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Uranium bioprecipitation mediated by yeasts utilizing organic phosphorus substrates

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Abstract

In this research, we have demonstrated the ability of several yeast species to mediate U(VI) biomineralization through uranium phosphate biomineral formation when utilizing an organic source of phosphorus (glycerol 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P.xH_2O$ (G2P)) or phytic acid sodium salt hydrate ($C_6H_{18}O_{24}P_6.xNa^+.yH_2O$ (PyA))) in the presence of soluble $UO_2(NO_3)_2$. The formation of metaankileite ($K_2(UO_2)_2(PO_4)_2.6(H_2O)$), chernikovite ($(H_3O)_2(UO_2)_2(PO_4)_2.6(H_2O)$), bassetite ($Fe^{++}(UO_2)_2(PO_4)_2.8(H_2O)$), and uramphite ($(NH_4)(UO_2)(PO_4).3(H_2O)$) on cell surfaces was confirmed by X-ray diffraction in yeasts grown in a defined liquid medium amended with uranium and an organic phosphorus source, as well as in yeasts pre-grown in organic phosphorus-containing media and then subsequently exposed to $UO_2(NO_3)_2$. The resulting minerals depended on the yeast species as well as physico-chemical conditions. The results obtained in this study demonstrate that phosphatase-mediated uranium biomineralization can occur in yeasts supplied with an organic phosphate substrate as sole source of phosphorus. Further understanding of yeast interactions with uranium may be relevant to development of potential treatment methods for uranium waste, utilization of organic phosphate sources, and for prediction of microbial impacts on the fate of uranium in the environment.

Introduction

Uranium contamination of the environment occurs from a number of sources including the nuclear industry, weathering of uranium-containing natural rocks and minerals, and the extensive use of uranium-containing phosphate fertilizers (Llorens et al. 2012). One potential strategy to inhibit the spread of uranium in the environment consists of inducing uranium precipitation via a biogenic or non-biogenic process. As previous research has demonstrated, many microorganisms can accumulate large amounts of toxic metals and generate crystalline minerals: toxic metals can precipitate with ligands generated from chemical and/or enzymatic processes, such as sulfide, carbonate, phosphate and oxalate (Macaskie et al. 1992; 2000; Gadd 2010; Sivaswamy et al. 2011). Many studies have focused on uranium reduction processes in bacteria, which can play an important role in uranium bioremediation (Lovley and Phillips 1992; Llorens et al. 2012; Martinez et al. 2007; 2014). Several Gram-positive and Gram-negative bacteria, such as *Cupriavidus metallidurans* CH34 (Ray et al. 2011), *Rhodopseudomonas palustris* (Llorens et al. 2012), *Thermoterrabacterium ferrireducens* (Khijniak et al. 2005), *Mycobacterium smegmatis* (Andres et al. 1993; 1994), *Bacillus subtilis* (Fowle et al. 2000), *Rahnella* sp. (Martinez et al. 2007), and *Shewanella oneidensis* MR-1 (Sheng and Fein 2013) can reduce U(VI) to U(IV) which precipitates as U(IV)-carbonate. *Citrobacter sp*. can precipitate U(VI)-phosphate minerals as a result of phosphatase-mediated hydrolysis of an organic source of phosphorus in the presence of U(VI) (Macaskie et al. 1992; 1994; 2000).

Fungi show some variation in cell wall chemical composition, which leads to a broad metal biosorption capacity range across a variety of fungal species (Gadd 2009; 2010; Fomina and Gadd 2014). Most proposed U bioremediation applications of fungi have concentrated on biosorption of uranium, sometimes using waste biomass generated from large scale industrial fungal

fermentations (Lovley and Phillips 1992; Andres et al. 1993; Macaskie et al. 2000; Fowle et al. 2000; Sakamoto et al. 2005; Aytas et al. 2011; Llorens et al. 2012). A composite adsorbent consisting of *Jania rubens* (marine macroalga) and *Saccharomyces cerevisiae* immobilized on silica gel showed good biosorption properties in removing uranium from dilute aqueous solution (Aytas et al. 2011). However, little attention has been paid to uranium biomineralization by fungal systems. Previous research has demonstrated that fungi exhibit uranium tolerance and can solubilize uranium oxides and depleted uranium and reprecipitate secondary uranium phosphate minerals of the meta-autunite group, uramphite and/or chernikovite, which can encrust fungal hyphae to high accumulation values (Fomina et al. 2007; 2008; Gadd and Fomina 2011). Such minerals may be capable of long-term U retention (Fomina et al. 2007; 2008; Gadd and Fomina 2011). Fungi, like bacteria, also show the ability for phosphatase-mediated uranium precipitation during growth on an organic phosphorus source, and extensively precipitated uranium and phosphorus-containing minerals on fungal hyphal surfaces (Liang et al. 2015a). *S. cerevisiae* also shows some properties of uranium biomineralization through formation of a U(IV)-bearing precipitate during growth in a high-phosphate medium (Ohnuki et al. 2005). Uranium removal by yeasts has mainly focused on *S. cerevisiae*, while other yeast species have received little attention (Soares et al. 2002; Ohnuki et al. 2005; Sakamoto et al. 2005; 2007; Sarri et al. 2009).

