Effects of the Anaerobic Respiration of *Shewanella oneidensis* MR-1 on the Stability of Extracellular U(VI) Nanofibers

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Uranium (VI) is considered to be one of the most widely dispersed and problematic environmental contaminants, due in large part to its high solubility and great mobility in natural aquatic systems. We previously reported that under anaerobic conditions, *Shewanella oneidensis* MR-1 grown in medium containing uranyl acetate rapidly accumulated long, extracellular, ultrafine U(VI) nanofibers composed of polycrystalline chains of discrete meta-schoepite ([UO$_2$]$_2$[OH]$_2$H$_2$O). Wild-type MR-1 finally transformed the uranium (VI) nanofibers to uranium (IV) nanoparticles via further reduction. In order to investigate the influence of the respiratory chain in the uranium transformation process, a series of mutant strains lacking a periplasmic cytochrome MtrA, outer membrane (OM) cytochrome MtrC and OmcA, a tetraheme cytochrome CymA anchored to the cytoplasmic membrane, and a trans-OM protein MtrB, were tested in this study. Although all the mutants produced U(VI) nanofibers like the wild type, the transformation rates from U(VI) nanofibers to U(IV) nanoparticles varied; in particular, the mutant with deletion in tetraheme cytochrome CymA stably maintained the uranium (VI) nanofibers, suggesting that the respiratory chain of *S. oneidensis* MR-1 is probably involved in the stability of extracellular U(VI) nanofibers, which might be easily treated via the physical processes of filtration or flocculation for the remediation of uranium contamination in sediments and aquifers, as well as the recovery of uranium in manufacturing processes.

**Key words:** *Shewanella*, uranium, nanofibers, nanoparticles, mutant

Uranium is a radioactive and chemically toxic element that is widespread in the environment, primarily due to anthropogenic activity (25). Hexavalent uranium (VI) is considered to be one of the most widely dispersed and problematic environmental contaminants, due in large part to its high solubility and great mobility in natural aquatic systems (9). Investigations concerning the mineralization and transformation of hexavalent uranium complexes are of critical importance in terms of the long-term management of spent nuclear fuels, the remediation of uranium-contaminated environments and the recovery of uranium minerals. The immobilization of uranium, primarily through the reduction of uranium (VI) to uranium (IV), has relatively lower solubility and mobility, has long been thought a viable remediation strategy.

Due to its marked respiratory versatility, *Shewanella* sp. strains have received more recent attention since they are capable of reducing iron (III), uranium (VI), technetium (VII), and many other metals (3, 7–9, 15, 27). The product of bacterial uranium reduction is commonly reported to be tetravalent uraninite (UO$_2$) nanoparticles, with subtly different morphologies, that are located either outside the cell or in the periplasm (10, 21). Although the bacterial reduction of U(VI) and immobilization of U(IV) as uraninite nanoparticles has been well studied in recent years, the deposition of uranium (VI) by microorganisms is poorly understood. The uranium (VI) mineral schoepite ([UO$_2$]$_2$[OH]$_2$H$_2$O)$_2$ or UO$_2$.2.25H$_2$O was first described in 1923 (24). While a series of related minerals, including meta-schoepite was obtained through a variety of chemical routes, and their structure and composition have been characterized (5, 19, 26), the biogenic formation of uranium (VI) minerals remains rarely investigated. Considering the 1-dimensional morphology and physicochemical properties, hexavalent uranium nanoparticles could be the preferred form for uranium bio-remediation rather than the 0-dimensional U(IV) nanoparticles. We previously reported that wild-type *S. oneidensis* MR-1 produces extracellular uranium (VI) nanofibers; however, the U(VI) nanofibers finally transformed to U(IV) nanoparticles via the complete reduction of U(VI) (6).

*S. oneidensis* MR-1 contains 42 putative c-type cytochromes (1, 10) and mutagenesis studies have shown that some of them are essential for metal reduction. Among these, periplasmic, decaheme cytochrome (MtrA), decaheme cytochromes (MtrC and OmcA) exposed on the outer membrane (OM), and a tetraheme cytochrome (CymA) anchored to the cytoplasmic membrane, have all been known to be required for uranium reduction (3, 10, 22). In addition, a trans-OM protein MtrB, as part of a membrane-spanning protein complex (MtrABC), is needed for uranium reduction (4, 18). In order to investigate the respiratory activities influencing U(VI) reduction and further transformation, a series of mutants lacking CymA, MtrA, MtrB, or both MtrC and OmcA were tested in this study for the controllable formation of extracellular uranium (VI) nanofibers.

