The Journal of Microbiology, September 2002, p.193-198

Vol. 40, No. 3

Transformations of 2,4,6-Trinitrotoluene in Various Conditions by *Klebsiella* sp. Strain C1 Isolated from Activated Sludge

Chong-Suk Chang[1], Hyoun-Young Kim[1], Yang-Mi Kang[1], Kyung Sook Bae[2], and Hong-Gyu Song[1]*

[1]*Division of Biological Sciences, Kangwon National University, Chuncheon 200-701, Korea*
[2]*Korean Collection for Type Cultures, KRIBB, P.O. Box 115, Yusong, Taejon 305-600, Korea*

(Received August 12, 2002 / Accepted September 6, 2002)

Several 2,4,6-Trinitrotoluene (TNT) degrading bacteria were isolated from an activated sludge by an enrichment culture technique, and their TNT removal activities were examined. Among the isolates, strain C1 showed the highest degrading capability, and completely removed 100 or 200 mg l^{-1} of TNT within 6 hours of incubation. This bacterium was identified as *Klebsiella* sp. The effects of different carbon sources on the removal of the parent TNT by *Klebsiella* sp. C1 were negligible, but the transformation rates of TNT metabolites such as amino-dinitrotoluenes and diamino-nitrotoluenes were higher with fructose addition compared to glucose addition. When nitrate was used as the nitrogen source, the degradation rates of TNT and hydroxylamino-dinitrotoluenes were higher than those with the ammonium addition. Although the TNT removal rate of *Klebsiella* sp. C1 was slightly higher in anaerobic conditions, the further transformations of TNT metabolites were more favorable in aerobic conditions.

Key words: 2,4,6-trinitrotoluene biodegradation, aerobic degradation, anaerobic degradation

Nitroaromatic compounds such as nitrobenzenes, nitrobenzoates, nitrotoluenes, 2,4-dinitrobenzene, and 2,4,6-trinitrotoluene (TNT) have multiple applications in the synthesis of pharmaceuticals, pesticides and explosives, and comprise an important group of environmental pollutants (Spain, 1995; Lewis *et al.*, 1997). The biological degradation of explosives, such as TNT is an important research area because TNT has contaminated various sites for the manufacturing and decommisioning of munitions, and TNT and its metabolites have toxicity and mutagenicity to various organisms (Won *et al.*, 1976; Honeycutt *et al.*, 1996). TNT is more recalcitrant than mono- and dinitrotoluenes, and cannot be degraded by classic mono- and dioxygenase enzymes involved in the microbial metabolism of aromatic compounds due to the effect of the nitro groups in reducing the electron density of an aromatic ring (Vorbeck *et al.*, 1994; Esteve-Núñez *et al.*, 2001). Nevertheless, microbial transformations of TNT under aerobic, anaerobic and combined conditions has been well demonstrated (Walker and Kaplan, 1992; Boopathy *et al.*, 1993; Spain, 1995; French *et al.*, 1998).

The initial metabolism of TNT in aerobic conditions is divided into two reductive pathways. One pathway involves a reduction of one or two nitro groups into a hydroxylamino group followed by the formation of various amino-substitued metabolites such as amino-dinitrotoluenes and diamino-nitrotoluenes (Haïdour and Ramos, 1996; French *et al.*, 1998). The other metabolic pathway is initiated by a hydride attack on the aromatic ring, and the transformation of TNT into dinitrotoluene with the release of nitrite via the hydride-Meisenheimer complex of TNT (Vorbeck *et al.*, 1994; Haïdour and Ramos, 1996). This metabolism has been reported to be affected by the type of nitrogen source (Martin *et al.*, 1997). In anaerobic conditions, TNT is reduced to triaminotoluene (TAT). TAT can be further transformed into *p*-cresol or toluene, but may be a dead-end product in some cases (Hawari *et al.*, 1998). The anaerobic metabolism of TNT has the potential advantages of rapid reduction and the minimization of the oxidative polymerization of metabolites (Esteve-Núñez *et al.*, 2001).

