RESEARCH LETTER

Ruminal bioremediation of the high energy melting explosive (HMX) by sheep microorganisms

Hillary L. Eaton[1], Lia D. Murty[2], Jennifer M. Duringer[3] & A. Morrie Craig[4]

[1]Department of Microbiology, Oregon State University, Corvallis, OR, USA; [2]Department of Pharmaceutical Sciences, Oregon State University, Corvallis, OR, USA; [3]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, USA; and [4]Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR, USA

Correspondence: A. Morrie Craig, Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA.
Tel.: +1 541 737 3036;
fax: +1 541 737 2730;
e-mail: a.morrie.craig@oregonstate.edu

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Abstract

The ability of ruminal microorganisms to degrade octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (high melting explosive, HMX) as consortia from whole rumen fluid (WRF), and individually as 23 commercially available ruminal strains, was compared under anaerobic conditions. Compound degradation was monitored by high-performance liquid chromatography, followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) for delineation of the metabolic pathway. In WRF, 30 μM HMX was degraded to 5 μM HMX within 24 h. Metabolites consistent with *m/z* 149, 193 and 229 were present throughout the incubation period. We propose that peaks with an *m/z* of 149 and 193 are arrived at through reduction of HMX to nitroso or hydroxylamino intermediates, then direct enzymatic ring cleavage to produce these HMX derivatives. Possible structures of *m/z* 229 are still being investigated and require further LC-MS/MS analysis. None of the 23 ruminal strains tested were able to degrade HMX as a pure culture when grown in either a low carbon or low nitrogen basal medium over 120 h. We conclude that microorganisms from the rumen, while sometimes capable as individuals in the bioremediation of other explosives, excel as a community in the case of HMX breakdown.

Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, otherwise known as HMX for high melting explosive, is a man-made nitrate munition which explodes violently at high temperatures (534 °F and above), making it ideal for use in nuclear devices, plastic explosives, rocket fuels, and burster chargers (Sunahara *et al.*, 2009). Soil adsorption and vapor pressure of HMX are low, which can cause it to leach into ground water from contaminated soil near manufacturing, storage, or testing sites, and also into terrestrial and aquatic plants (Bhadra *et al.*, 2001; Groom *et al.*, 2001). While HMX has not been linked to phytotoxicity in plants such as lettuce and barley (Robidoux *et al.*, 2003), HMX caused reproductive problems in earthworms (Robidoux *et al.*, 2001) and decreased hatching success by 50% in lizard eggs that were incubated in an environment near maximum environmental concentrations (McMurry *et al.*, 2012). Inhaling contaminated dust particles and swallowing contaminated ground water are possible routes of exposure for military personnel and residents living near places that manufacture or use HMX. Information on the adverse health effects of HMX is limited, but studies in rats, mice, and rabbits indicate that HMX is harmful to the liver and central nervous system if it is swallowed or has contact with the skin (Sunahara *et al.*, 2009; Agency for Toxic Substances and Disease Registry, 2010).

HMX in soil and ground water is noticeably recalcitrant to degradation with half-lives of up to 2300 and 8000 days, respectively (Jenkins *et al.*, 2003; Agency for Toxic Substances and Disease Registry, 2010). Because HMX remains in the soil and ground water for long periods of time, we can conclude that microorganisms in these environments cannot remediate the compound to any large extent under natural conditions. Some studies have shown biodegradation of HMX in sewage sludge (Hawari *et al.*, 2000; Boopathy, 2001) and cold marine sediments (Zhao *et al.*, 2004), which are typically oxygen-poor environments. Conclusions from studies with

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soil-dwelling bacteria and fungi under aerobic conditions indicate that, in many instances, selection and addition of an appropriate substrate to enhance the growth and biodegradation of contaminants in soil by indigenous microorganisms is a superior strategy to the introduction of nonindigenous microorganisms (Axtell *et al.*, 2000; Monteil-Rivera *et al.*, 2003; Crocker *et al.*, 2006).

