

Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by *Clostridium* sp. EDB2

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Abstract

Cyclic nitramine explosives, RDX, HMX, and CL-20 are hydrophobic pollutants with very little aqueous solubility. In sediment and soil environments, they are often attached to solid surfaces and/or trapped in pores and distribute heterogeneously in aqueous environments. For efficient bioremediation of these explosives, the microorganism(s) must access them by chemotaxis ability. In the present study, we isolated an obligate anaerobic bacterium *Clostridium* sp. strain EDB2 from a marine sediment. Strain EDB2, motile with numerous peritrichous flagella, demonstrated chemotactic response towards RDX, HMX, CL-20, and NO_2^- . The three explosives were biotransformed by strain EDB2 via N-denitration with concomitant release of NO_2^- . Biotransformation rates of RDX, HMX, and CL-20 by the resting cells of strain EDB2 were 1.8 ± 0.2 , 1.1 ± 0.1 , and $2.6 \pm 0.2 \text{ nmol h}^{-1} \text{ mg wet biomass}^{-1}$ (mean \pm SD; $n = 3$), respectively. We found that commonly seen RDX metabolites such as TNX, methylenedinitramine, and 4-nitro-2,4-diazabutanol neither produced NO_2^- during reaction with strain EDB2 nor they elicited chemotaxis response in strain EDB2. The above data suggested that NO_2^- released from explosives during their biotransformation might have elicited chemotaxis response in the bacterium. Biodegradation and chemotactic ability of strain EDB2 renders it useful in accelerating the bioremediation of explosives under in situ conditions.

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Increasing use of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) has severely contaminated both marine and terrestrial environments around the world. The main sources of contamination are, dumping of huge amounts of unexploded ordnance (UXO), effluents from explosive manufacturing plants, military training, ordnance waste disposal by open burning/open detonation (OB/OD), land mines, and commercial use of explosives in propellants and mining [1–4]. Department of defense (DoD) and energy (DoE), USA, alone has over 21,000 contaminated sites and most of them are contaminated with explosives [5]. DoD of Canada has estimated 103 training sites and 3 OB/OD sites which are contaminated with RDX, HMX, and 2,4,6-trinitrotol-

uene (TNT) [6]. Cyclic nitramine explosives such as RDX and HMX cause adverse effects on biological systems, environment, and human health [7–11]. US environmental protection agency (USEPA) has recommended a lifetime health advisory for RDX [12] and HMX [13]. It is likely that due to its structural similarity with RDX and HMX, CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) (Fig. 1), a future generation high performance explosive, may also cause similar environmental problem. The cleanup of explosive(s) contaminated sites is a matter of growing concern among environmental protection groups.

Cyclic nitramines lack the electronic stability of aromatic compounds such as TNT and therefore an initial attack, whether biological or chemical, on the molecule(s) leads to their rapid spontaneous decomposition [3,14–18]. Bioremediation of contaminants, with little aqueous solubility, has always been a challenging task.

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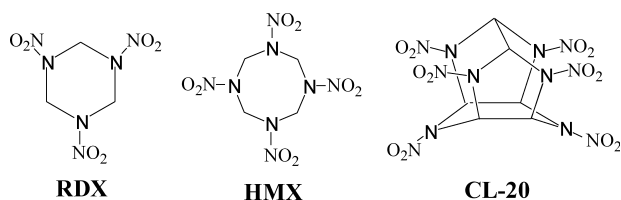


Fig. 1. Molecular structures of RDX, HMX, and CL-20.

Rates of biodegradation of such chemicals are limited by the rates of their mass-transfer from non-aqueous phase. In sediments and soil environments, hydrophobic pollutants are often attached to solid surfaces and/or trapped in pores. Cyclic nitramine explosives such as RDX, HMX, and CL-20 (Fig. 1) constitute one such class of chemicals with very little water solubilities of 40.0, 6.6, and 3.6 mg L⁻¹, respectively [19], and therefore distribute heterogeneously in marine and terrestrial environments. In order to degrade the explosive, the potential microorganism(s) must come in contact with the molecule. Bacterial chemotaxis is one such process that brings microbes closer to the contaminated sites and thus enhances the rate of biodegradation [20,21]. Motile and chemotactic microorganisms have advantage over non-motile and non-chemotactic ones by having the ability of sensing the explosive and thus move to form high-population densities around the chemical [22]. The dense microbial population can tolerate higher concentration of toxic chemicals [23] and reproduce more rapidly thus stimulating a rapid cleanup. Bacterial chemotaxis to a variety of organic pollutants and their subsequent degradation has been studied extensively [20,21,24–28] however no report is available so far with regard to cyclic nitramine explosives.

