Binding of cobalt and zinc by organic acids and culture filtrates of *Aspergillus niger* grown in the absence or presence of insoluble cobalt or zinc phosphate

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Received 23 January 2001; accepted 19 June 2001.

The ability of commercially-available citric, oxalic and gluconic acids to bind Co$^{3+}$ and Zn$^{2+}$ was investigated and compared with culture filtrates from *Aspergillus niger*, a fungus capable of citric, gluconic and oxalic acid production, grown in the presence and absence of cobalt or zinc phosphate. This work demonstrated that citric and oxalic acid and the culture filtrates of *Aspergillus niger* produced in the presence and absence of cobalt or zinc phosphate are capable of binding these metals. Gluconic acid did not bind Zn$^{2+}$ in some cases, the culture filtrates were more efficient than commercial organic acids. Gluconic acid did not bind Co$^{3+}$ or Zn$^{2+}$ under the conditions used in this study. The presence of insoluble metal phosphates in the growth medium was found to markedly influence the production of organic acids and, while large concentrations of gluconic acid were produced in the presence of Co$_2$(PO$_4$)$_3$, the culture filtrate was unable to bind Zn$^{2+}$. The production of oxalic acid by *A. niger* when grown in the presence of Zn$_2$(PO$_4$)$_3$ led to the precipitation of insoluble zinc oxalate, a phenomenon with implications for metal tolerance and toxicity. The significance of these findings in relation to the environmental mobility of metals and phosphate, and the role of fungi in such transformations are discussed.

INTRODUCTION

Human activities release many pollutants into the environment, including potentially toxic metals that interact with its microbial and abiotic components. The nature of these interactions requires better resolution to fully understand the role of microorganisms in the environmental fate of introduced metals. The speciation, or form, of metals in natural environments affects biological availability, toxicity and geochemical reactivity (Varney *et al.* 1984). Complexation of metals by organic molecules is an important determinant of speciation and organic acids play fundamental roles in the environmental mobility of metals (Gadd 1999, Sayer *et al.* 1999). Citrate anions form stable complexes with a number of metal ions, for example copper and zinc, and highly mobile complexes may be resistant to biodegradation (Francis, Dodge & Gillow 1992). In contrast, oxalate anions precipitate many metal ions, e.g. calcium, as insoluble metal oxalates (Sayer & Gadd 1997, Gharieb *et al.* 1998, Gadd 1999). Most oxalate salts are immobile, possibly conferring tolerance on oxalate-producing organisms (Purvis 1984), and fairly resistant to degradation by anaerobic bacteria, aerobic actinomycetes, aerobic bacteria and fungi (Allison *et al.* 1995). Toxic effects of metals can also be ameliorated by complexation with a variety of other naturally-occurring organic ligands including humic substances, amino acids and extracellular products (Jardim & Allen 1984).

Fungi predominate in most acidic soils and often comprise the largest biomass. In such locations, metals may be speciated into more mobile forms (Gadd 1993). Many fungi are capable of solubilizing metals from insoluble forms, thereby increasing their availability to plants and other organisms (Asea, Kucey & Stewart 1988, Gadd 1993, Banks *et al.* 1994a, Banks, Waters & Schwabb 1994b, Sayer, Raggett & Gadd 1995, Sayer *et al.* 1999). Organic acid production is a major determinant of such solubilization phenomena (Gadd 1999, Gadd & Sayer 2000). *Aspergillus niger* has received considerable attention since it can produce significant quantities of citric, gluconic and oxalic acids, along with other organic acids (Mattey 1992, Burgstaller & Schinner 1993, Wolschek & Kubicek 1999). When the growth medium is deficient in manganese (less than 10$^{-8}$ M), *A. niger* produces large quantities of citric acid (Kisser, Kubicek & Röhrl 1980) and is used commercially to produce approximately 400000 tons of citric acid per year for use in food, confectionery, beverages and pharmaceuticals (Mattey 1992). Gluconic acid is also produced by *A. niger* and, using invert syrup or fructose as a carbon source, the process has been adapted to produce a non-carcinogenic sweetener containing gluconic acid, fructose and oligosaccharides (Mattey 1992). Oxalic acid...
production by *A. niger* can precipitate metal cations as insoluble metal oxalates (Sayer & Gadd 1997, Sayer, Kierans & Gadd 1997, Gharieb, Sayer & Gadd 1998, Sayer et al. 1999). This fungal-mediated process is of both physiological and biogeochemical significance (Gadd 1999, Gadd & Sayer 2000).

