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2,4,6-Trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria

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Several bacterial strains were examined for their ability to degrade the nitroaromatic explosive 2,4,6-trinitrotoluene (TNT). The strains examined included various clostridial strains isolated from a 4-year-old munition enrichment, related clostridial strains obtained from a culture collection, two enteric bacteria, and three lactobacilli. All *Clostridium* species tested were able to reduce TNT rapidly in a complex medium. In cell suspension experiments, these strains were also able to reduce 2,4-diamino-6-nitrotoluene (DANT) to 2,4,6-triaminotoluene (TAT) and to produce a compound that is not yet identified; thus, they could not be distinguished from one another with regard to the pathway of transformation. The enteric strains and the lactobacilli were able to perform the initial reduction of TNT, but none was capable of reducing DANT in cell suspensions.

Keywords: anaerobic TNT degradation; clostridia; enteric bacteria; lactobacilli

Introduction

The polynitroaromatic compound 2,4,6-trinitrotoluene (TNT) is the primary explosive used in munitions production. Due to past disposal practices, TNT can be found as a contaminant in ground water and soils at explosive manufacturing and processing facilities. TNT is toxic and mutagenic [22,24], and relatively stable in the environment. Because of its toxicity, TNT-contaminated environments must be remediated. Thus far, the only generally accepted technology for elimination of nitroaromatic compounds from soil is incineration, which is prohibitively expensive. Thus, bioremediation, ie, composting and bio-slurry technologies, represents an important alternative approach to cleaning up contaminated areas and is being investigated intensively [5,7].

Only a few naturally occurring nitroaromatic chemicals have been identified; for example, chloramphenicol, nitrosporin, and pyrolnitrin. Therefore, it was not surprising when early research indicated that mineralization of TNT did not occur under aerobic or anaerobic conditions [7]. Several aerobic and facultative organisms partially reduce TNT to 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene as well as convert it to azoxytetranitrotoluenes [10].

Though other routes of transformation by aerobic organisms are now known [4,14,23], previous data suggested that only partial reductions and polymerization could be achieved. Therefore, anaerobic systems for TNT degradation have been studied in order to give more efficient reduction and stabilization for further transformation. The ultimate fate of TNT solely through nitro-reductive routes of transformation is 2,4,6-triaminotoluene (TAT) [13,16,17], an unstable, electron-rich intermediate. Under anaerobic and acidic conditions TAT is converted into unknown products accompanied by ammonia release [17]. Boopathy and Kulpa [2] isolated a *Desulfovibrio* sp which reportedly used TNT as a sole nitrogen source. They proposed reductions of TNT to give TAT, followed by the reductive elimination of the amino groups of TAT, ultimately yielding toluene. Unusual aspects of this report include an excess stoichiometry of toluene production, and growth before the release of nitrogen from aromatic intermediates. In another examination of TNT transformation by a *Desulfovibrio* sp, Preuss *et al* [17] showed the conversion of TNT to TAT by cell suspensions and isolated enzymes from this organism, *Clostridium pasteurianum*, and *Clostridium thermoaceticum*, but did not detect toluene. They also showed that under aerobic conditions, particularly in the presence of manganese (II) or nickel (II), TAT is subject to autoxidation and polymerization [17]. Funk *et al* [5] reported TAT as well as methyl phloroglucinol and *p*-cresol as transient products of a mixed culture of anaerobes derived by enrichment from sewage sludge and soil exposed to nitroaromatic herbicides.

To characterize the TNT transformation processes reported by Funk *et al* [5], we studied bacteria isolated from their bioreactor. To date, isolates determined to belong to the genus *Clostridium* have been examined most extensively [19,21]. These produce a prominent, but unidentified, transformation product after an initial reduction of TNT [11]. While working to identify this unknown transformation product, we undertook a comparative study intended to determine: 1) whether rapid reduction of TNT was an exclusive property of strict anaerobes possessing ferredoxin-type redox mediators; and 2) whether the activity of

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the isolated clostridia arose from long-term exposure to the nitroaromatic compounds during enrichment, or was a fortuitous reaction carried out by constitutively expressed metabolic activities of anaerobic microorganisms.

