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Involvement of cytochrome *c* CymA in the anaerobic metabolism of RDX by *Shewanella oneidensis* MR-1

Nancy N. Perreault, Fiona H. Crocker, Karl J. Indest, and Jalal Hawari

Abstract: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a cyclic nitramine explosive commonly used for military applications that is responsible for severe soil and groundwater contamination. In this study, *Shewanella oneidensis* MR-1 was shown to efficiently degrade RDX anaerobically ($3.5 \mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{g protein})^{-1}$) via two initial routes: (1) sequential N-NO₂ reductions to the corresponding nitroso (N-NO) derivatives (94% of initial RDX degradation) and (2) denitration followed by ring cleavage. To identify genes involved in the anaerobic metabolism of RDX, a library of ~2500 mutants of MR-1 was constructed by random transposon mutagenesis and screened for mutants with a reduced ability to degrade RDX compared with the wild type. An RDX-defective mutant (C9) was isolated that had the transposon inserted in the *c*-type cytochrome gene *cymA*. C9 transformed RDX at ~10% of the wild-type rate, with degradation occurring mostly via early ring cleavage caused by initial denitration leading to the formation of methylenedinitramine, 4-nitro-2,4-diazabutanal, formaldehyde, nitrous oxide, and ammonia. Genetic complementation of mutant C9 restored the wild-type phenotype, providing evidence that electron transport components have a role in the anaerobic reduction of RDX by MR-1.

Key words: RDX, explosive, nitramine, biotransformation, *Shewanella*.

Résumé : La nitramine cyclique hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) est un explosif couramment utilisé pour des applications militaires et qui cause une contamination sévère de sols et eaux souterraines. *Shewanella oneidensis* MR-1 a dégradé efficacement le RDX en condition anaérobie ($3,5 \mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{g protéine})^{-1}$) via deux routes initiales : (1) la réduction séquentielle du N-NO₂ en dérivés nitroso correspondants (N-NO) (94 % de la dégradation initiale du RDX); (2) dénitrification suivie du clivage de l'anneau du RDX. Afin d'identifier des gènes impliqués dans le métabolisme anaérobie du RDX, une banque d'environ 2500 mutants MR-1 fut construite par mutagenèse aléatoire par transposition et criblée à la recherche de mutants présentant une diminution de l'activité de dégradation du RDX par rapport à la souche sauvage. Un mutant (C9) fut isolé avec un transposon inséré dans le gène du cytochrome *c* *cymA*. C9 transformait le RDX à ~10 % du taux de dégradation de la souche sauvage. La dégradation s'effectuait principalement par dénitrification suivie du clivage de l'anneau et conduisait à la formation de méthylènedinitramine, 4-nitro-2,4-diazabutanal, formaldéhyde, oxyde nitreux et ammoniac. La complémentation génétique du mutant C9 a permis de restaurer le phénotype sauvage et d'ainsi confirmer que des composants de la chaîne de transport d'électrons ont un rôle à jouer dans la réduction anaérobie du RDX par MR-1.

Mots-clés : RDX, explosif, nitramine, biotransformation, *Shewanella*.

[Traduit par la Rédaction]

Introduction

The cyclic nitramine hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a major component of military high explosives and is used in a wide variety of munitions. The toxicity of RDX necessitates that contaminated soil and groundwater be remediated, preferably by low cost, environmentally friendly biological processes. Some aerobic bacteria of the order *Actinomycetales*, such as *Rhodococcus rhodochrous* 11Y (Seth-Smith et al. 2002) and *Rhodococcus* sp. strain DN22 (Fournier et al. 2002), are able to use RDX as the sole nitro-

gen source for growth, while RDX appears to be used as a source of energy, carbon, and nitrogen for the growth of *Williamsia* sp. strain KTR4 and *Gordonia* sp. strain KTR9 (Thompson et al. 2005). RDX was also used as the sole N source for the anaerobic growth of *Desulfovibrio* spp. (Boopathy et al. 1998). RDX-degrading *Shewanella* strains have been isolated from RDX-contaminated marine sediments (Zhao et al. 2005, 2006). *Shewanella halifaxensis* was shown to degrade RDX anaerobically, under trimethylamine *N*-oxide (TMAO)-respiring conditions. TMAO is a naturally occurring osmolyte found in marine environments that serves as a ter-

