

Redox-reactive membrane vesicles produced by *Shewanella*

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ABSTRACT

This manuscript is dedicated to our friend, mentor, and coauthor Dr Terry Beveridge, who devoted his scientific career to advancing fundamental aspects of microbial ultrastructure using innovative electron microscopic approaches. During his graduate studies with Professor Robert Murray, Terry provided some of the first glimpses and structural evaluations of the regular surface arrays (S-layers) of Gram-negative bacteria (Beveridge & Murray, 1974, 1975, 1976a). Beginning with his early electron microscopic assessments of metal binding by cell walls from Gram-positive bacteria (Beveridge & Murray, 1976b, 1980) and continuing with more than 30 years of pioneering research on microbe–mineral interactions (Hoyle & Beveridge, 1983, 1984; Ferris *et al.*, 1986; Gorby *et al.*, 1988; Beveridge, 1989; Mullen *et al.*, 1989; Urrutia Mera *et al.*, 1992; Mera & Beveridge, 1993; Brown *et al.*, 1994; Konhauser *et al.*, 1994; Beveridge *et al.*, 1997; Newman *et al.*, 1997; Lower *et al.*, 2001; Glasauer *et al.*, 2002; Baesman *et al.*, 2007), Terry helped to shape the developing field of biogeochemistry. Terry and his associates are also widely regarded for their research defining the structure and function of outer membrane vesicles from Gram-negative bacteria that facilitate processes ranging from the delivery of pathogenic enzymes to the possible exchange of genetic information.

The current report represents the confluence of two of Terry's thematic research streams by demonstrating that membrane vesicles produced by dissimilatory metal-reducing bacteria from the genus *Shewanella* catalyze the enzymatic transformation and precipitation of heavy metals and radionuclides. Under low-shear conditions, membrane vesicles are commonly tethered to intact cells by electrically conductive filaments known as bacterial nanowires. The functional role of membrane vesicles and associated nanowires is not known, but the potential for mineralized vesicles that morphologically resemble nanofossils to serve as palaeontological indicators of early life on Earth and as biosignatures of life on other planets is recognized.

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INTRODUCTION

Many Gram-negative bacteria form outer membrane vesicles as part of their natural growth cycle (Beveridge, 1999; Rothfield & Pearlman-Kothencz, 1969; Wensink & Witholt, 1981; Nowotny *et al.*, 1982; Grenier & Mayrand, 1987; Deslauriers *et al.*, 1990). Membrane vesicles (MVs), which measure between 50 and 250 nm in diameter, are compositionally similar to the outer membranes of bacteria from which they emerge and contain lipopolysaccharides, outer membrane proteins, and phospholipids (Knox *et al.*, 1966; Kadurugamuwa & Beveridge, 1996). The lumens of MVs are filled with periplasmic proteins and can contain cytoplasmic components,

such as DNA (Renelli *et al.*, 2004). Some research suggests that disturbances in growth, exposure to antibiotics, or simply turnover in cell-wall components initiate vesicle formation (Knox *et al.*, 1966; Kadurugamuwa & Beveridge, 1995; Zhou *et al.*, 1998). Recent work has also suggested that production of bacterial outer MVs is a fully independent, general envelope stress response (McBroom & Kuehn, 2007).

The functional role and environmental significance of MVs have not been conclusively resolved, although some research clearly demonstrates the potential of MVs as vehicles of pathogenicity (Kadurugamuwa & Beveridge, 1996; Li *et al.*, 1998). Vesicles often contain peptidoglycan hydrolase, an enzyme that degrades peptidoglycan. MVs released from

Pseudomonas aeruginosa can fuse with outer membranes of other gram-negative bacteria and release hydrolase enzyme into the periplasmic region leading to cell lysis. Pathogenic bacteria also release MVs as a strategy to deliver virulence factors, such as protease, proelastase, phospholipase C, and alkaline phosphatase (Kadurugamuwa & Beveridge, 1995), or potent toxins, such as enterotoxin (Kolling & Matthews, 1999; Horstman & Kuehn, 2000), to eukaryotic cells and tissues.

Although membrane vesicle formation appears to be important for transmission of materials involved in pathogenicity, very little is known about the potential roles these components play in nonpathogenic, environmental organisms. We report the formation of MVs by the Gram-negative dissimilatory metal-reducing bacterium *Shewanella putrefaciens* strain CN32 and *S. oneidensis* strain MR-1 under controlled laboratory conditions. The morphology, composition, enzymatic activity, and possible environmental significance of these structures are discussed.

