Journal of Industrial Microbiology & Biotechnology (1997) 18, 198–203

Anaerobic transformation of 2,4,6-TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*

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The transformation of TNT and related aminated nitrotoluenes by *Clostridium acetobutylicum* was investigated. 2,4,6-trinitrotoluene (TNT) was rapidly reduced (537 nM min^{-1} mg $protein^{-1}$) to undetermined end products via monohydroxylamino derivatives. TNT reduction was more rapid than that of 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene. The metabolic phase of clostridial cultures affected rates and extents of transformation of TNT and its intermediates. Acidogenic cultures showed rapid transformation rates and the ability to transform TNT and its primary reduction products to below detection limits; solventogenic cultures did not transform TNT completely, and showed accumulation of its hydroxylamino derivatives. Carbon monoxide-induced solventogenesis was capable of slowing the transformation of TNT and intermediates. Studies employing [ring-U-^{14}C]-TNT demonstrated that no significant mineralization occurred and that products of transformation were water-soluble.

Keywords: TNT; *Clostridium acetobutylicum*; transformation kinetics; transformation extent; mass balance

Introduction

Numerous and diverse bacteria are capable of transforming 2,4,6-trinitrotoluene (TNT) [2–5, 9, 14, 15, 19, 21, 28]. The most commonly cited metabolic pathway is the reduction of one or more nitro substituent(s) to the corresponding amine, via nitroso and hydroxylamino intermediates [15, 19]. While the reduction of nitro groups in TNT has been observed under aerobic conditions, reduction beyond the diamino-nitrotoluenes is rarely cited [20]. The aromatic ring of TNT may be mineralized [5,6,26], though this is not routinely observed [24], and many of the metabolic and environmental conditions required for such biocatalysis remain unknown.

Anaerobic conditions are conducive to a greater extent of reduction of TNT than aerobic [22]; Preuss and Reiger [20] have recently reviewed this field extensively. Anaerobic or sequential anaerobic-aerobic process schemes for the biotreatment of TNT are being evaluated for ring cleavage and eventual mineralization [8, 9]. To develop such systems effectively, the initial reductive stages must be understood. Incomplete reduction can yield potentially toxic aminated-nitrotoluenes or reactive intermediates that form recalcitrant products, eg condensation of nitroso- and hydroxylamino-intermediates forming azoxy-dimers, and the covalent binding of hydroxylamines to soils [7, 12, 15, 27, 28].

In this paper, we report the reduction of TNT and related aminated-nitrotoluenes by *Clostridium acetobutylicum*, an obligate, saccharolytic anaerobe with the reported ability to catalyze the reduction of nitroaromatics [1, 15, 19, 21]. The focus of these studies was to evaluate the effect of culture conditions on the rate and extent of biotransformation observed.

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Abbreviations: 2,4,6-trinitrotoluene: TNT; 2-amino-4,6-dinitrotoluene; 2A46DNT; 4-amino-2,6-dinitrotoluene: 4A26DNT; 2-hydroxylamino-4,6-dinitrotoluene: 2hA46DNT; 4-hydroxylamino-2,6-dinitrotoluene: 4hA26 DNT; 2,4-diamino-6-nitrotoluene: 24DA6NT; 2,6-diamino-4-nitrotoluene: 26DA4NT; 2,4,6-triaminotoluene: TAT; and retention time: RT.

Received 6 November 1995; accepted 15 August 1996

Materials and methods

Chemicals

2,4,6-trinitrotoluene (ChemService, Westechester, PA, USA); [ring-U-^{14}C]-2,4,6-trinitroluene (Chemsyn Science, Lenexa, KS, USA); 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (AccuStandard Inc, New Haven, CT, USA); 2-hydroxylamino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene (Ron Spanggord, SRI International, Menlo Park, CA, USA); 2,6-diamino-4-nitrotoluene, 2,4,6-triaminotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (Deborah Roberts, University of Houston, Houston, TX, USA); 2-propanol (EM Science, Gibbstown, NJ, USA).

