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ORIGINAL PAPER

Cosubstrate independent mineralization of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a *Desulfovibrio* species under anaerobic conditions

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Abstract Past handling practices associated with the manufacturing and processing of the high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has resulted in extensive environmental contamination. In-situ biodegradation is a promising technology for remediating RDX contaminated sites but often relies on the addition of a cosubstrate. A sulfate-reducing bacterium isolated from an RDX-degrading enrichment culture was studied for its ability to grow on RDX as a sole source of carbon and nitrogen and for its ability to mineralize RDX in the absence of a cosubstrate. The results showed the isolate degraded 140 μM RDX in 63 days when grown on RDX as a carbon source. Biomass within the carbon limited culture increased 9-fold compared to the RDX unamended controls. When the isolate was incubated with RDX as sole source of nitrogen it degraded 160 μM RDX in 41 days and exhibited a 4-fold increase in biomass compared to RDX unamended controls. Radiolabeled studies under carbon limiting conditions with ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine confirmed mineralization of the cyclic nitramine. After 60 days incubation 26% of the radiolabel was recovered as $^{14}CO_2$, while in the control bottles less than 1% of the radiolabel was recovered as $^{14}CO_2$. Additionally, ~2% of the radiolabeled carbon was found to be associated with the biomass. The 16S rDNA gene was sequenced and identified the isolate as a novel species of *Desulfovibrio*, having a 95.1% sequence similarity to *Desulfovibrio desulfuricans*. This is the first known anaerobic bacterium capable of mineralizing RDX when using it as a carbon and energy source for growth.

Keywords *Desulfovibrio* · Explosive · Hexahydro-1,3,5-trinitro-1,3,5-triazine · RDX degradation · Sulfate-reducing bacteria

Abbreviations

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
MDNA	Methylenedinitramine

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a secondary high explosive that is widely used by the

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U.S. military in shells, bombs and demolition charges (Gorontzy et al. 1994). Past handling practices at Army facilities which manufacture, process and dispose of RDX have resulted in extensive environmental contamination (Funk et al. 1993; Rieger and Knackmuss 1995; Jeger and Woodhull 2000). The Department of Defense has identified more than 1,200 sites within the U.S. contaminated with RDX that require cleanup (Scmelling et al. 1997). The most highly impacted areas are ammunition plants which have reported RDX groundwater concentrations in excess of 10 mg l^{-1} (Best et al. 1997). The treatment of these sites are of major environmental concern for the Army, particularly those sites scheduled for decommission.

RDX has moderate water solubility, a low absorption coefficient and is resistant to degradation under aerobic conditions (Talmage et al. 1999). These characteristics facilitate the compound's transport through the environment. RDX has been shown to be toxic to a variety of aquatic and terrestrial species (Robidoux et al. 2000; Talmage et al. 1999). In addition, the explosive has been classified as a potential human carcinogen (US Environmental Protection Agency 2006). Due to the compounds toxic and possible carcinogenic affects to humans and wildlife, contaminated environments must be remediated. Typical RDX remediation strategies include composting, pump-and-treat and incineration technologies (Jeger and Woodhull 2000; US Environmental Protection Agency 2001). All involve the translocation of the contaminant in order for treatment to take place. Although these techniques are effective in removing RDX from the environment, the reallocation processes makes them very costly (Jeger and Woodhull 2000).

In-situ bioremediation holds great potential as a cost-effective alternative to classical treatment technologies. Because soils and groundwater are not physically sequestered from the environment, remediation costs are kept low. However, in order to efficiently mineralize RDX traditionally a cosubstrate is needed to stimulate the process (Kitts et al. 2000; Young et al. 1997). Typically, substrates rich in organic matter are used to promote the microbial degradation process (Adrian et al. 2003; Adrian and Arnett 2007; Gorontzy et al. 1994; Hawari 2000). Starch (Funk et al. 1993), nutrient broth (McCormick et al. 1981), yeast extract (Kitts et al. 1994), glucose (Hawari et al. 2000), propylene glycol and ethanol (Adrian and Arnett 2007) have all been used to stimulate RDX-degrading activity in bacterial cultures. However, the addition of carbon rich substrates into the environment can be problematic.

Compounds high in organic carbon can serve as energy sources for a variety of chemoorganotrophic bacteria resulting in capricious microbial growth. Biomass accumulation can restrict natural water flow through soils and sediments and dramatically increases the probability of aquifer plugging, especially around the point of introduction (Stewart and Fogler 2002). Identifying bacteria capable of utilizing RDX as an energy source could eliminate the need to add rich cosubstrates to the environment leading to less invasive remediation technologies. Here we report the isolation and characterization of the first known anaerobic bacterium capable of mineralizing RDX when using it as a carbon and energy source for growth.

Materials and methods

Chemicals

RDX was obtained from the Holston Army Ammunition Plant (Kingsport, TN) and had a purity of 99% or greater. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) and methylenedinitramine (MDNA) were obtained from SRI International (Menlo Park, CA). Solvents used were of HPLC grade or greater and all other chemicals and analytical standards were obtained from major chemical suppliers and were of the highest purity obtainable.

