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BIOREMEDIATION OF TRINITROTOLULENE BY A RUMINAL MICROORGANISM

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INTRODUCTION

2,4,6-trinitrotoluene (TNT) has been widely used for the production of explosives because of its low boiling point, high stability, low impact sensitivity, and safe manufacture. TNT is known to produce adverse health effects from occupational exposure including increased incidences of aplastic anemia, liver damage, dermatitis, ocular disorders, and gastrointestinal distress. In addition, TNT is of ecological concern based on its toxicity to certain aquatic organisms. More than 1,100 military facilities, each potentially contaminated with munitions waste, are expected to require treatment of more than one million cubic yards of contaminated soils. The cost associated with remediation of these sites has been estimated to be in excess of $1.5 billion.

Recently, researchers have studied ruminal microorganisms in relation to their ability to degrade xenobiotic compounds. Many of these organisms are strict anaerobes with optimal redox potentials as low as -420 mV . Ruminal organisms have been shown capable of destroying some pesticides, such as parathion, p-nitrophenol, and biphenyl-type compounds; thiono isomers, (8, 33); and nitrogen-containing heterocyclic plant toxins such as the pyrrolizidine alkaloids (5). Many of these compounds have structures similar to TNT.

A TNT-degrading ruminal microorganism has been isolated from goat rumen fluid with successive enrichments on triaminotoluene (TAT) and TNT. The isolate, designated *G.8*, utilizes nitrate and lactate as the primary energy source. *G.8* was able to tolerate and metabolite levels of TNT up to the saturation point of 125 mg/l.

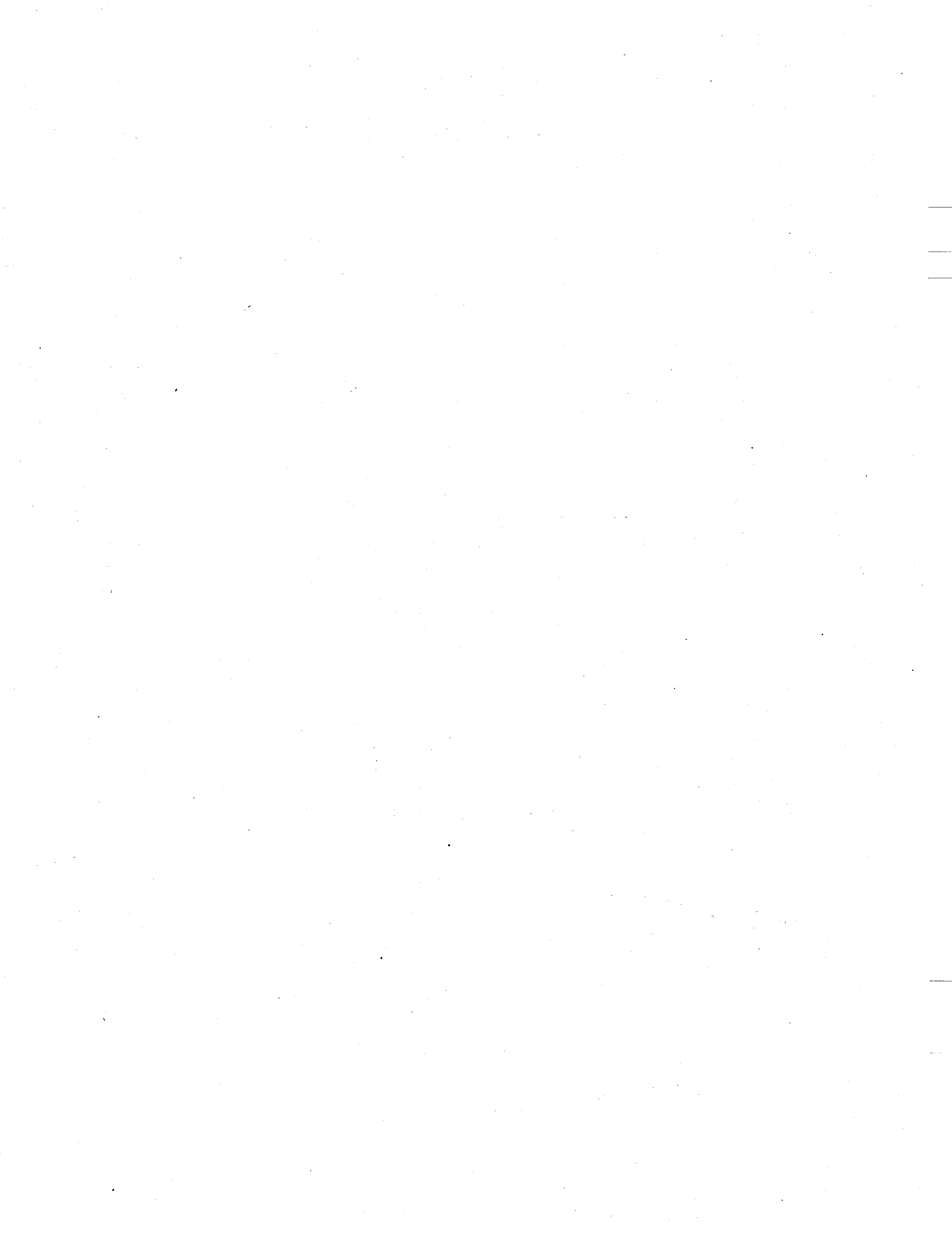
Objectives

Based upon the results of previous studies, the present research was directed at understanding the process of TNT degradation by the denitrifying ruminal microorganism *G.8* (5). The objectives of this study were: 1) to identify specific metabolites and define TNT destruction pathways and 2) to understand TNT and the metabolites transformation mechanisms on the different primary electron acceptors.