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Fig. 1. Schematic representation of the proposed pathway for anaerobic transformation of RDX. Route A consists of the sequential reduction of the N-NO₂ groups to produce the corresponding mono-, di-, and tri-nitroso derivatives MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine), DNX (hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine), and TNX (hexahydro-1,3,5-trinitroso-1,3,5-triazine), respectively. Route B consists of one or two denitration steps followed by ring cleavage to produce MEDINA (methylenedinitramine) or NDAB (4-nitro-2,4-diazabutanal), respectively. Route C involves denitrohydrogenation leading to the formation of the unstable 3,5-dinitro-1,3,5-triazacyclohexane (in brackets) whose decomposition leads to the formation of MEDINA. Adapted from Fournier et al. (2002) and Bhushan et al. (2002).

minimal electron acceptor in various bacteria (Barrett and Kwan 1985). Under these conditions, *S. halifaxensis* degraded RDX via a major route consisting of the sequential reduction of the N-NO₂ groups to produce the corresponding mono-, di-, and tri-nitroso derivatives hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), respectively (Fig. 1, route A), and a minor route involving denitration followed by ring cleavage (Fig. 1, route B) to produce methylenedinitramine (MEDINA) and its decomposition products formaldehyde (HCHO) and nitrous oxide (N₂O) (Halasz et al. 2002). A cytochrome *c*-552 purified from *S. halifaxensis* was shown to degrade RDX anaerobically via initial denitration followed by ring cleavage to produce MEDINA (Zhao et al. 2008), but other enzymes or cytochrome *c* proteins responsible for nitrosation have not been identified.

In a recent paper, the authors showed that polymerase chain reaction (PCR) failed to detect genes associated with RDX degradation (including the cytochrome P-450 gene *xplA*, hydrogenase gene *hydA*, organic nitrate reductase gene *onr*, and *Pseudomonas* flavoprotein xenobiotic reductases genes *xenA* and *xenB*) from RDX-contaminated groundwater samples (Fuller et al. 2010). Despite progress made towards understanding the degradation routes of RDX, the genetic determinants dictating the fate of RDX, particularly under anaerobic conditions, are still unclear. By analyzing the genomes

of 15 *Shewanella* strains, Zhao et al. (2010) observed that the RDX-degrading strains had a higher number of genes for cytochromes and nitrate or nitrite reductases. The iron-reducing bacterium *Shewanella oneidensis* MR-1 (Myers and Nealson 1988) has a complex electron transfer network and can utilize a variety of electron acceptors for anaerobic respiration, including Fe(III), Mn(IV), fumarate, nitrate, TMAO, and a variety of metals (Nealson and Saffarini 1994; Burns and DiChristina 2009). Because of its exceptional metabolic versatility and its potential use for the bioremediation of environmental contaminants, MR-1 has been extensively studied and its genome has been sequenced (Heidelberg et al. 2002). Although MR-1 was not isolated from an RDX-contaminated environment, it was recently proposed that MR-1 can also use RDX as an electron acceptor (Kwon and Finneran 2008) but the pathway for RDX degradation was not determined. Other Fe(III)-reducing bacteria such as *Geobacter metallireducens* and *Geobacter sulfurreducens* were shown to directly reduce RDX to its nitroso derivatives (Kwon and Finneran 2006). Additionally, cyclic nitramine transformation mediated by extracellular electron shuttles, such as humic substances or humic substances analog anthraquinone-2,6-disulfonate, is ubiquitous among the keystone Fe(III)-reducing microbial genera (Kwon and Finneran 2008, 2010). Electron shuttling compounds were shown to mediate RDX degradation via initial nitro group reduction (Bhushan et al. 2006; Kwon and Finneran 2006).

This study was undertaken to identify genes involved in the anaerobic metabolism of RDX by *S. oneidensis* MR-1. Transposon insertion mutants of strain MR-1 were isolated that had a reduced rate of RDX transformation compared with the wild-type MR-1 strain. The gene that was interrupted by the transposon insertion as well as the rate of RDX transformation and product distribution in the mutant were determined.

