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13. ABSTRACT *(Maximum 200 words)*

This work has contributed to the development of processes for bioremediation of explosives-contaminated soils and waters. We examined the role of microbial consortia and pure strains of *Clostridium* spp. in the biodegradation of TNT and other nitroaromatic contaminants. *C. bifermentans,* an anaerobe isolated from an enrichment of munitions-contaminated soil, degraded TNT co-metabolically. We identified reductive TNT transformations that produced triaminotoluene (TAT) and phenolic products of TAT hydrolysis. Since clostridia are common in soils, the addition of fermentable sugars to TNT-contaminated soils should stimulate the reactions we observed. Examination of the ability of clostridial strains isolated from a munition enrichment, non-adapted clostridia, and other bacterial strains to degrade TNT indicated that the ability to reduce TNT anaerobically is a general phenomenon and that the TNT degradative pathways are not inducible, but are associated with constitutively expressed metabolic functions of *Clostridium* spp. We showed that bulk production of clostridial spores is clearly achievable for use in bioaugmentation or bioremediation systems. Overall, our work with anaerobic consortia and with pure cultures derived from the consortia indicates a complex TNT biodegradation process in soil, which involves multiple organisms acting synergistically and probably sequentially.

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Physiology, Biochemistry, and Genetics of a Pure Culture of an Obligatory Anaerobic Bacterium That Utilizes 2,4,6-Trinitrotoluene (TNT)

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Introduction

2,4,6-trinitrotoluene (TNT), whose annual production is estimated at one thousand tons, is the primary explosive used in munitions manufacture. Manufacturing and decommissioning operations have generated and continue to generate large quantities of TNT as a waste product. Much of this waste has been deposited in soil and groundwater through leaching and from disposal in unlined lagoons. The management of munitions waste and the remediation of contaminated sites is critical to public health, since TNT is both mutagenic and acutely toxic. As public concerns mandate the proper disposal and cleanup of hazardous materials, the destruction of TNT from contaminated media has become an important industrial process that must be evaluated in terms of end-product acceptability as well as cost.

The biological destruction of hazardous organic compounds is often suggested for waste management and remediation processes since it offers the potential for effective removal of the target compound with relatively inexpensive technology. The amenability of a toxic compound to biological remediation depends upon the existence of metabolic activities which can render it non-toxic. The continued presence of TNT in soils contaminated during World War II shows that it can persist in the environment for long periods. Its persistence is not caused by a lack of reactivity in biological systems, for TNT would not be toxic if this were so. The nitro group, due to its electrophilic character, readily oxidizes biological reductants, causing toxicity directly or by formation of other reactive products such as nitroarene radicals, and retarding further transformation at high concentrations. Since nitro-substituted compounds are relatively rare in nature, the ability to metabolize them productively is probably correspondingly rare. Microbial activities have been described which lead to the removal of nitro substituents from the aromatic rings of other nitroaromatic compounds and allow metabolism of the remaining carbon skeleton through more conventional pathways. These activities can be classified into the following general categories:

1. ring oxygenation followed by release of nitrite
2. nucleophilic attack by a hydride ion to form a hydride-Meisenheimer complex, which may be followed by release of nitrite
3. reduction to form a hydroxylamine that is further metabolized.

The first type of activity has been described for the metabolism of mono- and dinitrotoluenes, however, oxygenolytic transformations have not been described for TNT. The increased degree of nitro substitution apparently renders the aromatic ring electron-deficient to the point that it no longer acts as a substrate for the electrophilic oxygenation mechanism. The initial steps of the other types of transformation, i.e., hydride-complex formation and, do seem to operate to various extents in examples of TNT transformation found in the literature. However, cleavage of the aromatic nucleus usually has not been demonstrated, or it occurs at low efficiency. For substantial mineralization by a single bacterial strain, it has required recruitment of genes from another organism. Apparently the unique substitution pattern of TNT is such that the occurrence of all genes required for productive metabolism (i.e., mineralization with energy conservation and carbon assimilation) is rare in a single organism, or else special conditions are required to allow their expression in one organism or consortium of organisms.