In view of reported differential effects of fungi on the dissolution of insoluble metal compounds (Sayer et al. 1995), and the paucity of work on insoluble metal phosphates, we examined the influence of zinc and cobalt phosphate on organic acid excretion by *A. niger* in liquid culture. This has allowed examination and quantification of changes in the nature and levels of organic acid production, as well as the release of soluble metal and phosphate species. Metal binding by fungal culture filtrates was compared with commercially-obtained organic acids (citric, oxalic and gluconic) to accurately assess the relative role of organic acids in these phenomena, as other substances in culture filtrates may also contribute to changes in metal speciation. The findings presented further understanding of fungal physiology and the roles of fungi in metal biogeochemistry and in bioremediation.

**MATERIALS AND METHODS**

**Organism and growth conditions**

*Aspergillus niger* (ATCC 210373) was maintained on malt extract agar (MEA, Lab M) at 25 °C. Liquid culture medium contained, per l ddH₂O: 5 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 0.2 g MgSO₄, 7H₂O, 0.05 g CaCl₂, 6H₂O, 0.1 g NaCl, 0.0025 g FeCl₃, 6H₂O, 0.004 g ZnSO₄·7H₂O, 0.004 g MnSO₄·4H₂O, 0.0004 g CuSO₄·5H₂O, 20 g D-glucose. *Aspergillus niger* was grown in 5 l aerated liquid medium (control) or liquid medium amended with 0.5 mM potassium phosphate (pH 6–7) and 5 mM cycloheximide. The electrolyte (with and without experimental additions) was sparged at 25°C with N₂ for 3 min before polarograms were recorded, and each polarogram was obtained from where consecutive voltage sweeps. Computer analysis provided values for evolution potential and current potential of the derived peaks. For analysis of Co²⁺ and Zn²⁺ binding by organic acids and *A. niger* culture filtrates, the desired metal compound (Co(NO₃)₂ or Zn(NO₃)₂) was added to 24.75 ml electrolyte in 5 × 50 µl aliquots, to ensure adequate mixing, from 10 mM stock solutions to a final concentration of 100 µM. Organic acids were added from stock solutions in 10 µl aliquots to the desired final concentration. *Aspergillus niger* culture filtrates were added in 10 × 100 µl aliquots.

Culture filtrates were analysed for available phosphate by ion chromatography (IC). The system comprised a Metrohm 690 ion chromatograph, a Metrohm 697 IC pump and a SP 4400 integrator (Thermoseparation products, Spectra Physics). The eluant was 2.5 mM p-hydroxybenzoic acid, 1 mM sodium benzoate and 2.5% methanol, pH 8.5, with a flow rate of 2 ml min⁻¹. Ten µl of filtrate was injected onto a PRP-X100 anion column in duplicate, and analysed for 15 min. Co and Zn content of the culture filtrates was determined by atomic absorption spectrophotometry (AAS) (SP9, Pye Unicam) and compared with appropriate standards in acidified (1% HNO₃) ddH₂O (White & Gadd 1995).

