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ORIGINAL ARTICLE

Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains

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Keywords

biodegradation, explosive, microaerophilic, RDX, *Rhodococcus*.

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Abstract

Aim: The goal of this study was to compare the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains under anaerobic, microaerophilic (<0.04 mg l⁻¹ dissolved oxygen) and aerobic (dissolved oxygen (DO) maintained at 8 mg l⁻¹) conditions.

Methods and Results: Three *Rhodococcus* strains were incubated with no, low and ambient concentrations of oxygen in minimal media with succinate as the carbon source and RDX as the sole nitrogen source. RDX and RDX metabolite concentrations were measured over time. Under microaerophilic conditions, the bacteria degraded RDX, albeit about 60-fold slower than under fully aerobic conditions. Only the breakdown product, 4-nitro-2,4-diazabutanal (NDAB) accumulated to measurable concentrations under microaerophilic conditions. RDX degraded quickly under both aerated and static aerobic conditions (DO allowed to drop below 1 mg l⁻¹) with the accumulation of both NDAB and methylenedinitramine (MEDINA). No RDX degradation was observed under strict anaerobic conditions.

Conclusions: The *Rhodococcus* strains did not degrade RDX under strict anaerobic conditions, while slow degradation was observed under microaerophilic conditions. The RDX metabolite NDAB was detected under both microaerophilic and aerobic conditions, while MEDINA was detected only under aerobic conditions.

Impact and Significance of the Study: This work confirmed the production of MEDINA under aerobic conditions, which has not been previously associated with aerobic RDX degradation by these organisms. More importantly, it demonstrated that aerobic rhodococci are able to degrade RDX under a broader range of oxygen concentrations than previously reported.

Introduction

Soil and groundwater contamination with explosive compounds has generated significant concern because of their mobility and persistence (Yamamoto *et al.* 2004). The extent of contamination, which occurs during munition production and military training operations, is currently being assessed (Jenkins *et al.* 2001; USDOD 2003; Clausen *et al.* 2004).

One of the important energetic compounds of concern is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). RDX

biodegradation has been observed under conditions ranging from fully aerobic to strictly anaerobic (Binks *et al.* 1995; Coleman *et al.* 1998; Hawari *et al.* 2000; Maloney *et al.* 2002; Adrian *et al.* 2003; Pudge *et al.* 2003; Adrian and Arnett 2004; Bhatt *et al.* 2005; Fuller *et al.* 2009).

The aerobic degradation of RDX by rhodococci has been previously characterized (Seth-Smith *et al.* 2002; Jackson *et al.* 2007). As these organisms are considered strict aerobes (Holt *et al.* 1994), RDX degradation under conditions with low or no oxygen was not examined over periods longer than 3 h (Coleman *et al.* 1998).

Degradation of RDX by the purified XplA/XplB enzymes of *Rhodococcus rhodochrous* strain 11Y under aerobic conditions yielded 4-nitro-2,4-diazabutanal (NDAB) (as seen with whole cells), while under anaerobic conditions, methylenedinitramine (MEDINA) was observed (Jackson *et al.* 2007). Enzymes with a high similarity to XplA/B have also been detected in strains of *Williamsia* and *Gordonia* that are able to utilize RDX as a sole source of carbon, nitrogen and energy (Indest *et al.* 2007).

During our research, we noticed RDX disappearance under conditions with low or no oxygen by two known RDX-degrading rhodococci (strains DN22 and 11Y) that were being used as controls. RDX degradation occurred in sealed serum bottles that were initially aerobic, but in which the oxygen was being utilized as the cells grew on the added succinate (1 g l^{-1}). This work was undertaken to further examine the observed RDX degradation by whole cells of three known RDX-degrading *Rhodococcus* strains under static aerobic, microaerophilic and anaerobic conditions.

Materials and methods

Chemicals and media

Technical-grade RDX (with octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) as a production impurity at 7% (w/w)) was a gift from James Phelan at Sandia National Laboratories (Albuquerque, NM, USA). All other chemicals were of reagent grade or purer. Basal salts medium (BSM) was prepared according to Hareland *et al.* (Hareland *et al.* 1975), which contained (g l^{-1}) $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 4.25; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1; NH_4Cl , 2; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 0.20; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.012; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.003; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; nitroloacetic acid, 0.123; nitrogen-free BSM (BSM-N) was prepared similarly, except that no ammonium chloride or nitroloacetic acid was added.

