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MINI-REVIEW

# Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20

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**Abstract** Cyclic nitramine explosives are synthesized globally mainly as military munitions, and their use has resulted in environmental contamination. Several biodegradation pathways have been proposed, and these are based mainly on end-product characterization because many of the metabolic intermediates are hypothetical and unstable in water. Biodegradation mechanisms for cyclic nitramines include (a) formation of a nitramine free radical and loss of nitro functional groups, (b) reduction of nitro functional groups, (c) direct enzymatic cleavage, (d) α-hydroxylation, or (e) hydride ion transfer. Pathway intermediates spontaneously decompose in water producing nitrite, nitrous oxide, formaldehyde, or formic acid as common end-products. In vitro enzyme and functional gene expression studies have implicated a limited number of enzymes/genes involved in cyclic nitramine catabolism. Advances in molecular biology methods such as high-throughput DNA sequencing, microarray analysis, and nucleic acid sample preparation are providing access to biochemical and genetic information on cultivable and uncultivable microorganisms. This information can provide the knowledge base for rational engineering of bioremediation strategies, biosensor development, environmental monitoring, and green biosynthesis of explosives. This paper reviews recent developments on the biodegradation of cyclic nitramines and the potential of genomics to identify novel functional genes of explosive metabolism.

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## Introduction

Surveys of the extent and distribution of explosive contamination on military ranges in the United States (U.S.) and Canada have shown that explosive residues can be widely and heterogeneously dispersed in soil. Generally, the concentrations of explosives are relatively low (<1 to around 50 μg/kg) except near low-order detonations, tank targets, and firing points where concentrations can be quite high (1,000 to >100,000 μg/kg) and large chunks of explosive formulations can be found (Hewitt 2002; Jenkins et al. 1998, 2001; Pennington et al. 2001, 2005; Walsh et al. 2001). The extent of contamination is not well known outside of North America because only a limited number of site characterizations have been completed at training or manufacturing facilities in Germany, Australia, and the United Kingdom (Spain 2000). A recent survey of the Älvdalen Shooting Range in Sweden found generally low levels of explosive and propellant residues in soil samples, with a few samples exhibiting concentrations as high as 4,200 μg/kg for octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 59,000 μg/kg for 2,4,6-trinitrotoluene (TNT), and 6,500 μg/kg for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Wingfors et al. 2006). Explosive residues have the potential to move into surface water and groundwater (Clausen et al. 2004) and to impact various ecological and human receptors. The U.S. Environmental Protection Agency (2004) has listed the cyclic nitramine RDX as a priority pollutant and HMX as a contaminant of concern. Many microorganisms have been shown to transform RDX and HMX, including aerobic bacteria, aerobic fungi, and anaerobic bacteria (Table 1). Thus, biological remediation is favored as a cost-effective ex situ or in situ treatment method for the removal of these compounds from contaminated environments.

**Table 1** Cyclic nitramine-degrading bacteria and possible biochemical mechanisms for degradation

| Strain | RDX[a] | HMX[b] | CL-20[c] | Mechanism[d] | Reference |
|---|---|---|---|---|---|
| *Serratia marcescens* | +[e] | NT | NT | A | Young et al. 1997 |
| *Enterobacter cloacae* | + | NT | NT | A | Kitts et al. 2000 |
| *Clostridium* sp. HAW-G3 | + | NT | NT | A, F | Zhao et al. 2003b |
| *Clostridium acetobutylicum* | + | NT | NT | C | Zhang and Hughes 2003 |
| *Desulfovibrio* sp. HAW-ES2 | + | NT | NT | A, F | Zhao et al. 2003b |
| *Acetobacterium malicum* | + | NT | NT | E, F | Adrian and Arnett 2004 |
| *Acetobacterium paludosum* | + | NT | NT | ND | Sherburne et al. 2005 |
| *Shewanella sediminis* HAW-EB3 | + | – | NT | A, F | Zhao et al. 2004c, 2005 |
| *Shewanella halifaxensis* HAW-EB4 | + | – | NT | A, F | Zhao et al. 2004c, 2006 |
| *Shewanella* sp. HAW EB1 | + | – | NT | A, F | Zhao et al. 2004c |
| *Shewanella* sp. HAW EB2 | + | – | NT | A, F | Zhao et al. 2004c |
| *Shewanella* sp. HAW-EB5 | + | – | NT | A, F | Zhao et al. 2004c |
| *Stenotrophomonas maltophila* PB1 | + | NT | NT | ND | Binks et al. 1995 |
| *Rhodococcus* sp. DN22 | + | NT | NT | G | Coleman et al. 1998 |
| *Rhodococcus rhodochrous* sp. 11Y | + | NT | NT | G | Seth-Smith et al. 2002 |
| *Williamsia* sp. KTR4 | + | NT | – | G, ND | Thompson et al. 2005 |
| *Gordonia* sp. KTR9 | + | NT | – | G, ND | Thompson et al. 2005 |
| *Methylobacterium* sp. JS178 | + | NT | NT | A | Fournier et al. 2005 |
| *Morganella morganii* B2 | + | + | NT | A, H | Kitts et al. 1994 |
| *Citrobacter freundii* NS2 | + | + | NT | A, ND | Kitts et al. 1994 |
| *Providencia rettgeri* B1 | + | + | NT | A, ND | Kitts et al. 1994 |
| *Klebsiella pneumoniae* SCZ1 | + | + | NT | A, E, F, J | Zhao et al. 2002, 2004b |
| *Clostridium bifermentans* HAW-1 | + | + | NT | A, F, J | Zhao et al. 2003a,b, 2004b |
| *Clostridium* sp. HAW-G4 | + | + | NT | A, F, H, J | Zhao et al. 2003b, 2004b |
| *Clostridium* sp. HAW-E3 | + | + | NT | A, F, H, J | Zhao et al. 2003b, 2004b |
| *Clostridium* sp. HAW-HC1 | + | + | NT | A, F, H, J | Zhao et al. 2003b, 2004b |
| *Clostridium* sp. HAW-EB17 | + | + | NT | A, F, H | Zhao et al. 2004c |
| *Desulfovibrio* sp. HAW-EB18 | + | + | NT | A, F, H | Zhao et al. 2004c |
| Fusobacteria isolate HAW-EB21 | + | + | NT | A, F, H, J | Zhao et al. 2004b,c |
| *Methylobacterium* sp. BJ001 | + | + | NT | A, H | Van Aken et al. 2004 |
| *Methylobacterium extorquens* | + | + | NT | A, ND | Van Aken et al. 2004 |
| *Methylobacterium organophilum* | + | + | NT | A, ND | Van Aken et al. 2004 |
| *Methylobacterium rhodesianum* | + | + | NT | A, ND | Van Aken et al. 2004 |
| *Gordonia* sp. KTC13 | – | NT | + | ND | Crocker et al., unpublished |
| *Pseudomonas* sp. FA1 | NT | NT | + | K | Bhushan et al. 2003 |
| *Agrobacterium* sp. JS71 | – | – | + | ND | Trott et al. 2003 |
| *Irpex lacteus* | NT | NT | + | ND | Fournier et al. 2006 |
| *Clostridium* sp. EDB2 | + | + | + | F, J, K, L, M | Bhushan et al. 2004c, 2005a |
| *Phanerochaete chrysosporium* | + | + | + | ND, H, J, K | Sheremata and Hawari 2000; Fournier et al. 2004b, 2006 |

[a] RDX hexahydro-1,3,5-trinitro-1,3,5-triazine
[b] HMX octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
[c] CL-20 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane
[d] Mechanisms are described using the same letter codes in Figs. 2, 3, and 4, which are used to describe the different pathways of biodegradation for RDX, HMX, and CL-20
[e] +, Positive for degradation of the explosive; –, does not degrade the explosive; *NT*, not tested; *ND*, not determined due to lack of information on metabolites

The biochemical details of the metabolic pathways involved in the degradation of the cyclic nitramines RDX and HMX have been reviewed (Hawari et al. 2000a). Since then, research into the biological fate of a new cyclic nitramine explosive 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) has been initiated. Unlike RDX and HMX, CL-20 is composed of a rigid and highly strained cage formed from two five-member rings and one six-member ring (Fig. 1). It also differs from RDX and HMX in that it has three carbon–carbon (C–C) bonds, and the repeating structural unit is CH–N–$NO_2$ instead of $CH_2$–N–$NO_2$. As with RDX and HMX, biological transformation of CL-20 has been reported in soils (Crocker et al. 2005; Strigul et al. 2006; Trott et al. 2003) and by pure cultures of bacteria and fungi (Table 1). However, CL-20 was more susceptible to abiotic transformation than RDX and HMX

