Novel Pathways of Nitroaromatic Metabolism: Hydroxylamine Formation, Reactivity and Potential for Ring Fission for Destruction of TNT - CU1214

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List of Acronyms

2HA4ANT	2-Hydroxylamino-4-amino-6-nitrotoluene
2ADNT	2-Amino-4,6-dinitrotoluene
4HADNT	4-Hydroxylamino-2,6-dinitrotoluene
BLK	Bruhn, Lenke, and Knackmuss minimal medium
DHANT	2,4-Dihydroxylamino-6-nitrotoluene
DHANT	2,4-Dihydroxylamino-6-nitrotoluene
DNT	Dinitrotoluene
2,4-DNT	2,4-Dinitrotoluene
DoD	Department of Defense
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB	Luria-Bertani Broth
NB	Nitrobenzene
NOM	Natural Organic Matter
SPE	Solid Phase Extraction
TAT	2,4,6-Triaminotoluene
TNT	2,4,6-Trinitrotoluene
UV	Ultraviolet
YM	Yellow Metabolite

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Acknowledgements

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Executive Summary

Contamination by 2,4,6-trinitrotoluene (TNT) is widespread at many sites where explosives have been manufactured and stored. TNT contamination occurs in the environment predominantly in surface and shallow subsurface soils. The presence of this contamination in easily accessible surficial soils makes it amenable to treatment by either *in situ* or ex situ engineered systems. Bioremediation has come into favor as the treatment of choice for munitions contamination because of the prohibitively high cost of the treatment alternative, incineration. To this end, this research was designed to provide information required for development of bioremediation systems to treat TNT contamination. This research investigated biological transformation of TNT with the primary goal of furthering the understanding of the fundamental biochemical mechanisms responsible for transformation of TNT and its fate in the environment. This research explored the products of novel TNT transformation pathways and determined the mechanisms of TNT transformation and identified the enzymes responsible. The fate of TNT transformation products in natural systems was investigated along with strategies to direct TNT metabolism to ring fission products and mineralization of TNT. Studies were primarily focused on transformation of TNT by the anaerobic organism *Clostridia acetobutylicum* and the aerobic organism *Pseudomonas pseudoalcaligenes* strain JS45.

The reactivity of partially reduced metabolites of 2,4,6-trinitrotoluene (TNT) produced by *C. acetobutylicum*, namely arylhydroxylamines and nitrosoarenes, was evaluated with a simple biological system and with components of soil natural organic matter (NOM). In the simple bioreduction system of *Clostridium acetobutylicum* cell-

free extract/molecular hydrogen (electron donor), 10% of the initial ^{14}C was found bound to solid proteinaceous material following sequential anaerobic/aerobic treatment. A review of the nitroso and hydroxylamino functional group chemistry revealed that the nitroso-thiol reaction was most likely responsible for the reaction with proteins. The introduction of a model thiol, 1-thioglycerol, into an anaerobic mixture of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 2,4-dihydroxylamino-6-nitrotoluene (DHANT) resulted in the formation of a new product, only when the reaction mixture was exposed to air. The reactivity of arylhydroxylamines and nitrosoarenes with standard humic acids was investigated using 4HADNT and nitrosobenzene as model compounds, respectively. Contrary to results reported by others, 4HADNT was found to be nonreactive towards humic acid at humic acid concentrations in excess of dissolved organic matter concentrations found in nature. Conversely, nitrosobenzene reacted rapidly with humic acids, with the extent of reaction being highest for humic acids that had a high protein content. Humic acids that were pretreated with a thiol derivatizing agent showed diminished capacity for reaction with nitrosobenzene. Since nitroso intermediates from TNT reduction are difficult to synthesize and are rarely observed in nature due to their high instability, their electrophilic characteristics were evaluated using a molecular modeling approach. Molecular models of potential TNT nitroso intermediates were compared with those of the strongly electrophilic nitrosobenzene. The comparison revealed that 2-nitroso-4-hydroxylamino-6-nitrotoluene was more likely to react similarly to nitrosobenzene than to 4-nitroso-2,6-dinitrotoluene.

Studies which investigated the enzymatic mechanism responsible for transformation of TNT by *C. acetobutylicum* were focused on determination of whether

the Fe-only hydrogenase is the primary enzyme in *C. acetobutylicum* responsible for reduction of TNT. Using puified enzyme, it was determined that the hydrogenase is capable of reducing TNT in an H_2 atmosphere in the absence of alternate electron donors, which subsequently allowed the kinetic parameters to be measured. Further studies were carried out to examine a causative relationship between the activity of hydrogenase present in a cell system and the corresponding rates of TNT reductase activity. The results of the studies demonstrated that the hydrogenase enzyme proposed is responsible for the major nitroreductive capability of *C. acetobutylicum*.

Further studies with C. acetobutylicum investigated the potential for mineralization of TNT using a two-stage anaerobic-aerobic incubation. We report that CO_2 production (confirmed using ^{14}C-TNT) occurred during a two-stage microbial incubation as a result of a combined biological-chemical process. ^{14}C-TNT was transformed by actively growing *C. acetobutylicum* and subsequently exposed to active aerobic cultures known to metabolically degrade 2,4-DNT. Aerobically-maintained reactors with unrestricted growth converted 13.3 to 14.1% of the initial radiolabeled metabolite to $^{14}CO_2$. Active reactors produced significantly more $^{14}CO_2$ than aerobic assays in which growth was restricted (via autoclaving, mineral omission, or biocide amendments). Production of $^{14}CO_2$ in these restricted-growth reactors ranged from 7.9 to 9.7%, suggesting that an abiotic pathway was responsible for greater than 56% of the observed mineralization yield. It was determined that incubation under anaerobic conditions in the second stage of the dual-step process limited mineralization (1.1 to 1.7% $^{14}CO_2$) by blocking growth and/or auto-oxidation pathways, while the addition of structurally similar compounds to TNT did not lead to mineralization. It is postulated

that the clostridial pathway that yields hydroxylated intermediates is key to providing more suitable starting compounds for initiation of the mineralization process.

We also investigated the mechanism and enzymes responsible for transformation of TNT by *P. pseudoalicaligenes* JS45, a soil organism isolated for its ability to grow on nitrobenzene under aerobic conditions. Cells grown on nitrobenzene were previously reported to transform TNT by partial reduction of the nitro groups catalyzed by nitrobenzene nitroreductase. The nitroreductase produces only hydroxylamino derivatives of TNT and the final product is an unidentified yellow metabolite. Here we discovered that *P. pseudoalcaligenes* JS45 contains multiple nitroreductases, including a unique constitutively expressed nitroreductase that converts TNT to 2HADNT. A wide range of bacteria, including *E.* coli, can catalyze the conversion of TNT to amino derivatives. All of the nitroreductases that have been purified, however, seem to catalyze only the reduction to the hydroxylamino derivatives. It has been a mystery how the conversion of the hydroxylamino compounds to the amines is carried out. In JS45 we have clearly demonstrated the activity of the enzyme that produces amino compounds from TNT. The existence of the enzyme is crucial to avoid accumulation of the much more reactive hydroxylamino compounds. Purification and characterization of the novel enzyme will reveal whether it is widespread and whether its activity is responsible for the often observed accumulation of monoamino derivatives of TNT under aerobic conditions. The advances in understanding from JS45 will allow prediction and enhancement of the activity in a variety of systems.

2ADNT is further transformed by the action of the same reductase, but the product of the reaction has not been isolated. The currently available evidence indicates that 2-amino-4-hydroxylaminonitrotoluene is formed and subsequently converted by the mutase enzyme to the corresponding aminophenol which accumulates transiently and then disappears. When the experiments were carried out with ^{14}C-TNT the products eventually became unextractable and bound to soil when it was included in the reaction mixture.

Enzymes of the nitrobenzene degradation pathway that effect transformation of TNT and its metabolites were also identified, and experiments with purified enzymes demonstrated that a mutase enzyme converts DHANT to the same yellow metabolite that is produced by cells grown on succinate. Experiments with ^{14}C-TNT demonstrated that JS45 can mineralize TNT when the nitrobenzene degradation pathway is expressed, but mineralization is suppressed by the presence of soil because of binding of polar metabolites of TNT or their precursors by soil components.

Nitrobenzene is more acutely toxic than TNT and thus is unlikely to be used as a primary growth substrate in TNT remediation systems. However, this project has shown that constitutive enzymes, including the nonspecific nitroreductase(s) and the constitutively expressed HabA will transform TNT almost as well as the fully expressed enzymes of the nitrobenzene-degradation pathway. *P. pseudoalcaligenes* JS45 is a natural environmental isolate and therefore faces few regulatory restrictions on its use in remediation systems. With the idea that the constitutively expressed enzymes of JS45 could be useful in TNT remediation systems *in situ*, we examined alternative carbon sources for cometabolism of TNT by JS45.

Tests with glucose, succinate, and molasses revealed that the best growth and TNT transformation resulted from molasses as carbon source. The molasses-grown cells took less than 24 hours to transform 100 μM TNT. Molasses is a cheap and readily available carbon source with demonstrated value for cometabolism. The difference between the system described here and previously described systems using molasses as electron donor is that the final product is not a polyamino compound due to the action of the mutase enzyme in JS45. Remediation systems based on cometabolism of TNT by JS45 during growth on molasses could be investigated for field application where the bulking of the contaminated material inherent in composting is to be avoided.

Objective

The goals of the studies were to examine the biochemical mechanism of TNT transformation in novel degradation pathways, and to use this fundamental information to develop strategies that harness the activity in remediation systems.

The specific objectives of the research were to:

1. Identify the products of novel TNT transformation pathways
2. Determine the mechanism of TNT transformation and identify the enzymes responsible. Characterize the properties of the enzymes and their regulation.
3. Examine the fate of TNT transformation products in natural systems.
4. Develop strategies to direct TNT metabolism to ring fission products.
5. Examine the potential for mineralization of novel TNT metabolites.

Background

Nitroaromatic compounds are widespread contaminants at DOD facilities. Over 700,000 cubic yards of soil and 10 billion gallons of groundwater require treatment (SERDP, 1993). The cost to complete cleanup of Military Munitions Response Program sites is estimated to be $18.7 billion through 2010 (DEP, 2004). TNT is the primary contaminant at these sites, along with dinitrotoluenes (DNT), and the other nitro substituted explosives (i.e., RDX and HMX). Current approaches used for site remediation typically involve excavation of contaminated soils, followed by incineration or composting, and pump-and-treat for contaminated groundwater.

The development of *in situ* bioremediation processes for the treatment of TNT and other nitroaromatics would greatly improve DOD's ability to restore contaminated sites in a more cost-effective manner (Anderson et al., 1999). The factor that has limited the development of *in situ* bioremediation processes for treatment of TNT contaminated soils or groundwater is the inability of bacteria to use TNT as a growth substrate.

Characteristics common to nitroaromatic comounds (i.e., electron deficient pi-orbitals and high redox potentials) can reduce the ability of oxygenase enzymes to catalyze an initial electrophilic attack on the aromatic ring and can cause reductive metabolic pathways to become favorable. Aryl nitro groups are labile and can undergo reduction reactions catalyzed by a range of electron transfer proteins found in microorganisms. In general, the propensity for reduction increases with the degree of nitro-substitution. For example, aerobic bacteria can use initial oxidative metabolism to metabolize 2,4-dintrotoluene and 2,6-dinitrotoluene, but reductive metabolism is predominant with TNT.

Reduction of the nitro groups makes the molecule more resistant to subsequent reduction and more susceptible to oxidation. For this reason, current ex situ treatment systems (i.e., composting and slurry reactors) have focused on co-metabolic transformations that lead to binding (also referred to as sequestration or humification) of metabolites to soil and amendments. This approach to TNT bioremediation does not result in ring fission, and because the products are difficult to characterize or monitor, the treatment endpoint remains controversial. The application of similar techniques for *in situ* treatment is unlikely as these processes use overwhelming amounts of co-substrate in heavily engineered systems to maintain very low redox potentials for extended periods. More recently, it has been suggested (Lenke et al., 2000) that partial reduction under anaerobic conditions can lead to binding and humification during subsequent aerobic treatment. The amount of carbon source and electron donors required are still daunting and the treatment does not result in destruction of the aromatic ring. Therefore, alternate technologies which alter the base molecular structure of TNT (i.e., loss of aromaticity, mineralization) are required.

Multiple organisms share intermediates in TNT transformation pathways that could lead to further transformation products which no longer display aromatic characteristics (Figure 1). The central metabolite in the pathways of *C. acetobutylicum* and *P. pseudoalcaligenes* JS45 is 2,4-dihydroxylamino-6-nitrotoluene (Fiorella and Spain, 1997), which is hydroxylated via rearrangement (Hughes et al., 1998) and then degraded to polar products. In the systems we have studied, the rearrangement of DHANT serves as a gateway to a novel pathway for TNT transformation.

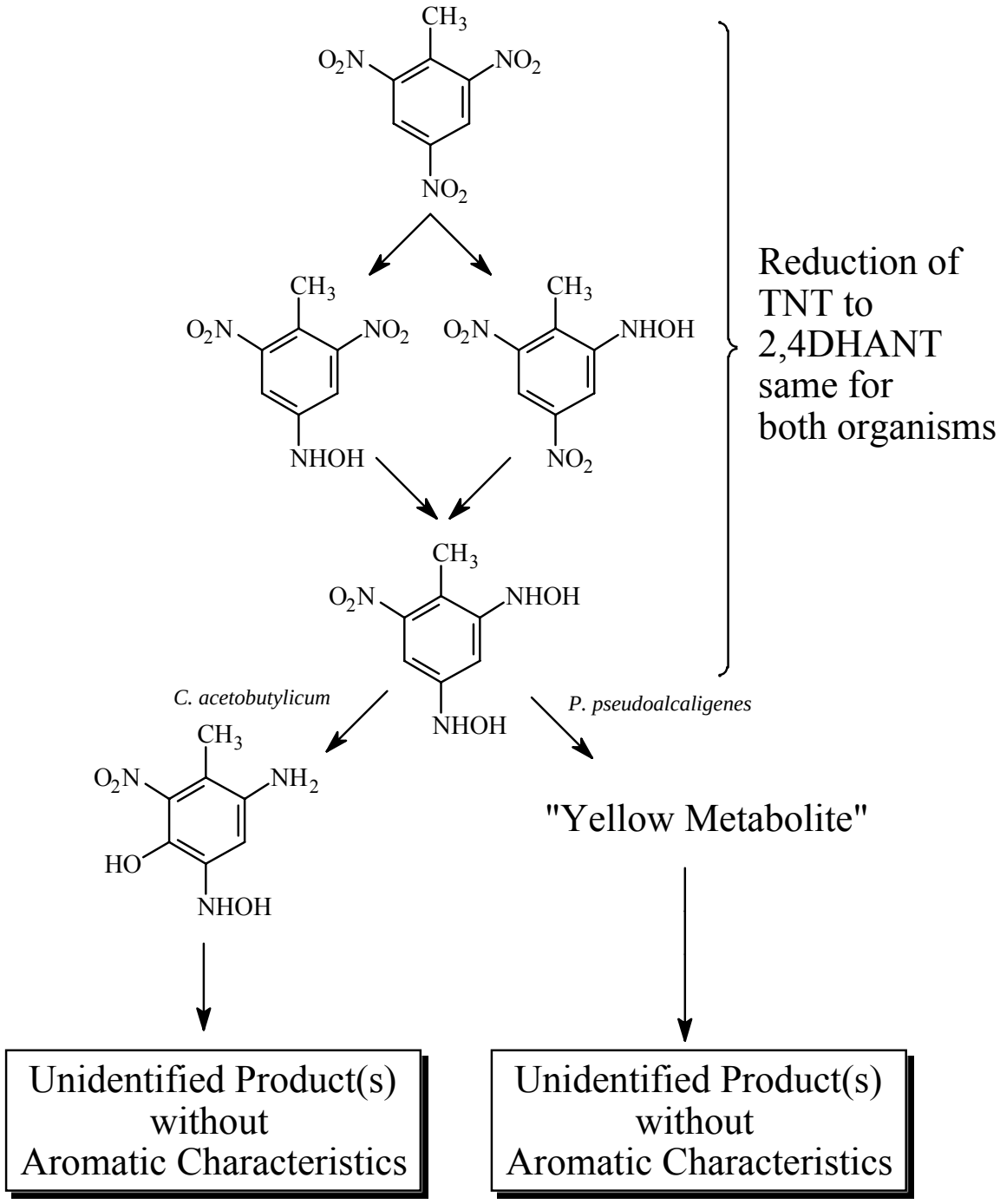

Figure 1. Novel pathway of TNT transformation through 2,4-dihydroxylamino-6-nitrotoluene (DHANT) for *C. acetobutylicum* (Hughes et al., 1998) and *P. psuedoalcaligenes* (Fiorella and Spain, 1997) that results in products that do not display aromatic characteristics (no UV absorbance, not extractable with organic solvent, highly polar). The "yellow metabolite" is believed to be a rearrangement product similar to that detected with *C. acetobutylicum.*

Aerobic Biodegradation. In recent years, it has been demonstrated that a number of nitroaromatic compounds are susceptible to aerobic microbial degradation and a considerable body of knowledge has been developed about the catabolic pathways of nitroaromatic metabolism during aerobic biodegradation (Nishino, Spain *et al.* 2000; Nishino and Spain 2004). Of particular interest in these studies are the pathways used by aerobic bacteria to remove the nitro group during conversion of the nitroaromatic compounds to central metabolites. The nitro group can be released as nitrite during aerobic microbial metabolism by three distinct mechanisms: a) dioxygenation of the aromatic ring to a dihydroxy intermediate (Spanggord, Spain *et al.* 1991; Ecker, Widmann *et al.* 1992; Nishino and Spain 1992; Haigler, Wallace *et al.* 1994; Nadeau and Spain 1995; Nishino and Spain 1995; Nishino, Paoli *et al.* 2000); b) monooxygenation to an epoxide (Zeyer and Kearney 1984; Spain and Gibson 1991; Schäfer, Harms *et al.* 1996); or, c) hydride-Meisenheimer complex formation (Lenke and Knackmuss 1992; Lenke and Knackmuss 1996; Behrend and Heesche-Wagner 1999; Rieger, Sinnwell *et al.* 1999). Alternatively, the nitro group can be released in the form of ammonia when pathways proceed via initial reduction and formation of an aryl hydroxylamine. (To date there is little evidence for an aerobic pathway that involves complete reduction to the amine prior to ring fission by the same bacterium). Because direct oxygenase attack on TNT had not been observed until recently (Tront and Hughes, 2005), and since it is uncertain whether productive TNT metabolism can occur from TNT hydride-Meisenheimer complexes (Vorbeck, Lenke *et al.* 1994; Haïdour and Ramos 1996; French, Nicklin *et al.* 1998; Vorbeck, Lenke *et al.* 1998; Rieger, Sinnwell et al. 1999; Pak, Knoke

et al. 2000; Weiß, Geyer *et al.* 2004), pathways involving initial reduction to hydroxylamines were of particular interest in this project.

The elimination of aryl nitro groups as ammonia via hydroxylamino-compounds occurs via two distinct mechanisms. In one mechanism, the hydroxylamino-compound is attacked by an enzyme described as a hydroxylaminolyase (Groenewegen and de Bont 1992), which produces a corresponding catechol and eliminates ammonia. Although details of the reaction mechanism itself remain unclear it is known to be involved in the metabolism of 4-nitrotoluene (Haigler and Spain 1993; Rhys-Williams, Taylor *et al.* 1993), 4-nitrobenzoic acid (Groenewegen, Breeuwer *et al.* 1992; Yabannavar and Zylstra 1995), and 3-nitrophenol (Meulenberg, Pepi *et al.* 1996). The second pathway that includes a hydroxylamino-intermediate (depicted in Figure 2) involves a mutase catalyzed intramolecular rearrangement of the hydroxylamino-compound to an *o*-aminophenol. The *o*-aminophenols serve as *meta*-ring cleavage substrates for highly specific dioxygenases, and the reactions result in the formation of aminomuconic semialdehydes. The amino group is released as ammonia in subsequent reactions. Nitrobenzene (Nishino and Spain 1993), 2-chloro-5-nitrophenol (Schenzle, Lenke *et al.* 1999), 4-chloronitrobenzene (Katsivela, Wray *et al.* 1999), 4-nitrotoluene (Spiess, Desiere *et al.* 1998) as well as 3-nitrophenol (Schenzle, Lenke *et al.* 1997; Schenzle, Lenke *et al.* 1999) are degraded by the mutase mediated pathway via *o*-aminophenols.

Interestingly, partial reduction of TNT to hydroxylamino-dinitrotoluenes and amino-dinitrotoluenes takes place prior to oxidation during its metabolism by fungi (Fritsche, Scheibner *et al.* 2000). As in other systems, initial reduction results in

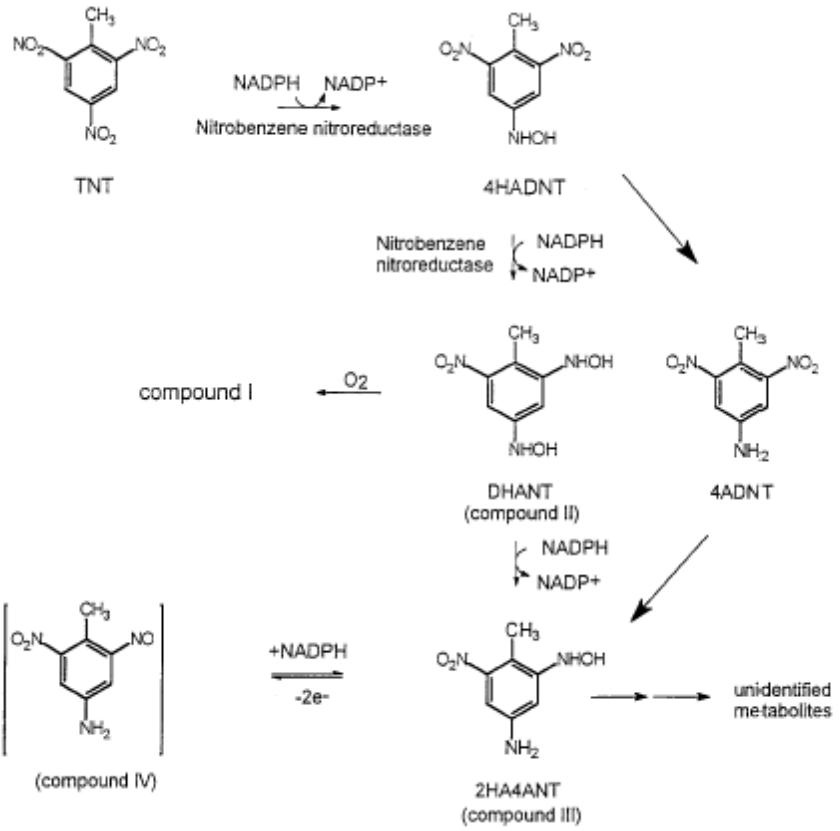

Figure 2. Proposed pathway for TNT transformation by nitrobenzene-grown cultures of *P. pseudoalcaligenes* (Fiorella and Spain 1997).

compounds that are more prone to oxidative attack. Evidence of analogous forms of TNT metabolism in bacterial culture has been reported, although little mechanistic information exists (Alvarez, Kitts *et al.* 1995).

Pseudomonas pseudoalcaligenes strains JS52 and JS45 grow aerobically on nitrobenzene using an initial reductive pathway (Figure 3) (Nishino and Spain 1993; He and Spain 1999). In this case, nitrobenzene nitroreductase converts nitrobenzene to hydroxylaminobenzene, which is then converted to 2-aminophenol by the action of hydroxylaminobenzene mutase. The 2-aminophenol is then subject to oxidative attack and ring fission. The mutase does not require cofactors and catalyzes an intramolecular rearrangement of the molecule (He, Nadeau *et al.* 2000). The reaction leads to the substitution of a hydroxyl group on the aromatic ring without the action of an oxygenase and without the addition of water. 2-Aminophenol is the substrate for a novel ring-fission dioxygenase that catalyzes the opening of the aromatic ring and the formation of aminomuconic semialdehyde. Several other strains that use analogous pathways for the degradation of 3-nitrophenol (Schenzle, Lenke et al. 1997), 4-nitrotoluene (Spiess, Desiere et al. 1998), and chloronitrobenzene (Katsivela, Wray et al. 1999) have been discovered in the past few years. The results described above suggest that the enzymes used for the reductive conversion of nitroaromatic compounds to ring-fission substrates are widespread. They may have different substrate preferences, but the mechanisms are probably the same.

These results have since led to the discovery that the nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* can catalyze the transformation of TNT to 4-hydroxylamino-2,6-dinitrotoluene and then to 2,4-dihydroxylamino-6-nitrotoluene

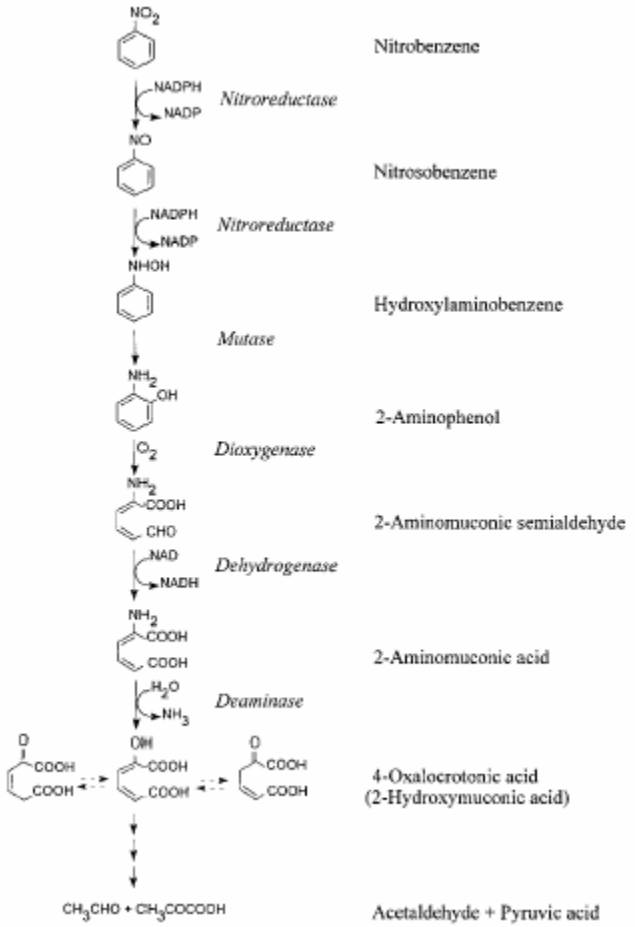

Figure 3. Partially reductive pathway for degradation of nitrobenzene (modified from (Nishino and Spain 1993; He and Spain 1999)).

(DHANT) (Fiorella and Spain 1997). DHANT is also a central intermediate in the metabolism of TNT by clostridia. The nitrobenzene nitroreductase does not transform DHANT, but other reductases in crude cell extracts catalyze the slow reduction of DHANT to 2-hydroxylamino-4-amino-6-nitrotoluene (2HA4ANT). Both DHANT and 2HA4ANT are transformed to more polar metabolites by enzymes in crude cell extracts. The transformations require oxygen, which suggests that the reactions involve incorporation of molecular oxygen. DHANT is also converted to a polar yellow metabolite (YM) that cannot be extracted from the aqueous phase with organic solvents. It is sensitive to acid and base and the UV/vis spectrum reveals maxima at 258, 325, and 405 nm. The mass spectrum gave a molecular ion of 183. The properties of the molecule are consistent with the structure of a toluene ring with two amino groups, a hydroxyl group, and a nitro group. Such a compound could be formed by a mutase-catalyzed rearrangement of DHANT in a reaction similar to the conversion of hydroxylaminobenzene to 2-aminophenol. The fact that the conversion of DHANT to the yellow metabolite requires air suggests on the other hand that an oxygenase is involved in the reaction. An alternative explanation would be a nonenzymatic oxidation of one of the functional groups of the molecule. Enzymes in cell extracts further metabolize the YM and nitrite is released during the process. It is not clear whether nitrite is released during the formation or the subsequent metabolism of the yellow metabolite. In any case, the YM and 2HA4ANT seem to be the gateway compounds to the subsequent conversion of the TNT metabolites to molecules that have lost their aromatic character and are no longer recognizable as TNT derivatives. Such compounds can bind to humic material or could be further degraded. Such binding and conversion to non-identifiable polar

products can cause the TNT related toxicity to disappear completely (Lenke, Achtnich *et al.* 2000). Alternatively, it may be possible to engineer pathways for the mineralization of the extensively modified TNT metabolites once they are identified.

Reductive Cometabolism. Fortuitous reduction of the aryl nitro groups of poly-nitroaromatics is commonly observed in cultures of aerobic and anaerobic bacteria (Ramos, Caballaro *et al.* 2004). Reductive pathways proceed through two intermediate forms before the process is complete, thus the reduction of TNT (or other poly-nitroaromatic compounds), has the potential to yield a variety of intermediates and products. Specifically, the groups formed during nitro group reduction include the nitroso-group (R-NO), the hydroxylamino-group (R-NHOH), and the amine ($R\text{-}NH_2$). Nitroso-groups are rarely observed in microbial metabolism, because further reduction to hydroxylamines is strongly favored. Complete reduction to the amine can occur, although some organisms appear to be deficient in this ability and the accumulation of hydroxylamines has been observed frequently even under strong reducing conditions Most of the nitroreductases studied to date catalyze the conversion of the nitro group to the hydroxylamine (Somerville, Nishino *et al.* 1995; Koder, Haynes *et al.* 2002). The enzymes responsible for the subsequent reduction to the amine are a mystery.

Reductive reactions can be involved in the complete degradation of nitroaromatic compounds by aerobic bacteria. However, cometabolic reduction processes appear to be a more widely distributed form of reductive metabolism – particularly with TNT (Rieger and Knackmuss, 1995). The rate and extent of the cometabolic reduction of nitroaromatic compounds is strongly influenced by other substituents on the ring in

addition to the nitro group(s) (Haderlein and Schwarzenbach, 1995). For example, the electron withdrawing characteristics of two additional nitro groups facilitates the initial reduction of one nitro group of TNT. The presence of ring activating groups (amines, hydroxyl groups, etc.) will produce the opposite effect. For this reason, cometabolic TNT reduction under aerobic conditions rarely proceeds beyond the reduction of a single nitro group. The stronger reducing conditions present under anaerobic conditions may lead to the reduction of two or more nitro groups of TNT.

The ability to reduce nitroaromatic compounds is also related to organism-specific properties including the expression of low-redox electron transfer proteins that exhibit nitroreductase activity, and the rate of electron transfer within the organism. Based upon these two organism-specific factors, considerable interest has been focused on the propensity of fermentative bacteria to reduce nitroaromatic compounds (Ederer et al., 1997). Clostridia, in particular, rapidly reduce aryl nitro groups due to the high expression by clostridia of low redox Fe-S electron transfer proteins, and the need to recycle NADH rapidly during anaerobic glycolysis (i.e., The Pasteur effect). Interestingly, the products of fermentative TNT reduction appear to be predominantly hydroxylamino-derivatives that undergo rearrangement to substituted aminophenols (Hughes et al., 1998; Hughes et al., 1998b; Hughes et al., 1999). This rearrangement reaction is similar to those discussed in the previous section, although it appears that differences in the mechanism involved may exist (*para*-rearrangement instead of *ortho*-rearrangement is observed).

Research focused on the role of hydroxylamines in the rapid anaerobic metabolism of TNT, 2,4-DNT, and 2,6-DNT by clostridia has demonstrated that

dihydroxylamino-forms of each nitroaromatic were central intermediates of metabolism. In the case of TNT, further transformation yielded an aminophenol product via a Bamberger rearrangement (Hughes et al., 1998) as depicted in Figure 1. Subsequent studies have concluded that the reduction of the nitro group is due to a Fe-S containing hydrogenase, which also appears to catalyze rearrangement and hydroxylation. The Fe-S clusters present in this hydrogenase is common to a number of enzymes in clostridia and other bacteria. Carbon monoxide dehydrogenase from *C. thermoaceticum* containing identical Fe-S clusters also transformed TNT to the same aminophenol product (Huang et al., 1999). While the formation of hydroxylamino groups was observed in cell cultures and purified enzyme systems for all nitroaromatics tested, the formation of aminophenols was only observed from TNT. Whether this is due to steric effects (i.e., the shape of the molecules involved) or electronic effects (i.e., the electron withdrawing characteristics of substituent groups) is not known. The final products of TNT and DNT transformation via hydroxylamine pathways were highly oxygen sensitive (Wang et al., 2000) and products of decomposition do not exhibit aromatic characteristics. In particular, they do not have a UV absorbance in the aromatic region, can not be extracted into organic solvent, display characteristics of a zwitterion (expected for an aminated organic acid), and are highly polar. The long term fate, toxicity, and potential for mineralization of these products have not yet been investigated. Ames tests have confirmed that these products are not mutagenic (Padda et al., 1999).

An interesting component to these studies is the rapid nature of transformation observed in the early growth phase of the organism. After the organism's metabolism

switches from acidogenic (fermentation to acetic acid and butyric acid) to solventogenic (fermentation to ethanol and butanol), the ability to catalyze TNT transformation disappears (Khan et al., 1997). We have demonstrated that this loss of activity results from strict metabolic control of hydrogenase activity through testing of mutant strains deficient in the genes required for solventogenic growth and with selective inhibitors of the hydrogenase. These results imply that it will be important to understand the regulation of pathway expression to maintain desired activity *in situ.*

The product of complete TNT reduction is 2,4,6-triaminotoluene (TAT). TAT has been postulated as a central intermediate in the anaerobic treatment of TNT (Crawford, 1995), even when it has not been detected. It has been demonstrated that reduction under iron-reducing conditions leads to rapid production of TAT (Heijman et al., 1995). The amino groups of TAT are strong ring activators, making the ring susceptible to electrophilic attack. As reviewed by Lenke, et al., (2000) TAT is readily oxidized by oxygen in abiotic reactions catalyzed by metal ions such as Mn^{2+}. The products of this process appear to be polymers that are at best difficult for bacteria to degrade. Studies have also shown that TAT will "disappear" in anaerobic systems, possibly forming tetraaminoazobenzenes or polynuclear azo compounds. Thus it does not appear that the complete reduction of TNT to TAT will result in the formation of ring fission precursors that are amenable to complete destruction. Conversion to TAT and subsequent humification might be an acceptable strategy for treatment of excavated soil contaminated with TNT in anaerobic reactors where high concentrations of additional carbon sources can be added and low redox potentials can be maintained.

Anaerobic-Aerobic Treatment of TNT. Because the initial metabolism of TNT involves reduction, and reduced products should be more amenable to oxygenase attack, two-stage treatment of TNT contaminated soils has been investigated. There are several process configurations that achieve this two-stage approach, including composting (Breitung et al., 1996; Bruns-Nagel et al., 1998), slurry reactors (Funk et al., 1993; Manning et al., 1996; Greist et al., 1998), and in some cases natural attenuation. Regardless of the system employed, anaerobic-aerobic treatment appears to yield a high degree of bound residues and does not result in destruction of the aromatic ring (Lenke et al., 2000). Because the products are difficult to characterize or monitor, and because the process is only useful for excavated soil, the effectiveness of anaerobic-aerobic treatment processes remains controversial. The process of binding is an area of continued study (Lenke et al., 2000), but it appears to result from reactions of partially reduced intermediates with the organic matrix of soil or compost amendments (Daun et al., 1998; Lenke et al., 1998). Despite the strong reducing conditions present, the rate of binding reactions in these processes appears to be slow, relative to the rate of TNT transformation and it should be possible to route product distribution away from bound residues in *in situ* processes. It is our hypothesis that this could best be achieved for TNT through pathways of initial reductive metabolism that yield the rapid formation of aerobic ring fission precursors (i.e., hydroxylated rearrangement products). Such strategies involving reduction only to the hydroxylamine would require far less carbon addition and less dramatic shifts in redox potential and so would be considerably less energy intensive. The properties of such a system would be much more amenable for *in situ* treatment of

contaminated soil and groundwater using optimization of “amendments” to induce desired pathways, bioaugmentation, or through metabolic engineering.

Materials and Methods

An expansion of materials used and experimental methodology for work completed with *C. acetobutylicum* are described with data in Appendix A. The methodology used for experimentation with *P. pseudoalcaligenes* JS45 is described here.

Bacteria and culture methods. *P. pseudoalcaligenes* JS45 was grown in a nitrogen free minimal medium (BLK) (Bruhn, Lenke *et al.* 1987) with NB provided as the sole carbon, nitrogen and energy source (Nishino and Spain 1993). To grow high density cultures ($1 < A_{600} < 11$), the amount of MgSO4 • 7H2O in BLK was increased 10-fold. The strain was maintained on BLK agar plates provided with NB vapor as previously described (Nishino and Spain 1993). Small shake flask cultures (100 ml) were inoculated with JS45 and provided 2.5 mM nitrobenzene, and grown overnight at 30 °C, with shaking at 200 rpm. Shake flask cultures were used to inoculate a 2 L (1.3 L working volume) bioreactor (New Brunswick Scientific, Edison, NJ). NB was fed to the bioreactor via a syringe pump. The NB flow rate was high enough to support cell growth, but low enough so that there was no detectable nitrobenzene in the culture medium by high performance liquid chromatography (HPLC) analysis. Cultures were monitored for growth by measuring A_{600} on a Cary 3E spectrophotometer.

Transformation of TNT. TNT (100 mM) dissolved in HPLC grade methanol, was added to NB-grown cultures of JS45 either in shake flasks or in the 2 L bioreactor to give a final TNT concentration of 200 to 1000 μM. Disappearance of NB and TNT and appearance of metabolites were monitored by HPLC.

Analytical methods. HPLC was performed on a Hewlett Packard HP-1090 system equipped with a diode array detector. Analyses were performed with a Spherisorb C8 column as previously described (Fiorella and Spain 1997) or with a Merck Chromolith Performance RP-18e column (100 mm x 4.6 mm I.D.) using a combined flow and solvent gradient. The initial flow rate was 0.75 ml/min and the mobile phase consisted of a 95:5 ratio of part A (13.5 mM trifluoroacetic acid in water) and part B (6.75 mM trifluoroacetic acid in acetonitrile). At 3.01 min the flow was stepped up to 1 ml/min and the mobile phase changed to 65:35 part A:part B and the flow rate was increased in a linear gradient from 1.0 to 4.0 ml/min over 4 min then held at 4.0 ml/min for 0.5 min. NB, TNT, and metabolites were monitored at A_{254} and the yellow metabolite at A_{420}.

Metabolite purification. Culture fluids were clarified by centrifugation at 25000 x g then pumped through Bond Elut C-18 solid phase extraction (SPE) cartridges (Varian Inc.). The fluids that passed through the column and material extracted from the column with deionized water, which included the YM, were collected and extracted onto an Envi-Carb graphitized nonporous carbon (Supelco) SPE column. The cartride was prepared and extracted according to the manufacturer's method for extraction of base-neutral and acidic pesticides (Supelco 1997). The YM and one other compound eluted in the acidic fraction. The solvents were removed by flash evaporation. Material that remained bound to the C-18 column was sequentially eluted with 30%, 50% and 70% methanol in deionized water. The methanol was removed from the fractions by flash evaporation and the aqueous phase was extracted with ethyl acetate, which was in turn removed by flash evaporation to concentrate the metabolites.

Preparation of DHANT. DHANT was produced by incubating partially purified nitrobenzene nitroreductase in phosphate buffer with TNT (200 μM) and NADPH (500 μM) for 1 h at room temperature. Complete transformation of TNT to DHANT was confirmed by HPLC. The DHANT was used without further purification.

Protein extraction and fractionation. *P. pseudoalcaligenes* JS45 cells were grown in BLK with succinate and ammonium chloride. Beginning with cell harvest, all manipulations were carried out at 4 °C. Cells were harvested from mid- to late-exponential phase by centrifugation at 8,000 x *g*. Pellets were washed twice in phosphate buffer (10 mM, pH 7) and then suspended in 20 ml of the same buffer containing a protease inhibitor cocktail. The cells were disrupted by three passages through a French press at 16,000 psi and the lysate was centrifuged for 20 minutes at 10,000 x *g* to remove debris and unbroken cells. The crude extract was partitioned into soluble, weakly-membrane associated, and tightly membrane-bound fractions as follows. The crude extract was centrifuged at 200,000 x *g*. The supernatant contained the soluble protein fraction. The pellet was suspended in 50 mM glycylglycine, 2 M NaBr, 200 mM sucrose buffer with protease inhibitor cocktail and extracted by gentle stirring for 30 minutes. An equal volume of a 50 mM glycylglycine buffer was added and the mixture was centrifuged at 200,000 x *g*. The supernatant contained the weakly membrane-associated proteins. The pellet, was suspended in 10 mM phosphate buffer pH 7, with Triton X100 0.5% (vol/vol) and protease inhibitor cocktail. Proteins were solubilized by gentle stirring for 60 minutes before centrifugation at 200,000 x *g* to pellet insoluble materials. The supernatant constituted the membrane-bound protein fraction.

Enzyme assays. The reductase activity was monitored spectrophotometrically as the decrease in A_{340} due to the oxidation of NADPH or NADH in a reaction mixture containing the protein fraction (1 mg ml^{-1}), NADPH or NADH (500 μM) and substrate (TNT or NB, at 100 μM) in 1 ml of phosphate buffer (10 mM, pH 7). The mutase activity was assayed by formation of 2-aminophenol as described previously (Davis, Paoli et al. 2000).

Results and Accomplishments

Novel metabolic products in TNT transformation pathway.

Transformation of TNT by *Clostridium acetobutylicum.* Previous work completed in the Hughes laboratory identified a hydroxylaminiotoluene as an intermediate in the anaerobic transformation of TNT by *C. acetobutylicum*. All efforts to expand the understanding of this transformation pathway demonstrated that further products gained polarity and were extremely oxygen sensitive. No further contaminant transformation products were identified despite attempts using ^{13}C labeled TNT in conjunction with nuclear magnetic resonance and mass spectrometry. Data in Figure 4 represent an example UV profile of a sample taken from a reactor where *C. acetobutylicum* was exposed to ^{14}C labeled-TNT. The experiment was allowed to proceed past the point of known metabolites where transformation was verified with the presence of transient hydroxylaminotoluenes and the formation a red colored metabolite. The UV profile showed absorbance that corresponded to aminodinitrotoluenes (ADNTs) which eluted at 10.8 and 11.0 min and the solvent front. Corresponding radioactivity measurements confirmed that all TNT transformation products were associated with ADNTs or with the polar solvent front.

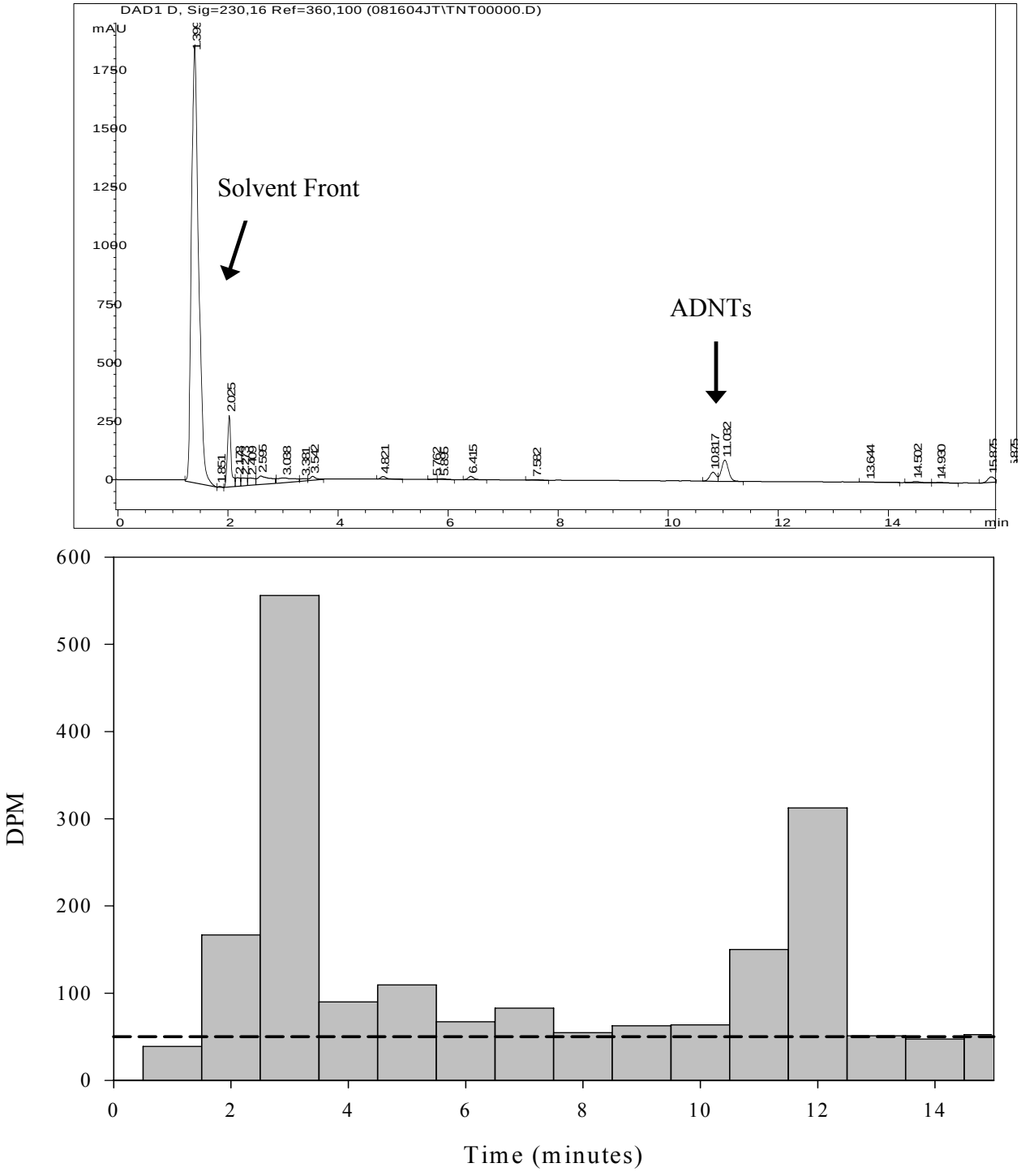

Figure 4. UV profile and radioactivity of efflent of ^{14}C labeled sample from a reactor where C. *acetobutilcum* was exposed to TNT for 14 d. The dotted line represents background radioactivity.

Transformation of TNT by *P. pseudoalcaligenes* JS52. *P. pseudoalcaligenes* JS52, a spontaneous mutant of JS45, was previously reported to produce a polar yellow metabolite from 2,4,6-trinitrotoluene (TNT) after growth on nitrobenzene (NB) (Fiorella and Spain 1997). The metabolite was one of four (Figure 2) distinguished by HPLC retention times and spectra during the initial experiments, but was the only metabolite that required molecular oxygen for its production, during the course of which nitrite was released. The compound was also reported to be persistent once formed in batch cultures. A working hypothesis is that the yellow metabolite is a rearrangement product of 2,4-dihydroxylamino-6-nitrotoluene similar to one detected as an end product in *Clostridium acetobutylicum* transformations of TNT (Hughes, Wang *et al.* 1998). The YM along with the *C. acetobutylicum* product was believed to be a key intermediate in the TNT transformation pathway that leads to unidentified metabolites that no longer display aromatic characteristics.

We combined large experiments in bioreactors with simpler shake flask experiments to determine the time course and optimize the conditions for TNT transformation. Because we believed the yellow metabolite was only produced by NB grown cultures in the presence of NB and TNT we developed a strategy to grow strain JS45 to high cell densities on NB before the addition of TNT for transformation. The toluene-degrading isolate *Acinetobacter* sp. strain F4 will continuously convert diphenylacetylene (not a growth substrate) to a *meta*-ring fission product when a low level of toluene is continuously provided to the culture (Spain, Nishino *et al.* 2003) resulting in the accumulation of high levels of the ring fission product. We attempted to apply a similar strategy to JS45 in order to accumulate the YM from TNT.

Preliminary experiments. The work described with JS52 (Fiorella and Spain 1997) involved preparation of the YM in small batch cultures of resting cells. NB and TNT were added to the resting cells with NB in a 4-10 fold excess over TNT. Under those conditions (A_{600} = 1, TNT = 100-200 μM) production of the YM required a 40 min incubation to reach the maximum concentration, and the metabolite was stable for several hours in the culture medium. In an initial experiment, JS45 was grown in a bioreactor in which NB was pumped in at a high enough rate for cell growth, but slowly enough so that NB was never detectable in the culture fluid. When TNT was added, the YM reached the maximum concentration within 25 min, concomitant with the complete disappearance of TNT, after which the concentration declined (Figure 5). A second addition of TNT resulted in a second spike in the yellow metabolite concentration. The preliminary work showed that the yellow metabolite can be further transformed by active cultures, and that transformation of TNT is much more rapid in active cultures than in resting cells.

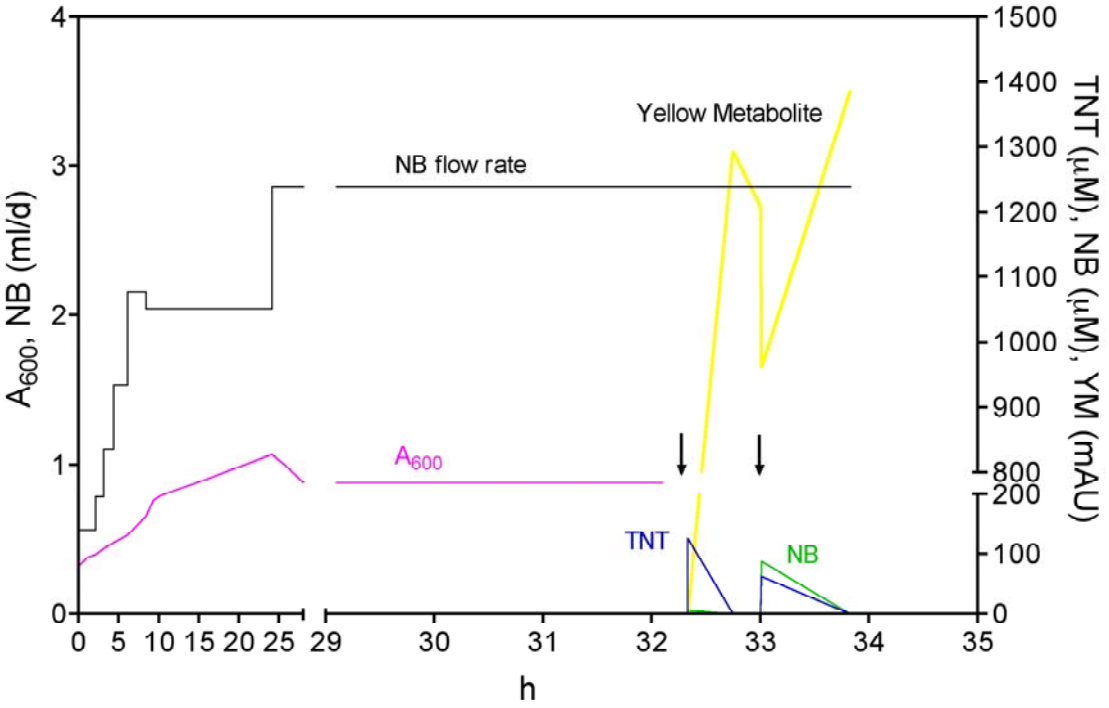

Figure 5. Cultures of *P. pseudoalcaligenes* JS45 actively growing on NB converted TNT (addition indicated by arrows) to the yellow metabolite, which began to disappear after the TNT was exhausted.

Shake flask experiments. Studies in shake flasks were designed to optimize the conditions for transformation of TNT. The studies revealed that TNT transformation by resting cells can introduce a lag period during which metabolite accumulation differs from metabolite accumulation in more active cultures. Metabolites that disappeared in active cultures accumulated in shake flask experiments. Shake flask experiments also established a minimum requirement for 2 mol of NB per mol TNT transformed. In practice, higher concentrations of NB are required due to volatilization of NB.

TNT transformation in bioreactor cultures. During a TNT transformation experiment in bioreactor culture, 24 metabolites, identified by HPLC retention time and UV-Vis spectra, were detected. The metabolites detected included all the pathway intermediates proposed by Fiorella (Figure 2) (Fiorella and Spain 1997) as well as 2-hydroxylamino-2,6-dinitrotoluene (2HADNT).

Transformation time courses. P. pseudoalcaligenes JS45 and *E. coli* JS995 were used in time courses for the transformation of unlabelled TNT and 2ADNT as well as ^{14}C-U-ring-labelled TNT (American Radiolabelled Chemicals, Inc. specific activity = 8 mCi/mmol, radiopurity = 99.14% by HPLC analysis). JS45 was grown on nitrobenzene so that the partially reductive nitrobenzene pathway was induced (Nishino and Spain 1993), and JS995 was grown in LB with 1 mM IPTG and 100 μg/ml of ampicillin. Controls included succinate-grown cells of JS45 and *E. coli* C43(DE), the host strain used to construct JS995. JS995 (Kadiyala, Nadeau *et al.* 2003) contains the genes for nitrobenzene nitroreductase and hydroxylaminobenzene mutase from the nitrobenzene degradation pathway of JS45. When nitrobenzene-grown cells of JS45 were given TNT

or 2ADNT, the yield of YM from 50 uM of the initial substrate was 161 and 220 mAU from TNT and 2ADNT, respectively. Uninduced cells did not convert the substrates. When IPTG-induced cells of JS995 were given TNT or 2ADNT, the YM yield was markedly different, with only 23 mAU from TNT, and 330 mAU from 2ADNT. *E. coli* C43(DE) made no YM from either substrate, but approximately 10% of the initial TNT was converted to 2ADNT. Previous experiments showed that nitrobenzene nitroreductase converts TNT to DHANT (Fiorella and Spain 1997); and that hydroxylaminobenzene mutase (HabA) does not convert DHANT to the YM.

Strain JS995 converted TNT mostly to DHANT, and DHANT was not converted to the YM, but disappeared from the culture fluid over several hours in the presence of oxygen. The small amounts of YM made from TNT were probably the result of the formation of 2ADNT by a nonspecific nitroreductase in the *E. coli* C43(DE) host as observed in the control experiments. That the control lacking the genes from JS45 did not convert 2ADNT to the YM while JS995 did, suggests that HabA is involved in the transformation of 2ADNT to the YM. The results further suggest that the wild-type, JS45 converts TNT to the YM only through 2ADNT because the JS995 clone only made the YM from 2ADNT. The yield of YM from TNT and 2ADNT by JS45 further supports the interpretation. One might expect a higher yield of YM from 2ADNT over TNT because some of the TNT is diverted to DHANT. The ratio of the yield of YM from TNT and 2ADNT in JS45 is 0.73 which might approximate the flux of TNT through 2ADNT vs. 0.27 through DHANT in the wild-type bacteria.

The existence of a nitroreductase in *P. pseudoalcaligenes* JS45 that is involved in the conversion of TNT to 2ADNT is also inferred from the above results because nitrobenzene nitroreductase exclusively attacks TNT at the 4-position. Such a nitroreductase is unique in two respects: it preferentially attacks TNT at the 2-position, and 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) is reduced to the amine. No nitroreductase has been characterized to date with those properties.

Time courses with ^{14}C-labelled TNT and strain JS45 showed that 27% of the initial radioactivity accumulated in the YM peak and 21% accumulated in a second peak with a retention time of 2.1 min by HPLC. Using *E. coli* JS995, the radioactivity initially accumulated in 4HADNT, then shifted to DHANT. As the DHANT disappeared, the radiolabel became dispersed throughout the HPLC fractions, with no distinct peaks of radiolabel. The experiments confirmed that the YM is a significant metabolite of TNT, and also revealed a second less polar metabolite with strong radiolabel. The UV-Vis signal associated with the 2.1 min is not very strong, but the strong radiolabel indicates its importance as a transformation product. An average of 94% of the initial radiolabel was recovered in the HPLC fractions, no mineralization was detected, and less than 3% was associated with the cell mass. Based upon the transformation studies carried out under this project, the TNT degradation pathway used by JS45 has been modified to reflect the major pathway of TNT degradation going through 2ADNT (Figure 6).

6).

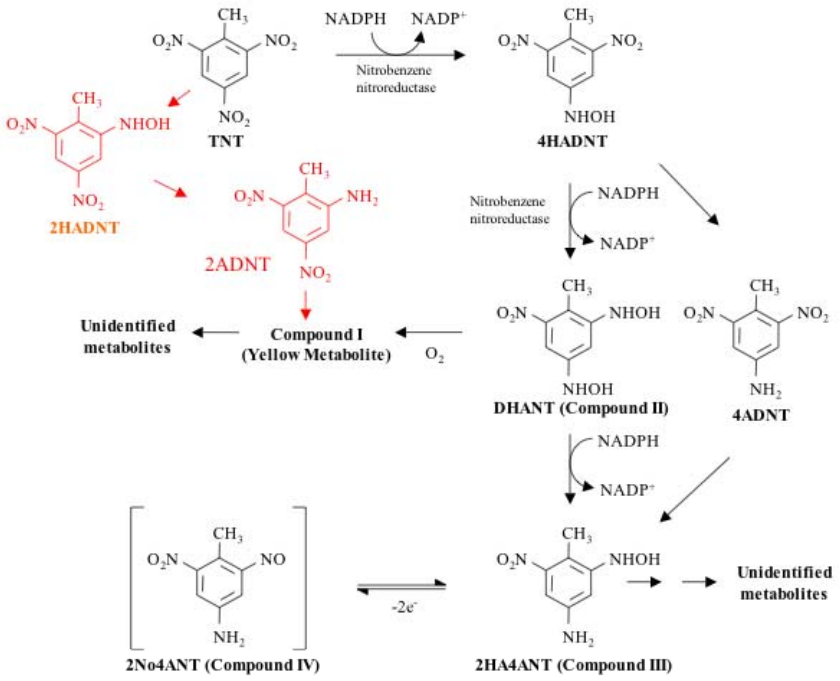

Figure 6. Modified TNT transformation pathway in *P. pseudoalcaligenes* JS45.

Characterization of the yellow metabolite. Attempts to purify and concentrate the YM in quantity failed because the YM does not partition into organic solvents (Fiorella and Spain 1997) or form stable derivatives. We were, however; able to produce quantities of YM suitable for LC-MS analysis using an *in vitro* system with purified enzymes. TNT was converted to DHANT by NbzA (Fiorella and Spain 1997). Then partially-purified immobilized HabB (Luckarift, Nadeau *et al.* 2005) was added to the freshly prepared DHANT. The reaction mixture was incubated for 15 minutes at room temperature then briefly centrifuged to remove the immobilized enzyme.

Because HabB rearranges hydroxylamines to *o*-aminophenols (Nadeau, He *et al.* 2000) only a limited number of structures are possible for the YM (Figure 7). LC-MSMS analysis of the reaction mixture showed that the YM yielded a deprotonated molecular mass ion [M-H] at 198 Da (MW, 199 Da). Relevant LC-MS fragmentation patterns (181, 166, 153 and 135 Da) are consistent with an aromatic ring substituted with the following groups: $-NO_2$, NHOH and NH_2 (data not shown). The results exclude structure D (Figure 8) and also indicate clearly that the YM is not a ring-fission product. We were unable to isolate the YM in sufficient quantity and purity for NMR analysis because it was unstable.

Although we could not determine the isomeric structure of the YM, it is clear that it is an aromatic compound produced by the rearrangement of DHANT into aminophenol. A similar polar compound (same molecular weight) was produced by a Bamberger rearrangement of DHANT during the degradation of TNT by *Clostridium acetobutylicum* under anaerobic conditions (Hughes, Wang et al. 1998). The product produced by *C. acetobutylicum* results from a rearrangement of the *2*-postion hydroxylamino group to an

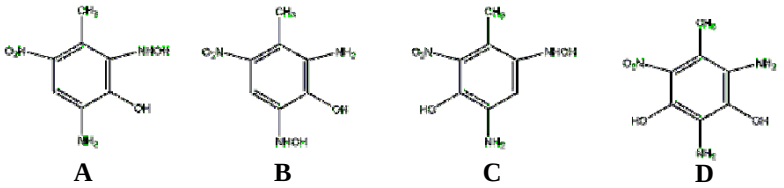

Figure 7. Possible structures resulting from the hydroxylaminobenzene mutase reaction on DHANT. **A**: 2-hydroxylamino-3-hydroxyl-4-amino-6-nitrotoluene; **B**: 2-amino-3-hydroxyl-4-hydroxylamino-6-nitrotoluene; **C**: 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene and **D**: 2,4-diamino-3,5dihydroxyl-6-nitrotoluene.

amine and the subsequent formation of a hydroxyl group at the 5-position. Furthermore, the products from the transformations of TNT by *P. pseudoalcaligenes* and *C. acetobutylicum* have substantially different HPLC retention times and UV-spectra which supports the conclusion that they are different isomers. HabB, which catalyzes the rearrangement of DHANT to the YM in *P. pseudoalcaligenes*, adds hydroxyl groups at the *ortho*-position exclusively (Nadeau, He et al. 2000; Nadeau, He *et al.* 2003) whereas an Fe-only hydrogenase has been implicated in the conversion of DHANT to a phenolic metabolite by *C. acetobutylicum* (Watrous, Clark *et al.* 2003).

Characterization of mechanism and enzymes responsible for TNT transformation.

2,4,6-Trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum.*

The role of hydrogenase in the reduction of TNT by *Clostridium acetobutylicum* was evaluated. An Fe-only hydrogenase was isolated and identified 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 = 152 μM. Comparisons between wild type and a mutant strain lacking the region encoding an alternate 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 over-expressed the hydrogenase activity resulted in maintained TNT reduction during late growth phases when it is not typically observed in wild type strains. Strains exhibiting under-expression 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 is 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 details of this work were published in Applied and Environmental Microbiology (Watrous et al., 2003) as listed in Appendix B and an expanded description

of this work is included in Appendix A1, including tables presenting data supporting conclusions drawn.

Enzymes responsible for transformation of TNT by *P. pseudoalcaligenes* JS45**.** Fiorella established that NB nitroreductase catalyzes the conversion of TNT to DHANT but not to 4ADNT (Fiorella and Spain 1997). Therefore, some nonspecific but possibly inducible nitroreductase is present in NB-grown cells of JS45 that can catalyze the reduction of 4HADNT to 4ADNT. The significance of another nitroreductase is that 2ADNT has been discovered as another route to the YM. Whether the compound is identical to the YM from DHANT has not been determined. The compounds are indistinguishable by HPLC by retention time and UV-Vis spectra. Both early and late forms are produced. Both intact cells and cell extracts catalyze the transformation. Time course experiments suggest that the route through 2ADNT might be the major route to the YM, but a route that goes through 2ADNT cannot go through DHANT.

Two genes that encode HAB mutases are carried by strain JS45. Both genes have been cloned and expressed in *E. coli*; but only HabA is expressed in JS45 during growth on NB (Davis, Paoli *et al.* 2000). HabB, the protein that is not expressed during growth on NB, however; converts DHANT to the YM, while HabA does not. To clarify some of the anomalies, the JS45 genes that encode nitrobenzene nitroreductase and HabA were cloned into *E.coli* and placed under the control of an IPTG-inducible promoter (Kadiyala, Nadeau *et al.* 2003). Cells and cell extracts of *E. coli* strain JS995 (Figure 8) were incubated with 2ADNT and TNT after induction with IPTG and compared with transformations by nitrobenzene-grown *P. pseudoalcaligenes* JS45. 2ADNT was converted to the YM, whereas

TNT disappeared without the formation of UV-Vis detectable products. During time course experiments with induced cells of JS995, TNT was converted to 4HADNT which then began to disappear, and only traces of YM were identified. A time course with 2ADNT revealed the accumulation and disappearance of the early YM, followed by the appearance of the late YM. An additional unidentified peak (2.4 min peak) not detected during TNT transformation also accumulated and began to disappear. Finally, a similar time course with 4ADNT also showed the accumulation and disappearance of the early YM followed by the appearance of the late YM, and the additional 2.4 min peak accumulated, but did not disappear. The rate of disappearance of 4ADNT was about half that of 2ADNT and TNT. The amount of YM and 2,4 min peak accumulated from 4ADNT was less than half the amounts accumulated from 2ADNT. The time course experiments with JS995 support the interpretation that a pathway through 2ADNT is the major route to the YM. The lack of accumulation of YM from TNT confirms that HabA does not convert DHANT to the YM.

Based on the preliminary results, studies were done to determine the enzyme activities responsible for transformation of TNT, 2ADNT, and DHANT.

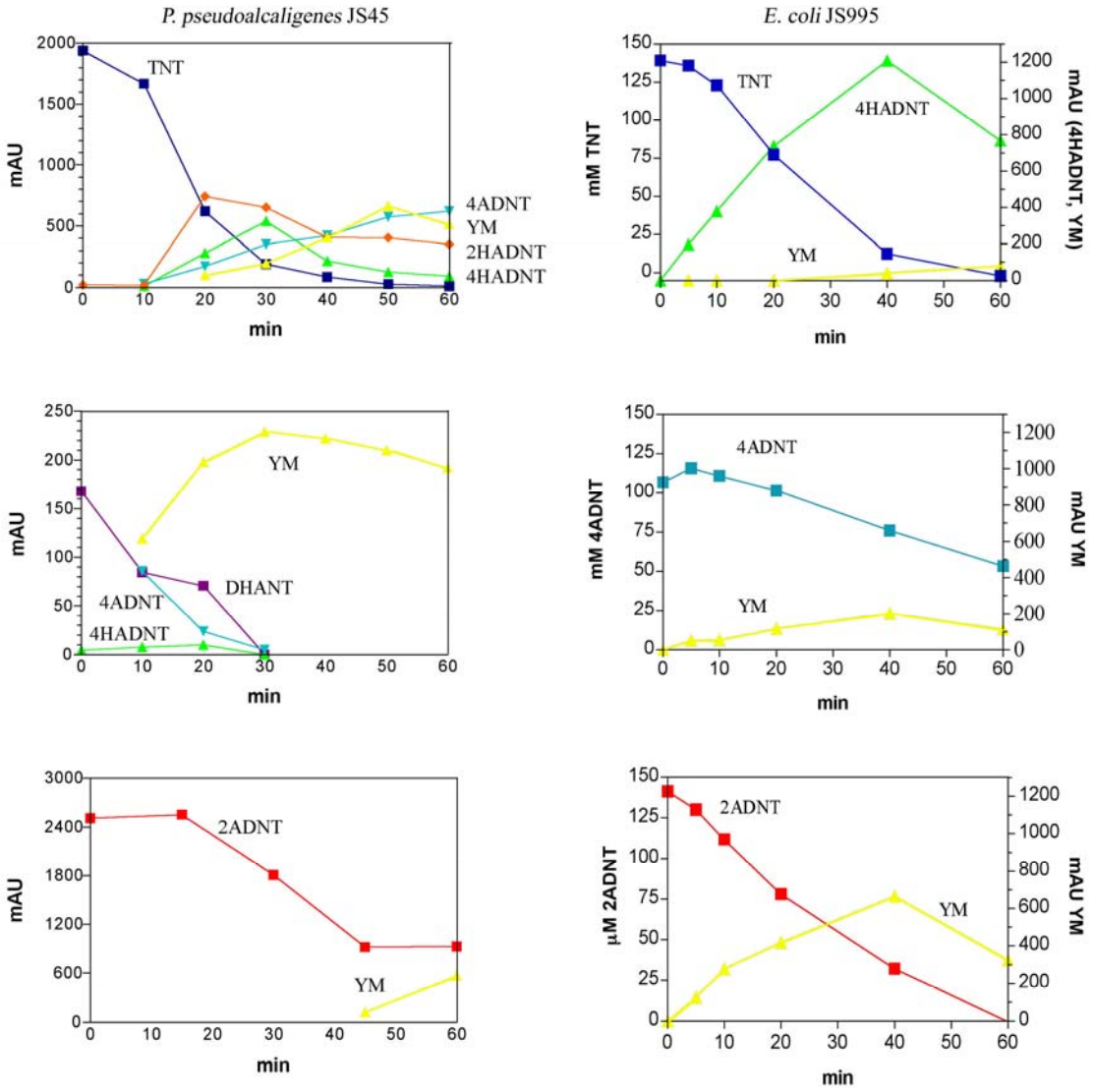

Figure 8. Transformation of A) TNT, B) DHANT, and C) 2ADNT by NB-grown JS45, and transformation of D) TNT, E) 4ADNT, and F) 2ADNT by E. coli JS995 after growth in LB and induction by IPTG.

Enzymes involved in the formation of the yellow metabolite. In order to determine whether the hydroxylaminobenzene mutase isoenzymes HabA and/or HabB are involved in the formation of the YM, we tested *E. coli* C43 (DE) strains that express *hab*A and *hab*B genes under an IPTG inducible promoter, as well as *P. pseudoalcaligenes* JS45 strains ΔA and ΔB containing deletions of *hab*A and *hab*B, respectively.

Resting cell experiments were performed with TNT, 2-ADNT and DHANT as substrates. The transformation activities of the succinate-grown JS45 wild type and of JS45 ΔB lacking *habB* were very similar. Both converted TNT and 2ADNT to the YM, but not DHANT. JS45 ΔA strain lacking *habA* converted neither TNT nor 2ADNT to the YM. The results indicated that HabA, but not HabB was required for transformation of 2ADNT to the YM.

The *E. coli* strain that expressed HabB converted DHANT but not 2ADNT or TNT to the YM. The *E. coli* strain that expressed HabA converted 2ADNT but not DHANT or TNT to the YM. Strain JS995 converted TNT to DHANT but did not convert DHANT to the YM. JS995 only made the YM from 2ADNT.

Nitrobenzene pathway enzymes from JS45 were partially purified and tested against TNT, 2ADNT, and DHANT. Partially purified nitrobenzene nitroreductase converted TNT to DHANT via 4HADNT as previously reported (Fiorella and Spain 1997). Partially purified HabB converted DHANT, but not 2ADNT to the YM. Partially purified HabA converted neither DHANT nor 2ADNT to the YM.

Previous work indicated that the reduction of TNT to DHANT is catalyzed by NB nitroreductase in cells of *P. pseudoalcaligenes* JS45 and JS52 (Fiorella and Spain 1997).

The current work shows that DHANT can be rearranged to the YM by the hydroxylaminobenzene mutase HabB . However, the rearrangement does not take place *in vivo* because HabB mutase is not expressed by *P. pseudoalcaligenes* JS45 after growth on NB or succinate (Davis, Paoli et al. 2000). Fiorella reported that the YM was formed from DHANT by extracts of NB-grown cells (Fiorella and Spain 1997). The conclusion was based on the results of experiments with crude cell extracts and without a mass balance. We now believe that the cultures used by Fiorella to make the cell extracts likely contained a second nitroreductase that reduced TNT at the 2-nitro position. We infer the lack of induction from the relatively slow TNT transformation rates seen in the earlier work. The results from strain *E. coli* JS995 are consistent with Fiorella's results. TNT was mostly converted to DHANT, and DHANT was not converted to the YM, but disappeared from the culture fluid over several hours in the presence of oxygen. The result was also consistent with radiolabelled experiments, where the radiolabelled DHANT peak disappeared from the *E. coli* JS995 culture leaving only low levels of radiolabeled products over the entire LC chromatogram.

Hydroxylaminobenzene mutase HabA is required to convert 2-ADNT to the YM, but because hydroxylaminobenzene mutases can convert only hydroxylamines to aminophenols (Nadeau, He *et al.* 2003) 2-ADNT cannot be the direct substrate for HabA. 2-ADNT must first be converted to a hydroxylamino compound that can then be rearranged by HabA. The activity that converts 2ADNT into a substrate for HabA is also likely to be a nonspecific activity, being present in both *E. coli* strains and in nitrobenzene-grown as well as succinate-grown JS45 cultures.

Preliminary studies on the TNT nitroreductase. The ability of succinate-grown *P. pseudoalcaligenes* JS45 cells to transform TNT to the YM indicated the participation of a nitroreductase distinct from NB nitroreductase. NB nitroreductase is induced by NB and attacks TNT exclusively at the *para*-position. The nonspecific nitroreductase is part of a constitutive, second pathway in which TNT is converted to 2-ADNT rather than DHANT. The purification of this nitroreductase is currently in progress. Preliminary experiments indicate that the nitroreductase is NADPH-dependent, localized in the soluble protein fraction and has a higher activity with TNT than with NB. Partially purified extracts of succinate-grown JS45 convert radiolabelled TNT to 2HADNT in the presence of NADPH.

Our results suggest that a novel pathway, via 2-ADNT is the predominant TNT transformation pathway in strain JS45. The finding of only traces of radiolabel in DHANT, but significant accumulation of label in the YM supports the findings of the enzyme work. The nitroreductase enzymes involved in the pathway are expressed during growth on NB or succinate and are therefore not induced by NB. The identity of the nitroreductase for reduction of TNT to 2-ADNT has not yet been determined but the nitroreductase is singular in that it attacks TNT at the *ortho* position.

The initial transformation is not specific as indicated by the fact that it took place in both *P. pseudoalcaligenes* and *E. coli*. The reaction was also too rapid to allow detection of the postulated amino-hydroxylamino-nitrotoluene by HPLC.

Fate of TNT transformation products in natural systems.

The reactivity of partially reduced metabolites of 2,4,6-trinitrotoluene in natural systems (*C. acetobutylicum*). The reactivity of partially reduced metabolites of TNT, namely arylhydroxylamines and nitrosoarenes, was evaluated with a simple biological system and with components of soil natural organic matter (NOM). This study was carried out to determine the impact of irreversible binding to soil NOM and biomass, commonly observed during the reductive transformation of polynitroaromatic contamination. The study focused on partially reduced metabolites rather than the completely reduced arylamine metabolites that have already been extensively investigated for their role in binding to soil NOM.

In the simple bioreduction system of *Clostridium acetobutylicum* cell-free extract/molecular hydrogen (electron donor), 10% of the initial ^{14}C was found bound to solid proteinaceous material following sequential anaerobic/aerobic treatment. A review of the nitroso and hydroxylamino functional group chemistry revealed that the nitroso-thiol reaction was most likely responsible for the reaction with proteins. The introduction of a model thiol, 1-thioglycerol, into an anaerobic mixture of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 2,4-dihydroxylamino-6-nitrotoluene (DHANT) resulted in the formation of a new product, only when the reaction mixture was exposed to air. The results from the model reaction confirmed that thiols could act as competing nucleophiles for nitroso compounds, which are readily formed from hydroxylamino compounds upon exposure to air.

The reactivity of arylhydroxylamines and nitrosoarenes with standard humic acids was investigated using 4HADNT and nitrosobenzene as model compounds, respectively. Contrary to results reported by others (Achtnich, Pfortner *et al*. 1999), 4HADNT was found to be nonreactive towards humic acid at humic acid concentrations in excess of dissolved organic matter concentrations found in nature. Conversely, nitrosobenzene reacted rapidly with humic acids, with the extent of reaction being highest for humic acids that had a high protein content. Humic acids that were pretreated with a thiol derivatizing agent showed diminished capacity for reaction with nitrosobenzene. Since nitroso intermediates from TNT reduction are difficult to synthesize and are rarely observed in nature due to their high instability, their electrophilic characteristics were evaluated using a molecular modeling approach. Molecular models of potential TNT nitroso intermediates were compared with those of the strongly electrophilic nitrosobenzene. The comparison revealed that 2-nitroso-4-hydroxylamino-6-nitrotoluene was more likely to react similarly to nitrosobenzene than 4-nitroso-2,6-dinitrotoluene.

The details of this work were published in Environmental Science & Technology (Ahmad and Hughes, 2002) as listed in Appendix B and an expanded description of this work is included in Appendix A2, including tables presenting data supporting conclusions drawn.

Fate of *P. pseudoalcaligenes* JS45 transformation products in natural systems. The fate of ^{14}C-TNT in soil microcosms in the presence of *P. pseudoalcaligenes* JS45 was evaluated. JS45 cultures were grown on succinate or nitrobenzene. The cells were washed in minimal media then suspended in minimal medium with ^{14}C-spiked TNT (100 μM) plus the growth substrate (200 μM nitrobenzene or 1 mM succinate). Cultures were divided and freshly collected garden soil was added to one culture (10% wet weight/volume). Cultures were shaken at 200 rpm at 30 °C. Samples were taken at 0, 30, 60, and 120 min. At 60 min, cultures without added soil were again divided and garden soil was added to one of the portions. Samples were centrifuged to remove the soil, and the culture fluid was analysed by HPLC. 10 second fractions were collected and analysed by scintillation counting. Each experiment was run twice.

Figures 9 and 10 show the distribution of radiolabel in aqueous components at each sampling time, with the initial samples on the left. Top rows show cultures without soil, middle rows show cultures with soil, and the bottom row is the culture with soil added after a delay. In all cultures, all the initial radiolabel was in the TNT peak. By 30 min, most of the TNT was converted to 2HADNT, 4HADNT, and 4ADNT. In cultures without soil, most of the radiolabel accumulated in polar products, including the YM as the HADNTs disappeared. 4ADNT was the principal non-polar product that persisted. In cultures with soil, few radiolabelled polar products were detected, and no YM was found in the aqueous phase. Again, 4ADNT was the principal persistent non-polar product.

Following prolonged incubation (3 weeks), roughly half of the radiolabel recovered was found in the acetonitrile-washed soil pellet in cultures with soil added (Table 1). In cultures without soil most of the radiolabel remained in the aqueous fraction.