Bacterial strains

Rhodococcus strains capable of RDX biodegradation were acquired from the following sources (reference describing explosive degradative abilities in parentheses): *R. rhodochrous* 11Y (NCIMB 40820), Dr Neil C. Bruce, University of York, GB (via NCIMB Ltd, Aberdeen, UK) (Seth-Smith *et al.* 2002); *Rhodococcus* sp. DN22, Dr Diane Fournier, National Research Council, Canada, originally isolated by Dr Nick Coleman (Coleman *et al.* 1998); *Rhodococcus* sp. Strain A, Dr Diane Fournier. Strains were maintained on R2A agar and minimal agar (BSM-N solidified with noble agar) supplemented with succinate (1000 mg l^{-1}) as the carbon source and RDX (5 mg l^{-1}) as the sole nitrogen source.

RDX degradation screening

Rhodococcus strains were grown overnight in BSM with succinate. All solutions were purged with nitrogen for 10 min then equilibrated in the anaerobic glove bag (Coy Laboratory Products, Grass Lake, MI, USA) overnight with stirring prior to use. Cells were pelleted by centrifugation ($3400 \text{ rev min}^{-1}$, 4°C), washed twice and resuspended in anaerobic BSM-N. Triplicate vials (35 ml total volume in 40-ml glass vials with teflon-lined silicone rubber septa caps) of BSM-N amended with RDX (c. 4 mg l^{-1} or $19 \mu\text{mol l}^{-1}$, with 0.4 mg l^{-1} HMX) and succinate (1000 mg l^{-1}) were inoculated in a glove bag to achieve an optical density at 550 nm (OD_{550}) of 0.02 (corresponding to 10, 13 and $32 \mu\text{g}$ of total initial cell protein per vial for strains DN22, 11Y and A, respectively). Anaerobic vials were incubated with gentle shaking in a glove bag equipped with a palladium oxygen stripper. Microaerophilic vials were incubated with gentle shaking in a glove bag without an oxygen stripper or added hydrogen gas. The atmosphere in the glove bag was monitored and purged to keep the atmosphere at approximately 0.1% (v/v), which translated to 0.04 mg l^{-1} dissolved oxygen in the liquid in the vials, assuming full equilibration. Both glove bags had a temperature of $25 \pm 1^\circ\text{C}$. Aerobic treatments were purged with filtered air for 2 min, then incubated in vials equipped with vents (18-gauge needles and sterile $0.2\text{-}\mu\text{m}$ syringe filters). Samples were removed periodically and analysed for RDX, HMX and metabolites. Growth in the microaerophilic and anaerobic treatments was assessed by measuring the optical density at 550 nm (OD_{550}) at the beginning and end of the experiment. Culture liquid streaked onto agar plates (BSM agar with succinate and RDX; R2A agar), indicated cultures remained pure.

The production of MEDINA, NDAB and formaldehyde was determined at the end of the incubation when at least 50% of the initial RDX had degraded (or over 90% for the aerobic cultures). Ten millilitres of the cultures was filtered through sterile $0.45\text{-}\mu\text{m}$ glass microfibre syringe filters, while another 10 ml was preserved by the addition of 10% (w/v, final concentration) sea salt, which has been shown to stabilize MEDINA for up to a month when stored at 4°C (J. Hawari, unpublished data). Samples were shipped to the Biotechnology Research Institute, National Research Council Canada (Montreal, Canada) and analysed according to previously described methods (Hawari *et al.* 2000; Bhushan *et al.* 2003). Detection limits for these analytes were ($\mu\text{g l}^{-1}$) RDX, 0.2; MEDINA, 10; HCHO, 20; NDAB, 10.

An additional experiment was performed to confirm the formation of MEDINA under aerobic conditions using a single strain, *Rhodococcus* DN22. Cells were grown

and handled as described earlier, except that all steps were performed under ambient (e.g. aerobic) as opposed to anaerobic conditions. Four 1-l amber glass bottles with 500 ml of nitrogen-free BSM amended with RDX (10 mg l^{-1}) and succinate (1 g l^{-1}) were inoculated with washed DN22 cells to achieve an initial OD_{550} of 0.04. Two bottles were stirred at 500 rev min^{-1} and aerated with sterile air, while the other two bottles were incubated without stirring or aeration. The dissolved oxygen in each bottle was measured using a dissolved oxygen probe placed in the culture medium, and samples were removed for analysis of RDX and breakdown products (NDAB, MEDINA, formaldehyde) as described earlier. Samples were also removed for analysis of nitrite according to EPA Method 300.0 (Clescerl *et al.* 1998).

