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We have determined that an organism able to degrade both RDX and TNT in a pure culture is a strain of Clostridium bifermentans. The consortium from which this organism is derived also degrades these compounds, and we suspect that C. bifermentans is also the responsible organism within that consortium. The bioconversion of RDX and TNT occurs under anaerobic conditions both in the consortium and in pure culture without the need of an added reductant. The presence of co-metabolites speeded these biotransformations.

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Technical Report

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Augmentation to *In Situ Biodegradation of Nitroaromatic Compounds in Soil*

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Augmentation to In Situ Biodegradation of Nitroaromatic Compounds in Soil

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Characterization of *Clostridium bifermentans* and its Biotransformation of 2,4,6-Trinitrotoluene (TNT) and 1,3,5-Triaza-1,3,5-Trinitrocyclohexane (RDX)

Summary

We have determined that an organism able to degrade both RDX and TNT in a pure culture is a strain of *Clostridium bifermentans*. The consortium from which this organism is derived also degrades these compounds, and we suspect that *C. bifermentans* is also the responsible organism within that consortium. The bioconversion of RDX and TNT occurs under anaerobic conditions both in the consortium and in pure culture without the need of an added reductant. The presence of co-metabolites speeded these biotransformations.

Introduction

Bacteria in the genus *Clostridium* have long been known for their ability to carry out novel bioconversions of unusual substrates. The products of these bioconversions are as varied as the substrates themselves. We have confirmed this metabolic versatility by isolating clostridia from an anaerobic digester fed munitions compounds as its sole source of carbon and energy (Funk *et al.*, 1993). One isolate, a strain of *Clostridium bifermentans*, was able to transform the primary components of explosives, and was also able reproduce the sequence of events previously observed in the digester. That sequence was characterized by an initial reduction in the concentration of 2,4,6-trinitrotoluene (TNT) followed by concurrent reductions in the concentrations of both TNT and 1,3,5-triaza 1,3,5-trinitrocyclohexane (RDX).

Experimental Approach

To isolate the bacterium, 1 ml of our anaerobic consortium was used to inoculate 100 ml of anoxically prepared, sterile brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with ~50 ppm RDX. The inoculated enrichment was reduced with dithionite at a final concentration of 0.001% and incubated at 35°C overnight. The enrichment flask was streaked for isolation of pure cultures on BHI broth solidified with Bacto agar (Difco) to a finalconcentration of 2%. BHI agar plates used for the isolation of potential TNT/RDX degraders contained no RDX. They were incubated at 35°C overnight in an anaerobic glove box. Individual colonies showing different macroscopic morphologies were then streaked to anoxically prepared BHI slants containing 50 ppm of RDX. The inoculated slants were incubated 24 hours at 35°C to obtain good growth and sporulation of any sporogenic cultures, and stored at 4°C until analyzed.

Morphological types were tested alone and in mixtures for their ability to biotransform RDX in BHI. Of the isolates and mixtures tested, one isolate, a sporulating bacillus, removed RDX from the media more rapidly than the other isolates and mixtures (data not shown). This isolate was then tested for its ability, as compared to that of the batch fed anaerobic consortium from which it was isolated, to simultaneously transform TNT and RDX. These experiments were carried out in

anaerobically prepared BHI media containing both TNT(~30 ppm) and RDX (~50 ppm). Inoculated flasks were prepared under one of three reducing conditions: non-reduced, cysteine-reduced, and dithionite-reduced. An isolate designated KMR-1, which showed the highest calculated percentage change DX after 72 hours, was physiologically characterized and identified by the AN-IDENT m (API Systems).