Materials and methods

Bacterial growth: Unless otherwise noted, bacteria were grown in Brain Heart Infusion broth (BHI, Difco, Detroit, MI, USA), supplemented with 0.5% Yeast Extract (YE, Difco) under anaerobic conditions at 37°C. TNT was added in appropriate concentrations from a 100× stock solution in methanol.

Bacterial strains and sources: See Table 1.

Chemicals: 4-Amino-2,6-dinitrotoluene (ADNT) and 2,4-diamino-6-nitrotoluene (DANT) were obtained from Ron Spanggord, SRI International, Menlo Park, CA, USA. 2,4,6-Triaminotoluene trihydrochloride was obtained from Chem Service, West Chester, PA, USA. TNT (>99% purity) was synthesized in our laboratory by S Goszczynski.

TNT degradation by growing cultures: Bacteria were subcultured at late exponential-phase into BHI medium containing TNT. Optical density (OD) at 600 nm was used to monitor growth. Samples (0.5 ml) were taken for analysis by reverse phase high-performance liquid chromatography (HPLC). The samples were prepared by adding 1/10 volume 6 M trichloroacetic acid, mixing, and incubating them on ice for at least 1 h. Following centrifugation in a microcentrifuge for 15 min, 450 μl of the supernatant phase were placed into HPLC vials containing 200 μl H_2O : acetonitrile (1 : 3, v/v) and stored at 4°C in the dark until analysed. TNT and its intermediates were separated on a Hewlett Packard model 1090 HPLC with a diode array detector using a Hewlett Packard ODS Hypersil, 5 mm, 100×4.6 mm, C_{18} reverse phase column with a H_2O/acetonitrile gradient. Detection was at 210 nm.

Table 1 Bacterial strains used in the analyses presented and their source, optical densities of cell suspension cultures, and change in glucose concentration during the course of the experiment (48 h). Δ [gluc] = mg per 100 ml

Strain	Source	OD_{600} (nm)	Δ [gluc]
C. bifermentans LJP-1	bioreactor	0.397	140
C. bifermentans SBF-1	bioreactor	0.264	128
C. bifermentans 638	ATCC	0.193	155
C. sordellii 9714	ATCC	0.259	86
C. sporogenes 11437	ATCC	0.489	196
C. acetobutylicum	[25]	0.110	196
L. acidophilus	lab collection	0.118	63
L. casei	lab collection	0.267	155
L. lactis	lab collection	0.235	172
E. coli	lab collection	0.207	169
S. typhimurium	lab collection	0.230	183

Experiments using cell suspensions: Bacteria were grown anaerobically overnight on BHI agar at 25°C in an anaerobic chamber, unless otherwise stated. The cells were then scraped off the agar plate with a sterile inoculating loop and resuspended in 5 ml of M9 anaerobic mineral salts-glucose medium [12], without ammonium chloride, in the anaerobic chamber. The media were supplemented with 25 μl of a 37.7 mg ml^{-1} DANT stock solution in anaerobic methanol to give a final concentration of 1.1 mM and stoppered with sterile Teflon™-faced butyl rubber stoppers (West Co, Phoenixville, PA, USA). A 0.5-ml sample was removed to determine the optical density (OD) of the cell suspension at 600 nm. Samples (0.5 ml) were taken anaerobically at 0, 24, and 48 h, filtered through syringe filters (0.2-μm pore size, Acrodisk, Gelman, Ann Arbor, MI, USA) into HPLC vials, and stored anaerobically at 25°C. HPLC analysis was done on a Hewlett Packard model 1090 HPLC with a Spherex 5 μm, C_{18} (250×2.0 mm) reverse phase HPLC column (Phenomenex, Torrance, CA, USA). The diode array detector was set to monitor at 220 and 317 nm. The mobile phase consisted of 15 mM ammonium acetate (pH 7.1) : acetonitrile gradient elution system and the flow rate was 0.3 ml min^{-1}.