Maintenance and growth of C. acetobutylicum

Clostridium acetobutylicum (ATCC 824) was maintained as axenic spores in a 'corn mash meal' (50 g L^{-1} corn mash, 5 g L^{-1} glucose and 0.5 g L^{-1} l-cysteine) at 4°C. The growth medium employed for the culture of *C. acetobutylicum* was NYG, composed of 8 g L^{-1} nutrient broth (Difco, Detroit, MI, USA), 1 g L^{-1} yeast extract (Difco) and 4 g L^{-1} glucose. Active cell cultures of *C. acetobutylicum* were initiated from spores with a 2-ml (10% v/v) inoculum added to 18 ml NYG medium in a 100-ml serum bottle (Wheaton, Millville, NJ, USA), which was then heated at 70–75°C for 10 min, crimp-sealed, purged with pre-purified N_2 (Trigas, Irving, TX, USA) and incubated at 37°C. After purging serum bottles with N_2 to evacuate O_2, the excess pressure was always released to restore atmospheric pressure.

Analytical

An HPLC with diode array UV-visible detection was employed to separate and quantify TNT and related nitroaromatics. The Waters system (Milford, MA, USA) used for HPLC analysis consisted of a fluid pump (Model 600E), a photodiode array detector (Model 996), a sample injector/autosampler (Model 717) and a programmable fraction collector. The system was controlled by a PC-based workstation (NEC Image 466es) equipped with the Millenium Chromatography Manager software. Spectra were acquired continuously between 200 to 400 nm and chromatograms extracted at 230 nm for quantification. The identification of analyte(s) was based on UV spectra and HPLC retention times. Analytes were separated on a reverse-phase Waters Nova-Pak C_8 column (3.9×150 mm) at room temperature. Detection limits for TNT and related nitroaromatic compounds were 0.05 mg L^{-1}. An isocratic mobile phase mixture of 82% water and 18% 2-propanol (Milli-Q H_2O, Millipore Co, Bedford, MA, USA) at 1 ml min^{-1} was employed for TNT and most related nitroaromatics. For TAT and azoxy compounds not optimally resolved with the previous mobile phase, a 99:1 mixture of water and 2-propanol at 1 ml min^{-1} and a 50:50 mixture of water and 2-propanol at 0.8 ml min^{-1}, respectively, were used. Measurements of ^{14}C were performed with a Beckman LS 3801 liquid scintillation counter (LS). Protein concentration was measured colorimetrically using a diagnostic kit (P5656; Sigma, St Louis, MO, USA).

Experimental

Cultures of *C. acetobutylicum* were grown at 37°C as described in a previous section. Stock solutions of TNT, 2A46DNT, 4A26DNT or 24DA6NT dissolved in methanol (at a concentration of 20 g L^{-1}) were added to cultures or medium and heat-inactivated controls to obtain an initial target concentration of 0.44 mM. Heat-inactivated controls were prepared by autoclaving cultures prior to addition of nitroaromatics. Since *C. acetobutylicum* undergoes a distinct metabolic shift during exponential growth (ie acidogenesis to solventogenesis), individual nitroaromatics were added to active cultures that were in the same phase of growth, specifically the acidogenic phase (5.5–6.5 h after germination in fresh NYG). The metabolic activity, specifically acidogenesis, was monitored by measuring gas production; acidogenic fermentation is accompanied by the production of H_2 and CO_2 [11]. Gas production increased from atmospheric pressure at the start of culture to roughly 1.3 times atmospheric pressure during the acidogenic phase of growth, which spans approximately 11 h following germination in fresh NYG. Gas production rates in solventogenic growth phase decrease markedly, perhaps since H_2 is no longer produced by the culture [11]. Media samples for HPLC quantification of nitroaromatics were withdrawn from serum bottles with sterile syringes, immediately centrifuged at $13000 \times g$ for 7 min, and the supernatant phase was retained for analysis.