MATERIALS AND METHODS

Strains and culture conditions

Shewanella putrefaciens strain CN32, a facultative metal-reducing bacteria that was isolated from New Mexico subsurface sediments (Fredrickson *et al.*, 1998), was cultured either aerobically in 1-L shake flasks (150 r.p.m) containing 500 mL of tryptic soy broth (TSB) or anaerobically in a 3-L Bioflow 3000 vessel containing 1.5 L of TSB supplemented with 20 mM lactate as the electron donor and 30 mM fumarate as the electron acceptor. Anaerobic cultures were continuously bubbled with nitrogen gas to maintain anaerobic conditions. *S. oneidensis* strain MR-1, originally isolated from lake sediments (Myers & Neelson, 1988), was cultivated in chemostat cultures using a chemically defined medium with O₂ limitation as previously described (Gorby *et al.*, 2006). MR-1 was cultivated as biofilms on the surfaces of specular hematite fixed to the bottom of the wells of a 12-well tissue culture plates (BD Biosciences, San Jose, CA USA). Briefly, a loopful of MR-1 cells was transferred from tryptic soy agar plates into 250 mL flasks containing 50 mL of the chemically defined medium with 65 mM lactate as the electron donor. Cultures were incubated at 30 °C for 4 h at 150 r.p.m. A multiwell plate (12-well polystyrene tissue culture treated, BD Biosciences) containing small pieces of specular hematite attached to a glass cover slips using aquarium grade silicon received 0.5 mL per well of this 4-h culture. Following a 5-min incubation, unattached cells and culture medium were replaced with 2 mL of sterile medium containing 18 mM lactate as the sole electron donor. The plate was incubated at room temperature with gentle mixing on a plate shaker for 24 h. The plate was then fitted with a media drip line that delivered sterile medium into the sealed wells containing developing biofilm. The

sample was incubated for 500 h before being fixed with 2% paraformaldehyde, critical point dried with liquid CO₂, and imaged by scanning electron microscopy.

Membrane vesicle isolation/purification

MVs were isolated using a slightly modified method described by Horstman and Kuehn (2000). Anoxic conditions were maintained at each step of the purification. Cells of CN32 from aerobic or anaerobic cultures were sedimented by centrifugation (10 000 × *g*, 10 min). The supernatant fluid was concentrated 100-fold using a 70-kDa tangential filtration device (Pall-Gelman, Ann Arbor, MI, USA). Cells remaining in the retentate were removed by centrifugation (6000 × *g*, 10 min) followed by filtration through a 0.45-µm 90-mm filter unit. The resulting filtrate was centrifuged at 150 000 × *g* for 2 h to sediment the MVs and other extracellular material. Sedimented material was washed three times with anaerobic 30 mM PIPES buffer to remove culture medium and extracellular debris. Each preparation was examined by transmission electron microscopy (TEM) to evaluate the purity of the sample.

Gel electrophoresis

To prepare MVs for gel electrophoresis, purified membrane vesicle samples were boiled for 1 min in two times sample buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, and 0.02% bromophenol blue, pH 6.8), applied to 12.5% precast acrylamide minigels (Jule, Inc., Milford, CT, USA) and run at constant voltage (100 V) for 2.5 h. Duplicate samples were loaded so that one lane gel could be silver-stained and the other subjected to heme staining according to the method of Thomas *et al.* (Brock & Gustafson, 1976). Biorad low molecular weight standards were used with the silver stain and prestained standards were used with the heme stain.

LPS isolation and analysis

Lipopolysaccharide (LPS) was isolated from CN32 by the method of Darveau and Hancock (Darveau *et al.*, 1983), yielding a precipitated product. LPS (100 mg) was kept at 100 °C for 4 h in 4 M NaOH (4 mL) containing a small amount of NaBH₄, cooled, and neutralized to pH 8–9 with 2 M HCl (7.5 mL). The precipitate was removed by centrifugation, and the components of the resulting pellet were separated on a Sephadex G50 SF gel (Amersham Pharmacia, Uppsala, Sweden) column (2.5 × 80 cm) using pyridine-acetic acid buffer (4 mL of pyridine and 10 mL of acetic acid in 1 L of water) as the mobile phase. Preparations of oligosaccharides 3 and 4 LPS (80 mg) were hydrolyzed with 2% acetic acid (100 °C, 4 h). The resulting precipitate was removed by centrifugation, and the supernatant was separated on a Sephadex G50 column to give a mixture of the oligosaccharides 3 and 4 (25 mg) and D-galactose (3 mg).

Polysaccharide (1 mg) was hydrolyzed with TFA (0.5 mL of 3 M, 100 °C, 2 h), evaporated to dryness under stream of nitrogen, dissolved in water (0.5 mL), reduced with NaBH₄ (~5 mg, 30 min), treated with acetic acid (0.5 mL of 99.7%), dried and resuspended twice in MeOH (1 mL). The resulting dried precipitate was acetylated with acetic anhydride (0.5 mL, 100°, 30 min), dried, and analysed by gas liquid chromatography (GLC) using a DB-17 capillary column (25 m × 0.25 mm) with flame ionization detector (HP 5890 instrument) in a temperature gradient 180–240 °C, 2 °C/min, or on Varian Saturn 2000 instrument (Varian Instruments, Walnut Creek, CA, USA) equipped with ion-trap mass spectral detector. Retention times were compared with those of standard samples prepared from L-rhamnose, N-acetyl-L-fucosamine, malic acid and 3-hydroxybutyric acid with (*R*)- and (*S*)-2-butanol. The content of 2-keto-3-deoxyoctonate in LPS was determined using the method of Karkhanis *et al.* (1978).

Nuclear magnetic resonance (NMR) spectroscopy

¹H and ¹³C NMR spectra were recorded using a Varian Inova 500 spectrometer in D₂O solutions at 60 °C with acetone standard (2.225 p.p.m. for ¹H and 31.5 p.p.m. for ¹³C) using standard pulse sequences as defined in Korenevsky *et al.* (2002). Spectra were assigned with the help of PRONTO program (Kjaer *et al.*, 1994).

