REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

Public Reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comment regarding this burden estimates or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188,) Washington, DC 20503.

1. AGENCY USE ONLY (Leave Blank)

2. REPORT DATE 8/4/04

3. REPORT TYPE AND DATES COVERED
Final Report 7/1/2001-6/30/2004

4. TITLE AND SUBTITLE
Genetic and biochemical Analysis of anaerobic TNT Biodegradation

5. FUNDING NUMBERS
DAAD 19-01-1-0524

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle park, N.C. 27709-2211

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
41897.4-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

20040914 070

13. ABSTRACT (Maximum 200 words)

Nitroaromatic compounds are widely used in the synthesis of dyes and munitions and have become common contaminants. The main nitroaromatic contaminant found at various Department of defense facilities is 2,4,6-trinitotoluene (TNT). This project investigated the metabolism of nitroaromatic compounds using cultures of Clostridium acetobutylicum ATCC824. The studies included (1) the characterization of products of TNT degradation, (2) investigation of RDX and DNT degradation, (3) analysis of enzymes involved in the reduction of TNT, (4) genetic studies related to TNT degradation. The report describes cloning and expression of two nitroreductases from C. acetobutylicum. TNT transformation of about the same rate was found in strain M5, a mutant of C. acetobutylicum lacking the large plasmid which bears the solvent-producing genes. This confirms the finding that TNT degradation is chromosomally encoded and is largely due to the Fe-hydrogenase. Studies on the purification and expression of this hydrogenase also suggest its importance in TNT degradation. Antisense studies also confirmed this finding. The mutagenicity of several degradation compounds of TNT and DNT was also examined.

14. SUBJECT TERMS
Clostridium, nitroaromatic, TNT, enzyme, biodegradation

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

TITLE AND SUBTITLE

Genetic and Biochemical Analysis of Anaerobic TNT Biodegradation

REPORT DATE
Aug. 4,2004

REPORT TYPES AND DATES COVERED
Final Report

FUNDING NUMBERS
DAAD 19-01-1-0524

AUTHORS
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SPONSORING/MONITORING AGENCY NAME

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ABSTRACT

Contamination by 2,4,6-trinitrotoluene (TNT) is widespread at many sites where explosives have been manufactured and stored. Due to concerns regarding toxicity and human as well as environmental health effects of TNT and its reduced metabolites, much current research has focused on remediation by biological processes. Predominant explosives of environmental concern include 2,4,6-trinitrotoluene (TNT), 2,4 Dinitrotoluene (2,4-DNT), 2,6 Dinitrotoluene (2,6-DNT) hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), which were often used in combination, as well as octahydro-1,3,5,7- tetranitro-1,3,5,7-tetrazocine (HMX).

Over the past decade, efforts focusing on the cleanup of contamination resulting from herbicides and explosives has stimulated an interest in anaerobic systems due to their ability to completely reduce polynitroaromatics. A substantial amount of research has been conducted with mixed anaerobic cultures, which forms the basis of commercially available biological processes. The cleanup strategies include developing appropriate remedial actions to address site contamination and treat the contaminated soil and groundwater to established cleanup standards. This necessitates research

investigations regarding the fundamental aspects of TNT metabolism, which would aid in development and monitoring of effective bioremediation technologies.

The present report describes studies suggesting that the major TNT transforming enzyme in *Clostridium acetobutylicum* ATCC 824 is Fe- hydrogenase. The *C. acetobutylicum* hydrogenase is closely related to that of *C. pasteurianum;* and can be fitted to the X- ray crystal structure with a root mean square deviation of 1.18 angstroms for the Cα atoms of the generated 3D simulation model. This report also describes the specific involvement of the catalytic domains and the Fe-S centers in the reactions of TNT transformation by Fe-hydrogenase enzyme. Antibodies were generated to three peptides of the hydrogenase sequence. Inhibition studies using antibodies against Fe-hydrogenase from *Clostridium acetobutylicum* indicated that the transformation of TNT by crude cell extracts was completely inhibited by Hyd2 antibody (to amino acid 415-428) whereas antibodies Hyd1 (to residues 1-16) and Hyd 3 (to amino acid 424 - 448) inhibited less effectively. The Hyd2 antibody reacted with both the hydrogenase protein in cell extracts and with *Clostridium acetobutylicum* hydrogenase expressed in *E. coli* as demonstrated by Western blotting. The transition metal Cu (II) at 0.5 mM completely inhibited the transformation of TNT (100 uM) suggesting the destruction of [4Fe-4S] centers which are essential for transfer of electrons from the H2-activating site to TNT.

