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Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB

Mark E. Fuller · Kevin McClay · Jalal Hawari · Louise Paquet · Thomas E. Malone · Brian G. Fox · Robert J. Steffan

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Abstract The transformation of explosives, including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), by xenobiotic reductases XenA and XenB (and the bacterial strains harboring these enzymes) under both aerobic and anaerobic conditions was assessed. Under anaerobic conditions, *Pseudomonas fluorescens* I-C (XenB) degraded RDX faster than *Pseudomonas putida* II-B (XenA), and transformation occurred when the cells were supplied with sources of both carbon (succinate) and nitrogen (NH_4^+), but not when only carbon was supplied. Transformation was always faster under anaerobic conditions compared to aerobic conditions, with both enzymes exhibiting a O_2 concentration-dependent inhibition of RDX transformation. The primary degradation pathway for RDX was conversion to methylenedinitramine and then to formaldehyde, but a minor pathway that produced 4-nitro-2,4-diazabutanal (NDAB) also appeared to be active during transformation by whole cells of *P. putida* II-B and purified XenA. Both XenA and XenB also degraded the related nitramine explosives octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane. Purified XenB was found to have a broader substrate range than XenA, degrading more of the explosive compounds examined in this study. The results show that these two xenobiotic reductases (and their respective bacterial strains) have the capacity to transform RDX as well as a wide variety of explosive compounds, especially under low oxygen concentrations.

Keyword Pseudomonas · RDX · Explosive · Biodegradation · CL-20 · HMX

M. E. Fuller (✉) · K. McClay · R. J. Steffan
Shaw Environmental, Inc,
17 Princess Road,
Lawrenceville, NJ 08648, USA
e-mail: mark.fuller@shawgrp.com

J. Hawari · L. Paquet
Biotechnology Research Institute,
National Research Council Canada,
6100 Royalmount Ave,
Montreal, PQ H4P 2R2, Canada

T. E. Malone
Molecular and Environmental Toxicology Program,
University of Wisconsin,
433 Babcock Drive,
Madison, WI 53706, USA

B. G. Fox
Department of Biochemistry, University of Wisconsin,
433 Babcock Drive,
Madison, WI 53706, USA

Introduction

Past and current activities at sites where munitions are manufactured and tested have resulted in the release of munition-related compounds. The environmental fate of these contaminants is an issue of significant concern to the United States Department of Defense (DoD), regulators, and the public because their mobility and persistence allow them to contaminate ground water supplies (Tipton et al. 2003; Yamamoto et al. 2004). Recently, information describing the extent of soil and groundwater contamination at military training ranges has been published (Jenkins et al. 2001; Clausen et al. 2004; Pennington et al. 2006). Several of these compounds have been placed on the U.S. Environmental Protection Agency's Contaminant Candidate List (http://www.epa.gov/safewater/ccl/ccl3.html#chemical).

Extensive research has examined the biological transformation of explosive compounds by pure cultures of bacteria and mixed consortia in soil and groundwater (see review of Hawari et al. 2000a). Most research has focused on the dinitrotoluenes (DNT) and 2,4,6-trinitrotoluene (TNT), with interest in hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) increasing in recent years. RDX biodegradation has been observed under conditions ranging from fully aerobic (Binks et al. 1995; Coleman et al. 1998) to strictly anaerobic (Hawari et al. 2001; Maloney et al. 2002; Adrian et al. 2003; Pudge et al. 2003; Adrian and Arnett 2004; Bhatt et al. 2005), and at least three major degradation pathways have been elucidated (Fig. 1). Anaerobic processes involve either a direct attack on the ring structure or the successive reduction of the pendant nitro groups followed by ring cleavage (McCormick et al. 1981; Hawari et al. 2000a). Many bacterial strains can utilize RDX as a sole nitrogen source (Boopathy et al. 1998; Zhao et al. 2003; Thompson et al. 2005), but only recently has the use of RDX as a sole source of carbon, nitrogen, and energy by a single organism (Thompson et al. 2005) and mixed cultures (Adrian and Arnett 2006) been reported. Several bacterial enzyme systems that degrade RDX have been identified, including the XplA/XplB system of a number of geographically dispersed rhodococci (Jackson et al. 2007; Seth-Smith et al. 2008), the diaphorase of clostridia (Bhushan et al. 2002), and the type I nitroreductases of two enterobacteria (Kitts et al. 2000).

The degradation of nitroglycerin and TNT by the xenobiotic reductases (XenA and XenB) from the aerobes *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C has been explored (Blehert et al. 1999; Pak et al. 2000). Though XenA and XenB are both members of the Old Yellow Enzyme family (flavoprotein oxidoreductases) and catalyze similar reactions, there are significant differences in the catalytic rates and substrate specificities between the two. For example, purified XenB catalyzes the transformation of TNT ~5-fold faster than XenA, whereas the catalytic rates with nitroglycerin (NG) are approximately equal. However, XenA preferentially denitrates NG at the terminal positions (1 and 3 positions), whereas XenB preferentially denitrates NG at the interior position (2 position). Furthermore, the rate of TNT transformation by XenB was only slightly enhanced under anaerobic conditions, but the