Glucose determination: Metabolic activity was measured by determining the glucose content of M9 cell suspensions used for the HPLC analyses at various time intervals. A diagnostics glucose kit (Sigma Chemical Company, St Louis, MO, USA) was used for the analysis.

Results

Since previous work had indicated that the removal of nitrogen from the TNT aromatic nucleus took place in the long-term munition-fed bioreactor [5], we wished to determine what types of organisms were responsible for the many steps necessary to carry out this chemical transformation. Initial isolation and screening focused on those organisms showing the most rapid transformation of the specific munition compounds. These proved to be clostridia identified as *C. bifermentans* [19,21].

Because TNT is toxic to bacteria [8], our strains from the bioreactor were examined for their sensitivity to TNT. In addition, if these strains were specially 'adapted' to these compounds, ie, if specific metabolic pathways or traits had evolved in our bioreactor-derived organisms, those pathways might be expected to be regulated in response to the presence of nitroaromatics. In examining the kinetics of growth of and TNT transformation by *C. bifermentans* KMR-1 (Figure 1), we found that when TNT was added at a final concentration of 100 ppm to a growing culture at $OD_{600} = 0.05$, growth was inhibited. After about 4 h growth recovered to a rate approximately equal to the growth rate of cultures without added TNT (Figure 1a). Simultaneous monitoring of TNT removal from the culture showed that TNT was removed immediately, becoming undetectable after 3.5 h. Similar growth inhibition and TNT removal was seen in the same type of experiments with *C. bifermentans* ATCC 638, *C. sordellii*, and *C. sporogenes* (not shown). We suspect that TNT inhibited bacterial growth due to general oxidation-related toxicity, such as occurs when cultures of these anaerobes are exposed to oxygen.