The objective of this study was to evaluate the potency of several yeast strains, some originating from metal-polluted environments, to accumulate and immobilize uranium through phosphatase-facilitated uranium phosphate precipitation. Fundamental understanding of the interactions of yeasts with uranium may be helpful in further understanding yeast eco-physiology in polluted habitats, and for developing radioactive waste treatments, short-term and long-term waste management strategies, and for better predicting

microbial impacts on the fate of uranium in the environment and in waste repositories.

Materials and Methods

Organisms and media

Yeast strains used in the experiments were *Kluyveromyces lactis* IFO1267 (Dombrowski) Van der Walt and *Pichia acaciae* (NRRL 18665) Van der Walt (kindly supplied by Professor Mike Stark, University of Dundee) (Worsham and Bolen 1990); *Cryptococcus podzolicus* PYCC 4488^T (= CBS 6819^T) (Babeva & Reshetova) Golubev, originally isolated from a disused arsenic mine in Devon; *Cryptococcus filicatus* Golubev & Samp JP, originally isolated from a disused Cornish copper mine; *Candida sake* (Saito & Oda M) van Uden & Buckley HR, originally isolated from a lead-polluted area in Wales (strain details are found in Holland et al. 2014) and *Candida argentea* (NCYC 3753^T) S.L. Holland, S.V. Avery & P.S. Dyer sp. nov., originally isolated from a metal-polluted site in Wales (kindly supplied by Dr Sara Holland, University of Nottingham). These yeast strains were chosen for their demonstrated abilities in mineral and toxic metal biotransformations (Holland et al. 2011; 2014; Fernandes et al. 2014; Liang et al. 2015b). All yeast cells were grown in Modified Burkholder's medium (MBM) in 250 ml Erlenmeyer conical flasks containing 100 ml nutrient medium on an orbital shaking incubator (Infors Multitron Standard, Rittergasse, Switzerland) at 180 rpm for 48 h at 30°C in the dark. Modified Burkholder's medium (MBM) consists of dextrose 20 g, $(NH_4)_2SO_4$ 4 g, asparagine 2 g, KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 2H_2O$ 0.33 g, KI 7.6 mg, $ZnSO_4 \cdot 7H_2O$ 0.7 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, $MnSO_4 \cdot 4H_2O$ 0.1 mg, $Na_2B_4O_7 \cdot 10H_2O$ 0.1 mg, $CuSO_4 \cdot 5H_2O$ 0.1 mg, $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ 0.1 mg, inositol 10,000 µg, nicotinic acid 200 µg, pyridoxine 200 µg, thiamine HCl 200 µg, pantothenic acid 200 µg, *p*-amino benzoic acid 50 µg and biotin 2 µg per 1000 ml sterile Milli-Q water. Cells at 48 h were harvested by centrifugation at 4000 rpm (4880 g) for 30 min and were then aseptically transferred to a sucrose- and P-free equivalent nutrient solution and grown for a further 48 h

under the same conditions to deplete the phosphorus in MBM before use for further experiments.

Preparation of MBM amended with organic phosphorus sources and uranium

One batch of yeast cells in the stationary growth phase was harvested by centrifugation at 4000 rpm (4880 g) for 30 min and then aseptically transferred to and grown in MBM substituting 30 mM glycerol 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P.xH_2O$ (G2P)) or 5 mM phytic acid sodium salt hydrate ($C_6H_{18}O_{24}P_6.xNa^+.yH_2O$ (PyA)) for KH_2PO_4 as the sole phosphorus source. Test yeast strains were grown in three different media (MBM with 30 mM G2P, MBM with 5 mM PyA and MBM without any phosphorus source as a control) to examine uranium biomineral formation in yeasts pre-grown in the presence of source of organic phosphorus and then exposed to $UO_2(NO_3)_2$. All yeast strains were grown at 30°C in 250-ml flasks containing 100 ml MBM on an orbital shaking incubator at 180 rpm for 120 h in the dark. All phosphorus sources were separately sterilized by membrane filtration (cellulose nitrate, 0.2 µm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved MBM medium (121°C, 15 min) at room temperature, to give a final concentration of 30 mM G2P or 5 mM PyA. MBM without any phosphorus source was the control and uninoculated medium served as an abiotic control for each set of experiments. The organic phosphorus substrates produced no significant precipitation on reaction with uranyl nitrate in the absence of yeasts.

To examine uranium biomineral formation in cultures growing in the presence of uranium, another batch of yeast cells in the stationary growth phase at 96 h were harvested by centrifugation at 4000 rpm (4880 g) for 30 min and then aseptically transferred to and grown in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA as sole phosphorus sources in 250-ml conical flasks containing 100 ml nutrient medium on an orbital shaking

incubator at 180 rpm at 30°C in the dark. $UO_2(NO_3)_2$, G2P and PyA were dissolved separately in Milli-Q water and sterilized by membrane filtration (cellulose nitrate, 0.2 µm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved MBM (121°C, 15 min) at room temperature, to give 0.2 or 1 mM $UO_2(NO_3)_2$, 30 mM G2P and 5 mM PyA final concentrations. MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ without any phosphorus source was the control and uninoculated medium served as an abiotic control for each set of experiments.

