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A derivative of the menaquinone precursor 1,4-dihydroxy-2-naphthoate is involved in the reductive transformation of carbon tetrachloride by aerobically grown *Shewanella oneidensis* MR-1

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Abstract Transformation of carbon tetrachloride (CT) by *Shewanella oneidensis* MR-1 has been proposed to involve the anaerobic respiratory-chain component menaquinone. To investigate this hypothesis a series of menaquinone mutants were constructed. The *menF* mutant is blocked at the start of the menaquinone biosynthetic pathway. The *menB*, *menA* and *menG* mutants are all blocked towards the end of the pathway, being unable to produce 1,4-dihydroxy-2-naphthoic acid (DHNA), demethyl-menaquinone and menaquinone, respectively. Aerobically grown 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. We conclude that a derivative of DHNA, that is distinct from menaquinone, is involved in the reduction of CT by aerobically grown *S. oneidensis* MR-1. When cells were grown anaerobically with trimethylamine-N-oxide as the terminal electron acceptor, all the menaquinone mutants showed wild-type levels of CT reduction. We conclude that *S. oneidensis* MR-1 produces two different factors capable of dehalogenating

CT. The factor produced under anaerobic growth conditions is not a product of the menaquinone biosynthetic pathway.

Introduction

Carbon tetrachloride (CT) is a persistent environmental contaminant that can be reductively dehalogenated under anaerobic reducing conditions, either abiotically or biologically. The role of microorganisms in these processes has been studied and suggests that the dehalogenation mechanism may be species-, as well as condition dependent. Picardal et al. (1993) showed that CT could be degraded by the facultative anaerobe *Shewanella putrefaciens* 200, a microbe that can link respiratory proton translocation to the reduction of a variety of compounds, including nitrate, iron (III) oxides, trimethylamine-N-oxide (TMAO) and fumarate (Arnold et al. 1986; Picardal et al. 1995). The effect of growth with different electron acceptors was studied with respect to dehalogenation of CT in both *S. putrefaciens* 200 and *Shewanella oneidensis* MR-1 (previously *S. putrefaciens* MR-1). Dehalogenation of CT by *S. putrefaciens* 200 was inhibited in the presence of oxygen and nitrate but was unaffected when cells were grown on Fe(III), fumarate or TMAO (Picardal et al. 1995). In *S. oneidensis* MR-1 the highest rate of transformation was observed when cells were grown under iron (III)-respiring conditions. However, growth on nitrate, TMAO or fumarate did not inhibit the transformation process (Petrovskis et al. 1994). Petrovskis et al. (1994) also reported transformation of CT by *S. oneidensis* MR-1 using aerobically grown cells, although further conditions were unspecified.

In certain bacterial species the dehalogenation of CT results in the formation of carbon dioxide and other non-hazardous products. Reduction of CT by *S. oneidensis* MR-1 results in the formation of 20–25% chloroform (CF) (Petrovskis et al. 1994), a toxic compound that can persist in the environment. Consequently, understanding the different mechanisms of CT reduction by diverse

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bacterial species assumes considerable importance. Petrovskis et al. (1995) investigated the hypothesis that an anaerobic respiratory-chain component is responsible for the transformation of CT by *S. oneidensis* MR-1 and demonstrated that menaquinone-deficient mutants lost 90% of CT-transformation activity. We have investigated the role of menaquinone in the reductive dehalogenation of CT by strain MR-1 by constructing mutants unable to complete different steps in the menaquinone biosynthetic pathway. We provide evidence that a derivative of the menaquinone precursor 1,4-dihydroxy-2-naphthoate (DHNA) is responsible for the CT transformation activity observed in this organism after aerobic growth. This derivative is not menaquinone but an alternative end product of the menaquinone biosynthetic pathway. Our data also suggest that the reduction of CT by *S. oneidensis* MR-1 under anaerobic conditions involves a different molecule.