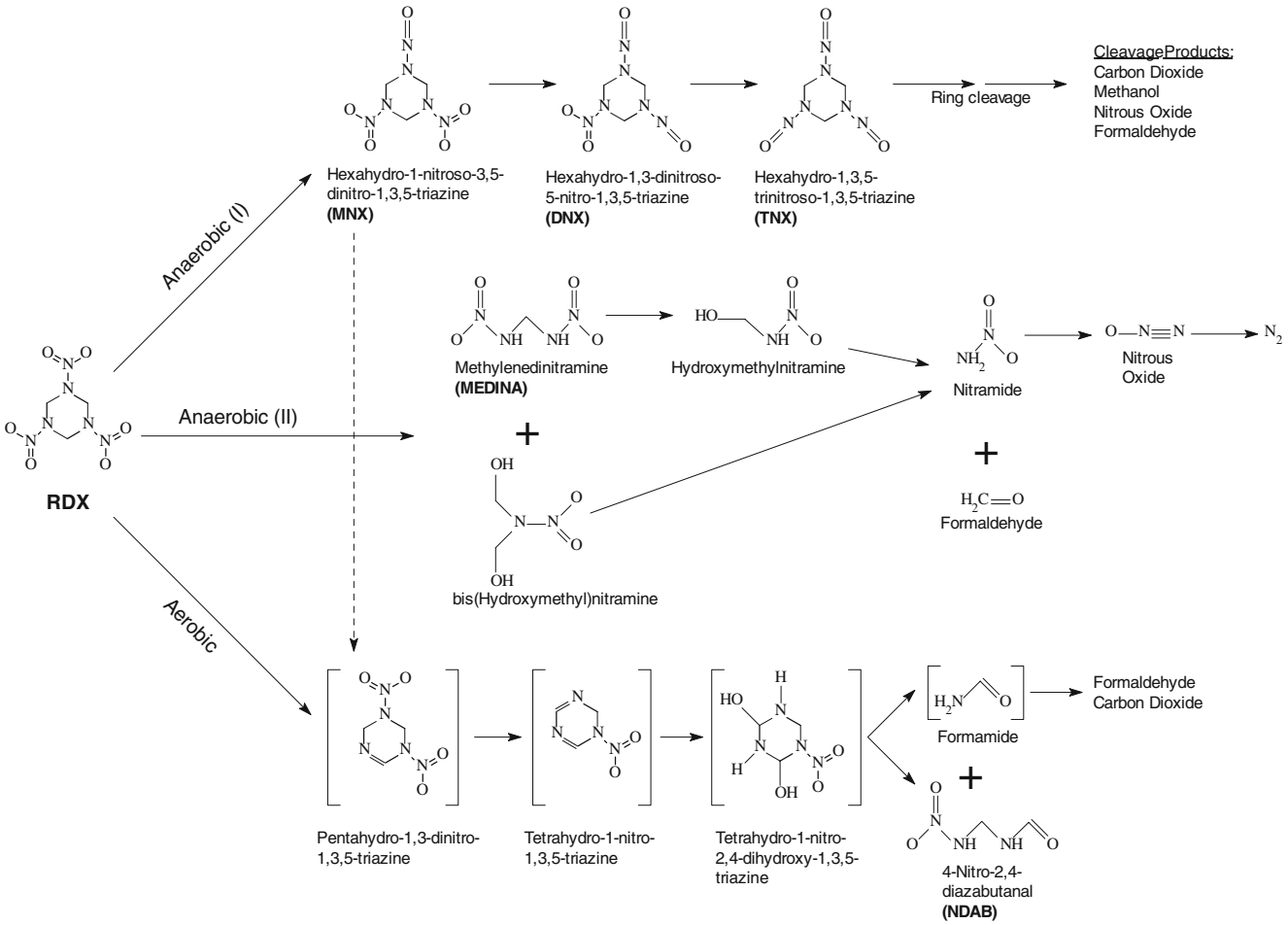

Fig. 1 Known degradation pathways for RDX. Pathways derived/adapted from Hawari et al. (2000b) and Zhao et al. (2003)

product distribution resulting from TNT transformation varied greatly under anaerobic conditions. Transformation of RDX by these enzymes was not characterized.

In the present study, the effect of decreasing O_2 tension on the catalytic characteristics of XenA and XenB expressed in their native bacterial hosts and as purified enzymes were explored. The results reveal that both enzymes are capable of degrading RDX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and a suite of related energetic compounds under reduced O_2 concentrations, but not necessarily under fully aerobic conditions. The observation that RDX can be degraded by aerobic organisms under reduced oxygen tensions could lead to enhanced bioremediation technologies and a better understanding of natural attenuation process.

Materials and methods

Chemicals All chemicals were reagent grade or purer. The three nitroso-containing metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine; and hexahydro-1,3,5-trinitroso-1,3,5-triazine) of RDX were purchased from SRI International (Menlo Park, CA, USA). RDX (7% HMX as a manufacturing impurity) was a gift from James Phelan at Sandia National Laboratories (Albuquerque, NM, USA). HMX was a gift from Herb Fredrickson at the U.S. Army Engineer Research and Development Center (Environmental Laboratory, Vicksburg, MS, USA). [^{14}C]-RDX (specific activity= 60.0 mCi/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). The manufacturer stated the [^{14}C]-RDX radiochemical purity of 99.9% based on radiochromatography. CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) was obtained from ATK Launch Systems (Corinne, Utah, USA). Neat standards of nitroaromatic compounds were purchased from ChemService (West Chester, PA, USA).

