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Association of uranyl with the cell wall of *Pseudomonas fluorescens* inhibits metabolism

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Abstract—Citric acid is found along with uranyl in the subsurface of former nuclear facilities because of its use as a decontamination agent in the nuclear industry. Citrate's metal chelating properties affect the mobility of uranyl in the subsurface and consequently, citrate biodegradation may significantly impact uranyl fate and transport. Under the non-growth conditions considered, low (micromolar) uranyl concentrations inhibit the biodegradation of citrate by *Pseudomonas fluorescens*, a common subsurface denitrifying bacterium. Additionally, uranyl is found readily associated with the cell envelope of *P. fluorescens*. The observed inhibition appears to be linked to the binding of uranyl to the cell surface and is reversible by desorbing cell-bound uranyl. This study establishes a link between uranyl association with the cell surface and the observed inhibitory effect of uranyl on cell metabolism. *Copyright © 2003 Elsevier Ltd*

1. INTRODUCTION

Uranium is a contaminant of concern in the subsurface environment of a majority of former nuclear fuel and weapons manufacturing facilities of the Department of Energy (DOE) (Riley et al., 1992). In the 1950s and 1960s, wastes containing a mixture of radionuclides and chelators such as citric acid, oxalic acid, ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA) were disposed of in shallow trenches (Department of Energy, 1997, 1998; Hartman et al., 2000). In several cases, the radionuclides have reached the water table and are threatening water supply wells or surface waters. The chelators are not contaminants in their own right (with the notable exception of NTA) but are important because they have the potential to significantly alter uranyl mobility in the subsurface.

Specifically, citric acid is known to affect uranyl adsorption to mineral surfaces. Citrate has been shown to enhance uranyl adsorption on goethite and to reduce the adsorption of uranyl on kaolinite (Redden et al., 1998). Thus, the mobility of uranyl in the subsurface is crucially linked to the persistence of citric acid and other chelators in the subsurface.

However, citric acid is readily metabolized by a variety of microorganisms, both aerobically and anaerobically (Hugenholtz, 1993; Fuller and Scow, 1997; Coates et al., 1999). An understanding of the propensity of citrate to be biodegraded in the presence of uranyl would be useful in assessing its persistence in uranyl-contaminated environments and thus its role in uranyl fate and transport.

The goal of this study is to investigate the inhibitory effect of uranyl on the biodegradation of citrate by *Pseudomonas fluorescens*, a common subsurface denitrifier, at environmentally relevant concentrations and under non-growth conditions. The present study differs from others in several ways: (1) The uranyl concentrations used are considerably lower than those in other studies (<10 μmol/L vs. >400 μmol/L). This is significant because measured uranyl concentrations in contaminated aquifers at circumneutral pH rarely exceed 30 μmol/L (Department of Energy, 1997). Although DOE sites are heavily contaminated, uranyl sorbs strongly at circumneutral pHs and the majority of uranyl is associated with the sediments at those pHs. (2) Non-growth conditions were used and are representative of often oligotrophic subsurface environments. Previous studies were conducted under growth conditions in the presence of ample supply of a carbon source. In this study, we examine the metabolism of *P. fluorescens* under non-growth conditions, in the presence of low, environmentally relevant, uranyl and citrate concentrations. (3) Lastly, and most importantly, an additional novelty of this study resides in the fact that it focuses on the relationship between uranyl biosorption and the inhibition of citrate degradation by uranyl and establishes a link between the two.

Several researchers have studied citrate degradation by bacteria in the presence of uranyl but exclusively under growth conditions. Joshi-Tope and Francis (1995) and Francis et al. (1992) showed that, in the presence of equimolar uranyl (0.52 mmol/L), there was no degradation of citrate by *P. fluorescens*. However, in cell-free extract, citrate was fully biodegraded under similar conditions. The authors concluded that the persistence of citrate stemmed from the inability of the cells to transport the uranyl-citrate complex across the cell membrane. Another study by Banaszak et al. (1999) suggests that the uranyl-citrate complex itself is toxic to *P. fluorescens*. Finally, Huang et al. (1998) show that there is incomplete citrate (0.55 mmol/L) degradation by a mixed cell culture in the presence of uranyl (0.42 mmol/L) at pH 6.

This study shows that uranyl inhibits cell metabolism at low, environmentally relevant concentrations and that the inhibition is associated with the binding of uranyl to the cell envelope. This inhibitory effect is reversible by desorbing cell-bound uranyl.

2. MATERIALS AND METHODS

2.1. Thermodynamic Calculations

Equilibrium speciation calculations were conducted using the chemical equilibrium program HYDRAQL (Papelis et al., 1988). The thermodynamic data used are shown in Table 1. Briefly, the citrate com-

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Table 1. Thermodynamic constants used in calculation of chemical speciation. The values shown are formation constants adjusted to an ionic strength of 0.

Species	logK	Reference
$4\ UO_2^{2+} + 7\ H_2O = (UO_2)_4(OH)_7^- + 7H^+$	–21.9	Grenthe et al. (1992)
$2\ UO_2^{2+} + 3\ H_2O + CO_3^{2-} = (UO_2)_2(OH)_3 \cdot CO_3^- + 3H^+$	1.01	Grenthe et al. (1992)
$UO_2^{2+} + 3\ CO_3^{2-} = UO_2(CO_3)_3^{4-}$	21.6	Grenthe et al. (1992)
$UO_2^{2+} + 2\ CO_3^{2-} = UO_2(CO_3)_2^{2-}$	16.94	Grenthe et al. (1992)
$UO_2^{2+} + CO_3^{2-} = UO_2CO_3^0$	9.72	Grenthe et al. (1992)
$UO_2^{2+} + CO_3^{2-} = UO_2CO_3(s)$	14.4	Smith et al. (1997)
$UO_2^{2+} + 2\ H_2O = UO_2(OH)_2(s) + 2H^+$	–4.93	Grenthe et al. (1992)
$H^+ + Cit^{3-} = H_3Cit$	14.285	Smith et al. (1997)
$2\ H^+ + Cit^{3-} = H_2Cit^-$	11.157	Smith et al. (1997)
$H^+ + Cit^{3-} = HCit^{2-}$	6.396	Smith et al. (1997)
$3\ UO_2^{2+} + 7\ H_2O = (UO_2)_3(OH)^- + 7H^+$	–31	Grenthe et al. (1992)
$2\ H^+ + CO_3^{2-} = H_2CO_3$	16.681	Smith et al. (1997)
$H^+ + CO_3^{2-} = HCO_3^-$	10.329	Smith et al. (1997)
$2\ UO_2^{2+} + 2\ Cit^{3-} = (UO_2)_2Cit_2^{2-}$	20.8	Smith et al. (1997)
$UO_2^{2+} + Cl– = UO_2Cl^+$	0.21	Smith et al. (1997)
$UO_2^{2+} + Cit^{3-} = UO_2Cit^-$	8.00	Lenhart et al. (2000)
$3\ UO_2^{2+} + 4\ H_2O = (UO_2)_3(OH)_4^{2+} + 4H^+$	–33	Grenthe et al. (1992)
$3\ UO_2^{2+} + 5\ H_2O = (UO_2)_3(OH)_5^+ + 5\ H^+$	–15.5	Grenthe et al. (1992)
$3\ UO_2^{2+} + 4\ H_2O = (UO_2)_3(OH)_4^{2+} + 4H^+$	–11.9	Grenthe et al. (1992)
$2\ UO_2^{2+} + 2\ H_2O = (UO_2)_2(OH)_2^{2+} + 2H^+$	–5.62	Grenthe et al. (1992)
$UO_2^{2+} + 2\ H_2O = UO_2(OH)_2^0 + 2H^+$	–12.0	Langmuir (1997)
$2\ UO_2^{2+} + H_2O = (UO_2)_2OH^{3+} + H^+$	–2.7	Grenthe et al. (1992)
$UO_2^{2+} + 3\ H_2O = UO_2(OH)_3– + 3H^+$	–19.2	Grenthe et al. (1992)
$UO_2^{2+} + H_2O = UO_2OH^+ + H^+$	–5.2	Grenthe et al. (1992)
$K^+ + Cit^{3-} = KCit^{2-}$	1.22	Smith et al. (1997)