For the removal of TNT from the contaminated sites, the utilization of microorganisms which can transform and mineralize TNT efficiently is very important. In this study several bacterial strains were isolated by the enrichment of an activated sludge to screen the bacteria which can transform TNT rapidly, and the isolate showing a high TNT removal rate was identified. The initial metabolism of TNT in different environmental conditions by the isolated strain was investigated to examine the optimum conditions for the TNT degradation.

* To whom correspondence should be addressed.
(Tel) 82-33-250-8545; (Fax) 82-33-241-4627
(E-mail) hgsong@kangwon.ac.kr

Materials and Methods

Chemicals

Analytical-grade TNT, 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,6-diamino-4-nitrotoluene (2,6-DANT), 2,4-diamino-6-nitrotoluene (2,4-DANT), 2,4-dinitrotoluene (2,4-DNT), and 2,6-dinitrotolene (2,6-DNT) were purchased from Supelco Co. (Bellefonte, PA, USA) and AccuStandard Inc. (New Haven, CN, USA). All solvents were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, USA).

Isolation and identification of TNT degrading bacteria

The activated sludge obtained from the municipal sewage treatment plant in Chuncheon, Korea was added to a mineral salt basal (MSB) medium (Stanier *et al.*, 1966) supplemented with 2.5 g/l of glucose and 1 g/l of ammonium sulfate, and cultured in a continuous reactor system. The culture was maintained for 6 months with an increase of the TNT level (10 to 300 mg/l, final concentration) at room temperature. The hydraulic retention time was fixed at 36 h. After the enrichment of the activated sludge, the culture was spread on a solid MSB medium containing 200 mg/l of TNT, and incubated at 30°C. Different colonies were selected and subcultured several times to isolate pure cultures. Four different isolated strains showing fast growth were selected. A bacterial strain showing the fastest TNT removal rate among the isolates was identified by Gram staining, cell morphology, the full sequencing and the similarity analysis of 16S ribosomal DNA (Saitou and Nei, 1987), and the lipid profile analysis (Midi Inc., Newark, DE, USA).

Bacterial degradation of TNT

Four isolates were grown on MSB broth medium (20 ml) with glucose 0.1% (w/v) as the carbon source. The bacterial cultures were harvested and washed twice with 20 mM phosphate buffer (pH 7.0). An aliquot (2 ml) of the diluted bacterial biomass with phosphate buffer was inoculated into 18 ml of the same MSB medium resulting in the population number of approximately 10^6 cells/ml. After one day of incubation (30°C, 130 rpm, dark condition) TNT dissolved in methanol was added (final conc. of 100 or 200 mg/l) into the bacterial cultures. For the analysis of residual TNT and its metabolites at certain time intervals during further incubation, the whole bacterial culture was mixed with an equal volume of methylene chloride in a 50-ml centrifuge tube. This mixture was mixed vigorously with a vortex mixer for 30 sec. and then centrifuged (24,900 g, 15 min). After centrifugation, the methylene chloride phase containing the residual TNT and its metabolites was analyzed by HPLC.

To compare the effects of some environmental factors on TNT biodegradation, different carbon sources [final conc. 1% (w/v) of glucose, fructose and sucrose] and nitrogen sources (1 g/l of ammonium sulfate and sodium nitrate) were added, and the TNT degradation patterns were examined. TNT metabolism under aerobic and anaerobic conditions by facultative anaerobic TNT degrading bacterium were also compared. The anaerobic work was carried out in an anaerobic system (Forma Scientific, Marietta, OH, USA).

Analytical methods

Residual TNT and its metabolites were analyzed by Waters (Milford, Mass, USA) HPLC with a reverse-phase column (5 μm, 4.6×250 nm, Luna 5 ODS; Phenomenex, Torrance, CA, USA). Elution was by a linear gradient of 20% acetonitrile in water increased to 90% in 60 min. Both solvents contained 1% acetic acid. Flow rate was 1 ml min^{-1} and column temperature was 38°C. The retention times of TNT and its metabolites were determined by monitoring the effluent at 235 nm and the confirmation of target compounds was accomplished by comparison of the retention times of commercially available reference compounds.

All experiments were carried out in duplicate or triplicate, and the mean values are presented. The coefficients of variation are noted as error bars when larger than the symbols.