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Materials and Methods

Bacterial growth conditions

Shewanella strains used in this study were *S. oneidensis* MR-1, and a series of mutants lacking either cymA, mtrA, mtrB, or both mtrC and omcA genes (2, 3, 10, 14, 16, 22). The mutant strains of *S. oneidensis* MR-1 were gifts from Dr. James K. Fredrickson in Pacific Northwest National Laboratory, Richland, USA. The culture medium and incubation conditions were in accordance with our previous study (6). Briefly, the culture media contained 30 mM NaHCO₃, 10 mM sodium DL-lactate as the electron donor, and ~2 mM uranyl acetate (UO₂(CH₂COO)₂·2H₂O) as an electron accepter for the synthesis of uranium nanofibers. The pH value of the medium was adjusted to 7.0. The *Shewanella* strains were inoculated into sealed serum bottles with the 30 mM N₂ purged culture medium at a final cell density of 2×10⁸ cells mL⁻¹. All the cultures were incubated anaerobically in the dark at 30°C for 120 h.

Measurements of U(VI) and lactate in the culture media

The samples were collected at selected times during incubation for the detection of soluble uranium and lactate consumption in aqueous medium. To measure the concentration of uranium, the culture supernatants were filtered through a 0.2 µm membrane filter (MFS-25; Advantec MFS, Dublin, CA), and the filtrates were diluted for analysis using inductively-coupled plasma mass spectrometry (7500ce, ICP-MS; Agilent Technologies, Palo Alto, CA). The concentrations of the organic acids were detected by HPLC (Shimazu, Tokyo, Japan), which was equipped with a SPD-10A UV detector (Shimazu) and a Shodex RSpak KC-811 (8.0 mm ID*300 mm) column (Shodex, Tokyo, Japan). The mobile phase was 5 mM sulfuric acid with a flow rate of 0.5 mL min⁻¹, and UV detection was performed at 210 nm.

Characterization of materials

Transmission electron microscopy (TEM) was performed with samples collected to observe the mineralogical morphologies. To prepare the electron microscopy specimen, the precipitates in the bacterial culture media were collected and washed with DI water three times. Such specimens were then dried on a Cu grid under ambient conditions for TEM (TEM; JEOL, Tokyo, Japan).

For mineralogical analysis, the minerals produced by bacteria were collected from culture bottles with syringes after incubation and washed with DI water three times. Samples were then treated with 30% HNO₃, evaporated to a dry residue, and identified using X-ray diffraction (XRD) analysis (Rigaku D/MAX Ultima III high resolution X-ray diffractometer) and a Rigaku Powder Diffraction File (PDF #43-0364), and indicated that the mineral was likely meta-schoepite (UO₂·2H₂O, orthorhombic) (6). As the incubation time proceeded, the nanofibers were further transformed into particulate shapes in the sample collected at 120 h (Fig. S3) and XRD patterns indicated that the nanoparticles were composed of tetravalent uraninite (UO₃), as previously reported (10, 20, 21, 23) (Fig. 1B). In contrast, although the mutants with deletions in MtrC/OmcA, MtrA, MtrB or CymA also formed similar uranium nanoparticles at an early stage, the final products produced by the former three mutants were a mixture of U(VI) nanofibers and U(IV) nanoparticles. The CymA⁻ mutant stably maintained U(VI) nanofibers up to 120 h of incubation (Fig. 2). The results of XRD analysis confirmed that the final product at 120 h in the culture with CymA⁻ mutant was likely meta-schoepite (Fig. 1A).

Mechanisms of the transformation of U(VI) nanofibers to U(IV) nanoparticles

Kinetic analyses indicated that 70% of the soluble uranium in the culture medium of wild-type *S. oneidensis* MR-1 rapidly decreased over 12 h. Similarly, the concentra-
accumulation of uranium (VI) nanofibers at an early stage.
(Fig. 3). However, after the 12 h incubation period, lactate
stoichiometric, especially during the first 12 h of incubation
and the electron donor lactate by the strains was not
blocked in electron transport (3) (Fig. 3A).
uranium (VI) to uranium (IV), 3) accumulation of UO
surfaces, 2) fast dynamic reduction of a small amount of
culture solution than was seen with the wild-type strain (Fig.
phenomenon, although less U(VI) were precipitated from the
reduction of uranium (VI) to uranium (IV), rather than the
that lactate consumption by strain MR-1 was coupled to the
medium color changed from brown to black, indicating
inoculated with wild-type and mutant strains under anaerobic condi-
tions. Data points from three parallel independent incubations.