Phytoremediation of HMX has also been examined. Aquatic plants (Bhadra *et al.*, 2001), and several indigenous and agricultural species demonstrated no transformation of the parent compound, but only translocation into the aerial tissues (Groom *et al.*, 2001). We have been developing a technology called *Phytoruminal bioremediation*, in which cool-season grasses (accustomed to high levels of nitrogen) can be seeded over explosives-containing soil to accumulate energetic compounds into the shoots (Duringer *et al.*, 2010) for grazing by sheep, where ruminal microorganisms then complete degradation of the explosives (Fleischmann *et al.*, 2004; Smith *et al.*, 2008; De Lorme & Craig, 2009; Eaton *et al.*, 2011; Perumbakkam & Craig, 2012; Eaton *et al.*, 2013). This technique combines aspects of both *in situ* and *ex situ* bioremediation technologies by leaving the contaminated soil *in situ*, but utilizing grasses and grazing sheep to remove the compounds to the *ex situ* rumen, which is a cheap and controlled anaerobic environment.

In this study, we compared the metabolic fate of HMX between ovine whole ruminal fluid and 23 commonly isolated and commercially available bacterial strains from the rumen. We hypothesized that HMX would be degraded in whole rumen fluid (WRF), which contains a consortium of bacteria, faster and more completely than by the strains based on past experience with other explosives; but that, by examining the strains, we would better understand which organisms may be crucial for identifying novel genes responsible for HMX breakdown. These objectives were accomplished by high-performance liquid chromatography (HPLC) analysis of spent culture supernatants to identify possible degraders, followed by identification and quantitation of metabolites by liquid chromatography–tandem quadrupole mass spectrometry (LC-MS/MS).

Materials and methods

Chemicals and reagents

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; 99% purity) was purchased from ChemService (West Chester, PA). Methylenedinitramine (98% purity) was provided by R.J. Spanggord from SRI International (Menlo Park, CA). Solvents were of HPLC and LC-MS/MS grade. Reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO). An ELGA Ultra PureLab (Cary, NC) reverse osmosis water purification system was used to generate Milli-Q (resistance > 18.2 MΩ-cm)-quality water for all aqueous solutions.

Organisms, media, and growth conditions

Pure culture strains listed in Table 1 were obtained from the American Type Culture Collection (Rockville, MD) or the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). Some strains required species-specific media, instead of a general complex medium, for optimal growth. These included *Desulfovibrio* medium (DSMZ medium 63), *Clostridium polysaccharolyticum* (DSMZ medium 140), and *Lactobacillus ruminus* (DSMZ medium 232). The remaining cultures were grown in a complex medium (Eaton *et al.*, 2011). All media were prepared anaerobically and immediately placed into an anaerobic glove box H_2/CO_2 (10 : 90). All media were dispensed into Balch tubes, which were sealed with butyl rubber stoppers and aluminum crimp caps and autoclaved for 35 min at 120 °C, then stored until use. Anaerobically prepared and sterilized reducing agent (1.25% cysteine sulfide) and B-vitamins solution (Eaton *et al.*, 2011) were added to media prior to inoculation. Cultures were grown in the dark at

Table 1. Strains and sources of ruminal bacteria tested for HMX degradation ability

Organism	Strain	Source
Anaerovibrio lipolyticus		ATCC 33276
Butyrivibrio fibriosolvens	D1	ATCC 19171
	nyx	ATCC 51255
Clostridium bifermentans		ATCC 17836
Clostridium pasteurianum	5	ATCC 6013
Clostridium polysaccharolyticum	B	ATCC 33142
Desulfovibrio desulfuricans subsp. *desulfuricans*	MB	ATCC 27774
Eubacterium ruminantium	GA 195	ATCC 17233
Fibrobacter succinogenes	S85	ATCC 19169
Lactobacillus ruminus	RF1	ATCC 27780
Lactobacillus vitulinus	T185	ATCC 27783
Megasphaera elsdenii	T-81	ATCC 17753
Peptococcus heliotrinreducens		ATCC 29202
Prevotella albensis	M384	DSMZ 13370
Prevotella bryantii	B14	DSMZ 11371
Prevotella ruminicola		ATCC 19189
Selenomonas ruminantium	HD4	ATCC 27209
	PC18	ATCC 19205
Streptococcus bovis	IFO	ATCC 15351
	JB1	ATCC 700410
Streptococcus caprinus	2.2	ATCC 700065
Succinovibrio dextrinosolvens	554	ATCC 19716
Veillonella parvula	TE3	ATCC 10790

ATCC: American Type Culture Collection; DSMZ: Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

39 °C with shaking (150 r.p.m.) for 18–24 h between transfers. Cultures were transferred at least three times before beginning degradation experiments.

