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

Mary M. Watrous,¹ Sandra Clark,² Razia Kutty,² Shouqin Huang,² Frederick B. Rudolph,² Joseph B. Hughes,¹ and George N. Bennett^{2*}

Civil and Environmental Engineering¹ and Department of Biochemistry and Cell Biology and the Institute of Biosciences and Bioengineering,² Rice University, Houston, Texas 77005-1892

Received 26 August 2002/Accepted 23 December 2002

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 μ M. 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. ace-*

tobutylicum 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

* Corresponding author. Mailing address: Department of Biochemistry and Cell Biology and the Institute of Biosciences and Bioengineering, Rice University, Houston, TX 77005-1892. Phone: (713) 348-4920. Fax: (713) 348-5154. E-mail: gbennett@bioc.rice.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) ^a	Source and/or reference ^b
Strains		
<i>E. coli</i> DH10B	<i>mcrA</i> , Δ <i>mcrBC</i> , <i>recA1</i>	NEB, 25
<i>C. acetobutylicum</i> ATCC824	Wild type	ATCC
<i>C. acetobutylicum</i> M5	Absence of pSOL1	7
Plasmids		
pAN1	p15A ori; Cm ^r , Φ 3I	26
pPTB	ColE1 ori, ORF II ori; Em ^r , <i>ptb</i> promoter	17
pSOS84	ColE1 ori, ORF II ori; Em ^r , Ap ^r , <i>ptb</i> promoter	39
pMFH1	ColE1 ori; Ap ^r , <i>ptb</i> promoter, <i>hydA</i>	13
pHTB	ColE1 ori, ORF II ori; Em ^r , <i>ptb</i> promoter, <i>hydA</i>	This study
pASH1	ColE1 ori, ORF II ori; Em ^r , <i>ptb</i> promoter, <i>hydA</i> -asRNA	This study
pASH2	ColE1 ori, ORF II ori; Em ^r , <i>ptb</i> promoter, <i>hydA</i> -asRNA	This study
pASH3	ColE1 ori, ORF II ori; Em ^r , <i>ptb</i> promoter, <i>hydA</i> -asRNA	This study

^a pSOL1, 210-kb plasmid containing the *hydA* gene; p15A ori, p15A origin of replication; Cm^r, chloramphenicol resistance; Φ 3I, Φ 3I methyltransferase gene; ColE1 ori, 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, ampicillin 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 H₂-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% CO₂, 9.9% H₂, and 85% N₂ 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-sulfonyl 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 Na₂SO₄ 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 Na₂SO₄ (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'-CGCGGATCCAGGAGGATAACATGAAAACAATAATC-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 ATCCTTCATTGCCATTAA), containing a *Bam*HI site, and AS5 (5'-CCCC CGGGTAATGTAATTACTTTTGT), containing an *Ava*I site, were used to

construct pASH1. Primers AS4 (5'-GCCGATCCGAGTGTGGGATATC), containing a *Bam*HI site, and AS5 (as above) were used in construction of pASH2. Primers AS3 (as above) and AS6 (5'-CTAGGTAATGTAATTACTTT TACCGGGAGGATAAACA 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 H₂. 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 O₂ 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 predominantly 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 MKTIILNG-NEVHTDKDITIL, 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, experi-

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.

ments 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.

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 H₂ 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 μ g) added to 110 μ M TNT solution. Aliquots (100 μ l) 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 solvotogenic 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 H₂ 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.

ACKNOWLEDGMENTS

This material is based upon work supported by, or in part by, the U.S. Army Research Laboratory and the U.S. Army Research Office under the grant number DAAD 19-01-1-0524. Support was also obtained from the Strategic Environmental Restoration Development Program (SERDP). Grants from the Robert A. Welch Foundation, C-1268 (G.N.B.) and C-1372 (F.B.R.), are gratefully acknowledged.