METHODOLOGY

Growth Medium

The media used to incubate the isolate in serum bottles consisted of (mg/l) $CH_3CHOHCOONa$ (500), $MnSO_4.H_2O$ (8.5) $FeSO_4.7H_2O$ (10), KNO_3 (2000), $ZnSO_4.7H_2O$ (5), $CaCl_2.2H_20$ (24), $Na_2HPO_4.H_2O$ (2550), H_3BO_3 (1.5), $NaMo_4.2H_2O$ (1.5), $NaH_2PO_4.H_2O$ (975), $CoCl_2.6H_2O$ (0.6),



Na_2EDTA (21.5), yeast extract (100), $CuCl_2.2H_2O$ (0.05), $MgSO_4.7H_2O$ (30), and $NiCl_2.6H_2O$ (1). The nutrients were mixed and well-stirred. pH was adjusted to 7.0 with 0.1 N NaOH, then boiled for 3 minutes under argon gas flow and dispensed into serum bottles, which were then stoppered and autoclaved for 25 minutes. To prevent the precipitation of salts from the medium, 0.3 ml of 800 ppm $CaCl_2 \cdot 2H_2O$ was injected following autoclaving. An 0.2 % inoculum was used for all experiments. Serum bottles were cultivated anaerobically at 37°C for given periods of time using a mechanical shaker. Ethanol was used to prevent contamination at the contact points for the inoculant and samples, and sample aliquots were indexed and frozen at -14°C.

Analytical Methods

TNT, 4-amino,2,6-dinitrotoluene (4A26DNT), 2,6-dinitrotoluene (26DNT), 2-amino,6-nitrotoluene (2A6NT), and 2-nitrotoluene (2NT) were measured by high performance liquid chromatography (HPLC),. Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett-Packard Model 5988A connected to a Model 5890 gas chromatography (GC) with a XTI-5 fused-silica capillary column. A Dionex 4000i ion chromatography (IC) with a HPIC-CS3 anion column was used to measure anions such as nitrate and nitrite. Culture turbidity in the serum tubes was measured using a spectrophotometer at 660 ηm absorbance (Bausch and Lomb, Spectronic 20D) for cell-growth analysis.

Microorganism

A TNT degrader was isolated from rumen fluid with successive enrichment of TNT and TAT.. The isolated microorganisms from a fistulated goat was named *G.8*. The gram negative *G.8* was identified based fatty acid analysis as a *Escherichia Coli* with similarity index (SI) of 91.4 percents.

RESULTS

G.8 growth was monitored by optical density using different energy sources including nitrates, nitrites, or TNT to investigate the relation of TNT to *G.8* metabolism. *G.8* growth occurred in the presence of only nitrate, but not on nitrite, or TNT, indicating that nitrate serves as a viable terminal electron acceptor. Fermentation was not observed with the presence of lactate only in the medium. *G.8* growth was detected in the absence of lactate (TNT and nitrate present), suggesting that TNT could have been potentially used as the carbon source and electron donor. *G.8* growth was observed in the medium without lactate (only nitrate present) as much as in the medium containing TNT and lactate, probably from the use of the yeast extract as the electron donor and carbon source.

Additional experiments were conducted to investigate the influence of TNT on the utilization of primary energy source with *G.8* growth. Nitrate was converted to nitrite completely within 10 hours, followed by further reduction of the nitrite. Nitrate was converted to nitrite and then less transformation of the nitrite in the medium was observed when TNT is present.

In the preliminary experiments,*G.8* growth was not observed with nitrite or TNT as the primary electron acceptor, indicating *G.8* does not initially utilize the nitrite or TNT. When TNT was absent from the medium, further transformation of the nitrite from nitrate was observed and less transformation of the nitrite with presence of TNT in the medium. Thiese results demonstrate that nitrite or TNT conversions takes place in the presence of nitrate. It suppose that the nitrite and TNT appear to compete as cometabolic electron acceptors, with the TNT being preferrentially used.

G.8 Experiments to Determine Metabolic Pathways

Based on evidence of co-metabolic TNT transformation, we decided to attempt to identify the TNT biotransformation pathways and limiting factors. TNT and each of its transformation

products were incubated individually with a *G.8* medium containing lactate as the electron donor and nitrate or nitrite as the primary electron acceptor. A medium containing lactate and TNT metabolites was also prepared.