plexation data were obtained from Lenhart et al. (2000) and Smith and Martell (1997), the hydrolysis and carbonato complexes data from Grenthe et al. (1992) except for the $UO_2(OH)_2^0$ complex taken from Langmuir (1997).

2.2. Cell Growth Conditions

P. fluorescens is ubiquitous in the subsurface and has been used previously for studies of the biodegradation of metal-citrate complex (Francis et al., 1992; Joshi-Tope and Francis, 1995). *P. fluorescens* ATCC 55241 biovar II was kindly provided by A. J. Francis (Brookhaven National Laboratory, Upton, NY). Cells of *P. fluorescens* were grown at 25°C in a sterile defined mineral salts medium dubbed MM2 and amended with filter-sterilized (Nalgene 0.2-μm PES syringe filter, Nalge Co., Rochester, NY) citric acid (sodium citrate dihydrate, Mallinckrodt, Phillipsburg, NJ) at a final concentration of 1 mmol/L. One liter of MM2 contains 35.8 mg of NH_4Cl, 2.75 mg of $CaCl_2 \cdot 2H_2O$, 6.25 mg of $MgCl_2 \cdot 6H_2O$, 5.5 mg of KH_2PO_4, 2.585 mg $Fe(SO_4)_2NH_4 \cdot 12H_2O$, 1.155 mg of $MnSO_4 \cdot H_2O$, 0.101 mg of $CuCl_2 \cdot 2H_2O$, 0.095 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.165 mg of $ZnSO_4 \cdot 7H_2O$, 0.151 mg of $CoCl_2 \cdot 6H_2O$, 2.13 g of MES (2-[N-morpholino]ethanesulfonic acid, Sigma Chemical Co., St. Louis, MO), 7.4 g of KCl (Sigma) and was adjusted to pH 6.0 $\pm$ 0.1. After 16 to 18 h (exponential phase), the cells were harvested by centrifugation at 10,800*g* for 30 min at 25°C, washed twice with buffer BS1 and concentrated in BS1 (1 L of cells to 100 mL). BS1 consists of 0.1 mol/L KCl (7.4 g/L) and 10^{-2} mol/L MES (2.13 g/L) at pH 6.0 $\pm$ 0.1. This cell concentrate was used for all experiments.

2.3. Citrate and Glucose Degradation

Degradation experiments were conducted in 500-mL polycarbonate (PC) bottles containing 50 mL of sterile BS1. An adequate volume of filter-sterilized 1 mmol/L uranyl solution (uranyl nitrate, Johnson Matthey, Ward Hill, MA) and 1 mmol/L filter-sterilized citrate or 10 mmol/L filter-sterilized glucose (D-[+]-glucose anhydrous, Sigma Chemical Co., St. Louis, MO) solution were added to the bottles. In addition, 10 μL (0.1 μCi) of filter-sterilized [1,5]^{14}C-citrate (DuPont, DE) or 10 μL (0.1 μCi) uniformally labelled ^{14}C-glucose (ICN Pharmaceuticals, Irvine, CA) were added to each bottle. Each bottle was prepared in triplicates. The addition of ^{14}C labeled citrate or glucose changes the total concentration by $< 1\%$.

After 30 min, cells were added to each bottle to yield a final OD_{600} of 0.1 ($\sim$5 mg protein/L^{-1} or 1.4×10^{11} cells/L^{-1}). The capped bottles were incubated at 25°C on an orbital shaker at 100 rpm, in the dark. pH remained within 0.1 unit of pH 6.0 in all cases. During the degradation experiment, samples were collected aseptically from the bottles, filtered through a 0.22-μm GVHP filter (Millipore Corporation, Bedford, MA) and the concentration of uranyl and citrate determined. To quantify uranyl, the filtrate was diluted five-fold with nitric acid (final HNO_3 concentration 0.2%; Optima grade, Fisher Scientific, Pittsburgh, PA) and analyzed on an HP 4500 series Inductively Coupled Plasma-Mass Spectrometer (ICP-MS). To quantify ^{14}C-citrate, 1 mL of the filtrate, 1 mL of MilliQ water and 20 μL of concentrated hydrochloric acid (A.C.S. Plus grade, Fisher Scientific, Fair Lawn, NJ) were mixed in a 20-mL scintillation vial and purged with nitrogen gas for 20 min to remove CO_2. The vial was analyzed on a TriCarb 2500 TR/AB Packard Liquid Scintillation Analyzer with 5 mL of Ultima Gold XR liquid scintillation solution (Packard, Meriden, CT). Cell lysis was monitored by collecting samples, centrifuging them (at 16,000*g* for 30 min) and analyzing the supernatant for protein by the colorimetric Bradford method (Biorad Laboratories, Hercules, CA). There was no interference from citrate or uranyl and the method was sensitive enough to detect cell lysis (as defined by passage through a French Pressure Cell (SLM Aminco) at 1000 psi once). For heat-killed cells, the cell concentrate was subjected to 60°C for 1 h before the addition to each bottle. The heat treatment was sufficient to halt citrate degradation. The integrity of the cells was verified by the protein assay described above.

Control experiments were conducted ([citrate] = 1 μmol/L, [uranyl] = 0, 3 or 5 μmol/L) to verify the complete biodegradation of citrate to CO_2. The experiments were identical to the ones described above with the difference that a scintillation vial containing 1 mL of 1N NaOH was suspended in the headspace of the PC bottle using Teflon tape. As ^{14}C-citrate was being metabolized, the ^{14}C-CO_2 evolved and was captured in the base trap. The bottles were destructively sampled after 25 h. Measuring ^{14}C-CO_2 in the base trap produced identical results to measuring ^{14}C remaining in solution. Thus, it was concluded that citrate was fully biodegraded to CO_2 and the latter experimental design was adopted for the remainder of the study.

