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Biodegradation of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by *Phanerochaete chrysosporium*: New Insight into the Degradation Pathway

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Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is a recalcitrant energetic chemical that tends to accumulate in soil, close to the surface. The present study describes the aerobic biodegradability of HMX using *Phanerochaete chrysosporium*. When added to 7 day old static *P. chrysosporium* liquid cultures, HMX (600 nmol) degraded within 25 days of incubation. The removal of HMX was concomitant with the formation of transient amounts of its mono-nitroso derivative (1-NO-HMX). The latter apparently degraded via two potential routes: the first involved N-denitration followed by hydrolytic ring cleavage, and the second involved α-hydroxylation prior to ring cleavage. The degradation of 1-NO-HMX gave the ring-cleavage product 4-nitro-2,4-diazabutanal (NDAB), nitrite (NO_2^-), nitrous oxide (N_2O), and formaldehyde (HCHO). Using [^{14}C]-HMX, we obtained $^{14}CO_2$ (70% in 50 days), representing three C atoms of HMX. Incubation of real soils, contaminated with either HMX (403 μmol kg^{-1}) (military base soil) or HMX (3057 μmol kg^{-1}), and RDX (342 μmol kg^{-1}) (ammunition soil) with the fungus led to 75 and 19.8% mineralization of HMX (liberated $^{14}CO_2$), respectively, also via the intermediary formation of 1-NO-HMX. Mineralization in the latter soil increased to 35% after the addition of glucose, indicating that a fungus-based remediation process for heavily contaminated soils is promising. The present findings improve our understanding about the degradation pathway of HMX and demonstrate the utility of using the robust and versatile fungus *P. chrysosporium* to develop effective remediation processes for the removal of HMX.

Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is a toxic energetic compound (*1*−*4*), and its wide use in commercial and military applications has led to contamination of soil and water (*5*, *6*). Although HMX is found to have a low affinity for soils (*7*), its low aqueous solubility (5 mg/L at 25 °C) as compared to that of 2,4,6-trinitrotoluene (TNT, 145 mg/L) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX, 60 mg/L) (*2*) and its low vapor pressure contributed to its persistence in surface soil (*5*, *6*). Although both RDX and HMX are oligomeric cyclic nitramines composed of the same structural unit CH_2-N-NO_2, trimer and tetramer, respectively, HMX has thus far shown unique recalcitrance behavior as compared to RDX, especially under aerobic conditions.

Several studies have reported the biodegradation of HMX under anaerobic conditions (*8*, *9*). Shen et al. (*9*) reported 4% mineralization of HMX in soil by indigenous anaerobic degraders, which in the presence of anaerobic sludge increased to 65% in 5 months. In a subsequent study, Hawari et al. (*10*) found that anaerobic sludge transformed HMX via the intermediary formation of the ring cleavage products methylenedinitramine (MEDINA) and bis(hydroxymethyl)-nitramine, which finally degraded to nitrous oxide (N_2O), formaldehyde (HCHO), and CO_2. The initial reactions that lead to ring cleavage were not known.

Using a commercial xanthine oxidase (XO) under anaerobic conditions, Bhushan et al. (*11*) recently reported the occurrence of an initial N-denitration reaction prior to ring cleavage of HMX to MEDINA, 4-nitro-2,4-diazabutanal (NDAB), HCHO, and formic acid (HCOOH). Decomposition of HMX via the N-denitration mechanism was also reported to occur under abiotic conditions during the oxidation of HMX using Fenton's reagents (*12*) or alkaline hydrolysis (*13*).

In contrast, limited research has been published on the aerobic degradation of HMX (*14*, *15*). Spanggord et al. (*15*) reported that HMX can be aerobically degraded in contaminated river water samples amended with yeast extract but did not succeed in the enrichment of any bacterial or fungal strains. The three previously reported *Rhodococcus* strains, known for their ability to degrade RDX (*16*−*18*), did not remove HMX from a contaminated soil amended with nutrients (*19*). Although the white rot fungus *Phanerochaete chrysosporium* is found to successfully degrade RDX (*20*−*23*) and HMX (*24*), no data on products or degradation routes are described.