Materials and methods

Chemicals and culture media

RDX (99% pure), TNX, and MEDINA were provided by Defense Research and Development Canada, Quebec. MNX (98% pure), DNX, and 4-nitro-2,4-diazabutanal (NDAB) were purchased from SRI International, Menlo Park, California. All other chemicals were of reagent grade. *Shewanella oneidensis* MR-1 was purchased from the American Type Culture Collection (ATCC 700550). MR-1 was routinely maintained on Luria–Bertani (LB) medium. The minimal medium MMR2 used in this study was a modification of the medium described by Tang et al. (2007) and consisted of (per L of distilled water): 1.34 mmol·L⁻¹ KCl, 5 mmol·L⁻¹ NaH₂PO₄, 0.7 mmol·L⁻¹ Na₂SO₄, 1 mmol·L⁻¹ MgSO₄·7H₂O, 0.2 mmol·L⁻¹ CaCl₂·2H₂O, 50 mmol·L⁻¹ NaCl, 20 mmol·L⁻¹ PIPES, a vitamin mixture (Tang et al. 2007), and trace elements SL-10 (Widdel et al. 1983). MMR2 contained 28 mmol·L⁻¹ NH₄Cl and 25 mmol·L⁻¹ sodium lactate as the nitrogen and carbon source, respectively; TMAO (30 mmol·L⁻¹) was used as the terminal electron acceptor. Fumarate, Fe³⁺ (FeCl₃), and nitrate (NaNO₃) at 15 mmol·L⁻¹ were also tested individually as the sole electron acceptor. The pH was adjusted to 7.0 before sterilization through a 0.2 µm filter.

Construction and screening of the transposon library

A transposon library of MR-1 was generated by random mutagenesis using the EZ-Tn5 <R6Kγori/KAN-2> Tnp Transposome kit (Epicentre) according to the manufacturer's instructions. Briefly, freshly prepared MR-1 cells were transformed with 1 µL of the Tnp transposome. Transformants were initially selected on LB agar containing 50 µg kanamycin·mL⁻¹ (Kan 50) and then grown aerobically overnight in 96-well plates containing 1 mL of LB Kan 50. Glycerol was added at 15% v/v final concentration and the cultures were flash-frozen and kept at -80 °C until used. For the phenotypic screening, a MULTI-BLOT Replicator (V&P Scientific) was used to transfer culture inocula to a new 96-well plate filled with 1.5 mL of LB supplemented with 90 µmol·L⁻¹ RDX. Controls consisted of two wells inoculated with the wild-type MR-1 and two noninoculated wells. The plates were sealed with sterile breathable membranes and placed in anaerobic jars. The jars were purged with oxygen-free N₂ and incubated for 48 h at 30 °C in the dark. After centrifugation at 4000g for 10 min, the supernatants were transferred to 1 mL glass vials for RDX quantification. Kanamycin-resistant MR-1 strains showing a reduced RDX degradation activity were further characterized by identification of the disrupted gene, by RDX degradation rate, and by product distribution (described below).

Determination of the transposon insertion site

Genomic DNA was extracted from the mutant strain using the QIAGEN DNeasy Blood & Tissue kit. Five micrograms of purified DNA was added to 100 µL of Tris–EDTA (pH 8.0) and submitted to 200 passages through a 30-gauge hypodermic needle. DNA fragments of 6–8 kb in length were purified from agarose gel using the illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and eluted in MilliQ water. One microgram of purified DNA was blunt ended and 5'-phosphorylated using the End-it DNA End-Repair kit (Epicentre). The repaired DNA was self-ligated at 14 °C overnight with the T4 DNA ligase (Promega). Ligation products were transformed into freshly prepared electrocompetent *Escherichia coli* EC100D *pir*-116 (Epicentre) and plated on LB agar Kan 50. The transposon insertion sites from a number of kanamycin-resistant rescue clones were amplified by PCR using the transposon-specific primers KAN-2 FP-1 and R6KAN-2 RP-1 (Epicentre). Sequencing of the amplicons was performed at the McGill University Genome Quebec Innovation Centre (Montréal, Quebec, Canada). The identity of the DNA sequences was determined by a search of the NCBI nonredundant database using the DNA–DNA BLAST (blastn). The search was limited to records matching the genome of *S. oneidensis* MR-1 (genome AE014299, taxid 211586).