In the present study, we isolated an obligate anaerobic bacterium *Clostridium* sp. strain EDB2 from a marine sediment collected from a shipwreck site near Halifax Harbor in Canada. The strain demonstrated chemotaxis response towards the three cyclic nitramine explosives, RDX, HMX, and CL-20, and successfully degraded them. The present study is thus a model system to understand the environmental significance of chemotactic bacteria in accelerating the biodegradation of cyclic nitramine explosives under in situ conditions.

Materials and methods

Chemicals

Commercial grade RDX, HMX (chemical purity >99% for both explosives), [UL-¹⁴C]RDX (chemical purity >98%, radiochemical purity 97%, and specific radioactivity 28.7 μCi mmol⁻¹), and [UL-¹⁴C]HMX (chemical purity >94%, radiochemical purity 91%, and specific radioactivity 101.0 μCi mmol⁻¹) were provided by the Defense Research and Development Canada (DRDC), Valcartier, Quebec. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and 4-nitro-2,4-diazabutanol were obtained from SRI International (Menlo Park,

CA). Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) was synthesized according to the method described by Brockman et al. [29]. Methylenedinitramine (MDNA) was obtained from the rare chemical department of Aldrich, Oakville, ON, Canada. ε-CL-20 (99.3% purity) was provided by ATK Thiokol Propulsion, Brigham City, UT, USA. Microcapillaries (1 and 5 μl), low-melting-point agarose, and NADH were purchased from Sigma chemicals, Canada. Nitrous oxide (N₂O) was purchased from Scott specialty gases, Sarnia, ON, Canada. All other chemicals were of highest purity grade.

Marine sediment samples were collected from a shipwreck site (200 m deep-sea bed) at 50 nautical miles east of the Halifax harbor, Canada. Chemical analysis showed that sediment was composed of silt, clay, sand, and total organic matter at a concentration of 90.45%, 8.25%, 1.30%, and 1.90%, w/w, respectively. Major metals were Fe, Ca, Al, and Na at a concentration of 40, 24, 15, and 10 g kg⁻¹, respectively. pH of sediment was 7.7.

Media composition

Medium M1 was composed of (per liter): NaCl, 10.0 g; NaHCO₃, 2.5 g; NaH₂PO₄, 0.6 g; KCl, 0.2 g; NH₄Cl, 0.5 g; and Fe(III)-citrate, 12.25 g. Ingredients were mixed in hot water. pH was adjusted to 7.0 with 10 N NaOH. One liter medium was bubbled with N₂:CO₂ (80:20) for 45 min. After autoclaving at 121 °C for 20 min, we added filter-sterilized anaerobic solutions of lactate and glucose, 25 mM each; peptone, 1.0 g; trace elements, 10 ml; and vitamin mixture, 10 ml. Trace elements and vitamin mixture were same as reported [30]. Medium M2 was composed of (per liter): peptone, 8 g; yeast extract, 2 g; NaCl, 10 g; and glucose and lactate, 25 mM each. For making solid agar slants or plates, 1.8% agar powder (Difco) was added to the medium.

Isolation of chemotactic bacteria

Enrichments were carried out by adding RDX, HMX, and CL-20 together (30 μM each) and sediment (1% w/v) to medium M1 followed by incubation at room temperature for about 2 weeks under strict anaerobic conditions (N₂:CO₂, 80:20). The enriched culture (2.0 ml) was suspended in 3.0 ml phosphate-buffered saline (PBS) (100 mM, pH 7.0) supplemented with glucose (5 mM) as carbon and energy source. The cell-suspension was sealed in 10 ml vials under strict anaerobic conditions. Microcapillaries, sealed at one end, were then charged with 5 μM solution of either RDX, HMX or CL-20, inserted into the above sealed vials by piercing through the butyl rubber septum, and partly dipped into the enriched culture as shown in Fig. 2. After every 24 h of incubation, one capillary was taken out, plated onto the agar slants of both media M1 and M2, and incubated at room temperature under anaerobic conditions. This exercise was repeated every 24 h for about two weeks to allow isolation of a motile and chemotactic bacterial strain. The isolated strain formed small, black colonies (≤1 mm diameter) on solidified medium M1, and small, white, shining colonies (≤1 mm diameter) on solidified medium M2.

