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Reduction of U(VI) by the deep subsurface bacterium, *Thermus scotoductus* SA-01, and the involvement of the ABC transporter protein

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ABSTRACT

In this study we investigated the effect of uranium on the growth of the bacterium *Thermus scotoductus* strain SA-01 as well as the whole cell U(VI) reduction capabilities of the organism. Also, site-directed mutagenesis confirmed the identity of a protein capable of a possible alternative mechanism of U(VI) reduction. SA-01 can grow aerobically in up to 1.25 mM uranium and has the capability to reduce low levels of U(VI) in under 20 h. TEM analysis performed on cells exposed to uranium showed extracellular and membrane-bound accumulation of uranium. The reductase-like protein was surprisingly identified as a peptide ABC transporter, peptide-binding protein. This study showcases the concept of protein promiscuity, where this protein with a distinct function *in situ* can also have the unintended function of a reactant for the reduction of U(VI).

1. Introduction

Uranium is the forty-ninth most abundant metal in the earth's crust and thus natural uranium contamination is quite ubiquitous in nature (Wall and Krumholz, 2006). Although higher concentrations of uranium are highly toxic to living organisms, studies done over the last decade have shown that these uranium-containing extreme environments are populated by a large variety of bacteria (Ehrlich, 1998; Francis, 1998; Pedersen, 1998). These bacteria have shown the ability to tolerate radioactivity (Chicote et al., 2005), to bioaccumulate uranium (Selenska-Pobell et al., 1999; Merroun et al., 2003a,b) and even to biotransform it (DiSpirito and Tuovinen, 1982; Lovley et al., 1991). These interactions encouraged research pertaining to the potential of such bacteria to bioremediate uranium-contaminated sites by *in situ* biostimulation (Holmes et al., 2002; Anderson et al., 2003; Nevin et al., 2003). Previous studies described the toxicity of uranium to microorganisms (Leduc et al., 1997; Suzuki and Banfield, 1999), and Sakamoto et al. (2005) evaluated the effect of uranium on the growth of *Saccharomyces cerevisiae*. However, very little work has described the effect(s) of uranium on, and its interaction, with extremophiles including thermophiles. It has been established that predominantly mesophillic dissimilatory Fe(III)-reducing (Lovley et al., 1991) and sulfate-reducing microorganisms (Lovley and Phillips, 1992; Lovley et al., 1993a) are able to reduce U(VI) to U(IV), *Geobacter, Shewanella* and *Desulfovibrio* genera are the most intensively studied due to their remarkable respiratory versatility, which among others, includes the ability to utilize U(VI) as a terminal electron acceptor (Lovley et al., 1993b, 2004; Nealson and Saffarini, 1994). Utilization of such a wide spectrum of terminal electron acceptors is largely due to the diversified respiratory network found in these organisms (Marshall et al., 2006). It has been found that *c*-type cytochromes form an integral part of the terminal reductase complexes (Myers and Myers, 1992).

The thermophilic bacterium, *Thermus scotoductus* SA-01, was isolated in 1999 by Kieft et al. (1999) from groundwater sampled at a depth of 3.2 kmbls in Mponeng, Witwatersrand Basin, Republic of South Africa. Lin et al. (2004) found that fracture water collected in the basin contained highly elevated levels of H_2 which coincides with levels predicted for the radiolytic dissociation of H_2O during radioactive decay of U, Th, and K in the host rock. Thus *T. scotoductus* SA-01, which was isolated in this environment could be well equipped to deal with exposure to heavy metals like U and as such has shown the ability to reduce certain metals under growth and non-growth conditions, including Mn(IV), Co(III)-EDTA, Cr(VI) (Opperman and van Heerden, 2007) and, most importantly for this study, U(VI) (Kieft et al., 1999). However, Kieft only reported on whole cell reduction of U(VI) with lactate as an electron donor in a very limited fashion thus, to date, there is no information on the effect of uranium on actively growing cells as well as its

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capabilities to reduce U(VI) with other electron donors and the proteins involved. This led us to explore the full extent of the microbe's capabilities to interact with uranium and the mechanisms involved.

2. Materials and methods

2.1. Bacterial strain and culture conditions

T. scotoductus strain SA-01 (ATCC 700910; American Type Culture Collection) was routinely cultured aerobically in a complex organic medium, TYG (5 g L^{-1} tryptone, 3 g L^{-1} yeast extract and 1 g L^{-1} glucose, pH 7.0) at 65 °C with shaking (160 rev min^{-1}) (Opperman and van Heerden, 2007).

Growth was furthermore evaluated under aerobic conditions in TYG medium supplemented with U(VI) concentrations ranging from 0 (control) to 1.5 mM for 24 h at 65 °C with shaking (160 rev min^{-1}) and the optical density (600 nm) was recorded at 2 h intervals on a Spectronic® Genesys™ 5. U(VI) was prepared by dissolving $UO_2(CH_3COO)_2{\cdot}2H_2O$ in deinonized water. Growth was initiated in 500 mL Erlenmeyer flasks containing 100 mL of medium using standardized inocula (5% v/v) to approximately 0.06 g of cells (dry weight) per liter.

2.2. Hexavalent uranium reduction assay

U(VI) reduction was analysed spectrophotometrically according to the 2-(5-bromo-2-pyrdulazo)-5-diethylaminophenol (Bromo-PADAP) method as described by Johnson and Florence (1970). The formed uranyl-bromo-PADAP complex absorbs light at 578 nm in the presence of a mixed complexing solution.