Since *C. acetobutylicum* undergoes a distinct metabolic shift during exponential growth, studies were conducted to assess the influence of the metabolic stage on the rate and extent of TNT transformation observed. Additions were made in the acidogenic phase (5.5–6.5 h after germination in fresh NYG), solventogenic phase (12 h after germination in fresh NYG) or stationary phase (38 h after germination in fresh NYG). Experiments were also conducted under conditions of induced solventogenesis. This was achieved by the addition of CO [13, 17] to cultures during early-acidogenic phase (4 h after germination in fresh NYG) by flushing the headspace for 20 min with CO.

Mass balance studies employing ^{14}C-labeled TNT and LS analysis were conducted to quantify the extent of TNT mineralization, sorption to biomass, and final product distribution. TNT (0.44-mM including [ring-U-^{14}C]-TNT to a level of $1–1.3 \times 10^4$ dpm ml^{-1}) was added to cultures in their acidogenic phase and allowed to incubate for 34 h. CO_2 traps containing Carbon 14 Cocktail (RJ Harvey, Hillsdale, NJ, USA) were inserted in serum bottles and counted directly. Media samples were centrifuged and the supernatant phase analyzed following the addition of Ready Gel scintillation cocktail (Beckman, Fullerton, CA, USA). The ^{14}C associated with the pellet was determined by washing it with methanol. The distribution of end products was evaluated by fractionation of HPLC eluant and LS analysis.

Results

Transformation of TNT and aminated nitrotoluenes

The transformation of TNT and related aminated nitroaromatics was investigated in *C. acetobutylicum* cultures during the acidogenic growth phase. Results of these experiments are shown in Figure 1. Heat-inactivated controls showed no transformation during the 5 h of incubation (no data were taken after 5 h). In comparison, active cultures transformed TNT and 2A46DNT to concentrations below detection limits (<0.05 mg L^{-1}) within 25 min. Slower transformation rates were observed for 4A26DNT and 24DA6NT. 4A26DNT was transformed from 0.44 mM to 0.01 mM in 45 min, and then remained at detectable levels for an additional 100 min. 24DA6NT was reduced from 0.51 mM

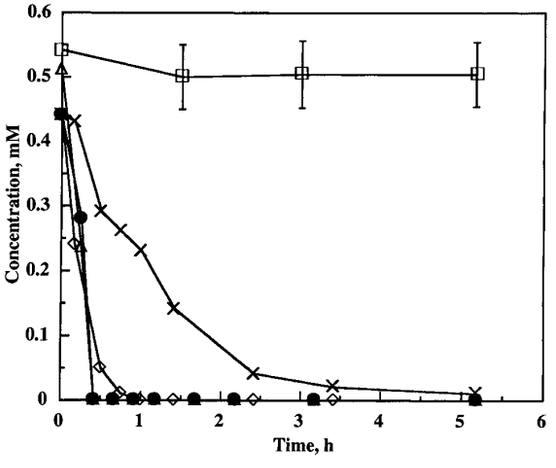

Figure 1 Transformation of TNT (●), 2A46DNT (△), 4A26DNT (◇) and 24DA6NT (×) by acidogenic-phase cultures of *C. acetobutylicum*. The fate of TNT in heat-killed culture controls (□) is included for comparison. The *x*-axis (elapsed time) represents time following the application of nitroaromatic compound. The data for TNT in killed controls (□) are the mean of triplicate cultures, while that of TNT and 2A46DNT in viable cultures are the mean of duplicate cultures. The remainder are representative single experiments. Error bars are standard deviations.

to 0.05 mM in 2 h, and remained at detectable levels for the duration of the experiment. The zero-order specific rate constants of these and related transformations are summarized in Table 1 after normalizing to protein concentrations.