Under this USAFOSR grant we have examined the role of microbial consortia and several pure strains of species of *Clostridium* in the biological destruction of TNT and

other nitroaromatic environmental contaminants. Our progress has been reported in a number of publications in the peer-reviewed literature. In the following summary report, we discuss the general aspects of this progress.

C. bifermentans: An Obligatory Anaerobic Bacterium That Degrades TNT and RDX

Clostridium bifermentans is an anaerobe isolated from a four-liter bioreactor enriched from municipal waste and munitions-contaminated soil (Funk *et al.,* 1993a; Regan and Crawford, 1994; Funk *et al.,* 1993b; Shin and Crawford, 1995). This organism was capable of transforming 50 mg/liter TNT to the transient intermediates 4A26DNT and 24DA6NT in the presence of yeast extract or tripticase soy and a fermentable co-substrate. *C. bifermentans* was not able to grow on TNT as a sole source of carbon or nitrogen, but degraded it co-metabolically. No other aromatic intermediates were detectable after the disappearance of 24DA6NT. The benzene ring may have been cleaved, leading to the formation of volatile organic acids, a conclusion that is not yet confirmed but is supported by indirect evidence (Shin and Crawford, 1995).

Products of Anaerobic TNT Transformation by C. bifermentans

Our experiments on the TNT-transforming activity of *Clostridium bifermentans* strain LJP-1 identified reductive TNT transformations that ultimately produced as end products triaminotoluene (TAT) and phenolic products of TAT hydrolysis. An adduct of TAT, apparently formed by condensation of TAT and pyruvic aldehyde (methyl glyoxal), was also detected (**Figure 1**). Our studies with cell suspensions and growing cultures of clostridia allow us to speculate on the fate of TNT in environments where clostridial activities prevail, and on their role in our munition-fed bioreactor. Since clostridia are common in soils, the addition of fermentable sugars to soils contaminated with TNT would be expected to stimulate the types of reactions observed in our experiments, which would lead primarily to the formation of TAT with some phenolic and Schiff base derivatives. The likely fate of TAT has been proposed by others to include polymerization, due to auto-oxidation, and sorption to soil particles. The secondary products described here may not differ greatly from TAT in these respects, since we noticed a rapid darkening of purified material upon exposure to air, indicating their susceptibility to oxidative polymerization (unpublished observation). The sorptive character imparted by amine substitution also remains in these species. In consortia, however, TAT, the more oxidized species that precede it, and the material derived from it, might be amenable to aerobic mineralization, a possibility supported by studies by others showing TNT mineralization in soils in which nitro group reduction products were the only intermediates identified. Thus, in obligately anaerobic mixed cultures fed fermentable substrates, clostridia probably play an important role in the complete or near complete reduction of TNT. Other microbial groups, however, appear to influence the ultimate transformation of reduced TNT metabolites to their final products.