Materials and methods

Strains and growth conditions

Shewanella oneidensis MR-1 (ATCC no. 700550) was originally isolated from Lake Oneida, N.Y., USA (Myers and Nealson 1988). *S. oneidensis* MR-1 α , a spontaneous ampicillin-resistant derivative of MR-1, was isolated during this study. Cells for genetic manipulations were grown aerobically in LB broth (Miller 1972), with shaking at 30°C. Cells for use in CT transformation assays were grown either aerobically, or anaerobically at 30°C in LM medium [200 mg/l yeast extract, 100 mg/l peptone, 10 mM NaHCO₃, 10 mM HEPES pH 7.4 (Myers and Nealson 1988)]. The electron donor was 12 mM sodium lactate under either condition, while 4 mM TMAO was used as the anaerobic electron acceptor. TMAO was used under anaerobic conditions as mutants deficient in menaquinone were previously shown to retain growth using this electron acceptor (Myers and Myers 1993). Media was made anaerobic by gas exchange in an air-lock chamber filled with N₂. Ampicillin was included in the media at 50 μ g/ml, kanamycin at 50 μ g/ml and tetracycline at 10 μ g/ml, as required.

Construction of mutants

Preliminary sequence data for *S. oneidensis* MR-1, obtained from The Institute for Genomic Research (Rockville, Md., USA), was used to design primers for the amplification of internal fragments of the menaquinone genes by polymerase chain reaction. The *menA* forward (menAF) 5'-CGGGATCCCAAAGTGGCATGATCCCA-CC and reverse (menAR) 5'-CGGAATTCTACAGTACTTTGGC-CTGCGC primers were used to amplify a 410 bp fragment of *menA*. The *menB* forward (menBF) 5'-GGGGTACCAATACTC-GTTCTCCTCCGGC and reverse (menBR) 5'-CGGAATTC-TTATTGACCATGCCCATGGC primers were used to amplify a 400 bp fragment of *menB*. The *menF* forward (menFF) 5'-CGGGATCCAGATACCCCTAAGGTTGTCC and reverse (menFR) 5'-CGGAATTCAGATGGTGGCTTCTTCTTCG primers were used to amplify a 390 bp fragment of *menF*. Lastly, the *menG* forward (menGF) 5'-CGGGATCCCTTTTGGCGGTCAATCA-GC and reverse (menGR) 5'-CGGAATTCACAGGAATAGAG-GCAAGG primers were used to amplify a 260 bp fragment of *menG*. Restriction enzyme sites located at the 5'-end of each primer are shown underlined. These restriction sites were used to clone the amplified fragments into the 'suicide' vector pJP5603 (Penfold and Pemberton 1992). The resultant plasmids, pJPmenA, pJPmenB, pJPmenF and pJPmenG were transformed into *Escherichia coli*

S17-1 λ pir, then introduced into *S. oneidensis* MR-1 α by conjugation. Transconjugants were selected for on LB agar containing kanamycin and ampicillin. The constructs were designed such that a single homologous recombination event would insert the entire plasmid into the chromosome, thereby interrupting the targeted *men* gene. Insertions within the correct gene were confirmed by Southern blotting using the appropriate digoxigenin-labeled *men* gene as the probe.

Menaquinone determinations

S. oneidensis MR-1 and *men* mutant cells were grown either aerobically or anaerobically in 100 ml volumes. Cells were harvested by centrifugation for 10 min at 12,000 g after the cultures reached an OD₆₀₀ $\approx$ 2.0. Pellets were resuspended in 6 ml chloroform:methanol (2:1, v/v) and sonicated for 10 min. The solvent was removed and transferred to another vial. After three repetitions the extracts for individual samples were pooled. The solvent was then evaporated to dryness and the product resuspended in chloroform for thin layer chromatography (TLC). TLC was carried out by spotting samples from various extractions alongside a menaquinone (vitamin K₂) standard (Aldrich, Milwaukee, Wis., USA) on Silica Gel 60 F254 aluminum-backed plates (EM Science, Gibbstown, N.J., USA). Samples were eluted with a mixture of n-pentane and diethyl ether (80:20, v/v).

