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ORIGINAL PAPER

Simple menaquinones reduce carbon tetrachloride and iron (III)

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Abstract Cell-free supernatant from *Shewanella oneidensis* MR-1 reduced carbon tetrachloride to chloroform, a suspension of Fe(III) and solid Fe(III) to iron (II). The putative reducing agent was tentatively identified as menaquinone-1 (MQ-1)—a water-soluble menaquinone with a single isoprenoid residue in the side chain. Synthetic MQ-1 reduced carbon tetrachloride to chloroform and amorphous iron (III) hydroxide to iron (II). To test the generality of this result among menaquinones, the reductive activities of vitamin K_2 (MQ-7)—a lipid-associated menaquinone with 7 or 8 isoprenoid residues—was evaluated. This molecule also reduced carbon tetrachloride to chloroform and iron (III) to iron (II). The results indicate that molecules within the menaquinone family may contribute to both the extracellular and cell-associated reduction of carbon tetrachloride and iron (III).

Keywords Carbon tetrachloride · Iron reduction · Biodegradation · Bioremediation · *Shewanella oneidensis* MR-1 · Menaquinones · Secreted molecules

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Introduction

Shewanella oneidensis MR-1 plays important biogeochemical roles in metal reduction due to its versatile respiratory chain (Nealson and Saffarini 1994). The mechanisms of Fe(III) reduction have received particular attention (Lovley et al. 2004; Maier and Myers 2004). Biochemical systems and molecules implicated in this process include: (i) outer membrane (OM) cytochromes components (Picardal et al. 1993; Beliaev et al. 2001; Myers and Myers 2002); (ii) electron transport component menaquinone-7,8 (vitamin K_2) (Petrovskis et al. 1995; Saffarini et al. 2002); (iii) secreted electron shuttle molecules (Newman and Kolter 2000; Rosso et al. 2003); and (iv) extracellular pillin appendages coated with decaheme-cytochromes (Gorby et al. 2005; Dohnalkova et al. 2005). Thus, in response to different environmental cues, cells of strain MR-1 and likely many cell types are able to create and manage networks of electron transfer that include both parallel and serial circuits and families of electron carriers that diffuse within the cell membrane, protrude from it, or are secreted. This article adds to this understanding by establishing that simple menaquinones may be secreted and can contribute to environmentally important reductions.

Molecules with quinone functional moieties have been previously implicated in the reduction of Fe (III). Newman and Kolter (2000) reported that addition of a menaquinone precursor, dihydroxynaphthoic acid

(DHNA), restored growth of *Shewanella oneidensi* MR-1 mutants deficient in menaquinone biosynthesis. They speculated that there exists a common biochemical mechanism for reduction of anthraquinone-2,6-disufonic acid ((AQDS) and humic acid by possibly a secreted small quinone-type compound either functions as an electron shuttle or as an intermediate in synthesis of secreted menaquiones. Nevin and Lovley (2002) proposed that some *Shewanella* species use secreted quinones for Fe(III) oxide reduction based on circumstantial evidence. Rosso et al. (2003) reported that *Shewanella* species could reduce Fe(III) oxides even when the oxides spatially separated from the cells as evidenced from atomic force microscopy images, indicating the presence of a soluble electron shuttle.

Molecules with quinone/hydroquinone functional moieties can transfer electrons from sulfide and iron species to humic compounds and nitroaromatics and to chlorinated molecules. Schwarzenbach et al. (1990) found that natural napthoquinone compounds, such as juglone and lawsone, are capable of reducing nitroaromatic compounds. Curtis and Reinhard (1994) discovered AQDS, pre-reduced by hydrogen, reductively transformed carbon tetrachloride.