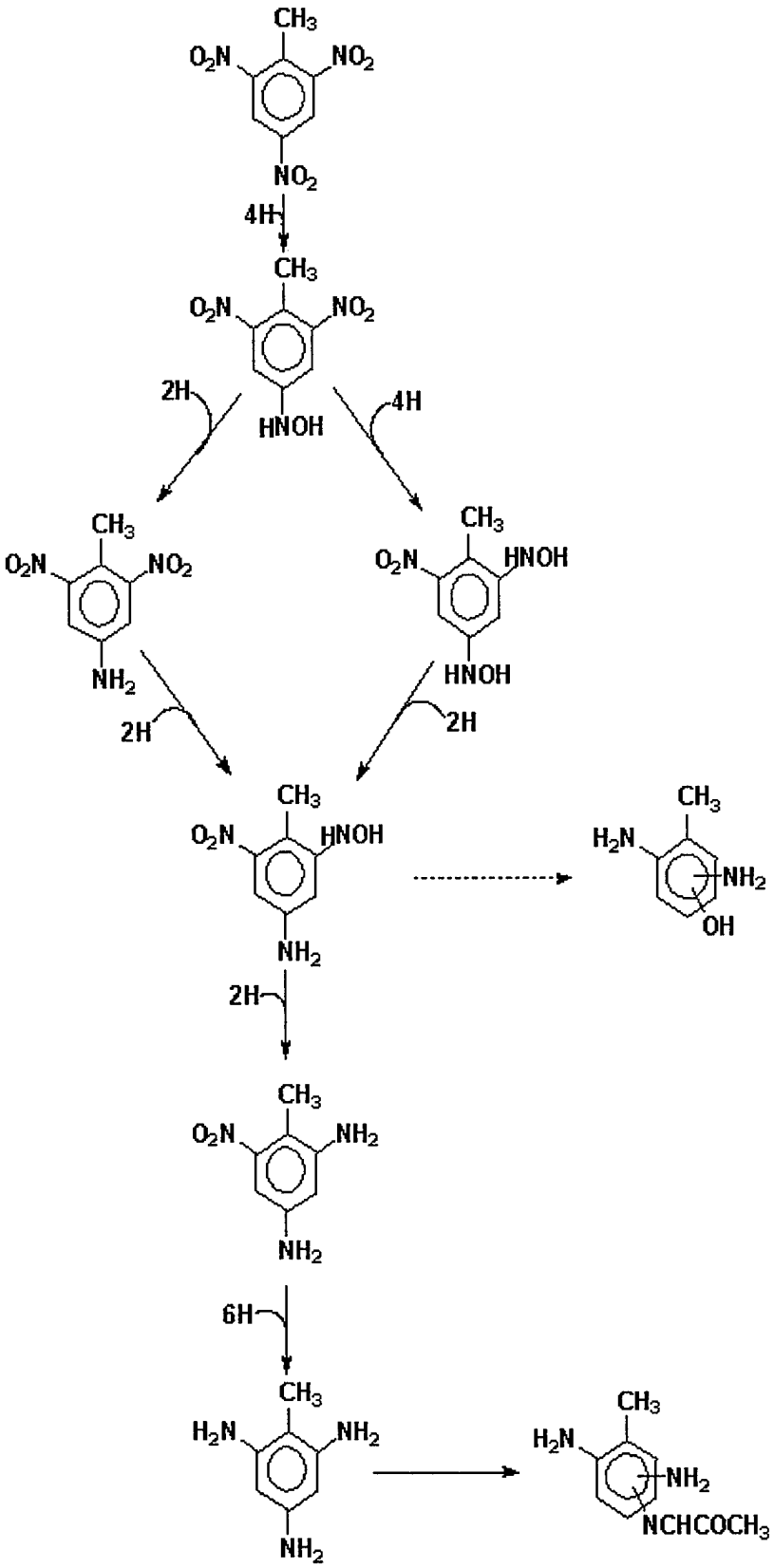

FIGURE 1: TRANSFORMATIONS OF TNT BY *CLOSTRIDIUM BIFERMENTANS*

TNT Transformation by Adapted and Non-Adapted Clostridia

We examined the ability of different clostridial strains isolated from a four-year-old munition enrichment, related clostridial strains obtained from a culture collection, two enteric bacteria, and three lactobacilli to degrade the nitroaromatic explosive 2,4,6-trinitrotoluene (TNT). All *Clostridium* species tested were able to rapidly reduce TNT in a complex medium. These were also able to reduce 2,4-diamino-6-nitrotoluene (DANT) to 2,4-diamino-6-hydroxylaminotoluene (DAHAT) and to produce an as yet unidentified compound in cell suspension experiments and thus could not be distinguished from one another with regard to the pathway of transformation. The enteric strains and the lactobacilli were able to perform the initial reduction of TNT, but none were capable of reducing DANT in cell suspensions. These characterization experiments have shown that the clostridia isolated from a long-term munition-fed bioreactor do not show increased resistance to TNT and display constitutive transformation activities. Our observations are consistent with previous studies indicating that the ability to reduce TNT anaerobically is a general phenomenon and that the TNT degradative pathways are not encoded by inducible genes, but could be associated with constitutively expressed metabolic functions of different *Clostridium* spp. Our experience shows that strains of *C. bifermentans* the were most easily isolated from long-term enrichments performed in the presence of TNT, probably because they are fast growers relative to other clostridia. Another explanation might be that the ability of the clostridia to sporulate aided their survival in our bioreactor and permitted prolonged exposure to the nitroaromatic compounds present. In our experiments, even the microaerophilic, ferredoxin-free lactobacilli were able to transform TNT to a substantial degree. Our observations confirm that non-ferredoxin-dependent mechanisms for anaerobic nitroaromatic degradation do operate in bacterial cultures.