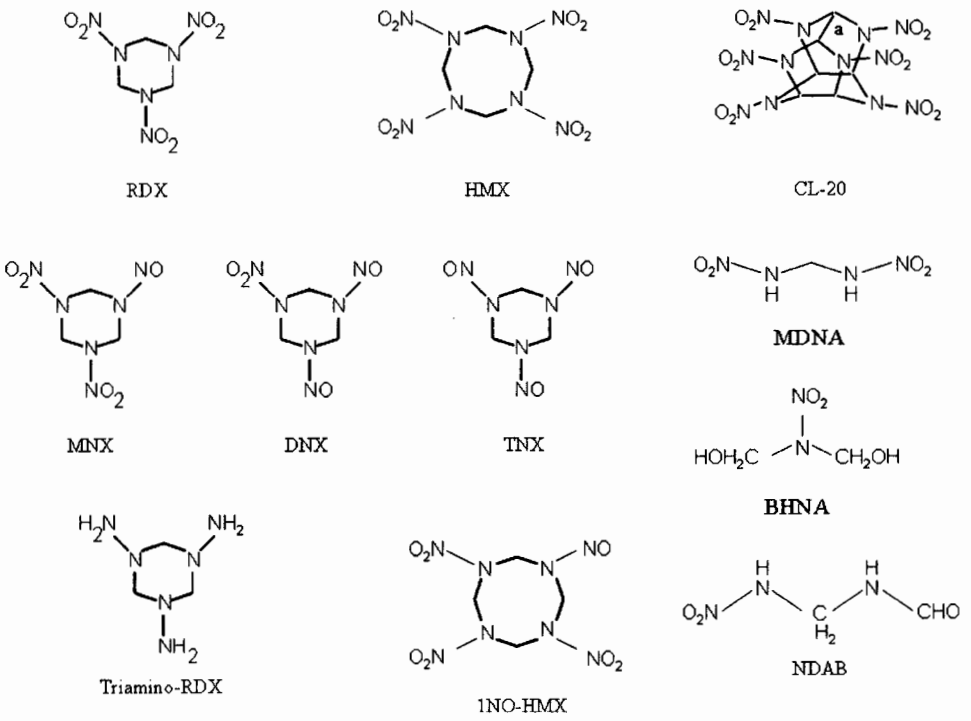


**Fig. 1** Molecular structures of hexahydro-1,3,5-trinitro-1,3,5-triazine (*RDX*), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (*MNX*), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (*DNX*), hexahydro-1,3,5-trinitroso-1,3,5-triazine (*TNX*), methylenedinitramine (*MDNA*), *bis*-(hydroxymethyl)nitramine (*BHNA*), 4-nitro-2,4-diazabutanal (*NDAB*), 1,3,5-triamino-1,3,5-triazine, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (*HMX*), octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (*1NO-HMX*), and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (*CL-20*)

(Balakrishnan et al. 2003; Monteil-Rivera et al. 2004; Szecsody et al. 2004).

CL-20 has been shown to be nontoxic to bacteria, a green alga, or terrestrial plants at levels up to 10,000 mg/kg soil (Gong et al. 2004) but lethal to earthworms at levels as low as 90.7 mg/kg soil (Robidoux et al. 2004). Abnormal developmental effects in Japanese quail embryos (*Coturnix coturnix japonica*) may be caused by CL-20 at concentrations above 11 mg/kg feed, but few effects were observed in adults (up to 5,304 mg/kg body weight; Bardai et al. 2005). In comparison, RDX and HMX exerted little to no effects on soil microbial activities up to 12,500 mg/kg soil (Gong et al. 2001, 2002). RDX and HMX were shown to be reproductive toxicants to earthworms at concentrations ≥46.7 and ≥15.8 mg/kg, respectively, and yet survival was not affected by RDX concentrations up to 167.3 mg/kg or HMX up to 711.0 mg/kg (Robidoux et al. 2002).

Cyclic nitramines can be biodegraded by several mechanisms that produce a similar distribution of end-products. Enzymatic reactions with the nitramines can lead to the reduction of the molecule to nitroso- or hydroxylamino- derivatives, formation of a free radical and loss of a nitro group, direct ring cleavage at N–N or C–N bonds, α-hydroxylation at C–H bonds, or a hydride ion transfer. These initial enzymatic reactions destabilize the nitramine ring structure such that the intermediates are thought to spontaneously decompose in water. Some of the predicted secondary intermediates are also unstable in water and spontaneously decompose. Thus, cyclic nitramines are biodegraded by a combination of biotic and abiotic reactions that produce nitrite, nitrous oxide, ammonia, and formaldehyde (RDX and HMX) or formic acid and glyoxal (CL-20) as common end-products (Bhushan et al. 2003c, 2004a,b, 2005a; Fournier et al. 2002; Hawari et al. 2000b, 2001; Zhao et al. 2004b). These end-products may in turn be utilized by the same nitramine-degrading microorganisms or by other microorganisms that coexist in the soils, etc. For example, nitrite and ammonia may be utilized as nitrogen sources for growth (Coleman et al. 1998; Seth-Smith et al. 2002; Thompson et al. 2005), or the nitrous oxide may be converted to nitrogen gas by denitrifying bacteria (Halasz et al. 2002). Formaldehyde has been shown to be subsequently metabolized to formic acid, methanol, carbon dioxide, or methane by various microorganisms (Fournier et al. 2002, 2004a; Hawari et al. 2000b, 2001; Van Aken et al. 2004; Zhao et al. 2002, 2003a). Similarly, the formic acid and glyoxal produced from CL-20 have been shown to be mineralized to carbon dioxide (Fournier et al. 2006). Thus, the distribution and yield of metabolites and end-products generated from the biotransformation of cyclic nitramines will likely depend on the concerted activities of various microorganisms present in the environment and the specific combination of biological and chemical reactions that react with the nitramine and pathway intermediates. This review includes a discussion of the current knowledge of biodegradation mechanisms for the cyclic nitramines RDX, HMX, and CL-20. This is followed by a discussion of how recent advances in genomics may be used to identify novel functional genes/genetic pathways involved in the metabolism of cyclic nitramine explosives. This

information can provide the knowledge base for rational engineering of explosive biotechnologies in bioremediation, biosensor development, environmental monitoring, and green biosynthesis.

## RDX

RDX biotransformation occurs in a variety of environments from surface and subsurface soils (Crocker et al. 2005; Oh et al. 2001; Ringelberg et al. 2003), aquifers (Beller and Tiemeier 2002; Bradley and Dinicola 2005; Davis et al. 2004; Waisner et al. 2002), sewage sludges (Hawari et al. 2000b), and cold marine sediments (Zhao et al. 2004a). In some of these examples, RDX biodegradation was stimulated by the addition of carbon amendments, which suggests that RDX biodegradation may be a carbon or electron donor limited in the environment. RDX biodegradation occurs under aerobic (Coleman et al. 1998; Seth-Smith et al. 2002; Thompson et al. 2005), nitrate-reducing (Bradley and Dinicola 2005; Freedman and Sutherland 1998), sulfate-reducing (Boopathy et al. 1998), manganese-reducing (Bradley and Dinicola 2005), acetogenic (Adrian and Arnett 2004; Beller 2002; Sherburne et al. 2005) anaerobic (Hawari et al. 2000b), and methanogenic conditions (Adrian et al. 2003). Three mechanisms for the transformation of RDX have been proposed: two-electron reduction, single-electron reduction/denitration, and direct enzymatic cleavage (Fig. 2). Reduction refers to the addition of two or more redox equivalents ($2e^-/2H^+$) to RDX leading to reduced nitroso- and postulated hydroxylamino- or amino-triazine derivatives. Denitration involves the addition of a single electron to RDX