Table 2. Calculated speciation of uranyl and citrate in solutions with 1 μmol/L uranyl and 1 μmol/L citrate, 3 μmol/L uranyl and 1μmol/L citrate or 1 μmol/L uranyl and 10 μmol/L glucose at pH 6.0 and atmospheric P_{CO2}. Free citrate refers to protonated and deprotonated citrate. UO_2^{2+} refers to uranyl in the free ion form, UO_2^{2+} hydroxides and UO_2^{2+} carbonates refer to the sum of all uranyl hydroxide solution complexes and all solution uranyl carbonate species respectively. Carbonate complexes do not become important until pH > 6.0.

	1 μmol/L citrate and 1 μmol/L uranyl		1 μmol/L citrate and 3 μmol/L uranyl		10 μmol glucose and 1μmol uranyl
Species	Percent uranyl as	Percent citrate as	Percent uranyl as	Percent citrate as	Percent uranyl as
UO_2Cit^-	22	22	12	37	
$(UO_2)_2(Cit)_2^{2-}$	1	1	1	3	
Free citrate		63		50	
$KCit^{2-}$		14		14	
UO_2^{2+}	11		8		14
UO_2^{2+} hydroxides	50		59		65
UO_2^{2+} carbonates	15		19		21

2.4. Uranyl Association With Cells

The samples intended for uranium analysis were filtered with GVHP filters selected specifically for their low uranyl binding capacity (<3% for 1 μmol/L uranyl at pH 6.0). To determine the localization of uranyl within the cells, we followed the standard method of cell fractionation. Briefly, cells were lysed using a French Press, the lysate (mixture of lysed and intact cells) centrifuged to remove intact cells and large membrane fragments. The supernatant was then ultracentrifuged to separate the cytoplasmic and membrane fractions. A more detailed protocol follows: Bottles were prepared as described above but without ^{14}C-citrate and with 3 μmol/L uranyl, incubated in the dark for 23 to 24 h and centrifuged for 30 min at 10,800*g*. The supernatant was removed and the pellet resuspended in 3 mL of fresh sterile BS1. The resulting cell concentrate was lysed by three passages through a French Pressure Cell at 1000 psi. The suspension was analyzed for total protein and total uranyl. The lysate was placed in two Eppendorf microcentrifuge tubes (dubbed A and B) and centrifuged at 10,800*g* for 30 min. The supernatants (containing the intracellular fraction and membrane fractions) were analyzed for protein (A) and uranyl (B) respectively. The pellets (containing unbroken cells and a portion of the membrane fraction), were resuspended in 0.5 mL of concentrated HNO_3 (A used for U analysis) or in 0.5 mL of MilliQ water (B used for protein measurement). Analysis of total protein before centrifugation and protein in the supernatant and in the pellet allowed the calculation of the efficiency of cell lysis. The supernatant (combined from A and B) was transferred to a polycarbonate ultracentrifuge tube and centrifuged at 208,000*g* for 2 h at 4°C. The supernatant from ultracentrifugation represents the cytoplasmic fraction and was analyzed for uranium. The pellet (membrane fraction) was resuspended in 1 mL of concentrated HNO_3 and measured for U.

2.5. Viability

To determine the effect of uranyl on cell viability, bottles were prepared as described above and sampled periodically. The samples were diluted 10^4- to 10^5-fold with sterile BS1 and 100 μL of the dilute solution plated onto tryptone glucose extract agar (Difco Laboratories, Detroit, MI) in triplicates. The plates were incubated at 30°C for 24 h and the colonies counted manually.

2.6. Reversibility of Inhibition

Bottles containing sterile BS1 (50 mL), 10 μmol/L citrate (with 10 nmol/L of ^{14}C tracer), 20 μmol/L uranyl and cells at a final OD_{600} of 0.3 were incubated in the dark for 4 h and centrifuged at 10,800*g* for 30 min. The supernatant was analyzed for U. The cells were resuspended in 50 mL of 0.1 mol/L of $KHCO_3$ and incubated in the dark for 1 h. The bottles were then centrifuged and the supernatant analyzed for uranyl and discarded. It was replaced by 50 mL of BS1 containing 10 μmol/L of citrate spiked with ^{14}C-citrate and citrate degradation was measured over time. At the end of the experiment, the bottles were centrifuged, the pellets dissolved in 1 mL of concentrated HNO_3 and analyzed for uranyl.

2.7. Electron Microscopy

Suspensions of freshly grown cells (final OD_{600} of 0.26 or 0.13) containing 10, 40 or 100 μmol/L uranyl and 1 μmol/L citrate were incubated in the dark for 24 h. The cells were pelleted (at 10,800*g* for 30 min), fixed in 2% glutaraldehyde (EM grade Polysciences Inc., PA), dehydrated in an ethanol gradient series (70, 80, 90 and 100% ethanol) and embedded in Spurr low viscosity resin (Polysciences Inc., PA). Subsequently, the polymerized resin was thin-sectioned using a Reichert Ultracut S microtome and imaged with a Philips Transmission Electron Microscope TEM300 (equiped with Gatan, Bioscan CCD camera) at 80 kV using a 20-μm aperture. The Energy Dispersive X-ray spectra were collected with an EDAX DX-4.

2.8. Spectrophotometry

To determine whether cells outcompete citrate for uranyl in BS1, the absorbance spectrum of the uranyl-citrate complex was monitored before and after the addition of heat-killed cells. The uranyl-citrate complex absorbs at 437 nm (Dodge and Francis, 1997) and the uranyl cation at 420 nm. Citrate does not absorb in the 400 to 500 nm range. Uranyl and citrate (each 100 μmol/L) were placed in BS1 buffer and a spectrum collected in the range 400 to 500 nm on a Perkin Elmer Lambda Bio spectrometer in a 10-cm path length cell. Heat killed cells (OD_{600} 0.2 or 0.5) were added and after a short equilibration (2 or 5 min), a sample was filtered and a spectrum collected.

3. RESULTS

3.1. Fate of Uranyl

Numerous uranyl-citrate complexes have been proposed in the literature: a 1:1 uranyl-citrate complex (Newman et al., 1951; Rajan and Martell, 1965; Markovits et al., 1972; Nunes and Gil, 1987; Lenhart et al., 2000), a 2:1 complex (Nunes and Gil, 1987), a 2:2 complex (Newman et al., 1951; Feldman et al., 1954; Rajan and Martell, 1965; Markovits et al., 1972; Nunes and Gil, 1987; Allen et al., 1996), a 2:3 complex (Dodge and Francis, 1997), a 3:2 complex (Feldman et al., 1954) and a hexameric complex (Rajan and Martell, 1965). In our speciation calculations, we consider only the complexes for which a formation constant exists: the mononuclear (1:1) and binuclear (2:2) complexes. Thermodynamic calculations indicate that at the micromolar concentrations used in our experiments, the mononuclear complex dominates (Table 2). A complete list of