Previously Petrovskis et al. (1995) tested a menaquinone-deficient mutant and found evidence that electron transport chain components played a role in the reduction and menaquinones were implicated as the active agents responsible for carbon tetrachloride reduction. Ward et al. (2004) tested carbon tetrachloride reduction by a suite of menaquinone-deficient mutants. For cells that were aerobically grown, a derivative of dihydroxynaphthoic acid (DHNA), a compounds derived from the menaquinone biosynthesis, was inferred as the likely agent of transformation. DHNA molecule is a precursor for menaquinone biosynthesis. Their result again pointed to the existence of a small molecule with simple quinone chemical structure active for electron transfer. They speculated that this simple quinone molecule might be involved for carbon tetrachloride transformation. However no structural information has been obtained on the simple menaquinone.

To gain insight into the mechanism of carbon tetrachloride reduction and iron (III) reduction by *Shewanella oneidensis* MR-1, we further investigated on the nature of the possible secreted quinone compound(s). In this article, we report reduction of carbon tetrachloride by reduced supernatant from aerobically grown MR-1 wild-type cells. In previous studies, analytical determination and comparison of the supernatant from the wild type and mutant cultures suggested that the secreted factor was a simple menaquinone (Ward et al. 2004). To confirm this activity, we synthesized and tested reduction activities of menaquinone-1 (MQ-1) and of menaquinone-7,8 (vitamin K_2). Both molecules were able to reduce carbon tetrachloride and soluble and insoluble Fe(III) oxides. The results have implications for bioremediation of metals and chlorinated solvents, and add to a rapidly growing body of knowledge regarding the physiology of *Shewanella oneidensis* MR-1.

Materials and methods

Extraction of culture supernatant and putative identification of the secreted menaquinone

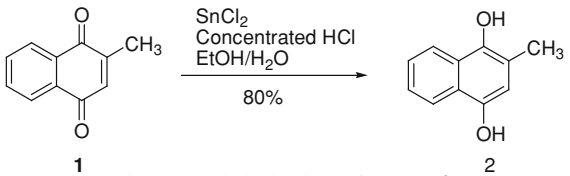
Shewanella oneidensis MR-1 (ATCC no. 700550) cultures were routinely maintained on LB agar. Strain MR-1 was grown aerobically in LB broth to an OD_{600} of 0.7–0.8. The cell suspension was then centrifuged at 10,000 rpm, and the supernatant filtered through a 0.2 μm filter. The filtered supernatant was then extracted with 2:1 chloroform:methanol (20 ml × 3) using a separatory funnel. Solvent aliquots used for extraction were pooled in a 500-ml round bottom flask. The aqueous solution was then extracted a second time with 1:2 chloroform:methanol (20 ml × 3). All extracts were combined in the 500 ml flask. Solvent was then removed using a Büchi rotorvapor (Brinkman Instruments). The residue remaining after solvent removal was then analyzed by thin layer chromatography (TLC) with 1,4-dihydroxynaphoic acid and menaquinone as reference compounds.

A UV active spot was observed between DHNA and menaquinone. This fraction that showed UV adsorption on fluorescent TLC plate was tested for transformation of carbon tetrachloride or iron reduction and subjected to further liquid chromatography-mass spectrometry (LC-MS) and time of flight mass spectrometry (TOF-MS) analysis. The putative structure was then synthesized to determine whether it would indeed exhibit activity for reduction of carbon tetrachloride and iron (III) species.

Synthesis of menaquinone-1 (MQ-1)

Based on the elucidated putative structure of MQ-1, the compound was synthesized and purified. Unless otherwise noted, materials were purchased from commercial suppliers without further purification. The following chemicals were purchased from Aldrich (Milwaukee, WI): sodium hydrosulfite, 1,4-naphthoquinone, 1,4-dihydroxynaphic acid (DHNA), 1-chloro-3-methyl-2-butene and menaquinone (vitamin K_2).

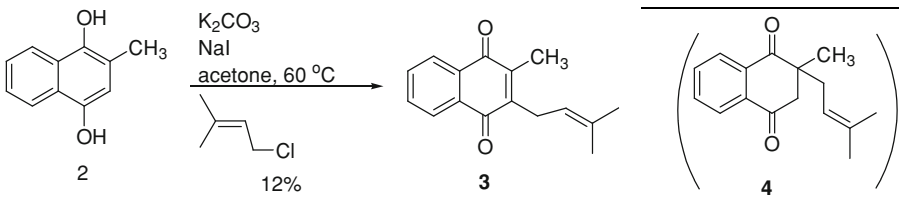
2-Methyl-1,4-naphthohydroquinone 2



1 2

2-methyl-1,4-naphthohydroquinone **2** was prepared from commercially available 2-methyl-1,4-naphthoquinone **1** according to literature procedure (Fieser et al. 1939).