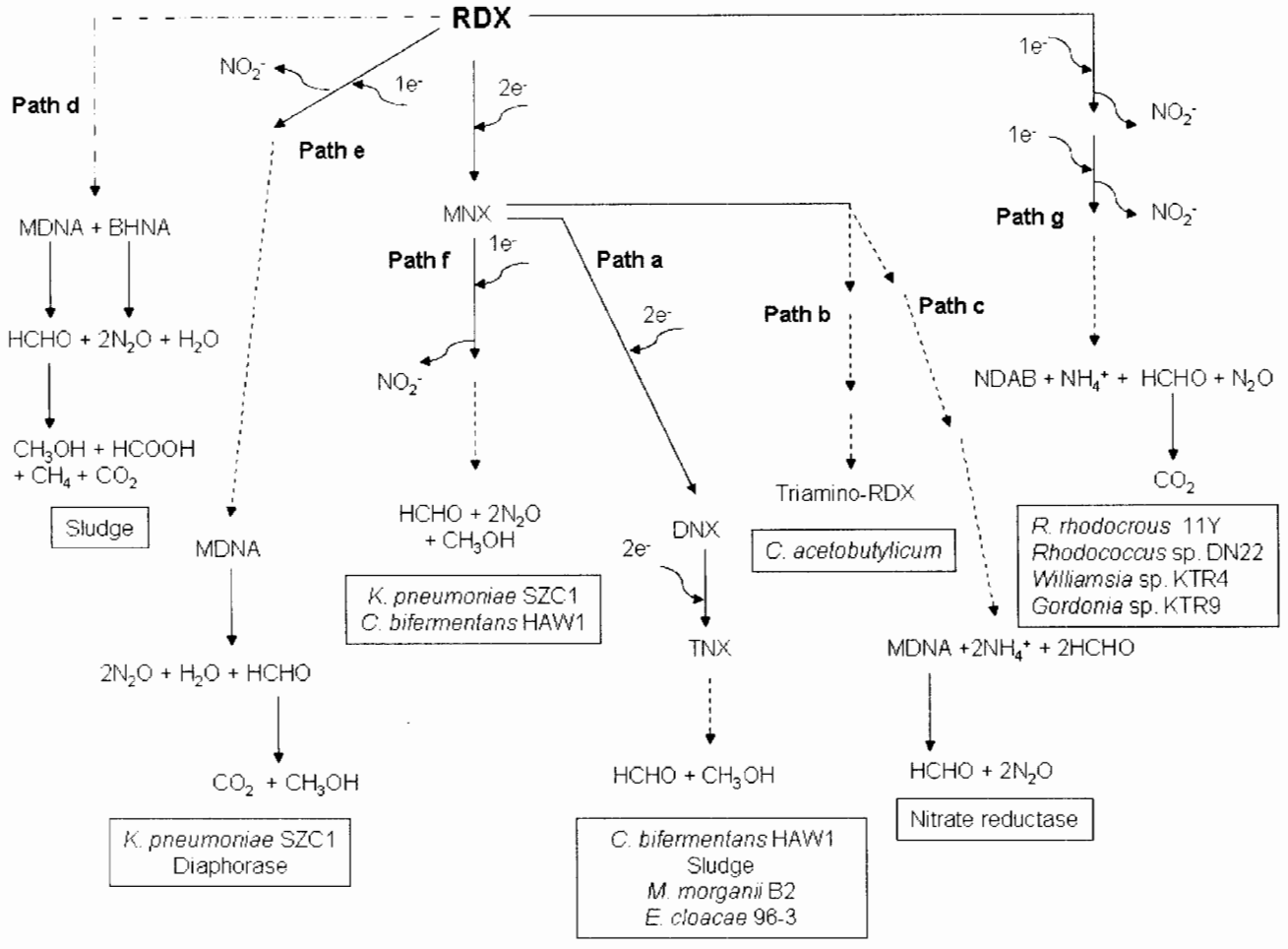


**Fig. 2** Proposed biodegradation pathways for hexahydro-1,3,5-trinitro-1,3,5-triazine (*RDX*). *Path a* Reduction of RDX to nitroso derivatives before ring cleavage (McCormick et al. 1981). *Path b* Reduction of RDX to 1,3,5-triamino-1,3,5-triazine (Zhang and Hughes 2003). *Path c* Reduction of RDX via *Aspergillus niger* nitrate oxidoreductase enzyme (Bhushan et al. 2002b). *Path d* Direct enzymatic cleavage of RDX (Hawari et al. 2000b). *Path e* Anaerobic denitration of RDX (Zhao et al. 2002). *Path f* Denitration of RDX via the reductive intermediate MNX (Zhao et al. 2003a). *Path g* Aerobic denitration of RDX (Bhushan et al. 2003c; Fournier et al. 2002). *Solid arrows* Reactions leading to actual intermediates or end-products. *Dashed arrows* Multiple pathway steps based on hypothetical intermediates, which are not shown. *Dashed plus dotted arrows* Hypothetical enzyme reactions

generating the anion radical ($RDX^{-}$) followed by ring cleavage. Denitration of mononitroso-RDX (MNX) is included here, although MNX is formed by an initial reductive event. Enzymatic cleavage refers to a direct enzymatic attack at the carbon–nitrogen (C–N), methylene (C–H), or nitrogen–nitrogen (N–N) bonds in the molecule.

The two-electron reductive pathway (Fig. 2, path a) as originally proposed by McCormick et al. (1981) follows the sequential reduction of RDX through mono-, di-, and trinitroso-RDX (MNX, DNX, and TNX). Further reduction of these compounds to hypothetical hydroxylamino derivatives was thought to destabilize the ring leading to hydrolytic cleavage and the formation of hydrazines, methanol, and formaldehyde. Others have failed to identify hydrazines but have frequently observed MNX, DNX, and TNX as transient intermediates and methanol and formaldehyde as end-products (Adrian and Chow 2001; Adrian et al. 2003; Halasz et al. 2002; Hawari et al. 2000b; Oh et al. 2001). The formation of methanol and formaldehyde indicates that the ring of the nitroso derivative can be cleaved but the steps in this mechanism still remain unclear. A hydroxylamino-DNX intermediate has been observed by Adrian and Chow (2001), and it was believed to have been formed from TNX, but no other hydroxylamino derivatives were observed in that study or in other studies with sludges and soils (Halasz et al. 2002; Hawari et al. 2000b; Oh et al. 2001). This two-electron reduction pathway may serve as a minor RDX degradation route in some bacteria (*Klebsiella pneumoniae* strain SCZ-1, *Clostridium bifermentans* strain HAW-1, *Shewanella halifaxensis* strain HAW-EB4, and *Shewanella* sp. HAW-EB5; Zhao et al. 2002, 2003a, 2004c, 2006), while in others, it may be the major route of degradation (*Methylobacterium* spp., enterobacteria, *Shewanella* sp. HAW-EB2; Fournier et al. 2005; Kitts et al. 1994; Van Aken et al. 2004; Zhao et al. 2004c).

The initiation of the two-electron reduction pathway by oxygen-insensitive type I nitroreductase enzymes has been suggested in studies with sewage sludge (Hawari et al. 2000b) and with enterobacteria (Kitts et al. 2000). The *nsfI* gene, a type I nitroreductase that was previously cloned, sequenced, and purified from *Enterobacter cloacae* strain 96-3 (Bryant et al. 1991), was shown to have RDX nitroreductase activity when expressed from a plasmid in *Escherichia coli* (Kitts et al. 2000). In addition, RDX nitroreductase activity was documented in an in vitro assay that followed NADPH oxidation as a function of RDX disappearance. While no metabolites were identified in these studies, the authors suggest that the likely mechanism of action for RDX transformation by *nsfI* is the predicted two-electron reduction of nitro groups to nitroso groups.

An alternate two-electron reduction pathway (Fig. 2, path b) that diverges from MNX in the McCormick pathway has been proposed. Cell-free extracts of *Clostridium acetobutylicum* in the presence of $H_2$ transform RDX through MNX, hydroxylamino, and amino intermediates to 1,3,5-triamino-1,3,5-triazine (Fig. 1; Zhang and Hughes 2003). Ring cleavage was not observed, and the triamino compound accumulated, accounting for 91% of the carbon mass balance. The presence of these reduced compounds suggested a pathway analogous to the reductive transformation pathways of TNT, 2,4-dinitrotoluene, and 2,6-dinitrotoluene previously described for this strain (Hughes et al. 1998, 1999). However, Zhao et al. (2003a) were unable to detect 1,3,5-triamino-1,3,5-triazine as an end-product after biotransformation by *C. bifermentans* strain HAW-1. Studies on cell-free systems may not accurately represent the types of reactions possible by whole cells, and so in vivo studies with *C. acetobutylicum* should be conducted to confirm this mechanism.