Growing cell assay

Bacterial growth and RDX degradation were followed in MMR2 medium supplemented with 90 µmol·L⁻¹ RDX. The medium was distributed in sterile glass serum bottles sealed with rubber septa and aluminum crimp caps. The bottles were made anaerobic by degassing for 30 min and then performing five cycles of degassing and recharging with argon. Bacterial cells were inoculated at an initial optical density of 0.04 at 600 nm (OD₆₀₀). Abiotic controls containing RDX in uninoculated culture medium and controls containing bacteria in medium without RDX were conducted in parallel. The bottles (in triplicate) were incubated for a period of 72 h in the dark at 25 °C with agitation at 150 r·min⁻¹. Growth was followed by monitoring the increase in OD₆₀₀. Samples were collected over time for analysis of RDX and its products.

Resting cell assay for metabolic studies

Time-course experiments were conducted on cells pre-grown anaerobically in MMR2 with TMAO and supplemented with 22.5 µmol·L⁻¹ RDX. The cells were harvested at late exponential phase, washed in cold 25 mmol·L⁻¹ sodium phosphate buffer (pH 7.0), and resuspended at a concentration of 2 mg cell protein·mL⁻¹ in the same buffer but containing ~90 µmol·L⁻¹ RDX. The reactions were performed in serum bottles that were sealed and made anaerobic by briefly degassing (1 min) and then purging with argon for 10 min at 5 psi (1 psi = 6.894757 kPa). The bottles were incubated at 25 °C in the dark. Uninoculated control bottles and controls without RDX were conducted in parallel. At selected times, aliquots of the cell suspensions were collected, centrifuged at 16 000g for 10 min, and the supernatant was used for chemical analysis of RDX and its breakdown products. The results are presented as the mean of triplicate assays with the standard deviation. Protein concentration was determined using the bicinchoninic acid method by following the instruction of the

BCA Protein Assay kit (Pierce, Rockford, Illinois). Some microcosms were spiked with L-[U- ^{14}C]RDX (0.038 μCi (1 Ci = 37 Gbq)) to measure mineralization (liberated $^{14}\text{CO}_2$) with a Tri-Carb 4530 liquid scintillation counter (model 2100 TR; Packard Instrument Company) as described previously (Zhao et al. 2002).

Southern blot and complementation

Southern blot was performed to confirm the insertion site of the transposon. The genomic DNA from the mutant and the wild-type strains was digested with three restriction enzymes (*Bst*BI, *Eco*RV, and *Nco*I, individually), and the resulting digested DNAs were separated by electrophoresis on a 0.5% agarose gel. DNA was transferred onto a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled probe targeting the kanamycin resistance (Km^r) gene of the transposon (Roche PCR DIG Probe Synthesis kit). Detection was performed by chemiluminescence. The expected sizes of the transposon-containing DNA bands were calculated using the web-based program Webcutter 2.0.

A complementing plasmid for mutant C9 (mutant described below) was constructed and used as a control to ensure that the phenotype observed was caused by disruption of the *cymA* gene. The complete *cymA* gene, plus 97 and 120 nucleotides of the upstream and downstream regions, respectively, was PCR-amplified from the wild-type MR-1 using the primers C9v2F_*Eco*RI (CGGCATTTTATAGGCTTGAATC-CAAACTTCT) and C9v2R_*Bam*HI (AATCGCCCTTAGCGCCGGATCCCGCAGAG). The 783 bp *Eco*RI-*Bam*HI fragment was cloned directionally into pBBR1MCS-5, a broad-host-range cloning vector with gentamicin resistance (Kovach et al. 1995), resulting in complementing plasmid pB14. The pB14 plasmid was electroporated in *E. coli* DH10B and the colonies were selected on LB plates with gentamicin at 20 $\mu\text{g}\cdot\text{mL}^{-1}$. The plasmid was then purified with the QIAprep Spin Miniprep kit and the proper cloning was confirmed by sequencing. The plasmid was finally electroporated in mutant C9.