Isolation procedure

The RDX-degrading isolate originated from a mixed culture capable of mineralizing RDX in the absence of a cosubstrate (Adrian and Arnett 2006). The bacterium was isolated by serially diluting the culture in Balch tubes containing a mineral medium and RDX. The mineral medium consisted of the following l^{-1}: NaCl, 0.8 g; NH_4Cl, 1.0 g; KCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; KH_2PO_4, 1.35 g; K_2HPO_4, 1.75 g; $NaHCO_3$, 1.5 g; TES buffer, 4.6 g; resazurin, 1.0 mg;

$Na_2S \cdot 7H_2O$, 0.5 g; trace metal solution, 10 ml; vitamin solution, 10 ml. Trace metal and vitamin solutions were made as described by Tanner (1989). The pH of the medium was adjusted to 7.2 and dispensed into Balch tubes using strict anoxic techniques. The headspaces were exchanged with H_2:CO_2 (80:20) and pressurized to 1.7 atm. Tubes were steam sterilized for 15 min at 120°C. A filter-sterilized (0.2 μm) solution of RDX was added to a final concentration of 25 μM. The mixed culture was serially diluted and 1 ml of each dilution was used to inoculate the tubes. All cultures were incubated at 28°C shaking in the dark. Tubes at the highest dilution factor exhibiting RDX degradation activity were plated onto solid mineral media containing 25 μM RDX and incubated in an anaerobe jar pressurized with H_2:CO_2 (80:20, 1.3 atm). After two weeks incubation random well isolated colonies were picked from the plates under anaerobic conditions and inoculated back into Balch tubes containing the mineral media described above. The culture exhibiting the greatest RDX-degrading activity, isolate EFX-DES, was chosen for further study. Purity was confirmed by streaking and incubating the culture on plate count agar under both aerobic and anaerobic conditions.

Growth on RDX as a sole source of carbon and nitrogen

Studies evaluating the growth of isolate EFX-DES on RDX as a sole source of carbon and energy were performed similar to earlier studies (Adrian and Arnett 2004), with the exceptions that the media was amended with RDX to a greater concentration. The limited solubility of RDX and its susceptibility to abiotic degradation during steam sterilization required the RDX to be added directly to the media. RDX was added to a concentration of 200 μM, followed by filter-sterilizing the media through a sterile 0.2 μm filter. Cultures were grown in the mineral media described previously with the exception that $NaHCO_3$, resasurin and TES buffer (possible sources of carbon) were omitted. Eighty ml of the media was dispensed into sterile 160 ml serum bottles and sealed with sterile butyl rubber stoppers and aluminum crimp seals. The headspace gas was exchanged with ultrahigh purity N_2 using sterile gas filters and pressurized to 1.7 atm. Twenty ml of the isolate was used to inoculate the bottles resulting in a final RDX concentration of ~160 μM. Sterile controls were prepared by autoclaving the cultures two consecutive times prior to inoculation.

The isolates ability to grow on RDX as a sole source of nitrogen was determined in 160 ml serum bottles containing 80 ml of mineral media described in the isolation procedure with the exception that NH_4Cl was excluded as a source of nitrogen. The media was filter-sterilized (0.2 μm), dispensed into sterile serum bottles and sealed with sterile butyl rubber stoppers. The headspace gas was exchanged with H_2:CO_2 (80:20) to 1.7 atm. Twenty ml of the isolate was used to inoculate the cultures resulting in a final RDX concentration of ~170 μM. Sterile controls were prepared as described previously.

Both RDX growth experiments were performed in triplicate and incubated at 28°C in the dark with shaking. Liquid samples were taken periodically and analyzed for RDX and common RDX degradation products by reverse phase high-pressure liquid chromatography (HPLC) and gas chromatography (GC). Growth of isolate EFX-DES on RDX as sources of carbon and nitrogen was determined by quantifying the microbial biomass by measuring the ester-linked phospholipid fatty acids (PLFA) as described below.

Ester-linked phospholipid fatty acid analysis

Ester-linked PLFA were extracted from isolate EFX-DES to quantify viable microbial biomass as described by White and Ringelberg (1998). Briefly, phosphate washed cells were suspended in 0.8 ml H_2O and extracted with 1 ml $CHCl_3$ and 2 ml MeOH overnight. Total soluble lipids were recovered and dried under N_2 then fractionated on an amino-propyl solid phase extraction column (Pinkart et al. 1998). Fatty acids from the polar lipid fraction were prepared for gas chromatography/mass spectrometry (GC/MS) by mild alkaline methanolic transesterification (Guckert et al. 1985) and dissolved in hexane containing methyl nonadecanoate (50 pmol μl^{-1}) as an internal standard prior to injection. Cell numbers based on PLFA concentrations were calculated as described by Sundh et al. (1995).

Sulfate as an electron acceptor

Growth of isolate EFX-DES on lactate, ethanol or H_2, with and without sulfate as an electron acceptor, was

determined by measuring the optical density of the culture at 600 nm (OD_{600}). Balch tubes containing 9 ml of the mineral medium described in the isolation procedure were amended with either 10 mM lactate, 10 mM ethanol or the headspace of the Balch tubes were pressurized with H_2:CO_2 (80:20) to 1.7 atm. Lactate and ethanol amended tubes were pressurized with N_2:CO_2 (80:20, 1.7 atm). Tubes were inoculated with 1 ml of the isolate and sterile controls were prepared by autoclaving prior to inoculation. All growth conditions were performed in triplicate and incubated at 28°C in the dark with shaking. After 8 days of incubation all tubes were spiked with Na_2SO_4 to a final concentration of 25 mM and optical densities were monitored for an additional 9 days.