Similarly, a nitrate oxidoreductase from *Aspergillus niger* (Bhushan et al. 2002b) has been shown to reduce RDX to ammonium (16.98% of N balance), nitrous oxide (57.37% of N balance), and formaldehyde (92.27% of C balance) in a cell-free system (Fig. 2, path c). The reduced intermediate, MNX, and a ring cleavage product, methylenedinitramine (MDNA; Fig. 1), were formed as transient intermediates, but DNX and TNX were not observed. MNX was shown to be the precursor of MDNA and, it was presumed to be reduced by additional reductive steps before ring cleavage because the reaction consumes three molecules of NADPH ($6e^{-}$). However, several intermediates in the pathway remain hypothetical, and the exact steps at which two of the NADPH molecules would be consumed are unclear.

A degradation pathway based on the direct enzymatic cleavage of inner C–N, $N–NO_2$, or methylene C–H bonds was proposed to explain the formation of MDNA and *bis*-(hydroxymethyl)nitramine (Fig. 1) as the major intermediates in the anaerobic biotransformation of RDX by sewage sludge (Fig. 2, path d; Halasz et al. 2002; Hawari et al. 2000b). Due to the complex bacterial consortia that can exist in sludge, it was unclear as to how these intermediates were produced from RDX. Halasz et al. 2002 confirmed that one molecule of RDX was transformed to one molecule of MDNA and that water was involved in the process at an unknown step. As a result, the cleavage of a C–N bond by a hydrolase or the enzymatic α-hydroxylation of a methylene bond was proposed as a mechanism that may produce MDNA. The importance of these mechanisms to the biodegradation of RDX is uncertain as later studies with pure cultures of *K. pneumoniae* strain SZC-1 (Zhao et al. 2002) and *C. bifermentans* strain HAW-1 (Zhao et al. 2003a), isolated from the same sludge, have shown that MDNA can be formed via denitration of RDX.

*Bis*-(hydroxymethyl)nitramine and MDNA did not accumulate in the sludge microcosms but disappeared to form

nitrous oxide (22.6% of N balance), nitrogen gas (trace), formaldehyde, formic acid, methanol, carbon dioxide (60% of C balance), and methane (Hawari et al. 2000b). The MDNA was shown to spontaneously decompose in water to 2 mol of nitrous oxide and 1 mol of formaldehyde (Halasz et al. 2002), and *bis*-(hydroxymethyl)nitramine was also proposed to spontaneously decompose in water to nitrous oxide and formaldehyde (Hawari et al. 2000b). Acetogenic and methanogenic bacteria in the sludge were presumed to be responsible for the conversion of formaldehyde to methanol and formic acid and then to carbon dioxide and methane.

Denitration, the loss of a nitro group through single-electron transfer and free-radical formation, appears to be a major route of RDX biotransformation (Fig. 2, paths e, f, and g). This reaction is thermodynamically favorable (Qasim et al. 2005b), and abiotic electron donors may also be used. Anaerobic and aerobic denitration mechanisms are both oxygen sensitive but occur by slightly different mechanisms. Anaerobic denitration involves a single-electron transfer that may occur by an oxygen-sensitive, type II nitroreductase generating a free-radical anion ($RDX^{\cdot-}$) and subsequently releasing a nitro group. The loss of the nitro group destabilizes the molecule leading to ring cleavage and the formation of one molecule of MDNA and other unknown intermediate(s). The MDNA spontaneously decomposes in water to 2 mol of nitrous oxide and 1 mol of formaldehyde (Halasz et al. 2002). The formaldehyde in turn can be biotransformed to methanol, formate, or carbon dioxide by various bacteria. *K.pneumoniae* strain SCZ-1 and *C. bifermentans* strain HAW-1 use denitration as the main pathway for biodegradation of RDX and MNX. In strain SCZ-1, the denitration of RDX is the major pathway (Fig. 2, path e), while reduction to MNX followed by denitration of MNX is a minor route (Fig. 2, path f; Zhao et al. 2002) because only a trace amount of MNX was formed from RDX. Carbon and nitrogen mass balances for strain SCZ-1 were 12% methanol, 72% carbon dioxide, and 60% nitrous oxide. In contrast, in strain HAW-1, the reduction of RDX to MNX occurs before denitration (Fig. 2, path f; Zhao et al. 2003a) because RDX was transformed by strain HAW-1 to 56% MNX before denitration. Biodegradation of RDX by strain HAW-1 yielded methanol (23% of C balance), formaldehyde (7.4% of C balance), carbon dioxide (3% of C balance), and nitrous oxide (29.5% of N balance). The mechanism of RDX biodegradation by a diaphorase enzyme from *Clostridium kluyveri* in a cell-free system (Bhushan et al. 2002a) was also shown to occur via denitration (Fig. 2, path e), producing nitrous oxide (55% of N balance), nitrite (15% of N balance), ammonium (13% of N balance), MDNA (8% of N balance), and formaldehyde (88% of C balance).

Denitration of RDX by aerobic bacteria involves two one-electron transfer steps and releases two nitro groups before ring cleavage (Fig. 2, path g). As a result, one molecule of 4-nitro-2,4-diazabutanal (Fig. 1, NDAB) is formed along with nitrous oxide, ammonium, formaldehyde, and carbon dioxide (approximately 30% of C balance) (Bhushan et al. 2003c; Fournier et al. 2002). The aerobic bacteria *Rhodococcus rhodochrous* strain 11Y (Seth-Smith et al. 2002), *Rhodococcus* sp. strain DN22 (Coleman et al. 1998; Fournier et al. 2002), *Williamsia* sp. strain KTR4 (Thompson et al. 2005), and *Gordonia* sp. strain KTR9 (Thompson et al. 2005) appear to mineralize RDX according to this mechanism and utilize the nitrite generated as a nitrogen source for growth. Strains KTR4 and KTR9 were the first bacteria isolated with the ability to utilize RDX as a source of energy, carbon, and nitrogen for growth (Thompson et al. 2005). The ability of strains KTR4 and KTR9 to utilize RDX as sole carbon, nitrogen, and energy sources shows that these strains have the ability to connect the catabolic RDX pathway with anabolic pathways for carbon and nitrogen assimilation and that they can conserve energy from the oxidation of RDX.

The NDAB produced accumulates (64% of C balance) and is apparently not further metabolized by strains 11Y, DN22, KTR4, or KTR9 (Fournier et al. 2002, 2004a; Thompson et al. 2005). However, it has recently been shown that NDAB may not be an environmentally recalcitrant end-product as it can be biodegraded by a methylotrophic bacterium and a white-rot fungus (Fournier et al. 2004a, 2005). *Methylobacterium* sp. strain JS178 degraded NDAB as a nitrogen source for growth producing 1 mol of nitrous oxide and 1 mol of carbon dioxide. While strain JS178 was unable to grow on RDX or HMX, it was able to transform RDX via reduction in nutrient broth (Fournier et al. 2005). Allantoin supported the biodegradation of NDAB by strain JS178, which suggests that enzymes from the purine catabolic pathway might be involved (Fournier et al. 2005). *Phanerochaete chrysosporium* which has previously been shown to mineralize RDX without the production of NDAB (Sheremata and Hawari 2000) also biodegraded NDAB to nitrous oxide (1 mole), carbon dioxide, and traces of nitramide (Fournier et al. 2004a). The extent of mineralization of $^{14}$C-RDX increased from 30% with *Rhodococcus* sp. strain DN22 to 76% when *P. chrysosporium* degraded the $^{14}$C-NDAB formed by strain DN22. The degradation of NDAB in *P. chrysosporium* was attributed to a manganese-dependent peroxidase because the enzyme was shown to be able to transform NDAB in vitro, but the end-products were not described (Fournier et al. 2004a).

There are now several studies that confirm the involvement of a cytochrome P450 enzyme in the transformation of RDX by *Rhodococcus* strains 11Y and DN22. Indirect evidence for the involvement of a cytochrome P450 in the biodegradation of RDX was first provided by Coleman et al. (2002) and later supported by Bhushan et al. (2003c) and

Seth-Smith et al. (2002). Using various inhibitors and inducers of cytochrome P450 enzymes, these studies documented decreases and increases in RDX biotransformation activity by DN22 and a rabbit liver cytochrome P450. Seth-Smith et al. (2002) were the first to successfully isolate a genetic locus involved in the transformation of RDX. The gene responsible for degrading RDX, *xplA*, was isolated from strain 11Y, and it encodes a constitutively expressed, fused flavodoxin-cytochrome P450 enzyme (Rylott et al. 2006; Seth-Smith et al. 2002). In vitro enzyme assays using purified XplA protein indicate that NADPH is the natural reductant in the cell supporting reduction by this enzyme (Rylott et al. 2006). Bhushan et al. (2003c) provided further biochemical evidence of cytochrome P450 involvement by proving that the same products of RDX biotransformation were generated by a rabbit liver cytochrome P450 enzyme and strain DN22. RDX biotransformation by the rabbit liver cytochrome P450 enzyme and the XplA protein was faster under anaerobic than aerobic conditions (Bhushan et al. 2003c; Rylott et al. 2006). This data suggests that these enzymes promote denitration of RDX by the oxygen-sensitive free-radical reduction of RDX.