Bacterial identification

Morphological, physiological, and biochemical characterization of the isolated strain was performed by standard methods described for gram-staining, spore-staining, catalase, oxidase, and H₂S production, and nitrate- and nitrite-reduction, in the manual of methods for general bacteriology [31]. 16S rRNA gene analysis (1200 bases) and mol% G+C content of the strain were performed by laboratory services division, University of Guelph, ON, Canada. The strain was named EDB2 (EDB stands for explosive degrading bacterium).

Following microscopy techniques were performed to determine morphology, motility, flagella, and cell count: (1) phase contrast microscopy was used to observe cell-motility by hanging drop method [31] using standard microscope glass slides with cavity. It was also used for bacterial cell count with Petroff–Hausser counter (Hausser

Sealed microcapillary

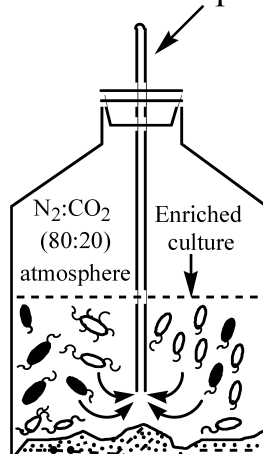


Fig. 2. Schematic representation of the technique used to isolate chemotactic bacteria. An air-tight 10 ml glass vial containing 5 ml enriched culture under anaerobic conditions. A 5 μ l microcapillary containing an explosive solution and sealed at outer end inserted into glass vial.

Scientific, Horsham, PA, USA); (2) transmission electron microscopy (TEM) was used to observe bacterial morphology and type of flagella. For TEM, bacterial cells were negatively stained as follows: a 20 μ l of mid-log-phase culture was placed onto a formvar (polyvinyl formaldehyde) coated grid of mesh size 400. After 4 min of incubation at room temperature the excess fluid was drained off with Whatman filter paper. The culture sticking to the grid was stained with 2% ammonium molybdate for about 30 s. Excess stain was drained and grid was washed with double-distilled water. The grid was air-dried and observed under a transmission electron microscope (Hitachi H7500).

Chemotaxis assays

Qualitative agarose-plug assay. This assay was conducted as described by Childers et al. [32] with some modifications using low-melting-point agarose (1.6% w/v, Sigma Chemicals, Canada). Briefly, a chemotaxis chamber of dimensions 20 \times 20 \times 1.5 mm was made with two plastic strips glued to a glass slide. A drop of low-melting-point agarose containing an explosive compound was placed on a glass coverslip and inverted onto the plastic strips. Bacterial cells, grown anaerobically in 100 ml of medium M2 supplemented with RDX, HMX, and CL-20 together (15 μ M each), were washed once with PBS buffer and resuspended in 8 ml PBS buffer containing 1 mM glucose as energy source before transferring to the chemotaxis chamber. The latter was then transferred to a plastic tube sealed with a rubber stopper followed by flushing with N₂:CO₂ (80:20) to create anaerobic conditions and then incubated at 30 °C. Bacterial ring formation around the agarose-drop was observed for 60 min. Two separate controls were used for comparison; the first contained agarose-drop with buffer alone, and the second contained autoclaved killed cells against a test chemical(s).

Quantitative microcapillary assay. A modified version of previously described method [24] was used. The cyclic nitramines, at a defined concentration, were charged into 1 μ l microcapillaries (Drummond Scientific Company, Broomall, PA, USA). The latter were inserted into a U-shaped chamber (similar to the chemotaxis chamber as mentioned above but without agarose-drop) already flooded with cell suspension of strain EDB2. The U-shaped chamber was then incubated under anaerobic conditions for 30 min. Microcapillaries were removed from the chamber and the cells sticking outside the capillaries were washed away with PBS buffer. Cells accumulated inside the capillaries were counted by Petroff–Hausser counter following serial dilutions. Controls were same as mentioned above.

Biotransformation assays for RDX, HMX, and CL-20

Biotransformation assays were performed in 6 ml air-tight glass vials under strict anaerobic conditions by purging the reaction mixture with argon for 20 min. Each assay vial contained (1 ml of assay mixture) either RDX (20 μ M), HMX (20 μ M) or CL-20 (20 μ M) and resting-cell preparation (5 mg wet biomass ml⁻¹) in a potassium phosphate buffer (50 mM, pH 7.0). To determine the effect of NADH on rate of biotransformation of explosive(s), NADH (200 μ M) was added to the reaction vial(s) and incubated at 30 °C. Three different controls were prepared by omitting either resting-cells, NADH or both from the assay mixture. Residual NADH was measured as described before [18]. The energetic chemicals and their biotransformed products were analyzed as previously described [15–18]. Explosive degradation activity of the cells was expressed as nmol h⁻¹ mg cell biomass⁻¹ unless otherwise stated.