WRF microcosms with HMX

Ovine WRF was collected from two cannulated male sheep fed a high forage diet of alfalfa twice daily from the Oregon State University (OSU) Sheep Center (Corvallis, OR) in accordance with International Animal Care and Use Committee regulations. WRF (7 mL) was inoculated into sterile, anaerobically prepared screw-capped tubes. HMX was added as a liquid solution to each tube for a final concentration of 30 μM, which is near the upper limit of solubility in water at room temperature (Hesselmann & Stenstrom, 1994; Agency for Toxic Substances and Disease Registry, 2010). An autoclaved control was run in parallel which consisted of 30 μM HMX added to 7-mL autoclaved WRF. Tubes were incubated anaerobically in the dark at 39 °C on a rotary shaker (150 r.p.m.); samples were taken at 0.25, 1, 2, 3, 4, and 24 h. All controls and tests were repeated in triplicate.

Ruminal strain incubations with HMX

Each strain was incubated with a concentration of 17 μM HMX, added as a liquid solution, which equaled roughly half of the dose in WRF microcosms, in low nitrogen basal (LNB) and low carbon basal (LCB) media (Eaton *et al.*, 2011; upon pilot testing a dose range of HMX, 17 μM was found to be the highest dose the cultures could tolerate for the 7-day incubation period). A media control consisted of 17 μM HMX in both LNB and LCB without the addition of test organism. A solvent control consisted of both types of media with 1.0 mL of overnight culture of the test organism and the addition of 0.1 mL acetonitrile. Cultures were incubated anaerobically, in the dark, at 39 °C on a rotary shaker (150 r.p.m.) for 120 h. Samples were collected at 0, 1, 4, and 5 days and processed for analysis by HPLC and LC-MS/MS as described below. Extracted samples were analyzed immediately by HPLC or frozen at −20 °C until LC-MS/MS analysis. All controls and tests were repeated in triplicate.

Sample preparation for chromatography

WRF samples were collected, then frozen at −20 °C until prepared for HPLC and LC-MS/MS analysis through solid-phase extraction using Waters Oasis HLB (3 mL/60 mg 30 μm) cartridges (Milford, MA), per the manufacturer's instructions, and modified as previously described (Eaton *et al.*, 2013).

HPLC and LC-MS/MS analyses

HPLC analyses were used to determine the HMX concentration of samples and were carried out using minor modifications (Eaton *et al.*, 2013) to Environmental Protection Agency method 8330A (U.S. Environmental Protection Agency, 2007).

LC-MS/MS analyses were performed on an ABI/SCIEX (Applied Biosystems, Foster City CA) 3200 QTRAP LC-MS/MS system using atmospheric pressure chemical ionization in the negative ion mode (Borton & Olson, 2006). A Phenomenex Ultracarb ODS (20) column (250 × 4.6 mm i.d., 5 μm particle size) was used to separate HMX and its metabolites at a flow rate of 0.75 mL min^{-1} over 20 min using mobile phases consisting of 0.6 mM ammonium acetate in water (A) and methanol (B) as follows: 0–5 min 90% A, decreasing linearly from 5 to 8 min to 80% A, then to 42% A from 8 to 20 min. Data were acquired using multiple reaction monitoring (MRM), using 46 → 355 and 147 → 355 ($HMX + CH_3COO^-$), 59.8 → 135 (methylenedinitramine), 61 → 118 (NDAB) as transitions. Source and gas parameters followed those in Eaton (2013). Declustering potential, entrance potential, collision entrance potential, collision energy, and collision exit potential were as follows: HMX (−15, −3.5, −24.8, −12, −4 for both transitions), methylenedinitramine (−10, −2.5, −10, −16, −58), 4-nitro-2,4-diazabutanal (NDAB; −5, −3.5, −6, −10, 0). Data used to identify possible new metabolites were acquired using an enhanced mass spectra (EMS) enhanced product ion scan via information-dependent acquisition experiments. HMX and possible metabolites were separated using the same conditions as in the MRM method, with the exception of the gradient which was 0–5 min held at 80% A, decreasing linearly from 5 to 30 min to 50% A, decreasing linearly to 0% A from 30 to 60 min, and then holding for 5 min, before equilibrating to 80% A for 5 min. Source and gas parameters followed those in Eaton (2013). Final EMS data were analyzed using LIGHTSIGHT 2.0 (Applied Biosystems) and CHEMDRAW ULTRA 12.0 (CambridgeSoft, Cambridge, MA) software to capture and interpret possible metabolites.