## HMX

The HMX is structurally similar to RDX (Fig. 1), containing the same monomeric units of $CH_2$–N–$NO_2$. Due to structural similarities between RDX and HMX, the mechanism of HMX biodegradation has been found to be similar to that of RDX, although HMX has been shown to be more recalcitrant to biodegradation than RDX. Electron donors and acceptors that support RDX biodegradation also support the biodegradation of HMX (Adrian et al. 2003; Boopathy 2001; Zhao et al. 2004b), and several microorganisms have been shown to cometabolize both RDX and HMX (Table 1; Bhushan et al. 2004c; Fournier et al. 2004b; Kitts et al. 1994; Van Aken et al. 2004; Zhao et al. 2004b,c). Biotransformation of HMX has been shown to occur in sewage sludge (Boopathy 2001; Hawari et al. 2001), soil (Monteil-Rivera et al. 2003), and cold marine sediments (Zhao et al. 2004a).

Similar to RDX, HMX has generally been thought to be recalcitrant to aerobic biodegradation (Zhao et al. 2004c). However, Van Aken et al. (2004) recently showed that aerobic *Methylobacterium* sp. strain BJ001 was able to cometabolize HMX under aerobic conditions with the production of 61% carbon dioxide after 55 days. HMX was not used as a carbon and/or nitrogen source for growth by strain BJ001. The high yield of carbon dioxide was believed to be related to the strain's ability to use methanol and formaldehyde, which may have been formed from the biodegradation of HMX. The likely mechanism of HMX biotransformation was proposed to be reductive based on how RDX and TNT were transformed by this strain. However, no other metabolites besides carbon dioxide were reported to be produced from HMX. Van Aken et al. (2004) also suggested that cometabolism of explosives may be a common trait among methylobacteria as they showed that three other *Methylobacterium* species were able to cometabolize HMX, RDX, and TNT.

Most studies on the anaerobic fate of HMX list nitroso derivatives as intermediates formed from the two-electron reduction of the nitro groups on the ring (Fig. 3, path h) Usually, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (1NO-HMX; Zhao et al. 2004a–c) is formed, but reports of octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine or octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine (2NO-HMX; Hawari et al. 2001; Zhao et al. 2004b), and occasionally octahydro-1,3,5-trinitroso-7-nitro-1,3,5,7-tetrazocine (3NO-HMX; Boopathy 2001; Zhao et al. 2004b) and octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine (4NO-HMX; Monteil-Rivera et al. 2003), are observed. The fate of these nitroso derivatives is unknown as they are produced transiently and in trace amounts, making it difficult to determine the mechanism for the transformation of these derivatives. Hydroxylamino-HMX derivatives and other proposed intermediates from the McCormick pathway (McCormick et al. 1981) have not been observed (Bhushan et al. 2003a; Hawari et al. 2001; Monteil-Rivera et al. 2003). Ring cleavage intermediates of HMX have also recently been reported (Hawari et al. 2001; Bhushan et al. 2003a; Zhao et al. 2004b) as is the suggestion of an anaerobic one-electron reduction/denitration route (Bhushan et al. 2003a).

Sewage sludge biotransformed HMX to the ring cleavage intermediates MDNA and *bis*(hydroxymethyl) nitramine, which later transformed to formaldehyde (trace), formic acid (trace), and nitrous oxide (50%) (Fig. 3, path i; Hawari et al. 2001). The possible presence of acetogens and methanogens, and denitrifying bacteria in the sludge were also responsible for the production of carbon dioxide (40%) and methane from formic acid, and nitrogen gas from nitrous oxide, respectively. In this study, the authors were unable to determine if ring cleavage intermediates were the result of a direct enzymatic cleavage of the C–N bond in the ring, α-hydroxylation of a C–H bond, or a single-electron reductive denitration mechanism as nitrite was not detected. The authors also do not discount the possibility that a mononitroso-HMX intermediate may have produced the ring cleavage product MDNA (Hawari et al. 2001).

Evidence for the single-electron reduction/denitration of HMX came from the enzymatic study with a xanthine oxidase from buttermilk (Fig. 3, path j; Bhushan et al. 2003a). The in vitro biotransformation of HMX was accompanied by the transient accumulation of the ring

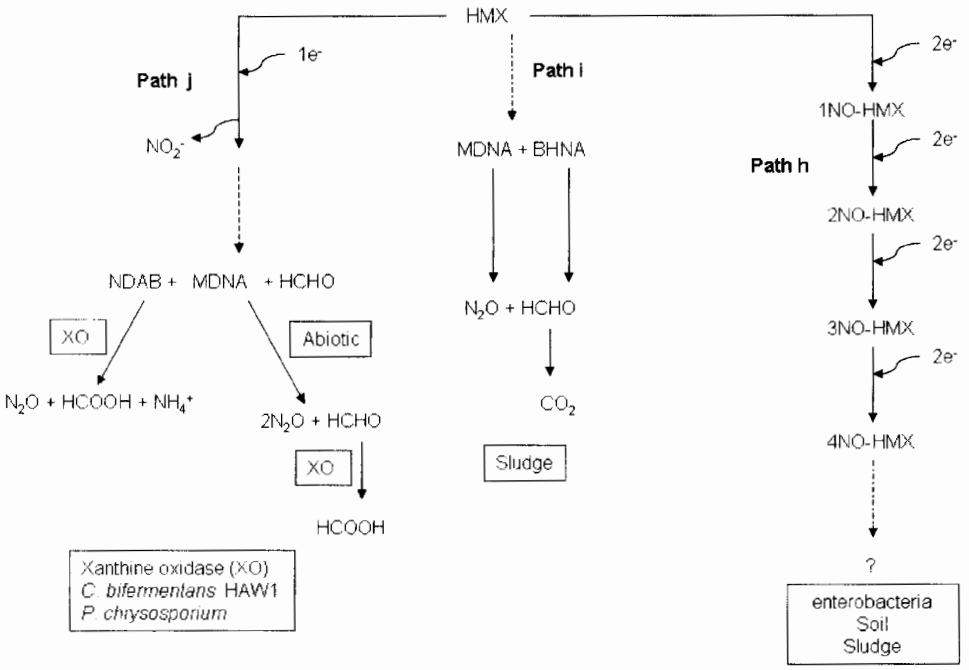


**Fig. 3** Proposed biodegradation pathways for octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (*HMX*). *Path h* Reduction of HMX to nitroso derivatives before ring cleavage (McCormick et al. 1981). *Path i* Direct enzymatic cleavage of HMX (Hawari et al. 2001). *Path j* Denitration of HMX via xanthine oxidase enzyme (Bhushan et al. 2003a). *Solid arrows* Reactions leading to actual intermediates or end-products. *Dashed arrows* Multiple pathway steps based on hypothetical intermediates, which are not shown. *Dashed plus dotted arrows* Hypothetical enzyme reactions

cleavage products MDNA and NDAB, and the end-products nitrite (0.6 mol), nitrous oxide (2.4 mol), ammonium (0.7 mol), formaldehyde (1.3 mol), and formic acid (2 mol). The yield of end-products accounted for a carbon recovery of 91% and a nitrogen recovery of 88%. The absence of nitroso intermediates and the presence of nitrite and NDAB were taken as evidence of a single-reductive denitration mechanism, analogous to RDX denitration. It is interesting to note that with xanthine oxidase, NDAB was biotransformed to nitrous oxide, ammonium, and formic acid, and formaldehyde was biotransformed to formic acid. Xanthine oxidase is a metalloflavo enzyme in which the active flavin moiety was active in transferring a single electron to HMX, causing the removal of a nitro group and the subsequent spontaneous decomposition of the ring (Bhushan et al. 2003a). Denitration as a mechanism for HMX biotransformation was supported by studies with *C. bifermentans* strain HAW-1 (Zhao et al. 2004b) and with *P. chrysosporium* (Fournier et al. 2004b). With strain HAW-1 nitrite, nitrous oxide (74% of total N in HMX), formaldehyde (22%), carbon dioxide (7.8%), and MDNA were produced (Zhao et al. 2004b), while with *P. chrysosporium* NDAB, nitrite, nitrous oxide, formaldehyde, and carbon dioxide (70%) were produced (Fournier et al. 2004b).