TNT Transformation

G.8 was incubated for three days in serum bottles containing 20 mg/l of TNT. From Bottle A (containing lactate and nitrate as primary energy sources), the metabolites were identified as 4A26DNT. The complete TNT transformation in Bottle A and the non-transformations of TNT in Bottles B (containing lactate and nitrite as primary energy sources) and C (containing only lactate) showed that TNT is co-metabolically degraded in the presence of nitrate, and not degraded in the presence of nitrite.

4A26DNT Transformation

G.8 was incubated for eight days in serum bottles containing 20 mg/l of 4A26DNT. In Bottle B, 4A26DNT was reoxidized to TNT and deaminated to 26DNT. In Bottle A, only negligible amounts of TNT and 26DNT were detected. No 4A26DNT transformation occurred in Bottle C. Nitrites in the medium appeared to stimulate the *G.8* reduction and oxidation systems.

26DNT Transformation

In the same manner described for the 4A26DNT incubations, 26DNT was incubated for 20 days with *G.8* in Bottles A, B, and C. The transformation of 26DNT to 2A6NT was not detected or was negligible in Bottles A and B. The reduction of the 26DNT ortho-position nitro group was detected in bottle C, suggesting that *G.8* utilized 26DNT as a primary electron acceptor.

2A6NT Transformation

During 18 days of incubation, 2A6NT in Bottle B was transformed to 2NT and 26DNT. Oxidation and reduction of 2A6NT trends were identical to those for the 4A26DNT incubation. The absence of a primary electron acceptor in Bottle C did not stimulate 2A6NT transformation, and the oxidation of 2A6NT was faster than the reduction processes.

2NT and 2AT Transformations

During 18 days of incubation, 2NT metabolites were not detected from either HPLC or GC/MS analysis, but it was noteworthy that 2-aminotoluene (2AT) completely disappeared from Bottles A and B, suggesting that 2AT transformed more rapidly than 2NT. It is reasonable to suppose that the 2AT transformation products converted rapidly to other metabolites, but that the transformation of 2NT to 2AT was too slow. The 2AT transformation products were identified as o-cresol, toluene, and small amounts of 2NT. These trends for amino group transformation were identical to those for 4A26DNT and 2A6NT.

TNT transformation pathways were established with series connections of each transformation products presented above. Proposed pathways for the TNT degradation are shown in Figure 1. The TNT biotransformation steps shown are the reduction process, wherein the nitro group is reduced to an amino group, followed by deaminization and oxidation when the amino group was present. As observed previously, para-nitro group reduction was the most susceptible transformation .

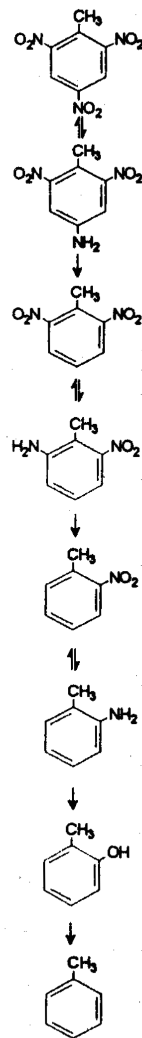


Figure 1. **Proposed pathways for TNT biotransformation with *G.8* incubation. The pathways are established with series connection of the identified transformation products by GC/MS and HPLC.**

CONCLUSIONS

The *G.8* isolate was capable of reducing the TNT nitro group to an amino group and was also involved in deaminization as a co-metabolite, resulting in nitrogen free compounds such as toluene or o-cresol.

The reduction (deaminization) and oxidation (hydroxylation) reactions took place simultaneously when the amino group was present. From previous studies, it had been reported that oxidation and reduction processes occurred jointly subject to anaerobic conditions. Vogel et al. (4) demonstrated that *Cytochrome* P450 could mediate both the oxidation and reduction reactions for degradation of halogenated aliphatic compounds. For *Pseudomonas* sp. strain PN-1, it was also found that oxidation steps proceeded under conditions of denitrification (1). CO_2 and chloroform formations from carbon tetrachloride in the culture of *Escherichia Coli* K-12 was found by Criddle et al. (1). These mechanisms are coincident with the present mechanisms of *G.8*.

Degradation of compounds is dependent upon the characteristics of the parent compounds, the microbial consortium, and environment factors (3). In the experiments described, the patterns of TNT metabolite transformation were dependent on the type of electron acceptors. The presence of nitrates in the medium stimulated the reduction of para-positioned nitro groups, and nitrites stimulated the deaminization and hydroxylation processes. The absence of such primary energy sources as nitrates or nitrites stimulated the reduction of the ortho-positioned nitro groups.

It is assumed that the incomplete TNT transformations are related to inadequate combinations of the terminal electron acceptors. Nitrate consumption in the medium was complete while TNT was transformed to 4A26DNT and 26DNT. For certain periods of time, nitrite accumulated in the medium does not support *G.8* metabolism and subject to termination of 26DNT reduction process, as was indicated at 26DNT transformations with presence of nitrite in the medium.

From these findings, it is concluded that an appropriately designed sequencing reactor system, or other alternatives to control such energy source as nitrates, or nitrites in the TNT degradation system, could result in obtaining the full transformation of TNT to o-cresol or toluene.

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