The chromosomal DNA was obtained from the clostridial isolate and its 16s ribosomal sequence amplified by PCR. The 16s amplified sequence was purified and cloned into pT7Blue T-vector (Novagen). This construct was introduced into Novablue competent cells (Novagen), and several colonies containing the insert were selected. Three colonies were grown in a rich, selective medium for isolation of the T7Blue plasmid containing the insert. The plasmid DNA was isolated and prepared for dideoxysequencing using M13-Forward and M13-Reverse primers labeled with an infrared fluorophore. A Sequitherm and the Sequitherm cycle sequencing protocol (Epicenter Technologies Corp.) were used for direct sequencing. The sequencing gel was analyzed via the Li-Cor DNA 4000 (Li-Cor. Inc.) from three separate sequencing experiments with three isolated colonies.

To characterize the antibiotic sensitivities of the clostridial isolate KMR-1, it was tested against batteries of both traditional and non-traditional anti-clostridial antibiotics, using methods as described by Sutter (1985). The growth of strain KMR-1 was monitored by measuring the optical density (OD) at 600 nm in the presence of explosives and different reducing agents.

RDX and TNT concentrations were determined by reverse phase HPLC according to EPA Method 8330. Analysis was based on the solute elution times and spectra analysis, as compared to authentic standards run under identical conditions. An Ultracarb 5 ODS(20) 250 x 4.6 mm column (Phenomenex) was used for analysis. The solutes were eluted from the column by an isocratic mobile phase of 55% (v/v) methanol and 45% (v/v) water, at a flow rate of 0.5 ml/min. TNT and RDX, synthesized in our laboratory, were >99% pure (S. Goszczynski, pers. comm.).

To obtain scanning electron micrographs, cells were fixed with glutaraldehyde, progressively dehydrated with ethanol and fixed on aluminum carriers with carbon tape. Cells were sputter-coated with gold prior to observation with a Hummer III (Techics). The preparations were examined using an AMRAY scanning election microscope at 15.0 kV.

Results and Discussion

Three morphological types were found among the isolates. Flat, translucent colonies with an entire edge were formed by strain KMR-1, a rod-shaped, gram-positive, obligately anaerobic strain that was motile, catalase-negative, and endospore-forming. It transformed TNT and RDX in BHI medium efficiently, and was chosen for further study. Physiological characterization by the API AN-IDENT system tentatively identified KMR-1 as a strain of *Clostridium bifermentans*. The four positive reactions obtained were indole production, leucine aminopeptidase, proline aminopeptidase, and motility; all others were negative. Using three colony isolates, the 16s ribosomal summarized sequence (Figure 1) containing 1024 bases was determined.

GCAGCAGTGG GGAATATTGC ACAATGGGCG AAAGCTGATG CAGCAACGCC GCGTGAGATG
AAGGCCTTCG GGTCGTAAAG CTCTGTCCTC AAGGAAGATA ATGACGGTAC TTGAGGAGGA
AGCCCCGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT AGGGGGCTAG CGTTATCCGG
AATTACTGGG CGTAAAGGGT GCGTAGGTGG TTTTTTAAGT CAGAAGTGAA AGGCTACGGC
TCAACCGTAG TAAGCTTTTG AAACTAGAGA ACTTGAGTGC AGGAGAGGAG AGTAGAATTC
CTAGTGTAGC GGTGAAATGC GTAGATATTA GGAGGAATAC CAGTAGCGAA GGCGGCTCTC
TGGACTGTAA CTGACACTGA GGCACGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
GTAGTCCACG CCGTAAACGA TGAGTACTAG GTGTCGGGGG TTACCCCCTC GGTGCCGCAG
CTAACGCATT AAGTACTCCG CCTGGGAAGT ACGCTCGCAA GAGTAAACTC AAAGGAATTG
ACGGGGACCC GCACAAGTAG CGGAGCATGT GGTTTAATTC GAAGCAACGA GAAGAACCTT
ACCTAAGCTT GACATCCCAC TGACCTCTCC CTAATCGGAG ATTTCTTCGG GGACAGTGGT
GACAGGTGGT GCATGGTTGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA AGTCCCGCAA
CGAGCGCAAC CCTTGCCTTT AGTTGCCAGC ATTAAGTTGG GCACTCTAGA GGGACTGCCG
AGGATAACTC GGAGGAAGGT GGGGATGACG TCAAATCATC ATGCCCCTTA TGCTTAGGGC
TACACACGTG CTACAATGGG TGGTACAGAG GGTTGCCAAG CCGCGAGGTG GAGCTAATCC
CTTAAAGCCA TTCTCAGTTC GGATTGTAGG CTGAAACTCG CCTACATGAA GCTGGAGTTA
CTAGTAATCG CAGATCAGAA TGCTGCGGTG AATGCGTTCC CGGGTCTTGT ACACACCGCC
CGTA

