Isolation of Three Hexahydro-1,3,5-Trinitro-1,3,5-Triazine-Degrading Species of the Family *Enterobacteriaceae* from Nitramine Explosive-Contaminated Soil

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Three species of the family *Enterobacteriaceae* that biochemically reduced hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) were isolated from nitramine explosive-contaminated soil. Two isolates, identified as *Morganella morganii* and *Providencia rettgeri*, completely transformed both RDX and the nitroso-RDX reduction intermediates. The third isolate, identified as *Citrobacter freundii*, partially transformed RDX and generated high concentrations of nitroso-RDX intermediates. All three isolates produced $^{14}CO_2$ from labeled RDX under O_2-depleted culture conditions. While all three isolates transformed HMX, only *M. morganii* transformed HMX in the presence of RDX.

The nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are used by the military in high-yield munitions, often in combination. Manufacture and testing of explosives have resulted in soils contaminated with mixtures of these nitramine explosives. Bioremediation is proposed as a safe and cost-effective means of site cleanup (9, 11, 15). Attempts to biodegrade RDX and HMX aerobically have not been successful (8, 12, 15, 19). However, biodegradation of both RDX and HMX has been reported to occur under anaerobic or anoxic conditions (6, 9, 12, 13, 16, 19).

The first step of biological RDX degradation involves reduction of the nitro groups to form nitroso compounds. Stable mono-, di-, and trinitroso derivatives of RDX have been isolated (12, 13). Mono-, di-, tri-, and tetranitroso intermediates of biological HMX reduction have also been observed (13, 19). McCormick et al. used results obtained with a sewage sludge inoculum to propose a degradation pathway for RDX wherein the biochemical reduction of mono-, di-, and trinitroso-RDX intermediates to unstable hydroxylamino compounds is followed immediately by hydrolytic ring cleavage (12). Hydrolysis of the remaining carbon-nitrogen bonds and reduction of all nitro groups to amines complete the postulated pathway. Strictly anaerobic cultures did not produce $^{14}CO_2$ from labeled RDX; however, $^{14}CO_2$ production was observed in composting experiments (9).

Previous work with RDX degradation used sewage sludge, horse manure, or amended soils; however, no microorganisms were isolated or identified (6, 9, 12, 13). In this report, we describe the isolation and identification of three individual members of the family *Enterobacteriaceae*, each of which degraded RDX in pure culture.

Isolation and identification of RDX-reducing bacterial strains. The numerically predominant, aerotolerant bacteria from a nitramine explosive-contaminated soil inoculum were the subject of this investigation. An aqueous extract of nitramine explosive-contaminated soil from Los Alamos National Laboratory (110 mg of HMX g of soil^{-1} and 16 mg of RDX g of soil^{-1}) was used to inoculate nutrient broth (Difco) containing 0.33 mM RDX and 0.05 mM HMX. The culture was shaken (150 rpm, 30°C) in a stoppered flask for 2 weeks, when 1 ml was transferred to fresh medium and incubated for 7 more days. During this time, both RDX and HMX were completely transformed. The predominant organisms from this culture were isolated by dilution plating onto nutrient agar (Difco) and subsequently tested for the ability to degrade RDX. Several of the isolated bacterial strains tested did not transform RDX after 2 weeks of growth under the conditions described below. However, three isolated species were able to transform RDX in pure liquid culture. These isolates were identified as *Providencia rettgeri* B1, *Morganella morganii* B2, and *Citrobacter freundii* NS2 with the Biolog system (2) and the API20E system (18) (Table 1). The identifications were confirmed by reference to *Bergey's Manual of Systematic Bacteriology* (3). Standard bacterial physiology tests (7) were also applied (Table 1). These strains were sent to the American Type Culture Collection for storage and dissemination.