Analytical methods

RDX, MNX, DNX, and TNX were analyzed by a reverse-phase high performance liquid chromatography (HPLC) instrument connected to a photodiode array detector. Briefly, the products were analyzed with a Waters HPLC system (Milford, Massachusetts, USA) using a Supelcosil LC-CN HPLC column (25 cm by 4.6 mm by 5 μm) (Oakville, Ontario, Canada) at 35 $^{\circ}\text{C}$. MEDINA and NDAB were determined on an AnionSep Ice-Ion-310 Fast organic acids HPLC column (St. Louis, Missouri, USA) at 225 nm and 35 $^{\circ}\text{C}$. The mobile phase was acidified water (pH 2.0) at a flow rate of 0.6 $\text{mL}\cdot\text{min}^{-1}$. Chromatograms were taken at a wavelength of 225 nm. HCHO was analyzed as its derivative with 2,4-pentanedione (Zhao et al. 2002). N_2O was analyzed in the headspace of the samples using an Agilent 6890 gas chromatograph equipped with an electron capture detector (ECD). The ammonium cation was analyzed using a TSP Spectra-SYSTEM HPLC system equipped with a Waters 431 conductivity detector and a Hamilton PRP-X200 analytical cation-exchange column (250 mm by 4.1 mm by 10 μm). Nitrite (NO_2^-), nitrate (NO_3^-), and formic acid (HCOO^-) were ana-

lyzed by ion chromatography using a Dionex DX 120 ion chromatograph (Sunnyvale, California, USA).

Results

Screening the transposon library for an RDX-defective mutant and identification of the disrupted gene

Random insertion mutagenesis was performed on MR-1, and ~2500 transformants (mutants) were subjected to phenotypic screening (RDX removal). After 48 h of incubation in LB medium amended with RDX, the cultures of five mutants had remaining concentrations of RDX similar to those of the controls without bacterial cells. RDX-defective phenotypes were verified by three additional rounds of screening. To exclude the possibility that the RDX-defective phenotype might be a result of severe growth defects, the growth of mutants in LB plus RDX was monitored by measuring the OD_{600} . Mutant C9 showed the RDX-defective phenotype in three rounds of screening, while its OD_{600} showed growth similar to the wild type. Sequencing of the rescued plasmid from the mutant strain identified the disrupted gene as *cymA* (locus SO_4591; transposon inserted ~145 nucleotides before the stop codon); the gene encodes a tetraheme cytochrome *c*. Another mutant, D6, lacked the orange pigmentation characteristic of the wild-type strain and the RDX-degradation phenotype; it grew in LB to an OD_{600} , which is ~30% lower than the wild type. The transposon was located in the naphthoate synthase gene *menB* (locus SO_4739; transposon inserted ~222 nucleotides before the stop codon). Southern blots showed the presence of a single insertion of the Km^r gene in the genomic DNA of C9 and D6, as well as its absence in MR-1. The size of the bands in *Bst*BI, *Eco*RV, and *Nco*I-digested genomic DNA was consistent with the expected (calculated) bands (data not shown). However, as we failed to complement strain D6 to confirm the phenotype, this strain was excluded from further analysis.

Anaerobic metabolism of RDX in *S. oneidensis* MR-1

RDX degradation by strain MR-1 in MMR2 medium was studied using growing and resting cells. Anaerobic growth of MR-1 in MMR2 minimal medium was not supported by the addition of RDX as the sole nitrogen source, carbon source, or electron acceptor. Growth of MR-1 in MMR2 with fumarate or TMAO as the sole terminal electron acceptor gave an OD_{600} of 0.36 ± 0.01 and 0.35 ± 0.02 after 24 h, respectively; when nitrate was used, the cells reached an OD_{600} of 0.09 ± 0.02 after 24 h.

Growth of MR-1 in MMR2 with TMAO was accompanied by the degradation of RDX at a rate of $4.3 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$. After 12 h of incubation, ~50% of RDX was degraded, producing mostly the three nitroso derivatives MNX (72%), DNX (4%), and TNX (traces). We were unable to analyze for other degradation products such as MEDINA and NDAB because of interference from the medium. Resting cells of MR-1 (2 mg cell protein $\cdot\text{mL}^{-1}$) degraded RDX at a rate of $3.54 \mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{g protein})^{-1}$ (Table 1). The disappearance of RDX was accompanied by the sequential formation of the three nitroso derivatives MNX (47.8%), DNX (38.5%), TNX (7.4%), and the degradation products MEDINA (1.1%), NDAB (0.4%), HCHO (2.2%), N_2O (0.3%), and ammonia

Table 1. Stoichiometry of nitrogen after degradation of $\sim 45 \mu\text{mol}\cdot\text{L}^{-1}$ RDX by the wild-type (MR-1) and mutant (C9) strains.