Growth rate, inorganic phosphate (P_i) release, tolerance indices (TI), and pH analysis

To examine the effect of uranium on phosphatase activity when grown with an organic phosphorus source, yeast growth was measured by optical density (OD) at 595 nm using a spectrophotometer (Anthos 2001 microplate reader) over 120 h culture in 0.2 or 1 mM $UO_2(NO_3)_2$-containing MBM amended with G2P or PyA. Calculations were carried out using the Windows-based control and evaluation software for Rosys Anthos microplate readers (Anthos Labtec Instruments, Wals, Austria). Background OD_{595} was determined by spectrophotometric measurement of uninoculated wells.

Inorganic phosphate (P_i) release into the medium during growth in 0.2 or 1 mM $UO_2(NO_3)_2$ containing MBM amended with G2P or PyA was determined spectrophotometrically using the malachite green assay (Irving and McLaughlin, 1990). 15 µl aliquots of supernatant were sterilized by membrane filtration (cellulose nitrate, 0.2 µm pore diameter, Whatman, Maidstone, Kent, UK), and added to each well, in a 96-well plate, with 185 µl Milli-Q water, followed by the addition of 100 µl malachite green reagent and left for 15 min after mixing. The absorbance at 620 nm was read on an Anthos 2001 microplate reader, and calculations were carried out as described above. For the malachite green background comparison, 200 µl Milli-Q water was mixed

with 100 µl malachite green reagent standard in the 96-well plate giving a final volume of 300 µl. After incubating the plate for 15 min, the absorbance at 620 nm was processed the same way as the inoculated wells. All experiments were conducted at least in triplicate.

To assess uranium tolerance, test yeast species were grown in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA as sole phosphorus source in 250-ml conical flasks containing 100 ml medium on an orbital shaking incubator at 180 rpm at 30°C in the dark. Yeast biomass was harvested at appropriate time intervals by centrifugation at 4000 rpm (4880 g) for 30 min. Biomass dry weights were used to obtain a tolerance index (TI) and the supernatant analysed for changes in pH. Metal tolerance was evaluated using a TI as follows: (dry weight of uranium-exposed biomass/dry weight of control biomass x 100%) (Wei et al. 2013; Liang et al. 2015a,b). For dry weight determination, biomass was harvested, washed three-times with 0.1 M NaCl, and dried to constant weight in a vacuum desiccator at room temperature for at least 30 days, and then ground to a powder using a pestle and mortar (Milton Brook, Dorset, UK). Supernatants were obtained by membrane filtration (0.45 µm pore diameter, Whatman, Maidstone, Kent, UK). The pH of supernatants was measured using a pH 210 Microprocessor pH Meter (Hanna Instruments, Woonsocket, RI, USA). All experiments were conducted at least in triplicate.

Analysis of biominerals produced by yeast

To investigate uranium bioprecipitation by yeast cell suspensions after pre-growth with organic phosphorus sources, test yeast cells were harvested from 30 mM G2P or 5 mM PyA amended MBM after 120 h by centrifugation at 4000 rpm (4880 g) for 30 min and washed three times with 0.1 M NaCl. 50 mg (wet weight) of harvested yeast cells were transferred into 2 ml microcentrifuge tubes (Starlab, Hamburg, Germany) to which 1 ml of 1 mM $UO_2(NO_3)_2$ solution was added. Duplicate samples were incubated for 48 h at 30°C. After this time,

samples were centrifuged at 4000 rpm (4880 g) for 20 min, washed three times with 0.1 M NaCl, and then dried in a vacuum desiccator at room temperature for at least 30 days. The elemental composition of crystals precipitated on yeast cell surfaces was analysed using a JEOL JSM-T300 SEM system equipped with a Princeton Gamma Tech EDX microanalysis spectrometer (Princeton Gamma-Tech Inc., Princeton, NJ, USA). For scanning electron microscopy (SEM), yeast cells mounted on stubs were sputter coated for 5 min with gold and palladium (30 nm) using a Cressington 208HR sputter coater (Ted Pella, Inc., Redding, CA, USA). Specimens were examined using an environmental scanning electron microscope (ESEM) (Hitachi s-4700) (Hitachi Ltd, Tokyo, Japan) operating at an accelerating voltage of 15 kV.

Secondary mineral formation on yeast cell walls after growth in media containing different concentrations of uranium and G2P or PyA was examined similarly after harvesting by centrifugation at 4000 rpm (4880 g) for 30 min. Biomass and supernatant were separated, and cells were dried in a vacuum desiccator at room temperature prior to examination by SEM as described above. Uncoated samples were examined for elemental composition using energy-dispersive X-ray analysis (EDXA) before Au/Pd coating the samples in order to exclude the Au/Pd peak which overlaps P/Cl peaks. Spectra were acquired using a Phoenix EDXA (EDAX Inc., Mahwah, NJ, USA) analysis system embedded within the environmental scanning electron microscope (ESEM) (Philips XL30 ESEM FEG) (FEI Company, Hillsboro, USA) operating at an accelerating voltage of 20 kV.