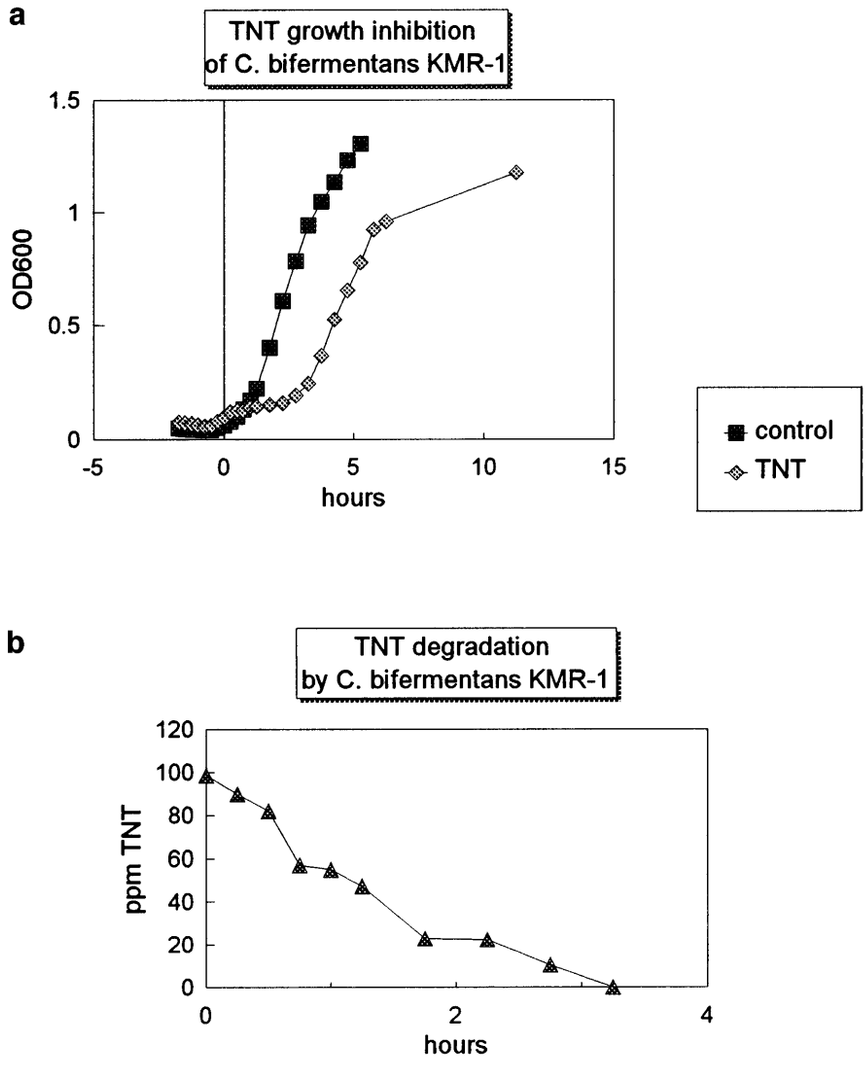

Figure 1 TNT degradation by *Clostridium bifermentans* KMR-1. (a) Growth inhibition of *C. bifermentans* KMR-1 by addition of 100 ppm TNT at OD_{600} = 0.05; TNT was added at time 0. (b) TNT degradation by *C. bifermentans* KMR-1; samples were taken starting immediately after TNT addition.

We also compared the kinetics of TNT transformation by organisms similar to our isolates, ie, other clostridia, that had not been previously exposed or specially adapted to TNT, and to organisms significantly divergent from ours, ie, facultative anaerobes and ferredoxin-free fermentors. These included the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, and the lactobacilli *L.acidophilus*, *L. casei*, and *L. lactis*. TNT removal was monitored in bacterial cultures grown anaerobically at 37°C in BHI medium supplemented with 0.5% YE, which supported growth of all organisms tested (Figure 2). TNT removal kinetics in *Clostridium bifermentans* strain ATCC 638, which to our knowledge had not been exposed to nitroaromatics prior to this experiment, were similar to those of our bioreactor-derived *Clostridium bifermentans* strains KMR-1 [19] (Figure 2a and b), LJP-1, and SBF-1 (data not shown). A similar pattern of TNT removal was observed for *Clostridium sordellii* (Figure 2c) and *Clostridium sporogenes* (Figure 2d), which were also strains from culture collections with no history of TNT exposure. TNT was reduced more quickly by the *Lactobacillus* cultures (Figure 2e, f, g) than it was in the sterile control experiment, which showed significant abiotic TNT reduction over 48 h (Figure 2j); however, only *L. casei* reduced at least two nitro groups of the TNT (Figure 2f). The facultative enteric bacteria *Escherichia coli* and *Salmonella typhimurium* transiently produced 4-amino-2,6-dinitrotoluene (ADNT) and accumulated less than stoichiometric amounts of 2,4-diamino-6-nitrotoluene (DANT, Figure 2h and i).

Clostridium acetobutylicum strain NCIMB 8052::Tn*1545* (AA010) was used in the same type of experiment. Surprisingly, when a late exponential-phase culture (24 h after inoculation) was subcultured into BHI/0.5% YE, growth seemed to be completely inhibited by the TNT, and no TNT degradation products were observed. However, when rapidly growing cultures were subcultured into medium containing TNT, no inhibition in growth was observed and TNT was rapidly reduced, with no detectable intermediates after 24 h. The sensitivity of this strain of *C. acetobutylicum* to TNT thus was markedly different from that of the other organisms tested.