Biomining of RDX and HMX in sediment microcosms

Sediment microcosms were prepared in four different combinations under anaerobic conditions (N₂) as follows: (1) 2 g native-sediment and 2 ml of 50 mM PBS (100 mM, pH 7.0); (2) 2 g sterile-sediment (gamma-irradiated) and 2 ml PBS; (3) 2 g native-sediment, 1 ml PBS, and 1 ml bacterial culture (2×10^8 cells ml⁻¹); and (4) 2 g sterile-sediment, 1 ml PBS, and 1 ml bacterial culture (2×10^8 cells ml⁻¹). Radiolabeled [UL-¹⁴C]RDX (33 μ g g⁻¹ sediment equivalent to 50,118 dpm) and [UL-¹⁴C]HMX (16 μ g g⁻¹ sediment equivalent to 54,310 dpm) were added to the microcosms through rubber septum using a syringe and needle. A small glass tube, placed inside microcosm, containing 0.5 ml of 0.5 M KOH was used as ¹⁴CO₂ trap. Evolution of ¹⁴CO₂ was determined in terms of dpm counts in KOH solution by using a liquid scintillation counter (Packard, Tri-Carb 4530, model 2100 TR, Packard Instruments Company, Meriden, CT). Experiments were performed in duplicate. *Note.* CL-20 was not included in this experiment because of unavailability of radiolabeled [UL-¹⁴C]CL-20.

Results and discussion

Isolation and identification of strain EDB2

We isolated an obligate anaerobic bacterial strain EDB2, from a marine sediment, by using a new technique devised to isolate chemotactic bacteria (see Materials and methods, and Fig. 2). Strain EDB2 was small rods of length 1.8–3.5 μ m and diameter 0.7–1.0 μ m, and exhibited gram-variable character. Spores were not seen in a stationary-growth-phase culture. The temperature and pH optima for the growth were 30 °C and 7.0, respectively. Strain EDB2 was motile with the help of numerous peritrichous flagella (Fig. 3). It was catalase and oxidase negative, and produced H₂S from S₂O₃²⁻. Also, it reduced nitrate and nitrite to N₂O using NADH as electron-donor. 16S rRNA gene analysis of 1200 bases (GenBank Accession No. AY510270) showed that strain EDB2 was 97% similar to *Clostridium xylanolyticum* (GenBank Accession No. X76739) and *Clostridium* strain DR7 (GenBank Accession No. Y10030). Strain EDB2 differed from *C. xylanolyticum* for having higher mol% G + C content of 53.6 compared to 40%, and for its potential to reduce nitrite and nitrate. No published description was available for *Clostridium* strain DR7.

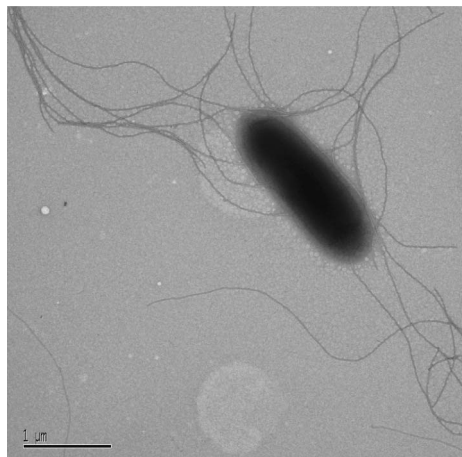


Fig. 3. Transmission electron micrograph of negatively stained cell of strain EDB2. Bar indicates 1 μ m.

The results showed that strain EDB2 belonged to genus *Clostridium* however it did not match with any closely related species.