## Hexanitrohexaazaisowurtzitane (CL-20)

The biodegradation of CL-20 has recently been shown to occur by acrobic soil bacteria that utilize CL-20 as a nitrogen source for growth (Bhushan et al. 2003b; Trott et al. 2003). The anaerobic bacterium *Clostridium* sp. EDB2 was shown to be able to transform CL-20 as well as RDX and HMX (Bhushan et al. 2004c) and is so far the only bacterium capable of this trait. In addition, CL-20 has been shown to be biodegraded in surface and subsurface soils under aerobic or anaerobic conditions (Crocker et al. 2005; Jenkins et al. 2003; Strigul et al. 2006; Trott et al. 2003). Aerobic soils have shown very slow rates of degradation of CL-20 under unsaturated conditions and at low concentrations of CL-20 (half-lives = 144–686 d; Jenkins et al. 2003) to essentially no degradation at concentrations in excess of 125 mg/kg (Strigul et al. 2006). In the latter case, CL-20 biodegradation only occurred if the soils were amended with starch or cellulose above 1,000 mg/kg. Extensive fungal growth was observed in these microcosms, suggesting a role of fungi in the biodegradation of CL-20 in these soils (Strigul et al. 2006). Fournier et al. (2006) have shown that the white-rot fungus, *P. chrysosporium*, transformed and mineralized CL-20 during lignolytic growth. Faster rates of biodegradation were observed in soil microcosms amended with CL-20 concentrations at or slightly above the water solubility (3.5 to 10 mg/l). First-order rates of biodegradation in biologically active surface soils amended with glucose were 0.068–1.222 $d^{-1}$ (Crocker et al. 2005). Anaerobic conditions favored the biodegradation of CL-20 in soils with CL-20 concentrations at 250 mg/kg, and starch amendments significantly reduced the lag phase (Strigul et al. 2006). In these soil studies, the microorganisms responsible for

degrading CL-20, or the intermediates or end-products of CL-20 biotransformation, were not described. Strigul et al. (2006) reported that degradation products were observed via HPLC but no attempt was made to identify the products at the current time, and Crocker et al. (2005) only reported the production of $^{14}CO_2$ from $^{14}C$–CL-20 under aerobic conditions.

Several enzymatic assays have suggested that CL-20 may be transformed by three different pathways that eventually generate a similar distribution of end-products. The first pathway involves denitration (Fig. 4, path k) and ring cleavage of the weak "attic" C–C bond, and this reaction is highly favored according to computational chemistry theory (Okovytyy et al. 2005). Salicylate 1-monoxygenase (Bhushan et al. 2004b), nitroreductase (Bhushan et al. 2004a), and dehydrogenase (Bhushan et al. 2005a) are believed to promote the transfer of a single electron to the molecule forming a free-radical anion that then releases a nitro group. This reaction is oxygen sensitive, as oxygen quenches the electron from the free-radical anion regenerating CL-20 (Bhushan et al. 2004a). Loss of one of the four nitro groups on the two five-member rings (Okovytyy et al. 2005) destabilizes the ring leading to spontaneous ring cleavage at the "attic" C–C bond which joins the two five-member rings (designated as "a" in Fig. 1; Okovytyy et al. 2005). Another single-electron transfer by these enzymes eliminates a second nitro group from one of the five-member rings from the molecule. This leads to the formation of two isomeric intermediates (II and III; Fig. 4, path k) containing two C=N bonds (Bhushan et al. 2004a,b). The use of both ring- and nitro-labeled [$^{15}N$]–CL-20 in enzyme assays has supported the formation of this intermediate having a molecular weight of 346 Da and the formula $C_6H_6N_{10}O_8$ (Bhushan et al. 2004b, 2005a). This mechanism has also been observed in *Pseudomonas* sp. FA1 (Bhushan et al. 2003b), by zero-valent iron (Balakrishnan et al. 2004), alkali (Balakrishnan et al. 2003; Qasim et al. 2004), and light (Hawari et al. 2004; Qasim et al. 2005a).

Two contrasting hypothetical reaction pathways for the fate of intermediate II have been proposed. The first pathway is based on the work done by Hawari's group both under abiotic and biotic conditions, the latter mostly in vitro enzyme assays. This pathway is based on the supposition that intermediate II is unstable in water and will spontaneously decompose to produce ring cleavage products such as nitrite (2 to 5 mol), nitrous oxide (0 to 0.3 mol), ammonium (1.4 to 2 mol), formate (2 to 5.3 mol), and glyoxal (trace) under abiotic conditions (Balakrishnan et al. 2003, 2004; Hawari et al. 2004). Ring cleavage products produced under biotic conditions included 1.5 to 2.3 mol of nitrite, 1.5 to 3.3 mol of nitrous oxide, 0 to 1.3 mol of ammonium, 0 to 1.7 mol of formate, and 0 to 1 mol of glyoxal (Bhushan et al. 2003b, 2004a,b, 2005a). The spontaneous decomposition metabolites formed from intermediate II were produced in trace amounts and were also proposed to spontaneously decompose in water. Furthermore, limited mass spectral data were published to support the molecular structure of these metabolites (Bhushan et al. 2004b, 2005a,b), and without data from other laboratories, the pathway will remain speculative.

The alternate pathway is based on computational predictions related to molecular structure and supported

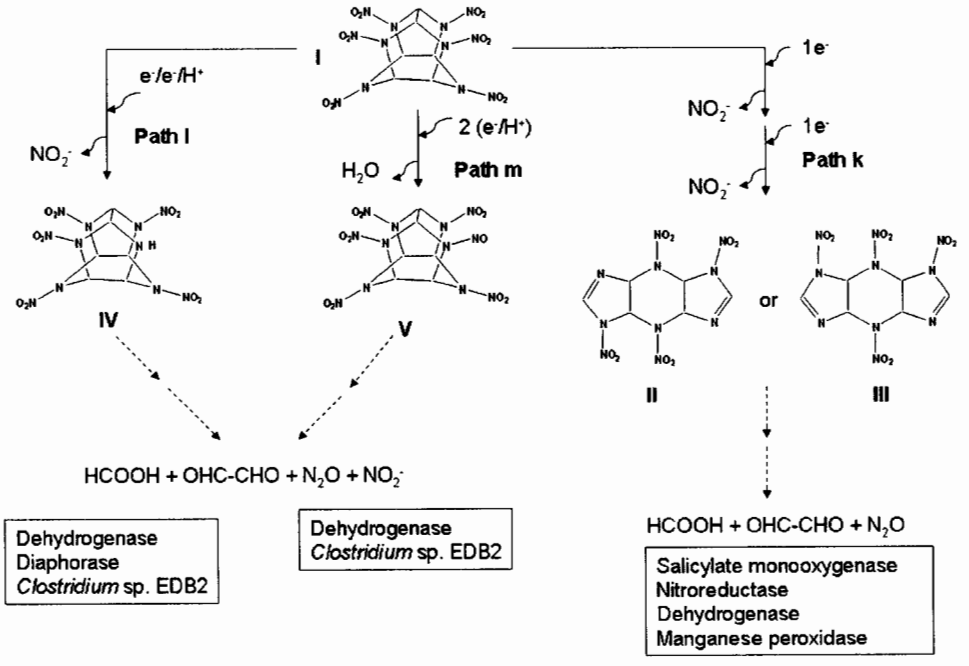


**Fig. 4** Proposed biodegradation pathways for 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (*CL-20*). *Path k* Denitration of CL-20 before ring cleavage (Bhushan et al. 2004a,b; Bhushan et al. 2005a). *Path l* Hydride transfer to CL-20 before ring cleavage (Bhushan et al. 2005a,b). *Path m* Reduction of CL-20 to nitroso derivative before ring cleavage (Bhushan et al. 2005a). *Solid arrows* Reactions leading to actual intermediates or end-products. *Dashed arrows* Multiple pathway steps based on hypothetical intermediates, which are not shown

by UV/VIS and FTIR spectra during alkaline hydrolysis and photodegradation of CL-20 (Qasim et al. 2004, 2005a; Okovytyy et al. 2005). Under these conditions, the favored pathway based on bond energies would be the continued loss of nitro groups and an increase in conjugated π bonds resulting in a stable pyrazine aromatic molecule. However, this pyrazine molecule has not been reported to be produced by microorganisms (Bhushan et al. 2004a,b, 2005a; Fournier et al. 2006), and so the relevance of this mechanism in biological systems is unknown at this time.