Nitramine explosive degradation in liquid culture. Explosive degradation was accomplished by aerobic culturing of the organisms followed by culturing under O_2-depleted conditions. YE medium (minimal salts [1] with 0.8% yeast extract [Difco]) was supplemented with either a mixture of RDX (0.33 mM) and HMX (0.05 mM) or HMX (0.07 mM) alone. Thirty-milliliter cultures were initially grown aerobically in 50-ml fluted flasks at 30°C with shaking (150 rpm). Although the aerobic growth rate of each bacterial isolate was unaffected by these concentrations of RDX and HMX, they were unable to transform either compound when grown aerobically. Therefore, once aerobic cultures reached an A_{560} of 1.0 (6 to 8 h of growth), the flasks were plugged with rubber stoppers to obtain O_2-depleted conditions. Each culture was momentarily exposed to air once every 2 to 3 days when samples were taken. Samples (400 μl) were centrifuged for 5 min in a microcentrifuge, and 350 μl of the culture supernatant was set aside. The cell pellet was resuspended in acetonitrile (400 μl) to recover any hydrophobic compounds that may have been attached to cell wall material. After centrifugation, 350 μl of acetonitrile supernatant was combined with the culture supernatant and filtered (0.2-μm-pore-size filter). The filtrate was analyzed for RDX, HMX, and the products of biotransformation. After 45 days of growth, cultures were plated on nutrient agar. Inspection of the colonies growing on these plates after 2 days of

TABLE 1. Results of genus and species identification tests

Strain	Oxidase	Catalase	Gram stain	Motility	Nitrate reductase	Lysine decarboxylase	Ornithine decarboxylase	Citrate utilization	Urease	H_2S production	β-Galacto-sidase	Conversion of tryptophan to indole	% Confidence of Biolog test
B2	−	+	−	+	+	−	+	−	+	−	−	+	92
B1	−	+	−	+	+	−	−	+	+	−	−	+	95
NS2	−	+	−	+	+	−	−	+	−	+	+	−	96

growth showed that each culture contained only the originally inoculated bacterial strain.

Quantitation of explosives and intermediates of biological reduction. RDX, HMX, and their biotransformation products were separated by using reverse-phase high-performance liquid chromatography (HPLC) with a 25-cm Dynamax C-18 column (Rainin) and a mobile phase of 28:72 MeOH-H_2O (Baker) at a flow rate of 0.5 ml min^{-1}. The explosives were quantified on the basis of their A_{230}. By this method, the minimum detectable concentration of RDX and nitroso-RDX intermediates was 1.3 μM. The molar extinctions at 230 nm of the nitroso derivatives of RDX and HMX are identical to those of the parent compounds (10). The nitroso derivatives of RDX and HMX were identified by comparison of their retention times during HPLC and their molecular weights to those of authentic compounds synthesized and generously shared with us by J. Oxely of the New Mexico Technical Institute. Liquid chromatography-mass spectrometry (Hewlett-Packard 5989A mass spectrometer) was performed with a thermospray interface and the HPLC method described above with addition of 0.1 M ammonium acetate to the mobile phase. For all nitramine explosives and their reduction products, this procedure gave a molecular ion mass of +59 (an acetate adduct) as the major peak in the negative-ion detection mode.

Experiments with [^{14}C]RDX. The fate of RDX carbons was monitored by using the culturing procedures described above with a few modifications. YE medium (120 ml in a 500-ml flask) was supplemented with a mixture of unlabeled RDX and [U-^{14}C]RDX (1.2 Ci/mol; radiochemical purity, >93%; Chemsyn Science Labs) to make a final RDX concentration of 0.15 mM (72 mCi mol^{-1}) and autoclaved to ensure sterility. Cultures were inoculated with the appropriate strain aerobically grown to an A_{560} of 1.0, at which point the flasks were plugged and incubated for 45 days. At this time, the labeled volatile organics and CO_2 were recovered and trapped by first sparging the headspace of each flask with air for 6 min and passing the exit gasses through a volatile organic trap (Supelco) and a solution of 5 N NaOH. The culture medium had become basic (pH 8 to 9), requiring that it be acidified (1 ml of H_2SO_4) to ensure complete recovery and trapping of $^{14}CO_2$. Cellular material was removed by centrifugation. The culture supernatant was extracted with an equal volume of ethyl ether to generate the aqueous and ether-soluble fractions. All fractions were then analyzed by liquid scintillation counting (in Packard Ultima Gold XR scintillation fluid) to determine the final distribution of radioactivity.