In addition to chemical reduction of TNT nitro groups by biochemical cofactors such as NADH, enzymatic nitroreductase activity has been described by others. In our TNT degradation study, *L. casei* was found to be able to remove TNT, ADNT, and DANT, whereas in basal medium (M9) cell suspension experiments the organism did not exhibit any activity on DANT, indicating that different types of degradation activities are possible in rich medium. The type of activity responsible for the removal of DANT by *L. casei* cultures is likely not reductive, since reduction products were not detected, and supports the notion that low-potential biochemical reductants present in strictly anaerobic bacteria such as ferredoxin, ferredoxin-reducing proteins, or dissimilatory sulfite reductase, are necessary for reduction of the nitro group of DANT. Another indication for the existence of alternate, possibly non reductive, degradation pathways are implied by the results that amounts of ADNT and DANT observed did not account for all the TNT transformed by the lactobacilli and the facultative organisms. Radiolabeling experiments by others using activated sewage sludge have shown transformation to a polyamide-like material. This type of transformation or other polymerizations to form insoluble material would have escaped our analytical techniques.

Production of the novel, unidentified product detected in the cell suspension experiments using a bioreactor-derived *Clostridium* sp. is not a result of a biochemical activity selected for during prolonged exposure to munitions compounds. Rather, it appears to be an activity common among clostridia (and possibly other obligate anaerobic bacteria not yet analyzed). According to the evolutionary tree constructed by Lawson *et al.* using the 16S rRNA sequences, *C. bifermentans* and *C. sordellii* are very closely

related, and *C. sporogenes* and *C. acetobutylicum* are more closely related to each other than to either of the first species. Therefore, the ability to transform TNT (and accumulation of the unidentified compound) is not only a feature of a small group of clostridia, but seems to be rather widely distributed in this bacterial genus.

Production of C. bifermentans *Spores as Inoculum for Bioremediation of Nitroaromatic Contaminants*

Spores of *Clostridium bifermentans* strain KMR-1 were produced for use as a microbial inoculum for bioremediation, preserved in both liquid and dry form. All spore formulations showed good viability and ability to biodegrade the target compound 2,4,6-trinitrotoluene (TNT) after 4 months of storage. For low-cost bulk spore production, several media compositions were tested, based on soy peptone, corn steep liquor, and meat peptone, yielding 10^7 spores per ml. A medium pH above 7.0, low glucose concentration, and sufficient protein concentration favored the sporulation of *C. bifermentans* KMR-1. Examinations of these various media encouraged the use of several of the complex media bulk sources, since these provided an acceptable spore yield of $\approx 10^7$ spores per ml. Acceptable constituents included corn steep liquor ($0.06/kg), soy peptone ($9.50/kg), and meat peptone ($21.00/kg). Casamino acids ($32.00/kg) were judged unacceptable because of cost. Such particulate-free media compositions are expected to be compatible with the ultrafiltration unit (downstream processing of spores), and would allow production of high spore densities after concentration by hollow fiber units.

Though acceptable spore yields and spore storability at reasonable cost were achieved here, continued work on optimization and scale-up of this process could yield even greater economy. Spore quality is very much dependent on the quality of sporulation medium, and further improvements are possible. Bulk spore production processes are clearly achievable for use in bioaugmentation of bioremediation systems.