2-Methyl-3-prenyl-1,4-naphthoquinone 3

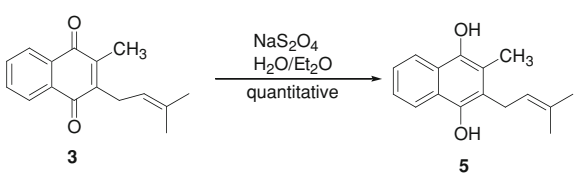


2 3

4

To a solution of 2-methyl-1,4-naphthohydroquinone **2** (500 mg, 2.87 mmol) in 20 ml of dry acetone was added sodium iodide (1.3 g, 8.61 mmol), potassium carbonate (400 mg, 2.87 mmol) and prenyl chloride (0.29 ml, 2.58 mmol). The reaction mixture was heated at 60 °C for 20 h. The organic solvent was removed under vacuum. Flash chromatography (petroleum ether/diethyl ether 9:1) yielded 74 mg (12%) of product **3** as yellow oil. The major by-product is tentatively assigned as stereoisomer **4.**

2-Methyl-3-prenyl-1,4-naphthohydroquinone 5



3 5

To a solution of 2-methyl-3-prenyl-1,4-naphthoquinone **3** (40 mg, 0.17 mmol) in 5 ml of diethyl ether was added a solution of sodium hydrosulfite (400 mg, 2.3 mmol). The biphasic solution was stirred until the yellow color disappeared (ca. 12 h). The reaction mixture was extracted with degassed diethyl ether (3 × 10 ml). The combined extracts were dried over sodium sulfate. The organic solvent was removed under vacuum to yield 40 mg (quantitative) of product **5** as a white solid. (Note: Product **5** must be stored under argon. Naphthohydroquinone **5** can be oxidized to naphthoquinone **3** when exposed to air. The yellow naphthoquinone **3** can be removed by washing with degassed hexane.)

Preparation of an amorphous ferric hydroxide suspension

To evaluate reduction of solid iron (III) species by *Shewanella oneidensis* MR-1, a suspension of amorphous ferric hydroxide or hydrous ferric oxide (HFO) was prepared using the protocol of McCormick and Adriaens (2004). Amorphous ferric hydroxide was synthesized by neutralizing a 0.17 M solution of $FeCl_3 \cdot 6H_2O$ to pH 7 with 5 M NaOH. A thick red slurry of amorphous ferric hydroxide formed. After the slurry had ripened for 2 h, it was centrifuged for 15 min at 7,000 rpm, the supernatant decanted, and the solids washed with Milli-Q water and re-centrifuged. The solids were washed seven times to remove chloride from the amorphous ferric hydroxide. The final slurry was kept in a refrigerator, and maintained in a closed container sealed with paraffin film. The Fe(III) content of the amorphous ferric hydroxide was analyzed according to a procedure described later.

Reduction of ferric iron by MQ-1 and vitamin K_2

Methanol stock solutions of MQ-1 (100 mM) or vitamin K_2 (1 mM) were prepared by dissolving

40 mg of synthetic MQ-1 in 1.67 ml oxygen-free methanol or 2 mg of purchased vitamin K_2 in 4.4 ml oxygen-free methanol respectively. Solutions were stored anaerobically in sealed amber glass vials. Prior to use, the vitamin K_2 stock solution was reduced by bubbling with hydrogen gas (99.99%) in the presence of palladium shot (Aldrich-Sigma) (Curtis and Reinhard 1994).