Effect of culture growth conditions on TNT transformation

The effect of growth conditions on the transformation of TNT was investigated with acidogenic-, solventogenic-, and stationary-phase cultures of *C. acetobutylicum*. Results of TNT disappearance and the formation of identifiable intermediates are shown in Figure 2a and b, respectively. Acidogenic cultures transformed TNT (0.44 mM) to below detection limits in 25 min accompanied by the transient appearance of 2hA46DNT and 4hA26DNT. Aminated nitrotoluenes were not detected. Slower rates of TNT transformation were observed in solventogenic and stationary phase cultures. Nominally 50% of the TNT added to the solventogenic culture was transformed over the 5 h of incubation. The only products identified under solventogenic conditions were hydroxylamines, which accumulated and remained throughout the experiment. The zero-order specific rate constant observed in the solventogenic culture was 111 nM min^{-1} mg^{-1}, almost a factor of 5 lower than that of the acidogenic culture (V_{max} = 537 nM min^{-1} mg^{-1}). Concentrations of TNT in stationary phase cultures decreased by only 4% (Figure 2a), without the accumulation of detectable concentrations of reduced products.

To evaluate further the influence of growth phase on the rate and extent of TNT transformation, experiments were conducted in cultures under induced solventogenic conditions. Results of experiments comparing TNT disappearance and hydroxylamine formation in solventogenic-induced systems and acidogenic controls are shown in Figure 3a and b, respectively. While TNT was transformed to concentrations below detection limits within 25 min in acidogenic-phase control cultures, 130 min elapsed before it was completely transformed in a solventogenic-induced culture. The monohydroxylamines, 4hA26DNT and 2hA46DNT, appeared transiently in both cultures to maxima of 0.24–0.26 mM and 0.08 mM, respectively (Figure 3). However, the maxima were attained in CO-treated cultures after 2 h, while in untreated controls within 30 min.

Table 1 Zero-order specific rate constants for disappearance of TNT and related aminated munitions compounds in actively growing suspension cultures of *C. acetobutylicum*. Single or replicate cultures of *C. acetobutylicum* were treated as indicated in Figure 1 during the acidogenic phase of growth

Treatment compound	Concentration (mM)	Zero-order rate constant, *k* (nM min^{-1} mg^{-1} protein)
TNT	0.44	537
2A46DNT	0.51	444
4A26DNT	0.44	267
24DA6NT	0.44	196

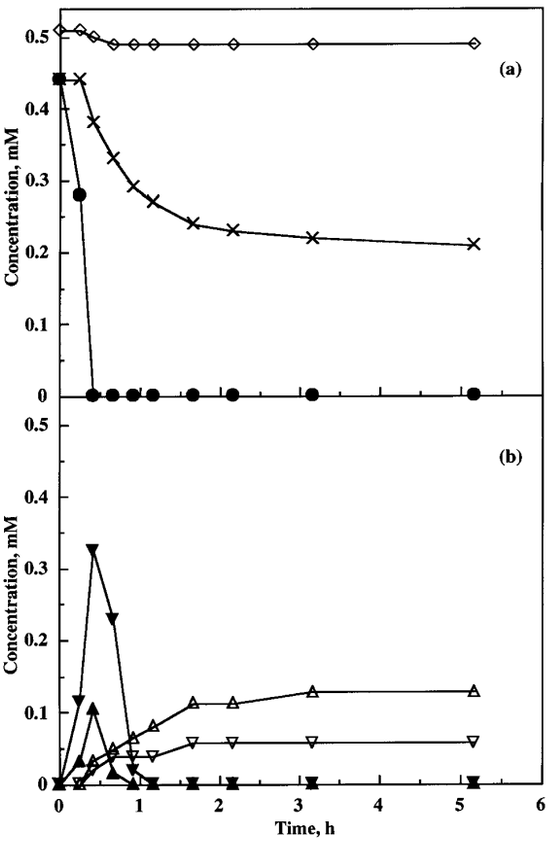

Figure 2 The effect of growth phase of *C. acetobutylicum* on: (a) transformation of TNT by acidogenic (●), solventogenic (×) and stationary (◇) phase cultures; and (b) formation and transformation of 2hA46DNT (▲,△) and 4hA26DNT (▼,▽) in acidogenic (filled symbols) and solventogenic (unfilled symbols) phase cultures. In each case, 0.44 mM TNT was added to cultures. The *x*-axis (elapsed time) represents time following the application of TNT. Data are from representative single experiments.