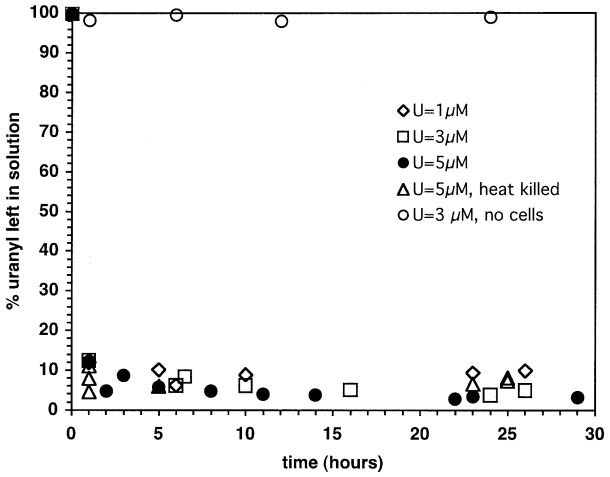

Fig. 1. Percent of initial uranyl remaining in solution after addition of *P. fluorescens* cells (added at $t = 0$ and with an OD_{600} 0.1) in BS1 and in the presence of 1 μmol/L citrate. Initial uranyl is 1, 3 or 5 μmol/L at pH 6.0. Controls with heat-killed cells or no cells are shown.

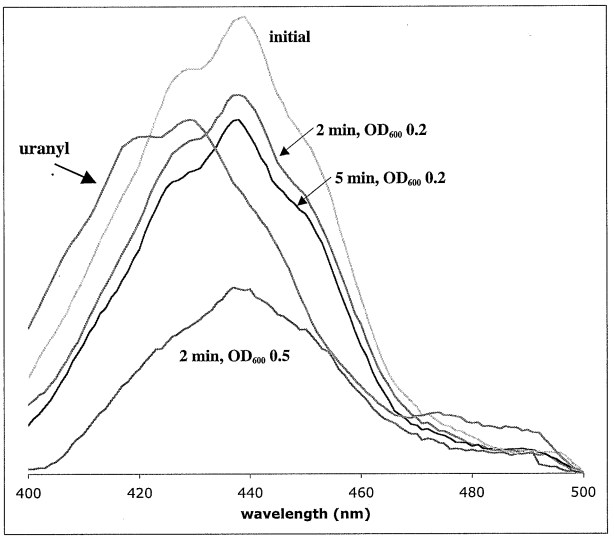

Fig. 2. Absorbance of the uranyl-citrate complex in BS1 before and after addition of *P. fluorescens* cells. The spectra 2 and 5 min after the addition of heat-killed cells (OD_{600} 0.2) are shown. Also, the spectrum 2 min after addition of cells (OD_{600} 0.5) and the spectrum with no citrate are shown. $[uranyl]_T = [citrate]_T = 100$ μmol/L, pH 6.0.

the thermodynamic data used in the speciation calculations is given in Table 1.

The empirical determination of a formation constant for the mononuclear U:cit complex by three independent research groups resulted in three different values: $\log \beta_{1,1} = 7.4$, 6.7 and 6.3 at $I = 0.1$ (respectively Rajan and Martell, 1965; Lenhart et al., 2000; and Markovits et al., 1972). Lenhart et al. (2000) studied the formation of the mononuclear uranyl:citrate complex in $NaClO_4$ at $I = 0.1$ at $U_T = 1$ μmol/L and $[citrate]_T = 20$ μmol/L to 0.6 mmol/L at pH 4.0 and 5.0. The experimental conditions in our study were similar with $U_T = 1$ to 5 μmol/L, $[citrate]_T = 1$ μmol/L and pH 6.0. In contrast, the complexation constants determined by Rajan and Martell (1965) were obtained at considerably higher uranyl and citrate concentrations 0.68 to 17 mmol/L at the same $I = 0.1$ Markovits et al. (1972) obtained their constants at $I = 1.0$ and U and citrate concentrations = 30 μmol/L to 1.2 mmol/L. Therefore, we used the complexation constant determined under experimental conditions most akin to ours (Lenhart et al., 2000) as is reflected in Table 1.

The thermodynamic calculations predict that 100% of the uranyl present is in solution (Table 2). This prediction is confirmed by experimental data collected in the absence of cells and showing ~100% uranyl in solution over the time frame of our experiments (Fig. 1). However, in the presence of cells, < 10% of uranyl is measured in solution (Fig. 1). These data suggest a strong association of uranyl with cells. The association of uranyl with cells is likely to be due to sorption of uranyl to cells rather than intracellular uptake of uranyl because heat-killed cells scavenge uranyl as effectively as metabolically active cells (Fig. 1).

Thermodynamic calculations predict that 22% of citrate will be bound in a uranyl-citrate complex (Table 2) for [U] = [cit] = 1 μmol/L. However, due to the uncertainty in the thermodynamic data, it is not possible to state with certainty what fraction of the ~90% uranyl scavenged by cells (Fig. 1) was initially bound with citrate. Also, the uranyl and citrate concentrations are too low to directly measure the initial presence of the uranyl-citrate complex. At higher uranyl and citrate concentrations, at which it is possible to monitor the uranyl-citrate complex(es) by spectrophotometry (Fig. 2), the dissociation of the uranyl-citrate complex in the presence of cells is clearly observed. The uranyl-citrate complex has a characteristic absorption spectrum with a peak at 437 nm (Dodge and Francis, 1997). The peak appears immediately upon mixing 100 μmol/L uranyl and 100 μmol/L citrate in a 0.1 mol/L KCl solution buffered at pH 6.0 with 10 mmol/L MES. Addition of heat-killed cells (OD_{600} 0.2), equilibration for 2 to 5 min followed by the removal of cells by filtration show a steady disappearance of the peak. The uranyl-citrate peak decreases by 17% after 2 min of equilibration with cells and by 22% after 5 min. Moreover, the addition of a higher cell concentration (OD_{600} 0.5) results in a more dramatic disappearance (59%) of the uranyl-citrate peak. The characteristic uranyl peak at 420 nm (Fig. 2) does not appear, suggesting that uranyl is not released into solution. These data show that, under these conditions, the uranyl-citrate complex initially present dissociates in the presence of metabolically inactive cells and that the uranyl released from the complex binds to cells. Thus, under these conditions, cells outcompete citrate for uranyl and strip off uranyl from the preformed uranyl-citrate complex. These results suggest that *P. fluorescens* cells act as a ligand for uranyl and may participate in the speciation of the cation.

We have conducted an empirical study of the kinetics of formation of the uranyl-citrate complexes to confirm their formation within the equilibration time. The results are shown in Figure 3. The spectrum obtained immediately upon mixing of uranyl and citrate is identical to that obtained after 1 week of equilibration under sterile conditions. The kinetics of formation of the complex appear to be extremely rapid and the complex(es) can be assumed to form instantaneously.