Results and discussion

Degradation of HMX in ovine WRF

LC-MS/MS analysis of ovine WRF samples showed near complete anaerobic degradation of HMX from 30 to 5 μM at 24 h; autoclaved controls showed little change in HMX concentration over 24 h (Fig. 1). To identify

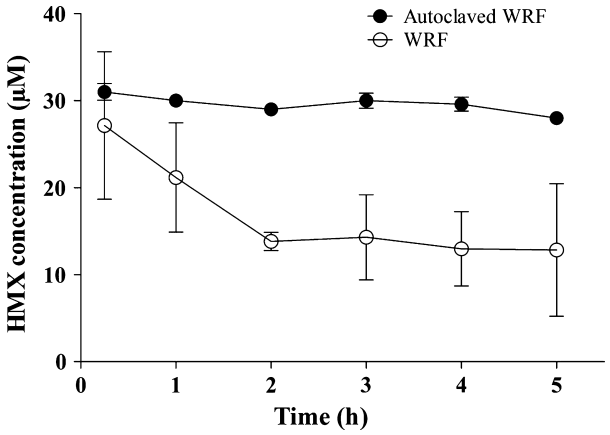

Fig. 1. HMX concentration over 5 h in ovine WRF microcosms, as determined by LC-MS/MS. Error bars represents the standard deviation of three replicate samples per time point.

metabolic products in HMX degradation by WRF, an enhanced mass spectrometry (EMS) scan was performed (Fig. 2). At 1 h, the HMX concentration had decreased to 22 μM and metabolite peaks consistent with an *m/z* of 149, 193 and 229 appeared (Figs 2c and 3). After 4 h, the HMX concentration decreased to 14 μM, while metabolite peaks consistent with *m/z* 149 and 193 increased and the peak consistent with an *m/z* of 229 showed a slight decrease. From 4 to 24 h, peaks consistent with *m/z* 193 and 229 continued to increase, while the peak consistent with *m/z* 149 decreased slightly (Figs 2 and 3). At 24 h, EMS analysis showed a second, additional product consistent with an *m/z* of 149, which suggests ring cleavage from either a reduction product or a hydroxylamino derivative of HMX (Fig. 4). Peaks visible after 40 min in Fig. 2 were found in the method blank in addition to samples, with the exception of peaks with an *m/z* of 227