Observed intermediates of nitroaromatic transformation

The major intermediates observed in these studies of TNT transformation by *C. acetobutylicum* were 2hA46DNT and 4hA26DNT. 4A26DNT was observed in some cases, but at levels never exceeding 3% of the TNT added. Reduced forms of TNT that were not observed include 2A46DNT, azoxy compounds, or TAT. An unknown intermediate was observed occasionally during TNT transformation studies, as well as in 2A46DNT and 4A26DNT experiments. Its UV spectrum (λ_{max} = 214.6 nm) was similar to that of 24DA6NT (λ_{max} = 209.8 nm), but it eluted 1 min earlier than 24DA6NT during HPLC analysis.

Mass balance studies were conducted with [ring-U-^{14}C]-TNT to evaluate the distribution of transformation products. Table 2 summarizes the distribution of ^{14}C radiolabel 34 h after the addition of TNT to acidogenic phase *C. acetobutylicum*. These results demonstrate that the products of TNT transformation are primarily soluble, and mineralization is negligible. HPLC with UV detection yielded no quantifiable levels of TNT or known reduction products. Fractionation of HPLC eluant showed that 56% of the ^{14}C injected was present in the bulk solvent front (Figure 4). An additional 29% eluted in the tail of the solvent front, in a

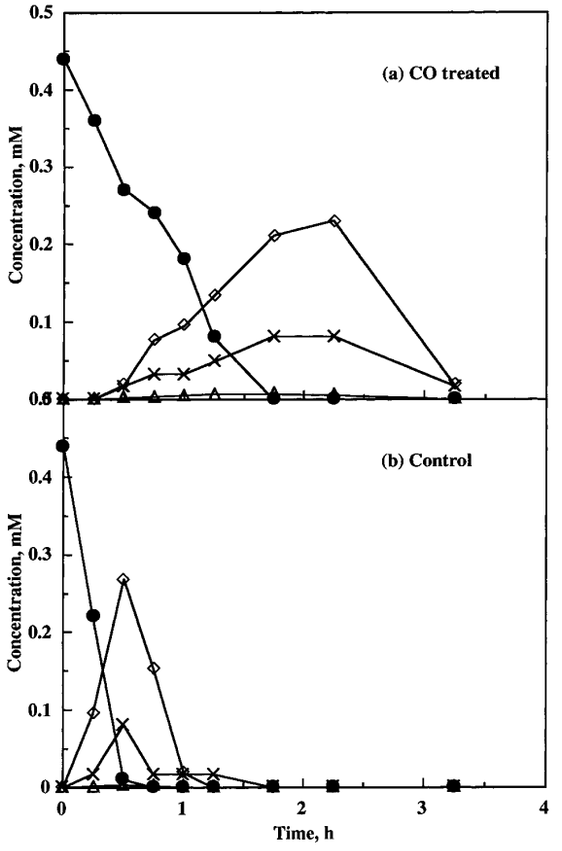

Figure 3 Transformation of 0.44 mM TNT (●) and the transient formation of 2hA46DNT (×), 4hA26DNT (◇) and 4A26DNT (△) in acidogenic-phase cultures of *C. acetobutylicum* that were: (a) exposed to CO and (b) not exposed to CO. The *x*-axis (elapsed time) represents time following the application of TNT. Data are from representative single experiments.