Metabolite	Wild type	C9
Rate ($\mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{g protein})^{-1}$)	3.54	0.32
MNX (%)	47.8 (± 0.3)	2.7 (± 0.6)
DNX (%)	38.5 (± 0.7)	BDL
TNX (%)	7.4 (± 0.2)	BDL
MEDINA (%)	1.1 (± 0.1)	21.0 (± 0.2)
NDAB (%)	0.4 (± 0.2)	14.3 (± 0.2)
NO_2^- (%)	0.0 (± 0.0)	3.4 (± 0.6)
NH_4^+ (%)	5.5 (± 0.4)	42.4 (± 2.1)
N_2O (%)	0.3 (± 0.1)	12.5 (± 3.1)
Total (%)	101.0	96.3

Note: Values were calculated based on the percentage of reacted RDX using total theoretical numbers of N atoms. BDL, below detection level. RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; DNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine; MEDINA, methylenedinitramine; NDAB, 4-nitro-2,4-diazabutanal; HCHO, formaldehyde.

(5.5%) (Fig. 2a; Table 1). The C- and N-mass balances were calculated at 97% and 101%, respectively.

Anaerobic metabolism of RDX in MR-1 mutants

Mutant C9 grew in MMR2 with TMAO as the sole electron acceptor. C9 cells started to remove RDX at a rate of $0.75 \mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ after the cells reached the stationary phase at an OD_{600} of ~ 0.35 . C9 grew poorly on fumarate, Fe^{3+} (FeCl_3), or nitrate (NaNO_3) as the sole electron acceptor (data not shown). Therefore, TMAO was used as the terminal electron acceptor in the MMR2 medium for the metabolic studies. The RDX transformation products were identified in resting cells. RDX removal by C9 occurred at a rate of $0.32 \mu\text{mol}\cdot\text{h}^{-1}\cdot(\text{g protein})^{-1}$ (Table 1). RDX was degraded mostly via initial denitration leading to ring cleavage and decomposition to MEDINA (21.0%), NDAB (14.3%), NO_2^- (3.4%), N_2O (12.5%), and ammonia (42.4%) (Fig. 2b; Table 1). Traces of MNX were also detected. MEDINA declined over time as it decomposed to HCHO (12% of the C) and N_2O . The N- and C-mass balances were 96% and 36%, respectively. The carbon mass balance was finalized by the detection of $^{14}\text{CO}_2$ from incubations with uniformly labelled ^{14}C -[RDX]: 63% of $^{14}\text{CO}_2$ was recovered for a C-mass balance of 99%. Growth with fumarate as the sole electron acceptor was partially restored and the ability to transform RDX was completely restored in C9:pB14 (Fig. 3). The mutant strain containing pBBR1MCS-5 without insert was used as a control; pBBR1MCS-5 did not restore growth or RDX transformation.

Discussion

Shewanella oneidensis MR-1, a freshwater bacterium isolated from a noncontaminated site (Myers and Nealson 1988), degraded RDX efficiently and through the same metabolic pathways used by the marine *Shewanella* strains isolated from RDX-contaminated sediments. Similarly, MR-1 did not use RDX as a source of energy, N, or C for growth; RDX transformation proceeded via co-metabolism. MR-1 degraded RDX via two initial routes as illustrated in Fig. 1: (1) a major route (route A) represented by the sequential re-

Fig. 2. Time course of anaerobic transformation of RDX ($\sim 90 \mu\text{mol}\cdot\text{L}^{-1}$) at 25°C in $25 \text{ mmol}\cdot\text{L}^{-1}$ sodium phosphate buffer (pH 7.0) for wild-type strain MR-1 (a) and for mutant strain C9 (b). The cell protein concentration was $2 \text{ mg}\cdot\text{mL}^{-1}$. RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; MNX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; DNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; TNX, hexahydro-1,3,5-trinitroso-1,3,5-triazine; MEDINA, methylenedinitramine; NDAB, 4-nitro-2,4-diazabutanal; HCHO, formaldehyde.

duction of the N- NO_2 groups to the corresponding N-NO groups to produce MNX, DNX, and TNX and (2) a minor route (route B) involving initial denitration followed by ring cleavage as demonstrated by the detection of key products such as MEDINA, NDAB, N_2O , and HCHO. Previously, we identified denitrohydrogenation as the initial step leading to RDX decomposition to MEDINA (Bhushan et al. 2002). In the present study, RDX could have undergone denitrohydrogenation, leading to the formation of the unstable 3,5-dinitro-1,3,5-triazacyclohexane whose decomposition would also lead to the formation of MEDINA (Fig. 1, route C) (McHugh et al. 2002). The present finding was in line with what we observed during degradation of RDX with the marine strain *S. halifaxensis* (Zhao et al. 2008); also for both strains, RDX was used as a weak electron acceptor.