Radiolabeled studies

Experiments to determine the ability of the isolate EFX-DES to mineralize ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine (^{14}C-RDX) to $^{14}CO_2$ when added as the sole source of carbon and energy was carried out as previously described (Adrian and Arnett 2006). The radiolabeled tracer for this study was uniformly labeled ^{14}C-RDX that had a specific activity of 0.035 μc μg^{-1} (New England Nuclear, MA). Briefly, 40 μl of 3.4 μc ml^{-1} ^{14}C-RDX acetone solution was added to sterile 160 ml serum bottles yielding an average of 0.15 μc or 4.3 μg ^{14}C-RDX per bottle. All acetone was allowed to evaporate prior to the addition of the anaerobic growth media. Cultures were incubated at 26°C on an orbital shaker and 1 ml samples were periodically taken to determine RDX concentrations. At specific time intervals, the serum bottles were sacrificed for determining the carbon mass balance as described by Adrian and Arnett (2006).

16S ribosomal DNA sequencing

DNA was extracted from isolate EFX-DES using a MoBio Power Soil Extraction Kit (Carlsbad, CA) per the manufactures instructions. The 16S rDNA gene of the isolate was amplified using a 27F 1492R primer pair. Polymerase chain reaction (PCR) thermocycling parameters consisted of 10 min at 94°C, then 34 cycles of 94°C for 3 s, 60°C for 45 s and 72°C for 45 s, followed by a 5 min extension at 72°C. Reactions were carried out using a BD Biosciences Clontech Advantage 2 Polymerase Mix (San Jose, CA) supplemented with 10 pmol of each primer listed above. The total amplification reaction volume was 25 μl. The PCR amplicon was confirmed by visualization on a 1% agarose gel and the product was purified using a MoBio Ultra Clean PCR Clean-up DNA Purification Kit prior to DNA sequencing. The PCR product was sequenced at the University of Tennessee, Molecular Biology Resource Facility (Knoxville, TN) using a Big Dye Terminator reaction (Applied Biosystems, Forest City, CA). Sequence reads were manually edited and compared to sequences compiled in the Ribosomal Database Project II (RDP) Release 9.51 (http://rdp.cme.msu.edu/index.jsp) for taxonomic identification. The RDP *Classifier* program (http://rdp.cme.msu.edu/classifier/classifier.jsp) was used to assign the 16S rDNA sequence to the taxonomical hierarchy based on a naïve Bayesian rRNA classifier.

Phylogenetic analysis

Related sequences and a preliminary alignment were obtained using the RDP Release 9.51 *Seqmatch* Version 3 (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Both type and non-type strains of uncultured and isolated organisms were searched with near full length sequences consisting of greater than 1200 bases. A phylogenetic tree was created with the RDP *Tree Builder* (http://rdp.cme.msu.edu/treebuilder/treeing.spr) program. Weighbor Joining and evolutionary distance was estimated using the Jukes-Cantor distance matrix model and the final tree was rooted by using *Desulfovibrio fairfieldensis* as an outgroup.

Microscopy

Light and phase-contrast microscopy was performed using a Nikon Eclipse 4000 light microscope (Melville, NY). Cells were Gram stained using a Fisher Scientific Protocol Gram Stain Kit (St. Louis, MO) and compared to positive and negative control stains using a BBL Gram Stain Slide (Becton Dickenson, Spark, MD). Cells were observed by scanning electron microscopy (SEM) as previously described by Adrian and Arnett (2004).

Analytical methods

RDX, MNX, DNX, TNX and MDNA were analyzed by HPLC as previously described (Adrian and Arnett

2004). Formic acid production was determined by HPLC using an anion exclusion column, 300 × 7.8 mm (Alltech, Deerfield, IL) and a UV absorbance detector. The following conditions were used: mobile phase, 0.003 N H_2SO_4; wavelength, 210 nm; injection volume, 20 µl; flow rate, 0.5 ml min^{-1}. Formaldehyde was quantitated by colorimetric Hantzsch assays as described by Nash (1953). Methanol was analyzed for by GC using a Varian 3800 GC equipped with a flame ionization detector. Samples for analysis were filtered using a 0.2 µm syringe filter and 1–5 µl was injected onto a J & W Scientific DB-624 column (75 m × 0.53 mm × 3.00 µm; Cobert Associates, St. Louis, MO). The carrier gas was helium at a flow rate of 30 ml min^{-1}. The initial oven temperature was 50°C for 2 min and ramped to 275°C at 10°C min^{-1} and held for 5.5 min. The injector temperature was 260°C and detector temperature was set at 280°C.