Results and Discussion

Isolation and identification of TNT degrading bacteria

Many researchers have attempted to isolate TNT degrading bacteria from various munition-contaminated environments, and examined the effective TNT removal conditions. However, most TNT degrading bacteria have the capability of biotransformation rather than effective mineralization of TNT (Kalafut *et al.*, 1998; Hawari *et al.*, 2000). In this study we tried to isolate bacteria which can transform and mineralize TNT effectively. Several TNT-degrading bacteria were isolated from activated sludge from a sewage treatment plant in Chuncheon city by an enrichment culture technique. Although a high concentration of TNT is toxic to most microorganisms (Spiker *et al.*, 1992; Spain, 1995), these isolates grew fast on the solid minimal medium in the presence of 200 mg/l and even with 500 mg/l of TNT. TNT degradation rates of the isolates were measured in the liquid medium. Among 4 isolates, C1 and C2 showed the highest removal rates of TNT. One hundred mg/l of TNT was completely degraded in both C1 and C2 cultures within 6 hours of incubation (Fig. 1A). The TNT degrading capabilities of C1 and C2 were similar in the presence of 100 mg/l of TNT; however, the C1 isolate showed a higher removal rate than that of the C2 isolate when adding 200 mg/l of TNT (Fig. 1B). Strain C1

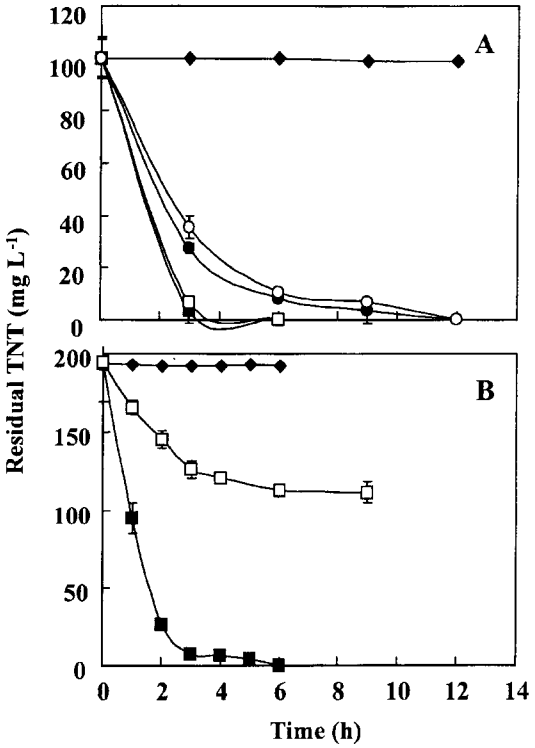

Fig. 1. Removal of TNT by several TNT-degrading bacteria isolated from activated sludge; 100 mg/l (A) and 200 mg/l (B) of TNT was added. Symbols: (◆), uninoculated control in 100 (A) and 200 mg/l TNT mg/l (B); (■), strain C1; (□), strain C2; (●), strain S3; (○), strain S4.

is a facultative anaerobic, Gram negative rod-shaped bacterium by the cultivation and morphological examination. It showed 99.5% similarity of full sequence of 16S rDNA (1434 bp) with *Klebsiella oxytoca* (GenBank Accession No. AY150697). However, the strain C1 showed the highest similarity (0.797) with *Klebsiella pneumoniae* in the lipid profile analysis. Further tests, such as a biochemical tests are necessary for the precise identification at the species level. This isolate was named *Klebsiella* sp. strain C1.

Effects of some environmental conditions on TNT degradation

Although TNT can be utilized as carbon and nitrogen sources by some bacteria, most TNT degrading bacteria co-metabolize TNT (Martin *et al.*, 1997; Kalafut *et al.*, 1998). Therefore, carbon and nitrogen sources are very important to TNT degradation. The removal rates of parent TNT were similar among the cultures of *Klebsiella* sp. C1 with different carbon sources (glucose, fructose, and sucrose). When glucose was added, the transformation rate of HADNTs was faster than those with fructose and glucose, but ADNTs and DANTs were transformed at the lowest rate among the three carbon sources (Fig. 2). Fructose seemed to be the best carbon source for the degradation of TNT and its metabolites among these sugars. Oh