To evaluate reduction of soluble Fe (III) by reduced MQ-1 and vitamin K_2, 8–20 μl of reduced MQ-1 and 100 μl of reduced vitamin K_2 stock solutions were added to a 1-ml oxygen-free water containing 50 μM ferric chloride and buffered at pH 7 by 5 mM HEPES to give final concentrations of reduced MQ-1 and reduced vitamin K_2 in the range of 100–1000 μM. To assay reduction of amorphous iron (III) hydroxide, 0.0015 g of the iron hydroxide suspension was mixed with 2 ml of oxygen-free water and spiked with MQ-1 or vitamin K_2 stock solutions, as described above. Reaction vials were then placed on a shaker inside the anaerobic chamber, and periodically sampled for Fe (II) assay. Control experiments are set up the same way except without the addition of the reduced MQ-1 or vitamin K_2. Fe (II) was measured by adding 100 μl of sample to 1 ml of ferrozine (1 g/l) in 0.05 M HEPES buffer. Absorbance was then measured at 562 nm using a UV-spectrophotometer ($UVIKON_{XL}$, BIO-TEK Instruments). A standard for Fe(II) was prepared from 1 g/l of $FeCl_2$. A standard curve was constructed for the range 0–100 mg Fe (II)/L. All Fe(II) measurement was done in triplicate.

The Fe(III) content of the amorphous ferric hydroxide was assessed after conversion to Fe(II) by the hydroxylamine method: 0.02 g of amorphous ferric hydroxide was digested in 5 ml of 0.5 M anoxic HCl for 1 h. 150 μl of the solution was added into 1,350 μl of hydroxylamine and bathed in 60°C water for 1 h to enable complete reduction. The measured Fe (III) content of the amorphous ferric hydroxide was 1.94 mmol Fe(III)/g.

Assay of carbon tetrachloride reduction

Cell-free supernatant of *Shewanella oneidensis* MR-1 was prepared from late exponentially grown culture. The cells were first removed by centrifuge and the supernatant was filtered again by 20 μm Amicon filters (Amicon, CA). The filtrate was reduced by reduced by bubbling with hydrogen gas in the presence of palladium shot before assay for carbon tetrachloride reduction. Control experiment was conducted using sterilized growth medium without cell incubation.

To test for reduction of carbon tetrachloride by the synthesized MQ-1 (in reduced form), 10 milliliter of anoxic phosphate-buffered water was added 100 μl of the extracted UV-active fractions (resolubilized in anoxic methanol) or to 10 μl of 1 mM solutions of MQ-1 in reduced form (which gives a 1 μM MQ-1 concentration in reaction vial) or pre-reduced vitamin K_2 in 11.6 ml serum vials, crimp sealed, then spiked with carbon tetrachloride stock solution to give an initial liquid phase concentration of carbon tetrachloride of $\sim$1 μM, and placed on a shaker.

Concentrations of carbon tetrachloride and chloroform were periodically determined by injecting 50 μl headspace gas samples from the sealed serum vials onto an Agilent 6890 N gas chromatograph outfitted with an HP-5 Siloxane column (30 m: 0.32 mm:0.25 μm), a flame ionization detector (FID) and ChemStation (Palo Alto, CA). The instrument was operated at the following settings: oven isochratic at 45°C, detector at 300°C, column pressure at 15 psi, nominal initial flow of 3.7 ml/min, and average velocity of 52 cm/s. The total concentration of carbon tetrachloride and chloroform in the reaction vial were calculated based on Henry's Law. Quantification of carbon tetrachloride and chloroform were based on five-point external calibration carves. The standards were prepared adding appropriate methanol stock solution to sterilized medium in vials having the same headspace-liquid ratio as the vials in the tests.