Results

Isolation and characterization

The RDX-degrading bacteria in the explosive degrading mixed culture were enriched by amending the culture with RDX and H_2. The enrichment was serially diluted and the culture having the greatest dilution factor that exhibited RDX degradation activity was streaked for isolation onto solid media containing RDX and incubated under a H_2 atmosphere. Several random well isolated colonies were picked from the plates and transferred back to the Balch tubes containing the mineral media supplemented with RDX and hydrogen. The culture exhibiting the greatest RDX-degrading activity was named isolate EFX-DES and became the focus of further study. Purity of the isolate was confirmed by streaking onto plate count agar and incubating under aerobic and anaerobic atmospheres. After 2 weeks of incubation, no growth was observed on the aerobic plates. The anaerobic plates consisted of completely homogeneous colonies averaging 1–2 mm in diameter and having a slight creamy white color. Light microscopy revealed all cells to be small Gram-negative rods which were spiral to vibrioid-shaped. When viewed under a phase-contrast microscope, the cells were morphologically uniform and motile, moving in a directional manner by apparent flagellar action. The cells did not autofluoresce under epifluorescence or produce spores during either log or stationary phases of growth. When viewed by SEM the cells were again of a homogeneous nature ranging in size from 0.3–0.5 × 2–3 µm (Fig. 1).

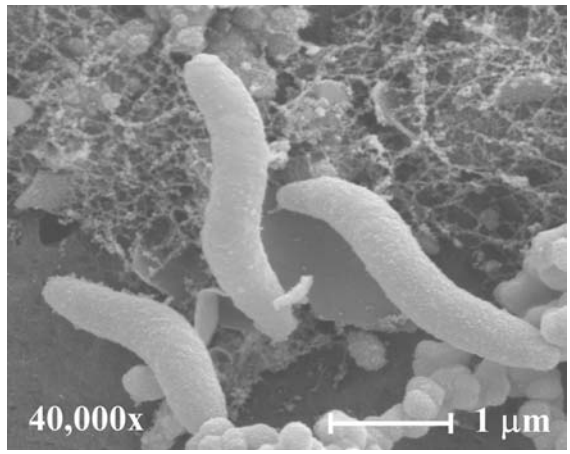

Fig. 1 Scanning electron micrograph of isolate EFX-DES at 40,000×. The scale bar represents 1 µm. The isolate was a motile, Gram-negative, non-spore forming, spirilium, having average dimensions of 0.3–0.5 × 2–3 µm

The isolate's ability to grow on lactate, ethanol or hydrogen as an energy source was examined. When incubated with each substrate as an electron donor, minimal or no growth took place (Fig. 2). After 3 days incubation lactate amended tubes grew to an

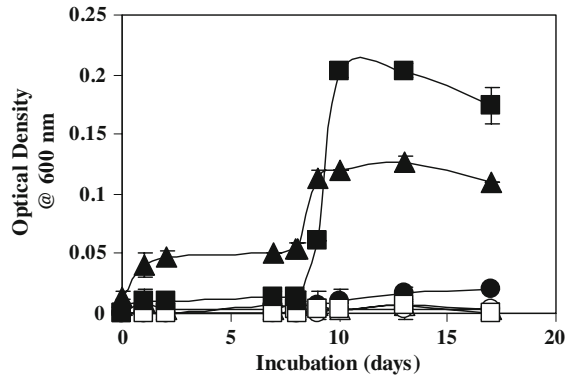

Fig. 2 Growth of isolate EFX-DES on lactate, ethanol and hydrogen. Cultures were grown in a minimal media amended with either 10 mM lactate, 10 mM ethanol, or pressurized to 1.7 atm with H_2:CO_2 (80:20). After 8 days of incubation all cultures were amended with 25 mM Na_2SO_4. Values are the means of three replicates ± standard deviations. Symbols: H_2:CO_2, ■; H_2:CO_2 sterile control, □; lactate, ▲; lactate sterile control, Δ; ethanol, ●; ethanol sterile control, ○

OD_{600} of 0.05, with no additional growth after 8 days. No growth, relative to the sterile controls, was observed in the ethanol or hydrogen amended tubes during this same period. On day 8 all cultures were spiked with 25 mM Na_2SO_4. The optical densities immediately increased in the lactate and hydrogen amended tubes. The lactate amended tubes increased to maximum OD_{600} of 0.13 and hydrogen amended tubes increased to a maximum OD_{600} of 0.2 after 13 days incubation. Minimal growth was observed in ethanol amended bottles after the addition of Na_2SO_4 relative to the sterile controls ($OD_{600} \leq 0.02$).

PCR amplification of the 16S rDNA gene using universal bacterial primers yielded a 1,469 bp amplicon. Subsequent DNA sequencing of the amplicon and comparison to near full length sequences in the RDP revealed isolate EFX-DES was a member of the δ-subclass of *Proteobacteria* and fell within to the tightly grouped genus *Desulfovibrio* (Fig. 3). With *D. fairfieldensis* serving as an outgroup isolate EFX-DES was found to be most closely related to *Desulfovibrio desulfuricans* strain CSN and *D. desulfuricans* strain 734, exhibiting a 95.1 and 94.0% sequence similarity to these species respectively.

