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(54) **Title**: ISOLATION OF A PROTEIN RESPONSIBLE FOR URANIUM (VI) REDUCTION

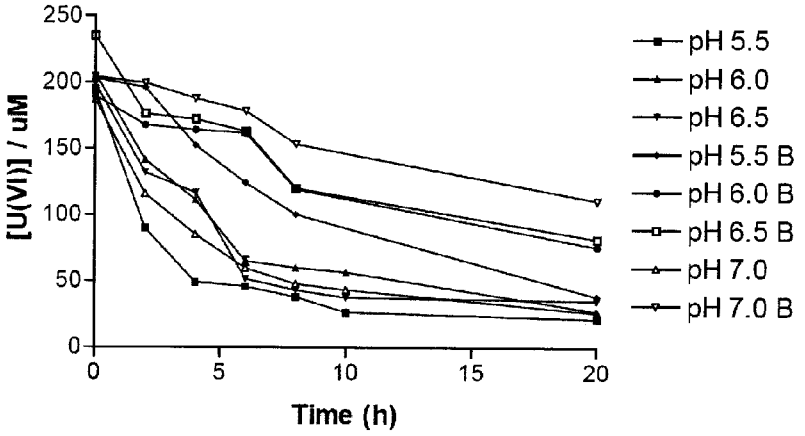


FIGURE 8

(57) **Abstract**: The present invention relates to the isolation and characterization of a protein responsible for the reduction of uranium (Vl) to uranium (IV) The present invention extends to the use of the isolated protein in the reduction of uranium (Vl) to uranium (IV) and further extends to a process for the bioremediation, or at least partial remediation, of a site contaminated with a source of U (Vl) According to a first aspect thereof, the present invention provides an isolated polypeptide derived from Thermus scotoductus strain SA-01 that is responsible for the reduction of uranium (Vl), in a source of uranium (Vl), to uranium (IV)1 wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 1.

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ISOLATION OF A PROTEIN RESPONSIBLE FOR URANIUM (VI) REDUCTION

Technical Field

The present invention relates to the isolation and characterization of a protein responsible for the reduction of uranium (VI) to uranium (IV). The present invention extends to the use of the isolated protein in the reduction of uranium (VI) to uranium (IV) and further extends to a process for the bioremediation, or at least partial remediation, of a site contaminated with a source of U (VI).

Background to the Invention

In the past decade, our concept of what conditions are compatible with life, have changed significantly. The earlier, anthropocentric view of nature has limited our capacity to access new microbes and their genomes, but the discovery that almost all environments on earth and even the subsurface (more than 4 km), or subzero temperatures and high levels of radiation are likely to contain specially adapted life forms, has made the notion to understand the microbial biodiversity very important.

One of the most amazing features of the microbial world is that even the most toxic and apparently recalcitrant of substances developed by (chemical) industry over the decades usually prove to be degradable by one micro-organism or another. Microorganisms can encounter a large variety of chemicals such as metals in contaminated environments, thus it is not surprising that they would interact with these metals (Nies and Sliver, 1995).

U (VI) resistant bacteria isolated from contaminated environments have been shown to possess the ability to successfully remove toxic U (VI) from the environment by either reduction (generally by bacteria) or biosorption (usually by fungi) (van Heerden *et al.*, 2008).

Recently, the microbial reduction of metals has attracted interest as these transformations can play crucial roles in the cycling of both inorganic and organic species and therefore have opened up new and exciting areas of research with potential practical application (Anderson *et al.*, 1998; Rooney-Varga *et al.*, 1999; Lovley and Lloyd, 2000; Anderson *et al.*, 2003; Lovley *et al.*, 2004). Dissimilatory metal reducing bacteria (DMRB) have been shown to gain energy to support anaerobic growth by coupling the oxidation of H_2 or organic matter to the reduction of a variety of multivalent metals. This metabolism can lead to the complete mineralization of organic matter or

to the precipitation and immobilization of metal contaminants under anaerobic conditions (Sani *et al.*, 2002).

For the bioremediation of uranium contaminated sites, the chemistry of the element offers an approach that has received much attention in the last 20 years. The oxidation state of uranium is crucial to its stability, mobility and bioavailability. The oxidized or hexavalent, (VI), state of uranium is highly soluble and therefore mobile, while the reduced or tetravalent, (IV), state is relatively insoluble. In waste, uranium is present primarily as soluble salts of the uranyl ion (UO_2^{2+}). When the uranyl ion is reduced from the U (VI) oxidation state to a lower oxidation state such as U (IV), the solubility decreases and it becomes immobilized.

The list of bacteria known in the art to reduce U (VI) is growing. When *Thermus scotoductus* SA-01 is incubated anaerobically with U (VI), U (VI) will precipitate out of solution indicating that *Thermus scotoductus* SA-01 has the ability to reduce U (VI). Studies have also shown that *Thermus scotoductus* SA-01 has the ability to reduce almost 100% of a 0.25 mM U (VI) solution under anaerobic conditions with lactate as an electron donor in less than 30 hours (van Heerden *et al.*, 2008).

However, very little is known about the mechanisms involved in U (VI) reduction and the proteins involved in these mechanisms and accordingly conclusive evidence as to which protein(s) are responsible for uranium reduction is still lacking.

For purposes of the present specification, "polypeptide" is understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

Summary of the Invention

According to a first aspect thereof, the present invention provides an isolated polypeptide derived from *Thermus scotoductus* strain SA-01 that is responsible for the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV), wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 1.

The isolated polypeptide is characterized in that it is a homogenous protein, having a molecular mass of 70 kDa, as shown by SDS-PAGE gel analysis.

The invention further provides for the isolated polypeptide to be a peptide ABC transporter, peptide-binding protein, as revealed by NCBI BLASTP analysis.

The Applicant believes that the isolated polypeptide identified herein is capable of performing more than one function, namely that of a peptide ABC transporter, peptide-binding protein and that of uranium reductase. Such proteins are commonly referred to in the art as "moonlighting proteins".

The isolated polypeptide identified herein possesses a disulphide bond, which when cleaved by a reducing agent, supplies a nucleation site for U (VI) reduction.

According to a second aspect thereof, the present invention provides isolated nucleic acid molecules coding for the amino acid sequence of SEQ ID No: 1 comprising a nucleotide sequence of SEQ ID No: 2.

For ease of reference, the amino acid sequence and nucleotide sequence referred to in this description and contained in the sequence listing filed herewith are also set out below. The underlined amino acids set forth in SEQ ID No: 1 represent the N-terminal amino acid sequence which was used to identify the polypeptide as a peptide ABC transporter, peptid-binding protein.

