suspension following 1 h of mixing. The amount of total organic carbon was measured by a wet oxidation-reduction titration method[19]. The procedure involves the oxidation of organic carbon with a dichromate solution and titration with a Fe^{2+} solution to determine the amount of dichromate remaining. Metals present in the soil, Cr, Fe, Cu, Pb, and V were determined by atomic absorption spectrophotometry (AAS) (Analyst 100, Perkin Elmer, USA) after the soil samples were digested with a mixture of boiling nitric and hydrochloric acids (1:3 v/v). All experiments were carried out in triplicate.

Artificially contaminated soils were prepared by adding 500 ml of 5.86 mM $CuCl_2$ to each of the 250 g samples of soil Y and soil S, and 500 ml of 3.70 mM

$FeCl_3$ was added to another 250 g sample of soil S. A sample of soil Y contaminated with additional iron was not prepared because the original soil was found to already contain the metal. The contaminated soil samples were shaken at 200 rpm for 1 month and left to stand for 2 months. Thereafter, the supernatant was removed by decantation and the residual soil was washed with excess deionized water (three times). Some colloidal particles were lost during decantation. The soil solution was filtered, dried at room temperature under vacuum, and stored in an air-tight container. Soil Y contaminated with Cu ions is referred to as YC and soil S contaminated with copper and iron are referred to as SC and SI, respectively. The Cu and Fe contents of the soil sample were determined by AAS after hot acid digestion with a mixture of nitric acid and hydrochloric acid (1:3 v/v).

Production of pyoverdin I

The strain *Pseudomonas aeruginosa* R′ was obtained from the Department of Microbiology, Faculty of Science, Khon Kaen University. It was isolated from a patient's urine at Srinagarind hospital, Khon Kaen University, Khon Kaen, Thailand. The bacterium was grown on a synthetic SA medium (modified from Sucrose Asparagine medium, SA) made up of 20 g of sucrose, 2.0 g of L-asparagine, 2.0 g of K_2HPO_4 and 1.0 g of $MgSO_4 \cdot 7H_2O$ per litre and adjusted to pH 7.0 by adding the required volume of 1N NaOH or 1N HCl prior to sterilization[20]. A preculture was prepared using a single colony of 48 h bacterial culture on a chrome azurol sulphonate agar plate. The colony was resuspended in 25 ml SA medium in a 125-ml Erlenmeyer flask. The culture was aerated by vigorous shaking at room temperature for 24 h. A 20-ml preculture was used to inoculate 1.5 l media in a 5-l culture flask. Incubation was carried out in an orbital shaker at 250 rpm, 27–30 °C. After 24 h, the proteins of a cell free solution were obtained by filtering through Whatman No. 42 filter paper, and were precipitated by saturation with sodium chloride. The cell-free supernatant was purified by column chromatography using an Amberlite XAD-4 resin column by adding it into the column slowly and then washing with water until the filtrate was clear. The crude pyoverdin I, appeared as green solution, was eluted with 90% methanol in water and concentrated using a rotary evaporator prior to further purification using a Bio-Gel P-2 column (40×2.5 cm, i.d.). Pyoverdin I was eluted with 0.02 M pyridine acetate buffer, pH 6.5 according to the method previously reported[21]. Fractions were collected, evaporated and lyophilized. The lyophilized samples
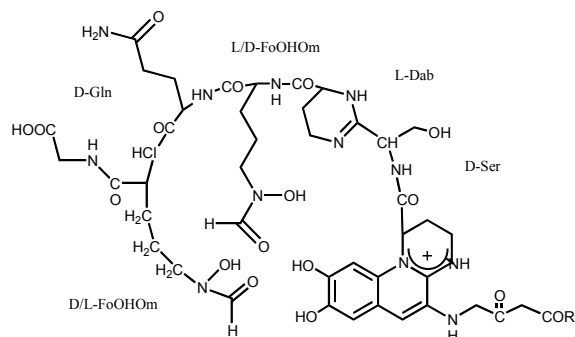
Fig. 1 Pyoverdin I (Ia: R = NH$_2$, Ib: R = OH, Ic: R = COOH) from a strain of *Pseudomonas aeruginosa* R′ (from Ref. 22).

were stored at –20 °C. The purity of pyoverdin I in the purified sample was analysed by RP-HPLC, and pyoverdin I (Fig. 1) was identified by spectroscopic techniques.

Effect of pH on metal-pyoverdin I complex formation

The complex formation between metals (Cu^{2+} and Fe^{3+} ions) and pyoverdin I was monitored using a spectrofluorometric method. First, 5 ml of pyoverdin I (478 nM) was dispensed into a 30-ml vial, and the pH was adjusted to 3.0, 5.0, 7.0, and 9.0 using 0.1 M HCl or 0.1 M NaOH. An equal volume of Cu^{2+} (5.68 mM) or Fe^{3+} (3.70 mM) was then added to give the pyoverdin I final concentrations of 147 nM and 92 nM, respectively. After 2 h, the fluorescence quenching due to metal-pyoverdin I complex formation was monitored by a Shimadzu spectrofluorometer at excitation and emission wavelengths of 400 and 460 nm, respectively[23].