Consortia Versus Pure Cultures

In determining pathways, pure cultures are easier to work with than are consortia, since consortia make it difficult to determine which organism is responsible for individual steps in the transformation process. Furthermore, consortia are usually found in environmental samples and may require unknown factors and minerals present in sediment in order for individual strains to coexist. If such factors are absent, a single strain that may not perform the complete transformation could dominate a culture. However, we have found that an anaerobic consortium kept under a selective pressure of TNT in a minimal medium was able to thrive on TNT in a bench-top bioreactor without the addition of a carbon co-substrate (Funk *et al.,* 1993b). When pure strains from this consortium were cultured, none of the isolates could grow in the same minimal medium with TNT as the only carbon source. Furthermore, the consortium was able to tolerate >100 mg/liter TNT while the isolates alone became somewhat inhibited at concentrations >50 mg/liter TNT.

We have followed the pathway of TNT degradation in the consortium using reverse phase high performance liquid chromatography (HPLC) and mass spectroscopy (MS). Analysis of the supernatant in the consortium showed sequential reduction of TNT to TAT; however, TAT only occasionally accumulated, and even then only in very low concentrations. In addition, methylphloroglucinol (MPG) and *p*-cresol transiently

appeared in the supernatant. All compounds were identified using HPLC by UV spectra and retention times as compared to authentic standards chromatographed under the same conditions. The mono- and diamino intermediates were also confirmed by the MS results.

Regan and Crawford (1994) examined a strain of *C. bifermentans* (KMR-1) isolated from our anaerobic bioreactor, which was capable of transforming RDX and TNT in a rich medium. TNT was selectively removed by KMR-1 prior to RDX degradation.

C. bifermentans strain CYS-1 was also isolated from our anaerobic bioreactor. Shin and Crawford (1995) examined the ability of CYS-1 to degrade TNT cometabolically in various defined media. This strain could overcome the toxicity of and degrade >150 ppm TNT in liquid media supplemented with a rich cosubstrate such as yeast extract or trypticase soy, given an appropriate inoculum ($\approx 10^7$ CFU/ml). Furthermore, it was found that CYS-1 could degrade TNT which contaminated a sandy loam soil. The degradation of TNT proceeded through the transient intermediates 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene.

Overall, the results obtained from research with anaerobic consortia, with pure cultures derived from the consortia, and with other pure bacterial cultures are indicative of a complex TNT biodegradation process in soil that involves multiple organisms acting synergistically and probably sequentially.

Enzymology

Funk *et al.* (1992) showed the reduction of TNT to 4-amino-2,6-dinitrotoluene (4A26DNT) and then to 2,4-diamino-6-nitrotoluene by a mixed anaerobic consortium in a medium that contained TNT and RDX. It was shown that the RDX concentration remained constant until the all of the TNT was converted to the diamino compound. Hence, RDX may require a lower redox potential for reduction than TNT and 4A26DNT. Similar results were observed by Regan and Crawford (1994).

The hypothesis stating that these various nitro-substituted compounds are reduced using reducing power stored in proteins such as ferrodoxins needs to be confirmed. Additional tests still need to be done to confirm which proteins are involved in the reduction of nitroaromatic compounds. Possibilities are oxidoreductase, ferrodoxin, flavodoxin, rubiboxin, and hydrogenase, to name just a few. These proteins may also require cofactors such as iron, FMN, FADH, and NAD(P)H that allow them to function.

Conclusions

Extensive research in our laboratory has contributed to the recent development of treatment processes for the bioremediation of soils and waters contaminated with nitro-substituted explosives. By elucidating the degradative pathways in both aerobic and anaerobic systems, we can determine the fate of the parent molecule and assess its effects on the environment. Further research into treating soil contaminated with various nitroaromatics is essential, since their incineration is not always a viable option, due to high cost and risk of pollution.

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