SEQ ID No: 1-

MetArgLysValGlyLysLeuAlaValPheGlyLeuAlaAlaLeuGlyLeuAlaLeuAlaGlyProGlnAspAsnSerLeuV
alIleGlyAlaSerGlnGluProArgValLeuAlaGlyAspPheLeuSerIleIleSerAsnGlnSerIleLysLeuGluIleGlu
GlnTyrLeuPheAlaProLeuIleGlyPheAsnAlaAsnSerGluAsnPheProValLeuValThrGluValProThrArg
GlnAsnGlyArgLeuArgValThrAspIleGlyGlyGlyLysLysArgLeuGluMetAspLeuThrIleArgProAspAlaArg
TrpSerAspGlyLysProIleThrThrGluAspValAlaPheTyrTyrGluValGlyLysAlaLysGlyMetProValLeuAsn
ProAspTyrTrpGluArgValAsnLeuArgValArgAspAlaArgAsnPheThrValIlePheGluProAlaTyrTyrTyr
AspThrTyrGlyGlyThrTyrGlySerProIleGlyTyrAlaProLysHisIleMetGlyAlaGluTrpGluLysValLysAlaAla
AlaArgAsnLeuAspProAspLysAspAlaGluArgLeuAsnGluLeuTyrArgAsnPhePheLeuLysPheAlaThr
ProGlnAlaLeuAsnArgGlyAlaMetValTyrSerGlyAlaPheLysLeuArgArgTrpValProGlyAsnSerIleGluMet
GluArgAsnProAsnPheProIleLysProGluGlyGlyGluSerArgTyrValGlnArgValValTyrArgPheIleGlnAsn
ThrAsnSerLeuLeuValAlaValLeuGlyGlySerIleAspAlaThrSerSerValSerLeuThrPheAspGlnGlyArg
SerArgGlnLeuThrSerArgAlaProGlyArgPheAspIleTrpPheValProGlyAlaIleTrpGluHisIleAspValAsnL
ysPheGluAsnCysGlnAlaValArgAspLeuGlyLeuAsnAspValArgThrArgArgAlaLeuLeuHisAlaLeuAsn
ArgGluGlyLeuValLysAlaPhePheAspGlyLeuGlnProValAlaHisThrTrpIleAlaProValAsnProLeuPheAs
nProAsnValArgLysTyrGluPheAspLeuLysLysAlaGluAlaLeuLeuAlaGluMetGlyTrpArgLysGlyProAsp
GlyIleLeuGlnArgThrValGlyGlyArgThrValArgPheGluIleGluPheValThrThrAlaGlyAsnAlaIleArgGluAr
gThrGlnGlnPhePheAlaGluAspLeuLysLysIleGlyIleAlaValLysIleAsnAsnAlaProSerAlaValValPheAla
AspAspTyrIleGlnArgAlaSerGluCysLysTrpThrGlyLeuPheGluPheAlaTrpValSerAsnLeuAlaGluAspG
lySerLeuPheGlnTyrLysAsnLeuAsnThrGlyAlaIleMetValProThrLysGluAsnAsnTyrGlnGlyGlnAsnIle
GlyGlyTrpArgAsnAspGluPheAspArgLeuThrSerGlnGlyValLeuGluPheAspGluAlaArgArgLysGlnLeu
PheTrpArgAlaGlnGluIleTrpAlaGluGluLeuProAlaLeuProLeuTyrPheArgAlaAsnProTyrValValArg
LysGlyLeuValAsnTyrValAlaSerAlaTyrAlaGlyGlyTyrGlyTyrProGlyTrpAsnAlaTrpGluIleGlyTrpGluSe
rArgGlyAlaValLysLysTrpAspGlnAlaLysTyrAlaLeuSerValLys

SEQ ID No: 2-

ATGAGAAAAGTAGGCAAGCTGGCTGTATTCGGTTTAGCCGCCCTGGGCTTGGCCCTGGCG
GGGCCCCAGGACAACAGCCTGGTCATAGGGGCTTCGCAGGAGCCCCGGGTTCTGGCGGG
GGACTTCCTAAGCATCATCTCCAACCAGTCCATCAAGTTGGAGATCGAGCAGTACCTCTTC
GCCCCCCTCATCGGTTTCAACGCCAACAGCGAAAACTTTCCCGTGCTGGTCACCGAGGTG
CCCACCCGGCAAAACGGGCGTTTGCGGGTGACGGACATCGGCGGGGGCAAGAAGCGCTTG
GAGATGGACCTCACCATCCGGCCCGATGCCCGCTGGTCCGACGGCAAGCCCATCACCACC
GAGGATGTGGCCTTCTACTACGAGGTGGGCAAGGCCAAGGGGATGCCGGTGCTCAACCCG
GACTACTGGGAGCGGGTGAACCTCCGGGTCAGGGACGCCCGCAACTTCACCGTGATCTTT

GAGCCCGCCTACTACTACGACACCTACGGCGGCACCTACGGCTCCCCCATCGGCTACGCT
CCCAAGCACATCATGGGCGCCGAGTGGGAGAAGGTGAAAGCGGCGGCCCGGAACCTGGAT
CCCGATAAGGATGCGGAGAGGCTCAACGAGCTCTACCGCAACTTCTTCCTCAAGTTCGCC
ACTCCCCAGGCCCTAAACCGGGGAGCCATGGTCTACTCGGGGGCCTTCAAGCTGCGGCGC
TGGGTGCCGGGGAACTCCATTGAGATGGAGCGGAACCCCAACTTCCCCATCAAGCCCGAG
GGTGGGGAGAGCCGGTACGTGCAGAGGGTGGTCTACCGCTTCATCCAGAACACCAACTCC
CTCCTGGTGGCCGTCCTGGGCGGGAGCATTGACGCCACCTCCAGCGTCTCCCTCACCTTT
GACCAAGGCCGTAGCCGCCAGCTCACCTCCCGGGCCCCTGGCCGCTTTGACATCTGGTTC
GTGCCCGGGGCCATCTGGGAGCACATTGACGTCAACAAGTTTGAGAACTGCCAGGCGGTC
CGCGACTTGGGCCTGAACGACGTCCGCACCCGTCGGGCCCTCCTCCACGCTCTGAACCGC
GAGGGGTTGGTCAAGGCCTTCTTTGACGGCCTCCAGCCCGTGGCCCACACCTGGATCGCC
CCCGTCAACCCCCTCTTCAACCCCAATGTGCGGAAGTACGAGTTTGACCTGAAGAAGGCG
GAGGCGCTCTTGGCGGAGATGGGCTGGAGGAAGGGGCCGGACGGCATCCTTCAGCGCAC
CGTGGGTGGCCGCACCGTGCGCTTTGAGATTGAGTTCGTCACCACCGCGGGCAACGCTATC
CGGGAGCGCACCCAGCAGTTCTTCGCCGAGGACCTGAAGAAGATCGGCATCGCCGTCAAG
ATCAATAACGCCCCCAGCGCCGTGGTCTTCGCCGACGACTACATCCAGCGGGCCAGCGAG
TGCAAGTGGACCGGGCTGTTTGAGTTCGCTTGGGTTTCCAACCTGGCCGAGGATGGCTCC
CTCTTCCAGTACAAGAACCTGAACACCGGGGCCATCATGGTGCCCACCAAGGAGAACAAC
TACCAGGGGCAGAACATCGGCGGCTGGCGCAACGACGAGTTTGACCGTCTGACGAGCCAG
GGTGTCCTGGAGTTTGACGAGGCCAGGCGGAAGCAGCTCTTCTGGAGGGCCCAGGAGATC
TGGGCCGAGGAGCTGCCTGCCTTGCCCCTCTACTTCCGCGCTAACCCCTACGTGGTGCGG
AAGGGCCTGGTCAACTACGTGGCCAGCGCTTACGCGGGCGGCTACGGTTACCCCGGCTGG
AACGCTTGGGAGATCGGCTGGGAGAGCCGCGGCGCCGTGAAGAAGTGGGACCAGGCGAA
GTACGCTCTTTCCGTCAAGTAA

In an embodiment of the invention, the polypeptide indentified herein is isolated from a culture of *Thermus scotoductus* strain SA-01, recovered and purified. Exemplary procedures suitable for such recovery and purification include column chromatographic methods and size exclusion techniques of the type known and described in the art. In an embodiment thereof, the present invention provides for purification of the protein to be achieved by nickel affinity column chromatography followed by gel filtration. In an alternative embodiment thereof, the present invention provides for the protein to be purified by heat denaturation followed by gel filtration.

In an alternative embodiment, the polypeptide of SEQ ID No: 1 is produced recombinantly by expressing the nucleotide sequence of SEQ ID No: 2 encoding the polypeptide in a host cell. With

the aid of an expression vector, the nucleic acid molecules containing the nucleotide sequences of SEQ ID No: 2 may be transfected and expressed in a host cell.