Biotic dechlorination assays

Dechlorination assays were performed using cell suspensions from both aerobically and anaerobically grown cultures. All manipulations were done in an anaerobic chamber (N₂:CO₂:H₂, 80:10:10) (Coy Laboratories, Ann Arbor, Mich., USA) unless otherwise indicated. Aerobically grown cells were harvested by centrifugation at an OD₆₀₀ of 1.8–2.0. Anaerobically grown cells were harvested by centrifugation in gas-tight centrifuge bottles at an OD₆₀₀ of 1.7–2.3. The cells were washed and resuspended in anaerobic LM medium. The resuspended cells from both cultures contained between 1.2 and 1.5 mg protein/ml. For dechlorination assays, 9.95 ml aliquots of the resuspended cells were dispensed into 10 ml serum vials (actual volume 11.6 ml) and sealed with a Teflon rubber stopper and an aluminum crimp cap. The dechlorination assays were initiated by addition of a CT stock solution (0.5 mM in methanol) (99.9+%, HPLC grade, Aldrich, Milwaukee, Wis., USA) to each vial to give a final CT concentration of 15 μ M. Concentrations of CT and CF were monitored over time by removing 50 μ l samples of vial headspace with a 250 μ l Pressure-Lok (Supelco, Bellefonte, Pa., USA) precision analytical gas-tight syringe. Samples were injected onto a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a J&W DB-1701 (30 mm x 0.32 mm x 0.25 μ m) column (Agilent, Folsom, Calif., USA), and operated isothermally at 40°C. Quantification of CT and CF was based on five-point external calibration curves. Standards were prepared by adding appropriate methanol stock solutions to sterilized anaerobic medium in vials having the same headspace:liquid ratio as the vials used in the assays.

The protein concentration of the resuspended cultures was determined based on a modified procedure of Bradford (Bollag et al. 1996). A 1 ml aliquot of resuspended culture was taken, 0.4 ml of 5 N NaOH was added and then the tube was heated for 10 min at 98°C. The liquid was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13,000 rpm for 10 min. Protein concentrations were then measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif., USA) using bovine serum albumin standards.

Abiotic dehalogenation assay

Abiotic dechlorination assays were carried out in 10 ml serum vials containing 9.95 ml LM medium. DHNA (Aldrich, Milwaukee,

Wis., USA) was added to the solution from a 5 mM stock solution (in methanol) to various final concentrations and the vials sealed with Teflon rubber stoppers and aluminum caps. CT was added outside the anaerobic chamber to a final concentration of 10 μ M using a syringe. Measurements were performed as above.

Results

Identification of the genes required for menaquinone biosynthesis in *S. oneidensis* MR-1

Studies on the enzymology of menaquinone biosynthesis have been performed predominantly in *E. coli*, where seven enzyme activities are currently known to be required (Fig. 1). The first enzyme in the pathway, isochorismate synthase (MenF), converts chorismate into iso-chorismate (Daruwala et al. 1996). Isochorismate, in the presence of α -ketoglutarate, is then converted into 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid

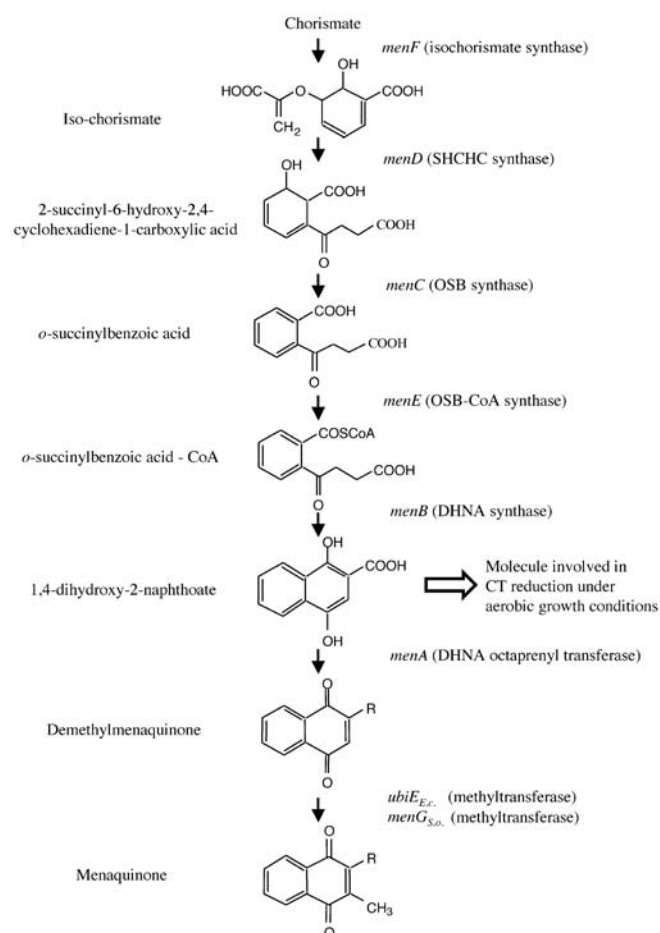


Fig. 1 Menaquinone biosynthetic pathway showing the main intermediate compounds and the gene/enzyme required for each step. The structures of the intermediates and the final product menaquinone are shown. A potential branch point in the pathway, where our results suggest that 1,4-dihydroxy-2-naphthoic acid (DHNA) may be utilized for the synthesis of other redox active molecules, is indicated

(SHCHC) by SHCHC synthase (MenD) (Palaniappan et al. 1992). The aromatic intermediate *o*-succinylbenzoic acid (OSB) is formed by the action of OSB synthase (MenC) (Sharma et al. 1993). OSB is then converted into DHNA via an OSB-CoA intermediate. These reactions are catalyzed by the OSB-CoA synthase (MenE) and DHNA synthase (MenB) enzymes, respectively (Sharma et al. 1992, 1996). DHNA octaprenyltransferase (MenA) then converts soluble DHNA into the membrane-associated, demethylated form of menaquinone (Suvama et al. 1998), which is ultimately converted into menaquinone by a methyltransferase (UbiE). This final methylation was previously thought to be performed by the MenG methyltransferase. The genes encoding the MenA-G enzymes were identified from the genome sequence of *S. oneidensis* MR-1 using the *E. coli* MenA-G proteins in homology searches. An *ubiE* homologue was also found in *S. oneidensis* MR-1 but was not analyzed further in this study.

The *menA* gene of *S. oneidensis* is predicted to encode a 291 amino acid protein with 32% identity to *E. coli* MenA. A protein of 300 amino acids, showing 42% similarity to *E. coli* MenB, is the predicted product translated from the *S. oneidensis* *menB* gene. The *menA* and *menB* genes are not located in close proximity to each other, or to the other *men* genes. The *menA* gene does not appear to be part of an operon. Surrounding the *menB* gene are an upstream open reading frame (ORF), transcribed in the same direction as *menB*, and a downstream gene transcribed divergently from *menB*. While the downstream gene showed similarity to the *glmS* gene of *E. coli*, the upstream gene showed no homology to known sequences.

The *menC*, *menD* and *menE* genes from *S. oneidensis* MR-1 appear to be part of an operon. The first gene in this operon shows homology to genes encoding type-*c* cytochromes and is followed by *menD*. The gene downstream of *menD* shows strong similarity to *yfbB*, which is located in the *men* operons of both *E. coli* and *Bacillus subtilis*. This gene was proposed to encode an enzyme required for menaquinone biosynthesis, albeit with a currently unknown function (Sharma et al. 1996). Downstream of the *yfbB* gene are the *menC* and *menE* genes, respectively. The *menE* gene is then followed by another ORF in the same transcriptional orientation. However, the proposed transcriptional start for this ORF is nearly 400 bp downstream of *menE*, suggesting that these genes might not be transcriptionally linked. The proposed MenC, MenD and MenE proteins from *S. oneidensis* are 374, 573 and 481 amino acids in length, and show 29%, 36% and 27% similarity to the *E. coli* proteins, respectively.