Electron and atomic force microscopy

Whole mounts for TEM were prepared by placing a small drop of a washed MV suspension on a formvar/carbon-coated grid. Excess solution was wicked away using a piece of Whatman filter paper. Vesicles were negatively stained using a 1% solution of uranyl acetate. Samples were fixed for 1 h by the addition of an anoxic solution of glutaraldehyde (final concentration of 2.5%) and then dehydrated using a graded ethanol series and embedded in LR White embedding medium. All samples were fixed, embedded, and sectioned under anaerobic conditions to avoid oxidations of redox-sensitive components, as previously described (Wildung *et al.*, 2000). Whole mounts and thin sections of MVs were examined using a JEOL 2010 TEM instrument (Tokyo, Japan) operating at a 200-kV accelerating voltage. Low-magnification and high-resolution images were digitally recorded using a 1024 × 1024 charge-coupled device camera (Gatan Instruments, Pleasantville, CA, USA). Lattice fringe images were generated at high magnification to assist in the identification of solids and their crystallinity. The d-spacings were measured directly from the digital images and compared to those of standard Tc solids available from the PDF database.

Euhedral tabular single crystal platelets of pure hematite were synthesized by the method of Sapiaszko and Matijevic (Sapiaszko & Matijevic, 1980). The samples averaged 20 μm wide by 2 μm thick. Hematite platelets were incubated with CN32 under anoxic conditions in bicarbonate-buffered

medium (pH = 6.8) using lactate as the electron donor (Rosso *et al.*, 2003) and hematite as the sole electron acceptor. Abiotic control samples were run in parallel. Over the course of a few days, samples of the bacteria–hematite suspensions were obtained, the solid phase was filtered, washed, extracted with dilute HCl to remove sorbed Fe(II), neutralized in buffer, washed, and air-dried. Hematite platelets were collected from air-dried filtrates and dry mounted on TempfixTM-coated (Electron Microscopy Sciences, Hatfield, PA, USA) microscope slides. Samples were imaged by atomic force microscopy (AFM) using a Digital Instruments, Inc. (Santa Barbara, CA, USA) BioScopeTM under ambient conditions with standard Si₃N₄ contact mode tips. Imaging performed in contact mode used low force cantilevers with contact forces minimized; contact mode did not disturb soft biological structures and tapping mode did not improve the image quality. Scan rates between 0.5 and 1.0 Hz were used. Images were background subtracted using standard zero-order planar fits. Samples from these runs were also later transferred into a vacuum chamber for scanning Auger analysis of the surface chemical composition using line scans across residual cell and MV material on the hematite crystals.

Samples for topographical and conductive atomic force microscopy were cultivated in chemostat cultures under O₂-limited conditions, which promotes the formation of cell aggregates and electrically conductive nanowires (Gorby *et al.*, 2006). Cell aggregates were gently transferred from the bioreactor to a coupon of highly ordered pyrolytic graphite (HOPG) and allowed to sit for 1 min. Media and unattached cells were carefully removed using a needle and syringe. Attached cells and extracellular material were gently washed with 10 mL of anaerobic deionized water, wicked dry with filter paper, and air dried. Topographical images were acquired in AC mode using an MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA). Topography, amplitude error maps, and phase images were acquired simultaneously in 512 × 512 pixel images, at a typical line scan rate of 1–3 Hz.

Metal reduction assays

Enzymatic reduction of Fe(III), U(VI), and Tc(VII) was evaluated in sealed Balch tubes containing anaerobic solutions of 30 mM PIPES buffer (pH 7) with ferric citrate, uranyl acetate, or ammonium pertechnetate as the terminal electron acceptor (0.5 mM each). Hydrogen served as the electron donor and was supplied to sealed reaction vials through the stopper using a needle and syringe. Anaerobic techniques were used to recover duplicate samples at regular intervals. Ferrozine was used as a colorimetric reagent for monitoring reduction of iron (Stookey, 1970). Uranium reduction was evaluated using a Kinetic Phosphorescence Analyser (ChemCheck Instruments, Richland, WA, USA) (Gorby & Lovley, 1992). Reduction of technetium was determined by recording the distribution of radioactivity in the aqueous and

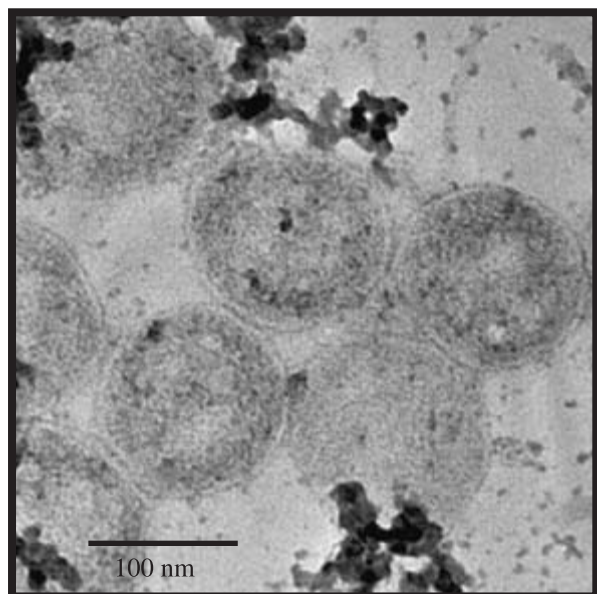


Fig. 1 Transmission electron microscopy image of membrane vesicles recovered from an aerobic culture of *Shewanella putrefaciens* strain CN32. Uranyl acetate served as the contrasting agent in this whole mount preparation.

nonaqueous phase following extraction of complexation of TcO_4^- with tetraphenyl arsonium chloride (TPAC) and chloroform (Wildung *et al.*, 2000).