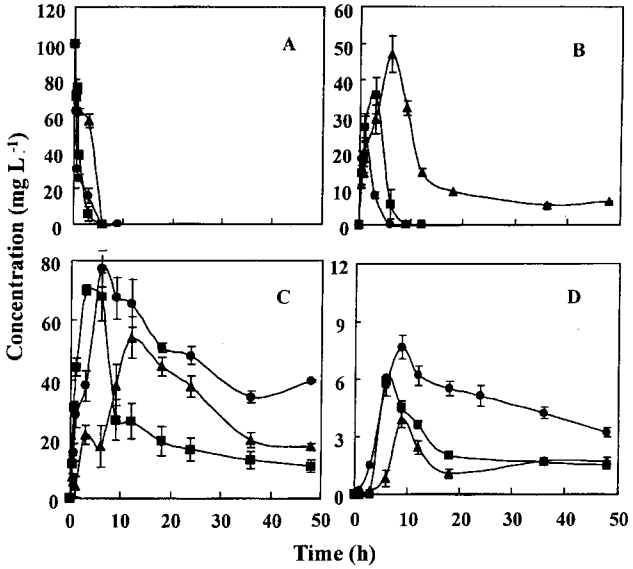

Fig. 2. Effects of addition of three different carbon sources on degradation of TNT and its metabolites by *Klebsiella* sp. strain C1: A; TNT, B; HADNTs, C; ADNTs, D; DANTs. Symbols: (●), glucose; (■), fructose; (▲), sucrose.

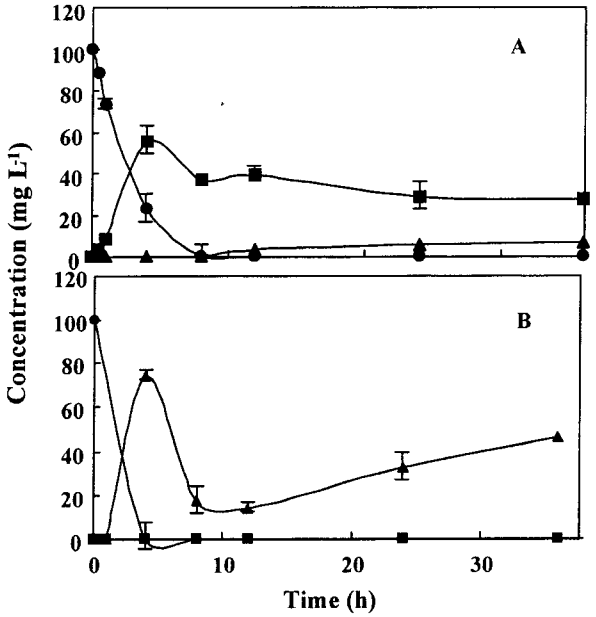

Fig. 3. Effect of addition of two different nitrogen sources on degradation of TNT and its metabolites by *Klebsiella* sp. strain C1: A; ammonium sulfate, B; sodium nitrate. Symbols: (●), TNT; (■), HADNTs; (▲), ADNTs.

et al. (1998) also reported a higher removal rate of TNT with fructose rather than glucose by *s*-triazine degrading bacterium.

The addition of sodium nitrate showed a higher transformation rate of TNT and HADNTs than those with ammonium sulfate (Fig. 3). In a study with *Pseudomonas savastanoi*, addition of nitrite enhanced the denitration