Figure 1. 16s ribosomal sequence of *Clostridium bifermentans* KMR-1.

This 16s ribosomal sequence was compared to published 16s ribosomal RNA sequences of both the eubacteria and archaebacteria via PCGENE (Table 1) computer software. The eubacterial comparisons were made to two strains of *Clostridium bifermentans*, one strain of *Lactobacillus bifermentans*, and one strain each of *Clostridium sordelli* and *C. difficile*. The archaebacterial comparisons were made to *Methanococcus voltae*, *M. jannaschii*, and *M. thermolithotrophicus*; *Halobacterium halobium* and *H. volcanii*; *Halococcus morrhuae*; and *Sulfolobus acidocaldarius*. The greatest sequence homology was found among the known *C. bifermentans* strains and our putative *C. bifermentans*, strain KMR-1, and the least among the archaebacteria and *C. bifermentans* KMR-1 (Table 1).

Table 1. Comparison of 16s ribosomal sequence homology of *C. bifermentans* KMR-1 to species of eubacteria and archaebacteria.

Bacterial Strain	NCBI Seq. ID[a]	Identity Value[b]	% Homology[b]
C. bifermentans	443826	1000	97.66
C. bifermentans	437746	1008	98.44
C. difficile	437749	975	95.21
C. sordelli	174132	993	96.97
L. bifermentans	175017	836	81.64
H. halobium	43554	667	66.11
H. volcanii	174702	668	65.23
H. morrhuae	43618	679	66.31
S. acidocaldarius	460149	718	70.12
M. voltae	175444	684	66.80
M. thermolithotrophicus	175445	687	67.09
M. jannaschii	175446	682	66.60

[a] National Center for Biotechnology Information, NCBI

[b] Myer and Miller 1988, Open Gap Cost = 10; Unit Gap Cost = 10.

Of the traditional antibiotics tested (ampicillin, carbenicillin, cephalothin, chloramphenicol, clinamycin, penicillin G, and tetracycline), KMR-1 showed resistance only against tetracycline. Of the non-traditional antibiotics tested (kanamycin, erythromycin, and gentamicin), KMR-1 showed resistance to all but erythromycin.

Electron micrographs showed strain KMR-1 to be a pure culture (Figure 2). The organism, a motile, urease-negative, gram-positive, anaerobic bacillus, has properties similar to those of other strains isolated from the munitions-degrading consortium by our research group (data not shown).

Figure 2. Electron micrograph of *C. bifermentans* KMR-1.

In non-reduced media without explosives, strain KMR-1 grew rapidly and maintained a very high OD, while on media with explosives, it grew more slowly (Fig. 3). Under all reducing conditions, there was a significant lag time for strain KMR-1 grown in media containing explosives. Data on the concurrent biotransformation of explosives by strain KMR-1 under different reducing conditions are shown in Figures 3-6. For clarity, graphed data are mean values of replicate experiments and were not plotted with their associated standard errors of the mean. In all three media, TNT degradation occurred before RDX degradation. Cell numbers did not increase until TNT concentrations approached their minimum. Once the TNT concentrations decreased, an increase in cell number occurred with concurrent metabolism of RDX. As the concentration of RDX reached its minimum, the cell number in the culture reached a maximum. Only the cysteine-reduced medium exhibited regrowth within the time range of this experiment (Fig. 5). In abiotic controls, concentrations of RDX did not decrease in any of the reducing treatments, but concentrations of TNT decreased in all treatments. A trend was seen in the amount of abiotically transformed TNT and the theoretical Eh potential of the media: the more reduced the medium, the greater the abiotic reduction of TNT.