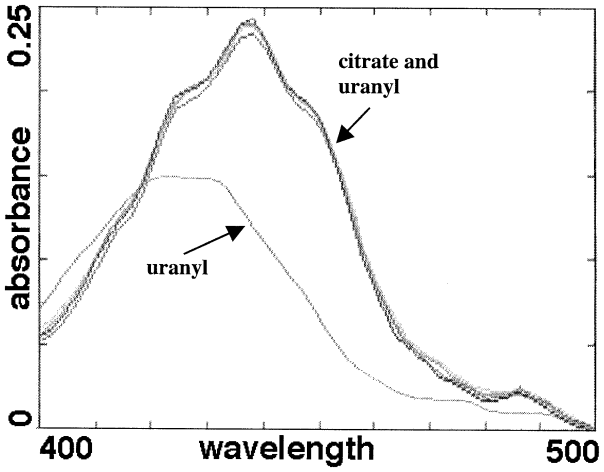

Fig. 3. UV-VIS spectra of uranyl and citrate (5 and 12.5 mmol/L respectively at pH 5.2) upon mixing, after 1 h, 24 h and 1 week are identical. The spectrum of 5 mmol/L uranyl at pH 4.16 is also shown.

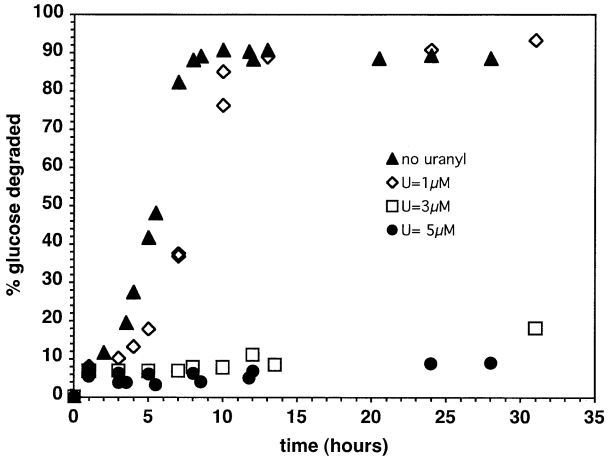

Fig. 5. Degradation of glucose (10 μmol/L) by *P. fluorescens* (OD_{600} 0.1) in BS1 in the absence of uranyl, in the presence of 1, 3 5 μmol/L uranyl at pH 6.0.

3.2. Inhibitory Effect of Uranyl

The ability of *P. fluorescens* to degrade citrate under non-growth conditions and in the presence of low, environmentally relevant, concentrations of uranyl was evaluated. *P. fluorescens* degrades citrate (1 μmol/L) at an initial rate of 0.12 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ under non-growth conditions (Fig. 4) as indicated by the rapid drop in the solution concentration of ^{14}C-citrate. In the presence of equimolar uranyl, the initial rate of citrate degradation is similar at 0.1 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ (Fig. 4). At increasing uranyl concentrations, citrate degradation is slower (initial degradation rate of 0.035 and 0.025 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ for 3 and 5 μmol/L uranyl respectively) and ceases at a higher remaining citrate concentration (Fig. 4). In the presence of 3 μmol/L uranyl, 45% of citrate is degraded after 25 h whereas only 18% is consumed in the presence of 5 μmol/L uranyl in the same time interval (Fig. 4). In the presence of heat-killed cells, citrate is not degraded and remains in solution (Fig. 4).

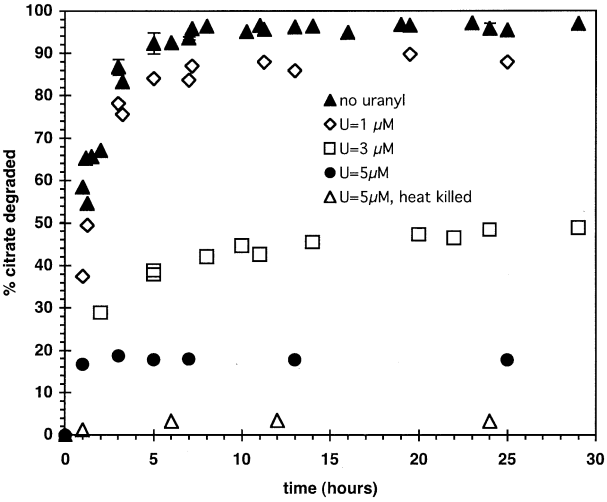

Fig. 4. Citrate (1 μmol/L) degradation in BS1 by *P. fluorescens* (OD_{600} 0.1) in the absence of uranyl and in the presence of 1, 3 or 5 μmol/L uranyl at pH 6.0. The control with heat-killed cells is included.

There are two plausible explanations for the observed decrease in the rate of citrate degradation with increasing uranyl concentration: (1) at higher (3 or 5 μmol/L) uranyl concentrations, the concentration of the bioavailable citrate species (assuming that uranyl-citrate complex itself is not bioavailable) is significantly lower than the half-velocity coefficient (K_m) for citrate degradation and thus the degradation rate of citrate is minimal; or (2) uranyl inhibits the metabolism of *P. fluorescens* and increasing uranyl concentrations shut down citrate metabolism with increasing efficiency. The former (1) is unlikely because there is at least 0.5 μmol/L citrate (50% of the total citrate) that is not complexed to uranyl in the presence of 5 μmol/L uranyl, yet only 18% is degraded (Fig. 4). At least 0.5 μmol/L citrate is not complexed to uranyl because, according to Figure 1, only 0.5 μmol/L uranyl remains in solution in the presence of cells. The maximum concentration of complexed citrate equals the total concentration of uranyl: 0.5 μmol/L citrate.

Glucose was used as a substrate to unambiguously deconvolute a potential inhibitory effect from the effect of a low substrate concentration. Glucose is not known to complex uranyl and therefore uranyl is only present as the free ion, the hydroxy and the carbonato complexes (Table 1) and the amount of bioavailable glucose can be measured directly.

The effect of uranyl on glucose degradation by *P. fluorescens* is similar to that on citrate degradation (Fig. 5). In the presence of 1 μmol/L uranyl, the initial rate of glucose degradation is slightly lower than in the absence of uranyl (0.16 vs. 0.11 $\mu mol\ glucose \cdot h^{-1} \cdot mg\ protein^{-1}$). At higher uranyl concentrations (3 and 5 μmol/L), glucose degradation does not exceed 15% after 30 h (and the initial rate is 0.04 $\mu mol\ glucose \cdot h^{-1} \cdot mg\ protein^{-1}$). We deduce that, under the present conditions, micromolar uranyl has an inhibitory effect on the metabolism of glucose and citrate by *P. fluorescens*. To confirm that deduction, we conducted viability tests for cells exposed to uranyl and citrate or uranyl and glucose.