RESULTS

Morphology and composition of membrane vesicles

Strain CN32 produced MVs during growth in aqueous suspensions or in the presence of solid phase iron oxides. The vesicles shown in Fig. 1 were isolated from the growth medium of an aerobic culture at late exponential phase of growth. The vesicles exhibited lipid bilayer morphology typical of MVs from other Gram-negative bacteria. Silver-stained gels from sodium dodecyl sulphate-polyacrylamide gel electrophoresis

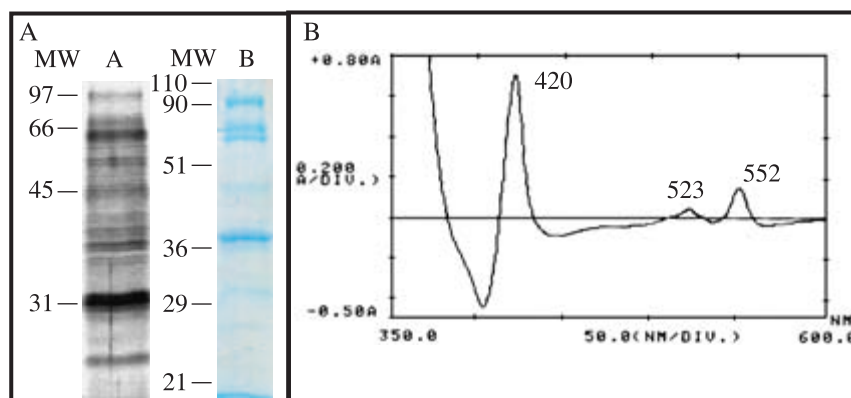
(SDS-PAGE) showed that the MVs contained LPS that were compositionally similar to those detected in the outer membrane of intact cells (data not shown). Extraction, chemical hydrolysis, and ^1H and ^{13}C NMR analyses of CN32 LPS revealed that there was no O-side chain and the core consisted of only a β -Gal-(1-3)- β -Gal-(1-4)- β -Glc-(1-4)- α -DDHep2PetN-(1-5)- α -Kdo4P-(1-6)- β -GlcN4P-(1-6)- α -GlcN1P backbone. Although many Gram-negative bacteria possess long O-side chains on their surface LPS, which can extend more than 40 nm above the outer membrane, *S. putrefaciens* CN32 possesses only 'rough' LPS consisting of a short core oligosaccharide above the lipid A substituent. MVs also contained α -ketodeoxyoctonate (KDO), a component found in the outer membrane of all Gram-negative bacteria (Karkhanis *et al.*, 1978).

Examination of MVs by SDS-PAGE (silver stain) clearly revealed the presence of a select number of proteins. (Fig. 2A, lane A). Heme-containing proteins with molecular weights of approximately 94, 78, 71, 44, 37, 30, and 26 kDa were observed by staining gels using a peroxidase activity stain (Brock & Gustafson, 1976) (Fig. 2A, lane B). Optical absorption spectra of the MVs indicated the presence of c -type cytochromes with characteristic α , β , and the Soret peaks at 552, 523, and 420 nm, respectively (Fig. 2B). Cytochromes that were chemically reduced by sodium dithionite were oxidized by anaerobic solutions of ferric citrate, sodium fumarate or sodium nitrate, suggesting that these cytochromes may be involved in the reduction of a variety of terminal electron acceptors.

Enzymatic activity

Membrane vesicles from CN32 were examined for their ability to reduce Fe(III) and other multivalent heavy metals. Results collected from duplicate samples demonstrated that isolated MVs reduced ferric citrate when hydrogen was supplied as an electron donor (Fig. 3). Iron was not reduced in control preparations that received no vesicles or in suspensions of MVs that received no hydrogen. Although whole cells couple the

Fig. 2 (A) SDS-PAGE of proteins from membrane vesicles (MV) isolated from *Shewanella putrefaciens* strain CN32. Silver-stained proteins appear in lane A. Heme-containing proteins appear in lane B. Molecular weight standards are illustrated in the lane labelled MW. (B) Dithionite-reduced minus air-oxidized difference spectrum of membrane vesicles (MV) isolated from strain CN32.



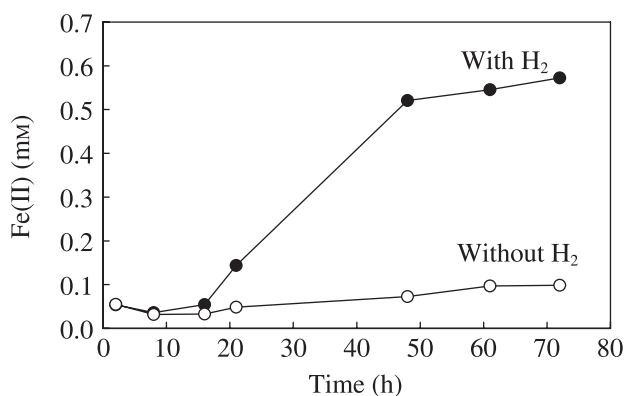


Fig. 3 Reduction of ferric citrate by membrane vesicles isolated from CN32.