The mineralogy of the biominerals was determined using a Hiltonbrooks X-ray diffractometer (XRD) (HiltonBrooks Ltd., Crewe, UK) fitted with a monochromatic CuKα source and curved graphite, single Seiko crystal chronometer (30 mA, 40 kV). The finely ground samples obtained were firmly compacted on the reverse side of an aluminium 15 x 20 x 2 mm^3 specimen holder, later held against a clean glass side. After compaction, the minerals

stay firm on the back cover of the specimen holder, which was then snapped into place and the glass side removed from the holder. Duplicate samples were analysed over the range 3-60° 2-θ at a scan rate of one degree/min in 0.1 degree increments.

Statistical analysis

All data presented are the means of at least three replicates and error bars represent one standard error either side of the mean. SigmaPlot, version 12.5, was used to perform statistical analyses. One-way ANOVA tests on means were performed for dry weight, the malachite green P_i assay, pH and growth rate measurements.

Results

Effect of uranium on yeast growth and P_i released in MBM amended with G2P or PyA

The optical density after growth of yeasts for 120 h in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA is shown in Table 1. Most of the test yeasts showed some ability to grow in uranium-amended media except for *C. sake*, *C. argentea* and *P. acaciae* in the present of PyA (Table 1). Growth of the yeasts was affected by the presence of 0.2 and 1 mM $UO_2(NO_3)_2$ in MBM amended with G2P or PyA (Table 1). The optical density was reduced compared to that of cells grown in U-free media, and the higher the concentration of U, the lower the extent of yeast growth (Table 1). Growth in U and organic phosphorus amended medium showed a reduction in cell yield and the inhibitory effect with PyA was greater than that with G2P (Table 1). The optical densities of the test strains were mostly higher than 1.0 in the presence of 0.2 mM $UO_2(NO_3)_2$ in MBM amended with 30 mM G2P, while in 5 mM PyA amended MBM, the optical densities dropped below 1.0 in the presence of uranium. The presence of 0.2 and 1 mM $UO_2(NO_3)_2$ in 5 mM PyA MBM exerted strong inhibition of *C. sake*, *P. acaciae* and *C. argentea* (Table 1).

The P_i released into the medium after yeast growth for 120 h in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA is shown in Table 2. The fraction of P_i released was reduced compared to that of cells grown in uranium-free medium, and the higher the concentration of uranium in the medium, the lower was the fraction of P_i released (Table 2). More than 50% of P_i was released in 30 mM G2P medium after exposure of cells to medium containing 0.2 mM $UO_2(NO_3)_2$ except for *P. acaciae* (Table 2). A lower proportion of P_i was released in 5 mM PyA medium in the presence or absence of U in most of the test yeasts with ~ 10 - 39% P_i being released (Table 2).

Medium pH values and tolerance indices of yeast strains grown in MBM amended with uranium and G2P or PyA

After growth of the yeasts, the pH of MBM amended with 30 mM G2P or 5 mM PyA with or without 0.2 or 1 mM $UO_2(NO_3)_2$ varied between different yeast species and U concentrations (Table 3). In the absence of uranium, the initial pH of control medium amended with 30 mM G2P or 5 mM PyA were pH 6.9 and pH 3.8 respectively. The pH of the medium dropped after inoculation of yeast species from pH 6.9 to around pH 5.5 in medium amended with 30 mM G2P, and from pH 3.8 to around pH 3 in 5 mM PyA, except for *C. sake* (pH 3.8) in the present of PyA. The changes in pH were similar when MBM was amended with $UO_2(NO_3)_2$, falling to around pH 5.7 for 0.2 mM $UO_2(NO_3)_2$, and around pH 5.5 for 1 mM $UO_2(NO_3)_2$ except for *P. acaciae* (pH 5.8) and *C. sake* (pH 7.1) with 0.2 mM $UO_2(NO_3)_2$, and *C. sake* (pH 5.7) and *C. podzolicus* (pH 5.6) with 1 mM $UO_2(NO_3)_2$ (Table 3). In the presence of 5 mM PyA, the pH dropped from pH 3.5, as in the control, to pH 3.2 after inoculation with yeasts while for 1 mM $UO_2(NO_3)_2$, the pH dropped from pH 3.5 to around pH 3 for most of the test yeasts except for *C. sake* (pH 3.5) and *P. acaciae* (pH 3.2) with 0.2 mM $UO_2(NO_3)_2$ and *C. sake* (pH 3.4) with 1 mM $UO_2(NO_3)_2$ (Table 3).

Tolerance indices (TI) were used to compare biomass yields of all test yeast species grown in MBM with or without 0.2 and 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA (Table 4). A TI value < 100% indicates growth inhibition, while a TI > 100% indicates growth stimulation. All biomass yields of the test yeast species were reduced in the presence of uranium at both concentrations. The TI values varied among species and between different uranium concentrations. In the presence of 0.2 mM $UO_2(NO_3)_2$, TI values showed less reduction in MBM amended with 30 mM G2P or 5 mM PyA, with most TI values over 70%. However, growth of *P. acaciae* and *C. podzolicus* was inhibited in the presence of 1 mM $UO_2(NO_3)_2$ in MBM amended with 5 mM PyA, with TI values of 54.5% and 57.1% respectively. Since negligible growth was

observed in MBM amended with 5 mM PyA, TI values for *C. sake* were negligible (Table 4). In the presence of 1 mM $UO_2(NO_3)_2$ in MBM amended with 30 mM G2P, *C. sake* and *C. argentea* showed TI values of 61.5% and 53.3% respectively (Table 4).