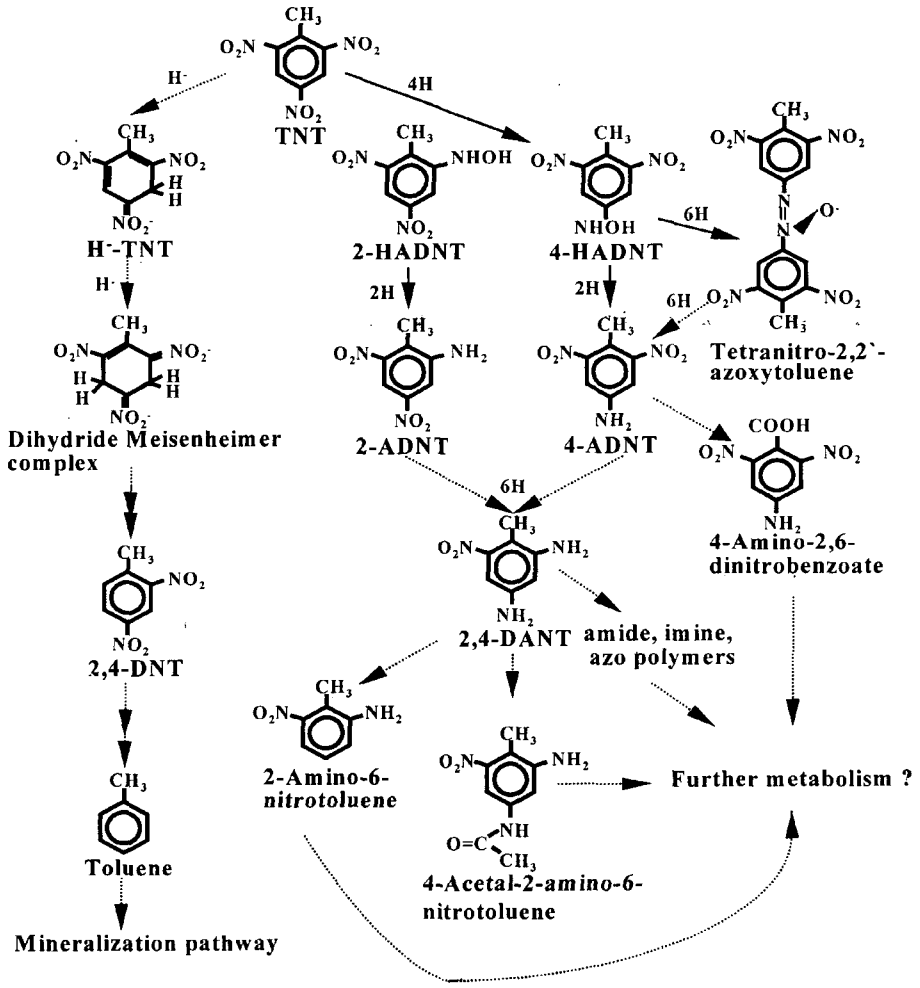

Fig. 4. Transformation pathways of TNT. All structures shown have been identified in various examples of TNT transformation. Solid arrows connect compounds occurring in TNT metabolism by *Klebsiella* sp. strain C1. Dotted arrows show speculative pathways.

pathway of TNT, but ammonium reduced it (Martin *et al.*, 1997). The same phenomenon could happen in the culture of *Klebsiella* sp. C1 which also uses the denitration pathway together with the nitro group reduction pathway (Fig. 4) for the initial TNT metabolism (Song and Kim, 2002). The white rot fungus *Irpex lacteus* also showed the same pattern of TNT metabolism (Kim and Song, 2000). Ammonium might reduce the denitration pathway of TNT by *Klebsiella* sp. C1, thus the overall transformation rates of TNT and its metabolites could be decreased. As the dependence on the nitro group reduction pathway increased, the concentrations of TNT and HADNTs in the nitro group reduction pathway became higher than those with nitrite (Fig. 3A). The concentration of ADNTs with nitrate addition was always higher than that with ammonium because of the higher transformation rate of HADNTs (Fig. 3B). However, it slightly increased again after 12-h incubation, and this might be due to the decrease of cell growth and overall TNT metabolism. Although the bacterial growth was not measured in this study, the changes of TNT and its metabolites were quite similar to the culture of aerobic *Serratia* sp. in which the bacterial growth began to decrease after 24 hours of incubation (Drzyzga *et al.*, 1998).

Although the aerobic degradation of most aromatic compounds generally begins with an oxidative attack on the ring structure by electrophilic oxygenase, reductive degradation occurs more easily in TNT metabolism (Fig. 4) because of its electrophilic character due to the nitro group (Shelley *et al.*, 1996). TNT disappeared very rapidly in the cultures of *Klebsiella* sp. C1 under both aerobic and anaerobic conditions (Fig. 5A). The transformation rate of initial reduction from TNT to HADNTs under anaerobic conditions seemed to be slightly higher than that in aerobic conditions. Since the initial transformations of TNT are the reductive metabolic reactions, anaerobic processes have the potential advantages at low redox potential (Esteve-Núñez *et al.*, 2001). However, HADNTs and following metabolites were transformed quickly and maintained lower levels in the aerobic culture than those