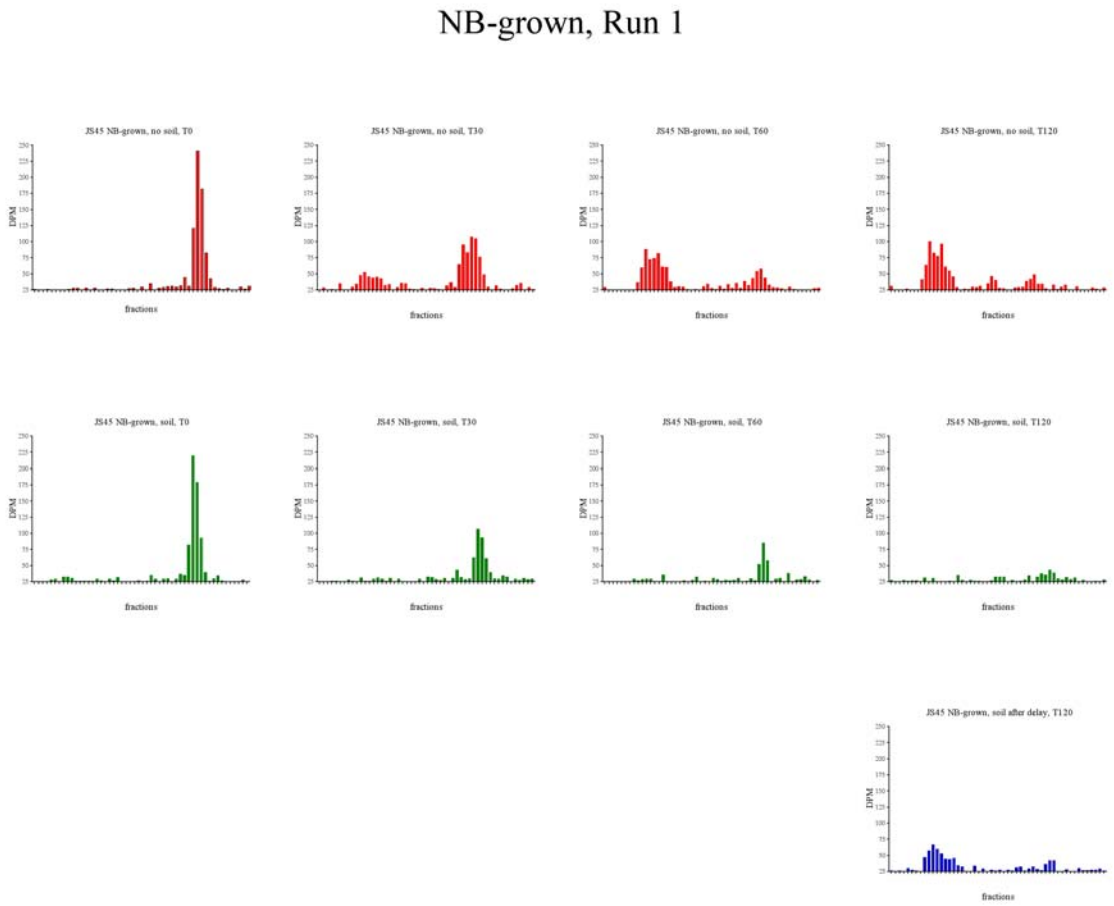

Figure 9. Transformation of ^{14}C-TNT by nitrobenzene-grown cultures of *P. pseudoalcaligenes* JS45. All figures show DPM plotted on the same scale with 0, 30, 60, and 120 min samples plotted from left to right.

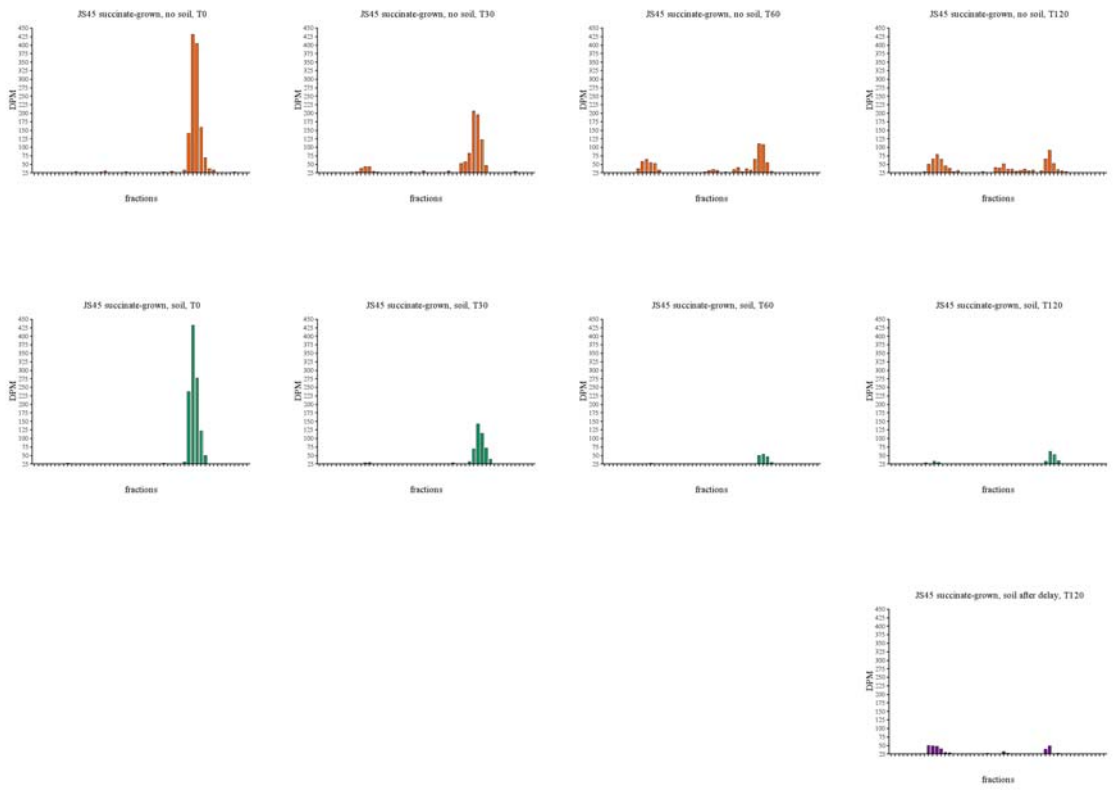

Figure 10. Transformation of ^{14}C-TNT by succinate-grown cultures of *P. pseudoalcaligenes* JS45. All figures show DPM plotted on the same scale with 0, 30, 60, and 120 min samples plotted from left to right.

Recovery of total radiolabel from cultures with soil was much lower than the total recovered in cultures without soil. Poor recovery was likely due to the inability to sample the larger/heavier soil components in the bottom of the flasks.

The lack of polar metabolites in the aqueous phase of cultures with soil added and the large percentage of radiolabel in the soil, suggests that polar metabolites or precursors to polar metabolites (nitroso- or hydroxylamino- compounds) were bound to the soil in our aerated and stirred systems. Earlier workers found that such compounds became covalently bound to soil components in anaerobic-aerobic composting systems (Daun, Lenke *et al.* 1998; Achtnich, Fernandes *et al.* 1999) and the binding was essentially irreversible (Achtnich, Sieglen *et al.* 1999; Weiß, Geyer *et al.* 2004).

Although both nitrobenzene-grown and succinate-grown cultures rapidly transformed TNT to the same metabolites, nitrobenzene-grown cultures mineralized more TNT than did the succinate-grown cultures (Table 1). The bulk of the mineralization occurred not during the period of rapid TNT transformation, but sometime during the immediate 2 weeks following the initial transformation. The percent of mineralization achieved over a 3 week period is relatively high for bacterial cultures (Weiß, Geyer *et al.* 2004) and the higher mineralization with the nitrobenzene-grown cultures without added soil indicates that some portion of the more polar TNT metabolites are susceptible to the enzymes of the nitrobenzene degradation pathway.

Table 1. Distribution of radiolabel in nitrobenzene- and succinate-grown cultures after 3 weeks incubation as the percent of total radiolabel added.

Fraction	$^{14}CO_2$				Aqueous	Solids	Total
	Initial	2 weeks	3 weeks	Total			
NB 1	1.1	5.9	1.0	8.0	91.8	0.5	100.3
NB 2	1.1	6.8	1.3	9.2	78.9	1.2	89.3
NB-S 1	0.5	1.8	0.5	2.8	27.7	29.5	60.0
NB-S 2	0.4	2.4	0.5	2.4	20.6	24.0	47.0
NB-DS 1	0.6	5.2	1.4	7.2	40.5	21.5	69.2
NB-DS 2	0.7	4.5	0.4	5.6	30.2	24.1	59.9
Su 1	0.9	1.7	0.4	2.9	66.3	4.0	73.2
Su 2	0.9	2.1	0.5	3.4	65.3	15.2	83.9
Su-S 1	0.7	1.7	0.6	3.0	17.7	23.4	44.0
Su-S 2	0.5	1.8	0.6	2.9	17.3	27.0	47.0
Su-DS 1	0.6	2.2	0.9	4.0	20.5	21.3	45.5
Su-DS 2	0.6	2.6	0.8	4.0	17.1	19.6	40.7

NB, nitrobenzene grown; Su, succinate grown; S, soil added; DS, soil added after 1 h

Develop strategies to direct TNT metabolism to ring fission products.

The project was begun with the assumption that the YM was a ring-fission product, based on its behavior in solvent systems and the UV-Vis spectrum. We now know that the YM is a highly substituted aromatic compound with one of the three structures shown in Figure 7A, 7B, or 7C. Because the YM is not a ring-fission product we redirected our efforts towards determination of how to most effectively use *P. pseudoalcaligenes* JS45 in a field remediation system.

Nitrobenzene is more acutely toxic than TNT and thus is unlikely to be used as a primary growth substrate in TNT remediation systems. However, this project has shown that constitutive enzymes, including the nonspecific nitroreductase(s) and the constitutively expressed HabA will transform TNT almost as well as the fully expressed enzymes of the nitrobenzene-degradation pathway. *P. pseudoalcaligenes* JS45 is a natural environmental isolate and therefore faces few regulatory restrictions on its use in remediation systems. With the idea that the constitutively expressed enzymes of JS45 could be useful in TNT remediation systems *in situ*, we examined alternative carbon sources for cometabolism of TNT by JS45.

P. pseudoalcaligenes JS45 was incubated in minimal with i) 50 μM succinate as the carbon source and 50 μM TNT as the nitrogen source, ii) 50 μM TNT as the carbon source and 50 μM NH_4Cl as the nitrogen source and iii) 50 μM TNT as the sole source of carbon and nitrogen. No growth occurred under these conditions: *P. pseudoalcaligenes* JS45 was unable to use TNT as the sole source of carbon and/or nitrogen. When *P. pseudoalcaligenes* JS45 was grown in the presence of a carbon and nitrogen source (succinate and NH_4Cl or NB), TNT was transformed.

To develop a co-substrate for field implementation, various co-substrates were screened with JS45. Minimal medium + NH_4Cl was tested with the following carbon sources: glucose 0.5%, succinate 0.5% and molasses 0.1%. Growth and TNT degradation were monitored at different times.

Best growth resulted from molasses as carbon source (Figure 11). Similarly, TNT transformation was most rapid with molasses as carbon source (Figure 12). The molasses-grown cells took less than 24 hours to transform 100 μM TNT, the succinate-grown and NB-grown ones 42 and 52 hours respectively. Interestingly, JS45 was not able to use glucose as carbon source and no TNT transformation was observed in cultures with glucose. Molasses is a cheap and readily available carbon source with demonstrated value for cometabolism of TNT (Manning, Boopathy *et al.* 1995; Widrig, Boopathy *et al.* 1997). Remediation systems based on cometabolism of TNT by JS45 during growth on molasses could be feasible for field application.

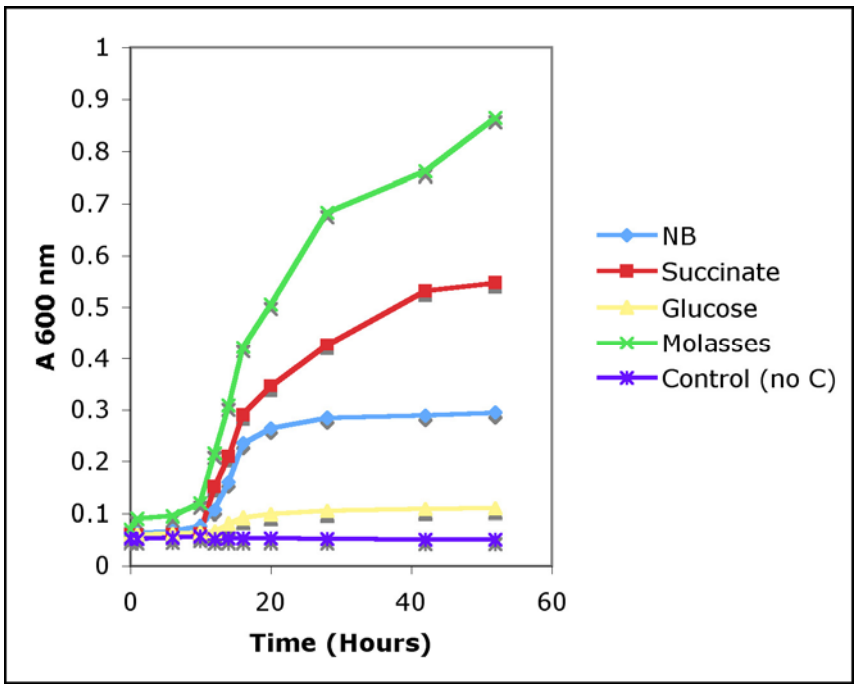

Figure 11. Growth of JS45 with alternate carbon sources.

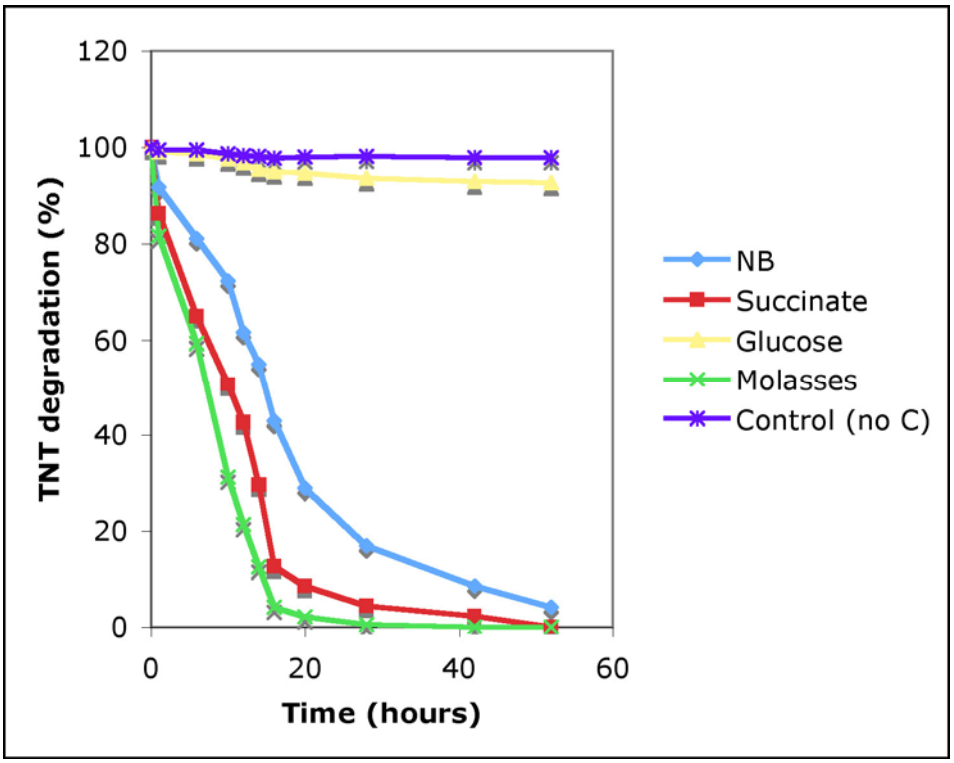

Figure 12. Transformation of TNT by JS45 grown on alternate carbon sources.

Mineralization of TNT.

Mineralization of TNT using a two-stage anaerobic-aerobic process. Complete mineralization is the desired endpoint of explosives remediation processes because the production of CO_2 represents an unequivocal elimination of the contaminant and all potentially toxic intermediates. Initial studies completed in our laboratory demonstrated that TNT mineralization occurred via combined biotic-abiotic mechanisms during a two-stage anaerobic-aerobic treatment process. Anaerobic incubations of *Clostridium acetobutylicum* with ^{14}C-TNT produced a reduced hydroxylated metabolite that served as a more favorable starting point for mineralization in subsequent aerobic treatment. Total ^{14}C recovery during this second stage ranged between 95 and 101% of the initial activity. It was determined that aerobically-maintained reactors with unrestricted growth converted 13.3 to 14.1% of the initial radiolabeled metabolite to $^{14}CO_2$. This was significantly higher than in the aerobic reactors in which growth was restricted (via autoclaving, mineral omission, or biocide amendments). Production of $^{14}CO_2$ in the restricted-growth reactors ranged from 7.9 to 9.7%, suggesting that an abiotic pathway was responsible for greater than 56% of the observed mineralization yield. Extended anaerobic incubation in place of the aerobic second stage of the dual-step process limited mineralization (1.1 to 1.7% $^{14}CO_2$) by blocking growth and/or auto-oxidation pathways. The addition of structural analogs to TNT failed to induce mineralization. Inoculation with a consortium enriched on 2,4-dinitrotoluene indicated that mineralization was not tied to the stimulation of specific degrading organisms. The key difference appeared to be the production of a reduced TNT metabolite that proved particularly susceptible to further

transformation and eventual mineralization. This represents the most successful outcome reported to date for an anaerobic-aerobic dual-stage process.

However, results which demonstrated mineralization of TNT were not readily repeatable and therefore were not published in peer-reviewed literature. Subsequent studies showed no significant difference between mineralization observed in active systems and jn inactivated controls. Expanded details of studies which demonstrated mineralization and those where mineralization was not present are included in Appendix A3.

Mineralization of TNT by *P. pseudoalcaligenes* JS45. Several short-term experiments with *P. pseudoalcaligenes* JS45 measured mineralization of ^{14}C-TNT. In all experiments in which incubation times were 3 days or less, very little mineralization was detected (less than 2%). Only during prolonged incubation (discussed above under transformation in natural systems) was significant mineralization detected during a brief period after TNT was no longer detected in the cultures. Mineralization required highly active nitrobenzene-grown cultures and the absence of soil which apparently bound the polar metabolites or their precursors that went on to be mineralized in soil-free systems. Thus, it is unlikely that JS45 could effect much mineralization of TNT in a field remediation system. The strain might, however, be an effective bioremediation agent for TNT-contaminated water in a bioreactor where it could be grown with nitrobenzene under controlled conditions.

Conclusions

This research examined the biochemical mechanism of TNT transformation in novel aerobic and anaerobic degradation pathways in an effort to advance our understanding of the fundamentals of biological transformation of TNT. Conclusions are summarized for research which investigated reactivity of reduced metabolites of TNT with humic substances and in cell extract systems, and for work which established the enzymes responsible for transformation of TNT in aerobic and anaerobic systems. The unresolved nature of work on mineralization of TNT is described. This fundamental information about biological transformation of TNT will aid in developing strategies that harness the biological transformation of TNT in remediation systems.

The following general conclusions were reached regarding the reactivity of partially reduced metabolites of TNT in a *C. acetobutylicum* cell extract system (a simple bioreduction system):

- Partially reduced metabolites of TNT, namely DHANT and 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol, bind to proteinaceous materials following sequential anaerobic/aerobic treatment.
- The more reduced arylhydroxylamino metabolite of TNT, DHANT, oxidizes upon exposure (to most likely 2-nitroso-4-hydroxylamino-6-nitrotoluene) and readily reacts with model thiols such as 1-thioglycerol.
- Binding between reduced TNT metabolites and proteins occurs most likely by the nitroso-thiol reaction owing to the aerobic requirements of the reaction.

The following conclusions regarding reactive functionalities and substituent effects were drawn from the studies involving the reaction of partially reduced metabolites of nitroaromatics and various IHSS standard humic acids:

- 4HADNT shows no appreciable reactivity towards a standard humic acid (i.e., IHSS peat humic acid standard) under anaerobic conditions. When conditions are made aerobic at pH 7 the 4HADNT precipitates out of solution in the form of 4,4',6,6'-tetranitro-2,2'-azoxytoluene.
- Nitrosobenzene readily reacts with various humic acids.
- The extent of nitrosobenzene binding increases as the protein content of the humic acid increases.
- Thiol derivatization of humic acids adversely affects their reaction with nitrosobenzene with greatest effect shown by the humic acid having the highest protein content.
- Molecular modeling analyses predict that the more reduced nitroso metabolites of TNT such as 2-nitroso-4-hydroxylamino-6-nitrotoluene should be closer in electrophilic character and reactivity to nitrosobenzene than the less reduced 4-nitroso-2,6-dinitrotoluene.

The Fe-only hydrogenase was determined to be the primary enzyme responsible for TNT reduction in *C. acetobutylicum* systems and conclusions regarding the enzyme and mechamism responsible for TNT transformation by *C. acetobutylicium*.

- The Fe-only hydrogenase was primarily considered as the catalyst for TNT reduction as opposed to the Ni-Fe hydrogenase, which is typically associated with hydrogen uptake.

- Characterization of the purified enzyme allowed determination of the N-terminal peptide identical to the 67 Kda Fe-only hydrogenase.
- The enzyme exhibits saturation kinetics with Km for TNT of 152 μM.
- Sequence analysis of the hydrogenase gene identified the enzyme as a soluble protein with 4Fe-4S clusters, characterized by the iron-sulfur binding region signatures at amino acid positions 121-132.
- A transmembrane spanning region occurs at amino acid positions 150-200.
- The sequence shows 99% homology to periplasmic (Fe) hydrogenase large subunit of *Desulfovibrio vulgaris,* which may be involved in hydrogen uptake for the reduction of sulfate to hydrogen sulfide in the electron transport chain.

The following conclusions apply to the aerobic degradation of TNT by *P. pseudoalicaligenes* JS45, a soil organism with potential for use in bioremediation systems.

- *P. pseudoalcaligenes* JS45 contains multiple nitroreductases that are specific for nitro-groups substituted at different positions on the benzene ring, and thus direct TNT transformation towards different end products. Metabolic engineering to enhance the activity of selected nitroreductases and/or to silence the expression of nitroreductases that cause unwanted transformations might result in an organism that could be used in a more predictable or robust treatment system.

A constitutively expressed nitroreductase converts TNT to 2ADNT which is subsequently converted to a polar, transient yellow metabolite by the action of the reductase and mutase.

A wide range of aerobic bacteria, including *E.* coli, can catalyze the conversion of TNT to amino derivatives. All of the nitroreductases that have been purified, however, seem to catalyze only the reduction to the hydroxylamino derivatives. It has been a mystery how the conversion of the hydroxylamino compounds to the amines is carried out. In JS45 we have clearly demonstrated the activity of the enzyme that produces amino compounds from TNT. The existence of the enzyme is crucial to avoid accumulation of the much more reactive hydroxylamino compounds. Purification and characterization of the novel enzyme will reveal whether it is widespread and whether its activity is responsible for the often observed accumulation of monoamino derivatives of TNT under aerobic conditions. The advances in understanding from JS45 will allow prediction and enhancement of the activity in a variety of systems. Experiments with radiolabelled TNT reveal that a significant fraction of the TNT can be mineralized while the remainder is converted to polar products or bound to soil. The details of the reactions and the rigorous identification of the yellow metabolite remain to be worked out. Complete understanding of the pathway will provide the basis for enhancing its activity.

- The discovery that *E. coli* strain JS995 containing nitrobenzene nitroreductase and mutase B can extensively transform TNT clearly indicates the efficacy of the two enzymes for elimination of TNT. Additional strain improvement and further

investigation of the process might yield a more effective strain for practical application, particularly in closed systems.

- Nitrobenzene grown cells transform TNT to the yellow metabolite via 2HADNT and DHANT. Nitrobenzene nitroreductase and mutase catalyze the reactions in nitrobenzene grown cells, but not in succinate grown cells.
- JS45 can mineralize TNT when the nitrobenzene degradation pathway is expressed, but mineralization is suppressed by the presence of soil because of binding of polar metabolites of TNT or their precursors. Elucidation of which specific metabolites are mineralized might enable development of strategies to increase the rate and extent of mineralization. Alternatively, the binding to soil might be taken as an effective endpoint.
- JS45 could be used in a bioremediation system to cometabolize TNT with molasses as the primary carbon source. The inclusion of nitrobenzene as an inducer might slightly enhance the extent of mineralization, but not sufficiently to warrant the additional technical difficulties of using a toxic compound in a treatment system. A variety of other systems based on cometabolism of TNT have been used in the past. The advantage of the aerobic system based on JS45 would be that the products are not polyamines and additional bulking of the soil is not required.

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Appendix A

Supporting Data

Appendix A1

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

RICE UNIVERSITY

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

by

Mary Margaret Watrous

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IN PARTIAL FULFILLMENT OF THE
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APPROVED, THESIS COMMITTEE:

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HOUSTON, TEXAS

JUNE, 2002

ABSTRACT

2,4,6 –Trinitrotoluene Reduction by Hydrogenase in *Clostridium acetobutylicum*

by

Mary Watrous

Unique genetic modifications to *C. acetobutylicum* altered the level of hydrogenase expression, allowing study of the resulting effects on the 2,4,6-trinitrotoluene (TNT) reduction rates to be possible. A strain designed to over-express the hydrogenase gene resulted in maintained TNT reduction during late growth phases when it is not typically observed. Strains exhibiting under-expression of hydrogenase produced slower TNT rates of reduction correlating to the expected inhibition of each strain type. Hydrogenase activity, measured by hydrogen production, in *Clostridium acetobutylicum* correlates strongly (R^2=0.89) to TNT reduction rates. Indications suggested that hydrogenase potentially played an integral role in catalysis of TNT transformation by reducing its nitro substituents to the corresponding hydroxylamines. A mechanistic pathway is proposed by which this transformation takes place and may enhance the understanding of commonly found hydrogenases in other microorganisms and their ability to transform nitroaromatic compounds.

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basis of my work, as well as for developing an HPLC method to quantify TNT and its metabolites.

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CHAPTER 1 – INTRODUCTION

Contamination by 2,4,6-trinitrotoluene (TNT) is widespread at many munitions 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 (*1-3*), much current research has focused on remediation by biologic process development targeted at removal of TNT from the subsurface (*4*). Microbial reduction of TNT has been well established by a wide variety of aerobic and anaerobic microorganisms (*5,6*), and reduction by clostridia species has been extensively reviewed (*7*).

In many clostridia systems and in particular, for the purpose of this research, in *Clostridium acetobutylicum* systems, TNT reduction to hydroxylamino intermediates is observed without further reduction to corresponding amines (*8-11*). Historically, accumulation of this intermediate has not been observed in typical anaerobic reductive cultures (*5,12,13*). Elucidation of the mechanism by which this transformation takes place has thus become a research area of interest.

Evidence to date has suggested biocatalysis, by a constitutive enzyme, as the probable force driving this reduction (*9*). Several additional factors have indicated the possible role of hydrogenase in initial TNT transformation. For example, *C. acetobutylicum* reduces TNT rapidly only during initial stages of growth when acid production is high and hydrogen is being produced (*11*). Hydrogenase reversibly oxidizes hydrogen and is responsible for the observed hydrogen production; thus, the hydrogenase enzyme is thought to be active during this primary stage of *C. acetobutylicum* metabolism when TNT reduction is primarily reduced. Additionally, it

has been reported that TNT is reduced by crude extracts only when H_2 is a constituent in the atmosphere in which the assay is conducted (*11*) further implicating the role of hydrogenase. Both carbon monoxide and oxygen are well known inhibitors of the hydrogenase enzyme effectively blocking its activity by binding to the hydrogenase catalytic center (*14,15*). Accordingly, carbon monoxide has been shown to slow TNT reductive reactions (*11*) and oxygen irreversibly inhibits the capability of active crude extracts to reduce TNT. Another evidence directing focus on hydrogenase is that an enzyme with similar mechanism, carbon monoxide dehydrogenase, from a similar organism, *Clostridium thermoaceticum*, has been purified and identified as capable of TNT reduction to identical intermediates to the ones observed in the *C. acetobutylicum* cultures (*16*).

Having identified an enzyme on which to focus research allows for genetic approaches to be applied to effectively alter hydrogenase levels expressed in cell cultures. The specific techniques, termed metabolic engineering, take advantage of recombinant DNA methods in order to increase expression of hydrogenase when it would not typically be observed in wild type cells (*17*) as well as to inhibit the level of hydrogenase expression as compared to the wild type (*18*). As stated by Spain in his introduction to Biodegradation of Nitroaromatic Compounds and Explosives, "Recent advances in molecular biology pave the way for pathway engineering that would allow for complete degradation [of TNT] in a variety of organisms. Success would reduce the cost of bioremediation considerably for major explosives, including TNT" (*6*).

This work is thus focused on determining the nature of the role of hydrogenase in TNT reduction using a genetics approach with the following specific objectives:

- Characterize and sequence existing genetically manipulated hydrogenase plasmids to verify construct and expected effectiveness at altering hydrogenase expression levels.
- Transform plasmids into *C. acetobutylicum* and determine solvent production levels of each strain type to better understand hydrogenase effect on clostridia metabolism.
- Determine the effectiveness of each plasmid type in producing expected results regarding hydrogenase produced and TNT reducing capacity.
- Ascertain if a correlation exists between the hydrogenase activity of each strain and its corresponding ability to reduce TNT.
- Propose a reasonable mechanism by which TNT is reduced by hydrogenase if a correlation exists.

The format of this thesis is as follows: Chapter 2 provides a review of previous research regarding TNT reduction by *C. acetobutylicum,* the structure of hydrogenase and its suspected role in transformation of TNT, as well as genetic tools available to test the hypothesis proposed, providing the motivation behind this research; Chapter 3 details the materials and methods used to perform the research presented in this thesis; Chapter 4 contains the results of the studies; Chapter 5 provides a discussion of the results presented and describes their significance; Chapter 6 is a summary of the conclusions and findings of this research; Chapter 7 explains the significance of this work in a broader engineering context; and finally, Chapter 8 suggests areas for future work to be conducted on this topic.

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CHAPTER 2 - LITERATURE REVIEW

2.1 Introduction

This chapter provides background information needed to understand the motivation and interpretation of studies presented in subsequent chapters. It includes information on TNT contamination and a review of research examining its remediation. Detail is provided regarding metabolic mechanisms that may ultimately be responsible for TNT reduction in microbial systems. Finally material is presented regarding recent developments in the fields of biochemistry and molecular biology, specifically the use of genetic manipulations, which will be useful in this research to further elucidate a key enzyme involved in the catalysis of TNT reduction.

2.2 Transformation of TNT by *Clostridium*

Nitrated organic compounds, including explosives, are common contaminants in the environment. The most contaminated sites, typically army ammunition sites, exist where explosives have been manufactured, handled, and stored over the past century. In particular, wide spread contamination of 2,4,6-trinitrotoluene (TNT) is observed at these locations. Due to TNT's prevalence and persistence in the environment, concerns with its toxicity and human health effects have grown in recent years. Previous work has shown that TNT and its reduced metabolites, specifically 2,4-diamino-6-nitrotoluene (2,4-DANT); 2-amino-4,6-dinitrotoluene (2-ADNT); and 4-amino-2,6-dinitrotoluene (4-ADNT), strongly affect cells important to the human immune response (*1*). Studies conducted by the United States EPA indicate that TNT holds the ability to cause liver damage including cirrhosis and hepatic swelling (*2*). Evidence also reveals that these nitro compounds display cytotoxicity towards human cells and display mutagenicity in

bacterial strains, classifying these compounds as possible carcinogens (*3*). It is thus important to effectively remove these constituents from the soils they contaminate.

TNT can be reduced by a wide variety of aerobic and anaerobic microorganisms as has been discussed in collaboratively published review articles (*4,5*). High electronegativity of TNT's aromatic nitro groups ($R\text{-}NO_2$) causes high susceptibility to reductive attack. Reduction of aryl nitro groups to corresponding amines ($R\text{-}NH_2$), through nitroso (R-NO) and subsequently hydroxylamino (R-NHOH) intermediates, is often sited as the TNT transformation pathway in many systems (*4,6-8*). Current evidence suggests that TNT is only completely reduced to 2,4,6-triaminotoluene (TAT) under strictly anaerobic conditions (*7*). Upon further examination of anaerobic cultures, in attempt to identify those capable of TNT transformation, many microorganisms belonging to the genus *Clostridium*; which are obligate, saccharolytic anaerobes; have proven to be rather effective at reducing nitro groups of various compounds (*9*). In particular, *Clostridium acetobutylicum* has been employed in several experiments to transform TNT into metabolites (*8,10-12*). Although formation of aminodinitrotouenes and diaminotoluenes are often cited as initial intermediates of TNT reduction in anaerobic systems, studies utilizing *C. acetobutylicum* observed accumulation of the hydroxylamino intermediates, specifically 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT) and 2,4-dihydroxylamino-6-nitrotoluene (24DHA6NT), without formation of commonly observed amines (*11*). Further reduction of these metabolites, by *C. acetobutylicum,* results in formation of a phenolic amine through Bamberger rearrangement (*10*). This transformation product quickly decomposes in aqueous medium in anaerobic systems. The catalysis mechanism by which metabolites are

formed in these systems has not been clarified to date, although evidence for bio-catalysis has been presented and probable key enzymes have been indicated in reduction steps. However, much research exists that provides understanding and characterization of this organism, both metabolically and genetically, using the specific strain *C. acetobutylicum* ATCC 824, which is utilized in the research presented in this thesis.

The metabolism of clostridia has been the subject of extensive reviews particularly as it relates to solventogenesis, as clostridia naturally produce solvents, specifically acetone and butanol, which are important feed-stocks in the chemical industry (*13,14*). Clostridia undergo two distinct phases of metabolism. Initially, during exponential growth phase, clostridia produce high levels of the acids acetate and butyrate. It has been shown that *C. acetobutylicum* rapidly reduces TNT only in the acidogenic, or acid production, phase of growth. Cultures allowed to proceed into the solvent production phase, which typically occurs after several hours when pH has decreased significantly due to high acid levels, incompletely transform TNT (*12*).

Data also establishes that during periods of high acid production, increased levels of hydrogen production are observed (*15*). Clostridia cells are not capable of converting all of the available electron supply, stored as NADH, by the known pathways that proceed during acidogenis. Due to the presence of hydrogenase, an enzyme that reversibly oxidizes hydrogen, both excess electrons, from NADH supply, and protons, from acid production, can be utilized simultaneously to produce hydrogen. Hence increased hydrogen production, as is observed during acidogenisis, is typically associated with amplified activity of the hydrogenase enzyme.

It is also evident from previous work that during acidogenisis, it is theoretically impossible for hydrogen to be produced, from an electron equivalant balance standpoint on glucose breakdown to pyruvate resulting in NADH formation, in the concentrations at which it is observed. NADH, however, can be cyclically converted through reaction coupling with ferredoxin, allowing NADH to be a continuous source of electron donor (*16*). Ferredoxin-hydrogenase systems have previously been shown to reduce nitro-groups of other compounds. For example, an assay for ferredoxin level was developed in which metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] reduction by H_2 via hydrogenase is measured (*17*). Additionally, ferredoxin-hydrogenase systems in *Clostridium* have been shown to be responsible for the reduction of nitro groups of metronidazole when used as a bactericidal antibiotic as well (*18*). It thus seems likely, or at least possible, that this hydrogenase catalysis is also involved in TNT nitro group reduction.

2.3 Mechanism of the Hydrogenase Enzyme

In order to understand the probable role of hydrogenase in TNT reduction, it is important to more fully describe the mechanism of the hydrogenase enzyme and its variety of roles in microbial cells. As stated earlier, hydrogenase enzymes catalyze the reversible oxidation of hydrogen, which results in either uptake of or production of hydrogen in microbial systems in which it is active. Hydrogenase enzymes are present in and have been purified from at least 20 different microbial species. As a group, the enzymes display much heterogeneity with varying chemical composition, catalytic activity in producing and oxidizing hydrogen, levels of inactivation by oxygen and

carbon monoxide, and specificity for electron carriers. For extensive review of many hydrogenases see Adams et al., 1981 and Adams, 1990 (*19,20*).

All hydrogenases contain iron-sulfur clusters, however two main subgroups exist within the hydrogenase category: those that contain a nickel center, Ni-hydrogenases; and those that contain iron but lack nickel, Fe-only hydrogenases. Ni-hydrogenases are much more commonly observed and provide an array of functions in organisms. They are typically associated with systems in which hydrogen is consumed; here hydrogen is used as a reductant to generate energy supplies. However, our focus will lie in the understanding of Fe-only hydrogenases, as they are typically associated with anaerobic bacteria, such as clostridia, that use non-oxygen compounds as a terminal electron acceptor in substrate level phosphorylation. In these systems, hydrogen is produced as a disposal method for excess reductant in the absence of electron acceptors other than protons (*19*).

Fe-only hydrogenases are remarkably more efficient at producing hydrogen than those in the Ni-hydrogenase category. As the role of Ni-hydrogenases is typically H_2 uptake, low values for hydrogen production are expected; they have been shown to produce H_2 in concentrations from 1-150 μmol H_2/min per mg of protein (*19*). In contrast, Fe-only hydrogenases of strict anaerobes evolve hydrogen on the order of 5,500-10,400 μmol H_2/min per mg of protein (*20*).

The elucidation of the Fe-only hydrogenase structure reveals a series of 5 iron-sulfur [Fe-S] clusters which work in series to produce these observed, high levels of hydrogen (*21*). It should be noted that although the Fe-only hydrogenase primarily studied and characterized belongs to *Clostridium pasteurianum*, its function and thus

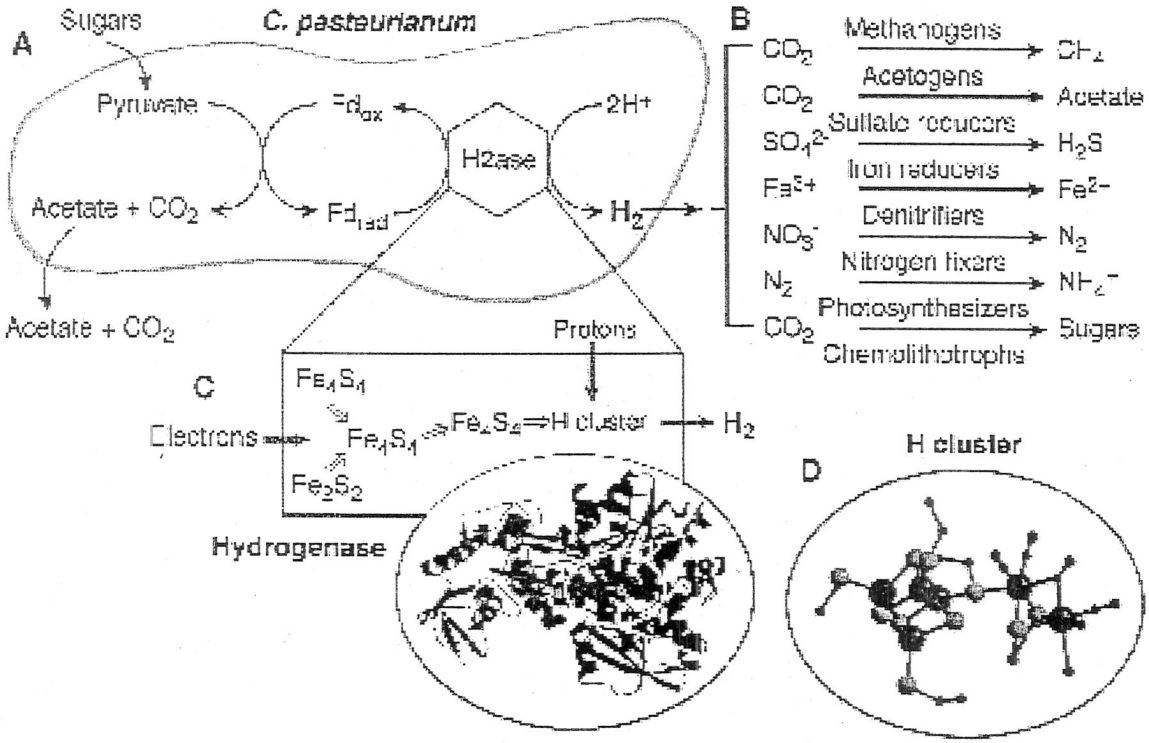
structure resemble that of *C. acetobutylicum*. It appears that the four initial clusters compose an electron transfer chain that lead to the fifth center, termed the H-cluster. The H-cluster is the center of catalytic activity and although the folded protein structure causes this center to be internal, it is accessible to electrons through the electron transfer chain and to protons through a hydron channel (*22*). The hydron channel is composed of an amino acid chain and a water molecule, in conjunction capable of proton transfer; unseen in the Ni-hydrogenases, this difference plays a vital role in direction in which the reversible hydrogen oxidation is carried out (*21*).

The structure of the hydrogenase gives insight into its catalytic functionality. The obligate anaerobe clostridium lacks a cytochrome system and the typical aerobic mechanism for oxidative phosphorylation. They instead obtain energy, in the form of ATP, from substrate level phosphorylation during fermentation as a means of oxidizing the reduced carriers. The redox balance is maintained by channeling electrons from reduced substrate to H_2 via the hydrogenase and its specific electron carrier without need for a terminal electron acceptor other than protons (*19*). Fermentative organisms, for example *C. acetobutylicum,* utilize this process to regenerate oxidized electron carriers such as the protein ferredoxin in order to sustain sugar oxidation through pyruvate (*23*). See Figure 2.1 for a schematic of hydrogenase and hydrogen metabolism in clostridia.

2.4 Hydrogenase's Suspected Role in TNT Reduction

It is hypothesized that an Fe-only hydrogenase coupled with a ferredoxin protein, is responsible for catalyzing the breakdown of TNT in *C. acetobutylicum*. Several lines of evidence exist which indicate hydrogenase. For example, Hughes et. al. have shown that TNT is actively reduced by *C. acetobutylicum* cultures which have been grown

Figure 2.1 – Hydrogenase and Hydrogen Metabolism in Clostridia



(**A**) The cell of *C. pasteurianum* whose metabolism involves oxidation of sugars and evolution of hydrogen by the iron-only hydrogenase designated as a hexagon. (**B**) The range of organisms that use hydrogen as a reductant and use the nickel-iron uptake hydrogenase. (**C**) Schematic of iron-only hydrogenase enzyme showing paths for electron and proton transfer converging at the H-cluster. (**D**) Schematic of the H-cluster showing the six-iron cluster with a two-iron subcluster bound to five CO or CN^- ligands.

This figure is taken from Adams, 1998 (*23*).

without any nitroaromatic compound present (*10*). This result suggests that the enzymes involved in TNT reduction by *C. acetobutylicum* are constitutive and do not require a nitroaromatic-inducing substrate to stimulate activity. Hydrogenase, as was established previously, is an active enzyme in many microorganism system, and thus fits the prescribed characteristic as constitutive. Additionally, Hughes et. al. further strengthen the observation that, as discussed in a previous section, correlation appears to exist between rates of TNT reduction and hydrogen production by hydrogenase in the acidogenic phase (*12*) by reporting that TNT is only reduced by crude cell extracts when H_2 is a constituent of the anaerobic atmosphere in which the TNT reduction assay is preformed.

Additional evidence becomes apparent when inhibition studies are compared between hydrogenase activity and TNT reduction. Both carbon monoxide (CO) and oxygen (O_2) are well-established inhibitors of hydrogenase and it has been shown that these molecules also slow or cease TNT reduction when introduced into a TNT assay system. One study observed slowing rates of TNT transformation to intermediates, by four fold, during solventogenesis when carbon monoxide was introduced (*12*). It was later shown that, mechanistically, a single CO molecule binds to the oxidized H-cluster, the center of catalytic activity, of hydrogenase effectively inactivating it (*24*). Similarly, assays for *C. acetobutylicum* reduction of TNT are obligatorily carried out anaerobically due to oxygen sensitivity and hydrogenase's irreversible inactivation by O_2 has been well established. Consistency between irreversible inactivation of the hydrogenase enzyme in *C. acetobutylicum* and slowed or ceased rates of TNT reduction further links this enzyme to its proposed catalytic role.

Recently, Huang et. al. purified and characterized a carbon monoxide dehydrogenase (CODH), from *Clostridium thermoaceticum*, similar in structure and function to the hydrogenase of interest (*25*). The purified CODH enzyme reduced TNT to the same intermediates that accumulate in *C. acetobutylicum* TNT reduction. By showing that CODH transforms TNT in the presence of CO and without the presence of ferredoxin or viologen, common electron acceptors, and that cyanide, an inhibitor of CODH, also inhibited TNT reduction in their system, Huang et. al. proposed CODH as the preliminary catalytic enzyme involved in TNT reduction by *C. thermoaceticum*. Hydrogenase enzymes from *C. acetobutylicum* have been purified in a similar manner, using TNT reduction activity as the basis for purification (*26*). This study also found the purified hydrogenase to transform TNT in the presence of H_2 without the addition of the electron acceptor, ferredoxin. This work, however, has not been published and in conjunction with the research presented in this thesis, hopes to provide substantial verification of hydrogenase as the preliminary catalytic mechanism by which TNT is reduced in *C. acetobutylicum*.

2.5 Genetic Tools

In order to determine direct effect of hydrogenase activity levels on the reduction of TNT, it is useful to examine systems in which these levels are varied in order to correlate enzyme activity with TNT response. Thus this section will examine current possibilities in the field of molecular biology that make this possible.

In recent years, developments in the area of biochemistry have led to the new field of metabolic engineering. In metabolic engineering, recombinant DNA methods, such as insertion, deletion, or other genetic modifications, allow for controlled alteration

of cellular metabolism. These alterations provide the possible application of inducing desirable cellular traits in microorganisms that play an important environmental or industrial role. For example, metabolic engineering of solventogenic clostridia has been the focus of much current research in order to develop strains with enhanced solvent producing capabilities (*13*). Genetic tools also hold the capability to provide useful means for closely examining effects of particular enzymes on compounds of interest. These tools can be employed in this instance to observe the effects of manipulating levels of hydrogenase in *C. acetobutylicum* on the capacity for this organism to reduce TNT.

In order to effectively utilize these strategies for *C. acetobutylicum*, altered DNA must be introduced into the host microorganism and subsequently expressed. This is done by a method termed transformation, in which altered DNA is incorporated into the microorganisms in the form of a plasmid which has been produced in sufficient quantitiy using *E. coli* as a substitute host due to its insensitivity to oxygen. Attempts to transform *C. acetobutylicum* in the past failed due to the presence of a restriction enzyme in *C. acetobutylicum*, which recognizes a site commonly found in *E. coli* plasmids often used in the transformations (*27,28*). Subsequent studies achieved successful transformation by protecting plasmid DNA from restriction cutting using methylation techniques in which a second phage plasmid, a methyltransferase, is introduced in conjunction with the plasmid DNA containing the gene of interest (*29*). Plasmids transformed in this manner proved to maintain stability and to produce in sufficiently high copy numbers (*30*) thus providing the field with an effective tool for genetic alterations of *C. acetobutylicum*.

Furthermore, the genes that encode expression of the enzyme of interest must be identified and characterized in order to successfully alter them in wild type strains. To

date the gene encoding region for hydrogenase, the *hydA* region, of several clostridia strains, in particular: *C. perfringens* (*31*), *C. acetobutylicum* P262 (*16*), and most importantly for this study, *C. acetobutylicum* ATCC 824 (*32*) have been established. In the gene characterization of *C. acetobutylicum* ATCC 824 *hydA*, Gorwa, et. al. verify that the expression of hydrogenase is in fact regulated at the level of transcription (*32*). Knowing that *hydA* levels can be controlled during the process of transcribing DNA to mRNA, as opposed to during protein synthesis, provides confidence that alterations of this gene will be an effective means for further study of hydrogenase effects. Gorwa et. al.'s study also provides a developed plasmid, pMFH 1, containing the entire isolated sequence of the *hydA* gene in *C. acetobutylicum* ATCC 824 (*32*). Availability of this plasmid, kindly provided by Philippe Socaille (Institut National des Sciences Appliquées, Centre de Bioingénierie G. Durand, Toulouse, France) allows for further examination of the enzyme under altered conditions.

In order to examine the effects of hydrogenase production on resulting TNT degradation, specific genetic tools can be used to induce either amplified or inhibited *hydA* levels. Several studies have successfully increased production of genes involved in clostridia solventogeneis (*27,33,34*). Through enzyme assays, these studies were able to show amplified activity of genes targeted by metabolic engineering. However, until recently, no effective gene reporter system for *C. acetobutylicum* existed by which to determine promoter strength and regulation. In order to observe increased enzyme production over natural production, plasmid DNA containing a promoter sequence upstream of the gene to be transcribed is transformed into the wild type microorganism. Different promoters possess differing qualities as to how effective they are at heightening

gene expression levels. A recent study tests the *lacZ* gene from another microorganism as a proposed reporter gene for *C. acetobutylicum* ATCC 824 and indicates that promoters can be characterized through this method (*35*). The study also examines several promoters for their ability to amplify gene expression and found that the promoter of the phosphotransbutyrylase gene (*ptb* promoter) effectively increases enzyme production. Inserting the *ptb* promoter into plasmids upstream of a gene of interest allows for over-expression of the gene and can be used in further metabolic studies.

Another way in which expression of an enzyme is often genetically regulated is through formation of antisense RNA (asRNA), RNA containing a complimentary sequence to regularly expressed mRNA . Binding of asRNA to target mRNA inhibits mRNA translation by either blocking ribosome binding sites and thus not allowing cellular mechanics to proceed or by altering the mRNA structure so that ribonucleases will degrade it. It is known that asRNA regulates gene expression in this manner in some naturally occurring systems (for a review see Wagner and Simons, 1994 (*36*)) and has been implicated in regulation of glutamine synthetase, an ammonia production catalyst, in *C. acetobutylicum* (*37*). Thus it is thought, by artificially producing asRNA through metabolic engineering, the effect will result in downregulation of targeted enzyme levels.

Strategies for developing asRNA technology in clostridia have been summarized and reviewed by Tummala et. al. (*38*), however the only study to employ these methods in clostridia was performed by Desai and Papoutsakis (*39*). In their study, they utilized asRNA strategies to regulate the expression of two enzymes involved in the formation of butyrate. Previous studies, on other types of prokaryotic cells, place focus on identifying the role of the structural genes targeted for downregulation (*40,41*), however the goal of

Desai and Papoutsakis' study was to redirect the primary metabolism of *C. acetobutylicum* ATCC 824. This study and at least one other suggest that artificial production of asRNA is capable of controlling primary metabolism in prokaryotes (*39,42*). It is this type of asRNA regulation which may prove useful in determining enzymes responsible for the reduction of TNT.

2.6 Specific *hydA* Genetically Altered Plasmids for *C. acetobutylicum*

The strategies described in the previous section have been employed, in part, in unpublished work by Bennett et. al. (Rice University, Department of Biochemistry) with the *hydA* gene in *C. acetobutylicum*. Specifically, Dr. Shouqin Huang preformed work to construct a plasmid to over-express hydrogenase through enhanced promotion of *hydA*, and Dr. Ranjit Padda contributed to the work by utilizing several technologies in attempt to inactivate or lower the expression of *hydA*.

In one report, Huang discusses construction of a *hydA* over-expression vector which is formed by PCR amplification of the entire *hydA* gene inserted into a vector plasmid with a promoter known to enhance gene expression (*43*). The vector pPTB, previously constructed and used for over-expression of other genes by Bennett et. al., contains the *ptb* promoter (*44*), which as discussed in a previous section, when used in combination with a *lacZ* reporter system has been shown to amplify gene expression (*35*). Preliminary experimentation with this recombinant plasmid, named pHTB, showed decreased levels of solvent production as compared to the wild type. Initial colorimetric assays for TNT reduction showed more rapid reduction of TNT by the pHTB transformed strain as compared to wild type, however more detailed HPLC analysis was not

performed for determination of metabolites. The plasmid also has not been characterized genetically and has not been assayed for hydrogenase activity levels.

In several studies, Padda examines possibilities of *hydA* inactivation or reduced expression in order to enhance solvent production in *C. acetobutylicum* ATCC 824 (*45*). In one of these studies, attempts to inactivate expression of the *hydA* gene through homologous recombination failed, indicating that hydrogenase activity is likely necessary during cell harvesting, plating, or early growth phases of the experiment. Therefore Padda utilized alternate technologies in order to reduce *hydA* expression by two to three fold through use of various hydrogenase promoters significantly differing from those found in wild types and through use of asRNA technologies, which have been previously discussed. Padda refers to two different approaches used to clone antisense strands of *hydA*. In one approach the entire *hydA* gene is amplified and cloned in opposite orientation in the promoter vector pPTB, which contains the *ptb* promoter. In the second approach, only small portions of the N-terminal region of the *hydA* gene is amplified and cloned in antisense in a vector plasmid pSOS84, which also contains the *ptb* promoter. The antisense plasmids were examined for the resulting effects of hydrogenase expression on solvent production. From preliminary experimentation, it appears that solvent production is increased in the strains in which an antisense strand has been transformed as well as those in which vector only controls have been transformed. The research identified future goals as characterization of these plasmids and determination of the effects of hydrogenase expression on TNT reduction in this organism. It is unclear however, which asRNA technique was used and thus which plasmid or plasmids were constructed successfully.

The studies carried out by these researches have been left incomplete and unpublished for several years. It is the goal of this study to continue this work, specifically to characterize existing plasmids both genetically and quantitatively for measure of hydrogenase expression. This study will address the additional goal of examining a more detailed analysis of TNT reduction by transformed strains for reduction rates and presence of metabolites.

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CHAPTER 3 - MATERIALS AND METHODS

3.1 Materials

This section details specific chemicals, media, bacterial strains and plasmids as well as any other materials used in the process of carrying out this research.

Chemicals

All chemicals used for media preparation were reagent grade unless otherwise noted. All enzymes were obtained from New England Biolabs. Gases used consist of: hydrogen, nitrogen, argon, and a mixture of 5.1% CO_2, 9.9% H_2, 85% N_2 and were obtained in the highest available purity from Trigas (Iving, TX). Dry chemicals used include: TNT (Chemsyn Science, Lenexa, KS) purified (98.6%), erythromycin (Sigma), sodium dithionite (85%, Acros), and methyl viologen (hydrate 98%, Acros). The solvent used, acetonitrile (99.9%, Fisher Scientific), was HPLC grade.

Nutrient Media

Clostridial growth medium (CGM) pH 7 was prepared as described by Hartmanis and Gatenbeck (*1*) and was modified to contain the following constituents $(NH_4)_2SO_4$ (2 g/l), K_2HPO_4 (1 g/l), KH_2PO_4 (0.5 g/l), $MgSO_4 \cdot 7\ H_2O$ (0.1 g/l), $FeSO_4 \cdot 7\ H_2O$ (0.015 g/l), $CaCl_2$ (0.01 g/l), $MnSO_4 \cdot H_2O$ (0.01 g/l), $CoCl_2$ (0.002 g/l), $ZnSO_4 \cdot 7\ H_2O$ (0.0034 g/l), tryptone (2 g/l), yeast extract (1 g/l), and glucose (50 g/l). CGM agar plates were prepared with exact concentrations above with the addition of agar (12 g/l).

2xYTG medium consisted of yeast extract (10g/l), tryptone (16 g/l), glucose (5 g/l), and NaCl (4 g/l). This media replaced CGM media in situations in which enriched clostridial growth was needed, particularly during transformation stages when cell strength was lowered due to electroporation shocking.

Luria-Bertani medium (LB) contained yeast extract (5 g/l), tryptone (10g/l), NaCl (5 g/l), and NaOH (2 ml, 1 M) and was used for *E. coli* growth. LB agar plates were prepared using the concentrations above with the addition of agar (15 g/l). All media were sterilized by autoclave.

Additional Solutions

Electroporation buffer (EPB), used in *C. acetobutylicum* transformations, consisted of sucrose (270 mM) and NaH_2PO_4 (1.26 mM) and was filter sterilized. Hydrogenase assay solution (HAS) was made up of the buffer Tris· HCl (50mM, pH 8), terminal electron donor, sodium dithionite (60 mM), and electron donor, methyl viologen (1 mM).

Bacterial Strains and Plasmids

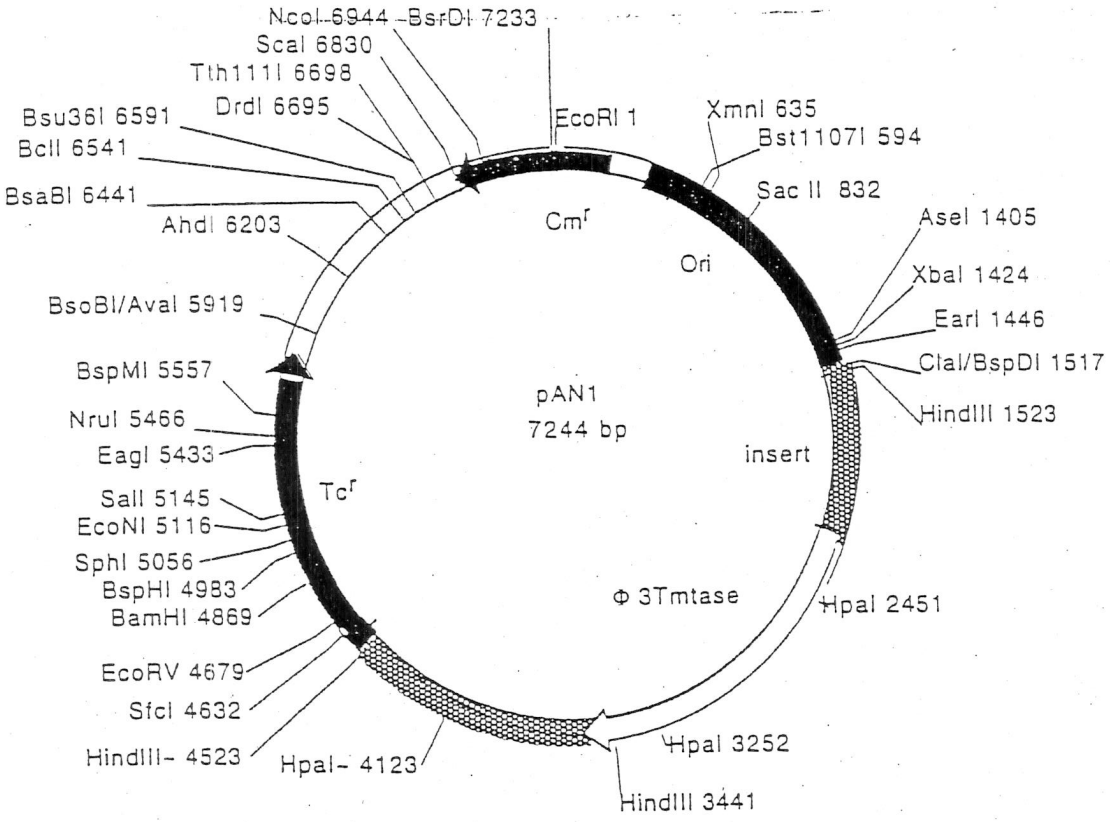
The bacterial strains and plasmids used in this study are listed in Table 3.1. The pAN 1 plasmid (Figure 3.1) is a methylating plasmid used for transformation of other plasmids into *C. acetobutylicum* (*2*). Plasmid pPTB (Figure 3.2) is an *E. coli-C. acetobutylicum* shuttle vector containing the *ptb* promoter region (*3*) and was used as a control *C. acetobutylicum* strain to account for host-plasmid interactions as well as to construct pHTB. Plasmid pSOS84 (Figure 3.3) is also an *E. coli-C. acetobutylicum* shuttle vector containing the promoter region of the *ptb* gene (*4*) and was used in construction of the hydrogenase A gene, antisense RNA plasmids (*hydA*-asRNA); pASH1, pASH 2, pASH 3, and pASH 4 (*5*). Plasmid pPMFH1, kindly provided by Philippe Soucaille (Institut National des Sciences Appliquées, Centre de Bioingénierie G. Durand, Toulouse, France), was used to obtain the hydrogenase gene for PCR amplification in plasmid construction (*6*). Plasmid pHTB, developed by Shouqin Huang

Table 3.1 – Bacterial Strains and Plasmids

Strain or Plasmid	Relevant Characteristics[a]	Source/Reference[b]
Strains		
E. coli DH10B	mcrA, ΔmcrBC, rec A1	11
C. acetobutylicum ATCC 824	Wild type	ATCC
Plasmids		
pAN 1	p15A ori; Cm^r, Φ*3t*I	2
pPTB	ColE1 ori, ORF II ori; Em^r, *ptb* promoter	3
pSOS84	ColE1 ori, ORF II ori; Em^r, Ap^r, *ptb* promoter	4
pMFH 1	ColE1 ori; Ap^r, *ptb* promoter, *hyd*A	6
pHTB	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hyd*A	This study
pASH 1	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hyd*A-asRNA	This study
pASH 2	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hyd*A-asRNA	This study
pASH 3	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hyd*A-asRNA	This study
pASH 4	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hyd*A-asRNA	This study

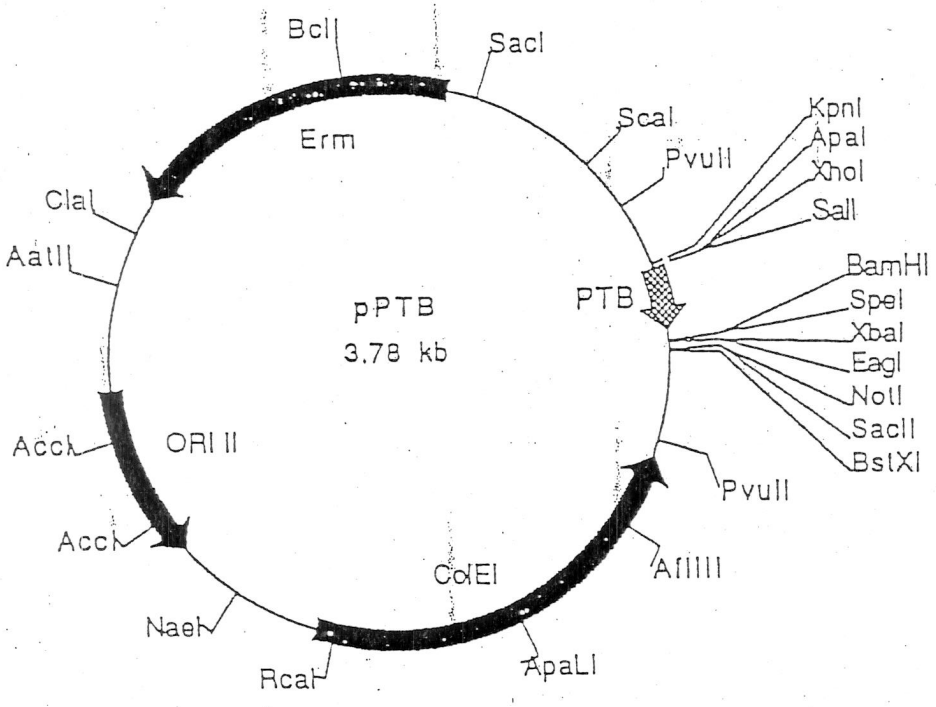
[a]p15A ori – p15A origin of replication, Cm^r – chloramphenicol resistance, Φ*3t*I - Φ*3t*I methyltransferase gene, ColE1 oriI - ColE1 origin of replication (recognized by *E. coli*) ORF II ori – ORF II origin of replication (recognized by *C. acetobutylicum*), Em^r – erythromycin resistance, Ap^r – ampicilllin resistance, *ptb* promoter – promoter of phosphotransbutyrylase in *C. acetobutylicum, hydA* – hydrogenase A gene, *hyd*A-asRNA – hydrogenase A gene antisense RNA insert
[b]NEB – New England Biolabs, Beverly, Mass.; ATCC – American Type Cultures Collection, Manassas, Va.

Figure 3.1 – pAN 1, methylating plasmid



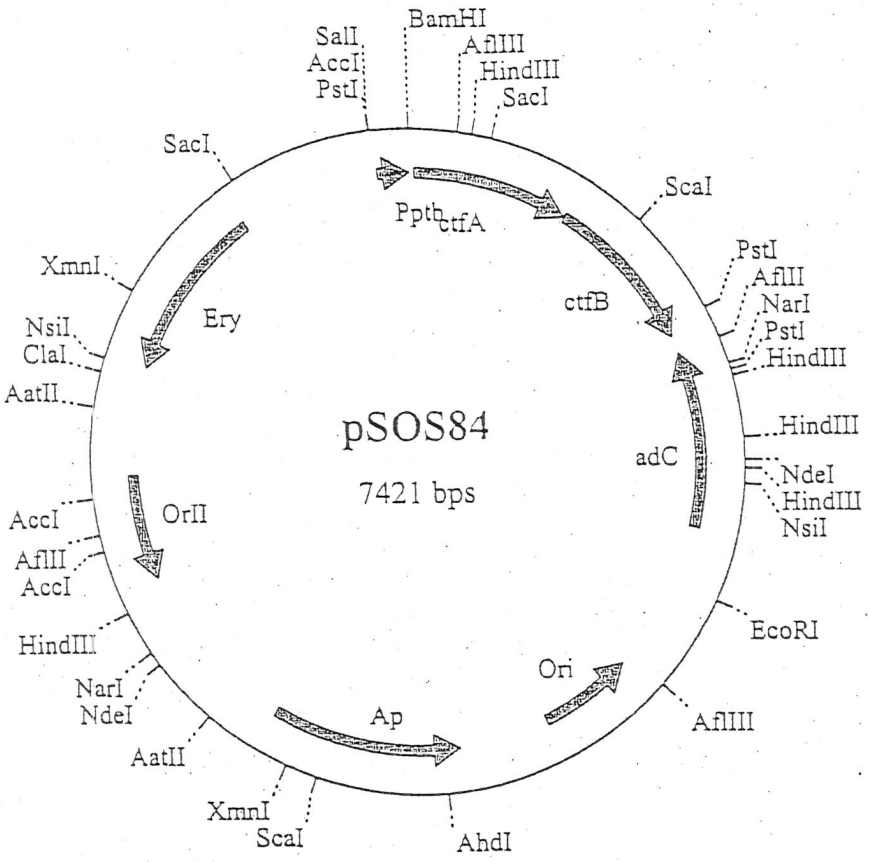
Relevant characteristics include: Cm^r, chloramphenicol resistance marker used for selection; Φ3Tmtase, Φ3T I methyltransferase encoding region; Tc^r, tetracycline resistance marker inactivated due to promoter disruption; Ori, origin of replication of pACYC184, a P15A type origin [2].

Figure 3.2 – pPTB, *E. coli-C.acetobutylicum* shuttle vector



Relevant characteristics include: Em^r, erythromycin resistance marker used for selection; ORI II, origin of replication recognized by *C. acetobutylicum*; ColEI, origin of replication recognized by *E. coli*; PTB, promoter of phosphotransbutyrylase (*ptb* gene) in *C. acetobutylicum* [3]

Figure 3.3 – pSOS84, *E. coli-C.acetobutylicum* shuttle vector



Relevant features include: ORF II, origin of replication recognized by *C. acetobutylicum*; ORI, ColE1 origin of replication from pUC18 recognized by *E. Coli*; Ery, erythromycin resistance marker used for selection; Ap^r, ampicillin resistance marker used for selection; *ctfA*, *ctfB* and *adc*, genes composing an artificial acetone production operon (*ctfA* and *ctfB* encode subunits of acetyl-CoenzymeA:acetate/butyrate:CoenzyemeA transferase; *adc* encodes acetoacetate decarboxylase, it is in opposite orientation thus acetone operon is inactive); PTB Pro, *ptb* gene encoding the phosphotransbutyrylase promoter in *C. acetobutylicum* [4].

(Rice University, Department of Biochemistry), contains a PCR amplified insert of the entire *hydA* gene in the pPTB vector, due to its strong *ptb* promoter, for enhanced production of the hydrogenase enzyme (*7*).

3.2 Analytical Methods

This section contains detailed information regarding specific techniques used in producing and transforming altered DNA plasmids as well as methods used in characterizing these plasmids. Preparation and cell culture conditions surrounding the preparation of cell extracts and storage of differing strains is also discussed.

DNA Production and Transformation

Transformation of plasmid DNA into *E. coli* DH10B cells was performed as described by the May 1994 edition of the New England Biolabs (NEB) Transcript (*8*). Mid-scale preparation of plasmid DNA from *E. coli* was performed using a Quiagen Plasmid Midi Prep Kit. *E. coli* was grown aerobically in LB medium and all plasmids were selected for, using 200 µg/l erythromycin (Sigma) as the selection antiboitoic.

Electrotransformation of pAN 1 methylated plasmids into *C. acetobutylicum* was performed in an anaerobic chamber (Forma Scientific, under an atmosphere of 85% N_2, 9.9% H_2, and 5.1% CO_2) using the general method of a previously published procedure (*2,9*). Four colonies (10 ml) were grown overnight in CGM for approximately 18 hours and then transferred into 75 ml 2xYTG to grow to exponential phase (OD_{600}=1.0-1.2). Cells were then centrifuged for 10 minutes at 10,000 k and decanted. Pellets were resuspended in cold EPB for a combined volume of 20 ml. After the suspension was chilled for 10 minutes, the cells were then repelleted by centrifugation for 10 minutes at 10,000 k and decanted. Pellets were then resuspended in cold EPB (0.6 ml for each

transformation). The suspension was then aliquoted in 0.6 ml units to chilled 0.4 cm electroporation cuvettes containing 15 µl plasmid DNA to be transformed. After chilling an additional 5 minutes, the bacteria were electrotransformed (Bio Rad Electroporator) by applying a pulse (25 µFD capacitance, 2 kv pulse, and ∞ ohms resistance). The contents of each cuvette were immediately transferred to 10 ml of 37°C 2xYTG and incubated for 4 hours at 37°C. Each sample was then pelleted as above and resuspended in ~0.3 ml 2xYTG and then plated on CGM agar plates with 40 µg/l erythromycin selection antibiotic and left to incubate at 37°C for 48 hours.

Plasmid Characterization

Maps of previously constructed plasmids were assembled through plasmid characterization by restriction mapping as well as by sequencing with various primers. Plasmid preparations were initially characterized by restriction enzyme cutting using Bam HI and Not I to identify the presence of the hyd A gene insert in pHTB and Bam HI and Sac II or Bam HI and Ava I to gain insight on inserts present in pASH1, pASH 2, pASH 3 and pASH 4. After digestion, agarose gel electrophoresis was carried out at 100 volts for one hour and DNA fragments were compared to Gene Choice DNA Ladder I standards.

Sequencing was done by Lone Star Labs using universal primers M13F and M13R as well as developed primers PTBF, ADCR, hyd 6F, hyd 7F, hyd 8R and hyd 9F (Table 3.2).

Cell Extract Preparation

Clostridia cultures grown overnight within the anaerobic chamber in CGM with antibiotic selection of 40µg/l erythromycin for plasmid containing strains were harvested

Table 3.2 – Primers Used for Plasmid Characterization

Primer Name	Sequence[a]	Origin of Primer[b]	Reference
PTBF	CCT CGA GGT CGA CTG TGG ATG G	*ptb* promoter	13
ADCR	CTT CAG CTC TAG GCA ATA TTA TAT CTG C	reverse compliment *adc* gene (925-952)	12
hyd 6F	TAC GTA ATA TTT ACG TTG ATT AAA CGT	*hyd* A gene (598-635)	6
hyd 7F	GAC AAA GGG CTG TTG GAA CTG TTG ATG	*hyd* A gene (1200-1227)	6
hyd 8R	GTT GAC CTC CAC CAT TTA TAC ATC CAC	reverse compliment *hyd* A gene (2210-2173)	6
hyd 9F	TTA TAG AAG TAA TGG CTT GCC CTG GTG	*hyd* A gene (2161-2188)	6

[a] all primers in direction of 5' to 3'
[b] sequences of *ptb* promoter, *acd* gene, and *hyd A* gene can be found in Appendix 1

after 18 hours by centrifugation (16,000 x g for 10 minutes at 4° C). The cells were then resuspended in 10 mM Tris· HCl (ph 7.9) buffer for washing followed by additional centrifugation (16,000 x g for 10 minutes at 4° C). The washed pellets were then resuspended in 10 ml 10mM Tris· HCl (pH 7.9), 10 mg lysozyme and 2.5 mg AESBF protease inhibitor for each gram of recovered cells. The suspension was left to incubate for 1 hour. The lysate was then sonicated (Ultrasonics, Inc.) for 20 minutes at 60% duty cycle, at an output of 7 on pulse mode. Finally, the sonicate was centrifuged twice at 20k for 15 minutes and again at 45k for 30 minutes all at 4° C to remove cellular debris. The remaining supernatant was the crude cell extract. The Bradford assay method (BioRad) was used to determine protein content of the extracts.

Storage

All of the *E. coli* strains were stored at 80° C in glycerol solution (50% glycerol v/v). *C. acetobutylicum* strains were lypholyzed in horse serum under vacuum and kept in glass ampoules at room temperature.

3.3 Experimental Procedures

Solvent Production Quantification

Gas chromatography was used to determine concentrations of the aqueous phase fermentation products ethanol, acetate, acetone, butanol, and butyrate produced by growing *C. acetobutylicum* cultures. Samples taken from growing cell cultures during different stages of cell growth were centrifuged to remove cellular components. The supernatant, containing fermentation products, was then acidified with 50% H_2SO_4 (20µl per 1 ml of sample). Aqueous samples (5µl) were directly injected into a GC (Hewlett Packard 5890) equipped with a flame ionization detector (FID) and a glass column (6' x

¼" x 2mm ID) packed with porous polystyrene, 80/100 mesh Poropak QS (Alltech). The operating parameters were as follows: initial temperature = 125° C, hold 5 min., ramp at 30° C/min. to 190° C, hold 6 min., ramp at 30° C/min. to 220° C, hold 22 min. The injector temperature was set at 215° C and the detector temperature was set at 245° C. Nitrogen was used as the carrier gas at pressure 50psi and flow rate 30ml/min. The flame was maintained by hydrogen gas and air at pressures of 40psi and 45psi respectively and flow rates of 30 ml/min and 400 ml/min respectively. Standards were prepared by adding known volumes of fermentation products of interest to DI water and sample concentration were determined by the peak height method.

Hydrogenase Assay

Hydrogenase activity was determined in the hydrogen evolution direction with the gas chromatography method described by Jungermann et al. (*10*) and was modified as described. The assay was carried out at 25° C. The hydrogenase assay solution (HAS) was made anaerobic either by equilibrating with the atmosphere of an anaerobic chamber overnight or by sparging with argon for 20 minutes. The HAS was then transferred in 2 ml volumes into vials (20 ml), which had been sealed with a butyl rubber stopper and an aluminum cap and was then flushed with argon. The addition of crude cell extract (100 µl) using a gas tight syringe started the reaction. Hydrogen production was then measured in the headspace by injecting samples (0.25 ml) into a Gow-Mac Series 600 GC with a TDC detector at 120° C over time. Separation was obtained with a molecular sieve column (length 8 ft) at an oven temperature of 80° C using argon as the carrier gas. Hydrogen was quantified by the peak height method. Hydrogen concentrations in assays

with HAS and no addition of cell extract were measured for background H_2 production; none was observed.

TNT Reduction Study

TNT reduction assays were carried out under anaerobic conditions in a hydrogen atmosphere. Each experiment contained 10 ml TNT stock solution (100 mg/L TNT in milli-q water), which was augmented with crude cell extract (100 µl) to begin the reaction. The assay was sampled over time (10 minutes at approximately 1 minute intervals) for TNT rate reduction determination. As samples were taken, the reaction was stopped by exposure of the assay solution to oxygen, which inhibits the hydrogenase enzyme. TNT concentrations were measured with The Waters System (Milford, MA) for HPLC analysis, which consisted of a separation module (Model 2690), a diode-array UV-visible detector (Model 996) and a thermabeam Mass Detector. The system was controlled by a PC-based workstation (digital) equipped with the Millenium Chromatography Manager software. Spectra were acquired continuously between 200 to 400 nm and chromatograms extracted at 230 nm for quantification. Analytes were separated on a reverse-phase Waters Nova-Pak-C18 column (2 x 150 mm) at room temperature with a variety of gradient mobile phases consisting of water/acetonitrile (75/25 - 85/15) (v/v) at 0.25 ml/min.

3.4 Data Analysis

Sequencing Analysis.

Results of each sequencing run were input separately into the Biology Workbench 3.2 on-line program (http://workbench.sdsc.edu/). The nucleic tools, BL2SEQ was used for comparing nucleotide sequences to the published sequence of the *hyd* A gene

(Genbank database accession number U15277) as well as to vector plasmids pSOS84 and pPTB. The data from different sequencing runs on the same plasmid were also aligned using BL2SEQ in order to compile overlapping sequences. TACG was used in order to identify restriction enzyme cutting sites on each plasmid map.

Rate Determinations

Hydrogen production rates were determined by examining graphs of hydrogen produced versus time for each cell extract assayed. The slope of the first order production was taken to be used in future correlations and was expressed as μmol H_2 produced/min/μl cell extract. TNT reduction rates were determined by examining first order reduction of TNT to degradation products over time for each cell extract assayed and were expressed in μmol TNT degraded/min/μl cell extract.

3.5 References

(1) Hartmanis, M. G. N.; Gatenbeck, S. 1984. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. *Applied and Environmental Microbiology* 47:1277-1283.

(2) Mermelstein, L. D.; Papoutsakis, E. T. 1993. In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Applied and Environmental Microbiology* 59:1077-1081.

(3) Huang, K-X. 1998. Unpublished report. Rice University, Department of Biochemistry, Houston, TX.

(4) Soucaille, Unpublished.

(5) Padda, R. S. 1999. Reducing the expression of hydrogenase. Unpublished Report; Rice University, Department of Biochemistry, Houston, TX.

(6) Gorwa, M. F.; Croux, C.; Soucaille, P. 1996. Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. *Journal of Bacteriology* 178:2668-2675.

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(9) Mermelstein, L. D.; Welker, N. E.; Bennett, G. N.; Papoutsakis, E. T. 1992. Expression of cloned homologous fermentation genes in *Clostridium acetobutylicum* ATCC 824. *Bio/Technolgy* 10:190-195.

(10) Jungermann, K.; Thauer, R.; Rupprecht, E.; Ohrloff, C.; Decker, K. 1969. Ferredoxin mediated hydrogen formation from NADPH in a cell-free system of *Clostridium kluyveri*. *FEBS Letters* 3:144-146.

CHAPTER 4 – RESULTS

4.1 Plasmid Characterization and Sequencing

Plasmid characterization through gel electrophoresis gave insight into the size of each plasmid and the size of the inserts they contain. From single digests with Bam HI restriction enzyme, plasmids pHTB, pASH 1, pASH 2, pASH 3, and pASH 4 all appear to be approximately 5.5 kilo-bases (kb) in size and plasmid pPTB appears to be approximately 3.8 kb in size (Figure 4.1).

One proposed method existed for the construction of plasmid pHTB (*1*); thus, digestions were carried out to verify the construct. Single digestion of pHTB was carried out with Bam HI and Not I respectively to compare single cut bands. Each restriction enzyme preformed only one cut as expected resulting in one band, 5.5 kb in size. Double digestion of pHTB using restriction enzymes Bam HI and Not I, which appear to have been used for PCR amplification of the entire *hydA* gene for construction of this plasmid, produced two distinct bands approximately 1.7kb and 3.8 kb in size, as expected. Digestion patterns are shown on gel electrophoresis in Figure 4.2. Figure 4.3 schematically shows the identified method used in previous studies to create the over-expression plasmid, pHTB.