Growth of isolate EFX-DES on RDX as sources of carbon and nitrogen

Without the addition of a cosubstrate, isolate EFX-DES degraded 140 μM RDX in 63 days (Fig. 4a). Ninety percent of the initial RDX was depleted in this period and the subsequent formation of small amounts of nitroso-RDX reduction products was observed. MNX increased to maximum of 8.4 μM after 42 days of incubation and slowly decreased to ~7.1 μM by the end of the study. Over the same time period DNX increased to a maximum of 1.5 μM. No TNX was observed in any of the experimental bottles throughout the study. We observed approximately a 30% loss of RDX in the sterile controls during the same time period and the production of less than 1.2 μM MNX, but no DNX or TNX was observed. Commonly reported RDX ring-cleavage intermediates methanol, formaldehyde, formic acid and MDNA were not detected under any condition throughout the study.

Sustained growth of isolate EFX-DES in the mineral medium was found to be dependant upon the presence of RDX (Fig. 4b). In cultures amended with 160 μM RDX the biomass increased from 1.5×10^6 to a maximum of 1.2×10^7 cells ml^{-1} after 42 days incubation. In the absence of RDX isolate EFX-DES grew to a maximum cell concentration of 5.9×10^6 cells ml^{-1} after 21 days incubation and slowly decreased over the course of the study to a final concentration of 1.2×10^6 cells ml^{-1}. After 63 days incubation isolate EFX-DES exhibited a 9-fold increase in biomass compared to the RDX unamended control bottles, 1.1×10^7–1.2×10^6 cells ml^{-1} respectively. The biomass concentration within the sterile controls dropped from an initial concentration of 8.6×10^5 to less than 9.0×10^4 cells ml^{-1} after 63 days incubation indicating abiotic factors were responsible for the loss of RDX in these cultures.

When RDX was supplied as the sole source of nitrogen, 93% of the RDX was degraded in 41 days incubation (Fig. 5a). MNX production peaked on day 13 at 16.0 μM and slowly decreased to 7.8 μM by the end of the study. DNX production peaked on day 34 at

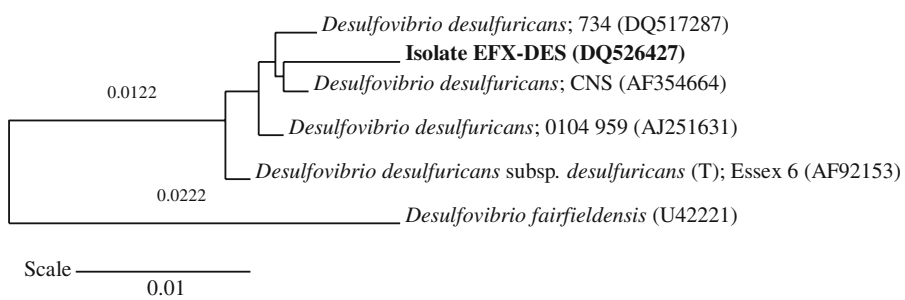

Fig. 3 Phylogenetic tree of isolate EFX-DES based on the sequence of a 1,469 base pair amplicon of the 16S rDNA gene. The sequence of *D. fairfieldensis* served as the outgroup. Values listed are the evolutionary distances. The scale bar indicates 1 nucleotide difference per 100 nucleotide positions. In brackets are the GenBank accession numbers of the 16S rDNA sequences

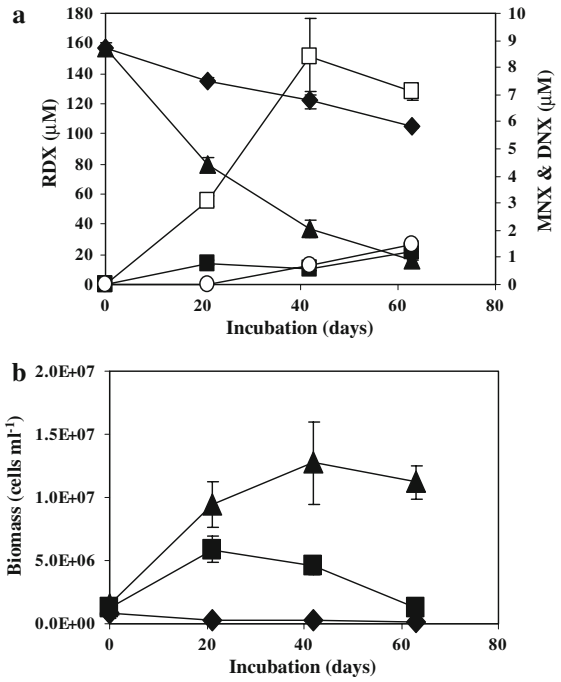

Fig. 4 Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation and biomass formation by isolate EFX-DES where 160 μM RDX was added as the sole source of carbon and energy. (**a**) RDX degradation and nitroso-RDX formation. Symbols: RDX, ▲; MNX, □; DNX, ○; sterile control RDX, ◆; sterile control MNX, ■. (**b**) Total biomass formation. Symbols: cells + RDX, ▲; cells only, ■; sterile control + RDX, ◆. All values are the means of three replicates ± standard deviations