Each of the three isolates released $^{14}CO_2$ when incubated with [^{14}C]RDX (Table 2). Most of the label from *P. rettgeri* and *M. morganii* cultures remained in the aqueous phase. The ether-soluble fractions contained residual amounts of RDX and its nitroso derivatives, as shown by HPLC analysis. The *C. freundii* culture had the highest percentage of radioactive label remaining in the ether-soluble fraction. None of the isolates produced significant amounts of volatile compounds, and intermediates produced during RDX degradation did not appear to adhere to cell material.

In contrast to results obtained previously with strictly anaerobic liquid culture (12), CO_2 was evolved in this study by using an O_2-depleted liquid culture. Each of the three isolates tested released $^{14}CO_2$ when incubated with [^{14}C]RDX (Table 2). Thus, our isolates were all able to break the RDX ring because $^{14}CO_2$ could be released only if the ring carbons of RDX were completely metabolized. This finding is consistent with a previous report of $^{14}CO_2$ being released from RDX degraded in a compost where a mixture of aerobic, anaerobic, and anoxic conditions could have existed (9). The distribution of label in the organic phase also corresponds well to the amount of RDX and nitroso-RDX intermediates remaining after 45 days in the unlabeled experiments described below.

Biochemical reduction of RDX by three individual strains. All three isolates transformed RDX in pure culture during the stationary phase of growth. *P. rettgeri* and *M. morganii* transformed all of the RDX, whereas *C. freundii* did not (Fig. 1). Mono- and dinitroso derivatives of RDX accumulated to maximum concentrations within 10 days. *M. morganii* accumulated the lowest concentrations of these nitroso compounds. *P. rettgeri* and *M. morganii* removed each of these nitroso-RDX intermediates to levels below 5% of the initial RDX concentrations (Fig. 1A and B). As corroborated by the radiolabel experiments described above, disappearance of the nitroso intermediates also implies ring breakage. Assuming that the pathway suggested by McCormick et al. (12) applies, these bacteria may reduce the nitroso groups to unstable hydroxylamino groups, which decompose, thus hydrolyzing the ring.

TABLE 2. Distribution of radioactivity[a] from metabolism of [^{14}C]RDX

Organism	Avg % radioactivity						
	Total at start	Total at end	Cell-associated fraction	Volatile organics	CO_2	Ether-soluble fraction	Aqueous fraction
M. morganii B2	100	75 (5)	1 (0.2)	<1	5 (1)	7 (1)	62 (7)
P. rettgeri B1	100	75 (7)	<1	≪1	8 (2)	14 (2)	52 (5)
C. freundii NS2	100	65 (9)	<1	1 (0.2)	9 (2)	33 (4)	22 (3)

[a] Distribution by percentage of initial [^{14}C]RDX added (total, 2.8×10^7 cpm). The values shown are averages of duplicate samples with one standard deviation in parentheses.