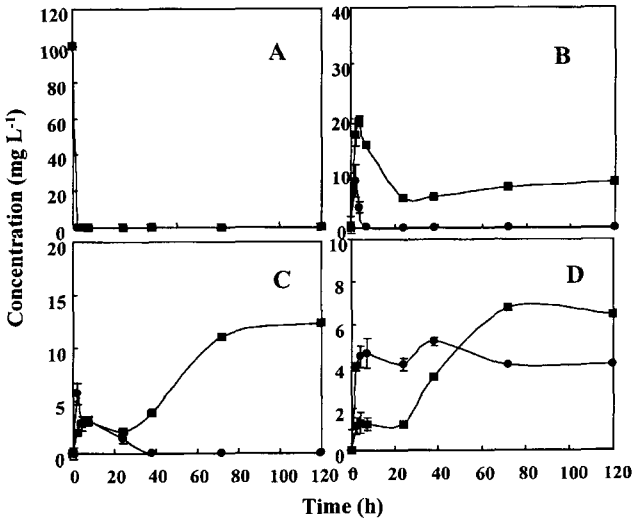

Fig. 5. Comparison of degradations of TNT and its metabolites in aerobic and anaerobic conditions by *Klebsiella* sp. strain C1: A; TNT, B; HADNTs, C; ADNTs, D; DANTs. Symbols: (●), aerobic conditions; (■), anaerobic conditions.

in the anaerobic culture (Fig. 5B, C, D). The biodegradation can be also influenced by cell growth, thus the concentrations of TNT metabolites seemed to remain at the higher levels in the anaerobic conditions which does not support the faster cell growth than those in the aerobic conditions. TNT may be reduced to triaminotoluene (TAT) in anaerobic conditions, and TAT often acted as a dead-end metabolite (Lewis *et al.*, 1997; Hawari *et al.*, 1998; Esteve-Núñez *et al.*, 2001). In general, the overall biodegradation can be faster and more complete under aerobic conditions. The involvement of oxidative transformation of aminodinitrotoluenes by dioxygenases can be one of the reasons for faster degradation of TNT metabolites under aerobic conditions (Johnson *et al.*, 2001). Fiorella and Spain (1997) reported that dihydroxylamino-nitrotoluene and 2-hydroxylamino-4-amino-6-nitrotoluene accumulated in the culture of *Pseudomonas pseudoalkaligenes* under anaerobic conditions, and suggested that their further metabolism was oxygen dependent. However, in our study *Klebsiella* sp. C1 produced diamino-nitrotoluenes, and seemed to transform them to the next metabolite in anaerobic conditions (Fig. 5D).

Some researchers tested the sequential anaerobic/aerobic process for the treatment of TNT contaminated soil because the initial reduction is more favorable in the anaerobic conditions (Roberts *et al.*, 1996; Knicker *et al.*, 1999). Since *Klebsiella* sp. C1 transforms TNT under both aerobic and anaerobic conditions and the initial TNT reduction seems to be slightly higher under anaerobic conditions, this characteristic may be utilized for the effective removal of TNT. The carbon and nitrogen sources have to be selected to achieve the high transformation and mineralization of TNT because they can affect the transformation rates of TNT and its metabolites, and nitrogen sources may affect the mineralization of TNT.

Acknowledgments

This work was supported by Grant No. R02-2000-00106 from the Basic Research Program of the Korea Science & Engineering Foundation.

References

Boopathy, R., M. Wilson, and M. Kulpa. 1993. Anaerobic removal of 2,4,6-trinitrotoluene (TNT) under different electron accepting conditions: laboratory study. *Water Environ. Res.* 65, 271-275.

Drzyzga, O., D. Bruns-Nagel, T. Gorontzy, K. Blotevogel, D. Gemsa, and E. von Löw. 1998. Mass balance studies with 14C-labeled 2,4,6-trinitrotoluene (TNT) mediated by an anaerobic *Desulfovibrio* species and an aerobic *Serratia* species. *Curr. Microbiol.* 37, 380-386.