Results

Carbon tetrachloride transformation by cell-free supernatant

Cell free supernatant was tested for existence of agents capable of carbon tetrachloride transformation. Carbon tetrachloride was added to supernatant prepared by filtration of cell cultures collected at late exponential growth and subsequently reduced by hydrogen gas. Carbon tetrachloride reduction occurred with concurrent and approximately stoichiometric production of chloroform (Fig. 1). Control experiment with sterilized

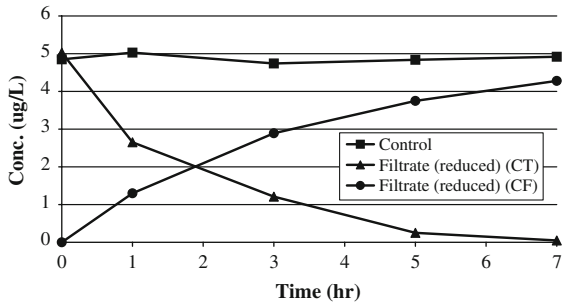


Fig. 1 Transformation of carbon tetrachloride by cell free supernatant of *Shewanella oneidensis* MR-1 (control experiment used sterilized growth medium without cell inoculation)

growth medium without cell incubation did not show activity for carbon tetrachloride transformation.

Putative identification of the secreted compound as a simple menaquinone

In order to characterize the identity of the active agent(s) in the supernatant that exhibited carbon tetrachloride reduction activity, a combination of several analytical techniques were utilized. Thin layer chromatography (TLC) was conducted using Silica Gel F_{254} plate. Spotting was performed with both ultra violet light (254 nm) and ceric ammonium molybdate solution. The remaining residue was then subjected to silica gel flash column separation with methanol:ethyl acetate (1:1) as solvent. The elution was then fractionated and analyzed a second time with TLC, and fractions with the same Rf, the distance a compound traveled divided by the distance of the eluting solvent front, were combined. The TLC results of the extract were similar to that of Newman and Kolter (2000). TLC revealed a UV active spot that migrated between menaquinone (vitamin K_2) and 1,4-dihydroxynaphic acid (DHNA). This fraction was tested for transformation of carbon tetrachloride or iron reduction and subjected to further LC-MS analysis for structural characterization.

Mass spectroscopy was first conducted on a ThermoFinnigan LCQ ion trap LC-MS with a Surveyor analytical HPLC and photodiode array UV detector (PDA). A strong peak was detected 14 minutes into the LC run, with adsorption maxima at 220, 260 and 350 nm confirming detection of isoprenoid quinones (Collins 1985). The electrospray mass spectrum contained several mass peaks with $m/z < 245$. In order to determine the accurate molecular weight of the UV active peak, the UV active fraction collected from column chromatography that also corresponded to the UV active spot on the TLC plate was analyzed further with a Micromass Q-TOF hybrid quadruple-time of flight LC-MS. The molecular weight determination was determined as 240.12 and the database equipped with the instrument give a molecular formula of $C_{16}H_{16}O_2$. Time of flight measurement provides precise mass of the analyzed compound and in this case only one possible formula matched the molecular weight. A structure that matches all the analytical characterization is 2-methyl-3-prenyl-1,4-naphthoquinone, MQ-1 (Fig. 2).

Carbon tetrachloride transformation by MQ-1 and vitamin K_2

Synthesized MQ-1 was assayed for reduction of carbon tetrachloride. As shown in Fig. 3, the reduced form of MQ-1, a hydroquinone, reduced carbon tetrachloride, with concomitant formation of chloroform.

The ability of the reduced MQ-1 to reduce carbon tetrachloride led us to question whether the final product of the menaquinone biosynthetic pathway—menaquinone-7,8 (vitamin K_2)—might possess similar activity. As indicated in Fig. 3, approximately the

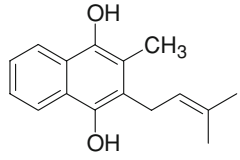


Fig. 2 Structure of menquinone-1 (MQ-1) (reduced form)