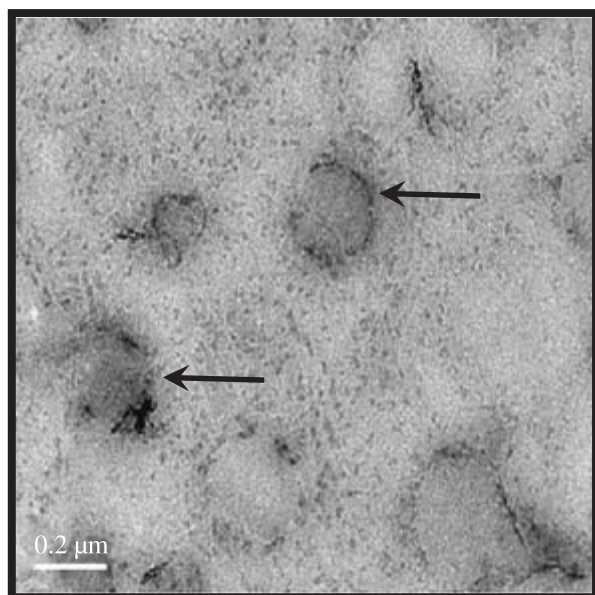


Fig. 4 Transmission electron micrograph of a thin section of vesicles following reduction of 1 mM ferric citrate with H₂ as the electron donor. Arrows indicate the location of membrane vesicles encrusted with Fe precipitates.

oxidation of lactate to the reduction of metals, results from a single experiment demonstrate that purified MVs could not use lactate as an electron donor.

Membrane vesicles that reduced dissolved ferric citrate became encrusted in an iron precipitate. Unstained thin sections of these vesicles demonstrated that the precipitate accumulated on the outside of each vesicle and was present in the surrounding medium (Fig. 4). High-resolution TEM revealed the crystalline nature of the precipitate and d-spacings obtained from the lattice fringe image were consistent with ultra fine-grain magnetite (data not shown).

Membrane vesicles isolated from strain CN32 were also tested for their ability to reduce multivalent heavy metals and

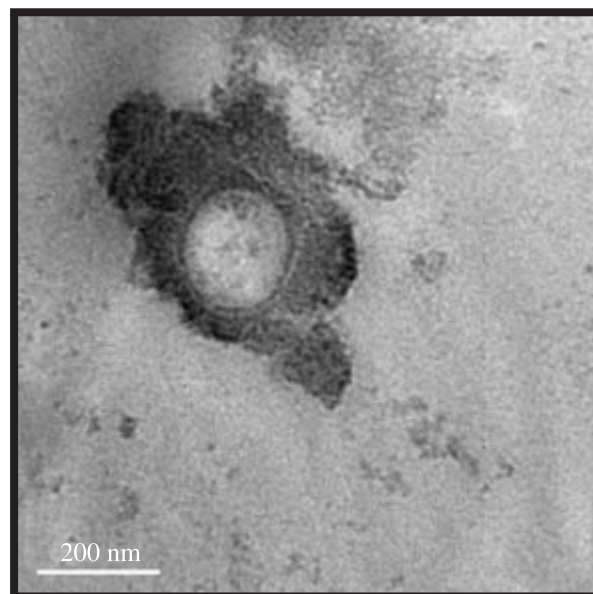


Fig. 5 Transmission electron micrograph of a thin section of membrane vesicles following reduction of 0.5 mM sodium pertechnetate. Reduced technetium accumulated as poorly crystalline TcO₂ precipitate on the external surface of the vesicles.

radionuclides that are known to be reduced by intact cells of iron-reducing bacteria (Gorby & Lovley, 1992; Wildung *et al.*, 2000). As with Fe(III), oxidized, dissolved forms of uranium and technetium were reduced in anaerobic suspensions of MVs when H₂ was provided as the electron donor (data not shown). Precipitation of uranium and technetium solids accompanied reduction, as expected under the circumneutral pH conditions of the experiment. Images from unstained thin sections of vesicles incubated with 0.5 mM sodium pertechnetate with H₂ as an electron donor illustrate that precipitates formed on the outer surface of the vesicles (Fig. 5). The precipitate consisted almost entirely of Tc and oxygen, as determined by energy dispersive X-ray spectroscopy (EDS). The precipitates lacked any detectable crystallinity, as determined by high resolution TEM or selected area electron diffraction, which is consistent with the amorphous Tc(IV) hydrous oxide that formed on the external surfaces and in the periplasmic region (area between the outer and the inner membranes) of intact cells of CN32 (Wildung *et al.*, 2000).

MVs and iron oxides

Cells of strain CN32 produced MVs while attached to the surface of synthetic tabular hematite. In an anaerobic, chemically defined medium, cells attached to the (001) surface of hematite, such as those observed by Rosso *et al.* (2003), were studded with particles of the same size and morphology as MVs from aqueous cell suspensions. Atomic force microscopy indicated that the vesicles, as well as a thin layer of