The effect of pyoverdin I on copper and iron mobility in artificially contaminated soils at different pH conditions

The potential availability by pyoverdin I extraction of copper and iron was investigated by comparison with the control samples. The effect of pH on the mobilization of these metals by pyoverdin I was carried out in the pH range 3.0–9.0. The buffers used in the experiment were 0.1 M citric acid-phosphate buffer, pH 3.0, 5.0, and 7.0, including 0.1 M Tris-HCl buffer, pH 9. To 1.0 g of each contaminated soil in a polyethylene bottle was added 25 ml of a solution containing 72 nM of pyoverdin I to obtain the optimum concentration as reported in the previous studies (data not shown). The pH of the suspension was adjusted with 0.1 M HCl or 0.1 M NaOH. The soil suspension samples were shaken for 24 h to ensure that an equilibrium was reached. The suspension was centrifuged at 250 rpm and the clear solution was filtered through Whatman No. 42 paper filter. The total extractable metal concentrations in the filtrates were measured by AAS. Deionized water was used as a control for the study.

## RESULTS AND DISCUSSION

Soil characterization

The physico-chemical characterizations of the soil samples are shown in Table 1. Soil pH can be measured in an aqueous matrix such as water or in a dilute salt solution. The pH measurement in the matrix of 0.01 M CaCl$_2$ against water has advantages for agricultural soils, but the addition of the salt lowers the pH by about 0.5 compared to the soil pH in water.

The amounts of copper and iron in the contaminated sea sand were 0.1109 and 0.3892 mg/g, respectively. This result shows that the amount of heavy metals retained in the soils depended not only on the soil type, but also on the heavy metals involved. This was in agreement with several earlier studies[24,25]. The mechanisms suggested in several studies on heavy-metal retention include precipitation as solid phase oxides, hydroxides, carbonates, ion exchange, and complexation reactions[26,27].

Table 1 Chemical and physical characteristics of the soil samples.

Characteristics	Soil Y	Soil YC	Soil S	Soil SC	Soil SI
Organic matter (mg/g)	19.88	18.76	ND	ND	ND
pH-water	7.26	3.78	8.55	6.91	8.07
pH-CaCl$_2$	7.10	3.29	7.98	6.70	7.42
Heavy metals (mg/g)					
Chromium (Cr)	ND	ND	ND	ND	ND
Iron (Fe)	0.0142	0.0138	ND	ND	0.3892
Copper (Cu)	ND	0.1649	0.01	0.1109	ND
Lead (Pb)	ND	ND	ND	ND	ND
Vanadium (V)	ND	0.005	ND	ND	ND

ND: Not detectable
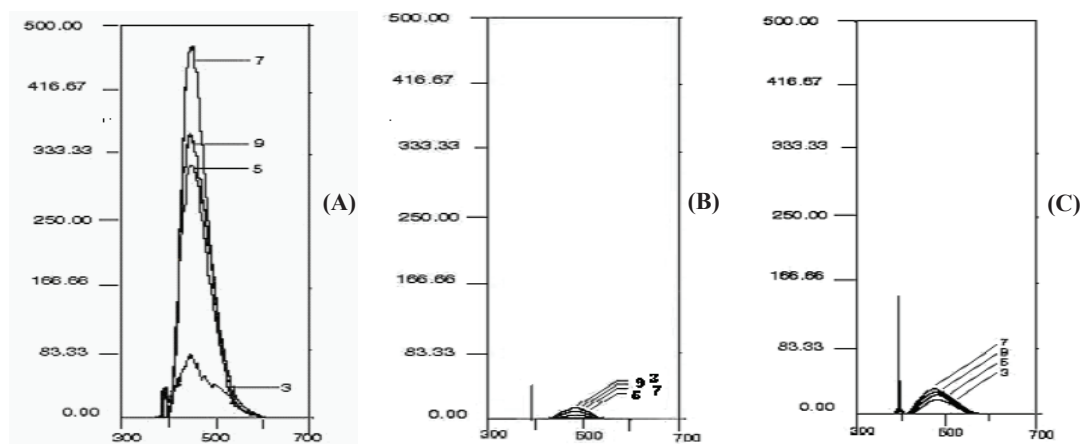
Fig. 2 Fluorescence spectra (λ_{ex} = 400 nm, λ_{em} = 460 nm) at pH 3, 5, 7, and 9 of (A) pyoverdin I, (B) pyoverdin I-Fe complex, (C) pyoverdin I-Cu complex.