Bioprecipitation of uranium by yeast biomass harvested from organic phosphorus-amended MBM after reaction with $UO_2(NO_3)_2$

Most test yeasts previously grown in organic P-amended medium showed uranium bioprecipitation when subsequently reacted with 1 mM $UO_2(NO_3)_2$ (Fig.1-2B,E,H). Compared to the large amounts of precipitation found on *C. podzolicus* (Fig.1H), *C. sake* (Fig.2B) and *K. lactis* (Fig.2E) pre-grown in G2P-amended MBM, other yeast strains showed relatively poor abilities in precipitating uranium biominerals, and only a minor proportion of the population exhibited electron-dense precipitation. After growth in PyA, *P. acaciae* precipitated abundant biominerals on the cell walls. Little precipitation was observed on *C. podzolicus* grown in PyA-amended MBM after mixture with 1 mM $UO_2(NO_3)_2$ (data not shown). SEM revealed the presence of electron-dense clusters with distinct crystalline shapes on the surface of some of the yeast species in the early stages of growth. Large electron-dense deposits were observed on the yeast cells after longer reaction times (Fig.1-2 B,E,H).

Formation of uranium-containing secondary minerals by yeasts grown in MBM amended with uranium and G2P or PyA

Most test yeasts showed uranium bioprecipitation when grown in G2P or PyA-amended media with 0.2 or 1 mM $UO_2(NO_3)_2$ (Fig.1C,F,I). *C. sake* hardly grew in PyA amended MBM and therefore mineral precipitation was not observed. Compared to the large amount of precipitation found with *C. podzolicus* grown in G2P-amended MBM with $UO_2(NO_3)_2$ (Fig.1I), only a small amount of precipitation occurred when grown in PyA amended MBM with 0.2

or 1 mM $UO_2(NO_3)_2$. Precipitation occurred in varying amounts with the various P sources with the different yeast species (Fig. 1-2). Differences were found between the secondary minerals precipitated in these growth experiments to the previous experiments where pre-grown control cells were reacted with $UO_2(NO_3)_2$ solutions, regarding their morphologies and the occurrence of nanoscale particles.

Energy-dispersive X-ray analysis (EDXA) of uranium-containing secondary minerals

Energy-dispersive X-ray analysis (EDXA) revealed the elemental composition of the secondary minerals formed by yeast cells harvested from G2P or PyA amended MBM and then reacted with $UO_2(NO_3)_2$, and yeasts grown in uranium-amended MBM with G2P or PyA. Control yeast cells from MBM amended with G2P showed carbon, oxygen, sodium and phosphorus as the main elements with occasional detection of sulfur and magnesium. The minerals precipitated on yeast cells grown with an organic phosphorus source and then reacted with uranium nitrate, showed carbon, oxygen, phosphorus and uranium as the main elements and sometimes sodium and potassium (Table 5). The minerals formed with yeasts grown in uranium-G2P or PyA amended MBM showed carbon, oxygen, phosphorus and uranium as the main elements detected and sometimes aluminium and sulfur (Table 5). Most of the uranium-containing minerals precipitated on the yeast cells shared similar crystalline morphologies.

X-ray diffraction (XRD) of minerals produced after yeast growth

XRD showed the formation of metaankileite ($K_2(UO_2)_2(PO_4)_2.6(H_2O)$), chernikovite ($(H_3O)_2(UO_2)_2(PO_4)_2.6(H_2O)$), bassetite ($Fe^{++}(UO_2)_2(PO_4)_2.8(H_2O)$), and uramphite ($(NH_4)(UO_2)(PO_4).3(H_2O)$) in MBM amended with 1 mM $UO_2(NO_3)_2$ and 30 mM G2P after yeast growth (Fig. 3-4). All of these minerals are uranium- and phosphorus-containing minerals.

Metaankileite and chernikovite were found with all yeast species (Fig. 3-4). Uramphite was found in most of the yeasts except *C. podzolicus* (Fig. 3-4). Bassetite only appeared in MBM amended with $UO_2(NO_3)_2$ and G2P after growth of *C. argentea* and *K. lactis*. Metaankileite and chernikovite were the only minerals found in MBM amended with $UO_2(NO_3)_2$ and G2P after growth of *C. podzolicus* (Fig. 4).