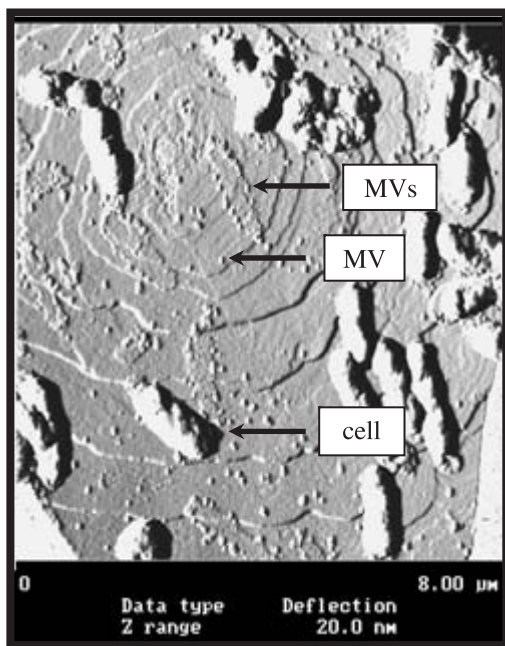


Fig. 6 Deflection-mode atomic force micrograph of the (001) surface of crystalline tabular hematite that was incubated anaerobically with cells of *Shewanella putrefaciens* strain CN32. Whole cells and arrays of membrane vesicles (MVs) can be observed on the hematite surface. Size analysis and scanning Auger compositional analysis of sites previously occupied by cells confirms that the structures are MVs along with a thin residual layer of organic material.

biological material, remained adhered to the mineral surface after the bulk of the cell had detached (Fig. 6). Scanning Auger spectroscopy line scans across this residual cell 'footprint' and MV material confirmed its biogenic origin and that it was not a mineralization feature.

Other *Shewanella* species produced MVs when cultivated with hematite. *S. oneidensis* strain MR-1 produced numerous MVs that typically assumed curvilinear arrangements around the periphery of cells (Fig. 7A) in a very similar pattern as seen by AFM in Fig. 6. MVs were tethered to each other and to intact cells by electrically conductive bacterial nanowires (Fig. 7B). MVs were typically associated with hematite surfaces but most abundantly associated with secondary mineralization features present on the surfaces of natural specular hematite crystals (Fig. 7C). Biofilms attached directly to the surface of the glass microscope slide to which tabular hematite was attached did not show attached MVs (Fig. 7D). Reasons for this difference are not yet clear.

Electrical conductivity of MVs and the nanowires that tethered them to cells was confirmed by scanning probe microscopy using a conducting probe (C-AFM). Topographical AFM was applied to map MVs and associated branched appendages (Fig. 8A). The tip raster and tapping oscillation was stopped over locations of interest on the appendages and MVs where the electrical current (I) passed between the

underlying graphite substrate and the tip is measured as a function of the applied bias voltage (V). The IV curve presented in Fig. 8(B) was collected over a membrane vesicle and shows the double-exponential behavior characteristic of a non-Ohmic junction. Measurable current over this bias range demonstrates that MVs from MR-1 are likely electrically conductive. The IV behavior collected using MVs resembles IV curves generated for bacterial nanowires, suggesting that these structures share electron transport components that appear to serve as semiconductors. Additional analyses using a variety of techniques are required to more fully characterize the conductive properties of MVs. A more complete description of the conductive properties of nanowires that tether MVs to intact cells is currently under review and therefore not included in this manuscript.

DISCUSSION

Membrane vesicles are common in Gram-negative bacteria where they apparently serve multiple roles, including delivery of hydrolytic enzymes involved in microbial pathogenesis and transfer of genetic information (Kadurugamuwa & Beveridge, 1995; Kadurugamuwa & Beveridge, 1996; Kolling & Matthews, 1999; Yaron *et al.*, 2000; Renelli *et al.*, 2004). Most research targeting bacterial MVs has focused on organisms of pathogenic concern (Nowotny *et al.*, 1982; Kadurugamuwa & Beveridge, 1997; Keenan & Allardyce, 2000). Our study suggests that MVs produced by metal-reducing bacteria such as *Shewanella* demonstrate redox reactivity with potentially significant biogeochemical and palaeontological implications.

Our work represents the first report of the enzymatic reduction and transformation of heavy metals and radionuclides by MVs produced by metal-reducing bacteria. Results from metal reduction assays demonstrate that MVs recovered from strain CN32 using established purification protocols contained proteins and cytochromes necessary for coupling the oxidation of hydrogen to the reduction of multivalent metals. SDS-PAGE and in-gel heme staining confirmed the presence of numerous cytochromes that may facilitate electron flow (Wigginton *et al.*, 2007) to heavy metals and radionuclides. The precipitation of reduced iron, uranium and technetium on the external surface of purified MVs suggest that terminal reductases are located on these extracellular structures, although additional studies are needed to confirm this hypothesis.

Controversy remains regarding the formation of biogenic nanofossils and, indeed, whether nanofossils exist at all (McKay *et al.*, 1996; Bradley *et al.*, 1997; Steele *et al.*, 1998). A full understanding of the many possible biogeochemical processes leading to formation of putative nanofossils is obviously lacking. Although laboratory results and observations presented here are certainly limited with regard to their environmental relevance, they allow us to hypothesize that MVs produced by *Shewanella* and perhaps other metal-reducing