The *menF* and *menG* genes from *S. oneidensis* MR-1 do not appear to be part of operons. The genes both upstream and downstream of *menF* are transcribed in the opposite orientation to *menF*. No ORF was identified directly upstream of *menG* but one with similarity to *argI* was identified downstream of *menG*, transcribed in the same orientation. However, the start codon for this gene

was located approximately 130 bp downstream of the stop codon for *menG*, suggesting that the two genes are transcribed separately. In addition, a likely transcriptional terminator, with the sequence 5'-AAAGCCAGCTA-AGCTGGCTTT, was identified just downstream of the *menG* stop codon. The *menF* and *menG* genes are predicted to encode proteins of 359 and 161 amino acids, respectively. These putative MenF and MenG enzymes show 31% and 68% identity to the *E. coli* proteins, respectively.

Construction of *men* mutants

To study the effects of mutations that interrupt synthesis of menaquinone at different steps in the pathway, mutants in the *menF*, *menB*, *menA* and *menG* genes were constructed after cloning internal fragments of these genes into the kanamycin-resistance encoding 'suicide' vector pJP5603. The resultant constructs were then introduced into *S. oneidensis* MR-1 α by conjugation. Since the vector pJP5603 is unable to replicate in this background, kanamycin-resistant transconjugants could only be obtained if the constructs had inserted into the chromosome. The presence of an internal fragment of one of the *men* genes on each construct allows for the integration of the entire plasmid within the genomic copy of the *men* gene by a single recombination event. This approach results in two truncated copies of the targeted gene, interrupted by pJP5603, in the transconjugants. Since insertion mutations constructed in this manner may cause polar effects on downstream genes, those *men* genes known to be present in the *men* operon (*menC*, *menD* and *menE*) were not mutated using this method. Southern blots were performed on the *S. oneidensis* MR-1 α *menA*, *menB*, *menF* and *menG* mutants to ensure that the insertions had occurred at the correct sites. Growth of the mutants under aerobic conditions was shown to be similar to that of both the wild-type MR-1 and MR-1 α strains. To confirm that the *menF*, *menB*, *menA* and *menG* mutants were defective in menaquinone production, aerobic and anaerobic cell extracts were analyzed using TLC in comparison with wild-type extracts and a commercially available menaquinone standard (vitamin K₂). A spot that ran similarly to the menaquinone standard was present in the wild-type extracts but absent in the *menF*, *menB*, *menA* and *menG* mutant extracts under both conditions (data not shown).

Dehalogenation assays performed on cells grown aerobically

Assays were performed in triplicate with the wild-type MR-1 strain, the ampicillin-resistant derivative strain MR-1 α and the *menF*, *menB*, *menA* and *menG* mutants, grown aerobically. The MR-1 and MR-1 α strains reduced CT similarly under all conditions and therefore the results for the MR-1 α strain are not shown. While the *menA* and

Table 1 Observed pseudo second-order rate coefficients [$-\frac{dC}{dt} = k'CX$ where C = CCl₄ conc, X = protein conc (mg/ml), t = time(h)] for carbon tetrachloride transformation by *S. oneidensis* MR-1 and *men* mutants

Growth condition	Strain	k' (ml/mg protein-h)
Aerobic	MR-1	0.219±0.003
	MR-1 <i>menF</i>	0.011±0.001
	MR-1 <i>menF</i> + DHNA	0.104±0.002
	MR-1 <i>menB</i>	0.009±0.002
	MR-1 <i>menB</i> + DHNA	0.126±0.004
	MR-1 <i>menA</i>	0.216±0.005
	MR-1 <i>menG</i>	0.187±0.004
Anaerobic	MR-1	0.110±0.004
	MR-1 <i>menF</i>	0.093±0.005
	MR-1 <i>menB</i>	0.090±0.002
	MR-1 <i>menA</i>	0.087±0.005
	MR-1 <i>menG</i>	0.089±0.003

menG mutants had CT degradation capabilities similar to that of the MR-1 wild-type, the *menB* and *menF* mutants exhibited reduced CT transformation (Fig. 2a). Pseudo second-order rate coefficients are shown in Table 1. Control reactions performed with autoclaved cells showed no transformation of CT under any condition. Both the wild-type and the *menA* and *menG* mutant strains were shown to produce some CF during the assay (13–26%) (Fig. 2b). These results suggested that DHNA, an intermediate in menaquinone biosynthesis that is not produced by the *menF* or *menB* mutants, might be involved in the reduction of CT.