Bacterial strains *P. putida* II-B and *P. fluorescens* I-C were maintained on R2A agar. The two strains were deposited in the open collection of the Agricultural Research Service (ARS) Culture Collection, National Center for Agricultural Utilization Research of the U.S. Department of Agriculture. The accession number of *P. putida* II-B is NRRL B-50270, and the accession number of *P. fluorescens* I-C is NRRL B-59269.

Whole cell biotransformation assays *P. putida* II-B and *P. fluorescens* I-C possessing the *xenA* and *xenB* genes, respectively, were screened for transformation of RDX, HMX, and CL-20. A basal salts medium (BSM) (Hareland et al. 1975) was used for screening. The carbon source was succinate. Inocula were prepared by growing the strains in BSM plus succinate overnight, followed by concentration and washing of the cells twice with nitrogen-free BSM. The washed cells were used to inoculate vials (40 mL) of BSM medium (20 mL) amended with sodium succinate (6.2 mM) and RDX (22 μM) or HMX (~3 μM). The initial optical density of the cultures at 550 nm (OD_{550}) was approximately 0.15 (corresponding to 0.3 mg total cell protein per vial). Vials were incubated at room temperature with shaking, and samples were removed periodically, passed through 0.45 μm glass microfiber filters into 2-mL glass sample vials, and analyzed for RDX, HMX, and breakdown products by high-performance liquid chromatography (HPLC; see below). Aerobic vials were equipped with vents to allow 0.2-μm filtered air to enter and maintain oxygen concentrations. Experiments performed under anaerobic conditions were prepared, incubated, and sampled in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA).

Transformation of CL-20 (~2 μM initial concentration) by the *Pseudomonas* strains was performed similarly, except that the screening was performed in polypropylene tubes instead of glass to prevent abiotic loss of CL-20 (Monteil-Rivera et al. 2004). Anaerobic treatments were prepared and incubated in an anaerobic glove box. Samples were removed periodically and centrifuged in polypropylene microfuge tubes to remove biomass, and the supernatant was transferred to polypropylene HPLC vials for analysis.

The effect that changes in the RDX concentration had on the rate and extent of transformation by *P. putida* II-B and *P. fluorescens* I-C was examined by adding washed cells to anaerobic BSM plus succinate amended with RDX at concentrations of 3, 13, 31, 63, and 126 μM. Samples were removed periodically and analyzed by HPLC. Direct toxicity of RDX (at 0, 86, and 153 μM) to these two strains was examined by monitoring cell density at 550 nm during aerobic growth in BSM plus succinate (a condition under which RDX was not degraded).

Production of nitrous oxide (N_2O) and nitrite (NO_2^-) from RDX were determined by incubating cultures of *P. putida* II-B and *P. fluorescens* I-C with RDX and periodically removing samples of the headspace and liquid for analysis (see analytical section below). To identify the less common RDX breakdown products, cultures were incubated with ~90 μM of RDX at room temperature with shaking, then frozen at −70°C after approximately 50% of the initial RDX had degraded. Frozen samples were shipped on dry ice to the Biotechnology Research Institute, National Research Council Canada for more extensive analysis of RDX breakdown products according to previously described methods (Hawari et al. 2000b).

Cell-free enzyme assays Several experiments were performed to assess the catalytic properties of the xenobiotic reductases of *P. putida* II-B and *P. fluorescens* I-C, which were isolated and purified as previously described (Blehert et al. 1997; Pak et al. 2000). The explosive transformation assays were performed with the test compounds dissolved in sodium phosphate buffer (100 mM, pH 7.4). NADPH was added to a final concentration of 1.5 to 4.5 mM. Vials were purged with at least 20 volumes of O_2-free N_2 bubbled through the liquid, then transferred to an anaerobic chamber where 1 mL of the solutions were transferred to 2-mL glass screw cap auto-sampler vials (or polypropylene vials in the case of the explosive CL-20) and sealed with Teflon lined septa. To examine the effect of O_2 on the rate of RDX and HMX transformation, pure O_2 gas was added via a syringe needle inserted through the septum of the vial to bring the headspace O_2 concentration up to the desired percentage on a (*v*/*v*) basis with the headspace and vigorously shaken. An assay was initiated by injecting 1 μL of purified XenB (0.017 mg) or 1 to 5 μL of purified XenA (0.014 to 0.070 mg) through the septum. For kinetic assays, the vials were automatically and repeatedly analyzed via HPLC (see below). Negative controls composed of substrate, buffer, and NADPH were included in all experiments and were used to detect and adjust for any non-enzymatic substrate losses.

The aerobic and anaerobic degradation rates of a range of explosive compounds with XenB were examined. Assays were performed as described, except that the amount of XenB protein was adjusted as needed to assure that degradation did not occur faster than could be measured based on the HPLC analysis times for a given compound. Assays were performed in duplicate, and the initial linear rates were calculated as micromoles compound degraded per milligram XenB protein per minute. Non-kinetic experiments were performed with the XenA enzyme in which only the 24-h endpoint result (degradation/no degradation) was measured. Degradation of CL-20 by both XenA and XenB was also assessed by an endpoint assay.