Fig. 3. Consumption of the electron acceptor uranium (VI), and
lactate, the electron donor. (A) The time-dependent concentration of
soluble uranium (VI), and (B) lactate remaining in culture media
inoculated with wild-type and mutant strains under anaerobic condi-
tion of soluble uranium in culture media inoculated with the
MtrC−/OmcA−, MtrB− and MtrA− mutants decreased by 50,
40 and 30% after 12 h, respectively. In contrast, <10% of
uranium (VI) was removed after 12 h in culture medium
inoculated with the CymA− mutant, which appears to be
blocked in electron transport (3) (Fig. 3A).
The consumption of the electron acceptor uranium (VI),
and the electron donor lactate by the strains was not
stoichiometric, especially during the first 12 h of incubation
(Fig. 3). However, after the 12 h incubation period, lactate
consumption by the wild-type strain noticeably increased as
the medium color changed from brown to black, indicating
that lactate consumption by strain MR-1 was coupled to the
reduction of uranium (VI) to uranium (IV), rather than the
accumulation of uranium (VI) nanofibers at an early stage.
The mutant strains tested in this study also showed a similar
phenomenon, although less U(VI) were precipitated from the
culture solution than was seen with the wild-type strain (Fig.
3). These results suggested that the accumulation of uranium
(VI) nanofibers is not predominantly associated with U(VI)
reduction by lactate consumption. The different rates of
soluble uranium (VI) decrease and uranium (VI) nanofiber
formation by the wild type and mutants might be due to
different amounts of the initially-formed U(IV) nanoparticles
on the cell surface, which acts as a nucleus for the growth of
U(VI) nanofibers.
The normalized U K-edge X-ray absorption near edge
structure (XANES) spectra obtained in our previous study
(6) suggested the transformation of the uranium nano-
structures by wild-type MR-1 involving several steps as
follows: 1) initial absorption of uranium (VI) to bacterial cell
surfaces, 2) fast dynamic reduction of a small amount of
uranium (VI) to uranium (IV), 3) accumulation of UO₂
precipitates on the bacterial cell surfaces and/or in the
periplasm that appear to play a role in triggering the formation
of extracellular uranium (VI) nanofibers, and 4) reduction of
the uranium (VI) nanofibers to uranium (IV) nanoparticles
with the concurrent consumption of lactate.
The mutants also showed a similar process but the
accumulated U(VI) nanofibers were not finally reduced to
U(IV) nanoparticles (Fig. 2). Although the mutants lack
various cytochromes or outer membrane protein involved in
metal reduction, slow, but continuous reduction of uranium
occurred by the mutant strains. Alternative proteins or other
unknown factors likely active and participate in a low level
of activity that is required for initial reduction of soluble
U(VI) to U(IV) nanoparticles on the cell surface, which acted
as a nucleus for the growth and accumulation of U(VI)
nanofibers. Under a defined set of conditions, the U(VI)
nanofibers appeared to be formed and accumulated as long
as the minimum amount of U(IV) formed by either wild-type
MR-1 or the mutants as a nucleus on the cell surface at the
initial stage, regardless of the further reduction of the formed
U(VI) nanofibers. The initially minimum reduction of U(VI)
might be due to the activities of alternative cytochromes or
other unknown factors. However, after the U(VI) nanofibers
formed and accumulated, the mutant strains showed much
less transformation of U(VI) to U(IV) than that by the
wild-type strain. It should be noted that the CymA− mutant,
which appeared to be disabled from the further reduction of
insoluble U(VI) fibers to insoluble U(IV) nanoparticles, can
stably maintain U(VI) nanofibers. However, it has been
reported that the CymA− mutant did not completely abolish
U(VI)-reducing activity due to the presence of possible
multiple pathways for soluble U(VI) reduction (3). This
discrepancy in U(VI) reduction by the CymA− mutant is
probably due to the blockage of alternative pathways or
factors affecting electron transfer to extracellular solid U(VI)
nanofibers. The results obtained in this study strongly
suggested that the cytochromes and OM proteins significantly
influence uranium reduction and the formation of U(VI)
nanofibers, and subsequent further transformation to U(IV)
nanoparticles.

Environmental implication
The contamination of surface and ground water with
uranium is a serious environmental concern. Results of the
current study suggest that it may be possible to immobilize
soluble uranium (VI) into uranium (VI) nanofibers by using
facultatively anaerobic Shewanella strains and a series of
mutants. Although the biogenic production of uraninite (UO₂)
nanoparticles has previously been suggested as a remediation
strategy to remove soluble uranium (VI) from the environ-
ment, this mineral form is likely to be mobile in porous
sediments and rapidly re-oxidized due to its nanometer-scale
size (12, 17, 23). In contrast, 1-dimensional uranium (VI)
nanofibers may offer another method for the removal of
soluble uranium (VI) from the environment via physical
processes of filtration and flocculation. The mutant strains
of S. oneidensis MR-1, especially CymA−, allows the
synthesis and stabilization of unique, long, and ultrafine
uranium (VI) nanofibers, which are distinct from previously
reported biogenic tetravalent uraninite nanoparticles. The
biological transformation of uranium (VI) nanofibers may
also provide an alternative biological tool for “Yellow Cake”
manufacturing processes, which currently use diverse harsh
physicochemical treatments to extract and leach uranium from
ores (11, 13).

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References


