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**Uranium bioprecipitation mediated by yeasts utilizing organic
phosphorus substrates**

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1 **Abstract**

2 In this research, we have demonstrated the ability of several yeast species to
3 mediate U(VI) biomineralization through uranium phosphate biomineral
4 formation when utilizing an organic source of phosphorus (glycerol
5 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P \cdot xH_2O$ (G2P)) or phytic acid
6 sodium salt hydrate ($C_6H_{18}O_{24}P_6 \cdot xNa^+ \cdot yH_2O$ (PyA))) in the presence of soluble
7 $UO_2(NO_3)_2$. The formation of metaankileite ($K_2(UO_2)_2(PO_4)_2 \cdot 6(H_2O)$),
8 chernikovite ($(H_3O)_2(UO_2)_2(PO_4)_2 \cdot 6(H_2O)$), bassetite
9 ($Fe^{++}(UO_2)_2(PO_4)_2 \cdot 8(H_2O)$), and uramphite ($(NH_4)(UO_2)(PO_4) \cdot 3(H_2O)$) on cell
10 surfaces was confirmed by X-ray diffraction in yeasts grown in a defined liquid
11 medium amended with uranium and an organic phosphorus source, as well as
12 in yeasts pre-grown in organic phosphorus-containing media and then
13 subsequently exposed to $UO_2(NO_3)_2$. The resulting minerals depended on the
14 yeast species as well as physico-chemical conditions. The results obtained in
15 this study demonstrate that phosphatase-mediated uranium biomineralization
16 can occur in yeasts supplied with an organic phosphate substrate as sole
17 source of phosphorus. Further understanding of yeast interactions with
18 uranium may be relevant to development of potential treatment methods for
19 uranium waste, utilization of organic phosphate sources, and for prediction of
20 microbial impacts on the fate of uranium in the environment.

21

1 **Introduction**

2 Uranium contamination of the environment occurs from a number of sources
3 including the nuclear industry, weathering of uranium-containing natural rocks
4 and minerals, and the extensive use of uranium-containing phosphate
5 fertilizers (Llorens et al. 2012). One potential strategy to inhibit the spread of
6 uranium in the environment consists of inducing uranium precipitation via a
7 biogenic or non-biogenic process. As previous research has demonstrated,
8 many microorganisms can accumulate large amounts of toxic metals and
9 generate crystalline minerals: toxic metals can precipitate with ligands
10 generated from chemical and/or enzymatic processes, such as sulfide,
11 carbonate, phosphate and oxalate (Macaskie et al. 1992; 2000; Gadd 2010;
12 Sivaswamy et al. 2011). Many studies have focused on uranium reduction
13 processes in bacteria, which can play an important role in uranium
14 bioremediation (Lovley and Phillips 1992; Llorens et al. 2012; Martinez et al.
15 2007; 2014). Several Gram-positive and Gram-negative bacteria, such as
16 *Cupriavidus metallidurans* CH34 (Ray et al. 2011), *Rhodopseudomonas*
17 *palustris* (Llorens et al. 2012), *Thermoterrabacterium ferrireducens* (Khijniak et
18 al. 2005), *Mycobacterium smegmatis* (Andres et al. 1993; 1994), *Bacillus*
19 *subtilis* (Fowle et al. 2000), *Rahnella* sp. (Martinez et al. 2007), and
20 *Shewanella oneidensis* MR-1 (Sheng and Fein 2013) can reduce U(VI) to U(IV)
21 which precipitates as U(IV)-carbonate. *Citrobacter* sp. can precipitate
22 U(VI)-phosphate minerals as a result of phosphatase-mediated hydrolysis of
23 an organic source of phosphorus in the presence of U(VI) (Macaskie et al.
24 1992; 1994; 2000).