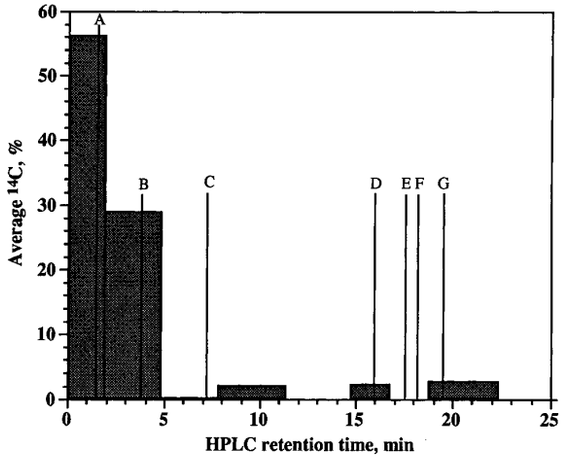

Figure 4 The distribution of ^{14}C radiolabel obtained by the HPLC fractionation of a 34-h sample of TNT-treated acidogenic-phase culture of *C. acetobutylicum*. The culture was treated with 0.44 mM TNT and 1.3×10^4 dpm ml^{-1} (U-ring-^{14}C)-TNT in acidogenic phase. The retention times of likely intermediates are labeled as: (A) TAT; (B) 24DA6NT; (C) TNT; (D) 2hA46DNT; (E) 4hA26DNT; (F) 2A46DNT; and (G) 4A26DNT. Total recovery of ^{14}C radiolabel after fractionation of HPLC eluant was 91%. Bars for first three ^{14}C-radiolabel fractions have no identifiable products.

Table 2 Mass balance on ^{14}C-labeled 2,4,6-trinitrotoluene applied to an acidogenic phase cell suspension culture of *C. acetobutylicum*. 0.44 mM TNT and 1×10^4 dpm ml^{-1} [U-ring-^{14}C]-TNT were applied to early acidogenic phase cultures of *C. acetobutylicum*. Total recovery of ^{14}C radiolabel after 34 h was 98.7%

Time (h)	Radiolabel location	^{14}C recovery (%)
0+	Culture	100
34	Supernatant medium	96.5
	Pellet	2.1
	Mineralization (base traps)	0.1

region of the chromatogram where no discernible peaks were observed by the UV detector and the ^{14}C in this fraction could not be attributed to known aminated nitrotoluenes. Approximately 4.5% of the ^{14}C injected was recovered in the monoamino- and hydroxylamino regions of the chromatogram, and 8.6% of ^{14}C injected was not accounted for.

Discussion

The ability of microorganisms, both aerobic and anaerobic, to reduce the aryl nitro groups of TNT has been well documented [2–5, 9, 14, 15, 19, 21, 28]. As reviewed by Preuss and Reiger [20], the reduction of TNT is often not complete (ie beyond diaminonitrotoluenes), but can occur with certain anaerobic cultures. Identifying classes of anaerobes that are capable of efficiently mediating such reactions is critical to the operation of TNT biotreatment systems to avoid incomplete metabolism or the formation of unwanted end products. Previous studies have demonstrated that *Clostridium* species are capable of nitroaromatic transformation [1, 15, 19, 21], and recently it has been shown that certain clostridia are capable of transforming diaminonitrotoluenes [19]. The studies reported in this paper investigated the ability of *C. acetobutylicum* to transform TNT and commonly cited TNT reduction products (eg aminated nitrotoluenes). Experiments demonstrated that active *C. acetobutylicum* cultures were capable of rapid nitroaromatic transformation, without a lag phase, under optimal laboratory conditions with no previous exposure to TNT or other nitroaromatic compound.