To determine the structure of and methods used to develop antisense plasmids: pASH 1, pASH 2, pASH 3, and pASH 4; a series of double digestions were conducted. One approach used to produce antisense plasmids in early experiments conducted by Padda (*2*), utilized PCR amplification to clone the entire *hyd A* gene in antisense orientation to the *ptb* promoter using restricition sites Bam HI and Sac II. Double digests of antisense plasmids using these restriction enzymes produce one band 5.5 kb in size. If

Figure 4.1 – Gel Electrophoresis of Experimental Plasmid DNA Digested with Bam HI

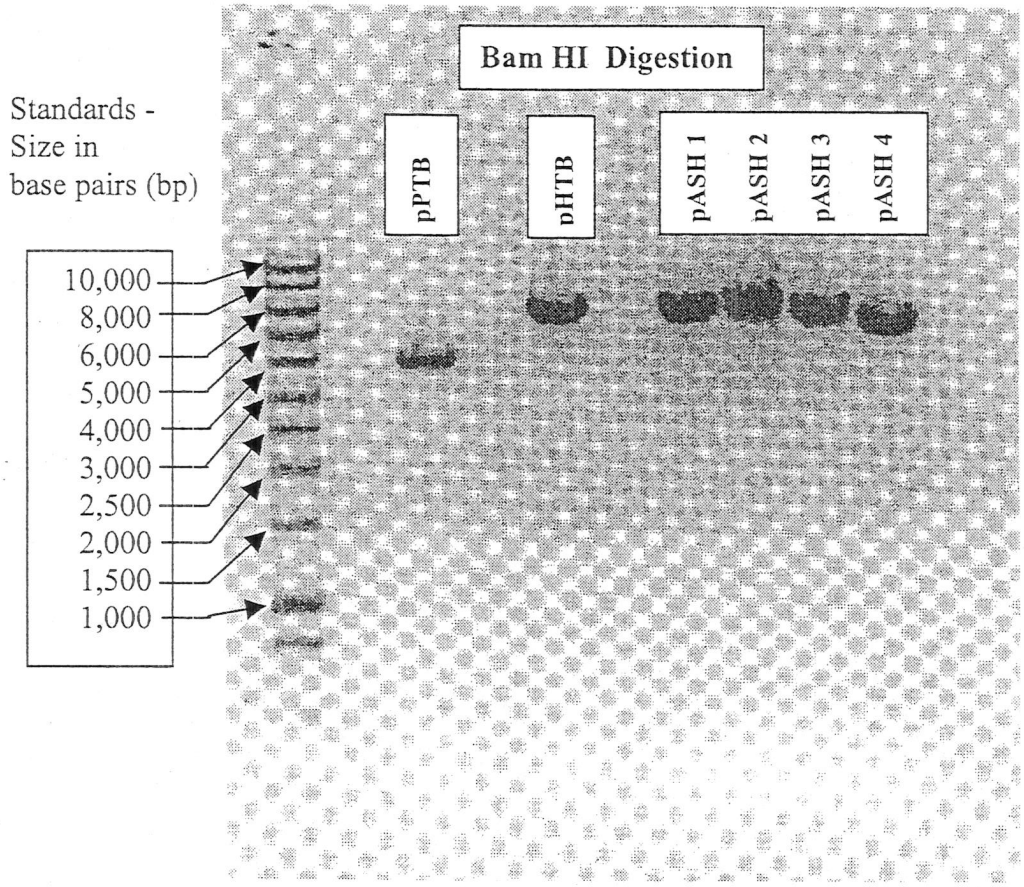


Figure 4.2 – Gel Electrophoresis of Over-expression Plasmid DNA (pHTB); Undigested, singly digested with Bam HI and Not I respectively, doubly digested with Bam HI and Not I

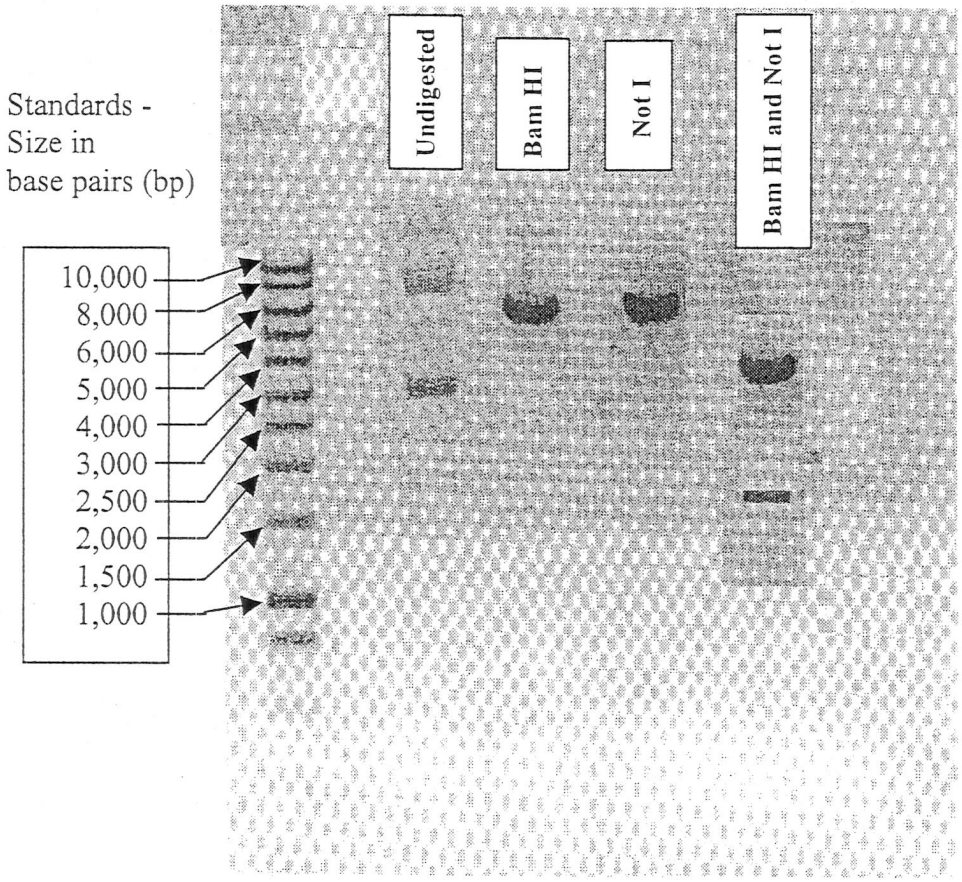
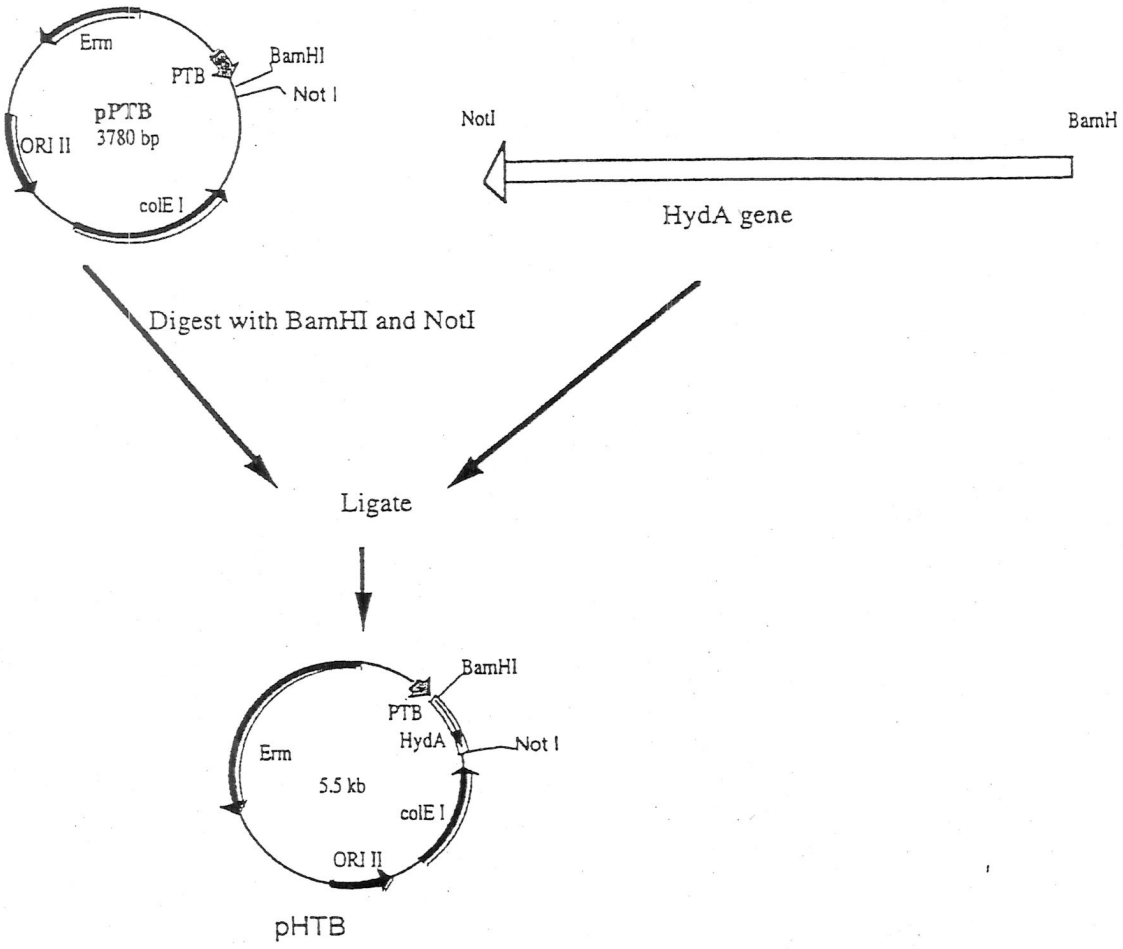


Figure 4.3 – Schematic of Method Used to Clone *hyd A* Gene into Plasmid pHTB



the antisense plasmids were constructed with this method, one would expect to see two distinct bands, the insert being 1.7 kb and the remainder of the vector plasmid, pPTB in this case, being 3.8 kb; however, this is not observed. Additionally, single digestion of the antisense plasmids with Sac II alone failed to produce the expected, single band. The pattern of digestion is that of undigested plasmids where several bands are observed due to differential folding of uncut plasmid DNA. Figure 4.4 compares singly digested, with Sac II, antisense plasmids and doubly digested, with Bam HI and Sac II, antisense plasmids.

A second approach, previously discussed in reports by Padda (*2*) for constructing antisense plasmids, PCR amplifies and clones only small N-terminal *hyd A* segments in antisense direction to the *ptb* promoter using restriction enzymes Bam HI and Ava I. Double digestion of antisense plasmids using Bam HI and Ava I produce single bands. Plasmids pASH 1, pASH 2, and pASH 3 bands are approximately 5.5 kb and pASH 4 is slightly lighter around 5.3 kb. This result is expected for digestion of plasmids constructed in this manner, given that the inserts in this construct are likely 45 to 150 base pairs (or 0.045 to 0.150 kb) and would be to small to identify by the gel electrophoresis carried out. The fact that the antisense plasmids produced a single band when digested with Ava I, further suggests that they were constructed with small N-terminal inserts of the *hydA* gene; the vector plasmid itself does not contain the Ava I restriction site and thus it has been inserted into the plasmids through PCR amplification techniques. Figure 4.5 contains the results of digestions of antisense plasmids with Ava I alone and with Bam HI in conjunction with Ava I. Figure 4.6 schematically shows the identified method used in previous studies to create the antisense plasmids.

Figure 4.4 – Gel Electrophoresis of Antisense Plasmid DNA;
Digested singularly with Sac II and doubly with Bam HI and Sac II

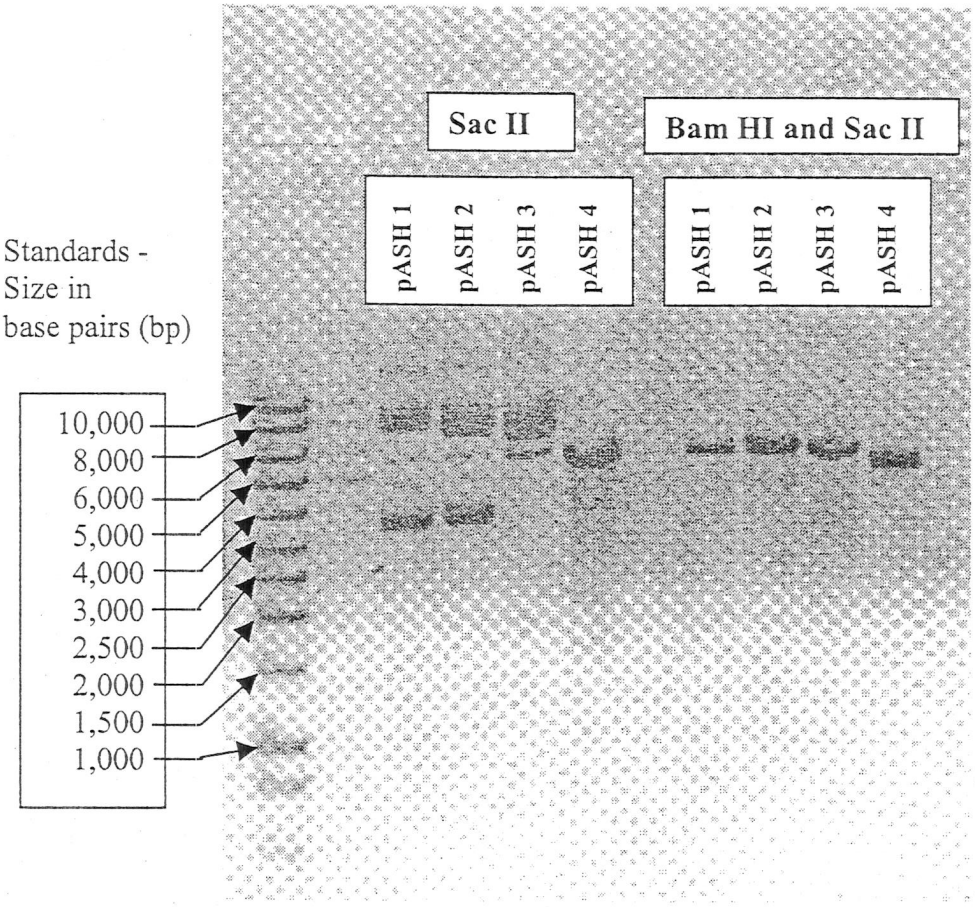


Figure 4.5 – Gel Electrophoresis of Antisense Plasmid DNA;
Digested Singularly with Ava I and doubly with Bam HI and Ava I

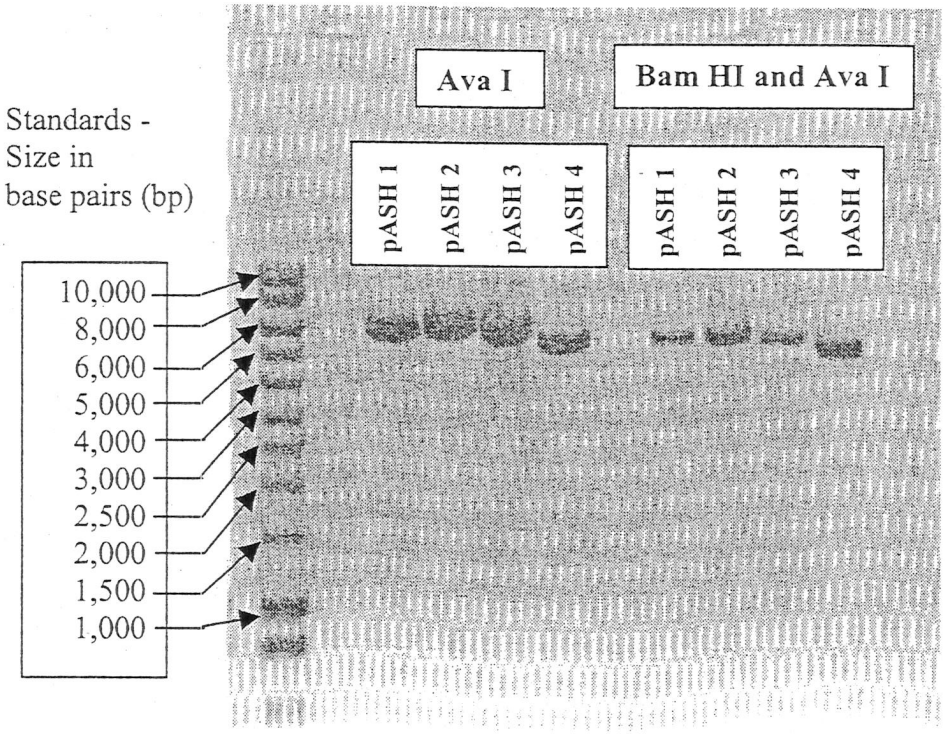
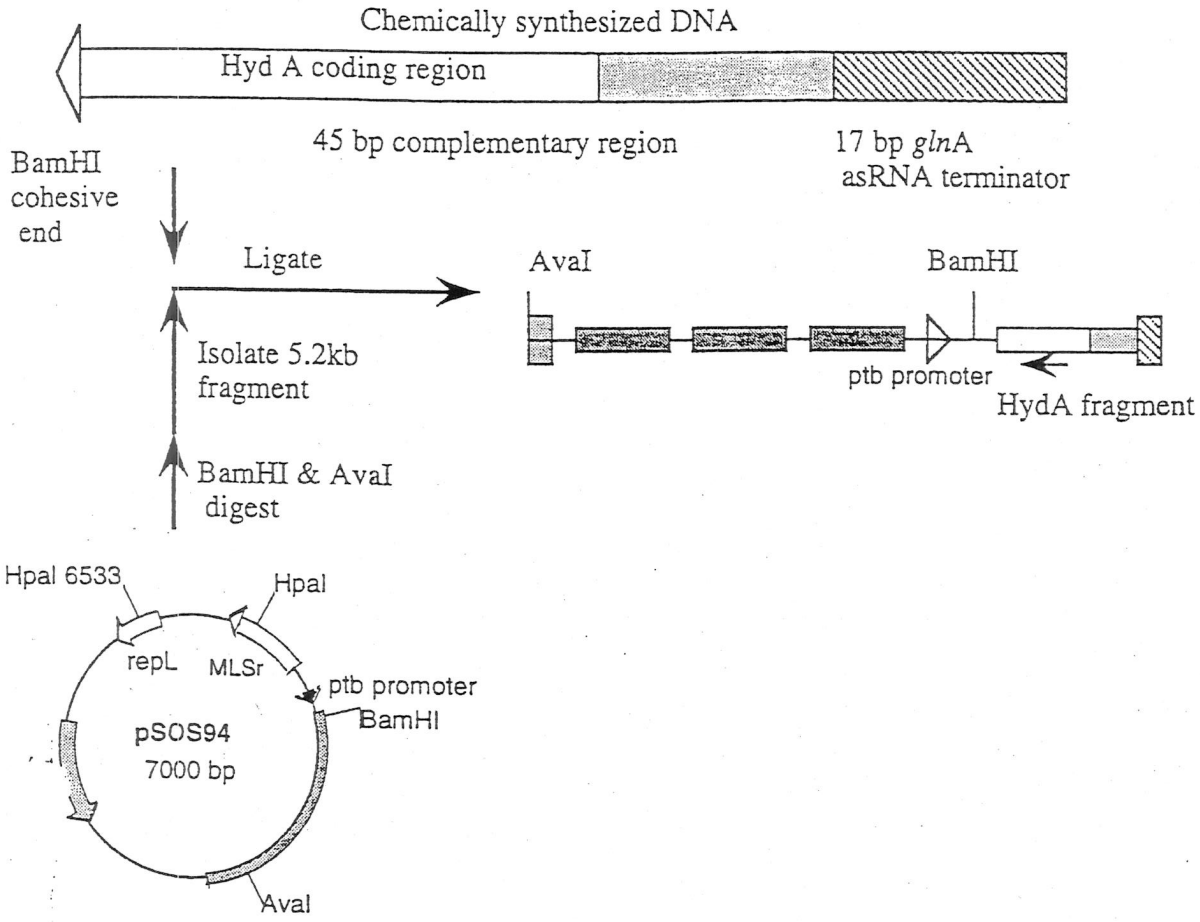


Figure 4.6 – Schematic of Method Used to Create Antisense Plasmids

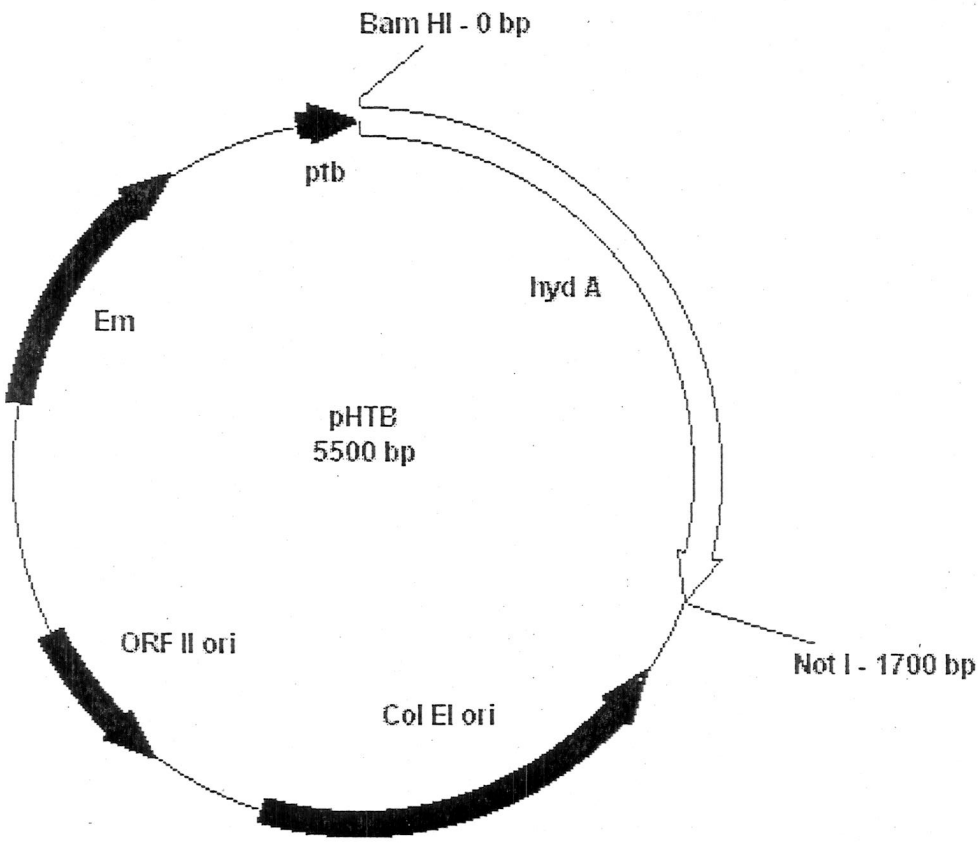


Control plasmids pPTB and pSOS84 were not further digested as they have been well characterized previously (*3,4*). Plasmid pMFH 1 has also been previously characterized by Gorwa et al. (*5*); however, due to uncertainty in this plasmid's construct during studies in which the *hydA* gene was obtained for cloning, further characterization of this plasmid was performed to ensure purity and to solidify the construct since it served previously as the source for the *hyd A* gene in experiments to create the plasmids of interest. Digestion patterns obtained for pMFH1 where unclear due to missing information as to the exact restriction enzyme sites it contained. This plasmid was thus characterized almost entirely through genetic sequencing.

Nucleotide sequences of the experimental plasmids served to further elucidate genetic alterations in the existing plasmids. Plasmid pHTB was sequenced with several developed primers to determine if the entire *hyd A* gene was present as it exists in published pMFH 1. Presence of the *ptb* promoter sequence was also identified from these sequences. A plasmid map of pHTB is presented as Figure 4.7. Appendix A contains the nucleotide sequence of the entire *hyd A* gene as published by Gorwa et. al. and Appendix B contains nucleotide sequencing data for this plasmid with matches to *hyd A*, *ptb* promoter, and vector plasmid pPTB identified where possible.

Sequencing was particularly necessary for antisense plasmids to determine the size and sequence of the antisense segments to ensure that they were in fact segments of the *hyd A* gene and to ensure that the sequence inserts were in the antisense direction to the plasmid promoter. Sequencing of these plasmids determined that pASH 1, pASH 2, and pASH3 all contained segments of the *hyd A* gene in antisense direction, which should encode for antisense RNA that will effectively bind to genomicly produced mRNA and

Figure 4.7 – pHTB Plasmid Map, experimental *hyd A* over-expression plasmid



Relevant characteristics include: ptb, promoter of phosphotransbutyrylase in *C. acetobutylicum*; hyd A, cloned *hyd A* gene; ColE1 ori, origin of replication recognized by *E. coli*; ORF II ori, origin of replication recognized by *C. acetobutylicum*; Em, erythromycin resistance marker used for selection

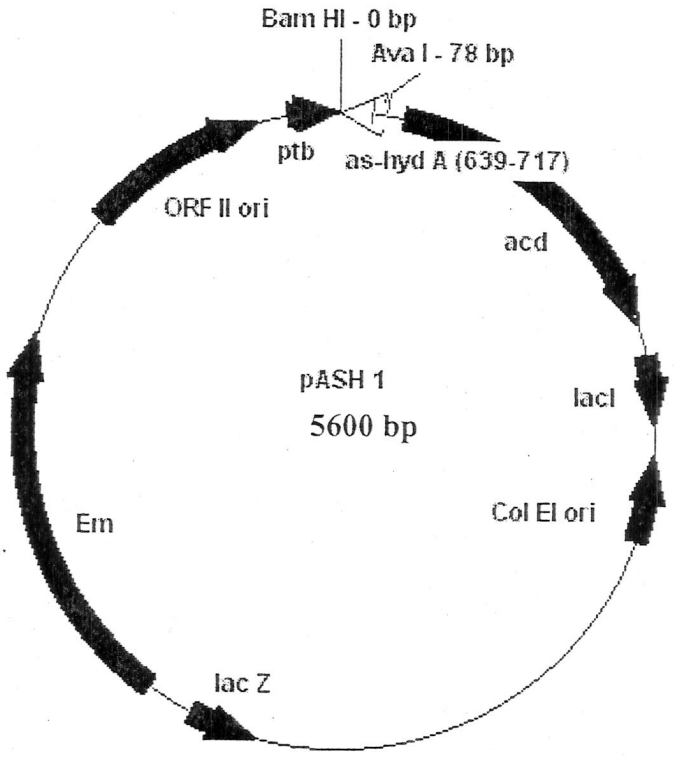
cause inhibition of hydrogenase production. Plasmid pASH 4 however, contained an insert fragment of the vector plasmid; thus, pASH 4 is not an effective antisense plasmid. It was therefore disregarded and not used in further experimentation. Sequencing results also further confirmed pSOS84 as the vector for antisense inserts. Figures 4.8 through 4.10 represent the plasmid maps of the effective antisense plasmids: pASH 1, pASH 2, and pASH 3 respectively, as determined by sequencing and restriction enzyme digestion. Appendix C contains nucleotide sequences obtained for experimental antisense plasmids and identifies matches with published *hyd A* data as well matches with sequences available for vector plasmid pSOS84.

To compare published pMFH 1 results with the plasmid pMFH 1 available for study, sequencing was carried out using several developed primers. Plasmid pMFH 1 is mapped in Figure 4.11 and Appendix D contains exact nucleotide sequence obtained for experimental pMFH 1, which identifies completely with published pMFH 1 data including open reading frames as described in the literature.

4.2 Solvent Production

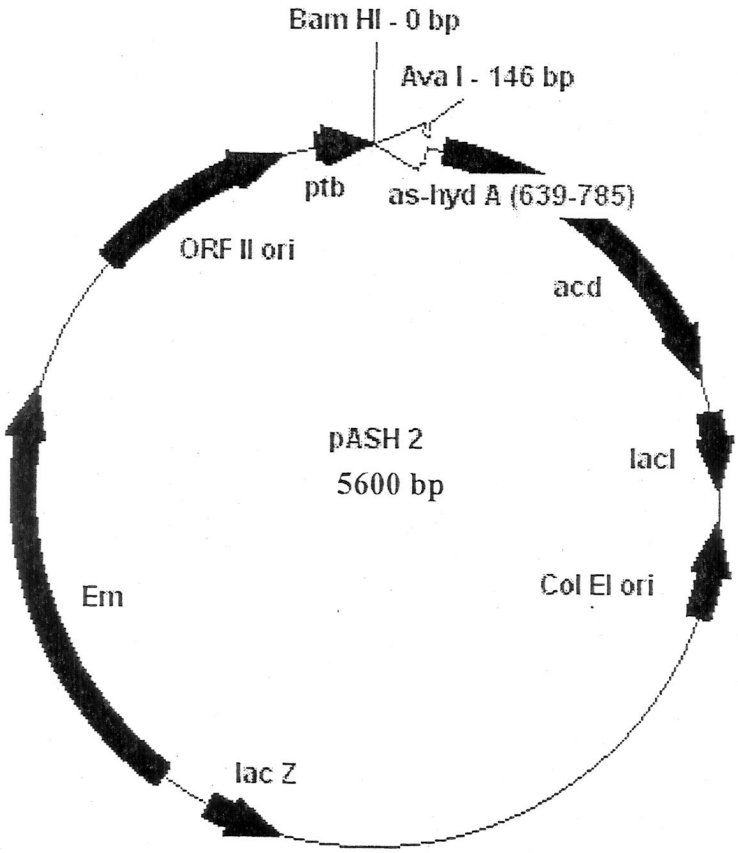
Solvent production was assessed for each *C. acetobutylicum* strain during cell extract preparation, which was after approximately 18 hours of cell growth and represents solvent production during the exponential growth phase known as acidogenesis. Solvent production was measured again after 48 hours of growth, during the stationary growth phase, when solvent production is active. Table 4.1 presents averaged concentrations in mM of the acids: acetate and butyrate; and the solvents: ethanol, acetone, and butanol produced by each strain. Optical density of cellular growth media at the time of solvent extraction is given at a wavelength of 600 nm.

Figure 4.8 – pASH 1 Plasmid Map, experimental *hyd A* antisense plasmid 1



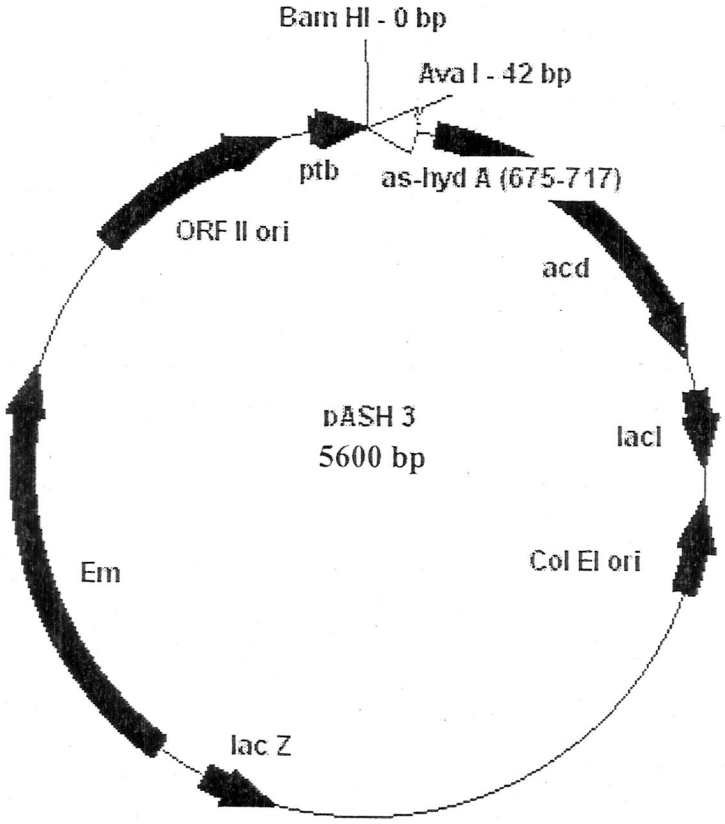
Relevant characteristics include: ptb, promoter of phosphotransbutyrylase in *C. acetobutylicum*; as-hyd A (639-717), *hyd A* gene nucleotide segment 639-717, in antisense direction to promoter; acd, gene encoding acetoacetate decarboxylase; ColE1 ori, origin of replication recognized by *E. coli*; Em, erythromycin resistance marker used for selection; ORF II ori, origin of replication recognized by *C. acetobutylicum*

Figure 4.9 – pASH 2 Plasmid Map, experimental *hyd A* antisense plasmid 2



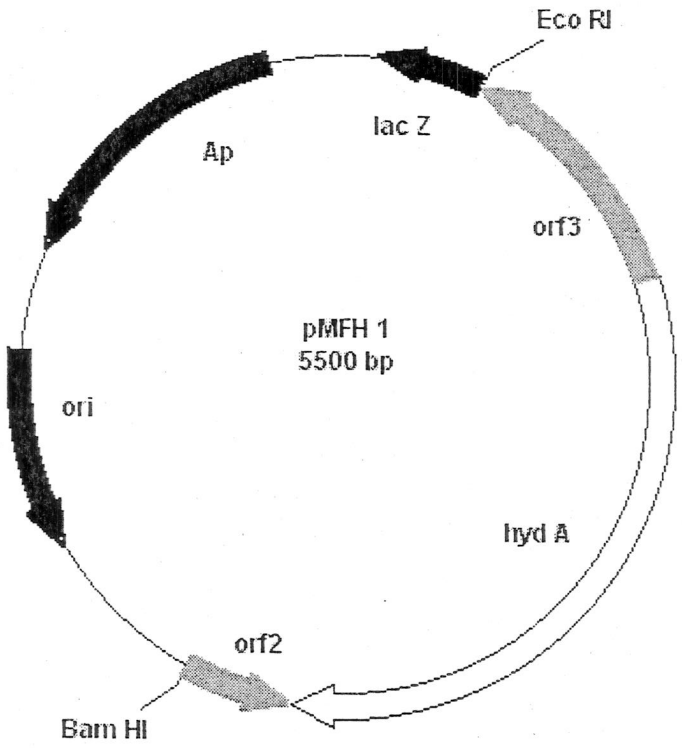
Relevant characteristics include: ptb, promoter of phosphotransbutyrylase in *C. acetobutylicum*; as-hyd A (639-785), *hyd A* gene nucleotide segment 639-785, in antisense direction to promoter; acd, gene encoding acetoacetate decarboxylase; ColE1 ori, origin of replication recognized by *E. coli*; Em, erythromycin resistance marker used for selection; ORF II ori, origin of replication recognized by *C. acetobutylicum*

Figure 4.10 – pASH 3 Plasmid Map, experimental *hyd A* antisense plasmid 3



Relevant characteristics include: ptb, promoter of phosphotransbutyrylase in *C. acetobutylicum*; as-hyd A (675-717), *hyd A* gene nucleotide segment 675-717, in antisense direction to promoter; acd, gene encoding acetoacetate decarboxylase; ColE1 ori, origin of replication recognized by *E. coli*; Em, erythromycin resistance marker used for selection; ORF II ori, origin of replication recognized by *C. acetobutylicum*

Figure 4.11 – pMFH 1 Plasmid Map



Relevant characteristics include: orf3, partial open reading frame surrounding *hyd* A gene, transcribed in opposite direction; hyd A, *hyd A* gene in its entirety; orf2, partial open reading frame surrounding *hyd* A gene, transcribed in opposite direction; ori, origin of replication recognized by *E. coli*; Ap, ampicillin resistance marker used for selection

Table 4.1 – Acid and Solvent Production for Each Strain Type During Acidogenisis and Solventogenisis

	Strain Type	OD_{600}	Ethanol (mM)	Acetone (mM)	Acetate (mM)	Butanol (mM)	Butyrate (mM)
18 hr Late-Exponential Growth	Wild Type	1.6	3	2	25	23	51
	pPTB (pHTB control)	1.8	10	5	24	25	46
	pHTB (over-expressor)	1.5	19	2	23	14	47
	pSOS84 (as control)	1.6	9	10	12	16	19
	pASH 1 (as plasmid 1)	1.9	10	2	23	24	53
	pASH 2 (as plasmid 2)	1.2	11	1	20	4	32
	pASH 3 (as plasmid 3)	1.6	9	5	20	19	47
48 hr Steady-State Growth	Wild Type	2.6	6	6	24	101	39
	pPTB	3.1	18	22	15	133	16
	pHTB	2.4	9	2	23	7	54
	pSOS84	NA	14	14	14	15	24
	pASH 1	2.9	12	8	23	68	29
	pASH 2	2.8	11	9	25	62	50
	pASH 3	2.9	11	6	25	49	50

4.3 Hydrogenase Production and TNT Reduction Assays

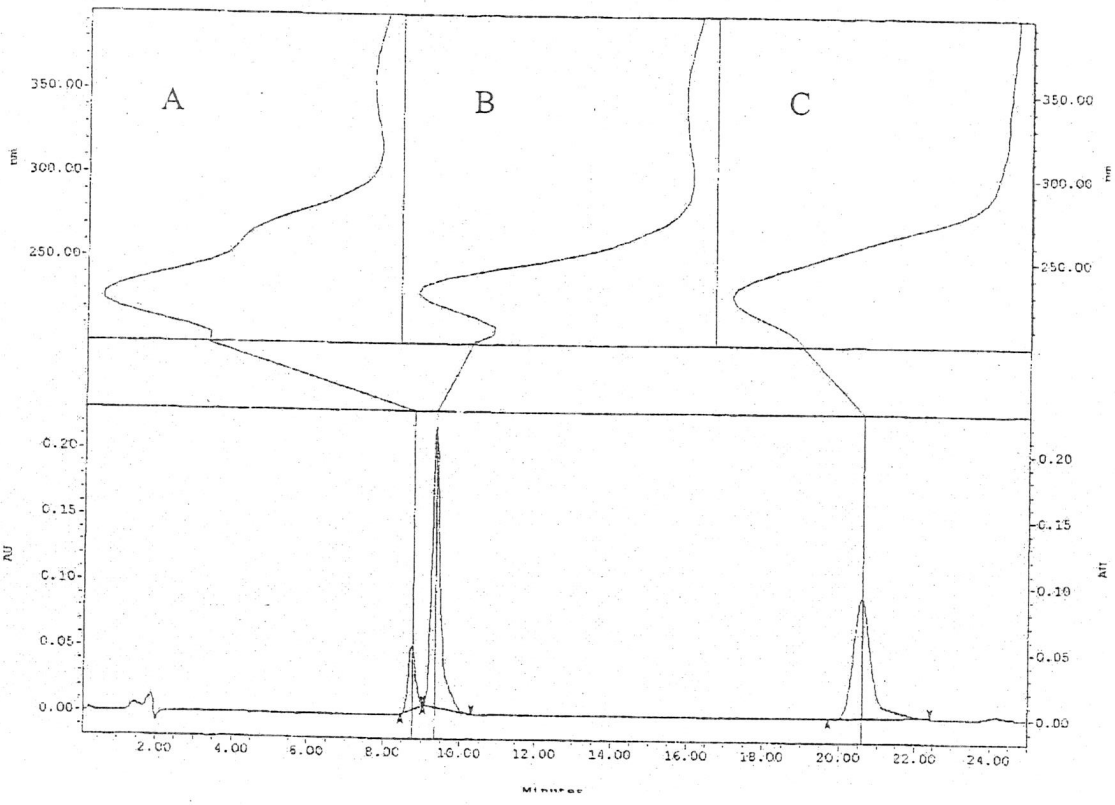
Cell extracts prepared for each plasmid were characterized for activity through two sets of assays, one for hydrogenase activity through hydrogen production rates and another for TNT reduction rates. Data gathered for each rate were taken during zero order reactivity and thus plotting of hydrogen production or TNT reduction over time produced slopes which could be taken as first order rates of reaction in each case. Rates for average hydrogenase activity for each plasmid strain extract are available in Appendix E reported in mol H_2 produced/liter/minute. These values, through a series of conversions based on assay volume and protein concentration in cell extract preparations, are later expressed in μmol H_2 produced/minute/μg of cell extract, for correlation purposes. Rates of TNT reduction are expressed similarly in Appendix F in mg TNT reduced/liter/minute; these values are also later expressed as μg TNT reduced/minute/μg of cell extract, for correlation purposes. Each value expressed in the tables was taken for one assay run of each strain's cell extract, over a period of one hour for hydrogen production and a period of 10 minutes for TNT reduction.

TNT metabolites, observed in TNT reduction assays, show accumulation of 4-hydroxlyaminno-2,6-dinitrotoluene (4HA26DNT) and 2,4-dihydroxylamino-6-nitrotoluene (24DHA6NT) as expected according to previous results for reduction by *C. acetobutylicum* (*6-9*). Figure 4.12 shows the HPLC chromatogram and UV-VIS spectra, at 230nm, for TNT reduction products observed during the assays.

4.4 Correlations

For each cell extract preparation, the highest rates of TNT reduction and hydrogenase activity were extracted. These values represent maximum activity obtained,

Figure 4.12 – HPLC Chromatogram and UV-VIS Spectra of TNT and Observed Metabolites During TNT Reduction Assay
(A) 2,4-dihydroxylamino-6-nitrotoluene, (B) 4-hydroxylamino-2,6-dinitrotoluene, (C) 2,4,6-trinitrotoluene



disregarding effects of oxygen contamination or other inhibitory factors. The values plotted against each other as a correlation are presented in Figure 4.13 with an R^2 value of 0.89, displaying a reasonably high level of correlation between the two variables. Table 4.2 contains data of TNT reduction capabilities averaged for each strain type. This data has been normalized for protein content of cell extracts and is expressed in μmol TNT reduced/min/μg protein content. The values are listed in order of decreasing TNT reduction capability. Values given for antisense plasmid strains are representative of TNT reduction during acidogenic phase. Cell extracts prepared for these strains during solventogenesis showed no TNT reduction (nor hydrogenase activity) through the duration of the assay as compared to control assays run with no cell extract added. Figure 4.14 shows graphically, the difference in wild type loss of activity between acidogenic and solventogenic phases compared to pHTB retention of activity between the two phases.

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Figure 4.13 – Correlation of TNT Reduction Capability vs. Hydrogenase Activity

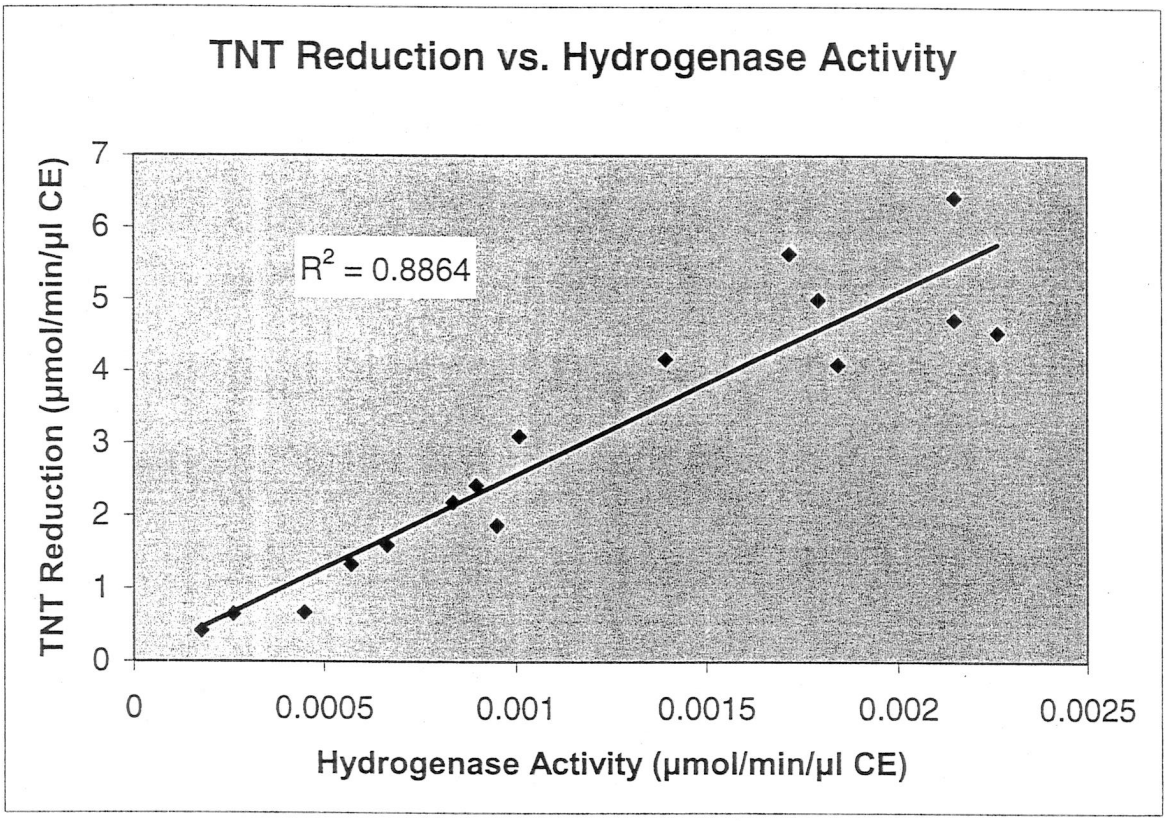
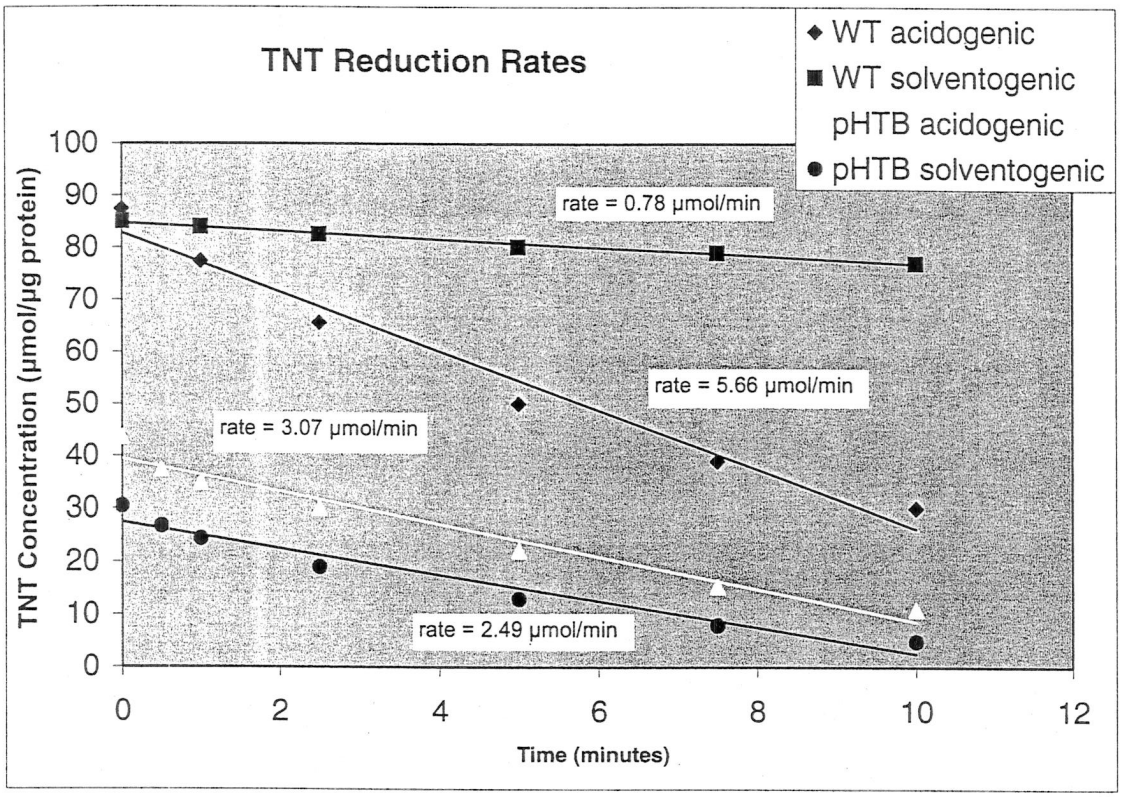


Table 4.2 – TNT Reduction Capability for Each Strain Type Normalized to Protein Content of Cell Extract Preparation

Cell Extract ID	TNT Reduction Rate (µmol/min/µg protein)
Wild Type –acidogenic	5.37
pPTB	3.78
pHTB – acidogenic	2.76
pSOS84	2.69
pHTB – solventogenic	2.49
pASH 3	2.40
pASH 1	2.16
pASH 2	1.30
Wild Type – solventogenic	0.78

Figure 4.14 – TNT Reduction Rates for Wild Type and pHTB Strain Cells During Acidogenic and Solventogenic Stages of Growth



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CHAPTER 5 – DISCUSSION

Much work has been done to understand the array of TNT reduction products in anaerobic clostridia systems (*1-6*), and focus has recently shifted to include elucidation of enzymes responsible for these transformations (*7,8*). The purpose of this study was to continue previously unfinished and non-definitive research in pursuit of utilizing metabolic engineering techniques to clarify the role of an Fe-only hydrogenase in the reduction of TNT by *C. acetobutylicum*.

5.1 Identification of Successful Plasmid Formation

As discussed in earlier chapters, several plasmids containing DNA encoding all or partial *hyd* A gene sequences in *C. acetobutylicum* were constructed to alter the expression level of the hydrogenase enzyme as compared to wild type cells. Results obtained from characterization and sequencing studies reveal successful formation of plasmid pHTB, the over-expression plasmid, containing the *ptb* promoter as well as the entire sequence of the *hyd A* gene. This combination of genes in sequence produces an amplified amount of the hydrogenase enzyme over control strain cells. The sequencing results suggest expected enhanced rates of TNT reduction.

Characterization also identified three successful antisense plasmid constructs: pASH1, pASH 2, and pASH 3, which all contain short antisense encoding regions of the *hyd A* gene. These antisense regions are preceded by the *ptb* promoter, which has been shown to increase expression of genes in plasmid DNA (*9*). These two gene segments in sequence produce large amounts of antisense RNA expected to bind to mRNA responsible for transcribing the genomic hydrogenase enzyme; thus, effectively inhibiting cells from producing normal levels of hydrogenase activity. Due to differences in length

and region of the *hyd A* gene encoded, the antisense strands produced from each of the three plasmids bind differentially to mRNA and cause varying levels of hydrogenase production inhibition.

5.2 Solvent Production by Varying Strain Types

Data collected for solvent production rates allow for comparison of solvent and acid production between strain types and give some indication of expected hydrogenase activity. Cells grown over 18 hours were harvested in the late exponential growth phase, evidenced by optical densities ranging from 1.2 to 1.9. During this stage, solvent production has begun to increase but has not yet reached maximum rates of production. The shift in *C. acetobutylicum* metabolism from acidogenic stage to solventogenic phase is not well defined thus solvent production is observed throughout cell growth, excluding very early acidogenic stages. Increasing acid levels and resulting low pH induce clostridia to undergo the shift to solvent production, and in later phases acid concentrations remain fairly constant given that an alternate pathway for uptake of acids exists during the solventogenic stage (*10*). Cells grown over 48 hours are in the stationary phase with optical densities ranging from 2.4 to 3.1. During this phase solvent concentrations reach their maximum levels.

When analyzing data, it is important to compare genetically altered strains of interest with appropriate control strains. The wild type *C. acetobutylicum* has not been exposed to the conditions and alterations that genetically modified strains have and thus do not adequately represent a control for evaluation. The wild type data is presented to provide expected levels in unaltered activity of *C. acetobutylicum*. Strain pPTB, wild type *C. acetobutylicum* containing only the vector plasmid used to construct pHTB

without the *hyd A* insert, functions as the control for suitable comparison to strain pHTB. This allows for effects of plasmid transformation as well as antibiotic selection criteria to be disregarded as influential in observed results. Similarly, strain pSOS84, wild type *C. acetobutylicum* containing the vector plasmid used in construction of all antisense plasmids with no antisense encoding region present, serves as a suitable control for examining pASH 1, pASH 2, and pASH 3.

Cells containing plasmid pHTB produce considerably lower levels of solvents in both early and late stages of growth as compared to the pPTB control. Strain pHTB produced 14 mM butanol in contrast to 25 mM butanol produced by pPTB in late exponential growth and 7 mM butanol in contrast to 133 mM butanol produced by pPTB in stationary growth. As established previously, increased levels of hydrogen production, and thus hydrogenase activity, are associated with acidogenic periods when acid production is greater than solvent production (*11*). Furthermore, when hydrogenase is active, lower levels of NAD(P)H are available to drive the required reductions which take place during solvent production (*10*). Thus these results suggest that hydrogenase activity in the pHTB strain is maintained throughout all stages of growth and that the plasmid is effectively expressing the *hyd A* gene even during stationary growth when hydrogenase activity in wild type cultures declines.

Upon examining results of solvent production data for antisense plasmid strains, an increase of solvents is evident over the pSOS84 control strain. Butanol production after 18 hours of growth was measured at 24 mM in the pASH 1 strain, 4 mM in the pASH 2 strain, and 19 mM in the pASH 3 strain compared to 16 mM in the pSOS84 strain. Plasmid pASH 2 does not exibit heightened solvent production, which can

possibly be attributed to samples for this strain representing earlier stages of exponential phase; here the optical density is 1.2 whereas other strains were harvested at optical densities ranging from 1.6 to 1.9. The differences in solvent production between strains is minimal after 18 hours of growth; however, during stationary growth, after 48 hours, butanol levels reach 68 mM in pASH 1 strains, 62 mM in pASH 2 strains, and 49 mM in pASH 3 strains. In contrast, the pSOS84 control strain only produced 15 mM butanol. Increased levels of solvent production in the antisense strains, due to the same mechanism described above, are evidence of inhibited hydrogenase activity in these cells over the comparable control.

5.3 Mechanistic Role of Hydrogenase in TNT Reduction

A high degree of correlation ($R^2 = 0.89$) exists between hydrogenase activity in *C. acetobutylicum* and TNT reduction by these organisms. This correlation exists despite the possible confounding factors such as high oxygen sensitivity of the cell extracts as well as other intrinsic experimental variability. This correlation suggests a causative relationship between the level of hydrogenase active in a cell system and the ability of that system to reduce TNT.

Previous work identified a carbon monoxide dehydrogenase (CODH) in *C. thermoaceticum* as the enzyme catalytically responsible for TNT reduction by that organism (*8*). This study went on to state that due to identical reaction intermediates produced by *C. thermoaceticum* CODH and those observed in *C. acetobutylicum* TNT reduction, that "this organism [i.e. *C. acetobutylicum*] may contain CODH or an enzyme exhibiting a similar mechanism." The study also points out that since TNT reduction was stimulated by H_2 in *C. acetobutylicum* as compared to a CO driven reaction as in their

research, that possibilities other than CODH should be considered. In light of the research carried out in this thesis, it appears that hydrogenase catalyzed TNT reduction in *C. acetobutylicum*, which accounts for the observed H_2 driven reaction. Due to the similarity in structure of the CODH and hydrogenase enzymes, this result in not unexpected and furthermore it is likely that mechanistically the two enzymes reduce TNT similarly.

The structure and role of hydrogenase in cell systems aids in the mechanistic understanding of TNT reduction by this enzyme (for a schematic of the hydrogenase enzyme see Figure 2.1). The transformation of TNT to hydroxylamino-intermediates (R-NHOH) requires the four-electron reduction of TNT. Since hydrogenase activity is typically coupled with ferredoxin in whole cell systems, and the TNT reduction assays were carried out in this research in the absence of any additional electron shuttle, it is probable that TNT interacts with the hydrogenase enzyme in a manner that resembles ferredoxin coupling. Hydrogenase is composed of 5 iron-sulfur clusters, one of which is termed the H-cluster and is the center of catalytic activity (*12*). This active H-cluster couples H_2 oxidation with reduction of ferredoxin or in this case TNT. The proposed mechanism, consequently, is that through nucleophilic attack by the fully reduced state of the hydrogenase enzyme, followed by two protonations and loss of water, TNT undergoes a two-electron reduction to nitroso (R-NO) intermediate. This intermediate then immediately undergoes a similar attack by reduced state hydrogenase followed by two protonations to complete the four-electron reduction of TNT to form R-NHOH. This is postulated mechanism only and requires further research to test its validity.

5.4 Reduction of TNT by Varying Strain Types

By examining the TNT reduction capabilities of each strain type, it is possible to assess the effectiveness of the genetic alterations preformed on *C. acetobutylicum*. Table 4.2 lists strain types in order of decreasing TNT reduction rates. The data is normalized to μg of protein content of each cell extract since the preparation techniques varied in yield of protein content, and thus hydrogenase concentration, extracted from cells. It is evident that the unaltered wild type cells, during the acidogenic growth stage are most effective at TNT reduction. Upon comparing the over-expression pHTB plasmid to the suitable control, pPTB, in the acidogenic phase, decreased activity is observed. This result is not consistent with expected activity for the plasmid as identified through characterization. It is plausible that inconsistency exists between preparations of the sets of extracts and that the result is due to oxygen inhibition, which occurred during cell extract isolation. Another possible explanation might be that a regulation mechanism is occurring which does not allow hydrogenase levels in these cells to be vastly greater than normal expression levels. This mechanism is not known to exist in this system, however regulation of enzyme expression is a well-known phenomenon.

Although the pHTB plasmid did not induce heightened TNT reduction activity, results suggest that the plasmid is effective in producing hydrogenase during phases when it is not normally observed. As stated earlier, decreased levels of solvent production during stationary growth give indication that hydrogenase is active in these systems. Furthermore, rates of TNT reduction for pHTB strains in the solventogenic stage resemble those occurring in the acidogenic stage. In wild type cell systems, a significant

decline in TNT reduction activity is observed in late stages of growth. Upon comparing the observed effect in pHTB cell systems to what is observed with unaltered wild type cell systems, it is evident that hydrogenase activity persists into late phases of growth in the pHTB strain. This implies that expression of hydrogenase other than that which is genetically encoded is produced in these systems due to effective expression of hydrogenase by the genetically modified plasmid.

All antisense plasmids resulted in decreased TNT reduction activity compared to the pSOS84 antisense control plasmid. Plasmid pASH 2 was particularly effective at reducing hydrogenase levels and TNT reduction capability even though results of solvent production analysis suggest this strain appears to be in earlier stages of acidogenesis than the other antisense plasmids at the time of cell harvest. Sequencing of the antisense plasmids identifies differences between the three, which may account for this difference in effective inhibition. Plasmids pASH 1, pASH 2, and pASH 3 contain antisense *hyd A* inserts 78 bp, 146 bp, and 42 bp in size respectively. Corresponding TNT reduction capabilities for the strains containing these plasmids are 2.16 μmol/min/ μg protein, 1.30 μmol/min/ μg protein, and 2.40 μmol/min/ μg protein respectively. With increasing size of antisense insert, a corresponding decrease in TNT reduction rates is observed.

The mechanism by which antisense inhibition occurs explains this phenomenon. As described earlier, the region encoded in the plasmid DNA forms segments of asRNA which effectively bind to mRNA involved in translation of genomic DNA. This approach inhibits translation at varying degrees and does not entirely cease the production of targeted proteins. Longer segments of antisense RNA, as encoded by larger plasmid inserts, more effectively bind with mRNA due to higher probability of

contact as well as increased binding locations. It thus is reasonable for pASH 2 to display the highest level of hydrogenase inhibition followed by pASH 1 and pASH 3.

5.5 References

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CHAPTER 6 – CONCLUSIONS

The following conclusions are drawn based on the results of the research presented in this thesis:

- Plasmids developed in previous work were successfully constructed according to metabolic engineering techniques available for alteration of enzyme expression levels. Plasmid pHTB contains a genetic sequence expected to result in increased production of hydrogenase. Plasmids pASH 1, pASH 2, and pASH 3 contain genetic information to encode for antisense RNA effective in inhibiting hydrogenase expression levels.
- Solvent production by altered strains corresponded with expected levels. Plasmid pHTB produced lower solvent concentrations than control pPTB, suggesting heightened hydrogenase activity. Antisense plasmids produced increased levels of solvents compared to control pSOS84 thus indicating decreased hydrogenase activity.
- A high degree of correlation exists between hydrogenase activity in *C. acetobutylicum* and TNT reduction by these organisms. This result, provided in the context of previous works, strongly suggests a causative relationship between the two variables. The hydrogenase enzyme is mechanistically capable of transforming TNT to hydroxylamino intermediates.
- Genetic alterations to increase levels of hydrogenase activity were not effective during acidogenisis, when hydrogenase is typically active. However, hydrogenase activity was maintained into stationary growth phase, when

hydrogenase is typically known to decrease activity, due to plasmid-encoded hydrogenase.

- Genetic alterations to inhibit hydrogenase production through the antisense approach were successful. Increasing length of plasmid antisense encoded region corresponded to more effective inhibition of hydrogenase production and accordingly TNT reduction capabilities.

CHAPTER 7 – ENGINEERING SIGNIFICANCE

The emerging field of metabolic engineering has recently been employed to utilize recombinant DNA methods to allow for controlled alteration of cellular metabolism. Several studies have successfully increased production of genes expressed in clostridia solventogenesis (*1-3*); however, antisense strategies have only been implemented once before to alter metabolism in clostridia (*4*). Extending this technology further, by varying levels of enzymes thought to be catalytic in environmental contaminant bioremediation, can help to identify organisms and mechanisms responsible for biodegradation. This study successfully applied antisense strategies to decrease rates of TNT reduction and although it did not enhance TNT reduction rates with an over-expression plasmid, TNT reduction activity was able to be maintained into stationary growth phase when it is not ordinarily observed. Metabolic engineering techniques thus proved useful in ascertaining the role of specific enzymes in environmentally engineered systems.

Furthermore, identification of the hydrogenase enzyme as catalytically responsible for TNT reduction in this system may be extrapolated to other systems of concern. As stated earlier, the hydrogenase enzyme is commonly found in a variety of microbial species (*5,6*) and has previously been determined to be responsible for nitro group reduction of compounds other than TNT (*7,8*). It is likely that due to hydrogenase structure and function and the highly electronegative reductive potential of the nitro group that this enzyme would function in alternate systems in a similar manner.

Existing technologies may allow for antibody probes to be developed in order to identify presence of specific enzymes *in situ*. The probes can only provide an appropriate

assay for enzymes that have been previously identified and characterized. Determination of hydrogenase as a catalyst in TNT reduction is a preliminary step to constructing a probe capable of assessing nitro-reduction potential of cell systems where contamination is present.

The fields of molecular biology and environmental engineering are rapidly becoming intertwined in the area of contaminate fate in natural and engineered systems. It is important that the two disciplines are exploited simultaneously to fully understand the context of bioremediation complexity. This study, in addition to other previous works in the area of TNT remediation, have fully incorporated microorganism metabolism and characterization into the understanding of engineered systems, thus contributing to the meshing of the two disciplines.

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CHAPTER 8 – FUTURE RESEARCH RECOMMENDATIONS

Considerable research has been conducted prior to these studies suggesting the role of hydrogenase enzymes in TNT reduction. This research successfully correlated the two variables of hydrogenase activity and TNT reduction capacity determining an observed causative nature between the two. It is important to consider this result in a broader context of microbial nitroaromatic reduction. Doing so raises significant unanswered questions.

Hydrogenase belongs to a class of enzymes grouped collectively as nitroreductases, based on observed reduction of nitroso groups by these enzymes (*1*). As stated by Riefler and Smets, experiments in which isolated enzymes have been used to transform TNT result in production of hydroxylamino-intermediates without observation of the corresponding amines (*2*). They go on to suggest that "typical nitroreductases may be unable to reduce hydroxylaminoarenes and that other enzymes are required to complete the conversion to an aminoarene." However, to date, enzymes have not been isolated or studied which carry out this transformation. Due to the activation energy required to carry out this final reductive step being greater than the two steps performed by enzyme catalysis, it is possible that transformation to amines is carried out by abiotic reductants. Currently there is no evidence to confirm the mechanism by which hydroxylamino-intermediates are transformed or why the nitroreductase enzymes do not complete the reduction. Further work focusing on answering these types of questions may shed light on what processes are occurring in natural and engineered systems where nitro-reduction is observed.

Furthermore, it is interesting to consider the implication of an enzyme, such as hydrogenase as well as other nitroreductases, so central to cellular metabolism and function displaying a great deal of versatility and non-specificity towards substrate binding. Characterization of these enzymes and examination into qualities that allow them to transform nitro groups without prior acclimation periods may provide insight into chemical fate in bioremediation systems. A broader understanding of enzyme interaction with substrates and contaminants of interest will enhance the field of environmental engineering and provide a useful tool particularly in the area of environmental biotechnology.

8.1 References

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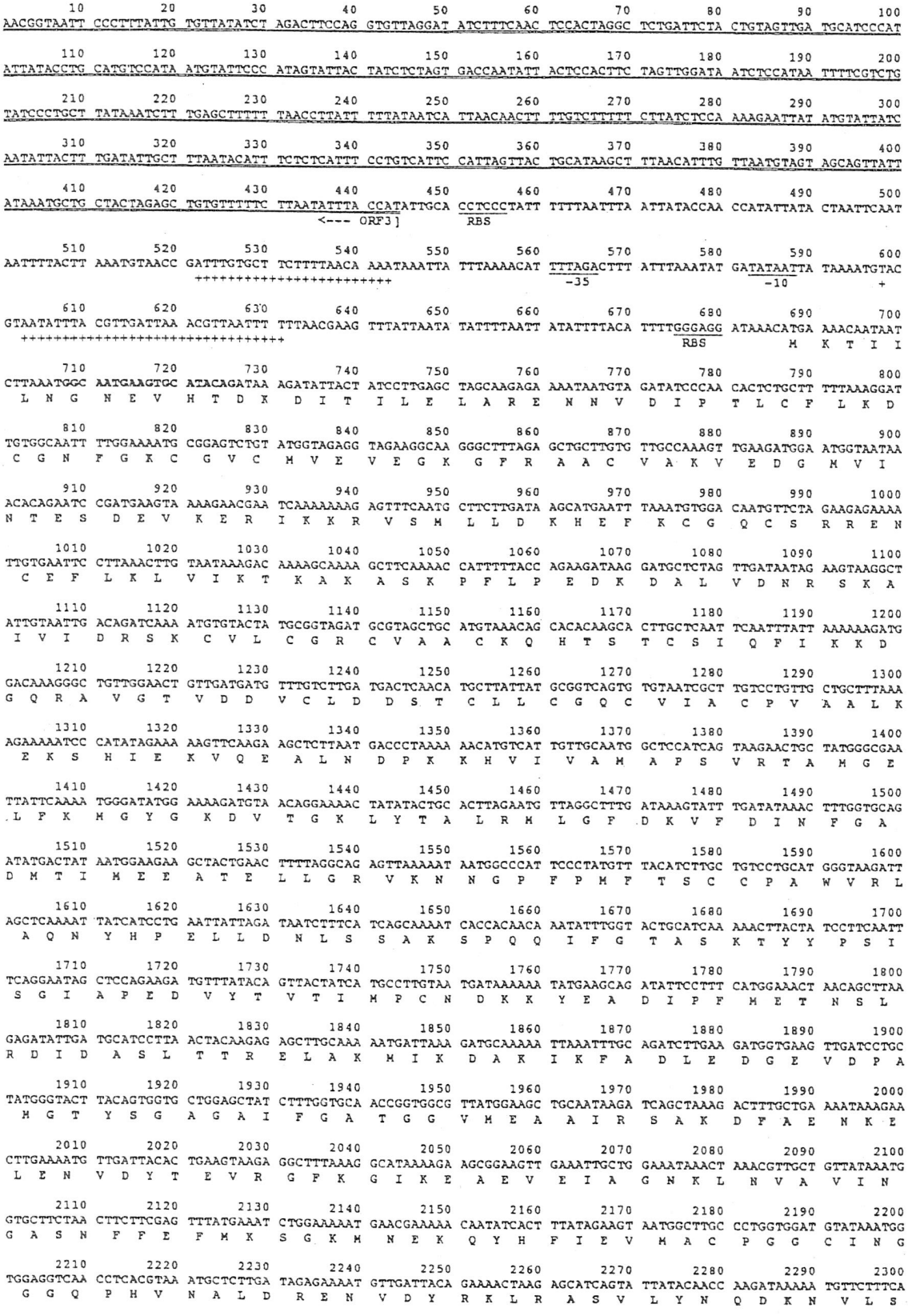
CHAPTER 9 – APPENDICES

9.1 Appendix A – Published *hyd A* Sequence



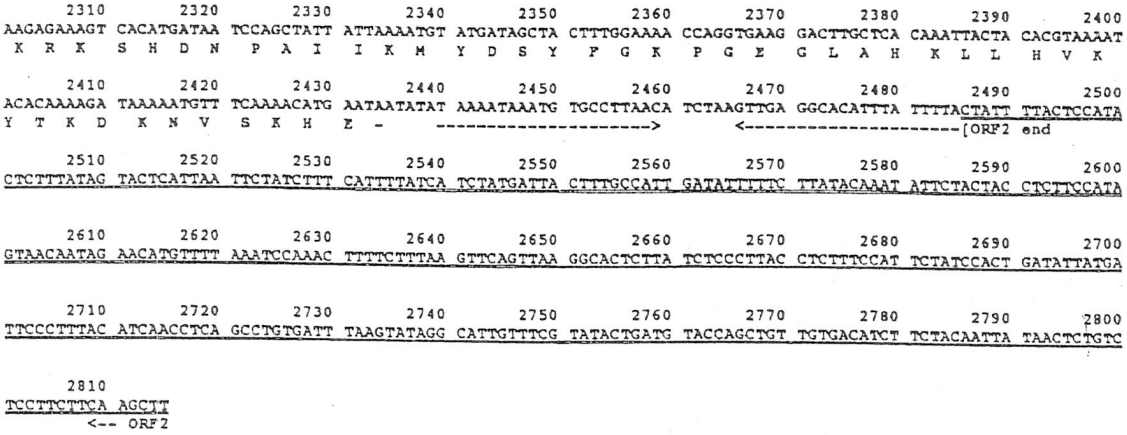
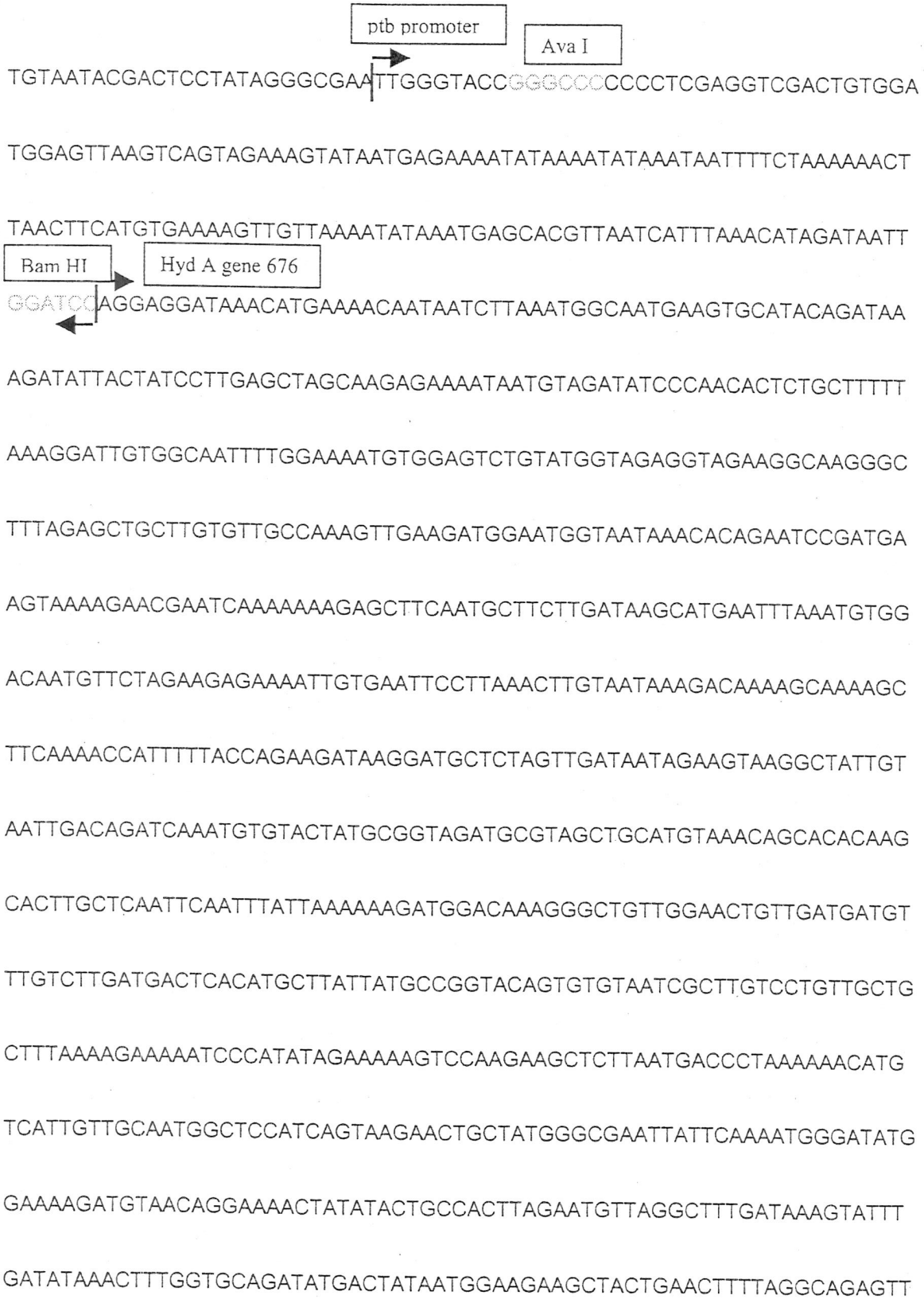


FIG. 2. Nucleotide sequence of the DNA fragment containing the entire transcribed region of the *C. acetobutylicum* ATCC 824 *hydA* gene. ORF2 and ORF3 are heavily underlined. The deduced amino acid sequence of hydrogenase is represented in single-letter code below the nucleotide sequence. The major transcriptional start site of *hydA* is indicated (+). The corresponding −10 and −35 regions of *hydA* as well as the putative ribosome-binding sites (RBS) for *hydA* and ORF3 are underlined. The inverted-repeat sequences upstream of the −35 promoter region and downstream of the −10 promoter region are underscored (+). The putative rho-independent transcriptional terminator (for *hydA* and ORF2) is indicated by opposing arrows. The complementary sequence of the HYDPE primer (bp 726 to 708) is written in bold letters.