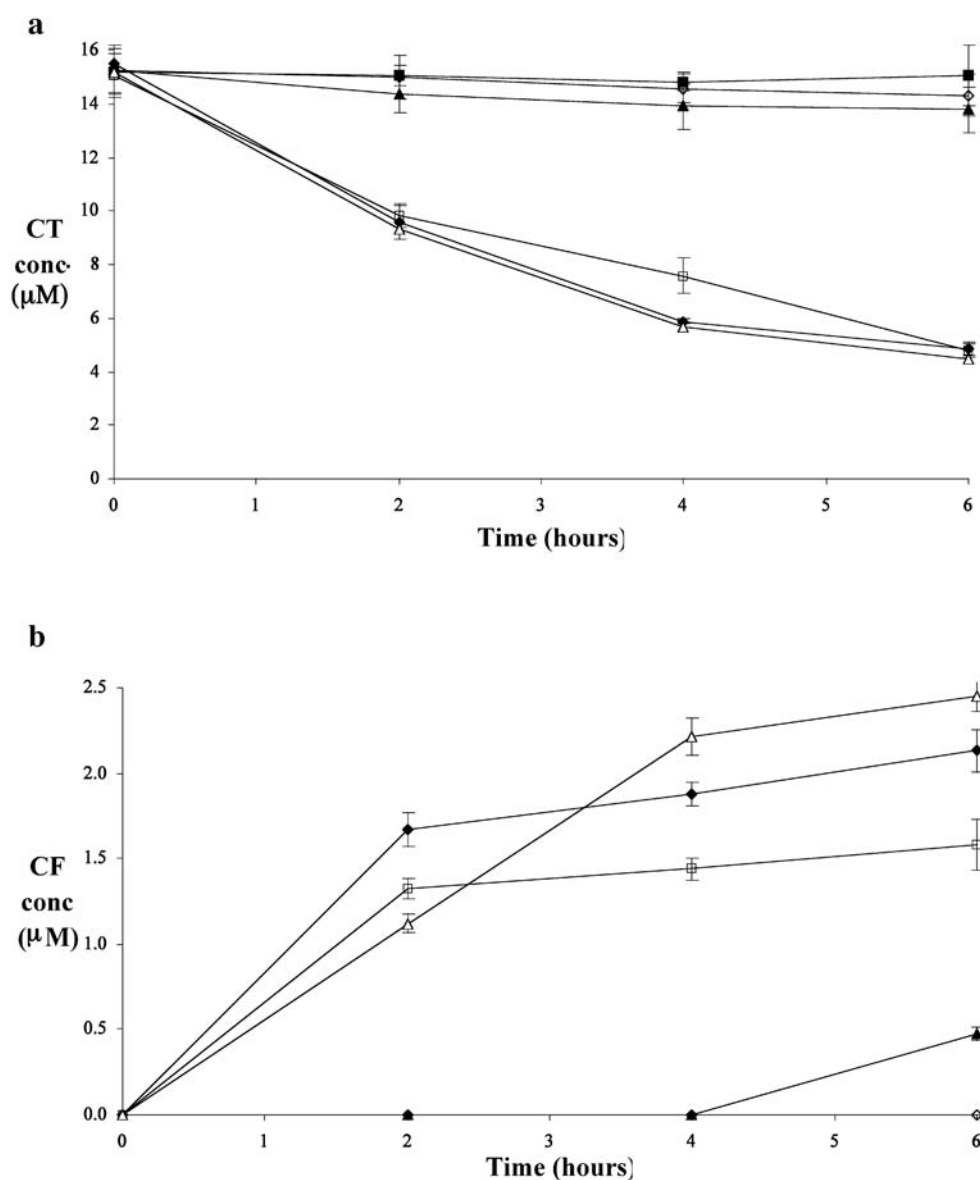
DHNA does not transform CT in an abiotic assay