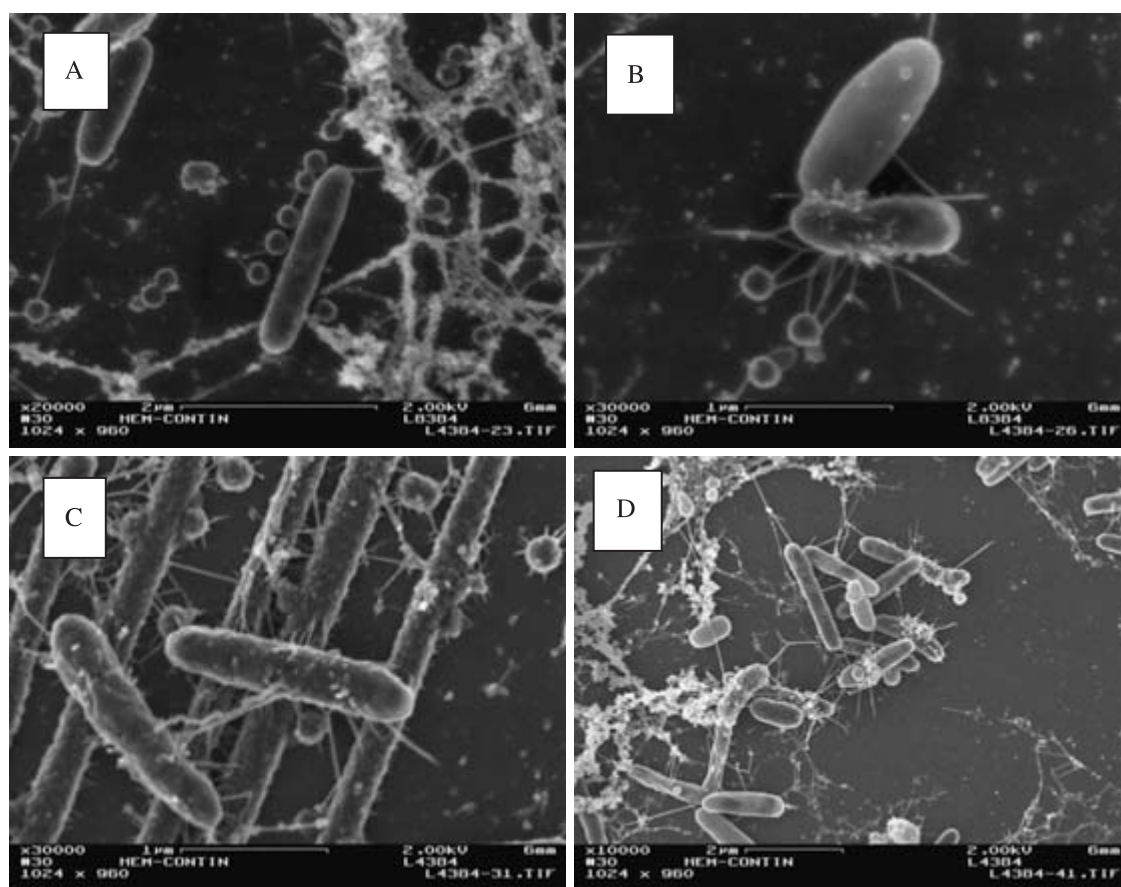


Fig. 7 Scanning electron microscopy images of *Shewanella oneidensis* strain MR-1 cultivated with tabular hematite. (A) Membrane vesicles (MVs) were commonly associated with cell surfaces. (B) Nanowires typically tethered membrane vesicles to each other or to intact cells. (C) MVs appeared to preferentially associate with secondary precipitation features found on surfaces of the natural tabular hematite crystals. (D) MVs were typically absent from areas of the biofilm that colonized the surface of the glass microscope slide that supported the specular hematite.

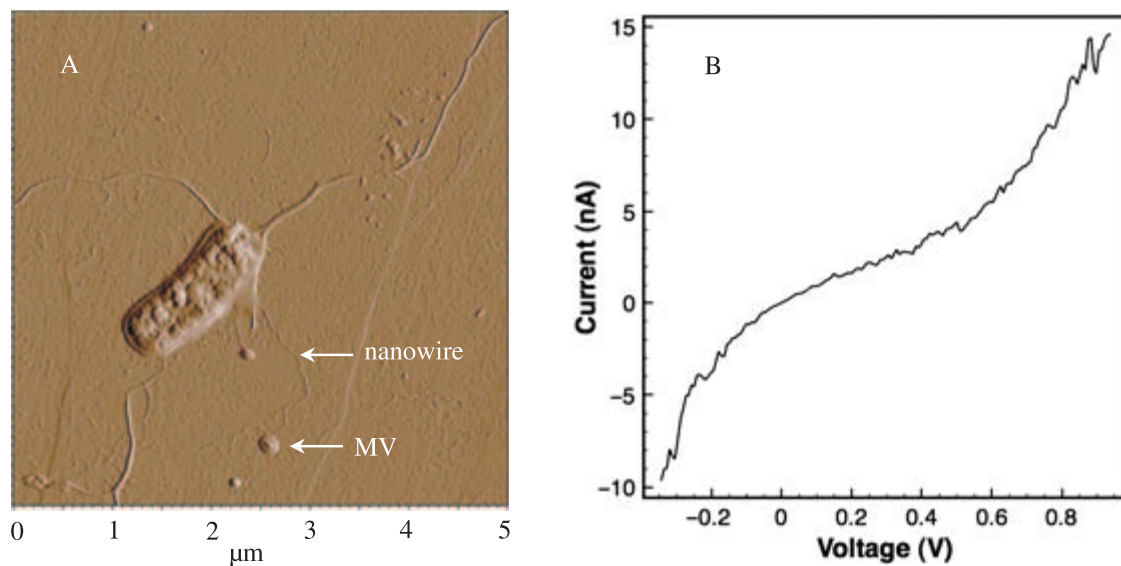


Fig. 8 'AFM tapping mode amplitude error' image of *Shewanella oneidensis* strain MR-1 on graphite. (A) Here a Membrane vesicle is connected to an intact cell by nanowires. (B) A representative IV curve collected using C-AFM.

bacteria may play a role in reductive transformation of heavy metals and radionuclides and may contribute to the formation of nanofossils in natural environments.