The aim of the present study is to investigate the potential of *P. chrysosporium* to degrade HMX in a synthetic culture medium and to understand the degradation pathway. We will also demonstrate the ability of *P. chrysosporium* to degrade HMX in two different contaminated soils so the data can be used to develop a fungal-based bioremediation technology.

Experimental Section

Chemicals. Commercial grade (>99% purity) HMX, RDX, and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) were provided by the Defense Research and Development Canada (DRDC, Valcartier, Quebec, Canada). Uniformly labeled UL-[^{14}C]-HMX (chemical purity 94%, radiochemical purity 91%, specific activity 93.4 μCi/mmol) and ring-labeled [^{15}N]-HMX (>98% purity) were also obtained from DRDC (*25*). NDAB was obtained from R. Spanggord (SRI International, Menlo Park, CA). Methylenedinitramine (MEDINA) was obtained from the rare chemical department of Aldrich, Oakville, Ontario, Canada. All other chemicals used were of reagent grade.

Soil Characteristics. Two soils contaminated with explosives were used. The first was sampled from an old manufacturing plant for explosives in Valleyfield (Quebec, Canada) and is referred to as the ammunition soil. The second was sampled at a military base in Gagetown (New Brunswick,

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TABLE 1. Characteristics of the Soils Used

composition	Gagetown soil	ammunition soil
total organic C (%)	14	0.8
total N (mg·kg^{-1})	4800	1100
HMX (μmol.kg^{-1})	403	3057
RDX (μmol.kg^{-1})	none	342
pH	6.8	6.5

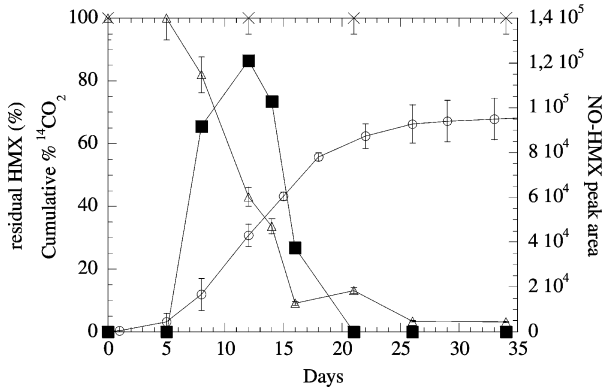

FIGURE 1. Percentage of residual HMX (Δ), production of 1-NO−HMX (■), and mineralization of [^{14}C]-HMX to $^{14}CO_2$ (○) with 7 day old *P. chrysosporium* in nitrogen-limited culture medium; percentage of residual HMX in noninoculated controls (X). Values represent the average and standard deviation of triplicate experiments.

Canada) and is referred to as Gagetown soil. The nitramine content of the two soils was analyzed according to the U.S. EPA Method 8330 (*26*), and their C and N content is summarized in Table 1. Prior to usage, the soils were passed through a 2 mm sieve, and residual humidity was removed by air-drying in a fume hood.

Biotransformation of HMX with Fungi. *P. chrysosporium* ATCC 24725 was maintained on YPD plates (per liter: yeast extract, 5 g; peptone, 10 g; dextrose, 20 g; agar, 20 g; at pH 5.5 adjusted with H_2SO_4) and cultivated in a nitrogen-limited medium as previously described (*19*). Biotransformation experiments were conducted in nitrogen-limited liquid medium (10 mL) and were started by adding HMX (18 μL from a 10 000 mg/L acetone stock solution) to 7 day old mycelium (precultivated in 125 mL serum bottle). The degradation in soil was performed by preparing slurries with 2 g of either ammunition or Gagetown soil (both γ-irradiated), 10 mL of nitrogen-limited medium, and 2×10^5 fungal spores/mL. Liquid cultures and soil slurries were incubated statically at 37 ± 2 °C and were aerated every three to four days. Mineralization of HMX was performed by adding to 7 day old fungal liquid cultures both HMX (60 μM) and [U-^{14}C]-HMX (0.048 μCi, 27 μM). Mineralization assays performed in soils were started by adding [U-^{14}C]-HMX (0.048 μCi, 27 μM) and 2×10^5 fungal spores/mL to the slurries. Formation of $^{14}CO_2$ was monitored as described previously (*27*).