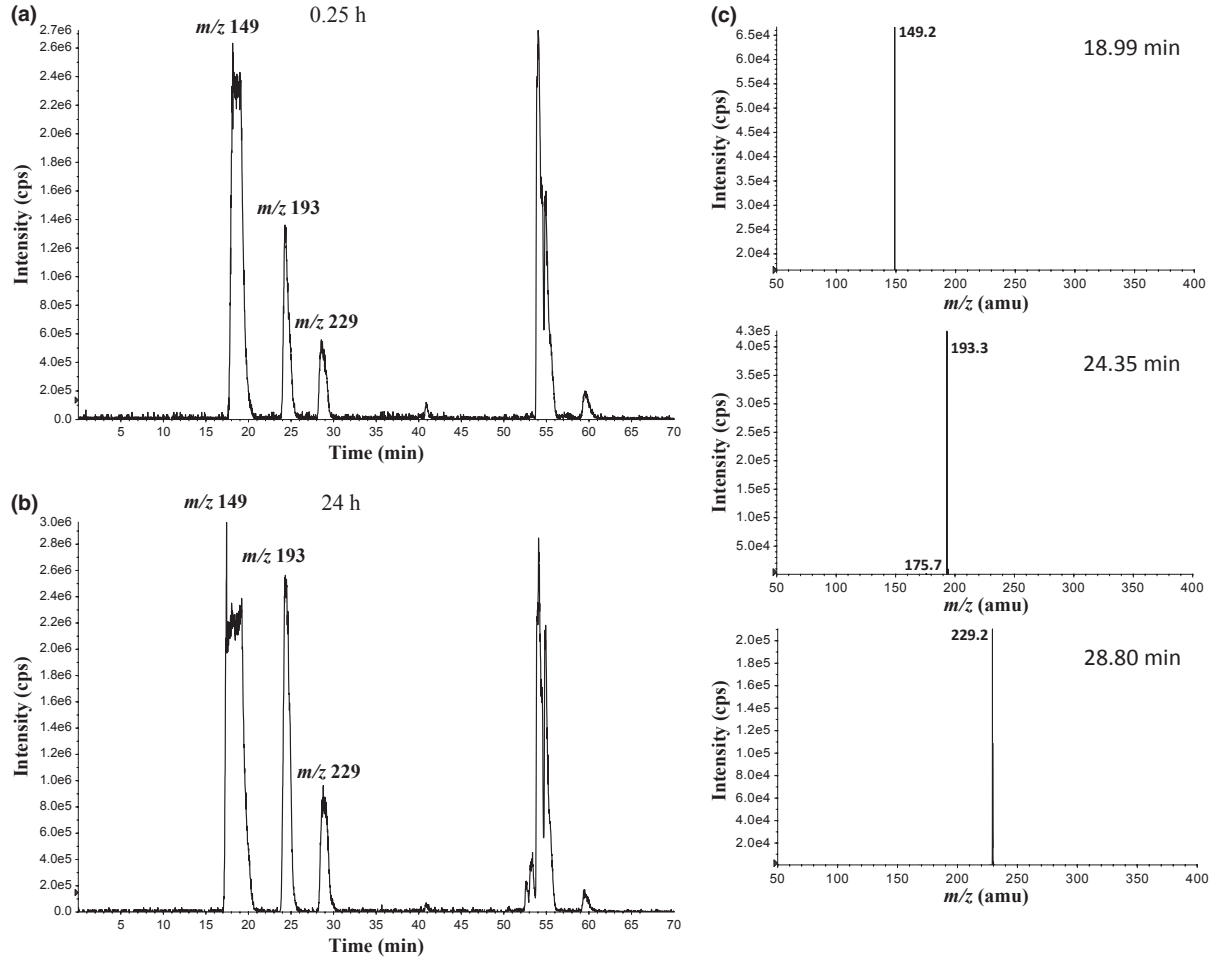

Fig. 2. Total ion chromatograms (TIC) of WRF incubated with 30 μM HMX at 0.25 h (a) and 24 h (b), obtained using an EMS scan. (c) LC-MS spectra of the most abundant peaks at 18.99 min (*m/z* 149.2), 24.35 min (*m/z* 193.3), and 28.80 min (*m/z* 229.2).

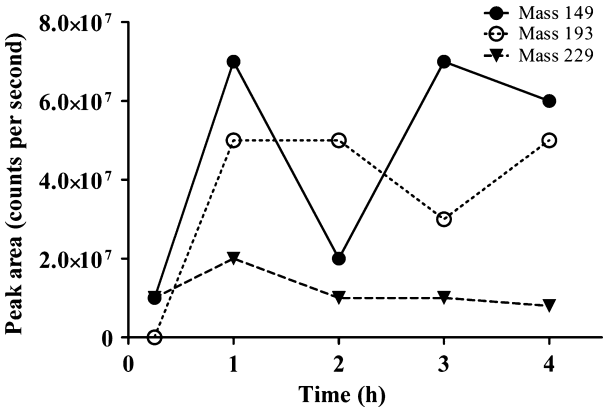

Fig. 3. Peak areas of the three most abundant metabolite peaks detected in ovine WRF incubated with 30 μM HMX from 0.25 to 5 h.

and 241 at 52.5 and 53 min, respectively. Fluctuations in the occurrence of these possible metabolites were noted and will need further separation and analysis to clarify the chemical composition. Neither methylenedinitramine nor NDAB were detected in the MRM or EMS scans of the WRF microcosm samples. Overall, it appears that HMX degradation occurs more slowly in WRF than degradation of TNT (Fleischmann *et al.*, 2004; Smith *et al.*, 2008; De Lorme & Craig, 2009) or RDX (Eaton *et al.*, 2011, 2013). Any toxic metabolites left in the rumen beyond 20 h could be cause for concern if they were passed into the bloodstream and transported to fat, organs, and tissues. Thus, future studies should examine whether these HMX metabolites are toxic to the host ruminant.