Thus the present invention also relates to vectors that include the nucleotide sequence of SEQ ID No: 2, host cells that are genetically engineered with one or more recombinant expression vectors, and the production of the polypeptide of SEQ ID No: 1 as indentified herein by recombinant techniques as is well known in the art.

The present invention further provides a method for producing at least one polypeptide, as indentified herein, that is responsible for the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV), the method including the steps of:

a) transfecting the nucleic acid molecules of SEQ ID NO: 2 into a host cell;
b) culturing the host cell so as to express the polypeptide of SEQ ID NO: 1 in the host cell; and
c) optionally, isolating and purifying the polypeptide of SEQ ID NO: 1.

Also according to the invention, there is provided a microorganism transformed with a U (VI) resistant gene obtained from the host cell described above, or any U (VI) resistant functional part thereof.

For purposes of the present invention, uranium reductase activity is determined by measuring a decrease in hexavalent uranium. Uranium reductase activity is measured spectrophotometrically using 2-(5-bromo-2-pyrdulazo)-5-diethylaminophenol.

According to a third aspect thereof, the present invention provides a process for the bioremediation, or at least partial bioremediation, of a site contaminated with a source of U (VI), the process comprising the steps of introducing an electron donor to the contaminated site in order to stimulate the proliferation of *Thermus scotoductus* strain SA-01 to reduce the U (VI) in the source of U (VI) present therein, to U (IV), or the step of removing environmental media from a U (VI) contaminated site and introducing an electron donor to such environmental media for a sufficient period of time so as to allow *Thermus scotoductus* strain SA-01 to reduce U (VI), in the source of U (VI) present therein, to U (IV).

In an embodiment of the instant invention, *Thermus scotoductus* strain SA-01 is derived from the Mponeng mine located on the north-western rim of the Witwatersrand Basin in the North West Province of South Africa which mine is operated by Western Deep Levels, Inc., or from environmental media obtained from this site.

For purposes of the present specification, the term "environmental media" denotes solid and liquid wastes, soils, sediments, water bodies, or a combination of one or more thereof.

In an embodiment of the present invention, the source of U (VI) is selected from the group consisting of $UO_2(CH_3COO)_2.2H_2O$ and $UO_2(NO_3)_2$. It will be appreciated that the source of uranium of the present invention is not limited to the foregoing and accordingly may be any suitable source of hexavalent uranium.

In one embodiment of the present invention, reduction takes place under aerobic and/or anaerobic conditions. Preferably, reduction takes place under anaerobic conditions so as to prevent the reduced U (IV) from being oxidized to U (VI).

As mentioned herein before, the reduction of U (VI) to U (IV) is initiated by an electron donor. It will be appreciated that the electron donor may be any suitable electron donor of the type known and described in the art. In one embodiment of the invention, the electron donor is selected from the group consisting of H_2, reduced quinone (in particular hydroquinone), acetate, lactate, citric acid, and pyruvate.

The aforesaid process can be employed for the bioremediation, or at least partial bioremediation, of a site contaminated with a source of hexavalent U that can be practiced *in situ*, *ex situ*, or both.

According to a fourth aspect thereof, the present invention provides for the use of an isolated polypeptide of SEQ ID No: 1, as identified and characterized herein, in the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV).

Furthermore, the present invention provides for the use of *Thermus scotoductus* strain SA-01 in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI) contaminated environmental media.

The invention provides further for the use of *Thermus scotoductus* strain SA-01 in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI)

contaminated environmental media, wherein said *Thermus scotoductus* strain SA-01 is derived from the U (VI) contaminated site or the U (VI) contaminated environmental media that is to be remediated, or at least partially remediated.

Also according to the invention, there is provided the use of a microorganism transformed with a U (VI) resistant gene obtained from the host cell described above, or any U (VI) resistant functional part thereof, in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI) contaminated environmental media.

These and other objects, features and advantages of the invention will become apparent to those skilled in the art following the detailed description of the invention.

Brief Description of the Drawings

Figure 1: is a graph depicting growth curves for *Thermus scotoductus* in different concentrations of uranium. TYG medium (■) amended with 0.25 mM (▲), 0.5 mM (▼), 0.75 mM (♦), 1.0 mM (●), 1.25 mM (□) and 1.5 mM (△) U(VI) during inoculation (t = 0);

Figure 2: is a graph depicting a standard curve of the optical density reading of the specific diluted uranium sample *vs* the known U (VI) concentration for the specific diluted uranium sample;

Figure 3: is a graph showing the reduction of uranium (VI) by *T. scotoductus* SA-01 under non-growth conditions. Cells harvested in late exponential phase with assay solution containing 0.25 mM U(VI) and 10 mM lactate as electron donor (■), control assay solution of cells harvested in late exponential phase containing 0.25 mM U(VI) and no electron donor (▲), cells harvested in early exponential phase with assay solution containing 0.25 mM U(VI) and 10 mM lactate as electron donor (▼), control assay solution of cells harvested in early exponential phase containing 0.25 mM U(VI) and no electron donor (♦), cells harvested in late exponential phase with assay solution containing 0.25 mM U(VI) and 10% hydrogen as electron donor (●), control assay solution with autoclaved cells with no electron donor (□), control assay solution lacking cells with 10 mM lactate (△) and 10% hydrogen (▽) as electron donors;

Figure 4: is a graph depicting uranium (VI) reduction activity of the combination of the membrane and periplasmic fractions from *T. scotoductus* SA-01 after dialysis and being purged with 10% H_2 gas and hydroquinone as electron donors;

Figure 5: is a graph depicting an elusion profile for Super-Q Toyopearl pertaining to uranium (VI) reduction activity;

Figure 6: is a graph depicting an elusion profile for SP Toyopearl pertaining to uranium (VI) reduction activity;

Figure 7: is a SDS-PAGE gel analysis depicting the isolated uranium reductase protein;

Figure 8: is a graph depicting the reduction of uranium (VI) at different pH values. pH values (■) 5.5, (▲) 6.0, (▼) 6.5, (△) pH 7.0, (♦) 5.5 protein free control, (●) pH 6.0 protein free control, (□) pH 6.5 protein free control, (▽) pH 7.0 protein free control;

Figure 9: is a graph depicting the reduction of uranium (VI) at different pH values. pH values (▲) 7.5, (■) 8.0, (▼) 8.5, (♦) 9.0, (□) pH 7.5 protein free control, (●) pH 8.0 protein free control, (△) pH 8.5 protein free control, (▽) pH 9.0 protein free control; and

Figure 10: is a graph depicting the reduction of uranium (VI) at 55 °C (■) and 65°C (▲) with the blank rate subtracted.

The presently disclosed subject matter will now be described more fully hereinafter with reference to the accompanying Examples, in which representative embodiments are shown. The presently disclosed subject matter can, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the embodiments to those skilled in the art.

Examples

The invention was performed in accordance with the following steps.

Growth of Thermus scotoductus SA-01

The thermophilic bacterium, *Thermus scotoductus* strain SA-01, employed herein, was isolated in 1999 by Kieft *et al.* from groundwater sampled at a depth of approximately 3.2 km in the Mponeng gold mine which is located on the north-western rim of the Witwatersrand Basin in the North West Province of South Africa operated by Western Deep Levels, Inc.

Thermus scotoductus SA-01, deposited under ATCC Accession No. 700910; American Type Culture Collection, was plated out from a glycerol stock on solid TYG medium and allowed to grow for 24 hours at 65°C. This was then replated again on solid TYG medium and allowed to grow for 24 hours at 65°C. A pre-inocculum was then prepared by inoculating a loop of growth from the plate in 50 ml of liquid TYG medium. This was grown for 8 hours at 65°C after which 10 ml of growth was transferred to 90 ml of liquid TYG medium. This was then grown for 8 hours at 65°C after which 5 ml of growth was transferred to 95 ml of liquid TYG medium.