Discussion

Uranium bioimmobilization by yeasts has been widely studied, with sorption of uranium species to cell surfaces as the first step and subsequent uranium precipitation through complexation with various anions present in the system (Langmuir 1978; Panak et al. 2000; Haas et al. 2001; Francis et al. 2004). Uranium complexation with both organic and inorganic substrates may reduce uranium toxicity (Newsome et al. 2014). Previous research has shown the formation of surface complexes of uranium carbonate and uranium phosphate as a result of uranium sorption by *Shewanella putrefaciens* (Haas et al. 2001). Under calcium-rich conditions, thermodynamic modelling revealed that uranyl carbonates, calcium uranium carbonates and uranyl hydroxides can also form stable cell surface complexes on *Bacillus subtilis* (Gorman-Lewis et al. 2005). The formation of H-autunite by a *Citrobacter* sp., inner-sphere uranium complexes with phosphate groups in a *Bacillus* sp. and formation of needle-like fibrils of uranium-containing minerals in *S. cerevisiae* have also demonstrated the capacity for uranium precipitation by various microorganisms (Yong and Macaskie 1995; Volesky and May-Philips 1995; Macaskie et al. 1992; 1994; 2000; Panak et al. 2000; Ohnuki et al. 2005). SEM observations of yeast cells grown in MBM containing a source of organic phosphorus after subsequent exposure to a uranium nitrate solution showed that the resulting uranium-containing minerals mainly accumulated on cell surfaces. Such uranium phosphate precipitation may be mediated by both electrostatic forces and binding to sites such as carboxylic and phosphate groups (Gorman-Lewis et al. 2005) as well as phosphatase-mediated uranium biomineralization, phosphatase activities releasing free phosphate (P_i) from the organic P source which precipitates with soluble uranium species as a uranium phosphate (Macaskie et al. 1992; 2000).

The presence of cell surface-associated uranium phosphate precipitation suggested that the phosphatase activity that mediated cell-associated uranium

precipitation was located at the cell periphery. Previous research has demonstrated that PhoY and phytase (CCNA-01353) in *Caulobacter crescentus* (Yung and Jiao 2014; Yung et al. 2014), and PhoK (alkaline phosphatase) in *Sphingomonas* sp. BSAR-1 were responsible for mediating uranium biomineralization (Nilgiriwala et al. 2008). Therefore, the uranium bioprecipitation process not only depends on uranium sorption, but also the release of free phosphate (P_i) as a result of phosphatase activity (Macaskie et al. 1992; 2000; Yong and Macaskie 1995; Martinez et al. 2007). Furthermore, mineral precipitation is also influenced by the presence of other metal cations (Murphy et al. 1989). Thus, it seems the organic phosphorus sources added to the medium were hydrolysed by phosphatase activity and UO_2^{2+} associated with the cell surface could react immediately with the liberated P_i. That more secondary minerals were precipitated on yeast cell surfaces after growth in media with G2P rather than PyA may be the result of PyA requiring a specific phytase for hydrolysis while G2P can be hydrolysed by a range of phosphatase enzymes. Concentrations of P_i released by the yeasts from G2P or PyA were higher in the absence than in the presence of uranium. This may be due to growth inhibition in the presence of uranium as well as the formation of uranium-containing minerals, the released P_i being consumed by the formation of the uranium phosphates. The morphology of the minerals precipitated on the yeast cell walls was variable and this can be influenced by many factors, such as the presence of other metal cations, pH and solubility of different mineral species. The formation of uranium phosphate minerals has been considered to be a more durable process than uranium biosorption since insoluble minerals can remain in an insoluble state even after cell lysis (Ohnuki et al. 2005).

This work has demonstrated the ability of several yeast species to mediate U(VI) biomineralization through uranium phosphate biomineral formation via phosphatase activity in the presence of an organic phosphorus source as sole

source of phosphorus. Uranium- and phosphate-containing bioprecipitation was detected on the surfaces of yeast cells after interaction with uranium and the minerals metaankoleite, chernikovite, bassetite and uramphite were confirmed by X-ray diffraction (Fig. 3-4). This work has demonstrated the potential of yeasts in the utilization of organic phosphate sources for transformation of soluble metal species into insoluble minerals via phosphatase-mediated bioprecipitation.

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Compliance with ethical standards

The authors declare that they have no competing interests and confirm that ethical principles have been applied in this study.

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Table 1. Growth of test yeasts in MBM containing 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA as sole P source.

	Optical density at 595 nm					
	30 mM G2P	30 mM G2P + 0.2 mM $UO_2(NO_3)_2$	30 mM G2P + 1 mM $UO_2(NO_3)_2$	5 mM PyA	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$	5 mM PyA + 1 mM $UO_2(NO_3)_2$
C. sake	1.67	1.11	0.65	0.21	0.13	0.1
P. acaciae	2.07	1.04	0.79	0.65	0.21	0.17
K. lactis	2.03	1.41	1.24	1.06	0.55	0.43
C. filicatus	1.98	1.63	1.55	1.72	0.89	0.78
C. podzolicus	2.01	1.14	0.78	1.85	0.65	0.55
C. argentea	1.79	1.32	0.98	0.42	0.22	0.19

The optical densities of yeast culture were measured at 595 nm after 120 h growth in MBM amended with G2P or PyA, and $UO_2(NO_3)_2$ at 30°C in the dark at 180 rpm. The initial OD_{595} of the yeast suspension was approximately 0.1. Measurements are the means of at least three replicate measurements with typical relative standard deviations of about 5%.