9.2 Appendix B – pHTB Sequencing Results



AAAAATAATGGCCCATTCCCTATGTTTACATCTTGCTGTCCTGCATGGGTAAGATTAGCTCAA

AATTATCATCCTGAATTATTAGATAATCTTTCATCAGCAAAATCACCACAACAAATATTTGGTA

CTGCATCAAAAACTTACTATCCTTCAATTTCAGGAATAGCTCCAGAAGATGTTTATACAGTTA

CTATCATGCCTTGTAATGGATAAAAAATATGAAGCANATATTCCTTTCATGGAAACTAACAGC

TTAAGAGATATTGATGCATCCTTACTACAAGAAAGCTTGCAAAAATGATTAAAGAGCAAAAAT

TAAATTTGCANATCTTGAAAAAGGGGGAAGTGGATCCTGCTATGG Hyd A gene 1905

Hyd A gene 1921

CTGGAGCTATCTTTGGGCAACCGGTGGCGTTATGGAAGCTGCAATAAGATCAGCTAAAGACT

TTGCTGAAAATAAAGAACTTGAAAATGTTGATTACACTGAAGTAGAGGCTTTAAAGGCATAAA

AGAAGCGGAAGTTGAAATTGCTGGAAATAAACTAAACGTTGCTGTTATAAATGGTGCTTCTAA

CTTCTTCGAGTTTATGAAATCTGGAAAAATGAACGAAAAACAATATCACTTTATAGAAGTAATG

GCTTGCCCTGGTGGATTGTATAAATGGTGGAGGTCAACCTCACGTAAATGCTCTTGATAGAG

AAAATGTTGATTACAGAAAACTAAGAGCATCAGTATTATACAACCAAGATAAAAATGTTCTTTC

AAAGAGAAAGTCACATGATAATCCAGCTATTATTAAAATGTATGATAGCTACTTTGGAAAACC

AGGTGAAGGACTTGCTCACAAATTAATACACGTAAAATACACAAAAGATAAAAATGTTTCAAA

ACATGAATAATATATAAAATAAATGTGCCTTAACATCTAAGTTGAGGCACATTTATTTTACTAT

Hyd A gene 2517 Not I

TTTACTCCATACTCTTTATAGTACTCAT GCGGCCGC CACCGCGGTGGAGCTCCAGCTTTTGT

TCCCTTTAGGAGGGTTAATTTCGAGCNGGCGA

9.3 Appendix C – Antisense Plasmid Sequencing Results

pASH 1

TATATGAGAAATATAAAATATAAATAATTTTCTAAAAAACTTAACTTCATGTGAAAAGTTTGTTA

Bam HI → *hyd A* gene; as (639-717)

AAATATAAATGAGCACGTTAATCATTTAACATAGATAATTGGATCCTTCATTGCCATTTAAGAT

TATTGTTTTCATGTTTATCCTCCCAAAATGTAAAATATAATTAAATATATTAATAAACTAAAAGT

Ava I

AATTACATTACTCGGGTATCCAAAGCTTTTTGTGGATTCAGATACTTTAGTAGGAACTTTAGA

pSOS84 vector

CTATGGAAAACTTAGAGTTGCGACAGCTACAATGGGGTACAAACATAAAGCCTTAGATGCTA

ATGAAGCAAAGGATCAAATTTGTCGCCCTAATTATATGTTGAAAATAATACCCAATTATGATG

GAAGCCCTAGAATATGTGAGCTTATAAATGCGAAAATCACAGATGTTACCGTACATGAAGCTT

GGACAGGACCAACTCGACTGCAGTTATTTGATCACGCTATGGCGCCACTTAAATGATTTGCC

AGTAAAAGANATTGTTTCTAGCTCTCACATTCTTGCAGATATAATATTGCCCTAAAGCTGAAG

TTATATATGGATTTATCTTTAAGTAAATNAAAAATAAAGAAGTTAACCTTTNAAATGGGTAACC

TCCTTAATTTTTTTTTTAATGGGCCCACCTCCATTAAAAAATTTCCGTAAAATCCATTGGGGNCA

ATAANCCTTGGGTTTTCCCCTGGGGGGNNGAAAAAATTTGGTTTATTNCCCCNCCTCCACAA

AANTTTNCCCCCCCCCCAAACCATTTTACCGAAANCCCCCGGGNAAAAGCCANTTTAAAAAG

GNGGGTAAAAAAAGCCCCCTGGGGGGGGGNGGNCCCTAAAAATTGGNAANTGGGAAGCCC

TAAACCCCTCCCCCCNTTTAAAATTTGGGCCGNTTTGGCCGCCCTCCNCCTGGGNCCCCCC

CTTTTTTTCCCAAGNNCNGGGGGAAAAACCCTTGGNCCCGNNGGCCCANCCTGGCCATTTT

AAATGGAAAACCCGGGCCCAAACCCCCCCCGGGGGGNAAAAAGGGCCGGNTTTTTTCCNTA

TTTTGGGGGGNGCTTCNTTCCCNNNTTTTCCNCCCNCNTTCNCTTGAAACTCCCCNTTGCCC

CNCCGGGNCNCTTTCNGGNTGGCGGCNAAAACNGGNAACCCCCCCCCCCCCCAAANGNNC

pASH 2

1 CATTAATGCA GCTGNNCGAC AGGTTTNCCG ACTGNAAAGC

41 GGCAGTGAG CGCAACGCAA NTAATGTGAG TTAGCTCACT

81 CATTAGGCAC CCCAGGCTTT ACACTTTATG CTTCCGGCTC

121 GTATGTTTGG TGGAATGTGA GCCGATAACA ATTTCACCCA

161 GGAANCAGCT ATGACCATGA TTACGAATTC TATGAGTCGA

201 CATTAAAAAA ATAAGAGTTA CCATTTAAGG TAACTCTTAT

241 TTTTATTACT TAAGATAATC ATATATAACT TCAGCTCTAG

281 GCAATATTAT ATCTGCAAGA ATGTGAGAGC TAGAAACAAT

321 CTCTTTTACT GGCAAATCAT TAAGTGGCGC CATAGCGTGA

361 TCAAATAACT GCACTCGAGT TGGTCCTGTC CAAGCTTCAT

401 GTACGGTAAC ATCTGTGATT TTCGCATTTA TAAGCTCACA

441 TATTCTAGGG CTTCCATCAT AATTGGGTAT TATTTTCAAC

481 ATATAATTAG GGCGACAAAT TTGATCCTTT GCTTCATTAG

521 CATCTAAGGC TTTATGTTTG TACCCCATTG TAGCTGTCGC

561 AACTCTAAGT TTTCCATAGT CTAAAGTTCC TACTAAAGTA

pSOS84 vector | Ava I

601 TCTGAATCCA CAAAAAGCTT TGGATACCCG GGTAATGTAA

hvd A 639-785

641 TTACTTTTAG TTTATTAATA TATTTTAATT ATATTCTTTT

681 ACATTTTGGG AGGATAAACA TGAAAACAAT AATCTTAAAT

721 GGCAATGAAG TGCATACAGA TAAAGATATT ACTATCCTTG

761 AGCTAGCAAG AGAAAATAAT GTAGATATCC CAACACTCGG

Bam HI

pSOS84 vector

801 ATCCAATTAT CTATGTTAAA TGATTAACGT GCTCATTTAT

841 ATTTTAACAA ACTTTTCACA TGAAGTTAAG TTTTTTAGAA

881 AATTATTTAT ATTTTATATT TTCTCATTAT ACTTTCTACT

921 GACTTAACTC CATCCACAGT CGACCTGCAG GAGCTTTTTC

961 TAATTCACAT AAGCGTGCAG GTTTAAAGTA CATAAAAAAT

1001 ATAATGAAAA AAAGCATCAT TATACTAACG TTATACCAAC

1041 ATTATACTCT CATTATACTA ATTGCTTATT CCAATTTCCT

1081 ATTGGTTGGA ACCAACAGGC GTTAGTGTGT TGTTGAGTTG

1121 GTACTTTCAT GGGATTAATC CCATGAAACC CCCAACCAAC

1161 TCGCCAAAGC TAGCTTTGGC TAACACACAC GCCATTCCAA

1201 CCAATAGTTT TCTCGGCATA AAGCCATGCT CTGACGCTTA

1241 AATGCACTAA TGCCTTAAAA AAACATTAAA GTCTAACACA

1281 CTAGAACTTA TTTACTTCGT AATTAAGTCG TTAAACCGTG

1321 TGCTCTACGA CCAAAAGTAT AAAACCCTTT AAGAACTTTC

1361 TTTTTTTCTT GAAAAAAAAG AAACTAGATA AAATCTCTCA

1401 TATCNTTTTA TTCCAATAAT CGCATCAGAT TGCCGNATAA

1441 ATNTTAACGA TCCCTCATCA TGTTCATAAT TTATNNAGAG

1481 CTNGTGCCTN AANTATACTA AANNGTANAG GGAGGAAAAA

1521 ATNANGGNGG GCTCTNATNA AACNGAAAAA ATTTNAAACC

1561 CCCGNCAAAA CCTTNTTACT NCNAAACANA ATAAT

pASH 3

TCGTNGTCAGTAGAAAGTATAATGAGAAAATATAAAATATAAATAATTTTCTAAAAAACTTAAC

pSOS84 vector

TTAACTTCATGTGAAAGTTTGTTAAAATATAAATGAGCACGTTAATCATTTAACATAGATAATT

Bam HI *hyd A* gene; as (675-717) Ava I

GGATCCTTCATTGCCATTTAAGATTATTGTTTTCATGTTTATCCTCCCGGGTAAAAGTAATTAC

pSOS84 vector

ATTACCTAGTCGGGTATCCAAAGCTTTTTGTGGATTCAGATACTTTAGTAGGAACTTTAGACT

ATGGAAAACTTAGAGTTGCGACAGCTACAATGGGGTACAAACATAAAGCCTTAGATGCTAAT

GAAGCAAAGGATCAAATTTGTCGCCCTAATTATATGTTGAAAATAATACCCAATTATGATGGA

AGCCCTAGAATATGTGAGCTTATAAATGCGAAAATCACAGATGTTACCGTACAATTGAAGCTT

GGACAGGACCAACTCGACTGCAAGTTATTTGATCACGCTATGGCGCCCTTAATGATTTGCCA

GTAAAAGAGATTGTTTCTAGCTCTCACATTCTTGCAGATATAATATTGCCTAGAGCTGAAGTT

ATATATGATTATCTTAAGTAATAAAAATAAGAGTTCCTTAAATGGTAACTCTTATTTTTTTAATG

TCGACTCAATAGAATTCGTAATCAATGGGTCATAGCT

pASH 4

CNAGTCTTGCTGCCNGCTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACTG

TGGATGGAGTTAAGTCAGTAGAAAGTATAATGAGAAAATATAAAATATAAATAATTTTCTAAAA

pSOS84 vector

AACTTAACTTCATGTGAAAAGTTTGTTAAAATATAAATGAGCACGTTAATCATTTAACATAGAT

pSOS84 vector insert

AATTGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCT

pSOS84 vector

TTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTG

TTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG

CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGA

AACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTA

TTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG

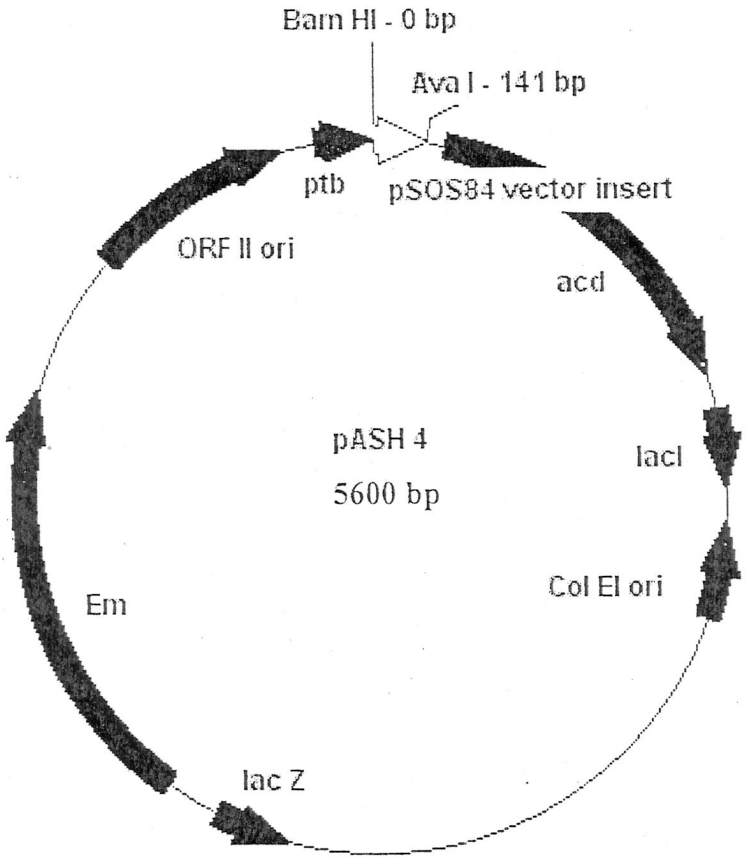
AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA

GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTG

CTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA

GAGGTGGCGAAACCCCGACAGGACTNTNAAGATACCAGGCGNTTNCCCCTGGAAGCTNC

pASH 4 Plasmid Map, experimental *hyd A* antisense plasmid 4



Relevant characteristics include: ptb, promoter of phosphotransbutyrylase in *C. acetobutylicum*; pSOS84 vector insert, segment of pSOS84 vector plasmid, ineffective as an antisense plasmid

9.4 Appendix D – pMFH1 Sequencing Results

hyd A 1 - 651

AACGGTAATTCCCTTTATTGTGTTATATCTAGACTTCCAGGTGTTAGGATATCTTTCAACTCCA

CTAGGCTCTGATTCTACTGTAGTTGATGCATCCCATATTATACCTGCATGTCCATAATGTATT

CCCATAGTATTACTATCTCTAGTGACCAATATTACTCCACTTCTAGTTGGATAATCTCCATAAT

TTTCGTCTGTATCCCTGCTTATAAATCTTTGAGCTTTTTTAACCTTATTTTTATAATCATTAACA

ACTTTTGTCTTTTTCTTATCTCCAAAAGAATTATATGTATTATCAATATTACTTTGATATTGCTTT

AATACATTTCTCTCATTTCCTGTCATTCCATAAGTTACTGCATAAGCTTTAACATTTGTTAATGT

AGTAGCAGTTATTATAAATGCTGCTACTAGAGCTGTGTTTTTCTTAATATTTACCATATTGCAC

CTCCCTATTTTTTTAATTTAATTATACCAACCATATTATACTAATTCAATAATTTTACTTAAATGTA

ACCGATTGTGCTCTTTTAACAAAATAAATTATTTAAAACATTTTAGACTTTATTTAAATATGATA

TAATTATAAAATGTACGTAATATTTACGTTAAACGTTAATTTTTAACGAAGTTATTAATAT

hyd A 671 - 2160

TTTTGGGAGGATAAACATGAAAACAATAATCTTAAATGGCAATGAAGTGCATACAGATAAAGA

TATTACTATCCTTGAGCTAGCAAGAGAAAATAATGTAGATATCCCANCACTCTGCTTTTTAAA

GGATTGTGGCAATTTTGGAAAATGCGGAGTCTGTATGGTAGAGGTAGAAGGCAAGGGCTTT

AGAGCTGCTTGTGTTGCCAAAGTTGAAGATGGAATGGTAATAAACACAGAATCCGATGAAGT

AAAAGAACGAATCAAAAAAAGAGTTTCAATGCTTCTTGATAAGCATGAATTTAAATGTGGACA

ATGTTCTAGAAGAGAAAATTGTGAATTCCTTAAACTTGTAATAAAGACAAAAGCAAAAGCTTC

AAAACCATTTTTACCAGAAGATAAGGATGCTCTAGTTGATAATAGAAGTAAGGCTATTGTAAT

TGACAGATCAAAATGTGTACTATGGGTAAATGCGTANCTGCATGTAAACAGCCACACAAGCA

CTTGCTCAATTCAATTTATTAAAAAAAGATGGGACAAAGGGCTGTTGGGAACTGTTGATGATGT

TGGTCTTGATGACTCCAACATGCTTATTATGCGGTCAGTGTGTAATCGCTTGTCCTGTTGCTG

CTTTAAAAGAAAAATCCCATATAGAAAAAGTTCAAGAAGCTCTTAATGACCCTAAAAAACATG

TCATTGTTGCAATGGCTCCATCAGTAAGAACTGCTATGGGCGAATTATTCAAAATGGGATATG

GAAAAGATGTAACAGGAAAACTATATACTGCACTTAGAATGTTAGGCTTTGATAAAGTATTTG

ATATAAACTTTGGTGCAGATATGACTATAATGGAATAAGCTACTGAACTTTTAGGCAGAGTTA

AAAATAATGGCCCATTCCCTATGTTTACATCTTGCTGTCCTGCATGGGTAAGATTAGCTCAAA

ATTATCATCCTGAATTATTAGATAATCTTTCATCAGCAAAATCACCACAACAAATATTTGGTAC

TGCATCAAAAACTTACTATCCTTCAATTTCAGGAATAGCTCCAGAAGATGTTATACAGTTACTA

TCATGCCTTGTAATGATAAAAATATGAACANATATCCTTTCTGGAACTACACTTAGANATTTGA

GCTCTTACTCANANACTTGCAAATGATTAAGAGCAAATAATTCGACTGAAAGGGAGTGACCG

CTATGGNNTNCGGGGGCTGACTACTTGGGCACNGGGCNTNTGGAACTGATATGAAGCAGAT

ATTCCTTTCATGGAAACTAACAGCTTAAGAGATATTGATGCATCCTTAACTACAAGAGAGCTT

GCAAAAATGATTAAAGATGCAAAAATTAAATTTGCAGATCTTGAAGATGGTGAAGTTGATCCT

GCTATGGGTACTTACAGTGGTGCTGGAGCTATCTTTGGTGCAACCGGTGGCGTTATGGAAG

CTGCAATAAGATCAGCTAAAGACTTTGCTGAAAATAAAGAACTTGAAAATGTTGATTACACTG

AAGTAAGAGGCTTTAAAGGCATAAAAGAAGCGGAAGTTGAAATTGCTGGAAATAAACTAAAC

GTTGCTGTTATAAATGGTGCTTCTAACTTCTTCGAGTTTATGAAATCTGGAAAAATGAACGAA

AAACAATATCACT

hyd A 2197-2798

ATGGTGGAGGTCAACCTCACGTAAATGCTCTTGATAGAGAAAATGTTGATTACAGAAAACTAA

GAGCATCAGTATTATACAACCAAGATAAAAATGTTCTTTCAAAGAGAAAGTCACATGATAATC

CAGCTATTATTAAAATGTATGATAGCTACTTTGGAAAACCAGGTGAAGGACTTGCTCACAAAT

TACTACACGTAAAATACACAAAAGATAAAAATGTTTCAAAACATGAATAATATATAAAATAAAT

GTGCCTTAACATCTAAGTTGAGGCACATTTATTTTACTATTTTACTCCATACTCTTTATAGTAC

TCATTAATTCTATCTTTCATTTTATCATCTATGATTACTTTGCCATTGATATTTTTCTTATACAAA

TATTCTACTACCTCTTCCATAGTAACAATAGAACATGTTTTAAATCCAAACTTTTCTTTAAGTTC

AGTTAAGGCACTCTTATCTCCCTTACCTCTTTCCATTCTATCCACTGATATTATGGATTCCCTT

TACATCAACCTCAGCCTGGGGATTTAAGTATAGGCATTGTTTCCTNATACTGGAGGGAACCA

GCTGGTGGGGACATCTTCTACAATTATAACTCTG

9.5 Appendix E -Rates of Hydrogen Production

Cell Extract ID	H2 Produced (mol/l/min)	H2 Produced (umol/min)	H2 Produced (umol/min/ul CE)	Averages
WT 110101 A1	0.00000903	0.17157	0.0017157	0.0016226
WT 110101 A3	0.00000805	0.15295	0.0015295	
WT 110101 B1	0.00000923	0.17537	0.0017537	0.0016359
WT 110101 B3	0.00000799	0.15181	0.0015181	
WT 18 hr -041402	0.0000113	0.2147	0.002147	
WT 48 hr -041502	0.00000138	0.02622	0.0002622	
pPTB A1	0.00000915	0.17385	0.0017385	0.0020083
pPTB A2	0.0000102	0.1938	0.001938	
pPTB A3	0.0000135	0.2565	0.002565	
pPTB A4	0.00000943	0.17917	0.0017917	
pPTH 041402	0.00000531	0.10089	0.0010089	
pASH 1 A1	0.00000733	0.13927	0.0013927	0.0010317
pASH 1 A2	0.00000353	0.06707	0.0006707	
pASH 1 041402	0.000003	0.057	0.00057	
pASH 2 A1	0.00000111	0.02109	0.0002109	0.000329175
pASH 2 A2	0.00000221	0.04199	0.0004199	
pASH 2 A3	0.00000205	0.03895	0.0003895	
pASH 2 A4	0.00000156	0.02964	0.0002964	
	0.0000044	0.0836	0.000836	
pASH 2 041402	0.00000094	0.01786	0.0001786	
pASH 3 A1	0.00000158	0.03002	0.0003002	
pASH 3 A2	0.00000232	0.04408	0.0004408	0.000383167
pASH 3 A3	0.00000215	0.04085	0.0004085	
pASH 3 041402	0.000005	0.095	0.00095	
pHTB 020202 CM	0.0000123	0.2337	0.002337	
pHTB 031402 (18 hr	0.0000097	0.1843	0.001843	
pHTB 031402 (48 hr	0.0000113	0.2147	0.002147	
pHTB 041402	0.00000472	0.08968	0.0008968	0.00081795
	0.00000389	0.07391	0.0007391	
pSOS84	0.0000119	0.2261	0.002261	
	0.00000236	0.04484	0.0004484	
pMFH1	0.00000349	0.06631	0.0006631	0.00062605
	0.0000031	0.0589	0.000589	

H2 Produced umol/min/ug protein
0.001040314
0.000987835
0.002903398
0.00031371
0.001431046
0.001311764
0.00068964
0.000671853
0.000234435
0.000595391
0.000512848
0.000312933
0.001306952
0.001822077
0.001669898
0.00148747
0.001206454
0.001671109
0.001774665
0.00068359

9.6 Appendix F - Rates of TNT Reduction

Cell Extract ID	TNT degraded (mg/l/min)	TNT degraded (umol/min)	TNT degraded (umol/min/ul CE)	Averages
WT 110101 A2	11.488	505.7829554	5.057829554	5.345766577
WT 110101 A3	12.796	563.3703601	5.633703601	
WT 110101 B1	15.564	685.2372839	6.852372839	6.734380297
WT 110101 B2	15.028	661.6387755	6.616387755	
WT 041402 18 hr	14.577	641.7825679	6.417825679	
WT 041502 48 hr	1.4826	65.27453078	0.652745308	
pPTB A1	11.142	490.5495899	4.905495899	4.949963237
pPTB A2	11.344	499.4430576	4.994430576	
pPTB 041402	7.045	310.1706929	3.101706929	
pASH 1 A1	9.2	405.0490241	4.050490241	4.10772543
pASH 1 A2	9.46	416.4960618	4.164960618	
pASH 1 041402	3.038	133.7542321	1.337542321	
pASH 2 A1	4.9604	218.3918673	2.183918673	1.937279039
pASH 2 A2	3.84	169.0639405	1.690639405	
pASH 2 041402	0.965	42.48612047	0.424861205	
pASH 3 A1	8.4509	372.0683476	3.720683476	2.732077681
pASH 3 A2	3.96	174.3471887	1.743471887	
pASH 3 041402	4.251	187.1590654	1.871590654	
pHTB 020202 CM	6.336	278.9555018	2.789555018	2.284212334
	4.0404	177.8869649	1.778869649	
pHTB 031402 (18hr)	5.5321	243.562142	2.43562142	3.235255702
	7.2299	318.3112978	3.183112978	
	9.283	408.7032708	4.087032708	
pHTB 031402 (48hr)	6.9254	304.9050556	3.049050556	3.591361302
	6.8291	300.665249	3.00665249	
	10.717	471.8380861	4.718380861	
pHTB 18hr 041502	5.499	242.1048461	2.421048461	
pSOS84	6.2528	275.2924498	2.752924498	3.646277732
	10.311	453.9630965	4.539630965	
pSOS84 - 041402	1.536	67.6255762	0.676255762	
pMFH1 041402	3.644	160.4346352	1.604346352	

TNT degraded (umol/min/ug protein)
3.427385545
4.06654123
8.678855247
0.78097967
3.527175494
4.03281664
2.745807944
1.5765477
1.379710708
1.219984429
2.231293216
2.574820333
1.780920257
2.931387107
2.488142307
3.570980331
2.694970661
2.67646562
1.751800839

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

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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 K_m of 152 μM. 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 resulted in 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 is proposed. Due to the prevalence of hydrogenase in soil microbes, this research may enhance the understanding of nitroaromatic compound transformation by common microbial communities.

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 (4, 11, 16), much recent research has focused on remediation by biological processes (34). Microbial reduction of TNT has been well established by a wide variety of aerobic and anaerobic microorganisms (36, 37), and reduction by anaerobic clostridia species has been recently reviewed (3).

Reduction of aryl nitro groups to corresponding amines has been reported for anaerobic systems (8, 10, 32, 36). *C. acetobutylicum* transformed TNT with accumulation of the hydroxylamino intermediates, specifically 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT) and 2,4-dihydroxylamino-6-nitrotoluene (24DHA6NT), without formation of commonly observed amines (8, 32, 36). Further reduction of these metabolites by *Clostridium acetobutylicum* results in the formation of a phenolic amine through a Bamberger rearrangement (19). TNT is only completely reduced to 2,4,6-triaminotoluene under strictly anaerobic conditions (32). The catalytic process for these systems has not been clarified to date, although evidence for biocatalysis has been presented (19) and probable key enzymes, including hydrogenases, have been implicated in reduction steps (17).

Several additional findings support the possible role of hydrogenase in initial TNT transformation. For example, *C. acetobutylicum* reduces TNT rapidly only during the initial stages of growth when acid production is high and hydrogen is being produced (22). Additionally, it has been reported that TNT is reduced by crude extracts only when H_2 is a constituent in the atmosphere in which the assay is conducted (22), further implicating the role of hydrogenase. Both carbon monoxide and oxygen are known inhibitors of the hydrogenase enzyme effectively blocking its activity by binding to the hydrogenase catalytic center (2, 24). Accordingly, carbon monoxide has been shown to slow TNT reductive reactions (22), and oxygen irreversibly inhibits the capability of active crude extracts to reduce TNT. A purified enzyme with a similar mechanism, carbon monoxide dehydrogenase, from *Clostridium thermoaceticum* is responsible for TNT reduction to intermediates identical to the ones observed in the *C. acetobutylicum* cultures (17).

The primary function of the hydrogenase enzyme in whole-cell systems is to catalyze the reversible oxidation of H_2, which results in the uptake or production of hydrogen in systems in which it is active. Two classes of hydrogenase exist which are present in *C. acetobutylicum*, the Fe-only and the Fe-Ni hydrogenases. The role and function of each type of hydrogenase have been discussed in previous reviews (1, 2).

In *C. acetobutylicum* the Fe-only hydrogenase is located on the microbial chromosome, whereas the genetic information for the Fe-Ni hydrogenase is located on a separate plasmid (8, 29). The comparison of TNT-reducing activity in wild-type and mutant strains lacking the plasmid would indicate the contribution of each hydrogenase in the TNT reduction.

The purpose of these studies was to determine whether the Fe-only hydrogenase is the primary enzyme in the catalytic

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source and/or reference[b]
Strains		
E. coli DH10B	*mcrA*, Δ*mcrBC, recA1*	NEB, 25
C. acetobutylicum ATCC824	Wild type	ATCC
C. acetobutylicum M5	Absence of pSOL1	7
Plasmids		
pAN1	p15A ori; Cm^r, *Ø3t*I	26
pPTB	ColE1 ori, ORF II ori; Em^r, *ptb* promoter	17
pSOS84	ColE1 ori, ORF II ori; Em^r, Ap^r, *ptb* promoter	39
pMFH1	ColE1 ori; Ap^r, *ptb* promoter, *hyd*A	13
pHTB	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hydA*	This study
pASH1	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hydA*-asRNA	This study
pASH2	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hydA*-asRNA	This study
pASH3	ColE1 ori, ORF II ori; Em^r, *ptb* promoter, *hydA*-asRNA	This study

[a] pSOL1, 210-kb plasmid containing the *hydA* gene: p15A ori, p15A origin of replication; Cm^r, chloramphenicol resistance; *Ø3t*I, *Ø3t*I methyltransferase gene; ColE1 oriI, ColE1 origin of replication (recognized by *E. coli*); ORF II ori, ORF II origin of replication (recognized by *C. acetobutylicum*); Em^r, erythromycin resistance; Ap^r, ampicilllin resistance; *ptb* promoter, promoter of phosphotransbutyrylase in *C. acetobutylicum*; *hydA*, hydrogenase A gene; *hydA*-asRNA, hydrogenase A gene antisense RNA insert.

[b] NEB, New England Biolabs; ATCC, American Type Culture Collection, Manassas, Va.

ability of *C. acetobutylicum* to reduce TNT. The H_2-dependent reduction of TNT by the purified Fe-hydrogenase enzyme and the kinetic constants of TNT reduction has been described. Further studies were carried out to examine a causative relationship between the activity of hydrogenase present in a cell system and the corresponding rates of TNT reductase activity. The results of the studies demonstrate that the hydrogenase enzyme proposed is responsible for the major nitroreductive capability of *C. acetobutylicum*.

MATERIALS AND METHODS

Chemicals. All chemicals used for media preparation were reagent grade unless otherwise noted. All restriction enzymes were obtained from New England Biolabs (Beverly, Mass.). Gases used consisted of hydrogen, nitrogen, argon, and an anaerobic mixture of 5.1% CO_2, 9.9% H_2, and 85% N_2 and were obtained in the highest available purity from Trigas (Irving, Tex.). Solid chemicals used include TNT (Chemsyn Science, Lenexa, Kans.) purified to 98.6%, erythromycin (Sigma, St. Louis, Mo.), 4-(2-aminoethyl)benzene-sylfonyl fluoride (95%; Sigma), sodium dithionite (85%; Acros, Pittsburgh, Pa.), methyl viologen (hydrate 98%; Acros), ferredoxin (Sigma), and sodium sulfite (Mallinckrodt Inc., St. Louis, Mo.). Chemicals used for enzyme purification were Reactive Red 120-agarose (Sigma), Phenyl Sepharose (Amersham Pharmacia, Piscataway, N.J.), and Superdex-200 (Amersham Pharmacia). The solvent used, acetonitrile (99.9%; Fisher Scientific, Pittsburgh, Pa.), was high-performance liquid chromatography (HPLC) grade.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The pAN1 plasmid bears a specific methylase used for treatment of other plasmids prior to transformation into *C. acetobutylicum* (27). Plasmid pPTB is an *Escherichia coli-C. acetobutylicum* shuttle vector containing the *ptb* promoter region (K.-X. Huang, unpublished data) and was used to prepare a control *C. acetobutylicum* strain to account for host-plasmid interactions as well as to construct pHTB. Plasmid pSOS84 is also an *E. coli-C. acetobutylicum* shuttle vector containing the promoter region of the *ptb* gene (39; P. Soucaille, unpublished data) and was used in construction of the *hydA* antisense RNA plasmids (*hydA*-asRNA) pASH1, pASH2, and pASH3. Plasmid pPMFH1, provided by Philippe Soucaille (Institut National des Sciences Appliquées, Centre de Bioingénierie G. Durand, Toulouse, France), was used to obtain the *hydA* gene for PCR amplification and plasmid construction (12). *C. acetobutylicum* M5 lacks solvent-producing genes, including the region encoding an Fe-Ni hydrogenase (7, 29, 38).

Bacterial growth conditions. Cultures of *C. acetobutylicum* and all strains were grown and maintained at 37°C on clostridial growth medium, pH 7, as described by Hartmanis and Gatenbeck (15). Clostridia strains containing plasmids were selected by using 40 μg of erythromycin/liter. Mutant strain M5 was grown as described by Clark et al. (6).

Cell extract preparation. The cell extracts were prepared entirely by the anaerobic procedure as described by Hughes et al. (19). The protein content was determined by the Bradford assay method (Bio-Rad, Philadelphia, Pa.).

Detection of TNT reduction enzymatic activity by colorimetric measurement. TNT reduction activity was screened during enzyme purification steps through a modification of a method for the analysis of soil samples described by Jenkins et al. (20). TNT reacts with Na_2SO_4 to form a yellow color, which is stable for at least 24 h, with maximum absorbance at 420 nm. The absorbance at 420 nm is linearly dependent on the concentration of TNT up to 440 μM. A reaction mixture composed of Tris buffer (pH 7.2), enzyme, and TNT was prepared, and after a defined time aliquots (0.5 ml) were removed and added to an equal volume of Na_2SO_4 (0.2 g/ml). The color complex formed, resulting from addition of sulfite with the aromatic ring of TNT, was analyzed at 420 nm by using a UV-Vis spectrometer.

Isolation, purification, and identification of enzymes involved in TNT reduction. All column separations were carried out in an anaerobic chamber. Cell extract (40 ml) was applied to a Reactive Red 120 column (Sigma type 3000-CL, 2.25 by 9 cm) preequilibrated with 10 mM Tris buffer (pH 7.9). The column was washed with 10 ml of 0.5 M NaCl in Tris buffer (pH 7.9). The enzyme was eluted with 2 M NaCl in Tris buffer containing methyl viologen (0.25 g/liter). Fractions containing TNT-reducing activity, quantified by colorimetric measurements at 420 nm, were pooled and concentrated by ultrafiltration (Amicon columns MWCO 10K; Beverly, Mass.). The concentrate was then applied to a Phenyl Sepharose column (1 by 28 cm; Amersham Pharmacia), preequilibrated with 2 M KCl in 50 mM phosphate buffer (pH 7.0) containing methyl viologen (0.25 g/liter). The enzyme was eluted with 10 mM Tris buffer (pH 7.9) containing methyl viologen (0.25 g/liter). The active fractions containing TNT-reducing activity were pooled and concentrated to a volume of 1 ml by ultrafiltration. The concentrate was applied to a Superdex-200 column (Amersham Pharmacia) preequilibrated with 0.15 M NaCl in 50 mM phosphate buffer (pH 7.0) containing methyl viologen (0.25 g/liter). The active fraction eluted from this column was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (31). The major and minor protein bands were subjected to N-terminal sequence analysis by using an Applied Biosystems Procise sequencer, courtesy of Richard G. Cook, Baylor College of Medicine (Houston, Tex.).

Plasmid construction. *C. acetobutylicum* ATCC 824 genomic DNA, prepared as previously described (14), was used as a template for PCR. Two primers, H5 (5′-CGCGGATCCAGGAGGATAAACATGAAAACAATAATC-3′), containing a *Bam*HI site, and H3 (5′-GCTGGATCCGCGGCCGCATGAGTACTAT AAAGAGTATGGAGT), containing a *Not*I site, were used to amplify the *hydA* gene by using Pfu polymerase (Stratagene, Cedar Creek, Tex.). The amplified PCR product (2.1 kb) was purified by gel electrophoresis and was subcloned into the corresponding sites of pPTB to form pHTB. Antisense RNA techniques (9) were used to produce plasmids pASH1, pASH2, and pASH3 containing *hydA*-asRNA inserts 78, 146, and 42 bp in size, respectively. Primers AS3 (5′-CGGG ATCCTTCATTGCCATTTAA), containing a *Bam*HI site, and AS5 (5′-CCCC CGGGTAATGTAATTACTTTTAGT), containing an *Ava*I site, were used to

construct pASH1. Primers AS4 (5′-GCCGATCCGAGTGTTGGGATATC), containing a *Bam*HI site, and AS5 (as above) were used in construction of pASH2. Primers AS3 (as above) and AS6 (5′-CTAGGTAATGTAATTACTTT TACCCGGGAGGATAAACA TGAAAAC), containing an *Ava*I site, were used to construct pASH3. All as-RNA insert segments were amplified by using pMFH1 as a template (12). Plasmid constructs were verified by restriction enzyme digests followed by DNA sequencing of the appropriate segment by Lone Star Labs (Houston, Tex.).

DNA production and transformation. Transformation of plasmid DNA into *E. coli* DH10B cells was performed by standard procedures (33). Electrotransformation of pAN1-methylated plasmids into *C. acetobutylicum* was performed in an anaerobic chamber by using a previously published procedure (27, 28).

Solvent production quantification. Gas chromatography was used to determine concentrations of the aqueous-phase fermentation products ethanol, acetate, acetone, butanol, and butyrate produced by growing *C. acetobutylicum* cultures to determine metabolic growth phase (13).

Hydrogenase assay. Hydrogenase activity was determined at 25°C via hydrogen evolution by using a modified gas chromatography method described by Jungermann et al. (21). The hydrogenase assay solution (HAS) was made up of the buffer Tris · HCl (50 mM, pH 8), terminal electron donor, sodium dithionite (60 mM), and the electron donor methyl viologen (1 mM) and was made anaerobic either by equilibration in an anaerobic chamber or by sparging with argon for 20 min. The HAS was then transferred in 2-ml volumes into vials (20 ml) which had been sealed with a butyl rubber stopper and an aluminum cap and then flushed with argon. The addition of crude cell extract (100 μl) by using a gas-tight syringe started the reaction. At appropriate time intervals hydrogen production was measured in the headspace by injecting samples (0.25 ml) into a Gow-Mac Series 600 Gas Chromatograph (Bethlehem, Pa.) with a thermal conductivity detector at 120°C. Separation was obtained with a molecular sieve column (length, 2.43 m; Gow-Mac) at an oven temperature of 80°C by using argon as the carrier gas at a flow rate of 20 ml/min. Hydrogen evolution in HAS controls with no addition of cell extract was never observed.

TNT reduction study. TNT reduction assays were carried out under anaerobic conditions in 1 atm of H_2. Each experiment contained 10 ml of TNT stock solution (100-mg/liter concentration of TNT in deionized water), which was augmented with crude cell extract (100 μl) to begin the reaction. Samples of 20 μl taken over time (10 min at approximately 1-min intervals) were exposed to O_2 to quench the reaction for determination of the TNT reduction rate. TNT concentrations were measured as described by Huang et al. (17) with the following modification: analytes were separated on a reverse-phase Waters Nova-Pak-C18 column (2 by 150 mm) with a variety of gradient mobile phases consisting of water/acetonitrile (75/25 to 5/15 [vol/vol]) at 0.25 ml/min.

RESULTS

Isolation, purification, and identification of major enzymes involved in TNT reduction. The colorimetric method used for detecting TNT is specific to TNT; no significant absorption was observed at 420 nm for TNT reduction intermediates. The specific activity of the enzyme portion reducing TNT increased by 10-, 17-, and 23-fold after purification by using Reactive Red agarose-120, Phenyl Sepharose, and Superdex-200, respectively. The final fraction showing TNT reduction activity predominately contained two proteins (Fig. 1) identified as acetoacetyl-coenzyme A (CoA) thiolase (46 kDa) and hydrogenase (67 kDa) by N-terminal protein sequencing. The N-terminal amino acid sequence of the hydrogenase was MKTIILNGNEVHTDKDITIL, corresponding to that of the Fe-hydrogenase of *C. acetobutylicum* (12). The minor band on the gel had the N-terminal sequence of MKEVVIASAV, which is that of the previously purified acetoacetyl-CoA thiolase (40). No evidence has been reported that a thiolase enzyme can engage in a redox reaction (25, 30); therefore, the acetoacetyl-CoA thiolase is not expected to reduce TNT. Thus, the role of hydrogenase as the catalytic enzyme in *C. acetobutylicum* responsible for reducing TNT was proposed. To further examine the possibility that an alternative *C. acetobutylicum* Fe-Ni hydrogenase contributed to TNT reduction in whole-cell cultures, experiments comparing the transformation of TNT in growing cells at an optical density at 600 nm of 0.4 for the wild type versus mutant strain M5, in which the Fe-Ni hydrogenase encoding region is absent, demonstrated 0.256 and 0.245 mM TNT being transformed, respectively, during 4 h of incubation in a culture volume of 5 ml (data not shown). Heat-inactivated cells of each type showed negligible TNT transformation. This data reinforced the proposed role of the Fe-only hydrogenase in TNT reduction, whereas the Fe-Ni hydrogenase does not play a very significant role in the reduction steps under the growth conditions examined.

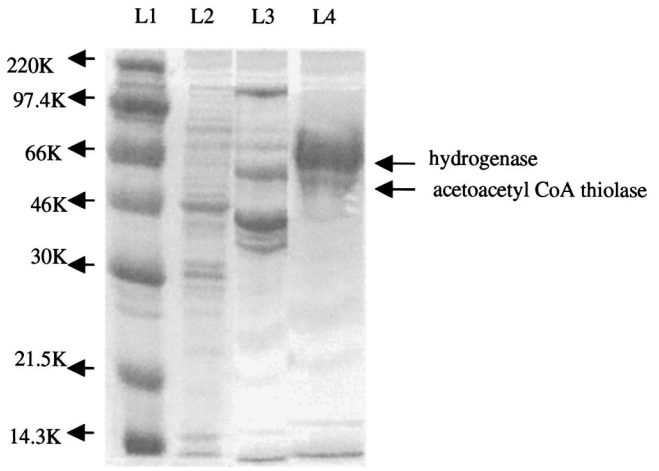

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of hydrogenase enzyme protein at each purification step. Samples (25 μg) were analyzed by electrophoresis on a 12% gel. The gel was stained with Coomassie blue R-250. L1, molecular size markers; L2, Reactive Red column fraction; L3, Phenyl Sepharose fraction; L4, Superdex-200 column fraction showing 43- and 67-kDa proteins.

Reduction of TNT catalyzed by isolated Fe-only hydrogenase. The purified hydrogenase was tested for catalytic ability to reduce TNT in the absence of additional electron donors, such as ferredoxin, under an H_2 atmosphere. The HPLC chromatogram, detected at 230 nm, and UV-VIS spectra of the individual peaks are shown in Fig. 2. After 35 min of hydrogenase addition, 2-hydroxylamino-4,6-dinitrotoluene (2HA46DNT) and 4HA26DNT were detected at concentrations approximately equal to the amount of decrease in TNT concentration. At 90 min the monohydroxylamino derivatives are converted to 2,4-dihydroxylamino-6-nitrotoluene (24DHANT) and the subsequent intermediate, a polar species resulting from a Bamberger rearrangement of 24DHANT, which was previously reported (18).

Kinetics of the first step in hydrogenase catalyzed TNT reduction. The apparent K_m of hydrogenase for TNT was determined by graphical analysis to be 152 μM under the conditions of the experiment.

Correlation of hydrogenase production and TNT reduction. In order to identify the Fe-only hydrogenase as the primary enzyme responsible for TNT reduction in whole *C. acetobutylicum* cell systems, cell extracts were prepared for strains containing plasmids developed to vary the hydrogenase expression level. The extracts were characterized for activity through two sets of assays, one for hydrogenase activity through hydrogen

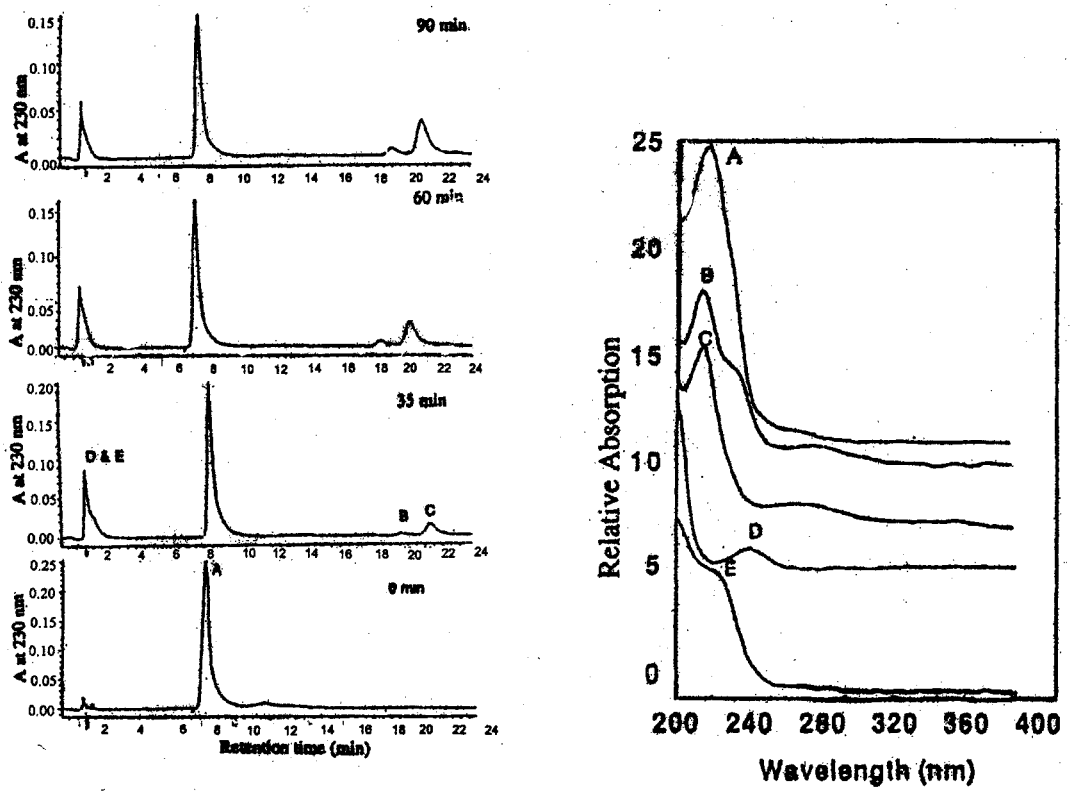

FIG. 2. HPLC chromatogram (left panel) and UV-VIS spectra (right panel) of TNT reduction products catalyzed by purified hydrogenase enzyme (7.8 μg) added to 110 μM TNT solution. Aliqots (100 μl) were analyzed by HPLC at 0, 35, 60, and 90 min after reaction start and then were monitored at 230 nm. UV-VIS spectra were obtained for the peak fractions TNT (A), 2HA46DNT (B), 4HA26DNT (C), 24DHANT (D), and polar Bamberger rearrangement product (E).

production rates and another for TNT reduction rates. Data gathered for each rate were taken during the initial zero order region of the reaction. TNT metabolites observed in TNT reduction assays by cell extracts of each strain show accumulation of 4HA26DNT, 2HA46DNT, and 24DHA6NT, as expected according to previous results for reduction by *C. acetobutylicum* (17–19). For each cell extract preparation values of TNT reduction and hydrogenase activity were plotted against each other, resulting in the appearance of a relationship between the two variables displaying a correlation coefficient (R^2) of 0.89 (Fig. 3). As the level of hydrogenase activity increases in a cell system, there is a corresponding increase in ability to transform TNT.

In order to determine the effectiveness of alterations to each strain, TNT reduction capability data was collected during different phases of growth (Table 2). Values given for antisense plasmids pASH1, pASH3, and pASH3 as well as for control plasmids pPTB and pSOS84 are representative of TNT reduction during acidogenic phase. The solvetogenic phase showed neither TNT reduction nor hydrogenase activity.

DISCUSSION

The isolated hydrogenase catalyzed the H_2-dependent reduction of TNT to 2HA46DNT and 4HA26DNT and subsequent reduction of these compounds to 24DHANT. The previous report on Bamberger rearrangement during TNT metabolism in cell extracts of *C. acetobutylicum* has demonstrated that this rearrangement occurred in the presence of cell extract and H_2 but did not occur in cell extract free controls, indicating that hydrogenase active during acidogenic metabolism by this organism may be involved in this transformation.

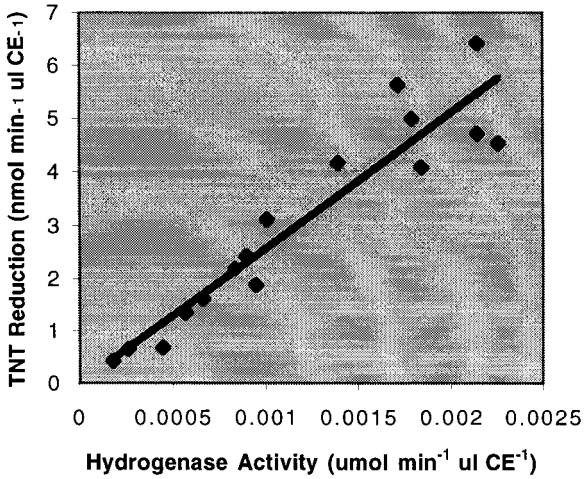

FIG. 3. Correlation of TNT-reducing capability with the hydrogenase activity of each cell extract (CE) with the values normalized to the volume of cell extract used in each assay. A high correlation exists, with an R^2 value of 0.89.

TABLE 2. TNT reduction capability for each strain type normalized to protein content of cell extract preparation

Cell extract		TNT reduction rate (nmol min^{-1} μg $protein^{-1}$)
Strain	Growth phase	
ATCC 824	Acidogenic	5.37 ± 0.50
ATCC 824 pPTB	Acidogenic	3.78 ± 0.17
ATCC 824 pHTB	Acidogenic	2.76 ± 0.37
ATCC 824 pSOS84	Acidogenic	2.69 ± 0.38
ATCC 824 pHTB	Solventogenic	2.49 ± 0.39
ATCC 824 pASH3	Acidogenic	2.40 ± 0.40
ATCC 824 pASH1	Acidogenic	2.16 ± 0.39
ATCC 824 pASH2	Acidogenic	1.30 ± 0.11
ATCC 824	Solventogenic	0.78 ± 0.16

However, the acid catalyzed rearrangement of 24DHA6NT also resulted in the formation of identical product (19). Presently, conclusive evidence indicating the role of hydrogenases in Bamberger rearrangement is lacking.

The Fe-only hydrogenase was determined to be the primary enzyme responsible for TNT reduction in *C. acetobutylicum* systems. Characterization of the purified enzyme (67 kDa) allowed determination of the N-terminal peptide which was identical to the N terminus of Fe-only hydrogenase. The enzyme exhibits saturation kinetics with a K_m for TNT of 152 μM.

The Fe-only hydrogenase is typically associated with hydrogen production in clostridia. Due to the fact that *C. acetobutylicum* rapidly reduces TNT only in the acidogenic, or acid production, phase of growth when increased levels of hydrogen production are observed (35), this hydrogenase was reported as the catalyst for TNT reduction (26). The M5 mutant strain displayed no significant decrease in TNT reduction activity, indicating that there was no observable contribution to TNT reduction by this Ni-Fe hydrogenase enzyme under the culture conditions.

Hydrogenase is composed of five iron-sulfur clusters, one of which is termed the H-cluster and is the center of catalytic activity (5). This active H-cluster couples H_2 oxidation with reduction of ferredoxin, or in this case, TNT. The proposed mechanism, consequently, is that through nucleophilic attack by the fully reduced state of the hydrogenase enzyme followed by two protonations and loss of water, and TNT undergoes a two-electron reduction to nitroso (R-NO) intermediate. This intermediate then immediately undergoes a similar attack by reduced-state hydrogenase followed by two protonations to complete the four-electron reduction of TNT to form R-NHOH. This is only a postulated mechanism, and it requires further research to confirm its validity (37).

In a comparison of the overexpression pHTB plasmid to the suitable control, pPTB, in the acidogenic phase, decreased activity is observed. This result is not consistent with expected activity based on the plasmid structure and may possibly be explained by a regulation mechanism that does not allow hydrogenase levels in these cells to be measurably greater than normal expression levels. However, the rates of TNT reduction for pHTB strains in the solventogenic stage resemble those occurring in the acidogenic stage. In wild-type cell systems a significant decline in TNT reduction activity is observed in late stages of growth. In a comparison of the observed effect in pHTB cell systems to what is observed with unaltered wild-type cell systems, it is evident that hydrogenase activity persists into late phases of growth in the pHTB strain.

To alter levels of hydrogenase in whole cells during normal growth, an antisense strategy was investigated (9). All antisense plasmids resulted in decreased TNT reduction activity compared to that of the pSOS84 antisense control plasmid. Plasmid pASH2 was particularly effective at reducing hydrogenase levels and TNT reduction capability. The antisense plasmids contain different as-*hydA*-encoding regions of differing sizes, which may account for this difference in effective inhibition. Plasmids pASH1, pASH2, and pASH3 contain antisense *hydA* inserts 78, 146, and 42 bp in size, respectively. The plasmid pASH2 possesses the longest segment of antisense RNA and displays the highest level of hydrogenase inhibition, followed by pASH1 and pASH3.

The information obtained from this study will aid in the development of an effective bioremediation approach for remediation of TNT-contaminated sites. This paper demonstrates that TNT-reducing activity is related to hydrogenase activity; thus, maintaining higher hydrogenase activity would more effectively transform TNT. The efficient field condition for TNT reduction can be achieved by maintaining strictly anaerobic conditions by providing saccharolytic fermentation conditions, which allow production of higher molecular hydrogen because of a high level of expression of ferredoxin-reducing enzymes. Earlier studies have reported remediation of TNT-contaminated soil. Williams et al. (41) observed significant TNT disappearance from contaminated soil in both thermophilic and mesophilic composting conditions. A similar study on TNT remediation has also been reported for groundwater aquifer slurries by Kromholt et al. (23). If physiological conditions optimal for enzymatic reactions favoring TNT reduction can be maintained, the remediation would be more effective. Methods for monitoring hydrogenase may allow the reductive capacity of sites to be evaluated.

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Appendix A2

Fate of TNT Transformation Products in Natural Systems.

RICE UNIVERSITY

The Reactivity of Partially Reduced Metabolites of 2,4,6-Trinitrotoluene in Natural Systems

by

Farrukh Ahmad

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE

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HOUSTON, TEXAS
APRIL, 2001

ABSTRACT

The Reactivity of Partially Reduced Metabolites of 2,4,6-Trinitrotoluene in Natural Systems

by

Farrukh Ahmad

The reactivity of partially reduced metabolites of 2,4,6-trinitrotoluene (TNT), namely arylhydroxylamines and nitrosoarenes, was evaluated with a simple biological system and with components of soil natural organic matter (NOM). This study was carried out to address the long-standing problem of irreversible binding to soil NOM and biomass, commonly observed during the reductive transformation of polynitroaromatic contamination. The study focused on partially reduced metabolites rather than the completely reduced arylamine metabolites that have already been extensively investigated for their role in binding to soil NOM.

In the simple bioreduction system of *Clostridium acetobutylicum* cell-free extract/molecular hydrogen (electron donor), 10% of the initial ^{14}C was found bound to solid proteinaceous material following sequential anaerobic/aerobic treatment. A review of the nitroso and hydroxylamino functional group chemistry revealed that the nitroso-thiol reaction was most likely responsible for the reaction with proteins. The introduction of a model thiol, 1-thioglycerol, into an anaerobic mixture of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) resulted in the formation of a new product, only when the reaction mixture was exposed to air.

The results from the model reaction confirmed that thiols could act as competing nucleophiles for nitroso compounds, which are readily formed from hydroxylamino compounds upon exposure to air.

The reactivity of arylhydroxylamines and nitrosoarenes with standard humic acids was investigated using 4HADNT and nitrosobenzene as model compounds, respectively. Contrary to results reported by others, 4HADNT was found to be nonreactive towards humic acid at humic acid concentrations in excess of dissolved organic matter concentrations found in nature. Conversely, nitrosobenzene reacted rapidly with humic acids, with the extent of reaction being highest for humic acids that had a high protein content. Humic acids that were pretreated with a thiol derivatizing agent showed diminished capacity for reaction with nitrosobenzene. Since nitroso intermediates from TNT reduction are difficult to synthesize and are rarely observed in nature due to their high instability, their electrophilic characteristics were evaluated using a molecular modeling approach. Molecular models of potential TNT nitroso intermediates were compared with those of the strongly electrophilic nitrosobenzene. The comparison revealed that 2-nitroso-4-hydroxylamino-6-nitrotoluene was more likely to react similarly to nitrosobenzene than 4-nitroso-2,6-dinitrotoluene.

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I. INTRODUCTION AND PROBLEM STATEMENT

Contamination by secondary explosive compounds ("secondary" based on a susceptibility to initiation), including 2,4,6-trinitrotoluene (TNT), is found around the world at current and former military installations. In the United States, such contamination exists mainly because of incomplete ordnance combustion in military open detonation ranges, and because of past disposal practices of high volumes of pink water (46, 99). Pink water is the waste product of shell packing operations that use hot water to wash off debris from the outside of sealed shells. TNT, like other polynitroaromatic explosive compounds with low aqueous solubility, precipitates out of solution once the temperature of the washwater cools down. Other sources of explosive contamination such as red water (byproduct of TNT manufacturing) are prevalent in explosives manufacturing countries other than the United States, which ceased production of TNT in the early nineteen-eighties (99).

TNT contamination occurs in the environment predominantly in surface and shallow subsurface soils. The presence of this contamination in easily accessible surficial soils makes it amenable to treatment by either *in situ* or ex-situ engineered systems. Bioremediation has come into favor as the treatment of choice for munitions contamination because of the prohibitively high cost of the treatment alternative, incineration.

The electrophilic nature of the nitro groups of TNT makes them prone to reductive attack, even under aerobic conditions (107, 114). The complete reduction of a single nitro group involves a six-electron transfer resulting in the formation of an amino

group. This reduction pathway proceeds sequentially via the potentially stable two- and four-electron transfer intermediates of nitroso and hydroxylamino groups, respectively (30). Despite their thermodynamic stability (when compared to nitro groups), nitroso and hydroxylamino groups are chemically reactive and can undergo a variety of reactions, including rapid abiotic condensation reactions with each other to form toxic azoxy compounds (30, 73). The complete reduction of polynitroaromatics such as TNT progressively gets slower with the reduction of each nitro group. This phenomenon is largely due to the destabilization of the aromatic ring with each reduction, and the subsequent decrease in the electrophilic nature of the remaining nitro substituents. In biological systems, the complete reduction of TNT to 2,4,6-triaminotoluene (TAT) has been observed only under strictly anaerobic conditions (107).

Over the past decade, efforts focusing on the cleanup of contamination resulting from herbicides (72) and explosives (46, 106) has stimulated an interest in anaerobic systems due to their ability to completely reduce polynitroaromatics. Both mixed anaerobic cultures and pure cultures of anaerobic organisms have been used in laboratory-scale polynitroaromatic remediation studies. Recently, clostridia (soil obligate anaerobes) have been isolated from a commercially-available anaerobic consortium capable of reducing TNT (111). This finding has revitalized research in the ability of clostridia to reduce nitroaromatics. Clostridia have long been known to possess enzymes that are capable of rapid nitro group reduction ("nitroreductase" activity). Interestingly, the rates of nitro group reduction achieved in studies with pure clostridial cultures, cell-free extracts, and purified enzymes have been at least an order of magnitude faster than the commercially-available anaerobic consortia (based on an equal degree of

reduction). On the other hand, evidence for the complete reduction of polynitroaromatics by clostridial enzymes has been inconclusive; the enzymes isolated so far have instead shown an accumulation of the chemically reactive hydroxylamino compounds (65, 106) and, at times, their Bamberger Rearrangement products (66). Therefore, one of the challenges remaining in this area is to determine which factors, either biological or environmental, control the complete reduction of nitro groups of polynitroaromatic compounds.

Perhaps the biggest challenge facing the application of TNT anaerobic remediation technology to the field is the accounting of competing sinks for the reduced metabolites of TNT. For example, numerous bench- and field-scale studies conducted with TNT-contamination over the years have resulted in a disparity in the molar balance between TNT and its reduced (or partially reduced) products. This has been especially true of anaerobic processes that have been followed by an aerobic remediation phase. The use of radiolabeled TNT has demonstrated that binding of the reduced metabolites occurs to the solid matrix. Further investigations to determine which fraction of the solid matrix binds the TNT metabolites have pointed in the direction of biomass and natural organic matter (NOM) (24, 34, 37, 40, 73, 119). The binding has been deemed "irreversible" or covalent because a variety of extraction techniques with common organic solvents have yielded poor recoveries of the reduced metabolites. Covalent binding has been proven using novel spectroscopic techniques such as cross polarization magic angle spinning (CPMAS) solid-state ^{15}N-NMR (19, 83). Recently, this irreversible binding has even been proposed as a post-remediation immobilization technique of TNT

contamination in soil (1, 3, 4, 19). The binding is believed to occur at the nitrogens of the reduced metabolites (as demonstrated by ^{15}N-NMR (1, 19)).

The functional group (on the metabolite) typically held responsible for such reactions is the aromatic amino group primarily because of the abundance of evidence regarding its fate in model systems. From model studies, three pathways for the irreversible binding of aromatic amines to soil natural organic matter have been elucidated: the nonenzymatic 1,4-nucleophilic or Michael addition of amino groups to quinoid rings of humic acid constituents (62, 63, 103); phenol oxidase (e.g., laccase) mediated free radical addition reactions (35, 36); and, metal catalyzed reactions between aromatic amines and humic substances (134). However, little is known about the fate of the nitroso and hydroxylamino metabolites generated from the partial reduction of nitroaromatic compounds in environmental systems. A recent study has implied that the reactive hydroxylamino groups of partially reduced TNT metabolites are responsible for abiotically initiating the covalent binding to humic acids under anaerobic conditions (3). However, conclusive proof of this hypothesis does not exist. Questions that remain unanswered at the current time include the following:

1. Specifically, which TNT intermediate(s) (i.e., functional group) is responsible for initiating the covalent binding?
2. Which specific chemical functional group(s) or site(s) on the sorbent organic matter participates in the binding reaction?
3. What is the mechanism of the chemical binding reaction?
4. What external environmental (or system) parameters/conditions control the binding reaction?

II. OBJECTIVES

The overall objective of this work is to investigate possible covalent binding between partially reduced metabolites of TNT and components of natural organic matter. In particular, interactions between two types of sorbate functionalities (namely, arylhydroxylamino and nitrosoarene groups) and components of soil natural organic matter (namely, humic acids and biomass) and cells will be investigated. The specific goals that will be pursued during the course of the research will be as follows:

1. Investigate the fate of TNT in a purely biological system (*Clostridium acetobutylicum* anaerobic cell extract/molecular hydrogen TNT reduction system) following sequential anaerobic/aerobic system.

2. Test the most current binding hypothesis, which is:

   Arylhydroxylamino groups spontaneously and abiotically bind to humic acids under anaerobic conditions.

3. Investigate the interactions of nitrosoarene and arylhydroxylamino groups with the proteinaceous fraction of humic acids.

III. LITERATURE REVIEW

1. THE REDUCTION/OXIDATION PATHWAY

The electrophilic nature of the nitro groups of TNT makes them prone to reductive attack, even under oxidative conditions (107, 114). The complete reduction of a single nitro group involves a six-electron transfer resulting in the formation of an amino group. This pathway proceeds sequentially via the potentially stable two- and four-electron transfer intermediates of nitroso and hydroxylamino groups, respectively (Figure 1). The reduction pathway does not preclude odd numbered electron transfers that lead to the formation of highly unstable anion radicals that are rarely observed in natural systems (30). Despite their thermodynamic stability (when compared to nitro groups), nitroso and hydroxylamino groups are chemically reactive and can undergo a variety of reactions, including rapid abiotic condensation reactions with each other to form toxic azoxy compounds (30, 73). The nitroso and the hydroxylamino substituents can be considered to exist in a “pseudo equilibrium” with each other since the nitroso substituents predominate under oxidative conditions whereas hydroxylamino substituents are favored under reductive conditions (30, 93). The complete reduction of a single nitro group to an amino group entails the energy-intensive cleavage of the N-O bond of the hydroxylamino intermediate.

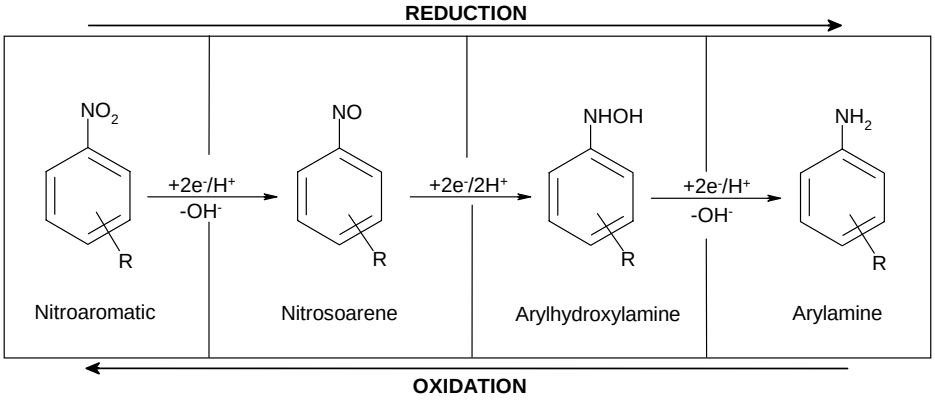

Figure 1. The redox pathway between nitroaromatic compounds and corresponding arylamines involving the relatively stable nitrosoarene and arylhydroxylamine intermediates.

The complete reduction of all the nitro groups of a polynitroaromatic compound such as TNT progressively gets slower with the reduction of each nitro group (114). This phenomenon is largely due to the destabilization of the aromatic ring with each reduction, and the subsequent decrease in the electrophilic nature of the remaining nitro substituents. This is evident in the progressively lower redox requirements published for the reduction of consecutive nitro groups of polynitroaromatic compounds (60). In biological systems, the complete reduction of TNT to 2,4,6-triaminotoluene (TAT) has been observed only under strictly anaerobic conditions (107).

The reoxidation of an aromatic amine to a hydroxylamine, also known as N-oxidation, involves a high activation energy barrier that can be overcome under natural conditions only with the aid of biological catalysis (30). In higher animals, N-oxidation occurs in the liver, kidneys, and lungs with the help of flavin-containing monooxygenases or cytochrome P450, and is the major route of toxicity for the aromatic amines (102).

2. TOXICOLOGY OF ARYLHYDROXYLAMINES AND NITROSOARENES

2.1 Assessing the Toxicity of Xenobiotic Compounds

Upon exposure to xenobiotic chemicals, lipophilic chemicals are easily absorbed by an organism whereas more hydrophilic ones are readily excreted in urine or egested in faeces (84). As a general rule, higher plants and animals biotransform xenobiotic compounds in order to convert the compound's lipophilic properties to more hydrophilic ones (102). Hence, biotransformation allows animals and plants to maintain homeostasis by excreting or sequestering, respectively, the parent xenobiotic chemical.

The transformation of xenobiotic compounds is performed by a limited number of enzymes, each of which typically has a broad substrate specificity. Some chemicals can also induce a reversible enzyme-formation response in an organism. More often, the biotransforming enzyme is synthesized in the organism without prior exposure to the chemical and plays some role in its metabolism (102).

Unfortunately, in some cases the biotransformation may form reactive products that have a considerably higher toxicological effect than the parent xenobiotic. Keeping this phenomenon in mind, biotransformation reactions have been divided into two distinct "phases" in toxicology literature (84, 102). Phase I reactions typically precede phase II reactions and include hydrolysis, reduction, and oxidation reactions. Such reactions generally result in a moderate increase in the polarity of the xenobiotic compound. Phase II reactions lead to the formation of highly hydrophilic products.

These reactions include sulfation, acetylation, methylation, glucuronidation, and conjugation with glutathione and amino acids.

Before proceeding any further, the definition of key terms commonly used in toxicology literature may be in order. The two major classes of toxic response are termed cytotoxic and genotoxic. The term cytotoxic means that an organism experiences a toxic response on a cellular level upon exposure to a xenobiotic chemical. This term implies that the xenobiotic or its transformed product interacts with cellular proteins. The term genotoxic means that an organism's genetic elements are being modified or that a mutation occurs because of the exposure. Hence, the terms genotoxic and mutagenic can be used interchangeably (84). The term tumorogenenic refers to chemicals that cause the formation of tumors in test species during toxicological tests. Tumors occur in either benign or malignant forms. The distinguishing characteristic between these two forms is the ability of the tumor to spread or to metastasize. Malignant or metastasizing tumors are also known as cancer and, therefore, chemicals or substances leading to the formation or promotion of malignant tumors are referred to as carcinogens (84). However, it should be stressed that all carcinogenic substances are not genotoxic (or mutagenic); carcinogenic substances that promote cancer but do not cause mutations are referred to as epigenetic substances.

2.2 Toxicological Effects of Arylhydroxylamines and Nitrosoarenes

Arylhydroxylamines and nitrosoarenes are believed to have both cytotoxic (affecting proteins) and genotoxic (affecting DNA) effects on plants and animals (30, 49, 102, 130), albeit via different mechanisms. The phase II bioorganic reactions involved in

producing the toxic effects are described at length in the next subsection; here, only the dominant mechanistic trends leading to toxic effects are discussed for each compound.

The toxicity of arylhydroxylamines is generally mediated via acylation or sulfate activation at the nitrogen atom of the hydroxylamino substituent, after cleavage of the N-O bond (termed O-acylation or O-sulfation due to the re-formation of the N-O bond). The sulfate or acyl activation is believed to eventually lead to the formation of protein and DNA adducts (102), in reactions where nucleophilic sites on the protein or DNA trap the nitrenium ion or displace its associated functionality (30). The structure of one of the major known DNA adducts produced via the transformation of 2-aminofluorene to 2-hydroxylaminofluorene (15, 30) is presented in Figure 2.

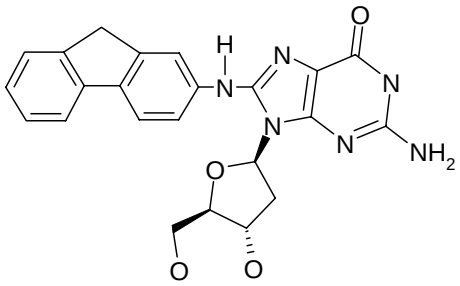

Figure 2. 2-Aminofluorene adduct to DNA showing binding to ring position 8 of a guanine base.

Nitrosoarenes are also believed to manifest cytotoxic effects in living organisms (42). A spontaneous abiotic reaction with thiols is predominantly responsible for such effects. This reversible reaction produces a semimercaptal compound that can undergo further irreversible reactions to form covalent adducts with cysteine-rich proteins (41, 42, 80, 81). Such reactions are believed to be responsible for physiological effects such as a depletion of glutathione levels from the liver and ferrihemoglobin formation in the blood (41, 74). In addition, nitroso intermediates can also undergo abiotic condensation

reactions with hydroxylamino intermediates to form highly toxic azoxy compounds (30, 73).

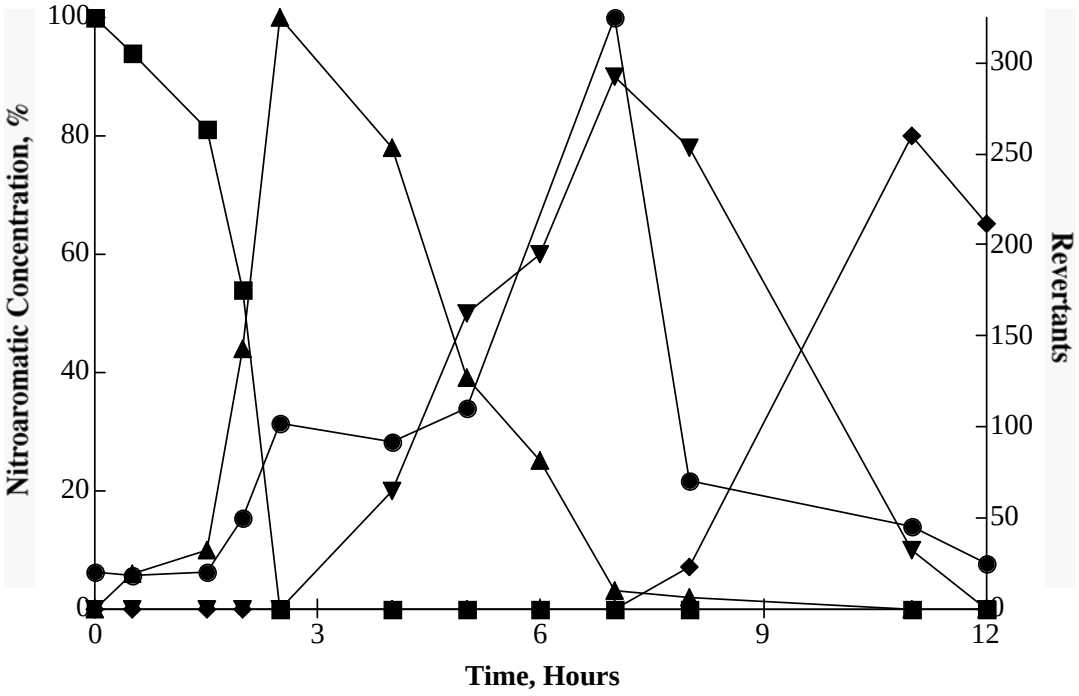

Figure 3. Ames mutagenicity testing results of the transient profile of TNT metabolites from transformation by *C. acetobutylicum* crude cell extracts. Nitroaromatics are plotted on the left axis scale in percent of initial (TNT(n), Sum of monohydroxylaminodinitrotoluenes (s), 2,4-dihydroxylamino-6-nitrotoluene (t), and the proposed Bamberger rearrangement product(u). Concurrent Ames Test results are plotted on the right axis (Revertants (l)) (101).

Owing to the inherent unstable nature of the hydroxylamino and nitroso intermediates of TNT in the environment, such intermediates cannot be used in traditional long-term chronic toxicity tests. However, when *C. acetobutylicum* cell extracts were incubated with TNT solution the mutagenicity, as measured by the Ames assay, was maximum when the 2,4-dihydroxylamino-6-nitrotoluene concentration was maximal (101) (Figure 3). Aminophenolic products showed only a fraction of the mutagenicity displayed by the hydroxylamino intermediates of TNT. This evidence

suggests that the toxicity of nitroaromatic compounds is expressed via the phase I reactions to form hydroxylamino and nitroso compounds.

2.3 Relevance of Arylhydroxylamino and Nitroso Toxicity to the Toxicity of Aromatic Amines and Nitroaromatic Compounds

Today, it is a commonly accepted fact that the toxicity of aromatic amino and nitro compounds occurs via phase I reactions that yield arylhydroxylamine and nitrosoarene intermediates (58, 130). These phase I reactions are the N-oxidation of amino groups and the reduction of nitro groups. N-oxidation is perhaps the better studied of these two types of phase I reactions because aromatic amines have been implicated as the causative factors of bladder cancer since the late nineteenth century when the dye industry began to flourish in Europe (49). The toxicological link between aromatic amines and their corresponding arylhydroxylamino and nitroso intermediates has been extensively reviewed by Gorrod and Manson (49), and the reader is directed to this work for further review on this subject. Historically, this link has been established by two important toxicological studies. The first of these studies was the work of Heubner (57) in which aniline was studied as the causative factor for methaemoglobinaemia. In this study it was suggested that arylamines were N-hydroxylated by a toxification reaction. The second set of historically important studies were performed by Kiese (76, 77). In these studies, Kiese reported the detection of nitrosobenzene in the blood of dogs that had been receiving aniline. The observations of these earlier studies led researchers to search for physiological and natural enzymes responsible for the N-oxidation phase I reaction.

To date, cytochrome P450 (49), chloroperoxidase (30), and flavin-containing monooxygenases (102) have been implicated as the enzymes catalyzing this reaction.

The establishment of a link between the toxicity of nitroaromatics and the toxicity of their partially reduced hydroxylamino and nitroso products has been a more recent occurrence. Perhaps one of the reasons responsible for this delay is the widely held view that nitroreductases completely reduce nitroaromatics to their corresponding aromatic amines. The discovery of arylhydroxylamines in test organisms possessing nitroreductase activity, such as the discovery of monohydroxylamino intermediates of 2,6-dinitrotoluene in the rat liver, has prompted a reevaluation of the toxicological mechanisms of nitroaromatic compounds. Nitroaromatic compounds have historically yielded a mixed results in conventional toxicity tests (e.g. the mutagenicity of 2,6-DNT is not revealed in short-term genotoxicity assays (102)) . An important study illustrating the necessity of nitroreductase activity to the expression of nitroaromatic toxicity is the study by Corbett et al. (33). In this study, 1,4-dinitrobenzene displayed strong mutagenic action only in nitroreductase-containing strains of *Salmonella typhimurium*. Therefore, it appears that the toxicity of nitroaromatics, just like aromatic amines, is expressed only after phase I transformations to hydroxylamino and nitroso intermediates.

Like other nitroaromatic compounds, TNT toxicity studies have produced a variety of seemingly contradictory results. For example, Won et al. (142) found TNT to be a frameshift mutagen in certain strains of *Salmonella typhimurium* and Smock at al. (126) found TNT to inhibit growth in algae and fathead minnows at concentrations as low as 5 mg/L; however, Klausmeier et al. (79) found many microorganisms to grow readily in TNT concentrations of upto 20 mg/L, and Gram negative bacteria to grow well

in TNT concentrations as high as 100 mg/L. Studies to date have not correlated TNT toxicity with the nitroreductase activity of test organisms. A more recent study by Liu et al. (89) investigated the fate of radiolabeled TNT in rats. Radiolabel was recovered in highest concentrations in plasma and renal samples where it was found to be covalently bound to proteins. From their findings Liu et al. postulated that the cytotoxicity of TNT was mediated by a rapid reduction to hydroxylamino intermediates, followed by bioactivation to form covalent adducts with proteins.

3. TRANSFORMATION OF TNT BY CLOSTRIDIA

Over the past decade, efforts focusing on the cleanup of contamination resulting from herbicides (72) and explosives (46, 106) 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 two commercially available biological processes. Both of these processes attain the complete reduction of TNT but provide little insight into the biochemistry employed by the organisms involved. The recent isolation of clostridia from a commercially-available anaerobic consortium capable of reducing TNT (111) has revitalized research in the ability of clostridia to reduce nitroaromatics. Clostridia have long been known to possess enzymes that are capable of rapid nitro group reduction ("nitroreductase" activity). Interestingly, the rates of nitro group reduction achieved in studies with pure clostridial cultures, cell-free extracts, and purified enzymes have been at least an order of magnitude faster than the commercially-available anaerobic consortia (based on an equal degree of reduction). On the other hand, evidence for the complete reduction of polynitroaromatics by clostridial enzymes has been inconclusive; the enzymes isolated so far have instead shown an accumulation of the chemically reactive hydroxylamino compounds. Discrepancies such as these warrant further investigation of TNT biotransformation by clostridia so that the true fate of TNT with these organisms can be determined and subsequently, their role in the nitro group reduction by anaerobic fermentative consortia can be assessed.

Clostridia are generally classified as gram-positive endospore-forming obligate anaerobes that are incapable of the dissimilatory reduction of sulfate (9, 25, 26). With close to one hundred species (25, 26), the genus *Clostridium* is one of the largest genera among prokaryotes (9). Clostridia are ubiquitous in nature due to their fermentative diversity and due to their ability to form resistant spores (50, 61). They have been isolated from soils, decomposing biological materials, and from the lower gut of mammals. In addition, they have been widely studied because some proteolytic species (e.g., *C. tetani*) produce strong toxins and because other species (e.g., *C. acetobutylicum*) are of industrial importance due to their solvent producing ability. Clostridia are known to possess strong nitroreductase properties that have been extensively investigated with two purified enzymes and cell cultures (10, 11, 23, 27, 96, 100, 106). In addition, clostridia have been isolated from an anaerobic consortium that was used to transform nitroaromatic compounds in the presence of a suitable carbohydrate carbon source (46, 111). Therefore, saccharolytic clostridia, typically non-pathogenic organisms, are especially well-suited for the study of biotransformations of TNT in anaerobic engineered systems.

3.1 Overview of Clostridial Metabolism

This section provides only a brief overview of clostridial metabolic diversity and metabolic pathways. Many facets of this topic have been the subject of extensive reviews: namely, acid production or acidogenesis (90); solvent production or solventogenesis (14, 69, 70); proteolytic and purinolytic fermentations (9); and acetate production via multiple substrates or homoacetogenesis (90). Readers are guided to these

publications for detailed discussions on specific topics. The purpose of this section is to provide background material on the broader metabolic capabilities of clostridia that are relevant to their nitroreductase activity.

3.1.1 Fermentative Metabolism

Clostridia are unable to completely oxidize an electron donor due to the lack of an electron-transport phosphorylation system, and are limited to substrate-level phosphorylation (SLP) for energy generation. SLP is the formation of intermediates containing high-energy phosphoryl bonds (from the reaction of an inorganic phosphate with an activated organic substrate) that are enzymatically coupled to the production of ATP (50). Consequently, fermentative organisms including clostridia suffer from an energy limitation and must rely on a high throughput of substrate for active growth (also known as the "Pasteur Effect"). As a result, anaerobic fermenters relying on SLP for growth tend to overproduce reducing power in the form of reduced carriers (e.g., NADH or reduced ferredoxin) (50, 61), which can be partially recovered for biosynthesis in the form of NADPH (69). The reoxidation of the reduced carriers (hereafter termed "dissipation of reducing power") is essential for maintaining a constant supply of energy in fermentative organisms because these carriers are present in limited quantities within a cell.

In pure cultures, fermentative pathways serve two main purposes. These are the generation of ATP and the dissipation of reducing power. As mentioned earlier, energy is obtained from SLP reactions that are mediated by dehydrogenses (e.g., glyceraldehyde 3-phosphate dehydrogenase of the Embden-Meyerhoff-Parnas glycolytic pathway) and

from reactions mediated by kinases that convert high-energy intermediates to organic acids. In clostridia, reducing power in excess of biosynthesis requirements can be dissipated by the evolution of hydrogen gas (via the ferredoxin/hydrogenase system), the formation of a second high-energy intermediate such as butyryl-CoA from acetyl-CoA, or the generation of reduced fermentation end-products such as solvents. In saccharolytic fermentations, the reduction of an unrelated electrophilic compound such as TNT can provide an additional means for reoxidizing reduced electron carriers that are needed for energy generation via SLP.

Table 1. Fermentative diversity of some common clostridia that have been utilized in nitroreduction studies (9, 14, 69, 70, 90).