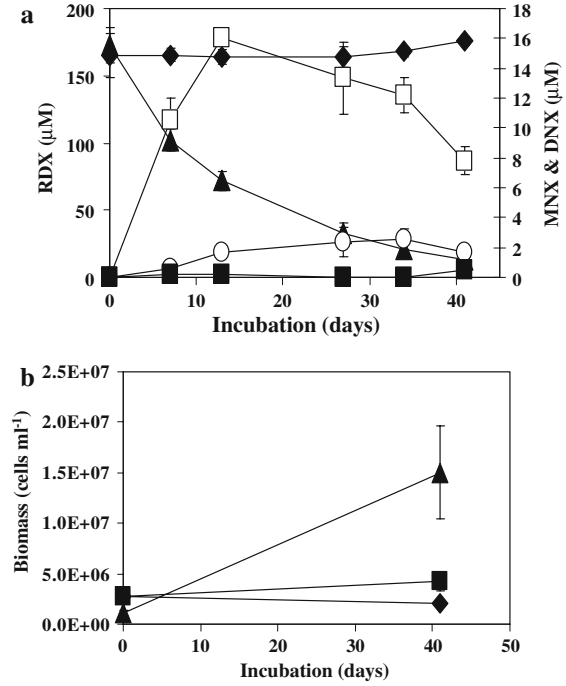

Fig. 5 Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation and biomass formation by isolate EFX-DES where 160 μM RDX was added as the sole source of nitrogen. (**a**) RDX degradation and nitroso-RDX formation. Symbols: RDX, ▲; MNX, □; DNX, ○; sterile control RDX, ◆; sterile control MNX, ■. (**b**) Total biomass formation. Symbols: cells + RDX, ▲; cells only, ■; sterile control + RDX, ◆. All values are the means of three replicates ± standard deviations

2.6 μM and no TNX was observed. Interestingly, in the absence of NH_4Cl no loss of RDX was observed in the sterile control bottles, which suggested that NH_4Cl may play a role in the abiotic degradation of RDX. Only trace amounts of MNX were formed (< 0.5 μM) and DNX and TNX were not detected in the sterile controls. During the 41 days incubation period the biomass increased in the RDX amended bottles from 1.1×10^6 to 1.5×10^7 cells ml^{-1}, a 13-fold increase (Fig. 5b). Biomass increased only 0.6-fold in the RDX unamended culture from 2.7×10^6 to 4.2×10^6 cells ml^{-1}. No increase in biomass was observed in the sterile control bottles over the same incubation period.

Cosubstrate independent mineralization of RDX

Studies conducted with ^{14}C-RDX confirmed isolate EFX-DES was capable of mineralizing RDX when grown in a mineral media lacking a cosubstrate. In 60 days, more than a 2-fold increase in RDX degradation was observed in the media containing the live cells compared to the uninoculated and sterile controls (Table 1). Ninety-seven percent of the RDX was depleted in the bottles containing live cells during this period and there was a 41% loss in the sterile controls

Table 1 Anaerobic biodegradation of radiolabeled ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by isolate EFX-DES when serving as a sole source of carbon and energy for the culture

Condition	RDX (μM)		% degraded
	Initial	Final[a]	
No cells	36 ± 5	21 ± 1	42
Sterile control	34 ± 10	20 ± 0.5	41
Live cells	37 ± 5	1 ± 0.5	97

[a] Final RDX concentrations were determined after 60 days incubation

Table 2 Radiocarbon distribution of ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine (^{14}C-RDX) in serum bottles at time zero

Condition	Initial DPM	Final DPM[a]			Volatile carbon	% recovered
		Nonvolatile	Solid phase	CO_2		
No cells	371,967 ± 3,750	345,030 ± 16,813	1,724 ± 212	1,145 ± 407	24,068	94
Sterile control	376,833 ± 2,108	357,750 ± 5,818	1,721 ± 203	3,685 ± 2,067	13,677	96
Live cells	378,900 ± 4,513	354,180 ± 7,841	2,164 ± 139	878 ± 110	21,678	94

[a] DPM, disintegrations per minute

Table 3 Radiocarbon distribution of ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine (^{14}C-RDX) in serum bottles at time 60 days

Condition	Initial DPM	Final DPM[a]			Volatile carbon	% recovered
		Nonvolatile	Solid phase	CO_2		
No cells	371,800 ± 6,067	333,900 ± 5,369	1,769 ± 147	198 ± 257	35,933	90
Sterile control	372,767 ± 2,259	329,130 ± 1,820	2,354 ± 177	4,678 ± 7,142	36,605	90
Live cells	372,300 ± 7,139	186,840 ± 12,964	6,048 ± 549	98,927 ± 25,245	80,485	78

[a] DPM, disintegrations per minute

and a 42% loss in the uninoculated controls. A mass balance was made on day 0 and again on day 60 to determine the distribution of the radiocarbon between $^{14}CO_2$, volatile carbon, nonvolatile carbon and solids. At time zero, 94% of the radiolabel added to the serum bottles containing the live EFX-DES cells and the uninoculated controls was recovered (Table 2). Ninety-six percent of the radiolabel was recovered in the sterile controls. All but ~1% of the radiolabel that was recovered was associated with the liquid phase as RDX or nonvolatile carbon. After 60 days incubation 26% of the radiocarbon was recovered as $^{14}CO_2$ in the serum bottles amended with live cells (Table 3). Fifty percent of the radiolabel was associated with the aqueous phase as nonvolatile carbon. Approximately 2% of the radiolabel was associated with the biomass or solid phase. We recovered 78% of the radiolabel added to the serum bottles. In both the uninoculated and sterile control bottles ~90% of the radiolabel was associated with the aqueous phase as RDX and other unidentified nonvolatile degradation products and ~1% or less of the radiolabel was recovered as $^{14}CO_2$. Ninety percent of the radiolabel was recovered from the serum bottles for both controls.