Culture filtrates were analysed for organic acid content by gas chromatography-mass spectrometry (GC-MS) after drying. The dried samples (containing 25 mM nor-leucine (Sigma) as internal standard) were redissolved in pyridine (20 µl), derivatised using N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (20 µl), gently mixed and heated to 70°C for 30 min. One µl of each sample was injected onto a VG organic GC-MS MD800, separated on a BPX capillary column and analysed directly by the mass spectrometer. The samples were scanned from masses 50–450 m/z and compared to known standards. For evaluation of gluconic acid, the bis (trimethylsilyl) tri-fluoroacetamide derivative was used to accommodate the higher molecular mass of gluconate, ensuring the molecule was volatile under the conditions specified and within the range of analytical limits. Organic acid quantification was achieved using high performance liquid chromatography (HPLC), the system comprising a Waters (Watford) 600E system controller, a Waters 490E programmable multi-wavelength detector, a Waters U6K pump controlled by Millipore (Watford) Millennium software and a Techsphere 55C8 octyl bonded phase column with a Techsphere 55C8 guard column. The eluant was 0.2% (v/v) orthophosphoric acid and 1% (v/v) acetonitrile. Samples and eluant were pre-filtered through 0.45 µm pore diameter membrane filters and 20 µl duplicate samples were analysed for 10 min.

**Analytical methods**

Metal binding was assessed by differential pulse polarography (DPP) using a Metrohm 663 VA stand (Metrohm) incorporating a dropping mercury electrode. Analysis was controlled by general purpose electrochemical software (Version 3.2 GPE53, Ecochemie). The electrolyte was 0.1 M KNO₃ in ddH₂O, buffered with 5 mM MES (Sigma) and adjusted to pH 6.0 using solid tetramethylammonium hydroxide pentahydrate. The electrolyte (with and without experimental additions) was sparged at 25°C with N₂ for 3 min before polarograms were recorded, and each polarogram was obtained from where consecutive voltage sweeps. Computer analysis provided values for evolution potential and current potential of the derived peaks. For analysis of Co²⁺ and Zn²⁺ binding by organic acids and *A. niger* culture filtrates, the desired metal compound (Co(NO₃)₂ or Zn(NO₃)₂) was added to 24.75 ml electrolyte in 5 × 50 µl aliquots, to ensure adequate mixing, from 10 mM stock solutions to a final concentration of 100 µM. Organic acids were added from stock solutions in 10 µl aliquots to the desired final concentration. *Aspergillus niger* culture filtrates were added in 10 × 100 µl aliquots.

**Scanning electron microscopy (SEM)**

SEM was used to find out whether any precipitation of insoluble metal oxalates occurred and, if so, to enable metal composition to be confirmed using X-ray microprobe analysis. Mycelial pellets from growth cultures at 18 and 30 d were analysed by SEM. The samples were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide and dehydrated through a 50–100% ascending series of ethanol in distilled water. Samples were critical point dried, mounted on 10-mm diameter aluminium stubs. For SEM, the samples were sputter coated with carbon using double-sided carbon tape onto 10-mm diameter aluminium stubs. For SEM, the samples were sputter coated with carbon using double-sided carbon tape onto 10-mm diameter aluminium stubs.
fitted with a Au/Pd target. X-ray microprobe analysis was carried out for at least 100 s on a minimum of five uncoated samples, to verify elemental composition.

**Chemicals and glassware**

$\text{Zn}_3(\text{PO}_4)_2$ and $\text{Co}_3(\text{PO}_4)_2$ were obtained from Alfa (Johnson Mathey). Citric, oxalic and gluconic acids were obtained from Sigma-Aldrich Chemical (Poole, UK). $\text{Co(NO}_3)_2$ and $\text{Zn(NO}_3)_2$ were obtained from BDH (Poole, UK). All glassware was rinsed in 1 M HCl followed by three rinses in ddH$_2$O prior to use.