To determine if RDX was converted to MNX during transformation by XenB, an experiment utilizing radiolabeled RDX was conducted. Briefly, the enzyme assay procedure described above was followed, except that the XenB and XenA enzymes were mixed with 78 μM of MNX and 54 μM [^{14}C]-RDX. Unlabeled MNX was included in the assay so that if very small amounts of MNX were being formed and subsequently degraded by XenB during the transformation of RDX, the large pool of unlabelled MNX would slow down the degradation of the enzymatically formed [^{14}C]-MNX, which could then be detected using scintillation counting. The reaction vial was repeatedly sampled, and the loss of the target substrates was monitored via HPLC as described below, except that the HPLC eluant was also collected at 20-s intervals into scintillation vials pre-filled with 3 mL of Optiphase HiSafe scintillation cocktail (Perkin-Elmer, Boston MA, USA). The time of elution of the radioactive peaks was compared with the elution time of the known explosive compounds and metabolites (RDX, MNX, DNX, and TNX) to determine if any of the [^{14}C]-RDX was being converted to [^{14}C]-MNX or other related compounds. Under the analytical conditions described below, there is more than a full minute separating the elution of MNX and RDX, which would be easily resolved with the described protocol.

Analytical Other than the analysis for the less common breakdown products of RDX performed by Biotechnology Research Institute noted above, the concentrations of the explosives and their common breakdown products in all experiments were determined using HPLC according to a modified EPA Method 8330A (http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/8330a.pdf) using a Hewlett-Packard 1100 HPLC equipped with a Allure C18 column (Restek, Bellefonte, PA, USA) and a UV detector (230 nm). The mobile phase was 50:50 methanol/water at a flow rate of 0.9 mL/min. The column temperature was 25°C. The lower detection limit was approximately 0.1 μM for RDX and 0.25 μM for the RDX breakdown products. CL-20 was analyzed on the same system, except that the mobile phase was adjusted to 55:45 methanol/water, and detection was at performed at 228 nm. Analytical standards of the parent explosive compounds (except CL-20) were obtained from Restek (Bellefonte, PA, USA). The standard for CL-20 was prepared from the neat material obtained from ATK Launch Systems (Corinne, Utah, USA). The standards for MNX, DNX, and TNX were prepared from the neat material obtained from SRI International (Menlo Park, CA, USA). Identity of peaks in the samples was based on retention time matching to peaks in the standards.

Nitrous oxide was measured using GC-TCD. Nitrite was determined colorimetrically (Hach Company, Loveland, CO, USA). Ammonia was measured spectrofluorometrically (Holmes et al. 1999). Hydrogen peroxide production by purified enzymes in the presence of oxygen was detected and quantified using either the Quantofix Peroxide Detection Kit (Macherey-Nagel, Bethlehem, PA, USA) or the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA, USA) with fluorometric analysis using SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA).

Results

Transformation of RDX and other explosives by whole cells During initial experiments, the two *Pseudomonas* strains examined here were able to degrade TNT under aerobic conditions, but no aerobic transformation of RDX, HMX, or CL-20 was observed (data not shown). Under anaerobic conditions, transformation of RDX was observed with *P. putida* II-B and *P. fluorescens* I-C (Fig. 2a). HMX was degraded anaerobically only by *P. fluorescens* I-C (Fig. 2b). The apparent first-order rate for RDX disappearance was about 14-fold higher for *P. fluorescens* I-C as compared to *P. putida* II-B (0.0084/h vs. 0.0006/h) at an initial RDX concentration of 31 μM. The RDX transformation rates of *P. putida* II-B and *P. fluorescens* I-C appeared to be concentration dependent. The transformation rate decreased 3- and 10-fold for *P. putida* II-B and *P. fluorescens* I-C, respectively, as the initial RDX concentration increased from 3 to 126 μM. However, the aerobic growth of these two strains was not affected by the presence of RDX even at 153 μM. When incubated under conditions in which an initially aerobic medium was allowed to become O_2-depleted during the growth of the culture, both *P. putida* II-B and *P. fluorescens* I-C degraded RDX, but only *P. fluorescens* I-C degraded HMX. Transformation of CL-20 was observed by pure cultures under anaerobic conditions, with *P. fluorescens* I-C degrading the compound much faster than *P. putida* II-B (Fig. 2c). These findings suggested that O_2 either inhibited the expression or the activity of the catalytic enzymes in these strains.