Analytical Procedures. HMX and its mono-nitroso derivative were analyzed by HPLC and by LC/MS as described previously (*19*). Analyses of nitrite (NO_2^-), formaldehyde (HCHO), and nitrous oxide (N_2O) were performed as described in Hawari et al. (*28*). The detection of 4-nitro-2,4-diazabutanal (NDAB) and methylenedinitramine (MEDINA) was performed as described in Fournier et al. (*19*).

Results and Discussion

Biotransformation of HMX in N-Limited Culture Medium. Figure 1 shows the disappearance of HMX after its addition to 7 day old *P. chryososporium* incubated in nitrogen-limited medium. In the noninoculated controls, the added HMX did not degrade throughout the incubation period (Figure 1). In the presence of the fungus, we observed a rapid degradation phase of HMX (600 nmol), which began on the sixth day of incubation and lasted 10 days, leading to more than 90% removal of HMX. After this period, HMX continued to slowly degrade, reaching 97% removal in 25 days. Figure 1 shows that the removal of HMX was accompanied with the formation of 1-NO-HMX. The identity of 1-NO-HMX was confirmed by the detection of the nitrite-adduct of its deprotonated molecular mass ion [M + NO_2 − H] at 325 Da. Such adducts have been frequently observed during mass analysis of cyclic nitramines (*29*). No traces of the other HMX-nitroso derivatives were observed. The concentration of 1-NO-HMX reached a maximal value at day 12 of incubation, after which it degraded to CO_2 (liberated as $^{14}CO_2$). Mineralization of [^{14}C]-HMX took place at the fifth day of incubation reaching a stationary phase at day 44 (69.2 ± 7.1% of $^{14}CO_2$), representing three C of the total four C atoms of HMX. The formation of $^{14}CO_2$ was concurrent with the formation and disappearance of 1-NO-HMX (Figure 1), indicating that a ring cleavage of this intermediate was responsible for producing CO_2. The removal of 1-NO-HMX, which was completed in 21 days, was accompanied with the detection of small amounts of the ring cleavage metabolite NDAB. The latter was previously shown to degrade by the same fungus (*19*). Likewise, relatively high mineralization amounts (ca. 66%) were previously observed with RDX when incubated with the fungus under similar conditions (*20*, *23*).

In addition, we were able to detect both nitrite and nitrous oxide, but we were not able to quantify them due to interference from unidentified artifacts. However, when we used the ring-labeled [^{15}N]-HMX, we detected N_2O with two mass ions at 44 and 45 Da, corresponding to $^{14}N^{14}NO$ (produced from -NO_2) and $^{15}N^{14}NO$ (produced from N-NO_2), respectively. Also, incubation of NO_2^- with *P. chrysosporium* gave N_2O.

Proposed HMX Degradation Pathway. On the basis of the metabolites observed, we suggest that after the reduction of HMX to 1-NO-HMX, two different mechanisms could occur. First, as shown in Figure 2 , path a, 1-NO-HMX could undergo N-denitration, leading to the formation of an intermediate with a reactive imine linkage (−C═N−), which upon reaction with one molecule of water should produce an α-hydroxy-alkylnitramine intermediate (*30*). In the second mechanism (Figure 2, path b), 1-NO-HMX might be subjected to an α-hydroxylation, as frequently observed during enzymatic degradation of dialkylnitrosamines (*31*). The α-hydroxy-alkylnitramine intermediates (Figure 2, paths a and b), being unstable, should decompose to produce the ring-cleavage intermediates NDAB, N_2O, and HCHO (*32*). In the case of path a, decomposition should also produce N_2. In fact, we detected ^{15}N-^{14}N (*m*/*z* 29) and confirm its production from HMX using [^{15}N]-HMX. However, since N_2O and N_2 were not quantified, we cannot determine the relative contribution of each path.