8 hours was determined beforehand to be the time needed for the organism to grow to the late exponential phase.

As can be seen from Figure 1, *Thermus scotoductus* SA-01 has the ability to grow in uranium concentrations of up to 1.25 mM. However, as can further be seen from said Figure, an increase in the concentration of uranium above 1.25 mM leads to a decrease in the ability of *Thermus scotoductus* SA-01 to grow effectively.

Spectrophotometric determination of U (VI)

All reagents were of analytical reagent grade. Deionized distilled water was used for the preparation of standard solutions. 100 mM was prepared by dissolving $UO_2(CH_3COO)_2.2H_2O$ in water. The prepared solution was stored in the dark and used for sequential dilution. 5-Br-PADAP was used to prepare a 0.05% solution by dissolving the reagent in ethanol. The complexing ligand solution (pH 7.8) was prepared by dissolving 1 g of NaF and 13 g of sulphosalicyclic acid in 40 ml water, the pH was then adjusted with NaOH and the solution was diluted to 100 ml. The buffer solution (pH 7.8) was prepared by diluting 14 g of TEA in 80 ml of water, the pH was then adjusted with perchloric acid, and the solution was left to stand overnight. Before use, the pH of the buffer solution was adjusted to 7.8 with perchloric acid and the solution diluted to 100 ml. All optical density measurements were made on a Spectronic® Genesys™ 5 at 600 nm (Johnson and Florence, 1971).

U (VI) dilutions were made by diluting the stock solution with water. 100 µl of the U (VI) dilution was taken in a 1.5 ml eppendorf tube containing 25 µl of complexing solution. To the above, the 100 µl of the buffer solution and 80 µl of Br-PADAP solution was added and made up with 620 µl ethanol and 75 µl water. This coloured solution was allowed to stand for about 2 h and absorbance was measured at 578 nm against a reagent blank (Johnson and Florence, 1971).

A standard curve was constructed, as depicted in Figure 2, by plotting the optical density reading of the specific diluted sample *vs* the known U (VI) concentration for the specific diluted sample.

U (VI) reduction by Thermus scotoductus SA-01 under non-growth conditions

T. scotoductus SA-01 cells were harvested from growth standardized inoculum. The cells were washed three times with 20 mM Tris-HCl buffer, pH 7.0, and the cell suspension purged with O_2-free N_2. To initiate the assay, a sample of the culture was added to a tube containing the assay solution (uranyl acetate in Tris-HCl buffer, pH 7.0, plus Na lactate as electron donor) to a final concentration of 0.25 mM U (VI) and 10 mM electron donor and subjected to analysis as described below. This was all done in an anaerobic chamber to prevent reduced U (IV) from being oxidized to U (VI). Along with the cell-free control, an electron donor free control was also prepared.

Uranium reductase activity was determined by measuring the decrease of hexavalent uranium. U (VI) was analyzed spectrophotometrically using 2-(5-bromo-2-pyrdulazo)-5-diethylaminophenol (Johnson and Florence, 1971).

When *Thermus* SA-01 is incubated anaerobically with U (VI), U (IV) will precipitate out of solution as indicated by a black precipitate (Haas and Northop, 2004) formed in the cell pellet. Most of the uranium (VI) was transformed to U (IV) in under 20 hours, as can be seen in Figure 3, which coincides with what was previously described in literature (Kieft *et al.*, 1999).

It was observed that even without an electron donor, the cells were still able to reduce the hexavalent uranium. No chemical reduction was observed in the cellfree control indicating that the reduction has to be due to cellular activity.

After the experiment was completed, the cells used were exposed to oxygen overnight which led to the disappearance of the black precipitate. Uranium (VI) determination was also done after the exposure to air and it was found that most of the U (IV) in the sample was oxidized to U (VI). This indicates that the reduction of U (VI) to U (IV) occurred since if uranium complexes, for instance U (VI) phosphate-type complexes, were formed by bioprecipitation or bioaccumulation, it would not have resulted in the reappearance of U (VI) under aerobic conditions.

Uranium (VI) reduction with different electron donors

To determine the most effective electron donor for whole cell uranium (VI) reduction, various reduction assays were performed with electron donors determined to be most relevant. In this regard, H_2, reduced quinone (particularly hydroquinone), acetate, lactate, citric acid, and pyruvate were employed. It was observed that whilst each electron donor is capable of producing a set amount of reducing equivalents (some electron donors more than others), uranium (VI) reduction is not directly coupled to the produced reducing equivalents. Accordingly, it can be concluded that the specific electron donor does not have any significant effect on uranium (VI) reduction.

Uranium (VI) reduction at different pH and temperature values

Whole cell uranium (VI) reduction under non-growth conditions was performed over a pH range of 5 to 9. The whole cell uranium (VI) reduction activity seemed to prefer a more neutral pH with maximum activity being observed at a pH of between 7 and 8. Very low reaction rates were

observed at pH values below 7. Since all appropriate controls were evaluated, this might be due to the fact that the assay does not function in this pH region.

The optimum temperature for whole cell uranium (VI) reduction was determined over a range of 35°C to 75°C. It was observed that temperature did not seem to have any significant effect on whole cell reduction.

Preparation of subcellular fractions

Subcellular fractions were prepared as described by Kaufmann and Lovley (2001). *T. scotoductus* SA-01 cells were harvested from growth standardized inoculum at 8 h of growth and washed three times with 50 mM Tris-HCl buffer, pH 7.8. Cells were then resuspended in 50 mM Tris-HCl buffer, pH 7.8, containing 25 % (w/v) sucrose. To accomplish cell wall lysis, lysozyme (20 mg) was added to the cell suspension (approximately 1 g wet weight) and stirred for 20 min at 37°C. Na_2-EDTA was added to a final concentration of 5 mM and stirred for another 15 min at 37°C. Finally $MgCl_2$ was added to a final concentration of 13 mM and the suspension was stirred for 15 min at 37°C. Separation of the spheroplast from the periplasmic fractions was obtained by centrifugation (20 000 x g, 30 min). Spheroplasts were resuspended in 50 mM Tris-HCl buffer, pH 7.8.

To obtain the membrane and cytoplasmic fraction, the protocol as described by Gaspard *et al.*, (1998), was used. DNAse and RNAse was added to final concentrations of 5 µg/ml and 10 µg/ml respectively as well as protease inhibitors and the cells were broken by ultrasonic treatment (3 times, 75 W, 5 min) with a sonifier (Branson Sonic Power Cell Disruptor B-30) in an ice-water bath. The suspension was then centrifuged (4000 x g, 10 min) at 4°C to remove cellular debris. To separate the membrane fraction from the cytoplasmic fraction, the supernatant was centrifuged (100 000 x g, 90 min). The pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.8.

The membrane, periplasmic and cytoplasmic fractions were all dialysed against 20 mM MOPS buffer, pH 7.0, with Snakeskin®Pleated Dialysis Tubing (10 000 MWCO) at 4°C with 3 x 2L buffer changes.

Determination of U (VI) reduction activity in subcellar fractions

With H_2 and reduced quinones as electron donors

The periplasmic and membrane fractions were subjected to uranium (VI) reduction experimentation as described above with the exception that the fractions were purged with O_2-free $N_2/H_2/CO_2$ (90%/10%/10%) mixed gas and supplemented with 2 mM hydroquinone to introduce 10% H_2 and reduced quinones as electron donors. To initiate the assay, uranyl acetate was added to a sample of the fraction to a final concentration of 0.25 mM U (VI) and subjected to analysis. This was all done in an anaerobic chamber to prevent reduced U (IV) from being oxidized to U (VI).

The combination of the periplasm and membrane fractions was screened for U (VI) reduction activity, since this combination has shown the most promise pertaining to the presence of the uranium reductase. Hydroquinone and H_2 were utilized as electron donors as these are the electron donors preferred from literature for this type of protein.