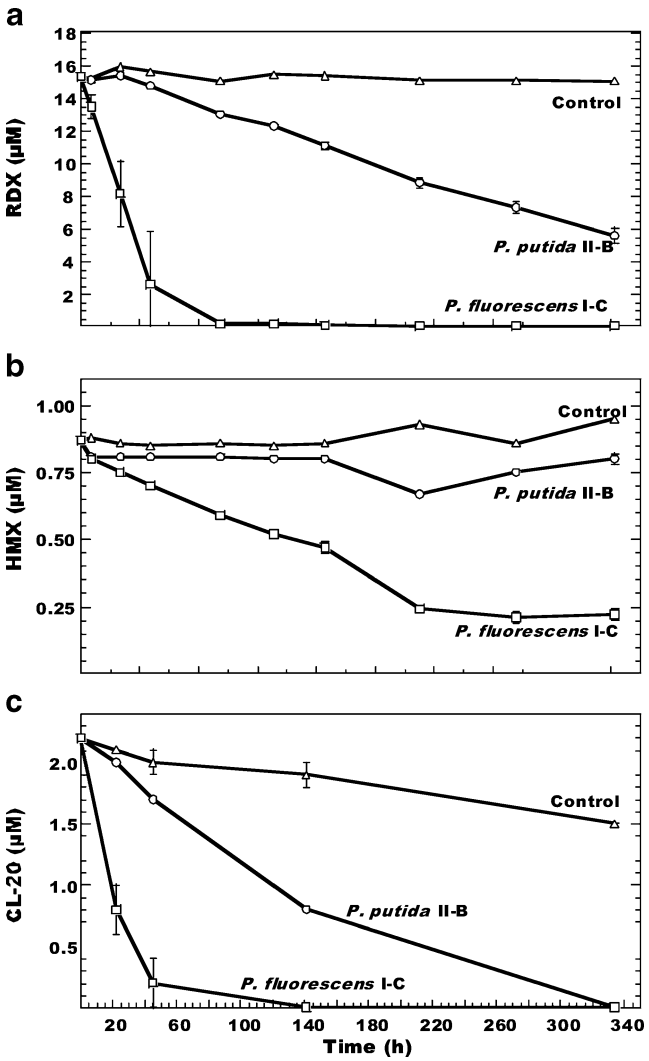

Fig. 2 Degradation of **a** RDX, **b** HMX (in the presence of RDX), and **c** CL-20 by pure cultures of *Pseudomonas* spp. under anaerobic conditions. Sterile control (*triangles*); *P. putida* II-B (*circles*); *P. fluorescens* I-C (*squares*). Data points for live cultures represent average of two replicate vials; a single sterile control vial was used during RDX and HMX experiments. Error bars represent one standard deviation of the mean. Note difference in *y*-axis scales

RDX transformation by purified XenA and XenB enzymes In order to assure that results of the whole cell degradation assays could be attributed to specific enzyme activities, experiments using purified XenA and XenB were conducted. Initial studies indicated that RDX was not degraded via a direct reduction of the nitro group (i.e., no nitroso-containing products were detected by HPLC), so a more detailed analysis of the transformation products was performed (Table 1). The product distribution resulting from RDX degradation differed not only between the XenA and XenB, but also between the purified enzymes and their source organisms. With both purified enzymes, the major products that accumulated indicated that RDX was degraded via the methylenedinitramine (MEDINA) pathway (Fig. 1, Anaerobic II pathway), yet MEDINA did not accumulate and was not detected in whole cell incubations. Formaldehyde was a major product of RDX metabolism by purified XenA and by XenB, whether assays were performed with pure enzymes or in whole cells. The carbon mass balances for the degradation of RDX by the enzymes and whole cells ranged from 60% to 100% (mole C basis). With purified XenA, production of trace amounts of 4-nitro-2,4-diazabutanal (NDAB) and MNX suggested that minor reactions occurred with this enzyme that did not occur with XenB. However, detection of MNX was not observed using the modified EPA Method 8330 and is therefore not believed to be produced from RDX by these enzymes. Indeed, the [^{14}C]-RDX/MNX experiment gave no evidence that XenA or XenB produced MNX during the breakdown of RDX. All of the RDX-derived radioactivity was contained in a broad peak that eluted well before the unlabelled MNX peak. Furthermore, the rate of degradation of RDX by XenB is ~10-fold faster than the degradation of MNX, which would have been expected to result in a buildup of [^{14}C]MNX from [^{14}C]RDX. This experiment,

Table 1 Product distribution during degradation of RDX by purified XenA and XenB enzymes (average of duplicate assays) and by whole cells of *P. putida* II-B and *P. fluorescens* I-C (single replicates)

Assay	RDX (μmol)		Products (μmol)				Mass balance (%)	
	Initial	Residual	MNX	MEDINA	NDAB	HCHO	C	N
P. fluorescens I-C	21.5	15.0	0.0	1.4	0.0	13.2	92	74
P. putida II-B	21.5	11.7	0.0	0.4	0.3	2.7	60	56
XenB	69.1	29.2	0.0	37.4	0.0	82.2	100	78
XenA	69.1	28.9	0.2	23.5	1.5	76.1	98	66

Samples were collected for analysis after about half the initial RDX had been degraded (corresponding to approximately 100 and 400 h for whole cell assays with *P. fluorescens* I-C and *P. putida* II-B, respectively, and 4 and 24 h for XenB and XenA, respectively)

therefore, showed conclusively that MNX was not a typical product of RDX breakdown by XenB.