HMX displays mass spectrum fragmentation characteristics of both nitro compounds and nitrogen substituted cyclic amines. Using known fragmentation patterns of these classes of compounds, structures of detected metabolites were proposed and are shown in Fig. 4. Peaks at *m/z*149 and *m/z* 193 suggest ring cleavage through the mono-nitroso intermediate 1-NO-HMX, a reduction

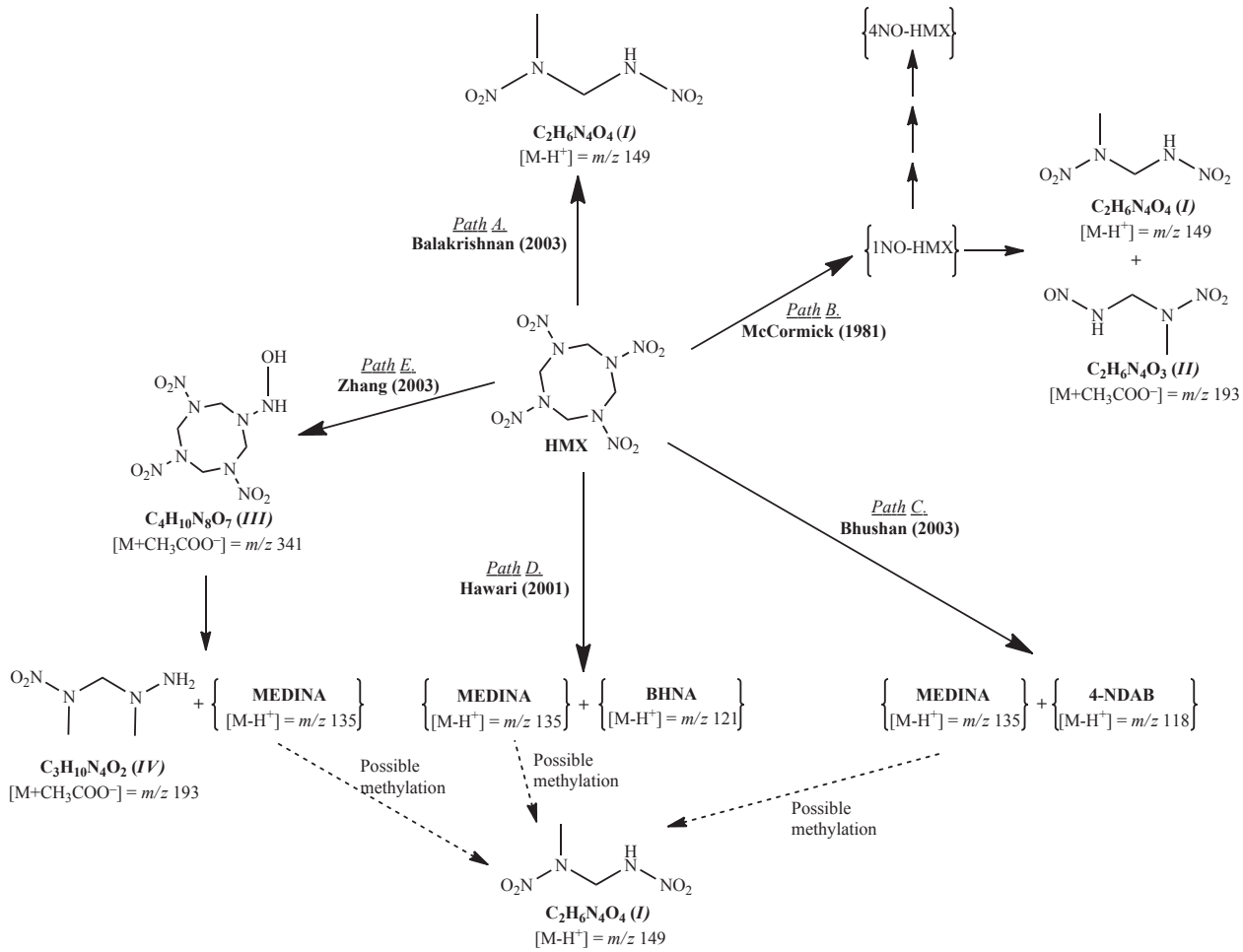

Fig. 4. Proposed biodegradation pathway for octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by ovine ruminal microorganisms under anaerobic conditions, as determined by EMS and LightSight analysis. Brackets represent hypothetical transformations.