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

1 fermentations (Lovley and Phillips 1992; Andres et al. 1993; Macaskie et al.
2 2000; Fowle et al. 2000; Sakamoto et al. 2005; Aytas et al. 2011; Llorens et al.
3 2012). A composite adsorbent consisting of *Jania rubens* (marine macroalga)
4 and *Saccharomyces cerevisiae* immobilized on silica gel showed good
5 biosorption properties in removing uranium from dilute aqueous solution (Aytas
6 et al. 2011). However, little attention has been paid to uranium
7 biomineralization by fungal systems. Previous research has demonstrated that
8 fungi exhibit uranium tolerance and can solubilize uranium oxides and
9 depleted uranium and reprecipitate secondary uranium phosphate minerals of
10 the meta-autunite group, uramphite and/or chernikovite, which can encrust
11 fungal hyphae to high accumulation values (Fomina et al. 2007; 2008; Gadd
12 and Fomina 2011). Such minerals may be capable of long-term U retention
13 (Fomina et al. 2007; 2008; Gadd and Fomina 2011). Fungi, like bacteria, also
14 show the ability for phosphatase-mediated uranium precipitation during growth
15 on an organic phosphorus source, and extensively precipitated uranium and
16 phosphorus-containing minerals on fungal hyphal surfaces (Liang et al. 2015a).
17 *S. cerevisiae* also shows some properties of uranium biomineralization through
18 formation of a U(IV)-bearing precipitate during growth in a high-phosphate
19 medium (Ohnuki et al. 2005). Uranium removal by yeasts has mainly focused
20 on *S. cerevisiae*, while other yeast species have received little attention
21 (Soares et al. 2002; Ohnuki et al. 2005; Sakamoto et al. 2005; 2007; Sarri et al.
22 2009).

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

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

1 **Materials and Methods**

2 **Organisms and media**

3 Yeast strains used in the experiments were *Kluyveromyces lactis* IFO1267
4 (Dombrowski) Van der Walt and *Pichia acaciae* (NRRL 18665) Van der Walt
5 (kindly supplied by Professor Mike Stark, University of Dundee) (Worsham and
6 Bolen 1990); *Cryptococcus podzolicus* PYCC 4488^T (= CBS 6819^T) (Babeva &
7 Reshetova) Golubev, originally isolated from a disused arsenic mine in Devon;
8 *Cryptococcus filicatus* Golubev & Samp JP, originally isolated from a disused
9 Cornish copper mine; *Candida sake* (Saito & Oda M) van Uden & Buckley HR,
10 originally isolated from a lead-polluted area in Wales (strain details are found
11 in Holland et al. 2014) and *Candida argentea* (NCYC 3753^T) S.L. Holland, S.V.
12 Avery & P.S. Dyer sp. nov., originally isolated from a metal-polluted site in
13 Wales (kindly supplied by Dr Sara Holland, University of Nottingham). These
14 yeast strains were chosen for their demonstrated abilities in mineral and toxic
15 metal biotransformations (Holland et al. 2011; 2014; Fernandes et al. 2014;
16 Liang et al. 2015b). All yeast cells were grown in Modified Burkholder's
17 medium (MBM) in 250 ml Erlenmeyer conical flasks containing 100 ml nutrient
18 medium on an orbital shaking incubator (Infors Multitron Standard, Rittergasse,
19 Switzerland) at 180 rpm for 48 h at 30°C in the dark. Modified Burkholder's
20 medium (MBM) consists of dextrose 20 g, (NH₄)₂SO₄ 4 g, asparagine 2 g,
21 KH₂PO₄ 1.5 g, MgSO₄·7H₂O 0.5 g, CaCl₂·2H₂O 0.33 g, KI 7.6 mg,
22 ZnSO₄·7H₂O 0.7 mg, FeSO₄·7H₂O 0.5 mg, MnSO₄·4H₂O 0.1 mg,
23 Na₂B₄O₇·10H₂O 0.1 mg, CuSO₄·5H₂O 0.1 mg, (NH₄)₆MO₇O₂₄·4H₂O 0.1 mg,
24 inositol 10,000 µg, nicotinic acid 200 µg, pyridoxine 200 µg, thiamine HCl 200
25 µg, pantothenic acid 200 µg, *p*-amino benzoic acid 50 µg and biotin 2 µg per
26 1000 ml sterile Milli-Q water. Cells at 48 h were harvested by centrifugation at
27 4000 rpm (4880 g) for 30 min and were then aseptically transferred to a
28 sucrose- and P-free equivalent nutrient solution and grown for a further 48 h

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

3 **Preparation of MBM amended with organic phosphorus sources and** 4 **uranium**