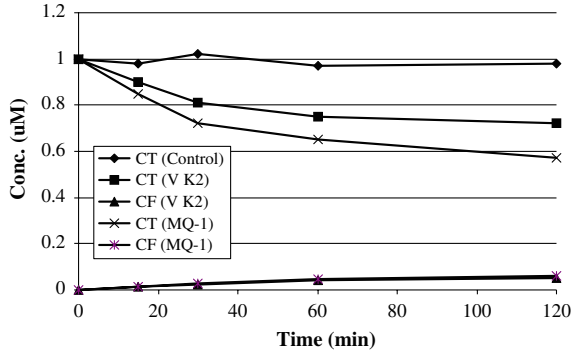


Fig. 3 Transformation of carbon tetrachloride by menaquinone-1 (MQ-1) and vitamin K_2

same fraction of the carbon tetrachloride was recovered as chloroform (18% for vitamin K_2; 15% for MQ-1). While no effort was made to identify other products, carbon tetrachloride is normally reduced by parallel and competing pathways, so multiple end products would be expected (Criddle and McCarty 1991).

Fe (III) reduction by MQ-1 and vitamin K_2

Iron (III) reduction was first tested for soluble iron (III) species. The reduction of Fe (III) was monitored through the formation of iron (II) by ferrozine. The results indicated that reduction of Fe (III) to Fe (II) does occur (Fig. 4). Moreover, iron (II) formation increased with increasing MQ-1 concentration.

MQ-1 was then tested for its ability to reduce amorphous iron (III) oxide and was found able to do so. Iron (II) production was detected with ferrozine (Table 1). A decline in Fe(II) followed its production is likely duo to adsorption to amorphous oxides (Roden and Zachara 1996; Urrutia et al. 1998). As observed for soluble Fe(III), increasing concentration of added MQ-1 resulted in increased production of iron (II).

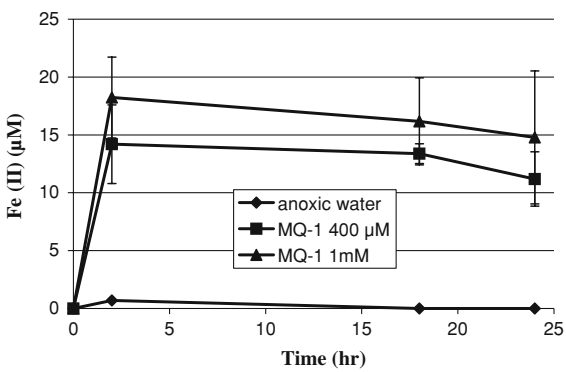


Fig. 4 Reduction of soluble iron (III) by MQ-1 (error bars represent one standard deviation of triplicate measurements)

Table 1 Fe(II) production from reduction of amorphous iron (III) oxide reduction by MQ-1

Samples	Fe (II) (μM)	
	2 h	4 h
Anoxic water (control)	1.9[a] ± 1.1[b]	2.3 ± 0.8
MQ-1 400 μM	74.3 ± 28.0	64.2 ± 21.9
MQ-1 1000 μM	136.7 ± 19.0	122.2 ± 18.8

Note: Time zero samples did not contain Fe(II). [a] Average value of triplicate measurements; [b] one standard deviation

Discussion

Previously Ward et al. (2004) found that aerobically grown wild type MR-1 cells reduced carbon tetrachloride, but mutants unable to produce the menaquinone precursor DHNA (*menF* and *menB* mutants) showed a distinctly different CT transformation profile than mutants able to produce DHNA, but unable to produce menaquinone (*menA* and *menG* mutants). While DHNA did not reduce CT in an abiotic assay, the addition of DHNA to the *menF* and *menB* mutants restored normal CT transformation activity. The authors concluded that a compound derived from the menaquinone pathway or DHNA derivative was likely involved and that it was probably a quinone type compound.

In subsequent experiments with wild-type cells, we found that cell-free supernatant could mediate the reduction for carbon tetrachloride, indicating that the activity was extracellular (Fig. 1). Since no such transformation was observed using sterilized growth medium, this result indicated that the reducing agent(s) existed in the supernatant obtained from incubation of *Shewanella oneidensis* MR-1 and that such reducing agent(s) was excreted from the cells. Thin layer chromatography analysis of the extract of wild-type supernatant revealed a UV active spot that migrated between menaquinone (vitamin K_2) and 1,4-dihydroxynaphic acid (DHNA).