Esteve-Núñez, A., A. Caballero, and J. Ramos. 2001. Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.* 65, 335-352.

Fiorella, P.D. and J.C. Spain. 1997. Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. *Appl. Environ. Microbiol.* 63, 2007-2015.

French, C.E., S. Nicklin, and N.C. Bruce. 1998. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Appl. Environ. Microbiol.* 64, 2864-2868.

Haïdour, A. and J. Ramos. 1996. Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. *Environ. Sci. Technol.* 30, 2365-2370.

Hawari, J., A. Halasz, L. Paquet, E. Zhou, B. Spencer, G. Ampleman, and S. Thiboutot. 1998. Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge: Role of triaminotoluene. *Appl. Environ. Microbiol.* 64, 2200-2206.

Hawari, J., S. Beaudet, A. Halasz, S. Thiboutot, and G. Ampleman. 2000. Microbial degradation of explosive; biotransformation versus mineralization. *Appl, Microbiol. Biotechnol.* 54, 605-618.

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.

Johnson, G.R., B.F. Smets, and J.C. Spain. 2001. Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. *Appl. Environ. Microbiol.* 67, 5460-5466.

Kalafut, T., M.E. Wales, V.R. Rastogi, R.P. Naumova, S.K. Zaripova, and J.R. Wild. 1998. Biotransformation patterns of 2,4,6-trinitrotoluene. *Curr. Microbiol.* 36, 45-55.

Kim, H.-Y. and H.-G. Song. 2000. Simultaneous utilization of two different pathways in degradation of 2,4,6-trinitrotoluene by white rot fungus *Irpex lacteus*. *J. Microbiol.* 38, 250-254.

Knicker, H., D. Bruns-Nagel, O. drzyzga, E. von L w, and K. Steinbach. 1999. Characterization of ^{15}N-TNT residues after an anaerobic/aerobic treatment of soil/molasses mixtures by solid-state ^{15}N NMR spectroscopy. 1. Determination and optimiza-

tion of relevant NMR spectroscopy parameters. *Environ. Sci. Technol.* 33, 343-349.

Lewis, T.A., M.M. Ederer, R.L. Crawford, and D.L. Crawford. 1997. Microbial transformation of 2,4,6-trinitrotoluene. *J. Ind. Microbiol. Biotechnol.* 18, 89-96.

Martin, J.L., S.D. Comfort, P.J. Shea, T.A. Kokjohn, and R.A. Drijber. 1997. Denitration of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. *Can. J. Microbiol.* 43, 447-455.

Oh, K.-H. and Y.-J. Kim. 1998. Degradation of explosive 2,4,6-trinitrotoluene by *s*-triazine degrading bacterium isolated from contaminated soil. *Bull. Environ. Contam. Toxicol.* 61, 702-708.

Roberts, D.J., F. Ahmad, and S. Pendharkar. 1996. Optimization of an aerobic polishing stage to complete the anaerobic treatment of munitions-contaminated soils. *Environ. Sci. Technol.* 30, 2021-2026.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.

Shelley, M.D., R.L. Autenrieth, J.R. Wild, and B.E. Dale. 1996. Themodynamic analysis of trinitrotoluene biodegradation and mineralization pathways. *Biotechnol. Bioeng.* 50, 669-672.

Song, H.-G. and H. Kim. 2002. Fate of 2,4,6-trinitrotoluene during degradation by *Enterobacter* sp. and characterization of nitroreductase involved. 102nd General Meeting of American Society for Microbiology. Abstract book p. 391.

Spain, J.C. 1995. Biodegradation of nitroaromatic compound. *Annu. Rev. Microbiol.* 49, 523-525.

Spiker, J.K., D.L. Crawford, and R.L. Crawford. 1992. Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58, 3199-3202.

Stanier, R.Y., N.J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* 43, 159-171.

Vorbeck, C., H. Lenke, P. Fischer, and H.-J. Knackmuss. 1994. Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. *J. Bacteriol.* 176, 932-934.

Walker, J.E. and D.L. Kaplan. 1992. Biological degradation of explosives and chemical agents. *Biodegra.* 3, 369-385.

Won, W.D., L.H. DiSalvo, and J. Ng. 1976. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Appl. Environ. Microbiol.* 31, 576-580.