The amount of copper retained in soil Y was slightly higher than that in soil S due to the higher content of organic matter. The mechanisms involved in the binding of metals by organic matter include complexation and adsorption, i.e. inner sphere reactions may occur as well as ion exchange[28,29]. The active sites in metal binding by organic matter are the negatively charged functional groups such as phenolate or carboxylate groups. Furthermore, the complexes formed with unidentate ligands were less stable than those formed with multidentate ligands. As expected, the natural organic components of the soil constituents have a high affinity for the heavy metal cations because of the presence of ligands or groups that can form chelates with them.

Complexation of metals and pyoverdin I at different pH values

Fig. 2A shows that the pyoverdin I produces fluorescence spectra similar to the previously reported ones of *Pseudomonas putida* type A[30]. The fluorescence intensity is decreased due to a quenching by these metals (Figs. 2B and 2C). Higher

fluorescence quenching corresponds to the stronger binding of the metal ion. It is well known that the formation of the Fe^{3+}-pyoverdin I complex results in a higher fluorescence quenching[23]. These results are due to the binding of the metals to the catecholate group of the chromophore moiety and to the hydroxamate groups provided by N-hydroxy ornithine derivatives[31]. These results show that the complex formation between pyoverdin I and either metal is highest near neutral pH and decreases with increasing pH. This may contribute to the precipitation of metal. The Fe^{3+}-pyoverdin reaction was pH dependent. The reaction rate increased with decreasing pH.

Effect of pH on the availability of metals by pyoverdin I

The investigation of the mobilization of metals at various pH and soil types is necessary to obtain more details in an environmental impact assessment. In this case, the implementation of natural conditions would be useful. The extractable efficiency of heavy metals depends on the pH values. At pH 3, 0.06–0.08 mg/g of copper was extracted from the soils by treatment
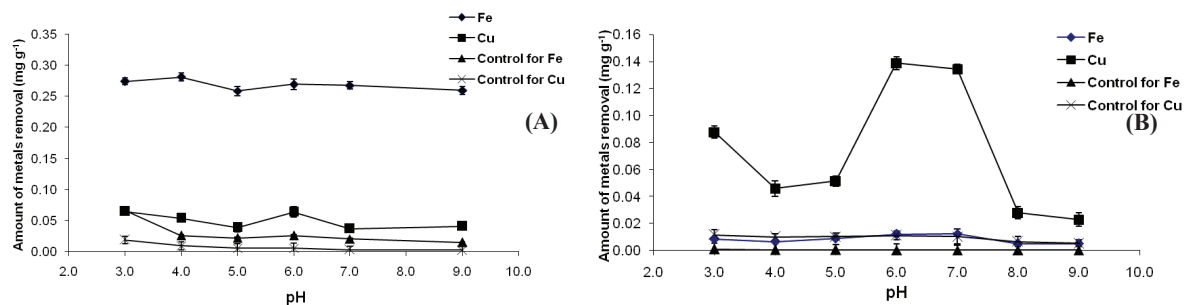
Fig. 3 Effect of pH of pyoverdin I on the removal of Cu^{2+} and Fe^{3+} from (A) soils SI and SC, (B) soil YC. Each point is the average of triplicate measurements.

with pyoverdin I. The amount of copper removal was 0.0877 mg/g (53.6%) for soil YC and 0.0647 mg/g (57.0%) for soil SC at pH 3, and the amount of iron removal was 0.0085–0.2741 mg/g for soil YC and SI. Fig. 3 shows that at higher pH, the activity of pyoverdin I decreases. From the fluorescence intensities of the metal-pyoverdin I complex at various pH values, it can be seen that the highest pyoverdin I activity occurs near pH 7. Pyoverdin I was more effective at removing iron than copper from soil samples. This result is due to the electronegativity of iron. Therefore, the mobility of copper was substantially lower than that of iron which is consistent with the stronger chemisorption of copper by oxides and humus in soils[32]. Pyoverdin I mobilized the heavy metals from soil SC and SI more effectively than those from soil YC. Lower pH typically leads to the protonation of the ionized chelate species. A drop in pH results in competition for binding sites between the hydrogen ion and metal ions resulting in a net decrease in metal solubilization. The extraction yield of the contaminated soil with water as a control at various pH values was not higher than 20%. This demonstrates the low solubility in water of the metals studied and confirms the need to add a chelating agent to the solution in order to increase its extractive capacity.

CONCLUSIONS

Most of the metals are bound to mineral or organic matter and as such they are insoluble or unavailable to microorganisms and plants. In nature, metals become available through mineral weathering and organic matter decomposition. Our results suggest that the metal desorption is significantly affected by the soil type and pH variation. Furthermore, the observed 2–4 fold increase in dissolved Cu and Fe in the presence of pyoverdin I indicates that naturally present bacterial pyoverdin I in the soil may have an influence on the metal mobility. The present results clearly indicate that pyoverdin I mobilizes both Fe and Cu in soils. Irrespective of their organic matter content and pH-values, pyoverdin I binds to Fe more strongly than to Cu, and has a high capacity for expediting weathering processes.

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