**RESULTS**

**Binding of $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ by citric, gluconic and oxalic acids**

Fig. 1a shows typical polarograms of the binding of $\text{Co}^{2+}$ by citric acid, while Table 1 summarises $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ binding data for all three organic acids. Stepwise additions of 100–1000 µM citric acid to solutions of $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ decreased the metal ion peak height (Table 1). A total of 10 × 100 µM additions of citric acid bound 78% of the Zn and 63.8% of the Co. Additions of 100–1000 µM oxalic acid to $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ solutions also decreased the metal ion peak height (Table 1). The 10 × 100 µM additions of oxalic acid bound 41% of the Zn and 26% of the Co. Oxalic acid, therefore, bound $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ approximately half as efficiently as citric acid. Gluconic acid did not result in any apparent binding/complexation of $\text{Co}^{2+}$ and $\text{Zn}^{2+}$ (Table 1).

**Organic acid production in the absence and presence of cobalt and zinc phosphate**

GC-MS (results not shown) and HPLC confirmed the presence of citric, gluconic and oxalic acids (Fig. 2). In the control

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### Table 1. Zinc and cobalt binding by citric, oxalic and gluconic acids. Final [Zn] and [Co] in the polarograph vessel (25 ml) were 100 µM (total amount 2.5 µmol). Organic acids were added to a final concentration of 1 mM (total amount = 25 µmol). Values show the amount and % bound ± the range of duplicate measurements.

<table>
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<tr>
<th></th>
<th>$\text{Zn}^{2+}$ bound</th>
<th>$\text{Co}^{2+}$ bound</th>
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<tr>
<td></td>
<td>µmol</td>
<td>% bound</td>
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<tr>
<td>Citric acid</td>
<td>1.94 ± 0.1</td>
<td>77.5 ± 3.6</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>1.04 ± 0.02</td>
<td>41.4 ± 0.7</td>
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<tr>
<td>Gluconic acid</td>
<td>No binding</td>
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**Fig. 1.** Polarograms of cobalt and zinc binding. (a) Binding of $\text{Co}^{2+}$ (100 µM final concentration in vessel, bold line) by 10 × 10 µl aliquots of citric acid to give a final concentration of 1 mM. (b) Binding of $\text{Zn}^{2+}$ (100 µM, bold line) by 10 × 100 µl additions of 15 d *Aspergillus niger* control culture filtrate. Typical polarograms are shown from one of at least three replicate determinations.

**Fig. 2.** Growth and organic acid production by *Aspergillus niger* in liquid medium. (■) citric acid; (▲) gluconic acid and (●) oxalic acid. (a) Growth of *A. niger* in liquid medium, (○) unamended and (□) amended with 5 mM $\text{Zn}_3(\text{PO}_4)_2$ or (△) 5 mM $\text{Co}_3(\text{PO}_4)_2$. Organic acid content of (b) control culture filtrate, (c) $\text{Zn}_3(\text{PO}_4)_2$-amended culture filtrate, (d) $\text{Co}_3(\text{PO}_4)_2$-amended culture filtrate. Absence of data points indicates no detection of the relevant organic acid at that time point: error bars show the range of duplicate samples and when not shown were smaller than symbol dimensions.
Metal binding by fungal culture filtrates

Solubilization of metal phosphates

The concentration of soluble phosphate in the control filtrate remained at a low level throughout (3 mM) (Fig. 3a). However, an increase in available phosphate was detected in both Zn$_3$(PO$_4$)$_2$ and Co$_9$(PO$_4$)$_2$-amended filtrates. Phosphate increased rapidly in the Co$_9$(PO$_4$)$_2$-amended filtrate, reaching a concentration of 16 mM by d 49. The increase in available phosphate in the Zn$_3$(PO$_4$)$_2$-amended filtrate was slower, reaching a concentration of 13 mM by d 49. In the Zn$_3$(PO$_4$)$_2$-amended filtrate, the soluble Zn concentration rose to 10.4 mM at 25 d while in the Co$_9$(PO$_4$)$_2$-amended filtrate, soluble Co rose to a maximum of 16.4 mM at 20 d (Fig. 3b). Control filtrates contained no cobalt, while the concentration of zinc remained at practically zero throughout (Fig. 3b).