In the second mechanism for biotransformation of CL-20, a hydride ion transfer occurs across an N–N bond removing nitrite and generating the denitrohydrogenated intermediate IV with formula $C_6H_7N_{11}O_{10}$ and molecular weight of 393 Da (Fig. 4, path l). This pathway has been observed with a dehydrogenase enzyme isolated from *Clostridium* sp. EDB2 (Bhushan et al. 2005a) and with a purified diaphorase enzyme (Bhushan et al. 2005b). In the third pathway, the mononitroso derivative of CL-20 (V; $C_6H_6N_{12}O_{11}$; molecular weight 422 Da) is formed via reduction with two redox equivalents (Fig. 4, path m). This pathway is also catalyzed by the dehydrogenase enzyme of strain EDB2 (Bhushan et al. 2005a). The fate of intermediates IV and V was not followed because they were transiently produced in small amounts. These initial enzymatic reactions were postulated to destabilize the CL-20 molecule and promote ring cleavage with the production of the same range of end-products as observed with denitration and ring cleavage (Bhushan et al. 2005a,b). Biotransformation of CL-20 by the dehydrogenase enzyme from strain EDB2 was postulated to occur primarily by denitration with the mechanisms of N-denitrohydrogenation and reduction being minor routes of degradation (Bhushan et al. 2005a).

CL-20 is mineralized by the white-rot fungus *P. chrysosporium* possibly via the action of manganese peroxidase (MnP) (Fournier et al. 2006). Nitrous oxide (45% of N balance) and carbon dioxide (>80% of C balance) were the only end-products observed during lignolytic growth on *P. chrysosporium*. A doubly denitrated metabolite of mass 346 Da and molecular formula $C_6H_6N_{10}O_8$ was produced by MnP in an enzyme assay along with nitrite, glyoxal, and small amounts of nitrous oxide and nitrate. Nitrite was assumed to be consumed for growth, and glyoxal was shown to be mineralized by *P. chrysosporium* but not transformed by MnP.

## Advancing the science of cyclic nitramine degradation: from genetics to genomics

As discussed earlier, most of the biochemistry of cyclic nitramine degradation has been determined from observed and theoretical degradation products resulting from degradation studies with microbial enrichments, microbial isolates, and/or in vitro enzyme studies. The lack of fundamental information regarding microbial genetic and biochemical pathways involved in cyclic nitramine transformation/mineralization can be partially attributed to the fact that less than 1% of all microorganisms present in the biosphere are amenable to laboratory cultivation thus far (Amann et al. 1995). Of the 40 microorganisms representing approximately 19 genera that have been isolated, which are capable of degrading cyclic nitramines (Table 1), limited strain-specific genetic tools, screens, or assays are available for identifying the genes responsible for cyclic nitramine degradation. Under these restrictive conditions, there are a few genetic strategies (summarized in Table 2) that one can employ to aid in the identification of genes coding for enzymes involved in cyclic nitramine degradation. In those instances where the strategy has not been employed for interrogation of cyclic nitramine catabolism, an appropriate reference is instead presented illustrating the strategy (Table 2).

The most straightforward approach toward gene discovery is to select or screen for a specific function. In general, a genomic library of interest is directly expressed in clones of a surrogate host, and each clone is screened for the desired activity. Thus far, Seth-Smith et al. (2002) have been the only ones to publish a genetic selection that successfully isolated a gene target involved in cyclic nitramine degradation. The authors took advantage of the fact that nitrite is produced during RDX biodegradation by *R. rhodochrous* strain 11Y to devise a strategy to clone the gene(s) responsible for this activity. A genomic plasmid library from strain 11Y was constructed in *E. coli* and transferred to *R. rhodochrous* strain CW25, a strain that cannot use RDX as a source of nitrogen but can grow on nitrite. Strain CW25 clones capable of reacting with RDX and subsequently generating an endogenous source of nitrite were enriched for their ability to grow in the presence of RDX as a sole nitrogen source. Individual CW25 clones exhibiting RDX-degrading activity were screened for by a zone-of-clearance method using RDX-layered agarose plates. This strategy led to the identification of the *xplA* gene, which codes for a unique fused flavodoxin-cytochrome P450 enzyme. While the overall in vivo contribution of the *xplA* gene toward RDX transformation/degradation remains circumstantial, barring isolation of an *xplA* knockout mutant, the authors later show that purified recombinant XplA protein is capable of catalyzing the degradation of RDX in vitro (Rylott et al. 2006). Utilizing the work of Seth-Smith et al. (2002), we developed an alternative high-throughput HPLC-based liquid screening method to screen for genes involved in the RDX breakdown (Athow and Indest 2004). The assay

**Table 2** Discovery strategies for isolating genes involved in cyclic nitramine transformation or mineralization

| Strategy | Description | Advantage | Disadvantage | References |
|---|---|---|---|---|
| Direct expression | Genomic DNA from a microbial isolate or a metagenomic community is expressed in a surrogate microbial host and screened for the ability to transform/mineralize cyclic nitramines. | Direct functional screen | Surrogate microbial host may not express gene; screening assays for cyclic nitramines lacking | (Seth-Smith et al. 2002; Athow and Indest 2004) |
| Genetic homology | Genetic homologs of existing genes involved in cyclic nitramine transformation/mineralization are identified from microbial isolates and or communities by hybridization analysis, i.e., Southern analysis, PCR, Microarray analysis. | Effective when gene target is conserved (>80% identity). | Gene discovery limited to genes previously characterized | (Indest et al. 2006) |
| Mutagenesis | Microbial isolate was subjected to random genome mutagenesis (transposon-mediated), and the resulting mutants were screened for defects in the ability to transform/mineralize cyclic nitramines. | Potential to isolate multiple gene targets and reconstruct transformation/mineralization pathways | Genetic tools lacking for microbial isolates that transform/mineralize cyclic nitramines | (Fernandes et al. 2001; Banh et al. 2005) |
| Gene profiling | Differences in mRNA expression profiles are compared between a control culture and an experimental culture metabolizing cyclic nitramines using an assortment of genomic methods such as differential display, suppressive subtractive hybridization, or shotgun microarray gene profiling. | Potential to isolate multiple gene targets and elucidate relevant metabolic and regulatory pathways/networks involved in cyclic nitramine catabolism | Gene targets must be induced/repressed to be identified; gene function inferred by association and requires independent validation; relies on emerging bioinformatic analyses | (Walters et al. 2001; Parro and Moreno-Paz 2003; Denef et al. 2004) |
| Stable isotope analysis | Microbial consortium incubated with $^{15}N$ or $^{13}C$ stable isotope-labeled cyclic nitramine and "heavy"-labeled genomic fragments derived from metabolism of these compounds are enriched for and characterized. | Identifies in situ microorganisms actively catabolizing cyclic nitramines; provides a linkage between taxonomy and function | Metabolic cross-feeding can complicate interpretation; chemical synthesis of heavy-labeled cyclic nitramines is expensive. | (Gallagher et al. 2005) |

is based on our observation that recombinant strains of *E. coli* Mach I, containing the *xplA* gene cloned on the high-copy plasmid pBlueScript II SK, will degrade 55 ppm of RDX in 48 h when grown in LB broth. Under these same conditions, *E. coli* Mach I cells containing only the plasmid vector do not degrade RDX. Currently, we are using this assay to screen plasmid genomic libraries from various RDX degraders for genes involved in RDX transformation.

Another functional approach toward gene discovery involves screening for mutants of cyclic nitramine degraders that have been rendered defective in the ability to transform or mineralize cyclic nitramines. Various genetic tools, such as transposon mutagenesis, have been recently developed for *Rhodococcus* (Fernandes et al. 2001) and *Gordonia* (Banh et al. 2005), which contain species capable of degrading cyclic nitramines. Gene targets subjected to mutagenesis are subsequently identified by genetic complementation studies and/or DNA sequencing. This approach is likely to be labor-intensive due to isolation of high background levels of auxotropic mutants. Furthermore, for those strains that cometabolize cyclic nitramines, the current lack of good high-throughput methods for assaying cyclic nitramines will make screening laborious. As a result, HPLC assays aimed at looking for the disappearance of cyclic nitramines will be required to identify likely gene candidates. The advantage of this method, however, is that additional genes involved in the downstream metabolism of cyclic nitramines may be identified.