The kinetic studies indicate that TNT and metronidazole compete for the same binding site, possibly the H cluster in Fe- hydrogenase. The Ki for metronidazole was found to be 30 uM. The triazine dye procion red inhibits TNT reduction suggesting that there are distinct binding domains for different substrates in Fe-hydrogenase. This study demonstrates distinct functional domains and redox centres involved in TNT reduction. The site specific inhibition studies suggest site H is essential for TNT biotransformation.

The present study is also engaged in the cloning, expression and purification of nitroreductase enzymes *nit*2 (MW 31.1 kDa) and *nit*9 (MW 23.25 kDa) encoded by nitroreductase genes (CAC 0718) and (CAC 3555) respectively from the *Clostridium acetobutylicum* ATCC 824 chromosome. The *nit*2 enzyme was further characterized biochemically to determine the kinetic parameters, substrate specificity and effect of inhibitors. The transcription of the nitroreductase gene *nit2* and *nit*9 of *Clostridium acetobutylicum* was examined by RT-PCR analysis.

1. List of papers published/communicated

I Padda RS, Wang C, Hughes JB, Kutty R and Bennett GN. Mutagenicity of nitroaromatic degradation compounds. Environ. Toxicol. Chem. 2003 Oct;22(10):2293-7.

II. Watrous MM, Clark S, Kutty R, Huang S, Rudolph FB, Hughes JB and Bennett GN. 2,4,6-trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 2003 Mar;69(3):1542-7.

III Kutty R, Rudolph FB and Bennett GN
Transformation of 2,4,6 - Trinitrotoluene by Fe-hydrogenase from *Clostridium acetobutylicum*: Relevance to mechanism of catalysis. Accepted in proceedings: The Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds held in Monterey, California, May 24-27, 2004

IV Ahmad, F, Hughes, JB, and Bennett, GN Biodegradation of hazardous materials by Clostridia, in press Handbook on Clostridia, CRC Press. Boca Rotan, FL.

V Kutty R, Rudolph FB and Bennett GN
Mechanism of Transformation of 2,4,6 - Trinitrotoluene by Fe-hydrogenase from *Clostridium acetobutylicum* (in preparation)

V I Kutty, R, Rudolph FB and Bennett GN
Cloning Expression and Characterization of an NADH: Flavin Mononucleotide nitroreductase from *Clostridium acetobutylicum*. (in preparation)

2. Razia Kutty
3. Inventions- none
4. Scientific progress and accomplishments

Over the past decade, efforts focusing on the cleanup of contamination resulting from herbicides and explosives have stimulated an interest in anaerobic systems due to their ability to completely reduce polynitroaromatics. Because of the scope of the problem and the staggering economic and environmental costs, there is great interest in developing alternative, low cost, efficient treatment technologies. On site bioremediation (using either bacteria or plants) is inherently cost effective and holds promise for solving explosives contamination problems. Both bacteria and plants have

shown to metabolize explosives. Effective implementation of this general information requires details about the metabolites formed and the enzyme activities involved in the transformation process.
A substantial amount of research has been conducted with anaerobic cultures, which catalyze the reduction of TNT but more insight is needed into the biochemistry employed by the organisms involved.

2,4,6-Trinitrotoluene Reduction by an Fe-only Hydrogenase in *Clostridium acetobutylicum*

The role of hydrogenase on the reduction of 2,4,6-trinitrotoluene (TNT) in *Clostridium acetobutylicum* was evaluated. An Fe-only hydrogenase was isolated and identified by using TNT reduction activity as the selection basis. The formation of hydroxylamino intermediates by the purified enzyme corresponded to expected products for this reaction, and saturation kinetics were determined with a Km of 152 microM. Comparisons between the wild type and a mutant strain lacking the region encoding an alternative Fe-Ni hydrogenase determined that Fe-Ni hydrogenase activity did not significantly contribute to TNT reduction. Hydrogenase expression levels were altered in various strains, allowing study of the role of the enzyme in TNT reduction rates. The level of hydrogenase activity in a cell system correlated (R^2 =0.89) with the organism's ability to reduce TNT. A strain that overexpressed the hydrogenase activity maintained TNT reduction during late growth phases, which it is not typically observed in wild type strains. Strains exhibiting underexpression of hydrogenase produced slower TNT rates of reduction correlating with the determined level of expression. The isolated Fe-only hydrogenase is the primary catalyst for reducing TNT nitro substituents to the corresponding hydroxylamines in *C. acetobutylicum* in whole-cell systems. A mechanism for the reaction was proposed. Due to the prevalence of hydrogenase in soil microbes, this research may enhance the understanding of nitroaromatic compound transformation by common microbial communities.