Analytical

The concentrations of the explosives and their breakdown products were monitored during incubation using HPLC according to a modified EPA Method 8330 (<http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/8330a.pdf>; accessed 15 June 2010), using a Dionex 3000 Ultimate HPLC with an Explosives E1 column, variable wavelength detector (reading at 230 nm) and a photodiode array detector collecting peak spectral data. The mobile phase was 43:57 methanol/water at a flow rate of 0.95 ml min^{-1} . The column temperature was 32°C . The lower method detection limit was approximately $2 \mu\text{g l}^{-1}$ for RDX and $5 \mu\text{g l}^{-1}$ for the RDX breakdown products.

Results

All three strains degraded RDX quickly under aerobic conditions when supplied with succinate as the carbon source (Fig. 1). Although the degradation rates were much slower, all the strains also degraded RDX under microaerophilic conditions. Fitting simple linear curves to the data indicated that the average first-order degradation rate coefficient (defined as the change in the aqueous RDX concentration per unit time) for the three strains under aerobic conditions was about 65 times larger than observed under microaerophilic conditions ($5.4 \text{ vs } 0.08 \text{ days}^{-1}$). A simple two-sample *t*-test assuming equal variances indicated that the observed microaerophilic RDX biodegradation curve for strain A and DN22 was not significantly different ($P > 0.05$), while that of 11Y was significantly different from both strains A ($P < 0.1$) and DN22 ($P < 0.05$). The culture OD in the microaerophilic vials increased by about 40% above the initial values for strains 11Y and A, whereas no increase was observed with DN22. This amount of growth is lower than the 5- to 10-fold increase in OD usually observed

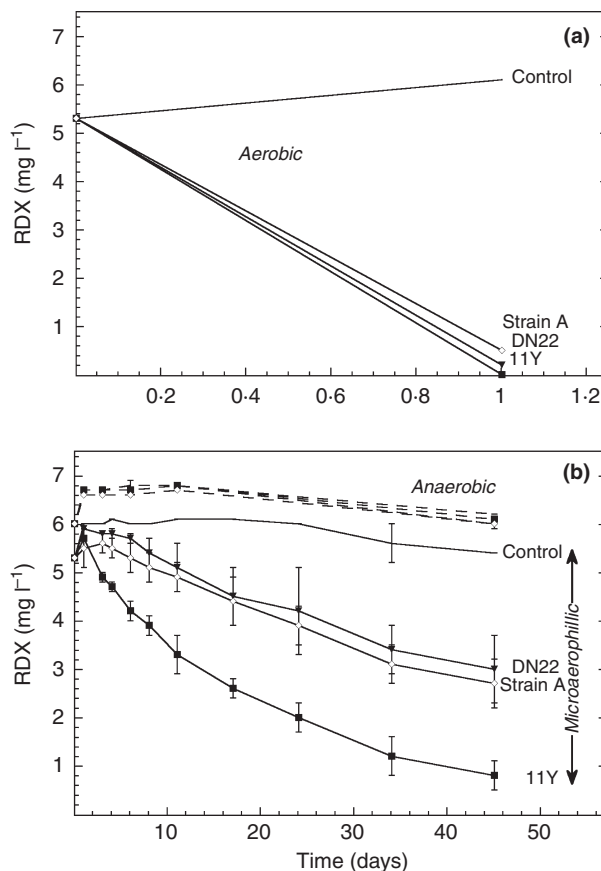


Figure 1 Degradation of RDX by three *Rhodococcus* strains under (a) aerobic and (b) microaerophilic (solid lines) or anaerobic (dashed lines) conditions. Data represent average and standard deviation of three replicate vials per treatment. (—) Control; (▼) DN22; (■) 11Y and (◇) Strain A. Note different scale on x-axis for upper and lower graphs.

for these strains under aerobic conditions. No degradation or OD increase was observed under strict anaerobic conditions. No degradation of HMX occurred under any of the test conditions.

The breakdown products from RDX after 1 day of aerobic incubation and 45 days of microaerophilic incubation (in terms of nitrogen and carbon mass balances) by the whole cells of the three rhodococci strains are presented in Table 1. Under microaerophilic conditions, NDAB was produced, but no MEDINA was detected. Additionally, the amount of residual formaldehyde was lower in the microaerophilic compared to the aerobic conditions.