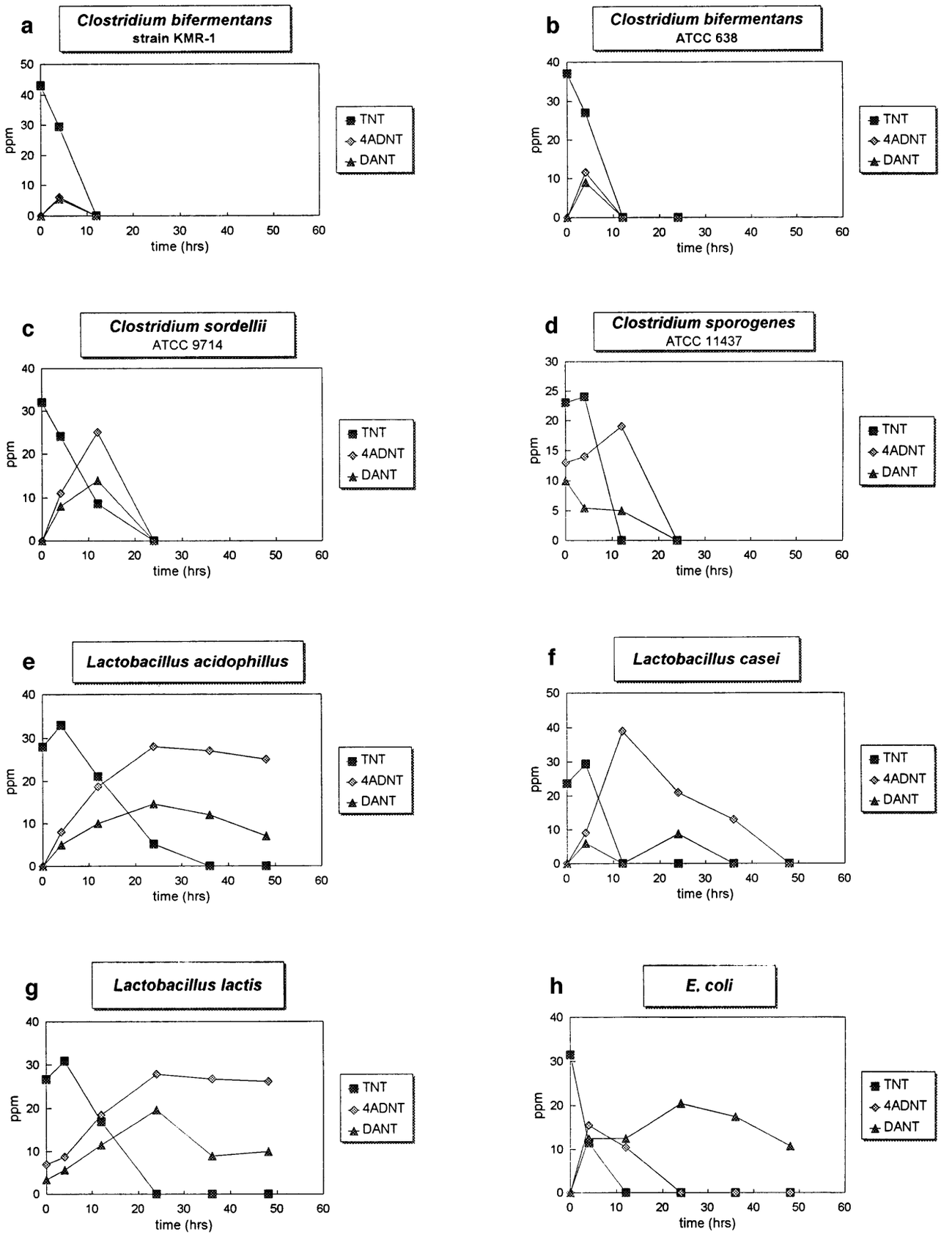

Figure 2 *Caption overleaf.*

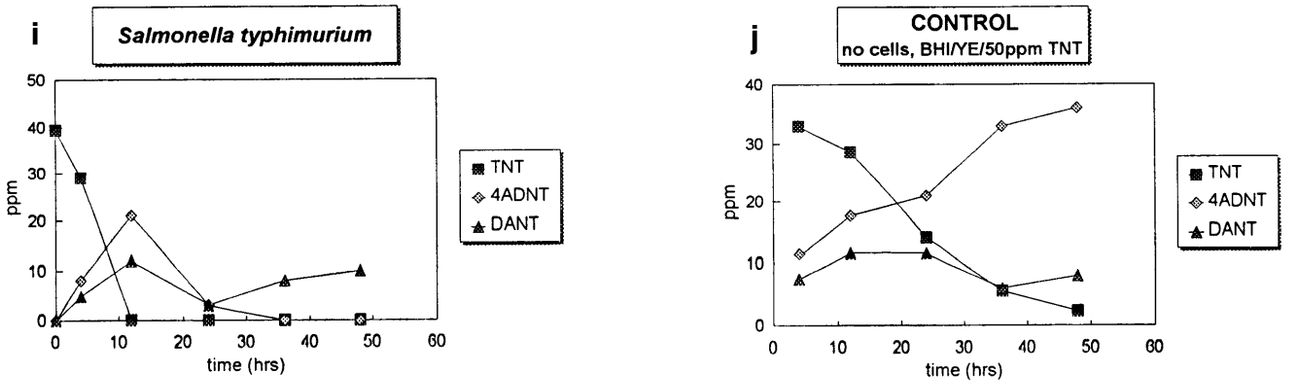

Figure 2 TNT degradation by different organisms. (a,b) *Clostridium bifermentans* spp; (c,d) *Clostridium sordellii* and *Clostridium sporogenes*; (e,f,g) *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus lactis*; (h,i) The enteric bacteria *Escherichia coli* and *Salmonella typhimurium*; (j) Sterile control: TNT (2,4,6-trinitrotoluene); 4ADNT (4-amino-2,6-dinitrotoluene); and DANT (2,4-diamino-6-nitrotoluene).

Cell suspensions: To detect additional soluble products that may have been co-eluted and thus obscured by UV-absorbing components of the medium, we used cell suspensions in a simpler medium that did not contain such components. We sought to determine whether the clostridia had a greater capacity than the other bacterial strains to reduce TNT and form products with lower standard midpoint redox potential, and whether the biotransformation products seen were the same for all or if unique pathways were evident.

Cells were suspended in an anaerobic, buffered medium (M9) that included glucose as an energy source but did not support growth of the clostridia or lactobacilli. Since it was found that all the organisms could reduce TNT, this set of experiments was designed to determine whether the different bacteria exhibit differential activity on DANT, a more reduced derivative of TNT. Previously, Lewis *et al* [11] reported that *C. bifermentans* strain LJP-1 accumulated an unidentified product when resuspended in modified M9 medium supplemented with DANT or DAHAT. We have since determined that the material referred to as DAHAT was actually TAT (unpublished data). We found that all clostridial strains tested (including *C. acetobutylicum*) accumulated the unknown product after 24–48 h. Figure 3 represents a chromatogram from a sample taken 48 h after addition of DANT; in the control (Figure 3a) no conversion of DANT can be detected, whereas *C. bifermentans* LJP-1 transformed DANT into TAT (retention time 8.5 min, Figure 3b) and an as-yet-unidentified compound with a retention time of 15.8 min and an absorption maximum at 317 nm. The peak eluting at 10.1 min represents an abiotically produced degradation product of TAT, since it also appears with time in sterile solutions of TAT.

For the lactobacilli and the enteric bacteria, no trace of the novel metabolite could be detected, and after 48 h DANT was still present in the suspensions at initial concentrations, indicating that these organisms cannot transform DANT under these conditions (not shown). All organisms were metabolically active, as evidenced by their consumption of glucose (Table 1).