Chemotaxis response of strain EDB2 towards explosives

Agarose-plug assays showed qualitative chemotaxis of strain EDB2 in the form of visible bright rings around the agarose-plug(s) containing RDX, HMX, CL-20 or nitrite. Control agarose-plug containing only buffer did not show chemotaxis response (Fig. 4). Whereas, microcapillary assays showed quantitative chemotaxis response of strain EDB2 towards the three explosives and the nitrite ion (Table 1). Since we found that biotransformation of RDX, HMX, and CL-20 occurred via an initial N-denitration (discussed below), we presumed that nitrite released from the explosive molecules was

Table 1
Quantitative chemotaxis assay with strain EDB2 by microcapillary method

Compound	Concentration (μ M)	Chemotaxis index ^a
Buffer ^b	—	1.0 ± 0.1
RDX	10	5.0 ± 0.4
HMX	10	2.4 ± 0.2
CL-20	8	7.2 ± 0.5
MNX	10	5.9 ± 0.4
TNX	10	1.3 ± 0.1
Nitrite	10	7.9 ± 0.6
	100	18.5 ± 1.3

^a Ratio of the number of cells accumulated inside the capillary containing test compound to the number of cells accumulated inside the capillary containing only buffer (i.e., 850 ± 90 cells); data are means \pm SD ($n = 3$).

^b Negative control without compound.

responsible for eliciting a chemotaxis response in strain EDB2. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), a RDX metabolite that also released nitrite on reaction with strain EDB2, elicited a chemotaxis response in strain EDB2 (Fig. 4 and Table 1). On the other hand, HMX, being the most recalcitrant (discussed below), elicited the least chemotaxis response (Table 1). Our hypothesis was strengthened when hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), a RDX metabolite without a nitro group [33], did not elicit chemotaxis response (Table 1). Additionally, methylenedinitramine and 4-nitro-2,4-diazabutanal, both ring-cleavage metabolites from RDX and/or HMX [15–18], neither released nitrite during their reaction with resting cells of strain EDB2 nor they elicited chemotaxis response in strain EDB2. Other known carbon products of cyclic nitramines, i.e., HCHO and HCOOH which lack NO_2^- elicited a poor chemotaxis response in bacterium (data

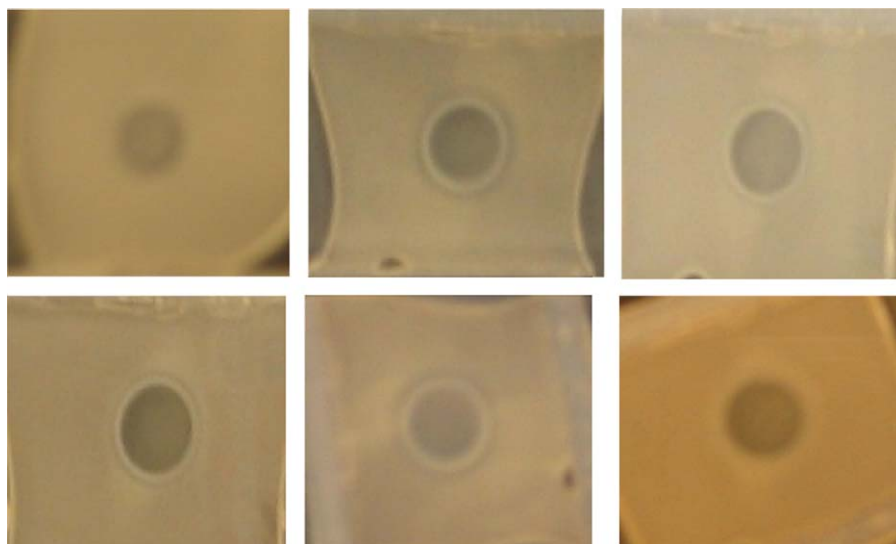


Fig. 4. Qualitative chemotaxis assay with strain EDB2 by agarose-plug method. Bright ring of bacterial cells around the plug indicated chemotaxis. Top line (left to right): buffer alone, nitrite, and RDX; bottom line (left to right): MNX, CL-20, and HMX.

not shown). The above data confirmed that nitrite released from the explosives was primarily responsible for inducing chemotaxis response in strain EDB2. Once gathered around the contaminated site, strain EDB2 may form high population density and reproduce more rapidly as demonstrated by other bacteria [22,23]. As a result, a higher degradation rate of energetic chemicals can be achieved by strain EDB2 as previously reported with regard to bacterial degradation of naphthalene [20,21].