The observation of 1-NO-HMX during the incubation of HMX with *P. chryososporium* is similar to the previous observation of transient amounts of MNX during the incubation of RDX with the same fungus (*22*). However, we confirmed that HMX was more recalcitrant than RDX because it did not transform by any of the following known RDX degradation systems: a commercial cytochrome P-450 (*33*), a RDX-induced *Rhodococcus* sp. strain (*27*), a manganese peroxidase assay performed by using either commercial or MnP rich *P. chrysosporium* extracellular fluid (*34*), or an abiotic system composed of manganese (III) oxalate and oxygen (*35*).

This study did not allow us to identify the enzymatic system responsible for the initial attack on HMX that leads

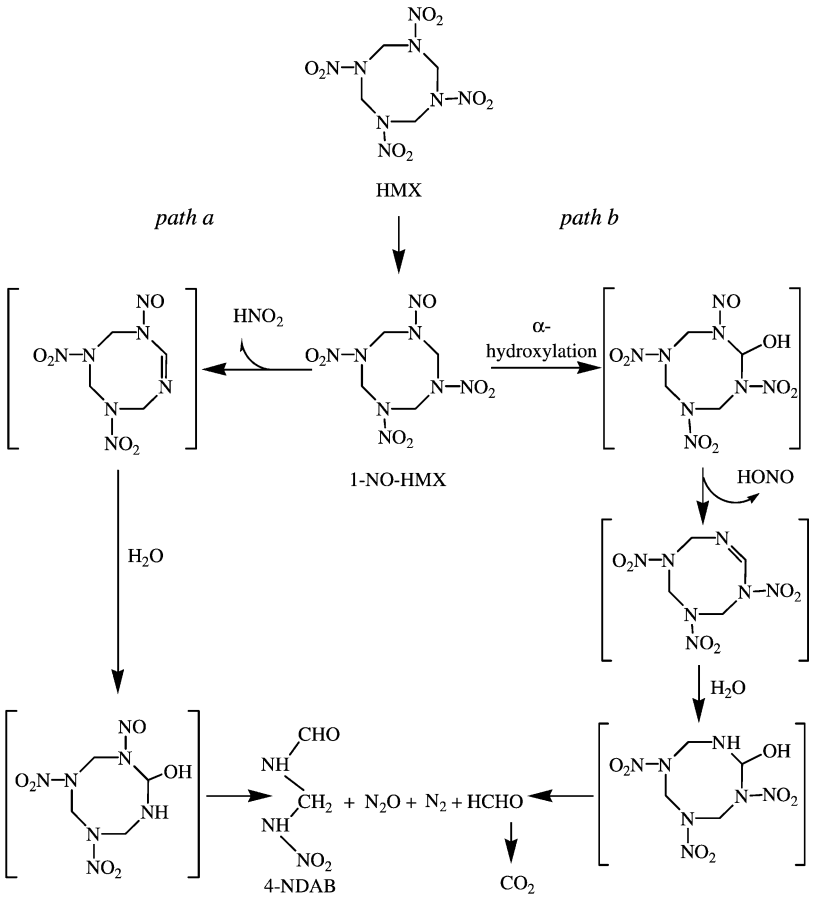

FIGURE 2. Proposed HMX degradation pathways using *P. chrysosporium*. Path a: denitration of 1-NO-HMX followed by hydrolytic decompostion and path b: α-hydroxylation followed by decompostion. N_2 is more likely a product from path a. As reported in Fournier et al. (*19*), the ring cleavage metabolite NDAB was found to degrade, producing N_2O, HCHO, and CO_2.

to its activation and ring cleavage. However, we found that the reduction of HMX to 1-NO-HMX required the presence of live intact mycelium. As reported for the nitroaromatic compound TNT, HMX could also be transformed by a similar reduction-based mechanism, dependent on a nonspecific plasma membrane redox system (*36*) or catalyzed by a membrane-associated nitroreductase (*37*).