RDX degradation by strain DN22 under well-mixed, aerated conditions and under static aerobic conditions was essentially identical, in terms of both the product distribution and the relative rates, even though the dissolved oxygen concentration decreased from 8 mg l^{-1} to $<1 \text{ mg l}^{-1}$ during static incubation (Fig. 2). Even as the

Table 1 Product distribution from RDX degradation by three *Rhodococcus* strains under static aerobic and microaerophilic conditions after 45 days of incubation. Values of zero indicate that the given analyte was below the detection limits; therefore, nitrogen and carbon masses could not be calculated. Data represent average and standard deviation of three replicates

		% Initial RDX				
Condition	Strain	RDX	MEDINA	NDAB	HCHO	Total
Nitrogen mass balance						
Microaerophilic	DN22	54.6 ± 13.8	0.00 ± 0.00	14.5 ± 5.4	—*	69.1 ± 14.8
	11Y	13.8 ± 5.3	0.00 ± 0.00	34.5 ± 2.1	—	48.3 ± 5.7
	Strain A	49.1 ± 9.1	0.00 ± 0.00	20.3 ± 3.8	—	69.4 ± 9.8
Aerobic	DN22	0.0 ± 0.0	3.43 ± 0.07	34.4 ± 0.2	—	37.8 ± 0.2
	11Y	0.0 ± 0.0	3.50 ± 0.08	34.2 ± 0.1	—	37.7 ± 0.1
	Strain A	5.1 ± 1.7	3.40 ± 0.03	30.2 ± 0.6	—	38.7 ± 1.9
Carbon mass balance						
Microaerophilic	DN22	54.6 ± 13.8	0.00 ± 0.00	19.4 ± 7.2	0.0 ± 0.0	74.0 ± 18.6
	11Y	13.8 ± 5.3	0.00 ± 0.00	46.0 ± 2.8	0.0 ± 0.0	59.8 ± 6.6
	Strain A	49.1 ± 9.1	0.00 ± 0.00	27.1 ± 5.1	0.0 ± 0.0	76.2 ± 12.4
Aerobic	DN22	0.0 ± 0.0	1.71 ± 0.03	45.9 ± 0.2	0.0 ± 0.0	47.6 ± 2.1
	11Y	0.0 ± 0.0	1.75 ± 0.04	45.6 ± 0.1	13.4 ± 0.2	60.7 ± 2.0
	Strain A	5.1 ± 1.7	1.70 ± 0.01	40.2 ± 0.8	14.2 ± 0.3	61.2 ± 4.4

MEDINA, methylenedinitramine; NDAB, 4-nitro-2,4-diazabutanal; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine.

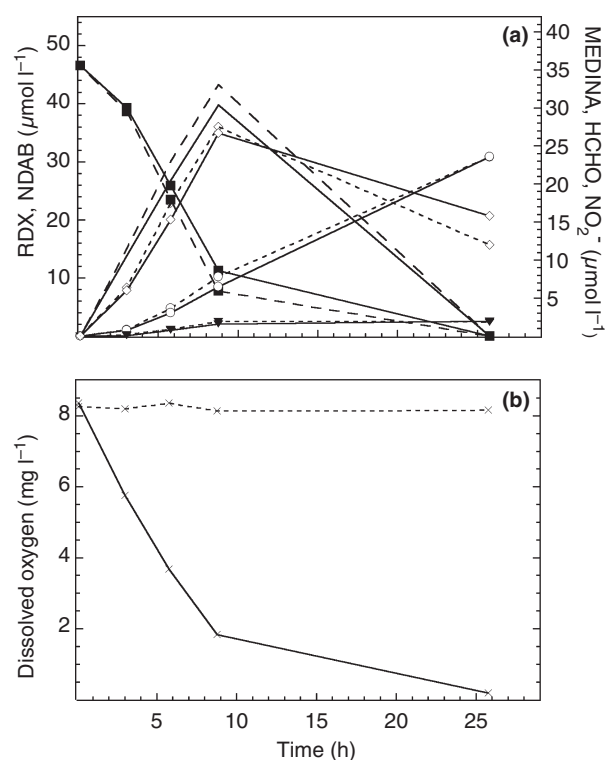
*Not applicable.

oxygen concentration decreased, it did not approach the low levels seen in the microaerophilic experiment (e.g., $<0.04 \text{ mg l}^{-1}$), and hence, RDX degradation proceeded rapidly and MEDINA was formed. As with the previous experiments, both NDAB and MEDINA were detected even under the fully aerated conditions.

