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

CHRISTOPHER L. KITTS, DARYL P. CUNNINGHAM, AND PAT J. UNKEFER
CST-18, Los Alamos National Laboratory, Los Alamos, New Mexico

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 14CO2 from labeled RDX under O2-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 trinitro 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 14CO2 from labeled RDX; however, 14CO2 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. Expansive degradation was accomplished by aerobic culturing of the organisms followed by culturing under O2-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 A560 of 1.0 (6 to 8 h of growth), the flasks were plugged with rubber stoppers to obtain O2-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
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-H2O (Baker) at a flow rate of 0.5 ml min⁻¹. The explosives were quantified on the basis of their A₂₃₀. 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 [¹⁴C]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-¹⁴C]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 A₅₆₅ of 1.0, at which point the flask was plugged and incubated for 45 days. At this time, the labeled volatile organics and CO₂ 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 H₂SO₄) to ensure complete recovery and trapping of ¹⁴CO₂. 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 ¹⁴CO₂ when incubated with [¹⁴C]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), CO₂ was evolved in this study by using an O₂-depleted liquid culture. Each of the three isolates tested released ¹⁴CO₂ when incubated with [¹⁴C]RDX (Table 2). Thus, our isolates were all able to break the RDX ring because ¹⁴CO₂ could be released only if the ring carbons of RDX were completely metabolized. This finding is consistent with a previous report of ¹⁴CO₂ 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 dinitro 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.

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**TABLE 1. Results of genus and species identification tests**

| Strain | Oxidase | Catalase | Gram stain | Motility | Nitratreductase | Lysinedecarboxylase | Ornithinedecarboxylase | Citrateutilization | Urease | H₂Sproduction | β-Galactosidase | Conversion of 
teophan to 
indole | % Confidence of 
Biolog test |
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<td>B2</td>
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<td>+</td>
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**TABLE 2. Distribution of radioactivity from metabolism of [¹⁴C]RDX**

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<tr>
<th>Organism</th>
<th>Avg % radioactivity</th>
<th>Total at start</th>
<th>Total at end</th>
<th>Cell-associated fraction</th>
<th>Volatile organics</th>
<th>CO₂</th>
<th>Ether-soluble fraction</th>
<th>Aqueous fraction</th>
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<td>M. morganii B2</td>
<td>100</td>
<td>75 (5)</td>
<td>1 (0.2)</td>
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<td>5 (1)</td>
<td>7 (1)</td>
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<tr>
<td>P. rettgeri B1</td>
<td>100</td>
<td>75 (7)</td>
<td>&lt;1</td>
<td>8 (2)</td>
<td>14 (2)</td>
<td>52 (5)</td>
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<tr>
<td>C. freundii NS2</td>
<td>100</td>
<td>65 (9)</td>
<td>&lt;1</td>
<td>1 (0.2)</td>
<td>9 (2)</td>
<td>33 (4)</td>
<td>22 (3)</td>
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*Distribution by percentage of initial [¹⁴C]RDX added (total, 2.8 × 10⁷ cpm). The values shown are averages of duplicate samples with one standard deviation in parentheses.*
C. freundii transformed the least RDX and accumulated the most nitroso-RDX compounds (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.

**REFERENCES**

methods for general bacteriology. American Society for Microbiology, Washington, D.C.