MVs are derived from blebs in the outer membrane in Gram-negative bacteria. Hence, a compositional relationship with the outer member should be expected and a functional role could be inferred. The external surface of *Shewanella* species has received recent scientific attention that has yielded insights into the possible roles of MVs in cell respiration and metabolism. Sokolov *et al.* (2001) reported that the cell surface of strain CN32 is characterized by a heterogeneous distribution of charged groups. Electrostatic force microscopy revealed that negatively charged areas of approximately 50 nm were distributed over the surface of the bacteria at pH 7 and above. Topographical examination using atomic force microscopy confirmed that these negatively charged areas corresponded with raised areas or blebs. Presumably, these negatively charged areas would be preferential sites for sorption of cations such as ferrous iron, the product of iron respiration. Recent results by Liu *et al.* (2001) confirmed this hypothesis. When Fe(II) was added to anaerobic suspensions of CN32 at pH 7, iron precipitates formed on cell surfaces that mimicked the size and distribution of negatively charged areas documented by Sokolov *et al.* (2001). The nature of the precipitates has yet to be determined, but low temperature Mössbauer spectra quantified the Fe(II):Fe(III) ratio at about 95:5 (R. Kukkadapu, Pacific North-west National Laboratory, unpubl. results). The presence of Fe(II) precipitates on cell surfaces partially inhibited iron reduction and growth of CN32 for a period of about 30 h (Liu *et al.*, 2001). Notably, cells released MVs, which were laden with Fe precipitates, as they recovered from the 30-h lag. These results suggest that MVs play a role in freeing cells of Fe(II) formed during iron respiration, but this has yet to be confirmed.

MVs may serve functional roles for metal-reducing bacteria. Prior to separating from the cell as fully developed MVs, blebs on the outer membrane could provide improved contact area with mineral surfaces, which are quite often topographically complex and heterogeneous with respect to charge distribution. Crystal defects, compositional imperfections, and natural weathering processes undoubtedly provide very rough terrains for attachment and reduction by metal-reducing bacteria. In principle, the large radius of curvature of bacterial surfaces relative to small length scales of roughness typically present at natural metal oxide surfaces would limit contact between the enzymatically active cell surfaces and their terminal electron acceptors.

Strain CN32 and the associated MVs possess only 'rough' LPS consisting of a short core oligosaccharide above the lipid A substituent, which suggests that the LPS mediates close bacterial attachment to metal oxides. The short core oligosaccharide could allow the outer membrane to closely associate with metal oxide surfaces while the phosphoryl and carboxyl groups of the core and lipid A moieties could electrostatically interact with available surface functional groups on the mineral.

Cytochromes *c* thought to be embedded in the outer membrane of *S. oneidensis* strain MR-1 are implicated in electron transport to solid phase iron and manganese (Myers & Myers, 2001, 2003) and apparently are present in preparations of MVs where they would serve a similar role. However, free MVs released from cells would not support cellular bioenergetics without some mechanism for transferring electrons from bacteria to the vesicles. Most metal-reducing bacteria can use exogenous sources of soluble redox-reactive compounds, such as anthraquinone disulphonate or natural humic substances, to mediate electron transfer between cells and metal oxides (Hernandez & Newman, 2001; Nurmi & Tratnyek, 2002; Royer *et al.*, 2002). Some reports reveal that *Shewanella* species produce small redox-reactive metabolites that might serve as electron shuttles to iron oxides (Newman & Kolter, 2000; Turick *et al.*, 2002, 2003; Lies *et al.*, 2005). Although many questions remain concerning the identity, production, release and use of electron shuttles by *Shewanella*, the potential for such compounds to transfer electrons from bacteria to redox reactive components of MVs does exist and warrants further investigation.

Shewanella produces electrically conductive appendages called bacterial nanowires, which have been hypothesized to provide an alternative strategy for extracellular transfer of electrons from cells to metal oxides and other solid phase electron acceptors (Gorby *et al.*, 2006). The microscopic analyses reported in the current study reveal that MVs are often linked to bacterial nanowires, and therefore it is possible to hypothesize further that MVs may facilitate electron transfer from bacteria to MVs to oxide surfaces. However, it is important to point out that conductivity of bacterial nanowires has only been reported perpendicular to the long axis. Measurements that confirm that electrons can flow along the length of a nanowire are needed to support the exciting hypothesis that nanowires can carry electrons from cells to MVs as part of a solid state extracellular electron transfer system.

The production of MVs by *Shewanella* in a nature or within mixed microbial communities has not yet been reported. However, MVs are common particulate features of the matrix of *Pseudomonas aeruginosa* biofilms (Schooling & Beveridge, 2006). Indeed, evidence for MVs in natural, mixed-community biofilms is now well established (Schooling & Beveridge, 2006) and may have been recognized for some time. Still, sentiments expressed by Dr Beveridge nearly a decade ago (Beveridge, 1999) remain true to all of us today: 'Because of their complexity, they must have slowly evolved into the systems we see today . . . we have just not recognized their importance until recently!' Much work remains before compelling conclusions can be drawn.

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