Discussion

The degradation of RDX by whole cells of these *Rhodococcus* strains under extremely microaerophilic conditions is interesting, given that *Rhodococcus* spp. are usually considered to be strict aerobes (Holt *et al.* 1994). *Rhodococcus* species have variable abilities to reduce nitrate, and some have been shown to grow and/or degrade specific compounds under denitrifying conditions (Yoon *et al.* 2000; Travkin *et al.* 2002; Ngugi *et al.* 2005). Reports of activity and growth (albeit slow) under anaerobic conditions are sparse (De Wever *et al.* 1998; Nikodinovic *et al.* 2006), and this work also demonstrated a lack of RDX degradation by whole cells under strictly anaerobic conditions.

The production of NDAB under aerobic conditions is consistent with previous results with strain DN22 (Fournier *et al.* 2002) and by purified XplA/B in cell-free aerobic assays (Jackson *et al.* 2007). However, the aerobic accumulation of measurable amounts of MEDINA by whole cells of these strains was unexpected, as MEDINA was only seen with purified XplA/B enzymes under anaerobic conditions by Jackson *et al.* (Jackson *et al.*

**Figure 2** (a) Degradation of RDX and production of metabolites by three *Rhodococcus* strain DN22 under stirred/aerated (dashed lines) and static aerobic (solid lines) conditions. Data represent average of duplicates. (■) RDX; (○) NDAB; (▼) MEDINA; (◇) HCHO and (—) nitrite. (b) Dissolved oxygen under stirred/aerated (dashed lines) and static aerobic (solid lines) conditions.

2007), and is generally considered to be a marker for anaerobic RDX degradation processes (Hawari *et al.* 2000; Halasz *et al.* 2002). It should be noted that the detection of MEDINA in these experiments does not necessarily imply a biological mechanism of formation. MEDINA can form through spontaneous ring cleavage of the triazine ring after the initial denitration of the RDX molecule (Fournier *et al.* 2002).

These results confirmed the production of MEDINA from RDX did occur under aerobic conditions. Previous work examining aerobic RDX degradation by strain DN22 by Fournier *et al.* (Fournier *et al.* 2002), while not specifically analysing for MEDINA, did detect nitrous oxide (N₂O). During this previous study, mass spectroscopy using ring-labelled [¹⁵N]RDX as a starting material indicated that nitrous oxide was formed as the nitrogen from the nitro moieties of RDX were assimilated and was postulated to form from the later abiotic degradation of some RDX breakdown product that contained part of the ring structure of RDX. The unknown breakdown product was later identified as MEDINA (NHNO₂CH₂NHNO₂), which can produce N₂O according to the reactions (Lamberton *et al.* 1949a,b; Hawari *et al.* 2001):



This work used preservation procedures that allowed the stabilization and detection of MEDINA arising from RDX degradation. Therefore, it confirmed that the previous detection of N₂O during RDX degradation by strain DN22 resulted from the production and subsequent abiotic degradation of MEDINA.

These results raise the possibility that the degradation of RDX by these and related rhodococci is more complex than has been reported. The differences between previously reported aerobic RDX products and what is presented here may represent shifts in RDX metabolism when oxygen is at less than saturated concentrations (i.e., air sparged incubation *vs* static vials) but is not completely anaerobic. The fact that MEDINA did not accumulate under microaerophilic conditions likely indicates that either MEDINA was not formed in the presence of even very small amounts of oxygen or that it was degraded further in these whole cell experiments. However, the latter explanation seems more probable given that MEDINA was observed in the aerobic treatments.

In the larger context of environmental fate and remediation of RDX, this work indicates that RDX degradation under suboxic and/or microaerophilic conditions may be attributed to a wider range of bacterial species than would be normally assumed, and that the role of

organisms that are classified as strict aerobes under less than fully aerobic conditions should not be overlooked. This work also indicates that the use of MEDINA as a 'signature' of anaerobic RDX breakdown, and, conversely, the use of NDAB as a signature of aerobic RDX breakdown need to be carefully applied, taking the entire geochemical data set into consideration. Although the RDX degradation rates we observed under microaerophilic conditions were much slower than those under aerobic conditions, they are not insignificant over the timescales considered for natural attenuation processes.

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