The research from our laboratories has demonstrated a potential of *Clostridium acetobutylicum* ATCC 824 to degrade TNT. The high rates of TNT transformation and absence of amino end products may represent a significant advance in our ability to remediate TNT-contaminated materials. The result from the study also suggests potential future applications to environmental monitoring and assessment of bioremediation processes that involve anaerobic soil microbes.
Previous investigations in our laboratory have shown rapid transformation of TNT in *Clostridium acetobutylicum* ATCC 824. Previous investigations have suggested the role of ferridoxins, hydrogenases, CO dehydrogenases, pyruvate-ferridoxin oxido-reductases and sulfite reducatses. The major enzyme involved in TNT degradation by *Clostridium acetobutylicum* is found to be Fe-hydrogenase. However the sequence analysis of Clostridial genome suggests the presence of nitroreductase family proteins which would possibly contribute to TNT transformation. This study is also engaged in

cloning and expression of genes encoding nitroreductases capable of transforming TNT from *Clostridium acetobutylicum* ATCC 824. The hydrogenase enzyme degrading TNT in *Clostridium acetobutylicum* ATCC 824 has been demonstrated to transform TNT to reduced intermediates. One of the experiments in the present study aimed at determining the contribution of Fe-Ni hydrogenase to the TNT transformation activity in *C. acetobutylicum* ATCC 824 by testing TNT transformation in strain M5 which has lost the plasmid bearing solvent producing genes and the Fe-Ni hydrogenase gene.
The experiments performed checked transformation of TNT in growing cells (0.4 O.D at 600nm) of *Clostridium acetobutylicum* wild type and mutant strain M5 demonstrated 0.256 and 0.245 mM of TNT being transformed respectively during 4 hrs incubation in culture volume of 5 ml. Heat inactivated cells showed negligible TNT transformation. The transformation of TNT by strain M5 a mutant of *Clostridium acetobutylicum* ATCC 824 indicates that chromosomally encoded Fe-hydrogenase enzyme possesses the major TNT transformation activity in *Clotridium acetobutylicum* atleast under normal growth conditions. This is also indicative of the fact that the other hydrogenase enzyme (Ni-Fe hydrogenase) contributes little to the TNT reduction in *Clostridium acetobutylicum* ATCC 824.

Simulation model of Fe-hydrogenase. The sequence analysis of the Fe- hydrogenase gene suggests that the hydrogenase enzyme is a soluble protein with 4Fe-4S ferridoxins which are the iron sulfur binding region signatures at aminoacid positions 121-132. Based on the X-ray structure of *Clostridium pasteurianum* we modelled the iron hydrogenase of *Clostridium acetobutylicum*. The structure closely fits to the X-ray crystal structure of the *Clostridium pasteurianum* hydrogenase reported in the literature.

Differential site specific inhibition using inhibitors of iron hydrogenase.
We have demonstrated the inhibition of TNT transformation by antibodies against the conserved sequence motifs of Fe-hydrogenase. Further studies using site specific inhibitors were carried out to investigate the involvement of different domains and redox centers of hydrogenase in TNT transformation.

Effect of transition metal ions

The inhibitory effect of transition metals on TNT transformation using cell extracts of *Clostridium acetobutylicum* indicate that Cu and Hg at 0.5 mM concentrations could inhibit TNT transformation whereas the divalent cations, Ni and Co had no inhibitory effect.

Effect of procion red dye

The triazine dye procion red immobilized onto agarose has been used successfully as a ligand for the affinity chromatography purification of certain hydrogenases. However the inhibitory effect of this and other triazine dyes on several hydrogenases has been also demonstrated. The inhibition of TNT reduction increased with an increase in the concentration of procion red.

Effect of metronidazole

TNT reduction experiments by cell extracts of *Clostridium acetobutylicum* in the presence of the drug metronidazole was carried out to investigate if TNT competes with metronidazole for binding to the same site on hydrogenase (H cluster). This experiment evaluates if the kinetic interaction between the two are competitive. The apparent Km of TNT reduction was found to be 800 uM which is higher than the Km in the absence of metronidazole (45.45 uM) indicating the characteristic of competitive inhibition. The Ki for metronidazole was 30 uM. This observation indicates that TNT and metronidazole possibly bind to the same site in the hydrogenase enzyme.

Cloning, purification and characterization of nitroreductases *nit*2 and nit9.

The nitroreductase genes (CAC 0718) and (CAC 3555) were amplified by PCR using primers *nit*2 : Forward - ATG AAT AAT ACA ATA GAT ACA ATG AAA AAT CAT AG; Reverse - TTT AGT TTT TAG TCC TTG TTT ATT AAT AGC GCC and *nit*9:Forward ATG ATA GAT TTA AAA ACT AGA AGA AGC ATA AG; Reverse: TTT AGA ATA TTT GTC GTA ATG AAG TTT ATT TAA AG . The PCR products were cloned into pTrcHis2 TOPO expression vector with a C-terminal His -tag (Invitrogen) producing pTrcHis2-*nit2* and pTrcHis2-*nit*9. TOP10 *E. coli* strain was transformed with the recombinant plasmids. Expression was induced with 1mM isopropyl-β-D-galactoside. Purification was carried out by probond nickel column (Invitrogen) according to the supplier's protocol.