2.3. Reduction of U(VI) by resting cells under non-growth conditions

The ability to reduce uranium by whole cells under non-growth conditions was determined by measuring the decrease in hexavalent uranium as described above. To prepare cells for the reduction assay, early and late stationary- phase cultures (with an optical density at 600 nm of about 1.0 when grown in TYG medium) were harvested by centrifugation at 6000*g* for 10 min and washed once in an equal volume of anaerobic 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.0. This buffer was always freshly prepared the day before the assay and purged with O_2-free N_2 before being taken into an anaerobic chamber (atmosphere of N_2–H_2, 95:5; Coy Laboratory Inc., Grass Lake Michigan). The washed pellet was resuspended in this buffer inside the anaerobic chamber. To initiate the assay, an identical sample of the cells was added to a tube containing the assay solution (0.25 mM uranyl acetate in anaerobic MOPS buffer, pH 7.0, with 10 mM electron donor) and subjected to the spectrophotometric assay. Electron donors tested were sodium lactate, sodium acetate, glucose, sodium pyruvate and H_2, with the latter tested by purging the assay solution with 10% H_2. Two control samples were prepared namely a cell-free control, as well as electron donor free control.

2.4. Transmission electron microscopy

T. scotoductus SA-01 cells exposed to 1.25 mM of U(VI) were harvested in late exponential growth phase and analyzed by transmission electron microscopy (TEM). The cells were subjected to fixation, dehydration and polymerization. Blocks were trimmed using an ultramicrotome and then cut into thin sections of approximately 0.2 μm. Sections were collected on a copper grid, subjected to lead staining and Electron micrographs taken with a Philips CM 100 (The Netherlands) TEM. Elemental analysis was performed with energy dispersive spectroscopy (EDS) attached to a Shimadzu SSX550 SEM (Van Wyk and Wingfield, 1991).

2.5. Purification and identification of the U(VI) reductase

Harvested cells of *T. scotoductus* SA-01 were fractionated into subcellular fractions as described by Kauffmann and Lovley (2001). Separation of the spheroplast from the periplasmic fraction was obtained by centrifugation (20 000*g*, 30 min). The spheroplasts were resuspended in 20 mM MOPS buffer, pH 7.0. To separate the membrane fraction from the cytoplasmic fraction, the supernatant was centrifuged (100 000*g*, 90 min). The extracted membrane, periplasmic and cytoplasmic fractions were all dialyzed against 20 mM MOPS buffer, pH 7.0, with Snakeskin®Pleated Dialysis Tubing (10 000 MWCO) at 4 °C with 3 × 2 L buffer changes (Gaspard et al., 1998).

Both the periplasmic and membrane fractions displayed U(VI) reduction, determined as previously described. These factions were combined and applied to a Super-Q Toyopearl (8 cm × 2.8 cm) column previously equilibrated with 20 mM MOPS buffer, pH 7.0. A salt gradient of 0–1.0 M NaCl at a flow rate of 5 mL min^{-1} was used to elute bound proteins that were evaluated for U(VI) reduction activity. Dialyzed (against 20 mM MOPS buffer, pH 7.0) active fractions were pooled and the dialyzate was applied to a SP Toyopearl (8 cm × 2.8 cm) column previously equilibrated with 20 mM MOPS buffer, pH 7.0. A salt gradient of 0–1.0 M NaCl at a flow rate of 5 mL min^{-1} was used to elute bound proteins and assayed for activity. SDS–PAGE electrophoresis was performed on active fractions (Laemmli, 1970).

2.6. N-terminal sequencing and mass spectrometry

Following results from the ion exchange chromatography, activity assays and reducing SDS–PAGE, a purified PVDF blotted gel band of ±70 kDa protein was sent to the Protein Chemistry Facility of the Centro de Investigaciones Biológicas (CSIC; Madrid, Spain). N-terminal sequencing was performed by automated Edman degradation with an Applied Biosystems 477A gas-phase sequencer (Foster City, California). A second analysis was performed by MS/MS analysis. The band in question was excised, lyophilized and sent to the Centre for Proteomic and Genomic Research (Cape Town, South Africa) for on an ESI/MALDI QToF, ToF/ToF (Agilent, Applied Biosystems) (Wattenberg et al., 2002). The protein was digested with trypsin before being analysed by mass spectrometry. A total of nine tryptic digest fragment spectra were obtained from the MS/MS as .T2D files, these were analyzed and used to generate peak lists using the Mascot Distiller (Matrix Science) software.

2.7. Expression of a recombinant T. scotoducts SA-01 'reductase'

Oligonucleotides for the mature protein were designed using the protein sequencing results as well as the gene sequence obtained from the *T. scotoductus* SA-01 genome database. The latter was completed using a combination of GS20 and FLX-pyrosequencing (GenBank: CP001962). The recombinant 'reductase' was expressed in Rosetta-Gami 2(DE3)pLysS (Novagen, Madison, WI) cells containing the plasmid, pET-28 b (+) (Novagen), in which the gene for the wild-type enzyme was incorporated. The mutants, Cys^{337} to Ala, Cys^{481} to Ala and $Cys^{337\&481}$ to Ala were also recombinantly expressed using pET-28 b (+), all in the same *Escherichia coli* strain. Mutants of the protein were generated with the Phusion® *Site-Directed Mutagenesis* Kit, and all mutants were verified by DNA sequencing. The transformants were inoculated into kanamycin-containing (30 μg mL^{-1}) LB media and cultured until an OD_{600} of 0.8 was reached before IPTG was added to a final concentration of 1 mM to induce expression. The cells were incubated a