Figures 3-6. Growth of *Clostridium bifermentans* under different growth conditions with munitions.

Figure3. Growth in BHI under different reducing conditions.

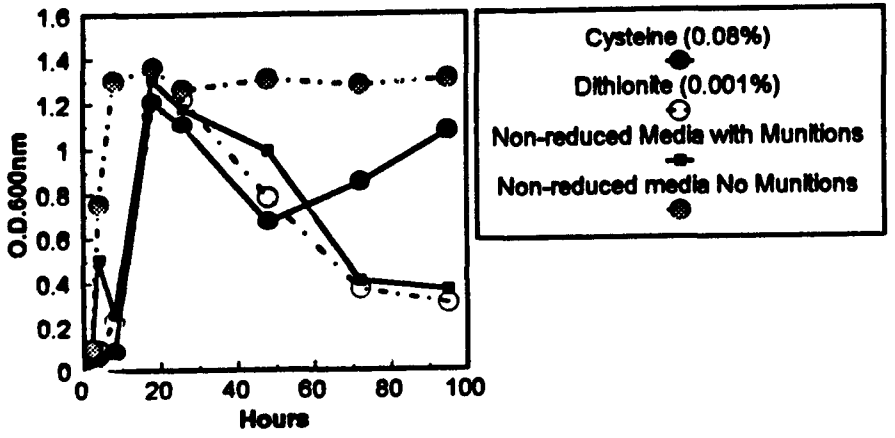


Figure 4. Non-Reduced BHI Media

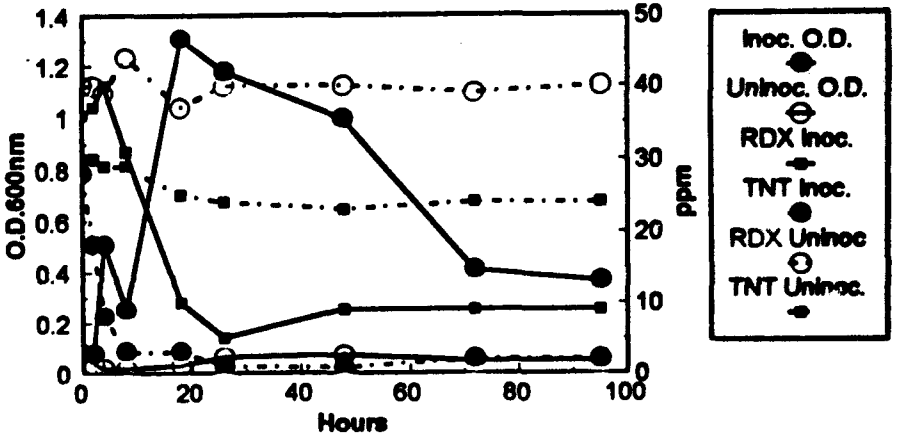


Figure 5. Cysteine (0.08%) reduced media with munitions.