SDS-PAGE electrophoresis analysis indicated that recombinant proteins *nit*2 and *nit*9 of molecular weights 31.17 and 23.25 kDa respectively were over expressed in *E coli* clones. Cell extracts of *E. coli* clones exhibit prominent bands that comigrated with pure nitroreductase protein. In contrast, lysates from control *E. coli* harbouring the plasmid pTrcHis2- TOPO lacking the DNA insert did not exhibit protein bands that comigrated with *nit*2 or *nit*9. In vitro activity assays conducted with cell extracts confirmed that the expressed proteins *nit*2 and *nit*9 had catalytic activity and exhibited higher specific activity than cell extracts from *E. coli* harbouring the control plasmid.

The nitroreductase enzymes could transform TNT in the presence of NADH as electron donor. The enzyme *nit*2 exhibits the greatest rates of NADH oxidation when TNT was provided as an electron acceptor, although 2,4 DNT and 2,6 DNT were also transformed. The catalytic efficiency was highest towards 2,4 DNT followed by TNT and 2,6 DNT. The purified *nit*2 from *E. coli* had K_m values of 8.51 μM for TNT, 3.75 μM for 2,4 DNT, 657 μM for 2,6 DNT. The K_m for NADH was 555. The V_{max} values for TNT, 2,4 DNT and 2,6 DNT were 44, 50 and 7.07 $\mu mol.min^{-1}.mg^{-1}$ of protein respectively. The *nit*2 enzyme had highest efficiency towards 2,4 DNT compared to that towards TNT and 2,6 DNT as determined by the V_{max}/K_m values.

The specific inhibition studies indicate that no metal cofactors are required and that sulphhydryl groups are present at the active site.

Database comparisons and sequence analysis. The deduced amino acid sequences of the nitroreductases *nit*2 was compared with the sequences in Gen bank using the NCBI BLASTP program. The flavoproteins identified included hypothetical oxidoreductase ycnD from Bacillus subtilis Accession P94424 showing 46% identity and 68% similarity, Nitro/flavin reductase from *Bacillus subtilis* Accession P39605 showing 40% identity and 58% similarity and Cr (VI) reductase Accession P96977 showing 33% identity and 51% similarity to nitroreductase *nit*2.
Putative NADH dehydrogenase/NAD(P)H nitroreductase from Archaeoglobus fulgidus Accession AF0226 showed showed 25% identity and 51% similarity to *nit*2 enzyme.
The *nit*2 and *nit*9 are oxygen insensitive NADH nitroreductases comprised of single nitroreductase domain. The members of this family utilize FMN as a cofactor and are often found to be homodimers.
RT-PCR analysis was performed with total RNA isolated from *C. acetobutylicum* grown with and without TNT. In negative controls, the reactions were performed in the absence of reverse transcriptase or RNA template. The level of transcript appeared to increase in the presence of TNT indicating the induction of the nitroreductase by TNT.

Mutagenicity of nitroaromatic Compounds during Anaerobic Transformation by *Clostridium acetobutylicum*

The mutagenicity of 2,4-dinitrotoluene (24DNT) and 2,6-dinitrotoluene (26DNT) and their related transformation products such as hydroxylamine and amine derivatives which are formed by *Clostridium acetobutylicum* were tested using *Salmonella typhimurium* TA100. A previous publication already reported the mutagenic activities of 2,4,6-trinitrotoluene (TNT) and its related hydroxylamine derivatives in this test system. A time course of the mutagenicity during the anaerobic transformation of TNT, 24DNT and 26DNT was also investigated in the same condition to compare with the results from the pure compounds. The monohydroxylamino intermediates 2-hydroxylamino-4-nitrotoluene (2HA4NT), 4-hydroxylamino-2-nitrotoluene (4HA2NT) and 2-hydroxylamino-6-nitrotoluene (2HA6NT) formed during anaerobic transformation of dinitrotolunes were proved to be mutagenic in the Ames test using *Salmonella typhimurium* TA100. 24DNT and 26DNT and their final metabolites 2,4-diaminotoluene (24DAT) and 2,6-aminotoluene (26DAT) appeared non-mutagenic. In a time course study of TNT degradation, the temporal sample containing 85% of 2,4-dihydroxylamino-6-nitrotoluene (24HA6NT) is most mutagenic.