5 One batch of yeast cells in the stationary growth phase was harvested by
6 centrifugation at 4000 rpm (4880 g) for 30 min and then aseptically transferred
7 to and grown in MBM substituting 30 mM glycerol 2-phosphate disodium salt
8 hydrate ($\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} \cdot x\text{H}_2\text{O}$ (G2P)) or 5 mM phytic acid sodium salt hydrate
9 ($\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6 \cdot x\text{Na}^+ \cdot y\text{H}_2\text{O}$ (PyA)) for KH_2PO_4 as the sole phosphorus source.
10 Test yeast strains were grown in three different media (MBM with 30 mM G2P,
11 MBM with 5 mM PyA and MBM without any phosphorus source as a control) to
12 examine uranium biomineral formation in yeasts pre-grown in the presence of
13 source of organic phosphorus and then exposed to $\text{UO}_2(\text{NO}_3)_2$. All yeast
14 strains were grown at 30°C in 250-ml flasks containing 100 ml MBM on an
15 orbital shaking incubator at 180 rpm for 120 h in the dark. All phosphorus
16 sources were separately sterilized by membrane filtration (cellulose nitrate, 0.2
17 μm pore diameter, Whatman, Maidstone, Kent, UK) and added to autoclaved
18 MBM medium (121°C, 15 min) at room temperature, to give a final
19 concentration of 30 mM G2P or 5 mM PyA. MBM without any phosphorus
20 source was the control and uninoculated medium served as an abiotic control
21 for each set of experiments. The organic phosphorus substrates produced no
22 significant precipitation on reaction with uranyl nitrate in the absence of yeasts.

23 To examine uranium biomineral formation in cultures growing in the presence
24 of uranium, another batch of yeast cells in the stationary growth phase at 96 h
25 were harvested by centrifugation at 4000 rpm (4880 g) for 30 min and then
26 aseptically transferred to and grown in MBM amended with 0.2 or 1 mM
27 $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA as sole phosphorus sources in
28 250-ml conical flasks containing 100 ml nutrient medium on an orbital shaking

1 incubator at 180 rpm at 30°C in the dark. $\text{UO}_2(\text{NO}_3)_2$, G2P and PyA were
2 dissolved separately in Milli-Q water and sterilized by membrane filtration
3 (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone, Kent, UK) and
4 added to autoclaved MBM (121°C, 15 min) at room temperature, to give 0.2 or
5 1 mM $\text{UO}_2(\text{NO}_3)_2$, 30 mM G2P and 5 mM PyA final concentrations. MBM
6 amended with 0.2 or 1 mM $\text{UO}_2(\text{NO}_3)_2$ without any phosphorus source was the
7 control and uninoculated medium served as an abiotic control for each set of
8 experiments.

9 **Growth rate, inorganic phosphate (P_i) release, tolerance indices (TI), and** 10 **pH analysis**

11 To examine the effect of uranium on phosphatase activity when grown with an
12 organic phosphorus source, yeast growth was measured by optical density
13 (OD) at 595 nm using a spectrophotometer (Anthos 2001 microplate reader)
14 over 120 h culture in 0.2 or 1 mM $\text{UO}_2(\text{NO}_3)_2$ -containing MBM amended with
15 G2P or PyA. Calculations were carried out using the Windows-based control
16 and evaluation software for Rosys Anthos microplate readers (Anthos Labtec
17 Instruments, Wals, Austria). Background OD_{595} was determined by
18 spectrophotometric measurement of uninoculated wells.

19 Inorganic phosphate (P_i) release into the medium during growth in 0.2 or 1 mM
20 $\text{UO}_2(\text{NO}_3)_2$ containing MBM amended with G2P or PyA was determined
21 spectrophotometrically using the malachite green assay (Irving and
22 McLaughlin, 1990). 15 μl aliquots of supernatant were sterilized by membrane
23 filtration (cellulose nitrate, 0.2 μm pore diameter, Whatman, Maidstone, Kent,
24 UK), and added to each well, in a 96-well plate, with 185 μl Milli-Q water,
25 followed by the addition of 100 μl malachite green reagent and left for 15 min
26 after mixing. The absorbance at 620 nm was read on an Anthos 2001
27 microplate reader, and calculations were carried out as described above. For
28 the malachite green background comparison, 200 μl Milli-Q water was mixed