Species	Substrate(s)	Product(s)
C. acetobutlicum	Starch, disaccharides, simple sugars	Acetate, butyrate, acetone, butanol, ethanol
C. bifermentans	Glucose, proteins, amino acids	Acetate, isocaproate, valerate, isovalerate, other acids
C. clostridiiforme	Hexoses, xylose	Acetate, lactate
C. kluyveri	Ethanol, propanol, succinate, acetate+carbon dioxide	Butyrate, caproate
C. paraputrificum	Hexose, disaccharide, starch, steroids	Acetate, propionate, lactate
C. pasteuranium	Hexoses, disaccharides	Acetate, butyrate
C. perfringens	Hexoses, disaccharides, starch	Acetate, butyrate, lactate, ethanol
C. thermoaceticum	Glucose, fructose, xylose, C-1 compounds	Acetate

Clostridia demonstrate enormous fermentative diversity by utilizing a wide variety of substrates (Table 1) and are commonly classified by the type of substrate they ferment. They are generally divided into four categories: (1) saccharolytic, (2)

proteolytic, (3) both saccharolytic and proteolytic, and (4) possessing specialized metabolism (e.g., purine fermenters such as *C. acidiurici* and ethanol fermenters such as *C. kluyveri*). However, it should be noted that regardless of the substrate, fermentation pathways usually proceed through common high-energy intermediates such as acetyl-CoA and butyryl-CoA (61). Clostridia are also casually classified by the type of fermentation end-products they produce, e.g., solventogenic (alcohol and acetone producers), acidogenic (organic acid producers), and acetogenic (acetate producers). Some saccharolytic clostridia such as *C. acetobutylicum* are typically acidogenic but undergo a switch in their metabolism to solventogenesis under conditions of stress (Figure 4a and b).

3.1.2 Autotrophic Metabolism

All clostridia are heterotrophs. However, some are capable of autotrophic metabolism (90, 110). Examples of such species are *C. aceticum* and *C. thermoaceticum*. These organisms can derive carbon for cell growth from the reduction of carbon dioxide via carbon monoxide to acetate. This autotrophic pathway (Figure 5) is mediated by the enzyme carbon monoxide dehydrogenase (CO-DH). The reducing power for the pathway is directly generated via the uptake of molecular hydrogen by hydrogenase or reduced ferredoxins formed from the fermentation of an electron donor (90).

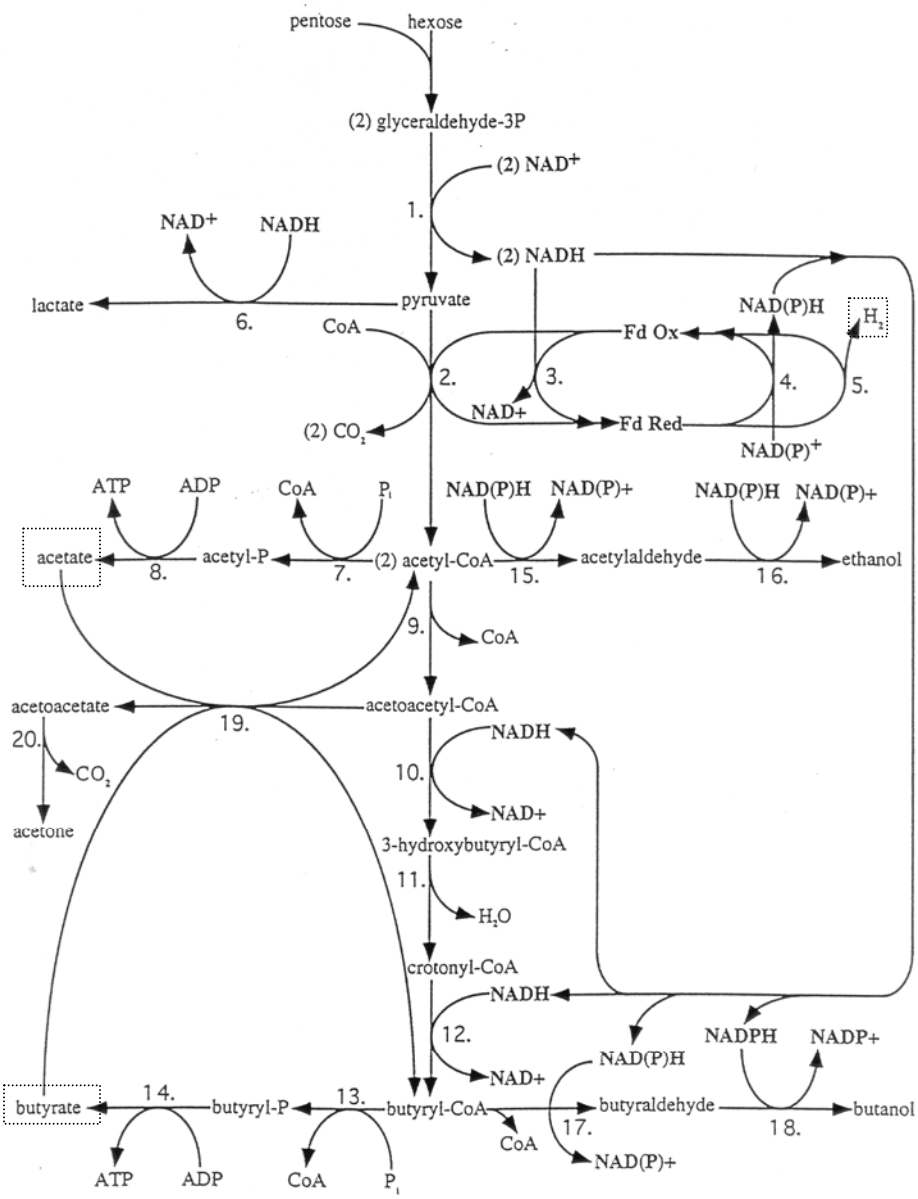

Figure 4a. Saccharolytic fermentative pathway of the butyric acid clostridia, *C. acetobutylicum*. Reactions for both the acidogenic phase (acetate and butyrate products) and the solventogenesis phase (acetone, butanol, and ethanol products) are shown; however, major products formed during the acidogenic phase are highlighted with boxes. Enzyme numbering is as follows: 1. Glyceraldehyde-3-phosphate dehydrogenase, 2. Pyruvate-ferredoxin oxidoreductase, 3. NADH-ferredoxin oxidoreductase, 4. NADPH-ferredoxin oxidoreductase, 5. Hydrogenase, 6. Lactate dehydrogenase, 7. Phosphate acetyltransferase, 8. Acetate kinase, 9. Thiolase (Acetyl-CoA acetyltransferase), 10. 3-hydroxybutyryl-CoA dehydrogenase, 11. Crotonase, 12. Butyryl-CoA dehydrogenase, 13. Phosphate butyltransferase, 14. Butyrate kinase, 15. Acetaldehyde dehydrogenase, 16. Ethanol dehydrogenase, 17. Butyraldehyde dehydrogenase, 18. Butanol dehydrogenase, 19. Acetoacetyl-CoA:acetate/butyrate:CoA transferase, 20. Acetoacetate decarboxylase. Enzymes not shown include granulose (glycogen) synthase and granulose phosphorylase which become active during the stationary phase. (Adapted from Jones and Woods (69)).

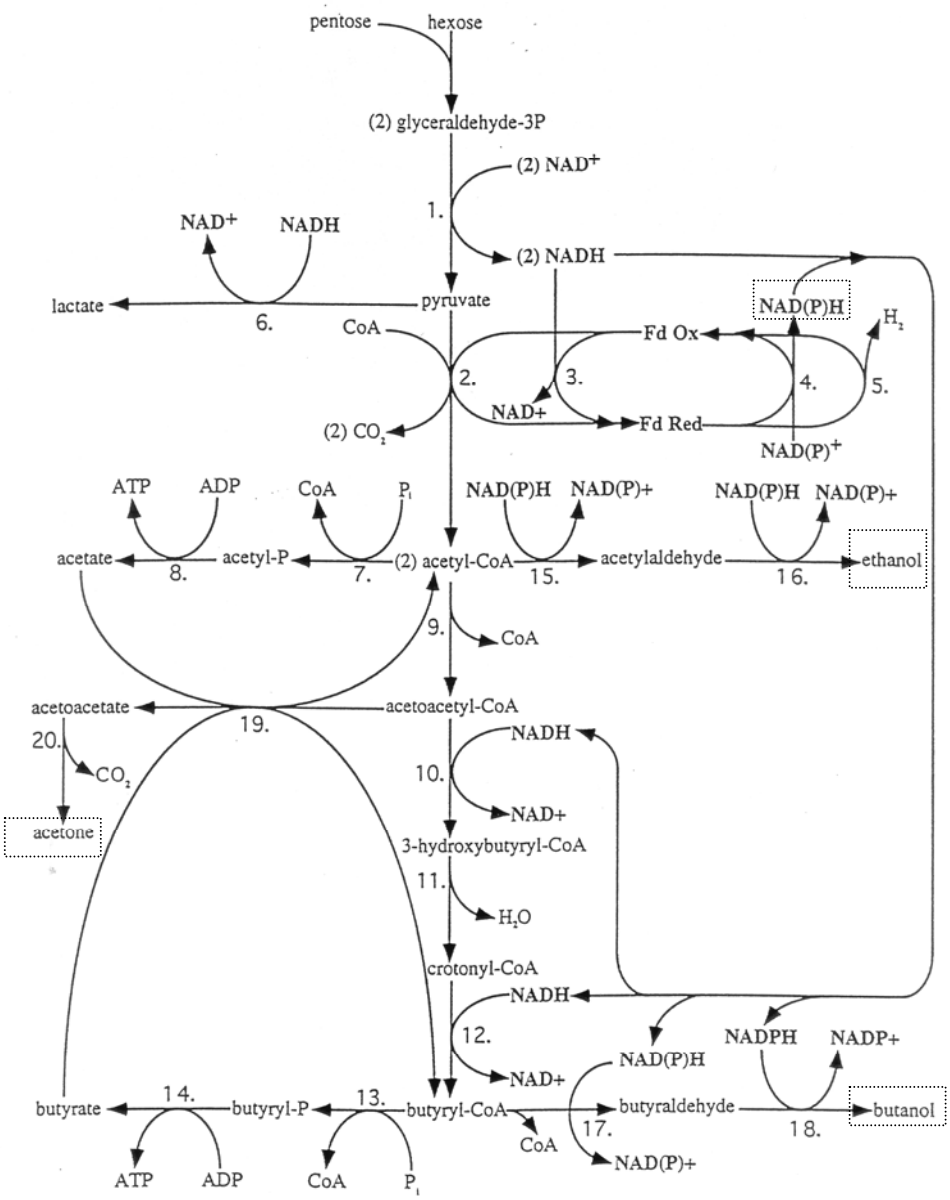

Figure 4b. Saccharolytic fermentative pathway of the butyric acid clostridia, *C. acetobutylicum*. Reactions for both the acidogenic phase (acetate and butyrate products) and the solventogenesis phase (acetone, butanol, and ethanol products) are shown; however, major products formed during the solventogenic phase are highlighted with boxes. Enzyme numbering is the same as in Figure 7a.

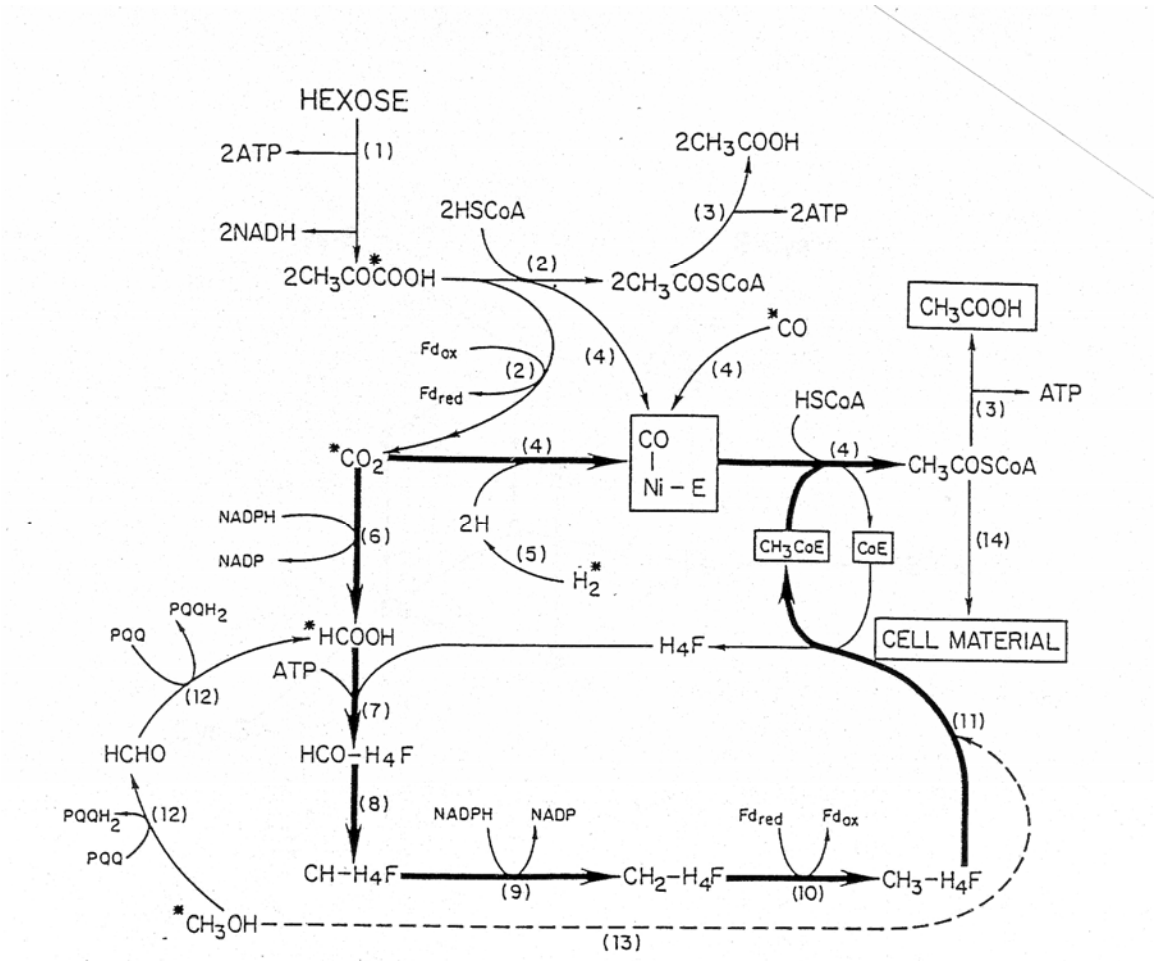

Figure 5. The autotrophic acetyl-CoA pathway (heavy arrows) and connected metabolism of hexoses, methanol, and CO in *C. thermoaceticum*. Symbols key: H_4F, tetrahydrofolate; CoE, corrinoid enzyme; CO-Ni-E, carbon monoxide dehydrogenase (CO-DH) with CO moiety bound to nickel; PQQ, pyrroloquinoline quinone; Fd, ferredoxin. Enzymes or reaction sequences are numbered as follows: 1. Glycolysis, 2. Pyruvate-ferredoxin oxidoreductase, 3. Phosphotransacetylase and acetate kinase, 4. CO-DH, 5. Hydrogenase, 6. Formate dehydrogenase, 7. Formyl-H_4 folate synthetase, 8. Methenyl-H_4 folate cyclohydrolase, 9. Methylene-H_4 folate dehydrogenase, 10. Methylene-H_4 folate reductase, 11. Transmethylase, 12. Methanol dehydrogenase, 13. Methanol cobamide methyltransferase, 14. Anabolism. (From Ljungdahl et al. (90))

3.2 Clostridial Enzymes Catalyzing Transformation of Nitro Substituents

Over the years, interest in the "nitroreductase" capability of clostridial enzymes has been driven by several pragmatic reasons. Originally, when 2,4-dinitrophenol (DNP) was implicated as an uncoupler of phosphorylation (10), researchers discovered that DNP could undergo photochemical reduction in ferredoxin-rich systems such as chloroplasts of photosynthetic plants (12, 91, 140). This discovery led del Campo et al. (23) to explore dark reactions that could also reduce DNP. They found that DNP could be effectively reduced to 2,4-diaminophenol by H_2 (established by H_2 uptake stoichiometry) in the presence of the hydrogenase/ferredoxin system of *C. pasteurianum.* Later in an unrelated study, medical researchers attempting to halt the protein synthesis of a rapidly growing culture of *C. acetobutylicum*, were unable to do so using chloramphenicol (100), a broad-spectrum antibiotic in common use at the time. Earlier, chloramphenicol, a nitroaromatic compound, had been shown to be an effective antibiotic against a wide variety of clostridia (78) via a mechanism that involved the inhibition of protein synthesis. Hence, the nitroreduction of this antibiotic by clostridia served as a potential mechanism for antibiotic resistance, and became a cause for concern owing to the pathogenic nature of certain proteolytic clostridia. More recently, attempts to remediate soil contaminated with di- and poly-nitroaromatics, have prompted an interest in anaerobic biotransformation; these types of compounds undergo complete reduction only under strictly anaerobic conditions (107). Therefore, the enzyme systems of clostridia, a common soil anaerobe, are being investigated for their ability to rapidly transform compounds like TNT to potentially less harmful products.

The reduction of nitro groups by clostridia has been mainly attributed to the gratuitous activity of oxidoreductases that have the well-defined metabolic function of reducing electron carriers with an extremely low mid-point redox potential (these enzymes are also referred to as “ferredoxin-reducing enzymes” and include hydrogenase, pyruvate-ferredoxin oxidoreductase, and NADH-ferredoxin oxidoreductase). Table 2 presents a comparative overview of studies that have utilized clostridial crude cell extracts or purified cell components (e.g., enzymes or electron carriers) to successfully reduce the nitro groups of various compounds. The clostridial enzymes conclusively identified as having nitroreductase activity, namely hydrogenase and carbon monoxide dehydrogenase (CO-DH), are different from the classical Type I (oxygen insensitive) and Type II (oxygen sensitive) nitroreductases purified from aerobic and facultative bacteria. Classical bacterial nitroreductases are flavin mononucleotide (FMN) requiring flavoproteins that operate at a suitably high mid-point redox that allows them to accept electrons from either NADH or NADPH (for a more detailed review of classical bacterial nitroreductases see Bryant and McElroy (21)). In contrast, clostridial hydrogenase and CO-DH have highly reducing iron-sulfur redox centers that allow transfer of electrons to electron carriers at a redox near that of the hydrogen electrode. Conversely, recent research suggests that other transformations such as the rearrangement of hydroxylamino compounds to aminophenols (66) can also be catalyzed by clostridial enzymes. Such enzymes can potentially divert the partially reduced intermediates of nitroaromatic compounds away from the reduction pathway catalyzed by nitroreductases.

Table 2. Reduction of compounds containing nitro substituents by crude cell extracts or purified components from clostridia.

Species	Biological Component			Electron Donor/ Reductant	Electron Carrier	pH	Starting Compound	Intermediates	Final Product	Reference
	Extract	Enzyme	Electron Carrier							
C. acetobutylicum	X			PYR	MV/FD	7.0	Chloramphenicol	NR	NR	O'Brien and Morris (100)
	X			PYR	MV	7.0	2/3-NP	NR	NR	(*)
	X			PYR	MV	7.0	2/3/4-NBA	NR	NR	
	X			PYR	MV	7.0	2NBD	NR	NR	
	X			PYR	MV	7.0	NF	NR	NR	
	X			PYR	MV	7.0	3NAn	NR	NR	
	X			PYR	MV	7.0	24DNP	NR	NR	
	X			H_2	-	7.8	TNT	DHA6NT	Aminophenol	Hughes et al. (66)
				H_2	-	7.8	24DNT,26DNT	HANTs, HAATs	DHATs	Hughes et al. (67)
C. kluyveri	X			H_2/NADH	-	8.5/11.5	pNB	-	pHAB	Angermaier et al. (11), and
		X		H_2	FD	8.5	pNB	-	pHAB	Angermaier and Simon (10)
C. pasteuranium		X		H_2	FD/FMN/ BV	8.0	24DNP	NR	NR ($6e^-/NO_2$)	del Campo et al. (23) (*, **)
	X			H_2	FD	7.5	TNT	4HADNT	TAT	McCormick et al. (96)
			X	Dithionite	FD/MV	7.0	Metronidazole	NR	NR ($4e^-/NO_2$)	Lindmark and Mueller (88) (*, **)
		X		H_2	FD/FLV	8.0	Metronidazole	NR	NR	Chen and Blanchard (27) (*)
	X			H_2	FD	8.5	pNB	-	pHAB	Angermaier et al. (11), and Angermaier and Simon (10)
		X		H_2	MV	8.0	DA6NT	-	DA6HAT	Preuss et al. (106)
C. sporogenes	X			H_2	FD	8.5	pNB	-	pHAB	Angermaier and Simon (10)
C. thermoaceticum		X		CO	MV	8.0	DA6NT	-	DA6HAT	Preuss et al. (106)

Electron Carriers: FD = Ferredoxin; FLV = Flavodoxin; FMN = Flavin mononucleotide; BV = Benzyl viologen; MV = Methyl viologen. Electron Donors: PYR = Pyruvate; H_2 = Hydrogen gas; NADH = Reduced nicotinamide adenine dinucleotide; CO = Carbon monoxide. Compounds: DA6HAT = 2,4-diamino-6-hydroxylaminotoluene; DAP = Diaminophenol; DNP = Dinitrophenol; 24DNT = 2,4-dinitrotoluene; 26DNT = 2,6-dinitrotoluene; HAATs =hydroxylamino-aminotoluenes; HANTs = hydroxylaminonitrotoluenes; 4HADNT = 4-hydroxylamino2,6-dinitrotoluene; NAn = Nitroaniline; NBA = Nitrobenzoic acid; NBD = Nitrobenzaldehyde; NF = Nitrofurantoin; NP = Nitrophenol; pHAB = para-Aminobenzoate; pNB = para-Nitrobenzoate; TAT = 2,4,6-triaminotoluene; TNT = 2,4,6-trinitrotoluene. Other: NR = Not reported; * = Loss of reactant monitored; ** = Hydrogen uptake monitored.

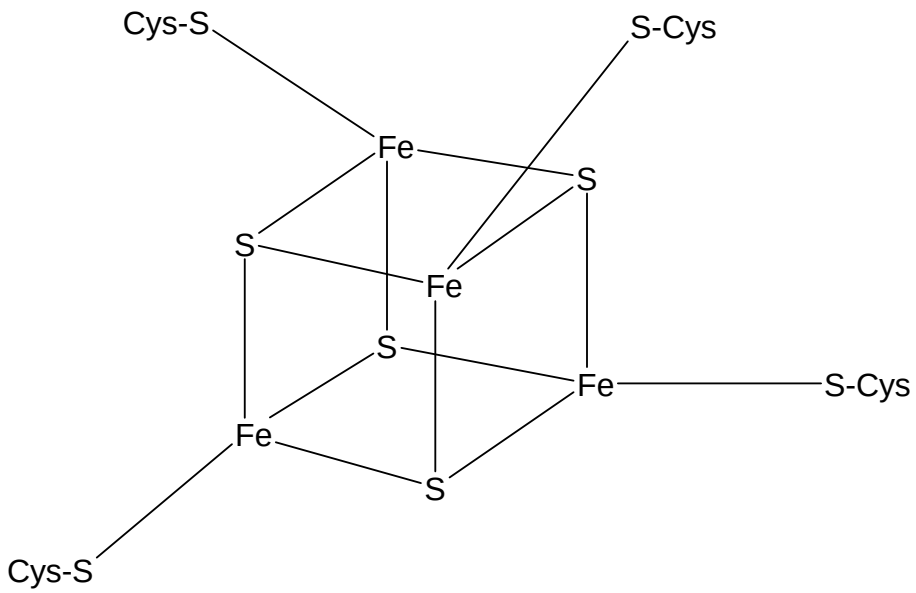

Figure 6. Structure of [4Fe-4S] non-heme iron/acid labile sulfur cluster in clostridial ferredoxin, hydrogenase, and carbon monoxide dehydrogenase.

3.2.1 The Role of the Hydrogenase/Ferredoxin System in Nitro Group Reduction

Clostridial hydrogenases and ferredoxins are proteins that contain highly reducing inorganic iron-sulfur centers that allow them to participate in electron transfer reactions at extremely low redox potentials (143). In clostridia, the iron-sulfur centers of hydrogenase are believed to contain three iron-sulfur clusters of the general form [4Fe-4S] (Fig. 6) (5, 13). Unlike the hydrogenases of aerobic hydrogen bacteria, the hydrogenases purified from clostridia are usually "bidirectional" (5, 143). This term means that they can catalyze the transfer of electrons to and from an electron carrier as shown by the equation:

$$(\text{Electron Carrier})_{\text{reduced}} + 2H^{+} \xrightleftharpoons{\textbf{Hydrogenase}} H_2 + (\text{Electron Carrier})_{\text{oxidized}}$$

Exposure to oxygen irreversibly inactivates hydrogenase, whereas exposure to carbon monoxide reversibly inhibits hydrogenase activity (5).

In obligate anaerobes, hydrogenases interact with an electron carrier that has a suitably low midpoint redox potential and that can be reduced by other oxidoreductases. In clostridia, this role is fulfilled by ferredoxins. Clostridial ferredoxins generally possess two [4Fe-4S] clusters that can each cycle between +3 and +2 oxidation states (excluding charge contributed by bound cysteine residues), thereby allowing each ferredoxin molecule to transfer one or two electrons (97, 143). The midpoint redox potentials of clostridial ferredoxins are in the range of -390 to –434 mV (143) based on the particular species from which they are extracted. In saccharolytic fermentations, ferredoxins can be reduced by oxidoreductases involved in the pyruvate phosphoroclastic reaction (108) or directly by NADH via the enzymatic action of NADH-ferredoxin oxidoreductase. In vitro, electron transfer with hydrogenase can be maintained when ferredoxins are replaced with other biological or non-biological electron carriers such as flavodoxins and redox dyes, respectively (5, 23). Table 3 compares the midpoint redox potentials for a number of electron carriers. Together, hydrogenase and ferredoxin form an electron transport system that plays a critical part in clostridial metabolism. During fermentation, clostridia as well as other fermentative anaerobes, use this system to dissipate excess reducing power by reducing protons to liberate hydrogen gas. On the other hand, clostridia such as *C. thermoaceticum* use the system to acquire reducing power that is used in the autotrophic synthesis of acetyl-CoA from CO_2.

Table 3. Midpoint redox potentials (E'_o) of clostridial electron carriers that have been shown to mediate nitroreduction.

Electron Carrier	**E'_o (mV)**	**Reference**
Methyl Viologen	-440	(88, 100, 106)
Ferredoxin	-410	(10, 11, 23, 27, 88, 100)
Benzyl Viologen	-385	(23)
Flavodoxin	-370	(27)
Flavin Mononucleotide (FMN)	-190	(23)

Clostridia exhibit an interesting regulatory mechanism under iron-limiting or iron-deprived conditions. Under such conditions, the formation of ferredoxin ceases, the iron of existing ferredoxin molecules is recycled for use in the production of essential enzymes (115), and the formation of a small flavoprotein, flavodoxin, is induced (92). Flavodoxins require FMN as cofactor, lack iron and labile sulfide, and have a low enough mid-point redox potential to substitute for ferredoxins as electron carriers. It should be noted, however, that flavodoxins are single electron carriers. Therefore, the change in electron carrier under iron limiting conditions can result in different reduction kinetics that may help to explain some of the variation in research findings regarding the reduction of TNT by clostridia.

The ability of the hydrogenase/ferredoxin system to reduce nitro groups was first observed by del Campo et al. (23) when they reduced DNP using hydrogen gas as the electron donor in the presence of purified hydrogenase and ferredoxin from *C. pasteurianum*. They also showed that spinach ferredoxin, benzyl viologen, and FMN were suitable replacements for the ferredoxin electron carrier. O'Brien and Morris (100), using crude cell-free extracts of *C. acetobutylicum* and pyruvate as the electron donor, expanded earlier findings to include various other nitroaromatic compounds, including the antibiotic chloramphenicol. In addition, using ferredoxin-free extracts, they were

able to demonstrate that ferredoxin or a similar electron carrier was essential for the nitro reduction in *C. acetobutylicum*. Furthermore, they demonstrated the inability of FMN, FAD, NADH, and NADPH to interact with the hydrogenase/ferredoxin system, contradicting some of the earlier findings of del Campo et al. In a non-enzymatic reaction, Lindmark and Muller (88) were able to partially reduce the antibiotic metronidazole, a nitroimidazole (2-methyl-5-nitroimidazole-1-ethanol), with electron carriers that had been previously reduced using dithionite. However, rates of the non-enzymatic reduction were much slower than rates with the hydrogenase/electron carrier system in the earlier studies. Building on the findings of the earlier studies, Chen and Blanchard (27) developed a hydrogenase-linked electron carrier assay using the reduction of metronidazole in the H_2/hydrogenase/ferredoxin or flavodoxin system. They went on to note that this system was more sensitive to the concentration of electron carrier as compared to that of the electron donor.

The first experiment that utilized the H_2/hydrogenase/ferredoxin system to reduce TNT was performed by McCormick et al. (96). They catalyzed the formation of TAT via 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) using ferredoxin-rich cell extracts of *C. pasteurianum*. The study was the first to monitor the formation of the partially reduced hydroxylamino intermediates. They used thin layer chromatography followed by the application of various dyes for separating and visualizing the reaction products. Earlier studies had only monitored the loss of nitroaromatic reactants and the stoichiometric consumption of electron donor to postulate the identity of the reduced product. Prediction of reduced products based on hydrogen uptake assays can lead to an

overestimation of the degree of reduction, especially when other ferredoxin-oxidizing enzymes (e.g., clostridial NADPH-ferredoxin oxidoreductase) are present in a system.

The accumulation of hydroxylamino products in two separate studies conducted with purified hydrogenase, suggests that the hydrogenase/ferredoxin system is inefficient at achieving the complete reduction of nitro substituents to amino substituents. Angermaier and Simon (10, 11) used purified components of the H_2/hydrogenase (*C. kluyveri*)/ferredoxin (*C. sp. La1*) system to partially reduce *para*-nitrobenzoate (pNB) to *para*-hydroxylaminobenzoate (pHAB) via a dianion radical that was detected using electron paramagnetic resonance (EPR) spectroscopy. The presence of the dianion radical intermediate confirmed that odd-numbered electron reductions do occur in these systems (30). They obtained similar results with cell extracts of *C. kluyveri*. However, they noted that during H_2 uptake studies with clostridial cell cultures there was a slow uptake of the third mole of hydrogen needed for the complete reduction to an amino substituent. Angermaier and Simon concluded that in the H_2/hydrogenase/ferredoxin system, the pHAB product must be a poor substrate for the reoxidation of the reduced ferredoxin. On a similar note, Preuss et al. (106) discovered that 2,4-diamino-6-hydroxylaminotoluene (DAHAT) accumulated as the final product when 2,4-diamino-6-nitrotoluene (DANT) was incubated with purified hydrogenase from *C. pasteurianum* and methyl viologen as electron carrier. In the same study they were able to catalyze the complete reduction of DANT to TAT with intact cells of *C. pasteurianum*. Based on these findings, Preuss et al. suggested that a second enzyme might be involved in the complete reduction of the nitro group of DANT to an amino group.

A recent study by Hughes et al. (66), performed with cell extracts of *C. acetobutylicum*, also showed partial reduction of a nitroaromatic compound. Hughes et al. provided rigorous proof of the accumulation of an aminophenolic product (2-hydroxylamino-4-amino-5-hydroxy-6-nitrotoluene) formed from TNT via a partially reduced 2,4-dihydroxylamino-6-nitrotoluene (DHANT). The results suggested that the DHANT underwent a Bamberger rearrangement (30, 98, 123, 128, 141) to form the aminophenolic product (Figure 7). A similar biologically-mediated rearrangement has been reported to occur with hydroxylaminobenzene in aerobic cultures of *Pseudomonas pseudoalcaligenes* (98, 128). Unlike transformations in clostridia, the reduction of the nitro group followed by the Bamberger rearrangement in *P. pseudoalcaligenes* was part of a productive catabolic sequence. It should be noted, however, that aminophenol formation was not detected when clostridial cell extracts were incubated with 2,4-dinitrotoluene (DNT) and 2,6-DNT as starting compounds (67).

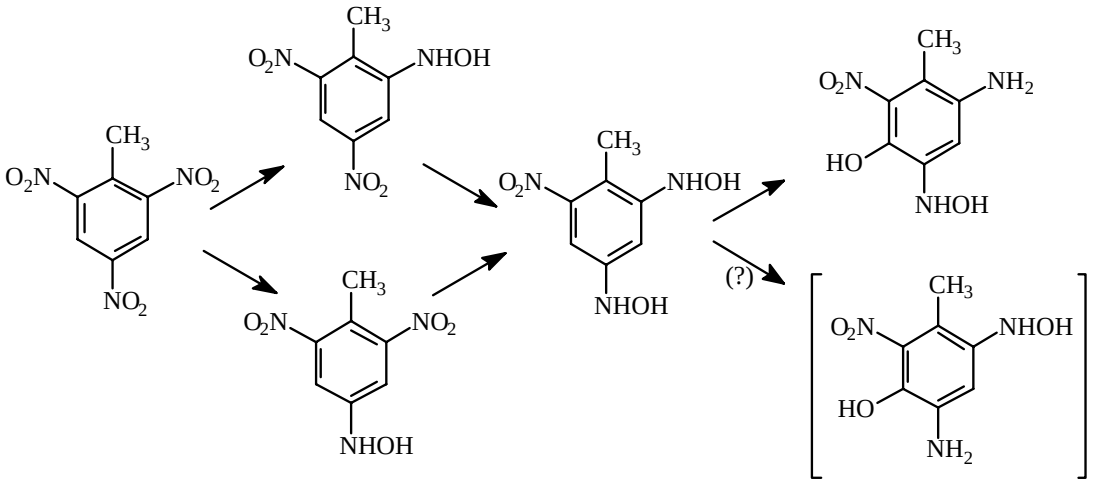

Figure 7. Proposed pathway of TNT transformation catalyzed by *C. acetobutylicum* crude cell extracts. The predominant product formed from the Bamberger rearrangement of 2,4-hydroxylamino-6-nitrotoluene is most likely 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol. Brackets around 6-amino-4-hydroxylamino-3-methyl-2-nitrophenol indicate that the formation of this structure is less likely. (From Hughes et al. (66)).

The transient or permanent presence of hydroxylamino products has repeatedly been detected in studies involving the reduction of nitro compounds with clostridial hydrogenase/ferredoxin systems, ever since these compounds have been monitored. Studies with purified hydrogenase/ferredoxin systems suggest the involvement of other factors during the complete reduction that has been observed in active clostridial cell cultures. Further studies are needed in order to elucidate whether these factors are biological (other enzymes or other reduced electron carriers) or environmental (culture conditions). Difficulties encountered with complete reduction of nitroaromatic compounds by the hydrogenase/ferredoxin system may facilitate the occurrence of competing biologically-mediated reactions such as Bamberger rearrangements that can form products amenable to aerobic mineralization by other organisms (98, 128).

3.2.2 Other Enzymes Catalyzing Reductions of Nitro Substituents

In studies with nitroaromatic compounds, two other clostridial enzymes besides hydrogenase have been implicated for their nitroreductase activity. In a study that focused on the nitroreductase activity of the hydrogenase/ferredoxin system, Agermaier et al. (10) made the unusual observation that the nitroreductase activity of cell extracts of *C. kluyveri* was NADH dependent and was maximum at a pH of 11.5. The observation was surprising because hydrogenase cannot interact directly with the NAD^+/NADH couple and typically has a much lower optimal pH (5). The authors ruled out several enzymes by performing multiple enzymatic assays on fractions collected from the separation of the extract on a DEAE-Sepharose column. They concluded that there was a high probability that the enzyme responsible for the nitroreductase activity was butyryl-

CoA dehydrogenase, an NADH-dependent enzyme that participates in the formation of butyryl-CoA from crotonyl-CoA in butyric acid clostridia. It should be noted that the authors did not rule out NADH-ferredoxin oxidoreductase, another ferredoxin reducing enzyme common in clostridia.

Preuss et al. (106) reported results similar to their work with the H_2/hydrogenase/methyl viologen system, when they employed the CO/CO-dehydrogenase/methyl viologen system to reduce DANT. An accumulation of DAHAT was also observed in the CO/CO-DH/methyl viologen system. The CO-dehydrogenase had been partially purified from *C. thermoaceticum*. A more recent study employing purified CO-DH showed that an electron carrier was unnecessary to produce dihydroxylaminonitrotoluenes from TNT (64) when CO was used as the electron donor. The highly reducing redox center of CO-DH contains nickel atoms, iron atoms and [4Fe-4S] clusters (90). The CO electron donor directly forms a complex with the nickel in the redox center. This complex is essential to the homoacetogenic pathway of *C. thermoaceticum* (Figure 8).

Incubations of nitroaromatic compounds with purified clostridial enzymes, in the presence of a suitable electronic carrier, have resulted in the rapid accumulation of hydroxylamino compounds. From these studies it appears that the initial reduction of nitroaromatic compounds is the fortuitous property of enzymes involved in the fermentative and homoacetogenic metabolic pathways of clostridia. However, the complete reduction of a nitro substituent to an amino substituent might involve other factors besides the nitroreductases evaluated to date.

3.3 Transformation of TNT by Clostridial Whole Cell Systems

O'Brien and Morris (100) discovered that the nitro substituent of chloramphenicol could be readily reduced by actively growing cultures of *C. acetobutylicum* using pyruvate as the electron donor. McCormick et al. (96) expanded the findings of O'Brien and Morris to include the reduction of TNT in the presence of a pure culture of *C. pasteurianum* with molecular hydrogen as the electron donor. Together the above findings implicated ferredoxin-reducing enzymes as having nitroreductase activity and prompted the exploration of various practical applications. Such applications included the biocatalysis of optically active aliphatic hydroxylamines to their corresponding amines (16), and the treatment of soil contaminated with nitroaromatic explosive compounds. The recent identification of *Clostridium* as the predominant organism in a commercially-available anaerobic consortium that had rapidly reduced TNT- and RDX-contaminated soil (111), has regenerated an interest in remediation studies with pure cultures of this organism. Consequently, a variety of such studies utilizing different species, nutrients (synthetic media/suspensions vs. complex media/active cultures), growth phases (active cells vs. resting cells), and parent nitroaromatic compounds have been conducted. Table 4 provides a listing of such pure culture biotransformation studies. An evaluation of trends evident among the studies follows.

Table 4. Reduction of nitroaromatic compounds by intact cells of clostridia.

Clostridial Species	Nitroaromatic or Intermediate Transformed	References (listed chronologically)
C. acetobutylicum	Chloramphenicol	O'Brien and Morris (100)
	TNT	Khan et al. (75)
	TNT, DA6NT	Ederer et al. (38)
	TNT, DHA6NT	Hughes et al. (65)
	TNT	Hughes et al. (66)
	24DNT,26DNT	Hughes et al. (67)
C. bifermentans	TNT, RDX	Regan and Crawford (111)
	TNT	Shin and Crawford (121)
	TNT, DA6NT, TAT	Lewis et al. (87)
	TNT	Shin et al. (122)
	TNT, DA6NT	Ederer et al. (38)
C. clostridiiforme	1NPy, pNBA, 1,3DNPy, 1,6DNPy	Rafii et al. (109)
C. kluyveri	pNB	Angermaier et al. (11)
	pNB	Angermaier and Simon (10)
C. leptum	1NPy, pNBA, 1,3DNPy, 1,6DNPy	Rafii et al. (109)
C. paraputrificum	1NPy, pNBA, 1,3DNPy, 1,6DNPy	Rafii et al. (109)
C. pasteuranium	TNT	McCormick et al. (96)
	pNB	Angermaier et al. (11)
	pNB	Angermaier and Simon (10)
	TNT, DA6NT	Preuss et al. (106)
	pNP, mNP, 2,4DNP, pNB, pNA	Gorontzy et al. (47)
C. perfringens	6NC	Manning et al. (95)
C. sordellii	TNT, DA6NT	Ederer et al. (38)
C. sp.	1NPy, pNBA, 1,3DNPy, 1,6DNPy	Rafii et al. (109)
C. sporogenes	pNB	Angermaier and Simon (10)
	TNT, DA6NT	Ederer et al. (38)
C. sp. W1	pNP, mNP, 2,4DNP, pNB, pNA	Gorontzy et al. (47)
C. thermoaceticum	TNT, DA6NT	Preuss et al. (106)

Compound Abbreviations

DA6NT = 2,4-diamino-6-nitrotoluene; DHA6NT = 2,4-dihydroxylamino-6-nitrotoluene; DAP = Diaminophenol; 2,4DNP = 2,4-dinitrophenol; 1,3DNPy = 1,3-dinitropyrene; 1,6DNPy = 1,6-dinitropyrene; 24DNT = 2,4-dinitrotoluene; 26DNT = 2,6-dinitrotoluene mNP = meta-nitrophenol; 6NC = 6-nitrochrysene; pNA = para-nitroaniline; pNB = para-nitrobenzoate; pNBA = para-nitrobenzoic acid; pNP = para-nitrophenol; pNPy = para-nitropyrene; RDX = Research Department Explosive (Hexahydro-1,3,5-trinitro-s-triazine); TAT = 2,4,6-triaminotoluene; TNT = 2,4,6-trinitrotoluene.

3.3.1 Cometabolic Nitro Substituent Reduction by Clostridial Cultures

Clostridia have diverse nutritional requirements for growth (e.g., salts, trace metals, vitamins, amino acids, etc.). In the laboratory, such requirements are best and most conveniently met by complex media such as peptone-yeast extract-glucose (PYG) or chopped meat-carbohydrate (CMC) media (9). Because of the intricate nutritional requirements of pure clostridial cultures, it is inherently difficult to design traditional nutrient limitation studies (18) to evaluate the role of nitroaromatic compounds as a sole source of carbon and/or nitrogen for growth. Results can be further confounded if internal changes in the distribution of a nutrient occurs while growth of the pure culture continues unhampered. For example, the observation that an iron-deprived culture of *Clostridium pasteurianum* showed little change in growth, or in the level of its iron-containing enzymes while its ferredoxin levels plummeted, indicated that internal cycling of iron from ferredoxin to essential enzymes (115) was occurring. Therefore, attempts to simplify the media in transformation studies involving nitroaromatic compounds have inevitably led to drastic reductions in growth (38, 65, 87, 122). Furthermore, the slow growth has been accompanied by correspondingly slower rates of reduction and often with partial reduction of the parent nitroaromatic compound (65, 122), especially when resting cells with low acidogenic activity (122) or synthetic media supplemented with inadequate carbon sources (87, 122) have been used. Hence, in lieu of effective nutrient limitation studies with clostridia, other lines of evidence must be examined to determine whether nitroaromatic compounds can serve as primary nutritional and/or energy substrates, or whether their metabolism is strictly cometabolic.

Transformation studies performed with ^{14}C-labelled nitroaromatic compounds have failed to show the generation of radiolabeled carbon dioxide, indicating that parent nitroaromatic compounds are not mineralized by pure clostridial cultures (65, 66, 75, 87, 121). In addition, mass balances performed in radiolabeled studies have shown the majority of the radioactivity to reside in solution (75, 121), suggesting that components of the nitroaromatic compounds are not incorporated into biomass. It should be pointed out, however, that transformation studies with labeled nitrogen in the nitro group have yet to be performed.

Given the plethora of nitroreduction data for ferredoxin reducing enzymes, it comes as no surprise that clostridial cells, either in cultures (actively growing cells) or suspensions (resting cells), can carry out at least the initial reductions of nitroaromatic compounds without any prior acclimation (75, 100). This observation suggests that the initial reduction of nitro groups is the gratuitous activity of clostridial enzymes that are already present in significant concentrations prior to exposure of the organism to nitroaromatic compounds. The absence of an acclimation phase, together with the lack of mineralization evidence, provide ample proof for the cometabolism of nitroaromatic compounds under fermentative conditions. Although the reduction of nitroaromatic compounds provides no nutritional benefit to clostridia, the reduction serves to reoxidize reduced electron carriers needed for energy production via substrate-level phosphorylation.

3.3.2 Significance of Acidogenic Conditions in the Reduction of Nitro Substituents

The reduction of nitroaromatic compounds has been catalyzed most rapidly and effectively by actively growing cultures of fermentative acidogenic clostridia (38, 48, 65, 75, 87, 121). Such organisms usually dissipate excess reducing power (i.e., in excess of biosynthesis requirements) generated in substrate level phosphorylation by employing one or more of three reaction pathways. The first of these dissipation pathways during clostridial fermentations is the reduction of protons via the ferredoxin/hydrogenase system to liberate hydrogen gas. Under acidogenic conditions, the generation of molecular hydrogen serves as the only means of maintaining the redox balance offset by the production of a large quantity of partially oxidized fermentation products. To initiate this reaction, ferredoxin is reduced by either pyruvate-ferredoxin oxidoreductase or NADH-ferredoxin oxidoreductase. The second pathway is common to all butyric acid clostridia that constitute greater than fifty percent of all clostridia isolated to date. In this pathway butyryl-CoA is generated from acetyl-CoA via several NADH dependent reactions. Butyric acid clostridia can utilize the first two pathways simultaneously as indicated by the fact that high levels of hydrogenase activity can be maintained under acidogenic conditions (8, 53, 69). The third pathway is present in species that can induce enzymes capable of producing solvents or "solventogenesis," thereby moving the organism away from acidogenic metabolism. A common example of such a species is the acetone, ethanol, and butanol producing *C. acetobutylicum* which undergoes a switch in its metabolism from producing acetate and butyrate to producing the more reduced solvents (Figure 4b). Although it has been widely shown that the onset of solventogenesis occurs in batch cultures of *C. acetobutylicum* during late exponential

growth phase, the exact mechanism for the transformation is unclear. However, low extracellular pH, the difference between intracellular and extracellular pH, and intracellular undissociated butyric acid concentration have been cited as important factors in triggering solventogenesis under non-nutrient limiting conditions (14, 69). Morphological changes have also been associated with the onset of solventogenesis (68, 69). It should also be noted that the first and third reaction pathways for dissipating reducing power are mutually exclusive; a large drop in hydrogenase activity occurs with the onset of solventogenesis (8, 53, 69, 70).

The metabolism of *C. acetobutylicum* is ideally suited for studies on the role of the different pathways responsible for reducing power dissipation, because this species is capable of utilizing any one of the three pathways. Also, the metabolism of this species is perhaps the best studied of all clostridia owing to its industrial importance as an alternative means of producing solvents. Studies that are carefully designed to inhibit one or more dissipation pathways at key branch points can help to reveal which enzymes are incapable of nitroreductase activity. One study that attempted such an approach was reported by Khan et al. (75). When solventogenesis was induced by carbon monoxide, a common inhibitor for hydrogenase activity TNT was reduced to hydroxlamino compounds much more slowly than in acidogenic controls (Figure 8). The result suggested that enzymes involved in solvent production and the acetyl-CoA to butyryl-CoA pathway are incapable of effective reduction of nitroaromatic groups, thereby re-establishing the importance of hydrogenase and acidogenic enzymes in nitroaromatic reductions.

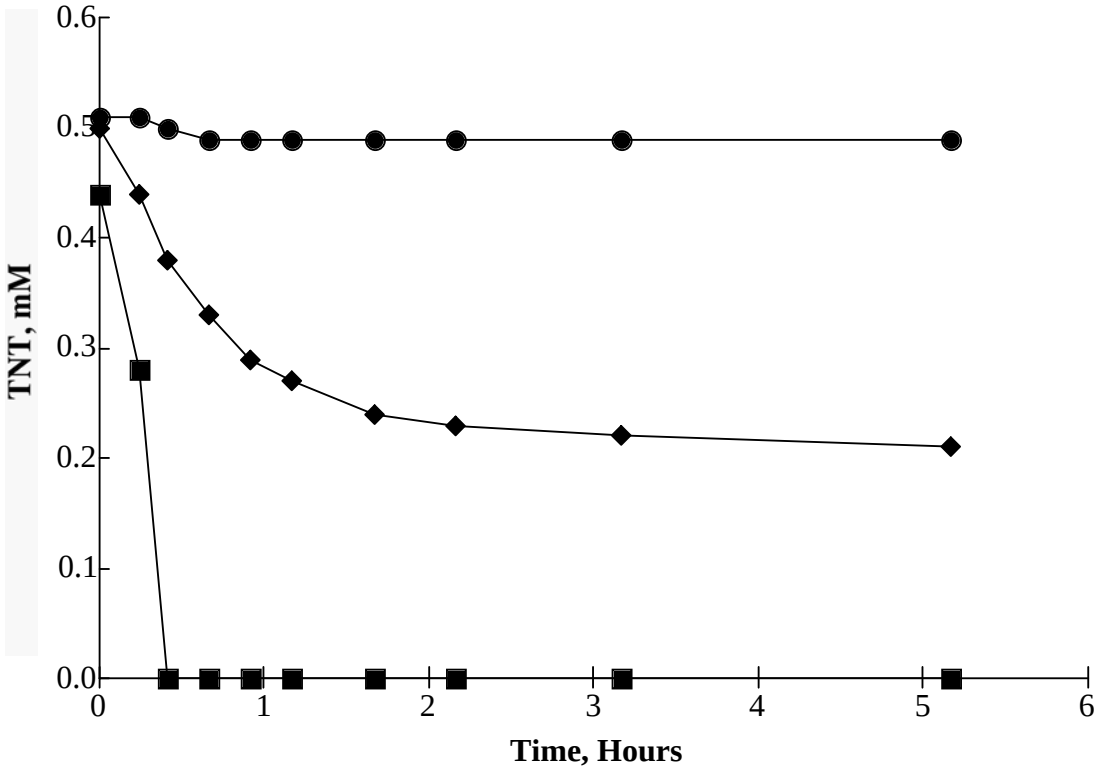

Figure 8. The transformation of TNT by cell cultures of *C. acetobutylicum* in acidogenic (n), solventogenic (u) and stationary (l) phases of growth. (From Khan et al. (75))

A few researchers have attempted nitroaromatic compound reduction with stationary phase or resting cells (75, 122). Clostridial cells in stationary phase generally have low acidogenic activity because their metabolism is diverted to the accumulation of granulose before the onset of sporulation (69). Such experiments have not demonstrated reduction of TNT beyond the monohydroxylamino stage.

3.3.3 Incomplete Nitro Substituent Reduction and the Formation of Aminophenols

Recent studies involving the reduction of nitroaromatic compounds (48, 65, 66, 75, 87, 121, 122), especially those conducted with radiolabeled TNT in the presence of

active clostridial cultures (65, 75, 87, 121, 122), have conclusively demonstrated the formation of large quantities of polar products other than TAT (Figure 9). In one study, the polar product formed was identified as an aminophenol (66) formed by a Bamberger-type rearrangement (30, 114, 123, 141) of a hydroxylamino intermediate of TNT. Furthermore, in an earlier study with active cultures, a reasonable mass balance could not be established between the aminonitrotoluene reduction intermediates and the completely reduced TAT product (96), indicating the potential existence of alternative pathways.

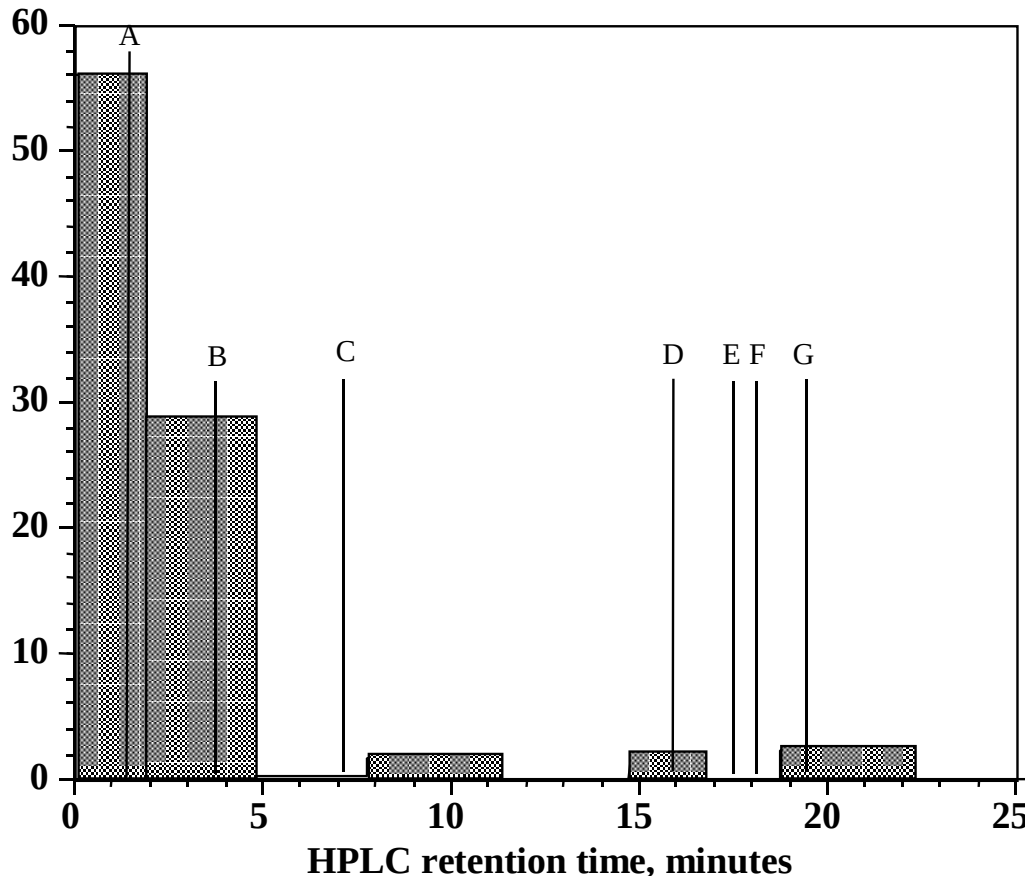

Figure 9. The distribution of radioactivity initially added as ^{14}C-radiolabeled TNT in HPLC fractions of a sample collected after the complete transformation of TNT by an acidogenic culture of *C. acetobutylicum*.

Corresponding HPLC retention times of standards are labeled as: (A) TAT; (B) DA6NT; (C) TNT; (D) 2HADNT; (E) 4HADNT; (F) 2ADNT; and (G) 4ADNT. Note that the UV spectra of authentic standards of TAT, 24DA6NT and TNT did not match those of observed polar products. (From Khan et al. (75))

Studies that have revealed aminophenol formation have been performed with active clostridial cultures grown in batch systems without any pH control. Since the pH in such cultures drops substantially over time due to acidogenic activity, it is difficult to discern whether the Bamberger rearrangement is biologically-mediated or is abiotic because of the low pH (114, 123, 141). However, more recent studies conducted with extracts prepared from acidogenic cells at neutral pH have duplicated the results obtained from growing cultures (66), indicating that the Bamberger rearrangement is enzyme catalyzed. Further evidence supporting this finding is the fact that rearrangement cannot be reproduced from arylhydroxylamino starting compounds in the absence of cell extract or active cell cultures (66).

Although the formation of polar products other than TAT has been observed in a number of studies, TAT has not been detected as an intermediate in such studies (65, 66, 75). Incubations of cell cultures with TAT have not produced any other polar products (87), indicating that TAT is a dead-end product. Similar results have been obtained with mixed anaerobic cultures (55). These results suggest that it is possible that more than one type of aminophenol may form from more than one hydroxylamino precursor, depending on the degree of reduction achieved in a system. Therefore, it can be inferred from these findings that the formation of aminophenols is a biologically-mediated pathway that competes with the complete reduction of TNT to TAT. Further studies are needed to determine the factors controlling the degree of TNT reduction and the extent of

competing reaction pathways such as aminophenol formation and various covalent binding reactions to organic components.

4. THE BINDING OF TNT BIOTRANSFORMATION PRODUCTS TO THE SOLID MATRIX

Over the years, numerous bench- and field-scale studies conducted with polynitroaromatic compounds such as TNT have resulted in a disparity in the molar balance between TNT and its reduced (or partially reduced) products. The use of radiolabeled TNT has demonstrated that binding of the reduced metabolites occurs to the solid matrix. Further investigations to determine which fraction of the solid matrix binds the TNT metabolites have pointed in the direction of biomass and natural organic matter (NOM) (24, 34, 37, 40, 73, 119). The binding has been deemed “irreversible” or covalent because a variety of extraction techniques with common organic solvents have yielded poor recoveries of the reduced metabolites. Recently, this irreversible binding has been proposed as a post-remediation immobilization technique of TNT contamination in soil (1, 3, 4, 19).

Sorption studies conducted with nitroaromatic compounds have demonstrated a relatively weak ion exchange mechanism of interaction between the nitro group and the soil mineral matter (51, 52). However, interactions with soil NOM have been all but absent. The interaction of nitroaromatics with minerals does not explain the irreversible binding to the solid matrix that is so commonly observed in anaerobic/aerobic and composting remediation systems. Conversely, studies conducted with aromatic amines have yielded strong binding to soil NOM and sediment under quite specific conditions.

The aminated products of TNT reduction have been reported to irreversibly bind with sediment when anaerobic reaction mixtures are made aerobic (39, 120). From model studies, three pathways for the irreversible binding of aromatic amines to soil natural organic matter have been elucidated: the nonenzymatic 1,4-nucleophilic or Michael addition of amino groups to quinones present in humic acid constituents (62, 63, 103); phenol oxidase (e.g., laccase) mediated free radical addition reactions (35, 36); and, metal catalyzed reactions between aromatic amines and humic substances (134). Further studies focusing on the conditions necessary to increase the extent of binding have indicated two important factors. They are the degree of reduction achieved in the initial anaerobic phase, and the need for an aerobic stage following the initial anaerobic reduction phase (2, 4, 37). These findings are believed to strengthen the evidence for the nucleophilic addition mechanism because the greater degree of reduction in the anaerobic phase increases the nucleophilic character of the amino nitrogen. Furthermore, the aerobic stage requirement allows for the reoxidation/tautomerization of the hydroquinone back to the quinone, thereby promoting further nucleophilic additions possibly leading to the formation of nitrogen heterocycles (103, 134). However, this mechanism does not adequately address the extensive binding observed in purely biological systems (89, 132) or the binding occurring to biomass in engineered remediation systems (24, 40, 119, 136) since arylamines undergo N-oxidation in biological systems prior to binding covalently with biomolecules (49). Moreover, N-oxidation of the amino groups to either the hydroxylamino or nitroso groups, is a distinct possibility with the class of soil enzymes known as phenol oxidases (36) prior to covalent binding (35).

Little is known about the fate of the nitroso and hydroxylamino metabolites generated from nitroaromatic reduction in environmental systems, in lieu of further reduction or azoxy formation. Part of the reason for this dearth of information regarding the covalent binding characteristics of partially reduced metabolites is the high reactivity of these compounds, which in turn makes them quite difficult to work with. A recent preliminary study conducted with 4HADNT and humic acids under apparent abiotic anoxic conditions showed rapid irreversible binding between these compounds (3). This finding appears to confirm that covalent binding to soil NOM occurs via hydroxylamino or nitroso intermediates of TNT.

The purpose of this section is to present a chronological survey of the remediation studies that have confirmed binding of reduced TNT metabolites to soil NOM. Because the studies conducted to date have employed different remediation conditions and extraction procedures, it is impossible to compare one study to another. Hence, a summary of facts of each study is presented in the table that follows.

Table 5. Summary of TNT Biotransformation Studies in which Binding of Metabolites to Organic Matter was Confirmed.

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
1978	Carpenter et al. (24)	Mixed culture under aerobic conditions (Activated Sludge from two different sources).	Use of uniformly labeled ^{14}C-TNT, followed by extraction of radiolabel from temporal samples. Liquid scintillation count (LSC) performed on samples.	Sample Preparation Centrifugation of sludge floc, followed by washing of pellet with DI water. Carbohydrate Fraction Pellet suspended in ice-cold 10% trichloroacetic acid (TCA) solution, followed by centrifugation. Carbohydrates in supernatant. Lipid Fraction Pellet produced in carbohydrate step suspended in (v:v=1:1) ethanol/ether solution at 50-55 °C, followed by centrifugation. Lipids in supernatant. Nucleic Acid Fraction Pellet produced in lipid step suspended in 5% TCA solution at 100 °C, followed by centrifugation. Nucleic acids in supernatant. Protein Fraction Pellet produced in nucleic acid step suspended in 10N NaOH at 25 °C for 18 hours, followed by centrifugation. Denatured proteins in supernatant.	1st Reactor Aqueous samples yielded 50% of the total radiolabel in the sludge floc pellet. Of this 50% radiolabel, the distribution of total radiolabel was as follows: 0.8% carbohydrates, 30.6% lipids, 1.0% nucleic acids, and 7.8% proteins. The remainder of the total radiolabel to make up the 50% of the sludge pellet was in the washwater and the residue. 2nd Reactor Aqueous samples yielded 52.4% of the total radiolabel in the sludge floc pellet. Of this 52.4% radiolabel, the distribution of total radiolabel was as follows: 4.7% carbohydrates, 20.5% lipids, 1.9% nucleic acids, and 15.8% proteins. The remainder of the total radiolabel to make up the 50% of the sludge pellet was in the washwater and the residue.

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
1982	Kaplan and Kaplan (73)	Mixed culture enriched for thermo- and mesophiles. Aerobic-Anaerobic (Composting at 55 °C) treatment. Samples taken at 24 and 91 days.	Use of uniformly labeled ^{14}C-TNT, followed by extraction of radiolabel from temporal samples. LSC performed on samples.	<u>Sample Preparation</u> Drying in 50 °C oven . <u>Solvent Extractions</u> Ether: Reflux, 3x300 mL, 24 hours. Ethanol: Reflux, 3x300 mL, 24 hours. Water: Reflux, 1x300 mL, 24 hours. Acetone: Reflux, 3x300 mL, 24 hours. <u>Humus or Humic Substances</u> Insoluble fraction of the samples collected were washed with 0.1N HCl, followed by the addition of 0.5N NaOH and 0.1N $Na_4P_2O_7$. The insoluble fraction remaining after this procedure was classified as humin. The soluble fraction was further acidified to a pH of 3 in order to precipitate out the humic acids. The soluble fraction that remained after acidification was classified as fulvic acid.	<u>Solvent Extractions</u> The percentage of total radioactivity introduced at the start of the experiment for the 24 and 91 day samples, respectively, were as follows: Ether: 67.4% (24d), 48.3% (91d). Ethanol: 2.1% (24d), 1.5% (91d). Water: 13.5% (24d), 9.7% (91d). Acetone: 3.6% (24d), 2.0% (91d). <u>Humic Substances</u> The percentage of total radioactivity introduced at the start of the experiment for the 24 and 91 day samples, respectively, were as follows: Humin: 1.3% (24d), 13.9% (91d). Fulvic acid: 0.4% (24d), 0.4% (91d). Humic acid: 4.0% (24d), 7.8% (91d).
1998	Drzyzga et al. (37)	Sequential Anaerobic (5 weeks) - Aerobic (4 weeks) treatment with a mixed culture; molasses used as carbon source to drive system to	Use of uniformly labeled ^{14}C-TNT, followed by recovery of radiolabel from 5 week and 9 week samples for each experimental setup.	<u>Recovery of Unbound Radiolabel (TNT and its Metabolites)</u> Solid samples were dried overnight (12 hours) at 60 °C and were then extracted sequentially by solvents in an ultrasonic bath for 30 minutes. Solvents used were water, methanol, and ethyl acetate. Drying was performed before and after each step.	<u>Experiment A Anaerobic (5 weeks)</u> -Unbound (Extracts): 6.0% in water 34.5% in methanol 13.0% ethyl acetate -Bound to Solid Matrix: 35.5% in humin 4.3% in humic acid

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
		anaerobic conditions. Two reactor setups employed. There was headspace present and the soil was not compacted in the setup used for Experiment A. In Experiment B, the soil/molasses mixture was compacted under a helium flush to render the system oxygen-free and to reduce pore space.		<u>Recovery of Radiolabel by Humic Substance Fractions</u> Dried samples after the solvent extractions were first split into two homogenized parts. The first part was bio-oxidized at 900 °C to release ^{14}C-CO_2. The radiolabeled CO_2 was trapped and counted by LSC. The second split sample was subjected to alkaline hydrolysis (50mL of 50% NaOH per 25g solid sample, reflux 2hrs) to release humic and fulvic acids. After this procedure the hydrolysates were vacuum filtered. The solid fraction, or humin, remaining was dried overnight at 60 °C and was bio-oxidized to release ^{14}C-CO_2 which was trapped and counted by LSC. The liquid hydrolysates were acidified to pH 1 in order to precipitate out the humic acids. The radiolabel of these samples was counted in liquid form, after dilution, using LSC.	1.0% in fulvic acid <u>Experiment A Anaerobic/Aerobic (9 weeks)</u> -Unbound (Extracts): 4.8% in water 27.9% in methanol 12.0% ethyl acetate -Bound to Solid Matrix: 28.8% in humin 13.1% in humic acid 4.9% in fulvic acid <u>Experiment B Anaerobic (5 weeks)</u> -Unbound (Extracts): 8.6% in water 22.1% in methanol 9.5% ethyl acetate -Bound to Solid Matrix: 47.0% in humin 4.5% in humic acid 5.3% in fulvic acid <u>Experiment B Anaerobic/Aerobic (9 weeks)</u> -Unbound (Extracts): 1.7% in water 4.4% in methanol 3.3% ethyl acetate -Bound to Solid Matrix: 62.0% in humin 11.1% in humic acid 9.5% in fulvic acid

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
1998	Esteve-Nunez and Ramos (40)	Pure culture of *Pseudomonas* sp. JLR11 under anaerobic, nitrogen-limiting conditions. Minimal media with 0.1% glucose was used. Sealed reactors were incubated at 30 °C with shaking.	Use of uniformly labeled ^{14}C-TNT, followed by extraction of radiolabel from temporal samples.	Culture supernatant was analyzed by LSC after TCA precipitation/centrifugation separation of the biomass. The biomass pellet was dissolved in 200 µL of water, after washing with clean culture medium and re-centrifugation, and analyzed by LSC.	Recovery of total radiolabel as a percentage of the initial radiolabel introduced into the system was as follows: -45% in biomass -15% in culture supernatants -1% in respired CO_2 -Remainder claimed to be trapped in the form of "volatile acids" in the NaOH traps used for trapping the ^{14}C-CO_2.
1998	Shen et al. (119)	Aerobic and anaerobic mixed culture microcosm (bioslurry) studies with molasses as the carbon source. Aerobic studies used activated sludge inoculum, whereas anaerobic studies used anaerobic digester sludge inoculum. Incubations at 28 °C on a rotary shaker	Use of uniformly labeled ^{14}C-TNT, followed by recovery of radiolabel from temporal samples. LSC performed on samples.	<u>Unbound Radiolabel (TNT and Metabolites)</u> Each samples was extracted with 20 mL acetonitrile (overnight on a rotary shaker) twice, followed by an extraction with water. After each extraction step, samples were filtered, the filtrates from each step were combined, and analyzed by LSC. <u>Bound Radiolabel</u> The filter cake from the solvent extractions (contained biomass and soil) was digested with a potassium chromate/sulfuric acid/phosphoric acid mixture. The 14C-CO2 liberated was trapped potassium hydroxide (KOH) traps. The KOH was analyzed by LSC.	<u>Aerobic Microcosm Study</u> Nonextractable/or bound radiolabel increased sharply to about 35% in 2 days and remained at that level for the remainder of the 30-day monitoring period. <u>Anaerobic Microcosm Study</u> Nonextractable/or bound radiolabel kept increasing temporally throughout the 30-day monitoring period. After 30 days of incubation it was at 72% of the total radiolabel introduced at the start of the experiment.

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
1999	Achtnich et al. (4)	Sequential Anaerobic (60 days) - Aerobic (20 days) bioslurry reactor with different mixed cultures for each phase; glucose used as the carbon source in the anaerobic phase. At the end of the anaerobic phase the reactor was inoculated with an aerobic culture from a sewage treatment plant to initiate the aerobic phase. The aerobic culture was capable of degrading glucose fermentation products. Temperature was maintained at 30 °C and pH at 7.3 for the entire course of the study.	Contaminated soil was spiked with uniformly labeled ^{14}C-TNT, followed by recovery of radiolabel from temporal samples. LSC performed on sample supernatant and extracts. Dried soil samples were also bio-oxidized for ^{14}C-CO_2 recovery at the end of the treatment.	<u>Unbound Radiolabel (TNT and Metabolites)</u> Supernatant from the sample was collected after centrifugation and analyzed by LSC. The soil-biomass pellet was extracted twice with methanol (1 hour each time at 30 °C). The methanol extracts from each step were combined, and analyzed by LSC. <u>Bound Radiolabel</u> Soil samples were air dried and homogenized after the anaerobic-aerobic treatment. Five, 300 mg aliquots were analyzed by bio-oxidation. Fifty grams of the soil was subjected to alkaline hydrolysis (suspended in 1000 mL of 0.5M NaOH overnight) to recover the humic and fulvic acid fractions. The humic acid was precipitated out of the centrifuged solution by dropping the pH to about 1.5 (fulvic acid remained in solution at pH 1.5). The organic fraction remaining in the soil was assumed to be humin. The remaining soil was subjected to a variety of extraction techniques. These were: (a) HCl (0.5M) followed by MeOH (b) HCl (5M) followed by MeOH	<u>Unbound Radiolabel (TNT and Metabolites)</u> At the end of anaerobic phase 13% of the total radiolabel introduced initially was in the supernatant/methanol extract fraction. At the end of the anaerobic-aerobic phase this value dropped to only 2%. <u>Bound Radiolabel</u> At the end of the treatment the radiolabel distribution in humic substances was as follows: humic acid (7.2%), fulvic acid (7.4%), and humin (85.4%). Among the great variety of extractions attempted, only the extractions using the higher concentrations of HCl (b) and EDTA (i), followed by MeOH were able to recover appreciable amounts of radiolabel; 12.5% and 5.9%, respectively.

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
				(c) MeOH only (d) MeOH at elevated temperatures (e) MeOH with soil crushing (f) MeOH with sonication (g) Water followed by MeOH (h) EDTA (25mM) followed by MeOH (i) EDTA (100mM) followed by MeOH	
1999	Achtnich et al. (1)	Same setup as described in the previous entry (4) except that the anaerobic phase was 51 days long, followed by an aerobic phase of 32 days.	Contaminated soil was spiked with $^{15}N_3$-TNT and ^{14}C-TNT, followed by recovery of radiolabel from temporal samples. LSC performed on sample supernatant and MeOH extracts. Dried soil samples were also bio-oxidized for ^{14}C-CO_2 recovery at the end of the treatment. In addition, ^{15}N NMR was performed on MeOH extracts, and humic substance fractions, including silylized	The procedures used for unbound and bound radiolabel were the same as those described earlier (4). However, the 9 detailed extraction procedures described were not performed. Instead, silylation was performed on soil samples to release humin for ^{15}N NMR analysis. The silylation procedure involved treatment of the sample with DMSO/trimethylchlorosilane/pyridine mixture, followed by evaporating to dryness. Samples had to be redissolved in an appropriate solvent (either dimethyl-d_6 sulfoxide or 1% deuterated NaOD) prior to ^{15}N NMR analysis.	<u>Radiolabel Distribution After Anaerobic Phase</u> MeOH Extract/Supernatant 17.4% Fulvic Acid 8.9% Humic Acid 5.7% Humin 64.6% <u>Radiolabel Distribution After Anaerobic-Aerobic Phase</u> MeOH Extract/Supernatant 1.1% Fulvic Acid 6.2% Humic Acid 7.1% Humin 71.0% <u>^{15}N NMR Results</u> At the end of the anaerobic-aerobic incubation, the NMR results suggested that nitrogen was covalently bound to humic acid fractions as substituted amines and amides. The NMR results for fulvic acids and silylated humin were inconclusive; however, humin results

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
			humin fractions.		implied the formation of azoxy and imine linkages.
2000	Bruns-Nagel et al. (19)	Sequential Anaerobic (13 weeks) - Aerobic (7 weeks) composting treatment with a mixed culture; molasses was used as the carbon source to drive the system anaerobic. The soil was ground with a pestle and mortar during the $^{15}N_3$-TNT spiking. The reactor was incubated in the dark at 30 °C. Soil moisture content was maintained between 30-40%. During the aerobic phase, the solids were mixed daily.	Contaminated soil was spiked with $^{15}N_3$-TNT, followed by recovery of radiolabel from temporal samples. The $^{15}N/^{14}N$ ratios for all samples were determined using pyrolysis capillary gas chromatography - atomic emission detection (GC-AED). In addition, cross polarization magic angle spinning (CPMAS) solid state ^{15}N NMR was performed on the soil and various humic substance extracts.	<u>Unbound Radiolabel (TNT and Metabolites)</u> The soil samples collected were air dried and were then extracted with acetonitrile in 1:10 solids to solvent ratio. The soil samples were re-dried to remove any solvent residue. <u>Bound Radiolabel/Humic Substances Extraction</u> Soil samples from which unbound radiolabel had been extracted were subjected to alkaline hydrolysis (referred to as a mild extraction) and involved suspending the soil sample in 0.5M NaOH (4 mL/ gram of soil) for 24 hours while shaking. The humic acid was precipitated out of the centrifuged supernatant from the first step by dropping the pH to <1 (fulvic acid remained in solution at the low pH). The organic fraction remaining in the soil was assumed to be humin. The humin remaining on the soil was subjected to a "drastic" or hot extraction procedure to remove additional humic and fulvic acids. The drastic extraction involved refluxing the sample in 150 mL of 0.5 N NaOH at 95 °C for three hours.	<u>Radiolabel Distribution After Anaerobic-Aerobic Composting</u> Solvent Extract: 33% Fulvic Acid: 5% (mild), 9% (hot) Humic Acid: 7% (mild), 12% (hot) Humin: 23% Note: the hot extraction procedure caused, a loss of nitro groups via alkaline hydrolysis. This was confirmed in the NMR results of these fractions. <u>CPMAS Solid State ^{15}N NMR Results</u> The CPMAS ^{15}N NMR results were considerably more conclusive than earlier studies (1) and proved covalent binding of the radiolabel to humic substances, especially humin. The chemical shifts suggested that the radiolabel was covalently bound predominantly by either imidazole/indole/ pyrrole/carbazole/quinolone/anilide/ amide/enaminone linkages or by condensed linkages with other radiolabel (i.e., nitroanilines, anilinohydroquinone, phenoxazones, or hydrazines). The exact functionality can not be determined due to the superimposition of their chemical shifts in ^{15}N NMR.

Year	Author(s)	Culture Conditions	Confirmation Technique	Extraction/Confirmation Procedure	Results & Conclusions
2000	Achtnich et al. (2)	Two Sequential Anaerobic (short-term = 8 days; Long-term = 51 days) - Aerobic (20 days) bioslurry reactors were set up similarly to earlier setups by Achtnich et al. (1, 3, 4). The purpose of the study was to determine the effect of the duration of the anaerobic phase on the extent of ^{14}C binding.	Contaminated soil was spiked with uniformly labeled ^{14}C-TNT, followed by recovery of radiolabel from temporal samples using rigorous extraction methods (HCl, EDTA, CH_3OH). LSC performed on sample supernatant and extracts. Dried soil samples were also bio-oxidized for ^{14}C-CO_2 recovery at the end of the treatment.	Performed in a similar fashion as earlier studies (4).	The main result of this effort was that 40% of the ^{14}C appeared to be covalently bound after short-term anaerobic/aerobic treatment, whereas 98% was bound after the long-term anaerobic/aerobic treatment. The results clearly indicated the importance of the duration of the anaerobic phase in ensuring irreversible binding.

5. HUMIC SUBSTANCES AS COMPONENTS OF SOIL NATURAL ORGANIC MATTER

Humic substances are ubiquitous in nature and constitute the dominant fraction of natural organic matter in soils (7). The classical definitions of humic substances and their component parts or fractions come from a 1985 publication by Aiken at al. (7). Aiken et al. defined humic substances as:

> "A general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in color, of high molecular weight, and refractory."

This broad definition has largely stood the test of time; however, the high molecular weight and refractory properties have been called into question by recent research (56). Research into the fractionation of humic substances by molecular size distribution (e.g., by gel permeation chromatography) and charge density (e.g., electrophoresis) have revealed the polydisperse nature of humic substances (43, 56, 131). The resistance of humic substances to microbial degradation (i.e., their refractory nature) in natural environments is attributed mainly to self association properties of their molecules, and to their close association with soil minerals which leads to their formation of colloids and their entrapment in soil aggregates.

Over the years, a wide array of spectrometric techniques have been applied to determine the structure of humic substances. The techniques that have achieved some measure of success include proton NMR, CPMAS ^{13}C-NMR, Fourier Transform Infrared

(FTIR) spectroscopy, fluorescence spectroscopy, electron spin resonance (ESR, also known as electron paramagnetic resonance or EPR), and mass spectrometry (56) (129). New techniques holding some promise include a variety of 2-dimensional NMR techniques, pyrolysis-GCMS, and surface enhanced Raman spectroscopy.

Molecular structural elucidation efforts have revealed isolated humic substances to be polyanions rich in carboxylate and phenolic functionalities (94, 131). However, differences in structure are commonly reported for humic substances from different sources, and even different depths (e.g., those extracted from peat). Although earlier protocol in this field distinguished between macromolecules commonly observed in recognizable plant/animal debris and humic substances (56), such structural differences have become less clear over time. To date, proteinaceous and lignaceous moieties, as well as carbohydrate and lipid structures, have been regularly reported to be present in humic substances (43).

5.1 Humic Acids

The three fractions of humic substances, including humic acids, are defined operationally on the basis of their aqueous solubility with respect to solution pH. More specifically, Aiken et al. (7) defined humic acids as:

> "That fraction of humic substances that is not soluble in water under acidic conditions (below pH 2), but becomes soluble at greater pH."

The above definition also serves as a basis for the isolation of humic acids, namely, the acidification of solutions to precipitate humic acids. A humic acid precipitation pH of 2 is favored by water scientists, whereas a pH value of 1 is favored by soil scientists (56). Structurally, humic acids isolated from soil have a 25 to 45 percent aromatic character. Aquatic humic acids are more highly oxidized and have a less aromatic nature. Unlike fulvic acids, a substantial portion of the oxygen in humic acids occurs as structural components that cannot be accounted for by the carbonyl, carboxyl, and phenolic functionalities (131). It occurs as ether and ester linkages. In addition, humic acids tend to have a higher average molecular weight than fulvic acids.

5.2 Fulvic Acids

Fulvic acid fractions are defined as (7):

> "That fraction of humic substances that is soluble under all pH."