Figure 6 shows the colony forming units (CFU) counted on

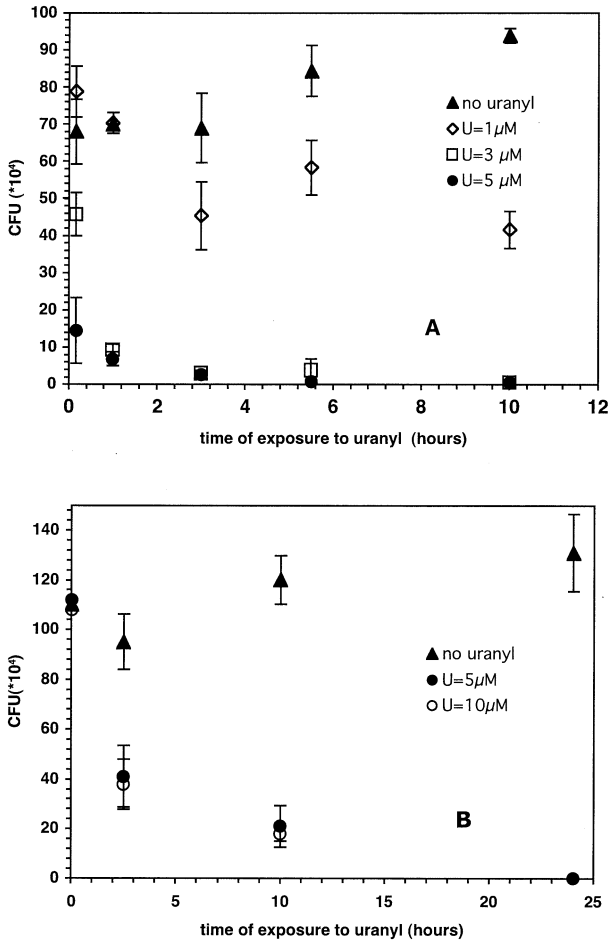

Fig. 6. Colony forming units (CFU) of *P. fluorescens* (1.4×10^{11} cells $\cdot$ L^{-1}) exposed to 1 μmol/L citrate (panel A) or 10 μmol/L glucose (panel B) and various concentrations of uranyl for the number of hours corresponding to the x-axis and diluted by 10^4 in BS1 before plating on tryptone glucose plates.

agar plates after *P. fluorescens* cells were exposed to uranyl (various concentrations) and 1 μmol/L citrate (panel A) or 10 μmol/L glucose (panel B) for varying durations. Total cell counts of cells not exposed to uranyl do not change significantly, whereas CFU of cells exposed to uranyl decrease over the duration of exposure. In particular, cells exposed to 3, 5 or 10 μmol/L uranyl show a precipitous decrease in CFU. Cells exposed to uranyl and citrate for 3 h and plated on a solid agar medium devoid of uranyl display a $>$ 70-fold decrease in viable cell counts (Fig. 6A). A similar behavior is observed for cells exposed to uranyl and glucose (Fig. 6B). These results confirm the above suggestion that uranyl inhibits both citrate and glucose metabolism.

The inhibition of *P. fluorescens* metabolism by uranyl can be reversed by adding a strong U(VI) complexing agent to the solution. Figure 7 shows the degradation of citrate (1) for cells never exposed to uranyl, (2) for cells exposed to uranyl and washed in 0.1 mol/L potassium bicarbonate and (3) for cells in the presence of uranyl. After washing the cells with a bicarbonate solution, a significant recovery in activity ensues (Fig. 7). Cells were lost during the wash procedure, thus the observed rate of citrate degradation after washing (seen on Fig. 7) is an underestimate relative to the rate normalized to cell mass. More accurately, the calculated rate of citrate degradation is 0.04 μmol $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ for unwashed cells as compared to 0.18 μmol $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ for washed cells. The rate of citrate degradation for washed cells remains well below the rate for cells never exposed to uranyl $\sim$0.6 μmol $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$.

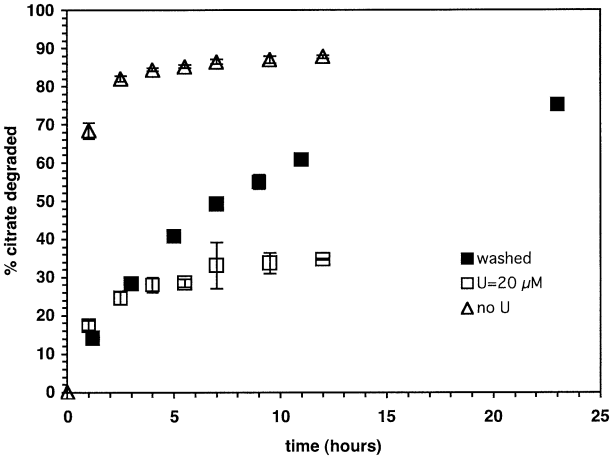

Fig. 7. Citrate (10 μmol/L) degradation in BS1 by *P. fluorescens* (OD_{600} 0.3) cells never exposed to uranyl, in the presence of 20 μmol/L uranyl, exposed to 20 μmol/L uranyl for 4 h and washed with 0.1 mol/L $KHCO_3$.

Cell washing with a bicarbonate solution correlates directly with the recovery of uranyl in solution. Figure 8 shows the amount of uranyl in solution after washing. Approximately 90% of the uranyl associated with cells is recovered by washing

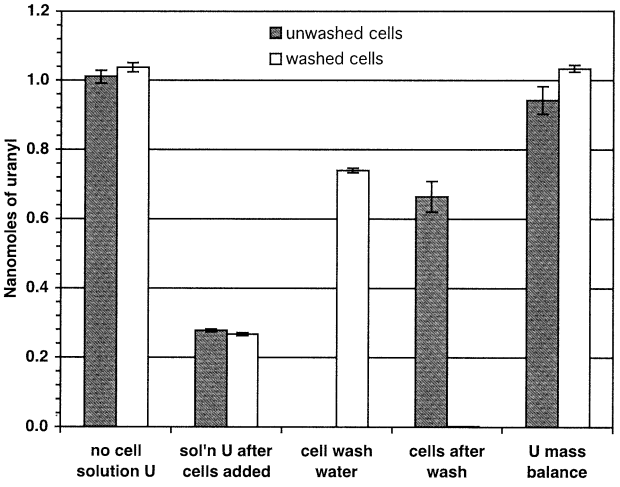

Fig. 8. Distribution of uranyl (20 μmol/L) in BS1 solution containing 10μmol/L citrate in the presence of unwashed cells (filled) and washed cells (blank). Column 1: initial uranyl concentration in solution before cell addition. Column 2: uranyl concentration in solution after cell addition. Column 3: uranyl in bicarbonate wash solution after the wash. Column 4: uranyl associated with the cells. Column 5: mass balance of uranyl is equal to the sum of uranyl left in solution after cell addition (column 2), uranyl recovered by washing (column 3) and uranyl associated with cells (column 4).