Table 2. Fraction of P_i (%) released into the medium by the test yeasts after 120 h growth in MBM amended with 30 mM G2P or 5 mM PyA and containing 0.2 or 1 mM $UO_2(NO_3)_2$.

	Fraction of P_i (%) released			
	30 mM G2P + 0.2 mM $UO_2(NO_3)_2$	30 mM G2P + 1 mM $UO_2(NO_3)_2$	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$
C. sake	70.1	50.6	--	--
P. acaciae	39.2	26.4	13.2	10.9
K. lactis	63.2	46.1	19.3	13.2
C. filicatus	54.6	48.1	29.6	22.1
C. podzolicus	60	55.3	39.4	28.6
C. argentea	62.4	45.6	19.6	15.6

Yeasts were grown for 120 h at 30°C in the dark at 180 rpm. P_i released was quantified using the malachite green assay. Measurements are the means of at least three replicate measurements with typical relative standard deviations of about 5%.

Table 3. pH of media after growth of yeast strains in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA.

	pH value					
	30 mM G2P	30 mM G2P + 0.2 mM $UO_2(NO_3)_2$	30 mM G2P + 1 mM $UO_2(NO_3)_2$	5 mM PyA	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$	5 mM PyA + 1 mM $UO_2(NO_3)_2$
Uninoculated control	6.9	6.8	7	3.8	3.5	3.5
C. sake	5.6	7.1	5.7	3.8	3.5	3.4
P. acaciae	5.5	5.8	5.5	3.1	3.2	3
K. lactis	5.5	5.7	5.5	3	3.3	3
C. filicatus	5.5	5.8	5.5	3	3.3	3
C. podzolicus	5.6	5.8	5.6	3	3.2	3
C. argentea	5.5	5.7	5.6	3.1	3.2	3

pH measurements were taken after 120 h incubation at 30°C in the dark on an orbital shaking incubator at 180 rpm. All values shown are means of at least three measurements with typical relative standard deviations of about 5%.

Table 4. Tolerance indices (TI), expressed as a percentage, of yeast species grown in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA.

	TI (%)			
	30 mM G2P + 0.2 mM $UO_2(NO_3)_2$	30 mM G2P + 1 mM $UO_2(NO_3)_2$	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$
C. sake	76.9	61.5	ND	ND
P. acaciae	94.1	82.3	81.8	54.5
K. lactis	87.5	75	85.7	71.4
C. filicatus	88.9	77.8	99.7	80
C. podzolicus	87.5	81.3	90.5	57.1
C. argentea	93.3	53.3	88.9	66.7

Values shown are tolerance indices derived from the biomass dry weight of yeast grown in the absence or presence of uranium for 120 h at 30°C in the dark on an orbital shaker at 180 rpm. The mean biomass dry weights of yeasts per 100 ml grown in MBM amended with 30 mM G2P in the absence of uranium were: *C. sake*, 130 mg; *P. acaciae*, 170 mg; *K. lactis*, 160 mg; *C. filicatus*, 170 mg; *C. podzolicus*, 140 mg; *C. argentea*, 140 mg. The biomass dry weights of yeasts per 100 ml grown in MBM amended with 5 mM PyA in the absence of uranium were: *C. sake*, 20 mg; *P. acaciae*, 220 mg; *K. lactis*, 100 mg; *C. filicatus*, 150 mg; *C. podzolicus*, 210 mg; *C. argentea*, 80 mg. All values shown are percentages derived by comparison with the mean control biomass yields. ND = not detectable. Averages from three measurements are shown with typical relative standard deviations of about 5%.

Table 5. Elemental and mineralogical composition of the biominerals produced by the yeast species grown in MBM amended with 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA.

		Elemental (EDXA) and mineralogical composition (XRD)			
		30 mM G2P + 0.2 mM $UO_2(NO_3)_2$	30 mM G2P + 1 mM $UO_2(NO_3)_2$	5 mM PyA + 0.2 mM $UO_2(NO_3)_2$	5 mM PyA + 1 mM $UO_2(NO_3)_2$
C. sake	EDXA	C, O, P, S, U	C, O, Al, P, S, U	C, O, Na, P, U	C, O, P, U
	XRD	Metaankoleite, Chernikovite, Uramphite			
P. acaciae	EDXA	C, O, P, S, U, K	C, O, Al, P, S, U	C, O, P, U	C, O, Na, P, U
	XRD	Metaankoleite, Chernikovite, Uramphite			
K. lactis	EDXA	C, O, Al, P, S, U	C, O, Al, P, S, U	C, O, P, U, K	C, O, P, U
	XRD	Metaankoleite, Chernikovite, Bassetite, Uramphite			
C. filicatus	EDXA	C, O, Al, P, S, U	C, O, Al, P, U	C, O, P, U	C, O, P, U
	XRD	Metaankoleite, Chernikovite, Uramphite			
C. podzolicus	EDXA	C, O, Al, P, S, U	C, O, Al, P, U	C, O, P, U, K	C, O, P, U
	XRD	Metaankoleite, Chernikovite			
C. argentea	EDXA	C, O, P, S, U, K	C, O, Al, P, S, U	C, O, P, U	C, O, P, U
	XRD	Metaankoleite, Chernikovite, Bassetite, Uramphite			

EDXA and XRD were carried out on samples obtained after 120 h growth of the yeasts at 30°C on an orbital shaker at 180 rpm in the dark in 0.2 or 1 mM $UO_2(NO_3)_2$ and 30 mM G2P or 5 mM PyA-amended MBM medium. Typical analyses are shown from one of at least three determinations.