Extraction methods for aquatic and soil fulvic acids rely on the separation by XAD-8 (polymethylmethacralate) resins after the removal of humic acids by precipitation (133, 135, 137). The highly polar acids remaining in solution after XAD-8 treatment are removed from solution by XAD-4 (styrene divinylbenzene) resins and are referred to as "XAD-4 acids" (56, 137). Fulvic acids tend to be smaller, more polar, more highly charged, and less aromatic than humic acids (56). The simplest known fulvic acid is called tannic acid (59).

5.3 Humin

Aiken et al. (7) defined humin as:

"That fraction of humic substances that is not soluble in water at any pH."

Based on this definition, humin can be extracted from soil with nonaqueous solvents once the humic and fulvic acids have been extracted from soil with a basic aqueous solvent. Other methods that have been applied to isolate humin include DMSO/HCl extraction (56) and methyl isobutyl ketone (MIBK) extraction (112). Humin isolation methods typically employ a final digestion of the sample with a mixture of HF and HCl in order to remove soil mineral matter, fats, and proteins.

The structure of humin is largely unknown because most of the soil organic carbon research has focused on humic and fulvic acids. Nevertheless, several hypotheses have been formulated on the structure and/or composition of humin. One of these hypotheses is that the structure of humin is similar to that of other humic substance fractions. This hypothesis attributes the inability of humin isolation via aqueous extraction to the close interaction of the "polar faces" of these molecules with inorganic colloids, which leaves the nonpolar portion of the molecules exposed to the solution. Other hypotheses suggest that the structure of humin is quite different than other humic substance fractions. This point of view is supported by CPMAS ^{13}C-NMR evaluation of soil humin and soil humic acid from the same source (54, 112). Rice and MacCarthy (112) have proposed that humin is an aggregation of bitumen, bound humic acid, bound lipids, and some insoluble inorganic material. They note that none of the components by

itself conforms to the operational definition of humin; however, the individual components can interact with each other to form humin.

IV. THE FATE OF TNT IN A SIMPLE LOW-ACTIVITY BIOREDUCTION SYSTEM

1. ABSTRACT

The *Clostridium acetobutylicum* cell extract/molecular hydrogen bioreduction system was used to determine the fate of ^{14}C-TNT in a cell-free purely biological system. Following anaerobic/aerobic treatment of ^{14}C-TNT, no recognizable products could be identified using reverse phase-HPLC/UV detection. The distribution of the total ^{14}C changed at the end of the treatment with approximately 10% of the ^{14}C bound to floating precipitate that had a high protein concentration. A review of the bioorganic chemistry of arylhydroxylamines and nitrosoarenes revealed the nitroso-thiol as the most likely reaction responsible for the binding of partially reduced TNT to proteinaceous material. The feasibility of this reaction was evaluated by reacting 1-thioglycerol with a mixture of 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) produced from the biotransformation of TNT. It was demonstrated that DHA6NT formed a new and relatively polar product with 1-thioglycerol only in the presence of oxygen. The oxygen requirement indicated that the nitroso functionality was responsible for the reaction.

2. INTRODUCTION

The reduction of nitroaromatic compounds commonly occurs in both aerobic and anaerobic biological systems owing to the strong electrophilic nature of nitro groups (107, 114). The complete reduction of a single nitro group requires a six-electron

transfer to form an amino group (11, 107). The reduction pathway proceeds sequentially via the partially reduced nitrosoarene and arylhydroxylamine intermediates that are considered to be highly reactive in nature (29, 58). The nitroso and hydroxylamino functional groups exist in what can be considered a "pseudo redox equilibrium" due to the insignificant kinetic barrier for the interconversion between these two functionalities (30). As a consequence, reductive conditions favor arylhydroxylamine formation whereas oxidative conditions favor nitrosoarene formation. Although the presence of arylhydroxylamine intermediates is frequently reported in anaerobic remediation systems (3, 34, 44, 65, 66, 96, 122), the detection of nitrosoarenes is a rarity in bioremediation systems and nature, especially when arylhydroxylamines are also present. The presence of arylhydroxylamines in any system results in the scavenging of the nitrosoarenes to form relatively insoluble azoxy compounds via a rapid condensation reaction (30, 45, 130). Therefore, the production of nitroso compounds in a system is generally inferred from the presence of azoxy compounds.

Biotransformation studies conducted with ^{14}C radiolabeled TNT in engineered systems have demonstrated that the fate of the radiolabel resides in natural organic matter (2, 4, 24, 37, 73, 119) and biomass (24, 40, 119, 136) fractions of the system. More recently, the existence of covalent linkages have been established between reduced metabolites of TNT and humic substances using ^{15}N-NMR spectroscopy (1, 19, 20). The functional group (on the metabolite) typically held responsible for such reactions is the aromatic amino group primarily because of the abundance of evidence regarding its fate in model systems. From model studies, three pathways for the irreversible binding of aromatic amines to soil natural organic matter have been elucidated: the nonenzymatic

1,4-nucleophilic or Michael addition of amino groups to quinoid rings of humic acid constituents (62, 63, 103); phenol oxidase (e.g., laccase) mediated free radical addition reactions (35, 36); and, metal catalyzed reactions between aromatic amines and humic substances (134). However, little is known about the fate of the nitroso and hydroxylamino metabolites generated from nitroaromatic reduction in environmental systems, in lieu of further reduction or azoxy formation.

The acidogenic *Clostridium sp.* cell extract/molecular hydrogen system is a very simple cell-free anaerobic system that uses molecular hydrogen as an electron donor to reduce ferredoxin electron carriers via the action of the enzyme hydrogenase (10, 27). The reduction of ferredoxins results in a corresponding reduction of nitroaromatic compounds to mainly hydroxylamino compounds (65, 106) that can be readily oxidized to their nitroso counterparts upon exposure to air (130). Since the system is cell-free and utilizes the reversible enzyme hydrogenase, it has no growth supplement or organic electron donor requirements (6). Such requirements have the potential to confound interpretations of compound fate in complex natural and biological systems. This problem is related to the inherent difficulty in identifying the reacting site/functional group from the sea of available functional groups in a complex system. In the case of arylamine intermediates, the problem has been addressed by demonstrating covalent binding with model compounds via enzymatic (35) and non-enzymatic reactions (103, 134, 138). However, such reactions do not explain the binding to biomass because in purely biological systems arylamines undergo N-oxidation to form arylhydroxylamines and nitrosoarenes prior to undergoing reactions with biological molecules (57).

Unfortunately, model studies do not exist for nitrosoarene and arylhydroxylamine compounds in environmental literature.

In this study we report the fate of partially reduced ^{14}C-TNT metabolites in a simple low activity *C. acetobutylicum* cell extract system following sequential anaerobic-aerobic treatment. The results for the fate are examined in the context of the bioorganic chemistry of functional groups concerned and used to formulate a hypothesis for covalent binding of the metabolites. Finally, this hypothesis is evaluated using the reaction with a model thiol.

3. EXPERIMENTAL SECTION

3.1 Chemicals

The following chemicals were used in this study: 2,4,6-Trinitrotoluene, 99% purity (ChemService, Westchester, PA); [U-ring-^{14}C]-2,4,6-trinitrotoluene, specific activity of 21.6 mCi/mmole, 99.5% purity (Chemsyn Science, Lenexa, KS); 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4HADNT, 2,2',6,6'-tetranitro-4,4'-azoxytoluene, 2,4',6,6'-tetranitro-2',4-azoxytoluene, and 4,4',6,6'-tetranitro-2,2'-azoxytoluene (Ron Spanggord, SRI International, Menlo Park, CA); 0.1mg/mL analytical standard of 4HADNT in acetonitrile (AccuStandard Inc., New Haven, CT); 3-mercapto-1,2-propanediol (1-thioglycerol), 95% purity (Aldrich, Milwaukee, WI); biotech grade bovine serum albumin lyophilized powder (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ); protein assay dye reagent concentrate (Bio-Rad, Hercules, CA); monobasic and dibasic potassium phosphate (Sigma, St. Louis, MO); HPLC grade acetonitrile, methanol, methylene chloride, n-pentane, 1N HCl solution, 1N NaOH solution, ScintiSafe Plus^{TM}

50% cocktail (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ); Carbon-14 Cocktail for R. J. Harvey biooxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ).

3.2 Preparation of Cell Extract

Crude cell extracts of *Clostridium acetobutylicum* ATCC 824 were prepared using the lysozyme/sonication procedure as described previously (66). However, one step was added to remove larger structural proteins by ultracentrifuging at 45,000g. Also, the anaerobically sealed cell extract vials were stored at -20°C. Two batches of cell extract were produced. The first batch was kept in storage at -20°C for six months prior to experimentation and had a very low TNT transformation activity (0.33 mg TNT/Vol.% cell extract/minute). This extract was used in the first transformation reaction to determine the fate of the radiolabeled TNT following anaerobic-aerobic treatment.

The second batch was prepared similarly; however, its storage time was considerably less (approximately one week). This extract was used to rapidly convert TNT to predominantly DHA6NT for the reaction with the model thiol compound. The activity of this extract could not be measured with the TNT depletion assay, as TNT could not be measured within 2 minutes of cell extract addition (based on initial conditions the activity was >3.1 mg TNT/Vol.% cell extract/minute).

3.3 Analytical Methods

Temporal samples for TNT biotransformation reactions, as well as thiol reaction samples were monitored using the Waters (Milford, MA) HPLC system. The system consisted of 2690 separations module, 996 photodiode array detector, and a Nova-Pak C8

analytical column (3.9x150 mm) and guard column assembly. The method used for all HPLC analysis was a acetonitrile/water gradient method with a linear ramp from 35% to 75% acetonitrile in 12 minutes followed by a return to the original conditions in 6 minutes.

3.4 TNT Transformation

Both cell-extract-free transformation reaction solutions were degassed with nitrogen for 30 minutes followed by molecular hydrogen for 30 minutes. The cell extract was added after 30 minutes of hydrogen addition to mark the start of the anaerobic reaction phase. Temporal bulk aqueous samples were collected under gas purge (molecular hydrogen for anaerobic phase, air for aerobic phase, and nitrogen when anaerobic conditions were to be maintained without further reaction). The samples were analyzed for ^{14}C content by scintillation counting and for product formation by HPLC.

The starting conditions for the first TNT transformation reaction were 865 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 100 mg/L. The anaerobic phase or molecular hydrogen bubbling was continued for 22.5 hours (1,350 minutes), after which it was replaced with air for a period of one hour. At the end of the anaerobic phase the reaction mixture consisted of DHA6NT and aminophenols (66) only.

The initial conditions for the second reaction were 1,493 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 50 mg/L. This reaction was carried out in 10 mM (pH 7) phosphate buffer. Temporal samples were collected after 20 and 40 minutes of hydrogen addition and analyzed by HPLC (Note that a trial reaction

was run earlier to determine cell extract activity on the basis of TNT depletion). Fractions were also collected (one fraction/2minutes) for the 40-minute sample using a 100 μL injection. After 40 minutes of hydrogen addition all of the parent TNT had been transformed to DHA6NT with some traces of 4HADNT remaining. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas. Thiols were then added to the 40-minute reaction solution.

3.5 ^{14}C-Radiolabel Recovery and Protein Quantitation

At the end of the first transformation reaction (sequential anaerobic-aerobic conditions), a clear solution (i.e., without any visible precipitate) was decanted from the reaction vessel. Three milliliters of this solution was passed through a Gellman Sciences 0.2 micron PTFE filter. Both the filtrate and the prefiltered clear solution were subjected to liquid scintillation counting using ScintiSafe Plus^{TM} 50% cocktail (10 mL cocktail/1mL sample) on a Beckman LS6500 scintillation counter. Protein quantitation was also performed on both samples by the Bio-Rad protein assay (adapted from the Bradford Assay) which uses the Coomassie brilliant blue G-250 dye. Serial dilutions of a 2 mg/mL solution of bovine serum albumin were used with a 1:5 dilution of the dye reagent concentrate to generate a standard curve for the protein quantitation.

The long strands of the brown floc-like precipitate were removed from the reaction solution using a sterile spatula. The collected precipitate was processed for ^{14}C analysis by combusting it at 900 °C in the R. J. Harvey Instrument Corporation Biooxidizer Model OX600, trapping the radiolabeled CO_2 produced in the Carbon-14 Cocktail, and counting it by scintillation counting. A small fraction (0.12 gm wet

weight) was removed to conduct protein analysis and solubility testing. Acid hydrolysis was performed on 0.06 gm (wet weight) of the collected precipitate by first placing it in a microcentrifuge tube with 500 μL of DDI water and vortexeing it for 30 seconds. Following the mixing, the tube was centrifuged at 13,000 rpm for 5 minutes and the supernatant solution above the pellet was discarded after the centrifugation. Five hundred microliters of pH 2 HCl solution was added to the pellet and the tube was vortexed for 30 seconds to mix its contents, followed by centrifugation at 13,000 rpm for 5 minutes. The acidic solution above the pellet was used for protein analysis. Note that the acidic solution was very cloudy, especially after the vortexing step. Solubility testing was carried out with the remaining precipitate by vortexing the precipitate with n-pentane and methylene chloride.

3.6 Reaction with 1-Thioglycerol

Molecular hydrogen addition for the second TNT transformation reaction conducted with the higher activity cell extract (concentration of 8% v/v) in 10 mM (pH 7) phosphate buffer, was stopped at the 40-minute mark. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas. The reaction was monitored by HPLC (100 μL injection) and 2-minute fractions were collected for the 18-minute HPLC method. Following sampling, 1-thioglycerol stock in acetonitrile (6 μL of 200 mM) was added to the still anaerobic reaction mixture and allowed to react under anaerobic conditions for 20 minutes. Note that the thiol addition yielded a molar ratio of thiol groups to potential nitroso groups (calculated on the basis of initial TNT concentration) of 1:2. A sample was collected from the reaction mixture under the

nitrogen purge at the 20-minute anaerobic reaction time mark and immediately analyzed by HPLC. Injection and fraction collection were conducted as earlier. The reaction mixture with thiols was then exposed to air and a 20-minute sample was collected and analyzed by HPLC with fraction collection performed as earlier.

4. RESULTS AND DISCUSSION

4.1 Fate of TNT Following Anaerobic/Aerobic Treatment

The anaerobic phase was terminated when HPLC analysis showed that the initial TNT was in the form of DHA6NT and aminophenols, products that have been reported previously (66) (Figure 10). During the aerobic phase, creamy white strands formed that slowly turned into a brown floc-like precipitate. Unlike behavior expected from azoxy compounds, no dissolution of the precipitate was observed in either n-pentane or methylene chloride. However, the precipitate appeared to partially dissolve in acidic (pH 2) solution, turning the solution to a cloudy color. Note that no recognizable products (with the exception of the polar front) were observed on the reverse phase (RP) HPLC chromatogram following the aerobic stage. The final pH of the reaction mixture was estimated to be between 5 and 6 using pH paper.

An appreciable drop in the bulk aqueous ^{14}C concentration was not observed over the entire course of the anaerobic/aerobic treatment. Upon completion of the experiment an overall mass balance of 96.2% was obtained on the basis of the total initial ^{14}C count (Figure 11 and Table 6). At the end of the anaerobic/aerobic treatment the remaining ^{14}C

in the system was distributed as follows: 9.6% precipitate (biooxidized fraction), 26.6% aqueous filter reject, and 63.8% aqueous filtrate.

The protein assay established that the bulk of the protein mass was in the form of- or associated with- the precipitate. Binding of the Coomassie blue G-250 dye to protein becomes quite inefficient in acidic solutions resulting in a subsequent loss of sensitivity of the assay (116). Still, a high value of 0.34 mg/mL was obtained for the pH 2 hydrolysate of the precipitate (Table 6).

As reported earlier, the precipitate contained only about 10% of the remaining ^{14}C after the experiment. The bulk of the ^{14}C was in the aqueous phase, perhaps associated with the lower-concentration soluble protein fraction (Table 6). An alternative explanation for the absence of RP-HPLC/UV-VIS analyzed products with no loss in ^{14}C could possibly be the loss of aromatic character of the products formed upon aeration. This phenomena appears to be unlikely because the reaction mixture contained DHA6NT in addition to the aminophenols; although unstable under aerobic conditions, DHA6NT has never been reported to undergo ring fission upon exposure to air. Regardless of the explanation for this unusual phenomenon, a significant portion of the radiolabel was found to be associated with insoluble proteins. This observation warrants further research into the covalent reactions of the reactive functionalities (i.e., aromatic hydroxylamino and nitroso groups) that were present in the reaction mixture.

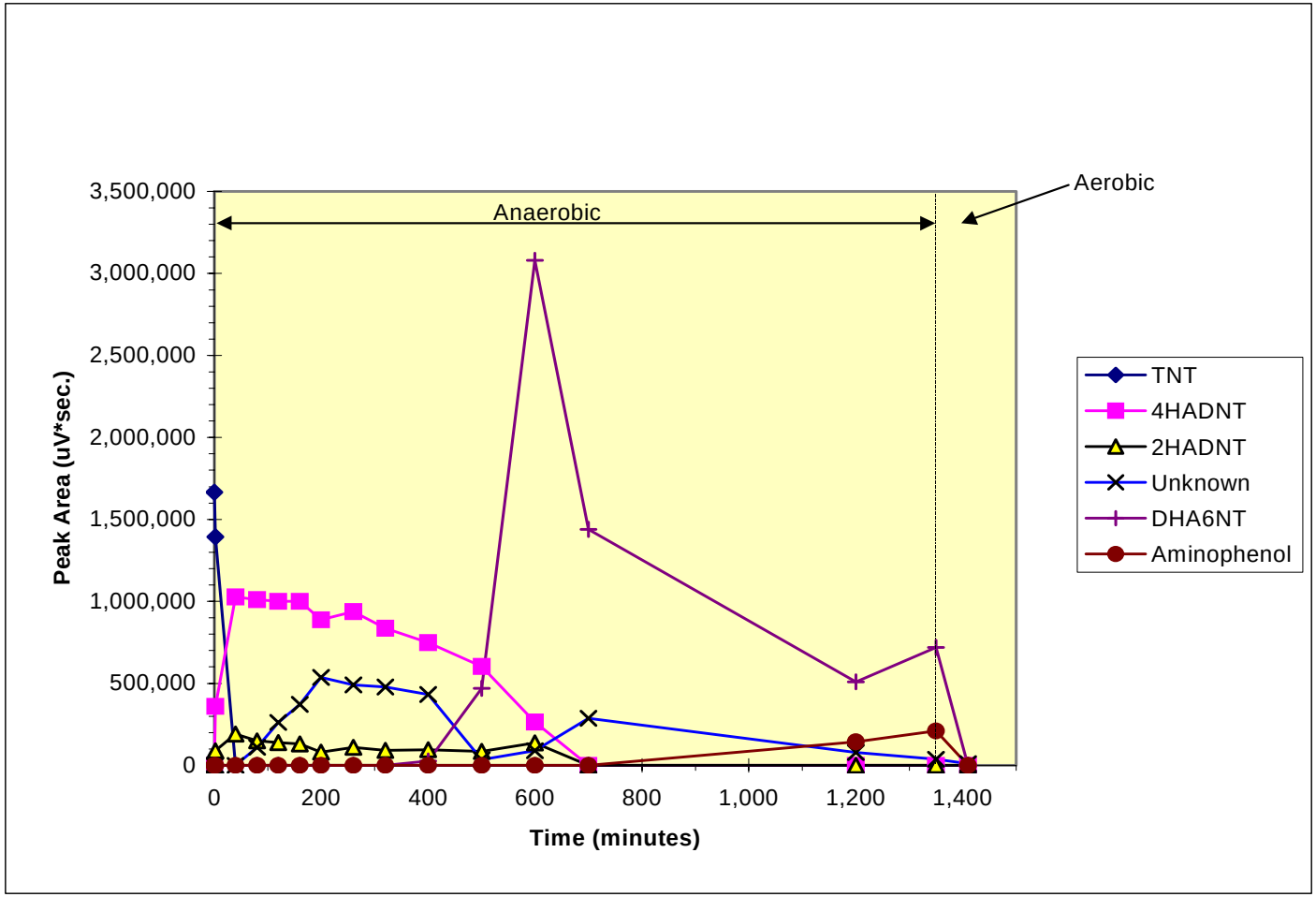

Figure 10. HPLC data for temporal samples from the first TNT transformation reaction (anaerobic/aerobic) with low activity *C. acetobutylicum* cell extract.

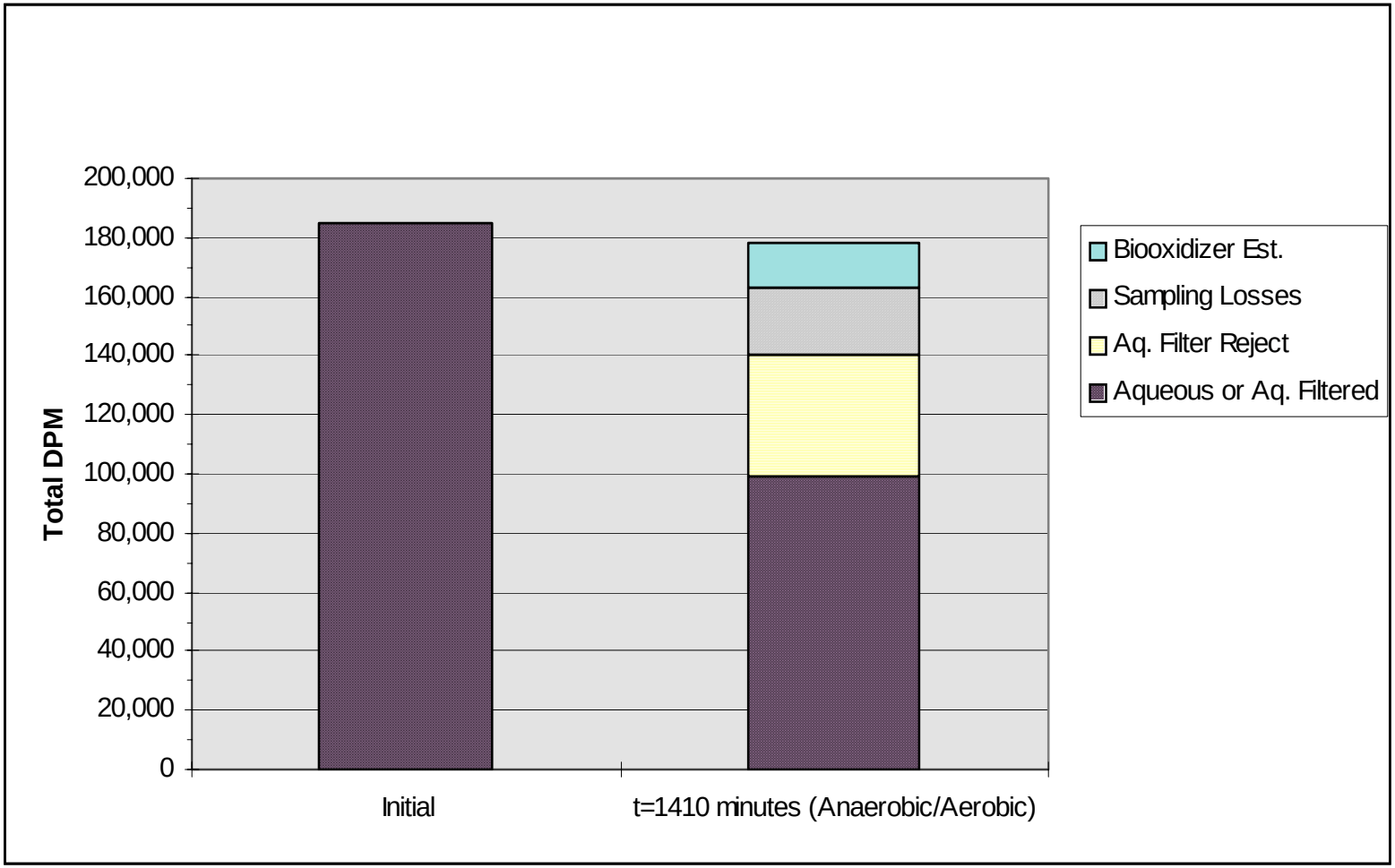

Figure 11. Distribution of ^{14}C-radiolabel at the beginning and end of the first TNT transformation reaction (anaerobic/aerobic) with low activity *C. acetobutylicum* cell extract.

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Table 6. ^{14}C radiolabel Distribution and Protein Concentrations for Various Fractions.

Description	Initial ^{14}C-dpm (% of total)	Post Treatment ^{14}C-dpm (% of total initial)	Initial Protein Conc. (mg/mL)	Post Treatment Protein Conc. (mg/mL)
Biooxidizer	-	8.1	-	0.34 *
Sampling Losses	-	12.2	-	-
Filter Reject	-	22.4	-	0.03
Filtrate	-	53.7	-	0.07
TOTAL	**100**	**96.2**	**0.18**	**-**

* Refers to a pH 2 hydrolysate

4.2 Reactions of Arylhydroxylamines

The known transformation products of arylhydroxylamines are the result of diverse biological and abiotic reactions (Figure 12). Reactions involving the heterolytic cleavage of the N-O bond at physiological pH, either by spontaneous ionization or nucleophilic displacement, generally tend to be enzyme catalyzed because this bond cleavage entails a high activation energy barrier (30, 45). The N-O bond cleavage often yields a strongly electrophilic nitrenium-iminium ion intermediate that can be rapidly trapped by an external nucleophile (141). The exact mechanism of the N-O bond cleavage is the subject of some debate as both spontaneous ionization to form a nitrenium ion (an SN1-type mechanism), and nucleophilic displacement of a suitable leaving group (an SN2-type mechanism), have been reported in literature (30). Nitrenium ions are relatively stable since they undergo extensive charge delocalization over the aromatic ring. Consequently, the formation of stable nitrenium ion intermediates can be hampered by the presence of aromatic ring deactivating/electron withdrawing substituents. The ability of an arylhydroxylamine or a related compound to undergo nucleophilic displacement is dependent on the "nucleofugacity" (i.e. ability of nucleophile to leave) of the potential leaving group (30, 104).

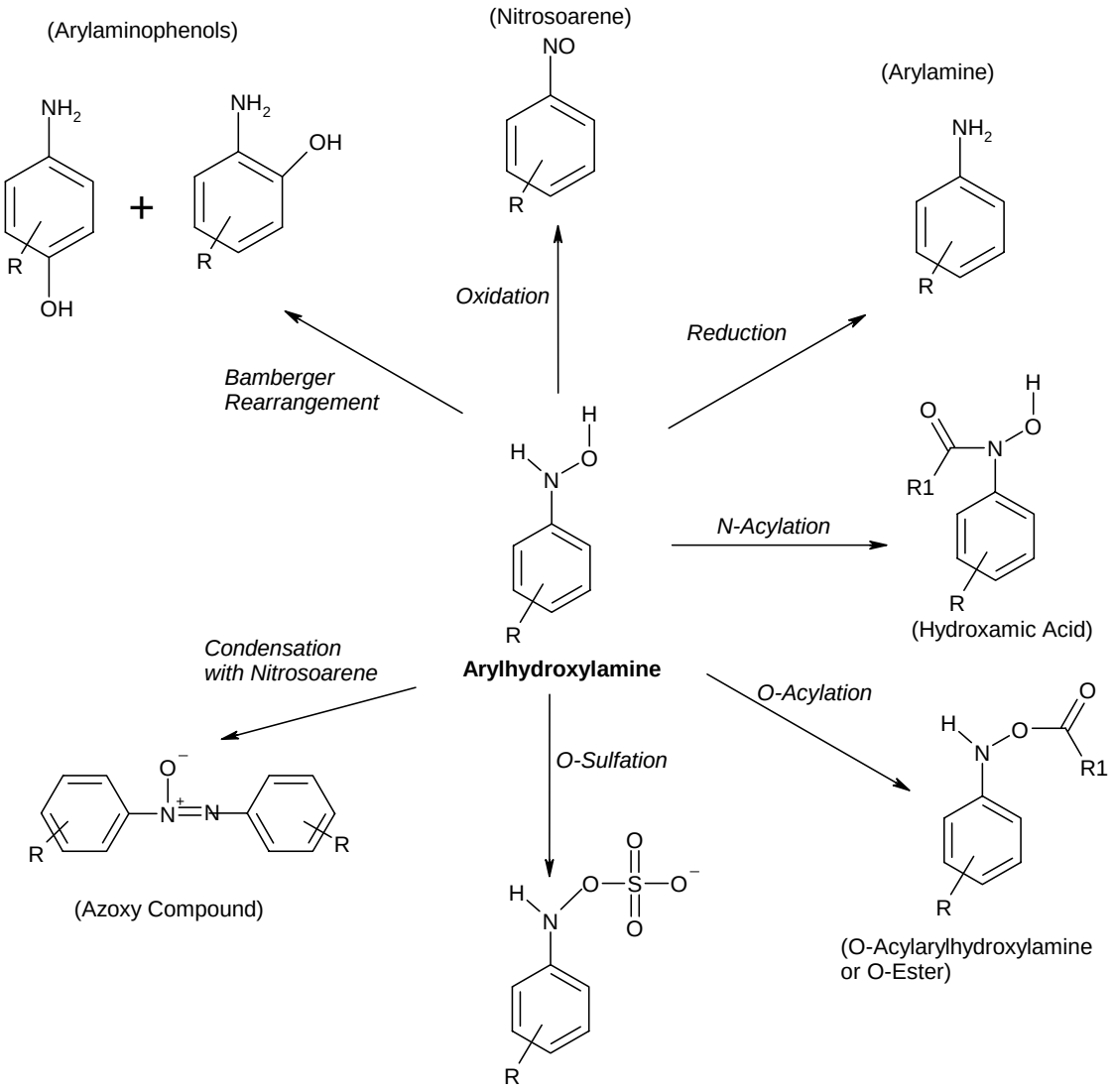


Figure 12. Potential reactions of arylhydroxylamines. Reactions involving the heterolytic cleavage of the N-O bond (i.e., reduction, O-acylation, O-sulfation, Bamberger Rearrangement, and N-acylation) are generally biologically catalyzed; however, the Bamberger Rearrangement can also be acid catalyzed.

A good example of an N-O bond cleavage reaction is the Bamberger Rearrangement reaction. This reaction involves the trapping of a nitrenium ion (SN1 mechanism) originating from an arylhydroxylamine, either in the ortho or para position (relative to the nitrogen), by a water molecule to form an arylaminophenol (Figure 13). The Bamberger Rearrangement can occur under acid-catalyzed conditions or can be biologically mediated. Due to the presence of lone pairs of electrons, both the nitrogen and the oxygen of an arylhydroxylamino group display nucleophilic characteristics, albeit the nitrogen is more nucleophilic than the oxygen. Therefore, under acidic conditions, the nitrogen is subject to more frequent protonation than the oxygen; however, the N-protonated intermediate rarely leads to further reaction and typically breaks down to form the parent arylhydroxylamine (30, 141). Conversely, the O-protonation results in the removal of water which allows for competing reactions that are largely irreversible, and eventually causes the depletion of the parent arylhydroxylamine. Note that nucleophiles other than water can also react with the nitrenium ion intermediate formed by the SN1-type mechanism, or by nucleophilic displacement via the SN2 mechanism (30).

Other reactions involving the nitrenium ion intermediate include the formation of hydroxamic acids. These are formed when the nitrenium ion undergoes hydration at the nitrogen. The formation of hydroxamic acids from hydroxylamines via this mechanism is a minor reaction (30); greater yields of hydroxamic acids can be obtained via pathways involving nitrosoarenes.

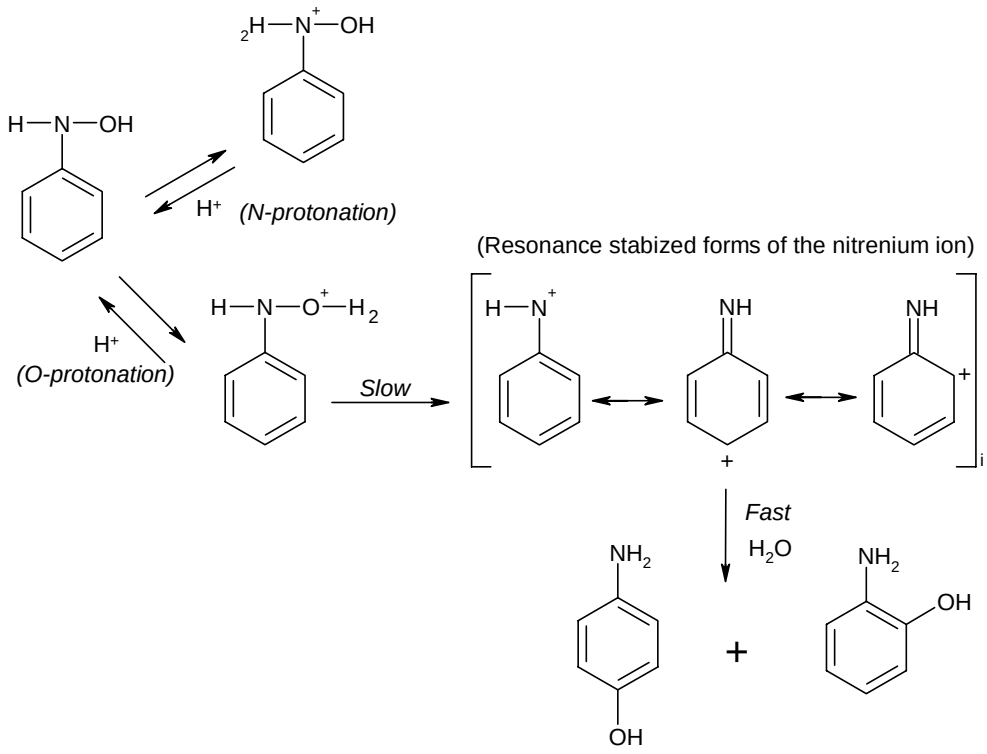


Figure 13. Mechanism for the acid catalyzed Bamberger Rearrangement of arylhydroxylamine.

Arylhydroxylamine reactions that do not involve N-O bond cleavage occur abiotically. However, the only two such reactions reported in literature are the oxidation of the hydroxylamino group to a nitroso group under aerobic conditions, and the condensation reaction with nitrosoarenes to form azoxy compounds. In the condensation reaction, the nucleophilic hydroxylamino nitrogen attacks the relatively electrophilic nitroso group. The azoxy compounds tend to be insoluble and precipitate.

4.3 Reactions of Nitrosoarenes

The majority of reactions of nitrosoarenes occur spontaneously as they are abiotic reactions that are favored, both, thermodynamically and kinetically (30). In these abiotic reactions, the nitroso group behaves as an electrophile that can be attacked by a variety of nucleophiles (Figure 14). The simplest of these reactions is the abiotic coupling of nitrosoarenes with the nucleophilic nitrogen of hydroxylamino groups to form azoxy compounds. Another such reaction is the nitroso-glyoxylate reaction where the glyoxylate ion acts as the nucleophile (28, 30). This mechanism becomes dominant at a pH of 3 (around the glyoxylic acid pKa of 3.30) and the reaction rate increases with increasing pH until it reaches a pH of 5, where it becomes largely independent of pH (30). An interesting facet of the nitroso-glyoxylate reaction is the fact that the roles of the nitroso group and the glyoxylic acid are reversed below a pH of 2; namely, the nitroso group behaves as a weak nucleophile that reacts with the electrophilic carbonyl carbon of the glyoxylic acid. The nitroso-glyoxylate reaction occurring at the lower pH is the only known reaction where the nitroso group acts as a nucleophile instead of an electrophile. N-formyl hydroxamic acid and carbon dioxide are formed for both the low

and elevated pH reactions. Additionally, the low pH mechanism is known to work with other α-oxoacids such as pyruvic acid (32).

Perhaps the best-studied and most important of all abiotic reactions under physiological conditions is the reaction of the nitroso group with sulfahydryl groups of thiols (30, 41). This reaction is often cited as the reaction most likely responsible for the cytotoxic behavior of nitroso-, amino-, and nitro-arenes (42). This reaction proceeds via the rapid, albeit reversible, formation of a semiacetal-like N-hydroxysulfenimide that is commonly termed a "semimercaptal" in literature (41, 42, 81). The semimercaptal is a relatively unstable and undergoes a variety of reactions depending on the ring stabilizing/destabilizing effects of the aryl substituents present (Figures 14 and 15) (42). If the aryl substituents are strongly electronegative and stabilize the ring by withdrawing electrons, then one of two products are possible: an aryl hydroxylamine from the cleavage of the N-S bond (in the presence of excess undissociated thiol) and a sulfinamide (or sulfinanilide) via the trapping of a sulfenium ion by a water molecule. The sulfenium ion is formed by the cleavage of the N-O bond. Unlike nitrenium ion formation from arylhydroxylamines, the rate of N-O bond cleavage of the semimercaptal is enhanced by a factor of 10^6 due to the presence of the adjacent sulfur atom (74). If the overall effect of the aryl substituents is to donate electrons to the ring (or destabilize the ring) then a variety of other side products are also formed in addition to the sulfinamide, especially when excess thiol is present. These side products include thioethers, arylamines, and sulfenylquinonimines (42).

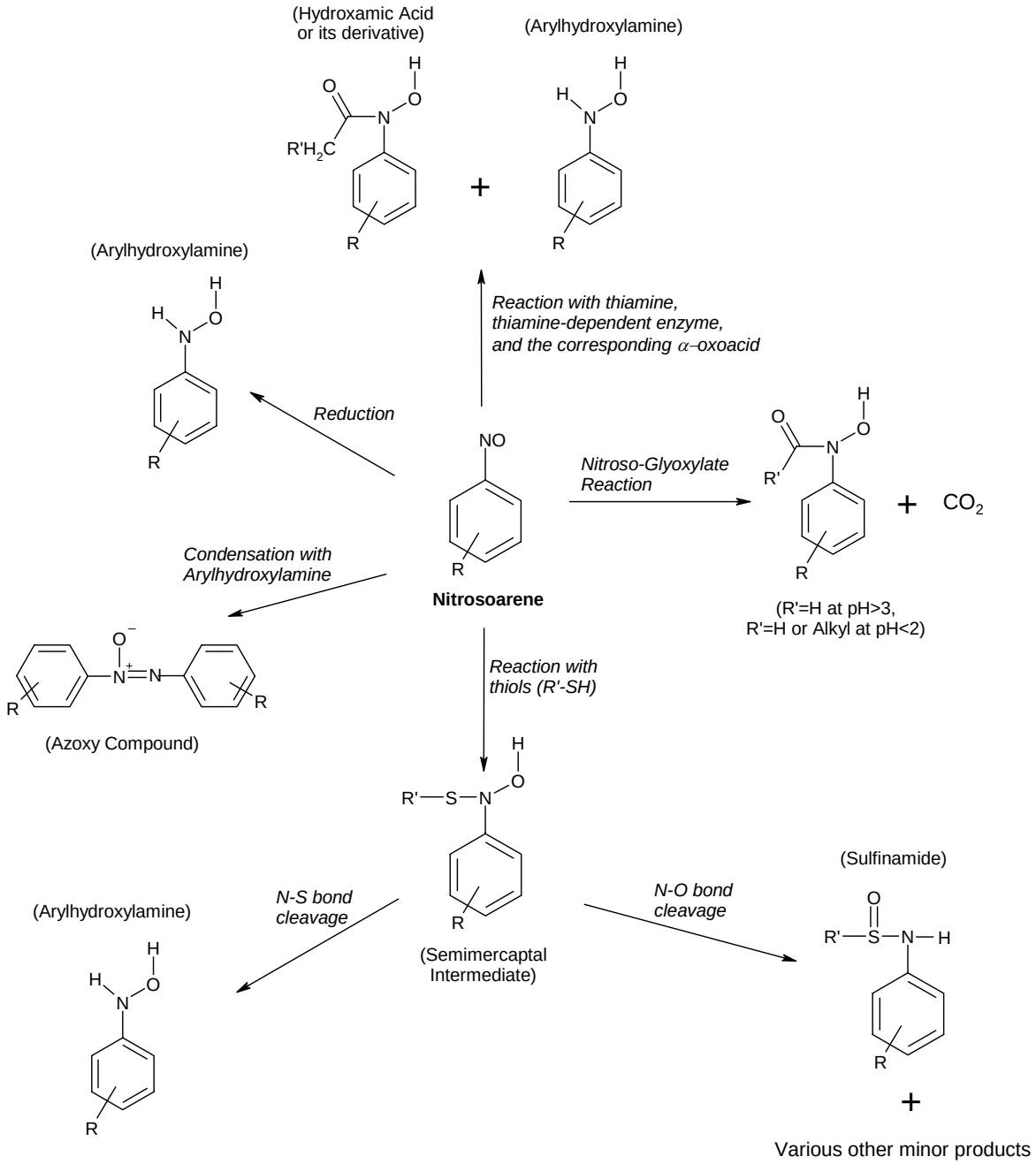


Figure 14. Potential reactions of nitrosoarenes. All reactions are of an abiotic nature, with the exception of reactions involving thiamine-dependent enzymes.

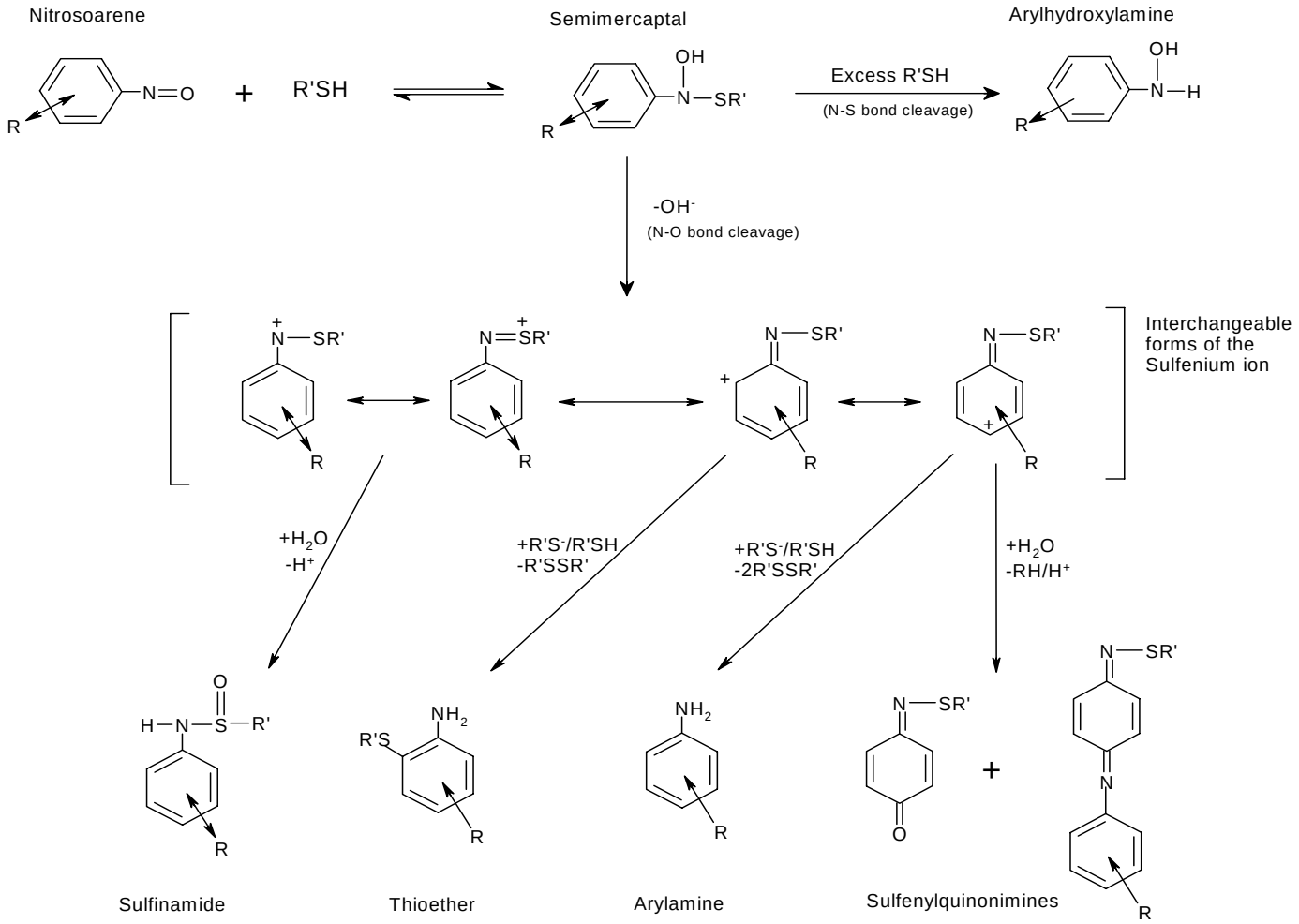


Figure 15. Reactions of nitrosoarenes with thiols (adapted from Eyer and Gallemann (42)).

The only known nitrosoarene reaction requiring a role for a biocatalyst is its interaction with a thiamine cofactor/α-oxoacid intermediate of one of the three thiamine-dependent enzymes (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, or transketolase). This reaction leads to the formation of hydroxamic acid (or its derivative) and, in some cases, arylhydroxylamine (30, 31). Note that the reaction catalyzed by transketolase does not yield any arylhydroxylamines, whereas the reaction involving α-ketoglutarate dehydrogenase yields both arylhydroxylamines and its Bamberger rearrangement products.

In summary, the nitroso-thiol reaction is the most likely candidate for explaining the covalent binding of reduced nitroaromatics to proteinaceous species. It is unlikely that hydroxylamino groups interact with biomass without the participation of a catalyst. The next section tests the feasibility of the nitroso-thiol reaction between 1-thioglycerol and an anaerobically reduced TNT solution.

4.4 Reaction of Anaerobically Biotransformed TNT with a Model Thiol

The second TNT transformation reaction yielded predominantly DHA6NT (retention time of 4.1 minutes) and trace levels of 4HADNT (retention time of 8.1 minute). The chromatogram and its percent ^{14}C distribution obtained from fraction collection are presented in Figure 16(a) and 16(b), respectively. As expected, the radiolabel was concentrated in the peaks of the reduced metabolites of TNT. The addition of approximately 1.2×10^{-3} mmole of 1-thioglycerol followed by 20 minutes of anaerobic incubation produced no significant change in the percent ^{14}C distribution of the chromatogram (Figure 17 (a) and (b)). Note that the moles of thiol added were roughly

half the number of moles of TNT present prior to the anaerobic biotransformation. The molar ratio of thiols to potential nitroso functionalities was deliberately kept below 1:1 because the presence of excess thiol reduces the nitroso functionalities back to hydroxylamino (81). This sets an undesirable situation where both nitroso and hydroxylamino functional groups are present in the system and a rapid condensation of azoxy products ensues (especially at neutral or mildly acidic pH). Following 20 minutes of aerobic incubation, a new more polar peak (retention time of 3.3 minutes) was identified (Figure 18 (a)) that had a virtually identical UV spectrum as DHA6NT. A corresponding shift in the percent ^{14}C distribution of the chromatogram (Figure 18 (b)) was also observed. The reaction profile matches that of the 1-thioglycerol/nitrosobenzene reaction reported in literature (41, 81). This reaction generates an acid labile sulfinamide as the dominant product. No new products having spectra similar to 4HADNT were identified. This could either be the result of the low initial concentration of 4HADNT making the identification of a trace product difficult or simply the inability of 1-thioglycerol to act as a competing nucleophile for the strongly ring deactivated 4HADNT molecule; peak area for 4HADNT decreased upon aerobic incubation, indicating that perhaps the remaining nucleophilic hydroxylamino groups were outcompeting the thiol nucleophile to produce azoxy compounds. In conclusion, the model reaction demonstrated the feasibility of the nitroso-thiol reaction for nitrosoarenes produced from the oxidation of DHA6NT. A similar conclusion could not be reached for 4HADNT.

The formation of a new product by the reaction of oxidized DHA6NT with 1-thioglycerol indicates that thiols can act as competing nucleophiles for partially reduced

TNT metabolites in sequential anaerobic/aerobic biotransformation systems. Such reactions can allow the thiol-containing cysteine amino acid residues of proteins to become potential sinks for the parent nitroaromatic contamination. In addition, if the protein undergoing binding is involved as an enzyme in the reduction pathway then binding can pose an inhibition threat for further transformation. In any case, the evaluation of the thiol concentration at any given time in the system may be of vital importance in determining the fate of the nitroaromatic contamination.

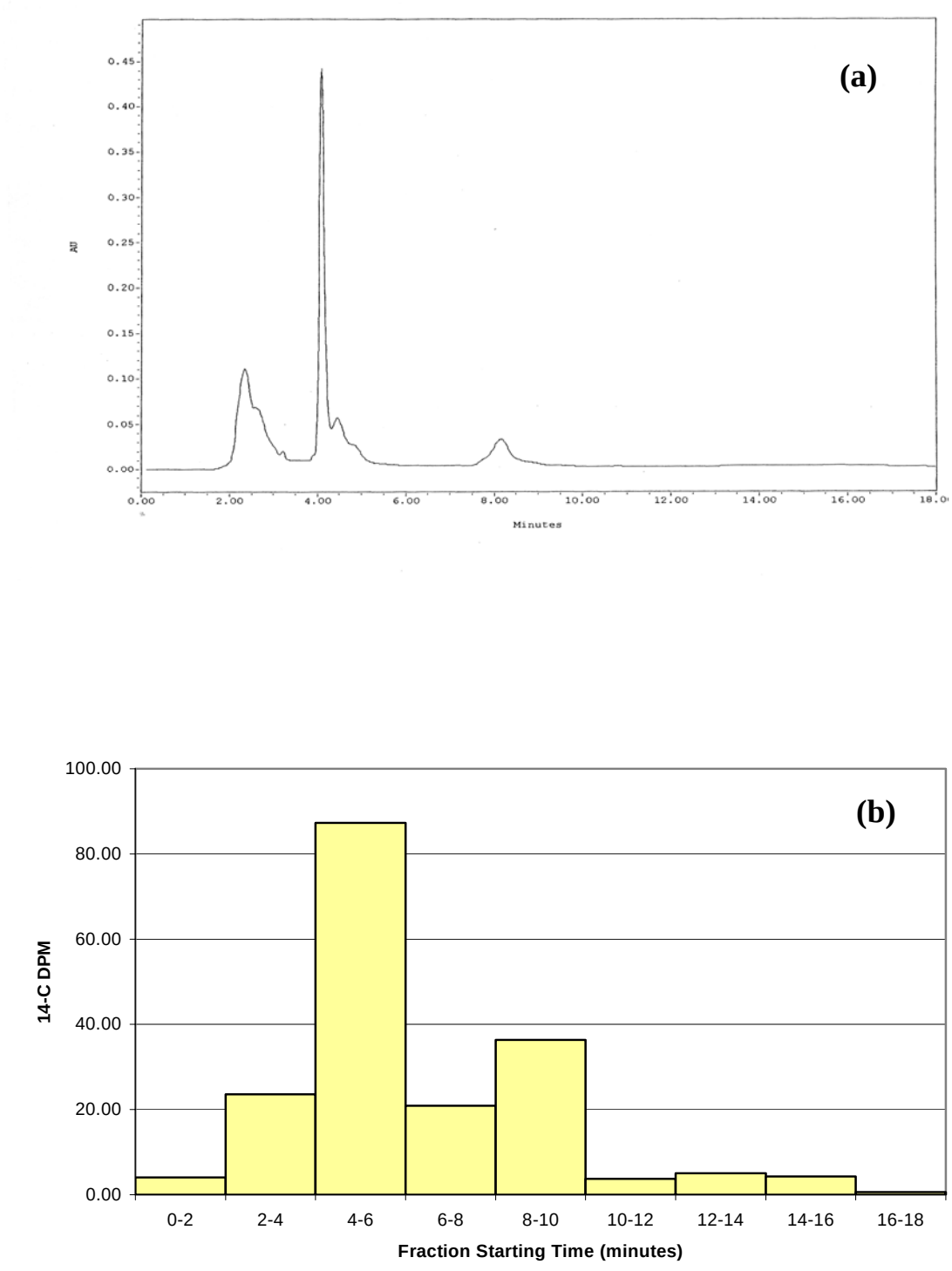
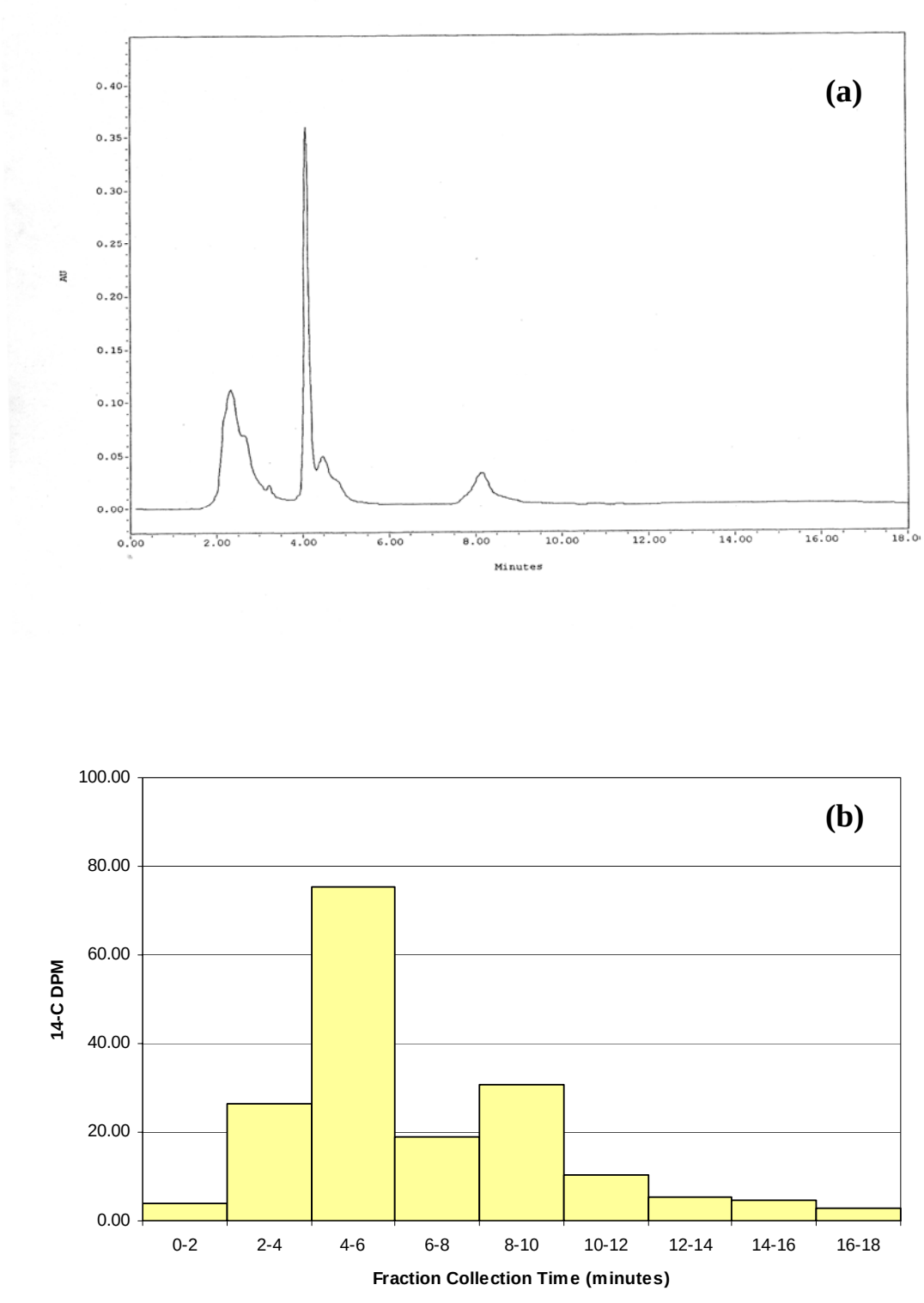
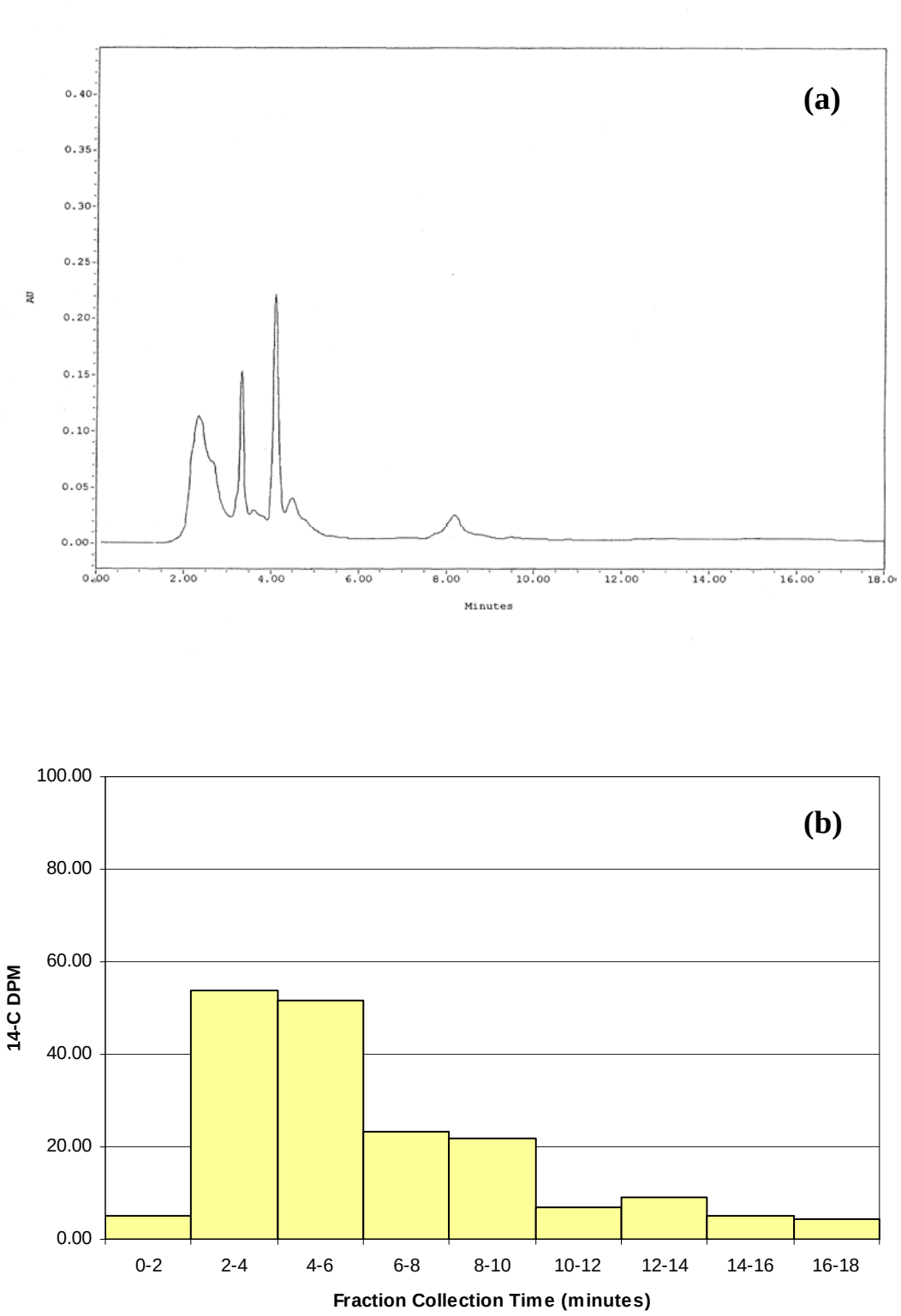


Figure 16 (a) and (b). Chromatogram and ^{14}C distribution under anaerobic conditions prior to thiol addition.



Figures 17 (a) and (b). Chromatogram and ^{14}C distribution under anaerobic conditions after thiol addition (20 minute anaerobic incubation time).



Figures 18 (a) and (b). Chromatogram and ^{14}C distribution under anaerobic conditions after thiol addition (20 minute aerobic incubation time).

V. THE REACTIVITY OF ARYLHYDROXYLAMINES AND NITROSOARENES TOWARDS HUMIC ACIDS

1. ABSTRACT

The reactivity of arylhydroxylamino and nitrosoarene functionalities towards International Humic Substance Society (IHSS) peat humic acid was evaluated under anaerobic and aerobic conditions, respectively. The model arylhydroxylamine, 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), showed no appreciable reactivity towards peat humic acid at a mass fraction (mass of arylhydroxylamine/mass of humic acid) of 0.01. Conversely, the model nitrosoarene compound, nitrosobenzene, showed rapid reactivity with peat humic acid (50% removal in 48 hours). When tested with two other humic acids, it was found that the proteinaceous fraction of the humic acids was responsible for at least part of the nitrosoarene's removal from solution. The pretreatment of the humic acids with a selective thiol derivatizing agent had a considerable effect on their ability to react with nitrosobenzene. Finally, molecular modeling tools were used to compare the electrophilic characteristics of nitrosobenzene with potential nitroso intermediates from the reduction pathway of the prevalent nitroaromatic contaminant, 2,4,6-trinitrotoluene.

2. INTRODUCTION

Over the years, numerous bench- and field-scale biotransformation studies conducted with polynitroaromatic contamination have resulted in a disparity in the molar balance between the parent compound and its reduced products (35, 66). The use of ^{14}C and ^{15}N radiolabeled nitroaromatic compounds has demonstrated that binding of the reduced metabolites occurs to the solid matrix. Further investigations to determine which fraction of the solid matrix binds the TNT metabolites have pointed in the direction of biomass (24, 40, 44, 119, 136) and natural organic matter (NOM) (2, 4, 24, 37, 73, 119). The observed binding has been deemed "irreversible" or covalent because a variety of extraction techniques with common organic solvents have yielded poor recoveries of the reduced metabolites (2, 4). Recovery of humic substances from the soil NOM has shown the radiolabel to be associated predominantly with the humin and humic acid fractions (1, 19, 37, 73). In recent studies, the covalent linkages have been confirmed using ^{15}N-NMR spectroscopy (1, 19, 20). The mechanism cited most often in literature for the covalent binding is the 1,4-addition of the nucleophilic aromatic amine intermediates to quinoid moieties in the humic substances (2, 19, 39, 103, 120, 134).

Further studies focusing on the conditions necessary to increase the extent of binding have indicated two important factors. They are the degree of reduction achieved in the initial anaerobic phase, and the need for an aerobic stage following the initial anaerobic reduction phase (2, 4, 37). These findings are believed to strengthen the evidence for the nucleophilic addition mechanism because the greater reduction achieved in the anaerobic phase leads to greater nucleophilic character of the amino nitrogen

produced. Furthermore, the aerobic stage requirement allows for the reoxidation/tautomerization of the hydroquinone back to the quinone, thereby promoting further nucleophilic additions possibly leading to the formation of nitrogen heterocycles (103, 134). However, this mechanism does not adequately address the extensive binding observed in purely biological systems (89, 132) or the binding occurring to biomass in engineered remediation systems (24, 40, 119, 136). First of all, quinones make up the structure of electron transfer proteins and oxidoreductases in biological systems, both of which are present in limited quantities in an organism (61). Secondly, studies that involve the recovery of humic substance fractions from soil NOM to establish a mass balance for the parent radiolabel, overlook the presence of co-extracted and co-precipitated molecules (56, 139). Soil NOM not only contains humic substances, but also has two other major components: namely, decomposable organic residues from plant and animal decay and the heterotrophic organisms that feed on the organic residue (124, 139). Since the various humic substance fractions (namely, fulvic acids, humic acids, and humin) are operationally defined on the basis of their aqueous solubility with respect to solution pH (i.e., following an alkaline extraction), they often contain varying degrees of biomolecular contaminants such as denatured proteins and carbohydrates that originate from the parent soil NOM (56). This point is further supported by evidence that the greatest degree of binding of reduced nitroaromatics occurs to the humin fraction (i.e., fraction insoluble at all pH) of soil NOM (1, 19); structurally, humin is considered to be an aggregation of various organic and inorganic molecules that include humic acids and biomolecules (112).

An alternative reaction mechanism that has requirements similar to those needed for irreversible binding (2) is the covalent binding of reduced nitroaromatic- or N-oxidized arylamine-metabolites to proteins via the nitroso-thiol reaction (74, 80, 81). This mechanism is often cited in literature to explain the cytotoxicity of nitroaromatics and aromatic amines in biological systems (71, 89, 132). In this mechanism the nitroso group acts as an electrophile and is trapped by the thiolate anion nucleophile (74). The reaction is dependent on a variety of factors including thiol concentration, pH, and substituent effects on the aromatic ring of the metabolite (42).

In this work, we evaluate the reactivity of aromatic hydroxylamino and nitroso functionalities towards IHSS (International Humic Substance Society) peat humic acid under anaerobic and aerobic conditions, respectively. Peat humic acid was selected because peaty soils tend to be water logged and, subsequently, their humic acids contain high concentrations of biological molecules due to a lack of aerobic decay (22). 4HADNT, a common metabolite found in the reduction pathway of TNT, was used as the model arylhydroxylamine. Attempts to generate nitroso formation and subsequent reactions by the aeration of aqueous 4HADNT in the presence of competing nucleophiles were unsuccessful. To the best of our knowledge the synthesis of nitroso derivatives of TNT has not been reported in literature, perhaps due their extremely high reactivity. The only two C-nitrosoarenes commercially available are nitrosobenzene and nitrosotoluene both of which have relatively activated (or electron rich) ring systems, unlike 4HADNT, which has an electron deficient ring due to the presence of two electron-withdrawing nitro substituents. For these reasons, a choice was made to use nitrosobenzene, the less ring activated of the two commercially available nitrosoarenes, as the model nitroso

compound. Using nitrosobenzene, we explore the dependence of the nitroso group reactivity on the proteinaceous content of humic acid and attempt to restrict the binding contribution from the nitroso-thiol reaction by pretreating the humic acids with a selective thiol derivatizing agent. Finally, we employ molecular modeling tools to compare the electrophilic nature of unstable nitroso intermediates of TNT with that of nitrosobenzene to determine whether the more unstable compounds would behave in a way similar to the model compound.

3. EXPERIMENTAL SECTION

3.1 Chemicals

The following chemicals were used: 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene, 2,4',6,6'-tetranitro-2',4-azoxytoluene, and 4,4',6,6'-tetranitro-2,2'-azoxytoluene (Ron Spanggord, SRI International, Menlo Park, CA); 0.1mg/mL analytical standard of 4-hydroxylamino-2,6-dinitrotoluene in acetonitrile (AccuStandard Inc., New Haven, CT); nitrosobenzene, 97% purity (Aldrich, Milwaukee, WI); EDTA (J. T. Baker, Phillipsburg, NJ); monobasic and dibasic potassium phosphate, and 90% pure N-dansylaziridine (Sigma, St. Louis, MO); IHSS peat humic acid standard, leonardite humic acid standard, and Summit Hill reference humic acid (Paul Bloom, University of Minnesota, St. Paul, MN); HPLC grade acetonitrile, methanol, methylene chloride, 1N HCl solution, 1N NaOH solution (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ).

3.2 Analytical Methods

Temporal samples for the reaction of humic acids with 4HADNT and nitrosobenzene were monitored using the Waters (Milford, MA) HPLC system. This system consisted of 2690 separations module, 996 photodiode array detector, and a Nova-Pak C8 analytical column (3.9x150 mm) and guard column assembly. The HPLC method used for all temporal analysis was an acetonitrile/water gradient method with a linear ramp from 40% to 70% acetonitrile in 10 minutes followed by a return to the original conditions in 4 minutes. 4HADNT experiments were monitored at a wavelength of 220 nm, whereas nitrosobenzene experiments were monitored at a wavelength of 305 nm. A more nonpolar gradient method was used to analyze the dissolved precipitate produced in the aerobic 4HADNT/peat humic acid experiment. This method involved a linear ramp of acetonitrile from 50% to 100% in 12 minutes, followed by a 100% plateau for one minute and a recovery to original conditions in 5 minutes.

3.3 Reactions with Humic Acids

All reactions were carried out in triplicate and in the dark in 10 mM phosphate buffer (pH of 7) under continuous stirring. 4HADNT was chosen as the model arylhydroxylamine compound and nitrosobenzene was chosen as the model nitrosoarene compound (note that nitrosobenzene and nitrosotoluene are the only two stable aromatic C-nitroso compounds commercially available). Experiments were designed to keep a low sorbate (e.g., 4HADNTor nitrosobenzene) to sorbent (humic acid) mass ratio at initial conditions in order to better estimate potential second order reactions with pseudo-first order kinetics (17, 86). The initial sorbate to sorbent mass ratio for 4HADNT

experiments was approximately 0.02 and for nitrosobenzene experiments was approximately 0.01; the difference in the ratio between the two compounds reflects the approximately 2:1 ratio of molecular weights for 4HADNT:nitrosobenzene even though these molecules have potentially one mole of reactive groups per mole of either compound. The humic acid concentration was fixed at 625 mg/L. This concentration reflects a less than tenfold increase (on the basis of carbon concentration) from maximum dissolved organic material concentrations of 50 mg Carbon/L observed in nature (138). As in past studies (134, 138), the higher than natural concentration of humic acid was selected to bring about a appreciable change in aqueous sorbate concentration over a reasonable period of time, which in our case was 48 hours.

The reaction between 4HADNT and IHSS peat humic acid, together with a control containing no sorbent, was conducted under anaerobic conditions. Freshly prepared 4HADNT stock in acetonitrile was added to vials containing degassed (30 minutes Helium followed by 30 minutes Nitrogen) buffer/humic acid solution to start the reaction. Temporal samples were collected under a nitrogen purge, passed through Accell^{TM} Plus QMA anion exchange cartridges (Waters, Milford, MA) into HPLC vials that had been flushed with nitrogen. A needle attached to a disposable check valve (Cole-Parmer, Vernon Hills, IL) was used to allow the nitrogen displaced by the sample to exit the HPLC vials. Samples were analyzed by HPLC immediately upon collection. An additional reaction was run with 4HADNT and IHSS peat humic acid. This reaction was set up with the same precautions as the earlier anaerobic reaction; however, the reaction mixture was exposed to the atmosphere in order to assess the ability of the humic acid to compete for the nitroso oxidation product of 4HADNT. The precipitate

formed in this reaction was extracted using methylene chloride, followed by air drying, redissolution in a 50/50 (v/v) acetonitrile/buffer mixture, and analysis by RP-HPLC/UV-Visible detection.

Reactions between nitrosobenzene and various humic acids were conducted in a closed aerobic system (i.e., no prior degassing) due to the mild volatility/sublimation characteristics of nitrosobenzene. Temporal samples were collected and passed through Accell^{TM} Plus QMA cartridges into HPLC vials. During sampling, creation of a vacuum in the reaction bottle was avoided by again using a needle/disposable-check-valve assembly to allow air in to replace the sample volume removed. An experiment with nitrosobenzene involved the doubling of the sorbent concentration. For this experiment the sorbate to sorbent mass ratios were approximately 0.01 and 0.005 for the regular and doubled concentrations of the sorbent, respectively. Additionally, controls with no sorbent were run for the experiment.

3.4 Thiol Derivatization and Recovery of Humic Acids

The thiol content of IHSS peat and Summit Hill humic acids was derivatized by adapting a thiol derivatizing procedure from literature (82, 85, 117, 118). This procedure utilized N-dansylaziridine, a highly selective thiol derivatizing agent. A ratio of 10 mg humic acid : 2.5 ml of pH 8.2 derivatization buffer (67 mM phosphate/0.2 mM EDTA) : 0.25 mL of a 5 mg/mL solution of N-dansylaziridine in methanol. The derivatization mixture was placed in a crimped top serum bottle and suspended in a water bath (60 °C) for one hour. The bottle was then removed from the water bath, the top was removed, and its contents were allowed to cool under stirring for another hour. The cooled

mixture was transferred into 500 Dalton (Molecular Weight Cutoff [MWCO]) cellulose ester dialysis membranes that were sealed using dialysis clips. The sealed membranes were placed in aluminum foil pouches containing 10 gm of Spectra/Gel™ absorbent powder to dewater membrane contents overnight (Note: all dialysis equipment and dewatering absorbents obtained from Spectrum Laboratories, Laguna Hills, CA). After overnight dewatering/volume reduction the contents were transferred into 500 Dalton MWCO cellulose ester Spectra/Por® Dispodialyzers®. The dispodialyzers were suspended in a DDI water reservoir for approximately 12 hours. During this step the reservoir water was replaced with fresh water at least twice. The dispodialyzer contents were dewatered as before using the absorbent powder; however, this time they were left in the absorbent bed for only 6-8 hours owing to their substantially higher surface area to volume ratio. The contents of the dewatered dispodialyzers were removed using a thin disposable pipette and transferred into preweighed aluminum weighing dishes. The weighing dishes were placed into a 60 °C oven for 6 hours, the time used to reach a constant weight using this procedure. After 6 hours the dishes were removed from the oven and allowed to cool in a dessicator overnight. The cooled dishes were weighed to calculate the weight of their contents (i.e., derivatized humic acid). The derivatized humic acid was ground using a pestel and mortar, transferred to a labeled vial, and placed in the dessicator for storage.

3.5 Molecular Modeling

Molecular modeling was carried out in CAChe™ software (Oxford Molecular/Pharmacopeia, Hunt Valley, MD). Models for nitrosobenzene, 4-nitroso-2,6-

dinitrotoluene (4NDNT, potential oxidation product of 4HADNT), and 2-nitroso-4-hydroxylamino-6-nitrotoluene (2N4HA6NT, potential oxidation product of 2,4-dihydroxylamino-6-nitrotoluene) were designed. The molecular models were then optimized to obtain their lowest energy conformations. Essentially two calculations were performed to assess the reactivity of the nitroso nitrogen: First, the partial charge on the nitroso nitrogen was calculated; secondly, the electrostatic potential averaged over the molecular surface was determined to assess the locations where the molecule would be susceptible to a nucleophilic attack.

4. Results and Discussion

4.1 Reactivity of 4HADNT with IHSS Peat Humic Acid

The anaerobic experimental series run to test the reactivity of 4HADNT towards IHSS peat humic acid at a sorbate/sorbent mass ratio of 0.02 yielded an approximately 30% loss in concentration over 48 hours (Figure 19). However, the difference between this loss and the loss observed in non-sorbent-containing controls was less than 6%. Loss of 4HADNT in the humic acid-free anaerobic control series was approximately 24%. The experiment was repeated and produced similar results (data not shown). Anaerobic reactivity of 4HADNT was also tested with derivatized IHSS peat humic acid in anticipation of the reactivity between 4HADNT and peat humic acid. This series also demonstrated virtually no difference between experimental systems and controls. The lack of reactivity of the model arylhydroxylamino compounds towards humic acid can be explained in terms of its bioorganic chemistry in natural systems.

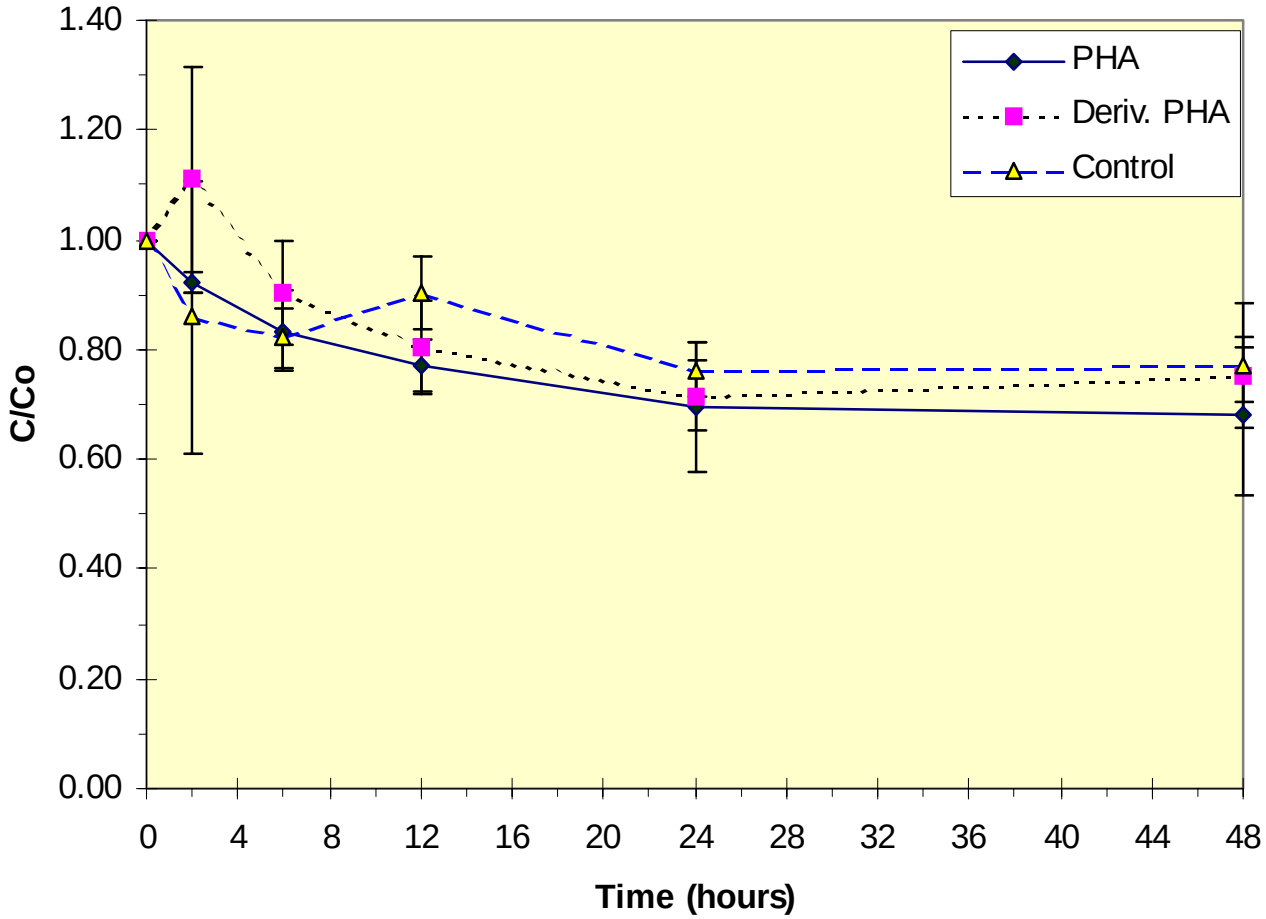


Figure 19. Reaction of 4HADNT with non-derivatized (PHA) and thiol-derivatized (Deriv. PHA) IHSS peat humic acid standard under anaerobic conditions. The plot also shows data for an anaerobic control of 4HADNT in the absence of any humic acid.

In a companion publication we mentioned that although the hydroxylamino nitrogen has reasonable nucleophilic characteristics, in all its observed reactions (with the exception of azoxy formation) it behaves as an extremely strong electrophile by first forming a nitrenium ion intermediate. The arylhydroxylamine can form the electrophilic nitrenium ion only when assisted by catalytic conditions to overcome the kinetic barrier for the N-O bond cleavage. Therefore, in the absence of catalysis, either acidic or biological, it is unlikely that the arylhydroxylamine will react with the multitude of potential nucleophiles present in the humic acid.