To understand the genes responsible for initiating RDX degradation, we constructed a transposon library of MR-1

Fig. 3. Complementation of mutant strain C9 with plasmid pB14 (i.e., *cymA* from MR-1 cloned in pBBR1MCS-5). (a) RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) removal, and (b) growth of the strains in MMR2 mineral medium with fumarate as the terminal electron acceptor.

and identified mutant C9 with a reduced ability to degrade RDX in comparison with the wild type. Specifically, the reduction of the N-NO₂ groups of RDX to the corresponding N-NO derivatives (Fig. 1, route A) was affected by disruption of *cymA*. Other studies have demonstrated that the absence of *cymA* significantly impedes the ability of MR-1 (i.e., 80%–100%) to use a range of substrates as terminal electron acceptors, including Fe(III)/Mn(IV) oxides, fumarate, nitrate, nitrite, and DMSO (Myers and Myers 1997; Schwalb et al. 2003; Lies et al. 2005). CymA and menaquinone participate in vanadium reduction (V(V) to V(IV)) (Myers et al. 2004) and arsenate reduction (As(V) to As(III)) in *Shewanella* spp. (Murphy and Saltikov 2007). CymA, a cytoplasmic membrane-bound *c*-type cytochrome, transfers electrons between the menaquinone pool of the inner membrane and periplasmic terminal reductases or periplasmic *c*-type cytochrome proteins, such as MtrA (Schuetz et al. 2009). Our results suggest that a system of electron transfer involving CymA and possibly other cytochrome *c*'s or electron transport proteins can reduce RDX in strain MR-1. Although RDX transformation was greatly repressed in mutant C9 (reduction of 90% compared with the wild type), it was not completely inhibited. Since *Shewanella* species have approximately 40 *c*-type

cytochromes (Fredrickson et al. 2008; Zhao et al. 2010), this versatility may be exploited by MR-1 in mutant C9 permitting a slower transformation of RDX mainly by denitration. For example, Cordova et al. (2011) have reported that SirCD can functionally replace CymA in several respiratory pathways of MR-1. CymA does appear to have an important role but the specific nature is still unknown due to the robust electron transport system and cytochrome pool in *Shewanella*. The downstream components involved in RDX transformation by MR-1 are also unknown, since CymA can couple to multiple terminal reductases and periplasmic electron transfer proteins (Cordova et al. 2011).

The screening of 2500 transposon mutants certainly did not identify all of the genes involved in the RDX metabolism by MR-1. However, the identification of the *cymA* gene provided evidence that electron transport components have a role in the anaerobic metabolism of RDX by MR-1. RDX and its *N*-nitroso derivatives are toxic (Zhang et al. 2006; Pan et al. 2007). On the other hand, the ring-cleavage product MEDINA is unstable and decomposes to HCHO or CO₂ and N₂O (Halasz et al. 2002), while NDAB can be degraded and mineralized by microorganisms such as *Methylobacterium* and *Phanerochaete chrysosporium* (Fournier et al. 2004, 2005). As already discussed, CymA is involved in the reduction of several natural substrates. This study demonstrated that *cymA* was also involved in the transformation of the xenobiotic compound RDX. Zhao et al. (2008) showed that the mechanism of electron transfer had an impact on the RDX transformation route in *S. halifaxensis*: cells grown with nitrate transformed RDX mainly by denitration while cells grown with TMAO transformed RDX mainly by *N*-nitroso formation. Similarly, the rate and type of RDX metabolites formed depended on whether electrons were transferred from biologically reduced Fe(III) or electron shuttles (Bhushan et al. 2006; Kwon and Finneran 2008, 2010). Therefore, supplementary data on the specific electron transfer mechanisms occurring in *Shewanella* (and other anaerobes) that increase the rate and extent of RDX ring-cleavage products is suitable to develop better remediation strategy for RDX contaminated soils and groundwater.

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