Alternatively, genes involved in cyclic nitramine transformation can be identified by their sequence homology to known genes involved in cyclic nitramine degradation. For this approach to be successful, the gene of interest needs to be moderately conserved. Thus far, this approach is limited due to the fact that only two genes, *xplA* (Seth-Smith et al. 2002) and *nsfI* (Kitts et al. 2000), have been shown to code for enzymes involved in cyclic nitramine transformation. A BLAST comparison of the translated *xplA* amino acid sequence with those deposited in the NCBI database revealed several highly similar homologs directly submitted to NCBI (Edwards and Bruce 2005). It is interesting to note that despite the fact that some of these homologs were isolated from geographically different areas, they still maintain a high similarity with one another, suggesting that they may be conserved. Taking advantage of this observation, we probed the genomes of two RDX-degrading bacteria isolated in our lab from China Lake surface soil, *Williamsia* sp. KTR4 and *Gordonia* sp. KTR9 (Thompson et al. 2005), with a random-primed labeled probe coding for the *xplA* gene (Indest et al. 2006). The results of the southern hybridization, under moderate to high stringency, reveal significant hybridization of the *xplA* gene probe with the genomes of both KTR9 and KTR4, suggesting the presence of *xplA* homologs in both strains (Indest et al. 2006). This observation is somewhat intriguing considering the different geographical origins of the isolates and that unlike the previously characterized RDX-degrading *Rhodococcus* strains 11Y (Seth-Smith et al. 2002) and DN22 (Coleman et al. 1998), strains KTR4 and KTR9 can use RDX as a sole source of nitrogen and carbon for growth (Thompson et al. 2005).

Other genomic approaches that are not so dependent on a specific bacterial genetic system or existing DNA sequence information but rather on comparisons of mRNA levels under different experimental conditions will likely provide significant insights into the metabolic networks governing cyclic nitramine catabolism. These approaches traditionally are referred to as differential display technologies (Liang and Pardee 1992) and have been successfully used to identify genes involved in the transformation of various xenobiotic compounds (Iimura and Tatsumi 1997; Walters et al. 2001; Brzostowicz et al. 2003). With respect to nitroaromatic explosive compounds, this technique was used to identify genes induced by 2,4-dinitrophenol (2,4-DNP), two of which were subsequently confirmed to be involved in the degradation of 2,4-DNP (Walters et al. 2001). The development of DNA microarray technology (Schena et al. 1995) along with the availability of over 331 completed microbial genomes has transformed differential display analysis into gene expression profiling (Schena et al. 1996), a method whereby small changes in mRNA expression in response to a condition or compound are globally measured at the genome level. For microorganisms in which complete genomic DNA sequencing information is available, gene expression profiling has, for example, implicated genome-wide metabolic networks involved in contaminant metabolism (Denef et al. 2004). Thus far, the technique has not been applied to decipher cyclic nitramine metabolism, in part, because limited genomic information is available for organisms that degrade these compounds. For poorly characterized organisms or organisms in which complete genome sequence information is lacking, alternative random shotgun-microarray methods have been developed. These methods have been used to identify nitrogen fixation genetic responses in the strict iron-oxidizing bacterium *Leptospirillum ferrooxidans* (Parro and Moreno-Paz 2003), and metabolic genetic responses of the archaeon *Haloferax volcanii* (Zaigler et al. 2003). We are currently investigating the feasibility of a shotgun microarray-assisted differential display method using custom shotgun DNA microarrays to interrogate *Gordonia* sp. KTR9 under conditions whereby RDX is metabolized as a sole carbon and nitrogen source.

Advances in high-throughput DNA sequencing and environmental sample preparation have now enabled access to biochemical and genetic information on environmental

microbes without the prerequisite of cultivation. Collectively, these uncultivable microbial genomes are referred to as the "metagenome" (Handelsman et al. 1998) and contain a wealth of unexplored genetic information. High-throughput metagenomic DNA sequencing and functional gene expression metagenomic studies have yielded some interesting results. For example, using a whole-genome shotgun sequencing approach, a total of 1.045 billion base pairs of nonredundant sequence was generated from an environmental sample collected from the Sargasso Sea (Venter et al. 2004). A total of 1.2 million previously unknown genes were discovered within this sequence dataset. Similarly, a microbial biofilm community collected from an acid-mine drainage site was sequenced, allowing the reconstruction of near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II, and the partial recovery of three other genomes. This sequencing dataset provided insight on microbial community structure and community metabolism (Tyson et al. 2004). Such an approach could be applied to advance an understanding of cyclic nitramine metabolism in circumstances where a defined microbial consortium of limited diversity has been identified, which metabolizes cyclic nitramines. Incubation of the consortium with $^{15}N$ or $^{13}C$ stable isotope-labeled explosives (Gallagher et al. 2005) could be used to produce a metagenomic library enriched with genome fragments from organisms that derived their nitrogen or carbon from the explosive and thus are central to the metabolism of the explosive. The stable isotope probing approach has recently been combined with metagenomics to document the isolation of the particulate methane monooxygenase gene from a forest soil using $^{13}C$-methane (Dumont et al. 2006).

Alternatively, functional gene studies could be conducted in which metagenomic DNA is expressed in a surrogate microbial host and screened for novel genes involved in cyclic nitramine metabolism. Similar functional studies have led to the discovery of novel metabolic genes encoding for an acyl-homoserinelactone synthase (Williamson et al. 2005), hydroxybutyrate dehydrogenases (Henne et al. 1999; Wang et al. 2006), enzymes involved in biotin synthesis (Entcheva et al. 2001), and alcohol oxidoreductase activity (Knietsch et al. 2003). Despite successes in functional metagenomics, the lack of rapid methods to assay for cyclic nitramine transformation along with the high number of metagenomic clones required for a successful screening has limited the practicability of this technique. As an alternative, a substrate-induced gene-expression screening based on an operon-trap *gfp* expression vector has been used to isolate genes induced by aromatic hydrocarbons from a groundwater metagenomic library (Uchiyama et al. 2005). The applicability of this approach toward cyclic nitramine metabolism may be limited because the genes so far identified that transform cyclic nitramines are not induced by the explosives (Seth-Smith et al. 2002).

By embracing the new tools and perspective of genomics, it will be possible to not only identify those individual genes involved in the transformation of cyclic nitramines but also to understand how microorganisms respond to and metabolize explosive compounds. This is particularly important for environmentally reactive contaminants such as explosives, which can be transformed by abiotic processes as well as a range of biologically mediated processes (cometabolism, as a nitrogen, carbon, or nitrogen plus carbon source for growth, or as an electron donor or acceptor). More specifically, the insights into what genes and genetic pathways are involved in (1) migration of bacteria toward cyclic nitramine compounds, i.e., chemotaxis, (2) transport of cyclic nitramines within the bacterial cell, (3) catabolic breakdown of cyclic nitramines, and (4) regulatory control points that modulate the above processes on cyclic nitramine metabolism will be realized.

In summary, basic information on the biodegradation of nitramine explosives provides a foundation on which microbially based biotechnologies can be developed. Current successful technologies that showed enhanced bioremediation of nitramine explosives in soils or groundwater via the addition of acetate (Davis et al. 2004), soybean oil and peat moss (Fuller et al. 2005), and zero-valent iron (Shrout et al. 2005) have progressed to field scale trials under the United States Environmental Security Technology Certification Program (http://www.estcp.org/Technology/ER-Energetics.cfm). While these results are encouraging, knowledge of the enzymes and genes involved in nitramine biodegradation could be used to engineer more efficient rational remediation strategies. For example, after demonstrating that the product of the *xplA* gene was active against RDX, the *xplA* gene was successfully cloned and expressed in *Arabidopsis thaliana*, a member of the mustard family of vascular plants (*Brassicaceae*; Rylott et al. 2006). The recombinant *A. thaliana* plants were shown to remove RDX from liquid media and to be resistant to RDX phytotoxicity, indicating that similar recombinant plants may be useful for phytoremediation of explosive-contaminated soils. Furthermore, basic information of the enzymes and genes involved in nitramine biodegradation could lead to the development of explosive-specific biosensors as well as facilitate the green biosynthesis of explosives or industrially important bioactive compounds that contain N–N bonds. To advance our basic knowledge of cyclic nitramine biodegradation, it appears that the best strategy will involve the use of traditional microbiological and genomic approaches, thus facilitating the development of efficient remediation and containment technologies for nitramine explosives in the environment.

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