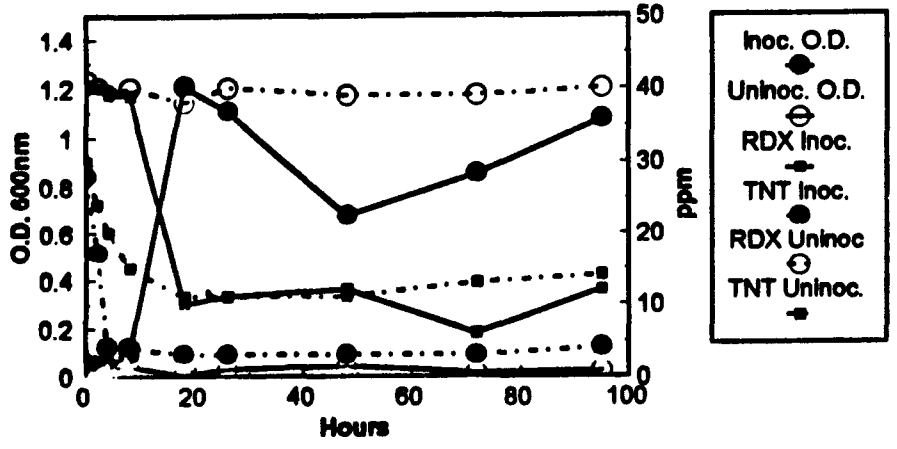
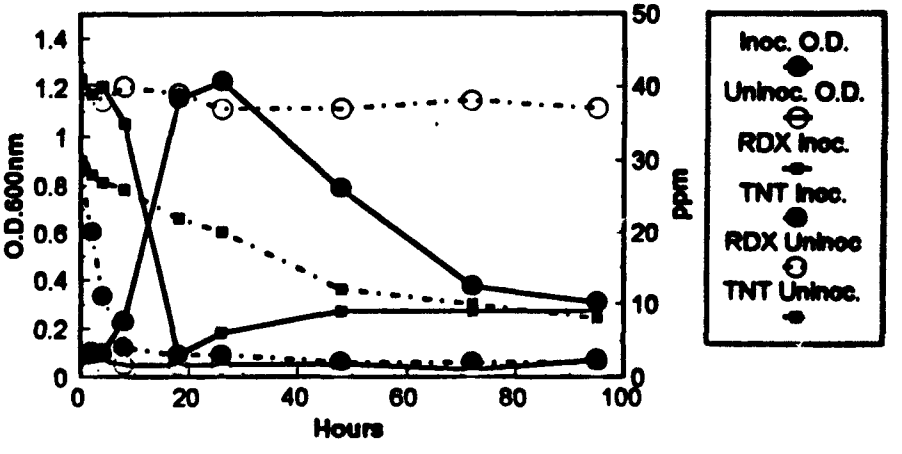


Figure 6. Dithionate (0.001%) reduced media.



In all treatments the amount of biotically transformed explosive was greater than that of abiotically transformed explosive. Funk *et al.* (1993) found that the biotransformation of TNT by their consortium occurred over a period of 4 days and RDX over 24 days. The long period required for the observed biotic transformation may have been due to the limited amount of co-substrate supplied to the consortium in these experiments, while the comparatively short time required for biotic transformation of TNT and RDX in our experiments, 4 h and 23 h respectively, was probably due to the rich, supplemented substrate supplied to strain KMR-1. In our experiments as in those of Funk *et al.* (1993), the biotransformation of TNT took place before that of RDX, separated by a short lag time of approximately 2 h, which may represent the time required for the bacteria to develop the cellular machinery to biotransform RDX. The biodegradation of RDX has been shown to yield several biological products under anaerobic conditions. McCormick *et al.* proposed a pathway and identified products including hexahydro-1-nitroso-3, 5-dinitro-1, 3, 5-dinitro-1, 3, 5-triazine; hexahydro-1, 3-dinitroso-5-nitro-1, 3, 5-triazine; hexahydro-1, 3, 5-trinitroso-1, 3, 5-triazine, hydrazine; 1,1-dimethylhydrazine; 1, 2-dimethylhydrazine; formaldehyde, and methanol. Various anaerobic products from biodegradation of TNT in both pure culture and by consortia have been described (Schackmann and Muller, 1991; Preuss *et al.*, 1993; Parrish, 1977; Funk *et al.*, 1993; McCormick *et al.*, 1976; Kaplan and Kaplan 1982; Boopathy *et al.*, 1993; Boopathy and Kulpa 1992). We are now identifying the intermediates associated with the biotransformation of both RDX and TNT by *C. bifermentans* KMR-1. Anaerobic biodegradation was previously shown to be an effective and economical approach for the remediation of soils contaminated with munition residues (EPA 1994). Our work suggests clostridia as prime facilitators of this process.

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