One of the more interesting findings from these experiments was the discovery that the TNT transformation activity of *C. acetobutylicum* decreases as it exits acidogenic growth and enters solventogenesis. This was evidenced by the decrease of TNT transformation rates and the accumulation of hydroxylamines. Of these two observations, the latter is more important in application of bioremediation systems. In soil systems, hydroxylamines are known to bind to soil organics, reducing bioavailability [7, 8, 27]. In addition, hydroxylamines can react with nitroso intermediates to form low solubility azoxy compounds [12, 15, 23, 28]. This was not observed in these cultures, acidogenic or solventogenic, presumably due to the low pH of the medium (4.1–4.5). The rates of azoxy formation decrease as pH decreases [8]. In a bioremediation system, where pHs may be closer to neutral (due to the buffering

capacity of soils or the activity of other organisms in a mixed culture), the accumulation of hydroxylamines during solventogenic growth may result in increased azoxy formation. Under acidogenic conditions, the hydroxylamines produced during the reduction of aryl nitro groups were transformed rapidly. During solventogenic conditions or under CO-mediated inhibition, hydroxylamines were more persistent, thus increasing the potential for these unwanted side reactions.

Thus it appears that it may be advantageous to operate initial reductive stages of TNT bioremediation systems under acidogenic conditions. This may, however, present a significant operational challenge. In the presence of excess carbohydrates, solventogenesis can begin within hours after start-up [10, 25], while complete dissolution of TNT from highly contaminated soils may require significant time periods [18]. In batch systems, it may be possible to sustain acidogenic conditions through careful regulation of carbohydrate additions. Alternatively, a continuous flow system where acid levels do not induce solventogenesis could sustain high levels of acidogenesis for extended periods [16]. A third possibility is to maintain a balanced mixed-culture where produced acids and H_2 are metabolized by non-fermentative bacteria in a manner analogous to anaerobic digestion.

Another interesting finding of this work was the absence of aminated nitrotoluenes as an intermediate or end product of TNT transformation, even during solventogenesis. Assuming the reduction pathway presented by Preuss [19] represents TNT transformation by *C. acetobutylicum*, and evaluating the rate coefficients observed in experiments presented herein, an accumulation of diamino nitrotoluenes would be expected. The rapid transformation of 2A46DNT and 4A26DNT may explain why they were not observed. The presence of TAT was not routinely evaluated due to the analytical difficulties involved with this compound. However, when analyzed for, TAT was never detected. It is impossible to say that TAT was not produced; TAT reacts with O_2, and samples were exposed to air during centrifugation and in the HPLC. However, TAT standards were stable for these time periods (less than an hour). In addition, if formed, TAT may have been subject to further transformation. Reported products of TAT transformation under anaerobic conditions include methylphloroglucinol [8] and toluene [3]. It is unlikely that toluene was produced in these experiments—^{14}C evaluation showed no partitioning losses into the headspace of serum bottles, and toluene does not elute in the polar solvent front of reverse phase HPLC analysis. Methylphloroglucinol standards were not available for these studies.

The only intermediates identified were hydroxylamines. An unknown compound was observed when 2A46DNT and 4A26DNT were added, and was occasionally observed when TNT was added. This compound had a UV spectrum similar to the diaminonitrotoluenes but was more polar (ie it eluted more rapidly in reverse phase HPLC). Both observations are consistent with the formation of a hydroxylamino aminonitrotoluene, but could not be confirmed.

For anaerobic, or anaerobic-aerobic bioremediation to mineralize TNT successfully, reductive stages are critical. The primary concern is the extent of reduction achieved. Merely converting TNT to corresponding aminated nitrotoluenes, which are also toxic, is insufficient. Furthermore, it is important to minimize unwanted side reactions that render the contaminant non-bioavailable. In these studies the rate and extent of TNT transformation in *C. acetobutylicum* were influenced by the growth phase of the cultures. Ongoing studies are currently evaluating the end product(s) of TNT reduction under acidogenic growth conditions.

Acknowledgements

We thank Dr George Bennett and Dr Edward Green for providing *Clostridium acetobutylicum* ATCC 824 and valuable information and guidance. Dr Ron Spanggord of SRI International was extremely generous in providing nitroaromatic standards. We also thank Adam Richardson for his invaluable assistance in the lab and Vicky Wynne for reviewing and editing the manuscript. This research was supported by US EPA HSRC-South/Southwest.

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