REFERENCES

- Adams, M. W. W. 1990. The structure and mechanism of iron-hydrogenases. *Biochim. et Biophys. Acta* **1020**:115-145.
- Adams, M. W. W., L. E. Mortenson, and J.-S. Chen. 1981. Hydrogenase. *Biochim. Biophys. Acta* **594**:105-176.
- Ahmad, F., and J. Hughes. 2000. Anaerobic transformation of TNT by *Clostridium*, p. 185-212. In J. C. H. Spain and H.-J. Knackmuss (ed.), *Bio-degradation of nitroaromatic compounds and explosives*. Lewis Publishers, Boca Raton, Fla.
- Bruns-Nagel, D., S. Scheffer, B. Casper, H. Garn, O. Drzyzga, E. Von Low, and D. Gerns. 1999. Effect of 2,4,6-trinitrotoluene and its metabolites on human monocytes. *Environ. Sci. Technol.* **33**:2566-2570.
- Cammack, R. 1999. Hydrogenase sophistication. *Nature* **397**:214-215.
- Clark, S. W., G. N. Bennett, and F. B. Rudolph. 1989. Isolation and characterization of mutants of *Clostridium acetobutylicum* ATCC 824 deficient in acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (EC 2.8.3.9) and in other solvent pathway enzymes. *Appl. Environ. Microbiol.* **55**:970-976.
- Cornillot, E., R. V. Nair, E. T. Papoutsakis, and P. Soucaille. 1997. The genes for butanol and acetone formation in *Clostridium acetobutylicum*

- ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. *J. Bacteriol.* **179**:5442–5447.
8. Crawford, R. L. 1995. Biodegradation of nitrated munition compounds and herbicides by obligately anaerobic bacteria, p. 87–98. *In* J. Spain (ed.), *Biodegradation of nitroaromatic compounds*, vol. 49. Plenum Press, New York, N.Y.
 9. Desai, R., and E. Papoutsakis. 1999. Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. *Appl. Environ. Microbiol.* **65**: 936–945.
 10. Ederer, M. M., T. A. Lewis, and R. L. Crawford. 1997. 2,4,6-Trinitrotoluene (TNT) transformed by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. *J. Indust. Microbiol. Bio/Technol.* **18**:82–88.
 11. Environmental Protection Agency 1993. Integrated risk information system data for TNT, vol. 2002. [Online.] www.epa.gov/iris/subst/0269.htm.
 12. Gorwa, M. F., C. Croux, and P. Soucaille. 1996. Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol.* **178**:2668–2675.
 13. Green, E. M., and G. N. Bennett. 1996. Inactivation of an aldehyde/alcohol dehydrogenase gene for *Clostridium acetobutylicum* ATCC 824. *Appl. Biochem. Biotechnol.* **57/58**:213–221.
 14. Green, E. M., Z. L. Boynton, L. M. Harris, F. B. Rudolph, E. T. Papoutsakis, and G. N. Bennett. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. *Microbiology* **142**:2079–2086.
 15. Hartmanis, M. G. N., and S. Gatenbeck. 1984. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. *Appl. Environ. Microbiol.* **47**:1277–1283.
 16. Honeycutt, M. E., A. S. Jarvis, and V. A. McFarland. 1996. Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. *Ecotoxicol. Environ. Safety* **35**:282–287.
 17. Huang, S., P. A. Lindahl, C. Wang, G. N. Bennett, F. B. Rudolph, and J. B. Hughes. 2000. 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. *Appl. Environ. Microbiol.* **66**: 1474–1478.
 18. Hughes, J. B., C. W. Wang, R. Bhadra, A. Richardson, G. N. Bennett, and F. Rudolph. 1998. Reduction of 2,4,6-trinitrotoluene by *Clostridium acetobutylicum* through hydroxylamino intermediates. *Environ. Toxicol. Chem.* **17**: 343–348.
 19. Hughes, J. B., C. Y. Wang, K. Yesland, A. Richardson, R. Bhadra, G. N. Bennett, and F. Rudolph. 1998. Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. *Environ. Sci. Technol.* **32**:494–500.
 20. Jenkins, T. F., M. E. Walsh, and P. W. Schumacher. 1995. Development of colorimetric field screening methods for munitions compounds in soil, p. 324–333. *In* T. Vo-Dinh and R. Niessner (ed.), *Proceedings, Environmental Monitoring and Hazardous Waste Site Remediation*, Munich, Germany, 19 to 21 June 1995.
 21. Jungermann, K., R. Thauer, E. Rupprecht, C. Ohloff, and K. Decker. 1969. Ferredoxin mediated hydrogen formation from NADPH in a cell-free system of *Clostridium kluyveri*. *FEBS Lett.* **3**:144–146.
 22. Khan, T. A., R. Bhadra, and J. Hughes. 1997. Anaerobic transformation of 2,4,6 TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*. *J. Indust. Microbiol. Bio/Technol.* **18**:198–203.
 23. Krumholz, L. R., J. Li, W. W. Clarkson, G. G. Wilber, and J. M. Sufilta. 1997. Transformations of TNT and related aminotoluenes in groundwater aquifer slurries under different electron accepting conditions. *J. Indust. Microbiol. Bio/Technol.* **18**:161–169.
 24. Lemon, B., and J. Peters. 1999. Binding of exogenously added carbon monoxide at the active site of the iron-only hydrogenase (Cpl) from *Clostridium pasteurianum*. *Biochemistry* **38**:12969–12973.
 25. Mathieu, M., Y. Modis, J. P. Zeelen, C. K. Engel, R. A. Abagyan, A. Ahlberg, B. Rasmussen, V. S. Lamzin, W. H. Kunau, and R. K. Wierenga. 1997. The 1.8 Å crystal structure of the dimeric peroxisomal 3-ketoacyl-CoA thiolase of *Saccharomyces cerevisiae*: implications for substrate binding and reaction mechanism. *J. Mol. Biol.* **273**:714–728.
 26. McCormick, N. G., F. E. Feeherry, and H. S. Levinson. 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Appl. Environ. Microbiol.* **31**:949–958.
 27. Mermelstein, L. D., and E. T. Papoutsakis. 1993. In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T1 methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* **59**:1077–1081.
 28. Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis. 1992. Expression of cloned homologous fermentation genes in *Clostridium acetobutylicum* ATCC 824. *Bio/Technology* **10**:190–195.
 29. Nolling, J., G. Breton, M. V. Omelchenko, K. S. Makarova, Q. Zeng, R. Gibson, H. M. Lee, J. Dubois, D. Qui, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, and D. R. Smith. 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J. Bacteriol.* **183**:4823–4838.
 30. Palmer, M. A., E. Differding, R. Gamboni, S. F. Williams, O. P. Peoples, C. T. Walsh, A. J. Sinskey, and S. Masamune. 1991. Biosynthetic thiolase from *Zoogloea ramigera*. Evidence for a mechanism involving Cys-378 as the active site base. *J. Biol. Chem.* **266**:8369–8375.
 31. Petersen, D. J., and G. N. Bennett. 1990. Purification of acetoacetate decarboxylase from *Clostridium acetobutylicum* ATCC 824 and cloning of the acetoacetate decarboxylase gene in *Escherichia coli*. *Appl. Environ. Microbiol.* **56**:3491–3498.
 32. Preuss, A., and P. G. Rieger. 1995. Anaerobic transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds, p. 69–86. *In* J. C. Spain (ed.), *Biodegradation of nitroaromatic compounds*, vol. 49. Plenum Press, New York, N.Y.
 33. Raleigh, L. A. 1984. RbCl transformation procedure for improved efficiency. *N. Engl. Biolabs Trans.* **6**:7.
 34. Rieger, P. G., and H. J. Knackmuss. 1995. Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil, p. 1–18. *In* J. C. Spain (ed.), *Biodegradation of nitroaromatic compounds*, vol. 49. Plenum Press, New York, N.Y.
 35. Saint-Amans, S., L. Girbal, J. Andrade, K. Ahrens, and P. Soucaille. 2001. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures. *J. Bacteriol.* **183**:1748–1754.
 36. Spain, J. (ed.). 1995. *Biodegradation of nitroaromatic compounds*, vol. 49. Plenum Press, New York, N.Y.
 37. Spain, J. C., J. B. Hughes, and H.-J. Knackmuss (ed.). 2000. *Biodegradation of nitroaromatic compounds and explosives*. Lewis Publishers, Boca Raton, Fla.
 38. Stim-Herndon, K. P., R. Nair, E. T. Papoutsakis, and G. N. Bennett. 1996. Analysis of degenerate variants of *Clostridium acetobutylicum* ATCC 824. *Anaerobe* **2**:11–18.
 39. Wardwell, S. A., Y. T. Yang, H. Y. Chang, K. Y. San, F. B. Rudolph, and G. N. Bennett. 2001. Expression of the *Klebsiella pneumoniae* CG21 acetoin reductase gene in *Clostridium acetobutylicum* ATCC 824. *J. Indust. Microbiol. Bio/Technol.* **27**:220–227.
 40. Wiesenborn, D. P., F. B. Rudolph, and E. T. Papoutsakis. 1988. Thiolase from *Clostridium acetobutylicum* ATCC 824 and its role in the synthesis of acids and solvents. *Appl. Environ. Microbiol.* **54**:2717–2722.
 41. Williams, R. T., P. S. Ziegenfuss, and W. E. Sisk. 1992. Composting of explosives and propellant contaminated solid under thermophilic and mesophilic conditions. *J. Indust. Microbiol.* **9**:137–144.