Interestingly, our results showing no significant removal of 4HADNT with humic acids are in direct contrast to findings reported by others (3). Achtnich et al. reported a complete removal 0.04 mM 4HADNT (8.5 mg/L) in less than 2 hours when humic acid was present at a concentration of 7,500 mg/L. Since we are not aware of all the details of their system, we can only offer speculations as to why the two experiments produced opposite outcomes. The most obvious of these differences is the humic acid concentration. We used 625 mg/L of IHSS peat humic acid that is approximately 56% carbon by mass (See Table 7 for the characteristics of the IHSS humic acids used in this study). These values translate into an effective carbon mass concentration of 350 mg/L, which is seven times the maximum DOM concentrations observed in nature. Similarly, the humic acid concentration used by Achtnich et al. translates into a DOM concentration of 3,750 mg carbon/L if a conservative carbon content of 50% by mass is assumed for their humic acid (note that characteristics of humic acids used were absent from their publication).

Table 7. Some chemical characteristics of IHSS peat and leonardite humic acid standards and IHSS Summit Hill reference humic acid (127).

IHSS Humic Acid*	Elemental Analysis									Neutral Hydrophobic					Neutral Hydrophilic			Acidic		Basic		S-cont.	
	(mass %)									(Nanomole Amino Acid/mg Humic Acid)													
	C	H	O	N	S	P	Total	H_2O	Ash	Val	Ile	Leu	Tyr	Phe	Thr	Ser	Gly	Asp	Glu	Arg	His	Met	Total
Leonardite	63.8	3.70	31.3	1.23	0.76	< 0.01	100.8	7.2	2.58	1.1	0.6	1.2	0.3	0.4	0.4	1.7	0.9	1.1	1.5	1.1	0.2	0.1	11
Peat	56.4	3.82	37.3	3.69	0.71	0.03	102.0	11.1	1.12	44	24	32	6.1	15	14	27	35	63	54	50	5.2	4.0	373
Summit Hill	54.0	4.84	37.9	5.13	0.64	0.40	102.9	8.1	1.41	146	61	66	33	40	33	183	225	160	178	232	34	26	1420

This value is 75 times the maximum concentrations of DOM in nature and most probably exceeded its aqueous solubility. At our relatively low humic acid concentrations of 625 mg/L we observed an average drop in pH of 0.4 units over the course of the 48-hour experiment duration even though the experiments were conducted in pH 7, 10 mM phosphate buffer. Not having any data on their humic acid's carboxylic and phenolic acidity components, we can only speculate that the drop of pH in their pH 7.3, 50 mM buffered systems was much greater. A significant drop in pH can produce conditions for the acid catalyzed cleavage of the arylhydroxylamine's N-O bond. Although the concentration of the nitrenium ions produced may not be significant when compared to the total concentration of arylhydroxylamine available, the presence of strong nucleophiles in the humic acid could lead to the depletion of arylhydroxylamines from the system.

The second major difference between our 4HADNT experimental systems was the anaerobic integrity. Our controls showed a 24% loss in concentration over 48 hours, whereas Achtnich et al.'s control showed a 75% concentration loss in less than 20 hours. The presence of trace levels of oxygen in the system could promote oxidation to the electrophilic nitroso functionalities that can subsequently be trapped by the nucleophilic nitrogen of the remaining arylhydroxylamines in solution.

In a second experiment with 4HADNT, the effects of converting the system from anaerobic to aerobic conditions was analyzed in the presence of IHSS peat humic acid. The purpose of this experiment was to determine whether the nucleophiles in the humic acid could compete with the arylhydroxylamines for the relatively electrophilic nitroso

compounds produced upon exposure of 4HADNT to air. No appreciable difference in the rates of removal of 4HADNT from the aqueous phase could be observed between the humic acid series and the control series (Figure 20). Both series resulted in the formation of precipitate that was identified as predominantly 4,4',6,6'-tetranitro-2,2'-azoxytoluene. The azoxy formation followed pseudo-first order kinetics as shown in Figure 21. From the results it was evident that the nucleophiles present in humic acid were unable to compete for the nitroso intermediates resulting from the oxidation of 4HADNT. Earlier in the companion publication, we demonstrated that thiols reacted more readily with the more ring activated 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) than they did with the more strongly ring deactivated 4HADNT. It is very likely that the cause for this phenomenon is rooted in the aromatic substituent effects of the nitroso compound formed.

4.2 Reactivity of Nitrosobenzene with Various IHSS Humic Acids

Three IHSS humic acids were picked for their widely varying amino acid/proteinaceous content (based on analytical data for 13 amino acids), as well as their relatively similar sulfur content (Table 7). Nitrosobenzene was exposed to three different humic acids and the trends in reactivity were compared to non-sorbent containing controls. All three humic acids displayed the ability to remove nitrosobenzene from the buffer solution (Figure 22). Moreover, the extent of removal achieved over the 48-hour period appeared at least in part to be dependent on the proteinaceous content of the humic acid. Going from highest to lowest protein content, Summit Hill, peat, and leonardite humic acids showed nitrosobenzene removals of 70%, 50%, and 35%, respectively.

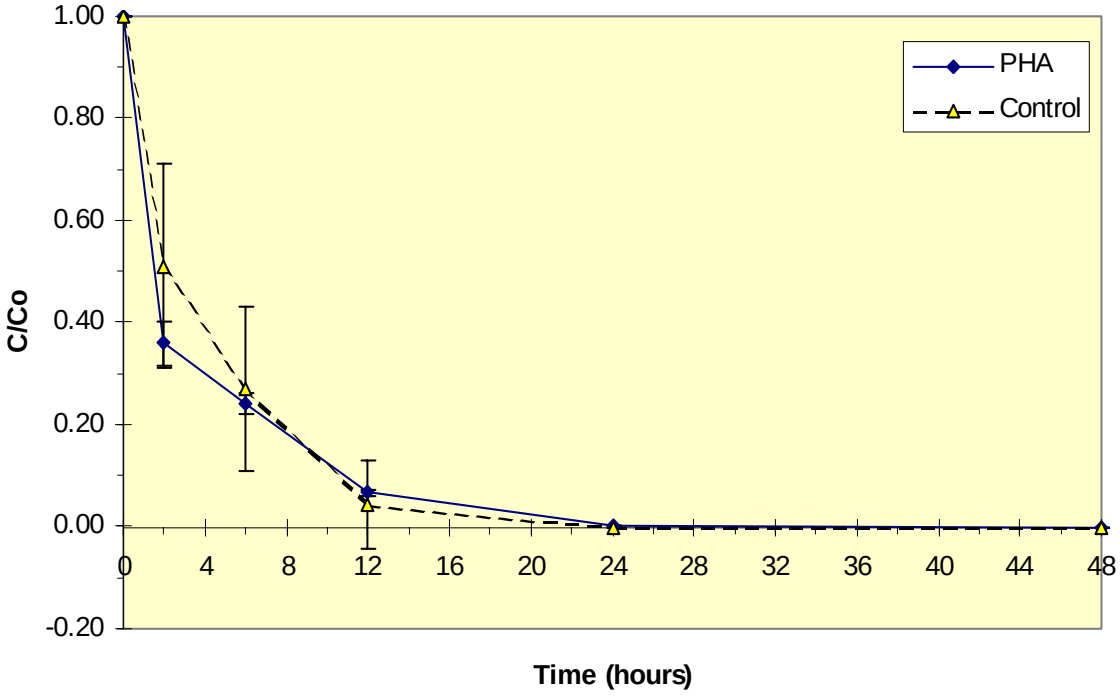


Figure 20. Aerobic reaction of 4HADNT with IHSS peat humic acid standard (PHA).

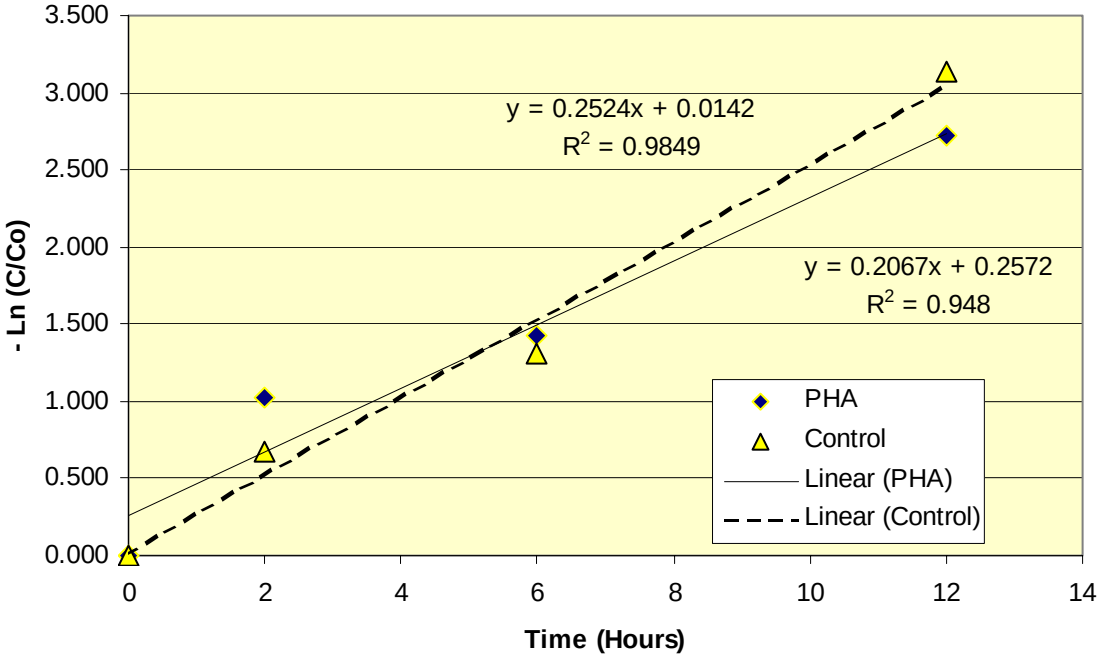


Figure 21. Pseudo-first order reaction analysis for data from the aerobic reaction of 4HADNT with IHSS peat humic acid standard.

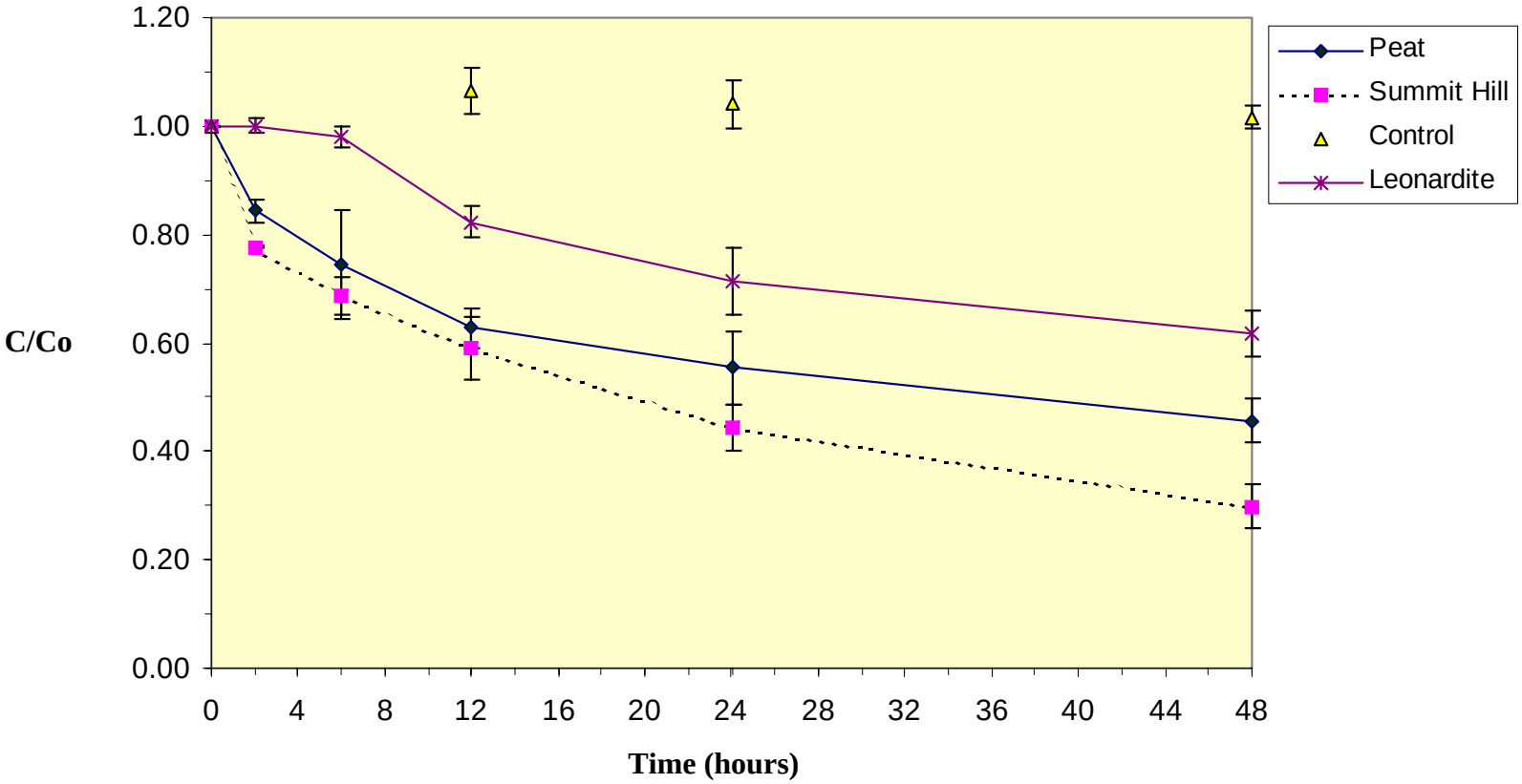


Figure 22. Data for the reaction of nitrosobenzene with non-derivatized IHSS peat and leonardite humic acid standards, and IHSS Summit Hill reference humic acid in a closed aerobic system. The plot also shows data for an aerobic closed system control of nitrosobenzene in the absence of any humic acid.

The results for the removal of nitrosobenzene with leonardite was somewhat unusual because it displayed an initial slow removal followed by a rapid increase in removal after the 6-hour sampling mark. This trend in the leonardite data may be explained by the poor initial solubility of the leonardite humic acid observed in the initial phase of the experiment.

Biphasic kinetic analyses were performed on the peat and Summit Hill data to obtain different pseudo-first order rate constants for the later slow removal and the initial fast removal of aqueous nitrosobenzene. The slower binding reaction showed good linear least square fits with correlation coefficients generally over 0.96 (Figure 23). The data in the faster phase was adjusted to remove any contributions from the slower phase and replotted on a semilog plot. Unfortunately, the early data did not demonstrate a good fit on this first order reaction analysis (Figure 24) possibly due to the limited number of data points available.

The final experiment in this section determined the effect of doubling the sorbent concentration on nitroso removal. As it can be seen from Figure 25, doubling the concentration of humic acid had a marked effect on the removal of aqueous nitrosobenzene. The nitrosobenzene removal increased by 22% and 18% for peat and Summit Hill humic acids, respectively.

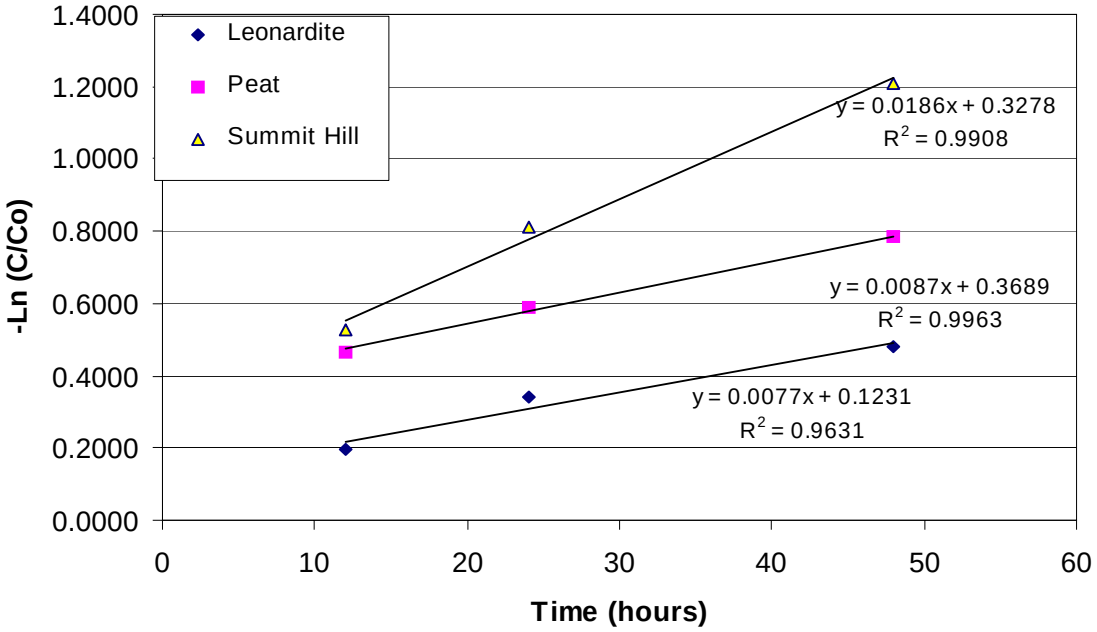


Figure 23. First order reaction analysis for the later slow phase of the nitrosobenzene/humic acid reaction.

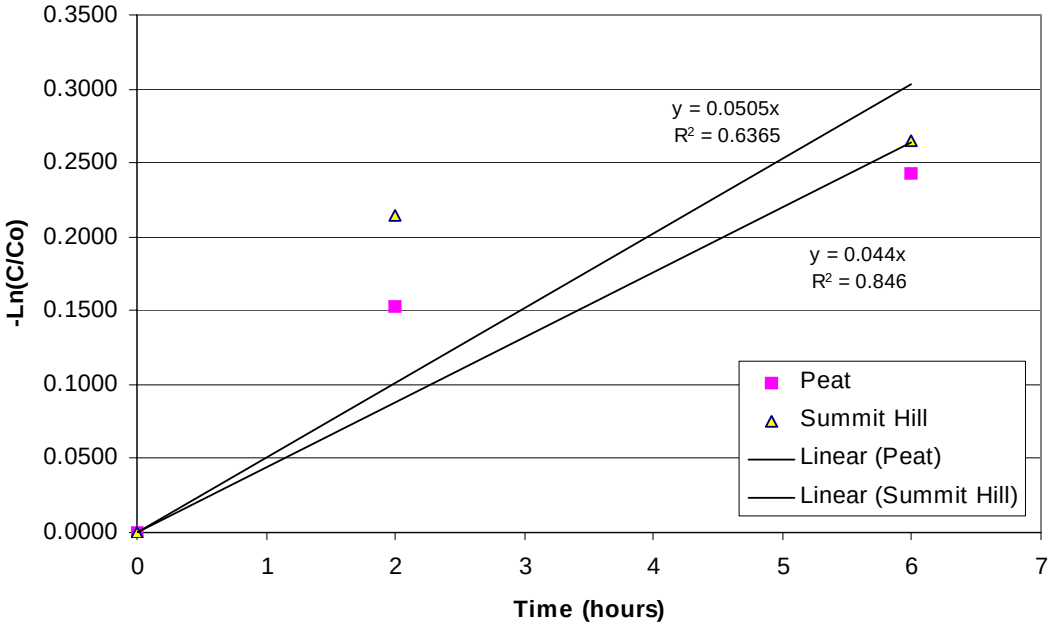


Figure 24. First order reaction analysis for the initial fast phase of the nitrosobenzene/humic acid reaction.

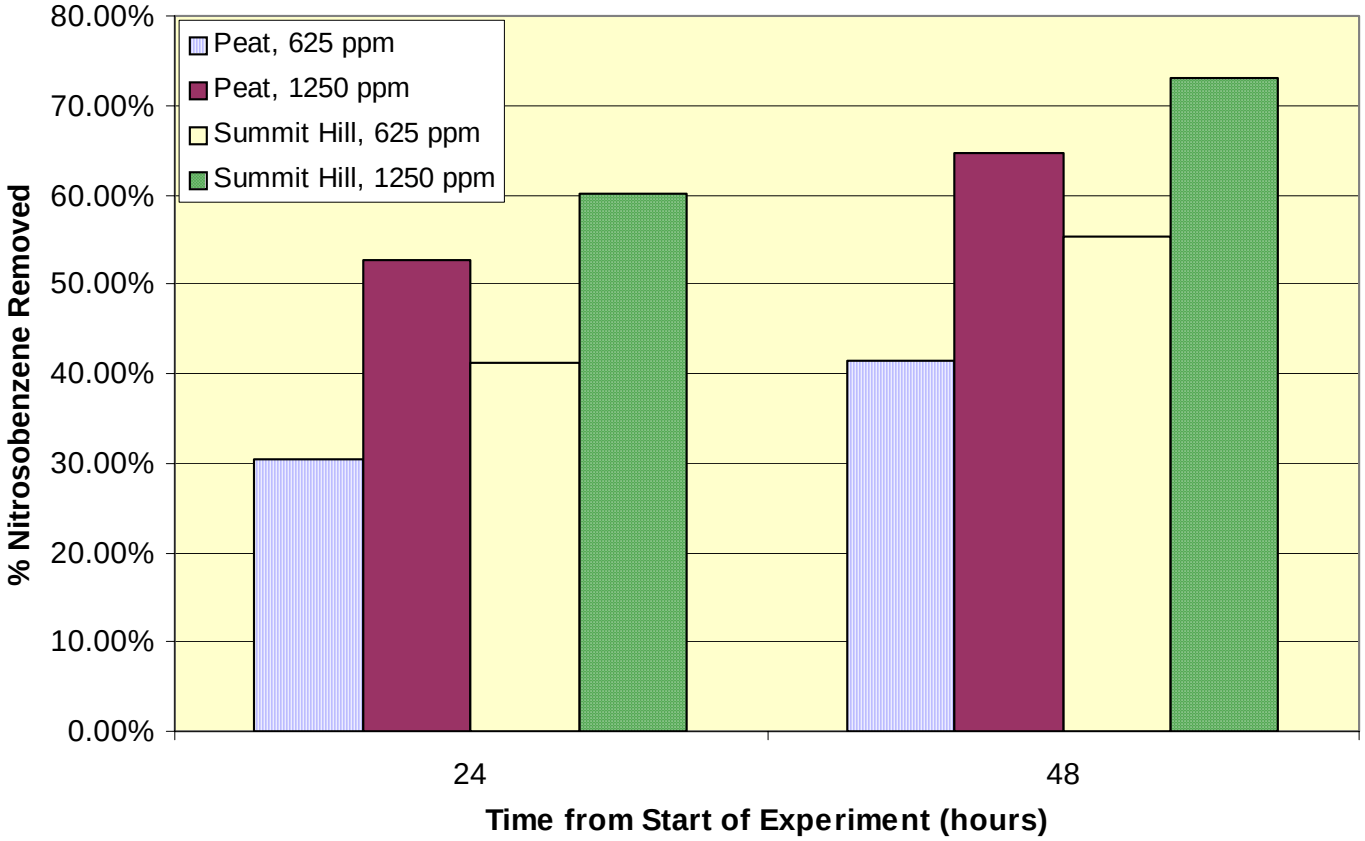


Figure 25. Effect of doubling humic acid concentration on nitrosobenzene removal.

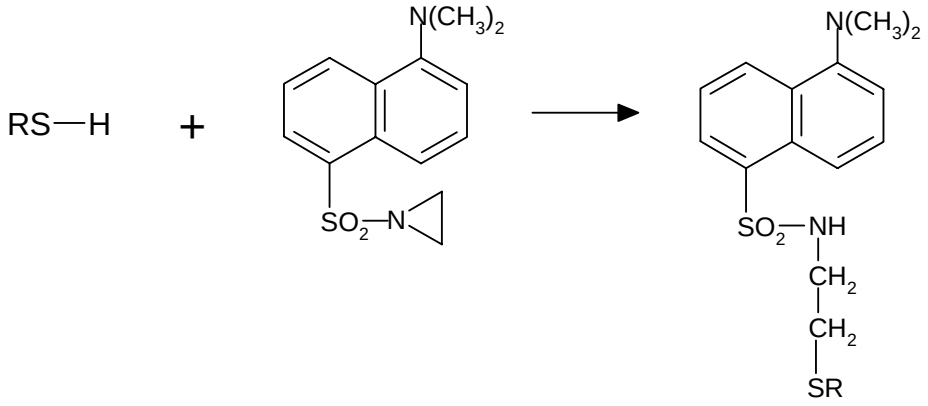


Figure 26. Derivatizing reaction of N-dansylaziridine with sulfhydryls.

4.3 Reactivity of Nitrosobenzene with Thiol Derivatized IHSS Humic Acids

IHSS peat and Summit Hill humic acids were selected for derivatization. Both of these sorbents showed a greater capacity for the removal of aqueous nitrosobenzene than the IHSS leonardite humic acid (Figure 22). N-Dansylaziridine was used as the thiol derivatizing agent. N-Dansylaziridine reacts only with strong nucleophiles such as thiols by forming thioether linkages (Figure 26). Unlike other thiol derivatizing compounds (e.g. N-substituted maleimides), N-dansylaziridine does not react with functionalities having weaker nucleophilic properties such as phenols, alcohols, and amines (117, 118). Figures 27 and 28 illustrate the effects of the thiol derivatization on aqueous nitrosobenzene removal by IHSS peat and Summit Hill humic acids, respectively. Pretreatment of the two humic acids showed an obvious difference in the effectiveness of nitrosobenzene removal, with the change being more prominent for the Summit Hill humic acid. Incidentally, the Summit Hill humic acid had approximately four times the

estimated amino acid concentration than the peat humic acid (Table 7). However, since the cysteine concentrations were not available a value for the thiol concentration in each system could not be accurately determined. The thiol concentration values were inferred from the mass fraction of sulfur and the sum of the concentrations of the 13 amino acids that were analyzed by the IHSS. Based on the estimated protein concentration of the Summit Hill humic acid alone, it appeared that a more drastic effect on nitrosobenzene removal was observed than could be explained by only the blocking of thiols. This observation could be explained by the possibility of reactions involving N-dansylaziridine and strong nucleophiles in the humic acids (other than thiols) that were also binding the electrophilic nitrosobenzene.

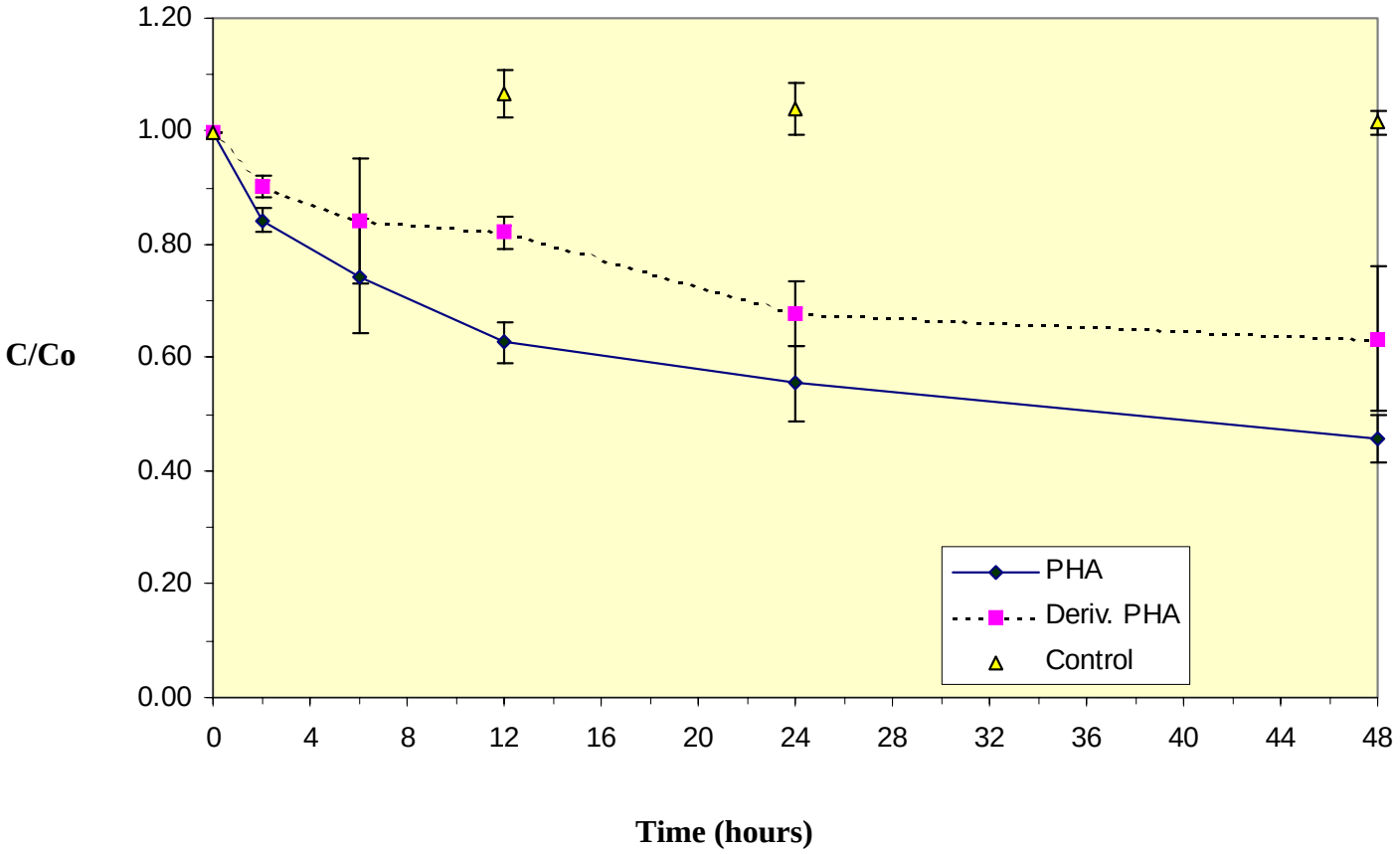


Figure 27. Effect of thiol-derivatization on IHSS peat humic acid's capacity to remove nitrosobenzene from the aqueous phase. Results for non-derivatized (PHA) and derivatized (Deriv. PHA) peat humic acid, as well as a non-humic-acid-containing control are shown.

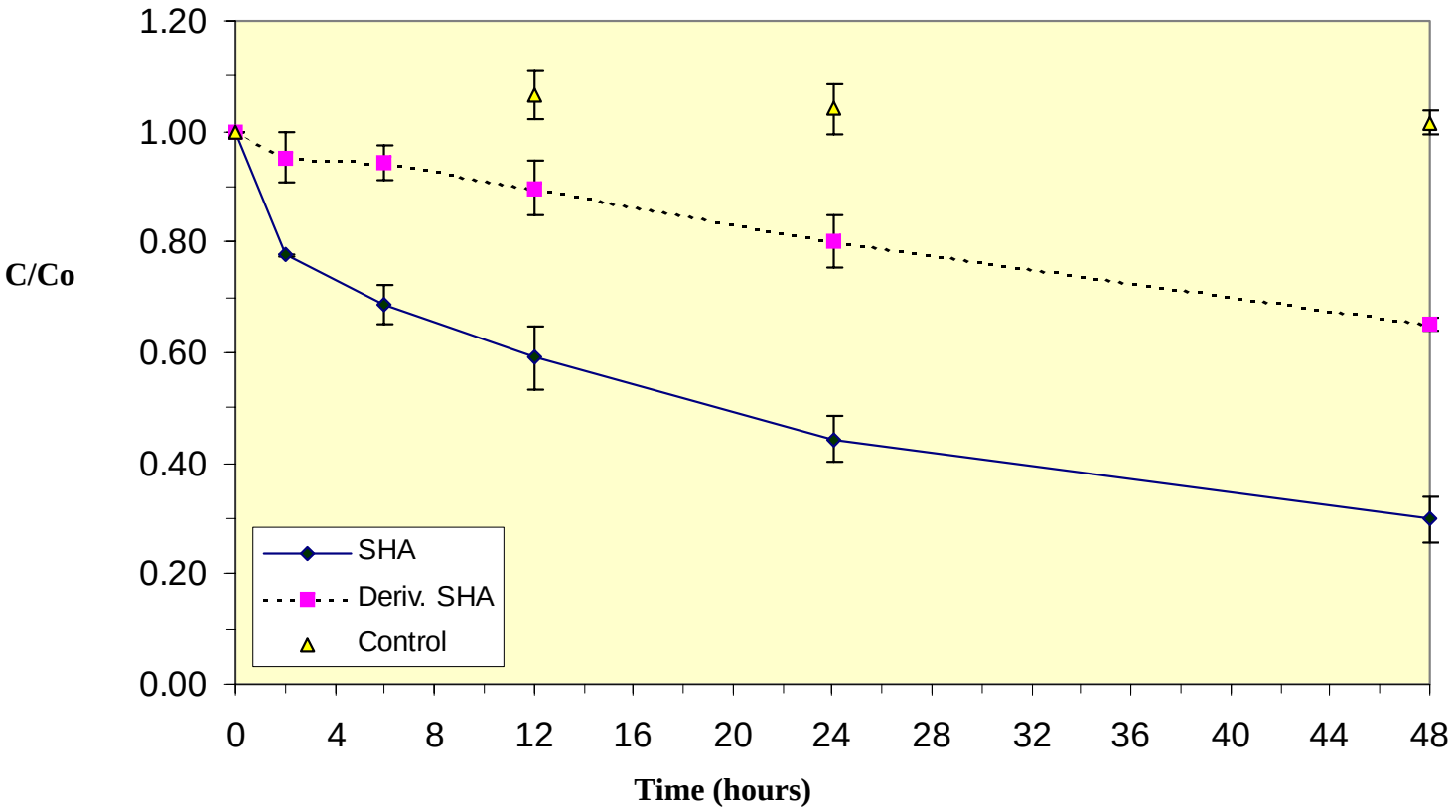


Figure 28. Effect of thiol-derivatization on IHSS Summit Hill humic acid's capacity to remove nitrosobenzene from the aqueous phase. Results for non-derivatized (PHA) and derivatized (Deriv. PHA) Summit Hill humic acid, as well as a non-humic-acid-containing control are shown.

4.4 Covalent Binding of the Potential Nitroso Intermediates of TNT via the Nitroso-Thiol Reaction

Molecular models were created for nitroso intermediates that can potentially form during the reduction of TNT, and were compared to the molecular model of nitrosobenzene. This comparison was performed to compare the molecules' electrophilic properties with nitrosobenzene so that patterns for their reactions with thiol containing systems could be predicted. Pictorial representations of the molecular models for nitrosobenzene, 4NDNT (potential oxidation product of 4HADNT), and 2N4HA6NT (potential oxidation product of DHA6NT) in their lowest energy conformations are shown in Figures 29-31. The figures also show the molecular surface envelope with a color-coded depiction of the surface electrostatic potential (or electron density isosurface) for the three molecules.

The partial charges on the nitroso-nitrogen of nitrosobenzene, 4NDNT, and 2N4HA6NT obtained from the lowest energy optimization were 0.311, 0.325, and 0.320, respectively. These values showed limited utility in predicting the electrophilic nature of the three compounds because of their narrow range. On the other hand, the calculated surface electrostatic potentials as depicted in Figures 29-31 produced some interesting results. This parameter has been shown in literature to be an effective parameter for the prediction of nucleophilic/electrophilic processes (105, 125). Looking at it another way, the surface maps in Figures 29-31 show the locations where the molecules are most susceptible to a nucleophilic attack (yellow>green>light blue>dark blue>pink).

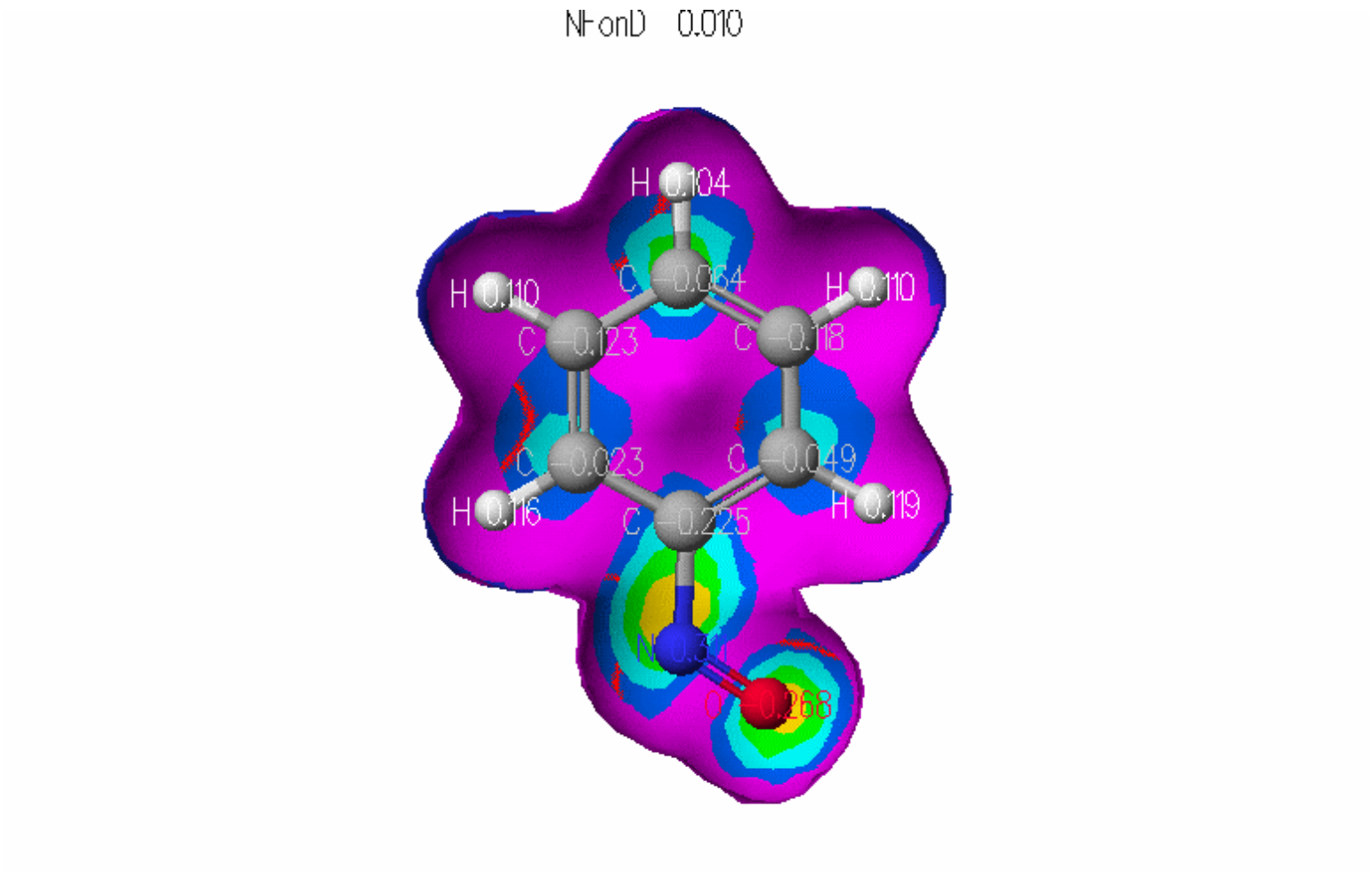


Figure 29. The chemical structure of nitrosobenzene in its lowest energy conformation, surrounded by its electron density isosurface. The colors of the surface depict the molecules susceptibility to nucleophilic attack (yellow>green>light blue>dark blue>pink).

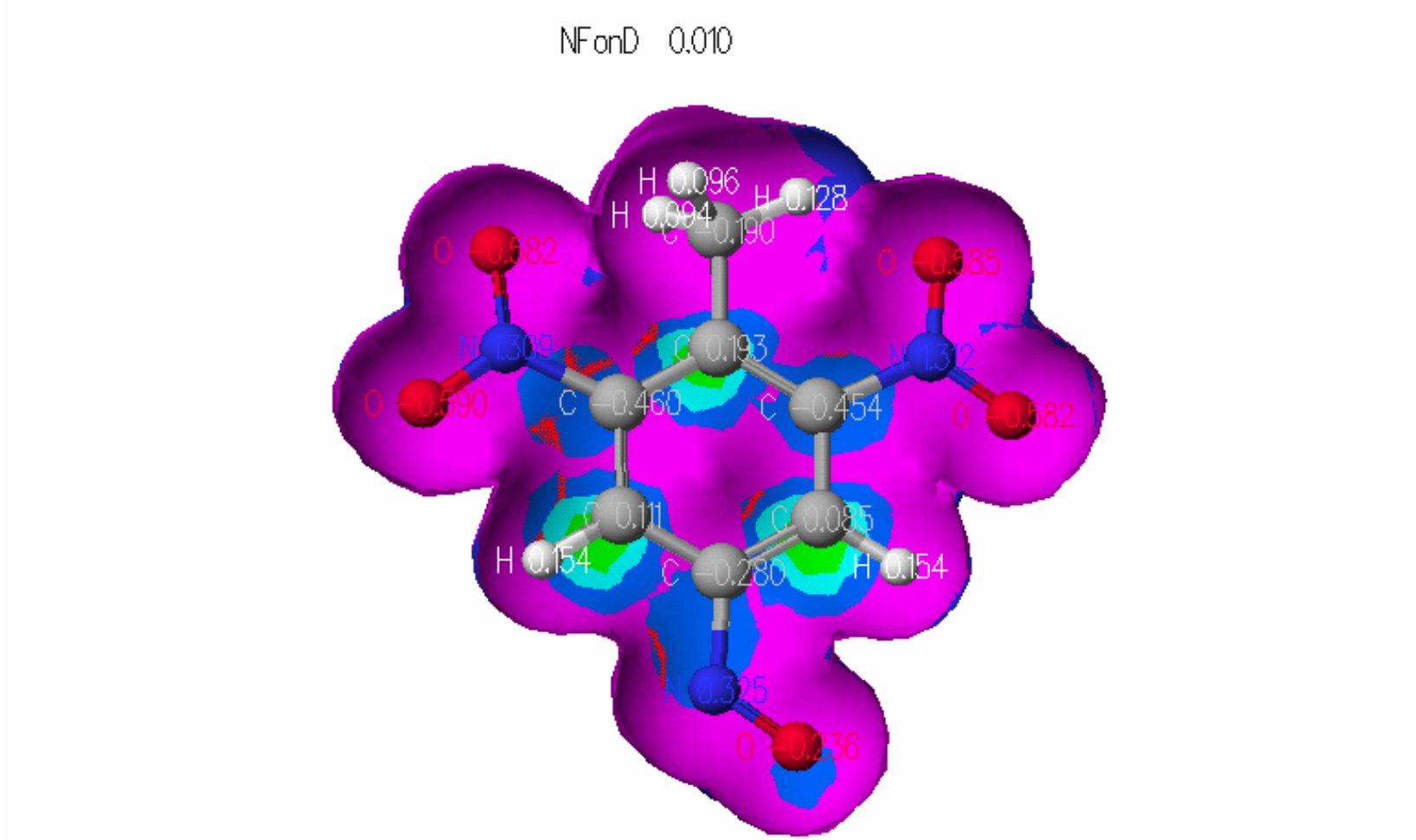


Figure 30. The chemical structure of 4-nitroso-2,6-dinitrotoluene in its lowest energy conformation, surrounded by its electron density isosurface. The colors of the surface depict the molecules susceptibility to nucleophilic attack (yellow>green>light blue>dark blue>pink).

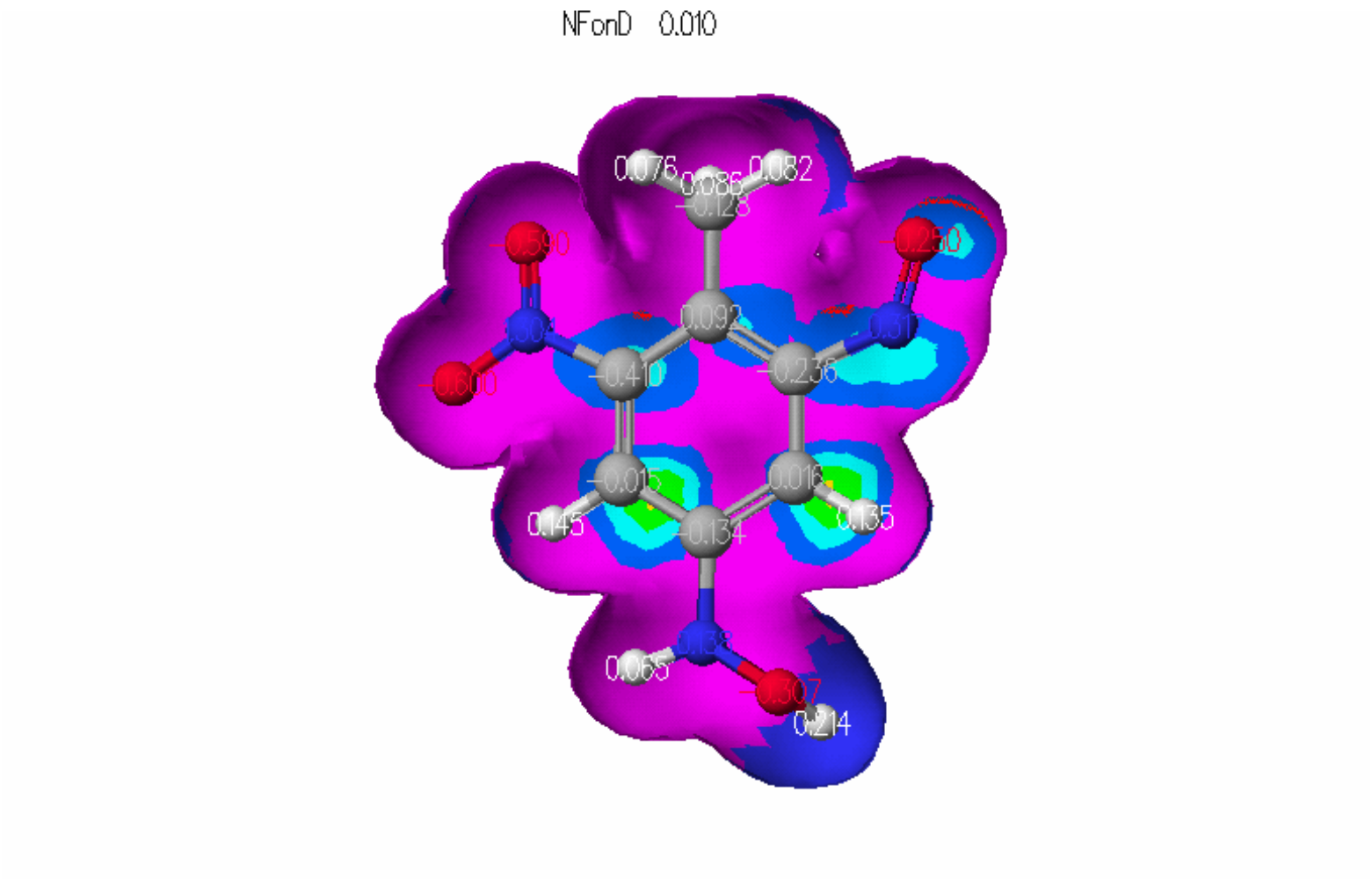


Figure 31. The chemical structure of 2-nitroso-4-hydroxylamino-6-nitrotoluene in its lowest energy conformation, surrounded by its electron density isosurface. The colors of the surface depict the molecules susceptibility to nucleophilic attack (yellow>green>light blue>dark blue>pink).

Comparing the electron density isosurface above the nitroso-nitrogens it becomes clear that the nitroso groups in nitrosobenzene (Figure 29) and 4NDNT (Figure 30) have a very different electrophilic character. The nitroso group in nitrosobenzene is a very strong electrophile, whereas the one in 4NDNT is a much weaker one. The difference in electrophilic character can be explained by the aromatic substituent effects of the two molecules. Nitrosobenzene has no other substituents besides the nitroso group. In contrast, the 4NDNT molecule has two very strong ring deactivating substituents in the form of nitro groups. The effect of these electron-withdrawing nitro groups is also visible in the electron density isosurface above its C3 and C5 ring positions, which show a strong electron deficiency. Moving onto the surface plot of the more reduced 2N4HA6NT molecule (Figure 31), it becomes apparent that the replacement of the nitro group at the C4 position with a hydroxylamino group allows the nitroso nitrogen at the C2 position to display a much stronger electrophilic character than the less reduced 4NDNT molecule. Therefore, it can be concluded that the more reduced hydroxylamino intermediates of TNT are favored to react via the nitroso-thiol reaction.

VI. CONCLUSIONS

The following general conclusions were reached regarding the reactivity of partially reduced metabolites of TNT in a *C. acetobutylicum* cell extract system (a simple bioreduction system):

- Partially reduced metabolites of TNT, namely DHA6NT and 4-amino-6-hydroxylamino-3-methyl-2-nitrophenol, bind to proteinaceous materials following sequential anaerobic/aerobic treatment.
- The more reduced arylhydroxylamino metabolite of TNT, DHA6NT, oxidizes upon exposure (to most likely 2-nitroso-4-hydroxylamino-6-nitrotoluene) and readily reacts with model thiols such as 1-thioglycerol.
- Binding between reduced TNT metabolites and proteins occurs most likely by the nitroso-thiol reaction owing to the aerobic requirements of the reaction.

The following conclusions regarding reactive functionalities and substituent effects were drawn from the studies involving the reaction of partially reduced metabolites of nitroaromatics and various IHSS standard humic acids:

- 4HADNT shows no appreciable reactivity towards a standard humic acid (i.e., IHSS peat humic acid standard) under anaerobic conditions. When conditions are made aerobic at pH 7 the 4HADNT precipitates out of solution in the form of 4,4’,6,6’-tetranitro-2,2’-azoxytoluene.
- Nitrosobenzene readily reacts with various humic acids.
- The extent of nitrosobenzene binding increases as the protein content of the humic acid increases.

- Thiol derivatization of humic acids adversely affects their reaction with nitrosobenzene with greatest effect shown by the humic acid having the highest protein content.
- Molecular modeling analyses predict that the more reduced nitroso metabolites of TNT such as 2-nitroso-4-hydroxylamino-6-nitrotoluene should be closer in electrophilic character and reactivity to nitrosobenzene than the less reduced 4-nitroso-2,6-dinitrotoluene.

VII. ENGINEERING SIGNIFICANCE

In recent years, there has been considerable interest and activity in the fate of TNT in engineered biotransformation systems. A large body of such work suggests that the reduced metabolites of TNT bind to soil NOM and biomass. Yet the majority of published work in this field has concentrated on studying the binding of arylamines in complex and model systems. Consequently, it has largely overlooked the interactions of partially reduced metabolites of TNT with biomass and soil NOM. Much of the work presented here demonstrates that the partially reduced nitroaromatic metabolites are highly reactive towards both biological materials and soil NOM components such as humic acids.

Perhaps, the main reason for ignoring the binding and reactivity of partially reduced metabolites of polynitroaromatics has been the inherent difficulty in working with these highly reactive compounds. For example, arylhydroxylamines are highly unstable under aerobic conditions and all manipulations with these compounds have to be performed under anoxic conditions. Similarly, nitrosoarenes are generally never observed under natural conditions due to their high reactivity, unless the overall effect of their aromatic substituents is strongly ring activating; their presence in natural systems is inferred from the formation of azoxy compounds. By contrast, the arylamine intermediates of nitroaromatics are highly stable and are commonly used in model binding studies. As researchers have pointed out, these compounds bind to quinoid moieties in natural systems by a variety of catalytic and noncatalytic mechanisms. However, in purely biological systems arylamines undergo N-oxidation prior to reactions

with biological molecules, a fact known by toxicologists by as early as 1913 (57). Binding reactions in biological systems can be mediated by enzymatically-catalyzed activation reactions of arylhydroxylamines (e.g., acylation or sulfation), or can be spontaneous such as the reaction of nitrosoarenes with thiols. Hence, one is left with no choice but to work with the specific arylhydroxylamine and nitrosoarene intermediates of the target nitroaromatic contaminant, or to use suitable surrogates with these reactive functionalities if one is to study the binding to biomass in natural and engineered systems.

The findings presented here could be of important relevance to two types of nitroaromatic bioremediation research reported in literature. First, researchers reporting the formation of only arylamine intermediates together with spontaneous binding to biomass upon aeration might be well advised to search for partially reduced metabolites in their systems. These compounds could either be formed in their organisms' metabolic pathway or could result via common biologically-mediated oxidizing reactions in soil (36). Secondly, researchers reporting extensive binding in weakly reducing and high biomass containing biological processes such as composting might be well advised to quantify the contribution of biomass to binding. The anaerobic/aerobic nature and the high concentration of biomass present in such systems could make the nitroso-thiol reaction the dominant irreversible binding mechanism.

VIII. FUTURE WORK

Future research related to this work should focus on developing assays to predict the potential for reaction of nitroso compounds to strong nucleophiles such as thiols in any given system. One way to proceed with this objective would be to adapt and optimize the thiol derivatization procedure employed in this work; N-dansylaziridine forms fluorogenic derivatives of thiols that can be detected to very low concentrations. A small solid phase sample from a bioremediation process could be subjected to an N-dansylaziridine assay followed by fluorescence detection to characterize the potential binding to thiols.

A line of research relating to the oxidation of arylamine intermediates of nitroaromatic compounds by common phenoloxidase soil enzymes could also be pursued. The focus of this line of research should be to determine whether N-oxidation of arylamines to arylhydroxylamines could occur with soil enzymes. Off course, model reactions in humus-free systems would have to be used in order to avoid reactions of arylamines with quinones.

Another interesting line of research relates to the disparity in the types of metabolites found in different anaerobic TNT biotransformation systems. Some of these systems yield only arylhydroxylamine intermediates, whereas others show the presence of only arylamine intermediates. The midpoint redox potentials for the formation of various aminated intermediates of TNT have been experimentally determined (113). The values obtained for some of the more reduced intermediates (e.g., 2,4-diamino-6-

nitrotoluene, E'_o = -502 mV) appear to be substantially lower than those measured in mixed culture systems or those theoretically possible (e.g., hydrogenase) with pure systems. In addition, the formation of 2,4,6-triaminotoluene has yet to be proven conclusively. A line of research to establish a link between the two pathways can be of vital interest to this field. A good starting point would be the determination of the azoxy compounds' role in reducing systems. Azoxy compounds form by a condensation reaction between hydroxylamino and nitroso functional groups. They are typically incompletely quantified due to their aqueous insolubility even though they often appear as transient intermediates in TNT biotransformation studies. Electrochemically, the azoxy linkage can be easily reduced to an azo linkage, followed by the formation of arylamines.

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Reactivity of Partially Reduced Arylhydroxylamine and Nitrosoarene Metabolites of 2,4,6-Trinitrotoluene (TNT) toward Biomass and Humic Acids

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Sequential anaerobic/aerobic treatment of 2,4,6-trinitrotoluene (TNT) generally results in the incorporation of residues into biomass and natural organic matter fractions of a system. To better understand the potential contribution of hydroxylamine and nitroso moieties in these reactions, studies were conducted using model systems taking advantage of the biocatalytic-activity of *Clostridium acetobutylicum* that does not produce aminated TNT derivatives. To evaluate binding to biomass only, systems containing cell-free extracts of *C. acetobutylicum* and molecular hydrogen as a reductant were employed. At the end of treatment, mass balance studies showed that 10% of the total ^{14}C was associated with an insoluble protein-containing precipitate that could not be extracted with organic solvents. Model reactions were conducted between a mixture of 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 1-thioglycerol to test the involvement of the nitroso-thiol reaction in binding to biomass. It was demonstrated that DHA6NT formed a new and relatively polar product with 1-thioglycerol only in the presence of oxygen. The oxygen requirement confirmed that the nitroso functionality was responsible for the binding reaction. The reactivity of arylhydroxylamino and nitrosoarene functionalities toward International Humic Substance Society (IHSS) peat humic acid was evaluated under anaerobic and aerobic conditions, respectively. 4HADNT showed no appreciable reactivity toward peat humic acid. Conversely, the nitrosoarene compound, nitrosobenzene, showed rapid reactivity with peat humic acid (50% removal in 48 h). When tested with two other humic acids (selected on the basis of their protein content), it became apparent that the proteinaceous fraction was responsible at least in part for the nitrosoarene's removal from solution. Furthermore, the pretreatment of the humic acids with a selective thiol derivatizing agent had a considerable effect on their ability to react with nitrosobenzene. Finally, molecular modeling tools were used to compare the electrophilic characteristics of potential nitroso intermediates forming from the oxidation of arylhydroxylamino metabolites of TNT. Molecular modeling analysis demonstrated that the more reduced TNT derivative containing nitroso groups were more likely to react with nucleophiles in humic substances than the less reduced nitroso intermediates.

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Introduction

Over the years, numerous bench- and field-scale biotransformation studies conducted with polynitroaromatic contaminants, particularly 2,4,6-trinitrotoluene, have resulted in a disparity in the molar balance between the parent compound and identifiable reduced products (*1, 2*). The use of ^{14}C and ^{15}N radiolabeled nitroaromatic compounds has demonstrated this discrepancy mostly results from the binding of reduced metabolites to the solid matrix. Further investigations to determine the identity of the solid matrix fraction binding the TNT metabolites have pointed in the direction of biomass (*3*−*7*) and natural organic matter (NOM) (*3, 6, 8*−*11*). The observed binding has been deemed "irreversible" or covalent because a variety of extraction techniques with common organic solvents have yielded poor recoveries of the reduced metabolites (*8, 9*). Studies with radiolabeled TNT focusing on the NOM binding have shown the radiolabel to be associated predominantly with the humin and humic acid fractions (*10*−*13*). In recent studies, the existence of covalent linkages has been confirmed using ^{15}N NMR spectroscopy (*12*−*14*). The functional group (on the metabolite) that is typically held responsible for such reactions is the aromatic amino group, primarily because of evidence regarding its fate in model systems. From model studies, three pathways for the irreversible binding of aromatic amines to soil NOM have been elucidated: the nonenzymatic 1,4-nucleophilic or Michael addition of amino groups to quinoid rings of humic acid constituents (*15*−*17*); phenol oxidase (e.g., laccase)-mediated free radical addition reactions (*1, 18*); and metal catalyzed reactions between aromatic amines and humic substances (*19*).

Unfortunately, little is known about the role of nitroso and hydroxylamino intermediates generated from partial reduction of TNT, despite their recognized reactivity. Several nitroaromatic biotransformations conducted with pure cultures of common soil anaerobic microorganisms have demonstrated an inability to produce the completely reduced amine intermediates (*20*−*24*). Instead, these studies demonstrate the formation of only partially reduced hydroxylamino intermediates. Model studies, such as those conducted with arylamines, have not been attempted with nitrosoarene and arylhydroxylamine compounds in environmental systems. This is largely due to the instability of such compounds and to the difficulty of the synthesis of their standards. A recent study has implied that the reactive hydroxylamino groups of partially reduced TNT metabolites are responsible for initiating an abiotic covalent binding to humic acids under anaerobic conditions (*25*). Questions that remain unanswered at the current time regarding the binding of partially reduced TNT metabolites include the following: (1) specifically, which functional group or groups on TNT metabolites are responsible for initiating the covalent binding; (2) which specific chemical functional group(s) or site(s) on the sorbent organic matter participates in the binding reaction; (3) what is the mechanism of the chemical binding reaction; and (4) what external environmental (or system) parameters/conditions control the binding reaction.

Recent studies focusing on the conditions necessary to increase the extent of binding to NOM have indicated two

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important factors. These are the degree of reduction achieved in the initial anaerobic phase and the need for an aerobic stage following the initial anaerobic reduction phase (*8*−*10*). These findings are believed to strengthen the evidence for the nucleophilic addition mechanism because the greater reduction achieved in the anaerobic phase leads to greater nucleophilic character of the amino nitrogen produced. Furthermore, the aerobic stage requirement allows for the reoxidation/tautomerization of the hydroquinone back to the quinone, thereby promoting further nucleophilic additions possibly leading to the formation of nitrogen heterocycles (*15*, *19*). However, this mechanism does not adequately address the extensive binding observed in purely biological systems (*26*, *27*) or the binding occurring to biomass in engineered remediation systems (*3*, *4*, *6*, *7*). First of all, quinones make up the structure of electron-transfer proteins and oxidoreductases in biological systems, both of which are present in limited quantities in a microorganism (*28*). Second, studies that involve the recovery of humic substance fractions from soil NOM to establish a mass balance for the parent radiolabel, overlook the presence of coextracted and coprecipitated molecules (*29*−*31*). Soil NOM not only contains humic substances but also has two other major components: namely, decomposable organic residues from plant and animal decay and the heterotrophic organisms that feed on the organic residue (*30*, *32*). Since the various humic substance fractions (namely, fulvic acids, humic acids, and humin) are operationally defined on the basis of their aqueous solubility with respect to solution pH (i.e., following an alkaline extraction), they often contain varying degrees of biomolecular contaminants such as denatured proteins and carbohydrates that originate from the parent soil NOM (*29*). This point is further supported by evidence that the greatest degree of binding of reduced nitroaromatics occurs to the humin fraction (i.e., fraction insoluble at all pH) of soil NOM (*12*, *13*); structurally, humin is considered to be an aggregation of various organic and inorganic molecules that include humic acids and biomolecules (*33*).

In this study we report the reactivity of arylhydroxylamino and nitrosoarene functionalities toward biomass and humic acids in model environmental systems. The reactivity toward biomass is studied under sequential anaerobic-aerobic conditions in a "low-activity" and cell-free *Clostridium acetobutylicum* cell extract/H_2 (electron donor) model system. In this portion of the work, the ^{14}C distribution was examined in the context of the protein distribution in the reaction mixture. In addition, reactions of the partially reduced TNT metabolites with model thiols were conducted under different conditions to test possible mechanisms for the binding to biomass. The reactivity toward humic acids was investigated in model systems with International Humic Substance Society (IHSS) peat humic acid under anaerobic and aerobic conditions, respectively. Peat humic acid was selected because peaty soils tend to be water logged and, subsequently, their humic acids contain high concentrations of biological molecules due to a lack of aerobic decay (*34*). 4-Hydroxylamino-2,6-dinitrotoluene (4HADNT), a common metabolite found in the reduction pathway of TNT, was used as the model arylhydroxylamine. One of the only two relatively stable and commercially available C-nitrosoarenes, namely nitrosobenzene, was used as the model nitroso compound. Using nitrosobenzene, we explored the dependence of the nitroso group's reactivity on the proteinaceous content of humic acid by attempting to restrict the binding contribution from strong nucleophiles such as thiols by pretreating the humic acids with a selective thiol derivatizing agent. Finally, we employed molecular modeling tools to compare the electrophilic nature of unstable nitroso intermediates of TNT to determine whether the binding behavior of these intermediates via nucleophilic substitution reactions.

Background Reactions

Although limited and sometimes conflicting information regarding the reactivity of partially reduced metabolites of TNT can be found in environmental literature, publications exist in chemistry and toxicology literature that review the various reactions of related functionalities (i.e., aryl-hydroxylamino and nitroso). These include publications on reactions with thiols (*35*, *36*), rearrangement reactions (*37*, *38*), and various enzyme-catalyzed reactions (*39*).

Figure 1 presents a summary of the reactions of arylhydroxylamino and nitrosoarene functionalities reported in the literature. Generally speaking, the nitrosoarene functionality undergoes reactions that are mostly spontaneous, whereas the arylhydroxylamino functionality requires catalysis to cleave the N−O bond to undergo further reaction. The nitroso and hydroxylamino functional groups exist in what can be considered a "pseudo redox equilibrium" due to the insignificant activation energy barrier for their interconversion (*39*). As a consequence, reductive conditions favor arylhydroxylamine formation, whereas oxidative conditions favor nitrosoarene formation. Although the presence of arylhydroxylamine reduction intermediates of nitroaromatic compounds is frequently reported in anaerobic remediation systems (*2*, *5*, *22*, *24*, *25*, *40*, *41*), the detection of nitrosoarenes is a rarity in natural and engineered systems, especially when arylhydroxylamines are also present. The presence of arylhydroxylamines in any system results in the scavenging of the nitrosoarenes to form relatively insoluble azoxy compounds via a rapid condensation reaction (*39*, *42*, *43*). Hence, the production of nitroso compounds in a system is typically inferred from the presence of the commonly detected and relatively insoluble azoxy compounds.

Experimental Section

Chemicals. The following chemicals were used in this study: 2,4,6-trinitrotoluene, 99% purity (ChemService, Westchester, PA); [U-ring-^{14}C]-2,4,6-trinitrotoluene, specific activity of 21.6 mCi/mmole, 99.5% purity (Chemsyn Science, Lenexa, KS); 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-4,6-dinitrotoluene (4HADNT), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 2,4′,6,6′-tetranitro-2′,4-azoxytoluene, and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (Ron Spanggord, SRI International, Menlo Park, CA); 0.1 mg/mL analytical standard of 4HADNT in acetonitrile (AccuStandard Inc., New Haven, CT); 3-mercapto-1,2-propanediol (1-thioglycerol), 95% purity, and nitrosobenzene, 97% purity (Aldrich, Milwaukee, WI); EDTA (J. T. Baker, Phillipsburg, NJ); biotech grade bovine serum albumin lyophilized powder (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ); protein assay dye reagent concentrate (Bio-Rad, Hercules, CA); monobasic and dibasic potassium phosphate (Sigma, St. Louis, MO); HPLC grade acetonitrile, methanol, methylene chloride, *n*-pentane, 1 N HCl solution, 1 N NaOH solution, ScintiSafe Plus 50% cocktail (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ); carbon-14 Cocktail for R. J. Harvey biooxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ); monobasic and dibasic potassium phosphate, and 90% pure N-dansylaziridine (Sigma, St. Louis, MO); IHSS peat humic acid standard, leonardite humic acid standard, and Summit Hill reference humic acid (Paul Bloom, University of Minnesota, St. Paul, MN).

Preparation of Cell Extract. Crude cell extracts of *Clostridium acetobutylicum* ATCC 824 were prepared using the lysozyme/sonication procedure as described previously (*2*). However, one step was added to remove larger structural proteins by ultracentrifugation at 45 000*g*. Also, the anaerobically sealed cell extract vials were stored at −20 °C. Two batches of cell extract were produced. The first batch was kept in storage at −20 °C for a long duration (i.e., 6 months)

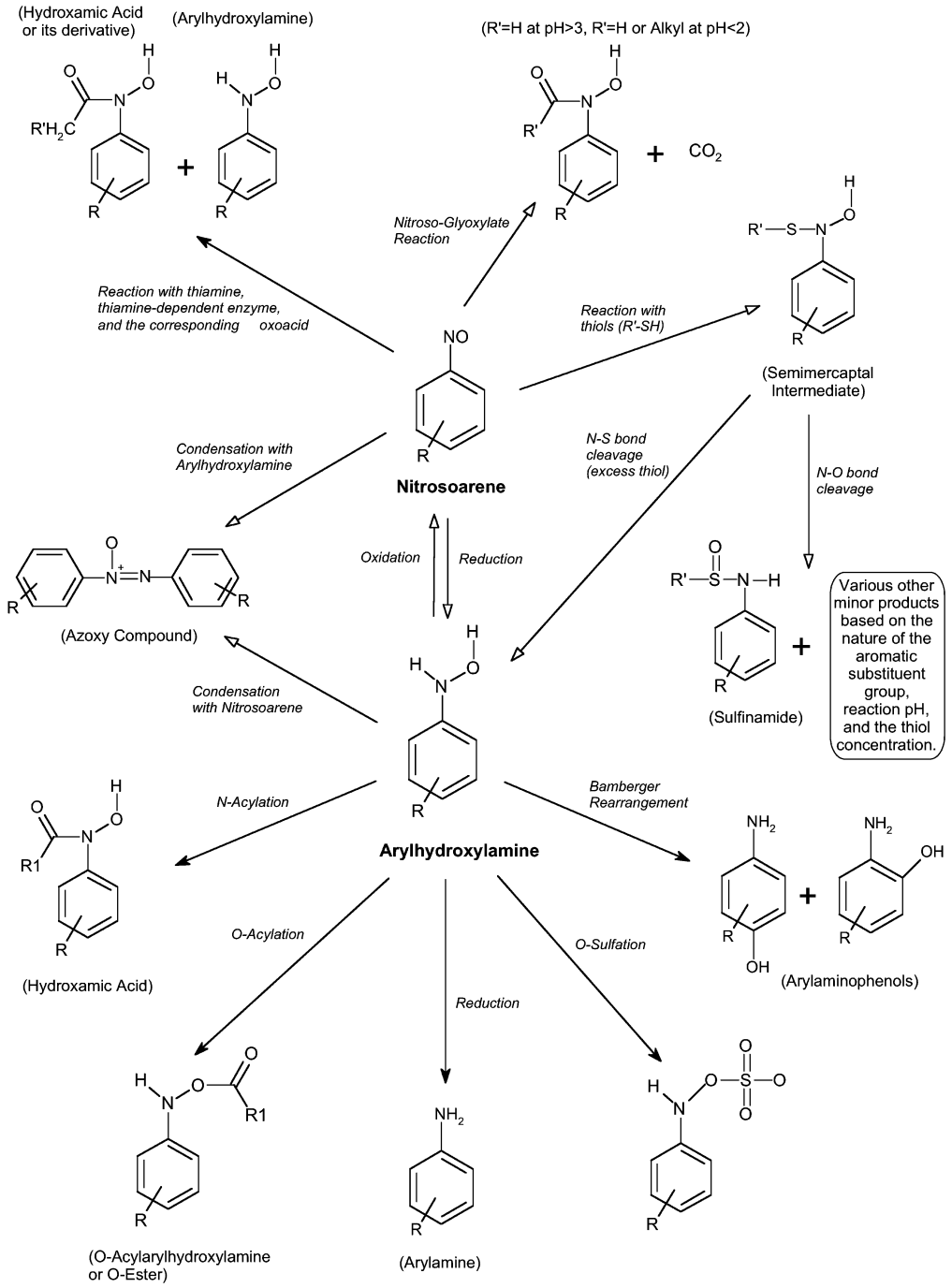

FIGURE 1. Reactions of arylhydroxylamines and nitrosoarenes reported in the literature. Reactions of arylhydroxylamines involving the heterolytic cleavage of the N−O bond (i.e., reduction, O-acylation, O-sulfation, Bamberger Rearrangement, and N-acylation) generally require catalysis. All reactions of nitrosoarenes are of a spontaneous nature, with the exception of reactions involving thiamine-dependent enzymes. Note that reactions requiring catalysis are depicted with solid arrowheads and spontaneous reactions are depicted with clear arrowheads.

prior to experimentation and had a very low TNT transformation activity (0.33 mg TNT/volume% cell extract/minute). The loss in enzyme activity in cell extracts of gram positive organisms can be substantial over long periods of time due to proteolytic degradation, even at storage temperatures of −20 °C (*44*). The low activity extract was used in the first transformation reaction in order to determine the fate of the radiolabeled TNT following anaerobic-aerobic treatment.

The second batch was prepared similarly; however, its storage time was considerably less (approximately 1 week). This extract was used to rapidly convert TNT to predominantly 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) for the reaction with the model thiol compound. The activity of this extract could not be measured with the TNT depletion assay, as TNT could not be measured within 2 min of cell extract addition (based on initial conditions the activity was >3.1 mg TNT/volume% cell extract/minute).

Analytical Methods. Temporal samples for TNT biotransformation reactions as well as thiol reaction samples were monitored using the Waters (Milford, MA) HPLC system. The system consisted of 2690 separations module, 996 photodiode array detector, and a Nova-Pak C8 analytical column (3.9 × 150 mm) and guard column assembly. The method used for all HPLC analysis was an acetonitrile/water

gradient method with a linear ramp from 35% to 75% acetonitrile in 12 min followed by a return to the original conditions in 6 min. Two TNT metabolites were identified based on UV spectra and relative retention times based on earlier work (*2*, *22*). These metabolites included 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) and the major aminophenol product resulting from the Bamberger rearrangement of DHA6NT.

Temporal samples for the reaction of humic acids with 4HADNT and nitrosobenzene were also monitored using the Waters (Milford, MA) HPLC system described earlier. The HPLC method used for analysis was an acetonitrile/water gradient method with a linear ramp from 40% to 70% acetonitrile in 10 min followed by a return to the original conditions in 4 min. 4HADNT experiments were monitored at a wavelength of 220 nm, whereas nitrosobenzene experiments were monitored at a wavelength of 305 nm. A nonpolar gradient method was used to analyze the dissolved precipitate produced in the aerobic 4HADNT/peat humic acid experiment. This method involved a linear ramp of acetonitrile from 50% to 100% in 12 min, followed by a 100% plateau for 1 min and a recovery to original conditions in 5 min. The mobile phase flow rate for all HPLC methods was 0.5 mL/min.

TNT Transformation. Both cell-extract-free transformation reaction solutions were degassed with nitrogen for 30 min followed by molecular hydrogen for 30 min. The cell extract was added after 30 min of hydrogen addition to mark the start of the anaerobic reaction phase. Temporal bulk aqueous samples were collected under gas purge (molecular hydrogen for anaerobic phase, air for aerobic phase, and nitrogen when anaerobic conditions were to be maintained without further reaction). The samples were analyzed for ^{14}C content by scintillation counting and for product formation by HPLC.

The starting conditions for the first TNT transformation reaction were 865 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 100 mg/L. The anaerobic phase or molecular hydrogen bubbling was continued for 22.5 h (1350 min), after which it was replaced with air for a period of 1 h. At the end of the anaerobic phase the reaction mixture consisted of DHA6NT and aminophenols (*2*) as the only identifiable products.

The initial conditions for the second reaction were 1493 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 50 mg/L. This reaction was carried out in 10 mM (pH 7) phosphate buffer. Temporal samples were collected after 20 and 40 min of hydrogen addition and analyzed by HPLC. Note that a trial reaction was run earlier to determine cell extract activity on the basis of TNT depletion. Fractions were also collected (one fraction/2 min) for the 40-min sample using a 100 μL injection. After 40 min of hydrogen addition all of the parent TNT had been transformed to DHA6NT with only traces of 4HADNT remaining. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas.

^{14}C-Radiolabel Recovery and Protein Quantitation. At the end of the first transformation reaction (sequential anaerobic-aerobic conditions), a clear solution (i.e., without any visible precipitate) was decanted from the reaction vessel. Three milliliters of this solution was passed through a Gellman Sciences 0.2 micron PTFE filter. Both the filtrate and the prefiltered clear solution were subjected to liquid scintillation counting using ScintiSafe Plus 50% cocktail (10 mL cocktail/1 mL sample) on a Beckman LS6500 scintillation counter. Protein quantitation was also performed on both samples by the Bio-Rad protein assay (adapted from the Bradford Assay) which uses the Coomassie brilliant blue G-250 dye. Serial dilutions of a 2 mg/mL solution of bovine serum albumin were used with a 1:5 dilution of the dye reagent concentrate to generate a standard curve for the protein quantitation.

The long strands of the brown floc-like precipitate remaining in the reaction solution after decantation were removed using a sterile spatula. The collected precipitate was processed for ^{14}C analysis by combusting it at 900 °C in the R. J. Harvey Instrument Corporation Biooxidizer Model OX600, trapping the radiolabeled CO_2 produced in the Carbon-14 Cocktail and counting it by scintillation counting. A small fraction (0.12 gm wet weight) was removed to conduct protein analysis and solubility testing. Acid hydrolysis was performed on half of this fraction by first placing it in a microcentrifuge tube with DDI water (500 μL) and vortexing it for 30 s. Following the mixing, the tube was centrifuged (13 000 rpm for 5 min), and the supernatant solution above the pellet was discarded after the centrifugation. HCl (500 microliters at pH 2) was added to the pellet, and the tube was vortexed for 30 s to mix its contents, followed by centrifugation (13 000 rpm for 5 min). The acidic solution above the pellet was used for protein analysis. Solubility testing was carried out with the remaining precipitate by vortexing the precipitate with *n*-pentane and methylene chloride.

Reaction with 1-Thioglycerol. Molecular hydrogen addition for the second TNT transformation reaction conducted with the higher activity cell extract (concentration of 8% v/v) in phosphate buffer (10 mM, pH 7) was stopped after 40 min. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas. The reaction was monitored by HPLC (100 μL injection), and 2-min fractions were collected for the 18-min HPLC method. Following sampling, 1-thioglycerol stock in acetonitrile (6 μL of 200 mM) was added to the still anaerobic reaction mixture to yield a molar ratio of thiol groups to potential nitroso groups (calculated on the basis of initial TNT concentration) of 1:2. This mixture was then allowed to react under anaerobic conditions for 20 min. A sample was collected from the reaction mixture under the nitrogen purge at the 20-min anaerobic reaction time mark and immediately analyzed by HPLC. Injection and fraction collection were conducted as earlier. The reaction mixture with thiols was then exposed to air, and a 20-min sample was collected and analyzed by HPLC with fraction collection performed as earlier.

Reactions with Humic Acids. All reactions were carried out in triplicate and in the dark in 10 mM phosphate buffer (pH of 7) with continuous stirring. 4HADNT was chosen as the model arylhydroxylamine compound and nitrosobenzene was chosen as the model nitrosoarene compound. Experiments were designed to keep a low sorbate (e.g., 4HADNTor nitrosobenzene) to sorbent (humic acid) mass ratio at initial conditions in order to better estimate potential second-order reactions with pseudo-first-order kinetics (*45*, *46*). The initial sorbate to sorbent mass ratio for 4HADNT experiments was approximately 0.02 and for nitrosobenzene experiments was approximately 0.01; the difference in the ratio between the two compounds reflects the approximately 2:1 ratio of molecular weights for 4HADNT:nitrosobenzene even though these molecules have potentially one mole of reactive groups per mole of either compound. The humic acid concentration was fixed at 625 mg/L. This concentration reflects a less than 10-fold increase (on the basis of carbon concentration) from maximum dissolved organic material concentrations of 50 mg carbon/L observed in nature (*47*). As in past studies (*19*, *47*), the higher than natural concentration of humic acid was selected to bring about an appreciable change in aqueous sorbate concentration over a reasonable period of time, which in this case was 48 h.