Biodegradation of cyclic nitramine explosives

Growing-culture of strain EDB2 biotransformed the three explosives, in medium M2, as a function of its growth with the following order, i.e., CL-20 > RDX > HMX (Fig. 5). In the resting-cell study, biotransformation rates of RDX, HMX, and CL-20 by strain EDB2 were 1.8 ± 0.2 , 1.1 ± 0.1 , and 2.6 ± 0.2 $\text{nmol h}^{-1} \text{mg biomass}^{-1}$ (mean \pm SD; $n = 3$), respectively, whereas, in the presence of $100 \mu\text{M}$ NADH, the biotransformation rates of the three explosives enhanced to 4.5 ± 0.3 , 2.5 ± 0.3 , and 7.2 ± 0.6 $\text{nmol h}^{-1} \text{mg biomass}^{-1}$ (mean \pm SD; $n = 3$), respectively. In contrast, a negligible response was observed upon addition of $100 \mu\text{M}$ NADPH indicating the involvement of an unidentified NADH-dependent enzyme(s). No degradation was observed in control experiments without resting cells within 1 h of assay time. We found that biotransformation of RDX or HMX was accompanied by the formation of NO_2^- , N_2O , HCHO , and HCOOH . Whereas, biotransformation of CL-20 produced NO_2^- , N_2O , and HCOOH . The products obtained from each explosive were similar to those observed previously [15–18].

Resting-cells of strain EDB2 also catalyzed NADH-dependent biotransformation of HCHO to HCOOH which did not accumulate (data not shown). In a subsequent experiment, strain EDB2 mineralized 40% of

H^{14}CHO (of the total $340 \mu\text{g H}^{14}\text{CHO/L}$ medium) with evolution of $^{14}\text{CO}_2$ after 4 days of incubation in medium M1 suggesting an intermediary formation of HCOOH .

To mimic the in situ conditions, mineralization experiments were performed in sediment-microcosms. When strain EDB2 (2×10^8 cells ml^{-1}) was incubated with native-sediment in the presence of either $[\text{UL-}^{14}\text{C}] \text{RDX}$ or $[\text{UL-}^{14}\text{C}] \text{HMX}$, we obtained 70% (of the total 33 mg RDX/kg sediment) and 46% (of the total 16 mg HMX/kg sediment) mineralization, respectively, after 18 days. In contrast, in native-sediment alone, only 17% and 8% mineralization was obtained for RDX and HMX, respectively. On the other hand, when strain EDB2 was inoculated in sterile-sediment, mineralization of RDX and HMX was only 11% and 2%, respectively. We found that compared to the sterile-sediment, the mineralization of $[\text{UL-}^{14}\text{C}] \text{RDX}$ and $[\text{UL-}^{14}\text{C}] \text{HMX}$ by strain EDB2 in the liquid medium M1 was 30% and 13%, respectively, after 18 days. The reason for rapid mineralization of RDX and HMX in liquid medium attributed to a better accessibility of explosive molecules to the bacterial cells. Taken together, the above data suggested that strain EDB2 seems to be a promising degrader of cyclic nitramine explosives under native-sediment (or in situ) conditions.

In conclusion, the present study demonstrated a chemotaxis-mediated biodegradation of cyclic nitramine explosives where local population of strain EDB2 first initiates biotransformation of cyclic nitramines with release of NO_2^- . The NO_2^- thus produced attracts other distantly located bacterial cells to help accelerate the biodegradation process. In comparison to the conventional microbial degradation where microorganism(s) fortuitously come in contact with the energetic chemicals, the present study emphasizes the use of chemotactic bacteria for efficient removal of explosives in contaminated sites. This is the first report which showed that a pure bacterial culture (strain EDB2) accessed the three hydrophobic cyclic nitramine explosives by chemotaxis and degraded them to innocuous products. The properties demonstrated by strain EDB2 renders it useful for the cleanup of sites contaminated with cyclic nitramines.

Acknowledgments

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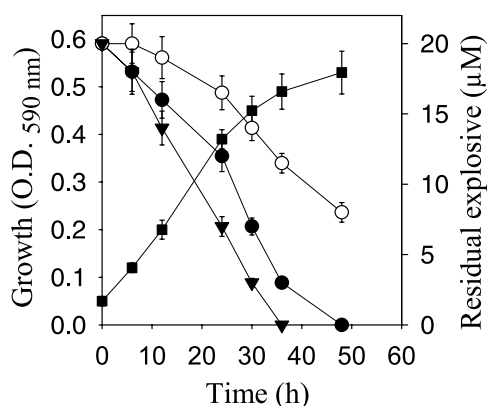


Fig. 5. Biotransformation of RDX (●), HMX (○), and CL-20 (▼) by strain EDB2 as a function of its growth (■). Data are means \pm SD ($n = 3$). Some error bars are not visible due to their small size.

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