Nitrogen mass balances ranged from 56% to 78%. As shown in Fig. 1 (Anaerobic II pathway), RDX can be converted to MEDINA and bis(hydroxymethyl)nitramine, and these compounds decay to form formaldehyde and nitramide, the latter of which may further break down to form nitrous oxide and nitrogen gas (Hawari 2004). Therefore, measurement of these inorganic nitrogenous products was performed, and percentages were calculated on the basis of the nitrogen present in the amount of RDX degraded during a given experiment. Nitrous oxide was not detected during RDX degradation with the purified enzymes, but small amounts of nitrous oxide (1 to 2 mol %) were detected during whole cell assays. Nitrite was detected during RDX degradation by purified XenB at a level of ~17 mol%. Nitrite was detected in whole cell assays with *P. putida* II-B and *P. fluorescens* I-C at levels ~2 and ~12 mol%, respectively. Ammonia was detected during transformation of RDX by whole cells at levels equal to ~15 mol% and during degradation of RDX by XenB (~23 mol%). However, the possibility that the assay was actually detecting one or more of the possible RDX breakdown products (e.g., nitramide) rather than ammonia could not be ruled out. Inclusion of these inorganic nitrogenous products increased the nitrogen mass balances of the products produced during RDX transformation by *P. putida* II-B and *P. fluorescens* I-C to 75% and 102%, respectively (compared to 56% and 74% based on only the organic products with nitrogen are considered; Table 1). Similarly, the overall nitrogen mass balance for RDX degradation by XenB was increased to 118% when both organic and inorganic nitrogenous products are considered.

Because HMX is a common contaminant of RDX preparations and because the two compounds are often found together in the environment, we investigated whether HMX was degraded sequentially or consecutively with RDX. With purified XenB, little to no HMX (present at approximately 10% the RDX concentration) was degraded in the presence of high concentrations of RDX. However, when RDX and HMX were present at approximately equal concentrations, HMX and RDX were degraded simultaneously by XenB. Similarly, in a mixture of RDX and the common breakdown products MNX, DNX, and TNX, XenB degraded all the compounds simultaneously.

Additional experiments were performed to determine the O_2 inhibition characteristics for RDX and HMX transformation. While XenB degraded RDX much faster than XenA (~30-fold), both enzyme systems had similar O_2 inhibition characteristics (Fig. 3a and b). A similar effect was noted when HMX served as a substrate for XenB (Fig. 3c). The percentage of saturation for O_2 that resulted in a 50% reduction in the initial linear degradation rates (derived from Fig. 3) were 1.5±0.3% and 1.6±0.3% for RDX degradation by XenA and XenB, respectively, and 2.3±0.4% for HMX degradation by XenB.

Aerobic and anaerobic transformation of various explosives by XenA and XenB A screening-level study of purified XenA and XenB was performed to assess their ability to degrade a range of nitroaromatic and nitramine explosive compounds under aerobic and anaerobic conditions. With XenB, ten of the compounds were degraded aerobically and 14 were degraded anaerobically (Table 2). The rates of degradation of the other compounds relative to the degradation rate of TNT varied considerably. The rate of aerobic TNT degradation by the XenB enzyme was 0.155 μmol/mg protein/min, which is within a factor of four of the specific activity based on previously published data (Pak et al. 2000). The degradation rate observed for TNB and tetryl were greater than for TNT under both aerobic and anaerobic conditions, while all the other nitroaromatic compounds were degraded slower than TNT. Among the nitramine compounds, the relative activity of XenB against

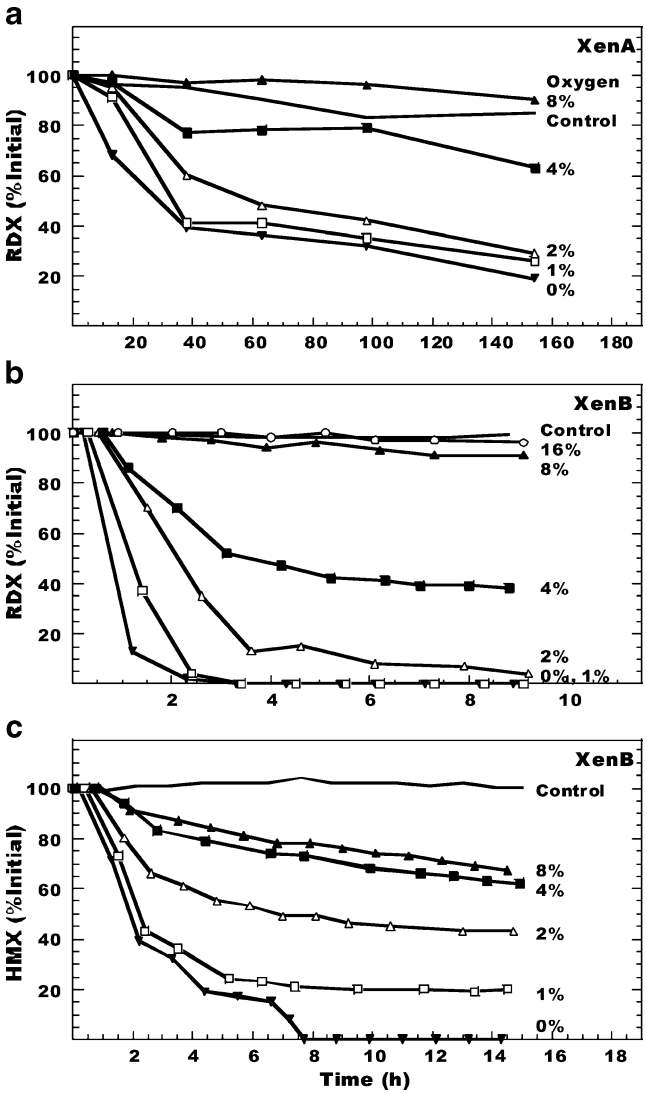

Fig. 3 Degradation of RDX by purified **a** XenA and **b** XenB enzymes and **c** HMX by purified XenB enzyme under different initial oxygen concentrations. The initial concentrations of RDX and HMX were 55, 83, and 9 μM in **a**, **b**, and **c**, respectively. Control contained ambient oxygen concentration (~20%). Each line represents data from two duplicate vials that were alternatingly sampled during the course of the experiment; therefore, no error bars were calculated. Note difference in *x*-axis scales