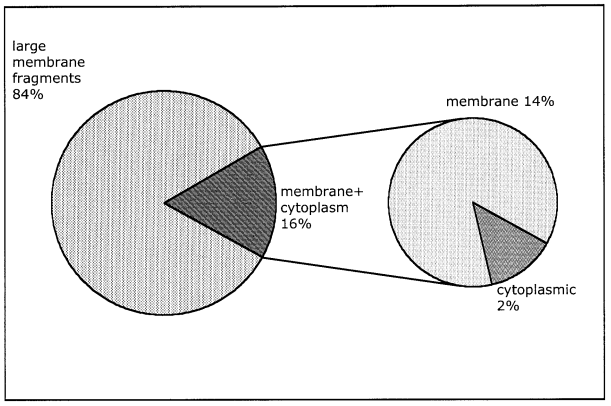

Fig. 9. Distribution of uranyl in cellular fractions after French Pressing and slow centrifugation (large pie) and after ultra-centrifugation of the supernatant from the slow centrifugation (small pie). Unlysed cells in the pellet after slow centrifugation were accounted for and their contribution to the slow centrifugation pellet subtracted.

with 0.1 mol/L bicarbonate. Carbonate is a strong complexing agent for uranyl and strips uranyl from the cell surface by forming uranyl-carbonate complexes in solution. The amount of uranyl associated with washed cells is negligible (Fig. 8). The metabolic inhibition of *P. fluorescens* by uranyl appears to be associated with the passive binding of uranyl to cells. The binding is passive (i.e., does not require the use of energy by cells) because heat-killed cells scavenge uranyl as effectively as live cells (Fig. 1).

The recovery of metabolic activity after treatment with bicarbonate is consistent with the results from the viability experiments (Fig. 6). Cells exposed to uranyl (3, 5 or 10 μmol/L) and plated on agar grew poorly (Fig. 6) because the dilution in BS1 buffer (0.1 mol/L KCl and 10 mmol/L MES) carried out before plating did not remobilize the uranyl into solution. Uranyl was still associated with cells when they were plated on agar and consequently, the cells were unable to metabolize the tryptone glucose substrate and could not grow.

3.3. Localization of Uranyl Within Cells

Treatment with bicarbonate recovers 85 to 95% of the initial uranyl (Fig. 8). This suggests that (1) most of the uranyl is associated with the outside of the cell and that (2) the uranyl-cell interaction is a relatively labile "complex." To confirm the former suggestion, we conducted cell fractionation and thin-section TEM (Transmission Electron Microscopy) analyses of cells exposed to uranyl. Cell fractionation demonstrated the presence of uranyl in the membrane fraction (Fig. 9). Approximately 98% of uranyl was associated with the membrane fraction (Fig. 9).

Thin-section TEM micrographs (Figs. 10 and 11) further confirm the localization of uranyl. Uranyl (the only electron-dense atom present in the sample) is clustered in the membrane(s) (Fig. 11). Uranyl is associated with both the outer and the cytoplasmic membranes and also deposits along the periplasm separating the two membranes (Fig. 10). Finally, Energy Dispersive X-ray (EDX) analysis confirms that the deposit is indeed U and shows no significant contribution from potential contaminants such as Fe (Fig. 12).

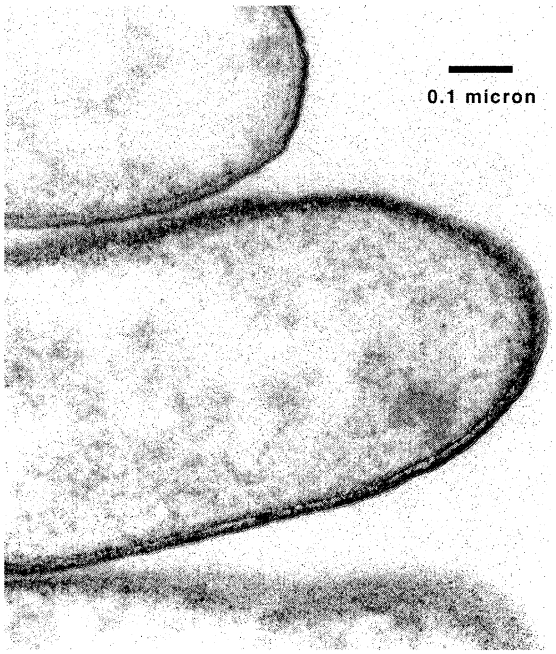

Fig. 10. Transmission electron micrograph of *P. fluorescens* cells exposed to 100 μmol/L uranyl. The samples have not been stained. High electron density corresponds to the presence of uranyl. The membranes (both outer and inner) are significantly darker than the cytoplasm which indicates that uranyl accumulates in the membranes and periplasm.

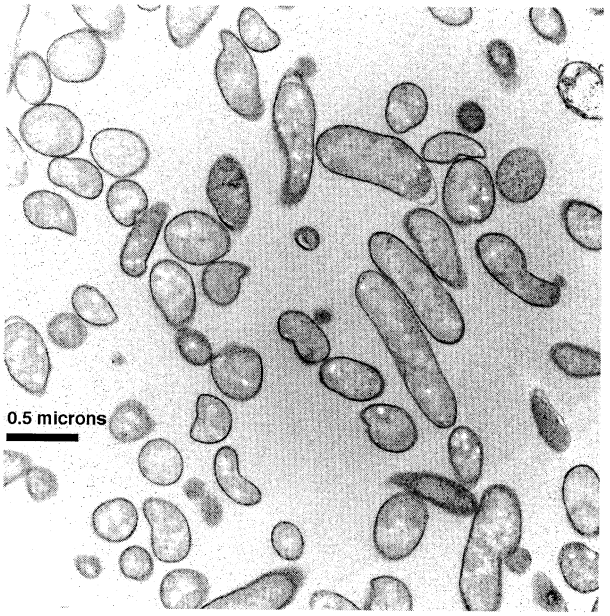

Fig. 11. Transmission electron micrograph of *P. fluorescens* cells exposed to 100 μmol/L uranyl. The samples have not been stained. High electron density corresponds to the presence of uranyl. The membranes (both outer and inner) are significantly darker than the cytoplasm which indicates that uranyl accumulates in the membranes and periplasm.

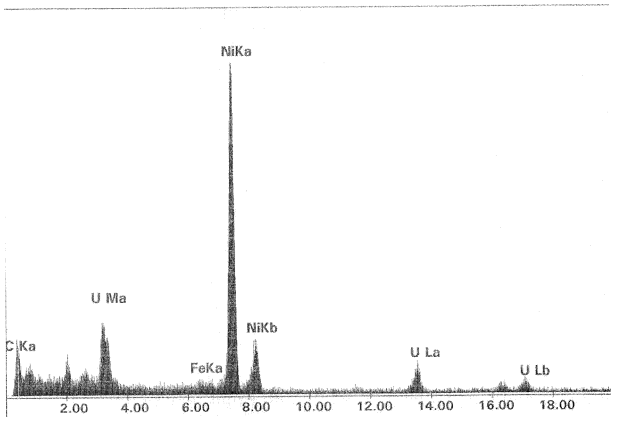

Fig. 12. Energy dispersive X-ray spectrum if the membrane of a cell exposed to uranyl. X-rays from the uranium L and M shells are clearly detectable, while no X-rays from Fe appear. The Ni signal comes from the grid.