A graph was then constructed, as shown in Figure 4, depicting uranium (VI) reduction activity of the combination of the membrane and periplasmic fractions from *T. scotoductus* SA-01, after dialysis and being purged with 10 % H_2 gas and hydroquinone as electron donors.

As can be seen from Figure 4, a combination of the H_2 and reduced quinones delivered the best reduction activity. Furthermore, a blackish-yellow precipitate was formed. Since it has been shown that proteins in the periplasm precipitates out a yellow U (VI) precipitate, it is no surprise that the formed precipitate was not completely black.

Optimization of the method for the isolation of the protein of interest by chromatographic methods

Extraction of ionically bound membrane proteins

The membrane pellet was resuspended in 20 mM MOPS buffer, pH 7.0, by stirring overnight at 5°C. The volume of the membrane fraction was doubled with 1 M KCl in 20 mM MOPS buffer, pH 7.0. The solution was then stirred for 2 h at room temperature to extract the peripheral membrane proteins and the resulting suspension was ultra-centrifuged at 100 000 *x g* for 90 min at 4°C to

pellet the membrane. The KCl extractable membrane fraction (the supernatant from the centrifugation step) was dialyzed against a 20 mM MOPS buffer pH 7.0 at 5°C with 3 x 2L buffer changes.

Isolation of the membrane/periplasmic fractions

The dialyzed KCl extracted membrane fraction in combination with the periplasmic fraction was applied to a Super-Q Toyopearl (8 cm x 2.8 cm) column previously equilibrated with 20 mM MOPS buffer, pH 7.0. The columns were washed with 20 mM MOPS buffer, pH 7.0, until the $A_{280\ nm}$ readings were less than 0.01. A salt gradient of 0 – 1.0 M NaCl at a flow rate of 5 ml/min was used to elute proteins.

Fractions determined to be active for uranium (VI) reduction activity were pooled and dialyzed against 20 mM MOPS buffer, pH 7.0, with Snakeskin®Pleated Dialysis Tubing (10 000 MWCO) at 4°C.

A graph depicting the elusion profile for the Super-Q Toyopearl was then constructed, as shown in Figure 5. The circled peaks in said Figure indicate the produced uranium (VI) reduction activity. The circled peaks were pooled and applied to the SP Toyopearl after dialysis.

The dialysate was applied to a SP Toyopearl (8 cm x 2.8 cm) column previously equilibrated with 20 mM MOPS buffer, pH 7.0. The column was washed with 20 mM MOPS buffer, pH 7.0, until the $A_{280\ nm}$ readings were less than 0.01. A salt gradient of 0 – 1.0 M NaCl at a flow rate of 5 ml/min was used to elute proteins. Selected fractions were collected (10 ml) and tested for uranium (VI) reduction activity.

A graph depicting the elusion profile for the SP Toyopearl was thereafter constructed, as shown in Figure 6. Arrow A represents fraction 11, arrow B represents fraction 16, arrow C represents fraction 19, and arrow D represents fraction 33. From this Figure, it can be seen that fractions 16, 19 and 33 indicate uranium (VI) reduction activity.

SDS-PAGE gel analysis was done on the selected fractions from chromatographic separation on the SP Toyopearl resin. As shown in Figure 7, lane M represents the molecular mass marker proteins; lane 1 represents fraction 11; lane 2 represents fraction 16; lane 3 represents fraction 19; whilst lane 4 represents fraction 33. Accordingly, the resulting SDS-PAGE gel analysis indicated that the only protein present in all three of these fractions was the +/- 70 kDa protein.

Sequence determination of unknown protein

N-Terminal Sequencing

Fraction 19 was loaded onto a 10% SDS PAGE gel and run at 100V. After the run was completed the gel was blotted onto a PVDF membrane according to the manufacturer's specification. The blotted membrane was then stained with Coomassie Brilliant Blue and the band of interest was cut out and sent for sequencing.

The results obtained revealed that the N terminal sequence is XPXDNSLVIG

BLASTP analysis in the NCBI web using the "DNSLVIG" sequence resulted in 100% identity with the following:

(i) a dipeptide-binding protein from *Thermus thermophilus* HB27;

(ii) an extracellular solute-binding protein family 5 (ABC-type dipeptide transport protein) from *T. aquaticus* Y51MC23; and

(iii) an oligopeptide binding protein from *T. thermophilus* Hhb8.

All of these proteins were identical in sequence.

The sequences from the proteins obtained from NCBI BLASTP analysis were blasted against the *Thermus scotoductus* SA-01 draft genome and resulted in 100% identity with a peptide ABC transporter protein, peptide binding protein. Also a BLASTP analysis of the N terminal sequence against the draft genome sequence of *Thermus scotoductus* SA-01 resulted in 100% identity with a peptide ABC transporter, peptide-binding protein.

Therefore, XPXDNSLVIG represents the first residues of the mature sequence of the protein. After the BLASTP analysis, it can be deduced from the chromatogram of the N-terminal sequencing that the first amino acid corresponds to G and the third residue corresponds to Q.

In view of the foregoing, it was deduced that the N-terminal sequence is GPQDNSLVIG

From the aforesaid, it can be concluded that the protein involved in uranium reduction is the peptide ABC transporter, peptide-binding protein. It can also be concluded that said protein moonlights as uranium reductase.

MS/MS sequencing

Fraction 19 was loaded onto a 10% SDS PAGE gel and run at 100 V. After the run was completed, the gel band for the +/- 70 kDa protein was cut out and freeze dried. The freeze dried sample was then sent to the Centre for Proteomic and Genomic Research in Cape Town for MS/MS analysis. A total of nine tryptic digested fragment spectra were obtained which was analyzed using the Mascot Distiller software.

Four of the spectra could then be also annotated to the peptide ABC transporter, peptide-binding protein.

The aforegoing therefore serves to verify that the protein in question is the peptide ABC transporter, peptide-binding protein.

Protein expression and purification

Vectors pET28, containing the sequence for the peptide ABC transporter, peptide-binding protein were constructed using methods known in the art. pET28 contains the sequence for attaching a histidine rich area to the N-terminus of the protein, thus enabling the protein to be purified with a nickel affinity column.

ABC/pET28 was then transformed into Rosetta-Gami 2(DE3)pLysS competent cells and inoculated into a LB medium containing an antibiotic, KAN for pET28. The cells were then cultured and grown to an OD of between 0.8 – 1.0 and protein expression was induced with the addition of 1 mM (final concentration) IPTG.

Four hours after induction, the cells were harvested and washed. The cells were broken by passage through a French pressure cell and the cytoplasmic fraction was harvested by ultracentrifugation (100 000 x *g*, 4°C, 90'). The resultant protein from pET28 was then purified by a nickel affinity column followed by a gel filtration step.

Characterization of the recombinant ABC proteins

The recombinant protein was evaluated for the ability to reduce uranium (VI). The recombinant protein was characterized using the same physio-chemical parameters as used for characterizing the whole cells.

In order to demonstrate that the disulphide bond, present in the protein, provides a nucleation site for U (VI) reduction, a reducing agent needed to be applied in order to reduce the thiol moeity. Out of the possible reducing agents, namely dithiothreitol, sodium dithionite and β-mercaptoethanol that were assayed with uranium (VI), β-mercaptoethanol produced the lowest level of chemical reduction of uranium (VI).

An excess of β-mercaptoethanol was thus utilized to reduce the disulfide bond present in the protein before experimentation.

Generation of cysteine mutants of the peptide ABC transporter, peptide-binding protein

A homology model of the ABC transporter peptide-binding protein of *T. scotoductus* SA-01 was compiled using Yasara Structure & Whatif. The template used was the oligopeptide-binding protein of *T. thermophilus* HB8 (2D5W-B) which has a 90% identity (95% similarity) with the ABC transporter, peptide-binding protein of *T. scotoductus* SA-01.