RDX was the highest and that of HMX was the lowest, in both the presence and absence of oxygen.

Of the 16 compounds tested by an endpoint assay with XenA (tetryl was not tested with XenA), six were degraded by XenA under aerobic conditions, and nine were degraded anaerobically (data not shown). RDX, TNT, 2,4-DNT, 1,3-DNB, and TNB were degraded by XenA under both aerobic and anaerobic conditions. The RDX breakdown products MNX, DNX, and TNX, as well as HMX, were only degraded under anaerobic conditions by XenA (within the timeframe of the assays). Additionally, both the XenA and XenB purified enzymes degraded the relatively new explosive compound CL-20.

Discussion

Only a single previous report has described the aerobic transformation of the nitramine explosive RDX by a *Pseudomonas* sp., though the enzymes involved and the degradation pathway were not discussed (Chang et al. 2004). In our study, RDX and HMX transformation by two *Pseudomonas* sp. strains occurred under strictly anaerobic conditions, as well as under "anoxic" conditions created as cells consumed dissolved O_2 while growing on succinate. The biodegradation pathway described herein for RDX by purified xenobiotic reductases and whole cells of *P. putida* II-B and *P. fluorescens* I-C leads to more labile products (formaldehyde) and less toxic (nitrous oxide) products, rather than the more toxic nitrosolated compounds like those produced during other anaerobic processes (Adrian and Sutherland 1999; Zhang and Hughes 2003) or dead-end products like NDAB that is produced during aerobic transformation by some *Rhodococcus* spp. (Fig. 1, Aerobic pathway). These products do not persist in the environment and thus are a more desirable end point for bioremediation applications.

Unlike previously described *Rhodococcus* spp. (Coleman et al. 1998; Nejidat et al. 2008), RDX transformation by pure cultures in this study was not inhibited, but rather was facilitated, by the presence of utilizable nitrogen (NH_4^+). Transformation rates by whole cells decreased with increasing RDX concentrations, whereas the RDX transformation rate from purified XenB increased with increasing RDX concentration. Additionally, the aerobic growth rates of *P. putida* II-B and *P. fluorescens* I-C were not inhibited with increasing RDX concentration. Taken together, these results suggest that although RDX itself is not toxic to either the cells or the degradative enzymes described here, the breakdown products may exert toxicity by an unknown mechanism. This finding is in general agreement with previous results showing toxicity in another pseudomonad during aerobic transformation of RDX (Chang et al. 2004).

Previous studies with xenobiotic reductases (and related enzymes) have shown that the presence of O_2 can impact the transformation of explosive compounds in more than one way. For example, Pak et al. (2000) noted that while TNT was degraded by XenB both aerobically and anaerobically, the presence of O_2 changed the product distribution. Most notably, certain TNT dimers accumulated, resulting in the release of nitrite only in the presence of O_2 (or other oxidants such as $NADP^+$) via an abiotic mechanism. In another study investigating degradation of RDX by three *Enterobacteriaceae* isolates, O_2 also played a key role in

Table 2 Degradation of nitroaromatic and nitramine explosive compounds by xenobiotic reductase XenB under aerobic and anaerobic conditions

Compound	Abbreviation	Initial concentration (μM)		Relative activity (%)	
		Aerobic	Anaerobic	Aerobic	Anaerobic
Nitroaromatics					
2,4,6-Trinitrotoluene	TNT	36	37	100	385
2-Nitrotoluene	2-NT	9	8	0	0
3-Nitrotoluene	3-NT	10	9	0	0
4-Nitrotoluene	4-NT	8	8	0	0
2,4-Dinitrotoluene	2,4-DNT	60	57	3.7	15
2,6-Dinitrotoluene	2,6-DNT	25	27	0.2	4.4
2-Amino-4,6-dinitrotoluene	2A-4,6-DNT	96	94	0	24
4-Amino-2,6-dinitrotoluene	4A-2,6-DNT	162	165	24	36
Nitrobenzene	NB	83	80	0	0.2
1,3-Dinitrobenzene	1,3-DNB	49	48	17	24
1,3,5-Trinitrobenzene	TNB	50	50	426	2243
N-methyl-*N*,2,4,6-tetranitroaniline	tetryl	5	13	1454	1520
Nitramines					
Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	47	47	1.5	21
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine	HMX	22	21	0	1.6
Hexahydro-1,3,5-trinitroso-1,3,5-triazine	TNX	49	50	0.4	6.6
Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine	DNX	41	41	0	3.7
Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine	MNX	29	27	0.1	2.1

Rates are presented relative to the rate obtained for TNT under aerobic conditions (0.155 μmol TNT degraded/mg XenB protein/min)

the final outcome, as RDX was degraded only under oxygen-depleted conditions (Kitts et al. 1994). Similarly, it was reported that RDX degradation by *Klebsiella pneumoniae* strain SCZ-1 was completely quenched by the presence of O_2, though the concentrations of O_2 required to stop RDX degradation was not reported (Zhao et al. 2002).