4. DISCUSSION

This study shows that *P. fluorescens*, a common subsurface denitrifier, can serve as a ligand for uranyl. Under the conditions investigated (Fig. 2), cells rapidly scavenge solution uranyl and even outcompete citrate for uranyl, leading to the dissociation of preformed uranyl-citrate complex(es). Metabolically inactive cells also cause the dissociation of uranyl-citrate complex(es) and scavenge uranyl. Thus, the dissociation of uranyl-citrate complexes can be attributed to the depletion of uncomplexed uranyl by binding to the cell envelope, rather than to the depletion of uncomplexed citrate by biodegradation.

The ability of *P. fluorescens* to outcompete citrate for uranyl under the conditions studied is probably due to the low concentration of citrate present and the absence of other uranyl-binding ligands (principally carbonate). As with all ligands, the amount of uranyl complexed by cells will vary according to the relative concentration of uranyl, cells, other ligands and pH. One situation in which cells may complex a significant amount of uranyl is during the bioremediation of a low-carbonate site, where high cell concentrations are expected due to the bioaugmentation or biostimulation strategies.

Electron micrographs (Figs. 10 and 11) show the association of uranyl with both the outer and the cytoplasmic membranes as well as with the periplasm. A study of uranyl biosorption on another strain of *P. fluorescens* shows the association of uranyl with the cell envelope with the exception of ~10% of the cells that accumulate uranyl intracellularly (Krueger et al., 1993). In that study, uranyl precipitates as platy crystals aligned in the periplasm so that their flat surfaces associate with the periplasm side of the outer and cytoplasmic membranes. It may be that those crystals are a uranyl phosphate phase formed during a cell wash with a phosphate buffer.

The present study scrupulously excluded phosphate. Spectroscopic and isotherm data both show no evidence of uranyl precipitation onto the cell surface (data not shown). Moreover, a rapid wash of uranyl-laden cells with bicarbonate released ~90% of uranyl suggesting the presence of a labile uranyl-cell "complex" rather than a precipitate. Thus, we characterize the association of uranyl with cells as complexation. A similar complexation was described by Haas et al. (2001) for the association of uranyl with another gram-negative bacterium *Shewanella putrefaciens*.

The precise nature of the uranyl-cell complex is unknown. Researchers have suggested the importance of carboxyl and phosphato functional groups in the biosorption of uranyl by both gram-positive (Fowle et al., 2000; Kelly et al., 2001) and gram-negative bacteria (Haas et al., 2001; Kelly et al., 2002). We are currently investigating the molecular details of uranyl binding to the surface of *P. fluorescens*.

Under our experimental conditions, a large fraction of uranyl present is biosorbed and, consequently, a large fraction of citrate is uncomplexed (Fig. 1). Therefore, citrate is expected to be readily degraded by cells in the presence of uranyl because citrate is present to a large extent as either free citrate or $Kcit^{2-}$, both biodegradable species. However, we observe little citrate degradation in the presence of some concentrations of uranyl (≥ 5 μmol/L). Similarly, there is little glucose degradation in the presence of 3 μmol/L uranyl or higher. These results reveal the inhibitory effect of uranyl on cell metabolism of both citrate and glucose even at micromolar concentrations.

The inhibition appears to be associated with the binding of uranyl to the cell envelope. A cell wash with bicarbonate recovers ~90% of uranyl and leads to a recovery (30%) in metabolic activity of cells. The link between uranyl binding to the cell surface and the inhibitory effect of uranyl is important because it suggests that biosorption inhibits metabolism. The ease of reversibility of the inhibition by washing with a complexing agent strengthens the argument that the inhibitory effect of uranyl is not associated with intracellular intake of uranyl but rather with binding to the cell surface. We hypothesize that the binding of U may alter the conformation of membrane proteins crucial for the metabolism of citrate and glucose or that uranyl biosorption reduces membrane fluidity and permeability, thus limiting citrate and glucose uptake. The cells recover only 30% of their activity after washing with bicarbonate even as 90% of uranyl is in solution, suggesting a hysteresis effect in the recovery from uranyl biosorption.

Several studies have described the toxic effect of uranyl on bacterial metabolism. An early study (Barron et al., 1948) documented the toxic effect of uranyl on yeast and bacteria, including *Pseudomonas aeruginosa*. Tuovinen and Kelly (1974a, 1974b) also recorded the toxic effect of uranyl on the iron oxidizer *Thiobacillus ferrooxidans* at acidic pH. They found that the presence of a chelator such as EDTA or a high concentration of competing cations reduced the inhibitory effect of uranyl on *T. ferrooxidans*.

Huang et al. (1998) studied the degradation of citrate in the presence of uranyl by a growing mixed culture. At pH 6, they found that 73% of citrate was degraded within 100 h when present in threefold excess but no further reduction took place in the following 140 h. At that pH, uranyl was sorbed to cells extensively (~50%). At higher citrate:uranyl ratios (8:1), complete citrate degradation was observed within 130 h at pH 6 but less uranyl was associated with cells. For pHs at which solution carbonate limits U sorption to cells (e.g., pH 8, 9), citrate degradation was unhindered and proceeded to 100% within 10 h even at low citrate:uranyl ratios. The authors attributed the difference in citrate degradation at pH 6 and 8 to changes in uranyl speciation (carbonate vs. citrate complexes). Nonethe-

less, there appears to be a correlation between incomplete citrate degradation and sorption of uranyl onto cells in their data. The study (Huang et al., 1998) was conducted under growth conditions, with a mixed bacterial community and with considerably higher uranyl and citrate concentrations ([uranyl] = 0.42 mmol/L and [citrate] = 0.55–3.5 mmol/L). Despite the major differences with conditions in our study, the effect of biosorbed uranyl on citrate biodegradation was also observed.

Other studies show different results. Joshi-Tope and Francis (1995) and Francis et al. (1992) found that *P. fluorescens* was unable to degrade citrate in the presence of equimolar uranyl. Cell free extract of the cells readily degraded 100% of citrate in the presence of equimolar uranyl. The authors suggested that the uranyl-citrate complexes had limited lability, rendering citrate non-bioavailable. Unfortunately, no uranyl measurements were made and thus was not possible to assess the fraction of uranyl sorbed to cells in those experiments.

The toxic species responsible for binding to cells and inhibiting metabolism was not identified in this study. However, due to the chemical simplicity of the system studied, only four species can be considered as possible inhibitors: UO_2^{2+}, UO_2Cl^+, UO_2OH^+ and $(UO_2)_2(OH)_2^{2+}$. Only two hydroxide species are considered because, the inhibitory species is likely to be a cation due to its binding to the negatively charged surface of cells. Banaszak et al. (1999) concluded that a uranyl-citrate complex was the toxic species. The design of our experiments does not allow a definitive conclusion on the toxicity of the uranyl-citrate complex. However, it is clear that there is at least one additional inhibitory species as inhibition was also observed in the absence of citrate.

5. CONCLUSIONS

This study shows that, under the conditions studied, the cellular envelope of *P. fluorescens* serves as a ligand for uranyl. Additionally, the binding of uranyl to the cell envelope results in the inhibition of cell metabolism. The inhibition is reversible by desorbing uranyl.

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