Binding of Co$^{2+}$ and Zn$^{2+}$ by culture filtrates

Typical polarograms of the binding of Zn$^{2+}$ by the 15 d control filtrate are shown in Fig. 1b. Uninoculated liquid medium did not bind either Zn$^{2+}$ or Co$^{2+}$ (Fig. 4, 0 h) and there was no binding of Zn$^{2+}$ by the control filtrate until 4 d. The 4 d filtrate bound 7% of the available Zn$^{2+}$, which increased to 70% at 10 d–30 d, and 77% at 49 d (Fig. 4a). The control filtrate was also able to bind Co$^{3+}$ and 1 d (13%), and
As A. niger solubilized zinc phosphate (Fig. 3), polarograms of Zn\(^{2+}\) showed increases in Zn\(^{2+}\) peak height from 4 d (Fig. 4c). The greatest increase in Zn\(^{2+}\) peak height occurred at 20 d where Zn\(^{2+}\) increased at approximately 0.9 µmol per 100 µl addition (equivalent to a Zn\(^{2+}\) concentration in the filtrate of 9.0 mM, Fig. 4c). After 20 d the Zn\(^{2+}\) peak height decreased. In this filtrate, the concentration of citric acid peaked at 25 d while the concentration of oxalic acid remained constant throughout (Fig. 2c). The final pH of this filtrate was 2.6. This filtrate was able to bind Co\(^{2+}\) after 12 h (23%), and was able to bind 100% of the Co\(^{2+}\) present by 5 d (Fig. 4c).

**Crystal formation in pellets**

Scanning electron micrographs of mycelial pellets from Zn\(_6\)(PO\(_4\))\(_2\)-amended growth medium of *Aspergillus niger* at 18 d and 30 d showed the presence of crystalline structures (Figs 5a, b) that X-ray microprobe analysis showed contained only Zn and no phosphorus (results not shown) and possessed similar disc-like morphologies to zinc oxalate crystals obtained from colonies of *A. niger* grown on Zn\(_6\)(PO\(_4\))\(_2\)-amended solid medium (Sayer & Gadd 1997). Crystals were not observed in pellets from control medium or from medium amended with Co\(_6\)(PO\(_4\))\(_2\).

**DISCUSSION**

This study demonstrates that Co\(^{2+}\) and Zn\(^{2+}\) are bound both by pure organic acids and by *Aspergillus niger* culture filtrates. Solubilization of cobalt and zinc phosphates by *A. niger* was confirmed by phosphate release and increased soluble Co and Zn species in the culture medium. The formation of insoluble zinc oxalate by *A. niger* in liquid medium amended with Zn\(_6\)(PO\(_4\))\(_2\) was also observed, but formation of cobalt oxalate was not detected in the medium amended with Co\(_6\)(PO\(_4\))\(_2\). *A. niger* produced large amounts of gluconic acid when the growth medium was amended with Co\(_6\)(PO\(_4\))\(_2\). A. niger produced large amounts of gluconic acid when the growth medium was amended with Co\(_6\)(PO\(_4\))\(_2\) increasing to \(\sim 56\) mM (equivalent to 11 gl\(^{-1}\) 49 d after inoculation. This is not unusually high as gluconic acid; concentrations for use in the food industry, of 67 gl\(^{-1}\) (Buzzini *et al.* 1993a) and 500 gl\(^{-1}\) have been achieved with *A. niger* by stepwise addition of grape must (Buzzini, Gobbetti & Rossi 1993b). In our work, however, culture filtrates containing large amounts of gluconic acid were not able to bind Zn\(^{2+}\). Commerially-available gluconic acid was also incapable of binding the metal ions under the same conditions. This was surprising as gluconic acid has a purported industrial use as a chelating agent for metal ions (Mattey 1992).