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

5 To assess uranium tolerance, test yeast species were grown in MBM amended
6 with 0.2 or 1 mM $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA as sole phosphorus
7 source in 250-ml conical flasks containing 100 ml medium on an orbital
8 shaking incubator at 180 rpm at 30°C in the dark. Yeast biomass was
9 harvested at appropriate time intervals by centrifugation at 4000 rpm (4880 g)
10 for 30 min. Biomass dry weights were used to obtain a tolerance index (TI) and
11 the supernatant analysed for changes in pH. Metal tolerance was evaluated
12 using a TI as follows: (dry weight of uranium-exposed biomass/dry weight of
13 control biomass x 100%) (Wei et al. 2013; Liang et al. 2015a,b). For dry weight
14 determination, biomass was harvested, washed three-times with 0.1 M NaCl,
15 and dried to constant weight in a vacuum desiccator at room temperature for at
16 least 30 days, and then ground to a powder using a pestle and mortar (Milton
17 Brook, Dorset, UK). Supernatants were obtained by membrane filtration (0.45
18 µm pore diameter, Whatman, Maidstone, Kent, UK). The pH of supernatants
19 was measured using a pH 210 Microprocessor pH Meter (Hanna Instruments,
20 Woonsocket, RI, USA). All experiments were conducted at least in triplicate.

21 **Analysis of biominerals produced by yeast**

22 To investigate uranium bioprecipitation by yeast cell suspensions after
23 pre-growth with organic phosphorus sources, test yeast cells were harvested
24 from 30 mM G2P or 5 mM PyA amended MBM after 120 h by centrifugation at
25 4000 rpm (4880 g) for 30 min and washed three times with 0.1 M NaCl. 50 mg
26 (wet weight) of harvested yeast cells were transferred into 2 ml microcentrifuge
27 tubes (Starlab, Hamburg, Germany) to which 1 ml of 1 mM $\text{UO}_2(\text{NO}_3)_2$ solution
28 was added. Duplicate samples were incubated for 48 h at 30°C. After this time,

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

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

24 The mineralogy of the biominerals was determined using a Hiltonbrooks X-ray
25 diffractometer (XRD) (HiltonBrooks Ltd., Crewe, UK) fitted with a
26 monochromatic CuK α source and curved graphite, single Seiko crystal
27 chronometer (30 mA, 40 kV). The finely ground samples obtained were firmly
28 compacted on the reverse side of an aluminium 15 x 20 x 2 mm³ specimen
29 holder, later held against a clean glass side. After compaction, the minerals

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

5 **Statistical analysis**

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

11

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 presence 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 $\text{UO}_2(\text{NO}_3)_2$ 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 presence of PyA. The changes in pH were similar when MBM was amended with $\text{UO}_2(\text{NO}_3)_2$, falling to around pH 5.7 for 0.2 mM $\text{UO}_2(\text{NO}_3)_2$, and around pH 5.5 for 1 mM $\text{UO}_2(\text{NO}_3)_2$ except for *P. acaciae* (pH 5.8) and *C. sake* (pH 7.1) with 0.2 mM $\text{UO}_2(\text{NO}_3)_2$, and *C. sake* (pH 5.7) and *C. podzolicus* (pH 5.6) with 1 mM $\text{UO}_2(\text{NO}_3)_2$ (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 $\text{UO}_2(\text{NO}_3)_2$, 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 $\text{UO}_2(\text{NO}_3)_2$ and *C. sake* (pH 3.4) with 1 mM $\text{UO}_2(\text{NO}_3)_2$ (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 $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, 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 $\text{UO}_2(\text{NO}_3)_2$ in MBM amended with 5 mM PyA, with TI values of 54.5% and 57.1% respectively. Since negligible growth was

1 observed in MBM amended with 5 mM PyA, TI values for *C. sake* were
2 negligible (Table 4). In the presence of 1 mM $\text{UO}_2(\text{NO}_3)_2$ in MBM amended
3 with 30 mM G2P, *C. sake* and *C. argentea* showed TI values of 61.5% and
4 53.3% respectively (Table 4).

5 **Bioprecipitation of uranium by yeast biomass harvested from organic** 6 **phosphorus-amended MBM after reaction with $\text{UO}_2(\text{NO}_3)_2$**

7 Most test yeasts previously grown in organic P-amended medium showed
8 uranium bioprecipitation when subsequently reacted with 1 mM $\text{UO}_2(\text{NO}_3)_2$
9 (Fig.1-2B,E,H). Compared to the large amounts of precipitation found on *C.*
10 *podzolicus* (Fig.1H), *C. sake* (Fig.2B) and *K. lactis* (Fig.2E) pre-grown in
11 G2P-amended MBM, other yeast strains showed relatively poor abilities in
12 precipitating uranium biominerals, and only a minor proportion of the
13 population exhibited electron-dense precipitation. After growth in PyA, *P.*
14 *acaciae* precipitated abundant biominerals on the cell walls. Little precipitation
15 was observed on *C. podzolicus* grown in PyA-amended MBM after mixture
16 with 1 mM $\text{UO}_2(\text{NO}_3)_2$ (data not shown). SEM revealed the presence of
17 electron-dense clusters with distinct crystalline shapes on the surface of some
18 of the yeast species in the early stages of growth. Large electron-dense
19 deposits were observed on the yeast cells after longer reaction times (Fig.1-2
20 B,E,H).