Discussion

In previous studies with a RDX-degrading mixed culture a substantial loss in RDX was observed over short incubation periods in microcosms not amended with a readily available electron donor (Adrian and Arnett 2006). It was originally believed that small amounts of reducing equivalents transferred during the inoculation process were serving as a source of energy for RDX transformation. When RDX degradation persisted after eliminating the inoculum of any possible reducing equivalents, it was hypothesized that bacteria present within the culture were utilizing RDX as a growth substrate. This was confirmed by growing the culture on RDX as a sole source of carbon for greater than 1 year. Over this period a 7-fold increase in biomass was observed in the RDX amended culture compared to the RDX unamended culture. In addition, ~35% of the ring carbon from RDX was accounted for as CO_2.

Serial diluting the mixed culture and plating it onto solid media resulted in a pure RDX-degrading culture. The homogeneity of the culture was confirmed by streaking onto plate count agar and by morphological analysis using SEM and light microscopy. Under anaerobic conditions colonies formed on both minimal media and plate count agar were identical in appearance, consisting of round 1–2 mm creamy white colonies. No colonies were formed on aerobically incubated plates signifying no facultative anaerobes were present. When viewed by bright-field, phase-contrast and SEM all cells appeared homologous, indicating the presence of a pure culture. PCR amplification of the isolates 16S

rDNA gene and subsequent DNA sequencing produced uncontaminated sequencing reads, which served as additional evidence that the culture was pure.

The harsh preparative conditions associated with SEM analysis did not allow the observation of flagella (Fig. 1), but due to the directional locomotion of the bacteria when viewed under phase-contrast microscopy, it was likely the cells were polarly flagellated. No spores were observed during either log or stationary phases and the cells stained Gram-negative, differentiating the organism from several RDX-degrading *Clostridium* species (Arnett and Adrian 2001; Regan and Crawford 1994; Zhang and Hughes 2003; Zhao et al. 2003a).

Growth studies with isolate EFX-DES on ethanol, lactate, and hydrogen yielded minimal to moderate growth in the absence of a readily available electron acceptor after 8 days incubation (Fig. 2). Moderate growth was observed on lactate and no growth was observed in the ethanol or hydrogen amended tubes relative to the controls. When sulfate was added as an electron acceptor the optical densities immediately increased in both the lactate amended tubes and hydrogen amended tubes. The isolate's ability to utilize lactate as an electron donor and sulfate as an electron acceptor classified it as a Group I sulfate-reducer (Brock et al. 1994). This was substantiated by the fact that isolate EFX-DES had the ability to grow chemolithotrophically with hydrogen serving as an electron donor, sulfate as an electron acceptor and CO_2 as a source of carbon. Only select species of *Desulfovibrio* are known to possess this unique growth trait (Brock et al. 1994).

Sequencing nearly the entire 16S rDNA gene and comparing it known sequences sequestered from the RDP database confirmed the initial assessment that EFX-DES belonged to the genus *Desulfovibrio* (Fig. 3). When the sequence was compared to both cultured and uncultured organisms in the RDP the isolate was found to be most closely related to *D. desulfuricans* strain CSN and *D. desulfuricans* strain 734 having 95.1 and 94.0% sequence similarities respectively. The 4.9–6.0% variation in 16S rDNA sequence classified the isolate as a novel species of *Desulfovibrio* (Stackebrandt and Goebel 1994). On the basis of growth on the tested substrates, morphology and the 16S rDNA sequencing data, we identified and named the isolate *Desulfovibrio* species EFX-DES. The 1,469 bp ribosomal gene sequence of species EFX-DES has been deposited in GenBank under the accession number DQ526427.

RDX was shown to support the growth of EFX-DES as a source of carbon and energy. When incubated with 160 μM RDX the isolate exhibited a 9-fold increase in biomass after 63 days incubation compared to the RDX unamended controls (Fig. 4b). The initial growth in the RDX unamended controls was likely due to trace amounts of carbon transferred during the inoculation process or from the mineral media vitamin solution. Of the ~140 μM RDX degraded, only 6% could be accounted for as MNX and DNX (Fig. 4a). McCormick et al. (1981) proposed a degradation pathway which involved the transfer of two-electrons to the nitro groups of RDX to sequentially form MNX, DNX and TNX. The authors found when anaerobic sludge was incubated with RDX that ~40% of the RDX was sequential reduced to MNX, 20% to DNX and 14% to TNX. These reduction products were found to be transitory in nature and further degraded to methanol, formaldehyde and hydrazine. Others have proposed a single-electron transfer involving denitration of RDX to produce MDNA, formaldehyde and ultimately methanol (Zhao et al. 2002). The lack of significant amounts of nitroso-RDX intermediates and the complete absence of TNX, methanol and formaldehyde suggested that a reductive pathway was not the major route for RDX transformation by the isolate.