In the present study, O_2 had a large impact on the activity of XenA and XenB. Several compounds that were not degraded (or degraded very slowly) aerobically were degraded under anaerobic conditions, and the rates of degradation observed during kinetic assays with XenB were always higher under anaerobic compared to aerobic conditions. RDX and HMX were among the compounds for which this was observed and studied in more detail. It is important to note that O_2 did not function as a binary on/off switch for the transformation of RDX and HMX, but rather it caused a gradual decrease in RDX transformation as a function of the initial O_2 concentration (Fig. 3). The mechanism by which oxygen interferes with the degradation of explosives has not yet been conclusively determined. Hydrogen peroxide was produced when XenB was incubated in the presence of NADPH and oxygen, but it was not observed in the absence of oxygen (data not presented). This would seem to indicate that oxygen is reduced by XenB in the absence of a more preferred substrate. Therefore, the concentration of oxygen would compete with explosive compounds for the active site of XenB and affect the degradation rates.

These results greatly expand the known substrate range of both XenA and XenB. Although only screening-level data were obtained and more mechanistic studies are warranted, some observations and possible explanations for the observed differences in degradation rates are presented here. Degradation rates increased as the degree of nitro-substitution increased for both the toluene (TNT > DNTs >> NTs) and the benzene (TNB > DNB >> NB) series under both aerobic and anaerobic conditions. Lack of activity of XenB against the mononitrotoluenes (2-, 3-, and 4-NT) was previously reported (Pak et al. 2000). It is interesting to note that the relative activity of XenB against 2,4-DNT was higher compared to 2,6-DNT under both aerobic and anaerobic conditions. These results also comport with the work of Pak et al. (2000), as well as previous literature reports indicating that the 2,4 isomer is generally more labile than the 2,6 isomer of DNT (Nishino et al., 1999; Nishino et al. 2000).

Of the nitramine explosives, RDX was degraded the fastest, followed by the nitroso-amines (in the order mononitroso > dinitroso > trinitroso) and then HMX. The decreasing degradation rate with increasing number of nitroso moieties seems to be counter-intuitive with respect

to steric controls on the rate since the nitroso groups (–NO) would be expected to be less bulky than the nitro groups ($–NO_2$), allowing the enzyme easier access. The observed rates may reflect the inherent specificity of some part of the XenB enzyme peripheral to the active site with preferences for nitro groups over nitroso groups. However, there must also be enough flexibility in these areas of the enzyme to allow some recognition of the nitroso moiety or TNX would not be expected to degrade at all. The difference in rates between RDX and HMX likely reflects the differences in the conformation of the heterocyclic rings, which affect the interaction of the active site of the enzyme with the molecule.

This work also adds to the information base for the new energetic compound CL-20. CL-20 was developed as more powerful and less sensitive replacement for RDX and HMX (Trott et al. 2003). In general, CL-20 has been found to be more labile than RDX in soil (Balakrishnan et al. 2004; Crocker et al. 2005). Our studies have demonstrated anaerobic transformation of CL-20 by purified xenobiotic reductase enzymes and whole cells. This new information adds to the few previously published reports, which demonstrated the transformation of CL-20 by monooxygenases (Bhushan et al. 2004b), nitroreductases (Bhushan et al. 2004a), and membrane-associated flavoenzymes (Bhushan et al. 2003).

The addition of RDX, HMX, and CL-20 to the list of known substrates for the xenobiotic reductases, under reduced O_2 tension, has important implications for bioremediation efforts, assuming that ability to degrade these explosives is a common characteristic among xenobiotic reductases. Basic research in environmental microbiology is often directed toward the isolation and characterization of bacterial strains that use a target compound as a sole source nutrient (for carbon, nitrogen, or energy). However, actual field-scale bioremediation is dominated by biostimulation approaches (i.e., addition of nutrients to stimulate the indigenous microbial community) rather than the addition of specific strains, which derive nutrients from a pollutant. The results reported here support the practice of general biostimulation approaches to effect remediation of explosives-contaminated sites as follows: (1) Transformation occurs under a relatively broad range of O_2 concentrations (anoxic to anaerobic); (2) transformation is not inhibited by the presence of utilizable nitrogen; and (3) transformation is performed by a class of enzyme that is widespread among bacterial genera. Several studies in our laboratory examining the microbial ecology of RDX biodegradation have detected *Pseudomonas* spp. 16S rDNA sequences in RDX-degrading enrichments were derived from groundwater from an explosives manufacturing site (unpublished data). Furthermore, given the widespread distribution of *Pseudomonas* spp. in the environment, it is likely that these organisms play a larger role in the transformation of nitramine explosives than previously thought, which could be further expanded when environmental conditions are manipulated to maximize their degradative potential.

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