21 **Formation of uranium-containing secondary minerals by yeasts grown in** 22 **MBM amended with uranium and G2P or PyA**

23 Most test yeasts showed uranium bioprecipitation when grown in G2P or
24 PyA-amended media with 0.2 or 1 mM $\text{UO}_2(\text{NO}_3)_2$ (Fig.1C,F,I). *C. sake* hardly
25 grew in PyA amended MBM and therefore mineral precipitation was not
26 observed. Compared to the large amount of precipitation found with *C.*
27 *podzolicus* grown in G2P-amended MBM with $\text{UO}_2(\text{NO}_3)_2$ (Fig.1I), only a small
28 amount of precipitation occurred when grown in PyA amended MBM with 0.2

1 or 1 mM $\text{UO}_2(\text{NO}_3)_2$. Precipitation occurred in varying amounts with the various
2 P sources with the different yeast species (Fig. 1-2). Differences were found
3 between the secondary minerals precipitated in these growth experiments to
4 the previous experiments where pre-grown control cells were reacted with
5 $\text{UO}_2(\text{NO}_3)_2$ solutions, regarding their morphologies and the occurrence of
6 nanoscale particles.

7 **Energy-dispersive X-ray analysis (EDXA) of uranium-containing** 8 **secondary minerals**

9 Energy-dispersive X-ray analysis (EDXA) revealed the elemental composition
10 of the secondary minerals formed by yeast cells harvested from G2P or PyA
11 amended MBM and then reacted with $\text{UO}_2(\text{NO}_3)_2$, and yeasts grown in
12 uranium-amended MBM with G2P or PyA. Control yeast cells from MBM
13 amended with G2P showed carbon, oxygen, sodium and phosphorus as the
14 main elements with occasional detection of sulfur and magnesium. The
15 minerals precipitated on yeast cells grown with an organic phosphorus source
16 and then reacted with uranium nitrate, showed carbon, oxygen, phosphorus
17 and uranium as the main elements and sometimes sodium and potassium
18 (Table 5). The minerals formed with yeasts grown in uranium-G2P or PyA
19 amended MBM showed carbon, oxygen, phosphorus and uranium as the main
20 elements detected and sometimes aluminium and sulfur (Table 5). Most of the
21 uranium-containing minerals precipitated on the yeast cells shared similar
22 crystalline morphologies.

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

24 XRD showed the formation of metaankileite ($\text{K}_2(\text{UO}_2)_2(\text{PO}_4)_2 \cdot 6(\text{H}_2\text{O})$),
25 chernikovite $((\text{H}_3\text{O})_2(\text{UO}_2)_2(\text{PO}_4)_2 \cdot 6(\text{H}_2\text{O}))$, bassetite
26 $(\text{Fe}^{++}(\text{UO}_2)_2(\text{PO}_4)_2 \cdot 8(\text{H}_2\text{O}))$, and uramphite $(\text{NH}_4)(\text{UO}_2)(\text{PO}_4) \cdot 3(\text{H}_2\text{O})$ in MBM
27 amended with 1 mM $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P after yeast growth (Fig. 3-4).
28 All of these minerals are uranium- and phosphorus-containing minerals.

1 Metaankileite and chernikovite were found with all yeast species (Fig. 3-4).
2 Uramphite was found in most of the yeasts except *C. podzolicus* (Fig. 3-4).
3 Bassetite only appeared in MBM amended with $\text{UO}_2(\text{NO}_3)_2$ and G2P after
4 growth of *C. argentea* and *K. lactis*. Metaankileite and chernikovite were the
5 only minerals found in MBM amended with $\text{UO}_2(\text{NO}_3)_2$ and G2P after growth of
6 *C. podzolicus* (Fig. 4).