Legend to figures

Fig. 1. Scanning electron microscopy of uranium-containing biominerals produced by *Candida argentea*, *Cryptococcus filicatus* and *Cryptococcus podzolicus*

To examine U biomineral formation in yeasts pre-grown in the presence of a source of organic phosphorus and then exposed to $UO_2(NO_3)_2$, *Candida argentea*, *Cryptococcus filicatus* and *Cryptococcus podzolicus* were grown in 30 mM G2P-amended MBM, harvested after 120 h and then mixed with (A,D,G) Milli-Q water or (B,E,H) 1 mM $UO_2(NO_3)_2$. In another experiment to examine U biomineral formation in cultures growing in the presence of uranium, *C. argentea*, *C. filicatus* and *C. podzolicus* were grown in (C,F,I) 1 mM $UO_2(NO_3)_2$-amended MBM with 30 mM G2P at 30°C at 180 rpm in the dark and harvested after 120 h. (A) *C. argentea* grown in MBM amended with 30 mM G2P, scale bar = 4 µm. (B) Uranium precipitates on *C. argentea* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 5 µm. (C) Uranium precipitates on *C. argentea* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 2 µm. (D) *C. filicatus* grown in MBM amended with 30 mM G2P, scale bar = 5 µm. (E) Uranium precipitates on *C. filicatus* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 1 µm. (F) Uranium precipitates on *C. filicatus* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 3 µm. (G) *C. podzolicus* grown in MBM amended with 30 mM G2P, scale bar = 5 µm. (H) Uranium precipitates on *C. podzolicus* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 5 µm. (I) Uranium precipitates on *C. podzolicus* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 2 µm. Typical images are shown from several examinations.

Fig. 2. Scanning electron microscopy of uranium-containing biominerals produced by *Candida sake*, *Kluyveromyces lactis* and *Pichia acaciae*

To examine U biomineral formation in yeasts pre-grown in the presence of a source of organic phosphorus and then exposed to $UO_2(NO_3)_2$, *Candida sake*, *Kluyveromyces lactis* and *Pichia acaciae* were grown in 30 mM G2P-amended MBM, harvested after 120 h and then mixed with (A,D,G) Milli-Q water or (B,E,H) 1 mM $UO_2(NO_3)_2$. In another experiment to examine U biomineral formation in cultures growing in the presence of uranium, *C. sake*, *K. lactis* and *P. acaciae* were grown in (C,F,I) 1 mM $UO_2(NO_3)_2$-amended MBM with 30 mM G2P at 30°C at 180 rpm in the dark and harvested after 120 h. (A) *C. sake* grown in MBM amended with 30 mM G2P, scale bar = 5 µm. (B) Uranium precipitates on *C. sake* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 4 µm. (C) Uranium precipitates on *C. sake* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 500 nm. (D) *K. lactis* grown in MBM amended with 30 mM G2P, scale bar = 5 µm. (E) Uranium precipitates on *K. lactis* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 5 µm. (F) Uranium precipitates on *K. lactis* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 3 µm. (G) *P. acaciae* grown in MBM amended with 30 mM G2P, scale bar = 5 µm. (H) Uranium precipitates on *P. acaciae* harvested from MBM amended with 30 mM G2P after reaction with 1 mM $UO_2(NO_3)_2$, scale bar = 3 µm. (I) Uranium precipitates on *P. acaciae* harvested from 1 mM $UO_2(NO_3)_2$ MBM amended with 30 mM G2P, scale bar = 2 µm. Typical images are shown from several examinations.

Fig. 3. X-ray diffraction of biominerals precipitated by *Cryptococcus filicatus*, *Kluyveromyces lactis* and *Pichia acaciae*

Diffraction patterns were collected from mineral particulates harvested from 30 mM $C_3H_7Na_2O_6P.xH_2O$-amended MBM containing 1 mM $UO_2(NO_3)_2$ after 120 h growth of (A) *C. filicatus* (B) *K. lactis* and (C) *P. acacia* at 30°C at 180 rpm in the dark. Patterns for dominant mineralogical components are shown. Typical diffraction patterns are shown from one of several determinations.

Fig. 4. X-ray diffraction of biominerals precipitated by *Candida argentea*, *Candida sake* and *Cryptococcus podzolicus*

Diffraction patterns were collected from mineral particulates harvested from 30 mM $C_3H_7Na_2O_6P.xH_2O$-amended MBM containing 1 mM $UO_2(NO_3)_2$ after 120 h growth of (A) *C. argentea* (B) *C. sake* and (C) *C. podzolicus* at 30°C at 180 rpm in the dark. Patterns for dominant mineralogical components are shown. Typical diffraction patterns are shown from one of several determinations.