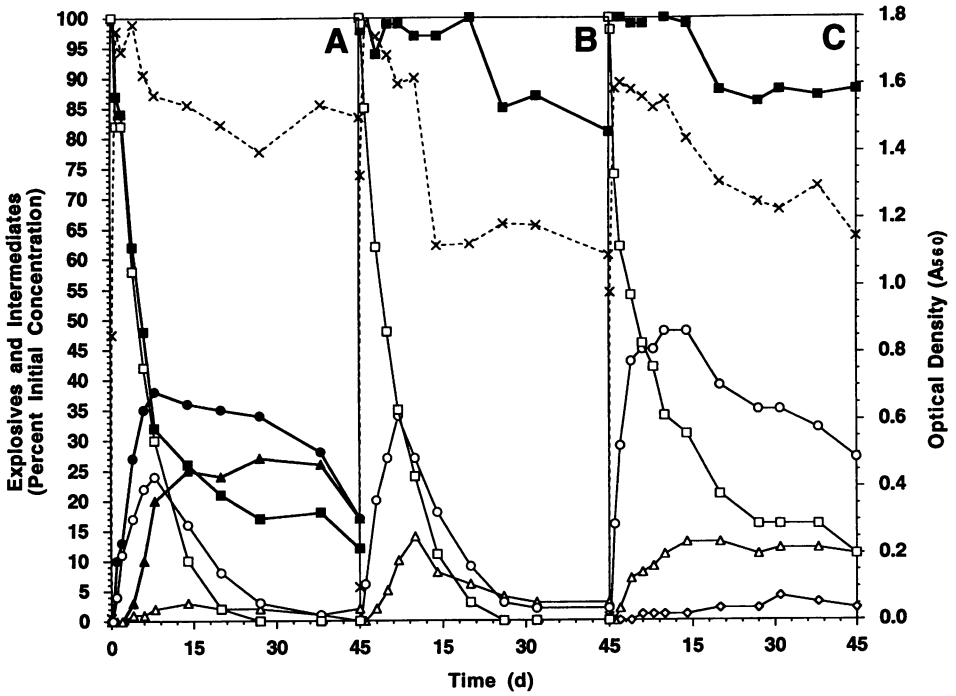

FIG. 1. Degradation of an RDX-HMX mixture in YE medium by *M. morganii* B2 (A), *P. rettgeri* B1 (B), and *C. freundii* NS2 (C). Each nitroso intermediate concentration is expressed as a percentage of the parent compound's initial concentration. Symbols: □, RDX; ○, mononitroso-RDX; △, dinitroso-RDX; ◇, trinitroso-RDX; ■, HMX; ●, mononitroso-HMX; ▲, dinitroso-HMX; ×, cell growth (A_{560}). Nota bene: because the initial concentration of HMX was an order of magnitude less than that of RDX, the absolute rate of HMX transformation by *M. morganii* was an order of magnitude less than that of RDX transformation.

C. freundii transformed the least RDX and accumulated the most nitroso-RDX compounds (Fig. 1C). Only this isolate produced detectable amounts of trinitroso-RDX, which may indicate a reduced ability to transform these nitroso compounds.

Biochemical reduction of HMX by three individual strains. Each isolate catalyzed the disappearance of another nitramine explosive, HMX, although the process was slower and less complete than that for RDX. After 45 days, *M. morganii* transformed approximately 60% of the initial HMX, *P. rettgeri* transformed ~60%, and *C. freundii* transformed ~50%. Mono- and dinitroso intermediates of HMX also accumulated in the media of all three cultures.

The extent of HMX transformation by *M. morganii* was not inhibited by RDX (Fig. 1A). In contrast, RDX strongly inhibited transformation of HMX by either *C. freundii* or *P. rettgeri* (Fig. 1B and C). *M. morganii* was probably able to further metabolize nitroso-HMX intermediates in the presence of RDX, as supported by accounting for the initial HMX. At the end of the incubation, 15% remained as HMX, 25% was mononitroso-HMX, and 25% was dinitroso-HMX, leaving about one-third further metabolized, presumably in a manner similar to the fate of the nitroso-RDX intermediates.

Summary. We have isolated three RDX-degrading members of the family *Enterobacteriaceae* from explosive-contaminated soil. All three isolates released $^{14}CO_2$ from [^{14}C]RDX under O_2-depleted conditions and were therefore able to break the RDX ring. All three isolates also transformed HMX; however, *P. rettgeri* and *C. freundii* were unable to do so when RDX was present. The best demonstrations of RDX degradation have been obtained with inocula derived from either sewage sludge (11, 12) or horse manure (9). Each of these inocula are good sources of members of the family *Enterobacteriaceae*, and therefore members of this family may be responsible for most of the nitramine explosive degradation witnessed under these circumstances. Coincidentally, many enteric bacteria are recognized for their aromatic nitroreductase activities (4, 5, 14, 17, 20). We are currently examining an aromatic nitroreductase activity in *M. morganii* to determine its involvement in nitramine reduction.

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