7

1 **Discussion**

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

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

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

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

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

8

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19

20 **Compliance with ethical standards**

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

23

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Table 1. Growth of test yeasts in MBM containing 0.2 or 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$	30 mM G2P + 1 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA	5 mM PyA + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 1 mM $\text{UO}_2(\text{NO}_3)_2$
<i>C. sake</i>	1.67	1.11	0.65	0.21	0.13	0.1
<i>P. acaciae</i>	2.07	1.04	0.79	0.65	0.21	0.17
<i>K. lactis</i>	2.03	1.41	1.24	1.06	0.55	0.43
<i>C. filicatus</i>	1.98	1.63	1.55	1.72	0.89	0.78
<i>C. podzolicus</i>	2.01	1.14	0.78	1.85	0.65	0.55
<i>C. argentea</i>	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 $\text{UO}_2(\text{NO}_3)_2$ 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 $UO_2(NO_3)_2$
<i>C. sake</i>	70.1	50.6	--	--
<i>P. acaciae</i>	39.2	26.4	13.2	10.9
<i>K. lactis</i>	63.2	46.1	19.3	13.2
<i>C. filicatus</i>	54.6	48.1	29.6	22.1
<i>C. podzolicus</i>	60	55.3	39.4	28.6
<i>C. argentea</i>	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 $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA.

	pH value					
	30 mM G2P	30 mM G2P + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	30 mM G2P + 1 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA	5 mM PyA + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 1 mM $\text{UO}_2(\text{NO}_3)_2$
Uninoculated control	6.9	6.8	7	3.8	3.5	3.5
<i>C. sake</i>	5.6	7.1	5.7	3.8	3.5	3.4
<i>P. acaciae</i>	5.5	5.8	5.5	3.1	3.2	3
<i>K. lactis</i>	5.5	5.7	5.5	3	3.3	3
<i>C. filicatus</i>	5.5	5.8	5.5	3	3.3	3
<i>C. podzolicus</i>	5.6	5.8	5.6	3	3.2	3
<i>C. argentea</i>	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 $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA.

	TI (%)			
	30 mM G2P + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	30 mM G2P + 1 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$
<i>C. sake</i>	76.9	61.5	ND	ND
<i>P. acaciae</i>	94.1	82.3	81.8	54.5
<i>K. lactis</i>	87.5	75	85.7	71.4
<i>C. filicatus</i>	88.9	77.8	99.7	80
<i>C. podzolicus</i>	87.5	81.3	90.5	57.1
<i>C. argentea</i>	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 $\text{UO}_2(\text{NO}_3)_2$ and 30 mM G2P or 5 mM PyA.

		Elemental (EDXA) and mineralogical composition (XRD)			
		30 mM G2P + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	30 mM G2P + 1 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 0.2 mM $\text{UO}_2(\text{NO}_3)_2$	5 mM PyA + 1 mM $\text{UO}_2(\text{NO}_3)_2$
<i>C. sake</i>	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			
<i>P. acaciae</i>	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			
<i>K. lactis</i>	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			
<i>C. filicatus</i>	EDXA	C, O, Al, P, S, U	C, O, Al, P, U	C, O, P, U	C, O, P, U
	XRD	Metaankoleite, Chernikovite, Uramphite			
<i>C. podzolicus</i>	EDXA	C, O, Al, P, S, U	C, O, Al, P, U	C, O, P, U, K	C, O, P, U
	XRD	Metaankoleite, Chernikovite			
<i>C. argentea</i>	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 $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, *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 $\text{UO}_2(\text{NO}_3)_2$. 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 $\text{UO}_2(\text{NO}_3)_2$ -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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 5 μm . (C) Uranium precipitates on *C. argentea* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 1 μm . (F) Uranium precipitates on *C. filicatus* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 5 μm . (I) Uranium precipitates on *C. podzolicus* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, *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 $\text{UO}_2(\text{NO}_3)_2$. 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 $\text{UO}_2(\text{NO}_3)_2$ -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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 4 μm . (C) Uranium precipitates on *C. sake* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 5 μm . (F) Uranium precipitates on *K. lactis* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{UO}_2(\text{NO}_3)_2$, scale bar = 3 μm . (I) Uranium precipitates on *P. acaciae* harvested from 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} \cdot x\text{H}_2\text{O}$ -amended MBM containing 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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 $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P} \cdot x\text{H}_2\text{O}$ -amended MBM containing 1 mM $\text{UO}_2(\text{NO}_3)_2$ 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.