Mineralization of HMX in Soil Slurries The ability of *P. chrysosporium* to mineralize HMX in soil was assessed by conducting experiments using a soil sampled from Gagetown military site, contaminated with HMX (403 μmol kg^{-1}), and a soil sampled from an old manufacturing plant for explosives, contaminated with RDX (342 μmol kg^{-1}), and HMX (3057 μmol kg^{-1}) (Table 1). To distinguish the role of *P. chrysosporium* from that of the soil microorganisms, the soils were sterilized by γ-irradiation. The sterile soils were then mixed with a nitrogen-limited medium (20 % wt/vol) and supplemented with [^{14}C]-HMX (0.048 μCi, 27 μM). In the noninoculated soil slurries prepared with Gagetown soil, the percentage of $^{14}CO_2$ remained insignificant, reaching 2% in 128 days (Figure 3). Figure 3 also shows that in slurries amended with the fungus, the extent of mineralization was approximately similar to that observed in the liquid cultures, reaching 74.9 ± 1.6% in 71 days. This confirms the carbon stoichiometry obtained using liquid cultures. Once again, 1-NO-HMX and NDAB were observed (not shown). We also detected HCHO, NO_2^-, and N_2O but were unable to quantify them due to severe interference. In controls prepared with sterilized ammunition soil and the fungal culture medium, less than 1% of $^{14}CO_2$ was obtained in 150 days (Figure 3).

When *P. chrysosporium* was added to the slurries prepared with ammunition soil, mineralization (liberated CO_2) of HMX

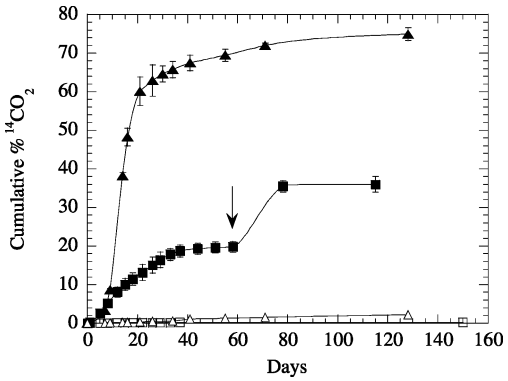

FIGURE 3. Mineralization of [^{14}C]-HMX added to 20% soil slurries (wt/vol) prepared with sterilized Gagetown soil contaminated with HMX (403 μmol kg^{-1}) in nitrogen-limited medium (Δ), in nitrogen-limited medium and *P. chrysosporium* (▲), sterilized ammunition soil contaminated with HMX (3057 μmol kg^{-1}) and RDX (342 μmol kg^{-1}) in nitrogen-limited medium (□), or in nitrogen-limited medium with *P. chrysosporium* (■) (the arrow indicates the time when glucose (6 g/L) was added to the cultures). Values represent the average and standard deviation of duplicate experiments.

reached only 19.8 ± 0.3% in 58 days, probably because of carbon limitation (Figure 3). As Table 1 indicates, the ammunition soil contains 17.5 times less organic carbon than Gagetown soil. Table 1 also indicates that compared to Gagetown soil, the ammunition soil contains 7.6 times more HMX. For instance, supplementation of the microcosms with glucose (6 g/L) on day 58 increased mineralization to 35.5% after 78 days (Figure 3). Thus, a fungus-based remediation

process for heavily contaminated soils is promising but would require adequate supplementation with growth substrates.

In conclusion, the results presented in this study show that *P. chrysosporium* degrades and mineralizes HMX, both in liquid culture and in soils slurries. The formation of the mono-nitroso derivative followed by ring cleavage and decomposition suggests that HMX can be activated by an initial reductive step. The cleavage of 1-NO-HMX can then be achieved via two potential routes: the first involves N-denitration followed by hydrolytic ring cleavage (Figure 2, path a), and the second involves α-hydroxylation prior to ring cleavage, as the case is with *N*-dialkylnitrosamines (Figure 2, path b). The observation of high mineralization in liquid cultures and in soil slurries may be useful for designing fungal-based remediation strategies for HMX-contaminated soils and sediments.

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