Discussion

These experiments have shown that the clostridia isolated from a long-term munition-fed bioreactor do not show increased resistance to TNT, and that they do display constitutive transformation activities. Our observations are consistent with the results of previous studies indicating that the ability to reduce TNT anaerobically is a general phenomenon and that the TNT degradative pathways are not encoded by inducible genes, but could be associated with constitutively expressed metabolic functions of different *Clostridium* spp. Gorontzy *et al* [6] reported nitro reductase activity in different, 'non-adapted' bacterial strains. It is very likely, however, that the conditions in the fermentor allowed for enrichment of clostridial species during its adaptation phase [5], with the result that *C. bifermentans* strains were most easily isolated. Another explanation to be considered is that the ability of the clostridia to sporulate aided their survival in the bioreactor under prolonged exposure to nitroaromatics.

In our experiments, even the microaerophilic, ferredoxin-free lactobacilli were able to transform TNT to a substantial degree. Our observations confirm that non-ferredoxin-dependent mechanisms for anaerobic nitroaromatic degradation do operate in bacterial cultures. In addition to chemical reduction of nitro groups by ferredoxin-reducing enzymes [1,15,17], enzymatic nitroreductase activity has been described. Rafii *et al* [18] determined that nitroreductase activity of different *Clostridium* spp was not only constitutive and extracellular but required flavin adenine dinucleotide as a cofactor. In our TNT degradation study, *L. casei* was found to be able to remove TNT, ADNT, and DANT, whereas in the M9 cell suspension experiment the organism did not exhibit any activity on DANT, indicating that different types of degradation activities are possible in a rich medium. The type of activity responsible for the removal of DANT by *L. casei* cultures is likely not reductive, since reduction products were not detected, and supports the notion that low-potential biochemical reductants present in strictly anaerobic bacteria, such as ferredoxin, ferredoxin-reducing proteins, or dissimilatory sulfite