Modelling of the protein revealed a disulphide bond which is present on the exterior of the protein, thereby supplying a possible nucleation site for U (VI) reduction once the bond has been reduced by the addition of a reducing agent such as β-mercaptoethanol.

The cysteine residues responsible for this bond are located at positions 337 and 481. Therefore, to probe the role of the cysteine residues in U (VI) reduction, the cysteine residues were each mutated to alanines by site-directed mutagenesis. A combination of both the two cysteine mutants was also devised.

The mutant proteins were thereafter purified using Ni-NTA affinity chromatography and were found to have greater than 98% purity on Coomassie-stained gels.

Other proteins with cysteine thiol-disulfide bridges have shown the ability to reduce U (VI), such as a thioredoxin from *Desulfovibrio desulfuricans* strain G20 (Li and Krumholtz, 2009).

U (VI) reduction by the cysteine mutants was thereafter performed in reactions containing 320 µg/ml of protein at pH 7 and 65°C.

Participation of the cysteine residues in enzymatic activity

Both the Cys-337 and Cys-481 mutants showed very little or negligible activity when compared to the wild-type. Also, the double mutant showed no significant activity. The results of these assays clearly indicate that Cys-337 and Cys-481 are required for U (VI) reduction.

The effect of pH on uranium (VI) reduction

In essence, reduction performed with a reduced disulfide bond can be seen as reduction by hydrogen sulphide. Hua and coworkers (2006) observed that the reduction of uranium (VI) by hydrogen sulphide happened optimally at neutral pH values and could best be represented by the following equation:

$$UO_2^{2+} + HS^- = UO_2 + S^0 + H^+ \qquad \text{(Hua } et\ al.\text{, 2006)}$$

The highest rate of reduction can be observed for pH values between 7 and 8 (Figures 8 and 9), which coincides with what was observed in literature for sulphide reduction (Hua *et al.*, 2006). At pH values below 7 and above 8, the rate of chemical reduction of uranium (VI) by β-mercaptoethanol in the samples lacking protein are very high. This might be due to pH inhibition of the reduction of the disulphide bond, producing increasing amounts of β-mercaptoethanol which are free to reduce the uranium (VI).

The effect of temperature on uranium (VI) reduction

The highest rate of reduction can be observed for temperature values between 55°C and 65°C (Figure 10), coinciding with the optimal growth temperature for the organism (Kieft *et al.*, 1999). At temperatures below 45°C, almost no activity was observed and above 65°C the rates of chemical reduction of uranium (VI) by β-mercaptoethanol in the samples lacking protein were very high.

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Claims

1. An isolated polypeptide derived from *Thermus scotoductus* strain SA-01 that is responsible for the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV), wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 1.

2. The polypeptide according to claim 1, wherein the polypeptide is characterized in that it is a homogenous protein, having a molecular mass of 70 kDa, as shown by SDS-PAGE gel analysis.

3. The polypeptide according to claim 1, wherein the polypeptide is a peptide ABC transporter, peptide-binding protein, as revealed by NCBI BLASTP analysis.

4. The polypeptide according to claim 1, wherein the source of U (VI) is selected from the group consisting of $UO_2(CH_3COO)_2.2H_2O$ and $UO_2(NO_3)_2$.

5. Isolated nucleic acid molecules coding for the amino acid sequence of SEQ ID No: 1 comprising a nucleotide sequence of SEQ ID No: 2.

6. A vector including the nucleic acid molecules according to claim 5.

7. A host cell including the vector according to claim 6.

8. A method for producing at least one polypeptide, according to any one of claims 1 to 4, that is responsible for the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV), the method including the steps of:
   a) transfecting the nucleic acid molecules of SEQ ID NO: 2 into a host cell;
   b) culturing the host cell so as to express the polypeptide of SEQ ID NO: 1 in the host cell; and
   c) optionally, isolating and purifying the polypeptide of SEQ ID NO: 1.

9. A microorganism transformed with a U (VI) resistant gene obtained from the host cell according to claim 7, or any U (VI) resistant functional part thereof.

10. A process for the bioremediation, or at least partial bioremediation, of a site contaminated with a source of U (VI), the process comprising the steps of introducing an electron donor to the contaminated site in order to stimulate the proliferation of *Thermus scotoductus* strain SA-01 to reduce the U (VI) in the source of U (VI) present therein, to U (IV), or the step of removing environmental media from a U (VI) contaminated site and introducing an electron donor to such environmental media for a sufficient period of time so as to allow *Thermus scotoductus* strain SA-01 to reduce U (VI), in the source of U (VI) present therein, to U (IV).

11. The process according to claim 10, wherein the *Thermus scotoductus* strain SA-01 is derived from the Mponeng mine located on the north-western rim of the Witwatersrand Basin in the North West Province of South Africa or from environmental media obtained from this site.

12. The process according to claim 10, wherein the source of U (VI) is selected from the group consisting of $UO_2(CH_3COO)_2.2H_2O$ and $UO_2(NO_3)_2$.

13. The process according to claim any one of claims 10 to 12, wherein reduction takes place under aerobic and/or anaerobic conditions.

14. The process according to claim 13, wherein reduction takes place under anaerobic conditions so as to prevent the reduced U (IV) from being oxidized to U (VI).

15. The process according to claim 10, wherein the electron donor is selected from the group consisting of H_2, reduced quinone (hydroquinone), acetate, lactate, citric acid, and pyruvate.

16. The process according to any one of claims 10 to 15, wherein the process is employed for the bioremediation, or at least partial bioremediation, of a site contaminated with a source of hexavalent U that can be practiced *in situ*, *ex situ*, or both.

17. Use of a polypeptide of SEQ ID No: 1 according to any one of claims 1 to 4 in the reduction of uranium (VI), in a source of uranium (VI), to uranium (IV).

18. Use of *Thermus scotoductus* strain SA-01 in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI) contaminated environmental media.

19. Use of *Thermus scotoductus* strain SA-01 in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI) contaminated environmental media, wherein said *Thermus scotoductus* strain SA-01 is derived from the U (VI) contaminated site or the U (VI) contaminated environmental media that is to be remediated, or at least partially remediated.

20. Use of a microorganism according to claim 9 in the bioremediation, or at least partial bioremediation, of a U (VI) contaminated site or of U (VI) contaminated environmental media.