To determine whether DHNA is directly involved in CT transformation, abiotic assays were conducted using different concentrations of DHNA (0.15–15 μ M). DHNA did not reduce CT in these abiotic assays. This result suggests that DHNA may not be the active dechlorinating agent. However, a derivative of DHNA could be the active molecule. This derivative could not be menaquinone since this molecule is not produced by the *menA* and *menG* mutants that transformed CT similarly to the wild-type MR-1.

Complementation of *men* mutants using DHNA

Since DHNA showed no CT transformation activity in the abiotic dechlorination assay, complementation tests were performed in order to determine if a derivative of DHNA might be responsible for CT transformation in aerobically grown cells. The addition of DHNA to aerobically growing cells restored the ability of the *menB* and *menF* mutants to degrade CT (Fig. 3). CF production was also restored to wild-type levels (not shown). These results indicate that a derivative of DHNA is most likely

Fig. 2a, b Transformation of carbon tetrachloride (CT) by aerobically grown cells. **a** Reduction of CT and **b** production of chloroform by: ■ no cells, △ *Shewanella oneidensis* MR-1, ◆ *menA* mutant, ◇ *menB* mutant, ▲ *menF* mutant, and □ *menG* mutant. Autoclaved cells did not show CT transformation activity (not shown). Error bars represent the standard deviation of triplicate samples



involved in the reduction of CT by *S. oneidensis* MR-1. An alternative possibility, that a cell-associated enzyme is responsible for DHNA-dependent CT transformation, was considered less likely based on preliminary results that indicated activity in a cell-free supernatant prepared from wild-type cells.

Dehalogenation assays performed on cells grown anaerobically

Additional CT transformation experiments were conducted in triplicate using cells grown anaerobically, with TMAO as the electron acceptor. Under these conditions all four of the menaquinone mutants, *menF*, *menB*, *menA* and *menG* demonstrated rates of dechlorination similar to those of wild-type MR-1 cells. The observed pseudo second-order rate constants with respect to CT concen-

tration are shown in Table 1. Chloroform was produced at similar levels (12–17%) by all strains. As previously, autoclaved cells showed no transformation activity.

Discussion

The reduction of the halogenated compound CT by different microorganisms involves different molecules. For example, corrinoids are responsible for CT transformation by the homoacetogen *Acetobacterium woodii* (Hashsham and Freedman 1999), while a secreted factor, pyridine-2,6-bis(thiocarboxylic acid) is responsible for CT dehalogenation in *Pseudomonas stutzeri* KC (Lee et al. 1999). Both cytochromes and menaquinone have been proposed to play a role in CT reduction by *Shewanella* species. The involvement of such different molecules is particularly important with respect to the products of the