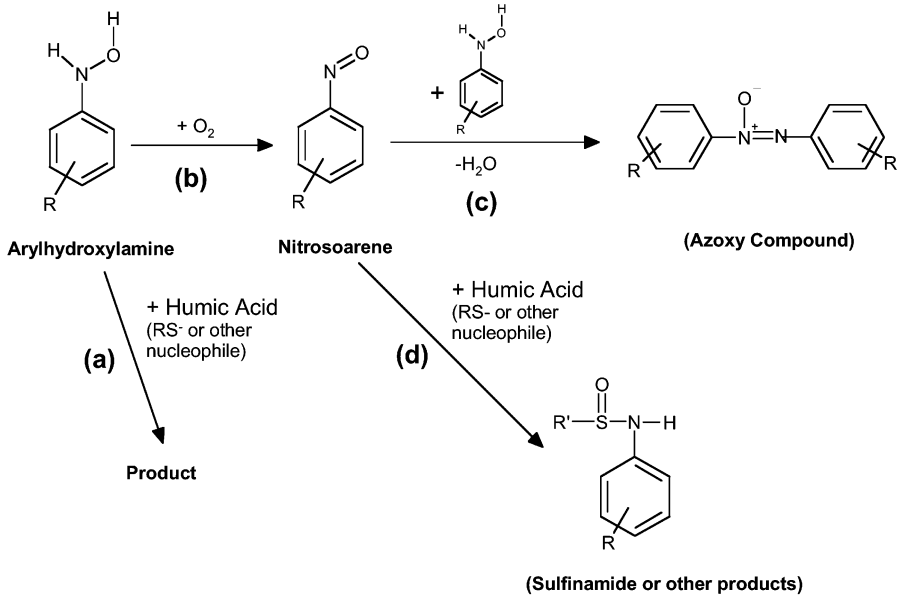

FIGURE 2. Reactions attempted during the course of this study. Note that arylhydroxylamines are stable only under anoxic conditions.

The reaction between 4HADNT and IHSS peat humic acid (Figure 2, reaction (a)), together with a control containing no sorbent, was conducted under anaerobic conditions. Freshly prepared 4HADNT stock in acetonitrile was added to vials containing degassed (sparged 30 min with Helium followed by 30 min with Nitrogen) buffer/humic acid solution to start the reaction. Temporal samples were collected under a nitrogen purge and passed through Accell Plus QMA anion exchange cartridges (Waters, Milford, MA) into HPLC vials that had been flushed with nitrogen. A needle attached to a disposable check valve (Cole-Parmer, Vernon Hills, IL) was used to allow the nitrogen displaced by the sample to exit the HPLC vials. Samples were analyzed by HPLC immediately upon collection. An additional reaction was run with 4HADNT and IHSS peat humic acid. This reaction was conducted with the same precautions as the earlier anaerobic reaction; however, the reaction mixture was exposed to the atmosphere in order to assess the ability of the humic acid to compete for the nitroso oxidation product of 4HADNT (Figure 2, reaction (b) followed by reactions (c) and (d) in parallel). The precipitate formed in this reaction was extracted using methylene chloride, followed by air-drying, redissolution in a 50/50 (v/v) acetonitrile/buffer mixture, and analysis by RP-HPLC/UV−visible detection.

Reactions between nitrosobenzene and various humic acids (Figure 2, reaction (d)) were conducted in a closed aerobic system (i.e., no prior degassing) due to the mild volatility/sublimation characteristics of nitrosobenzene. As before, temporal samples were collected and passed through Accell Plus QMA cartridges to remove humic acid and placed in HPLC vials for analysis. During sampling, creation of a vacuum in the reaction bottle was avoided by again using a needle/disposable-check-valve assembly to allow air in to replace the sample volume removed (note that the gas flow to equilibrate pressure was in the opposite direction when compared to Figure 2, reaction (a)). Additionally, controls with no sorbent were run for the experiment.

Thiol Derivatization and Recovery of Humic Acids. The thiol content of IHSS peat and Summit Hill humic acids was derivatized by adapting a thiol derivatizing procedure presented elsewhere (*48*−*51*). This procedure utilized N-dansylaziridine, a thiol derivatizing agent. A ratio of humic acid (10 mg) in 2.5 mL of derivatization buffer (67 mM phosphate/0.2 mM EDTA, pH = 8.2) to 0.25 mL of a solution of N-dansylaziridine in methanol (5 mg/mL) was used. The derivatization mixture was placed in a crimped top serum bottle and suspended in a water bath (60 °C) for 1 h. The bottle was then removed from the water bath, the top was removed, and its contents were allowed to cool under stirring for another hour. The cooled mixture was transferred into 500 Dalton (Molecular Weight Cutoff [MWCO]) cellulose ester dialysis membranes that were sealed using dialysis clips. The sealed membranes were placed in aluminum foil pouches containing 10 gm of Spectra/Gel absorbent powder to dewater membrane contents overnight (Note: all dialysis equipment and dewatering absorbents obtained from Spectrum Laboratories, Laguna Hills, CA). After overnight dewatering/volume reduction the contents were transferred into 500 Dalton MWCO cellulose ester Spectra/Por Dispodialyzers. The dispodialyzers were suspended in a DDI water reservoir for approximately 12 h. During this step the reservoir water was replaced with freshwater at least twice. The dispodialyzer contents were dewatered as before using the absorbent powder; however, this time they were left in the absorbent bed for only 6−8 h owing to their substantially higher surface area-to-volume ratio. The contents of the dewatered dispodialyzers were removed using a thin disposable pipet and transferred into preweighed aluminum weighing dishes. The weighing dishes were placed into a 60 °C oven for 6 h, the time used to reach a constant weight using this procedure. After 6 h the dishes were removed from the oven and allowed to cool in a desiccator overnight. The cooled dishes were weighed to calculate the weight of their contents (i.e., derivatized humic acid). The derivatized humic acid was ground using a pestle and mortar, transferred to a labeled vial, and stored in a desiccator.

Molecular Modeling. Chemical structures for nitrosobenzene, 4-nitroso-2,6-dinitrotoluene (4NDNT, potential oxidation product of 4HADNT), and 2-nitroso-4-hydroxylamino-6-nitrotoluene (2N4HA6NT, potential oxidation product of 2,4-dihydroxylamino-6-nitrotoluene) were created in CAChe software (Oxford Molecular/Pharmacopeia, Hunt Valley, MD) that utilizes semiempirical quantum mechanics routines to determine structural conformations with the lowest heat of formation. Eventually, two parameters were evaluated to assess the reactivity of the nitroso-nitrogen. These were the partial charge on the nitroso-nitrogen and the electrostatic potential averaged over a constant electron density molecular surface of the structure (this parameter correlates with a "susceptibilty to a nucleophilic attack" in CAChe (*52*, *53*)).

TABLE 1. ^{14}C Radiolabel Distribution and Protein Concentrations for Various Fractions

description	initial ^{14}C-dpm (% of total)	post-treatment ^{14}C-dpm (% of total initial)	initial protein concn (mg/mL)	post-treatment protein concn (mg/mL)
biooxidizer		8.1		0.34[a]
sampling losses		12.2		
filter reject		22.4		0.03
filtrate		53.7		0.07
total	100	96.2	0.18	

[a] Refers to a pH 2 hydrolysate.

Values for both these parameters were generated with MOPAC/PM3 wave function for each structure at a geometry determined by performing a preoptimization calculation in an empirical molecular mechanics force field model (an augmented version of Allinger at al.'s MM3 model (*54*) incorporated in CAChe), followed by an optimized geometry calculation in MOPAC using PM3 parameters (*55*).

Results and Discussion

Fate of TNT Following Anaerobic/Aerobic Treatment. The anaerobic phase was terminated when HPLC analysis showed that the initial TNT was in the form of DHA6NT and aminophenols, products that have been reported previously (*2*). During the aerobic phase, creamy white strands formed that slowly turned into a brown floc-like precipitate. Unlike behavior expected from azoxy compounds, no dissolution of the precipitate was observed in either *n*-pentane or methylene chloride. However, the precipitate appeared to partially dissolve in acidic (pH = 2) solution, turning the solution to a cloudy color. No recognizable products (with the exception of the polar front) were observed on the reverse phase (RP) HPLC chromatogram following the aerobic stage. The final pH of the reaction mixture was estimated to be approximately 5.5.

An appreciable drop in the bulk aqueous ^{14}C concentration was not observed over the entire course of the anaerobic/aerobic treatment. Upon completion of the experiment an overall mass balance of 96.2% was obtained on the basis of the total initial ^{14}C count (Table 1). At the end of the anaerobic/aerobic treatment the remaining ^{14}C in the system was distributed as follows: 9.6% precipitate (biooxidized fraction), 26.6% aqueous filter reject, and 63.8% aqueous filtrate. The protein assay established that the bulk of the protein mass was in the form of—or associated with—the precipitate. Binding of the Coomassie blue G-250 dye to protein becomes quite inefficient in acidic solutions resulting in a subsequent loss of sensitivity of the assay (*44*). Still, a high value of 0.34 mg/mL was obtained for the pH 2 hydrolysate of the precipitate (Table 1).

The precipitate contained approximately 10% of the remaining ^{14}C after the experiment. The bulk of the ^{14}C was in the aqueous phase, perhaps associated with the soluble protein fraction (Table 1). It must be noted, however, that the ultracentrifugaton step during the preparation of the cell extract excluded most of the larger structural proteins that could provide much higher levels of material for the binding and precipitation of ^{14}C. An alternative explanation for the absence of RP-HPLC/UV−Vis analyzed products without a corresponding loss in ^{14}C is the possible loss of aromatic character of the products formed upon aeration. This phenomena appears to be unlikely because the reaction mixture contained DHA6NT in addition to the aminophenols; although unstable under aerobic conditions, DHA6NT has never been reported to undergo ring fission upon exposure to air. Regardless of the explanation for this unusual phenomenon, a significant portion of the radiolabel was found to be associated with insoluble proteins.

Reaction of Anaerobically Biotransformed TNT with a Model Thiol. The second TNT transformation reaction yielded predominantly DHA6NT (retention time of 4.1 min) and trace levels of 4HADNT (retention time of 8.1 min). The chromatogram and its percent ^{14}C distribution obtained from fraction collection are presented in parts (a) and (b), respectively, of Figure 3. As expected, the radiolabel was concentrated in the peaks of the reduced metabolites of TNT. The addition of approximately 1.2×10^{-3} mmole of 1-thioglycerol followed by 20 min of anaerobic incubation produced no significant change in the percent ^{14}C distribution of the chromatogram (Figure 3c,d). The molar ratio of thiols to potential nitroso functionalities was deliberately kept below 1:1 because the presence of excess thiol reduces the nitroso functionalities back to hydroxylamino (*56*). This sets up an undesirable situation where both nitroso and hydroxylamino functional groups are present in the system and a rapid condensation of azoxy products ensues (especially at alkaline to mildly acidic pHs). Following 20 min of aerobic incubation, a new more polar peak (retention time of 3.3 min) was identified (Figure 3(e)) that had a virtually identical UV spectrum as DHA6NT. A corresponding shift in the percent ^{14}C distribution of the chromatogram to the new peak was also observed (Figure 3(f)). The reaction profile matches that of the 1-thioglycerol/nitrosobenzene reaction reported in the literature (*35*, *56*). This reaction generates an acid labile and more polar sulfinamide as the dominant product. No new products having spectra similar to 4HADNT were identified. This could be the result of the inability of 1-thioglycerol to act as a competing nucleophile for the strongly ring deactivated 4HADNT molecule (A more detailed discussion of this phenomenon is presented later in the molecular modeling subsection.). Upon aerobic incubation, the peak area for 4HADNT decreased, and only 57% of the ^{14}C associated with this peak was conserved (as compared to 98% percent conservation of ^{14}C between chromatographic retention times of 2 and 6 min), indicating that perhaps the remaining nucleophilic hydroxylamino groups were outcompeting the thiol nucleophile to produce azoxy compounds. In conclusion, the model reaction demonstrated the feasibility of the nitroso-thiol reaction for nitrosoarenes produced from the oxidation of DHA6NT; however, a similar result was not obtained for 4HADNT.

The formation of a new product by the reaction of oxidized DHA6NT with 1-thioglycerol indicates that thiols do act as competing nucleophiles for partially reduced TNT metabolites in sequential anaerobic/aerobic biotransformation systems. Such reactions can allow the thiol-containing cysteine amino acid residues of proteins to become potential sinks for the parent nitroaromatic contamination. In addition, if the protein undergoing binding is involved as an enzyme in the reduction pathway, then binding can pose an inhibition threat for further transformation. In any case, the evaluation of the thiol concentration at any given time in the system may be of importance in determining the fate of the nitroaromatic contamination. This could be especially true for TNT bioremediation processes where the biomass, and subsequently the protein concentrations, are considerably higher than the low concentrations utilized in this study. Furthermore, the pH of such anaerobic processes is typically quite acidic and consequently does not favor azoxy formation. Such conditions can allow other nucleophiles such as thiols to react with any nitrosoarene metabolites in the system. Finally, in light of the findings of this study, it becomes critical to determine the nature of the covalent bond(s) formed from sequential anaerobic-aerobic treatment before such binding can be proposed as a means of immobilizing nitroaromatic contamination (*10*, *40*). As Table 2 demonstrates with the

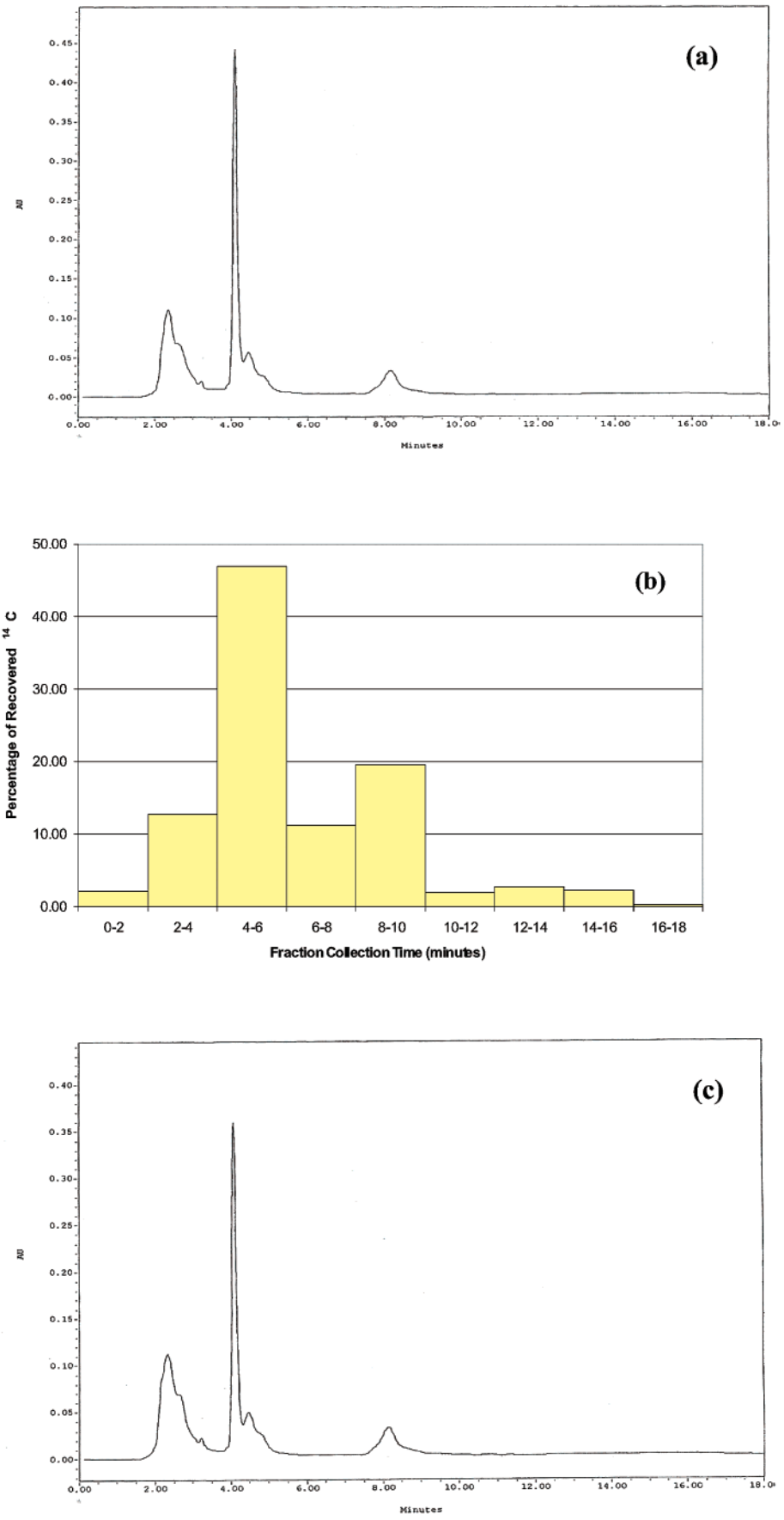

FIGURE 3. Parts (a) and (b) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions prior to thiol addition. Parts (c) and (d) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions after thiol addition

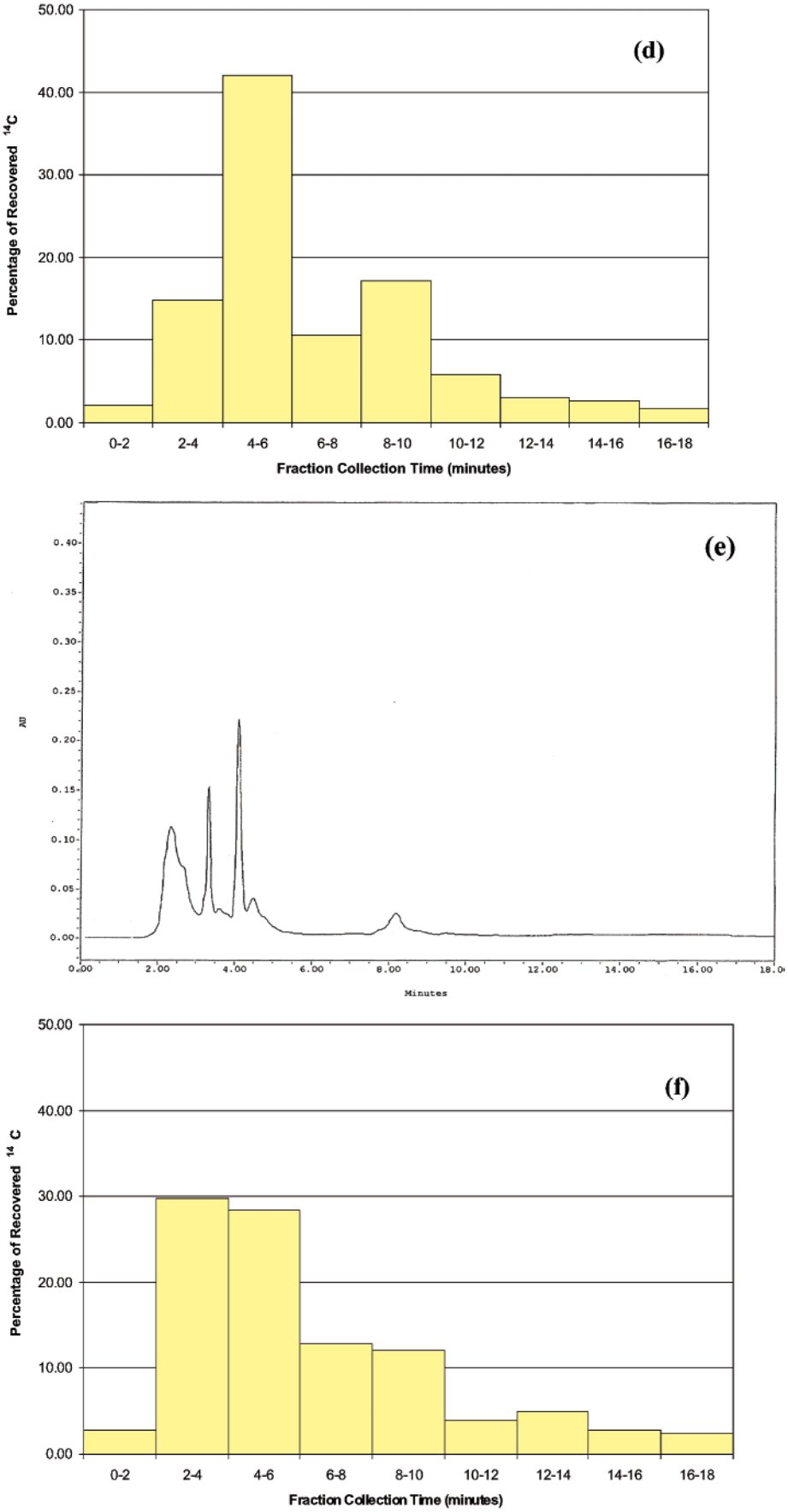

FIGURE 3. (Continued) (20 min anaerobic incubation time). Parts (e) and (f) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions after thiol addition (20 min aerobic incubation time).

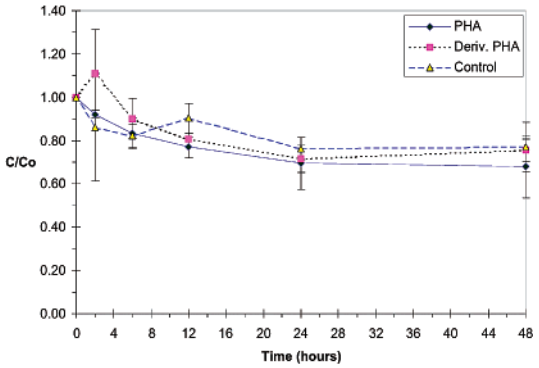

FIGURE 4. Reaction of 4HADNT with nonderivatized (PHA) and thiol-derivatized (deriv. PHA) IHSS peat humic acid standard under anaerobic conditions. The plot also shows data for an anaerobic control of 4HADNT in the absence of any humic acid.

TABLE 2. Bond Dissociation Energies at 298 K (*59*)

molecule	dissociation energy (kJ/mol)	molecule	dissociation energy (kJ/mol)
C−H	338	N−O	631
C−N	770	N−S	464

bond dissociation energy values of diatomic molecules, the strength of a N−S bond is only 60% the strength of a C−N bond.

Reactivity of 4HADNT with IHSS Peat Humic Acid. The anaerobic experimental series run to test the reactivity of 4HADNT toward IHSS peat humic acid at a sorbate/sorbent mass ratio of 0.02 yielded an approximately 30% loss in concentration over a 48-h duration (Figure 4). However, the difference between this removal and the removal observed in nonsorbent-containing controls was less than 6%. Loss of 4HADNT in the humic acid-free anaerobic control series was approximately 24%. The experiment was repeated and produced similar results (data not shown). Anaerobic reactivity of 4HADNT was also tested with nucleophile-derivatized IHSS peat humic acid in anticipation of the reactivity between 4HADNT and peat humic acid. This series also demonstrated virtually no difference between experimental systems and controls.

The lack of reactivity of the model arylhydroxylamino compounds toward humic acid can be explained in terms of its bioorganic chemistry in natural systems. The arylhydroxylamino nitrogen has reasonable nucleophilic characteristics. In all its observed reactions (with the exception of azoxy formation) it behaves as an extremely strong electrophile by first forming a nitrenium ion intermediate. However, arylhydroxylamines can form the electrophilic nitrenium ion only when assisted by catalytic conditions to overcome the kinetic barrier for the N−O bond cleavage. Therefore, in the absence of catalysis, either acidic or biological, it is unlikely that arylhydroxylamines will react with the multitude of potential nucleophiles present in the humic acid.

Interestingly, our results of no significant removal of 4HADNT with humic acids are in direct contrast to findings reported by others (*25*). Achtnich et al. reported a complete removal 0.04 mM 4HADNT (8.5 mg/L) in less than 2 h when humic acid was present at a concentration of 7500 mg/L. It is quite possible that the difference in outcomes for the two experiments could be attributed to a significantly difference in experimental design. The most obvious of these differences was the humic acid concentration; our experiments used a 625 mg/L concentration of IHSS peat humic acid that was approximately 56% carbon by mass (See Table A, Supporting Information, for the detailed characteristics of the IHSS humic acids used in this study.). This value translates into an effective carbon mass concentration of 350 mg/L, a value that is seven times the maximum DOM concentration typically observed in nature (*47*). Similarly, the humic acid concentration used by Achtnich et al. translates into a DOM concentration of 3750 mg carbon/L if a conservative carbon content of 50% by mass is assumed for their humic acid. This value is 75 times the maximum concentration of DOM in nature and probably exceeded the humic acid's aqueous solubility. At our relatively low humic acid concentrations of 625 mg/L we observed an average drop in pH of 0.4 units over the course of the 48-h experiment duration even though the experiments were conducted in pH 7, 10 mM phosphate buffer. Not having any data on their humic acid's carboxylic and phenolic acidity components, we can only speculate that the drop of pH in their pH 7.3, 50 mM buffered systems was most likely greater. A significant drop in pH can produce conditions for the acid-catalyzed cleavage of the arylhydroxylamine's N−O bond. Although the concentration of the nitrenium ions produced may not be significant when compared to the total concentration of arylhydroxylamine available, the presence of strong nucleophiles in the humic acid could lead to the depletion of arylhydroxylamines from the system.

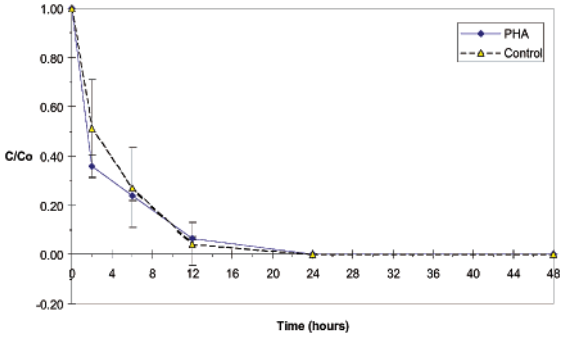

FIGURE 5. Aerobic reaction of 4HADNT with IHSS peat humic acid standard (PHA). The plot also shows data for a control reaction of 4HADNT performed under the same conditions but without any humic acid.

The second major difference between the two 4HADNT experimental systems may have been anaerobic "integrity". Our controls showed a 24% loss in concentration over 48 h, whereas Achtnich et al.'s control showed a 75% concentration loss in less than 20 h. The presence of trace levels of oxygen in the system could promote oxidation to the electrophilic nitroso functionalities that can subsequently be trapped by the nucleophilic nitrogen of the remaining arylhydroxylamines in solution to generate azoxy compounds.

In a second experiment with 4HADNT, the effects of converting the system from anaerobic to aerobic conditions were analyzed in the presence of IHSS peat humic acid. The purpose of this experiment was to determine whether the nucleophiles in the humic acid could compete with the arylhydroxylamines for the relatively electrophilic nitroso compounds produced upon exposure of 4HADNT to air (Figure 2, reaction b followed by reactions c and d in parallel). No appreciable difference in the rates of removal of 4HADNT from the aqueous phase could be observed between the humic acid series and the control series (Figure 5). Both series resulted in the formation of precipitate that was identified as predominantly 4,4′,6,6′-tetranitro-2,2′-azoxytoluene. The 4HADNT depletion/azoxy formation followed pseudo-first-order kinetics with a rate constant of 0.21/h. From the results it was evident that the nucleophiles present in humic acid were unable to compete for the nitroso intermediates resulting from the oxidation of 4HADNT. Earlier in the reaction of TNT metabolites with the model thiol, 1-thioglyc-

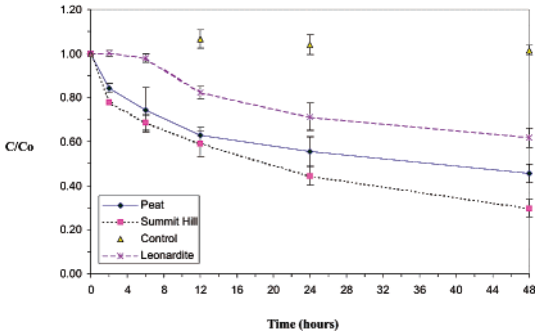

FIGURE 6. Data for the reaction of nitrosobenzene with nonderivatized IHSS peat and leonardite humic acid standards and IHSS Summit Hill reference humic acid in a closed aerobic system. The plot also shows data for an aerobic closed system control of nitrosobenzene in the absence of any humic acid.

erol, we demonstrated that thiols reacted more readily with the more ring destabilized (or electron rich) 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) than they did with the more strongly ring deactivated 4HADNT. It is very likely that the cause for this phenomenon is the aromatic substituent effects of the nitroso compound formed.

Reactivity of Nitrosobenzene with Various IHSS Humic Acids. Three IHSS humic acids were picked for their widely varying amino acid/proteinaceous content (based on the available analytical data for 13 amino acids) as well as their relatively limited range of sulfur content. (Total elemental sulfur content was 0.64, 0.71, and 0.76% by mass for Summit Hill, Peat, and Leonardite IHSS Humic Acids, respectively; see Table A, Supporting Information.) The organic sulfur content of the humic acids (based on the available information on the concentration of the sulfur-containing amino acid, methionine) correlated well with the protein content of the humic acids used. Nitrosobenzene was exposed to the three different humic acids, and the trends in reactivity were compared to nonsorbent containing controls. All three humic acids displayed the ability to remove nitrosobenzene from the buffer solution (Figure 6). Moreover, the extent of removal achieved over the 48-h period appeared at least in part to be dependent on the proteinaceous content of the humic acid. Going from highest to lowest protein content, Summit Hill, peat, and leonardite humic acids (1420, 373, and 11 nmole total amino acid/mg humic acid) showed nitrosobenzene removals of 70%, 50%, and 35%, respectively.

The results for the removal of nitrosobenzene with leonardite was somewhat unusual because it displayed an initial slow removal followed by a rapid increase in removal after the 6-h sampling mark. This trend in the leonardite data may be explained by the poor initial solubility of the leonardite humic acid observed in the initial phase of the experiment.

Biphasic kinetic analyses were performed on the peat and Summit Hill data using a "curve peeling" procedure (*45*). Different pseudo-first-order rate constants for the later slow removal (0.019, 0.009, and 0.008/h for Summit Hill, Peat, and Leonardite, respectively) and the initial fast removal (0.05 and 0.04 for Summit Hill and Peat, respectively) of aqueous nitrosobenzene were obtained using this procedure. The slower binding reaction showed reasonable least-squares fits with correlation coefficients generally over 0.96. The data in the faster phase, adjusted to remove any contributions from the slower phase, did not demonstrate as good fit on this first-order reaction analysis in large part due to the limited number of data points available for this phase of the reaction.

Reactivity of Nitrosobenzene with Thiol Derivatized IHSS Humic Acids. IHSS peat and Summit Hill humic acids were selected for derivatization. Both of these sorbents

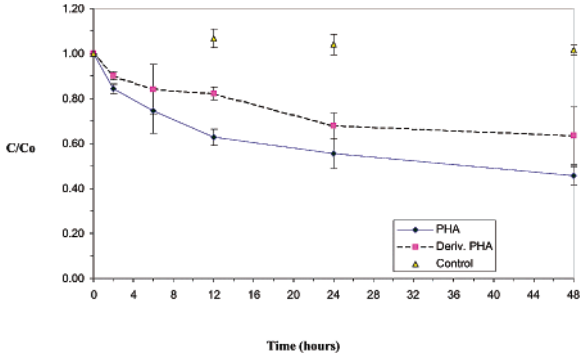

FIGURE 7. Effect of thiol-derivatization on IHSS peat humic acid's (PHAs) capacity to remove nitrosobenzene from the aqueous phase. Results for nonderivatized (PHA) and derivatized (deriv. PHA) peat humic acid as well as a nonhumic-acid-containing control of aqueous nitrosobenzene are shown.

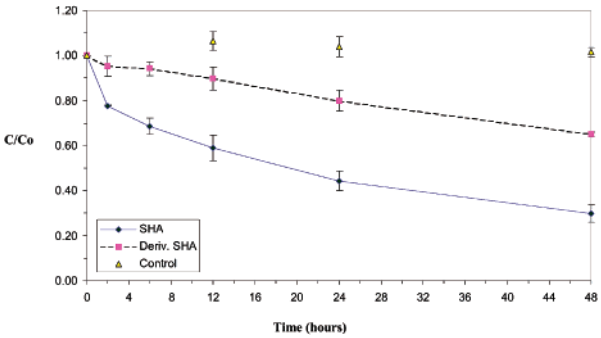

FIGURE 8. Effect of thiol-derivatization on IHSS Summit Hill humic acid's capacity to remove nitrosobenzene from the aqueous phase. Results for nonderivatized (SHA) and derivatized (deriv. SHA) Summit Hill humic acid as well as a nonhumic-acid-containing control of aqueous nitrosobenzene are shown.

showed a greater capacity for the removal of aqueous nitrosobenzene than the IHSS leonardite humic acid (Figure 6). N-Dansylaziridine was used as the thiol derivatizing agent. N-Dansylaziridine reacts only with strong nucleophiles such as thiols by forming thioether linkages. Unlike other thiol derivatizing compounds (e.g., N-substituted maleimides), N-dansylaziridine does not react with functionalities having weaker nucleophilic properties such as phenols, alcohols, and amines (*50*, *51*). Figures 7 and 8 illustrate the effects of the thiol derivatization on aqueous nitrosobenzene removal by IHSS peat and Summit Hill humic acids, respectively. Pretreatment of the two humic acids showed an obvious difference in the effectiveness of nitrosobenzene removal, with the change being more prominent for the Summit Hill humic acid. Incidentally, the Summit Hill humic acid had approximately four times the estimated amino acid concentration than the peat humic acid. (See Table A, Supporting Information.) However, since the cysteine concentrations were not available a value for the thiol concentration in each system could not be accurately determined. The thiol concentration values were inferred from the mass fraction of total sulfur, organic sulfur (i.e., methionine), and the protein content (i.e., sum of the concentrations of the 13 amino acids that were analyzed by the IHSS) of the humic acids. Based on the estimated total amino acid concentration of the Summit Hill humic acid alone, it appeared that a more drastic effect on nitrosobenzene removal was observed than could be explained by only the blocking of thiols. This observation could be explained by the possibility of reactions involving N-dansylaziridine and strong nucleophiles in the humic acids (other than thiols) that might have also bound the electrophilic nitrosobenzene.

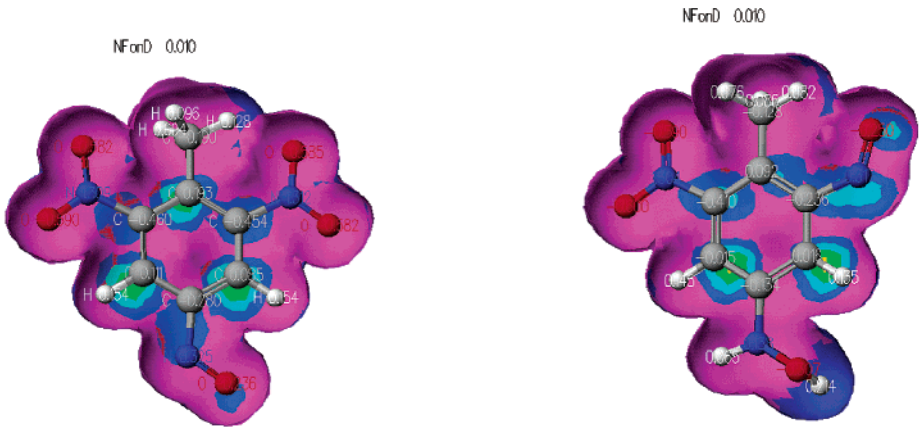

FIGURE 9. (a) Gas-phase molecular model of 4-nitroso-2,6-dinitrotoluene (4NDNT) in its lowest energy conformation, surrounded by its electron density isosurface. The colors of the surface depict the molecules susceptibility to nucleophilic attack (yellow > green > light blue > dark blue > pink). The red, blue, gray, and white atom colors represent oxygen, nitrogen, carbon, and hydrogen, respectively. (b) Molecular model of 2-nitroso-4-hydroxylamino-6-nitrotoluene (2N4HA6NT) in its lowest energy conformation, surrounded by its electron density isosurface.

Comparison of the Reactivity of Potential Nitroso Intermediates of TNT with Nitrosobenzene Using Molecular Modeling. Molecular models were created for nitroso intermediates that can potentially form from the oxidation of arylhydroxylamine metabolites of TNT (i.e., from the oxidation of 4HADNT and DHA6NT). The molecular modeling was performed to compare the two molecules' electrophilic properties so that patterns for their reactions with strong nucleophiles (e.g., thiol) could be predicted. Pictorial representations of the molecular models for 4NDNT (potential oxidation product of 4HADNT) and 2N4HA6NT (potential oxidation product of DHA6NT) in their lowest energy conformations are presented in parts (a) and (b), respectively, of Figure 9. The figures also show the color-coded depiction of the surface electrostatic potential on the electron density isosurface for the three molecules.

The partial charges on the nitroso-nitrogen (nitroso-N) of 4NDNT and 2N4HA6NT obtained from the lowest energy optimization were 0.325 and 0.320, respectively. These values showed limited utility in predicting the electrophilic nature of the three compounds because of their narrow range. On the other hand, the calculated surface electrostatic potentials as depicted in Figure 9 produced more interesting results. This parameter has been shown in the literature to be an effective parameter for the prediction of nucleophilic/electrophilic processes (*52*, *53*). Thus, the surface maps in Figure 9 show the locations where the molecules are most susceptible to a nucleophilic attack (yellow > green > light blue > dark blue > pink). Solvation effects were not accounted for in the molecular models; however, such effects in a polar solvent like water are expected to enhance the electrophilic nature of the nitroso-N of these molecules (*57*).

Comparing the electrostatic surface potentials above the nitroso-Ns it becomes clear that the nitroso group in 4NDNT (Figure 9(a)) has a very different electrophilic character than the one in 2N4HA6NT (Figure 9(b)). The nitroso group in 2N4HA6NT is a stronger electrophile than the one present in 4NDNT. The difference in electrophilic character can be explained by the aromatic substituent effects of the two molecules. The 4NDNT molecule has two very strong ring deactivating substituents in the form of nitro groups. The effect of these electron-withdrawing nitro groups is also visible in the electrostatic surface potentials above its C3 and C5 ring positions, which show a strong electron deficiency. Conversely, the surface plot of the more reduced 2N4HA6NT molecule shows that the replacement of the nitro group at the C4 position with a less electron withdrawing hydroxylamino group allows the nitroso nitrogen at the C2 position to display a much stronger electrophilic character than the less reduced 4NDNT molecule. Therefore, it would be expected that the more reduced hydroxylamino intermediates of TNT are favored to react via the nitroso-thiol reaction upon exposure of the reaction mixture to air. In addition, when more electron donating substituents are present on the ring, the increased electrophilic characteristic of the nitroso nitrogen is accompanied by a concomitant decrease in nucleophilic characteristics of the hydroxylamino nitrogen. This phenomenon may allow stronger nucleophiles such as thiols to outcompete arylhydroxylamines for the more reduced nitrosoarene intermediates, allowing reactions alternative to the azoxy reaction to occur.

Collectively, the studies presented herein demonstrate reactions that contribute to the binding of TNT reduction products to both biomass and natural organic matter. In both cases, the reactions center around the nitroso group formed directly through reduction reactions or indirectly through oxidation of amino or hydroxylamino forms with the reduced organic sulfur contained in either class of macromolecules. These findings help to explain the extent of binding of TNT that is often observed and can not be explained by reactions involving amino groups in organic matrices.

Acknowledgments

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Supporting Information Available

Chemical characteristics of IHSS peat and leanoardite humic acid standards and IHSS Summit Hill reference humic acid (Table A) and products resulting from the N−O bond cleavage of the semimercaptal intermediate formed by the nitroso-thiol reaction (Figure A). This material is available free of charge via the Internet at http://pubs.acs.org.

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TABLE A. Some chemical characteristics of International Humic Substances Society (IHSS) peat and leonardite humic acid standards and IHSS Summit Hill reference humic acid *.

IHSS Humic Acid*	Elemental Analysis (mass %)									Neutral Hydrophobic (Nanomole Amino Acid/mg Humic Acid)					Neutral Hydrophilic			Acidic		Basic		S-cont.	
	C	H	O	N	S	P	Total	H_2O	Ash	Val	Ile	Leu	Tyr	Phe	Thr	Ser	Gly	Asp	Glu	Arg	His	Met	Total
Leonardite	63.8	3.70	31.3	1.23	0.76	< 0.01	100.8	7.2	2.58	1.1	0.6	1.2	0.3	0.4	0.4	1.7	0.9	1.1	1.5	1.1	0.2	0.1	11
Peat	56.4	3.82	37.3	3.69	0.71	0.03	102.0	11.1	1.12	44	24	32	6.1	15	14	27	35	63	54	50	5.2	4.0	373
Summit Hill	54.0	4.84	37.9	5.13	0.64	0.40	102.9	8.1	1.41	146	61	66	33	40	33	183	225	160	178	232	34	26	1420

* Source: Product packet provided by the IHSS.

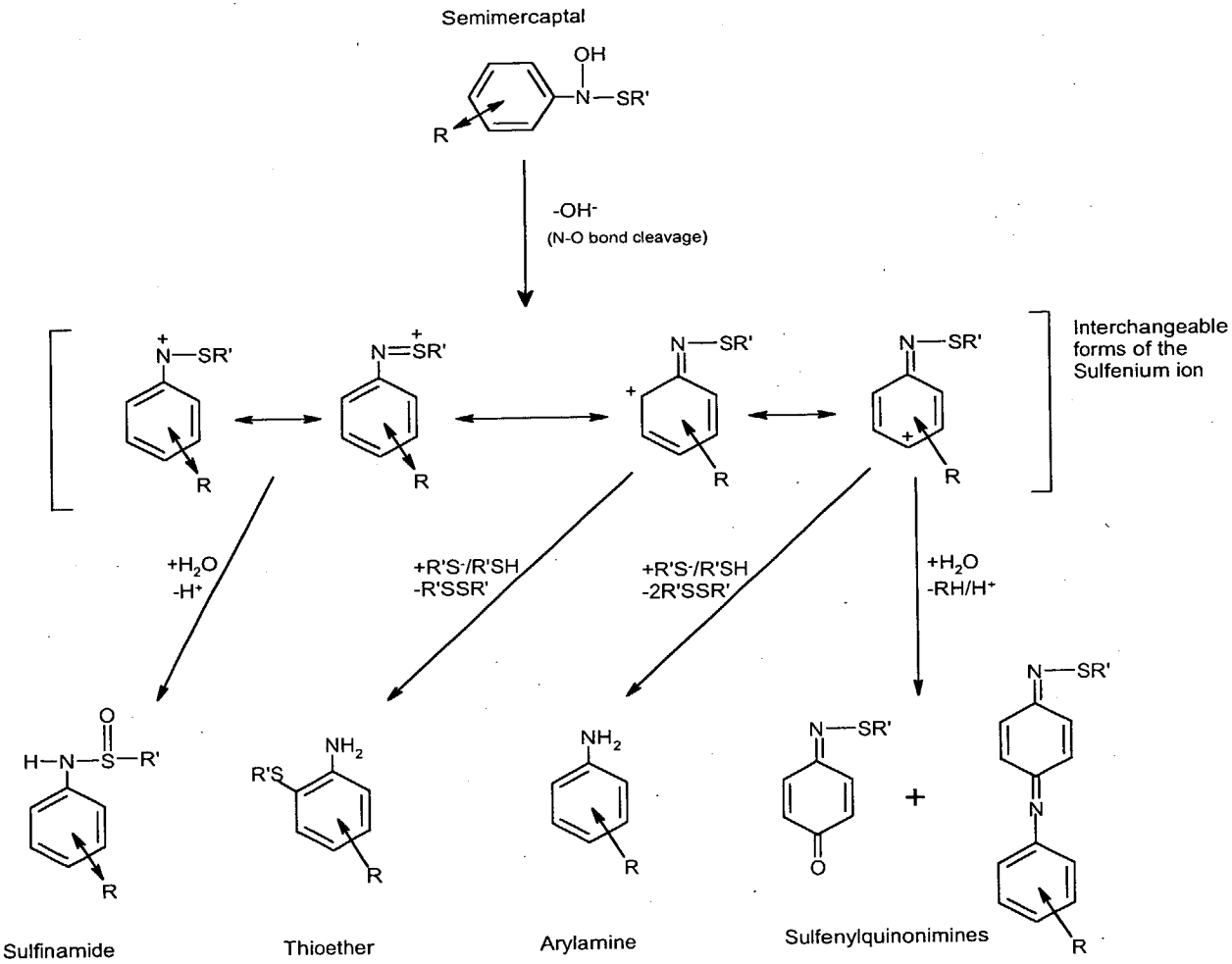

FIGURE A. Products resulting from the N-O bond cleavage of the semimercaptal intermediate formed by the nitroso-thiol reaction. Figure illustrates the large variety of products possible depending on thiol concentration and the overall aromatic ring-activating or electron donating substituent effects (depicted by the direction of the arrow away from the generic aromatic substituent "R").

Appendix A3

TNT Mineralization During a Two-Stage Anaerobic-Aerobic Process

Introduction

The development of effective processes for the remediation of TNT-contaminated sites remains a priority for stakeholders. While excavation and incineration are established methods for dealing with heavily contaminated subsurface materials (1), the fact that TNT is transformed biologically by a variety of microorganisms has led to an interest in developing potentially lower-cost *in situ* biological treatment technologies (2). A number of recent studies have increased the understanding of mechanisms involved in the transformation processes. The majority of known pathways involve initial reduction of the aryl nitro groups and do not appear to support growth of the mediating organisms (3-5). This attack has been demonstrated to occur both aerobically (6) and anaerobically (7, 8). The formation of reduced amino groups, particularly during the anaerobic transformation of TNT, yields compounds that are highly reactive and subject to covalent binding to soil (3, 9-14). In contrast, the biological transformation of TNT by *Clostridium acetobutylicum* yields intermediate compounds with multiple hydroxylamino substituents rather than the amino groups common to other anaerobic reductive pathways (15). This fermentation by *C. acetobutylicum* results in the formation of 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) with further transformation to aminophenolic compounds following a Bamburger rearrangement (16). This pathway does not result in the formation of reduction products such as aminodinitrotoluene, diaminonitrotoluene, or triaminotoluene, and binding of metabolites to natural organic matter occurs only when nitroso-containing intermediates formed following extensive oxygen exposure (17).

A two-step sequential strategy has been studied previously as a means for employing both the anaerobic and aerobic mechanisms for nitroaromatic transformation (3,4, 14). The

initial anaerobic reduction steps yield compounds that are more subject to oxidative attack. However, there is little evidence to suggest that either these aerobic or anaerobic pathways lead to significant mineralization of TNT (5, 12). Current biological remediation strategies rely on the formation of reduced metabolites followed by binding of these compounds to the surrounding soil matrix (2). This effectively serves to immobilize the contaminants and eliminates further transport off site. While toxicity is lessened when these intermediates are immobilized, the stability of these bound residues remains a subject of continued concern (5). Because the production of CO_2 represents an unequivocal elimination of TNT and all potentially toxic intermediates, complete mineralization is the goal of all explosives remediation processes.

The vast majority of nitroaromatic degradation studies conducted to date have not quantitatively demonstrated mineralization of TNT (3, 4, 6, 9, 11-53). The observation of fungal-mediated mineralization is the only significant pathway that clearly has a major biological component (54-62). This lignolytic attack by white-rot fungi and other species is assumed to be incapable of supporting growth, and the application of this type of catalysis to remediation systems is potentially difficult due to the formation of preferentially soil-bound intermediates (specifically aminonitrotoluenes) during microcosm studies. Mineralization of nitroaromatics with fewer nitro groups such as 2,4-dinitrotoluene (2,4-DNT) has been observed and appears linked to the recruitment of a number of dioxygenases to initiate the growth-supporting degradation process (24, 63, 64). Dioxygenases are well-known for their capabilities to attack a wide variety of structurally similar compounds, including aromatics such as the aminated products of anaerobic TNT transformation (24). Expression of these enzymes during enrichment on similar substrates may prove useful in promoting

mineralization of TNT biotransformation products. This study was designed to investigate how these factors contribute to mineralization potential. We report that CO_2 production (confirmed using ^{14}C-TNT) occurred during a two-stage microbial incubation as a result of a combined biological-chemical process. It is postulated that the Clostridial pathway that yields hydroxylated intermediates is key to providing more suitable starting compounds for initiation of the mineralization process.

Materials and Methods

Chemicals. 2,4,6-trinitrotoluene was obtained in both the unlabeled (99% purity, ChemService, Westchester, PA) and ^{14}C-labeled forms (Chemsyn Science, Lenexa, KS). The U-ring-^{14}C labeled compound had a purity of 99.5% and a specific activity of 22.6 mCi/mmol. Other chemicals used were: 2,4-DNT, 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), methanol, acetonitrile, 1 N KOH, Scinti-Safe Plus 50% cocktail (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ), carbon-14 Cocktail for R.J. Harvey biooxidizer (R.J. Harvey Instrument Corp., Hillsdale, NJ), 4-aminophenol, naphthalene, aniline, and mercuric chloride. All chemicals were HPLC grade or higher.

Cultures and Media. *Clostridium acetobutylicum* was obtained from Dr. George Bennett (Rice University, Houston, TX). Cultures were grown in CGM media as described previously (46). Briefly, cells were grown to an optical density (wavelength = 600 nm) of 1.1 to ensure that the organisms were harvested during the acidogenic phase when hydrogenase expression was near optimum as opposed to during the solventogenic phase.

A 2,4-dinitrotoluene degrading consortium was used as an inoculum for a series of mineralization assays. This culture was derived from DNT contaminated soil obtained from

the Badger Army Ammunition Plant (BAAP) as described by Fortner et al (68). Further enrichment on 2,4-DNT as a sole carbon source has resulted in consistent mineralization activity over time and a reduction in the number of species present to two (data not published). In addition to this enrichment culture, BAAP soil was used to develop a second mixed culture that was not enriched on any nitroaromatic compound.

The potassium phosphate buffer used contained per liter 0.7 g K_2HPO_4 and 0.3 g KH_2PO_4, pH 8.0. Minerals added for bacterial growth are per liter 0.5 g $(NH_4)SO_4$, 0.5 g NaCl, 0.05 g $MgSO_4$-$7H_2O$, 0.1 g $CaCl_2$-$2H_2O$, 0.003 g $FeSO_4$, and 1 mL trace elements (per liter 0.1 g H_3BO_3, 0.05 g $CaSO_4$-$5H_2O$, 0.05 g $ZnSO_4$-$7H_2O$, and 0.05 g Na_2MoO_2-$6H_2O$).

Two-stage TNT Mineralization Assays. After *C. acetobutylicum* were harvested by centrifugation, cultures were washed and resuspended in potassium phosphate buffer (0.2 to 0.8 L) and maintained at 37 ºC in strictly anaerobic conditions in the absence of an external carbon source. This incubation served as the first stage in the two-stage TNT transformation assays. Unlabeled TNT was added at a concentration of 25 mg/L. [U-ring-^{14}C]-2,4,6-TNT was added from a stock solution (7.36 μCi/mL) such that an initial activity in reactors was established within a range of 9.5 and 10.9 x 10^{-4} μCi/mL. Each day, the suspended *C. acetobutylicum* culture was sparged with 80% H_2/ 20% CO_2 for 15 minutes to maintain the desired oxidation-reduction environment (18). Aqueous samples were collected daily and analyzed via HPLC to confirm transformation of TNT. Anaerobic incubation continued until the suspension attained the rusty orange color characteristic of transformation beyond the Bamburger rearrangement product and the aminophenolic metabolite (Figure 1). It should be noted that the identity of this endproduct is not yet finalized, but preliminary analysis using a nitrosobenzene derivatization procedure has indicated that the compound contains hydroxyl

groups at both the 3- and 5-positions of the ring. This metabolite is non-detectable via HPLC due to its highly polar nature, but previous derivatization attempts have suggested that the compound contains both hydroxyl- and amino groups (unpublished results). This incubation period generally lasted between 3 and 14 days.

Following this endpoint, cells were removed via centrifugation under either aerobic or strictly anaerobic conditions and the supernatant was supplemented with additional mineral nutrients for 2,4-DNT culture growth. After storing overnight at 4 ºC, the combined mineral solution was filter-sterilized and divided into 25 mL aliquots and transferred to 125-mL shake flasks equipped with 3 cm deep center wells. These wells were filled with 1 mL of 1 N KOH to provide a CO_2 capture mechanism. Flasks were sealed with Teflon-lined butyl septa and incubated at room temperature (23 ºC) using no means of mixing or agitation.

In this second stage of incubation, reactors were maintained either aerobically or anaerobically. For those maintained in aerobic conditions, oxygen was allowed to enter the systems during transfer and sampling, but use of seals meant that oxygen exposure over time was limited. In anaerobic trials, reactors were stored in an anaerobic chamber where all transfers and sampling were conducted. Biotic systems received a 1% v/v inoculum of the 2,4-DNT degrading culture, while all other systems were not supplemented and were maintained as abiotic systems. In addition to an inoculum-free abiotic control, other abiotic systems included 1) autoclaving at 121°C for 30 minutes, 2) mercuric chloride amendments (100 mg/L) as a biocide, 3) growth media in which mineral salts were omitted, and 4) growth media that was mineral-free and autoclaved. None of these latter four treatments were inoculated. All treatments were conducted in duplicate.

An additional assay was performed to determine the ability of other aromatic hydrocarbons to stimulate mineralization activity following the Clostridial transformation step. This second stage was conducted aerobically using a number of initial conditions. Each reactor was amended with 25 mg/L of one of the following compounds: aniline, 4-aminophenol, 2,4-DNT, and naphthalene. Aliquots were transferred from filter-sterilized stock solutions, and reactors were re-amended periodically in an effort to maintain comparable levels of these structural analogs.

Analytical Methods. Temporal samples for all nitroaromatics were analyzed via HPLC as described previously (17). Optical density at a wavelength of 600 nm (OD600) on an SP830 Spectrophotometer (Turner) was used as a measure of growth. Aqueous-phase radiolabeled carbon (^{14}C) was determined by transferring 1 mL samples to 9 mL of ScintiSafe Plus 50% cocktail and then measuring on a Beckman LS6500 scintillation counter. Solids-associated ^{14}C determinations involved combusting samples in O_2 at a temperature of 900 C for 4 minutes using a Biooxidizer Model OX600 (R.J. Harvey Instrument Corporation) and capturing in Carbon-14 cocktail. Activity in this solution was also quantified using the LS6500 scintillation counter.

Results and Discussion

TNT Mineralization in Anaerobic-Aerobic Two-Stage Assays. Initial anaerobic transformation of TNT was mediated by *C. acetobutylicum* in the absence of an external carbon source. Following transfer to aerobic flasks, the amount of $^{14}CO_2$ produced was analyzed over the course of several months. Measurable mineralization occurred without lag in all reactors, and rates were relatively steady after an initial burst in the first 10 days of

monitoring (Figure 2). Following 67 days of incubation, the amount of $^{14}CO_2$ produced in these treatments ranged between 7.3 and 14.1% of the initial measured activity. The highest mineralization yields were observed in the inoculated reactors (14.1%) and the reactors containing no inoculum (13.3%). Both of these treatments had statistically significantly higher $^{14}CO_2$ production rates than those observed in the $HgCl_2$-amended (9.7%), mineral-free (9.2%), autoclaved (7.3%), and mineral-free/autoclaved (8.2%) controls. Based on the data from replicates, none of these control reactors were significantly different in terms of mineralization yield. The addition of a 2,4-DNT-degrading culture did not result in a measurable impact on the amount of $^{14}CO_2$ produced. The degree of growth observed in these reactors also appeared similar, as indicated by final OD600 readings of 0.36 and 0.47 for the inoculated and uninoculated reactors, respectively (Table 1). These absorbance readings were significantly higher than those in the remaining four treatments, all of which were only slightly above the initial background level (0.03). Therefore, the methods selected for controlling growth (mineral omission, autoclaving, biocide addition) appeared suitable and were likely responsible for the decreased production of $^{14}CO_2$ relative to the reactors where growth was not impeded. The growth observed in these reactors was unrelated to the addition of microbes and—while beneficial to mineralization—did not appear to be due to the stimulation of specific degrading organisms. Growth of non-specific organisms that entered the reactors during sampling likely contributed the higher absorbance readings in these treatments.

These rates of mineralization compare favorably with those observed in previous studies. Similar attempts at using a combined anaerobic-aerobic process have not resulted in mineralization yields higher than 1% (5, 64), regardless of whether soil slurries or

composting-type systems were employed. For the most part, the initial aerobic steps of biotransformation in these studies produced highly polar compounds that were susceptible to binding to soil and/or humic material. This behavior decreased the availability of the metabolites and presumably lessened the opportunity for potential mineralization. Biological transformation by a single organism has not reported; no measurable $^{14}CO_2$ production has been observed in extended incubations with *C. acetobutylicum* or any other pure bacterial species (65). Studies with various fungal species have been more promising. For example, *Phanaerochaete chrysosporium* has demonstrated the ability to mineralize up to 34% of added TNT, although low initial concentrations are necessary to avoid inhibition of this pathway (57). Other ligninolytic fungi grown in similar experimental conditions have yielded slightly lower percentages of $^{14}CO_2$, but mineralization becomes a significantly less productive pathway in soil (54, 61), presumably because the initial steps of fungal degradation yield products that are stable following covalent coupling to organic-rich soil matrices (61). Therefore, fungal-based remediation strategies suffer from the same limitations as previously described anaerobic-aerobic processes in terms of limited mineralization potential.

The only similar mineralization yields reported to date have been in soil microcosms maintained in predominantly microaerobic conditions (66, 67). In these studies, upwards of 11% of added ^{14}C-TNT was mineralized in samples containing soil and indigenous organisms from a nitroaromatic-contaminated site. However, establishment of accurate initial concentrations of TNT were either complicated by the presence of soil or not clearly stated. Amendments with O_2 significantly reduced mineralization. These studies, along with previous work with fungal-mediated TNT transformation, have demonstrated a strong inhibitory effect on mineralization at higher TNT concentrations. While this concentration

effect was not investigated here, it should be noted that initial concentrations (0.11 mM) in the assays are comparable to those typically observed in subsurfaces contaminated with TNT (2).

Like previous studies that have used combined anaerobic-aerobic stages as a transformation strategy, the initial process described in this paper (Clostridial transformation) resulted in the formation of a more polar endproduct. The result is an endproduct that has been shown to be less susceptible to binding to typical soil constituents, in part because it is the nitroso intermediates (rather than the hydroxylamines) that are prone to forming covalent linkages with natural organic matter (17). Therefore, the product has the potential to be more available relative to those formed via pathways that involve intermediates with multiple amino groups. The current study supports this theory since the overall pathway resulted in considerably more favorable CO_2 production than has been observed previously.

TNT Mineralization in Anaerobic-Anaerobic Two-Stage Assays. To determine the effect of exposure to O_2 on decomposition or degradation of the ^{14}C-labeled metabolite as well as on biological growth, a parallel set of assays were conducted in anaerobic conditions. All transfers to reactors were performed using the same batch of solution mixture, meaning that the concentration of the TNT metabolite and the ^{14}C activity were identical to the anaerobic-aerobic two-stage assays. Reactors were analyzed at similar intervals for $^{14}CO_2$ production (Figure 3). Autoclaving was not used as a control method for this anaerobic-anaerobic assay because the oxygen exposure could not be prevented during this sterilization procedure. Mineralization yields were noticeably lower in strictly anaerobic conditions relative to those observed in the aerobic incubation (Figure 2). None of the systems demonstrated greater than 1% mineralization until day 25, and even after 54 days of incubation the yields ranged between 1.1 and 1.7% for all reactors. Production of $^{14}CO_2$ in

the mineral-free treatment was slightly lower than the other three treatments, though this may have been the result of an initial lag in observed mineralization.

The presence of a 2,4-DNT degrading culture did not provide any benefit in terms of mineralization potential. In fact, it did not appear that growth of any type was stimulated in these conditions, as absorbance readings in all reactors were comparable to that obtained initially for the growth media on day 0. Growth of the 2,4-DNT degrading consortium was not expected because the catabolic pathway is aerobic (68).

The lack of oxygen also reduced the potential for decomposition of nitroaromatics that has been observed in previous analyses of these systems (17, 21). If this decomposition in the presence of oxygen leads to compounds that are more subject to mineralization, then subsequent oxygen exposure should lead to a sharp spike in the amount of $^{14}CO_2$ recovered. Reactors were transferred out of the anaerobic chamber on day 54 and opened to allow entry of ambient air. Within 10 days, the mineralization yield had increased by a factor of 2 to 4 in all systems, and yields continued to increase at similar rates over the course of the monitoring period. Growth of organisms may have contributed to the increased $^{14}CO_2$ production in these treatments. However, the absorbance readings in the mineral-free amendment remained relatively unchanged despite a rise in mineralization yield from 1.1% to 5.4% during the 20-day period after reactors were exposed to oxygen (data not shown). Therefore, the abiotic mineralization of aerobic decomposition products was likely a more important pathway in improving observed $^{14}CO_2$ production than any potential biological pathways in these conditions.

Sequential degradation has been perceived as a beneficial strategy in terms of exploiting the chemical characteristics of the parent compound and its reduced metabolites

(5). The electrophilic nitro groups provide favorable locales for reduction, but further electron transfer to these groups becomes progressively slower following reduction to less electrophilic groups such as nitroso, hydroxylamino, and amino groups. However, this change in the electrophilic nature results in an aromatic ring that is potentially less stable and more susceptible to oxidative attack (24, 65). Based on the finding that the endproduct of the clostridial biotransformation pathway is partially hydroxylated, further oxidative degradation and potential ring cleavage holds promise. The mineralization yields in the aerobic assays were roughly an order of magnitude higher than those observed in strictly anaerobic conditions. The absence of oxygen served to restrict growth of aerobic organisms, in particular the enrichment culture that requires oxygen to drive 2,4-DNT degradation. Growth of non-specific anaerobes was not observed, indicating that biological contamination did not occur. In addition to this restriction on biologically mediated transformation, the potential for non-biological aerobic decomposition was also minimized. Auto-oxidation via a non-productive pathway has long been established for reduced TNT products such as triaminotoluene (37, 69), and there is recent evidence that products such as 2HA46DNT and 4HA26DNT are unstable in the presence of oxygen (21). Formation of aerobic decomposition products such as nitroso intermediates and azoxy compounds would be limited in anaerobic assays, and therefore the potential for further mineralization of these by-products would be restricted. Formation of relatively insoluble azoxy compounds (17) may have contributed to the separable solid-phase fraction following centifugation (Table 1), although this was not confirmed analytically and there was no indication that this increased the measured absorbance in the reactors. Regardless, this combination of blocked auto-oxidation

and biological transformation pathways likely led to the low mineralization yields observed in anaerobic conditions.

However, it is clear that decomposition in the presence of oxygen was not solely responsible for the observed mineralization rates in the anaerobic-aerobic dual stage assays. Compared to the reactors in which growth was restricted, total $^{14}CO_2$ production was 37 to 78% higher in the inoculated and inoculum-free treatments. This correlated strongly with increased absorbance readings as an indicator of growth. All treatments were exposed to roughly the same oxygen levels over the course of the experiment, and this likely contributed to the similarity between the observed ^{14}C production in the control reactors ($HgCl_2$, mineral-free, autoclaved, and autoclaved/mineral-free). Likewise, autoclaving the reactors provided no significant benefit over omitting minerals from the assay. This normalization suggests that the contribution to mineralization via an abiotic pathway is represented by these four treatments (7.9 to 9.7% of initial ^{14}C addition) and that the incremental benefit observed in the treatments where growth was unrestricted is related to a biotic pathway. Based on the observed yields (Table 1), it is estimated that the biological component represented 27 to 44% of the total mineralization yield observed at the conclusion of the monitoring period.

TNT Mineralization in Presence of Additional Aromatic Co-Contaminants. The ability to stimulate TNT mineralization through the addition of structurally similar compounds was studied by adding a number of these compounds at similar concentrations. Reactors behaved relatively similarly in terms of $^{14}CO_2$ production over time, ranging from 7.8% to 11.1% after the conclusion of the monitoring period on day 48. The one system that demonstrated a statistically significantly higher mineralization yield was the reactor containing only the 2,4-DNT degrading culture. Mineralization in this system was higher

than even the reactor containing both the 2,4-DNT degraders and the growth substrate 2,4-DNT. While utilization of 2,4-DNT was not monitored, it appears that it served as a competitive substrate as opposed to one that served to stimulate expression of necessary enzymes for the degradation and mineralization of the ^{14}C labeled metabolite. In fact, there was a lag in the mineralization rate for the system containing both 2,4-DNT and the culture enriched on 2,4-DNT, indicating that 2,4-DNT was being used preferentially to the TNT metabolite. It has been postulated that enzymes involved in DNT transformation could be particularly useful in degrading TNT because 1) the two compounds are often present together in contaminated sites and 2) DNT degrading organisms have previously demonstrated an ability to attack TNT. For example, *Burkholdia cepacia* JS872 uses a 2,4-DNT enzymatic pathway to catalyze transformation TNT to aminated metabolites (Nishino and Spain, unpublished data). Similarly, the enzymes involved in the aerobic degradation of nitrobenzene in *Pseudomonas pseudoalcaligenes* JS45 have been shown to produce hydroxylated metabolites during TNT transformation (63). In the case of the experimental conditions described here, the ability to degrade DNT provided little benefit in promoting mineralization of TNT. The inhibition pattern further emphasizes that there was a biological contribution to $^{14}CO_2$ production, though it is unclear if that pathway can be stimulated.

All reactors containing the BAAP inoculum demonstrated similar mineralization yields, and this inoculum was slightly less successful at producing $^{14}CO_2$ than the 2,4-DNT degrading culture when no other compounds were present. After five days of incubation, slight variations could be noted in the patterns between treatments. For example, mineralization in the presence of naphthalene gradually increased after this point while mineralization in the presence of 4-aminophenol generally decreased, suggesting some degree

of acclimation (both positive and negative) over time. Mineralization rates were similar regardless of whether or not 2,4-DNT was supplied to the medium, suggesting that there was no inhibitory relationship between 2,4-DNT and the TNT product.

Growth was not comprehensively quantified in these reactors because of an inability to establish a base-line for initial absorbance, but all systems were characterized by an increase in the apparent number of cells (data not shown). A system containing no inoculum was run in parallel with the amended reactors, and the final mineralization yield of 8.1% was generally lower but comparable to those that were inoculated. Therefore, the addition of compounds with structural similarities to the nitroaromatic biotransformation product did not stimulate mineralization relative to unamended systems. While this trial attempted to promote the induction of catabolic enzymes via growth-related degradation of alternate cosubstrates, it did not appear that enrichment on these compounds supported secondary metabolism. Mineralization proceeded in the absence of cosubstrates, meaning it was unlikely that regulation was affected in these cultures. Transformation of cosubstrates was not monitored, but on-going research will address this as well as determine if lower concentrations and/or alternate analogs are more successful at recruiting the necessary enzymes to promote mineralization.

^{14}C Balance. A complete activity balance was conducted on the anaerobic-aerobic two-stage assay. In addition, activity balances were conducted on selected samples from the alternate assays to confirm that patterns were similar. All systems received similar initial levels of labeled TNT (9.5 to 10.9 x 10^{-4} μCi/mL), and aqueous samples (1 mL) analyzed for activity prior to and following centrifugation indicated that ^{14}C losses during the clostridial incubation period were generally less than 5%. The subsequent cell separation decreased the

activity by another 5-15%. Incorporation and/or sorption of radiolabeled compound to cell surfaces were confirmed to be responsible for this loss. This represented a loss of 6.8% (mean value) of the initial activity added to the Clostridial reactor in the form of ^{14}C-TNT.

Values for total ^{14}C recovery during the second stage of the assay ranged between 95 and 101% of the initial activity (Table 1), indicating that methods employed during this separation process were appropriate. The majority of systems in which growth was controlled had markedly lower ^{14}C in the solid-associated fraction, with the exception of the $HgCl_2$-amended reactor. The addition of $HgCl_2$ resulted in the formation of a fine precipitate that did not increase the absorbance of the suspension (Table 1) and did not appear to flocculate in a manner characteristic to growth of new organisms. This precipitate served as a surface for sorption of the labeled compound, as was demonstrated by a 70-75% reduction in ^{14}C following filtration (0.22 μm pore size) of the solution. The centrifugation step also resulted in recovery of a high portion (43.8%) of ^{14}C in this solid-associated fraction. Identification of this precipitate was not attempted.

The solids-associated fractions in the inoculated and inoculum-free reactors were slightly higher than those in the remaining treatments, suggesting that microbial growth provided a means for removing ^{14}C from solution. The formation of this type of solid phase in these reactors was previously observed in similar systems during an aerobic phase following Clostridial transformation (17), though the solids fraction in the current study did not form the same characteristic long brown strands. Protein assays during the previous study confirmed that the bulk of the protein in the systems was associated with this precipitate. This suggests that the solid phase observed in reactors and recovered following centrifugation was at least partially attributable to biological growth.

It was not determined whether organisms were capable of growth via the exploitation of a mineralization pathway. The steady production of $^{14}CO_2$ throughout the monitoring period suggests that the degradation was sustained and perhaps coupled to the presence of the nitroaromatic substrate, particularly if CO_2 was formed as a result of a central metabolic pathway. However, there were likely other alternate energy sources present in the medium in the form of soluble products of the clostridial incubation. In addition, small masses of methanol were added as part of the unlabeled TNT stock solution, and this provided a readily degradable substrate for growth. It is expected that utilization of these alternate substrates would have been complete within the initial stages of aerobic incubation, and that growth-supporting mineralization was a more promising prospect during the latter stages of the assay.

It has been suggested that there is little value in differentiating between abiotic and biotic processes in any type of two-stage process that results in mineralization (5). Subsurface environments are complex systems, and the presence of multiple microbial populations as well as a number of potential redox factors means that a variety of reactions can be feasible. The production of an innocuous and unambiguous compound such as CO_2 remains a desirable outcome for any remediation strategy, regardless of the mechanism. Obviously, the use of particular amendments shown to stimulate mineralization potential would represent a more engineering-intensive method for reaching this outcome than relying on natural soil elements to mediate abiotic or biotic reactions. Previous research has demonstrated that sequential anaerobic-aerobic strategies for remediation can serve to attenuate TNT via soil binding (10, 12, 34), but significant mineralization has remained elusive. This study demonstrated that the potential for mineralization exists, and that the rates of $^{14}CO_2$ production were only minimally influenced by any particular amendment or control strategy. The key difference appeared to

be the production of a reduced TNT metabolite that proved particularly susceptible to further transformation and eventual mineralization. This represents the most successful outcome reported to date for an anaerobic-aerobic dual-stage process.

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Table 1. ^{14}C Distribution and Activity Balances After 67 Days of Aerobic Incubation

	Supernatant	Solids-associated	CO_2	TOTAL	$O.D._{600}$
DNT-degrading inoculum	60.9	25.6	14.1	101	0.36
No inoculum	62.6	20.7	13.3	96.6	0.47
$HgCl_2$-amended	47.2	43.8	9.7	101	0.15
No salts	81.7	5.3	9.2	96.2	0.10
Autoclaved	79.2	14.1	7.9	101	0.098
No salts + Autoclaved	70.0	16.5	8.2	94.7	0.12

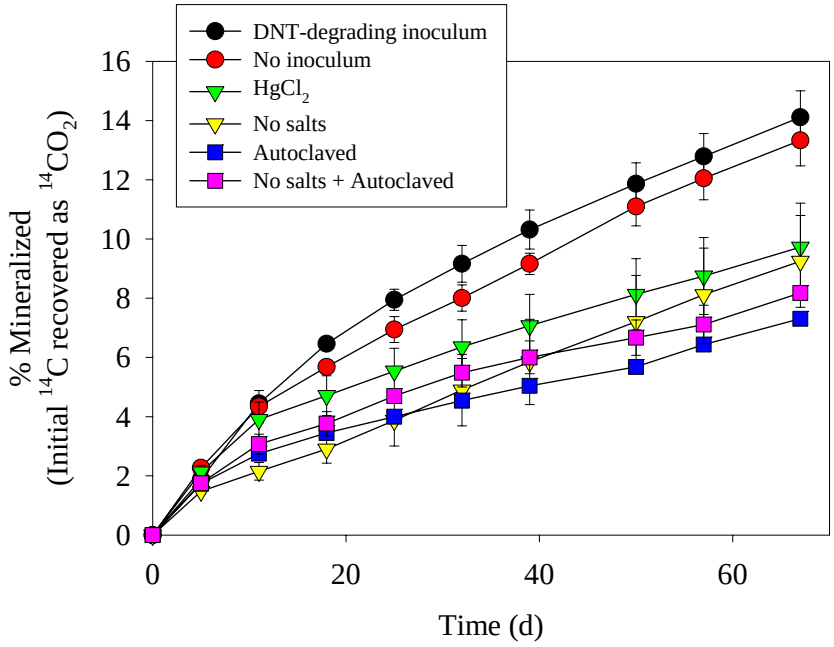
Note: The Clostridial incubation resulted in a 6.8% decrease in the initial ^{14}C activity following separation of the solids-associated fraction. This resulted in an initial ^{14}C activity in the aerobic incubations of 8.55×10^{-4} μCi/mL.

FIGURE LEGENDS

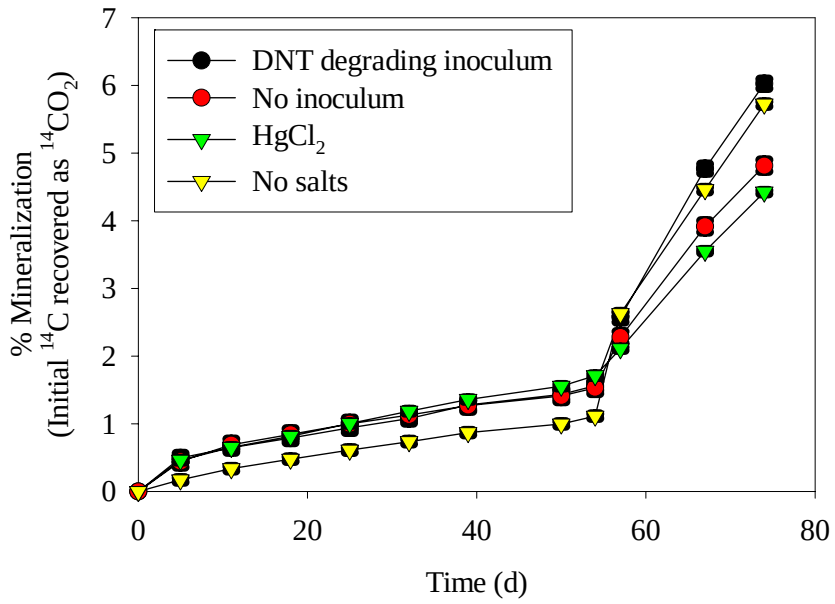
FIGURE 1. Mineralization of TNT during aerobic incubation stage of anaerobic-aerobic two-stage assay. Expressed as a percentage of initial ^{14}C added to each reactor recovered as $^{14}CO_2$.

FIGURE 2. Mineralization of TNT during anaerobic incubation stage of anaerobic-anaerobic two-stage assay. Reactors were exposed to oxygen on day 54. Expressed as a percentage of initial ^{14}C added to each reactor recovered as $^{14}CO_2$.

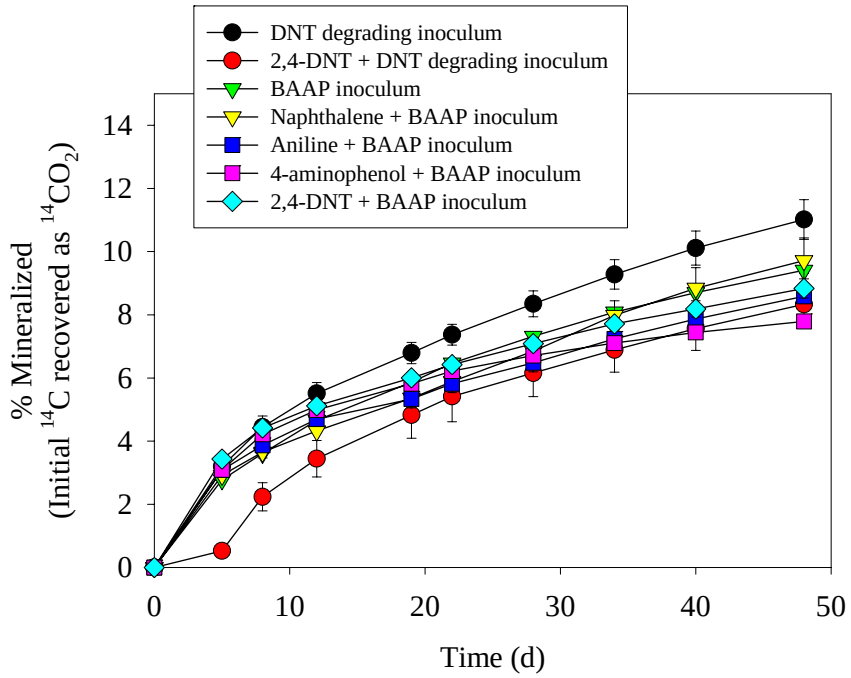
FIGURE 3. Mineralization of TNT during aerobic incubation stage of anaerobic-aerobic two-stage assay in the presence of structurally similar compounds. Expressed as a percentage of initial ^{14}C added to each reactor recovered as $^{14}CO_2$.


DNT-degrading inoculum
No inoculum
HgCl2
No salts
Autoclaved
No salts + Autoclaved
% Mineralized
(Initial 14C recovered as 14CO2)
0
2
4
6
8
10
12
14
16
0
20
40
60
Time (d)

1


DNT degrading inoculum
No inoculum
HgCl2
No salts
% Mineralization
(Initial 14C recovered as 14CO2)
Time (d)
0
1
2
3
4
5
6
7
0
20
40
60
80

1


DNT degrading inoculum
2,4-DNT + DNT degrading inoculum
BAAP inoculum
Naphthalene + BAAP inoculum
Aniline + BAAP inoculum
4-aminophenol + BAAP inoculum
2,4-DNT + BAAP inoculum
% Mineralized
(Initial ^{14}C recovered as $^{14}CO_2$)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
Time (d)

1

Appendix B

List of Technical Publications

Ahmad, F., and Hughes, J. B. 2002. Reactivity of partially reduced arylhydroxylamine and nitrosoarene metabolites of 2,4,6-trinitrotoluene (TNT) toward biomass and humic acids. *Environmental Science & Technology* 36**:**4370-4381.

Watrous, M. M., S. Clark, R. Kutty, S. Huang, F. B. Rudolph, J. B. Hughes, G.N. Bennett. (2003) 2,4,6-Trinitrotoluene Reduction by an Fe-Only Hydrogenase in*Clostridium acetobutylicum*. *Applied and Environmental Microbiology* 69 (3) p. 1542-1547.