pathway proposed by Zhao *et al.* (2004) and seen in preliminary work from our laboratory with WRF (Perumbakkam & Craig, 2012) and/or via hydroxylamino-HMX derivatives as proposed by McCormick *et al.* (1981) (McCormick *et al.*, 1981). The EMS scan for the peak consistent with *m/z* 193 suggests metabolite *II* in Fig. 4 is the most likely chemical structure to assign to this compound due to the mass loss of 16, equivalent to a single O atom, which is commonly seen in nitro-containing compounds (Pretsch *et al.*, 2000). A metabolite with an *m/z* of 149, labeled *I* in Fig. 4, could result from multiple degradation pathways, with the most likely pathway being ring cleavage through a methylenedinitramine intermediate (paths C, D, and E). However, the route proposed in path E has only been postulated in RDX and assumes that the nitro groups behave similarly under anaerobic conditions (Hawari *et al.*, 2001; Bhushan *et al.*, 2003; Zhang & Hughes, 2003). Metabolite *III* (*m/z* 341) represents a possible route of metabolism through reduction of one nitroso group, and then ring cleavage to metabolite *IV* (*m/z* 193) and methylenedinitramine, which would be metabolized to metabolite *I*. Possible structures of *m/z* 229 are still being investigated and will require LC-MS/MS analysis.

Degradation of HMX by ruminal bacterial strains

Twenty-three bacterial strains from the rumen were tested for their ability to degrade HMX in low carbon and LNB media over 120 h (Table 1). None of the strains were capable of HMX biotransformation or degradation, as compared to controls, within this time frame. No metabolites were identified by LC-MS/MS. In general, controls (reduced media without bacteria) resulted in a minor decrease in HMX concentration (5%) after 120 h (data not shown). Solvent controls did not appear to inhibit growth of any organism. We found these results surprising because many of the individual ruminal species tested in this study have been identified in the past as capable degraders of both TNT (De Lorme & Craig, 2009) and RDX (Eaton *et al.*, 2011, 2013). The concentration of HMX degraded by isolates in previous studies (Boopathy *et al.*, 1998; Hawari *et al.*, 2001; Zhao *et al.*, 2004) was more than double what we used in this study, so we do not suspect toxicity. The media used in this experiment may not have provided the appropriate conditions for degradation of HMX. These results demonstrated that HMX is more recalcitrant to degradation than the explosives TNT and RDX, which several ruminal organisms tested in this study have been able to biotransform or degrade previously (De Lorme & Craig, 2009; Eaton *et al.*, 2013). Future work will focus on enriching for organisms capable of HMX degradation in the complex consortia that comprises WRF to identify isolates, such as *Prevotella* species that were not tested in this study, that may possess the ability to degrade HMX (Perumbakkam & Craig, 2012).

This study, combined with past research, has shown that the differences in the chemical structure of TNT, RDX, and HMX lend them to be optimally degraded by different species of ruminal microorganisms. For example, *Prevotella ruminicola* is the most prevalent organism in the rumen and has been previously isolated from WRF enrichments for RDX degraders (Eaton *et al.*, 2011); in pure culture, it was shown to degrade 50% of 34 μM RDX within 7 days as a sole source of nitrogen, but was incapable of TNT or HMX degradation, despite previous research showing that the genus *Prevotella* increased significantly during an 8-h HMX incubation in WRF (Perumbakkam & Craig, 2012). Removal of TNT and all metabolites (< 5% of original TNT recovered as a metabolite) occurred for *Butyrivibrio fibriosolvens*, *Fibrobacter succinogenes*, *Lactobacillus vitulinus*, *Selenomonas ruminantium*, *Streptococcus caprinus*, and *Succinovibrio dextrinosolvens* (De Lorme & Craig, 2009). *Anaerovibrio lipolyticus* and *Desulfovibrio desulfuricans* were inhibited by TNT (De Lorme & Craig, 2009) and HMX (this study), but not by RDX (Eaton *et al.*, 2013). *Streptococcus caprinus* and the *Clostridia* organisms have shown a strong degradative ability for TNT and RDX, but not HMX (Zhao *et al.*, 2003; De Lorme & Craig, 2009). *Lactobacillus vitulinus* tends to favor TNT over RDX, although it can degrade both (De Lorme & Craig, 2009; Eaton *et al.*, 2013), while *L. ruminus* has not been found to be capable of degrading any energetic compound.

The general trend we have observed is that microorganisms from the rumen, while sometimes capable as individual strains/isolates, excel as a community in the bioremediation of explosives. *Phytoruminal bioremediation* is a technique that is proving to be viable for the remediation of energetic compounds, which includes TNT (Fleischmann *et al.*, 2004; Smith *et al.*, 2008; De Lorme & Craig, 2009), RDX (Eaton *et al.*, 2011, 2013), and now HMX (Perumbakkam & Craig, 2012).

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