Hawari et al. (2000), has proposed an alternative RDX degradation pathway involving direct ring-cleavage rather than the sequential reduction. The initial step in the pathway involves the enzymatic cleavage of RDX resulting in the formation of MDNA and *bis*-(hydroxymethyl)nitramine. Both compounds are transient in nature and undergo spontaneous transformation in aqueous solution to form stoichiometric amounts of formaldehyde (Halasz et al. 2002). Both MDNA and formaldehyde were analyzed for but at no point throughout the study were either detected. This does not eliminate the possibility of a direct enzymatic attack but does suggest an alternate RDX degradation pathway possibly involving novel enzymes.

In addition to growth on RDX as a carbon source, EFX-DES was also capable of growing on RDX as a sole source of nitrogen. The isolate exhibited a 13-

fold increase in biomass after 41 days of incubation, which was a 4-fold increase in total biomass relative to RDX unamended controls (Fig. 5b). Interestingly, ~125 μM RDX was degraded after 42 days incubation in the carbon unamended cultures and roughly 160 μM RDX was degraded after 41 days incubation in the nitrogen unamended cultures (Fig. 4a, 5a). Biomass concentrations for each condition were nearly equivalent after 6 weeks of incubation (Fig. 4b, 5b) suggesting EFX-DES preferentially utilized RDX as a nitrogen source. Of the ~160 μM RDX degraded ~12% could be accounted for as nitroso-RDX reduction products. Again, the lack of substantial amounts of MNX and DNX and the complete lack of TNX suggested that sequential reduction of RDX was not the primary degradative pathway under nitrogen limiting conditions.

When EFX-DES was grown with radiolabeled RDX under carbon limiting conditions, 26% of the ring carbon was recovered as $^{14}CO_2$ (Table 3). Less that 1% of the radiolabel was recovered as $^{14}CO_2$ in the uninoculated and sterile controls. This clearly demonstrated the isolate's ability to mineralize RDX in the absence of a cosubstrate. Furthermore, in the live cultures ~2% of the radiolabel was found to be associated with the solid phase in contrast to only 0.5% in the control cultures. This suggested 1.5% of the ^{14}C was incorporated into the biomass, supporting our contention that the isolate was in fact utilizing RDX for catabolic growth. To date, only two aerobic species, *Williamsia* species strain KTR4 and *Gordonia* species strain KTR9, have been identified that have this same metabolic trait (Thompson et al. 2005). Each species was capable of mineralizing ~30% of the depleted RDX to CO_2 when grown in the absence of a cosubstrate. In addition, both species were also capable of growing on RDX as a sole source of nitrogen. Others have demonstrated the ability of select *Clostridium* and *Desulfovibrio* species to utilize RDX as a source of nitrogen for growth (Boopathy et al. 1998; Zhao et al. 2003b), but no anaerobic organisms have been reported to grow on RDX exclusively as a source of carbon. To our knowledge EFX-DES is the first anaerobe isolated that not only grows on RDX, but also mineralizes the cyclic nitramine independent of a cosubstrate.

Select *Desulfovibrio* species have been shown to degrade explosives. Boopathy et al. (1998) demonstrated the degradation of 1,3,5-trinitrobenzene (TNB), octahydro-1,3,5,7-tranitro-1,3,5,7-tetraazocine (HMX) and RDX by a sulfate-reducing consortium consisting of two strains of *D. desulfuricans*, one strain of *Desulfovibrio gigas* and a strain of *Desulfovibrio vulgaris*. Zhao et al. (2003b) isolated the first RDX-degrading *Desulfovibrio* species HAW-ES2 from anaerobic sludge. HAW-ES2 was similar to EFX-DES in that both were phylogenetically related to *D. desulfuricans*. However, HAW-ES2 was capable of prolific growth on ethanol, which differentiated it from strain EFX-DES (Fig. 2). HAW-EB18 was another RDX-degrading *Desulfovibrio* species isolated from marine sediment (Zhao et al. 2004). The isolate was found to be closely related to *Desulfovibrio acrylicus* and when grown on marine media was capable of mineralizing RDX. Similar to EFX-DES both isolates produced minimal MNX and DNX and no TNX. This may indicate a common enzymatic RDX degradation pathway among the species.

Although only limited RDX degradation products were identified in this study, the ability of EFX-DES to degrade and grow on RDX as a sole source of carbon and nitrogen was clearly apparent. In addition, the production of $^{14}CO_2$ from ^{14}C-RDX verified the isolate's ability to mineralize RDX. To our knowledge this is the first report demonstrating cosubstrate independent mineralization of RDX by an anaerobic bacterium. Identification of similar bacteria in the environment could eliminate the need for cosubstrates to sustain RDX degradation activity. Because sulfate and sulfate-reducing bacteria are fairly ubiquitous in nature the potential for natural attenuation in carbon limited environments is likely given proper redox potentials exist. Allowing such an in-situ approach would be more cost-effective than tradition translocation processes and would have far less impact on the environment than cosubstrate supplementation.

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