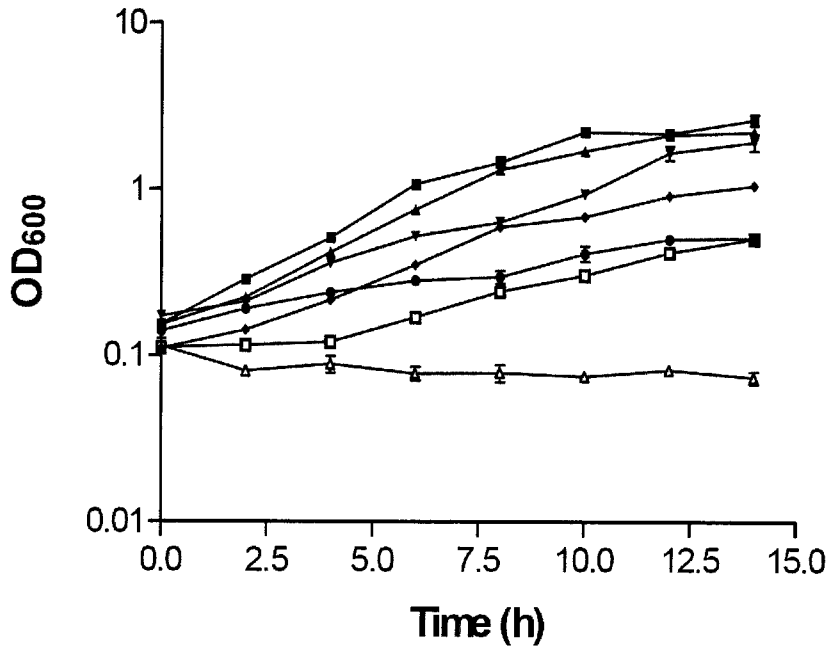


FIGURE 1

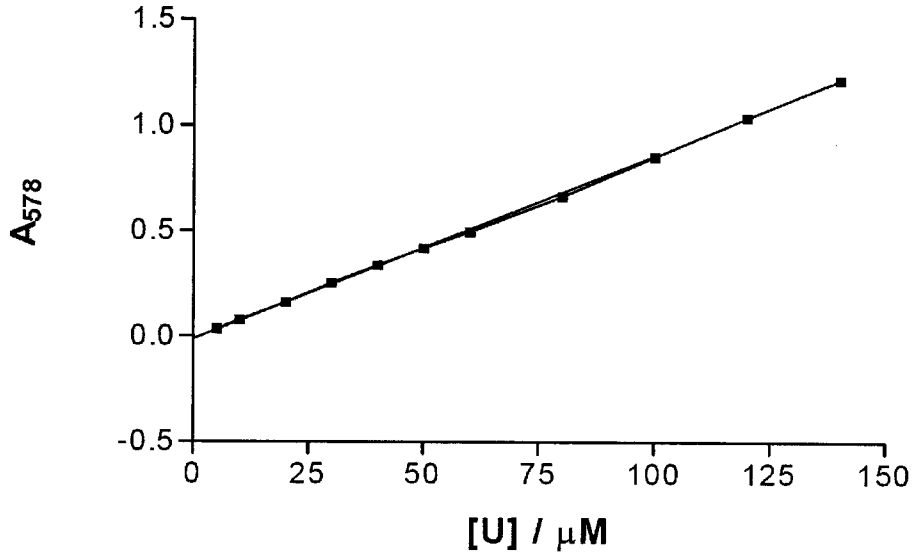


FIGURE 2

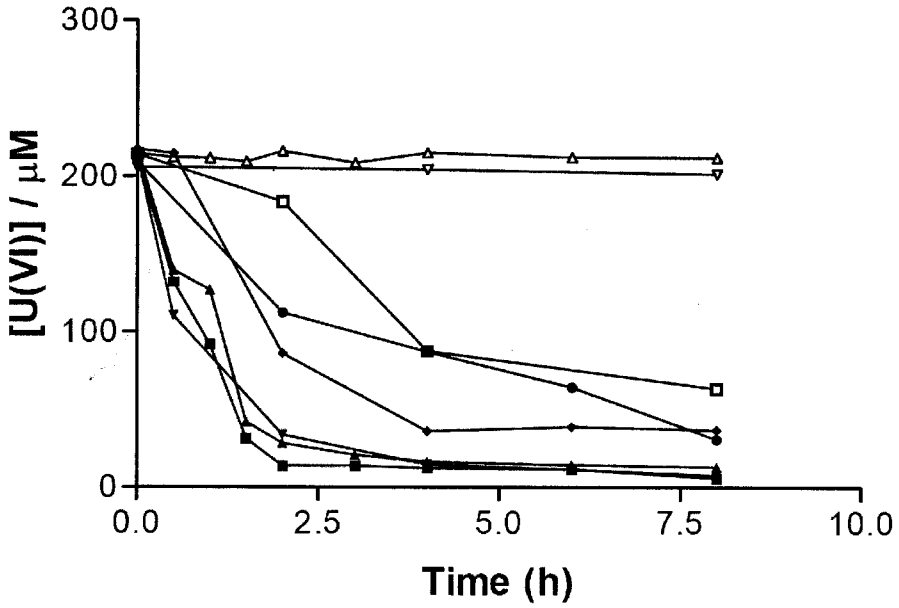


FIGURE 3

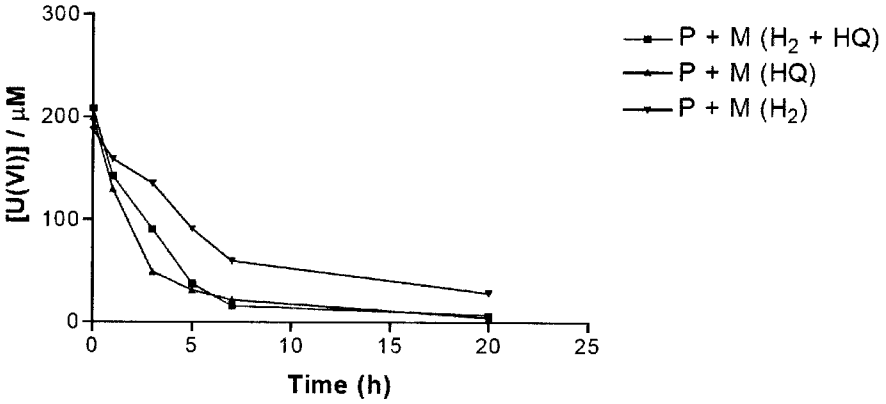


FIGURE 4

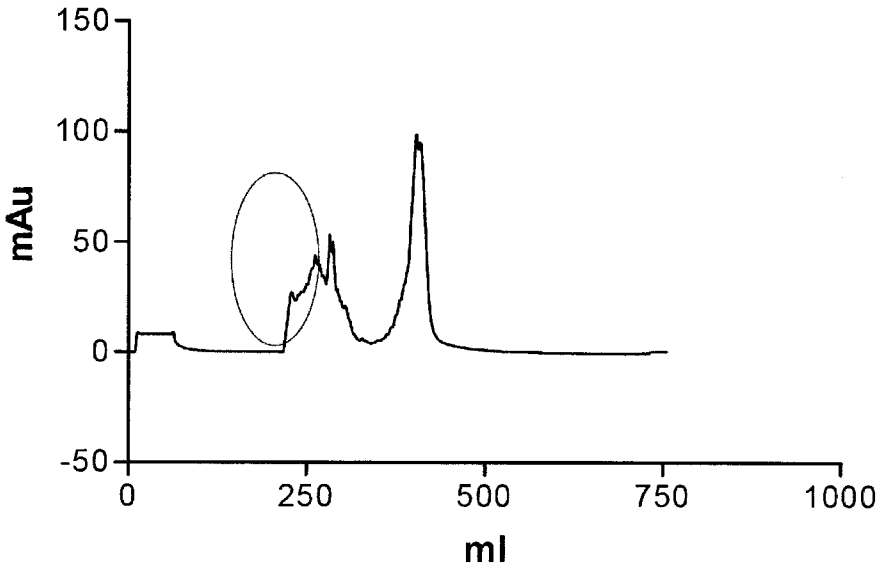


FIGURE 5

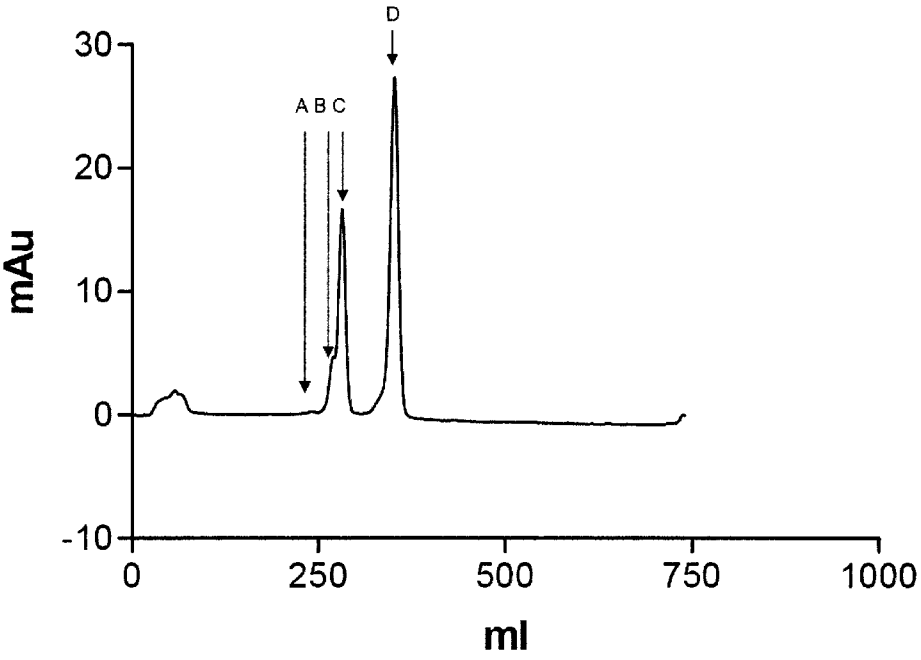


FIGURE 6

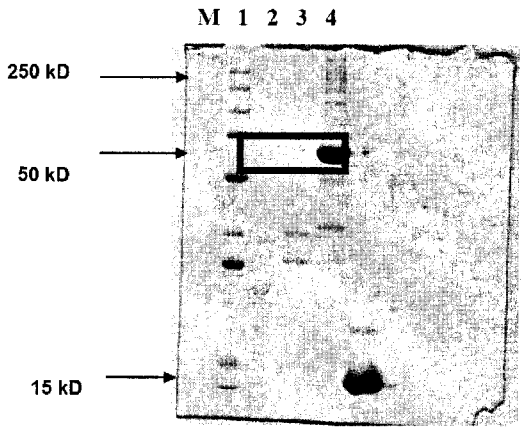


FIGURE 7

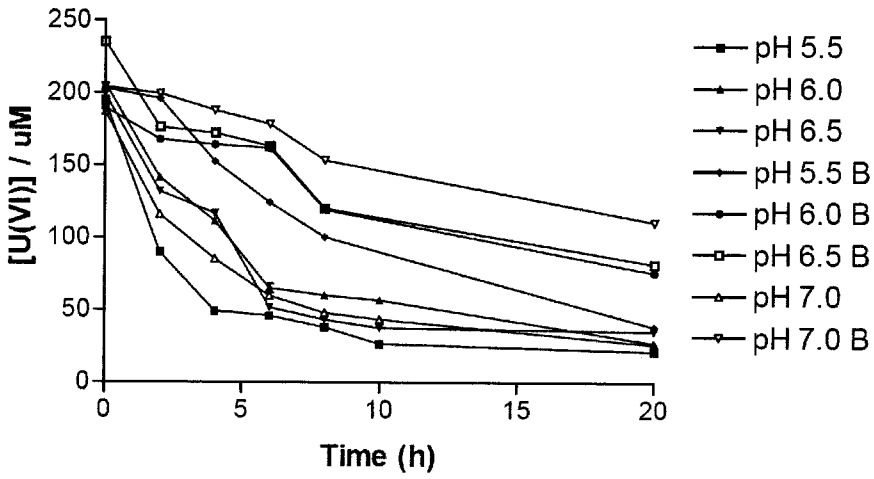

250
200
150
100
50
0
[U(VI)] / uM
0
5
10
15
20
Time (h)
pH 5.5
pH 6.0
pH 6.5
pH 5.5 B
pH 6.0 B
pH 6.5 B
pH 7.0
pH 7.0 B

FIGURE 8

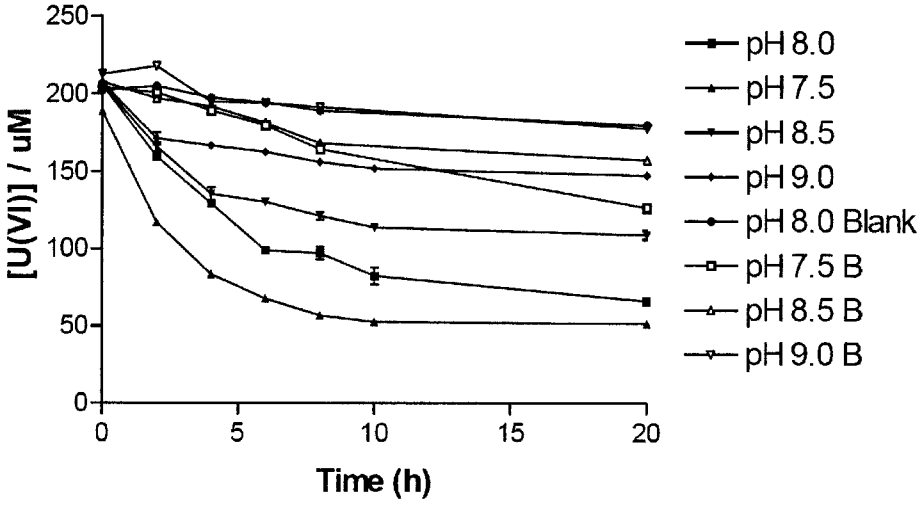

250
200
150
100
50
0
[U(VI)] / uM
0
5
10
15
20
Time (h)
pH 8.0
pH 7.5
pH 8.5
pH 9.0
pH 8.0 Blank
pH 7.5 B
pH 8.5 B
pH 9.0 B

FIGURE 9

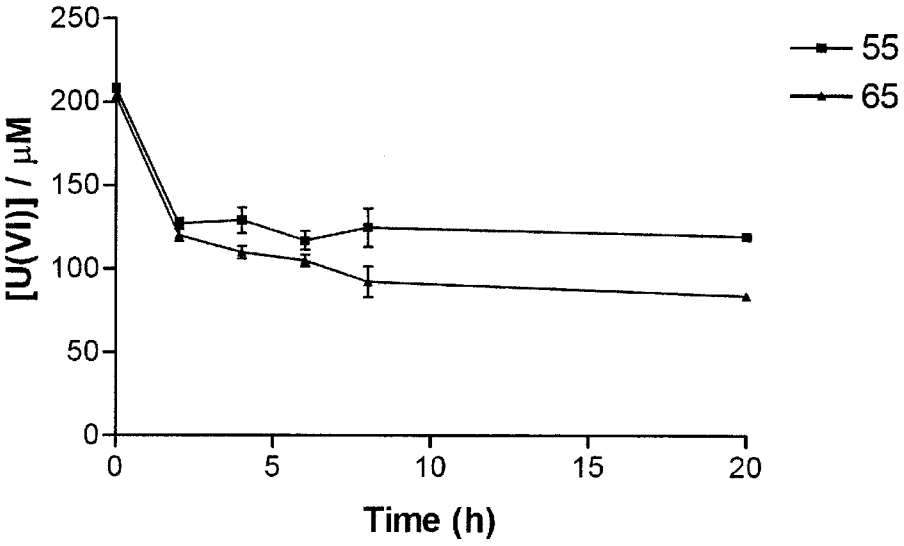


FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/054259

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 B09C1/10 C01G43/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K B09C C01G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VAN HEERDEN ET AL: "Metabolic promiscuity from the deep subsurface: a story of Survival or Superiority", PROCEEDINGS OF THE SPIE, SPIE, BELLINGHAM, WA, US, vol. 7097, 12 August 2008 (2008-08-12), pages S-1-S-12, XP002616795, DOI: 10.1117/12.801142 * abstract pages S-3, paragraphs 2,6 pages S-5, paragraph 1 pages S-8, paragraph 3 pages S-10, paragraph 4 ----- -/--	10-16, 18,19

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2011	28/01/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Tudor, Mark

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/054259

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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