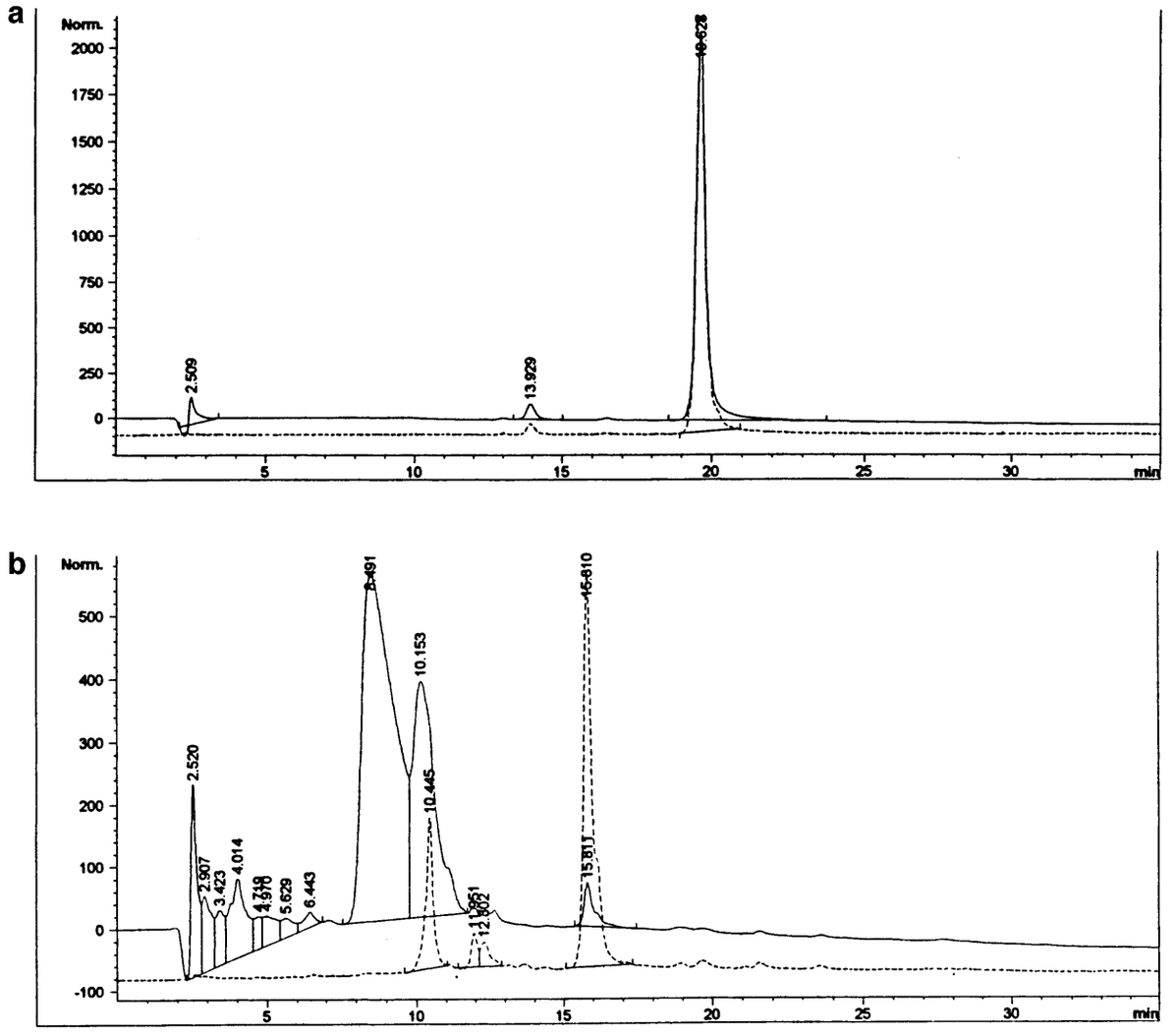

Figure 3 HPLC chromatograms representing the conversion of DANT into an unidentified compound by *C. bifermentans* LJP-1 (48 h after addition of DANT). The other clostridial strains tested yielded comparable chromatograms. (a) Sterile control; (b) *C. bifermentans* LJP-1. Solid line, A_{210}; dotted line, A_{317}.

reductase, are necessary for reduction of the nitro group of DANT [17]. The existence of alternate, possibly non-reductive, degradation pathways is further implied by the finding that the amounts of ADNT and DANT observed did not account for all the TNT transformed by the lactobacilli and the facultative organisms. Radiolabelling experiments using activated sewage sludge have shown transformation of TNT to a polyamide-like material [3]. This type of transformation or other polymerizations to form insoluble material would have escaped our analytical techniques.

McCormick *et al* [13] reported that cell-free extracts from *E. coli* were able to transform TNT to DANT and TAT, but no such activity could be detected in cell suspensions or cultures growing anaerobically. McCormick's results and ours indicate the existence of some nitroreduction activity in enteric bacteria. Previously, nicotinamide adenine dinucleotide and flavoproteins in cell-free extracts of *Neurospora* [26] and *E. coli* [20] were suspected of involvement in the first reduction of TNT leading to 4-amino-2,6-dinitrotoluene.

The novel unidentified product detected in the cell suspensions of a bioreactor-derived *Clostridium* sp is not the result of a biochemical activity selected for during prolonged exposure to munitions compounds. Rather, it appears to be produced by an activity common among clostridia and possibly other obligate anaerobic bacteria not yet analysed. According to the evolutionary tree constructed by Lawson *et al* [9] using 16S rRNA sequences, *C. bifermentans* and *C. sordellii* are very closely related, and *C. sporogenes* and *C. acetobutylicum* are more closely related to each other than to either of the first species. Therefore, the transformation path that results in the accumulation of the unidentified compound is a feature not restricted to a small group of clostridia, but seems to be rather widely distributed in this bacterial genus.

We are concentrating on the identification of the unknown compound accumulated in the cell suspensions

supplemented with DANT, with the goal of clarifying the TNT degradation pathway and the role of *Clostridium bifermentans* in the ecology of our bench-top reactor.

Acknowledgments

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