Newman and Kolter (2000) previously presented evidence that a molecule secreted by strain MR-1 was likely a small quinone. In previous study, analytical determination and comparison of the supernatant from the wild type and mutant cultures suggested that the secreted molecule is a simple menaquinone (Ward et al. 2004). Accurate measurement of the molecular weight by time of flight mass spectrometry led to the putative structural identification of this molecular as menaquinone-1, MQ-1.

Several organisms reductively transform carbon tetrachloride via cometabolic mechanisms involving secreted molecules. *Pseudomonas stutzeri* KC secretes pyridine-2,6-bis-thiocarboxylate, a molecule that when chelated with copper transforms carbon tetrachloride to

carbon dioxide and other dechlorinated end products (Criddle et al. 1990a, b; Dybas et al. 1995; Lee et al. 1999). Cell exudates from *Methanosarcina thermophila* also reduce carbon tetrachloride, with the chloroform as the major product. Metal chelators or porphorinogens are implicated as possible agents responsible for the carbon tetrachloride transformation (Novak et al. 1998; Koons et al. 2001; Baeseman and Novak 2001).

In general, chloroform is a common reduction product of carbon tetrachloride, and is observed with *Escherichia coli K12* (Criddle et al. 1990a, b) and other common bacteria (Criddle and McCarty 1991). This may be due to the ubiquity of menaquinone within microorganisms. Vitamin K_2 is widely distributed in the cellular membranes of microbial populations such as the *Enterobacteriaceae* family, for example *Bacteroides fragilis,* bifidobacteria, clostridia and *Staphylococcus faecalis* (Bentley and Meganathan 1982).

The carbon tetrachloride transformation activity by the wild-type MR-1 cell supernatant demonstrated the existence of reducing agent(s) excreted from the cells. Our results indicated that secreted simple menaquinone from MR-1 is capable of reducing carbon tetrachloride. The supernatant exhibited slight different transformation profile for carbon tetrachloride from that of MQ-1 and vitamin K_2. It is possible that other reducing factors exit in the supernatants as well.

A variety of bacterial species reduce iron (III) (Lovley et al. 2004). Circumstantial evidence indicated that small secreted quinone compounds functioned as extracellular electron shuttles between the solid mineral surface and cells. Added AQDS facilitated reduction of different metals by *Geobacter and Shewanella* species (Rosso et al. 2003; Bentley and Meganathan 1982). The reductive activity of MQ-1 and vitamin K_2 toward carbon tetrachloride led us to investigate whether this activity would also extend to iron (III) species.

While it has been reported that addition of vitamin K_2 restores the reduction of solid iron (III) by mutants of *Shewanella putrefaciens* deficient in menaquinone biosynthesis (Saffarini et al. 2002) demonstrating vitamin K2 can function as electron transfer molecule, tests of vitamin K_2 in this work indicate that reduced vitamin K_2 can directly reduce amorphous ferric hydroxide. As shown in Table 2, up to 35 μM ferrous iron was produced after two hours of reaction.

Table 2 Fe(II) production from reduction of amorphous ferric hydroxide by vitamin K_2

Samples	Fe (II) (μM)	
	1 h	2 h
Anoxic water (control)	0.5 ± 1.0	4.7 ± 1.6
Vitamin K2 100 μM	27.5 ± 17.8	34.7 ± 9.6

Note: Time zero samples did not contain Fe(II)

In this experiment, only soluble iron (II) was measured which undoubtedly underestimates the actual amount of Fe(II) produced. However the result clearly demonstrated that MQ-1 is capable of reducing iron (III).

We conclude that MQ-1 or a molecule structurally similar to MQ-1 is produced by strain MR-1. This molecule is able to reduce carbon tetrachloride, a suspension of Fe(III) and Fe(III) oxide solids. This capability extended to vitamin K_2, a lipid-associated menaquinone and a component of some membrane-associated electron transport chains. This suggests that these capabilities are likely a general property of molecules in the menaquinone family. The ubiquity of these molecules may help to explain the widespread ability of microorganisms to reduce carbon tetrachloride and iron oxides in the presence of an electron donor.

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