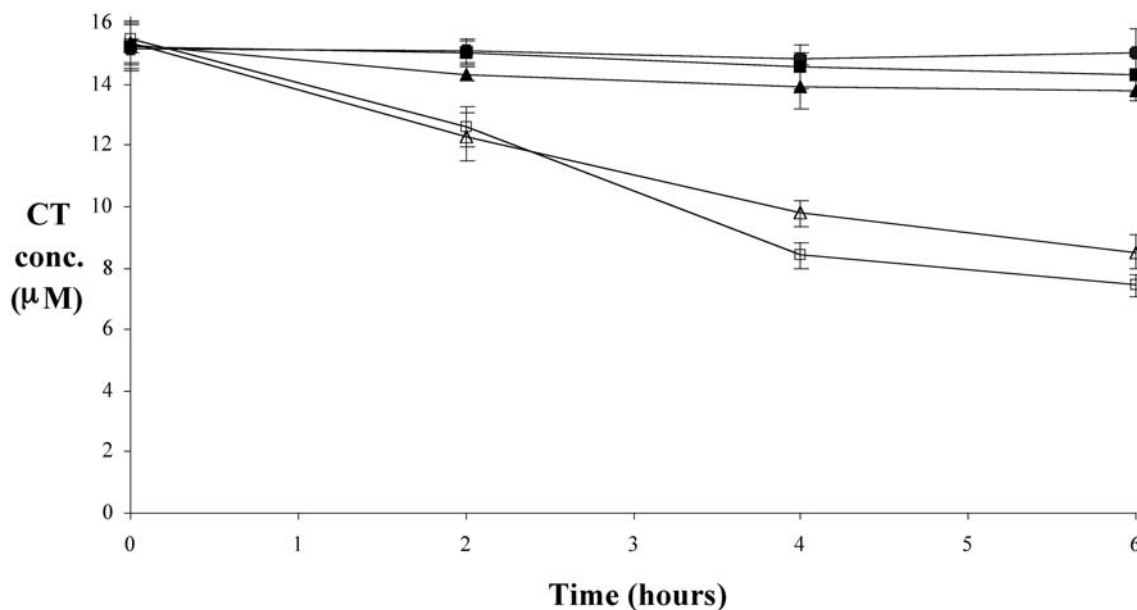


Fig. 3 Effect of DHNA on CT transformation by aerobically grown *menB* and *menF* mutants. The assays were conducted in the absence and presence of DHNA. ● no cells, ■ *menB* mutant, □ *menB*

mutant + DHNA, ▲ *menF* mutant, and Δ *menF* mutant + DHNA. Error bars represent the standard deviation of triplicate samples

transformation because many reactions convert an appreciable fraction of CT into the unwanted byproduct CF. Understanding the mechanism of this transformation might enable modification or manipulation of CT degradation to prevent or minimize CF formation. In these studies, we have focused on menaquinone because it was previously implicated in CT reduction by *S. oneidensis* MR-1 (Petrovskis et al. 1995).

The genes required for menaquinone biosynthesis in *S. oneidensis* MR-1 were identified from the whole genome sequence (made available by TIGR). Two methyltransferase genes (*menG_{S.o.}* and *ubiE_{S.o.}*), encoding the MenG and UbiE enzymes, were identified during this preliminary analysis. Our TLC analysis of extracts from the *S. oneidensis* MR-1 *menG* mutant indicated that this mutant does not produce menaquinone. Therefore, it appears likely that MenG, rather than UbiE, is required for menaquinone biosynthesis in *S. oneidensis* MR-1.

By constructing mutants that disrupt the menaquinone biosynthetic pathway at different steps, we demonstrated that the menaquinone precursor DHNA was a more likely candidate for transformation of CT than menaquinone by aerobically grown cells. This hypothesis is based on the observation that the *menF* and *menB* mutants (which do not produce DHNA) failed to transform appreciable amounts of CT, whereas the *menA* and *menG* mutants (which do produce DHNA but do not produce menaquinone), transformed CT similarly to the wild-type. However, DHNA (which is a reduced molecule) did not reduce CT in an abiotic assay, although, when added exogenously, it could complement both the *menF* and *menB* mutants to near wild-type levels of CT reduction (Table 1). Therefore, we propose that a derivative of

DHNA is required for the transformation of CT by *S. oneidensis* MR-1.

Our results implicate a DHNA derivative in the reduction of CT by *S. oneidensis* MR-1 cells grown aerobically. When cells were grown anaerobically, using TMAO as the terminal electron acceptor, the *men* mutants all transformed CT at rates similar to wild-type. Accordingly, we hypothesize that anaerobic growth induces production of a specific redox-active molecule (or more than one molecule) that can reduce CT. This molecule is presumably not produced under aerobic growth conditions, nor associated with products of the menaquinone biosynthetic pathway. We conclude that *S. oneidensis* MR-1 produces at least two molecules capable of dehalogenating CT. One molecule is produced under aerobic growth conditions and appears to be a derivative of the menaquinone precursor DHNA. The other molecule is produced under anaerobic growth conditions and its transformation activity is not dependent upon the products of the menaquinone pathway. The CT transformation mechanisms of both molecules resulted in the formation of CF.

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