The significance of citric and oxalic acids as powerful natural chelating agents with important roles in nutrient cycling and availability has often been described in the literature (see Gadd 1999). In this study, citric and oxalic acids were effective binding agents for Co\(^{2+}\) and Zn\(^{2+}\) with citric acid binding both ions more strongly than oxalic acid. There was no binding of Zn\(^{2+}\) by the control filtrate until 4 d after inoculation and no binding of Co\(^{2+}\) until 1 d after inoculation (Fig. 4a). Citric acid was detected in the filtrate 10 d after inoculation (Fig. 2b) at which time the filtrate bound so
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...strongly that the Co²⁺ peak disappeared (Fig. 4a). When the different metal phosphates were present in the growth medium, the organic acid profile of A. niger was markedly changed. Gluconic acid was produced in large quantities when the medium was amended with Co₃(PO₄)₂, but no binding of Zn²⁺ was observed. When the medium was amended with Zn₃(PO₄)₂ the filtrate was able to bind Co²⁺ after only 12 h, compared to 1 d for the control filtrate (Fig. 4c).

Microbial organic acid production is important in soil to release metals from minerals and nutrients such as phosphate (Bolan et al. 1994, Kpomblekou & Tabatabai 1994, Di Simine, Sayer & Gadd 1999, Gadd 1999). The inability of calcifuge plants to establish themselves on limestone soil was linked to organic acid production. The roots of acidifuge plants on limestone soil excrete large amounts of oxalate and citrate, while the roots of calcifuge plants on acidic soil exude large amounts of monocarboxylic acids such as formate, acetate and lactate but very small amounts of oxalate and citrate. This pattern of organic acid exudation inhibits growth on limestone soils where concentrations of iron and phosphate are low since oxalate has a high phosphate solubilization ability and citrate is a powerful iron chelator (Tyler & Strom 1995). Oxalic acid is further implicated in the leaching of aluminium in forest ecosystems where organic acids are abundant in the soil and rhizosphere and organic acid complexation of Al and Fe is involved in the podzolization process (Fox & Comerford 1990). Organic acid excretion in soil by fungi and plant roots can increase the mobility of metals in metal-contaminated sites and may enhance leaching of potentially toxic species to water systems, with hazardous implications for the biota (Francis et al. 1992). Such effects should be considered when old mining sites are re-vegetated to prevent erosion by wind and water. Organic acid production could increase the solubility of metals present by complexation (Banks et al. 1994a, b).

The production of organic acids and ensuing complexation and/or precipitation of free metal ions has been implicated in metal tolerance, as the mobility and toxicity of metals is related to speciation (Pettersson et al. 1993). This work demonstrated that the presence of different metal phosphates can influence both the nature and the extent of organic acid production by A. niger. The presence of cobalt phosphate stimulated gluconic acid production, which, although ineffective for Co²⁺ and Zn²⁺ complexation under the conditions used in this study, is a documented chelator of other metals such as Fe and Ca (Mattey 1992). However, other important metal-complexing organic acids, citric and oxalic, exhibited metal binding and these were produced in significant levels in the culture media. This clearly demonstrates the ability of fungi to change the speciation of metals in their growth environment and may be a significant determinant of metal toxicity and tolerance (Gadd & Sayer 2000). The culture filtrates were as efficient, if not better, at binding Co²⁺ and Zn²⁺ as individually-supplied commercial organic acids. In addition to solubilization by citric acid, complexation by oxalic acid can lead to formation of highly insoluble metal oxalates (Sayer & Gadd 1997, Sayer et al. 1997, Gharieb et al. 1998, Gadd 1999) and this occurred in the medium supplemented with Zn₃(PO₄)₂. Results of this research are relevant to an understanding of fungal responses towards metal species as well as the mechanisms fungi use in affecting metal and phosphate bioavailability, with obvious implications for the biogeochemical cycling of metals and phosphorus and the bioremediation of metal-contaminated soils and wastes by heterotrophic leaching.

ACKNOWLEDGEMENTS

G.M.G. gratefully acknowledges financial support received from the Biotechnology and Biological Sciences Research Council (SPC 02182, 94/MAF12243).

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*Corresponding Editor: M. Levy*